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Screening of Keratinase Producing Bacteria and Optimization of Culture Conditions for Its Production

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Absract

The primary screening of keratinase producing bacteria was carried out using different soil samples of local poultry farms of Anand district. 10 keratinase producing bacterial isolates were obtained and their morphological and cultural characteristics were studied. Upon further screening four isolates viz. AN2, AN5, AN6, AN8 were tested for keratinase production using shake flask culture. Among them AN8 showed the highest keratinase production of 21 U/ml after 48 Hrs of incubation at 30 °C temperature. Optimization of temperature and pH increased the enzyme production to 70.4 U/ml at 37 °C and at 7.0 pH. The keratinase production using raw substrates showed the highest production of 68 U/ml with feathers. The selected isolate AN8 showed the degradation of whole feather and polyethelene sheet in the medium. The optimum temperature and pH for enzyme activity was 50 ºC and 7.0, respectively.

Introduction

Keratin is the most abundant proteins in epithelial cells of vertebrates and represents the major constituents of skin and its appendages such as nail, hair, feather and wool. The main content of feather is keratin, which are insoluble proteins resistant to degradation by common proteolytic enzymes such as trypsin, pepsin and papain because of a high degree of crosslinkingby disulfide bonds, hydrogen bonding and hydrophobic interactions.Feathers, which consist of over 90% keratin, do not accumulate in nature because keratins can be degraded by keratinases [EC 3.4.21/24/99.11] produced by some microorganisms [Onifade *et.al.*(1998)] including bacilli [Cai and Zheng (2009), Cai *et.al.* (2008), Dozie *et.al*. (1994)], fungi [Naghy *et.al.* (1998), Friedrich *et.al.* (2005), Gradisˇar *et.al.* (2005)] and actinomycetes [Gradisˇar *et.al.* (2000), Grazziotin *et.al.* (2006)]. Feather degradation

and keratinase production by *Bacillus* spp. have been extensively studied in *B. licheniformis* and *B. subtilis*, and the keratinases belonging to the subtilisin family of serine proteases [Gupta and Ramnani (2006)].Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking not only destroy the amino acids but also consume large amounts of energy. Biodegradation of feathers by keratinase from microorganisms may provide a viable alternative. The ability of microbes to degrade keratin, and keratinase production levels, vary according to species, keratin substrates and culture conditions. Substrates utilized in keratinase activity determination including white guinea pig hair [Gushterova *et.al.* (2005)], stratum corneum [Ignatova *et.al.* (1999)], azo-keratin [Langeveld *et.al.* (2003)] and keratin azure [Lucas *et.al.* (2003)], etc., and the ways in which enzyme activity is documented varies based on the substrate selected. Keratinases from microorganisms have many applications in the feed, fertilizer, detergent, leather and pharmaceutical industries [Macedo *et.al.* (2005)]. For example, feather hydrolysates of *B. licheniformis* PWD-1 and *Vibrio sp.* strain kr2 [Reddy *et.al.*(2017), Riffel *et.al.*(2003)] can be used as animal food, keratinase from *B. licheniformis* PWD-1 can degrade the infectious form of prion, PrPsc, in the presence of detergents and heat treatment [Sanghvi *et.al.* (2016)]. In the leather industry, keratinase from *B. subtilis* S14 exhibits remarkable dehairing capabilities [Suntornsuk *et.al.* (2005)] without the degradation of collagen; this ecofriendly dehairing approach shows great utilization potential. Keratinolytic enzymes have important utilities in biotechnological process involving keratin containing wastes from poultry and leather industries, through the development of non-polluting process. After hydrolysis, the feather can be converted to feed stuffs, fertilizer, glues, films and the source of rare amino-acids such as serine, cysteine, and proline [Macedo *et.al.* (2005)].

Materials and Methods

Sample collection: 12 Different feather samples and 12 farm soil samples were collected from local poultry farms of Anand district, in sterile polyethylene bags and transported to our laboratory. Natural wool and plastic polyethylene films were procured from local market.

Isolation of keratinase producing microorganism: For primary screening of keratinase producing bacteria casein agar plates were used. To prepare the casein agar plates, Nutrient agar was added with 1.0 gm % casein and sterilized at 121°C for 20 minutes. For screening approximately 1.0 gm of different soil samples were added in sterile distilled water to prepare soil suspension. To get the better keratinase producing *Bacillus sp*. of bacteria, prepared soil suspensions were boiled at 70 °C for 15 minutes to kill other vegetative cells. Then, boiled soil suspension was serially diluted, 0.1 ml from each dilution was spreaded on casein agar plates and incubated at 37º C. Plates were regularly checked for colonies producing zone of clearance on the medium. Colonies showing zone of clearance were selected and further tested for keratinase production. Isolates were streaked on nutrient agar slants and stored in refrigerator by periodic transfer.

Morpholoical and cultural characterization: As per the standard protocol given in Bergie's Mannual of Diterminative Bacteriology, morpholoical and cultural characteristics of 10 isolates were recorded as Gram staining, motility test, endospore staining, colonial characteristics. Catalase test and caseinase activity were screened on plates.

Keratinase production: Prior to go for submerged production, these 10 isolates were screened using the method described by Paul *et.al.* (2013). Each isolate was inoculated as spot to

measure the ratio of colony diameter to zone of clearance. On the basis of results, AN2, AN5, AN6, AN8 were selected and tested for keratinase production.

Four selected isolates were tested for keratinase production using the method described by Zhang *et.al.* (2009). The inoculum culture was Luria-Bertani (LB) (10 g bactotryptone, 5 g yeast extract, 10 g NaCl, per liter; pH 7.2 – 7.6), and 20 ml of the feather meal medium (10 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, perliter; pH 7.0 – 7.2) in 100 ml Erlenmeyer ûasks were inoculated from 10^6 colony forming units (cfu)/ml culture and then cultivated at 37 ^oC temperature by shaking at 150 rpm for 72 h.. Samples were withdrawn at regular time interval of 24 h and tested for keratinase production.

Keratinase assay:

1.0 mL crude enzyme properly diluted in Tris–HCl buffer (0.05 mol/L, pH 8.0) was incubated with 1 mL keratin solution (HiMedia, India) at 50 ^oC in a water bath for 10 min, and the reaction was stopped by adding 2.0 mL 0.4 mol/L trichloro acetic acid (TCA). After centrifugation at 1,450 g for 30 min, the absorbance of the supernatant was determined at 280 nm using UV-Viz Spectrophotometer (Elico, India) against a control. The control was prepared by incubating the enzyme solution with 2.0 mL trichloro acetic acid (TCA) at 50 ^oC for 10 min before the addition of keratin solution.

One unit (U/mL) of keratinase activity was deûned as an increase of corrected absorbance at 280 nm (A280)[28] of 0.01 per minute under the conditions described [Thys *et.al.* (2004)].

Optimization of culture conditions:

Effect of temperature on keratinase production: The production medium of 100 ml Luria-Bertani (LB) in 250 ml Erlenmeyer flask. Inoculated flasks were incubated at different temperatures like 28 C, 37 C, 50 C and 60 C,150 rpm on rotary shaker and keratinase production was checked periodically.

Effect of pH on keratinase production: The production medium flasks containing 100 ml of above mentioned medium with different pH like 5.0, 7.0, 8.0 and 9.0 were inoculated with fresh inoculums. Inoculated flasks were incubated at 37 C, 150 rpm on rotary shaker and keratinase production was checked periodically.

Effect of substrates on keratinase production: Apart from pure nitrogenous sources, raw natural keratin sources like hair, wool and feather were checked for keratinase production. One gram each keratin source was added in the medium and keratinase production was checked for each substrate. Medium used for keratinase production contains 1 g raw keratin substrate, 0.1 g peptone, 0.3 g meat extract, 0.5 g NaCl. Inoculated flasks were incubated 37 C at 150 rpm in shaker and keratinase production was checked periodically.

Degradation of raw substrates: The selected culture was tested for degrading capacity of raw substrates like feathers, hairs and polyethylene plastic films in submerged condition.

Enzyme characterization: The optimum temperature and pH for keratinase activity was determined using crude enzyme.

To determine the optimum temperature for keratinase activity, 20 µl crude enzyme was added to 1.5 ml phosphate buffer (100 mM, pH 7.5) containing 15 mg powdered keratin and incubated at different temperatures of 28 ºC, 37 ºC, 50 ºC and 70 ºC for 20 minutes.

To determine the optimum pH for keratinase activity, 20 µl crude enzyme was added to 1.5 ml phosphate buffer (100 mM) with different pH of 5.0, 7.0, 8.0 and 9.0 containing 15 mg powdered keratin and incubated at 50 C for 20 minutes.

Effect of metal activators like Mg and inhibitor like EDTA was tested on crude keratinase. Both $MgCl_2$ and EDTA was tested at 5.0 mM concentration and added to 1.5 ml phosphate buffer (100 mM, pH 7.0) containing 15 mg powdered keratin with 20 µl crude enzyme and incubated at 50 ºC for 20 minutes. After incubation residual activity of the enzyme was determined.

Results and Discussion

Isolation of keratinase producing microorganism: For primary screening of keratinase producing bacteria different soil samples were screened using casein agar plates. Plates were regularly checked for colonies producing zone of clearance on the medium. Ten (10) bacterial colonies showing zone of clearance were observed on plates and they were further tested for keratinase production. Isolates were streaked on nutrient agar slants and stored in refrigerator by periodic transfer.

Morphological and cultural characterization: Morphological and cultural characteristics of these 10 bacterial isolates were studied. Colonial characteristics of these bacterial isolates are shown in table 1.

Table 1. Colonial characteristics of screened bacterial isolates

Isolate	Size	Shape	Margin	Elevation	Texture	Opacity	pigmentation
AN1	Small	Round	Entire	Convex	Smooth	Opaque	Off White
AN2	Big	Round	Entire	Slightly raised	Rough	Opaque	Cream
AN3	Large	Irregular	undulate	Flat	Rough	Opaque	Off White
AN4	Small	Round	Entire	Convex	Smooth	Opaque	Off White
AN5	Large	Irregular	undulate	Flat	Rough	Opaque	Off White
AN6	Big	Irregular	undulate	Slightly raised	Smooth	Opaque	Off White
AN7	Large	Irregular	undulate	Flat	Rough	Opaque	Off White
AN8	Medium	Irregular	undulate	Slightly raised	Smooth	Opaque	Off White
AN9	Small	Round	Entire	Convex	Smooth	Opaque	Off White
AN10	Small	Round	Entire	Convex	Smooth	Translucent	Pale yellow

Morphological characteristics were studied by Gram's staining, motility was observed by hanging drop preparation and endospore staining by Dornar's method. Two enzymes, catalase and caseinase were tested by the plate assay method.

Table 2. Morphological and biochemical characteristics of isolates

Isolate	Gram Staining	Motility	Endospores	Catalase Test	Caseinase test
AN1	Gram negative Short rods	Motile	Absent	Positive	Positive
AN2	Gram Positive Big rods	Motile	present	Positive	Positive
AN3	Gram Negative Short Rods	Highly Motile	Absent	Positive	Positive
AN4	Gram Positive Big Rods	Highly Motile	Present	Positive	Positive
AN5	Gram Positive Big Rods	Highly Motile	Present	Positive	Positive
AN6	Gram Positive Big Rods	Motile	Present	Positive	Positive
AN7	Gram Positive Big Rods	Motile	Present	Positive	Positive
AN8	Gram Positive Big Rods	Motile	Present	Positive	Positive
AN9	Gram positive Cocci	Motile	Absent	Positive	Positive
AN10	Gram Positive Big Rods	Motile	Present	Positive	Positive

Keratinase production: These 10 selected isolates were further tested for keratinase production on the skim milk agar plates by spot inoculation. Ratio of zone of clearance and diameter of the colonies were measured to get the better isolates. Out of these 10, four isolates were selected for submerged keratinase production.

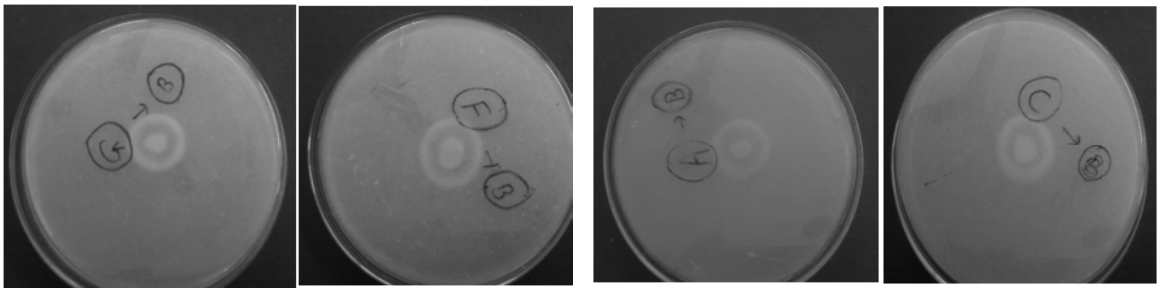

Figure 1: Zone of clearance produced by AN2, AN5, AN6 and AN8, spot inoculation

Keratinase production: Four selected isolates viz. AN2, AN5, AN6, AN8 were tested for keratinase production using the 100 ml medium in 250 ml flasks. The keratinase production of 12, 15, 16 and 21 U/ml was obtained after 48 h incubation at 30 ºC by AN2, AN5, AN6, AN8 respectively. The bacterial isolate AN8 showed the highest keratinase production hence was selected for further study. The AN8 is Gram positive, nonspore forming, motile rods.

Optimization of culture conditions:

Effect of temperature on keratinase production: The production medium flasks of 100 ml medium were inoculated by AN8 culture and flasks were incubated at different temperatures like 28 C, 37 C, 50 C and 60 C, 150 rpm on rotary shaker. The highest keratinase production of 70.4 U/ml was obtained at 37 ºC.

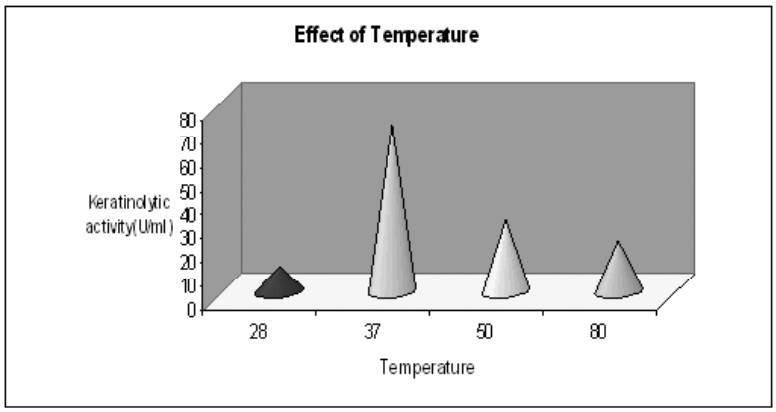

Figure 2: Effect of temperature on keratinase production by AN8

The optimal temperature and age of inoculum were found to be 28°C and 16 h, respectively, in previous studies in 2008 by Cai *et.al.* by using feather substrate.

Effect of pH on keratinase production: The production medium flasks containing 100ml of above mentioned medium with different pH like 5.0, 7.0, 8.0 and 9.0 were inoculated with fresh AN8 culture inoculums and incubated at 37 C, 150 rpm on rotary shaker. The pH of 7.0 found the optimum showing 21 U/ml of keratinase production.

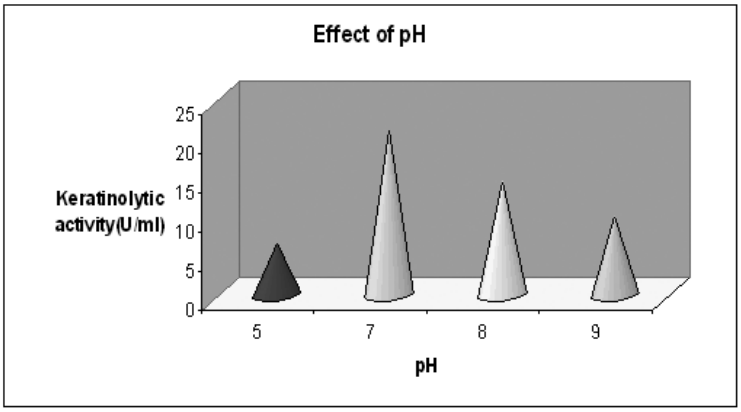

Figure 3: Effect of pH on keratinase production by AN8

Cai *et.al.* (2008) concluded that for feather substrate, the optimum initial pH value was 7.5 and the medium pH increased to a relatively stable level of about 8.5.

Effect of substrates on keratinase production: The isolate AN8 was tested for keratinase production using raw natural keratin sources like hair, wool and feathers. The keratinase production of 51, 68, 59,63 U/ml was obtained with wool, feather, keratin and hair, respectively. Feather meal waste found as the best raw source of keratin for AN8.

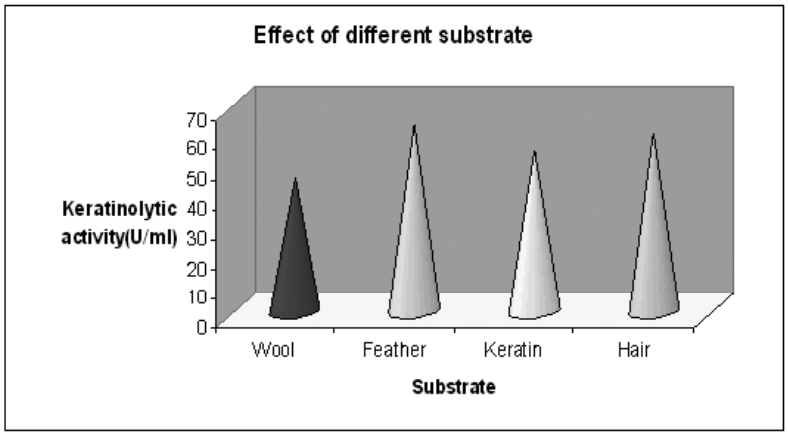

Figure 4: Effect of raw substrates on keratinase production by AN8

Degradation of raw substrates by AN8: The selected culture AN8 was tested for degradation of raw substrates like feathers and polyethylene plastic films in submerged condition along with medium. Whole feather and polyethylene sheet were degraded significantly after 5-10 days of incubation which is shown in figure 5.

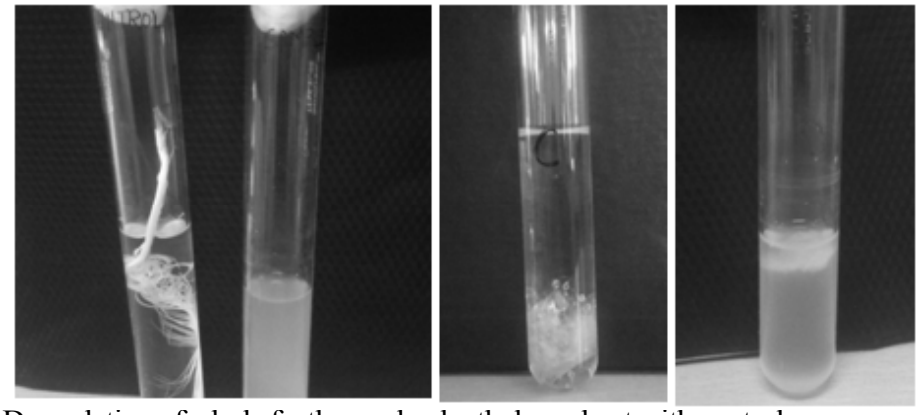

Figure 5. Degradation of whole feather and polyethelene sheet with control

In their experiment Suntornsuk*et.al* used different keratinous substrates where feather coffee was maximum degraded after 96 h of incubation at 37æ%C. However, In an another experiment Sanghvi*et.al.* achieved production of keratinase by adding feathers as source of keratin, which suggest that enzyme might be inducible by exogenous inducer in the culture condition. In some cases like *Chrysosporium keratinophylum*, keratinase was reported to be constitutively expressed as cell bound keratinolytic protease [Wang *et.al.* (2003)]. Cai *et.al.* (2003) used feather as substrate at a concentration of 10 g/L which was concluded to be optimum for keratinase production. In their experiment Cai and Zheng (2009) tested human hair at 2, 6, 10, 16 g/L and the results showed that, with the increase in hair concentration, the production of keratinase was increased to maximum at 16 g/L.

Enzyme characterization: The optimum temperature and pH for keratinase activity was determined using crude enzyme. The optimum temperature for keratinase activity was 50 ºC and the optimum pH for keratinase activity was 7.0. In presence of metal Mg and inhibitor EDTA, enzyme activity was decreased.

Conclusion

The keratinase producing bacteria were successfully isolated from different soil samples collected from poultry farms. Four better isolates were tested for submerged production of keratinase and isolate AN8 was selected. The keratinase production using AN8 was increased 3.35 times from 21 U/ml to 70.4 U/ml after optimization of physical parameters like temperature and pH. The isolate AN8 also showed degradation of raw substrates like feather, hair and wool as well as whole feather and polyethylene sheet. Therefore, enzymatic properties of the isolate AN8 could be further explored as this enzyme has many industrial applications.

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