Biotransformation of a single amino-acid L-tyrosine into a bioactive molecule L-DOPA

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Abstract- *Penicillium jensenii* (identified by Dept. of Mycology, Agharkar Research Institute, Pune) isolated from soil (rhizosphere zone) was found to be a potent tyrosinase producer. Tyrosinase is capable of bringing about biotransformation of a single amino acid tyrosine to L-DOPA (3, 4-dihydroxyphenylalanine) which is used as therapeutic agent in the treatment for Parkinson's disease. Maximum yield of tyrosinase was 13 U/ml at 30°C in tyrosine broth (3mg/ml of tyrosine), pH 7 with an inoculum size of 5% w/v after 24 hrs of incubation under shake-culture conditions (120 rpm).

Index Terms- Biotransformation, L-DOPA (3, 4-dihydroxyphenylalanine), *Penicillium jensenii*, Tyrosinase

I. Introduction

Biotransformation is the chemical modification (or alteration) of chemical compounds like amino acids[1], toxins [2], xenobiotics [3,4] and drugs [5-7], brought about by an organism to produce bioactive molecules. Enzymes are the catalysts for the biotransformation reactions [8]. Tyrosinases have been isolated from prokaryotes such as *Pseudomonas putida*, *Bacillus* sp. [9, 10]; fungi such as *Aspergillus oryzae* [11, 19], *Neurospora crassa* [12], *Agaricus bisporus* [13], *Pycnoporus* sp. [14] and *Acremonium rutilum* [21]; plants such as *Amorphophallus campanulatus* [15] and *Portulaca grandiflora* [16]; mammals [17]. Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase) EC 1.14.18.1; is a type III copper protein and is an important enzyme participating in the process of melanin biosynthesis [18]. One of the main applications of tyrosinase is biotransformation of a single amino acid L-tyrosine to a biologically active molecule L-DOPA (3,4-dihydroxy-L-phenylalanine) by hydroxylation reaction as shown in **Figure 1**. This biotransformation reaction was first reported in edible mushroom *Agaricus bisporus* [13]. L-DOPA is converted into dopamine in the brain and body by the enzyme L-aromatic amino acid decarboxylase which is further responsible for all kinds of the voluntary movements of the body. In clinical use, Levodopa is used as therapeutic for Parkinson's disease [20].

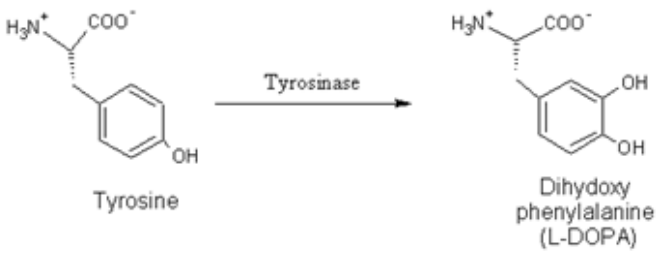

Figure 1: Biotransformation of L-tyrosine to L-DOPA by tyrosinase

The present study was undertaken to isolate and explore novel tyrosinase producing microorganisms present in the soil and obtain considerable amount of L-DOPA. There have been no reports of tyrosinase from *Penicillium jensenii* producing L-DOPA as far as we are aware. This paper deals with identification of the isolate producing tyrosinase and confirmation of the production of L-DOPA as well as optimization of the environmental parameters for maximum production of tyrosinase and L-DOPA.

II. Materials and Methods

Materials

Milk agar-

Peptone	1 %
NaCl	0.5 %
Yeast Extract	0.3%
Agar	2%
Milk separately autoclaved at 10 lbs pressure	10 %
pH	6.5-7.2

Tyrosine agar –

Peptone	0.5%
Beef extract	0.3%
Agar	2%
L-tyrosine	0.5%
pH	7

Tyrosine Broth-

0.1gm Tyrosine dissolved in 100 ml distilled water and a few drops of chloroform
pH 7.

Enrichment media for bacteria-

Yeast extract	0.3%
Peptone	1%
NaCl	0.5%
pH	7

Enrichment media for fungi and *Actinomyces*-

Glucose	3%
Yeast extract	0.5%
Peptone	0.5%
NaCl	0.5%
pH	8-8.2

Chemical detection of L-DOPA-
0.5 N Hydrochloric acid
Nitrite- molybdate reagent-
Dissolve 10 gm. of sodium nitrite and 10 gm. of sodium molybdate in 100 ml. of distilled water
1 N sodium hydroxide
Standard curve of L-DOPA (Sigma Aldrich) estimation using nitrite-molybdate reagent (Range: 0.01-0.1mg/ml)

Buffer systems for pH optimization-
0.2M Acetate buffers of pH- 4, 4.6, 5, and 5.6
0.2M Phosphate buffers of pH- 6, 6.6, 7, 7.6, and 8.

Tyrosinase assay-
0.2M Phosphate buffer (pH 6.6)
0.001M L-tyrosine (Sigma Aldrich)

HPLC-
Column C-18 250/4.6 mm length and diameter Cremarso prominence (THENOMENEF), mobile phase is 0.1 M Potassium phosphate buffer pH 3.5 with a flow rate of 0.6 ml/min. The detector potential is set at 0.8 V vs the Ag/AgCl electrode wavelength 215 nm and 270 nm. [28]
Alpha methyl DOPA (Sigma Aldrich) as standard

Enzyme purification-
Ammonium sulphate – analytical grade (60%)
Dialysis membrane (Hi-Media) (10kDa MWCO)
0.2M Phosphate buffer (pH 7)
SDS-PAGE (mid-range protein marker from Bio-Era)
Bradford's reagent for protein estimation

Methods
Isolation of tyrosinase producers from the soil sample
1gm of soil sample was serially diluted and 0.1 ml was spread plated on milk agar. Isolated colonies showing zone of casein hydrolysis were selected.

Qualitative detection of tyrosinase production on tyrosine agar and tyrosine broth-
Tyrosinase detection on Tyrosine agar [21]-
Qualitative selection of good tyrosinase producers by spot inoculation on tyrosine agar medium was done to check and compare their capacity to produce melanoidin pigments (brown to black). Incubation was done at 30^{o}C for 24 to 48 hrs.

Characterization and Identification of Isolates:
The organism showing maximum production of tyrosinase and L-DOPA was characterized on the basis of morphological characteristics.

Tyrosinase detection in Tyrosine broth-
A loopful of the each of the above isolates was inoculated in 0.1% tyrosine water having pH 7. Few drops of chloroform were added to the inoculated broth to prevent the loss of tyrosinase (if produced by the cultures) from the broth. Incubation was done at 30^{o}C for 24 to 48 hrs. If tyrosinase is produced, broth colour changes from colourless to pink to brown and ultimately black.

Tyrosinase Assay [29]-
Spectrophotometer was adjusted to 280 nm and 25°C.
Following solutions were pipetted into test tube in the following order:

Solution to be added	Amount (ml)
0.5 M phosphate buffer, pH 6.5	1.0
0.001 M L-tyrosine	1.0
Reagent grade water	0.9

The reaction mixture was oxygenated by bubbling oxygen through a capillary tube for 4-5 minutes. Absorbance was recorded at 280 nm for 4-5 minutes to achieve temperature equilibration and to establish blank. 0.1 ml of the supernatant of the enriched culture in tyrosine broth (presumably expected to be producing tyrosinase) was added to the reaction mixture and absorbance was recorded for 10-12 minutes. A non-linear "lag" of 2-3 minutes can be expected. Calculation of enzyme activity was done using the following formula:

Units of enzyme/ ml=

$$\frac{\left(\Delta A_{280\,nm}/\min \text{ Test} - \Delta A_{280nm}/\min \text{ Blank}\right)}{(0.001)(0.1)}(df)$$

Protein content: Bradford's method
Protein content was measured with Bovine Serum Albumin (BSA) as standard protein by Bradford's method [30]. 1 ml of sample was mixed with 5 ml of Bradford's reagent (Coomasie Brilliant Blue G-250) and incubated for 5 min. Absorbance was measured at 595 nm. Protein content was expressed as milligrams of protein per millilitre of sample. Following tyrosinase assay and Bradford's method, specific activity of tyrosinase was calculated using the following formula-

$$\text{Specific activity} = \frac{\text{Units of enzyme/ml/min}}{\text{mg of protein/ml}}$$

Biotransformation reaction: Quantitative detection of L-DOPA

L-DOPA Production was measured according to the method of Arnow for chemical detection [24]. The method has the following steps-

- Enriched culture incubated in tyrosine broth (0.1%) for 24 hrs at 30°C.
- Post filtration (Whatman filter paper No. 1) l ml of cell free broth was taken and the following reagents were added in the following order, mixing well after each addition.

Reagent	Amount (ml)
0.5 N hydrochloric acid	1
Nitrite-molybdate reagent	1
Yellow color results at this point	
1 N sodium hydroxide	1
Red colour develops at this point. Make up volume with distilled water to 5 ml	
Record the absorbance at 530nm	

The absorbance recorded is directly proportional to amount of L-DOPA present in the reaction mixture.

Time course of L-DOPA production during primary screening:
Isolates showing a brown colouration of melanin production on tyrosine agar were checked for production of L-DOPA in 1% tyrosine broth, over an incubation period of 6 days. At the end of every 24 hrs, chemical detection of L-DOPA was performed, as per the method explained above [24].

Qualitative detection of L-DOPA production by HPLC- [28]
Each of the isolates was inoculated in Tyrosine broth. After 48 hrs and 72 hrs sample were withdrawn and centrifuged. The supernatant was collected and was subjected to HPLC analysis for the detection of L-DOPA produced by 5 isolates (as it showed appreciable amount of L-DOPA production).

Optimization of the pH for enzyme production and enhanced L-DOPA production [11, 23, 24, 25]-
In order to further check the enhanced production of tyrosinase and L-DOPA, different buffer systems were taken having a specific pH which is as follows:

Buffer system	pH
0.2M Acetate buffer	4 4.6 5 5.6
0.2M Phosphate buffer	6 6.6 7 7.6 8

Cells were harvested from their respective enrichment media (5 gm) and inoculated into these buffers containing 0.1% tyrosine. The flasks were incubated on a shaker at 30°C for 24 hrs. The broth was then centrifuged and the supernatant was checked for enzyme units (enzyme assay) and concentration of L-DOPA produced (chemical detection).

Optimization of the temperature for enzyme production and enhanced L-DOPA production [11, 23, 24, 25]-
0.1% tyrosine broth inoculated with enriched culture and incubated at different temperatures ranging from 20°C to 60°C for 24hrs.
Following incubation, the broth was centrifuged and the supernatant was assayed for tyrosinase activity and L-DOPA production.

Optimization of the concentration of substrate for enzyme production and enhanced L-DOPA production [23]-
Tyrosine broth of different concentrations ranging from 1mg/ml to 10mg/ml was inoculated with enriched culture and incubated for 24 hrs at 30°C.
Following incubation, the broth was centrifuged and the supernatant was assayed for tyrosinase activity as well as L-DOPA production.

Purification of tyrosinase from *Penicillium jensenii*: [26]
Penicillium jensenii was enriched in appropriate enrichment medium. The enriched culture was then re-inoculated in 0.1% tyrosine broth and incubated for 24 hrs at 30°C. The culture was filtered and the tyrosinase was further purified from the filtrate, following the steps given below-

- **Ammonium sulphate precipitation:**

Analytical grade ammonium sulphate powder was added to the filtrate slowly with continuous stirring in an ice bath, until 60% saturation is achieved. The mixture was refrigerated overnight, followed by centrifugation at 6000-10,000g in a refrigerated centrifuge. The precipitate was dissolved in 0.2 M phosphate buffer (pH 7.0). The suspended precipitate was then checked for tyrosinase activity as well as total protein content.

- **Dialysis:**

Dialysis membrane of appropriate length was pre-treated in boiling water for 60 min and stored in 0.2 M phosphate buffer (pH7.0). The membrane was filled up with the suspended precipitate and sealed at both ends. The dialysis bag was then suspended overnight at 4°C in a glass cylinder containing 0.2 M phosphate buffer (pH 7.0) with continuous mixing using a magnetic stirrer. The dialysed sample was checked for tyrosinase activity as well as total protein content [29, 30].

SDS-PAGE- SDS-PAGE was carried out in a 12% polyacrylamide gel using Tris-glycine buffer (pH 8.3) by the method of Laemmli [27]. The cell free broth (crude extract), ammonium sulphate precipitated protein and dialysed sample obtained from *Penicillium jensenii* was loaded on to a denaturing polyacrylamide gel and compared with mid-range protein marker. Silver staining was performed in order to visualize the protein bands. The gel results were documented in Gel Doc EZ Imager, BioRad; Software: Image Lab 3.0.

III. RESULTS

From the soil sample 10 isolates were obtained that gave a zone of hydrolysis on (casein) milk agar. Out of these only 8 isolates showed positive result on Tyrosine agar plate. The occurrence of a distinct brown spot which gradually changed its color to black (melanin formation) was indicative of the fact that the above isolates were tyrosinase positive. The yield of L-DOPA obtained from the isolates over a period of 6 days has been compared in Table I. It was seen that the three bacterial isolates viz. DKP-1-B, DKP-2-B and DKP-3-B did not show appreciable production of L-DOPA even after 6 days of incubation. The two fungal isolates DKP-1-F and DKP-2-F produced a maximum of 0.038mg/ml of L-DOPA on the 6th day. The screening results have been graphically represented in Figure 2.

Table I: L-DOPA production by different soil isolates over an incubation period of 6 days

Organism	Days	L-DOPA (mg/ml)
DKP-1-F	Day 1	0.025
	Day 2	0.025
	Day 3	0.027
	Day 4	0.032
	Day 5	0.03
	Day 6	0.038
DKP-2-F	Day 1	0.015
	Day 2	0.016
	Day 3	0.023
	Day 4	0.03
	Day 5	0.032
	Day 6	0.038
DKP-1-A	Day 1	0.01
	Day 2	0.018
	Day 3	0.02
	Day 4	0.018
	Day 5	0.01
	Day 6	0.01
DKP-2-A	Day 1	0.015
	Day 2	0
	Day 3	0.003
	Day 4	0.005
	Day 5	0.015
	Day 6	0.013
DKP-3-A	Day 1	0.01
	Day 2	0
	Day 3	0
	Day 4	0
	Day 5	0.001
	Day 6	0.004
DKP-1-B	Day 1	0.01
	Day 2	0.01
	Day 3	0.01
	Day 4	0.015
	Day 5	0.01
	Day 6	0.028
DKP-2-B	Day 1	0
	Day 2	0
	Day 3	0
	Day 4	0
	Day 5	0.005
	Day 6	0.003
DKP-3-B	Day 1	0.01
	Day 2	0
	Day 3	0
	Day 4	0.015
	Day 5	0.002
	Day 6	0

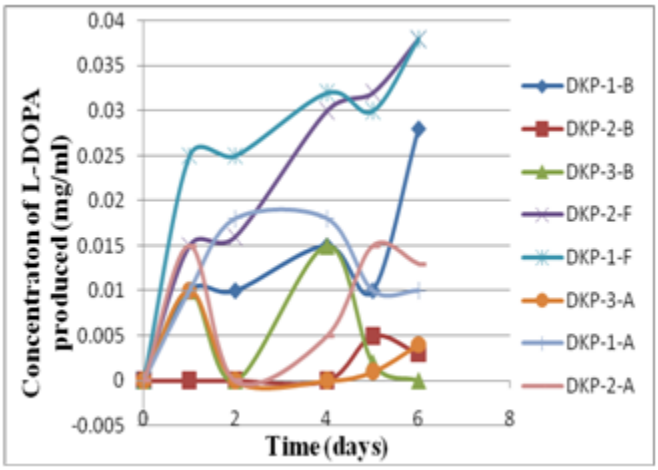

Figure 2: L-DOPA production by different soil isolates over an incubation period of 6 days

The fungal isolate DKP-1-F was identified on the basis of morphological techniques as Penicillium jensenii by ARI (Agharkar Research institute), Pune. The colony morphology and microscopic observation of P. jensenii have been demonstrated in Figure 3 a,b.

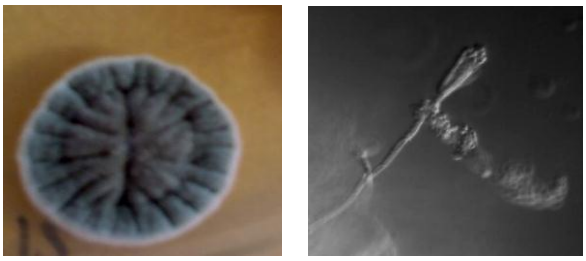

Figure 3: (a) *Penicillium jensenii* on tyrosine agar plate. (b) Wet mount of *Penicillium jensenii*, light microscope at 45X.

HPLC results:
The comparison of retention time of standard L-DOPA and standard alpha methyl DOPA with that produced by isolates is given below-

Retention time in minutes of standard compounds	**Retention time in minutes of test samples**
L-DOPA-3.5 to 3.8 min	3.765 (215 nm) DKP-1-F
Alpha methyl DOPA -5.5 min	5.544 (270 nm) DKP-1-F 5.508 (215 nm) DKP-1-F

The tyrosinase activity as well as L-DOPA production by *P. jensenii* (DKP-1-F) was optimized with respect to temperature, pH and substrate concentration.

pH:
The results summarized in **Table II** and **Figure 4** indicated that optimum pH for tyrosinase production was pH7.0, while that for L-DOPA production is pH 6.

Table II: Units of tyrosinase and amount of L-DOPA obtained per ml, at different pH values

pH	**Enzyme U/ml**	**L-DOPA (mg/ml)**
4	0	0
4.6	0	0
5	4	0
5.6	5	0.01
6	6	0.085
6.6	6	0.08
7	8	0.032
7.6	6	0.025
8	6	0

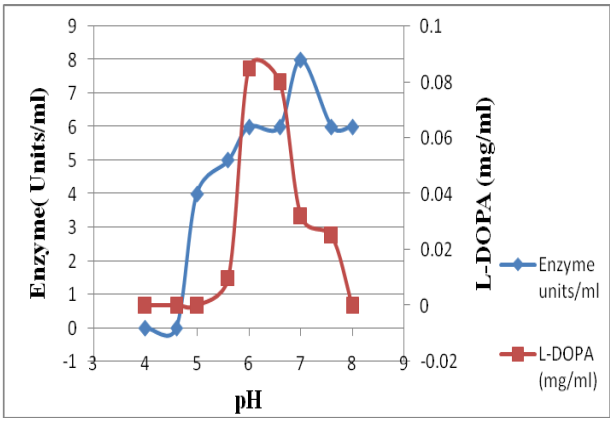

Figure 4: Optimization of pH conditions for tyrosinase and L-DOPA production by P. jensenii

Temperature:
Table III and Figure 5 indicate that the optimum temperature for both tyrosinase production as well as L-DOPA production is 30^0C.

Table III: Units of tyrosinase and amount of L-DOPA obtained per ml, at different temperature values

Temperature (^{0}C)	Enzyme (U/ml)	L-DOPA production (mg/ml)
20	5	0
30	13	0.025
40	5	0.005
50	3	0
60	4	0

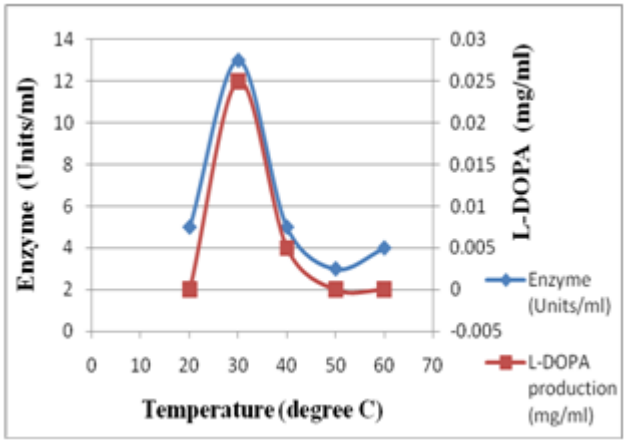

Figure 5: Optimization of temperature conditions for tyrosinase and L-DOPA production by *P. jensenii*

Substrate concentration:
It was observed that equal tyrosinase production was obtained at a substrate concentration of 1mg/ml and 3mg/ml. Maximum yield of L-DOPA was obtained at a concentration of 3mg/ml, as indicated in **Table IV** and **Figure 6**.

Table IV: Production of tyrosinase and L-DOPA at various substrate concentrations

Substrate concentration (L-tyrosine, mg/ml)	Enzyme (U/ml)	L-DOPA production (mg/ml)
1	11	0.001
3	11	0.014
5	0	0.002
7	0	0
9	0	0
10	0	0

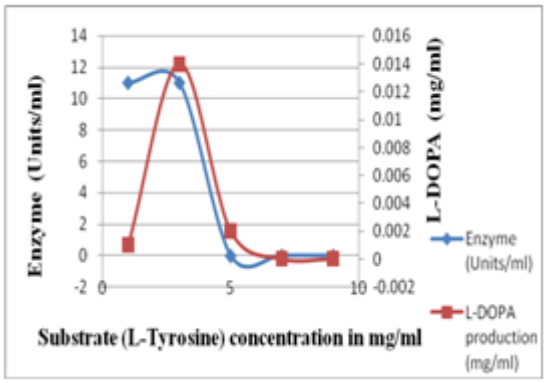

Figure 6: Optimization of tyrosinase and L-DOPA production at different substrate concentration

Purification of tyrosinase:
The comparison of specific activity and fold purification between the crude extract and the dialysed precipitate has been depicted in **Table V**. The specific activity of tyrosinase was shown to increase from 933.333U/mg to 3000U/mg after ammonium sulphate precipitation. The table also indicates that tyrosinase was purified by 3.214 fold.

The crude extract as well as the purified enzyme (post-dialysis) was allowed to run on a polyacrylamide gel under denaturing conditions, along with a mid-range protein marker. Results summarized in **Figure 7** and **Figure 8** revealed the presence of a single band corresponding to approximately 14.7 kDa, which is found to be smaller than the molecular weights of known fungal tyrosinases.

Table V: Partial purification chart of tyrosinase produced by Penicillium jensenii

Steps	Volume taken (ml)	Total protein (mg/ml)	Total enzyme activity (U/ml/min)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	800	0.015	14	933.333	1	100
60% $(NH_4)_2SO_4$ followed by dialysis	50	0.004	12	3000	3.214	85.714

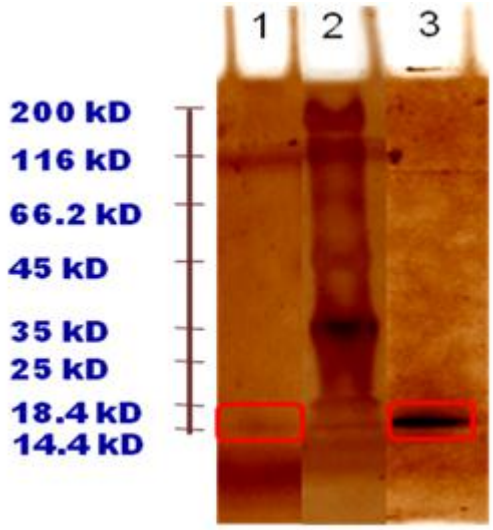

Figure 7: SDS-PAGE Gel Doc image of partially purified tyrosinase stained by silver staining technique. (1) crude extract (2) mid-range protein marker (3) sample post dialysis

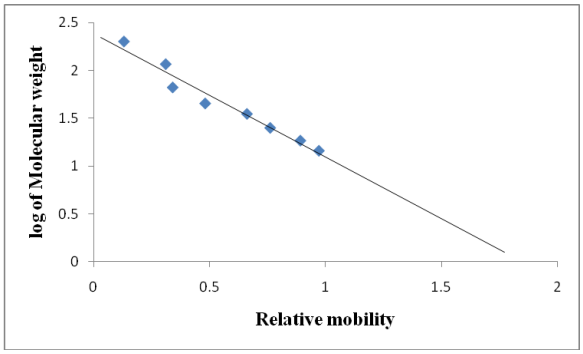

Figure 8: Determination of molecular weight of tyrosinase by SDS-PAGE

IV. Discussion

The tyrosinase activity of all eight soil isolates was determined qualitatively and quantitatively. Tyrosinase being a bifunctional enzyme catalyses both the orthohydroxylation of monophenols (tyrosine)- monophenolase activity or cresolase activity and subsequent oxidation of diphenols (L-DOPA) to orthoquinones (dopachrome)- diphenolase activity or catecholase activity. Quinones produced, are highly susceptible to non-enzymatic reactions, which lead to the formation of melanins and heterologous polymers [18]. The fungal isolate DKP-2-F produced appreciable amount of L-DOPA after 24 hrs which continued to increase up to the sixth day. As DKP-1-F produced considerable amount of L-DOPA within 24 hrs when compared to that of all the other isolates, it was selected for further study. *Penicillium jensenii* (DKP-1-F) remarkably produced 0.025 mg/ml of L-DOPA at the end of 24 hrs, which increased up to 0.038mg/ml by the end of 6^{th} day. Since *Penicillium jensenii* gave the fastest and maximum yield of L-DOPA as compared to the other isolates over a period of 6 days, it was further considered for optimization. Post optimization studies, maximum L-DOPA production by *Penicillium jensenii* was compared with those of known fungal tyrosinase producers [11-14, 21, 25].

Penicillium jensenii was checked for maximum tyrosinase and L-DOPA production by subjecting inoculated L-tyrosine broth to a range of temperature, pH and substrate concentration. It was found that the maximum yield of L-DOPA was obtained at 30^0C, pH 6 and 3mg/ml of substrate, while maximum units of tyrosinase was obtained at 30^0C, pH 7 and 1mg/ml and 3mg/ml of substrate.

Production of Alpha-methyl DOPA along with L-DOPA detected in HPLC results is reported for the first time in this paper. The detection of alpha methyl DOPA was purely qualitative and no further work concerning the estimation of alpha methyl DOPA was done in this study. The proposed biochemistry could be as shown in **Figure 9**.

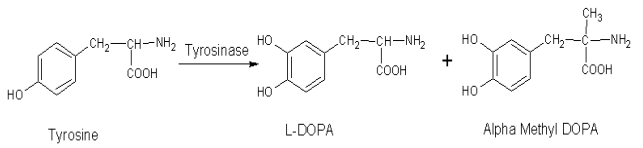

Figure 9: Two products obtained during the biotransformation of tyrosine by tyrosinase

Partial purification of tyrosinase was carried out. SDS-PAGE results revealed the presence of a single band corresponding to approximately 14.77 kDa. Purification of tyrosinase starting with a bulk volume of 800ml was reduced to 50 ml in just one step of purification (ammonium sulphate precipitation) followed by dialysis. A darker band after partial purification as compared to that obtained from crude extract was due to concentration of protein contents. A single band in the lane for crude extract itself could be because no other substrate other than tyrosine was present in the broth used for L-DOPA production. Thus a single enzyme- tyrosinase is inducibly produced during the biotransformation process.

V. CONCLUSION

Further purification of the enzyme tyrosinase with ultra membrane filtration from *Penicillium jensenii* followed by detailed study of kinetic parameters could be done. Antioxidant activity of L-DOPA towards the organs of the body including brain could be further investigated. Like other transformed nano molecules viz. aspartame, Indole 3 carbinol, Alpha methyl DOPA, L-DOPA also ushers the principle basis of molecular recognition theory of bio-nanotechnology paving the way for directed drug delivery in the injured substantia niagra part of the brain. This prevents the metabolism of L-DOPA, which is a significant drawback of L-DOPA therapy where L-DOPA gets metabolized by aromatic amino acid decarboxylase into dopamine in the peripheral tissue before actually crossing the blood brain barrier and reaching the dead nerve cells [20]. The genomic basis for tyrosinase production could also be studied. Once all parameters are determined, an attempt to scale-up the production of L-DOPA and Alpha methyl DOPA could be made, since the by-product Alpha methyl DOPA is equally commercially viable as an important anti-hypertensive and psychoactive drug.

ACKNOWLEDGMENT

I would like to thank Dr E.M Khan, Principal, Abeda Inamdar Senior College for providing us the necessary infrastructure conducive for research.

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