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ORIGINAL PAPER

Recombinant Strains of *Escherichia coli* for L-aspartic Acid Biosynthesis

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Abstract

The aspartase overproducing mutant B-715 was used as a donor of the aspartase gene for further construction of the aspartase-hyperproducing strains by molecular cloning. In preliminary experiments activity of transformants and their efficiency in L-aspartic acid biosynthesis were compared. The conditions for recombinant strain multiplication, biomass activation and L-aspartic acid biosynthesis were optimized. The optimum temperature for cells multiplication, their activation and for product biosynthesis was 37°C. Two-stage process of the multiplication of bacteria (first in LB medium, and then in FF medium) eliminates the appearing of the inclusion bodies of aspartase in the cells. The shaking during cell activation improved cells productivity. The change of pH in the course of the biosynthesis process was insignificant but did not influence the process.

Key words: L-aspartic acid biosynthesis, conditions optimization for biosynthesis of L-aspartic acid

Introduction

Aspartic acid can be produced as a racemate mixture using chemical technology (Mazo *et al*., 1999a; Mazo *et al.*, 1999b) or as its L form using enzymatic process with aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) (Virtanen and Tarnanen, 1932; Waller, 2001; Chibata *et al.*, 1974). In our laboratory a search for the aspartase-active bacteria among both the collection strains and new isolates of *E. coli* resulted in selection of a well known *E. coli* K-12 strain as the best producer of that enzyme (Papierz *et al*., 2007). Its UV-mutant B-715 with a significant overproduction of aspartase was used as a donor of the aspartase gene for further construction of aspartase-hyperproducing strain by molecular cloning. This paper deals with the clones construction and preliminary investigations in their culturing and stability, as well as their use for L-aspartic acid biosynthesis.

Experimental

Material and Methods

Bacteria. The strains used in this work were *E. coli* UV-mutant B-715 and four recombinant strains *E. coli* P-1, P-2, P-3 and P-4. The strain B-715 was obtained in our laboratory as a result of UV mutagenesis of parental strain *E. coli* K-12 (Papierz *et al*., 2007). Suspensions of bacteria were mixed with 50% glycerol (1:1), frozen and stored in –70°C for further use (stocks).

Construction of recombinants of *Escherichia coli*. Recombinants *E. coli* P1, P2, P3 and P4 include multiply copy aspartase gene localized in plasmid vector. As a source of the aspartase gene the mutant *E. coli* B-715 obtained in our laboratory (Papierz *et al.*, 2007) was used (Fig. 1). PCR techniques was applied for the gene multiplication using two primers: ASP1.SEQ[20]: 5’ GGT TCA TAT GCC AAA CAA CA 3’ and ASP2.SEQ[26]: 5’ AAA AAG CTT ACT GTT CGC TTT CAT TC 3’, which were synthesized at the Institute of Bioorganic Chemistry of the Polish Academy of Sciences (Poznań). PCR product was digested using endonucleases NdelI and HindIII, and thereafter inserted into the pBS+ vector. Resulting plasmid pBSASP1 was multiplied in *E. coli* BLD21 (DE3) and digested with NdelI and HindIII. The ASP gene was then introduced into a plasmid expression pT7-7.This plasmid includes ampicillin resistance gene as a marker and a strong promoter of RNA polimerase of phage T7. Recombinant plasmid pT7ASP1 was introduced into the ampicillin resistance strain *E. coli* BLD21(DE3). This strain is deficient in both Lon and

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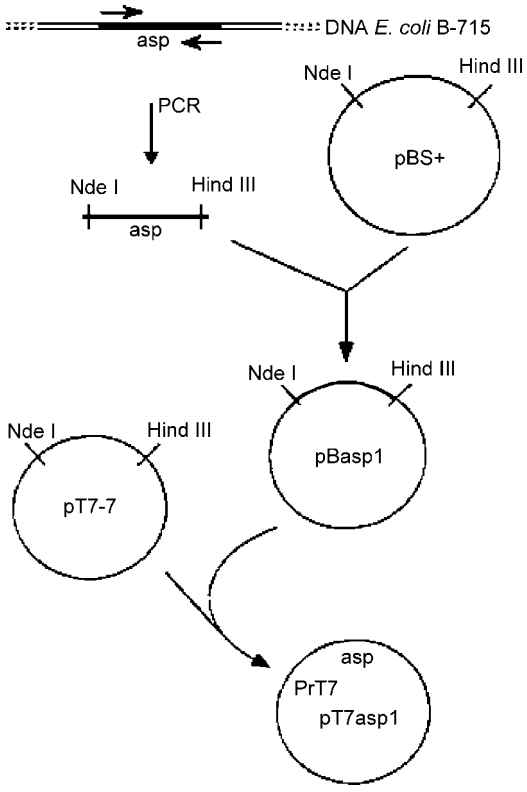

Fig. 1. Construction of expression vector with aspartase gene.

OmpT proteases and include the gene of RNA polymerase originated from phage T7 integrated to chromosome. The T7 RNA polymerase gene is under the control of *lac UV5* promoter and is induced by lactose or its analog isopropyl-1-thio-β-D-galactoside (IPTG).

Media. (1) LB medium; Trypton 10.0 g/l; Yeast Extract (Difico) 5.0 g/l; NaCl 10.0 g/l; pH 7.5; (2) FF medium for multiplication of biomass (our laboratory): Yeast Extract (Difico); 20.0 g/l, ammonium fumarate 5.0 g/l, KH_2PO_4 11.4 g/l, $MgSO_4 \times 7\ H_2O$ 0.5 g/l, pH 7,2. (3) Medium for cells activation (activate medium): ammonium fumarate 50.0 g/l, $MgSO_4 \times 7\ H_2O$ 0.25 g/1; 1% Triton 0.50 ml/l, pH 8.5. (4) Medium for production of L-aspartic acid (productive medium): ammonium fumarate 150.0 g/l; $MgSO_4 \times 7\ H_2O$ 0.25 g/l; pH 8.5. Chemicals, if not indicated otherwise, were purchased from POCh S.A, G (at analytical grade).

Conditions of bacteria multiplication and product biosynthesis. (1) Inoculum preparation: 100 µl of cells suspension from a stock were introduced into 100 ml of LB medium and cultivated in shaken 250 ml flask over 6 hours at 37°C. For growing of recombinant cultures the ampicillin at the concentration of 0.1 g/l as selection factor was added. **(2) Cell biomass multiplication.** 100 ml of FF medium in 250 ml Erlenmayer flasks was inoculated with pre-multiplied inoculum culture or with a cells stock (100 µl per 100 ml). For growing of recombinants cultures ampicillin was added (0.1 g/l). For an optimization of the cultivation conditions the shaken cultures of bacteria were carried out at 27 or 37°C for 16, 18, 20 or 24 hours. **(3) Cells activation.** The cell culture was centrifuged at 4000 rpm for 20 min and introduced into the activation medium (1 g of wet mass/20 ml). Tween 80 (0.5%) was added to obtain homogeneous cell suspension. The cell suspension was activated with or without shaking for 24 hours at the temperature of 27, 33, 37 or 40°C. Activated cell suspension was centrifuged at 4000 rpm for 20 min and biomass was washed twice with distilled water (1 g wet mass/ 20 ml water). **(4) Biosynthesis of L-aspartic acid**. Activated cells were mixed with the production medium (1 g wet mass/20 ml) in 100 ml flasks and shaken at the temperature of 27, 33, 37 or 40°C. After 15, 30 and 60 min of incubation the samples of 1 ml were withdrawn for analysis.

Aspartic acid analysis. For estimation of L-aspartic acid HPLC technique was applied using column 250–4 LiChrospher^{TM} 100 RP-18 (Merck) and Waters fluorence detector type 474. The details were described in proceding paper (Papierz *et al.*, 2007).

Results and Discussion

Comparison of recombinants. In preliminary experiments aspartase expression and activity in four transformants of *E. coli* BLD21 (DE3)/pT7ASP1, designed as P1, P2, P3 and P4, was estimated. Bacteria were multiplied in liquid FF medium with ampicillin at 37°C for 24 hours. Electrophoresis of cell protein showed intensive stimulation of cloning gene expression in the presence of IPTG (Fig. 2), however results in the aspartase activity tests were unsatisfied, because the level of L-aspartic acid biosynthesis was low. Microscopic observation showed occurrence of inclusion bodies inside the cells. Under those condi-

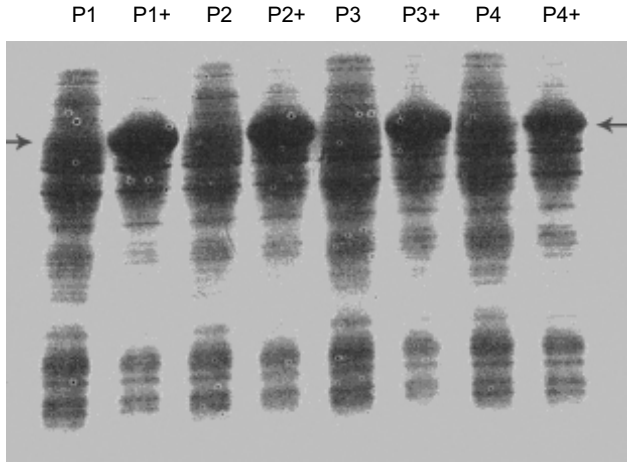

Fig. 2. Protein electrophoresis of cell lysate of recombinants of *E. coli* P1, P2, P3 i P4 cultured in FF medium with (+) or without (–) IPTG (1 mM).

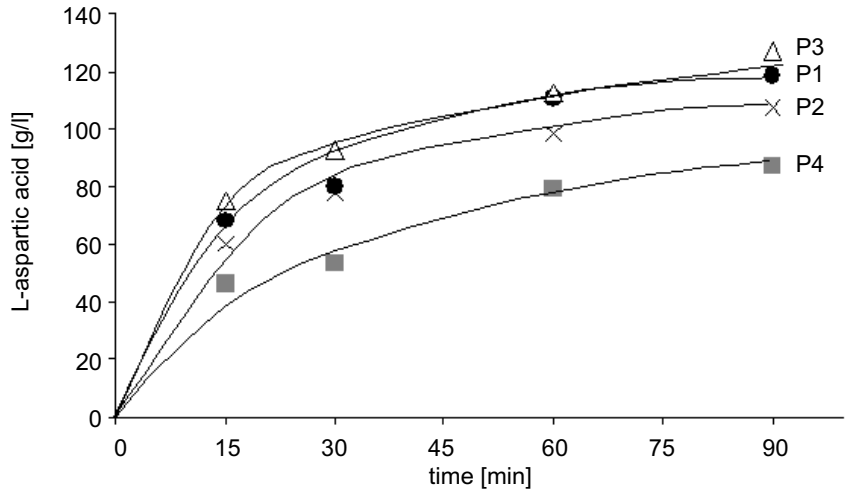

Fig. 3. Activity of recombinants *E. coli* P1, P2, P3 and P4 in productive medium

tions, the expression of the cloned aspartase gene effected synthesis of large amount of aspartase which was inactive and deposited in the inclusion bodies.

Conditions to obtain the cells with high aspartase activity were searched. Inoculum multiplication in LB medium was conducted for 3–8 hours but usually 6 hours incubation was sufficient to obtain a density with the absorption of about 0.25–0.3 at $\lambda = 540$ nm. FF growth medium without IPTG was inoculated using 2% of the inoculum cultures and incubated at 37°C for 16–24 hours. For three clones, *i.e.*: P1, P2 and P3 the cells cultivated 16–18 hours converted over 90% of substrate to L-aspartic acid already after 90 min of incubation (Fig. 3). Aspartase activity in the cells of recombinant P4 was considerably lower with statistical significance of $\alpha = 0.05$ in Manna-Whitney U-test. In further experiments we present the results obtained for recombinant *E. coli* P1 which was very active and stable during long-term investigations.

Optimization of recombinants multiplication. In the preliminary experiments we observed the presence of inclusion bodies after prolonging culture duration and especially in the presence of IPTG. The bodies are often present in recombinants cells of *E. coli* if cloned gene is expressed, and protein deposited in the bodies is biologically inactive. Long-term observations made by one of us show that by lowering temperature of bacteria cultivation it is possible to oppose creating of inclusion bodies in the transformed cells. To confirm this, the strain *E. coli* P1 was cultivated in FF growth medium without IPTG at 27 and 37°C. In this experiment inclusion bodies inside the cells of bacteria were not found at both temperaturs. Moreover decrease of temperature of bacteria cultivation caused considerable decrease in aspartase activity (Fig. 4). The product synthesis rate in the first 15 minutes of the process for bacteria multiplied at 37°C was 5.5 g/l/min and for bacteria multiplied at 27°C was

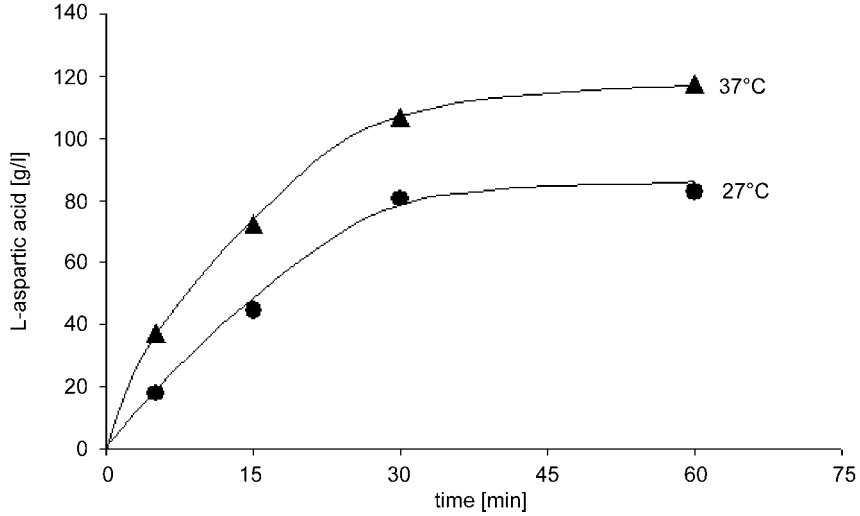

Fig. 4. Effect of temperature of biomass multiplication on biosynthesis of L-aspartic acid by activated cells of *E. coli* P1 (1 g of biomass per 10 ml of substrate solution)

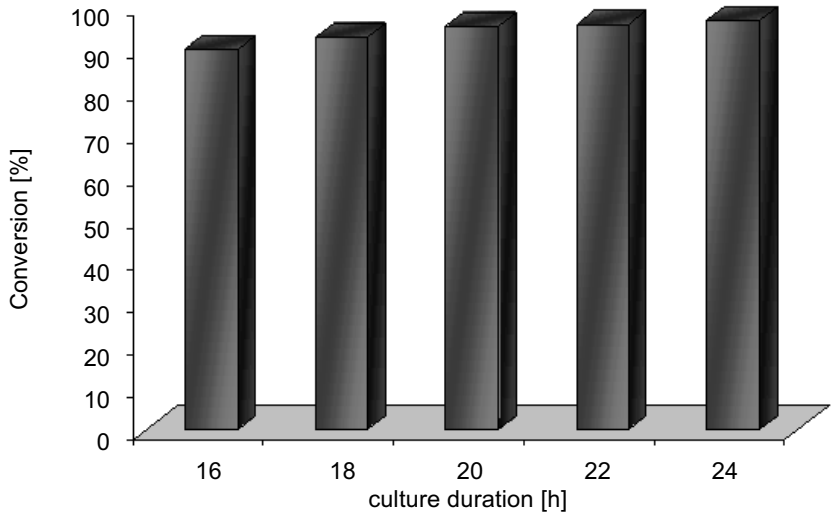

Fig. 5. Effect of culture duration of *E. coli* P1 on conversion of ammonium fumarate to aspartic acid.

only 3.5 g/l/min. Decrease of cultivation temperature was disadvantageous for aspartase activity.

For multiplication of biomass of recombinant P1, FF medium without IPTG was inoculated using the 6 hours culture of this strain in LB medium. Biomass was multiplied at the temperature of 37°C for 16, 18, 20, 22 or 24 hours, and then the cells were activated at 37°C for 24 hours and used for L-aspartic acid biosynthesis. Samples were taken after 60 minutes. The best conversion of ammonium fumarate to aspartic acid was observed after multiplication for 24 hours; however differences between the tested cultivation time variants were not significant (Fig. 5). Preliminary multiplication of bacteria in LB medium efficiently eliminate the problem of inclusion bodies, and prolonging of biomass cultivation even to 24 hours after applying this modification was advantageous.

Biomass activation. To optimize the process of cell activation for the biomass multiplied at 37°C for 24 hours both the cell suspension shaking and four variants of activation temperatures (*i.e.* 27, 33, 37, and 40°C) were tested. For process of biosynthesis of L-aspartic acid at temperature 37°C, samples were taken after 5, 15, 30 and 60 minutes. The shaking during cell activation improved cells productivity by more then a factor of two; product biosynthesis rate in first 15 minutes of process for cells that were not shaken was 1.6 g/l/min, and for shaken cells was 3.4 g/l/min. The highest value of the starting maximum rate of the product synthesis, of more then 5.5 g/l/min, and the most efficient total production of L-aspartic acid were found for the cells activated at 37°C (Fig. 6).

Optimization of temperature and pH for L-aspartic acid biosynthesis. As a reference, the process of production of L-aspartic acid was carried out at the temperature of 37°C. An effect of the decrease or increase temperature of 3°C was tested (Fig. 7). In this experiment the best conversion was observed at 37°C.

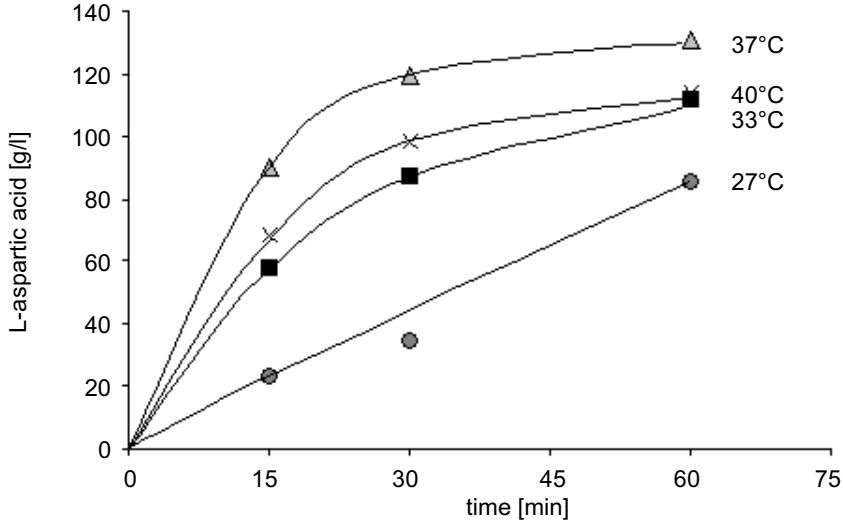

Fig. 6. Effect of cell activation temperatures for *E. coli* P1 on biosynthesis of L-aspartic acid.

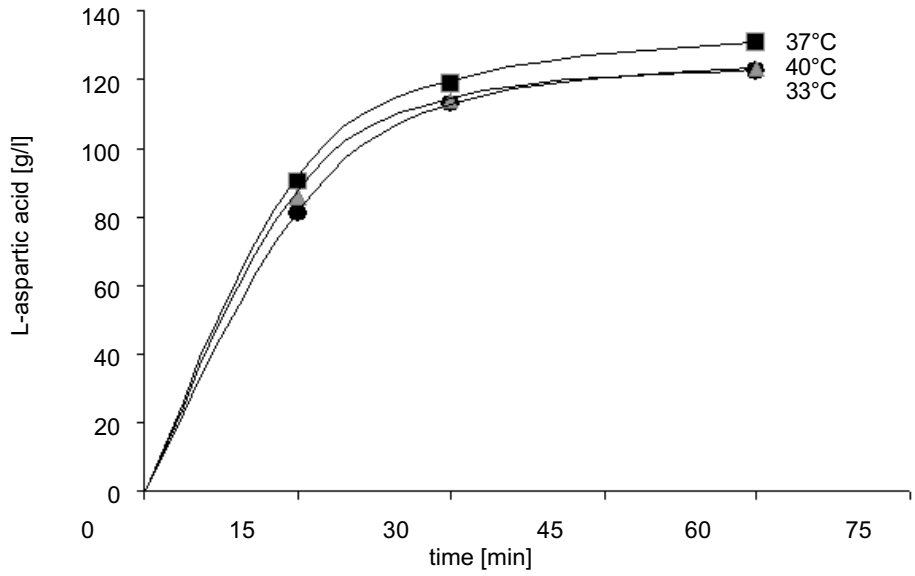

Fig. 7. Effect of biosynthesis temperature on L-aspartic acid production by *E. coli* P1

The starting maximum rate of the product synthesis was 6.0 g/l/min, while at the temperature of 33 or 40°C it was respectively 5.4 and 5.7 g/l/min respectively.

Optimum pH for efficient conversion ammonium fumarate to L-aspartic acid in the reaction catalyzed by aspartase is 8.5–10.5 (Chibata *et al.*, 1974; Tosa *et al*., 1974). In their studies, a production medium with pH 8.5 was used. In our experiment two starting values of pH 8.5 and 9.5 for the production medium were applied (Fig. 8). The change of pH during the process and substrate conversion to L-aspartic acid was investigated. Directly after adding wet mass to the production media pH decreased insignificantly. Constant uniform pH drift during the process was observed in both variants of the experiment but pH in the investigated range did not influence the conversion process.

The application of genetic engineering methods for improving strains is effective manner of rationalization for aspartic acid production. The bacteria with active aspartase were used for researches of L-aspartic acid biotechnology. The most often used were *Escherichia coli* and *Pseudomonas fluorescens* as well as representatives of genera *Enterobacter* and *Citrobacter* (Mukouyama and Komatsuzaki, 2001). The best producers (*E. coli strains* with high aspartase activity) were obtained by introducing aspartase gene using diverse plasmids vectors *e.g.*: pHC18, pSC101, pBR322, pBR325, pKK223-3, pACYC177, pACYC184 (Komatsubara *et al.*, 1986; Nishimura *et al*., 1987a; 1987b;

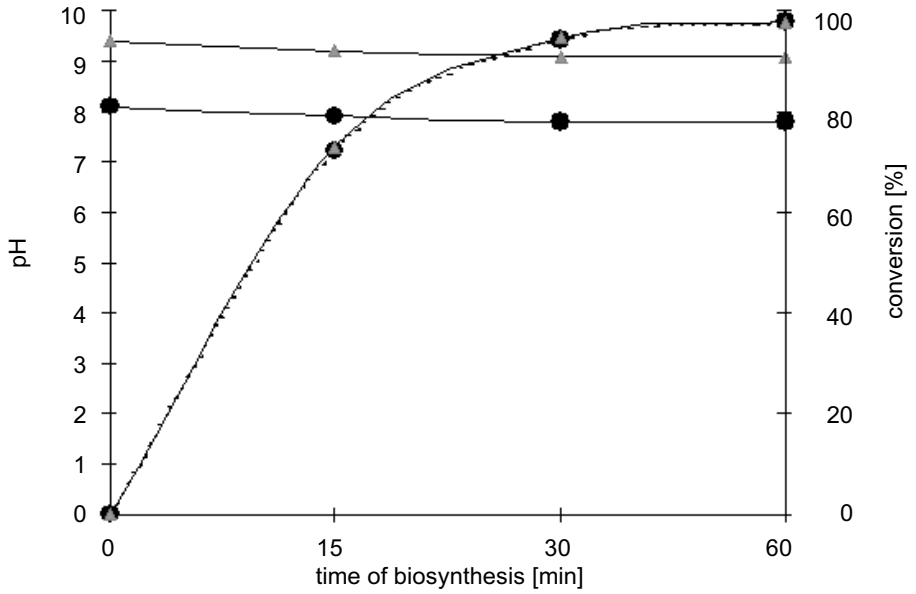

Fig. 8. pH and conversion substrate during biosynthesis of L-aspartic acid by cells *E. coli* P1 in media of pH 8.5 and 9.5

Nishimura *et al*., 1989; Mukouyama and Komatsuzaki, 2001; Mukouyama *et al*., 2004). Obtained recombinants have significantly better efficiency of L-aspartic acid production; for example the level of conversion of fumaric acid into L-aspartic acid by strain *E. coli* PUaspE2 was 20 times higher than for wild strain *E. coli* K-12 (Mukouyama and Komatsuzaki, 2001). *E. coli* P1 derivative constructed and used in our laboratory are over 12-times more effective than parents strain *E. coli* K-12 and over 3-times more effective than mutant *E. coli* B-715. Industrially application of production technology of L-aspartic acid required the immobilization of enzymes or cells. In our laboratory an efficient method for immobilization of *E. coli* B-715 and P1 strains was developed. The details are given in the proceding paper (Papierz *et al*., 2007).

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