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L-Lysine production at 65 °C by auxotrophic-regulatory mutants of *Bacillus stearothermophilus*

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SUMMARY

The amino acid L-lysine was produced from auxotrophic-regulatory mutants of *Bacillus stearothermophilus* at a temperature of 60–65 °C. One of the mutants (AEC 12 A5, S-(2-aminoethyl)-cysteine^r, homoserine$^-$), produced L-lysine at the concentration of 7.5 g/l in shaken flasks in minimal medium containing 5% glucose. Culture conditions for optimizing L-lysine production were not investigated. The aspartokinase activity of the wild strain *B. stearothermophilus* Zu 183 was inhibited by lysine alone and by threonine plus lysine. AEC resistant mutants showed an aspartokinase activity genetically desensitized to the feedback inhibition. Optimal temperature and pH of aspartokinase were 45 °C and 9.5, respectively. The data provide significant evidence that mutants of the species *B. stearothermophilus* have a potential value for amino acid production.

INTRODUCTION

L-Lysine, an essential amino acid primarily used in supplements for animal feeds, is usually obtained in batch or fed-batch fermentation processes by auxotrophic and regulatory mutants of glutamic acid producing organisms [20] which operate at temperatures below 35 °C. In order to lower energy consumption of the process the use of thermophilic microorganisms has been suggested. In 1981 Hagino et al. [7] proposed producing L-lysine by an auxotrophic-regulatory mutant of *Bacillus licheniformis* capable of growth at 46 °C. Recently Schendel et al. [16] proposed L-lysine production at 50 °C by mutants of a methylotrophic *Bacillus* sp. In order to explore the possibility of producing L-lysine by these and other thermophilic *Bacillus* it is crucial to know their complete L-lysine biosynthesis regulation mechanism. The aspartokinase enzyme, which catalyzes the first step in the synthesis of all amino acids belonging to the aspartate family (lysine, methionine, threonine and isoleucine), is the most important control point of this pathway.

In *Bacillus brevis* [9], *B. polymyxa* [15], *B. subtilis* [8], *B. licheniformis* [18], *B. cereus* [6], *B. megaterium* [4] and *B. stearothermophilus* [12] aspartokinase activity appears to be regulated mainly by either L-lysine alone or multivalent feedback inhibition by L-lysine and L-threonine. In particular, in *B. stearothermophilus* two isofunctional aspartokinases, one inhibited in a concerted manner by L-lysine and L-threonine [12] and the other by *meso*-diaminopimelate and involved in the sporulation process, were observed [13].

Recently, in a plasmid screening of thermophilic *Bacillus* we have found several strains of *B. stearothermophilus* and *B. coagulans* harbouring one or more plasmids ranging in size from 1.7 to 50 kb. The smallest plasmid pBC1 was used to construct a new vector for cloning industrial characters in thermophilic bacilli [5]. One of these strains, Zu 183, from a species of *B. stearothermophilus*, was used in the experiments described in this paper to obtain auxotrophic-regulatory mutants able to overproduce L-lysine.

MATERIALS AND METHODS

Organisms and cultivation. The following thermophilic strains were used: *B. stearothermophilus* ATCC 12980, ATCC 10149, Zu 183, Zu 184 and Zu 195; *B. coagulans* Zu 257; *B. licheniformis* NRS 918 (Institute of Hygiene, University of Perugia, Italy).
Zu strains, derived from the sugar industry, have been previously described [2].

G1 basal medium, used in selecting auxotrophic and regulatory mutants, was a modification of the medium described by Kuhn et al. [11] and had the following composition (per liter): glucose 10 g, $(NH_4)_2SO_4$ 5 g, Na

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glycerophosphate 3 g, $MgCl_2 \cdot 6\ H_2O$ 30 mg, $CaCl_2 \cdot 2\ H_2O$ 500 mg, yeast nitrogen base W/A (Difco) 3 g, B salt solution 0.3 ml. The pH of the medium was adjusted to 7.0–7.2. The B salt solution contained (per liter): $MnCl_2 \cdot 4\ H_2O$ 130 mg, H_3BO_3 2.5 g, $CuSO_4 \cdot 5\ H_2O$ 125 mg, $NaMoO_4 \cdot 2\ H_2O$ 125 mg, $CoNO_3 \cdot 6\ H_2O$ 230 mg. Fermentation medium G2 was medium G1 containing 5% glucose, 3% $(NH_4)_2SO_4$ and 3% $CaCO_3$ (the latter sterilized separately). All the original cultures and the mutants were cultivated at 60° or 65 °C for 24 h.

The complex medium TSA (BBL) was used to maintain wild-type strains.

Selection of S-(2-aminoethyl)-cysteine (AEC) resistant and homoserine auxotrophic mutants. Overnight cells, grown at 65 °C on 300 ml of G1 medium in 1 l baffled Erlenmeyer flasks, were washed with Tris-maleic acid buffer (pH 6) and resuspended in 10 ml of the same buffer containing 1.5 mg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). After incubation for 30 min at 37 °C shaking slowly, cells were harvested, washed twice and resuspended in 5 ml of the same buffer. 0.1–0.5 ml of NTG treated cell suspension were spread on medium G1 agar plates containing AEC (0.1–0.4–1.0 mg/ml) plus threonine (1 mg/ml) and cultured for 4 days at 65 °C. AEC resistant colonies were picked up and streaked on agar slants of G1 medium supplemented with 100 μg/ml AEC and threonine (1000 μg/ml) to prevent the reversion of mutants and tested for the L-lysine production.

The best lysine producing AEC resistant mutant was NTG (1.5 mg/ml) mutagenized, treated with penicillin (10 U/ml) and spread onto minimal G1 medium both without and with 0.1% yeast extract. Auxotrophs were selected from colonies which could grow on the latter medium but not on the former and used for further studies.

Analytical methods. Wild-type strains and mutants were cultivated for 4 days at 65 °C in G2 fermentation medium at 200 rpm and L-lysine production was determined by bioassay using *Leuconostoc mesenteroides* P 60.

Preparation of cell-free extracts. The cells were cultured for 6–8 h at 65 °C on a shaker in 1 l baffled Erlenmeyer flasks containing 300 ml of medium G1. Cells were harvested by centrifugation and washed twice in 0.05 M potassium phosphate buffer (pH 7.5). The washed cells were then suspended in the same buffer containing 1 mM DL-dithiothreitol (DTT) and disrupted with a sonicator (Branson Sonifier) in ice bath. After centrifugation at 20000 × *g* for 60 min at 0 °C, the crude extract was maintained at − 20 °C before use. Protein concentration was determined by the Lowry method [14].

Enzyme assay. Aspartokinase activity was determined by the Black method [1] slightly modified, measuring the amount of aspartohydroxamate formed. The assay mixture contained the following components (μmol) in a final volume of 1.5 ml: Tris-HCl buffer (pH 9.5) 100, L-aspartate 30, ATP 10, hydroxylamine 500, KCl 300, $MgCl_2$ 10, and enzyme extract (1–2 mg/ml). After incubation at 45 °C for 60 min, the reaction was stopped by adding 1.5 ml of $FeCl_3$ reagent. The centrifuged solution was filtered and the absorbance was measured at 540 nm. Specific activity was expressed as μmol aspartohydroxamate formed in 60 min per mg of protein.

Purification of aspartokinase. A 5% (w/v) solution of protamine sulfate was added to the crude extract until a protamine sulfate/protein ratio of 0.2 mg/ml was obtained. The precipitate formed was removed by centrifugation at 18000 × *g* for 20 min. Solid ammonium sulfate was added to the fluid surnatant to achieve 35–55% saturation. After being stirred for an additional 30 min, the precipitate formed was collected by centrifugation and dissolved in a small volume of 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM DTT; the precipitate was desalted with a Sephadex G-25 disposable column. The eluate was applied on a 70 × 2.6 cm Sephacryl S-300 column equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM DTT. The column was eluted with 400 ml of the above buffer. Five-ml fractions were collected.

RESULTS

Sensitivity to AEC and derivation of resistant mutants

As the existence of a concerted feedback inhibition by L-lysine and L-threonine in various *Bacillus* had previously been reported, we preliminarily studied sensitivity of *B. stearothermophilus* wild-type strains to AEC in presence or absence or L-threonine. The most sensible strains to AEC were found to be Zu 183, Zu 194 and NRS 918, inhibited at 100 μg/ml. The growth inhibition of Zu 183 by AEC was enhanced to 50 μg/ml by further addition of L-threonine (1000 μg/ml). A total of 350 AEC resistant colonies were picked up and transferred for an initial screening of L-lysine production on Lutri plates (Lutri Plates Inc., Starkville, MS, U.S.A.). After spreading over a lysine auxotroph (*Leuconostoc mesenteroides* P 60), halo-forming colonies were selected. 7.3% showed a L-lysine production ranging from 0.6 to 4.5 g/l in the fermentation medium G2. The best producer was AEC 12 (4.5 g/l).

Derivation of auxotrophs from AEC 12

In order to improve L-lysine production auxotrophic mutants were derived from AEC 12. After NTG and penicillin treatment, 615 colonies were picked up and examined for their auxotrophy. Among the mutants tested, five stable homoserine auxotrophs produced the highest amounts of L-lysine. In G2 fermentation medium

TABLE 1

L-Lysine production and aspartokinase activity in *Bacillus stearothermophilus*

Strains	Characters	Production	Specific activity[a]
Zu 183	Parental strain	0	1.97[b]
AEC 12	AEC^r	4.5	4.51[b]
AEC 12 A5	AEC^r, $homo^-$	7.5	1.17[c]

[a] Aspartokinase activity was expressed as μmol of aspartohydroxamate formed/h/mg protein.
[b] Aspartokinase activity was measured on cell extracts purified on Sephacryl S-300 column.
[c] Aspartokinase activity was measured on crude extract.

the best producer AEC 12 A5 showed the highest yield of L-lysine (7.5 g/l) (Table 1).

Specific activity and regulation of aspartokinase

Specific activity of aspartokinase was determined in cell-free extracts from cells harvested at various times from batch cultures grown in G1 medium. As shown in Fig. 1, the highest specific activity measured as μmol of aspartohydroxamate formed, was found at the earliest exponential phase (after 7–9 h), while it strongly decreased in the stationary phase. As shown in Fig. 2 maximal levels of aspartokinase activity were obtained at pH 9.5 and 45 °C; in addition this activity was enhanced by Cl^- ions in the form of potassium or magnesium salts and it was inhibited by $(NH_4)_2SO_4$. Aspartokinase was isolated and purified from Zu 183 and AEC 12 as

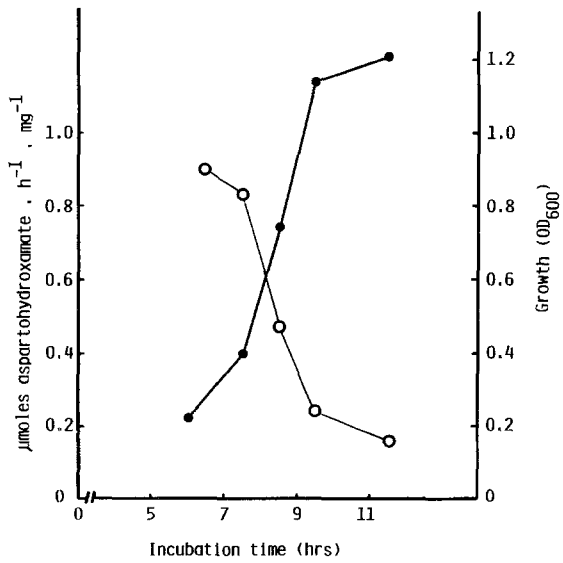

Fig. 1. Time course of growth of wild strain Zu 183 and growth-dependent aspartokinase activity measured on Zu 183 crude extract. ●, growth (OD_{600}); ○, aspartokinase activity.

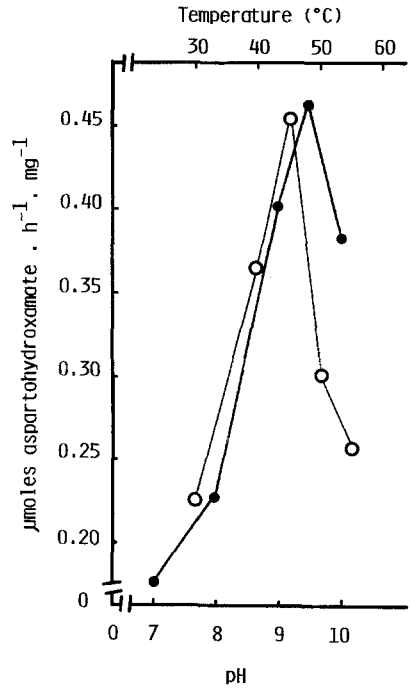

Fig. 2. Effects of temperature and pH on aspartokinase activity of Zu 183 crude extract. ○, temperature (°C); ●, pH.

described in MATERIALS AND METHODS. As shown in Table 1, the specific activity of aspartokinase from AEC 12 was markedly higher (4.51 μmol aspartohydroxamate formed per h per mg).

Effects of L-lysine and L-threonine on aspartokinase activity of the parental strains were examined under standard assay conditions except for the concentration of L-aspartate (15 μmol/1.5 ml) and the omission of KCl. As shown in Table 2 in purified extract of Zu 183 aspartokinase activity was inhibited by L-lysine (40%) and L-threonine (15%) added to the assay mixture; furthermore a weak concerted feedback inhibition by L-lysine plus L-threonine was also observed. In AEC 12 mutant aspartokinase activity was completely deregulated: no inhibition was found by single or simultaneous addition of L-lysine and L-threonine.

DISCUSSION

The bacteria currently used for the fermentative production of L-lysine are the glutamic acid producing organisms such as *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum* [19] and the overproduction of this metabolite is generally obtained by auxotrophic, regulatory and auxotrophic-regulatory mutants of the above indicated species [20] which operate well at a temperature of 30–35 °C. The industrial advantages of using thermophilic bacteria for large-scale

TABLE 2

Regulation of aspartokinase activity in the wild strain *Bacillus stearothermophilus* Zu 183 and in its *S*-(2-aminoethyl)-cysteine resistant mutant AEC 12

Strains	Relative aspartokinase activity (%)[a]														
	L-lysine (mM)					L-threonine (mM)					L-lysine (mM) + L-threonine (mM)				
	0	5	10	30	50	0	5	10	30	50	0	5	10	30	50
Zu 183	100	66	66	64	62	100	100	94	87	87	100	80	62	58	50
AEC 12	100	101	100	100	102	100	93	101	101	105	100	99	100	101	108

[a] Aspartokinase activity was assayed in cell extracts purified on Sephacryl S-300 column.

production of fermentation products, such as reduced risk of contamination, reduced consumption of cooling water and reduced viscosity and surface tension of water, have been outlined by Weimer [21], Brock [3] and Sonnleitner and Fiechter [17]. Moreover both the diffusion rate and the solubility of most nongaseous compounds are generally increased; these changes facilitate mass transfer and lower mixing costs.

Thermophiles produce enzymes capable of catalyzing biochemical reactions at temperatures markedly higher than those of conventional organisms. In addition, enzymes from these bacteria are more stable at conventional temperatures, thus prolonging the shelf life of commercial products. Industrial enzymatic process should thus occur more rapidly if it can be carried out by a thermostable enzyme.

Accordingly the use of thermophilic microorganisms for industrial processes has been advocated. At present only two different *Bacillus* strains have been proposed for lysine production at 46 °C [7] and 50 °C [16]: *Bacillus licheniformis* N-89 AEC^r homoserine$^-$ [7] growing at 46 °C which produced at 40 °C 15 g/l of L-lysine; *Bacillus* sp. [16], an AEC resistant mutant growing at 50 °C able to produce 7.8 g/l of L-lysine in presence of methanol as the sole carbon and energy source.

Our results also showed that true thermophilic *Bacillus* can overproduce L-lysine at a temperature above 60 °C. Although culture conditions for L-lysine production were not investigated, we have selected a *S*-(2-aminoethyl)-cysteine resistant and homoserine auxotrophic strain of *B. stearothermophilus* capable of producing 7.5 g/l of L-lysine in shaken flasks at 65 °C. These results appear quite comparable with those found by Schendel et al. [16] but are lower in respect to those obtained at 40 °C by Hagino et al. [7] on *B. licheniformis*.

Enzymatic studies concerning aspartokinase activity and its regulation mechanism in the wild-type strain showed that a concerted feedback inhibition of aspartokinase by L-lysine plus L-threonine or by L-lysine alone is operating. In AEC resistant mutant (AEC 12) the aspartokinase activity was completely deregulated: its specific activity was doubled in respect to parental strain and a strong desensitization of aspartokinase to the feedback inhibition by L-lysine plus L-threonine and/or by L-lysine alone was observed. This behaviour appears to be very similar to that observed in *B. licheniformis* [18].

We also noted, in accordance with other authors [10,18], that aspartokinase activity strongly decreases after exponential phase. This activity was optimal at pH 9.5 and 45 °C.

By submitting the mutant AEC 12 A5 to further mutations and by optimizing the culture conditions for L-lysine production, it appears likely that a good increase in the yield of L-lysine could be obtained.

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