J Ind Microbiol Biotechnol (2008) 35:1581–1586
DOI 10.1007/s10295-008-0401-2

ORIGINAL PAPER

Enhanced production of poly (γ-glutamic acid) from *Bacillus licheniformis* NCIM 2324 in solid state fermentation

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Received: 25 March 2008 / Accepted: 9 July 2008 / Published online: 25 July 2008

Abstract This work reports on the optimization of PGA production by *Bacillus licheniformis* NCIM 2324 in solid state fermentation (SSF). In the first step, the one factor-at-a-time method was used to investigate the effect of solid substrates, initial moisture content, pH, and additional carbon and nitrogen source on PGA production; subsequently, response surface methodology (RSM) was used to establish the optimum concentrations of the key nutrients for higher PGA production. In the second step, the effects of amino acids and TCA cycle intermediates on the production of PGA were studied. The final optimized medium gave a maximum yield of 98.64 ± 1.61 mg gds^{-1} of PGA, which is significantly higher than that reported in the literature.

Keywords Poly (γ-glutamic acid) · *Bacillus licheniformis* · Optimization · Solid-state fermentation · Response surface methodology

Introduction

Poly (γ-glutamic acid) (PGA), extracellular polymer produced as a slime layer of certain *Bacillus* species 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 [1, 2]. It is biodegradable, edible and non-toxic towards human and environment and hence has been suggested to be a good candidate for various applications [3–7].

Strategies for the production of PGA in submerged fermentation (SmF) have been studied by several researchers [2], but literature reports on the production of PGA in SSF are very scanty. Solid substrates like soybean cake powder, wheat bran, dairy manure, swine manure and sweet potato residues alone or in combination have been used for production of PGA. Xu et al. [8] investigated and optimized the nutritional and environmental conditions for SSF of PGA from *Bacillus subtilis* CCTCC 202048 and achieved 83.61 mg g^{-1} of dry substrate of PGA. Chen et al. [9, 10] studied production of PGA in swine manure and dairy manure and found lower yields of PGA as compared to the yield reported by Xu et al. [8]. No report is available on use of amino acids and TCA cycle intermediates as stimulator of PGA synthesis from *Bacillus licheniformis* in SSF.

This paper addresses the optimization of production of PGA from *Bacillus licheniformis* NCIM 2324 in SSF.

Materials and methods

Materials

All the chemicals used in present study were of the AR grade and were purchased from Hi-Media Limited, Mumbai, India. Dairy manure, soybean meal, wheat bran, coconut oil cake, olive oil cake, palm seed oil cake and oatmeal were collected from local market. Cottonseed meal (CSM) was a gift sample from Central Institute for Research on Cotton Technology (CIRCOT), Matunga, Mumbai. PGA was generously provided by Vedan Enterprise Corporation, Taiwan.

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Bacterial strain and inoculum preparation

Bacillus licheniformis NCIM 2324 was used in the present study. The growth and maintenance medium contained (g l^{-1}) peptone 5, yeast extract 1.5, beef extract 1.5, sodium chloride 5, and agar 20 (pH 7 ± 0.2). Bacterial cells in agar slants were incubated at 37 °C for 24 h and stored at 4 °C. A loopful of cells from a slant was transferred to 20 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. It was containing approximately 3×10^7 cells/ml.

Fermentation

Five gram of solid substrate was placed in 250 ml Erlenmeyer flasks and appropriate amount of supplement solution (5.0% L-glutamic acid, 2.5% citric acid, 0.3% $MgSO_4 \cdot 7H_2O$, 0.2% $MnSO_4 \cdot 2H_2O$) was added to produce moisture content of 65%. The flasks were then autoclaved at 121 °C, 15 lbs for 20 min. Inoculum (1 ml) was added and incubated at 37 ± 2 °C for 72 h. All the experiments were carried out at least in triplicate.

Optimization of fermentation medium using one factor-at-a-time method

Different solid substrates listed were checked for their suitability for PGA production. The effect of initial moisture content (50, 55, 60, 65, 70 or 75%) on PGA was investigated. Effect of pH on supplement solution on PGA production was evaluated by carrying out the fermentation at initial pH of 5–8.

Different carbon sources such as glucose, lactose, maltose, glycerol, sucrose, soluble starch, and galactose were added into the solid substrate separately to a final concentration of 1.0% and tested for their effect on the yield of PGA. Different nitrogen sources such as yeast extract, peptone, ammonium sulphate, ammonium chloride, sodium nitrate, ammonium nitrate and potassium nitrate were added separately at 0.5% and evaluated for PGA production.

Optimization of media by 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. The experiments were designed using the software, Design Expert Version 6.0.10 trial version (State Ease, Minneapolis, MN).

The medium components selected were glycerol, ammonium sulphate, L-glutamic acid and citric acid. 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 dependent variable. Response surface plots were also obtained using Design Expert Version 6.0.10.

Effect of amino acids on PGA production

The amino acids of glutamic acid family (L-glutamine, L-arginine, L-ornithine and L-proline) and amino acid involved in biosynthetic pathway of PGA (L-alanine and L-aspartic acid) were added individually at 0.05, 0.1 and 0.5% in the RSM optimized medium, and evaluated for their effect on PGA production.

Effect of TCA cycle intermediates on PGA production

TCA cycle intermediates, viz, α-keto glutaric acid, malic acid, succinic acid and pyruvic acid were added individually at 0.25, 0.5, and 1.0% in RSM optimized medium, and evaluated for their effect on PGA production.

Extraction of PGA

After termination of fermentation, a weighed quantity of fresh fermented substrate was entirely transferred to 500 ml conical flasks, 10 volumes of distilled water was added (w/v, based on initial dry weight of the substrate), mixed at room temperature (28 ± 2 °C) on a rotary shaker (200 rpm) for 2 h and then filtered through two-layer muslin cloth. The extracts so obtained were centrifuged at 10,000 rpm for 15 min. Clarified supernatants were collected for PGA determination.

Purification of PGA

PGA was purified by the method reported by Goto and Kuniko [11]. Ten millilitre of supernatant obtained by above method was poured in to four volumes of methanol and kept for 12 h at 4 °C. Crude PGA was collected by centrifugation for 30 min at 10,000 rpm and 4 °C, dissolved in distilled water and any insoluble impurity, if found, was removed by centrifugation. The aqueous PGA solution was desalted by dialysis (molecular weight cutoff 3,500) against 1 l of distilled water for 12 h with three water exchange, and finally lyophilized to prepare pure PGA.

Analytical methods

Determination of PGA

PGA concentration was determined by the method reported by Chen et al. [9]. 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 a 0.1 mM sodium chloride at a flow rate 1 ml/min and detected at 220 nm. The purified PGA was used as a standard.

Plate count of bacteria

Plate count of bacteria was carried out by the method described by Chen et al. [13]. The weighed quantity of fermented matter was adequately mixed with 10 volumes of sterilized water (w/v, based on initial dry weight of the substrate). The mixture was aseptically diluted to suitable concentration by serial dilution. A 0.1 ml of diluted mixture was spread on nutrient agar plates. The average colony was counted after incubating for 24 h at 37 °C. The number of CFU gds^{-1} (1 CFU = 1 colony forming unit) was calculated according to the dilution factor.

Characterization of PGA

Amino acid analysis

The PGA obtained from *B. licheniformis* NCIM 2324 was purified and hydrolyzed with 6 N HCl at 110 °C for 24 h in a sealed and evacuated tube and used for amino acid analysis. Thin-layer chromatography was performed on a cellulose plate (Merck) with solvent systems of butanol–acetic acid–water (3:1:1, w/w) and 96% ethanol–water (63:37, w/w). Amino acids were detected by spraying with 0.2% ninhydrin in acetone [7].

Total sugar content

The total carbohydrate content of the PGA produced by *B. licheniformis* NCIM 2324 was determined by the phenol–sulphuric acid method [12].

Results and discussion

Optimization using one factor-at-a-time method

Soybean meal supported maximum PGA production of 27.81 ± 1.10 mg g^{-1} of dried substrate (gds, i.e., gram of dried fermented matter) among all substrates used. Hence, for further study, soybean meal was used as the substrate. Highest production of PGA was observed at an initial moisture content of 60% and at an initial pH of 6.5. Among the different carbon and nitrogen sources supplemented, glycerol and ammonium sulphate supported maximum PGA production of 48.95 ± 1.15 mg gds^{-1} and 58.23 ± 1.24 mg gds^{-1}, respectively.

The results of optimization of PGA production using one factor-at-a-time in the present study are in agreement with those already know in the published literature [8–10, 13, 14].

Optimization by RSM

A CCRD matrix of independent variables in form of coded and actual values along with responses of each experimental trial is given in Table 1. The results were analyzed by Design Expert Version 6.0.10 (Table 2). The ANOVA of the quadratic regression model indicated the model to be significant ($P < 0.05$).

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. The coefficient estimates and the corresponding P values (Table 2) suggest A (glycerol), B (ammonium sulphate), C (L-glutamic acid), A^2 (Glycerol^2), B^2 (ammonium sulphate^2), C^2 (L-glutamic acid ^{2}) and D^2 (citric acid^2) to be significant model terms. Interactions between A (glycerol) and B (ammonium sulphate), A (glycerol) and D (citric acid); and B (ammonium sulphate) and C (L-glutamic acid) were significant.

The corresponding second-order response model found after analysis for the regression was PGA (mg gds^{-1}) = 75.15390 + 5.12846 × glycerol + 7.65719 × ammonium sulphate + 1.52160 × L-glutamic acid − 1.75079 × citric acid − 12.37254 × glycerol^2 − 11.89229 × ammonium sulphate^2 − 8.10535 × L-glutamic acid^2 − 7.10360 × citric acid^2 − 5.04591 × glycerol × ammonium sulphate + 0.71875 × glycerol × L-glutamic acid + 9.53844 × glycerol × citric acid − 4.62014 × ammonium sulphate × L-glutamic acid + 1.26527 × ammonium sulphate × citric acid − 0.62986 × L-glutamic acid × citric acid.

It was observed that medium containing (%) glycerol 2.0, ammonium sulphate 1.1, L-glutamic acid 5.4 and citric acid 2.5 yielded a maximum PGA of 76.18 ± 1.2 mg gds^{-1}.

Effect of amino acids and TCA cycle intermediates on PGA production

Figure 1 shows the effect of amino acids on PGA production. It was observed that among the different amino acid evaluated, L-glutamine at 0.05% supported maximum PGA production of 84.40 ± 1.2 mg gds^{-1}. At L-glutamine concentration below 0.05%, the yield of PGA was lower. PGA yields also decreased with increasing concentrations of L-glutamine. Effect of amino acid on PGA production in SSF has not yet been studied by any investigators. However, in SmF, Kunioka [15] observed higher yield of PGA without any by-products by using L-glutamine instead L-glutamic acid. Maximum PGA yield was obtained by using 0.1 g/l of

Table 1 The CCRD matrix of independent variables in coded form and actual values with their corresponding response in terms of production of PGA by *B. licheniformis* NCIM 2324

Standard run	Glycerol (%)	Ammonium sulphate (%)	L-Glutamic acid (%)	Citric acid (%)	PGA[a] (mg gds^{-1})
1	1.00 (2.0)	1.00 (1.5)	1.00 (6.0)	−1.00 (1.0)	31.77 ± 1.2
2	1.00 (2.0)	1.00 (1.5)	−1.00 (2.0)	−1.00 (1.0)	36.02 ± 0.6
3	1.00 (2.0)	−1.00 (0.5)	1.00 (6.0)	1.00 (3.0)	50.11 ± 1.1
4	−1.00 (1.0)	1.00 (1.5)	−1.00 (2.0)	1.00 (3.0)	37.58 ± 1.8
5	1.00 (2.0)	−1.00 (0.5)	−1.00 (2.0)	1.00 (3.0)	38.39 ± 1.5
6	−1.00 (1.0)	−1.00 (0.5)	1.00 (6.0)	−1.00 (1.0)	35.61 ± 0.8
7	−1.00 (1.0)	1.00 (1.5)	1.00 (6.0)	1.00 (3.0)	27.94 ± 0.7
8	−1.00 (1.0)	−1.00 (0.5)	−1.00 (2.0)	−1.00 (1.0)	24.25 ± 1.6
9	−1.68 (0.66)	0.00 (1.0)	0.00 (4.0)	0.00 (2.0)	32.19 ± 0.4
10	1.68 (2.34)	0.00 (1.0)	0.00 (4.0)	0.00 (2.0)	49.44 ± 1.4
11	0.00 (1.5)	−1.68 (0.16)	0.00 (4.0)	0.00 (2.0)	29.30 ± 1.3
12	0.00 (1.5)	1.68 (0.84)	0.00 (4.0)	0.00 (2.0)	55.05 ± 0.6
13	0.00 (1.5)	0.00 (1.0)	−1.68 (0.64)	0.00 (2.0)	49.44 ± 2.1
14	0.00 (1.5)	0.00 (1.0)	1.68 (7.36)	0.00 (2.0)	56.33 ± 0.3
15	0.00 (1.5)	0.00 (1.0)	0.00 (4.0)	−1.68 (0.32)	58.66 ± 1.7
16	0.00 (1.5)	0.00 (1.0)	0.00 (4.0)	1.68 (3.68)	52.77 ± 1.0
17	0.00 (1.5)	0.00 (1.0)	0.00 (4.0)	0.00 (2.0)	75.22 ± 2.3
18	0.00 (1.5)	0.00 (1.0)	0.00 (4.0)	0.00 (2.0)	73.33 ± 1.5
19	0.00 (1.5)	0.00 (1.0)	0.00 (4.0)	0.00 (2.0)	74.44 ± 1.3
20	0.00 (1.5)	0.00 (1.0)	0.00 (4.0)	0.00 (2.0)	75.88 ± 1.2
21	0.00 (1.5)	0.00 (1.0)	0.00 (4.0)	0.00 (2.0)	73.33 ± 0.9

Values within parenthesis indicate the actual values

[a] Results are mean of three determinations

Table 2 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	75.15	6139.28	0.77	137.09	<0.0001
A	5.13	148.78	0.63	46.51	0.0005
B	7.66	331.68	0.75	103.69	<0.0001
C	1.52	31.62	0.75	9.88	0.0200
D	−1.75	17.34	0.48	5.42	0.0588
A2	−12.37	2287.63	0.75	715.14	<0.0001
B2	−11.89	2113.49	0.46	660.70	<0.0001
C2	−8.11	981.78	0.46	306.91	<0.0001
D2	−7.10	754.10	0.46	235.74	<0.0001
AB	−5.05	84.37	0.98	26.38	0.0021
AC	0.72	4.13	0.63	1.29	0.2990
AD	9.54	301.49	0.98	94.25	<0.0001
BC	−4.62	170.77	0.63	53.38	0.0003
BD	1.27	5.30	0.98	1.66	0.2453
CD	−0.63	3.17	0.63	0.99	0.3577

A Glycerol, *B* ammonium sulphate, *C* L-glutamic acid, *D* citric acid

L-glutamine, and yield was found to decrease on either side of this concentration [15].

Among the TCA cycle intermediates, α-ketoglutaric acid at 0.5% supported maximum PGA production of 90.98 ± 2.5 mg gds^{-1} (Fig. 2). Other TCA cycle intermediates did not support PGA prediction. Effect of addition of α-ketoglutaric acid on PGA production in SSF has not been looked into by any workers, either in SSF or in SmF.

The yield of PGA in a medium containing both, L-glutamine (0.05%) and α-ketoglutaric acid (0.5%) was higher at 98.64 ± 1.61 mg gds^{-1} than that of medium containing either L-glutamine or α-ketoglutaric acid. The yield of PGA obtained in this study is significantly higher as compared to the values reported in the literature.

Biosynthesis of PGA in bacteria is carried out in two steps. In first step, synthesis of L- and D-glutamic acid takes

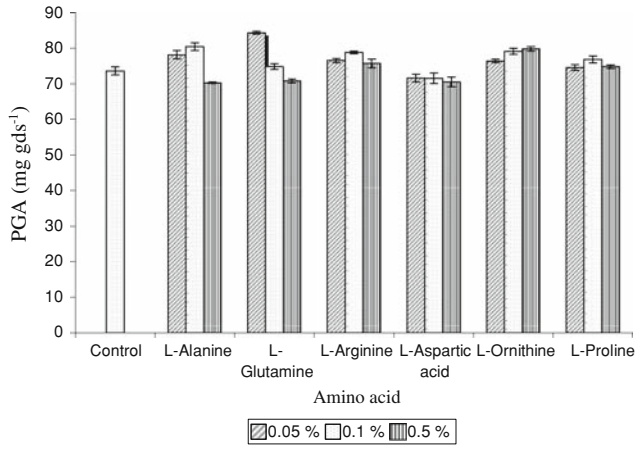

Fig. 1 Effect of amino acids on PGA production in SSF by *Bacillus licheniformis* NCIM 2324

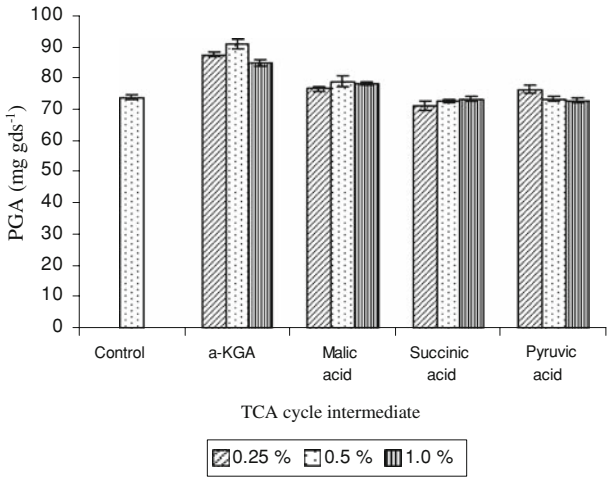

Fig. 2 Effect of TCA cycle intermediates on PGA production in SSF by *Bacillus licheniformis* NCIM 2324

place, whereas in the second step these D- and L-glutamic acid units are joined together to form PGA [16].

Endogenous L-glutamic acid is formed from α-ketoglutaric acid in two different ways. In the absence of glutamine, the glutamate dehydrogenase (GD) pathway is used, in which L-glutamic acid is synthesized from α-ketoglutaric acid and ammonium sulphate, this synthesis is catalyzed by GD [17]. In the presence of L-glutamine, another pathway involving glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT) is activated, in which formation of L-glutamic acid from α-ketoglutaric acid and L-glutamine is catalyzed by GOGAT and regeneration of glutamine from L-glutamic acid and ammonium sulphate is catalyzed by GS [2].

D-glutamic acid is presumably to be produced form L-glutamic acid through the indirect conversion mechanism.

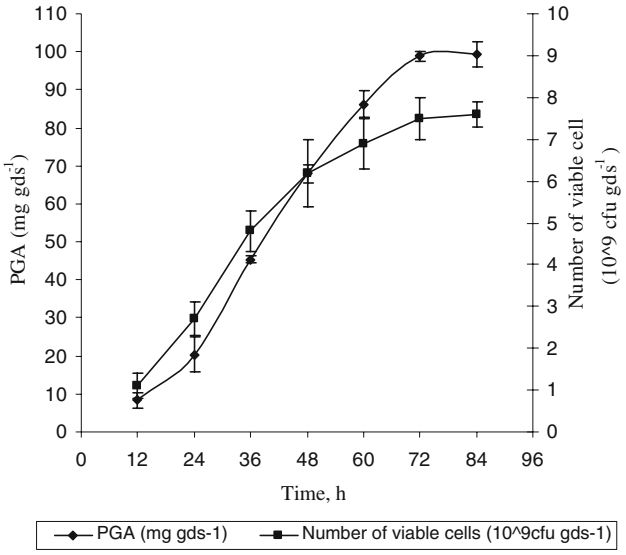

Fig. 3 Production profile of PGA in SSF by *B. licheniformis* NCIM 2324 on the optimized medium

Initially, L-alanine is formed by transamination between pyruvic acid and L-glutamic acid. L-alanine so formed is then converted to D-alanine by alanine racemase. D-amino acid aminotransferase (DAT) catalyses the transamination between α-ketoglutaric acid and D-alanine to produce D-glutamic acid and pyruvic acid [18, 19]. D- and L-glutamic acid is converted in to PGA in *B. licheniformis* by using PGA synthetase complex [20, 21].

Results obtained in present study suggests that endogenous L-glutamic acid synthesis in *B. licheniformis* NCIM 2324 may be carried out by both the GD as well as GOGAT pathway, depending on the medium composition. The GOGAT pathway is more effective for PGA production in *B. licheniformis* NCIM 2324 as the yield of PGA increased dramatically on addition of L-glutamine and α-ketoglutaric acid to the medium.

Figure 3 shows the profile of PGA production by *B. licheniformis* NCIM 2324 in SSF by using the optimized medium. The PGA production and the number of viable cells increased rapidly after 12 h, reached a maximum at 72 h and remained constant, thereafter. These results suggested that PGA production by *B. licheniformis* NCIM 2324 was associated partially with cell growth.

Characterization of PGA

Amino acid analysis

The 6 N HCl hydrolysate of the purified material was composed solely of glutamic acid as a single spot with R_f value identical to that of authentic glutamic acid was observed on cellulose thin layer plate.

Sugar content

Evaluation of purified PGA by the phenol–sulphuric acid method for polysaccharide content did not detect any sugar.

Conclusion

Soybean meal was found to be the best substrate for the production of PGA by *B. licheniformis* NCIM 2324 using SSF with initial moisture content having a significant effect. Supplementation of soybean meal with additional carbon and nitrogen sources was beneficial for PGA production. RSM could successfully optimize the concentrations of selected medium components for improving the yield of PGA. Supplementation of the fermentation medium with L-glutamine and α-ketoglutaric acid increased the PGA production in SSF. The PGA so produced was devoid of any polysaccharides as a by-product.

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