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Sustainable Seafood Processing: Reducing Waste and Environmental Impact in Aquatic Ecosystems

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ABSTRACT

The global seafood industry is crucial in food production, providing essential nutrition and contributing to food security. Beyond its traditional role, the industry holds significant potential for generating high-value products by utilizing seafood resources. This comprehensive review explores the diverse applications of seafood resources, focusing on fish, shellfish, and seaweeds, in producing high-value products. The review examines various technological processes in extracting and purifying bioactive compounds from seafood, highlighting the advancements in seafood processing areas such as nanoencapsulation, fermentation, and enzymatic hydrolysis. Furthermore, it also discusses these innovations' economic and environmental impacts, emphasizing the importance of sustainability and efficiency in utilizing seafood by-products and waste. The seafood industry can minimize environmental pollution and promote circular economy principles by repurposing these materials. The review provides a holistic view of the future directions in this field, advocating for continued research and development efforts to enhance the value and sustainability of seafood resources. Overall, this review underscores the significance of seafood-derived high-value products in addressing global challenges while fostering economic growth and environmental stewardship.

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1 Introduction

Seafood, encompassing a diverse range of marine organisms such as fish, shellfish, and seaweeds, has long been integral to human diets globally (Luo et al. 2024; Martins et al. 2024). Its appeal lies in its delicious taste and exceptional nutritional value, offering essential nutrients like high-quality proteins, omega-3 fatty acids, vitamins, and minerals crucial for maintaining overall health (Tas and El 2024; Edo et al. 2024). With the global population steadily increasing, the demand for nutritious food sources is rising, prompting the seafood industry to explore innovative methods to utilize seafood resources efficiently and sustainably (Dhandwal et al. 2024). This exploration is pivotal for enhancing food security, reducing waste, and adding economic value to the industry.

The significance of seafood in global food security cannot be overstated. It serves as a primary source of animal protein for over three billion people worldwide and significantly contributes to the livelihoods of millions, particularly in coastal communities. Fish alone provides approximately 17% of the global animal protein intake and is a vital part of traditional diets in many cultures (Magbanua and Ragaza 2024). Seafood's rich nutrient profile, particularly its high content of omega-3 fatty acids, is associated with numerous health benefits, including improved cardiovascular health, enhanced cognitive function, and reduced inflammation (Tacon et al. 2024).

Further, seafood is a rich source of bioactive compounds such as omega-3 fatty acids, peptides, collagen, and chitin, which have significant health benefits and are used in formulating dietary supplements, functional foods, and therapeutic products. For instance, fish oils rich in omega-3 fatty acids are marketed as supplements for heart health, while peptides derived from fish proteins are explored for their antihypertensive and antioxidant properties (Santos et al. 2020. The cosmetic industry also benefits from marine-derived ingredients like collagen and alginates, which are prized for their skin health benefits (Kalasariya et al. 2024). Collagen from fish skin is used in anti-aging creams and other skincare products.

The rising demand for seafood has led to overfishing and the depletion of many fish stocks, raising concern about the long-term sustainability of marine resources. This scenario necessitates a paradigm shift towards more sustainable seafood production and utilization practices. One promising approach is producing high-value products from seafood resources (Cadena et al. 2024) (Figure 1). These products extend beyond traditional food consumption, including nutraceuticals, pharmaceuticals, cosmetics, and industrial products (Zhang et al. 2024). They are often derived from parts of seafood that are typically considered waste, such as fish skin, bones, viscera, shellfish exoskeletons, and seaweed biomass (Naghdi et al. 2024). Beyond health and beauty, seafood resources are also utilized in various industrial applications. Chitosan, derived from chitin in shellfish shells, is used in water treatment, bioplastics, and as a food preservative due to its biodegradable and non-toxic properties (Venugopal 2022). Additionally, seaweed biomass is explored for biofuel production, offering a renewable energy source that can help reduce reliance on fossil fuels (Milledge et al. 2014). One key strategy for enhancing the value derived from seafood resources is the utilization of by-products and waste. The seafood processing industry generates substantial by-products, which, if not properly managed, can lead to environmental pollution. However, these by-products are often rich in valuable compounds that can be extracted and utilized in high-value applications. For example, fish processing generates by-products such as heads, frames, skin, and viscera, which can be processed into fish protein hydrolysates, fish oil, and collagen (Välimaa et al. 2019). Similarly, shellfish shells can be a source of chitin and chitosan, and seaweed trimmings can extract bioactive polysaccharides like alginates and carrageenan.

The efficient extraction and utilization of high-value products from seafood resources require advanced technological processes. Techniques such as supercritical fluid extraction, enzymatic hydrolysis, and membrane filtration are employed to isolate and purify bioactive compounds from seafood by-products (Bruno et al. 2019). These technologies enhance the yield and quality of the extracted products and ensure that the processes are environmentally sustainable. Moreover, biotechnological advancements, including fermentation and genetic modification, are being explored to improve the efficiency and functionality of seafood-derived products. For instance, fermentation processes can enhance the bioavailability and health benefits of bioactive compounds, while genetic modification can increase the production of desirable compounds in marine organisms.

The shift towards producing high-value products from seafood resources offers significant economic and environmental benefits (Veríssimo et al. 2021). Economically, it opens up new markets and revenue streams for the seafood industry, creating job opportunities and stimulating economic growth, especially in coastal and rural areas (Burbridge et al. 2021). Environmentally, it promotes sustainability by reducing waste and minimizing the ecological footprint of seafood processing activities. This approach aligns with the principles of a circular economy, where resources are used efficiently and waste is transformed into valuable products. Despite the promising potential, several challenges must be addressed to fully realize the benefits of utilizing seafood resources for high-value products (Cooney et al. 2023). These include ensuring sustainable harvesting practices, developing cost-effective and scalable extraction technologies, and establishing regulatory frameworks to ensure the safety and efficacy of seafood-derived products. Additionally, consumer acceptance and market development for these products are crucial for their

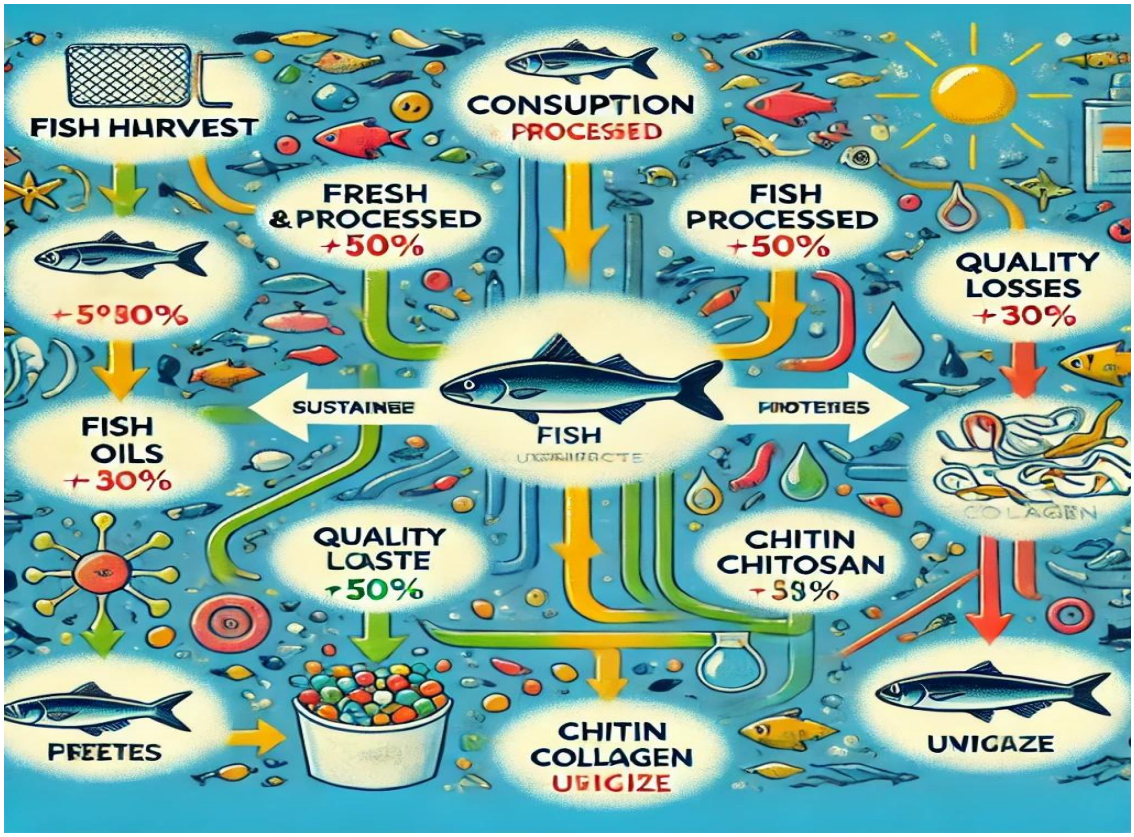

Figure 1 Seafood product description

successful commercialization. This review article aims to conduct an in-depth analysis of sustainable seafood processing practices, focusing on reducing waste and minimizing the environmental impact on aquatic ecosystems. The study seeks to identify innovative methods that transform fish by-products, traditionally discarded as waste, into valuable resources such as fish oils, proteins, enzymes, and collagen. By improving the efficiency of seafood processing, the research aims to maximize the use of harvested fish while significantly reducing the volume of waste generated. Additionally, the study aims to assess the current challenges related to quality and safety losses during seafood processing, which can lead to environmental harm and economic inefficiencies. By exploring advanced technological solutions, such as precision processing techniques, water recycling systems, and closed-loop operations, the research seeks to develop methods that address these issues while lowering the carbon footprint of the seafood industry. A core objective is to investigate how integrating sustainable practices, such as using renewable energy and eco-friendly packaging materials, can further reduce the environmental burden associated with seafood processing. The study will also emphasize the importance of responsible sourcing and traceability systems to promote sustainable fishing practices and ensure the long-term health of marine ecosystems. Ultimately, the study also provides practical recommendations for the seafood industry to adopt more sustainable processing methods, contributing to conserving marine biodiversity, protecting aquatic ecosystems, and achieving global food security. Through these efforts, the research aspires to align industry practices with environmental sustainability and the responsible management of marine resources.

2 Importance of seafood in global food security

The efficient use of seafood resources can enhance food security, reduce waste, and contribute to economic growth, particularly in coastal communities reliant on fishing industries (Farmery et al., 2022). Seafood plays a crucial role in global food security, serving as a primary source of protein for billions of people worldwide. Omega-3 fatty acids, for instance, are known for their benefits to cardiovascular health and cognitive function, while the vitamins and minerals found in seafood contribute to various bodily functions, including bone health and immune support (Stetkiewicz et al. 2022). Efficient utilization of seafood resources is important for nutritional, economic, and environmental reasons. By optimizing these resources, we can enhance food security by ensuring a steady and sustainable supply of nutrient-rich food. Reducing waste through better resource management can also bring significant economic benefits, especially in coastal communities that depend heavily on fishing industries.

3 Overview of high-value products from seafood

3.1 Bioactive compounds

High-value products derived from seafood include a variety of bioactive compounds that offer significant health and economic benefits. Omega-3 fatty acids, primarily found in fish oils, are one of the most well-known bioactive compounds. These essential fatty acids, particularly EPA and DHA, are crucial for maintaining cardiovascular health, supporting brain function, and reducing inflammation. Omega-3 supplements are widely consumed globally due to their proven benefits in reducing the risk of heart disease and improving mental health outcomes (Ozogul et al. 2021). Peptides derived from seafood are another valuable bioactive compound. These short chains of amino acids, obtained through the hydrolysis of fish proteins, possess various health-promoting properties. They have been shown to exhibit antioxidant, antihypertensive, and antimicrobial activities. Due to these properties, fish-derived peptides are increasingly used in functional foods and nutraceuticals to enhance health and wellbeing (Mutalipassi et al. 2021).

The health and beauty industries extensively utilize collagen extracted from fish skin, bones, and scales. Marine collagen is prized for its high bioavailability and efficacy in improving skin health, reducing wrinkles, and promoting joint health. Its applications in cosmetic products such as creams, serums, and supplements have made it a popular ingredient for maintaining youthful skin and overall physical vitality (Menon and Lele 2015).

Chitin and its derivative, chitosan, are extracted from the exoskeletons of shellfish like shrimp and crabs. These compounds are renowned for their versatility and functional properties and are used in various applications, including biomedical fields for wound healing and drug delivery systems, due to their biocompatibility and biodegradability (Hamed et al. 2016). They are also used in agricultural products, water treatment processes, and as natural preservatives in food products.

3.2 Pharmaceuticals and nutraceuticals

Seafood is a rich bioactive compound source that has garnered attention for their potential pharmaceutical and nutraceutical applications (Ashraf et al. 2020). Seafood-derived antioxidants, such as astaxanthin in shrimp and krill, exhibit potent antioxidant properties, making them valuable ingredients in pharmaceutical formulations and dietary supplements to promote overall health and wellbeing (Ashraf et al. 2020).

Furthermore, seafood is a reservoir of anti-inflammatory agents, which have been studied for their therapeutic potential in managing inflammatory conditions such as arthritis, inflammatory bowel disease, and asthma (D'Orazio et al. 2012). Compounds like omega-3 fatty acids, particularly EPA and DHA found in fish oils, possess anti-inflammatory properties that can help alleviate symptoms and reduce the severity of inflammatory diseases (D'Orazio et al. 2012). As a result, fish oil supplements have become increasingly popular as adjunctive therapy in managing inflammatory disorders.

Dietary supplements derived from seafood offer a convenient and effective means of delivering essential nutrients and bioactive compounds to consumers (Awuchi et al. 2022). These supplements may include fish oil capsules rich in omega-3 fatty acids, krill oil supplements containing astaxanthin and phospholipids, and collagen supplements derived from fish skin and bones. These products are marketed for various health benefits, including cardiovascular support, joint health, cognitive function, and skin rejuvenation. In addition to traditional pharmaceuticals and nutraceuticals, seafood-derived compounds are being investigated for their potential therapeutic applications in areas such as cancer treatment, wound healing, and immune modulation (Mutalipassi et al. 2021). The unique composition of bioactive compounds found in seafood presents exciting opportunities for developing novel pharmaceuticals and nutraceuticals that can address unmet medical needs and improve health outcomes for individuals worldwide. As research in this field advances, seafood is poised to play an increasingly important role in developing innovative therapies and preventive healthcare solutions.

3.3 Cosmetics

The cosmetics industry has long recognized the potential of marine-derived compounds for enhancing skin health and beauty. Seafood is a rich bioactive compound source that offers various benefits when incorporated into cosmetic formulations (Siahaan et al. 2022). One of the most sought-after ingredients is marine-derived collagen, extracted from fish's skin, bones, and scales. Marine collagen is prized for its high bioavailability and compatibility with human skin, making it an ideal ingredient for anti-aging skincare products. Collagen helps improve skin elasticity, reduce the appearance of wrinkles and fine lines, and promote overall skin hydration, resulting in a more youthful and radiant complexion (Mohiuddin 2019).

Alginates, derived from seaweed, are another valuable ingredient in cosmetics. These polysaccharides have unique gelling and hydrating properties, making them ideal for skincare products such as masks, creams, and serums (Pereira 2018). Alginate-based formulations help moisturize and soothe the skin, leaving it feeling soft, smooth, and refreshed. Additionally, alginates have been shown to have detoxifying and purifying effects, making them popular in skincare treatments designed to cleanse and rejuvenate the skin (López-Hortas et al. 2021). Other bioactive compounds found in seafood, such as astaxanthin and polyphenols, are also

gaining attention in the cosmetics industry for their antioxidant properties (Fernandes et al. 2019). Astaxanthin, a carotenoid pigment found in shrimp, crab, and salmon, is one of the most powerful antioxidants, neutralizing free radicals and protecting the skin from oxidative damage caused by environmental stressors such as UV radiation and pollution. Astaxanthin-based skincare products help improve skin tone, reduce hyperpigmentation, and enhance overall skin resilience, providing a natural defence against premature aging (Fernandes et al. 2019). Incorporating marine-derived bioactive compounds into cosmetics offers consumers a natural and sustainable alternative to traditional skincare ingredients (Fernandes et al. 2019). By harnessing the power of the ocean, cosmetics companies can develop innovative formulations that deliver tangible results while minimizing environmental impact. As consumer demand for clean, eco-friendly beauty products continues to rise, marine-derived cosmetics are poised to become increasingly popular in the global market.

3.4 Industrial applications

Seafood resources are valuable for human consumption and cosmetic purposes and hold immense potential for various industrial applications (Figure 2). These applications include the production of biofuels, bioplastics, and enzymes, offering sustainable alternatives to conventional materials and processes (Singh et al. 2022).

Biofuels derived from seafood biomass, particularly seaweeds, are gaining attention as renewable energy sources. Seaweeds have a high growth rate and can be cultivated using minimal land and freshwater resources, making them an attractive option for biofuel production. Additionally, seaweeds do not compete with food crops for agricultural land, mitigating concerns about food security and land use conflicts. Through processes such as fermentation and anaerobic digestion, seaweed biomass can be converted into biofuels like bioethanol and biogas, which can be used to power vehicles, generate electricity, and heat homes, reducing reliance on fossil fuels and mitigating greenhouse gas emissions (Maneein et al. 2018).

Bioplastics made from seafood-derived compounds offer a sustainable alternative to petroleum-based plastics. Chitosan, a polysaccharide derived from chitin found in shellfish shells, is one such compound that has garnered interest for its biodegradability, biocompatibility, and antimicrobial properties. Chitosan-based bioplastics have applications in packaging, agriculture, and biomedical fields, providing an eco-friendly solution to plastic pollution and environmental degradation. By replacing

Figure 2 Seafood industrial application overview

conventional plastics with biodegradable alternatives, seafood-derived bioplastics contribute to efforts to reduce plastic waste and promote a circular economy (Maneein et al. 2018).

Enzymes extracted from seafood organisms are valuable catalysts for various industrial processes. These enzymes exhibit unique properties such as high specificity, stability, and activity under extreme conditions, making them suitable for food processing, textile manufacturing, and wastewater treatment applications. For example, proteases and lipases from marine organisms are used in detergent formulations to enhance stain removal and fabric softening. Additionally, enzymes like cellulases and amylases are employed in biofuel production to break down biomass into fermentable sugars, increasing yield and efficiency (Maneein et al. 2018).

In summary, seafood resources offer numerous opportunities for industrial innovation and sustainability. By harnessing the biochemical properties of marine organisms, we can develop renewable energy sources, eco-friendly materials, and efficient biotechnological processes that contribute to a greener and more sustainable future. As research in this field continues to advance, seafood-derived industrial applications have the potential to revolutionize various sectors and drive positive environmental and economic outcomes.

4 Utilization of seafood resources

4.1 Application of Fish Resources

4.1.1 Fish oil

Fish is one of the most extensively utilized seafood resources globally, with applications beyond direct consumption. Various parts of the fish, such as skin, bones, and viscera, traditionally discarded as waste, are now repurposed into high-value products. Among these, fish oils stand out for their rich content of omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These essential fatty acids are widely recognized for their health benefits, especially in cardiovascular, cognitive, and anti-inflammatory functions. The oils are primarily extracted from fatty fish species such as salmon, mackerel, and sardines, rich sources of these bioactive compounds.

In the commercial sector, fish oils are a key component of dietary supplements and functional foods that promote heart health and brain function. The growing awareness of omega-3's health benefits has led to a significant increase in the demand for fish oil supplements in the nutraceutical industry. Advanced extraction techniques, such as supercritical fluid extraction and molecular distillation, have been developed to enhance these oils' yield, purity, and stability, ensuring their high quality for commercial use. These technologies also reduce contaminants, such as heavy metals and pollutants, further enhancing product safety. In addition to human health, fish oils are also used in aquaculture feed to improve the nutritional profile of farmed fish, thereby contributing to the sustainability of fish farming practices. The wide-ranging commercial applications of fish oils underscore their importance as a by-product of sustainable seafood processing practices.

4.1.2 Collagen and gelatin

Collagen and gelatin, derived from fish skin, scales, and bones, have gained significant commercial importance in various industries, including food, cosmetics, and pharmaceuticals. Collagen, a fibrous protein, is widely used for its health benefits, particularly in promoting skin elasticity, joint function, and wound healing. Gelatin, derived from the hydrolysis of collagen, is utilized as a gelling agent in food products, pharmaceuticals, and supplements. Fish-derived collagen has become an alternative to bovine or porcine sources due to its lower allergenicity and religious acceptability. The enzymatic hydrolysis process allows the production of collagen peptides, which are bioactive and easily absorbed by the body, making them a valuable ingredient in anti-aging and nutraceutical products. Additionally, collagen and gelatin have applications in regenerative medicine, such as in wound dressings and tissue scaffolding.

4.1.3 Fish protein hydrolysate

Fish protein hydrolysate (FPH) is produced by breaking fish proteins into smaller peptides through enzymatic hydrolysis, making it highly digestible and bioavailable. FPH has gained significant attention for its wide applications in the food, nutraceutical, and pharmaceutical industries. Due to its high nutritional value, FPH is commonly used as a functional ingredient in food products, offering emulsifying, foaming, and antioxidant properties. The peptides derived from FPH can also be bioactive compounds, exhibiting various health benefits such as antihypertensive, antimicrobial, and antioxidant activities. In the commercial market, FPH is incorporated into dietary supplements and specialized food products aimed at improving human health. It is particularly valuable in medical nutrition, where patients with compromised digestive systems require easily digestible proteins. Moreover, FPH is used in animal feed, especially aquaculture, to enhance farmed fish and shrimp's growth and immune response. The production of FPH also contributes to the sustainability of the seafood industry by utilizing fish by-products, which would otherwise be discarded as waste. Technological advances in the production of FPH have enabled better control of the degree of hydrolysis, allowing manufacturers to tailor the functional properties of the hydrolysates for specific applications. As the demand for sustainable, protein-rich ingredients grows, FPH remains a promising human and animal nutrition resource.

4.1.4 Bioactive Peptides

Bioactive peptides, derived from fish proteins, have emerged as valuable compounds in the nutraceutical and functional food industries due to their numerous health benefits. These short chains of amino acids exhibit various biological activities, including antioxidant, antimicrobial, antihypertensive, and anti-inflammatory properties. Bioactive peptides are produced through the enzymatic hydrolysis of fish proteins and have been shown to play a significant role in preventing and managing chronic diseases such as hypertension, cardiovascular disease, and diabetes (Power et al. 2013). Their ability to inhibit enzymes like angiotensin-converting enzyme (ACE) makes them potent antihypertensive agents. In the commercial sector, bioactive peptides are integrated into dietary supplements, functional foods, and beverages to enhance health and wellness. Additionally, they are being studied for their potential in cosmetic formulations for skin protection and anti-aging properties. The growing interest in bioactive peptides reflects their promising role as natural alternatives to synthetic compounds, with increasing applications in medical nutrition, particularly for aging populations. Advances in extraction and purification techniques, such as membrane filtration and chromatography, have improved the bioavailability and functionality of these peptides, further expanding their industrial use.

4.2 Utilization of shellfish resources

Shellfish, including shrimp, crab, and molluscs, are rich in bioactive compounds and chitin, which can be converted into high-value products (Azelee et al. 2023). The global shellfish industry generates large amounts of waste, primarily in exoskeletons and shells, rich in chitin, a biopolymer that can be converted into high-value products. Shellfish by-products are increasingly used in pharmaceuticals, food, and agriculture due to their bioactive compounds, which exhibit antioxidant, antimicrobial, and anti-inflammatory activities. These compounds are also used in cosmetics, contributing to anti-aging and skin health formulations. The commercial utilization of shellfish by-products helps to promote sustainability within the seafood industry by reducing waste and creating value-added products. In the food industry, shellfish-derived bioactive compounds are incorporated into functional foods, while in agriculture, they are used as biopesticides and soil conditioners. Additionally, the shellfish industry contributes to medical research, where bioactive compounds are being explored for their potential in drug development and wound healing applications (Islam et al. 2023). Recent innovations in processing and extraction technologies have further expanded the possibilities for utilizing shellfish by-products.

4.2.1 Chitin and chitosan

Chitin, a natural biopolymer found in the exoskeletons of crustaceans like shrimp and crabs, is one of the most abundant biopolymers on earth. Chitin is converted into chitosan, a highly versatile compound with various industrial applications through deacetylation. Chitosan's biocompatibility, biodegradability, and antimicrobial properties make it a valuable material in the biomedical field, where it is used in wound dressings, drug delivery systems, and tissue engineering. Chitosan's ability to form films and gels makes it an ideal candidate for water purification, agriculture, and food preservation (Azelee et al. 2023).

Commercially, chitosan is utilized in food packaging to extend shelf life by inhibiting microbial growth and in cosmetics for its skin-protective properties. In agriculture, chitosan is used as a natural pesticide and a plant growth enhancer. Its ability to form a protective barrier against pathogens has led to its use in biopesticides and seed coatings. The increasing demand for environmentally friendly and biodegradable materials has driven the growth of the chitosan industry, with ongoing research into new applications across various sectors, from pharmaceuticals to environmental management (Azelee et al. 2023; Akram et al., 2023).

4.2.2 Biomedical applications of chitosan

Chitosan is widely used in wound dressings for its unique properties that enhance wound healing. Its antimicrobial nature helps prevent infections, a critical aspect of wound management. Chitosan promotes hemostasis by aiding blood clotting, which is particularly beneficial for treating acute wounds. Furthermore, it facilitates the regeneration of tissues by providing a moist environment that supports cell proliferation and migration (Kankariya and Chatterjee 2023). Chitosan-based wound dressings are available in various forms, including films, hydrogels, and sponges, catering to different types of wounds and stages of healing. Chitosan plays a significant role in drug delivery due to its ability to form hydrogels, nanoparticles, and microspheres, which can encapsulate drugs and control their release. The biocompatibility and mucoadhesive properties of chitosan enhance the bioavailability of drugs, particularly those administered orally or through mucosal routes. Chitosan can be chemically modified to alter its solubility and degradation rate, providing customized drug delivery profiles (Haider et al. 2024). This makes chitosan an excellent carrier for many pharmaceuticals, including peptides, proteins, and vaccines, ensuring sustained and targeted drug release while minimizing side effects. Chitosan is extensively used in tissue engineering to develop scaffolds that mimic the extracellular matrix, supporting cell adhesion, proliferation, and differentiation (Wang et al. 2023). Its structural similarity to glycosaminoglycans, a component of the natural extracellular matrix, allows it to interact positively with cells and tissues. Chitosan scaffolds can be tailored to porosity, mechanical strength, and degradation rate to suit specific tissue engineering applications, such as bone, cartilage, skin, and nerve regeneration. Additionally, chitosan can be combined with other biomaterials

like collagen or hydroxyapatite to enhance its functionality and performance in tissue repair and regeneration (Xing et al. 2023).

4.2.3 Agricultural applications of chitosan

Chitosan is widely recognized as an effective biopesticide in agriculture. Its natural antimicrobial properties help protect plants from various pathogens, including bacteria, fungi, and viruses. When applied to crops, chitosan can induce a plant's defence mechanisms, known as systemic acquired resistance (SAR), enhancing the plant's ability to resist infections. Chitosan disrupts the cell walls of pathogens, inhibiting their growth and spread (Román-Doval et al. 2023). Unlike chemical pesticides, chitosan is non-toxic and biodegradable, making it an environmentally friendly alternative that reduces the chemical load on the ecosystem and helps maintain soil health and biodiversity (Román-Doval et al. 2023).

In addition to its role as a biopesticide, chitosan also acts as a potent plant growth enhancer. It promotes seed germination and improves root development, leading to stronger and more resilient plants (Sun et al. 2023). Chitosan enhances plant nutrient uptake, ensuring better growth and higher yields. Its application can increase chlorophyll production, improving photosynthesis and overall plant vigour. Moreover, chitosan can stimulate the production of phytohormones such as auxins and gibberellins, which play crucial roles in plant growth and development (Islam et al. 2023). This multifaceted action of chitosan helps farmers achieve higher productivity and better-quality crops.

4.2.4 Water treatment applications of chitosan

Chitosan is highly effective in removing heavy metals from wastewater due to its excellent chelating properties. The amino groups in chitosan's structure can bind with metal ions such as lead, mercury, cadmium, and arsenic, forming stable complexes easily separated from the water. This process helps detoxify industrial effluents and prevent harmful metals' release into natural water bodies. The efficiency of chitosan in heavy metal removal can be enhanced by modifying its structure or combining it with other materials, making it a versatile and powerful tool in water purification (Bhatt et al. 2023).

In addition to heavy metals, chitosan removes dyes from wastewater, particularly from textile and dyeing industries. Dyes are often toxic and resistant to degradation, posing significant environmental hazards. Chitosan's adsorption capacity effectively captures and removes various dyes, including acidic, basic, and reactive dyes (Bhatt et al. 2023). The adsorption process involves electrostatic interactions, hydrogen bonding, and van der Waals forces between chitosan and the dye molecules. Chitosan can be used in different forms, such as beads, flakes, or membranes, to treat dye-laden wastewater efficiently (Bhatt et al. 2023).

4.2.5 Other bioactive compounds from shellfish

Shellfish, such as shrimp and crabs, are rich in bioactive compounds, with astaxanthin being one of the most notable. Astaxanthin is a powerful antioxidant found in the shells of these marine creatures, known for its exceptional ability to neutralize free radicals and protect cells from oxidative damage. This compound is widely utilized in various industries due to its health benefits and functional properties (Khursheed et al. 2023). In the nutraceutical sector, astaxanthin is incorporated into dietary supplements to support cardiovascular health, enhance immune function, and reduce inflammation. Its skin-protective and anti-aging properties make it a valuable ingredient in the cosmetics industry, where it is used in skincare products to improve skin elasticity, reduce wrinkles, and protect against UV damage (Khursheed et al. 2023). Additionally, astaxanthin's vibrant red-orange color makes it an attractive natural food colorant, enhancing the appearance of food products and imparting antioxidant benefits. These diverse applications highlight the significance of astaxanthin as a multifunctional bioactive compound derived from shellfish.

4.2.6 Shellfish-derived proteins and enzymes

Due to their functional and bioactive properties, shellfish-derived proteins and enzymes play crucial roles in various industries. Enzymes such as proteases and lipases extracted from shellfish are extensively used in food processing to enhance food products' texture, flavor, and nutritional value. These enzymes help break down proteins and fats, facilitating the creation of specialized products like tenderized meats and improved dairy items (Yang et al. 2023). In bioremediation, shellfish enzymes contribute to the degradation of pollutants, offering an environmentally friendly solution for cleaning up contaminated sites. Additionally, producing bioactive peptides from shellfish proteins has gained attention for their health benefits, including antihypertensive, antioxidant, and antimicrobial properties (Azelee et al. 2023). These peptides are incorporated into functional foods and nutraceuticals to promote health and wellness, demonstrating the diverse and significant applications of shellfish-derived proteins and enzymes across multiple sectors (Yang et al. 2023).

4.3 Utilization of seaweed resources

Seaweeds, also known as marine macroalgae, hold immense promise as a renewable resource that yields valuable products across multiple industries. Their abundant presence in marine ecosystems and rapid growth rates make them highly sustainable sources of raw materials. Seaweeds offer a rich biochemical composition, containing essential nutrients, vitamins, minerals, and bioactive compounds. This diverse array of compounds opens up numerous avenues for their utilization in producing high-value

products (Jiménez-González et al. 2023). From food and nutritional supplements to pharmaceuticals, cosmetics, agricultural biostimulants, and even industrial materials like biofuels and bioplastics, seaweeds are being increasingly recognized for their versatility and potential (Jiménez-González et al. 2023).

4.3.1 Alginates, agar, and carrageenan

Seaweeds serve as a primary source of hydrocolloids, including alginates, agar, carrageenan, and polysaccharides, and they have diverse applications across several industries (Lomartire and Gonçalves 2023). In the food industry, these hydrocolloids act as essential additives, as thickeners, stabilizers, and gelling agents. Alginates, primarily extracted from brown seaweeds, are valued for their ability to form gels in the presence of calcium ions, making them ideal for use in products like dairy desserts, sauces, and bakery fillings (Lomartire and Gonçalves 2023). Agar, also derived from red seaweeds, forms strong and stable gels at relatively low concentrations, making it suitable for confectionery, desserts, and microbiological culture media applications. Carrageenan, obtained from red seaweeds, is widely used for its gelling, thickening, and stabilizing properties in various food products, including dairy, meat, and processed foods. Beyond the food industry, these seaweed-derived hydrocolloids find applications in pharmaceuticals, where they are utilized as excipients and drug delivery systems, and in cosmetics, where they serve as thickeners, emulsifiers, and moisturizers in various formulations (Lomartire and Gonçalves 2023). The versatility and functionality of alginates, agar, and carrageenan highlight the importance of seaweeds as valuable sources of hydrocolloids with widespread industrial applications.

4.3.2 Bioactive compounds from seaweeds

Seaweeds contain bioactive compounds like phycocyanins and fucoidans, known for their antioxidant, anti-inflammatory, and anticancer properties, contributing to their potential applications in pharmaceuticals, functional foods, and nutraceuticals.

4.3.2.1 Phlorotannins

Seaweeds are reservoirs of bioactive compounds, among which phlorotannins stand out for their remarkable antioxidant properties. These polyphenolic compounds are found abundantly in various species of seaweeds, contributing to their ability to scavenge free radicals and protect cells from oxidative damage. Phlorotannins have garnered significant attention due to their potential health benefits, including anti-inflammatory, anticancer, and antidiabetic properties (Perez-Vazquez et al. 2023). As potent antioxidants, they are crucial in maintaining cellular health and reducing the risk of chronic diseases. The presence of phlorotannins in seaweeds underscores their significance as valuable sources of natural antioxidants, with promising applications in pharmaceuticals, functional foods, and dietary supplements to promote human health and wellbeing (Gisbert et al. 2023).

4.3.2.2 Fucoidans

Seaweeds boast a rich repertoire of bioactive compounds, including fucoidans, renowned for their potent anti-inflammatory and anticancer activities (Rengasamy et al. 2020). These sulfated polysaccharides, prevalent in various species of seaweeds, possess remarkable biological properties that make them valuable in pharmaceutical and medical applications. Fucoidans exhibit anti-inflammatory effects by modulating immune responses and inhibiting inflammatory pathways, offering potential therapeutic benefits for arthritis and inflammatory bowel diseases. Additionally, their anticancer properties involve inducing apoptosis, inhibiting angiogenesis, and suppressing tumor cell proliferation, making them promising candidates for cancer prevention and treatment strategies. The multifaceted actions of fucoidans highlight their importance as natural compounds with significant health-promoting potential, positioning seaweeds as valuable resources for developing novel therapeutics and functional foods (Rengasamy et al. 2020).

4.3.2.3 Laminarins

Seaweeds are abundant sources of bioactive compounds, including laminarins, which exhibit notable immunomodulatory effects. Laminarins, a type of β-glucan polysaccharide found in various seaweed species, have garnered attention for their ability to regulate the immune system (Ha et al. 2024). These compounds can modulate immune responses by enhancing the activity of immune cells such as macrophages, natural killer cells, and dendritic cells. Laminarins stimulate the production of cytokines and other signaling molecules involved in immune regulation, promoting innate and adaptive immune functions. Their immunomodulatory properties have implications for health and disease, including potential applications in boosting immune function, combating infections, and managing immune-related disorders (Ha et al. 2024). The presence of laminarins in seaweeds underscores their significance as natural immunomodulators with promising therapeutic potential in immune health and disease management.

4.3.3 Seaweed-based biofuels

Seaweeds are increasingly being recognized as a sustainable and viable source for biofuel production. Their rapid growth rates and ability to thrive in marine environments without needing arable land or freshwater make them a highly eco-friendly alternative to traditional biofuel crops like corn or sugarcane. Unlike terrestrial biofuel sources, seaweeds do not compete with food crops for land and water resources, making them particularly valuable in addressing energy and food security challenges. Additionally,

seaweeds can absorb carbon dioxide from the atmosphere, helping mitigate the effects of climate change by acting as a carbon sink (Nakhate and Van Der Meer 2021). Seaweed-based biofuels are primarily produced through anaerobic digestion, fermentation, and pyrolysis processes. These processes convert the carbohydrate-rich biomass of seaweeds, including species such as *Saccharina* and *Laminaria*, into biogas, bioethanol, and biodiesel. The bioethanol production process, for instance, exploits the high carbohydrate content (e.g., alginate, mannitol, and laminarin) present in certain seaweeds, making it a promising source of renewable energy (Kumar et al. 2022). Research is ongoing to improve these processes' efficiency and make seaweed biofuels commercially competitive with fossil fuels and other bioenergy sources. Commercial utilization of seaweed biofuels is still in its early stages, but several pilot projects and collaborations have been initiated worldwide. Countries like Japan, Norway, and South Korea are exploring large-scale seaweed farming for biofuel production, taking advantage of their extensive coastlines. In addition to direct energy production, seaweed biomass can be used in a circular economy model. The by-products of biofuel production, such as proteins and minerals, can be utilized as animal feed, fertilizers, or bioplastics, further enhancing the commercial viability of seaweed cultivation (Nakhate and Van Der Meer 2021; Kumar et al. 2022). As seaweed farming requires fewer inputs than land-based crops, it offers a low-cost, low-impact solution for sustainable biofuel production, with potential to scale up in the coming decades as technological advancements continue reducing costs.

5 Technological processes in high-value product production

The production of high-value products from seafood involves various technological processes, each contributing to the efficiency and quality of the final product.

5.1 Extraction and purification techniques

Efficient extraction and purification techniques are crucial for isolating high-value compounds from seafood resources.

5.1.1 Solvent extraction

Solvent extraction is a traditional method widely used for extracting oils and lipid-rich compounds from seafood, such as fish and shellfish. In this process, organic solvents like hexane or ethanol dissolve and extract lipids from the raw material (Caruso et al. 2020). The solvent extracts are then separated from the solid residue through filtration or centrifugation, followed by evaporation to recover the solvent and obtain the desired oil or lipid fraction (Caruso et al. 2020). Solvent extraction is known for its simplicity and effectiveness in obtaining oils from seafood, making it suitable for large-scale industrial applications. However, concerns about solvent residues and environmental impact have led to the development of alternative methods (Caruso et al. 2020).

5.1.2 Supercritical fluid extraction

Supercritical fluid extraction (SFE) is an advanced technique that utilizes supercritical fluids, typically carbon dioxide (CO_2), at specific temperature and pressure conditions to extract high-purity compounds from seafood (Wang et al. 2021). In SFE, CO_2 is pressurized above its critical point to become a supercritical fluid exhibiting both liquid and gas-like properties. This supercritical CO_2 is then used as a solvent to selectively extract target compounds, such as omega-3 fatty acids, without leaving behind solvent residues (Wang et al. 2021). SFE offers advantages such as high selectivity, low environmental impact, and the ability to produce extracts with high purity and quality. It is particularly suitable for extracting thermally sensitive compounds from delicate seafood matrices (Wang et al. 2021).

5.1.3 Enzymatic hydrolysis

Enzymatic hydrolysis involves using enzymes to break down complex biomolecules, such as proteins, into smaller peptides and hydrolysates with specific bioactivities (Cruz-Casas et al. 2021). In seafood, enzymatic hydrolysis is commonly used to produce protein hydrolysates from fish or shellfish proteins. Proteolytic enzymes, such as proteases, are added to the raw material to catalyze the hydrolysis of proteins into peptides of varying sizes. The resulting protein hydrolysates exhibit enhanced solubility, digestibility, and bioavailability compared to the original proteins (Cruz-Casas et al. 2021). Depending on the source material and enzyme used, they may possess bioactive properties, such as antioxidant, antimicrobial, or antihypertensive activities. Enzymatic hydrolysis offers precise control over the degree of hydrolysis and allows the production of tailored peptide mixtures with specific functional characteristics.

5.1.4 Membrane filtration

Membrane filtration techniques are used to separate and concentrate bioactive compounds from seafood extracts based on differences in molecular size, shape, and charge (Abhari and Mousavi Khaneghah 2021). Common membrane filtration methods include ultrafiltration, nanofiltration, and reverse osmosis. These techniques involve passing the seafood extract through a semipermeable membrane, which selectively allows certain molecules to pass through while retaining others (Abhari and Mousavi Khaneghah 2021). Ultrafiltration separates proteins and peptides from larger molecules and impurities based on their molecular weight. Nanofiltration and reverse osmosis further concentrate and purify the desired compounds by removing smaller molecules and ions. Membrane filtration offers advantages such as mild processing conditions, scalability, and the ability to retain the bioactivity of the target compounds. It is often combined with other extraction methods to achieve higher purity and concentration of bioactive compounds from seafood resources.

5.2 Fermentation and biotechnological applications

Fermentation processes harnessing the metabolic activities of microorganisms are instrumental in improving the bioavailability and functionality of seafood-derived products (Adegoke and Tahergorabi 2021). Through fermentation, beneficial microorganisms such as bacteria, yeasts, and molds transform raw seafood materials into value-added products with enhanced nutritional profiles, flavors, and textures. Further, fermentation can be employed to produce fermented fish sauces, fish pastes, and seafood-based condiments that not only improve the taste and aroma but also increase the digestibility and bioavailability of nutrients (Adegoke and Tahergorabi 2021). Moreover, fermentation can lead to the synthesis of bioactive compounds such as peptides, organic acids, and enzymes, which contribute to the health-promoting properties of fermented seafood products. In addition to fermentation, biotechnology plays a pivotal role in advancing seafood processing and production. Genetic engineering techniques are utilized to develop genetically modified organisms (GMOs) with enhanced traits, such as increased yield of desired compounds, improved resistance to pathogens, and enhanced nutritional content (Adegoke and Tahergorabi 2021).

5.3 Nanoencapsulation

Nanoencapsulation technology involves encapsulating bioactive compounds within nanoscale carriers to protect them from degradation, improve their stability, and enhance their delivery and absorption in the body. This technology offers numerous benefits for incorporating sensitive compounds, such as omega-3 fatty acids, into various food and pharmaceutical products. By encapsulating these compounds within nano-sized particles, nanoencapsulation shields them from environmental factors, such as oxygen, light, and moisture, which can cause degradation and loss of potency (Pateiro et al. 2021). Additionally, nanoencapsulation facilitates controlled release of the encapsulated compounds, ensuring optimal delivery to the target site in the body and maximizing their bioavailability. This technology enables the development of functional foods, dietary supplements, and pharmaceutical formulations with enhanced efficacy and consumer acceptability. Nanoencapsulation holds great promise for improving the stability and effectiveness of bioactive compounds, paving the way for the development of innovative products with enhanced health benefits and therapeutic potential.

5.4 Waste Reduction

Waste reduction is a key objective in sustainable seafood processing, as it directly impacts the industry's environmental footprint and enhances the efficient use of marine resources. Traditionally, 50-70% of fish biomass, including heads, bones, skin, and viscera, is considered waste during seafood processing. However, with technological advancements and innovative approaches, these by-products can now be converted into high-value products such as fish oils, proteins, and collagen, significantly reducing the waste generated (Nakhate and Van Der Meer 2021). For instance, FPH, produced by hydrolyzing fish by-products, has reduced processing waste by 40-60% and provided functional ingredients used in the food and nutraceutical industries (Pacheco-Aguilar et al. 2021).

6 Reducing environmental impact

In addition to reducing waste, sustainable seafood processing minimizes environmental impact by lowering greenhouse gas emissions and conserving water. By optimizing resource utilization and adopting energy-efficient technologies, seafood processing facilities have reduced their overall carbon footprint by up to 30%. Water consumption, often a major environmental concern, can be reduced by 20-50% through closed-loop systems that recycle and purify water used during processing (Shavandi et al. 2019). Processing plants worldwide increasingly adopt these systems to conserve resources and improve environmental sustainability.

7 Case Study – Iceland's circular economy model

A notable example of waste reduction in seafood processing is Iceland, where strict regulations mandate that almost 100% of the fish biomass be utilized. This practice has reduced waste while increasing the economic value derived from fish by-products. By converting fish waste into valuable products such as fishmeal, oils, and fertilizers, Iceland has established a circular economy model that minimizes environmental impact and maximizes resource use (Kumar et al. 2022).

8 Challenges and future directions

Unlocking the full potential of seafood resources requires addressing key challenges while advancing sustainable practices. Sustainable harvesting is critical, necessitating strategies like quotas, seasonal closures, and marine protected areas to prevent overfishing and promote species recovery. Collaborative efforts between governments, industry, and conservation groups are essential to harmonize sustainable fisheries management. Simultaneously, technological advancements can maximize resource utilization by optimizing extraction methods and reducing waste. Innovations like supercritical fluid extraction and membrane filtration can enhance the industry's capacity to derive high-value compounds while minimizing environmental impacts. Strong regulatory frameworks are needed to ensure product safety, quality control, and market transparency. Harmonized regulations across jurisdictions, informed by science and collaboration among stakeholders, can foster responsible seafood utilization. A

coordinated, multidisciplinary approach will be vital for ensuring the seafood industry delivers nutritious and sustainable products for future generations.

Conclusion

The utilization of seafood resources to produce high-value products represents a promising pathway toward enhancing the sustainability and profitability of the seafood industry. Through the strategic application of advanced technologies and a steadfast commitment to sustainable practices, stakeholders in the seafood sector can unlock the full potential of marine resources, contributing to food security, economic prosperity, and environmental conservation. Adopting advanced technologies, such as nanoencapsulation, fermentation, and biotechnological approaches, offers opportunities to extract, purify, and enhance the bioavailability of valuable compounds from seafood. Nanoencapsulation, in particular, provides a means to protect sensitive bioactive compounds, such as omega-3 fatty acids, from degradation and enhance their delivery and absorption in the body, thereby enriching the functionality of food and pharmaceutical products. Furthermore, embracing sustainable practices is paramount to ensuring the long-term viability of seafood resources. Sustainable harvesting methods, informed by scientific research and guided by robust regulatory frameworks, are essential to prevent overfishing and mitigate environmental degradation. By prioritizing ecosystem health and biodiversity conservation, the seafood industry can safeguard marine ecosystems for future generations while maintaining a thriving seafood supply chain. Moreover, producing high-value products from seafood by-products and waste streams offers opportunities to minimize environmental pollution and promote circular economy principles. Through innovative approaches such as fermentation and enzymatic hydrolysis, valuable compounds can be extracted from seafood by-products, transforming waste into useful resources and reducing reliance on virgin raw materials. As the global demand for high-value seafood products escalates, the seafood industry faces challenges and opportunities. Technological advancements, regulatory frameworks, and collaborative initiatives will be crucial in navigating these complexities and realizing the full potential of seafood resources. By embracing innovation, fostering sustainable practices, and prioritizing environmental stewardship, the seafood industry can thrive in a rapidly evolving market landscape while contributing to the wellbeing of ecosystems and communities worldwide.

In summary, this review underscores the significance of using seafood resources for high-value products in the context of sustainability and economic development. By harnessing the potential of marine resources responsibly and innovatively, the seafood industry can play a pivotal role in addressing global challenges while creating opportunities for growth and prosperity.

References

Abhari, K., & Mousavi Khaneghah, A. (2020). Alternative extraction techniques to obtain, isolate and purify proteins and bioactive from aquaculture and by-products. *Advances in food and nutrition research*, *92*, 35–52. https://doi.org/10.1016/bs.afnr.2019.12.004.

Adegoke, S. C., & Tahergorabi, R. (2021). Utilization of seafood-processing by-products for the development of value-added food products. In R. Bhat (Ed.), *Valorization of agri-food wastes and by-products* (pp. 537-559). Academic Press. https://doi.org/10.1016/B978-0-12-824044-1.00012-X

Akram, S., Muzaffar, A., & Farooq, Q. (2023). Persistent organic pollutant fragile effects, their sources, transportation and state of the art technologies. *International Journal of Agriculture and Environment*, *2*(2), 26-45.

Ashraf, S. A., Adnan, M., Patel, M., Siddiqui, A. J., Sachidanandan, M., Snoussi, M., & Hadi, S. (2020). Fish-based bioactives as potent nutraceuticals: Exploring the therapeutic perspective of sustainable food from the sea. *Marine Drugs*, *18*(5), 265.

Awuchi, C. G., Chukwu, C. N., Iyiola, A. O., Noreen, S., Morya, S., Adeleye, A. O., ... & Okpala, C. O. R. (2022). Bioactive compounds and therapeutics from fish: Revisiting their suitability in functional foods to enhance human wellbeing. *BioMed Research International*, *2022*(1), 3661866.

Azelee, N. I. W., Dahiya, D., Ayothiraman, S., Noor, N. M., Rasid, Z. I. A., et al. (2023). Sustainable valorization approaches on crustacean wastes for the extraction of chitin, bioactive compounds, and their applications: A review. *International Journal of Biological Macromolecules*, *253*(Pt 2), 126492. https://doi.org/10.1016/j.ijbiomac.2023.126492

Bhatt, P., Joshi, S., Bayram, G. M. U., Khati, P., & Simsek, H. (2023). Developments and application of chitosan-based adsorbents for wastewater treatments. *Environmental Research*, *226*, 115530.

Bruno, S. F., Ekorong, F. J. A. A., Karkal, S. S., Cathrine, M. S. B., & Kudre, T. G. (2019). Green and innovative techniques for recovery of valuable compounds from seafood by-products and discards: A review. *Trends in Food Science & Technology*, *85*, 10-22.

Burbridge, P., Hendrick, V., & Rosenthal, H. (2001). Social and economic policy issues relevant to marine aquaculture. *Journal of Applied Ichthyology*, *17*(4), 194-206.

Cadena, E., Kocak, O., Dewulf, J., Iñarra, B., Bald, C., et al. (2024). Valorization of seafood side-streams through the design of new holistic value chains: WaSeaBi Project. *Sustainability*, *16*(5), 1846.

Caruso, G., Floris, R., Serangeli, C., & Di Paola, L. (2020). Fishery wastes as a yet undiscovered treasure from the sea: Biomolecules sources, extraction methods and valorization. *Marine Drugs, 18*(12), 622.

Cooney, R., de Sousa, D. B., Fernández-Ríos, A., Mellett, S., Rowan, N., et al. (2023). A circular economy framework for seafood waste valorization to meet challenges and opportunities for intensive production and sustainability. *Journal of Cleaner Production, 392*, 136283.

Cruz-Casas, D. E., Aguilar, C. N., Ascacio-Valdés, J. A., Rodríguez-Herrera, R., Chávez-González, M. L., & Flores-Gallegos, A. C. (2021). Enzymatic hydrolysis and microbial fermentation: The most favorable biotechnological methods for the release of bioactive peptides. *Food Chemistry: Molecular Sciences, 3*, 100047.

D'Orazio, N., Gammone, M. A., Gemello, E., De Girolamo, M., Cusenza, S., & Riccioni, G. (2012). Marine bioactives: Pharmacological properties and potential applications against inflammatory diseases. *Marine Drugs, 10*(4), 812-833.

Dhandwal, A., Bashir, O., Malik, T., Salve, R. V., Dash, K. K., et al. (2024). Sustainable microalgal biomass as a potential functional food and its applications in food industry: A comprehensive review. *Environmental Science and Pollution Research, 1*-19. https://doi.org/10.1007/s11356-024-33431-6

Edo, G. I., Samuel, P. O., Nwachukwu, S. C., Ikpekoro, V. O., Promise, O., Oghenegueke, O., Ongulu, J., Otunuya, C. F., Rapheal, O. A., Ajokpaoghene, M. O., Okolie, M. C., & Ajakaye, R. S. (2024). A review on the biological and bioactive components of Cyperus esculentus L.: insight on food, health and nutrition. *Journal of the science of food and agriculture*, 10.1002/jsfa.13570. Advance online publication. https://doi.org/10.1002/jsfa.13570

Farmery, A. K., Alexander, K., Anderson, K., Blanchard, J. L., Carter, C. G., et al. (2022). Food for all: Designing sustainable and secure future seafood systems. *Reviews in Fish Biology and Fisheries, 32*(1), 101-121.

Fernandes, S. S., Coelho, M. S., & de las Mercedes Salas-Mellado, M. (2019). Bioactive compounds as ingredients of functional foods: Polyphenols, carotenoids, peptides from animal and plant sources. In M. R. S. Campos (Ed.), *Bioactive compounds* (pp. 129-142). Woodhead Publishing.

Gisbert, M., Franco, D., Sineiro, J., & Moreira, R. (2023). Antioxidant and antidiabetic properties of phlorotannins from *Ascophyllum nodosum* seaweed extracts. *Molecules, 28*(13), 4937.

Ha, H. A., Aloufi, A. S., & Parveen, B. (2024). Essential bioactive competence of laminarin (β-glucan)/laminaran extracted from *Padina tetrastromatica* and *Sargassum cinereum* biomass. *Environmental Research, 252*, 118836.

Haider, A., Khan, S., Iqbal, D. N., Shrahili, M., Haider, S., et al. (2024). Advances in chitosan-based drug delivery systems: A comprehensive review for therapeutic applications. *European Polymer Journal, 210*, 112983. https://doi.org/10.1016/j.eurpolymj.2024.112983.

Hamed, I., Özogul, F., & Regenstein, J. M. (2016). Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. *Trends in Food Science & Technology, 48*, 40-50.

Islam, N., Hoque, M., & Taharat, S. F. (2023). Recent advances in extraction of chitin and chitosan. *World Journal of Microbiology and Biotechnology, 39*(1), 28.

Jiménez-González, C., Agrasar, A. M. T., Mallo, F., Rúa, M. L., & Fucinos, C. (2023). Red seaweed proteins: Valuable marine-origin compounds with encouraging applications. *Algal Research, 75*, 103262. https://doi.org/10.1016/j.algal.2023.103262

Kalasariya, H. S., Maya-Ramírez, C. E., Cotas, J., & Pereira, L. (2024). Cosmeceutical significance of seaweed: A focus on carbohydrates and peptides in skin applications. *Phycology, 4*(2), 276-313.

Kankariya, Y., & Chatterjee, B. (2023). Biomedical application of chitosan and chitosan derivatives: A comprehensive review. *Current Pharmaceutical Design, 29*(17), 1311-1325.

Khursheed, M., Ghelani, H., Jan, R. K., & Adrian, T. E. (2023). Anti-inflammatory effects of bioactive compounds from seaweeds, bryozoans, jellyfish, shellfish, and peanut worms. *Marine Drugs, 21*(10), 524.

Kumar, P., Yadav, N., Kumar, S., Bahadur, J., & Chauhan, P. K. (2022). Seaweed-based biofuels: Current status and prospects. *Bioresource Technology, 348*, 126618. https://doi.org/10.1016/j.biortech.2022.126618

Lomartire, S., & Gonçalves, A. M. (2023). Algal phycocolloids: Bioactivities and pharmaceutical applications. *Marine Drugs, 21*(7), 384.

López-Hortas, L., Flórez-Fernández, N., Torres, M. D., Ferreira-Anta, T., Casas, M. P., et al. (2021). Applying seaweed compounds in cosmetics, cosmeceuticals, and nutricosmetics. *Marine Drugs, 19*(10), 552.

Luo, J., Frank, D., & Arcot, J. (2024). Creating alternative seafood flavour from non-animal ingredients: A review of key flavour molecules relevant to seafood. *Food Chemistry, X*, 101400.

Magbanua, T. O., & Ragaza, J. A. (2024). Selected dietary plant-based proteins for growth and health response of Nile tilapia *Oreochromis niloticus*. *Aquaculture and Fisheries*, *9*(1), 3-19.

Maneein, S., Milledge, J. J., Nielsen, B. V., & Harvey, P. J. (2018). A review of seaweed pre-treatment methods for enhanced biofuel production by anaerobic digestion or fermentation. *Fermentation*, *4*(4), 100.

Martins, T. R., de Andrade, M. R., de Alcantara, M., Martins, I. B. A., Sant'Anna, L. J., et al. (2024). Exploring the consumer perception of seafood: A look at Brazilians. *International Journal of Gastronomy and Food Science*, *36*, 100938.

Menon, V. V., & Lele, S. S. (2015). Nutraceuticals and bioactive compounds from seafood processing waste. In S. K. Kim (Eds.), *Springer handbook of marine biotechnology* (pp. 1405-1425). Springer Handbooks. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-53971-8_65

Milledge, J. J., Smith, B., Dyer, P. W., & Harvey, P. (2014). Macroalgae-derived biofuel: A review of methods of energy extraction from seaweed biomass. *Energies*, *7*(11), 7194-7222.

Mohiuddin, A. (2019). Skin aging and modern age anti-aging strategies. *International Journal of Clinical Dermatology & Research*, *7*, 209-240.

Mutalipassi, M., Esposito, R., Ruocco, N., Viel, T., Costantini, M., & Zupo, V. (2021). Bioactive compounds of nutraceutical value from fishery and aquaculture discards. *Foods*, *10*(7), 1495.

Naghdi, S., Rezaei, M., Heidari, M. G., Tahergorabi, R., Lorenzo, J. M., & Mirzaei, F. (2024). Insights into fishery by-product application in aquatic feed and food: A review. *Aquaculture International*,*32*, 5851-5910. https://doi.org/10.1007/s10499-024-01447-x

Nakhate, P., & Van Der Meer, Y. (2021). A systematic review on seaweed functionality: A sustainable bio-based material. *Sustainability*, *13*(11), 6174.

Ozogul, F., Cagalj, M., Šimat, V., Ozogul, Y., Tkaczewska, J., et al. (2021). Recent developments in valorization of bioactive ingredients in discard/seafood processing by-products. *Trends in Food Science & Technology*, *116*, 559-582.

Pacheco-Aguilar, R., Gaxiola-Cortés, M. G., Velázquez-Gutiérrez, M. C., Hernández-Ledesma, B., & Montes-Montes, R. (2021). Seaweed bioactive compounds in animal and human health and food applications: Potential uses of seaweeds in sustainable food production systems. *Journal of Applied Phycology*, *33*(3), 1819–1832. https://doi.org/10.1007/s10811-020-02268-9

Pateiro, M., Gómez, B., Munekata, P. E., Barba, F. J., Putnik, P., Kovačević, D. B., & Lorenzo, J. M. (2021). Nanoencapsulation of promising bioactive compounds to improve their absorption, stability, functionality, and the appearance of the final food products. *Molecules*, *26*(6), 1547.

Pereira, L. (2018). Seaweeds as a source of bioactive substances and skin care therapy: Cosmeceuticals, algotherapy, and thalassotherapy. *Cosmetics*, *5*(4), 68.

Perez-Vazquez, A., Carpena, M., Barciela, P., Cassani, L., Simal-Gandara, J., & Prieto, M. A. (2023). Pressurized liquid extraction for the recovery of bioactive compounds from seaweeds for food industry application: A review. *Antioxidants*, *12*(3), 612.

Power, A. M., Johnson, M. P., Marine, D. P., & Kelly, T. C. (2013). Seaweed as a resource for biofuels: An overview of technologies and challenges. *Marine Biotechnology*, *15*(4), 431-445. https://doi.org/10.1007/s10126-013-9502-7

Rengasamy, K. R., Mahomoodally, M. F., Aumeeruddy, M. Z., Zengin, G., Xiao, J., & Kim, D. H. (2020). Bioactive compounds in seaweeds: An overview of their biological properties and safety. *Food and Chemical Toxicology*, *135*, 111013.

Román-Doval, R., Torres-Arellanes, S. P., Tenorio-Barajas, A. Y., Gómez-Sánchez, A., & Valencia-Lazcano, A. A. (2023). Chitosan: Properties and its application in agriculture in context of molecular weight. *Polymers*, *15*(13), 2867.

Santos, H. O., Price, J. C., & Bueno, A. A. (2020). Beyond fish oil supplementation: The effects of alternative plant sources of omega-3 polyunsaturated fatty acids upon lipid indexes and cardiometabolic biomarkers: An overview. *Nutrients*, *12*(10), 3159.

Shavandi, A., Hu, Z., Teh, S., Zhao, J., Carne, A., Bekhit, A. E.-D. A., & Bekhit, A. A. (2019). Seaweed polysaccharides as prebiotics, wound dressings, and drug delivery systems: A review. *Journal of Functional Foods*, 64, 103587. https://doi.org/10.1016/j.jff.2019.103587

Siahaan, E. A., Agusman, Pangestuti, R., Shin, K. H., & Kim, S. K. (2022). Potential cosmetic active ingredients derived from marine by-products. *Marine Drugs*, *20*(12), 734.

Singh, S., Negi, T., Sagar, N. A., Kumar, Y., Tarafdar, A., et al. (2022). Sustainable processes for treatment and management of seafood solid waste. *Science of the Total Environment*, *817*, 152951.

Stetkiewicz, S., Norman, R. A., Allison, E. H., Andrew, N. L., Ara, G., et al. (2022). Seafood in food security: A call for bridging the terrestrial-aquatic divide. *Frontiers in Sustainable Food Systems*, *5*, 703152.

Sun, W., Shahrajabian, M. H., Petropoulos, S. A., & Shahrajabian, N. (2023). Developing sustainable agriculture systems in medicinal and aromatic plant production by using chitosan and chitin-based biostimulants. *Plants*, *12*(13), 2469.

Tacon, A. G., Coelho, R. T., Levy, J., Machado, T. M., Neiva, C. R., & Lemos, D. (2024). Annotated bibliography of selected papers dealing with the health benefits and risks of fish and seafood consumption. *Reviews in Fisheries Science & Aquaculture*, *32*(2), 211-305.

Tas, A. A., & El, S. N. (2024). The food system with optimum nutrition vision. In A. A. Tas, & S. Nehir El (Eds.), *Smart Food Industry: The Blockchain for Sustainable Engineering* (pp. 3-24). CRC Press.

Välimaa, A. L., Mäkinen, S., Mattila, P., Marnila, P., Pihlanto, A., Mäki, M., & Hiidenhovi, J. (2019). Fish and fish side streams are valuable sources of high-value components. *Food Quality and Safety*, *3*(4), 209-226.

Venugopal, V. (2022). Green processing of seafood waste biomass towards blue economy. *Current Research in Environmental Sustainability*, *4*, 100164.

Veríssimo, N. V., Mussagy, C. U., Oshiro, A. A., Mendonça, C. M. N., de Carvalho Santos-Ebinuma, V., et al. (2021). From green to blue economy: Marine biorefineries for a sustainable ocean-based economy. *Green Chemistry*, *23*(23), 9377-9400.

Wang, Q., Wang, X., & Feng, Y. (2023). Chitosan hydrogel as tissue engineering scaffolds for vascular regeneration applications. *Gels*, *9*(5), 373.

Wang, W., Rao, L., Wu, X., Wang, Y., Zhao, L., & Liao, X. (2021). Supercritical carbon dioxide applications in food processing. *Food Engineering Reviews*, *13*, 570-591. https://doi.org/10.1007/s12393-020-09270-9

Xing, X., Han, Y., & Cheng, H. (2023). Biomedical applications of chitosan/silk fibroin composites: A review. *International Journal of Biological Macromolecules*, *240*, 124407. https://doi.org/10.1016/j.ijbiomac.2023.124407

Yang, L., Shu, T., Wang, K., Yuan, Z., & Zhang, X. (2023). Roles of marine shellfish proteins with high contents of angiotensin-converting enzyme (ACE)-binding peptides in nutrition support for hypertension. *Applied Sciences*, *13*(8), 4654.

Zhang, J., Ahmmed, M. K., Regenstein, J. M., & Wu, H. (2024). Recent advances of recycling proteins from seafood by-products: Industrial applications, challenges, and breakthroughs. *Trends in Food Science & Technology*, *149*, 104533. https://doi.org/10.1016/j.tifs.2024.104533

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Genome Editing Technologies towards Tomato Improvement: Recent Advances and Future Perspectives

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ABSTRACT

Tomato (*Solanum lycopersicon* L.) is the world's second major vegetable crop and a superior model plant for studies on fruit biology. However, the changing climatic conditions are hugely impacting the yield and quality of tomato. CRISPR/Cas9 technology has been widely used in tomato breeding for enhanced disease resistance, herbicide tolerance, domestication and urban farming of wild tomato, and improved fruit yield and quality. Furthermore, new and advanced editing systems like Cas12a, Cas12b, base editing, and prime editing have been recently applied for high-precision tomato improvement. CRISPR variants, PAM-less genome editing, advanced transformation protocols, and gene delivery systems have played a critical role in fast breeding. This review offers an informative summary of recent progress in various genome editing methods and applications for improving tomatoes. It also focuses on critical issues, regulatory concerns, and prospects of genome editing platforms to improve tomato and allied crops.

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1 Introduction

Tomato (*Solanum lycopersicon* L.) is the second most crucial agronomic crop grown worldwide. With global production of more than 182 million metric tons in approximately 50 million hectares, tomato contributed to a trade value of 19.5 billion USD in 2018 (Khan et al. 2021). In addition to the bioactive compounds, it has significant physiological properties like anti-allergenic, antithrombotic, anti-inflammatory, antioxidant, antimicrobial, and vasodilatory (Kumar et al. 2022). However, many environmental factors highly affect tomato production, affecting global food security (Mishra et al. 2021). Over the last few decades, research has been focused on breeding toward tolerance to environmental stresses. It has resulted in identifying multiple stress-tolerant genes that can be used for tomato breeding programs (Gupta et al. 2022). Among the available tools, genetic engineering is the most important tool for crop improvement. Technological advances in functional genomics have made it possible to introduce multiple stress-responsive genes into plants and make them climate resilient. Although transgenic breeding has been overcome, most of the bottlenecks related to genetic recombination faced by traditional approaches, public concerns, and expensive regulatory processes continue to impede commercialization.

While the acceptance of gene-modified crops is debatable, genome editing techniques via sequence-specific nucleases (SSNs) have the potential to resolve these limitations. They could help develop multi-trait-modified crops. The SSNs cleave the double-stranded DNA at specific sites, which is healed through the cell's endogenous repair mechanism, resulting in a precise genetic mutation. Since 1988, when the first tobacco gene targeting experiment was conducted, multiple gene targeting tools, including the meganucleases, ZFNs (Zinc Finger Nucleases), TALENs (Transcription Activator Like Effector Nucleases), and the CRISPR system, have been developed and utilized in the improvement of multiple crop varieties (Ahmar et al. 2020). Among others, the CRISPR/Cas system has wide acceptance in the scientific community due to its simplicity, non-requirement of complex protein chemistry, and the ability for simultaneous introduction of double-stranded breaks (DSBs) at multiple sites (Mishra et al. 2019).

Since its implementation in 2014, gene modification has enormously aided precision breeding in tomato (Brooks et al. 2014). More insight into the intricacies of tomato fruit ripening genes has been reevaluated using targeted mutagenesis gene-editing machinery (Wang et al. 2019; Gao et al. 2020). CRISPR/Cas9 generated knockout mutations of numerous master regulators of tomato ripening have resulted in reduced ripening inhibition phenotype as compared to their naturally occurring mutations or RNAi lines, indicating that the fruit ripening regulation network is more intriguing than previous knowledge (Wang et al. 2021). Genome editing technologies significantly enhance fruit productivity and quality, stress resistance, and domestication (Xu et al. 2019; Kwon et al. 2020). Most recently, the base and prime editors have greatly improved the editing efficacy, expanding the scope for sustainable agricultural development (Mishra et al. 2019; Anzalone et al. 2020). In other words, CRISPR/Cas9 has revolutionized the basic research in plants for crop improvement. This review attempts to summarize the genome editing platforms and applications of CRISPR/Cas-based genome editing tools in tomato improvement, as well as the future perspectives of the editing approaches concerning basic research and crop improvement.

2 Advanced gene editing systems applied in tomato

Genome editing helps create a DNA mutation by deletions, insertions, or base substitutions in the target sequences. Such modifications can be performed using editing tools like TALENs, ZFNs, and CRISPR/Cas9 system (Wei and Li 2023). ZFNs and TALENs are first-generation genome editing tools that are costly, time-consuming, and difficult to design. In contrast, the CRISPR/Cas9 system is inexpensive, time-saving, and simple to build. Over time, several new Cas9 variants, such as SacCas9, NmCas9, and StCas9, have been discovered, which helped to improve target specificity and editing efficiency and decreased the off-target cleavage of this editing platform (D'Ambrosio et al. 2018).

2.1 CRISPR/Cas9

The discovery of CRISPR/Cas9 genome modification in 2012 has dramatically altered the field of plant science (Jinek et al. 2012). This tool is based upon the bacterial-acquired immune system acting upon exogenous invading genes or factors. These foreign gene fragments are retained as memory as spacer sequences comprising a CRISPR array (Koonin et al. 2017). The Cas protein and the spacer sequence are being used as surveillance systems to recognize and degrade foreign DNA or RNA. Adaptation, expression, and interference are three major steps in this process. Cas1 and Cas2 mediate the adaptation step to integrate foreign DNA fragments into the CRISPR locus of the host, followed by the production and maturation of transcribed gRNA by expression step. The last interference phase is completed by cleaving invaded DNA by Cas proteins, which are complex with mature gRNA (Monn et al. 2019).

The two essential elements of the CRISPR/Cas9 system necessary for genome modification are an adaptable single-stranded RNA (sgRNA) and the DNA endonuclease (Cas9) protein from *Streptococcus pyogenes* (Figure 1a). A sizeable globular recognition lobe and a tiny nuclease lobe make up the bilobed Cas9 protein with two nuclease domains, RuvC and HNH, and

these two domains are specific for each cut in a particular DNA strand. Effective editing requires a PAM (Protospacer adjacent motif) sequence close to the target location. For example, Cas9, derived from *S. pyogenes*, recognizes 5'-NGG-3' as PAM. Various types of Cas proteins have been discovered to overcome this limitation. Furthermore, Cas 12 identifies the sequence 5'-TTTN-3' or 5'-TTN-3' as a PAM. However, additional Cas9 variations (VQR, EQR, VRER) have been created to recognize different PAMs. As a result, there will be more opportunities to alter any target sequence in the genome (Asmamaw and Zawdie 2021).

2.2 CRISPR/Cas12a

CRISPR/Cas12a, formerly named/called CRISPR/Cpf1 (Figure1a), derived from *Prevotella* and *Francisella* 1, is one of the advanced forms of CRISPR used in plant gene modification (Endo et al. 2016). A significant difference between CRISPR/Cas9 and CRISPR/Cas12a is its location and reorganization sequence of PAM. Various monocot and dicot plants have been modified/improved using CRISPR/Cas12a. CRISPR/Cas9 needs a PAM sequence that is rich in G at the 3' end of the target sequence (5'-NGG-3'), whereas in the case of CRISPR/Cas12a, it targets the T-rich PAM sequence (5'-TTTN-3' or 5'-TTN-3') at the 5'end which results in high efficiency for cleavage (Zetsche et al. 2015). CRISPR/Cas12a functions without a trace RNA for cleavage. The complex of Cas12a-crRNA can cleave the targeted DNA efficiently. The crRNA containing the repeat of 40-45 nucleotides long along with the spacer can edit the genome more effectively when compared with the sgRNA of CRISPR/Cas9, which contains nearly 100 nucleotides (Zetsche et al. 2015). Cas12a includes dual enzymatic activity like RNAase and nuclease. The RNAase activity helps process pre-crRNA into crRNA, and the nuclease activity helps cleavage dsDNA. CRISPR/Cas12a can generate multiple crRNAs by the involvement of a single promoter, which makes it simpler when compared with CRISPR/Cas9. The off-target cleavage activity of Cas12a is also low compared to CRISPR/Cas9. Furthermore, the RuvC and Nuc domains cut the targeted sequence at the 25th base and the non-targeted sequence at the 17th base, resulting in cohesive ends with five base-pair overhangs (Zetsche et al. 2015). Genome editing in the tomato geminiviral replicon using the CRISPR/Cpf1 system was up to three times more effective than the CRISPR/Cas9 system. A single replicon system by CRISPR/Cas9 was converted into a multi-replicon system using CRISPR/LbCas12a-based HDR (Vu et al. 2020). Three orthologs of Cas12a, namely, the *AsCas12a* from *Acidaminococcus* sp.BV3L6, *FnCas12a* from *Francisella tularensis* subs *pnovicida* U112, and *LbCas12a* from *lachnospiraceae* bacterium ND2006 are currently in use for precise modification in Tobacco, Arabidopsis, tomato, cotton, and rice (Endo et al. 2016; Kim et al. 2017).

2.3 Base editing

The base editing system includes two DNA base editors, i.e., adenine base editors (ABEs) and cytosine base editors (CBEs); these two do not require a double-stranded break or a donor template for an immediate and irreversible change of one targeted base pair into another (Komor et al. 2016; Nishida et al. 2016). These base editors are created by joining a dormant Cas9 domain, cytosine deaminase domain, and an inhibitor of uracil glycosylase (Eid et al. 2018) (Figure 1b). Base editors recognize a specific NGG PAM sequence and function only when the base editing window adheres to the target region (Komor et al. 2016; Gaudelli et al. 2017). The utilization of specific PAM sequences lowers editing efficiency in this system. To overcome this issue, several base editors with PAM flexibility have been engineered, and novel ABEs and CBEs have been developed by using variants of Cas9 with recognition of different PAM sequences other than NGG (Endo et al. 2019; Qin et al. 2019). These enhanced base editors offer the opportunity to boost base editing's effectiveness and broaden its use by targeting different plants. The SpCas9 variants like EQR-BE3, SaKKH-BE3, VQR-BE3, and VRER-BE3 with target NGCG, NRT, NGAN, and NGCG PAMs, respectively, increased the efficiency by about 2.5 folds (Kim et al. 2017).

CBEs help in the conversion of Cytosine-Guanine (C-G) base pairs into Thymine-Adenine (T-A) base pairs (Li et al. 2017; Lu et al. 2017). In 2017, the CBEs were first applied in tomato to edit two hormone-signaling genes, i.e., ETR1 and DELLA, showing a base editing efficiency of 26.2% to 53.8% (Shimatani et al. 2017). Target-AID base editing technology targeted three genes related to carotenoid accumulation: SlDDB1, SlCYC, and SlDET1. It was observed that allelic variation occurs due to base substitution from cytidine to thymine, which results in the difference in the accumulation of carotenoid content (Hunziker et al. 2020). The branched-chain amino acid biosynthesis pathway includes the acetolactate synthase (ALS) genes. The mutation of the *ALS1* gene in the tomato plant successfully created resistance towards chlorsulfuron, and 12.9% of these plants were transgene-free (Veillet et al. 2019). In the tomato plant, the *ALS* genes were also involved in causing mutation of proline 186 residue, and the base editing efficiency of *ALS*1 pro-186-residue codon was up to 71.4 %. Three additional tomato genes, viz., mDNA Damage UV Binding Protein 1, Deetiolated 1, and Lycopene beta cyclase, were subjected to CBE-mediated nucleotide substitutions, and the results demonstrated a considerable rise in total lycopene, carotenoid, and carotene level (Hunziker et al. 2020).

2.4 Prime editing

To improve precision in genome modification, prime editing is the most recently developed tool implemented for specific mutations in crop plants (Anzalone et al. 2019). The prime editing system

comprises three components, including the prime editing guide RNA (pegRNA), an engineered Moloney murine leukemia virus (M-MuLV) reverse transcriptase, which fused with the C terminal end of Cas9 (H840A), and a nickase that helps to avoid double-stranded break formation (Scholefield and Harrison 2021) (Figure 1c). Cas9 nickase cuts in the non-complimentary strand of DNA just after three nucleotides upstream to the PAM site. After the cut, a DNA flap is exposed, generating a 3'OH group that binds with the primer binding site (PBS) of the RNA template and serves as a primer for the reverse transcriptase enzyme, which is responsible for extending the 3' flap by copying the edited sequence of pegRNA. Comparatively to the unedited 5' flap, it is less preferable to hybridize with the unedited complementary strand after extending the 3' flap. The endonuclease FEN1 helps in the excision of the 5' flap, which favors the hybridization of the edited 3' flap (Scholefield and Harrison et al. 2021). The PE system has been tested in several crop species, such as rice (Butt et al. 2020; Xu et al. 2020a; Li et al. 2020), tomato (Lu et al. 2021), maize (Hua et al. 2020; Jiang et al. 2020), wheat (Lin et al. 2020; 2021; Li et al. 2022a) and potato with promising results (Perroud et al. 2022).

Parameters that define the effectiveness of prime editing include thermostability, source of reverse transcriptase enzyme, primer binding site sequence length, length of reverse transcriptase template, and nicking sgRNA in unmodified strand (Lin et al. 2020). Mutations that make reverse transcriptase more thermostable and capable of attaching to its target location also increase the editing efficiency up to 3-fold (Anzalone et al. 2019). Different RT sources show varying efficiency in editing, such as the Cauliflower Mosaic Virus (CaMV) derived RT demonstrated low editing compared to that from Molony Murine Leukemia Virus (MMuLV). Furthermore, the efficiency of prime editors was strongly affected by the length of the RT template but not significantly by the primer binding site's size and the sgRNA nicking site (Anzalone et al. 2019). The pegRNA secondary structure and the G/C composition of the primer binding site may impact the effectiveness of prime editing. Prime editors have low off-target editing frequency compared to the CRISPR/Cas9 system. Its hybridization occurs between the spacer region of pegRNA and the target DNA, the primer binding site of pegRNA, and the edited DNA flap (Marzec et al. 2020). In prime editors, the efficiency of mutation types varies. It was reported recently that the deletion frequency of 6 bp can be up to 21.8%, insertion of 3 bp frequency can be up to 19.8%, and the frequency of point mutation can be up to 0.03-18.75% in rice (Xu et al. 2020b; Lin et al. 2020). In the case of plants, there is also a decrease in the indels with the increase of targeted deletions or insertion (Lin et al.2020).

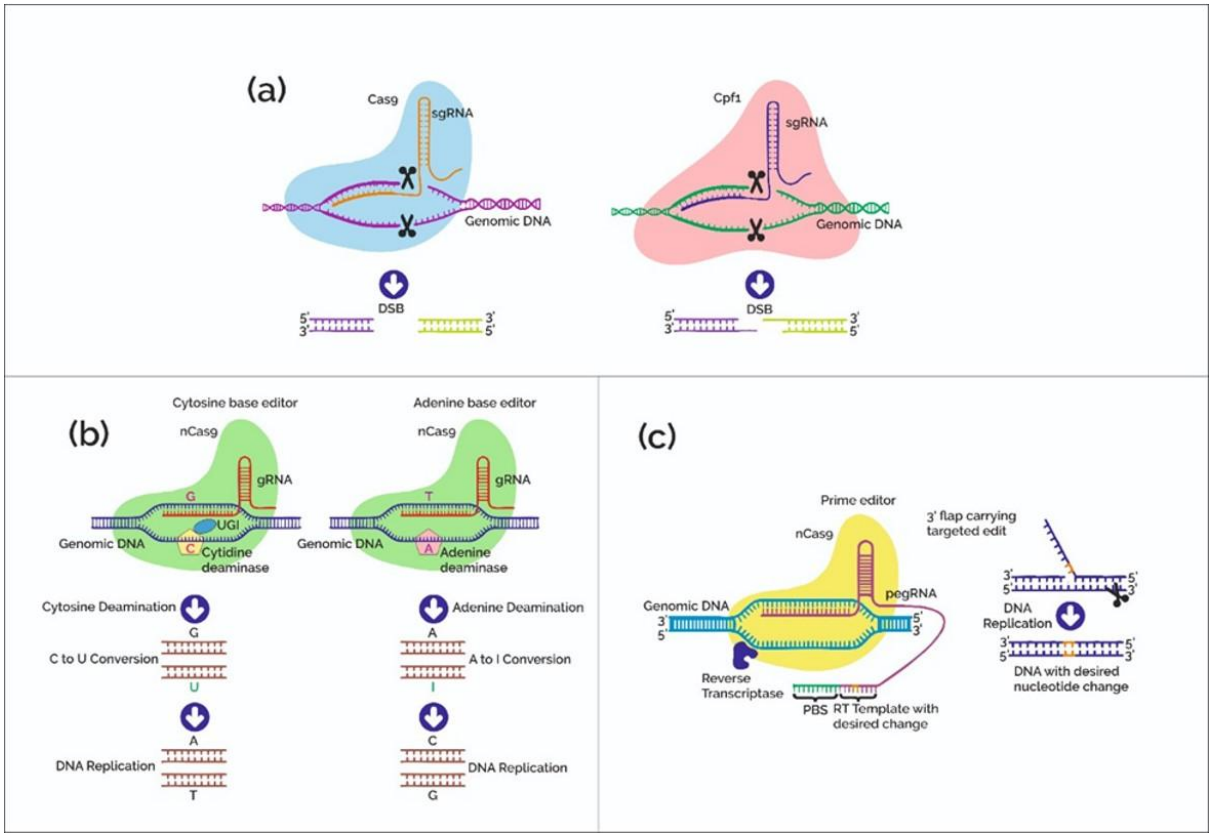

Figure 1a: A schematic diagram showing CRISPR/Cas9 and Cas12 (Cpf1) genome editing system, 1b: Showing the schematic diagram for cytosine base editing and adenine base editing, and 1c: is the schematic diagram for Prime editing

In plants, prime editing adequacy is usually low compared to human cells. All the prime editors in plants so far reported using RNA polymerase III promoters like U6 promoter for the expression of pegRNA. Previous studies in CRISPR/Cas9 systems use RNA polymerase II promoters such as CmYLcv to improve editing efficiency up to 2 folds in plants (Li et al. 2022b). So, an alternative RNA polymerase like CmYLcv can be used to improve the efficiency of prime editing, or for the expression of pegRNA U3 promoter can be used. In another study, by combining different optimization techniques, such as changing the PE design to PEmax and expressing engineered pegRNA with a structured motif under the direction of a composite promoter, the plant prime editor 2 (enpPE2) was created (Li et al. 2022c). The resultant T_0 rice plants exhibited higher editing frequencies (64.58% to 77.08%) than the unmodified pPE2. The study indicates that the enpPE2 system can be a solid and effective technique for altering plant genomes precisely.

3 Impact of CRISPR/Cas9-based genome editing on plant productivity and stress tolerance

CRISPR/Cas9 Genome editing is a highly efficient tool for improving tomato varieties concerning biotic and abiotic stresses, high yield potential, and enhanced shelf life (Figure 2). Furthermore, the technology has effectively met demands by improving fruit quality and yield (Ito et al. 2015; Ueta et al. 2017).

3.1 Yield and quality improvement

Tomatoes are an important agronomic crop, and their productivity is determined by flowering speed, flowering number, size, and fruit number. CRISPR/Cas9 induced mutation in the promoter of *CLV3* signaling peptide, alteration in *COMPOUND INFLORESCENCE (S)* gene governing inflorescence architecture known as *SELF PRUNING (SP),* and cis-regulatory frameshift through CRISPR/Cas9 has enhanced the number and size of the floral organs in the fruit, resulting in a boost in tomato yield (Rodriguez-Leal et al. 2017). Thus, CRISPR/Cas9-induced mutations in the promoter region can be exploited to develop a new cultivar with changes in quantitative traits to boost agricultural yield. Color, shape, size, nutrition, sweetness, scent, acidity, and shelf life are all fruit-quality components. Consumers increasingly demand higher fruit quality, and genome engineering has been effectively utilized to increase tomato fruit quality genetically. Fruit color is due to the accumulation of pigments such as carotenoids and flavonoids and the degradation of chlorophyll content during the ripening of fruits. Since red-colored tomatoes are more prevalent, other hues of tomatoes are also in demand in the market for different consumer groups. Furthermore, mutation of multiple genes like *PSY1, SGR1, and MYB12* produced green tomatoes by only affecting the biosynthesis or accumulation of pigments without affecting the yield and fruit quality (Yang et al. 2023). Knockout of *slsp/sler and slsp5g* multiplex mutation increased the compactness and yield of tomato plants (Kwon et al. 2020). Likewise, a group of five genes in the carotenoid metabolism pathway of tomatoes has been engineered using a multiplex CRISPR/Cas9 system (Li et al. 2018b). Surprisingly, multiplexed tomato fruit shows an increase in lycopene concentration of 5.1 times. Total carotenoid, lycopene, and carotene levels also dramatically increased due to CBE-mediated sequence alterations in three more tomato genes implicated in the accumulation of carotenoids (Hunziker et al. 2020). *NAC* transcriptional factors play a critical role in fruit ripening, and *SNAC9* is involved in various metabolic pathways of ethylene and abscisic acid. CRISPR/Cas9 mutation of *SNAC9* affected the carotenoid metabolism, which decreases carotenoid

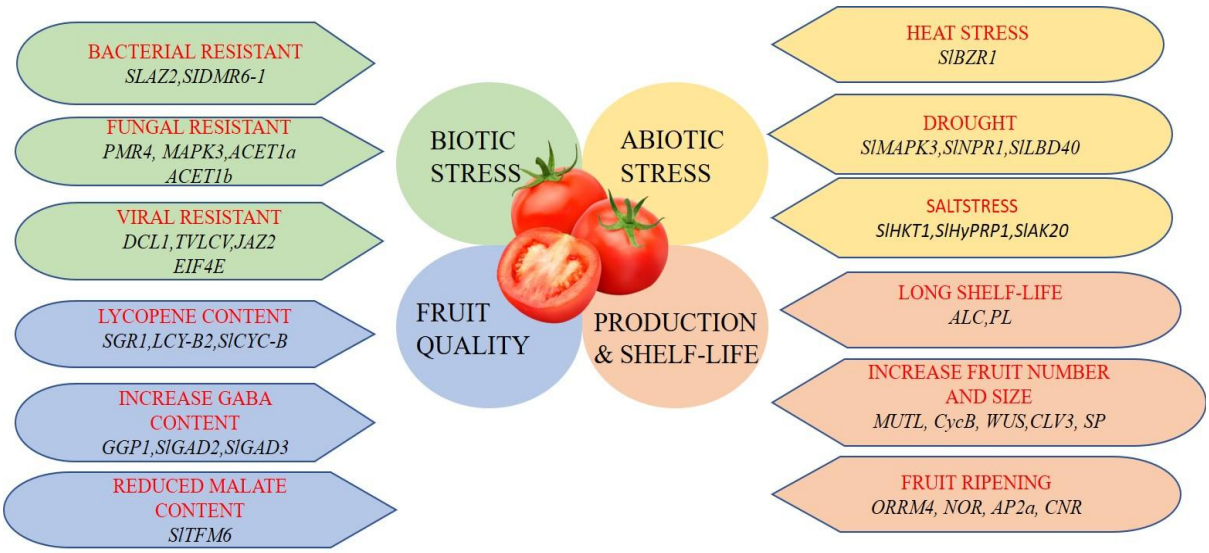

Figure 2 CRISPR/Cas9 genome editing of genes involved in tomato improvement.

and lycopene content as well as degradation of chlorophyll content and mutant *SNAC9* delayed fruit ripening by altering the gene expression levels responsible for metabolism of pigment *PSY1, PDA, CRTISO, LCYE, Z-ISO, SGR1, DXS2, LCYB* and *CrtR-b2* genes (Feng et al. 2023).

3.2 Improving nutritional value

Tomato fruit has a high concentration of gamma-aminobutyric acid (*GABA*), a human functional component that acts as an inhibitory neurotransmitter (Bachtiar et al. 2015). Knockout of *GABA-TP1*, *GABA-TP3*, *CAT9*, and *SSADH* increased the *GABA* content by 19 folds in tomato with high oxalic acid content, which has anti-nutrient metabolite (Li et al. 2018b). It has been proven that vitamin D3 is more beneficial than vitamin D2, and vitamin D3 is present in tomato leaves but not in fruits. It is stored as an intermediate in the form of SGAs in the fruits. Multiplex-editing of five genes, including three GABA transaminase genes, a cationic amino acid transporter *(CAT9)*, and a succinate semialdehyde dehydrogenase (*SSADH)* gene, led to a significant increase in the GABA content of tomato (Figure 3) (Li et al. 2018a). A key enzyme in the synthesis of GABA is glutamate decarboxylase (GAD), and induced mutations in GAD genes *SlGAD2* and *SlGAD3* in tomato increased GABA buildup in tomato fruit by 7 to 15-fold (Nonaka et al. 2017).

3.3 Production of seedless tomato

Seedless fruit production without fertilization is an important trait that confers various benefits in agriculture. When compared to the wild-variety plant, mutations in the auxin/indole-3-acetic acid (Aux/IAA) producing gene *SlIAA9* and two *AUXIN RESPONSE FACTORS (ARFs)-SlARF7* and *SlARF5*- led to seedless tomato and morphological alterations in leaves (Ueta et al. 2017). *SLAGL6* regulates the change from the ovarian arrest state that prevents anthesis to the fertilization-triggered fruit set. Parthenocarpic fruit, a fertilization-independent fruit, can be considered a valuable goal as problems are faced during fertilization due to global warming. Even in heat stress, CRISPR/Cas9 mutation of *SlAGL6* led to the formation of parthenocarpic fruit (Klap et al. 2017).

3.4 Genome editing for fruit ripening/fruit quality

Fruit ripening is a complex biological process that involves multiple biochemical, physiological, organoleptic, and metabolic changes. Ripening of fruit causes sugar accumulation, softening of fruits, color and volatile chemical accumulation, and a decrease in organic acid concentration. Fruit quality and quantity are the two main important factors for crop improvement. Tomato is one of the model plants for fruit biology research, and CRISPR/Cas9 knockout technology has been effectively used to study various

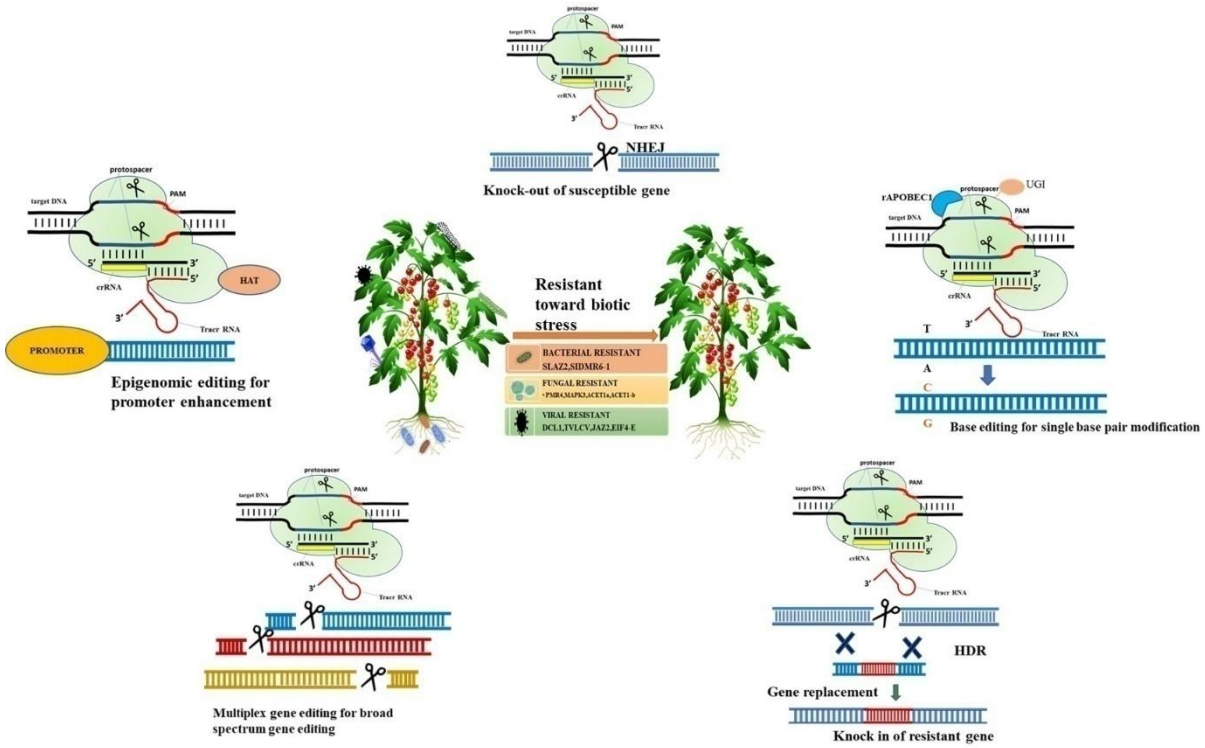

Figure 3 Strategies of genome editing towards plant tolerance to multiple diseases and crop improvement by knocking out susceptible genes through epigenomic editing for promoter enhancement, multiplex genome editing for targeting numerous genes, gene replacement by homologous direct repair, base editing for particular base change and repair by non homologous end joining method.

functions regulated by transcriptional factors and signaling pathways for architecture, growth, development, ripening of fresh fruits, and for the shelf life of tomato (Wan et al. 2021). Since 2014, genome editing has been used for precision engineering in tomato (Brooks et al. 2014) and revisiting the earlier work performed for fruit ripening by RNA silencing. It was noticed that CRISPR/Cas 9 editing system shows weaker ripening inhibitory symptoms when compared with wild-type or RNAi plants (Wang et al. 2020). CRISPR/Cas9 mediated modification of the *RIPENING INHIBITOR (RIN)* gene has contributed to the suppression of fruit ripening in tomatoes (Xu et al. 2020b) (Figure 3). Knock out the polygalacturonase gene in tomato *(SlPG),* which is responsible for fruit firmness, and the mutant *slug* delayed the softening of tomato fruit (Nie et al. 2022). The sweetness of tomatoes is essential for providing a saucy taste due to the amount of fructose, glucose, and TSS content. Gene editing of specific genes like *SlINVINH1* and *SlVPE5* improves the sweetness by increasing the amount of glucose, fructose, and TSS (Wang et al. 2021). Ascorbate is one of the essential products of tomatoes and is used as a supplement for the human diet. A negative regulator of the producing gene *SlAPX4* was knockout, resulting in high ascorbate production in ripened tomatoes (Do et al. 2022). Mutation of *SlAS2* or *SlAS2L* decreased the thickness of the pericarp by reducing the cell layer and cell area, which decreases fruit size. Stamens and leaves also exhibited several morphological defects in single and double mutants (Dong et al. 2023).

3.5 Targeting photoperiodic response

Flowering at the correct time is critical not just for reproductive potential but also for yield optimization. Different genes are responsible for regulating tomato flowering in terms of day length. *SP5G*, a Blooming LOCUS T-like gene, acts as a flower repressor, controlling the flowering state for the day. SELF-PRUNING GENE (*SP gene*) is an Arabidopsis *(TFL1)* TERMINAL FLOWER 1 ortholog that encodes for a flowering repressor gene in Tomato. Double mutations on *sp5g sp* resulted in early fruit ripening and flowering bursts (Soyk et al. 2017). Knockout of *SP5G* shows quick flowering and increased compact and determinate growth of tomato (Soyk et al. 2017). Domestication of tomato has been done by using CRISPR/Cas knock out of specific genes like the Self-pruning *SP* gene for determinate plant growth, *FAS MULT, and FW2.2* gene for increasing size, number, and weight of the fruit (Agustin et al. 2018).

A knockout mutation of *HEL* via CRISPR/Cas9 generated vine-like fruits (Yang et al. 2020). In contrast, *CRABS CLAW(CRC*) orthologues are crucial in determining floral meristem and gynoecium formation across angiosperms, which help perfect flower and fruit formation. So, for a clear understanding of the mechanism of CRC-mediated flower meristem regulation, CRISPR/Cas9 mediated knock out of *SlCRCa* was performed, and indeterminate floral meristem was formed, and it was clear that CRC mediates the floral meristem termination (Castaneda et al. 2022). *SlDOF9* negatively modulates the floral differentiation in tomatoes, and the knockout of *SlDOF9* significantly influences the differentiation of inflorescence and floral meristem (Hu et al. 2022). Additionally, the knockout of a set of kinase-inducible domain interacting genes, i.e., *SlKIX8* and *SlKIX9*, resulted in enlarged fruit with increased pericarp due to cell expansion and dome-shaped leaves (Swinnen et al. 2022). B-box transcription factor *BBXs* plays a major role in the development of plants, and SlBBX4 in tomatoes has a role in photomorphogenesis. Mutation of *slbbx4* resulted in a hypersensitive response towards red light and a normal response towards far-red, blue, and UV-B light in hypocotyl length assays. Mutant *slbbx4* under long and short-day conditions delayed flowering (Xu et al. 2023).

3.6 Engineering biotic stress resistance in tomato

Plants are continually assaulted with biotic and abiotic stresses, and various genes are associated with plant stress responses. Tomato breeders' primary goal is always to improve tomatoes' tolerance to environmental stress (Tieman et al. 2017).

3.6.1 Development of bacterial-resistant tomato

The bacterial pathogen *Pseudomonas syringae* causes a broad range of diseases in tomatoes. So, it was a great challenge to solve this problem. *P.syringae* Pv.tomato DC3000 (PtoDC3000), the causal agent of bacterial speck disease, helps the pathogen produce coronatine, which stimulates stomata opening and facilitates the colonization of bacteria in the leaf. In *Arabidopsis*, *AtJAZ2* acts as a co-repressor for COR, and the dominant mutation of *AtJAZ2* Jas repressor is resistant to proteasomal degradation and prevents the opening of stomata by COR. In tomato, an orthologue of AtJAZ2 was identified named *SlJAZ2*, and CRISPR/Cas9 mutation of *SlJAZ2* (Figure 3) was used for generating JAZ2 repressor, which lacks the C terminal of JAS and prevents reopening of stomata. This resulted in the resistance of tomatoes to the pathogen, which causes bacterial specks. Besides this, grey mold disease remained unaltered in mutated *SlJAZ2* plants and helped in broad-spectrum resistance in tomatoes (Ortigosa et al. 2019). *Clavibacter michiganensis(Cm)* causes bacterial cancer, one of the most destructive diseases in tomatoes. *SlWAT1* showed susceptibility in tomato, and knockout of *SlWAT1* in tomato decreased the free auxin and ethylene synthesis in the stem of tomato, suppressing the expression of bacterial virulence factor (Koseoglou et al. 2023).

3.6.2 Tweaking CRISPR/Cas9 to enhance fungal resistance in tomato

The fungal pathogen has an excessive impact on agricultural fields, which causes various diseases like Smut, mildew, etc. Powdery

mildew is a calamitous fungal disease caused by the obligate biotrophic fungus *Oidium neolycopersici* that affects tomatoes worldwide. Susceptible genes are the main factors that cause disease by responding to pathogens. *MILDEW-RESISTANT LOCUS O* (*Mlo*) is a conserved S gene found in monocots and dicots that confers susceptibility to powdery mildew fungus (Acevedo-Garcia et al. 2014). Knockdown of *SlMlo1* reduced susceptibility to powdery mildew (Nekrasov et al. 2017). Powdery Mildew Resistant 1 (*PMR1* to *PMR4)* are four *S*genes in *Arabidopsis* that confer susceptibility to the fungus that causes powdery mildew. The orthologue of *PMR4* has been identified in tomatoes, and CRISPR/Cas9 modification of *SlPMR4* enhances disease resistance against the powdery mildew pathogen *O. neolycopersici* (Santillán Martínez et al. 2020). Another susceptible gene, *DMR6*, belongs to the 2-ODDs 2-oxoglutarate Fe(II)-dependent dioxygenases and is upregulated during pathogenic infection in *Arabidopsis thaliana*. Orthologous *DMR6* has been found in tomato *SlDMR6-1 and SlDMR6-2*, of which *SlDMR6-1* increased the SA level during pathogen infection, and *SlDMR6-2* helped balance SA levels in fruits and flowers. (Table 1, Figure 3) Knockout of *SlDMR6-1* shows resistance toward bacterial spots (Thomazella et al. 2021). *Fusarium* wilt caused by *Fusarium oxysporum f. sp. lycopersici (Fol)* is one of the destructive diseases. *XSP10 (Xylem sap protein 10) and SlSAMT* (*Salicyclic acid methyl transferase*) are the two susceptible genes for fusarium wilt (Debbarma et al. 2021). CRISPR/Cas9 mutation of *XSP10* and *SlSAMT* showed high tolerance towards fusarium wilt when dual editing was performed compared to single gene mutation (Debbarma et al. 2023).

Table 1 List of *Solanum lycopersicum* genes targeted for genome modification via CRISPR/Cas9 genome editing

Traits	Target genes	Target gene function	Editing Strategy	Genetic effects	References
Heat stress	*SlBZR1*	developmental process and stress response	CRISPR/Cas9	heat stress tolerance	Yin et al. 2018
	SlHyPRP1	Involved negatively in multi-stress responses	CRISPR/Cas9	Multi-stress is tolerant like stress tolerant, high quality, high yield, and susceptible to fusarium wilt	Tran et al. 2021
Drought stress	*SlMAPK3*	*MAPKs* (Mitogen-activated protein kinases) are the signaling molecule that responds to drought stress.	CRISPR/Cas9	Induced through drought stress	Wang et al. 2017
	SlNPR1	*NPR1* is the regulator of plant defense mechanism towards pathogens and SlNPR1 response towards both biotic as well as abiotic stress	CRISPR/Cas9	Mutant plant shows reduced drought tolerance	Li et al. 2019
	SlLBD40	*LBD40* is involved in jasmonic acid signaling and acts as a negative regulator of drought tolerant.	CRISPR/Cas9	Boost drought tolerant	Liu et al. 2020a
Cold stress	*SlCBF1*	*CBFs* are highly conserved C-repeat binding factors are the cold response components.	CRISPR/Cas9	Reduced chilling tolerance	Li et al. 2018a
Salt stress	*SlHKT1, 2*	*SlHKT1;2* is a salt tolerant allele.	LbCpf1	Targeted salt-tolerant gene1;2 and was tolerant towards salt tolerance	Vu et al. 2020
	SlHyPRP1	*HyPRP1* is a negative regulator for salt stress.	CRISPR/Cas9	Salinity tolerance	Tran et al. 2021
Herbicide	*SlALS1*, *SlALS2*	*ALS* is the key enzyme for the biosynthesis of amino acids like leucine, isoleucine, and valine, and also it targets commercial herbicides.	CBE	Herbicide-resistant	Veillet et al. 2019
Bacterial	*SlJAZ2*	*JAZ2* is expressed constitutively on stomata and hijacked by COR, which is produced by bacteria that help in the suppression of SA-dependent closure of stomata and promote penetration of bacteria	CRISPR/Cas9	Bacterial speck resistant	Ortigosa et al. 2019
	SlDMR6-1	*DMR 6-1* gene is upregulated during pathogen infection.	CRISPR/Cas9	Resistance towards bacterial pathogens like *Xanthomonas spp, P. syringes, P. capsicum,* and different oomycete	Thomazella et al. 2021

Traits	Target genes	Target gene function	Editing Strategy	Genetic effects	References
Fungal	*SlMPR4*	*MPR4* negatively regulates biotic stress	CRISPR/Cas9	Resistance towards powdery mildew fungus *Oidium neolycopersi*	Santillán Martínez et al. 2020
	SlMYC2	*MYC2* is a transcription factor and a main regulator of the MeJA signaling pathway	CRISPR/Cas9	Decrease in disease resistance towards *B. cinerea*	Shu et al. 2020
Viral	*DCL2b*	*DCL2b* is a key player in the biogenesis of small RNA and antiviral defense	CRISPR/Cas9	Susceptibility towards Tobacco mosaic virus (TMV), Potato virus X, Tobacco mosaic virus (ToMV)	Wang et al. 2018
	JAZ2	*JAZ2* is expressed constitutively on stomata and hijacked by COR, which is produced by bacteria that help in the suppression of SA-dependent closure of stomata and promote penetration of bacteria	CRISPR/Cas9	Resistance against banana streak virus	Ortigosa et al. 2019
	EIF4E1	*eIF4E* and its isoforms are the recessive resistance gene for potyviruses	CRISPR/Cas9	Resistance towards potyvirus PepMoV	Yoon et al. 2020
	slosca4.1	*OSCA4.1* are the key plant drought resistance regulators involved in pathogen infection. SlOSC4.1 contributes to the proper regulation of calcium homeostasis, which is required for PepMV infection	CRISPR/Cas9	Resistance towards PePMV	Ruiz-Ramon et al. 2023
Weed	*More Axillary Growth1*	*MAX1*are involved in the synthesis of strigolactones, which are the inhibitors of branching that are needed for germination of root parasitic weed	CRISPR/Cas9	Resistance towards root parasitic weed *Pheliancheaegyotiaca*	Bari et al. 2021a
Harvest quality	*Rin*	*RIN* has an important role in fruit ripening	NHEJ	Incomplete ripening of fruit	Ito et al. 2015
	Self-pruning 5G(SlSP5G)	*SP5G* is involved in photoperiodic sensitivity in plants	CRISPR/Cas9	Day-length-sensitive flowering	Soyk et al. 2017
	CRTISO and PSY1	*CRTISO and PSY1* genes are involved in the biosynthesis of the carotenoid pathway	CRISPR/Cas9	Yellow and orange tomatoes	Dahan-Meir et al. 2018
	SlPSY1(Phytoene synthase1)	*PSY1* genes are important genes of carotenoid biosynthesis	CRISPR/Cas9	Yellow fresh tomatoes	D'Ambrosio et al. 2018
	MYB12	*SlMYB*is a transcription factor that is involved mainly in the flavonol biosynthesis branch	CRISPR/Cas9	Pink tomatoes	Deng et al. 2018
	SlGAD2 and SlGAD3	*SlGAD2 and SlGAD3* are the key enzymes in the biosynthesis of GAB, A C terminal auto-inhibitory domain is present, which regulates enzymatic function, and removing this domain regulates enzymatic function.	CRISPR/Cas9	Increase of GABA accumulation from sevenfold to 15-fold	Nonaka et al. 2017
	NOR	*NOR* gene is mostly involved in fruit ripening	CRISPR/Cas9	The ripening process was partially affected	Wang et al. 2019
	AP2a	*SlAPA2a* acts as a negative regulator of fruit ripening.	CRISPR/Cas9	Initiated ripening of fruit in early stage but did not ripen fully	Wang et al. 2019
	FUL1/FUL2	*FUL1 and FUL2* are the transcription factors of the MADS-domain and are involved in regulating flowering time, fruit ripening, and architecture of inflorescence	CRISPR/Cas9	Double mutants did not show a ripe red color	Wang et al. 2019

Traits	Target genes	Target gene function	Editing Strategy	Genetic effects	References
	BZR1	*BZR1* is the regulator of brassinosteroid and is involved in developmental as well as stress tolerance	Loss of function	Decrease in heat stress tolerance	Yin et al. 2018
	CBF1	*CBF1(C-repeat binding factors)* are involved in cold response system	Loss of function	Decrease in chilling stress tolerance	Li et al. 2018a
	SlMAPK3	*MAPK s* (Mitogen-activated protein kinases) are the signaling molecules involved in drought stress	Loss of function	Decrease in drought stress tolerance	Wang et al. 2017
	Carotenoid cleavage dioxygenase8(CCD 8) More Auxiliary Growth1 (MAX1)	*CCD8 and MAX1* genes help synthesize strigolactones, which are branching inhibitory hormones and are required for germination of root parasitic weed	Loss of function	Resistance against *phelipanche aegytiaca*	Bari et al. 2021a
	MPK20	*SlMPK20* regulates the post-meiotic development of pollen by modulating sugar as well as auxin signaling and metabolism	Loss of function	Repression of genes controlling sugar and auxin metabolism	Chen et al. 2018
	Enzymes pectate lyase *(PL)*, polygalacturonase 2a (*PG2A*), and β −galactanase *(TBG4)*	*PL*, *PG2a*, and *TBG4* work on separate cell wall domains and are involved in shelf life.	Generation of a range of CRISPR alleles	Pectin degradation control	Wang et al. 2019
	ARF7	*ARF7* is the key repressor of the initiation of fruit in tomato	Loss of function	Parthenocarpic	Hu et al. 2018
	SlSTK	Serine/Threonine kinase domain regulates the signaling of glucose, which is essential for the plant for stress response, growth, and development	Loss of function	Attenuated sensitivity of glucose	Lu et al. 2023

Phospholipase C2 gene *SlPLC2* regulates the effect of pathogens in plants, resulting in resistance or susceptibility of plants towards different diseases. There are six members of PLC present in tomato *SlPLC1-SlPLC6*. CRISPR/Cas9 knockout of the *SlPLC2* gene decreased the production of ROS upon *B.cinerea* infection, which increased resistance towards the pathogen by reducing the pathogen proliferation as proliferation requires the production of ROS for cell death. Moreover, the *SlPLC2* mutant produced small necrotic areas (Perk et al. 2023). Late blight is one of the fungal diseases caused by *Phytophthora infestans*. Knockout of *SlMYBS2* reduced the resistance towards *P. infestans* by increasing the necrotic cells, disease index, and lesion size and decreasing the expression of the pathogenesis-related (PR) gene in the mutated plant, which concluded that *SlMYBS2* is a positive regulator for the *P. infestans* in tomato (Liu et al. 2021). Besides this, MicroRNA has a great role in susceptibility by targeting the resistant gene. CRISPR/Cas9 multiplex knockout of *miR482b* and *miR482C* shows more resistance towards *P. infestans* than the single knockout of *miR482b* (Hong et al. 2021). In potato, mutation of the *PMR4* gene reduced susceptibility to powdery mildew. An orthologue of the *PMR4* gene was found in tomatoes, and four different guide RNAs were designed to target different regions of the *SlPMR4* gene in two different varieties of tomatoes. Four sgRNAs successfully knocked out the *PMR* locus (Figure 3) and induced resistance towards *P.infestans* (Li et al. 2022a). Knock out of *NUCLEOREDOXIN* gene in tomato *SlNRX1* and *SlNRX2* exhibited resistance towards the pathogen *Pseudomonas syringae pv. maculicola (Psm) ES42326*, which causes bacterial leaf as well as towards the fungal pathogen *Alternaria brassicicola* in mutant *slnrx*. However, a mutation in *slnrx2* showed no resistance (Cha et al. 2023). Mutant *slnrx1* increased endogenous salicylic acid and decreased the jasmonic acid level after infection with *Psm* compared with *slnrx2* and wild-type plants. In the mutant *slnrx1*, the SA biosynthesis gene *SlICS1(ISOCHORISMATE SYNTHASE1)* and *SlEDS5 ENHANCED DISEASE SUSCEPTIBILITY 5* were upregulated compared to wild type plant. However, the *PR1* gene, a systemic acquired resistance gene, increased in *slnrx1* compared to the wild type (Cha et al. 2023). CRISPR activation of *SlPR-1 PATHOGENESIS-RELATED GENE 1* showed enhanced disease resistance against *Clavibacter michiganesis subsp.* without changing their agronomic character (García-Murillo et al. 2023). PTI (Pattern effector-triggered immunity) and ETI (effector-triggered immunity) are the two tired immune response systems in plants towards pests and pathogens. NLR receptor link with PTI and ETI, which is required for plant immune response. Mutation in *h-NLR SlNRC4a* and *SlNCR4b* resulted in a gain of constitutive

function defense activation and broad-spectrum disease resistance. The double mutant of *SlNRC4a* and *SlNRC4b* increased resistant against the fungal pathogen compared to a single mutation (Leibman-Markus et al. 2023). 3-Dehydroquinate dehydratase/shikimate dehydrogenase*(DQD/SDH)* is involves in the synthesis of shikimate. Knock out of *SlDQD/SDH2*, which is a ripening associated factor, lowers the content of flavonoids and shikimate by down-regulating flavonoid biosynthesis genes and shows resistance towards *Botrytis cinerea* (Wang et al. 2023).

3.6.3 Harnessing CRISPR/Cas9 to engineer virus resistance in tomato

Viruses that affect plants are usually obligate parasites that completely depend upon the host for survival. Moreover, the susceptible factors, also called recessive genes, present in the plant are the main factors that regulate viral disease in plants. Tomato yellow leaf curl virus (TYLCV) is from the Geminiviridae family. It causes one of the devasting viral diseases for tomatoes. *Ty-5* (*SlPelo*) is one of the susceptibility factors (S gene) for TYLCY. Knockdown of SlPelo through CRISPR/Cas genome editing confers host-mediated immunity against the pathogen (Praminik et al. 2021). Interestingly, a tomato plant with a stably designed CRISPR/Cas9 platform targeting viral genes encoding coat protein (*CP*) or replicase (*Rep*) demonstrated improved resistance to TYLCV infection. Translation initiation factor (*eIF4E*), a cap-binding protein, is one of the prominent susceptible factors to potyvirus. The recent CRISPR/Cas9-based knockdown of the eIF4E1 gene in tomatoes showed enhanced resistance to multiple viruses, including the cucumber mosaic virus, the pepper mottle virus, and the potato virus Y N strain (Yoon et al. 2020; Atarashi et al. 2020). The Pepino mosaic virus (PepMV) was first identified in 1999 as a tomato pathogen in the Netherlands. Since then, it has spread worldwide and created a pandemic in tomato crops, causing great economic loss. *SlGSTU38* is the susceptible gene for *PepMV* mutation, and this gene triggers the accumulation of oxygen species in leaves and deregulates stress-responsive genes (Mendez - Lopez et al. 2023).

3.6.4 Weed control in tomato

The obligate and facultative are the two characterized parasitic plants that adopt different forms for invading host plants by attaching them through their roots or shoots. Most economically significant crops are infected by root parasite weeds, which reduces yield and yield quality. Important agronomic crops are severely harmed by the parasitic weeds *Orobanche* and *Phelipanche* spp., which are entirely dependent on the host for their nutritional value. Strigolactones are plant hormones produced from plant carotenoids by cleavage of the CCD7 and CCD8 enzymes. These are necessary for germinating parasitic root weeds and function as a branching inhibitory hormone. CRISPR/Cas9 genome editing of two homologs of ATP binding cassette transporter (ABC) genes *Solyc08g067610* and *Solyc08g067620* in tomato decreased the growth of parasitic weeds *P. aegyptiaca*. It reduced the primary stem length, increased branching, and increased axillary bud growth. Moreover, the expression of two strigolactone biosynthetic genes, i.e., *CCD8* and *MAX1*, was significantly decreased in the ABC mutant lines and resulted in an alteration in root extract orobanchol (Bari et al. 2019). The mutated ccd8 in the second exon shows some morphological changes, like adventitious root formation and increased shoot branching. Additionally, *CCD8* mutants with SL-deficient show reduced parasite infection. It was observed that in the *CCD8* mutation, orobanchol (SL) content was reduced, and carotenoid and expression of genes that participate in carotenoid biosynthesis increased (Bari et al. 2019).

Further, the CRISPR/Cas 9 strategy has been successfully used to mutate the SL biosynthetic gene More Auxiliary Growth1 *(MAX1)* (Bari et al. 2021b). Due to lowered orobanchol levels, the *Slmax1* edited lines showed resistance to *P. aegyptiaca*. The study offers a fresh insight into creating an effective management strategy that might be utilized to prevent the growth of root parasite weeds, significantly impacting the agricultural economy (Bari et al. 2021a).

3.7 Engineering abiotic stress tolerance in tomato

Abiotic stresses include high or low temperature, excessive or inadequate water conditions, high salt concentration, accumulation of heavy metals, etc., affecting the plant's growth and development (Liu et al. 2022). Modifying the genes involved in the regulatory pathway of certain stress hormones, reactive oxygen species, etc., can create artificially resistant plants. CRISPR/Cas technologies help understand the crucial role of complex mechanisms of various abiotic stresses in plants and develop new climate-resilient crop varieties (Illouz-Eliaz et al. 2020).

3.7.1 Heat stress

Primarily due to global warming, as the temperature increases, it significantly affects the growth and development of plants. An increase in the production of ROS, cellular damage, and unwanted biological compound formation leads to a disturbance in plant cellular metabolism under heat stress (Chaudhury and Sidhu 2022). CRISPR/Cas9 strategy has been effectively utilized to tackle the heat stress response in tomatoes. Knocked out of *the SlBZR1 gene of the tomato showed susceptibility* to heat stress by reducing the quantum efficiency of photosystem II (Yin et al. 2018). Similarly, mitogen-activated protein kinases (MAPKs) are involved in multiple signaling pathways that lead to abiotic stress tolerance (Yu et al. 2019). CRISPR/Cas9-mediated mutation of *SlMAPK3* reported less cell membrane damage and ROS production, less

wilting, and more expression of heat shock proteins (HSPs) and heat shock factors (HSFs) (Yu et al. 2019).

3.7.2 Osmotic stress

Most abiotic stress like drought, salinity, and cold stress causes the plant's osmotic stress, where the water and electrolyte imbalance occurs. Osmotic stress damages the cellular and DNA labels, which has a major effect on the physiology of plant cells (Chen et al. 2021a). The tomato plant requires a lot of water, and any imbalance limits growth, germination, and elongation (Liu et al. 2018). As per the previous report, auxin response factors (ARFs) have an influential physiological role in plants (Roosjen et al. 2018). A gene of the ARF family *SlARF4*, expressed in guard cells and tomato's vascular bundle, was studied under water deficit conditions. In a study, it was reported that abscisic acid treatment and deficiency in water reduced the expression of the *SlARF4* gene (Bouzroud et al. 2018). CRISPR/Cas9 mediated loss of function mutation of the *SlARF4* gene resulted in enhanced tolerance to water stress and dehydration in the mutated lines (Chen et al. 2021b). This indicates that the *SlARF4* gene might be associated with the ABA signaling pathway by modulating the expression of *SlABI5/ABF* and *SCL3* genes, helping the tomato plant resist water deficit conditions (Chen et al. 2021a).

Similarly, in tomatoes, it was found that the PROCERA gene encodes the DELLA protein (a negative growth regulator) associated with the tomato resistance towards osmosis stress. Loss of function mutation of DELLA protein using CRISPR/Cas9 and a sgRNA provides several dominant dwarf mutations and loss-of-function mutations (Tomlinson et al. 2019). In 2019, the dominant dwarf PROCERA allele was first reported, showing partial responsiveness towards exogenously applied gibberellins. The intermediate phenotype was observed in heterozygotes at the seedling stage, but later, during their adult stage, the heterozygotes were dwarfed as homozygotes (Tomlinson et al. 2019). Based on these studies, it can be assumed that specific genetic networks regulate abiotic factors that monitor the osmotic stress in plants well, and with the advent of gene editing, resistant tomato varieties can be generated.

3.7.3 Drought stress

Multiple genes control drought stress in tomato CRISPR/Cas9 system was used to edit *SlNPR1* (Stable tomato *NPR1*) gene from tomatoes to study its role in regulating tomato drought tolerance response (Li et al. 2019). Reduced drought tolerance was evident in the *slnpr1* mutants, and it was further supported by the expression of genes associated with drought, such as *SlGST*, *SlDREB, and SlDHN*, being downregulated. *SlNPR1* mutants, by reducing the expression of multiple drought tolerance genes, also concurrently resulted in increased levels of malondialdehyde (MDA) and hydrogen peroxide, larger stomatal aperture, higher electrolytic leakage, and lower activity of antioxidant enzymes in the mutant lines (Table 1, Figure 3) (Li et al. 2019). In yet another study, CRISPR/Cas9 mutated *SlLBD40* knockouts were found to be less responsive to drought than WT tomato plants by increasing the water-holding ability (Liu et al. 2020b).

3.7.4 Chilling stress

The tomato plant is sensitive to chilling. In many species, it was found that highly conserved CBFs (C-repeat binding factors) are responsible for cold-response systems. The CRISPR-Cas9 generated *slcbf1* mutant demonstrated a more chilling-injury response than the wild type with high malondialdehyde and hydrogen peroxide contents, increased antioxidant enzymes, and higher electrolyte leakage (Li et al. 2018a). Low temperatures induce gene expression and protein content of SlNPR1. Mutation of *SlNPR1* resulted in chilling stress by increasing oxidative damage and synthesis of ferulic acid (Shu et al. 2023).

4 Challenges and Future prospects of tomato genome editing

Since the last decade, CRISPR/Cas9 genome editing technology has emerged as an evolutionary tool in agricultural biotechnology for crop improvement. However, the technology faces some major challenges that need to be addressed for further improvement and adoption in crop improvement.

4.1 Overcoming PAM Constraint

Mutation frequency is enhanced by minimalizing the distance between the cut or PAM sites and the edit sites. To address this problem, the idea of engineered Cas9 protein free from PAM site restriction has been developed. Recently developed Cas9 variants, SpG can efficiently cut at NGN sites where SpRY can act in almost every location near the target sites. SpRy is designed from wild variety Cas9 protein by changing 11 amino acids in its peptide chain. SpRy is potentially more efficient on NRN (R=A, G) PAM sites than NYN (Y=C, T) PAM sites in human cells. Genome editing in rice protoplast by SpRY revealed a similar fact with human cells that SpRy has the potential to edit all NNN PAM sites as well as a relatively high potential to edit at NRN PAM sites instead of NYN PAM sites (Ren et al. 2021).

4.2 Delivery Methods

Genome editing in plants has a few constraints, like unwanted insertion or deletion, off-target activity, sgRNA mismatch, and overexpression of Cas9 (Shen et al. 2019). Delivering editing machinery, i.e., gRNA or CRISPR/Cas9 components, into the host plant is still challenging. The common approaches are floral-dip-mediated transfer, Particle bombardment, Protoplast transformation, and *Agrobacterium* transformation. All these

methods have particular benefits as well as disadvantages. Dipping flower buds execute the floral dip method in a buffer containing *Agrobacterium*, then collecting seeds and selecting a positive transformant by growing them in a selection media. Similarly, *Agrobacterium*-mediated plant transformation is also executed by co-cultivating explants followed by positive transformant selection in a selection media. However, both methods require a thorough screening for T-DNA and Cas9-free mutant homozygous lines. Although particle bombardment and PEG-protoplast-mediated approaches are efficient, they also generate transgenic plants that must pass through extensive screening.

Viral vectors are most widely used in plant cells for gene silencing and the expression of inserted foreign proteins. Transgenic plants have already been edited with the expression of Cas9 nucleoprotein by using a viral vector system (Hu et al. 2019). However, transgene-free genome editing proves to be complicated due to certain virus restrictions. DNA and +ve strand RNA viruses were used in the plant genome for editing but are limited due to their cargo capacities (Liu et al. 2020a). The use of negative-strand RNA virus avoided the difficulties faced due to the use of positive-strand RNA virus as vector due to the large cargo capacity in negative-strand RNA virus vector. After continued effort, viral vectors are now intriguing tools for the transgenesis-free genome editing approach, which uses host machinery for their multiplication, enabling a broad level of expression (Cody and Scholthof 2019). Prior endeavors to express sgRNA using plant RNA virus vectors, such as tobacco mosaic virus, tobacco rattle virus, barley stripe mosaic virus, beet necrotic yellow vein virus, pea early browning virus, and foxtail mosaic virus, were successful in introducing mutations into host genomes (Ellison et al. 2020). However, due to the massive size of Cas9 and the negative correlation between the stability of plant viral vectors and the length of foreign gene insert, expression of virus vector-mediated Cas9 is difficult. Although the delivery of SYNV vector is possible, plant regeneration has certain limitations as plant rhabdoviruses are difficult to invade into plant germline or meristem cells (Liu et al. 2020b).

4.3 Transgene free genome editing

In conventional genome editing methods, transfer and a combination of DNA cassettes are needed to modify the host genome, which causes indels and changes in the DNA sequence and generates detrimental effects (Kim et al. 2014). However, transgene-free gene editing is fast and expanding due to its advantages. This technique targets genome alteration without any conflict with the gene and creates an opportunity for the delivery of non-genetically modified organisms (Rather et al. 2022). However, it has the same issue of transformation as in the conventional gene editing approach. The transformation approach mainly includes the invitro RNP complex comprising Cas9 and gRNA, which different approaches like microinjection, electroporation, particle bombardment, protoplast-mediated transformation, etc, can deliver (Zhang et al. 2021a). The other approach for transformation includes virus-mediated delivery and type IV secretory system of *A. tumefaciens* for Cas/gRNA delivery into plant cells (Tsanova et al. 2021). Ribonucleoprotein(RNPs) mediated genome editing is one of the methods for generating transgene-free genome editing plants in which the RNPs are composed of Cas9 nuclease protein and sgRNA, which will be delivered into the plant cell by using protoplast mediated transformation by using PEG, and calcium in rice and *Arabidopsis* (Toda et al. 2019). Particle bombardment was transformed to transfer the RNP into embryonic maize and wheat (Woo et al. 2015). However, the major disadvantage is full-length plant regeneration, and the selection process is time-consuming and expensive. Recently, DNA-free gene editing through protoplast-mediated transformation was performed in potatoes with 95% editing efficiency (Rather et al. 2022).

4.4 Grafting-mediated transformation of Cas9 and sgRNA cassette

Protoplast-mediated transformation is unsuitable in some plant species, or it takes a long time for a generation to overcome this problem. Designed Cas9 and gRNA transcripts are transferred from transgenic roots to shoots of wild-type plants (Scions) (Yang et al. 2023). Using such grafting, wild-type scions can be transferred into genome-edited mutants by transferring the gRNA and Cas9 transcript from the transgenic root. A tRNA-like sequence or TLS motifs were added along with Cas9 and gRNA transcript for movement from root to shoot.

4.5 Regulatory aspects of genome-edited tomato

Few nations, such as South and North America, have removed regulatory barriers to encourage the commercialization of GE crops. Furthermore, Australia and Japan have reviewed and updated their normative approval processes for genetically engineered organisms and goods, including site-directed nuclease 1 (SDN-1) type alteration (Kaul et al. 2020). Genome-edited crops developed using SDNs are categorized as SDN 1, 2, and 3. Most countries follow the SDN terminology to categorize the SDN applications legally. Oligonucleotide-directed mutagenesis (ODM) is also an alternative approach for targeted mutagenesis, and the outcome is mainly considered an SDN-2 event. In the last few years, several countries have adopted legislation and introduced guidelines for clarifying genome-edited products' legal status (Menz et al. 2020). CRISPR/Cas9 is subject to strict regulation in Europe, and the European Court of Justice included genome-edited crops in the rules governing genetically modified crops. Meanwhile, Australia allows genome editing without the involvement of foreign particles (Zhang et al. 2021b). Asia, China,

and Japan have relaxed restrictions on genome-edited crops, and there have been reports of such crops being grown in the field. The first gene-edited food that entered the market was GABA-enriched tomato. GABA is a health-promoting compound like vitamin C and a neurotransmitter that helps block impulses between nerve cells in the brain (Alamgir 2018). Tokyo-based Sanatech Seed has sold CRISPR/Cas9 genome-edited Sicilian Rouge tomatoes since September 2021. This genome-edited food contains a high amount of γ-aminobutyric acid (GABA), which helps to lower blood pressure and promote relaxation (Waltz 2022). A commercialized startup, Sanatech, from the University of Tsukuba, started sending free genome-edited tomato seedlings at the request of 4200 home gardeners in September 2021. More than 400 GABA-enriched food and beverage items, including chocolates, are already available in the Japanese market (Milon et al. 2024).

Conclusion

CRISPR/Cas9 is widely used as a promising tool for plant breeding. Its accuracy, simplicity, and precision make it a versatile system for modification of a wide range of crop plants. Recently developed prime editing system has advantages over base replacement and holds great promise in the precise modification of crops. Research endeavours must be adapted to successfully implement the CRISPR/Cas9 genome editing system in crop improvement, and the grand challenges must be addressed. Overcoming challenges like off-target activity, PAM constraint, genotype dependency transformation, and delivery methods can greatly increase crops' editing efficiency. Furthermore, the regulatory issues and ethical concerns associated with the technology must be debated globally, and scientific frameworks must be developed to widen the acceptance of edited crops.

Reference

Acevedo-Garcia, J., Kusch, S., & Panstruga, R. (2014). Magical mystery tour: MLO proteins in plant immunity and beyond. *New Phytologist*, *204(2)*, 273-281.

Agustin, Z., Tomas, C., Rezende, N. E., & Morato, N. M. (2018). Edel Kai H, Weinl Stefan, Freschi Luciano, Voytas Daniel F, Kudla Jörg, Peres Lázaro Eustáquio Pereira. De novo domestication of wild tomato using genome editing. *Nature Biotechnology*, *36(12*), 1211-1216.

Ahmar, S., Saeed, S., Khan, M. H. U., Ullah Khan, S., Mora-Poblete, F., et al. (2020). A revolution toward gene-editing technology and its application to cropimprovement. *International Journal of Molecular Sciences*, *21*(16), 5665.

Alamgir, A. N. M. (2018). Vitamins, nutraceuticals, food additives, enzymes, anesthetic aids, and cosmetics. In A. N. M. Alamgir (Ed.) *Therapeutic Use of Medicinal Plants and their Extracts: Volume 2: Phytochemistry and Bioactive Compounds* (pp. 407-534). Springer, Cham. https://doi.org/10.1007/978-3-319-92387-1_5.

Anzalone, A. V., Koblan, L. W., & Liu, D. R. (2020). Genome editing with CRISPR–Cas nucleases, base editors, transposases, and prime editors. *Nature Biotechnology*, *38*(7), 824-844.

Anzalone, A. V., Zairis, S., Lin, A. J., Rabadan, R., & Cornish, V. W. (2019). Interrogation of eukaryotic stop codon readthrough signals by in vitro RNA selection. *Biochemistry*, *58*(8), 1167-1178.

Asmamaw, M., & Zawdie, B. (2021). Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. *Biologics : targets & therapy*, *15*, 353–361. https://doi.org/10.2147/BTT.S326422.

Atarashi, H., Jayasinghe, W. H., Kwon, J., Kim, H., Taninaka, Y., Igarashi, M., Ito, K., Yamada, T., Masuta, C., & Nakahara, K. S. (2020). Artificially Edited Alleles of the Eukaryotic Translation Initiation Factor 4E1 Gene Differentially Reduce Susceptibility to Cucumber Mosaic Virus and Potato Virus Y in Tomato. *Frontiers in microbiology*, *11*, 564310. https://doi.org/10.3389/fmicb.2020.564310

Bachtiar, V., Near, J., Johansen-Berg, H., & Stagg, C. J. (2015). Modulation of GABA and resting state functional connectivity by transcranial direct current stimulation. *eLife*, *4*, e08789. https://doi.org/10.7554/eLife.08789.

Bari, V. K., Nassar, J. A., & Aly, R. (2021a). CRISPR/Cas9 mediated mutagenesis of MORE AXILLARY GROWTH 1 in tomato confers resistance to root parasitic weed *Phelipanche aegyptiaca*. *Scientific reports*, *11*(1), 3905. https://doi.org/10.1038/s41598-021-82897-8.

Bari, V. K., Nassar, J. A., Kheredin, S. M., Gal-On, A., Ron, M., et al. (2019). CRISPR/Cas9-mediated mutagenesis of CAROTENOID CLEAVAGE DIOXYGENASE 8 in tomato provides resistance against the parasitic weed *Phelipanche aegyptiaca*. *Scientific Reports*, *9*(1), 11438.

Bari, V. K., Nassar, J. A., Meir, A., & Aly, R. (2021b). Targeted mutagenesis of two homologous ATP-binding cassette subfamily G (ABCG) genes in tomato confers resistance to parasitic weed *Phelipanche aegyptiaca*. *Journal of Plant Research*, *134*(3), 585-597.

Bouzroud, S., Fahr, M., Bendaou, N., Bouzayen, M., Zouine, M., et al. (2018). SlARF4 Under Expression Improves Tolerance to Salinity in Tomato (Solanum lycopersicum). *International Journal of Agricultural and Natural Sciences*, *1*(3), 242-244.

Brooks, C., Nekrasov, V., Lippman, Z. B., Van Eck, J. (2014). Efficient gene editing in tomato in the first generation using the

clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant physiology*, *166*(3), 1292-1297.

Butt, H., Rao, G. S., Sedeek, K., Aman, R., Kamel, R., et al. (2020). Engineering herbicide resistance via prime editing in rice. *Plant Biotechnology Journal*, *18*(12), 2370.

Castañeda, L., Giménez, E., Pineda, B., García-Sogo, B., Ortiz-Atienza, A., et al. (2022). Tomato CRABS CLAW paralogues interact with chromatin remodelling factors to mediate carpel development and floral determinacy. *The New phytologist*, *234*(3), 1059–1074. https://doi.org/10.1111/nph.18034.

Cha, J. Y., Uddin, S., Macoy, D. M., Shin, G. I., Jeong, S. Y., et al. (2023). Nucleoredoxin gene SINRX1 negatively regulates tomato immunity by activating SA signaling pathway. *Plant Physiology and Biochemistry*, *200*, 107804.

Chaudhry, S., & Sidhu, G. P. S. (2022). Climate change regulated abiotic stress mechanisms in plants: A comprehensive review. *Plant Cell Reports*, *41*(1), 1-31.

Chen, L., Yang, D., Zhang, Y., Wu, L., Zhang, Y., et al. (2018). Evidence for a specific and critical role of mitogen-activated protein kinase 20 in uni-to-binucleate transition of microgametogenesis in tomato. *New Phytologist*, *219*(1), 176-194.

Chen, M., Zhu, X., Liu, X., Wu, C., Yu, C., et al. (2021a). Knockout of auxin response factor SlARF4 improves tomato resistance to water deficit. *International Journal of Molecular Sciences*, *22*(7), 3347.

Chen, T., Cohen, D., Itkin, M., Malitsky, S., & Fluhr, R. (2021b). Lipoxygenase functions in 1O2 production during root responses to osmotic stress. *Plant Physiology*, *185*(4), 1638-1651.

Cody, W. B., & Scholthof, H. B. (2019). Plant virus vectors 3.0: transitioning into synthetic genomics. *Annual Review of Phytopathology*, *57*, 211-230.

D'Ambrosio, C., Stigliani, A. L., & Giorio, G. (2018). CRISPR/Cas9 editing of carotenoid genes in tomato. *Transgenic research*, *27*(4), 367-378.

Dahan-Meir, T., Filler-Hayut, S., Melamed-Bessudo, C., Bocobza, S., Czosnek, H., et al. (2018). Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. *The Plant Journal*, *95*(1), 5-16.

Debbarma, J., Saikia, B., Singha, D. L., Das, D., Keot, A. K., et al. (2023). CRISPR/cas9-mediated mutation in XSP10 and slSAMT genes impart genetic tolerance to fusarium wilt disease of tomato (*Solanum lycopersicum* L.). *Genes*, *14*(2), 488.

Debbarma, J., Saikia, B., Singha, D. L., Maharana, J., Velmuruagan, N., et al. (2021). XSP10 and SlSAMT, Fusarium wilt disease responsive genes of tomato (*Solanum lycopersicum* L.) express tissue specifically and interact with each other at cytoplasm in vivo. *Physiology and Molecular Biology of Plants*, *27*(7), 1559-1575.

Deng, L., Wang, H., Sun, C., Li, Q., Jiang, H., et al. (2018). Efficient generation of pink-fruited tomatoes using CRISPR/Cas9 system. *Journal of Genetics and Genomics= Yi chuanxue bao*, *45*(1), 51-54.

Do, J. H., Park, S. Y., Park, S. H., Kim, H. M., Ma, S. H., et al. (2022). Development of a genome-edited tomato with high ascorbate content during later stage of fruit ripening through mutation of SlAPX4. *Frontiers in Plant Science*, *13*, 836916.

Dong, R., Yuan, Y., Liu, Z., Sun, S., Wang, H., et al. (2023). ASYMMETRIC LEAVES 2 and ASYMMETRIC LEAVES 2-LIKE are partially redundant genes and essential for fruit development in tomato. *The Plant Journal*, *114*(6), 1285-1300.

Eid, A., Alshareef, S., & Mahfouz, M. M. (2018). CRISPR base editors: genome editing without double-stranded breaks. *Biochemical Journal*, *475*(11), 1955-1964.

Ellison, E. E., Nagalakshmi, U., Gamo, M. E., Huang, P. J., Dinesh-Kumar, S., et al. (2020). Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nature plants*, *6*(6), 620-624.

Endo, A., Masafumi, M., Kaya, H., & Toki, S. (2016). Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from Francisellanovicida. *Scientific Reports*, *6*(1), 38169.

Endo, M., Mikami, M., Endo, A., Kaya, H., Itoh, T., et al. (2019). Genome editing in plants by engineered CRISPR–Cas9 recognizing NG PAM. *Nature Plants*, *5*(1), 14-17.

Feng, Y., Kou, X., Yuan, S., Wu, C., Zhao, X., et al. (2023). CRISPR/Cas9-mediated SNAC9 mutants reveal the positive regulation of tomato ripening by SNAC9 and the mechanism of carotenoid metabolism regulation. *Horticulture Research*, *10*(4), uhad019.

Gao, Y., Wei, W., Fan, Z., Zhao, X., Zhang, Y., et al. (2020). Re-evaluation of the nor mutation and the role of the NAC-NOR transcription factor in tomato fruit ripening. *Journal of Experimental Botany*, *71*(12), 3560-3574.

García-Murillo, L., Valencia-Lozano, E., Priego-Ranero, N. A., Cabrera-Ponce, J. L., Duarte-Aké, F. P., et al. (2023). CRISPRa-mediated transcriptional activation of the SlPR-1 gene in edited tomato plants. *Plant Science*, *329*, 111617.

Gaudelli, N. M., Komor, A. C., Rees, H. A., Packer, M. S., Badran, A. H., et al. (2017). Programmable base editing of A• T to G• C in genomic DNA without DNA cleavage. *Nature*, *551*(7681), 464-471.

Gupta, S. K., Sharma, M., & Mukherjee, S. (2022). Buckeye rot of tomato in India: Present status, challenges, and future research perspectives. *Plant Disease*, *106*(4), 1085-1095.

Hong, Y., Meng, J., He, X., Zhang, Y., Liu, Y., et al. (2021). Editing miR482b and miR482c simultaneously by CRISPR/Cas9 enhanced tomato resistance to *Phytophthora infestans*. *Phytopathology*, *111*(6), 1008-1016.

Hu, G., Wang, K., Huang, B., Mila, I., Frasse, P., et al. (2022). The auxin-responsive transcription factor SlDOF9 regulates inflorescence and flower development in tomato. *Nature Plants*, *8*(4), 419-433.

Hu, J., Israeli, A., Ori, N., & Sun, T. P. (2018). The interaction between DELLA and ARF/IAA mediates crosstalk between gibberellin and auxin signaling to control fruit initiation in tomato. *The Plant Cell*, *30*(8), 1710-1728.

Hu, J., Li, S., Li, Z., Li, H., Song, W., et al. (2019). A barley stripe mosaic virus-based guide RNA delivery system for targeted mutagenesis in wheat and maize. *Molecular Plant Pathology*, *20*(10), 1463-1474.

Hua, K., Jiang, Y., Tao, X., & Zhu, J. K. (2020). Precision genome engineering in rice using prime editing system. *Plant Biotechnology Journal*, *18*(11), 2167.

Hunziker, J., Nishida, K., Kondo, A., Kishimoto, S., Ariizumi, T., et al. (2020). Multiple gene substitution by Target-AID base-editing technology in tomato. *Scientific Reports*, *10*(1), 20471.

Illouz-Eliaz, N., Nissan, I., Nir, I., Ramon, U., Shohat, H., et al. (2020). Mutations in the tomato gibberellin receptors suppress xylem proliferation and reduce water loss under water-deficit conditions. *Journal of Experimental Botany*, *71*(12), 3603-3612.

Ito, Y., Nishizawa-Yokoi, A., Endo, M., Mikami, M., & Toki, S. (2015). CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. *Biochemical and Biophysical Research Communications*, *467*(1), 76-82.

Jiang, Y. Y., Chai, Y. P., Lu, M. H., Han, X. L., Lin, Q., et al. (2020). Prime editing efficiently generates W542L and S621I double mutations in two ALS genes in maize. *Genome Biology*, *21*, 1-10.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), 816-821.

Kaul, T., Sony, S. K., Verma, R., Motelb, K. F. A., Prakash, A. T., Eswaran, M., Bharti, J., Nehra, M., & Kaul, R. (2020). Revisiting CRISPR/Cas-mediated crop improvement: Special focus on nutrition. *Journal of Biosciences*, *45*, 137.

Khan, N., Ray, R. L., Sargani, G. R., Ihtisham, M., Khayyam, M., et al. (2021). Current progress and future prospects of agriculture technology: Gateway to sustainable agriculture. *Sustainability*, *13*(9), 4883.

Kim, H., Kim, S. T., Ryu, J., Kang, B. C., Kim, J. S., et al. (2017). CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nature Communications*, *8*(1), 14406.

Kim, S., Kim, D., Cho, S. W., Kim, J., & Kim, J. S. (2014). Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Research*, *24*(6), 1012-1019.

Klap, C., Yeshayahou, E., Bolger, A. M., Arazi, T., Gupta, S. K., et al. (2017). Tomato facultative parthenocarpy results from Sl AGAMOUS-LIKE 6 loss of function. *Plant Biotechnology Journal*, *15*(5), 634-647.

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, *533*(7603), 420-424.

Koonin, E. V., Makarova, K. S., & Zhang, F. (2017). Diversity, classification and evolution of CRISPR-Cas systems. *Current Opinion in Microbiology*, *37*, 67-78.

Koseoglou, E., Hanika, K., Mohd Nadzir, M. M., Kohlen, W., Van Der Wolf, J. M., et al. (2023). Inactivation of tomato WAT1 leads to reduced susceptibility to *Clavibacter michiganensis* through downregulation of bacterial virulence factors. *Frontiers in Plant Science*, *14*, 1082094.

Kumar, M., Chandran, D., Tomar, M., Bhuyan, D. J., Grasso, S., et al. (2022). Valorization potential of tomato (*Solanum lycopersicum* L.) seed: nutraceutical quality, food properties, safety aspects, and application as a health-promoting ingredient in foods. *Horticulturae*, *8*(3), 265.

Kwon, C. T., Heo, J., Lemmon, Z. H., Capua, Y., Hutton, S. F., et al. (2020). Rapid customization of Solanaceae fruit crops for urban agriculture. *Nature Biotechnology*, *38*(2), 182-188.

Leibman-Markus, M., Gupta, R., Schuster, S., Avni, A., & Bar, M. (2023). Members of the Tomato NRC4 h-NLR family augment

each other in promoting basal immunity. *Plant Science*, *330*, 111632.

Li, H., Li, J., Chen, J., Yan, L., & Xia, L. (2020). Precise modifications of both exogenous and endogenous genes in rice by prime editing. *Molecular Plant*, *13*(5), 671-674.

Li, J., Sun, Y., Du, J., Zhao, Y., & Xia, L. (2017). Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system. *Molecular plant*, *10*(3), 526-529.

Li, Q., Feng, Q., Snouffer, A., Zhang, B., Rodríguez, G. R., et al. (2022a). Increasing fruit weight by editing a cis-regulatory element in tomato KLUH promoter using CRISPR/Cas9. *Frontiers in Plant Science*, *13*, 879642.

Li, R., Liu, C., Zhao, R., Wang, L., Chen, L., et al. (2019). CRISPR/Cas9-Mediated SlNPR1 mutagenesis reduces tomato plant drought tolerance. *BMC Plant Biology*, *19*, 1-13.

Li, R., Maioli, A., Yan, Z., Bai, Y., Valentino, D., et al. (2022b). CRISPR/Cas9-Based Knockout of the PMR4 Gene Reduces Susceptibility to Late Blight in Two Tomato Cultivars. *International Journal of Molecular Sciences*, *23*(23), 14542.

Li, R., Zhang, L., Wang, L., Chen, L., Zhao, R., et al. (2018a). Reduction of tomato-plant chilling tolerance by CRISPR–Cas9-mediated SlCBF1 mutagenesis. *Journal of Agricultural and Food Chemistry*, *66*(34), 9042-9051.

Li, X., Wang, X., Sun, W., Huang, S., Zhong, M., et al. (2022c). Enhancing prime editing efficiency by modified pegRNA with RNA G-quadruplexes. *Journal of Molecular Cell Biology*, *14*(4), mjac022.

Li, X., Wang, Y., Chen, S., Tian, H., Fu, D., et al. (2018b). Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. *Frontiers in Plant Science*, *9*, 559.

Lin, Q., Jin, S., Zong, Y., Yu, H., Zhu, Z., et al. (2021). High-efficiency prime editing with optimized, paired pegRNAs in plants. *Nature Biotechnology*, *39*(8), 923-927.

Lin, Q., Zong, Y., Xue, C., Wang, S., Jin, S., et al. (2020). Prime genome editing in rice and wheat. *Nature Biotechnology*, *38*(5), 582-585.

Liu, D., Shen, Z., Zhuang, K., Qiu, Z., Deng, H., et al. (2022). Systematic annotation reveals CEP function in tomato root development and abiotic stress response. *Cells*, *11*(19), 2935.

Liu, H., Meng, F., Miao, H., Chen, S., Yin, T., et al. (2018). Effects of postharvest methyl jasmonate treatment on main health-promoting components and volatile organic compounds in cherry tomato fruits. *Food Chemistry*, *263*, 194-200.

Liu, J., Wang, S., Wang, H., Luo, B., Cai, Y., et al. (2021). Rapid generation of tomato male-sterile lines with a marker use for hybrid seed production by CRISPR/Cas9 system. *Molecular Breeding*, *41*, 1-12.

Liu, L., Zhang, J., Xu, J., Li, Y., Guo, L., et al. (2020a). CRISPR/Cas9 targeted mutagenesis of SlLBD40, a lateral organ boundaries domain transcription factor, enhances drought tolerance in tomato. *Plant Science*, *301*, 110683.

Liu, M., Liang, Z., Aranda, M. A., Hong, N., Liu, L., et al. (2020b). A cucumber green mottle mosaic virus vector for virus-induced gene silencing incucurbit plants. *Plant Methods*, *16*, 1-13.

Lu, H. P., Liu, S. M., Xu, S. L., Chen, W. Y., Zhou, X., et al. (2017). CRISPR-S: an active interference element for a rapid and inexpensive selection of genome-edited, transgene-free rice plants. *Plant Biotechnology journal*, *15*(11), 1371.

Lu, H., Fan, Y., Yuan, Y., Niu, X., Zhao, B., et al. (2023). Tomato SlSTK is involved in glucose response and regulated by the ubiquitin ligase SlSINA4. *Plant Science*, *331*, 111672.

Lu, Y., Tian, Y., Shen, R., Yao, Q., Zhong, D., et al. (2021). Precise genome modification in tomato using an improved prime editing system. *Plant Biotechnology Journal*, *19*(3), 415.

Marzec, M., Brąszewska-Zalewska, A., & Hensel, G. (2020). Prime editing: a new way for genome editing. *Trends in Cell Biology*, *30*(4), 257-259.

Mendez-López, E., Donaire, L., Gosálvez, B., Díaz-Vivancos, P., Sánchez-Pina, M. A., et al. (2023). Tomato SlGSTU38 interacts with the PepMV coat protein and promotes viral infection. *New Phytologist*, *238*(1), 332-348.

Menz, J., Modrzejewski, D., Hartung, F., Wilhelm, R., & Sprink, T. (2020). Genome edited crops touch the market: a view on the global development and regulatory environment. *Frontiers in Plant Science*, *11*, 586027.

Milon, R. B., Hu, P., Zhang, X., Hu, X., & Ren, L. (2024). Recent advances in the biosynthesis and industrial biotechnology of Gamma-amino butyric acid. *Bioresources and Bioprocessing*, *11*(1), 32.

Mishra, R., Rout, E., & Joshi, R. K. (2019). Identification of resistant sources against anthracnose disease caused by *Colletotrichum truncatum* and *Colletotrichum gloeosporioides* in *Capsicum annuum* L. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *89*, 517-524.

Mishra, R., Zheng, W., Joshi, R. K., & Kaijun, Z. (2021). Genome editing strategies towards enhancement of rice disease resistance. *Rice Science*, *28*(2), 133-145.

Moon, S. B., Kim, D. Y., Ko, J. H., & Kim, Y. S. (2019). Recent advances in the CRISPR genome editing tool set. *Experimental & Molecular Medicine*, *51*(11), 1-11.

Nekrasov, V., Wang, C., Win, J., Lanz, C., Weigel, D., et al. (2017). Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Scientific Reports*, *7*(1), 482.

Nie, H., Shi, Y., Geng, X., & Xing, G. (2022). CRISRP/Cas9-mediated targeted mutagenesis of tomato polygalacturonase gene (SlPG) delays fruit softening. *Frontiers in Plant Science*, *13*, 729128.

Nishida, K., Arazoe, T., Yachie, N., Banno, S., Kakimoto, M., et al. (2016). Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science*, *353*(6305), aaf8729.

Nonaka, S., Arai, C., Takayama, M., Matsukura, C., & Ezura, H. (2017). Efficient increase of ɣ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. *Scientific Reports*, *7*(1), 7057.

Ortigosa, A., Gimenez-Ibanez, S., Leonhardt, N., & Solano, R. (2019). Design of a bacterial speck resistant tomato by CRISPR/Cas9-mediated editing of Sl JAZ 2. *Plant Biotechnology Journal*, *17*(3), 665-673.

Perk, E. A., Arruebarrena Di Palma, A., Colman, S., Mariani, O., Cerrudo, I., et al. (2023). CRISPR/Cas9-mediated phospholipase C 2 knockout tomato plants are more resistant to *Botrytis cinerea*. *Planta*, *257*(6), 117.

Perroud, P. F., Guyon-Debast, A., Veillet, F., Kermarrec, M. P., Chauvin, L., et al. (2022). Prime Editing in the model plant *Physcomitrium patens* and its potential in the tetraploid potato. *Plant Science*, *316*, 111162.

Pramanik, D., Shelake, R. M., Park, J., Kim, M. J., Hwang, I., et al. (2021). CRISPR/Cas9-mediated generation of pathogen-resistant tomato against tomato yellow leaf curl virus and powdery mildew. *International Journal of Molecular Sciences*, *22*(4), 1878.

Qin, R., Li, J., Li, H., Zhang, Y., Liu, X., et al. (2019). Developing a highly efficient and wildly adaptive CRISPR-SaCas9 toolset for plant genome editing. *Plant Biotechnology Journal*, *17*(4), 706.

Rather, G. A., Ayzenshtat, D., Teper-Bamnolker, P., Kumar, M., Forotan, Z., et al. (2022). Advances in protoplast transfection promote efficient CRISPR/Cas9-mediated genome editing in tetraploid potato. *Planta*, *256*(1), 14.

Ren, Q., Sretenovic, S., Liu, S., Tang, X., Huang, L., et al. (2021). PAM-less plant genome editing using a CRISPR–SpRY toolbox. *Nature Plants*, *7*(1), 25-33.

Rodríguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E., & Lippman, Z. B. (2017). Engineering quantitative trait variation for crop improvement by genome editing. *Cell*, *171*(2), 470-480.

Roosjen, M., Paque, S., &Weijers, D. (2018). Auxin response factors: output control in auxin biology. *Journal of Experimental Botany*, *69*(2), 179-188.

Ruiz-Ramón, F., Rodríguez-Sepúlveda, P., Bretó, P., Donaire, L., Hernando, Y., et al. (2023). The tomato calcium-permeable channel 4.1 (SlOSCA4. 1) is a susceptibility factor for pepino mosaic virus. *Plant Biotechnology Journal*, *21*(10), 2140-2154.

Santillán Martínez, M. I., Bracuto, V., Koseoglou, E., Appiano, M., Jacobsen, E., et al. (2020). CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene PMR4 for resistance against powdery mildew. *BMC Plant Biology*, *20*, 1-13.

Scholefield, J., & Harrison, P. T. (2021). Prime editing–an update on the field. *Gene Therapy*, *28*(7), 396-401.

Shen, W., Zhang, J., Geng, B., Qiu, M., Hu, M., et al. (2019). Establishment and application of a CRISPR–Cas12a assisted genome-editing system in *Zymomonas mobilis*. *Microbial Cell Factories*, *18*, 1-11.

Shimatani, Z., Kashojiya, S., Takayama, M., Terada, R., Arazoe, T., et al. (2017). Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nature Biotechnology*, *35*(5), 441-443.

Shu, P., Li, Y., Xiang, L., Sheng, J., & Shen, L. (2023). SlNPR1 modulates chilling stress resistance in tomato plant by alleviating oxidative damage and affecting the synthesis of ferulic acid. *Scientia Horticulturae*, *307*, 111486.

Shu, P., Li, Z., Min, D., Zhang, X., Ai, W., et al. (2020). CRISPR/Cas9-mediated SlMYC2 mutagenesis adverse to tomato plant growth and MeJA-induced fruit resistance to *Botrytis cinerea*. *Journal of Agricultural and Food Chemistry*, *68*(20), 5529-5538.

Soyk, S., Müller, N. A., Park, S. J., Schmalenbach, I., Jiang, K., et al. (2017). Variation in the flowering gene SELF PRUNING 5G promotes day-neutrality and early yield in tomato. *Nature genetics*, *49*(1), 162–168. https://doi.org/10.1038/ng.3733.

Swinnen, G., Mauxion, J. P., Baekelandt, A., De Clercq, R., Van Doorsselaere, J., et al. (2022). SlKIX8 and SlKIX9 are negative regulators of leaf and fruit growth in tomato. *Plant Physiology*, *188*(1), 382-396.

Thomazella, D. P. D. T., Seong, K., Mackelprang, R., Dahlbeck, D., Geng, Y., et al. (2021). Loss of function of a DMR6 ortholog in

tomato confers broad-spectrum disease resistance. *Proceedings of the National Academy of Sciences*, *118*(27), e2026152118.

Tieman, D., Zhu, G., Resende Jr, M. F., Lin, T., Nguyen, C., et al. (2017). A chemical genetic roadmap to improved tomato flavor. *Science*, *355*(6323), 391-394.

Toda, E., Koiso, N., Takebayashi, A., Ichikawa, M., Kiba, T., et al. (2019). An efficient DNA-and selectable-marker-free genome-editing system using zygotes in rice. *Nature Plants*, *5*(4), 363-368.

Tomlinson, L., Yang, Y., Emenecker, R., Smoker, M., Taylor, J., et al. (2019). Using CRISPR/Cas9 genome editing in tomato to create a gibberellin-responsive dominant dwarf DELLA allele. *Plant Biotechnology Journal*, *17*(1), 132-140.

Tran, M. T., Doan, D. T. H., Kim, J., Song, Y. J., Sung, Y. W., et al. (2021). CRISPR/Cas9-based precise excision of SlHyPRP1 domain (s) to obtain salt stress-tolerant tomato. *Plant Cell Reports*, *40*, 999-1011.

Tsanova, T., Stefanova, L., Topalova, L., Atanasov, A., &Pantchev, I. (2021). DNA-free gene editing in plants: a brief overview. *Biotechnology & Biotechnological Equipment*, *35*(1), 131-138.

Ueta, R., Abe, C., Watanabe, T., Sugano, S. S., Ishihara, R., et al. (2017). Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Scientific Reports*, *7*(1), 507.

Veillet, F., Perrot, L., Chauvin, L., Kermarrec, M. P., Guyon-Debast, A., et al. (2019). Transgene-free genome editing in tomato and potato plants using agrobacterium-mediated delivery of a CRISPR/Cas9 cytidine base editor. *International Journal of Molecular Sciences*, *20*(2), 402.

Vu, T. V., Sivankalyani, V., Kim, E. J., Doan, D. T. H., Tran, M. T., et al. (2020). Highly efficient homology-directed repair using CRISPR/Cpf1-geminiviral replicon in tomato. *Plant Biotechnology Journal*, *18*(10), 2133-2143.

Waltz, E., (2022). GABA-enriched tomato is first CRISPR-edited food to enter market. *National Biotechnology*, *40*(1), 9-11.

Wan, L., Wang, Z., Tang, M., Hong, D., Sun, Y., et al. (2021). CRISPR-Cas9 gene editing for fruit and vegetable crops: strategies and prospects. *Horticulturae*, *7*(7), 193.

Wang, D., Samsulrizal, N. H., Yan, C., Allcock, N. S., Craigon, J., et al. (2019). Characterization of CRISPR mutants targeting genes modulating pectin degradation in ripening tomato. *Plant Physiology*, *179*(2), 544-557.

Wang, L., Chen, L., Li, R., Zhao, R., Yang, M., et al. (2017). Reduced drought tolerance by CRISPR/Cas9-mediated SlMAPK3 mutagenesis in tomato plants. *Journal of Agricultural and Food Chemistry*, *65*(39), 8674-8682.

Wang, R., Angenent, G. C., Seymour, G., & de Maagd, R. A. (2020). Revisiting the role of master regulators in tomato ripening. *Trends in Plant Science*, *25*(3), 291-301.

Wang, R., Liu, K., Tang, B., Su, D., He, X., et al. (2023). The MADS-box protein SlTAGL1 regulates a ripening-associated SlDQD/SDH2 involved in flavonoid biosynthesis and resistance against *Botrytis cinerea* in post-harvest tomato fruit. *The Plant Journal*, *115*(6), 1746-1757.

Wang, T., Deng, Z., Zhang, X., Wang, H., Wang, Y., et al. (2018). Tomato DCL2b is required for the biosynthesis of 22-nt small RNAs, the resulting secondary siRNAs, and the host defense against ToMV. *Horticulture Research*, *5*, 62. https://doi.org/10.1038/s41438-018-0073-7

Wang, Z., Hong, Y., Li, Y., Shi, H., Yao, J., et al. (2021). Natural variations in SlSOS1 contribute to the loss of salt tolerance during tomato domestication. *Plant Biotechnology Journal*, *19*(1), 20.

Wei, J., & Li, Y. (2023). CRISPR-based gene editing technology and its application in microbial engineering. *Engineering Microbiology*, *3* (4), 100101. https://doi.org/10.1016/j.engmic.2023.100101.

Woo, J. W., Kim, J., Kwon, S. I., Corvalán, C., Cho, S. W., et al. (2015). DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nature Biotechnology*, *33*(11), 1162-1164.

Xu, D., Liu, X., Guo, C., Lin, L., & Yin, R. (2023). The B-box transcription factor 4 regulates seedling photomorphogenesis and flowering in tomato. *Scientia Horticulturae*, *309*, 111692.

Xu, J., Hua, K., & Lang, Z. (2019). Genome editing for horticultural crop improvement. *Horticulture Research*, *6*, 113.

Xu, R., Li, J., Liu, X., Shan, T., Qin, R., & Wei, P. (2020a). Development of Plant Prime-Editing Systems for Precise Genome Editing. *Plant communications*, *1*(3), 100043. https://doi.org/10.1016/j.xplc.2020.100043

Xu, X., Yuan, Y., Feng, B., & Deng, W. (2020b). CRISPR/Cas9-mediated gene-editing technology in fruit quality improvement. *Food Quality and Safety*, *4*(4), 159-166

Yang, Q., Wan, X., Wang, J., Zhang, Y., Zhang, J., Wang, T., Yang, C., & Ye, Z. (2020). The loss of function of HEL, which encodes a cellulose synthase interactive protein, causes helical and vine-like growth of tomato. *Horticulture research*, *7*(1), 180. https://doi.org/10.1038/s41438-020-00402-0.

Yang, T., Ali, M., Lin, L., Li, P., He, H., et al. (2023). Recoloring tomato fruit by CRISPR/Cas9-mediated multiplex gene editing. *Horticulture research*, *10*(1), uhac214. https://doi.org/10.1093/hr/uhac214.

Yin, Y., Qin, K., Song, X., Zhang, Q., Zhou, Y., et al. (2018). BZR1 transcription factor regulates heat stress tolerance through FERONIA receptor-like kinase-mediated reactive oxygen species signaling in tomato. *Plant and Cell Physiology*, *59*(11), 2239-2254.

Yoon, Y. J., Venkatesh, J., Lee, J. H., Kim, J., Lee, H. E., et al. (2020). Genome editing of eIF4E1 in tomato confers resistance to pepper mottle virus. *Frontiers in Plant Science*, *11*, 1098.

Yu, W., Wang, L., Zhao, R., Sheng, J., Zhang, S., Li, R., & Shen, L. (2019). Knockout of SlMAPK3 enhances tolerance to heat stress involving ROS homeostasis in tomato plants. *BMC Plant Biology*, *19*, 1-13.

Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., et al. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell*, *163*(3), 759-771.

Zhang, S., Shen, J., Li, D., & Cheng, Y. (2021a). Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. *Theranostics*, *11*(2), 614.

Zhang, Y., Restall, J., Crisp, P., Godwin, I., & Liu, G. (2021b). Current status and prospects of plant genome editing in Australia. *In Vitro Cellular & Developmental Biology-Plant*, *57*(4), 574-583.

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Unveiling the positive impacts of the genus *Rhodococcus* on plant and environmental health

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ABSTRACT

Organic farming has emerged as a sustainable solution to the adverse effects (diminished nutritional value, compromised food quality, environmental contamination, and public health hazards) that are usually associated with harmful chemical pesticides. To overcome such loss, one must explore the plant-associated microbes that are the naturally occurring root commensal and could positively improve crop health. In this review, we highlight the importance of the bacterial genus Rhodococcus, a subset of Actinobacteria that carries immense potential in enhancing crop yield and is associated with bioremediation of toxic pesticides and other chemicals to improve soil health. However, it has been noticed that few species of *Rhodococcus* are pathogenic for the plant (*R. fascians*) as well as humans/animals (*R. equi*). But still, the majority of *Rhodococcus* isolates are found to be non-pathogenic and carry substantial beneficial traits. Here, we have attempted to comprise those beneficial traits of the different members of the genus *Rhodococcus*. The main emphasis of this review article is to explore the major areas such as enzyme production, phytohormone synthesis, growth regulation, siderophore production, bioremediation, organic compound degradation, and environmental pollution control. Opinions towards the applications of advanced methodologies for utilizing the cumulative prospective potential of the genus *Rhodococcus* have also been discussed in the different sections of the review. Conclusively, this article gathers the scattered information from the past and recent literature about this bacteria and provides the future direction about how it can improve plant/soil health and eliminate toxic chemicals and environmental pollutants.

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1 Introduction

Modern agriculture practices have reached a state where the food demands are not easy to fulfil due to the increasing global population and agricultural land crisis. Therefore, no other option was left to overuse chemical fertilizers and harmful pesticides to increase crop yield. However, these hazardous chemicals can adversely affect human and environmental health (Islam and Karim 2020). Therefore, a pressing need is required to draw attention to organic farming and put a hold on the over-usage of these kinds of chemicals. Recent reports have indicated the role of symbiotic microbes in tackling abiotic and biotic stress in plants (Sharma et al. 2024) and approaching a healthy environment (Hartman et al. 2023). The present review has discussed the diverse roles played by the different members of the genus *Rhodococcus* (an aerobic, nonsporulating, nonmotile Gram-positive bacteria closely related to *Mycobacterium* and *Corynebacterium*) for various applications in the improvement of plant/soil health as well as biodegradation of toxic chemical fertilizers, pesticides, and environmental pollutants. Marginal pathogenicity of certain *Rhodococcus* species (*R. equi* and *R. fascians*) has also been reported, but predominantly existing Rhodococcus species are found non-pathogenic (Vázquez-Boland and Meijer 2019). The major highlights of this review is on (i) aniline (hazardous component of chemical pesticides) bioremediation by the *Rhodococcus* species (*A-deg 1* and *A-deg 2*) (Pande et al. 2022), (ii) p-nitrophenol and 2,4-dinitrophenol (2-DNP) degradation by *Rhodococcus RKJ300T* strain (Ghosh et al. 2010) as well as 2,4-DNP by the *Rhodococcus XM24D* strain (Hu et al. 2021), (iii) *Rhodococcus*-mediated breakdown of neonicotinoids, glyphosate, pesticides, and herbicides (Pang et al. 2020), (iv) role in siderophore-mediated metal detoxification, and role of quorum sensors and quenchers in microbiome (Saeed et al. 2021), and (v) enzymatic degradation of plastic (Zampolli et al. 2022). Conclusively, mechanistic investigation of the role of different *Rhodococcus* species in such important microbe-plant as well as microbe-environmental bio-processes can provide clues about the better understanding of genetic/molecular counterparts that will help advance the utility of *Rhodococcus* in sustainable agriculture and also in environmental bioremediation. In this direction, the present review has been written to gather recent and relevant information about the role of *Rhodococcus* in these versatile biological processes involved in maintaining plant health, bioremediation of toxic chemicals from soil/water, and environmental eco-balancing.

2 *Rhodococcus* in pesticide and insecticide degradation

Chemical-derived micronutrients, fertilizers, insecticides, and pesticides have been used widely to enhance crop productivity and fulfil the food demands of modern agriculture practices. This over-usage of such chemical products leads to their bioaccumulation in the plant product, soil, water, and environment, and ultimately could cause disastrous hazards for the animal kingdom in many ways. Among these chemicals, aniline is one of them, which is a breakdown product of several pesticides and herbicides (Chaturvedi 2022).

2.1 Aniline compounds degradation by *Rhodococcus*

One must target the utility of *Rhodococcus* bacteria in microbial bioremediation, where this bacterium can alternatively use the aniline as a source of nitrogen and carbon. Two strains of *Rhodococcus* (*R.A-deg11* and *R.A-deg-2*) can degrade aniline, as summarized in Figure 1 (Krivoruchko et al. 2023). However, the upper limit of aniline concentrations that Rhodococcus can detoxify is still unknown, which makes it difficult to use *Rhodococcus* strains for microbial-based aniline degradation. Therefore, we believe targeting the successful application of these bacteria in the agriculture sector as a set of safety and toxicity assessments is always necessary.

2.2 Nitro/para-nitro phenols degradation by *Rhodococcus*

Rhodococcus bacteria can also degrade p-nitrophenol and 4-NP (Takeo et al. 2018). However, in pesticide-affected areas, *R. imtechensis (RKJ300T)* efficiently reduces p-nitrophenol (PNP) and 2,4-dinitrophenol (2,4-DNP), demonstrating the ability of such microbes to decontaminate the soil from these toxic chemicals as shown in Figure 1 (Ghosh et al. 2010). Four enzymes in the *R. imtechensis RKJ300T* strain catalyze the conversion of 4-NP to maleylacetate (Santillan et al. 2020), and *R. rhodochrous* degrades malathion, but the involved genes and mechanisms are still unknown (Wrońska et al. 2016). Therefore, a better understanding of the mechanisms by which *R. imtechensis (RKJ300T)* degrades PNP and 2,4-DNP is required (Mawang et al. 2021). 4-nitrophenol (4-NP) is a chemical which is present in insecticides and fungicides, can cause headaches, tiredness, nausea, and cyanosis (a bluish colouring of the lips, ears, and fingernails), and it can irritate the eyes. The *Rhodococcus BUPNP1* strain produces an enzyme that can break down and utilize 4-NP as a carbon source for its growth and survival when no carbon and nitrogen source is available. Bacterial enzymes that can capably degrade the chemical substances (4-NP, 4-nitrocatechol (4-NC) and 1,2,4-benzenetriol) as carbon source belongs to the category of monooxygenases (MO). These MO enzymes in bacteria can capably convert 4-NP into 4-NC and trigger the release of nitrite ions to initiate the breakdown of 4-NP. Therefore, bacteria that contain MO can grow in the presence of 4-NP, 4-NC, and 1,2,4-benzenetriol. A progressive degradation primarily degrades 4-NP and subsequently carries out 4-NC degradation (Sengupta et al. 2019a). However, the cellular enzymes responsible for such processes stepwise have been identified earlier (Brookbank et al. 2021). But, in a recent study, MO enzymes have been mentioned as a responsible

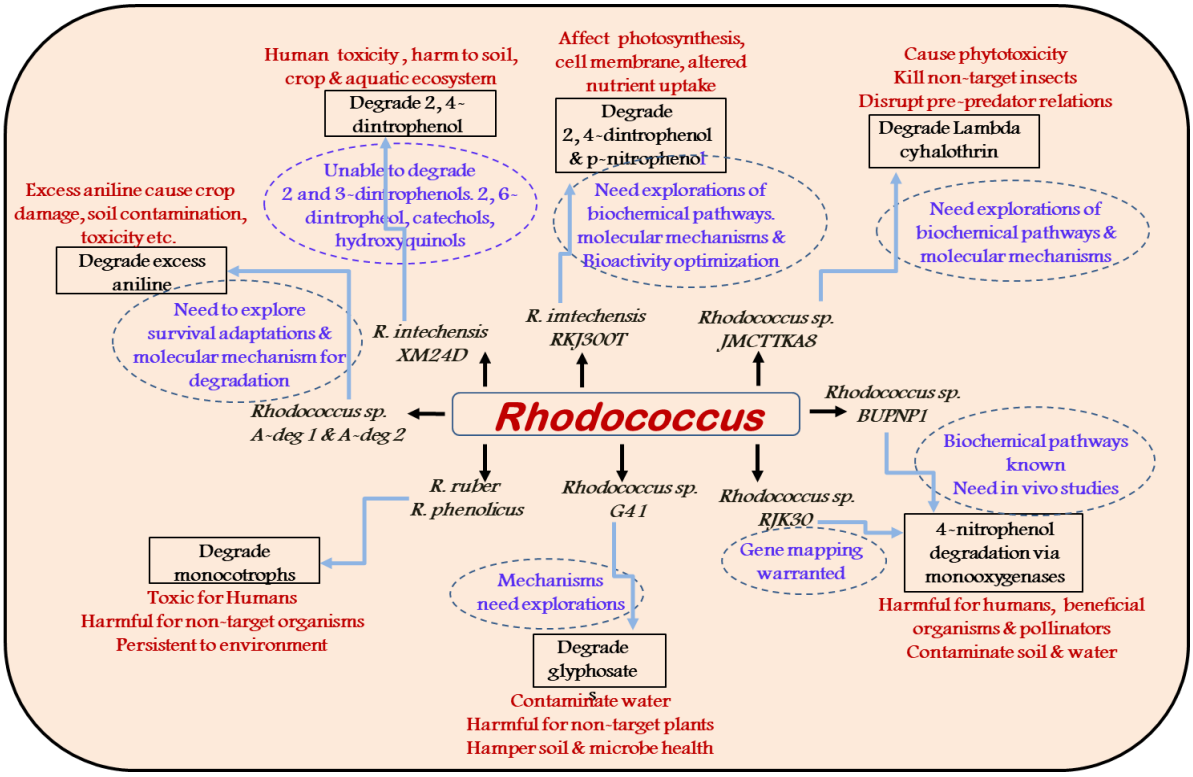

Figure 1 Schematic representation of the role of *Rhodococcus* species in pesticide and insecticide degradation as discussed briefly in the above section

molecule for the conversion of 4-NP into the less toxic, water-soluble compound (4-NC) and releases nitrite ions via synthesizing an unstable intermediate, which is essential for the process of 4-NP breakdown (Hecko et al. 2023). Interestingly, it was reported by Sengupta et al. (2019a) that the highest activity of MO was observed during the early exponential growth phase. In contrast, the abundance of alcohol dehydrogenase (ADH) was observed during the exponential growth phase of the *Rhodococcus BUPNP1* strain, and this enzyme can cause the breakdown of 4-NP. However, a deep understanding of these enzymes and associated pathways involved in bacteria-mediated degradation of 4-NP can be investigated by looking into the protein-protein interactions, pathway analysis, and conservancy (Kaushik et al. 2022). A better understanding of the concept of developing microbial consortia to enhance the potential of the 4-NP degradation can be achieved by assembling a variety of bacterial strains to break down contaminants collectively in a much better way (Zhang and Zhang 2022). The degradation of 4-NP by *Rhodococcus FXJ9.536* strain at different temperatures was evaluated, and it was observed that the degradation was optimal at 28°C but less effective at 10°C. During the degradation process, intermediates 4-nitrocatechol and 1,2,4-benzenetriol have been identified (Huang et al. 2022). The *R. tibetensis FXJ9.536* genes peg.5460, and peg.3921 were involved in 4-NP degradation (Figure 1). The lower degradation at 10°C may be due to decreased enzyme activity. The breakdown of 4-NP by *Rhodococcus* bacteria is dependent on temperatures between 28-30°C. The gene encoding 4-hydroxyphenyl acetate 3-monooxygenase was found in cold-adapted *Rhodococcus* strains, including *R. erythropolis* and *R. qingshengii*, possibly associated with the degradation of 4-NP (Bordin et al. 2021).

It has been reported that in the pesticides and industrial waste polluted areas, two chemical compounds (PNP and 2,4-DNP) are present predominantly (Hu et al. 2021). The strain *Rhodococcus RKJ300* was isolated from pesticide-laden Punjab State soils at 30°C via a serial dilution plating method. These bacteria grow well on minimal agar media supplemented with 0.5 mM PNP or 2,4-DNP as carbon and energy sources, representing that the *Rhodococcus RKJ300* strain can capably degrade PNP and 2,4-DNP (Ghosh et al. 2006). Compound 2,4-DNP contains two nitrogen groups on its benzene ring, the most often used pesticides, herbicides, and fungicides (Aslam et al. 2023). Meanwhile, the *Rhodococcus XM24D* strain utilizes 2,4-DNP as a source of carbon and nitrogen (Figure 1). Interestingly, the *Rhodococcus XM24D* strain can break down 4-PNP and 2C4NP efficiently but is incapable of degrading 2,6-NP, 2-NP, 3-NP, 4-nitrocatechol, hydroxyquinol, catechol and hydroquinone (Xiang et al. 2022). Research on the *Rhodococcus XM24D* strain that exposed to 2,4-DNP stress has revealed that 12 out of 17 genes in the relevant pathway were remain unregulated, including NADPH-dependent

F420 oxidoreductase (dnpB), hydrolase (dnpC), and coenzyme F420 biosynthesis pathway genes. The gene cluster comprises five PNP degradation genes of the *Rhodococcus XM24D* strain. The PNP and 2C4NP degradation pathways predominantly enhance the expression of these genes. Like the *R. imtechensis* and *R. RKJ300* strains, the *R. XM24D* strain degrades PNP and mineralizes 2C4NP (Alam and Saha 2022). Furthermore, the ecological consequences of *R. XM24D* must be addressed to ensure its mutually beneficial coexistence in the environment. In-depth molecular biology studies may reveal the breakdown processes of 2,4-DNP, PNP, and 2-C-4-NP to understand and exploit metabolic regulation (Kim et al. 2018; Swangjang 2022). However, using *R. JMCTTKA8* strain is promising in approaching the bioremediation of a persistent insecticide (lambda-cyhalothrin) (Sakr and Rashad 2023). Therefore, exploring the use of microbes in bioremediation processes under variable environmental conditions can be approached, which is certainly achieved by using *Rhodococcus* bacteria (Raffa and Chiampo 2021; Thi Mo et al. 2022). However, better utilization of such chemicals in minimal amounts for pest management can be achieved by the adoption of a new approach (Yousef et al. 2023), which can help to synthesize the advanced active formulation of such chemicals ingredients and thus reduce hazardous effects of chemicals on the environment (Camara et al. 2019). Conventional pest control methods are not enough to decompose such chemicals, and the risk of bioaccumulation in the environment of these chemicals remains on high alert (Okoye et al. 2022).

2.3 Monocrotophos degradation by *Rhodococcus*

Neonicotinoids are another popular class of pesticides used in more than 120 countries globally. They destroy pests by targeting their central nervous system (Costas-Ferreira and Faro 2021), and most of the neonicotinoid treatments also protect plant seedlings, increasing Bt toxin tolerance(D'Ambrosio et al. 2020). According to Parte and Kharat (2019) imidacloprid and clothianidin have their respective half-lives of 3000 and 6931 days. Meanwhile, thiamethoxam and acetamiprid have 353 and 450 days of soil half-live, respectively (Bhattacherjee et al. 2020). These chemicals enter the water sources and subsequently enter the food chain. The long environmental lifespan and negative impacts of these chemicals on unspecific insects raised concerns about their applications in the agricultural sector. It also affects the population of essential pollinators, including bees, butterflies, dragonflies, and wild bees, which have lost their behaviour, immunity, and survival. Increased neonicotinoid use has also been connected to bird population reduction. Only a portion of the active chemical protects the crops, while the remaining compound is dispersed in the environment, harming soil microbial populations and their living creatures, including earthworms, amphibians, and aquatic insects (Glen-Karolczyk et al. 2021). Recent studies have shown that certain bacteria can break down the neonicotinoids (imidacloprid and acetamiprid) (Thi Mo et al. 2022). Bacteria (*R. phenolicus and R. ruber*), isolated from pesticide-contaminated soils can break down monocrotophos and mineralize acetochlor in six days (Ahirwar 2023). Acetamiprid biodegradation is efficiently increased by adding carbon and nitrogen sources (Xu et al. 2020; Rasool et al. 2022). *Rhodococcus* bacteria can metabolize chemical substances such as neonicotinoids. However, the capacity of *Rhodococcus* bacteria to break down the pesticides and its adaptation to the soil environment, as well as its interactions with other microorganisms in the soil, can only determine its utility for neonicotinoid bioremediation (Dai et al. 2021).

2.4 Glyphosates degradation by *Rhodococcus*

Many organophosphate herbicides in agriculture and forestry use glyphosate to suppress broadleaf weeds and grasses. However, environmental concerns have been raised because of its impact on soil health. Response surface methodology (RSM) involves optimizing glyphosate degradation to decrease environmental and health issues (Firdous et al. 2020). There are two major pathways for degrading glyphosate. One of the pathways involves glyphosate oxidoreductase, which breaks the C-N bond and releases glyoxylate. The second pathway uses C-P bond lyase to convert glyphosate into sarcosine and inorganic phosphorus. Sarcosine oxidase converts it to glycine, an amino acid that can be further utilized for protein synthesis (Singh et al. 2020). Inorganic phosphate in the soil can prevent glyphosate's bacterial degradations and generate toxic aminomethylphosphonic acid (AMPA) (Sun et al. 2019). Therefore, efforts are required to find solutions for safe and efficient glyphosate degradation and removal of AMPA residues (Castrejón-Godínez et al. 2021). Interestingly, reports have demonstrated that the *R. soli G41* strain degraded 42.7% of glyphosate in seven days, and this bacterium can grow very well and attain maximum optical density at 30°C and pH 7.0. The *R. soli G41* strain reduced glyphosate in nonsterile soil and enhanced weeds' germination by 10% in contaminated soil, where weeds have shown soil healing potential (Nguyen et al. 2022). Another study also revealed that *R. soli G41* contains an enzyme (sarcosine oxidase) and utilizes sarcosine instead of AMPA, which is more environmentally friendly (Pérez Rodríguez et al. 2019). Sarcosine oxidase is essential for converting glyphosate to phosphate by C-P lyase. The soxA gene in *R. soli G41* was 98.9% similar to that in *R. opacus* and included sarcosine oxidase; potentially, this newly identified *R. soli G41* strain may help remediate glyphosate-contaminated soil (Pérez Rodríguez et al. 2019).

3 *Rhodococcus* in AHL degradation mediated quorum quenching

In microbes, N-acyl homoserine lactone (AHL) degradation has been reported as a novel method for quorum quenching (QQ) by using the enzyme (lactonases and acylases) (Kusada et al. 2017).

Exploring the QQ pathway in disease-specific pathogenic bacteria may be beneficial in reducing bacterial resistance. Importantly, the *Rhodococcus* bacteria can break down AHL through certain enzymatic degradative pathways (Figure 2). However, targeting the QQ employing *Rhodococcus* enzymes (lactonases and acylases) may help inhibit the biofilm formation in pathogenic bacteria (Murugayah and Gerth 2019; Sikdar and Elias 2020). This strategy has been meaningful for reducing the disease burden caused by pathogenic microbes and maintaining the balance between pathogenic and non-pathogenic bacterial populations. Because biofilms are essential for the colonization and survival of bacteria, these QQ mechanisms in *Rhodococcus* species have demonstrated a great potential for inhibiting biofilm formation (Paluch et al. 2020). *Rhodococcus* species enriched with AHL-degrading enzymes have demonstrated predominant expression of oxidoreductases (Ryu et al. 2020). AHLs are involved in the quorum sensing-mediated pathogenicity of certain pathogenic bacteria and biofilm formation, and the ability of the *R. erythropolis* W2 strain to destroy these bacteria depends on the simultaneous activity of different QQ enzymes (Figure 2) (Chakraborty et al. 2023). When the production of AHL in a pathogenic species, such as *E. caratovora*, is hampered, survival diminishes, decreasing its pathogenicity. Interestingly, *Rhodococcus* strains (*LS31* and *PI33*) have shown strong inhibitory effects on OHHL and pectate lyase activity when grown together with *E. carotovora* (Sarveswari and Solomon 2019). In the *Rhodococcus* strain *LS31*, one can more clearly understand the suppression mechanism of quorum sensors by lactonases and acylases-mediated AHL breakdown (Park et al. 2006). In other species of *Agrobacterium*, AHLases such as AttM can digest c-butyrolactone, but the mechanism is still elusive. Therefore, it was earlier directed that a deeper mechanistic understanding of the relationship between lactonase and acylase enzymes must be explored (Kumar et al. 2022). The ability of the *R. erythropolis W2* strain to degrade AHLs reflects the relevance of AHL turnover under varying environmental conditions where the length of the acyl chain affects the level of AHL degradation (Utari et al. 2017). Biochemical pathways analysis of the enzymes produced by different *Rhodococcus* strains can develop new approaches to control the AHL mediated pathogenicity in microbes (Rehman et al. 2022). However, LC-ESI/MS employed investigations on AHL degradation by *Rhodococcus* strains (*LS31* and *PI33*) demonstrated that the *Rhodococcus LS31* synthesize several byproducts from N-hexanoyl-L-homoserine lactone (HHL) more rapidly and enhance AHL degradation (Taşkan and Taşkan 2021). Such observations indicated the putative role of AHLases in releasing homoserine lactone rings that indirectly affect the density of the final product.

On the other side, bioremediation traits of *Rhodococcus* bacteria were demonstrated by agar-based immobilization assays carried out on synthetic polymers where biphenyl metabolization was mediated with the help of limonene (Tyumina et al. 2023). Cumin oil containing cumin aldehyde and cumene initiates the co-metabolization process of trichloroethylene by Rhodococcus strain

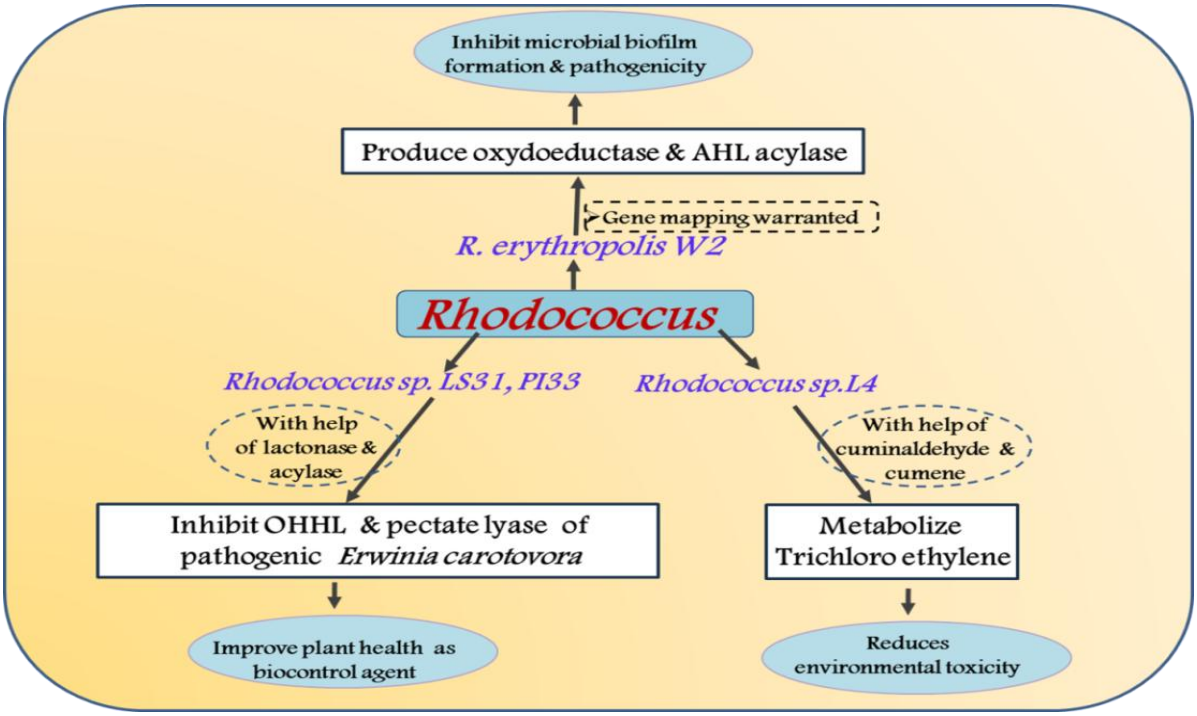

Figure 2 Illustration of the role of the symbiotic bacterium *Rhodococcus* in providing plant benefits and eco-protection via enhanced quorum quenching.

L4, indicating that one pathway could be targeted for environmental detoxification (Suttinun et al. 2010). If we overlook in total, enzymes (lactonases and acylases) have been the key players in QQ processes (Dubert et al. 2017). The complicated enzymatic pathways of *Rhodococcus* strains highlight their potential in modulating quorum sensing, microbial behaviour, and responses to environmental stressors (Barbey et al. 2018). The degradation of AHL has several potential uses, from preventing disease spread to cleaning polluted areas by *Rhodococcus* bacteria (Prazdnova et al. 2022).

4 *Rhodococcus* as a siderophore producer and metal scavenger

Siderophores are complex molecules with remarkable metal-binding abilities, and they play a crucial role in many biological processes, such as metal-ion metabolism and degradation (Gomes et al. 2024). Siderophore production by different *Rhodococcus* species (*R. qingshengii*, *R. erythropolis*, and *R. jostii*) has taken the lead in revealing the complex gene clusters and metabolic pathways that could control the metabolism of metal-containing compounds (Khilyas et al. 2021). The reported literature has demonstrated that the *Rhodococcus* metabolites (heterobactins and rhequichelin) are hydroxamate- and catecholate-type siderophores, respectively. Two nonribosomal peptide synthases (NRPSs) and siderophore-producing clusters have sequence homology (da Silveira et al. 2020). The heterobactin gene clusters of *R. qingshengii BKS* 20-40, the *Rhodococcus* strain *ADH*, and *R. erythropolis SK121* have 100% identity, while the erythrochelin gene cluster shares 57%. *R. qingshengii S10* produces siderophores in liquid M9 medium and on chrome azurol S (CAS) agar under iron-deficient conditions. The bacteria grow and produce catalase and urease in the presence of NaCl at pH values ranging from 5 to 9. The *R. qingshengii S10* genome contains twelve trehalose-producing genes that protect against desiccation, temperature variations, and high salinity (Khilyas et al. 2021). However, a deeper understanding of the functional differences between these clusters, especially genes with different sequences, could shed light on the factors that lead to siderophore diversity and their distinct roles. Rhodochelin, a principal secondary metabolite, was identified, and genome analysis revealed the responsible gene clusters. The rhodochelin-producing cluster (rhc) contains the entire bimodular NRPS synthetase, rhcB, and genes for export and import (rhcC, D, F). The crucial enzyme isochorismatase (rhcA) is in the rhc cluster. In an operon with rhcA, a dhbE-like gene is near two additional 2,3-DHB-producing genes, dhbC and dhbA. This operon covers the 2,3-DHB pathway from chorismate to adenylate (Puja et al. 2023). Siderophore synthesis was reduced in a gene knockout study targeting key genes in clusters (rhcB, dhbE, rmo, and rft) (Yin et al. 2023). *R. erythropolis B7g* has shown promise in siderophore synthesis and metal interactions (Retamal-Morales et al. 2018). Genome investigation of *R. erythropolis B7g* revealed two siderophore biosynthesis-related gene clusters. One cluster produces heterobactins and mixed catecholate-hydroxamate siderophores, while the other produces requichelin siderophores, whose nature is unknown. When. *R erythropolis B7g* was grown in a specialized medium to produce apo-heterobactin S2 and heterobactin B as siderophores. Optimizing the growth conditions with supplementation with glucose, n-hexadecane, and casamino acids increased the production of siderophores. For instance, adding 30 mM glucose and 1% casamino acids to 1 L culture medium increased the siderophore concentration and improved production efficiency (Srimathi and Suji 2019). Complete genomic and transcriptomic analyses of both symbiotic partners (plants and microbes) that take up nutrients via siderophores are necessary (Hofmann et al. 2021).

A study investigated siderophore production by the bacterium *R. jostii RHA1* under various growth conditions revealed. It was observed that *R. jostii RHA1* produced siderophores in response to iron deficiency, as demonstrated by a positive CAS assay (Srimathi and Suji 2019). In the subsequent step, CAS-positive supernatant was purified using ion exchange chromatography to identify siderophores. These iron-chelating chemical peaks were observed in the HPLC chromatogram. These peaks were misplaced under iron-rich growth conditions, confirming their association with iron scarcity. Liquid chromatography-mass spectrometry (LC–MS) revealed the behaviours of these peaks and their expression patterns. One peak resembled that of rhodochelin (a secretory bacterial siderophore), whereas a companion peak resembled that of rhodochelin, which lacked a modified ornithine residue (Pathak et al. 2024). Thus, *Rhodococcus* bacteria play a pivotal role in sustainable agriculture through siderophore production. The siderophore activity of *R. jostii* was enhanced by its interaction with 4HBA (Yasin et al. 2023). *R. rhodochorus GD02* can produce siderophores essential for iron uptake and metabolism. Interestingly, enzyme 4HBA plays a significant role in siderophore synthesis, particularly under iron-limited conditions. When iron is abundantly available, siderophore production is inhibited (Pathak et al. 2024). It was also evident that the *R. jostii* RHA1 gene, which produces rhodochelin, was similar to the gene cluster that produces 2,3-DHB in *R. rhodochorus GD02* but had different siderophore expression profiles. NRPS genes, which are important for siderophore production, were identified in *R. rhodochorus GD02* but are poorly characterized. Additionally, anti-SMASH analysis could identify secondary metabolite biosynthesis-related gene clusters, which seem to be involved in metallophore synthesis and could also be responsible for synthesizing other compounds, such as siderophores. This suggests that *R. rhodochorus GD02* has a complex genetic landscape related to siderophores that needs to be studied in more depth to understand siderophore production fully (Ward et al. 2018). One must target the recent approaches of OMICs that include transcriptomics, proteomics, metabolomics

and integrative bioinformatics platforms for elucidation of pathways, protein-protein interaction and other metabolites cross-talk for a thorough investigation of siderophore production as well as respective metabolic processes.

5 *Rhodococcus* and plastic degradation

Rhodococcus bacteria also have a peculiar ability to degrade plastic material and offer hope to solve the non-degradable plastic burden-related environmental issues (Bacha et al. 2023). In a comparative bioinformatics study, analysis of 669 *Rhodococcus* genomes revealed genetic components involved in plastic and polymer breakdown, where 24% of bacteria target the CC backbone and 18% target heteroatomic polymer degradation, with 57% of these bacteria possessing enzymes for degrading poly-3 hydroxybutyrate (PHB) and polybutylene adipate-co-terephthalate (PBAT). Further in-depth analysis showed that *R. pyridinivorans*, *R. qingshengii*, and *R. hoagii* degraded the CC backbone of the plastic (Figure 3) (Zampolli et al. 2023). *Rhodococcus* species with enzymes (multicopper oxidases, alkane monooxygenases, and cytochrome P450 hydroxylases) may break down plastic (Mohanan et al. 2020). Initial studies suggested that extracellular secretion signals and enzyme types such as cutinase and PU esterase degrade heteroatomic backbone plastic. Polyester breakdown enzymes (carboxylesterase, PLA-depolymerase, and PHB-depolymerase) were consistently prevalent in *R. triamomae* (Ke et al. 2017). Understanding the molecular mechanisms underlying the degradation of various polymers is essential, as elucidating the biochemical pathways and enzymatic reactions can provide insights into the substrate selectivity, kinetics, and structural characteristics of these enzymes and elucidate the molecular basis of the plastic-degrading capabilities of the genus *Rhodococcus* (Cai et al. 2023). A study showed that the textile, pesticide, and fertilizer industries waste materials in waste materials that are enriched in anthraquinone (AC) compounds, which are ultimately deposited gradually in agricultural land. Unfortunately, these ACs contain an anthraquinone ring that harms microbes, plants, and animals (Safiarian et al. 2023). The *R. pyridinivorans GF3* strain degrades AC by breaking the anthraquinone ring into catechol and salicylic acid. However, the enzymes and genes that drive this process remain elusive and must be investigated in detail. So far, *R. pyridinivorans GF3* strain has been tested for the capacity to degrade 1-aminoanthraquinone-2-sulfonic acid (ASA-2) (Wang et al. 2022). ASA-2 breakdown produced catechol, 3-amino-4-sulfophthalic acid, salicylic acid, and 3-amino-4-sulfosalicylic acid. Gene analysis revealed that CYP450 and short-chain dehydrogenase/reductase are upregulated in *R. pyridinivorans GF3* during ASA-2 exposure, demonstrating their roles in AC oxidation and hydroxylation. The expression of catechol metabolism genes, which break down the AC compound and produce ATP through the TCA cycle, was upregulated (Figure 3) (Wang et al. 2022). The strains *R. pyridinivorans X1* and *X2* exhibited enhanced tolerance to phenolic contaminants, attributed to mutations identified through genome sequencing. These mutations were detected in genes associated with phosphotransferase, MFS transporter, AcrR regulator, and GlpD regulator, which collectively contribute to increased phenol tolerance and degradation capabilities in the transformed strains

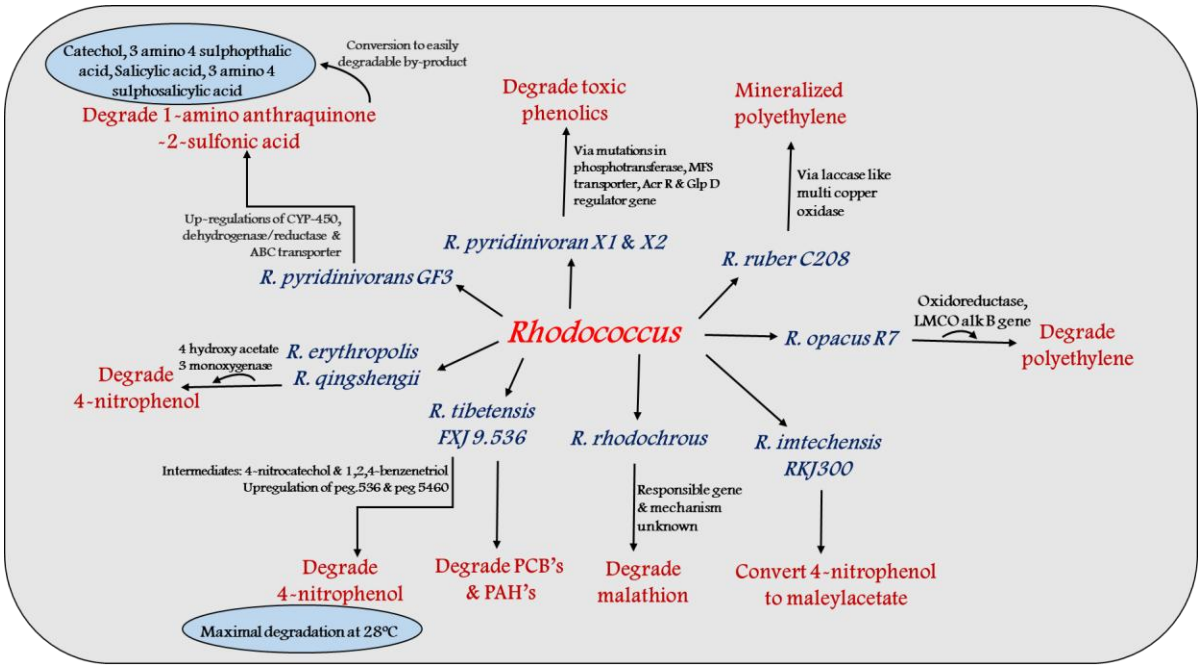

Figure 3 Highlights the roles of the bacterium *Rhodococcus* in plastics and toxin degradation.

(Wang et al. 2022). Notably, bacteria's polyethylene (PE) degradation can be influenced by acidity, photooxidation, and heat (Ghatge et al. 2020). The *R. ruber C208* strain mineralizes PE without external stimuli, and bacterial laccase-like multicopper oxidase (LMCO) decreases the molecular weight of PE (Zampolli et al. 2024). Similar to *R. ruber C208*, another strain, *R. opacus R7*, exhibit growth and survival capabilities by degrading PE and using it as a carbon source. *R. opacus R7* RNA sequencing revealed elevated expression of genes responsible for oxidoreductase activity, hydrocarbon catabolism type and other activities (Figure 3). The overexpression of three LMCO genes (LMCO1, 2, 3) suggested that these genes play an important role in early PE oxidation. The highly expressed alkB gene degrades medium-chain n-alkanes and shows that *R. opacus R7* can degrade PE (Zampolli et al. 2021). The *R. tibetensis FXJ9.536* strain was discovered on the Qinghai–Tibet Plateau (QTP). This strain degrades QTP organic pollutants such as polychlorinated biphenyl (PCBs) and polyaromatic hydrocarbons (PAHs) (Huang et al. 2022). Our attention must be directed towards approaches which help identify new plastic-degrading enzymes from *Rhodococcus* bacteria. One must explore global proteomics analysis to discover the uncharacterized unique enzymes from the *Rhodococcus* bacteria.

6 *Rhodococcus* and environmental stress adaptation

Rhodococcus bacteria can also encounter environmental stressors that result in adaptation and resilience against abiotic stress (Kuhl et al. 2021). A mechanistic study between the fungal pathogen, abscisic acid (ABA) levels in sugar beet plants, and counter-current mechanisms of *Rhodococcus* have been investigated in the past (Rangel et al. 2020). *C. beticola* is a pathogenic fungus that can elevate the ABA levels in the sugar beet plants during the infection cycle. This ABA level enhancement in sugar beet can make plants more susceptible to other pathogens. Because ABA hampers the immune system of plants, excess ABA leaches into the soil through dead shoot tissues and root turnover, thus changing the amount of ABA in the soil (Figure 4) (Brookbank et al. 2021). This delicate equilibrium between ABA synthesis and breakdown contributes to oxidative changes within the system, with CYP707A2 monooxygenase emerging as a crucial player in this cyclic process (Rai et al. 2021). Therefore, a deep understanding of the modulating interactions between plants and fungi and their respective resultant effects on the ABA availability in the soil has been important while considering plant health and resistance to pathogenic diseases (Begum et al. 2019).

The *Rhodococcus P1Y* strain uses ABA as a carbon and energy source (Yuzikhin et al. 2021). Phaseic and dehydrophaseic acids are predominantly derived from the modification of cyclohexene by ABA. Researchers have shown that the *Rhodococcus P1Y* strain can degrade ABA to dehydrovomifoliol in a synthetic nutrient medium using ABA as the sole carbon and energy source (Bordin et al. 2021). However, the process of converting ABA to dehydrovomifoliol is still unknown. By analyzing the genome of the

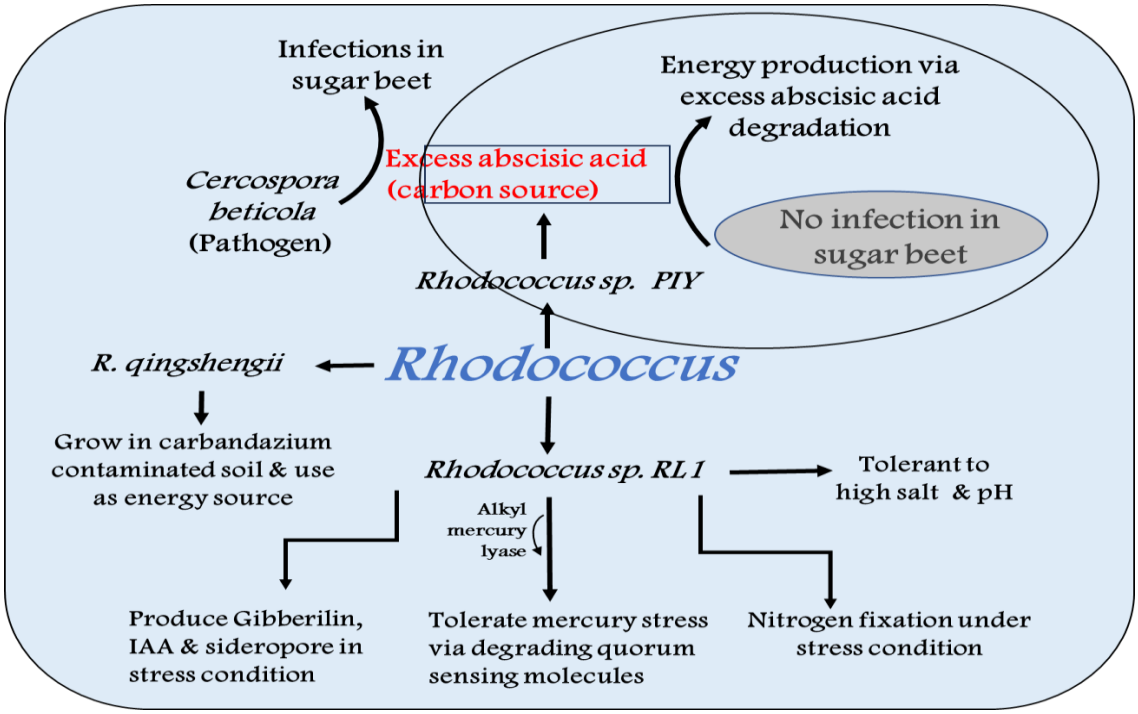

Figure 4 Explaining the *Rhodococcus* sp.-mediated stress adaptation and resilience mechanisms to establish healthy symbioses between plants and soil microbes

Table 1 Different roles of *Rhodococcus* species in the bioremediation and metabolism processes

S. N.	Strain	Application	Mechanism	Reference
1	*Rhodococcus* sp. *A-deg 1* and *A-deg 2*	Degrade aniline	Degradation of aniline and consumption as a single carbon source. Mechanism unknown	Krivoruchko et al. 2023
2	*R. imtechensis RKJ300T* strain	Degrade p-nitrophenol (PNP) and 2,4-dinitrophenol (2,4-DNP)	Degradation of PNP and 2,4-DNP by consuming as a single carbon source. Mechanism unknown	Ghosh et al. 2006
3	*Rhodococcus JMCTTKA8* strain	Degrade Lambda cyhalothrin (pesticide)	Breakdown and consumption of Lambda cyhalothrin byproducts as major source of energy. Mechanism unknown	Djouaka et al. 2018
4	*R. phenolicus* and *R. ruber*	Degrade monocrotophos and mineralized acetochlor	Monocrotophos (MCP-1 and MCP-2) degradation and acetochlor mineralization by a known mechanism	Srinivasulu et al. 2017
5	*Rhodococcus BUPNP1*strain	4-NP metabolization	Monooxygenase dependent degradation of 4-NP, 4-NC, and 1,2,4-benzenetriol to use as single carbon source and release nitite ions.	Sengupta et al. 2019b
6	*Rhodococcus RKJ300* strain	Degrade PNP or 2,4-DNP	Degradation via oxidative and reductive processes creates chlorohydroquinone and generates hydroquinone as an intermediate.	Ghosh et al. 2010
7	*R. soli G41* strain	Glyphosate degradation	Glyphosate degradation via sarcosine oxidase and C-P lysis turns glyphosate into phosphate.	Nguyen et al. 2022
8	*Rhodococcus XM24D* strain	Degrade 2,4-DNP and its analogue	Biosynthetic genes involved in 2,4-DNP degradation include dnpB and dnpC, F420 oxidoreductases, and F420 coenzyme	Hu et al. 2021
9	*R. erythropolisW2* strain	Quorum quencher	Quenching of quorum sensing by N-acylhomoserine lactone (AHL) degradation and inhibiting bacterial cross-talk.	Uroz et al. 2005
10	*Rhodococcus* strain (*LS31* and *PI33I*)	Inhibition of AHLs type OHHL and Pectate lysin	Lowering of AHLs, especially OHHL (N-(3-oxohexanoyl)-L-homoserine lactone) in *Erwinia carotovora* via pectate lyase activity.	Park et al. 2006
11	*Rhodococcus L4* strain	Metabolize trichloroethylene (TCE)	TCE degradation via enzyme cumene aldehyde and cumene.	Suttinun et al. 2010
12	*R. pyridinivorans, R. qingshengii*, and *R. hoagii*	Degrade plastic C-C backbone of plastics	Enzymatic degradation of C-C backbone by para-nitrobenzylesterase and carboxylesterase.	Mohanan et al. 2020
13	*R. triatomae*	Polyester breakdown	Polyester degradation mediated by enzymes: Carboxylesterase, PLA-depolymerase, and PHB-depolymerase	Zampolli et al. 2022
14	*R. pyridinivorans GF3* strain	Degrade anthraquinone compounds	Breakdown of anthraquinone compounds by enzymatic cleavage of anthraquinone ring.	Wang et al. 2022
15	*R. pyridinivorans X1* and *X2* strain	Phenol tolerance and degradation	Enzymatic metabolization of phenol by phosphotransferase, MFS transporter, AcrR regulator, and GlpD regulator.	Peng et al. 2022
16	*R. ruber C208* strain	Polyethylene (PE) mineralization	Mineralizes of PE without external stimuli where the bacterial laccase-like multicopper oxidase (LMCO) decreases the PE molecular weight.	Zampolli et al. 2021
17	*R. tibetensis* FXJ9.536 strain	Degrade polyaromatic hydrocarbon (PAH) polychlorinated biphenyl (PCB) sand	Breakdown of organic pollutants such as PCBs and PAHs by generating intermediates (4-nitrocatechol and 1,2,4-benzenetriol).	Huang et al. 2022
18	*RhodococcusP1Y* strain	Degrade excess Abscisic acid (ABA)	ABA is utilized as its only carbon source, and energy is converted into specific metabolites, such as dehydrophaseic acid.	Yuzikhin et al. 2021
19	*Rhodococcus RL1* strain	Stress tolerance	Removal of harmful compounds from the environment. Such as quorum-quenching, mercury resistance, high-salt, extreme pH, osmotic stress resilience, biofilm formation, and the production of compounds that help plants grow and the soil stay fertile. Mechanisms Unknown	Kuhl et al. 2021

Rhodococcus RL1 strain, scientists have identified a large number of genes related to stress tolerance, bioremediation of toxic compounds, and colonization of rhizospheric biota to ensure the establishment of beneficial interactions between plants and microbes (Figure 4) (Kuhl et al. 2021). This genetic profile suggests that the *Rhodococcus RL1* strain is a tool required for interactions with other organisms, including plants, and can survive in various abiotic stress situations. *In vitro* tests were performed to analyze the practical implications of the genomic potential of the *Rhodococcus RL1* strain. The *Rhodococcs RL1* strain broke down quorum-quenching signals (important for bacterial cross-talk) and tolerated mercury stress. The *Rhodococcus RL1* strain contains genes encoding an enzyme (alkyl-mercury lyase) and a DNA-binding protein (merR family), which allows it to grow quickly in the presence of mercury (Kuhl et al. 2019). *Rhodococcs RL1* was also reported to be resistant to high salt levels (up to 7.5% NaCl), extreme acidic pH ranges (pH 2-5), and osmotic stress conditions. The strain *Rhodococcs RL1* can also form biofilms, which are important for initiating many microbial interactions (Kuhl et al. 2021). The ability of *R. cholerae RL1* to produce IAA and siderophores that collect iron from the environment helps improve soil fertility and helps in healthy plant growth. A summary of the prospective contributions of the genus *Rhodococcus* to the phytoremediation and metabolism of complex environmental pollutants is provided in Table 1.

Conclusions And Future Prospectus

The multifaceted capabilities of the bacterium *Rhodococcus* have suggested that it is an important microbe for improving plant and soil health and balancing the ecological environment. By harnessing their diverse enzymatic pathways and genetic traits, one can look forward to their respective synchronized coexistence between the agriculture sector and environmental pollution control, which could help us achieve sustainable development goals. However, through bioremediation and eco-friendly pest control, the genus *Rhodococcus* also contributes to mitigating the adverse effects of chemical toxic materials and promoting a healthy ecosystem. Concurrently, because of their ability to degrade hazardous pollutants and plastics by modulating quorum sensing ability and assisting in plant-fungal interactions, *Rhodococcus* species have been highlighted as a combination of beneficial traits because of their remarkable adaptability and resilience for improving the health of soils, plants and ecosystems. However, to gain more knowledge about the molecular mechanism, biological pathways, and protein-protein interactions responsible for such unusual traits of genus *Rhodococcus*, a detailed study will be required by employing diverse OMICs platforms. One must plan the genomics, transcriptomics, proteomics, and metabolomics investigations about the above-discussed *Rhodococcus* strains to get deeper insight into exclusive properties of genus *Rhodococcus*, and should integrate the independent outcomes by in-depth bioinformatics analysis to get more holistic bio-molecular view of multidisciplinary functional traits of genus *Rhodococcus*.

Abbreviations

2,4-DNP--2,4-Dinitrophenol; 4-NC--4-Nitrocatechol; 4-NP--4-Nitrophenol; ABA--Abscisic acid; ACs-- Anthraquinone compounds; ADH--Alcohol dehydrogenase; AHL--N-acyl homoserine lactone; AMPA--Aminomethylphosphonic acid; ASA-2--1-aminoanthraquinone-2-sulfonic acid; CAS--Chrome azurol S; HHL--N-hexanoyl-L-homoserine lactone; MO--Monooxygenase; NRPS--Nonribosomal peptide synthase; PBAT--Poly(butylene adipate-coterephthalate); PNP--Para-nitrophenol; QQ--Quorum quenching; QTP--Qinghai–Tibet Plateau; TCE--Trichloroethylene;

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Writing and original draft preparation- BS, SAM, writing, review and editing SAM, SA, BS, and supervision- B.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declare no competitive or financial conflicts of interest.

Consent to subjects and ethics statement

Not applicable

Patent

Not applicable.

Ethical approval

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Consent to publish

Not applicable.

Data availability statement

The data presented in this study are available upon request from the corresponding author.

References

Ahirwar, N. K. (2023). Advanced Pesticide Nano Formulations and Understanding Their Breakdown by Bacteria. *Indian Journal of Microbiology Research, 10*(4), 193–201. doi: 10.18231/j.ijmr.2023.035.

Alam, S. A., & Saha, P. (2022). Microbial Biodegradation of Nitrophenols and Their Derivatives: A Review. *Journal of Experimental Biology and Agricultural Sciences, 10*(4):743–66. doi: 10.18006/2022.10(4).743.766.

Aslam, S., Jing, Y., & Nowak, K. M. (2023). Fate of glyphosate and its degradation products AMPA, glycine and sarcosine in an agricultural soil: Implications for environmental risk assessment. *Journal of hazardous materials*, *447*, 130847. https://doi.org/10.1016/j.jhazmat.2023.130847.

Bacha, A. U., Nabi, I., Zaheer, M., Jin, W., & Yang, L. (2023). Biodegradation of macro- and micro-plastics in environment: A review on mechanism, toxicity, and future perspectives. *The Science of the total environment*, *858*(Pt 3), 160108. https://doi.org/10.1016/j.scitotenv.2022.160108.

Barbey, C., Chane, A., Burini, J. F., Maillot, O., Merieau, A., et al. (2018). A Rhodococcal Transcriptional Regulatory Mechanism Detects the Common Lactone Ring of AHL Quorum-Sensing Signals and Triggers the Quorum-Quenching Response. *Frontiers in microbiology*, *9*, 2800. https://doi.org/10.3389/fmicb.2018.02800.

Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., Ahmed, N., & Zhang, L. (2019). Role of Arbuscular Mycorrhizal Fungi in Plant Growth Regulation: Implications in Abiotic Stress Tolerance. *Frontiers in plant science*, *10*, 1068. https://doi.org/10.3389/fpls.2019.01068.

Bhattacherjee, A. K., Shukla, P. K., & Dikshit, A. (2020). Microbial Biotransformation of Neonicotinoid Insecticides in Soil – A Review. *International Journal of Current Microbiology and Applied Sciences, 9*(7), 3255–3277. doi: 10.20546/ijcmas.2020.907.380.

Bordin, A. I., Cohen, N. D., Giguère, S., Bray, J. M., Berghaus, L. J., Scott, B., Johnson, R., & Hook, M. (2021). Host-directed therapy in foals can enhance functional innate immunity and reduce severity of *Rhodococcus equi pneumonia*. *Scientific reports*, *11*(1), 2483. https://doi.org/10.1038/s41598-021-82049-y.

Brookbank, B. P., Patel, J., Gazzarrini, S., & Nambara, E. (2021). Role of Basal ABA in Plant Growth and Development. *Genes*, *12*(12), 1936. https://doi.org/10.3390/genes12121936.

Cai, Z., Li, M., Zhu, Z., Wang, X., Huang, Y., Li, T., Gong, H., & Yan, M. (2023). Biological Degradation of Plastics and Microplastics: A Recent Perspective on Associated Mechanisms and Influencing Factors. *Microorganisms*, *11*(7), 1661. https://doi.org/10.3390/microorganisms11071661.

Camara, M. C., Campos, E.V.R, Monteiro, R.A., Pereira, A.E.S., Proença, P.L.F., & Fraceto, L.F. (2019). Development of Stimuli-Responsive Nano-Based Pesticides: Emerging Opportunities for Agriculture. *Journal of Nanobiotechnology, 17*(1), 100. doi: 10.1186/s12951-019-0533-8.

Castrejón-Godínez, M. L., Tovar-Sánchez, E., Valencia-Cuevas, L., Rosas-Ramírez, M. E., Rodríguez, A., & Mussali-Galante, P. (2021). Glyphosate Pollution Treatment and Microbial Degradation Alternatives, a Review. *Microorganisms*, *9*(11), 2322. https://doi.org/10.3390/microorganisms9112322.

Chakraborty, S., Bashir, Y., Sirotiya, V., Ahirwar, A., Das, S., & Vinayak, V. (2023). Role of bacterial quorum sensing and quenching mechanism in the efficient operation of microbial electrochemical technologies: A state-of-the-art review. *Heliyon*, *9*(5), e16205. https://doi.org/10.1016/j.heliyon.2023.e16205.

Chaturvedi, N. K. (2022). Comparison of Available Treatment Techniques for Hazardous Aniline-Based Organic Contaminants. *Applied Water Science, 12*(7), 173. doi: 10.1007/s13201-022-01695-3.

Costas-Ferreira, C., & Faro, L. R. F. (2021). Neurotoxic Effects of Neonicotinoids on Mammals: What Is There beyond the Activation of Nicotinic Acetylcholine Receptors?-A Systematic Review. *International journal of molecular sciences*, *22*(16), 8413. https://doi.org/10.3390/ijms22168413.

da Silveira, B. P., Gressler, L. T., Cargnelutti, J. F., Bordin, A. I., & de Vargas, A. C. (2020). GAPDH, rhbC, and vapA gene expression in *Rhodococcus equi* cultured under different iron concentrations. *Microbial pathogenesis*, *139*, 103885. https://doi.org/10.1016/j.micpath.2019.103885.

Dai, Z. L., Yang, W. L., Fan, Z. X., Guo, L., Liu, Z. H., & Dai, Y. J. (2021). Actinomycetes *Rhodococcus ruber* CGMCC 17550 degrades neonicotinoid insecticide nitenpyram via a novel hydroxylation pathway and remediates nitenpyram in surface water. *Chemosphere*, *270*, 128670. https://doi.org/10.1016/j.chemosphere.2020.128670.

D'Ambrosio, D. A., Kennedy, G.G., & Huseth, A.S. (2020). *Frankliniella fusca* and *Frankliniella occidentalis* Response to Thrips-Active Cry51Aa2.834_16 Bt Cotton with and without Neonicotinoid Seed Treatment. *Crop Protection, 129*, 105042. doi: 10.1016/j.cropro.2019.105042.

Djouaka, R., Soglo, M. F., Kusimo, M. O., Adéoti, R., Talom, A., et al. (2018). The Rapid Degradation of Lambda-Cyhalothrin

Makes Treated Vegetables Relatively Safe for Consumption. *International journal of environmental research and public health*, *15*(7), 1536. https://doi.org/10.3390/ijerph15071536.

Dubert, J., Barja, J. L., & Romalde, J. L. (2017). New Insights into Pathogenic Vibrios Affecting Bivalves in Hatcheries: Present and Future Prospects. *Frontiers in microbiology*, *8*, 762. https://doi.org/10.3389/fmicb.2017.00762.

Firdous, S., Samina, I., & Samina, A. (2020). Optimization and Modeling of Glyphosate Biodegradation by a Novel *Comamonas odontotermitis* P2 through Response Surface Methodology. *Pedosphere*, *30*(5), 618–27. doi: 10.1016/S1002-0160(17)60381-3.

Ghatge, S., Yang, Y., Ahn, J.H., & Hur, G.H. (2020). Biodegradation of Polyethylene: A Brief Review. *Applied Biological Chemistry*, *63*(1), 27. doi: 10.1186/s13765-020-00511-3.

Ghosh, A., Khurana, M., Chauhan, A., Takeo, M., Chakraborti, A. K., & Jain, R. K. (2010). Degradation of 4-nitrophenol, 2-chloro-4-nitrophenol, and 2,4-dinitrophenol by *Rhodococcus imtechensis* strain RKJ300. *Environmental science & technology*, *44*(3), 1069–1077. https://doi.org/10.1021/es9034123.

Ghosh, A., Paul, D., Prakash, D., Mayilraj, S., & Jain, R. K. (2006). *Rhodococcus imtechensis* sp. nov., a nitrophenol-degrading actinomycete. *International journal of systematic and evolutionary microbiology*, *56*(Pt 8), 1965–1969. https://doi.org/10.1099/ijs.0.63939-0.

Gleń-Karolczyk, K., Boligłowa, E., Gospodarek, J., Antonkiewicz, J., & Luty, L. (2021). Effect of Seed Dressing and Soil Chemical Properties on Communities of Microorganisms Associated with Pre-Emergence Damping-Off of Broad Bean Seedlings. *Agronomy*, *11*(9):1889. doi: 10.3390/agronomy11091889.

Gomes, A. F. R., Sousa, E., & Resende, D.I.S.P. (2024). A Practical Toolkit for the Detection, Isolation, Quantification, and Characterization of Siderophores and Metallophores in Microorganisms. *ACS Omega*, *9*(25), 26863–77. doi: 10.1021/acsomega.4c03042.

Hartman, K., Schmid, M. W., Bodenhausen, N., Bender, S. F., Valzano-Held, A. Y., Schlaeppi, K., & van der Heijden, M. G. A. (2023). A symbiotic footprint in the plant root microbiome. *Environmental Microbiome*, *18*(1), 65. https://doi.org/10.1186/s40793-023-00521-w.

Hecko, S., Schiefer, A., Badenhorst, C. P. S., Fink, M. J., Mihovilovic, M. D., Bornscheuer, U. T., & Rudroff, F. (2023). Enlightening the Path to Protein Engineering: Chemoselective Turn-On Probes for High-Throughput Screening of Enzymatic Activity. *Chemical reviews*, *123*(6), 2832–2901. https://doi.org/10.1021/acs.chemrev.2c00304.

Hofmann, M., Heine, T., Malik, L., Hofmann, S., Joffroy, K., Senges, C. H. R., Bandow, J. E., & Tischler, D. (2021). Screening for Microbial Metal-Chelating Siderophores for the Removal of Metal Ions from Solutions. *Microorganisms*, *9*(1), 111. https://doi.org/10.3390/microorganisms9010111.

Hu, F., Yang, L., Wang, Z., & Wang, J. (2021). Genome and transcriptome sequencing of a newly isolated 2,4-dinitrophenol-degrading strain *Rhodococcus imtechensis* XM24D. *Genes & genomics*, *43*(7), 829–835. https://doi.org/10.1007/s13258-021-01101-3.

Huang, J., Ai, G., Liu, N., & Huang, Y. (2022). Environmental Adaptability and Organic Pollutant Degradation Capacity of a Novel *Rhodococcus* Species Derived from Soil in the Uninhabited Area of the Qinghai-Tibet Plateau. *Microorganisms*, *10*(10), 1935. https://doi.org/10.3390/microorganisms10101935.

Islam, S.M.F., & Karim, Z. (2020). World's Demand for Food and Water: The Consequences of Climate Change. In M. H. D. A. Farahani, V. Vatanpour, & A. H. Taheri (Eds.) *Desalination - Challenges and Opportunities*. IntechOpen. DOI: 10.5772/intechopen.85919

Kaushik, S., He, H., & Dalbey, R. E. (2022). Bacterial Signal Peptides- Navigating the Journey of Proteins. *Frontiers in physiology*, *13*, 933153. https://doi.org/10.3389/fphys.2022.933153

Ke, X., Guan-Jun, D., Bin-Xiang, M., Zhi-Qiang, L., Jin-Feng, Z., & Yu-Guo, Z. (2017). Characterization of a Novel CYP51 from RhodococcusTriatomae and Its NADH-Ferredoxin Reductase-Coupled Application in Lanosterol 14α-Demethylation. *Process Biochemistry*, *62*, 59–68. doi: 10.1016/j.procbio.2017.07.030.

Khilyas, I. V., Sorokina, A. V., Markelova, M. I., Belenikin, M., Shafigullina, L., et al. (2021). Genomic and phenotypic analysis of siderophore-producing *Rhodococcus qingshengii* strain S10 isolated from an arid weathered serpentine rock environment. *Archives of microbiology*, *203*(2), 855–860. https://doi.org/10.1007/s00203-020-02057-w.

Kim, D., Choi, K. Y., Yoo, M., Zylstra, G. J., & Kim, E. (2018). Biotechnological Potential of *Rhodococcus* Biodegradative Pathways. *Journal of microbiology and biotechnology*, *28*(7), 1037–1051. https://doi.org/10.4014/jmb.1712.12017.

Krivoruchko, A., Kuyukina, M., Peshkur, T., Cunningham, C. J., & Ivshina, I. (2023). *Rhodococcus* Strains from the Specialized Collection of Alkanotrophs for Biodegradation of Aromatic Compounds. *Molecules (Basel, Switzerland)*, *28*(5), 2393. https://doi.org/10.3390/molecules28052393.

Kuhl, T., Chowdhury, S. P., Uhl, J., & Rothballer, M. (2021). Genome-Based Characterization of Plant-Associated *Rhodococcus*

qingshengii RL1 Reveals Stress Tolerance and Plant-Microbe Interaction Traits. *Frontiers in microbiology*, *12*, 708605. https://doi.org/10.3389/fmicb.2021.708605.

Kuhl, T., Felder, M., Nussbaumer, T., Fischer, D., Kublik, S., Paul Chowdhury, S., Schloter, M., & Rothballer, M. (2019). *De Novo* Genome Assembly of a Plant-Associated Rhodococcus qingshengii Strain (RL1) Isolated from Eruca sativa Mill. and Showing Plant Growth-Promoting Properties. *Microbiology Resource Announcements*, *8*(46), e01106-19. https://doi.org/10.1128/MRA.01106-19.

Kumar, L., Patel, S. K. S., Kharga, K., Kumar, R., Kumar, P., et al. (2022). Molecular Mechanisms and Applications of N-Acyl Homoserine Lactone-Mediated Quorum Sensing in Bacteria. *Molecules (Basel, Switzerland)*, *27*(21), 7584. https://doi.org/10.3390/molecules27217584.

Kusada, H., Tamaki, H., Kamagata, Y., Hanada, S., & Kimura, N. (2017). A Novel Quorum-Quenching *N*-Acylhomoserine Lactone Acylase from Acidovorax sp. Strain MR-S7 Mediates Antibiotic Resistance. *Applied and environmental microbiology*, *83*(13), e00080-17. https://doi.org/10.1128/AEM.00080-17.

Mawang, C. I., Azman, A. S., Fuad, A. M., & Ahamad, M. (2021). Actinobacteria: An eco-friendly and promising technology for the bioaugmentation of contaminants. *Biotechnology reports (Amsterdam, Netherlands)*, *32*, e00679. https://doi.org/10.1016/j.btre.2021.e00679.

Mohanan, N., Montazer, Z., Sharma, P. K., & Levin, D. B. (2020). Microbial and Enzymatic Degradation of Synthetic Plastics. *Frontiers in microbiology*, *11*, 580709. https://doi.org/10.3389/fmicb.2020.580709.

Murugayah, S. A., & Gerth, M. L. (2019). Engineering quorum quenching enzymes: progress and perspectives. *Biochemical Society transactions*, *47*(3), 793–800. https://doi.org/10.1042/BST20180165.

Nguyen, N. T., Vo, V. T., Nguyen, T. H. P., & Kiefer, R. (2022). Isolation and optimization of a glyphosate-degrading *Rhodococcus soli* G41 for bioremediation. *Archives of microbiology*, *204*(5), 252. https://doi.org/10.1007/s00203-022-02875-0.

Okoye, C. O., Addey, C.I., Oderinde, O., Okoro, J.O., Uwamungu, J.Y., et al. (2022). Toxic Chemicals and Persistent Organic Pollutants Associated with Micro-and Nanoplastics Pollution. *Chemical Engineering Journal Advances*, *11*, 100310. doi: 10.1016/j.ceja.2022.100310.

Paluch, E., Rewak-Soroczyńska, J., Jędrusik, I., Mazurkiewicz, E., & Jermakow, K. (2020). Prevention of biofilm formation by quorum quenching. *Applied microbiology and biotechnology*, *104*(5), 1871–1881. https://doi.org/10.1007/s00253-020-10349-w.

Pande, V., Pandey, S. C., Sati, D., Bhatt, P., & Samant, M. (2022). Microbial Interventions in Bioremediation of Heavy Metal Contaminants in Agroecosystem. *Frontiers in microbiology*, *13*, 824084. https://doi.org/10.3389/fmicb.2022.824084.

Pang, S., Lin, Z., Zhang, W., Mishra, S., Bhatt, P., & Chen, S. (2020). Insights Into the Microbial Degradation and Biochemical Mechanisms of Neonicotinoids. *Frontiers in microbiology*, *11*, 868. https://doi.org/10.3389/fmicb.2020.00868.

Park, S. Y., Hwang, B. J., Shin, M. H., Kim, J. A., Kim, H. K., & Lee, J. K. (2006). N-acylhomoserine lactonase producing *Rhodococcus* spp. with different AHL-degrading activities. *FEMS microbiology letters*, *261*(1), 102–108. https://doi.org/10.1111/j.1574-6968.2006.00336.x.

Parte, S. G., & Kharat, A. S. (2019). Aerobic Degradation of Clothianidin to 2-Chloro-methyl Thiazole and Methyl 3-(Thiazole-yl) Methyl Guanidine Produced by *Pseudomonas stutzeri* smk. *Journal of environmental and public health*, *2019*, 4807913. https://doi.org/10.1155/2019/4807913.

Pathak, D., Suman, A., Sharma, P., Aswini, K., Govindasamy, V., Gond, S., & Anshika, R. (2024). Community-forming traits play role in effective colonization of plant-growth-promoting bacteria and improved plant growth. *Frontiers in plant science*, *15*, 1332745. https://doi.org/10.3389/fpls.2024.1332745.

Peng, F., Ye, M., Liu, Y., Liu, J., Lan, Y., Luo, A., Zhang, T., Jiang, Z., & Song, H. (2022). Comparative genomics reveals response of *Rhodococcus pyridinivorans* B403 to phenol after evolution. *Applied microbiology and biotechnology*, *106*(7), 2751–2761. https://doi.org/10.1007/s00253-022-11858-6.

Pérez Rodríguez, M., Melo, C., Jiménez, E., & Dussán, J. (2019). Glyphosate Bioremediation through the Sarcosine Oxidase Pathway Mediated by *Lysinibacillus sphaericus* in Soils Cultivated with Potatoes. *Agriculture*, *9*(10), 217. doi: 10.3390/agriculture9100217.

Prazdnova, E. V., Gorovtsov, A. V., Vasilchenko, N. G., Kulikov, M. P., Statsenko, V. N., et al. (2022). Quorum-Sensing Inhibition by Gram-Positive Bacteria. *Microorganisms*, *10*(2), 350. https://doi.org/10.3390/microorganisms10020350.

Puja, H., Mislin, G. L. A., & Rigouin, C. (2023). Engineering Siderophore Biosynthesis and Regulation Pathways to Increase Diversity and Availability. *Biomolecules*, *13*(6), 959. https://doi.org/10.3390/biom13060959.

Raffa, C. M., & Chiampo, F. (2021). Bioremediation of Agricultural Soils Polluted with Pesticides: A Review. *Bioengineering (Basel, Switzerland)*, *8*(7), 92. https://doi.org/10.3390/bioengineering8070092.

Rai, K. K., Pandey, N., Rai, N., Rai, S.K., & Pandey-Rai. S. (2021). Salicylic Acid and Nitric Oxide: Insight Into the Transcriptional Regulation of Their Metabolism and Regulatory Functions in Plants. *Frontiers in Agronomy*, 3. doi: 10.3389/fagro.2021.781027.

Rangel, L. I., Spanner, R. E., Ebert, M. K., Pethybridge, S. J., Stukenbrock, E. H., de Jonge, R., Secor, G. A., & Bolton, M. D. (2020). *Cercospora beticola*: The intoxicating lifestyle of the leaf spot pathogen of sugar beet. *Molecular plant pathology*, *21*(8), 1020–1041. https://doi.org/10.1111/mpp.12962.

Rasool, S., Rasool, T., & Gani, K.M. (2022). A Review of Interactions of Pesticides within Various Interfaces of Intrinsic and Organic Residue Amended Soil Environment. *Chemical Engineering Journal Advances*, *11*, 100301. doi: 10.1016/j.ceja.2022.100301.

Rehman, Z. U., Momin, A. A., Aldehaiman, A., Irum, T., Grünberg, R., & Arold, S. T. (2022). The exceptionally efficient quorum quenching enzyme LrsL suppresses *Pseudomonas aeruginosa* biofilm production. *Frontiers in microbiology*, *13*, 977673. https://doi.org/10.3389/fmicb.2022.977673.

Retamal-Morales, G., Mehnert, M., Schwabe, R., Tischler, D., Zapata, C., Chávez, R., Schlömann, M., & Levicán, G. (2018). Detection of arsenic-binding siderophores in arsenic-tolerating Actinobacteria by a modified CAS assay. *Ecotoxicology and environmental safety*, *157*, 176–181. https://doi.org/10.1016/j.ecoenv.2018.03.087.

Ryu, D. H., Lee, S. W., Mikolaityte, V., Kim, Y. W., Jeong, H. Y., et al. (2020). Identification of a Second Type of AHL-lactonase from *Rhodococcus* sp. BH4, belonging to the α/β Hydrolase Superfamily. *Journal of microbiology and biotechnology*, *30*(6), 937–945. https://doi.org/10.4014/jmb.2001.01006.

Saeed, Q., Xiukang, W., Haider, F. U., Kučerik, J., Mumtaz, M. Z., et al. (2021). Rhizosphere Bacteria in Plant Growth Promotion, Biocontrol, and Bioremediation of Contaminated Sites: A Comprehensive Review of Effects and Mechanisms. *International journal of molecular sciences*, *22*(19), 10529. https://doi.org/10.3390/ijms221910529.

Safiarian, M. S., Ugboya, A., Khan, I., Marichev, K. O., & Grant, K. B. (2023). New Insights into the Phototoxicity of Anthracene-Based Chromophores: The Chloride Salt Effect†. *Chemical research in toxicology*, *36*(7), 1002–1020. https://doi.org/10.1021/acs.chemrestox.2c00235.

Sakr, S., & Rashad, W. A. (2023). Lambda-cyhalothrin-induced pancreatic toxicity in adult albino rats. *Scientific reports*, *13*(1), 11562. https://doi.org/10.1038/s41598-023-38661-1.

Santillan, J. Y., Rojas, N.L. Ghiringhelli, P.D., Nóbile, M.L. Lewkowicz, E.S., & Iribarren, A.M. (2020). Organophosphorus Compounds Biodegradation by Novel Bacterial Isolates and Their Potential Application in Bioremediation of Contaminated Water. *Bioresource Technology*, *317*, 124003. doi: 10.1016/j.biortech.2020.124003.

Sarveswari, H. B., & Solomon, A. P. (2019). Profile of the Intervention Potential of the Phylum Actinobacteria Toward Quorum Sensing and Other Microbial Virulence Strategies. *Frontiers in microbiology*, *10*, 2073. https://doi.org/10.3389/fmicb.2019.02073.

Sengupta, K., Alam, M., Pailan, S., & Saha, P. (2019a). Biodegradation of 4-Nitrophenol by a *Rhodococcus* Species and a Preliminary Insight into Its Toxicoproteome Based on Mass Spectrometry Analysis. *Journal of Environmental Biology*, *40*(3), 356–362. doi: 10.22438/jeb/40/3/MRN-931.

Sengupta, K., Swain, M. T., Livingstone, P. G., Whitworth, D. E., & Saha, P. (2019b). Genome Sequencing and Comparative Transcriptomics Provide a Holistic View of 4-Nitrophenol Degradation and Concurrent Fatty Acid Catabolism by *Rhodococcus* sp. Strain BUPNP1. *Frontiers in microbiology*, *9*, 3209. https://doi.org/10.3389/fmicb.2018.03209.

Sharma, V., Mohammed, S. A., Devi, N., Vats, G., Tuli, H. S., Saini, A. K., Dhir, Y. W., Dhir, S., & Singh, B. (2024). Unveiling the dynamic relationship of viruses and/or symbiotic bacteria with plant resilience in abiotic stress. *Stress biology*, *4*(1), 10. https://doi.org/10.1007/s44154-023-00126-w.

Sikdar, R., & Elias, M. (2020). Quorum quenching enzymes and their effects on virulence, biofilm, and microbiomes: a review of recent advances. *Expert review of anti-infective therapy*, *18*(12), 1221–1233. https://doi.org/10.1080/14787210.2020.1794815.

Singh, S., Kumar, V., Gill, J. P. K., Datta, S., Singh, S., et al. (2020). Herbicide Glyphosate: Toxicity and Microbial Degradation. *International journal of environmental research and public health*, *17*(20), 7519. https://doi.org/10.3390/ijerph17207519.

Srimathi, K., & Suji, H.A. (2019). Siderophores Detection by Using Blue Agar CAS Assay Methods. *International Journal of Scientific Research in Biological Sciences*, *5*(6), 180–85. doi: 10.26438/ijsrbs/v5i6.180185.

Srinivasulu, M., Nilanjan, P. C., Chakravarthi, B. V. S. K., Jayabaskaran, C., Jaffer, M. G., et al. (2017). Biodegradation of Monocrotophos by Bacteria Isolated from Soil. *African Journal of Biotechnology*, *16*(9), 408–17. doi: 10.5897/AJB2015.14885.

Sun, M., Li, H., & Jaisi, D. P. (2019). Degradation of glyphosate and bioavailability of phosphorus derived from glyphosate in a soil-water system. *Water research*, *163*, 114840. https://doi.org/10.1016/j.watres.2019.07.007.

Suttinun, O., Müller, R., & Luepromchai, E. (2010). Cometabolic degradation of trichloroethene by *Rhodococcus* sp. strain L4 immobilized on plant materials rich in essential oils. *Applied and environmental microbiology*, *76*(14), 4684–4690. https://doi.org/10.1128/AEM.03036-09.

Swangjang, K. (2022). Linkage of Sustainability to Environmental Impact Assessment Using the Concept of Ecosystem Services: Lessons from Thailand. *Sustainability*, *14*(9), 5487. doi: 10.3390/su14095487.

Takeo, M., Yamamoto, K., Sonoyama, M., Miyanaga, K., Kanbara, N., Honda, K., Kato, D. I., & Negoro, S. (2018). Characterization of the 3-methyl-4-nitrophenol degradation pathway and genes of *Pseudomonas* sp. strain TSN1. *Journal of bioscience and bioengineering*, *126*(3), 355–362. https://doi.org/10.1016/j.jbiosc.2018.04.001.

Taşkan, B., & Taşkan, E. (2021). Inhibition of AHL-mediated quorum sensing to control biofilm thickness in microbial fuel cell by using *Rhodococcus* sp. BH4. *Chemosphere*, *285*, 131538. https://doi.org/10.1016/j.chemosphere.2021.131538.

Thi Mo, L., Irina, P., Natalia, S., Irina, N., Lenar, A., et al. (2022). Hydrocarbons Biodegradation by *Rhodococcus*: Assimilation of Hexadecane in Different Aggregate States. *Microorganisms*, *10*(8), 1594. doi: 10.3390/microorganisms10081594.

Tyumina, E., Bazhutin, G., Kostrikina, N., Sorokin, V., Mulyukin, A., & Ivshina, I. (2023). Phenotypic and metabolic adaptations of *Rhodococcus cerastii* strain IEGM 1243 to separate and combined effects of diclofenac and ibuprofen. *Frontiers in microbiology*, *14*, 1275553. https://doi.org/10.3389/fmicb.2023.1275553.

Uroz, S., Chhabra, S. R., Cámara, M., Williams, P., Oger, P., & Dessaux, Y. (2005). N-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. *Microbiology (Reading, England)*, *151*(Pt 10), 3313–3322. https://doi.org/10.1099/mic.0.27961-0.

Utari, P. D., Vogel, J., & Quax, W. J. (2017). Deciphering Physiological Functions of AHL Quorum Quenching Acylases. *Frontiers in microbiology*, *8*, 1123. https://doi.org/10.3389/fmicb.2017.01123.

Vázquez-Boland, J. A., & Meijer, W. G. (2019). The pathogenic actinobacterium *Rhodococcus equi*: what's in a name?. *Molecular microbiology*, *112*(1), 1–15. https://doi.org/10.1111/mmi.14267

Wang, X., Lu, H., Li, Q., Zhou, Y., & Zhou, J. (2022). Comparative genome and transcriptome of *Rhodococcus pyridinivorans* GF3 for analyzing the detoxification mechanism of anthraquinone compounds. *Ecotoxicology and environmental safety*, *237*, 113545. https://doi.org/10.1016/j.ecoenv.2022.113545.

Ward, A. L., Reddyvari, P., Borisova, R., Shilabin, A. G., & Lampson, B. C. (2018). An inhibitory compound produced by a soil isolate of *Rhodococcus* has strong activity against the veterinary pathogen *R. equi*. *PloS one*, *13*(12), e0209275. https://doi.org/10.1371/journal.pone.0209275.

Wrońska, N., Brzostek, A., Szewczyk, R., Soboń, A., Dziadek, J., & Lisowska, K. (2016). The Role of fadD19 and echA19 in Sterol Side Chain Degradation by *Mycobacterium smegmatis*. *Molecules (Basel, Switzerland)*, *21*(5), 598. https://doi.org/10.3390/molecules21050598.

Xiang, W., Liang, Y., Hong, S., Wang, G., You, J., Xue, Y., & Ma, Y. (2022). Degradation of long-chain n-alkanes by a novel thermal-tolerant *Rhodococcus* strain. *Archives of microbiology*, *204*(5), 259. https://doi.org/10.1007/s00203-022-02872-3.

Xu, B., Xue, R., Zhou, J., Wen, X., Shi, Z., et al. (2020). Characterization of Acetamiprid Biodegradation by the Microbial Consortium ACE-3 Enriched From Contaminated Soil. *Frontiers in microbiology*, *11*, 1429. https://doi.org/10.3389/fmicb.2020.01429.

Yasin, R., Rashid, G.M.M., Ali, I., & Bugg, T.D.H (2023). Engineering of *Rhodococcus jostii* RHA1 for Utilisation of Carboxymethylcellulose. *Heliyon* *9*(9), e19511. doi: 10.1016/j.heliyon.2023.e19511.

Yin, Y., Zhang, Q., & Peng, H. (2023). Retrospect and prospect of aerobic biodegradation of aniline: Overcome existing bottlenecks and follow future trends. *Journal of environmental management*, *330*, 117133. https://doi.org/10.1016/j.jenvman.2022.117133.

Yousef, H. A., Fahmy, H.M., Arafa, F.N., Abd Allah, M.Y., Tawfik, Y.M., et al. (2023). Nanotechnology in Pest Management: Advantages, Applications, and Challenges. *International Journal of Tropical Insect Science*, *43*(5), 1387–1399. doi: 10.1007/s42690-023-01053-z.

Yuzikhin, O. S., Gogoleva, N. E., Shaposhnikov, A. I., Konnova, T. A., Osipova, E. V., et al. (2021). Rhizosphere Bacterium *Rhodococcus* sp. P1Y Metabolizes Abscisic Acid to Form Dehydrovomifoliol. *Biomolecules*, *11*(3), 345. https://doi.org/10.3390/biom11030345.

Zampolli, J., Mangiagalli, M., Vezzini, D., Lasagni, M., Ami, D., et al. (2023). Oxidative Degradation of Polyethylene by Two Novel Laccase-like Multicopper Oxidases from *Rhodococcus opacus* R7. *Environmental Technology & Innovation*, *32*, 103273. doi: 10.1016/j.eti.2023.103273.

Zampolli, J., Orro, A., Manconi, A., Ami, D., Natalello, A., & Di Gennaro, P. (2021). Transcriptomic analysis of *Rhodococcus opacus* R7 grown on polyethylene by RNA-seq. *Scientific reports*, *11*(1), 21311. https://doi.org/10.1038/s41598-021-00525-x.

Zampolli, J., Orro, A., Vezzini, D., & Di Gennaro, P. (2022). Genome-Based Exploration of *Rhodococcus* Species for Plastic-Degrading Genetic Determinants Using Bioinformatic Analysis. *Microorganisms*, *10*(9), 1846. https://doi.org/10.3390/microorganisms10091846.

Zampolli, J., Vezzini, D., Brocca, S., & Di Gennaro, P. (2024). Insights into the biodegradation of polycaprolactone through genomic analysis of two plastic-degrading *Rhodococcus* bacteria. *Frontiers in microbiology*, *14*, 1284956. https://doi.org/10.3389/fmicb.2023.1284956.

Zhang, T., & Zhang, H. (2022). Microbial Consortia Are Needed to Degrade Soil Pollutants. *Microorganisms*, *10*(2), 261. https://doi.org/10.3390/microorganisms10020261.

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Examining the adaptability of soil pH to soil dynamics using different methodologies: A concise review

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ABSTRACT

Soil pH is crucial to soil health, influencing nutrient availability, microbial activity, and plant growth. This review aims to assess the adaptability of soil pH under changing soil conditions by analyzing natural and human factors. Information was gathered from various sources, including peer-reviewed articles, field studies, and recent advances in soil science. The study explores how natural factors such as parent material, climate, and vegetation establish baseline soil pH, while human activities such as intensive farming and land-use changes further modify it, often leading to soil acidification or alkalinization. Traditional management methods like lime application, organic amendments, and crop rotation are reviewed for their effectiveness in stabilizing soil pH and their limitations under varying soil conditions. The review also explores modern technological innovations like precision agriculture, which uses soil sensors and variable rate technology for targeted pH management, and biological approaches, such as microbial inoculants, to enhance nutrient cycling and organic matter decomposition. Integrating these traditional and contemporary approaches is essential for sustainable soil pH management and long-term

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productivity. The findings highlight the need for a holistic approach that combines historical knowledge with emerging technologies to promote sustainable agricultural practices and environmental conservation.

1 Introduction

The pH of the soil is a crucial factor that significantly affects its chemical, biological, and physical properties, ultimately determining its suitability for plant growth and overall ecosystem health. Soil comprises mineral particles, organic matter, water, and air, varying proportions based on the type of rock beneath the soil, climate, and land use. The mineral fraction consists of sand, silt, and clay, which determine the soil's texture and affect water retention and nutrient availability. Organic matter, which comes from decomposed plant and animal material, enriches the soil with essential nutrients and improves its structure. The soil's pH is influenced by the interactions among these components and their reaction with various natural and human-induced factors, such as the breakdown of minerals, decomposition of organic matter, and farming practices. Understanding the basic composition of soil and its pH dynamics is essential for effective soil management and sustainable agricultural practices.

A combination of natural and human factors influences soil pH. The type of rock beneath the soil, climate, local vegetation, and farming practices significantly affect soil pH. Additionally, changes in land use, such as urbanization, deforestation, and mining, disrupt the natural soil formation processes and introduce pollutants, altering the soil pH. Understanding these interconnected factors is crucial for effective soil management and sustainable agricultural practices.

The importance of soil pH lies in its direct impact on nutrient availability (Neina 2019). Most essential nutrients are accessible to plants within specific pH ranges (Melese et al. 2015). For example, macronutrients such as nitrogen, phosphorus, and potassium are highly available in soils with a pH range of 6 to 7.5. In contrast, in highly acidic soils (pH < 5.5), the availability of phosphorus and other essential nutrients decreases significantly, leading to nutrient deficiencies that can hinder plant growth and reduce crop yields (Brady and Weil 2008). Conversely, in alkaline soils (pH >7.5), micronutrients such as iron, manganese, and zinc become less available, adversely affecting plant health and productivity (Schmidt and Ellsworth 2000).

Traditional methods for managing soil pH include the application of lime to neutralize acidity and organic amendments like compost and manure to stabilize pH fluctuations (Yenesew et al. 2024). Crop rotation and cover crops can also help maintain balanced soil pH by improving soil structure and increasing organic matter content (Johnston and Goulding 1990; Head 2024). These practices have been used for centuries and are effective in many agricultural systems.

Recent technological innovations have introduced more precise and efficient soil pH management methods. Precision agriculture techniques such as soil sensors and variable rate technology (VRT) enable real-time monitoring and targeted application of soil amendments, which help optimize pH management (Brady and Weil 2008). Biological approaches, including microbial inoculants, are also gaining attention due to their potential to enhance soil health and regulate pH by improving nutrient cycling and organic matter decomposition (McCauley and Jacobsen 2009).

This review aims to comprehensively outline the strategies for effectively managing soil pH under changing environmental and agricultural conditions. It emphasizes combining traditional methods such as lime application, organic amendments, and crop rotation with modern technologies like precision agriculture, soil sensors, and biological interventions. By examining the strengths and limitations of these diverse approaches, the study aims to provide insights into how integrated soil pH management can enhance soil health, optimize plant growth, and contribute to sustainable agricultural practices and environmental conservation. The goal is to equip stakeholders with a nuanced understanding of adaptive soil pH management strategies responsive to natural and human-induced changes, ensuring long-term soil productivity and ecosystem stability.

2 Importance of soil pH

It's crucial to emphasize the significance of soil pH, as it directly influences the availability of vital nutrients for plant growth and their chemical form and solubility. In highly acidic soils with a pH below 5.5, the availability of important nutrients such as phosphorus decreases significantly (Brady and Weil 2008; McCauley and Jacobsen 2009). Additionally, acidic soils can release harmful elements like aluminium and manganese, damaging plant roots and hindering nutrient absorption (Sparks 2003).

The availability of key nutrients such as nitrogen, phosphorus, potassium, iron, manganese, zinc, and copper varies with soil pH (Figure 1). Each nutrient has an optimal pH range where it is most available to plants (Toor and Naeem 2023). Similarly, microbial activity is also affected by pH, being highest around a neutral pH (7.0), indicating that soil microbes are most active and beneficial at this pH level. The shaded area between pH 6 and 7 indicates the optimal pH range for most plant growth, where nutrient availability and microbial activity are generally balanced and favourable (Figure 1).

On the other end of the spectrum, alkaline soils (pH above 7.5) can result in deficiencies of essential micronutrients such as iron, manganese, zinc, and copper. These micronutrients are crucial for various physiological functions in plants, including enzyme activation, chlorophyll synthesis, and maintaining cellular integrity. In alkaline conditions, these nutrients become less soluble and, therefore, less available to plants, leading to symptoms such as chlorosis (yellowing of leaves), reduced growth, and lower crop quality (Schmidt and Ellsworth 2000).

The optimal pH range for most crops lies between 6.0 and 7.5, where the availability of most essential nutrients is maximized, and toxic elements remain insoluble. This range also optimizes microbial activity, which is crucial for organic matter decomposition, nitrogen fixation, and nutrient cycling processes. Microorganisms, like nitrifying bacteria, are also sensitive to pH changes and perform best in slightly acidic to neutral soils. Hence, maintaining soil pH within this optimal range is crucial for sustaining soil fertility and promoting healthy plant growth (Wei et al. 2024).

3 Natural Factors Affecting Soil pH

Several natural factors determine the initial pH of the soil and its changes over time. These include the parent material, climate, and vegetation type. Each factor plays a critical role in shaping the soil's chemical environment and influences its suitability for various plant species and agricultural practices (Wang et al. 2024).

3.1 Parent Material

The mineral composition of the parent material from which soil is formed significantly impacts its pH. Parent material is the underlying geological material (generally bedrock or a superficial or drift deposit) forming soil horizons (Whiteman 2024). The type of minerals present in the parent material largely dictates the initial chemical characteristics of the soil, including its pH. For example, soils that develop from limestone or other calcareous rocks tend to be more alkaline due to calcium carbonate. This compound reacts with water and carbon dioxide to form bicarbonate, which raises the soil pH (Brady and Weil 2008). Conversely, soils derived from acidic parent materials like granite or sandstone typically have a lower pH. Granite, rich in quartz and feldspar, contributes to the formation of acidic soils due to the lack of basic cations like calcium, magnesium, potassium, and sodium (Jing et al. 2024; Subramani et al. 2024). These acidic parent materials make soils more prone to leaching, enhancing their acidity (Sparks 2003). Additionally, the rate of weathering of parent material also influences soil pH. Rapid weathering of certain minerals can release ions that alter the soil's acidity or alkalinity. For instance, the weathering of feldspar minerals releases potassium ions, increasing soil pH, while the breakdown of sulfide minerals can

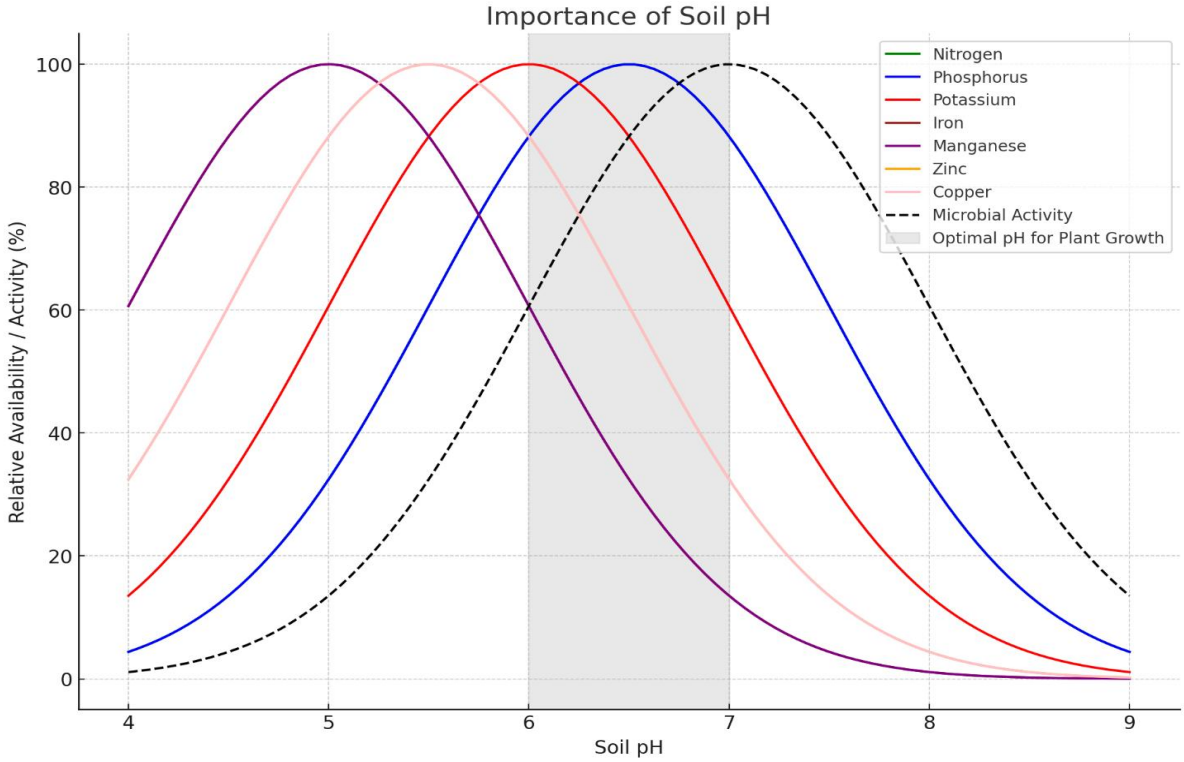

Figure 1 This graph illustrates how soil pH impacts soil health and plant productivity (Based on information from Brady and Weil 2008).

release sulfuric acid, contributing to soil acidity (McCauley and Jacobsen 2009).

3.2 Climatic Conditions

Climatic conditions are another significant factor affecting soil pH, primarily through its influence on weathering processes, leaching, and decomposition of organic matter (Philippot et al. 2024). In regions with high rainfall, acidic conditions are often prevalent. This might be due to the excessive precipitations that lead to leaching, where water percolates through the soil profile and carries away the basic cations such as calcium, magnesium, and potassium. As these cations are leached out, they are replaced by hydrogen ions and aluminium, leading to increased soil acidity (Chatterjee et al. 2024). In contrast, arid and semi-arid regions tend to have more alkaline soils. Limited rainfall reduces leaching, allowing salts and basic cations to accumulate in the soil. These conditions favour the formation of alkaline soils, often characterized by high sodium, calcium, and magnesium carbonate levels. Additionally, the evaporation rate in these regions often exceeds precipitation, concentrating these basic cations in the soil and further increasing alkalinity (Si and Li 2024).

In addition to rain, temperature plays a pivotal role in soil pH dynamics. Warmer temperatures generally enhance the rate of organic matter decomposition and their release, ultimately lowering the soil's pH (Galluzzi et al. 2024). However, in colder climates, slower decomposition rates result in the accumulation of organic matter, which can produce organic acids and contribute to soil acidity over time (Brady and Weil 2008).

3.3 Vegetation Type

The type of vegetation growing on the soil significantly influences its pH through organic matter deposition, root exudation, and nutrient uptake. Different plant species contribute varying amounts and types of organic matter to the soil, and decomposition of these organic matters can raise or lower the soil pH (Yuan et al. 2024). For instance, coniferous forests tend to produce more acidic soils. The needles of coniferous trees, such as pines and spruces, decompose slowly and release organic acids into the soil. These acids contribute to the acidification of the soil, leading to lower pH levels (Mueller et al. 2024). Additionally, the deep root systems of conifers can bring up acidic compounds from deeper soil layers, further contributing to surface soil acidity (Sparks 2003). In contrast, deciduous forests, composed of trees like oaks and maples, lead to the formation of more neutral or slightly acidic soils. The leaf litter from deciduous trees decomposes rapidly and releases fewer organic acids than coniferous needles. This reduces acidification and often more balanced soil pH levels (Joshi et al. 2024).

Grasslands and prairies also impact soil pH differently. The dense root systems of grasses contribute substantial organic matter to the soil, which helps buffer pH changes. Grassland soils often maintain a relatively neutral pH due to the rapid decomposition of organic matter and the cycling of nutrients within the root zone (McCauley and Jacobsen 2009).

4 Anthropogenic factors affecting soil pH

Human activities influence soil pH through agricultural practices, land use changes, and pollution (Figure 2).

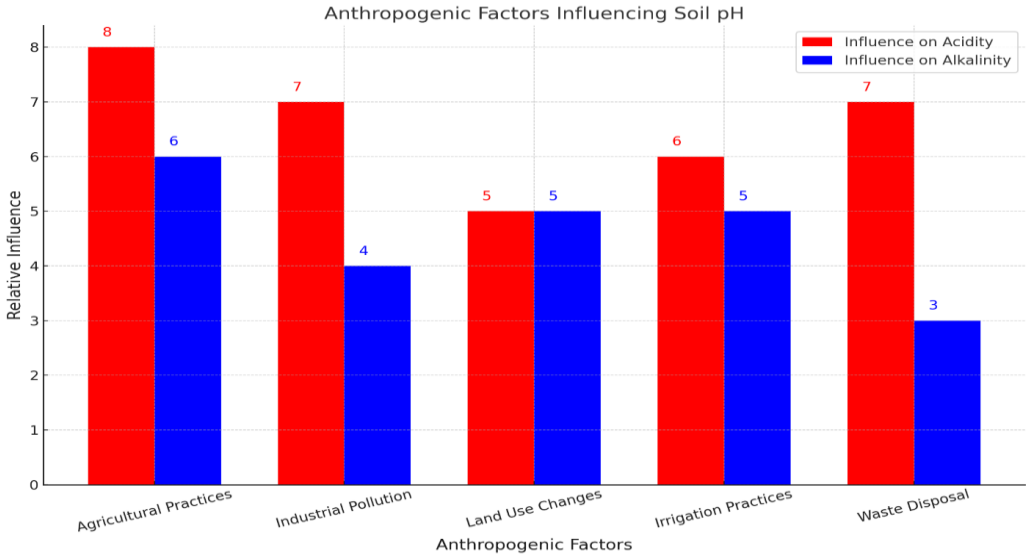

Figure 2 illustrates how each human-induced factor can influence soil pH, leading to either acidity or alkalinity. This figure was created for the current manuscript using data from Brady and Weil (2008).

4.1 Agricultural practices

Agricultural practices such as fertilization, crop rotation, and tillage techniques greatly influence soil pH. Nitrogen fertilizers, especially those containing ammonium, are known for their soil acidification properties. These fertilizers release hydrogen ions during nitrification, lowering soil pH levels (Toor and Ramzan 2023). On the other hand, lime application is a common method to counteract soil acidity and increase pH levels. Lime, usually in calcium carbonate or hydroxide, reacts with hydrogen ions and helps neutralize acidity, promoting a more alkaline soil environment. Crop rotation also plays a significant role in managing soil pH. Certain crops, such as legumes, can fix atmospheric nitrogen through symbiotic relationships with nitrogen-fixing bacteria. This reduces the need for nitrogen fertilizers, thus reducing the risk of soil acidification (Akram et al. 2023). Additionally, tillage practices can impact soil pH by influencing factors such as soil aeration and mixing (Liu et al. 2023; Akram et al. 2023). While these agricultural practices are essential for maintaining soil fertility and crop productivity, careful management is crucial to prevent adverse pH shifts and sustain soil health in the long term.

4.2 Land use changes

Land use changes, including urbanization, deforestation, and mining, disrupt natural soil-forming processes and introduce pollutants that can alter soil pH levels. Urban expansion often results in soil sealing, limiting water infiltration and disrupting soil microbial activity, which affects soil pH (Abakumov et al. 2023). Soil sealing through pavement and construction can lead to localized changes in soil pH, often contributing to increased acidity (Akram et al. 2023). Deforestation reduces organic matter inputs, leading to soil degradation and potential acidification, while mining activities can induce severe soil acidification by releasing acidic compounds such as sulfuric acid. Removing vegetative cover reduces organic matter inputs into the soil and diminishes its buffering capacity against acidity. Additionally, deforestation increases the risk of soil erosion, which can expose acidic subsoil layers, further contributing to soil acidification. The disturbance of soil microbiota and nutrient cycling processes due to forest removal can also impact soil pH dynamics (Tsegaye et al. 2023). Pollution from various sources, including industrial activities and improper waste disposal, can directly contaminate soils with acidic substances, further exacerbating pH imbalances (Wen et al. 2023). Urban areas are sources of various pollutants, including heavy metals and industrial emissions, which can directly acidify the soil or induce chemical reactions leading to pH alterations (Franco-Pesantez and Torres 2023). Mining activities, particularly surface and open-pit mining, also profoundly affect soil pH. Soil disturbance during mining operations exposes acidic subsoil and bedrock materials to the surface, leading to significant soil acidification. Moreover, mining activities often result in acid mine drainage, where sulfuric acid is generated through sulfide minerals oxidizing surrounding soils and waterways (Cao et al. 2024). Chemicals used in mining processes can also contribute to soil acidification through direct contamination and alteration of soil chemistry. Therefore, understanding and mitigating the impacts of these human-induced factors are essential for maintaining soil health and sustaining agricultural productivity in the face of environmental challenges.

5 Approaches to manage soil pH

Various approaches can be used to manage and adjust soil pH under changing conditions, ensuring optimal soil health and productivity (Figure 3).

5.1 Lime application

Lime application is crucial for managing soil pH and creating the best conditions for crop growth. Calcium carbonate ($CaCO_3$) is a commonly used lime source due to its effectiveness in neutralizing soil acidity. Another form of lime, calcium hydroxide ($Ca(OH)_2$), has similar benefits but reacts more quickly with soil acidity, leading to faster increases in pH levels (Charak et al. 2024). This rapid response can be advantageous for crops needing immediate pH adjustments. However, calcium hydroxide is often pricier than calcium carbonate, and its use may result in temporary pH spikes if overused. Careful management is essential to prevent excessive pH fluctuations and maximize its benefits.

Dolomitic lime containing magnesium and calcium carbonate is an effective solution in magnesium-deficient soils. Dolomitic lime raises soil pH and corrects magnesium deficiencies, promoting balanced nutrient availability for plants (Cano-Franco et al. 2024). Overall, lime application, whether in the form of calcium carbonate, calcium hydroxide, or dolomitic lime, is a valuable tool for maintaining soil pH within optimal ranges for crop production. However, proper application rates, timing, and considerations for soil type and crop requirements are essential to maximize its effectiveness while minimizing potential drawbacks.

5.2 Application of appropriate fertilizers

Proper management of fertilizers is crucial for maintaining soil pH balance. Ammonium-based nitrogen fertilizers, commonly used in agriculture, can contribute to soil acidification due to nitrification. During nitrification, soil bacteria convert ammonium ($NH4^+$) into nitrate ($NO3^-$), releasing hydrogen ions (H^+) in the process, which lowers soil pH (Akram et al. 2023). The following methods can be used to manage the acidifying effects of nitrogen fertilizers: (i) Properly managing the application rates and timing of nitrogen fertilizers is crucial. Avoiding excessive nitrogen applications helps minimize the accumulation of ammonium in the soil, reducing the potential for soil acidification, (ii) Utilizing

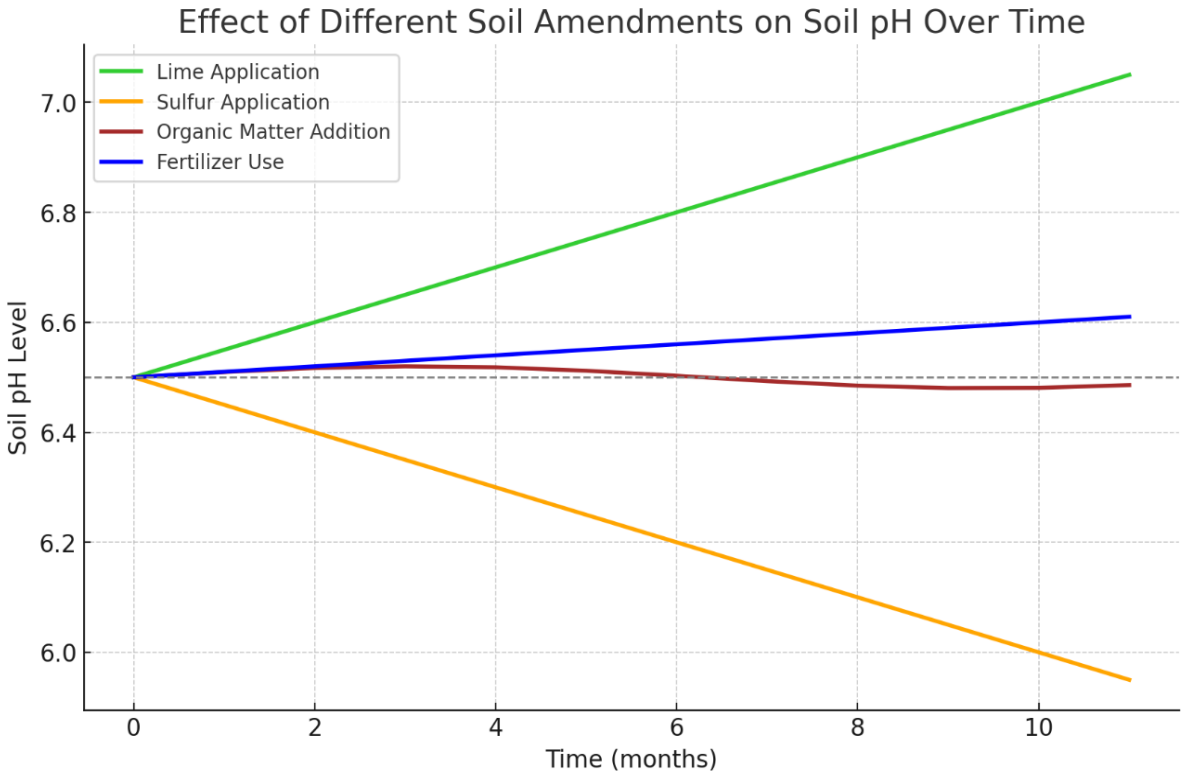

Figure 3 The different approaches can manage soil pH, comparing their effects over one year.

nitrification inhibitors such as nitrapyrin, dicyandiamide (DCD), or 3,4-dimethylpyrazole phosphate (DMPP) can be an effective strategy to slow down the conversion of ammonium to nitrate. These inhibitors work by inhibiting the activity of nitrifying bacteria, thereby reducing the release of hydrogen ions and mitigating soil acidification (Chen et al. 2008), (iii) Choosing nitrogen fertilizers with a higher proportion of ammonium over nitrate can also help minimize soil acidification. Ammonium-based fertilizers release fewer hydrogen ions during nitrification compared to nitrate-based fertilizers, thereby exerting less acidifying pressure on the soil (Chen et al. 2008), (iv) Sulfur-containing fertilizers, such as ammonium sulfate, elemental sulfur, or sulfur-coated urea, can contribute to soil acidification over time through the oxidation of sulfur compounds. Sulfur oxidation produces sulfuric acid, which lowers soil pH. To manage the acidifying effects of sulfur-based fertilizers (Akram et al. 2023), Regularly monitoring soil pH levels helps farmers assess changes over time and detect signs of soil acidification. Timely adjustments to fertilizer management practices can help to prevent or mitigate soil pH imbalances (Lashari 2022), and (vi) Soil buffering capacity should also be considered when using sulfur-based fertilizers. Soils with higher buffering capacity can resist pH changes more effectively, whereas soils with lower buffering capacity are more susceptible to pH fluctuations in response to fertilizer applications (Akram et al. 2023).

5.3 Proper selection of crops and their rotation

Crop selection and rotation strategies are crucial for managing soil pH and promoting overall soil health. By carefully choosing and rotating crops, farmers can enhance nutrient cycling, reduce dependence on fertilizers, and mitigate soil acidification (Mohanty et al. 2024). Two key approaches to crop selection and soil pH rotation incorporate leguminous and deep-rooted crops.

5.3.1 Leguminous crops

Legume crops such as beans, peas, clover, and alfalfa have a special ability to form partnerships with nitrogen-fixing bacteria called rhizobia (Chakraborty 2023). These bacteria live in nodules on the roots of leguminous plants and convert atmospheric nitrogen (N_2) into ammonia (NH_3), which the plants can easily absorb. Farmers can effectively "fix" atmospheric nitrogen by including leguminous crops in crop rotations, reducing the need for synthetic nitrogen fertilizers. This is important because nitrogen fertilizers, especially those containing ammonium, can contribute to soil acidification. Farmers can reduce their reliance on these fertilizers by using legume crops and help mitigate soil acidification (Chakraborty 2023). Additionally, when leguminous crops decompose, they add organic matter to the soil, which improves soil structure and microbial activity, helping to manage pH.

5.3.2 Deep-rooted crops

Certain grasses, grains, and some vegetables have deep root systems that reach into the soil to access nutrients and moisture from lower soil layers (Sharma et al. 2024). These deep-rooted crops can affect soil pH at different depths by reaching deeper soil layers. They play a crucial role in redistributing nutrients, especially those that may have leached below the root zone of shallow-rooted crops. Incorporating deep-rooted crops into crop rotations can help maintain more balanced soil pH profiles throughout the root zone. Additionally, deep-rooted crops contribute to the accumulation of soil organic matter through root turnover and residue deposition, which enhances soil fertility and its ability to resist pH fluctuations (Kumar and Hegde 2023).

5.3.3 Cover crops and Organic amendments

Planting cover crops during fallow periods or between cash crop rotations is an effective strategy for managing organic matter and stabilizing soil pH. Cover crops, such as legumes, grasses, or brassicas, contribute to soil organic matter accumulation through root exudates, root turnover, and aboveground biomass production. As these cover crops decompose, organic matter is incorporated into the soil, enriching soil organic carbon content and enhancing pH buffering capacity (Fageria et al. 2005). Cover crops also play a vital role in nutrient cycling, scavenging residual nutrients from the soil and recycling them upon decomposition. By maintaining soil cover and promoting biological activity, cover crops help sustain soil pH stability, reduce erosion, suppress weeds, and improve soil health (Fageria et al. 2005).

Adding organic materials such as compost, manure, or crop residues to the soil is widely practised to improve soil structure, fertility, and pH buffering capacity. These organic amendments contain various organic compounds that undergo decomposition and release organic acids, which act as buffers against soil acidity, helping to neutralize excess hydrogen ions and mitigate pH fluctuations (Bolan et al. 2023).

Additionally, incorporating organic matter enhances soil aggregation and porosity, improving water retention, nutrient availability, and root penetration. Soils amended with organic matter exhibit greater resilience to pH changes and provide a more favourable environment for plant growth and microbial activity. Soils amended with organic matter can either increase or decrease soil pH, depending on the type of organic matter used and the initial soil conditions. Organic matter, such as compost, manure, or plant residues, generally helps buffer soil pH, making it more resistant to rapid changes. If the organic matter has a high pH (e.g., wood ash), it can increase soil pH, making it more alkaline. Conversely, if the organic matter is more acidic (e.g., peat or certain composts), it can lower the pH, making the soil more acidic. Therefore, the effect on soil pH depends on the specific properties of the organic matter being applied.

Overall, organic matter management through adding organic amendments and using cover crops is essential for maintaining soil pH stability and fertility in agricultural systems (Singh et al. 2024). These practices contribute to long-term soil sustainability by enhancing soil structure, nutrient cycling, and microbial diversity, supporting resilient and productive crop production systems.

6 Technologies used for real-time pH monitoring and management

6.1 Precision agriculture technologies

Precision agriculture technologies provide advanced tools for efficiently and effectively managing soil pH. These technologies

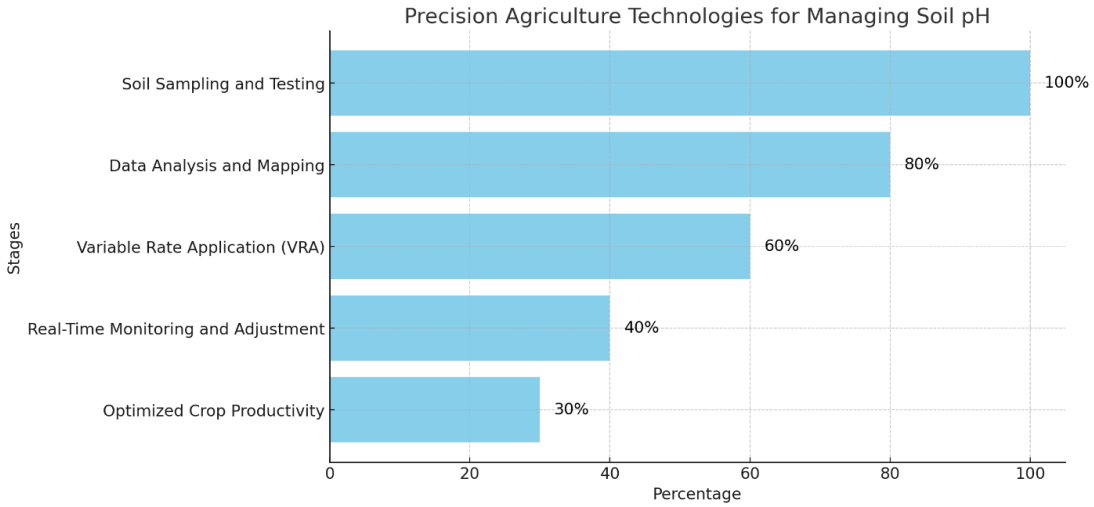

Figure 4 illustrates the progression of precision agriculture technologies from initial soil sampling to optimized crop productivity, showcasing effectiveness and adoption rates at each stage.

optimize crop productivity while minimizing environmental impacts. Two key precision agriculture technologies for soil pH management are Variable Rate Application (VRA) and Soil Sensors and Monitoring Systems (Figure 4).

6.1.1 Variable rate application

The VRA technology allows farmers to apply inputs such as lime and fertilizers at variable rates across fields based on specific soil conditions. This is determined by soil pH maps generated through soil testing (Pawase et al. 2023). By integrating soil pH data into precision agriculture systems, farmers can accurately identify field areas with pH imbalances and adjust lime and fertilizer application rates accordingly. In regions where soil pH varies spatially, VRA ensures that inputs are applied where needed, optimizing pH management, reducing input costs, and minimizing environmental impacts. This targeted approach to soil pH management enhances nutrient use efficiency, reduces the risk of overapplication or underapplication, and promotes more uniform crop growth and yield across fields.

6.1.2 Soil sensors and monitoring systems

Continuous monitoring of soil pH using sensors and monitoring systems provides real-time data on soil pH levels. This enables timely interventions to adjust pH to maintain optimal crop growing conditions (Das et al. 2024). Soil pH sensors can be installed at various depths in the soil profile to monitor changes in pH over time and detect trends or anomalies that may require management action. Additionally, soil pH monitoring systems can be integrated with other precision agriculture technologies, such as automated irrigation systems or crop management software, to facilitate data-driven decision-making and optimize pH management practices (Soussi et al. 2024). By providing insights into soil pH dynamics and trends, soil sensors and monitoring systems empower farmers to implement proactive strategies for pH management, ensuring that crops receive the appropriate pH conditions for optimal growth and productivity.

6.2 Water management

Effective water management, especially irrigation, is essential for regulating soil pH, particularly in areas where water quality can impact pH levels.

6.2.1 Irrigation water quality influence

The quality of irrigation water, including its pH, mineral content, and salinity, can directly influence soil pH. In areas with alkaline water sources, irrigation water may naturally have a high pH due to high concentrations of dissolved minerals such as calcium carbonate or bicarbonates. When alkaline irrigation water is used on soils, it can lead to soil alkalinity and increased soil pH levels over time. This effect is especially common in dry and semi-dry regions where water sources often come from limestone aquifers or contain high levels of dissolved carbonates (Benadela et al. 2022).

6.2.2 Appropriate Irrigation Practices

Appropriate irrigation practices are crucial for managing soil pH and minimizing the potential impacts of alkaline irrigation water. In the following sections, we will look in-depth at irrigation practices and their effect on soil pH.

6.2.2.1 Leaching

Leaching uses excess water to flush out salts and alkaline compounds from the root zone, preventing them from building up in the soil. During leaching, soluble salts and alkaline minerals are carried downward and removed from the root zone, which helps maintain optimal soil pH levels. This process is especially important in saline or sodic soils, where excessive salt accumulation can cause soil alkalinity and pH imbalances (Osman 2018).

6.2.2.2 Water pH adjustment

Adjusting the pH of irrigation water on a large scale is feasible and has been used in various agricultural practices, especially in regions with high water alkalinity or soils prone to becoming overly alkaline. By using acidifying agents such as sulfuric acid or citric acid in irrigation water, alkaline compounds can be neutralized, thus reducing their impact on soil pH when applied to the field (Neupane and Guo 2019; Rakibuzzaman et al. 2024). Precision water management systems, which include pH adjustment, are increasingly being adopted to improve water use efficiency and prevent soil degradation in large-scale farming operations (Neupane and Guo 2019; Rakibuzzaman et al. 2024).

6.2.2.3 Selection of Water Source

When possible, choosing irrigation water sources with lower alkalinity and mineral content can help reduce the risk of soil pH increase. Assessing water quality parameters such as pH, electrical conductivity (EC), and bicarbonate concentrations before irrigation can provide valuable information for water management decisions and help prevent potential soil pH problems (Rakibuzzaman et al. 2024).

6.3 Integration with Soil Management Practices

Combining effective irrigation management with soil management practices is crucial for achieving optimal soil pH levels and enhancing crop productivity. Soil testing and monitoring can help evaluate soil pH status and guide irrigation scheduling and water management decisions. In addition, soil amendments such as gypsum or elemental sulfur can help reduce soil alkalinity and

maintain soil pH within the desired range. Moreover, pH-adjusting fertilizers, including controlled-release fertilizers (CRFs) and pH-balanced fertilizers, provide innovative solutions for managing soil pH while supplying essential nutrients to crops. These fertilizers are designed to release nutrients slowly and steadily over time, which helps maintain optimal soil pH levels and nutrient availability. The controlled release of nutrients aligns more closely with the plant's uptake needs, reducing nutrient losses and minimizing environmental impacts. This is particularly beneficial in managing soil pH as it prevents sudden changes in pH levels that could negatively impact soil health and crop productivity (Babadi et al. 2021; Li and Li 2024).

6.3.1 Controlled-release fertilizers

Controlled-release fertilizers (CRF) such as polymer-coated urea, sulfur-coated urea, polymer-sulfur-coated urea (PSCU), urea-formaldehyde (UF), isobutylidene diurea (IBDU), biochar-based coated fertilizers, and starch-based coated fertilizers are formulated with coated or encapsulated nutrient granules. These granules release nutrients slowly and continuously over an extended period. The release rate of nutrients from CRFs is controlled by factors such as temperature, moisture, and microbial activity in the soil. By providing a steady supply of nutrients to crops over time, CRFs help optimize nutrient uptake efficiency and reduce nutrient losses through leaching or volatilization. Additionally, the gradual release of nutrients from CRFs minimizes the risk of rapid pH changes in the soil, promoting more stable soil pH conditions conducive to optimal crop growth (Greenhouse Grower 2024).

6.3.2 pH-balanced fertilizers

pH-balanced fertilizers are specially formulated to provide essential nutrients without significantly altering soil pH levels. For example, calcium nitrate supplies calcium and nitrogen while maintaining a neutral to slightly alkaline pH, and potassium nitrate offers potassium and nitrogen without impacting soil acidity. Another pH-neutral option is magnesium sulfate, or Epsom salt, which provides magnesium and sulfur. Monoammonium phosphate (MAP) is frequently used for its balanced pH effect, making it ideal for crops with moderate pH requirements. While not a traditional fertilizer, gypsum adds calcium and sulfur without altering soil pH, making it useful for neutralizing excess sodium in soils. These pH-balanced fertilizers are suitable for sensitive crops and soils, helping to maintain optimal growing conditions (Warner et al. 2023).

These fertilizers typically contain buffering agents or additives that help stabilize soil pH and prevent pH fluctuations caused by nutrient applications. By balancing nutrient delivery with pH management, these fertilizers ensure that crops receive adequate nutrition while promoting soil health and fertility (Rahmat et al. 2023). Additionally, pH-balanced fertilizers are compatible with various soil types and cropping systems, providing flexibility and convenience for farmers in managing soil pH. Some of the most common benefits of using these fertilizers are given in the subsequent section of this article.

6.3.2.1 Optimized nutrient availability

Utilizing pH-adjusting fertilizers can help ensure that crops receive a consistent and balanced supply of nutrients, thus optimizing nutrient uptake and utilization. This is particularly crucial as soil pH significantly affects the availability of essential nutrients such as nitrogen, phosphorus, and potassium. Maintaining an optimal pH range enables plants to more effectively absorb these nutrients, leading to healthier growth and higher yields (Miller 2021).

6.3.2.2 Stable soil pH

pH-adjusting fertilizers help to maintain stable soil pH levels by incorporating pH-buffering agents and releasing nutrients gradually. This reduces the risk of pH imbalances, which can have a negative impact on crop productivity and soil health. The stability in soil pH provided by these fertilizers is crucial for sustaining long-term soil fertility and avoiding the problems associated with rapid pH changes, such as nutrient lock-up or toxicity (Potash Development Association 2021).

6.3.2.3 Reduced environmental impact

The controlled-release fertilizers (CRFs) in pH-adjusting formulations help minimize nutrient leaching and runoff, significantly contributing to water pollution. By slowly releasing nutrients, these fertilizers reduce the potential for nitrogen and phosphorus to enter water bodies, promoting environmental sustainability and protecting aquatic ecosystems (Oshunsanya 2019).

6.4 Technological innovations

6.4.1 Soil sensors and monitoring systems

Continuous monitoring of soil pH using sensors and monitoring systems provides real-time data on pH levels. This enables timely interventions to adjust pH as needed (Narayana et al. 2024), ensuring optimal crop growing conditions and facilitating data-driven decision-making in soil management.

6.4.2 Remote sensing and GIS technologies

6.4.2.1 Satellite imagery

Remote sensing technologies offer valuable insights into soil characteristics, including pH levels, across extensive agricultural landscapes. Satellite imagery can delineate spatial variations in soil pH, enabling targeted management interventions.

6.4.2.2 GIS mapping

Geographic Information Systems (GIS) allow for integrating soil pH data with other spatial information, including topography, land use, and weather patterns. This integration helps identify the factors that influence soil pH variability and enables the development of customized management strategies

6.5 Smart farming solutions

6.5.1 IoT and cloud computing

Integrating IoT and cloud computing in agriculture has transformed traditional farming methods by enabling real-time monitoring, data-driven decision-making, and automation. IoT devices such as sensors and drones continuously monitor soil moisture, temperature, humidity, and crop health. This data is then transmitted to cloud-based platforms, processed and analyzed to provide actionable insights for optimizing resource use, such as water and fertilizers. For instance, smart irrigation systems can automatically adjust water levels based on real-time soil moisture data, reducing water wastage and ensuring crops receive the optimal amount of water (Friha et al. 2021; Randazzo et al. 2022).

In precision farming, IoT and cloud computing allow farmers to apply inputs like fertilizers and pesticides with pinpoint accuracy tailored to the needs of individual crops or specific field areas. This targeted approach minimizes input use and maximizes yield, contributing to more sustainable agricultural practices (Mohammed et al. 2021). Additionally, cloud computing facilitates the processing of large datasets from multiple sources, such as weather forecasts and satellite imagery, to provide farmers with recommendations on planting and harvesting times, helping them adapt to changing climatic conditions and mitigate risks associated with unpredictable weather (Hori et al. 2010; Thilakarathne et al. 2023).

Studies have shown the effectiveness of these technologies in improving operational efficiency and productivity in agriculture. Friha et al. (2021) reviewed various IoT-based applications, including smart water management and disease management, and highlighted the potential of cloud and fog computing for real-time data processing. Similarly, Randazzo et al. (2022) explored the architecture of IoT-enabled smart agriculture, identifying opportunities for integrating big data and cloud computing to enhance agricultural sustainability. These advancements pave the way for more efficient, resilient, and sustainable farming practices (Friha et al. 2021; Randazzo et al. 2022; Thilakarathne et al. 2023).

6.5.2 Machine learning and AI

Machine learning (ML) and artificial intelligence (AI) are increasingly used in agriculture to enhance productivity, optimize resource utilization, and promote sustainable farming practices. These technologies are applied in various areas, including crop management, soil health monitoring, and predictive analytics for weather and pest control. For example, ML algorithms analyze data from sensors and drones to monitor crop health, detect diseases, and assess soil conditions in real-time. This allows farmers to make well-informed decisions regarding irrigation, fertilization, and pest management, ultimately leading to improved yields and reduced environmental impact (Benos et al. 2021). AI and ML are also employed in automated machinery and robotics in agriculture. Autonomous tractors and harvesters equipped with AI can perform tasks such as planting, weeding, and harvesting with minimal human intervention. This reduces labour costs and ensures precision in farming activities, resulting in higher efficiency and productivity (Jha et al. 2018). Additionally, AI-driven platforms are being developed for supply chain management to help predict market demands, optimize logistics, and reduce food wastage. These platforms use predictive analytics to anticipate supply and demand trends, enabling better planning and distribution of agricultural products (Hassan et al. 2021). These technologies hold great potential for transforming traditional farming into a more data-driven, precise, and sustainable system. The adoption of AI and ML in agriculture is expected to grow as new applications and technologies emerge, further enhancing agricultural productivity and sustainability (Pallathadka et al. 2023).

6.6 Advanced soil amendments

6.6.1 Nano- and microscale amendments

Nano- and microscale amendments, such as nanofertilizers and nanopesticides, offer significant agricultural productivity and sustainability advancements. These amendments are designed to improve nutrient use efficiency, reduce environmental impacts, enhance plant resilience to stress conditions, and help maintain soil pH. Nanofertilizers are engineered to deliver nutrients more effectively to plants by utilizing nanoparticles that provide a larger surface area and improved solubility than traditional fertilizers. This leads to more efficient nutrient uptake and reduced losses due to leaching or volatilization, indirectly affecting soil pH. These nanomaterials ensure a controlled release of active ingredients, enhancing their effectiveness while lowering environmental risks associated with pH change (Victoria et al. 2023). The application of these nano- and microscale technologies is not limited to nutrient delivery and pest control. They also play a role in soil and water remediation, helping to remove contaminants and improve soil health (Behera et al. 2022). These innovative solutions are part of a broader move towards sustainable agriculture, where precision and efficiency are prioritized to meet the growing global food demand without compromising environmental integrity (Akhtar et al. 2022).

6.6.2 Biochar and soil conditioners

Biochar is a carbon-rich product made from the pyrolysis of organic materials like agricultural waste and biomass. It is increasingly recognized as an effective soil conditioner in sustainable agriculture. Its application offers multiple benefits, including improved soil fertility, enhanced nutrient retention, increased water-holding capacity, long-term carbon sequestration, and pH maintenance. Biochar's porous structure allows it to retain nutrients and water, making it more available to plants and reducing nutrient leaching. This can significantly improve crop yields, especially in degraded soils or areas with poor soil fertility (Alotaibi and Schoenau 2019). Additionally, biochar's ability to act as a habitat for beneficial soil microbes promotes a healthy soil ecosystem, indirectly affecting soil pH, which enhances the soil's capacity to support plant growth and resist diseases. Moreover, biochar is highly stable and potentially sequesters carbon. However, the effectiveness of biochar can vary based on factors such as the type of feedstock used, the pyrolysis process, and the specific soil and climatic conditions. While biochar generally improves soil properties and crop yields, some studies have reported inconsistent results in different environmental settings, emphasizing more region-specific research and long-term field trials to understand better its impacts (Galinato et al. 2011; Pandit et al. 2018). In addition to biochar, other soil conditioners such as gypsum, lime, and organic amendments enhance soil structure, pH balance, and nutrient availability. Integrating biochar with these conditioners can offer synergistic benefits, improving soil health and agricultural productivity (Anawar et al. 2015).

6.6.3 Biological approaches

Microbial inoculants, such as specific bacteria and fungi, can help regulate soil pH by enhancing organic matter decomposition and nutrient cycling. These biological approaches offer sustainable solutions for long-term soil health (Johnston and Goulding 1990).

Conclusion and Future Prospects

It is crucial to manage soil pH for sustainable agriculture and ecosystem health. Soil pH directly affects nutrient availability, microbial activity, and soil fertility, influencing crop growth and productivity. Farmers can maintain soil pH balance and fertility using traditional methods like liming, crop selection and rotation, and organic matter management. Modern technological innovations, such as precision agriculture technologies and biological approaches like microbial inoculants and plant-microbe interactions, offer efficient ways to manage soil pH at a finer spatial scale. Future research should focus on creating integrated approaches that combine these strategies to maximize soil resilience, productivity, and sustainability.

The biochar and soil conditioners study shows significant potential for enhancing soil health and sustainable agricultural practices. Biochar, a carbon-rich material produced through the pyrolysis of organic waste, has shown promise in improving soil properties such as fertility, water retention, and nutrient availability. Future studies should focus on large-scale, long-term field trials to better understand the interactions between biochar, soil, and crops over time. It is also important to explore the integration of biochar with other soil conditioners, such as compost and gypsum, to assess their synergistic effects on soil health and productivity. Furthermore, research should address biochar production's economic and environmental feasibility, considering cost, scalability, and potential carbon sequestration benefits. Understanding the role of biochar in mitigating climate change by capturing atmospheric carbon and reducing greenhouse gas emissions from agriculture is crucial for developing sustainable farming practices. Addressing these research gaps will contribute to formulating effective biochar-based strategies for improving soil management and ensuring long-term agricultural sustainability.

Conflict of Interest

Authors declared no conflict of interest with each other.

Ethical Clearance

It is certified that no animal or human model was used during the study, so there is no need for any ethical clearance.

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References

Abakumov, E., Yuldashev, G., Mirzayev, U., Isagaliev, M., Sotiboldieva, G., et al. (2023). The Current State of Irrigated Soils in the Central Fergana Desert under the Effect of Anthropogenic Factors. *Geosciences*, *13*(3), 90.

Akhtar, N., Ilyas, N., Meraj, T. A., Pour-Aboughadareh, A., Sayyed, R. Z., & Mashwani, Z. U. (2022). Improvement of plant responses by nano-biofertilizer: A step towards sustainable agriculture. *Nanomaterials*, *12*, 965. https://doi.org/10.3390/nano12060965

Akram, S., Muzaffar, A., Farooq, Q., & Lashari, M. W. (2023). Soil pH and its functions in plant nutrient uptake and restoration. *International Journal of Agriculture and Environment*, *2*(1), 1-5.

Alotaibi, K. D., & Schoenau, J. J. (2019). Addition ofBiochar to a sandy desert soil: effect on crop growth, water retention, and selected properties. *Agronomy,* 9(6), 327; https://doi.org/10.3390/agronomy9060327

Anawar, H. M., Akter, F., Solaiman, Z. M., & Strezov, V. (2015). Biochar: An emerging panacea for remediation of soil contaminants from mining, industry, and sewage wastes. *Pedosphere*, *25*(5), 654–665. https://doi.org/10.1016/S1002-0160(15)30046-1

Babadi, F. E., Yunus, R., Soltani, S. M., & Shotipruk, A. (2021). Release mechanisms and kinetic models of Gypsum–Sulfur–Zeolite-coated urea sealed with microcrystalline wax for regulated dissolution. *ACS Omega*, *6*, 11144-11154. https://doi.org/10.1021/ACSOMEGA.0C04353

Behera, K. K., Alam, A., Vats, S., Sharma, H. P., & Sharma, V. (2022). Advancing sustainable agriculture: Enhancing crop nutrition with next-generation nanotech-based fertilizers. *Nano Research*, *16*, 13205-13225. https://doi.org/10.1007/s12274-022-4396-3

Benadela, L., Bekkoussa, B., &Gaidi, L. (2022). Multivariate analysis and geochemical investigations of groundwater in a semi-arid region, case of superficial aquifer in Ghriss Basin, Northwest Algeria. *Journal of Groundwater Science and Engineering*, *10*(3), 233-249 doi: 10.19637/j.cnki.2305-7068.2022.03.003

Benos, L., Tagarakis, A.C., Dolias, G., Berruto, R., Kateris, D., & Bochtis, D. (2021) Machine Learning in Agriculture: A Comprehensive Updated Review. *Sensors*, *21*, 3758. https://doi.org/10.3390/s21113758

Bolan, N., Sarmah, A. K., Bordoloi, S., Bolan, S., Padhye, L. P., et al. (2023). Soil acidification and the liming potential ofBiochar. *Environmental Pollution*, *317*, 120632.

Brady, N. C., & Weil, R. R. (2008). *The Nature and Properties of Soils*, 14 ed. Prentice Hall. Upper Saddle River, New Jersey.

Cano-Franco, J. C., Hernández-García, H. F., & Álvarez-Láinez, M. L. (2024). Fast-dissolving calcium carbonate particles as a high-performance lime for amelioration of agricultural acidic soils. *Journal of Environmental Chemical Engineering*, *12*(3), 112607.

Cao, Y., Ai, Z., Dang, X., Hou, M., Liu, H., et al. (2024). Effects of different reclamation measures on soil quality restoration in open-pit mines: A meta-analysis based on the Chinese Loess Plateau. *Ecological Engineering*, *203*, 107257.

Chakraborty, A. (2023). *Symbiotic Nitrogen Fixation and Soil Fertility*. Integrated Publications TM New Delhi, pp. 127.

Charak, R., Bhardwaj, A., & Gautam, S. (2024). Calcium-based hydroxide: Synthesis and applications. In S. S. Nanda, J. P. Singh, S. Gautam, & D. K. Yi (Eds.), *Calcium-Based Materials: Processing, Characterization, and Applications* (pp. 71-87). CRC Press. https://doi.org/10.1201/9781032419558

Chatterjee, D., Das, S. R., Saha, S., Sarkar, A., & Pathak, H. (2024). Impacts of climate change on soil processes. In H. Pathak, D. Chatterjee, S. Saha, & B. Das (Eds.), *Climate Change Impacts on Soil-Plant-Atmosphere Continuum* (pp. 3-36). Springer Nature Singapore. https://doi.org/10.1007/978-981-19-9572-1_1

Chen, D., Suter, H., Islam, A., Edis, R., Freney, J.R., & Walker, C.N. (2008). Prospects of improving efficiency of fertilizer nitrogen in Australian agriculture: a review of enhanced efficiency fertilizers. *Australian Journal of Soil Research*, 46(4), 289-301. doi: 10.1071/SR07105.

Das, H., Singh, S. K., Priyadarshini, E., Maurya, C. L., Tiwari, A. K., et al. (2024). Nanotechnology Facilitated Real Time Soil Monitoring for Optimized Crop Production. *Asian Journal of Soil Science and Plant Nutrition*, *10*(2), 582-607.

Fageria, N. K., Baligar, V. C., & Bailey, B. A. (2005). Role of cover crops in improving soil and row crop productivity. *Communications in soil science and plant analysis*, *36*(19-20), 2733-2757.

Franco-Pesantez, F., & Torres, M. E. C. (2023). Organic fertilizers enhance soil water and nutrients holding capacity and their mechanism. *International Journal of Agriculture and Environment*, *2*(1), 21-25.

Friha, O., Ferrag, M.A., Shu, L., Maglaras, L., & Wang, X. (2021). Internet of Things for the Future of Smart Agriculture: A Comprehensive Survey of Emerging Technologies. *IEEE/CAA Journal of Automatica Sinica*, *8*, 718-752.

Galinato, S. P., Yoder, J. K., & Granatstein, D. (2011). The economic value ofBiochar in crop production and carbon sequestration. *Energy Policy*, *39*(10), 6344-6350.

Galluzzi, G., Plaza, C., Priori, S., Giannetta, B., & Zaccone, C. (2024). Soil organic matter dynamics and stability: Climate vs. time. *Science of the Total Environment*, *929*, 172441.

Greenhouse Grower. (2024). Understanding Plant Nutrition: Controlled- And Slow-Release Fertilizers. Retrieved from Greenhouse Grower. https://www.greenhousegrower.com/production/fertilization/understanding-plant-nutrition-controlled-and-slow-release-fertilizers/

Harter, J., Weigold, P., El-Hadidi, M., Huson, D. H., Kappler, A., & Behrens, S. (2016). Soil biochar amendment shapes the composition of N2O-reducing microbial communities. *Science of the Total Environment*, *562*, 379–390. https://doi.org/10.1016/j.scitotenv.2016.03.220

Hassan, S.I., Alam, M.M., Illahi, U., Al Ghamdi, M.A., Almotiri, S.H., & Su'ud, M.M. (2021). A Systematic Review on Monitoring

and Advanced Control Strategies in Smart Agriculture. *IEEE Access, 9*, 32517-32548.

Head, K. V. K. (2024). Soil Health and Nutrient Management. In S. Bhardwaj, A. Kumar, & J. Singh (Eds.), *Sustainable Agricultural Technologies* (pp. 177-203). Springer Nature. https://doi.org/10.1007/978-3-030-70555-2_8

Hori, M., Kawashima, E., & Yamazaki, T. (2010). Application of Cloud Computing to Agriculture and Prospects in Other Fields. *Fujitsu Scientific & Technical Journal*, *46*(4), 446-454.

Jha, S.K., & Ahmad, Z. (2018) Soil microbial dynamics prediction using machine learning regression methods. *Computers and Electronics in Agriculture, 147*, 158–165

Jing, T., Li, J., He, Y., Shankar, A., Saxena, A., et al. (2024). Role of calcium nutrition in plant Physiology: Advances in research and insights into acidic soil conditions-A comprehensive review. *Plant Physiology and Biochemistry*, *210*, 108602. DOI: https://doi.org/10.1016/j.plaphy.2024.108602.

Johnston, A. E., & Goulding, K. W. T. (1990). Soil Acidification and its Reversal. *Agriculture, Ecosystems & Environment*, *30*(1-2), 49-74.

Joshi, S. R., Tfaily, M. M., Young, R. P., &Mcnear Jr, D. H. (2024). Root exudates induced coupled carbon and phosphorus cycling in a soil with low phosphorus availability. *Plant and Soil*, *498*(1), 371-390.

Kumar, K. A., & Hegde, R. (2023). Crop management practices for carbon sequestration. In S. K. Meena, A. de Oliveira Ferreira, & V. S. Meena (Eds.), *Agricultural Soil Sustainability and Carbon Management* (pp. 27-48). Academic Press. https://doi.org/10.1016/B978-0-323-95911-7.00003-5

Lashari, M. W. (2022). Management of plant soil health with nanoparticles: an efficient approach to multiple issues. *International Journal of Agriculture and Environment*, *1*(1), 1-12.

Li, X., & Li, Z. (2024). Global Trends and Current Advances in Slow/Controlled-Release Fertilizers: A Bibliometric Analysis from 1990 to 2023. *Agriculture*, *14*(9), 1502.

Liu, Z., Zhao, Y., Zhang, B., Wang, J., Zhu, L., & Hu, B. (2023). Deterministic effect of pH on shaping soil resistome revealed by metagenomic analysis. *Environmental science & technology*, *57*(2), 985-996.

McCauley, A., Jones, C., & Jacobsen, J. (2009). Soil pH and Organic Matter. *Nutrient Management Module No. 8*. Montana State University Extension.chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.certifiedcropadviser.org/files/certifications/certified/education/self-study/exam-pdfs/38.pdf

Melese, A., Gebrekidan, H., Yli-Halla, M., &Yitaferu, B. (2015). Phosphorus status, inorganic phosphorus forms, and other physicochemical properties of acid soils of Farta district, Northwestern highlands of Ethiopia. *Applied and Environmental Soil Science*, *2015*(1), 748390.

Miller, J.O. (2021). Soil pH Affects Nutrient Availability. Retrieved from https://extension.umd.edu/sites/extension.umd.edu/files/publications/FS-1054%20Soil%20pH%20and%20Nutrient%20Availbility_Update_12_2021.pdf

Mohammed, M., Riad, K., & Alqahtani, N. (2021). Efficient IoT-Based Control for a Smart Subsurface Irrigation System to Enhance Irrigation Management of Date Palm. *Sensors (Basel, Switzerland)*, *21*(12), 3942. https://doi.org/10.3390/s21123942

Mohanty, L. K., Singh, N. K., Raj, P., Prakash, A., Tiwari, A. K., Singh, V., & Sachan, P. (2024). Nurturing Crops, Enhancing Soil Health, and Sustaining Agricultural Prosperity Worldwide through Agronomy. *Journal of Experimental Agriculture International*, *46*(2), 46-67.

Mueller, C. W., Baumert, V., Carminati, A., Germon, A., Holz, M., et al. (2024). From rhizosphere to detritusphere–Soil structure formation driven by plant roots and the interactions with soil biota. *Soil Biology and Biochemistry*, *193*, 109396. DOI: https://doi.org/10.1016/j.soilbio.2024.109396.

Narayana, T. L., Venkatesh, C., Kiran, A., Kumar, A., Khan, S. B., Almusharraf, A., & Quasim, M. T. (2024). Advances in real-time smart monitoring of environmental parameters using IoT and sensors. *Heliyon*, *10*(7), e15606. https://doi.org/10.1016/j.heliyon.2024.e15606

Neina, D. (2019). The role of soil pH in plant nutrition and soil remediation. *Applied and environmental soil science*, *2019*(1), 5794869.

Neupane, J., & Guo, W. (2019). Agronomic basis and strategies for precision water management: A review. *Agronomy*, 9(2), 87. https://doi.org/10.3390/agronomy9020087

Oshunsanya, S. (Ed.). (2019). Soil pH for Nutrient Availability and Crop Performance. IntechOpen. doi: 10.5772/68057

Osman, K. T. (2018). Saline and sodic soils. In K. T. Osman (Eds.) *Management of soil problems* (pp. 255-298). Springer, Cham. https://doi.org/10.1007/978-3-319-75527-4_10

Pallathadka, H., Mustafa, M., Sanchez, D.T., Sajja, G.S., Gour, S., & Naved, M. (2023) Impact of Machine learning on

Management, healthcare and Agriculture. *Materials Today: Proceedings*, *80* (3), 2803-2806

Pandit, N. R., Mulder, J., Hale, S. E., Zimmerman, A. R., Pandit, B. H., & Cornelissen, G. (2018). Multi-year double cropping biochar field trials in Nepal: Finding the optimal biochar dose through agronomic trials and cost-benefit analysis. *The Science of the total environment*, *637-638*, 1333–1341. https://doi.org/10.1016/j.scitotenv.2018.05.107

Pawase, P. P., Nalawade, S. M., Bhanage, G. B., Walunj, A. A., Kadam, P. B., Durgude, A. G., & Patil, M. G. (2023). Variable rate fertilizer application technology for nutrient management: A review. *International Journal of Agricultural and Biological Engineering*, *16*(4), 11-19.

Philippot, L., Chenu, C., Kappler, A., Rillig, M. C., & Fierer, N. (2024). The interplay between microbial communities and soil properties. *Nature Reviews Microbiology*, *22*(4), 226-239.

Potash Development Association (PDA). (2021). Influence of soil pH on plant nutrient availability. Retrieved from https://www.pda.org.uk/wp/wp-content/uploads/2015/12/PDA-lf24.pdf

Rahmat, Z., Sohail, M. N., Perrine-Walker, F., & Kaiser, B. N. (2023). Balancing nitrate acquisition strategies in symbiotic legumes. *Planta*, *258*(1), 12.

Rakibuzzaman, M., Li, D., Wu, Z., Xue, Y., Xiao, F., Zhang, L., Song, Y., Zheng, Y., & Cui, Y. (2024). A review of precision irrigation water-saving technology under changing climate for enhancing water use efficiency, crop yield, and environmental footprints. *Agriculture*, *14*(7), 1141. https://doi.org/10.3390/agriculture14071141

Randazzo, G., Muzirafuti, A., & Forcén-Muñoz, L. (2022). IoT-Enabled Smart Agriculture: Architecture, Applications, and Challenges. *Applied Sciences*, *12*(7), 3396.

Schmidt, J. P., & Ellsworth, T. R. (2000). Soil pH and Plant Nutrients. University of Illinois Extension.https://extension.illinois.edu/soil/interpreting-test-results

Sharma, A., Saini, P., Saini, P., Tyagi, V., Sharma, S., Ahmed, N., & Sheikh, I. (2024). Root system architecture in cereals: Exploring different perspectives of the hidden half. *Brazilian Journal of Botany*, *47*(1), 925-943. https://doi.org/10.1007/s40415-024-00991-3.

Si, L., & Li, Z. (2024). Atmospheric precipitation chemistry and environmental significance in major anthropogenic regions globally. *Science of The Total Environment*, *926*, 171830.

Singh, N. K., Sachan, K., BP, M., Panotra, N., &Katiyar, D. (2024). Building Soil Health and Fertility through Organic Amendments and Practices: A Review. *Asian Journal of Soil Science and Plant Nutrition*, *10*(1), 175-197.

Soussi, A., Zero, E., Sacile, R., Trinchero, D., & Fossa, M. (2024). Smart Sensors and Smart Data for Precision Agriculture: A Review. *Sensors*, *24*(8), 2647.

Sparks, D. L. (2003). *Environmental Soil Chemistry* (2nd ed.). Academic Press.

Subramani, T., Gopinathan, P., Satheeskumar, V., & Karunanidhi, D. (2024). Role of Geochemistry of Rocks and Soils for Groundwater Quality in the Chithar River Basin, South India—An Approach Using Enrichment Factor (EF) of Trace Elements and Chemical Index of Alteration (CIA). In S. Satheeshkumar, V. Thirukumaran, & D. Karunanidhi (Eds.), *Modern River Science for Watershed Management: GIS and Hydrogeological Application* (pp. 381-402). Springer Nature Switzerland. https://link.springer.com/book/10.1007/978-3-031-54704-1

Thilakarathne, N. N., Bakar, M. S. A., Abas, P. E., & Yassin, H. (2023). Towards making the fields talks: A real-time cloud enabled IoT crop management platform for smart agriculture. *Frontiers in plant science*, *13*, 1030168. https://doi.org/10.3389/fpls.2022.1030168

Toor, M. D., & Naeem, A. (2023). Recent developments in nano-enabled fertilizers for environmental and agricultural sustainability. *International Journal of Agriculture and Environment*, *2*(2), 62-66.

Toor, M. D., & Ramzan, H. (2023). A brief description on the use of organomineral phosphorus in sustainable agriculture. *International Journal of Agriculture and Environment*, *2*(2), 67-71.

Tsegaye, N. T., Negewo, D. A., & Mitiku, S. T. (2023). Effect of Deforestation on the Status of Soil Fertility. *East African Journal of Forestry and Agroforestry*, *6*(1), 137-147.

Victoria, J., Tiwari, K., Rana, S., Tripathi, D. K., Sharma, S., &Sahi, S. (2023). Recent advances and perspectives of nanomaterials in agricultural management and associated environmental risk: A review. *Nanomaterials*, *13*, 1604. https://doi.org/10.3390/nano13101604

Wang, L., Xiong, W., Chen, H., Meng, F., & Tan, Y. (2024). Exploring the spatial distribution and influencing factors of soil pH value of cultivated land in Sichuan Province. *Environmental Earth Sciences*, *83*(1), 10.

Warner, J. M., Mann, M. L., Chamberlin, J., & Tizale, C. Y. (2023). Estimating acid soil effects on selected cereal crop

productivities in Ethiopia: Comparing economic cost-effectiveness of lime and fertilizer applications. *Plos One*, *18*(1), e0280230.

Wei, X., Xie, B., Wan, C., Song, R., Zhong, W., Xin, S., & Song, K. (2024). Enhancing soil health and plant growth through microbial fertilizers: mechanisms, benefits, and sustainable agricultural practices. *Agronomy*, *14*(3), 609.

Wen, J., Wu, C., Bi, X., Zhang, S., Ouyang, H., et al. (2023). Soil pH change induced by smelting activities affects secondary carbonate production and long-term Cd activity in subsoils. *Applied Geochemistry*, *152*, 105663.

Whiteman, C. A. (2024). Periglacial landforms and landscape development in southern England. *Proceedings of the Geologists' Association*. https://doi.org/10.1016/j.pgeola.2024.04.006.

Yenesew, S. A., Selassie, Y. G., Ejigu, W., Abere, T., Lewoyehu, M., &Adegeh, A. (2024). Effectiveness of micro-dosing of lime on selected chemical properties of soil in Banja District, North West, Ethiopia. *Open Agriculture*, *9*(1), 20220272.

Yuan, Y., Yin, Y., Adamczyk, B., Liang, D., Gu, D., et al. (2024). Nitrogen addition alters the relative importance of roots and mycorrhizal hyphae in regulating soil organic carbon accumulation in a karst forest. *Soil Biology and Biochemistry*, *195*, 109471.

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Potential Nitrogen Fixing Rhizobia Isolated from Some Wild Legumes of Nagaland Based on RAPD with *Nif*-directed Primer and Their Biochemical Activities

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ABSTRACT

Wild legumes are widely dispersed and can survive in challenging environments as bacteria dwell in their nodules and help each other. Although Nagaland is home to many wild legume varieties, research on the microbial diversity that goes along with them is still in its infancy. This work aimed to characterize several wild legume root nodules and distinguish possible rhizobial isolates using RAPD and *nif*-directed RPO1 primer. Nodule bacteria were isolated in Yeast extract culture media. Based on their colony morphology, 150 isolates were selected for performing RAPD with *nif*-directed RPO1 primer. Eighty-four isolates were bonded with RPO1 primer, and a few biochemical tests were conducted on RPO1-positive isolates. Activities that promoted plant development were also investigated for these isolates. Of all the isolates, 18 exhibited phosphate solubilization capacity, while 38 isolates were found to produce IAA. This study entails a large variety of rhizobia in the nodules, which were able to promote growth. Hence, these isolates promise to be bio-fertilizers that could improve agricultural operations.

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1 Introduction

Microbes are cosmopolitan and found in almost every region of the world. Soil is considered one of the greatest reservoirs for microbial biodiversity, predominated by bacteria and fungi followed by Archaea and about 107-109 virus particles per gram (Banerjee and Van Der Heijden 2023). A single gram of soil is estimated to harbour up to 8 million microbial species, which are responsible for pollutant degradation, nutrient transformation, litter decomposition, and biosynthetic reactions (Jia et al. 2020; Rosselló-Móra and Stackebrandt 2021). Soil microbial diversity is prevalently studied because of its role in crop productivity and improving soil quality. Soil microbial communities represent probably the most known biological diversity reservoirs (Labouyrie et al. 2023). Microorganisms are quite inactive in bulk soil but show increased activities in the rhizosphere, a microbial soil hot spot, especially during the plant developmental stages (Schloter et al. 2018). Free-living soil bacteria beneficial for plant growth are typically called 'Plant Growth Promoting Rhizobacteria' (PGPR) as they can promote plant growth by colonizing the plant root (Tatung and Deb 2023). The PGPR is divided into two groups based on their residing hubs: (i) intracellular PGPR (iPGPR) (i.e., symbiotic bacteria), which live inside the plant cells and are localized inside the specialized structures called nodules and (ii) extracellular PGPR (ePGPR) (i.e., free-living rhizobacteria), which live outside the plant cells and do not form nodules, but they still prompt plant growth (Giannelli et al. 2023). Rhizobia that infects legumes produces nodules and develops a symbiotic relationship with the host, making legume-rhizobia interaction a prime example of iPGPR. Legume-*Rhizobium* symbiosis is the most economical and environment-friendly method and results in the incorporation of atmospheric N_2 into organic compounds, which is thought to account for around 200 Tg of organic N per year (Peoples et al. 2009). It is generally acknowledged that interactions between legumes and rhizobia fix more nitrogen than free-living bacteria. Typically, their connection fixes 25-60 kg of N_2 per year, whereas non-symbiotic species fix less than 5 kg ha^{-1} (Hopkins and Hüner 2014).

Over 17,000 legume species have been reported, of which 20% were found to form nodules (Hopkins and Hüner 2014). A nodule is an integral structure formed due to the exchange of signals between the host legume and compatible rhizobia. It is the site where rhizobia fixes nitrogen after differentiating into bacteriods. Nodules are morphologically categorized into two types: determinate and indeterminate. Determinate nodules are spherical because of their loss of meristematic activity. Indeterminate nodules have an active meristematic activity and are found in most legumes (Green et al. 2019). They are cylindrical and are branched. Rhizobia are one of the many PGP microbes that are vital for enriching soil to enhance plant growth and yield, which will help to cope with the growing demands of food of the increasing population in a sustainable way (Dabo et al. 2019; Ibny et al. 2019). Colony morphology and molecular techniques like Randomly Amplified Polymorphic DNA (RAPD) are generally applied to preliminary screening potential Rhizobial isolates. Williams et al. (1991) performed an initial study on Rhizobia with a gene-directed primer RPO1. Gene-directed RAPD primers are not random and bind to a specific sequence adjacent to a particular locus of the primer, such as RPO1 RAPD primer selectively binds to a sequence adjacent to the *nif* gene locus of bacteria if present. Based on the absence or presence of band(s), Rhizobial and non-rhizobial isolates can be distinguished. Different isolates exhibit different banding patterns; based on that, similar isolates of Rhizobia can be grouped. The homology in the banding patterns of the isolates indicates its primer annealing sites and similarity in the length of the amplified region. This provides a preliminary genetic fingerprint of the target gene and aids in distinguishing the genomes of a wide range of bacterial strains (Bassam et al. 1992), including *Rhizobium* (Richardson et al. 1995).

Nagaland is a part of the North-eastern region of India situated between 93°20″E - 95°5″E and 25°12′N-26°3′N. With 8,633,000 hectares of forest cover, the state occupies 16,579 Km^2 (Ritse et al. 2020). It is a state rich in biodiversity, with sub-tropical and tropical evergreen forests predominating (Achumi et al. 2014). Much research hasn't been done on Rhizobia in this state despite its diverse agroclimatic zones supporting enormous biodiversity, particularly in the flora (Chouhan et al. 2022; Megu et al. 2024). The fact that rhizospheric bacteria have been shown to support host survival in various environmental conditions is evidence of this. The present study aimed to isolate and differentiate potential rhizobial isolates from some wild legumes from Nagaland based on their RAPD fingerprints. The study also aimed to record the morphological structures of nodules and perform some biochemical tests on bacteria isolated from them.

2 Materials and Methods

2.1 Sampling sites

Nodules of wild legumes were collected from six sites in Zunheboto district, Nagaland, India: Alaphumi, Akuluto, Lumami, Sumi Setsii, Zaphumi and Zunhebhoto town. The details of the sites are given in Table 1.

2.2 Soil characterization

Basic soil properties were tested at different sampling sites (Table 1). One g of soil was suspended in 100ml distilled water, and the suspension was allowed to settle down for 3-4 h, and then *p*H was recorded. The widely used wet combustion method of rapid titration protocol by Walkley Blake (Anantha et al. 2020) was used with standard protocols for organic carbon estimation. To determine soil phosphorus, Bray's no 1 extraction method was followed by using a UV-Vis spectrophotometer (Bray and Kurtz 1945). Available potassium was estimated using the atomic absorption photometric

Table 1 Sampling sites and their soil characterization

S. N.	Collection Sites	*p*H	Phosphorus (Kg/ha)	Potassium (Kg/ha)	Organic Carbon (%)	N (Kg/Ha)
1	Sumi Setsu	6.66	11.854	106.265	6.18%	87.44
2	Alaphumi	6.23	10.612	103.110	6.03%	94.03
3	Zaphumi	5.92	12.158	172.102	7.20%	96.21
4	Lumami	6.00	13.251	149.430	8.32%	101.46
5	Akuluto	5.78	10.215	130.112	5.89%	95.71
6	Zunhebhoto	5.81	14.394	134.848	7.32%	97.056

method using a flame photometer (Trivedy and Goel 1984). The Kjeldahl Nitrogen Analyzer (Kelplus Nitrogen Estimation system) estimated soil nitrogen using the Kjeldahl method (Kjeldahl 1883).

2.3 Collection of legumes and root nodule characterization

The native wild legumes from the different collection sites were carefully uprooted, and nodules were collected. An average of 5-6 plants of each legume species were collected from the sites for nodule characterization. They were then taken to the laboratory, washed, and photographed. The number of nodules was counted and recorded for each plant. Nodulation types in collected legumes were recorded as well. Nodule characteristics were recorded and presented in Table 2.

2.4 Isolation of root nodulating bacteria

About 8-10 pink and healthy nodules of each sample were surface sterilized and considered for bacteria isolation. The protocols followed were as given by Somasegaran and Hoben (1985). The nodules were washed with pure water followed by 70% (v/v) ethanol for 2-3 min. The nodules were washed with 1% (w/v) Bavistin (anti-fungal agent) for 2-3 minutes, followed by 0.1% $HgCl_2$ for 5-8 min. This was followed by washing with pure water 5-6 times to remove the traces of $HgCl_2$. The Nodules were then placed on a sterile glass Petri plate, and 2-3 drops of distilled water were added. The nodules were then crushed with a pair of sterile forceps and streaked on Yeast Extract Mannitol Agar (YEMA) media plates [Yeast extract ($0.5gL^{-1}$), Mannitol ($10gL^{-1}$), K_2HPO_4 ($0.5gL^{-1}$), $MgSO_4$ ($0.2gL^{-1}$), NaCl ($0.1gL^{-1}$) and agar ($15gL^{-1}$)]. Congo red dye was used as an indicator, as Rhizobia does not tend to take the dye during the initial stage of culture. The plates were then incubated at 28°C -30°C for 8-10 days. Repeated streaking of isolates was done to obtain pure cultures and for further study.

2.5 DNA extraction of bacterial isolates

Bulky, white, and translucent colonies were preliminary and phenotypically selected as Rhizobia. DNA of the selected isolates was extracted by following the protocol of Sambrook and Russell (2001). The quality of DNA was then checked by running it in 1% (w/v) agarose gel electrophoresis.

2.6 RAPD analysis

For nodule screening, 12 wild legumes were collected from the six sampling sites (Alaphumi, Akuluto, Lumami, Sumi Setsii, Zaphumi, and Zunhebhoto) of Zunheboto district, Nagaland. One hundred fifty bacterial strains in total were isolated from the root nodules. The RAPD analysis of the isolated bacterial isolates was carried out using *nif*-directed RPO1 primer 5'AATTTTCAACGCTCGTGCCA 3' (Richardson et al. 1995). The PCR mixture was made up of 2.5µl 10X TE buffer, 2.5µl of dNTPs, 5µl of RPO1 primer, 0.2µl of *Taq* polymerase, 2µl of template, and 13.8µl dH_2O. For the PCR cycle, the denaturation temperature was set at 94°C, annealing at 54°C, and elongation at 72°C for 30 cycles (BIO-RAD T100™ Thermal cycler). The PCR product was subjected to 1.5% (w/v) agarose gel electrophoresis, and the banding pattern was visualized in Bio-RadChemi Doc.

2.7 Biochemical characterization of root nodulating bacteria

Biochemical characterization of bacterial isolates was done based on their plant growth-promoting activities and biochemical characterization. Only fresh cultures (24-48 h old) were used to perform the tests.

2.7.1 Plant growth promoting (PGP) activities

2.7.1.1 Phosphate solubilization activity

For qualitative phosphate solubilizing activity assay, fresh culture was spot inoculated in Pikovskaya agar medium. The plates were then incubated at 28-30°C for 2-3 days. The occurrence of a halo zone around the spot indicates phosphate solubilizing activity.

2.7.1.2 IAA production

The protocol given by Gang et al. (2019) was used for the IAA test. Fresh cultures were inoculated in Tryptone-Yeast broth with added tryptophan as a precursor and incubated for 24-48 h. In a clean 2ml centrifuge tube, 500(l of Salkowski reagent and 50(l) fresh cultures were added. The tube was incubated in the dark for 30 min. The change of the reagent colour to pink indicates IAA production by the isolates.

Table 2 Wild legumes collected with their nodule characters and cultures isolated

Wild legumes	Nodule Characteristics				Total Isolates	RPO1 Positive	Unique Isolates
	Nodule Type	Avg. Nodule Size (l x w in mm)	Nodule colour	Number of nodules per plant			
A. americana L.	Determinate, stem and root, small sized and many nodules	3x2	Pink	20-30	11	AIS1, AIS3, AIS4, AIS5, AIS6, AIS7, AIS8, AIS9, AIS10, AIS12, AIR14 (Total positive isolates 11)	AIS1, AIS3, AIS5, AIS7, AIS8, AIS9, AIS10, IS12, AIR14 (Total Unique Isolates 9)
A. chinensis (Osbeck) Merr.	Determinate, primary and secondary root, small and medium-sized nodules, few nodules	3x1	Pink	3-6	13	LUMAC1, LUMAC2, LUMAC5, LUMAC6, LUMAC7, LUMAC8, LUMAC9, LUMAC10, LUMAC11, LUMAC12 (Total positive isolates 10)	LUMAC1, LUMAC5, LUMAC6, LUMAC9, LUMAC10, LUMAC11, LUMAC12 (Total Unique Isolates 7)
C. mysorensis Roth	Unbranched Indeterminate, primary roots, medium-sized nodules, high in number	3x2	Pink	15-20	8	LUMCF1, LUMCF2, LUMCF3, LUMCF5, LUMCF7, LUMCF10, LUMCF11, LUMCF12) (Total positive isolates 8)	LUMCF1, LUMCF2, LUMCF3, LUMCF7, LUMCF10, LUMCF11, LUMCF12 (Total Unique Isolates 7)
D. heterocarpum (L.) DC.	Determinate, small nodules, primary and secondary roots, many nodules	2x1	Pink	20-30	12	LUMDes1, LUMDes3, LUMDes4, LUMDes5, LUMDes6, LUMDes9, LUMDes10, LUMDes11) (Total positive isolates 8)	LUMDes1, LUMDes3, LUMDes5, LUMDes9, LUMDes11 (Total Unique Isolates 5)
D. triflorum (L.) DC.	Determinate, primary and secondary roots, small-sized nodules, few nodules	1x1	Pink	5-8	9	LUMDT2, LUMDT3, LUMDT6, LUMDT9, LUMDT11, LUMDT12, LUMDT16 (Total positive isolates 7)	LUMDT2, LUMDT3, LUMDT9, LUMDT11, LUMDT16 (Total Unique Isolates 5)
E. stricta Roxb.	Determinate, primary roots, medium-sized nodules, few nodules	4x3	Pink	6-8	7	LUMESA, LUMESB, LUMES1, LUMES3, LUMES4, LUMES5 (Total positive isolates 6)	LUMESA, LUMESB, LUMES4, LUMES5 (Total Unique Isolates 4)
L. leucocephala (Lam.) de Wit	Indeterminate and unbranched, large nodules, primary roots, few nodules.	3x2	Pink	2-4	15	LUMLL1, LUMLL2, LUMLL3, LUMLL6, LUMLL8, LUMLL9, LUMLL10, LUMLL11, LUMLL12, LUMLL13, LUMLL14, LUMLL15 (Total positive isolates 12)	LUMLL1, LIMLL2, LUMLL6, LUMLL8, LUMLL9, LUMLL11, LUMLL12, LUMLL13 (Total Unique Isolates 8)

Wild legumes	Nodule Characteristics				Total Isolates	RPO1 Positive	Unique Isolates
	Nodule Type	Avg. Nodule Size (l x w in mm)	Nodule colour	Number of nodules per plant			
M. diplotricha C. Wright	Branched Indeterminate, primary and secondary root, medium-sized nodules, several nodules	2x1	Pink	7-10	15	LUMMD2, LUMMD3, LUMMD5, LUMMD6, LUMMD9, LUMMD12, LUMMD13, LUMMD15, LUMMD16, LUMMD18, LUMMDa, LUMMDb, LUMMDc, LUMMDd, LUMMDG, LUMMDm (Total positive isolates 14)	LUMMD2, LUMMD3, LUMMD5, LUMMD6, LUMMD9, LUMMD12 LUMMD13, LUMMD15, LUMMD16, LUMMDb, LUMMDc, LUMMDd, LUMMDm (Total Unique Isolates 13)
M. pudica L.	Indeterminate, primary and secondary roots, medium-sized, several nodules	3x1	Pink	10-15	15	LUMMP1, LUMMP2, LUMMP4, LUMMP10, LUMMP16, LUMMP19, LUMMPF, LUMMPO (Total positive isolates 8)	LUMMP1, LUMMP2, LUMMP4, LUMMP10, LUMMPF (Total Unique Isolates 5)
T. candida DC.	Indeterminate globular nodules, primary roots, few nodules	4x2	Pink	3-5	16	LUMTC1, LUMTC3, LUMTC4, LUMTC7, LUMTC8, LUMTC9, LUMTC10, LUMTC11, LUMTC12, LUMTC13, LUMTC14, LUMTC15, LUMTCA (Total positive isolates 13)	LUMTC1, LUMTC3, LUMTC11, LUMTC15, LUMTCA (Total Unique Isolates 5)
V. nepalensis Tateishi & Maxted	Determinate, small-sized nodules, primary and secondary roots, many nodules	2x1	Pink	30-50	18	LUMVRW1, LUMVRW2, LUMVRW3, LUMVRW5, LUMVRW6, LUMVRW7, LUMVRW8, LUMVRW9, LUMVRW10, LUMVRW11, LUMVRW12, LUMVRW13) (Total positive isolates 12)	LUMVRW1, LUMVRW2, LUMVRW8, LUMVRW9, LUMVRW10, LUMVRW11, LUMVRW12 (Total Unique Isolates 7)
V. vexillata (L.) A. Rich	Determinate, medium-sized, globular nodules, primary roots, several nodules	3x2	Pink	10-12	11	LUMVV1, LUMVV2, LUMVV4, LUMVV9, LUMVV10, LUMVV11, LUMVV13, LUMVVO, LUMVVx) (Total positive isolates 9)	LUMVV1, LUMVV2, LUMVV4, LUMVV9, LUMVV10, LUMVV11, LUMVV13, LUMVVO, LUMVVx (Total Unique Isolates 9)
B. variegate L.	Non-nodulating	-	-	-	-		
C. pulcherrima (L.) Sw.	Non-nodulating	-	-	-	-		
P. speciosa Hassk.	Non-nodulating	-	-	-	-		

2.7.2 Biochemical characterization

2.7.2.1 Catalase test

A colony from fresh culture was picked and then smeared on a glass slide. 2-3 drops of 0.5% (v/v) H2O2 were added to the smear. The appearance of bubbles on the smear marks positive catalase activity (Shoaib et al. 2020).

2.7.2.2 Citrate test

The test was performed by streaking the pure and fresh colony on Simmon's citrate agar slants and incubated for 24-48 h at 28°C - 30°C. Positive results showed a change of colour from green to blue (Shoaib et al. 2020).

2.7.2.3 Carbon utilization test

Carbon utilization or fermentation tests of six sugars, namely dextrose, fructose, glucose, maltose, mannitol, and sucrose were performed. Respective sugar broths (peptone, sugar, NaCl, water) were made with phenol red as an indicator, and a fresh colony was inoculated in each sugar broth. Broths were then inoculated with fresh cultures and incubated at 28°C for 18-24 h. All of the tests were performed in triplicates. The carbon broth is usually pink in colour because of the phenol indicator. The broth changes colour from pink to yellow as a sign of positive fermentation of the respective sugar (Shoaib et al. 2020).

2.7.2.4 Starch hydrolysis

Starch hydrolysis was done to assay the amylase production activity by isolates. The bacterial isolates were spot inoculated on starch agar medium [Beef extract (3 gL^{-1}), peptone (5 gL^{-1}), soluble starch (2 gL^{-1}), agar (15 gL^{-1})] plates and incubated at 30°C for 48 h. The plates were flooded with freshly prepared grams of iodine solution, kept for a minute and then poured off the excess iodine solution. Iodine reacts with starch to form a blue colour compound. This blue colour fades rapidly. Hence, the colourless zone resembling a halo surrounding colonies indicates amylase production (Shoaib et al. 2020).

2.7.3 Stress tolerant properties

2.7.3.1 *p*H tolerance test

Tolerance to acidic or alkaline media was assessed by spot inoculating fresh cultures in YEMA plates with varying *p*H 3, 5, 9, and 11. The *p*H of the media was taken using a *p*H meter before adding agar. The media was then boiled, autoclaved for 15 minutes, and poured into Petri plates in the laminar hood. These experiments were performed in triplicates for each isolate for the respective *p*H. The plates were then incubated at 28-30°C, and the growth of cultures was checked after 3 days (Bissa et al. 2020).

2.7.3.2 Salinity tolerance test

For the salt tolerance study, YEMA media without salt was prepared, and varying salt concentrations (w/v), i.e., 1-4%, were added to each medium and autoclaved for 15 min. Media was poured into Petri plates in the laminar chamber, cooled, and treated with UV for 15 min. Salt tolerance of the bacterial isolates was determined by spot-inoculating fresh cultures in YEMA plates supplemented with different concentrations of NaCl. The experiment was performed in triplicates for each isolate in each NaCl concentration. The inoculated plates were incubated at 28°C for 3 days, and growth was checked (Bissa et al. 2020).

2.7.3.3 Temperature tolerance

The ability of bacterial isolates to survive in different temperatures was assessed by inoculating fresh cultures and incubating them in different temperatures (10, 20, 30, 40, and 50°C) for 3 days. Fresh YEMA media was prepared, and in the laminar hood, the media plates were spot inoculated with isolates in triplicates for each temperature at which they would be incubated. The growth chambers were adjusted at different temperatures, and the plates were incubated (Bissa et al. 2020). The results were then recorded for their survivability.

3 Results

3.1 Collection of legumes and their nodule characteristics

A total of 15 legume species were identified and collected (Figure 1). Wild legumes are notably diverse, and some of them, such as *Mimosa pudica*, *Desmodium heterocarpum*, *Tephrosia candida*, *Albizia chinensis*, *Mimosa diplotricha*, *Leucaena leucocephala*, were found to be extensively cosmopolitan and are found in almost all the sites like higher altitude, temperature, and other abiotic conditions. *Crotalaria mysorensis*, recorded as native to India (POWO 2024), was found in the Lumami of Zunhebhoto district. Further, *Desmodium* species, *D. heterocarpum* and *D. triflorum* were of sub-shrub habit and commonly found in all the sites. *Erythrina stricta*, a deciduous tree legume, was also recorded in Lumami. *Vigna* species, *V. vexillata* and *V. nepalensis* were commonly found in all surveyed sites. Further, determinate and indeterminate nodule types commonly occur in wild legumes (Figure 2). Seven legumes formed determinate nodules, while five were formed indeterminate type (Table 2). The nodules of some leguminous species like *Albizia chinensis* (Figure 2b) and *Vigna vexillata* (Figure 2i) had nodules with rough outer texture, which may protect against microbes or pests.

3.2 Isolation of root nodulating bacteria

A total of 150 bacteria were isolated from root nodules of different legumes. The isolates took 3-8 days to grow in the YEM medium,

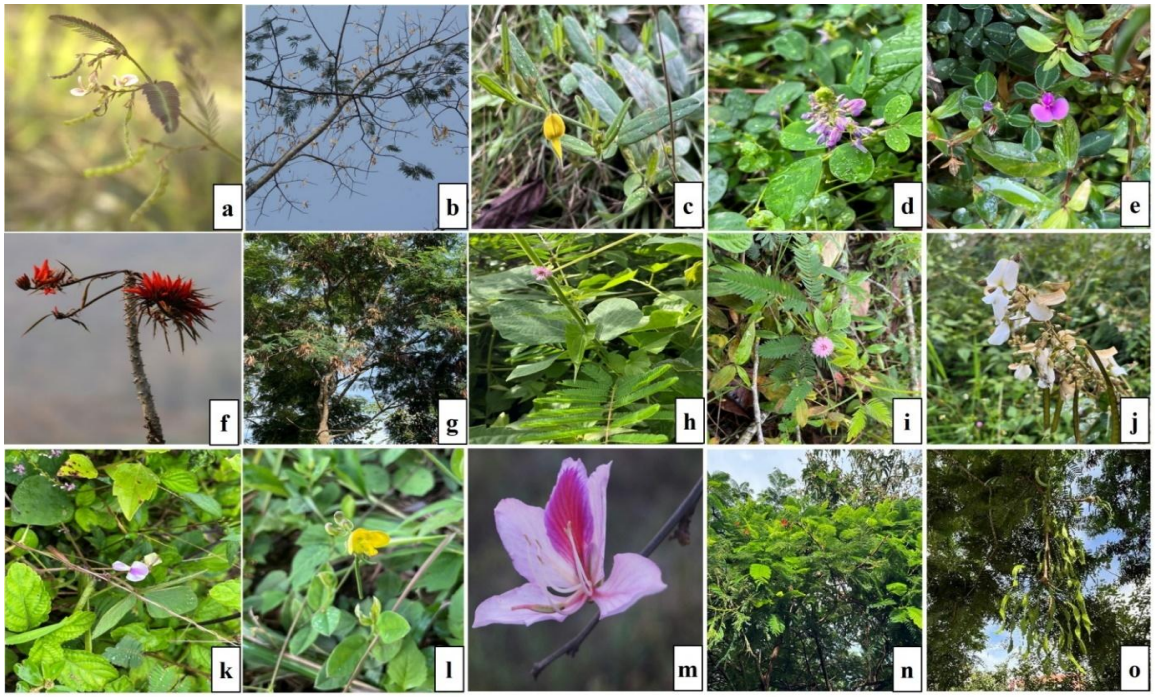

Figure 1 Wild legumes collected from Zunheboto district, a. *Aeschynomene indica*, b. *Albizia chinensis*, c. *Crotalaria mysorensis*, d. *Desmodium heterocarpum*, e. *Desmodium triflorum*, f. *Erythrina stricta*, g. *Leucaena leucocephala*, h. *Mimosa diplotricha*, i. *Mimosa pudica*, j. *Tephrosia candida*, k. *Vigna vexillata*, l *Vigna nepalensis*, m. *Bauhinia variegata*, n. *Caesalpinia pulcherrima*, o. *Parkia speciosa*.

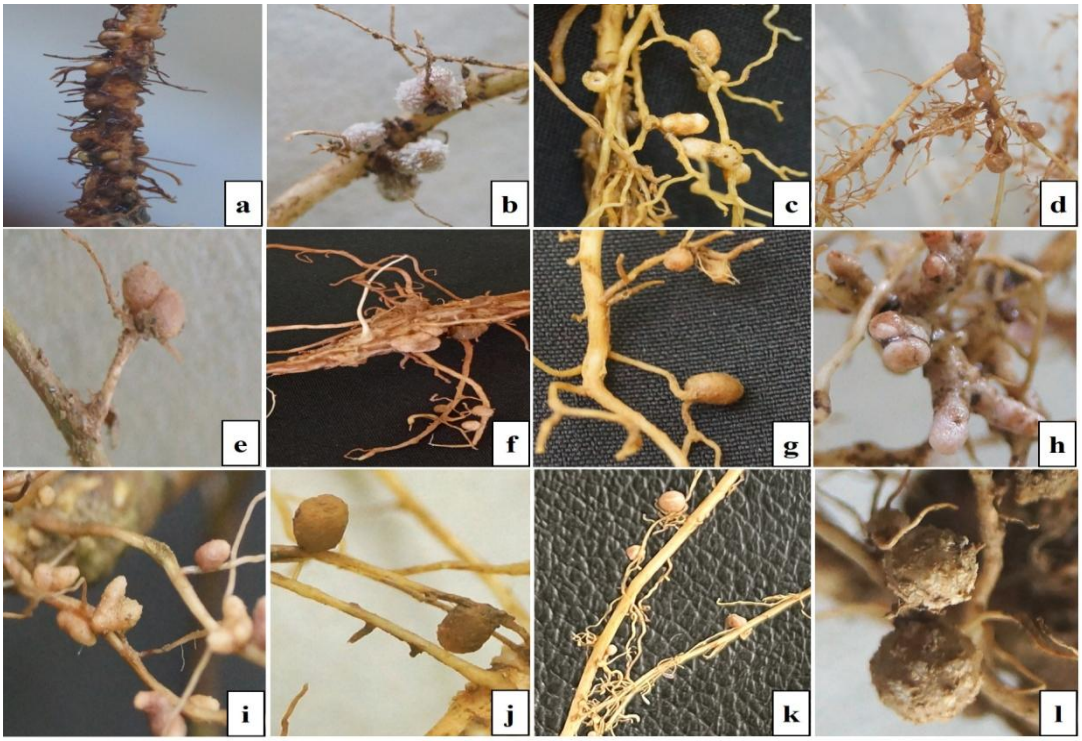

Figure 2 Nodule morphology of collected wild legumes, a. *Aeschynomene indica*, b. *Albizia chinensis*, c. *Crotalaria mysorensis*, d. *Desmodium heterocarpum*, e. *Desmodium triflorum*, f. *Erytherina stricta*, g. *Leucaena leucocephala*, h. *Mimosa diplotricha*, i. *Mimosa pudica*, j. *Tephrosia candida*, k. *Vigna nepalensis*, l. *Vigna vexillata*.

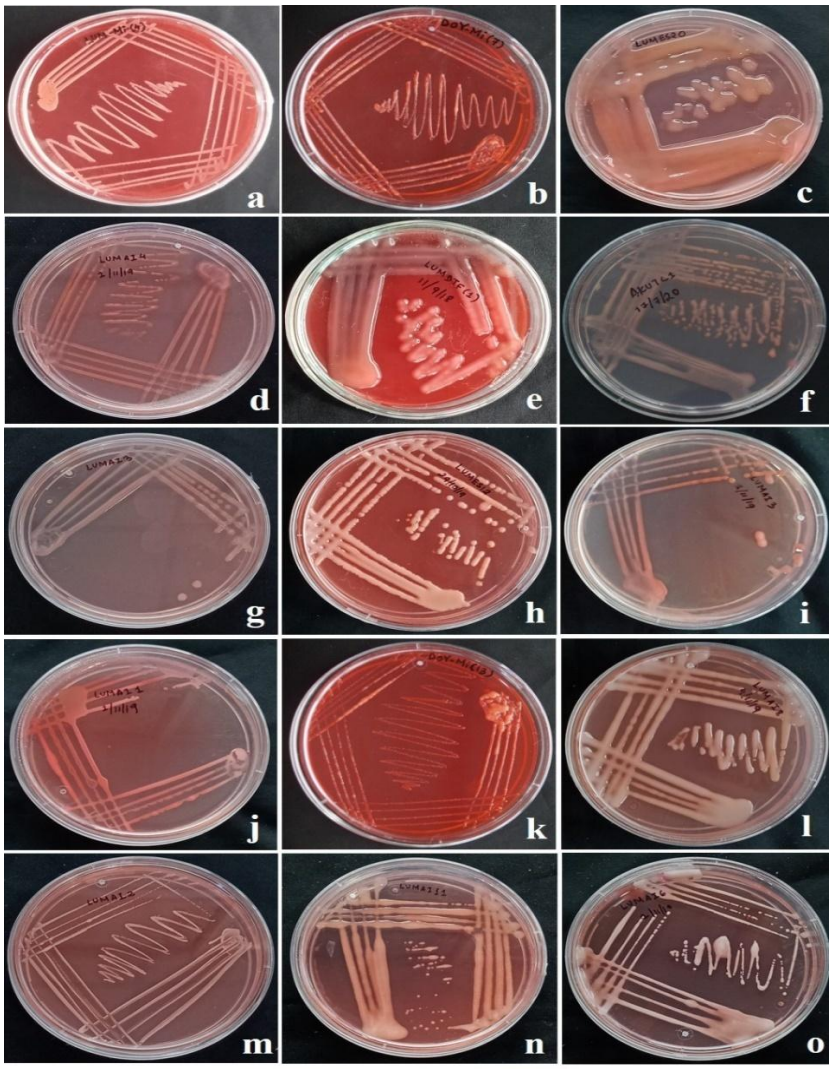

Figure 3 Some root nodulating bacterial endophytes isolated from wild legumes in YEMA-CR medium. A. LUMMi, b. DOYMi7, c. LUMES20, d. LUMAI4, e. LUMDTF1, f. AKUTC1, g. LUMAI3, h. LUMES12, i. LUMAI5, j. LUMAI1, k. DOYMi13, l. LUMAI8, m. AIS9, n. LUMAI1, o. LUMAI6.

showing that some were fast and slow-growing. Some pure cultures obtained were observed to be translucent white, while some were opaque. The cultures were predominantly white, but some tended to be pink after absorbing the Congo red dye and incubating for longer days. Bacterial colonies were also observed to be bulky, raised, and creamy. Excretion of exopolysaccharides was also recorded from some bacteria due to their mucus colony and sticky nature. The colonies were commonly observed to have irregular and continuous borders (Figure 3).

3.3 RAPD analysis of the isolates

Potential rhizobia at the molecular level was identified through RAPD analysis. RAPD was performed on 150 isolates, and among these, 119 isolates confirmed the presence of *nif*-gene by binding to the RPO1 primer. The gel image showed various banding patterns, indicating that some isolates have the *nif* gene (Figure 4). Some isolates from the same host showed similar banding patterns in RAPD when visualizing the gel image. For instance, when RAPD was performed on isolates from *Aeschynomene indica*, band patterns in Lane 4 (L4) and Lane 5 (L5) were similar, possibly due to the genetic similarities between these two strains. Hence, they were considered the same species, and one was chosen to represent that group. It was helpful to group similar bacterial isolates and chose a representative to minimize the possibility of attaining repeated strains. A similar situation has been reported for *Desmodium triflorum*, where the prominent bands in L3, L5, and L9 are similar. A total of 11 isolates from *Aeschynomene indica* bound with RPO1 primer, and on excluding the occurrence of common bands in the banding pattern of isolates, nine of them

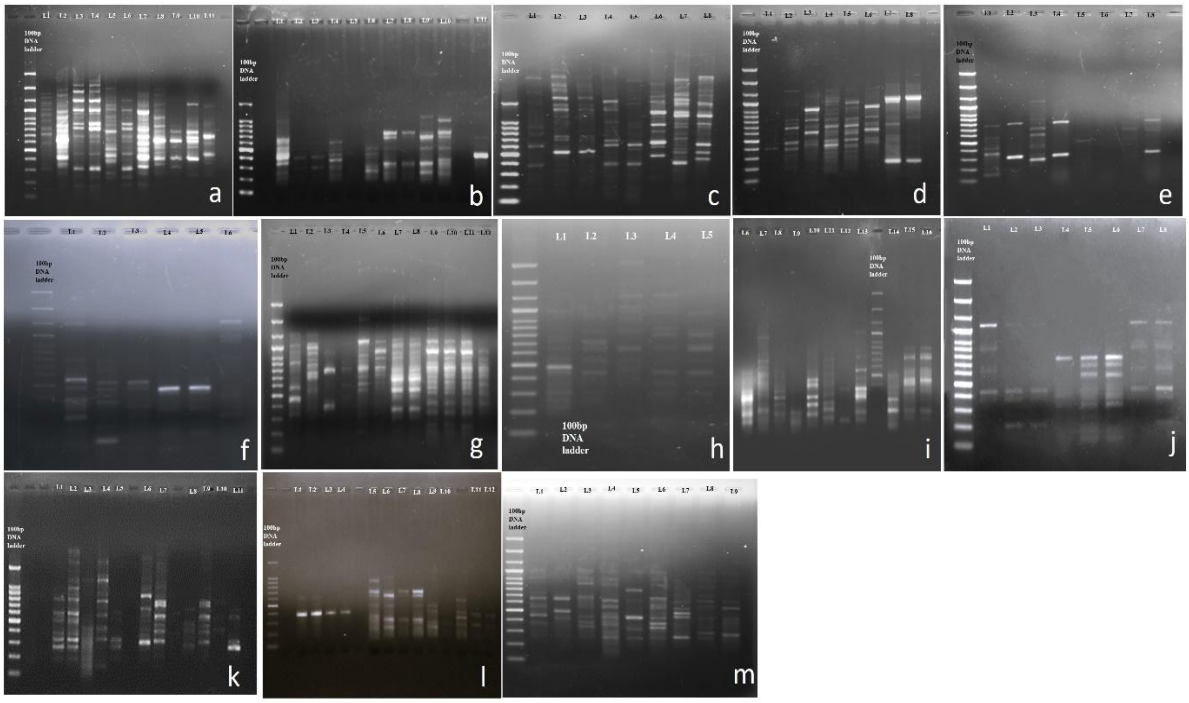

Figure 4 Gel images of RAPD of isolates with RPO1*nif*- directed primer, a. *Aeschynomene indica*, b. *Albizia chinensis*, c. *Crotalaria mysorensis*, d. *Desmodium heterocarpum*, e. *Desmodium triflorum*, f. *Erytherina stricta*, g. *Leucaena leucocephala*, h-i. *Mimosa diplotricha*, j. *Mimosa pudica*, k. *Tephrosia candida*, l. *Vigna nepalensis*, m. *Vigna vexillata*.

were found to be unique, which were L2, L3, L4, L6, L7, L8, L9, L10 and L11 (Figure 4a). From the nodules of *Albizia chinensis*, a total of 13 isolates were cultured, of which 10 were RPO1 positive and seven lanes, namely L2, L3, L5, L6, L7, L9, and L11 showed unique banding patterns (Figure 4b). *Crotalaria mysorensis* also showed unique banding patterns in seven of its isolates. L2, L3 and L4 were considered similar, while L5, L6, L7, L8 and L9 were observed to be unique (Figure 4c). *Desmodium heterocarpum* showed unique banding patterns in five isolates (L2, L3, L4, L5 and L8) out of eight **(**Figure 4d) and in *Desmodium triflorum* L2, L3 (similar to L5 and L9), L5, L6 and L8 were unique (Figure 4e). A total of six isolates were RPO1 positive from *Erythrina stricta*, of which four were unique (Figure 4f), namely L2, L3, L5, and L7. In the case of *Leucaena leucocephala*, 12 isolates bound with RPO1 primer, of which eight unique banding patterns in lanes L2, L3, L4, L5, L6, L7, L8, and L10 were observed in the gel images (Figure 4g). *Mimosa diplotrica* showed the highest Rhizobial diversity in isolates, with 13 out of 16 isolates showing unique RAPD banding patterns (Figure 4h-i). Lanes 5 and 6 had the same bandings; similarly, Lanes 11 and 12 showed similar patterns, as did Lanes 15 and 16. Five isolates were unique in *Mimosa pudica* (Figure 4j), which were L2, L4, L5, L6, and L8 out of eight. In *Tephrosia candida*, 7 isolates in lanes L2, L5, L6, L7, L8, L9 and L11 were deemed unique from 11 RPO1 positive bacterial isolates (Figure 4k). From wild *Vigna nepalensis* legume species, 13 RPO1 positive isolates were cultured, out of which seven unique band patterns (L1, L5, L6, L7, L9, L10, and L11) were observed (Figure 4l). Nine RPO1 positive isolates were isolated from *Vigna vexillata*, and all nine had unique bands (Figure 4m). On excluding similar banding patterns in the isolates, 84 unique Rhizobial isolates were deduced and considered diverse.

3.4 Biochemical characterization

3.4.1 Catalase test

A catalase test is done to screen the catalase enzyme. On adding drops of 0.5% (v/v) H_2O_2 to the bacterial smear, bubbles were formed for 28 isolates isolated from *A. americana, D. heterocarpum*, *A. chinensis*, *Crotalaria*, *Erytherina*, *Leucaena*, *Mimosa* species, *Tephrosia* and *Vigna* species confirming their ability to produce catalase enzyme (Table 3).

3.4.2 Citrate test

When pure cultures were streaked on Simon's citrate agar slants, out of 84 isolates tested, 23 were able to change the colour of media from green to blue, which included 1 each from *A. americana* and *A. chinensis*, 3 from *D. heterocarpum*, 5 from *D. triflorum*, 2 from *L. leucocephala*, 8 and 2 isolated from *M. diplotricha* and *M. pudica* respectively, 5 from *T. candida* and 6 from *V. nepalensis*. Colour change confirmed the activity of the citrate enzyme in those isolates (Table 3).

Table 3 Biochemical activities of isolates from root nodules of wild legumes

Host Species	Isolates	PGP Activities*		Biocontrol and Biochemical Analysis*			Sugar Fermentation*					
		IAA	PSB	Catalase	Citrate	Starch	Dextrose	Fructose	Glucose	Maltose	Mannitol	Sucrose
A. americana L.	AIS1	+	+	+	+	-	+	+	+	+	+	+
	AIS3	+	-	-	-	-	+	+	+	+	+	-
	AIS5	-	-	-	-	-	-	-	-	-	-	-
	AIS7	-	-	-	-	-	+	+	+	+	+	+
	AIS8	-	-	-	-	-	+	+	+	+	+	+
	AIS9	+	-	+	-	-	+	+	+	+	+	+
	AIS10	-	-	-	-	-	+	+	+	+	+	+
	AIS12	+	-	-	-	-	-	-	+	+	-	-
	AIR14	+	-	-	-	-	+	+	+	+	-	+
A. chinensis (Osbeck) Merr.	LUMAC1	+	-	-	-	-	+	+	+	+	-	+
	LUMAC5	-	-	-	-	-	+	+	+	+	-	+
	LUMAC6	-	-	+	-	+	+	+	+	+	+	+
	LUMAC9	+	-	-	-	-	+	+	+	+	-	-
	LUMAC10	-	+	+	+	-	+	+	+	+	+	+
	LUMAC11	-	-	-	-	-	+	+	+	+	-	-
	LUMAC12	-	-	-	-	-	+	+	-	-	-	-
C. mysorensis Roth	LUMCF1	-	+	-	-	-	+	+	+	+	+	+
	LUMCF2	+	+	-	-	-	+	+	+	+	+	+
	LUMCF3	-	-	-	-	+	-	+	+	+	+	+
	LUMCF7	+	-	+	-	-	+	+	+	+	+	+
	LUMCF10	-	+	+	-	-	+	+	+	+	+	+
	LUMCF11	-	-	-	-	-	-	-	-	-	-	-
	LUMCF12	-	+	-	-	-	+	+	-	-	+	+
D. heterocarpum (L.) DC.	LUMDes1	+	+	-	-	+	+	+	+	+	+	+
	LUMDes3	+	+	-	+	+	+	+	+	+	+	+
	LUMDes5	+	+	-	+	-	+	+	+	+	+	+
	LUMDes9	-	-	-	+	-	+	+	+	+	+	+
	LUMDes11	+	-	-	-	-	+	+	+	+	+	+
D. triflorum (L.) DC.	LUMDT2	-	+	-	+	-	+	+	+	+	+	+
	LUMDT3	+	+	+	+	-	+	+	+	+	+	+
	LUMDT9	+	+	+	+	-	+	+	+	+	+	+
	LUMDT11	-	-	-	+	+	+	+	+	+	+	+
	LUMDT16	+	+	+	+	-	-	+	+	+	+	-
E. stricta Roxb	LUMESA	-	-	+	-	-	+	+	+	+	-	-
	LUMESB	+	-	-	-		-	-	-	-	-	-
	LUMES4	+	-	+	-	-	+	+	+	+	-	-
	LUMES5	+	-	+	-	-	+	+	+	+	-	-

Host Species	Isolates	PGP Activities*		Biocontrol and Biochemical Analysis*			Sugar Fermentation*					
		IAA	PSB	Catalase	Citrate	Starch	Dextrose	Fructose	Glucose	Maltose	Mannitol	Sucrose
L. leucocephala (Lam.) de Wit	LUMLL1	-	-	-	-	-	-	-	-	-	-	-
	LIMLL2	-	-	-	-	-	-	-	-	-	-	-
	LUMLL6	-	+	-	-	-	+	+	+	+	+	+
	LUMLL8	-		-	-	-	+	+	+	+	+	+
	LUMLL9	+	-	+	+	-	+	+	+	+	+	+
	LUMLL11	+	-	-	+	-	+	+	+	+	+	+
	LUMLL12	+	-	-	-	-	+	+	+	-	-	-
	LUMLL13	+	-	-	-	-	+	+	+	+	+	+
M. diplotricha C. Wright	LUMMD2	-	-	-	-	-	+	-	+	+	+	-
	LUMMD3	+	+	+	+		+	-	+	+	-	+
	LUMMD5	+	+	+	+	+	-	-	+	-	-	-
	LUMMD6	-	-	-	+	-	-	+	+	-	-	-
	LUMMD9	-	+	-	-	-	+	+	-	+	+	+
	LUMMD12	+	-	-	+	-	-	-	+	+	+	+
	LUMMD13	+	-	+	+	+	+	+	+	+	+	+
	LUMMD15	-	-	-	-	-	-	-	-	-	-	-
	LUMMD16	+	-	-	+	+	-	+	+	-	-	+
	LUMMDb	-	-	-	-	-	+	+	+	+	-	+
	LUMMDc	-	-	-	-	+	+	-	+	+	+	-
	LUMMDd	-	-	-	+	+	+	-	+	+	+	-
	LUMMDm	-	-	-	+	-	+	+	+	+	-	+
M. pudica L.	LUMMP1	-	-	-	-	+	-	+	+	+	-	-
	LUMMP2	-	-	-	-	-	-	+	+	-	-	-
	LUMMP4	+	+	+	-	-	+	+	+	+	+	+
	LUMMP10	-	-	-	+	-	+	+	+	+	+	+
	LUMMPF	-	-	-	+	-	-	+	+	+	-	-
T. candida DC.	LUMTC1	+	-	+	+	+	+	+	-	+	-	+
	LUMTC3	+	-	+	+	-	+	-	+	+	-	+
	LUMTC11	+	-	+	+	-	+	-	-	+	-	+
	LUMTC15	-	-	+	+	-	+	+	+	+	-	+
	LUMTCA	+	-	+	+	-	+	+	+	+	-	+
V. nepalensis Tateishi & Maxted	LUMVRW1	+	-	+	-	-	-	+	-	+	-	-
	LUMVRW2	+	-	+	+	-	-	-	-	+	-	-
	LUMVRW8	-	-	-	+	-	-	-	+		-	-
	LUMVRW9	-	-	-	+	-	-	-	-	-	-	-
	LUMVRW10	-	-	-	+	-	-	-	-	-	-	-
	LUMVRW11	+	-	+	+	-	-	-	-	-	-	-
	LUMVRW12	-	-	-	+	-	-	-	-	-	-	-

Host Species	Isolates	PGP Activities*		Biocontrol and Biochemical Analysis*			Sugar Fermentation*					
		IAA	PSB	Catalase	Citrate	Starch	Dextrose	Fructose	Glucose	Maltose	Mannitol	Sucrose
V. vexillata (L.) A. Rich	LUMVV1	-	-	-	-	+	+	+	+	+	-	-
	LUMVV2	-	-	-	-	-	+	+	+	+	-	-
	LUMVV4	-	-	-	-	+	+	+	+	+	-	+
	LUMVV9	-	-	+	-	+	+	+	+	+	+	+
	LUMVV10	+	-	-	-	-	-	-	-	-	-	-
	LUMVV11	+	-	+	-	-	-	-	-	-	-	-
	LUMVV13	-	-	+	-	-	-	-	-	-	-	-
	LUMVVO	-	-	-	-	+	-	+	-	-	-	-
	LUMVVx	-	-	-	-	+	-	+	+	+	-	-

* Note: +: Positive activity; -: Negative activity.

3.4.3 Starch hydrolysis tests

For screening the biocontrol properties of the 84 isolates, an amylase production or starch hydrolysis test was done. A halo zone was formed by 19 isolates, of which 1 was from *A. chinensis*, *D. triflorum*, *M. pudica* and *T. candida*, 2 from *C. mysorensis* and *D. heterocarpum*, 5 from *M. diplotricha*, and 5 from *V. vexillata* confirming amylase production by them (Table 3).

3.4.4 Sugar fermentation tests

Sugar fermentation tests were done to screen the isolates based on their ability to form organic compounds after metabolizing the carbohydrates. Six sugars were used as carbon sources: dextrose, fructose, glucose, maltose, mannitol, and sucrose. Positive sugar fermentation was confirmed after the colour change of media from pink to yellow. On testing 119 strains, dextrose was fermented by 56 isolates, fructose by 60 isolates, 63 isolates fermented glucose, 62 maltose, 38 fermented mannitol, and 46 isolates that could ferment sucrose. It was found that the isolates from *E. stricta* could not ferment mannitol and sucrose, bacterial isolates from *T. candida* failed to ferment mannitol and isolates from *V. nepalensis* could not hydrolyze dextrose, mannitol and sucrose sugars (Table 3).

3.5 PGPR activities

The *nif* gene-positive isolates were screened for plant growth-promoting traits like phosphate solubilization and IAA production. Out of 84 bacterial strains tested, 38 isolates showed IAA production activity. These isolates include 5 from *A. americana* and *M. diplotricha*, 2 each from *A. chinensis*, *C. mysorensis*, and *V. vexillata*, 3 each from *D. triflorum*, *E. stricta* and *V. nepalensis*, 4 from *D. heterocarpum*, *L. leucocephala* and *T. candida*. Further, 18 isolates showed phosphate solubilizing activities forming a halo zone a, which included 1 each from *A. americana*, *A. chinensis*, *L. leucocephala* and *M. pudica*, 3 from *D. heterocarpum* and *M. diplotricha* and 4 from *C. mysorensis* and *D. triflorum* (Table 3).

3.6 Abiotic stress tolerance

The abiotic stress tolerant ability of the 84 isolates was based on *p*H, salt, and temperature tolerance (Table 4). To check for acidic

Table 4 Stress tolerance tests of bacteria isolated from root nodules of wild legumes.

Host Species	Isolates	*p*H Tolerance				Temperature Tolerance (°C)				Salinity Tolerance (NaCl, %)			
		3	5	9	11	10	20	40	50	1	2	3	4
A. americana L.	AIS1	+	+	+	+	+	+	+	+	+	+	+	-
	AIS3	-	-	+	+	-	-	+	+	+	+	+	-
	AIS5	-	+	+	+	+	+	+	+	+	+	+	+
	AIS7	-	-	+	+	-	-	+	+	+	+	+	+
	AIS8	-	-	+	-	-	+	+	+	+	+	+	+
	AIS9	-	-	+	+	-	+	+	+	+	+	+	+
	AIS10	-	-	+	-	-	-	+	+	+	+	+	+
	AIS12	-	-	+	-	-	+	+	+	+	+	+	+
	AIR14	-	-	+	+	-	+	+	+	+	+	+	+

Host Species	Isolates	*p*H Tolerance				Temperature Tolerance (°C)				Salinity Tolerance (NaCl, %)			
		3	5	9	11	10	20	40	50	1	2	3	4
A. chinensis (Osbeck) Merr.	LUMAC1	-	-	+	-	-	-	+	+	+	+	+	+
	LUMAC5	-	-	+	-	+	-	+	+	+	+	+	+
	LUMAC6	-	-	+	-	+	+	+	+	+	+	+	+
	LUMAC9	+	+	+	-	+	+	+	+	+	+	+	+
	LUMAC10	-	-	+	-	+	-	+	+	+	+	+	+
	LUMAC11	-	-	+	-	-	-	+	+	+	+	+	+
	LUMAC12	-	-	+	-	-	-	+	+	+	+	+	+
C .mysorensis Roth	LUMCF1	-	-	+	-	-	-	+	+	+	+	-	-
	LUMCF2	-	-	+	-	-	+	+	+	+	+	+	+
	LUMCF3	-	-	+	+	+	+	+	+	+	+	+	+
	LUMCF7	-	-	+	+	-	-	+	+	+	+	-	-
	LUMCF10	-	-	+	-	+	+	+	+	+	+	-	-
	LUMCF11	-	-	+	-	-	+	+	+	+	+	+	+
	LUMCF12	-	-	+	-	-	+	+	+	+	+	+	+
D. heterocarpum (L.) DC.	LUMDes1	-	-	+	-	-	+	+	+	+	+	+	+
	LUMDes3	-	-	+	-	+	+	+	+	+	+	+	+
	LUMDes5	-	-	+	-	+	-	+	+	+	+	+	+
	LUMDes9	-	-	+	-	-		+	+	+	+	+	+
	LUMDes11	-	-	+	-	-	+	+	+	+	+	+	+
D. triflorum (L.) DC.	LUMDT2	-	-	+	+	-	+	+	+	+	+	+	+
	LUMDT3	-	-	+	+		+	+	+	+	+	+	+
	LUMDT9	-	-	+	+	+	+	+	+	+	+	+	+
	LUMDT11	-	-	+	+	+	-	+	+	+	+	-	+
	LUMDT16		+	+	+	-	+	+	+	+	+	-	+
E. stricta Roxb	LUMESA	-	+	+	-	-	-	+	+	+	+	+	+
	LUMESB	-	-	+	-	-	+	+	+	+	+	+	+
	LUMES4	-	-	+	-	+	-	+	+	+	+	+	+
	LUMES5	-	-	+	-	+	-	+	+	+	+	+	+
L. leucocephala (Lam.) de Wit	LUMLL1	-	-	+	-	-	+	+	+	+	+	-	-
	LIMLL2	-	-	+	-	-	+	+	+	+	+	+	+
	LUMLL6	-	-	+	+	-	+	+	+	+	+	+	+
	LUMLL8	-	-	+	+	-	-	+	+	+	+	+	+
	LUMLL9	-	-	+	+	+	-	+	+	+	+	+	+
	LUMLL11	-	-	+	+	-	+	+	+	+	+	+	+
	LUMLL12	-	-	+	+	-	+	+	+	+	+	+	+
	LUMLL13	-	-	+	+	-	+	+	+	+	+	+	+

Host Species	Isolates	*p*H Tolerance				Temperature Tolerance (°C)				Salinity Tolerance (NaCl, %)			
		3	5	9	11	10	20	40	50	1	2	3	4
M. diplotricha C. Wright	LUMMD2	-	-	+	-	+	+	+	+	+	+	+	-
	LUMMD3	-	-	+	+	+	+	+	+	+	+	+	-
	LUMMD5	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD6	-	-	+	-	-	-	+	+	+	+	+	+
	LUMMD9	-	-	+	-	-	-	+	+	+	+	+	+
	LUMMD12	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD13	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD15	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMD16	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMDb	-	-	+	-	-	-	+	+	+	+	-	+
	LUMMDc	-	-	+	-	-	+	+	+	+	+	+	+
	LUMMDd	-	-	+	-	-	-	+	+	+	+	+	+
	LUMMDm	-	-	+	-	-	+	+	+	+	+	+	-
M. pudica L.	LUMMP1	+	-	+	-	+	-	+	+	+	+	+	-
	LUMMP2	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMP4	-	-	+	-	+	+	+	+	+	+	+	+
	LUMMP10	-	-	+	+	+	+	+	+	+	+		+
	LUMMPF	-	-	+	+	-	+	+	+	+	+	+	+
T. candida DC.	LUMTC1	-	-	+	-	-	+	+	+	+	+	-	-
	LUMTC3	-	-	+	-	+		+	+	+	+	+	+
	LUMTC11	-	-	+	-	-	+	+	+	+	+	+	-
	LUMTC15	-	-	+	-	+	+	+	+	+	+	+	+
	LUMTCA	-	-	+	-	-	+	+	+	+	+	+	+
V. nepalensis Tateishi & Maxted	LUMVRW1	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVRW2	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVRW8	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVRW9	-	-	+	-	-	+	+	+	+	+	+	+
	LUMVRW10	-	-	+	-	-		+	+	+	+	+	+
	LUMVRW11	-	-	+	-	-	+	+	+	+	+	+	+
	LUMVRW12	-	-	+	-	-	+	+	+	+	+		-
V. vexillata (L.) A. Rich	LUMVV1	-	-	+	-	+	+	+	+	+	+	+	-
	LUMVV2	-	-	+	-	+	+	+	+	+	+	+	-
	LUMVV4	-	-	+	-	+	+	+	+	+	+	-	-
	LUMVV9	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVV10	-	-	+	-	-	+	+	+	+	+	-	-
	LUMVV11	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVV13	-	-	+	-	+	+	+	+	+	+	+	+
	LUMVVO	-	-	+	-	+	+	+	+	+	+	+	+

tolerance, isolates were grown in media with *p*H 3 and 5, and for alkaline tolerance, they were incubated in media adjusted with *p*H 9 and 11. Of all the strains tested, 17 survived at pH 3 media, except 2 isolates survived in acidic *p*H of 5. All the isolates could thrive in media with an alkaline pH of 9, while 22 could grow in a *p*H11 medium. At lower temperatures 10°C, 36 isolates could grow and at 20°C growth, 60 isolates could survive. All the isolates survived at 40°C and 50°C, except 6 isolates that did not grow at 50 °C. All the isolates thrived at NaCl concentrations of 1% and 2%. In the salt concentration of 3%, 12 were unable to thrive, and in 4% salt concentration, 17 could not grow.

4 Discussions

Nagaland, home to numerous legume types, has long understood the value of legumes. Wild legumes have been traditionally utilized as medicines in several Nagaland districts due to their widespread prevalence. In the six sites explored, the distribution of legumes was mostly similar. It is to the findings by Rathi et al. (2018) and Pires et al. (2018) that the soil *p*H and other ecological factors play a significant role in the biogeography of rhizobial microsymbionts and influence the selection of them by the host in a particular ecological region hence deciding the diversity of legumes in that area.

Trap experiments are performed when nodules are not collected at the site. It was observed that some legumes did not form nodules in greenhouse conditions, although they formed nodules naturally. This might imply that even if the growth conditions are favourable for the host, it might not be true for rhizobia, hence the lack of nodules. It illustrates that environmental conditions play an important role in both the growth and efficient nodulation of legumes, and the present findings are in agreement with Choudhary et al. (2020), who argued that the biogeography of rhizobia is a result of interactions among the host legumes, the genomic backgrounds of compatible bacterial partners and their environments leading to co-evolution of the legume hosts and compatible rhizobia.

The nodules uprooted were found to be mainly of two common types, determinate and indeterminate, e.g., *Mimosa* species. The size of the nodules also varied from very small, like that of *Desmodium triflorum*, to large, like that of *Tephrosia candida*. When dissected and observed, the nodules were mostly pink due to leghaemoglobin indicating active nitrogen fixation, as described by Aung and Oo (2020).

Streaking of the root exudates of healthy and pink nodules produced cultures with varying phenotypic characteristics. Most of them were regular, raised, and bulky. Some isolates were slow growing and took 5-7 days to grow; these were probably *Bradyrhizobia* (Dinkwar et al. 2020; Padukkage et al. 2021). The slow-growing character of *Bradyrhizobium* was attributed to its distinct cell physiology that includes the display of cell aggregation rather than cell division during the lag and initial exponential growth phase, hindrance in their growth due to an increase in specific nutrient concentration (Medici et al. 2024). The culture obtained had varying morphological characteristics. Cultures obtained were pink, white, creamy, translucent, and sometimes yellow. Since wild legumes are infected by various symbionts with varying degrees of nodulation and nitrogen fixation efficiency, DNA fingerprinting could be an essential method for studying their microbial diversity. Further, performing RAPD is a vital method to assess their genetic diversity. A similar opinion was also expressed by Aoki et al. (2010), as the similarity in the banding patterns obtained after amplification indicated homogenous isolates.

Biochemical tests are performed when it is difficult to distinguish bacteria morphologically; hence, they are identified by differences in their metabolic activities. Various biochemical tests were performed to determine their biocontrol, enzyme activities, plant growth promoting, sugar fermentation, and stress-tolerant activities. Biochemical characterization of isolates suggested that starch hydrolysis was negative for most of the isolates, similar to the results obtained by Singha et al. (2015).

In the present study, out of 84 isolates, 18 showed phosphate solubilizing activity, and 38 were able to produce IAA. Sijilmassi et al. (2020) and Oparah et al. (2024) reported that *Rhizobium* was good for the solubilization of phosphate. Similar findings have been recorded by Sijilmassi et al. (2020) and Sharma and Goswami (2020) in Rhizobial species to produce IAA.

Growth of rhizobia in soils is sensitive to pH, and it has been shown to limit survival and persistence in soils (Kapembwa et al. 2016). The isolates obtained were all able to grow in an acidic medium (pH 3 to 5), who reported that the tested isolates exhibited very poor growth at acidic pH. Rhizobial cultures isolated in this study showed normal growth at pH 6.8. These results are similar to those of Jain et al. (2020), who reported the best rhizobial growth in media with a pH around neutral. LUMMP4, LUMTC4, LUMCF10, LUMAC1, AIS6, and LUMDes5 were some isolates out of 22 that could thrive in extremely alkaline pH11.

Several studies reported that most rhizobia are salt tolerant (Bissa et al. 2020; Oparah et al. 2024). In the present study, some isolates like LUMMD3, LUMESA, and LUMDes11 were able to grow profusely after 24h of spot inoculation, while colonies of other isolates like AIS9, LUMCF3, LUMCF11, LUMMDa, LUMMDc, LUMMPO were seen after 32h of inoculation in the varying salt concentrations. The isolates differed in their growth rate but were able to thrive in all salt concentrations irrespective of salt

concentration, which is in contrast to the reports of Ali et al. (2009) and Thrall et al. (2008), who reported that with increasing salt concentration there was a decrease in growth of rhizobial isolates. It has been stated that Salinization/ alkalization is known to limit nodulation and nitrogen fixation. Response of legumes varies greatly; some legumes, e.g., *Vicia faba* and *Phaseolus vulgaris*, are more salt tolerant than others, such as *Pisum sativum*. Other legumes like *Prosopis, Acacia,* and *Medicago sativa* are sensitive to high salt, but their rhizobia is more salt tolerant than the host plants (Zahran 1999).

Sugar fermentation tests are performed to investigate if the bacterial isolates can ferment different carbohydrates and form organic compounds. Six sugars were used: Dextrose, Fructose, Glucose, Maltose, Mannitol, and Sucrose. There was diversity in the ability of isolates to ferment different carbon sources because both fast and slow-growing Rhizobia were isolated. This corroborates with the study by Kapembwa et al. (2016), which found that fast-growing Rhizobia has a wider range for carbon utilization while slow-growing Rhizobia has a narrow range. Further, 63 isolates were able to ferment glucose, which is a confirmatory test for *Rhizobium*, as given by Rai and Sen (2015). Twenty-eight isolates were able to ferment all the carbohydrate sources, assuring their identity to be Rhizobia, as per Hossain et al. (2019).

Conclusions

This study uncovered the vast microbial diversity present in the nodules of wild legumes, positively asserting the wide scope of bacterial diversity study possible from legumes of Nagaland. Out of 150 isolates, 119 were bound with RPO1 primer preliminary, confirming their identity as nitrogen-fixing rhizobia. RAPD band polymorphisms among the isolates were observed, which indicated the huge bacterial diversity in the nodules. It affirms that nif-directed RAPD is beneficial in giving a Head Start for subsequent molecular works on the isolates. The present work also observed 11 isolates that could solubilize phosphate and produce IAA, certifying their ability to benefit plants. Remarkably, most of the isolates were also tolerant towards abiotic stress, which was considered in our study. Thus, the current research findings offer the initial examination of the phylogenetic diversity of native Rhizobia that nodulates several significant wild legumes in Nagaland. Research on native rhizobia in the wild without a history of rhizobial inoculation could be crucial for the selection of novel strains suitable for the regional environment. Also, since the rhizobial diversity of Nagaland is vast and still unexplored, there is a possibility of obtaining novel strains since PCR methods are designed to amplify only the target regions, and the known primers might not be applicable for the novel strains. Further molecular work with nitrogen fixation genes and other housekeeping genes is recommended to solidify the identity of rhizobia.

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Conflicts of Interest

Authors declare that no conflicts exist.

Consent of authorship

All authors consented to a part of the paper. Both CRD and MM have an equal share of authorship.

Authors Contributions

Fund arrangement, concept development, experimental design, supervision of the research work, data analysis and manuscript correction done by CRD, MM executed the research work, data analysis, manuscript draft preparation as a per of her Ph. D. research, AP is the Co-Supervisor of MM and partially supervised initial part of the research work.

References

Achumi, B., Phom, L., Zevelou, M.A., Lal, P., & Yenisetti, S.C. (2014). Ecogeographic biodiversity pattern of Genus *Drosophila* (Insecta, Diptera: Drosophilidae) in Nagaland state, India. *Nagaland University Research Journal*, *7*, 332–337.

Ali, S.F., Rawat, L.S., Meghvansi, M. K., & Mahna, S.K. (2009). Selection of stress-tolerant rhizobial isolates of wild legumes growing in dry regions of Rajasthan, India. ARPN *Journal of Agricultural and Biological Science*, *4*(1), 13-18.

Anantha, K. C., Majumder, S. P., Badole, S., Padhan, D., Datta, A., Mandal, B., & Sreenivas, C. H. (2020). Pools of organic carbon in soils under a long-term rice–rice system with different organic amendments in hot, sub-humid India. *Carbon Management*, *11*(4), 331-339.

Aoki, S., Kondo, T., Prévost, D., Nakata, S., Kajita, T., & Ito, M. (2010). Genotypic and phenotypic diversity of rhizobia isolated from *Lathyrus japonicus* indigenous to Japan. *Systematic and Applied Microbiology*, *33*(7), 383-397.

Aung, T.M., & Oo, P.P. (2020). Isolation and characterization of *Rhizobium* from root nodules of *Arachis hypogaea* L.(Groundnut). *Journal of the Myanmar Academy of Arts and Science*, *18*, 197-210.

Banerjee, S., & Van Der Heijden, M.G. (2023). Soil microbiomes and one health. *Nature Reviews Microbiology*, *21*(1), 6-20.

Bassam, B.J., Caetano-Anollés, G., & Gresshoff, P.M. (1992). DNA amplification fingerprinting of bacteria. *Applied Microbiology and Biotechnology*, *38*, 70-76.

Bissa, G., Tak, N., & Gehlot, H.S. (2020). Phenotypic Characterization of Rhizobial strains symbiotically associated with *Prosopis* species in India. *Plant Archives*, *20* (2), 5951-5957.

Bray, R.H., & Kurtz, L.T. (1945) Determination of total, organic and available forms of phosphorus in soils. *Soil Science*, *59*, 39–45.

Choudhary, S., Tak, N., Bissa, G., Chouhan, B., Choudhary, P., Sprent, J. I., James, E.K. & Gehlot, H. S. (2020). The widely distributed legume tree *Vachellia (Acacia) nilotica* subsp. indica is nodulated by genetically diverse *Ensifer* strains in India. *Symbiosis*, *80*, 15-31.

Chouhan, B., Tak, N., Bissa, G., Adhikari, D., Barik, S. K., et al. (2022). Evolution of novel strains of *Ensifer* nodulating the invasive legume *Leucaena leucocephala* (Lam.) de Wit in different climatic regions of India through lateral gene transfer. *FEMS Microbiology Ecology*, *98*(9), fiac086.

Dabo, M., Jaiswal, S.K., & Dakora, F.D. (2019). Phylogenetic evidence of allopatric speciation of *Bradyrhizobia* nodulating cowpea (*Vigna unguiculata* L. Walp) in South African and Mozambican soils. *FEMS Microbiology Ecology*, *95*, 1–14.

Dinkwar, G. T., Thakur, K., Bramhankar, S., Pillai, T., Isokar, S., Ravali, T., & Kharat, V. (2020). Biochemical and physiological characterization of *Bradyrhizobium japonicum*. *International Journal of Chemical Studies*, *8*(2), 1589-1594.

Gang, S., Sharma, S., Saraf, M., Buck, M., & Schumacher, J. (2019). Analysis of indole-3-acetic acid (IAA) production in *Klebsiella* by LC-MS/MS and the Salkowski method. *Bio-protocol*, *9*(9), e3230-e3230.

Giannelli, G., Potestio, S., & Visioli, G. (2023). The contribution of PGPR in salt stress tolerance in crops: Unravelling the molecular mechanisms of cross-talk between plant and bacteria. *Plants*, *12*(11), 2197.

Green, R. T., East, A. K., Karunakaran, R., Downie, J. A., & Poole, P. S. (2019). Transcriptomic analysis of *Rhizobium leguminosarum* bacteroids in determinate and indeterminate nodules. *Microbial Genomics*, *5*(2), e000254.

Hopkins, W.G., & Hüner, N.P. (2014). *Introduction to Plant Physiology* (Fourth Edition). John Wiley & Sons, New York, Pp. 195-211.

Hossain, A., Gunri, S.K., Barman, M., Sabagh, A.E., et al. (2019). Isolation, characterization and purification of *Rhizobium* strain to enrich the productivity of groundnut (*Arachis hypogaea* L.). *Open Agriculture*, *4*(1), 400-409.

Ibny, F.Y.I., Jaiswal, S.K., Mohammed, M., & Dakora, F.D. (2019). Symbiotic effectiveness and ecologically adaptive traits of native rhizobial symbionts of Bambara groundnut (*Vigna subterranea* L. Verdc.) in Africa and their relationship with phylogeny. *Scientific Reports*, *9*(1), 12666.

Jain, D., Kumari, A., Saheewala, H., Sanadhya, S., Maheshwari, D., Meena, R.H., Singh, A., Gera, R. & Mohanty, S.R. (2020). Biochemical, functional and molecular characterization of pigeon pea rhizobia isolated from semi-arid regions of India. *Archives of Microbiology*, *202*, 1809-1816.

Jia, Z.J., Myrold, D.D., & Conrad, R. (2020). Soil biodiversity in a rapidly changing world. *Pedosphere*, *30*, 1–4.

Labouyrie, M., Ballabio, C., Romero, F., Panagos, P., Jones, A., et al. (2023). Patterns in soil microbial diversity across Europe. *Nature Communications*, *14*(1), 3311.

Kapembwa, R., Mweetwa, A.M., Ngulube, M., & Yengwe, J. (2016). Morphological and biochemical characterization of soybean nodulating rhizobia indigenous to Zambia. *Sustainable Agriculture Research*, *5*, 84.

Kjeldahl, J.G.C.T. (1883). Neue method ezurbestimmung des stickstoffs in organischenkörpern. *Zeitschrift Für AnalytischeChemie*, *22*(1), 366-382.

Medici, I. F., Bartrolí, L., Guaimas, F. F., Fulgenzi, F. R., Molina, C. L., et al. (2024). The distinct cell physiology of *Bradyrhizobium* at the population and cellular level. *BMC Microbiology*, *24*(1), 129.

Megu, M., Paul, A., & Deb, C.R. (2024). Isolation and screening of stress tolerant and plant growth promoting root nodulating rhizobial bacteria from some wild legumes of Nagaland, India. *South African Journal of Botany*, *168*, 260-269.

Oparah, I.A., Hartley, J.C., Deaker, R., Gemell, G., Hartley, E., & Kaiser, B.N. (2024). Symbiotic effectiveness, abiotic stress tolerance and phosphate solubilizing ability of new chickpea root-nodule bacteria from soils in Kununurra Western Australia and Narrabri New South Wales Australia. *Plant and Soil*, *495*(1), 371-389.

Padukkage, D., Geekiyanage, S., Reparaz, J. M., Bezus, R., Balatti, P. A., & Degrassi, G. (2021). *Bradyrhizobium japonicum, B. elkanii* and *B. diazoefficiens* interact with rice (*Oryza sativa*), promote growth and increase yield. *Current Microbiology*, *78*, 417-428.

Peoples, M.B., Brockwell, J., Herridge, D.F., Rochester, I.J., Alves, B.J.R., et al. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis*, *48*, 1-17.

Pires, R.C., Reis Junior, F.B., Zilli, J.E., Fischer, D., et al. (2018). Soil characteristics determine the rhizobia in association with different species of *Mimosa* in Central Brazil. *Plant and Soil*, *423*(1–2), 411–428.

POWO (2024). Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://www.plantsoftheworldonline.org/ Retrieved 10th January, 2024.

Rai, R., & Sen, A. (2015). Biochemical characterization of French bean associated rhizobia found in North Bengal and Sikkim. *Journal of Academia and Industrial Research*, *4*, 10-18.

Rathi, S., Tak, N., Bissa, G., Chouhan, B., et al. (2018). Selection of *Bradyrhizobium* or *Ensifer* symbionts by the native Indian caesalpinioid legume *Chamaecrista pumila* depends on soil *p*H and other edaphic and climatic factors. *FEMS Microbiology Ecology*, *94*(11), fiy180.

Richardson, A.E., Viccars, L.A., Watson, J.M., & Gibson, A.H. (1995). Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. *Soil Biology and Biochemistry*, *27*(4-5), 515-524.

Ritse, V., Basumatary, H., Kulnu, A.S., Dutta, G., et al. (2020). Monitoring land use land cover changes in the Eastern Himalayan landscape of Nagaland, Northeast India. *Environmental Monitoring and Assessment*, *192*(11), 1-17.

Rosselló-Móra, R., & Stackebrandt, E. (2021). Bridging 200 years of bacterial classification. In B. Paul, S. David, S. Erko (Eds.), *Trends in the Systematics of Bacteria and Fungi* (pp. 1-20), CABI Digital Library. Doi: 10.1079/9781789244984.0001

Sambrook, J., & Russell, D. W. (2001). Detection of DNA in agarose gels. *Molecular Cloning, A Laboratory Manual, (3rd Ed.)* (pp. 5-14) Cold Spring Harbor Laboratory Press, New York.

Schloter, M., Nannipieri, P., Sørensen, S.J., van Elsas, J.D. (2018). Microbial indicators for soil quality. *Biology and Fertility of Soils*, *54*(1), 1-10.

Sijilmassi, B., Filali-Maltouf, A., Fahde, S., Ennahli, Y., Boughribil, S., Kumar, S., & Amri, A. (2020). *In vitro* plant growth promotion of rhizobium strains isolated from lentil root nodules under abiotic stresses. *Agronomy*, *10*(7), 1006.

Sharma, L., & Goswami, G.K. (2020). Identification and Biochemical Characterization of β-Rhizobia isolated from Root Nodules of *Mimosa pudica*. *Journal of Academia and Industrial Research*, *8*(11), 173.

Shoaib, M., Muzammil, I., Hammad, M., Bhutta, Z.A., & Yaseen, I. (2020). A mini-review on commonly used biochemical tests for identification of bacteria. *International Journal of Research Publications*, *54*(1), 1-7.

Singha, B., Das, P., & Mazumder, P.B. (2015). Morphological and biochemical characterization of rhizobia isolated from root nodule of *Crotolaria junceae* L. grown in Assam. *International Journal of Science and Research*, *4*(4), 1928-1931.

Somasegaran, P., & Hoben, H. J. (1985). *Methods in Legume-Rhizobium Technology*. Hawaii Institute of Tropical Agriculture and Hunan Resources, pp. 1-52.

Tatung, M., & Deb, C.R. (2023). Isolation, characterization, and investigation on potential multi-trait plant growth promoting rhizobacteria from wild banana (*Musa itinerans*) rhizospheric soil. *Journal of Pure and Applied Microbiology*, *17*(3), 1578-1590.

Thrall, P.H., Bever J.D., & Slattery, J.F. (2008). Rhizobial mediation of Acacia adaptation to soil salinity: evidence of underlying trade-offs and tests of expected pattern. *Journal of Ecology*, *96*(4), 746-755.

Trivedy, R.K & Goel, P.K. (1984). *Chemical and biological methods for water pollution studies*. Environmental Publications, Karad, India, pp. 248.

Williams, M.N.V., Pande, N., Nair, S., Mohan, M., et al. (1991). Restriction fragment length polymorphism analysis of polymerase chain reaction products amplified from mapped loci of rice (*Oryza sativa* L.) genomic DNA. *Theoretical and Applied Genetics*, *82*, 489-498.

Zahran, H.H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in acid climate. *Microbiology and Molecular Biology Reviews*, *63*, 968-989.

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The impact of the Russian-Ukrainian War on the food security of the Kingdom of Saudi Arabia

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ABSTRACT

The economic and social impact of the Russian-Ukrainian war was the focus of this study, which aimed to assess its effect on the food security of the Kingdom of Saudi Arabia. To achieve its objectives, the study utilized published data, food security index measures, and standard economic analysis. The findings revealed that the Kingdom's food security environment index increased from 58.1% in 2012 to 69.9% in 2022, indicating moderate food security throughout this period. The index was lower than the estimated counterpart for the rest of the Gulf Cooperation Council countries but exceeded the global average (113 countries) by 12.38% in 2022. Furthermore, the study demonstrated that a 10% increase in the estimated food production index and real per capita income led to a 2.72% and 6.55% increase in the food security index, respectively. Conversely, a 10% rise in the estimated consumer price index for foodstuffs resulted in a 1.74% decrease in the food security index. Despite the challenges posed by the Russian-Ukrainian war, the food security index is projected to improve for the Kingdom of Saudi Arabia, expected to increase from 72.4% in 2024 to 75.6% in 2030, attributed to the country's policy of investing in agriculture abroad and focusing on local agricultural investments such as vertical expansion, protected agriculture, and agricultural practices. This strategic approach ensures high-quality produce and facilitates significant financial surpluses, enhancing the country's capacity to import goods from overseas.

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1 Introduction

Due to the outbreak of the Russian-Ukrainian war on February 24, 2022, there have been significant impacts on growth rates, economic development, and the prices of goods and services. Inflationary pressures have intensified in the Middle East and North Africa region, mainly due to the impact of the war on the quantity and value of food imports, leading to higher prices in international markets. In 2021, the inflation rate in the Middle East and North Africa reached 14.8%. Countries such as Iran, Lebanon, and Yemen are experiencing concerning inflation levels, with rates reaching 43%, 154%, and 30%, respectively (Acevedo et al. 2022). According to a study by the Ministry of Planning and International Cooperation, Republic of Yemen (2022), the Russian-Ukrainian war increased the inflation rate to 45% in 2022, possibly driven by the rise in oil and food prices. The high food prices and the risk of food insecurity are particularly harmful to low-income families, as they spend a significant portion of their income on food and energy, unlike wealthier families. The FAO Food Price Index for cereals, vegetable oils, sugar, meat, and dairy products has significantly increased. Specifically, the FAO Food Price Index rose from 135.8 in January 2022 to 160.3 in March 2022, then declined to 117.3 in February 2024 (Figure 1).

The data in Figure 1 shows that the Russian-Ukrainian war has significantly impacted foreign trade and supply chains for food commodities. This is a major concern for global food security because Russia is the world's largest wheat exporter. Russia and Ukraine account for over a third of global grain exports (FAO 2024). More than 50 countries, including Saudi Arabia, Libya, Djibouti, Yemen, Lebanon, and Tunisia, rely on Russia and Ukraine for at least 30% of their wheat imports. As a result of the war, food prices have risen by 40 to 60 percent. Global supplies of food products such as wheat, barley, corn, and sunflower oil are anticipated to decrease by 10-50% (Abdel Shafi 2022). The war has also led to a decline in foreign trade for Central Asian countries with Russia and Ukraine, exacerbating the deficit in their trade balance. Additionally, the war has posed challenges for these countries regarding benefiting from their natural and petroleum resources (Abdel Nabi 2022).

Some economic studies have focused on the impact of the Russian-Ukrainian war on the Kingdom of Saudi Arabia. For instance, a study by Ghanem et al. (2023a) measured the impact of the war on consumer prices for food products. This study showed an increase in the world food price index and total population of Saudi Arabia by 10%, leading to a 1.22% and 4.95% increase in the consumer price index for food products, respectively. The consumer price index for food products is expected to continue to increase, reaching 137.7 in 2022, which is 12.2% higher than the 122.78 index in 2021.

In a study by Ghanem et al. (2023b), the impact of the Russian-Ukrainian war on the value of imports and the food trade balance of the Kingdom of Saudi Arabia was examined. The study found that a 10% increase in the world food price index leads to a 6.98%

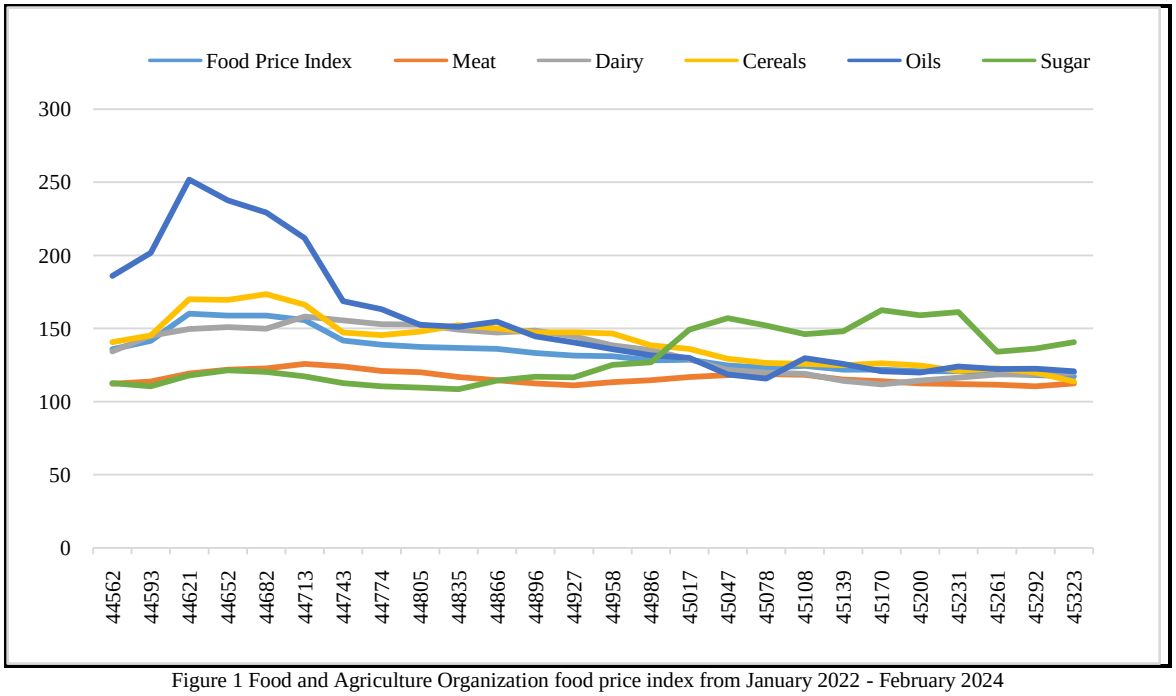

Figure 1 Food and Agriculture Organization food price index from January 2022 - February 2024

(Source: Food and Agriculture Organization (FAO), March 8, 2024).

rise in the value of food imports and a 7.87% increase in the food trade deficit. Additionally, a 10% increase in the food production index results in a 1.88% decrease in the value of food imports. Furthermore, increasing the value of food exports by 10% reduces the food trade deficit by 5.24%. With the food price index reaching 145.8, the value of food imports and the food trade deficit have increased by 37.1% and 44.5%, respectively, compared to the current situation in 2021. Al-Bashabsheh (2023) also highlighted that the COVID-19 pandemic and the conflict in Ukraine have reduced global food supply and increased food prices. The conflict in Ukraine has particularly impacted European countries that rely on Russian gas passing through Ukraine. Furthermore, in a study by Meligi and Salem (2023), it was revealed that the Russian-Ukrainian war has had negative repercussions on the Arab Republic of Egypt, and it is expected that the value of the food security index will decline by about 24% between 2022 and 2027.

Despite the positive outlook for producing basic food commodities, climate change, increasing geopolitical tensions, and sudden policy changes pose risks to global food production systems. These risks could upset the balance of supply and demand and weaken the expected performance of foreign trade and food security. The foreign trade volume in coarse grains, rice, vegetable oils, fats, sugar, milk products, meat, and fish is expected to decrease between 2023 and 2024. Additionally, the value of global food imports is expected to increase to $2 trillion in 2023, which is a $35.3 billion, or 1.8%, increase over its 2022 level (FAO, 2023). The continuation of the Russian-Ukrainian war has impacted the supply of food commodities, leading to increased costs of obtaining them. As a result, the periods of production adequacy and import coverage for domestic consumption decrease, reducing the surplus directed to developing strategic stocks and food security factors. Maintaining a safe strategic stock that allows the continued flow of food commodities to local markets can help the state control inflation and rising prices for food commodities. This study uses an econometric methodology to estimate the impact of the Russian-Ukrainian war on the future of food security for the Kingdom of Saudi Arabia. The research also includes a comparative economic analysis of the food security index between the Kingdom of Saudi Arabia and the rest of the Gulf Cooperation Council countries and the global average. Furthermore, the study estimates the proposed model to assess the impact of the Russian-Ukrainian war on the level of food security in the Kingdom of Saudi Arabia from 2000-2022 and predicts the future of food security for the Kingdom of Saudi Arabia until 2030.

2 Materials and Methods

To achieve the objectives of this study, we relied on published data from the Food and Agriculture Organization (FAO) and the General Authority for Statistics in Saudi Arabia. We also used data from the Global Food Security Environment Index (GFSI) issued by the Economist Impact Foundation in England. This index depends on several metrics, including:

Table 1 The relative weight of the criteria for measuring the food security environment index.

Measure	Relative weight%	Measure	Relative weight%
Affordability**:**	30.00	Food security and access obligations	12.61
Change in average food cost	23.85	Quality and safety**:**	22.5
Percentage of population below the global poverty line	19.23	Dietary diversity	19.50
Inequality-adjusted income index	16.92	Nutritional standards	20.33
Agricultural trade	19.23	Availability of micronutrients	19.51
Food safety net program	20.77	Protein Quality	20.33
Availability**:**	25.0	Food Safety	20.33
Access to agricultural inputs	11.71	Sustainability and resilience**:**	22.5
Agricultural research and development	11.71	Exposure to risks	17.00
Farm infrastructure	9.01	water	16.50
Agricultural production fluctuation	11.26	land	16.50
Food loss	11.26	Oceans, rivers and lakes	15.50
Supply chain infrastructure	9.91	Political commitment to resilience	19.00
Adequacy of supply	11.71	Disaster risk management	15.50
Political and social barriers to access	10.81		

Source: Economist Impact (2023), Global Food Security Index (2023), and the GFSI website navigation guide

Table 2 The range used to describe the food security situation.

Range	Describe the food security situation
80- 100	Very Good
70- 79.9	Good
55- 69.9	Moderate
40- 54.9	Weak
0- 39.9	Very Weak

Source: Economist Impact (2023), Global Food Security Index (2023), the GFSI website navigation guide

1. Affordability: Measures the ability of consumers to purchase food, exposure to rising prices, and the availability of programs and public policies to control prices.
2. Availability: Measures the adequacy of national food supplies, the risk of supply disruption, and the country's ability to provide food and support scientific research efforts to expand production.
3. Quality and Safety: Measures the variety and quality of diets and food safety.
4. Natural Resource Sustainability and Resilience: Measures the country's exposure to the impact of climate change and natural resource risks and its ability to adapt to them.

The Food Security Environment Index is calculated based on the relative weight of the metrics mentioned above, as represented in Table 1. The food security situation is described in Table 2.

This study also relied on the proposed model consisting of three behavioural equations, which can be expressed as follows:

$$Y_1 = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + e_1 \ldots\ldots\ldots\ldots\ldots (1)$$

$$Y_2 = b_0 + b_1\hat{Y}_1 + b_2X_5 + b_3X_6 + e_2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$Y_3 = c_0 + c_1\hat{Y}_1 + c_2\hat{Y}_2 + c_3X_7 + e_3 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

The model we're proposing includes the following variables:

I. Endogenous Variables: These depend on three variables indicating the country's ability to produce food. They are expressed by the food production index (Y1), the consumer price index for food commodities (Y2), and the global food security index for the Kingdom of Saudi Arabia (Y3).
II. Exogenous Variables: There are seven of these: cropped area in thousand hectares (X1), agricultural labour in thousand workers (X2), amount of water used for agricultural purposes in billion m3 (X3), value of agricultural investments in million riyals (X4), World Food Price Index (X5), total population in million people (X6), and real per capita income in thousand riyals (X7).

The proposed model was estimated using the ordinary least squares method. This was chosen because the matrix of internal variables has a diameter of one, and all numbers above this diagonal take the number zero as shown in below table (Gujarati, translated and reviewed by Odeh 2015).

Since the model used is based on time series data, the problem of autocorrelation of the residuals may arise. It is detected using several tests, and the most important of which are: (i) the Durbin-Watson test, whose value ranges between zero and four ($0 \leq DW \leq 4$), (ii) the Breusch- Godfrey Serial Correlation LM test, if the probability value (P-value) is greater than the level of significance (α), this indicates that there is no autocorrelation between the random errors, but if the probability value is less than the level of significance, this indicates the presence of autocorrelation between the random errors.

3 Results and Discussion

3.1 The disparity in the level of food security between the KSA and the rest of the Gulf Cooperation Council countries and the global average

The Global Food Security Index (GFSI) has been published by Economist Impact for 113 countries worldwide since 2012. The index considers factors such as food costs, availability, quality, and safety, natural resources, and resilience. Analyzing the food security level in the Kingdom of Saudi Arabia from 2012 to 2022,

External variables							Internal variables		
X_7	X_6	X_5	X_4	X_3	X_2	X_1	Y_3	Y_2	Y_1
0	0	0	$-a_4$	$-a_3$	$-a_2$	$-a_1$	0	0	1
0	$-b_3$	$-b_2$	0	0	0	0	0	1	$-b_1$
$-c_3$	0	0	0	0	0	0	1	$-c_2$	$-c_1$

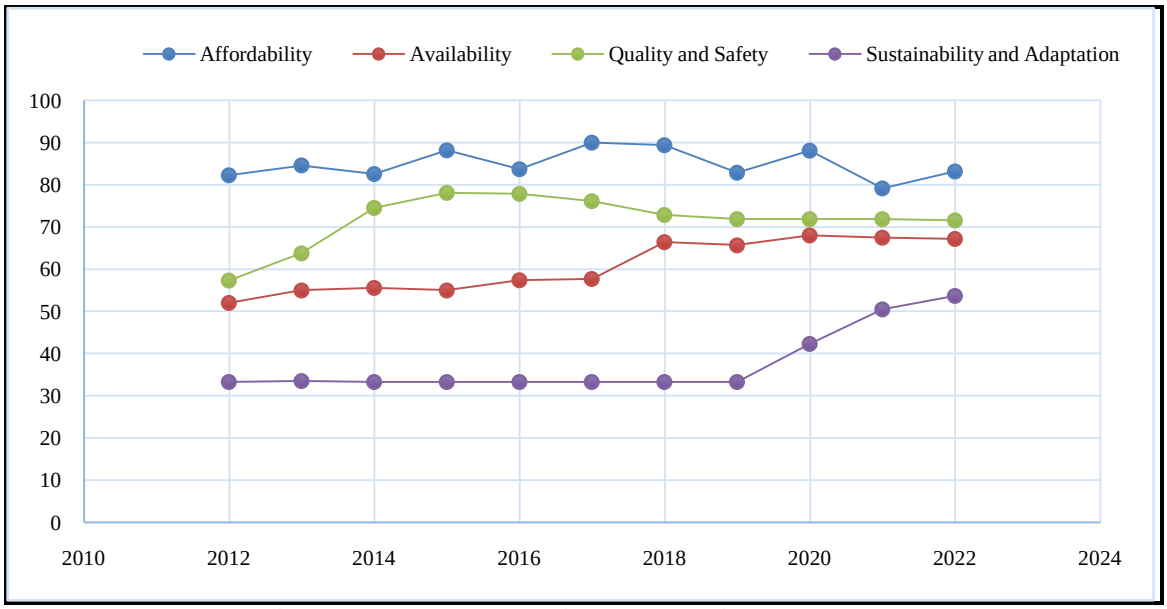

Figure 2 Components of the Global Food Security Index for the Kingdom of Saudi Arabia during the period 2012-2022 (Source: Economist Impact, Global Food Security Index: Country Ranking 2021, Retrieved on June 3, 2022, from https:// impacteconomist.com/sustainability/project/food-security-index/Index)

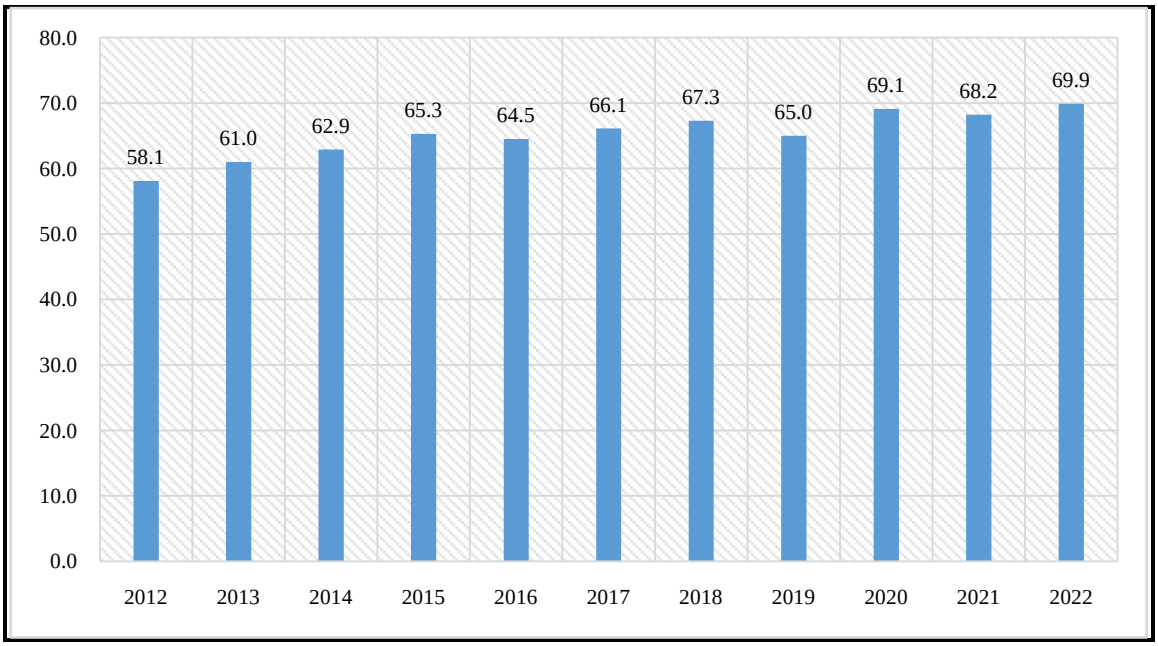

Figure 3 Global food security index for the Kingdom of Saudi Arabia during the period 2012- 2022 (Source: Data contained in Figure 2).

we can see that the ability to afford food ranged from a low of 79.2% in 2021 to a high of 90.0% in 2017, averaging 84.9%. Food availability ranged from a low of 52.0% in 2012 to a high of 68.0% in 2020, averaging at 60.7% annually. The measure of food quality and safety ranged from a low of 57.3% in 2022 to a high of 78.1% in 2015, with an average of 71.6% annually. The measure of natural resources sustainability and resilience ranged from 33.3% between 2012 and 2019 to a high of 53.7% in 2022, averaging 37.6% annually. Overall, the food security environment index for the Kingdom of Saudi Arabia ranged from a low of 58.1% in 2012 to a high of 69.9% in 2022, with an average of 65.2% from 2012 to 2022. This data reveals disparities in food security levels between Saudi Arabia and other Gulf Cooperation Council countries and the global average.

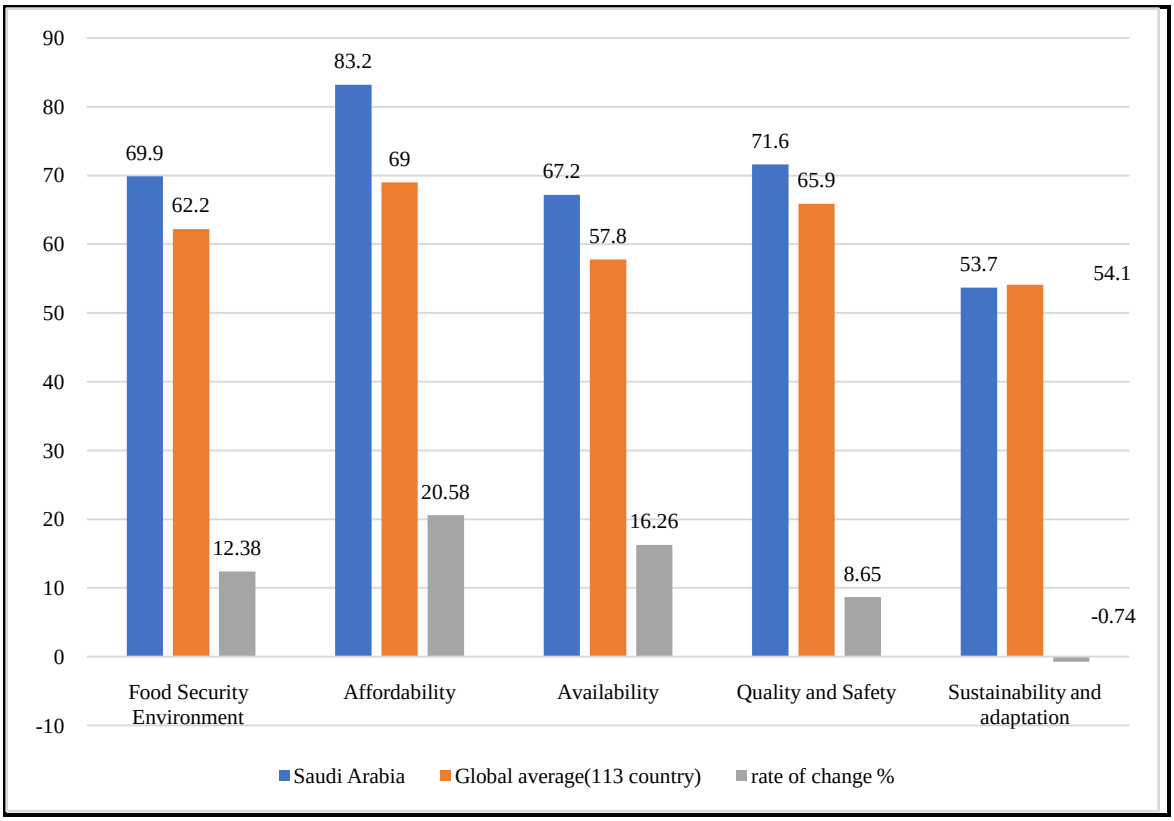

Figure 4 Comparison between global food security indicators for the Kingdom of Saudi Arabia and the global average (113 countries) in 2022 (Source: Economist Impact, Global Food Security Index: Country Ranking 2021, Retrieved on June 3, 2022, from https:// impacteconomist.com/ sustainability/project/food-security-index/ Index)

By comparing the measures of the Food Security Environment index for the Kingdom of Saudi Arabia with the global average (113 countries) in 2022, it is clear from the data presented in Figure 4 that the measure of food affordability, food availability, quality and food safety for the Kingdom of Saudi Arabia exceeds the global average (113 countries), at rates of 20.58%, 16.26%, and 8.65% for each of them, respectively, in 2022. The measure of natural resource sustainability and resilience for the Kingdom of Saudi Arabia falls short of the global average by a small rate of 0.74% in 2022. Finally, the Global Food Security Environment index for the Kingdom of Saudi Arabia exceeds the global average (113 countries), with a rate of 12.38% in 2022.

An economic analysis of the global food security environment index was conducted for the Gulf Cooperation Council (GCC) countries. The data in Table 3 shows that the United Arab Emirates had a moderate level of food security from 2012 to 2017, which improved to a good level by 2022. Qatar's food security situation was moderate in 2012 and became good from 2013 to 2022. The Sultanate of Oman had moderate food security from 2012 to 2015, which improved to a good level from 2016 onwards. Bahrain, Saudi Arabia, and Kuwait had moderate food security from 2012 to 2022, except for Bahrain, which had a neutral food security situation in 2022.

On average, the Global Food Security Environment Index ranked Qatar first among the GCC countries, followed by Oman, the UAE, Bahrain, Kuwait, and Saudi Arabia. In 2022, the United Arab Emirates ranked 23rd, Qatar 30th, Oman 35th, and Bahrain 38th globally, according to the Global Food Security Index. Saudi Arabia ranked 41st, and Kuwait ranked 50th. The food security situation in the GCC was relatively stable, with low variation in the Food Security Environment Index scores during the study period. The coefficient of variation ranged from 2.8% for Kuwait to 9.0% for the United Arab Emirates.

The data in Table 4 shows that the Global Food Security Index scores for the Gulf Cooperation Council countries in 2022 compared to 2012-2021 have changed. The scores remained positive for the United Arab Emirates, Kingdom of Saudi Arabia, and Bahrain, indicating an improvement in global food security for these countries in 2022. However, the score for the State of Qatar decreased to 72.4 in 2022 from its previous score between 2014 and 2017-2021. Similarly, the global food security index for the Sultanate of Oman decreased to 71.2 in 2022 from its score between 2017-2021. Kuwait's global food security index declined to 65.2 in 2022 from its score between 2012-2014 and 2018-2021.

Table 3 Global food security environment index of the Gulf Cooperation Council during the period 2012-2022

Year	United Arab Emirates	Qatar	Oman	Bahrain	Saudi Arabia	Kuwait
2012	63.2	69.9	57.4	64.7	58.1	65.7
2013	61.4	70.1	59.4	64.5	61.0	66.7
2014	62.1	72.8	64.6	65.9	62.9	67.2
2015	61.5	72.0	64.4	65.2	65.3	64.5
2016	60.3	72.0	70.1	65.5	64.5	63.1
2017	63.9	73.0	71.3	66.1	66.1	64.7
2018	71.6	73.0	73.3	69.5	67.3	68.0
2019	72.9	73.8	72.2	69.4	65.0	68.7
2020	73.7	74.0	72.0	68.6	69.1	68.4
2021	73.6	74.6	72.3	69.3	68.2	68.0
2022	75.2	72.4	71.2	70.3	69.9	65.2
average	67.22	72.51	68.02	67.18	65.22	66.38
Standard deviation	6.05	1.48	5.63	2.22	3.54	1.86
Coefficient of variation(%)	9.00	2.04	8.27	3.31	5.43	2.80
Rank among 113 countries in 2022	23	30	35	38	41	50

Source: Economist Impact, Global Food Security Index: Country Ranking 2021, Retrieved on June 3, 2022, from https://impact economist.com/sustainability/project/food-security-index/index.

Table 4 The food security index score change for the Gulf Cooperation Council countries in 2022 compared to 2012-2021.

.Year	United Arab Emirates	Qatar	Oman	Bahrain	Saudi Arabia	Kuwait
2012	12.0	2.5	13.8	5.6	11.8	-0.5
2013	13.8	2.3	11.8	5.8	8.9	-1.5
2014	13.1	-0.4	6.6	4.4	7.0	-2.0
2015	13.7	0.4	6.8	5.1	4.6	0.7
2016	14.9	0.4	1.1	4.8	5.4	2.1
2017	11.3	-0.6	-0.1	4.2	3.8	0.5
2018	3.6	-0.6	-2.1	0.8	2.6	-2.8
2019	2.3	-1.4	-1.0	0.9	4.9	-3.5
2020	1.5	-1.6	-0.8	1.7	0.8	-3.2
2021	1.6	-2.2	-1.1	1.0	1.7	-2.8

Source: Data in Table 3

3.2 Estimating the proposed model to measure the impact of the Russian-Ukrainian War on food security

The proposed model aims to analyze the impact of the Russian-Ukrainian war on food security in the Kingdom of Saudi Arabia from 2000 to 2022. The study used stepwise multiple regression analysis in both linear and double logarithmic forms, and the results indicated the superiority of the logarithmic equations presented in Table 5 (Gujarati, translated and reviewed by Odeh 2015).

It is evident from the estimated behavioural equations of the proposed model that:

1. A 10% change in each of the following factors: cropped area (X1), agricultural labour (X2), agricultural loans (X3), and the amount of water used for agricultural purposes (X4) results in a corresponding change in the Kingdom of Saudi Arabia's ability to produce food (food production index) by 1.95%, 3.41%, 1.24%, and 2.04% respectively.

Table 5 Estimated behavioural equations for the proposed model during the period 2000-2022

$\text{Ln}\hat{Y}_1 = 0.595 + 0.195\text{Ln}X_1 + 0.341\text{Ln}X_2 + 0.124\ \text{Ln}\ X_3 + 0.204\ \text{Ln}\ X_4\ (2.45)^{*}(3.82)^{**}(3.59)^{**}(2.43)^{*}(4.85)^{*}$ $R^2 = 0.95, F = 85.50, D.W = 1.46, \text{Arch test} = 0.01$
$\text{Ln}\ \hat{Y}_2 = 1.853 - 0.294\ \text{Ln}\hat{Y}_1 + 0.265\ \text{Ln}\ X_5 + 0.915\text{Ln}\ X_6 + 0.559\text{AR}(1)(6.20)^{**}(-2.47)^{*}(2.85)^{*}(5.75)^{**}(3.89)^{**}$ $R^2 = 0.91, F = 64.04\ \ D.W = 1.63, \text{Arch test} = 0.16$
$\text{Ln}\ \hat{Y}_3 = 0.703 + 0.272\text{Ln}\hat{Y}_1 - 0.174\ \text{Ln}\ \hat{Y}_2 + 0.655\text{Ln}\ X_7 + 0.432\text{AR}(1)\ (2.45)^{*}(2.38)^{*}(-4.16)^{**}(3.49)^{**}(2.21)^{**}$

Source: Statistical analysis of the data used in this study.

Table 6 Indicators measuring the efficiency of the estimated equations of the proposed model during the period 2000-2022.

Indicator	First	Second	Third
Root Mean Squared Error	0.081	0.131	0.043
Mean Absolute Error	0.052	0.108	0.032
Mean Abs. Percent Error	0.562	2.930	0.762
Theil Inequality Coef.	0.009	0.051	0.008

Source: Equations of the model estimated in this study.

2. Increasing the estimated food production index (Ŷ1) by 10% leads to a 2.94% decrease in the consumer price index for food in the Kingdom of Saudi Arabia. Additionally, a 10% change in the world food price index (X5) and the total population of the Kingdom of Saudi Arabia (X6) results in a corresponding change in the consumer price index for food by 2.65% and 9.15%, respectively.
3. A 10% change in the country's food production capacity (the estimated food production index) and the real per capita income (X7) leads to a corresponding change in the food security index by 2.72% and 6.55%, respectively. An increase in the estimated food consumer price index (Ŷ2) by 10% leads to a 1.74% decrease in the food security index.

According to the D.W test, the behavioural equations of the proposed model are free from the problem of autocorrelation of the residuals. Furthermore, according to the Arch Test, there is no autocorrelation in the series variance. The estimated model is also characterized by efficiency in representing the data used in the estimation, based on indicators measuring the efficiency of the models, including the U-Theil inequality coefficient, whose value is close to zero (Table 6).

3.3 Predicting the future of food security in the Kingdom of Saudi Arabia until 2030

The future of food security for the Kingdom of Saudi Arabia until 2030 was forecasted by analyzing internal and external variables in a proposed model to measure the impact of the Russian-Ukrainian war on food security. General trend equations were utilized to predict the external variables in Table 7, and their predictive ability was evaluated using the indicators listed in Table 8. It's important to note that the Ministry of Environment, Water and Agriculture has restructured the crop composition, excluding water-depleting

Table 7 Estimated general trend equations for the explanatory variables included in the proposed model from 2000-2022.

variable	F	R^2	equation
Cropped area	27.29	0.57	$\text{Ln}\hat{X}_1 = 7.121 - 0.024T$ $(112.85)^{**}(-5.22)^{**}$
Agriculture labor	24.52	0.71	$\hat{X}_2 = 364.94 + 51.77T - 2.22T^2$ $(8.81)^{**}(6.51)^{**}(-6.94)^{**}$
Agriculture loans	10.27	0.51	$\hat{X}_3 = 1959.25 - 277.84T + 14.04T^2$ $(4.00)^{**}(-2.95)^{**}(3.69)^{**}$
Water quantity used for irrigation	9.53	0.31	$\text{Ln}\hat{X}_4 = 3.038 - 0.023T$ $(29.36)^{**}(-3.04)^{**}$
World food price index	31.29	0.60	$\text{Ln}\hat{X}_5 = 4.083 + 0.035T$ $(47.61)^{**}(5.59)^{**}$
Total population	349.36	0.94	$\text{Ln}\hat{X}_6 = 3.028 + 0.021T$ $(201.32)^{**}(18.69)^{**}$
Real per capita income	84.59	0.80	$\text{Ln}\hat{X}_7 = 4.186 + 0.015T$ $(180.63)^{**}(9.20)^{**}$

**Significant at the 1% probability level (Source: Data contained in this study)

Table 8 Indicators for measuring the efficiency of the general trend equations estimated for the explanatory variables included in the proposed model.

variable	Root Mean Squared Error	Mean Absolute Error	Mean Abs. Percent Error	Theil Inequality Coef.
Cropped area	0.139	0.115	1.72	0.010
Agriculture labor	56.48	43.73	7.79	0.049
Agriculture loans	66.77	46.51	4.61	0.022
Water quantity used for irrigation	0.229	0.185	7.05	0.041
World food price index	0.190	0.168	3.71	0.021
Total population	0.033	0.028	0.863	0.005
Real per capita income	0.051	0.041	0.935	0.006

Source: equations listed in table 7

Table 9 Predictive values of the internal and external variables of the proposed model until 2030

Variable	2024	2025	2026	2027	2028	2029	2030
Cropped area in thousand hectares	534.5	534.5	534.5	534.5	534.5	534.5	534.5
Agriculture labour in thousand	400.0	400.0	400.0	400.0	400.0	400.0	400.0
Agriculture loans in a million riyals	3788.3	4226.4	4692.7	5187.1	5709.5	6260.1	6838.7
The amount of water used in irrigation in billion m3	7.24	7.06	6.89	6.72	6.55	6.37	6.20
World food price index	142.3	147.4	152.6	158.1	163.7	169.5	175.6
Total population in millions of people	34.92	35.66	36.42	37.19	37.98	38.78	39.61
Real individual income in thousand riyals	95.68	97.13	98.59	100.08	101.60	103.13	104.69
Food production index	198.0	199.7	201.3	202.8	204.2	205.3	206.5
Consumer price index for food	129.4	132.8	136.4	140.0	143.7	147.6	151.7
Food security index	72.4	73.0	73.5	74.1	74.6	75.1	75.6

Source: Equations in tables 5, 7

crops, with the most significant being green fodder. As a result, the cropped area decreased to 534.5 thousand hectares in 2022. Therefore, it was assumed that the cropped area and agricultural labour would remain unchanged from 2022 until 2030.

The endogenous variables were predicted by substituting the exogenous variables' predictive values into the proposed model's equations. According to the model, real per capita income is expected to increase from 95.68 thousand riyals in 2024 to 104.69 thousand riyals in 2030. Despite the decline in crop area, which reached 534.5 thousand hectares in 2022 and led to a decrease in the plant production index, it is anticipated that the estimated index for food production will increase from 198.0 in 2024 to 206.5 in 2030. This is due to the state's efforts to develop livestock and increase the animal production index. Despite the impact of the Russian-Ukrainian war on the internal and external variables of the proposed model, the food security index is expected to improve for the Kingdom of Saudi Arabia, increasing from 72.4 in 2024 to 75.6 in 2030 (Table 9).

Conclusion

Due to the outbreak of the Russian-Ukrainian war on February 24, 2022, global and local prices of food products such as meat, dairy, grains, vegetable oils, and sugar have increased. A study of the current food security situation in the Kingdom of Saudi Arabia revealed that the food security environment index increased from 58.1% in 2012 to 69.9% in 2022, with an annual average of 65.2%. This indicates that the food security situation remained moderate during 2012-2022. When comparing the food security environment index among the Gulf Cooperation Council countries, it was found that the State of Qatar ranked first, followed by the Sultanate of Oman, United Arab Emirates, Bahrain, Kuwait, and the Kingdom of Saudi Arabia. The lower level of food security in the Kingdom of Saudi Arabia, compared to the rest of the Gulf Cooperation Council countries, can be attributed to several factors, the most important of which are: (i) the Kingdom of Saudi Arabia has the largest population among the Gulf countries, (ii) a decrease in the average real per capita income of 92.75 thousand riyals compared

to its counterparts in the other Gulf Cooperation Council countries in 2022, (iii) a decrease in the food affordability index of 83.2% compared to its estimated counterpart in the other Gulf Cooperation Council countries, except for the State of Kuwait in 2022, (iv) A decrease in the food availability index of 67.2% compared to its estimated counterpart in the States of Qatar and United Arab Emirates in 2022, (v) a decrease in the food quality and safety index of 71.6% compared to its estimated counterpart in the other Gulf Cooperation Council countries, except for the State of Kuwait in 2022, and (vi) a decrease in the natural resources sustainability and resilience index of 53.7% compared to its estimated counterpart in the United Arab Emirates at 55.2% in 2022. Despite the impact of the Russian-Ukrainian war on the internal and external variables of the proposed model, the food security index is projected to improve for the Kingdom of Saudi Arabia. It is expected to increase from 72.4 in 2024 to 75.6 in 2030 due to the state adopting the Saudi agricultural investment policy abroad and directing local agricultural investments towards vertical expansion, protected agriculture, and good agricultural practices, in addition to achieving significant financial surpluses, leading to an increase in the state's ability to import from abroad.

Acknowledgement

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Ethical Clearance

It is certified that no animal or human model was used during the study, so there is no need for any ethical clearance.

References

Abdel Nabi, M. A. (2022). The Economic Impacts of the Russian-Ukrainian War on the Central Asian Countries. *Asian Horizons Magazine, Arab Republic of Egypt*, *6*, (10), 275-289.

Abdel Shafi, E. (2022). The Russian-Ukrainian War and the Future of the International Order, Al Jazeera Center for Studies, Doha, State of Qatar, May 3, pp. 1-23.

Acevedo, G. L., Nguyen, M. C., Mohamed, N., and& Hoogeveen, J. (2022). How rising inflation in MENA impacts poverty, World Bank, Published on Arab Voices, June 30.

Al-Bashabsheh, A.A.B. (2023). The Impact of the Russo-Ukrainian War on Food and Energy Security. Journal of Al-Quds Open University for Humanities and Social Sciences, *64*, 1-15. DOI: 10.33977/0507-000-064-003.

Central Bank of Saudi Arabia (2022). Annual statistics 2021, publication date 5/31/2022.

Economist Impact (2023). Global Food Security Index, 2023, the GFSI website navigation guide

Economist Impact, Global Food Security Index: Country Ranking. (2021). Retrieved on June 3, 2022, from https:// impact economist.com/ sustainability/project/food- security- index/ Index.

Food and Agriculture Organization. (2024). FAO Food Price Index, website, release date March 8.

Food and Agriculture Organization of the United Nations. (2023). Food Outlook Biannual Report on Global Food Markets, Rome, November 10.

Ghanem, A. M., Alrwis, K.N., Alaagib, S.B.; Aldawdahi, N.M., Ibrahim O. Al-Nashwan, I.O., & Ghanem, H.A. (2023a). Effects of the Russian-Ukrainian war on the value of imports and the food trade balance for the Kingdom of Saudi Arabia. DOI 10.1108/AGJSR- 02-2023-0088.

Ghanem, A. M., Al-Nashwan, O.S., Moneimkamara, S.A., & Ahamada, S.A. (2023b). The impact Russian-Ukrainian war on consumer prices of food products in Saudi Arabia. *International Journal of Food, Nutrition and Public Health,13*, (1- 2), 67- 79.

Gujarati, Damodar N., translated and review by Ghaffar, O.H.A. & Al-Dash, A. A. H. (2015). *Basic Econometrics*, the Second Part, Marekh House of Publishing, Cairo, pp. 983- 986.

Meligy, A., & Salem, N. (2023). (2023). Measuring the expected impacts of the Russian-Ukrainian war on food security in Egypt until 2027: An applied study. *Egyptian Journal of Development and Planning*, *31* (4), 58-86.

Ministry of Planning and International Cooperation of the Republic of Yemen, Economic Studies and Forecasts Sector (2022). Economic and Social Repercussions of the Russian-Ukrainian War on Yemen, Analytical Paper (3), June.

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Extraction of Flavonoids from Parasitic plant *Macrosolen cochinchinensis* using Ultrasound-Assisted Extraction: An Optimization Approach

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KEYWORDS

Macrosolen cochinchinensis

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ABSTRACT

The parasitic plant *Macrosolen cochinchinensis* (Lour.) VAN Tiegh, commonly found parasitizing mango trees, contains flavonoid compounds with potential anticancer properties. This study aims to optimize the extraction of flavonoids from *M. cochinchinensis* using the Ultrasonic Assisted Extraction (UAE) method. Three extraction parameters were investigated to determine the best conditions for maximizing extract yield and flavonoid concentration. The parameters considered for the UAE technique were different ethanol concentrations (30%, 70%, and 96%), extraction times (15, 30, and 45 minutes), and solvent-to-sample ratios (1:10, 1:20, and 1:30). The study used Response Surface Methodology (RSM) to identify the optimal extraction conditions. The analysis using RSM indicated that the highest extraction yield (10%) was achieved with a sample-to-solvent ratio of 1:30, 30% ethanol concentration, and an extraction time of 45 minutes. The highest flavonoid content (457.96 mg QE/g extract) was obtained with a solid-to-liquid ratio between 1:20 and 1:30, using 65 to 80% ethanol solvent and an extraction time of 45 minutes. These results suggest that these parameters extract flavonoid compounds from *M. cochinchinensis* leaves.

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1 Introduction

Mistletoe (*Macrosolen cochinchinensis*) is a hemiparasitic plant from the Loranthaceae Family known for its various bioactivities, including antioxidant, antidiabetic, anticancer, and antihyperglycemic properties (Mutiah et al. 2018; Shin and Lee 2018; Firmata Sari 2020; Kumal et al. 2021). This plant commonly parasitizes trees such as jackfruit (*Artocarpus heterophyllus*), sapodilla (*Manilkara zapota*), and mango (*Mangifera indica*) (Nigam 2022). Previous research focused on four fractions of *M. cochinchinensis* ethyl acetate extracted and isolated from the jackfruit host plant for their anticancer properties. The study revealed that the ethyl acetate extract of *M. cochinchinensis* possesses anticancer activity against the T47D breast cancer cell line, with an IC_{50} of 314.8 µg/mL (Indradmojo 2016). Although a relatively high concentration is required for the anticancer effect, the study suggested that the mechanism involves inhibiting cell cycle phases. The *M. cochinchinensis* extract also induced early apoptosis and disrupted the proliferation of cancer cells (Indradmojo 2016). Further research showed that a combination of *Eleutherine palmifolia* (L.) Merr and *M. cochinchinensis* (Lour.) inhibited cell cycle progression and increased HeLa cell apoptosis (Mutiah et al. 2018). Another study compared the total phenolic and flavonoid compounds in several *M. cochinchinensis* species and reported that this extract contains a total flavonoid content of 24.9 ± 2.3 mg QE/g and a phenolic content exceeding 30 mg GAE/g in the methanolic extract. Additionally, they found that *M. cochinchinensis* exhibits significant DPPH radical inhibition with an IC50 of 65.9 ± 2.8 µg/mL (Kumal et al. 2021).

The interactions between plant parasites and different host species can directly affect the performance of both the host and the parasite. *M. cochinchinensis* contains phytochemical compounds such as terpenoids and flavonoids. Specifically, the terpenoids found in *M. cochinchinensis* are identified as thymol, while the flavonoids are identified as rutin, quercetin, and quercitrin (Santosa et al. 2022).

Flavonoids are a group of secondary metabolites characterized by a three-ring structure responsible for many herbal medicines' colour and therapeutic effects (Iwashina 2000; Samanta et al. 2011). The extraction of flavonoids is challenging due to their wide range of medicinal properties. Several factors, such as the duration of extraction, type and pH of solvents, particle size of the materials, and the selection of extraction methods, may significantly affect the level of obtained flavonoids. Flavonoids possess physicochemical properties that range from polar to nonpolar; therefore, various types of solvents (e.g., water, acetone, methanol, ethanol, or their water mixtures) have been reported for their extraction (Sharma and Janmeda 2017; Chaves et al. 2020). One advanced extraction technique used to extract flavonoids is Ultrasound-assisted extraction. This technique allows researchers to extract flavonoids quickly and with relatively few solvents. Furthermore, it is known to produce higher yields by the capability of ultrasound to induce the breaking of cell walls, enabling the movement of phytochemical compounds from the cell to the solvents. The sound vibration and bubbling during extraction prevent the early saturation of soluble compounds in the solvent, allowing for more compounds to be extracted (Pico 2013; Chemat et al. 2017; Medina-Torres et al. 2017).

Several studies have investigated the best ways to extract flavonoids from plants. For example, Azahar et al. (2017) studied the extraction of flavonoids from *Curcuma zedoaria* using the

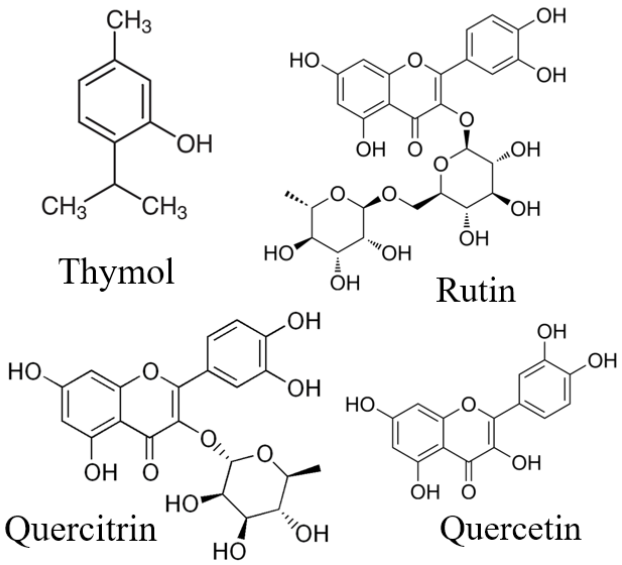

Figure 1 Some flavonoid derivatives reported to be found in *M. cochinchinensis* (Santosa et al. 2022)

reflux method to find the best time and temperature for the process. The results showed that the best conditions for flavonoid extraction were using 90% ethanol at a temperature of 75°C for 92 minutes (Azahar et al. 2017). Similarly, another study found that the best way to extract phenolic, flavonoid and antioxidant compounds from *Clitoria ternatea* was through maceration at 45°C, combined with agitation using 37% ethanol for 90 minutes (Jaafar et al. 2020). These studies demonstrate that the best extraction conditions depend on the plant species, likely due to plant flavonoid composition differences. Therefore, studying the ideal extraction process to maximize flavonoid yield from different plants is important. The present research aims to investigate the best extraction conditions for flavonoids from *M. cochinchinensis* (MC) using a response surface methodology to analyze three parameters: ethanol concentration, extraction time, and sample-to-solvent ratio in the solid state.

2 Materials and Methods

2.1 Plant material

The plant material was gathered from the mango tree, which was the host. The leaves of *M. cochinchinensis* (MC) were dried in an oven for three days, and the resulting dried material was then stored in a tightly sealed container.

2.2 Experimental Design

Respond Surface Methodology (RSM) using Design Expert 7.1.5 and a three-level factorial Box Behnken Design (BBD) was used to find the best extraction conditions for MC. Three independent variables (ethanol concentration, extraction time, and sample-to-solvent ratios) were tested to determine the total flavonoid content (TFC) in *M. cochinchinensis* plant extract. Graphical and numerical optimization techniques were applied to identify the ideal extraction condition. The complete design, with a rotatable alpha, included 20 experimental runs. Six replicate runs were performed at the centre points of the design to estimate the pure error. All experiments were randomly conducted to minimize the influence of unexplained variability in the observed responses due to systematic errors.

2.3 Extraction

The mistletoe leaves, weighing 10 grams, were ground and then extracted with 100 mL of ethanol using the ultrasound-assisted extraction method. Three different concentrations of ethanol (30%, 70%, and 96%) in water were used for the extraction, with three different extraction times (15, 30, and 45 minutes) and three different solid-to-liquid ratios (1:10, 1:20, and 1:30). The extracts were filtered through Whatman no. 40 filter paper. The filtrates were concentrated using a rotary evaporator until a thick extract was obtained (Pan et al. 2012).

2.4 Spectrophotometric determination of total flavonoids

The extracts' total flavonoid content (TFC) was determined using a modified spectrophotometric method with aluminum chloride reagent. The technique was calibrated against quercetin, which served as the reference standard. The TFC was quantified based on a calibration curve prepared by diluting a quercetin stock standard with ethanol p.a to obtain concentrations of 2, 4, 6, 8, and 10 µg/mL. The results were calculated using the calibration curves for quercetin, and the total flavonoids were expressed as milligrams of quercetin equivalents (QE) per 100 grams of extract based on duplicate analysis. The values were reported as means (N=3) ± standard deviations (S.D.).

2.5 Statistical analysis

All analyses were conducted twice, and the results were presented as means ± standard deviation. This study examined the significance of the quadratic model and the interactions between the independent variables using analysis of variance (ANOVA) in the RSM, with a significance level set at $p<0.05$. The experimental R2 assessed in the laboratory was compared to the predicted models.

3 Results and Discussion

3.1 Influence of Different Extraction condition on total yield

Three independent variables, sample-to-solvent ratio, extraction time, and ethanol concentration, were assessed and optimized using the Box-Behnken Design (BBD) to determine the best extraction conditions for maximizing yield and flavonoid content (Table 1). The yield of MC extracts ranged from 1.45% to 8.50%, while the flavonoid content varied from 161.18 mg/g to 430.79 mg/g QE. This study investigated the impact of three independent variables on the total yield and flavonoid content extracted from MC leaves: ethanol concentration, extraction time, and sample-to-solvent ratio. Maximizing extraction yield is essential to meet the standards of Indonesian traditional medicine. Furthermore, optimizing these factors is important for increasing flavonoid content, as different types of flavonoids offer various pharmacological benefits, including cancer prevention, diabetes management, and potent antioxidant properties (Mohan and Nandha Kumar 2014; Batra and Sharma 2013; Abotaleb et al. 2018; Manavi et al. 2021). The models assessed in this study were created using the Box-Behnken Design in MINITAB 18. This software generated 15 models to analyze the effects of ethanol concentration, extraction time, and sample-to-solvent ratio as independent variables. The results of these models are presented in Table 1.

The results in Table 1 indicate that the highest yield achieved was 8.50%. This was obtained under the following conditions: simplicial-

Table 1 The mass fraction of TFC extracted from MC using an Ultrasound-assisted extraction method under different conditions

No.	Sample code	X_1	X_2	X_3	Extract weight (g)	Average of Yield (%)	Average of Flavonoid content (mg/g)
1	A1	-1	1	0	0.66	6.75	341.26±1.45
	A2	-1	1	0	0.69		
2	B1	1	0	1	0.27	2.75	427.37 ± 8.21
	B2	1	0	1	0.28		
3	C1	0	0	-1	0.26	2.35	429.77 ± 11.59
	C2	0	0	-1	0.21		
4	D1	0	-1	-1	0.20	1.90	327.59 ± 23.68
	D2	0	-1	-1	0.18		
5	E1	0	0	-1	0.22	2.15	422.93 ± 8.70
	E2	0	0	-1	0.21		
6	F1	1	1	0	0.44	4.25	333.74 ± 0.48
	F2	1	1	-1	0.41		
7	G1	-1	0	-1	0.61	6.45	293.76 ± 33.83
	G2	-1	0	-1	0.68		
8	H1	0	1	-1	0.34	3.50	402.43 ± 7.73
	H2	0	1	-1	0.36		
9	I1	1	0	-1	0.36	3.35	396.62 ± 0.48
	I2	1	0	-1	0.31		
10	J1	0	-1	1	0.19	1.65	429.08 ± 26.09
	J2	0	-1	1	0.14		
11	K1	0	0	0	0.28	2.55	430.79 ± 10.15
	K2	0	0	0	0.23		
12	L1	0	-1	0	0.16	1.75	161.18 ± 15.46
	L2	0	-1	0	0.19		
13	M1	0	0	1	0.82	8.50	252.07 ± 12.56
	M2	0	0	1	0.88		
14	N1	0	1	1	0.56	5.60	419.86 ± 2.42
	N2	0	1	1	0.56		
15	O1	1	-1	-1	0.18	1.45	316.66 ± 7.25
	O2	1	-1	-1	0.11		

Independent Variables	Code Unit	Coded Variable Level		
		-1	0	1
Ethanol Concentration (%)	X_1	30	70	90
Ratio of simplicial to solvent	X_2	01:10	01:20	01:30
Time (minutes)	X_3	15	30	45

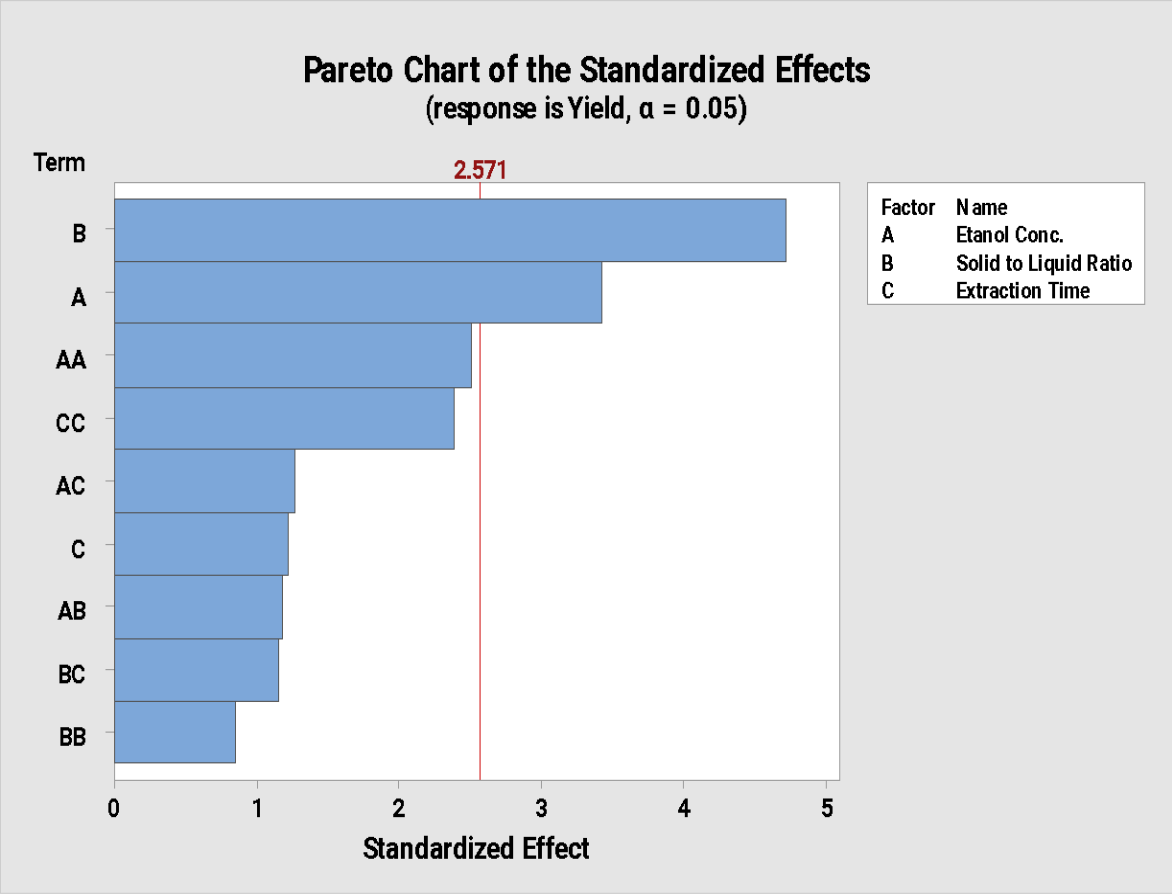

Figure 2 Pareto chart on the effect of ethanol concentration, extraction time, and simplicial: solvents ratio to % yield of MC leaves

solvent ratio of 1:20, 45-minute extraction time, and 30% ethanol concentration as the solvent. This suggests that the extract contains a significant number of hydrophilic compounds that are highly soluble in solvents with a higher water concentration. On the other hand, the lowest yield was 1.45%, obtained from the model with a simplicial-solvent ratio of 1:10, 30-minute extraction time, and 96% ethanol as the solvent. This result shows that a low solid-liquid ratio and a high ethanol concentration produced less extract.

The yield data was analyzed using the MINITAB application to assess the impact of the simplicial-solvent ratio, extraction time, and ethanol concentration on the yield percentage. The resulting Pareto chart is depicted in Figure 2.

Figure 2 indicates that the solid-liquid ratio and ethanol concentration parameters have a standardized effect value of 2.571, suggesting that they significantly influence the yield percentage from MC leaf extraction.

The relationship between the independent variables and the yield was shown in contour plots created by the model (Figure 3). The dark green colour on the plot indicates the interactions between the variables being tested. Figure 3a presents the impact of the solid-liquid ratio and ethanol concentration on the yield of MC extracts. It was observed that an increase in the solid-liquid ratio and the water content of the solvent (30% ethanol) led to a rise in yield, indicated by the dark green region. This result is consistent with Prasad et al. (2012), who found that a higher yield is achieved with a higher solid-to-liquid ratio and water concentration in ethanol. A solid-liquid ratio between 1:20 to 1:30 and 30% ethanol concentration resulted in a 6-7% yield range. Additionally, Figure 3b demonstrates the effect of extraction time and ethanol concentration on MC extract yield. It can be seen that a longer extraction time and higher water content of the solvent (30% ethanol) increased yield. Extraction times exceeding 40 minutes and using 30% ethanol as the solvent led to a 6-7% yield range. This finding aligns with other research that reported that more polar solvents and longer extraction times result in an optimal yield (Nawaz et al. 2018). Furthermore, Figure 3c shows the correlation between extraction time, solid-liquid ratio, and the yield of MC extract. This figure illustrates that increased extraction time and solid-liquid ratio led to a higher extract yield. The results confirm an increased contact period between the sample and solvents leads to more compounds being extracted.

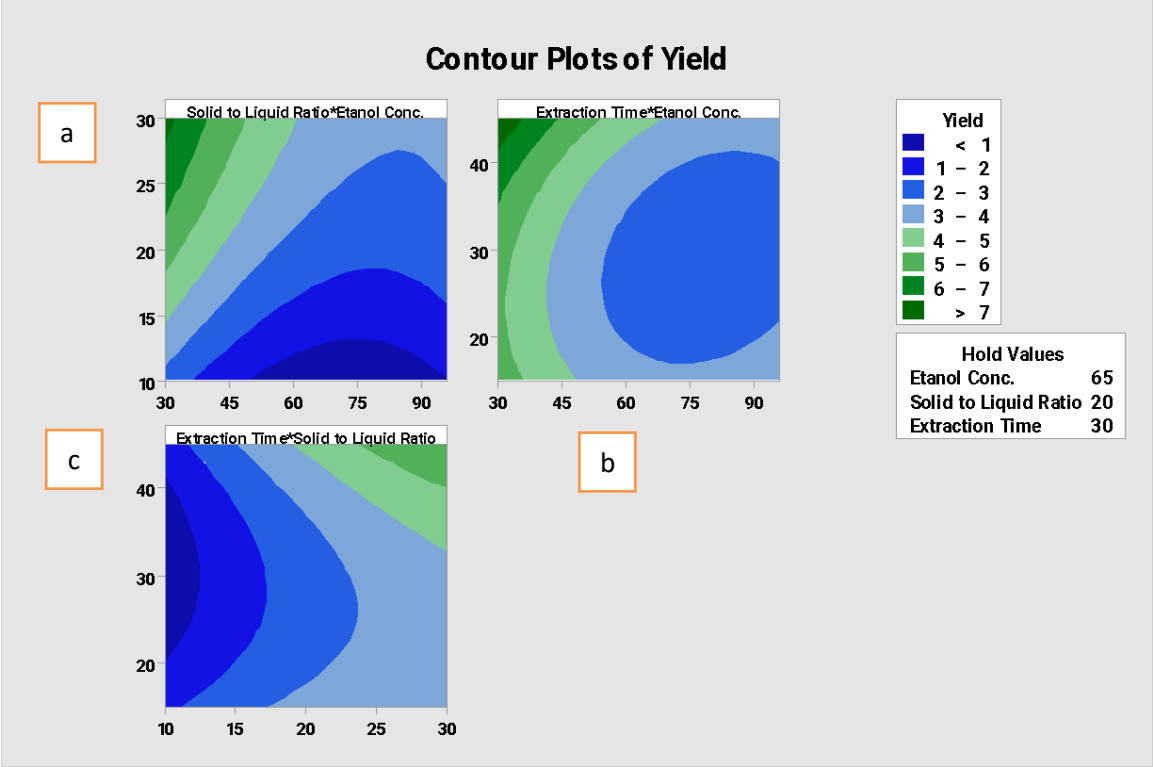

Figure 3 Counterplots of the correlation between the independent variables (including solid-liquid ratio, extraction time, and ethanol concentration) and MC extracts yield

3.2 Influence of different extraction conditions on total flavonoids

The ultrasonic-assisted extraction method successfully extracted a high concentration of flavonoids from MC. However, the flavonoid content varied in each extract due to different extraction conditions, such as ethanol concentration, extraction time, and solvent ratios. To improve extraction efficiency, in this study, different ethanol concentrations (30%, 70%, and 95%), extraction times (15, 30, and 45 minutes), and solvent ratios (1:10, 1:20, and 1:30) have been used. Furthermore, Response Surface Methodology (RSM) was used to evaluate the total flavonoids of MC and fitted all the independent variables into a second-order model equation.

Flavonoid content = -403 + 13.21 X1 + 33.67 X2 – 1.95X3 – 0.0814 X1X1 – 0.437 X2X2 + 0.0470 X3X3 – 0.1311 X1 X2 + 0.0426 X1 X3 – 0.140 X2 X3

Where X1 is the ethanol concentration, X2 is the solid-to-liquid ratio, and X3 is the extraction time.

The model was found to be significant, with a p-value of 0.01. The lack of fit value was 0.011 ($p < 0.05$), indicating significance. The significant models included X1, X2, X1X2, X1X1, and X2X2 with p-values < 0.05.

Furthermore, the concentration data for flavonoids was analyzed using the MINITAB application to assess the impact of solid-to-liquid ratio, extraction time, and ethanol concentration on flavonoid content. The Pareto chart obtained is depicted in Figure 4.

In Figure 4, it is evident that the interactions between ethanol concentration and solid-liquid ratio, and ethanol concentration and extraction time, as well as solid-liquid ratio and extraction time, substantially affect the flavonoid content extracted from MC. The reported value of 2.571 confirms the significant impact of these parameter interactions. However, it was observed that the extraction time does not significantly impact the flavonoid content.

The study investigated the relationship between the independent variables and the flavonoid content of MC extract using surface and contour plots generated by the model (Figure 5). The contour plot shapes (ellipse or round) indicated the significance of interactions between the variables being tested. Figure 5a illustrates the effect of solid-to-liquid ratio and ethanol concentration on the flavonoid content of MC extracts. The round shape in green indicates that solid-to-liquid ratios between 1:20 to 1:30 and ethanol concentrations between 50% to 90% resulted in a high flavonoid content (400 - 450 mg/g QE). This finding is consistent with Prasad et al. (2012), who reported that a 68% ethanol concentration and a liquid-to-solid ratio of 20.2 mL/g resulted in

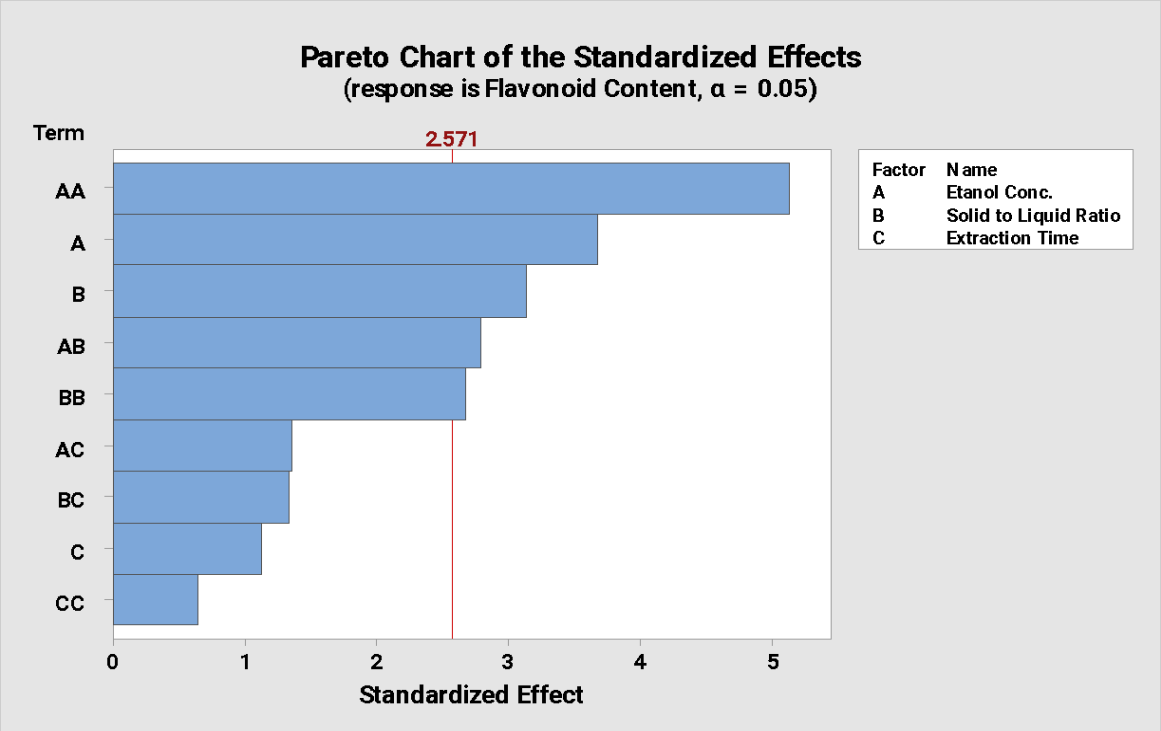

Figure 4 Pareto chart on the effect of ethanol concentration, extraction time, and solid-to-liquid ratio on flavonoid content of MC leaves

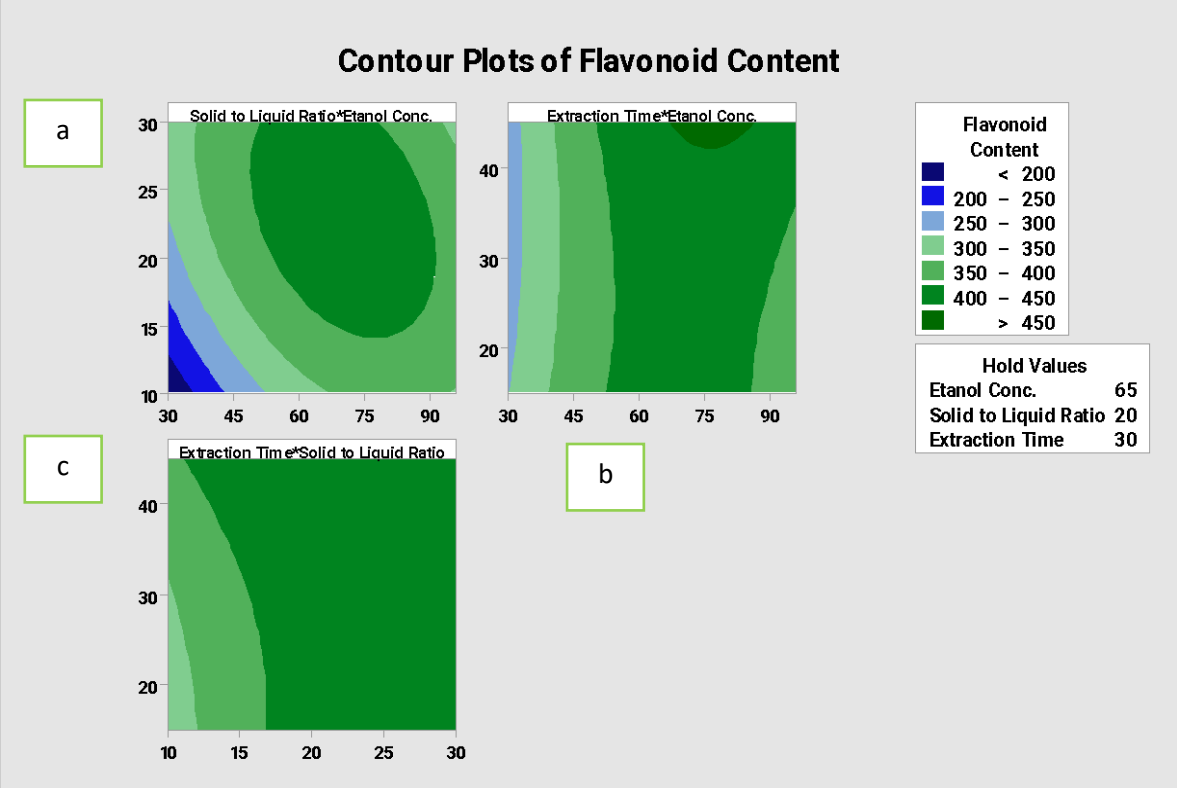

Figure 5 Counter plots of the correlation between the independent variables (including solid-to-liquid ratio, extraction time and ethanol concentration) and the flavonoid content of MC extracts

optimal flavonoid content. Additionally, Figure 5b shows the effect of extraction time and ethanol concentration on the flavonoid content of MC extract. This figure indicates that ethanol concentrations between 65% and 80% and extraction times exceeding 40 minutes resulted in a flavonoid content of more than 450 mg/g QE. This confirms that prolonged extraction time and a mixture of ethanol and water as the solvent increase the flavonoid content of the extract. This result is in line with Pan et al. (2012), who reported that 72% ethanol and 1.5 hours of extraction time resulted in the optimal extraction of flavonoid content from hawthorn seed extract. Figure 5c demonstrates the effect of extraction time and solid-to-liquid ratio on the flavonoid content of MC extract. This figure indicates that increasing the solid-to-liquid ratio (1:20 to 1:30) resulted in a higher flavonoid content (400-450 mg/g QE). The result confirms that increasing the solid-to-liquid ratio increases the amount of compound extracted. However, further confirmation experiments of these optimal parameters are still needed to understand the actual results better and achieve the optimum conditions for flavonoid extraction from MC leaves.

Conclusion

The study found that Ultrasound-assisted extraction (UAE) was effective and cost-effective in increasing the extract yield and flavonoid content from MC leaves. The optimal conditions for UAE of flavonoid content from MC leaves, as determined by Response Surface Methodology (RSM), were a solid-to-liquid ratio of 1:20 to 1:30, an ethanol concentration of 65-80%, and an extraction time of more than 45 minutes. This research also highlighted that MC can be considered one of the best sources of flavonoids.

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Ethical Clearance

It is confirmed that no animal or human model was utilized in the study; therefore, no ethical clearance is necessary.

Conflict of interest

The authors declare no conflict of interest in this study.

References

Abotaleb, M., Samuel, S. M., Varghese, E., Varghese, S., Kubatka, P., Liskova, A., & Büsselberg, D. (2018). Flavonoids in Cancer and Apoptosis. *Cancers*, *11*(1), 28. https://doi.org/10.3390/cancers11010028

Azahar, N. F., Gani, S. S. A., & Mohd Mokhtar, N. F. (2017). Optimization of phenolics and flavonoids extraction conditions of Curcuma Zedoaria leaves using response surface methodology. *Chemistry Central journal*, *11*(1), 96. https://doi.org/10.1186/s13065-017-0324-y

Batra, P., & Sharma, A. K. (2013). Anticancer potential of flavonoids: recent trends and future perspectives. *3 Biotech*, *3*(6), 439–459. https://doi.org/10.1007/s13205-013-0117-5.

Chaves, J. O., de Souza, M. C., da Silva, L. C., Lachos-Perez, D., Torres-Mayanga, P. C., Machado, A. P. D. F., Forster-Carneiro, T., Vázquez-Espinosa, M., González-de-Peredo, A. V., Barbero, G. F., & Rostagno, M. A. (2020). Extraction of Flavonoids From Natural Sources Using Modern Techniques. *Frontiers in chemistry*, *8*, 507887. https://doi.org/10.3389/fchem.2020.507887.

Chemat, F., Rombaut, N., Sicaire, A. G., Meullemiestre, A., Fabiano-Tixier, A. S., & Abert-Vian, M. (2017). Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics sonochemistry*, *34*, 540–560. https://doi.org/10.1016/j.ultsonch.2016.06.035.

Firmata Sari, A. A. (2020). *Isolasi Dan Karakterisasi Komponen Utama Dari Ekstrak Daun Benalu (Macrosolen cochinchinensis (Lour.) Van Tiegh) Dengan Inang Pohon Mangga (Mangifera Indica L.)*. [Doctoral thesis, Hasanuddin University, Makassar Indonesia].

Indradmojo, C. (2016) Aktivitas Antikanker Dan Mekanisme Farmakologi Ekstrak Dan Fraksi Benalu Nangka (MacrosolenCochinchinensis) Pada Sel Kanker Payudara T47D.

Iwashina, T. (2000). The Structure and Distribution of the Flavonoids in Plants. *Journal of Plant Research*, *113*(3), 287-299.

Jaafar, N. F., Ramli, M. E., & Mohd Salleh, R. (2020). Optimum Extraction Condition of *Clitorea ternatea* Flower on Antioxidant Activities, Total Phenolic, Total Flavonoid and Total Anthocyanin Contents. *Tropical life sciences research*, *31*(2), 1–17. https://doi.org/10.21315/tlsr2020.31.2.1

Kumal, K., Pant, D. R., Aryal, B., Tripathi, G. R., & Joshi, G. P. (2021). Phytochemical and Antioxidant Properties Of Traditionally Used Mistletoes In Nepal. *Scientific World*, *14*(14), 83–89. https://doi.org/10.3126/sw.v14i14.34999.

Manavi, S. P., Amiri, T., & Mozafaryan, M.J. (2021). Role of Flavonoids in Diabetes. *Journal of Reviews in Medical Sciences*, *1*(3), 149–61.

Medina-Torres, N., Ayora-Talavera, T., Espinosa-Andrews, H., Angeles Sánchez-Contreras, A., & Pacheco, N. (2017). Ultrasound Assisted Extraction for the Recovery of Phenolic Compounds from Vegetable Sources. *Agronomy*, *7*(3), 47. https://doi.org/10.3390/agronomy7030047

Mohan, S., & Nandhakumar, L. (2014). Role of Various Flavonoids: Hypotheses on Novel Approach to Treat Diabetes. *Journal of Medical Hypotheses and Ideas*, *8*(1), 1–6.

Mutiah, R., Anik, L., Arief, S., Annisa, R., Hakim, A., Anggraini, W., Susilowati, R. (2018) Activity of inhibit of the cell cycle and induct apoptosis in HeLa cancer cell with combination of Sabrang onion (*Eleutherine palmifolia* (L.) Merr) and Starfruit Mistletoe *Macrosolen cochinchinensis* (Lour.) Tiegh extracts. *Journal of Applied Pharmaceutical Science* 8(10), 122–28.

Nawaz, H., Shad, M. A., & Rauf, A. (2018). Optimization of extraction yield and antioxidant properties of Brassica oleracea Convar Capitata Var L. leaf extracts. *Food chemistry*, *242*, 182–187. https://doi.org/10.1016/j.foodchem.2017.09.041

Nigam, Y. P. (2022). The Bornean Mistletoes as Versatile Parasites: A Systematic Review. *Systematic Reviews in Pharmacy*, *13*(1), 42–47.

Pan, G., Yu, G., Zhu, C., & Qiao, J. (2012). Optimization of ultrasound-assisted extraction (UAE) of flavonoids compounds (FC) from hawthorn seed (HS). *Ultrasonics sonochemistry*, *19*(3), 486–490. https://doi.org/10.1016/j.ultsonch.2011.11.006

Pico, Y. (2013). Ultrasound-Assisted Extraction for Food and Environmental Samples. *TrAC Trends in Analytical Chemistry*, *43*, 84–99. DOI: https://doi.org/10.1016/j.trac.2012.12.005.

Prasad, K. N, Weng Kong, K., Ramakrishnan Nagasundara Ramanan, R.N., Azlan, A., & Ismail, A. (2012). Determination and Optimization of Flavonoid and Extract Yield from Brown Mango Using Response Surface Methodology. *Separation Science and Technology*, *47*(1), 73–80.

Samanta, A., Das, G., & Das, S.K. (2011). Roles of Flavonoids in Plants. *Carbon*, *100*(6), 12–35.

Santosa, D., Widyastuti, S. M., Rosyidah, U., & Silmia, B. (2022). Detection of Terpenoid and Flavonoid of Five Species of Mistletoes at *Stelechocarpus burahol* (Bl.) Hook. f. & Th and *Lagerstroemia speciosa* (L.) Pers. by Using Thin Layer Chromatography Method. In A. Linggawati, A. A. P. Anfa, S, A. Linatoc, C. K. Fusianto, D. Lawrie, et al. (Eds.) *7th International Conference on Biological Science (ICBS 2021)* (pp. 388–393). Atlantis Press. DOI: https://doi.org/10.2991/absr.k.220406.055.

Sharma, V., & Janmeda, P. (2017). Extraction, Isolation and Identification of Flavonoid from Euphorbia Neriifolia Leaves. *Arabian Journal of Chemistry*, *10*(4), 509–14.

Shin, H. W., & Lee, N. S. (2018). Understanding plastome evolution in Hemiparasitic Santalales: Complete chloroplast genomes of three species, Dendrotrophe varians, Helixanthera parasitica, and Macrosolen cochinchinensis. *PloS one*, *13*(7), e0200293. https://doi.org/10.1371/journal.pone.0200293

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Discovery of Active Antibacterial Fractions of Different Plant Part Extracts of clove (*Syzigium aromaticum*) Against *Streptococcus mutans*

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Antibacterial

Cloves

Dental caries

Syzigium aromaticum

Streptococcus mutans

ABSTRACT

Nowadays, dental cavities caused by *Streptococcus mutans* are a major focus of research in Indonesia. While several antibiotics are available to combat this bacterium, concerns about antibiotic resistance have prompted researchers to explore natural remedies. Clove (*Syzigium aromaticum*) is a commonly studied natural remedy against dental cavities and S. mutans. Among the different parts of the clove plant, clove bud is the most widely used against dental cavities or *S. mutans*, and the potential of other clove parts has not been thoroughly explored. Identifying which parts of the clove plant have higher concentrations of active ingredients and exhibit the strongest antibacterial activity is important. Therefore, this study evaluated the antibacterial activity of three different parts, i.e., leaf, stems, and buds of the clove plant ethanolic extracts against *S. mutans*. The ethanolic extracts of clove leaf, stems, and buds were prepared using the maceration method with 70% ethanol, and their activity against *S. mutans* was tested using the disc diffusion method at three different concentrations (10%, 5%, 2.5% b/v). Fractionation was carried out using hexane and water to obtain two fractions: hexane and water fraction. These fractions were then subjected to antibacterial assays. The ethanolic leaf, stems, and bud extracts exhibited varying antibacterial activity levels. The best activity was observed with the 10% clove bud ethanolic extract, which produced an inhibition zone of 20.83 ± 0.77 mm. The leaf and stem extracts showed inhibition zones of 16.38 ± 3.84 mm and 17.95 ± 5.15 mm, respectively. Furthermore, the hexane-soluble fraction of the clove bud displayed the highest activity with an inhibition zone diameter of 23.7 ± 3.21 mm at 10%. This activity was twice as high as ampicillin, used as the positive control. In conclusion, clove bud remains the best source of antibacterial compounds against *S. mutans*.

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Fractionation of the bud extract using hexane can significantly enhance its activity. Further investigation should be conducted to optimize the effectiveness of this active fraction for use as an anti-dental caries treatment.

1 Introduction

Dental caries (DC) is an oral infection characterized by tooth decay, primarily caused by *S. mutans*, and is a highly prevalent disease among children. According to the International Caries Detection and Assessment System (ICDAS), the prevalence of caries exceeds 90% among children aged 6-12 years (Aripin et al. 2024). DC affects both children and adults, targeting primary and permanent teeth. The starch and sugar residue around teeth are fermented by microorganisms, forming acids that cause the decalcification of enamel and dentin, ultimately resulting in caries (Sivapathasundharam and Raghu, 2020).

Pharmacological treatment and prevention of dental caries range from the use of anti-cariogenic compounds such as fluoride, chlorhexidine, or xylitol to antibiotics such as amoxicillin, amoxicillin-clavulanate, clindamycin, cephalexin, and metronidazole (Cui et al. 2019; Qiu et al. 2020; Ahmadi et al. 2021). However, several studies have reported the development of resistance in *S. mutans* to various anti-cariogenic compounds (Lee et al. 2012; Liao et al. 2017; Cieplik et al. 2019), as well as resistance to most commonly used antibiotics (Haque et al. 2019). Furthermore, antibiotics might disrupt the balance of the oral microflora community or cause side effects such as allergies, nephritis, and gastrointestinal disorders (Cheng et al. 2022; Heta and Robo 2018).

When treating dental cavities, natural compounds could be used as an alternative to antibiotics. Several studies have demonstrated that a wide range of natural compounds exhibit antibacterial activity against the growth of *S. mutans* (Dwivedi and Singh 2015; Jacob and Nivedhitha 2018; Folliero et al. 2022). The essential oil from the clove plant *S. aromaticum* is often empirically and clinically used. A previous study by Moon et al. (2011) combined ampicillin and clove oil and found that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were decreased approximately fourfold against *S. mutans*, resulting in a synergistic influence which accounted for a fractional inhibitory concentration index (FICI) of less than 0.5. Another study revealed a synergistic activity of eugenol, clove oil, and clove methanol extract in combination with azithromycin to inhibit biofilm formation resulting from the growth of *S. mutans* (Jafri et al. 2021). Clove oil showed a strong inhibitory effect as fluoride-free toothpaste against *S. mutans* and its biofilm (Dhamodhar et al. 2014; de Oliveira Carvalho et al. 2020).

Nurdjannah et al. (2016) evaluated phytochemical research on the clove plant and found that the highest concentrations of clove essential oil were found in clove buds (10-20%), followed by the stems (5-10%) and leaf (1-4%). In another study, steam distillation of clove resulted in different concentrations of phytochemical compounds from buds, leaf, and stem parts, and it was found that the clove leaf contained a higher percentage of eugenol (82.97%) than the buds (75.30%). However, stems contain the highest amount of eugenol (97.75%). Furthermore, the antibacterial activity test of those three parts showed that the stem possesses the highest inhibitory zone (15.05± 0.200) against *Escherichia coli*, followed by *Salmonella typhimurium* (13.67 ± 0.764) (Sohilait et al. 2018; Mak et al. 2019). The results indicate the potential for other parts of the clove plant to be utilized since clove buds take about four years to grow (Cortés-Rojas et al. 2014). Therefore, this research was carried out to evaluate the potential activity of stems, leaves, and buds extracted from clove plants.

Additionally, the most active part of the plant extract will be subjected to fractionation to separate the polar and nonpolar compounds. This process aims to obtain the most active fraction. In a previous study, Mann (2012) described three different fractions (hexane, chloroform, and methanol fraction) of clove basil (*Ocimum gratissimum*) and revealed varying antimicrobial activities. The hexane soluble fraction exhibited the highest inhibitory activity against *Niesseria gonorrheae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella penumoniae*, and *Vibrio cholerae* compared to the other fractions. This demonstrates the significant benefits of fractionating and separating an active extract to explore the more concentrated active compounds from the plant extract (Silvestre et al. 2009).

This study compared the antibacterial activity of a 70% ethanol extract of clove leaves, stems, and buds against *S. mutans*. Subsequently, the active extract was fractionated, and its antibacterial activity against *S. mutans* was investigated. The results of this study were expected to address the need for active fractions in treating diseases caused by resistant microorganisms, such as dental caries.

2 Materials and Methods

2.1 Sample Preparation

The cloves used in this study were obtained from Sadar Village, Bone Regency, South Sulawesi, Indonesia (Figure 1). The buds, leaf, and stems were dried in an oven at 60°C for three days and then crushed using a blender to obtain the samples.

Figure 1 Figure illustrates Different Parts of the Clove Plant

2.2 Sample Extraction

Two hundred grams of coarse powder from clove buds, leaves, and stems were soaked in 70% ethanol (1:10 ratio). The soaking lasted three days with occasional stirring, and then the mixture was filtered through filter paper. The residue underwent two additional soaking processes. The resulting extracts were evaporated using a Buchi® rotary evaporator until they became thick and viscous. The extracts were then stored in separate amber vials and weighed to determine their yield percentage (Rasul 2018).

2.3 Antibacterial Assay of Clove Extracts

The antibacterial assay was conducted using the agar diffusion method outlined in CLSIM100-S22 (Clinical Laboratory Standard Institute 2012) with some adjustments. Before the assay, *S. mutans* ATCC®25175 was cultured for 24 hours in a Nutrient Agar medium (Merck®). Afterwards, the bacteria were suspended in saline solution and adjusted to the turbidity of the McFarland 0.5 standard (Himedia®) (equivalent to approximately $1.5x10^8$ CFU/mL). Ethanol extracts of clove buds, leaves, and stems were dissolved in 10% DMSO (Merck®) and diluted to obtain various concentrations (10%, 5%, 2.5% v/v). Twenty microliters of the extracts were transferred onto blank disks (Oxoid®) and then dried in a desiccator for 15-30 minutes. A swab of bacterial suspension was spread on the surface of the Mueller Hinton Agar medium (Merck®), followed by the placement of the extract-containing disks. An Ampicillin disk (Oxoid®) served as the positive control, while a disk containing 10% DMSO was used as the negative control. Each assay was carried out in triplicate. The plates were then incubated at 37°C for 24 hours, and the diameter of the inhibition zone was observed and measured using a calliper (Krisbow®). The extract demonstrating the highest activity was subjected to liquid-liquid extraction.

2.4 Liquid-liquid Extraction and Antimicrobial Assay of Fractions

Based on the results of the antibacterial assay, the ethanol extract of clove bud exhibited the most potent activity in inhibiting the growth of *S. mutans*. As a result, this extract underwent further fractionation to separate the polar and nonpolar compounds. Hexane and water (1:1) were solvents to obtain the hexane and water fractions. The obtained fractions were concentrated using a rotary evaporator and air-drying using a fan and water bath (Abubakar and Haque 2020). The dried extract was then subjected to an antibacterial assay using the same method as described in the previous section

2.5 Qualitative Screening of Phytochemical Compounds of Clove Extracts and Fractions

A small quantity of clove extracts, fractions, and eugenol marker was placed into vials, dissolved using acetone, and then spotted on a silica (Merck®) thin-layer chromatography plate. The TLC plate was placed as the eluent in a toluene acetone chamber (3:1). After elution, the plate was dried and observed under a 254 nm UV lamp. The spots were visible after spraying with an anisaldehyde-sulfuric acid reagent (Kumar et al. 2010).

2.6 Statistical analysis

All assays were performed in triplicate, and the data was presented as the mean of three independent experiments ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to

Table 1 Extraction Result of Various Clove Plant Pats

Sample	Sample (g)	Average of Extract (g)	Average of %Yield
Buds	200	19.3±0.04	9.65
Leaf	200	18.9±0.08	9.45
Stems	200	17.8±0.122	8.9

Data are the mean of three replicates, ± Standard Deviation

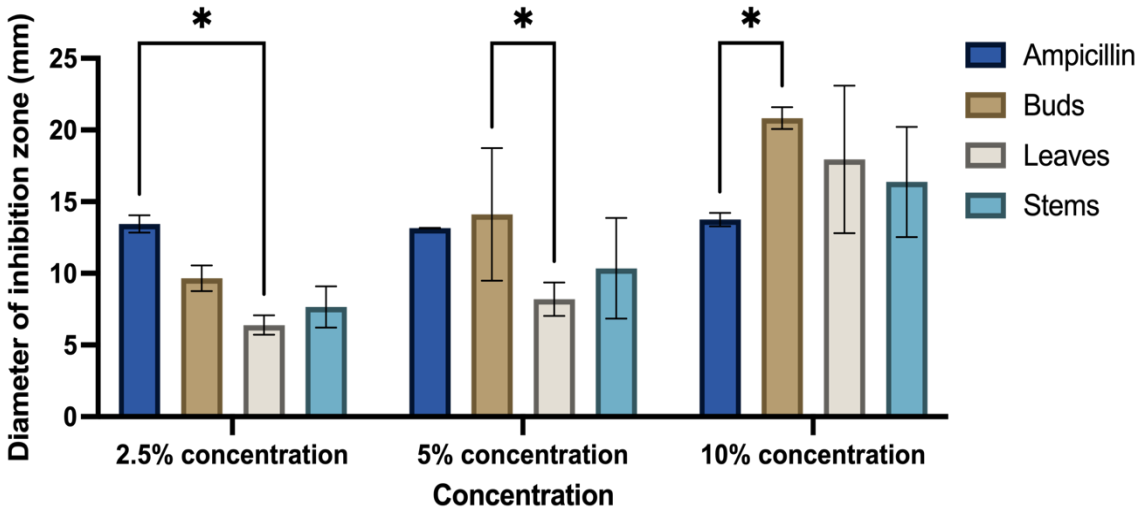

Figure 2 Correlation between plant parts and antibacterial activity of its extracts (mean ± SD, n = 3), significance levels are conceived by stars: * = significant (P < 0.05)

compare the antibacterial activities of individual extracts and fractions, followed by the Tukey HSD test.

3 Results

3.1 Extracted Sample Yield

The quantification results for the extract weight and yield percentage are outlined in Table 1. The findings indicated that the highest extract amount was obtained from clove buds (9.65%), followed by the leaf (18.9%) and stem (17.8%) extract. However, this difference was not significant (Table 1).

3.2 Antibacterial Assay of Clove Extracts

The disk diffusion method is commonly used to test the antibacterial activity of plant extracts. This method measures the diameter of the clear zone that forms around a sample after it has been incubated with bacteria. Different plant extracts may inhibit bacterial growth to varying degrees.

The study found that the bud extract of clove at a concentration of 10% showed the highest activity, with an inhibition zone diameter of 20.83 ± 0.77 mm. This was followed by the clove leaf extract (16.38 ± 3.84 mm) and the stem extract (17.95 ± 5.15 mm). The activity of these extracts varied at different concentrations (Figure 2).

Different plant parts may possess different antibacterial potency. Figure 3 shows the difference in the clear zone size of each plant part of the clove. It can be observed that clove bud extract possesses the widest clear zone compared to positive control (ampicillin), stem extract, and leaf extract.

3.3 Liquid-liquid Extraction and Antimicrobial Assay of Fractions

Out of the three tested extracts, the clove bud extract exhibited the highest inhibitory activities and was thus selected for further fractionation using the liquid-liquid extraction (LLE) method. This process resulted in two fractions: water and hexane. Both fractions underwent antibacterial assays to confirm their potency in inhibiting the growth of *S. mutans*. The results of the antibacterial activity test revealed a significant difference between the two fractions (p-value < 0.001), with the hexane fraction exhibiting the highest inhibitory activity (23.70±3.22 mm). This indicates that the nonpolar compounds in the clove bud have a strong activity in inhibiting the growth of *S. mutans*. When the concentration of the hexane fraction was increased from 2.5% to 5% and 10%, the diameter of the clear zone also increased to 9.66 ± 0.9 mm, 13.4 ± 4.28 mm, and 20.83±0.77 mm, respectively. Figure 4 illustrates the significant difference in antibacterial activity between each experiment. Notably, at a concentration of 10%, the hexane fraction exhibited

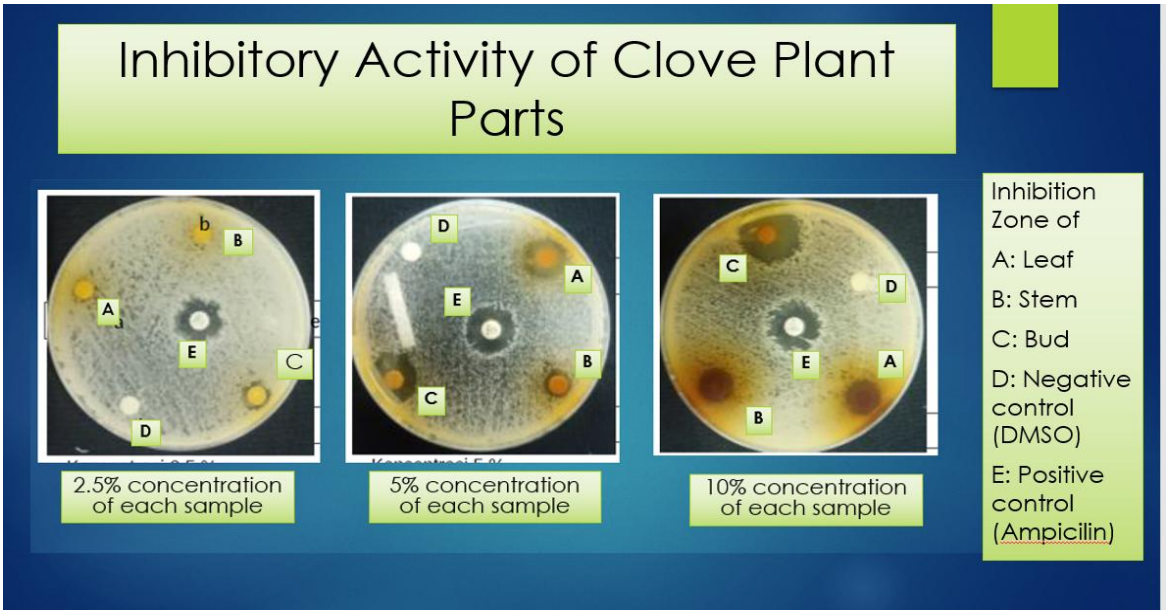

Figure 3 Inhibition activities of different extracts against *S. mutans*

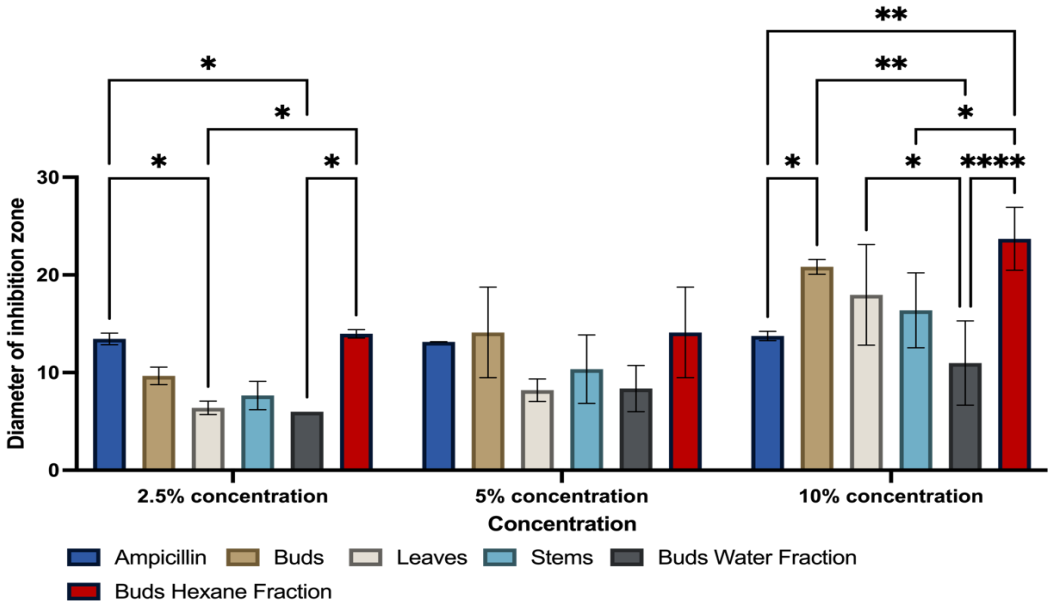

Figure 4 Correlation between plant parts and antibacterial activity of its extracts (mean + SD, n = 3), the Significance grade obtained from two-tailed t-tests are conceived by stars: * = significant ($P < 0.05$), ** = highly significant ($P < 0.01$), *** = very high significant ($P < 0.001$).

higher activity compared to the water fraction and the positive control (ampicillin) with $p< 0.001$ and $p< 0.01$, respectively.

3.4 Qualitative Screening of Phytochemical Compounds of Clove Extracts and Fractions

The results of the phytochemical screening using TLC (thin-layer chromatography) showed that the extracts and fractions contained eugenol. This was indicated by a dark blue spot at a similar Rf value with eugenol when observed at UV 254 (Figure 5). All extracts and fractions contain eugenol with an Rf value of 0.8, which aligns with the eugenol standard. This finding is supported by Pathak et al. (2004), who reported that eugenol was detected with TLC densitometry using toluene: ethyl acetate: formic acid (3:2:0.4) with an Rf value of 0.77. However, in the hexane fraction of clove bud extract, a prominent spot was observed under the eugenol marker, indicating the presence of compounds other than eugenol in the extract with high concentrations.

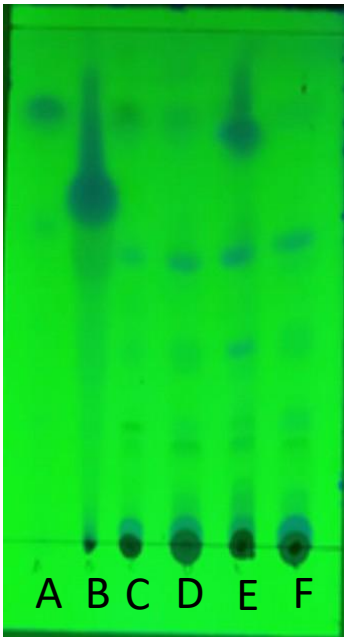

Figure 5 Chromatogram of *S. aromaticum* plant part extracts: (a) eugenol, (b) hexane fraction of clove bud extract, (c) clove leaf extract, (d) clove stem extract, (e) clove bud extract, (f) water fraction of clove bud extract

4 Discussion

4.1 Sample Extraction

This study used the maceration method to extract substances from the cloves plant's buds, leaves and stems. Maceration is an extraction process that dissolves plant substances into solvents. It has several advantages: it is easy to do, requires simple equipment, and can safely extract thermolabile compounds (Rasul 2018). The early extraction process used 70% ethanol to obtain polar and nonpolar compounds from clove plant parts (Sucipto et al. 2022). Ethanol was chosen because it is self-preservative at a concentration above 20%, nontoxic at low concentrations, and requires minimal heat to concentrate the extract (Abubakar and Haque 2020). Additionally, ethanol can prevent the growth of fungi and bacteria in the extract and has an excellent performance of diffusing into plant cell walls and dissolving almost all phytochemical compounds (Utami and Putri 2020). Previous research suggested that the eugenol content in clove extract with ethanol was approximately 87.18%, higher than in clove extracted with n-hexane solvent (76.30%). This study obtained the highest extract yield (9.65%) from the clove bud. The percentage of extract yield can be influenced by the extraction method and the timing of harvesting plant parts (Sulaiman et al. 2015).

4.2 Antibacterial Assay of Clove Extracts

The clove bud extract exhibited the highest inhibition zone against *S. mutans* growth at a concentration of 10%, with a diameter exceeding 20 mm, indicating strong antibacterial activity. There was a significant difference ($p < 0.05$) in the diameter of the inhibition zone between ampicillin (used as the positive control) and the clove bud extract, suggesting that the clove bud extract was highly effective in inhibiting the growth of *S. mutans* bacteria and could be a potential antibacterial agent. The positive control showed a zone of inhibition of less than 14 mm, indicating that the bacteria likely developed resistance to ampicillin (Clinical Laboratory Standard Institute 2012).

While there was no significant difference in the clove plant part at a concentration of 10%, a significant difference was observed between the clove bud and leaf extract at a concentration of 5%. Previous research has shown that 1% essential oil of clove leaf possesses inhibition against *S. mutans* (22.1 ± 1.55 mm). Various studies have reported that the clove bud of *S. aromaticum* has antibacterial and antibiofilm activity against *S. mutans*. Gupta and Prakash (2021) reported intense activity of clove flower extract and clove oil (15-17 mm) against *S. mutans*. In another study, clove bud extract inhibited the growth of multi-drug-resistant *S. mutans* isolated from dental plaque, with an inhibition zone diameter of > 20 mm (Gupta and Prakash 2021). Specifically, the clove extract was reported to be able to damage the cell membrane of *S. mutans* (Suhendar and Sogandi 2019).

4.3 Liquid-liquid Extraction and Antibacterial Assay of Fractions

In this study, clove bud extract was fractionated using the liquid-liquid extraction method and then subjected to an antibacterial activity assay to determine whether the active compound was polar

or nonpolar. The results showed that the compound of clove bud dissolved in the hexane fraction had higher antibacterial activity and was reported to be twice as high as ampicillin. Statistical analysis showed that the hexane fraction significantly differs from ampicillin ($p < 0.05$). Furthermore, the hexane fraction expresses significantly different activity ($p < 0.001$) from the water extract. In previous research, the hexane extract of *Zanthoxylum piperitum* seed also expressed antibacterial activity against *S. mutans* with an inhibitory zone of 15.6 mm. This is higher than the methanol and ethyl acetate extracts, which had 11.6 mm and 13.2 mm, respectively (Park et al. 2008). Based on phytochemical compounds, the major phytochemical constituents of clove were eugenol, eugenyl acetate, caryophyllene, and pyrogallol (Hemalatha et al. 2016). In a review, Hiwandika et al. (2021) reported that the hexane extract contains eugenol, eugenol acetate, β-caryophyllene, and flavonoids. Furthermore, it has been reported that eugenol inhibits the growth of *S. mutans* by preventing bacterial adhesion and biofilm formation. Apart from eugenol, this high activity could also be due to the presence of β-caryophyllene, a nonpolar sesquiterpene compound, which has been reported to have antibacterial activity against *S. mutans* (Pieri et al. 2016).

4.4 Qualitative Screening of Phytochemical Compounds of Clove Extracts and Fractions

The thin layer chromatography method was used to qualitatively analyze phytochemical compounds in clove extract and fractions. It was found that all extracts and fractions contain eugenol, as indicated by a spot with an Rf value of about 0.8, which corresponds to the eugenol spot. This finding is consistent with the results of Pathak et al. (2004), who detected eugenol using TLC densitometry with toluene:ethyl acetate:formic acid (3:2:0.4) and an Rf value of 0.77. Another study identified eugenol and β-caryophyllene in clove acetonic and ethanolic extracts. The different polarity of compounds in the extracts or fractions may result in different spots being observed under UV light. This is due to the flow rate of the spot along the silica plate being dependent on the polarity of the compounds in the extracts or fractions and the polarity of the eluent (Pathak et al. 2004; Hemalatha et al. 2016; Hiwandika et al. 2021; Mostafa et al. 2023).

Conclusion

According to the results of this study, it can be concluded that the clove bud extract showed the highest inhibitory activity against *S. mutans* ($p < 0.05$) compared to the leaf extract, stem extract, and the positive control (ampicillin). Additionally, the activity of the hexane and water fractions against *S. mutans* growth was significantly different ($p < 0.001$). Clove bud is the best source of antibacterial compounds against *S. mutans*. Fractionation of the ethanol extract using hexane can substantially enhance its activity more than ampicillin as the positive control. This suggests that the hexane fraction could be a promising source for treating diseases caused by *S. mutans* infection.

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Ethical Clearance

It is confirmed that no animal or human model was utilized in the study; therefore, no ethical clearance is necessary.

Conflict of interest

The authors declare no conflict of interest in this study.

References

Abubakar, A. R., & Haque, M. (2020). Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. *Journal of Pharmacy & bioallied sciences*, *12*(1), 1–10. https://doi.org/10.4103/jpbs.JPBS_175_19

Ahmadi, H., Ebrahimi, A., & Ahmadi, F. (2021). Antibiotic Therapy in Dentistry. *International journal of dentistry*, *2021*, 6667624. https://doi.org/10.1155/2021/6667624

Aripin, D., Suwargiani, A.A., Susilawati, S., Putri, F.M., Hamdani, A.M., Yolanda, Y., Suryanti, N., & Mohd Yusof, Z.Y. (2024). Detection of Caries Coronal Condition Prevalence of Permanent Teeth in Children Using the International Caries Detection and Assessment System for Measuring Dental Caries: A Cross-Sectional Study. *The Open Dentistry Journal*, *18*, e18742106275531. DOI: 10.2174/0118742106275531240219044930.

Cheng, X., He, F., Si, M., Sun, P., & Chen, Q. (2022). Effects of Antibiotic Use on Saliva Antibody Content and Oral Microbiota in Sprague Dawley Rats. *Frontiers in Cellular and Infection Microbiology*, *12*, 721691. https://doi.org/10.3389/fcimb.2022.721691

Cieplik, F., Jakubovics, N. S., Buchalla, W., Maisch, T., Hellwig, E., & Al-Ahmad, A. (2019). Resistance Toward Chlorhexidine in Oral Bacteria - Is There Cause for Concern?. *Frontiers in microbiology*, *10*, 587. https://doi.org/10.3389/fmicb.2019.00587

Clinical Laboratory Standard Institute. (2012). *Performance standards for antimicrobial susceptibility testing: twenty-second informational supplement* (M100-S22 V ed.). Clinical Laboratory Standard Institute.

Cortés-Rojas, D. F., de Souza, C. R., & Oliveira, W. P. (2014). Clove (*Syzygium aromaticum*): a precious spice. *Asian Pacific*

journal of tropical biomedicine, *4*(2), 90–96. https://doi.org/10.1016/S2221-1691(14)60215-X

Cui, T., Luo, W., Xu, L., Yang, B., Zhao, W., & Cang, H. (2019). Progress of Antimicrobial Discovery Against the Major Cariogenic Pathogen *Streptococcus mutans*. *Current issues in molecular biology*, *32*, 601–644. https://doi.org/10.21775/cimb.032.601

de Oliveira Carvalho, I., Purgato, G. A., Píccolo, M. S., Pizziolo, V. R., Coelho, R. R., Diaz-Muñoz, G., & Alves Nogueira Diaz, M. (2020). In vitro anticariogenic and antibiofilm activities of toothpastes formulated with essential oils. *Archives of oral biology*, *117*, 104834. https://doi.org/10.1016/j.archoralbio.2020.104834

Dhamodhar P, Murthy, S., Channarayappa, Ramesh, K., Gopinath, N., Shantha kumar, S.S., & Varuvelil, G.J. (2014). Antibacterial efficacy of *Syzygium aromaticum* extracts on multi-drug resistant *Streptococcus mutans* isolated from dental plaque samples. *Journal of Biochemical Technology*, *3*, 155–157.

Dwivedi, D., & Singh, V. (2015). Effects of the natural compounds embelin and piperine on the biofilm-producing property of Streptococcus mutans. *Journal of traditional and complementary medicine*, *6*(1), 57–61. https://doi.org/10.1016/j.jtcme.2014.11.025

Folliero, V., Dell'Annunziata, F., Roscetto, E., Amato, A., Gasparro, R., Zannella, C., Casolaro, V., De Filippis, A., Catania, M. R., Franci, G., & Galdiero, M. (2022). Rhein: A novel antibacterial compound against Streptococcus mutans infection. *Microbiological research*, *261*, 127062. https://doi.org/10.1016/j.micres.2022.127062

Gupta, C., & Prakash, D. (2021). Comparative Study of the Antimicrobial Activity of Clove Oil and Clove Extract on Oral Pathogens. *Dentistry Open Journal*, *7*(1), 12–15. https://doi.org/10.17140/DOJ-7-144

Haque, M., Sartelli, M., & Haque, S. Z. (2019). Dental Infection and Resistance-Global Health Consequences. *Dentistry journal*, *7*(1), 22. https://doi.org/10.3390/dj7010022

Hemalatha, R., Nivetha, P., Mohanapriya, C., Sharmila, G., Muthukumaran, C., & Gopinath, M. (2016). Phytochemical composition, GC-MS analysis, in vitro antioxidant and antibacterial potential of clove flower bud (*Eugenia caryophyllus*) methanolic extract. *Journal of food science and technology*, *53*(2), 1189–1198. https://doi.org/10.1007/s13197-015-2108-5

Heta, S., & Robo, I. (2018). The Side Effects of the Most Commonly Used Group of Antibiotics in Periodontal Treatments. *Medical sciences (Basel, Switzerland)*, *6*(1), 6. https://doi.org/10.3390/medsci6010006

Hiwandika, N., Sudrajat, S.E., & Rahayu, I. (2021). Antibacterial and Antifungal Activity of Clove Extract (*Syzygium Aromaticum*). *Eureka Herba Indonesia*, *2*(2): 86–94.

Jacob, B., & Nivedhitha, M.S. (2018) Comparative Assessment of the Antibacterial Efficacy of Natural Products and Chlorhexidine Mouthwash against *Streptococcus mutans*: A Systematic Review. *Journal of Clinical & Diagnostic Research*, *12*(12), ZE01-ZE07.

Jafri, H., Ahmad, I., Jafri, H., & Ahmad, I. (2021). In Vitro Efficacy of Clove Oil and Eugenol against *Staphylococcus* spp and *Streptococcus mutans* on Hydrophobicity, Hemolysin Production and Biofilms and their Synergy with Antibiotics. *Advances in Microbiology*, *11*(2), 117–143. https://doi.org/10.4236/AIM.2021.112009

Kumar, U., Kumar, B., Bhandari, A., & Kumar, Y. (2010). Phytochemical investigation and comparison of antimicrobial screening of clove and cardamom. *International Journal of Pharmaceutical Sciences and Research*, *1*(12), 138–147.

Lee, S. H., Choi, B. K., & Kim, Y. J. (2012). The cariogenic characters of xylitol-resistant and xylitol-sensitive Streptococcus mutans in biofilm formation with salivary bacteria. *Archives of oral biology*, *57*(6), 697–703. https://doi.org/10.1016/j.archoralbio.2011.12.001

Liao, Y., Brandt, B. W., Li, J., Crielaard, W., Van Loveren, C., & Deng, D. M. (2017). Fluoride resistance in *Streptococcus mutans*: a mini review. *Journal of oral microbiology*, *9*(1), 1344509. https://doi.org/10.1080/20002297.2017.1344509

Mak, K.K., Kamal, M. B., Ayuba, S. B., Sakirolla, R., Kang, Y.B., et al. (2019). A Comprehensive Review on Eugenol's Antimicrobial Properties and Industry Applications: A Transformation from Ethnomedicine to Industry. *Pharmacognosy Reviews*, *13*(25), 1-9. https://doi.org/10.4103/phrev.phrev_46_18

Mann, A. (2012). Phytochemical constituents and antimicrobial and grain protectant activities of clove basil (*Ocimum gratissimum* L.) grown in Nigeria. *International Journal of plant research*, *2*(1), 51-58.

Moon, S. E., Kim, H. Y., & Cha, J. D. (2011). Synergistic effect between clove oil and its major compounds and antibiotics against oral bacteria. *Archives of Oral Biology*, *56*(9), 907–916. https://doi.org/10.1016/J.ARCHORALBIO.2011.02.005

Mostafa, A. A. F., Yassin, M.T., Al–Askar, A.A., & Al-Otibi, F.O. (2023). Phytochemical Analysis, Antiproliferative and Antifungal Activities of Different *Syzygium aromaticum* Solvent Extracts. *Journal of King Saud University-Science*, *35*(1), 102362.

Nurdjannah, N., Besar, B., Dan, P., Pasca, P., & Pertanian, P. (2016). Diversifikasi Penggunaan Cengkeh. *Perspektif*, *3*(2), 61–70. https://doi.org/10.21082/p.v3n2.2004.61-70

Park, H. S., Jun, D.Y., Fang, Z., Woo, M.H., & Kim, Y.H. (2008). Antimicrobial Activity of Seeds of *Zanthoxylum piperitum* against Oral Pathogen *Streptococcus mutans*. *Journal of Life Science*, *18*(2), 167–74.

Pathak, S. B., Niranjan, K., Padh, H., & Rajani, M. (2004). TLC densitometric method for the quantification of eugenol and gallic acid in clove. *Chromatographia*, *60*(3), 241-244.

Pieri, F. A., Souza, M. C. de C., Vermelho, L. L. R., Vermelho, M. L. R., Perciano, P. G., Vargas, F. S., Borges, A. P. B., da Veiga-Junior, V. F., & Moreira, M. A. S. (2016). Use of β-caryophyllene to combat bacterial dental plaque formation in dogs. *BMC Veterinary Research*, *12*(1), 1–8. https://doi.org/10.1186/S12917-016-0842-1/FIGURES/3

Qiu, W., Zhou, Y., Li, Z., Huang, T., Xiao, Y., et al. (2020). Application of Antibiotics/Antimicrobial Agents on Dental Caries. *BioMed research international*, *2020*, 5658212. https://doi.org/10.1155/2020/5658212

Rasul, M. G. (2018). Extraction, Isolation and Characterization of Natural Products from Medicinal Plants. *International Journal of Basic Sciences and Applied Computing*, *2*(6), 1-6.

Silvestre, C. I., Santos, J. L., Lima, J. L., & Zagatto, E. A. (2009). Liquid-liquid extraction in flow analysis: A critical review. *Analytica chimica acta*, *652*(1-2), 54–65. https://doi.org/10.1016/j.aca.2009.05.042.

Sivapathasundharam, B., & Raghu, A. R. (2020). Dental caries. In *Shafer's Textbook of Oral Pathology, 9th Edition* (pp. 369–403). Elsevier RELX India Pvt Ltd.

Sohilait, H. J., Kainama, H., Nindatu, M., Sohilait, H. J., Kainama, H., & Nindatu, M. (2018). Chemical Composition and Antibacterial Activity of the Essential Oils from Different Parts of *Eugenia caryophylata*, Thunb Grown in Amboina Island. *International Journal of Organic Chemistry*, *8*(2), 229–239. https://doi.org/10.4236/IJOC.2018.82017

Sucipto, R. K. H., Kuswandi, K., & Wibawa, G. (2022). Ternary liquid-liquid equilibrium for eugenol + tert-butanol + water and eugenol + 1-octanol + water systems at 303.15 K and atmospheric pressure. *IOP Conference Series: Earth and Environmental Science*, *969*(1), 012051. https://doi.org/10.1088/1755-1315/969/1/012051

Suhendar, U., & Sogandi, S. (2019). Identifikasi Senyawa AktifEkstrak Daun Cengkeh (*Syzygium aromaticum*) sebagai Inhibitor *Streptococcus mutans*. *Al-Kauniyah: Jurnal Biologi*, *12*(2), 229–239. https://doi.org/10.15408/KAUNIYAH.V12I2.12251

Sulaiman, N., Idayu, M.I., Ramlan, A.Z., Fashya, M.N., Farahiyah, A.N.N., Mailina, J., & Azah M.A.N. (2015). Effects of extraction methods on yield and chemical compounds of gaharu (*Aquilaria malaccensis*). *Journal of Tropical Forest Science*, *27* (3), 413–419

Utami, L. A., & Putri, D. H. (2020). The Effect of Ethanol Solvent Concentration on Antimicrobial Activities the Extract of Andalas Endophytic Bacteria (*Morus macroura* Miq.) Fermentation Product. *Eksakta :BerkalaIlmiah Bidang MIPA, 21*(1), 1–6. https://doi.org/10.24036/EKSAKTA/VOL21-ISS1/210

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Assessment of Physico-Chemical Properties of Biogas Slurry as an Organic Fertilizer for Sustainable Agriculture

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ABSTRACT

Chemical fertilizers have been extensively used for growing crops and controlling plant diseases, but they pose potential hazards to the environment, soil health, plants, and people. The current world situation highlights the need to implement eco-friendly agricultural practices for sustainable crop production. Using environmentally friendly manure, such as biogas slurry, can help reduce the negative effects of chemical fertilizers. Biogas slurry is an efficient waste material and organic fertilizer, making it an ideal supplement for sustainable crop production and waste management. An experiment was conducted at IARI, New Delhi, to explore the nutrient potential of biogas slurry. The main objective of this study was to assess biogas slurry's physico-chemical characteristics and nutrient contents. Samples of biogas slurry were collected in three replications and analyzed using standard methods for macro and micronutrients. The data revealed that biogas slurry has a pH of 7.2-8.5, EC of 1.06 to 1.12 dS/m, and organic carbon content of 41.7 to 45.8%. In terms of fertility, it contains significant amounts of nitrogen (1.98-2.17%), phosphorus (0.97 to 1.15%), and potassium (1.98 to 2.17%). Additionally, biogas slurry contains micronutrients such as Zn (0.023-0.027 ppm), Cu (0.005-0.009 ppm), Fe (0.32-0.38 ppm), and Mn (0.089-0.094 ppm). Statistical analysis using ANOVA and Post Hoc tests indicated that the mean data values among all three replications do not differ significantly. Therefore, it can be concluded that the nutritive value of biogas slurry is sufficient to reduce the reliance on chemical fertilizers in agriculture. It represents an optimal long-term organic remedy for developing fertile soil, ensuring enduring agricultural productivity, and mitigating the negative environmental impacts associated with waste management.

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1 Introduction

The agriculture sector plays a significant role in India's economy and significantly impacts the country's GDP. According to the Food and Agriculture Organization (FAO), there is projected to be a 60% increase in the demand for agricultural commodities by 2030, with over 85% of this increase attributed to developing economies (Mia and Shamsuddin 2010). The use of chemical fertilizers has increased globally, leading to higher farm productivity but also posing environmental challenges (Faheed and Abd-El Fattah 2008; Thorat and More 2022). In India, the use of chemical fertilizers significantly rose in the 1960s following the introduction of high-yielding crop varieties. However, the excessive use of chemical fertilizers can lead to soil degradation, such as acidification and reduced fertility, impacting crop yields over time (Yadav et al. 2023).

Nitrogen (N) fertilizers are the main source of N_2O emissions from agricultural soil, with the agricultural sector contributing 65–80% of the world's N_2O emissions (Ghosh et al. 2022; Mosier and Kroeze 2000; IPCC 2007). Overusing nitrogen-based fertilizers can lead to nitrate leaching, contaminating groundwater and causing adverse effects on the ecosystem (Sharma et al. 2022). This can result in nitrate and phosphate runoff from agricultural fields, leading to eutrophication in nearby water bodies, harming fish populations and posing health risks to humans and animals (Agarwal et al. 2010; Rahman et al. 2008).

Inorganic fertilizers contain poisonous elements that plants absorb, pass through to vegetables and grains, and enter the food chain. This can result in financial losses and environmental harm (Tirado and Allsopp 2012). Fertilizers include various heavy metals such as lead, mercury, uranium, and cadmium, which can severely damage the kidneys, lungs, and liver and, at higher concentrations, can be a cause of cancer (WHO 1992). A higher dose of potassium may cause kidney dysfunction, diabetes, hypertension, heart disease, coronary artery disease, adrenal insufficiency, and pre-existing hyperkalemia (APHA 1998). Cadmium toxicity, known as "Itai-Itai", was first identified in Japan in 1912. When associated with human tissues, cadmium may cause harm, especially to kidneys, bones, and lungs. Therefore, if chemical fertilizer is not handled properly, it can put farmers at risk for poisoning, and straw made from crops fertilized with chemical fertilizer can cause animals to grow thin (Berihu 2012).

The shift towards environmentally sustainable practices is crucial, moving away from conventional methods and prioritizing organic farming over chemical fertilizers to improve soil quality and ecological well-being. Organic farming differs significantly from chemical fertilizers by reducing nutrient loss, enhancing soil fertility (Gattinger et al. 2012), lowering global warming potential (Cavigelli et al. 2013), and increasing crop yields (Seufert et al. 2012). Organic residues are key to soil fertility (Ayuso et al. 1996). While inorganic fertilizers contain higher levels of plant nutrients than organic fertilizers, including organic fertilizers is essential for improving soil fertility and agricultural productivity (Sanwal et al. 2007; Adeleye et al. 2010). Organic agriculture, a sustainable farming method, has gained global interest, but specific challenges must be addressed to expand these practices (Kumar et al. 2023). The utilization of organic residues, especially biogas slurry (BGS), may provide a potential solution (Malav et al. 2015) and serve as a viable alternative to synthetic fertilizers for ecologically sustainable agricultural practices (Tang et al. 2022).

Anaerobic digestion of crop residues and animal manure produces biogas slurry, often used as a substitute to reduce mineral fertilizer input (Sun et al. 2024). Cow dung (CD) is commonly used as a feedstock for biogas production and a source of BGS (Younessi et al. 2023). The discharge of biosolids (BS) currently presents significant environmental challenges. Therefore, it is crucial to explore alternative strategies for the effective utilization of biosolids (Wang et al. 2023). Biogas slurry, a byproduct of the anaerobic digestion of livestock manure, is a highly efficient method for the management and disposal of livestock manure and agricultural byproducts (Kang et al. 2020; Ferdous et al. 2020; Liang et al. 2023; He et al. 2024). It contains significant nutrients compared to other organic manures, such as farmyard manure (FYM) and composts (Table 1). Due to its high nutrient content, the use of biogas slurry as a fertilizer has significantly increased in China and many other Asian nations (Gupta et al. 2016). In India, the generation of biogas slurry has increased, leading to significant environmental disposal issues (Khan et al. 2015).

Table 1 Nutritional comparison of biogas slurry and other compost

Slurry	Oven-dried slurry	Slurry compost	Biogas effluent	Ordinary compost	Biogas sludge	Farmyard manure (FYM)
Nitrogen %	1.6 - 3.7	0.57 - 2.23	0.03 - 0.08	0.5 - 1.0	0.8 - 1.5	0.3 - 0.5
Phosphorus %	1.6 - 2.2	0.072 - 2.11	0.02 - 0.06	0.1 - 0.3	0.4 - 0.6	0.1 - 0.2
Potassium %	0.8 - 3.6	0.0 - 5.1	0.5 - 0.10	0.5 - 0.7	0.6 - 0.12	0.5 - 0.7
References	Karki 2004	Vaidya et al. 2007	Warnars and Oppenoorth 2014	Meena and Biswas 2013	Yakout et al. 2014	Bandyopadhyay et al. 2010

(Source: Mukhtiar et al.2024)

The government of India has recently implemented substantial measures to promote the utilization of biogas slurry as an organic fertilizer. The livestock population in India is estimated to be 512.05 million (Dikshit and Pratap 2010). Each year, 76.8 million tons of slurry and approximately 1.15 metric tons of nitrogen are produced in India (Kumar et al. 2015). It has been estimated that using slurry instead of chemical fertilizer can reduce nitrogen use by around 8.78%, phosphorus use by about 11.01%, and potassium use consumption by roughly 14% (Devarenjan et al. 2019). By using biogas slurry, farmers can lower their production costs and increase soil fertility and productivity while reducing the use of chemical fertilizers.

Biogas slurry is a product of the anaerobic digestion of organic material, primarily used for cooking, lighting, and running machinery (Kamp and Forn 2016). It is a low-cost, environmentally friendly, and safe option for people and animals (Islam et al. 2016). According to Devarenjan et al. (2019), biogas slurry consists of 93 percent water, 4.50 percent dry matter, and 2.50 percent inorganic matter. It contains significant levels of macronutrients (N, P, K) and micronutrients (Zn, Mn, B) essential for plant growth, making it a valuable source of organic fertilizer (Alam 2006). The biogas slurry contains 1.50 percent nitrogen, 1.10 percent phosphorus, and 1.0 percent potassium. Additionally, Vinh (2010) suggested that one cubic meter of biogas slurry contains 0.16 to 1.05 kg of nitrogen, equivalent to 0.35 to 2.5 kg of urea. The growth rate of crops can be significantly improved by using biogas slurry (Ahmad and Jabeen 2009) and enhancing the nutrient content in crops (Vishwakarma et al. 2023). Most research has shown that using biogas slurry resulted in maximum crop yields. For example, the anaerobic digestate from beef cows increased nitrogen and phosphorus intake in barley fodder (Tang et al. 2021). Jothi et al. (2023) also found a significant enhancement in tomato yield and the number of fruits produced per plant when they utilized biogas slurry obtained through an anaerobic digestion process. Similarly, optimal rice and rapeseed production levels were achieved by applying biogas slurry at 165.10 and 182.10 t ha^{-1} (Zhang et al. 2022). Garg et al. (2005) reported an increase in maize grain yields (6.21 Mg ha^{-1}) and an enhancement of root length density (1.10 cm cm^{-3}) by using biogas slurry at the rate of 15 Mg ha^{-1}. Kumar et al. (2015) and Feng et al. (2024) reported that biogas slurry can reduce chemical fertilizer use by 15% to 20%. These studies suggest that the efficient use of organic fertilizers promotes economic development, improves agricultural sustainability, and positively affects the environment by reducing reliance on imported chemical fertilizers. This information was gathered during a study conducted at the Indian Agricultural Research Institute (IARI), New Delhi, to explore the benefits of biogas slurry in agriculture and to estimate its physico-chemical characteristics and nutrient contents.

2 Materials and Methods

2.1 Biogas sample collection, preparation and analysis

The experiment occurred at CESCRA, ICAR-Indian Agricultural Research Institute (IARI) in New Delhi, India. Biogas slurry samples were collected from biogas plants at the IFS Agronomy Division of IARI farm in New Delhi. The collected samples, with a volume of 1 L and containing between 92 and 93% water content, underwent a 15-day sun-drying process. After drying, the samples were crushed and sieved through a 2 mm sieve (Figure 1). Subsequently, the biogas slurry was analyzed at the Laboratory of CESCRA, ICAR-IARI, in New Delhi to determine various parameters using standard methods (Table 2).

The biogas slurry samples were analyzed for their physicochemical parameters. Each analysis was conducted in three replications. To determine the total solids content in the biogas slurry, the samples were subjected to oven drying at 105°C until a consistent weight was achieved, and then the weight of the dried residue was calculated. The pH and electrical conductivity are important for characterizing biogas slurry, so they were measured in fresh samples using a pH meter and an electrical conductivity meter.

Figure 1 Biogas slurry sample collection and preparation

Table 2 Physico-chemical parameters and their estimation methods

Parameter	Methods
Soil pH (1:2.5 soil: water)	Piper1966
Electrical conductivity (dS/m)	Jackson1967
Organic carbon (%)	Walkley and Black1934
Nitrogen (%)	Kjeldahl 1883
Phosphorus (%)	Vanado molybdate phosphoric yellow colour method, Jackson 1973
Potassium (%)	Flame photometer method, Jackson 1973
Micronutrients (Zinc, Copper, Iron and Manganese)	Lindsay and Norvell 1978

Table 3 Physico-chemical characterization of biogas slurry

Nutrient content in biogas slurry				
Parameters	Total solids (%)			
Range	5.2-6.5			
Average ± Stand. dev.	5.83±0.651			
Parameters	pH	Electric conductivity (dS/m)	Organic Carbon (%)	
Range	7.2-8.5	1.06-1.12	41.7-45.8	
Average ± Stand. dev.	7.80 ±0.656	1.09 ±0.030	43.80 ±2.052	
Macronutrients (%)				
Parameters	Nitrogen	Phosphorus	Potassium	
Range	1.98-2.17	0.99-1.15	0.91-1.16	
Average ± Stand. dev.	2.09 ± 0.100	1.07 ± 0.091	1.03 ± 0.126	
Micronutrients (ppm)				
Parameters	Zinc	Copper	Iron	Manganese
Range	0.023-0.027	0.005-0.009	0.32-0.38	0.089-0.094
Average ± Stand. dev.	0.03 ±0.002	0.01 ±0.002	0.36±0.032	0.09 ±0.003

2.2 Statistical analysis

The data obtained from three replications of biogas slurry were analyzed using statistical methods. The statistical technique used for this analysis was an analysis of variance (ANOVA) and a Post Hoc test. These tests were used to identify any significant differences between the means of the three replications (Zheng et al. 2017; Xu et al. 2021). In this study, each parameter represents a dependent group, and the replications of biogas slurry (R-I, R-II, R-III) are the independent groups. The Null Hypothesis (H_0) in the analysis of variance states that the means of the total composition of biogas slurry for the three replications are equal, while the Alternative Hypothesis (H1) states that at least one of the means is different among all the replications.

3 Results and Discussion

3.1 Physico-chemical characteristics of biogas slurry

The biogas slurry samples were collected and taken to the laboratory for analysis. The nutrient components of biogas slurry can vary depending on factors such as the constituents of the feedstock, efficiency of the digestion process, any additives or treatments used, etc. The physico-chemical factors that determine whether biogas slurry is suitable to use as manure for crops include total solids, pH, electrical conductivity, organic carbon, nitrogen, phosphorus, potassium, and micronutrients (such as Zn, Cu, Fe, and Mn). Three replications of biogas slurry have been analyzed for their physico-chemical properties (Table 3).

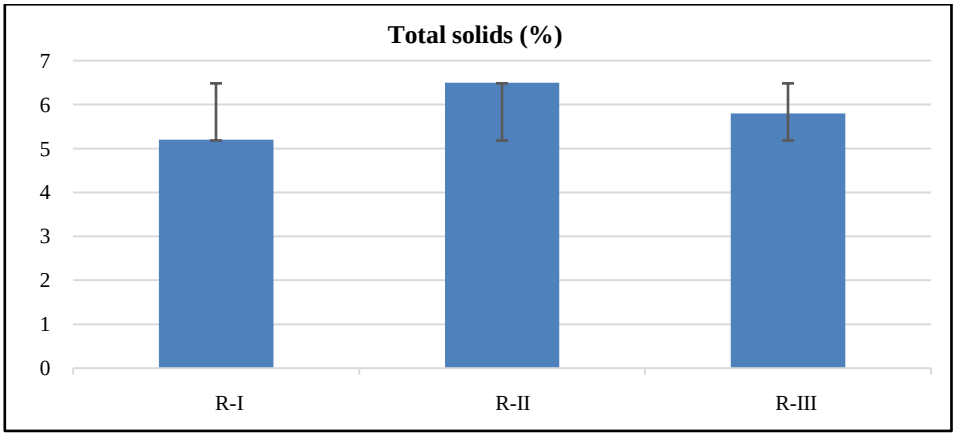

Figure 2 Total solids in the current biogas slurry sample

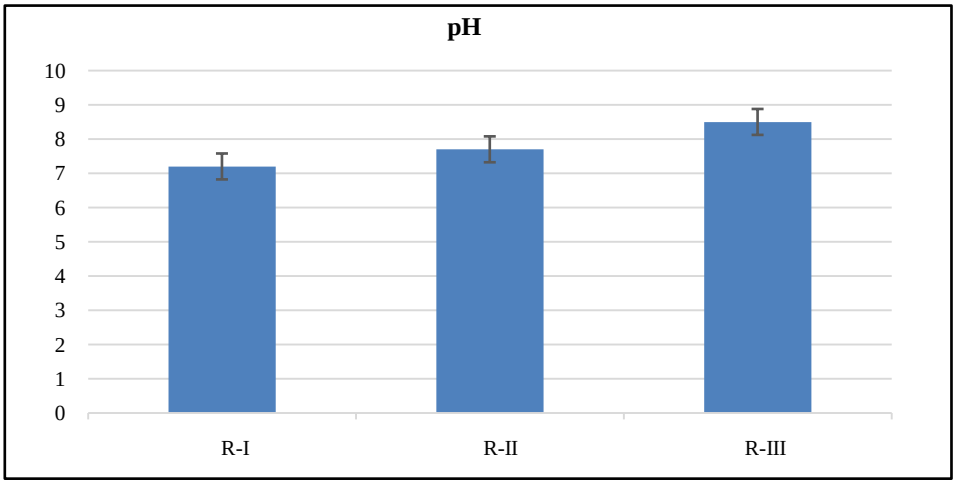

Figure 3 pH in biogas slurry

3.1.1 Total solids

The amount of solid materials present in biogas slurry can vary depending on the specific sample and the type of feedstock used in biogas production and digestion. According to the current study, the total solids content ranges from 5.2 to 6.5 percent (Figure 2). These findings are consistent with the results reported by Barasa et al. (2020), who indicated that a total solids concentration of 6 to 8 percent is beneficial for stabilizing anaerobic digestion processes. Research on biogas production from bovine excreta has explored a wide range of dilutions (2:1–1:19) and associated total solids concentrations (ranging from 1% to 13.5%), as well as extremely low total solids levels (TS < 4%) (Jeppu et al. 2022).

3.1.2 pH and Electric conductivity

Monitoring and controlling the pH of biogas slurry is crucial to maintaining optimal conditions for the microbial activity responsible for biogas production. However, this range can fluctuate due to the specific conditions of the biogas reactor and the quality of the feedstock being processed. The pH level in the soil directly impacts the availability of the nutrients required for plant growth. The data related to pH monitoring revealed that the pH of the biogas slurry ranges from 7.2 to 8.5 (Figure 3). These results are consistent with earlier findings that the pH value of biogas slurry falls within the range of 7.69 to 8.29 (Malav et al. 2015; Naihui et al. 2023). Studies have indicated that different pH levels can influence biogas production from bovine excreta, with a pH of about 8.52, resulting in an alkaline condition (Bahira et al. 2018). Additionally, biogas slurry can neutralize acidic soils and improve soil pH (Feng et al. 2024).

It is crucial to regularly monitor and manage the electrical conductivity to maintain the stability and efficiency of biogas production processes. The biogas slurry's electric conductivity (EC) ranged from 1.06 to 1.12 dS/m, as shown in Figure 4. The concentration of electric conductivity is dependent on the concentrations of dissolved ions and salts. In a previous experiment, Yadav et al. (2023) demonstrated that the electric conductivity of biogas slurry ranged between 1.72 and 1.83.

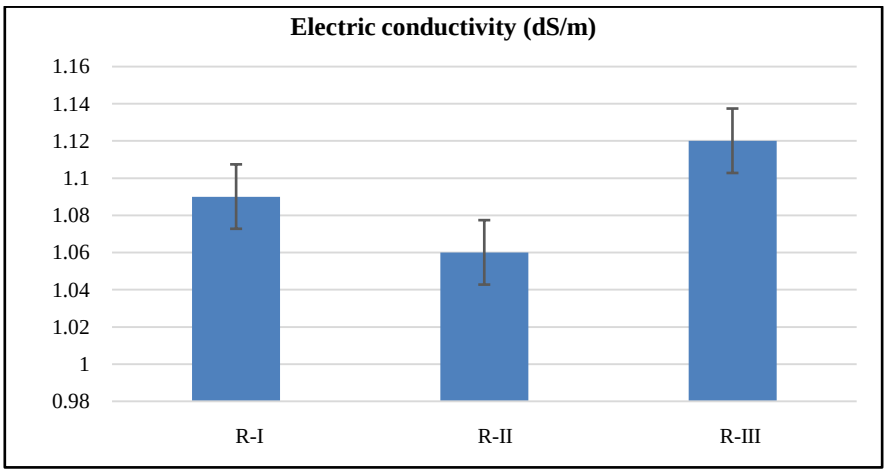

Figure 4 Electric conductivity in biogas slurry

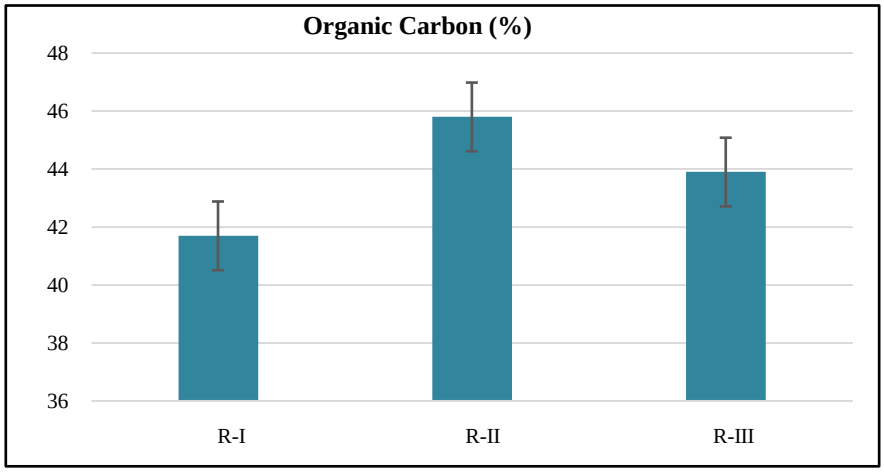

Figure 5 Organic carbon in biogas slurry

3.1.3 Organic carbon

The biogas slurry samples were analyzed for their organic carbon content, crucial for determining their nutrient value and potential use as a nutrient-rich fertilizer for agriculture or soil improvement. The study showed that the biogas slurry contains approximately 41.7 to 45.8 percent organic carbon (Figure 5). Biogas slurry (BS) is widely acknowledged as a significant source of organic matter and essential nutrients for enriching soil organic carbon (Chen et al. 2024).

The organic carbon in biogas slurry is crucial for maintaining microorganism stability, which in turn sustains continuous decomposition activity and enhances the nutritional dynamics of the digestate.

3.1.4 Nitrogen, Phosphorus and potassium (NPK)

The anaerobic breakdown of cow excrement leads to elevated levels of ammonium nitrogen while also increasing the durability of organic compounds. However, this process also significantly reduces the carbon-nitrogen ratio, leading to a higher presence of Nitrogen (Gutser et al. 2005). Given the high costs associated with phosphorus and potassium, which are important environmental concerns, using bio-slurry as an alternative source is expected to reduce agricultural production costs substantially. Data shows that biogas slurry's nitrogen, phosphorus, and potassium content ranges from 1.98 to 2.17 percent, 0.97 to 1.15 percent, and 0.91 to 1.16 percent, respectively (Figure 6). With its NPK content, biogas slurry can be used as a nutrient-rich organic fertilizer due to its three essential nutrients for plant growth. The current study indicates slightly higher NPK values than those of Skrzypczak et al. (2023), who reported approximately 2.55 percent nitrogen, 0.57 percent phosphorus, and 1.77 percent potassium. Some research findings suggest that the combined use of liquid bio-slurry (20.6 mha^{-1}) and nitrogen (41 kg N ha^{-1}) has the potential to improve the physico-chemical characteristics of soil (Musse et al. 2020). This study is supported by research indicating that biogas slurry has a higher NPK content than chemical fertilizers (Vishwakarma et al. 2023).

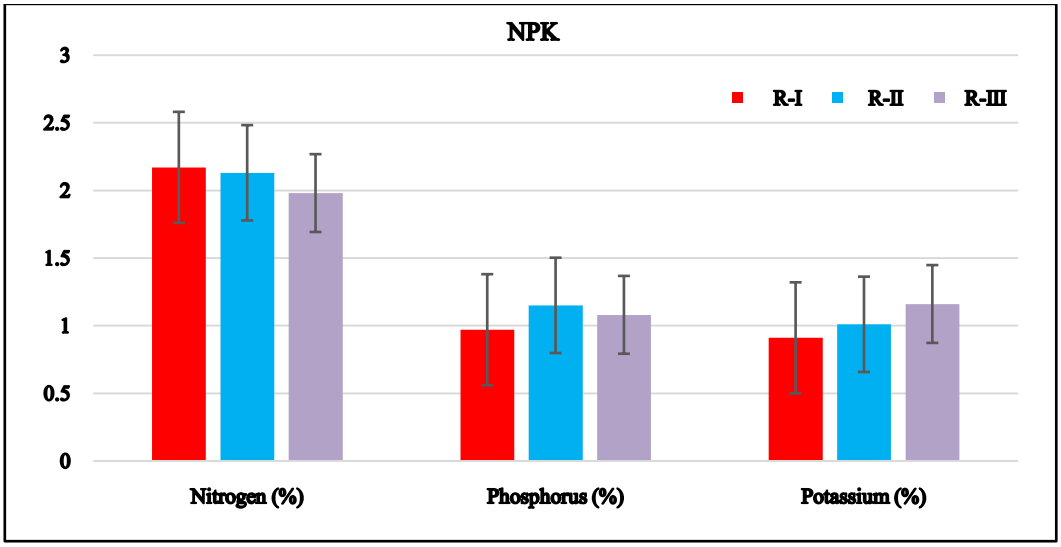

Figure 6 NPK in biogas slurry

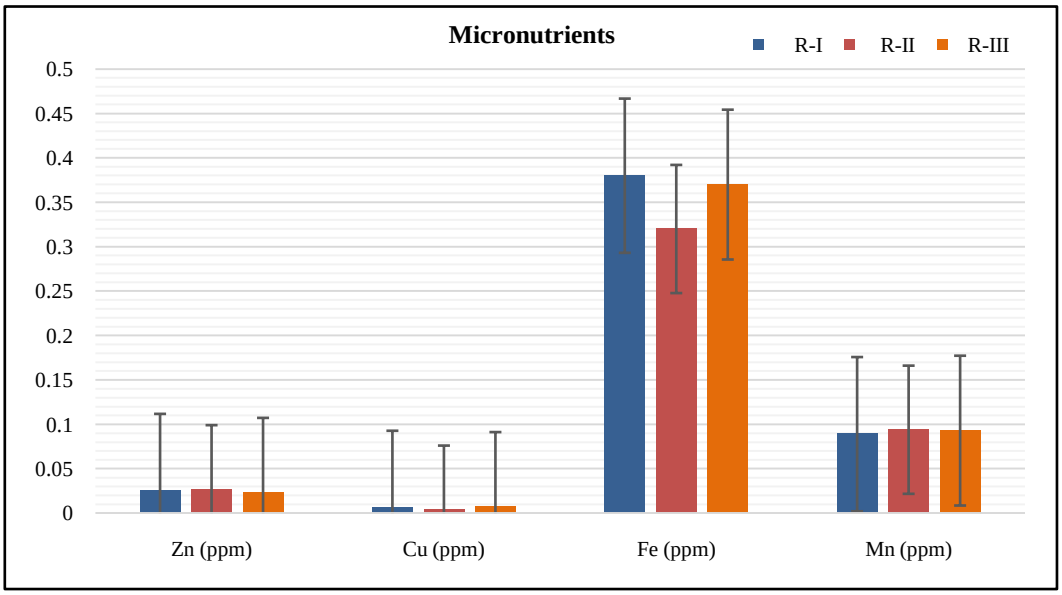

Figure 7 Micronutrients in biogas slurry

3.1.5 Micronutrients

Plant growth is heavily dependent on micronutrients. Plants require a precise balance of essential nutrients for optimal development and productivity. As a result, the micronutrient content in biogas slurry has been analyzed using AAS. The study data revealed that the micronutrients zinc, copper, iron, and manganese range from 0.023-0.027 ppm, 0.005-0.009 ppm, 0.32-0.38 ppm, and 0.089-0.094 ppm, respectively (Figure 7). Previous research has also confirmed that biogas slurry provides a rich supply of micronutrients essential for plant growth, including Zn, Cu, Fe, and Mn (Liu et al. 2023). Malav et al. (2015) reported that biogas slurry contains Zn (0.023 ppm), Cu (0.004 ppm), Fe (0.34 ppm), and Mn (0.088 ppm). Therefore, the results of the current study are consistent with those of these researchers.

3.2 Statistical analysis

3.2.1 Analysis of variance (ANOVA) among three replications of biogas slurry

The results from the physicochemical analysis of three sets of biogas slurry were statistically interpreted using the ANOVA method. This statistical approach will help identify significant differences in the parameter means across all three sets (Table 4). The P value was greater than 0.05, indicating no significant

Table 4 ANOVA test among three replications of biogas slurry

	Sum of the squares	df	Mean of the square	F	Sig.
Between the Groups	1.770	2	0.885	0.005	0.995
Within the Groups	4983.173	30	166.106		
Total	4984.943	32			

Table 5 Post Hoc Tests among three replications of biogas slurry

Replications		Mean Differences (I-J)	Standard Errors	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
R-I	R-II	-0.551364	5.495548	0.994	-14.09938	12.99665
	R-III	-0.391182	5.495548	0.997	-13.93920	13.15684
R-II	R-I	0.551364	5.495548	0.994	-12.99665	14.09938
	R-III	0.160182	5.495548	1.000	-13.38784	13.70820
R-III	R-I	0.391182	5.495548	0.997	-13.15684	13.93920
	R-III	-0.160182	5.495548	1.000	-13.70820	13.38784

difference between all three replications of biogas slurry. The composition of parameters in each replication is equally impacted. This study demonstrates that there is no significant difference among the replications.

3.2.2 Comparison of replications of biogas slurry through Post Hoc Test

The data from all three replications were analyzed using a post hoc test to gain a better understanding of the differences among them. This test is done after an ANOVA to compare the means of the different replications and identify where the significant differences are. After comparing the treatment replications, it was found that there were no significant differences among the different replications of biogas slurry (Table 5).

In this experiment, the biogas slurry was considered the dependent variable, while the nutrients in the biogas slurry were treated as the independent variable. The null hypothesis (H_0) suggests no significant difference between the means of the replications, whereas the alternative hypothesis (H_1) indicates that the means of at least one replication significantly differ from the others. The means of the three biogas slurry replications do not vary significantly, as indicated by a P value greater than 0.05. The correlations among pH, electric conductivity, organic carbon, nitrogen, phosphorus, potassium, and micronutrients were positive and statistically significant. The results of this investigation suggest that biogas slurry has the potential to be an effective soil amendment compared to cow dung, as it can regulate soil pH, enhance nutrient accessibility, and gradually release phosphorus fertilizer according to specific plant requirements. The nutrients in the biogas slurry were statistically insignificant (>0.05), indicating that biogas slurry is a superior organic fertilizer for soil amendment compared to other organic fertilizers. In addition to significant amounts of organic matter, the biogas slurry under investigation contains sufficient macro and micronutrients crucial for plant development.

Conclusion

The study of biogas slurry in three replications showed that this organic manure contains a significant amount of macro and micronutrients. Statistical techniques such as ANOVA and Post Hoc tests were used to validate these results. The results indicated no significant difference in the mean values of any physico-chemical parameters of the biogas slurry between the three replications, with a P value larger than 0.05. This suggests that biogas slurry can provide enough nutrients to reduce India's reliance on chemical fertilizers, which benefits the country's economy and ecology. Farmers are encouraged to incorporate biogas slurry into their agricultural practices for environmentally friendly crop development and sustained crop growth.

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Conflict of Interest

Authors have no conflicts of interest.

References

Adeleye, E.O., Ayeni, L.S., & Ojeniyi, S.O. (2010). Effect of poultry manure on soil physico-chemical properties, leaf nutrients content and yield of yam (*Discorea rotundata L.*) on Alfisol in

South Western Nigeria. *Journal of American Science*, *6*(10), 871-878.

Agrawal, A., Pandey, R.S., & Sharma, B. (2010).Water pollution with special reference to pesticides contamination in India. *Journal of Water Resources Protection*, *2*(5), 432-448.

Ahmad, R., & Jabeen, N. (2009). Demonstration of growth improvement in sunflower (*Helianthus annuus* L.) by the use of organic fertilizers under saline conditions. *Pakistan Journal of Botany*, *41*(3), 1373-1384.

Alam, S. (2006). *Production of organic manure in Bangladesh, Bangladesh Livestock Research Institute's Report, Savar, Dhaka, Bangladesh:* Science Publishing Group.

APHA. (1998). *Standard methods for the examination water and waste water*. Washington DC: 20th edition American Public Health Association.

Ayuso, M., Hernández, T., García, C., & Pascual, J.A. (1996). Biochemical and chemical-structural characterization of different organic materials used as manures. *Bioresource Technology*, *57*(2), 201-207.

Bahira, B.Y., Baki, A.S., & Bello, A. (2018). Effect of Varying pH on Biogas Generation using Cow Dung. *Direct research journal of biology and biotechnology*, *4*(3), 28-33.

Bandyopadhyay, K.K., Misra, A.K., Ghosh, P.K., & Hati, K.M. (2010). Effect of integrated use of farmyard manure and chemical fertilizers on soil physical properties and productivity of soybean. *Soil Tillage Research*, *110*(1), 115-125.

Barasa, H.M., Daudi, M.N., Musa, R.N., & Joseph, W.M. (2020). Effect of total solids on biogas production in a fixed dome laboratory digester under mesophilic temperature. *Annals of Advanced Agricultural Sciences*, *4*(2), 26-33.

Berihu, A. (2012). *Biogas-bio-slurry: A package for narrowing gender disparity in the rural households – the case of Hintalo-Wajirat and Ofla woredas, Tigray region. [Master's Thesis*, Addis Ababa University, Addis Ababa Ethiopia].

Cavigelli, M.A., Mirsky, S.B., Teasdale, J.R., Spargo, J.T., & Doran, J. (2013). Organic grain Cropping systems to enhance ecosystem services. *Renewable Agriculture and Food Systems*, *28*(2), 145-159.

Chen, Z., Ma, J., Ma, J., Ye, J., Yu, Q., Zou, P., et al. (2024). Long-term biogas slurry application increases microbial necromass but not plant lignin contribution to soil organic carbon in paddy soils as regulated by fungal community. *Waste management*, *175*, 254-276.

Devarenjan, J., Joselin Herbert, G.M., & Amutha, D. (2019), Utilization of Bioslurryfrom Biogas Plant as Fertilizer. *International Journal of Recent Technology and Engineering*, *8*(4), 12210-12213.

Dikshit, A. K., & Pratap, S. (2010). Environmental value of dung in mixed crop-livestock systems. *Indian Journal of Animal Sciences*, *80*(7), 679–682.

Faheed, F., & Abd-El Fattah, Z. (2008). Effect of *Chlorella vulgaris* as Bio-fertilizer on Growth Parameters and Metabolic Aspects of Lettuce Plant., *Journal of agriculture and social sciences*, *4*, 165-169.

Feng, G., Hao, F. He, W., Ran, Q., Nie, G., et al. (2024). Effect of Biogas Slurry on the Soil Properties and Microbial Composition in an Annual Ryegrass-Silage Maize Rotation System over a Five-Year Period. *Microorganisms*, *12*(4), 716.

Ferdous, Z., Ullah, H., Datta, A., Attia, A., Rakshit, A., & Molla, S. H.(2020). Application of Biogas Slurry in Combination with Chemical Fertilizer Enhances Grain Yield and Profitability of Maize (*Zea mays* L.). *Communications in Soil Science and Plant Analysis*, *51*(19), 2501–2510.

Garg, R.N., Pathak, H., Das, D.K., & Tomar, R.K. (2005). Use of Flyash and Biogas Slurry for improving Wheat Yield and Physical Properties of Soil. *Environmental Monitoring and Assess*ment, *107*(1-3), 1-9.

Gattinger, A., Muller, A., Haeni, M., Skinner, C., Fliessbach, A., Buchmann, N., et al.(2012). Enhanced top soil carbon stocks under organic farming. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 18226-18231.

Ghosh, S., Sarkar, A., Hazra, A., & Bagdi, T. (2022). Organic Farming and Digested Biogas Slurry for Sustainable Agriculture in India: A Review. *Journal of Social Work and Social Development*, *12* (2), 81-96.

Gupta, K.K., Aneja, K.R., & Rana, D. (2016). Current status of cow dung as a bioresource for sustainable development. *Bioresource and Bioprocess*, *3*(28), 1-11.

Gutser, R., Ebertseder, T., Weber, A., Schram, M., & Schmidhalter, U. (2005). Short-term and residual availability of nitrogen after long-term application of organic fertilizers on arable land. *Journal of plant Nutritional and Soil science*, *168*, 439-446.

He, W.Q., Fan, Y.F., Zhang, X.F., & Xiang, T.Y. (2024). Effects of biogas slurry as the replacement for chemical fertilizers on chinese cabbage yield and soil quality. *Applied Ecology and Environmental Research*, *22*(3):2359-2366.

IPCC. (2007). Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. *Geneva, Switzerland: Teri Press.*

Islam, R., Nur Hossain Md., Fakruddin, Md., Rabbi K., & Abdul Baten, Md. (2016). Effect of solid waste slurry from biogas plant on soil parameters and yield of spinach (*Spinacia oleracea L.*). *Journal of Agriculture and Ecology Research International, 5*(1), 1-11.

Jackson, M. L. (1967). *Soil Chemical Analysis*. *Bombay, India*: *Oxford and IBHP Publishers.*

Jackson,M.L.(1973). *Soil Chemical Analysis*. New delhi, India: *Prentice Hall of India Pvt., Ltd.*

Jeppu, G.P., Janardhan, J., Kaup, S., Janardhanan, A., Mohammed, S., & Acharya S. (2022). Effect of feed slurry dilution and total solids on specific biogas production by anaerobic digestion in batch and semi-batch reactors. *Journal of material cycles and waste Management, 24*, 97–110.

Jothi, G., Pugalendhi, S., Poornima, K., & Rajendran, G. (2003). Management of root-knot nematode in tomato *Lycopersicon esculentum*, Mill., with biogas slurry. *Bioresource Technol*ogy, *89* (2), 169–170.

Kamp, L.M., & Forn, E.B. (2016). Ethiopia's emerging domestic biogas sector: Current status, bottleneck and drivers. *Renewable and sustainable energy reviews*, *60*, 475-488.

Kang, Q., Zhao, J., Zhu, P., Gong, Q., Wang, L., & Li, Z. (2020). The Growth Characteristics of Three Terrestrial Plants Cultivated with Biogas Slurry as a Hydroponic Medium. *American Journal of Plant Sciences*, *11*, 819-831.

Karki, K.B. (2004). Estimation of plant nutrients content in biogas slurry and losses during handling. *Agricultural research for enhancing livelihood of Nepalese people*, *30*, 330-334.

Khan, S.A., Malav, L.C., Kumar, S., Malav, M.K., & Gupta, N. (2015). Resource Utilization of Biogas Slurry for Better Yield and Nutritional Quality of Baby Corn. *Advances in Environmental and Agricultural Science*, *32*, 382-394.

Kjeldahl, J. (1883). A New Method for the Determination of Nitrogen in Organic Matter. *Zeitschrift für Analytische Chemie*, *22*, 366-382.

Kumar, A., Verma, L.M., Sharma, S., & Singh, N. (2023). Overview on agricultural potentials of biogas slurry (BGS): Applications, challenges and solution. *Biomass conversion and biorefinery*, *13*, 13729-13769.

Kumar, S., Malav, L.C., Malav, M.K., & Khan, S.A. (2015). Biogas slurry: source of nutrients for eco-friendly agriculture. *International Journal of Extensive Research*, *2*, 42-46.

Liang, F., Shi, Z., Wei, S., & Yan, S. (2023). Biogas slurry purification-lettuce growth nexus: Nutrients absorption and pollutants removal. *Science of the total environment*, *890*, 164383.

Lindsay, W.L. and Norvell, W.A. (1978) Development of a Dtpa Soil Test for Zinc, Iron, Manganese, and Copper. *Soil Science Society of America Journal*, *42*, 421-428. https://doi.org/10.2136/sssaj1978.03615995004200030009x

Liu, Z., Jia, M., Li, Q., Lu, S., Zhou, Z., Feng, L., Hou, Z., & Yu, J. (2023). Comparative analysis of the properties of biochars produced from different pecan feedstocks and pyrolysis temperatures. *Industrial crops and products*, *197*: 116638.

Malav, L.C., Shakeel A.K., Gupta, N., Kumar, S., Bhattacharyya, R., & Malav, M.K. (2015). Effect of Biogas Slurry and Urea on Soil Health. *Journal of Agricultural Physics*, *15*(1), 55-62.

Meena, M., & Biswas, D. (2013). Residual effect of rock phosphate and waste mica enriched compost on yield and nutrient uptake by soybean. *Legume Research*, *36*(5),406-413.

Mia, M. A. B., & Shamsuddin, Z. H. (2010). Rhizobium as a crop enhancer and biofertilizer for increased cereal production. *African Journal of Biotechnology*, *9*(37), 6001–6009.

Mosier, A.R., & Kroeze, C. (2000). Potential impact on the global atmospheric N2O budget of the increased nitrogen input required to meet future global food demands. *Chemosphere-Global Change Sci*ence, *2*, 465–473.

Mukhtiar, A., Mahmood, A., Zia, M.A., Ameen, M., Dong, R., et al. (2024). Role of biogas slurry to reclaim soil properties providing an eco-friendly approach for crop productivity. *Bioresource Technology Reports*, *25*(10), 101716, 1-14.

Musse, Z. A., Yoseph Samago, T., & Beshir, H. M. (2020). Effect of liquid bio-slurry and nitrogen rates on soil physico-chemical properties and quality of green bean (*Phaseolus vulgaris* L.) at Hawassa Southern Ethiopia. *Journal of Plant Interactions*, *15*(1): 207–212.

Naihui, L., Yang, X., Liu, J., Liu, Y., Chen, Q., Wu, F., & Chang, R. (2023). Effect of raw material and application rate of biogas slurry on Cucumber growth, *Fusarium* wilt suppression, and soil properties. *Environmental Technology & Innovation*, *32*, 103396.

Piper, C.S. (1966). *Soil and plant analysis*. Bombay: Hans Publishers.

Rahman, S. M. E., Islam, M. A., Rahman, M.M., & Oh, D.H. (2008). Effect of cattle slurry on growth, biomass yield and

chemical composition of maize fodder. *Asian-Austtralasian Journal of Animal Sciences*, *21*(11), 1592- 1598.

Sanwal, S.K., Lakminarayana, K., Yadav, R. K., Rai, N., Yadav, D.S., & Bhuyan, M. (2007). Effect of organic manures on soil fertility, growth, physiology, yield and quality of turmeric. *Indian Journal of Horticulture*, *64*(4), 444-449.

Seufert, V., Ramankutty, N., & Foley, J.A. (2012). Comparing the yields of organic and conventional agriculture. *Nature*, *485*(7397), 229–232.

Sharma, G.K., Khan, S.A., Srivastava, M., Bhattacharyya, R., Sharma, A., et al. (2022). Phycoremediated N-fertilization approaches on reducing environmental impacts of agricultural nitrate leaching, *Journal of Cleaner Production*, *354*, 1-21.

Skrzypczak, D., Izydorczyk, G., Taf, R., & Moustakas, K. (2023). Cellulose-based fertilizers for sustainable agriculture: Effective methods for increasing crop yield and soil health. *Industrial Crops and Products*, *205*(1429):117500.

Sun, H., Shi, K., Ding, H., Ding, C., Yang, Z., et al. (2024). The effect of biogas slurry application on biomass production and the silage quality of corn. *Animal Biosciene*, *36*(12): 1918-1925.

Tang, J., Yin, J., Davy, A.J., Pan, F., Han, X., Huang, X., & Wu, D. (2022). Biogas Slurry as an Alternative to Chemical Fertilizer: Changes in Soil Properties and Microbial Communities of Fluvo-Aquic Soil in the North China Plain. *Sustainability*, *14*(22), 15099.

Tang, Y., Luo, L., Carswell, A., Misselbrook, T., Shen, J., & Han, J. (2021). Changes in soil organic carbon status and microbial community structure following biogas slurry application in a wheat-rice rotation. *Science of the Total Environment*, *757*, 143786.

Thorat J. C., & More A. L. (2022). The effect of chemical fertilizers on environment and human health. *International Journal of Scientific Development and Research*, *7*(2), 99-105.

Tirado, R., & Allsopp, M. (2012). Phosphorus in agriculture problems and solutions. Technical Report (Review). Netherland: *Greenpeace Research Laboratories Technical Report*. Retrieved from https://www.greenpeace.to/greenpeace/wp-content/uploads/2012/06/tirado-and-allsopp-2012-phosphorus-in-agriculture-technical-report-02-2012.pdf.

Vaidya, G.S., Shrestha, K., & Wallander, H. (2007). Function of organic matter (green manure) and the effect on soil properties. *Banko Janakari*, *17*(2), 62-69.

Vinh, N. Q. (2010). Utilization of liquid bio-slurry as fertilizer for green mustards and lettuces in Dong Nai province.*Vietnam*: *Vietnam Publication*.

Vishwakarma, M., Bangre, J., Khandkar, U.R., Tiwari, S.C., & Vishwakarma, M. (2023). Effect of Inorganic fertilizers and biogas slurry on content and uptake of NPK in wheat grown under sodic vertisols. *International Journal of Plant and Soil Science*, *35*(20), 1197-1260.

Walkley, A. J., & Black, I. A. (1934). Estimation of soil organic carbon by the chromic acid titration method. *Soil Science*, *37*, 29–38.

Wang Z., Sanusi I.A., Wang, J., Ye, X., Kana, E.B.G. Olaniran, A.O., & Shao, H. (2023). *Developments and Prospects of Farmland Application of Biogas Slurry in China—A Review. Microorganisms*, *11*(11), 2675.

Warnars, L., & Oppenoorth, H. (2014). A study on bioslurry results and uses. Hauge: *Deltahage.*

World Health Organization (1992). Environmental Health Criteria 134: Cadmium; World Health Organization: Geneva. Switzerland: *WHO Library Cataloguing in Publication Data.*

Xu, W., Zhu, Y., Wang, X., Ji, L., Wang, H., Yao, L., & Lin, C. (2021). The Effect of Biogas Slurry Application on Biomass Production and Forage Quality of *Lolium multiflorum*. *Sustainability*, *13*(7), 3605.

Yadav, R., Sudhishri, S., Khanna, M., Lal, K., Dass, A., et al. (2023). Temporal characterization of biogas slurry: a pre-requisite for sustainable nutrigation in crop production. *Frontiers in Sustainable Food System*, *7*, 1234472.

Yakout, T., Mostafa, D.M., & Youssef. G. (2014). Utilization of dried biogas digester residue as an organic fertilizer with mineral and bio-fertilizer on growth and yield of sweet peppers. *Alexandria Science Exchange Journal*, *35*(4), 325-333.

Younessi, H.S., Bahramara, S., Adabi, F., & Golpîra, H. (2023). Modeling the optimal sizing problem of the biogas-based electrical generator in a livestock farm considering a gas storage tank and the anaerobic digester process under the uncertainty of cow dung. *Energy*, *270*(1): 126876.

Zhang, H., Xu, B., Zhao, C., Liu, J., Zhao, Y., Sun, S., & Wei, J. et al. (2022). Simultaneous biogas upgrading and biogas slurry treatment by different microalgae-based technologies under various strigolactone analog (GR24) concentrations. *Bioresource Technology*, *351*, 127033.

Zheng, X., Fan, J., Xu, L., & Zhou, J. (2017) Effects of Combined Application of Biogas Slurry and Chemical Fertilizer on Soil Aggregation and C/N Distribution in an Ultisol. *PLoS ONE*, *12*(1), e0170491.

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Impact of Incorporating Argan Cake (*Argania spinosa* L.) and Desalted Anchovy Waste (*Engraulis encrasicolus*) on the Productive Performance of Broiler Chickens (*Gallus gallus*)

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ABSTRACT

Desalted anchovy bones (*Engraulis encrasicolus*) and argan cake are important sources of minerals and animal proteins. The objective of this study was to analyze the respective consequences of their addition to the diet of broiler chickens (*Gallus gallus*). Four groups, each containing 15 chickens, were organized according to the following factorial scheme: four feed treatments (including a control group) x five chickens per treatment x three repetitions (4x5x3). The groups were fed four different feed rations containing varying proportions of desalted anchovy bones (DAB) and argan cake (AC): T (0%DAB/0%AC), L01 (1%DAB/1%AC), L02 (2%DAB/2%AC), and L03 (3%DAB/3%AC). Results of the study revealed a significant difference ($p>0.05$) in weight gain during the start and end of the study for L02 (2144.46g), which was higher compared to the control T (2140.56g). Regarding the feed conversion ratio, L02 (1.54) was lower than the control T (1.65). Conversely, the other feed combinations, including 1% (L01) and 3% (L03), negatively affected weight gain and feed conversion ratio due to the addition of DAB and AC. From the results of the study, it can be concluded that at a low rate of 2% (L02: 2%DAB/2%AC), both desalted anchovy bones and argan cake appear to be effective substitutes for other fish meals and soybean cake in the diet of broiler chickens.

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1 Introduction

Poultry production has grown significantly globally, increasing from 101 million tonnes in 2014 to 133 million tonnes by 2021 (FAO 2021). The broiler poultry sector provides more than 98,000 direct and 225,000 indirect jobs (Abdelmajid et al. 2021). Poultry meat production has experienced an annual growth rate of more than 4% in recent years (Govoni et al. 2021). Poultry is one of the most consumed animal products in the world and plays a crucial role in guaranteeing food security and nutrition (Mottet and Tempio 2017). Current estimates suggest that by 2025, poultry meat production and consumption will surpass that of beef, pork, and mutton (Belkhanchi et al. 2023). Global poultry meat production is projected to reach 331 million tons by 2028 (Oladokun and Adewole 2020). The growing consumption of poultry can be attributed to its affordability and accessibility as a protein source (Belkhanchi et al. 2023). The poultry sector remains crucial as a provider of high-quality proteins, vitamins, and micronutrients essential for human consumption (Oladokun and Adewole 2020). The increased production of white meat demands a significant quantity of feed (Govoni et al. 2021).

Animal feed manufacturing has undergone numerous advancements, transitioning from simple manual formulations to computerized formulations (Alhotan 2021). Animal feed formulations are evidence to trace quality or address consumer claims (Bouchand et al. 2020). Plant by-products are increasingly considered nutrient-rich sources with useful compounds, offering cost-effectiveness and versatility (Taarji et al. 2018). Argan cake (*Argania spinosa* L.) is commonly used in the agri-food sector (Mirpoor et al. 2022). In Morocco, argan cake is indispensable to meet the protein requirements of livestock (Lakram et al. 2019). The argan forest in Morocco covers approximately 800,000 hectares and contains over 20 million trees (Sinsin et al. 2020). Meanwhile, argan oil production volume reached 5,640 tons in 2019 (MAMFRDWF 2020), with exports experiencing a nearly 55% increase from 871 tons in 2012 to 1,348 tons in 2019 (MAMFRDWF 2020). Traditionally, argan cake is used by the Berber community to feed and warm livestock (Mechqoq et al. 2022), including lambs (Moutik et al. 2021). Due to its nutritional properties, the argan tree is highly valued (Koufan et al. 2020) but rarely used in poultry feed. It is proposed that incorporating argan cake could improve the quality of chicken meat while reducing production costs (Boumendil et al. 2023). Therefore, it will be used as an ingredient in our broiler chicken formulation.

On the other hand, desalted anchovy bones present a significant mineral source that can also be repurposed as another ingredient in innovative broiler chicken formulations (Boumendil et al. 2023). National fishery production recorded a volume increase of more than 10% compared to 2021 (Driouichi 2023), with anchovy being one of the most frequently caught species worldwide (Fernandez et al. 2018). In-depth studies on the use of salted fish meal in poultry have not yet been conducted (Hasan et al. 2019). These local resources used in broiler chicken formulations are tested on three groups with 1%, 2%, and 3% ratios.

The composition of poultry feed is determined by several factors, including the nutritional needs of birds at different stages of their lives, available ingredients, costs, and production goals (Belkhanchi et al. 2023). The success of quality feed formulation heavily depends on a good understanding of the physicochemical characteristics of raw materials (Hertrampf and Piedad-Pascual 2012). It is necessary to know the chemical composition, physical characteristics, and digestibility of ingredients used in animal feed. However, the raw materials used for feed formulation depend on the country or region and their potential (Brah et al. 2015). It is recommended that each formulator build their database based on available local or natural feed resources (Burel et al. 2000). The number of nutrients to be considered for formulation varies. Still, the most commonly used for formulation are metabolizable energy, crude protein (Brah et al. 2015), amino acids (Sterling et al. 2005), fatty acids, calcium, and phosphorus (Burel et al. 2000). The maximum and minimum limits of each ingredient must be known to avoid toxicity (Brah et al. 2015). Determining nutrient requirements, including metabolizable energy, crude protein, amino acids, calcium, and phosphorus of poultry feed, is essential for its successful formulation (Belkhanchi et al. 2023). Further, poorly adjusted feed formulation can negate the profit margin of chicken production. It is thus necessary to optimize the feed contents of each essential amino acid (methionine, lysine, threonine, tryptophan) (Alagawany et al. 2021). This step involves calculations to determine the combination of different ingredients that cover the recommended needs for poultry.

Previously, formulation techniques such as empirical and manual methods (Chiba 2009; Omidiora et al. 2013) were employed. Mathematical programming techniques such as linear, nonlinear, multiobjective, and quadratic programming are also employed (Peña et al. 2009; Heydari 2014; Brah et al. 2015). The objective of this study was to meet the nutritional needs of broilers (*Gallus gallus*) and improve production performance using local and natural resources: desalted anchovy bones (*E. encrasicolus*) and argan cake, following a profitable, practical, and less expensive formulation.

2 Materials and Methods

The study was conducted on a certified farm located in the Tnine Chtouka region, El Jadida province, Morocco (N°295 23/VV/09/EL/POU/Ch). Sixty (ROSS 308) broiler chickens (n = 60) were randomly divided into 4 groups according to a factorial design: 4 dietary treatments (including a control group), 5 chickens per treatment, and 3 repetitions (4x5x3). The chickens, selected

from a hatchery at one day old with an initial weight of approximately 40 grams, were fed with starter and grower feed.

The feed used as the control (T) was a commercial feed produced by OMNIA INTAJ, which specializes in manufacturing compound feed for poultry. The poultry houses had ventilation and lighting systems provided by mesh-covered wall openings placed 60 cm above the ground. Temperature and humidity were regularly monitored to ensure an optimal environment for the chickens. Nipple drinkers, brooders, and feeders were installed to provide the chickens with ad libitum feeding and continuous access to drinking water. Unconsumed feed was collected and weighed at regular intervals.

The formulation of the diets was developed using the ALLIX[3] software in collaboration with the CCPA GROUP, a French company specializing in animal nutrition and health. The experimental diets included standard components such as corn and soybean meal, different levels of desalted anchovy bone waste (DAB) and argan cake (AC). The feed rations prepared for the experiment were control T (0% DAB/0% AC), L01 (1% DAB/1% AC), L02 (2% DAB/2% AC), and L03 (3% DAB/3% AC). The chemical compositions of the DAB, AC, and the experimental diets calculated in this study are presented in Tables 1 and 2.

Statistical analysis was performed using IBM SPSS.21 software. Analysis of variance (ANOVA) was conducted to assess zootechnical performance, and differences were considered significant at $p< 0.05$.

3 Results and Discussion

3.1 Composition of the Ingredients Used in This Study

The argan tree, *Argania spinosa* (L.) Skeels, is an endemic forest species in Morocco, widely cultivated for the significant nutritional qualities of its oil (Chakhchar et al. 2022). However, argan cake, a by-product of oil extraction, remains underutilized by farmers despite its nutrient richness. The biochemical composition of argan cake, rich in protein, essential amino acids, and other nutritional elements, makes it a valuable feed ingredient in poultry diets (Lakram et al. 2019).

Broiler chicken farming has experienced significant growth, which affects the birds' skeletal development and leads to leg disorders, resulting in economic losses. Calcium and phosphorus are essential components of chicken bones, and their presence in the diet is crucial, with recommended amounts of 6 to 6.5 g/kg for calcium and 2 to 3.5 g/kg for phosphate (Matuszewski et al. 2020). Anchovy bones are considered an excellent source of organic minerals, particularly calcium at 7.07% and phosphorus at 3.65% (Table 1). Therefore, introducing anchovy bones into broiler chicken diets could improve skeletal health and reduce economic losses related to leg disorders (Savitri et al. 2021).

To evaluate their effectiveness as poultry feed, it is imperative to conduct rigorous experimental studies to determine their impact on poultry growth. The results of these studies will provide precise recommendations regarding the optimal proportions of argan cake

Table 1 Chemical Composition of Various Ingredients Used in the Diets

Compositions	Nutrients	DAB	AC
Chemical (%)	Crude protein (%)	34.50	39.50
	Dry matter (%)	90.90	89.90
	Total phosphorus (%)	03.65	00.65
	Fat (%)	16.10	17.00
	Ash (%)	24.80	04.00
	Crude fiber (%)	00.00	17.20
Mineral (%)	Potassium(%)	01.20	01.00
	Phosphorus(%)	03.65	00.65
	Calcium (%)	07.07	00.50
	Sodium chloride (%)	04.70	00.30
Amino acid (%)	Digestible isoleucine (%)	01.33	00.98
	Digestible lysine (%)	02.37	01.26
	Digestible threonine (%)	01.30	01.04
	Digestible valine (%)	01.56	01.49

DAB: Desalted Anchovy Bones, AC: Argan cake

(AC) and desalted anchovy bones (DAB) to include in broiler chicken diets. The chemical compositions of AC and DAB used in this study are presented in Table 1.

3.2 Formulation and Composition of the Experimental Diets

Formulation software is crucial to optimize the use of raw materials and ensure better incorporation of new ingredients. In this study, a linear program was deemed essential to formulate balanced diets regarding essential nutrients, aiming to improve zootechnical performance and reduce economic losses (Mallick et al. 2020). The design of the feed formulas was carried out using the ALLIX 3 software, developed in collaboration with the CCPA GROUP, a French company specializing in animal nutrition and health.

The formulation results highlighted the variations between the different prepared diets, ensuring that these diets were isocaloric, thereby emphasizing the importance of maintaining consistent energy while optimizing ingredient combinations. Table 2 details the different feed compositions for each phase of broiler chicken production (*Gallus gallus*).

This research indicates that the protein concentration increases from 17.5% in the control group to 17.80% for L01, 19.32% for L02, and 20.52% for L03. In the starter phase, the protein concentration is 20.8% in the control group, while it is 21.2%, 23%, and 23% for groups L01, L02, and L03, respectively. The increase in AC concentration in the diets can also raise the levels of essential minerals, such as calcium, from 0.57% in the control group to 0.62% in the three groups. Potassium levels, however, are 0.817% for L01, 0.806% for L02, and 0.726% for L03, remaining lower than the control group in the growth phase at 0.823%.

In summary, increasing the AC concentration appears to positively impact protein concentration, while calcium levels increase and potassium levels slightly decrease. Based on these observations, it is possible that increasing the AC concentration has distinct effects on the various nutrients present in the diets.

Table 2 Chemical composition of the diets

Period	Composition	Startup Composition	T	L01	L02	L03
Startup	Chemical (%)	Crude protein (%)	20.80	21.20	23.00	23.00
		Total phosphorus (%)	0.636	0.602	0.599	0.562
		Crude fat (%)	3.00	3.231	3.562	2.944
		Ash (%)	5.981	5.806	6.149	6,061
		Crude fiber (%)	4.829	3.063	3.238	2.573
	Mineral (%)	Calcium (%)	0.970	0.970	0.970	0.970
		Sodium chloride (%)	0.150	0.150	0.150	0.150
	Aminoacid (%)	Digestible lysine (%)	1.060	1.100	1.100	1.207
		Digestiblemethionine(%)	0.483	0.508	2.400	30.826
		Digestible valine (%)	0.868	0.902	0.969	0.978
Growth	Chemicals (%)	Crude protein (%)	17.50	17.801	19.321	20.527
		Total phosphorus (%)	0.509	0.468	0.474	0.500
		Crude fat (%)	3.044	2.773	3.385	2.593
		Ash (%)	4.522	4.294	4.561	4.172
		Crude fiber (%)	4.299	3.437	3.861	2.451
	Minerals (%)	Calcium (%)	0.570	0.620	0.620	0.620
		Potassium (%)	0.823	0.817	0.806	0.726
		Sodium chloride (%)	0.162	0.140	0.140	0.140
	Amino acids (%)	Digestible lysine (%)	0.910	0.910	0.968	0.910
		Digestible methionine (%)	0.408	0.401	0.385	0.382
		Digestible valine (%)	0.717	0.735	0.814	0.840

T: 0% DAB/0% AC, L01:1% DAB/1% AC, L02: 2% DAB/2%AC, L03: 3% DAB3%AC

3.3 Analysis of Broiler Chicken (*Gallus gallus*) Zootechnical Performance

Broiler chickens were monitored for 35 days. Figures 1 (initial stage of broilers) and 2 (growth stage of broilers) show the progression of parameters (weight, weight gain, feed consumption, and feed conversion ratio). The results of this study, illustrated by Figure 1, show that the initial average weight of L03 (926.06 g) is almost identical to that of the control group T (926.70 g), unlike L01 (925.70 g), which is slightly lower. However, the average weight of L02 (929.78 g) exceeds the standard value of T (926.70 g). On the 35th day (Figure 2), it was observed that the average weight of chickens in L01 (2137.58 g) and L03 (2137.23 g) was lower than that of chickens in T (2140.56 g). In contrast, the average weight of chickens in L02 at the end of the experiment was higher (2144.46 g) than that of chickens in T (2140.56 g). These results indicate that incorporating DAB and AC into broiler chicken diets in a proportion of L02 (2% DAB/2% AC) can increase chicken weight.

The results of this study show that the total feed consumption during the starter phase varies from 1215.07 g to 1242.44 g. Feed consumption ranges from 3386.05 g to 3443.97 g during the growth phase. Chickens fed with L01 (3386.05 g) and L03 (3443.97 g) had the highest consumption compared to L02 (3304.43 g), which was lower than the control group T (3333.30 g). These results suggest that chickens fed with the L02 diet can grow normally compared to control chickens (T).

Regarding the feed conversion ratio of the three studied groups, L02 (1.54) has the lowest conversion ratio compared to L01 (1.58) and L03 (1.61) and is close to the standard T (1.56). These results are positive, as the weight of L02 is higher than that of the control group T, with a lower feed conversion ratio than the other two diets (L01 and L03).

The obtained results showed that a balanced diet with proteins, essential amino acids, and minerals has a greater advantage in achieving better results than diets with amounts below or above the standards. This can be explained by further research conducted by Aftab et al. (2006), showing that protein percentage is always closely related to the composition of essential amino acids. The same observation was made by Garcia Neto et al. (2000), who found that a diet with 17% protein instead of 24% over 21 days led to a decline in zootechnical performance. This observation is consistent with the composition of our diets, as L02 has the best protein percentage in the starter phase at 23% compared to the control group at 20.8% and L01 at 21.2%. Similarly, in the growth phase, L02's protein percentage (19.32%) is higher than that of the control group and L01, which have 17.05% and 17.8% protein percentages, respectively. Following the recommendations of the producers of the strain used in this investigation(ROSS308 2022), L02 remains within the acceptable protein percentage range, as they recommend 22% in the starter phase and 19-20% in the growth phase (ROSS308 2022).

On the other hand, the desalination method used for DAB allowed the detoxification of argan cake from 4.56 to 0.4 mg/g (reduction

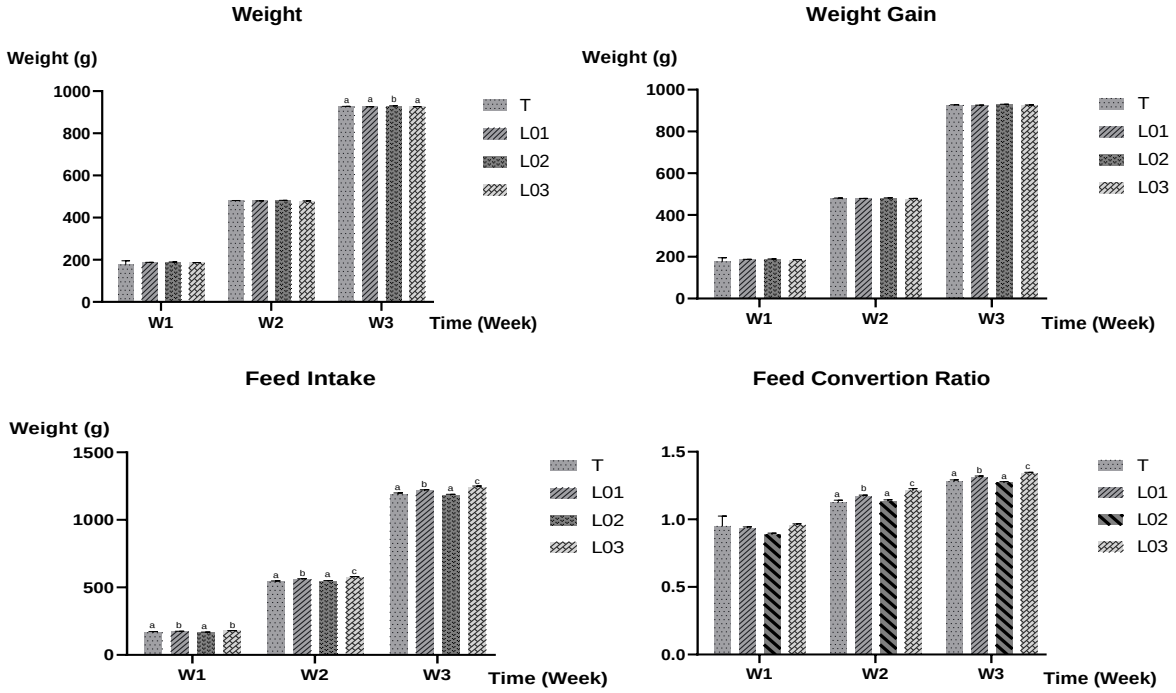

Figure 1 Initial stage of broilers (*G. gallus*)

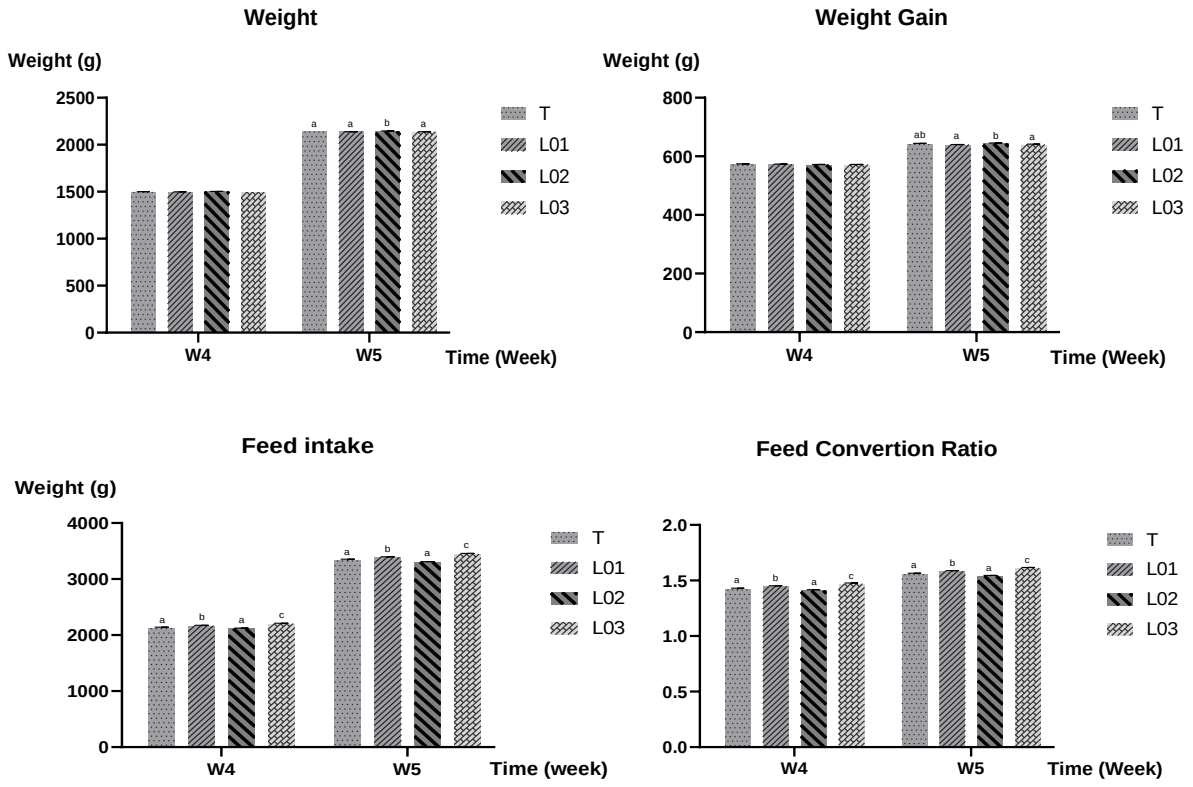

Figure 2 Growth stage of broilers (*G. gallus*)

of antinutritional substances such as saponins)(Lakram et al. 2019). The study of the physicochemical parameters of the by-products and the method of preserving semi-finished fish bones also show that our product meets the poultry needs according to the studied parameters (Boumendil et al. 2019). Indeed, the analyses conducted have demonstrated that our product meets the essential nutritional criteria for poultry growth and health.

Conclusion

The results of this study show that chickens fed diets enriched with AC and DAB at 1% (L01), 2% (L02), and 3% (L03) achieved notable outcomes. The 1% (L01) and 3% (L03) ratios, however, were negatively affected by the addition of DAB and AC, with the average weights of L01 (2137.58 g) and L03 (2137.23 g) being lower than that of the control group T (2140.56 g). In contrast, L02 showed two positive outcomes: an average weight of 2144.46 g, higher than T (2140.56 g), and a lower feed conversion ratio of 1.54 compared to the control group's ratio of 1.56. These results suggest that L02 chickens have better feed conversion efficiency, indicating improved utilization of the nutrients in their diet. Based on these findings, continuing studies exploring the effects of different proportions of DAB and AC in broiler chicken diets would be valuable. Further research could also focus on assessing the nutritional quality of the produced meat.

Reference

Abdelmajid, S., Mukhtar, A., Baig, M. B., & Reed, M. R. (2021). Climate change, agricultural policy and food security in Morocco. In: M. Behnassi, M. Barjees Baig, , M. El Haiba, M.R. Reed (Eds) *Emerging Challenges to Food Production and Security in Asia, Middle East, and Africa: Climate Risks and Resource Scarcity* (pp. 171-196). Springer, Cham. https://doi.org/10.1007/978-3-030-72987-5_7

Aftab, U., Ashraf, M., & Jiang, Z. (2006). Low protein diets for broilers. *World's Poultry Science Journal*, *62*, 688-701.

Alagawany, M., Elnesr, S. S., Farag, M. R., Tiwari, R., Yatoo, M. I., Karthik, K., Michalak, I., & Dhama, K. (2021). Nutritional significance of amino acids, vitamins and minerals as nutraceuticals in poultry production and health - a comprehensive review. *The veterinary quarterly*, *41*(1), 1–29. https://doi.org/10.1080/01652176.2020.1857887

Alhotan, R. (2021). Commercial poultry feed formulation: Current status, challenges, and future expectations. *World's Poultry Science Journal*, *77*, 279-299.

Belkhanchi, H., Ziat, Y., Hammi, M., & Ifguis, O. (2023). Formulation, optimization of a poultry feed and analysis of

spectrometry, biochemical composition and energy facts. *South African Journal of Chemical Engineering*, *44*, 31-41.

Bouchand, C., Nguyen, D., Secretan, P.H., Vidal, F., Guery, R., et al. (2020). Voriconazole topical cream formulation: evidence for stability and antifungal activity. *International Journal of Antimicrobial Agents*, *56*, 106083.

Boumendil, I., Baggar, A., Yassif, F. Z., Fechtali, T., & Safi, A. (2019). Physicochemical parameters of moroccan fish salted waste desalting of natural process. In M. Ezziyyani, (Eds), *Advanced Intelligent Systems for Sustainable Development (AI2SD'2019)*: *Advanced Intelligent Systems for Sustainable Development Applied to Environment, Industry and Economy, 2020* (pp 514-519). Springer, Cham. https://doi.org/10.1007/978-3-030-36671-1_46.

Boumendil, I., Baggar, A., Sisouane, M., El Haimer, Y., Bounouar, N., et al. (2023). Desalting of anchovy residues salted with argan cake for use in poultry feed: use of surface response methodology (RSM). *Chinese Journal of Medical History / Zhong Hua Yi Shi Za Zhi*, *53* (4).

Brah, N., Houndonougbo, M., & Issa, S. (2015). Etapes et méthodes de formulation d'aliment de volaille: Une synthèse bibliographique. *International Journal of Biological and Chemical Sciences*, *9*, 2924-2931.

Burel, C., Boujard, T., Tulli, F., & Kaushik, S. J. (2000). Digestibility of extruded peas, extruded lupin, and rapeseed meal in rainbow trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima*). *Aquaculture*, *188*, 285-298.

Chakhchar, A., Ben Salah, I., El Kharrassi, Y., Filali-Maltouf, A., El Modafar, C., & Lamaoui, M. (2022). Agro-fruit-forest systems based on argan tree in Morocco: A review of recent results. *Frontiers in Plant Science*, *12*, 783615.

Chiba, L. I. (2009). Animal nutrition handbook. *Section*, *12*, 316-331.

Driouichi, Z. (2023). Guide D'investissement Dans Le Secteur De La Peche Maritime Et De L'aquaculture Au Maroc. In D. D. L. P. Maritime (ed.) 1 ed.: Ministere De L'agriculture Et De La Peche Maritime.

FAO (2021). Gateway to poultry production and products. 2021 ed. Retrived from https://www.fao.org/poultry-production-products/production/en/: FAO.

Fernandez, G., Cabral, A., Andreoli, M. F., Labarthe, A., M'Kadmi, C., et al. (2018). Evidence Supporting a Role for Constitutive Ghrelin Receptor Signaling in Fasting-Induced Hyperphagia in Male Mice. *Endocrinology*, *159*(2), 1021–1034. https://doi.org/10.1210/en.2017-03101

Garcia Neto, M., Pesti, G. M., & Bakalli, R. I. (2000). Influence of dietary protein level on the broiler chicken's response to methionine and betaine supplements. *Poultry science*, *79*(10), 1478–1484. https://doi.org/10.1093/ps/79.10.1478

Govoni, C., Chiarelli, D. D., Luciano, A., Ottoboni, M., Perpelek, S. N., Pinotti, L., & Rulli, M. C. (2021). Global assessment of natural resources for chicken production. *Advances in Water Resources*, *154*, 103987.

Hasan, B., Putra, I., Suharman, I., Iriani, D., & Muchlisin, Z. A. (2019). Growth performance and carcass quality of river catfish Hemibagrus nemurus fed salted trash fish meal. *The Egyptian Journal of Aquatic Research*, *45*, 259-264.

Hertrampf, J. W., & Piedad-Pascual, F. (2012). *Handbook on ingredients for aquaculture feeds*, Springer Science & Business Media.

Heydari, S. G. (2014). Effect of linear and random nonlinear programming feed formulating on performance of broilers. *Journal of Novel Applied Sciences*, *3*, 1426-1429.

Koufan, M., Belkoura, I., Mazri, M. A., Amarraque, A., Essatte, A., Elhorri, H., Zaddoug, F., & Alaoui, T. (2020). Determination of antioxidant activity, total phenolics and fatty acids in essential oils and other extracts from callus culture, seeds and leaves of *Argania spinosa* (L.) Skeels. *Plant Cell, Tissue and Organ Culture*, *141*, 217-227.

Lakram, N., Moutik, S., Mercha, I., Maadoudi, E. H. E., Kabbour, R., et al. (2019). Effects of the inclusion of detoxified argan press cake in the diet of dairy goats on milk production and milk quality. *Turkish Journal of Veterinary & Animal Sciences*, *43*, 323-333.

Mallick, P., Muduli, K., Biswal, J. N., & Pumwa, J. (2020). Broiler poultry feed cost optimization using linear programming technique. *Journal of Operations and Strategic Planning*, *3*, 31-57.

Mamfrdwf (2020). Filière Arganier.

Matuszewski, A., Łukasiewicz, M., & Niemiec, J. (2020). Calcium and phosphorus and their nanoparticle forms in poultry nutrition. *World's Poultry Science Journal*, *76*, 328-345.

Mechqoq, H., Hourfane, S., El Yaagoubi, M., El Hamdaoui, A., Da Silva Almeida, J. R. G., Rocha, J. M., & El Aouad, N. (2022). Molecular docking, tyrosinase, collagenase, and elastase inhibition activities of argan by-products. *Cosmetics*, *9*, 24.

Mirpoor, S. F., Giosafatto, C. V. L., Mariniello, L., D'agostino, A., D'agostino, M., Cammarota, M., Schiraldi, C., & Porta, R. (2022). Argan (*Argania spinosa* L.) seed oil cake as a potential source of protein-based Film Matrix for Pharmaco-Cosmetic Applications. *International Journal of Molecular Sciences*, *23*, 8478.

Mottet, A., & Tempio, G. (2017). Global poultry production: current state and future outlook and challenges. *World's Poultry Science Journal*, *73*, 245-256.

Moutik, S., Benali, A., Bendaou, M., Maadoudi, E. H., Kabbour, M. R., El Housni, A., & Es-Safi, N. E. (2021). The effect of using diet supplementation based on argane (*Argania spinosa*) on fattening performance, carcass characteristics and fatty acid composition of lambs. *Heliyon*, *7*(2), e05942. https://doi.org/10.1016/j.heliyon.2021.e05942

Oladokun, S., & Adewole, D. I. (2020). In ovo delivery of bioactive substances: an alternative to the use of antibiotic growth promoters in poultry production—a review. *Journal of Applied Poultry Research*, *29*, 744-763.

Omidiora, E. O., Adegoke, B., & Aderounmu, G. (2013). Review of livestock feed formulation techniques. *Journal of Biology, Agriculture and Healthcare*, *3* (4), 69-76.

Peña, T., Lara, P., & Castrodeza, C. (2009). Multiobjective stochastic programming for feed formulation. *Journal of the Operational Research Society*, *60*, 1738-1748.

ROSS308 (2022). Normes Et Valeurs Nominales Pour Le Poulet De Chair ROSS308 Belgique: AVIAGEN EPI NV.

Savitri, I., Tohata, V., Sormin, R., Lalopua, V., Mailoa, M., & Rieuwpassa, F. (2021) Chemical properties of dried anchovy (*Stolephorus* sp) from Buru Island. *IOP Conference Series: Earth and Environmental Science*, *797*, 012022. doi:10.1088/1755-1315/797/1/012022

Sinsin, T. E., Mounir, F., & El Aboudi, A. (2020). Conservation, valuation and sustainable development issues of the Argan Tree Biosphere Reserve in Morocco. *Environmental & Socio-economic Studies*, *8*, 28-35.

Sterling, K., Vedenov, D., Pesti, G., & Bakalli, R. (2005). Economically optimal dietary crude protein and lysine levels for starting broiler chicks. *Poultry Science*, *84*, 29-36.

Taarji, N., Da Silva, C. A. R., Khalid, N., Gadhi, C., Hafidi, A., et al. (2018). Formulation and stabilization of oil-in-water nanoemulsions using a saponins-rich extract from argan oil press-cake. *Food Chemistry*, *246*, 457-463.