EFFICIENCY OF ENDOPHYTIC BACTERIAL ISOLATES AND THEIR ROLE IN DECOMPOSING AGRICULTURAL RESIDUE

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Abstract

The environment faces a significant threat due to the presence of agricultural residue that is difficult to break down. In order to address this issue, the current research focuses on decomposing harmful waste materials like feathers, human hairs, plastic, and epoxy. This decomposition process is facilitated by newly isolated endophytic bacteria in squash fruit. These bacteria, which form colonies in the intercellular space, were obtained from the melon fruit *Benincasa hispida* (Thunb) Cogn. A total of 22 bacterial colonies were isolated from the fruit using a highly alkaline medium with a pH of 14.0 and at a temperature of 37 °C. 12 bacterial populations were selected out of these colonies based on their growth conditions. These populations were named KARE_P1, KARE_P2, KARE_P3, KARE_P5, KARE_P8, KARE_P9, KARE_P11, KARE_P12, KARE_P14, KARE_P15, KARE_K1, KARE_K2, and KARE_W1. KARE_P3 and KARE_P8 were identified as non-pathogenic based on their 16S RNA sequence. Further characterization of these bacteria involved studying their growth under different conditions, including temperatures of 18°C, 37°C, and 55°C, and pH values of 5.0, 7.0, and 14.0. The protein extracted from these bacteria was analyzed using scanning electron microscopy (SEM) to assess their proteolytic and keratinolytic feather degradation capabilities. Additionally, a zymogram assay was conducted to evaluate their degradative potential.

Keywords: Biodegradation, Feather, Alkaline, Proteolytic, Keratinolytic

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DOI: 10.53555/ecb/2022.11.11.99

INTRODUCTION

Plants are commonly associated with a variety of microorganisms, especially endophytes that colonize the internal tissues of the plant and show no signs of infection or adverse effects on the host (Schulz & Boyle, 2006). Plants form vast and diverse niches for endobionts. All the approximately 300,000 plant species present on the Earth are hosts of one or more endophytes (Strobel et al., 1993). However, in most cases, there is not a single plant species without endophytes. Few of these plants have been thoroughly studied for their endophytes. As a result, the potential for discovering new and useful symbiotic micro-organisms among plant diversity in different ecosystems is greatly increased. Endophytes have been isolated from a wide variety of plants for Organisms such as *Bacillus, Enterobacter, Klebsiella, Pseudomonas, Burkholderia, Panthea, Agrobacterium, and Methylobacterium* species. It constitutes an endophyte commonly found in a variety of plants such as rice, wheat, maize, cotton, clover, potato, sugar cane, tomato, cucumber, and wildflowers (Bacon and Hinton, 2006).

The exact role of endophytes in plants is still unknown. However, the ability to thrive within host tissues far from microbial competition and environmental degradation makes endophytes potential candidates for agricultural applications. The roles of endophytic microorganisms in plants can be divided into two categories, growth promotion and disease control, based on the type of activity. Although research on keratinolytic microorganisms has focused primarily on biotechnological applications involving the hydrolysis of keratin-containing by-products, promising new applications related to drug delivery and hydrolysis of prion proteins have been described. (Brandelli, 2008; Brandelli *et al*., 2010). However, the ecological relevance of these organisms in natural systems and the prevalence of their ability to degrade feathers by bacteria are largely still unstudied issues (Lucas et al., 2003).

The aim of this study is the selection and identification of new culturable degrading bacteria from the fruit of important bacterial isolates from pumpkin. A potential medicinal plant wax gourd contains an endophytic bacterium (Benincasa hispida), from which 22 isolates of endophytic bacteria were screened for enhanced proteolytic activity and potential benefits. A feather depletion effect was demonstrated by them. Among these, KARE_P3 and KARE_P8 were screened for their spring depletion capacities. Further characteri-zation was performed by proteolytic and keratinolytic activity, different growth conditions – temperature, pH, total protein from crude extracts, SEM analysis, and zymography. Interestingly, KARE_P3 degraded 100% within 3 days. Feather utilization mechanisms including colonization, reducing agent and protease secretion, peptide/amino acid import and cellular metabolism, oxygen consumption, and iron uptake during fermentation were also investigated.

MATERIALS AND METHODS

Collection of samples

The wax gourd, which is prized in India for its therapeutic qualities in the Ayurvedic medical system, was chosen for the current study. It is also significant in Indian and Yoga spiritual traditions, where it is recognized as a rich source of prana. The wax gourd was obtained from the central market in Madurai, India and then transferred to sterile polythene bags for further processing.

Fruit preparation and surface sterilization

Fruits that were readily accessible in *Benincasa hispida* were purchased and stored for no longer than 10 days at room temperature (20 °C) before being processed. Fruits were properly cleaned with soap and water, surface-sterilized for five minutes with 70% ethanol, and then allowed to dry in order to prevent contamination by environmental bacteria. By plating samples of the fruit surface on an agar medium, the sterility of the surface was confirmed. Because the naturally occurring species' first sterilization phase did not completely get rid of all surface associated bacteria, the fruits were also submerged in 1% sodium hypochlorite solution for five minutes before being thoroughly rinsed three times with sterile distilled water.

Screening, Characterization, and Isolation

To isolate endophytic bacteria from the fruits for the current investigation, we had to devise two distinct procedures. The first approach entailed dividing samples into two halves, impregnating each half on LB Agar plates with pH 7.0, and then incubating each half at 37 °C for 24 hours. The second technique entailed using a sterile pestle and mortar to macerate the surface sterilized samples. The macerated sample of randomly chosen 1–5 g of tissues was serially diluted, and the dilutions were then plated on LB Agar media with 1.2% KCl supplementation and kept at a pH 14.0 in an incubator for 24 hours at 37 °C. 22 colonies were isolated, and they underwent pH testing at a high 14.0. 12 of these were chosen and used for further study.

KARE isolates' keratinase activity was analysed
1.0 mL of the cell-free supernatant and 0.01 g of chicken keratin powder (prepared in 1 mL of 50 mmol citrate buffer pH 5.0) made up the reaction mixture. For 60 minutes, the mixture was incubated at 50 °C in a water bath. By adding 2.0 mL of 10% trichloroacetic acid (TCA), the process was stopped. Centrifugation was used to separate the resultant precipitate for 10 minutes at 10,000 g. Finally, 5.0 mL of the alkaline copper reagent (40 g of sodium carbonate, 7.5 g of tartaric acid, 4.5 g of copper sulphate, and 1000 mL of distilled water; ultimate pH 9.0) was added after 0.2 mL of the supernatant was diluted to 1.0 mL with purified water. Afterward, to create the blue colour, 0.5 mL of the Folin-Ciocalteu reagent was added, and the tubes were left in the dark for 30 minutes. By mixing 2 mL of 10% TCA without keratin with the enzyme solution, a negative control was created. Tyrosine served as the standard for the absorbance measurement at 660 nm (UV-visible spectrophotometer Sistronic). According to the L-tyrosine standard curve, one unit of keratinolytic activity is equal to the enzyme concentration that releases 1 mol tyrosine mL–1 min–1 under standard test conditions. Bovine serum albumin (BSA) was used as the reference standard for measuring total protein content, and the specific keratinase activity per mg of protein was computed.

Total protein content was determined through analysis of KARE isolates.
Using bovine serum albumin (BSA) as a protein standard, the Bradford technique (MM. Bradford, 1976) was used to calculate the crude extract's protein concentration.

Analysis of the effects of pH and temperature on the synthesis of keratinase in KARE isolates
White chicken feathers were purchased from a nearby market, thoroughly cleaned by rinsing in double-distilled water, dried, and then preserved for future research. A pH 12.5 was maintained for the FDB enrichment media, which comprised (g L^1) NH_4Cl 0.5 g, NaCl 0.5 g, K_2HPO_4 0.3 g, KH_2PO_4 0.4 g, $MgCl_2$ 0.1 g, yeast extract 1.0 g, and chicken feather 10.0 g. Crude chicken feather 1.0 g, basal medium (BM) 0.5 g NaCl, 0.4 g KH_2PO_4, and 0.3 g K_2HPO_4; pH values of 5.0, 7.0, and 14.0; and varied temperatures of 18 °C, 37 °C, and 55 °C.

Analysis of *KARE isolates* on *Proteolytic activity*
On bovine haemoglobin 1 mg/mL (Sigma-Aldrich), in Tris-HCl buffer pH 9.5 (0.05 M), at 55 °C, proteolytic activity was assessed. Trichloroacetic Acid (TCA) 8% was used to stop the process. The mixture was centrifuged (12,000g, 10 min), chilled for 20 min, and the absorbance at 280 nm wavelength was measured. One mol of released tyrosine computed per 1 mL of culture fluid within 1 min was used to express one unit of proteolytic activity.

KARE isolates are tested for the feather hydrolysis assay
The examined bacteria's level of feather hydrolysis was evaluated using Nnolim et al.'s [26] weight-loss method. To recover the undegraded feathers, the fermentation broth was filtered using Whatman® qualitative filter paper, Grade 1, Maidstone, UK. The soup was then oven dried at 50 ^oC for 24 hours to produce the constant weight. The following equation was used to calculate the degree of feather hydrolysis:

$$\%\ of\ hydrolysis = \left(\frac{IM - FM}{IM}\right) \times 100$$

Where, (IM) is the initial dry mass of the intact feather before the fermentation process, and (FM) is the dry mass of the residual feather after the fermentation process.

Study of the zymogram
The culture supernatant of the chosen bacterial isolate was subjected to zymographic examination. The sample and buffer sample (Tris-HCl0.32 M; pH 6.8; glycerol 48%; SDS 8%; bromophenol blue 0.06%) were combined in a 1:1 ratio. The mixture sample was put onto a 12% polyacrylamide gel (5% staking gel) that included 0.1% copoly-merized casein in amounts of 5 or 10 L. PAGE As a reference marker, ruler pre-stained (Thermo Scientific) was applied. At a steady 18 mA and 2 °C, electrophoresis was conducted. The gel was then incubated for 24 h at 28 °C in the same buffer (Tris-HCl 0.05 M, pH 7.5, containing CaCl2 2 mM and NaN3 0.02%), washed three times with Triton-X 2.5%, and once with the incubation buffer. Bands of proteolytic activity were visualized by Coomassie Blue staining and colour removal using methanol, acetic acid, and water (50:10:40).

Identification and phylogeny research using molecules
The sequence analysis of the 16S rDNA genes served as the basis for the identification of certain bacterial isolates. Following the usual technique, the product was amplified by PCR using the following specific primers: (27 F) AGAGTT TGATCGTGGCTCAG and (1492l R) GGTTACCTTGTTACGACT. Using the same primers, the PCR product was separated from

reaction by-products and sequenced. The retrieved sequences were searched for similar nucleotide sequences using the Ribosomal Database Project (RDP) release 10 databases. Using MAFFT version 6 and Archaeopteryx version 0.9914, the sequence alignment and phylogenetic analysis were carried out (Cole et al. 2014). Under the accession numbers shown in Table 2, the nucleotide sequences were added to the GenBank database of the National Centre for Biotechnology Information (NCBI).

Feather Degradation Analysis Using Scanning Electron Microscopy (SEM)

Feather samples were collected for SEM analysis at different time intervals (0, 8, 16, 24, and 32 h) in order to identify morphological changes that take place throughout distinct stages of feather deterioration. The samples were put on aluminium stubs after being dehydrated. The specimens were then sputter-coated which were then examined and captured on camera using a scanning electron microscope (Zeiss, EOS18).

RESULTS AND DISCUSSION

The identifying of issues

Benincasa hispida (Thunb.) was utilized for the repeated isolation and characterization of the endophytic microbial community. It was observed that Cogn belongs to the Cucurbitaceae family. Of the numerous isolates, only 12 were selected for further investigation based on their high pH of 14.0 (Fig. 1) in the medium composition. These isolates were screened to assess their degrading abilities towards the plant. After 24 hours of culture in the liquid medium, the colonial morphology was as follows: the bacteria exhibited yellow and orange colors, indicating the presence of endophytic microorganisms. They appeared as moist and diamondshaped cocci, with a smooth, convex surface and distinct edge. SEM images of the *Staphylococcus lentus* strain KARE_P3 (Fig. 2) revealed a coccus form with 8.0mm in width and 1.0-1.2mm in length. Additionally, Fig. 3 displayed the Gram's staining technique, providing a composite microscopic picture of the strain.

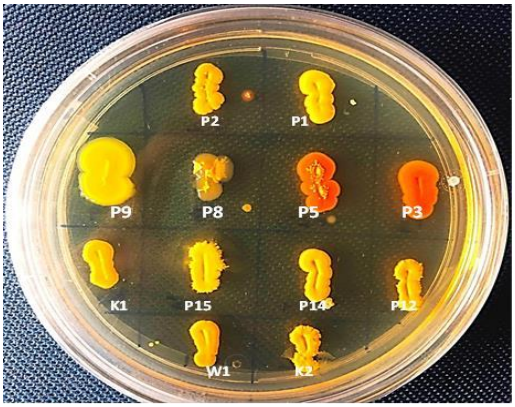

Fig 1. Shown on different endophytic isolates from *Benincasa hispida (Pumpkin) on agar plate at pH14.0*

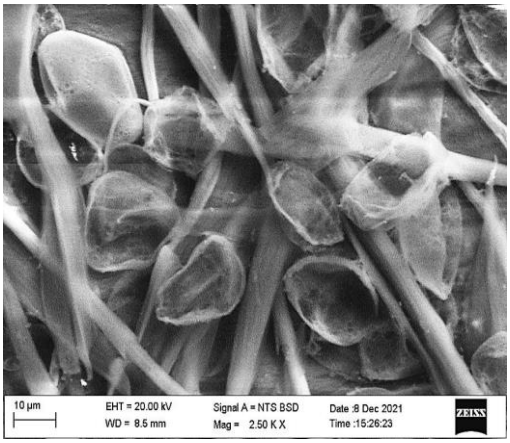

Fig-2 SEM image of strain KARE_P3 (*Staphylococcus Lentus*)

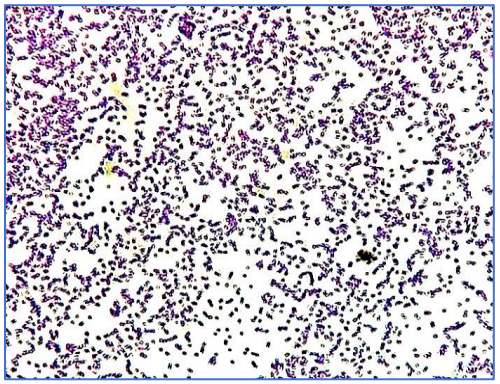

Fig-3 showed that gram's staining method of strain KARE_P3 (*Staphylococcus Lentus*)

Study of the 16S rRNA sequencing from studies on endophytic bacteria

The 16S rRNA sequencing of various strains of endophytic bacteria was studied. Yaazh Genomics found that the National Library of Medicine owns the trademark for BLAST. The length of the 16S rRNA sequences of the strains KARE_P1, KARE_P2, KARE_P3, KARE_P5, KARE_P8, KARE_P9, KARE_P11, KARE_P14, KARE_P15, KARE_K1, KARE_K2, and

KARE_W1 ranged from 786 to 1311 base pairs. The results of the BLAST analysis for the 16S ribosomal RNA gene in bacterial strains are presented in Table 1 and Fig. 4 and 5. The phylogenetic tree was created using the BLASTN 2.13.0+ program. Through the analysis of the 16S rRNA, twelve different isolates of gram-positive bacteria were identified. Two of these isolates not only help in reducing environmental pollution but also have the potential to affect human health.

P3 tree

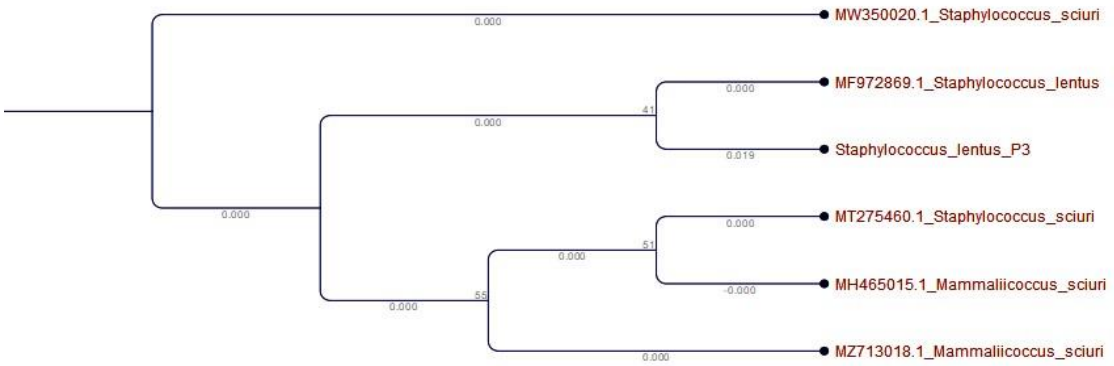

Fig 4 & 5. Shown KARE_P3 sequence tree

Staphylococcus lentus strain VMS-4 16S ribosomal RNA gene, partial sequence
Sequence ID: **MF972869.1**Length: 1400Number of Matches: 1
Range 1: 118 to 1361

Fig-12 shown in sequence report

Score	**Expect**	**Identities**	**Gaps**	**Strand**	**Frame**
2167 bits(1173)		0.0()	1220/1244(98%)	0/1244(0%)	Plus/Plus

Features:

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Query   1    GATAATATTTTGAGCCGCATGGGTCTATAGTGAAAGACGG   60
Sbjct   118  CTTCGGATGTCATTTATAAA                     177
             ||||||||||| ||||||| || |||||||||||| ||||| ||||| | |
             GATAATATTTTGAACCGCATGGTTCAATAGTGAAAGACGG
             TTTCGGCTGTCACTTATAGA
Query   61   TGGACCCGCGCCGAATTAACTAGTTGGTAAGGTAACGGCT   120
Sbjct   178  TACCAAGGCGACGATACGAA                     237
             ||||||||||| |||| |||||||||||||||||||||||||||||||| |
             TGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCT
             TACCAAGGCGACGATACGTA
Query   121  GCCGACCTGAGAGGGTGATCGGACACACTGGAACTGAGA   180
Sbjct   238  CACGGACCAGACTCCTACGGG                    297
             |||||||||||||||||| |||||||||||||||| ||||||||||||
             GCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGA
             CACGGTCCAGACTCCTACGGG
Query   181  AGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCT   240
Sbjct   298  GACGGAGCAACGCCGCGTGAG                    357
             |||||||||||||||||||||||||||||||||||||||||||||||||||
             AGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCT
             GACGGAGCAACGCCGCGTGAG
Query   241  TGATGAAGGTCTTCGGATGGTAAAACTCTGTTGTTAGGGA   300
Sbjct   358  GGACCAACTTTGTTAGTAAC                     417
             |||||||||||||||| |||||||||||||||| || ||| ||||||||||
             TGATGAAGGTCTTCGGATCGTAAAACTCTGTTGTTAGGGA
             AGAACAAATTTGTTAGTAAC
Query   301  TGACCAAGTCTTGACGGTACCTACCCAGAAAGCCACGGGT   360
Sbjct   418  AACTACGTGCCAGCAGCCGC ||| |||||||||||||||| |||||||||||| ||||||||||||||||||| 477
             TGAACAAGTCTTGACGGTACCTAACCAGAAAGCCACGGC
             TAACTACGTGCCAGCAGCCGC
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Query  361  GGTAATACGTAGGTGGCAAGCGTTATCCGGTAATTATTGG  420
Sbjct  478  GCGTAAAGCGCGCGTAGGCG                      537
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            GGTAATACGTAGGTGGCAAGCGTTATCCGGNAATTATTGG
            GCGTAAAGCGCGCGTAGGCG
Query  421  GTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCATCCGTG  480
Sbjct  538  GAGGGTCATTGGAAACTGGG                      597
            || |||||||||||||||||||||| |||||||||||||||||||
            GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTG
            GAGGGTCATTGGAAACTGGG
Query  481  AAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGT  540
Sbjct  598  AGCGGTGAAATGCGCAGAGAT                    657
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            AAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGT
            AGCGGTGAAATGCGCAGAGAT
Query  541  ATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTG  600
Sbjct  658  TAACTGACGCTGATGTGCGAA                    717
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            TAACTGACGCTGATGTGCGAA
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Sbjct  718  ACGCCGTAAACGATGAGTGCT                    777
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Query  721  GGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACG  780
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Query  841  GCTCTAGAGATAGAGTCTTCCCCTTCGGGGGACAAAGTGA  900
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Sbjct 1258 AGTAATCGTAGATCAGCATG 1317

ATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCT
AGTAATCGTAGATCAGCATG

Query 1201 CTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCG
TCAC 1244

Table-1 BLAST analysis of the endobacterial isolates of bacterial strain from *Benincasa hispida (Pumpkin).*

S.NO	**Strains**	**Accession**	**Length**	**Homology**	**Name of the bacteria**
1	KARE_P1	MN894559.1	859bp	100%	*Staphylococcus sciuri subsp. carnaticus*
2	KARE_P2	MK629787.1	855bp	100%	*Staphylococcus xylosus*
3	KARE_P3	DQ279389.1	1247bp	99%	*Staphylococcus lentus*
4	KARE_P5	KR1091881.1	786bp	100%	*Staphylococcus sp. JSM 101067*
5	KARE_P8	MT367809.1	1313bp	100%	*Exiguobacterium acetylicum*
6	KARE_P9	DQ279389.1	1311bp	100%	*Staphylococcus sciuri*
7	KARE_P11	MF972869.1	1311bp	99%	*Staphylococcus sciuri*
8	KARE_P14	MN894563.1	1029bp	100%	*Staphylococcus sciuri*
9	KARE_P15	MK606065.1	1029bp	99.8%	*Staphylococcus sciuri*
10	KARE_K1	LC484818.1	1029bp	100%	*Staphylococcus sciuri*
11	KARE_K2	MK414841.1	1029bp	100%	*Staphylococcus sciuri*
12	KARE_W1	MN865948.1	1311bp	100%	*Staphylococcus sciuri*

Endophytic bacteria's Morphological, Biochemical, and Physical identifying feature of endophytic isolates

In agricultural fields, the wild wax gourd (*Benincasa hispida*) harbours a significant number of culturable endophytic bacteria, which could potentially have negative effects. Consequently, when the pH level was high (14.0), twelve endophytic bacteria were found in the fruits. Each isolate was subjected to various tests including examination of morphology, gram staining, protease, and keratinase activity, as well as assessments of pH and temperature values (refer to Tables 2 & 3) and biochemical investigations. Among the isolates, KARE_P3 and KARE_P8 exhibited the highest levels of protease and keratinase activity, as well as tolerance to different temperatures and pH levels, along with notable biochemical activity. Similar findings were observed in the degradation of contaminated soil by Jie Jiang et al 2019.

Table 2: shows an evaluation of different endobacterial isolates analysis in morphological and physical factors.

S No	**Name Of The Isolates**	**Morphology**		**Grams Staining**	**Protenase Enzyme Activity (U/mg^{-1})**	**Keratinase Enzyme Activity (U/mg^{-1})**	**Growth At Different pH**			**Zone formation in (diameter mm)**
		COLONY COLOR	**COLONY SHAPES**				**5.0**	**7.0**	**14.0**	**72hr**
1	KARE_P1	Yellow	Round	+	92.80	122.96	-	+	++	17.88
2	KARE_P2	Orange	Round	+	97.45	120.64	-	+	++	26.00
3	KARE_P3	Orange	Round	+	129.93	157.77	+	+	++	16.62
4	KARE_P5	Orange	Round	+	99.76	116.00	-	+	++	16.70
5	KARE_P8	Pale yellow white	Rod	+	104.40	92.80	+	+	++	17.14
6	KARE_P9	Greenish Yellow	Round	+	102.08	120.64	-	+	++	18.17
7	KARE_P12	Orange	Round	+	90.48	122.96	-	+	++	18.27
8	KARE_P14	Orange	Round	+	102.08	120.64	-	+	++	19.45

9	KARE_P15	Orange	Round	+	99.76	132.25	+	+	++	24.31
10	KARE_K1	Orange	Round	+	95.12	125.29	-	+	++	16.96
11	KARE_K2	Orange	Round	+	88.16	116.00	-	+	++	21.55
12	KARE_W1	Orange	Round	+	88.16	134.57	-	+	++	16.81

"+, - "Sign indicate positive and negative response

Table 3: Shows studies on biochemical characteristics of the endophytic KARE isolates

S No	***KARE isolates***	**Gram staining**	**Gelatin hydrolysis**	**Oxidase**	**Catalase**	**Amylolytic enzyme**	**Methyl red**	**Indole**	**Voges-Proskauer**	**Citrate**
1	KARE_P1	*Positive*	-	-	+	+	-	+	+	+
2	KARE_P2	*Positive*	-	-	+	+	-	+	+	+
3	KARE_P3	*Positive*	+	+	+	+	+	+	+	+
4	KARE_P5	*Positive*	-	-	+	+	-	-	+	+
5	KARE_P8	*Positive*	+	+	+	+	+	+	+	+
6	KARE_P9	*Positive*	-	-	+	+	-	+	+	+
7	KARE_P11	*Positive*	-	-	+	+	-	-	+	+
8	KARE_P14	*Positive*	-	-	+	+	-	-	+	+
9	KARE_P15	*Positive*	-	-	+	+	-	-	+	+
10	KARE_K1	*Positive*	-	-	+	+	-	-	+	+
11	KARE_K2	*Positive*	-	-	+	+	-	-	+	+
12	KARE_W1	*Positive*	-	-	+	+	-	-	+	+

"+, - "Sign indicate positive and negative response

Effects of various cultural conditions and Effects of pH and temperature.

In this study, Figure 6 (a and b) examines the impact of temperature and pH on bacterial growth and the rate at which feathers break down. The results showed that at a temperature of 37°C and a pH of 7.0, many KARE isolates experienced a significant increase in both bacterial growth and the rate of feather degradation. Compared to the control group, KARE_P3 and KARE_P8 showed positive increases and a strong correlation with the OD600. The growth and degradation rates of the KARE_P3 strain reached their peak, with the feather degradation rate reaching 89.0%. Furthermore, the strains were able to grow well and effectively degrade feathers within a temperature range of 18 to 42°C. This suggests that the KARE_P3 strain is capable of withstanding a wide range of temperatures, making it suitable for real-world applications. Similarly, an increase in pH, which is common in microbes that thrive on protein substrates, had similar effects. The specific mechanism of keratin degradation, however, still remains somewhat mysterious. It is believed that deamination, which creates an alkaline environment necessary for substrate swelling, along with sulphites and proteolytic attack, maybe the initial steps in keratin degradation (Kunert, 2000).

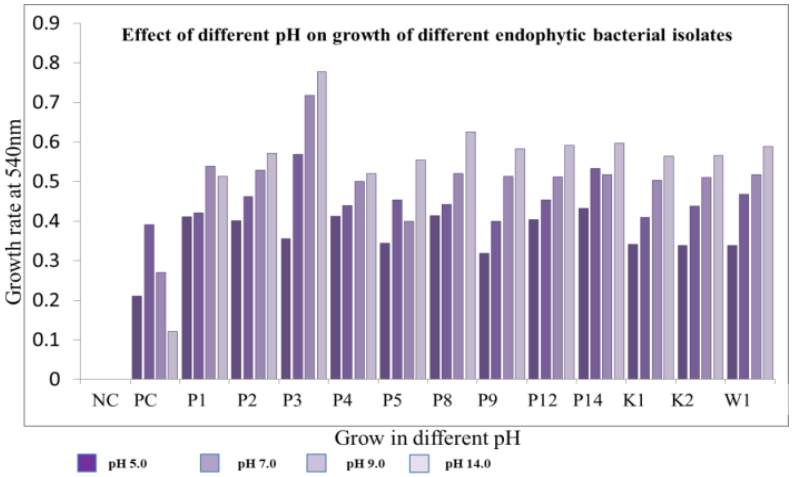


Fig 6a. Shown in effect of different pH analysis on different endophytic bacterial isolates

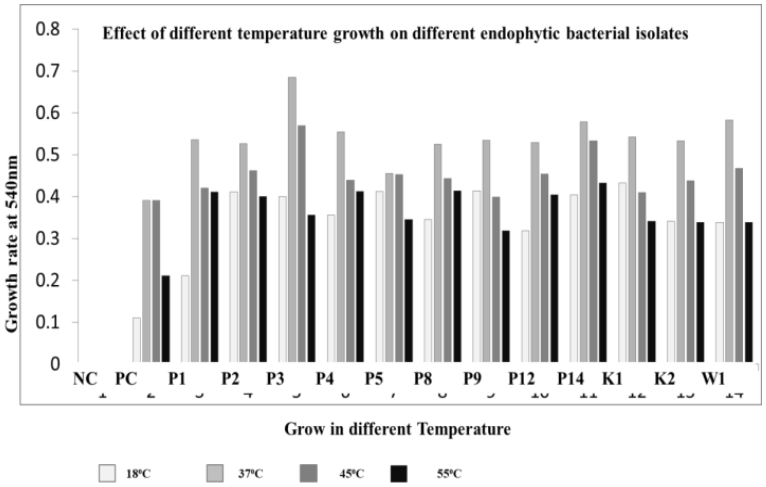


Fig 6b. Shown in the effect of different temperature analyses on different endophytic bacterial isolates

Screening of endophytic bacterial isolates with keratinolytic and proteolytic activity

The study utilized the endophytic bacterial population found in *Benincasa hispida* wax gourd as a practical source of proteolytic bacteria that may possess keratinolytic characteristics. A total of 12 proteolytic bacterial isolates were evaluated through spot testing on skim milk agar, which revealed several isolates with remarkable proteolytic activity and clear zone width around colonies. Two of the isolates were selected for liquid cultures in a medium with feathers as the primary food source, and the resulting substrate degradation byproducts and proteolytic activity were analysed. The concentration of hydrolysis products varied greatly among the examined isolates. Previous studies on keratinolytic bacteria have found that their growth and ability to break down feathers are most active at higher temperatures. One particular isolate, Ktn-1, was able to completely degrade feathers and significantly increase the pH of the medium, indicating its strong keratinolytic nature. Organisms with higher keratinolytic activity tend to alkalize the media more than those with lower activity. This alkalization is caused by the production of ammonia when keratin is broken down into peptides and amino acids. As a result, the medium becomes more alkaline.

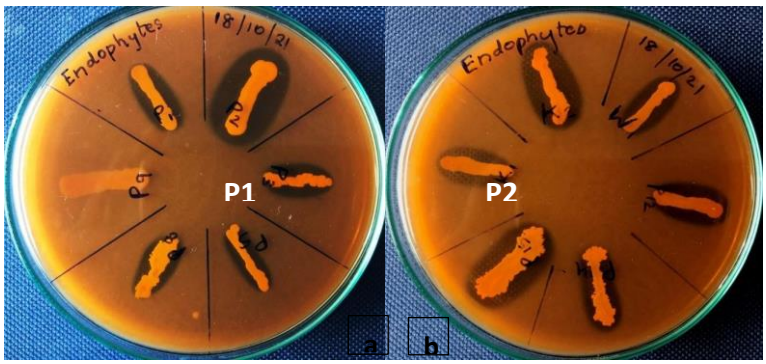


Fig 7 a & b. Analysis of *KARE isolates* Proteolytic activity

No obvious zone formation was observed except for KARE_P9 when the proteolytic activity was tested using the halo formation test on SMA plates. These plates were incubated at different temperatures for up to 48 hours. To confirm the hydrolysis of feather meal and feather, the bacteria that showed halo formation were cultured in FMB and FB at 37°C for up to 5 days. The results supported the visual observations, and the keratinolytic activity was measured by the degradation of extracellular feathers. The total protein content increased when comparing samples without feathers to those supplemented with feather meal, as indicated by Lowry et al. (1951) (Table 4).

ith the exception of KARE_P9, no obvious zone formation was seen when the proteolytic activity was tested in duplicate using the test of halo formation on SMA (Skim Milk Agar) plates that were incubated at 30, 37, 44, and 55 ºC for up to 48 h (Pillai and Archana, 2008). To confirm visual hydrolysis of feather meal and feather, the bacteria that were positive for halo formation were seeded

in duplicate in FMB (Feather meal broth) and in FB and cultured at 37 ºC in an orbital shaker (200 rpm) for up to 5 days. A similar outcome was obtained to support the visual outcome, and the keratinolytic activity was measured by extracellular feather degradation (Szabo et al., 2000). The amount of protein increased as indicated by Lowry *et al.* (1951) when measuring the total protein without feathers (12.9930.017 g/ml) and comparing it to feather meal supplemented (15.7770.001 g/ml) (Table 4).

Table 4: Analysis of extracellular protein from different endophytic bacterial isolates under the supplementation of with and without feather substrate

S.No	Name of the Isolates	Extra cellular protein in µg/ml	
		Without feather	With feather
1	NC	0.0	0.0
2	PC	8.816±0.01	9.048±0.01
3	KARE_P1	9.280±0.012	10.296±0.033
4	KARE_P2	9.745±0.032	10.064±0.089
5	KARE_P3	12.993±0.017	15.777±0.001
6	KARE_P5	9.976±0.073	9.600±0.01
7	KARE_P8	10.440±0.022	11.280±0.001
8	KARE_P9	6.208±0.023	8.064±0.04
9	KARE_P12	9.048±0.009	9.296±0.01
10	KARE_P14	10.208±0.01	10.064±0.09
11	KARE_P15	9.976±0.0123	9.225±0.077
12	KARE_K1	4.512±0.091	5.529±0.01
13	KARE_K2	8.816±0.065	8.600±0.052
14	KARE_W1	6.816±0.01	7.957±0.01

“± **“**Sign indicate standard deviation and deviation of the error response (minimum 3 replicate)

Finding effective KARE isolates that degrade feathers

The bacteria that can degrade feathers were obtained from a pumpkin fruit and then diluted. They were then placed in an agar medium with a high pH of 14.0 and incubated overnight at 37 °C. After that, the bacterial colonies were transferred to plates with feather powder and cultivated for 5 more days at 37 °C. Finally, single colonies were selected from strains with strong growth and streaked onto feather meal plates at 37 °C. The capacity of the isolates to degrade feathers was next tested by transferring them to 1% CFM. There is still some mystery around the specific mechanism of keratinolytic (Fig 8a-c). Deamination, which produces an alkaline environment required for substrate swelling, sulfites, and proteolytic attack, has been postulated as the initial step in keratin degradation (Kunert, 2000).

Degradation of non-synthetic material evaluation

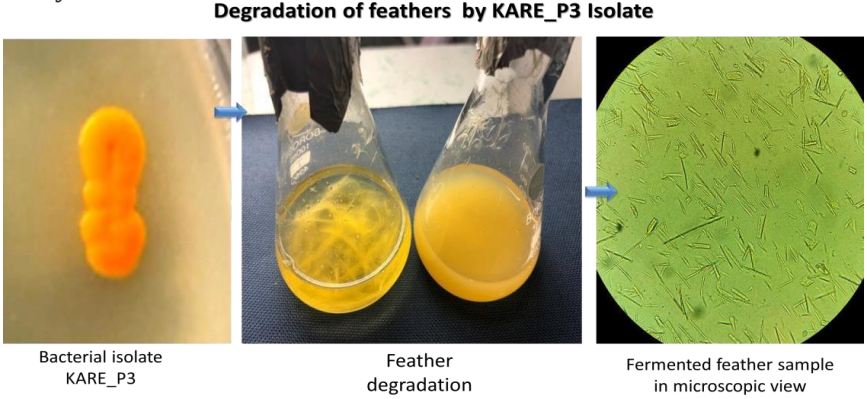


Fig 8 a-c. Shown in Feather degradation as preliminary studies on (a) Control (without inoculation of bacteria) (b) KARE_P3 isolates.

Evaluation of the degradation of natural materials

The assessment of the breakdown of natural materials has been conducted through the examination of the percentage of degrading activity in the broth culture and the biomass of disintegrated feathers. Based on the results presented in Figure 8 a-c and Table 5, it is suggested that the bacterial strains may have released synthesized enzymes through extracellular proteins to completely degrade the feathers within a short period of 3 days. The study also involved the measurement of extracellular protein as total protein analysis of KARE_P3 15.7770.001 and KARE_P8 11.2800.001, and the SEM examination of the degraded feather sample in various areas, as shown in Figure 9a-f. Mursheda Akhter *et al.* (2020) discovered that Bacillus cereus had the highest keratinase activity at 60 U/ml after 96 hours using a chicken feather, while Pseudomonas sp had the highest at 44 U/ml after 72 hours. Jahan et al. (2010) also conducted similar research and found that isolate Z4 had the highest keratinase activity of 22.3 U/ml after 72 hours and total feather disintegration after 7 days, as well as the highest recorded keratinase activity of 50 U/ml from the Bacillus genus. Additionally, Radha and Gunasekaren's (2008) report showed that Bacillus licheniformis MKU released a significant amount of keratinase within 48 hours of growth. These findings demonstrate that feathers serve as an enzyme inducer in the feather growth medium.

Table 5: Shown percentage of feather degradation biomass of dry weight under the fermentation at 45 days incubation

S.NO	Name of the Isolates	Feather sample *gm* /ml of broth		% of feather degradation
		The initial weight of a dry mass of the intact feather	After fermentation residual feather	
1	NC	0.0	0.0	
2	PC	0.500	0.490	2.0
3	KARE_P1	0.500	0.498	4.0
4	KARE_P2	0.500	0.483	3.4
5	KARE_P3	0.500	0.080	84.0
6	KARE_P5	0.500	0.490	2.0
7	KARE_P8	0.500	0.160	68.0
8	KARE_P9	0.500	0.493	1.4
9	KARE_P12	0.500	0.467	6.6
10	KARE_P14	0.500	0.451	9.8
11	KARE_P15	0.500	0.466	6.8
12	KARE_K1	0.500	0.499	0.2
13	KARE_K2	0.500	0.475	5.0
14	KARE_W1	0.500	0.490	2.0

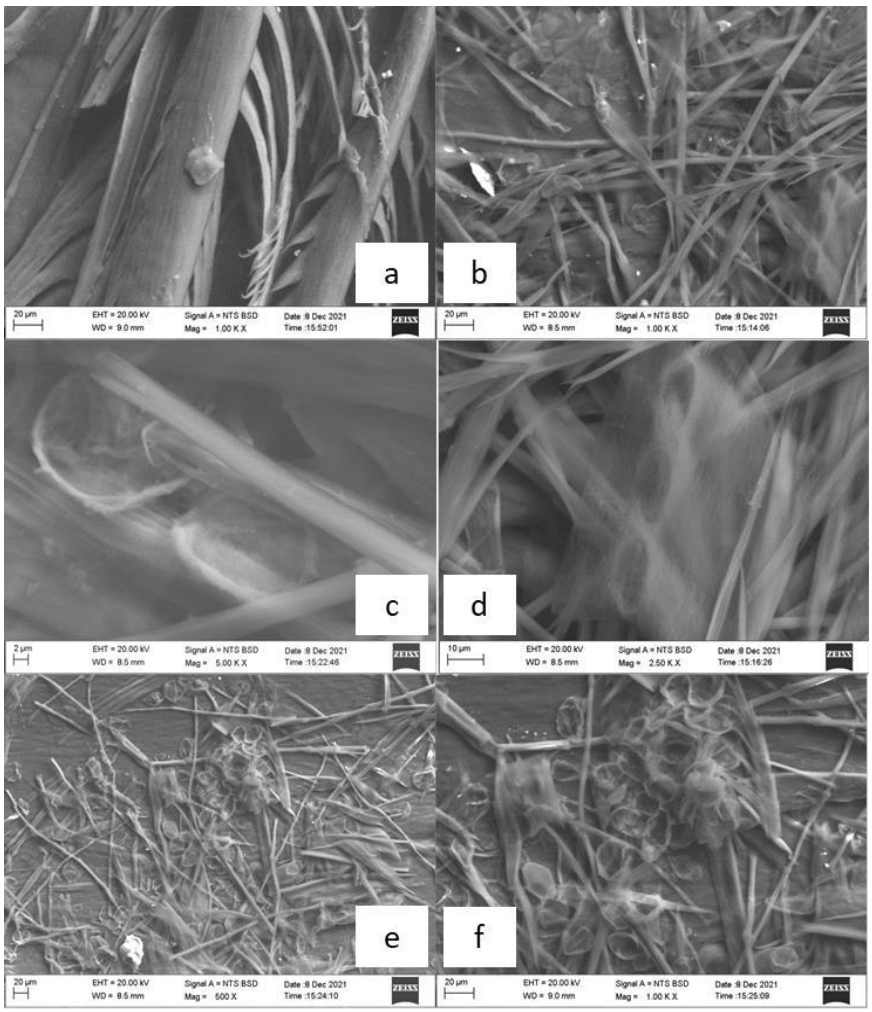
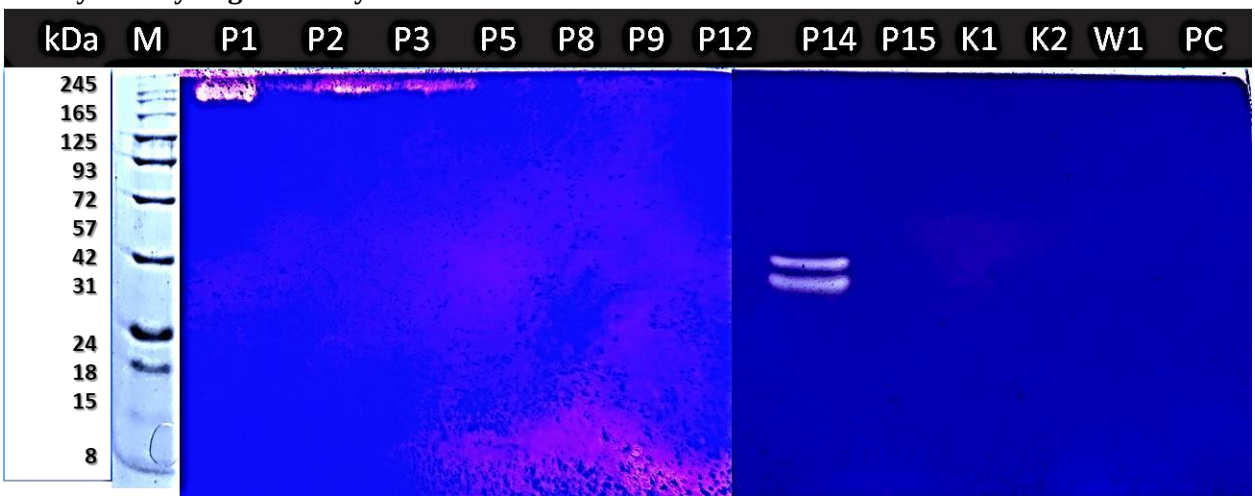


Fig 9a-f. *Scanning* electron microscope analysis of chicken-feather degradation by KARE_P3 in basal medium (A) Untreated chicken feather; (B, C) barb and barbule degradation after 816 h; (D) barbule degradation after 24 h; (E, F) feather shaft degradation after 32-40 h.

Zymogram assay: proteolytic and keratinolytic assay on gelatin SDS-PAGE

In this study, we observed changes in the electrophoresis patterns of all the extracellular enzymes produced by endophytic organisms when the integrated substrate was introduced to the liquid medium, with or without the presence of feathers. Unlike the 38 kDa and 42 kDa protein bands observed in KARE_P14, the samples KARE_P1, KARE_P2, KARE_P3, and KARE_P14 only showed a high molecular weight protein band. Additionally, the 250-kDa band was more prominent in the KARE_P1, KARE_P2, and KARE_P3 samples (refer to Figure 10). We further investigated the effect of feathers added to the broth by analysing the keratinolytic activity using gelatin substrate electrophoresis profiles and comparing it with the proteolytic zymogram assay. Interestingly, the intensities of some protein bands varied in the presence of feathers (refer to Figure 10). The electrophoresis profiles of the co-polymerized protein substrates, namely KARE_ P1, KARE_P2, KARE_P3, KARE_P5, KARE_P9, and KARE_P15, were found to be similar when BSA was used as a positive control. Interestingly, the 250-kDa band was observed to increase in the endophytic isolates. However, the KARE_P14 isolates that did not express the low molecular protein in the keratinolytic assay were not detected in the profiles of the peptidases that were examined with co-polymerized casein. Nonetheless, this isolate exhibited a new band of approximately 250 kDa, as shown in Figure 11. To determine the molecular weight of the pure keratinase, a zymogram analysis was conducted using a chicken feather as the enzyme's hydrolysis substrate. This was done to obtain a more accurate measurement of the keratinase's molecular weight (Jendri Mamangkey *et al*. 2020; Cai CG, Lou BG, Zheng XD, 2008).

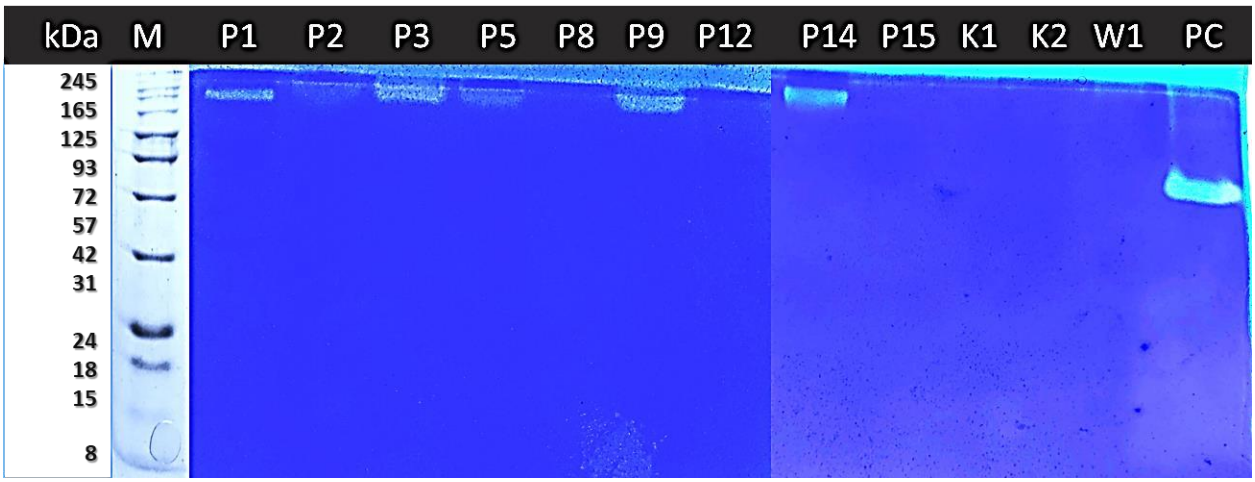
Proteolytic in zymogram assay on SDS-PAGE



1. kDa; M-Marker; PC-Positive control; P1-W1 different endophytic isolates

Fig 10. Shown in proteolytic analysis on zymogram assay in different endophytic bacterial isolates

Keratinolytic in zymogram assay on SDS-PAGE



1.kDa; M-Marker; PC-Positive control; P1-W1 different endophytic isolates

Fig 11. Shown in keratinolytic analysis on zymogram assay in different endophytic bacterial isolates

Endophytic bacterial isolates PCR analysis

The study has developed a PCR assay that can identify and isolate endophytic bacteria. This assay will be combined with other PCR assays that focus on the gene's ability to degrade. By using quick and easy sample preparation techniques, these PCR assays can be modified for direct detection from the isolating efficiency degrading. However, due to the presence of Staphylococcus sp components, these assays would have limited value for degradation for Staphylococcus sp screening of all 22 isolates unique after detection.

The PCR assays developed in this study can provide a quicker determination of the effectiveness and efficiency of gram-positive endophytic bacteria isolated for degrading activity when used for direct detection from endophytic isolates. The findings of the PCR assay using Primer-1 and Primer-2 were different, with most of the isolates showing a single pattern of around 1700 bp with Primer-2. Out of the 22 isolates, 16 were randomly paired with Primer2. However, the study focused on the biological and industrial waste feather degradation process using only 12 isolates from the 16 matched with Primer-2.

Molecular assay: Studies on PCR analysis of 22 endophytic isolates on 1% agrose gel for specific primer -1 and 2.

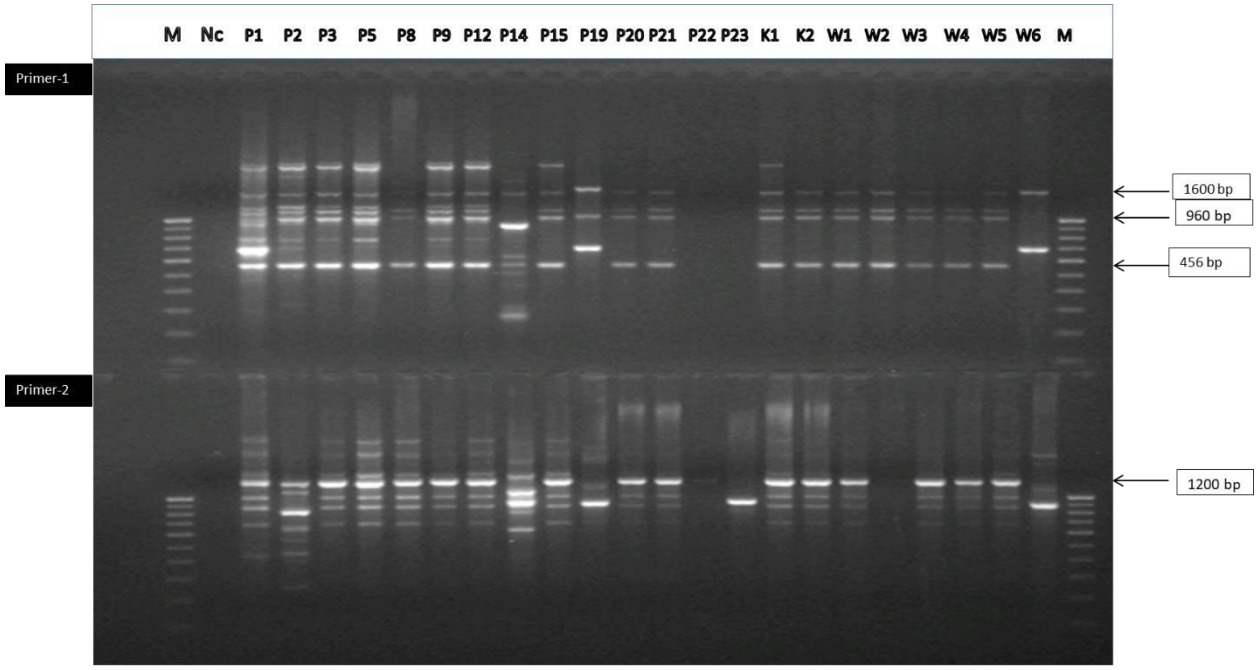


Fig 12. Shown in PCR analysis on 1% agarose in different endophytic bacterial isolates

CONCLUSIONS

The results of this investigation clearly demonstrate that 22 endophytic isolates from the KARE series were tested on feathers that had undergone degradation in a highly alkaline medium with a pH of 14.0. Through sequencing analysis, the pathogenic and non-pathogenic nature of these bacteria was determined. Based on the sequencing data, it was found that KARE_P3 and KARE_P8 were non-pathogenic. These two selected isolates were then used to break down the feathers into a high-protein feather meal that could be incorporated into animal feed. The proteolytic, keratinolytic, total protein, pH, temperature, zymogram, and PCR analyses of the 12 isolates were thoroughly examined. The ability of KARE_P3 to degrade feathers within a span of three days is a significant advancement in waste management, as it contributes to pollution reduction. Furthermore, this microbial approach not only addresses economic and environmental concerns but also generates value-added bioproducts that hold considerable industrial potential.

ACKNOWLEDGEMENT

We appreciate the ongoing encouragement to complete this job from the Vice President, Chancellor, and Vice-Chancellor of Kalasalingam University at Krishnankoil, Viruthunagar District, Srivilliputhur - 626 126 Tamil Nadu, India.

Conflicts of Interest

The authors do not have any conflicts of interest.

REFERENCES

1. Acmite Market Intelligence. Global Epoxy Resin Market, 3rd ed.; Acmite Market Intelligence: Ratingen, Germany, 2017.
2. May, Clayton (2018). Epoxy Resins: Chemistry and Technology (2nd ed.). CRC Press. p. 65. ISBN 978-1-351-44995-3.
3. Shah, A.A.; Hasan, F.; Hameed, A.; Ahmed, S. Biological degradation of plastics: A comprehensive review. Biotechnol. Adv. 2008, 26, 246–265. [CrossRef] [PubMed]
4. Pangallo, D.; Buckova, M.; Krakova, L.; Puskarova, A.; Sakova, N.; Grivalsky, T.; Chovanova, K.;
5. Zemankova, M. Biodeterioration of epoxy resin: A microbial survey through culture independent and culture-dependent approaches. Environ. Microbiol. 2015, 17, 462–479. [Cross Ref] [PubMed]
6. Little, B.J.; Lee, J.S. Microbiologically Influenced Corrosion; John Wiley & Sons: Hoboken, NJ, USA, 2007.
7. Wang, G.; Chai, K.; Wu, J.; Liu, F. Effect of Pseudomonas putida on the degradation of epoxy resin varnish coating in seawater. Int. Biodeterior. Biodegrad. 2016, 115, 156–163. [Cross Ref]
8. Chen, X.; Schauder, S.; Potier, N.; Van Dorsselaer, A.; Pelczer, I.; Bassler, B.L.; Hughson, F.M. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 2002, 415, 545–549. [Cross Ref][PubMed]
9. Ivanova, K.; Fernandes, M.M.; Francesko, A.; Mendoza, E.; Guezguez, J.; Burnet, M.; Tzanov, T.Quorum-quenching and matrix-

degrading enzymes in multilayer coatings synergistically prevent bacterial biofilm formation on urinary catheters. ACS Appl. Mater. Interfaces 2015, 7, 27066–27077. [Cross Ref] [PubMed]

10. Papenfort, K.; Bassler, B. Quorum-sensing signal-response systems in Gram-negative bacteria. Nat. Rev. Microbiol. 2016, 14, 576–588. [CrossRef] [PubMed].
11. 1.Castresana, J. (2000). Selection of conserved blocks from multiple alignments for theiruse in phylogenetic analysis. Molecular Biology and Evolution 17, 540- 552.
12. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, GuindonS, Lefort V, Lescot M, Claverie JM, Gascuel O (2008). Phylogeny.fr: robust phylogene-ticanalysis for the non-specialist. Nucleic Acids Res.1:36. Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high through put. Nucleic Acids Res 2004, 32 (5):1792-1797.
13. Talavera, G., and Castresana, J. (2007). Improvement of phylogenies after remov ingdivergent and Ambiguously aligned blocks from protein sequence alignments. Systematic Biology 56, 564-577.
14. Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989) Isolation and directcomplete nucleotide determination of entire genes: characterization of a gene coding for16S ribosomal RNA. Nucleic Acids Res 17: 7843–7853.
15. D. Kumar, L. Kumar, S. Nagar, C. Raina, R. Parshad, V.K. Gupta, Screening, isolation and production of lipase/esterase producing Bacillus sp. strain DVL2 and its potential evaluation in esterification and resolution reactions, Arch. Appl. Sci. Res.4 (2012) 1763–1770.
16. F.N. Niyonzima, S.S. More, Screening and identification of a novel alkaline lipase producing bacterium, Int. J. Pharm. Bio. Sci. 4 (2013) 1037–1045.
17. M. Veerapagu, A.S. Narayanan, K. Ponmu-rugan, K.R. Jeya, Screening selection identification production and optimization of bacterial lipase from oil spilled soil,Asian J. Pharm. Clin. Res. 6 (2013) 62–67.
18. Jendri Mamangkey, Dwi Suryanto, Erman Munirand Apon Zaenal Mustopa, Keratinase Activity of A Newly Keratinolytic Bacteria, Azotobacter chroococcum B4, J Pure Appl Microbiol | 14(2):1203-1211 | June 2020
19. Cai CG, Lou BG, Zheng XD. Keratinase production and keratin degradation by a mutant strain of Bacillus subtilis. J Zhejiang Univ Sci B 2008; 9: 60-67
20. Mursheda Akhter , Lolo Wal Marzan , Yasmin Akter and Kazuyuki Shimizu, Microbial Bioremediation of Feather Waste for Keratinase Production: An Outstanding Solution for Leather Dehairing in Tanneries, Microbiology Insights Volume 13: 1–12 https://doi.org/10.1177/1178636120913
21. Jahan Z, Khan SN, Mozammel Hoq M. Screening of keratinolytic bacteria from Poultry Wastes Bangladesh. Journal of Scientific and Industrial Research. 2010;45(3):261-266. doi: 10.3329/bjsir.v45i3.6535
22. Rhadha S, Gunasekaren P. Sustained expression of keratinase gene under PxyLA and PamyL promotores in the recombinant Bacillus megaterium MS941. Bioresour Technol. 2008; 99:5528-5537. doi: 10.1016/j. biortech. 2007. 10.052
23. Kunert J. 2000. Physiology of Keratinolytic Fungi. In Kushwaha, RKS, Guarro J (Eds), Biology of Dermatophytes and other Keratinophilic Fungi. *Revista Iberoamericana de Micologia,* Bilbao, p77-85.
24. Williams, C.M., Richter, C.S., Mackenzie, J.M., Jason, J.R. and Shih, C.H. 1990.Isolation, identification and characterization of a feather-degrading bacterium.*Appl. Environ. Microbiol.* 56: 1509-1515.
25. Nam GW, Lee DW, Lee HS, Lee NJ, Kim BC, Choe EA, Hwang JK, and Pyun YR. 2002. Native feather degrading by *Frvidobacteium islandicum* AW-1, a newly isolated keratinase producing thermophilic anaerobe. *Arch. Microbiol.* 178: 58-547.
26. Kaul S, and Sumbali G. 1997. Keratinolysis by poultry farm soil fungi. *Mycopathologia.* 139: 137-140.
27. Elias, R.J., Kellerby, S.S. and Decker, E.A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition*, 48(5), 430-441. https://doi.org/10.1080/10408390701425615
28. Tang, X., He, Z., Dai, Y., Xiong, Y.L., Xie, M. and Chen, J. (2009). Peptide fractionation and free radical scavenging activity of zein hydrolysate. *Journal of Agriculture and Food Chemistry*, 58(1), 587-593. https://doi.org/10.1021/jf9028656
29. Acmite Market Intelligence. Global Epoxy Resin Market, 3rd ed.; Acmite Market Intelligence: Ratingen, Germany, 2017.
30. Shah, A.A.; Hasan, F.; Hameed, A.; Ahmed, S. Biological degradation of plastics: A comprehensive review. Biotechnol. Adv. 2008, 26, 246–265. [Cross Ref] [PubMed]

31. Pangallo, D.; Buckova, M.; Krakova, L.; Puskarova, A.; Sakova, N.; Grivalsky, T.; Chovanova, K.; Zemankova, M. Biodeterioration of epoxy resin: A microbial survey through culture independent and culture-dependent approaches. Environ. Microbiol. 2015, 17, 462–479. [CrossRef] [PubMed]