EXPERIMENTAL DESIGN FOR SIMULTANEOUS PRODUCTION OF PHB, MESOPHILIC PROTEASES AND LIPASES

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ABSTRACT: *Bacillus* species such as *Bacillus subtilis, Bacillus pumilus* and *Bacillus thuringiensis* are known to produce PHB, proteases and lipases. This study employs the Plackett-Burman and Box-Behnken experimental design with the application of Excel solver for optimized production of PHB, proteases and lipases using *Bacillus* mixed culture. The statistical analysis of the results proved an insignificant relationship between the media compositions and the responses. The results clearly proved a competition between the production of PHB, Proteases and Lipases. Meanwhile a systematic experimental design succeeded in minimizing this competition. The maximum production of PHB, proteases and lipases gained were 16.48 g/l/48 hr, 534 Units/ml/48 hr and 22.56 Units/ml/48 hr respectively. The strategies used in this study are recommended for simultaneous production of PHB, proteases, and to some extend lipases.

ABSTRAK: Spesies Bacillus seperti *Bacillus subtilis*, *Bacilus pumilus* dan *Bacillus thuringiensis* boleh menghasilkan PHB, Proteases dan Lipase. Dalam kajian ini, kaedah rekabentuk ujikaji Plackett-Burman dan Box-Behnken telah digunapakai dengan penyelesai Excel untuk mengira kadar optimum pengeluaran kultur Bacilus bercampur. Keputusan analisis statistik telah membuktikan hubungan yang longgar di antara komposisi media dan tindakbalas media. Hasil kajian jelas membuktikan persaingan antara pengeluaran PHB, Proteas dan Lipases. Sementara itu, suatu reka bentuk uji kaji yang sistematik berjaya untuk mengurangkan persaingan ini. Perolehan maksimum PHB, proteas dan lipase yang diukur dalam kajian ini ialah masing-masing 16.48 g/l/48 jam, 534 Unit/ml/48 jam dan 22.56 Unit/ml/48 jam. Strategi yang digunakan dalam kajian ini disyorkan untuk pengeluaran serentak PHB, proteas dan pada kadar tertentu lipase.

KEY WORDS: *Plackett-Burman, Box-Behnken, Excel Solver, PHB, Proteases, Lipases, optimization*

1. INTRODUCTION

Perhaps, Beijerinck wrote the first report about lucent granules of polyhydoxy alkanoates (PHAs) in bacterial cells in 1888 (Reported in Chowdhury, 1963) [1]. The father of polyhydroxybutyrate (PHB) is the French scientist Maurice Lemoigne who was able to characterize these inclusion bodies. Lemoigne made his first experiment on Bacillus. His work was published in 27 publications from 1923 until 1951 [2]. The Imperial Chemical Industries in Billingham, in the United Kingdom produced copolymers of PHB-*co*-PHV on a large scale [3]. The market penetration is rather scarce, products are known as Biopol trademark. In 1990, the German company Wella used Biopol-made flasks for a new shampoo. PHA is approximately, five to ten times more expensive than its competitors, polypropylene or polyethylene. PHAs' price at that time was $17 to $22/-kg [4].

Major expenses in the production of PHAs are determined by the cost of their related substrates, and the extraction method used [3]. The production cost of PHAs is the main commercialization-limiting factor. PHAs' accumulation requires special growth conditions and it is usually produced during imbalanced nutrition that causes slow growth. Development of better fermentation and purification technologies and the use of genetic engineering lowered the price, which becomes $4/ kg, but even then it is still expensive [4].

Yamane *et al.* (1993), reported that the substrate is the major cost in PHA production [5]. It is important to highlight that if microbial cells in their optimum PHA accumulation condition are able to accumulate PHA, they will not exceed certain limit based on their size capacity [4]. Unfortunately, usually big microbial cells have long generation time [4]. The cheapest substrate cost is $0.22/ kg of PHA while the cost of polypropylene is $0.185/ kg [6]. The substrate cost affects the overall cost but the cheapest substrate is not always the ideal choice concerning the downstream processes. When the PHB productivity increased from 1.98 to 3.2 g/ h, the PHB production cost decreased from $5.37/ kg to $4.91/ kg [7]. In a laboratory fed-batch system using *Alcaligenes latus*, the highest reported productivity was 4.94 g/ h with cost about $2.6 kg [7]. Page and Cornish (1993) commented that beside the substrate cost, the PHA extraction method from inside the cells, and the treatment of the fermentation wastes are the major problem [8].

The early history of PHAs research focused on the use of the polymer as a packaging material to substitute petroleum plastics. After many un-successful trials, recent researches on PHAs have been directed towards medicinal applications [4].

Aiming in relation to a wide range of industrial applications and attempting to establish a method for changing PHAs compositions as well as PHAs overproduction Amara et al. (2001, 2002) described a new simple strategy for *in vivo* random mutagenesis and mutants selection using $phaC_{Ap}$ synthase gene employing the mutator strain *Escherichia coli* XL1-Red and Nile-red plates [9-10]. Taguchi *et al.* (2001, 2002)

used another strategy based on *in vitro* mutagenesis for $phaC_{Re}$ using PCR [11-12]. It is clear from the amounts of research about PHA that this biopolymer is very promising. PHA is biodegradable, biocompatible, biovaluable, and has good mechanical properties.

Experimental design is a versatile tool for the optimization of different parameters and conditions usually attained by randomizing the used values [13-14]. In this study Plackett-Burman and Box-Behnken experimental design as well as Excel solver were used in the optimization of the nutritional condition for the productions of the PHB, proteases and lipases in one fermentation flask and the result analyzed statistically using different statistical methods. The results of the optimization proved to be promising.

2. MATERIALS AND METHODS

2.1 Microorganisms

Four Bacillus strains were used in this study. The strains were isolated from the Egyptian ecosystem and identified using standard criteria as *Bacillus subtilis*, *Bacillus pumilus, Bacillus thuringiensis* and *Bacillus sp.* They grow routinely in LB medium (Luria-Bertani) at 37°C and maintained at -70°C by adding 300 µl glycerol to each 1 ml culture in suitable plastic container [15].

2.2 PHB, Proteases and Lipases Production Medium

The four microbial strains were used for the production of PHB, proteases and lipases using cultivation media designed in this study containing: Glucose; Tryptone Soyabean; KCl; Tween-20; cottonseed oil; KH_2PO_4; Trace elements solution; $FeSO_4$; Yeast extract and Skim milk. The medium constituents and the Bacillus strains have been used in amounts represented as +1, 0 and -1 according to the Plackett-Burman and Box-Behnken design in quantities as in Table 1.

The Trace element solution consists of: 10 mg/l $ZnSO_4.7H_2O$; 3 mg/l $MnCl_2.4H_2O$; 30 mg/l H_3BO_3; 20 mg/l $CoCl_2.6H_2O$; 1 mg/l $CuCl_2.2H_2O$; 2 mg/l $NiCl_2.6H_2O$; and 3 mg/l $Na_2MoO_4.2H_2O$ [16]. The trace elements solution and $FeSO_4$ solution [0.012 g/l] were sterilized using 0.22 µm sterilized filter system. The media constituents and the Bacillus strains have been used in amounts represented as +1, 0 and -1 according to the Plackett-Burman and Box-Behnken design in quantities as in Table 1.

2.3 In-Flask Fermentation Condition

PHB, proteases and lipases production were conducted using 250 ml Erlenmeyer-Flask containing 100 ml medium, with the shaking rate of 250 rpm at 37°C. The medium composition for each experiment has been prepared according to the Plackett-Burman and Box-Behnken design as described in Table 3 and 13.

Table 1: Media constituents represented equal to +1, 0 and 1 values.

Variables Name	1	0	-1	Units/100 ml
B. subtilis	100	55	10	µl
B. pumilus	100	55	10	µl
B. thuringiensis	100	55	10	µl
B. sp.	100	55	10	µl
Glucose	2	1.2	0.4	gm
Tryptone Soyabean	10	7.5	5	mg
KCl	0.3	0.165	0.03	gm
Tween-20	1.5	0.825	0.15	gm
Cottonseed Oil	8	5	2	gm
KH_2PO_4	0.1	0.055	0.01	gm
$FeSO_4$	25	15	5	(12 mg/l) µl
Trace elements solution	5	3	1	µl
Yeast extract	0.4	0.24	0.08	gm
Skim milk	10	5.1	0.2	mg

2.4 Proteases Analysis

2.4.1 Preparation of L-Tyrosine Standard Curve

L-tyrosine (1.1 mM) was dissolved in 100 ml deionized water by heating gently (without boiling). After complete dissolving of the L-tyrosine the standard curve was generated.

2.4.2 Preparation of Casein-Universal Buffer

The Universal buffer was prepared according to Britton and Robinson (1931) consisting 40 mM H_3PO_4, 40 mM acetic acid and 40 mM H_3BO_3 [17]. The pH was adjusted to 8 using 0.2 M NaOH.

Casein, 0.325 mg (Hydrolysate-Hy-Case®Amino-Fluka® Protein) was weighted and dissolved in 50 ml of the Universal buffer. The mixture was dissolved by heating gently

to 80-90°C without boiling. The mixture was either used immediately or preserved in -20°C for further usage.

2.4.3 Protease Activity

Supernatant (300 µl), which contains the crude enzymes was added to the same volume of the Casein-Universal buffer (pH 8). The enzymes-substrate mixture for each pH was incubated at 40°C for 30 min. After the incubation period, the enzymes reaction was stopped by adding 600 µl of 10% Trichloroacetic acid. The mixture was allowed to stand at room temperature for 15 min then centrifuged at 10^4 rpm for 10 min (Biofuge 15 - Heraeus Sepatech). The absorbance of each sample was determined spectrophotometrically at 280 nm (PerkinElmer-UV/VIS Spectrometer Lambda) and their tyrosine content derived from the tyrosine standard curve. Each enzyme activity was identified as Units/ml.

2.5 Lipase Activity

To determine the lipase activity of each experiment extracellular lipase was assayed according to the following method:

The substrate was prepared by adding 200 µl Tween-20 to 40 mg *p*-nitrophenyl palmitate dissolved in 10 ml DMSO. 500 µl from the substrate mixture was added to 500 µl buffer (50 mM Tris HCl, pH 8.0) and the enzyme reaction started by adding 500 µl from the supernatant, which contains the crude enzymes. The buffer-substrate mixture and the supernatant were warmed to 30°C before starting the assay.

The extracellular lipase activity for each experiment was determined by the rate of *p*-nitrophenol production (*p*NP) measured at 405 nm spectrophotometerically (PerkinElmer-.UV/VIS Spectrometer Lambda). The increase in the absorbance against time was measured. The extinction coefficient under the conditions described was 14500 L mol cm^{-1}.

One unit (U) was defined as the amount of enzyme catalyzing the liberation of 1 µmol *p*-nitrophenol/ min at 30 °C under the given conditions.

2.6 PHB Characterization

A spectrophotometric assay was performed after modification as described by Law and Slepecky (1961) (PerkinElmer-.UV/VIS Spectrometer Lambda) to determined PHB as crotonic acid [18]. Two ml from each cultivation was centrifuged at 13000 rpm for 15 min. One ml H_2SO_4 was then added to the precipitate, which contained the cells. The solution was incubated in 70°C for 20 min. After cooling, 10 µl was taken and added to 990 µl H_2O and the absorbance of the solution was measured at 235 nm. The PHB

amount for each experiment was determined by calculating the amount of crotonic acid against crotonic acid standard curve reproduced from standard PHB authentic sample.

2.7 Experimental Designs

2.7.1.1 Plackett-Burman

The different variables (X_1 to X_{14}) with amounts represented as +1, 0 and -1 (as presented in Table 1) are:

A. Four microbial strains consisting of
   - (X_1) *B. subtilis*,
   - (X_2) *B. pumilus,*
   - (X_3) *B. thuringiensis*, and
   - (X_4) *B. sp.*

B. Ten different constituents of the used medium:

(X_5)	Glucose;	(X_{10})	KH_2PO_4 ;
(X_6)	Tryptone Soyabean;	(X_{11})	Trice elements solution;
(X_7)	KCl;	(X_{12})	$FeSO_4$;
(X_8)	Tween-20;	(X_{13})	Yeast extract; and
(X_9)	Cottonseed oil;	(X_{14})	Skim milk

Twenty experiments containing +1 and -1 values following Plackett-Burman design have been conducted as in Table 2 to optimize fourteen variables represented at two levels, high and low, which are denoted by +1 and -1. In addition, two centre points – experiments containing only the medium level, denoted by 0 values, were conducted.

Three responses representing PHB, proteases and lipases have been measured for each experiment. All of the twenty-two experiments were conducted using 250 ml Erlenmayer-Flasks containing 100 ml medium at 37°C and 250 rpm for 48 h. Three different amounts: 100 µl/100ml (+1); 55 µl/100ml (0); and 10 µl/100ml (-1) of each *Bacillus* strain was added. *Bacillus* species were cultivated overnight in test tubes containing LB media.

The OD_{600} for all of the strains were standardized using a spectrophotometer where 10 µl from each cultivation were added to 990 ml water to give absorbance equal to 0.025 nm. Sterile distilled water was used to adjust the absorbance before the start of the experiment under aseptic conditions. All quantities calculated as x/100 ml culture as in Table 1.

2.7.2 Multiple Linear Regression Analysis of Plackett-Burman Design

The results of the Plackett-Burman design experiments were applied to linear multiple regression analysis using Micosoft Excel 2002. The linear multiple regression analysis was conducted for each of PHB, proteases and lipases respectively. The statistical analysis of the data in Table 3 has been summarized in Table 7, 9 and 11. The variables whose confidence levels % were ≥ than 90 % were considered to be significantly affecting the PHB, proteases and lipases productions, whilst variables with confidence level % between 90 % and 70 % were considered to be effective [19]. i.e.: Confidence level ≥ 90 %: Significant effect on PHB, proteases and lipases production 90 % ≥ Confidence level ≥ 70 % : Effective on PHB, proteases and lipases production

2.7.3 Generating 1st Order Model.

The model created from the analysis of Plackett-Burman experimental design using multiple regression analysis is based on the 1st order-model:

$$Y=\beta_0+\sum\beta_i\ X_i \qquad (1)$$

Where Y is the predicted response, β_0 model intercept, and β_i variables linear coefficient. ANOVA test was generated for each response to determine the relationship between the variables at the 90 % or higher confidence level.

2.7.4 Response Surface Experimental Design

Response surface design using Box-Behnken experimental design, modeling and analysis were carried out using Microsoft Excel 2000 and Essential Exp., Version 2.205 software [20]. Box-Behnken experimental design was used to optimize three variables represented at three levels: High, Medium and Low; which are denoted by +1, 0 and -1 respectively. The selected variables are Cottonseed oil (X_1), Glucose (X_2) and Skim milk (X_3). Fifteen experiments were conducted as in Table 6.

The other medium constituents were used in the same quantities as in the experiments that gave the highest amounts of PHB.

Table 2: Values of the variables randomized in Box-Behnken design

Media constituents	**+1**	**0**	**-1**	**x/100 ml**
Glucose	2	1.2	0.4	gm
Cottonseed Oil	8	5	2	gm
Skim milk	10	5.1	0.2	mg

2.7.5 Multiple Linear Regression Analysis of Box-Behnken Experimental Design

The PHB produced from the different experiment results were analyzed by multiple regression analysis.

2.7.6 Generating 2nd Order Model

The created model was applied using the coefficient result of each variable. For the three randomized variables (X_1, X_2 and X_3) this equation was used:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (2)$$

Where:

X_1, X_2 and X_3 are independent variables;
β_1, β_2, and β_3 are linear coefficients;
β_{12}, β_{13} and β_{23} are cross product coefficients; and
β_{11}, β_{22} and β_{33} are the quadratic coefficients.

The various response surface and counter plots for each two variables and the response (PHB g/l) were done using Excel 2000 and Essential Exp., Version 2.205 software [20].

2.7.7 Microsoft Excel Solver Optimization

Cottonseed oil (X_1), Glucose (X_2) and Skim milk (X_3) were further optimized to calculate the best Y (PHB production) value using Microsoft excel 2002 solver. The experiment with calculated optimum Cottonseed oil X_1, Glucose X_2 and Skim milk X_3 was conducted practically in the laboratory.

2.7.8 Determination of the 2nd Order Model Accuracy

To prove the accuracy of the model; the % accuracy was calculated from the formula:

$$\% \text{ Model Accuracy} = [Y_{Experiment} / Y_{Calculated}] \times 100 \quad (3)$$

3. RESULTS AND DISCUSSION

Plackett-Burman Design

PHB is a biodegradable bioplastic. PHB has been used in various important applications most of which are protected by patents. Notwithstanding researches' continuous quest and investigations on PHB ever since the time of their discovery, However, PHB has yet to be commercialized.

PHB has many positive points over petroleum plastics [4]. However, its production cost did not encourage investors for long term market penetration. The researchers have been concentrating on increasing its production in an attempt at reducing the production cost. Despite numerous efforts at improvements (the fermentation conditions; the media; the strains; the cell density; the biochemical engineering; enhancement of the PhaC synthase activity; controlling the metabolic pool and directing it to produce PHB etc.); all these strategies failed at succeeded in PHB's cost reduction and commercialization [4]. Still, there are yet many opportunities for PHB commercialization. Amara (2008) suggests directing the research concerning PHB to medicinal applications [4].

The current study aims to produce other products alongside PHB to reduce the overall production costs. It has produced proteases, lipases, and as well as PHB using mixed *Bacillus* strains culture in different mediums with ten different components as described earlier. These strains and constituents have been randomized through Plackett-Burman design to map the best conditions for the production of PHB, proteases and lipases [13-14]. Statistical and logical analyses of the yielded data were used to evaluate the best variables for further usein Box-Behnken design. Plackett-Burman and Box-Behenken proved to be powerful tools for PHB optimization, while Excel solver has been used for extra-optimization. The results were analysed mathematically and statistically to investigate the role of each variable [13].

3.1.1 Plackett-Burman Mean effect

The Plackett-Burman experimental data was yielded using +1, 0 and -1quantitative denotions for each variable (Table 3) where twenty-two experiments were conducted. The variations in results illustrate the importance of using the above experimental design in media optimization. The means of (+1) experiments and (-1) experiments were calculated using the following formulae:

$$\text{Mean}_{(+1)\,\text{experiments}} = \sum_{(+1)} / n_{(+1)} \quad (4)$$

$$\text{Mean}_{(-1)\,\text{experiments}} = \sum_{(-1)} / n_{(-1)} \quad (5)$$

The Main Effect of both of +1 and -1 for each variable was taken as the difference between the two means above:

$$\text{Main Effect} = [\sum_{(+1)} / n_{(+1)}] - [\sum_{(-1)} / n_{(-1)}] \quad (6)$$

The Main Effects of the different variables for PHB, proteases and lipases were calculated and summarized in Figure 1, 2 and 3 as well as Table 4, 5 and 6 respectively.

The experiments with Main Effect values above the x-axis in the graphs as shown in Fig. 1, 2 and 3; have positive effects on the production of PHB, proteases and lipases respectively; whilst those under the x-axis have negative effects *B. thuringiensis* has the highest positive effect on PHB and Proteases productions while K_2HPO_4 and trace elements solution have the highest negative effect. Cottonseed oil has the highest positive effect on Lipases production while *B. subtilis* has the highest negative effect.

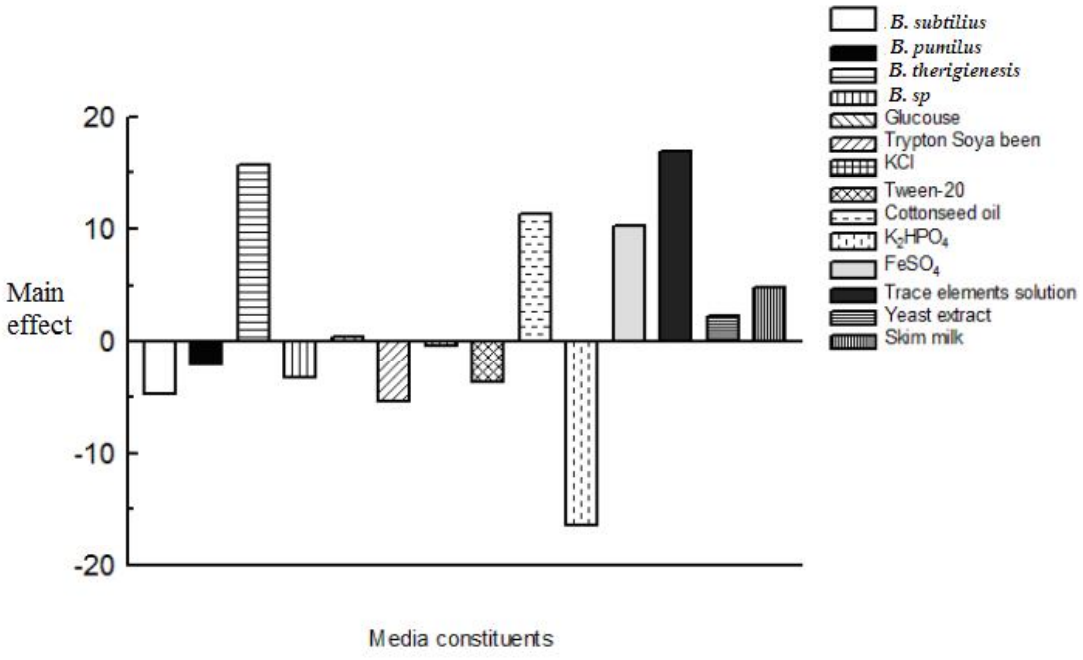

Fig. 1: Main effect of medium compositions on PHB production.

Table 3: Plackett-Burman design. Experiments 13, 18 and 1 show high PHB, Protease and Lipases respectively. Experiments 3 and 11 contain only 0 value as a central points *(Y=βo)*

Exp. No.		*Bacillus strains*				*Media constituents Units/100 ml*										*Responses*		
		***B. subtilius* (X_1)**	***B. pumilus* (X_2)**	***B. therigienesis* (X_3)**	***B. sp.* (X_4)**	***Glucose* (X_5)**	***Trypton Soya been* (X_6)**	***KCl* (X_7)**	***Tween-20* (X_8)**	***Cotto-nseed oil* (X_9)**	***K_2HPO_4* (X_{10})**	**$FeSO_4$ (X_{11})**	**Trace element solution (X_{12})**	***Yeast extract* (X_{13})**	***Skim milk* (X_{14})**	***PHB g/l***	***Protease Units/ml***	***lipase Units/ml***
Plackett-Burman Experiments	1	1 (100)	1 (100)	1 (100)	-1 (10)	-1 (0.4)	1 (10)	1 (0.3)	-1 (0.15)	1 (8)	1 (0.1)	-1 (1)	-1 (1)	-1 (0.08)	-1 (0.2)	2.18	230.2	0.22
	2	1 (100)	1 (100)	1 (100)	1 (100)	-1 (0.4)	-1 (5)	1 (0.3)	1 (1.5)	-1 (2)	1 (0.1)	1 (5)	-1 (1)	-1 (0.08)	-1 (0.2)	0.94	99.8	0.02
	3	0 (55)	0 (55)	0 (55)	0 (55)	0 (1.2)	0 (7.5)	0 (0.165)	0 (0.825)	0 (5)	0 (0.055)	0 (3)	0 (3)	0 (0.24)	0 (5.1)	4.42	175.4	0.91
	4	-1 (10)	-1 (10)	1 (100)	-1 (10)	1 (2)	-1 (5)	1 (0.3)	1 (1.5)	1 (8)	1 (0.1)	-1 (1)	-1 (1)	1 (0.4)	1 (10)	4.26	333.6	8.59
	5	-1 (10)	1 (100)	1 (100)	-1 (10)	1 (2)	1 (10)	-1 (0.03)	-1 (0.15)	-1 (2)	-1 (0.01)	1 (5)	-1 (1)	1 (0.4)	-1 (0.2)	2.75	83.2	0.00
	6	-1 (10)	1 (100)	-1 (10)	1 (100)	-1 (0.4)	1 (10)	1 (0.3)	1 (1.5)	1 (8)	-1 (0.01)	-1 (1)	1 (5)	1 (0.4)	-1 (0.2)	2.11	114.0	5.86
	7	-1 (10)	1 (100)	-1 (10)	1 (100)	1 (2)	1 (10)	1 (0.3)	-1 (0.15)	-1 (2)	1 (0.1)	1 (5)	-1 (1)	1 (0.4)	1 (10)	2.18	44.7	6.42
	8	1 (100)	-1 (10)	-1 (10)	-1 (10)	-1 (0.4)	1 (10)	-1 (0.03)	1 (1.5)	-1 (2)	1 (0.1)	1 (5)	1 (5)	1 (0.4)	-1 (0.2)	3.17	104.4	0.90
	9	1 (100)	1 (100)	-1 (10)	-1 (10)	1 (2)	1 (10)	-1 (0.03)	1 (1.5)	1 (8)	-1 (0.01)	-1 (1)	-1 (1)	-1 (0.08)	1 (10)	1.85	64.2	7.66
	10	1 (100)	-1 (10)	1 (100)	1 (100)	1 (2)	1 (10)	-1 (0.03)	-1 (0.15)	1 (8)	1 (0.1)	-1 (1)	1 (5)	1 (0.4)	-1 (0.2)	3.76	99.1	1.47
	11	0 (55)	0 (55)	0 (55)	0 (55)	0 (1.2)	0 (7.5)	0 (0.165)	0 (0.825)	0 (5)	0 (0.055)	0 (3)	0 (3)	0 (0.24)	0 (5.1)	2.86	165.0	2.09
	12	1 (100)	1 (100)	-1 (10)	-1 (10)	-1 (0.4)	-1 (5)	1 (0.3)	-1 (0.15)	1 (8)	-1 (0.01)	1 (5)	1 (5)	1 (0.4)	1 (10)	6.68	38.1	0.72
	13	-1 (10)	1 (100)	1 (100)	1 (100)	1 (2)	-1 (5)	-1 (0.03)	1 (1.5)	1 (8)	-1 (0.01)	1 (5)	1 (5)	-1 (0.08)	-1 (0.2)	8.47	35.0	2.79
	14	-1 (10)	-1 (10)	1 (100)	1 (100)	-1 (0.4)	1 (10)	1 (0.3)	-1 (0.15)	-1 (2)	-1 (0.01)	-1 (1)	1 (5)	-1 (0.08)	1 (10)	5.40	70.6	9.67
	15	-1 (10)	1 (100)	1 (100)	-1 (10)	-1 (0.4)	-1 (5)	-1 (0.03)	1 (1.5)	-1 (2)	1 (0.1)	-1 (1)	1 (5)	1 (0.4)	1 (10)	2.68	49.2	0.78
	16	-1 (10)	-1 (10)	-1 (10)	-1 (10)	1 (2)	-1 (5)	1 (0.3)	-1 (0.15)	1 (8)	1 (0.1)	1 (5)	1 (5)	-1 (0.08)	-1 (0.2)	2.44	92.3	8.04
	17	1 (100)	-1 (10)	1 (100)	-1 (10)	1 (2)	1 (10)	1 (0.3)	1 (1.5)	-1 (2)	-1 (0.01)	1 (5)	1 (5)	-1 (0.08)	1 (10)	4.60	91.2	0.53
	18	1 (100)	-1 (10)	1 (100)	1 (100)	-1 (0.4)	-1 (5)	-1 (0.03)	-1 (0.15)	1 (8)	-1 (0.01)	1 (5)	-1 (1)	1 (0.4)	1 (10)	4.55	687.4	9.60

	19	-1 (10)	-1 (10)	-1 (10)	-1 (10)	-1 (0.4)	-1 (5)	-1 (0.03)	-1 (0.15)	-1 (2)	-1 (0.01)	-1 (1)	-1 (1)	-1 (0.08)	-1 (0.2)	2.75	48.4	0.00
	20	-1 (10)	-1 (10)	-1 (10)	1 (100)	-1 (0.4)	1 (10)	-1 (0.03)	1 (1.5)	1 (8)	1 (0.1)	1 (5)	-1 (1)	-1 (0.08)	1 (10)	1.06	41.9	3.16
	21	1 (100)	-1 (10)	-1 (10)	1 (100)	1 (2)	-1 (5)	1 (0.3)	1 (1.5)	-1 (2)	-1 (0.01)	-1 (1)	-1 (1)	1 (0.4)	-1 (0.2)	0.78	230.2	0.00
	22	1 (100)	1 (100)	-1 (10)	1 (100)	1 (2)	-1 (5)	-1 (0.03)	-1 (0.15)	-1 (2)	1 (0.1)	-1 (1)	1 (5)	-1 (0.08)	1 (10)	0.85	99.8	0.00

Table 4: Main effect of each variable using Plackett-Burman design on PHB production.

Variable	**Values**		**Main effect** $[\sum_{(+1)} / n_{(+1)}] - [\sum_{(-1)} / n_{(-1)}]$
	$\sum_{(+1)} / n_{(+1)}$	$\sum_{(-1)} / n_{(-1)}$	
B. subtilis	29.4	34.14	-4.74
B. pumilus	30.74	32.8	-2.05
B. thurigiensis	39.63	23.91	15.71
B. sp.	30.13	33.40	-3.26
Glucose	31.97	31.56	0.41
Tryptone Soyabean	29.10	34.44	-5.33
KCl	31.61	31.93	-0.32
Tween-20	29.96	33.57	-3.60
Cottonseed oil	37.41	26.12	11.28
K_2HPO_4	23.55	39.98	-16.42
$FeSO_4$	36.88	26.65	10.23
Trace elements solution	40.20	23.33	16.86
Yeast extract	32.95	30.58	2.37
Skim milk	34.16	29.37	4.79

Table 5: Main effect of each variable using Plackett-Burman design on proteases production

Variables	Values		Main effect
	$\sum_{(+1)} / n_{(+1)}$	$\sum_{(-1)} / n_{(-1)}$	$[\sum_{(+1)} / n_{(+1)}] - [\sum_{(-1)} / n_{(-1)}]$
B. subtilis	174.44	91.29	83.15
B. pumilus	85.82	179.91	-94.09
B. thuringiensis	177.93	87.8	90.13
B. sp.	152.25	113.48	38.77
Glucose	117.33	148.4	-31.07
Tryptone Soyabean	94.35	171.38	-77.03
KCl	134.47	131.26	3.21
Tween-20	116.35	149.38	-33.03
Cottonseed oil	173.58	92.15	81.43
K_2HPO_4	119.5	146.23	-26.73
$FeSO_4$	131.8	133.93	-2.13
Trace elements solution	79.37	186.36	-106.99
Yeast extract	178.39	87.34	91.05
Skim milk	152.07	113.66	38.41

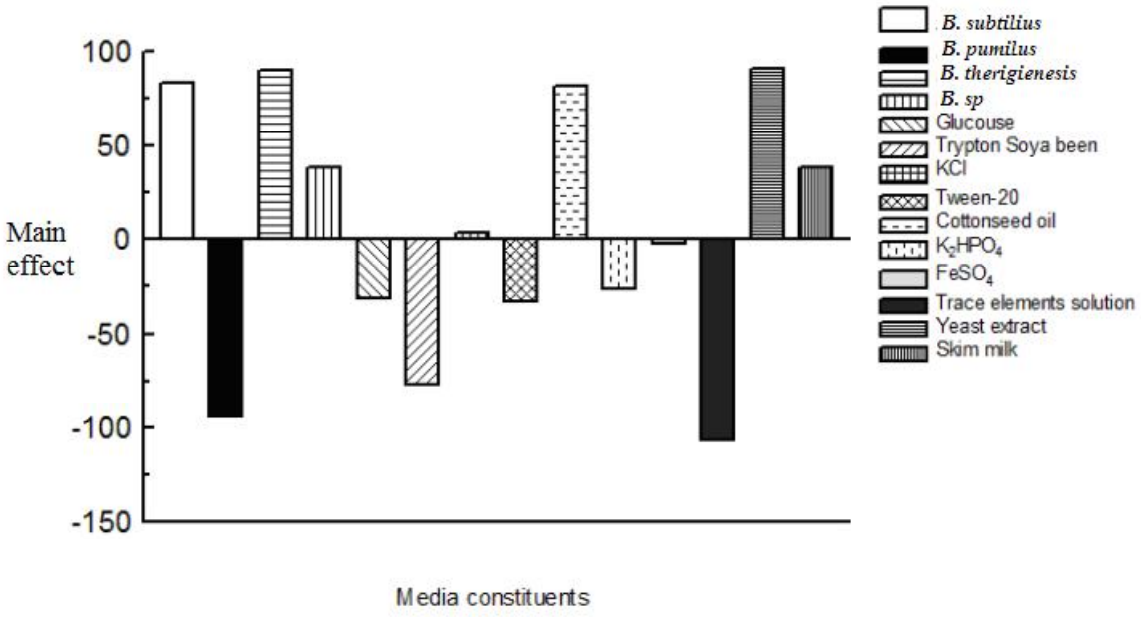

Fig. 2: Main effect of medium compositions on proteases production.

Table 6: Main effect of each variable using Plackett-Burman design on lipases production

Variable	Values		Main effect
	$\sum_{(+1)} / n_{(+1)}$	$\sum_{(-1)} / n_{(-1)}$	$[\sum_{(+1)} / n_{(+1)}] - [\sum_{(-1)} / n_{(-1)}]$
B. subtilis	2.112	4.531	-2.419
B. pumilus	2.447	4.196	-1.749
B. thuringiensis	3.367	3.276	0.091
B. sp.	3.899	2.744	1.155
Glucose	3.55	3.093	0.457
Tryptone Soyabean	3.589	3.054	0.535
KCl	4.007	2.636	1.371
Tween-20	3.029	3.614	-0.585
Cottonseed oil	4.811	1.832	2.979
K_2HPO_4	2.96	3.683	-0.723
$FeSO_4$	3.218	3.425	-0.207
Trace elements solution	3.076	3.567	-0.491
Yeast extract	3.434	3.209	0.225
Skim milk	4.713	1.93	2.783

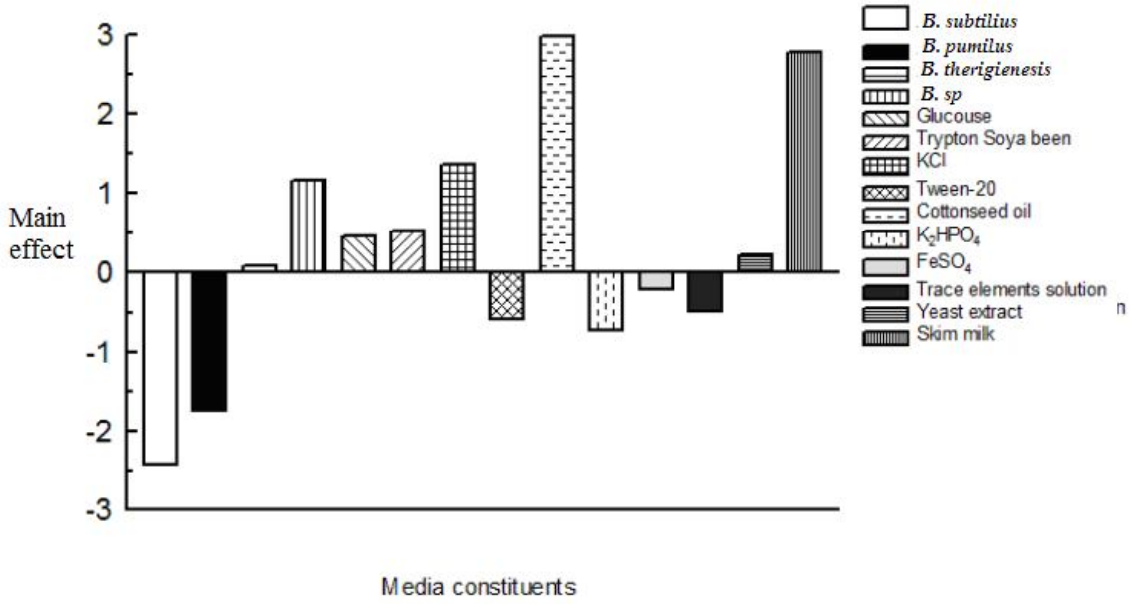

Fig. 3: Main effect of medium compositions on lipases production.

3.1.2 Plackett-Burman Linear Multiple Regression Analysis and ANOVA Test

A multiple linear regression analysis for PHB, proteases and lipases results has been performed to study the relationship between different variables and their level of significance regarding each response. The Coefficient, Standard error, T Statistic, P-Value and Confidence level % for each has been calculated as in Tables 7, 9 and 11. The confidence level has been calculated from the formula

$$\textit{The confidence level \%} = 100*(1 - \textit{P-value}).$$

The P-value from the ANOVA analysis for each response was determined to analyse the relationship between the variables at 90 % or higher confidence level as in Table 8, 10 and 12.

Table 7: Linear multiple regression analysis results of Plackett-Burman design [PHB]

Variables	**Coefficient**	**Standard Error**	**T Statistic**	**P-Value**	**Confidence level %**
Intercept	3.21973	0.377774	8.52289	0.0001	99.99
Cottonseed oil	0.5643	0.396213	1.42423	0.1974	80.26
Glucose	0.0208	0.396213	0.052497	0.9596	4.04
K_2HPO_4	-0.8212	0.396213	-2.07262	0.0769	92.31
KCl	-0.0161	-0.396213	0.040635	0.9687	3.13
Skim milk	0.2398	0.396213	0.60523	0.5641	43.59
B. subtilis	-0.2371	0.396213	-0.59842	0.5684	43.16
B. pumilus	-0.1029	0.396213	-0.25971	0.8026	19.74
B. thuringiensis	0.7859	0.396213	1.98353	0.0877	91.23
B. sp.	-0.1634	0.396213	-0.41241	0.6924	30.76
$FeSO_4$	0.5115	0.396213	1.29097	0.2377	76.23
Trace elements solutions	0.8435	0.396213	2.12891	0.0708	92.92

Tryptone Soyabean	-0.2669	0.396213	-0.67363	0.5222	47.78
Tween-20	-0.1803	0.396213	-0.45506	0.6628	33.72
Yeast extract	0.1187	0.396213	0.299586	0.7732	22.68

Table 8: ANOVA statistical analysis of variance of Plackett-Burman Model [PHB]

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	57.062	14	4.07586	1.3	0.3786
Residual	21.9778	7	3.13969		
Total (Corr.)	79.0399	21			

R-squared = 72.194 %; R-squared = 16.5819 %;
Standard Error = 1.77192 and Mean absolute error = 0.875939

The relationship between PHB and fourteen independent variables were fitted using linear multiple regression analysis and the model has been generated.

$$PHB = 3.21973 + 0.5643 * Cottonseed\ oil + 0.0208 * Glucose - 0.8212 * K_2HPO_4 - 0.0161 * KCl + 0.2398 * Skim\ milk - 0.2371 * B.\ subtilis - 0.1029 * B.\ pumilus + 0.7859 * B.\ thuringiensis - 0.1634 * B.\ sp. + 0.5115 * FeSO_4 + 0.8435 * Trace\ elements\ solution - 0.2669 * Tryptone\ Soyabean - 0.1803 * Tween\text{-}20 + 0.1187 * Yeast\ extract$$

The P-value from the ANOVA analysis is greater or equal to 0.10. There is no statistically significant relationship between the variables at the 90 % or higher confidence level. The standard error of the estimate shows the standard division of residuals to be 1.77192. The R-Squared statistic indicates that the model as fitted explains 72.194 % of the variability in PHB. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 16.5819 %. The mean absolute error of 0.875939 is the average value of the residuals. The highest P-value on the independent variables is 0.9687, belonging to KCl which is not significant and could be deleted from the model.

Table 9: Linear multiple regression analysis results of Plackett-Burman design [Proteases]

Variables	Coefficient	Standard Error	T Statistic	P-Value	Confidence level %
Intercept	136.259	28.7436	4.7405	0.0021	99.79
Cottonseed oil	40.715	30.1465	1.35057	0.2189	78.11
$FeSO_4$	-1.065	30.1465	-0.03533	0.9728	2.72
Glucose	-15.535	30.1465	-0.51532	0.6222	37.78
K_2HPO_4	-13.365	30.1465	-0.44334	0.6709	32.91
KCl	1.605	30.1465	0.05324	0.959	4.1
B. subtilis	41.575	30.1465	1.3791	0.2103	78.97
B. pumilus	-47.045	30.1465	-1.56054	0.1626	83.74
B. thuringiensis	45.065	30.1465	1.49487	0.1786	82.14
B. sp.	19.385	30.1465	0.643026	0.5407	45.93

Skim milk	19.205	30.1465	0.637055	0.5444	45.56
Trace elements solution	-53.495	30.1465	-1.7745	0.1193	88.07
Tryptone Soyabean	-38.515	30.1465	-1.27759	0.2421	75.79
Tween-20	-16.515	30.1465	-0.54782	0.6008	39.92
Yeast extract	45.525	30.1465	1.51012	0.1748	82.52

Table 10: ANOVA statistical analysis of variance of Plackett-Burman Model [Proteases]

Source	**Sum of Squares**	**Df**	**Mean Square**	**F-Ratio**	**P-Value**
Model	309779	14	22127.1	1.22	0.4145
Residual	127234	7	18176.3		
Total (Corr.)	437013.0	21			

R-squared = 70.8856 %; R-squared = 12.6567 %;
Standard Error = 134.819 and Mean absolute error = 58.3017

The relationship between the amount of proteases enzymes as Units/ ml and fourteen variables fitted using multiple linear regression analysis and the following model has been generated.

$$Protease = 136.259 + 40.715 * Cottonseed\ oil - 1.065 * FeSO_4 - 15.535 * Glucose - 13.365 * K_2HPO_4 + 1.605 * KCl + 41.575 * B.\ subtilis - 47.045 * B.\ pumilus + 45.065 * B.\ thuringiensis + 19.385 * B.\ sp. + 19.205 * Skim\ milk - 53.495 * Trace\ elements\ solution - 38.515 * Tryptone\ Soyabean - 16.515 * Tween\text{-}20 + 45.525 * Yeast\ extract$$

The P-value from the ANOVA analysis is greater or equal to 0.10. There is no statistically significant relationship between the variables at the 90 % or higher confidence level. The R-Squared statistic indicates that the model as fitted explains 70.8856 % of the variability in Protease. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 12.6567 %. The standard error of the estimate shows the standard deviation of the residuals to be 134.819. The mean absolute error of 58.3017 is the average value of the residuals. The highest P-value on the independent variables is 0.9728, belonging to $FeSO_4$ thus could be deleted from the model.

Table 11: Linear multiple regression analysis results of Plackett-Burman design [Lipases]

Variables	Coefficient	Standard Error	T Statistic	P-Value	Confidence level %
Intercept	3.15916	0.861809	3.66573	0.008	99.2
Cottonseed oil	1.49043	0.903873	1.64894	0.1431	85.69
$FeSO_4$	-0.10381	0.903873	-0.11485	0.9118	8.82
Glucose	0.22825	0.903873	0.252525	0.8079	19.21
K_2HPO_4	-0.36047	0.903873	-0.39881	0.7019	29.81
KCl	0.6857	0.903873	0.758625	0.4728	52.72
B. subtilis	-1.20956	0.903873	-1.3382	0.2227	77.73
B. pumilus	-0.87408	0.903873	-0.96704	0.3657	63.43
B. thuringiensis	0.04671	0.903873	0.051678	0.9602	3.98
B. sp.	0.5777	0.903873	0.639139	0.5431	45.69
Skim milk	1.39143	0.903873	1.53941	0.1676	83.24
Trace elements solution	-0.24533	0.903873	-0.27142	0.7939	20.61
Tryptone Soyabean	0.26767	0.903873	0.296137	0.7757	22.43
Tween-20	-0.29219	0.903873	-0.32327	0.7559	24.41
Yeast extract	0.11281	0.903873	0.124807	0.9042	9.58

The relationship between the amount of Lipases as Units/ml and fourteen variables were fitted using multiple regression analysis and the following model has been generated.

*Lipase = 3.15916 + 1.49043 * Cottonseed oil - 0.10381 * $FeSO_4$ + 0.22825 * Glucose - 0.36047 * K_2HPO_4 + 0.6857 * KCl - 1.20956 * B. subtilis - 0.87408 * B. pumilus + 0.04671 * B. thuringiensis + 0.5777 * B. sp. + 1.39143 * Skim milk – 0.24533 * Trace elements solution + 0.26767 * Tryptone Soyabean - 0.29219 * Tween-20 + 0.11281 * Yeast extract*

Table 12: ANOVA statistical analysis of variance of Plackett-Burman Model [Lipases]

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	152.267	14	10.8762	0.67	0.7555
Residual	114.378	7	16.3397		
Total (Corr.)	266.645	21			

R-squared = 57.1048 %; R-squared = 0.0 %;
Standard Error = 4.04224 and Mean absolute error = 1.92388

The P-value from the ANOVA analysis is greater or equal to 0.10. There is no statistically significant relationship between the variables at the 90 % or higher confidence level. The R-Squared statistic indicates that the model as fitted explains 57.1048 % of the variability in lipase. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 0.0 %. The standard error of the estimate shows the standard deviation of the residuals to be 4.04224. The mean absolute error of 1.92388 is the average value of the residuals. The highest P-value on the independent variables is 0.9602, belonging to *B. thuringiensis* which is not significant and could be deleted from the model.

From reading the results of the linear multiple regression analysis of each of PHB, Proteases and Lipases as well as ANOVA tests, it is clear that there are insignificant relationships between variables included in the *Bacillus* strains and media constituents as in Tables 7-12. Meanwhile there is a significant increase in the PHB production in the Plackett-Burman design. The PHB amount in the experiment number 13 was 8.47 g/l while in experiment number 21 it was 0.71 g/l. Plackett-Burman succeeded in mapping the points where PHB production increased. Statistically, trace elements solution is the most effective variable in production of both of PHB and Proteases; and Cottonseed oil in case of Lipases.

In the case of Lipases the results is perfectly logical since cottonseed oil is the main substrate for lipases. In the case of PHB, trace elements solution plays significant roles in PHB accumulations in the microbial cells but it is not the main PHB substrate. Unfortunately and unexpectedly, Glucose, which is the main substrate for PHB, gives only 4.04 confidence level %. It is clear that PHB amounts could be increased if factors affecting the Glucose consumption have been removed, in which instance providing an opportunity to gain more PHB quantities.

The same results were observed in the case of proteases production where trace elements solution also was the highest in its confidence level percentage. Meanwhile in the case of Proteases further optimization was biased to Skim milk which is (from our experience) more suitable as substrate for *Bacillus* strains in comparison to the Tryptone Soyabean (which is higher in its confidence level %), as well as being less expensive [21-22].

3.2 Box-Behnken Design

Cottonseed oil, Glucose and Skim milk were used for further optimization in Box-Behnken design as in Table 13 [14]. Meanwhile to get the maximum benefit from the results obtained from Plackett-Burman design the media constituents in experiment number 13 (which gave the maximum PHB amount) was used instead of the substrates randomized in Box-Behnken as in Table 13.

Table 13: Box-Behnken design experiments and the different PHB values.

Experiment No.	*Randomized media constituents variables*			*Responses*		
	Cottonseed oil (X_1)	*Glucose (X_2)*	*Skim milk (X_3)*	*PHB g/l*	*Protease Units/ ml*	*lipase Units/ ml*
1	0 (5)	0 (1.2)	0 (5.1)	3.668	133.62	3.01
2	0 (5)	1 (2)	-1 (0.2)	11.117	685.26	15.23
3	1 (8)	-1 (0.4)	0 (5.1)	0.297	762.91	10.65
4	0 (5)	0 (1.2)	0 (5.1)	1.736	733.79	15.05
5	1 (8)	0 (1.2)	-1 (0.2)	0.651	782.32	26.27
6	1 (8)	1 (2)	0 (5.1)	3.306	149.80	5.77
7	0 (5)	-1 (0.4)	1 (10)	1.304	830.85	22.49
8	-1 (2)	1 (2)	0 (5.1)	4.945	172.45	3.11
9	0 (5)	1 (2)	1 (10)	14.726	1005.57	10.78
10	1 (8)	0 (1.2)	1 (10)	0.986	175.68	2.39
11	-1 (2)	0 (1.2)	-1 (0.2)	6.545	182.15	2.14
12	-1 (2)	0 (1.2)	1 (10)	3.971	177.30	1.28
13	0 (5)	0 (1.2)	0 (5.1)	1.489	1005.57	3.15
14	0 (5)	-1 (0.4)	-1 (0.2)	0.978	689.79	5.51
15	-1 (2)	-1 (0.4)	0 (5.1)	0.607	138.48	4.41

The other media constituents are: *B. subtilis* 100 µl (X_1); *B. pumilus* 10 µl (X_2); *B. thuringiensis* 100 µl (X_3); *B. Sp.* 100 µl (X_4); Tryptone Soyabean 5 mg/100 ml (X_6); *KCl* 0.03 g/100ml (X_7); Tween-20 1.5 g/100ml (X_8); K_2HPO_4 0.01 g/100 ml (X_{10}); trace elements solution (X_{11}); $FeSO_4$ 5 µl (X_{12}) and Yeast extract 0.08 g/l (X_{13})

3.2.1 Box-Behnken Design Multiple Regression Analysis and ANOVA Test

Table 14: Linear multiple regression analysis result of Box-Behnken design [PHB]

Variables	Coefficient	Standard Error	T Statistic	P-Value	Confidence level %
Intercept	2.29767	1.76024	1.30531	0.2486	75.14
X_1	-1.3535	1.07792	-1.25565	0.2647	73.53
X_1X_1	-2.00096	1.58666	-1.26111	0.2629	73.71
X_1X_2	-0.33225	1.52442	-0.21795	0.8361	16.39
X_1X_3	0.72725	1.52442	0.477068	0.6534	34.66
X_2	3.8635	1.07792	3.5842	0.0158	98.42
X_2X_2	1.99204	1.58666	1.25549	0.2648	73.52
X_2X_3	0.82075	1.52442	0.538403	0.6134	38.66
X_3	0.212	1.07792	0.196674	0.8518	14.82
X_3X_3	2.74154	1.58666	1.72787	0.1446	85.54

Where X_1= Cottonseed oil, X_2=Glucose and X_3= Skim milk

Table 15: ANOVA statistical analysis of variance of Box-Behnken [PHB]

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	199.641	9	22.1824	2.39	0.1755
Residual	46.4768	5	9.29537		
Total (Corr.)	246.118	14			

R-squared = 81.116 %; R-squared = 47.1249 %; Standard Error = 3.04883 and Mean absolute error = 1.49464

The relationship between the amount of PHB and three variables and their interaction were fitted using multiple regression analysis and the following model has been generated.

$$PHB = 2.29767 - 1.3535 * X_1 - 2.00096 * X_1X_1 - 0.33225 * X_1X_2 + 0.72725 * X_1X_3 + 3.8635 * X_2 + 1.99204 * X_2X_2 + 0.82075 * X_2X_3 + 0.212 * X_3 + 2.74154 * X_3X_3$$

The P-value from the ANOVA analysis is greater or equal to 0.10. There is no statistically significant relationship between the variables at the 90 % or higher confidence level.

The R-Squared statistic indicates that the model as fitted explains 81.116 % of the variability in PHB. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 47.1249 %. The standard error of the estimate shows the standard deviation of the residuals to be 3.04883. The mean absolute error of 1.49464 is the average value of the residuals. The highest P-value on the independent variables is 0.8518, belonging to X_3 which could be deleted from the model.

Table 16: Linear multiple regression result of Box-Behnken design [Proteases]

Variables	Coefficient	Standard Error	T Statistic	P-Value	Confidence level %
Intercept	624.327	202.021	3.0904	0.0271	97.29
X_1	150.041	123.712	1.21282	0.2794	72.06
X_1X_1	-395.961	182.099	-2.17442	0.0817	91.83
X_1X_2	-161.77	174.956	-0.92464	0.3976	60.24
X_1X_3	-150.448	174.956	-0.85992	0.4291	57.09
X_2	-51.1187	123.712	-0.41321	0.6966	30.34
X_2X_2	77.5442	182.099	0.425834	0.6879	31.21
X_2X_3	44.8125	174.956	0.256137	0.808	19.2
X_3	-18.765	123.712	-0.15168	0.8854	11.46
X_3X_3	100.997	182.099	0.554624	0.603	39.7

Where X_1= Cottonseed oil, X_2=Glucose and X_3= Skim milk

Table 17: ANOVA statistical analysis of variance of Box-Behnken Model [Proteases]

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	1.09E+06	9	120718	0.99	0.5373
Residual	612189	5	122438		
Total (Corr.)	1.70E+06	14			

R-squared = 63.9602 %; R-squared = 0.0 %; Standard Error. = 349.911 and
Mean absolute error = 161.929

The relationship between the amount of Proteases enzymes as Units/ ml and three independent variables and their interaction were determined using linear multiple regression analysis and the following model has been generated.

$$Proteases = 624.327 + 150.041 * X_1 - 395.961 * X_1X_1 - 161.77 * X_1X_2 - 150.448 * X_1X_3 - 51.1187 * X_2 + 77.5442 * X_2X_2 + 44.8125 * X_2X_3 - 18.765 * X_3 + 100.997 * X_3X_3$$

The P-value from the ANOVA analysis is greater or equal to 0.10. There is not a statistically significant relationship between the variables at the 90 % or higher confidence level. The R-Squared statistic indicates that the model as fitted explains 63.9602 % of the variability in Proteases. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 0.0 %. The standard error of the estimate shows the standard deviation of the residuals to be 349.911. The mean absolute error of 161.929 is the average value of the residuals. The highest P-value on the independent variables is 0.8854, belonging to X_3 which could be deleted from the model.

Table 18: Linear multiple regression analysis result of Box-Behnken design [lipases]

Variables	Coefficient	Standard Error	T Statistic	P-Value	Confidence level %
Intercept	7.07	4.50726	1.56858	0.1775	82.25
X_1	4.2675	2.76012	1.54613	0.1827	81.73
X_1X_1	-3.28375	4.06279	-0.80825	0.4557	54.43
X_1X_2	-0.895	3.9034	-0.22929	0.8277	17.23
X_1X_3	-5.755	3.9034	-1.47435	0.2004	79.96
X_2	-1.02125	2.76012	-0.37	0.7265	27.35
X_2X_2	2.19875	4.06279	0.541192	0.6116	38.84
X_2X_3	-5.3575	3.9034	-1.37252	0.2283	77.17
X_3	-1.52625	2.76012	-0.55297	0.6041	39.59
X_3X_3	4.23375	4.06279	1.04208	0.3451	65.49

Where X_1= Cottonseed oil, X_2=Glucose and X_3= Skim milk

Table 19: ANOVA statistical analysis of variance of Box-Behnken Model [Lipases]

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	554.692	9	61.6324	1.01	0.525
Residual	304.731	5	60.9462		
Total (Corr.)	859.423	14			

R-squared = 64.5424 %; R-squared = 0.718618 %; Standard Error = 7.8068 and
Mean absolute error = 4.09333

The relationship between the amount of lipases enzymes as Units/ml and three independent variables and their interaction were determined using multiple regression analysis and the model has been generated.

$$Lipase = 7.07 + 4.2675 * X_1 - 3.28375 * X_1X_1 - 0.895 * X_1X_2 - 5.755 * X_1X_3 - 1.02125 * X_2 + 2.19875 * X_2X_2 - 5.3575 * X_2X_3 - 1.52625 * X_3 + 4.23375 * X_3X_3$$

The P-value from the ANOVA analysis is greater or equal to 0.10. There is no statistically significant relationship between the variables at the 90 % or higher confidence level. The R-Squared statistic indicates that the model as fitted explains 64.5424 % of the variability in Lipases. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 0.718618 %. The standard error of the estimate shows the standard deviation of the residuals to be 7.8068. The mean absolute error of 4.09333 is the average value of the residuals. The highest P-value on the independent variables is 0.8277, belonging to X_1X_2 which could be deleted from the model.

The various response surface and counter plots for each two variables and the response (PHB g/l/ 48 h) were drown using Excel 2000 and Essential Exp. Version 2.205 software as shown in Fig. 4 to 9 [20]. The surface responses and counter plots show the interaction between variables. The shape of response surface and counter plot as in Fig. 4 and 5 prove the weak interaction between the cottonseed oil and the Glucose. The results obtained from Fig. 6 and 8 concerning the interaction between Cottonseed and Skim milk shows some interaction. In the case of Fig. 8 and 9 the interaction between Glucose and Skim milk also shows some interaction. The results obtained from Fig. 4 to 9 agree with that in Table 14.

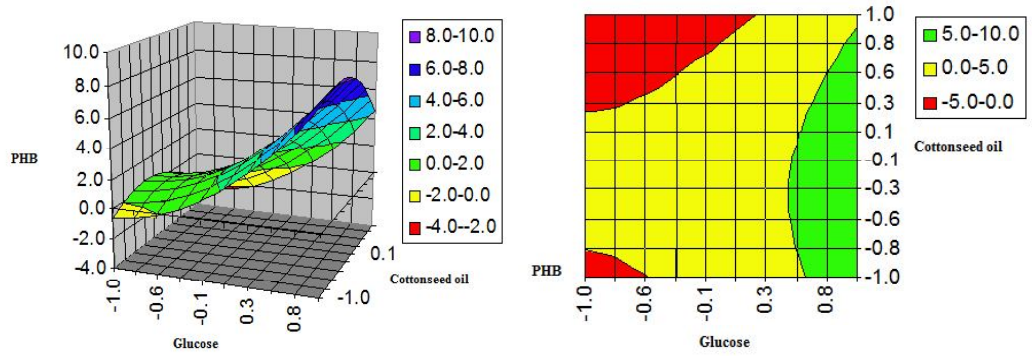

Fig. 4 and 5: The response surfaces plot and its corresponding contour plot showing the effects of Cottonseed oil and Glucose on the amount of PHB.

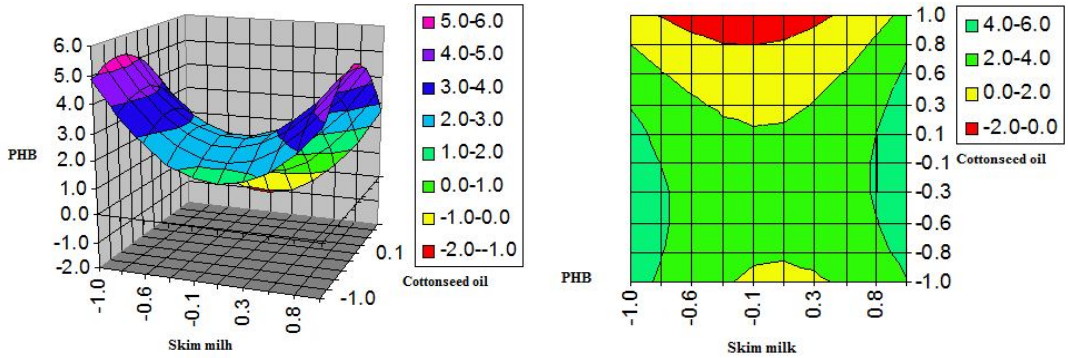

Fig. 6 and 7: The response surfaces plot and its corresponding contour plot showing the effects of Cottonseed oil and Skim milk on the amount of PHB.

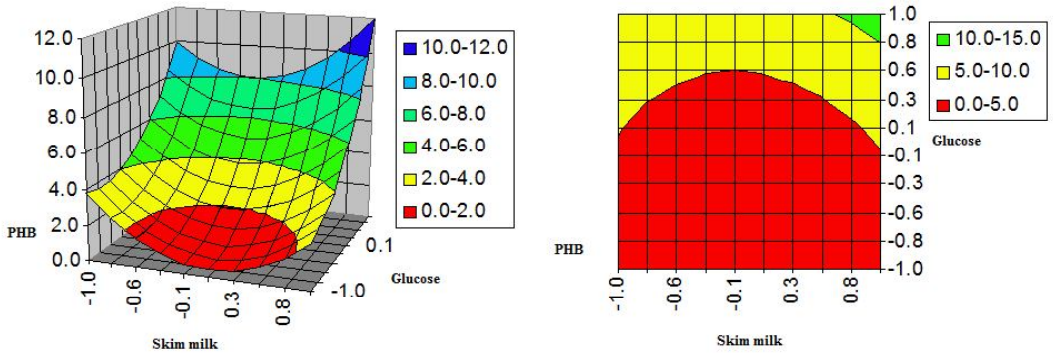

Fig. 8 and 9: The response surfaces plot and corresponding contour plot showing the effects of Glucose and Skim milk on the amount of PHB.

It is quite apparent from the results obtained from Box-Behnken experiments as well as the response surface and counter plots that there is a chance for further optimization.

3.3 Excel Solver For Optimization Box-Behnken Design

The best levels of the three variables as obtained from the maximum point of polynomial model were estimated using the solver function of Microsoft Excel 2000 tools and found to be for Cottonseed oil = 4.28 g/100 ml; for Glucose = 2 gm/100 ml and for Skim milk = 10 mg/100 ml, with a prediction calculated PHB equal to 12.04 g/ L/ 48 hr.

Table 20: Excel 2002 Solver optimization for X_1, X_2 and X_3

Term	***Cottonseed oil (X_1) gm/100 ml***	***Glucose (X_2) g/100 ml***	***Skim milk (X_3) mg/100 ml***
Data Minimum	-1 (2)	-1 (0.4)	-1 (0.2)
Data Average	0 (5)	0 (1.2)	0 (5.1)
Data Maximum	1 (8)	1 (2)	1 (10)
Data Solver	-0.23951 (4.28)	1 (2)	1 (10)

3.3.1 Confirming The Model Accuracy

The Y value which optimization from the model was calculated using Microsoft Excel and PHB amount found to be 12.04 g/ l/ 48 hr. The *in vivo* experiments show that Y value is 16.48 g/ l/ 48 hr. By calculating the model accuracy from the formula in material and methods section, the model accuracy was 136.8 %. The Proteases activities determined to be 534 Units/ ml while the Lipases activities were 22.56 Units/ ml.

The three variables selected were Cottonseed oil, Glucose and Skim milk. Glucose was responsible for PHB production, Skim milk for protease production and cottonseed oil for proteases production. The other medium constituents were used in quantity as in experiment number 13 (Plackett-Burman design) which show the best PHB production. Enzyme activity was used to evaluate different lipases and proteases from each experiment in both of Plackett-Burman and Box-Behnken methods. The result clearly proves that products other than PHB are an additional lode on the microbial cells. Meanwhile experimental design systematically succeeded in normalizing the production conditions. Regarding Plackett-Burman design the maximum amount from PHB was 8.478 g/ l/ 48 h. In case of Box-Behnken the maximum produced PHB was 14.726 g/ l/ 48 h. In case of using optimization conditions from the Excel solver, the maximum produced, PHB was 16.48 g/ l/ 48 hr. Regarding Plackett-Burman, Box-Behnken design and the Excel solver the maximum produced Proteases 35.0, 1005.57, 534 Units/ ml/ 48 h and for Lipases were 2.79, 3.15 and 22.56 Units / ml/ 48h respectively as in Table 21.

Table 21: Summarization of the maximum amounts of PHB, Proteases and Lipases gained from Plackett-Burman, Box-Behnken and Excel solver in one flask.

	Plackett-Burman	**Box-Behnken**	**Excel-Solver**	**Unit**
PHB	8.478	14.726	16.48	g/ l/ 48 h
Protease	35.0	1005.57	534	Units / ml/ 48 h
Lipase	2.79	3.15	22.56	Units / ml/ 48 h

PHB and Lipase gains have been systematically improved during various experimental designs and Excel solver as in Table 21. Only proteases have been reduced after optimization using Excel solver even though Skim milk has been used in quantities equal to its +1 range. The reduction in proteases has been expressed as increases in both of PHB and Lipases. By comparing experiment number 9 which shows maximum PHB production in Box-Behnken experiments with Excel solver, the only difference was in the amount of the cottonseed oil which was 5 g/ l/ 100 ml in Box-Behnken and 4.28 g/ l/ 100 ml in the Excel solver. The decrease for cottonseed oil leads to an improvement in the PHB and Lipases amounts.

4. CONCLUSION

It is worth noting from the study that experimental design proves to be powerful tools in improving complicating conditions. It is clear that more cottonseed oil has been utilized by the *Bacillus* strains as proven from the increasing Lipases activity. It must also be highlighted here that proteases have a negative effect on lipases.

Based on Table 21 which summarizes the overall outcome obtained from different optimization strategies, the results achieved from the Box-Behnken experiment where a high amount of proteases have been produced, is more economically and industrially sound. Meanwhile a significant increase in the amount of PHB which is the main target of this study has been produced as a result of the application of Excel solver. As well established tools as the Plackett-Burman and Box-Behnken experimental designs; the modified implementations used in this study are recommended for optimal outcomes. The valuable knowledge accumulated from researches over the years support the selection of variables during optimizations in the present study.

The fact that no proteases inhibitors of any kind used served our main aim which is to introduce real data and practical conditions towards feasible industrial applications. A series of limiting factors have been excluded from this study - environmental factors such as pH, temperature, physical shaking, etc stand as variables of concern. Other issues including limitations due to the use of Erlenmeyer flasks and the absence of a well controlled fermentating device may as well skew the results in one way or another. These are variables that should be taken into considerations, or steps taken to ensure a certain level of control when conducting further investigations.

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