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# Sequential Optimization Approach for Enhanced Production of Poly(γ-Glutamic Acid) from Newly Isolated *Bacillus subtilis*

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**Summary**

A bacterial strain of marine origin showing production of poly(γ-glutamic acid) (PGA) has been identified by taxonomical and 16S rRNA studies as *Bacillus subtilis*. A sequential optimization approach was applied for improving the PGA production. The effect of carbon sources, nitrogen sources and pH on the production of PGA was investigated by one factor-at-a-time method. Plackett-Burman design was then adopted to select the most important nutrients influencing the yield of PGA. After identifying the most significant nutrients, response surface methodology (RSM) was used to develop a mathematical model to identify the optimum concentrations of the key nutrients for higher PGA production, and confirm its validity experimentally. PGA production was further improved by supporting the medium with α-ketoglutaric acid. The PGA production increased from 7.64 to 25.38 g/L by using the sequential optimization methods.

*Key words*: poly(γ-glutamic acid), *Bacillus subtilis*, biopolymer, response surface methodology

## Introduction

Poly(γ-glutamic acid) (PGA) is an anionic, naturally occurring, water-soluble homo-polyamide consisting of D- and L-glutamic acid monomers connected by amide linkages between α-amino and γ-carboxyl groups. It is biodegradable, edible and nontoxic towards humans and environment. Potential applications of PGA and its derivative have been of interest in the past few years in a broad range of industrial fields like foods, pharmaceuticals, and medicine (*1*).

Most of the PGA-producing strains reported in literature are able to grow in high saline conditions. PGA is used by some microorganisms to decrease high local salt concentrations, enabling them to survive in a hostile environment (*2*). Therefore, marine bacteria are considered as ideal targets and potential source for production of PGA.

PGA-producing bacteria usually use glucose, glutamic acid, citric acid and/or glycerol for the production of PGA (*3*,*4*). Citric acid and glutamic acid act as precursor substrates for polymer production (*4*,*5*). Literature on the use of amino acids and tricarboxylic acid (TCA) cycle intermediates as stimulators of PGA synthesis is scant. Supplementation of the fermentation medium with L-glutamine and α-ketoglutaric acid increased the PGA production by *Bacillus licheniformis* NCIM 2324 in solid-state fermentation (*6*). However, reports on the study of TCA intermediates as stimulators of PGA syntheses by *B. subtilis* in submerged fermentation are not available.

The use of statistical experimental design in the optimization of fermentation processes or media is well documented, but very few papers have applied all the experimental designs well. Sequential strategy includes the screening of a large number of medium components

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followed by the optimization of a subset of those components using response surface methodology (*7*).

This paper addresses screening of marine bacteria for PGA production, identification of the strain giving maximum production by 16S rRNA, and optimization of PGA production applying sequential optimization strategy and by using amino acids and TCA cycle intermediates.

## Materials and Methods

### *Materials*

All the chemicals used in the present study were of the AR grade and were purchased from HiMedia Ltd, Mumbai, India. L-glutamine, L-arginine, L-ornithine, L-proline, L-alanine, L-aspartic acid, α-ketoglutaric acid, malic acid, succinic acid and pyruvic acid were purchased from S.D. Fine Chemicals Limited, Mumbai, India. PGA was generously provided by Vedan Enterprise Corporation, Taiwan.

### *Screening of marine bacteria for PGA-producing strain*

About 350 marine strains were screened for PGA production. Marine strains were spread on the medium containing (in g/L): glucose 10, ammonium chloride 5, L-glutamic acid 10, $KH_2PO_4$ 0.5, $MgSO_4{\cdot}7H_2O$ 0.1 and agar 15 (pH=6.5) and incubated at 37 °C. After 48 h of incubation, highly mucoid colonies on the plates were picked up, inoculated into 20 mL of screening medium and incubated at 37 °C in a rotary shaker at 220 rpm for 48 h. The screening medium was prepared by combining the composition of the medium reported by Du *et al*. (*8*) for production of PGA and artificial seawater (*9*), which is necessary for growth of marine organisms. The composition of the screening medium was (in g/L): glycerol 25, ammonium chloride 6, citric acid 12, L-glutamic acid 20, NaCl 24.7, KCl 0.66, $K_2HPO_4$ 1, $MgSO_4{\cdot}7H_2O$ 6.8, $CaCl_2{\cdot}2H_2O$ 0.2, $NaHCO_3$ 0.18, $MgCl_2{\cdot}6H_2O$ 4.7 and $MnSO_4{\cdot}7H_2O$ 0.05 (pH=6.5). The relative viscosity of culture broth was measured by Haake VT550 rotoviscometer with a sensor system NV (cup) and MV (rotor). The PGA produced in highly viscous culture broth was confirmed by using HPLC profile and product characterization. Taxonomical characterization of PGA-producing bacteria was performed according to Bergey's Manual of Bacteriology (*10*). 16S rRNA study was carried out at National Centre for Cell Science (NCCS), Pune, India.

### *Fermentative production of PGA*

Composition of the production and seed medium was similar to that of the screening medium. A loopful of cells from a slant was transferred to 25 mL of the seed medium in a 100-mL conical flask, incubated at 37 °C and 200 rpm for 16 h, and used as the inoculum. Fermentation was carried out in 250-mL Erlenmeyer flasks, each containing 50 mL of the sterile production medium. The medium was inoculated with 1 % (by volume) of 16-hour culture containing approx. $3{\cdot}10^7$ cells/mL. The flasks were inoculated on a rotary shaker at (37±2) °C and 200 rpm.

### *Primary optimization stage: One factor-at-a-time method*

For evaluation of the effect of different carbon sources on the production of PGA, glycerol was substituted with different carbon sources, *viz*. glucose, sucrose, maltose, lactose, galactose, sorbitol or mannitol at 25 g/L.

To study the effect of different nitrogen sources on PGA production, ammonium chloride was replaced with organic nitrogen sources like peptone, yeast extract, soybean meal, corn steep liquor and malt extract; and inorganic nitrogen sources like sodium nitrate, potassium nitrate, ammonium chloride, ammonium sulphate and ammonium nitrate at 6.0 g/L.

In order to monitor the effect of pH on PGA production, fermentation runs were carried out at initial pH varying from 5.0–7.0.

### *Screening stage: Plackett–Burman design*

In this study Plackett–Burman design was applied for screening medium components with respect to their main effects and not their interaction (*11*). These variables screened with a twelve-run Plackett–Burman design are shown in Table 1. The effect of each variable was determined by the following equation:

$$E(x_i)=2(\Sigma M_i^{+}-M_i^{-})/N \qquad /1/$$

where $E(x_i)$ is the concentration effect of the tested variable, $M_i^+$ and $M_i^-$ are the total production from the trials where the measured variable ($x_i$) was present at high and low concentrations, respectively; and $N$ is the number of trials. Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{eff}=\Sigma(E_d)^2/n \qquad /2/$$

where $V_{eff}$ is the variance of the concentration effect, $E_d$ is the concentration effect for the dummy variable and $n$ is the number of dummy variables. Standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (p-value) of each concentration effect was determined using Student's *t*-test:

$$t(x_i)=E(x_i)/SE \qquad /3/$$

where $E(x_i)$ is the effect of variable $x_i$.

### *Superior optimization stage: RSM*

A central composite rotatable design (CCRD) for four independent variables was used to obtain the combination of values that optimizes the response within the region of three-dimensional observation spaces, which allows one to design a minimal number of experiments. The experiments were designed using the software Design Expert trial version 6.0.10 (State Ease, Minneapolis, MN, USA).

The medium components (independent variables) selected for the optimization were glucose, citric acid, sodium chloride and ammonium chloride. Regression analysis was performed on the data obtained from the design experiments. The second order polynomial coefficients were calculated to estimate the responses of the

Table 1. Plackett–Burman design with coded values along with observed results for PGA production

| Serial no. | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | $X_7$ | $X_8$ | $X_9$ | $X_{10}$ | $D_1$ | $\gamma$(PGA)[a]/(g/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 6.21±0.62 |
| 2 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 3.26±0.21 |
| 3 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | 3.25±0.38 |
| 4 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 5.92±0.61 |
| 5 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 3.78±0.35 |
| 6 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 11.28±0.82 |
| 7 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | 4.82±0.15 |
| 8 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | 1.64±0.19 |
| 9 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | 2.64±0.31 |
| 10 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 2.71±0.14 |
| 11 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | 2.44±0.11 |
| 12 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | 0.85±0.02 |

$X_1$–$X_{10}$ are independent variables: $X_1$ glucose, $X_2$ citric acid, $X_3$ ammonium chloride, $X_4$ glutamic acid, $X_5$ $MgSO_4 \cdot 7H_2O$, $X_6$ NaCl, $X_7$ $K_2HPO_4$, $X_8$ KCl, $X_9$ $CaCl_2$, $X_{10}$ $MnSO_4 \cdot 7H_2O$

$D_1$ is dummy variable

[a]Results are mean±SD of at least three determinations

dependent variable. Response surface plots were also obtained using Design Expert v. 6.0.10.

### *Optimization using precursors: Effect of the addition of amino acids and TCA cycle intermediates*

To study the effect of the addition of amino acids on PGA production, amino acids of glutamic acid family (L-glutamine, L-arginine, L-ornithine and L-proline) and amino acids involved in biosynthetic pathway of PGA (L-alanine and L-aspartic acid) were added individually at 0.5, 1.0 and 1.5 mM to the production medium. Fermentation medium devoid of amino acids was used as the control.

To study the effect of the addition of different TCA cycle intermediates on PGA production, α-ketoglutaric acid, malic acid, succinic acid or pyruvic acid were added individually at 5.0, 10 and 15 mM to the production medium. Fermentation medium devoid of TCA cycle intermediate served as control.

### *Purification of PGA*

PGA was purified by the method reported by Goto and Kunioka (*3*). Culture broth was appropriately diluted and cells were separated from the broth by centrifugation at 10 000 rpm and 4 °C for 20 min. The supernatant containing PGA was poured into four volumes of methanol and kept at 4 °C for 12 h. Crude PGA was collected by centrifugation at 10 000 rpm and 4 °C for 30 min, then dissolved in distilled water and any insoluble impurity was removed by centrifugation. The aqueous PGA solution was desalted by dialysis (molecular mass cutoff of 3500) against 1 L of distilled water for 12 h with three water exchanges, and finally lyophilized to prepare pure PGA.

### *Analytical methods*

PGA concentration was determined by the method of Xiong *et al*. (*12*). Jasko HPLC system fitted with PL aquagel-OH gel permeation chromatogram column (7.8×300 mm, Polymer Laboratories Ltd., UK) and UV detector was used for PGA analysis. Samples were eluted with 0.1 mM sodium chloride at a flow rate of 1 mL/min and detected at 220 nm. The purified PGA was used as a standard (*13*).

A volume of 20 mL of properly diluted culture broth was centrifuged at 10 000 rpm and 4 °C for 20 min to separate the cell mass. The detained cell pellet was then dried in hot air oven at 80 °C to a constant mass for dry cell mass.

Glucose in the fermentation broth was estimated by a dinitrosalicylic acid method (*14*). Concentration of citric acid was determined by a method described by Marrier and Boulet (*15*), while the concentration of glutamic acid was determined by HPLC with the conditions identical to those for quantification of PGA.

### *Characterization of PGA*

#### Molecular mass determination

Jasko HPLC system fitted with PL aquagel-OH gel permeation chromatogram columns (2×PL aquagel-OH MIXED-H 8 μm, 300×7.5 mm, Polymer Laboratories Ltd., UK) and refractive index (RI) detector was used for determination of molecular mass of PGA. Polyethylene oxide standards were used to construct a standard curve. The eluant was 0.2 M $NaNO_3$ (pH=7) and the flow rate was set at 1 mL/min (*13*).

#### Amino acid analysis

The PGA produced from *B. subtilis* R 23 was purified and hydrolyzed using 6 M HCl at 110 °C for 24 h in a sealed and evacuated tube and then used for amino acid analysis. Thin layer chromatography was performed on a cellulose plate (Merck, USA) with solvent systems of butanol/acetic acid/water (3:1:1, by mass) and 96 % ethanol/water (63:37, by mass). Detection of amino acids was done by spraying with 0.2 % ninhydrin in acetone (*16*).

Total sugar content

The total carbohydrate content of the PGA produced by *B. subtilis* R 23 was determined by the phenol–sulphuric acid method (*17*).

## Results and Discussion

### *Screening for PGA-producing strain*

Microbes can sense, adapt and respond to their environment quickly and can compete for defense and survival by generation of unique metabolites. These metabolites are produced in response to stress, and many have shown value in biotechnological or pharmaceutical applications. The marine environment contains high salt levels and poor nutrition, leading to smaller microbial growth. The ecology of marine natural products actually reveals that many of these compounds are utilized by the marine organisms for survival (*2*).

About 350 marine strains were screened for PGA production, initially on the basis of relative viscosity of fermentation broth. Amongst these, one strain produced sufficient amount of PGA, and hence was selected for further study. Taxonomical characterization of PGA-producing bacteria was performed according to Bergey's Manual of Bacteriology (*10*). It was an aerobic, Gram-positive, motile and rod-shaped organism. Furthermore, nitrate reduction, Voges-Proskauer, nitrate utilization, casein utilization and catalase production tests were positive, and starch utilization test was negative. On the basis of these characteristics, the bacterium was considered as belonging to genus *Bacillus*. Study including 16S rRNA showed the PGA-producing strain to have 98 % 16S rRNA sequence similarity with *Bacillus subtilis* JK-1, and hence was named *Bacillus subtilis* R 23. The 16S rRNA sequence of *Bacillus subtilis* R 23 was deposited in GenBank under the accession number EU 598267.

### *One factor-at-a-time method*

Among the evaluated carbon sources, glucose supported maximum production of PGA and biomass of (7.64±0.5) and (2.17±0.18) g/L, respectively (Fig. 1). The biosynthetic pathway for PGA synthesis, which most likely involves the TCA cycle, suggests that carbon sources like glucose are useful for both cell growth and PGA production (*1*). Glucose has also been reported to be a better carbon source than sucrose, maltose, lactose, or starch for PGA production in *B. subtilis* NX-2 and *B. subtilis* F-2-01 (*18,19*).

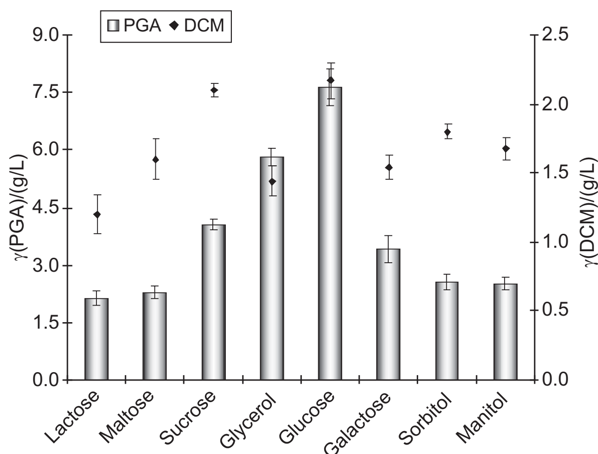


**Fig. 1.** Effect of different carbon sources on PGA production by *B. subtilis* R 23

Among the studied nitrogen sources, ammonium chloride supported maximum PGA production (Fig. 2). Biomass production was high but PGA yield was lower with organic nitrogen sources compared to inorganic nitrogen sources. Goto and Kunioka (*3*) suggested that free $NH_4^+$ ions are required for PGA production. The availability of free $NH_4^+$ ions from inorganic nitrogen sources is higher than that from organic nitrogen sources. Most researchers use inorganic nitrogen sources for production of PGA (*3,8,20*).

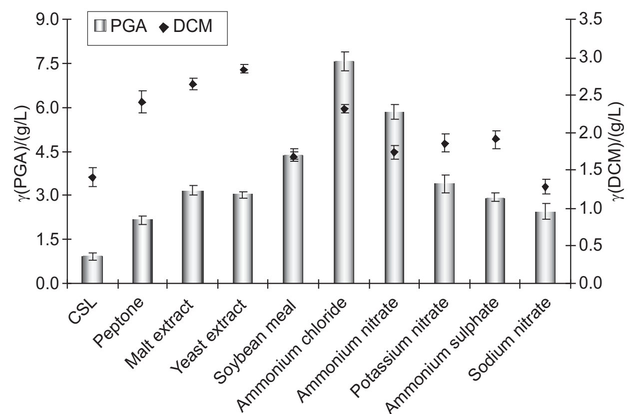


**Fig. 2.** Effect of different nitrogen sources on PGA production by *B. subtilis* R 23

An initial pH of 6.5 supported maximum PGA (7.81±0.21) and biomass (2.36±0.09) production (Fig. 3). The final pH value at the end of the cultivation was 5.7±0.3. Most studies on fermentative production of PGA were carried out at pH=6.5 (*8*). pH is a significant factor that influences the physiology of a microorganism by affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology, by-product formation and oxidative-reductive reactions. During production of PGA, citrate utilization occurs more rapidly and to a greater extent at pH=6.5 (*21*).

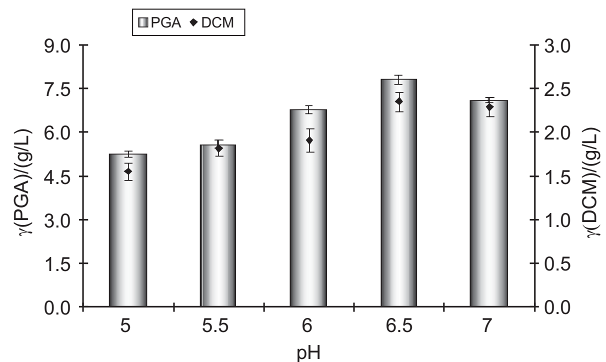


**Fig. 3.** Effect of pH on PGA production by *B. subtilis* R 23

### *Screening using Plackett–Burman design*

Plackett–Burman design was adopted to select the most significant medium components. High and low limits of variables are shown in Table 2. The experimental design protocol (recipe) along with responses of different experimental trials is shown in Table 1.

Table 2 shows ANOVA for the results of Plackett-Burman design.

Contrast coefficients allow the determination of the effect of each constituent. A large contrast coefficient, either positive or negative, indicates that a factor has a large impact on titre; while a coefficient close to zero means that a factor has little or no effect. The p-value is the probability that the magnitude of a contrast coefficient is due to random process variability and it serves as a tool for checking the significance of each of the coefficients. A low p-value indicates a real, or significant effect. The significance of each variable was determined by applying the Student's *t*-test.

Analysis of p-value showed that among the tested variables, glucose, citric acid, ammonium chloride, glutamic acid, sodium chloride, $K_2HPO_4$, KCl, $CaCl_2$ and $MnSO_4 \cdot 7H_2O$ had significant effect on PGA production ($p<0.05$) (Table 2). Contrast coefficients allow the determination of the effect of each constituent. Positive contrast coefficient suggests a factor to have a large impact on titre, while a negative contrast coefficient indicates it to be less effective on the titre. The contrast coefficient of glutamic acid, $K_2HPO_4$ and $MnSO_4 \cdot 7H_2O$ was negative (Table 2). By considering both facts, the four most significant components (glucose, citric acid, sodium chloride and ammonium chloride) were selected for further optimization by RSM.

### *Optimization using RSM*

Based on the Plackett–Burman design, glucose (A), citric acid (B), sodium chloride (C) and ammonium chloride (D) were selected for further optimization by RSM. To examine the combined effect of these medium components (independent variables) on PGA production, a central composite factorial design of $2^4=16$ plus 6 centre points and ($2\times4=8$) star points leading to a total of 30 experiments was performed. A CCRD matrix of independent variables in form of coded and actual values along with responses of each experimental trial is given in Table 3. The results of the ANOVA test of the quadratic regression model indicate that the model is significant at $p<0.05$ (Table 4).

The p-values were used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. Along with the coefficient estimate, p-values are given in Table 4. The smaller the magnitude of p, the more significant the corresponding coefficient. Values of p lower than 0.05 indicate that model terms are significant. The coefficient estimates and the corresponding p-values suggest that among the test variables used in the study, A (glucose), B (citric acid), C (sodium chloride), $A^2$ (glucose squared), $B^2$ (citric acid squared), $C^2$ (sodium chloride squared) and $D^2$ (ammonium chloride squared) were significant model terms. Interactions between A and B, A and C, and B and D were also significant. The corresponding second-order response model that was found after the analysis of regression was:

$$\mathrm{PGA}/(\mathrm{g/L})=4.89-0.78\times A+1.51\times B-1.43\times C+0.12\times D+1.00\times A^2-0.87\times B^2-0.50\times C^2-0.65\times D^2+0.63\times A\times B-0.90\times A\times C-0.39\times A\times D-0.39\times B\times C+0.51\times B\times D+0.089\times C\times D \quad /4/$$

The fit of the model was also expressed by the coefficient of regression $R^2$, which was found to be 0.95, indicating that 95 % of the variability in the response (PGA yield) could be explained by the model.

Other parameters of ANOVA for response surface quadratic model were also studied. The Pred R-Squared of 0.80 was in reasonable agreement with the Adj R-Squared of 0.91. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable (22). Here, the ratio of 19.10 indicated an adequate signal.

The special features of the RSM tool, 'contour plot generation' and 'point prediction' were also studied to find optimum value of the combination of the four medium constituents for the maximum production of PGA. These predicted values were experimentally verified. It was observed that medium containing (in g/L): glucose 52.5, citric acid 15.5, sodium chloride 20 and ammonium chloride 4.75 yielded a maximum of (20.23±1.2) g/L of PGA.

Table 2. Assigned concentrations of variables at different levels in Plackett–Burman design and ANOVA of the results for production of PGA by *B. subtilis* R 23

| Factor | γ/(g/L) | | Mean square | Coefficient estimate | Standard error | *F*-value | p-value |
|---|---|---|---|---|---|---|---|
| | Lower | Higher | | | | | |
| Glucose ($X_1$) | 10.00 | 50.0 | 25.58 | 1.46 | 0.027 | 2997.56 | 0.0116 |
| Citric acid ($X_2$) | 4.00 | 16.0 | 6.54 | 0.74 | 0.027 | 766.60 | 0.0230 |
| Ammonium chloride ($X_3$) | 2.00 | 8.0 | 25.35 | 1.45 | 0.027 | 2970.25 | 0.0117 |
| Glutamic acid ($X_4$) | 5.00 | 20.0 | 1.83 | –0.39 | 0.027 | 213.89 | 0.0435 |
| $MgSO_4 \cdot 7H_2O$ ($X_5$) | 0.50 | 1.0 | 0.27 | 0.15 | 0.027 | 31.64 | 0.1120 |
| NaCl ($X_6$) | 10.00 | 40.0 | 9.76 | 0.90 | 0.027 | 1143.29 | 0.0188 |
| $K_2HPO_4$ ($X_7$) | 0.50 | 1.0 | 2.61 | –0.47 | 0.027 | 306.25 | 0.0363 |
| KCl ($X_8$) | 0.75 | 3.0 | 3.14 | 0.51 | 0.027 | 368.16 | 0.0331 |
| $CaCl_2$ ($X_9$) | 0.10 | 0.5 | 0.76 | –0.25 | 0.027 | 89.07 | 0.0672 |
| $MnSO_4 \cdot 7H_2O$ ($X_{10}$) | 0.05 | 0.2 | 8.94 | –0.86 | 0.027 | 1048.14 | 0.0197 |

Table 3. The CCRD matrix of independent variables in coded form and actual values with their corresponding response in terms of production of PGA by *B. subtilis* R 23

| Standard run | γ(glucose) g/L | γ(citric acid) g/L | γ(sodium chloride) g/L | γ(ammonium chloride) g/L | γ(PGA)[a]/(g/L) |
|---|---|---|---|---|---|
| 1 | –1.00 (25) | –1.00 (8) | –1.00 (20) | 1.00 (12) | 16.58±1.1 |
| 2 | 1.00 (50) | –1.00 (8) | –1.00 (20) | –1.00 (6) | 16.13±0.8 |
| 3 | –1.00 (25) | 1.00 (16) | –1.00 (20) | –1.00 (6) | 18.11±0.7 |
| 4 | 1.00 (50) | 1.00 (16) | –1.00 (20) | 1.00 (12) | 20.24±1.0 |
| 5 | –1.00 (25) | –1.00 (8) | 1.00 (40) | –1.00 (6) | 15.87±1.3 |
| 6 | 1.00 (50) | –1.00 (8) | 1.00 (40) | 1.00 (12) | 10.19±0.5 |
| 7 | –1.00 (25) | 1.00 (16) | 1.00 (40) | 1.00 (12) | 17.82±0.4 |
| 8 | 1.00 (50) | 1.00 (16) | 1.00 (40) | –1.00 (6) | 13.89±0.7 |
| 9 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 15.30±0.9 |
| 10 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 16.38±0.4 |
| 11 | –1.00 (25) | –1.00 (8) | –1.00 (20) | –1.00 (6) | 11.07±0.8 |
| 12 | 1.00 (50) | –1.00 (8) | –1.00 (20) | 1.00 (12) | 9.40±0.2 |
| 13 | –1.00 (25) | 1.00 (16) | –1.00 (20) | 1.00 (12) | 15.18±1.3 |
| 14 | 1.00 (50) | 1.00 (16) | –1.00 (20) | –1.00 (6) | 15.74±0.8 |
| 15 | –1.00 (25) | –1.00 (8) | 1.00 (40) | 1.00 (12) | 11.42±0.6 |
| 16 | 1.00 (50) | –1.00 (8) | 1.00 (40) | –1.00 (6) | 7.52±0.3 |
| 17 | –1.00 (25) | 1.00 (16) | 1.00 (40) | –1.00 (6) | 11.77±0.4 |
| 18 | 1.00 (50) | 1.00 (16) | 1.00 (40) | 1.00 (12) | 11.40±0.8 |
| 19 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 14.35±0.7 |
| 20 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 14.05±1.2 |
| 21 | –2.00 (12.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 20.20±1.5 |
| 22 | 2.00 (62.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 17.45±1.4 |
| 23 | 0.00 (37.5) | –2.00 (4) | 0.00 (30) | 0.00 (9) | 8.83±0.2 |
| 24 | 0.00 (37.5) | 2.00 (16) | 0.00 (30) | 0.00 (9) | 13.91±0.8 |
| 25 | 0.00 (37.5) | 0.00 (12) | –2.00 (10) | 0.00 (9) | 15.76±0.4 |
| 26 | 0.00 (37.5) | 0.00 (12) | 2.00 (50) | 0.00 (9) | 9.91±0.2 |
| 27 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | –2.00 (3) | 12.04±0.1 |
| 28 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | 2.00 (15) | 12.40±0.5 |
| 29 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 14.76±0.4 |
| 30 | 0.00 (37.5) | 0.00 (12) | 0.00 (30) | 0.00 (9) | 14.49±0.6 |

Values within parentheses indicate the actual values
[a]Results are mean±SD of at least three determinations

Table 4. Analysis of variance (ANOVA) for the experimental results of the central composite design (quadratic model)

| Factor | Coefficient estimate | Sum of squares | Standard error | *F*-value | Prob>*F* p-value |
|---|---|---|---|---|---|
| Model | 14.89 | 221.11 | 0.36 | 20.77 | <0.0001 |
| A | –0.78 | 14.80 | 0.18 | 19.40 | 0.0007 |
| B | 1.51 | 54.40 | 0.18 | 71.60 | <0.0001 |
| C | –1.43 | 48.90 | 0.18 | 64.40 | <0.0001 |
| D | 0.12 | 0.30 | 0.18 | 0.40 | 0.5174 |
| $A^2$ | 1.00 | 27.30 | 0.17 | 35.90 | <0.0001 |
| $B^2$ | –0.87 | 20.60 | 0.17 | 27.10 | 0.0002 |
| $C^2$ | –0.50 | 6.80 | 0.17 | 9.00 | 0.0103 |
| $D^2$ | –0.65 | 11.70 | 0.17 | 15.40 | 0.0018 |
| AB | 0.63 | 6.40 | 0.22 | 8.40 | 0.0125 |
| AC | –0.90 | 13.00 | 0.22 | 17.20 | 0.0012 |
| AD | –0.39 | 2.40 | 0.22 | 3.20 | 0.0966 |
| BC | –0.39 | 2.40 | 0.22 | 3.20 | 0.0985 |
| BD | 0.51 | 4.10 | 0.22 | 5.50 | 0.0362 |
| CD | 0.09 | 0.10 | 0.22 | 0.20 | 0.6890 |

A=glucose, B=citric acid, C=sodium chloride, D=ammonium chloride

Accordingly, three-dimensional graphs were generated for the pair-wise combination of the four factors, while keeping the other two at their centre point levels. Graphs for the three significant interactions are given here to highlight the roles played by these factors (Figs. 4a–c). From the central point of the contour plot the optimal process parameters were identified.

### *Effect of the addition of amino acids and TCA cycle intermediates*

Fig. 5 shows the effect of amino acids on PGA production. It was observed that none of the amino acids supported PGA production in *B. subtilis* R 23. Fig. 6 shows the effect of TCA cycle intermediates on PGA production. It was observed that α-ketoglutaric acid at 5 mM supported maximum PGA production of 25.21 g/L. Other TCA cycle intermediates did not support PGA production. These results are in accordance with the results reported for PGA production (*8*,*18*).

Biosynthesis of PGA in bacteria is carried out in two steps. In the first step synthesis of L- and D-glutamic acid takes place, whereas in the second step these L- and D-glutamic acid units are joined together to form PGA. Endogenous L-glutamic acid is formed from α-ketoglutaric acid in two different ways. In the absence of L-glutamine, the glutamate dehydrogenase pathway is used, in which L-glutamic acid is synthesized from α-ketoglutaric acid and ammonium ions, with the synthesis being catalyzed by glutamate dehydrogenase (*23*). In the presence of L-glutamine, another pathway involving glutamine synthetase and glutamine-2-oxoglutarate aminotransferase is activated, in which formation of L-glutamic acid from α-ketoglutaric acid and L-glutamine is catalyzed by glutamine-2-oxoglutarate aminotransferase, and regeneration of glutamine from L-glutamic acid and ammonium sulphate is catalyzed by glutamine synthetase (*1*). The D-glutamic acid is presumed to be produced from L-glutamic acid through the indirect conversion

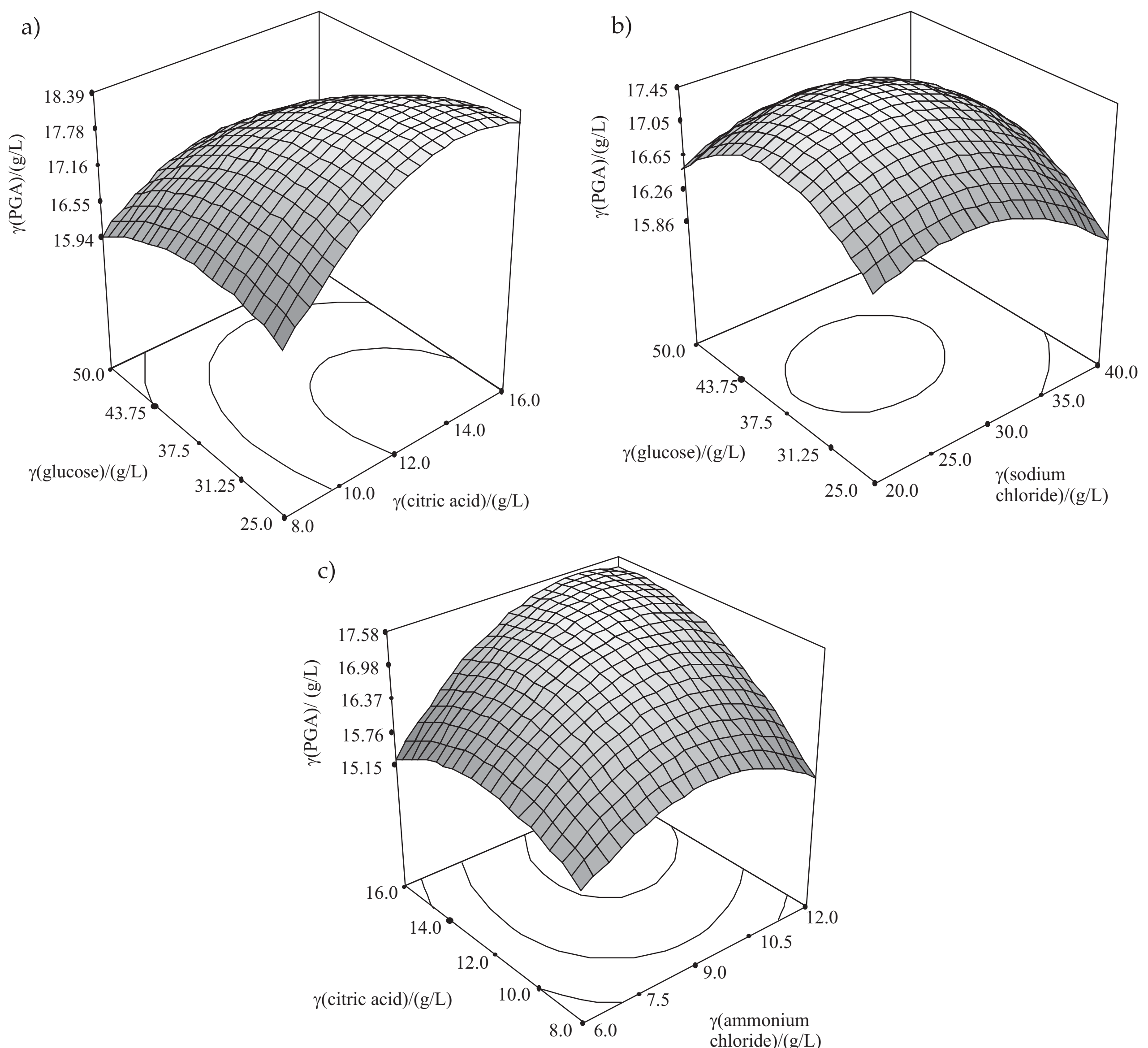


**Fig. 4.** Contour plot for PGA production: a) effect of glucose and citric acid, b) effect of glucose and sodium chloride, and c) effect of ammonium chloride and citric acid

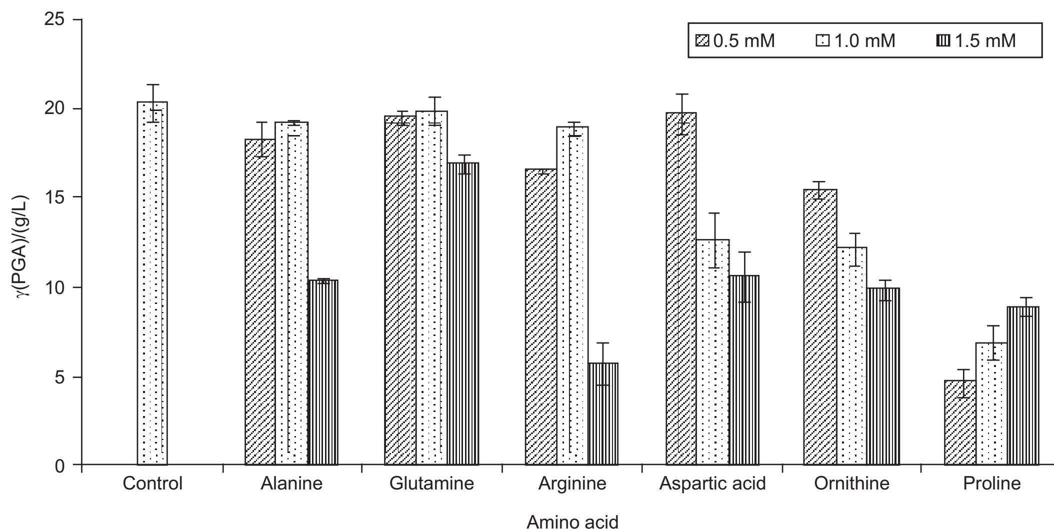


**Fig. 5.** Effect of amino acids on PGA production by *Bacillus subtilis* R 23

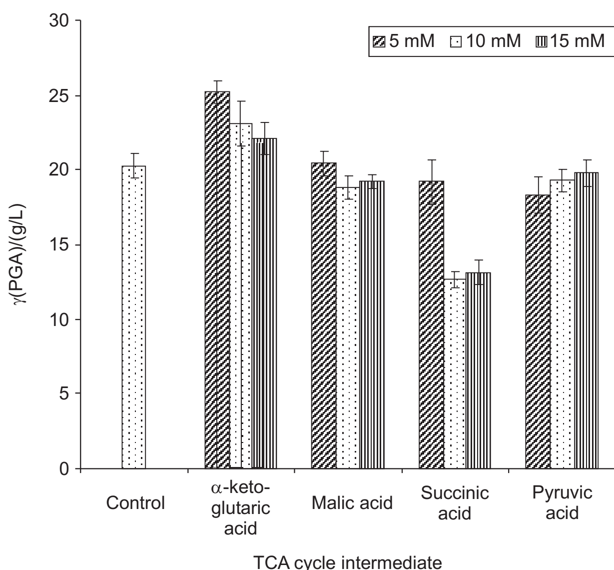


**Fig. 6.** Effect of TCA cycle intermediates on PGA production by *Bacillus subtilis* R 23

mechanism. In the first step, L-alanine is formed by transamination between pyruvic acid and L-glutamic acid. L-alanine thus formed is then converted into D-alanine by alanine racemase in the second step. In the third step, D-amino acid aminotransferase catalyses transamination between α-ketoglutaric acid and D-alanine to produce D-glutamic acid and pyruvic acid (*24,25*). D- and L-glutamic acids are converted into PGA by using PGA synthatase complex (*26*).

It is also presumed that L-glutamic acid is produced from citric acid through isocitric and α-ketoglutaric acids in the tricarboxylic acid (TCA) cycle, and PGA is polymerized from this glutamic acid (*27*). Kambourova *et al.* (*28*) suggested α-ketoglutaric acid as the direct precursor for glutamate and PGA synthesis.

Results obtained in the present study suggest that endogenous L-glutamic acid synthesis in *B. subtilis* R 23 may be carried out by glutamate dehydrogenase pathway. Pathway involving glutamine synthetase and glutamine-2-oxoglutarate aminotransferase may not be effective for PGA production in *B. subtilis* R 23 as the yield of PGA did not increase after the addition of L-glutamine to the medium. Fig. 7 shows the profile of PGA production using the optimized medium. The maximum PGA production (25.38 g/L) was obtained after 48 h, after which the yield decreased. This could be attributed to the depletion of important medium constituents like glutamic acid, citric acid and glucose.

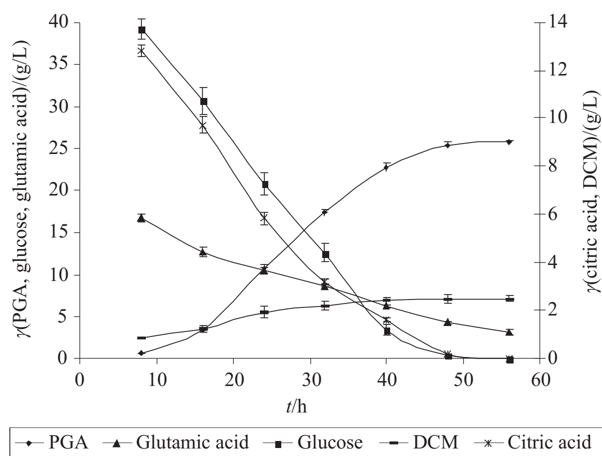


**Fig. 7.** Production profile of PGA by *B. subtilis* R 23 in the optimized medium

Since PGA is an extracellular, high molecular mass polymer, the culture medium becomes highly viscous with the progress of polymer production. This increased viscosity is likely to decrease the volumetric oxygen mass transfer, leading to oxygen limitation. Birrer *et al.* (*29*) reported the presence of 2,3-butanediol in cultures during fermentative production of PGA, indicating that the levels of oxygen in the medium were unsupportive of a fully aerobic mode of metabolism. The time-course of dry cell mass (DCM) in Fig. 7 also suggests oxygen limitation during fermentative production of PGA using

*B. subtilis* R 23. However, Richard and Margaritis (*30*) did not find oxygen limitation to influence PGA production. According to these researchers, peak oxygen demand for PGA formation occurs during the early exponential growth phase but before PGA appears in the culture broth. As the broth viscosity and PGA concentration reach their maximum values during the late exponential growth phase and early stationary phase, very little oxygen is required thereafter. A decrease in production of PGA in the late stationary phase is mainly due to the decrease in mass transfer, attributable to high viscosity.

The PGA yield (25.38 g/L) with productivity of 0.53 g/(L·h) in the present study is quite high compared to the reports until now. The maximum PGA production reported until now by the most widely used strain *B. lichenifomis* ATCC 9945A is 23 g/L with productivity of 0.24 g/(L·h) (*21*). *B. subtilis* IFO 3335, which is also extensively studied for PGA production, could produce a maximum of 20 g/L of PGA with productivity of 0.4 g/(L·h) (*31*). PGA production reported from *B. subtilis* (*chungkookjang*), isolated from the traditional Korean seasoning, is 15.6 g/L with productivity of 0.21 g/(L·h) (*32*). Maximum PGA production reported by Shih *et al*. (*20*) with *B. lichenifomis* CCRC 12826 was 19.62 g/L with productivity of 0.20 g/(L·h). PGA yield obtained by using a mutated strain of *Bacillus licheniformis* CICC10099 was 16.9 g/L with productivity of 0.21 g/(L·h) (*33*).

### *PGA characterization*

#### Molecular mass determination

The molecular mass of PGA obtained from *B. subtilis* R 23 was determined by gel permeation chromatography (GPC) and found to be over $2.1 \cdot 10^6$ Da. It is known that the molecular mass of PGA varies from $10^5$ to $10^6$ Da depending on the species and the cultivation conditions used for its production (*1*). Environmental parameters and medium components used during fermentation have also been indicated to affect it. Under the conditions used in this study, the molecular mass of PGA produced by *B. subtilis* R 23 is comparable to the results obtained so far with other species. Whether the above factors affect the molecular mass of PGA produced by *B. subtilis* R 23 is yet to be determined.

#### Amino acid analysis

Thin layer chromatography (TLC) was used to characterize the amino acids in the PGA obtained from *B. subtilis* R 23. Glutamic acid was the sole component of the 6 M HCl hydrolysate of the purified material. TLC of the hydrolysate performed on a cellulose plate and visualized with 0.2 % ninhydrin indicated a single spot with $R_f$ value of 0.33, which was identical to that of authentic glutamic acid.

### *Sugar content*

Polysaccharide content in the purified PGA was tested by the phenol–sulphuric acid method. No sugar was detected in PGA, indicating that under the optimized fermentation conditions, *B. subtilis* R 23 did not produce polysaccharides as a by-product.

## Conclusions

By screening of various marine strains for PGA production, a good producer was identified as belonging to *Bacillus subtilis* sp. by 16S rRNA study, and named *B. subtilis* R 23. Sequential optimization of PGA production from *B. subtilis* R 23 was carried out. Plackett–Burman design was used to determine the effect of medium components on PGA production by *B. subtilis* R 23. Further optimization of the most significant factors by RSM showed complex nutrient interactions among them, and also increased the production of PGA from 7.64 to 25.38 g/L. Results of this study clearly indicate that optimization by sequential optimization approach is an effective way of optimizing PGA production.

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