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Screening and Identification of Keratinase Producing Marine Actinobacteria from Chennai Sea Coast.

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ABSTRACT

Actinobacteria are virtually unlimited sources of novel compounds with many industrial and therapeutic applications. The present study was focussed on the isolation and identification of marine Actinobacteria producing keratinase enzyme from marine sediments.A total of four marine actinobacterial cultures were isolated on Actinomycetes Isolation Agar (AIA) and Starch Casein Agar (SCA) which were designated as SDS1, SDS2, SDS3 and SDS4. The Actinobacterial isolatesobtained were sub cultured on ISP- 7 media.Primary screening of the isolates for keratinase enzyme activity was done on Skim Milk Agar. Based on the clearing of the media, colonies weresubjected to secondary screening on modified basal medium which was supplemented with raw chicken feathers. Amongst the four isolates, only SDS3 showed maximum percentage of keratinase enzyme production that resulted in the loss of 78.16% of the feathers' dry weight. The potential strain SDS3 was identified as belonging tothe genus Streptomyces sp.by microscopic and biochemical tests.

Keywords: Marine Actinobacteria, Enzyme production, SDS3, Chicken feathers, Keratinase activity, Streptomyces sp.

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INTRODUTION

Keratin is an insoluble protein which cannot be degraded by peptidases class of enzymes, such as trypsin, pepsin and papain [1, 2]. It is the key structural material making up the outer layer of human skin, hair, nails and horns. The monomers of Keratin assemble into bundles to form intermediate filaments, which are tough and form strong mineralized tissues found in birds, amphibians, reptiles, and mammals. Keratin is a polymer of type I and type II intermediate filaments found in Chordates whereas type V intermediate filament are found in Non-chordates. The keratin consist of α and β keratin. α-keratin is found in hairs, nails and horns of mammals and the β-keratin is found in feathers, claws and beaks [3]. The β-keratin contains β-pleated sheets present in the epidermal layer of the skin making it waterproof and preventing from desiccation. In addition to intra and intermolecular H-bonds, keratin has large amounts of sulfur containing amino acids like Cysteine which is required for the disulfide bridge and adds permanent strength and rigidity [4]. The only other biological matter known to approximate the toughness of keratinized tissue is chitin. Wastes rich in keratin are feathers, hair, nails, and horn which are the by-products of agricultural and industrial processing [5-7].

Keratinase is an extracellular enzyme used for the degradation of keratin[8]. The tight packing of the production chains is an important factor for the mechanical stability of keratin and its resistance to biochemical degradation [9]. In α – Helix (α-keratin), or β – Sheet (β – keratin) structures, there are disulfide linkages which causes folding into initial 3- dimensional form [10]. The enzyme hence attacks the disulfide bond of keratin to degrade it. Keratinase enzymes are produced by many fungi, actinobacteria and bacteria which have been frequently isolated from soils where keratinous materials are deposited. Besides hydrolysis of prion proteins is an upcoming useful novel application of the enzyme. Recently, actinobacterial strains were isolated from Antartica[11]. It was found that keratinase in fungi, *Streptomyces* and bacteria were produced in nearly at alkaline pH and almost thermophilic temperatures. These enzymes have wide range of substrate specificity such as it can degrade other fibrous protein fibrin, elastin, collagen and other non-fibrous protein like casein, bovine serum albumin gelatine etc. These strains were able to grow on wastes containing keratin [12].

Actinobacteria are non-motile, aerobic and Gram-positive bacteria with high guanosine–cytosine (G+C) content in their DNA. They are phylogenetically related to the bacteria based on 16S ribosomal RNA sequencing studies [13]. Marine Actinobacteria are potential sources of bioactive compounds and the richest sources of secondary metabolites. Due to their magnificent diversity and their proven ability to produce bioactive compounds and antitumor agents in addition to enzymes of industrial interest, marine actinobacteria hold a prominent position as target in screening programs [14, 3]. Hence, marine Actinobacteria have characteristics different from those of their terrestrial counterparts since the marine environmental conditions are extremely different from that of the terrestrial environment. Therefore, the chances of producing different types of bioactive compounds are higher in marine actinobacterial species than when compared to their terrestrial counterparts[15]. Marine actinobacteria are exploited for the discovery of novel secondary metabolites. They are well known to produce enzymes with good stability at higher temperature and alkaline conditions. There is a greater demand for new bioactive compounds like enzymes including keratinase, which are synthesized by actinobacteria from various marine sources.

In the current study we focused on the isolation and identification of keratinase enzyme from feather degrading marine actinobacteria to assess the keratinase production. The aim of this study was to detect organisms with the best ability to degrade feathers by means of keratinolytic activity.

MATERIALS AND METHODOLOGY

Chemicals

All the chemicals and media used in the study were purchased from Sisco Research Laboratories (SRL) Private Limited, Mumbai, India and Hi Media Laboratories, Mumbai, India.

Sample collection

The marine sedimentsample wascollected from Chennai sea coast, Tamil Nadu, India [13.0542° N, 80.2837° E].The sample was transferred aseptically in sterile bags and was brought to Molecular and Microbiology Research Laboratory, VIT University,Vellore, Tamil Nadu, India for further processing.

Isolation of Marine Actinobacteria

Isolation of marine actinobacteria was performed by serial dilution and plating method of the marine sediments on Actinomycetes Isolation Agar (AIA) and Starch Casein agar (SCA). AIAand SCA media were prepared in 50% marine water and 50% sterile distilled water. 10g of marine sediment sample was suspended in 100 ml of sterilized distilled water and mixed properly. Serial dilution was made up to 10^{-6}. Aliquots from 10^{-2} to 10^{-6} were taken for plating. Serially diluted samples were inoculated in the respective media in duplicates.To minimize fungal and bacterial contamination, all agar plates are supplemented with nalidixic acid and potassium dichromate respectively. Plates were incubated in room temperature for 7-28 days. The visible morphologically distinct colonies were selectedand isolated on ISP-7 medium. The suspected actinobacterial isolates were maintained on ISP-7 slants at 4ºC for further studies. [16].

Actinobacterial extract production

The sub cultured actinobacterial isolates were inoculated in 100ml of the production broth (gl^{-1}) (Starch- 25g, Glucose- 100g, Yeast extract- 2g, $CaCl_2$- 3g and trace elements- 0.1 ml/100ml) for further screening procedures. The pH was adjusted to 7.5. The medium was incubated in a rotary shaker at a speed of 150 rpm in room temperature for 7-10 days. After incubation, the cells were removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant was collected. The supernatant was used to study the keratinolytic activities.

Primary Screening of keratinolytic actinobacteria

Primary screening for the keratinolytic marine actinobacteria was done on Skim Milk Agar using stabbing and the well diffusion method. The isolated actinobacteria was stab inoculated on to skim milk agar plates and incubated in growth chamber for 5-7 days. In well diffusion method, wells were made on to skim milk agar plates and the cell free supernatant obtained after centrifugation was transferred into the wells in the concentration of 50µl, 100µl, 150µl and 200µl. The plates were incubated at room temperature for 7 days to check for zone of hydrolysis. After the incubation period, the plates were observed for the crude extract activity. Based on the zone of clearance, actinobacterial isolates were chosen for secondary screening.[12]

Source of keratin

The chicken feathers were collected from a dumping site of the poultry waste in Vellore. The feathers were first extensively washed in running tap water followed by washing in double distilled water. The feathers were then dried in hot air oven at 60ºC.[17].

Feather degradation using potential actinobacterial strains

Each of the selected actinobacterial strain from the primary screening was inoculated in fresh production broth. Feather degradation by the selected actinobacterial strain was carried out in 250 ml Erlenmeyer flasks containing 50 ml of the Modified Basal Medium (g/l) (K_2HPO_4: 0.4g, $MgSO_4.7H_2O$: 0.05g,NaCl: 0.05g, $FeCl_3$: 0.01g, pH was maintained at 7.0) supplemented with 1g chicken feathers. 1ml of the actinobacterial extract was used as the inoculum in modified basal media. The flasks were incubated in room temperature for 6-7days to check the degradation capacity of the keratinolytic actinobacteria. After the incubation period, the residual feathers in the culture broth were harvested by filtration using Whatman number 1 filter paper. The feathers were washed extensively with double distilled water and then dried in hot air oven at 65ºC.Following this, the final weight of the degraded feathers was noted down.

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Determination of feather degradation

The percentage of feather degradation by the marine actinobacteria was determined by calculating the difference in residual feather dry weight between control (Feather without actinobacterial inoculation) and treated sample.[18] The percentage of weight loss was calculated using the following formula:

$$\text{Percentage of weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Identification of Marine Actinobacteria:

The potential isolate was characterized up to genus level by observing the aerial mass colour, melanoid pigment, reverse side pigment, soluble pigment and spore chain morphology. Morphological characterization was done using microscopic observation by cover slip culture method [19, 20]. The potential strain that was showing good keratinolytic activity was identified using Nonomura key [21, 22] and Bergey's manual of determinative bacteriology [9].

RESULTS AND DISCUSSION

Isolation and primary screening of keratinase enzyme producing marine actinobacteria:

A total of four Actinobacterial cultures designated as SDS1, SDS2, SDS3 and SDS4were isolated from marine sediments collected from the Chennai sea coast.The Actinobacterial isolates obtained were sub cultured on ISP- 7 media(Fig-1).

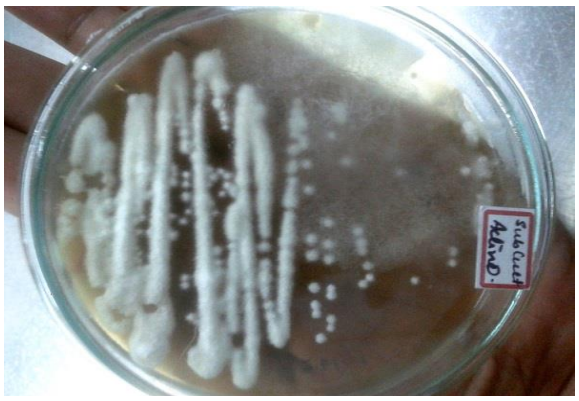

Figure 1: Actinobacterial sub cultured colony on ISP-7 media

All the isolates (SDS1 to SDS4) were then screened for Keratinase activity on Skim Milk Agar. The organisms producing zone of hydrolysis in Skim Milk Agar plates were considered as keratinolytic organisms. Among the four actinobacterial isolates only SDS3 isolate showed maximum zone of hydrolysiswhereas SDS2 showed the least zone of hydrolysis (Table 1).Based on the clearing of the media, SDS3 was chosen for further studies.In one study it was seen that eight bacteria were isolated from the soil samples collected from different feather processing areas in Vellore, TN, India, out of which only H5 isolate showed zone of hydrolysis of 26mm while other isolates didn't show any zone.

Table 1: Zone of hydrolysis in skim milk agar method.

ISOLATES	ZONE OF HYDROLYSIS (diameter measured in mm)			
	50µl	100µl	150µl	200µl
SDS1	1mm	3mm	4mm	5mm
SDS2	1mm	2mm	3mm	5mm
SDS3	6mm	17mm	19mm	22mm
SDS4	3mm	6mm	5mm	8mm

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Keratinase Assay

The degradation of raw chicken feathers by the keratinolytic isolateswere checked by secondary screening in Modified basal liquid media supplemented with processed raw chicken feathers and the actinobacterial extracts obtained after centrifugation. The set up was kept undisturbed for 7 days. After the period of incubation,the residual feathers were weighed after repeated washing and drying. The final weight was obtained as 0.218g. Applying the formula, the feather degradation was determined. Strain SDS3 showed maximum degradation of the chicken feathers i.e. 78.16% weight loss.

Identification of the potential strain

The potential keratinolytic action bacterial strain degrading the chicken feathers was identified as belonging to the genus *Streptomyces.* It has been reported thatmost of the actinobacteria producing potent antibiotics and enzymes belong to the genus *Streptomyces*[23].According to Kutzner (1972), for proper identification of genera and species of actinomycetes, besides morphological (Fig-2) and physiological properties, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be determined.The identification result of the potential keratinolytic marine actinobacteria SDS3 is given in Table-2 and Table-3.

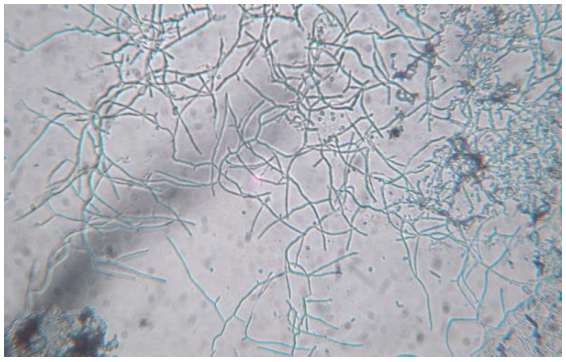

Figure 2: Spore chain morphology of genus *Streptomyces* observed

Table 2: Characterization of keratinolytic marine actinobacteria

STRAIN	SDS3
AERIAL MASS COLOUR	Creamy white
MELANOID PIGMENT	_
REVERSE SIDE PIGMENT	+
SOLUBLE PIGMENT	_
SPORE STAINING	Spore forming
SPORE CHAIN	Rectiflexible
MOTILITY	Non-motile

Result: - (Negative) and + (Positive)

Table 3: Carbon Utilization test.

CARBON SOURCES	SDS3
Arabinose	+
Xylose	+
Inositol	+
Mannitol	+
Fructose	+

CONCLUSION

The result of our current study indicates that the SDS3 strain which was isolated from the marine sediments is a potential keratinase enzyme producing marine actinobacteria. It showed the maximum keratin

degradation capacity compared to other strains used in the study. On characterization, SDS3 strain was proved to be belonging to the *Streptomyces sp*. Keratinase enzyme produced from SDS3can be used to address challenging issues of solid waste management, fuel scarcity and prion contamination in meat products by attacking keratin residues. Besides, they can be instrumental in refining the already existing leather biotechnology and in upgrading feed, detergents and pharmaceuticals.

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