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Isolation and Purification of Keratinase from *Streptomyces matensis* (MPLS-1)

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ABSTRACT

A new keratinase extracted from *Streptomyces matensis* (MPLS-1) was isolated and purified in this study. Solid ammonium sulfate was selected to precipitate the enzyme. Its proper adding mass was also determined. Through solid ammonium sulphate precipitation and liquid chromatography via the DEAE-Sephadex-LH 100 using the feather powder as substrate, 2.4-fold purification with a yield of 26.66% was obtained. The purification effect was determined through SDS-PAGE, and the molecular weight of enzyme was found at 34kDa. It is simple purification step and high yield using a cheap medium attest to the great biotechnological potential of keratinase, especially in environment protection and in recycling valuable materials from wastes.

I. INTRODUCTION

Keratin belongs to a family of fibrous, insoluble structural polypeptides, and constitutes the major component of the epidermis and its appendages such as hair, nails, feathers, wool and horns. According to secondary structure, keratins are grouped into α-keratin or β-keratin. On the basis of sulfur content, keratins are classified into hard (feather, hair, hoof and nail) and soft (skin and callus) keratin [7]. The high degree of cross-linking of disulfide bonds and several hydrophobic interactions confer structural rigidity and mechanical stability of keratinous materials and make them resistant to proteolytic enzymes such as trypsin, pepsin or papain [16, 1]. Feathers are generated in large quantities as a byproduct of poultry industry. Billions of chickens are killed annually and near about 8 billion tons of poultry feathers are produced. Nowadays feather waste is utilized as a dietary supplement for animal feedstuffs. Use of microbial keratinase for keratin degradation is the innovative solution for recycling feather waste and reducing pollution conversion of feathers into feather meal, dietary protein for animal feed by using physical and chemical treatment is significant. These methods can destroy certain amino acids and decrease protein quality and digestibility. Physical and chemical methods can lead to destruction of amino acids as well as decrease the protein content and digestibility. The utilization of agro-industrial residues may increase energy conservation and recycling. To overcome the loss of amino acids due to keratin hydrolysis microbial keratinases are used. However, the mechanism of keratin biodegradation by microorganisms is not yet completely understood. Comprehensive reviews about keratinases have been published in this article presents recent advances on keratinases from *Streptomyces* origin with emphasis on their biochemical properties and discussion on their current and potential applications in soils and poultry wastes[16].

Keratinase belong to class hydrolase. These are metalloproteins and efficient proteolytic enzymes. The enzyme keratinase is a potential enzyme for removing hair and feathers in poultry industry [21]. A number of keratinolytic microorganisms have been reported including species of fungi such as *Microsporum* [6], *Aspergillus* [3, 8], *Bacillus* [3, 8], *Streptomyces* [3, 19] and other Actinomycetes [3]. In this study, we report an efficient method for the isolation and purification of keratinase from the strain culture.

II. MATERIALS AND METHODS

Chemicals

Chicken feathers were supplied by a local poultry processing factory. The feather was ground by a ball mill to a feather powder. Standard proteins and important chemicals were purchased from Sigma Company.

Screening of *actinomycetes*

Collection of samples

In the present study, samples were collected in sterile containers from different sites of poultry waste soils in and around Visakhapatnam, Andhra Pradesh, India. Poultry soil was collected from Gullepalli village where feather wastes were dumped. From these samples, *actinomycetes* were isolated and screened for keratinolytic activity, followed by extraction of the bioactive compound from the selected isolates and identification of isolates.

Isolation of *actinomycetes*

About one gram of poultry waste soil was taken separately and transferred each to a sterile conical flask each containing 50mL of sterile water, mixed well and then placed on rotary shaker at 150 rpm for 30 min. After 30 minutes, serial dilutions were made from this solution up to 10^{-10} order dilution.

One ml of each dilution was added to 49 ml of sterile casein agar medium containing rifampicin (25µg/ml medium) and cycloheximide (50µg/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 5 days.

Test for keratinase production

A full loop of each pure culture was added to 50 ml liquid medium containing (%, w/v): NH_4Cl 0.05, NaCl 0.05, K_2HPO_4 0.03, KH_2PO_4 0.04, $MgCl_2.6H_2O$ 0.024, yeast extract 0.01 and feather meal 1, pH 7.5 in a 250 ml-Erlenmeyer flask. The culture was grown on a rotary shaker at 150 rpm and incubated at 37°C for 24 h and used as an inoculum. The enzyme production was done

by inoculating 1 ml of each *Streptomycete* inoculum into 500 ml-Erlenmeyer flasks containing the same 100 ml liquid medium. The flasks were shaken at 150rpm and incubated at 28°C for 5 days. The submerged cultures were carried out in duplicate. After 5 days of incubation, the samples were then centrifuged at 10000rpm for 10 min, after which the supernatant fluid was used for the crude enzyme preparation.

Enzyme assays

Keratinase activity was assayed with feather powder as a substrate, in accordance with the modified method described by Dozie *et al*. 1994 [4].

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 1h. After incubation, the reaction was terminated by adding 4 ml of 5% trichloroacetic 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 280nm. A control sample was prepared by adding TCA to the reaction mixture before incubation.

Protein assay

The protein content of the enzyme preparation was determined with bovine serum albumin as the standard protein according to the method previously described by Lowry [13]. After performing column chromatography, protein concentration estimation was undertaken by measuring the absorbance at 660nm.

Determination of solid ammonium sulfate powder saturation

Four saturation stages were selected to determine the properly added quantity. Solid ammonium sulfate powder (0–30% saturation) was initially added to precipitate the protein in the supernatant. Next, the mixture was centrifuged at 10000rpm for 10 min at 4°C, after which the first precipitate was collected. Solid ammonium sulfate powder (30–40% saturation) was then continuously added to the resulting supernatant. After conducting centrifugation under the same conditions, the second precipitate was collected. The solid ammonium sulfate powder (40–80%

saturation) was continuously added to the resulting supernatant, and the third precipitate was thereby collected. Finally, the 80–90% saturation of solid ammonium sulfate powder was added, also by centrifugation at 10000rpm for 10 min at 4°C, after which the fourth precipitate was collected. Afterward, the four collected precipitates were respectively dissolved and dialyzed against the Tris HCl buffer (10 mM, pH 8.5). The keratinase activity and the protein content were then assayed.

Sephadex- LH-100 chromatography

The proper dialyzed solution was applied to a column of Sephadex-LH-100 (Pharmacia, Sweden). It was then equilibrated with 10 mM Tris HCl buffer, with a pH of 8.5. Subsequently, the column was eluted with a gradient of 0-1.0M NaCl in 10 mM Tris HCl buffer with a pH of 8.5, after which 3 ml fractions were collected at a flow rate of 0.5 ml/min. Fractions with enzyme activity were pooled, dialyzed against distilled water, freeze-dried, and stored at -20°C until use. Chromatographic procedures were performed at 4°C.

Electrophoretic analysis

As described by Laemmli (1970), the protein purity and molecular mass of the enzyme were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 1 mm – thick slab gels containing 14.5% (w/v) polyacrylamide resolving gels and 4% (w/v) stacking gels. The lyophilized enzyme samples were solubilized in 65 mM Tris buffer, pH 6.8 and were then boiled for 5 min at 100°C upon the addition of 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) β- mercaptoethanol. After staining the gels with Coomassie brilliant blue R-250, the electrophoretic migration of the protein was compared with that of low-molecular-mass protein markers [10].

III. EXPERIMENT AND RESULT

Keratinase production

Streptomycetes matensis was isolated from poultry litter soil showing high keratinase activity. After 48 h of culturing on native chicken feather as the sole carbon, nitrogen, and energy source,

we found that it could completely degrade the chicken feather. At the same time, we also found that the keratinase reached 53.05 IU/ml at 28°C with an initial pH level of 9.0.

Determination of the precipitation reagent

The solid ammonium sulfate powder (90% saturation) had the best precipitation effect. Protein also had the highest keratinase activity after precipitating by ammonium sulfate.

Determination of the ammonium sulfate saturation

To remove unwanted proteins from the crude enzyme solution, the proper amount of ammonium sulfate to be added was determined in the experiment. The specific activity of precipitated keratinase indicated that 40-80% saturation of $(NH_4)_2SO_4$ had the best effect on enzyme purification. Although the 0–30% saturation of precipitate had enzyme activity, it contained much unwanted proteins. In contrast, at 80–90% saturation stage, no proteins were detected.

Purification through DEAE-Sephadex-LH chromatography

The protein from the crude enzyme extract was 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 60.0 and 88.0 IU/mg after dialysis and Sephadex LH-100 column chromatography respectively. In Sephadex LH-100 purification steps, Purity of keratinase increased by 2.4 folds with 26.6% recovery. The purification steps for keratinase are summarized in Table 1.

Table 1: Purification of keratinase from the isolate *Streptomycetes matensis* MPLS-1

Purification steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Fermented broth	396	11	36	1.0	100
Ammonium sulphate precipitation	153.6	3.2	48	1.3	38.78
Dialysis	120.0	2.0	60	1.6	30.30
Sephadex LH-100 chromatography	105.6	1.2	88	2.4	26.66

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 34 kDa as shown in Fig1.

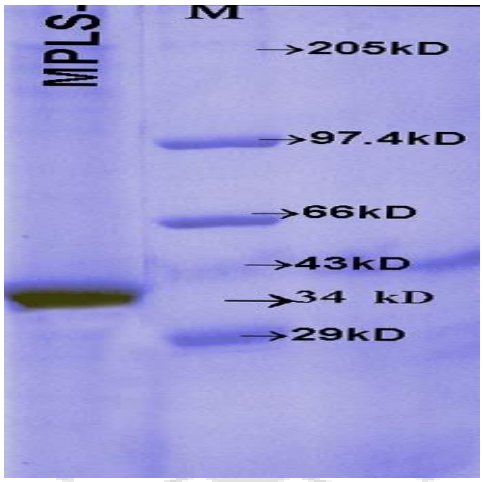

Fig 1: SDS-PAGE image of purified enzyme from the isolate *Streptomycetes matensis* (MPLS-1)

M indicates: Standard protein marker.

IV. DISCUSSION

The newly isolated *Streptomycetes matensis* is a novel strain which can degrade feather keratin. Compared to most other keratin-degrading strains, it can degrade the native feather in 120h and has a keratinase activity (53.05 IU/ml) under the same conditions [11, 15, 18, 5]. This suggests its potential use in biotechnological processes involving environment protection. The strain isolated from poultry litter soil showed the highest keratinase activity, which could be considered a microorganism of environmental origin.

The keratinase precipitated by the ammonium sulfate powder had been reported in many previous studies [22, 20, and 23]. However, the selection of proper precipitating reagents and proper ammonium sulfate saturation to precipitate the enzyme had not been reported so far. It is important to add the proper mass of ammonium sulfate to isolate the keratinase from crude culture solutions, because it can remove many unwanted proteins in the culture, simplifying the following processes and improving efficiency.

The detection of a unique band through the SDS-PAGE confirmed the purification of the keratinase from *Streptomyces* sp. The proteolytic activity of the keratinase after the final purification (88 IU/mg) increased approximately 2.4 fold compared to that of the crude extract (36 IU/mg). The level of purification is comparable to those reported in similar papers [9, 14, 17, 20] and the purification procedure also had a recovery rate (26.66%). This indicates its potential use in biotechnological processes that hasten the period of recycling the feather keratin from industrial wastes. The study also identified the molecular weight (MW) of the *Streptomyces* sp. keratinase as 34000 Da. It belongs to the molecular weight range of major keratinases, varying from 20 to 50 KDa [2]. Likewise, it is similar to the keratinase MW of *Bacillus pseudofirmus* FA30-01 at 27 KDa [9] and *Bacillus licheniformis* PWD-1 at 30 KDa [12].

V. CONCLUSION

The production of keratinase from *Streptomyces* sp. is straight forward and easy to scale up; the organism grows on simple media with feathers as its sole carbon, nitrogen, and energy source. Hence, it is possible to culture an organism with great commercial potential using an inexpensive substrate, resulting in low production cost. At the same time, it transforms a kind of industrial waste (chicken feather) into the required nutritional feed additives, thereby protecting the environment by minimizing wastage. Further studies should therefore be carried out in order to evaluate the biotechnological potential of this keratinase in processes involving keratin hydrolysis.

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