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Research Article

Optimization of production of Extracellular proteolytic keratinase using *Streptomyces sp.* isolated from poultry plant soil

Mohanasundaram S*[1], Doss VA[2], Prabhaharan CTA [1], Joseph J[1], Venugopal K[1], Agilandeshwari P[3], Sudansuhaa C[1], Balaganesh R[1], Ramkumar R [1], Rajaruban MDS [1], Balaji B[1]

[1]Department of Biotechnology, Karpaga Vinayaga College of Engineering and Technology, Maduranthagam, Tamil Nadu, India
[2]Department of Biochemistry, PSG College of Arts and Science, Coimbatore, Tamil Nadu, India
[3]Department of Biotechnology, Vivekananda College of Engineering for Women, Tiruchengode, Tamil Nadu, India

ABSTRACT

In this study, Proteolytic keratinase was observed from Streptomyces sp., isolated from poultry plant soil and purified by ammonium sulfate fractionation method followed by Gel filtration chromatography. The purified enzyme was further characterized partially which covers the influence of pH, temperature, incubation time, nitrogen sources and metal ions on the activity of proteolytic keratinase. From the results, it was observed that, proteolytic keratinase isolated from the Streptomyces sp. enumerated from the poultry plant soil showed maximal activity at pH 8.0 and at temperature 50oC within 55mins. It was also found that, at the minimum concentration of 2mM $BaCl.2H_2O$, $CuSO_4.5H_2O$ and $MgCl_2$, it showed highest keratinase activity.

Keywords: fermentation; Proteolytic keratinase; Streptomyces sp.; stability.

INTRODUCTION

Wool, bristle, horns, feathers, hoof, etc are some example waste materials containing huge level of keratin discharged from animal waste, leather and poultry plants (Gousterova *et al*., 2005). Since these wastes are rich sources of protein and amino acids, they can be utilized as an essential component in animal feed preparations. Few years before, these materials were subjected into high temperature and subsequent milling for some other uses after degradation (Wang and Parson, 1977). Keratin based wastes can be competently degraded by a few bacteria and fungi due to the secretion of keratinolytic peptidases into the culture medium (Onifade *et al*., 1998). Keratin-like materials cannot be successfully degraded by the peptidases currently included in detergents. Hence, enzyme with high activity has to be finding out to meet the industrial requirements and for other applications. So far, wide range of microorganisms with proteolytic keratinase activity has been found, including some species of Bacillus (Williams *et al*., 1990), Actinomycetes (Boeckle *et al*., 1995) and fungi (Santos *et al*., 1996). Always, there is a constant demand for thermophilic organisms because of their high thermal stability and resistance towards denaturation by acidic and alkaline factors. The most important feature of thermophilic organisms is production of enzymes that catalyze biochemical reactions at higher levels of temperatures and these enzymes are widely used in many industrial areas in modern day of science. In recent years, many enzymes from these thermophilic bacteria have been effectively purified, characterized and used in industrial applications (Berna *et al*., 2015). The aim of this present study is to isolate the Streptomyces species producing extracellular proteolytic keratinase from the poultry plant soil and subsequent purification and characterization of the enzyme.

* Corresponding Author
Email: sbmohan2007@gmail.com
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MATERIALS AND METHODS

Microorganism

Streptomyces sp. producing extracellular proteolytic keratinase was isolated from the poultry plant soils from in and around Thirukkalukundram, Kanchipuram district, Tamil Nadu, South India (Dey et al., 1992 and Hinge et al., 1989).

Chemicals

Solvent and other chemicals which were used in this study were purchased from Himedia, Merck and s.d. Fine-Chemicals, Mumbai, India.

Methods

Enzyme Production

Submerged fermentation was performed by inoculating pure culture of isolate into the production medium

containing feather meal (1%), yeast extract (0.01%), NaCl (0.05%), KH_2PO_4 (0.03%), K_2HPO_4 (0.04%) and $MgCl_2$ (0.01%), pH 8.0. The incubation was carried out at 37ºC for 3 days. The broth was centrifuged at 10,000 rpm for 10 min and the supernatant was used as crude enzyme. Bacteria for keratinase isolation were grown in a 250ml flask with 50 ml of medium at 37°C and pH 8.0 for 20h on a rotator shaker (250 rpm). The inoculum (10%) was grown for 6 h in the same medium. During the fermentation, subsamples were removed at various time intervals and the bacteria were checked for lysis and also stained for the detection of endospores (Dorner, 1926). Cells were separated from the 50ml culture broth by centrifugation (10,000 x g for 10 min), and the supernatant fluid was treated as extracellular crude enzyme.

Keratinase activity assessment

The proteolytic activity was determined (Sangali and Brandelli, 2000) with slight modification. Briefly, 100 µl of enzyme extract was added to 400 µl of 10 mg/ml azokeratin in 0.1 M phosphate buffer at pH 8.0. The reaction mixture was incubated for 30 min at 40ºC and then stopped by addition of trichloroacetic acid to reach a final concentration of 10% (w/v). Absorbance at 440 nm was measured after centrifugation at 10,000 x g for 5 min. The protein concentration was measured by Bradford assay (Bradford, 1976).

Partial enzyme Purification by chromatography

Ammonium sulphate was added to the crude enzyme extract to 45% saturation, incubated for an hour at 4ºC with gentle mixing. The precipitate was collected by centrifugation at 10,000 x g for 20 min at 4ºC and dissolved in 0.1M Tris HCl buffer (pH 7.0). The ammonium sulphate concentration was increased stepwise and finally to 90% saturation and the precipitates were collected accordingly (MohanaSundaram *et al*., 2013). The fraction containing proteolytic keratinase activity was pooled and dialyzed overnight against 0.1M Tris HCl buffer (pH 7.0). Then, a Sephacryl 200 column was prepared and equilibrated with 0.05 M Tris HCl buffer. The dialyzed enzyme was applied to the column and eluted with the same buffer. Fraction containing proteolytic keratinase activity was collected and pooled with ammonium sulphate and dialyzed against 0.1M Tris HCl buffer.

Effect of pH on Enzyme on stability

The optimum pH for proteolytic keratinase was determined by using acetate buffer (pH 4 to 5), citrate phosphate buffer (pH 5 to 6) potassium phosphate buffer (pH 6 to 8), Tris HCl buffer (pH 8 to 9) and glycine sodium hydroxide buffer (pH 9 to 14). The enzyme was incubated at various pH values at 40°C for 45 min in the absence of substrate and the optical density was measured at 280nm using spectrophotometer.

Effect of Temperature on Enzyme stability

Thermostability was examined by incubating the enzyme solution at pH 7.0 at various temperatures ranges from 10^oC to 90^oC for 45 min and measuring the optical density at 280nm using spectrophotometer (MohanaSundaram *et al*., 2013).

Effect of metal ions on Enzyme activity

Proteolytic keratinase purified from Streptomyces sp. was treated with 5mM- EDTA at 45^oC for 1h, and then washed with double-distilled water at 4°C to achieve ion-free enzyme preparation. The influence of metal ions on enzyme activity was determined by measuring proteolytic keratinase activity under optimum assay conditions after 15 min pre-incubation at 50oC in the presence of various metal ions in varying concentrations (2, 5 and 10mM). The effect of metal ions on enzyme activity was determined (MohanaSundaram *et al*., 2013).

Effect of Incubation time on Enzyme activity

The effect of incubation time on the activity of proteolytic keratinase was assessed. After the successful identification of influence of pH, temperature and metal ion concentration desirable for the finest activity, a typical reaction mixture was primed with all the crucial ingredients with optimal pH and temperature. Then standard proteolytic keratinase assay was performed and the % of enzymatic activity was calculated in every ten min to discover the sway of incubation time on the doings of the enzyme (MohanaSundaram *et al* ., 2013).

Effect of Carbon Sources on enzyme production

To realize the role and the necessity of various carbon sources in the growth media for proteolytic keratinase was studied by adding different carbon sources like glucose, sucrose, galactose, maltose, fructose, lactose and soluble starch (each 1% separately) along with the basal medium used for the study (MohanaSundaram *et al* ., 2013).

Effect of Nitrogen Sources on enzyme production

The necessity of various nitrogen sources in the growth media for keratinase was also studied by mixing different nitrogen sources like ammonium nitrate, ammonium molybdate, urea, peptone, tryptone, soyabean meal, casein and yeast extract at 1% concentration along with the growth medium.

RESULTS

Effect of Nitrogen Sources on enzyme production

The enzyme was purified from culture supernatant after centrifugation by ammonium sulphate fractionation step followed by Gel filtration chromatography as described in materials and methods. The maximum enzyme activity of broth was obtained after 96h (4days) of cultivation in media. Fraction collected during 90% saturation of ammonium sulphate showed

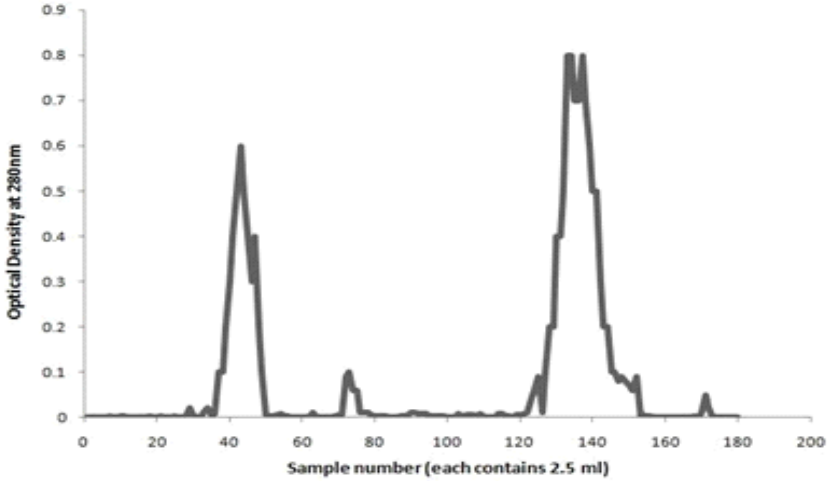

Figure 1: Elution profile of Streptomyces sp. targeting proteolytic keratinase using sephacryl s-200

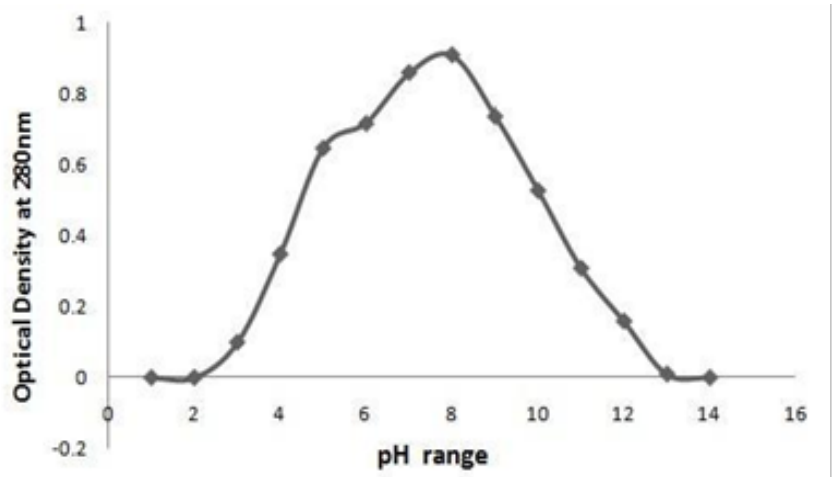

Figure 2: Effect of pH on Proteolytic keratinase stability

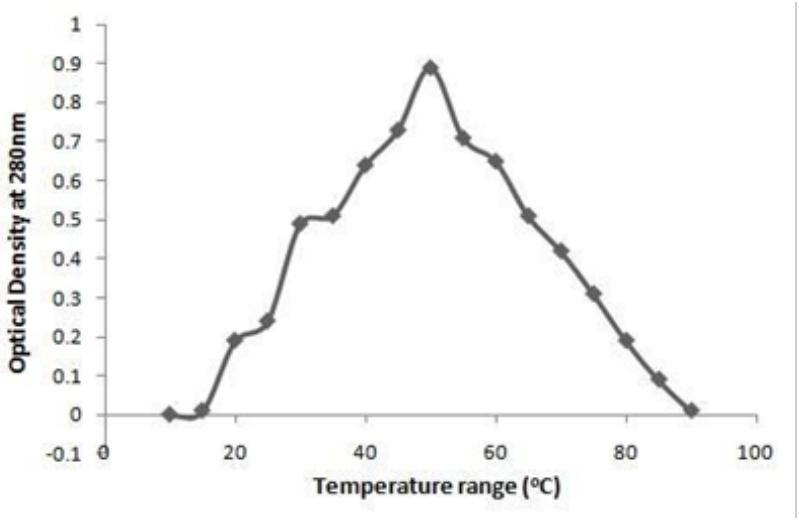

Figure 3: Effect of Temperature on Proteolytic keratinase stability

Table 1: Effect of metal ions on proteolytic keratinase activity

Effect of metal ions	% of relative enzyme activity					
	$ZnCl_2$	$MgCl_2$	$CaCl_2$	$CuSO_4.5H_2O$	$BaCl.2H_2O$	$MnCl_2$
2mM	68.3	99.2	79.3	98.1	98.1	84.7
5mM	57.6	90.1	68.8	88.3	90.4	71.8
10mM	48.3	81.3	59.3	81.7	81.5	63.7

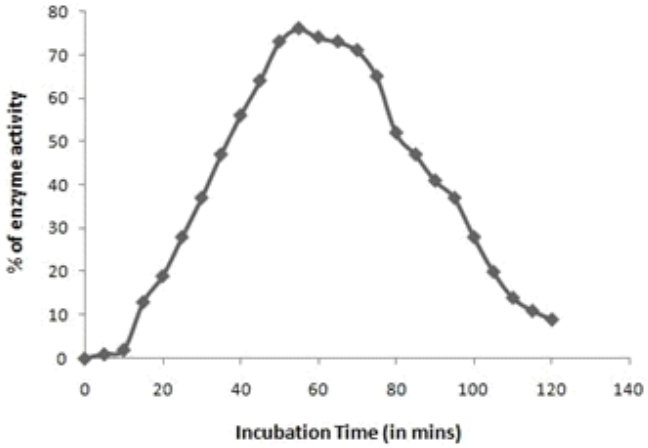

Figure 4: Effect of Incubation time on Proteolytic keratinase activity

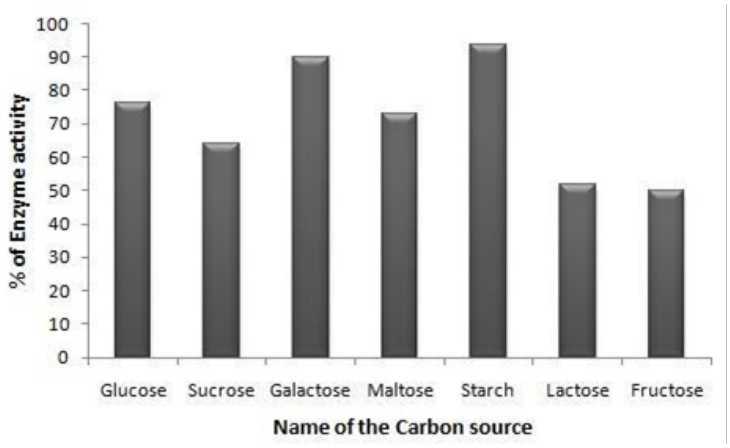

Figure 5: Effect of Carbon sources on Proteolytic keratinase activity

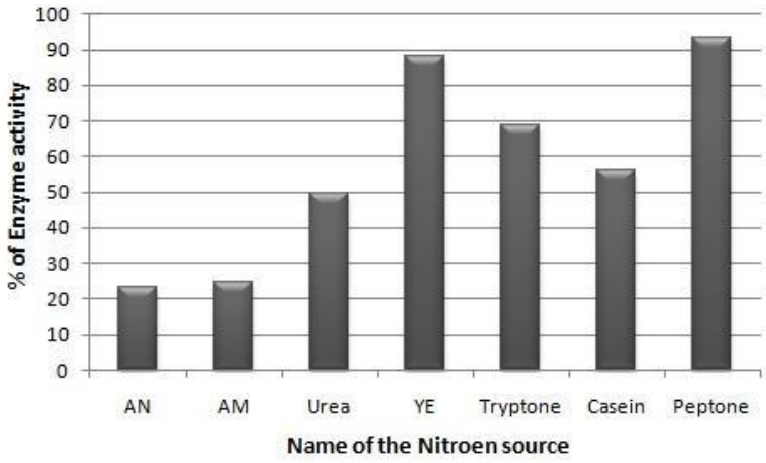

Figure 6: Effect of Nitrogen sources on Proteolytic keratinase activity

significant proteolytic keratinase activity and was further purified by chromatography using sephacryl S-200 column (Figure 1).

Totally 180 fractions (each contains 2.5ml) were eluted and observed for optical density at 280nm. From the graph, it was observed that, two different peaks were obtained from the fractions 37-48 and 136-148. By performing standard Proteolytic keratinase assay (Young and Smith, 1975), it was found that fractions 136-148 showed the maximum enzyme activity and those fractions were mixed and utilized for further studies.

Effect of pH on Enzyme on stability

To know the effect of pH on the stability of Proteolytic keratinase, it was incubated with different buffers of varying pH and the enzyme was found to be stable at pH 8.0 (Figure2). It was also observed that a gradual significant increase in activity at the pH range of 4 to 7 and a major decline at the range of 9 to 12.

Effect of Temperature

As like pH optimization, to understand the effect of temperature on the stability of Proteolytic keratinase, the enzyme mixture was incubated at different temperatures (from 10 to 90^{o}C) and the enzyme was found to be highly stable at 50^{o}C (Figure 3).

Effect of metal ions

When proteolytic keratinase was pre incubated with different metal ions at different concentrations (2, 5 and 10mM), it was found that $MgCl_2$, $CuSO_4.5H_2O$ and $BaCl.2H_2O$ were found to be essential for the maximal activity of proteolytic keratinase. The metal ions were taken in three different ranges (2, 5 and 10mM) along with the production media and it was observed that the above said three metal ions enhances the maximal activity of keratinase at very low concentration (2mM) when compared to other ions (Table 1).

Effect of Incubation time

The standard proteolytic keratinase assay was performed with necessary metal ions at pH 8 and at temperature 50^{o}C. Every five min once, the % of enzyme activity was calculated. From this attempt, it was observed that, when the catalytic reaction of proteolytic keratinase begins, within 55 min of incubation period (Figure 4), it expressed its maximal activity and a slow but sure decline in the activity was observed after 60 min of incubation.

Effect of carbon sources

When we mixed different carbon sources to the production media at 1% separately, it was observed that starch addition enhanced the maximal activity (Figure 5) of proteolytic keratinase enzyme rather than other sources and least activity was observed with the addition of fructose.

Effect of Nitrogen sources

When compared to other nitrogen sources in the production media, it was clear that peptone increased the activity (Figure6) of proteolytic keratinase enzyme rather than other sources and least activity was observed with the addition of ammonium nitrate.

DISCUSSION

In this study, a microorganism with feather degrading activity was observed and isolated successfully from the soil of poultry plants. Later, the organism isolated was found to be Streptomyces sp. using standard characterization methods. It has already been proven that Streptomyces pactum has the ability to release such keratinase with proteolytic activity (Boeckle *et al*., 1995). This paper also exemplifies the description of expression of proteolytic keratinase from Streptomyces sp. Proteolytic keratinase has been reported to be produced from with Streptomyces fradiae and S. pactum (MohanaSundaram *et al*., 2013). The amount of keratinase production by Streptomyces sp. is variable and is purely depend on the media composition. According to some early studies (Sangali and Brandelli, 2000), the production of keratinase can be induced by introducing some significant quantity of feather based ingredient to the medium extracellularly. The ideal temperature (according to our study, 50ºC) for the proteolytic activity was comparable to those of the enzymes from Streptomyces S.K1-02 (Letourneau *et al*., 1998), Streptomyces sp. 594 (Azeredo *et al*., 2006) and Streptomyces thermoviolaceus strain SD8 (Chitte *et al*., 1999). From the above results, it is very clear that, these keratinase enzymes are not thermophilic in nature and at 50^{o}C, these enzymes will have their maximal stability and activity. In this study, the optimal pH required for the complete activity of keratinase was 8.0. This has also been proved in an earlier attempt (Boeckle *et al*., 1995). For the production of keratinases, wide range of organisms can be tried and each of them are having their own optimal conditions like pH and temperature. There is no clear data was found to discuss the essential role of carbon sources on proteolytic keratinase yield and activity. But, our study explains that among other sugars, starch has the most positive influence towards proteolytic keratinase production. To select, a particular nitrogen source to be utilized in the production media for keratinase is a typical task. There are many earlier studies explaining that, different nitrogen sources can be used for production while using other organism excluding Streptomyces sp. In this attempt, we came to know that, when compared to other costlier and rare nitrogen sources, peptone was found to be the most suitable one for the production of keratinase in case of Streptomyces sp. When consider the necessity of metal ions in the production media, $BaCl.2H_2O$, $MgCl_2$ and $CuSO_4.5H_2O$ are showing some positivity towards the successful activity of keratinase at a concentration of 2mM. As per the results of some earlier study (Korkmaz *et al*., 2003) using Streptomyces strain BA7, it is again been proved that there is a need of some metal ions in the production media of keratinase. From our results, it was showing that, $CaCl_2$ and $ZnCl_2$ showed some recession in the activity of keratinase if they are a part of the media.

CONCLUSION

From the results we obtained, it is concluded that, the enzyme proteolytic keratinase can be produced in agreeable level cleanly with Streptomyces sp., which can also be enumerated from the natural soil easily. The results of characterization study experiments, it is again proved that, this enzyme can function maximum at pH (8.0) and temperature (50^{o}C) and it showed it's maximal catalytic activity in media containing starch rather than other sugars and the essential co factors includes $MgCl_2$, $CuSO_4.5H_2O$ and $BaCl.2H_2O$.

CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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