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Enhancement of Keratin Degrading Ability of *Bacillus* sp. Through Optimization of Submerged Fermentation

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Abstract. Keratinases have a broad range of bio-technological, pharmaceutical, industrial, agricultural and eco-friendly waste management applications owing to their ability of degrading insoluble keratin waste. The present study aims to enhance keratinase production by optimizing some production parameters during sub-merged fermentation, using *Bacillus* sp. In this study, total 26 bacterial strains were isolated from four different localities in Lahore *i.e*. Tollinton market, chicken shops in Faisal town, Johar town and Walton cantt and screened for proteolytic activity on skim milk agar. After screening for keratinolytic activity using chicken feathers as a substrate, five strains were selected. These selected strains were identified as *Bacillus* sp. based on their micro-morphology and biochemical tests. Out of all the selected FK-22 exhibited the maximum keratinase activity (6.86 U/mL) which was further optimized for enhanced keratinase production. The highest yield of 19.04 U/mL was obtained using medium 1 at pH 8 after 7 days of incubation at 40 °C.

Keywords: keratinolytic activity, bacterial keratinase, chicken feathers, submerged fermentation, optimization

Introduction

Keratinases (E.C. 3.4.99.11) are proteolytic enzymes capable of degrading highly stable and insoluble protein, keratin. They are bio-technologically important class of proteases as they cause the hydrolysis of highly cross-linked and rigid polypeptide keratin. They belong to subtilisin family and are characterized as serine or metal proteases (Brandelli *et al*., 2010). The molecular weight of keratinase varies between the range of 14-240 kDa, depending upon the source from where it has been isolated (Lopes *et al*., 2008).

Keratin is hard, fibrous and insoluble protein that is resistant to some proteases owing to their structural stability and configuration. Generally, keratins are categorized as type-I (acidic) and type-II (basic) keratins. Structural stability of keratin is due to the highly packed protein chains in α-helix and β-sheets by the crosslinking of disulfide bridges present between the cystine residues which are present in high concentration in the keratin *i.e*. 3-15%. Due to these strong interactions, keratins are difficult to degrade (Lange *et al*., 2016).

Poultry farms or poultry processing industries produce million tons of feathers as waste worldwide. The keratinous waste can be degraded both chemically and biologically. The biological degradation of feathers by keratinase producing micro-organisms is more effective and eco-friendly than chemical degradation (Arokiyaraj *et al*., 2019). Two main stages are involved in the degradation of keratin, sulphitolysis and proteolysis. During sulphitolysis, disulphide bonds crosslinking the polypeptide chains break and release cysteine and S-sulphocysteine residues. The S-sulphocysteine produced is further oxidized to sulfite and sulphates. The sulphitolysis provides active sites to alkaline proteases. Proteolysis, the second stage, involves the degradation of keratin by the action of other proteases (Yamamura *et al*., 2002; Kunert *et al*., 1989).

Keratinases are produced from diverse bacterial and fungal sources (Kachuei *et al*., 2012). Keratinolytic bacteria have advantage over the fungi as they can grow faster than fungi. Mostly reported keratinase producing bacteria are *Bacillus* sp. including *B. subtilus* and *B. licheniformis* (Kornillowicz and Bohacz, 2011). The *Bacillus* species have advantage over the other bacterial strains in enzyme production that they can act on keratin more efficiently and secrete high amounts (20-25 g/L) of extracellular enzyme. Their ability to produce extracellular enzyme in acidic, neutral and alkaline environment and at high temperature made them important enzyme producers (Schallmey *et al*., 2004).

There are many applications of keratinases and their related products. Keratinase from different *Bacillus* sp.

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is extensively used for de-hairing purpose in leather industry (Kalaikumari *et al*., 2019). Keratinases isolated from *Bacillus licheniformis* (PWD-1) have reported to degrade the infectious form of prions in the presence of detergent and heat that are used in animal feed (Parashar *et al*., 2018). Keratinase enzyme is also used in poultry industry for the treatment of waste, in production of cosmetics and medicines and used for plants as a nitrogenous fertilizer (Zerdani *et al*., 2004). Apart from this, the feather hydrolysate produced by keratinase action is also used to improve the quality of animal feed, fertilizers, films, glues and act as a source of some amino acids like serine, cysteine and proline (Cai and Zheng, 2009).

Due to its extensive applications, there is a need to enhance the yield of keratinase by optimizing several production parameters. The main production source in medium is keratin that meets the nitrogen and carbon requirement for bacterial growth. The addition of other carbon and nitrogen sources have been used to increase the enzyme's yield. The optimum conditions may vary for different micro-organism. Different parameters such as temperature, pH, time, aeration and media composition need to be optimized to enhance the production of an enzyme (Brandelli *et al*., 2010). This paper presents the optimization of production parameters including medium selection, medium pH, incubation temperature and incubation time for intensified keratinase production under sub-merged fermentation we have evaluated the optimum conditions for keratinase production by using the chicken feathers as a sole nitrogen and carbon source in the production medium.

Materials and Methods

Isolation and screening of micro-organism. Soil samples were collected from four distinct poultry sites of Lahore *i.e*. Tollinton market, chicken shops in Faisal town, Johar town and Walton cantt. Collected soil samples were used for the isolation of keratinolytic bacteria on skim milk agar (skim milk 3 g/L, casein 5 g/L, glucose 1 g/L, yeast extract 2.5 g/L, agar 12.5 g/L, pH 7) for proteolytic activity as primary screening. The skim milk was autoclaved separately for 10 min to prevent coagulation of milk protein. The plates were incubated for 24 h at 37 °C in an incubator. Clear zones appeared around the bacterial isolates after incubation and they were selected by comparing their zone diameter. Sub-culturing was performed at a regular interval to maintain the viability of cultures. Isolated bacterial strains were further screened to select the most potent keratinolytic strain using submerged fermentation with chicken feathers as a substrate.

Morphological and biochemical studies of bacterial isolates. The selected bacterial isolates were identified by their morphology and some preliminary biochemical tests *i.e*. gram's staining, catalase test, starch hydrolysis, casein hydrolysis and gelatin hydrolysis test. The colony characteristics of selected bacterial isolates were observed on skim milk agar plates and their micro-morphology were examined microscopically.

Inoculum preparation. Sterilized nutrient broth medium was inoculated with a loopful of bacterial culture from the preserved slants and incubated at 37 °C for 24 h in a shaking incubator. This 24 h old culture was used as inoculum for fermentation.

Pre-treatment of substrate. Chicken feathers were pre-treated before using them as a substrate in fermentation medium. They were washed extensively with anionic detergent. Then, tap water was used to remove the detergent. Washed feathers were dried in a hot air oven at 50 °C for overnight (Mazotto *et al*., 2011).

Fermentation procedure. The fermentation medium described by Mukhtar and Haq (2013) was used initially for screening of keratinolytic bacteria. 2% inoculum was added in 50 mL of the fermentation medium, aseptically. The flasks were then incubated at 37 °C and 160 rpm in a shaking incubator for 7 days.

Optimization of keratinase production conditions. The optimum culture conditions for keratinase production were determined by using three different fermentation media *i.e*.

- Medium 1 (protease medium) contained 1% substrate (chicken feathers), 1% glucose, 1% polypeptone, 0.5% Na_2CO_3 and 1% KH_2PO_4, adjusted at pH 7.5 (Mukhtar and Haq, 2013)
- Medium 2 (feather basal medium) contained 1% substrate, 1% yeast extract, 0.05% NH_4Cl, 0.05% NaCl, 0.04% KH_2PO_4, 0.02% $MgCl_2$ adjusted at pH 7.5 (Pandian *et al*., 2012)
- Medium 3 (Horikoshi medium) contained 1% substrate, 0.5% starch, 0.5% peptone, 0.5% glucose, 0.1% Na_2CO_3, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.5% yeast extract adjusted at pH 7.5 (Agrahari and Wadhwa, 2010).

The fermentation flasks were incubated for different time interval *i.e*. 5, 6, 7, 8 and 9 days with pH ranges

7, 7.5, 8, 8.5 and 9, and at different temperature *i.e*. 30, 37, 40 and 45 °C.

Down streaming of keratinase. The fermented broth was filtered using a muslin cloth after fermentation. Residual feathers were obtained and dried in a hot air oven at 50 °C for overnight. The filtrate obtained, was centrifuged at 6000 rpm for 10 min in a bench top centrifuge. The crude enzyme was obtained in a supernatant as keratinase is an extracellular enzyme. The supernatant was then analyzed for enzyme activity.

Analytical methods. ***Determination of feather degradation.*** The extent of feather degradation was calculated by determining its weight loss. Dried weight of feathers was considered as an initial feather weight, whereas the residual weight after fermentation was the final feather weight. The percentage of weight loss can be calculated by using the following formula (Yuhong *et al*., 2015):

$$\% \text{ degradation} = \frac{\text{initial feather weight - final feather weight}}{\text{initial feather weight}} \times 100$$

Enzyme assay. The keratinase activity was estimated by the protocol based on protease assay devised by McDonald and Chen (1965). 1% solution of Casein were prepared in a 0.1 M phosphate buffer of pH 8. Two reactions *i.e*. control and experimental were carried out separately. 1 mL enzyme and 1 mL casein were taken in experimental tube, while 1 mL enzyme, 1 mL casein and 5 mL TCA were taken in a control tube. Both tubes were incubated at 40 °C for 30 min in a water bath. After incubation, 5 mL TCA was added in experimental tube to stop the reaction. Both tubes were centrifuged at 6000 rpm for 10 min in a bench top centrifuge. 1 mL supernatant was taken from both tubes separately and 5 mL alkaline reagent was added in them. After that, 1 mL NaOH was added to the tubes and incubated at room temperature for 10 min. After 10 min of incubation, 1 mL Folin and Ciocalteau reagent in a ratio of (1:1) with distilled water was added in both tubes and incubated for 30 min to produce blue colour. The absorbance of mixture was measured through spectrophotometer at 660 nm. 1 mL distilled water was used instead of supernatant as blank, while rest of the agents were same.

One unit of keratinase activity is defined as, the amount of enzyme required to produce an increase 0.1 in optical density at 660 nm under defined conditions.

Results and Discussion

Isolation and screening of organism. Twenty six bacterial strains were isolated. Out of the total isolates, the diameter of the ten strains with maximum clearance zone are recorded in Table 1. Bacteria having the proteolytic activity hydrolyzed the milk protein and formed clear zones. Out of ten selected isolates, five strains *i.e*. FK-11, FK-18, FK-19, FK-22 and FK-23 showed greater proteolytic activity during fermentation. Among those five strains, FK-22 showed maximum keratinolytic activity, *i.e*. 6.86 U/mL with 67.06% feather degradation (Fig. 1).

Morphological and biochemical studies of isolates. Based on the morphological and biochemical testing,

Table 1. Diameter of clearance zones on skim milk agar by bacterial isolates from different poultry sites

Sample sites	Bacterial isolates	Diameter of zones of clearance (mm)
SK-1	FK-2	10 ± 0.01
SK-2	FK-8	10 ± 0.02
SK-2	FK-11	12 ± 0.01
SK-3	FK-13	10.5 ± 0.01
SK-3	FK-15	10 ± 0.01
SK-3	FK-17	10 ± 0.02
SK-4	FK-18	12 ± 0.02
SK-4	FK-19	12 ± 0.01
SK-4	FK-22	14 ± 0.02
SK-4	FK-23	15 ± 0.01

where: SK-1 = Faisal town; SK-2 = Johar town; SK-3 = Walton cantt; SK-4 = Tollinton market; ± = Standard deviation.

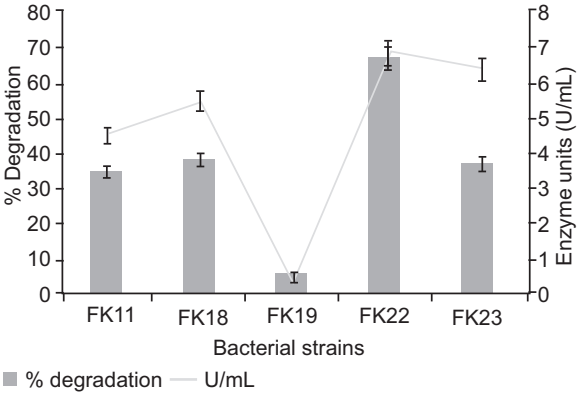

Fig. 1. Screening of bacterial isolates. Y-error bars represented the standard deviation (SD ≤ ± 0.05) between three replicates.

all the tested strains were identified to be *Bacillus* sp. The results are shown in Table 2. Henceforth, FK-22 was selected for further experimentation which involved optimization of keratinase production using chicken feathers as a substrate.

Selection of fermentation media. The effect of three different media on keratinase production was studied. Medium 1 gave the highest yield (11. 91 U/mL), while the other two media *i.e*. medium 2 and medium 3 gave 11.03 U/mL and 3.59 U/mL, respectively (Fig. 2). Medium 1 was selected due to high keratinase units and feather degradability *i.e*. 41%. The glucose present in medium 1 may have acted as an inducer that enhanced enzyme activity as glucose and peptone are reported to have a positive effect on keratinase production Ramnani and Gupta (2004). This medium gave the best results for protease production, as described by Mukhtar and Haq (2013). However, Agrahari and Wadhwa (2010) reported maximum keratinase activity and feather degradation with feather basal medium.

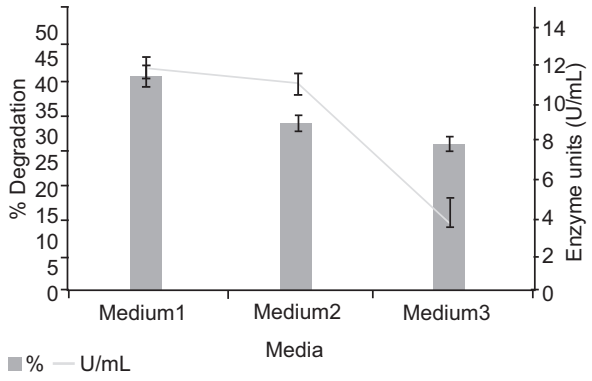

Fig. 2. Enzyme activity and % degradation using different fermentation medium for keratinase production by FK- 22. Y-error bars represented the standard deviation (SD ≤ ± 0.05) of three replicates.

Effect of incubation time. The effect of incubation time was studied and maximum keratinase activity was observed in 7 days old incubated flask *i.e*. 12.08 U/mL having 67.06% feather degradation. An increasing trend was observed from 5-7 days and then there was a decrease in activity from 7-9 days as shown in Fig. 3. The decrease in enzyme activity by increasing the incubation time was might be due to the depletion of nutrients and decrease in substrate concentration. However, feather degradation showed an increasing trend from 7-9 days. This increase in degradation can be attributed to the fact that keratinolysis can also takes place due to disruption of keratin's disulphide bridges in the presence of thiol, which tends to increase in the medium during cultivation (Bach *et al*., 2011). Similar results were reported by Sahoo *et al*. (2012) with *B. weihenstephanensis* and Dhiva *et al*. (2020) with *Bacillus pumilus*. However, results reported by Saibabu *et al*. (2013) were contrasting, showing maximum keratinase production by *B. megaterium* at 72 h.

Effect of pH. During the pH optimization for keratinase activity, maximum activity, *i.e*. 14.35 U/mL and feather

Table 2. Morphological and biochemical identification of selected strains

Tests	Bacterial strains				
	FK- 11	FK- 18	FK- 19	FK- 22	FK- 23
Colony characteristics					
Colour	White	White	White	White	White
Shapes	Rounded	Rounded	Rounded	Rounded	Rounded
Transparency	Opaque	Opaque	Opaque	Opaque	Opaque
Margin	Entire	Entire	Undulate	Undulate	Entire
Elevation	Raised	Raised	Flat	Raised	Raised
Gram's behaviour	positive	positive	positive	positive	positive
Bacterial morphology	Rods	Rods	Rods	Rods	Rods
Biochemical characteristics					
Catalase test	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+
Starch hydrolysis test	+	+	+	+	+
Gelatin hydrolysis test	+	+	-	+	+

degradation (50%) were observed at pH 8. Above pH 8, a decline trend in enzyme activity was noted as shown in Fig. 4. This may be due to an irreversible inactivation of enzyme which takes place by change in pH that alters the enzyme structure by ionization or deionization of acidic or basic groups (Robinson, 2015). Keratinase was found to be active at neutral to alkaline pH because between this range cystine reacts with sulphite, releasing cysteine and S-sulphocystein. Our results are in agreement with those reported by Nnolim and Nwodo (2020) *i.e*. maximum keratinase activity of *Bacillus* sp. at pH 8. Abdel-Fattah *et al*. (2018) reported similar trend, *i.e*. maximum keratinase activity of *B. licheniformis* from pH ranges 7-9 days.

Effect of incubation temperature. The effect of temperature on keratinase production was studied and increasing trend was observed from 30 to 40 °C. There was a decrease in activity at 45 °C as shown in Fig. 5. Keratinase activity was observed maximum at 40 °C *i.e*. 19.04 U/mL with 83.2% feather degradation. The maximum feather degradation under optimized conditions is shown in Fig. 6(d). The reason behind this might be that the temperature of the system affects the kinetic energy of the reacting molecules which increases by increasing the temperature of the system and thus,

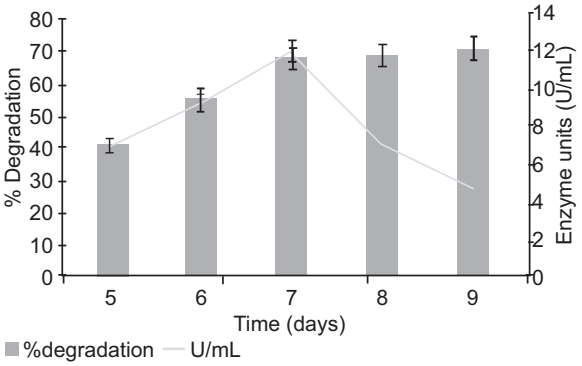

Fig. 3. Effect of incubation time for keratinase production by FK- 22. Y-error bars represented the standard deviation (SD ≤ ± 0.05) of three replicates.

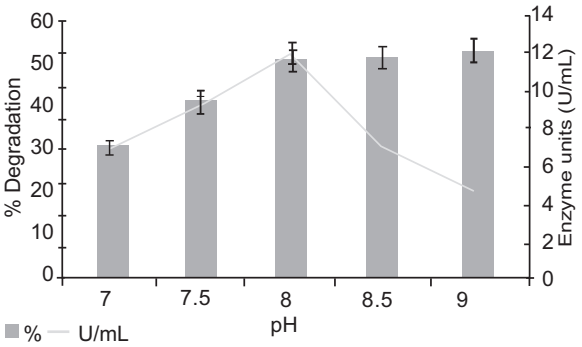

Fig. 4. Effect of medium pH for keratinase production by FK- 22. Y-error bars represented the standard deviation (SD ≤ ± 0.05) of three replicates.

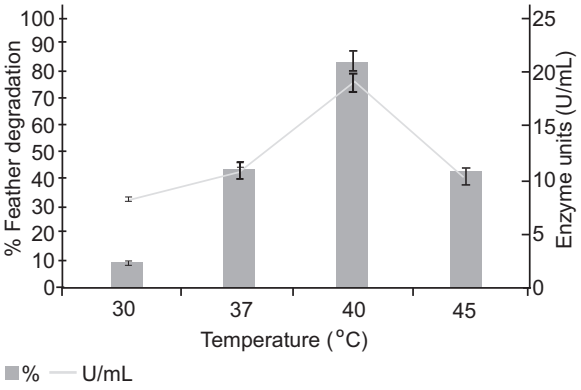

Fig. 5. Effect of incubation temperature for keratinase production by FK- 22. Y-error bars represented the standard deviation (SD ≤ ± 0.05) of three replicates.

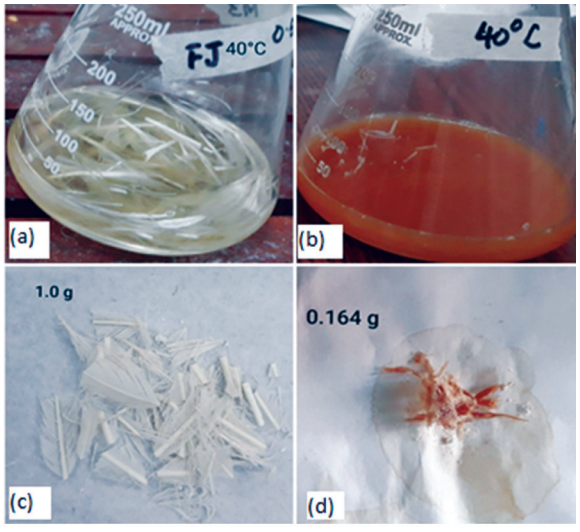

Fig. 6. (**a**) Medium before fermentation, (**b**) fermented broth at 40 °C, (**c**) feathers before fermentation and (**d**) degraded feathers after fermentation.

require by the reacting molecules to convert into products. However, enzyme activity decreases above the optimum temperature because enzyme loses its structure and becomes denatured (Robinson, 2015). Singh *et al*. (2017) reported similar results with *B. subtilis,* having best activity at 40 °C. Contrasting result was reported by Gafar *et al*. (2020) with *Bacillus* sp. that gave maximum keratinase activity at 30 °C. Another study reported *B. licheniformis* and *B. brevis* to show maximum activity between 40 and 45 °C (Sivakumar *et al*., 2011).

Conclusion

In the present study, the keratinolytic strains were isolated from soil samples of poultry sites. After optimization of production parameters, keratinase activity of FK-22 was increased upto 3 folds. Hence, we can conclude that the yield of keratinases can be further enhanced to several folds by optimizing some other production parameters. As keratin degraders are eco-friendly, therefore, scaling up the production of keratinase aided by molecular cloning or expression of keratinase gene into a suitable host organism will enhance its value in various industrial processes. Moreover, molecular sequencing is an important step for the identification of strain to the species level for future use. Further development of more effectual methods to produce keratinase will broad its applications in environmental waste management. Novel applications of keratinases continue to emerge as research advances.

Conflict of Interest. The authors declare they have no conflict of interest.

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