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OPTIMIZATION, PURIFICATION AND CHARACTERIZATION OF TYROSINASE FROM NOVEL STRAIN OF *STREPTOMYCES BIKINIENSIS*

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ABSTRACT

Nineteen actinomycetes were detected as tyrosinase producers among the seventy actinomycete isolates from soil samples. Among these, five were further sorted for their enzyme activity and among them isolate C15 was found to be most potent with enzyme activity of 4.1 IU. Its molecular analysis indicated it as a novel strain of *Streptomyces bikiniensis.* Tyrosinase assay with time interval of 24 h during fermentation was carried out and the maximum activity of 4.1 IU was found at 120 h of incubation. The production was enhanced 3.70 folds in medium with set of optimum parameters. The purification of enzyme was carried out by ammonium sulphate precipitation (fold purity 1.8), dialysis (fold purity 2.0), ion exchange chromatography (fold purity 33) and gel filtration chromatography (fold purity 720). The molecular weight was determined by polyacryl amide gel electrophoresis and was found approximately 33 kD. pH 8 and temperature 35°C were most effective for the activity of pure tyrosinase. Further, it was stable at 35°C for 5 minutes. Maximum velocity (*Vmax*) and *Km* value of reaction was 7.04 S^{-1} and 3.01 mM respectively. 1% SDS was the most suitable inhibitor whereas CuSO4 was the most suitable stimulator.

Keywords: Actinomycetes, Tyrosinase, *Streptomyces bikiniensis,* TGM medium.

1. INTRODUCTION

Actinomycetes are aerobic, spore forming, Gram positive bacteria grouped as branching unicellular organisms, belonging to order actinomycetales highlighted with substrate and aerial mycelial growth. The genus *Streptomyces* is largest group in class *Actinobacteria* of the order *Actinomycetales* and falls under the family Streptomycetaceae [1]. *Streptomyces species* are filamentous, aerobic, chemoorganotrophic, Gram positive bacteria, and non-acid-alcohol fast with GC content 69-78%. They account for about 40% of soil bacterial flora. Among the known microbial secondary metabolites, 70% are produced by actinomycetes and two thirds of them are contributed by *Streptomyces*. Therefore, the role of actinomycetes in biotechnology and medicine is well known and these industries are always looking for novel bioactive compounds [2]. The *Streptomyces* genus has been studied with respect to bioactive compounds such as antibiotics, pigments and many extracellular enzymes such as glucose isomerase, amylase, cellulases and proteases.

Melanin or melanin like pigments synthesis is a common character among *Streptomyces species* [3] and tyrosinase takes part in the formation of melanin. Tyrosinase (monophenol, L-dopa: oxygen oxidoreductase, EC 1.14. 18.1) is a copper-containing monooxygenase enzyme that comes under the family oxidoreductases and also considered as polyphenol oxidase. It utilizes molecular oxygen to catalyze two different enzymatic reactions such as monophenolase or cresolase and diphenolase or catecholase activity. The monophenolase or cresolase activity is expressed by catalyzing *o*-hydroxylation of monophenols to the corresponding catechols while diphenolase or catecholase is expressed by catalysing the oxidation of diphenols or catechols to the corresponding *o*-quinones.

Tyrosinase takes part in biosynthesis of melanin in which monophenolase or cresolase activity of an enzyme catalyses substrate tyrosine into 3,4-dihydroxy-phenylalanine or DOPA (o-diphenol) by ortho-hydroxy-lation reaction and diphenolase or catecholase activity catalyses substrate 3,4-dihydroxyphenylalanine or DOPA into dopaquinone (o-quinone) by oxidation reaction. This is followed by conversion of o-quinone to melanin pigments through series of enzymatic and nonenzymatic reactions [4, 5]. Along with tyrosine and L-DOPA,

numerous other substrates such as phenols and diphenols are also converted to corresponding diphenols and quinones. In pharmaceutical industries tyrosinase is used for the production of *o*-diphenols such as L-DOPA and dopamine. Melanin produced by tyrosinase has unique characters like skin protection effect from radiation, free radical scavenging and metal chelating activities and therefore they are used in medical, agricultural, industrial and waste treatment processes. The soluble melanin comprises an emerging class of pharmacologically active substances. Bioremediation of radioactive waste like uranium can be conducted by using melanin producing organism [6].

Microbial tyrosinase available in the market is not used in food or pharma industries due to its multicatalytic reaction. Such tyrosinase enzyme is in association with enzymes peroxidase and laccase that cause side reactions. However, certain microbial strains have potential to produce tyrosinase without peroxidase and laccase activities and they are isolated from soil samples [7].

Tyrosinase produced in *Streptomyces* is laccase and peroxidise free non-modified monomeric protein with a low molecular mass of 30 kD. Its extracellular nature makes its easy separation from mixture with simple methods. However a very little data is available on tyrosinase from *Streptomyces*. The present work was undertaken with the objective of optimization, purification and characterization of tyrosinase from a novel strain of *Streptomyces bikiniensis*.

2. MATERIAL AND METHODS

A total nine soil samples collected from the different villages in Sangli and Kolhapur District, M.S., India were used for the isolation of actinomycetes. All the components required for culture media were purchased from standard Hi media Laboratory, Mumbai. The reagents used in experimental work were of analytical grade and purchased from Molychem Laboratory, Mumbai.

2.1. Isolation of actinomycetes

Initially, the enrichment of soil samples was carried out in Bennett's broth supplemented with Cycloheximide (100µg/ml). A 10-fold serial dilution of the enriched sample was prepared up to 10^{-6} and 0.1ml of each dilution was inoculated on glycerol asparagine agar supplemented with cycloheximide (100µg/ml) that prevents the growth of fungal contaminants. The plates were incubated at room temperature (28°C) and monitored periodically over 5 to 7 days. After incubation, colonies showing tough leathery or chalky texture, dry or folded appearance were selected as colonies of actinomycetes. Pure isolates were transferred on slants of same medium and preserved at 4±2°C for further study.

2.2. Screening of tyrosinase producers

The primary screening of tyrosinase producing actinomycetes was carried out by spot inoculation on sterile skimmed milk media and incubation at an ambient temperature for 2 to 3 days. After incubation, the plates were examined for the clear zone around the colonies of isolates. Secondary screening of these isolates was carried out by using different media such as peptone water, tyrosine broth, peptone yeast extract iron agar and tyrosine gelatine beef extract agar (TGB agar). Potential tyrosinase producers were selected on the basis of zone of catalysis on tyrosine gelatine beef extract agar and tyrosinase assay of crude extract obtained from tyrosinase broth inoculated with isolates and incubated at 30°C for 5-7 days.

2.3. Tyrosinase assay

The tyrosinase present in crude enzyme extract was estimated by dopachrome spectrophotometric method. The reaction mixture was prepared by adding 0.1 ml of crude extract, 4.9 ml of 0.1 M (pH 6.8) sodium phosphate buffer and 4 mg DL-Dopa as substrate. It was kept for 5 minutes in water bath adjusted to 30°C for reaction. The UVvisible spectrophotometer (Systronics 2201) was set at 30°C with wavelength of 475 nm and absorbance values were recorded continuously for five minutes. The absorbance recorded at 1st minute was considered as initial absorbance (A_0) and absorbance recorded at 5th minute was considered as final absorbance (A_f). The actual absorbance (A_a) was calculated by subtracting final absorbance (A_f) from initial absorbance (A_0). The molar extinction coefficient of dopachrome was 3600 $M^{-1}.cm^{-1}$. 1 unit of tyrosinase activity was referred to as the amount of enzyme required to catalyze 1 µM of DL-dopa per minute under the above mentioned conditions. The tyrosinase enzyme activity was estimated in µmol/min/ml by using formula -

$$\mu mol/\ min\ /ml\ = \frac{(A_{f475nm}\ -\ A_{o475nm}\ ) \times \text{assay volume (ml)} \times \text{dilution factor} \times\ 10{,}000}{\varepsilon\ \text{nm}\ (l \times\ mol^{-1}cm^{-1}) \times 1\ cm\ \times\ \text{enzyme volume (ml)} \times \text{reaction time}}$$

2.4. Identification of isolates

The genus level identification of the isolates was carried out on the basis of microscopic and cultural characters

whereas species level identification was carried out by using biochemical and physiological characters. The characters considered for identification were spore chain morphology, pigmentation on various media, enzymatic activities, growth tolerance and nutritional requirements. Probabilistic identification of bacteria (PIB) software which is a modification of DOS based MICRO-IS software was used for the identification. It contains matrices of known strains and provides identification of unknown strains attaining minimum ID score.

2.5. Molecular identification of *Streptomyces*

Potent isolate of *Streptomyces* was identified by using 16 S rRNA sequencing. The genomic DNA was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit. Using below 16S rRNA universal primers, gene fragment was amplified using MJ Research Peltier Thermal Cycler. Primer used for forward sequencing was 27F with sequence AGAGTTTGATCMTGGCTCAG (20 bases) while primer used for reverse sequencing was 1492R with sequence TACGGYTACCTTGTTACGACTT (22 bases). Single pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The phylogeny analysis of sequence with the closely related sequence of BLAST result was performed. This was followed by multiple sequence alignment.

2.6. Submission of nucleotide sequences to DDBJ (DNA Data Bank of Japan)

Nucleotide sequences of potential tyrosinase producing organism along with forward and reverse primers were submitted to DDBJ for obtaining accession numbers.

2.7. Development of inoculums for tyrosinase fermentation

The inoculum for tyrosinase fermentation was developed by inoculating potential isolate in flask containing sterile peptone yeast extract iron broth medium and incubation at 30°C for 3-5 days on a 100 rpm rotating shaker till achievement of approximately 1.4×10^6 spores/ml.

2.8. Fermentation of tyrosinase by potential isolate

The fermentation of tyrosinase was carried out in 500 ml of sterile basal fermentation medium. It was inoculated with 2% (v/v) of 24 hrs old inoculum of potential isolate and incubated at 30°C for 144 hours on a 100 rpm rotating shaker. A 20 ml of fermented broth was collected and processed for tyrosinase assay at every 24 hours' interval and tyrosinase activity in terms of IU at 475 nm was recorded.

2.9. Optimization of fermentation parameters and production of tyrosinase using set of optimum parameters:

The optimization of various physicochemical parameters such as pH, temperature and agitation speed and nutritional parameters such as carbon sources, nitrogen sources and minerals was carried out to achieve the maximum tyrosinase production. It was done by the one factor at a time method where one factor is varied at a time with rest of factors kept constant.

The tyrosinase production was tested by using set of optimum parameters in 500 ml of fresh medium containing optimum concentrations of glucose (0.4%) as carbon source, yeast extract (0.6%) as a nitrogen source and $CuSO_4$ (0.02%) as a mineral source and pH 8.5. The medium was inoculated with 2% of 24 hrs old inoculum of potential isolate and incubated at optimum temperature of 35°C on rotatory shaker with optimum speed of 150 rpm upto 144 hours. The content of flask was taken for tyrosinase assay at every 24 hours' interval upto 144 hours and tyrosinase activity in terms of IU at 475 nm was recorded. The fermented broth thus obtained was further processed for purification of tyrosinase using different techniques in sequential manner.

2.10. Stepwise purification of tyrosinase and determination of activity

Tyrosinase enzyme from fermented broth was purified by techniques such as precipitation (salting out), dialysis, ion exchange chromatography, gel filtration chromatography and SDS polyacrylamide gel electrophoresis sequentially. Each method, except SDS polyacrylamide gel electrophoresis, was studied with respect to enzyme activity, protein concentration, specific activity and fold of purification. All methods mentioned above were standardized for purification and were carried out at 4°C. Increase in purity of tyrosinase at each step was estimated by comparing with protein content and enzyme activity. The enzyme activity was checked by spectrophotometric method and protein content was estimated by Bradford method.

2.11. Characterization of tyrosinase

2.11.1. Effect of pH

The effect of pH on pure tyrosinase activity was studied with DL-Dopa as substrate. It was determined by use of buffers of different pH such as acetate buffer pH 4.0, citrate buffer pH 5.0 and 6.0, phosphate buffer pH 7.0, Tris-HCl buffer pH 8.0, Glycine-NaOH buffer pH 9.0 and 10. They were prepared with 50 mM concentration of each in separate beaker with 20 ml volume.

2.11.2. Effect of temperature

Effect of temperature on enzyme activity was also studied with DL-dopa as substrate. The reaction mixture was prepared with 4.9 ml of 0.1 M phosphate buffer supplemented with 4 mg DL-dopa as a substrate in seven test tubes. This reaction mixture was added with 0.1 ml of purified and diluted enzyme and incubated at different temperatures 30, 35, 40, 45, 50, 55 and 60°C for 5 minutes.

2.11.3. Stability of pure tyrosinase

Stability of enzyme was determined with DL-dopa as a substrate. The reaction mixture was containing 4.9 ml of 0.1 M phosphate buffer supplemented with 4 mg of DL-dopa and 0.1 ml of diluted and purified enzyme. It was incubated at 35°C for 5 min, 15 minute, 30 minutes and 60 minutes respectively.

2.11.4. Kinetic studies

A kinetic study of tyrosinase was carried out in terms of Km and Vmax. The 16 mM DL-Dopa was used as a substrate for determination of Km and Vmax. A stock solution of 100 ml of 16 mM DL-Dopa as a substrate was prepared and used for dilutions. Blank was prepared by addition of 4 ml of phosphate buffer and 0.5 ml of purified enzyme solution. A total eight tubes were labelled as 16 mM, 8 mM, 4 mM, 2 mM, 1 mM, 0.5 mM, 0.25 mM and 0.125 mM.

2.11.5. Effect of inhibitors on pure tyrosinase

The effects of various inhibitors such as phenyl-methylsulfonyl fluoride (PMSF), 1-10 phenan-throline, p-chloromercuryl benzoate and EDTA at 2 mM concentration and H_2O_2 (1%bleaching agent), NaCl (1 M salt solution) and SDS (1% detergent) were tested on purified tyrosinase with spectrophotometric method. Four tubes were labelled as S (sample), IC (inhibitor control), SC (solvent control) and EC (enzyme control). The percent inhibition was calculated by the formula:

$$\% \text{ Inhibition} = \frac{(IC - SC) - (SC - EC)}{(IC - SC)} \times 100$$

2.11.6. Effect of metal ions on tyrosinase activity

The effect of various metal ions such as $HgCl_2$, $CaCl_2$, $FeCl_3$, $AgNO_3$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, and $CuSO_4$ at 20 mM concentration on tyrosinase activity was studied by using spectrophotometric method. Four tubes were labelled as S (sample), MC (Metal control), SC (solvent control) and EC (enzyme control). The percent inhibition was calculated by using the same formula.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of tyrosinase producing actinomycetes

A total seventy actinomycetes isolates were obtained on glycerol asparagine agar. They were coded with alphabets and serial numbers and further screened on skimmed milk agar medium. Among these, nineteen isolates showing clear zone around the colony on skimmed milk agar were used for secondary screening since they were potential tyrosinase producers. The primary screening of tyrosinase producing actinomycetes on skimmed milk agar has also been attempted [8] and found only two strains as potential producers from among the twenty actinomycete strains. The present work reports that among 70 isolates, 19 (27.14%) were maximum tyrosinase producers.

During the secondary screening, the intensity of blackish brown pigment in tyrosine broth and zone of catalysis on TGB medium was observed visually. It was maximum by the isolates C7, C15, S14, Kd8 and Kd14. Therefore these five isolates were considered as potential producers of tyrosinase. The use of tyrosine gelatin beef extract (TGB) agar as the most efficient medium for secondary screening of potential tyrosinase producing actinomycetes has also made previously [9]. In this study, additionally attempt of using peptone water as a new screening medium has been made. The isolates showing strong blackish brown pigmentation in peptone water also show maximum zone of catalysis on TGB medium.

3.2. Selection of most potential tyrosinase producer

The selection of most potential tyrosinase producer was done on the basis of tyrosinase activity of potential isolates as determined by tyrosinase assay. It is presented in Table 1.

It can be noted from the table that the isolate C15 shows maximum tyrosinase activity of 4.1 IU and therefore it was considered as the most potential and used for further study. Previous reports on the selection of potent

tyrosinase producers on the basis of tyrosinase assay are also available [10, 11].

3.3. Molecular identification of most potent isolate C15

Molecular identification of isolate C15 was attempted by gene amplification followed by sequencing, bioinformatic analysis and phylogenetic analysis (Fig.1) which indicated it as a novel strain of *Streptomyces bikiniensis*.

Table 1: Tyrosinase activity of isolates

Sr. No.	Isolates	Enzyme activity (IU) (Absorbance at 475 nm)
1	C7	2.5
2	C15	4.1
3	S14	3.6
4	Kd8	1.11
5	Kd14	2.22

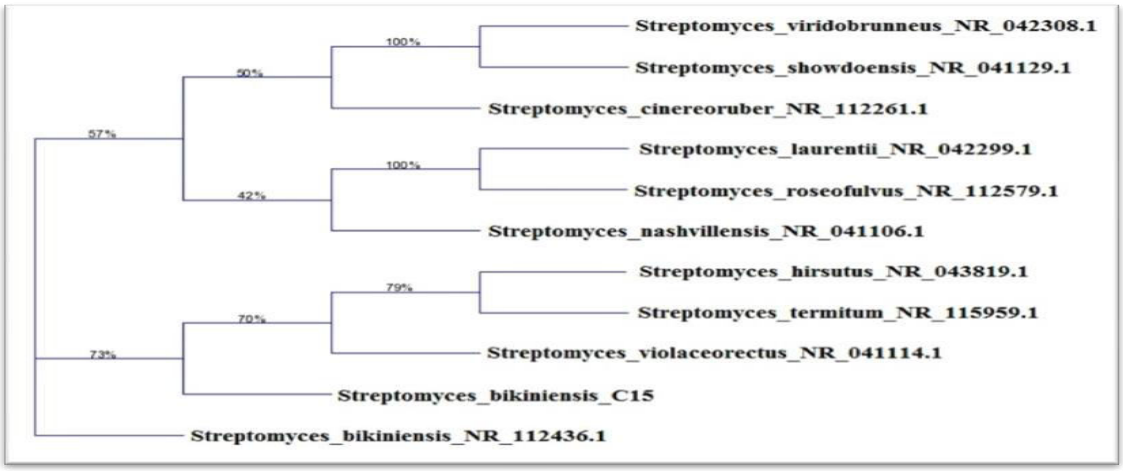

Fig 1:Phylogenetic tree of C15 isolate

3.4. Accession number from DDBJ

Nucleotide sequence of novel strain of *Streptomyces bikiniensis* was submitted to DDBJ and accession number was obtained as LC072711.

3.5. Optimization of fermentation parameters (pH, temperature, agitation speed, carbon, nitrogen and mineral source) and production of tyrosinase using optimum parameters

The enzyme activity was 3.4, 3.6, 4.0, 5.11 and 1.94 IU at pH 7.0, 7.5, 8.0, 8.5 and 9.0 respectively at 120 h of incubation indicating pH 8.5 as most suitable. The activity at 30, 35, 40 and 45°C temperature was 1.70, 4.5, 2.0 and 0.89 IU respectively indicating 35°C temperature as the most suitable. The activity at agitation speed of 100, 125, 150, 175 and 200rpm was 3.8, 4.2, 4.7, 3.6 and 3.2 IU respectively indicating 150 rpm as the most suitable. Maximum tyrosinase activity using mannitol, sucrose and starch at 0.6% concentration was 3.08, 3.98 and 3.72 IU respectively while for glucose and arabinose it was 4.66 and 3.94 IU respectively at 0.4% concentration. This indicated glucose as the most suitable carbon source. Similarly, the maximum activity using yeast extract at 0.6% concentration was 5.05 IU, using asparagine and arginine at 0.4% concentration was 3.91 and 2.78 IU respectively whereas for casein and gelatine it was 4.05 and 3.82 IU at 1% concentration. Thus, the yeast extract is found to be the most suitable nitrogen source. Further, the maximum activity using $CuSO_4$, $MgSO_4$, $FeSO_4$, $MnSO_4$, KH_2PO_4 and K_2HPO_4 at 0.02% concentration was 4.2, 2.1, 3.4, 2.57, 2.89 and 2.01 IU respectively. No activity was observed for all minerals at 0.1% concentration. Thus, $CuSO_4$ was the most suitable mineral source for optimum activity.

Increase in secondary metabolite production by the presence of a non-preferred carbon sourcehas been reported [12].Temperature 30°C, agitation speed 120 rpm, pH 7, inoculum size 5% w/v and 24 h incubation period was optimum for tyrosinase production using *Penicillium jensenii* [13]. pH7.5, 160 rpm, 24 hour incubation time, 1.25% sodium chloride concentration, 0.2% peptone and 0.75% maltose increased tyrosinase production upto 25% in *Streptomyces antibioticus* RSP-T1 [14]. The results in present work indicate maximum tyrosinase activity at pH 8.5, temperature 35°C, agitation speed 150 rpm, glucose 0.4%, yeast extract

0.6% and $CuSO_4$at 0.02% concentration. Maximum tyrosinase activity (15.2 IU) was at 120 h of duration while it was minimum (3.6 IU) at 24 h of incubation.

3.6. Estimation of protein

Protein content in fermented broth for C15 isolate was 10.6 mg/ml for 1:10 dilution and 1 mg/ml for 1:100 dilution. Thus, concentration of proteins with two different dilutions (1:10 and 1:100) was confirmed.

3.7. Purification of tyrosinase

The data on the purification of tyrosinase is as presented in Table 2.

The fraction number 13 obtained by ion exchange chromatography was having maximum tyrosinase activity of 11.7 IU and protein content of 0.07 mg/ml. The estimated values of specific activity, yield and fold purity using the obtained values of tyrosinase activity and protein concentration were 167 U/mg/min, 77% and 33 fold respectively (Fig.2).

The fraction number 11 obtained by gel filtration was having maximum tyrosinase activity of 10.3 IU and protein content of 0.01 mg/ml. The estimated values of specific activity, yield and fold purity using the obtained values of tyrosinase activity and protein concentration were 1030 U/mg/min, 68% and 720 fold respectively (Fig.3).

Table 2: Purification data

Purification Step	Tyrosinase activity (IU)	Protein concentration (mg/ml)	Specific activity U/mg/min	Yield (%)	Fold purity
Crude	15.2	10.6	1.43	100	1
Ammonium sulphate (75%)	14.1	5.6	2.51	92	1.8
Dialysis	12.4	2.4	5.1	81.6	2.0
Ion exchange chromatography	11.7	0.07	167	77	33
Gel filtration	10.3	0.01	1030	68	720

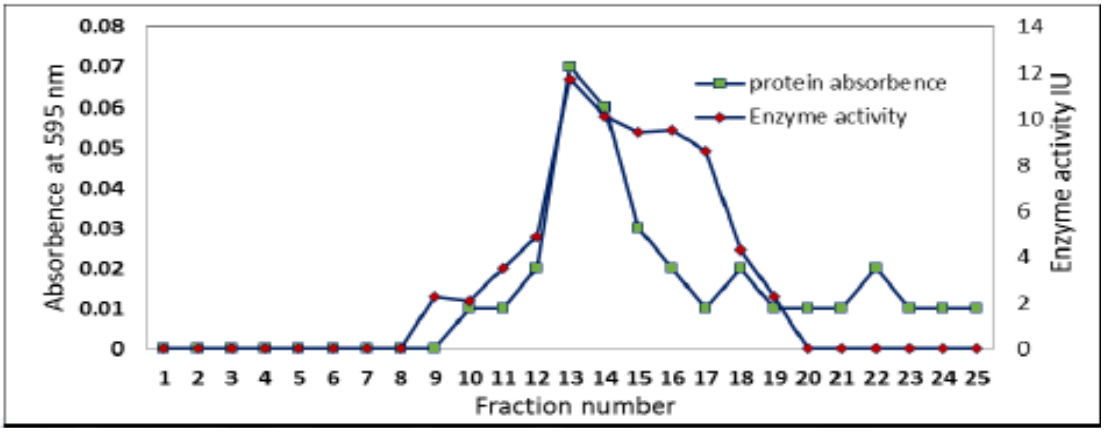

Fig. 2: The elution profile of tyrosinase enzyme using ion exchange column

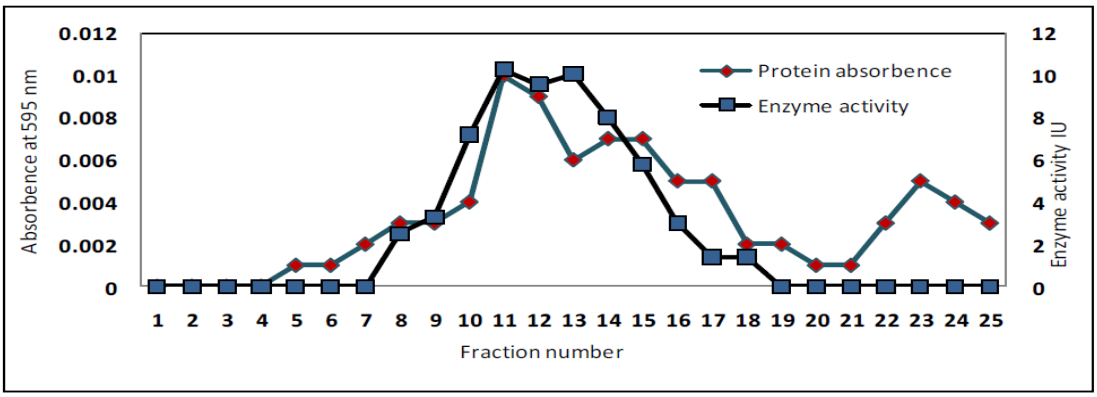

Fig. 3: The elution profile of tyrosinase enzyme using gel chromatography

3.8. Polyacrylamide gel electrophoresis (PAGE)

The bands of sample components and molecular marker on polyacrylamide gel electrophoresis (PAGE) are represented in Plate 1. It was noticed that bands of gel filtration, ion exchange chromatography fractions and crude filtrate are near to 33 kD of known molecular marker. Thus the molecular weight of tyrosinase as determined by comparing sample fractions with known molecular weight molecular markers was approximately 33 kD.Number of earlier workers reported the molecular weight of tyrosinase as determined by polyacrylamide gel electrophoresis in the range of 18 to 34kD.

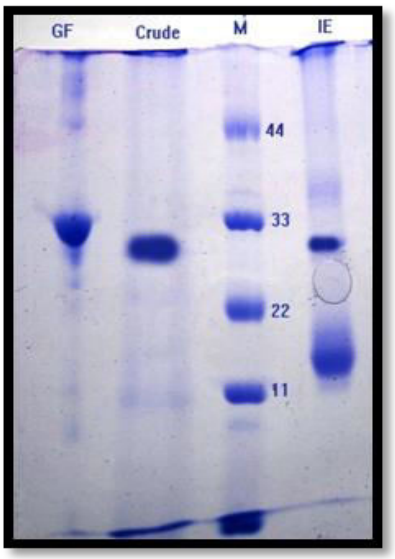

Plate1: Bands of sample components and molecular marker on PAGE

3.9. Characterization of tyrosinase:

3.9.1. Effect of pH

The maximum tyrosinase activity of 11.1 IU was at pH 8 and minimum was 4.7 IU at pH 5. Thus, pH 8 was the most suitable pH for optimum tyrosinase activity. Earlier workers [15-17] had reported pH 7 as most favourable pH for maximum tyrosinase activity indicating tyrosinase obtained from *Streptomyces bikiniensis* is active even at slightly alkaline condition.

3.9.2. Effect of temperature

The maximum tyrosinase activity of 9.44 IU was at temperature 35°C and minimum was 2.2 IU at temperature 35°C. Further, there was decrease in activity with increase in temperature above 35°C. Previous report indicated 40°C and 35°C as the most suitable temperature for maximum tyrosinase activity [16, 17].

3.9.3. Stability

Decrease in activity and thereby decrease in stability of tyrosinase was observed with increase in time period from 5 to 60 min. Percent stability of tyrosinase was 100, 81, 66 and 55% for 5, 15, 30 and 60 min respectively.

3.9.4. Kinetic studies

The maximum velocity (V_{max}) and *Km* value for tyrosinase reaction was 7.04 S^{-1} and 3.01 mM respectively as calculated by Lineweaver and Burk plot. The K*m* and V*max* values for substrate L-dopa was 3.13 mM and 92.87 µM/ min in *Actinomycetes* [18].This report supports our kinetic studies.

3.9.5. Effect of Inhibitors

The absorbance value of inhibitor control and 2 mM PMSF inhibitor was same. Hence, 0% inhibition was shown by inhibitor PMSF. The absorbance and inhibitory activity of inhibitors such as p-chloromercuryl benzoate, 1-10 phenanthroline and EDTA at 2 mM concentration was 0.32, 0.27 and 0.07 and 6.06, 21.21 and 81.81% respectively. The absorbance and inhibitory activity of inhibitors as H_2O_2 (1%), NaCl (1M) and SDS (1%) was 0.26, 0.19 and 0.02 and 24.24, 45.45 and 96.96% respectively. Residual activity of inhibitors for PMSF (2 mM), p-chloromercuryl benzoate (2 mM), 1-10 phenanthroline (2 mM) , EDTA (2 mM), H_2O_2 (1%), NaCl (1M) and SDS (1%) were 100, 93.94, 78.79, 18.19, 75.76, 54.55 and 3.04 respectively. Thus, maximum inhibition of 96.96 % was at SDS (1%) concentration and minimum inhibition of 6.06 % was at 2 mM p-chloromercuryl benzoate concentration. No inhibition was observed for inhibitor PMSF (2 mM). Thus, SDS (1%) was the most suitable inhibitor for inhibition of tyrosinase activity. The tyrosinase in actinomycete was more than 80% inhibited in the presence of 25 mM p-arbutin, NaCl and SDS [18]. Thus, our results of inhibition of tyrosinase by SDS are in accordance with earlier results.

3.9.6. Effect of metal ions on tyrosinase activity

100% inhibition at 20 mM concentration was shown by metal ions $HgCl_2$ and $AgNO_3$. The inhibitory activity of

metals such as $FeCl_3$, $ZnSO_4$ and $MgSO_4$ was 0.23, 0.30 and 0.29 and 33.33, 12.12 and 15.15% respectively at same concentration. The stimulatory effect of metals such as $CaCl_2$, $MnSO_4$, and $CuSO_4$ was 0.37, 0.36, and 0.42 and 9.09, 6.06 and 24.24% respectively at 20 mM concentration. Thus, maximum stimulatory activity was for metal ion $CuSO_4$ and minimum was for metal ion $MnSO_4$. Thus, $CuSO_4$ was the most suitable stimulator for stimulation of tyrosinase activity. Induction of tyrosinase of actinomycetes in the presence of 5 mM Ca^{2+}, Mg^{2+}, Mn^{2+} and Cu^{+2} is reported [18]. Our results of stimulation of tyrosinase in presence Ca^{2+}, Mg^{2+}, Mn^{2+} and Cu^{+2} were in accordance.

Thus in conclusion the isolate C15 is a novel strain of *Streptomyces bikiniensis* having potential to produce tyrosinase at 120 h of incubation, pH 8.5, temperature 35°C, agitation speed 150 rpm, 0.4 % glucose as carbon source, 0.6 % yeast extract as a nitrogen source and 0.02 % $CuSO_4$ as mineral source. The production gets enhanced 3.70 folds in fermentation medium with set of optimum parameters. The purification of tyrosinase can be carried out by various methods viz; ammonium sulphate precipitation (fold purity 1.8), dialysis (fold purity 2.0), ion exchange chromatography (fold purity 33) and gel filtration chromatography (fold purity 720). The molecular weight of tyrosinase was approximately 33 kD. The pH 8 and temperature 35°C are most effective for the activity of pure tyrosinase. The enzyme is stable at 35°C for 5 minutes and maximum velocity (*Vmax*) of reaction was 7.04 S^{-1} and *Km* value was 3.01 mM. 1 % SDS was the most suitable inhibitor for inhibition of tyrosinase activity and CuSO4 was the most suitable stimulator.

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Conflict of interest

The authors have no conflict of interests.

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