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PARTIAL PURIFICATION AND CHARACTERIZATION OF A KERATINOLYTIC ENZYME FROM *PSYCHROBACTER PULMONIS* AND ITS APPLICATIONS

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ABSTRACT

This study focused on isolating and characterizing bacteria that produce the keratinase enzyme. Enrichment technique was employed using natural sources, including chicken feathers, soil, and compost. Screening was performed on 14 soil isolates, and the most efficient isolates were selected. Hydrolysis zones were observed on agar-based media at 30°C for 72 hours. Through morphological, cultural, biochemical, and molecular characterization, the potent keratinolytic isolate was identified as *Psychrobacter pulmonis*. The optimum pH and temperature for bacterial growth and enzyme activity were 7 and 30°C, respectively. Keratinolytic activity was detected during growth in liquid basal media containing 1% of the feathers, which were completely degraded within 8-9 days. The crude keratinase was partially purified by 40% ammonium sulfate precipitation at pH 7. SDS-PAGE analysis revealed a single band, indicating a pure enzyme with a molecular weight of 63 kDa. The total protein concentration in the concentrated keratinase was 3.8 mg/ml, which was 12.7 times higher than the crude keratinase based on the Bradford assay.

Keywords: Chicken feather; Keratinolytic activity; Biodegradation; Bacterial Keratinase; feather degradation; partial purified keratinase*; Psychrobacter pulmonis*

INTRODUCTION

Keratin and other insoluble proteins are generally not recognized as substrates by common proteases due to their high stability and slow degradation rates. The mechanical stability of keratin and its resistance to microbial degradation is attributed to the tight packing of protein chains in α-helical (α-keratin) or β-sheet (β-keratin) structures linked by disulfide bridges **(Brandelli *et al*., 2010; McKittrick *et al*., 2012)**. Keratin-rich protein waste can serve as a valuable source of protein, amino acids, and uncommon amino acids like serine, cysteine, proline, leucine, threonine, and tyrosine through recycling **(Riffel *et al*., 2003)**.
Keratinase is a specific protease that hydrolyzes keratin, a protein found in wool, hair, collagen and feathers, to clear obstructions in sewage and wastewater systems (**Verma *et al*., 2017; Onifade & Abu, 1998**). Microorganisms represent a very important source for the production of this enzyme (**Jani *et al*., 2017**). Selecting the right organism is key for high yield of the desired enzyme (**Kainoor & Naik, 2010**). Keratinolytic enzymes have diverse biotechnological applications in processing wastes containing keratin from poultry and leather industries through non-polluting processes (**Brandelli, 2008; Salwan & Sharma, 2019; Onifade & Abu, 1998; Gupta & Ramnani, 2006; Sharma & Devi, 2018**). After hydrolysis, the feather can be used to produce feed, fertilizer, glue, films, and uncommon amino acids including serine, cysteine, proline, leucine, threonine, and tyrosine (**Riffel *et al*., 2003**). Potential applications are in the biomedical, pharmaceutical, cosmetic, textile industries for shrink proofing wool, etc. (**Gradisar *et al*., 2000; Brandelli, 2008; Salwan & Sharma, 2019; Onifade & Abu, 1998; Gupta & Ramnani, 2006**).
This investigation centers on the isolation, screening, and characterization of keratinolytic bacteria derived from local sources of feather waste, agricultural soil, and compost. Clear zone assays on keratin agar were employed to evaluate the keratin-degrading capability of isolates. Promising strains were identified through a combination of morphological, cultural, and biochemical tests, complemented by 16S rRNA sequencing. Subsequently, these strains were analyzed to determine optimal growth conditions, substrate specificity, and enzyme production profiles. Partially purified keratinases were further examined by studying the effects of temperature, pH, and inhibitors. The efficient utilization of keratinous wastes by such isolates might enable safe recycling processes to recover valuable by-products such as amino acids, peptides, and nitrogenous fertilizers. This screening endeavor aimed to augment the repertoire of known feather-degrading microbes and keratinases available for green biotechnology and industrial biocatalysis applications. The novelty of this research is accentuated by the heightened focus on proteolytic enzymes known as keratinases, which have garnered significant attention due to their capacity to convert keratinous feather wastes, known contributors to substantial environmental pollution. In this study, bacterial isolates from soil, compost, and feathers were systematically selected based on their ability to produce potent keratinolytic proteases (keratinases). These isolates exhibited high efficiency in transforming feather wastes into valuable products, including feedstuffs, fertilizers, adhesives, films, and serving as a source for rare amino acids such as serine, cysteine, proline, leucine, threonine, and tyrosine.

MATERIALS AND METHODS

Chemicals used were analytical grade and purchased from fine chemical suppliers.

Basal medium (BM)

The prepared basal salts –chicken feather liquid culture medium used for fermentation and enzyme production was as recommended by **El- Fadaly *et al.* (1996)**. The medium ingredients (g/L) were: potassium chloride 0.2 g, ammonium dihydrogen phosphate 1.0 g, magnesium sulphate 0.2 g and 1 % dried feather. The pH was adjusted to 7.

Isolation of feather degrading microorganisms using enrichment technique

Microorganisms capable of degrading feather were enriched from soil, compost and degraded feather. 1 g of soil was suspended in 98 ml basal medium. The medium was supplemented with 1% w/v feather suspension to shorten the incubation time. Cultures were incubated at 30°C and 150 rpm for 3 weeks. After 1 week, a volume of 10 ml of broth culture was transferred to a fresh 90 ml basal medium with 1% w/v feather suspension. This was repeated four times (after 6 weeks total). Dilution series up to 10^6 were prepared from the final enrichment culture. 100 µl from the last three dilutions were spread on basal agar media containing 1% casein using the Drigalski triangle. After 72 hr, single colonies showing clear zone formation were picked as feather degrading microorganisms (**El-Fadaly *et al*., 2002**).

Isolation and Screening of feather degrading bacteria

Screening was carried out on 14 soil isolates, and efficient isolates were selected based on high keratinase production as indicated by clear zone formation **(El-Fadaly *et al.*, 2002)**.

Identification of Keratinase producing microorganisms

Keratinase producing strains were identified and characterized by morphological and biochemical characterization according to the Bergey's Manual of Systematic Bacteriology (**Holt *et al*., 1994**) and Genetically Identified by 16S rRNA sequencing.

Morphological Characterization Biochemical Characterization

Keratinase producing strains were identified by morphological and biochemical tests according to Bergey's Manual of Systematic Bacteriology (**Holt *et al*., 1994**). Tests included Gram staining, catalase, oxidase, pigment production, etc.

16S rRNA gene sequencing.

The most effective keratinase producing isolate was identified by polymerase chain reaction (PCR) sequencing at Sigma Scientific Services Co., Giza, Egypt. Sequencing was carried out using the automated ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Japan). Genomic DNA extraction used hexadecyltrimethylammonium bromide (CTAB) and integrity was verified by agarose gel electrophoresis before PCR and sequencing analysis according to **Minamisawa *et al*. (1992)**. The CTAB DNA extraction method was adapted from Russell & Sambrook method **(Russell & Sambrook, 2001)**.
PCR reaction conditions: 25 µL MyTaq Red Mix, 8 µL DNA template, 1 µL each primer (20 pmol), 15 µL nuclease free water.
Thermal cycling: initial denaturation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 15 sec, extension at 72°C for 10 sec; final extension at 72°C for 5 min.

Optimization of cultural parameters for keratinase production

Factors influencing keratinase production were optimized for selected isolates.

Effect of different solid media

Basal media **(El-Fadaly *et al*., 1996)**, King B **(King *et al*., 1954)**, synthetic **(Wang *et al*., 2007)**, nutrient **(Manual, 1984)**, potato dextrose agar - PDA **(Okon *et al*., 1977)**, and Burdman's media **(Burdman *et al*., 1998)** with 1% casein were tested. Cultures were grown in each medium at 30°C and 150 rpm for 72 hr. Keratinase was quantified by clear zone diameter (mm). The experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Effect of different initial pH

Basal agar media with 1% casein were prepared at pH 5, 6, 7, 8 and 9. Cultures were incubated at 30°C and 150 rpm for 72 hr. Keratinase was quantified by clear zone diameter **(El-Fadaly *et al.*, 2002)**. The experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Effect of different temperatures

Basal agar media with 1% casein were prepared at optimal pH. Cultures were incubated at 20, 30 and 40°C and 150 rpm for 72 hr. Keratinase was quantified by clear zone diameter **(El-Fadaly *et al.*, 2002)**. The experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Effect of different incubation periods

Cultures were grown in basal media with 1% casein at optimal pH and temperature for 24, 48, 72 and 96 hr. Keratinase was quantified by clear zone diameter **(Sabri & Borhan Aldeen, 2014; Miller, 1972)**. The experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Effect of different carbon sources

Glucose, fructose, lactose, maltose, dextrose, starch and molasses (2% w/v) were added to basal media with 1% casein. Cultures were incubated at 30°C and 150 rpm for 72 hr. Keratinase was quantified by clear zone diameter **(Shirling & Gottlieb, 1966)**. The experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Effect of different nitrogen sources

Ammonium chloride, ammonium sulfate, yeast extract, casein and peptone (g/L concentrations) according to **Sabri and Borhan Aldeen (2014)** were added to basal media with 1% casein. Cultures were incubated at 30°C and 150 rpm for 72 hr. Keratinase was quantified by clear zone diameter **(Sabri and Borhan Aldeen, 2014)**. The experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Effect of different heavy metals

Cobalt, zinc, nickel, copper, cadmium and lead (1, 5 and 10 ppm) were added to basal media with 1% casein. Cultures were incubated at 30°C and 150 rpm for 72 hr. Keratinase was quantified by clear zone diameter **(Ahmad *et al*., 2014).** Each experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Effect of shaking on keratinase production

Cultures were grown in liquid basal media with 1% of the feathers with and without shaking at 30°C and 150 rpm for 24, 48, 96, 120, 144, 168, 192, 216 and 240 hr. Keratinase was quantified by clear zone diameter **(Rajesh *et al.*, 2016)**. Each experiment was conducted in triplicate, and the mean and standard deviation were subsequently computed.

Relationship between keratinase activity and growth

Bacterial growth (CFU/ml) was determined under optimal conditions at different incubation periods to assess the relationship between keratinase activity and biomass production over time **(Macedo & Termignoni, 2014).**

Partial purification of keratinase

Ammonium sulphate precipitation and dialysis

The culture medium was filtered, and the enzyme precipitated with 20, 40 and 60% ammonium sulfate saturation for 24 hr at 4°C **(Lee *et al*., 2008)**. Precipitates were collected by centrifugation and dialyzed against different pH buffers **(Gozali *et al*., 2022; Peshin & Mathur, 1999)**.

Determination of molecular weight.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).
Crude and purified keratinase samples were analyzed by 12% SDS-PAGE to determine purity and molecular weight **(Laemmli, 1970)**. Gels were photographed scanned, analyzed using Gel Doc VILBER LOURMAT system.

Native PAGE and zymogram staining

Native PAGE was performed to detect keratinase activity, using a discontinuous buffer system without SDS and 1% CMC in the gel **(Laemmli, 1970).**

Protein quantification

The Bradford assay was used to determine protein concentration in crude and purified keratinase based on Comassie blue binding. the choice of the Bradford assay over the Lowry assay was due to Bradford assay compatibility with the sample matrix as the Bradford assay is less susceptible to interference from certain compounds or reagents that may be present in the protein sample, such as detergents, reducing agents, or certain buffer components. The Bradford assay has a larger linear range and can be more sensitive for detecting low protein concentrations compared to the Lowry assay, the Bradford assay is generally considered a simpler and faster protocol, requiring fewer steps and reagents than the Lowry assay **(Bradford, 1976)**.

Enzyme Assay

Crude and purified keratinase activities were compared at different incubation periods for *P. pulmonis.*

Enzyme Characterization.

Purified keratinase was characterized as described by **Coral *et al*. (2002)**. All determinations were carried out in triplicates and measured against blank samples.

Effect of temperature stability on keratinase activity

Keratinase activity was measured after incubating the enzyme with 1% casein substrate at pH 7 and various incubation temperatures: 30, 40, 60 and 100°C for

30 min. Keratinase activity was quantified by clear zone diameter **(Sangali & Brandelli, 2000)**.

Effect of cooling and freezing on concentrated keratinase activity

Enzyme was stored at 4°C and -2°C for 1 month and activity was measured as previously mentioned **(Lin *et al.*, 1992).**

Feather degradation by partial purified keratinase

1 g feather, 10 ml buffer pH7 and 2 ml purified keratinase were incubated for 8, 16 and 24 hr. Feather degradation was assessed by weighing dried filter papers before and after filtering the reaction mixtures. Controls without enzyme were performed **(Ali *et al.*, 2011).**

RESULTS & DISCUSSION

Isolation and Screening of feather degrading bacteria

Fourteen soil isolates were screened, and isolate 12 showed the highest clear zone diameter as showed in figure 1, indicating potent keratinase production. Members of the Bacillus genus are rich sources of commercial enzymes including keratinases, making them suitable candidates for processing keratinous wastes **(Hoq *et al.*, 2005;Tamreihao *et al.*, 2019; Saleem *et al.*, 2012)**.

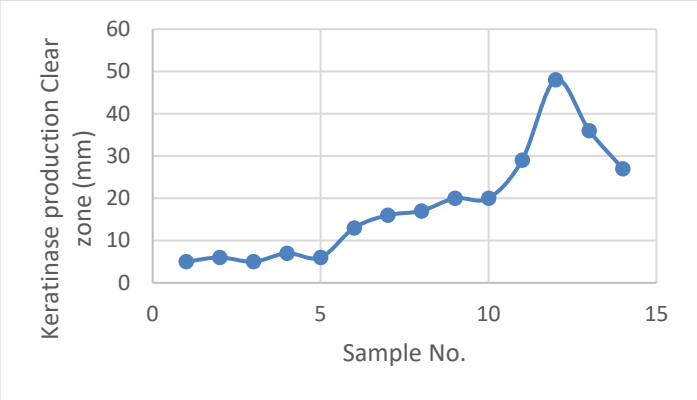

Figure 1 Isolation and Screening of degrading feather bacteria.

Identification of Keratinase producing microorganisms

Morphological and biochemical characterization

Table 1 summarizes the morphological and biochemical characterization of Isolate 12. Based on the results, the following can be observed:

Morphological Characteristics: The isolate is a non-spore-forming coccus (spherical-shaped bacterium), Gram-negative and non-motile.

Biochemical Tests: The isolate is positive for catalase and oxidase tests, it does not produce pigments or hydrogen sulfide (H2S), cannot grow anaerobically and cannot tolerate high salt concentrations (5% and 6% NaCl).

Growth Conditions: The isolate can grow at 20°C and 30°C but not at 40°C.

Antibiotic Sensitivity: The isolate is sensitive to gentamicin, penicillin, and tetracycline but resistant to kanamycin.
Overall, the results indicate that Isolate 12 is a Gram-negative, non-motile, catalase and oxidase-positive coccus that can grow at moderate temperatures and has varying sensitivity to different antibiotics.

Table 1 Morphological and biochemical characterization of Isolate 12

Test characteristic	Result
Shape of cell	coccus
Spore forming	-
Gram reaction	-ve
Motility	Non motile
Catalase test	+
Oxidase test	+
Anaerobic growth	-
Pigment production	-
Tolerance of NaCl	
5%	-
6%	-
Growth Temperature 20 C°	+
30 C°	+
40 C°	-
H2S production	-
Sensitivity to antibiotics	
Gentamycin (10)	+
penecillin	+
Kanamycin (30)	-
Tetracyclin (30)	+

16S rRNA gene sequencing

The isolate showed 88.54% sequence similarity to *Psychrobacter pulmonis*, as showed by figure 2. The following nucleotide sequence alignment between a Query sequence and a Subject sequence as the Subject sequence is labeled as "*Psychrobacter pulmonis* strain S-606 16S ribosomal RNA, partial sequence" showed the Query and Subject sequences along with various symbols indicating matches, mismatches, and gaps between the two sequences. The vertical bars (|) represent positions where the nucleotides are identical between the Query and Subject sequences, while spaces indicate gaps or insertions/deletions. The alignment covers a portion of the 16S ribosomal RNA gene sequence, which is commonly used for bacterial identification and phylogenetic analysis. The 16S rRNA gene is highly conserved among bacteria and contains both conserved and variable regions, making it useful for taxonomic studies. Based on the alignment, the Query sequence shares a significant similarity with the 16S rRNA sequence of the *Psychrobacter pulmonis* strain S-606, suggesting that the Query sequence belong to a closely related or potentially the same bacterial species.
Nucleotide sequence alignment between a Query sequence and a Subject sequence (*Psychrobacter pulmonis* strain S-606 16S ribosomal RNA, partial sequence):

```
Query  1     CGCACCAGCGCGTGGAGCATGTGGTTTATTTTG-GGCAA-ACGAAGAACCTTACCCAGT-  57
              ||||| |||| ||||||||||||||| || |  ||||  ||||||||||||  ||
Sbjct  856   CGCACAAGCG-GTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTC  914

Query  58    TCGACATATCCTGAATCAACCAGAGATGCGGGAGT-CTCTCGGGAATTAGAATACACATG  116
              | |||||||  |||||  ||||||||||||| |  ||||||||||||||  ||
Sbjct  915   TTGACATATCTAGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATTAGAATACAGGTG  974

Query  117   TGGCATGTGGGTGTTCAGCTCGTGTGTTGAGATGTTGTGTTAAGTCCCGCAACGAGCGCA  176
              |||||  ||  ||||||||||  ||||||||| ||||||||||||||||||||
Sbjct  975   CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA  1034

Query  177   ACCC-CGTCATTAGTTACCCATC-GTTTGGCCGGGCACT-TAAGGATACTGCCAGTGACA  233
              ||||  ||| |||||||| | | ||||||||||| ||| ||||||||||||||||||
Sbjct  1035  ACCCTTGTCCTTAGTTACC-AGCGGTTTGGCCGGGAACTCTAAGGATACTGCCAGTGACA  1093

Query  234   AACTGG-GGAA-GC-GGGACGACGTCAAGTCATCATGGCCCTT-CGACCAGGGCTACACA  289
              |||||| |||| || |||||||||||||||||||||||| ||||||||||||||
Sbjct  1094  AACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACA  1153

Query  290   CGTGCTACAATGGTAGGTACAGAGGGCAGCTACACAGCGAGGTGAAGCGAATCTCAAAAA  349
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Query  350   GCCGTTCTTAGTCCAGATTGGAGTCTGCAACTCGCCTCCATGAAGTAGGAATCGCTAGTA  409
              |||  || |||||||||||||||||||| |||||||||||||||||||||
Sbjct  1214  GCCTATCGTAGTCCAGATTGGAGTCTGCAACTCGACTCCATGAAGTAGGAATCGCTAGTA  1273
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Query 410 ATCGCGGATCAGCATGCCGCGGTGA 434
Sbjct 1274 ATCGCGGATCAGAATGCCGCGGTGA 1298

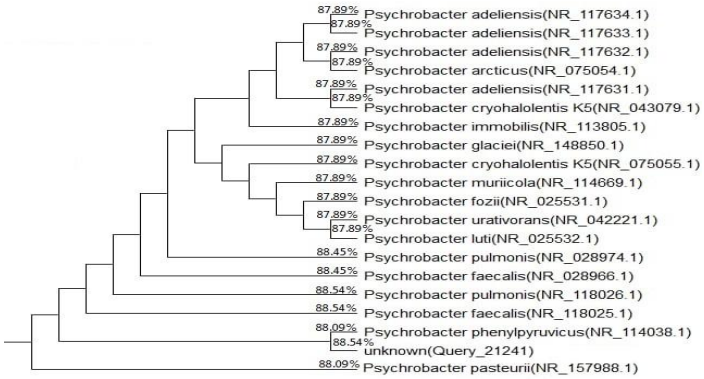

Figure 2 Phylogenetic tree of *Psychrobacter pulmonis*

Optimization of cultural conditions for keratinase production

Effect of different solid media

Based on Figure 3, the results show that the basal medium led to the highest keratinase production by *P. pulmonis*, as indicated by the largest clear zone diameter observed on this medium compared to the other solid media tested.
These findings are in line with previous studies that have investigated the effect of different solid media compositions on keratinase production by various microbial sources. For instance, **Anbu *et al.* (2007)** reported that a basal medium containing feather meal, yeast extract, and other nutrients supported optimal keratinase production by Scopulariopsis brevicaulis. Similarly, **Rajput *et al.* (2010)** found that a medium composed of feather meal, glucose, and inorganic salts was most favorable for keratinase production by *Bacillus pumilus*.
The composition of the basal medium likely provided an optimal balance of carbon, nitrogen, and other essential nutrients required for the growth and metabolic activities of *P. pulmonis*, leading to enhanced keratinase synthesis and secretion. Keratinolytic enzymes, such as keratinases, are typically induced by the presence of keratin-rich substrates in the culture medium, and the specific nutrients present can significantly influence enzyme production (**Gupta & Ramnani, 2006**).
It is worth noting that the optimal solid medium composition may vary among different microorganisms due to their diverse nutritional requirements and metabolic pathways. Therefore, optimizing the solid medium composition is crucial for maximizing keratinase production by a specific microbial strain. Further research could involve investigating the individual components of the basal medium and their respective contributions to keratinase production.
The use of casein, a milk-derived protein, in the basal media serves as a substrate or inducer for the production of the keratinase enzyme. Keratinases are proteolytic enzymes that specifically break down keratin, a structural protein found in feathers, hair, nails, and wool. While keratin is the natural substrate for keratinases, it is often insoluble and recalcitrant, making it challenging to utilize as the sole substrate for enzyme production or assays. By supplementing the basal media with a more readily available and soluble protein source like casein, the researchers likely aimed to induce or promote the production of keratinase enzymes by the bacterial cultures. The clear zone formation on the casein-containing media can then be used as an indirect measure of keratinase activity, as the enzyme would hydrolyze the casein, creating a clear zone around the bacterial colonies. Therefore, using keratin basal media alone without the supplementary casein may not provide an optimal environment for inducing or detecting keratinase production, as keratin itself can be more difficult for the bacteria to utilize initially. The addition of casein serves as an easily metabolizable protein source that can stimulate keratinase production and facilitate the screening process (**Gupta & Ramnani, 2006; Brandelli *et al*., 2010; Rajput *et al*., 2010**).

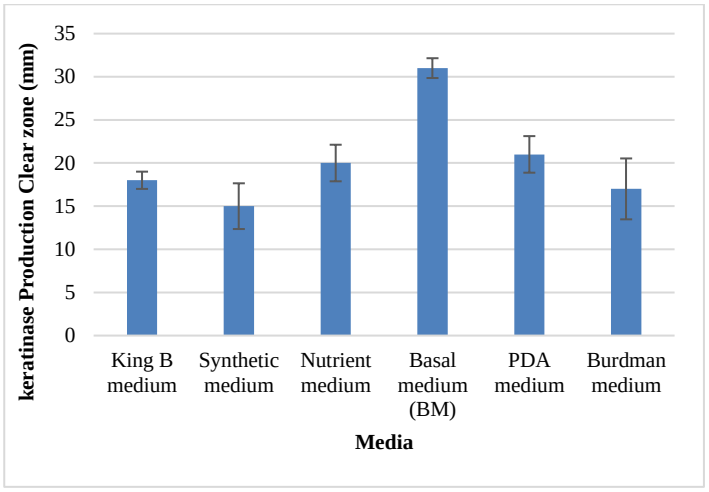

Figure 3 Effect of different solid media for keratinase production

Effect of different initial pH

Enzyme production was highest at pH 7 as showed by figure 4. Many keratinase producers show optimal activity at neutral to alkaline pH **(Reddy *et al.*, 2017)**. **El-Refai *et al*. (2005)** found pH 8 to be optimum when tested in the pH range 6-9 for *B. pumilus* FH9 bacterium. Medium pH for keratinolytic enzyme production by *B. megaterium* F7-1 was in the pH range 4.0–11.0 with an optimum at pH 6.5 – 11.

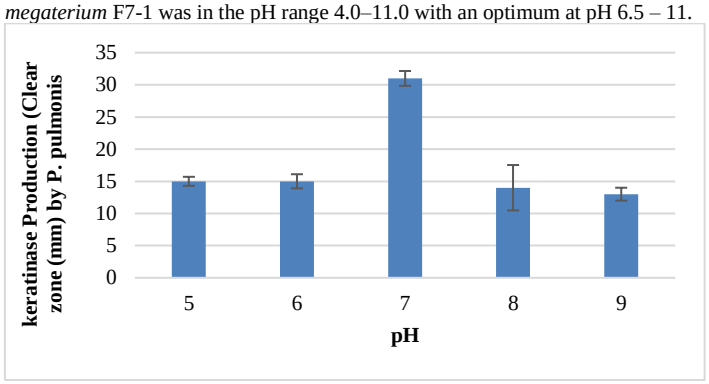

Figure 4 Effect of different pH for production of keratinase on solid BMA (Basal Agar medium)

Effect of different Temperatures

Incubation at 30°C resulted in the largest clear zone diameter, data were presented in figure 5. Keratinolytic bacteria like *Bacillus* sp. kr16 and *S. maltophilia* L1 also favor 30-40°C for enzyme production **(Werlang & Brandelli, 2005; Cao *et al*., 2009; Brandelli *et al*., 2010; Friedrich & Antranikian, 1996; Nam *et al*., 2002)**.

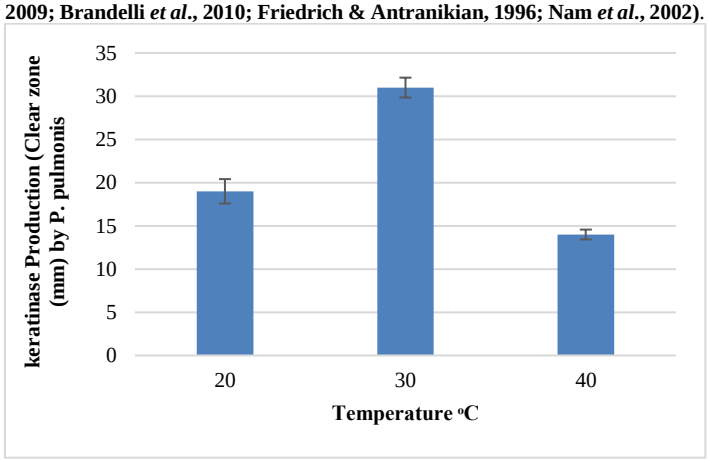

Figure 5 Effect of different Temperature at pH 7 for keratinase production on solid BMA (Basal Agar medium)

Effect of different incubation periods

Maximum enzyme production occurred after 72 hr, as showed in figure 6 which aligns with previous reports; **El-Refai *et al.* (2005)** reported that keratinase enzyme secretion by *B. pumilus* FH-9 was optimum after 48 hr of incubation when production was recorded for 12-96 hr of incubation. **Balaji *et al* (2008)** observed

keratinase production by *B. subtilis* MTCC 9102 for 24-120 hr of incubation and found that 48 hr of incubation was found optimum.

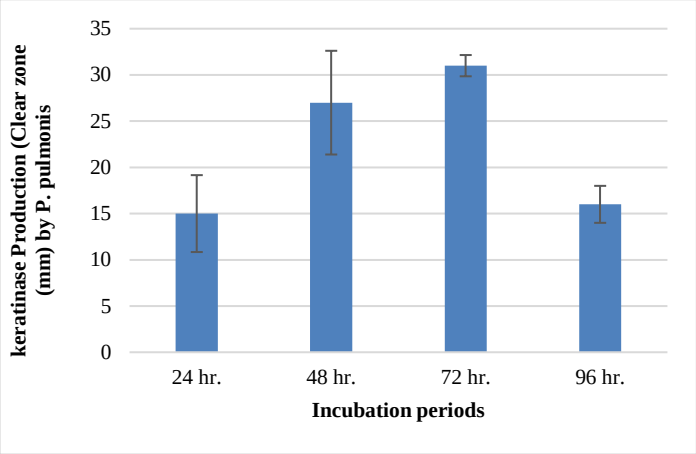

Figure 6 Effect of different incubation periods for production of keratinase on solid BMA (Basal Agar medium)

Effect of different carbon sources

Data presented in figure 7 showed that additional carbon sources decreased keratinase production, likely due to catabolite repression. *B. megaterium* F7-1 decreased keratinolytic enzyme production in media containing different carbon sources including fructose, galactose, glucose, glycerol, lactose, maltose, mannitol, sorbitol and sucrose each at 0.1% w/v to the basal salt medium with or without feather **(Gessesse *et al.*, 2003)**. In a different study, various concentrations of glucose ranging from 0% to 0.5% were added to the medium. Keratinolytic enzyme production was increased with an increase in glucose concentration from 0% to 0.1%. Further increase of glucose up to 0.5% inhibited the keratinolytic enzyme production by *Stenotrophomonas maltophila* R-13 strain **(Jeong *et al.*, 2010)**.

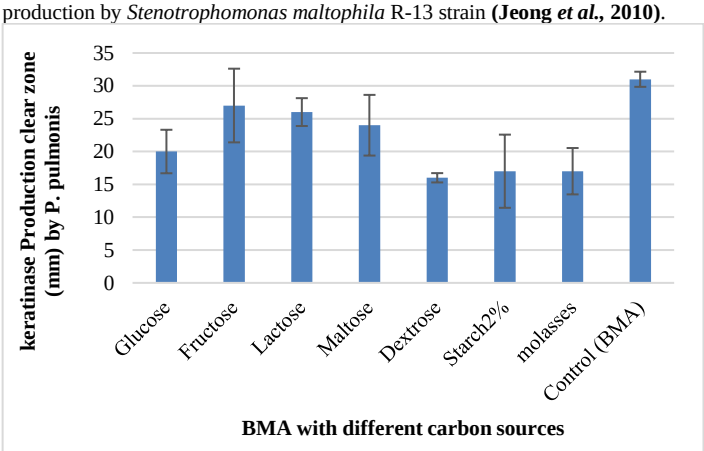

Figure 7 Effect of different carbon sources for production of keratinase on solid BMA (Basal Agar Medium)

Effect of different nitrogen sources

Data presented in figure 8 showed that additional nitrogen sources decreased keratinase production by *P. pulmonis* likely due to catabolite repression. **Gessesse *et al.* (2003)** described that *Nesternkonia* spp. AL-20, *B. pseudofirmus* AL-89 strains produced protease when grown using feather or casein as nitrogen and carbon sources. **Brandelli *et al.* (2010)** conversely reported that the addition of supplementary substrates (carbohydrates; inorganic and/or organic nitrogen sources) often decreased enzyme production by some microorganisms mainly due to catabolize repression mechanisms. Production of keratinase was increased using nitrogen source (potassium nitrate) and shaking at 200 rpm, **Jeong *et al.* (2010)** reported similar results.

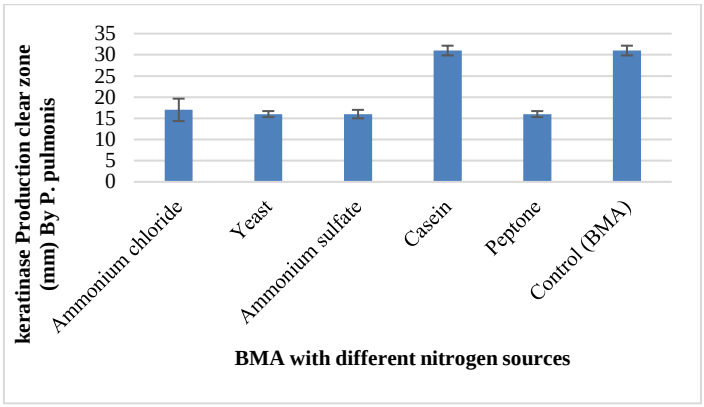

Figure 8 Effect of different nitrogen sources for production of keratinase on solid BMA (Basal Agar Medium)

Effect of different heavy metals

Low concentrations of Co^{2+}, Cu^{2+} and Cd^{2+} increased keratinase production by *P. pulmonis* while Zn^{2+}, Ni^{2+} and Pb^{2+} had inhibitory effects as showed in figure 9. These results agree with previous studies as **Brandelli *et al.*, (2010)** reported that keratinolytic protease activity was stimulated in the presence of divalent metal ions like Ca^{2+}, Mg^{2+}, and Mn^{2+} and on the other hand, heavy metals like Cu^{2+}, Ag^{2+}, Hg^{2+}, and Pb^{2+} caused inhibition of keratinolytic enzymes. **Jeddi *et al.*, (2021)** reported that the keratinolytic activity by *Bacillus Mojavensis* FUM125 was increased over 50% at different concentrations of Ca^{2+}, Mg^{2+}, K^{+}, and Ca^{2+} and strongly inhibited (over 80 %) with different concentrations of Co^{2+}, Fe^{2+}.

The heavy metals cobalt (Co), zinc (Zn), nickel (Ni), copper (Cu), cadmium (Cd) and lead (Pd) were chosen to evaluate their influence on keratinase production because previous studies have shown that certain divalent metal ions can stimulate keratinolytic protease activity, whereas transition metals or heavy metals often have an inhibitory effect **(Gupta & Ramnani, 2006; Tiwary & Gupta, 2012; Jaouadi *et al.*, 2010)**. Specifically, divalent metals such as calcium (Ca^{2+}), magnesium (Mg^{2+}), and manganese (Mn^{2+}) have been reported to enhance the activity of some keratinases **Gupta & Ramnani, 2006; Tiwary & Gupta, 2012)**. In contrast, cationic transition metals like copper (Cu^{2+}), silver (Ag^{2+}), mercury (Hg^{2+}) and lead (Pb^{2+}) are known to cause inhibition of these enzymes **(Riffel *et al.*, 2003; Jaouadi *et al.*, 2010)**. Therefore, this study selected a range of heavy metals, including stimulation factors cobalt and zinc as well as inhibitory metals like copper and lead, in order to elucidate their effect across a spectrum of influence on keratinase production by the isolated bacterial strains. The inclusion of multiple divalent and heavy metal ions allowed for comparative analysis to determine which factors induce or repress enzymatic activity. The findings would help characterize the nature of the keratinases produced by these newly isolated feather-degrading bacteria. Evaluating the impact of different metals also facilitates identification of conditions that may promote greater enzymatic yields for potential industrial-scale applications. In summary, the investigators strategically chose metals with previously reported enzyme stimulating or inhibiting properties in order to fully assess their effects on keratinase biosynthesis across the bacterial strains in this study.

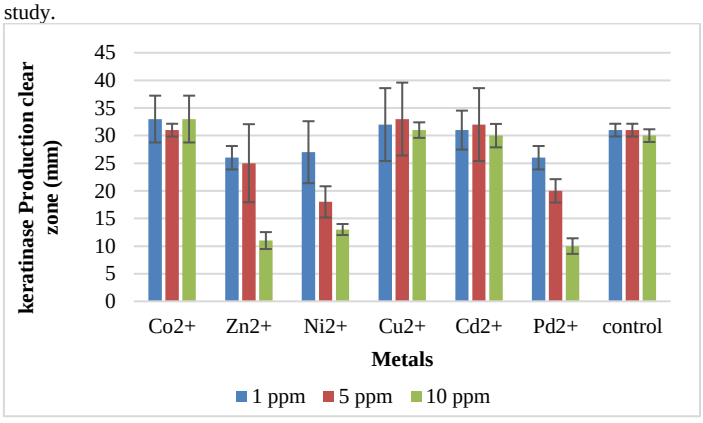

Figure 9 Effect of different Heavy metals for production of keratinase on solid BMA (Basal Agar Medium)

Effect of shaking on keratinase production

Enzyme production was higher with shaking at 216 hr compared to stationary cultures as showed by figure 10. Keratinolytic proteases are often produced under submerged shaking conditions. The results suggest that shaking or agitation during submerged fermentation enhances keratinase production by *Psychrobacter pulmonis* compared to stationary cultures. Specifically, the data in figure 10 showed higher keratinase activity after 216 hours in shaken cultures versus static

cultures. This finding aligns with previous studies reporting improved keratinase yields under shaking conditions for various bacterial strains. For instance, **Gupta and Ramnani (2006)** reported a 2.5-fold increase in keratinase production by Cronobacter sakazakii MTCC 5522 in a shaken flask compared to a static flask culture. Similarly, **Singh *et al.* (2017)** found that agitation at 150 rpm resulted in maximum keratinolytic activity by *Bacillus subtilis* Strain S1 in submerged State Fermentation Using Feather Waste and the lowest production was found at 300 rpm. Increased agitation speed led to enhanced oxygen transfer rates, thereby facilitating cell proliferation. However, excessive agitation, typically around 300 rpm, although conducive to bacterial growth, detrimentally impacted keratinase production. This adverse effect is attributed to elevated dissolved oxygen levels and heightened shear stress, which likely suppressed the synthesis and secretion of keratinase. Conversely, lower agitation speeds, such as 100 rpm, resulted in reduced keratinase production. This outcome may be attributed to inadequate mixing of bacterial cells and substrate due to the heterogeneous distribution caused by the lower agitation speed, consequently leading to diminished dissolved oxygen levels and, subsequently, reduced keratinase production **(Singh *et al.*, 2017)**. The enhanced keratinase production with shaking is likely due to improved oxygen transfer, substrate dispersion, and overall mass transfer in the shaken culture. Proper agitation and aeration are crucial for efficient microbial growth and enzyme secretion during submerged bioprocesses.

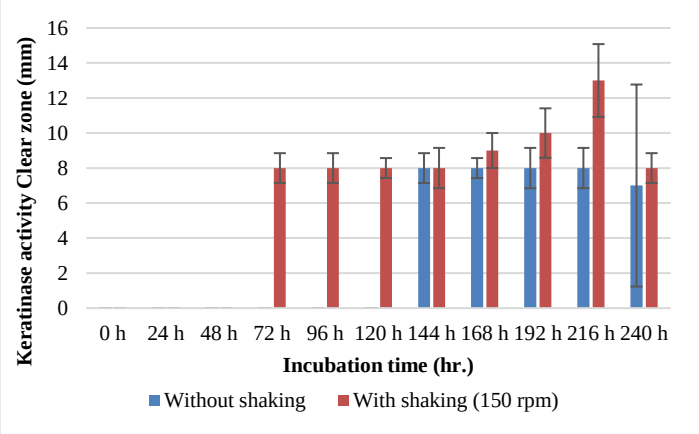

Figure 10 Effect of shaking process on production of keratinase by *Psychrobacter pulmonis*

Relationship between keratinase activity and growth

Keratinase activity and biomass increased steadily over time, reaching maximal levels after 216 hr., results were presented in figure 11. Similar trends have been reported in other keratinase producing fungi such as *Endothia parastica* **(Melzer & Boland, 1999)**, *Trichophyton simii* **(Singh, 1997)**, *Malbranchea gypsea* **(Singh, 1998)**, and *Trichophyton vanbreuseghemii* **(Moallaei *et al.*, 2006)**, the rate of keratinase production reached a maximum concentration after 21 days of incubation and the concentration of extracellular keratinase produced by *Lysobacter* NCIMB 9497 **(Allpress *et al.*, 2002)** was maximal after 29 days of grow.

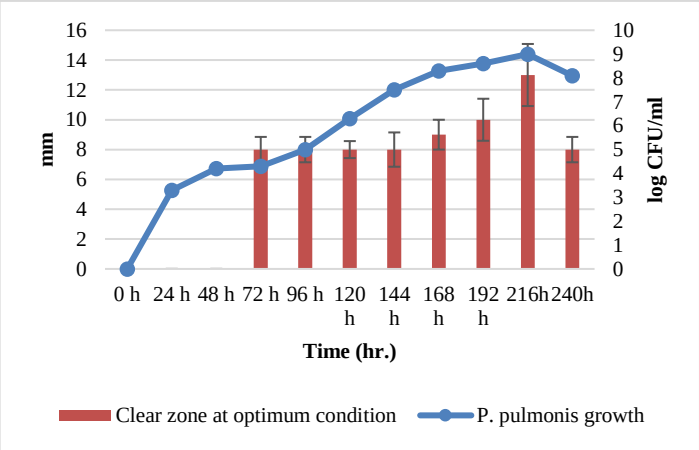

Figure 11 Relationship between keratinase activity and growth activity (biomass) of *P. pulmonis* at different incubation periods

Partial purification of keratinase

Ammonium sulphate precipitation and dialysis

Purification with 40% ammonium sulfate at pH 7 resulted in the highest recovery of enzyme activity as showed in table 2, which agrees with previous studies; **Cai *et al.* (2008)** studied the keratinase produced by *Bacillus subtilis* KD-N2 strain which was purified using ammonium sulphate precipitation. **Chitte *et al.* (1999)** detected the keratinolytic activity in the broth culture of feather degrading thermophilic *Streptomyces thermoviolaceus* SD8. The crude enzyme was concentrated by precipitation with 80% saturation of ammonium sulphate. **Zhang *et al.* (2009)** reported that an alkaline keratinase extracted from *Bacillus sp*. was purified and characterized and solid ammonium sulfate was selected to precipitate the enzyme, the results of the purification showed a final purity of 6.13 times higher with a yield of 19.4 %, which was consistent with the results of **Jaouadi *et al.* (2015)**.

Table 2. Partial purification of keratinase enzyme by different concentrations of ammonium sulfate

Isolate	Control	20 % amm. sulfate			40 % amm. sulfate			60 % amm. sulfate		
		Clear zone (cm)			Clear zone (cm)			Clear zone (cm)		
		PH4	PH7	PH9	PH4	PH7	PH9	PH4	PH7	PH9
P. pulmonis	6 ± 0	7 ± 10	7 ± 1	8 ± 1.15	7 ± 0.10	13 ± 2.08	9 ± 1.15	7 ± 1.0	8 ±0.70	9±1.0

Determination of molecular weight

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified keratinase showed a single band at ~63 kDa by SDS-PAGE (on a 12 % gel), indicating near homogeneity as showed in figure 12. **Cai *et al.* (2008)** studied that keratinase purification by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and found that the purified keratinase generated by *Bacillus subtilis* KD-N2 strain has a molecular mass of 30.5 kDa. **Chitte *et al.* (1999)** have detected keratinolytic activity in the broth culture of feather degrading thermophilic *Streptomyces thermoviolaceus* SD8 by precipitation with 80% saturation of ammonium sulphate, the crude enzyme was concentrated and desalted by SephadexG-10–120 gel chromatography followed by lyophilization. The specific activity of the enzyme was enhanced 50-fold. PAGE analysis indicated a monomeric form with a molecular weight of 40 kDa. The 16 molecular weight of reported keratinases is around 18 KDa and 280 KDa **(Brandelli *et al.*, 2010)**. The range of molecular weight of the other keratinases was reported, between 16 kDa **(Page & Stock, 1974)** and 440 kDa **(Ruey *et al.*, 1971)**.

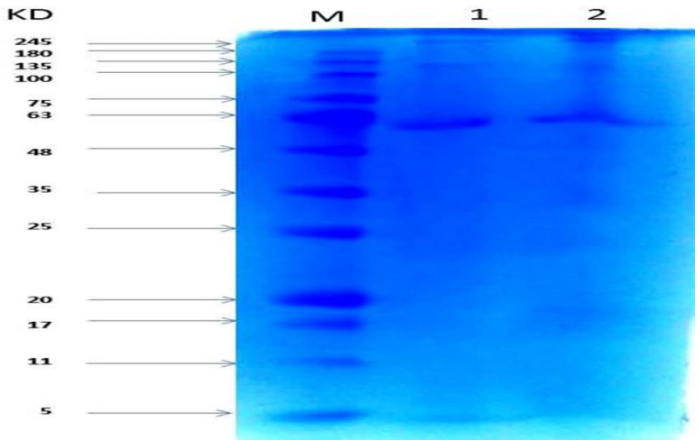

Figure 12 SDS-- polyacrylamide gel electrophoresis

Native PAGE and Zymogram staining for checking keratinase bands.

To detect the activity of keratinase in gel, Native gel electrophoresis was used and casein was used as a substrate to protein. Figure 13 showed that there were no bands as the denaturation of protein was not performed.

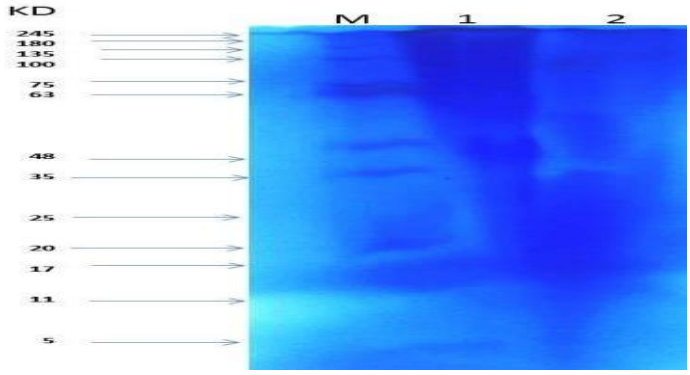

Figure 13 Native gel electrophoresis

Based on the information provided in Figure 13, the results showed no visible bands on the native polyacrylamide gel electrophoresis (PAGE) when casein was used as the substrate to detect keratinase activity. The lack of bands in the native PAGE is likely due to the absence of protein denaturation during sample preparation, as mentioned in the text. Native PAGE separates proteins in their folded, native conformations without prior denaturation or treatment with reducing agents. As a result, the keratinase enzyme likely retained its native structure, hindering its separation and visualization on the gel. This observation aligns with previous studies that highlight the importance of protein denaturation for effective keratinase detection via zymography. For example, **Ramnani and Gupta (2007)** reported successful visualization of keratinolytic activity bands on SDS-PAGE gels after treating the samples with β-mercaptoethanol and SDS to denature and dissociate the enzymes.

Similarly, **Farag and Hassan (2004)** employed SDS-PAGE zymography with gelatin as the substrate to detect keratinase activity from *Aspergillus oryzae*, demonstrating clear bands only after denaturing the enzyme samples. The authors emphasized the necessity of protein denaturation to facilitate enzyme entry into the gel matrix and subsequent renaturation during the zymogram staining process. In summary, the lack of visible bands in Figure 13 is likely due to the absence of protein denaturation, which prevented the keratinase from entering and resolving properly on the native PAGE gel. Appropriate denaturation steps, such as treatment with SDS and reducing agents, are typically required for successful detection and visualization of keratinase activity using zymogram techniques.

Protein concentration

The purified enzyme showed a 12.7-fold higher protein concentration compared to the crude extract according to data presented in table 3. According to **Aravind *et al.* (2016),** the total quantity of protein produced by *Pseudomonas florescence sp.* After 24 hours of incubation was found 262.85 µg/ml. **Kumar (2008)** also reported similar alkaline protease production from solid waste by *Pseudomonas aeruginosa*.

Table 3 relationship between the total quantity of protein and keratinase activity

sample	Concentration of total protein	volume	Activity of keratinase (clear zone cm)
Concentrated keratinase	3.8 mg/ml	13.5ml	13±2.08
Non-Concentrated keratinase	0.3 mg/ ml	1150 ml	7±10

Enzyme assay

Results presented in figure 14 showed that the purified keratinase degraded casein faster than the crude extract. The assessment of keratinase activity revealed that the crude keratinase enzyme was capable of hydrolyzing casein after a nine-day incubation, forming a wider clear zone after 20 hours. In contrast, the concentrated keratinase enzyme generated the same clear zone after only 8 hours, both attributable to *Psychrobacter pulmonis*.

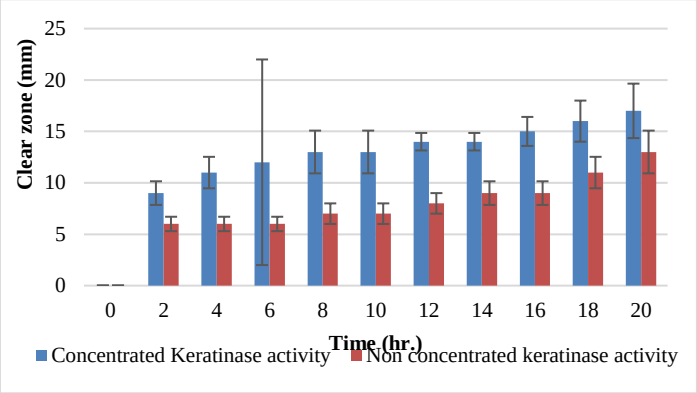

Figure 14 keratinase assay produced by *Psychrobacter pulmonis* for casein hydrolysis after 20 hr but conc. enzyme gives the same clear zone after 8 hr.

Keratinase enzyme characterization

Effect of temperature on keratinase activity

The enzyme was stable up to 60°C, with complete inactivation at 100°C due to protein denaturation as showed in figure 15. Similar thermal stabilities have been reported; **Su and Lee (2001)** reported enzyme stability up to 50°C followed by a decreased activity above 70°C. The enzyme retained more than 90 % and 15 % of its activity at 60°C and 70°C, respectively, however, the enzyme was inactivated at 80°C. The activity and stability of the enzyme was higher than salt-tolerant keratinase from *Aspergillus sp.* FC-10. **Chakrabarti *et al.* (2000)** showed maximum activity up to 60°C. The enzyme retained about 60 % at 70°C whereas at 80°C it retained only 20 % of its keratinolytic activity and at 90°C, it was inactive completely.

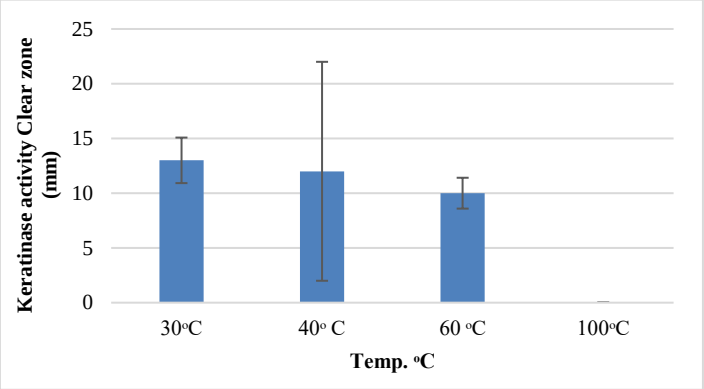

Figure 15 Effect of temperature on keratinase activity

Effect of cooling and freezing

Data presented in table 4 showed that storage at 4°C and -2°C increased enzyme activity compared to the crude extract, indicating improved stability. Other studies also showed retention of keratinase activity; *Bacillus licheniformis* N22 keratinase was stable at 4°C for up to 8 weeks compared to *Bacillus licheniformis* PWD-1 keratinase reported to loss 22 % of its activity after 19 days when stored at 4°C **(Shih & William, 1992)**. Keratinase of *Streptomyces fradiae* has also been reported to retain its activity for several weeks at 4°C when stored at pH 7 but rapidly lost its activity at pH 8.5 **(Nickerson & Noval, 1961)**.

Table 4 effect of cooling and freezing on keratinase activity

Temp.	Con. Keratinase (Clear zone mm)		Non conc. keratinase (control) (Clear zone mm)	
	24hr.	48hr.	24hr.	48hr.
30°C	13±2.08	12±10	7±10	7±10
4 °C	14±10	13±0.71	7±10	7±10
-2°C	14±0.85	14±0.85	7±10	7±10

Feather degradation by partial purified keratinase

Increased degradation was observed after 24 hr incubation with purified enzyme compared to crude extracts, highlighting its stronger keratinolytic activity according to data showed in table 5. Rapid feather degradation by other microbial keratinases has been documented such as *Streptomyces* BA7 **(Korkmaz *et al.*, 2003)**, *Streptomyces* S7 **(Tatineni *et al.*, 2008)** and *Bacillus licheniformis* ER-15 **(Tiwary & Gupta, 2010).**

Table 5 feather degradation by keratinase

Time (hr.)	Feather at pH7 (Control) Feather Weight loss	Feather + keratinase Feather Weight loss
8 hr.	0.18	0.85
16hr.	0.20	0.87
24hr.	0.23	0.9

CONCLUSION

Keratinase-producing bacteria were isolated from soil and identified as the species *Psychrobacter pulmonis*. This strain exhibited a remarkable capacity for keratinase production, demonstrating significant keratinolytic activity after a nine-day incubation period at 30°C. Purification of the enzyme was accomplished through ammonium sulfate precipitation at 40% saturation, followed by dialysis. The assessment of keratinase activity revealed that the crude keratinase enzyme was capable of hydrolyzing casein after a nine-day incubation, forming a wider clear zone after 20 hours. In contrast, the concentrated keratinase enzyme generated the same clear zone after only 8 hours, both attributable to *Psychrobacter pulmonis*. The production of this enzyme coincided with the degradation of chicken feather waste under aerobic conditions. The total protein content of the partially purified keratinase was found to be 12.7 times greater than that of the crude keratinase. Furthermore, the keratinase enzyme exhibited stability across a range of temperatures, from 30 to 60°C, while becoming inactivated at 100°C due to protein denaturation. Notably, keratinase activity increased upon cooling and freezing. The degradation of feathers by the partially purified keratinase exceeded that achieved by microorganisms. This keratinase-producing microorganism holds promise for diverse industrial applications, including bio-detergents, food processing, pharmaceuticals, leather processing, cosmetics, and textile industries, particularly in wool shrink-proofing.

Conflict of interest statement: Authors are not affiliated with or involved with any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this paper.

Data availability statement: The primary research data that support the findings of this study are in the possession of the authors of this manuscript only and are not available online other than for publication in this journal.

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