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Short Communication

Biosynthesis of L-Aspartic Acid from Maleic Anhydride Waste Residues by Immobilized Aspartase from Genetically Engineered Bacteria

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Abstract

An economical biosynthetic preparation of L-aspartic acid from maleic anhydride waste residues by immobilized aspartase from genetically engineered bacteria was investigated. Here, a feasible utilization strategy for the synthesis of L-aspartic acid from maleic anhydride waste residues was demonstrated. The immobilized recombinant aspartase from genetically engineered bacteria was prepared by using sodium alginate and glutaraldehyde as an immobilized matrix. The immobilized recombinant aspartase could efficiently convert maleic anhydride waste residues to L-aspartic acid at pH 8.0 and 45°C in the presence of Tween 80. In a scale-up study, the conversion ratio of maleic anhydride waste residues reached up to 95.7% with a final L-aspartic acid concentration of 51.7 g/L.

Keywords*:* L-aspartic acid; Maleic anhydride; Aspartase; Immobilization

Introduction

L-aspartic acid is an important compound which is extensively used in the manufacturing industry. It is a synthetic sweetening agent and contains amino acid derivatives of four carbon, such as butylene glycol and butylene oxide (Patel *et al.* 2017). The United States Department of Energy qualifies it as one of the high value-added products obtained from biomass. Biological process strengthening was performed for successful and economical, and vast production of immobilized aspartase from genetically engineered bacteria (Jayasekera *et al.* 1997; Shi *et al.* 1997). Because of large markets of commodities like NutraSweet®, the demand for L-aspartic acid has increased greatly in recent years. These demands have made it necessary to carry out important research for the biosynthesis of L-aspartic acid.

Aspartase catalyzes the invertible amination of fumaric acid to L-aspartic acid. In the present study, one of the main ways of comprehensive utilization of maleic anhydride waste residue to produce fumaric acid using an immobilized aspartase has been evaluated. Aspartase is a very important enzyme in microbial metabolic pathways and is purified from various bacteria. Most of the studies have focused on AspB obtained from *Bacillus* spp., and YM55-1 and aspartase obtained from *Escherichia coli* (Kawata *et al.* 1999; 2000; Fujii *et al.* 2003; Weiner *et al.* 2008; Puthan *et al.* 2009). The crystallographic structure of aspartase enzyme has been analyzed (Fibriansah *et al.* 2011). Aspartase is found in all types of bacteria and can be purified from *E. coli* (Kartsen *et al.* 1985; Takagi *et al.* 1985), *Pseudomonas fluorescens* (Takagi *et al.* 1984; 1986), *Bacillus subtilis* (Sun and Setlow, 1991), *Hafnia alvei* (Nuiry *et al.* 1984; Yoon *et al.* 1995), and a thermophile, *Bacillus sp*. YM55-1 (Kawata *et al*. 1999). Aspartase activity can be affected due to factors such as the concentration, temperature, and pH of the substrate.

A change in pH could lead to the disaggregation of groups on the aspartase active site. Enzymatic reactions for immobilization of aspartase cells require appropriate pH and environment. This study provides us with an alternative maleic anhydride waste residues utilization strategy to synthesize L-aspartic acid.

Materials and Methods

Materials

The maleic anhydride waste residues were provided by Suzhou Biological Engineering Co. Ltd. (Jiangsu, China). Physicochemical characteristics of maleic anhydride waste residues showed the presence of 23.6% maleic anhydride. All reagents used in the experiment were of analytical grade.

Microorganisms and fermentation

The aspartase gene L20944.1 was cloned from *E. coli* k-12 W3110 obtained from China General Microbiological Culture Collection Center. The *E. coli* BL21 strain carrying the plasmid Duet was used. An inoculating loop of

microorganism was used to inoculate a 250 mL conical flask containing with 100 mL of LB culture medium. The conical flasks were incubated on an oscillating incubator at 210 rpm for 12 h at 28°C. The conical flasks containing 100 mL of sterilized main medium contained 1% (m/v) bacto-tryptone, 3% (m/v) corn steep liquor, and 1% (m/v) lactobiose. The conical flasks were incubated on an oscillating incubator at 210 rpm for 15 h at 32°C. The aspartase producing genetically engineered bacteria were centrifuged at 10,000 rpm for 10 min at 4°C to obtain wet aspartase cells.

Immobilization of aspartase cells

Sodium alginate (25 g/L), glutaraldehyde (1 g/L), and aspartase cells were mixed in a beaker. To the mixture, calcium chloride solution (20 g/L) was added dropwise. The mixture immobilized the aspartase cells in a solution containing calcium chloride.

Enlarge cultivation and biosynthesis

The preparatory fermentation of aspartase was studied in concussion incubators of different volumes (0.25−1.0 L). Fermentation of aspartase genetically engineered bacteria was carried out in a 20 L fermentor (Baoxing biotech., Shanghai, China) which was filled with 14 L of media containing 5% (m/v) corn steep liquor, 1.0% (m/v) peptone, and 1% (m/v) lactobiose. The fermentation temperature was set at 30°C and the zymotic fluid pH was maintained at a pH of 8.0 ± 0.2. The zymotic fluid was centrifuged at 10,000 rpm at 4°C for 10 min to obtain the aspartase immobilized genetically engineered bacterial wet cells. Aspartase cell, sodium alginate (25 g/L), and glutaraldehyde (1 g/L) were mixed in a beaker.

Calcium chloride solution (20 g l/L) was added to the mixture dropwise resulting in immobilization of the aspartase cells. The immobilized aspartase cells were directly mixed with maleic anhydride waste residues in a 40 L reactor at pH 8.0 and 45°C in the presence of Tween 80 for biosynthesis (Baoxing Biotech, Shanghai, China). The immobilized aspartase cell reaction was carried out in the presence of 200 g/L maleic anhydride waste residues and Tween 80 (0.04%, m/v) at 45°C and pH of 8.0.

Aspartase activity assay

By analyzing L-aspartic acid biosynthesis from maleic anhydride waste residues, aspartase activity was analyzed by AAA (Hitachi L-8900, Japan). The reaction of the immobilized recombinant aspartase from genetically engineered bacteria was carried out in the presence of 10 mL of maleic anhydride waste residues, Tween 80 (0.04%, m/v), and 0.5 g Aspartase cell at 45°C for 10 h.

Statistical analyses

All experiments were performed in triplicates. Analysis of variance (ANOVA) was performed using S.A.S. program version 8.1 (S.A.S. Institute Inc., Cary, NC, USA).

Results

Effects of substrate concentration, temperature and pH on aspartase activity

In this study, the pH-dependency of L-aspartic acid by aspartase was studied in a pH range of 5 to 11. The reaction mixture in 1 L containing 200 g L^{-1} maleic anhydride waste residues and 1 g immobilized aspartases cells (Fig. 1). The mixture was shaken at 220 rpm, 10 h and 37°C. The optimal initial pH required for the synthesis of L-aspartic acid was found to be 8.0 (Fig. 2A). The effect of temperature on L-aspartic acid synthesis was studied for a temperature range of 20°C−60°C (Fig. 2B). Reaction mixture in 1 L containing 200 g L^{-1} maleic anhydride waste residues and 1 g immobilized aspartases cells. The mixture was shaken at 220 rpm, 10 h and pH 8. The best yield of L-aspartic acid was observed at 45°C. The change in conversion at different concentrations of the substrate is shown in Fig. 2C. The reaction mixture in 1 L containing 200 g L^{-1} maleic anhydride waste residues and 1 g immobilized aspartases cells. The mixture was shaken at 220 rpm, 10 h, 37°C and pH 8. The results showed that maleic anhydride waste residues could be converted to L-aspartic acid even at a high substrate concentration of maleic anhydride waste residues (200 g/L).

Preparation of L-aspartic acid

The zymotic fluid was centrifuged at 10,000 rpm for 10 min at 4°C. The aspartase cells in the calcium chloride solution were immobilized. Scanning electron microscopic image of immobilized aspartase cells is shown in Fig. 3. The immobilized cells adsorbed many aspartase on the surface (Fig. 3B). The surface of immobilized cells without aspartase was smooth (Fig. 3A). The immobilized aspartase cells were mixed with maleic anhydride waste residues at 45°C and pH 8. The synthesis of L-aspartic acid was catalysed by immobilized aspartases cells (5 g) and used maleic anhydride waste residues (200 g L^{-1}) as an economical substrate. The mixture was shaken at 220 rpm, 45°C and pH 8. The concentration of L-aspartic acid increased at 10 h (Fig. 4). Activated carbon was added at 60°C for 4 h. HCl was added to adjust the pH to 2.8 required for the synthesis of the product.

After cooling the filtrate at 15°C, the crystals of L-aspartic acid were separated by filtration. After drying the crystals, 2,014.6 g of L-aspartic acid was obtained. The melting point of L-aspartic acid was found to be 230°C (decomposed). The infrared spectrum of L-aspartic acid included the spectra of amide groups showing vibrational absorption (1500−1800/cm) (Fig. 5).

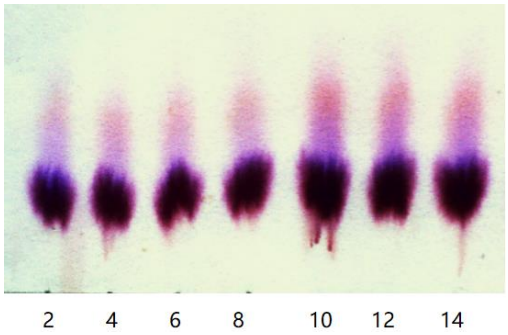

Fig. 4: Effect of time on aspartases from *Escherichia coli* k-12 catalysed synthesis L-aspartic acid

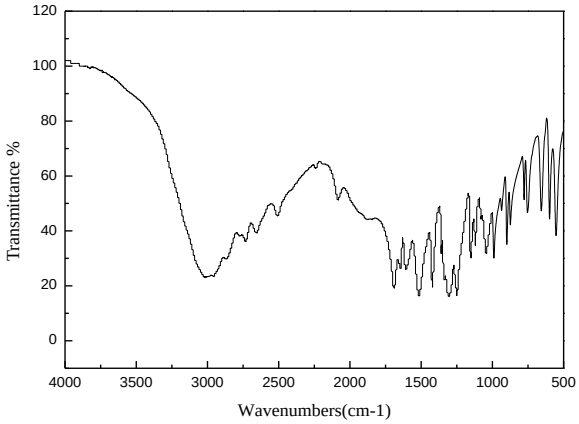

Fig. 5: Infrared spectrum of L-aspartic acid

Discussion

Aspartase catalyzes the reversible conversion of fumarate and ammonia to L-aspartate. Aspartase is used for the production of L-aspartic acid using immobilized cells and hyperproducing strains of *E. coli* (Chibata *et al.* 1974; Sato *et al.* 1979; Nishimura *et al.* 1989). Aspartase (EC 4.3.1.1) plays an important role in bacterial nitrogen metabolism by catalyzing the reversible conversion of L-aspartate to fumarate and ammoniumion (Viola 2000).

It is among the most specific enzymes and catalyzes the reaction with high specificity and catalytic efficiency (Ram and Mukesh 2013). Typically, aspartase is a bacterial enzyme and has been reported from *Bacillus* spp. (Singh *et al.* 2005). The enzyme from *E. coli* has been studied most extensively, and its crystal structure has been elucidated (Shi *et al.* 1997; Tomomi *et al.* 2003). L-Aspartase from *E. coli* is composed of four identical subunits of molecular weight 52,200 Da. Each monomer of aspartase is composed of three domains (Wang *et al.* 2000).

Maleic anhydride is an important organic raw material. In the process of production, purification, and equipment cleaning, wastewater containing a large number of acidic by-products is produced. The solid residues obtained from concentrated crystallization of wastewater are generally referred to as maleic anhydride waste residue, which contain maleic acid, fumaric acid, succinic acid, and phthalic acid in abundance.

Fig. 1: The immobilization of aspartase cells from *Escherichia coli* k-12. Sodium alginate (25 g L^{-1}), glutaraldehyde (1 g L^{-1}) and aspartase cell were mixed in beaker

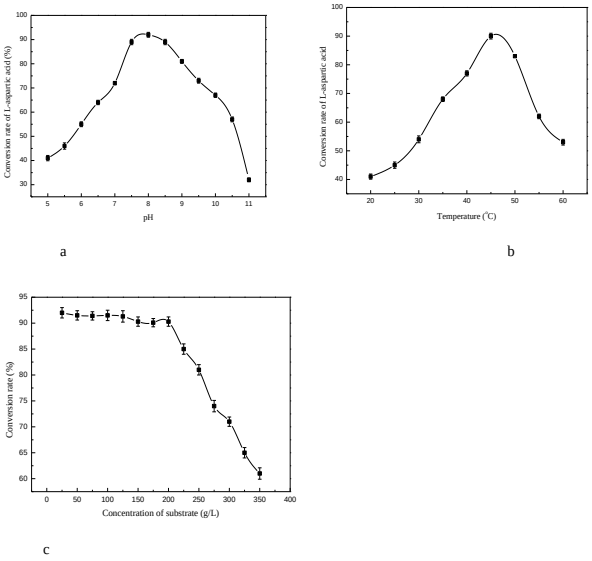

Fig. 2: Effect of factors on aspartases from *Escherichia coli* k-12 catalysed synthesis L-aspartic acid. (**a**: pH; **b**: temperature; **c**: substrate concentration)

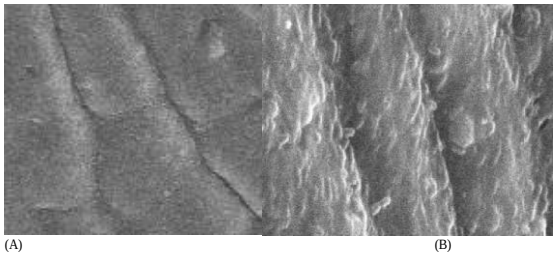

Fig. 3: Scanning electron microscopy of immobilization of aspartase cells from *Escherichia coli* k-12 (10 μm). Scanning electron microscopy of immobilization cells without aspartase (**A**). Scanning electron microscopy of immobilization cells with aspartase (**B**)

In China, a large amount of maleic anhydride waste residue needs to be treated annually. The immobilized aspartase from genetically engineered bacteria show not only indurative storage and reaction stability but also high activity recovery. Immobilized enzyme separation recovery has been previously widely reported (Garcia-Galan *et al.* 2011; Rafael *et al.* 2012; Lyu *et al.* 2019).

The research results show that after the above processing, the immobilized recombinant aspartase could efficiently convert maleic anhydride waste residues to L-aspartic acid at pH 8.0 and 45°C in the presence of Tween 80. In a scale-up study, the conversion ratio of maleic anhydride waste residues reached up to 95.7% with a final L-aspartic acid concentration of 51.7 g/L.

Conclusion

On the basis of the enzymatic synthesis of L-aspartic acid from maleic anhydride waste residues, it can be stated that biosynthesis is a feasible and economical method. Through scaling up fermentation, the conversion ratio of maleic anhydride waste residues reached up to 95.7% with a final L-aspartic acid concentration of 51.7 g/L. These results also lay a foundation for the use of industrial maleic anhydride waste residues.

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