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ORIGINAL ARTICLE

Bioconversion of L-tyrosine to L-DOPA by a novel bacterium *Bacillus* sp. JPJ

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Abstract L-DOPA is an amino acid derivative and most potent drug used against Parkinson's disease, generally obtained from *Mucuna pruriens* seeds. In present communication, we have studied the in vitro production of L-DOPA from L-tyrosine by novel bacterium *Bacillus* sp. JPJ. This bacterium produced 99.4% of L-DOPA from L-tyrosine in buffer (pH 8) containing 1 mg ml^{-1} cell mass incubated at 40°C for 60 min. The combination of $CuSO_4$ and L-ascorbic acid showed the inducing effect at concentrations of 0.06 and 0.04 mg ml^{-1}, respectively. The activated charcoal 2 mg ml^{-1} was essential for maximum bioconversion of L-tyrosine to L-DOPA and the crude tyrosinase activity was 2.7 U mg^{-1} of tyrosinase. Kinetic studies showed significant values of $Y_{p/s}$ (0.994), Q_s (0.500) and q_s (0.994) after optimization of the process. The production of L-DOPA was confirmed by analytical techniques such as HPTLC, HPLC and GC–MS. This is the first report on rapid and efficient production of L-DOPA from L-tyrosine by bacterial source which is more effective than the plant, fungal and yeast systems.

Keywords L-DOPA · Parkinson's disease · L-Tyrosine · *Bacillus* sp. JPJ · Bioconversion

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Introduction

The potential drug L-DOPA (3,4-dihydroxyphenyl-L-alanine) is an effective remedy for the treatment of Parkinson's disease (Kofman 1971), which is a common neurodegenerative disorder, having prevalence of 160/100,000 in Western Europe comprising 4% of the population over 80. This drug has been regarded as the standard drug for the treatment of this serious disease (Davie 2008). One of the features of Parkinson's disease is reduced dopamine level; however, the administration of dopamine directly is not useful since dopamine does not cross the blood–brain barrier, but L-DOPA a precursor of dopamine can cross the blood–brain barrier and be converted to dopamine thus giving relief to the Parkinson's patients (Kofman 1971). The demand for L-DOPA is about 250 tons per year and approximately half of it is produced by an enzymatic method (Koyanagi et al. 2005).

Several biological sources produce L-DOPA in various quantities; however, L-DOPA is also synthesized by chemical methods (Knowles 2003; Valdes et al. 2004). Conventional production of L-DOPA involves the extraction of L-DOPA from *Mucuna pruriens* seeds and it is marketed as tablets under various brand names such as Sinemet®, Atamet®, Parcopa® and Stalevo® (Ali and Haq 2006a) by pharmaceutical companies. In the United States, million of Parkinson disease patients are reported and expend about $5.6 billion annually (Rani et al. 2007). It is also reported using plant sources mainly from *M. pruriens* cell suspension (Chattopadhyay et al. 1994), callus cultures of banana (Bapat et al. 2000) and *Portulaca grandiflora* (Rani et al. 2007). L-DOPA is also produced from *Aspergillus oryzae*, a fungal biomass in buffer containing L-tyrosine (Ali and Haq 2006a) and *Acremonium rutilum* produced in a potato dextrose broth (Krishnaveni et al. 2009).

The yeast (*Yarrowia lipolytica*) biomass is used to produce the L-DOPA in a buffer containing L-tyrosine provided with enhancing material diatomite (Ali et al. 2007). The bacterial species reported to synthesize L-DOPA are *Escherichia intermedia* cells immobilized in polyacrylamide gel using pyrocatechol (Para and Baratti 1988) and *Erwinia herbicola* using catechol as a substrate (Koyanagi et al. 2005). It is also produced from mutant strains of actinomycetes in a broth containing L-tyrosine (Sukumaram et al. 1979).

The genus *Bacillus* is a large and diverse genus of bacteria in the family *Bacillaceae* and has variety of phenotypically heterogeneous species exhibiting a wide range of nutritional requirements, physiological and metabolic diversity and DNA base composition (Dong and Charles 2003). The bacterial cells of genus *Bacillus* are endospore-forming Gram positive rods with peritrichous flagella, and colony morphology and size are variables and some species of genus *Bacillus* produce pigments on specific media (Claus and Berkeley 1986). *Bacillus thuringiensis* and *Bacillus cereus* are reported earlier for the synthesis of tyrosinase and melanin (Liu et al. 2004; Chen et al. 2004; Zhang et al. 2007). Several enhancing materials such as illite, Celite and diatomaceous earth are previously used to improve the bioconversion of L-tyrosine to L-DOPA (Ali and Haq 2006a, b; Ali et al. 2007). Like other enhancing materials previously described, activated charcoal is a microporous adsorbent and has unique adsorption properties resulting from the high-surface area, microporosity, and broad range of surface functional groups (Abdel and El-Hendawy 2005).

Tyrosinases (E.C. 1.14.18.1) are copper-containing enzymes widely distributed in plants, animals and microorganisms. They are involved in two steps of melanin synthesis that is from L-tyrosine to L-DOPA and then L-DOPAchrome. Tyrosinase catalyzes two successive reactions: one is cresolase and another is catecholase (Claus and Decker 2006).

The chemical synthesis of DOPA is rapid, but the resultant racemic DL-mixture is inactive and to obtain enantiomerically pure L-DOPA from this mixture is very difficult and cumbersome. D-DOPA interferes with the activity of DOPA decarboxylase, the enzyme involved in the production of dopamine in the brain (Rani et al. 2007); hence, a one-step economical bioconversion of L-tyrosine into L-DOPA is highly imperative and essential.

The present work is on optimization of L-DOPA production using the cells of bacterium *Bacillus* sp. JPJ (NCBI Genbank accession number FJ545652.1) in a reaction mixture containing L-tyrosine. This is the first report of rapid and efficient in vitro production of L-DOPA (99.4%) from L-tyrosine using bacterial system. The bacterial source for industrial fermentation is most favorable system as compared to plant, fungal and yeast; hence, present study is an efficient alternative for industrial scale production of L-DOPA.

Materials and methods

Chemicals

L-Tyrosine, L-DOPA, catechol and L-ascorbic acid were obtained from Sigma (USA), whereas activated charcoal cupric sulfate and other chemicals were procured from Himedia (India) having highest purity available and of an analytical grade.

Screening and isolation of microorganism

The L-DOPA producing bacterium was isolated using soil samples collected from Kolhapur, India and by serial dilution technique on a medium having composition (g l^{-1}) L-tyrosine 5, beef extract 3, tryptone 5 and agar–agar 20 with pH 7 that were taken in 500 ml of Erlenmeyer flask and autoclaved at 15 psi (121°C). The agar plates were incubated at 30°C for 48 h. The bacterial cultures were maintained on same medium at 4°C.

Identification and phylogenic analysis of microorganism

The isolated bacterial strain was identified by 16S rRNA sequencing, carried out at Genombio Technologies Pvt. Ltd., India. The partial nucleotide sequence (16S rRNA) was obtained and submitted to Genbank, and the strain was identified as a novel bacterium *Bacillus* sp. JPJ with accession no. FJ545652.1. After release of the sequence by database of NCBI, it was aligned with non-redundant database present in NCBI using BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the homologous sequences of species were used for phylogenic analysis. The phylogenic tree was constructed with MEGA4 software (Arizona, USA). The evolutionary history was inferred using the neighbor z-joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length was 28.66236616. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches (Felsenstein 1985). The phylogenic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki et al. 1995). The clock calibration to convert distance to time was 1 (time/node height). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the

Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 783 positions in the final dataset. Phylogenic analyses were conducted in MEGA4 (Tamura et al. 2007).This type of phylogenic analysis was earlier reported by Dhanve et al. (2009).

Growth conditions and inoculum preparation

The medium for the cultivation of the isolated bacterium consisted (g l^{-1}) L-tyrosine 1, beef extract 0.5 and tryptone 4 at pH 7 that were taken in a 250 ml of Erlenmeyer flask, autoclaved at 15 psi (121°C). A single colony of *Bacillus* sp. JPJ was inoculated in the medium and incubated at 30°C in shaking incubator (Remi, India) with 120 revolutions per minute (rpm). The inoculum was prepared on the basis of the maximum crude tyrosinase activity, for this cell mass and crude tyrosinase activity were determined with 3-h time interval and the cells were harvested when crude tyrosinase activity was maximum. One milliliter of cell suspension after 6 h of growth having absorbance 0.50 at 530 nm measured on colorimeter (Erma, India) was inoculated in the same medium. The cells after the growth of 18 h having absorbance 0.75 at 530 nm were harvested by centrifugation at 10,000 rpm and the supernatant was decanted followed by resuspension of cells in potassium phosphate buffer (0.1 M, pH 7).

Production of L-DOPA

Initially, the biochemical reaction of L-tyrosine to L-DOPA was carried out in the reaction mixture containing 50 ml of potassium phosphate buffer (0.1 M, pH 7) and 1 mg ml^{-1} of L-tyrosine in 250 ml of Erlenmeyer flask, autoclaved at 15 psi (121°C). The cell mass of 0.5 mg ml^{-1} was resuspended in the sterilized reaction mixture and incubated at 30°C for 50 min at 120 rpm on an incubator shaker. The samples were then assayed after incubation for the produced L-DOPA and residual L-tyrosine. These reaction conditions were considered as initial reaction conditions which were maintained further in optimization studies of the cell mass, pH, temperature and L-tyrosine.

Effect of various factors on L-DOPA production

Effect of cell mass

The cell mass of densities 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mg ml^{-1} was used for studying its effect on L-DOPA production in a reaction mixture containing 1 mg ml^{-1} L-tyrosine, pH 7 and was incubated at 30°C for 50 min.

Determination of pH and temperature optima

The pH necessary for maximum bioconversion of L-tyrosine to L-DOPA was determined by changing pH of reaction to 3, 4, 5, 6, 7, 8, 9, and 10 and incubated at 30°C for 50 min with 0.5 mg ml^{-1} of cell mass and 1 mg ml^{-1} of L-tyrosine. The temperature (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50°C) was also varied and incubated for 50 min with pH 7, 0.5 mg ml^{-1} of cell mass and 1 mg ml^{-1} of L-tyrosine.

Effect of L-tyrosine

The effect of various L-tyrosine concentrations (1, 2, 3, 4, 5 mg ml^{-1}) on L-DOPA production was studied and incubated at 30°C for 50 min with pH 6.8, 0.5 mg ml^{-1} of cell mass.

Effect of $CuSO_4$, L-ascorbic acid and activated charcoal

The effect of $CuSO_4$ and L-ascorbic acid on the bioconversion of L-tyrosine to L-DOPA was studied with optimum cell mass, pH, temperature and L-tyrosine concentration and reaction mixture was incubated for 50 min. The concentration of $CuSO_4$ and L-ascorbic acid was varied (mg ml^{-1}): 0.02, 0.04, 0.06, 0.08, 0.1. The effect of activated charcoal was studied by varying the concentrations (mg ml^{-1}): 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0.

Effect of incubation time

For studying the effect of incubation time on L-DOPA production, the reaction mixture was incubated for 30, 60, 90, 120, and 150 min with optimum cell mass, pH, temperature, L-tyrosine, $CuSO_4$, L-ascorbic acid and activated charcoal concentrations.

L-DOPA and L-tyrosine assay

The L-DOPA produced in the reaction mixture was determined according to Arnow's method (Arnow 1937). The reaction mixture was centrifuged at 5,000 rpm for 15 min, and 1 ml supernatant was added with 1 ml of 0.5 N HCl, 1 ml of nitrite molybdate reagent and 1 ml of 1 N NaOH, final volume was adjusted to 5 ml by distilled water. The absorbance was measured at 530 nm using double beam UV-Visible spectrophotometer (Hitachi UV 2800, Japan) and concentration of L-DOPA was determined from Arnow's standard curve of L-DOPA. The L-tyrosine utilized was measured by estimating residual L-tyrosine in the

reaction mixture by Arnow's method, 1 ml of supernatant from the same reaction mixture was added with mercuric sulfate reagent and kept in boiling water bath for 10 min, cooled at room temperature, 1 ml of nitrite reagent was added and volume was adjusted to 5 ml by distilled water. The absorbance was measured at 530 nm. The L-tyrosine concentration was determined from Arnow's standard curve of L-tyrosine.

Tyrosinase assay

The tyrosinase activity was determined by the previously described method (Ali et al. 2007). The final assay concentration in 3 ml reaction mixture contained 50 mM potassium phosphate (pH 7.4), 0.17 mM catechol, 0.070 mM L-ascorbic acid equilibrated to 25°C. The $\Delta A_{265\ nm}$ was monitored until constant, and then 0.1 ml of the supernatant from the reaction mixture was added. The decrease in the $\Delta A_{265\ nm}$ was recorded for 1 min. The $\Delta A_{265\ nm}$ was obtained using the maximum linear rate for both the test and control. One unit of tyrosinase activity was equal to a $\Delta A_{265\ nm}$ of 0.001 per min at pH 7.4 at 25°C in a 3.0 ml reaction mixture containing L-catechol and L-ascorbic acid. Protein content in the reaction mixture and growth medium was determined using Lowry's method (Lowry et al. 1951).

Analysis of L-DOPA

High-performance thin layer chromatography (HPTLC) analysis was performed using HPTLC system (CAMAG, Switzerland). The 2 µl of standard L-tyrosine (1 mg ml^{-1}), standard L-DOPA (1 mg ml^{-1}) was prepared in potassium phosphate buffer (pH 7) and reaction mixture supernatant after the completion of reaction at optimized conditions was loaded on pre-coated HPTLC plates (Silica gel 60 F 254, Merck, Germany), using spray gas nitrogen and TLC sample loading instrument (CAMAG LINOMAT 5). The HPTLC plates were developed in solvent system *n*-butanol:acetic acid:water (4:1:2; Krishnaveni et al. 2009). After development, the plate was observed in UV chamber and scanned at 280 nm with slit dimension 5 × 0.45 mm using TLC scanner. The results were analyzed using HPTLC software WinCATS 1.4.4.6337.

High-performance liquid chromatography (HPLC) analysis was carried out (Waters model no. 2690) on C 8 column (symmetry, 4.6 mm × 250 mm) using methanol as mobile phase with flow rate of 1 mg ml^{-1} for 10 min and UV detector at 280 nm. The standard L-tyrosine, standard L-DOPA and reaction supernatant were prepared in HPLC grade water and injected in HPLC column.

Gas chromatography–mass spectroscopy (GC–MS) analysis was carried out with a QP2010 gas chromatography coupled with mass spectrometer (Shimadzu). The analysis was performed in the temperature programming mode at an ionization voltage 70 eV. Temperature of the Restek column (0.25 mm, 60 m; XTI-5) was kept at 80°C for initial 2 min, and raised up to 280°C with rate of 10°C min^{-1}, and held for 7 min. The temperature of injection port and the GC–MS interface was maintained at 280 and 290°C, respectively. The flow rate for helium as a carrier gas was 1.0 ml min^{-1}. NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC–MS was used for detection of mass peaks.

Kinetic studies

Kinetic parameters for the bioconversion of L-tyrosine to L-DOPA with initial and optimized reaction conditions were compared and studied as previously reported (Ali et al. 2007). The kinetic parameters are product yield coefficient ($Y_{p/s}$) = mg of L-DOPA produced mg^{-1} of substrate consumed, volumetric rate for substrate utilization (Q_s) = mg of substrate consumed ml^{-1} h^{-1} and specific substrate consumption rate (q_s) = mg of substrate consumed mg^{-1} of cells h^{-1}. All the experiments were carried out in triplicates and data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test, using GraphPad InStat Software, and readings were significant when $P < 0.05$.

Results and discussion

Bacterial strain identification and phylogenic position

The L-DOPA producing bacterial species was isolated and confirmed as *Bacillus* sp. JPJ (NCBI Genbank accession no. FJ545652.1). The phylogenic position of *Bacillus* sp. JPJ (FJ545652.1) in relation to other species of this genus is illustrated in Fig. 1. The digits adjacent to nodes are the statistical frequency of the indicated species. The numbers shown in parentheses are accession numbers of different species.

Inoculum preparation and tyrosinase activity

The crude tyrosinase activity in broth after 3 h was 0.05 U mg^{-1} of tyrosinase with 1.2 g l^{-1} of cell mass, after 18 h of incubation was 3.2 U mg^{-1} of tyrosinase with 4.98 g l^{-1} of cell mass (Fig. 2). The incubation time increases the tyrosinase activity up to 18 h and then decreased to 2.8 U mg^{-1} of tyrosinase after 24 h of incubation, which could be of conversion to DOPAchrome and other metabolites. The incubation time for obtaining the

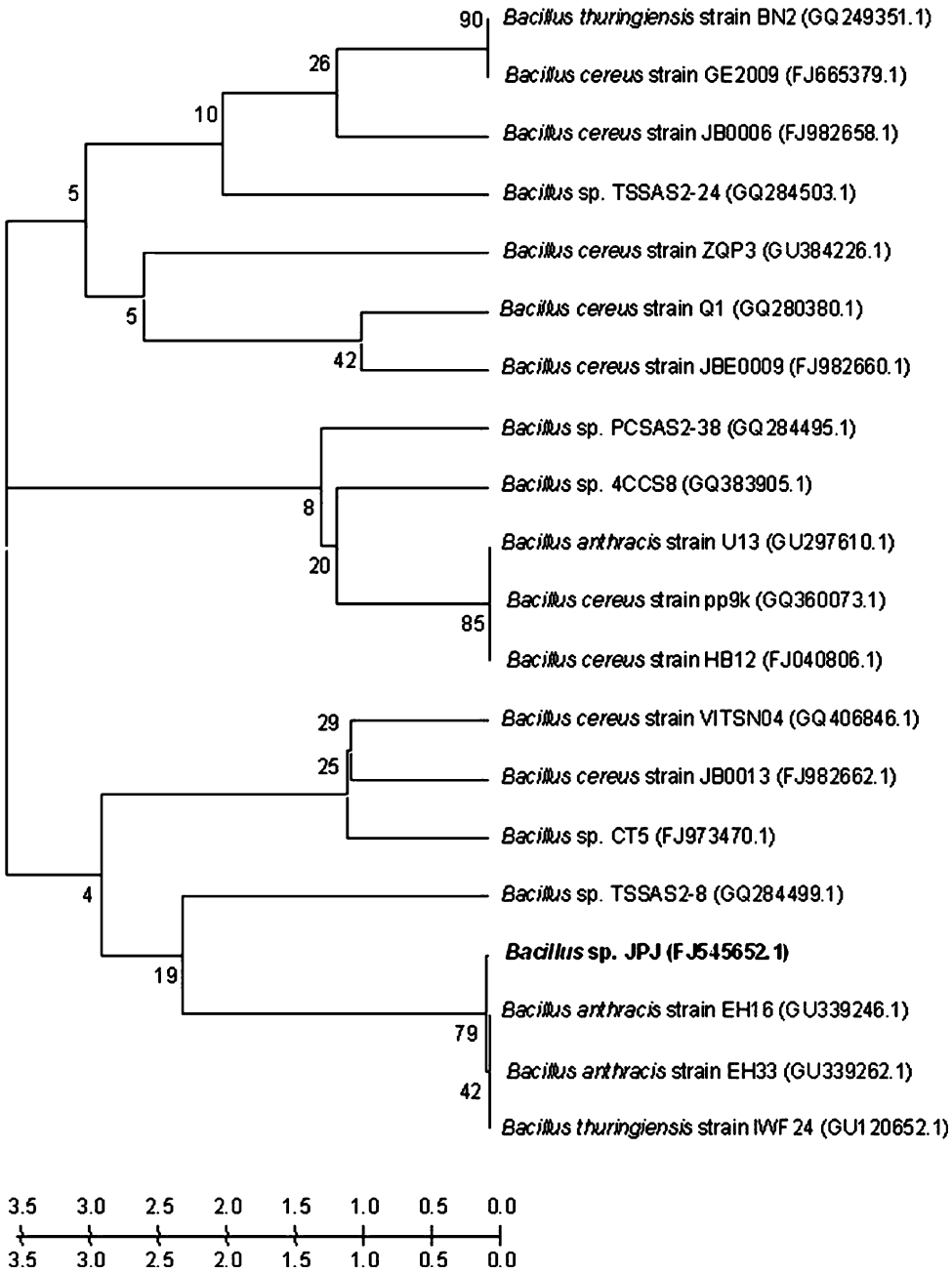

Fig. 1 Phylogenic tree of the *Bacillus* sp. JPJ and related organisms were aligned based on 16S rRNA sequences (neighbor-joining tree). *Scale bar* Number of nucleotide changes per sequence position. *The number* at nodes shows the bootstrap values obtained with 1,000 resampling analysis

cell mass for L-DOPA production in the present study was very less as compared to previously reported plant, fungal and yeast sources (Table 1). Hence, it has an advantage of fast growth over these sources and it will be useful for large scale production within less time.

Optimum cell mass

1 mg ml^{-1} cell mass was optimum which produces 0.695 mg ml^{-1} of L-DOPA by consuming 0.754 mg ml^{-1} of L-tyrosine (Fig. 3), less cell mass resulted in less L-DOPA production of 0.420 mg ml^{-1}, as cell mass increased up to 1 mg ml^{-1} the L-DOPA production increased but further increase in the cell mass to 5 mg ml^{-1}, decreased the L-DOPA production to 0.163 mg ml^{-1}. In earlier report, plant and fungal biomass required in large quantity than bacterial cell mass used here, while slightly large quantity of yeast cell mass required than present study for bioconversion of L-tyrosine to L-DOPA (Table 1). This will prove that the bacterial species reported here is efficient than previously used sources.

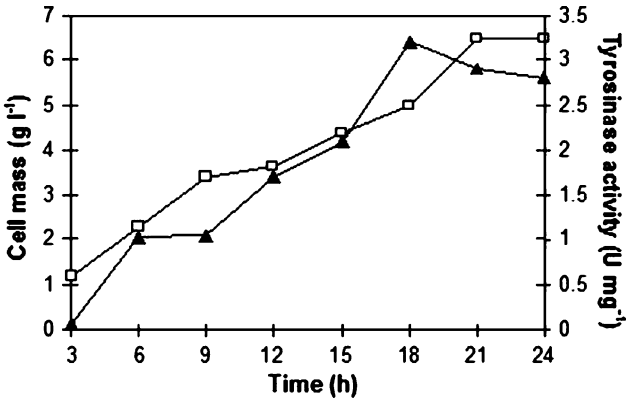

Fig. 2 Cell mass (*squares*) and crude tyrosinase activity (*triangles*) observed in growth medium after 3-h time interval for inoculum preparation

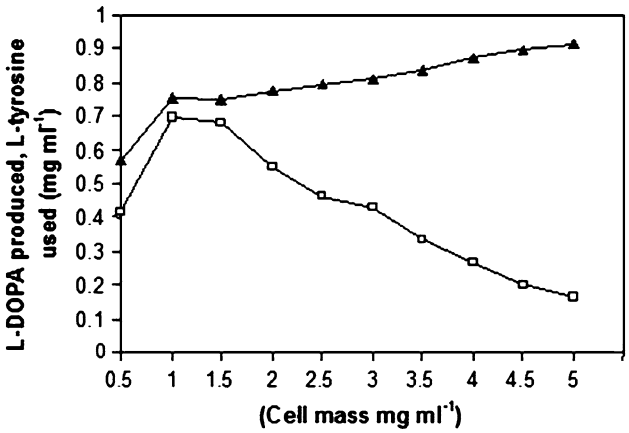

Fig. 3 Effect of cell mass on the bioconversion of L-tyrosine to L-DOPA with initial reaction conditions (1 mg ml^{-1} L-tyrosine with pH 7 and incubated at 30°C for 50 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

Optimum pH and temperature

At pH 8, there was maximum production (0.715 mg ml^{-1}) of L-DOPA by utilizing 0.812 mg ml^{-1} of L-tyrosine (Fig. 4). When pH decreased toward acidic condition, the L-DOPA production decreased, at pH 3, L-DOPA was not produced but 0.174 mg ml^{-1} of L-tyrosine was consumed and when pH increased toward alkaline, L-DOPA production decreased. At pH 10, the L-DOPA produced was very less (0.164 mg ml^{-1}), but comparatively tyrosine utilized was higher (0.870 mg ml^{-1}). This might be due to a faster conversion of L-DOPA to other metabolites at alkaline condition. Previously used pH for bioconversion of L-tyrosine to L-DOPA was acidic, while the pH required in present study was slightly alkaline (Table 1).

The effect of temperature on the bioconversion of L-tyrosine to L-DOPA showed the optimum temperature was 40°C at which 0.728 mg ml^{-1} of L-DOPA produced by utilizing 0.935 mg ml^{-1} of L-tyrosine (Fig. 5). L-DOPA produced was too low (0.134 mg ml^{-1}) at 5°C but as the temperature increased L-DOPA production increased up to 40°C and afterwards decreased to 0.297 mg ml^{-1} at 50°C. This might be because of tyrosinase that showed maximum activity at higher temperature. The literature survey revealed that the optimum temperature required for L-DOPA production from plant culture was less while for fungal and yeast biomass was same which was slightly higher than temperature required for bacterial cell mass used in present investigation (Table 1).

Optimum L-tyrosine concentration

The optimum L-tyrosine concentration was found to be 0.5 mg ml^{-1}, but cells utilize only 0.477 mg ml^{-1} (95.4%) of L-tyrosine from that and produced 0.461 mg ml^{-1} (92.2%) of L-DOPA (Fig. 6). The L-DOPA produced at lower concentration of L-tyrosine (0.25 mg ml^{-1}) was slightly less (82.6%) than the optimum. As L-tyrosine concentration increased up to 0.5 mg ml^{-1}, L-DOPA production increased but further increase in the concentration of L-tyrosine up to 5 mg ml^{-1} the production decreased to 17.6%. This might be because of the cell mass used and

Table 1 Comparison of L-DOPA production from biological sources

Biological source	*Portulaca grandiflora* (Rani et al. 2007)	*Aspergillus oryzae* (Ali and Haq 2006a, b)	*Yarrowia lipolytica* (Ali et al. 2007)	*Bacillus* sp. JPJ (present study)
Incubation time for harvesting cells	20–25 days	48 h	48 h	18 h
Cell mass (mg ml^{-1})	50	15	3	1
pH	5.8	3.5	5.4	8
Temperature (°C)	25	50	50	40
L-Tyrosine (mg ml^{-1})	0.9	2.5	3.5	0.5
L-Ascorbic acid (mg ml^{-1})	0.425	5	5	0.04
Incubation time for bioconversion	16 h	50 min	30 min	60 min
L-DOPA production (mg ml^{-1})	0.488	1.686	2.960	0.497
L-Tyrosine consumption (mg ml^{-1})	0.900	1.525	2.680	0.5

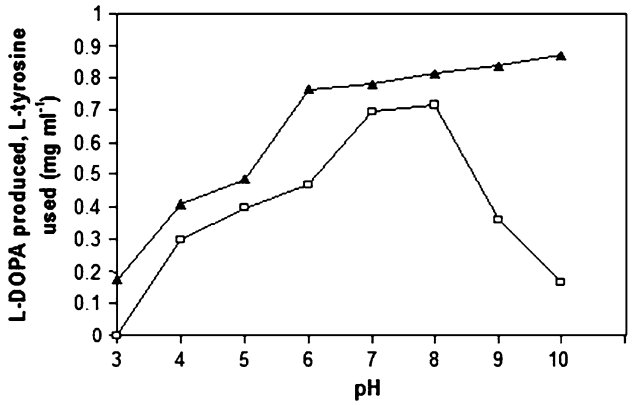

Fig. 4 pH optimization of the reaction mixture for maximum bioconversion of L-tyrosine to L-DOPA with initial reaction conditions (1 mg ml^{-1} L-tyrosine and 0.5 mg ml^{-1} cell mass and incubated at 30°C for 50 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

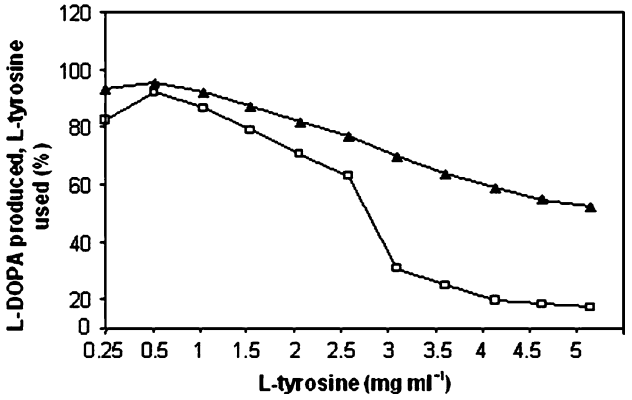

Fig. 6 Effect of L-tyrosine concentration on the bioconversion of L-tyrosine to L-DOPA with initial reaction conditions (0.5 mg ml^{-1} cell mass with pH 7 and incubated at 30°C for 50 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

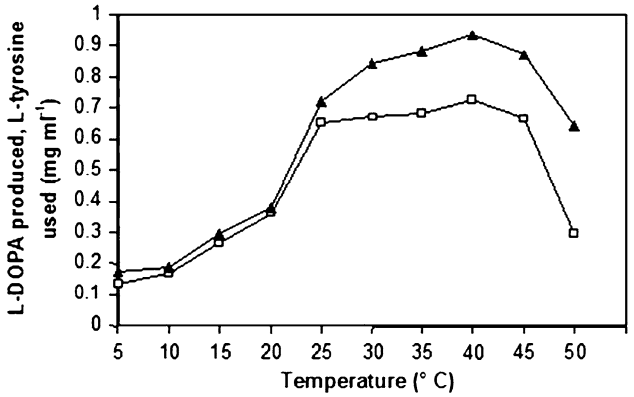

Fig. 5 Temperature optimization of the reaction mixture for maximum bioconversion of L-tyrosine to L-DOPA with initial reaction conditions (1 mg ml^{-1} L-tyrosine and 0.5 mg ml^{-1} cell mass with pH 7 for 50 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

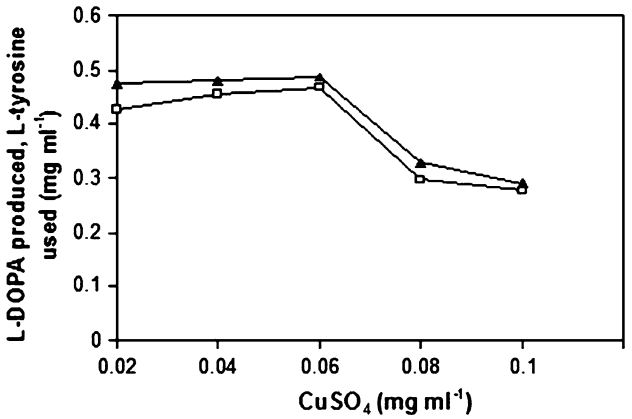

Fig. 7 Effect of $CuSO_4$ on bioconversion of L-tyrosine to L-DOPA with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 and incubated at 40°C for 50 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

incubation period provided is not sufficient to convert the higher concentrations of L-tyrosine, so it requires more time. The *Bacillus* sp. JPJ reported here produced maximum L-DOPA at lower concentrations of L-tyrosine as compared to previous reports (Table 1), but it has an advantage of less incubation time for harvesting the cells, less cell mass and slightly alkaline pH which make it suitable for cost-effective large-scale production.

Optimum concentrations of $CuSO_4$ and L-ascorbic acid

The $CuSO_4$ showed inductive effect and the maximum L-DOPA production obtained was 0.468 mg ml^{-1} by consuming 0.487 mg ml^{-1} of L-tyrosine using 0.06 mg ml^{-1} of $CuSO_4$ (Fig. 7). As the $CuSO_4$ concentration increased to 0.1 mg ml^{-1}, the L-DOPA production decreased to 0.279 mg ml^{-1}, at lower concentration of $CuSO_4$ (0.02 mg ml^{-1}) and the L-DOPA produced was 0.428 mg ml^{-1} demonstrating that lower concentrations of $CuSO_4$ resulted in sudden increase in the L-DOPA production. This might be because tyrosinase is a copper-containing enzyme; hence, the lower concentrations of $CuSO_4$ acted as inducer for it and ultimately lead to the increased L-DOPA production and its higher concentration may be toxic to the cell that results in lower L-DOPA production.

The optimum concentration of L-ascorbic acid was found to be 0.04 mg ml^{-1} which resulted in maximum L-DOPA production of 0.480 mg ml^{-1} by utilizing 0.485 mg ml^{-1} of L-tyrosine (Fig. 8). The L-DOPA production was 0.381 mg ml^{-1} at 0.02 mg ml^{-1} concentration of L-ascorbic acid and increased up to 0.04 mg ml^{-1}. The higher concentration of L-ascorbic acid that is at 0.1 mg ml^{-1}

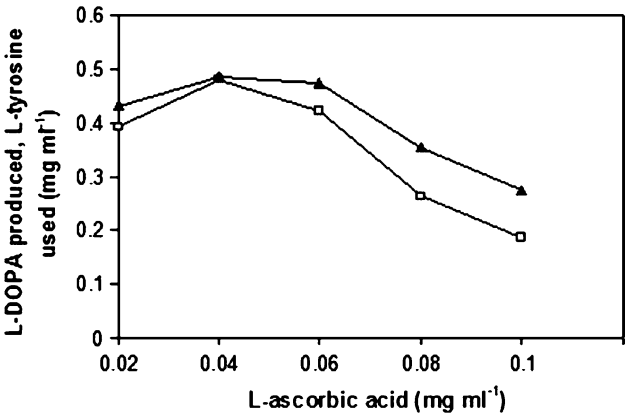

Fig. 8 Effect of L-ascorbic acid on bioconversion of L-tyrosine to L-DOPA with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 and incubated at 40°C for 50 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

lowered the L-DOPA production to 0.186 mg ml^{-1}, this may be due to L-ascorbic acid acted as an inhibitor of tyrosinase but at lower concentration it may control the further conversion of L-DOPA to L-DOPAchrome by inhibiting the excess tyrosinase produced from the cells. As compared with earlier reports (Table 1), here *Bacillus* sp. JPJ required very small amount of L-ascorbic acid which is economically feasible for large scale production.

Inductive effect of activated charcoal

This is the first time report on studying the effect of activated charcoal on the bioconversion of L-tyrosine to L-DOPA by this technique, which explains the inductive effect of activated charcoal at lower concentrations. The maximum L-DOPA production obtained was 0.497 mg ml^{-1} by consuming 0.5 mg ml^{-1} of L-tyrosine with 2 mg ml^{-1} activated charcoal (Fig. 9). The L-DOPA production with lower concentration 1 mg ml^{-1} of activated charcoal was 0.230 mg ml^{-1}. The L-DOPA production enhanced as the activated charcoal concentrations increased up to 2 mg ml^{-1} and then decreased to 0.2 mg ml^{-1} with increase in activated charcoal up to 5 mg ml^{-1}. The activated charcoal particles might be prevented the clumping of bacterial cells offering more surface area to absorb the L-tyrosine from buffer to inside the cell and so make it available for the cells for maximum production of L-DOPA, like another enhancing materials used previously (Ali et al. 2007).

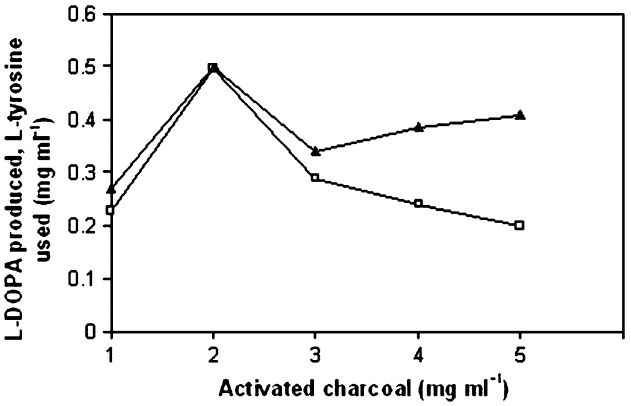

Fig. 9 Effect of activated charcoal on bioconversion of L-tyrosine to L-DOPA with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 and incubated at 40°C for 50 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

Optimized incubation time

The effect of incubation time on L-DOPA production with 30-min time intervals was carried out at optimized reaction conditions that are with the optimum cell mass, pH, temperature and with optimized concentrations of L-tyrosine, $CuSO_4$, L-ascorbic acid and activated charcoal. The maximum L-DOPA obtained after 60 min of incubation time was 0.497 mg ml^{-1} (99.4%) by utilizing 0.5 mg ml^{-1} of L-tyrosine (Fig. 10). The effect of incubation time on L-DOPA production showed that as the incubation time increased up to optimum the L-DOPA production increased and then decreased to 0.03 mg ml^{-1} after 150 min of incubation time. This might be due to further conversion of L-DOPA to other metabolites. The time required for optimum bioconversion of L-tyrosine to L-DOPA in the earlier reports is much more for plant biomass, slightly less for fungal biomass. Although the yeast species reported earlier required half time than bacterium reported here (Table 1), it produced maximum L-DOPA using less incubation time for harvesting the cells, less cell mass and slightly alkaline pH for maximum L-DOPA production which will be

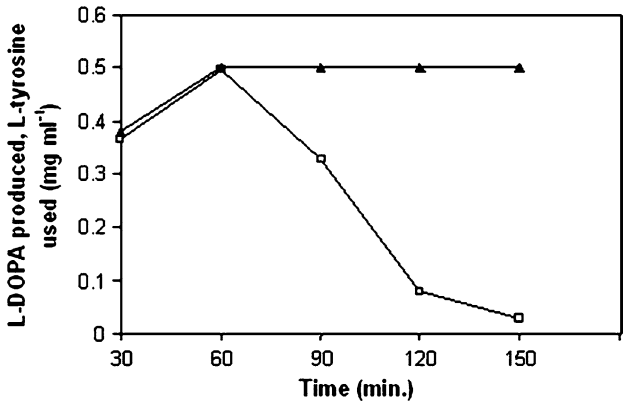

Fig. 10 Effect of incubation time with 30-min time interval on bioconversion of L-tyrosine to L-DOPA with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 incubated at 40°C and optimum concentrations of $CuSO_4$ 0.06 mg ml^{-1}, L-ascorbic acid 0.04 mg ml^{-1} and activated charcoal 2 mg ml^{-1}); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

advantageous over yeast species for cost-effective industrial scale production.

The comparative L-DOPA production at 10 min of incubation time intervals with initial reaction condition showed L-DOPA production increased after 30 min of incubation time, and maximum L-DOPA production was 0.428 mg ml^{-1} (42.8%) by consuming 0.624 mg ml^{-1} (62.4%) of L-tyrosine (Fig. 11), while with optimized reaction condition L-DOPA production increased after 20 min of incubation and maximum L-DOPA production observed was 0.497 mg ml^{-1} (99.4%) by utilizing 0.500 mg ml^{-1} (100%) of L-tyrosine after 60 min (Fig. 12).

The L-DOPA production reported using plant source *P. grandiflora* was 0.488 mg ml^{-1} by consuming 0.900 mg ml^{-1} L-tyrosine (Rani et al. 2007). L-DOPA

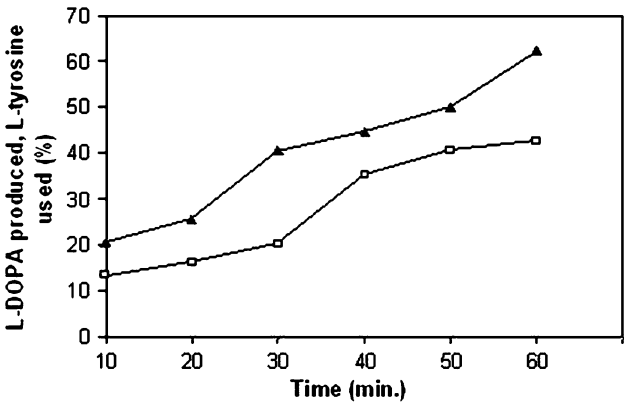

Fig. 11 Bioconversion of L-tyrosine to L-DOPA with 10 min of time interval with initial reaction conditions (1 mg ml^{-1} L-tyrosine and 0.5 mg ml^{-1} cell mass with pH 7 incubated at 30°C for 60 min); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

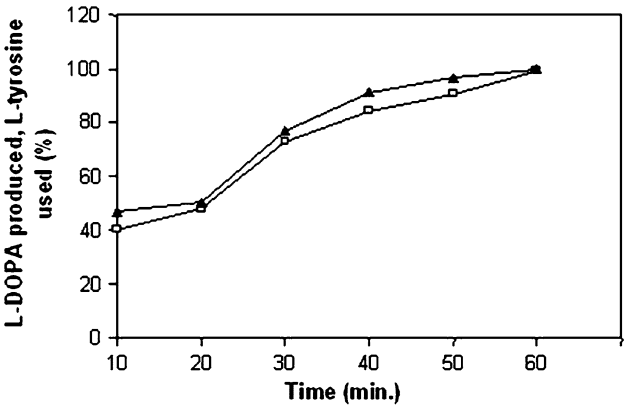

Fig. 12 Bioconversion of L-tyrosine to L-DOPA with 10 min of time interval with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 incubated at 40°C and optimum concentrations of $CuSO_4$ 0.06 mg ml^{-1}, L-ascorbic acid 0.04 mg ml^{-1} and activated charcoal 2 mg ml^{-1}); L-DOPA produced (*squares*), L-tyrosine used (*triangles*)

produced from fungal species like *A. oryzae* was 1.686 mg ml^{-1} with consumption of 1.525 mg ml^{-1} L-tyrosine using additional enhancing material Celite (Ali and Haq 2006a, b) and *A. rutilum* produced L-DOPA in a potato dextrose broth was 0.890 mg ml^{-1} with 5 mg ml^{-1} L-tyrosine (Krishnaveni et al. 2009), from yeast *Y. lipolytica* biomass the L-DOPA produced in buffer containing L-tyrosine and enhancing material diatomite was 2.960 mg ml^{-1} by utilizing 2.680 mg ml^{-1} L-tyrosine (Ali et al. 2007). The production of L-DOPA was reported from the bacterial species that includes immobilized *E. intermedia* cells in polyacrylamide gel from pyrocatechol and the productivity was 0.180 mg ml^{-1} of L-DOPA with 1 mg ml^{-1} pyrocatechol (Para and Baratti 1988). It is also produced from mutant strains of actinomycetes in a broth with 1.4 mg ml^{-1} using 2 mg ml^{-1} of L-tyrosine (Sukumaram et al. 1979).

As compared with earlier reports, the novel bacterial species *Bacillus* sp. JPJ produces 0.497 mg ml^{-1} (99.4%) of L-DOPA by consuming 0.5 mg ml^{-1} (100%) of L-tyrosine in reaction mixture containing 1 mg ml^{-1} cell mass, 0.06 mg ml^{-1} $CuSO_4$, 0.04 mg ml^{-1} L-ascorbic acid and 2 mg ml^{-1} of activated charcoal incubated at 40°C with pH 8 for 60 min. The crude tyrosinase activity of the reaction mixture observed after the incubation at optimized reaction conditions was 2.7 U mg^{-1} of tyrosinase. The tyrosinase activity reported earlier in *Y. lipolytica* (Ali et al. 2007) was 1.55 U mg^{-1} of tyrosinase which was less than bacterial strain used in this study, so this strain could be proved potential for large-scale production of L-DOPA.

The bioconversion of L-tyrosine to L-DOPA using bacterial system is very rapid and could be efficient system than plant fungal and yeast systems, as these systems are not appropriate for industrial scale production of L-DOPA due to its requirements of complex nutrients, longer incubation time and susceptibility to contamination, also they are not cost effective. The bacterial system used here is also superior over the chemical synthesis, as it produces optical active L-DOPA and no synthesis of side products. The bacterial cells can be harvested for production with short incubation time, also the cell mass required was very less than plant, fungal and yeast species reported earlier. The L-DOPA produced earlier by bacterial system like *E. intermedia* cells (Para and Baratti 1988) used pyrocatechol as substrate which is a toxic phenolic compound, it also requires polyacrylamide gel which is an expensive chemical; hence, this method is not suitable for industrial scale production. The another bacterial system used previously for L-DOPA production was *E. herbicola* cells (Koyanagi et al. 2005) which required catechol which is a toxic chemical compound, in addition to this it required ammonium chloride, ammonium nitrate, sodium nitrite and disodium ethylenediaminetetraacetic acid, so all these

chemicals required for reaction mixture make it costly process which is not feasible industrially. Thus, the bacterial system of *Bacillus* sp. JPJ used in this study is novel and first time reported for the production of L-DOPA from L-tyrosine by this technique which is advantageous than bacterial systems reported earlier. Also, the L-DOPA produced by this method is more pure than produced in the medium, which may contain other amino acids, short peptides and proteins that may interfere and makes difficult the final purification of L-DOPA.

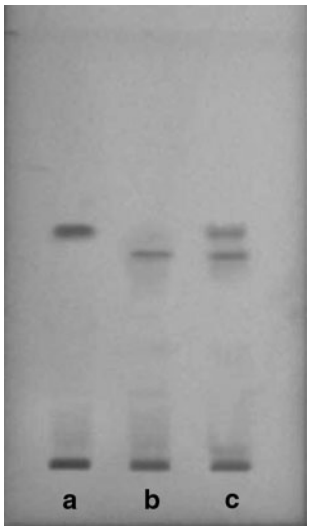

Fig. 13 HPTLC sheet loaded with *a* standard L-tyrosine, *b* standard L-DOPA and *c* reaction mixture with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 incubated at 40°C and optimum concentrations of $CuSO_4$ 0.06 mg ml^{-1}, L-ascorbic acid 0.04 mg ml^{-1} and activated charcoal 2 mg ml^{-1})

Detection of L-DOPA

The dark single band of standard L-tyrosine and standard L-DOPA was observed and two bands of reaction mixture supernatant were observed on fluorescent green HPTLC plate (Fig. 13). The three-dimensional graph of Rf values versus absorbance generated after scanning of HPTLC plate showed distinct peaks of standard L-tyrosine at Rf 0.30, standard L-DOPA at Rf 0.23 and reaction mixture supernatant showed two distinct peaks at Rf 0.29 and Rf 0.22, this confirms the production of L-DOPA in the reaction mixture (Fig. 14).

The HPLC elution profile of standard L-DOPA showed peak at retention time 2.723 min, and HPLC elution profile of the reaction mixture confirmed the production of L-DOPA with peak at retention time 2.724 min (Fig. 15).

The GC–MS analysis of the reaction mixture supernatant indicated a distinct mass peak (MW 197, *m/z* 197) that corresponded to molecular weight of L-DOPA (Fig. 16). Kinetic parameters were calculated and showed significance at $P < 0.0001$ (Table 2).

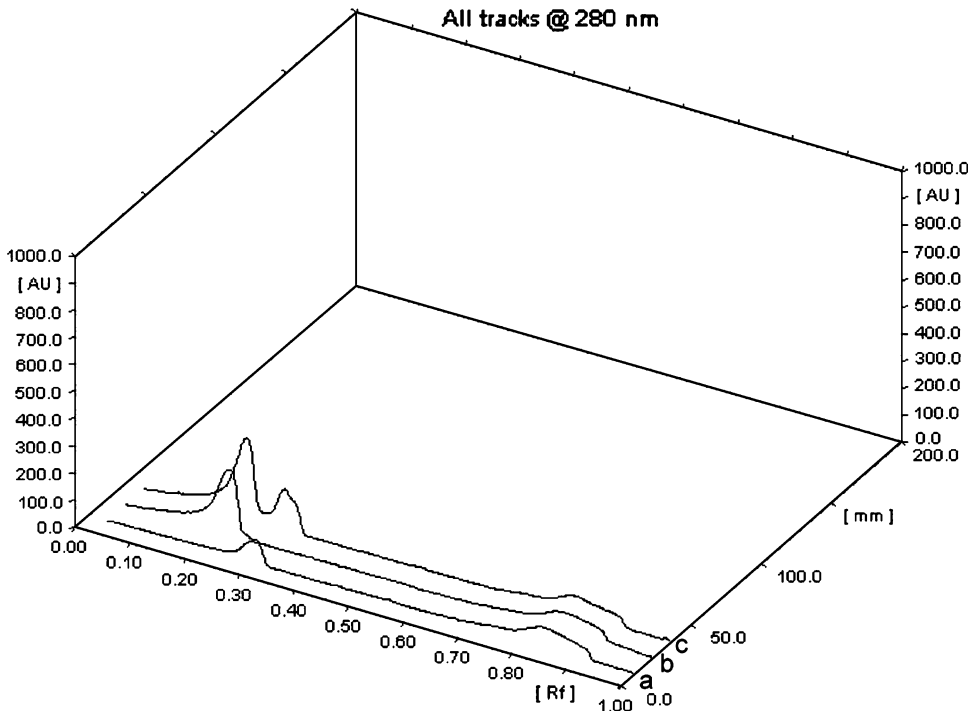

Fig. 14 Three-dimensional graph generated after scanning of HPTLC plate *a* standard L-tyrosine, *b* standard L-DOPA and *c* reaction mixture with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 incubated at 40°C and optimum concentrations $CuSO_4$ 0.06 mg ml^{-1}, L-ascorbic acid 0.04 mg ml^{-1} and activated charcoal 2 mg ml^{-1})

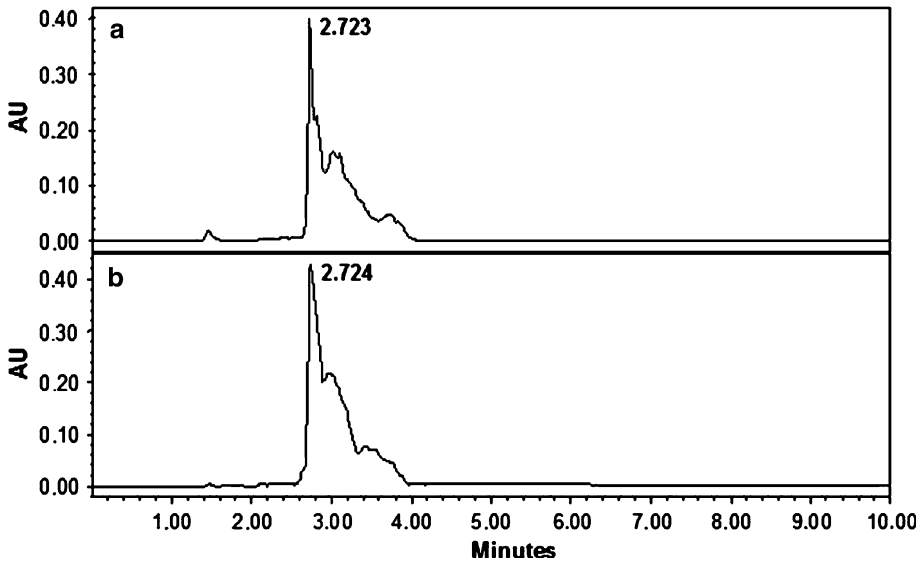

Fig. 15 **a** HPLC elution profile of standard L-DOPA and **b** HPLC elution profile of reaction mixture with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 incubated at 40°C and optimum concentrations $CuSO_4$ 0.06 mg ml^{-1}, L-ascorbic acid 0.04 mg ml^{-1} and activated charcoal 2 mg ml^{-1})

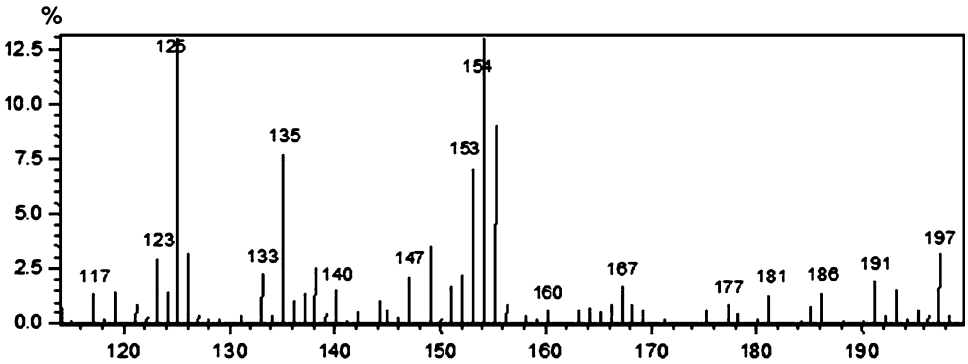

Fig. 16 GC–MS analysis of reaction mixture with optimized reaction conditions (0.5 mg ml^{-1} L-tyrosine and 1 mg ml^{-1} cell mass with pH 8 incubated at 40°C and optimum concentrations $CuSO_4$ 0.06 mg ml^{-1}, L-ascorbic acid 0.04 mg ml^{-1} and activated charcoal 2 mg ml^{-1})

Table 2 Comparison of kinetic parameters of L-DOPA production with initial reaction condition and optimized reaction condition

Kinetic parameters[a]	Initial reaction conditions[b]	Optimized reaction conditions[b]
$Y_{p/s}$	0.684 ± 0.0008*	0.993 ± 0.0008*
Q_s	0.576 ± 0.001*	0.500 ± 0.0008*
q_s	0.860 ± 0.0008*	0.994 ± 0.0008*

* $P < 0.0001$ by one-way ANOVA with Tukey–Kramer comparison test

[a] Product yield coefficient ($Y_{p/s}$) = mg of L-DOPA produced mg^{-1} of substrate consumed, volumetric rate for substrate utilization (Q_s) = mg of substrate consumed ml^{-1} h^{-1}, specific substrate consumption rate (q_s) = mg of substrate consumed mg^{-1} of cells h^{-1}

[b] Values are mean of three experiments ± SEM significantly different from initial reaction condition at * $P < 0.0001$ by one-way ANOVA with Tukey–Kramer comparison test

Conclusion

The *Bacillus* sp. JPJ reported here was observed to produce maximum L-DOPA 0.497 mg ml^{-1} by utilizing 0.5 mg ml^{-1} L-tyrosine. Thus, bacterial system used here carried out efficient bioconversion (99.4%) of L-tyrosine to L-DOPA. The optimization of physical parameters and optimum addition of inducers will lead to the cost effective and industrially feasible production of L-DOPA.

Conflict of interest The authors declare that they have no conflict of interest.

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