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Full Length Research Paper

Enhanced production of poly glutamic acid by *Bacillus* sp. SW1-2 using statistical experimental design

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***Bacillus* sp. SW1-2 producing poly glutamic acid (PGA), locally isolated from Eastern province in Saudi Arabia, was characterized and identified based on 16S rRNA gene sequencing. Phylogenetic analysis revealed its closeness to *Bacillus megaterium*. The homopolymer consists mainly of glutamic as indicated in the analysis of amino acid. Preliminary optimization of PGA production, through a series of one-variable-at-a-time (OVAT) experiments, revealed maximum PGA production of 10.5 (g/L). Statistically based experiments were applied to optimize culture conditions for production of PGA. Effect of 15 variables were examined for their significance on PGA production using Plackett-Burman factorial design. Among those variables; Na-citrate, $(NH_4)_2SO_4$, glutamic acid and $CaCl_2.2H_2O$ were most significant variables that encouraged PGA production, while those negatively affected were; glycerol, glucose and phosphate buffer. Significant parameters were further investigated using Box-Behnken design to define the optimal medium composition. Based on statistical analysis, maximal PGA production on optimized medium was 36.5 (g/L) which was more than 4-folds the basal production medium. Verification experiment was carried out to examine model validation and revealed more than 95% validity.**

Key words: Poly glutamic acid, *Bacillus megaterium,* statistical experimental design, optimization, one-variable-at-a-time (OVAT).

INTRODUCTION

Poly (γ-glutamic acid) (γ-PGA) is a homopolymer of D- and L-glutamic acid units naturally produced by several *Bacillus* species as an extracellular polymer (Cheng et al., 1989; Kunioka and Goto, 1994; Young and Gross, 1997; Ashiuchi and Misono, 2002; Oppermann-Sanio and Steinbüchel, 2002; Yong et al., 2011). It is biodegradable, edible, and non-toxic to human and the environment. Hence, it has been suggested to be a good candidate for various industrial applications including thickener, drug carrier, biodegradable fibers, highly water absorbable hydrogels and heavy metal absorbers (Chio et al., 1995; Bhattacharyya et al., 1998; Li et al., 1998).

Bacillus subtilis and *Bacillus licheniformis* are the most conspicuous bacteria producing PGA (Ko and Gross, 1998; Yoon et al., 2000; Ashiuchi et al., 2001; Hoppensack et al., 2003; Soliman et al., 2005; Berekaa et al., 2006). In *B. anthracis* and *B. megaterium*, PGA exists as a capsular exocellular polymer and serves as structural component (Hanby and Rydon, 1946; Roelants and Goodman, 1968). In *B. subtilis, B. licheniformis* and *Natrialba aegyptiaca*, PGA is excreted into the medium to increase survival when exposed to environmental stresses (Gross, 1998; Hezayen et al., 2000). Production of γ-PGA was extensively studied, and work has been carried out on the nutritional requirements to increase the yield (Birrer et al., 1994; Goto and Kunioka, 1992; Kunioka and Goto, 1994; Ko and Gross, 1998). There is a general practice of determining optimal concentration of media components by varying one-variable-at-a-time (OVAT). However, experimental design techniques present more balanced alternative to one-variable-at-a-

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time (OVAT) approach to fermentation improvement in PGA production (Soliman et al., 2005; Berekaa et al., 2006). In this study, PGA-producing *Bacillus* sp. SW1-2 was isolated, characterized and identified by 16S rRNA gene analysis. Series of OVAT experiments were carried out to determine critical variables level affecting PGA production. The influence of various culture conditions on PGA production was closely investigated using statistical experimental design namely Plackett-Burman. Special emphasis was given to the impact of significant variables interactions on biopolymer production applying Box-Behnken design.

MATERIALS AND METHODS

Microorganism, isolation and identification

Screening was carried out by enrichment and isolation of spore-forming bacilli from sewage sample obtained from aeration tank of a sewage treatment unit in Dammam, Saudi Arabia. Sewage sample was treated for 20 min at 90°C and subsequently cultivated on nutrient agar medium (composition (g/L): Beef, 3; peptone, 5 and NaCl, 5). Purified strains were tested for PGA production by cultivation on basal production medium supplemented with glutamic acid or ammonium sulfate as nitrogen source. Viscous colonies were selected for further experiments. Potent biopolymer producing bacterium (*Bacillus* sp. strain-SW1-2) was chosen for further analysis. The isolate was maintained on nutrient agar slant composed of (g/L): peptone; 5, beef extract; 3, NaCl; 2 and agar; 20. Stock culture was subcultured at regular intervals of one month and stored under refrigeration.

The bacterium was characterized and identified by 16S *rRNA* gene sequencing using universal primers as descirbed by Soliman et al. (2005). The forward and reverse primers were of the following sequences, respectively: AGAGTTTGATCMTGGCTCAG and TACGGYACCTTGTTACGACTT. The 16S *rRNA* sequence was analysed using BLAST sequence analysis tool available in NCBI, to test for similarity with other 16S rDNA sequences. Subsequently, the sequence was deposited in the GenBank under the accession number HQ124332.

Growth and production conditions

The bacterium grew in 50 ml aliquot of nutrient broth dispensed in 250 ml Erlenmeyer flask and incubated at 37°C for 24 h at 150 rpm. 1.5% (v/v) of overnight culture was used to inoculate the basal production medium E1 of the following composition (g/L): Na-citrate; 12, glycerol; 40, K_2HPO_4; 0.5, KH_2PO_4; 0.2, $MgSO_4.7H_2O$; 0.2 and 1 ml of trace element solution ($FeSO_4.4H_2O$, $CaCl_2.2H_2O$, $MnSO_4.4H_2O$, $ZnCl_2$ 1 mM each) at 37°C. Series of OVAT experiments were established to provide information about parameters used in experimental design experiments, in this concern different concentrations of ammonium sulfate (4 to 10 g/L) and glycerol (20 to 80 g/L) were tested. Furthermore, phosphate buffer was added to medium E1 at concentrations ranging from one to 5-fold increase. On the other hand, different nitrogen sources were tested individually (g/L): casein hydrolysate, 4; $(NH_4)_2SO_4$, 7; and mixture of casein hydrolysate or corn steep liquor and $(NH_4)_2SO_4$, 4 (g/L) each; glutamic acid, 20 (g/L) in presence or absence of yeast extract 0.5 (g/L). Generally, 50 ml medium in 250 ml Erlenmeyer flasks were inoculated with 750 µL of pre-culture. PGA was determined in culture supernatants after clarifying cultures by centrifugation and the cell dry weight was estimated. Recovery of polyglutamic acid was carried out according to the method described by Ashiuchi et al. (1999). For estimation of cell dry weight, the cells were harvested (30 min, 5000 rpm, 4°C) and washed once with phosphate buffer pH 7.0. The obtained pellet was dried and weighed.

Polyglutamic acid analyses

In order to analyze the produced polymer, the dried material was dissolved in 1 ml of distilled water and hydrolyzed with an equal volume of 6 M HCl at 100°C overnight. The hydrolysate was neutralized with 6 M NaOH and chromatographed on TLC against glutamic as authentic using *n*-butanol-acetone-water (12:3:5) as a solvent system. The plates were dried and sprayed with acetone containing 0.2% Ninhydrin to visualize the amino acid (Kambourova et al., 2001). On the other hand, the precipitated polymer material was hydrolyzed at 110°C for 24 h in a sealed evacuated tube, and amino acid composition was determined with Beckman system analyzer (Kambourova et al., 2001; Berekaa et al., 2006).

Fractional factorial design

Plackett-Burman experimental design

Plackett-Burman experimental design (1946) was applied to investigate the significance of various medium components on PGA production. 15 culture variables were tested in two levels: –1 for low and +1 for high level based on Plackett-Burman matrix design. Table 1 represents the lower and higher levels of each variable. In this study, the independent variables were screened in 22 combinations according to the matrix shown in Table 2. The main effect of each variable was calculated simply as the difference between average of measurements made at high setting (+1) and average of measurements observed at low setting (-1) of that factor. Plackett-Burman experimental design was based on the first order model (Equation 1):

$$Y=\beta_0+\sum \beta_i x_i \quad (1)$$

Where, Y is the predicted response, β_0 and β_i are constant coefficients, and x_i is the coded independent variables estimates or factors.

Box-Behnken design

To describe the nature of the response surface in the experimental region, Box-Behnken design (BBD) (Box and Behnken, 1960) was applied. As presented in Table 3, factors of highest confidence levels, elucidated through Plackett–Burman experimental design, were prescribed into 3 levels, coded -1, 0, +1. Table 4 represents the design matrix of 27 trials experiment. For predicting optimal point, second-order polynomial function was fitted to correlate relationship between independent variables and response represented by the amount of PGA produced. For the four factors the equation is:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{12}X_{12}+\beta_{13}X_{13}+\beta_{14}X_{14}+\beta_{23}X_{23}+\beta_{24}X_{24}+\beta_{34}X_{34}+\beta_{11}X_{11}+\beta_{22}X_{22}+\beta_{33}X_{33}+\beta_{44}X_{44} \quad (2)$$

Where, Y is the predicted response, β_0 the model constant; X_1, X_2, X_3 and X_4 independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are cross product coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients.

Table 1. Variables and their levels employed in Plackett-Burman design for screening of culture conditions affecting on PGA production by *Bacillus* sp. SW1-2.

Variable code	Variable	Value	
		-1	+1
X_1	Citrate (g/L)	2	16
X_2	Glycerol (g/L)	20	80
X_3	Glucose (g/L)	5	25
X_4	Casein hydrolysate (g/L)	0.8	8
X_5	Ammonium sulfate (g/L)	4	8
X_6	Glutamic acid (g/L)	5	20
X_7	K_2HPO_4 (g/L)	0.5	1.5
X_8	KH_2PO_4 (g/L)	0.2	0.6
X_9	$MgSO_4.7H_2O$ (g/L)	0.5	1.5
X_{10}	$FeCl_3.6H_2O$ (g/L)	0.04	0.1
X_{11}	$MnSO_4.H_2O$ (g/L)	0.2	0.8
X_{12}	$CaCl_2.2H_2O$(g/L)	0.2	0.8
X_{13}	Yeast extract	0.5	1
X_{14}	Agitation (rpm)	80	200
X_{15}	Inoculum (%)	0.5	2

Table 2. Plackett-Burman experimental design for evaluation of factors affecting poly-glutamic acid (PGA) and cell dry weight (CDW) production by *Bacillus* sp. SW1-2.

Trial No.	Variable code															PGA (g/L)	CDW (g/L)
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15		
1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	6.75	5.6
2	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	6	3.6
3	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	12.25	4
4	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	5.5	4.6
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.75	3
6	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	17	9.2
7	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	3.75	1
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	3.75	3.5
9	-1	1	1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	9	12
10	-1	1	-1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	4.75	4
11	-1	1	1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	9.5	5
12	1	1	-1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	12	5.2
13	-1	-1	1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	7.5	2.8
14	-1	1	-1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	1.75	3.8
15	1	1	-1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	7.25	5.7
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.75	3
17	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	14.75	4.3
18	1	-1	1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	9.75	3.6
19	1	1	1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	11.75	3.3
20	1	-1	-1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	23	3.5
21	1	-1	-1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	20.25	3.2
22	1	-1	1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	16	3.2

X_1, Na-citrate; X_2, glycerol; X_3, glucose; X_4, casein hydrolysate; X_5, ammonium sulfate; X_6, glutamic acid; X_7, K_2HPO_4; X_8, KH_2PO_4; X_9, $MgSO_4.7H_2O$; X_{10}, $FeCl_3.6H_2O$; X_{11}, $MnSO_4.H_2O$; X_{12}, $CaCl_2.2H_2O$; X_{13}, yeast extract; X_{14}, agitation; X_{15}, inoculum; PGA, polyglutamic acid; CDW, cell dry weight.

Table 3. Variables and Their settings employed in Box-Behnken design for optimization of PGA production by *Bacillus* sp. SW1-2.

Variable	Code	Level		
		-1	0	+1
Na-Citrate	X1	10	12	16
Glutamic acid	X2	15	20	25
Ammonium sulfate	X3	8	12	16
$CaCl_2.2H_2O$	X4	0.6	0.8	1.2

Table 4. Box-Behenken matrix representing the effect of significant variables affecting poly-glutamic acid (PGA) by *Bacillus* sp. SW1-2.

Trial number	Na-citrate	Glutamic acid	Ammonium sulfate	$CaCl_2.2H_2O$	PGA (g/L)
1	0	-1	-1	0	11
2	0	1	1	0	20
3	0	-1	0	1	18.25
4	0	1	-1	0	10
5	1	0	0	-1	19
6	0	0	1	-1	17.5
7	0	1	0	-1	16
8	0	1	0	1	15.25
9	-1	0	1	0	13.5
10	1	0	1	0	26
11	-1	0	0	-1	18
12	1	1	0	0	24.75
13	0	0	0	0	18.25
14	0	0	1	1	22.25
15	-1	-1	0	0	13.5
16	-1	0	-1	0	13
17	-1	0	0	1	15.5
18	0	-1	1	0	18.25
19	0	-1	1	1	21.75
20	1	0	0	1	28
21	0	0	-1	-1	19.5
22	0	0	-1	1	20.25
23	1	0	-1	0	3.25
24	0	-1	0	-1	3.25
25	1	-1	0	0	9.75
26	0	0	0	0	18.75
27	-1	1	0	0	0.5

Microsoft Excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Statistical analysis of the data

Data on PGA production were subjected to multiple linear regressions using Microsoft Excel 97 to estimate *t*-value, *P*-value and confidence level. Significance level (*P*-value) was determined using Student's *t*-test. Factors having highest *t*-value and confidence level over 95% were considered to be highly significant on PGA production. Optimal value of activity was estimated using the solver function.

RESULTS AND DISCUSSION

Isolation, characterization and taxonomic classification

In a program for exploring the potential production of PGA biopolymers from bacteria enriched and isolated

Table 5. Summary of one-variable-at-a-time (OVAT) experiments to optimize PGA production by *Bacillus* sp. SW1-2.

Component (g/L)	Initial concentration (g/L)	Final concentration (g/L)	PGA (g/L)	CDW (g/L)
Ammonium sulfate	7	8	8.55	4.6
Glycerol	40	60	9	3.9
Glucose	0	15	10.25	4.25
Phosphate buffer (K_2HPO_4:KH_2PO_4)	0.5:0.2	1:0.8	10.5	5.8

from aeration tank in a sewage treatment unit in Dammam, Saudi Arabia, six bacilli strains showed viscous growth on basal production medium E1. Isolate SW1-2 was the most potent biopolymer producer. Morphological and physiological characteristics of the isolate SW1-2 showed that it is rod shaped, spore forming Gram-positive bacterium, capable of growing on different sugars such as: glucose, fructose, mannose, xylose and arabinose. In addition, the isolate showed amylase, protease and lipase activities. To investigate phylogenetic affiliation of this strain, complete 16S *rRNA* gene was amplified, sequenced and deposited in the GenBank (accession number HQ124332). Comparison of the obtained sequence with other sequences available at NCBI database revealed the greatest similarity to the corresponding sequence of *B. megaterium*, a known producer of PGA (Shimizu et al., 2007).

Running of the hydrolysed polymer on TLC indicated that glutamic is the major product (data not shown). Furthermore, amino acid analysis of the polymer hydrolysate indicated that glutamic monomer is the dominant residue in precipitated polymer (approximately 56%) indicating that the precipitated polymer material is polyglutamic acid.

OVAT experiments to optimize PGA production

Preliminary, OVAT experiments were carried out to address the most effective ammonium sulfate, glycerol and phosphate concentrations. Results in Table 5 show that *Bacillus* sp. strain SW1-2 cultivated on medium E1 varied in its potentiality to produce PGA, with varying ammonium sulfate, glycerol and phosphate concentrations. Maximum PGA production (8.55 and 9 g/L) was obtained in the presence of final concentrations of 60 and 8 g/L of glycerol and ammonium sulfate, respectively. Moreover, small amount of PGA (4.25 and 3.5 g/L) was produced in the presence of 20 and 5 g/L of glycerol and ammonium sulfate, proving that ammonium sulfate is an important factor for PGA production. These results are supported by findings of other scientists (Kunioka and Goto, 1994; Du et al., 2005; Wu et al., 2010). They mention that large amount of PGA was produced in the presence of citric acid and ammonium sulfate. Furthermore, 4-fold increase in phosphate concentration led to the increase in PGA production to 10.5 g/L. Promotion of PGA production by phosphates can be attributed to the mechanism of PGA synthesis in some bacteria (Troy, 1973; Gardner and Troy, 1979).

Effect of different nitrogen sources

One of the most critical variables affecting PGA biopolymer production is the nitrogen source. In this concern, the effect of different nitrogen sources on PGA production by *Bacillus* sp. strain SW1-2 was closely investigated. Results in Figure 1 reveal that maximum PGA production (13.75 g/L) was obtained when glutamic acid was used as nitrogen source. Dramatic decrease in PGA production was recorded when yeast extract was used in the presence of glutamic acid (approxi. 70%), indicating that PGA production by *Bacillus* strain SW1-2 is enhanced by glutamic acid which is supported by the results obtained by Shimizu et al. (2007).

Evaluation of factors affecting PGA production

For screening purpose, various medium components as well as environmental factors were evaluated. The different factors were prepared in two levels, -1 for low level and +1 for high level, based on a Plackett–Burman statistical design (Plackett and Burman, 1946). Independent variables were examined and their settings are shown in Table 1. Design plan and averages of PGA for different trials are given in Table 2 and main effect of each variable was estimated. Data in Table 2 show wide variation in PGA production ranging from 1.75 to 23 g/L. This variation reflected the importance of medium optimization to attain higher production. Analysis of data obtained from Plackett–Burman experiments involved a first order model. The main effect of the examined factors on PGA production was calculated and illustrated graphically in Figure 2a. On the bases of regression coefficients analysis and *t*-value of 15 ingredients presented in Table 6, Na-citrate, glutamic acid, ammonium sulfate and $CaCl_2.2H_2O$ were most significant variables that encourage PGA production, while the

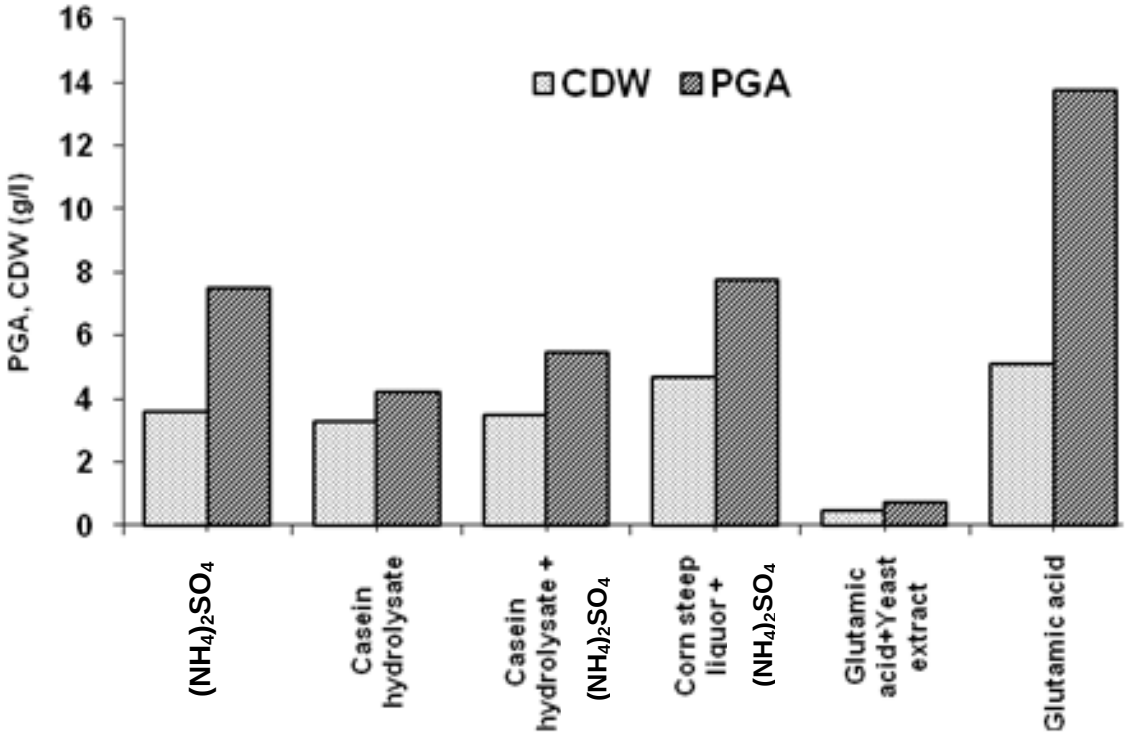

Figure 1. Effect of different nitrogen sources on growth (CDW) and poly-glutamic acid (PGA) production in culture of *Bacillus* sp.SW1-2.

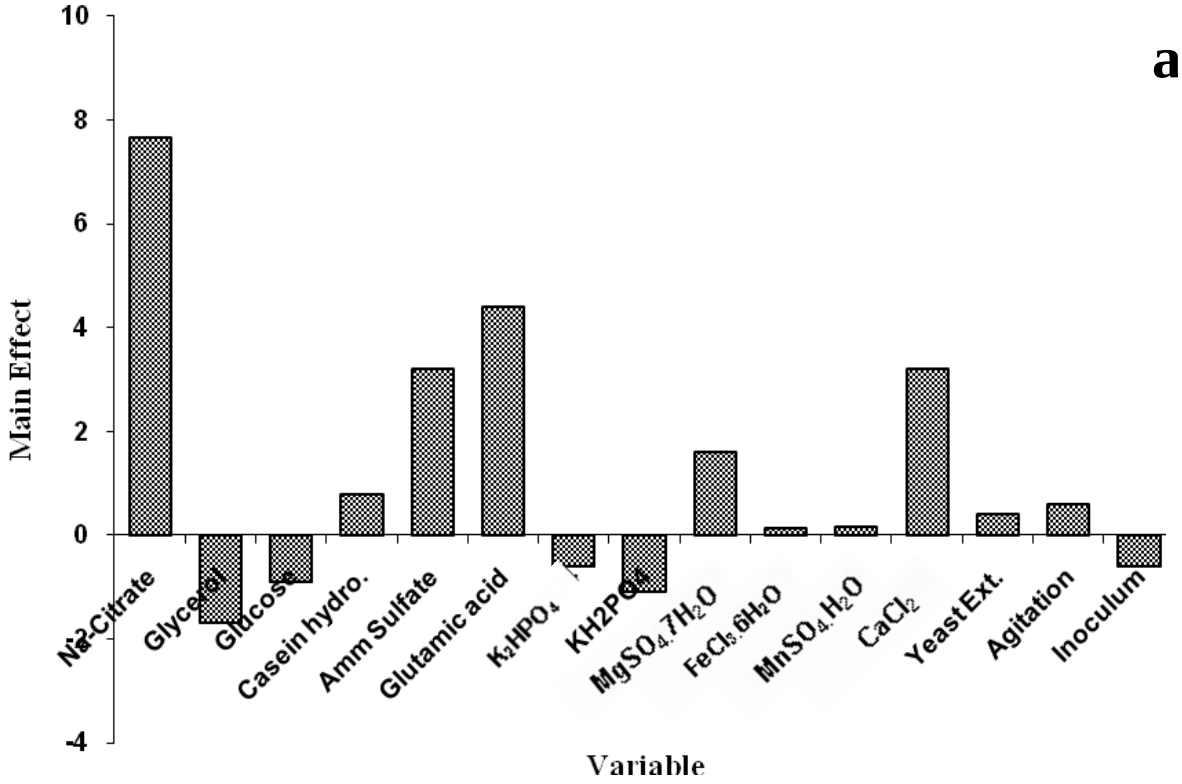

Figure 2a. Effect of environmental and nutritional factors on PGA production by *Bacillus* sp. SW1-2 based on Plackett-Burman design results.

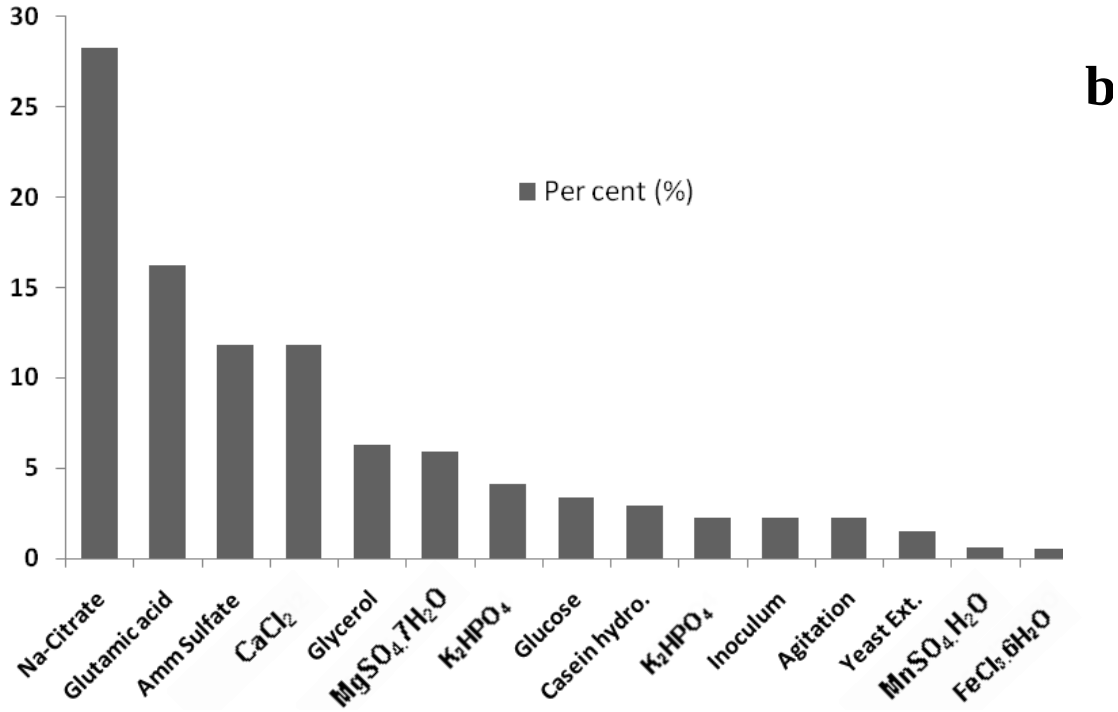

Figure 2b. Pareto plot for Plackett-Burman parameter estimates of PGA production by *Bacillus* sp. SW1-2.

Table 6. Statistical analysis of Plackett-Burman design showing coefficient values, *t*- and *P*-values for each variable.

Variable	Coefficient	*tStat*	*P*-value	Confidence level %
Intercept	10.19	-	-	-
X_1	4.24	7.85	0.0002	100
X_2	-0.83	-1.54	0.17	83
X_3	-0.43	-0.54	0.6	40
X_4	0.39	0.67	0.53	47
X_5	1.5	2.79	0.03	96
X_6	2.18	3.73	0.009	99.1
X_7	-0.31	-0.52	0.62	38
X_8	-0.46	-0.43	0.43	57
X_9	0.78	1.44	0.2	80
X_{10}	0.07	0.13	0.9	10
X_{11}	0.08	0.15	0.9	10
X_{12}	1.57	2.7	0.04	96
X_{13}	0.17	0.29	0.78	22
X_{14}	0.31	0.57	0.59	41
X_{15}	-0.28	-0.15	0.62	38

X_1, Na-citrate; X_2, glycerol; X_3, glucose; X_4, casein hydrolysate; X_5, ammonium sulfate; X_6, glutamic acid; X_7, K_2HPO_4; X_8, KH_2PO_4; X_9, $MgSO_4.7H_2O$; X_{10}, $FeCl_3.6H_2O$; X_{11}, $MnSO_4.H_2O$; X_{12}, $CaCl_2.2H_2O$; X_{13}, yeast extract; X_{14}, agitation; X_{15}, inoculum.

negatively affected were; glycerol, glucose and KH_2PO_4.

The *t*-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. In these experiments, variables with confidence levels greater than 95% were considered as significant. The quality of fit of the polynomial model equation was

expressed by the coefficient of determination R^2. The determination coefficient R^2 of the full model for PGA was 0.97. Neglecting the insignificant terms (p> 0.05%), the model equation for PGA can be written as:

$$Y_{PGA} = 10.19 + 4.24X_1 + 1.5X_5 + 2.18X_6 + 1.57X_{12} \quad (3)$$

Where, X_1, X_5, X_6 and X_{12} are the Na-citrate, ammonium sulfate, glutamic acid and $CaCl_2.2H_2O$, respectively.

One of the advantages of the Plackett-Burman design is to rank the effect of different variables on the measured response independent on its nature. Figure 2b shows the ranking of factor estimates in a Pareto chart. The chart displayed the magnitude of each factor estimate and is a convenient way to view results of Plackett-Burman design (Strobel and Sullivan, 1999). It has been reported that variable range of PGA production was attained among different strains of *B. licheniformis* strains when ammonium sulfate was used as a nitrogen source (Kambourova et al., 2001). In contrast, *B. subtilis* strains produced PGA only when growing on casein hydrolysate and did not produce PGA in the presence of $(NH_4)_2SO_4$ (Kambourova et al., 2001). Interestingly, PGA-producing bacteria were divided into two groups: glutamate-dependent and glutamate-independent producers (Kunioka and Goto, 1994; Ito et al., 1996).

In the former, the PGA yield increased upon addition of glutamate or glutamic acid to the medium, but the bacteria can produce considerable PGA even in the absence of glutamic acid because of the operation of the de novo pathway of L-glutamate synthesis (Kunioka and Goto, 1994). Cheng et al. (1989) reported that *B. licheniformis* A35 is a glutamate independent bacteria where it uses glucose as a major carbon source. As it could be seen in Figure 2a, PGA production by *B. megaterium* SW1-2 was induced on using glutamic acid as a nitrogen source. Consequently, it could be classified as glutamic-dependent PGA producer.

Application of Box-Behnken design and data analysis

Further experiments were carried out to obtain a quadratic model consisting of 27 trials. As presented in Table 3, factors of highest confidence levels were prescribed into 3 levels, coded -1, 0, +1. The design of this experiment is given in Table 4 together with the experimental results. Regression analysis was performed to fit the response function (PGA production) with the experimental data. Analysis of variance for the four variables (Na-citrate, glutamic acid, ammonium sulfate and $CaCl_2.2H_2O$) indicates that PGA production can be well described by a polynomial model with relatively high coefficient of determination (R^2 = 0.85). Statistical analysis of the full model proved that Na-citrate, glutamic acid, ammonium sulfate and $CaCl_2.2H_2O$ had a significant effect on PGA production (data not shown).

When presenting experimental results in the form of surface plot (Figure 3), it can be seen that high levels of Na-citrate, ammonium sulfate, $CaCl_2.2H_2O$ and moderately high levels of glutamic acid supported high PGA production. For predicting the optimal point, within experimental constrains, a second-order polynomial function was fitted to the experimental results of PGA production:

$$Y_{PGA} = 18.7 + 4.7X_1 + 1.1X_2 + 3.3X_3 + 2.1X_4 + 7X_{12} + 9.5X_{13} + 2.9X_{14} + 0.9X_{23} - 3.8X_{24} + 0.8X_{34} - 1.9X_{11} - 5X_{22} - 0.8X_{33} + 1.5X_{44} \quad (4)$$

Where, X_1, X_2, X_3 and X_4 represent codified values for Na-citrate, glutamic acid, ammonium sulfate and $CaCl_2.2H_2O$ respectively. At the model level, correlation measures for estimation of regression equation were the multiple correlation coefficients R and the determination coefficient R^2. The closer the value of R is to 1, the better the correlation between the observed and the predicted values. In this experiment, the value of R was 0.923 for PGA production. This value indicates a high degree of correlation between experimental and predicted values. The value of determination coefficient R^2 = 0.85 being a measure of fit of the model, indicates that about 15% of the total variations are not explained by the production model.

On analyzing Equation 4 with solver, based on non-linear optimization algorithm, maximum production was found to be 41.7 g/L when concentrations of tested variables reached to the maximum. Interestingly, results presented in Figure 3a, b and f clearly indicates that PGA production was enhanced due to interaction between Na-citrate with glutamic acid, ammonium sulfate and $CaCl_2.2H_2O$, respectively. Generally, some *Bacillus* strains such as *B. licheniformis* ATCC9945a need glutamic acid in the medium to produce PGA, others such as *B. subtilis* IFO3335 do not (Kunioka and Goto, 1994). In this work, PGA production by *B. megaterium* SW1-2 was enhanced in the presence of glutamic acid or glutamate which is supported by the results obtained by Shimizu et al. (2007). They reported that *B. megaterium* requires large amount of L-glutamate for polymer production as does a group of *B. subtilis* overproducers. Furthermore, large amount of PGA is produced from citric acid and ammonium sulfate (Kunioka, 1997; Kunioka and Goto, 1994).

Furthermore, increased utilization of glutamic and citrate corresponded with higher PGA production as was anticipated based on the fact that glutamate and citrate are known precursors of PGA synthesis (Young and Gross, 1997). It is presumed that L-glutamic acid is produced from citric acid through isocitrate and alpha-ketoglutarate in TCA cycle, and PGA is polymerized from glutamic acid (Kunioka, 1997). Verification of the calculated optimum was done with a culture medium representing the optimal points and yielding 36.5 g/L.

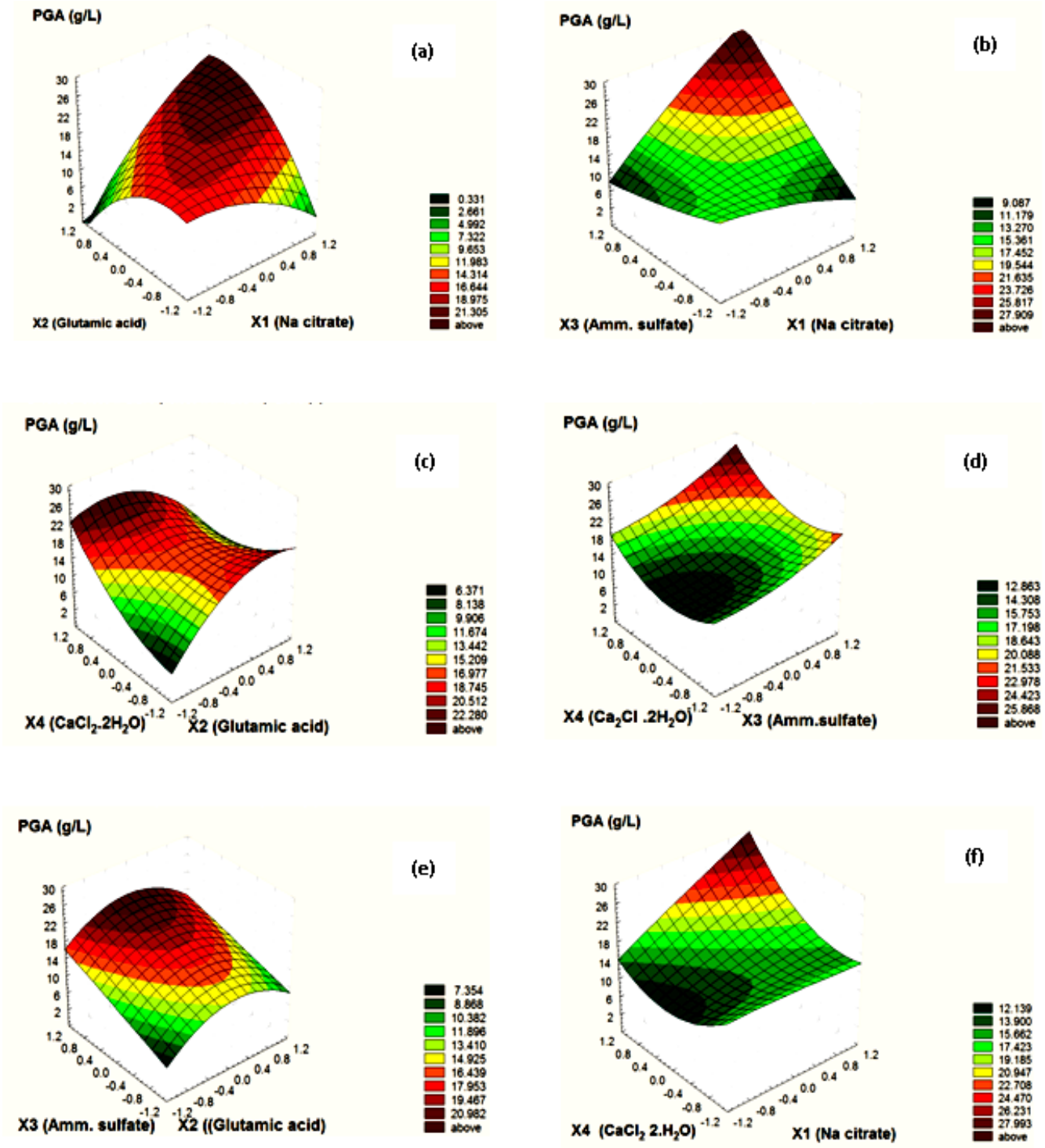

Figure 3a-f. Three dimensional response surface graphics showing the behavior of PGA production as affected by different culture conditions.

Results collectively indicated that PGA production by *B. megaterium* SW1-2 was about 4-folds increased when cultivated in the optimal medium developed by BBD, as compared to basal salts production medium E1. Therefore, statistical experimental design proved to be a powerful and useful tool for enhancing PGA production and confirm the necessity of the optimization process.

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