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INHIBITION OF SOME HUMAN BACTERIAL PATHOGENS USING *Streptomyces* sp. SD5 OBTAINED FROM SOIL SAMPLE FROM JEDDAH

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ABSTRACT

For decades, antibiotics are wonder drugs which treat different microbes and human pathogens such as pneumonia, tuberculosis and gonorrhea which are harder and difficult to treat. Due to miss use of antibiotics in agriculture and animal husbandry, antibiotics are becoming less effective and microbes became more resistant. This resistant increased every second, thus this study aimed to produce active antibiotic from soil actinomycetes which might play a highly significant role in medicine. About 15 bacterial isolates were obtained on starch nitrate agar medium from different soil samples. They were screened for antibacterial production against 5 different human pathogens, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis* and Methicillin-resistant *Staphylococcus aureus* (MRSA). The most active isolate was SD5 which showed the highest inhibition against *E. coli, E. faecalis* and MRSA was morphologically examined and characterized. Using molecular identification technique, it was reported that isolate SD5 belonging to genus *Streptomyces* and was similar to *Streptomyces geysiriensis* with 97% similarity and to *Streptomyces* sp. JSM147777 with 95% similarity. Maximum production of the antimicrobial agent was determined by measuring the diameter of inhibition zone using starch nitrate broth, prepared at pH 6.5 and incubation temperature at 30°C for 5 days. The antimicrobial agent was extracted by using three different organic solvents (ethyl acetate, n-Butanol and Petroleum ether). The best solvent was ethyl acetate which gave maximum inhibition against *E. faecalis, E. coli* and Methicillin-resistant *S. aureus.* In conclusion, actinomycetes especially

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genus *Streptomyces* obtained from soil still contained new isolates with excellent antimicrobial activities. Ethyl acetate is a good solvent for antimicrobial agent extraction (MIC ranged from 75-100 µg/ml) and no toxicity was recorded for all tested organic extracts at the different concentrations using *Artemia salina* as test organism. Purification and characterization of the antimicrobial agent must be carried out to obtain new material active against Methicillin-resistant *S. aureus*.

1 Introduction

Bacterial infection and antibiotic resistance are major health problems that cause human death. It is very important to use antibiotics in a rational manner to improve patient outcomes and minimize the appearance of antimicrobial resistance. Several reasons made the inappropriate use of antibiotic is costly because it was inefficacy, increased morbidity and mortality, extra drugs are used unnecessarily for long period of time and increased rates of resistance in bacteria (Luna et al., 2010). Antibiotic consumption is correlated with the level of bacterial resistant. In recent years, Methicillin-resistant *S. aureus* (MRSA) became resistant to almost all antibiotic, thus cause serious skin and underlying tissue infections (Peacock & Paterson, 2015). Alternative therapies with new antibiotics from natural sources are needed to solve these problems. One of the most famous antibiotics is Streptomycin, produced from Actinomycetes, played a significant role in drug discovery programs and often leads to discoveries of newer antimicrobial agents from different isolates, versatility and immense economic value (Berdy, 2005). Further, it was reported that the most promising resource for antibiotics is actinomycete groups which undoubtedly considered as important resources of active by products (Agwa et al., 2000). Scientist considered Actinomycetes, especially genera *Streptomyces* and *Micromonospora* as the most economically and biotechnologically useful microorganisms (Torsvik & Ovreas, 2002; Pandey et al., 2004; Frieden, 2013). They are extremely noteworthy and considered a sustain supply of new antibiotics that kill pathogens without disturbing the host cells (Ghanem & Aly, 2003; Aly & Sabbagh, 2004; Amer et al., 2006). These antibiotics have different mode of action and poses antibacterial, antifungal antitumor and wound healing properties and each one has unique mode of action (Amer et al., 2006; Rabbah et al., 2006; Rabbah et al., 2007; Jiao et al., 2013; Janardhan et al., 2014). More than two hundreds of antibiotics such as Beta-lactam peptide antibiotics, macrolide, tetracyclines, aminoglycosides, daptomycin and tigecycline were used and half of them are obtained from actinomycetes (Kekuda et al., 2010; Naine et al., 2011; Kaur & Narayan, 2014; Kaur & Chate 2015). Actinomycetes act as factory microbes for production of many important antibiotics, different vitamins, enzymes, enzyme inhibitors, and siderophores in addition to many secondary products with pharmaceutical and clinical applications (Koehn & Carter, 2005; Aly et al., 2013; Tork et al., 2018). The discovery of new antibiotics for bacterial resistance especially to MRSA, which caused serious public health problem, has continued in many countries. This paper aimed to isolate an antibiotic from actinomycetes with emphasis on the isolation source, extraction and antimicrobial activity.

2 Materials and Methods

2.1 Soil sample collection

Ten different soil samples were collected from hospital, house and University gardens, located at Jeddah, Saudi Arabia. These soil samples were dried and used to isolate some actinomycetes on starch nitrate agar (Shirling & Gottlieb, 1966) at 30°C.

2.2 Tested bacterial pathogens

Standard bacterial isolates that are known for being involved in the pathogenesis of human were collected from King Faisal Hospital and Research Center, Jeddah, Saudi Arabia. These bacterial isolates were *E. feacalis*, *S. aureus* (MRSA), *P. aeruginosa*, *E. coli* and *K. pneumonia*.

2.3 Isolation of actinomycetes from soil samples:

From collected soil samples, different actinomycete isolates were obtained on starch nitrate agar (SNA) medium. One gram of the soil sample was suspended in 9.0 ml of distilled water, the obtained suspension was mixed well and serial dilution was carried out up to the 10^{-3}. From this suspension, 0.5 ml of suspension was spread on plates of medium. All plates were incubated at 30°C for 4 days. All the obtained colonies were purified on the same medium until pure colonies were obtained. All the purified colonies were transferred to slants of starch nitrate agar and refrigerated at 4°C to preserve the isolates from 3 to 6 months. For longer preservation (more than six months), the selected pure isolates were kept in Tryptic soy broth with glycerol and stored in deep freezer at -80°C until used.

2.4 Screening of Actinomycetes isolates for antibacterial activities on solid medium

Screening of actinomycete isolates for antibacterial activities was done by agar disc diffusion method on Mueller Hinton agar, obtained from Sigma-Aldrich, (Hindler & Inderlied, 1985). A 7 mm diameter disk of the tested actinomycete isolate was bored by cork- borer from the SNA medium that were incubated at 30°C for 4 days. This disc was placed on MHA plate which was previously

inoculated with the tested bacterium. Then, all plates were incubated at 37°C for 24 hrs. After incubation of the Mueller Hinton agar plates, the obtained inhibition zone was measured three times in mm and mean value was recorded for each bacterium. The isolates of actinomycetes that gave the largest inhibition zone were selected and grown in starch nitrate broth medium for 4 days and culture filtrates were tested for any antimicrobial activates against the tested bacterial pathogens Westley et al. (1979).

2.5 Preparation of the preculture

Preculture was used to inoculate the main medium with a constant number of the tested bacterium. Starch nitrate broth medium was used as preculture medium for the growth of bacterial isolates. In 250 ml Erlenmeyer flasks containing 50 ml of the fresh sterile starch nitrate broth, 2 ml of the selected bacterium suspension were added. The flasks were incubated at 30°C on shaker incubator for 4 days. Each 2 ml of the preculture was used to inoculate each flask containing 48 ml of the prepared medium.

2.6 Bacterial growth in liquid medium

All bacterial isolates were named and numbered, SD1 to SD15, then all isolates were screened for antibacterial agent production and isolate SD5 was the most active. Each flask of starch nitrate broth was inoculated with the selected isolate SD5 and the flasks were incubated at 30°C for a period of 4 days on shaking incubator (120 rpm). Cells were collected after centrifugation at 5000 rpm for 15 min. the culture filtrate was sterilized using 0.22 bacterial filter and its antibacterial Activity was determined using agar well diffusion assay.

2.7 Determination of the Antibacterial Activity of chosen isolate SD5

Cells of all tested bacterial pathogens were suspended in sterile normal saline, adjusted at 0.5 McFarland turbidity standards and 100 µl of this suspension was used to inoculate on MH agar plates using sterilized cotton swabs. More than one well was done in each plate using sterile cork borer and 100 µl from the culture filtrate of the isolate SD5 were added to each well (7 mm diameter holes cut by cork borer in the MH agar). All plates were incubated at 37°C for 24 h. After incubation, bacterial growth was observed and inhibition zone diameter was measured and recorded in mm.

2.8 Determination of minimum inhibitory concentration (MIC) by Broth microdilution method

The minimum inhibitory concentrations (MICs) were determined using Broth microdlution method as described by Bonnavero et al. (1998) with some modifications. This test was carried out to determine MIC of the tested cultured extract against the selected bacteria pathogens. Nutrient broth was used to grow the bacteria overnight and the growth was diluted to approximately 10^4 cfu/ml and 7 drops of phenol red as a colorimetric indicator was added to clarify the end point by color change from yellow to pink. Nutrient broth with some drops of phenol red indicator was added into 12 wells in a micro titer plate (125µl/well), then 125µl of the selected culture extract along with DMSO was added to well no. 1 and the mixture was mixed. For serial dilution, about 125µl of the well no.1 was transferred to well 2 and so on and keep diluting the mixture, in this manner through well no. 11. No culture extracted mixture was transferred to well no. 12 (control). Three replicates were prepared and the microtiter plate was incubated at 37°C overnight. MIC was determined by the concentration (µg/ml) changing in color of the broth from yellow to pink (NCCLS, 2002).

2.9 Measuring of bacterial growth in liquid medium

Detection of bacterial growth in culture filtrates by measuring the optical density at 520 nm using UV spectrophotometer by adding 3 ml from the fresh filtrate in sterile cuvette. All observations were carried out in triplicate and averages were calculated.

2.10 Optimization factors of culture conditions and producing of antimicrobial activities

Growth estimation (determined by the optical density at 625 nm using UV spectrophotometer) and antibacterial activities (measured by agar well diffusion method on MH agar plates) were determined in the end of study. Effect of different broth media, pH value, temperate and incubation time on growth and antibacterial production was determined (Agwa et al., 2000). The tested medium was prepared in 250 ml flasks with 50 ml of the selected medium and inoculated with 2 ml of the prepared preculture of SNB and incubated in shaking incubator (120 rpm). Three replicates were maintained for each factor. Effect of different culture media such as starch nitrate broth (Shirling & Gottlieb, 1966), GBA-3 broth medium (Agwa et al., 2000), Emerson medium (Agwa et al., 2000), Omura medium (Omura et al., 1982), yeast extract starch peptone medium (ATCC 435) and Nutrient broth on microbial growth or antibiotic production was detected. The effect of different initial pH values, (pH 5, 5.5, 6. 6.5, 7 and 7.5) different incubation temperatures (20-45°C) and different incubation periods (2-7 days) on biomass and production of antimicrobial activity was determined.

2.11 Extraction of the antibacterial compound produced by the selected isolate

Extraction of the antimicrobial compound from the supernatant with equal volumes of different solvents (Ethyl acetate, Petroleum ether or n-Butanol) was carried. The solvent was evaporated, the resulting material was dissolving in one ml of DMSO and the antimicrobial activity against of gram-positive and gram-negative bacteria was determined using agar well diffusion assay. The antibacterial activities of DMSO (negative control) and Ampicillin (positive control) were also determined.

2.12 Identification of the selected bacterial isolate SD5

2.12.1 Morphological and physiological characterizations of the bacterial isolate SD5

The best antibacterial activities was selected and identified. The selected bacterial isolate SD5 was cultivated on Starch nitrate agar and identified according to morphology, physiology and biochemical characters. The cellular morphology of the bacterial isolate SD5 was examined under light microscope and scanning electron microscope. Cell shape, colony color and spore shape were recorded (Aly et al., 2013).

2.12.2 Isolation of genomic DNA and PCR amplification of 16S rRNA

The DNA of the isolate SD5 was extracted by the method given by Otsuki et al. (1996). For this, a loop full of cells from SD5 bacterial colony grown on an overnight LB agar plate (Hi media) at 37°C was transferred to 1.5 ml eppendorf tube with 200 µl sterile distilled water. It was boiled for 20 min, frozen and thawed twice centrifugation was performed at 12,000 rpm for 5 min. Crude DNA extract of cells (fresh preparation) from this strain was obtained and subjected to PCR amplification using DNA thermal cycler (Perkin Elmer, USA). The primers were designed based on the highly conserved region of 16S rRNA from various bacteria (Weisburg et al., 1991). The following primers 5′- AGTTTGATCATGGTCAG-3′ and 5′-GGTTACCTTGTTACGACT 3’ were used to amplify 16S rRNA gene. The purified PCR product of 1,517 bp was sequenced and analyzed by using DNA sequencer ABI PRISM 310 genetic analyzer (Perkin Elmer, USA). The DNA sequence was compared to the Gen Bank database in the National Center for Biotechnology Information (NCBI) using the BLAST program.

2.13 Cell toxicity using *Artemia salina* as test organism

Toxicity of the culture filtrate was determined against *Artemia salina* (test larva). The toxicity of the obtained extract in DMSO was conducted using larvae of a microcrustacean species, *A. salina* as test organism as described by Sangian et al. (2013). Eggs of *A. salina* were suspended in sterile sea water (pH 8.5). Eggs were allowed to hatch under light at 25ºC with continuous aeration. After 48 hr of cyst hatching, ten living larvae were collected and put in different concentrations of the tested material and every treatment was prepared three times. DMSO was used as negative control while 0.1% solution of $CuSO_4$ was used as positive control. The percentages of surviving or dead larvae were determined after 8 hr and lethal dose (LD50) was determined according to Meyer et al. (1982) and Aly & Gumgumjee (2011).

2.14 Statistical analyses

The statistical data are expressed as means plus standard deviation and one-way ANOVA was used for statistical to compare the results. Tukey test (t- test) was considered significant at $p \leq 5\%$.

3 Results and Discussion

Out of ten soil samples, 15 isolate of actinomycetes were obtained from soil of the hospital garden, house garden and university garden, located in Jeddah, Saudi Arabia (Table 1). These isolates have different colors and produced different pigments. The growth

Table 1 Source, color, growth and pigment production of the actinomycete isolates, obtained on starch nitrate agar medium

Isolate	Source	Colony color	Growth	Pigment	Antimicrobial activities *(E. coli)*
SD1	Hospital garden	Gray to white	Heavy	Black	+
SD2	Hospital garden	White to gray	Heavy	No pigment	+
SD3	Hospital garden	Off white	Heavy	Pink	-
SD4	Hospital garden	Off white	Heavy	Dark pink	-
SD5	Hospital garden	Gray	Moderate	Orange	++
SD6	Hospital garden	Gray	Heavy	Orange	+
SD7	Hospital garden	Gray	Moderate	yellow	+
SD8	University garden	White	Light	No pigment	++
SD9	University arden	Gray	Heavy	White	-
SD10	Hospital garden	Gray to pink	Heavy	Dark pink	+
SD11	Hospital garden	black	Heavy	Bright green	++
SD12	Hospital garden	Dark gray	Heavy	Green	-
SD13	Hospital garden	white	Moderate	Light yellow	-
SD14	House garden	Orange	Heavy	Yellow	+
SD15	House garden	White	Moderate	Dark pink	++

-: no inhibition, +: Moderate inhibition (8-10 mm), ++: Good inhibition (11-21 mm)

Table 2 The antimicrobial activities of 4 isolates of Actinomycetes against different bacterial pathogens

Tested isolate	Mean diameter of inhibition zone measured by mm				
	Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	MRSA
SD5	20±0.22*	14±1.23*	13±0.29*	21 ±0.42*	19 ±0.29*
SD9	11±0.42*	11±0.67*	11±0.89*	12±0.44*	14±1.34
SD11	15±0.27*	14±0.99*	14±0.77*	11±0.67*	11±0.79
SD15	14±1.02*	11±0.45*	12±0.46*	11±0.88*	11±1.95
Ampicillin (control)	39±0.44	34±1.21	43±0.20	29 ±0.31	11 ±0.11

*: significant results at $p < 0.05$ compared to control, MRSA: Methicillin-resistant Staphylococcus aureus

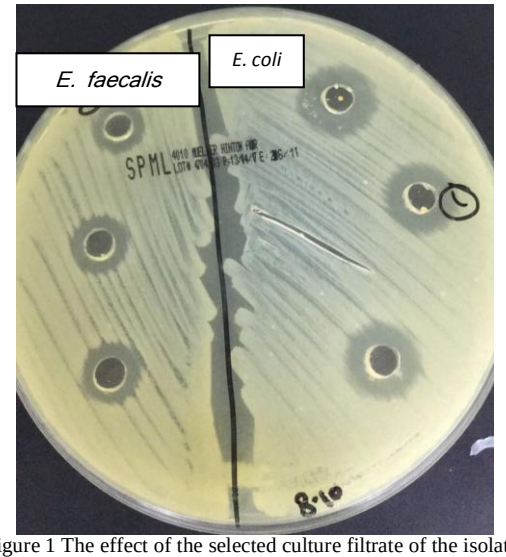


Figure 1 The effect of the selected culture filtrate of the isolate SD5 on two human bacterial isolates, *E. coli* and *E. faecalis*

of the isolated microorganisms on starch nitrate agar was heavy, moderate or poor. In many clinical microbiology laboratories, agar disk-diffusion method is the official method used for routine antimicrobial susceptibility testing. The disk diffusion method was used where agar disc of heavy growth was transferred to a solid medium, inoculated with the test pathogen, appearance of an inhibiting growth halo around the disc, meaning positive results. The diameter of the inhibition zone is proportional to the activity of the tested isolate in antibiotic production (Salami, 2004). Ebadi et al. (2018) used the same technique for isolation of active actinomycetes in antibiotics productions. The isolates SD 5, 9, 11 and 15 showed excellent antibacterial activities against the five tested bacteria (Table 2). The isolate SD5 was the most active isolate against *E. coli, E. faecalis K. pneumoniae, P. aeruginosa*, and MRSA. Figure 1 showed the activities of the isolate SD5 against *E. coli* and *E. faecalis*. The selected actinomycete was identified using morphological and physiological characteristics. Isolated microorganism was Gram positive filamentous bacterium, bearing aerial and substrate mycelia, grew well on starch nitrate medium (Figure 2). It belongs to the grey series

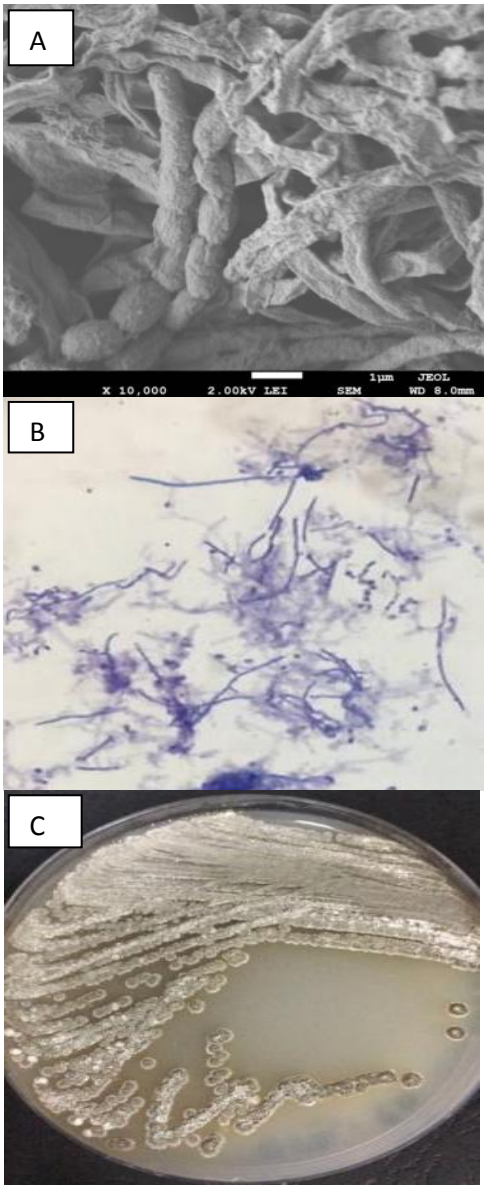


Figure 2 The selected actinomycete SD5 under scanning electron microscope (A), Light microscope (B) and on starch nitrate agar (C)

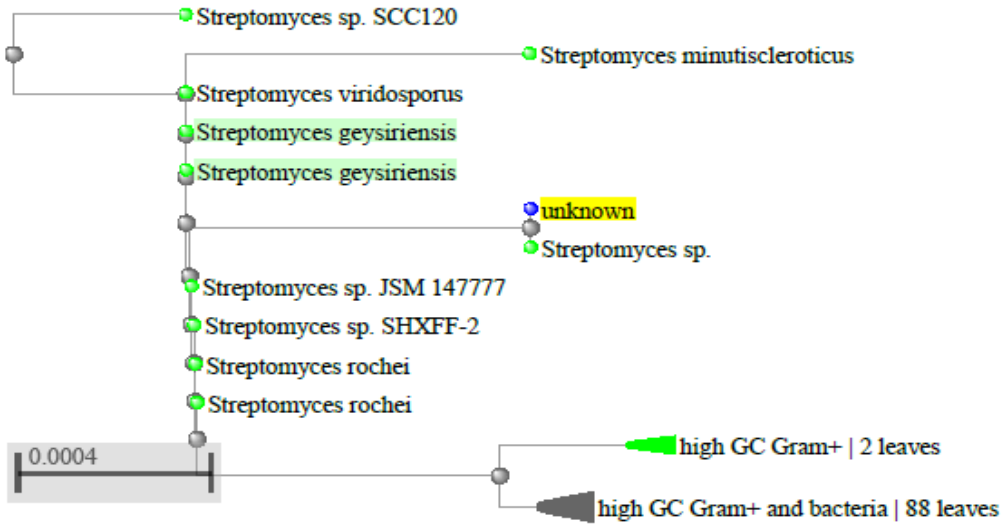


Figure 3 Phylogenetic tree of the selected isolate SD5 with the most related genera

Table 3 Effect of different culture media, medium pH, incubation temperature and incubation period on the growth (A_{520nm}) of *Streptomyces* SD5

Media	Biomass (A520nm)	pH	Biomass (A520nm)	Temp. (°C)	Biomass (A520nm)	Incubation period	Biomass (A520nm)
Yeast extract starch peptone	1.29±0.06	5.0	1.16±0.06	20°C	0.768±0.06	2 days	0.22±0.36
GBA-3	1.70±0.12*	5.5	0.97±0.16*	25°C	0.911±0.30*	3 days	1.54±0.41*
Emerson	1.67±0.25*	6.0	1.26±0.26*	30°C (control)	1.90±0.64	4 days (control)	1.85±0.33
Omura	0.81±0.23*	6.5	1.80±0.11	37°C	0.916±0.09*	5 days	1.63±0.21*
Starch nitrate	1.85±0.26*	7.0 (control)	1.85±0.42	40°C	0.828±0.08*	6 days	1.49±0.04*
Nutrient broth (control)	1.05±0.56	7.5	1.17±0.11*	45°C	0.928±0.17*	7 days	1.38±0. 63*

*: significant results at $p \leq 0.05$

actinomycetes. It was identified as species belong to genus *Streptomyces*. Results of molecular identification revealed that the identified was very similar to *Streptomyces* sp. SCC120, *S. minutiscleroticus*, *S. viridosporus*, *S geysiriensis*, *Streptomyces* sp. JSM 147777, *Streptomyces* sp. SHXFF-2 and *S. rochei* (Figure 3). Aliero et al. (2018) used molecular characterizations for identification of actinomycete isolates with antimicrobial activities. The unique group of gram-positive, spore forming bacteria with abilities of mycelia formation which named actinomycetes is the richest reservoir of secondary metabolites especially medicinal antibiotics (Ebadi et al., 2018). The most important antibiotics, streptomycin, gentamycin, rifamycin and erythromycin are obtained from these bacteria and many pharmaceutical and agricultural industries are depending on these well established antibiotic drugs (Kumar et al., 2010). These actinomycetes were mainly isolated from soil, wastes and waters (Jeffrey, 2008; Aly et al., 2012; Aly et al., 2013; Aly et al., 2015). The best genus of soil actinomycetes for antibiotics production is genus *Streptomyces* where all species of this genus produced potent secondary metabolites.

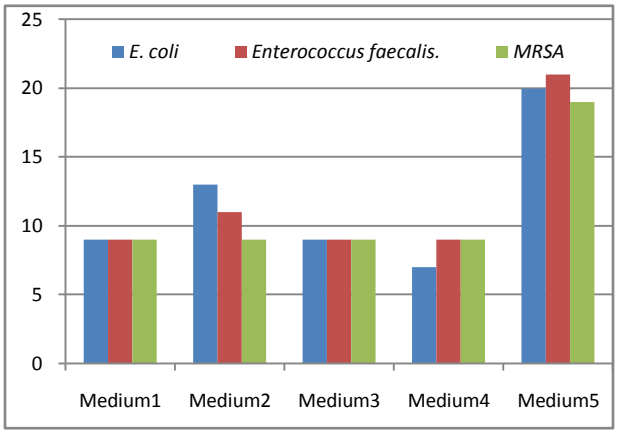
Factors affecting growth and antibiotic productions were studied for optimization the growth conditions for maximum antibiotic production. The growth of the selected bacterium was determined in different media, incubation temperature, pH values and incubation period. At the end of the growth period, the growth was determined by the absorbance at 520 nm (Table 3). High growth was recorded in starch nitrate broth, GBA-3 broth, Emerson medium and yeast extract starch peptone medium while poor growth was recorded in Omura medium and moderate growth was observed in nutrient broth medium. It was found that the selected bacterium showed maximum growth on starch nitrate medium at pH 6.5 and pH 7 and incubation temperature at 30°C for incubation period of 4 days (Table 3).

Similarly, the antimicrobial activities of the isolate SD5, determined by the diameter of the inhibition zone (mm) on the agar plate, previously inoculated of the selected bacterial pathogen, was affected by growth conditions. The growth inhibition was recorded against *E. coli*, *E. faecalis* (Gram negative) and MRSA (Gram positive). It was well reported that *E.*

faecalis considered as predominant species but now in many US hospitals 90% of clinical isolates were reported to cause nosocomial and healthcare associated infections (Laverde Gomez et al., 2011). Infection with *E. coli* usually associated with bacteraemia and is the common cause of urinary tract infections which may cause tachycardia, fever, tachypnoea and delirium (Lee et al., 2018). Although, *E. coli* infections in some cases cause uremia, liver failure, acute respiratory infections, coma and death, endotoxin led to intravascular coagulation and death (Madappa, 2014; Woodward, 2015; Zaman et al., 2015). Similarly, MRSA is a potent bacterial infection caused many pneumonia cases which were acquired during hospital and healthcare stay. Initial empiric therapy considered MRSA as possible etiologic agents (American Thoracic Society, 2005; Shittu et al., 2009). MRSA may cause Healthcare-related pneumonias, hospital-acquired pneumonia and ventilator-associated pneumonia. Strains of *S. aureus* have resistance against some commonly used classes of antibiotics including penicillins and cephalosporins, which used to treat both clinic and hospital patients (Moran et al., 2006; Hyun et al., 2009). For the treatment of MRSA, the current arsenal of antibiotics available are limited and used intravenously. In Latin American hospitals between 2003 and 2008, Sader et al. (2009) studied the resistant pattern of antibiotic among Gram-positive bacteria obtained from bloodstream, skin and infected soft tissue. The antibiotic, mupirocin inhibited synthesis of protein and ribonucleic acid (RNA) in cells of bacteria prolonged and intense use of mupirocin (including for decolonization purposes) has resulted in certain MRSA strains developing resistance to this antibiotic (Shittu et al., 2009). According to Helmi et al. (2013), discovering new agents for MRSA resistant isolates is very important.

Five different growth medium were tested but starch nitrate broth medium was the best medium for antimicrobial agent production by the isolate SD5 against the three tested bacterial pathogens, MRSA, *E. faecalis* and *E. coli* (Figure 4). Previous study suggested that starch nitrate broth medium enhanced enzyme and antimicrobial production against many pathogens (Agwa et al., 2000; Tork et al., 2018). Aliero et al. (2018) reported that optimization of culture conditions including medium components, initial pH value, incubation temperature and time are important for the production of antimicrobial agents from actinomycetes obtained from soil of Western Uganda. Using shake flask cultures and eight different media, modified nutrient broth supplemented with soluble starch and glycerol supported bioactive compound production by actinomycetes. Aqueous and ethanol extracts gave optimum bioactive activity for all the three organisms. The identification of 16SrDNA gene showed that, the three isolates belong to phylum actinobacteria into the genus Streptomycetes. This study showed that media compositions, cultural conditions and solvents for extraction play an important role in bioactive compound production in these actinomycetes isolates.

It was found that the best initial pH value was pH 6.5 at which the highest diameter of inhibition zone was recorded against the three tested bacterial pathogens (Figure 5). Throughout the growth period, pH value of the medium was changed while the best growth and antibiotic production are noticed at initial pH 7.0 which became pH 5.9 or pH 9.75 depending to the strain (Wang et al., 2011). Isaacson & Webster (2002) noticed that *Xenorhabdus* sp. RIO showed the best growth in culture broth at pH 7.07 which decreased to pH 6.89 during the first few hours of growth and the best pH value for growth varied from 6.5 to 8.5. Similarly, Yang et al. (2001) establish that initial pH value played an important role in the antibiotic production by *Xenorhabdus nematophila*. Moreover, incubation of the flasks at 30°C showed the best growth inhibition of the three tested bacterial pathogens



M1: Yeast extract starch peptone, M2: GBA-3, M3: Emerson medium, M4: Omura Medium, M5: Starch nitrate

Figure 4 Effect of different culture media after 4 days of incubation on production of antimicrobial agent by *Streptomyces* SD5 against three tested bacterial pathogens

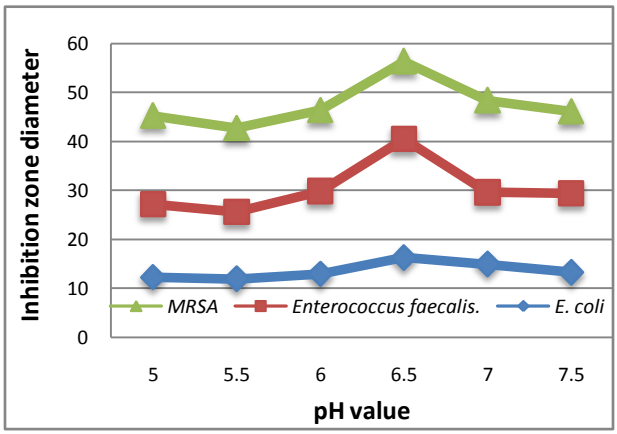


Figure 5 Effect of different pH value on the production of antimicrobial activity by the selected bacterium *Streptomyces* SD5 against three tested bacterial pathogens

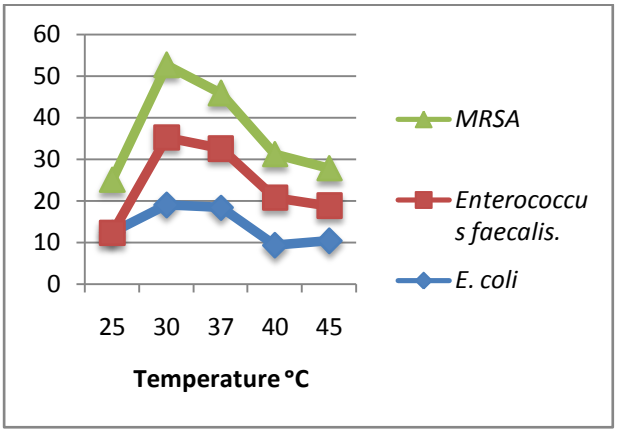


Figure 6 Effect of different incubation temperatures on production of antimicrobial activity by *Streptomyces* SD5 against three tested bacterial pathogens

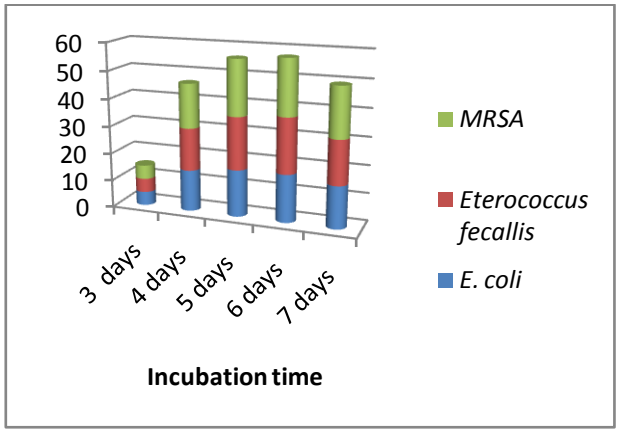


Figure 7 Effect of incubation period on antimicrobial agent production by *Streptomyces* SD5, used against three tested bacterial pathogens.

Table 4 Antibacterial activities (diameter of inhibition zone, mm) and Toxicity (LD50, µg/ml) of the different organic extracts obtained from the selected isolate *Streptomyces* SD5

Tested extract	MRSA	*Enterococcus faecalis*	*Escherichia coli*	Toxicity (LD50, µg/ml)
Ethyl acetate extract	33.00*	25.33*	19.33*	>400
n- Butanol extract	10.67*	13.67*	19.33*	>400
Petroleum ether extract	-ve	-ve	20.00*	>400
Control antibiotic (Ampicillin)	-ve	43	33	ND
CuSO4 (1%)	ND	ND	ND	400

ND: not detected, -ve: negative results *: Significant results, MRSA: Methicillin-resistant *Staphylococcus aureus*

while at higher or lower temperature, the activity decreased (Figure 6). Similarly, the effect of incubation period on antibacterial activity of the culture filtrate of the selected isolate SD5 was shown in Figure 7. After 5-6 days maximum inhibition was recorded against the three tested bacterial pathogens. The bioactive metabolite production started only after 48 hrs of growth and reached a maximum inhibition zone (19-20 mm) against *E. coli*, *E. faecalis* and MRSA after 5-6 days. Effect of incubation period on antibiotic production was statistically significant where increasing time enhancing the antibacterial activity up to 6 days, then there was significant decreased in the antibacterial activity and biomass. Aliero et al. (2018) reported that incubation of the culture in shaking incubator at temperature range of 30-35°C, pH (7.0-7.5) and incubation period (168 hr) were the best conditions for bioactive compound production. For extraction of the active material, laboratory scale production (1500 ml) was prepared using starch nitrate broth which enhanced antimicrobial production by the isolate SD5 against MRSA, *E. faecalis* and *E. coli*. In 30 Erlenmeyer flasks (250 ml), each contained 100 ml of the growth medium with optimum pH 6.5; these flasks were inoculated with the tested bacterium SD5 and incubated at the optimum temperature, determined from the previous experiment (30°C) for 4 days. The culture filtrate was collected and extracted with the same volume of the different organic solvents (ethyl acetate, n-butanol or Petroleum ether). Extraction with ethyl acetate was the best where it gave the highest inhibition compared to n-butanol or petroleum ether (Table 4). From the tested strains, MRSA, *E. faecalis* and *E. coli* have been inhibited more than other tested isolates. Dhanaskaran et al. (2005) isolated *Actinobacteria* from soils of different regions of India with antimicrobial activities which were extracted with ethyl acetate, aniline, chloroform and pyridine but ethyl acetate extract was the best for bacterial pathogen inhibitions which was in accordance with the results of the current study. No toxicity was recorded up to 400 µg/ml for the different extracts obtained (Table 4) compared to the control, $CuSO_4$ solution, which showed acute toxicity at 400 µg/ml. Previous experimental analysis by Gamakaranage et al. (2011) reported the toxicity of $CuSO_4$ at all

Table 5 Minimal inhibitory concentration of the extracted material from *Streptomyces* SD5 using ethyl acetate and compared to control.

Solvent	MRSA	*Enterococcus faecalis*	*Escherichia coli*
SD5	75*	75*	100*
Control antibiotic (Ampicillin)	-ve	5	5

-ve: negative results *: Significant results, MRSA: Methicillin-resistant *Staphylococcus aureus*

tested concentrations against animal cells and added that copper sulphate cause intravascular haemolysis, acute kidney injury and rhabdomyolysis. The lethal dose can be as small as 0.1 g/kg. *A. salina* was used as the test animal. Lucas et al. (2019) used *A. salina* and the same method to determine the toxic effect of some plant extracts and their results proved that this technique is the best for toxicity experiments. The detected MIC was ranged from 75-100 µg/ml while it was 5 µg/ml for the standard antibiotic (Table 5). Furthermore, species of genus *Streptomyces* produced 80% of the total used antibiotics and plant pathogen inhibitors (Oskay et al. 2004; Jeffrey, 2008; Arifuzzaman et al., 2010). Actinomycetes present in any specific soil and their numbers were influenced by the soil type, geographical location, cultivation and organic matter (Arifuzzaman et al., 2010). Various studies have been done by scientists to isolate actinoacteria for antibiotic discoveries. Although actinomycetes occurred widely in nature, only a small numbers of actinomycetes have been screened for antibiotics productions (Kumar et al., 2010). Many antibiotic producing actinomycete strains were generally isolated from soil. In conclusions, resistance to antibiotic is increased and actinomycetes is excellent source of new and active antibiotics

Conflict of interest

No potential conflict of interest was reported by the authors.

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