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"ISOLATION AND CHARACTERIZATION OF KERATINOLYTIC *STREPTOMYCE COELICOFLAVUS*."

Jadhav R.S.[1] and Kulkarni S.W.[2*]

[1]Department of Microbiology, Vishwasrao Naik Art's, Commerce and Baba Naik Science Mahavidyalaya, Shirala 415408, Dist-Sangli M.S, India.

[2*]Research Department of Microbiology, Shriman Bhausaheb Zadbuke Mahavidyalaya, Barshi 413 401, Dist-Solapur M.S, India.

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***Correspondence for Author**
Kulkarni S.W.
Research Department of Microbiology, Shriman Bhausaheb Zadbuke Mahavidyalaya, Barshi 413 401, Dist-Solapur M.S, India.

ABSTRACT

The present study was focused on isolation and characterization of *Streptomyces* producing keratinase enzyme. A total of eight soil samples were collected from the sangli district region. The total 51 *Actinomycete* isolates were obtained from soil by performing serial dilution technique and using Glycerol asparagine agar supplemented with Cycloheximide (80µg/ml). The primary screening of 51 isolates to protease production was carried out on skimmed milk agar. 14 isolates showed zone of hydrolysis and these were taken for secondary screening by using feather meal agar medium. The zone of hydrolysis was reported on feather meal agar. Among 14 isolates, 5 were reported as potential keratinase producers on the basis of size of zone of hydrolysis on feather meal agar and milk agar. Zone of hydrolysis were shown on feather meal agar at 30^0C for 36 hr. Of the 5, isolate No.2 (SH1) was identified on the basis of morphological, cultural, biochemical and 16S rRNA gene sequencing potent keratinolytic *Streptomyces coelicoflavus.*

KEYWORDS: *Actinomycetes*, *Streptomyces*, Keratinase, Feather.

INTRODUCTION

Keratin as well as other insoluble proteins are generally not recognized as a substrate for common proteases (Korkmaz *et al.,* 2003). Keratin is an insoluble protein macromolecule with very high stability and low degradation rate (Samuel pandian *et.al.,* 2012). Keratin is mainly present in hair, feather, nail, wool and horns. The mechanical stability of keratin and

its resistance to microbial degradation depend on tight packing of protein chain in α-helix(α-keratin) or β-sheet (β-keratin) structures and their linkage by cystine bridges (Brigitte and Bockle *et al.*,1995). Of the two types of keratin, β-keratin is rich in glycine, alanine, serine and specifically lysine (Lehninger, 1988) so with the help of keratinase, hair, wool and feather can be used as sources of valuable amino acid (Wyman and Goodman, 1993) and can consequently be used in animal feed preparation (Lahl and Braun, 1994). High protein complex of keratin waste can be used as a good source of protein and amino acid by systemic recycling. Keratinase (E.C.no .4.3.99.11) is a specific protease hydrolysing keratin which is a protein found in feathers, wool, hair and collagen to clear a obstructions in the sewage system in waste water system (Godfrey 1996, Chitte *et al.*,1999).

This enzyme has been produced by bacteria *Bacillus lichaniformis* (Zerdani *et al.2004*, Korkmaz *et al.*, 2003, Tamilmani *et al.*, 2008*)*, fungi including the species of *Aspergillus*, *Onygena*, and *Rhizomucor* (Friedrich *et al.*,1999), some species of dermatophytes including *Trichophyton mentagrophytes*, *T.rubrum*, *Microsporum canis* and *M. gypseum* (Wawrzkiewicz *et al.*, 1991*)* and *Actinomycetes* (Korkmaz *et al.*, 2003).

The *Actinomycetes* are Gram positive bacteria having high G+C (>55%) content in their DNA. *Actinomycetes* are recognized as prokaryotic organisms. The majority of *Actinomycetes* are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plant material. Several species of *Streptomyces* produces bioactive molecules like antibiotics, pigments and many extracellular enzymes as glucose isomerase, amylase, cellulose and protease. The production and characterization of keratinase from *Actinomycetes* has not been fully exploited and explored.

Keratinolytic enzymes have found, important utilities in biotechonological processes involving keratin containing wastes from poultry and leather industries, through the development of non polluting processes. After hydrolysis, the feather was converted to feed stuffs, fertilizers, glues, film and as the source of rare amino acid such as serine, cysteine and proline (Pandian *et al.*,2012), leucine, threonine, and tyrosin (Chitte R.R.1999). In this study we have reported *Streptomyces coelicoflavus* and its production of keratinolytic enzymes, which can efficiently degrade chicken feather within 48 to 96 hrs. This particular property can be used for degradation of highly stable poultry feathers.

MATERIALS AND METHODS

Materials

1. Soil samples- 8 soil samples were collected from the villages around Shirala, Sangli district M.S. India and used in this study for isolation of *Actinomycetes.*
2. Glycerol asparagine broth, Glycerol asparagine agar with Cycloheximide.
3. Skimmed milk agar, Basal salt medium, Feather meal agar.
4. Solvent system, Chromatographic paper.

METHODS

1. Isolation of *Actinomycetes*

The soil samples were collected from the villages around Shirala Dist.-Sangli, M.S. India and enrichment of soil samples were carried out in Glycerol asparagine broth supplemented with Cycloheximide (80µg/ml). A 10-fold serial dilutions of the sample were prepared up to 10^{-6} and 0.1ml aliquots of each dilution was inoculated into Glycerol asparagine agar (L-asparagine- 0.1g, K_2HPO_4-0.1g, glycerol- 1g, trace salt solution- 0.1ml, agar- 2.5g, distilled water-100ml pH-7.4). To avoid the growth of fungal contaminant, medium was supplemented with Cycloheximide (80µg/ml). Plates were incubated at room temperature (30°C) and monitored periodically over 5 to 7 days. Pure isolates were transferred to slants of Glycerol aspargin agar and preserved at 4°C for further study.

2 Identificationof *Streptomyces*

Morphological characteristics were studied with cover slip culture technique. Cultural characteristics were recorded on Glycerol asparagines agar medium. Biochemical characters were recorded on the basis of sugar utilization potential, enzymatic activities and growth under inhibitory substances. On the basis of spore mass colour, the substrate mycelium color, the shape of the spore chain, morphological and cultural characters the isolate were tentatively identified as *Streptomyces*. Biochemical characterisations of *Streptomyces* producing keratinase were carried out (Williams *et al;* 1983)

3 Primary Screening

The primary screening of keratinase enzyme producing *Streptomyces* were carried on Skimmed milk agar plates (pH 6.5–7.2) containing peptone-1%, sodium chloride-0.5%, yeast extract- 0.3%, agar-2% and skim milk-10%. All the plates were incubated at 30°C for 2–5 days. After incubation, the plates were observed for the zone of clearness around the colony.

4 Secondary Screening of keratinilytic *Streptomyces*

Keratinolytic *Streptomyces* were screened on keratin basal salt agar and in broth. (C. Vigneshwaran *et al;* 2010, Ghosh *et al;* 2008)

Keratine basal salt agar

The isolates were streaked on keratine basal salt agar (pH 7.2) containing K_2HPO_4-0.5%, $MgSO_4.7H_2O$-0.4%, NaCl-1.0 %, $CaCl_2$ H_2O- 0.02%, $NaNO_3$-1%, Na_2CO_3 -1%, Yeast extract-0.5% feather- 1%. All the plates were incubated at 30°C for 48 hrs. The occurrence of clear zone around the colonies was considered as keratinase positive.

Keratinase broth

The isolates were inoculated into 100 ml of basal salt medium containig 1% feather in 250mL Erlenmeyer flask and incubated at 30^0C for 48 to 96 hrs. Visual detection method was used to determine keratin feather degradation.

Molecular Identification of *Streptomyces*

One of the potent keratinase producing *Streptomyces* was identified by using 16s rRNA Sequencing. Name of the primer used for forward sequencing was 27F with sequence details AGAGTTTGATCMTGGCTCAG having number of Base 20. Name of the primer used for reverse sequencing was 1492R with sequence details TACGGYTACCTTGTTACGACTT having number of Base 22. 16S rRNA gene fragment was amplified using universal primers such as above mentioned. The phylogeny analysis of sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

RESULTS AND DISCUSSION

Isolation of keratinase producing *Streptomyces*

A total 51 *Actinomycetes* were isolated from soil samples. All 51 isolates were belonging to genus *Streptomyces* on the basis of morphological and cultural characteristics. Among 51 isolates, 14 isolates showed positive proteolytic activity in skimmed milk agar and were selected for further studies. On keratine basal salt agar (Fig. 1).,14 isolates showed clear zone around the colony and gave positive indication for the keratinase production. The zone of diameter, of isolates No.2, 6,11,12 and,14 were much higher than rest of isolates. Isolate No. 2(SH1) was selected for further study.

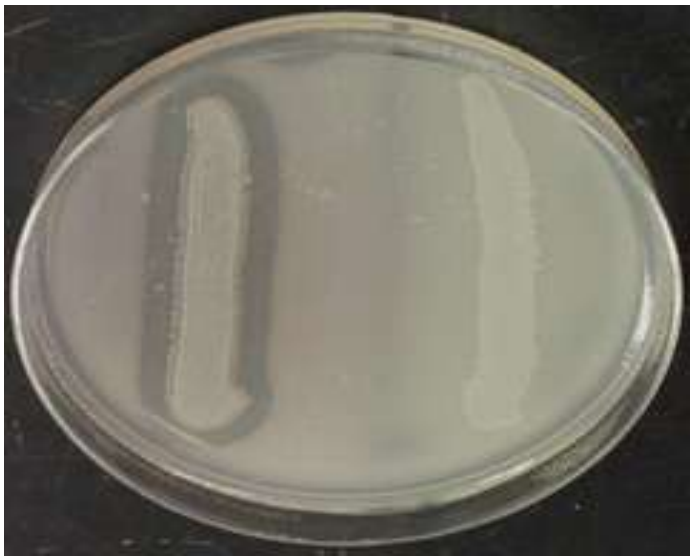
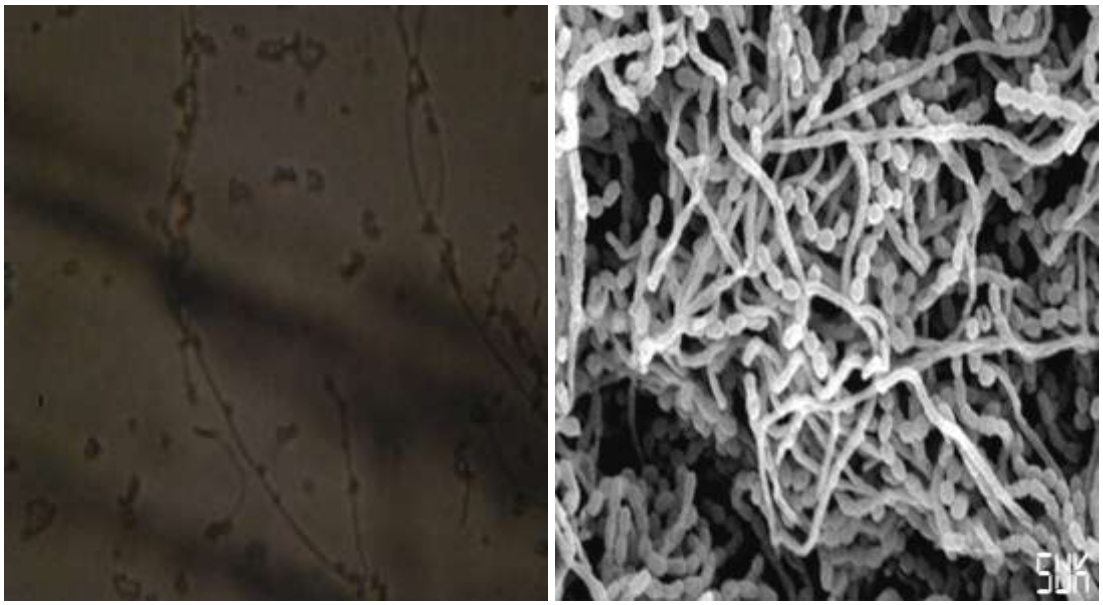


Fig No.1: Clear zone of keratin hydrolysis

Identification of *Streptomyces*

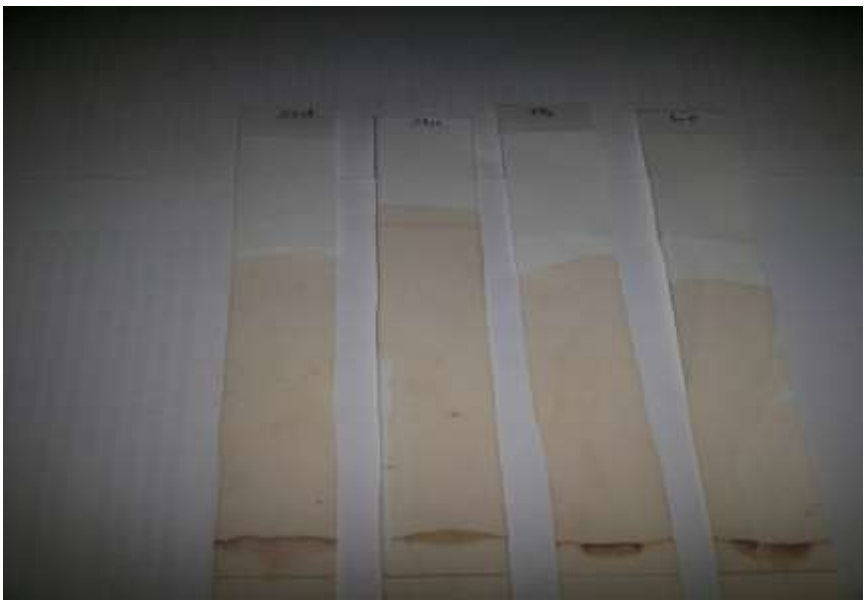
Based on morphological, cultural, biochemical and 16S rRNA the isolate No.2(SH1) was identified as *Streptomyces coelicoflavus.* Morphological aspect using light and scanning electron microscopy are shown in fig.2 and 3. Biochemical characters of Keratinase producing isolate No.2(SH1) was reported(Table No.1). 16S rRNA analysis of isolate No.2(SH1) was carried out.



"Fig.2 Spore chain morphology under Light MicroscopeisolateNo.2(SH1)""Fig.3 Scanning Electron Microscope image of isolate No.2 (SH1)"

"Fig.4.Isolate No.2(SH1) showing feather degradation "

Streptomyces coelicoflavus utilized feather in both distilled and tap water when incubated at pH 7.2 and 30^0 C in rotary shaker for 24 to 72 hrs. The paper chromatogram revealed the presence of leucin (4), threionine (3) in comparison with standard amino acids (Jayraman 1992).



1 2 3 4

Fig.6: Paper chromatography showing presence of amino acid in the broth. 1.,Standerd mixed amino acid(positive control) 2., Broth sample Sh1, 3.,Threonine, 4., Leucine

Table1.Characterization of isolate 2(SH1) (Williams *et al;* 1983a)

Sr.No.	Isolate	Characteristic			Result
1	2 (SH1)	**Morphological characters**	Spore chain morphology (spirals)		+
			Pigmentation of substrate mycelium (colony reverse) Yellowish brown.		+
		Pigmentation characters	Pigmentation on PYIA (Blackish brown)		+
		Carbon utilization	Glucose		+
			Sucrose		+
			Mannitol		+
			Xylose		+
			Arabinose		+
			Lactose		-
		Nitrogen utilization	L-phenylalanine		+
			L-Cysteine		-
			L-Histidine		+
			DL-Valine		+
		Enzyme activity	Catalase		+
			Oxidase		+
			Lecithinase		+
			Lipolysis		+
			Proteolysis		+
			Nitrate reduction		+
			H_2S production		-
		Degradation activity	Gelatin		+
			Starch		+
			L-Tyrosine		+
			Urea		+
		Growth temperatures	4^0C		-
			10^0C		-
			37^0C		+
			50^0C		-
		Growth in presence of inhibitory compounds	Crystal violet (0.0001%)		-
			Phenol (0.1%)		+
			Sodium azide	0.001%	+
				0.002%	-
			Sodium chloride	4%	+
				7%	-
				10%	-
				13%	-

**** Where + = positive - = negative***

Maximum keratinase production of *Streptomyces coelicoflavus* was observed between the 48h to 72h of culture. Chicken feather degradation by *Streptomyces spp.*, was demonstrated by De Azeredo, 2006 and Bockle *et al.*, 1995. Most authors have reported complete or partial degradation of different keratin by diverse microbes in the range of 4 -16 days (Gupta, Ramnani, 2006) other microorganism also produces keratinase enzyme such as *Bacillus spp*., (Laba, 2010), Fungi (Friedrich *et al.*,2005). Isolate No. 2(SH1) showed higher keratinolytic activity compared with other isolate. Further purification and characterization of the enzyme is being carried out.

CONCLUSION

The Actinomycete *Streptomyces coelicoflavus* isolated from soil sample was found to be the potential producer of keratinase.

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