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Enhanced production of poly(γ-glutamic acid) from a newly isolated *Bacillus sp*. using statistical approaches

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ABSTRACT

The *Bacillus* strain having the potential for the production of γ-polyglutamic acid (γ-PGA) isolated from soil samples was used in this study. Statistical experimental methods were used to study the effects of various cultural components on the production of γ-PGA. Primarily, the Plackett-Burman experimental design was used to examine the effect of 11 variables and to find the significant variables. The four most significant factors (glutamic acid, glucose, glycerol and $(NH_4)_2SO_4$) determined on the basis of the results of the Plackett-Burman experimental design were further studied by a 2^3 full factorial Central composite design (CCD) and Response surface methodology (RSM) to find their true values and the interaction effect. On the basis of the P-values ($P<0.005$) the linear and the squared coefficients of all the four factors were found to be significant. The interaction effect of glucose and glutamic acid was also found to be significant. The optimized composition of the four medium components derived from RSM regression was (g/l) glutamic acid, 60; glucose, 39; glycerol, 25; and $(NH_4)_2SO4$, 7.5. With this composition γ-PGA production reached 35.54 g/l. The Analysis of variance (ANOVA) showed a high coefficient of determination ($R^2= 0.9725$) which indicated a good adequacy of the quadratic model with the experimental data. The molecular weight of the purified γ-PGA produced by *Bacillus licheniformis* MTCC 10520 was estimated at 173.98 kDa.

KEYWORDS

γ-Polyglutamic acid;
Bacillus licheniformis MTCC 10520;
Plackett-Burman experimental design;
Response surface methodology.

INTRODUCTION

γ-Polyglutamic (γ-PGA) is a water-soluble, naturally available and biodegradable polymer. It is made up of D- and L-glutamic acid units linked by an amide linkage between α-amino and γ-carboxylic acid groups. Several bacteria produce γ-PGA as an extracellular viscous material majority of which belongs to genus *Bacillus*[1,2]. It is included in the traditional Japanese food, *natto*, made from soybeans fermented by *Bacillus* strains. A wide range of multifarious applications of γ-PGA in food, cosmetics, medicinal industries and agriculture have developed a great deal of interest in γ-PGA and its derivatives. For medical applications, the

special chemical properties of the γ-PGA polymer legislates it to fulfill several requirements: renders the drug water soluble, transport the drug to tumor sites and control release of drug over a period of time as the polymer degrades[3]. γ- PGA is used as a drug carrier for sustained release material. PG-TXL, prepared by covalent bonding of paclitaxel to γ-PGA is being used to target tumors[4]. γ- PGA in combination with other components has been used as medical adhesive (surgical glues), anticoagulant and nanoparticles for the delivery of drugs[5,6]. γ- PGA may be important as a therapeutic tool in the treatment of osteoporosis because it can increase Ca^{2+} solubility *in vivo* and *in vitro* thereby enhancing intestinal Ca^{2+} absorption[7]. γ- PGA is reported to be used in food industry[8], skin care products[9] and in fertilizers[10]. It is also anticipated that γ-PGA will be utilized in the areas of wastewater treatment, drinking water processing and downstream processing in food and fermentation industry because it is harmless toward human and environment[1].

The bacterial strains producing γ-PGA have been classified into two categories depending on their glutamic acid requirement (i) glutamic acid dependent strains, those that require glutamic acid for γ-PGA production, for example, *B. subtilis* strain IFO 3335[11], F-2-01 and *B. licheniformis* 9945[12], and (ii) strains that do not require glutamic acid for γ-PGA production, such as *B. licheniformis* A35[13]. For commercial applications of γ-PGA in large amounts, it is necessary to enhance the production through novel strain discovery and bioprocess optimization. A number of factors, such as carbon source, nitrogen source, metal ions, temperature, and pH have a significant effect on the γ-PGA production and these factors are found to vary according to the strain used. The different statistical design for medium optimization has been recently employed for many enzymes[14,15], antibiotics[16] and metabolites. Recently, we have reported that a newly isolated species *Bacillus licheniformis* MTCC 10520 produced γ-PGA in the presence of L-glutamic acid[17]. The enhanced production of γ-PGA from this species has been further attempted. We have applied statistical experimental methods to screen the significant medium components affecting γ-PGA production and to evaluate the optimal levels of the significant variables. First, Plackett-Burman screening design was applied to address the most significant variables affecting γ-PGA production. Second, a central composite design was used to investigate the individual crucial component of the medium that significantly affected the polymer yield. Statistical methods offer several advantages over conventional methods. The statistical method is a versatile technique for investigating multiple process variables because it makes the process easily optimized with fewer experimental trials and enables interactions between variables to be readily identified[18,19]. The molecular weight of the purified γ-PGA was estimated by SDS-PAGE[20].

MATERIALS AND METHODS

Materials

All the chemicals used in this study were purchased from Hi-Media Limited, Mumbai, India. The silica gel-60 plates and high range molecular weight (29kDa-205kDa) protein markers were purchased from Merck (Merck private limited, Mumbai, India).

Microorganism and medium

Bacillus licheniformis MTCC 10520 isolated from soil samples was used in this study[17]. The strain was maintained on agar-slants and was subculture over a period of 30 days. A medium containing (g/l) peptone, 5; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5; and agar 20 at pH 7.0 was used for growth and maintenance.

For the production of γ-PGA a medium optimized by us in one earlier study using one-factor-at-a-time method was used[17]. This medium contained (g/l) glucose, 25.0; glycerol, 20; citric acid 12.0; L-glutamic acid, 40.0; ammonium sulphate, 6.0; K_2HPO_4,1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$ 0.2, $FeCl_3 \cdot 6H_2O$ 0.02, $MnSO_4 \cdot 7H_2O$ 0.05. Fermentation was carried out in 250 ml Erlenmeyer flask, each containing 50 ml of production medium. This medium was used after autoclaving for 20 min at 121^0C and adjusting the final pH at 6.5. This medium was inoculated with 3% (v/v) of 16 h old *B. licheniformis* MTCC 10520 culture and the fermentation was carried out for 90 h.

Analytical methods

γ- PGA was purified using the methanol precipitation method proposed by Goto and Kunioka[11]. Cells

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were separated from the culture broth by centrifugation for 20 min at 12,000 rpm and 4^{0}C temperature. The culture supernatant was then poured into four volumes of cold methanol and gently stirred. It was left undisturbed overnight. The resulting precipitate was collected by applying centrifugation to the supernatant for 30 min at 12,000 rpm and 4^{0}C temperature. The precipitate was then dissolved in distill water and any insoluble impurity was removed by centrifugation. The aqueous γ-PGA solution was desalted by dialysis (mol wt cutoff 10,000) against 1 l volume of distilled water for 12 h with three water exchanges. The final solution was lyophilized, and the dry matter was determined to be γ-PGA.

Cell density was determined by measuring the optical density at 660 nm (OD 660). The absence of polysaccharides was confirmed by phenol-sulphuric acid method[21] and the absence of protein was confirmed by Lowry Folin method[22]. For the analysis of the produced polymer thin layer chromatography was performed. Thin layer chromatography of the hydrolyzed polymer was performed on a silica gel-60 plate (Merck) using n-butanol-acetic acid–water (12:3:5) against glutamate as authentic[23]. The molecular weight of γ-PGA was determined by SDS-PAGE using the method given by Laemmli[24]. Purified PGA was mixed with SDS sample buffer (2 % SDS, 30 % glycerol, 1M Tris HCl, pH 6.8) and boiled for 5 min. Ten µl of sample solution was loaded on the 8 % polyacrylamide gel and the electrophoresis was carried out. The protein markers were stained in a staining solution containing Coomassie brilliant blue R-250 and a staining solution containing a basic dye methylene blue was used to stain γ-PGA[20].

Plackett-Burman design

This study was done by Plackett-Burman design for screening medium components with respect to their main effects and not their interaction effects. The purpose of the first optimization step was to identify which ingredient(s) of the medium has a significant effect on the γ-PGA production. Based on Plackett-Burman factorial design, each variable was examined in two levels: (-1) for low level and (+1) for high level[25]. TABLE 1 shows the experimental design and TABLE 2 shows the variables under investigation as well as the level of each variable used in the experimental design. The impact of each variable on γ-PGA production was estimated based on the mean between the high level (+)

TABLE 1 : Plackett-Burman experimental design with coded values and observed results for γ-PGA production.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	D	γ-PGA (g/l)
1	1	1	1	-1	1	1	-1	1	-1	-1	-1	24.76±0.87
2	1	1	-1	1	-1	1	-1	-1	1	1	-1	16.18±0.26
3	-1	1	1	1	-1	-1	1	1	1	-1	-1	13.21±0.52
4	-1	-1	-1	1	1	1	-1	1	1	-1	1	12.89±0.21
5	1	-1	1	-1	-1	-1	-1	1	1	1	1	10.67±0.39
6	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	12.31±0.15
7	1	-1	-1	1	1	-1	1	1	-1	1	-1	16.43±0.37
8	-1	-1	1	-1	1	1	1	-1	1	1	-1	14.77±0.81
9	-1	1	1	1	1	-1	-1	-1	-1	1	1	19.89±0.49
10	-1	1	-1	-1	-1	1	1	1	-1	1	1	8.92±0.05
11	1	1	-1	-1	1	-1	1	-1	1	-1	1	18.58±0.87
12	1	-1	1	1	-1	1	1	-1	-1	-1	1	16.08±0.51

X_1: Glucose; X_2: Glycerol; X_3: $(NH_4)_2SO_4$; X_4: Citric acid; X_5: Glutamic acid; X_6: K_2HPO_4; X_7: $MgSO_4 \cdot 7H_2O$; X_8: $CaCl_2$; X_9: $FeCl_3 \cdot 6H_2O$; X_{10}: $MnSO_4 \cdot 7H_2O$; D: Agitation rate.

TABLE 2 : Assigned concentrations of variables at different levels in Plackett-Burman design and analysis of results.

Factors	Level(g/l)		Mean of H-level	Mean of L-level	Difference	Mean Square	Variance effect	F-values
	Lower	Higher						
Glucose (X_1)	10	40	17.11	11.99	5.12	26.21	2.18	2725.0
Glycerol (X_2)	10	30	16.92	12.18	4.73	22.37	1.86	2325.0
$(NH_4)_2SO_4$ (X_3)	4	8	16.55	12.55	4.0	16.0	1.33	1662.5
Citric acid (X_4)	8	16	15.78	13.32	2.44	5.95	0.49	612.5
Glutamic acid (X_5)	20	60	17.87	11.22	6.64	44.08	3.67	4587.5
K_2HPO_4 (X_6)	0.5	2.0	15.59	13.51	2.08	4.32	0.36	450.0
$MgSO_4 \cdot 7H_2O$ (X_7)	0.05	0.1	14.65	14.45	0.2	0.04	0.003	3.75
$CaCl_2$ (X_8)	0.1	0.4	14.48	14.62	-0.14	0.0196	0.0016	2.0
$FeCl_3 \cdot 6H_2O$ (X_9)	0.01	0.05	14.37	14.73	-0.36	0.129	0.0108	13.5
$MnSO_4 \cdot 7H_2O$ (X_{10})	0.02	0.1	14.46	14.63	-0.17	0.0289	0.0024	3.0
Agitation rate (D)	200	240	14.50	14.60	-0.10	0.01	0.0008	1.0

and low level (-). Plackett-Burman experimental design is based on the first order polynomial model:

$$E_{(Xi)} = \Sigma M_{(H)}/6 - \Sigma M_{(L)}/6 \quad (1)$$

Where, $E_{(Xi)}$ is the concentration effect of the tested variable. $M_{(H)}$ and $M_{(L)}$ are the total productions from the experimental trials where the variable X_i measured was present at high and low concentrations respectively. Variance effect of the variable was estimated by the following equation.

$$V_{(Xi)} = (\Sigma M_{(H)} - \Sigma M_{(L)})^2/12 \quad (2)$$

Experimental error was calculated by averaging the variance effect of dummy variable. F-effect was calculated as follows.

F effect = Factor mean square / Error mean square

This model does not describe interaction among different variables and is used to screen and evaluate the important variables that influence the response. In the present work, 11 assigned variables were screened in 12 experimental designs. All experiments were carried out in duplicate and the averages of the γ-PGA yield were taken as response. The significant level of the effect of each variable was determined by *F- test*. The experimental design and statistical analysis of the data were done by using MINITAB 15 statistical software.

Response surface methodology

Response surface methodology is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously[26]. Based on the results obtained from Plackett- Burman design, CCD was performed in the optimum vicinity to locate the true optimum concentrations of glutamic acid, glucose, glycerol and ammonium sulphate. MINITAB 15 statistical software was used to design and analyze the experimental results. Each variable was studied for five different levels (-2, -1, 0, 1 and 2). A total of 31 experiments were performed and γ-PGA productions were calculated. The analysis of variance (ANOVA) was conducted with the response functions, and the relationship and interaction between the variables were determined by the following second-order polynomial equation:

$$Y = \beta o + \sum_{i=o}^{n} \beta i\, xi + \sum_{i=o}^{n} \beta n\, xi^2 + \sum_{i>j}^{n} \beta ij\, xi\, xj \quad (3)$$

Where Y = Predicted response, βo = Intercept, βi = Linear coefficient, βn = Squared coefficient, βij = Interaction coefficient, xi, xj = Independent variables

The data obtained was analyzed using MINITAB 15 statistical software, and the response surface and contour plots were constructed to evaluate the optimal value of each variable and the interaction effect of parameters.

RESULT AND DISCUSSION

Screening of significant variables based on Plackett-Burman experimental design

PBD for 11 factors made a total of 12 experimental treatments. The PBD factors and the averages of γ-PGA production (g/l) for 12 different trials of the 11 different components are presented in TABLE 1. The significance of a variable was determined on the basis of F-test, higher the F-value and higher was the effect of the variable.

Analysis of F-value showed that among the variables evaluated, glutamic acid, glucose, glycerol, $(NH_4)_2SO_4$, citric acid and K_2HPO_4 had significant effect on γ-PGA production (TABLE 2). Out of these, the four most significant variables glutamic acid, glucose, glycerol and $(NH_4)_2SO_4$ were selected for further optimization by RSM. Various statistical experimental design are mentioned in the literature for the optimization of fermentation processes, but very few researchers have applied such design for optimizing the production of γ-PGA. To the best of our knowledge it is the first report showing that glucose, glycerol, glutamic acid and $(NH_4)_2SO_4$ have the most significant effect on γ-PGA production.

Optimization of γ-PGA production based on response surface methodology

Based on the results from Plackett-Burman design, four most significant variables, glutamic acid (A), glucose (B), glycerol (C), and $(NH_4)_2SO_4$ (D) were selected for the further optimization by RSM. TABLE 3 shows the maximum and minimum level of variables chosen in CCD. A central composite factorial was designed with eight axial points and seven replicates at the centre points leading to a total of 31 experiments. TABLE 4 shows the CCRD matrix of independent variables in the form of coded and actual values along with

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the observed and predicted response of each experiment trial. The mathematical model relating the yield of γ-PGA with the independent variables is given in Equation 4 and the second-order polynomial co-efficient for each term of the equation was determined through multiple regression analysis using the MINITAB 15 statistical software. The results of the regression analysis of the CCD and of the model fitting in the form of ANOVA (analysis of variance) are given in TABLE 4 and TABLE 5 respectively. The fit of the model was expressed by the coefficient of regression R^2, which was found to be 0.9725, explaining 97% of the variability in the response.

TABLE 3 : Coded and assigned concentrations of variables of different levels of the central-composite design.

Independent variables	Levels				
	-2	-1	0	+1	+2
Glutamic acid	40	50	60	70	80
Glucose	20	30	40	50	60
Glycerol	5	10	20	30	40
$(NH_4)_2SO_4$	2	4	6	8	10

TABLE 4 : The central composite design of independent variables in coded and actual values with their observed and predicted responses of γ-PGA production.

Runs	Glutamic acid		Glucose		Glycerol		$(NH_4)_2SO_4$		γ-PGA production	
	Coded values	Actual values	Coded values	Actual values	Coded values	Actual values	Coded values	Actual values	Observed	Predicted
1	0	60	0	40	-2	5	0	6	15.65	19.14
2	0	60	2	60	0	20	0	6	24.15	24.58
3	0	60	0	40	0	20	2	10	26.50	27.88
4	0	60	0	40	0	20	0	6	31.20	30.25
5	-1	50	1	50	-1	10	1	8	21.28	19.40
6	1	70	1	50	1	30	-1	4	20.75	22.60
7	-1	50	1	50	1	30	-1	4	17.80	16.20
8	2	80	0	40	0	20	0	6	32.60	30.73
9	1	70	-1	30	-1	10	1	8	28.15	29.09
10	0	60	0	40	0	20	0	6	31.00	30.25
11	0	60	0	40	0	20	-2	2	8.90	7.70
12	0	60	0	40	2	40	0	6	27.40	25.62
13	-2	40	0	40	0	20	0	6	10.50	12.55
14	-1	50	-1	30	1	30	1	8	22.50	21.88
15	0	60	0	40	0	20	0	6	29.85	30.25
16	1	70	-1	30	-1	10	-1	4	16.23	16.24
17	0	60	0	40	0	20	0	6	30.10	30.25
18	-1	50	-1	30	-1	10	-1	4	9.30	7.68
19	-1	50	1	50	-1	10	-1	4	9.15	9.67
20	1	70	1	50	1	30	1	8	32.20	33.16
21	-1	50	-1	30	1	30	-1	4	12.85	14.66
22	0	60	-2	20	0	20	0	6	25.20	24.95
23	1	70	-1	30	1	30	-1	4	23.85	25.07
24	-1	50	-1	30	-1	10	1	8	18.70	17.30
25	1	70	-1	30	1	30	1	8	35.60	35.52
26	1	70	1	50	-1	10	-1	4	14.25	14.21
27	1	70	1	50	-1	10	1	8	28.53	27.17
28	0	60	0	40	0	20	0	6	29.90	30.25
29	0	60	0	40	0	20	0	6	30.50	30.25
30	0	60	0	40	0	20	0	6	30.80	30.25
31	-1	50	1	50	1	30	1	8	23.10	23.54

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TABLE 5 : Results of the regression analysis of the CCD

Factors	Coefficient	t- value	P-values
Constant	-165.042	-7.602	0.000*
A	3.105	6.575	0.000*
B	1.705	4.096	0.001*
C	1.381	3.520	0.003*
D	9.990	5.060	0.000*
A*A	-0.022	-6.406	0.000*
B*B	-0.014	-4.082	0.001*
C*C	-0.028	-6.906	0.000*
D*D	-0.779	-9.270	0.000*
A*B	-0.010	-2.228	0.041
A*C	0.005	1.028	0.319
A*D	0.040	1.790	0.092
B*C	-0.001	-0.247	0.808
B*D	0.001	0.061	0.952
C*D	-0.030	-1.327	0.203

A: glutamic acid; B: glucose; C: glycerol; D: $(NH_4)_2SO_4$.
***Statistically significant at 95% of probability level.**

The value of the adjusted R^2 was also found to be very high (0.9484) indicating a high significance of the model. The corresponding second-order response model for equation 3 that was found after analysis for the regression was-

$$Y = -165.042 + (3.105)A + (1.705)B + (1.381)C + (9.990)D + (-0.022)A^2 + (-0.014)B^2 + (-0.028)C^2 + (-0.779)D^2 + (-0.010)AB + (0.005)AC + (-0.040)AD + (-0.001)BC + (0.001)BD + (-0.030)CD \quad (4)$$

The significance of each coefficient was determined by t-values and P-values. The larger magnitude of t-values and the smaller P-values mean the high significance of the corresponding coefficient[27]. The low probability P-value (< 0.005) indicated the model terms to be significant. On this basis, A (glutamic acid), B (glucose), C (Glycerol) and D ($(NH_4)_2SO_4$) were found to be significant. The squared coefficients of A^2 (glutamic

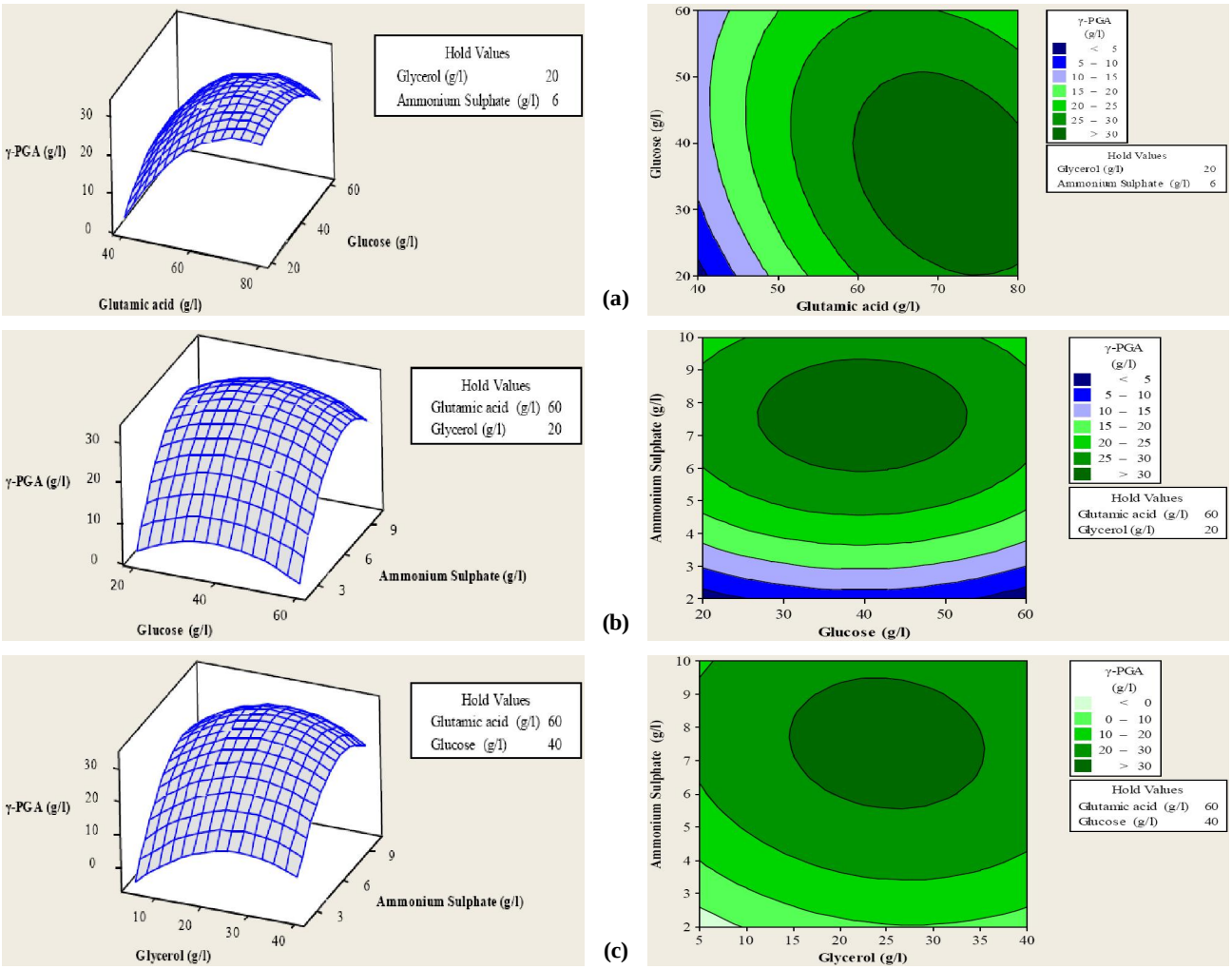

Figure 1 : Response surface and contour plots for γ-PGA production showing mutual interactions between (a) glutamic acid and glucose, (b) glucose and ammonium sulphate, and (c) glycerol and ammonium sulphate.

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TABLE 6 : Analysis of variance (ANOVA) for response surface quadratic model

Sources	Degree of freedom	Sum of square	Mean square	F-value	P-value
Regression	14	1839.20	131.371	40.36	0.000*
Linear	4	1284.12	52.385	16.09	0.000*
Square	4	519.10	129.775	39.87	0.000*
Interaction	6	35.98	5.997	1.84	0.154
Residual error	16	52.08	3.255		
Lack of fit	10	50.32	5.032	17.06	0.001*
Pure error	6	1.77	0.295		

***Statistically significant at 95% of probability level.**
R^2 = 0.9725; Adj R^2 = 0.9484

acid^2), B^2 (glucose^2), C^2 (glycerol^2), and D^2 ($(NH_4)_2SO_4)^2$ were also found to be having a remarkable effect on γ-PGA production. The interaction between A (glutamic acid) and B (glucose) was also found to significant. Other interaction terms were insignificant. The three dimensional response surface graphs and contour plots were generated for the various combinations of four factors, while keeping the other two at the middle values. The graphs are given in Figure 1. The main objective of the response surface analysis is to efficiently find the optimum value of the process variables. The response surface tool and the contour plots were further studied to find the optimum values of the combination of four variables for the maximum production of γ-PGA. These predicted values were experimentally verified. A maximum γ-PGA of 35.54 g/l was obtained when the concentration of the four media components was (g/l) glutamic acid, 60; glucose, 39; glycerol, 25; and $(NH_4)_2SO_4$, 7.5. In this study a wide variation in the γ- PGA production was observed (8.90-35.54). It shows the need and importance of the optimization of the fermentation medium in the development of a fermentation process.

Molecular weight

Under the conditions used in this study for the statistical optimization for the production of γ-PGA, the molecular weight of γ-PGA was found to be 173.98 kDa (Figure 2). For the estimation of molecular weight the relative mobility of each protein was plotted versus molecular weight and the molecular weight of γ-PGA was calibrated. From the earlier studies it is known that the molecular weight of γ-PGA varies from 100 kDa to 1000 kDa depending upon the species, the cultivation conditions and the enzyme PGA depolymerase which accumulates in the culture medium as the incubation time for the production of γ-PGA increases[28,29].

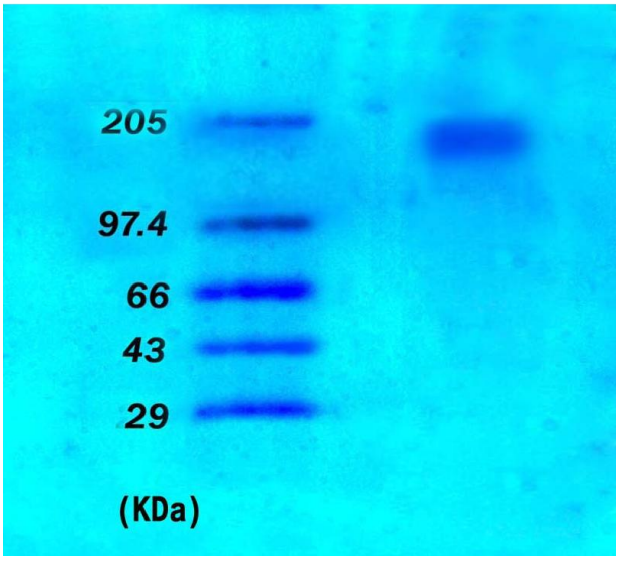

Figure 2 : SDS-PAGE of the γ-PGA produced from *Bacillus licheniformis* MTCC 10520

CONCLUSIONS

γ- PGA production was optimized using response analysis, which was found to be an efficient tool. From PBD experiments glutamic acid, glucose, glycerol, and $(NH_4)_2SO_4$ were shown to be critical components for γ-PGA production by *Bacillus licheniformis* MTCC 10520. The CCD experiment estimated the optimum values of the critical components for maximum γ-PGA production. Under the following conditions: A (glutamic acid) = 60 g/l, B (glucose) = 39 g/l, C (glycerol) = 25 g/l, D ($(NH_4)_2SO_4$) = 7.5 g/l the γ-PGA production achieved was 35.54 g/l. The γ-PGA production by *Bacillus licheniformis* MTCC 10520 could be increased by about 140% from 25.4 to 35.54 g/l, when an optimized medium developed by RSM was used as compared to the medium optimized by one-factor-at-a-time-method.

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