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Isolation and Characterization of Alkaline Protease Producing Streptomyces tendae SO-13 from Rhizosphere Soils of Western Ghats

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ABSTRACT

Microbial alkaline proteases are among the salient hydrolytic enzyme and used extensively as biocatalysts, Extracellular alkaline protease is of great importance due to its applications in detergent, food and leather industries by the prokaryotic actinomycetes. The present investigations were focusing on soil actinomycetes. Screening for alkaline protease and characterization of specific strain from rhizosphere soil from Western Ghats of Karnataka, India. The zones of hydrolysis were observed on skim milk agar medium under pH 10.0 at 30±02 0C for 5 to 7 days and produced 117.67 U/mL of alkaline protease by the fermentation. Morphological and biochemical characterization of the isolate was carried out and found that the isolate belongs to the *Streptomyces* genus. Further species confirmation of the *Streptomyces* was done by 16S rRNA gene sequencing. The obtained nucleotide sequences SO-13 were submitted to the GenBank database and the accession number assigned is MW130237. The results reveal the isolate SO-13 was identified as *Streptomyces tendae*. The present data reveal that the isolate SO-13 represents *Streptomyces tendae*. The potentiality of the eco-friendly enzyme could be used for various industrial applications. The alkaline protease production from *Streptomyces tendae* SO-13 is the potent strain for commercial use. Further investigation at the commercial level and novel applications of alkaline proteases to be carried out.

KEY WORDS: WESTERN GHATS, RHIZOSPHERE SOIL, STREPTOMYCES TENDAE, ALKALINE PROTEASE.

INTRODUCTION

The Western Ghats of India has been considered as one of thirty-four biodiversity hot spots in the world, with rich flora and fauna. The Western Ghats of Karnataka popularly known as Sahyadri hills are treasure houses of endangered species but less studied concerning microbial biodiversity. Actinomycetes are widely distributed in soil, and constitute a significant part of soil microflora (Bawazir and Manjula 2018). Soil actinomycetes are prokaryotes with various metabolic activities (Chavan et al., 2013). Actinomycetes are gram-positive, filamentous, bacteria, characterized by the formation of aerial mycelium, and spores on solid media, with DNA high in G+C content of 60-70 mol% (Shirling and Gottlieb 1966; Subbaraju and Onkarappa 2018).

Among the microorganisms, actinomycetes acquire special importance as the power source of antibiotics and, other bioactive primary and secondary metabolites such as enzymes (Chavan et al., 2013). Sources of commercial enzymes cover a wide range from microorganisms then animals and plants sources. Fungi and yeast contribute about 50%, bacteria 25%, animal 8% and plant 4% of the total in commercial enzymes production (Azmi et al., 1999). Proteases of neutrophilic as well as alkaliphilic

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bacterial and fungal origins are utilized for commercial exploitation (Pathak and Rothad 2018). Several microbial species are reported for the alkaline protease productions. The most copiously studied the alkaline protease producers among bacteria are the genera of *Bacillus* (Keshavamurthy et al., 2018) and *Pseudomonas* (Alexander et al., 2012), Streptomyces species (Sarkar and Suthindhiran 2020).

Alkaline proteases are also utilized for therapeutic agent's development. The oral administration of proteases from *Aspergillus oryzae* to aid in digestion and rectify lytic enzyme deficiency syndromes is already in practice (Mikawlrang, 2016). More recently, actinomycetes are an auspicious source of a wide range of important enzymes. *Streptomyces* protease preparations that are profit-oriented used include FRADIASE 7M *(S. fradiae*) and PRONASE 7M (S. griseus). While alkaline proteases from bacteria are vastly characterized, similar attention has not been paid to actinobacteria. Currently, antibiotics are the major bioactive compounds from the actinobacteria. However, in these prokaryotes, the ability to produce a variety of enzymes may be an attractive phenomenon (Govindharaj et al., 2016). In the present investigation, the new isolate SO-13 capable of producing the alkaline protease. The study includes morphological, biochemical and molecular identifications.

MATERIAL AND METHODS

Isolation Proteolytic actinomycetes from soil samples: The rhizosphere soil samples were collected in a sterile Ziplock plastic cover from a depth of 10-15 cm from the Western Ghats in Karnataka, India (Around the latitude 140 13l 07.3ll N and longitude 740 49l 57.2ll E). The samples were air-dried for 6 to 7 days and ground in a mortar using a pestle (Lingakumar et al., 2014; Subbaraju and Onkarappa, 2018). The samples were serially diluted and 0.1 ml of 10^{-2} to 10^{-6} dilutions were plating on Starch Casein agar medium (g/ml: Starch 10; Casein 3; KNO_3 2; NaCl 2; K_2HPO_4 2; $MgSO_4$ 0.05; $CaCl_2$ 0.02; $FeSO_4.7H_2O$, 0.01; agar 20, distilled water 1000ml pH=7.0± 0.2) described by Guravaiah (2016). The plates were incubated at 30±20 ^{0}C for 5 to 7 days. The isolates were grown on starch casein nitrate agar medium at 30±20 ^{0}C and stored at 4^{0}C for short term storage.

Screening for alkaline proteolytic activity: Proteolytic activity from isolated pure cultures was screened by plating on Skim milk agar Supplement with Na_2CO_3 for detection alkaline protease producing isolate (g/L Skim milk, 100.0; Yeast exact, 5.0: Na_2CO_3, 10.0; Agar, 20; distilled water, Final pH=10). Incubate for 5 to 7 days at 30±02 ^{0}C (Davoudi et al., 2014).

Characterization of potent alkaline producing isolate SO-13: The classifications of actinomycetes were originally based upon the morphological observations. Preferably, the *Streptomyces* species were identified and recorded using the Bergey's Manual of Systematic Bacteriology. Primarily, the characterizations of actinomycetes isolates were done by their colony morphology, spore colour, aerial mass colour and substrate mycelium, pigmentation appearance on the medium. The isolate was subjected to grams and acid-fast staining procedures. The isolate was identified to genus level based on their spore chain arrangement by coverslip technique and spore surface ornamentation by SEM analysis (Gautham et al., 2012).

Biochemical tests of isolate: Standard biochemical tests were employed, the Indole test, Methyl red test, Voges – Proskauer test, Citrate utilization test, Urease test, Catalase test, Degradation of Cellulose, hydrolysis of Casein, Gelatin and Starch to determine the potent alkaline protease producing Strain (Shirling and Gottlieb 1966; Gautham et al., 2012; Subbaraju and Onkarappa 2018).

16S rRNA gene sequencing of the isolate: The strain was further subjected for identification by 16S rRNA sequence using universal primers and genomic DNA as a template. The genomic DNA extracted from the isolate by using spin column kit (HiMedia, India). Bacterial 16S rRNA gene (1500 bp), (Clarridge JE, 2004) was amplified using polymerase chain reaction (PCR) in a thermal cycler and were purified using Exonuclease I -Shrimp Alkaline Phosphatase (Exo-SAP) (Darby et al., 2005). Purified amplicons were sequenced by Sanger method in ABI 3500xL genetic analyzer (Life Technologies, USA). Sequencing files (.ab1) edited using CHROMASLITE (version 1.5) and further analyzed by Basic Local Alignment Search Tool (BLAST) with closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences (Altschul et al., 1990). A duly annotated partial nucleotide sequence of the strain was deposited with NCBI Genbank (https://www.ncbi.nlm.nih.gov). Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software to construct the Phylogenetic tree using the neighbour-joining method (Tamura et al., 2011).

Alkaline protease production and enzyme assay: Alkaline Protease production by the selected Strain SO-06 was carried out by One ml of fresh isolate inoculum were added 100 ml of production medium (Glucose,10.0g/L; casein, 5.0 g/L; yeast extract, 5.0 g/L; $K_2H\ PO_4$, 2.0 g/L; KH_2PO_4, 2.0 g/L; $MgSO_4$. 7H2O, 1.0 g/L and at pH 9.0-9.5) into 250ml Erlenmeyer flask. The flask was placed in a rotary shaker incubator at 150 rpm at 30 °C for 5 to 7 days. The fermentation broth was centrifuged at 8,000rpm for 20 min at 4 ^{0}C to obtain the crude culture filtrate (Hosseini et al., 2016). The alkaline protease activity of the crude enzyme was done by taking 0.5 ml of culture filtrate was added to 0.5 ml of 1% casein (a substrate) in 0.1 M Phosphate buffer (pH 7.0) and then incubated for 10 min at room temperature.

To stop reaction 3ml of 10% (w/v) trichloroacetic acid and the mixture was centrifuged at 5000 rpm for 10 min. The supernatant, 5 ml 0.5M $Na2CO_3$ solution and 0.5ml of two Folin Ciocalteau reagent was added and mixed thoroughly, incubated for 30 min at room temperature in

dark condition. The optical density of blue colouration was measured using the UV-VIS spectrophotometer at 660 nm and the blank (Keshavamurthy et al., 2018). The amount of the released amino acids was calculated using the tyrosine standard. One unit of enzyme activity represents the amount of the enzyme required to release 1µg of tyrosine per ml per min (Pant et al., 2015).

RESULTS AND DISCUSSION

Fifteen rhizosphere soil samples were collected in sterile Zip lock plastic cover from at different Latitude and longitude of the Western Ghats in Karnataka, India (Figure 1) and air-dried soil samples were subject to isolation and based on the zone of hydrolysis on agar plates, SO-01 to SO-16 visible colonies were obtained on 10^{-2} to 10^{-6} dilution Petri plates (Figure 2).

Figure 1: The map showing sampling stop of Western Ghats, Karnataka.

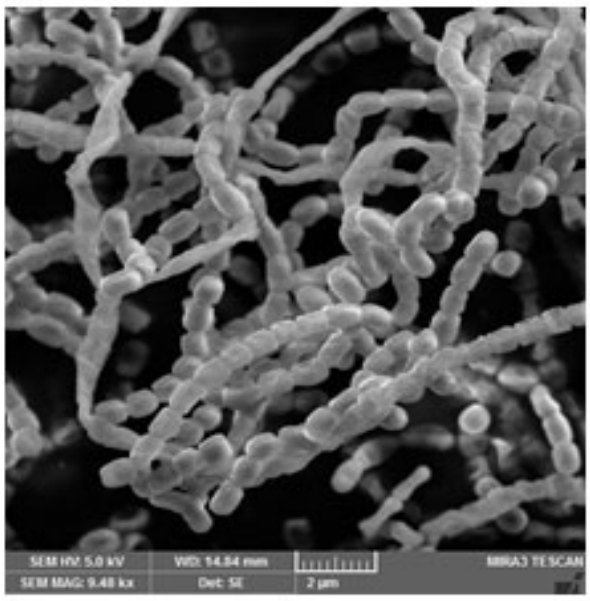
Figure 2: Visible colonies on 10^{-4} dilution petriplate

Isolate SO-13 was found to have alkaline protease activity depend on diameter zone of hydrolysis of proteolytic on the plate (Figure 3). Similar result found in *Streptomyces griseorubens* E44G (Rashadb et al., 2015) and *Streptomyces Indus* (Guravaiah 2016) for proteolytic activity. Hence the isolate SO-13 was selected for further studied.

Characterization of the potent alkaline protease producing isolate SO-13: The result showed a diverse morphological characteristic with spore colour-grey, colony morphology-radial, substrate-white and aerial mycelium-grey (Figure 3). The spore-bearing hyphae and spore chains show straight-flexuous with open and spore surface Ornamentation-smooth (Figure 4). Isolate shown the gram-positive, filamentous, rod structure, identified by gram staining and Nonacid fast. The biochemical characteristics as potent alkaline protease isolate SO-13 exhibit positive for methyl red, voges-proskauer, citrate, urease, catalase, hydrolysis of casein, starch and gelatin (Table 1). Based on the spore chain arrangements the isolate was assigned to the genus *Streptomyces* sp. Similar results were observed in *Streptomyces* sp. (Takeuchi et al., 1996) and *Streptomyces tendae* AR1 (Laidi et al.,2006).

Figure 3: Zone of hydrolysis by the isolate *Streptomyces tendae* SO-13

Figure 4: SEM of the isolate *Streptomyces tendae* SO-13



The 16S rRNA gene was amplified through PCR which showed 1500 kb band on 2% agarose gel (Figure 5). Subsequently, 16S rRNA gene sequence analyses were carried out to elucidate the taxonomic relationships among closely related *Streptomyces* species. The strain

SO-13 has been matriculated into a cluster containing *Strepomyces tendae*. Based on the phylogenetic analysis the strain was closely related to *Streptomyces tendae* strain ATCC 19812 exhibiting high similarity (99.88 %) (Takeuchi et al., 1996). So it is assigned as *Streptonyces tendae* SO-13 (Figure 6). Similar results were noticed in *Streptonyces tendae* AR1 (Laidi et al., 2006) and *Streptonyces tendae* 1 & 2 (Bahamdain et al., 2020). The obtained nucleotide sequences SO-13 were submitted to the GenBank database and the accession number assigned is MW130237. The results reveal the isolate SO-13 was identified as *Streptomyces tendae*.

Table 1. Morphological and Biochemical Characterization of isolate *Streptomyces tendae* SO-13

Morphological identification	
Media	Starch Casein Nitrate
Growth	Abundant
Colony Morphology	Radiating
Aerial Mycelium	Grey
Substrate Mycelium	White
Diffusible Pigment	No
Spore Arrangement	Straight-flexuous
Spore surface Ornamentation	Smooth
Biochemical tests	
Indole	-
Methyl Red	+
Voges – Proskauer	+
Citrate	+
Urease	+
Catalase	+
Casein	+++
Starch	++
Cellulose	-
Gelatin	+ +
pH	7.0-10.0
NaCl	5%
Tentative genera	*Streptomyces* sp.

Streptomyces tendae SO-13 was subjected to secondary screening for quantitative protease production. The protease produces 117.67 U/mL during 5 to 7days at pH 9.0. Similar results were observed in *Saccharomonospora viridis* SJ-21 (Hosseini et al., 2016) and *Streptomyces flavogriseus* HS1 (Sofiane et al., 2014). *Streptomyces* were industrially important organisms for commercial production of Protease. Alkaline proteases have been produced from numerous *Streptomyces* like thermoalkaline proteases by marine *Streptomyces* sp. D1 (Madanrao et al., 2013). *Streptomyces*. sp LL-DAP (Karthik et al., 2010) and *Streptomyces flavogriseus* HS1 (Sofiane et al., 2014) are also reported for protease production.

Figure 5: Agarose gel electrophoresis of *Streptomyces tendae* SO-13
L-Step-up 1 Kb DNA Ladder
Lane 1- 16S rRNA amplicon

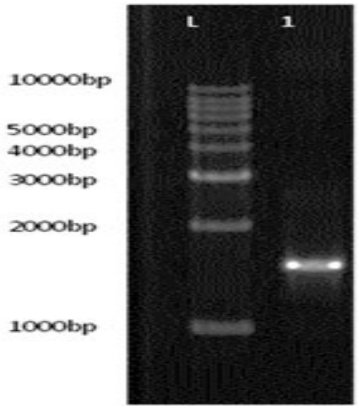
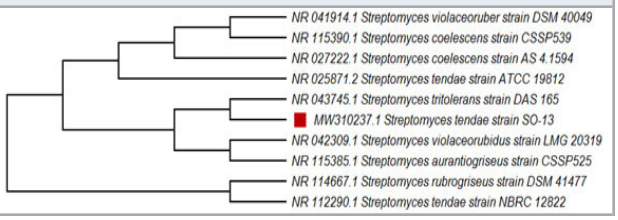


Figure 6: Phylogram obtained based on phylogenetic analysis of 16S rRNA gene sequence data showing the phylogenetic positions of isolate *Streptomyces tendae* SO-13



CONCLUSION

The Western Ghats are the treasure house for novel actinomycetes species associated with the rhizosphere soil. The characterizations of alkaline protease *Streptomyces tendae* SO-13 was confirmed by morphology, biochemical and 16S rRNA sequencing. The alkaline protease producing from *Streptomyces tendae* SO-13 is the potent strain for commercial production. Further investigation at the commercial level and novel applications of alkaline proteases to be carried out.

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