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Isolation and screening of keratinase producing bacteria from soil

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ABSTRACT

Keratinase (E.C 3.4.99.11) is an extracellular protease having efficient ability to degrade keratin found in feathers and hair. It has various applications including leather, textile, pharmaceutical, detergent and animal feed industries. In current study, keratinolytic bacteria were isolated from the soil of poultry farm and different cultural conditions were optimized to obtain maximum enzyme yield. The isolates were initially screened by formation of zone of clearance on the skimmed milk agar and then evaluated for keratinase production by using raw feathers as substrate in submerged fermentation. Two isolates, showing maximum enzyme production, were identified as *Bacillus* species on their morphological and biochemical basis. Crude enzyme revealed that *Bacillus* sp. IIB-B5 produced maximum keratinase (28.13 U/mL) at pH 7.0, at 40^{o}C after 72 h incubation. While *Bacillus* sp. IIB-B9 showed maximum enzyme production (26.59 U/mL) at pH 8.0, after an incubation period of 96 h at 50^{o}C. This study showed that these isolates have the potential to be used in keratin hydrolysis.

Keywords: Keratinase, Keratin, Feathers, Keratinolytic bacteria

INTRODUCTION

Keratin is an abundant biopolymer in the world. It is tough and fibrous protein well known as the main structural constituent of nails, hair, feathers, claws, hides, bristles and scales (Gopinath *et al*., 2015). It is unreactive against chemicals as well as common proteolytic enzymes like pepsin, papin and trypsin (Kanchana, 2012). Compared to other proteases, keratinases are capable of degrading and hydrolyzing keratin efficiently.

Keratinase enzyme (E.C. 3.4.99.11) is a protease, having ability to convert insoluble and rigid keratin molecules into a fairly soluble protein or peptides (George *et al*., 2013). The mechanism of degradation of keratin by keratinolysis involves the steps such as sulfitolysis, proteolysis and deamination (Ramnani *et al*., 2005). A group of these enzymes can be produced by various insects and frequently produced by various species of different bacteria, different fungi and some actinomycetes, that may be found in places where keratinous material is deposited (Riffel & Brandelli, 2006). For almost all keratinases production, keratinous substances such as chicken feathers, wool and hair can be used as substrates (Gupta & Ramnani, 2006). However, non-keratinous substrates such as skimmed milk, casein, gelatin, soy bean meal or soy flour can also act as keratinase inducers (Casarin *et al*., 2008). Microbial keratinase production is affected by certain factors such as pH, temperature, nature of carbon and nitrogen sources, aeration in the medium, culture conditions and medium composition. These factors can be optimized to increase the production of keratinases (Subathra Devi *et al*., 2018).

Keratinases can degrade feathers and other keratin substances very efficiently, therefore it can be utilized for economical generation of animal feed and fertilizers. Their applications can be extended to leather, detergent, textile, pharmaceutical (Gradisar *et al*., 2005), cosmetic industries, prion decontamination, and biogas production, and also to improve the quality of silk and wool (Arokiyaraj *et al*., 2019 and Sousa *et al*., 2015). The present work aims at isolation, and screening of keratinase producing bacteria from poultry farm soils.

MATERIALS AND METHODS

Soil sampling

Fifteen different soil samples were collected from poultry farms and feather processing areas, near Kasur, and Lahore, Pakistan, for isolation of keratinase producing bacteria.

Author's Contribution: M. A., Carried out the research work; **Y. A.**, Helped in manuscript writing; **H. M.**, Supervised the research work

Substrate pretreatment

Chicken feather were taken from chicken slaughtering shops and were washed thoroughly using boiling water, followed by washing with tap water. After that they were dried at 60°C using hot air oven for 2 days and then they were stored under room temperature for further use.

Isolation and primary screening of keratinolytic bacteria

For isolation of Keratinase producing bacteria, samples of soil were serially diluted up to 10^{-7} in sterile distilled water, and 0.1 ml of each dilution of sample were inoculated on skimmed milk agar (peptone 0.5%, yeast extract 0.3%, Skimmed milk powder 5% and Agar 1.5%) of pH 7. The media plates were then incubated at 37^{o}C for the period of 24 h. After 24 h, plates were observed for the zone of hydrolysis. The diameter of each zone was measured. The bacterial colony which made larger zones of hydrolysis were selected for secondary screening and were maintained on nutrient agar slants (beef extract 0.3%, peptone 0.5g, and agar1.5%) of pH 7 at 4^{o}C for further study.

Secondary screening of bacteria for keratinase production

Twenty mL of nutrient broth was inoculated with single colony of the selected isolate, under sterile conditions. The culture was then incubated overnight (16 h), at 37 °C using 160 rpm. After 16 to 18 h 1 ml of the bacterial inoculum was added in basal salt medium, enriched with chicken feathers medium (NaCl 0.05%, $MgCl_2$. $6H_2O$ 0.01%, KH_2PO_4 0.03%, K_2HPO_4 0.04%, Glucose 0.5%, Yeast extract 0.2%, raw chicken feathers 1%) under aseptic conditions. The medium was incubated for the period of 72 h at 37°C in rotary shaking incubator, at 160 rpm. Following 72 h of incubation, centrifugation was done at 6000 rpm for 10 min and supernatant was collected to be used as a crude enzyme.

Keratinase assay

Enzyme activity was determined using the procedure described by MacDonald & Chen (1965), using casein as substrate (Gowdhaman & Ponnusami, 2014). A reaction mixture was prepared by adding 1ml of 1% casein and 1 ml of enzyme extract in the test tube and was then incubated at 40°C temperature, for 30 min. Reaction between an enzyme and its substrate was stopped by adding 5ml of 5% TCA (Tri-chloroacetic acid). The untreated casein was separated by centrifuging at 6000 rpm for 10 min. 5 ml of alkaline reagent, 1 ml of 1N NaOH was then added in 1 ml of supernatant and 1 ml of Folin & Ciocalteau (FC) reagent was added to produce dark blue color. A control tube was also prepared in which 5 ml of 5% TCA was added, before incubation. The optical density of the mixture was measured at 660 nm by using UV-spectrophotometer. One unit of enzyme is defined as amount of the enzyme needed to increase 0.1 optical density under optimally defined conditions.

Identification of keratinolytic strains

The isolates were identified based on their colony morphology and biochemical tests, including Catalase test, Gelatin hydrolysis test, Starch hydrolysis test, and Carbohydrate fermentation tests (Harrigan & MacCance, 2000).

Optimization of cultural conditions

To gain maximum keratinase from bacterial isolates, various cultural parameters were optimized. To check the effect of incubation time, the bacterial isolates were inoculated in fermentation media (already mentioned as “basal salt medium, enriched with chicken feathers medium”) and were allowed to grow at different pH values ranging from 5.0 to 8.5, with an interval of 0.5. The effect of time was studied by allowing the bacteria to grow for various time periods such as 24, 48, 72 and 96 h. The effect of changing temperature, on keratinase enzyme production, was also studied using the range of 30^{o}C to 65^{o}C with 5^{o}C intervals.

RESULT

Screening of keratinase producing isolates

In the present study, 25 bacterial strains were isolated from soil samples collected from different poultry farms. In order to find the most productive strains for keratinases production, primary screening of these strains was carried out on the basis of forming zones of hydrolysis on skimmed milk agar. Six bacterial isolates made relatively larger zones of hydrolysis. Among six bacterial isolates, two strains named as IIB-B5 and IIB-B9 showed maximum clearance zones measuring 12mm and 9mm, respectively, while

other strains 11B-B1, IIB-B24, IIB-B15 and IIB-B20 formed zones of 8.5mm, 7mm, 6.5mm and 6mm, respectively as shown in Fig. 1.

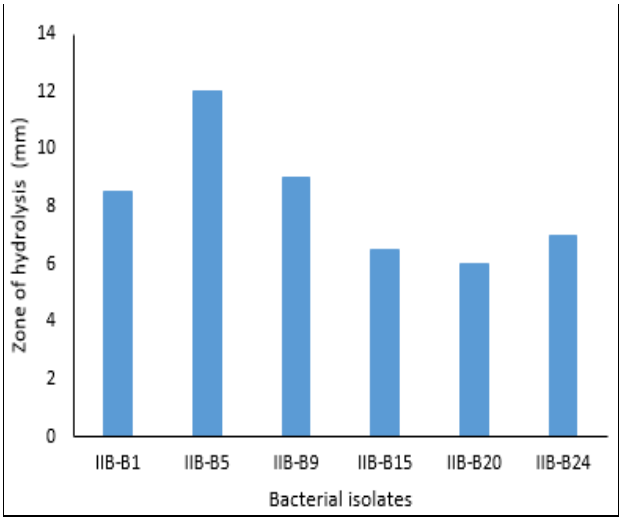

Fig. 1: Diameters of zone of hydrolysis formed by different bacterial isolates on skim*med* milk agar.

The cultivation was performed at initial pH 7.0, temperature 37°C for 24 h.

Secondary screening for keratinase production

Secondary screening of isolates was carried out in submerged conditions in which raw chicken feather were used as sole source for nitrogen and carbon. The result revealed that the bacterial strain IIB-B20 showed lowest production i.e., 8.36 U/mL. However, production of keratinase 17.8, 16.01, 14.24 U/mL from strains IIB-B1, IIB-B24 and IIB-B15 respectively was quite encouraging. A marked increase of 22.81 U/mL and 19.12 U/mL in enzyme activity was observed with IIB-B5 and IIB-B9, respectively (Table I). Out of six isolates, IIB-B5 and IIB-B9 were considered as best enzyme producers and were selected for further study and identification.

Identification of keratinolytic bacterial isolates

Based on biochemical and morphological characteristics the bacterial strain IIB-B5 and IIB-B9 were Gram positive, straight rods, and endospore formers, aerobes, motile, catalase positive and also hydrolyzed starch. Complete biochemical characters are shown in (Table II).

Table I: Screening of bacterial isolates for the production of Keratinase under submerged fermentation (SMF)

Sr. No of isolates	Strain coding	Enzyme units (U/mL)
1	IIB-B1	17.8
2	IIB-B5	22.81
3	IIB-B9	19.12
4	IIB-B15	14.24
5	IIB-B20	8.36
6	IIB-B24	16.0

Table II: Morphological and Biochemical characteristics of bacterial isolates for identification

Details of experiment	Observation	
	IIB-B5	IIB-B9
Shape of bacteria	rod	Rod
Endospore formation	+ve	+ve
Gram character	+ve	+ve
Colony characteristics		
Growth	Rapid	Rapid
Shape	flat	Circular flat
Margin	Undulate	Smooth entire
Color	Creamy white	Off white
Opacity	Opaque	Opaque
Biochemical characteristics		
Catalase	+ve	+ve
gelatinase	+ve	+ve
Caseinase	+ve	+ve
Starch hydrolysis	+ve	+ve
Carbohydrates fermentation		
Glucose	+ve	+ve
Maltose	+ve	+ve
fructose	+ve	+ve
Sucrose	-ve	+ve
Lactose	-ve	-ve

+ve = positive result
-ve = negative result

Optimization of culture parameters for keratinase production

Effect of pH

Effect of pH on keratinases production by both *Bacillus* species is depicted in fig. 2.

Keratinase production was studied using different pH values ranging between 5.0 and 8.5. Optimum keratinase production (23.8 U/mL) from *Bacillus* sp. IIB-B5 at pH 7.0 and (25.19 U/mL) from *Bacillus* sp. IIB-B9 at pH 8.0 was observed. While least enzyme production was noticed at pH value 5.0.

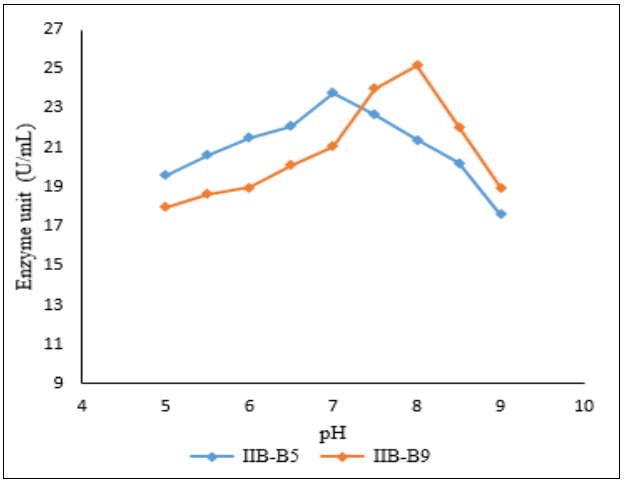

Fig. 2: Effect of pH on Keratinase production by *Bacillus* sp. IIB-B5 and IIB-B9

The cultivation was performed at 37°C and 160 rpm for 24 h with 1% 16-h-old inoculum in 10 g/L chicken feathers.

Effect of incubation time

The incubation time effect on enzyme production is shown in fig. 3. The keratinase secretion was started after 24 h but the optimum enzyme production (28.13 U/mL and 26.59 U/mL) was observed at an incubation period of 72 h and 96 h by *Bacillus* sp. IIB-B5 and IIB-B9, respectively.

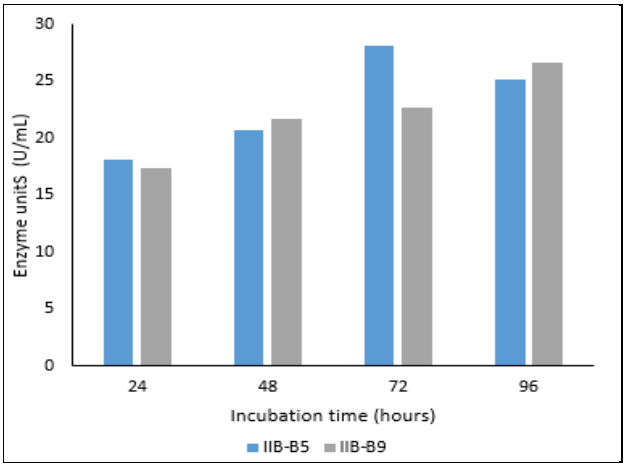

Fig. 3: Effect of incubation time on keratinase production by *Bacillus* sp. IIB-B5 and IIB-B9

The cultivation was performed at pH 7.0 and 8.0 for strain IIB-B5 and strain IIB-B9, 37°C and 160 rpm, with 1% 16-h-old inoculum in 10 g/L chicken feathers.

Effect of temperature

Keratinase production by *Bacillus* sp. IIB-B5 and IIB-B9 was observed at a wide range of temperature (30-65°C) and it was found to be maximum (28.16 U/mL and 25.2 U/mL) by *Bacillus* sp. IIB-B5 and IIB-B9 at temperature 40°C and 50°C respectively (Fig. 4).

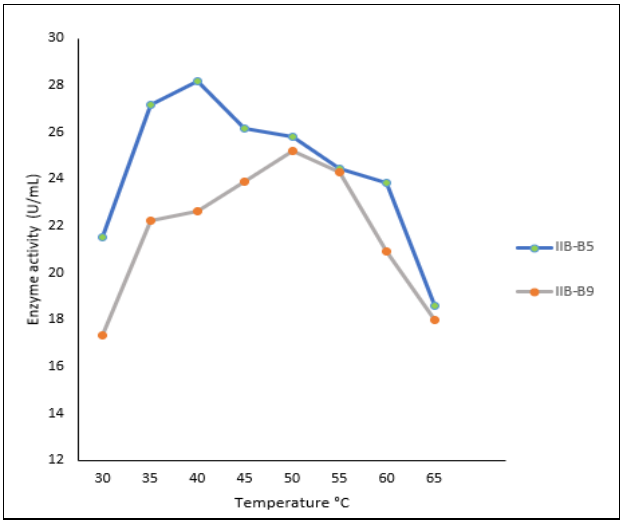

Fig. 4: Effect of temperature on keratinase production by *Bacillus* sp. IIB-B5 and IIB-B9

The cultivation was performed at pH 7.0 and 8.0 for strain IIB-B5 and strain IIB-B9, at 160 rpm, for 3 to 4 days, with 1% 16-h-old inoculum in 10 g/L chicken feathers.

DISCUSSION

Twenty five bacterial strains, producing keratinases, were isolated from soil samples. These isolates were primarily screened on the basis of formation of clear zone of hydrolysis on skimmed milk agar. The isolates which made clear zones on skimmed milk agar were considered as best protease producing isolates. The two bacterial isolates IIB-B5 and IIB-B9 made maximum zone of clearance of 12 mm and 9 mm respectively. Secondary screening of these species was done in order to find most productive strain for extracellular keratinase. Bacterial isolate IIB-B5 and IIB-B9 were observed with the highest enzyme production (22.81 U/mL and 19.12 U/mL, respectively). The

morphological and biochemical characteristics of these bacterial isolates were similar with features of species of genus *Bacillus* as described in Bergey's Manual of Systematic Bacteriology (Suntornsuk et al., 2005), which suggested that the bacterial isolates under study belonged to genus *Bacillus* (Sahoo *et al*., 2012).

Keratinase production was studied using different pH values ranging from 5.0 to 8.5. Maximum keratinase production (23.8 U/mL) using *Bacillus* IIB-B5 at pH 7.0 and (25.19 U/mL) using *Bacillus* IIB-B9 at pH 8.0 was observed. The increase in the keratinase production might be due to that raw feather more accessible for degradation at this optimum pH. The enzyme production by both strains started to decline after optimum pH. This could be due to the fact that a mild change in hydrogen ion concentration caused the change of polarity of the cell membrane due to ionization of certain amino acids present in bilayer of cell membrane. The results were in accordance to those reported by Jeevana *et al*. (2013) in which they isolated two bacterial strains, *Bacillus subtilis* and *Bacillus cereus*, which produced enzyme at optimum pH 8.

Different incubation periods for maximum production of keratinase were studied in raw chicken feather medium. *Bacillus* IIB-B5 and *Bacillus* IIB-B9 produced maximum enzyme (28.13 U/mL and 26.59 U/mL) at an incubation period of 72 h and 96 h, respectively. Further increase in incubation period caused decrease in enzyme production. This decrease in keratinase production might be due to the depletion of nutrients in medium which caused the cell to enter stationary phase in which secondary metabolites were synthesized. These metabolites usually interfere with enzyme conformation. The results were in accordance with results reported by Ire & Onyenama (2017), with maximum enzyme production at 72 h for *B. licheniformis*. While Sivakumar *et al*. (2012) reported maximum keratinase production after 72 h by *Bacillus thuringiensis*.

Production of keratinase is greatly affected by temperature change. The production of enzyme was also studied on different temperatures from 30 to 65^{o}C. The maximum enzyme (28.16 U/mL) was produced by *Bacillus* sp.IIB-B5 at temperature 40^{o}C and maximum enzyme (25. 2 U/mL) was produced by *Bacillus* sp. IIB-B9 at temperature 50^{o}C. Further increase in temperature caused the decrease in enzyme production, this might be due to that the higher temperature caused the denaturation of enzyme or microorganism could not survive at this temperature. This showed that both isolates might be mesophiles and higher temperature was not favorable for the secretion of keratinase. The results were in accordance with the results described by Sahoo *et al*. (2012) with optimum enzyme production at 40^{o}C for *Bacillus weihenstephanensis*, while Silva *et al*. (2014) also isolated bacterial strains among which *Bacillus subtilus* was the one, which showed maximum enzyme activity at 50^{o}C.

The study showed that isolates IIB-B5 and IIB-B9 had good capacity for keratinase production. These microbes completely degrade chicken feathers within 3 to 4 days. Hence these isolates may be the potential candidates for keratinase production at industrial scale.

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