Isolation, Identification and Characterization of Keratinolytic Streptomyces coelicoflavus

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Abstract

Fourteen isolates were obtained from poultry and beam house region soils of Sangli district. An isolate showing maximum keratinolysis was identified as Streptomyces coelicoflavus. The ability of Streptomyces coelicoflavus to utilize chicken feather as a substrate was tested. Crude extract of keratinase enzyme from feather meal broth was used for characterization. It was found that maximum enzyme activity was 49U/ml. Crude keratinase was optimally active in the pH range from 7.5 to 8 and at temperatures from 35°C to 40°C. The various divalent metal ion were tested, Ca++ and Zn++ were found to enhance the activity whereas Mg++, Cu++, Mn++ inhibit the enzyme activity. Also reducing agents and inhibitors were tested. Reducing agent such as Dithiothreitol (DTT) inhibit the enzyme activity whereas sodium sulphide, SDS increases the enzyme activity. 1,10phenanthroline and EDTA were increases the enzyme activity.

Keywords
Actinomycetes, Streptomyces, Keratinase, Feather meal.

Introduction

Feather is almost pure keratin protein consisting of amino acids (Mukesh kumar, 2012). In nature, keratin occurs mainly in the form of hair, horn, nail and cornified tissue. Worldwide about 24 billion chickens are killed annually and totally about 8.5 billion tonnes of poultry feather are produced (Agrahari, 2010). According to recent report India’s contribution alone is about 250 million tonnos (Agrahari, 2010). Feather contains over 90% protein. The main component being β-keratin, a fibrous and insoluble structural protein extensively crossed-linked with disulphide bond, hydrogen bond, hydrophobic interaction, resulting in the mechanical stability, of keratin and resistance to common proteolytic enzyme like as pepsin, trypsin and papain (Onifad et al., 1998). Proteases constitute one of the most important groups of enzyme and their annual sales account for
60% of the world enzyme market (Turk, 2006). Keratinase (E.C.3.4.95%) belongs to
the class hydrolase which are able to hydrolyze insoluble keratins more efficient
than other protease (Vigneshwaran et al., 2010). Feather degradating keratinase
producing bacteria are Bacillus licheniformis (Zerdani et al., 2004). There
are several reports that show Streptomyces produces keratinase enzyme e.g.
S. albidoflavus (Bressolier et al., 1999). Streptemces spp., 5a4 (DeAzeredo et al.,
2006) and Streptomyces spp., CN 902 (Lazim etal; 2009). Keratinolytic activities
were also demonstrated in saprophytic fungi like as Acta Mycologia (Kornillowicz,
1994).

In this study we identified Streptomyces coelicoflavus and then partially purified and
characterized the secreted keratinase enzyme. The ability of this enzyme to
degrade keratin based substrates selectively which was greater than the ability of other
Streptomyces spp.

Materials and Methods

Materials

Soil sample: 8 Soil samples were collected from the sangli district M.S., India and used
for isolation of Actinomycetes.

Glycerol asparagine broth and Glycerol
asparagine agar with cycloheximide(80
µg/ml). Skimmed milk agar, Basal salt
medium, Feather meal agar, Gelatine agar,
Christensen’s medium, Egg yolk agar,
PY1A, Peptone water, Starch agar, Peptone
nitrate broth, Sugar fermentation medium.
Keratin solution.

Buffer: Acetate Buffer (pH- 4 to 5.5),
Phosphate Buffer (pH- 6 to 7.5), Tris- HCl
Buffer (pH -8 to 9).

Metal: Zn++, Mg++, Cu++, Mn++, Ca ++.
Reducing and inhibitory agents: 1,10
Phenanthroline, SDS, EDTA, Sodium
Sulphied, Dithiothreitol(DTT). Dimethyl
Sulfoxide, Acetone, Trichloroacetic acid,
Bradford reagent.

Methods

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 10⁶ and 0.1ml
aliquots of 10⁻⁵ and10⁻⁶ dilution was
inoculated into Glycerol asparagine agar (L
asparagine- 0.1g, K₂HPO₄-0.1g, glycerol
1g, trace salt solution- 0.1ml, agar- 2.5g,
distilledwater-100 ml pH-7.4). To avoid
the growth of fungal contaminant, medium was
supplemented with Cycloheximide
(80µg/ml). Plates were incubated at room
temperature and monitored periodically over
5 to 7 days. Pure isolates were transferred to
slants of Glycerol asparagine agar and
preserved at 4°C for further study.

Identification of Isolate

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

**Primary Screening**

The primary screening of Keratinolytic 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.

**Secondary Screening**

Keratinolytic Streptomyces were screened on keratin basal salt agar. (Ghosh et al., 2008).

**Molecular Identification of Streptomyces**

One of the potent keratinase producing Streptomyces was identified by using 16SrRNA Sequencing. Name of the primer used for forward sequencing was 27F with sequence details AGAGTTTGATCMTG GCTCAG 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.

**Preparation of Crude Enzyme**

Keratinolytic Streptomyces colony was transfer on basal salt medium containing feather meal and kept for incubation at 35°C for 120 to 160 hrs. with shaking at 200 rpm. The feather meal broth was mixed thoroughly with a 50ml tris-HCl buffer (50mM, pH 8). The mixture was shaken for 30min at room temperature in an orbital shaker (155strokes/min) and the process was repeated two to three times. The filtrate thus obtained was used as a crude enzyme for protease activity (Ponnuswamy et al., 2012; Vigneshwaran et al., 2010).

**Preparation of Keratin solution**

Keratinolytic activity was measured with soluble keratin (0.5% w/v) as substrate. Soluble keratin was prepared from white / black chicken feat hers by the method of Wawrzkiewicket al.,(1997). Native chicken feathers (10 gm) in 500 ml of dimethyl sulfoxide were heated in a hot air oven at 100°C for 2 hrs. Soluble keratin was then precipitated by addition of cold acetone (1L) at Freezer temperature for 2 hrs, followed by centrifugation at 10,000xg for 10 min. The precipitate was washed two times with distilled water and dried at 40°C in a vacuum dryer. 1gm of quantified precipitate was dissolved in 20 ml of 0.05M NaOH. The pH was adjusted to 7.0 with 0.1 M HCl and the solution was diluted to 200 ml with 0.05 mol/L Phosphate buffer(pH 7.0) (Vigneshwaran et al., 2010).

**Keratinase Assay**

Keratinase activity was determined by the method as described by Krishna Rayudu (2014) with modification. 2ml of reaction mixture contain of 1 ml of keratin solution and 1ml crude enzyme of supernant and then reaction mixture incubated for 10 min. at 40°C in shaking water bath at 100 rpm agitation. 2 ml of (10%) chilled TCA was added and the mixture was kept for 20 min., followed by centrifugation 5000 rpm for 10 min. 0.1 ml of the supernant was added with 0.9 ml D/W and 0.5 ml of 500 mM sodium carbonate and incubated 35°C for 10 min. 2
ml Folin-Ciocalteu reagent (1:3 v/v) was added and incubated for 20 min. followed by reading absorbance at 660 nm for the developed blue color. A control was processed by adding the enzyme after incubation and TCA was immediately added. A standard graph was generated using standard tyrosine solution of 10-100µg/µl. One unit of keratinolytic activity is defined as the amount of enzyme that liberates 1 µg of tyrosine equivalent per min. under the described assay condition.

**Protein Determination**

Protein contain was measured by the method of Bradford (1976) using bovin serum albumin (BSA) as standard.

**Amino acid analysis by HPLC**

The method of amino acid analysis of Robert and Stephen (1984) and Jeong *et al.*, (2010) was used to determine the amino acid contents for feather degradation broth. High Performance Liquid Chromatography (HPLC) was used for amino acid analysis. In this technique quantified the amino acids by using phenylisothiocynate (PITC) as a coupling reagent. The quantitative pre column derivatization of amino acids and several reverse phase HPLC systems for separation of the resulting phenylthiocarbamyl (PTC) derivatives. During the process, 3.5µl Agilent columns (4.6 × 150 mm) were employed.

**Activity of keratinase with different Parameters**

**Effect of pH**

The optimum pH was determined at 40°C for 1 hrs. Using following buffers (50mM/l): Sodium phosphate buffer (pH 6-7.5), Tris-HCl buffer (pH 7.5-9).

**Effect of Temperature**

To determine optimum temperature for keratinolysis, enzyme reaction carried out at different temperature for 1hrs. Temperature ranging Acetate Buffer (pH- 4 to 5.5), from 30°C to 50°C were used. Enzyme activity was determined by standard enzyme assay.

**Effect of metal divalent ion**

Five metal divalent ion were used to determine effect on keratinase activity, such as (5mM) Ca++, Zn++, Cu++, Mn++, Mg++ activity of metal an keratinase was determined by standard enzyme assay.

**4) Effect of reducing agent and inhibitors**

The effect of SDS, sodium sulphide, dithiothreitol (DTT), 1,10 phenanthroline and EDTA on enzymolytic activity was determined. A control was kept with the enzyme and the substrate (without above agent) and the value of the control activity was consider as 100%

**Results and Discussions**

**Isolation of Keratinolytic *Streptomyces***

A total 14 caseinase producing isolates were screened and used in feather meal basal salt agar medium for feather degrading property it was found that the SH1 strain was shown feather degradation at 30°C within 94 hrs. The isolate SH1 strain belonging to genus *Streptomyces* was tentatively identified on the basis of morphological and biochemical characteristics were studied (Table 1).

**Identification of *Streptomyces***

On the basis of morphological and biochemical characteristic (Table 1) the isolate, was identified up to the genus level
and using 16s rRNA sequence the isolates further identified up to species *Streptomyces coelicoflavus*.

**Degradation of Feather**

*Streptomyces coelicoflavus* LC072737 was able to grow and produce keratinase in feather meal basal salt broth medium (Cai *et al.*, 2012) and resulted in maximum degradation of feather after 7 days incubation at 40°C at 150 rpm (fig.5). Keratinase activity measured was 49 U/ml in the absorbance at 660nm by standard enzyme assay method (Rayudu, 2014).

Amino acids analysis from feather degradation broth of *Streptomyces coelicoflavus* was quantified by High Performance Liquid Chromatography (HPLC). During the process Agilent columns (4.6x150mm) 3.5µl were employed.

A total 18 amino acids were found in the feather degradation broth. Cysteine was found in high concentration (3.3569µl/ml) followed by leucine (2.02558µl/ml). Total concentration of amino acid in broth was found 13.4986µl/ml. In feather degradation broth released essential amino acids such as threonine (0.4140µl/ml), methionine (1.7487µl/ml), 1-methyl histidine (0.5909µl/ml) and lysine (1.4014µl/ml).

Saha (2012) and Rayudu (2014) reported 15 and 16 amino acids from feather degradation broth respectively.

**Activity of keratinase with different parameters**

**Effect of pH**

The enzyme keratinase was active in pH 6 to pH 9. Enzyme has an optimum pH 8. The enzyme activity was high for alkaline pH, similar finding was reported in *Streptomyces spp.* (Azeredo *et al.*, 2006). Increasing activity were reported as there is increase in pH 6 to 8 and reduced slightly from pH 8 to 9.

**Effect of Temperature**

The effect of temperature on keratinase catalytic activity of enzyme increased with increasing temperature up to 40°C and beyond 40°C reduced the enzyme activity. Similar results were also reported by Jaouadi *et al.*, in 2010 of *Streptomyces spp.*, strain AB1. Similar kind of properties were studied on *S. thermoviolaceus* (Chitte, 1999), *B. subtilis* (Setyorine *et al.*, 2006), *Streptomyces spp.*, (Tapia and Simoes 2008), *B. Licheniformis* (Vigneshwaran 2010). Some microorganism show optimum activity at the temperature of 60°C.

![Fig.1 Spore chain morphology under light microscope (isolate SH1)](image-url)
# Table 1: Characterization of isolate 2 (SH1) (Williams et al., 1983a)

| Sr. No. | Characteristic                          | Result          |
|---------|----------------------------------------|-----------------|
| 1       | Morphological Characters               |                 |
|         | Spore chain morphology (spirals)       | +               |
| 2       | Pigmentation Characters                |                 |
|         | Pigmentation on PYIA (Blackish brown)  | +               |
| 3       | Carbon Utilization                     |                 |
|         | Glucose                                | +               |
|         | Sucrose                                 | +               |
|         | Mannitol                                | +               |
|         | Xylose                                  | +               |
|         | Arabinose                               | +               |
|         | Lactose                                 | -               |
| 4       | Nitrogen utilization                   |                 |
|         | L-phenylalanine                         |                 |
|         | L-Cysteine                              | -               |
|         | L-Histidine                             | +               |
|         | DL-Valine                               | +               |
| 5       | Enzyme activity                        |                 |
|         | Catalase                                | +               |
|         | Oxidase                                 | +               |
|         | Lecithinase                             | +               |
|         | Lipolysis                               | +               |
|         | Protease                                | +               |
|         | Nitrate reductase                       | +               |
|         | Gelatinase                              | +               |
|         | Amylase                                 | +               |
|         | Urease                                  | +               |
|         | H2S production                          | -               |
| 6       | Growth Temperatures                     |                 |
|         | 4°C                                    | -               |
|         | 10°C                                   | -               |
|         | 37°C                                   | +               |
|         | 50°C                                   | -               |
| 7       | Growth in presence of inhibitory       |                 |
|         | compounds                              |                 |
|         | Crystal violet (0.0001%)                | -               |
|         | Phenol (0.1%)                           | +               |
|         | Sodium azide                           | 0.001%          | +               |
|         |                                         | 0.002%          | -               |
|         | Sodium chloride                         | 4%              | +               |
|         |                                         | 7%              | -               |

* Where + = positive - = negative
Table.2 Amino acids from feather degradation using *Streptomyces coelicoflavus* in basal salt broth medium

| Sr.No. | Amino acids                  | µg/ml of sample |
|--------|------------------------------|-----------------|
| 1      | Aspartic acid                | 0.47547         |
| 2      | OH Proline                   | 0.34055         |
| 3      | Phosphoenolamine             | 0.1993          |
| 4      | Serine                       | 0.30448         |
| 5      | Aspargine                    | 0.27947         |
| 6      | Taurine                      | 0.76002         |
| 7      | Threonine                    | 0.4140          |
| 8      | Proline                      | 0.2004          |
| 9      | Arginine                     | 1.0853          |
| 10     | 1-Methyl histidine           | 0.59093         |
| 11     | Tyrosine                     | 0.29792         |
| 12     | Valine                       | 0.07203         |
| 13     | Methionine                   | 1.74876         |
| 14     | Cystathionine                | 0.09125         |
| 15     | Cysteine                     | 3.35692         |
| 16     | Leucine                      | 2.02558         |
| 17     | Phenylalanine                | 0.25544         |
| 18     | Lysine                       | 1.40148         |
|        | **Total**                    | **13.4986**     |

Table.3 Effect of metal on enzyme activity

| Sr. No. | Divalent ions | Concentration | Relative activity (%) |
|---------|---------------|---------------|-----------------------|
|         | Control       | -             | 100                   |
| 1       | Ca ++         | 5mM           | 108                   |
| 2       | Zn ++         | 5mM           | 101                   |
| 3       | Mg ++         | 5mM           | 108                   |
| 4       | Cu ++         | 5mM           | 101                   |
| 5       | Mn ++         | 5mM           | 101                   |

Fig.2 Spore chain morphology and arrangement under SEM
Table 4 Effect of Reducing agent and inhibitors on enzyme activity

| Sr. No. | Reducing Agent   | Concentration | Relative activity (%) |
|---------|------------------|---------------|-----------------------|
|         | Control          | -             | 100                   |
| 1       | Dithiotreitol (DTT) | 5mM          | 101                   |
| 2       | SDS              | 5mM           | 109                   |
| 3       | Sodium sulphide  | 5mM           | 111                   |
| 4       | DMSO             | 5mM           | 111                   |
| Inhibitors |                 |               |                       |
| 5       | 1,10 Phenanthroline | 5mM          | 106                   |
| 6       | EDTA             | 5mM           | 105                   |

Fig. 3 Clear zone around colony in Skimmed milk agar (isolate SH1).

Phylogenetic tree
**Fig. 5** Chromatogram of HPLC (reverse phase) Amino acid analysis.

**Amino acid analysed by HPLC**

**Fig. 6** Effect of pH

**Fig. 7** Effect Temperature

**Effect of Divalent ion on enzyme activity**

The result of enzyme activity of keratinase towards divalent ion (5mM) such as Ca$^{++}$, Zn$^{++}$, Mg$^{++}$, Cu$^{++}$, Mn$^{++}$, were stimulatory. The effect of heavy metal ions such as Cu$^{++}$ (Nam et al., 2002), Mn$^{++}$, Mg$^{++}$ on *B. subtilis* KD-N$_2$ strain was similarly reported by Bockel *et al.*, (1995).

**Effect of Reducing agent and Inhibitors on keratinase enzyme**

The keratinase activity was partially affected
by reducing agent Dithiotreitol (DTT) at a concentration 5mM. Sodium sulphide enhance the keratinase activity throughout the cleavage of disulphide bond reported by Letourneau in 1988. The SDS, 1,10Phenanthroline and EDTA (each 5mM) enhance effect on keratinase but less than sodium sulphide and DMSO. Similar results were observed by Streptomyces pactum DSMS 40536 (Bockle et al., 1995).

In conclusion, according to these results, we conclude that the Keratinolytic proteainase of Streptences coelicoflavus LC072737 might be suitable for processing of keratinase containing waste like feather and hairs in leather industry under appropriate conditions. The crude keratinase was active at broad range of temperature (30°C to 50°C) and pH values (6 to 9) with optima at 40°C and pH 8.

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