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Keratinase activity (Feather) of *Streptomyces cacaoi* subsp. *cacaoi*-M20

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Abstract
The actinomycete isolate M20 was isolated by dry heat (70 ºC) pre-treatment method on Starch casein agar media, from the soil sample that was collected nearer to the root region of the mangrove *Avicennia marina* from the back water area, Ariyankuppam, Puducherry (UT). It was identified as *Streptomyces cacaoi* subsp*. cacaoi* M20 by the 16S RNA sequencing technique. The isolate was keratinase and protease positive. The isolate utilized the small whole chicken feathers within 10-15 days. It was witnessed that the initial pH of the medium was 7.5, but the pH of the medium was raised from the day when the isolate started to utilize the keratin. Finally the pH of medium was measured as 8.8, more alkali, due to conversion of complex keratin into ammonia.

Keywords: Actinomycetes, Mangroves, Keratinase, Protease, 16S RNA sequencing, *Streptomyces cacaoi* subsp *cacaoi*. M20.

1. Introduction

One of the major problems related to environment in our country is dumping of chicken feathers. The dumped feathers in the soil are not degraded by the soil bacteria fastly, so that they remain in the soil for long time and cause environmental pollution. Keratinases are the group of serine hydrolases that are capable of degrading keratin, a fibrous and insoluble structural protein extensively cross-linked with disulfide, hydrogen and hydrophobic bonds (Anbu *et al*., 2006.,) Keratins, which are among the hardest-to-degrade animal proteins, are the major component proteins in poultry feathers and are characterized by a tightly packed form in α- helixes and β sheets with a high degree of disulfide bonds (Yasushi shigeri *et al*., 2009) [11]. Keratinase is an extra cellular enzyme used for the bio degradation of keratin. Keratinase is produced only in the presence of keratin substrate. Keratinase attacks the disulfide bond of keratin to degrade it. Some microbes have been reported to produce keratinase in the presence of keratin substrate. Keratinase producing microorganisms have ability to degrade chicken feathers, hairs, nails, wool etc. (Gradišar *et al.* 2005, Cai *et al.* 2008) [3, 2]. Mostly protease positive actinomycetes are useful for this study. Microbial alkaline proteases for manufacturing uses are produced mostly from streptomyces and bacillus. Actinomycetes particularly streptomycetes are known to secrete multiple proteases in culture medium (Sharmin 2005) [8]. The promising applications of keratinolytic proteases include enzymatic dehairing of leather, detergent industry and development of biodegradable films (Jin-Ha Jeong *et al*., 2010) [5]. In order to activate the degradation and decomposition process of chicken feathers in the environment fast, this research has been initiated. Actinomycetes are group of organisms; share the characteristics of both fungi and bacteria, responsible for degradation of the chicken feathers in soil fast and better.

2. Materials and methods

Collection of soil sample

Soil sample near root region of the mangrove plant*, Avicennia marina (Forsk). Vierh – (Avicenniaceae)* in Ariyankuppam back water estuary, Puducherry (Lat 11º46´03'' to 11º53´40'' North and Longi 79º49´45" to 79º48´00" East) was collected, packed in sterile plastic containers and transported immediately to the laboratory. The pH of the fresh soil sample was determined (Reed and cummmings, 1945) [7]. Then the soil sample was air dried for 7-10 days at 40 ºC, Crushed and sieved to remove the shells and debris and stored.

Soil analysis

Physio-chemical nature of soil sample was analysed in soil testing laboratory, Department of Agriculture, Puducherry, India.

Isolation of mangrove actinomycetes

The soil sample was subjected to dryheat (70 ºC for 15 min) (Hayakawa *et al*., 1991) [4] pretreatment to enhance the chances of isolating rare and novel actinomycetes. After pretreatment, one gram soil was mixed and serially diluted in sterile water blanks. 0.1 ml of last two dilutions ($10^{-5}$and 10^{-6}) was inoculated by pour plate method (Zheng *et al*., 2000) using Starch casein agar (Kuster and Williams, 1964) [6] supplemented with Fluconazole 80µg/ml and Nalidixic acid 75µg/ml. Plates were incubated at 30 ± ºC for up to 30 days. Plates were periodically examined for actinomycetes colonies. Selected colonies were transferred to Yeast Malt extract agar slants and maintained in the same medium.

Protease

The 100 ml potato dextrose agar with 4 g of caesin was dispensed into required number of petriplates and allowed to solidify. A loop of inoculum of the isolate M20 was streaked in the centre of the petriplates, incubated at room temperature for 4-5 days. A clear zone around the colony indicates protease activity (Smibert and Krieg, 1994) [9].

Molecular characterization

Template DNA was prepared by using standard procedure. 1 µl of template DNA was added 20 µl of PCR reaction solution. By using 2 universal primers: 518F/ 800R, performed 35 amplification cycles at 94 ºC for 45 sec, 55 ºC for 60 sec, and 72 ºC for 60 sec. The purified PCR products of approximately 1,400 bp were sequenced by using 2 universal

primers: 518F 5'CCAGCAGCCGCGGTAATACG 3', 800R 5' TACCAGGGTATCTAATCC 3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The isolate M20 was identified and phylogenetic tree was constructed.

Keratinase activity

Keratinase activity of isolate M20 was determined by its action on chicken feathers. Five boiling tubes with 15 ml mineral salt medium and a clean chicken feather (50-60mg) were sterilized. Four tubes were inoculated with a loop full of spores of the isolate M20. One tube without inoculum served as control. All the tubes were incubated under laboratory condition (28±2 ºC) for 15-20days. Keratinase activity was confirmed when the feather completely disintegrated.

The basal medium – composition

Ammonium sulphate	2.46 g/l
Potassium di hydrogen phosphate	2.38 g/l
Di-potassium hydrogen phosphate	5.65 g/l
Magnesium sulphate	1.0 g/l
Trace element solution	1 ml
Zinc sulphate	1.50 mg
Sea water	300 ml
Distilled water	700 ml
p H	7.5

3. Results and Discussion

The soil analysis results showed that there were very low available Nitrogen, P_2O_5 and Cu. Micro-Nutrients like Zn and Fe were high in their available form, Mn was medium.

Isolation and maintenance of actinomycetes

Totally 25 actinomycetes were isolated from soil sample that was collected near the root region of *Avicennia marina* by after dry heat (70 ºC for 15 min) pretreatment method. Dry heat method yielded bioactive actinomycetes for antimicrobial activity. The isolated actinomycetes were subcultured in yeast malt extract agar-ISP2.

Protease activity

The isolate M20 was protease positive, chosen for further identification for keratinase activity.

Plate 1: Clear zone around the colony (Indicates that it is protease positive)

Molecular characterization

The sequence was submitted to Gene Bank with the accession No. KP872910. Phylogenetic analysis of 16S rRNA gene (1400bp) of M20, species of *Streptomyces* was carried out with 18 different reference species of *Streptomyces* available in the Gene Bank database. The isolate M20 branched along with *Streptomyces cacaoi subsp cacaoi* (NRBC 12748(T)-AB184115 in the analysis. The phylogenetic tree was constructed by neighbour joining analysis. 16sRNA sequencing results revealed that the isolate shared 98.6% similarity with the *Streptomyces cacaoi subsp. cacaoi.* Therefore the isolate M20 was designated as *Streptomyces cacaoi subsp. cacaoi.*

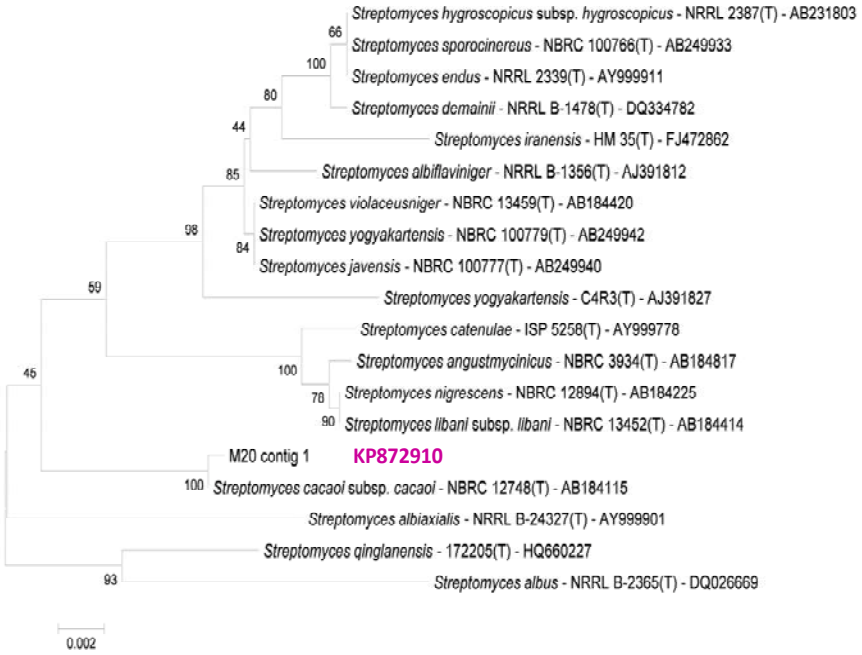


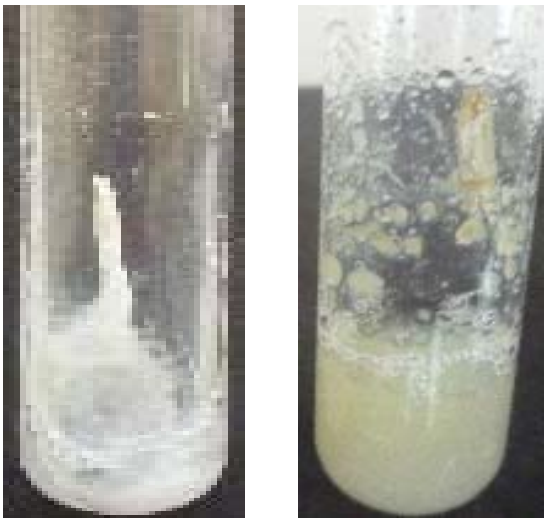
Fig 1: Phylogenetic tree based on the 16S rRNA sequence homology of M20

Neighbour-joining tree based on 16S rRNA sequence showing the phylogenetic relationship between the isolate M20 and other closely related species of the genus *Streptomyces*

Keratinase activity of isolate M20

It was observed that the growth of isolate M20 was slow in the basal medium initially, because of no carbon source in the medium. The growth of isolate M20 was started only after the 5th day of inoculation in the basal media. Due to lack of any carbon sources in the medium, it started to utilize the keratin from the chicken feathers. The isolate utilized the small whole chicken feathers within 10-15 days (Plate 2).

It was witnessed that the initial pH of the medium was 7.5, but the pH of the medium was raised from the day when the isolate started to utilize the keratin. Finally the pH of medium was measured as 8.8, more alkali, due to conversion of complex keratin into ammonia.



Control **degraded feather**

Plate 2: Keratinase activity with chicken feathers by isolate M20

Keratinases are largely produced in the presence of keratinous substrates in the form of hair, feather, wool, nail horn etc. during their degradation. Amongst the industrially important enzymes, actinomycetes keratinases are stimulating tremendous interests in the enzyme market owing to the fact that there is a great demand for developing biotechnological alternatives for recycling of keratin wastes, converting unused chicken feather to useful value added products (Williams *et al*., 1990) [10].

4. Conclusion

Keratinases occupy its centre of attraction in the leather industries and also keratinase producing microorganisms have the important industrial application in fermentation technology. Submerged fermentation of chicken feathers by Streptomyces producing keratinase helps in the conversion of nonsoluble keratin (feather) into soluble protein. Microbial keratinase has become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross linked structural polypeptide keratin recalcitrant to commonly known proteolytic enzymes: trypsin, pepsin and papain.

5. References

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