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### Degradation of Poly- $\beta$ -hydroxybutyrate BY *Trichoderma asperellum*

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#### KEYWORDS

*Trichoderma asperellum*

PHB

Degradation

Plackett Burman

Box Behnken

#### ABSTRACT

In this work, sequential optimization strategy, based on statistical designs, was employed to enhance the degradation of PHB by *Trichoderma asperellum* through the measurement of PHB depolymerase specific activity. For PHB degradation screening, two-level Plackett–Burman design was used. Result of study revealed that fermentation medium composition significantly influencing the PHB depolymerase specific activity. Further, it was reported that time of incubation, levels of PHB and concentration of glucose were found to be the major factors influencing the enzyme specific activity. A second three-level Box–Behnken design was applied to acquire the best process conditions for PHB depolymerase specific activity. In this study, the initial basal medium showed a PHB depolymerase specific activity of 40 ng / l / hr / g dry wt and through the two successive statistical design, the final level of PHB depolymerase specific activity was 3230 ng / l / hr / g drywt, this means a nearly of 80 fold increase.

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## 1 Introduction

Recently, daily used things based on Petro-based Plastics are widely used in modern life, because of their distinctive characteristics (Momani, 2009). However, Petro-based plastics have various serious negative threats on environment and human beings (Gervet, 2007). Among these, emissions of heat and CO<sub>2</sub> as a greenhouse gas from plastic industry are the some common global warming phenomena (Gielen et al., 2008). According to Gielen et al. (2008), releases of CO<sub>2</sub> from plastic industry has been increased by 160% from 1971-2004, resulting of a release of 1.0 Gt/year. As plastic products are increasing, it is obvious that CO<sub>2</sub> production will also increased obviously (Nordell, 2003).

Regarding the effects of plastic on health, bio-monitoring studies have demonstrated the presence of steady concentration of plastics' components in the human body (Calafat et al., 2005). According to Vandenburg et al. (2007), 90% people in the United States have great levels of bis-phenol A in urine. Nowadays, several animal experiments have been performed concerning the health effects of plastic components. Associations were found between exposure to these compounds and severe effects on health and reproduction (Safe, 2000; Richter et al., 2007; Diamanti-Kandarakis et al., 2009; Halden, 2010).

Plastic also threat for aquatic life where every square mile of ocean has 46,000 pieces of plastic floating in it. That is why over a million sea birds, whales, seals, dolphins, sea turtles, and so on die from plastic. Also, 100,000 sharks are also part of the death toll. The impact of plastic pollution in our oceans and ecosystems creates chaos for the environment (Law, 2017).

Negative impacts of Petroleum-based plastics on the economy and the environment have urged researchers to develop and investigate biodegradable plastics derived from renewable resources as an environmentally-friendly alternative. Degradation of P(3HB) and its copolymer has been investigated in different natural environments such as soils (Suyama et al., 1998), composts (Mergaert et al., 1994) and natural waters (Mergaert et al., 1995). PHB depolymerases have been studied in Gram-positive and Gram-negative bacteria (Schüber et al., 2000) and moulds (Oda et al., 1997).

Statistical experimental designs such as Plackett-Burman and response surface methodology (RSM) (Kennedy & Krouse, 1999) can collectively optimize all the affecting parameters to eliminate the limitations of a single-factor optimization process. Plackett-Burman is known as an effective way to identify the important factors, therefore, saving time and maintaining information on each parameter (Abdel-Fattah et al., 2005). RSM, which includes factorial design and regression analyses, helps in evaluating the important factors, building models to study the interactions

between the variables or desirable responses (Ghanem et al., 2010). Plackett-Burman design and RSM have been successfully employed to optimize some bioprocesses (Lotfy et al., 2007; Mohana et al., 2008; Ghanem et al., 2011; Ghanem et al., 2012). The main objective of this study is to isolate fugal isolates capable of degrading PHB and maximize the degradation by statistical designs.

## 2 Materials and Methods

### 2.1 Chemicals

All experiments were performed using PHB powder and were obtained from Biomer Inc., Germany. Other substrates were purchased from Aldrich Chemical Co. or Sigma (St. Louis, USA).

### 2.2 Sample collection

Twenty soils and 10 wastewater samples were collected from the four different plastic factories in the industrial zone in Jeddah, Saudi Arabia. Soil samples were collected in sterile plastic bags while water samples were collected in sterile plastic bottles.

### 2.3 Fugal identification

Isolated fungal species were identified on the basis of cultural and morphological characteristics and taxonomic key developed by Frey et al, (1979), Watanabe (2002) and CBS (2006).

### 2.4 Media and Fungal culturing

Many fungal isolates were obtained on Sabouraud dextrose agar and all the pure culture were screened on mineral salt medium containing PHB as carbon source. The most active isolate was selected and identified as *T. asperellum*.

*T. asperellum* was maintained on Sabouraud dextrose agar (SDA), where the fungus was grown for 7 days at 25° C. The stocks were kept in the refrigerator and subcultures at monthly intervals were done. Each after four-month intervals, fresh cultures were prepared by streaking out from stock slopes onto Sabouraud agar plates to check for purity and then sub-culturing onto fresh Sabouraud agar slants, both plates and slants were incubated at 25°C. Spores suspension of *T. asperellum* was prepared by washing 6 days old culture slants with sterilized saline solution (0.9% NaCl) and shaking vigorously for 1min. Spores were counted by a haemocytometer to adjust the count approximately to 6x10<sup>7</sup> spores/ml.

The medium for PHB degradation included (g/L): PHB (0.5), KH<sub>2</sub>PO<sub>4</sub> (0.7), K<sub>2</sub>HPO<sub>4</sub> (0.7), MgSO<sub>4</sub> (0.7), Yeast extract (2.5), Glucose (1), NaCl (0.005), FeSO<sub>4</sub> (0.002) and ZnSO<sub>4</sub> (0.007) at PH 7 and Inoculum size 5ml. In case of preparing solid medium, 15 g/l Agar was added.

Batch mode shake flask experiments were conducted in 250ml Erlenmeyer flasks containing 50ml of the basal Mineral salt PHB medium. Flasks were inoculated with standard inoculums ( $6 \times 10^7$  spores/ml) and incubated in shaking incubator (120rpm) at 25° C for 7days.

## 2.5. PHB depolymerase assay

PHB depolymerase assay was performed according to the method described by Kobayashi et al.,1999. About 0.3% Poly (3-hydroxybutyrate) was suspended in 50mM Tris-HCl buffer, pH 7.5 and the suspension was sonicated for 20min in a 300ml flask immersed in an ultrasonic water bath (35KHz, 285W) prior to the dilution to 0.03% in the same buffer. Culture supernatant 0.1ml was added to 0.9ml of the substrate suspension and incubated for 24 hours at 30°C. Activity was measured as the decrease in OD, at 650 nm through spectrophotometer, against substrate buffer blanks (Ali et al., 2017).

$$\text{Enzyme activity} = \frac{\text{OD} \times \text{Factor (1.9 mg/l)} \times \text{amount of enzyme (0.1 ml)}}{\text{Time (24 hr)}}$$

$$\text{Specific activity} = \frac{\text{Enzyme activity}}{\text{Dry Weight}} \text{ expressed in ng / 1/ hr / g drywt}$$

## 2.6. Plackett-Burman design

The Plackett-Burman experimental design (Plackett & Burman, 1946; Liu et al., 2003; Wang & Lu, 2005; Lee et al., 2006; Hegde et al., 2013; Suwanposri et al., 2014) was used to identify the major parameters that affect PHB degradation in liquid cultures. According to the Plackett-Burman matrix in the results section. Eleven independent variables were screened in twelve combinations organized For each variable, a high (+) and (-) level was investigated. All experiments were performed in duplicates and the averages of PHB depolymerase specific activity were treated as the responses.

Analysis for the Plackett-Burman experiment was carried out as follows. First, for all the components, their effect on the response was calculated, which was the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at the low setting (-1) of that factor. The main effect of each variable can be determined using the following equation:

$$\text{Main effect} = (\sum Mi^+ - \sum Mi^-) / N$$

Where  $Mi^+$  and  $Mi^-$  are the observations of trials and  $N$  is the number of trials divided by 2.

The factor that had no effect would give a value of zero. The value of main effect with a positive sign indicates that the high concentration of this variable is nearer to optimum and a value of negative sign indicates that the low concentration of this variable

is nearer to optimum. The experimental results were analyzed to extract independently the main effects of these factors; the analysis of variance technique was then applied to determine which factors were statistically significant. The controlling factors were identified, with the magnitude of effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. The Student's  $t$ -test was employed in order to check the statistical significance of the regression coefficients of the variables (Ghanem et al., 2010; Farag et al., 2015).

## 2.7.Box-Behnken design

In order to describe the nature of response surface in the experimental region and to elucidate the optimal concentrations of the most significant independent variables, a Box-Behnken design (Box & Behnken, 1960; Ghanem et al., 2013) was applied, which is a response surface methodology. Factors of highest confidence levels namely; were tested in three levels (low, basal, and high) coded (-1, 0, and +1). Accordingly, fifteen treatment combinations were executed. For predicting the optimal point, the following second order polynomial model was fitted to correlate relationship between independent variables and response:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_{12} + \beta_{13} X_{13} + \beta_{23} X_{23} + \beta_{111} X_1^2 + \beta_{222} X_2^2 + \beta_{333} X_3^2$$

As presented in Table 1, factors of highest confidence levels namely; The time of incubation ( $X_1$ ), the PHB concentration ( $X_2$ ), and glucose concentration ( $X_3$ ) were prescribed into three levels (low, basal, high) coded, (-, 0, +).Where,  $Y$  is the dependent

Table 1 Box-Behnken design matrix and response for *Trichoderma asperellum*

Factor	Symbol	Level		
		-	0	+
Poly-β-hydroxybutyrate (g/l)	PHB	0.25	0.5	0.75
K <sub>2</sub> HPO <sub>4</sub> (g/l)	K2	0.35	0.7	1.05
KH <sub>2</sub> PO <sub>4</sub> (g/l)	K	0.35	0.7	1.05
MgSO <sub>4</sub> (g/l)	M	0.35	0.7	1.05
Yeast extract (g/l)	Y	1.25	2.5	3.75
Glucose (g/l)	G	0.50	1.0	1.5
NaCl (g/l)	N	0.0025	0.005	0.0075
FeSO <sub>4</sub> (g/l)	F	0.001	0.002	0.003
ZnSO <sub>4</sub> (g/l)	Z	0.0035	0.007	0.0105
Time (days)	T	4	7	10
Inoculum size (ml/l)	I	2.5	5	7.5

variable (PHB depolymerase specific activity), X1, X2 and X3 are the levels of the independent variables ;  $\beta_0$  is regression coefficient at the center point;  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  are linear coefficients;  $\beta_{12}$ ,  $\beta_{13}$  and  $\beta_{23}$  are the second order interaction coefficients; and  $\beta_{11}$ ,  $\beta_{22}$  and  $\beta_{33}$  are quadratic coefficients. The quality of the polynomial model equation was expressed by coefficient,  $R^2$ . The optimal value of PHB degradation was estimated using the solver of Microsoft Excel tool. Three-dimensional graphical representations were also constructed using Statistica 6.1 program, in order to determine the effects and the interactions of independent variables on the objective.

### 3 Results and Discussion

#### 3.1. Optimization of PHB degradation by *T.asperellum* using statistical designs

Optimization of culture conditions for maximum degradation was planned via two approaches viz., first dealt with evaluating the different medium constituents using the Plackett-Burman design and the second was to optimize the most important factors that significantly affected the degradation process using the Box-Behnken design.

##### 3.1.1 Evaluating the significance of medium constituents using the Plackett-Burman design

The Plackett-Burman (Plackett & Burman, 1946) provided an effective way to identify the significant factors. This design was applied in this study to reflect the relative importance of different medium constituents with respect to degradation of PHB compound. The chosen levels of 11 variables are presented in Table 2. All experiments were performed in duplicates and the results (average of the observations) were presented also in Table 3. The results indicate a wide variation in PHB depolymerase specific activity ranging from 10 to 120 ng/ l/hr/ g dry wt in the 12 trials. The variation suggests that the optimization process was important for improving the degradation efficiency of PHB. The results of study revealed that the levels of factors at

Table 2 Factors affecting PHB degradation by *T. asperellum* and their levels in the Plackett-Burman experiment design

Trials	Independent variables		
	Time(days) ( X1)	PHB conc.(g/l) ( X2)	Glucose(g/l) ( X3)
1	- ( 5)	- (0.125)	0 ( 0.5)
2	+ (15)	-	0
3	-	+ ( 0.375)	0
4	+	+	0
5	-	0( 0.25)	- ( 0.25)
6	+	0	-
7	-	0	+ ( 0.75)
8	+	0	+
9	0	-	-
10	0 ( 10 )	+	-
11	0	-	+
12	0	+	+
13	0	0	0
14	0	0	0
15	0	0	0

trial (12) was the best achieving a degradation of 120 ng/ l/hr/ g dry wt. The main effect of each variable upon PHB degradation was estimated as the difference between both averages of measurements at the high level (+1) and the low level (-1) of that factor. The main effect results of each medium component are presented graphically in (Figure 1). Main effect analysis revealed that three variables out of the twelve variable included in this study were found to have a significant influence on PHB degradation, indicating the increase of the time of incubation was ideal for enhancing PHB degradation, whereas the low level of

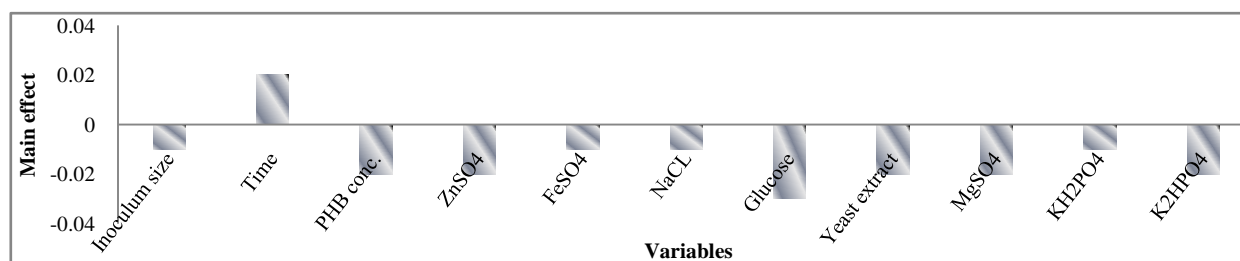


Figure 1 Main effect showing effect of different factors on PHB degradation for *Trichoderma asperellum*

Table 3 Plackett-Burman design matrix and response for *T. asperellum*

Trials	Independent variables											Enzyme Activity ( $\mu\text{g l}^{-1} \text{hr}^{-1}$ )	Dry Weight (gram)	Specific Activity ( $\mu\text{g l}^{-1} \text{hr}^{-1} \text{g dry wt}^{-1}$ )	Specific Activity ( $\text{ng l}^{-1} \text{hr}^{-1} \text{g dry wt}^{-1}$ )
	$\text{K}_2\text{HPO}_4$	$\text{K H}_2\text{PO}_4$	$\text{MgSO}_4$	Yeast extract	Glucose	NaCl	$\text{FeSO}_4$	$\text{ZnSO}_4$	PHB conc.	Time	Inoculum size				
1	+	+	-	+	+	+	-	-	-	+	-	0.00327	0.0594	0.06	60
2	+	-	+	+	+	-	-	-	+	-	+	0.00145	0.1034	0.01	10
3	-	+	+	+	-	-	-	+	-	+	+	0.00316	0.0480	0.07	70
4	+	+	+	-	-	-	+	-	+	+	-	0.00317	0.0439	0.07	70
5	+	+	-	-	-	+	-	+	+	-	+	0.00121	0.0480	0.03	30
6	+	-	-	-	+	-	+	+	-	+	+	0.00306	0.0554	0.06	60
7	-	-	-	+	-	+	+	-	+	+	+	0.00320	0.0443	0.07	70
8	-	-	+	-	+	+	-	+	+	+	-	0.00301	0.0621	0.05	50
9	-	+	-	+	+	-	+	+	+	-	-	0.00145	0.0789	0.02	20
10	+	-	+	+	-	+	+	+	-	-	-	0.00175	0.0527	0.03	30
11	-	+	+	-	+	+	+	-	-	-	+	0.00131	0.0355	0.04	40
12	-	-	-	-	-	-	-	-	-	-	-	0.00196	0.0161	0.12	120
13	0	0	0	0	0	0	0	0	0	0	0	0.00222	0.0526	0.04	40

glucose and PHB concentration in experimental range were favourable for increasing PHB depolymerase specific activity hence PHB reduction. Variables with the confidence levels greater than 80% were considered as significant. Time of incubation, PHB concentration and Glucose level were considered the most significant factors (above 80% confidence level). The range of the levels of variables (Table 4), which were based on t-value showed that negative level of glucose, negative level of PHB concentration and positive level of time of incubation showed all significant effect (above 80%). Other variables had no significant effect on PHB degradation such as inoculum size (71%),  $\text{ZnSO}_4$  (80%),  $\text{FeSO}_4$  (61%), NaCl (71%), Yeast extract (80%),  $\text{MgSO}_4$  (76%)  $\text{KH}_2\text{PO}_4$  (61%), and  $\text{K}_2\text{HPO}_4$  (80%).

A verification experiment was performed comparing the initial control medium with the optimized medium. The results revealed that optimized medium showed PHB degradation with 7 fold increase than both controls (Figure 2). The control 1 medium g/l [PHB (0.5),  $\text{KH}_2\text{PO}_4$  (0.7),  $\text{K}_2\text{HPO}_4$  (0.7),  $\text{MgSO}_4$  (0.7), Yeast extract (2.5), Glucose (1.0), NaCl (0.005),  $\text{FeSO}_4$  (0.002) and  $\text{ZnSO}_4$  (0.007), Time 7 days; Inoculum size 5 ml; all the rest of the media is kept as in basal concentration at pH 7.5 and 25 °C] is the initial starting medium with the variables in zero level. The optimized 1 medium [PHB (0.25),  $\text{KH}_2\text{PO}_4$  (0.7),  $\text{K}_2\text{HPO}_4$  (0.7),

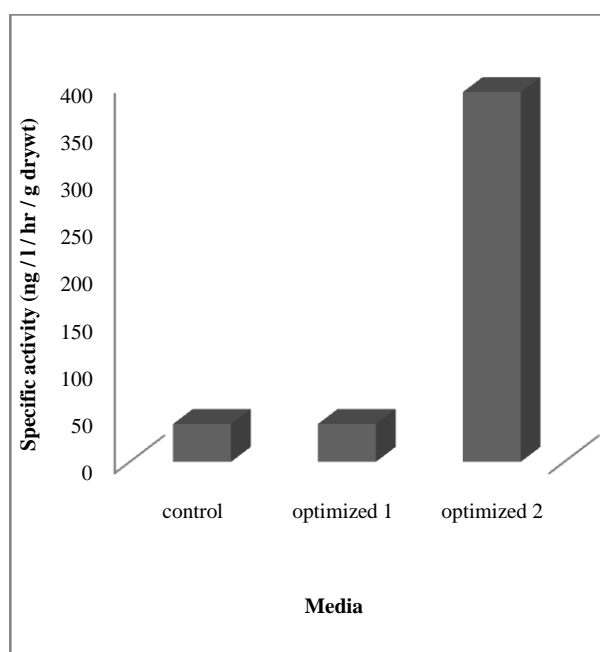


Figure 2 Verification of Plackett-Burman design comparing Specific activity of depolymerase of *Trichoderma asperellum* in control and optimized media

Table 4: Statistical analysis of Plackett-Burman experimental results for *T. asperellum*

	Coefficients	Standard Error	t <sub>Stat</sub>	P-value	Significance level	Main effect
<b>Intercept</b>	0.051692308	0.002797928	18.47520861	0.034424465		-0.11
<b>Inoculum size</b>	-0.005833333	0.002912176	-2.003084042	0.294775047	71%	-0.01
<b>Time</b>	0.010833333	0.002912176	3.720013221	0.167181564	83%	0.02
<b>PHB conc.</b>	-0.010833333	0.002912176	-3.720013221	0.167181564	83%	-0.02
<b>ZnSO<sub>4</sub></b>	-0.009166667	0.002912176	-3.147703494	0.19582925	80%	-0.02
<b>FeSO<sub>4</sub></b>	-0.004166667	0.002912176	-1.430774316	0.388339509	61%	-0.01
<b>NaCl</b>	-0.005833333	0.002912176	-2.003084042	0.294775047	71%	-0.01
<b>Glucose</b>	-0.0125	0.002912176	-4.292322947	0.145716667	85%	-0.03
<b>Yeast extract</b>	-0.009166667	0.002912176	-3.147703494	0.19582925	80%	-0.02
<b>MgSO<sub>4</sub></b>	-0.0075	0.002912176	-2.575393768	0.235785558	76%	-0.02
<b>KH<sub>2</sub>PO<sub>4</sub></b>	-0.004166667	0.002912176	-1.430774316	0.388339509	61%	-0.01
<b>K<sub>2</sub>HPO<sub>4</sub></b>	-0.009166667	0.002912176	-3.147703494	0.19582925	80%	-0.02

MgSO<sub>4</sub> (0.7), Yeast extract (2.5), Glucose (0.50), NaCl (0.005), FeSO<sub>4</sub> (0.002) and ZnSO<sub>4</sub> (0.007), Time of incubation (10 days); Inoculum size (5 ml); all the rest of the media is kept as in basal concentration at pH 7.5 and 25 °C] with the significant variables, PHB concentration and glucose concentration in negative levels, time of incubation in positive level, and all other variables in negative levels. The optimized 2 medium has following composition (PHB, 0.25; KH<sub>2</sub>PO<sub>4</sub>, 0.35; K<sub>2</sub>HPO<sub>4</sub>, 0.35; MgSO<sub>4</sub>, 0.35; Yeast extract, 1.25; Glucose, 0.50; NaCl, 0.0025; FeSO<sub>4</sub>, 0.001 and ZnSO<sub>4</sub>, 0.0035; time of incubation, 10 days; Inoculum size 2.5 ml; ) with the significant variables, PHB concentration and glucose concentration in negative levels, time of incubation in positive level, and all the rest of the media is kept as in zero basal concentration at pH 7.5 and 25 °C. Optimized medium 2 resulted in 8 fold increase in PHB depolymerase specific activity (400 ng / l / hr / g dry wt ) than control media and optimized medium 1. The resulted optimized medium 2 will be used for further optimization using upcoming experiment. In order to optimize enzyme production, the Plackett- Burman design was applied, which has been demonstrated as an efficient approach to screen for medium components and/or factors affecting PHB degradation. According to Ghanem et al. (2005) increasing PHB level negatively affect the enzyme activity which led to suggest that the PHB depolymerase is constitutive. Hydrolytic activity of PHB depolymerase is strongly enhanced in presence of low concentrations of divalent cations such as calcium, magnesium or zinc. These results indicate that PHB depolymerase shows a non-essential activation by divalent cations (Hidalgo et al., 2013). The ability of the fungi to degrade PHB was pronounced in liquid medium containing the polymer as the sole carbon source.

Degradation increased with increasing period of incubation (Mabrouk & Sabry, 2001). Previous researchers showed that the percentage for PHB was increased as the incubation time increases in soil (Altaee et al., 2016). Decreasing the level of glucose or PHB, increased the degradation efficiency of PHB. The synthesis of PHB depolymerase seems to be highly regulated. The presence of low concentration of soluble carbon sources as glucose in addition to the polymer in the medium, this increased PHB depolymerase specific activity, indicating that PHB depolymerase expression is repressed in the presence of a high level of soluble carbon source (Mabrouk & Sabry, 2001). Results of present study are supported by other researchers who demonstrating that all other known PHB bacterial depolymerases are repressed in the presence of a soluble carbon source (Jendrossek et al., 1995). Results are in accordance with the findings of Steinbuechel & Hein (2001) who reported similar results with *Pseudomonas lemoignei*. These results are in agreement with a previous investigation which demonstrated the importance of carbon sources in the growth medium for enzyme production as the rate of polymer degradation was influenced by the degree and availability of secondary carbon and by the initial carbon source (Shivakumar, 2013).

### 3.1. 2. Optimization of medium composition and inoculum level by Box-Behnken design

In order to approach the optimum response region of PHB depolymerase specific activity significant independent variables (time of incubation, PHB concentration and glucose concentration) were further explored, each at three levels



Table 5 Statistical analysis of Box-Behnken experimental results for *Trichoderma asperellum*

Trial	Time = x1	PHB = x2	Glucose = x3	x1x2	x1x3	x2x3	x1x1	x2x2	x3x3	Specific Activity ng / l / hr / g drywt
1	5	0.125	0.5	0.625	2.5	0.0625	25	0.015625	0.25	70
2	15	0.125	0.5	1.875	7.5	0.0625	225	0.015625	0.25	1200
3	5	0.357	0.5	1.785	2.5	0.1785	25	0.127449	0.25	170
4	15	0.357	0.5	5.355	7.5	0.1785	225	0.127449	0.25	150
5	5	0.25	0.25	1.25	1.25	0.0625	25	0.0625	0.0625	100
6	15	0.25	0.25	3.75	3.75	0.0625	225	0.0625	0.0625	1380
7	5	0.25	0.75	1.25	3.75	0.1875	25	0.0625	0.5625	60
8	15	0.25	0.75	3.75	11.25	0.1875	225	0.0625	0.5625	240
9	10	0.125	0.25	1.25	2.5	0.03125	100	0.015625	0.0625	2680
10	10	0.357	0.25	3.57	2.5	0.08925	100	0.127449	0.0625	430
11	10	0.125	0.75	1.25	7.5	0.09375	100	0.015625	0.5625	250
12	10	0.357	0.75	3.57	7.5	0.26775	100	0.127449	0.5625	150
13	10	0.25	0.5	2.5	5	0.125	100	0.0625	0.25	10
14	10	0.25	0.5	2.5	5	0.125	100	0.0625	0.25	3
15	10	0.25	0.5	2.5	5	0.125	100	0.0625	0.25	3

according to Box and Behnken (Box & Behnken, 1960). Table 5 represented the variables levels and the statistical analysis together with the experimental results of PHB depolymerase specific activity. Presenting experimental results in the form of surface plots (Figure 3a-c) shows that zero levels of time of incubation (10 days), low level of glucose (0.25 g/l) and PHB concentration (0.125 g/l) support relatively high levels PHB depolymerase activity. On the other hand, low levels of PHB depolymerase specific activity clearly located at high concentration of glucose and PHB.

For predicting the optimal points mathematically, a second order polynomial function was fitted to the experimental results of PHB depolymerase specific activity by the help of Excel program and Statistica software.

$$Y = \text{Intercept (4.664)} + 0.310 X_1 - 21.873 X_2 - 11.764 X_3 - 0.488 X_1X_2 - 0.22 X_1X_3 + 18.989 X_2X_3 - 0.00081 X_1X_1 + 28.4358 X_2X_2 + 7.3573X_3X_3$$

Where Y is the PHB depolymerase specific activity (ng / l / hr / g drywt); and X1, X2, and X3 are the time of incubation, PHB and glucose concentration, respectively.

At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation coefficients R

and the determination coefficient  $R^2$ . The closer the value of R is to 1; the better is the correlation between the measured and the predicted values. In this experiment the value  $R^2$  were 0.93, for the yield of PHB depolymerase specific activity This value indicates a high degree of correlation between the experimental and the predicted values, it is also a measure of fit of the model.

The optimal levels of the three variables were estimated using Excel program and found that glucose level (0.25 g/l); PHB concentration, 0.125g/l and time of incubation up to 10 days showed an observed PHB depolymerase specific activity of 2680 ng / l / hr / g dry wt. It nearly 7 times increases than control (The optimum medium reached by Plackett Burman). Optimal conditions realized from the Box behnken were verified and compared with the predicted yield from the model. The experimental Box-Behnken (trial 9) media for *T. asperellum* was (g/L): PHB (0.125);  $\text{KH}_2\text{PO}_4$  (0.35);  $\text{K}_2\text{HPO}_4$  (0.35);  $\text{MgSO}_4$ , (0.35); Yeast extract (1.25); Glucose (0.25); NaCl (0.0025);  $\text{FeSO}_4$  (0.001) and  $\text{ZnSO}_4$  (0.0035); Time 10 days; Inoculum size 2.5 ml; all the rest of the media is kept as in basal concentration at pH 7.5 and 25 °C. This medium was compared with the predicted medium (g/L) PHB (0.125);  $\text{KH}_2\text{PO}_4$  (0.35);  $\text{K}_2\text{HPO}_4$  (0.35);  $\text{MgSO}_4$  (0.35); Yeast extract (1.25); Glucose (0.25); NaCl (0.0025);  $\text{FeSO}_4$  (0.001) and  $\text{ZnSO}_4$  (0.0035); Time 15 days; Inoculums size 2.5 ml; all the rest of the media is kept as in basal concentration at pH 7.5 and 25 °C.

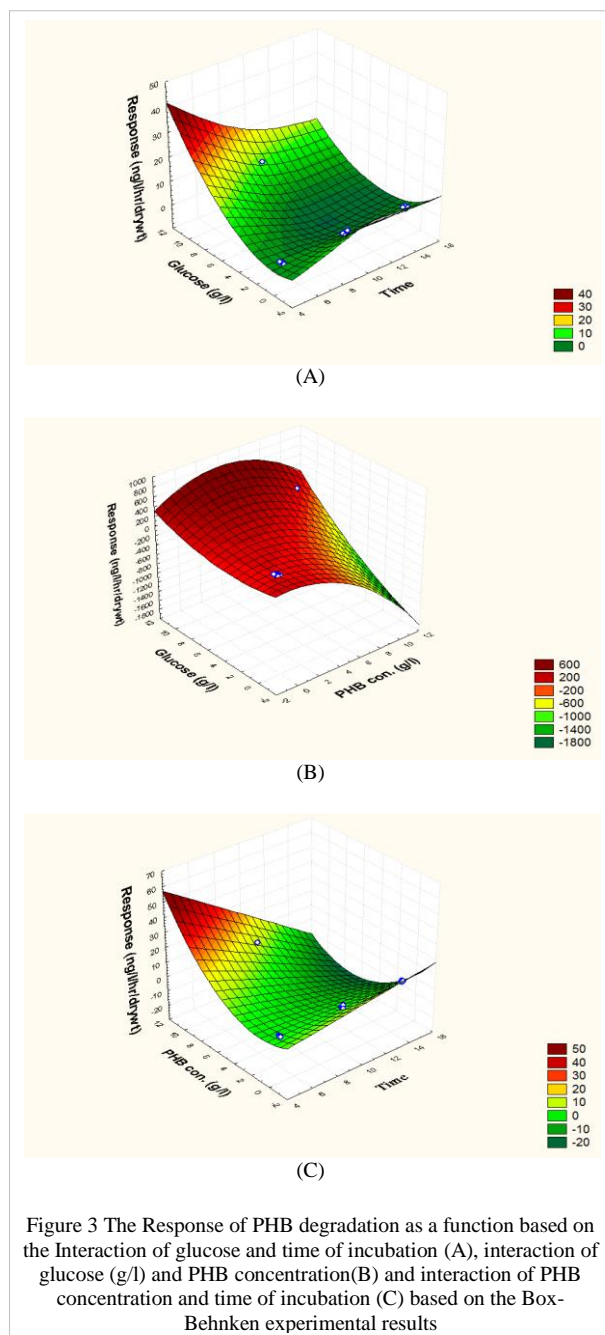


Figure 3 The Response of PHB degradation as a function based on the Interaction of glucose and time of incubation (A), interaction of glucose (g/l) and PHB concentration (B) and interaction of PHB concentration and time of incubation (C) based on the Box-Behnken experimental results

The predicted showed a PHB depolymerase specific activity of 3230 ng / l / hr / g dry wt compared with the observed experimental 2800 ng / l / hr / g dry wt (Figure 4). This shows higher similarity between the predicted and observed results reflects the accuracy and applicability of the Box-Behnken model in the optimization processes (Bloor & England, 1991; Teruel et al., 1997; Kimmel et al., 1998). In this study, the initial basal

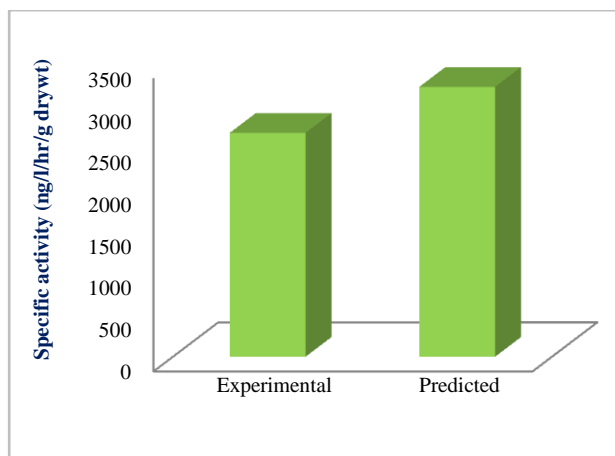


Figure 4 Verification of Box-Behnken showing the close relationship between the experimental and the predicted result by *Trichoderma asperellum*

medium showed a PHB depolymerase specific activity of 40 ng / l / hr / g dry wt and through the two successive statistical design, the final level of PHB depolymerase specific activity was 3230 ng / l / hr / g dry wt, this means a nearly of 80 fold increase. The Plackett-Burman and Box-Behnken designs have been successfully applied in many recent biotechnological applications (Haltrich et al., 1994; Lotfy, 2000; Mohamed, 2000).

Environment concerns and solid waste management problems increased the interest toward development of biodegradable plastics that possess the same physical and chemical properties of the conventional plastics (Bhagowati et al., 2015). The optimization work in this study has given a potent organism for PHB degradation. Meanwhile the results obtained from this study prove the powerful of the experimental design in optimizing complicated computational medium compositions. The use of culture will improve the biotechnological process especially those deal with expensive products such as PHB. The strategies included in this study are recommended to be used in optimizing complicated biotechnological process (Amara, 2013).

## Conclusion

Using Sequential optimization strategy, based on statistical designs, Plackett-Burman and Box-Behnken design, the PHB depolymerase specific activity in *T. asperellum* increased from 40 ng/l/hr/g to 3230 ng / l / hr / g dry wt, this means a nearly of 80 fold increase.

## Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.



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