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Advances in Bioresearch

Analysis of poultry wastes for keratinase producers, Isolation, Characterization, and Partial purification of keratinase

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ABSTRACT
Poultry industries produce vast quantities of feathers, which are usually buried in landfills or generated into electricity. A solid waste problem exists in the environment when chicken feathers are wasted in the poultry industry. Feather degradation can be caused by bacteria and fungi that degrade keratinous proteins. Keratinases degrade insoluble keratin among feathers, hair, horns, and hooves. The aims of this study include the isolation, screening, and identification of bacteria that produce keratinase and subsequently degrade feathers from the dumping site for feather waste. A total of sixty-three bacteria were isolated from feather waste dumping sites near Vadodara, Anand, and Kheda Districts, Gujarat, India, following the collection of soil samples from nine various poultry farms. To determine whether they were Proteolytic they were screened. Based on the Relative Enzyme Activity (REA) screened out eighteen isolates were selected to check keratinolytic activity. A feather containing a basic salt medium was used to isolate and identify the keratinolytic bacteria. Four isolates showed a greater ability to break down keratin than the other bacteria. The 16S rRNA gene sequence and morphological and biochemical characteristics led to their identification as Bacillus subtilis, Bacillus velezensis, Bacillus cabrialesii, and Bacillus paramycodies, respectively. Different substrates, temperature, pH, feather concentration, and feather concentration were studied to determine the effect on bacteria growth parameters. Several studies have been similar to the one presented in the present study. This study discovered that Bacillus strains produce a high amount of keratinase. They provide efficient biodegradation of chicken feather waste, making them promising organisms for managing chicken feather waste.
Keywords: Isolation, Proteolytic, REA, Feather degradation, Keratinolytic, Bacillus, Partial Purification.

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INTRODUCTION

A worldwide population explosion has made poultry an indispensable source of animal protein for the world's population [1]. As a consequence of eating large quantities of chicken, a great deal of waste is generated in slaughterhouses and poultry farms [2]. In poultry production, feathers are considered a by-product; untreated feather waste can harbor microorganisms and pollutants that can be harmful to health [3]. Worldwide, poultry feather waste is produced at a rate of 8.5 billion tons per year. By 2022, A total of USD 28.18 billion was spent on poultry in the Indian market. According to forecasts, as a result of a CAGR of 8.1%, the industry is expected to reach USD 44.97 billion by 2028. According to the last data available, The number of tons of broiler chicken produced in India was 4.6 million in 2018 [4] The annual amount of feather waste generated is approximately 350 million tons [5], A huge number of feathers are discarded in indiscriminately discarded in the environmental human ailments are caused by feather accumulation in the environment. This includes the following: the outbreaks of the H5N1 virus, fowl cholera, mycoplasmosis, chlorosis, and fowl cholera, [7, 8]. A variety of proteases, including papain, pepsin, or trypsin, will not degrade this feather. Due to keratin's disulfide bonds and resistance to degradation, chicken feathers are 90% keratin [9-11]. Keratin is insoluble in water due to extensive disulfide bonds and crosslinks. As a result

of the sulfur content of their keratins, There are several types of keratin, such as hard keratin, soft keratin, and feathery keratin [12]. The keratin content of sulfur is high as a result of sulfur-containing amino acids such as cystine, cysteine, methionine, and cysteine that are found in keratin [13]. When feathers are systematically recycled, they can provide essential amino acids and proteins [14]. Waste generations poultry farms and slaughterhouses in India have been managed in a variety of ways. Nevertheless, it remains a challenge to deal with feather waste in the future [15, 16]. Steam pressure cooking and acid/alkali hydrolysis are conventional methods of feather degradation that destroy an amino acid that is sensitive to heat, such as methionine and tryptophan. They consume a lot of energy while producing non-nutritive amino acids [17-19]. Using landfills and incinerators is likely to destroy the environment and release toxic gases [20]. Traditionally, feather waste is processed in a labour-intensive and collaborative nutrient. An amino acid polypeptide chain is hydrolysed by proteases hydrolysed due to the enzyme-catalysed reaction's efficiency and ease of selection, this reaction requires a mild reaction environment, less energy, and produces a high feather value. [20]. Some microorganisms and insects produce keratinases, which are secreted proteases that hydrolyse insoluble keratin more efficiently than other proteases [21]. This alternative method of feather processing is environmentally friendly because it utilizes microorganisms and their enzymes. Microbial keratinases are produced by a multitude of actinomycetes, bacteria, fungi, and algae [3, 23], offering an alternative possible way to bio convert keratin from poultry feathers. The disulfide bonds in keratin are broken by keratinase, an extracellular enzyme produced in response to the degradation of keratin.

The presence of keratinases in feather keratin can be attributed to the fact that they are often more efficient than other proteases when degrading bird feather keratin, despite the rigidity of their structure [11, 24]. As a result of soil keratinophilic and keratinolytic microorganisms that produce enzymes highly consistent in Conditions of high temperatures and alkaline solutions, a wide range of phylogenetically diverse microorganisms have been found there. [25]. In addition to bacteria (*Microbacterium sp.*, *Pseudomonas sp.*, *Bacillus pumilus*, *Micrococcus sp.*,), actinomycetes (*Thermoactinomyces candidus*, *Streptomyces sp.*), keratinase is produced by a variety of fungi, including *Alteraria radicina*, *Aspergillus*, *Doratomyces*, and *Rhizomucor sp*. [19]. Among the different types of Bacillus species that produce keratinase are *Bacillus licheniformis*, *Bacillus megaterium, Bacillus subtilis*, *Bacillus cereus,* and *Bacillus pumilus*, which can be distinguished based on the fact that different types of Bacillus species produce keratinase. [24]. As a member of the subtilisin group, serine proteases (S8 family) are subunits of the keratinase protein [26, 27]. In recent years, heterologous expression systems have been used to produce keratinases *Escherichia coli*, *Bacillus sp*. and *Pichia pastoris* a significant amount of research has been conducted on insect cells [28]. In general, microorganisms produce keratinases that are extracellular, inducible enzymes, and some of them are also intracellular. Exoproteases and Endoproteases are keratinases that are classified by their cleavage sites and have uses in leather, detergents, cosmetics, and pharmaceuticals. [29]. Thus, microbial keratinases could conquer the position of being the first proteases able to bioprocess keratinous waste without harming the environment. As a result of bacteria that convert keratin into amino acids and soluble proteins, chicken feather keratin plays an important role in the conversion of protein. It is, therefore, necessary to develop an innovative process that can valorise this resistant chicken feather waste ecologically and economically. To improve the degradation of chicken feathers, the present study is aimed at isolating and characterizing keratinolytic bacteria and optimizing their culture conditions.

MATERIAL AND METHODS

Media and Chemicals:

This study was carried out with materials and media obtained from Hi-media, Mumbai, India, SRL Chemicals, East Mumbai, India, and SD Fine Chemicals, Baroda, India, for this research.

Sampling:

In Vadodara, Anand, and Kheda provinces, soil samples were collected from nine different poultry waste dump sites near poultry farms. Sterile zippered bags were used to transport samples to the laboratory from a depth of 5 to 20 cm [21, 30].

Table: 1 Sample collecting sites and their GPS location

Sr No.	Districts	Samples	Sampling sites GPS Locations
1	Anand	N Poultry Farm, Singlav, road, Anand, Gujarat 388560	22° 27' 52.98113"N 72° 55' 9.58158" E
2		Ravi Poultry Farm, GX66+WQW, vidya dairy road, Anand, Gujarat 388355	22° 31' 3.11632"N 72° 57' 50.25663"E
3		Dharam Poultry Farm, Vidhya Dairy Road Survey No.	22° 30' 24.86673"N

		659/1, Navli, Gujarat 388355	72° 57' 31.7172"E
4	Vadodara	Foster Poultry Farm, Effluent Canal Project Road Lakhadikui, Jaspur, Vadodara, Gujarat 391440	22° 13' 21.24617"N 72° 56' 8.67605"E
5		Nasr Poultry Farm, 9R3F+C5M, Nani Rasli, Gujarat 391160	22° 22' 47.22582"N 73° 49' 52.15821"E
6		Noor Poultry Farm, 7GPG+F3X, Waghodia, Gujarat 391761	22° 20' 53.94129" N 73° 31' 45.29314 "E
7	Kheda	Royal Poultry Farm, QWXG+2RP, Kheda, Gujarat 387335	22° 48' 14.77903"N 72° 55' 43.07773" E
8		Navjivan Poultry Farm, MQXC+7QH, Vadiya Vistar, Palana Road, Dantali, Gujarat 387230	22° 42' 15.75158"N 72° 46' 20.71503" E
9		C.D. Patel Poultry Farm, JWQ7+77W, Nadiad-Anand Rd, Bhumel, Gujarat 387325	22° 38' 39.60047"N 72° 54' 45.94381"E

After they had been stored in the refrigerator for a while, they were removed from it. A skimmed milk agar plate was inoculated with soil samples (10^{-4}, $10^{-5}10^{-6}$, and 10^{-7}) following standard dilution procedures [31]. Table 1 predicted various soil sample sources and locations for primitive screening.

Screening, Isolation, and Measure of alkaline Protease producing bacteria on solid media:

It was found that the isolates were capable of producing alkaline proteases when they were plated on skimmed milk agar (pH 9.0) and incubated at 37 ± 2ºC for 24 hours to detect the production of alkaline proteases. The bacteria that produce caseinase, which is a protease, can be identified by finding clear zones of casein hydrolysis in the solution [32]. Fresh cultures were incubated on a skim milk agar plate for 5-6 days at optimum temperature. By measuring the zone of casein hydrolysis and the diameter of growth every 24 hours, the relative enzyme activity (REA) was calculated. [33]

$$REA = \frac{Diameter\ of\ Zone\ of\ casein\ hydrolysis\ in\ mm}{Diameter\ of\ colony\ in\ mm}$$

A protease production rating based on REA indicates that organisms are either excellent producers (REA >5), good producers (REA 2.0 to 5.0), or poor producers (REA 2.0).

Analysis of the efficiency of feather degradation by potent Isolate & Feather meal powder preparation:

A collection of chicken feathers (whole feathers) was collected from a poultry farm. In addition to extensive washing with tap water, feathers were also thoroughly cleaned with double distilled water. After steam sterilization, feathers were stored at 5°C until needed. Potent isolates were inoculated with fresh culture suspensions in basic salt (media composition consists of (g/l): 0.5 NH_4Cl; 0.5 $NaNO_3$; 0.3 K_2HPO_4; 0.4 KH_2PO_4; 0.1 $MgCl.6H_2O$) solution along with feathers [1]. Two feathers weighing the same amount total the volume of the tube. As a control, the same contents were maintained without inoculation. Kate et al. described the method of preparing the feather meal from native chicken feathers with modifications [34]. Several times, the feathers were washed with tap water after being cut into small pieces. To defeat feather pieces, they were soaked for 2 days in a mixture of chloroform: methanol equal amount, and then for 2 days in a mixture of quadrat amount of chloroform: acetone: methanol. There was a daily replacement of the solvent. Finally, several washes were performed on the feathers, dried at 40 ± 2 °C for 2-3 days, grinded using an electrical mixer blender, and obtained powder used as a feather meal. Additionally, all bacteria that exerted higher levels of activity were identified and taken for further examination. The physicochemical and morphological characteristics of the isolated microbes confirmed their ability to produce keratinase.

Identification of keratinase-producing bacteria:

The phenotypic characteristics of bacterial isolates were studied using microscopic observations and gram staining. To identify bacteria at this stage, tests such as Gram staining, Sporulation staining, catalase, oxidase, carbohydrates fermentation, motility test, starch, lecithin, gelatine and urea hydrolysis, and indole production, and other biochemical tests were achieved [35, 36].

Keratinase-producing bacteria identified by molecular methods:

DNA isolation from bacterial genomics:

Sankari and Khusro method was used to isolate bacterial genomic DNA [37]. The supernatant was discarded after centrifuging 2 ml broth culture for 5 min at 5000g. RNAse and UniFlexTM buffer 1 was added to the pellet and mixed well before being incubated for 30 minutes at 37°C. After that, 1 ml of a phenol-chloroform mixture (1:1) was added. A fresh vial (1.5 ml) was used to separate the aqueous layer after centrifugation for 15 minutes at 10,000 rpm. Add 1 ml UniFlex TM buffer 2 to the aqueous layer and mix well. In addition to centrifugation at 12,000 rpm for 15 min at room temperature, 500 microliters of

ethanol (70%) were added to the pellet and centrifuged at 10,000 rpm for 10 min at 4°C. Finally, the pellets were air-dried until ethanol evaporation was completed. DNA was recovered at -20°C after resuspending the pellet in Unflex TM elution buffer [19].

Amplification of 16S rRNA genes:
Universal primers were used for amplification of the 16S rRNA gene fragment (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-GGTTACCTTGTTACGACT-3') [38]. Genomic DNA (10ng total) and 10pmol of forward and reverse primers were included. Ethidium bromide staining was applied after PCR amplification a Gel documentation system was used to photograph stained gel. Hi- media Scientific (Mumbai) purified and sequenced the amplified fragments. NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare sequences to GenBank. Molecular Evolution MEGA version 11.0 was used to construct phylogenetic trees based on neighbour-joining.[19].

One-Variant Analysis for keratinase production:
The effects of various factors on keratinase production were examined during a variant analysis. This has been influenced by several factors, such as the incubation period, pH, carbon source, and nitrogen source.

Effect of Incubation Period:
Inoculating the production medium consisted of basal salt solution (pH 9) with 0.5% feather meal plus 1 ml of bacterial culture (OD 0.7 - 1 at 600nm). An incubation period of 120 hours is required for this medium. As a measure of the incubation period effect, enzyme activity was determined every 24 hours using a modified approach developed by Cai et al (2008) [39].

Effect of pH:
Inoculation was performed with 1ml of bacterial culture (OD 0.7 - 1 at 600nm) on a production medium, which included a baseline salt solution with pH 7/8/9/10 and 0.5% feather meal. At the optimum period, the medium is incubated and enzyme activity is measured using the modified Cai. et.al (2008) method at the optimal time [33] [40].

Effect of Carbon Sources:
An inoculum of 1ml of bacterial culture (OD0.7–1at 600nm) was inoculated in a production medium. The medium was composed of sucrose, glucose, mannose, lactose, and corn flour as various carbon sources (1%w/v) and 0.5 percent feather meal. At the optimum period, the medium is incubated and enzyme activity is measured using the modified Cai. et.al (2008) method at the optimal time [40] [41].

Effect of Nitrogen Sources:
A bacterial culture (OD 0.7–1 at 600nm) was inoculated into a production medium consisting of a basal salt solution (pH 9) containing yeast extract meal, ammonium chloride, sodium nitrate as various nitrogen sources (1% w/v) and 0.5 percent feather meal. For 72 hours, this medium is incubated. Enzyme activity was measured every 24 hours using modified Cai. *et .al* methods [40] [41].

RESULT AND DISCUSSION

Sampling, Screening, and Isolation of protease producing bacteria:
Poultry feathers are one of the solid wastes that piled up in huge quantities in developing countries like India. Because of the lesser knowledge on the remediation and conversion of this keratin waste into value-added products, the nutritional value of these huge feathers is being ignored or unnoticed. In recent years, keratinase has brought much attention due to its unique potential to degrade keratin waste. A total number of sixty-three bacterial isolates were isolated from poultry waste soil samples (Table 1) and screened for the alkaline protease-producing strain on skimmed milk agar plates. Figure 1 prediction corresponds to the observations that the majority of the gram-positive bacilli observed are the most potent isolates, while the minority of gram-negative short rods observed are relatively few.

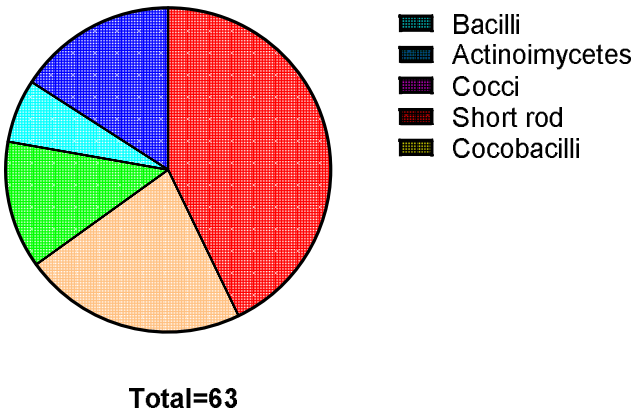


Figure 1: Microorganisms diversity as a keratinase producer.

Similarly, the report found that Bacillus stain, the predominant bacteria in the study, produced keratinases as well. In general, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus lichenifomis*, and *Bacillus cereus* are the species that are included in this group [42] [43] [44]. Some other bacteria can degrade feathers with high efficiency [45] [46, 47]. It might be important for the fungal infection to produce keratinase, a keratin-degrading enzyme, from some tissues [48] [49] [50]. It was shown that fungi such as *Chrysosporium* [48, 49] and *Trichophyton* [50] were able to degrade keratins through their produced keratinases [51] [52]. Due to the function of keratins, keratinases in some pathogenic fungi might be essential for the invasion by breaking the barrier between the tissue and the environment. Streptomyces is the predominant actinobacterium that can keratinases [22]. Quite a few reports have shown that keratinolytic actinobacteria can be isolated from different environments. Some actinobacteria can produce thermally stable keratinases which have great potential to be widely used in industry [53].

Secondary screening of isolates:

Eighteen isolates showed significant protease activity by forming a zone of casein hydrolysis on the skimmed milk agar plates. The diameter of the clear zone around the colony served as an index for the selection of strains with high protease production ability. An isolate designated as four isolates exhibited the largest zone of clearance and (Relative Enzyme Activity) REA on the screening plate which was later selected to test for feather degradation. Based on REA, RS1, RS4, RS5, and RS6 were found potent protease producers as compared to other isolates. (Figures 2 & 3). Based on calculated REA and spot test it was found that four isolates were giving the highest zone of casein hydrolysis after 72 hours compared to other isolates. Based on REA calculation, the isolates can be categorized into three groups. REA equals or more than 5mm is magnificent, REA equals and between 2 to 5 mm is satisfactory and REA equals and less than 2 is the deficient producer of protease [33, 54]. Similar reports were made by various Streptomyces species like, *Streptomyces exfoliates* CFS1068 (REA=4.0), *Streptomyces somaliensis* GS 1242 (REA= 8.8), *Streptomyces sampsonii* GS 1242 (REA-9.6) by the similar method [33]. Some Bacillus species like *Bacillus halodurans* RSCVS-PF(REA-2.86) [55].

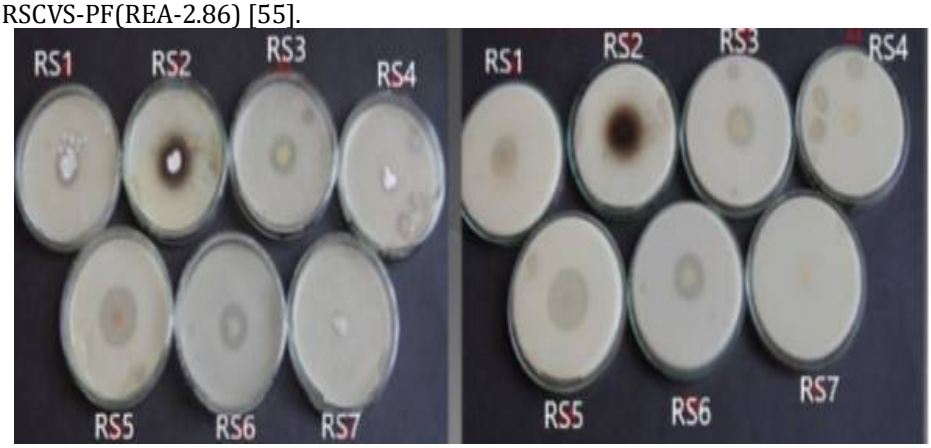


Figure 2: REA (Relative enzyme activity) by Isolates.

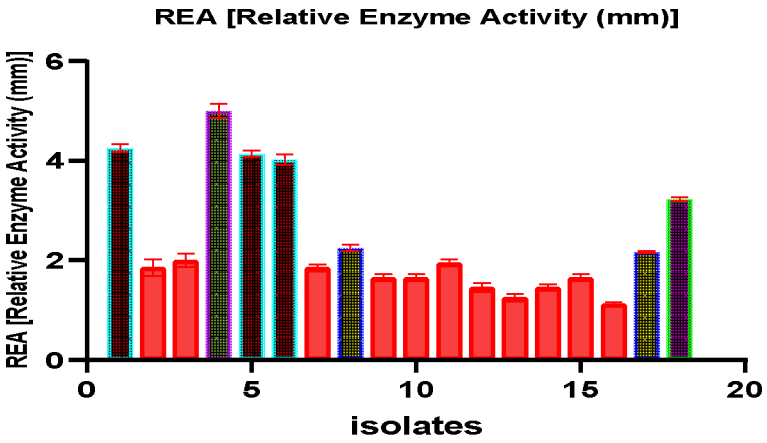


Figure 3: Relative Enzyme Activity by isolates

Analysis of the efficiency of feather degradation by potent isolates:

Fresh culture suspension of all four revived isolates of optical density between 0.7–1.0 at 600nm was inoculated in Basic Salt Solution containing 1gm feathers (as a sole source of carbon and nitrogen). The set was inoculated at 37±2^{0}C for 120 hours and observed for Feather degrading/Keratinolytic activity, result obtained were presented in Table 2 showing that isolate RS1 and RS6 degraded feather very efficiently within 72 hours and other isolates were very slow. Figure 4 shows the other degradation efficiency of isolates against control.

Bharti Agarwal *et.al*. have found similar observations for *Bacillus sp.* They choose two powerful isolates that degraded feathers in just four weeks [56]. Potent isolates RS1 and RS6 completely degraded the feathers within 72 hours. In terms of feather degradation, it can be concluded that the is ate has higher keratinase activity. After 96 hours of incubation, *Bacillus sp.* completely degraded the feathers reported by Sharad Sabugade *et.al* [12]. Feathers were entirely degraded within 120 hours of incubation with microorganisms from a chosen soil sample, according to Sarita Agrahari *et.al* [7].

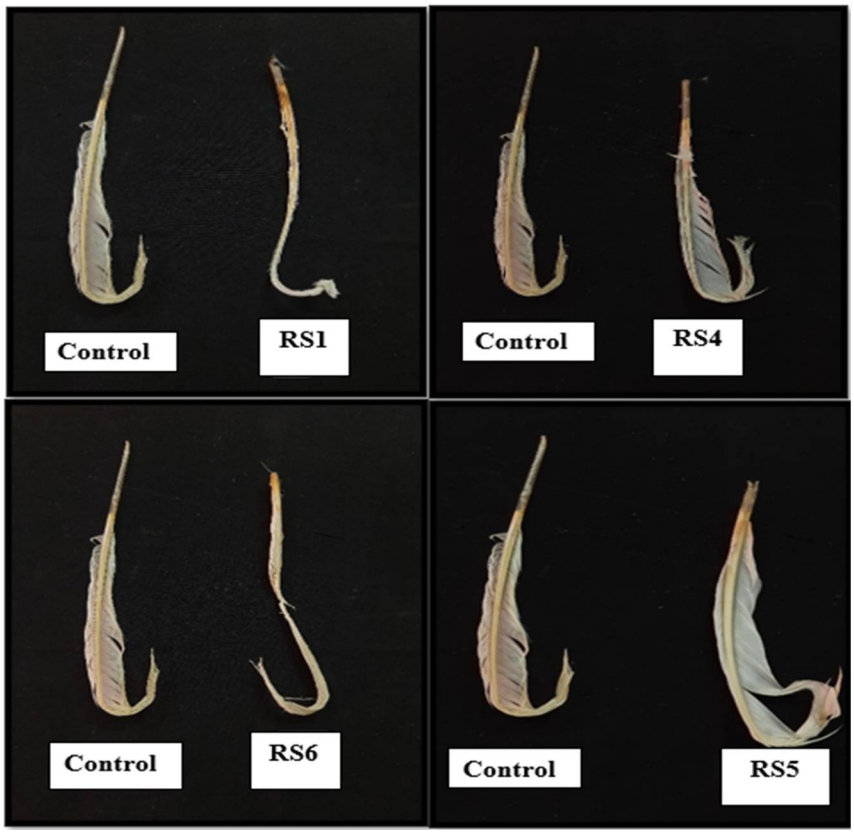


Figure 4: Feather degradation by potent isolates

Table 2: Feather degradation efficacy by isolate

Isolates	Feather Degradation Efficiency
RS1	++++ Within 72 hours
RS4	+++within 120 hours
RS5	++ Within 120 hours
RS6	++++ Within 72 hours

Identification of keratinolytic bacteria:

It has been determined that four potent isolates need to be investigated further for their keratinase activity, as well as their production and optimization. As shown in Tables 3 and 4, the characteristics of the isolates based on their morphological and colony characteristics are described.

Characters	RS1	RS4	RS5	RS6
Size	Medium	Big	Medium	Big
Shape	Circular	Circular	Irregular	Irregular
Margin	Uneven	Entire	Entire	Uneven
Elevation	Flat	Flat	Raised	Raised
Texture	Rough	Smooth	Smooth	Smooth with sticky
Opacity	Opaque	Opaque	Opaque	Opaque
Pigment	Fuzzy white	Chalky white	Creamy white	White
Appearance	Dull	Dull	Dull	Dull
Gram Nature	Gram Positive rod	Gram Positive rod	Gram Positive rod	Gram Positive rod

Table: 3 Morphology characteristics of Isolates.

Table 4: Biochemical characteristics of Isolates.

Sr. No	Bio-Chemical Test	RS1	RS4	RS5	RS6
1	Indole	Negative	Positive	Negative	Negative
2	MR	Negative	Negative	Negative	Positive
3	VP	Positive	Negative	Negative	Negative
4	Citrate	Positive	Positive	Negative	Negative
5	Oxidase	Negative	Positive	Negative	Negative
6	Catalase	Positive	Positive	Positive	Positive
7	Urease	Negative	Negative	Negative	Negative
8	Starch	Positive	Positive	Positive	Negative
9	Casein	Positive	Positive	Positive	Negative
10	Glucose	Positive	Positive	Positive	Positive
11	Xylose	Positive	Negative	Positive	Negative
12	Mannitol	Positive	Negative	Positive	Negative
13	Lactose	Positive	Only acid	Positive	Negative
14	Sucrose	Positive	Negative	Positive	Negative
15	Maltose	Positive	Negative	Positive	Negative
16	Motility	Positive	Negative	Positive	Negative
17	Spore	Positive	Positive	Positive	Positive

As a result of biochemical tests and analysis of 16S rRNA, it was observed that a wide range of Bacillus species was present. These species included *Bacillus subtilis*, *Bacillus velezensis*, *Bacillus cabrialesii*, and *Bacillus paramycodies* isolated from poultry waste soil samples.

Molecular identification of keratinolytic bacteria:

The Blastx program (BLAST), of the National Centre for Biotechnology Knowledge, was used to compare the sequences of DNA to those of unknown sequences Through this comparison, the program can identify homologous sequences of DNA. It is a powerful tool for biologists to identify the function of unknown sequences of DNA. It can be the RS1 bacteria was included in the genus *Bacillus* and is closely related to the

species *subtilis*. It showed the highest sequence similarities with *Bacillus subtilis strain SBMP4*. The *Bacillus subtilis strain RAS04* sequence was submitted to Gene Bank and had accession number **OQ519653.1**. RS4 bacteria were included in the genus *Bacillus* and closely related to the species *cabrialesii*. This similarity suggests that the RS6 bacteria are closely related to *Bacillus cabrialesii strain TE3* and are likely a subspecies of it. The Gene Bank accession number **OQ780760.1** confirms *Bacillus cabrialesii strain RAS13* is a member of this species, providing further evidence for this relationship. RS5 bacteria were included in the genus *Bacillus* and closely related to the species *velezensis*. It showed the highest sequence similarities with *Bacillus velezensis strain FZB42*. The *Bacillus velezensis strain RAS05* sequence was submitted to Gene Bank and had accession number **OQ625744.1**. RS6 bacteria were included in the genus *Bacillus* and closely related to the species *paramycoides*. It showed the highest sequence similarities with *Bacillus paramycoides strain MCCC 1A04098*. The *Bacillus paramycoides strain RAS18* sequence was submitted to Gene Bank and had accession number **OQ780761.1**. The phylogenetic trees were established and demonstrated in Figures 5, 6, 7, and 8 [19].

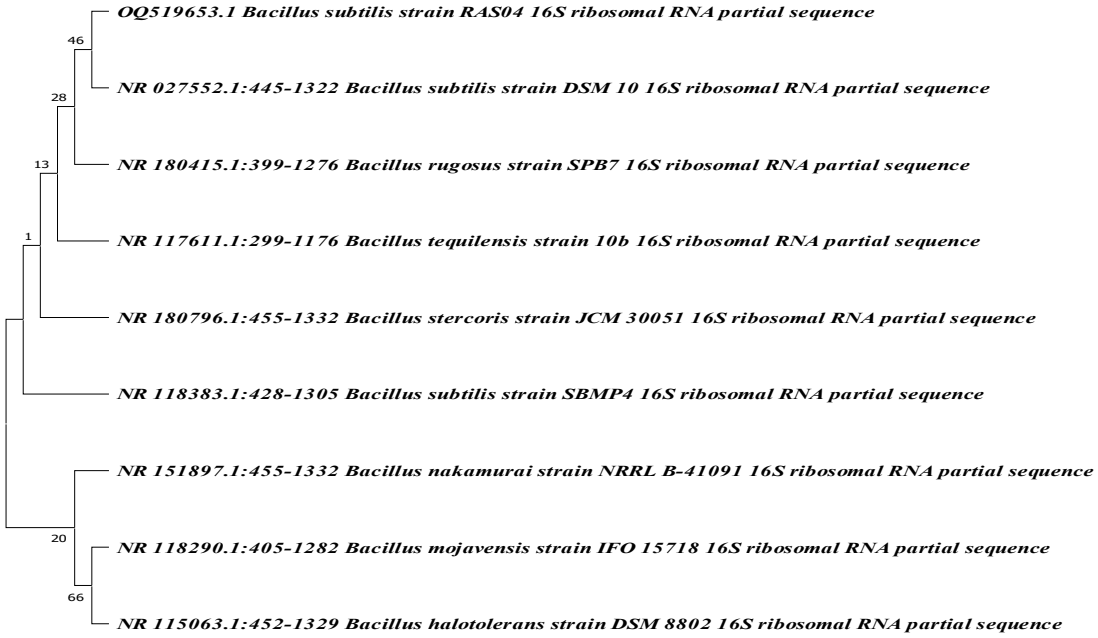
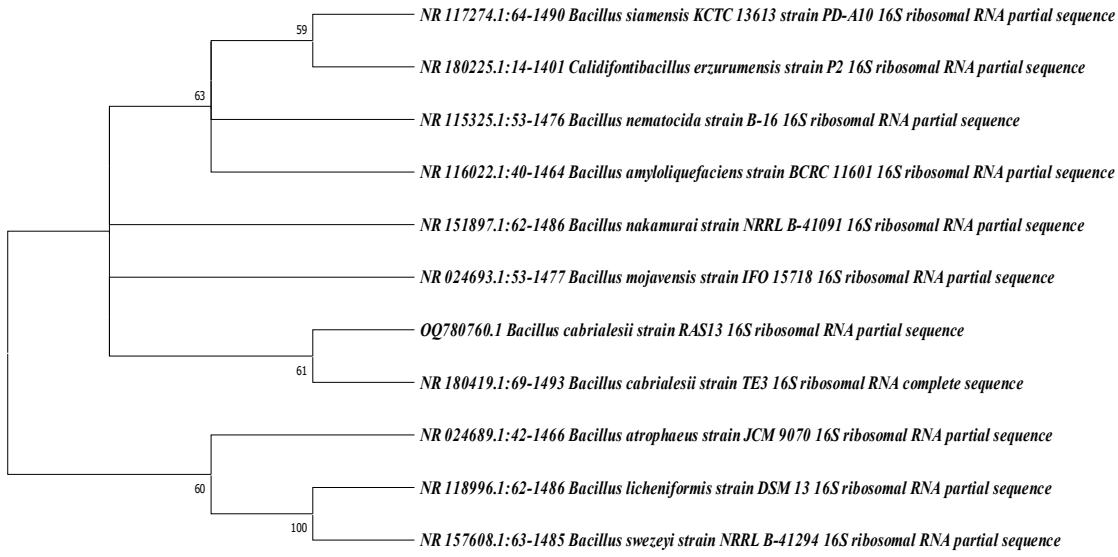


Figure 5: Phylogenetic tree of RS1 Isolate.

Figure 6: Phylogenetic tree of RS4 Isolate.



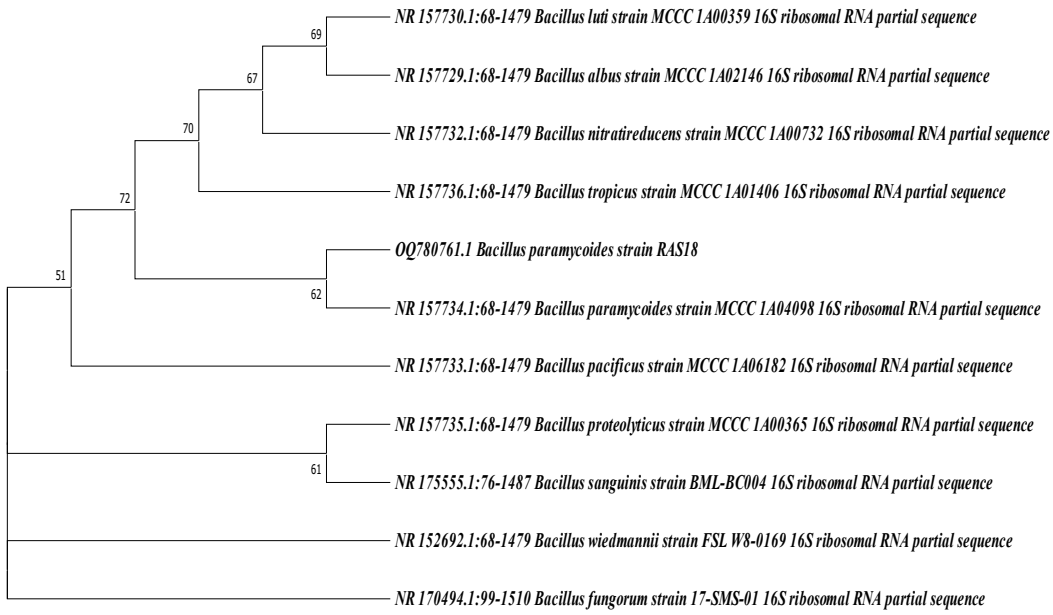


Figure 7: Phylogenetic tree of RS6 Isolate

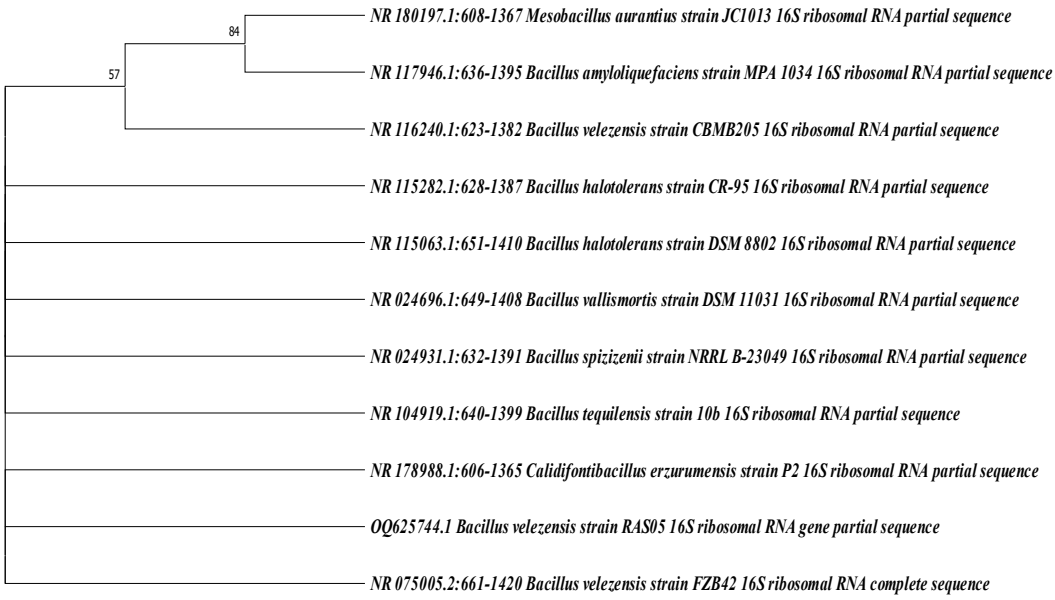


Figure 8: Phylogenetic tree of RS5

One-Variant Analysis for keratinase production:

Effect of Incubation Time:

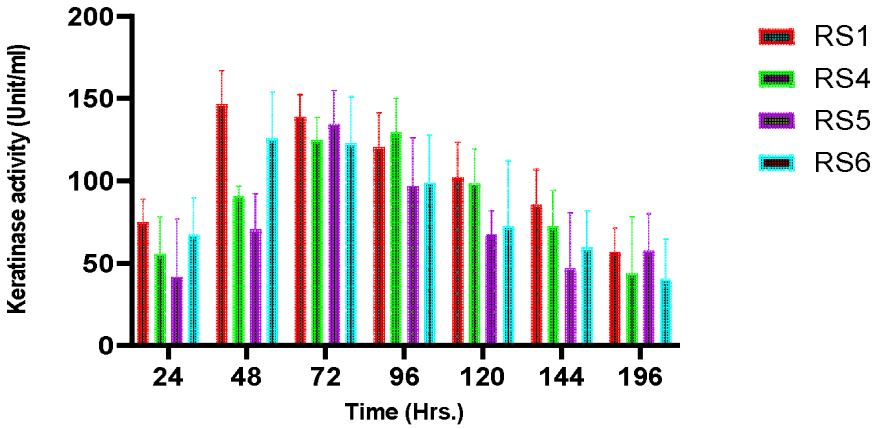


Figure 9: Effect of incubation time for keratinase production by Isolates.

The incubation period is one of the most significant parameters in keratinase production. The basal salt medium containing salt medium was used to determine the keratinase production of potent isolates after various incubation periods ranging from 24 to 196 hours. This experiment detected an enzyme activity peak in RS1 at 48 hours. As a result, RS4 gives 96 hours, RS5 gives 72 hours, and RS6 gives 48 hours. As shown in Figure 9, different enzyme activity levels were observed during the various incubation periods. This indicates that the keratinase production levels of the potent isolates vary depending on the incubation period. Similarly, *B. cereus LAU08* showed the highest enzyme production after 72 hours of incubation [57], while *B. cereus YQ15* obtained the highest enzyme output at 36 hours of incubation [58]. The result obtained by Kainoor, P. S., & Naik, G. R. (2010), represent that *Bacillus sp.* JB99 Showed maximum keratinase activity at 36 hours of incubation, after 36 hours it started decreasing [39]. Previously, similar work had been reported by [Jani.S.A.*et.al* 2014], where it was found that *Streptomyces sp.* (A1) and *Saccharothrix xinjiangenesis* (A2) produced a considerable amount of keratinolytic protease within 72 hours of incubation [33].

Effect of Incubation pH:

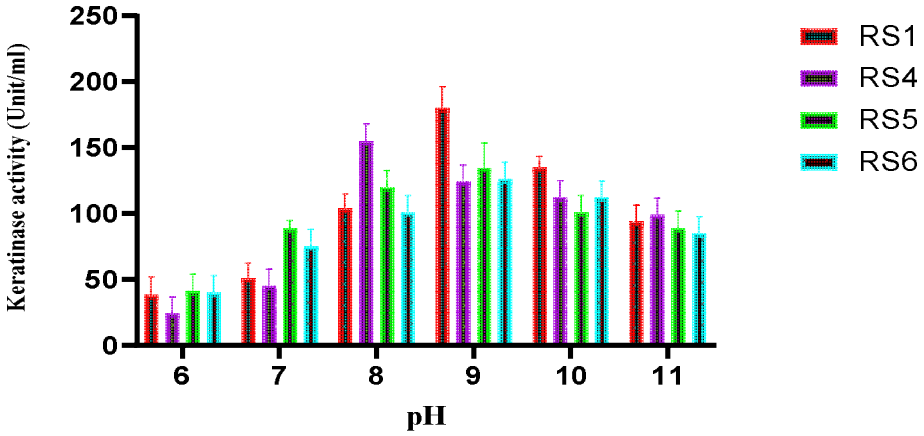


Figure 10: Effect of pH for keratinase production by Isolates.

pH of the production medium has also a great impact on enzyme production. pH levels that are too low or too high can reduce the activity of enzymes. It is important to optimize the pH level to maximize enzyme activity and production. Keratinase production by isolate was tested at various pH ranges of production medium such as pH 6, 7, 8, 9, 10, and 11. The results obtained represent that enzyme production was maximum at alkaline pH observed in Figure 10. It was observed that RS1 showed an enzyme activity peak at pH 9. For the other isolates, RS4 gave 8 pH, RS5 gave 9 pH, and RS6 gave 9 pH. Similarly, it was reported that the optimum pH for keratinase production by *B. tropicus Gxun-17* was neutral to weakly alkaline [59]. Moreover, *S. coelicoflavus* showed maximum keratinase production at pH 8.0 [60]. Generally, the most suitable pH for keratinase production from Actinomycetes, fungi, and bacteria was found to be neutral to the alkaline range [61]. Similar work had done, where the production of keratinolytic protease was highest at pH 8.5 for *Streptomyces sp.* (A1) and at pH 9.5 for *Saccharothrix xinjiangenesis* (A2) [33]. A similar result

was obtained when strain *Streptomyces gulbagensis* DAS 131 produced the highest amounts of keratinase at pH9 [62].

Effect of Various Carbon Sources:

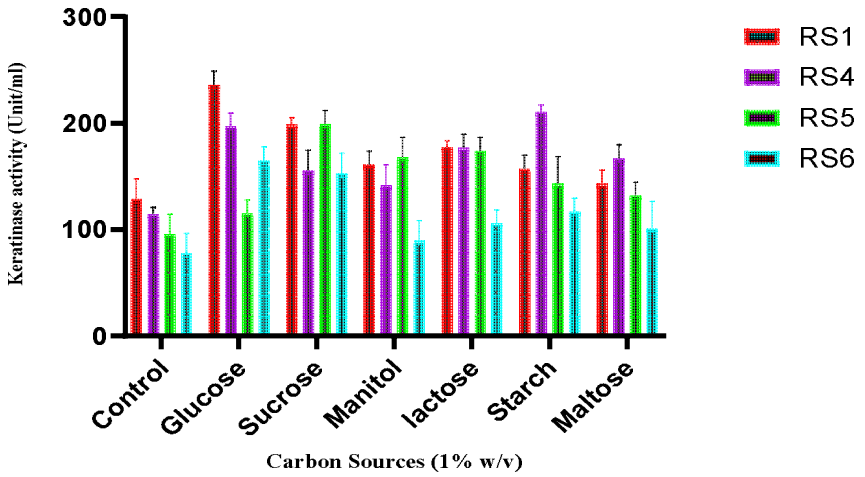


Figure 11: Effect of Various C Sources for keratinase production by Isolates.

Keratinase production by isolates was optimized by adding various carbon sources such as Glucose, Sucrose, Mannitol, Lactose, starch, and Maltose into a Basal salt solution containing feather meal. In a glucose-containing medium, isolate RS1 gave the highest enzyme activity. As a similar trend for another isolate, RS4 gave the maximum activity in starch, RS5 in sucrose, and RS6 in glucose. This suggests that the enzymes in the isolates are adapted to different carbon sources and that each isolate has different preferences for the sugar it can utilize. The result obtained for isolates is mentioned inFigure 11. Similar work reported shown that *Amycolatosis sp.* Strain MBRL 40 produced the highest amounts of keratinase in the presence of starch [41]. Similar work published by *Bacillus licheniformis* produced a high amount of bacitracin using glycol [63]. *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5 produced a high amount of keratinase in the presence of galactose in the production medium [64].

Effect of Various Nitrogen Sources:

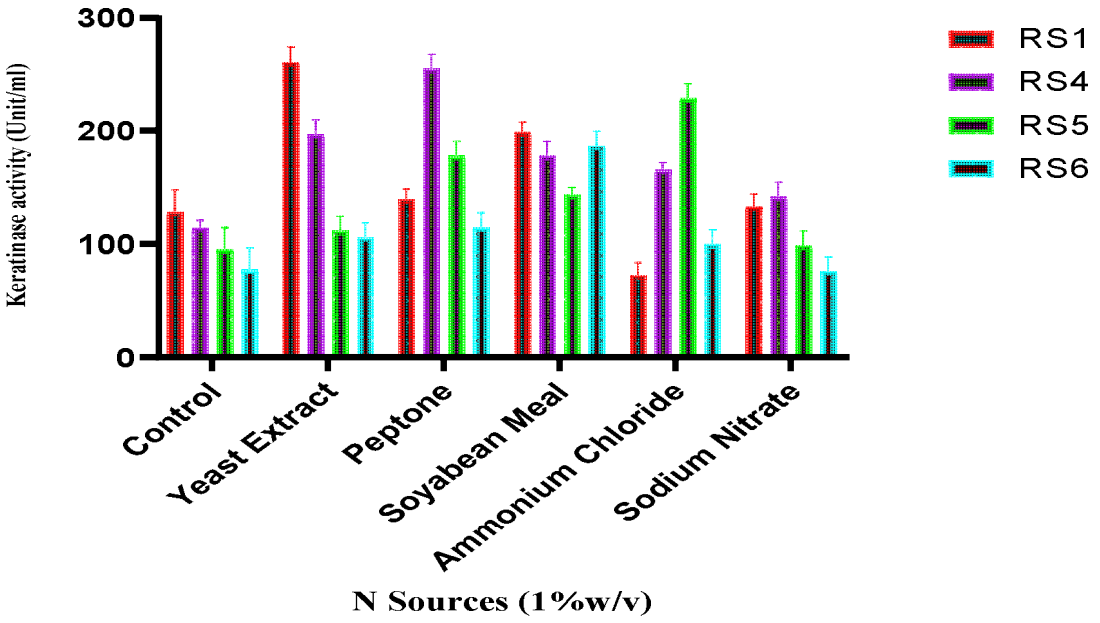


Figure 12: Effect of Various N Sources for keratinase production by Isolates.

Nitrogen sources in the production medium have a great impact on keratinase production Nitrogen sources are important for microbial growth, and if the right source is used, it can result in increased enzyme production. The right source can also increase the stability and activity of the enzyme. Keratinase production by isolates was optimized by adding various sources of nitrogen such as Yeast extract, Peptone,

Soybean meal, Ammonium chloride, and Sodium nitrate into a salt solution. As a detected results isolate RS1 gave the highest enzyme activity in yeast extract-containing activity. As a similar trend for another isolate, RS4 gave the maximum activity in peptone, RS5 in Ammonium chloride, and RS6 in soybean meal. This suggests that the enzymes in the isolates are adapted to different nitrogen sources and that each isolate has different preferences for the nitrogen it can utilize. The result obtained for isolates is mentioned in Figure 12. Similar results were obtained where it was found that in the presence of glutamic acid and alanine, *Bacillus licheniformis* produced a high amount of bacitracin [63]. Similar results were obtained by *Amycolatopsis sp.Strain MBRL 40* isolated from limestone habitat produced the highest amount of keratinase in the presence of Soybean meal [41]. *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5 produced a high amount of keratinase in the presence of NH_4NO_3 [64].

CONCLUSION

In this study, all four bacterial strains were isolated from soil containing keratin from poultry waste in natural habitats. The keratinase activity was evaluated by the hydrolysis of chicken feathers. The results showed that all the isolates were capable of producing keratinase. The isolates were identified as various *Bacillus* species by morphological, biochemical, and molecular characterization. They were identified as belonging to *Bacillus* species *viz. Bacillus subtilis*, *Bacillus velezensis*, *Bacillus cabrialesii*, and *Bacillus paramycodies,* respectively. The isolates *Bacillus subtilis* (RS1) and *Bacillus paramycodies* (RS6) show the highest feather degradation at 72 hours. The optimum temperature was 40°C for all the bacterial isolates. The optimum pH for RS1, RS5, and RS6 bacterial isolates was found to be 9 except for RS4 which was 8. Feather degradation to the extent of by isolates even under unoptimized conditions can be further exploited for efficient degradation of feathers under optimized conditions. Feather waste from the poultry industry can be processed using newly isolated bacteria that degrade feathers. To produce keratinase on large scales, additional research is required for purification, characterization, studying enzyme kinetics, testing different substrates, determining the effects of inhibitors and inducers, and submerged state fermentation. In addition, it is possible to examine whether a consortium of bacteria can enhance keratinolytic activity rather than using individual cultures. These studies can lead to the development of a cost-effective process for the production of keratinase on a large scale. This could potentially lead to the development of new products and applications derived from keratinase-mediated bioprocessing.

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