PURIFICATION AND MOLECULAR WEIGHT DETERMINATION OF KERATINASE ISOLATED FROM STREPTOMYCES MALAYSIENSIS

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ABSTRACT
Objective: The aim of the present study was to purify and determine the molecular weight of keratinase isolated from Streptomyces malaysiensis.
Methods: For that purpose purification was performed using ammonium sulphate and Sephadex-LH 100 column chromatography. Further, the fractions were pooled and subjected to molecular weight determination using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).
Results: The obtained results showed keratinase with 47.57% recovery, 3.5-fold purification and an estimated molecular mass of 27,000 Da. Keratinase showed an optimal activity at 60°C and pH 8. Keratinase activity of the purified product was assayed with feather powder as a substrate. The isolated strain was identified as Streptomyces malaysiensis based on phylogenetic tree analysis. The strain isolated from termite mound soil showed the highest keratinase activity, which could be considered a microorganism of environmental origin.
Conclusion: The production of keratinase on simple media with feathers as sole source allowing its production from the cheap substrate and a commercial production with low production cost. Stability in the presence of detergents, surfactants and solvents make this keratinase extremely useful for a biotechnological process involving keratin.
Keywords: Streptomyces, Keratinase, Purification, Feather powder, Environment protection
INTRODUCTION
Microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin". These enzymes are largely produced in the presence of keratineous substrates in the form of hair, feather, wool, nail, horn etc. during their degradation. The complex mechanism of keratinolysis involves the cooperative action of sulftolitic and proteolytic systems. Keratinases are robust enzymes with a wide temperature and pH activity.

The keratinous wastes largely comprise of insoluble structural protein "keratin". It is increasingly accumulating in the environment mainly from various industries. Today, it is also becoming a part of solid waste management since it is tough to degrade because of the high disulfide bond content and cross-linkage. Hence, there is a demand for developing biotechnological alternatives for recycling of such wastes.

Keratin forms a major component of the epidermis and its appendages viz. hair, feather, nails, horns, hoofs scales and wool. Keratins are also grouped into hard and soft keratins according to the sulfur content. Hard keratins found in appendages like feathers, hair, hoofs and nails have high disulfide bond content and are tough and inextensible. Whereas, soft keratins like skin and callus have a low content of disulfide bonds and are more pliable [1, 2]. The intensive cross-linkage in keratins hinders their degradation by commonly known proteolytic enzymes like trypsin, pepsin and papain [3]. Keratinases (EC 3.4.21/2499.11) are by and large serine or metalloproteases capable of degrading the structure forming keratinous proteins. Since most of the purified keratinases known to date cannot completely solubilize native keratin [4, 5], their exact nature and uniqueness for keratinolysis is still an enigma in the world of proteases. Although keratinases from dematophytic fungi have long been well known due to their notorious pathogenic nature [6] these enzymes have only recently gained biotechnological impetus. Their growing importance is mainly contributed to the isolation of keratinases from non-pathogenic microorganisms and their ability to degrade the tough insoluble keratin of feather and convert it into economically useful feather meal [7-9], nitrogenous fertilizers, biodegradable films, glues and foils [10, 11].

In the present study, we report an efficient method for the purification and molecular weight determination of keratinase from Streptomyces malaysiensis.
MATERIALS AND METHODS
Chemicals and reagents
All chemicals and reagents used in the present study are procured from "Sigma-Aldrich".
Collection of samples
In the present study, samples were collected in sterile containers from different sites of termite mound soils in and around Visakhapatnam, Andhra Pradesh, India. From these samples, actinomycetes were isolated and screened for keratinolytic activity, followed by extraction of the bioactive compound.
Isolation of actinomycetes
About 1g of termite mound soil was taken and transferred to a sterile conical flask each containing 50 ml of sterile water, mixed well and then placed on a rotary shaker at 150 rpm for 30 min. Serial dilutions were made from this solution up to 10-6 order dilution after 30 min.

One ml of each dilution was added to 49 ml of sterile casein agar medium containing rifampcin (25 µg/ml medium) and cycloheximide (50 µg/ml medium) to inhibit the growth of bacteria and fungi respectively and poured into sterile Petri plates aseptically and kept for solidification. After solidification plates were incubated for the growth of actinomycetes colonies at 28°C for 7 d [12].

Preliminary screening
The proteolytic activity of the actinomycetes isolates was determined by casein hydrolysis by Skim milk agar medium. Skim
After which 3 ml fractions were collected at a flow rate of 0.5 ml/min. A gradient of 0-1.0M NaCl in 10 mmol Tris HCl buffer with a pH of 8.5, HCl buffer, with a pH of 8.5. Subsequently, the column was eluted with the proper dialyzed solution was applied to a column of Sephadex-LH-100 (Pharmacia, Sweden). It was then equilibrated with 10 mmol Tris buffer (pH 7.5±0.2) for their keratinolytic activity [14].

**Secondary screening**

The promising isolates which showed caseinolytic activity obtained by primary screening was selected for secondary screening by shake flask studies. These selected isolates were subjected to shake flask fermentation experiment studies.

All the promising isolates were subjected to keratinase production by shake flask fermentation studies using the following modified medium in (% w/v) (NH₄Cl 0.05, CaCO₃ 0.022, MgSO₄ .7H₂ O 0.02, K₂ HPO₄ 0.03, yeast extract 1.0, sodium chloride 0.05, ferrous sulphate 0.001 chicken feather 2, pH7.5±0.2) for their keratinolytic activity [14].

**Enzymatic assay of keratinase**

About 20 mg of chicken feather powder was suspended in 4 ml of 0.05M glycine/NaOH buffer pH 10.09 to which 1 ml of culture filtrate was added. The reaction mixture was incubated at 60 °C for 1hr. After incubation, the reaction was terminated by adding 4 ml of 5% trichloracetic acid. The feather and insoluble residue were removed by filtration and the filtrate was centrifuged at 3000 rpm for 5 min. Proteolytic products in the supernatant were determined by reading the absorbance at 280 nm. A control sample was prepared by adding TCA to the reaction mixture before incubation [15].

(Unit definition: One unit of keratinase activity was defined as the amount of enzyme required to produce an absorbance increase of 0.01 under the described assay conditions)

**Confirmation of keratinase activity**

The medium was prepared, supplemented with 2% chicken feather and autoclaved. 20 ml of medium was then poured per plate and allowed to solidify. A loopful of culture growth was streaked on the centre of agar plates. After inoculation, the plates were incubated at 28 °C for 5 d. Regions of enzyme activity were detected as clear areas, indicating that the hydrolysis of the substrate had occurred.

**Extraction of crude enzyme**

A trial was run to determine the optimal concentration required for the precipitation of enzyme with various concentrations of ammonium sulphate. For this purpose, a portion of the supernatant obtained after centrifugation was subjected to ammonium sulphate precipitation. Ammonium sulphate was added at different concentrations ranging from 20-80% saturation. The precipitates so obtained were suspended in 50 mmol glycine/NaOH buffer pH 1.0.0 and tested for keratinase enzyme activity. The salting out the concentration of crude enzyme was established to be 80% on the basis of enzyme activity. 4.0 grams of crude precipitate so formed was separated by centrifugation for 15 min, dialyzed against 1L of 1.0M Tris buffer (pH 8.0) for 20 hr. After dialysis, the solution was centrifuged. The precipitate formed during dialysis was removed after centrifugation and was discarded. For the present study, dialysis membrane-100 (Hi-Media) was used. The supernatant obtained after centrifugation was subjected to Sephadex LH-100 column chromatography that was pre-equilibrated with 10 mmol Tris buffer (pH 8.5). Fractions showing keratinase activity were pooled and dialyzed against distilled water, freeze-dried, and stored at -20 °C until use [16].

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Sephadex LH-100 column chromatography fractions showing enzymatic activity were pooled, lyophilized and then subjected to SDS-PAGE. It was performed according to Laemmli (1970) using 12% acrylamide [17].

**Molecular identification of the isolate**

Molecular identification and characterization of the isolate TMS1a was carried out by 16S rRNA gene sequencing analysis of Streptomyces taxonomy to investigate the relationships at genus, species and strain level. This was performed at Institute of Microbial Technology (IMTECH), Chandigarh. Evolutionary history and evolutionary distances were inferred by using Neighbor-Joining method (Saitou and Nei, 1987) and Kimura 2-parameter method (Kimura, 1980) [18, 19].

**RESULTS**

**Screening and isolation of actinomycetes**

Soil samples were collected from termite mounds at different places in Visakhapatnam, Andhra Pradesh and named as TMS. A total of 50 colonies were isolated from the soil samples by serial dilution method. Based on the color of the colony, the pigment of the colony and aerial mycelium, similar colonies were pooled together and 32 colonies were selected and identified as actinomycetes as shown in fig. 1.

![Fig. 1: Screening and isolation of actinomycetes](image)

**Preliminary screening (casein hydrolysis)**

Proteolytic activity was studied with milk casein agar medium. The protease activity of the isolate was determined by the appearance of the zone of clearance on the plate. All the 32 actinomycete isolates which were isolated were subjected to primary screening by casein hydrolysis. Out of 32 isolates, 10 isolates showed clear zone around the growth of the isolates.

**Secondary screening (Keratinase assay)**

Keratinolytic activity was determined for all 10 isolates. It was found that the range of keratinase production was from 3.5-20.5 IU/ml. Out of the 10 isolates, TMS 1a (20.5 IU/ml) showed high keratinase activity as shown in table 1. Hence this isolate was selected for further studies.

**Testing on keratin agar plates**

The isolates TMS1a exhibited keratinolytic activity by hydrolyzing keratin on keratin agar plates. A Clear zone of hydrolysis was observed around the growth TMS1a isolate as shown in the fig. 2.

![Fig. 2: Keratinolytic activity by isolate TMS 1a on keratin agar plates](image)
Table 1: Production of keratinase by various isolates from termite mound soil (TMS)

| S. No. | Isolate   | Keratinase activity (IU/ml) |
|--------|-----------|----------------------------|
| 1.     | TMS1a     | 20.5±0.5                   |
| 2.     | TMS2a     | 16.0±1.0                   |
| 3.     | TMS3a     | 5.5±0.5                    |
| 4.     | TMS4a     | 14.5±0.5                   |
| 5.     | TMS5a     | 3.5±0.5                    |
| 6.     | TMS6a     | 14.0±1.0                   |
| 7.     | TMS7a     | 1.5±0.5                    |
| 8.     | TMS8a     | 5.8±0.7                    |
| 9.     | TMS9a     | 4.0±1.0                    |
| 10.    | TMS10a    | 3.5±0.5                    |

TMS1a 20.5±0.5, TMS2a 16.0±1.0, TMS3a 5.5±0.5, TMS4a 14.5±0.5, TMS5a 3.5±0.5, TMS6a 14.0±1.0, TMS7a 1.5±0.5, TMS8a 5.8±0.7, TMS9a 4.0±1.0, TMS10a 3.5±0.5.

Purification through DEAE-Sephadex-LH chromatography

The proteins from the crude enzyme extract were precipitated by the ammonium sulphate (80%) precipitation and most of the enzyme activity was retained in the precipitate. The specific activity of the enzyme increased to 46 and 70 IU/mg after dialysis and Sephadex LH-100 column chromatography respectively. The purity of keratinase increased by 3.5 folds with 47.57% recovery in Sephadex LH-100 purification step. The purification steps for keratinase were summarized in table 2.

Table 2: Purification of keratinase from the strain TMS1a

| Purification steps                     | Total activity (IU) | Total protein (mg) | Specific activity (IU/mg) | Purification fold | Yield (%) |
|----------------------------------------|--------------------|--------------------|---------------------------|-------------------|-----------|
| Fermented broth                        | 206                | 10.3               | 20                        | 1.0               | 100       |
| Ammonium sulphate precipitation        | 112                | 3.5                | 32                        | 1.6               | 54.36     |
| Dialysis                               | 119.6              | 2.6                | 46                        | 2.3               | 58.05     |
| Sephadex LH-100 chromatography         | 98                 | 1.4                | 70                        | 3.5               | 47.57     |

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Specifically, the purified enzyme was analyzed by polyacrylamide gel electrophoresis, and a unique protein band was visualized after ion exchange chromatography. The enzyme in the SDS-PAGE showed a molecular weight of approximately 27 kDa as shown in fig. 3.

Phylogenetic tree analysis

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method.
Keratinases from micro-organisms have attracted a great deal of attention in the last decades. In our investigation, we describe our keratinolytic S. malaysiensis (TMS1a) able to degrade chicken feather using keratin as substrate. This suggests its potential use in biotechnological processes involving environmental protection.

Proteins are made up of various amino acids linked together in long chains by means of peptide bonds. Many microorganisms can hydrolyze a variety of proteins into peptides (short chains of amino acids) and eventually into individual amino acids. They can then use these amino acids to synthesize their own proteins and other cellular molecules or to obtain energy. In the present study, the objective of the primary screening is to isolate protease producing actinomycetes based on the proteolytic activity of the isolates. As keratinase is one type of protease enzyme. As casein hydrolysis is an indication of protease production. Secondary screening was performed for the keratinolytic activity of the isolates. Keratinolytic activity was determined for all 10 isolates. Out of the 10 isolates, MTMS1a showed 100% homology to Streptomyces malaysiensis. Genebank accession number for all the type strains considered in the analysis are shown in the bracket as shown in fig. 4.

Keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin". Hence the actinomycete isolate MTMS1a which has exhibited positive result in secondary screening (keratinase assay) was further confirmed for keratinase activity by streaking on keratinase agar plates [28].

CONCLUSION

A novel Streptomyces malaysiensis isolate producing keratinase was isolated and purified. The production of keratinase on simple media with feathers as sole source allowing its production from the cheap substrate and a commercial production with low production cost. Further work is focused on the application of pure keratinase on the keratinous tissue to identify the keratinolytic activity.

AUTHORS CONTRIBUTION

Meetakoti Pavani and Karri Mallika carried out the work presented in the manuscript. Meetakoti Pavani wrote the manuscript. Prof. G. Girijasankar and Prof. G. Vidhya Sagar contributed in its correction.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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DISCUSSION

Keratinases from micro-organisms have attracted a great deal of attention in the last decades. In our investigation, we describe our keratinolytic S. malaysiensis (TMS1a) able to degrade chicken feather using keratin as substrate. This suggests its potential use in biotechnological processes involving environmental protection.

Actinomycetes colonies were recognized by their characteristic tough leathery colonies that adhered to agar surface, branched vegetative mycelia and when present, aerial mycelia and spore formation. Smriti et al., (2012) reported similar work of actinomycetes from poultry waste [20]. Of the different media employed for isolation and estimation of actinomycetes population, starch casein agar medium provided better recovery of actinomycetes. This medium has starch as the complex carbohydrate source and casein as a nitrogen source. A similar finding was reported by different investigators (Kuster and Williams, 1964; Ellaiah et al., 2004; Sujatha and Swethalatha, 2017) [21-23].

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According to Singh, the keratinase producing organism (Nocardopsis sp.) showed antimicrobial and anticaner activity. From this the future prospect of our work is anticancer activity [27].

Genebank accession number for all the type strains considered in the analysis are shown in the bracket as shown in fig. 4.
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