RESEARCH ARTICLE

Biotransformation of Phenol to L-tyrosine with Resting Cells of *Citrobacter freundii* MTCC 2424

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ABSTRACT- In this present study, the biotransformation of phenol to L-tyrosine was studied with resting cells of *Citrobacter freundii* MTCC 2424 containing high tyrosine phenol lyase activity. Different process parameters leading to synthesis of L-tyrosine were optimized. The L-tyrosine formed from biotransformation reactions was detected and quantified by HPLC technique. The maximum L-tyrosine conversion 69% (6.49 g/l) was obtained with ammonium chloride 0.25M, phenol 0.1M, and sodium pyruvate 0.2M in borate buffer (0.1M) of pH 8.5 at 35°C temperature for 45 min of incubation. The higher phenol concentration was found to be inhibitory for biotransformation due to phenol inactivation of catalyst.

Key-words- Biotransformation, *Citrobacter freundii*, L-tyrosine, Tyrosine phenol lyase

INTRODUCTION

L-tyrosine is an aromatic amino acid, one of the building blocks of protein and found in many proteins containing food products such as soy products, chicken, turkey, fish, peanuts, almonds, bananas, milk, cheese, yogurt, pumpkin seeds and sesame seeds. A number of studies on human have found tyrosine to be useful during condition of stress, cold, fatigue [1], loss of a loved one such as in death, prolonged work and sleep deprivation [2,3], improvements in cognitive and physical performance [4-6]. *C. freundii*, a member of the genus *Citrobacter* belongs to the family Enterobacteriaceae. These are areobic gram negative bacilli, long rod shaped bacteria (1-5 µm in length). Its habitat includes the environment (soil, water, and sewage), food, and the intestinal tracts of animals and humans [7].

Tyrosine phenol lyase (TPL) is an enzyme that catalyzes the synthesis of L-tyrosine. This enzyme has been found to exist in a number of bacteria, but some species in particular namely *C. freundii, Escherichia intermedia* and *Erwinia herbicola* have been recognized for high enzyme activity [8]. This enzyme catalyzes the multiple reactions such as α,β-elimination [9], reversal of α,β-elimination [10], β-replacement [11,12], and racemization reactions [13]. These reactions are important for enzymatic synthesis of L-tyrosine [14-16] and its related amino acids, including 3,4-dihydroxyphenylalanine or L-DOPA [17], treatments of phenolics in water [18] and for biotransformation of L-serine [15].

L-tyrosine has been used as nutritional supplements and mild antioxidants to alleviate the acute cases of Parkinson's symptoms [19]. L-tyrosine is required to make several neurotransmitters such as L-DOPA, dopamine, epinephrine and norepinephrine [20-22]. L-phenylalanine can also be converted into L-tyrosine utilizing the enzyme phenylalanine hydroxylase and in turn L-tyrosine is converted to levodopa (L-DOPA) by the enzyme tyrosine hydroxylase. This can be further converted into dopamine, epinephrine and norepinephrine. The derivatives of L-tyrosine in body fluids play regulatory roles in functions of the hormonal system in the adrenal, thyroid, and pituitary glands. The hormones epinephrine and norepinephrine have therapeutic use such as cardiostimulants in the treatment of acute circulatory insufficiency and hypotension [23]. Considering the importance of L-tyrosine and its use in the synthesis of molecules of therapeutic and industrial value the present study entitled "Biotransformation of phenol to L-tyrosine with resting cells of *C. freundii* MTCC 2424" was carried with objectives to develop a laboratory scale process for production of therapeutically and industrially important molecule L-tyrosine.

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MATERIALS AND METHODS

Microorganism and Maintenance of culture

The culture of *C. freundii* MTCC 2424 was procured from the Department of Biotechnology, Himachal Pradesh University, Shimla, India and used for this study. *C. freundii* MTCC 2424 was maintained on L-tyrosine

agar media containing (%, w/v) meat extract 0.5, yeast extract 0.5, peptone 0.25, L-tyrosine 0.1 and agar 2.0 [24]. The pH of media was maintained at 7.5. The plates were incubated at 30°C for 24 h after inoculation. Periodical sub-culturing was carried out and glycerol stocks of culture were prepared and stored at -40°C.

Estimation of cell mass

Cells of *C. freundii* MTCC 2424 were harvested by centrifuging the broth at 10,000 rpm for 10 min in a refrigerated centrifuge (4°C) and known amount of wet cell pellet was placed in oven at 80°C for overnight and corresponding absorbance of cell slurry was measured at 600 nm in a spectrophotometer. The known dried cell weight corresponding to their optical cell density was recorded and a standard graph was plotted between dry cell weight and $A_{600.}$ The cell mass in terms of dry cell weight (dcw) was measured from the standard curve.

Tyrosine Phenol Lyase (TPL) assay

The α,β-elimination reaction was used for assay of enzyme. TPL converts tyrosine to phenol, pyruvate, and ammonia. The amount of liberated ammonia was measured via spectrophotometer. Since TPL was found to be intracellular in nature, the resting cells suspended in borate buffer (0.1M, pH 8.5) were used for enzyme assay. Activity of TPL from whole cell of *C. freundii* MTCC 2424 was expressed in terms of units (U).

Estimation of Ammonia

The ammonia released from the hydrolysis of L-tyrosine was estimated by the Berthelot color reaction [25] for assay of enzyme activity.

HPLC analysis of L-tyrosine

The L-tyrosine formed from biotransformation reactions was detected and quantified by HPLC equipped with a reverse phase column and UV spectrophotometer. The reaction mixture was centrifuged (10000 rpm for 10 min) to remove all suspended particles. The supernatant was filtered through 0.20 µ filters and 10 µl of samples were loaded on HPLC column. The column was eluted with 0.01M ammonium acetate buffer (pH 3.5) at a flow rate of 1 ml/min and the detection was done at 280nm. The amount of L-tyrosine formed was calculated from the standard curve.

Preparation of seed and production culture

Seed and production medium used were of same composition containing (%, w/v) meat extract 0.5, yeast extract 0.5, peptone 0.25, L-tyrosine 0.1 and pH 7.5 [24]. Sterile seed culture (50 ml) was inoculated with a loopful of a culture grown on agar plates and incubated at 25°C in a temperature controlled incubator shaker at 150 rpm for 4h. The exponential phase cell mass (4 h old) was used as inoculum (6%, v/v) for 100 ml sterile production medium and flasks were incubated at 25°C in a temperature controlled incubator shaker at 150 rpm for 16h. After 16h, the broth was centrifuged at 10,000 rpm for 10 min and the cells pellet was washed three times with borate buffer (0.1M, pH 8.5). The washed pellet was suspended in 10ml borate buffer. The resting cells (30 OD, 0.48 mg/ml dcw) were used as catalyst for biotransformation reactions.

Optimization of various process parameters for biotransformation of phenol to L-tyrosine with resting cells of *C. freundii* MTCC 2424 in a Fermenter

Biotransformation studies were carried out in a 2l laboratory fermenter. The bioconversion percentage was calculated on the basis of phenol supplied and L-tyrosine formed (w/w).

Selection of suitable ammonium salt for Biotransformation

The different ammonium salts (ammonium chloride, ammonium sulfate, ammonium nitrate, ammonium acetate) were used (1M) in reaction mixture (500 ml) for biotransformation and amount of L-tyrosine formed was analyzed by HPLC technique. The reaction was carried out with resting cells of *C. freundii* MTCC 2424 in borate buffer (0.1M, pH 8.5), containing known amount of cell mass (48 mg, dcw), 0.05M phenol, 0.1M sodium pyruvate at 30°C (100 rpm) for 30min. The reaction was stopped by taking 2 ml of reaction mixture with 1ml of 1.0N HCl, centrifuged to recover the clear supernatant, filtered and injected into the HPLC.

Optimization of concentration of ammonium chloride for Biotransformation

The concentration of most suitable ammonium salt (ammonium chloride) was determined for biotransformation reaction by varying its concentration from 0.001M to 1.25M. The reactions with resting cells of *C. freundii* MTCC 2424 was carried out in borate buffer (0.1M, pH 8.5), containing known amount of cell mass (48 mg, dcw), 0.05M phenol, 0.1M sodium pyruvate at 30°C (100 rpm) for 30 min.

Optimization of concentration of phenol on its Biotransformation to L-tyrosine

The varying concentrations (0.05M to 0.25M) of phenol were used for biotransformation reaction along with 0.25M ammonium chloride and 0.1M sodium pyruvate at 30°C. The rest of reaction conditions were maintained same and L-tyrosine synthesized was analyzed by HPLC technique.

Optimization of concentration of sodium pyruvate for Biotransformation

Sodium pyruvate provides (-CH_2-CH-COOH) to tyrosine. Varying concentrations (0.1M to 0.5M) of sodium pyruvate were used along with 0.1M phenol. The rest of reaction conditions were maintained same.

Optimization of pH of borate buffer for Biotransformation

Reactions were carried out at various pH (7.5 to 9.5) of borate buffer to study its effect on biotransformation.

Optimization of incubation temperature for Biotransformation

To find out optimum temperature, biotransformation reactions were performed at different temperatures (25°C to 45°C).

Optimization of incubation time for Biotransformation

Biotransformation reactions were performed under previously described conditions for 75 min and samples were withdrawn at regular intervals of 15 min. In each sample L-tyrosine synthesized was analyzed by HPLC technique.

RESULTS

Colonies of *C. freundii* MTCC 2424 was observed on inoculated L-tyrosine agar media after incubation at 30°C for 24 h (Fig. 1). Biotransformation studies were carried out in the 2l laboratory fermenter (Fig. 2). Maximum conversion of Phenol to L-tyrosine was found to be 26% (1.22 g/l) when ammonium chloride was used (Fig. 3). Other ammonium salts used like ammonium sulfate, ammonium nitrate and ammonium acetate showed comparatively a lower conversion, which was 17%, 13%, and 7% respectively.

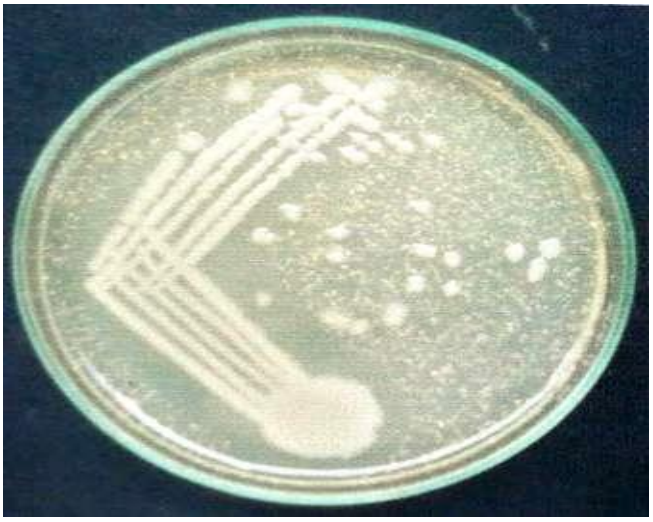

Fig. 1: Colonies of *C. freundii* MTCC 2424 cells

Fig. 2: Fermenter used for biotransformation

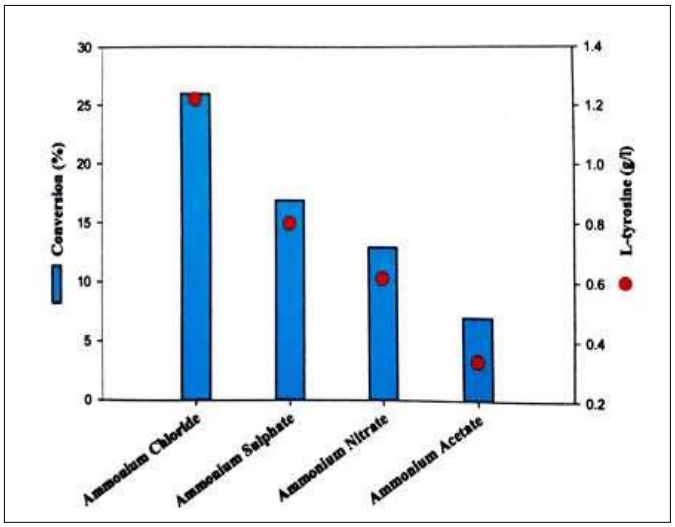

Fig. 3: Effect of different ammonium salts on Biotransformation

Resting cells of *C. freundii* MTCC 2424 were showed maximum conversion (39%) at 0.25M concentration of ammonium chloride. The maximum L-tyrosine biosynthesis was recorded to be 1.84 g/l (Fig. 4). Maximum biotransformation (48%) of phenol to L-tyrosine was obtained at 0.1M concentration of phenol with 4.52 g/l biosynthesis of L-tyrosine (Fig. 5). However, as the concentration of phenol was increased further to 0.25M in the reaction mixture, the conversion was reduced to 15%. Maximum biotransformation (54%) was observed at 0.2M concentration of sodium pyruvate with 5.08 g/l biosynthesis of L-tyrosine (Fig. 6). Maximum L-tyrosine conversion (55%) was observed with borate buffer (0.1M) at pH 8.5 with 5.18 g/l accumulations of L-tyrosine in the reaction mixture (Fig. 7). Maximum L-tyrosine conversion (61%) was observed at 35°C. L-tyrosine biosynthesis observed at 35°C was 5.74 g/l (Fig. 8). L-tyrosine production was found to increase initially with increasing incubation time (69% at 45min) and then attained constant value as the reaction proceeds. Maximum L-tyrosine biosynthesis (6.49 g/l) was observed at 45 min of incubation (Fig. 9).

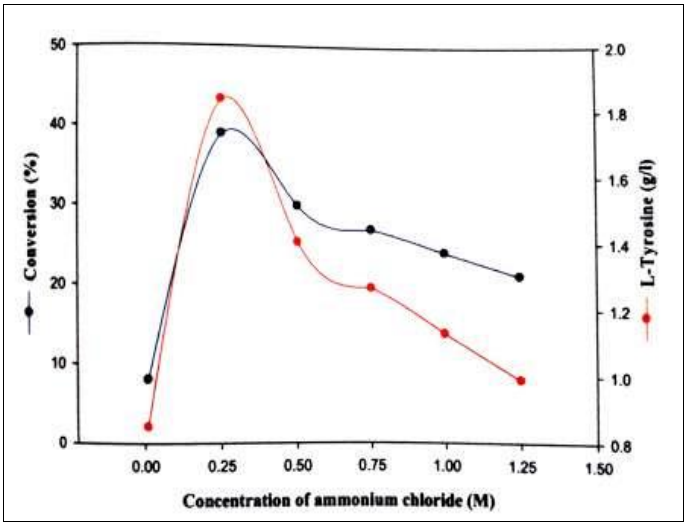

Fig. 4: Effect of ammonium chloride concentrations on biotransformation

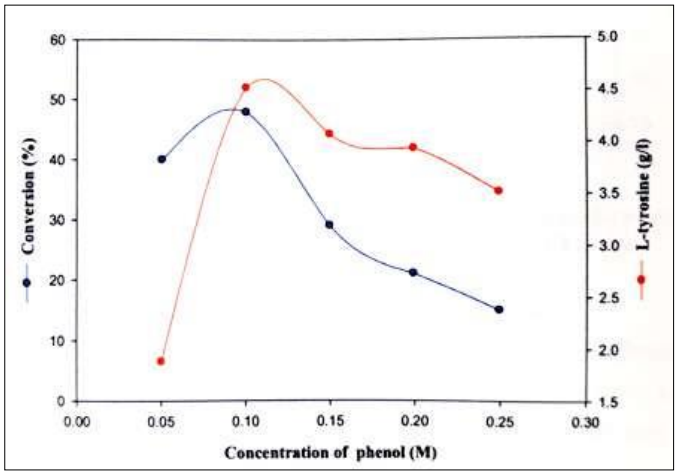

Fig. 5: Effect of phenol concentrations on biotransformation

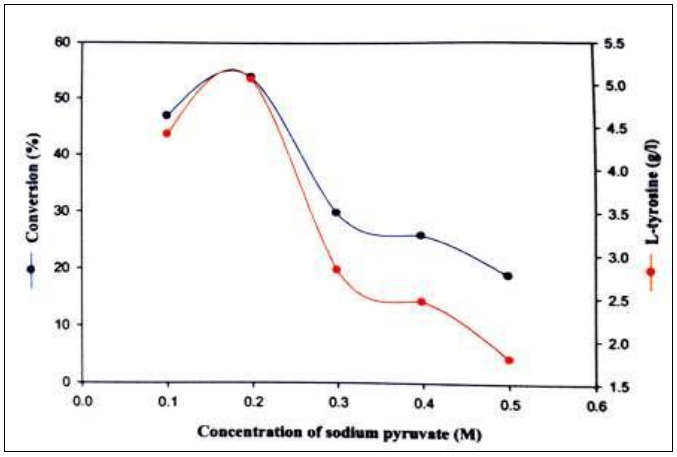

Fig. 6: Effect of sodium pyruvate concentrations on biotransformation

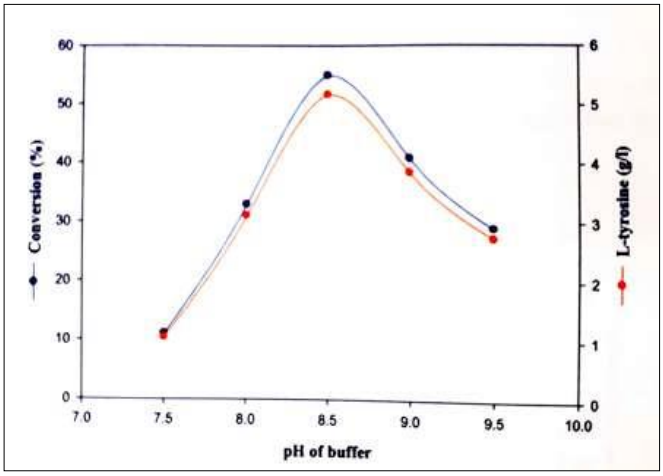

Fig. 7: Effect of buffer pH on biotransformation

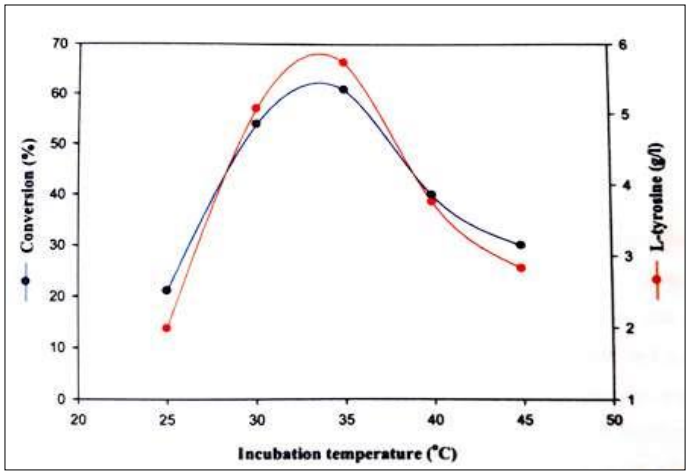

Fig. 8: Effect of incubation temperature on biotransformation

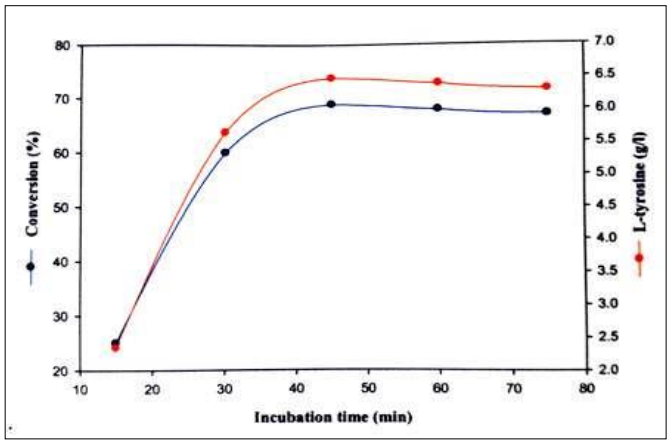

Fig. 9: Effect of incubation time on biotransformation

DISCUSSION

On the basis of our results, it was evident that intact cells of *C. freundii* MTCC 2424 prepared from the culture broth cultivated for 16 h contained high enzymatic activity. Enzymatic synthesis of L-tyrosine was first demonstrated by Yamada and Kumagai [17]. Ammonium chloride was found to be the best salt for biotransformation. Ammonium chloride (4M) was used as a source of ammonium ion for L-tyrosine production [26]. The 2M ammonium chloride was found to be optimum for biotransformation of phenol to L-tyrosine in a simulated waste water containing phenolics by a recombinant thermotolerant TPL of thermophillic *Symbiobacterium* sp. SMH-1 [18]. Many scientists were also reported ammonium acetate as a source of ammonium ions for L-tyrosine production [27,28]. Inhibition of TPL activity with phenol and its derivatives was studied [14]. They observed 83% inhibition in TPL activity at 1.0 mM concentration of phenol and its derivatives in the reaction mixture. It was observed that phenol concentration in reaction mixture should be as low as possible to avoid inhibition and inactivation of catalyst [29]. In addition, phenol, which can partially destroy cell walls and denature proteins, was often infused at a minimum concentration to avoid inhibition and inactivation of catalyst [30,31]. Optimum sodium pyruvate concentration was found to be 0.2M for

biotransformation. The same concentration of sodium pyruvate (0.2M) was used in a reaction mixture for L-tyrosine synthesis by *E. herbicola* ATCC 21433 [32]. Production processes using solubilized, intact cells of *E. herbicola* achieved a maximum concentration of 60.5 g/l L-tyrosine from 20 g/l sodium pyruvate added twice, 50 g/l ammonium acetate and a phenol concentration maintained at 10 g/l throughout the reaction [27]. Concentration of about 0.11M (20 g/l) L-tyrosine was reported with the addition of 0.30M (33 g/l) sodium pyruvate, 0.65M (50 g/l) ammonium acetate and 0.13M (12.4 g/l) phenol after 2 h [28].

In the present investigation, optimum buffer pH and temperature for biotransformation was found to be 8.5 and 35°C respectively. The incubation time for biotransformation was found to increase initially and then attained a constant valve as the reaction proceeds. This might be due to reason that as the reaction proceeds, the enzyme got saturated with substrate and the reaction rate becomes constant with time. L-tyrosine (14.5 g/l) synthesis from ammonium acetate (0.65M), phenol (0.1M) and sodium pyruvate (0.18M) was observed with intact cells of *E. herbicola* and optimum pH for reaction was around 8.0 and optimum temperature range was from 30°C to 37°C [33].

CONCLUSIONS

The present investigation attempts to find out the optimum reaction conditions for the synthesis of L-tyrosine with resting cells of *C. freundii* MTCC 2424. The various process parameters were individually optimized to maximize the biosynthesis of L-tyrosine. Out of different process parameters optimized, ammonium chloride 0.25M, phenol 0.1M, sodium pyruvate 0.2M, buffer 0.1M, pH 8.5, reaction temperature 35°C and incubation time 45min were found to be optimum for maximum production of L-tyrosine. Higher phenol concentration was found to be inhibited due to phenol inactivation of catalyst. Employing whole cells as biocatalyst for L-tyrosine synthesis offers clear advantages over *in vitro* enzymatic conversion because microbial synthesis proceeds under environmentally beneficial and non toxic conditions. In view of recent advances, it is clear that microbial fermentation becoming a very viable alternative option for L-tyrosine production.

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