Optimization of keratinase production from *Pseudomonas oleovorans* K1 and *Acinetobacter variabilis* K2, bacterial isolates obtained from poultry feather waste

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Abstract
Feathers, a major poultry industry by-product, present an environmental challenge due to improper disposal and their resistant nature. A primary method employed to degrade these feathers involves the use of keratinase, an enzyme that breaks down keratin, the principal component of poultry feathers. This study aimed to isolate keratinase-producing bacteria from poultry waste soil collected from Vazhayur, India. The process involved enrichment using feather meal broth followed by isolation and screening through skim milk agar, keratinase plate assays and feather degradation tests. Among the 10 isolates, three isolates designated as isolate 1, 2 and 10 exhibited high keratinase activity. These were identified as Pseudomonas oleovorans K1 (Isolate 1), Acinetobacter variabilis K2 (Isolate 2) and Bacillus tropicus K10 (Isolate 10) respectively.

The highest keratinase activity, 29.80±0.36 U/ml, was observed in P. oleovorans, while keratinase derived from A. variabilis demonstrated an activity of 26.23±0.39 U/ml. Optimization of keratinase production revealed that optimal conditions for maximum production included the presence of basal media supplemented with 1% keratin, at pH 7, incubated at 35 °C for 48 hours. Following optimization, an almost two-fold increase in keratinase production was observed highlighting the isolates potential to be used for large scale production of keratinase.

Keywords: Keratinase, feather degradation, optimization.

Introduction

Feathers account for approximately 8 billion tons of waste produced by the global poultry industry and are major environmental concerns[11]. A small percentage of this by-product is used as an insulating material, animal feed and fertilizer, although a significant portion of it is burnt or dumped as waste[19]. Chicken feathers contain 90% keratin, an insoluble fibrous structural protein enriched in disulfide bonds, which makes it recalcitrant to most proteases, thus delaying its degradation[10]. Although the protein-rich content of feather biomass makes it a cost-effective source of protein, its recalcitrant nature significantly reduces its quality[18].

Keratinase, an enzyme produced by various microbes, has considerable efficiency in degrading keratin when compared to many enzymes[14]. The superiority of keratinase-based methods over conventional physical and mechanical techniques, including hydrothermal degradation, in terms of sustainability, cost-effectiveness and yield, is evident. These traditional approaches are marred by environmental and cost concerns whereas keratinase application on feather waste presents a promising alternative[18]. A recent study highlighted the potential of keratinase-producing microbes to improve the synthesis of polyhydroxybutyrate (PHB), thus aiding in the conversion of feather waste into degradable bioplastics[20]. Nanomaterials composed of keratinase and reduced graphene oxide have been shown to exhibit antibiofilm activity against *Acinetobacter. baumannii*, in addition to their potential applications in the degradation of feather waste[2].

The utilization of keratinase produced by fungi and actinomycetes has been limited due to pathogenic concerns while bacterial isolates, particularly those belonging to *Bacillus* spp. have promising commercial potential[24]. Keratinases have been produced in *Pseudomonas aeruginosa*[16,23], *Acinetobacter* spp.[28] and *Serratia marcescens*[3]. Due to the extensive application of keratinase, the present study focused on optimizing keratinase production from bacterial isolates obtained from poultry farms and feather dumps in Vazhayur, Malappuram, Kerala.

Material and Methods

Isolation of bacterial isolates: The soil surrounding the dumped feather waste from a poultry outlet in Vazhayur, Kerala, was used as the sample. An initial enrichment of keratinase producing microbes was done by inoculating this soil in feather meal broth (10 g/l feather powder, 0.1 g/l yeast extract, 0.5 g/l NH_4Cl, 0.5 g/l NaCl, 0.3 g/l K_2HPO_4, 0.4 g/l $MgCl_2$, pH 7.5) and incubated for 24 hours. From this, 1 ml sample was used to prepare serial dilutions upto 10^{-5} and 100 µl of each dilution was spread plated on nutrient agar plates and incubated at 37°C for 48 hours. Distinct colonies from these plates were identified and were used for further screening.

Screening for proteolytic activity: Isolated colonies were streaked onto skimmed milk agar plates (0.5% peptone, 0.3% yeast extract, 5% skimmed milk powder and 1.5% agar) and incubated at 37ºC for 24 hours. Enzyme activity was calculated in terms of enzymatic index which is the ratio of zone of hydrolysis to colony diameter.

Screening for chicken feather degradation: Minimal basal media supplemented with 1% chicken feathers was inoculated with isolates that were positive for significant proteolytic activity. They were then incubated for 5 days at 37°C with shaking. After incubation, the residual feathers were washed, dried and weighed. The percentage of feather degradation was estimated using the given formula:

$$Percentage\ of\ dye\ degradation(\%) = \frac{(Weightofinitialfeather - weightofresidualfeather)}{weightofinitialfeather} \times 100$$

Keratinase plate assay: Modified keratin agar plates (0.3 g K_2HPO_4, 0.4 g KH_2PO_4, 0.5 g NaCl, 0.1 g $MgCl_2$,10 g keratin powder, 20 g agar per L) were spot-inoculated with the three isolates along with the control and incubated at 37°C for 72 h. Prominent zone formation upon the addition of Gram's iodine indicated keratinase production. In the case of Coomassie Brilliant Blue R-250 staining followed by destaining, halo formation indicated keratinase activity.

Estimation of keratinase activity: Basal media with composition in g/l (keratin powder (10 g), NH_4Cl (1 g), NaCl (1 g), K_2HPO_4 (0.6 g), KH_2PO_4 (0.8 g), $MgCl_2 \cdot 6H_2O$ (0.48 g) and yeast extract (0.2 g) with pH 7.5 was inoculated with bacterial isolate, incubated 37 °C for 72 hours with shaking. The cell-free supernatant collected after centrifugation of culture broth at 5000 rpm for 20 minutes at 4°C was used as the crude enzyme. Enzyme (0.25 ml) is incubated with 1.75 ml of substrate (1% keratin dissolved in 50 mM tris buffer pH 8) at 40 °C for 10 minutes, followed by the addition of 0.4 M trichloroacetic acid (2 ml). The solution was centrifuged and the amount of amino acids liberated in the supernatant was measured at 280 nm and estimated against the standard tyrosine graph. One unit (U/ml) of enzyme activity was defined as an increase of absorbance of 0.01 at 280 nm ($_{Abs}280$)[12] per minute under the assay conditions calculated using the following equation:

$$Enzyme\ activity\left(\frac{U}{ml}\right) = \frac{(\mu mol\ of\ tyrosine\ \times final\ volume)}{(sample\ volume \times reaction\ time)}$$

Molecular identification of isolates: Genomic DNA from the three isolates was isolated using the Mag Genome Express Bacterial DNA isolation kit. The isolated DNA was used to amplify the 16S rRNA gene using the primers 27F-5'-AGAGTTTGATCCTGGCTCAG-3' and reverse 1492R-5' GGTTACCTTGTTACGACTT-3'. PCR amplification was performed using the EmeraldAmp® GT PCR Master Mix. The 50 µl reaction consisted of 25 µl of 2X PCR master mix, 2 µl each primer, 5 µl of DNA and 16 µl of MilliQ water. The PCR was performed using the Eppendorf Gradient Master cycler system with an initial denaturation at 95°C for 2 minutes, followed by 30 cycles at 95°C for 10 s, 56°C for 30 s, 72°C for 60 and a final extension of 7 minutes at 72 °C. The amplified products were verified by agarose gel electrophoresis. They were then subjected to Sanger sequencing using universal primers which was performed at the Agri Genome, Kochi, Kerala. The sequencing data were analyzed using the BLASTn program to identify bacterial species. A phylogenetic tree was constructed using MEGA 11.0.

Optimization of keratinase production: Keratinase production was optimized by analyzing parameters such as incubation time, pH, temperature and substrate composition of the media. For the incubation time, basal media supplemented with keratin powder was inoculated with the bacterial isolates and incubated for time periods ranging–24-96 hours. The ideal pH of the production media was evaluated by growing the isolates in acidic (3,5), neutral (7) and alkaline pH (9,11). The effect of temperature on keratinase production was determined by growing cultures at various temperatures (15, 25, 35, 45 and 55 °C). Basal media were supplemented with 1% substrates such as keratin, casein, feather meal powder and skim milk powder and their effect on keratinase activity was analyzed.

Results and Discussion

Screening for keratinase-producing isolates: In this study, ten different bacterial strains were isolated from soil samples collected from poultry farms. Primary screening of these strains was carried out to identify those possessing protease activity based on zone formation by hydrolyzing the casein present in the medium. Six bacterial isolates exhibited large zones of hydrolysis (Fig. 1a). The enzymatic index was determined which is a measure of the enzyme activity. It was observed that an enzymatic index of 3.56, 2.17 and 2.0 was determined for isolates 1,2 and 10 respectively (Fig. 1 b). Previous studies employed similar screening techniques for proteolytic bacterial isolates[7,25]. Secondary screening of the selected isolates was carried out under submerged conditions, using raw chicken feathers as the sole source of nitrogen and carbon.

Secondary screening revealed that bacterial isolate 1 showed the highest keratinase production with 69% degradation, followed by isolates 2 and 10 with 63.90% and 57.00% respectively (Fig. 1c). Another round of screening on these isolates was performed by streaking them on modified keratin agar medium. The inoculated cultures displayed a clearly defined, discernible and prominent zone surrounding the colony growth. A clear zone around the growth of the bacteria was indicated as Keratinase activity (Fig. 1d).

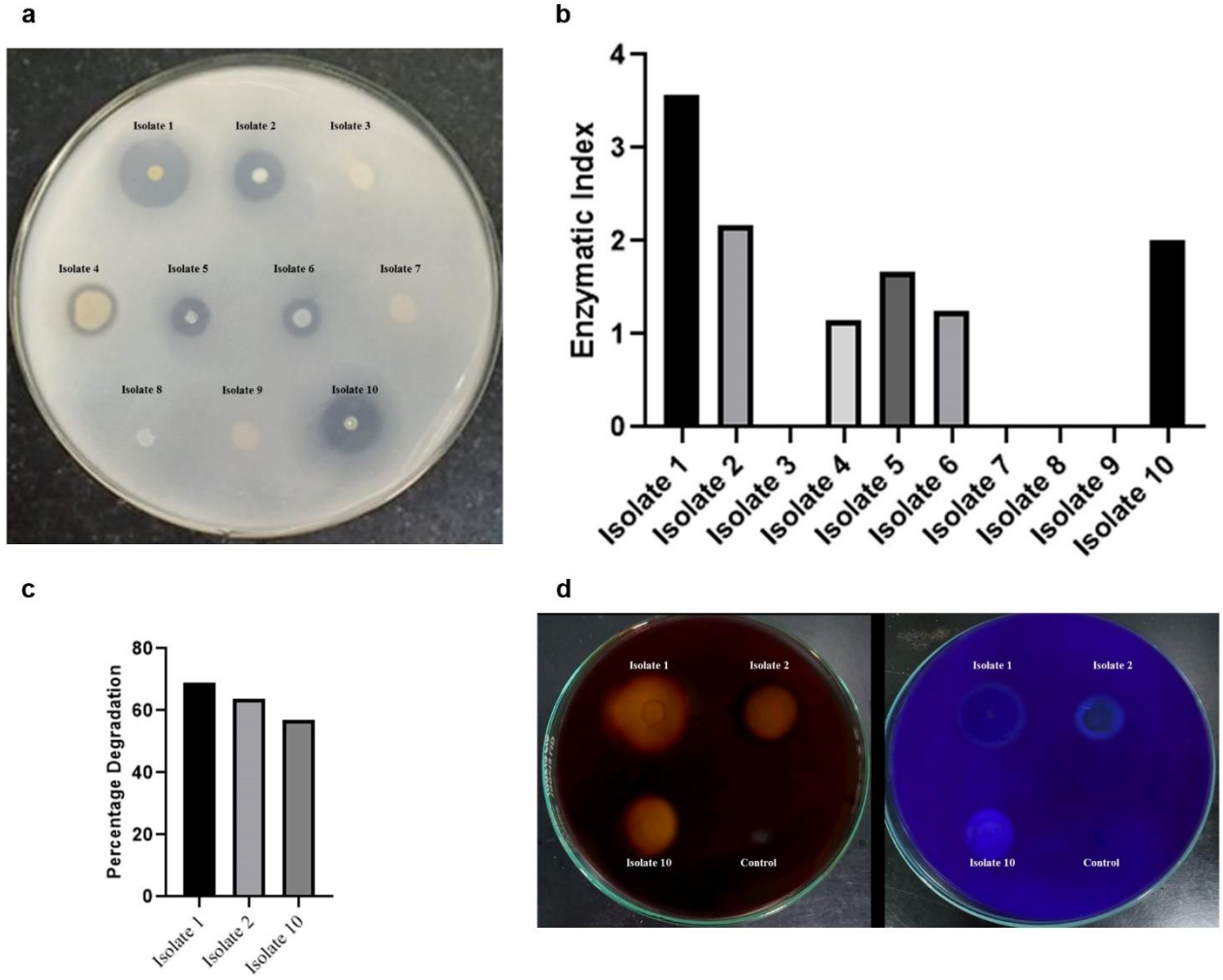

Figure 1: Screening for keratinase producing isolates
a) Isolates screened for protease activity on casein agar; b) Graph showing the enzymatic index of isolates for protease activity; c) Graph depicting the percentage of feather degradation; d) Keratinase activity of isolates on modified keratin agar plates stained with Gram's iodine or Coomassie Brilliant Blue R-250.

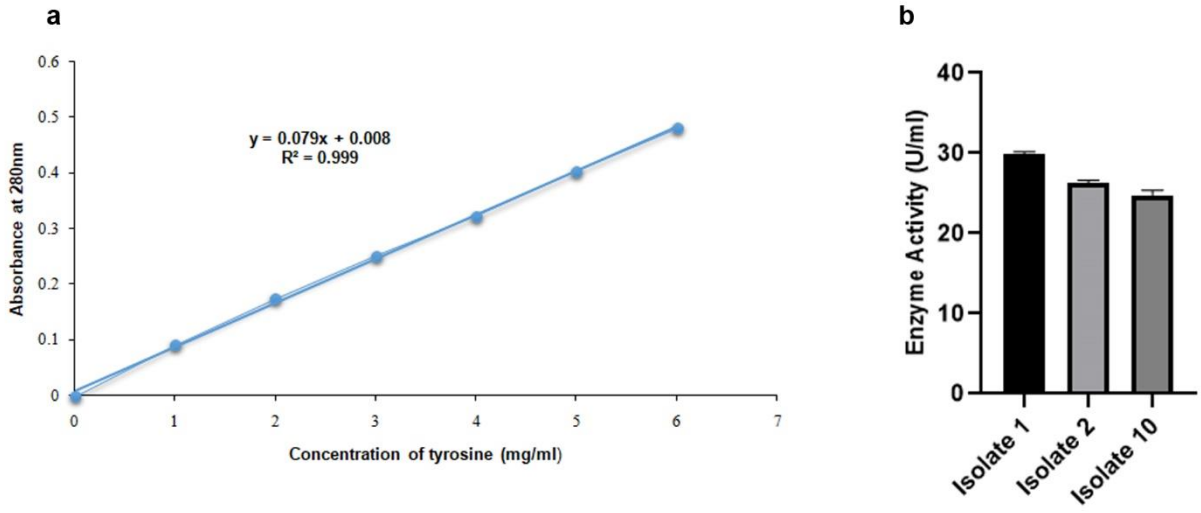

Figure 2: Estimation of keratinase production
a) Standard tyrosine graph; b) Graph depicting keratinase production by different isolates.

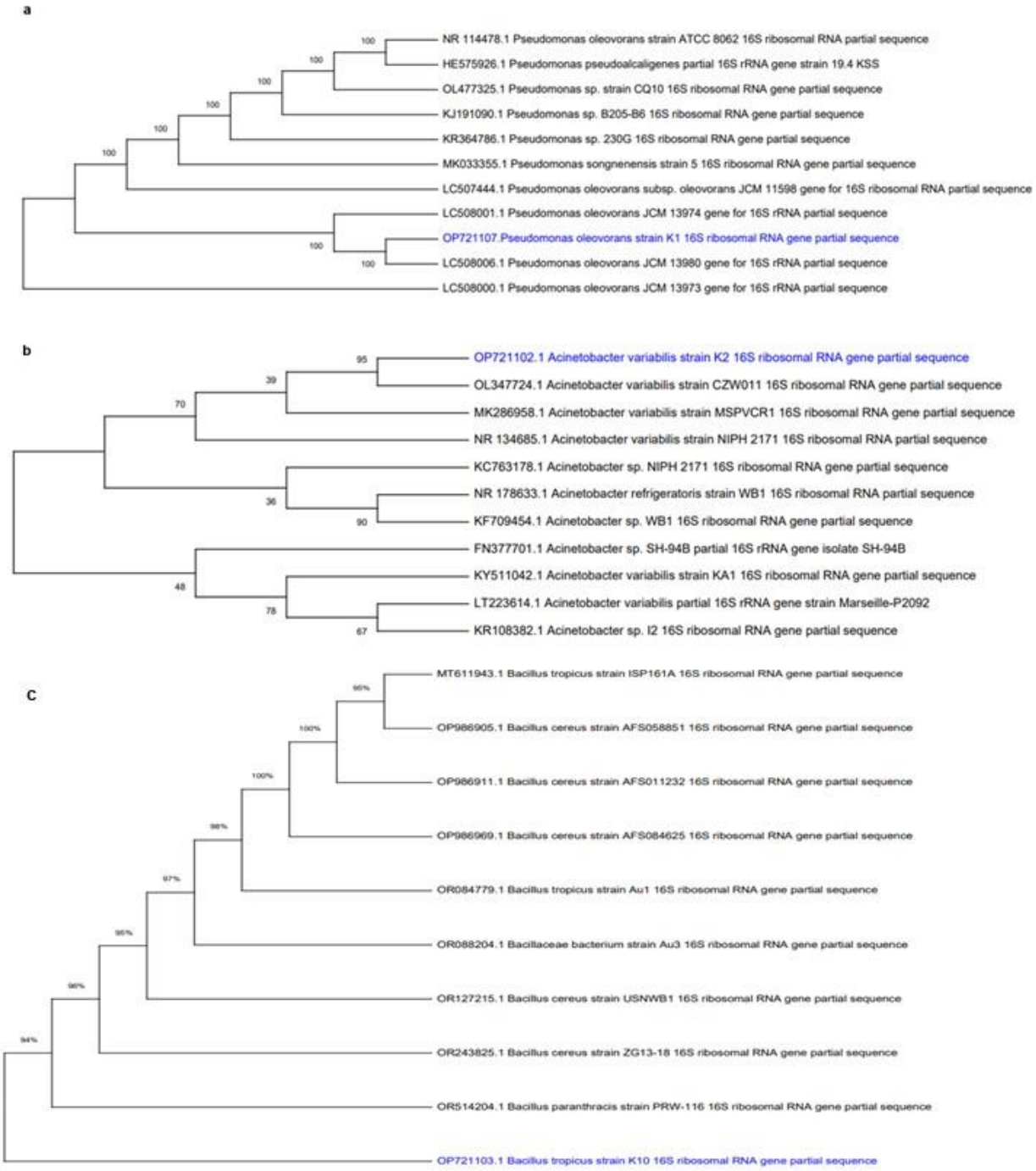

Figure 3: Phylogenetic analysis of the Isolate 1(a), Isolate 2(b) and Isolate 10 (c)

Estimation of keratinase production: Keratinase production by the selected isolates was evaluated under submerged conditions, utilizing keratin as the exclusive nitrogen and carbon source. Following the keratinase assay, the amount of tyrosine released by keratinase was determined from a standard tyrosine graph (Fig. 2a). The maximum keratinase activity (Fig. 2b) was observed in the case of isolate 1 (29.80±0.36) followed by isolate 2 (26.23±0.39) and isolate 10 (24.78±0.61).

Molecular identification of isolates: Identification of isolates 1,2 and 10 was identified by 16S rRNA sequencing of genomic DNA. Analysis of the sequencing data revealed isolate 1 as *Pseudomonas oleovorans* K1, isolate 2 as *Acinetobacter variabilis* K2 and isolate 10 as *Bacillus tropicus* K10 (Figure 3). The 16SrRNA gene sequence of these isolates has been submitted to the NCBI Gen bank database with the following accession numbers: *Pseudomonas oleovorans* K1(OP721107*), Acinobacter variabilis* K2 (OP721102) and *Bacillus tropicus* K10(OP721103). As higher keratinase activity was observed for *P. oleovorans* K1 and *A. variabilis* K2, production optimization was performed on these two isolates.

Multiple research groups have reported the production and purification of keratinase enzymes from *Pseudomonas aeruginosa*[9,16, 21,23] and other *Pseudomonas* species[20], although keratinase from *P. oleovorans* has not been reported. Similarly, although keratinase production by different *Acinetobacter* spp[2,28], has been reported, there are no reports on *Acinetobacter variabilis*.

On the other hand, *Bacillus tropicus* has been reported to produce keratinase[13,24] whereas other species of *Bacillus*, namely *B. licheniformis*, *B. subtilis*. *B. pumilis*, *B. cereus* and *B. halotolerans*[1,4,6,8,29] are well-known producers of keratinase.

Optimization of production conditions: The production conditions for enzymes from *P. oleovorans* K1 (Isolate 1) and *A. variabilis* K2 (Isolate 2) were optimized with respect to incubation time, pH, temperature and substrate. Evaluation of enzyme production across a time range of 24-120 hours showed maximum enzyme production at 72 hours with an activity of 39.851±0.171 and 34.492±0.171 for isolates 1 and 2 respectively (Table 1). Enzyme production in acidic (pH 3,5), neutral (pH 7) and basic (pH 9,11) was analyzed, which revealed that the highest production was achieved at neutral pH 7 for isolates 1 and 2 with an enzyme activity of 43.646±0.171 and 40.186±0.341 respectively (Table 2). The ideal temperature for keratinase production was determined to be 35 °C for isolate 1 (53.284±0.487) and isolate 2 (47.368±0.295) (Table 3).

Among the different substrates (keratin, casein, skim milk and feather meal powder) used at 1%, the maximum keratinase production was obtained with 1% keratin (Table 4) for both *P. oleovorans* K1 (56.485±0.232) and *A. variabilis* K2 (49.191±0.392). Approximately two-fold increase in enzyme production in isolate 1 (57.341±0.341) and isolate 2 (47.182±0.281) was observed after optimization when compared to initial enzyme activity. Keratinase production depends on the presence and concentration of keratin. Enzyme production may be reduced in the presence of a higher concentration of feather meal, indicating catabolic suppression[22]. Keratinase production is significantly influenced by nutritional and environmental factors[27]. Keratinase production was highest for both isolates at pH 7 and 35°C after 72 hours.

Using the Box-Behnken design, the *P. aeruginosa* Su 1 strain was optimized to produce keratinase at 30°C, pH 7, for an incubation time of 96 hours[9]. Similarly, the *Pseudomonas* sp. MS21 showed the highest keratinase production at pH 8, at 37°C after 24 hours[26], whereas *Pseudomonas* sp. LM19 showed the highest keratinase production at 30°C and pH 8 after 48 hours of incubation[15]. *Bacillus* spp. CSK2 was shown to produce keratinase at a pH 5 and 0.75% feather concentration after 48 hours of incubation at 30°C[30].

Table 1
Effect of incubation time on production of keratinase enzyme

	Enzyme activity (U/ml)	
Incubation time (hours)	**Isolate 1**	**Isolate 2**
24	0.443 ±0.064	0.443 ±0.064
48	29.989±0.129	26.045±0.112
72	39.851±0.171	34.492±0.171
96	30.250±0.064	27.310±0.171
120	21.170±0.232	20.612±0.171

Table 2
Effect of pH on production of keratinase enzyme

	Enzyme activity (U/ml)	
pH	**Isolate 1**	**Isolate 2**
3	13.802±0.064	10.267±0.465
5	27.720±0.129	23.254±0.697
7	43.646±0.171	40.186±0.341
9	30.734±0.064	26.566±0.171
12	9.262±0.232	7.178±0.112

Table 3
Effect of temperature on production of keratinase enzyme

	Enzyme activity (U/ml)	
Temperature (˚C)	**Isolate 1**	**Isolate 2**
15	0.480±0.112	0.554±0.064
25	51.015±0.341	44.726±0.281
35	53.284±0.487	47.368±0.295
45	29.320±0.423	32.148±0.451
55	7.699±0.359	3.122±0.171

Table 4
Effect of substrate on production of keratinase enzyme

	Enzyme activity (U/ml)	
Substrates	**Isolate 1**	**Isolate 2**
Keratin	56.485±0.232	49.191±0.392
Casein	30.697±0.503	26.566±0.281
Feather meal powder	53.284±0.487	47.517±0.171
Skimmed milk powder	31.329±0.503	27.980±0.232

Maximum keratinase production by *Chryseobacterium aquifrigidense* FANN1 was determined at a feather concentration of 1.5%, pH of 8 and temperature of 30°C after a period of 72 hours, as per the findings of Bokveld et al[5].

Conclusion

Screening for keratinase-producing microorganisms using skim milk agar, feather degradation and keratin agar assays led to the identification of three isolates. These isolates were identified as *Pseudomonas oleovorans* K1, *Acinetobacter variabilis* K2 and *Bacillus tropicus* K10 by 16S rRNA sequencing. Because *P. oleovorans* K1 and *A. variabilis* K2 produced higher keratinase, they were further used for media optimization studies. Optimization revealed that both bacterial isolates produced keratinase in the presence of keratin at pH 7.0 when incubated at 35 °C for 48 hours. These enzymes could potentially be used to degrade feathers and other keratin-containing wastes.

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