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Enhanced Production of Poly (γ-glutamic acid) from *Bacillus licheniformis* NCIM 2324 by Using Metabolic Precursors

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Abstract The aim of the present work was to study the effect of addition of different amino acids and tricarboxylic acid cycle intermediates as metabolic precursors on the production of poly (γ-glutamic acid) (PGA) by *Bacillus licheniformis* NCIM 2324. A maximum yield of 35.75 g/l was obtained with the medium supplemented with 0.5 mM L-glutamine and 10 mM α-ketoglutaric acid as compared to 26.12 g/l PGA achieved with the control in the absence of metabolic precursors. Addition of precursors also enhanced the utilization of L-glutamic acid during fermentation.

Keywords Poly (γ-glutamic acid) · Fermentation · *Bacillus licheniformis* · Precursors · α-Ketoglutaric acid

Introduction

Poly (γ-glutamic acid) (PGA) is an extracellular polymer produced as a slime layer of certain *Bacillus* species [1]. It 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 non-toxic towards human and environment. Hence, it has been suggested to be a good candidate for various applications including thickener, bitterness-relieving agent, cryoprotectant, sustained release material, drug carrier, curable biological adhesive, biodegradable fibers, highly water absorbable hydrogels, biopolymer flocculants, and heavy metal absorber [2].

A schematic pathway for the synthesis of PGA from L-glutamic acid, citric acid, and ammonium ions which is entirely based on a possible mechanism of PGA synthesis in *B. licheniformis* was proposed by Kunioka [3]. Biosynthesis of PGA in bacteria is carried out

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in two steps. The synthesis of L- and D-glutamic acid takes place in the first step, whereas in the second step these D- and L-glutamic acid units are joined together to form PGA.

It is presumed that L-glutamic acid is produced from citric acid through isocitric acid and α-ketoglutaric acid in the tricarboxylic acid cycle (TCA), and PGA is polymerized from this glutamic acid. A large amount of PGA is thus produced from citric acid and ammonium sulfate [3]. Two different pathways for formation of L-glutamic acid are reported. 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 sulfate, with the synthesis being catalyzed by GD [4]. 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 sulfate is catalyzed by GS [2].

The D-glutamic acid is presumed to be produced from L-glutamic acid through the indirect conversion mechanism in three steps. In the first step, L-alanine is formed by transamination between pyruvic acid and L-glutamic acid. L-Alanine so formed is then converted into D-alanine by alanine racemase in the second step. Alanine racemase is a pyridoxal 5′-phosphate (PLP)-containing enzyme, ubiquitous among most bacteria, and absent in humans. A lysine residue connected to the PLP cofactor by an internal aldimine bond acts as a base for the conversion of D-alanine to L-alanine while a nearby tyrosine from the second monomer acts as a base for the abstraction of a hydrogen from L-alanine [5]. According to the generally accepted mechanism, alanine racemase reaction is proposed to proceed in four steps: (1) transaldimination between Lys^{39} bound with PLP (I) and the α-amino group of L-alanine to produce an external aldimine II; (2) abstraction of the α-hydrogen from L-alanine to produce a resonance-stabilized quinonoid intermediate III; (3) reprotonation at the α-carbon of the quinonoid intermediate III on the side opposite to that where the α-hydrogen was abstracted; (4) the second transaldimination between IV and Lys^{39} to release D-alanine [6]. In the third step, D-amino acid aminotransferase (DAT) catalyzes transamination between α-ketoglutaric acid and D-alanine to produce D-glutamic acid and pyruvic acid [7–9]. D- and L-glutamic acid is converted into PGA in *B. licheniformis* by using PGA synthetase complex [10, 11].

Another pathway for PGA synthesis, including the direct conversion of L-glutamic acid to the D-isomer, has also been suggested [12]. The proposed reaction mechanism is divided into four steps. (1) L-glutamic acid is first activated by ATP to form L-glutamyl-γ-adenylate; (2) this activated L-glutamic acid is bound to catalytically essential sulfhydryl group in enzyme (or in acceptor) and isomerized into D-isomer; (3) the γ-D-glutamyl moiety was transferred to a growing poly-γ-D-glutamyl bound to another sulfhydryl group of the synthetase (or an acceptor); (4) the eventually elongated γ-D-PGA chain was transferred to NH_2 terminus of the elongated γ-D-PGA acceptor.

There is very little information available on the use of amino acids and TCA cycle intermediates as stimulators of PGA synthesis from *Bacillus licheniformis*. Kunioka [13] used L-glutamine instead of L-glutamic acid for production of PGA from *B. subtilis* IFO 3335. Higher yield of PGA without any by-products was observed in the L-glutamine/citric acid medium than in the L-glutamic acid/citric acid medium. Various intermediates of TCA cycle such as succinic acid, fumaric acid, and L-malic acid have been evaluated as carbon sources for PGA production [14, 15]. However, reports on study of TCA intermediates as stimulator of PGA synthesis are not available. There is no report in the literature on use of α-ketoglutaric acid, one of the TCA cycle intermediates, for improvement of PGA

production in *B. licheniformis*. The effect of α-ketoglutaric acid on the utilization and catabolism of amino acids by strains of nonstarter lactobacilli has been studied by Williams et al. [16], who showed that conversion of amino acid into cheese aroma and flavor compounds by nonstarter lactobacilli is enhanced in the presence of α-ketoglutaric acid. Tavaria et al. [17] also reported α-ketoglutaric acid to promote the uptake of amino acids by cheese lactobacilli.

This paper reports on the effect of addition of various amino acids and TCA cycle intermediates, alone and in combination on PGA production. The effect of addition of these precursors on the molecular weight of PGA was also evaluated.

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. HPLC grade sodium chloride was purchased from S.D. Fine Chemicals Limited, Mumbai, India. PGA was generously provided by Vedan Enterprise Corporation, Taiwan.

Bacterial Strain and Medium

A bacterial strain of *Bacillus licheniformis* NCIM 2324 was used in the present study. The medium used for the growth and maintenance contained (g/l) 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 48 h and stored at 4 °C.

For the production of PGA, a medium reported by Bajaj et al. [18] was used, which contained (g/l) glycerol, 62.4; citric acid, 15.2; L-glutamic acid, 20; ammonium sulfate, 8.0; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.2; $MnSO_4 \cdot 7H_2O$, 0.05. Initial pH of the medium was adjusted to 6.5 by using 2 N NaOH and/or 2 N HCl. The medium was sterilized in an autoclave for 15 min at 121 °C.

Inoculum and Fermentation

A loopful of cells from a slant was transferred to 20 ml of the seed medium in a 100-ml conical flask and incubated at 37 °C and 200 rpm for 16 h. This was 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% (*v*/*v*) of 16-h-old *B. licheniformis* culture containing approximately 3×10^7 cells/ml. The flasks were incubated for 96 h on a rotary shaker at 37±2 °C and 200 rpm. All the experiments were carried out at least in triplicate.

Effect of Addition of Amino Acids on PGA Production

To study the effect of 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 mM, 1 mM, and 1.5 mM in the production medium.

Effect of TCA Cycle Intermediated on PGA Production

To study the effect of addition of different TCA cycle intermediates on PGA production, α-ketoglutaric acid, malic acid, succinic acid, or pyruvic acid was added individually at 5.0, 10, and 1.5 mM in the production medium.

Effect of Addition of Combination of L-Glutamine and α-Ketoglutaric Acid

The effect of addition of combination of L-glutamine and α-ketoglutaric acid at various concentrations on the PGA production was studied by supplementing the fermentation medium with L-glutamine (0.25, 0.5, and 0.75 mM) in combination with α-ketoglutaric acid (7.5, 10, and 12.5 mM) (Table 1). The effect of addition of L-glutamine and α-ketoglutaric acid at various stages of fermentation was also studied.

Effect of L-Glutamine and α-Ketoglutaric Acid Addition on Molecular Weight of PGA

Molecular weight of the PGA sample isolated after 96 h fermentation was determined by using gel permeation chromatography. The effect of addition of L-glutamine and α-ketoglutaric acid on molecular weight of PGA was studied.

Isolation of PGA

PGA was purified by the method reported by Goto and Kunioka [19]. Culture broth was appropriately diluted and cells were separated from broth by centrifugation for 20 min at 10,000 rpm and 4 °C. The supernatant containing PGA was poured into 4 vol 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, then dissolved in distilled water and any insoluble impurity 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 exchanges, and finally lyophilized to prepare pure PGA.

Analytical Methods

PGA concentration was determined by the method of Chen et al. [20]. 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

Table 1 Effect of addition of combination of L-glutamine and α-ketoglutaric acid on the PGA production by *Bacillus licheniformis* NCIM 2324.

Run	L-Glutamine (mM)	α-Ketoglutaric acid (mM)	PGA[a] (g/l)
1	0.25	7.50	31.14±1.08
2	0.25	10.0	32.51±0.84
3	0.25	12.5	32.84±0.72
4	0.50	7.50	34.35±1.31
5	0.50	10.0	35.75±0.78
6	0.50	12.5	35.52±0.82
7	0.75	7.50	32.91±0.73
8	0.75	10.0	32.64±1.41
9	0.75	12.5	35.83±1.18

[a] Results are mean±SD of three determinations

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.

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

Glycerol in the fermentation broth was estimated by a colorimetric procedure described by Bok and Demain [21]. Concentration of citric acid was determined by the method described by Marrier and Boulet [22]. Concentration of glutamic acid was determined by HPLC under conditions that were identical to that for quantification of PGA [18].

Molecular weight of PGA was measured by gel permeation chromatography (GPC) using a Hitachi L6200 system controller equipped with Shodex KB800 series columns (two KB80M, one KB802.5) and a refractive index (RI) detector. Poly ethylene oxide standards were used to construct standard curve. The eluant was 0.2 M $NaNO_3$ (pH 7) and flow rate was set at 1 ml/min.

Results and Discussion

Effect of Addition of Amino Acids on PGA Production

Figure 1 shows the effect of amino acids on PGA production. It was observed that L-glutamine at 0.5 mM supported maximum PGA production of 31.40 g/l. Effect of lower concentrations of L-glutamine on PGA production was also studied, but yield of PGA was lower at L-glutamine concentration below 0.5 mM (results not shown). PGA yields decreased with increasing concentrations of L-glutamine (Fig. 1). Kunioka [13] had

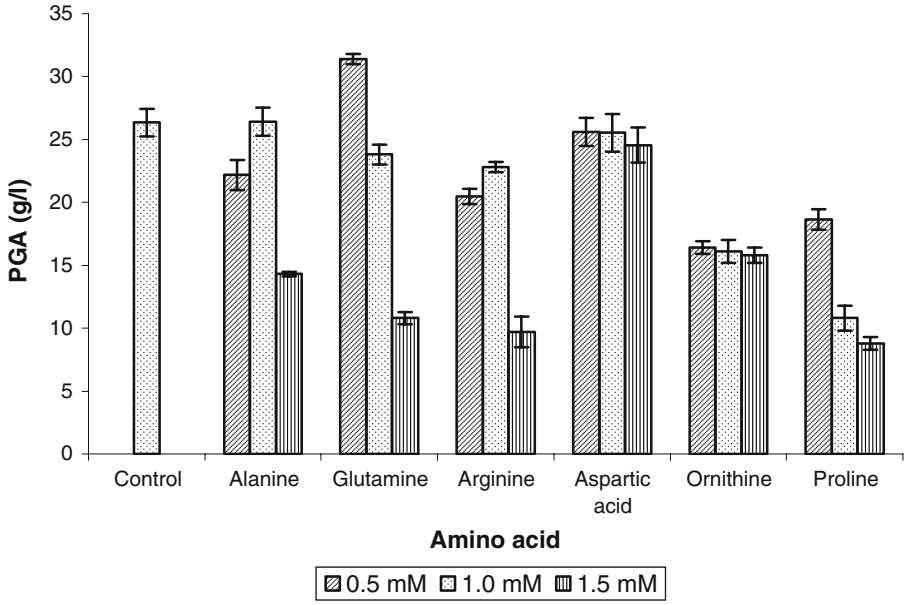

Fig. 1 Effect of amino acids on PGA production by *Bacillus licheniformis* NCIM 2324

observed higher yield of PGA without any by-products by using L-glutamine instead L-glutamic acid. Maximum yield of PGA was obtained by using 0.1 g/l of L-glutamine [13].

Effect of TCA Cycle Intermediated on PGA Production

Figure 2 shows the effect of TCA cycle intermediates on PGA production. It was observed that α-ketoglutaric acid at 10 mM supported maximum PGA production of 34.98 g/l. Other TCA cycle intermediates did not support PGA production. These results are in accordance with the results reported for production of PGA [14, 15].

Effect of Addition of Combination of L-Glutamine and α-Ketoglutaric Acid

Table 1 demonstrates the effect of addition of combination of L-glutamine and α-ketoglutaric acid at various concentrations on the PGA production. PGA yield was higher at two different combinations. L-Glutamine at 0.5 mM and α-ketoglutaric acid at 10 mM in the production medium supported production of 35.75±0.78 g/l of PGA, while addition of L-glutamine at 0.75 mM and α-ketoglutaric acid at 12.5 mM produced 35.83±1.18 g/l of PGA, indicating no significant improvement in the yield of PGA. Hence, L-glutamine at 0.5 mM and α-ketoglutaric acid at 10 mM were considered as optimum.

The yield of PGA obtained in this study is significantly higher as compared to the values reported in the literature. The maximum PGA production reported till date by using most widely used strain *B. lichenifomis* ATCC 9945A was 23 g/l [23]. *B. subtilis* IFO 3335,

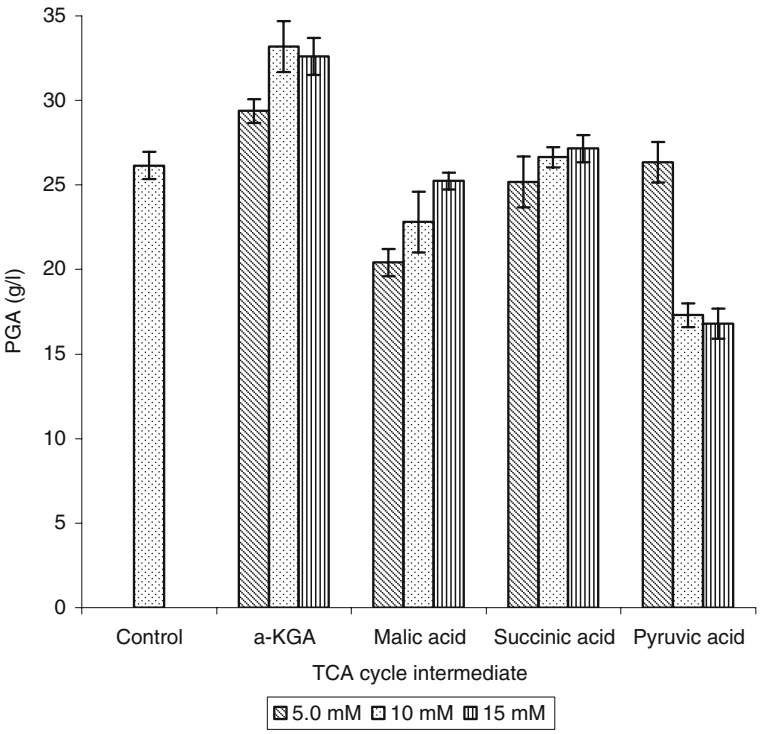

Fig. 2 Effect of TCA cycle intermediates on PGA production by *Bacillus licheniformis* NCIM 2324

Table 2 Effect of time of addition of precursors on the PGA and biomass production.

Time of addition (h)	PGA[a] (g/l)	DCW[a] (g/l)
0	35.79±0.35	2.48±0.11
12	34.41±1.24	2.41±0.26
24	29.12±0.64	2.38±0.15
36	26.29±0.54	2.29±0.08
48	26.78±1.16	2.38±0.12
60	27.08±0.88	2.34±0.25

[a] Results are mean±SD of three determinations

which is also extensively studied for PGA production, could produce maximum of 20 g/l of PGA [24]. PGA production reported from *B. subtilis* (*chungkookjang*), isolated from the traditional Korean seasoning was 15.6 g/l [25]. Maximum PGA production reported by *B. lichenifomis* CCRC 12826 was 19.62 g/l and *B. subtilis* NX-2 was 30.2 g/l [14, 26].

L-Glutamine and α-ketoglutaric acid were added at various stages of fermentation (Table 2). L-Glutamine (0.5 mM) and α-ketoglutaric acid (10 mM) added at the beginning of fermentation gave maximum PGA yield. Precursors added during fermentation did not increase PGA production significantly.

Results obtained in the present study suggests that endogenous L-glutamic acid synthesis in *B. licheniformis* NCIM 2324 may be carried out by glutamate dehydrogenase (GD) pathway as well as pathway involving glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT), depending on the medium composition. Pathway involving glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase

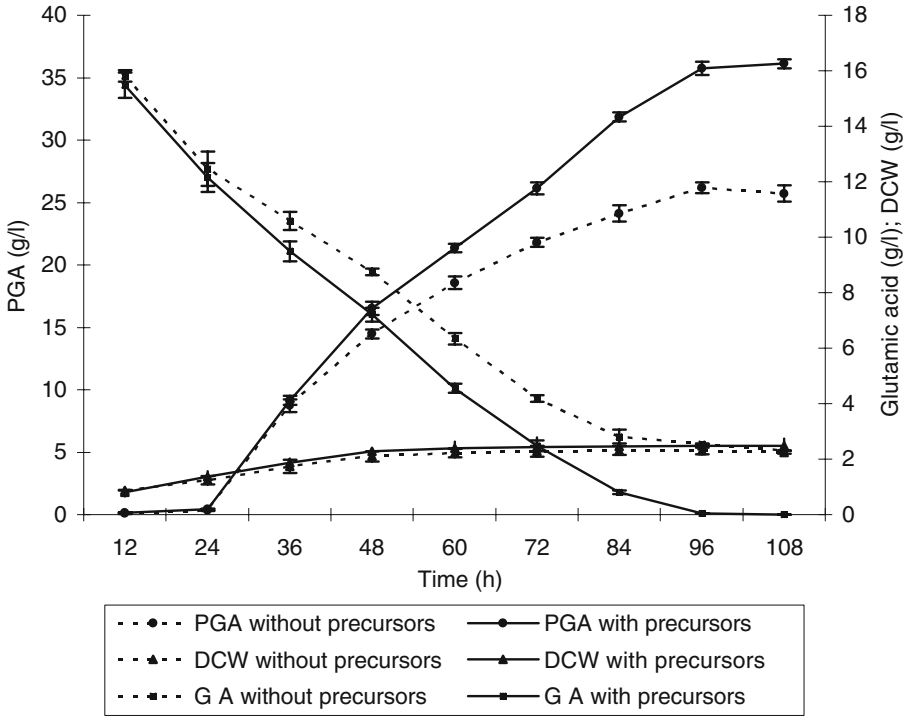

Fig. 3 Production profile of PGA in medium containing precursors and medium devoid of precursors

(GOGAT) 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 comparison of the profile of PGA production and glutamic acid utilization by *B. licheniformis* NCIM 2324 in a medium with precursors and that devoid of these precursors. It was observed that PGA production and glutamic acid utilization increased dramatically in the presence of precursors. In case of production of PGA in the medium containing precursors, glutamic acid was completely utilized after 96 h of fermentation. This is in accordance with the results obtained by Williams et al. [16] who reported an increase in the metabolism of amino acids by assimilation and/or catabolism in the presence of α-ketoglutaric acid in lactobacilli. Tavaria et al. [17] also reported α-ketoglutaric acid to promote the uptake of amino acids by cheese lactobacilli. There was no significant difference in utilization of glycerol and citric acid in medium with and without precursors. Addition of precursors also did not affect biomass production significantly.

Effect of Precursor Addition on Molecular Weight of PGA

The molecular weight of PGA obtained in a medium containing a combination of 0.5 mM L-glutamine and 10 mM α-ketoglutaric acid, and medium devoid of precursors was found to be 5.7×10^5 Da and 2.1×10^5 Da, respectively. The molecular weight of PGA with addition of only L-glutamine (0.5 mM) and only α-ketoglutaric acid (10 mM) was 5.3×10^5 Da and 5.6×10^5 Da, respectively. These results suggest that L-glutamine and α-ketoglutaric acid increased the molecular weight of PGA, when used either individually or in combination. Environmental parameters and medium components used during fermentation have been indicated to affect the molecular weight of PGA [2]. However, the exact reason for the increase in molecular weight in the presence of precursors is not clear.

Conclusions

L-Glutamine and α-ketoglutaric acid could serve as the metabolic precursors for the PGA production by *Bacillus licheniformis* NCIM 2324. Addition of precursors improved the yield and molecular weight of PGA, and also improved the utilization of glutamic acid.

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