OPTIMIZING PHB AND PROTEASE PRODUCTION BY BOX BEHNKEN DESIGN

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ABSTRACT: Mixed culture is more suitable to adapt more flexible fermentation process and also to produce different product simultaneously. In this study, a mixed Bacillus culture was investigated for their ability to produce the bioplastic "Polyhydroxybutyrate" and both of the mesophilic and the thermophilic proteases, in one flask. Box-Behnken experimental design was used in this investigation. The produced amount of PHB has been increased significantly. Meanwhile there is a competition between PHB and proteases. The maximum produced amount of PHB using Box-Behnken design was 2.82 g/l/48 h with protease activity equal to 41.9 Units/ml/48 h for thermophilic proteases and 99.65 Units/ml/48 h for mesophilic proteases. Excel solver was used for extra-optimization for the optimum conditions obtained from Box-Behnken experiments and its model. The maximum PHB obtained after using Excel solver was 2.88 g/l/48 h. The maximum mesophilic and thermophilic activities obtained at the same PHB production conditions were 175.68 and 243.38 Units/ml respectively. The model accuracy obtained from Excel solver was 118.8%, which prove that the power of the experimental design in optimizing such complicated process. The strategies used in this study are recommended for the production of PHB and different proteases simultaneously, using Bacillus mixed culture.

ABSTRAK: Kultur campuran adalah lebih sesuai bagi proses penapaian yang fleksibel dan ia boleh menghasilkan produk yang berbeza secara serentak. Dalam kajian ini keupayaan menghasilkan "Polyhydroxybutyrate" bioplastik serta mesofilik dan termofilik protease dalam satu flask oleh kultur *Bacillus* campuran telah disiasat. Eksperimen rekabentuk Box-Behnken telah digunakan. Jumlah PHB yang dikeluarkan meningkat dengan ketara dan terdapat persaingan antara PHB dan protease. Jumlah keluaran PHB maksima menggunakan rekabentuk Box-Behnken adalah 2.82 g/l/48 jam dengan aktiviti protease sama dengan 41.9 Unit/ml/48 jam untuk protease termofilik dan 99.65 Unit/ml/48 h untuk protease mesofilik. Solver Excel telah digunakan untuk memperolehi kadar optimum tambahan dari keadaan optimum yang diperolehi dari experimen dan model Box-Behnken. PHB maksimum diperolehi setelah menggunakan solver Excel adalah 2.88 g/l/48 jam. Aktiviti mesofilik dan termofilik maksima diperolehi daripada keadaan pengeluaran PHB yang sama adalah 175.68 dan 243.38 Unit / ml. Ketepatan model diperolehi daripada solver Excel adalah 118.8%, membuktikan kekuatan eksperimen tersebut bagi mengoptimumkan proses yang rumit. Strategi yang digunakan dalam kajian ini adalah disyorkan bagi pengeluaran PHB dan proteas berbeza secara serentak menggunakan kultur *Bacillus* campuran.

KEYWORDS: *PHB; proteases; mixed culture; Box-Behnken design.*

1. INTRODUCTION

Bacteria-produced PHB is approximately five to ten times more expensive than its competitors (e.g. polypropylene or polyethylene). Although this natural product is promising, its price at the early production time is rather expensive [1]. The cheapest substrate cost is $0.22/kg (sugar) of PHA while the cost of polypropylene is $0.185/kg [2]. The substrate cost affects the overall cost. When the PHB productivity was increased from 1.98 to 3.2 g/h, the PHB production cost was decreased from $5.37/kg to $4.91/kg [2]. In a laboratory fed-batch system using *Alcaligenes latus*, the highest reported productivity was 4.94 g/h with cost about $2.6 kg [3]. Thermostable enzymes can be produced by both of thermophilic and mesophilic microbes [4, 5]. *Bacilli* and particularly *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus pumilus* are the most used species [6]. Molecular biology tools were used to increase the PHB production. As an example random in vivo and in vitro mutageneses were performed [7-11]. Experimental design is another versatile tool could be used for optimizing different parameters and conditions. In this study Box-Behnken design and Excel solver were used to optimize the medium constituents[12]. PHB and the proteases were subjected to the optimization processes. The different obtained results from the conducted experiments were analyzed statistically using Excel 2000 and Essential Exp., version 2.205 software [13]. The results which have been obtained from Box-Behnken optimization in this study are promising.

2. MATERIALS AND METHODS

2.1 Microorganism

Five *Bacillus* species were used in this study. They were isolated from the Egyptian ecosystem and identified using standard criteria as *Bacillus subtilius*, *Bacillus pumilus*, *Bacillus thuringiensis*, *Bacillus licheniformis* and *Geobacillus stearothermophilus*. They are grown routinely in LB medium (Luria-Bertani) [14] at 40-37^{o}C and maintained at -70^{o}C. The *Bacillus* strains as mixed culture have been adjusted to have equal OD_{600} (OD_{600} = 0.025) (Sterilize distilled water has been used to adjust the OD). The used amount of each in the Box-Behnken design were: *B. subtilius* 100 µL; *B. pumilus* 100 µl; *B. thuringiensis* 10 µl; *B. lichenifermis* 10 µl and *Geobacillus stearothermophilus* 10 µl.

2.2 Cultivation Medium

The used medium has the following constituents: tryptone soy bean 10 mg; skim milk 10, 5.1 or 0.2 mg; lactose 2 g; glucose 2, 1.2 or 0.4 g; yeast extract 0.4, 0.24 or 0.08 g; KH_2PO_4 0.01 g; $FeSO_4$ solution 5 µl (12 mg/l); trace elements solution 5 µl/ml as described by Pfenning [15] and KCl_2 0.03 g (skim milk, glucose and yeast extract have been used in three different values represented as +1, 0 and -1 following Box-Behnken design). The trace elements and $FeSO_4$ solution were sterilized each separately using 0.22 µm sterilize filter system.

2.3 Preparation of Equal Cultures

Bacillus strains were pre-cultivated on LB agar plate for overnight at 37^{o}C. One loop from the fresh colonies from each *Bacillus* strain was taken and inoculated to test tube contains 5 ml LB broth medium. The cultures incubated overnight at shaker incubator at 200 rpm and 37^{o}C. The OD_{600} for each species was adjusted to 0.025 by adding sterilize distilled water to the culture under aseptic condition.

2.4 Shake Flask Fermentation Condition

PHB and proteases production under the different experimental conditions were conducted using 250 ml Erlenmeyer-Flasks each containing 100 ml medium. The shaking rate was 250 rpm at 37^{o}C. The medium constituents were changed when randomized according to Box-Behnken design [12] as described in Table 1 (see section 3.1).

2.5 Proteases Activities

The protease activities of mesophilic and thermophilic proteases have been determined at 37 and 60^{o}C respectively by the method described by Amara and Serour [16].

2.6 PHB Characterization

The assay was spectrophotometrically (PerkinElmer-UV/VIS Spectrometer Lambda) performed after modification as described by Law and Slepecky to determine PHB as crotonic acid [17]. Two milliliters from each cultivation was centrifuged at 13000 rpm for 15 min. One milliliter of H_2SO_4 then added to the precipitate, which contain the cells. The solution was incubated in 70^{o}C for 20 min. After cooling, 10 µl was taken and added to 990 µl of 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 Box-Behnken Design

The Box-Behnken experimental design, the model and ANOVA analysis were carried out using the Excel 2000 and Essential Exp., Version 2.205 software [13]. Box-Behnken experimental design was used to optimize three variables represented at three levels high, medium and low, which are donated by +1, 0 and -1, respectively. They represent Glucose (X1), Skim milk (X2) and Yeast extract (X3) g/l. The design contain fifteen experiments as in Table 1. The PHB produced from the different experiments were analyzed by multiple regression analysis. The created model was generated using the coefficient of each variable as described in detailed by Amara and Salem [18]. The various interactions between responses have represented in the surface plots.

2.8 Excel Solver Optimization

Considering that PHB is the main product, glucose (X1), skim milk (X2) and yeast extract (X3) were further optimized using Microsoft excel 2000 solver to calculate the best YPHB value. The experiment under mathematically calculated optimum glucose (X1), skim milk (X2) and yeast extract (X3) was practically conducted and the model accuracy was calculated using the following formula:

$$Accuracy\ \%\ of\ the\ model\ = \left[\frac{YExperiment}{YCalculated}\right] \times 100 \quad (1)$$

3. RESULTS AND DISCUSSION

3.1 Box-Behnken Design

Glucose, skim milk and yeast extract have been randomized according to Box-Behnken design while the other medium constituents as well as the amount of the used of each of the five *Bacilli* are constant as described above. The amount of the produced PHB, mesophilic and thermophilic proteases for each of the fifteen experiments were summarized in Table 1.

Table 1: Box-Behnken experiments.

Exp. no.	Glucose	Skim milk	Yeast extract	PHB g/100 ml/48h	Measophilic Protease Units/ml/48hr	Thermophilic protease Units/ml/48/hr
1	0 (1.2)	0 (5.1)	0 (0.24)	1.25	96.42	50.26
2	0 (1.2)	1 (10)	-1 (0.08)	1.45	80.24	29.16
3	1 (2)	-1 (0.2)	0 (0.24)	0.81	107.74	43.31
4	0 (1.2)	0 (5.1)	0 (0.24)	1.94	86.71	48.03
5	1 (2)	0 (5.1)	-1 (0.08)	2.82	99.65	41.90
6	1 (2)	1 (10)	0 (0.24)	1.25	98.03	43.31
7	0 (1.2)	-1 (0.2)	1 (0.4)	0.70	120.68	35.97
8	-1 (0.4)	1 (10)	0 (0.24)	1.49	102.89	27.14
9	0 (1.2)	1 (10)	1 (0.4)	1.01	115.83	21.27
10	1 (2)	0 (5.1)	1 (0.4)	0.99	114.21	25.99
11	-1 (0.4)	0 (5.1)	-1 (0.08)	1.56	175.68	243.38
12	-1 (0.4)	0 (5.1)	1 (0.4)	1.12	151.42	74.13
13	0 (1.2)	0 (5.1)	0 (0.24)	1.21	112.59	194.45
14	0 (1.2)	-1 (0.2)	-1 (0.08)	1.13	135.24	219.12
15	-1 (0.4)	-1 (0.2)	0 (0.24)	1.58	119.06	93.34

The best PHB produced amount was in experiment number five and was equal to 2.82 g/10 µl/48 hr with mesophilic and thermophilic proteases activities were 99.65 and 41.90 Units/ml/48 hr respectively. In case of proteases production, experiment number eleven shows the highest mesophilic and thermophilic activities which are equal to 175.68 and 243.38 Units/ml/48 hr respectively and the produced PHB amount was equal to 1.56 g/100 ml/48 hr.

Each response was analyzed statistically using the multiple regression analysis test and the estimate, standard error, T statistic, P-value and Coefficient % for each parameter was summarized (for each response) in Tables 2 (PHB), Table 4 (mesophilic proteases) and Table 6 (thermophilic protease). The model for each response was generated. The ANOVA test for PHB, mesophilic and thermophilic proteases were generated and the obtained results were summarized in Tables 3, 5 and 7, respectively.

3.2 PHB Model

PHB = 1.46667 + 0.015* Glucose + 0.182917* Glucose* Glucose + 0.1325* Glucose * Skim milk - 0.3475* Glucose* Yeast extract + 0.1225* Skim milk - 0.367083* Skim milk* Skim milk - 0.0025* Skim milk* Yeast extract 3 - 0.3925* Yeast extract - 0.0270833* Yeast extract* Yeast extract. The P-value in the ANOVA table is greater or equal to 0.10. There is no a statistically significant relationship between the variables at the 90% or higher confidence level as in Table 3.

Table 2: Linear Multiple regression analysis of the interaction between variables on PHB response.

Parameter	Estimate	Standard Error	T Statistic	P-Value	Confidence%
CONSTANT	1.46667	0.278582	5.26476	0.0033	99.67
Glucose	0.015	0.170596	0.087927	0.9333	6.67
Glucose* Glucose	0.182917	0.25111	0.728432	0.499	50.1
Glucose*Skim milk	0.1325	0.241259	0.549203	0.6065	39.35
Glucose*Yeast extract	-0.3475	0.241259	-1.44036	0.2093	79.07
Skim milk	0.1225	0.170596	0.718072	0.5049	49.51
Skim milk* Skim milk	-0.36708	0.25111	-1.46184	0.2036	79.64
Skim milk* Yeast extract	-0.0025	0.241259	-0.01036	0.9921	0.79
Yeast extract	-0.3925	0.170596	-2.30076	0.0697	93.03
Yeast extract* Yeast extract	-0.02708	0.25111	-0.10785	0.9183	8.17

Table 3: ANOVA test for PHB response.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	2.57144	9	0.285716	1.23	0.4321
Residual	1.16412	5	0.232823		
Total (Corr.)	3.73556	14			

R-squared = 68.8369 percent; R-squared (adjusted for d. f.) = 12.7433 percent; Standard Error of Est. = 0.482518; Mean absolute error = 0.252444

Table 4: Linear Multiple regression analysis of the interaction between variables on measophilic protease response.

Parameter	Estimate	Standard Error	T Statistic	P-Value	Confidence %
CONSTANT	98.5722	10.8994	9.04385	0.0003	99.97
Glucose	-16.177	6.67446	-2.42372	0.0598	94.02
Glucose* Glucose	15.3008	9.82454	1.55741	0.1801	81.99
Glucose * Skim milk	1.6177	9.43912	0.171383	0.8706	12.94
Glucose * Yeast extract	9.70623	9.43912	1.0283	0.351	64.9
Skim milk	-10.7173	6.67446	-1.60572	0.1692	83.08
Skim milk* Skim milk	-6.94265	9.82454	-0.70666	0.5113	48.87
Skim milk * Yeast extract	12.5372	9.43912	1.32822	0.2415	75.85
Yeast extract	1.41549	6.67446	0.212076	0.8404	15.96
Yeast extract* Yeast extract	21.3672	9.82454	2.17488	0.0816	91.84

Table 5: ANOVA test for mesophilic protease response.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*P*-Value
Model	6764.39	9	751.598	2.11	0.2128
Residual	1781.94	5	356.388		
Total (Corr.)	8546.32	14			

R-squared = 79.1497 percent; R-squared (adjusted for d.f.) = 41.619 percent; Standard Error of Est. = 18.8782; Mean absolute error = 9.47256

3.3 Mesophilic Protease Model

Mesophilic protease = 98.5722 - 16.177* Glucose + 15.3008* Glucose* Glucose + 1.6177* Glucose* Skim milk + 9.70623* Glucose* Yeast extract - 10.7173* Skim milk - 6.94265* Skim milk* Skim milk + 12.5372* Skim milk * Yeast extract + 1.41549* Yeast extract + 21.3672* Yeast extract* Yeast extract. 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 as in Table 5.

3.4 Thermophilic Protease Model

Thermophilic proteases = 97.58 - 35.435* Glucose - 12.9175* Glucose* Glucose + 16.55* Glucose* Skim milk + 38.335* Glucose - 33.8575* Skim milk - 32.8875* Skim milk* Skim milk + 43.815* Skim milk* Yeast extract - 47.025* Yeast extract + 11.6875* Yeast extract* Yeast extract. The P-value in the ANOVA table is greater or equal to 0.10. There is not a statistically significant relationship between the variables at the 90% or higher confidence level as in Table 7.

Table 6: Linear Multiple regression analysis of the interaction between variables on thermophilic protease.

Parameter	Estimate	Standard Error	T Statistic	*P*-Value	Confidence%
CONSTANT	97.58	38.5555	2.53089	0.0525	94.75
Glucose	-35.435	23.6104	-1.50082	0.1937	80.63
Glucose * Glucose	-12.9175	34.7535	-0.37169	0.7254	27.46
Glucose * Skim milk	16.55	33.3901	0.495656	0.6412	35.88
Glucose * Yeast extract	38.335	33.3901	1.1481	0.3029	69.71
Skim milk	-33.8575	23.6104	-1.43401	0.211	78.9
Skim milk* Skim milk	-32.8875	34.7535	-0.94631	0.3874	61.26
Skim milk * Yeast extract	43.815	33.3901	1.31222	0.2465	75.35
Yeast extract	-47.025	23.6104	-1.99171	0.103	89.7
Yeast extract* Yeast extract	11.6875	34.7535	0.336297	0.7503	24.97

Table 7: ANOVA test for thermophilic protease response.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*P*-Value
Model	56789.3	9	6309.92	1.41	0.3669
Residual	22297.9	5	4459.59		
Total (Corr.)	79087.2	14			

R-squared = 71.8059 percent; R-squared (adjusted for d.f.) = 21.0565 percent; Standard Error of Est. = 66.7802; Mean absolute error = 31.9153

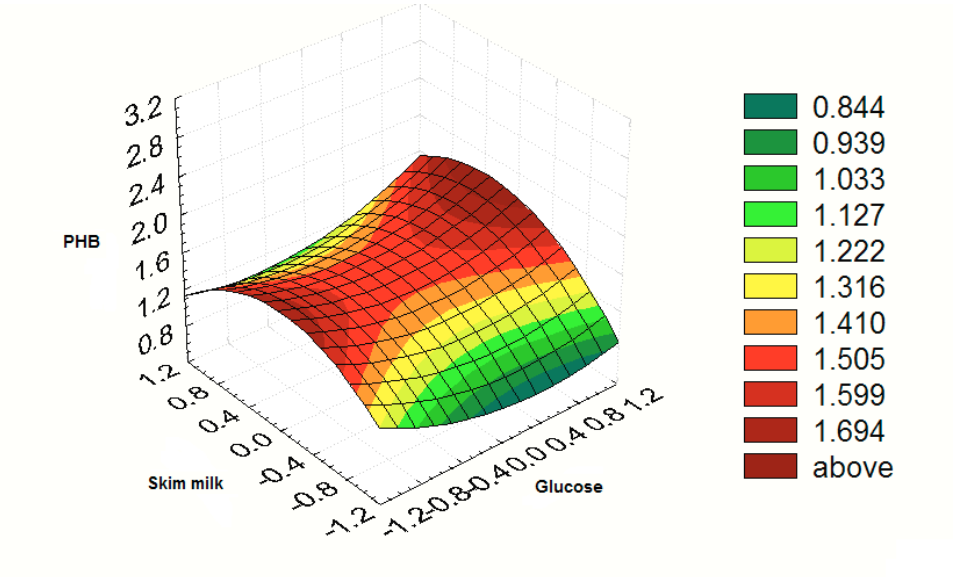

Fig. 1: Response surface for skim milk and glucose effect in PHB production.

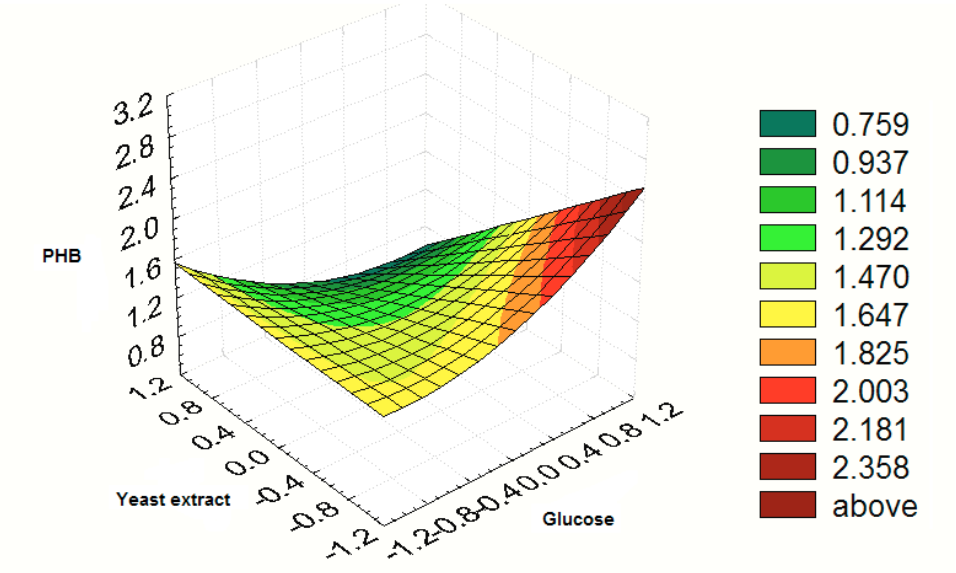

Fig. 2: Response surface for yeast extract and glucose effect in PHB production.

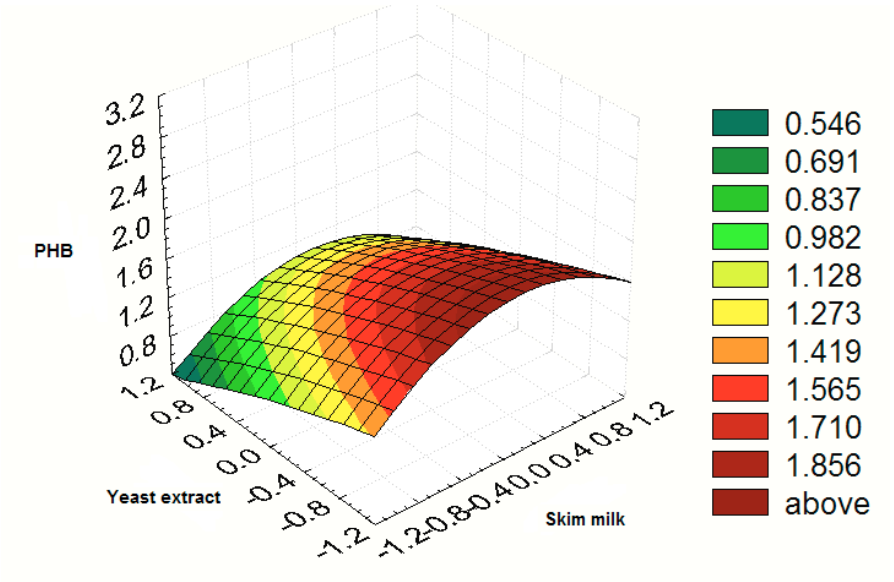

Fig. 3: Response surface for yeast extract and skim milk effect in PHB production.

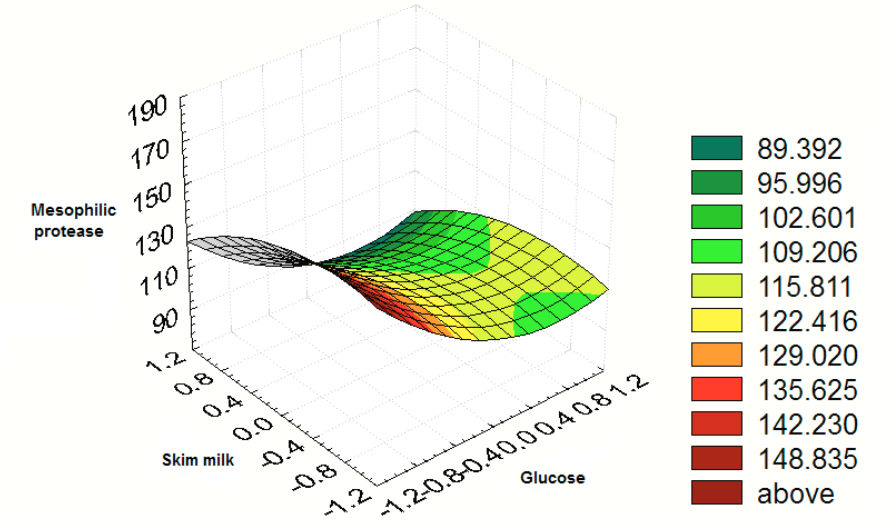

Fig. 4: Response surface for skim milk and glucose effect in mesophilic protease production.

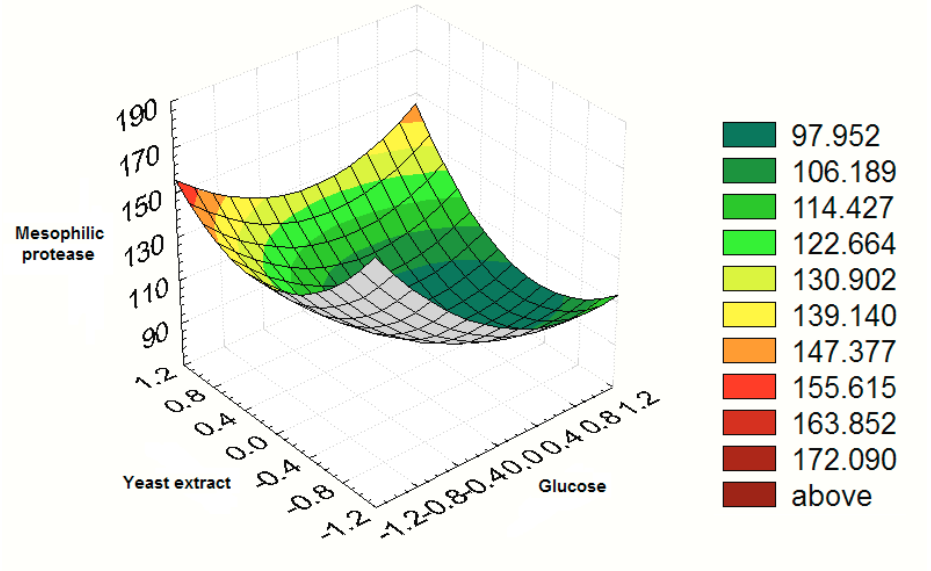

Fig. 5: Response surface for yeast extract and glucose effect in mesophilic protease production.

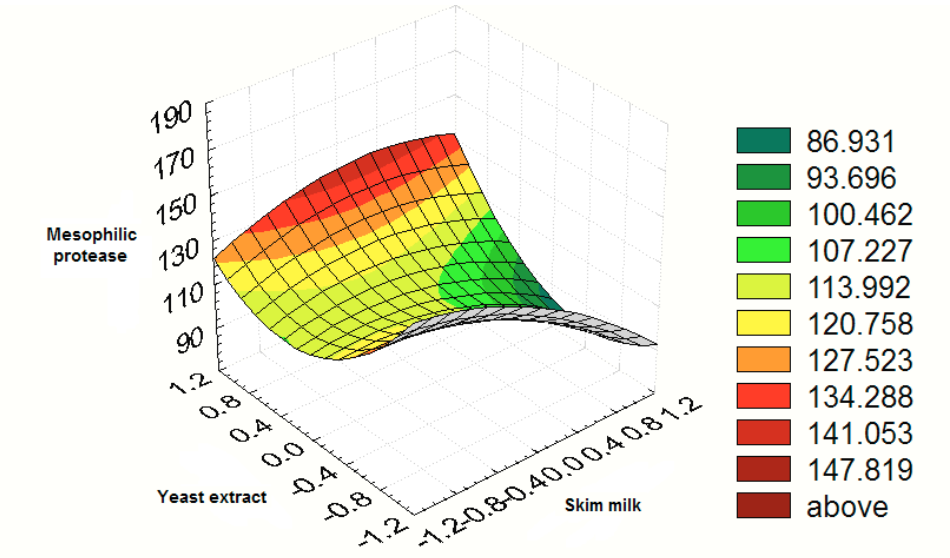

Fig. 6: Response surface for yeast extract and skim milk effect in mesophilic protease production.

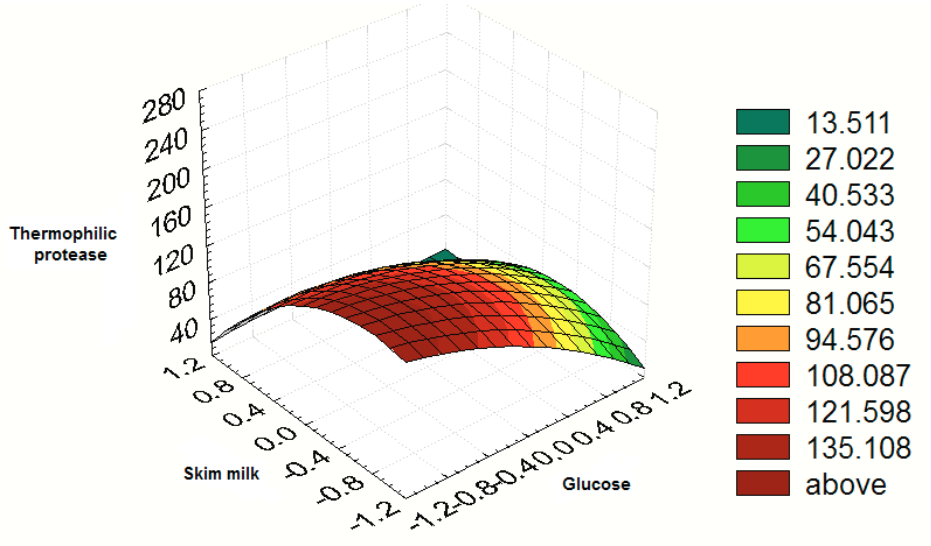

Fig. 7: Response surface for skim milk and glucose effect in thermophilic protease production.

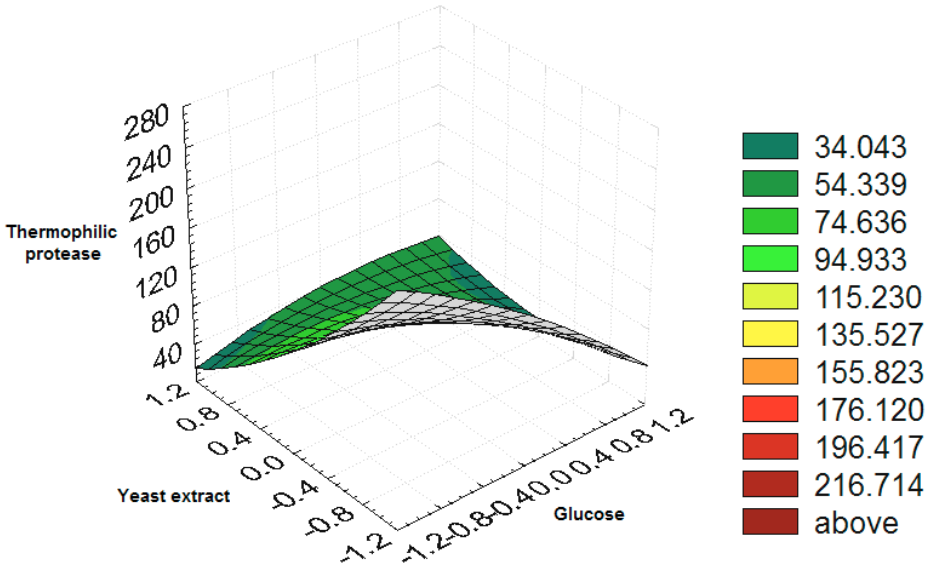

Fig. 8: Response surface for yeast extract and glucose effect in thermophilic protease production.

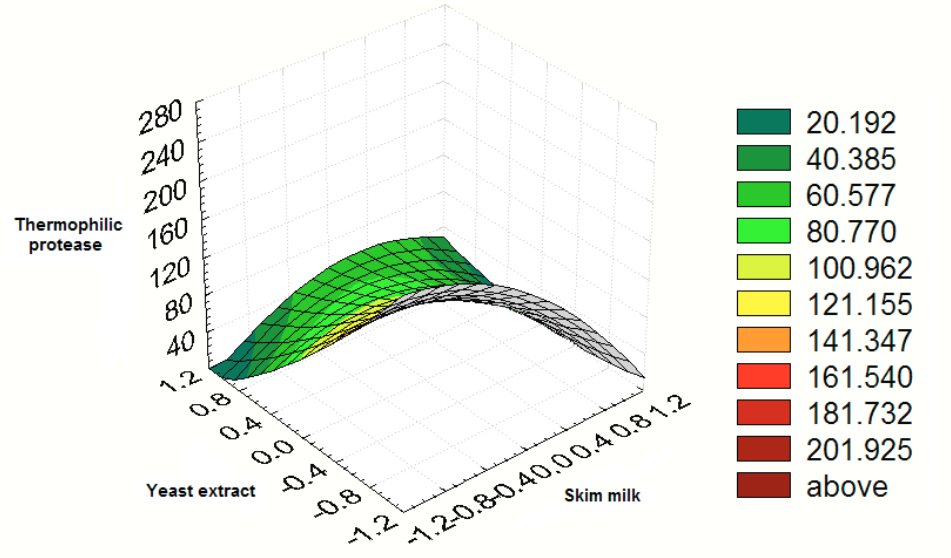

Fig. 9: Response surface for yeast extract and skim milk effect in thermophilic protease production.

3.5 Excel Solver Optimization

The best level of the three variable as obtained from the maximum point of polynomial PHB model was estimated using the solver function of Microsoft Excel 2000 tools and found to be for glucose = 2 g/100 ml, skim milk = 6.818 mg/100 ml and yeast extract = 0.08 g/100 ml, with a prediction calculated PHB equal to 24.2 g/l/48 h.

Table 8: Excel 2002 Solver optimization for X1, X2 and X3.

Term	Glucose (X_1) g/100 ml	Skim milk (X_2) mg/100 ml	Yeast extract (X_3) g/100 ml
Data Minimum	-1 (0.4)	-1 (0.2)	-1 (0.08)
Data Average	0 (1.2)	0 (5.1)	0 (0.24)
Data Maximum	1 (2)	1 (10)	1 (0.4)
Data Solver	1 (2)	0.353571 (6.818)	-1 (0.08)

3.6 Confirming accuracy of model

The Y value which was calculated using Microsoft Excel is equal to 24.2 g/l/48 h. The in vivo experiments show that Y value (PHB) is 28.8 g/l/48 h. By calculating the model accuracy from the formula in material and methods section, the model accuracy % was 118.8%. The measophilic and thermophilic proteases activities determined to be 175.68% and 243.38 Units/ml respectively.

4. DISCUSSION

Amara [7] suggests directing the research concerning the PHB production to the medicinal applications. This will greatly encourage the investors to give PHB researches

more concern. However studies which were concerning with the reduction of the PHB cost should not be stopped. This study introduces a new concept. It concern with the use of heterogeneous substrates and mixed Bacillus culture to produce valuable products beside PHB (the main product). mixed bacterial culture were used. Thermophilic and mesophilic proteases were additionally produced under mesophilic condition. PHB production is rather sensitive process usually conducted under nitrogen limitation and excess of carbon sources [7]. Proteases, which are protein in nature require considerable amount of carbon and nitrogen sources to fit its amino acids' monomeric structure [4-6]. PHB formed mainly from O_2, H and C [7]. To minimize the conflict between the PHB and proteases production conditions, Box-Behnken experimental design was used to map the points where PHB and the proteases could be produced simultaneously. Box-Behnken design was used to optimize three of the used medium constituents. Fifteen experiments were conducted according to the design. The three optimized medium constituents are glucose, skim milk and yeast extract. These three variables were selected based on literature review, experiments (data not shown) and basic science about PHB and proteases productions [4-7]. The maximum PHB amount was 2.82 g/100 ml/48 h as shown in experiment no 5 (Table 1). In experiment no. 5, glucose was in its +1 value, skim milk was in its 0 value and yeast extract was in its -1 value. The amount of measophilic proteases was 99.65 Units/ml and for thermophilic proteases was 41.90 Units/ml in the same experiment. Comparing the amount of proteases in experiment no 5 with that in experiments no 11 and 14 as well as the related amount of the produced PHB prove that there is a conflict between the PHB and Proteases production. The linear multiple regression analysis of the data as in Table 2 shows that under the experimental conditions, yeast extract was significant with confidence level equal to 93.03. The interaction between glucose and yeast extract as well as between skim milk and skim milk were effective with confidence level equal to 79.07 and 79.64 respectively. The glucose, which is the main carbon source for PHB accumulation, was insignificant with confidence % equal to 6.67. This is due to the existence of substrates contain nitrogen such as the skim milk suppress the PHB production. In the case of yeast extract which effect negatively on the PHB production, this is agree with a well-proved criteria that PHB produced under nitrogen limitation [7]. Moreover, the amounts of the used glucose (0.4, 1.2 and 2g/l) is sufficient for PHB production. In contrast, glucose gives confidence level % equal to 94.02 in case of mesophilic proteases. However, it is affect negatively on the mesophilic protease production. By analysis experiment number 11 which give the maximum mesophilic proteases activity {175.68 Units/ml} the amount of the used Glucose was in its minimal used amount (-1 value). It is concluded that glucose has a negative role in measophilic protease production and that the cell give a priority to proteases production over PHB production. Skim milk even it is not statistically significant but it is consider being effective and giving confidence level % equal to 83.08. However, skim milk is also negatively affect on the mesophilic protease production. It can be concluded that glucose which give a very low confidance level with PHB production (6.67%) was concumed by the cell for producing proteases and that mixed culture is more able to produc proteases in low level of skim milk. High quantity of skim milk might interfere with the ability of the cells to produce proteases. Nearly the same result obtained from statistical analysis of the themophilic proteases data where glucose gives confidence level % equal to 89.7. The maximum amount of Thermophilic proteases was in experiment no 14 where the amount of glucose was at its 0 value. The ANOVA test of each of PHB, measophilic and thermophilic proteases which were insignificant prove that there is a chance for further optimization and that it is still a conflict between the used substrates. The Response surface for the three calculated responces show different levels of interactions between the

responces and agree with the statistical analysis of the Box-Behnken results. For extra-optimization, Excel solver was used to optimize the model obtained from Box-Behnken experiments. The solver calculated Y value was 2.42 g/100 ml/48 h. Experimentally; the obtained Y value was equal to 2.88 g/100 ml/48 h. This gives a model accuracy % equal to 118.8%. The amounts of mesophilic and thermophilic proteases have been improved and the produced amount equal to 144.94 and 158 Units/ml respectively.

It is important here to highlight that Excel solver which optimize the model from Blacket-Burman design gives less calculated PHB amount than that produced in experiment number five in Box-Behnken design. However experimentally it gives more PHB. This might be explained that regression analysis could dilute the optimal result during fitting the best line pass through all the results. This problem should be considered by the mathematicians and perhaps can be solved by using manual fitting.

The responses of various interactions between the variables and responses have been visualized as response surface as in Figures from 1 to 9. The different response surface analysis results were agreed with the data obtained from the regression analysis and their models. It is important to highlight that there are some limitations concerning this study which did not included the environmental variables like pH, temperature and shaking rate. The fermentation process control is limited regarding to the use of Flasks rather than using a Fermentor. There are limitations in the size of the medium. Those factors should be considered in further studies. Meanwhile the results obtained from this study prove the powerful of the experimental design in optimizing complicated computational medium compositions. The use of mixed 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.

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