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Selection and Activation of *Escherichia coli* Strains for L-aspartic Acid Biosynthesis

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Abstract

The strain of *Escherichia coli* K-12 with high aspartase activity was irradiated with UV. After mutagenesis and selection, the mutant B-715 was isolated which was 4-times more active in L-aspartic acid biosynthesis than parental K-12 strain. The highest productivity was achieved while the strain was cultivated in the ammonium fumarate medium in 37°C for 18–30 hours. It was found that better results were obtained when before the main production step of biosynthesis of L-aspartic acid, the cells of *E. coli* B-715 were incubated in the activation medium with ammonium fumarate. Activation at 37°C was the most advisable for high efficiency of L-aspartic acid biosynthesis. The productivity of *E. coli* B-715 during 1 hour biosynthesis process was at the range 0.19–0.35 g of L-aspartic acid per 1 gram of dry mass (biomass) per minute.

Key words: L-aspartic acid biosynthesis, productive strains selection

Introduction

Aspartic acid is an amino acid used in pharmacy, cosmetics and food industry (Chibata *et al*., 1985; Mazo *et al*., 1999a; 1999b). It is produced from the fumaric acid and ammonia either by chemical synthesis or in biotechnological process, *i.e*. in enzymatic reaction. In the first case a racemate mixture is produced, while the enzymatic synthesis ensures production of the enantiomeric form L of aspartic acid. For the synthesis of L-(+)-aspartic acid L-aspartate ammonia-lyase EC 4.3.1.1 (aspartase), enzyme from *Escherichia coli* is applied. The catalytic activity of that enzyme in *E. coli* was described very early (Quastel and Woolf, 1926). Depending on the pH value two opposite directions of reaction are possible: in acidic conditions aspartic acid is deaminated to fumarate and ammonia, but in alkaline pH L-aspartic acid in solution of fumaric acid and ammonia (or ammonium fumarate) is produced. The first industrial application of aspartase for fermentative L-aspartic acid production was carried out with *E. coli* strain B by Tanabe Seiyaku Co. in 1960 (Kisumi *et al*., 1960), and further developments in the L-aspartic acid biotechnology mostly have been made in Japan. Important improvements of the biosynthesis process were developed using the immobilized cells (Chibata *et al*., 1974; Nishida *et al*., 1978; Sato *et al*., 1979), and the aspartase-hyperproducing strains of *E. coli* derived from the strain B and K-12 were introduced (Nishimura and Kisumi, 1984; Nishimura *et al*., 1989). Further technological improvement was made using recombined clones of *E. coli* (Takano and Kino, 1999; Komatsubara *et al*., 1986; Nishimura *et al*., 1987a; 1987b; Mukouyama and Komatsuzaki, 2001).

In Poland, aspartic acid (racemate mixture) is produced mainly by means of chemical synthesis. In our Department investigations on the enzymatic biosynthesis of L-aspartic acid have been started. This paper deals with the strain screening, selected strain mutagenesis and the aspartase-active mutants selection. Thereafter, *E. coli* recombinant strains characterized by L-aspartic acid overproduction were constructed. Regardless of a very wide distribution of aspartase in microbial world, according to the above cited papers *E. coli*, common organism populating intestines of humans and other mammalians, seems to be the best source of that enzyme. Our search for the aspartase

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overproducers involved both the known collection strains of *E. coli* and the strains newly isolated from humans. To induce the highly active strains mutagenesis was applied and a procedure for the cell cultivation and their subsequent activation before their use for L-aspartic acid production was standardized.

Experimental

Materials and Methods

Microorganisms. Bacteria used in this work were the collection strains of *E. coli* B and K-12, as well as new isolates from humans. The isolates were nonpathogenic variants of *E. coli* received from the Sanitary Epidemiological Laboratory in Łódź.

Media. For preliminary cultivation of bacteria, LB medium containing Tryptone (Difco) 10 g/l, Yeast Extract (Difco) 5 g/l, NaCl 10 g/l; pH 7.5, was used. A standard growth medium contained: Yeast Extract (Difco) 20 g/l, ammonium fumarate 5 g/l, fumaric acid 10 g/l, KH_2PO_4 2 g/l, $MgSO_4 \times 7\ H_2O$ 0.5 g/l; pH 7.2. For testing different nitrogen sources for active cells multiplication we used media containing $(NH_4)_2SO_4$ 1 g/l, aspartic acid 5 g/l or ammonium fumarate 5 g/l and K_2HPO_4 7 g/l, KH_2PO_4 3 g/l, $MgSO_4 \times 7\ H_2O$ 0.1 g/l; pH 7.2. Media for both – the cells activation and aspartase activity testing contained fumaric acid 5 and 100 g/l respectively, $MgSO_4 \times 7\ H_2O$ 0.25 g/l and Triton 0.5 ml/l; pH 8.5.

Mutagenesis procedure. Bacteria growing overnight in 250 ml shake flasks containing 50 ml of LB medium in temperature 34°C were centrifuged at 4500 rpm and suspended in 10 ml 0.9% NaCl solution. The cells were mutagenized using UV lamp with $\lambda = 254$ nm and different time of illumination to aim at a different cell survival.

Colony activity test. An agar-paper printing technique was developed. After colony culturing on the growth medium solidified with agar the pieces of agar medium (with diameter of 8 mm) containing tested colonies were put on the filter paper for several minutes. The correct time of exposition for sufficient extraction of product from medium pieces to paper was experimentally chosen. Thereafter, the paper was sprayed with 0.1% ethanolic solution of ninhydrin and placed in an air dryer for 5–7 min. Time of the blue spots appearance and their size and intensity were observed for choosing the best L-aspartic acid producers.

Cell suspension activity test. The cells were cultured in 50 ml of the growth medium in 250 ml shak flasks for 18, 24, 30 or 48 hours in 37°C and thereafter centrifuged at 4500 rpm for 20 min.. The pelleted cells were washed 3-times with 50 ml of sterile water. In preliminary investigations non-activated (*i.e.* nonpermeabilized) cells were used. For further studies the cells were activated in activation medium in 4, or 37°C for 24 or 48 h. After centrifuging and washing 1 g of fresh biomass was incubated in 10 ml of the production medium in 100 ml shake flasks for 3–4 h; every 1 h samples of 1 ml volume were withdrawn for an aspartic acid analysis.

Aspartic acid analysis. For semi-quantitative analysis of the product TLC analysis using DC-Alufolien Kiselgel 60 plates (Merck) was applied. Aliquots of 10 ml of the 100-time dilutions of the samples were placed on the plates and chromatographed in a mixture of ethanol and water (67:33 v/v) for 1.5 h. The plates were then sprayed with 0.1% ethanolic solution of ninhydrin and placed in an air dryer for 5–7 min. The single blue spots presented L-aspartic acid production. For precise estimation of the quantity of L-asparic acid HPLC technique was applied. The samples were deproteinised by with methanol. Methanol served as inactivation reagent of aspartase. Four volumes of methanol was added to the samples and the obtained mixture was centrifuged. After dilution and addition of OPT-thiol reagent, 20 ml sample was injected into HPLC system: Column 250-4 LiChrospher™ 100 RP-18 (5 microm.)-Merck, Waters pump type 600 and Waters fluorescence detector type 474 were used. A mobile phase was the following: 200 ml methanol (Backer HPLC analysed) in 800 ml 0.05 M sodium phosphate buffer. Retention time for aspartic acid was 8 min, flow-rate 1ml/min, pressure 2100 PSI. The system was operated at temperature 22°C.

Cell mass determination. Centrifugal tubes of known dry weight were used for centrifugation of 1 ml cell suspensions at 4500 rpm for 20 min. After removal of the supernatant by decantation the cells were washed with distilled water, centrifuged again, and then dried overnight at 60°C and subsequently in 105°C by 1 h. After cooling to room temperature the samples of dryed cells were weighed.

Results

Strains selection. For preliminary screening of the aspartase productivity two commonly known strains of *E. coli*; B and K-12, and ten new nonpathogenic isolates of *E. coli* were chosen. In 4 h incubation of cell suspension test two strains, *i.e.* K-12 and an isolate no. 44, presenting over 5-times higher product accumulation were selected (Table I). For further improvement of the productivity by mutagenization *E. coli* K-12 strain was selected.

Selection of the aspartase overproducing mutants for L-aspartic acid biosynthesis. UV-irradiation of the cells of *E. coli* K-12 was conducted as

Table I
L-aspartic acid production by tested *E. coli* strain in a cell suspension test in 4 h biosynthesis

Strain tested	L-aspartic acid [g/l]
23	0.34
38	0.29
40	0.31
41	0.28
42	0.28
43	0.22
44	1.90
93	0.21
94	0.29
95	0.25
B	0.32
K-12	2.18

Non-activated cells were used.

Table II
L-aspartic acid biosynthesis by the activated (permeabilised) cells of *E. coli* K-12 and their hyperproducing mutants in four-hour-process (through 4 h)

Strain tested	L-aspartic acid [g/l]
K-12	14.6
A-7	50.0
B-715	62.4

Before using for a biosynthesis test the cells were permeabilised in the activation medium at 27°C through 18 h.

described in Materials and Methods. After mutagenesis a single colony valuation test for the selection of high-L-aspartic acid-producing mutants was applied. In a ninhydrin test on a filter paper after imprinting of the colony-agar pieces, about 4000 colonies in four successive mutagenesis processes were selected. On the basis of the blue spots, their size and intensity as well as time of their appearance, dozen of colonies were selected. In comparison to the parental strain of *E. coli* K-12, the best UV-mutant (designated A-7) selected after the first mutagenesis step, presented more than 3-time higher aspartase activity in the cell suspension test for L-aspartic acid production. This mutant was used for subsequent mutagenesis steps, and as a result of two-stage selection procedure the mutant of *E. coli* B-715 was isolated which was more than 4-times as active as K-12 (Table II).

Table III
L-aspartic acid biosynthesis using the cells of *E. coli* B-715 activated through 48 h at 37°C

Biosynthesis time [min]	L-aspartic acid [g/l]
0*	1.3
30	66.1
60	103.5
120	107.4
180	117.1
240	121.9

* sample taken directly after mixing the cells with production medium; contact time about 2 min.

Standardization of the cell treatment for L-aspartic acid production. The cell activation, *i.e.* cell membrane permeabilization, apart from the metabolic potential of the strain used, is an important factor in the process of transformation of ammonium fumarate to L-aspartic acid; as it was found earlier by Japanese investigators (Chibata *et al*., 1974). Because this property is very important in a strain testing procedure as

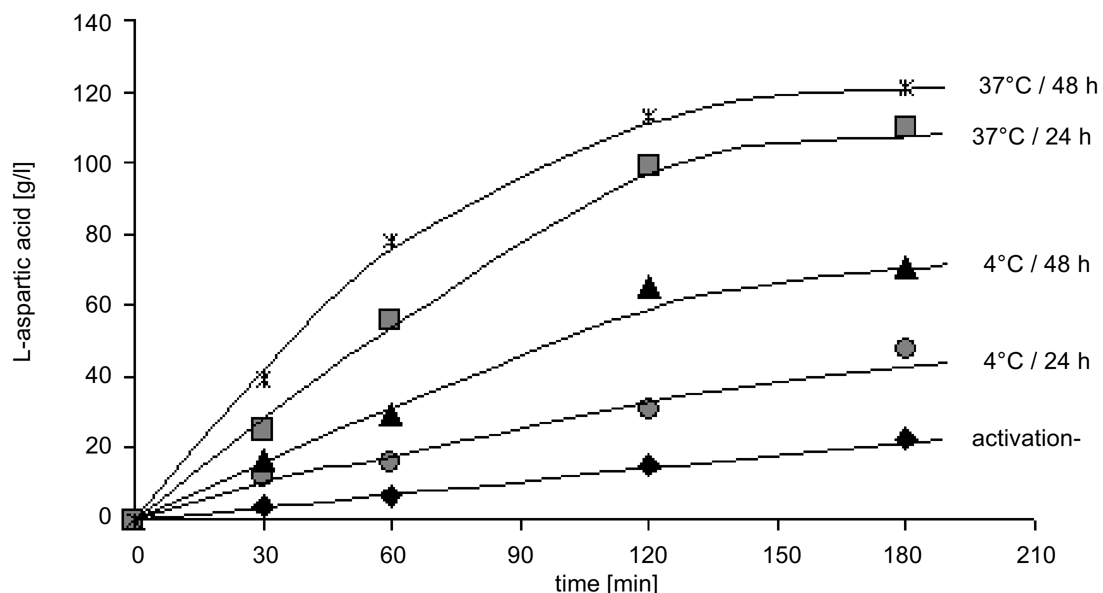

Fig. 1. Effect of temperature and activated time on L-aspartic acid biosynthesis.

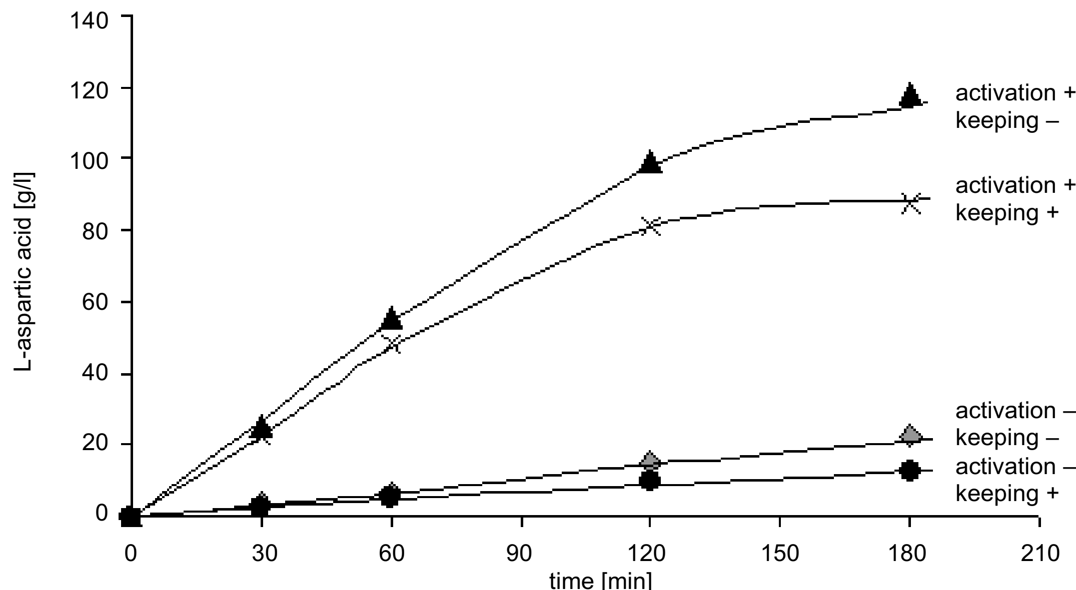

Fig. 2. Effect of keeping cells in the culture medium on L-aspartic acid biosynthesis.

well as for further development of a process of L-aspartic acid biosynthesis it was advisable to find the best method for the cell permeabilization. We have applied two different temperatures for the cell incubation in an activation medium: 4° or 37°C. Activation in temperature 37°C was the most advisable for efficiency of L-aspartic acid biosynthesis. The cells activated in 4°C were less productive. Further improvement of cell activity occurred after the prolongation of activation time to 48 h (Fig. 1).

The influence of keeping cells in a culture medium on biosynthesis of L-aspartic acid was also tested. The need to keep cells in a culture medium before their use for biosynthesis process sometimes occurs in technology process. The cells were kept in temperature about 4°C for 24 hours without shaking. This procedure was unprofitable for both the non-activated and activated cells of *E. coli*. The cells can be activated only in an activation medium with shaking (Fig. 2).

In the next experiment the effect of culture duration of mutant *E. coli* B-715 and also the effect of temperature of the cell activation were investigated. The cells were cultured at 37°C for 18, 24, 30 or 48 hours and subsequent they were suspended in the activated medium at 22, 27, 32 or 37°C for 24 h. The temperature 37°C was again the best activated tempera-

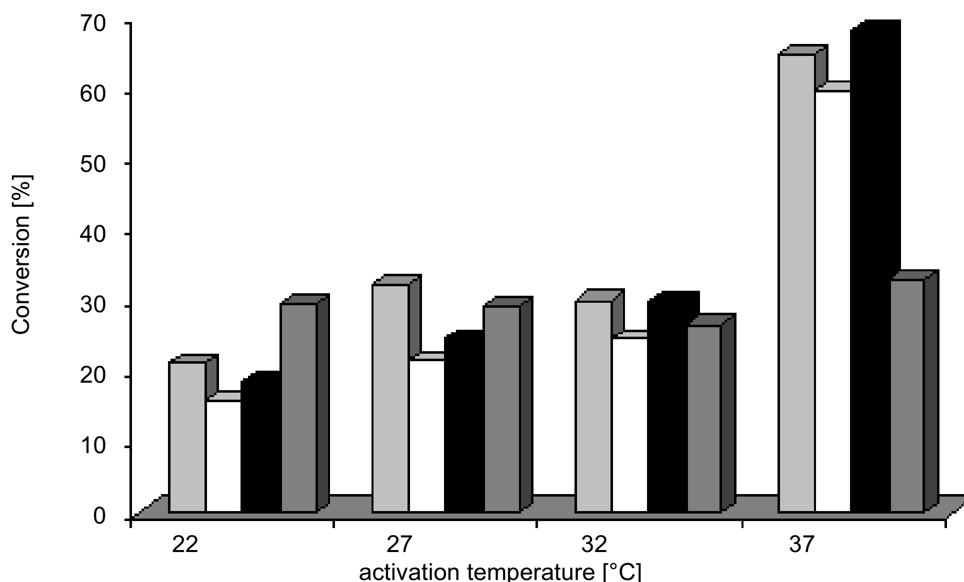

Fig. 3. Effect of culture duration (■ 18, □ 24, ■ 30 and ■ 48 hrs) and temperature of the cell activation (22°C, 27°C, 32°C, 37°C) on L-aspartic acid production.

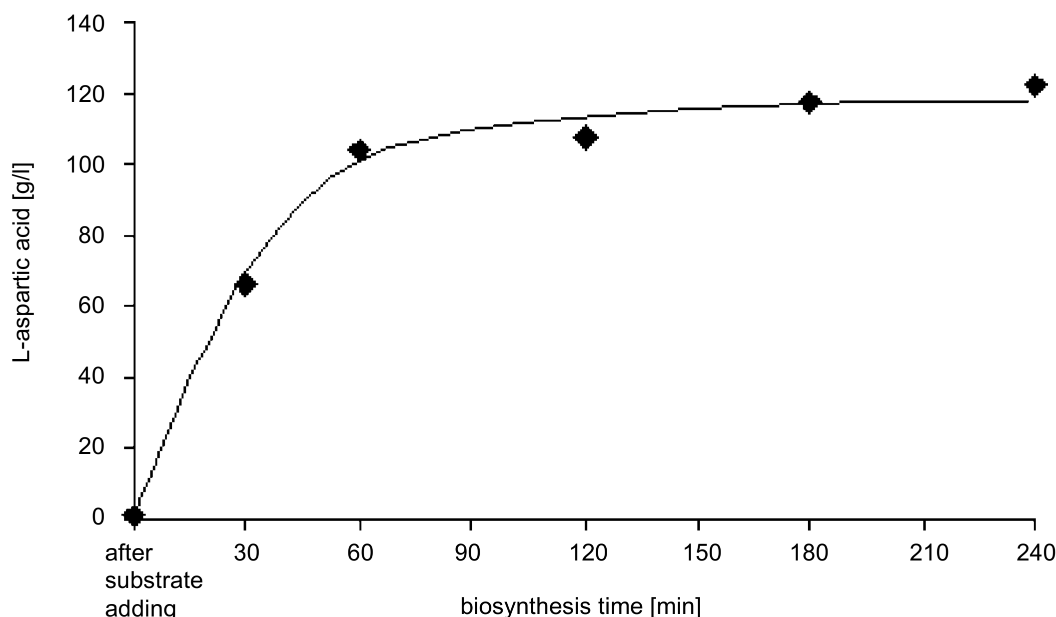

Fig. 4. L-aspartic acid biosynthesis using the cells of *E. coli* B-715, activated at 37°C through 48 h.

ture for L-aspartic acid production. An effect of culture duration on effective biosynthesis for 18–30 h was insignificant, but prolonging culture time to 48 h was unfavorable (Fig. 3). Partial cell autolysis during prolonged culture cannot substitute activation of cells in substrate solution.

This study stage was recapitulated following the experiment in which the bacteria were proliferating for 24 h at 37°C and activated at 37°C for 48 h (Fig. 4 and Table III).

Discussion

Different mutated and recombined strains of *Escherichia coli* have been used for production of L-aspartic acid from amonium fumarate. Other bacteria strains, for example: *Proteus vulgaris* OUT 8226, *Pseudomonas aeruginosa* OUT 8252, *Serratia marcescens* OUT 8259, *Bacterium succinium* IAM 1017 have been rarely proposed (Chibata *et al.*, 1974). In our laboratory, selection strains *E. coli* with high aspartase activity have been undrtaken. Out of ten new nonpathogenic isolates of *E. coli* and two collection strains of *E. coli* B and K-12, strain *E. coli* K-12 was chosen for further study. The strain chosen is very well characterized and was found to have high aspartase activity. The strain *E. coli* K-12 were irradiated with UV. Mutant of *E. coli* A-7 and better overproducing mutant of *E. coli* B-715 were isolated as the result of mutagenesis and investigatied for the yields of L-aspartic acid production.

E. coli B strain was used by Chibata *et al.* (1974). The yields of L-aspartic acid produced by this was tested. After 60 min and 180–240 min duration of the conversion process was at the of level 50% and about 90%, respectively. The strain *E. coli* ATCC 11303 was used in the study which resulted in the industrial technology. After 1 hour process, the cells of the strain (0.2 g dry mass and 30 ml production medium) produced 11 290 µmol L-aspartic acid. The productivity of 1 g dry cells was 0.125 g/g/min. This result cannot be compared with our results because authors did not determine the starting rate. In order to compare our results with those achieved by Chibata, productivity of strain *E. coli* B-715 was also calculated after 1 hour duration of biosynthesis. The calculated productivity was 0.19–0.35 g/g/min in different experiments. It was demonstrated that mutant of *E. coli* B-715 is considerably more active than the strain *E. coli* used in the first industrial technology of L-aspartic acid production.

Maximum value of biosynthesis velocity obtained in first minutes of a periodic process using mutant of *E. coli* B-715 was 2.2 g/g/min, so the productivity calculated on 1 g dry biomass of cells was 0.44 g/g/min.

The mutant of *E. coli* B-715 has been used as an aspartase gene source for further strain improvement using gene cloning procedure (Gadomska *et al.*, 2007).

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