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Research Article

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Application of pure keratinase on keratinous fibers to identify the keratinolytic activity

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ABSTRACT

Keratinases are very widespread in the microbial world and they can be identified from microorganisms of the three domains: Eukarya, Bacteria and Archea. These microorganisms have been isolated from the most distinct soil habitats, including aerobic and anaerobic environments. Therefore, microbial keratinases present a great diversity in their biochemical and biophysical properties. In addition to these species, keratinase production has been associated to an increasing number of bacteria. The biochemical properties of microbial keratinases may be diverse depending on the producer microorganism. Most of the microbial keratinases are alkaline or neutral showing optima pH ranging 7.5-9.0. The properties of microbial keratin degrading enzymes appear to differ according to the producing species of microorganism. Proteolytic enzymes are largely used in the industry for biotechnological applications involving the hydrolysis of protein substrates. However microbial keratinases are considered as promising biocatalysts for several pharmaceutical and biomedical applications. Bioconversion of Keratin-rich materials into amino acids, peptides and soluble proteins by keratinases is possible. The extracellular keratinase isolated from different microorganisms has a great potential for biotechnological applications. Based on the biochemical characterization, the keratinase producing species were analysed and reported for further classification of its application to the environment.

Key Words: Keratin, Proteolytic enzymes, biocatalysts, Biochemical properties, Keratinase.

INTRODUCTION

Biodegradation of feather keratin by microorganisms producing keratinases represents an alternative method to improve the nutritional value of feather waste and to prevent environmental contamination. There is growing interest in microbial proteases of commercial importance in many areas as environmental sciences, biomedicine and biotechnology. The use of microorganism to produce natural products and processes that benefits and improve our socio- economic lifestyles had been a part of our human history [31]. Only limited information regarding such microbial treatment of feathers is currently available [5] [18].

Environmental wastes are found in large quantities in many countries. Although some of them contain a considerable amount of protein and various carbon compounds, little attention is given to utilizing or recycling this waste in a technological way. Additionally, the accumulation of some of these wastes in nature is considered to be a serious source of pollution and health hazards. Therefore, their proper disposal may be considered as a means of avoiding environmental pollution. Recent works focused on the utilization of some polymeric wastes, mainly feather wastes. Feathers are generated in large amounts as a waste by product at commercial poultry-processing plants,

reaching millions of tons per year worldwide [12]. Since feathers are almost pure keratin protein, feather waste represents a potential alternative to more expensive dietary ingredients for animal foodstuffs.

Proteolytic enzymes are largely used in the industry for biotechnological applications involving the hydrolysis of protein substrates [8]. Proteases constitute an important fraction of the global enzyme sales, and a relevant part of this market is accounted by bacterial proteases [22]. Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates, and generally on a broad range of protein substrates [11]. These enzymes have been studied for de-hairing processes in the leather industry [21] and hydrolysis of feather keratin [11], which is a by-product generated in huge amounts by the poultry industry. A keratinase was produced by *Chryseobacterium* sp. kr6 under different growth conditions. This enzyme has been shown to be useful for biotechnological purposes such as hydrolysis of poultry feathers[23] and de-hairing of bovine pelts[24].Natural products have been our single most sucessful source of medicines[29].

The optimum conditions for keratinase synthesis by the *Chryseobacterium* strain kr6 were determined, which is an essential step for the production of adequate amounts for application in research of feed and other areas. The *Chryseobacterium* sp. strain kr6 was isolated from waste of a poultry industry and was capable to completely degrade chicken feathers. Its extracellular keratinase is a metalloprotease with great potential for biotechnological applications [23].

Discarded feathers are currently used to produce feather meal through thermal processing, resulting in a low nutritional value product [26]. Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed [27]. In addition, keratin hydrolysates have potential use as organic fertilizers, production of edible films and rare amino acids [4][3].

Moreover, feather waste represents a potential protein alternative to more expensive dietary ingredients for animal field [17] [6] and it is a byproduct of the domestic poultry industry and is 90% keratin[10]. It offers an interesting potential for the hydrolysis of keratinous wastes to be used as feed supplement or bioconversion to added value products [25]. Worldwide, commercial poultry processing generates million tons of feathers per year which are currently converted to feather meal through steam pressure and chemical treatment.

Keratinases have enormous potential applications in processing waste in the poultry and leather industries. The recent finding that *Bacillus licheniformis* PWD-1 keratinase cause enzymatic breakdown of prion protein PrP^{Sc} [9] leave open a novel relevant application for broad range keratinases.

However microbial keratinases are considered as promising biocatalysts for several pharmaceutical and biomedical applications. Bioconversion of Keratin-rich materials into amino acids, peptides and soluble proteins by keratinases is possible[1][13] stated that the utilization of keratin-degrading microorganisms has a brighter prospect for developing a new and durable biotechnique for the degradation of keratinous residues especially feathers.

Keratinases have recently gained biotechnological impetus because of their ability to act on hard-to-degrade proteins such as hair, feather, nail, etc and thus, becoming a part of solid waste management as recycling of these wastes is tough[2]. In nature, they have been continuously contributing to environmental cleanup of large amounts of feather by converting it into nitrogen rich feather meal.

Besides their use in traditional industrial sectors like detergent, medicine, cosmetics, leather and feed, they also find uses in dehairing for leather processing, as ungual enhancers to increase drug delivery[7][16][15][14][19]. We have earlier reported cloning, functional expression and biochemical characterization of keratinase from a feather degrading strain of *Bacillus pumilus* KS12 [20].

Hence it was thought interesting to carry out an analysis of the physico-chemical parameters such as temperature; pH etc., [28] During the last decade, the immobilization of enzymes has been often used in the production of pharmaceuticals, food and other biological products, and it is also essential for their application to industrial processes [30]

EXPERIMENTAL SECTION

Application studies and degradation activity analysis

Starch hydrolysis

Nutrient starch agar plates were prepared and sterilized. Then the medium was poured in to plates. After solidification of medium the culture was streaked on a single line and incubated. A positive test was indicated by the clearance of the medium around the Colonies, which was further visualized by addition of Lugol's iodine.

Casein Hydrolysis

Skim milk agar plates were prepared and sterilized. The plates were inoculated with the Actinomycetes isolate in a single line and incubated at 30^{o} C for 72 hours. The zone of clearance around the colonies indicated a positive result.

Gelatin test

Nutrient gelatin agar plates were prepared and sterilized. Then the medium was poured into plates. After solidifying, the actinomycete isolate was streaked as single line on the agar and incubated. A positive test was indicated by the clearance of the medium around the colonies. This was further visualized by flooding cultures with acidified $HgCl_2$.

Lipid Hydrolysis

Spirit blue agar was prepared and tributyrin was added as the substrate for lipase activity. The substrate mixture as homogenized in the magnetic thermal stirrer and sterilized. The medium was then inoculated with the Actinomycetes isolate in a zigzag manner and incubated. A positive lipase activity was determined by the reaction of dye round the colonies and on further incubation a zone of clearance around the colonies with dye concentrated around the colonies.

Lecithinase test

Preparation of agar plates: The basal medium was autoclaved in batches of 95 ml and then poured into plates,that were already poured with 5 ml of egg yolk emulsion.

Test: Agar plates were inoculated spot wise with 3 or 4 organisms. After 3, 5, and 8 days, the plates were checked in transmitted light for the occurrence of opaque zones around the colonies, which may be more than 10nm indicating the lecithinase activity.

Pectinase Assay

400 ml of dissolved agar powder was taken and sterilized. Pectin and yeast extract in another 100ml of distilled water was prepared. 500ml of mineral salt solution was dissolved and divided into 2 equal parts and set up at the pH of 5 and 7, both the solutions were autoclaved at 121^{o}C for 15mins. After sterilization the organisms were inoculated and the plates are incubated at room temperature for 5 days. After incubation, the plates were flooded with 1% aqueous solution of **hexadecyltrimethyl ammonium bromide (Sigma).**

RESULTS

The degradation activity shows the positive result for starch, gelatin, casein, pectin and negative result for lipid **(Table. 1, Fig. 1a, b).**

Table. 1. Degradation activity of Actinomycetes

S.NO	NAME OF THE TEST	CULTURE CODE				
		JRS18	JRS17	JRS16	JRS15	JRS14
1	Starch	+	+	-	-	+
2	Gelatin	+	+	+	+	+
3	Lipid	-	-	±	+	+
4	Casein	+	-	-	-	-
5	Pectin	+	-	±	-	+

+: Positive Reaction, ±: Neutral Reaction, -: No Reaction

Fig. 1(a).Casein hydrolysis test

Fig.1 (b).Gelatin hydrolysis test

DISCUSSION

The study revealed that the enzyme produced by *Streptomyces* sp. JRS 18 applied on chick feathers degrades keratin present in it, which reduces environmental pollution. The present investigation is correlated with many of the experimental work which includes that, the keratinolytic alkaline proteases from microbial sources have considerable potential in skin bioprocessing for leather production, offering effective biotreatments particularly for dehairing and bating. The enzyme is a suitable substitute instead of using chemical processes in tannery that causes environmental pollution.

The keratinolytic activity of the enzymes used in bating and other processes with the keratinase of *K. rosea* would be helpful for the evaluation of biotechnological application. On the other hand, more research on specific molecular characteristics of this interesting enzyme must be performed.Therefore, the utilization of keratinolytic enzyme might contribute to the production of high quality leather, also resulting in the improvement of waste water quality and reduced pollution in future.

CONCLUSION

Various investigations in our study showed that, keratinases are valuable enzymes to degrade the recalcitrant protein keratin. The knowledge on keratinolytic microorganisms and the biochemical properties of their keratinases are robustly increased, since keratin degradation is facilitated at high temperatures and hydrogen ion concentration. The thermostable hydrolases are employed in various industrial processes which are of great interest now-a-days. Therefore, the utilization of keratinolytic enzyme might contribute to the production of high quality leather, also resulting in the improvement of waste water quality and reduced pollution.

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