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Original Article

Production of L-tyrosinase from Novel Variants of *Streptomyces cellulosae*

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ABSTRACT

Background: Recently, a lot of interest has been focused on the discovery of high L-tyrosinase-yielding microorganisms that catalyze the production of enantiomerically pure L-DOPA, which has been recognized as the conventional treatment for Parkinson's disease. **Objectives:** The current work primarily focused on isolating novel, promising tyrosinase-producing isolates and performing taxonomical characterization on them. **Materials and Methods:** Initially six various actinomycetes isolates were used in present study. Primary screening and secondary screening of selected isolates were performed. By evaluating the morphological, cultural, physiological, biochemical and molecular characterization, promising isolates were identified. Using 16S rRNA gene sequencing, the isolates' Phylogenetic investigations were conducted. **Results:** *Streptomyces cellulosae* variants, isolates PD18 and PD26 were identified to be the potential actinomycete isolates and their respective maximal tyrosinase yields were reported to be 21.20 IU/mL and 24.19 IU/mL. **Conclusion:** According to the observations of the experiment, SS (Soluble starch) medium with 1mg/mL copper sulphate was most suitable medium for tyrosinase production from our promising isolates PD18 and PD26. The maximum yield was noticed after 168 hr of incubation. This work emphasizes the significance of finding novel variants of *Streptomyces cellulosae*, which were first used to produce tyrosinase as well as promising sources for potentially bioactive substances like antimicrobial metabolites, biosurfactants and enzymes like amylase, lipase, cellulase, uricase, urease, β- galactosidase and L- asparaginase.

Keywords: Tyrosinase, L- tyrosine, L-DOPA, SS medium, copper sulphate, *Streptomyces cellulosae.*

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INTRODUCTION

Actinomycetes have virtually completely different characteristics from bacteria and fungi, which is why they were given their own class. Branching spore-forming, Gram positive, aerobic unicellular organisms with aerial mycelia growth and substrate are found in the order Actinomycetales. Fission or unique spores or conidia are the two ways that actinomycetes reproduce. They have DNA that has a high (G+C) concentration of more than 55 mol%. The 16s ribosomal cataloguing and DNA evidence studies using rRNA pairing showed a Phylogenetic link between the actinomycete group.[1] Actinomycetes, one of the 18 fundamental lineages of the domain bacteria is a huge taxonomic category.[2] *Streptomyces* species generate a wide range of extracellular enzymes including glucose isomerase, amylase, cellulase, protease, lipase, xylanase, pectinase, glucose oxidase, keratinase and serratiopeptidase as well as antibiotics, pigment and other bioactive molecules. There has been less investigation on their capacity to manufacture tyrosinase.[3] Copper-containing tyrosinase (EC 1.14.18.1) is a diphenolase (catecholase) and monophenolase (cresolase) enzyme. When monophenols are o-hydroxylated to produce equivalent catechols, tyrosinase's monophenolase (cresolase) and diphenolase activities are active (catecholase). This enzyme is present in many different species, ranging from prokaryotes to mammals. Tyrosinase can play a significant impact in the biosynthetic pathway of skin pigment. Its monophenolase (cresolase) activity can catalyze the transformation of the material tyrosine into the o-diphenol 3,4-dihydroxyphenylalanine (DOPA) and its diphenolase (catecholase) activity can catalyse the transformation of the formed product DOPA into the quinone (o-quinone) through oxidization.[4,5] The significant capability of this enzyme for the food, medical (melanoma patients, pro drug activation), agricultural, analytical and environmental industries is supported by a wealth of data.[6-8]

The standard treatment for Parkinson's disease until 1967 was L-DOPA.[9] In cases of neurogenic damage, it is also employed to control the myocardium.[10] Each year roughly 250 tonnes of L-DOPA are sold worldwide.[11] The majority of L-DOPA used in commercial products is made from vanillin and hydantoin using an eight-step chemical procedure, one of which is an optical resolution.[12]

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Many investigations have been conducted to develop an alternative enzyme-based technique using free or immobilised tyrosinase to the chemical synthesis that L-DOPA is currently manufactured at industrial scale.[14-16]

MATERIALS AND METHODS

Primary screening

Initially, six plates of skim milk agar medium, which comprises peptone (1%), sodium chloride (0.5%), yeast extract (0.3%), agar (2%), and skim milk (10%) were streaked with five different actinomycetes isolates from kapuluppada plastic waste dumping yard, close to kommadi, Visakhapatnam, A.P and one actionomycetes isolate from Korangi mangrove forest, Korangi, Kakinada. All the plates were incubated at 27°C for 7days. Positive outcomes were seen as being very clear and negative outcomes as having no zone. Additionally, all six isolates were streaked on six Melanin medium plates that included yeast extract (0.1%), L-tyrosine (0.1%), NaCl (0.8%) and agar powder (1.5%) with an adjusted pH of 7. In contrast, control plates were created by producing screening media devoid of L- tyrosine. These plates served as test plates. At 27°C, all test and control plates were incubated for 7 days.[17]

Secondary Screening

The selected isolates were further tested for its ability to produce tyrosinase extracellular by submerged fermentation technique e using tyrosine medium arbitrarily. After 7 days of incubation at 27°C, the fermented flask was centrifuged at 4°C, 4000 rpm for 20 min. The obtained clear supernatant was used to check quantity of tyrosinase produced during fermentation by means of tyrosinase assay and protein assay.[19,20]

Tyrosinase enzyme Assay

Activity of tyrosinase as a monophenolase

Tyrosine was used as a substrate to assess the production of tyrosinase utilizing monophenolase activity.[21-25] The quantity of extracellular tyrosinase was determined by modified dopachrome method using molar extinction co-efficient.[26] The test solution was prepared by mixing 3mL of L- tyrosine (0.02M) substrate and 0.1mL of clear enzyme supernatant in a test tube where as in the blank the reaction mixture was prepared by mixing 3mL of L-tyrosine (0.02M) and 0.1mL of 0.05M sodium phosphate buffer. Both test and blank were incubated at 37°C for 5 min then absorbance of test is noted against blank using UV visible spectrophotometer at 475 nm.

An international unit (IU) of extracellular enzyme activity was established as the enzyme molecule required to catalyze the oxidation of 1Mmol of L-DOPA for each minute under the aforementioned circumstances. The dopachrome's 3600$M^{-1}cm^{-1}$ molar extinction co-efficient was used for calculation.[26]

Following equation was used to calculate enzyme activity in terms of IU/mL

$$\text{IU/mL Enzyme} = \frac{\text{absorption / min x assay volume x DF}}{\epsilon_{nm}\ (M^{-1}\ cm^{-1}.\ \text{cm.enzyme volume})} \times 10{,}000$$

ϵ_{nm} = absorption coefficient value 3600 M^{-1} cm^{-1}

DF= dilution factor

Lowry technique

The protein content was determined using the Lowry technique. 0.1mL of the sample or standard is mixed with 2N NaOH. Undergo hydrolysis at 100°C for ten min in a boiling water bath or heating chamber. Bring the hydrolyzate to ambient temperature before adding 1mL of newly prepared complex-forming reagent. Let the solution sit at ambient temperature for ten minutes. Folin reagent should be added using a mechanical stirrer and the mixture should be allowed to rest for 30 to 60 min at ambient temperature (do not exceed 60 min). If the protein concentration becomes less than 100 g/mL, read the optical density at 750 nm; if it was between 100 and 2000 g/mL, read the optical density at 550 nm.[20]

Preliminary screening of tyrosinase production medium

The suitable production medium composition was determined by adding the 1mL inoculums medium (Absorbance 0.9 OD) in different production media such as tyrosine broth [medium1-Peptone (0.5%), Beef extract (0.3), L-tyrosine (0.1%), NaCl (0.8%), pH 7.0],[22] tyrosine production medium [medium2 - Soluble starch (0.2%), Polypeptone (casein peptone (0.05%) + meat peptone (0.05%-0.1%), NaCl (0.1%), K_2HPO_4 (0.1%), $MgSO_4$ (0.005%), $FeSO_4$ (0.005%), $CuSO_4$ (0.005%), pH 7.0],[18] SS Medium (Soluble starch medium) with copper [medium3 - Soluble starch (2.5%), Glucose (1.0%), Yeast extract (0.2%), $CaCO_3$(0.3%), trace salt solution-100µL (0.005% of $FeSO_4$, Cu SO_4, $ZnSO_4$ and $MgSO_4$ were mixed in 100mL distilled water), L- tyrosine (0.1%), $CuSO_4$ (1mg/ mL), pH 7.0][22] and SS Medium Composition without copper (medium 4), ISP-7 [medium 5- Glycerol (1.5%), L-Tyrosine (0.05%), L-Asparagine(0.1%), K_2HPO_4 (0.05%), $MgSO_4.7H_2O$ (0.05%), $FeSO_4.7H_2O$ (0.001%), NaCl (0.005%), Trace salt solution (100µL), p^H (7-7.4) and distilled water upto 100 mL], ISP-5 [Medium 6- L- Asparagine (0.1%), Glycerol (1%), K_2HPO_4 (0.1%), Trace salt solution (100µL), p^H (7-7.4) and distilled water upto 100mL], ISP-4 [Medium 7- Soluble starch (1%), K_2HPO_4 (0.1%), $MgSO_4.7H_2O$ (0.1%), NaCl (0.1%), $(NH4)_2SO_4$ (0.2%), Ca_2CO_3 (0.1%), $FeSO_4.7H_2O$ (0.0001%), $MnCl_2.7H_2O$ (0.0001%), $ZnSO_4.7H_2O$ (0.0001%), p^H (7.0-7.4)] respectively and allowed to incubate at 27°C for 7 days. After incubation, enzyme was centrifuged and clear supernatant was used. Tyrosinase production was assayed in terms of monophenolase activity.[21-25]

Effect of copper concentration on production medium

In each flask, the specified medium was supplemented with different amounts of copper sulphate (1μM, 3.5 μM, 10 μM) as well as 1 mg, 2.5 mg, 5 mg and 10 mg of copper sulphate. The selected promising isolates were then added to the mixture in the appropriate amounts. Tyrosinase test was carried out using the clear enzyme supernatant following incubation.[21-25]

Polyphasic method for the identification of the promising isolate

Isolate features in terms of morphological, cultural, physiological, and biochemical

Cultural features, morphology, physiology, biochemical, and genetic analyses were employed to identify the potent actinobacterial strain. The type of the mycelium, colour and spore arrangement were taken into consideration during the microscopic evaluation using the direct method, inclined coverslip technique and slide culture technique.[27-29] To examine the strain's cultural traits such as the colour of the upper mycelium, the colour of the basal mycelium, the generation of pigment and the creation of spores, the strain was cultivated on seven International Streptomyces Project (ISP) media and four non-ISP media.[30] By growing the strain on tyrosine agar (ISP-7) media and screening medium, the synthesis of melanin pigment was evaluated.[31] Utilizing established procedures, biochemical characterization was done.[32-34] The growth of the isolates' physiological features, such as the impact of pH (5-10), temperature (25-40°C) and salinity were examined.[33,34]

Molecular Characterization and Phylogenetic analysis of the selected actinomycete isolates

The screened actinomycete isolated strains were identified by 16S rRNA gene sequencing (Sanger method). The sequencing was carried out at NCIM- Pune.

The evolutionary process was derived using a neighbor-joining technique.[35] An ideal tree has a total branch length of 0.15528769. The percentages of duplicate trees where related taxa were bootstrapped together (1000 repetitions) are shown beside the branches.[36] Using the same evolutionary distance measure that was used to estimate the tree, the branch lengths are calculated. Changes were computed after removing all places with holes and missing data. The final dataset contained 1428 sites altogether. At MEGA 6, an evolutionary study was conducted. It is determined using the Kimura two-parameter approach[37] and expressed as base substitutions per site. 18 nucleotide sequences were being investigated.[38]

Submission of nucleotide sequence to the National Center for Biotechnology Information (NCBI)

The isolates PD18 and PD26 have their nucleotide gene sequence [16S ribosomal RNA (rRNA)] data deposited in the Gene Bank database (NCBI).

Preliminary screening of actinomycetes for bioactive metabolites

Amylase activity

The starch agar medium which contains beef extract (0.3%), peptone (0.5%), soluble starch (0.2%) and agar (1.5%) was utilized for the amylase enzyme plate assay. The selected actinomycetes were streaked on a plate of starch agar and grown for seven days at 27°C. Following the incubation time 1% iodine solution was given to the starch agar plates and the presence of a clear zone of starch breakdown served as a marker for the presence of an amylase.[39]

Cellulase activity

For the cellulase enzyme plate assay, carboxymethylcellulose (CMC) agar was composed of yeast extract (0.005%) agar (1.5%), $NaNO_3$ (0.01%), K_2HPO_4 (0.01%), $MgSO_4$ (0.05%) and CMC (0.05%). On prepared media, promising actinomycetes were streaked and cultivated for seven days at 27°C. Congo red was applied to the plate after the incubation period, which was followed by 30 min rest period. The plate was then drained of additional Congo red, flooded with 1M NaCl and stand for 30 min. By obtaining a clean zone around the isolate, cellulolytic activity could be observed.[40,41]

Protease activity

The selected Actinomycete isolate was streaked on Skim Milk Agar (SMA), which comprises yeast extract (0.3%), NaCl (0.5%), agar (1.5%) and skim milk (1%). Plates were incubated for seven days at 30°C. A microorganism which produces proteases was observed in such a clear zone of hydrolyzed skim milk around the growth of microbe.[39,42]

Lipase activity

An modified version of the methodology used by Gopinath *et al.* For the purpose of detecting lipase enzyme plate assay, the selected actinomycetes were streaked on Tween-20/ Tween-80 agar which is composed of agar (2%), Tween-20/Tween-80 (1%), Peptone (1%), NaCl (0.5%) and $CaCl_2$ (0.01%). On tween-20 agar, selected actinomycetes were inoculated and the results were analyzed after seven days at room temperature. Around the colony, calcium salt precipitation revealed the presence of lipase enzyme.[43]

L-Asparaginase activity

On L-asparagines-glucose agar medium, the isolates were cultivated and incubated for 5-7 days. When an isolate produced positive results, pink colour media replaced yellow colour media and the colonies produced distinct pink zones.[44]

Gelatinase activity

Actinomycetes isolates were streaked on a gelatin agar medium made up of gelatin (12%), beef extract (0.3%), peptone (0.5%), agar (1.5%) in petri plates and the mixture was then incubated at 27°C for seven days. After seven days of incubation, the petri plates were spread with 10 mL of mercuric chloride solution and the area of hydrolysis around the isolates growth as well as their zone of growth zone's breadth were determined. From these measurements, the ratio between the hydrolyzing zone and growth zone was determined.[32-34]

Nitrate reductase activity

The promising isolates were put to 10 mL of nitrate broth and cultured for 5-7 days at 27°C as a control with nitrate broth that had not been inoculated. When 4- amino benzene sulfonic acid and α-naphthylamine are combined after incubation, an instantaneous cherry red colour results which is regarded as positive for such nitrate reductase enzyme assay. A little amount of zinc dust was also applied to the nitrate broth under investigation to further validate the existence or absence of nitrate reductase.[32-34]

ß- galactosidase activity

In comparison to lactose which is a synthetic substrate, O-nitrophenyl-beta-D-galactopyranoside (ONPG) has glucose replaced with an ortho-nitrophenyl group. The substrate ortho- nitrophenyl-beta-D-galactopyranoside (ONPG) able to penetrate into the microbe without the aid of permease unlike lactose. The organism is obtained from a medium with a high lactose concentration and inoculated into the ONPG medium using the broth technique of screening. If the organism has β-galactosidase, the enzyme will break down the β-galactosidase linkage and release the yellow chemical o- nitrophenol. This denotes a successful evaluation.[45]

Uricase Activity

By seeding the test organisms into agar plates with uric acid medium comprising uric acid (0.3%), beef extract (0.3%), peptone (0.5%), NaCl (0.2%) and agar (2.3%) at pH 7.5 incubation at 37°C, the primary screening of uricase from promising isolates was done. The development of clear zones surrounding the culture colonies is proof that uricase enzyme is being produced.[46]

Biosurfactant production

CTAB / Methylene blue agar plate technique

The CTAB agar plate assay is a semi-quantitative technique for detecting extracellular glycolipids and other anionic surfactants. Our isolates' crude extracts were put to cups of CTAB agar medium which contains the following: $NaNO_3$ (1.5%), KCl (0.11%), NaCl (0.11%), $FeSO_4$ (0.02%), KH_2PO_4 (0.34%), K_2HPO_4 (0.44%), $MgSO_4$ (0.05%), yeast extract (0.05%), glucose (2%), p^H (7) and others. Diffusion was permitted to occur for 24 hr at 28°C. The cups create a deep blue colour that denotes the presence of biosurfactants.[47]

Antimicrobial activity

Cross-streak method

The cross-streaking technique was used to assess the antibacterial spectrum of each actinomycete isolate against Gram-positive, Gram-negative and fungal bacteria. To incubate for seven days at 27°C, each isolate was streaked individually or separately along the middle of a plate containing starch casein agar (SCA) medium. An actinomycetes isolate that had fully matured and generated spores was streaked perpendicularly with a 48-hr-old fungus test organism and a 24-hr-old bacterium test organism. It was demonstrated that the actinomycete isolate might prevent the test organisms from growing.[48,49]

Agar overlay method

Isolates which exhibited a broad spectrum of antimicrobial activity were selected for agar overlay method. Selected isolates were centre stabbed on the starch casein media plates and incubated for 7 days at 28°C followed by overlay with nutrient agar inoculated with 24 hr fresh test bacterial culture or Sabouraud's dextrose agar containing 48 hr fresh fungal culture on the surface of foster medium having rich antimicrobial metabolites production with less agar content of 0.75%. In case of bacteria they are incubated for 18-24 hr at 37°C and for fungi 72-120 hr.[48,49]

RESULTS AND DISCUSSION

A copper metalloproteinase tyrosinase was selected for this study because of its potential applications in diverse fields. Basic structure of tyrosinase was conserved in various species and we attempted to isolate novel strains in order to produce the enzyme the industrially in a cost efficient manner. Therefore screening and isolation of tyrosinase producing actinomycetes were performed. As tyrosinase is a protease, so both proteolytic activity and melanin production test were performed with six cultures PD4, PD9, PD18, PD26, PD66, KMFA1 isolated earlier in our laboratory. Among them five strains PD4, PD9, PD18, PD26, KMFA1 were identified as protease producers, two isolate PD18 and PD26 were recognized as tyrosinase producers. The results of primary screening were shown in Figure 1. Screening of

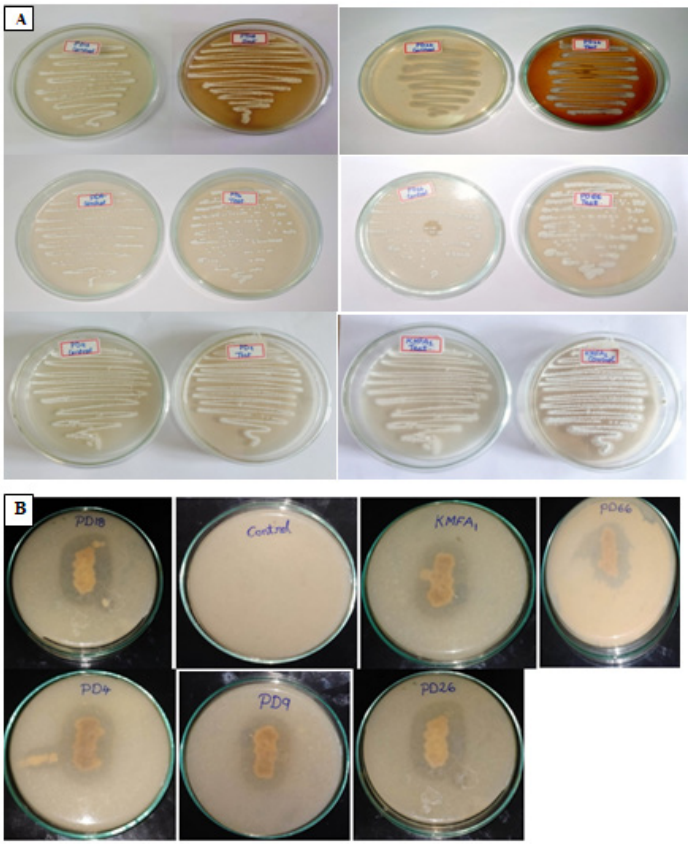

Figure 1: Preliminary screening A- melanin medium, B -skimmed milk agar medium.

suitable medium composition is a crucial step for the industrial production of metabolites so the selected isolates PD18 and PD26 were grow in different growth media such as tyrosine broth medium, tyrosine production medium, SS medium with copper and SS medium without copper. Among them SS medium with copper (medium 3) showed maximum growth and activity of enzyme. The results of the study were shown in Figure 2, similar results were also reported by Roy *et al*[16] and Bhaskara Rao *et al.*,[51] where extracellular tyrosinase was produced by *Streptomyces espinosus* strain LK4 and *Streptomyces aureofasciculus* respectively using SS medium with copper sulphate. Whereas, Subhash *et al*,[52] used peptone yeast extract iron broth for the production of tyrosinase where copper sulphate was omitted. By studying the impact of incubation period it was found tyrosinase production was started after 144 hr of incubation of culture broth of PD18 and PD26 respectively and reached maximum level after 168 hr and subsequently gradually decreased (Figure 3). The impact of copper on production medium was determined by supplementing different concentration of copper sulphate to production medium (medium 3) and it was found that PD18 and PD26 showed maximum monophenolase activity 21.20 IU/mL and 24.19 IU/mL respectively at a concentration of 1 mg/ mL of copper sulphate (Figure 4). Protein assay revealed that amount of protein concentration of PD18 and PD26 were found to be 264µg and 325µg after extrapolating the absorbance from standard graph of BSA (Figure 5). Our results revealed that SS medium with 1mg/mL copper was optimum for production of tyrosinase from cultures PD18 and P26 respectively whereas Roy *et al*[16] and Bhaskara *et al*[51] reported that addition of 10 mg/ mL of copper sulphate supplement to SS medium showed maximum tyrosinase yield from the cultures *Streptomyces espinosus* strain LK4 and *Streptomyces aureofasciculus* respectively. Study highlights the fact that new strains PD18 and PD26 required less supplement of copper sulphate for their growth to produce tyrosinase molecule. The morphology of aerial and substrate mycelium of promising isolates were shown in the Figure 6. The cultural characteristics of the strain are represented Table 1. The isolates PD18 and PD26 exhibited good growth on Tryptone yeast extract agar (ISP-1), YMD agar (ISP-2), Glycerol asparagine agar (ISP-5), Inorganic

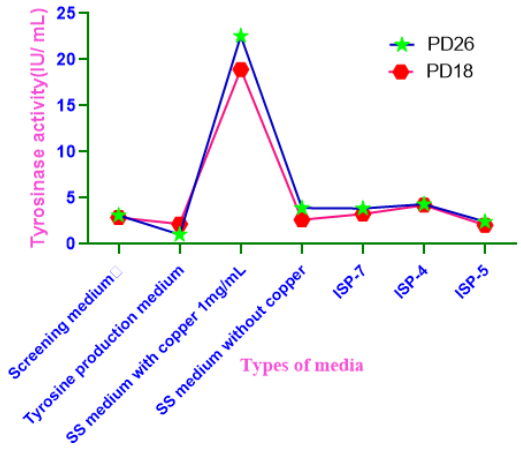

Figure 2: Effect of various media on tyrosinase activity.

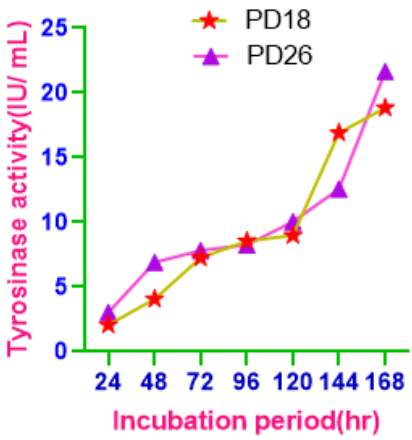

Figure 3: Effect of incubation period on tyrosinase activity.

Table 1: Cultural characteristics of the isolates PD18 and PD26.

Name of medium	Growth (PD26)	AM* (PD26)	SM** (PD26)	Pigmentation (PD26)	Growth (PD18)	AM* (PD18)	SM** (PD18)	Pigmentation (PD18)
Tryptone yeast -extract agar (ISP-1)	Good	White	Brown	Yes	Good	White	Brown	Yes
Yeast extract malt extract (ISP-2)	Good	White	White (Pale brown)	Yes	Good	Brown	Brown	Yes
Potato dextrose agar (ISP-3)	Poor	White	White	No	Poor	White	White	No
Inorganic salt starch agar (ISP-4)	Good	White	White	No	Poor	White	White	No
Glycerol Asparagine agar (ISP-5)	Good	White	Yellowish-brown	Yes	Good	White	Brown	Yes
Tyrosine agar (ISP-7)	Good	Brownish	Brown	Yes	Good	White	Brown	Yes
SAB	Moderate	White	White	No	Moderate	-	-	-
Nutrient agar	Good	White	White	No	Good	White	Pale Brown	No
Starch casein salts agar	Good	White	Brownish	Yes	Good	White	Brown	Yes (Light Brown)
Glucose Tryptone agar	Good	White	Yellowish-brown	No	Good	White	Yellow	No
Soluble Starch medium	Good	Grayish-brown	Brown	Yes	Good	Grayish-white	Pale Brown	Yes
Screening medium (melanin medium)	Good	Grayish-brown	Dark brown	Yes	Good	Grayish	Brown	yes

AM*-Aerial mycelium, SM**-Substrate mycelium and SAB-Sabouraud's dextrose agar medium.

salt starch agar (ISP-4) Tyrosine agar (ISP-7), Starch casein salts agar, Soluble Starch medium, Nutrient agar, Glucose tryptone agar and screening media. The growths were moderate on SAB medium. The poor growth or no growth was observed on Potato dextrose agar (ISP-3) medium. The colour of aerial mycelium was white and substrate mycelium were different on the different media tested. Soluble pigment production was observed on the Tryptone yeast - extract agar (ISP-1), Yeast extract malt extract (ISP-2), Glycerol Asparagine agar (ISP-5), Tyrosine agar (ISP-7), Starch casein salts agar, Soluble Starch and screening media tested. Isolate PD26 (*Streptomyces cellulosae)* also have capable to produce L- asparaginase, β- galactosidase, uricase, urease. Isolate PD18 (*Streptomyces cellulosae)* also have capable to produce L- asparaginase, β- galactosidase, urease but in less quantity. Actinomycetes have a reputation for marked nutritional versatility which is supported by the results of our analysis. In present study, the isolates were able to utilize and grow on variety of carbon and nitrogen sources (Table 2). The results of present study are correlated to previous results of carbohydrate utilization studies reported by Pridham and Gottlieb (1948) for the species differentiation of actinomycetes, hence the selected actinomycete strains were classified under the genera of *Streptomyces.* The 16S rRNA gene sequences of 1493 and 1495 nucleotides were generated for isolates PD18 and PD26 respectively after

Table 2: Physiological characteristics of the isolates PD18 and PD26.

Physiological Characterization		
Production of melanin pigment	+++	+++
Range of temperature for growth	25-40°C	25-40°C
Optimum temperature for growth	30°C	30°C
pH 5	+	+
pH 6	++	++
pH 7	+++	+++
pH 8	+++	+++
pH 9	++	++
pH 10	+	+
Optimum pH for growth Gram reaction	7	7
Utilization of Carbon Source		
Source Type	Isolates PD18	Isolates PD26
Starch	+++	+++
Glycerol	++	++
CMC	+	+
Lactose	++	++
Glucose	+++	+++
Arabinose	+	+
Mannitol	+	++
Sucrose	+	+
Utilization of Nitrogen Source		
Source Type	Isolates PD18	Isolates PD26
Casein	+++	+++
Gelatin	+	+
Tryptone	++	++
Meat extract	+	+
Beef extract	++	++
Malt extract	+	+
Yeast extract	+++	+++
Peptone	++	++

+++: Strong activity, ++: Moderate activity, +: Fair

removal of primer sequence. The BLAST similarity search and Phylogenetic analysis of the 16S rRNAgene revealed that the isolate PD18 shared 99.98% sequence similarity with the strain *Streptomyces cellulosae,* the isolate PD26 shared 100% sequence similarity with the strain *Streptomyces cellulosae.* The phylogenic

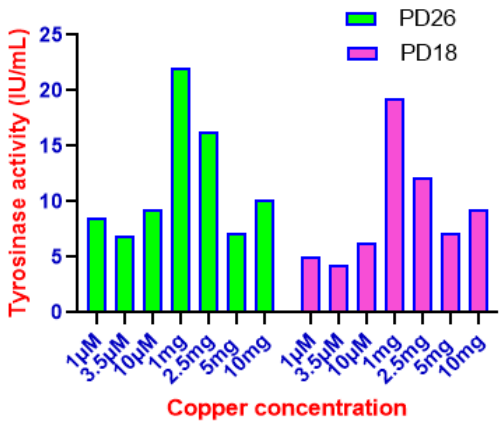

Figure 4: Effect of copper concentarion tyrosinase activity.

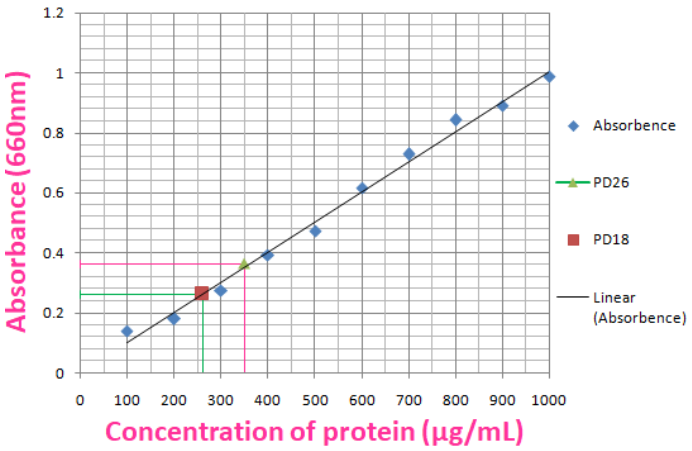

Figure 5: Standard graph of protein bovine serum albumin (BSA).

tree also suggested that the isolates were clustered with the other well characterized *Streptomyces* sp. In terms of sequence similarity in the range of 99.1% to 99.5%. The nucleotide sequences (16S rRNA) of both isolates PD18 and PD26 were submitted to Gene bank (NCBI) with Accession numbers OK652588.1 and OK655680.1. Consequently, the isolates PD18 and PD26 were designated as new variants of *Streptomyces cellulosae* strain PD18 and *Streptomyces cellulosae* strain PD26 respectively. According to earlier studies, the *Streptomyces cellulosae* strain was exclusively used to make antibiotics, biosurfactants and enzymes like lipase, amylase, cellulase, chitinase, and protease up until this point. This study highlights the contribution of discovering novel strains of *Streptomyces cellulosae* which were first used to produce tyrosinase as well as promising sources for potentially bioactive molecules such as antibiotics, antifungal metabolites, biosurfactants, and enzymes such as amylase, lipase, cellulase, uricase, urease, beta galactosidase and L- asparaginase (Figure 7, Table 3).

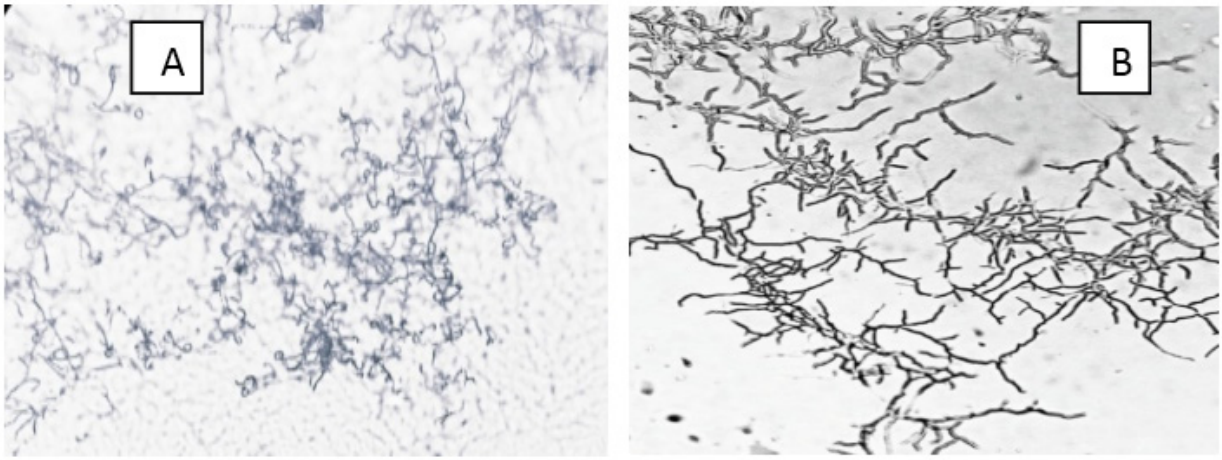

Figure 6: Aerial and substrate morphology of isolates (400X). A-Isolate PD18 and B- Isolate PD26.

Table 3: Morphological and biochemical characteristics of the isolates PD18 and PD26.

Character	Isolate PD 18	Isolate PD 26
Morphological characterization		
Sporophore morphology	Rectiflexible and short branched	Rectinaculiperti and long branched
Color of aerial mycelium	Greyish-white	Greyish-Brown
Color of substrate mycelium	Pale yellow to brown	Brown
Biochemical Characterization		
Amylase activity	+	+
Gelatin liquefaction	+	+
Lipase activity	+	+
Cellulase activity	+	+
Protease activity	+	+
Nitrate reduction	+	+
β-galactosidase activity	+	+
Carbohydrate fermentation	+	+
Indole test	+	+
Methyl red test	+	+
Voges Prosker test	-	-
Citrate utilization	+	+
Uriase activity	+	+
Uricase activity	-	+
Bio surfactant	+	+
Antibiotics	+	+
Antifungal metabolite	+	+

+: Positive and -: Negative

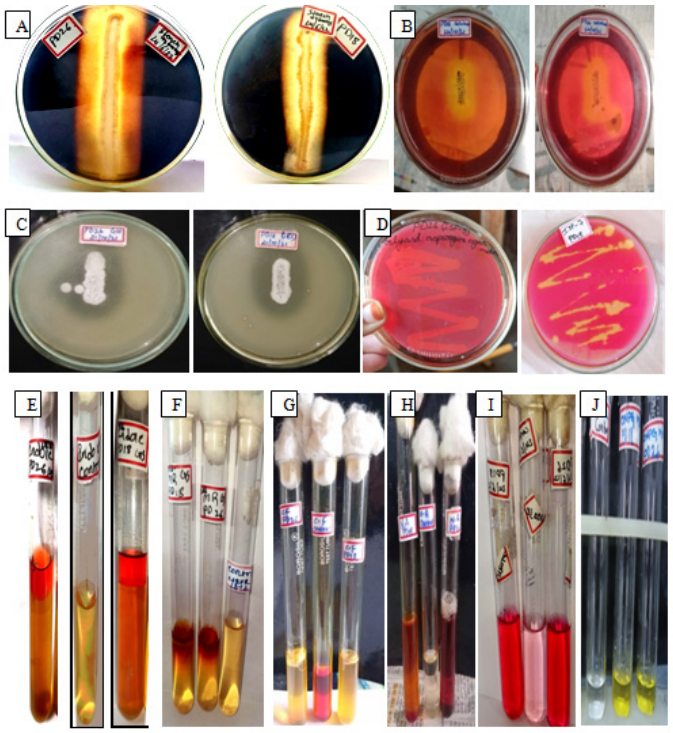

Figure 7: Biochemical characterization of isolates.
A- Amylase activity, B- Gelatinase activity, C- Cellulase activity, D- L-aspariginase activity, E- Nitrate reductase activity, F-Indole test, G- Methy red test, H- Carbohydrate fermentation test, I-uriase activity and J- β – galactosidase activity.

CONCLUSION

An attempt was made to isolate a tyrosinase producing actinomycetes from pre-existed cultures of our laboratory. Among six cultures, two strains PD18 and PD26 were found to be a novel source for tyrosinase production. Further, secondary screening revealed that SS medium was best for growth of isolates and tyrosinase production and maximum yield was noticed after 7 days (168 hr) of incubation. It was also found that presence of copper ion showed positive influence on the yield of tyrosinase

when compared to previous reports very less concentration (1mg/mL) is enough for the maximizing tyrosinase yield were found to be 21.20 IU/mL and 24.19 IU/mL by the cultures PD18 and PD26 respectively. The protein concentration of PD18 and PD26 were found to be 264µg and 325µg after extrapolating the absorbance from standard graph of BSA. As per previous research, the *Streptomyces cellulosae* strain was exclusively used to produce antibiotics, biosurfactants and enzymes like lipase, amylase, cellulase, chitinase, and protease up until this point. This work highlights the importance of discovering novel variants of *Streptomyces cellulosae* which were initially employed to produce tyrosinase, in addition to attractive sources for potentially bioactive compounds like antibiotics, antifungal metabolites, biosurfactants and enzymes like amylase, lipase, cellulase, uricase, urease, beta galactosidase and L- asparaginase. The BLAST similarity search and Phylogenetic analysis of the 16S rRNAgene revealed that the isolate PD18 shared 99.98% sequence similarity with the strain *Streptomyces cellulosae,* the isolate PD26 shared 100% sequence similarity with the strain *Streptomyces cellulosae.* Consequently, the isolates PD18 and PD26 were designated as new variants of *Streptomyces cellulosae* strain PD18 and *Streptomyces cellulosae* strain PD26 respectively.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool; **cm:** Centimeter; **mg:** Milligram; **µL:** Microliter; **µM:** Micromolar; **M:** Molar; **mL:** Milliliter; **rpm:** Revolution per minute; **hr:** Hours; **°C:** Degree Centigrade; **nm:** Nanometer; **IU:** International unit; **%:** Percentage; **NCIM:** National Collection of Industrial Microorganisms; **NCBI:** National Center for Biotechnological Information; **rRNA:** Ribosomal Ribonuclic acid.

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