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Research Article

TO DESIGN THE TECHNOLOGICAL COMBINATIONS AND FED BATCH APPROACH FOR BIOTRANSFORMATION OF PHENOL TO L-TYROSINE WITH RESTING CELLS OF *CITROBACTER FREUNDII* MTCC 2424

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ABSTRACT

Objective: The study was carried out with an objective to design technological combinations and fed batch approach for improved production of therapeutically and industrially important molecule L-tyrosine.

Methods: Technological combinations (2^3) were designed with optimized chemical parameters for the biotransformation of phenol to L-tyrosine with resting cells of *C. freundii* MTCC 2424. Eight combinations were obtained by combination of varying three optimized chemical parameters (ammonium chloride, phenol and sodium pyruvate). To observe the effect of phenol on L-tyrosine biosynthesis, two fed batch experiments were designed on the basis of its concentrations. The L-tyrosine formed was detected and quantified by HPLC technique.

Results: Maximum L-tyrosine conversion in technological combinations was observed with lower concentration of phenol than optimized value. In fed batch studies, higher phenol concentration was found to be inhibitory for L-tyrosine synthesis due to phenol inactivation of catalyst.

Conclusion: The present approach is helpful in comparing the fermentation processes and designing of better bioprocesses. Production of L-tyrosine at minimum cost and energy is helpful in meeting the challenging need of various industries.

Keywords: L-tyrosine, technological combinations, fed batch, *Citrobacter freundii* MTCC 2424.

INTRODUCTION

Aromatic amino acids possess considerable industrial interest, particularly for their use as pharmaceuticals, food and feed additives, dietary supplements and neutraceuticals [1]. The biosynthetic pathway of aromatic amino acids in microorganism is well known and has been subjected to various metabolic engineering approaches in past years. Tyrosine is an aromatic amino acid, one of the building blocks of protein. It is used as precursor in synthesis of some drugs [2], biodegradable polymers [3], melanin [4] and phenylpropanoids [5]. A number of studies on human have found tyrosine to be useful during condition of stress, cold, fatigue [6], loss of a loved one such as in death, prolonged work and sleep deprivation [7,8], improvements in cognitive and physical performance [9-11]. Tyrosine phenol lyase (TPL) is an enzyme that catalyzes the synthesis of L-tyrosine. TPL also referred as β-tyrosinase, is a pyridoxal enzyme [12]. This enzyme has been found to exist in a number of bacteria but some species in particular namely *Citrobacter freundii, Escherichia intermedia* and *Erwinia herbicola* have been recognized for high enzyme activity [13]. *Citrobacter freundii* belongs to family Enterobacteriaceae. These are aerobic gram negative bacilli, long rod shaped bacteria and present in environment (soil, water, and sewage), food, intestinal tracts of animals and humans [14].

L-tyrosine has been used as nutritional supplements and mild antioxidants to alleviate the acute cases of Parkinson's symptoms [15]. L-tyrosine is required to make several neurotransmitters such as L-DOPA, dopamine, epinephrine and norepinephrine [16-18]. L-tyrosine derivatives in body fluids play regulatory roles in functions of hormonal system in the adrenal, thyroid, and pituitary glands. The hormones epinephrine and norepinephrine have therapeutic use such as cardio stimulants in the treatment of acute circulatory insufficiency and hypotension [19]. Considering the importance of L-tyrosine and its use in synthesis of molecules of therapeutic and industria l value the present study entitled "To design the technological combinations and fed batch approach for biotransformation of phenol to L-tyrosine with resting cells of *Citrobacter freundii* MTCC 2424" was carried to enhance its production.

MATERIALS AND METHODS

Microorganism and Maintenance of culture

The culture of *C. freundii* MTCC 2424 was procured from Department of Biotechnology, Himachal Pradesh University, Shimla, India and used for this study. The culture 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 [20]. 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 standard curve.

Tyrosine phenol lyase assay and estimation of ammonia

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 (0.1 M, 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). Ammonia released from hydrolysis of L-tyrosine was estimated by Berthlot color reaction [21] 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 0.01 M ammonium acetate buffer (pH 3.5) at a flow rate of 1 ml/min and the detection was done at 280 nm. The amount of L-tyrosine formed was calculated from standard curve.

Preparation of seed and production cultures

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 with pH 7.5 [20]. Sterile seed culture (50 ml) was inoculated with a loopful of a culture grown an agar plates and incubated at 25 °C in a temperature controlled incubator shaker at 150 rpm for 4 h. 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 16 h. After 16 h, the broth was centrifuged at 10,000 rpm for 10 min and cells pellet was washed three times with borate buffer (0.1 M, pH 8.5). The washed pellet was suspended in 10 ml 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 2 l laboratory fermenter. The bioconversion percentage was calculated on the basis of phenol supplied and L-tyrosine formed (w/w). Before designing technological combinations, various process parameters viz. selection of suitable ammonium salt (ammonium chloride, ammonium sulphate, ammonium nitrate, ammonium acetate), concentration of ammonium chloride, concentration of phenol, concentration of sodium pyruvate, pH of borate buffer, incubation temperature, incubation time for biotransformation were optimized [22].

Technological combinations (2^3) with optimized chemical parameters for the biotransformation of phenol to L-tyrosine with resting cells of *C. freundii* MTCC 2424

In order to reduce the cost of reaction mixture and to find out the most suitable substrate concentration for biotransformation of phenol to L-tyrosine with resting cells of *Citrobacter freundii* MTCC 2424, technological combinations (2^3) were applied in designing the experiments. For this purpose, instead of one parameter being varied, different combination of optimum and next lower level of optimized substrate concentration was used. The experiments were carried out with optimized chemical parameters. Eight combinations were obtained by combination of varying three chemical parameters (ammonium chloride, phenol and sodium pyruvate). The biotransformation reactions were carried out at 35 °C for 45 min in borate buffer (0.1 M, pH 8.5). Designing the experiment with technological combinations is a statistical approach that examines all the possible combinations of independent variables at appropriate levels in a single experiment.

Fed batch studies for biotransformation of L-tyrosine

It has been reported that due to toxic nature of phenol, it cannot be used at higher concentration in reaction mixture for its biotransformation to L-tyrosine. Hence, in order to control the phenol concentration in reaction mixture, fed batch approach was tried. To achieve maximum product formation, two fed batch experiments were designed on the basis of phenol concentrations. The experiments were carried out at 35 °C, 100 rpm for 1 h. In one set of reaction, the initial reaction mixture (500 ml) in fermenter contained ammonium chloride (0.25 M), phenol (0.05 M), sodium pyruvate (0.2 M) and the biotransformation was carried out for 30 min. After 30 min of biotransformation in batch mode, 0.05 M phenol was added to the reaction mixture and the biotransformation was further continued for 30 min under the same conditions. In another set of reaction, double amount of phenol (0.1 M) was used in batch mode. Initially the fermenter contained ammonium chloride (0.25 M), phenol (0.1 M), sodium pyruvate (0.2 M) and the biotransformation was carried out in batch mode for 30min. An addition of 0.05 M phenol was done to observe its effects on L-tyrosine biosynthesis. Sampling was done at interval of 30 min and L-tyrosine formation was estimated by HPLC technique.

RESULTS AND DISCUSSION

Colonies of *C. freundii* MTCC 2424 were observed on inoculated L-tyrosine agar medium after incubation at 30 °C for 24 h (fig. 1). Biotransformation studies were carried out in 2l laboratory fermenter (fig. 2). Out of different process parameters optimized, ammonium chloride 0.25 M, phenol 0.1 M, sodium pyruvate 0.2 M, buffer 0.1 M, pH 8.5, reaction temperature 35 °C and incubation time 45 min were found to be optimum for maximum production of L-tyrosine.

Fig. 1: Colonies of *C. freundii* MTCC 2424 on L-tyrosine agar medium

Fig. 2: Fermenter used for biotransformation.

The maximum conversion of phenol to L-tyrosine with resting cells of *C. freundii* MTCC 2424 in technological combinations observed was 78 % (3.67 g/l) when reaction mixture (500 ml) contained 0.25 M ammonium chloride, 0.05 M phenol and 0.2 M sodium pyruvate. When phenol concentration was increased to 0.1 M in the reaction mixture keeping all other components constant, the conversion was 70 % with 6.59 g/l of L-tyrosine biosynthesis. Maximum L-tyrosine conversion in technological combinations was observed with lower concentration of phenol than optimized value (table 1).

Table 1: Design of technological combinations (2^3) with optimized chemical parameters for the biotransformation of phenol to L-tyrosine with resting cells of *C. freundii* MTCC 2424

S. No.	Ammonium chloride (M)	Phenol (M)	Sodium pyruvate (M)	L-tyrosine conversion (%)	L-tyrosine biosynthesis (g/l)
1	0.25	0.1	0.2	70	6.59
2	0.25	0.1	0.1	60	5.65
3	0.25	0.05	0.2	78	3.67
4	0.25	0.05	0.1	69	3.25
5	0.05	0.1	0.2	61	5.74
6	0.05	0.1	0.1	57	5.36
7	0.05	0.05	0.2	63	2.96
8	0.05	0.05	0.1	59	2.78

The results of fed batch studies showed that in first set of reaction (fig. 3), initially the L-tyrosine conversion was 49 % (2.3 g/l) and after supplementing phenol it increased to 68 % (6.40 g/l). The increase in bioconversion might be due to the lesser inhibition or denaturation effect of phenol on tyrosine phenol lyase activity. In another set of reaction (fig. 4), initially the L-tyrosine conversion was 54 % (5.08 g/l) and after feeding phenol it was 43 % (6.07 g/l). In second set of reaction, L-tyrosine production was found to decrease slightly after feeding phenol probably due to the loss of stability of the enzyme at it's higher concentration. A fed batch reactor with continuous feeding of phenol, pyruvate, and ammonia has been developed for efficient synthesis of L-tyrosine [23]. A thermo and chemo stable tyrosine phenol lyase from *S. toebii* was employed for this purpose. This biotransformation system produced 130 g/l of L-tyrosine (mostly as precipitated particles) within 30 h upon continuous feeding of substrates for 22 h and maximum conversion obtained on the basis of supplied phenol was 94 %.

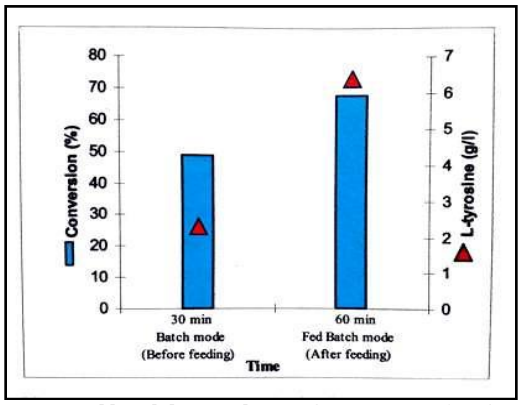

Fig. 3: Fed batch biosynthesis of L-tyrosine (0.05 M Phenol in batch mode and addition of 0.05 M phenol)

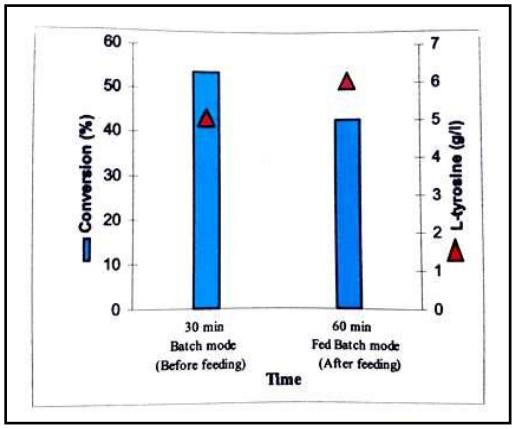

Fig. 4: Fed batch biosynthesis of L-tyrosine (0.1 M phenol in batch mode and addition of 0.05 M phenol)

CONCLUSION

In view of recent advances, it is clear that microbial fermentation is becoming a very viable alternative option for L-tyrosine production. In present study, technological combinations were designed to enhance the L-tyrosine synthesis. It was interesting to note that maximum L-tyrosine synthesized in technological combinations was with lower concentration of phenol than optimized values. This approach guarantees faster achievement of best combination with minimum production cost and time for L-tyrosine synthesis, thus helpful in comparing the fermentation processes and designing of better bioprocesses. Fed batch biosynthesis of L-tyrosine was also studied at different phenol concentration. Higher phenol concentration was found to be inhibitory due to phenol inactivation of catalyst. Production of L-tyrosine at minimum cost and energy is helpful in meeting the challenging need of various industries.

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