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# Effects of Some Cultural Conditions on Keratinase Production by *Bacillus licheniformis* Strain NBRC 14206

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author FSI designed the study and performed the statistical analysis. Author ACO wrote the protocol and the first draft of the manuscript. Both authors managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** Microbial keratinase is an extracellular enzyme capable of degrading keratin present in feathers, hair, and wool. They are widely used in chemical, medical industries as well as in animal feed industry and basic biological science. This study investigated the isolation and effects of some cultural conditions on keratinase production by *Bacillus licheniformis* strain NBRC 14206 using raw feather.
**Study Design:** One-factor-at-a-time strategy was adopted to evaluate the effects of some fermentation conditions on keratinase production.
**Place and Duration of Study:** Department of Microbiology, Faculty of Science, University of Port Harcourt, Nigeria, between September 2015 and June 2016.
**Methodology:** A total of twenty (20) bacterial isolates were isolated from three (3) feather dumping

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sites. Out of the 20 isolates screened, only one isolate with the highest zone of inhibition was selected. The isolate was morphologically and biochemically characterized based on Bergey's manual of determinative bacteriology flowchart as well as molecular analysis. In order to improve keratinase production by the selected isolate, culture medium and the effect of environmental parameters were individually screened and optimized using one-factor-at-a-time methodology. The following cultural parameters: pH, temperature, nitrogen sources, local carbon sources, carbon sources, substrate concentration and incubation time were evaluated for the production of keratinase by the bacterium.

**Results:** The highest keratinase producing bacteria, isolate KDB1 was found to belong to the genus *Bacillus*. The highest enzyme production was obtained at 72 h after incubation. The optimum pH and temperature for the production of keratinase by the bacterium were pH 9.0 and 40°C, respectively. The best carbon, local carbon and nitrogen sources were lactose, corn stalk and yeast extract at concentration of 1%, respectively. The highest activity was observed at 1% feather concentration.

**Conclusion:** Result obtained from this study showed that keratinase produced from isolate KDB1 could be useful in decomposition of keratin-waste (feather) and could find application in leather, pharmaceutical and cosmetics industries as well.

*Keywords: Keratinase; Bacillus licheniformis; feather waste; optimization.*

## 1. INTRODUCTION

Keratinases are extracellular enzymes used for the biodegradation of keratin and hydrolyzes both native and denatured keratin [1]. They are group of serine metalloproteases which release the free amino acids from keratinous proteins [2]. Keratin-containing wastes such as hair, hoves, and feather are abundant in nature but their use is limited, since they are insoluble and resistant to degradation by the common proteolytic enzymes [3,4,5]. Keratinases are produced by a large group of bacteria, actinomycetes and fungi [6]. Along with bacteria and fungi, some insects, including cloth mouth larvae, carpet beetle and chewing lice are known to digest keratin [5]. Keratinases are robust enzymes with wide temperature and pH activity range and are largely serine or metalloproteases. Bacterial keratinase are of particular interest because of their action on insoluble keratin substrate and generally on a broad range of protein substrates [7].

Keratinous wastes represent a source of important proteins and amino acids and could find application as additive for animals or nitrogen source for plants [3]. The enzymes are widely used not only in chemical and medical industries but also in animal feed industry and basic biological science [8]. Keratinase is applied in industrial and household including food, silk and pharmaceutical industries [4,9], biofertilizer production [10], biotechnological processes involving keratin containing waste from poultry [11], laundry, photography, agricultural and bioremediation process [5,12]. Keratinase is used in leather industry for dehairing, detergent addictives to aid in the removal of proteineous stains, to enhance drug delivery, preparation of protein hydrolysates of high nutritional value, bioprocessing of used x- ray or photograghic films for silver recovery, in agricultural waste degradation and in prion degradation [6]. Keratinase are used also in manufacturing textiles and production of essential amino acids for animal feed [7].

Keratinase producing microorganisms have been used to degrade feather [8], hair, nails and wool [1]. A total of 5-7% weight of a mature chicken comprises of feather [13]. Feather from commercial poultry processing are produced in excess of millions tons per year around the world [9,11,13,14]. Feather waste is very inexpensive [11,15], high in protein (over 90%) [16], highly resistant to the action of weak acids, alkalis, organic solvents or hydrolysis by common proteolytic enzymes such as trypsin, pepsin and papain due to a high degree of cross-linking by disulfide bonds, hydrogen bonds and hydrophobic interaction [17,18,19,20,21] and hence cannot be used directly in animal feed because of difficult indigestion [7]. Feather is currently converted to feather meal using steam and chemical treatments, but this destroys the essential amino acids and requires significant energy input. However, alternative use of keratinolytic microorganisms has been investigated and has been suggested as a better approach for efficient conversion of keratin to animal feed [9,11]. Thus, enzymatic utilization of

feather is regarded as the most promising method with the advantages of high specificity and efficiency and convenient operation in large scale commercial operation [22].

Several keratinolytic microorganisms have been isolated and characterized from feather dumping soils and feather processing units and includes Bacteria; *Bacillus sp.* FK46, *Bacillus licheniformis, Bacillus pumilis, Vibrio* sp. strain Kr2, *Actinobacteria, Streptomyces pactum,* and Saprophytic and Dermatophilic fungi, respectively such as *Aspergillus* spp., *Rhizomucor* spp., *Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton gallinae, Microsporum canis* and *Microsporum gypseum* [1,9,23]. Also, Fungi especially *Fungi imperfecti* have high keratinolytic activity and includes: *Aspergillus, Alternaria, Trichurus, Curvalaria, Gleomastis, Cladosporium, Fusarium, Geomyces, Monodictys, Myrothecium, Paecilomyces, Stachybotrys, Urocladium, Seopulariopsis, Penicillium and Doratomyces* [24,25], *Arthrobacter* sp., *Kocuria rosea* and *Microbacterium* sp. [5]. This study investigated the isolation and effects of some cultural conditions on keratinase production by *B. licheniformis* using raw feather.

## 2. MATERIALS AND METHODS

### 2.1 Source of Inoculum

Soil samples from feather-dumping sites were used for the study. The samples were collected from Rumuodumanya market feather dumping site, Sunny poultry farm, Port Harcourt, and Mile 3 market feather dumping site Port Harcourt, Rivers State, Nigeria. All the soil samples were collected from a depth of 2-6 cm using spade in a specimen bag and were transported to Industrial Microbiology laboratory of University of Port Harcourt, Rivers State Nigeria.

### 2.2 Processing of Keratinolytic Substrate

The substrate used for the study was raw feathers. The raw substrates collected from various locations were extensively washed with water. The feather was dried under sunlight and then oven dried at 60°C for 48 h. The dried feathers were milled to fine powder which served as a sole source of carbon, energy and nitrogen.

### 2.3 Isolation of Keratinolytic Bacteria

One gram of each soil sample was transferred into 9 ml sterile saline. The sample was serially diluted to $10^{-7}$ dilutions and 0.1 ml of each dilution was aseptically transferred to plates containing basal feather medium by spread plate techniques. The basal feather medium comprised of (g/100 ml): $NH_4Cl$ 0.5, NaCl 0.5, $K_2HPO_4$ 0.3, $KH_2PO_4$ 0.3, $MgCl_2.6H_2O$ 1, yeast extract 0.1, feather powder 1, agar 1.2, pH 9.0 [13]. Inoculated plates were incubated at 30°C for 2-5 days.

### 2.4 Screening of Isolates for Extracellular Keratinase Activity

Screening of the isolates was done by streaking 24 h cultures of the isolates on basal feather media plates. The cultures were incubated for 24 - 48 h for colony formation. Individual spot of colony formed was flooded with 0.0015% bromo cresol green dye and incubated for 30 – 40 min. Keratin degradation was indicated by the formation of cleared zone of hydrolysis around the colonies. Strains which showed maximum zones of clearance were selected for further studies [26].

### 2.5 Stock Culture

The screened and purified keratinolytic isolate was inoculated into 10 ml nutrient broth. The culture was incubated at 37°C for 24 h and re-streaked in basal medium incorporated with 1% feather and nutrient agar slants. The culture was incubated for 24 h at 37°C and stored in the refrigerator at 4°C for further use.

### 2.6 PCR Amplification of Bacterial 16S rRNA PCR Gene

The isolate was identified using molecular techniques (Polymerase Chain Reaction (PCR) and DNA sequencing) and biochemical characterization. The PCR amplification of the 16S rRNA gene was carried out using the primer set 27 F- 5′-AGA GTT TGA TYM TGG CTC AG-3', and 515R 5'-TTA CCG CGG CKG CTG GCA C-3'. The reaction was carried out in 20 ul reaction mixture containing 1X PCR buffer (Solis Biodyne), 1.5 mM Magnesium chloride (Solis Biodyne), 0.2 mM of each dNTP (Solis Biodyne), 2 U Taq DNA Polymerase (Solis Biodyne), 20 pMol of each primer and sterile water was used to make up the reaction mixture. PCR was carried out in an Eppendorf Nexus thermal cycler with the following cycling parameters; an initial denaturation step at 95°C for 5 min, followed by 30 consecutive cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension

at 72°C for 1 min. After this, a final extension at 72°C for 10 min was carried out. After the PCR reaction, PCR products were separated on a 1.5% agarose gel.100 bp DNA Ladder (Solis Biodyne) was used as DNA molecular weight marker. Electrophoresis was done at 80 V for 1 h 30 min, and the gel was viewed under UV light after ethidium bromide staining.

## 2.7 Construction of Phylogenetic Tree

The sequences were edited using the bioinformatics algorithm Bioedit, similar sequences were downloaded from the National Biotechnology Information Center (NCBI) data base using BlastN and these sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbour-Joining method [27]. The bootstrap consensus tree inferred from 500 replicates [28] was taken to represent the evolutionary history of the taxa analyzed [28]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Jukes-Cantor method [29] and were in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 864 positions in the final dataset. Phylogenetic tree was constructed using MEGA6 [30].

## 2.8 Determination of Enzyme Activity

One milliliter of 1% (keratin) feather powder was mixed with 0.2 ml of phosphate buffer (pH 8.0) and 0.5 ml of enzyme solution in test tubes. The solution was incubated for 30 min at 30°C. After incubation, the reaction was terminated by addition of 2 ml of 10% trichloroacetic acid (TCA). The untreated keratin (feather powder) precipitate was removed by centrifugation at 10000 rpm for 10 min. One milliliter (1 ml) of the supernatant was mixed with 5 ml of 4.2% sodium carbonate ($Na_2CO_3$) and 0.5 ml of Folin Ciocalteau phenol reagent. The reaction mixture was precipitated by standing in ice for 15 min and insoluble precipitate was removed by centrifugation at 10000 rpm for 10 min. Absorbance of the supernatant was read at 660 nm. A control assay, without the enzyme in the reaction mixture was done and used as the blank in all spectrophotometric measurements. All assays were done in duplicate. One unit of keratinase activity was defined as the amount of enzyme that released one microgram of tyrosine per min under standard assay conditions [2].

## 2.9 Optimization of Fermentation Culture Parameters

### 2.9.1 Effect of pH on keratinase production

Feather basal medium was prepared and the pH of the medium was adjusted to different pH (3, 5, 7, 9, 11 and 14 by drop-wise addition of either 1N NaOH or 1N HCl using pH meter. One millilitre (1 ml) of the culture from 24 h culture was inoculated into the medium and incubated in a shaker incubator at 150 rpm for 72 h at 37°C. After incubation, the broth was collected and used as crude enzyme to perform keratinase activity.

### 2.9.2 Effect of temperature on keratinase production

Feather basal medium was prepared and 1 ml of inoculum from 24 h culture of the isolate was inoculated into the 25 ml feather medium. The feather basal medium was incubated in shaker incubator (150 rpm) at various temperatures (20, 30, 40, 50, 60 and 70°C) for 72 h.

### 2.9.3 Effect of local carbon sources on keratinase production

Feather basal medium was prepared and incubated with various local carbon sources (0.25 g) (corn stalk, saw dust, cassava peel, groundnut shell and sugar cane baggasse). To the feather medium, 1 ml of inoculum from 24 h culture of isolate was inoculated and incubated at 37°C in shaker incubator at150 rpm for 72 h.

### 2.9.4 Effect of carbon sources on keratinase production

Feather basal medium was prepared and incubated with various carbon sources (0.25 g) (mannitol, sucrose, lactose, glucose and starch). To the feather medium, 1 ml of inoculum from 24 h culture of isolate was inoculated and incubated in shaker incubator at 150 rpm for 72 h at 37°C.

### 2.9.5 Effect of nitrogen sources on keratinase production

Feather basal medium was prepared and incubated with various nitrogen sources (0.25 g) (urea, $NaNO_3$, yeast extract, peptone and $NH_4Cl$). To the feather medium, 1 ml of inoculum

from 24 h culture of isolate was inoculated and incubated in shaker incubator (150 rpm) at 37°C for 72 h. Thereafter, the clear supernatant after centrifugation was used to assess keratinase activity as previously described.

#### 2.9.6 Effect of substrate concentrations on keratinase production

Feather basal medium was prepared with different substrate concentrations (%): 0.5, 1, 2, 3, 4 and 5. To the feather medium, 1 ml of inoculum from 24 h culture of isolate was inoculated and incubated in shaker incubator at 150 rpm for 72 h. After incubation broth was centrifuged and the supernatant was used to assay for keratinase activity.

#### 2.9.7 Effect of incubation time on keratinase production

In order to examine the effect of incubation period on keratinase production, two milliliters of the culture in fifty milliliters (50 ml) of designed feather medium (pH 9.0, temperature 40°C, feather concentration 1%, lactose 1% and yeast extract 1%) and incubated in a shaker incubator at 150 rpm for 5 day at 40°C. About 10 ml culture broth was removed periodically at an interval of 24 h and used to determine keratinase activity.

### 2.10 Statistical Analysis

All experiments were carried out in triplicates. The effects of various environmental factors on the production of the enzyme were evaluated using two way analysis of variance (ANOVA) and Student t-test at $\alpha = 0.05$ to identify means that differed significantly.

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation, Screening and Identification of Keratinase Producing Bacteria

Large amount of feathers are produced as waste at poultry processing plants, reaching millions of tons per year with potential environmental impact. In the present study, twenty (20) bacteria strains were isolated from soil collected from different feather dumping sites. The 20 bacteria colonies were screened for keratinase production by flooding with bromocresol green dye. All the twenty isolates showed potential for keratinase secretion (Fig. 1). The result revealed that isolate KDB1 had the highest zone of clearance, followed by isolate KDB2 while the least zone of clearance was observed in isolate KDB15 and KDB20. Out of the 20 isolates, only one with the highest zone of inhibition (isolate KDB1) was selected for further studies. The isolate with the highest zone of clearance was identified as *Bacillus licheniformis* strain NBRC 14206 16S ribosomal RNA gene based on morphological, biochemical and molecular identification. Phylogenetic tree of the isolate is shown in Fig. 2. Brandelli et al. [31] reported that keratinase are produced by variety of bacteria mainly *Bacillus* and *Streptomyces* species. Mohammed et al. [17] linked keratinase production by several species of *Bacillus* to the broad distribution of the enzyme among these genera. According to their report, highest enzyme production was recorded with *Bacillus amyloliquefaciens* and *Bacillus subtilis* using plate test method incorporated with wool as a sole carbon source. Keratinase production by *Bacillus licheniformis* have been reported by [7,32,33,34,35]. Venkata and Divakar [6], Armin et al. [36], Kainoor and Naik [37] reported highest enzyme production by *Bacillus megaterium, Bacillus sp.* and *Bacillus sp. JB*99.

### 3.2 Optimization of Culture Parameters for Keratinase Production by *Bacillus licheniformis*

#### 3.2.1 Effect of initial pH of medium on keratinase production

The effect of initial pH of medium on keratinase production by *B. licheniformis* is depicted in Fig. 3. Keratinase production by this bacteria progressed with increase in pH upto pH 9 after which the enzyme production declined. The optimum keratinase production was obtained at a starting pH of 9.0 whereas the least was obtained at pH 3.0. The increase in keratinase production at pH 9 could be that the accessibility of the raw feathers for degradation by the bacteria was more favoured at this pH. The result indicated that keratinase production by *B. licheniformis* is favoured more in alkaline environment than acidic range and extreme alkaline environments. The alkaline environment has been reported to make feathers more accessible for degradation by keratinase from microorganism and has been linked to partial dissolution of the feathers by the alkaline environment [38]. The report is in agreement with the observations by [39] and [40] with maximum enzyme production at pH 9 with alkaliphilic bacteria *Bacillus thuringenesis, Microsporum gypseum* and *Bacillus* sp. [40], recorded optimum enzyme production at pH of 8.5 by *Bacillus* sp. SAA5 while [4,6,37,41]

reported optimum enzyme production at pH of 10 with *Bacillus thuringiensis, Bacillus megaterium*, *Bacillus* sp. JB 99, *Bacillus cereus* and *Bacillus licheniformis* 1269. Additionally, [42] obtained maximum production of enzyme at pH of 11 with *Bacillus subtilis*.

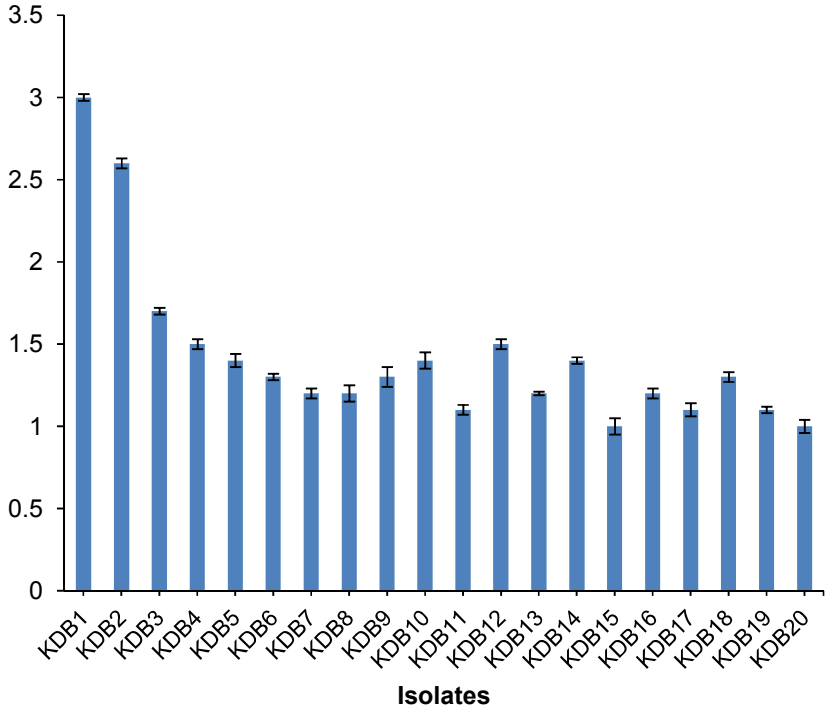


**Fig. 1. Diameter of zone of clearance of the 20 isolates**

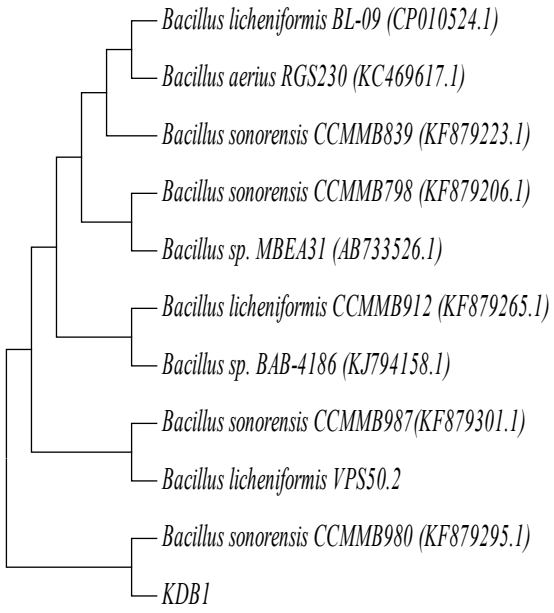


**Fig. 2. Phylogenetic tree of the isolate (Isolate KDB1) using neighbour joining method**

### 3.2.2 Effect of temperature of medium on keratinase production

Enzyme production was studied with *Bacillus licheniformis* over a broad range of temperature (20-70°C) and it was found to be optimal at 40°C (Fig. 4). Further increase in temperature to 70°C reduced the enzyme production due to destruction inactivation of the enzyme by high temperature. This result indicated that the isolate is a mesophilic organism and high temperatures may not favour the secretion of the keratinase. Our result is in agreement with results obtained by [11,41,42,43,44] with maximum keratinase production at 40°C for *Bacillus* sp., *B. licheniformis* 1269*, Bacillus licheniformis* KMBVP, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *B. cereus* 1268. Kansoh et al. [45] and [46] reported maximum keratinase production at 37°C. However, [6] recorded maximum production at 35°C for *B. megaterium*. Samuel et al. [11] reported that temperature has great impact on enzyme production. A higher temperature generally results in an increasing enzyme production but if the temperature rises beyond a certain point, however the enzyme production eventually leveled out and decline rapidly because at high temperatures most microorganisms may not survive. The result of analysis variance obtained revealed that the different temperature significantly affect (P<.05) keratinase production by *B. licheniformis*.

### 3.2.3 Effect of carbon sources and local carbon sources on keratinase production

Result of effect of carbon sources on enzyme production by *Bacillus licheniformis* is shown Fig. 5. In this study, highest keratinase production was observed with lactose followed by sucrose while the least was noticed when starch was used. However, the bacterial isolate was able to utilize the different local carbon sources (corn stalk, groundnut shell, saw dust, cassava peel and sugarcane bagasse in decreasing order) for keratinase production. Statistical analysis conducted indicated that there is no significant difference (P >.05) between the different carbon sources except for mannitol, glucose and starch on keratinase production by *B. licheniformis*. Other researchers working with keratinase have reported various carbon sources optima. Sivakumar et al. [4,37] reported optimum production with mannitol and starch, respectively for *Bacillus* sp. JB 99 and *B. thuringiensis*. Saber et al. [47] had highest keratinase production with starch and maltose for *Alternaria tenuissima* and *Aspergillus nidulans.*

### 3.2.4 Effect of nitrogen sources on keratinase production

Feather basal medium supplemented with 1% yeast extract as external nitrogen showed maximum keratinase production by

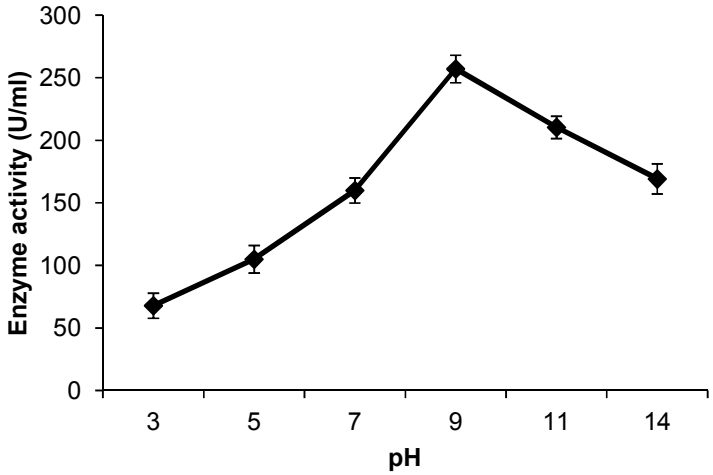


**Fig. 3. Effect of pH on keratinase production by *B. licheniformis***

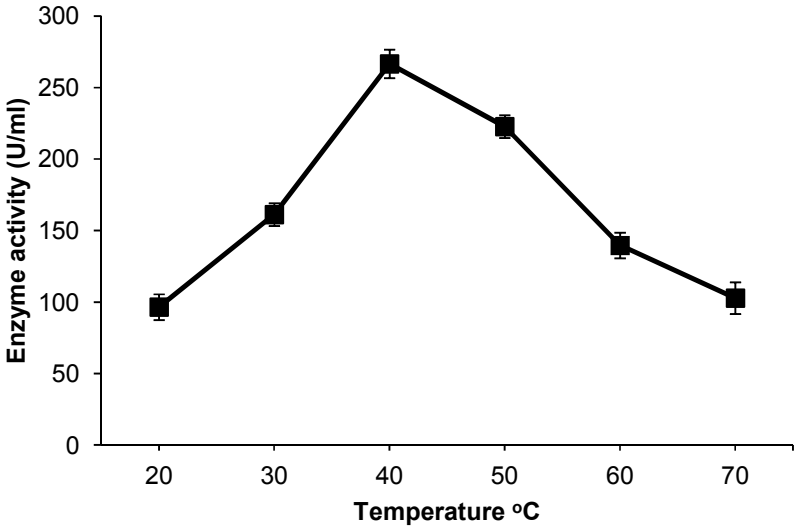


**Fig. 4. Effect of temperature on keratinase production by *B. licheniformis***

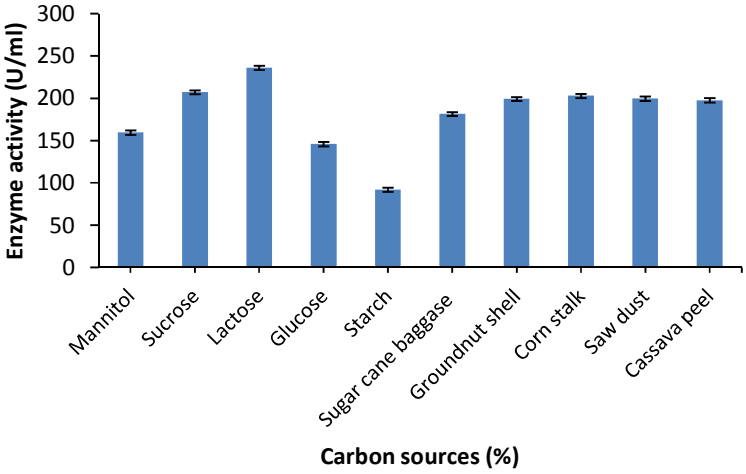


**Fig. 5. Effect of carbon sources on keratinase production by *B. licheniformis***

*B. licheniformis* (Fig. 6). This was closely followed by peptone and the least enzyme production was observed when ammonium chloride was used. The effects of urea, $NaNO_3$ and $NH_4Cl$ on production of the enzyme by the organism are not statistically significant. However, there is significant different between the effects of yeast extract, peptone with other nitrogen sources on keratinase production. Venkata et al. [6,37,43,47] recorded maximum keratinase production with 0.1% yeast extract for *B. megaterium, Bacillus* sp. JB 99, *B. licheniformis* KMBVP and *B. megaterium* while [4] reported optimum production with peptone for *B. thuringiensis*. [37] reported that in the presence of two different substrates, one which is structurally more compact and resistant (feather) and other which is more accessible and small protein supplement, the bacteria may preferentially use the latter. This would explain the comparative lower enhancement of keratinase activity measured in the presence of external nitrogen sources.

### 3.2.5 Effect of various feather concentrations on enzyme production

Fig. 7 shows the effect of various feather concentrations on keratinase production. The effect of various feather concentrations on

enzyme production by *B. licheniformis* showed optimum enzyme production with 1% feather concentration. This result is in agreement with reports [4,6,37] for *Bacillus* sp. JB 99, *B. megaterium* and *B. thuringiensis*. Lin and Yin [32] and [47] reported 1.5% and 0.5% feather concentration, respectively for *B. licheniformis* YJ4 and *B. megaterium*. The present result indicated that major regulatory mechanism is inductive. The increased concentration of feather in the medium decreased the enzyme production, suggesting catabolic repression of keratinase production [36]. A different result has been reported by [32], with 0.5% substrate concentration for *B. licheniformis*.

### 3.2.6 Effect of incubation time on keratinase production

The effect of incubation time on keratinase production is presented in Fig. 8. The organism started secretion of keratinase after 24 h of incubation. The result revealed that maximum enzyme production for isolate KDB1 was observed at 72 h and minimum enzyme production occurred at 24 h (Fig. 8). Enzyme

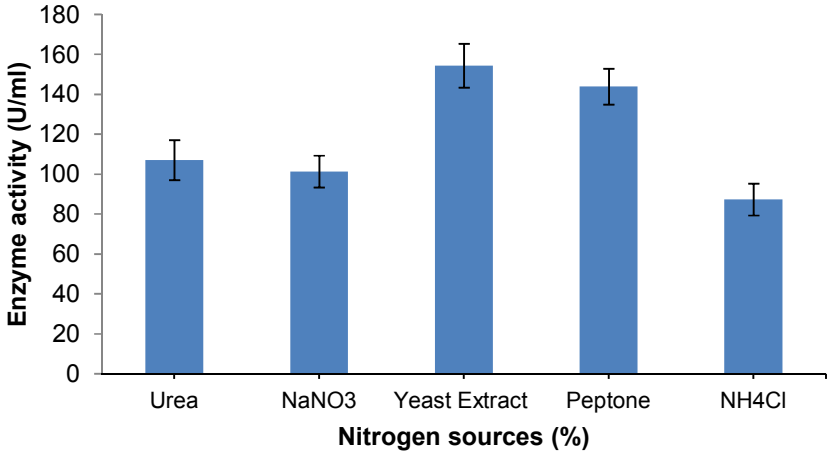


**Fig. 6. Effect of nitrogen sources on keratinase production by *B. licheniformis***

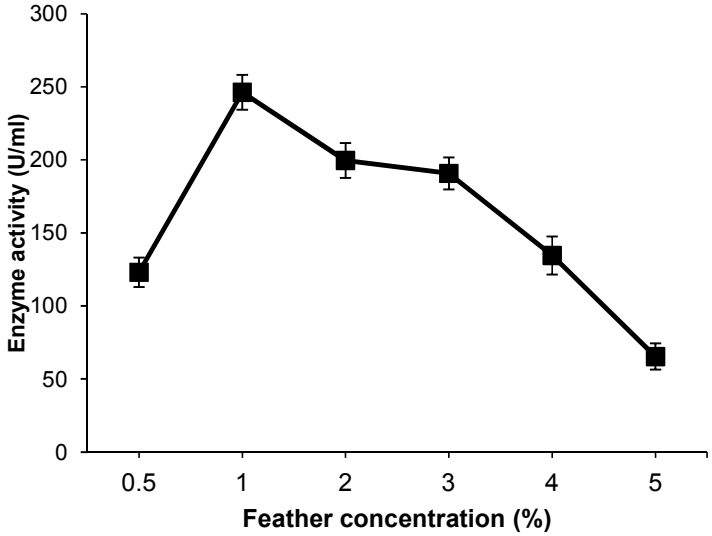


**Fig. 7. Effect of feather concentration on keratinase production by *B. licheniformis***

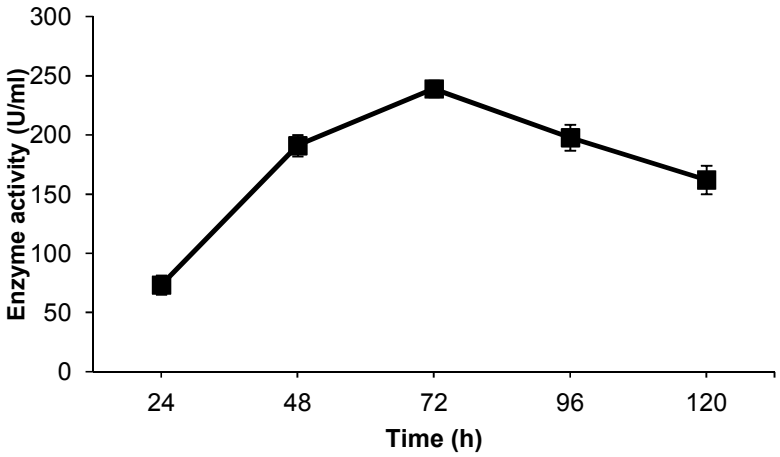


**Fig. 8. Effect of incubation time on keratinase production by *B. licheniformis***

production declined after 72 h of incubation. The decline in enzyme production after 72 h is because the production of the enzyme is growth associated. Secretion of most keratinase occurs at the growth phase of the organism. This result is in agreement with the reports by [6,32], with optimum keratinase production at 72 h for *Bacillus megaterium and B. licheniformis.* Kainoor and Naik [37] reported maximum keratinase production at 36 h incubation while [4] had optimum keratinase production at 96 h incubation time. Srivastava et al. [40,48] reported maximum keratinase production at 120 h with *Bacillus* strain SAA5 and *Bacillus thuringenesis* SN2 reported maximum keratinase production at 120 h.

## 4. CONCLUSION

The finding of this study indicated that isolate KDB1 possesses good capacity for keratinase production using cheap nutritional sources. The optimal fermentation conditions of keratinase production are as follows: pH 9.0, temperature 40°C, feather concentration 1%, lactose 1%, yeast extract 1% and incubation time 72 h. The results suggest that the keratinolytic enzyme from the isolate KDB1 strain belongs to alkaline serine protease family. Therefore, isolate KDB1 strain could be a potential candidate for the degradation of feather keratin and could be effectively used in the large scale production of keratinase for commercial purposes as well as in biotechnological applications. Attempt to purify and characterize the keratinase from this organism is currently ongoing in our laboratory.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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