Medium Optimization for PHB depolymerase production by *Stenotrophomonas maltophilia* using Plackett Burman design & Response Surface Methodology

Sonal J Wani, Sohel S Shaikh, Riyaz Z Sayyed

Abstract - The present study focuses on the optimization of the media component by the statistical way for the production of PHB depolymerase enzyme (E.C. no 3.1.1.75) by *Stenotrophomonas maltophilia.* The most important process parameters which enhance the PHB depolymerase production were screened using Plackett-Burman design. Among all the media components, PHB and pH were identified as significant variables. Further, optimal levels of these significant variables found by response surface methodology were, PHB; 0.13 g/100ml and pH 7.5. More than 2 fold increase in PHB depolymerase yield (1.501 U/ml) was obtained by using a statistical optimization approach vis-à-vis enzyme production under un-optimized conditions i.e. 0.721 U/ml activity. Moreover, validation of the model in response surface methodology reflected the accuracy and precision of the model and experimental set up. The predicted values of response surface methodology shows good correlation with the experimental values. Scale up study at bioreactor level revealed maximum PHB depolymerase production and yielded as 3.137U/ml activity.

Index Terms— Plackett Burman design, Poly-β-hydroxybutyrate, PHB, PHB depolymerase, Response surface methodology, RSM, Statistical optimization

1 INTRODUCTION

Poly-β-hydroxybutyrate (PHB) is the most common natural microbial polyesters [1]. A wide variety of micro-organisms accumulate PHB as intracellular granules in a highly reduced and insoluble polymer state [2]. Now a days PHB is of great interest as it is used as a substitute for synthetic polymers and it may provide solutions to global environmental problems caused due to synthetic polymer [3]. Since PHB has been widely accepted as biodegradable polymer, it is necessary to evaluate its biodegradation and the role of polymer-degrading microorganisms, environmental conditions and kind of micro-organism available in the environment. This is hoped to increase the commercial potential of such biopolymers. It has been previously reported that many microbes secreted extracellular PHB depolymerases which is responsible for degradation of microbial polymer [4, 5]. Optimization of the parameters by the statistical approach reduces the time and expenses.

In the present study *Stenotrophomonas maltophilia* was selected on the basis of its clear zone formation on an emulsified PHB agar plate. Furthermore the statistical optimization of media component for the production of PHB depolymerase was carried out by using Plackett Burman (PB) experimental design and response surface methodology (RSM).

2 Material and Methods

2.1 PHB

PHB was obtained from Sigma-Aldrich (Germany). All the experiment was carried out using PHB powder.

2.2 Isolation and identification of PHB degrading strain

The PHB degrading strain was isolated from the plastic contaminated site and dumping yard sites around Shahada (Maharashtra) and screened on minimal salt medium (MSM) having PHB as the only carbon source [6] at 28 ± 2^0C for 5-6 days and presence of a clear zone of colonies on an emulsified PHB agar plate was taken as a measure of PHB biodegradation. Identification was done by using 16s rRNA sequencing, GC FAME analysis and biolog as *Stenotrophomonas maltophilia* (data not shown). The gene sequence was submitted to NCBI as *Stenotrophomonas* sp RZS 7 under accession number KP862608.

2.3 Enzyme production

Production of PHB depolymerase was carried out at shake flask level by growing *Stenotrophomonas maltophilia* in minimal salt medium containing PHB, 0.15%; K_2HPO_4, 0.7 g; KH_2PO_4, 0.7 g; $MgSO_4$, 0.7 g; NH_4Cl, 1.0 g; $NaNO_3$, 1.0 g; NaCl, 5 mg; $FeSO_4$, 2 mg, $ZnSO_4$, 7 mg in 1 L of distilled water [7] at 120rpm for 4 days at 30^0C.

2.4 Statistical optimization to identify the interactive effect of variables on enzyme production using PB design

In the initial step of optimization, PB experimental design was used to identify the effect of media components on the production of PHB depolymerase activity in liquid culture medium [8]. In this experiment eleven independent variables were used in different combinations to identify the critical parameters for the production of PHB depolymerase. For each component, a high (+) and low (-) values were tested. All the trials were performed in triplicate and the average of PHB depolymerase activity was used as a response. The variables chosen to experiment with their code and their high (+) and low (-) values are given in Table 1. The PB experimental design was demonstrated in Table 2. Experimental error was estimated by calculating the variance among the dummy variables as [9].

$$Veff = \sum (ED)2/n$$

Where,

Veff is the variance of the effect of level; Ed is the effect of the level of the dummy variables; and n is the number of dummy variables used in the experiment.

The standard error (SE) for effect of concentration of variables was the square root of the variance of an effect and the significance level of each concentration effect was determined using the f test [10] and confidence level.

2.5 Response surface methodology (RSM)

The critical parameters which enhance the production of depolymerase was identified in the PB experiment. In a next step the optimum concentration of significant variables and the interactive effect of media ingredients were determined by response surface central composite design (CCD). Two variables viz: PHB and pH were chosen and thirteen set of experiments was designed by using design expert 9.0.3.1 software tool using central composite quadratic design (CCQD) keeping other variable constant. Each variable in the experimental

design was studied at five different levels as s. Furthermore the experimental design in concern with their actual and predicted values is given in table 3. The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software. The general form of the second order polynomial equation is

$$Yi = \beta 0 + \sum \beta iXi + \sum \beta iiXi^2 + \sum \beta ijXiXj$$

Where

Yi is the predicted response, XiXj are input variables which influence the response variable

Y, β0 is the offset term; βi is the *i*th linear coefficient; βii the ith quadratic coefficient and βij is the ijth interaction coefficient.

The adequacy of the model was evaluated by analysis of variance (ANOVA) (Table 4).

2.6 PHB depolymerase enzyme assay

To evaluate the PHB depolymerase activity, a uniform suspension of PHB granule in 50mM Tris-HCl buffer (pH 7.0) was prepared by sonication at 20 kHz for 15 min. The reaction mixture consisted of 50mM Tris-HCl buffer (pH 7.0), 150 μg/ml PHB and 2mM $CaCl_2$. This reaction was started by adding the culture supernatant and decrease in PHB turbidity was measured spectrophotometrically at 650nm. One unit of PHB depolymerase activity was defined as the quantity of enzyme required to decrease the OD (at 650nm) by 0.1/min [11, 12].

2.7 Bioreactor study for scale up of optimized process

After optimizing the parameters, the optimized protocol at shake flask level was used at bioreactor level to check the performance of the organism and to confirm the validity of different parameters optimized at shake flask level. Scale up was carried out in 5L capacity fully automated bioreactor (Murhopye Scientific Co., India, Model LF-5). The reactor was sterilized along with optimized medium (3L of working volume) at 121^0C for 20 min, cooled and then inoculated with inoculum. pH, temperature and dissolved oxygen level were maintained by their respective probes.

3. Results

3.1 Enzyme production

After 4 days of incubation under unoptimized conditions at 30^0C, the yield of enzyme production was less i.e. 0.721 U/ml. In order to increase the yield of enzyme statistical optimization of cultural conditions was used.

3.2 Statistical optimization to identify the interactive effect of variables on enzyme production using PB design

The experiment was carried out in 12 runs to study the effect of media component on enzyme production. Table 2 represents the design matrix of the experiment and their respective enzyme production yield. According to Stanbury et al. (2003) [10] f value and confidence level of the component which shows a value higher than other component was analyzed and chosen as significant variable for maximum enzyme production (Table 5). Among all the media component PHB and pH were identified as the most significant variable enhancing en-

zyme production yield. Thus, for further step of optimization, the optimal level of PHB and pH was checked by response surface methodology.

3.3 Response surface methodology

The RSM was employed to determine the optimal level of significant factors keeping other variable at a constant level. Multiple regression analysis was used to analyze the data and thus a polynomial equation was obtained from regression analysis as follows:

Enzyme activity = - (8.80974) - (37.23732 x PHB) + (3.24062 x pH) + (5.94167 x PHB x pH) + (3.27778 x PHB^2) – (0.26680 x pH^2)

ANOVA result showed that this regression is statistically significant for enzyme production (Table 4). The model F value 57.52 implies that the model is significant. The multiple correlation coefficient (R^2) is closer to 1 denotes better correlation between the observed and predicted values. In this study R^2 (0.9762) indicated the best correlation between the experimental and predicted values [13-15]. The lack-of-fit F-value 1.73 indicated that the lack of fit is not significant relative to the pure error. Non-significant lack of fit is good for the model to fit. The coefficient of variation (CV) indicated the degree of precision to which the experiments were compared. Higher the CV implies lower reliability of the experiment. In this case CV is 5.03 shows that experiment performed is reliable [16][9].

The interaction effects and optimal levels of the variables were determined by plotting the response surface curve. The contour plot (Fig 1) used to predict the optimal levels of component for different test variables. Three-dimensional response plot shown in fig 2 represents the surface interaction between a PHB and pH at different concentrations. Higher levels of both variables did not result in higher enzyme production yield. The shape of the response surface curve showed a moderate interaction between these tested variables. The combined effect of variables on enzyme production was also studied by perturbation plot at the mid-point (Fig 3). The curvature of the variables shows the sensitivity of the response to these factors. Fig 4 shows an overlay plot in which the concentration of (X1) PHB = 0.1299 g/100ml and (X2) pH = 7.497 indicate 1.494 U/ml enzyme activity.

3.4 Bioreactor study for scale up of optimized process

To understand the kinetics of PHB depolymerase production in batch cultivation under controlled conditions, lab scale bioreactor was used. Fig 5 shows the PHB depolymerase enzyme activity and their respective protein concentration at different time interval. Maximum PHB depolymerase enzyme activity was found to be 3.137U/ml at 60 h of fermentative production at bioreactor level.

4. Validation of the model

Validation of the model was carried out under conditions predicted by the model. The model was successfully validated as the values predicted by the model were good enough with the actual results obtained. The optimal levels of the process variables for depolymerase production by *Stenotrophomonas malto-*

philia were 0.13 g/100ml PHB and 7.5 pH concentration. The predicted enzyme production obtained from the model by using above optimal concentration of the component was 1.47961 U/ml. To validate the model experiments were carried out in triplicate using the optimal concentration of the component. This experiment yielded 1.501 U/ml enzyme activity. Thus, it is proved that the experimental model was successfully validated.

5. Discussion

In order to optimize enzyme production yield, the PB design and RSM was applied, which has been demonstrated as an efficient approach to screen for significant medium components and their optimal levels for maximum yield. Srividya (2012) [17] screened the media component for depolymerase enzyme production by *Penicillium expansum* using PB design. Bansal et al. (2014) [18] reported the production of depolymerase from *Aeromonas punctata* sp. and their optimization by PB design and RSM. Similarly, production of the PHB depolymerase from *E. minima* W2 (PhaZ*Emi*) was also studied [19]. Previous investigation demonstrated the importance of carbon sources in the growth medium for depolymerase enzyme production as the rate of polymer degradation was influenced by the carbon source [19]. In this study PHB depolymerase was produced and their optimization by PB design and RSM was carried out successfully. PHB and pH were investigated as significant variables in depolymerase production.

6. Conclusion

The statistical approach of experiment imparts an ideal platform to identify the significant component of the medium and to optimize the component within the minimum number of experimental sets for maximum enzyme production by using *Stenotrophomonas maltophilia.* The significant factors were screened by PB design and further were considered for the next stage in the media optimization by using response surface methodology. Also the validation of the model was carried out to check the accuracy and precision of the model. From this experimental set up it is concluded that the PB design and RSM was successfully used to define the optimal values of the significant variable for PHB depolymerase production by our potent isolate.

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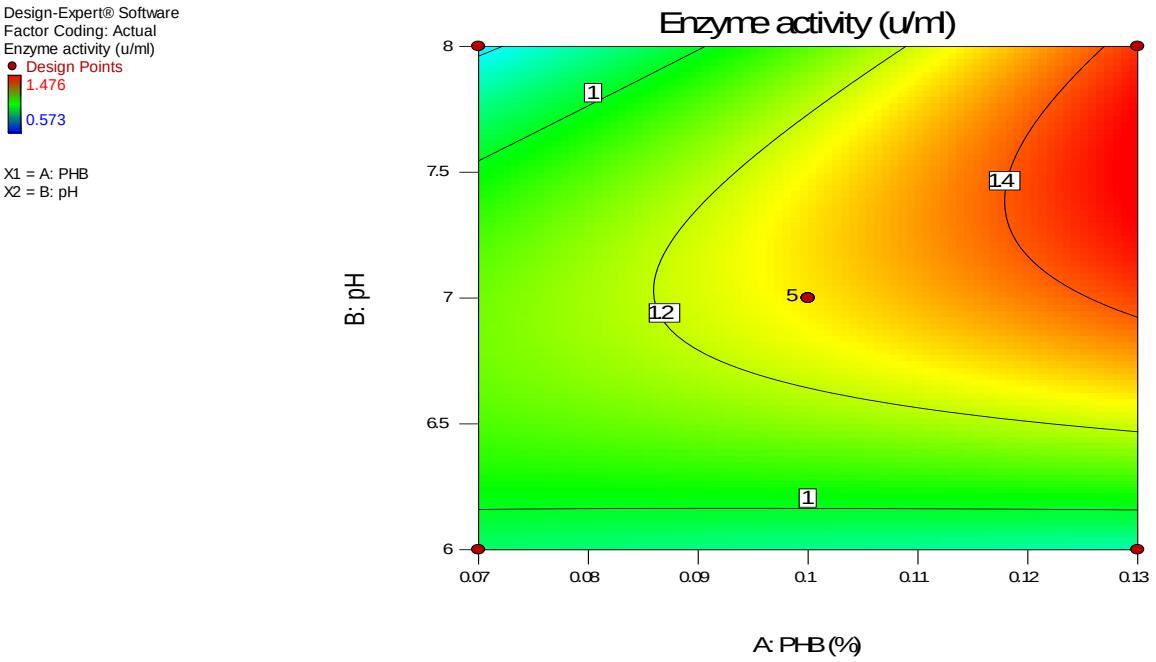

Fig. 1 Contour plot showing the interaction effect of PHB and pH concentrations on enzyme production.

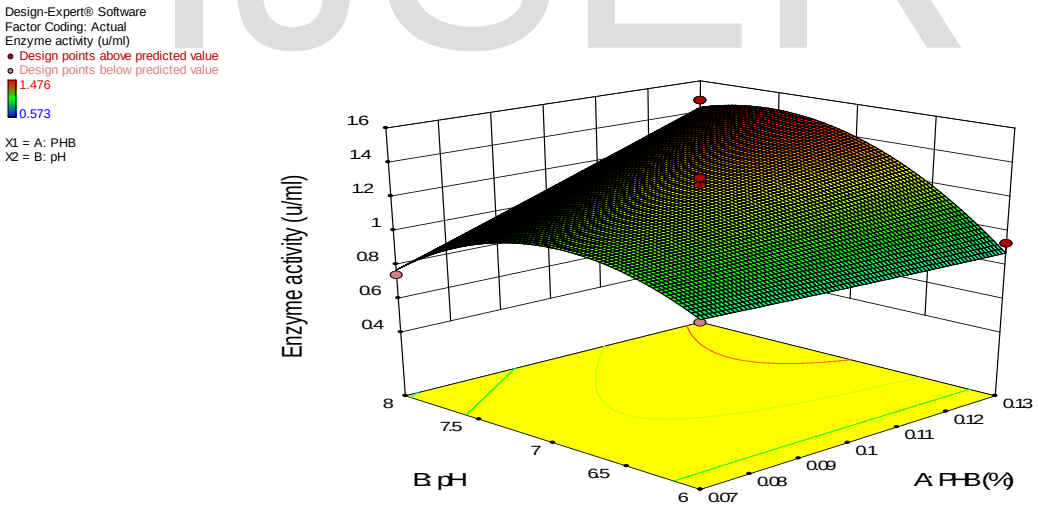

Fig. 2 Three dimensional response surface plots showing interaction between PHB and pH concentrations

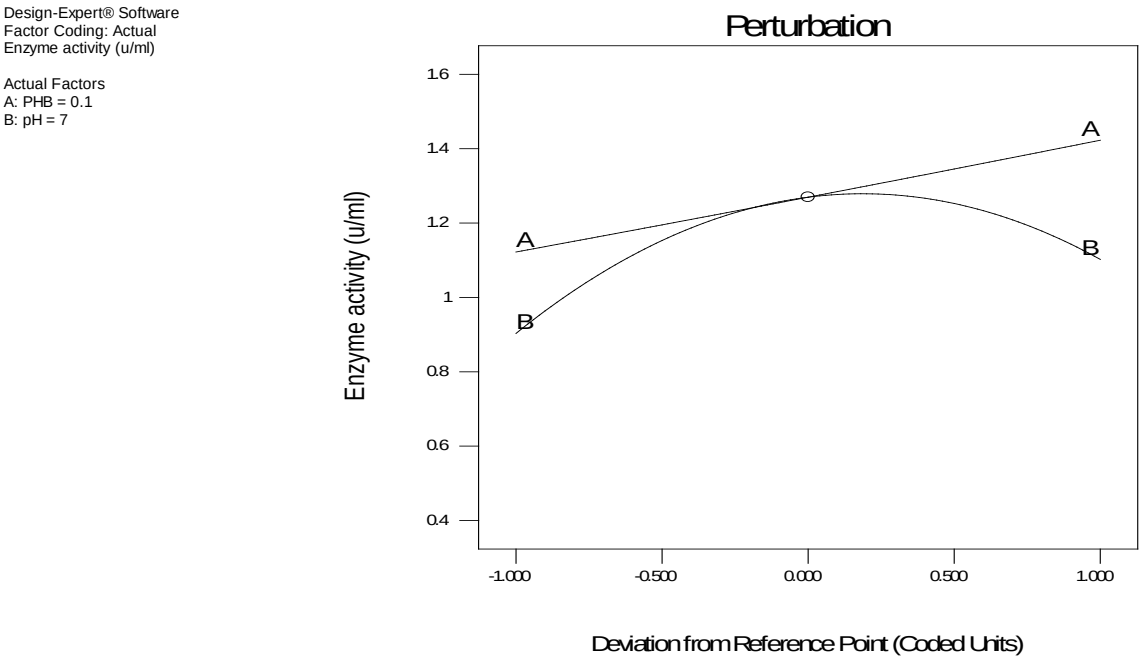

Fig. 3 Perturbation plot

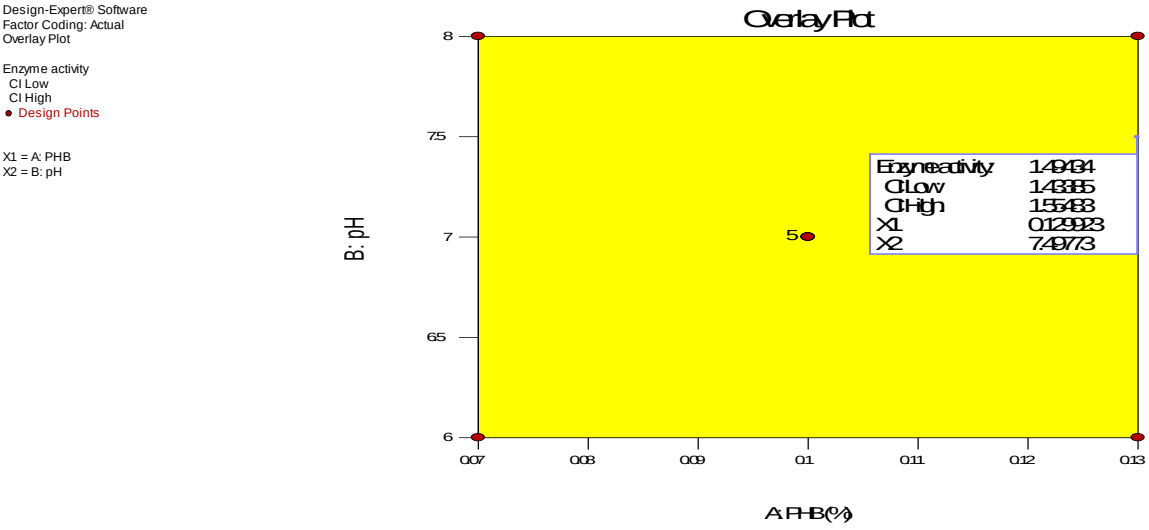

Fig. 4 Overlay plot of enzyme activity

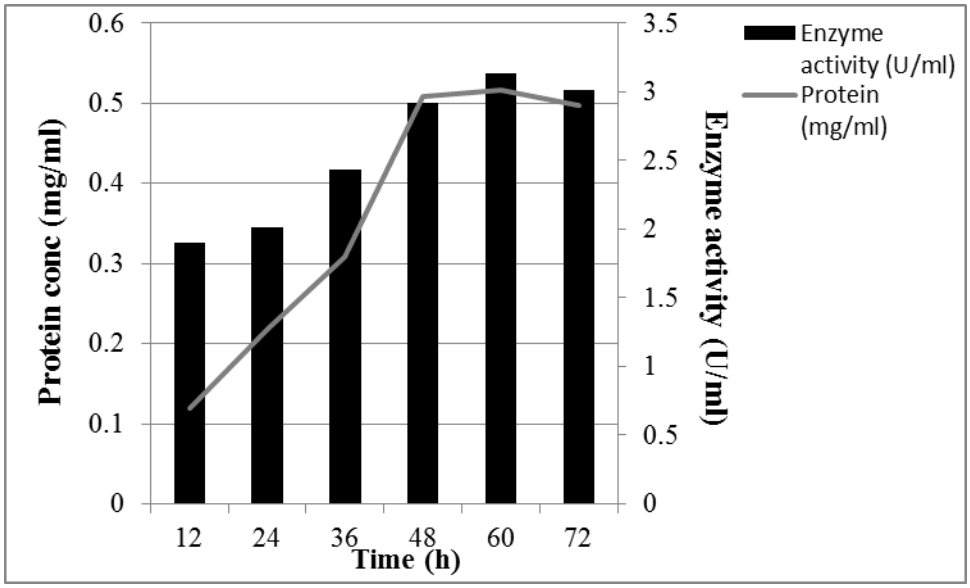

Fig. 5 Protein concentration and enzyme activity at different time interval in lab scale bioreactor

Figure caption

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Table titles

Table 1- Components used in Plackett Burman design

Table 2- Plackett- Burman experimental design

Table 3- Experimental design with their coded and predicted values and their response on enzyme activity

Table 4- ANOVA analysis of depolymerase enzyme activity in central composite design experiment

Table 5- Analysis of response by Plackett Burman design

Table 1- Components used in Plackett Burman design

Variable	Symbol	(+) High Value	(-) Low Value
$CaCl_2$	A	0.0026	0.0014
KH_2PO_4	B	0.0325	0.0175
$(NH_4)_2SO_4$	C	0.026	0.014
$MgSO_4$	D	0.026	0.014
PHB	E	0.13	0.07
$FeCl_3$	F	0.0065	0.0035
pH	G	8	6
Inoculum size (%)	H	5	1
Time (hr)	I	96	48
Dummy variable	J	-	
	K		

Table 2 - Plackett- Burman experimental design

Run	A	B	C	D	E	F	G	H	I	J	K	Yield (U/ml)
1	H	H	H	H	H	H	H	H	H	H	H	1.278
2	L	H	L	H	H	H	L	L	L	H	L	0.402
3	L	L	H	L	H	H	H	L	L	L	H	1.989
4	H	L	L	H	L	H	H	H	L	L	L	0.821
5	L	H	L	L	H	L	H	H	H	L	L	1.477
6	L	L	H	L	L	H	L	H	H	H	L	0.907
7	L	L	L	H	L	L	H	L	H	H	H	1.184
8	H	L	L	L	H	L	L	H	L	H	H	1.854
9	H	H	L	L	L	H	L	L	H	L	H	0.333
10	H	H	H	L	L	L	H	L	L	H	L	0.527
11	L	H	H	H	L	L	L	H	L	L	H	1.113
12	H	L	H	H	H	L	L	L	H	L	L	0.978

Table 3 -Experimental design with their coded and predicted values and their response on enzyme activity

PHB			pH		Enzyme activity (U/ml)	
Run	Actual	Coded	Actual	Coded	Experimental	Predicted
1	0.1	0	7	0	1.229	1.27
2	0.07	-1	6	-1	0.917	0.93
3	0.1	0	7	0	1.271	1.27
4	0.1	0	8.41421	+ α	0.874	0.88
5	0.1	0	5.58579	-α	0.573	0.60
6	0.1	0	7	0	1.314	1.27
7	0.142426	+ α	7	0	1.424	1.49
8	0.07	-1	8	+1	0.746	0.78
9	0.1	0	7	0	1.213	1.27
10	0.13	+1	6	-1	0.934	0.88

11	0.1	0	7	0	1.321	1.27
12	0.13	+1	8	+1	1.476	1.43
13	0.0575736	-α	7	0	1.102	1.06

Table 4 – ANOVA analysis of depolymerase enzyme activity in central composite design experiment

Source	**Sum of square**	**Degree of freedom**	**Mean square**	**F- value**	**P- value**	**Signifi-cance**
Model	0.89	5	0.18	57.52	< 0.0001	Significant
A-PHB	0.18	1	0.18	58.24	0.0001	
B-pH	0.079	1	0.079	25.57	0.0015	
AB	0.13	1	0.13	40.96	0.0004	
A^2	6.054E-005	1	6.054E-005	0.020	0.8928	
B^2	0.50	1	0.50	159.58	< 0.0001	
Residual	0.022	7	3.103E-003			
Lack of Fit	0.012	3	4.085E-003	1.73	0.2992	Not signifi-cant
Pure Error	9.467E-003	4	2.367E-003			
Cor Total	0.91	12				

Standard deviation = 0.056, Mean = 1.11, Coefficient of varience (CV) = 5.03, Press = 0.10, R^2 = 0.9762, Adj R-Squared = 0.9593, Pred R- Squared = 0.8885, Adeq Precision = 23.594

Table 5 – Analysis of response by Plackett Burman design

Compo-nent	**Effect**	**Mean square**	**Standard error**	**T- val-ue**	**F- val-ue**	**Confi-dence level (%)**
A	-0.3202	0.2051	0.7211	0.3581	0.4512	35.52
B	-0.6507	0.8469	0.7211	0.7278	1.8633	72.20
C	0.1802	0.0649	0.7211	0.2015	0.1427	19.99
D	-0.3277	0.2148	0.7211	0.3665	0.4726	36.36
E	0.7732	1.195	0.7211	0.8648	2.6292	85.80
F	-0.3507	0.2460	0.7211	0.3922	0.5412	38.91
G	-0.9745	1.899	0.7211	1.0900	4.1782	92.05
H	0.5092	0.5186	0.7211	0.5695	1.1410	56.50
I	-0.1372	0.076	0.7211	0.1534	0.0827	15.21

Note – Mean square for error = 0.4545

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