Journal of Industrial Microbiology & Biotechnology (2000) 25, 225–228

www.nature.com/jim

ORIGINAL PAPERS

Optimization of aspartate ammonia lyase production by *Bacillus cereus*

YG Garza[1,3], MG Rodríguez[1], CL Hernández[2] and JM Rodríguez[1]

[1]*Departamento de Biotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Coahuila, Blvd. V, Carranza y J. Cárdenas V. Saltillo, Coah, México CP 25000, Mexico;* [2]*Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Apartado postal F-16, Ciudad Universitaria, San Nicolás de los Garza, N.L. México 66450, Mexico*

In an attempt to clarify the function of L-aspartic acid and culture conditions in aspartate ammonia lyase induction, experiments were carried out on aspartase formation in *Bacillus cereus* cells. The enzyme was produced by microorganisms in response to L-aspartic acid, which is catabolized by direct deamination to fumarate. Enzyme synthesis by *B. cereus* was associated with physiological growth stages, which was confirmed by use of the protein synthesis inhibitor, chloramphenicol, whereas it did not influence synthesis when it was added directly to the reactor batch containing a biotransformation system. Aspartase activity was evaluated in a batch reactor by biotransformation of fumaric acid into L-aspartic acid catalyzed by whole *B. cereus* cells. The culture medium for the strain was optimized, which increased the initial aspartase activity threefold. *B. cereus* cells showed optimal aspartase activity at late log phase. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 225–228.

Keywords: aspartate ammonia lyase; induction; *B. cereus*

Introduction

Biotechnological transformations catalyzed by intracellular microbial enzymes have been investigated extensively with many types of natural and synthetic compounds [4,6,11,17,18,20,26]. As a common feature, these biotransformations involve slight changes of the molecular structure of an exogenus substrate without *de novo* molecular synthesis. In this way, aspartate ammonia lyase or aspartase or fumarate ammonia lyase (E.C. 4.3.1.1) from *Escherichia coli* cells immobilized in polyacrilamide and carrageenan gels have been used in the industrial production of L-aspartic acid [7,8,29–32]. Nevertheless, there is not enough information available about the regulatory aspects of intracellular aspartase enzyme production [12]. The use of whole microbial cells as biocatalyst and their applications have been the subject of increased interest [3–5,8,16,17,21,25–28]. In this respect regulatory mechanisms of enzyme synthesis are important for rational use of nutritive substances. Hence, it is necessary to have a detailed knowledge concerning the physiology of the producing microorganism [6,9,10,13,15,20,22,26].

We report here the ability of *Bacillus cereus* cells to produce intracellular aspartate ammonia lyase and a regulatory induction mechanism. Moreover, we present a full characterization of the culture conditions.

Correspondence: Dr JM Rodríguez, Departamento de Biotecnología. Facultad de Ciencias Químicas, Universidad Autónoma de Coahuila, Blvd. V, Carranza y J. Cárdenas V. Saltillo, Coah, México, CP 25000
[3]Also corresponding author.
Received 2 December 1999; accepted 9 August 2000

Materials and methods

Organisms and cultivation

B. cereus was obtained from the culture collection of the Department of Biotechnology of the Autonomous University of Coahuila. Microorganisms were precultivated on agar slants consisting of 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) KCl, 0.1% (w/v) NaCl, 2% agar supplemented with L-aspartic acid (0%, 0.05%, 0.1%, 0.2%, 0.3%). The medium was adjusted to pH 7.0 before being autoclaved at 121°C for 15 min. Cultures were incubated at 30°C for 24 h and were then stored at 4°C. Liquid medium contained 0.8% nutrient broth, 1.0% peptone, 0.25% yeast extract, 0.1% L-aspartic acid, pH 7.0. A 100-ml portion of the medium in a 1-l flask was sterilized by autoclaving it for 15 min at 120°C, inoculated with 0.1 ml cell suspension taken from a stock culture, and incubated for 2, 4, 6, 8, 10, 12, 14 and 16 h at 30°C on a reciprocal shaker operated at 250 strokes per minute with an 8-cm amplitude. After aerobic cultivation cells were harvested from culture broth by centrifugation (6370×*g*, 20 min at 4°C) and were washed twice, suspended with 0.01 M potassium sodium phosphate buffer (pH 7.0) and used as a suspension of intact cells.

Culture medium optimization

Optimization of the medium was performed testing each component for its ability to support growth and aspartase activity. Varying component concentrations were made and are shown in Table 1.

Cultures were incubated for the desired length of time at 30–32°C with reciprocal shaking. The influence of each medium component on aspartase activity was determined by biotransfor-

Table 1 Concentrations of liquid medium components

Component	Concentrations (%)
Nutrient broth	0, 0.1, 0.3, 0.5, 0.8, 1.0
Peptone	0, 0.1, 0.3, 0.5, 1.0
Yeast extract	0, 0.1, 0.2, 0.25, 0.3

mation assays using resting cells of *B. cereus* that were grown under the conditions described above.

Protein measurement

Cellular protein was measured by the modified Lowry technique [24] using bovine serum albumin as the standard.

Biotransformation assay

Bioconversion experiments were performed with intact resting cells in a batch and thermostated stirred reactor and monitored by the disappearance of ammonium fumarate at 240 nm in the reaction mixture consisting of 0.5 M of ammonium fumarate (pH 8.0, T=37°C), 1 mM $MgCl_2$, 1% toluene and 1–2 mg/ml cell protein [25,29,31]. The initial reaction volume was 33 ml. Samples (250 μl) were taken every 5, 10 and 15 min over a 2-h period. The reaction was stopped by immersion in a boiling water bath for 5 min. The remaining fumarate was determined spectrophotometrically. One unit of aspartase activity is defined in terms of micromoles of transformed substrate per milligram of protein per hour. Cells were removed from the batch reactor by centrifugation (6370×g, 15 min, 4°C) and the pH of the supernatant was adjusted to 2.5–3.0 with sulphuric acid. The precipitated product, L-aspartic acid, was separated by filtration and washed with water.

Effect of protein synthesis inhibitor

The ability *B. cereus* cells to form aspartase in fermentor cultures but not in the reaction system for bioconversion of fumaric acid into L-aspartic acid was verified by complementation experiments in which chloramphenicol was used. Cells were cultured for 6 h and at 2, 3, 4 and 5 h after the beginning of incubation at 30–32°C with reciprocal shaking, 300 μg ml^{-1} chloramphenicol was added and cultivation was continued for an established time after which cells were removed by centrifugation and used as biocatalyst for the bioconversion process. Chloramphenicol was also added to the reaction system in the reactor.

Analytical methods

Growth was monitored by plate counts and by following total cellular protein. L-Aspartase activity in the forward or amination direction was determined spectrophotometrically by measuring fumarate consumption at 240 nm. L-Aspartic acid was determined by HPLC on a reverse-phase column packed with 5 μm Lichrosorb RP-18 using acetonitrile/phosphate buffer as eluent.

Results

Induction of L-aspartate ammonia lyase activity

The best L-aspartic acid concentration (0.1%) as inducer was selected on a solid medium, with the other concentrations tested in the media (0.05%, 0.2% and 0.3% of L-aspartic acid), growth

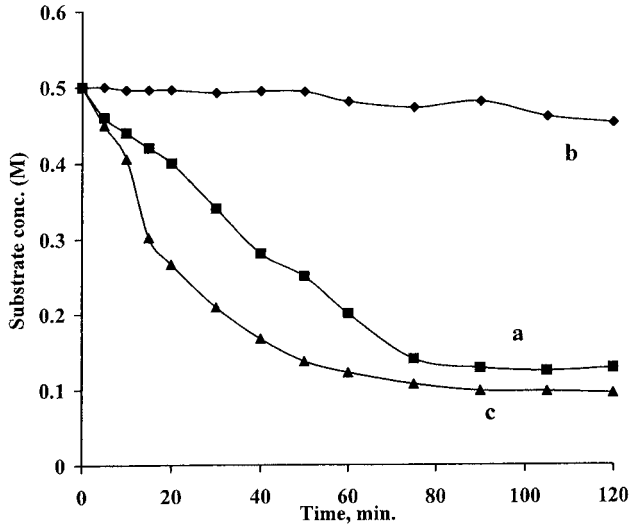

Figure 1 Comparison of the kinetics of fumarate consumption catalyzed by *B. cereus* grown in media: (a) (■) with inducer, (b) (◆) without inducer and (c) (▲) with optimal components and concentration.

appeared slowly after 24 h. Figure 1 shows the results of a kinetic study of substrate consumption when the amination reaction was catalyzed by resting cells grown in liquid media with (a) and without (b) L-aspartic acid as inducer. The curve for (b) shows no significant initial reaction rate (2.5×10^{-4} mM/min), calculated as slope in figures [S]=$f(t)$. The initial rate value for curve "a" was 5.2×10^{-3} mM/min. This showed that there was induced expression. The induction relation was 20.8 in terms of induced enzyme activity and basal enzyme activity.

Optimum incubation time

In a biotechnological process, if synthesis of a desired product is related to microbial growth, the basic route to increase productivity must be oriented to obtain optimal growth expressed in quantitative terms. If not, then it is necessary to correlate growth and the physiological condition of the microorganism [2,23]. The results obtained are shown in Figure 2. Aspartase

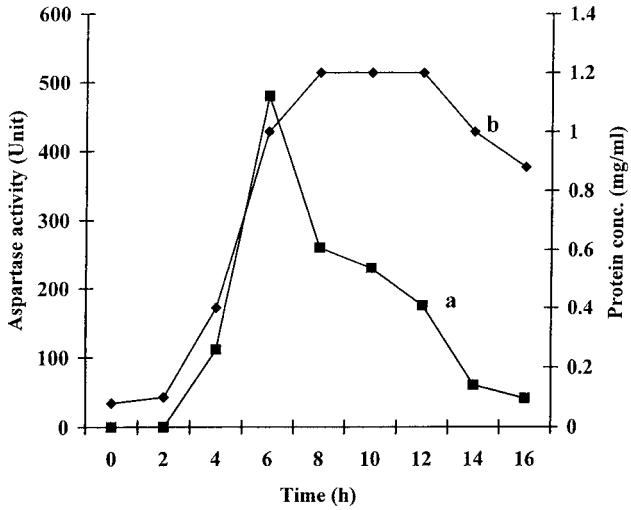

Figure 2 Influence of cultivation time on aspartase activity of *B. cereus*, (a) (■) and determination of cell protein, (b) (◆).

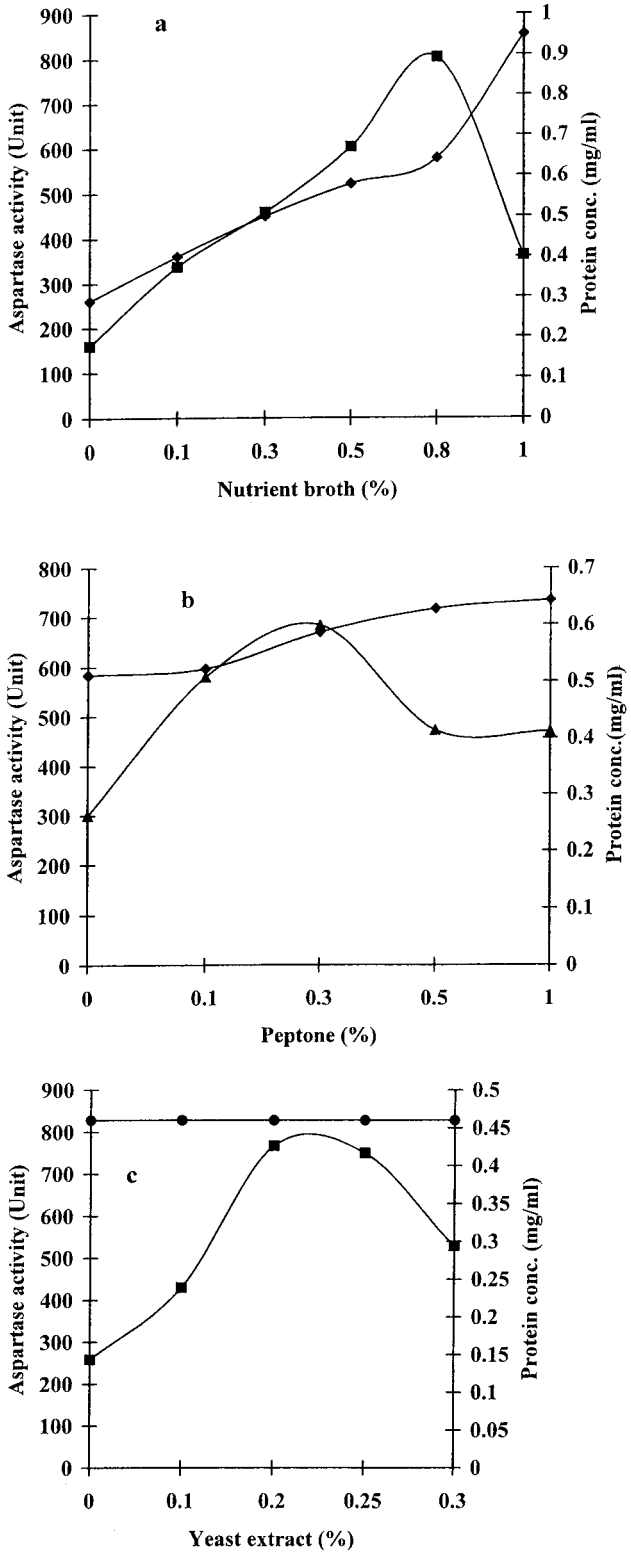

Figure 3 Effect of nutrient broth (a), peptone (b) and yeast extract (c) on aspartase activity of *B. cereus* as biocatalyst for the aspartate ammonia lyase reaction.

activity was associated with the physiological state in each growing phase of the *B. cereus* cells, reaching maximum activity (480 U) in a 6-h incubation (Figure 2a) and maximum protein production was reached 2 h later (Figure 2b). After 8-h incubation the aspartase activity diminished about 50% compared with the activity reached at 6 h.

Effect of nutrient broth

The formation of aspartase may be influenced by the concentration of nutrient broth in the medium. The effect of different nutrient broth concentrations was compared. An increase in nutrient broth concentration increased the amount of biomass determined by cell protein concentration (Figure 3a). The fundamental postulate of growth theory was done for conditions in which the growth rate is determined by a limited concentration of one of the medium compounds [14]. Thus, the limitation was analyzed as a phenomenon in which growth rate is decreased by decreasing the concentration of this component in the medium. Therefore, the nutrient broth in this medium is a limiting factor for *B. cereus* growth.

Effect of peptone

An organic nitrogen source may be useful not only for growth but also for acceleration of enzyme formation. Therefore, the effect of various peptone concentrations was investigated. The results are shown in Figure 3b. Peptone did not favor growth because the cell protein concentration did not change significantly between 0% and 1.0% peptone. The addition of 0.3% peptone enhanced aspartase formation as revealed by the initial rate reaction.

Effect of yeast extract

This component is not a growth-limiting factor although addition of different concentrations of yeast extract showed an appreciable effect on specific aspartase activity (Figure 3c). The best result was obtained when 2% or 2.5% yeast extract was added. In this case, the term "growth" is not related to an increment of cell population.

The course of aspartase formation

Initial aspartase formation was observed when chloramphenicol was added 4 h after the beginning of cultivation (Table 2). At near-optimum incubation time (6 h), the initial aspartase reaction rate increased whereas protein synthesis was inhibited. Aspartase activity was identical in the presence or absence of the protein synthesis inhibitor in the reaction system.

Finally, results obtained with cells grown in medium with optimal components and concentrations and optimal cultivation time (6 h), and used as biocatalyst in the biotransformation of fumaric acid into aspartic acid are shown in Figure 1c. The initial rate increased significantly and the aspartase activity was 541 U.

Table 2 Effect of chloramphenicol (300 μg l^{-1}) on aspartate ammonia lyase formation in *B. cereus* over cultivation time[a]

Time of addition of chloramphenicol (h)	Protein concentration (mg. ml^{-1})	Vo. EE+3 (mM/min)
6[a]	0.87	8.3
5[a]	0.53	6.35
4[a]	0.30	3.6
3[a]	0.09	0
2[a]	0.085	0
Control[a]	0.87	8.4

[a]Six-hour-incubation cells without chloramphenicol were used as control.

Discussion

It is suggested that L-aspartic acid induces aspartase formation in *B. cereus* when it's incorporated within the cell as fumarate into the tricarboxylic acid cycle as a result of direct deamination [9]. This route is not characteristic of all microorganisms [15]. As shown in Figure 2a, aspartase formation started immediately (3 h, early log phase, Figure 2b). However, maximum aspartase activity was reached after 6 h cultivation (Figure 2a), when cells were in late log phase (Figure 2b). These results suggest that in the incubation period of 8–12 h (stationary phase, Figure 2b) a very small fraction of the cell protein had aspartase activity. The rapid decrease in fumarate depends either on the rate of fumarate biotransformation by aspartase or on an increased rate of fumarate uptake, or on both. Previous studies [7,29–32] used other microorganisms that need more cultivation time and aspartase activity is minor.

It was confirmed that the concentrated cells used as biotransformation catalysts are nongrowing, live cells that retain most of the enzyme activities of growing cells [10], since the protein synthesis inhibitor, chloramphenicol, did not modify the aspartase activity of *B. cereus* cells in the reaction system. We suggest that the cell membrane of *B. cereus* suffers changes in its components when it is grown in the presence of chloramphenicol; during the biocatalytic process without the addition of toluene (plasmolizer), the bacteria showed the same catalytic activity (data not shown). Furthermore, aspartase production was sensitive to the concentration of medium components, since peptone and yeast extract were diminished 70% and 50%, respectively in the optimum medium and achieved a better initial reaction rate of about 8.3×10^{-3} mM/min (Figure 1c). Interest in working with intact microbial cells as biocatalysts is based on the relative ease of stimulating enzyme synthesis through changes of the media and with genetic manipulation. Moreover, enzymes in intact cells usually are more stable than their isolated counterparts. They are much cleaner than reactions with growing cells, resulting in easier product isolation [1,9,19,26,30].

References

1 Berezin IV and VI Yakovleva. 1980. Enzymatic methods for production of several aminoacids. *Dokl Acad Nauk SSSR Pushino*, 16–17.
2 Briukov UV and VM Kantere. 1985. Optimization of periodical processes in the microbiological synthesis. *J Microbiol* 11: 29–38.
3 Buikov VA, IA Krilov, MN Manakov, NS Markvichev, LM Orlova and NV Tarasova. 1987. Microbiological productions of physiological active substances and preparations. *Moscow, Russ Biotech Ser* 6: 10–20.
4 Buikov VA, MN Manakova, VI Panfilov, AA Svitsov and NV Tarasova. 1987. Proteic substances production. *Moscow, Russ Biotech Ser* 5: 105–134.
5 Cenci G and G Caldini. 1997. Catechol dioxygenase expression in a *Pseudomonas fluorescens* strain exposed to different aromatic compounds. *Appl Microbiol Biotechnol* 47: 306–308.
6 Cardillo R, S Servi and C Tinti. 1991. Biotransformation of unsaturated aldehydes by microorganisms with pyruvate decarboxylase activity. *Appl Microbiol Biotechnol* 36: 300–303.
7 Chibata I, T Tosa and T Sato. 1974. Immobilized aspartase containing microbial cells: preparation and enzymatic properties. *Appl Microbiol* 27: 878–885.
8 Chibata I, T Tosa and T Sato. 1986. Continuous production of L-aspartic acid. *Appl Biochem Biotechnol* 13: 213–240.
9 Debabov VG and VA Libshits. 1988. Modern methods for creation of industrial microbial strains. *Moscow, Russ Biotech Ser* 2: 6–68.
10 Elinov NP. 1989. Chemistry Microbiology. Moscow, Russ. ed. HighSch, pp. 108–109.
11 El-Sharkawy SH, L Dostal and JPN Rosazza. 1993. Microbiological transformations of lipids: acyl specific hydrolisis of lard by yeasts. *Appl Environ Microbiol* 59: 725–728.
12 Falzone CJ, WE Karsten, JD Conley and RE Viola. 1988. L-Aspartase from *E. coli*: substrate specificity and role of divalent metal ions. *Biochemistry* 27: 9089–9093.
13 Foor F, N Morin and KA Bostian. 1993. Production of L-DOPA in *Escherichia coli* with the tyrosine phenol-lyase gene cloned from *Erwinia herbicola*. *Appl Environ Microbiol* 59: 3070–3075.
14 Galovlev EL. 1980. Influence of Growth Limitation on Microbial Metabolism. Russian Academy Science Center Biological Research, pp. 22–23.
15 Goel A, J Ferrance, J Jeong and MM Atai. 1993. Analysis of metabolic fluxes in batch and continuous cultures of *Bacillus subtilis*. *Biotechnol Bioeng* 42: 686–696.
16 Gottschalk G. 1982. Bacterial Metabolism. Trad.Inglés-Ruso.red. E.N. Kondriateva. Ed. Mir., Moscú, Russia, pp. 26, 27, 132–134, 152–185.
17 Honorat A, F Monot and D Ballerini. 1990. Comparative study of the enzymatic synthesis of L-valine by purified enzymes, crude extract, intact or permeabilized cells from *Bacillus megaterium*. *Appl Microbiol Biotechnol* 34: 236–241.
18 Huang Z, L Dostal and JPN Rosazza. 1993. Microbial transformation of ferulic acid by *Saccharomyces cerevisiae* and *Pseudomonas fluorescens*. *Appl Environ Microbiol* 59: 2244–2250.
19 Lee J and SJ Parulaker. 1993. Enhanced production of α-amylase in fed batch cultures of *B. subtilis* TN106(pAT5). *Biotechnol Bioeng* 42: 1142–1150.
20 Larroche C, C Creuly and JB Gros. 1995. Fed-batch biotransformation of β-ionone by *Aspergillus niger*. *Appl Microbiol Biotechnol* 43: 220–227.
21 Nakamichi K, K Nabe, Y Nishida and T Tosa. 1989. Production of L-phenylalanine from phenylpiruvate by *Paracoccus denitrificans* containing aminotransferase activity. *Appl Microbiol Biotechnol* 30: 243–246.
22 Nakamura T, S Hoashi and S Natkatsu. 1978. Culture conditions for inulase production by *Aspergillus niger*: III. Studies on microbial inulase. *Nippon Nogei Kagaku Kaishi* 52: 105–110.
23 Pelczar JM, DR Reid and ECS Chan. 1992. Microbiology, 4th ed. McGraw-Hill, pp. 88–179.
24 Peterson GL. 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* 83: 346.
25 Rodríguez MJ and GY Garza. 1994. Investigación sobre la síntesis del aminoácido L-aspartico por células de *B. cereus* con actividad aspartatoamoníacoliasa. *Rev Soc Quim Mex* 38 (4): 162–168.
26 Shin HS and PL Rogers. 1995. Biotransformation of benzaldehyde to L-phenylacetilcarbinol, an intermediate in L-ephedrine production, by immobilized *Candida utilis*. *Appl Microbiol Biotechnol* 44: 7–14.
27 Buto S, L Pollegioni, D'Angiuro and MS Pilone. 1994. Evaluation of D-aminoacid oxidase from *R. gracilis* for the production of α-cetoacids. A reactor system. *Biotechnol Bioeng* 44: 1288–1294.
28 Tisichnaia IV, MJ Rodríguez, VI Yakovleva and IV Berezin. 1986. Some kinetic regularities of tyrosine synthesis catalyzed by free and immobilized bacterial cells. Effect of mass transfer of the reactants on the kinetics of tyrosine phenol lyase. *Bioquimia* 51: 1768–1775.
29 Sato T, Y Nishida, T Tosa and I Chibata. 1979. Immobilization of *E. coli* cells containing aspartase activity with k-carrageenan. Enzymatic properties and application for L-aspartic acid production. *Biochim Biophys Acta* 570: 179–186.
30 Tosa T, T Sato, T Mori and I Chibata. 1974. Basic studies for continuous production of L-aspartic acid by immobilized *E. coli* cells. *Appl Microbiol* 27: 886–889.
31 Tosa T, S Tadashi, N Yutaka and I Chibata. 1977. Reason for higher stability aspartase activity of immobilized *E. coli* cells. *Biochim Biophys Acta* 483: 193–202.
32 Umemura I, S Takamatsu, T Sato, T Tosa and I Chibata. 1984. Improvement of production of L-aspartic acid using immobilized microbial cells. *Appl Microbiol Biotechnol* 20: 291–295.
33 S VanSiujdam JC and JA Roels. 1993. Strategies of Biotechnological Industries. Press VI European Congress On Biotechnology, Firenze, Italy, June 13–17.
34 Yukawa H, T Nara and Y Takayama. 1980. Process for production of L-aspartic acid. Patent USA 4326029.