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RESEARCH ARTICLE

ISOLATION, OPTIMIZATION OF PRODUCTION CONDITIONS, CHARACTERISATION AND PARTIAL PURIFICATION OF KERATINASE ENZYME FROM *BACILLUS* SP.

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ABSTRACT

Keratinase producing bacteria were isolated from poultry farm soil and feathers from places located in and around Coimbatore, India. The isolated bacteria were screened for keratinase production using Feather Meal medium. The positive strains were then characterized and identified as *Bacillus sp*. Optimum conditions for the growth of the bacterium and production of keratinase enzyme has been identified as follows: pH 7.5, temperature 37°C, substrate concentration 1 %, inoculum concentration 4 % and incubation period 24 hours respectively. For enzyme activity optimum pH was found to be 7.5, temperature 45^{0}C.The enzyme was partially purified using filter sterilization. High degradation of keratin was observed in the degrading medium added with glucose as carbon source and inoculated with partially purified keratinase.

INTRODUCTION

Feathers are produced annually in huge amounts as waste products of commercial poultry-processing plants. Feathers are composed primarily of keratin. Because of a high degree of cross-linking by disulphide and other bonds keratin is an insoluble protein and is not degraded by normal proteases such as trypsin, pepsin and papain (Xu *et al.,* 2009). Despite their elevated resistance, keratins are recycled in nature and can be degraded by some microorganisms. A group of proteolytic enzymes which are able to hydrolyse insoluble keratins more efficiently than other proteases are called keratinases (Moniruzzaman *et al.,* 2007). It was classified as proteinase of unknown mechanism as recommended by the Nomenclature Committee on the International Union of Biochemistry with EC number 3.4.99 (Owen *et al*., 1983).Recently, some of the works defined keratinase as serine protease due to its 97% sequence homology with alkaline protease and it is also inhibited by the same inhibitor that inhibits serine protease (Bockle *et al*., 1995; Zaghloul *et al*., 1998 and Bressollier *et a1*., 1999). Keratinase is a protease capable of digesting keratins in chicken feathers as well as animal wool and hair (Selvam and Vishnupriya, 2012). Keratinolytic enzymes are produced by fungi, actinomycetes and bacteria and have been frequently isolated from soils where keratinous materials are deposited (Kaul and Sumbali 1997; Moallaei *et al.,* 2006, Xu *et al* 2009). Thus keratinolytic enzymes may have important uses in the biotechnological conversion of keratin containing wastes from poultry industry, through the development of non-polluting processes. Insoluble feather keratins can be converted, after enzymatic hydrolysis, to feedstuffs, fertilizers, and films (Gupta and Ramnani 2006; Brandelli *et al.,* 2009). Keratinases are mostly endo-proteases showing a broad spectrum of activity (Brandelli 2005), usually hydrolyzing soluble proteins (such as casein) more effectively than insoluble proteins such as keratins (Brandelli 2005; Suntornsuk *et al.,* 2005; Dastager *et al.,* 2009). Only few microbial keratinases show higher hydrolysis of keratins than soluble proteins (Balaji *et al.,* 2008). Besides some exceptions, purified keratinolytic enzymes are often ineffective to hydrolyse native keratin (Gupta and Ramnani 2006; Riffel *et al.,* 2003; Xie *et al.,* 2009), a behaviour that is mainly attributed to the high levels of disulfide bonds in the keratin molecules (Brandelli 2009). This study describes isolation, screening, optimization of production conditions of the organism and enzyme activity, partial purification.

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MATERIALS AND METHODS

Isolation of bacteria from soil and feather

Thirty kinds of soil and feather samples were collected from different poultry farms located at Coimbatore district, Tamil nadu, India. From each sample one gram was taken and added to 9ml of sterile saline solution and mixed well and named it as 10^{-1} dilution, from 10^{-1} dilution 1 ml of sample was taken and added to 9 ml of sterile saline solution in a test tube and named it as 10^{-2} dilution, in the similar process the samples were serially diluted using sterile saline solution up to10^{-9} dilutions. From each dilution 0.1ml of sample was spread using L-rod in

sterile conditions over Skimmed Milk agar plate. The plates were incubated at 37°C for 24 hours.

Screening on Feather meal agar plates

Feather meal agar was prepared, the soil and the feather isolated bacteria were streaked on the plates and incubated at 37°C for 24hrs. The soil isolated bacterial colony with a maximum clear zone was named as BS1. Similarly, the feather bacterial colony with a maximum clear zone was named as BF1.

Pure culturing of isolates

A well isolated colony forming a clear zone in feather meal agar plate was selected and transferred to a sterile nutrient broth for shake culturing at 37°C. A loop full of strain was taken from the broth and quadrant streaked on nutrient agar plates. The plates were incubated at 37°C for 24hours. After incubation the plates were observed for the growth and stored at 4°C for further usage.

Identification and Characterisation of Isolated bacteria

Microscopic examination

The isolates BS1 and BF1 was gram stained and observed under compound bright field microscope. Motility test was also done.

Cultural Characterisation

The isolates BS1 and BF1 were characterised based on the growth on nutrient agar, nutrient broth and gelatin stab.

Biochemical Characterisation

The isolates were biochemically characterised by Indole test, Methyl red test, Voges – Proskauer test, Citrate test, Catalase test, Starch hydrolysis and hydrolysis of gelatin test.

Inoculum prepartion

Isolated colonies of *BS1 and BF1* was taken from the nutrient agar plate and inoculated in 50ml nutrient broth separately and incubated at 37°C for 24 hours.

Production of keratinase from bacterial isolates (Matikeviciene *et al.*, 2009)

a) Enzyme production by feather meal as substrate

100 ml of feather meal medium was prepared and sterilized. 4 ml of *BS1* cultured in nutrient broth was used as inoculum to inoculate the feather meal medium. Similarly 4 ml of *BF1* cultured in nutrient broth was inoculated in another conical flask containing feather meal medium. The two flasks were incubated at 37°C for 24hrs with constant shaking at 200 rpm.

b) Enzyme production by soy meal as substrate

100 ml of soy flour medium was prepared and sterilized. 4 ml of *BS1*cultured in nutrient broth was inoculated in soy meal medium. Similarly 4 ml of *BF1* from the nutrient broth was transferred to another conical flask containing soy meal medium. The flasks were incubated at 37°C for 24 hours with constant shaking at 200 rpm.

Keratinase assay (Pissuwan *et al.*, 2001)

10 mg of feather meal powder suspended in 1 ml of 50mMTris-HCl buffer containing 1mM Calcium chloride and 1ml of culture filtrate was mixed and incubated at 45°C with shaking at 300 rpm for 30 minutes in shaker. The enzyme reaction was terminated by adding 2 ml of TCA solution into the reaction mixture. The mixture was then centrifuged at 6000rpm for 30 min at 4°C. The enzyme inactivated by TCA solution without the addition of substrate was used as control. The absorbance of the supernatant was spectrophotometrically measured at 275 nm.One unit (U/ml) of keratinolytic activity was calculated using the formula,

$$U=4\times n\times A275/ (0.01\times 30)$$

Where,
n is the dilution rate; 4 is the final volume (ml); 30 is the incubation time (min); 0.01 is the increase of correct absorbance per minute.

Determination of protein concentration

Protein concentration was determined by Lowry *et al.* (1951) methodology with Bovine serum albumin as standard. One ml of the enzyme aliquot was taken in a test tube and 5 ml of solution D was added. Mixed well and allowed to stand for 10 minutes. Then 0.5-1 ml of Folin-ciocalteau reagent was added and mixed using vortex. The solution was allowed to stand for 30 minutes and the absorbance was read at 660 nm against appropriate blank. The protein concentration of an unknown solution was determined by extrapolating the O.D. value on the standard graph.

Optimization of culture conditions for maximizing the enzyme production

pH

The keratinase production media with different pH (5.5, 6.5, 7.5, 8.5, and 9.5) were prepared. The bacterial culture was inoculated and incubated at 37°C for 24 hours with constant shaking at 200 rpm.

Temperature

The keratinase production medium was prepared. The bacterial culture was inoculated and incubated at different temperatures (17°C, 27°C, 37°C and 47°C) for 24 hours with constant shaking at 200 rpm.

Substrate concentration

The keratinase production medium was prepared with different percentages of feather meal substrate (2.5 mg/ml, 5.0 mg/ml, 10 mg/ml, 12.5 mg/ml, and 15.0 mg/ml). The bacterial culture was inoculated and incubated at 37°C for 24 hours with constant shaking at 200 rpm.

Inoculum percentage

The keratinase production medium was prepared. The bacterial culture was inoculated with different percentage of inoculum (2

%, 3 %, 4 %, 5 % and 6 %) and incubated at 37°C for 24 hours with constant shaking at 200 rpm.

Characterization of enzymes

Effect of ph on keratinase activity

The keratinase production medium was prepared. The bacterial culture was inoculated and incubated at 37°C for 24 hours with constant shaking at 200 rpm. The keratinase assay was carried out using 50mM Tris-HCl buffer of different pH (5.5, 6.5, 7.5, 8.5 and 9.5).

Effect of temperature on keratinase activity

The keratinase production medium was prepared. The bacterial culture was inoculated and incubated at 37°C for 24 hours with constant shaking at 200 rpm. The enzyme assay mixture was incubated at different temperatures (25°C, 35°C, 45°C, 55°C and 65°C).

Effect of substrate concentration on keratinase activity

The keratinase production medium was prepared. The bacterial culture was inoculated and incubated at 37°C for 24 hours with constant shaking at 200 rpm. The keratinase assay was carried out at different concentrations of substrate (0.01g/ml, 0.05 g/ml, 0.10g/ml, 0.15g/ml and 0.20g/ml).

Effect of chemicals on keratinase activity (Cortezi *et al,* 2008)

Triton x-100, Isopropanol were added to the enzyme preparation and incubated for 15 min at room temperature before being tested for keratinolytic activity at 275nm.

Partial purification of keratinase (Tamilmani *et al,* 2008)

After incubation, 50ml of *Bacillus sp.* cultured in feather meal was centrifuged at 10000rpm for 20 minutes and the supernatant was collected. The supernatant was prefiltered through Whatmann No.1 filter paper for removing the residual non-degraded particles. The filtrate was then filtered through a 0.45 μm pore size of sterile membrane filter to remove bacterial cells and other suspended particles.

Degradation of keratin rich waste using partially purified keratinase enzyme (Cortezi *et al,* 2008)

100 ml of medium-1, medium-2, medium-3 and medium-4 were taken in 4 different 500 ml conical flasks. 0.3g of raw feather was transferred into all four different media. 5 ml of partially purified keratinase was transferred into all four conical flasks. Flasks were incubated for 3 days at 40°C with 150 rpm. Degradation of feather was visually inspected. The degraded feather was dried and weighed.

RESULTS AND DISCUSSION

Screening on feather meal agar plates

The microbial growth in the serially diluted soil samples and feather samples were found to be 52 and 65 different colonies respectively. These colonies of microbes were further screened for the production of Keratinase by Feather meal agar plates out of which only 20 and 15 colonies produced zone of clearance. The maximum zone of clearance in soil microbe was 8 mm (BS1) and that of feather sample was 7 mm (BF1).

Identification, Characterisation of Isolated Bacteria

The selected bacterial strains BS1 and BF1 were characterised by microbial, cultural and biochemical examinations and the results were observed. In microscopic examination, the organisms were found to be Gram positive, motile in nature and rod shaped. In cultural characterisation, they formed irregular shape and smooth colonies on Nutrient agar and showed turbid growth in Nutrient broth and liquefaction in Gelatin stab. In the biochemical tests, the following tests were found to be positive: Methyl red test, Citrate utilization, Catalase test, Starch hydrolysis test, hydrolysis of Gelatin test, whereas Indole and Voges–proskauer tests were found to be negative. From the biochemical tests and based on Bergey's Manual of Determinative Bacteriology, the isolates were identified as *Bacillus Sp.*

Production of Keratinase from Bacterial isolates

The *Bacillus sp BS1* and *BF1* were cultured in keratinous and non-keratinous substrates like feather meal and soy meal medium respectively and were analyzed using spectrophotometer at 280 nm, it showed that keratinase producers grew fastidiously in feather meal medium. Hence the enzyme production is considered more in feather meal medium than in soy meal medium and also high keratinase production was found to be with *BS1* strain.

Determination of protein concentration

The protein concentration was determined using the estimation of protein by Lowry *et al.* method. The result showed that the protein concentration is more in feather meal medium than in soy meal medium and also with *BS1* strain. Therefore, further studies were done using feather meal medium and *Bacillus sp BS1* strain.

Optimization of culture conditions for keratinase production

pH

The optimum pH for keratinase production was found to be 7.5 (Fig. 1)

Temperature

The optimum temperature for keratinase production was found to be 37°C (Fig.2)

Substrate concentration

The enzyme production was found to be more at a substrate concentration of 10 mg/ml. (Fig.3)

Inoculum percentage

The enzyme production was found to be high at an inoculum percentage of 4. (Fig.4)

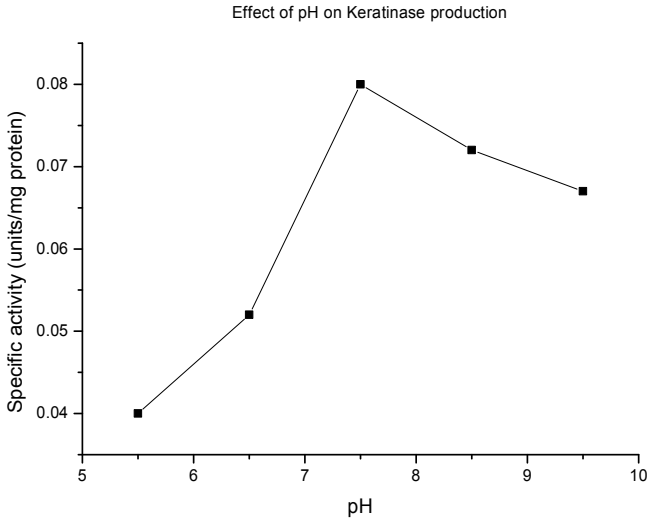

Fig. 1. Effect of pH on Keratinase production

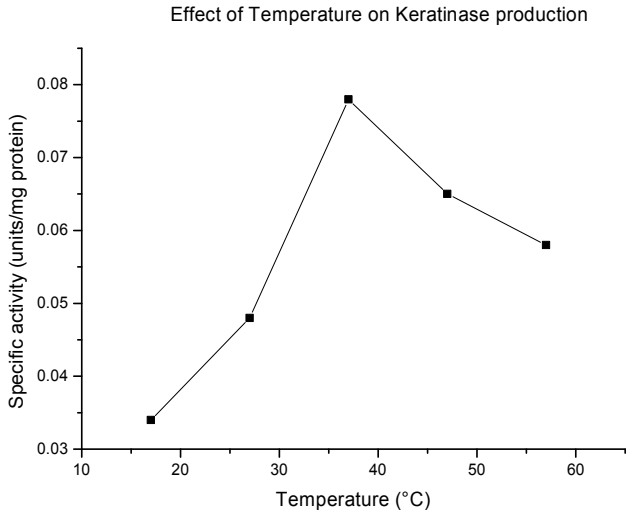

Fig. 2. Effect of Temperature on Keratinase production

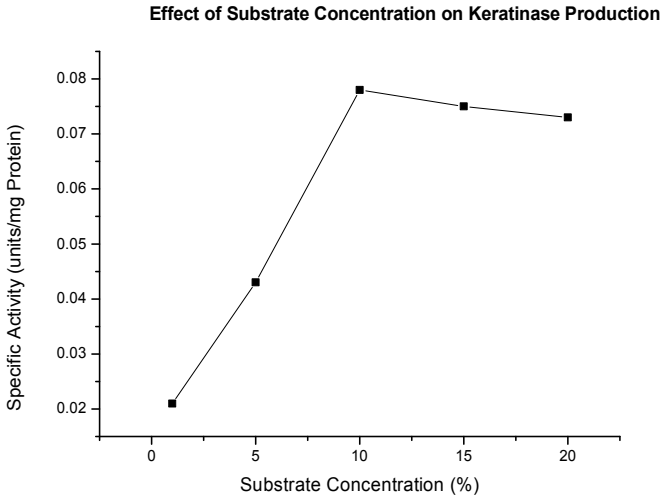

Fig. 3. Effect of Substrate concentration on Keratinase production

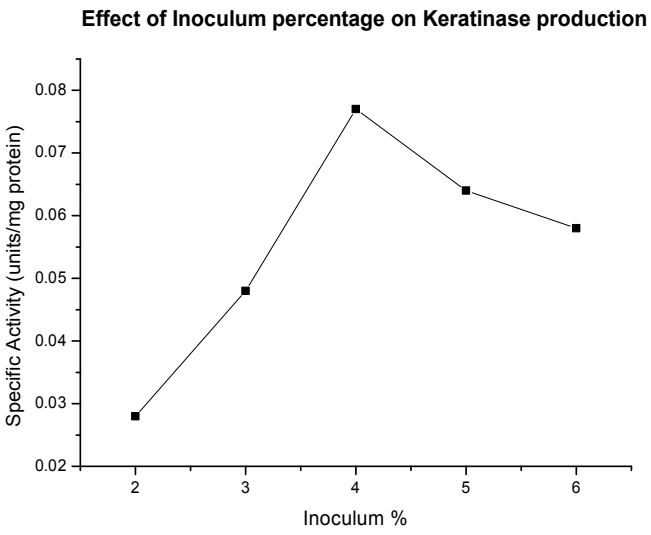

Fig. 4. Effect of Inoculum percentage on Keratinase production

CHARACTERIZATION OF KERATINASE

Effect of ph on Keratinase activity

The influence of pH on the activity of keratinase of *Bacillus sp* was observed. The buffer used was Tris-HCl buffer (pH 5.5 to 9.5) and the enzyme had an optimum pH of 7.5. (Fig.5)

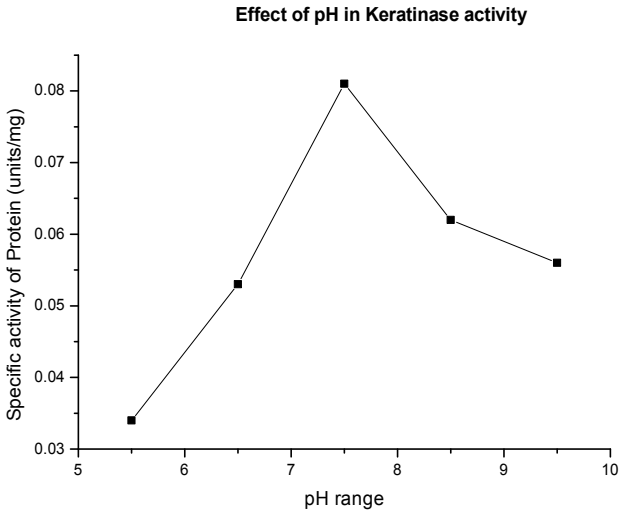

Fig. 5. Effect of pH on Keratinase activity

Effect of temperature on Keratinase activity

Keratinase activity was measured at each temperature. The optimum temperature was found to be 45°C. (Fig.6)

Effect of substrate concentration on Keratinase activity

Keratinase activity was measured at each substrate concentration. The maximum keratinase activity was found to be at 0.1 g/ml (Fig.7).

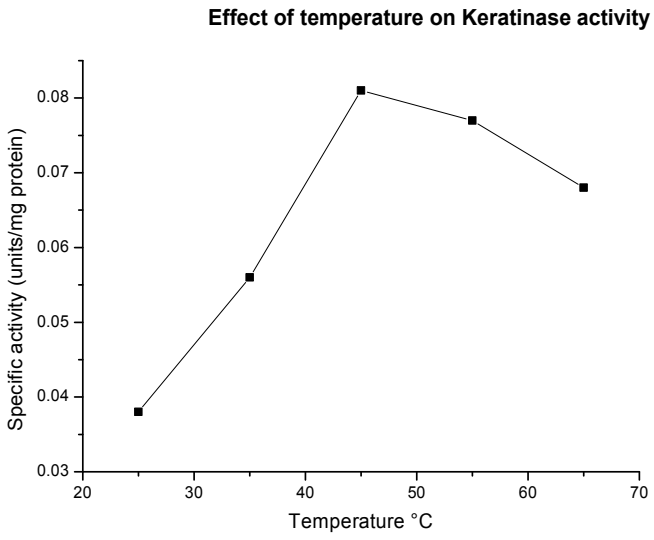

Fig. 6. Effect of Temperature on Keratinase activity

Table 1. Effect of chemicals on keratinase activity

Chemicals	*Bacillus sp.* Percentage of enzyme activity
Triton-X 100 (O.1%)	179%
Isopropanol (1%)	32%

From the Table1, it was evident that the chemical Triton-X 100 increases the enzyme activity while Isopropanol reduces enzyme activity. The addition of glucose (50mM) favoured the growth of the strain as well as the keratinolytic activity (Table 2). In the experiments carried out by Cortezi *et al.* (2008), glucose concentration of 50mM in the culture medium had a positive effect on keratinolytic activity. The addition of NH_4Cl (50mM) favoured the growth of cell than the enzyme production. The addition of casein (1%) and yeast extract (1%) results in moderate enhancement of cell growth and enzyme activity when compared with glucose and NH_4Cl. The medium with glucose as carbon source (50mM) favored the growth of *Bacillus* strains which also results in degradation of inoculated feathers (Table 3). In the present experiment, a glucose concentration of50mM in the culture medium had a positive effect. Comparatively, the experiment done by Cortezi *et al*., (2008) with 50 mM of glucose in culture medium showed a positive effect in production of enzyme.The usage of casein, yeast extract and ammonium chloride, influenced the growth and enzyme production but at low level than glucose.

Table 2. Degradation of keratin rich waste by partially purified Keratinase enzyme

Bacteria	Feather weight before degradation		Feather weight after degradation		Reduction % of weight	
Carbon source	G*	C*	G*	C*	G*	C*
Bacillus sp.	0.36	0.31	0.15	0.17	59%	46%

G*= Glucose (5OmM), C*= Casein (1%)

Table 3.

Bacteria	Feather weight before degradation		Feather weight after degradation		Reduction % of weight	
Nitrogen source	Y*	A*	Y*	A*	Y*	A*
Bacillus sp.	0.34	0.41	0.19	0.26	45%	37%

Y*=yeast extract (1%), A*= Ammonium chloride (5OmM).

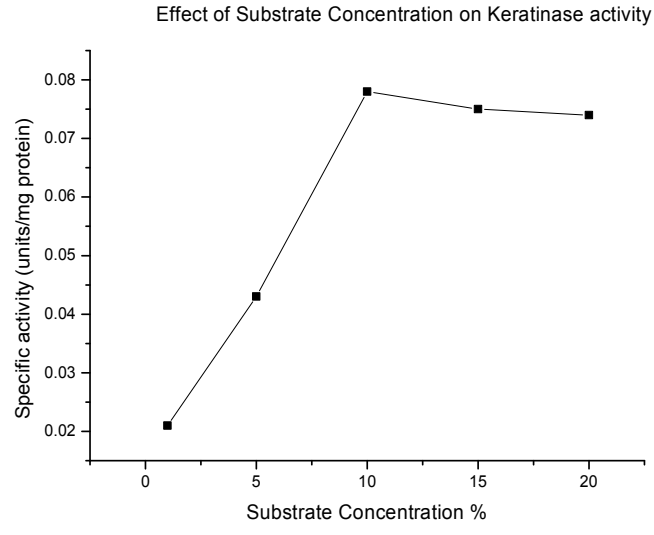

Fig. 7. Effect of Substrate concentration on Keratinase activity

Effect of chemicals on Keratinase activity

Based on the work of Cortezi (2008), two chemicals were selected for their effect on keratinase activity in utilizing keratin rich substrates. The effect of chemicals on keratinase activity was measured using the following formula:

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