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Hyperthermostable, Ca^{2+}-Independent, and High Maltose-Forming α-Amylase Production by an Extreme Thermophile *Geobacillus thermoleovorans*: Whole Cell Immobilization

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Abstract The synthesis of extracellular α-amylase in *Geobacillus thermoleovorans* was constitutive. The enzyme was secreted in metabolizable carbon sources as well as non-metabolizable synthetic analogues of glucose, but the titers were higher in the former than that in the latter. *G. thermoleovorans* is a fast-growing facultatively anaerobic bacterium that grows under both aerobic and anaerobic conditions and produces an extracellular amylolytic enzyme α-amylase with the by-product of lactic acid. *G. thermoleovorans* is a rich source of various novel thermostable biocatalysts for different industrial applications. α-Amylase synthesis was subject to catabolite repression in the presence of high concentrations of glucose. The addition of cAMP to the medium containing glucose did not result in the repression of α-amylase synthesis. The addition of maltose (1%) to the starch arginine medium resulted in a twofold enhancement in enzyme titers. Polyurethane foam (PUF)-immobilized cells secreted α-amylase, which was higher than that with the free cells. PUF appeared to be a better matrix for immobilization of the thermophilic bacterium than the other commonly used matrices. The repeated use of PUF-immobilized cells was possible over 15 cycles with a sustained α-amylase secretion. The use of this enzyme in starch saccharification eliminates the addition of Ca^{2+} in starch liquefaction and its subsequent removal by ion exchangers from the product streams.

Keywords Ca^{2+}-independent α-amylase · Whole cell immobilization and production

Introduction

α-Amylase (EC 3.2.1.1) is one of the enzymes of worldwide interest in food and fermentation industries. This enzyme is used in hydrolyzing starch to sugar syrups that are widely used in food, paper, and textile industries [1, 2]. Industrial processes of starch hydrolysis rely on inorganic acids or enzyme catalysis. The use of enzymes is preferred, as

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it offers a number of advantages including improved yields and favorable economics. Further, enzymatic hydrolysis allows greater control over amylolysis, the specificity of the reaction, and stability of the generated products [3]. Technological processes suggest the use of complex enzyme preparations resistant to high temperature, devoid of Ca^{2+} requirement, and capable of hydrolyzing starch at 95–100 °C [4, 5]. A high temperature reaction allows simultaneous gelatinization and enzymatic hydrolysis of starch, decreases the dose of enzymes, shortens the period of starch conversion, increases the yield of end products, and minimizes the cost of this process [6].

Most extracellular enzymes are affected by feedback inhibition or catabolite repression [7–11]. Generally, α-amylases are extracellular enzymes that are produced during growth of microbes on substrates containing starch and are subjected to catabolite repression by glucose and feedback inhibition by maltose [12]. Microbes are known to secrete low constitutive levels of α-amylase [13, 14].

A large number of biological processes using various biocatalysts such as enzymes, microorganisms, organelles, plant, and animal cells have been investigated to harness the advantages of immobilized biosystems. Extensive applications of immobilized cells and biocatalysts have been proposed in the industry [15, 16]. Immobilization of *Bacillus* sp. for the production of extracellular enzymes was accomplished using several immobilizing agents such as polyacrylamide [17, 18]. K-carrageenan gels [18] were found to be beneficial even though mass transfer complications were encountered [19, 20]. Subsequently Koshcheyenko et al. [21], Jamuna and Ramakrishna [22], and Dobreva et al. [23] have reported entrapment of *Bacillus* cells in alginate beads and agar and agarose blocks for the production of different metabolites and α-amylase. Immobilization matrices and conditions must be selected for each microbial strain [24]. We have been publishing several research papers on production, purification, and characterization of α-amylase from *G. thermoleovorans* [25–31]. However, there is still a need to develop a sustainable enzyme titer and continuous culture of *G. thermoleovorans* by using immobilization, aerobic, and anaerobic fermentation processes; for that, the present investigation was undertaken to understand the nature of synthesis of Ca^{2+}-independent, high maltose-forming, and hyperthermostable α-amylase in free as well as immobilized cells of *G. thermoleovorans* in aerobic and anaerobic fermentation.

Materials and Methods

Source and Maintenance of Strain

G. thermoleovorans NP 54 was isolated from a water sample collected from the hot water spring of the Waimangu volcanic valley (New Zealand) and maintained as described earlier [26–30]. The culture is deposited at Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India; MTCC 4220).

Seed Inoculum Preparation

The inoculum was prepared by transferring several loopfuls from a fresh culture of *G. thermoleovorans* into a 250-ml Erlenmeyer flask containing 50 ml starch–yeast extract–tryptone broth (g/1: soluble starch 20.0; yeast extract 3.0; tryptone 3.0; $MgSO_4 \cdot 7H_2O$ 0.2; K_2HPO_4 1.0; NaCl 1.0, pH 7) and incubated for 5 h in an incubator shaker at 70 °C and 200 rpm. To obtain cells for inoculation, the culture fluid was transferred aseptically to

sterilized centrifuge tubes and centrifuged at 8,000×*g* for 20 min (Sorvall RC 5C plus, Kendro Labs, USA). The cells thus sedimented were washed twice with sterile distilled water and resuspended in 50 ml sterile distilled water. This cell suspension was used as the inoculum for α-amylase production.

α-Amylase Production by Aerobic Fermentation

α-Amylase was produced in 250-ml Erlenmeyer flasks containing 50 ml of starch–arginine medium (g/l: starch 20; arginine 1.2; riboflavin 150 μg ml^{-1}; $MgSO_4 \cdot 7H_2O$ 0.2; NaCl 1.0, ph 7.0) by inoculating with 5-h-old bacterial inoculum ($1.\times10^8$ cfu/ml) and incubating in an incubator shaker at 70 °C for 12 h and agitated at 200 rpm. The bacterial strain was also grown in 50, 100, 200, and 400 ml of the starch–arginine medium with maltose (1%, *w*/*v*) in 0.25-, 0.5-, 1-, and 2-l Erlenmeyer flasks, respectively.

The growth curve of *G. thermoleovorans* was studied by growing the bacterium in the starch–arginine medium. Samples were withdrawn at regular intervals and the growth was monitored by recording absorbance at 600 nm. The enzyme activity was determined at the desired intervals.

Specific growth rate (μ) was calculated according to the equation:

$$\text{Specific growth rate}\,(\mu) = \frac{\mathrm{d}x}{\mathrm{d}t} \times \frac{1}{X}$$

where X is the mean biomass between two intervals during log phase. The apparent generation time (G) was calculated according to the equation:

$$G = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}.$$

The culture was harvested by centrifuging at 8,000×*g* for 12 min at 4 °C, and the cell-free supernatant was used as the source of extracellular α-amylase.

Intracellular α-Amylase

After cultivating *G. thermoleovorans* in 250-ml Erlenmeyer flasks containing 50 ml starch–arginine medium, the filtrate was separated by centrifugation and the pellet was weighed and thoroughly washed in phosphate buffer (0.1 M, pH 7.0) and resuspended in the buffer. The cell suspension was pretreated in buffer with lysozyme (50 U mg^{-1} dry biomass) and sonicated (Soniprep 150 MSE, USA) for 45 cycles of 45 s each with an intermittent cooling for 45 s. Then, the cell debris was removed by centrifugation and the supernatant used as the source of intracellular α-amylase and protein.

Different concentrations of glucose (0.5–1.5%, *w*/*v*) were incorporated into the starch–arginine medium and its influence on enzyme secretion was studied in order to understand catabolite repression. The possible repression of α-amylase biosynthesis by glucose and its derepression by cAMP (5 mM) were studied in starch–arginine medium containing 1.5% (*w*/*v*) glucose. Different concentrations of maltose (0.5–2%, *w*/*v*) were incorporated into starch–arginine medium in order to understand the role of maltose in enzyme synthesis and secretion. The synthesis and secretion of α-amylase was studied by growing the strain in starch–arginine medium by omitting each component of the medium and also adding deoxyglucose/methyl α-D-glucopyranoside (2%). Control cultures were run without any addition of inducers.

α-Amylase Production by Anaerobic Fermentation

Cells were grown in sealed anaerobic bottles containing 100 ml of starch–arginine medium with CO_2 as the gas phase. The medium was heat-sterilized (15 min at 121 °C) in anaerobic bottles with a nitrogen headspace. To the sterile media, concentrated H_2SO_4/1 N NaOH was added to adjust the pH to 7.2. The nitrogen head space was replaced by CO_2 and $Na_2S{\cdot}9H_2O$ (final concentration of 1 mg l^{-1}) was added to ensure strict anaerobic conditions. After 15 min, the reduced medium was inoculated with 2% of 5-h-old seed culture (1.8×10^8 cfu ml^{-1}) at 70 °C in an incubator shaker at 200 rpm for 12 h. The control (aerobic cultivation) flasks were used along with anaerobic cultivation and the α-amylase and organic acid levels compared. The cell-free supernatants were used as the source of extracellular α-amylase and organic acids. The organic acids were analyzed by high-performance liquid chromatography (HPLC; Shimadzu) as described earlier [32]. For this, culture broth was centrifuged at 8,000×*g* in Sorvall centrifuge (RC 5C Plus) for 15 min, filtered, and 20 μl of each sample was run on HPLC.

Assay of α-Amylase

The saccharogenic α-amylase activity was determined by the modified method of Bernfeld [33] by incubating the reaction mixture at 100 °C for 10 min (pH 8.0). One saccharogenic α-amylase unit is defined as the amount of enzyme required for the liberation of 1 μmol of reducing sugars, as maltose, per milliliter per minute under the assay conditions.

Effect of Ca^{2+} on Enzyme Activity

The same assay procedure was followed except that the reaction mixture included $CaSO_4{\cdot}2H_2O$ (10 mM)

Other enzymes were produced by *G. thermoleovorans* in extracellular broth.

Protease Assay

The reaction mixture comprised 0.5 ml of suitably diluted enzyme sample and 0.5 ml acetate buffer (pH 5.0). The control contained 0.5 ml diluted enzyme and 0.5 ml of 10% trichloroacetic acid (TCA) to denature the enzyme. One milliliter of 1% casein was added to each tube and incubated at 60 °C for 10 min. The reaction was stopped by adding 4 ml of 5% TCA to each tube. The tubes were kept at room temperature for an hour and the contents filtered. To 1 ml of filtrate, 5 ml of 0.4 M Na_2CO_3 was added. The tubes were kept at room temperature for 10 min and 0.5 ml of 1:1 diluted Folin's reagent was added. The tubes were kept in the dark for 30 min and A_{660} was recorded.

One unit (U) of protease is defined as the amount of enzyme required to liberate 1 μmol tyrosine per milliliter per minute under the assay conditions.

Lipase Assay

Lipase was assayed by adding 0.5 ml substrate (olive oil) to 2.5 ml of 0.01 M acetate buffer (pH 5.0) and kept at 45 °C for 10 min for priming in a water bath. To each tube, 0.5 ml of the enzyme sample was added and the reaction was carried out at 80 °C for 30 min. The free fatty acids released were titrated against 0.01 N KOH. The enzyme activity was expressed in terms of the amount of fatty acids released.

One unit of lipase is defined as the amount of enzyme required to liberate 1 μmol of fatty acids as oleic acid per milliliter per minute under the assay conditions.

Xylanase Assay

The reaction mixture containing 0.5 ml of 1% birchwood xylan in acetate buffer (0.1 M, pH 5.0) and 0.5 ml of enzyme sample was incubated at 80 °C for 10 min. After incubation, 1 ml of DNSA was added to all the tubes and were kept in a boiling water bath for 10 min, after which 0.4 ml of 33% potassium sodium tartrate was added and A_{540} was recorded.

One unit of xylanase is defined as the amount of enzyme required to liberate 1 μmol of reducing sugars as xylose per milliliter per minute under the assay conditions.

Cellulase Assay

The reaction mixture containing 0.5 ml of cellulose (fine pieces of filter paper) in acetate buffer (0.1 M, pH 5.0) and 0.5 ml of enzyme sample was incubated at 100 °C for 30 min. After incubation, 1 ml of DNSA was added to all the tubes and were kept in boiling water bath for 10 min, after which 0.4 ml of 33% potassium sodium tartrate was added and A_{540} was recorded.

One unit of cellulase is defined as the amount of enzyme required to liberate 1 μmol of reducing sugars as glucose per milliliter per minute under the assay conditions.

Pectinase Assay

The substrate was prepared (0.5% pectin) in phosphate buffer (0.1 M, pH 7.0) by constant stirring on a magnetic stirrer at 70 °C to ensure uniform distribution of substrate in buffer. The enzyme was assayed by incubating 0.5 ml of substrate prepared in phosphate buffer (pH 7.0) with 0.5 ml of appropriately diluted enzyme for 10 min, 60 °C, and determining the liberated reducing sugars using dinitrosalicylic acid reagent.

One unit of pectinase is defined as the amount of enzyme required to liberate 1 μmol of galacturanic acid per milliliter per minute under the assay conditions.

Tannase Assay

The reaction mixture containing 1 ml enzyme, 2 ml acetate buffer (0.1 M, pH 5.0), and 1 ml substrate tannic acid (1%) was incubated at 50 °C for 30 min. The reaction was stopped by adding 4 ml of 2% bovine serum albumin. The contents were centrifuged and the absorbance of the supernatant was measured at 260 nm.

One unit of tannase is defined as the amount of enzyme required to liberate 1 μmol of gallic acid per milliliter per minute under the assay conditions.

Phytase Assay

Phytase was assayed by measuring the amount of phosphate released using sodium phytate as the substrate. The reaction mixture comprised 0.5 ml of 0.1 M acetate buffer (pH 4), 0.5 ml of 1.5 mM sodium phytate, and 1 ml crude enzyme/cell suspension. The reaction was carried out at 100 °C for 15 min and then stopped by adding 2 ml 10% (*w*/*v*) trichloroacetic acid. One unit of phytase is defined as the amount of enzyme that liberates 1 μmol inorganic phosphate per milliliter per minute under the assay conditions.

Pullulanase Assay

The reaction mixture containing 0.5 ml of 1% pullulan in phosphate buffer (0.1 M, pH 7.0) and 0.5 ml of culture filtrate was incubated at 80 °C in a water bath for 30 min. After incubation, 1 ml of DNSA was added to all the tubes and were kept in boiling water bath for 10 min, after which 0.4 ml of 33% potassium sodium tartrate was added and A_{540} was recorded. One unit (IU) of pullulanase is defined as the amount of enzyme required to liberate 1 μmol of reducing sugars as maltose per milliliter per minute under the assay conditions.

α-Glucosidase Assay

The reaction mixture contained 0.2 ml of enzyme sample, 0.3 ml of buffer, and 0.5 ml of *p*-nitrophenyl α-D-glucopyranoside (1 mM) prepared in 100 mM buffer as the substrate. The reaction was terminated after 30 min of incubation at 70 °C by adding 2 ml of sodium carbonate–bicarbonate buffer (0.1 M, pH 10). The release of *p*-nitrophenol was measured at 400 nm and its yield was determined using a stand curve of *p*-nitrophenol (1–10 μg/ml) prepared in sodium carbonate–bicarbonate buffer (pH 8).

Immobilized Cell System

The effectiveness of polyurethane foam (PUF) as the matrix for immobilization of *G. thermoleovorans* was investigated according to Kapoor et al. [31]. PUF (1-cm^2 cubes) of density 32 kg m^{-3} and varying pore size (100 to 500 μm) were used. PUF cubes were pretreated by immersing in distilled water and autoclaving for 15–30 min to remove any toxic monomers or fillers which may otherwise leach out into the culture medium. They were later washed several times with distilled water and then autoclaved at 121 °C for 15 min before use for bacterial cell immobilization. The pretreated PUF cubes (0.25 g) were placed in 250-ml Erlenmeyer flasks containing 50 ml of starch–arginine medium with/without maltose (1%, *w*/*v*) and methyl α-D-glucopyranoside (2%, *w*/*v*). The medium was autoclaved and inoculated with 2% of 6-h-old seed cultures of *G. thermoleovorans* and incubated at 70 °C and 200 rpm. After every 12 h, the spent broth was replaced with the fresh medium for the next cycle and α-amylase was assayed in the cell-free supernatant. The process was repeated for 15 cycles in starch–arginine medium, while four cycles were run in a set of starch–arginine media prepared by substituting starch with maltose/methyl α-D-glucopyranoside.

Eighteen milliliters of k-carrageenan (4%, *w*/*v*) maintained at 40–50 °C was mixed with 2 ml of 5-h-old cell suspension (1.8×10^8 cfu/ml) and poured into sterile Petri plates and allowed to solidify. The solidified k-carrageenan was then cut into equal small blocks (approximately $5\times5\times4$ mm^3) and were cured in 2% KCl for an hour in the refrigerator. These blocks were washed thoroughly with sterile distilled water and used for the production of α-amylase. Six percent of agar and agarose in 0.1 M phosphate buffer at pH 7.0 were prepared and autoclaved separately in Erlenmeyer flasks. Two milliliters (1.8×10^8 cfu /ml) of 5-h-old bacterial cells were mixed with slurry at 50–55 °C and poured into plates rapidly. After solidification, approximately 100 equal-sized blocks ($0.9\times0.9\times0.33$ cm^3) of agar and agarose entrapped whole cells were cut aseptically and used in the production of α-amylase.

Two milliliters (1.8×10^8 cfu /ml) of 5-h-old bacterial cells were mixed with 50 ml of sterile sodium alginate (4%, *w*/*v*) slurry. The slurry was allowed to fall drop by drop into

chilled 0.2 M $CaCl_2$ solution with mild shaking. The calcium alginate beads (diameter, 3 mm) thus formed were cured by keeping them in 0.2 M $CaCl_2$ for 2 h at 4 °C. The beads were washed with sterile distilled water and used for the production of α-amylase.

Results

G. thermoleovorans is a fast-growing thermophilic bacterium with a lag phase of ~20 min followed by an exponential phase extended up to 9 h in starch–arginine medium. The generation time of the strain was 1 h (Fig. 1a). α-Amylase synthesis ceased in the shake

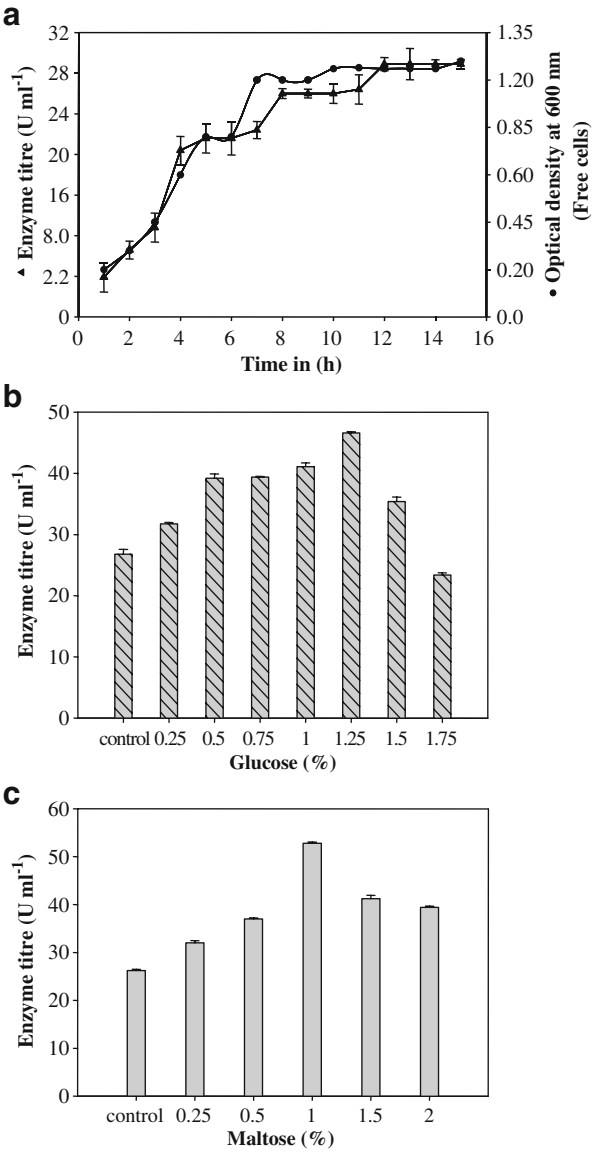

Fig. 1 **a** The Ca^{2+}-independent α-amylase production and growth curve in starch–arginine medium by *G. thermoleovorans* immobilized on polyurethane foam over 12 h incubation. **b** Effect of glucose on α-amylase production by an extreme thermophile *G. thermoleovorans* cultivated at 70 °C for 200 rpm in 12 h incubation. **c** Effect of maltose concentration on α-amylase production by an extreme thermophile *G. thermoleovorans* cultivated at 70 °C for 200 rpm in 12 h incubation

flasks before cessation of growth with concomitant secretion of other enzymes such as acid proteases and xylanase, as shown in Table 1.

Various Enzymes Produced by *G. thermoleovorans*

Other enzymes (including α-amylase) in the culture filtrate were assayed quantitatively. The activities of enzymes phytase and tannase were not detected in the culture filtrate. However, various levels of protease, xylanase, lipase, α-glucosidase, pullulanase, α-glucosidase, cellulase, and pectinase activities were detected in the cell-free culture filtrate as shown in Table 1.

The presence of the calcium ions even at low concentrations in the enzyme reaction mixture did not affect its enzyme activity (with/without Ca^{2+} ions ~28.2 U/ml in starch–arginine medium). Comparing production of α-amylase by *G. thermoleovorans* under aerobic and anaerobic cultivation conditions, the bacterium grew well both under aerobic and anaerobic cultivation conditions; α-amylase production was higher (28.0 U/ml) in the former than the latter (14.0 U/ml). Lactic acid production in anaerobic cultivation was much higher (2.5 g/L) in that in aerobic cultivation (0.6 g/L) was determined. >However, ethanol production was not determined in both cases. *G. thermoleovorans* produced an extracellular amylolytic enzyme in high yield (28.0 U/ml) than intracellular enzyme production (0.9 U/ml). However, cell-bound enzyme activity was not observed.

In order to understand the nature of α-amylase synthesis, *G. thermoleovorans* was grown in a medium containing different carbon sources. Active cell growth and α-amylase synthesis occurred in maltose- and starch-containing media. The cell growth and enzyme synthesis were very low in mannose, lactose, sucrose, and arabinose (Table 2). α-Amylase secretion in *G. thermoleovorans* was recorded in all the tested media, although it was higher in media which contained starch and maltose. *G. thermoleovorans* secreted α-amylase constitutively even in a medium containing only salts. The addition of inducers of α-amylase (non-metabolizable glucose analogues α-deoxy glucose, methyl-α-D-glucopyranoside) to the medium resulted in lower enzyme yields (Table 3).

Enzyme production was more in metabolizable carbon sources than in the nitrogen sources. Deletion of carbon source from the starch–arginine medium resulted in lower

Table 1 Extracellular different enzymes produced by an extreme thermophile *G. thermoleovorans* at 70 °C for 200 rpm in 12-h incubation.

Enzymes	Assay temperature (°C)	Titer (U/ml)
α-Amylase (Saccharogenic)	100	28.0±0.17
Dextrinizing activity	100	7.80±0.26
Xylanase	80	4.80±0.32
Lipase	80	1.74±0.62
Protease (acidic)	80	5.00±0.48
Pectinase	60	0.60±0.24
Pullulanase	80	2.37±0.77
α-Glucosidase	70	0.95±0.20
Phytase	60	–
Cellulase	100	0.10±0.35
Tannase	50	–

– Absent

Table 2 Growth and enzyme production in *G. thermoleovorans* in various carbon sources at 70 °C for 200 rpm in 12-h incubation.

Carbon source 2% (*w*/*v*) con.	Enzyme titer (U/ml)[a]	Growth (optical density at 600 nm)[a]
Arabinose	12.07±0.24	0.70
D-Glucose	26.04±0.33	1.27
Lactose	09.00±0.14	0.40
Maltose	24.00±0.74	1.90
Mannose	08.42±0.43	0.39
Raffinose	11.22±0.22	0.52
Starch	28.40±0.48	1.34
Sucrose	10.24±0.21	0.52

[a] Mean of three values and SD with in 10%

enzyme titers (Table 4). Amylase production increased with increasing concentrations of glucose in the starch–arginine medium up to 1.25% (*w*/*v*), and thereafter, it declined sharply (Fig. 1b). The addition of cAMP to the culture medium containing glucose (1.5%) did not influence the α-amylase synthesis (results not shown).

When starch–arginine medium was supplemented with maltose (1%), a twofold enhancement in enzyme titer was recorded, but beyond 1%, the enzyme production declined (Fig. 1c). The production of α-amylase with maltose (1%, *w*/*v*) was sustainable in flasks up to 1 l, and it declined in 2-l flasks (Table 5).

The cells of *G. thermoleovorans* immobilized on PUF secreted α-amylase in starch–arginine medium with/without maltose and methyl α-D-glucopyranoside (Figs. 2, 3, and 4). The enzyme production by the cells entrapped in calcium alginate, k-carrageenan, agar, and agarose was not sustainable beyond two batches (28.0 U/ml). The PUF-immobilized matrix was better than other matrices because the former could be reused over 15 batches with sustained α-amylase titers (~45.0 U/ml) and log phase cell growth was achieved.

Discussion

G. thermoleovorans is a facultatively anaerobic bacterium that grows under both aerobic and anaerobic conditions. The α-amylase of *G. thermoleovorans* was decreased when the cells entered into the stationary phase; this could be due to increasing lysis of the cells, depletion of nutrients after vigorous growth, or due to production of other biocatalysts such

Table 3 Enzyme production by *G. thermoleovorans* in 12 h in various liquid media.

	Enzyme titer (U/ml)[a]	Growth (optical density at 600 nm)
Only phosphate buffer (0.1 M)	2.30±0.12	0.12
Buffer + 2% starch	5.70±0.30	0.25
Buffer + 2% glucose	4.80±0.16	0.20
Buffer + 2% maltose	4.70±0.32	0.18
Buffer + 2%methyl α-d-glucopyranoside	4.00±0.22	0.15
Buffer + 2% deoxyglucose	3.02±0.26	0.14

[a] Mean of three values and SD with in 10%

Table 4 Growth and enzyme production by *G. thermoleovorans* in various media containing different components.

Media	Enzyme titer (U/ml)[a]	Optical density at 600 nm[a]
Control (starch–arginine medium)	28.04±0.46	1.27
Without arginine	22.0±0.58	0.72
Only salts	09.1±0.28	0.41
Without riboflavin	20.0±0.21	0.70
Without starch	13.0±0.33	0.57
Methyl α-d-glucopyranoside (2%)	19.0±0.55	0.67
Salts + methyl α-d-glucopyranoside (2%)	10.0±0.76	0.54
Salts + deoxyglucose (2%)	11.4±0.87	0.52

[a] Mean of three values and SD with in 10%

as acidic proteases [fall of pH from neutral (7.0) to acidic (4.5)] and xylanase, as observed in *Bacillus coagulans* [12]. Lactic acid was detected as a by-product in the fermentation broth of *G. thermoleovorans*, but ethanol was not detected as observed in *Thermoanaerobacter thermohydrosulfuricus* by Heitmann et al. [34]. The production of ethanol and acetic acid had been reported by Antranikian [35] from *Clostridium* sp. strain EM1 during continuous fermentation of α-amylase.

G. thermoleovorans produced an extracellular amylolytic enzyme in high yield than intracellular enzyme production, and it was categorized as an endo-acting α-amylase and exhibited significant thermostability ($t_{1/2}$ 3 h at 100 °C) [27].

G. thermoleovorans is a rich source of various other thermostable biocatalysts, is due to the fall of pH from neutral to acidic for the cultivation of α-amylase, and may induce the other biocatalysts. Most researchers have reported that the biosynthesis of amylolytic enzymes, particularly α-amylase, is induced only in the presence of starch [36]. The enzyme production by *G. thermoleovorans* was high in starch- and maltose-containing media as reported earlier in *Bacillus subtilis* [37]. α-Amylase was not, however, secreted by *B. subtilis* in the presence of non-metabolizable glucose analogues, α-deoxyglucose, and methyl-α-D glucose. The addition of non-metabolizable glucose analogues, α-deoxyglucose, and methyl-α-D-glucopyranoside to the buffer caused reduction in α-amylase production. In contrast, methyl-α-D-glucoside acted as an inducer of α-amylase in *Bacillus diastaticus* [38]. The amylase synthesis in *G. thermoleovorans* is constitutive, since it occurred not only in the presence of starch and non-metabolizable carbon sources but also in salt and phosphate buffered media, although the enzyme titers were low in the latter. The constitutive synthesis of α-amylase was also reported in *B. coagulans* [12].

Table 5 The α-amylase production in starch–arginine medium with maltose (1%, *w/v*) in increasing volumes of shake flasks.

Medium volume (ml)	Volume of flask (ml)	Enzyme titer (U/ml)[a]
50	250	55.1±0.23
100	500	57.0±0.43
200	1,000	57.5±0.54
400	2,000	48.5±0.44

[a] Mean of three values, SD within 10%

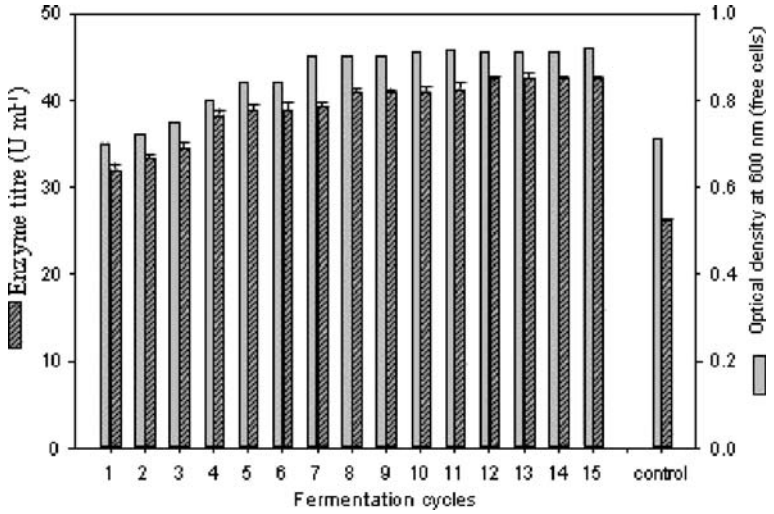

Fig. 2 Hyperthermostable, Ca^{2+}-independent α-amylase production in starch–arginine medium by *G. thermoleovorans* immobilized on polyurethane foam over 15 cycles

The enzyme production was more in metabolizable carbon sources than nitrogen sources. Deletion of carbon source from the glucose–arginine medium resulted in lower enzyme titers, as observed in *B. coagulans* [12]. Amylase production in *G. thermoleovorans* increased with increasing concentrations of glucose in the starch–arginine medium up to 1.25%, and thereafter, it declined sharply, which could be due to catabolite repression. The addition of cAMP to starch–arginine medium containing glucose did not influence α-amylase synthesis, suggesting the lack of catabolite repression at the 1.5% level of glucose. Thus, cAMP partially eliminated the repressive effect of glucose. Derepression of glucose repression by

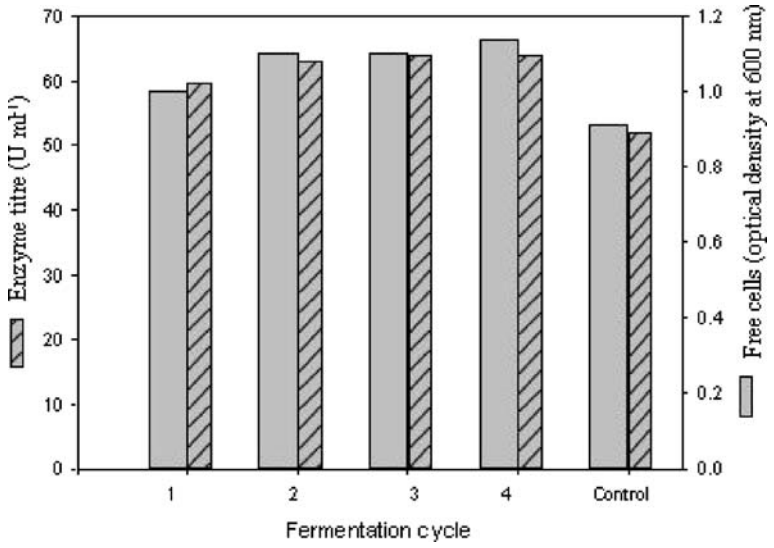

Fig. 3 α-Amylase production in starch–arginine medium containing maltose (1%, *w*/*v*) by *G. thermoleovorans* immobilized in polyurethane foam (control: free cells)

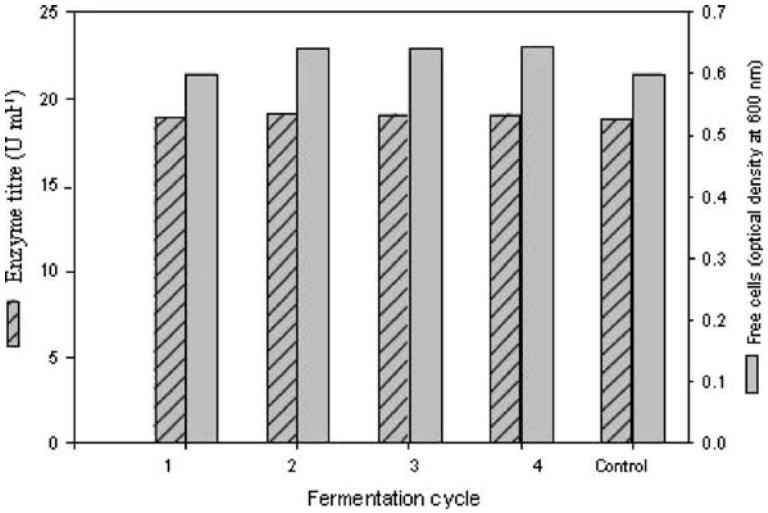

Fig. 4 α-Amylase production in starch–arginine medium containing methyl α-D-glucopyranoside (2%, *w*/*v*) by *G. thermoleovorans* immobilized in polyurethane foam (control: free cells cultivated in starch–arginine medium prepared by substituting starch with methyl α-D-glucopyranoside (2%, *w*/*v*)

cAMP was reported in *Bacillus stearothermophilus* [39] and *Endomycopsis fibuligera* [40], and stimulation of α-amylase secretion was observed in *Bacillus licheniformis* [41].

When starch–arginine medium was supplemented with maltose (1%), a twofold increase in enzyme titer was recorded, but there was reduction in enzyme production beyond 1%. At 1.5% maltose, the enzyme production in *G. thermoleovorans* was subjected to catabolite repression, in contrast to that in *Thermomonospora* where maltose was a good inducer of amylase even when used as sole carbon source [42].

The production of α-amylase in the starch–arginine medium containing maltose (1%, *w*/*v*) was not affected when *G. thermoleovorans* was grown in flasks up to 1 l, and thereafter, a decline in enzyme secretion was recorded. This could be due to improper mixing of nutrients and inadequate aeration on increasing the volume of the medium [27].

The PUF was preferred over other commonly used matrices (agar–agarose, calcium–alginate gels, and k-carrageenan) for immobilizing the extremely thermophilic *G. thermoleovorans* due to their low permeability and low stability at high temperature (70° C) for long-time cultivation. PUF could be reused for 15 batches in starch–arginine medium without any decline in α-amylase yield than commonly used matrices. The immobilized system proved to be advantageous over free cell cultivation as they secreted higher enzyme titers, which could be attributed to higher concentration of cells entrapped in the PUF that did not have any lag phase during their repeated use, as reported in the *Aspergilus sydowii* [43]. Kapoor et al. [31] achieved a 1.5-fold increase in polygalacturonase production by *Bacillus* sp. using PUF as the inert support matrix. Bacterial cells adhered to the surface of PUF and also partially infused into the pores as a consequence of bacterial growth. This technique has also been used successfully for the immobilization of *Streptomyces* sp. [44].

The thermostability of enzyme is an intrinsic property dictated by its primary structure; many external factors including ions influence increase in thermostability [12]. The presence of the calcium ions even at low concentrations in the enzyme reaction mixture containing α-amylase of *G. thermoleovorans* did not affect its enzyme activity.

In the present investigation, *G. thermoleovorans* is a fast-growing facultatively anaerobic bacterium that grows under both aerobic and anaerobic conditions and produces an extracellular amylolytic enzyme α-amylase with the by-product of lactic acid. This also reflects the environment of the biotope, as the temperature of the hot spring from which the organism was isolated and the organism is expected to get well adapted to anaerobic conditions. *G. thermoleovorans* is a rich source of various thermostable biocatalysts for different industrial applications. A substantial increase in α-amylase yield was recorded when the basal medium was supplemented with maltose. Enzyme production was more in metabolizable carbon sources than in non-metabolizable carbon sources. The repeated use of PUF-immobilized *G. thermoleovorans* over 15 batches was possible with sustained α-amylase production. The use of this enzyme in starch saccharification eliminates the addition of Ca^{2+} in starch liquefaction and its subsequent removal by ion exchangers from the product streams [45].

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