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Isolation and characterization of feather degrading bacteria from Mauritian soil

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This study is aimed at isolating and characterizing new culturable feather degrading bacteria from soils of the University of Mauritius Farm. Bacteria that were isolated were tested for their capability to grow on feather meal agar (FMA). Proteolytic bacteria were tested for feather degradation and were further identified according to their morphological and biochemical characteristics. All of the three isolates were from the *Bacillus* genus. Maximum enzyme activity was obtained from isolate DF1a. Maximum keratinolytic activity of 128 U/ml and maximum proteolytic activity of 373 U/ml were obtained. The optimal conditions for the keratinolytic activity of this strain were determined to be pH 8.0 and temperature of 70°C. In addition, this isolate was able to completely degrade a whole chicken feather within a period of 14 days at room temperature (28±2°C). These three *Bacillus* strains (DF1a, DF2b and DF3) are therefore promising strains for the management of chicken feather waste through biotechnological processes.

Key words: *Bacillus*, keratinolytic proteases, keratin waste management.

INTRODUCTION

Worldwide around 25 billion chickens are killed annually. In Mauritius, the annual production of poultry meat is about 50,000 tonnes, with an annual increase of about 4.5%. Feathers represent 5 to 7% of the total weight of mature chickens. As a result, poultry processing plants produce a substantial amount of feathers as waste by-product and represent a sizable waste disposal problem. At present these are either buried in landfills or incinerated in a power plant generator boiler. Although land application is an option, continued application can result in extreme high soil nitrogen levels with run-off contaminating streams and ground water with both chemicals and bacteria. A current value-added use for feather is its conversion, following treatment at high temperature and milling, to feather meal/ animal flour and used as a protein supplement into feed mixtures of domestic animals. However, production of feather meal is an expensive process which destroys certain amino acids, yielding a product with poor digestibility and

variable nutritional quality (Papadopoulos et al., 1986).

Keratins are highly specialized fibrous proteins also known as scleroproteins and occur abundantly in keratinized epidermis and corneous skin products such as hair, nails, claws, hooves, horns, scales, beaks and feathers. Feather is pure keratin protein and is insoluble and hard to degrade due to highly rigid structure rendered by extensive disulphide bond and cross-linkages. The keratin chain is insoluble, high stable structure tightly packed in the α -helix (α -keratin) and β -sheets (β -keratin) into super coiled polypeptide chain (Parry and North, 1998). 90% of the feather contains β -keratin by mass (Onifade et al., 1998) and β -keratin is extensively cross linked. Cross-linking of protein chains by cysteine bridges confers high mechanical stability and resistance to proteolytic degradation by pepsin, trypsin and papain. The disulphide bonds of β -keratin can be reduced by the enzyme disulphide reductase (Yamamura et al., 2002) followed by proteolytic keratinases (Gupta and Ramnani, 2006). Feather can be utilized so that it can be used as animal feed, this can prevent accumulation of feather in the environment and decrease the development of pathogenic strains. Biotechnological

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processing of feathers for the production of feather meal, instead of chemical processing is preferred as it preserves the essential amino acids (Methionine, Lysine, Histidine) (Riffel et al., 2003).

Keratinolytic microorganisms and their enzymes may be used to enhance the digestibility of feather keratin with important applications in processing keratin-containing wastes from the poultry industry and the nutritional upgrading of feather meal which could replace as much as 7% of the dietary protein for growing chicks (Odetallah et al., 2003). Following hydrolysis, the feathers can also be converted to glues, films and the source of rare amino acids, such as serine, cysteine and proline (Gupta and Ramnani, 2006; Cai et al., 2008; Cao et al., 2009).

The nutritional upgrading of feather meal through microbial or enzymatic treatment has been described. Feather meal fermented with *Streptomyces fradiae* and supplemented with methionine resulted in a growth rate of broilers comparable with those fed isolated soybean protein (Elmayergi and Smith, 1971). The use of feather-lysate from *Bacillus licheniformis* with amino acid supplementation produced a similar growth rate in chickens when compared to chickens fed with a diet that included soybean meal (Williams et al., 1991). The crude keratinase enzyme produced by *B. licheniformis* significantly increased the total amino acid digestibility of raw feathers and commercial feather meal (Lee et al., 1991).

In this study, we describe the isolation of bacteria from the University of Mauritius farm showing keratinolytic activity.

MATERIALS AND METHODS

Preparation of substrates and media

White chicken feathers were used in this study to prepare the pure feather meal powder. They were first washed extensively under tap water to remove blood and any dust particles. This was followed by washing with 0.1% Triton X-100 and then abundantly with distilled water. All the materials were later oven-dried at 75°C for 8 h. The dried keratin materials were chopped into pieces not exceeding 1.5 cm in length, and then milled to 60-mesh particle size. The powders were kept at room temperature and used for further studies. For the primary screening, skimmed milk agar was used comprising of 5.0 g/l peptone, 3.0 g/L yeast extract, 100 ml/l UHT skimmed milk, and 12 g/l agar. The minimal medium was prepared as follows (g/l): K₂HPO₄, 0.3; KH₂PO₄, 0.4; yeast extract, 0.1; NaCl, 0.5; MgSO₄·7H₂O, 0.16. The medium was sterilized at 121°C and 105 kPa for 15 min.

Isolation of microorganisms

Feather-degrading bacteria were isolated from soil samples originally from the same site where the keratin substrates were obtained. The soil samples were collected in dark polythene bags and stored at 4°C until further use. About 1 g of soil sample was dispersed in 9 ml of sterile distilled water. About 0.2 ml of the aliquot was used to inoculate milk agar for the selective growth of isolates.

The plates were incubated at 37°C for up to three days. Distinct colonies, observed using morphological features, were selected, isolated, and purified on keratin agar to obtain pure cultures. The pure cultures were identified by means of taxonomic schemes and descriptions (Buchanan and Gibbons, 1974).

Growth comparison of the isolates

The keratinolytic isolates obtained were inoculated in 10 ml peptone buffer in corning tubes and incubated at 37°C and 100 rpm for a total period of 120 h. Their absorbance at 600 nm was measured each 2 h for the first 6 h and then at 24 h of incubation, the absorbance was taken each 24 h interval for the five days of incubation. This experiment was performed in triplicate.

Enzyme production

The inoculum was prepared into a medium consisting of 1% keratin substrate, 0.2% yeast extract, pH 7.50. The culture was incubated at 37±1°C and 100 rpm for 24 h. This culture was transferred (5% v/v) into 50/250 ml flask of liquid minimal media identical to the one described earlier (in triplicates) and incubation was carried out at 37°C at 100 rpm for up to 120 h. At 24 h-intervals, whole flasks were taken out, the broth was centrifuged at 5000 rpm at 10°C for 20 min, and the supernatant served as crude extracellular keratinase, which was used without further purification. When not used immediately, the crude enzymes were stored at 4°C. The pH values of culture broths and the keratinolytic activities of supernatants were determined. The optimum pH, temperature and feather concentrations on keratinase production by isolate DF1a were also determined.

Optimisation of the pH for keratinase production

Five conical flasks with 100 ml of the feather meal broth (FMB) containing 15 g/L of powdered feather were prepared. The pH of each flask was adjusted to 6.0, 7.0, 8.0, 9.0 and 10.0, respectively and inoculated with 5 ml of DF1a. There were three replicates. One more flask with the same FMB was prepared at pH 7.0 and left un-inoculated to be used as control. The flasks were incubated at room temperature (30±1°C) for 72 h on a rotary shaker at 100 rpm. 10 ml of the broth were taken from each flask and centrifuged at 5000 rpm for 15 min. Absorbance of the supernatant was then measured at 280 nm against the supernatant of the sterile FMB as blank.

Optimisation of the temperature for keratinase production

Three different temperatures were investigated for their effects on the secretion of keratinase into the medium. Conical flasks with 100 ml of FMB containing 15 g/L keratin were prepared. They were inoculated with 5 ml of the inoculum and the control flasks were left un-inoculated. The flasks were incubated at three different temperatures - room temperature (30°C±1), 37 and 45°C on a shaking incubator at 100 rpm for 72 h after which the broths were centrifuged at 5000 rpm for 15 min and the supernatant analysed for keratinolytic activity.

Optimisation of the feather concentration for keratinase production

Different feather concentrations were tested for their effects on the keratinase secretion from the bacteria. 250 ml conical flasks with

100 ml minimal broth was prepared. Keratin powder was added to each flask to give five different concentrations of 5, 10, 15, 20 and 25 g/l, respectively. Following sterilisation, the flasks were inoculated with 10 ml of the DF1a except for the control. The flasks were incubated at 100 rpm at room temperature ($30^{\circ}\text{C}\pm 1$) for a period of 72 h. After the incubation period, the broth from each flask was immediately centrifuged and the supernatant measured for absorbance at 280 nm.

Enzyme assay

The keratinase activity was determined by the modified method of Cheng et al. (1995). About 0.5 ml of crude enzyme was incubated with 1.5 g of feather powder suspended in 2 ml of phosphate buffer (pH 7.5). The control experiment consisted of buffer and feather powder only. The reaction mixtures were incubated at 40°C for 3 h at 100 rpm. At the end of incubation, the reaction was quenched by adding 2 ml of 10% trichloro acetic acid (TCA). Centrifugation was carried out at 5000 rpm for 15 min at room temperature ($30\pm 2^{\circ}\text{C}$) to remove precipitated keratin. The increase in absorbance at 280 nm of the filtrate of the test sample relative to that of the control, a measure of release of protein was converted into keratinase units [1 U = 0.01 absorbance increase for 1 h reaction time (Ramnani and Gupta, 2004) and was measured.

Proteolytic activity was determined by an azocasein assay (Sarath et al., 1989). The reaction mixture consisted 30 ml of the enzyme, 270 ml of phosphate buffer (40 mM, pH 6.8), and 250 ml of 2% azocasein. Incubation was done at 55°C for 10 min at 100 rpm; the reaction was terminated by the addition of 2 ml of 10% trichloro acetic acid (TCA). Centrifugation at 5000 rpm for 20 min was done to remove undegraded azocasein. The supernatant was separated and absorbance determined at 440 nm. One unit of enzyme activity was the amount of enzyme that causes a change of absorbance of 0.01 under the conditions herein described. TCA was used as blank, while the mixture of azocasein, phosphate buffer, and TCA without enzyme was used as the control.

In situ degradation of keratin

The ability of the isolates to degrade native keratin substrate was investigated. The assay consisted of a whole chicken feather suspended in 100 ml of minimal medium (pH 7.0) sterilized at 121°C for 15 min and then inoculated with 5% (v/v) inoculum. Different sets were incubated at 100 rpm for 7, 14 and 21 days at room temperature ($30\pm 2^{\circ}\text{C}$), respectively. The control experiments were without inoculum. The set-ups were examined on a daily basis to determine the level of degradation of the whole feather.

Effects of pH and temperature on keratinolytic activities

The effect of the pH was studied by assaying the enzyme using 0.1 M citrate buffer, pH 5.0 and 5.5; sodium phosphate buffer, pH 6.0, 7.0 and 8.0; and bicarbonate buffer, 9.0 and 10.0. The effect of temperature was measured by incubating the enzyme at temperatures ranging from 35 to 80°C at the optimum pH. At the end of the reaction, the residual activity of the enzyme was determined.

RESULTS

Characterization of keratinolytic strains

Three isolates were able to grow on medium containing

feather meal as sole carbon and nitrogen source. The strains DF1a, DF2b and DF3 produced clearing zones when tested for proteolytic activity on milk agar (Figure 1). The largest clearing zones were observed for isolate DF1a. Diameter of clear area produced on the milk agar by the keratinolytic isolates DF1a, DF2b and DF3 is shown in Figure 2.

The identification of the keratinolytic bacteria was based on morphological, cultural and biochemical tests comparing the data with standard species (Hoq et al., 2005). Morphological and physiological characteristics of the bacteria were compared with the Bergey's Manual of Systemic Bacteriology. The results are shown in Table 1. All isolates were Gram positive, rod shaped and spore-former, and were able to utilize both glucose and sucrose but not lactose. They were also catalase and oxidase positive. The organisms were unable to utilize citrate and all were able to reduce nitrate to nitrite. All isolates showed typical characteristics of *Bacillus* sp. Reports of several workers showed that the *Bacillus* sp was considered as prime producer of keratinase (Lin et al., 1995).

In situ degradation of feather wastes

According to Kornitłowicz-Kowalska (1997), the mass loss of the keratin substrate is the most reliable indicator of microbial keratinolytic abilities. The strains were tested for their capacity to degrade feather wastes. Cultivation on different types of feathers resulted in the production of keratinase. After two weeks, strains DF1a (Figure 3a) degraded chicken feathers completely. Barbules and rachises were completely degraded to fine granulated forms settling at the bottom of test tubes. Isolates DF2a and DF3 disintegrated feather barbs and barbules but not all rachises (Figure 3b) after two weeks. However, after an extra seven days, complete degradation was observed (Figure 3c).

Growth comparison

Figure 4 shows the growth patterns of the three isolates over a period of five days. Based on results in Figure 4, subsequent experiments were carried out using isolate DF1a only.

Keratinolytic activity

Keratinolytic activity of the isolate DF1a was monitored during growth in feather meal broth. The keratinolytic activity increased in the first days. The keratinolytic activity in the range of 2.5 to 35.9 U/ml was observed during the five days of cultivation, with the highest activity occurring at day five of cultivation (Figure 5). However, the proteolytic activity reached a peak of 380 U/ml at 72

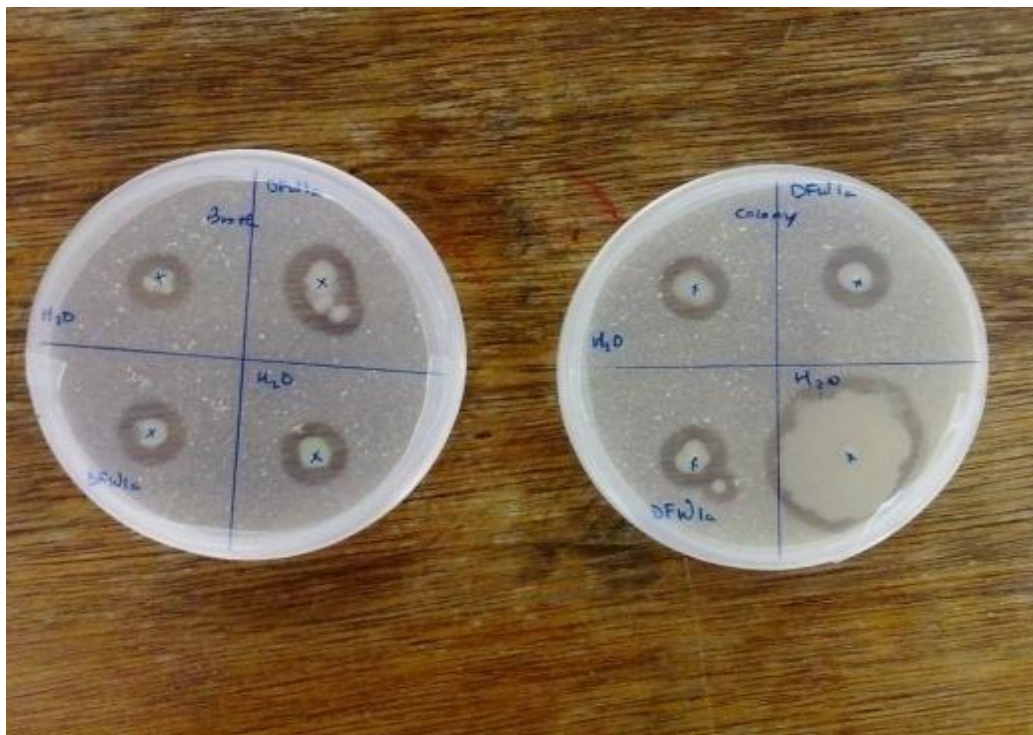


Figure 1. Production of clearing zones in milk agar plates by keratinolytic bacteria. Microorganisms were inoculated by stick and plates were incubated at 30°C for 24 h.

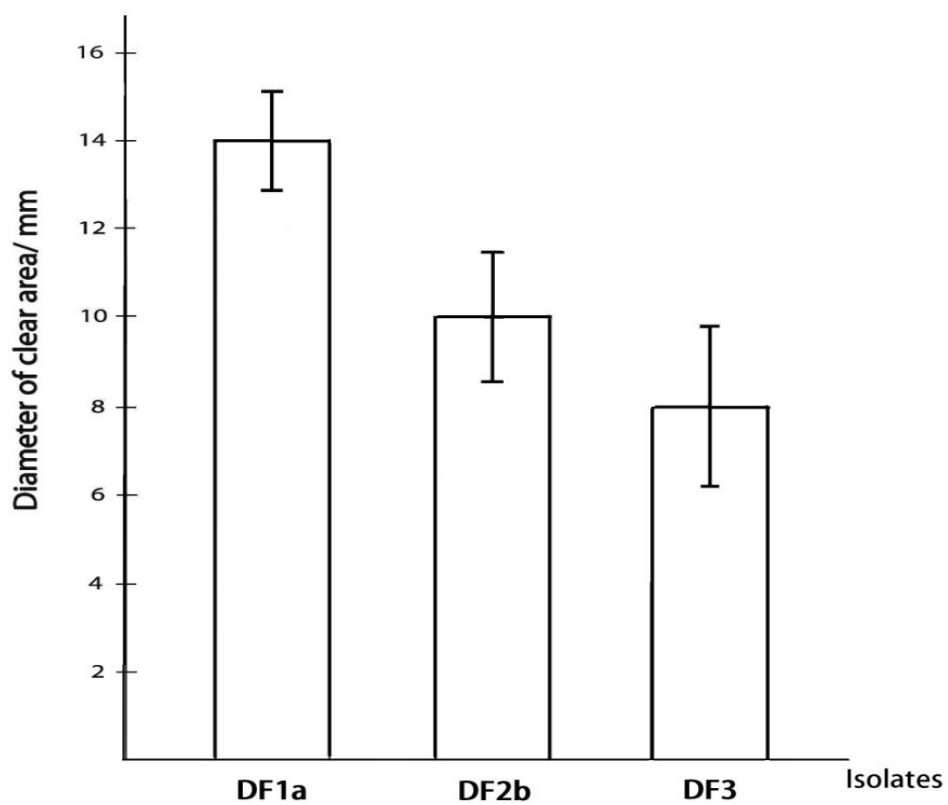


Figure 2. Diameter of clear area produced on milk agar.

Table 1. Results of morphological, physiological, cultural, biochemical characteristic of three isolated bacterial strain DF1a, DF2b, DF3 conducted.

Details of experiment	Observation		
	DF1a	DF2b	DF3
Shape of bacteria	Rod	Rod	Rod
Endospore formation	+	+	+
Motility	Highly motile	Motile	Motile
Gram character	+	+	+
Anaerobic growth	-	-	-
Colony characteristics			
Growth	Rapid	Rapid	Rapid
Shape	Irregular	Irregular	Irregular
Surface	Smooth	Smooth	Smooth
Margin	Entire	Entire	Entire
Colour	Cream	White	Cream
Elevation	Flat	Flat	Convex
Consistency	Buttery	Viscous	Buttery
Opacity	Opaque	Opaque	Opaque
Biochemical characteristics			
Glucose	A/-	A/-	-/-
Lactose	A/-	A/-	-/-
Mannitol	-/-	A/-	-/-
Indole production	-	-	-
Catalase	+	+	+

Collectively these characteristics indicated that the isolates were of genus *Bacillus*. +, Positive; -, negative; A/-, acid/no gas; -/-, no acid/no gas.



Figure 3. (a) Complete hydrolysis of chicken feather by strain DF1a after two weeks; (b) after 7 days the isolates could not hydrolyse the feather completely, and (c) took a further seven days for complete hydrolysis.

h. An increase in pH was always observed during growth shifting from an initial pH of 7.0 to an average of 8.8 after three days.

Effect of medium pH on the production of keratinase enzyme

The effect of the medium pH on the production of

keratinase by the DF1a isolate is shown in Figure 6.

Effect of temperature on the production of keratinase enzyme

The optimum temperature for keratinase production by isolate DF1a was found to be 37°C (Figure 7).

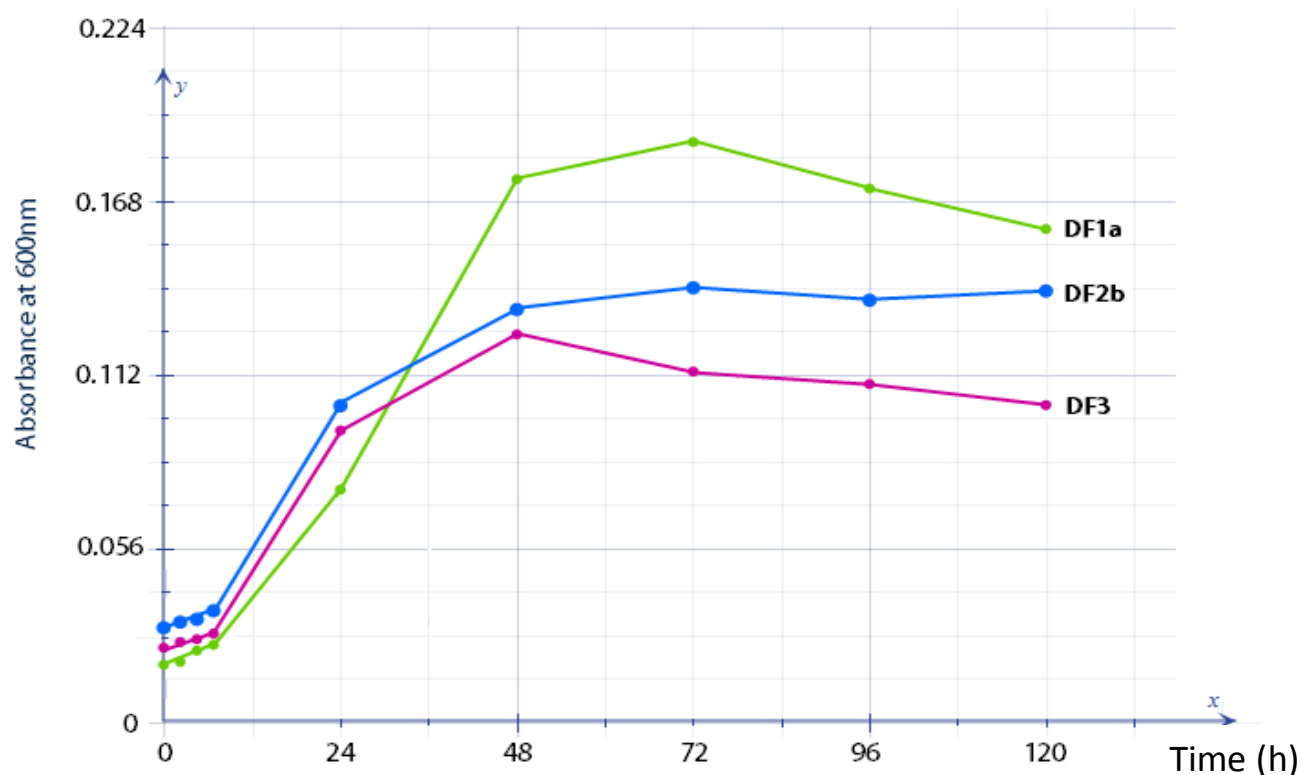


Figure 4. Growth comparison of the three isolates.

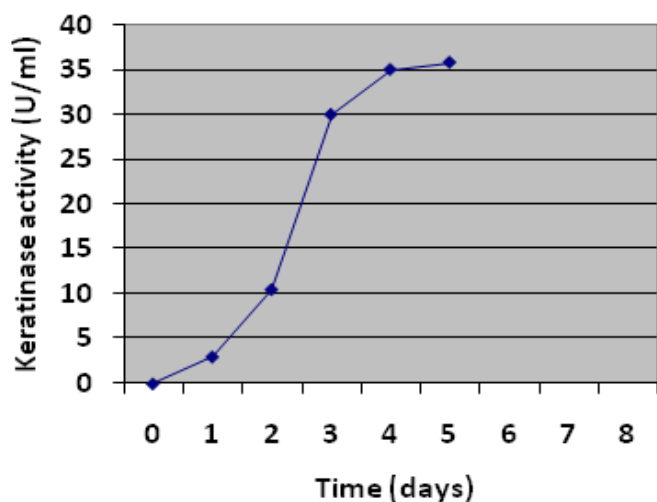


Figure 5. Keratinase activity of isolate DF1a during growth. Each point represents the mean of three independent experiments.

Effect of feather concentration on the production of keratinase enzyme

The optimum concentration of keratin in the medium for maximum keratinase secretion was found to be 20 g/l for isolate DF1a (Figure 8).

Effects of pH and temperature on keratinolytic activities

The effects of pH and temperature on the keratinolytic activities of crude extracellular keratinase of DF1a induced by feather showed that the enzyme was active over a broad range of pH of 5 to 10, with more than 50% activity occurring between a pH of 7 to 10 (Table 2). Its activity increased from pH of 5.0 to reach a maximum at the pH of 8.0. The enzyme was also active within the temperature range of 37 to 80°C that was investigated (Table 3). The keratinolytic activity increased gradually from 37°C to reach the optimum at 70°C. However, more than 50% activity was obtained between 45 and 70°C.

DISCUSSION

A screening programme was employed to obtain bacterial isolates capable of producing feather degrading extracellular keratinase enzyme. The potential isolates were then characterized and identified to its genus level. In this regard, samples were collected from various soil habitats and the feather pieces that were degraded in the aforementioned soil samples were inoculated into basal medium containing 1% ground feather as a sole carbon source and incubated at 37°C for the enrichment of feather degrading bacterial strains. A total of three

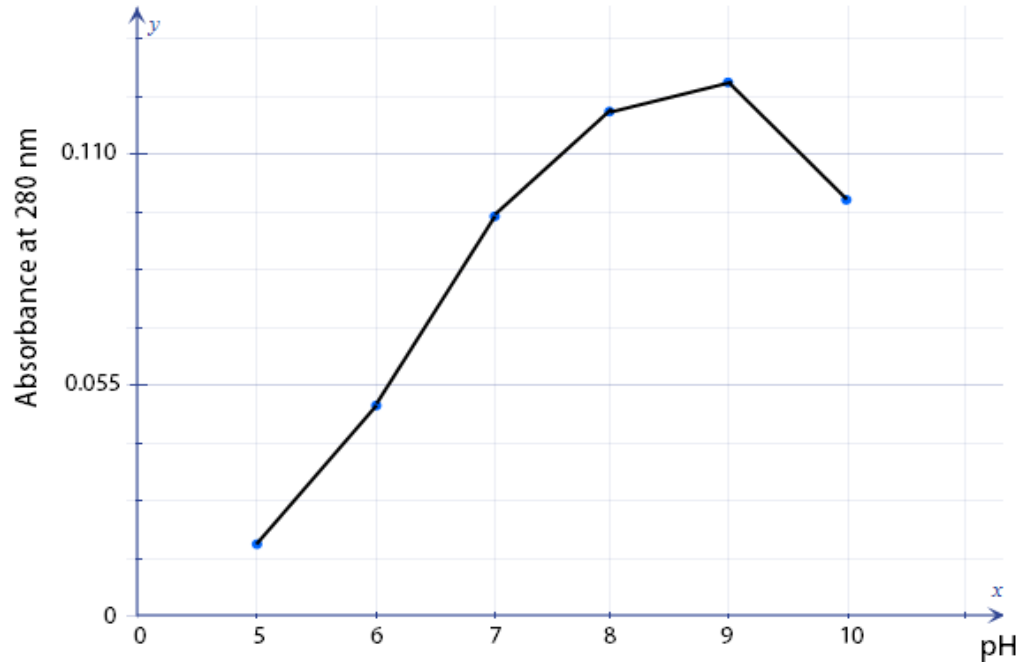


Figure 6. Effect of medium pH on keratinase production by isolate DF1a.

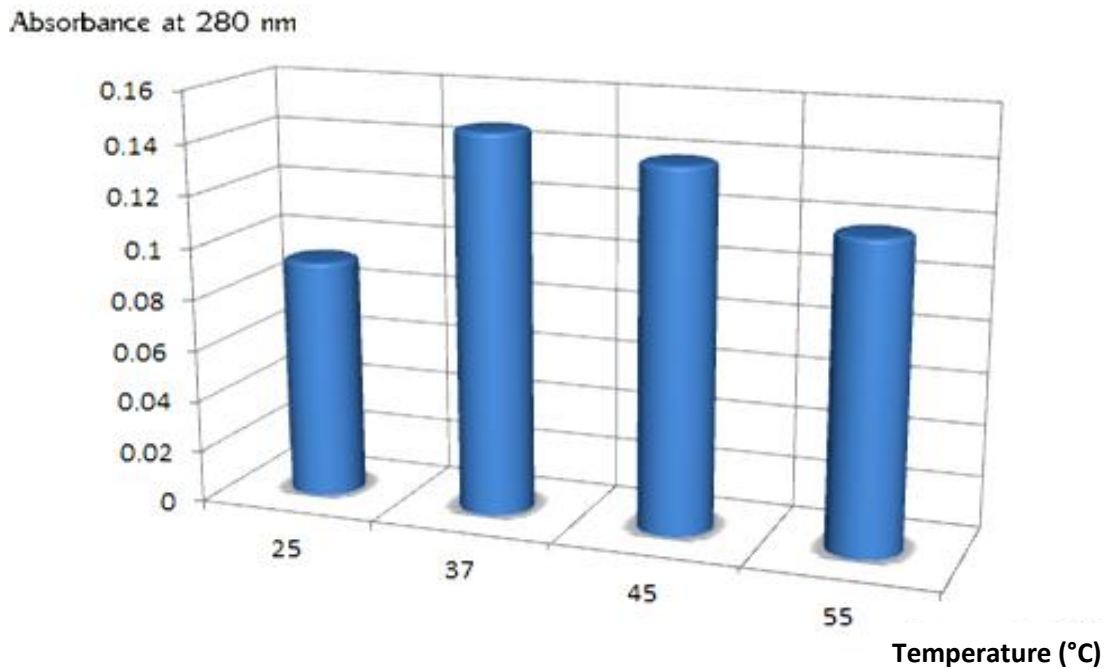


Figure 7. Effect of temperature on the production of enzyme keratinase.

isolates were obtained from two different habitats. All isolates showed good growth with distinct characteristics on nutrient agar and turned nutrient broth turbid. Morphological and physiological characteristics of the bacteria were compared with the Bergey's Manual of Systemic Bacteriology.

All isolates were Gram positive, rod shaped and spore-former and were able to utilize both glucose and sucrose but not lactose. They were also catalase and oxidase positive. All isolates showed typical characteristics of *Bacillus* sp. Reports of several workers showed that the *Bacillus* sp was considered as prime producer of

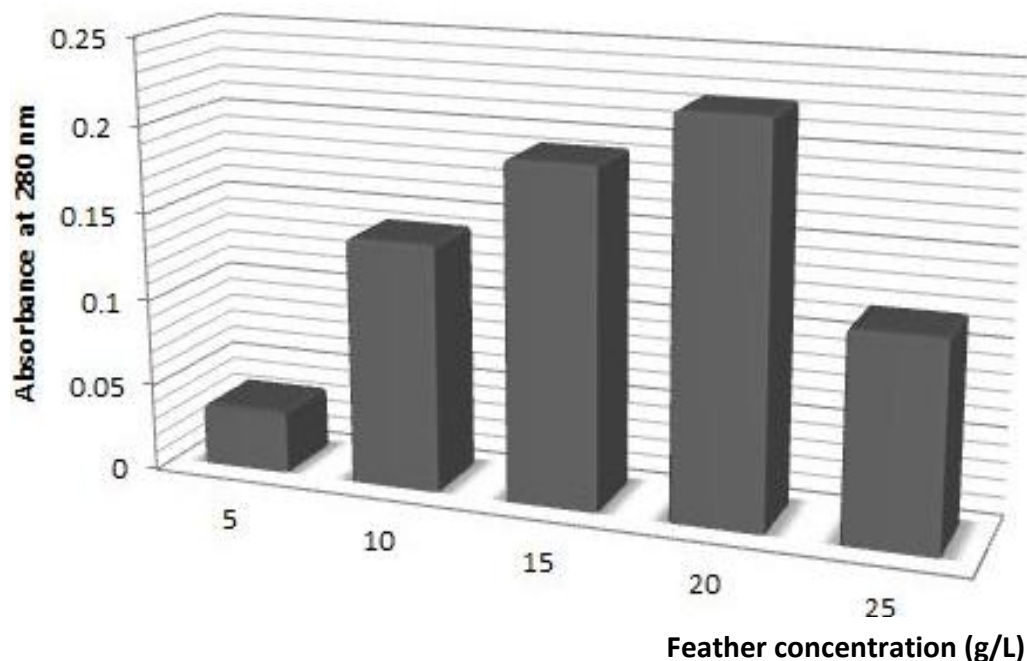


Figure 8. Effect of feather concentration on the production of keratinase enzyme.

Table 2. Effect of pH on keratinolytic activity produced by isolate DF1a.

pH	U/ml
5	17.0±0.76
6	45.5±1.50
7	61.0±3.50
8	63.5±2.50
9	57.5±1.50
10	50.5±3.61

Table 3. Effect of temperature on keratinolytic activity produced by isolate DF1a.

Temperature (°C)	U/ml
37	63.5±2.5
45	99±3.2
55	110±4.9
60	118±3.0
70	128±1.3
80	122±1.7

keratinase (Lin et al., 1995). Keratinolytic bacteria, particularly from the genus *Bacillus* have been isolated from the plumage and bird feathers (Burt and Ichida, 1999; Ichida et al., 2001), feather waste processed by fermentation (Williams and Shih, 1989; Williams et al., 1990) or composting (Kim et al., 2001). In bacteria,

feather keratin-degrading abilities have been observed mostly in strains of *Bacillus licheniformis* (Burt and Ichida, 1999; Lin et al., 1999), less frequently in populations of *Bacillus pumilis*, *Bacillus cereus* and *Bacillus subtilis* (Kim et al., 2001).

The *Bacillus* sp. showed good growth with a varying level of keratinolytic activity (Figure 1) on milk agar and the experiment was repeated for three times. The keratinolytic activity of all isolates increased with the increase of time however, the rates of increase of the enzyme activity were not similar in pattern for all the isolates (Figure 5). The highest activity was demonstrated by strain DF1a (35.9 U/ml) after 120 h of cultivation on feather meal. *Bacillus* spp. are known to produce a number of hydrolytic enzymes including keratinase, which is able to degrade feather, wool and hair (Daroit et al., 2009). Also, degradation of keratin has been reported to be mostly confined to Gram-positive bacteria including *Bacillus*, *Lysobacter*, and a few strains of Gram-negative bacteria, viz., *Vibrio* and *Xanthomonas* (Gupta and Ramnani, 2006). It has been reported that the more important bacteria involved in the degradation of wool and other animal fibres include *Bacillus putrificus*, *B. subtilis*, *B. cereus*, and *Bacillus mesentericus* (Lal et al., 1999). *Bacillus* strains, DF1a, DF2b and DF3 add to the growing list of keratinolytic bacteria.

An increase in pH values was observed during feather degradation, a trend similar to other microorganisms with large keratinolytic activities (Sangali and Brandelli, 2000). The increase in pH during cultivation is pointed as an important indication of the keratinolytic potential of

microorganisms (Kaul and Sumbali, 1997). It has been previously reported that alkaline pH from 6 to 9 supports keratinase production in most microorganisms (Gupta and Ramnani, 2006). Similarly, increase in alkalinity usually accompanies keratinolysis (Gupta and Ramnani, 2006), which is attributed to deamination reactions leading to the release of ammonium and thus increase in pH (Riffel et al., 2003), as obtained in this study. Furthermore, it has been reported that keratinases from most bacteria, actinomycetes, and fungi have pH optimal in neutral to alkaline range (Riffel et al., 2003; Anbu et al., 2005), and they are also known to be generally active and stable over a wide range of pH from 5 to 13 (Farag and Hassan, 2004). The optimum temperature of most keratinases has also been reported to be in the range of 30 to 80°C (Gupta and Ramnani, 2006). Similarly, the characteristics of the crude extracellular keratinase of isolates DF1a, DF2b and DF3 indicated that they were active over a broad range of pH and temperature.

Through the strategy of isolation of keratinolytic microorganisms utilized in this work, bacteria presenting high keratinolytic activity were selected. Considering that feather protein has been showed to be an excellent source of metabolizable protein (Klemersrud et al., 1998), and that microbial keratinases enhance the digestibility of feather keratin (Lee et al., 1991; Odetallah et al., 2003), these keratinolytic strains could be used to produce animal feed protein in addition to the biodegradation of poultry wastes, including composting (Korniłowicz-Kowalska and Bohacz, 2011). In fact, large-scale composting of keratin wastes, especially poultry feathers that are produced in the greatest amounts, may provide a solution to the problem of their utilization.

It will also prevent wasting of the material and the ecological hazards resulting from keeping those wastes on waste dumps. Although most of the subsequent experiments following isolation of the bacteria were done using isolate DF1a only, we believe that all three novel isolates present potential biotechnological use in processes involving keratin hydrolysis.

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