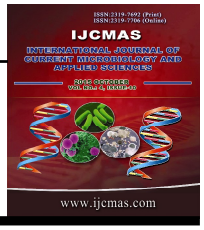
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 350-367**
http://www.ijcmas.com



Original Research Article

Role of Parametric Optimization on L-dopa and Cytosolic Tyrosinase Production under SmF from *A. rutilum*: its Purification and Characterization

R. Krishnaveni[1], Vandana Rathod[1*], Rajashekhar Nagur[3], Prema kulkarni[1] and Pramod desai[2]

[1]Department of Microbiology, Gulbarga University, Gulbarga -585106, Karnataka, India
[2]Department of Biotechnology, Gulbarga University, Gulbarga -585106, Karnataka, India
[3]Department of Animal Genetics and Breeding, KAVFSU, Bidar, Karnataka, India
**Corresponding author*

A B S T R A C T

In submerged fermentation system, Enriched potato Lactose Peptone Broth was found to be the best media for maximum expression of cyt tyr (282 U/ml) from *A.rutilum* belongs to deuteromycetes. By simultaneously varying physical and nutritional parameters this simple and economical methodology optimally produced L-dopa and cyt tyr. There is increase in the production of L-dopa with less cyt tyr and vice versa. Cyt tyr activity was induced when mycelium was stored at 0^{o} C for 10 days. Cyt tyr precipitated at 45–65% of ammonium sulphate yielded 45.16% recovery, was purified by Diethylacetate cellulose column showed 218 U/ml activity and 3.12 fold purification. The *A. rutilum* cyt tyr molecular mass has 43 and 29 kDa and is a latent enzyme activated by 2.5mM SDS. *A. rutilum* cyt tyr exhibits 25 mM km and 200 mM/ml/min Vmax with L-dopa as a substrate and high substrate specificity with pyrocatechol at acidic pH. *A. rutilum* cyt tyr is active from 4 to 25 ^{o}C at 5.5 pH indicating it to be a cold acidic enzyme. This feature is mandatory for applications such as protein cross linking, immobilization which can be exploited in food and biotechnological industries.

K e y w o r d s

Tyrosinase, L-dopa, *Acremonium rutilum*, Parametric optimization, Biotrans formation

Introduction

Tyrosinase (tyr), E.C. 1.14.18.1, is a rate limiting enzyme involved in the biosynthesis of melanin in the pathway called melanogenesis starting from L- tyrosine. The intermediate product L-dopa and the end product melanin have important functional role and pharmaceutical importance (Van Gelder et al., 1997). Recently, potential biotechnological applications of fungal tyrs are investigated. Tyr biosensors have applications in waste water treatment, bioremediation and detection, and quantification of phenols. Tyr as protein cross linker can be exploited in cosmetic and medical sector, cereal processing, dairy processing and texturization of meat. Tyrs can also be used in tailoring properties of food products, wool & silk fibers, and in the synthesis of antioxidants and polyphenolic polymers. Wool fibre proteins have been reported to be modified by tyr, and grafting of L-dopa to

wool fibres has also been successfully carried out. Tyrs are also used in the synthesis of high value added drugs such as L-dopa (3, 4-dihydroxyphenyl L-alanine) used in the treatment of Parkinson's disease. L-dopa, which undergoes two electron oxidation to form quinine, can be used to probe and manipulate electron transfer processes in proteins (Xie *et al.,* 2006), it also has prooxidant activity and stimulates a cellular antioxidant defense mechanism under certain conditions. At low concentrations it increases intracellular concentrations of the antioxidant glutathione, thereby enhancing free radical scavenging capacity of brain cells (Han et al., 1996). Tyrs was investigated in the fungi, *N. crassa* (Lerch, 1983), *A. bisporus* (Wichers et al., 1996), *A. oryzae* (Haneda, 1974), *Y. lipolytica* (Ali et al., 2007) and *A. rutilum* (Krishnaveni et al., 2009). Tyr plays a significant role in clinical studies such as marker for vitiligo and tool in treating melanoma. Tyr inhibitors can also be exploited as antibrowning agents of fruits and vegetables, insecticides and skin whitening agents.

To overcome, number of applications of tyr and L-dopa, there is a need to study in the area of bioprocess fermentations in order to increase maximum production which stimulated the research. Fungal tyrs from basidiomycetous fungi are thoroughly investigated in respect of purification, biochemical characterization and industrial applications, but the number of reported and well characterized ascomycetous, deuteromycetes tyrs were very less. Hence the research mainly focused on media composition and optimization for high yield. The present work describes the production of cyt tyr from *A. rutilum*, comparing the L-dopa yield by varying the substrate concentrations, different carbon and nitrogen sources, copper sulphate concentrations and their role in optimization. Purification, molecular characterization, stability and catalytic properties of cyt tyr were also reported.

Materials and Methods

Fungi and chemicals

A. rutilum, tyr producing fungi isolated in our laboratory was used for further study (Krishnaveni et al., 2008). L-tyrosine and Pyrocatechol were obtained from S.D. Fine Chem., Catechol, Gallic acid, L-dopa, Pyrogallol, Triton X-114 were obtained from Sigma, Triton X-100, PMSF, and PVP were purchased from Himedia Ltd. All reagents were of analytical grade, unless otherwise indicated.

Submerged fermentation

Effect of L-tyrosine concentration on L-dopa and cyt tyr yield was determined by amending 0.1-0.5% of L-tyrosine to enriched potato dextrose broth (EPB). 0.1mg/ml L-dopa containing 2.5ml of primary culture was inoculated to 100ml fresh media to check the effect of L-dopa as cofactor. Carbon sources (glucose, sucrose, maltose, fructose, lactose, starch, L-tyrosine and xylose) was evaluated first at same concentration (2%) and then at different concentrations (1–5%) under sterile conditions (pH 5.5) at 25 ^{o}C for 24 to 240 h .Next 0.1% each of malt extract, yeast extract, peptone, egg flakes, soyabean meal, casein and casein hydrolysate, ammonium chloride, ammonium nitrate, sodium nitrate and ammonium sulphate, were checked and copper sulphate concentrations (4–20mg/100ml) were also evaluated. Media in duplicates were inoculated aseptically with 2.5 ml inoculum and kept at 140 rpm on cooling shaker incubator. Flasks were withdrawn at predetermined time intervals

and the culture filtrate obtained after the filtration through muslin cloth was taken for estimation of L-dopa (Arnow, 1937), mycelium used for tyr assay (Fling et al.1963) and protein concentration was determined (Lowry *et al.,* 1951). Scale up was done by growing the culture under optimized physical and nutritional parameters.

Standardized protocol for Cyt tyr extraction

Mycelium was washed with cold sterile distilled water, PBS and stored in a conical flask at 0^{o} C for 10 days. It was thawed at 4^{o}C before extracting. 10ml sodium acetate buffer (5.5) containing 1mM ascorbic acid, 20% glycerol was added and the mycelium was homogenized using mortar and pestle with sterile glass beads. The homogenate was centrifuged at 10000 rpm, for 20min at 4^{o}C. The supernatant was subjected to 12% Triton–X114 (Peréz-Gilabert *et al.,* 2001) for cyt tyr extraction.

Purification and characterization of Cyt tyr

Ammonium sulphate precipitation was carried out stepwise, and tyr activity and total protein concentration were estimated. Dialysed extract was checked for its ionic binding by loading onto anion matrix DEAE-cellulose column. Activity staining was done by running the sample on native PAGE. The gel was then cut, washed with 0.1M acetate buffer (5.5) and treated with the same buffer containing 8mM L-dopa, 2mM L-tyrosine and 10mM pyrocatechol.

Molecular characterization by SDS-PAGE

The molecular weight standards used were Banglore Genei Protein Molecular Markers from 18.3 to 97.4 kDa. SDS-PAGE gels were stained with silver nitrate staining (Rosenberg, 1996).

Effect of pH, Temperature, SDS and substrate specificity (Km, Vmax) on cyt tyr activity

The optimum pH of cyt tyr was measured using the following buffers: 0.2 M Na acetate buffer (pH 4.0-5.5), Na phosphate buffer (pH 6.0-8.0) and glycine-NaOH buffer (pH 8.5-10.0). The effect of temperature on cyt tyr was studied in 0.1M acetate buffer (5.5) between 0 and 40^{o}C. For determining the stability, 0.5ml enzyme solutions were incubated at different temperatures (0 to 40°C) and cooled prior to measuring relative activity. The effect of different concentrations of SDS (0.1, 0.5, 1, 2.5, 5 mM) on tyr was also standardized. Effect of various substrates on the activity of purified cyt tyr was studied. L-tyrosine (0.5-50mM), catechol (2.5-100 mM), pyrocatechol (0.25–20 mM), pyrogallol (2.5–50 mM), L-dopa (0.5–32 mM), gallic acid (0.5–50 mM) and ABTS (0.5–5mM) were used to monitor cyt tyr activity (Zhou *et al.,* 1993). Reaction rates were plotted against L-dopa concentration to determine whether the enzyme obeys Michealis-Menten kinetics. Kinetic constants were determined (Line weaver, 1934).

Result and Discussion

L-tyrosine concentration

Maximum tyr production (130.2 U/ml) was observed with 0.3% L-tyrosine at 72h (Figure 1). The substrate concentration on tyr and L-dopa production by *A. rutilum* at 168 h has shown maximum activity of 170.2 U/ml and 0.28mg/ml respectively. L-dopa gradually increased with increase in L-tyrosine concentration (Figure 2). The

maximum accumulation of L-dopa (0.542–0.62mg/ml) was observed between 0.3–0.5% L-tyrosine at 72h. Maximum production of L-dopa was 0.89 mg/ml with 5 mg/ml L-tyrosine on 96h (Krishnaveni *et al.,* 2009).

L-dopa as cofactor

Tyr activity was 178 U/ml at 72 h with decrease in L-dopa (0.1 mg/ml). Tyr activity decreased and increased again (154U/ml) at 120 h, while L-dopa decreased steadily and was almost nil at 168h (Figure 3). The process of bioconversion of L-tyrosine to L-dopa in microorganisms is slow, but is accelerated by small amounts of L-dopa in the broth (Haq *et al.,* 2002). Tyr activity determined with di-phenolic compounds shows a lag phase only at low pH if L-dopa is used.

Carbon sources

According to the data starch (200U/ml) and lactose (163U/ml) showed maximum activity at 72h. L-dopa production with starch was 0.25 mg/ml whereas it was 0.1mg/ml with lactose (Figure 4). At 120 h, starch and lactose showed 222U/ml tyr activity and L-dopa production was 0.23 mg/ml with starch and 0.05 mg/ml with lactose. Activity of cyt tyr was 222.2 U/ml at 3% lactose with varying specific activity and decreased L-dopa (0.09 mg/ml) (Figure 5). Cyt tyr activity was 200 U/ml with 2% starch, hence 3% Lactose was used as optimum carbon source. L-dopa yield decreased at low concentrations of starch whereas at higher concentrations (3–5%) yield increased, due to the fact that starch adsorbs L-tyrosine and helps the fungus to assimilate faster.

Lactose and starch enhanced tyr activity in significant levels and hence, proved to be the best carbon source for cyt tyr production. This may be due to the fact that lactose and starch hydrolysis (maltose) exhibits mutarotation and reduces Cu^{++} ions to Cu^{+} ions creating a reducing environment. Complete aerobic metabolism of carbohydrates in simple media leads to the extrusion of protons and CO_2, which tends to depress culture pH by producing neutral or acidic products, and influences the enzyme and product yield. Disaccharides or high molecular weight substrates have been found to be the best supporters of intracellular enzymes (Halaouli *et al.,* 2006). This is confirmed by this study also as lactose supported tyr production significantly, at low concentrations, where as L-dopa production was induced by disaccharides or high molecular weight substrates at higher concentrations. However, 4% glucose showed highest L-dopa yield (0.89 mg/ml) followed by 3% starch (0.85 mg/ ml) with 52 U/ml tyr activity (Vandana and Krishnaveni, 2011). *Trichoderma* transformant produced the highest tyr activity (300 nkat/ml) cultivated with 4% lactose at 144h (US patent W0/2006/084953).

Nitrogen sources

Maximum titers of cyt tyr (244.2U/ml) were obtained from peptone amended medium at 72h (Figure 6) and 77.8 U/ml at 120h. Organic nitrogen sources exert a significant influence on growth and cyt tyr productivity as compared to inorganic nitrogen sources, which influences increased L-dopa production. However, 0.2% egg albumin flakes also influenced L-dopa (0.10 mg/ml) which may be due to the inhibition effect on tyr activity (44U/ml) (Vandana and Krishnaveni, 2011). Cyt tyr activity was 244.4 U/ml with 0.1% peptone, with decreased L-dopa (Figure 7). Minimum L-dopa production was probably due to

increased catecholase activity of tyr causing L-dopa to be used for quinone formation. Transformed *Streptomyces antibioticus* IMRU resulted in maximum production of 65.9 U/ml tyr with low casein media containing 0.4% Marcor Casein Peptone (US patent 5486351).

Effect of copper sulphate concentration

Tyr activity (260U/ml) was induced maximum with 16 mg/100ml of copper sulphate however, decrease in L-dopa yield (0.12mg/ml) was observed at 72 h (Figure 8). However, at 4 mg/100ml the yield of L-dopa was 1.09 mg/ml and tyr activity was 44U/ml (Vandana and Krishnaveni, 2011).

Overall process consistency for cyt tyr production

Under overall process consistency, the wet biomass was 5.06g/100ml and total protein content was 0.34mg/ml (Figure 9). Tyr activity and L-dopa at 72h was 284.4U/ml and L-dopa 0.05mg/ml, and slightly decreased and remain constant until 120 h (Figure 10). The methodology made simple by using the same natural raw material, potato extract as a basal media and then enriched by standardizing it for higher yield of both cyt tyr and L-dopa. ELPB was the economical standardized media which contained Potatoes-200g, Lactose -30g, Peptone-1g, L-Tyrosine-3g, $CuSO_4.5H_2O$-16mg, Tap water-1ltr (25^oC, pH 5.5, inoculum size-2.5ml), and was obtained after both optimized nutritional and physical parameters. Enriched potato Glucose Albumen Broth (EGAB) reported to be an efficient media for L-dopa yield with 1.12mg/ml (Vandana and Krishnaveni, 2011). Even the biomass increased with increase in L-dopa. The total protein content of the intracellular extract and the cyt tyr activity increased with decreased biomass which indicates the enzyme induced under starvation or stress. ELPB media significantly improved two fold cyt tyr activity, with reduced biomass and L-dopa, proving the methodology to be simple, economical and efficient. The media composition is similar with the work of Penttila et al. (1987) who cultivated *Trichoderma* transformants in standardized Trichoderma minimal medium containing 4% lactose, 2% distiller's spent grain and 2mM $CuSO_4$.

Purification of cyt tyr

Summary of the purification procedure and purity achieved for cyt tyr from *A. rutilum* is depicted in table 2. The 45–65% ammonium sulphate fraction resulted in 45.16% recovery, with 1.36 fold increase in specific activity. The dialysed sample showed 233.8 U/ml activity and 2.23 folds purification. Tyr revealed two black bands by specific activity staining after purification, one with in 15min, and another after 12h of incubation suspecting it is diphenolase and monophenolase activity respectively (Figure 11). The fractions (13–17) that exhibited activity after anion-exchange chromatography was pooled which showed cyt tyr activity of 218 U/ml with 3.12 folds purification (Figure 12).

Characterization of cyt Tyr

Molecular characterization of cyt tyr of *A. rutilum*

Silver nitrate staining showed two bands with molecular weight of 43 and 29 kDa indicating that tyr of *A. rutilum* contains 2 isoforms that are catalytically active (Figure 13). Tyr from *N. crassa* was found to consist of a single polypeptide chain of molecular weight of 44kDa (Lerch, 1976). Two monomeric 43–47 kDa isoforms of tyr were

isolated from the fruit-bodies of *A. bisporus* strain (Wichers *et al.,* 1996). The *P. sanguineus* CBS 614.73 tyr was monomeric with a molecular mass of 45 kDa and four isoforms could be observed after isoelectric focusing (IEF) (Halouli *et al.,* 2005).

Stability of cyt tyr from A.rutilum

The temperature optima as shown in figure 14a are in the range of 4–25^{o}C with 90-100% relative activity. At 0^{o}C and 35^{o}C, 72 to 76% of the original activity was retained. At 4°C cyt tyr retained all its activity for 30 min and reached half the maximum by 60 min (Figure 14b). Pure cyt tyr was found to lose its activity abruptly when incubated at temperatures above 30°C. At 35^{o} C, total activity was lost at the same time. Hence the cyt tyr is a cold enzyme and was stored at 4°C. Even during short incubations, 70–90°C is reported to inactivate tyrs completely (Kong *et al.,* 1998).

Catalytic characterization of cyt tyr from A.rutilum

Effect of PH

The maximum activity of the purified tyr with L-dopa was found between pH 5.0-5.5 (Figure 15a). The enzyme was relatively active at low pH. Half the maximal activity was still present at pH 4.5 and 6.5. L-dopa retains 30% of its reactivity towards tyr at pH 7.0. In *A. bisporus* the optimum pH is from 6.5-7.5 (Khan *et al.,* 1995) and *A. oryzae* tyr was reported to be 5.0–6.0 (Ichishima *et al.,* 1984). In case of *N. crassa,* the optimum pH was 5.0 but tyr activity was stable up to pH 8.0 (Sussman, 1961).

Table.1 Scale up and yield enhancement of cytosolic tyrosinase

parameters	Tyr activity (U/ml)			Specific activity(U/mg)		
	72h	120h	168h	72h	120h	168h
Screening (10th day incubation)	141.6	-		289	-	
Physical parameters						
Temperature (25^{0} C)	78.2	158.6		474	546.8	
pH (5.5)	78.4	160.5		179	697.8	
Inoculum size (2.5ml)	128.4	163.4		544.06	408	
Nutritional parameters						
L-Tyrosine conc. 3mg/ml	130.2	170.2		325.5	518.7	
L-DOPA (Cofactor)	178	124		482.4	476	
Carbon source						
3%Lactose	222.2			1010		
2%Starch	200			1694		
Nitrogen source	244.2			1222		
0.1%peptone						
Copper sulphate (16mg/100ml)	260			1300		
Overall cyt tyr production	284.4			1320		

Table.2 Summary of the purification procedure and purity achieved for cyt tyr from *A.rutilum*

Purification Steps	**Volume (ml)**	**Activity U/ml**	**Total activity (U)**	**Total protein content (mg/ml)**	**Specific activity U/mg**	**Rec. (%)**	**Purification (folds)**
Crude extract	200	310	62000	0.178 35.6	1741.5	100	1
Triton X-114	176	300	55264	0.12 21.2	2606.7	89.1	1.49
Ammonium salt precipitation 45-65%	100	280	28000	0.118 11.8	2372.8	45.16	1.36
Dialysis	50	233.8	11690	0.063	3896.6	19	2.23
DEAE-Cellulose column	5	218	1090	0.04 0.2	5450	18	3.12

Table.3 Substrate specificity of cytosolic tyrosinase

Sl.no	Substrate	Wavelength	Relative activity (%)	Km	Vmax
1	L-tyrosine	475	14.98	-	-
2	L-DOPA	475	82.4	25mM	2×102
3	Catechol	420	82.7	-	-
4	Pyrocatechol	334	100	-	-
5	Pyrogallol	300	85.6	-	-
6	ABTS	420	-	-	-
7	Gallic acid	420	-	-	-

Figure.1 Effect of substrate concentration on Cyt tyr and L-dopa productivity by *A.rutilum* at 72h. L-tyrosine concentration 0.1-0.5%, pH-5.5, Temperature-25^{o}C.Comparison of cyt tyr activity and specific activity with L-dopa yield by line graphs. As the concentration of L-tyrosine increased, L-dopa yield increased with decrease in monophenolase activity of cyt tyr (increased on 72h) and specific activity

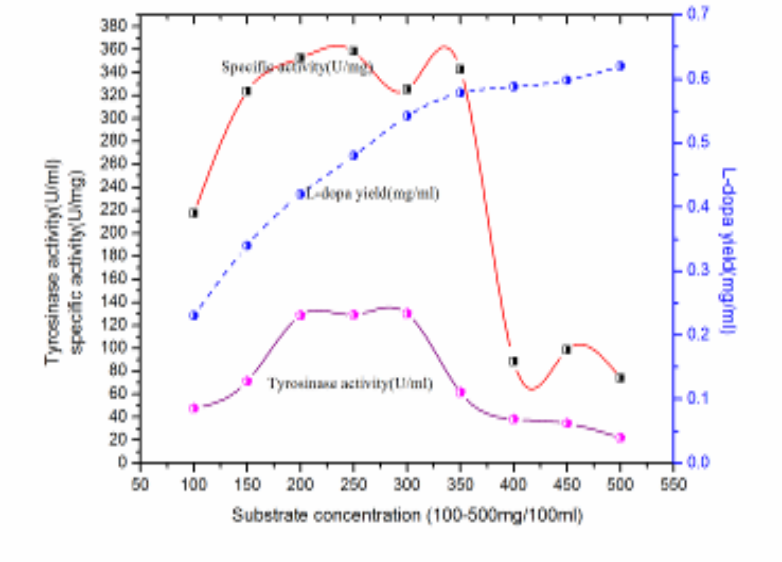


Figure.2 Effect of substrate concentration on cyt tyr and L-dopa productivity by *A.rutilum* at 168h (L-tyrosine concentration 0.1–0.5%, pH-5.5, Temperature-25^{o}C). Comparison of cyt tyr activity and specific activity with L-dopa yield by line graphs. As the concentration of L-tyrosine increased, L-dopa yield increased with decrease in o-diphenolase activity of cyt tyr (increased again on 168h) and specific activity

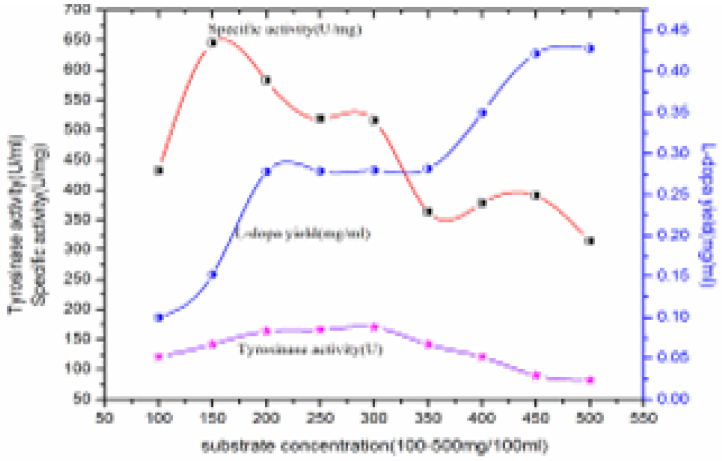


Figure.3 Effect of L-dopa as a co-factor on cyt tyr and L-dopa production by *A. rutilum* (0.3% L-tyrosine concentration, pH-5.5, Temperature-25^{o}C, 24-168h). Oxy form of tyr converted L-tyrosine to L-dopa and L-dopa acts as a cofactor coverts oxy form to deoxy form and hence maximum accumulation of L-dopa on 48h and maximum activity on 72h. Methoxy and oxy forms of tyr increased

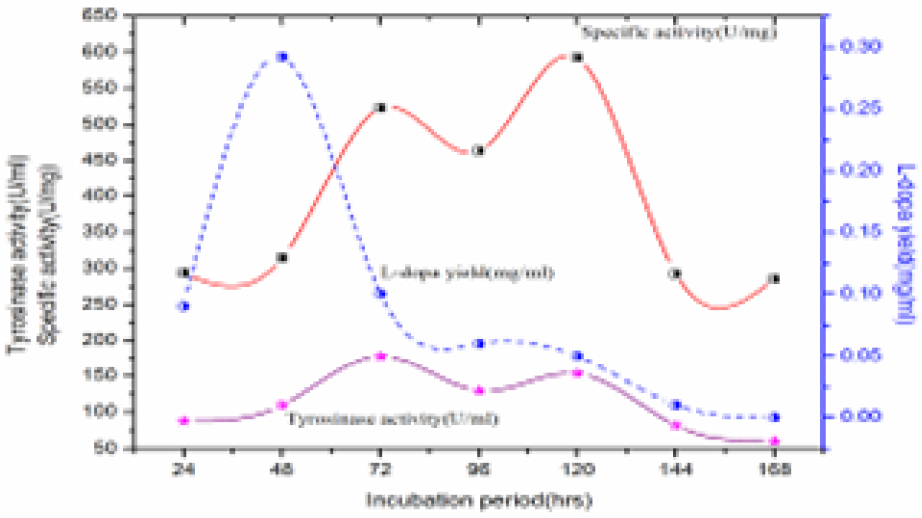


Figure.4 Effect of carbon sources on Cyt tyr and L-dopa production by *A. rutilum* (0.3% L-tyrosine concentration, 0.1% L-dopa, pH-5.5, Temperature-25^{o}C, 72h). Starch and Lactose influenced on cyt tyr activity however the latter responded less L-dopa yield, hence lactose was taken for further optimization of cyt tyr. Glucose, sucrose and starch are good sources of L-dopa production hence glucose taken for further optimization of L-dopa

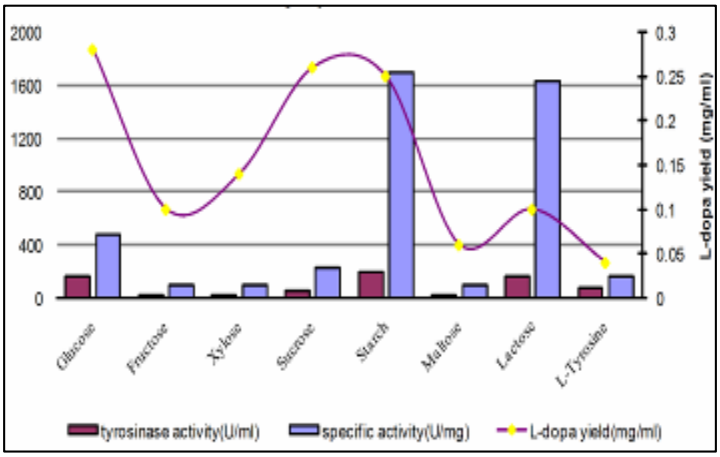


Figure.5 Effect of lactose concentration on Cyt tyr and L-dopa production by *A. rutilum* (0.3% L-tyrosine concentration, 0.1% L-dopa, Lactose (1-5%), pH-5.5, Temperature-25^{o}C, 72h). Cyt tyr activity was maximum (222.2 U/ml) with 3% lactose with less L-dopa (0.09 mg/ml)

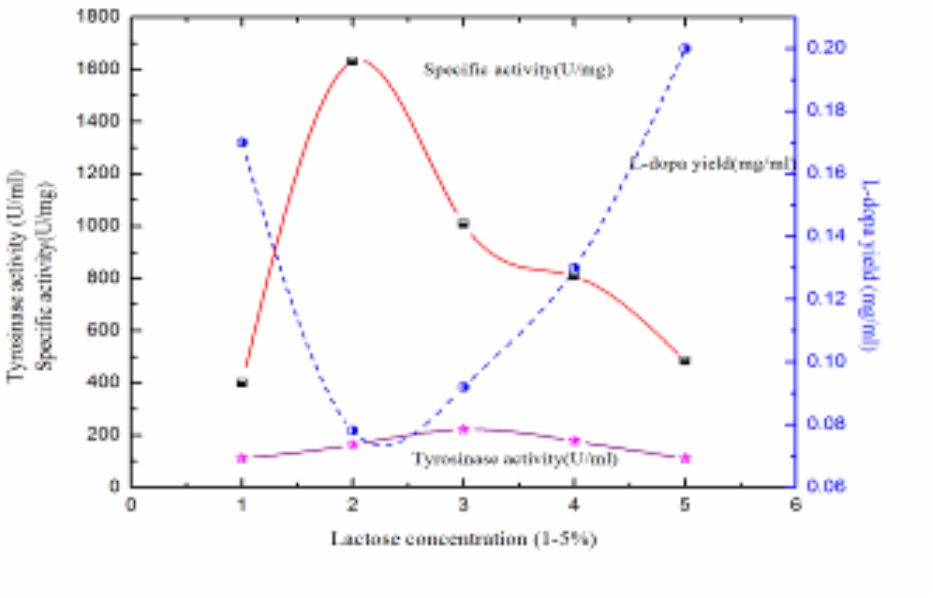


Figure.6 Effect of on organic nitrogen sources on Cyt tyr and L-dopa production by *A. rutilum* (0.3% L-tyrosine concentration, 0.1% L-dopa, 3%Lactose, pH-5.5, Temperature-25^{o}C, 72h). Cyt tyr activity was maximum (244.2 U/ml) with peptone with less L-dopa (0.07 mg/ml)

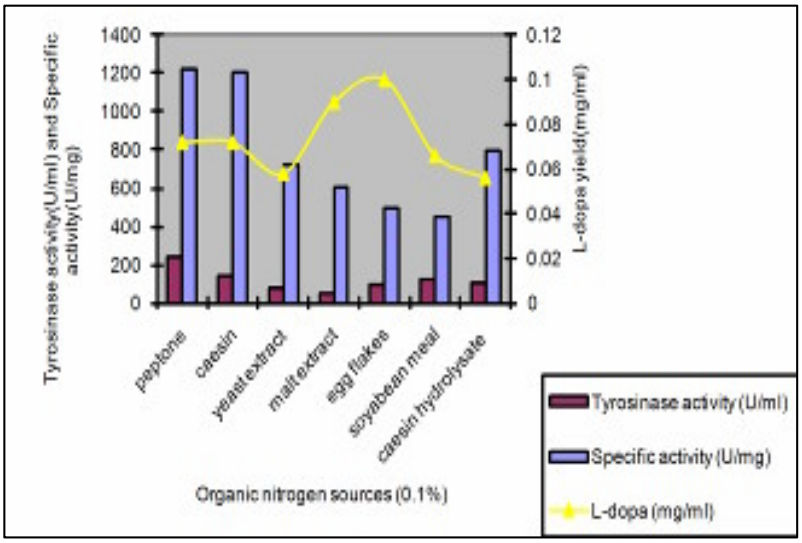


Figure.7 Effect of peptone concentration on Cyt tyr and L-dopa production by *A. rutilum* (0.3% L-tyrosine concentration, 0.1% L-dopa, 3% lactose, peptone (0.1-0.6%), pH-5.5, Temperature-25^{o}C, 72h).0.1% peptone showed maximum cyt tyr (244.4 U/ml) with less L-dopa (0.07 mg/ml)

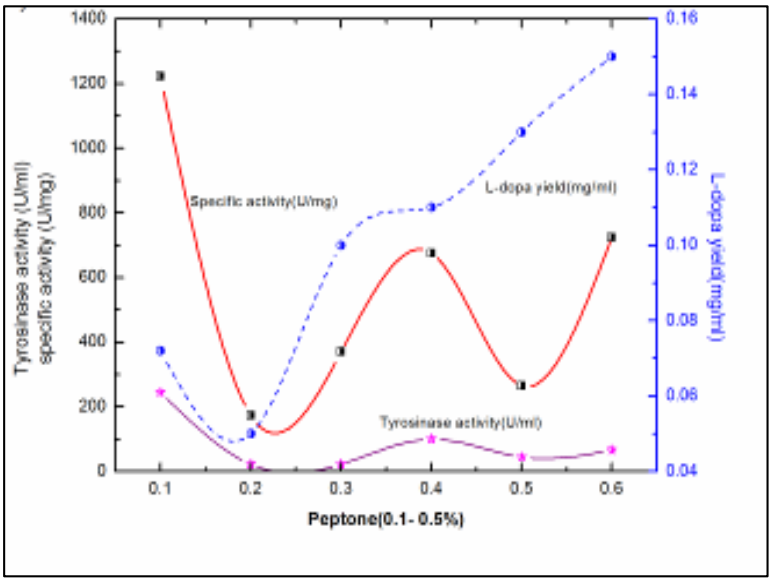


Figure.7 Effect of peptone concentration on Cyt tyr and L-dopa production by *A. rutilum.* (0.3% L-tyrosine concentration, 0.1% L-dopa, 3% Lactose, Peptone (0.1-0.6%), pH-5.5, Temperature-25^{o}C, 72h). 0.1% peptone showed maximum cyt tyr (244.4 U/ml) with less L-dopa (0.07 mg/ml)

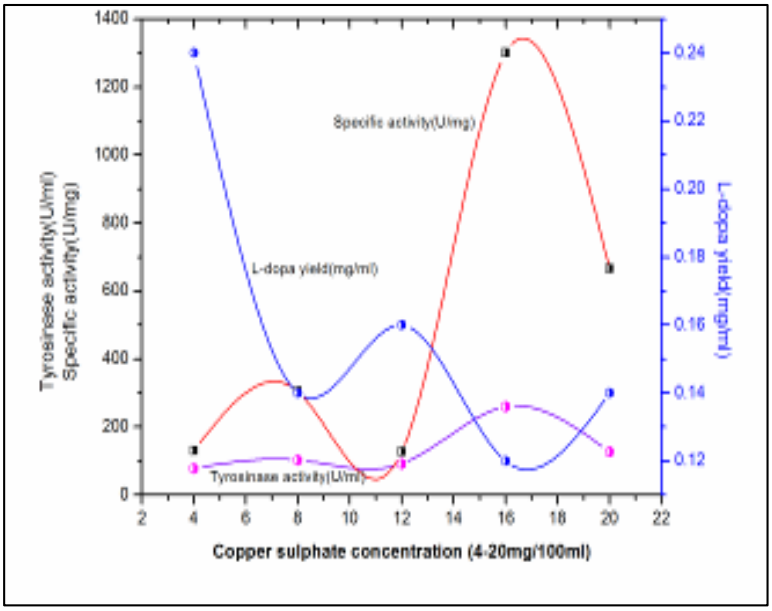


Figure 1.8 Effect of Copper Sulphate Concentration on Cyt tyr and L-dopa production by *A. rutilum* (0.3% L-tyrosine concentration, 0.1% L-dopa, 3% Lactose, Peptone (0.1%), pH-5.5, Temperature-25^{o}C, 72h). Cyt tyr activity was maximum (260 U/ml) with 16mg/ml of copper sulphate with less L-dopa (0.12 mg/ml)

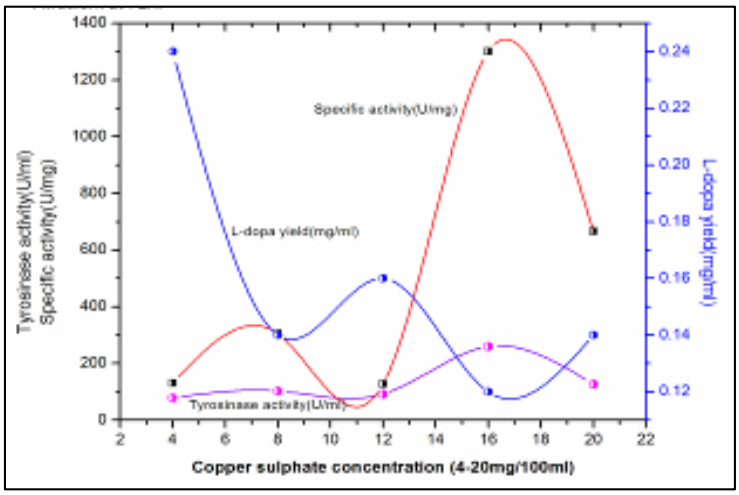


Figure.9 Biomass and cytosolic protein content of A.rutilum under overall parametric optimization (0.3% L-tyrosine concentration, 0.1% L-dopa, 3% Lactose, Peptone (0.1%), 16mg/ml copper sulphate, pH-5.5, Temperature-25°C, 12–120h). Biomass was higher 6.5g/100ml at 50h and cytosolic protein content was 0.42mg/ml. From 72h the biomass reduced from 5–4mg/100ml and cytosolic protein content was fluctuating

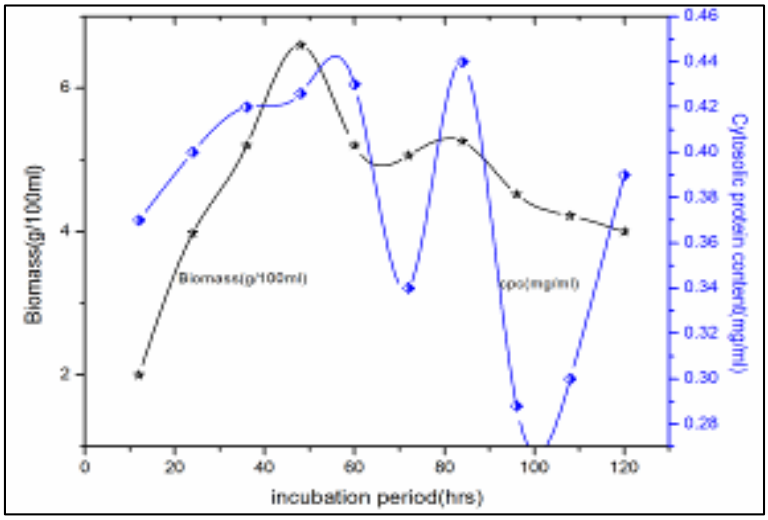


Figure.10 Cyt tyr and L-dopa production from *A. rutilum* under overall parametric optimization. (0.3% L-tyrosine concentration, 0.1% L-dopa, 3% Lactose, Peptone (0.1%), 16mg/ml copper sulphate, pH-5.5, Temperature-25°C, 12–120h). Cyt tyr yield was higher 284.4U/ml at 72h and L-dopa was 0.05 mg/ml

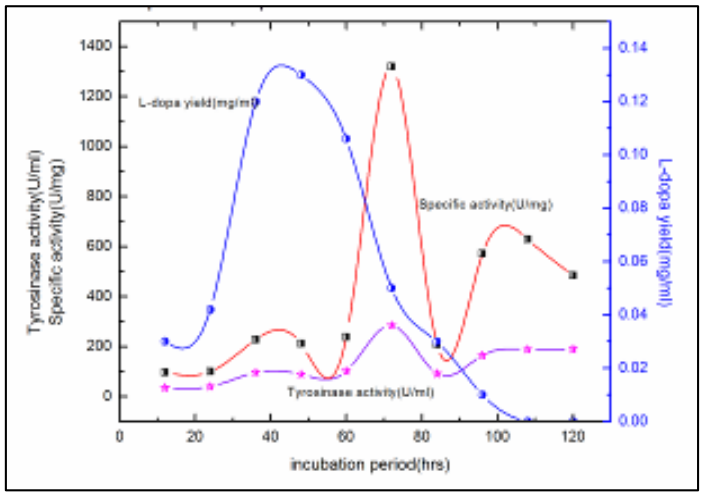


Figure.11 Activity staining of cyt tyr by Native PAGE. Gel showing black bands (i) at 5mins (ii) at 12 h incubation with 0.1M sodium acetate buffer containing 2mML-tyrosine, 8mML-dopa and10mM pyrocatechol, pH-5.5

Figure.12 Elution profile of Cyt tyr by DEAE cellulose column chromatography from *A. rutilum*.13 t0 17 fractions were pooled and the total activity was 218 U/ ml

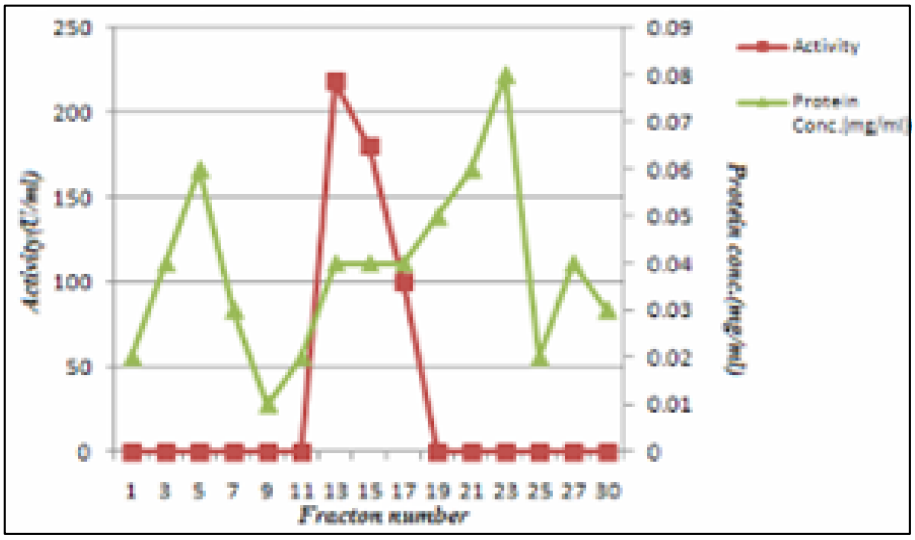


Figure.13 SDS-PAGE Gel: a. Marker (18.3–97.4KDa), b. crude extract, c. Triton X-114, d. Ammonium sulphate pptn + dialysed extract, e. DEAE cellulose purified sample, showing two bands with mol wt 43 and 29kDa by silver nitrate staining

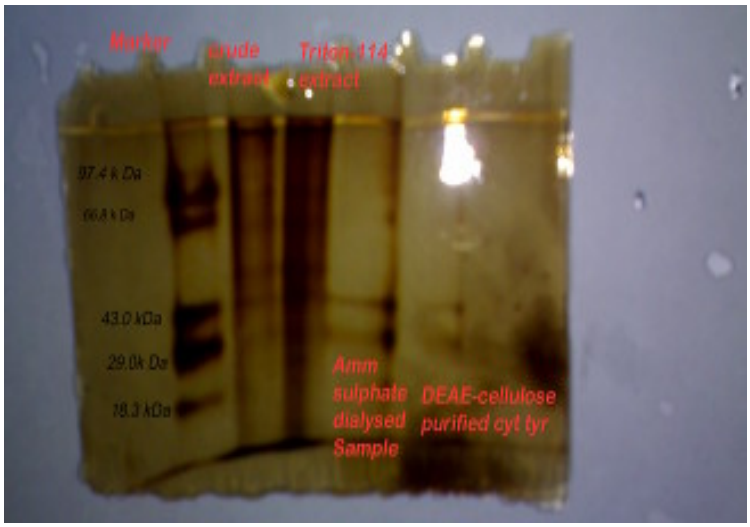


Figure.14 (a) Effect of temperature on cyt tyr from *A. rutilum* (b) Effect of temperature stability on Cyt tyr from *A. rutilum*

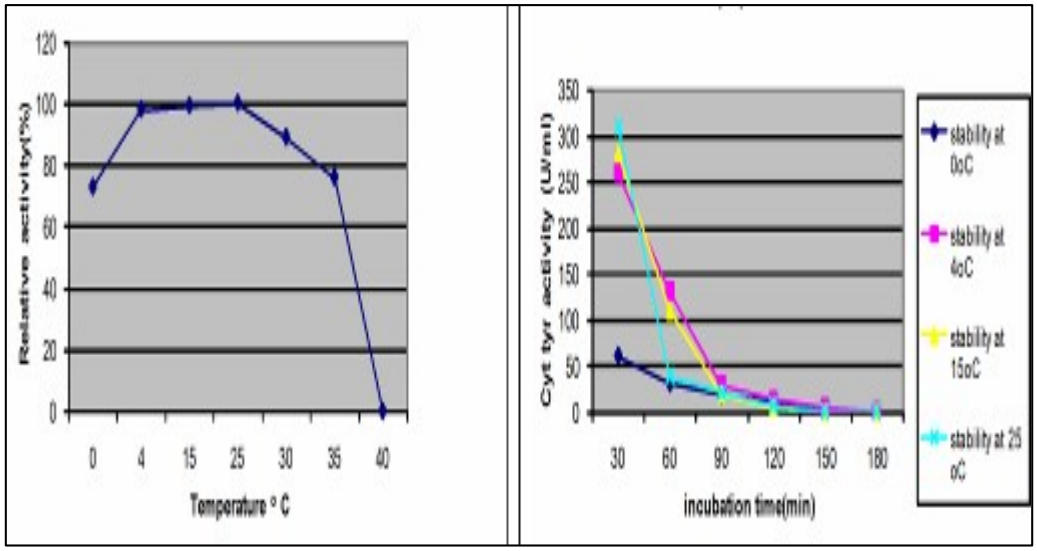


Figure.15 (a) Effect of pH on cyt tyr activity from *A. rutilum* (b) Effect of SDS on Cyt tyr from *A. rutilum*

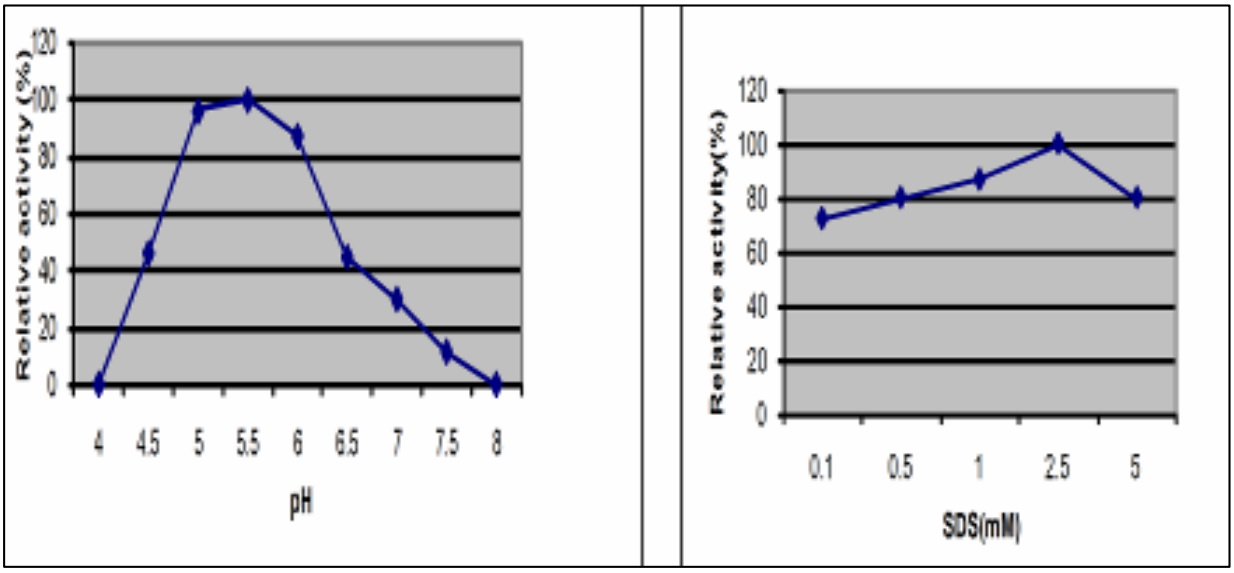
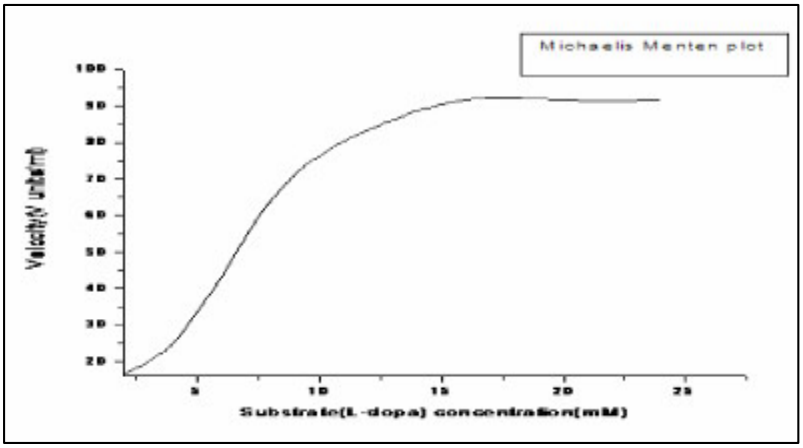


Figure.16 Micheals Menten plot of the effect of L-dopa concentration on Cyt tyr of *A. rutilum*



Effect of SDS concentration

The effect of SDS on purified cyt tyr and relative activity is presented (Figure 1.15b). At 0.5 mM and 5mM the relative activity was 80%. At 2.5 mM SDS, there was maximum (100%) increase in tyr activity which indicates that tyr existed in latent form. The activation process by SDS was found to involve a reorganization of protein tertiary structure.

Substrate specificity and its km and Vmax

L-tyrosine, used at concentrations of 0.5-50 mM, was catalysed by cyt tyr suggesting monophenolase activity. Cyt tyr was reactive towards catechol, pyrocatechol and L-dopa.It was highly reactive towards pyrocatechol (Table 3). Among the triphenols, pyrogallol was oxidized whereas gallic acid did not have any activity. The purified tyr displayed Michaelis-Menten kinetics (Fig.1.16). Double reciprocal Line weaver-Burk plot of L-dopa concentration and initial dopachrome formation yielded a straight line and cyt tyr exhibited k_m and V_{max} values of 25mM and 2×10^2 µM/ml/min.

In conclusion, the main findings of this study is that, nutritional parameters plays key role in improving both cyt tyr and L-dopa which are important for biotechnological applications. Not only the nutritional parameters, its varying concentrations also vary the yield of both tyr and L-dopa as, 0.1–0.3% of L-tyrosine concentrations improved cyt tyr yield however optimum L-tyrosine concentration was 0.3%, where as 0.4–0.5% concentration of L-tyrosine increased L-dopa production. Hence 0.3% L-tyrosine was taken for optimization of cyt tyr and 0.4% for L-dopa production. At 4mg/ml of $CuSO_4.5H_2O$, L-dopa yield was high 1.09 mg/ml and tyr activity was 44U/ml, at 16mg/ml $CuSO_4.5H_2O$ *A. rutilum* expressed optimum cyt tyr yield (0.12mg/ml).

The carbon and nitrogen source are two important factors affecting cell growth and product formation of microorganisms. High cytosolic tyr activity was induced by carbon source such as 3% lactose (222.4U/ml) and nitrogen source 0.1% peptone (244.4 U/ml), whereas the same sources repressed L-dopa yield and by the end of nutritional optimization its yield was almost nil. This indicates that due to high monophenolase

and 0-diphenolase activity of tyr, biotransformation of L-tyrosine to L-dopa occurred and converted to melanin, hence less yield of L-dopa. Carbon sources such as 4% glucose (0.89 mg/ml) and 3% starch (0.85mg/ml) with 52 U/ml tyr activity and nitrogen source such as 0. 2 % egg albumen flakes improved L-dopa yield 1.06 mg/ml by inhibitory effect on cyt tyr (44U/ml) by nutritional parameters. Hence the various byproducts of the carbon, nitrogen metabolism may enhance and/or inhibit the tyr production by decreasing the yields of tyr due to the inhibitory environment created by certain nutritional parameters, L-dopa production can be improved; whereas inducing environment created by certain nutritional parameters can improve tyr production. The simultaneous production of cyt tyr and L-dopa will be done efficiently by studying the effect of different nutritional parameters at different concentrations. ELPB and EGAB were two semi synthetic media used, which was the easiest methodology for efficient production of cyt tyr and L-dopa respectively. Tyr activity, induced by storing the mycelium at 0^{o}C for 10 days is easy to extract. Pure tyr was further used to screen and study tyr inhibitors from synthetic and microbial sources. The cold acidic tyr can be exploited in protein cross linking in food applications and can be stabilized by immobilization and genetic manipulation for wider industrial applications.

Abbreviations

Tyrosinase –tyr ,Cytosolic tyrosinase- cyt tyr, PMSF- Phenylmethanesulfonyl fluoride, PVP -polyvinyl pyrrolidone, ABTS -2, 2'-azino-bis, 3-ethylbenzothiazoline-6-sulphonic acid

Acknowledgement

Lead author is thankful for UGC for providing JRF to complete this work in the department of Microbiology, Gulbarga university and also to the registrar srinivas T for providing facilities in Navodya college of medical sciences Raichur, Karnataka, India.

Reference

Ali, S., Jeffry, S., Haq, I. 2007. High performance microbiological transformation of L-tyrosine to L-dopa by *Y. lipolytica*. NRRL-14. *BMC Biotechnol.*, Pp. 1–7.

Arnow, L.E. 1937. Colorimetric determination of the components of L-3, 4 dihydroxyphenylalanine-tyrosine mixture. *J. Biochem.*, 118: 531.

Fling, M., Horowitz, N.H., Heinemann, S.F., 1963. The isolation and properties of crystalline tyr from Neurospora. *J. Biol. Chem.*, 238: 2045–2053.

Garcia-Canvas, F., Garcia-Carmona, F., Lozano, A. 1981. Hysteresis of mushroom tyrs: lag period of cresolase activity. *Phytochem.*, 20: 1215.

Halaouli, S., Asther, M., Kruus, K., Guo, L., Hamdi, M., Sigoillot, J.C., Asther, M., Lomascolo, A. 2005. Characterization of a new tyr from *Pycnoporus* species with high potential for food technological applications. *J. Appl. Microbiol.*, 98: 332–343.

Halouli, S., Asther, M., Sigoillot, J.C., Hamdi, M., Lomascolo, A. 2006. Fungal tyrs: new prospects in molecular characteristics, bioengineering and biotechnological applications. *J. Appl. Microbiol.*, 100(2): 219–232.

Han, S.K., Mytilineou, C., Cohen, G. 1996. L-DOPA up-regulates glutathione and protects mesencephalic cultures against oxidative stress. *J. Neurochem.*, 66: 501–510.

Haneda, K., Takeda I. 1974. Activation of the enzyme of *A. oryzae* catalyzing the conversion of L-tyrosine to L-3, 4-

dihydroxyphenylalanine. *J. Ferm. Technol.,* 52: 106 –113.

Haq, I., Ali, S., Qadeer, M.A. 2002. Biosynthesis of L-dopa by *A. oryzae*. *Biores. Technol.,* 85: 25–29.

Ichishima, E., Maeba, H., Amikura, T., Sakata, H. 1984. Multiple forms of protyrosinase from *A. oryzae* and their mode of activation at pH 3.0. *Biochim. Biophys. Acta.,* 786: 25–31.

Kahn, V. 1995. Effect of kojic acid on the oxidation of DL-DOPA, norepinephrine and dopamine by mushroom tyr. *Pigment Cell Res.,* 8: 234–240.

Kong, K.-H., Lee, J.-L., Park, H.-J., Cho, S.-H. 1998. Purification and characterization of the tyr isozymes of pine needles. *Biochem. Mol. Biol. Int.,* 45: 717–724.

Krishnaveni, R., Vandana, R., Thakur, M.S., Neelgund. Y.F. 2009. Transformation of L-Tyrosine to L-dopa by a novel fungus *A. rutilum* under submerged fermentation. *Curr. Microbiol.,* 58: 122–128.

Krishnaveni, R., Vandana, R., Thakur, M.S., Neelgund. Y.F. 2008. Screening and characterization of a novel cytosolic tyrosinase producing fungus *A. rutilum w.gams*. *Bioscan,* 3(4): 523–528.

Lee, S.G., Hong, S.P., Sung, M.H. 1999. Development of an enzymatic system for the production of dopamine from catechol, pyruvate and ammonia. *Enzyme Microb. Technol.,* 25: 268–302.

Lerch, K. 1976. *Neurospora* tyr: molecular weight, copper content and spectral properties. *FEBS Lett.,* 61(1): 157–160.

Lineweaver, H., Burk, D. 1934. The determination of enzyme dissociation constant constant. *J. Am. Chem. Soc.,* 56: 658.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* 193: 265–275.

Penttila, M., Nevalainen, H., RattŒ, M., Salminen, E., Knowles, J.K.C. 1987. A versatile transformation system for the cellulolytic filamentous fungus *T. reesei*. *Gene,* 61: 155.

Peréz-Gilabert, M., Morte, A., Honrubia, M., García-Carmona, F. 2001. Partial Purification, characterization and histochemical localization of fully latent desert truffle polyphenol oxidase. *J. Agric. Food. Chem.,* 49: 1922–1927.

Rosenberg, I. 1996. Protein analysis and purification. Benchtop Techniques, Birkhauses Boston Library of congress cataloging-in- publication data.

Sussman, A.L. 1961. A comparison of the properties of two forms of tyr from *N. crassa. Arch. Biochem. Biophys.,* 95: 407.

US Patent 5486351. Method for making stable, extracellular tyr and synthesis of polyphenolic polymers there from.

US patent W0/2006/084953. novel microbial enzymes and their use.

Van Gelder, C.W.G., Flurkey, W.H., Wichers, H.J. 1997. Sequence and structural features of plant and fungal tyrs. *Phytochem.,* 45: 1309.

Vandana, R., krishnaveni, R. 2011. Transformation of L-tyrosine to L-dopa from *A. rutilum*: Effect of nutritional parameters. *Res. J. Biotech.,* 6(4): 7–11.

Wichers, H.J., Gerritsen, Y.A., Chapelon, C.G.J. 1996. Tyr isoforms from the fruit bodies of *A. bisporus*. *Photochem.,* 43: 333–337.

Xie, J., Schultz, P.G. 2006. A chemical tool kit for proteins an expanded genetic code. *Nature Rev.,* 775 Pp.

Zhou, P., Smith, N.L., Lee, C.Y., 1993. Potential purification and some properties of Monroe apple peel polyphenol oxidase. *J. Agric. Food. Chem.,* 41: 532–536.