J. Microbiol. Biotechnol. (2007), **17**(1), 116–122

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Development of Bioreactor System for L-Tyrosine Synthesis Using Thermostable Tyrosine Phenol-Lyase

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Received: August 28, 2006
Accepted: October 8, 2006

Abstract An efficient enzyme system for the synthesis of L-tyrosine was developed using a fed-batch reactor with continuous feeding of phenol, pyruvate, and ammonia. A thermo- and chemostable tyrosine phenol-lyase from *Symbiobacterium toebii* was employed as the biocatalyst in this work. The enzyme was produced using a constitutive expression system in *Escherichia coli* BL21, and prepared as a soluble extract by rapid clarification, involving treatment with 40% methanol in the presence of excess ammonium chloride. The stability of the enzyme was maintained for at least 18 h under the synthesis conditions, including 75 mM phenol at pH 8.5 and 40°C. The fed-batch system (working volume, 0.5 l) containing 1.0 kU of the enzyme preparation was continuously fed with two substrate preparations: one containing 2.2 M phenol and 2.4 M sodium pyruvate, and the other containing 0.4 mM pyridoxal-5-phosphate and 4 M ammonium chloride (pH 8.5). The system produced 130 g/l of L-tyrosine within 30 h, mostly as precipitated particles, upon continuous feeding of the substrates for 22 h. The maximum conversion yield of L-tyrosine was 94% on the basis of the supplied phenol.

Key words: Tyrosine phenol-lyase, *Symbiobacterium toebii*, fed-batch, bioconversion, L-tyrosine

L-Tyrosine is important to the structure of almost all proteins in nature. It is also required to make several neurotransmitters, such as L-DOPA, dopamine, epinephrine, and norepinephrine, which are required to regulate the brain functions that affect our moods [2, 6, 25]. Furthermore, its derivatives in body fluids play regulatory roles in the functions of the hormonal systems in the adrenal, thyroid, and pituitary glands. Consequently, L-tyrosine and its aromatic derivatives are utilized in primary medications for the treatment of brain diseases, such as Basedow's and Parkinson's.

In most living organisms, L-tyrosine is principally synthesized from L-phenylalanine. Yet, for the industrial production of L-tyrosine and its derivatives, attention has been focused on enzymatic synthesis using tyrosine phenol-lyase (TPL) from phenolics, such as hydroxylated or halogenated derivatives of phenol, 4-chlorophenol, 4-nitrophenol, and catechol [12, 24]. To date, TPL has been found in various enteric bacteria, including *Erwinia herbicola* [4, 10], *Escherichia intermedia* [19], and *Citrobacter freundii* [13], and also in the thermophilic bacteria *Symbiobacterium thermophilum* [22] and *S. toebii* [16]. However, the enzymes from mesophilic bacteria show a low stability in phenolic substrates, whereas those from thermophilic bacteria remain stable even in the presence of high concentrations of phenolic substances [15, 17].

In earlier studies, free and immobilized mesophilic TPLs were tested as a biocatalyst for the synthesis of L-tyrosine [5, 24], yet this requires costly preparation steps and a continuous supply of pyridoxal-5'-phosphate (PLP). The application of whole cells as the biocatalyst resulted in an improved productivity [3], although it raises other problems in the downstream process, including the generation of large clumps of cell aggregates during the precipitation of L-tyrosine. Finally, an immobilized cell preparation resulted in 10 g/l L-tyrosine in a batch process with a high yield [19]; however, this is also unsuitable for the efficient production of L-tyrosine, as microcrystalline precipitates accumulate in the immobilization matrix and interfere with the diffusion of the reactants.

Generally speaking, the enzyme costs are the most prominent factor determining the economical efficiency of

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enzyme-catalyzed synthesis. Thus, in a synthesis system with a toxic substrate, controlled feeding of the substrate would seem to offer a more efficient enzymatic process than a conventional batch method [18]. Accordingly, the present study developed a fed-batch system using a partially purified enzyme as the biocatalyst. The phenol and pyruvate concentrations were kept at a non- or weakly inhibitory level by controlled feeding of the denaturative substrates, thereby enabling the TPL to operate stably and at high reaction rates.

MATERIALS AND METHODS

Materials

The sodium pyruvate and pyridoxal-5'-phosphate (PLP) were obtained from Musashino Shoji (Japan) and Fluka (Japan), respectively. The methanol was purchased from Merck (NJ, U.S.A.), and the yeast extract and Bacto Casitone purchased from Difco (MO, U.S.A.). The other chemicals, including L-tyrosine, phenol, NH_4Cl, and polyethyleneglycol with an MW of 3,350 or 8,000, were all purchased from Sigma (MO, U.S.A.).

Plasmid and Enzyme Expression

Escherichia coli BL21 harboring the constitutive expression system pHCE19T(II)-TPL [9] was used for the production of *S. toebii* TPL [16]. For mass preparation of the biocatalyst, the *E. coli* was cultivated in a CPGY-ampicillin medium, consisting of 30 g Bacto-casitone, 5 g yeast extract, 5 g glutamic acid, 50 g glycerol, 3 g KH_2PO_4, 7 g Na_2HPO_4, 3 g NH_4Cl, 1 g $MgSO_4$, and 50 mg ampicillin per liter of distilled water. The main culture medium (working volume of 25 l) was inoculated with an overnight seed culture (1 l) of the *E. coli* in a Luria-Bertani broth. The temperature and pH were controlled at 37°C and 7.0, respectively. The agitation speed was 300 rpm and the air flow rate 1.5 v/v/m. The cell growth was monitored spectrophotometrically at 600 nm. The cultivation was completed approximately 2 h after the growth reached the stationary phase. The cells were then harvested by centrifugation at 4,000 rpm for 30 min, collected, and stored in a deep freezer.

Preparation of Enzyme Extract by Rapid Clarification Process

The harvested cell paste (wet weight 50 g) were thawed slowly at room temperature, suspended in a cold ammonium-cofactor solution (200 ml, pH 8.5), and disrupted using a homogenizer (APV-2000) (Invensys APV, Sussex, U.K.). The ammonium-cofactor solution contained 53.5 g NH_4Cl, 24.8 mg PLP, 0.2 ml 2-mercaptoethanol, 0.1 g DL-dithiothreitol, 1.0 g Na_2SO_3, and 0.4 g EDTA per liter of distilled water. The homogenated cells were gradually treated with pure methanol up to 40% (v/v), and then left

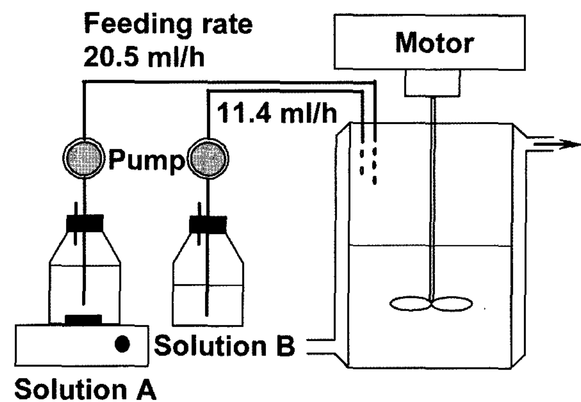

Fig. 1. Diagram of fed-batch bioreactor designed for enzymatic synthesis of L-tyrosine.
Solution A contained 2.2 M phenol and 2.4 M sodium pyruvate and/or 24% ethanol. Solution B contained 0.4 mM pyridoxal-5'-phosphate and 4 M NH_4Cl. The two substrate solutions were continuously fed into the bioreactor using a peristaltic pump. The temperature was controlled by a water jacket connected to a circulating water bath.

to stand for 2 h at 4°C to accelerate the aggregation of cell debris and unstable *E. coli* proteins. Finally, the methanol-treated cell homogenates were clarified by centrifugation at 4,000 rpm for 20 min and stored at 4°C until use.

Operation of Fed-Batch Reactor

The fed-batch system was comprised of a 2.5 l bioreactor (Korea Fermentor Co.) and two substrate reservoirs (0.5 l) to supply the toxic and unstable substrates (phenol and sodium pyruvate) and ammonium chloride to drive the synthesis reaction (Fig. 1). The headspace of the reactor was flushed with nitrogen gas to minimize the oxidation of the substrates by air. A mechanical stirrer was also included in the first reservoir that contained 2.2 M phenol and 2.4 M sodium pyruvate. In certain instances, absolute ethanol was added to the first reservoir at 24% (v/v). Vigorous stirring was continued throughout the feeding period to maintain the homogeneity of the substrate solution and avoid any phase separation between the phenol and water that would interfere with the balanced feeding of the substrates. The other reservoir contained 0.4 mM PLP and 4 M ammonium chloride dissolved in water (pH 8.5). Peristaltic pumps were used to precisely control the feeding rate at 20 ml/h for the first reservoir and 12 ml/h for the second reservoir. The feeding strategy was completed after 22–24 h, plus 6–8 h of prolonged incubation was allowed to reduce any remaining substrate. The agitation speed was controlled at 300 rpm.

Recovery and Recrystallization of L-Tyrosine

After the synthesis reaction was completed, the L-tyrosine precipitates were collected by filtration using a sintered glass funnel, washed once with cold water, and dried by air blowing. To purify the L-tyrosine, the crude powder (100 g)

was suspended in water (1 l) and mixed with 10 N HCl using a magnetic stirrer until the L-tyrosine particles were completely dissolved. After standing on ice for 1 h, the solution was filtrated using a sintered glass funnel to remove the protein aggregates. The filtrate was then collected and gradually titrated up to pH 7.0 with ammonia water (25–28%). Thereafter, the L-tyrosine particles that evolved during the ammonia titration were recovered by filtration, washed twice with ice-cold water, and dried at 60°C after being washed once more with ethanol. Finally, the purified L-tyrosine particles were recrystallized by either a temperature change or ammonia stripping [21].

Analysis

The L-tyrosine synthesis activity of the TPL was assayed by measuring the amount of synthesized L-tyrosine after terminating the reaction using 5 N HCl. The standard reaction mixture (2.0 ml) contained 650 mM NH_4Cl, 50 mM phenol, 50 mM sodium pyruvate, 0.1 mM PLP, 0.1% Na_2SO_3, and 0.5 units of the TPL, and the reaction was performed at 40°C for 30 min. One unit of L-tyrosine synthesis activity of TPL was defined as the amount of enzyme required to produce 1 μmol L-tyrosine per min. The amounts of L-tyrosine and phenol in the reaction medium were quantitatively determined using an HPLC system (Agilent Co., U.S.A.) equipped with an ODS18 column (Shimazu, Japan) and UV detector (265 nm). The elution was carried out using a co-solvent consisting of a 100 mM KH_2PO_4 buffer (pH 2.1) and acetonitrile (volume ratio=3:1) at a flow rate of 1.0 ml per min.

RESULTS

Preparation of Biocatalyst by Rapid Clarification Process

The cultivation of *E. coli* BL21 harboring pHCE19T(II)-TPL in a CPGY medium (25 l) produced 2.2 kg of cell paste (wet weight) during 24 h of cultivation. The expression level of the recombinant TPL gene was about 30% according to an SDS-PAGE analysis, and the total activity was calculated to be 52 kU. To isolate the TPL biocatalysts, approximately 50 g of the collected cells (wet weight) was suspended in 200 ml of an ammonium-cofactor solution (pH 8.5) and disrupted using a homogenizer, as described in Materials and Methods.

Dehydrating agents, such as alcohols and acetone, have been used for decades to increase protein precipitation and flocculate protein precipitates. Nucleic acids also tend to be insoluble in a polar solvent, such as alcohols. However, the solubility of membrane lipids in alcohols increases with the chain length of the alcohol, making them more insoluble in methanol than ethanol or larger alcohols. Thus, to enhance the aggregation of cellular matter and labile *E. coli* proteins, the disrupted cells were treated

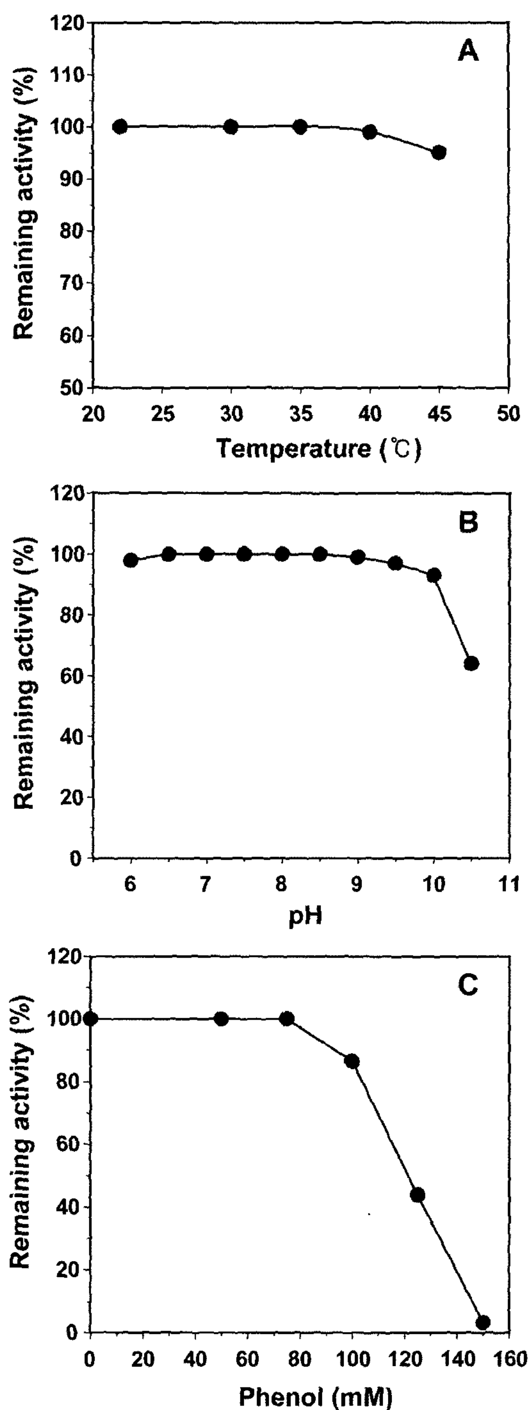

Fig. 2. Effect of temperature (**A**) and pH (**B**) on stability of TPL and effect of phenol concentration (**C**) on L-tyrosine synthesis activity of TPL.

The effect of temperature was determined by analyzing the remaining activity after incubating the enzyme under the synthesis conditions for 18 h at given temperatures, while the pH stability was determined after preincubation for 18 h at given pHs. The effect of the phenol concentration on the L-tyrosine synthesis activity of the TPL was determined using a reaction mixture (2 ml) containing 0.67 M NH_4Cl, 0.1% Na_2SO_3, 0.1 mM PLP, 0.5 U TPL, and the indicated concentrations of phenol. The reaction was started by the addition of 1.0 M sodium pyruvate (0.1 ml) after preincubating the reaction mixture for 3 h at 40°C.

with 40% (v/v) methanol for 2 h at 4°C. The methanol-enzyme mixture was then centrifuged and the supernatant collected for use as the biocatalyst. As a result, this rapid preparation of active TPL is very useful, reproducible, and showed the least amount of activity loss (<10%) during the process.

Stability of *S. toebii* TPL Under Operational Conditions

The stability of a biocatalyst under operational conditions is the most important requirement for the development of a biosynthesis strategy. Thus, to evaluate the stability of the *S. toebii* TPL preparations, the remaining activity was investigated after incubating the enzyme under different reaction conditions. Fig. 2A shows that the enzyme exhibited no activity loss for at least 18 h when incubated below 40°C, yet started to be inactivated at higher temperatures. The enzyme activity also remained stable at pHs between 6.0 and 9.5 (Fig. 2B). When investigating the phenol concentration (Fig. 2C), the enzyme maintained its original activity at phenol concentrations below 75 mM, yet became drastically inactivated at concentrations higher than 100 mM. Therefore, the operational conditions for the fed-batch reactor were preferentially optimized at 40°C and pH 8.5, while maintaining the phenol concentration below 75 mM during the enzyme reaction.

Fed-Batch Production of L-Tyrosine

The initial working volume of the fed-batch reactor was 0.5 l with 1.0 kU of the enzyme preparations (Table 1), and then increased up to 1.0–1.2 l as the phenol and auxiliary substrates were supplemented from the substrate reservoirs. Whereas the fed-batch reaction continued under the synthesis conditions, insoluble particles generally appeared in the bioreactor after approximately 30 min. To monitor the conversion process, samples of the reaction solution (1 ml) were sampled intermittently, dissolved in a 2.0 N HCl solution, centrifuged to recover the clear supernatant, and injected into an HPLC. As shown in Fig. 3, the phenol concentration in the fed-batch reactor was found to decrease during the initial reaction, and then increased as the synthesis rate slowed down.

Whereas the L-tyrosine precipitates were rapidly dissolved in the acid solution, a significant amount of insoluble aggregates was also observed, which increased as the reaction proceeded. These acid-insoluble impurities were then dissolved using 8 M urea and identified by Bradford assay as mainly proteins adsorbed to the L-tyrosine precipitates. Consistently, the amount of soluble proteins in the reaction

Table 1. Composition of starting solution for L-tyrosine synthesis.

Components	Amount in 0.5 l of starting solution
Phenol	2.3 g
Sodium pyruvate	2.7 g
Pyridoxal-5'-phosphate	12.4 mg
NH_4Cl	26.8 g
Na_2SO_3	0.5 g
Methanol	50.0 ml
Enzyme	1.0 kU

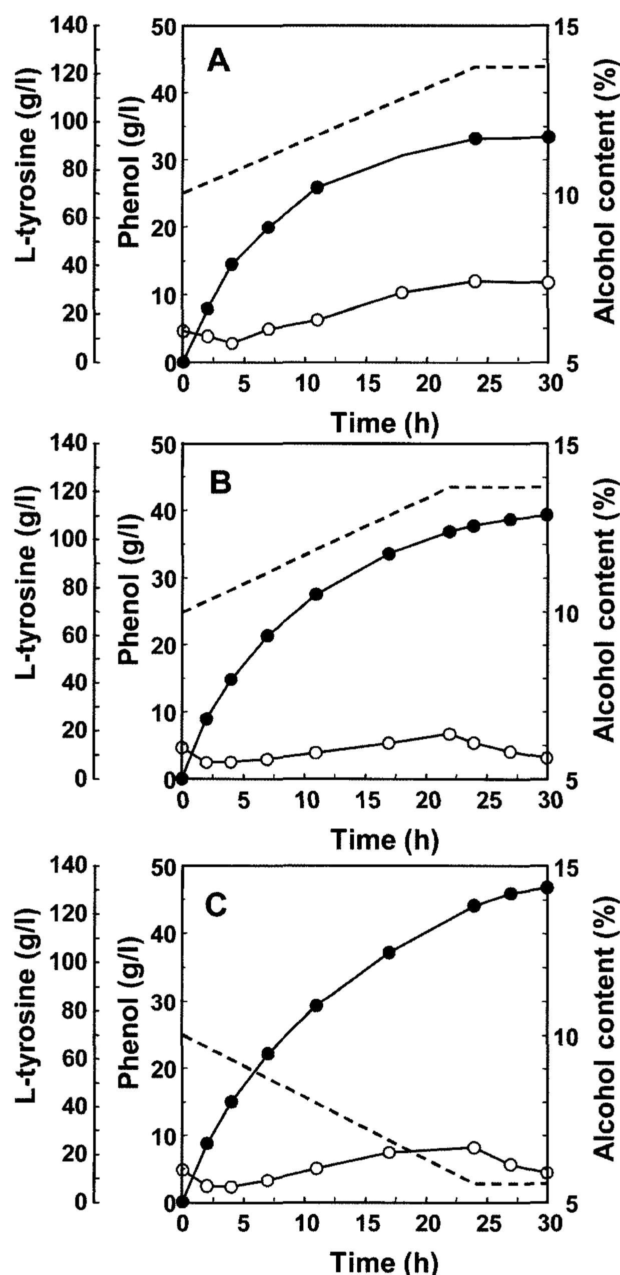

Fig. 3. Fed-batch production of L-tyrosine in a liter-scale bioreactor. **A.** Starting solution (see Table 1) supplemented with solution A and solution B containing 24% ethanol. **B.** Starting solution with 0.2% PEG supplemented with solution A and solution B containing 24% ethanol. **C.** Starting solution with 0.2% PEG supplemented with solution A and solution B without ethanol. The two substrate solutions were supplied at predetermined rates for 22–24 h. The symbols represent the amount of synthesized L-tyrosine (●) and residual amount of phenol (○), while the dotted line indicates the change of alcohol content in the reaction solution.

medium decreased continuously as the reaction proceeded, implying a loss of synthesis activity.

Polyethyleneglycol (PEG) and polyalcohol esters of fatty acids are used as effective bifunctional additives for many biotechnological applications, including the refolding and solubilization of insoluble proteins, and the suppression of protein aggregation and nonspecific adsorption during purification [11]. Therefore, to improve the synthesis of L-tyrosine, several types of bifunctional additives, including PEGs with different molecular weights, were tested as an ingredient of the fed-batch system. As a result, 0.2% PEG3350 was selected as the most effective additive, exhibiting a 15% increased productivity (Figs. 3A and 3B), whereas 0.4% PEG3350 and 0.2% PEG8000 with higher molecular weights did not improve the synthesis process, probably due to the concomitant increase in the viscosity of the synthesis solution (data not shown).

During the synthesis of L-DOPA by the TPL from *C. freundii* [14], alcoholic additives have been shown to be beneficial. Therefore, to investigate the effect of additional alcohol, a phenol-pyruvate solution containing 24% ethanol was prepared and fed continuously into the starting solution (0.5 l) that included 10% methanol. However, as evident when comparing Figs. 3A and 3C, the production of L-tyrosine was suppressed by the supplement of extra ethanol, possibly due to the dehydrating nature of ethanol, which may have induced a shrinkage of the L-tyrosine-protein co-aggregates, thereby inhibiting the functionality of the proteins.

Thus, when combining the positive effects of 0.2% PEG3350 and alcohol, the productivity of the fed-batch system increased up to 131 g/l/30 h, as shown in Fig. 3C, and the final conversion yield of L-tyrosine from the supplied phenol was evaluated to be 94%, with 46 mM phenol remaining in the reaction medium.

Purification and Recrystallization of L-Tyrosine

The crude particles were recovered by filtration of the reaction solution and purified according to the method described in Materials and Methods. The purification yield of L-tyrosine was approximately 70–75%, and the purity evaluated to be more than 98±0.5%, as determined by an HPLC analysis (Fig. 4). After ammonia stripping or recrystallization using temperature-dependent water solubility [21], L-tyrosine crystals were recovered at yields of over 80%. The morphological characteristics of the resulting crystals were thinner and longer (Fig. 4) than those of commercial L-tyrosine crystals (Sigma Co.). The conditioning of the crystallization pH (3.0, 7.0, 10.0) and inclusion of 20% (v/v) ethanol had no influence on the morphology of the L-tyrosine crystals during the recrystallization process.

DISCUSSION

Despite extensive efforts over the past decades, only a limited number of biocatalysts have been successfully applied to the biotechnological production of fine chemicals [20]. However, to actualize the promising potential of environment-friendly processes, enzymes must demonstrate not only superior performance characteristics, such as high activity and selectivity, but also cost-effectiveness and scalability. In addition, a successful biocatalytic process should be stable at high substrate and product concentrations, frequently in the presence of organic solvents.

Even though the biocatalytic synthesis of L-tyrosine by bacterial TPLs has already been demonstrated [3, 5, 19, 24], several problems remain that should be solved for the efficient production of L-tyrosine. Notwithstanding, the fed-batch reaction system proposed in the present study

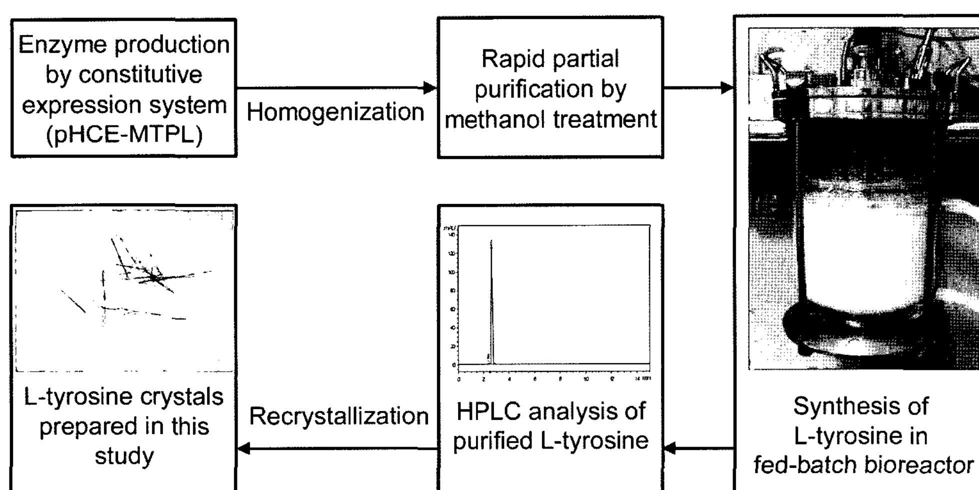

Fig. 4. Schematic representation of enzymatic synthesis, purification, and recrystallization of L-tyrosine, as used in the present study.

achieved a L-tyrosine productivity of 131 g/l (Fig 3C), although the L-tyrosine products tended to co-precipitate with proteins in the reaction medium as the reaction proceeded, causing a gradual decrease in the synthesis activity.

Thus, in an effort to suppress the protein aggregation and nonspecific adsorption during the biocatalytic process, PEGs were included in the reaction solution as a useful additive for the solubilization of proteins. PEGs are nontoxic and neutral substances that are used as solvent-dispersing agents in a variety of pharmaceuticals and medications. Of the PEGs tested, 0.2% (w/v) PEG3350 was found to be the most effective for L-tyrosine production. Additionally, the effect of the PEGs confirmed the fact that a nonionic polymer with good chain mobility can prohibit a hydrophobic interaction between certain proteins and hydrophobic substances [8]. However, in excess, the PEGs were found to act as a viscosity barrier against the diffusion of the enzyme and substrate molecules.

In summary, the enzymatic synthesis of L-tyrosine with a high space-time yield of over 100 g/l per day was successfully achieved by applying an efficient fed-batch strategy. Therefore, this high productivity of L-tyrosine is expected to encourage the application of *S. toebii* TPL as a biocatalyst for the industrial production of aromatic derivatives of L-tyrosine.

Acknowledgments

This project was supported by the Cleaner Production Program 10007946 of NCPC and a grant (code no. 20050301034476) from Biogreen 21 program.

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