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Isolation, Screening and Characterization of Poultry Feather Degrading Bacteria : A Path to Sustainable Feather Waste Management

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Abstract

Worldwide poultry feather waste is generating in large quantities, it leads to significant disposal challenges because it is made up of keratin protein, an insoluble protein with strong disulfide bonds, which makes it resistant to normal enzymatic degradation. Disposal methods like incineration, landfilling, burning and mechanical grinding were restricted due to the emission of harmful gases and degradation by chemical treatments, which are energy-intensive and damage protein quality by destroying essential amino acids. To overcome these problems, deploying keratinolytic microorganisms for maximum degradation of feathers is an economical, environmentally friendly approach. The present investigation focuses on the isolation and characterization of poultry feather-degrading bacteria from partially degraded feathers and poultry waste decomposing soil samples collected from Veterinary College, Bengaluru and Central Poultry Development Organization (CPDO), Hesaraghatta. A total of 54 morphologically distinct bacterial colonies were isolated, of which 16 isolates showed proteolytic activity on skim milk agar, indicating protease production. Morphological and biochemical characterization revealed that all screened isolates were Gram-positive and catalase-positive. Ten isolates capable of gelatin liquefaction (PWDS-2, PWDS-11, FWD-5, FES-1, FES-2, FES-3, FES-5, FES-8, FES-13 and FES-16), sixteen isolates were positive forcasein hydrolysis and eleven isolates positive for starch hydrolysis. Further, twelve isolates tested positive for citrate utilization and Voges-Proskauer tests (PWDS11, FWD3, FWD5, PDFSS3, FES1, FES2, FES5, FES8, FES9, PRB11, FES13 and FES16), while four isolates were positive for methyl red (PWDS2, FWD3, PDFSS3 and FES2) and five isolates were positive for urease tests (FWD3, PDFSS3, PDFSS4, PDFSS5 and FES1). Based on morphological and biochemical characterization as per Berges manual in the present study the bacterial isolates may be belonging to *Bacillus* sp.

Keywords : Feather degradation, Poultry waste, *Bacillus*, Keratinase, Protease, Biochemical characterization, Waste management

Poultry production has expanded rapidly, becoming a significant sector in India's economy. Annually, 24 billion chickens are slaughtered worldwide, generating approximately 8.5 billion tons of poultry feathers, with India's contribution alone reaching 350 million tons (Gupta *et al*., 2011). Feather waste, a byproduct of poultry processing, poses serious environmental challenges due to its recalcitrant nature and resistance to degradation by common proteolytic enzymes (Godheja *et al*., 2014).

Feathers constitute about 5-7 per cent of a poultry bird's total weight and comprise approximately 90 per cent keratin protein, making keratin one of the most abundant polymers in nature after cellulose and chitin (Lange *et al*., 2015). Structurally, keratin contains strong chemical bonds, including disulfide bonds, hydrogen bonds and hydrophobic interactions, contributing to its degradation resistance. Consequently, improper disposal of poultry feathers leads to environmental pollution.

Conventional disposal methods such as incineration, landfilling and mechanical grinding are limited due to harmful gas emissions and high environmental risks (Kumawat *et al*., 2018). Chemical treatments like alkali hydrolysis and steam pressure cooking, although effective, require substantial energy and compromise protein quality. Despite the high protein and nitrogen content in feathers, limited efforts have been made towards their efficient recycling and utilization.

Microbial degradation presents a sustainable and eco-friendly alternative for managing feather waste. Certain microorganisms, including *Bacillus* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Leuconostoc* sp. and *Xanthomonas* sp., have demonstrated the ability to degrade keratin (Gupta and Ramnani, 2006). Bacteria, in particular, are effective due to their rapid growth and production of extracellular proteolytic enzymes, such as keratinase, which hydrolyzes keratin into peptides and amino acids. Feathers treated with microbial keratinase have potential applications as dietary protein sources in animal feed (Lakshmi *et al*., 2013).

Given the environmental challenges posed by feather waste and the potential value of keratin degradation products, microbial degradation offers a viable and low-cost solution. The present study focuses on the isolation, screening and characterization of feather-degrading bacteria to advance sustainable feather waste management.

Material and Methods

Sample Collection

Four soil samples from chicken feather dumping sites and poultry waste decomposing soil were collected fromVeterinary College, Bengaluru and Central Poultry Development Organization Hesaraghatta Bengaluru. The samples were collected from a depth of 8-10 cm, using a spatula and transferred to pre-autoclaved sterile covers and stored at 4°C for further processing (Ray *et al*., 2021).

Isolation of Feather Degrading Bacteria

The collected samples were processed to isolate keratinolytic Bacteria. The homogenized feather waste decomposing soil sample of ten grams each was suspended in a 150 ml conical flask containing 90 ml sterile water and kept in a rotary shaker (180 rpm) for 2 hours, followed by allowing it to stand for 30 min at room temperature (Arati and Vendan, 2022). The suspensions were serially diluted up to 10^{-7}. Then, an aliquot of 100 ml of each dilution was spread on Nutrient agar (NA) plates and incubated at 30 ± 2°C for 24 hours. Morphologically distinct colonies were selected and purified by streaking on the agar plates De Oliveira *et al*. (2016). The bacterial isolates were maintained on nutrient agar slants and stored at 4°C.

Primary Screening

The isolated bacteria were first screened for extracellular protease production on skim milk agar (SMA) plates and incubated at 30°C for 24 hours. The plates were then examined for clear zones surrounding the colonies, indicating proteolytic enzyme production (Sekar *et al*., 2015).

Morphological Characterization of Poultry Feather Degrading Bacteria

Screened isolates were identified based on different colony characteristics such as Gram staining, cell shape, cell size, cell arrangement, colony, form, margin, surface, elevation and motility (Aneja, 2006).

Biochemical Characterization of Poultry Feather Degrading Bacteria

Biochemical tests are essential tools in microbiology for classifying bacteria and to understand their biology. The biochemical identification of poultry

feather degrading bacteria was carried out, such as catalase test, urease test, starch hydrolysis test, citrate utilization test, gelatin hydrolysis test, methyl red and Voges Proskauer's test (Aneja, 2006), to assess the metabolic characteristics of poultry feather-degrading bacteria and to understand their enzymatic abilities that helps to identify strains with potential biotechnological applications and enhances our knowledge of microbial ecology in waste management. By conducting these tests, we can confirm that the selected isolates possess the essential characteristics for efficient feather degradation, positioning them as optimal candidates for use in environmental sustainability and waste management initiatives.

Catalase Test

The presence of catalase enzyme was tested by transferring a bacteria culture onto separate glass slides. Hydrogen peroxide (30%) was added to the cells using a Pasteur pipette. The formation of bubbles indicated the presence of the catalase enzyme in the cells.

Casein Hydrolysis

Screened isolates were detected for hydrolytic enzymes. Skim milk agar plates were streaked with test culture and incubated for up to 3-7 at 30°C. After incubation, the clear zone around the colony against a black backdrop was considered positive for casein hydrolysis.

Starch Hydrolysis Test

The capacity of the isolates to hydrolyze starch in Petri plates containing starch agar was examined. Plates were inoculated with test cultures and incubated at 30°C for three days. Following incubation, the plates were filled with Lugol's iodine solution and allowed to stand for 15-20 minutes. For the test, the clear zone around the colony was considered positive.

Urease Test

The bacterial isolates were assessed for urease activity using urea broth as the testing medium. To begin, five milliliters of pre-sterilized urea broth, supplemented with phenol red as a pH indicator, were prepared. The bacterial cultures were inoculated into the broth and the tubes were incubated at 37°C for 24 to 48 hours for enzymatic activity. A positive result for urease activity was indicated by the development of a dark pink coloration in the medium, signifying an alkaline shift due to the hydrolysis of urea and the subsequent release of ammonia.

Citrate Utilization Test

The test was done to differentiate among enteric bacteria based on their ability to utilize citrate as a sole source of carbon. Citrate utilization depends on the presence of citrase enzyme produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. This test was performed by inoculating the microorganisms into Simmons's citrate agar slants and incubated at 28±2°C for 24 h. Change in colour from green to blue indicates a positive reaction for citrate utilization.

Gelatin Liquefaction Test

To the pre-sterilized nutrient gelatin deep tubes containing 13 g L^{-1} dehydrated Nutrient Broth and cultures were inoculated and tubes were incubated at 28±2°C for 24 hours. Following this, the tubes were kept in a refrigerator at 4°C for 30 minutes. The tubes with liquefaction of gelatin indicated positive for gelatinase enzyme.

Methyl Red Test

Sterilized glucose phosphate broth tubes were inoculated with the test culture and incubated at 28±2°C for 48h. After incubation five drops of Methyl red indicator were added to each tube and gently shaken. Red color production indicates isolate have the ability to produce and maintain stable acid end products from glucose fermentation and yellow color production indicates negative for the test.

Voges Proskauer's Test

Test cultures were inoculated and incubated at 37°C for 48h in the pre-sterilized glucose phosphate broth

tubes. After incubation ten drops of Baritt's reagent were added and gently shaken followed by the addition of ten drops of Baritt's reagent B. Development of pink colour in the broth was considered as positive for the test it denotes that the isolates have the ability to detect acetoin (acetyl methyl carbinol) a neutral end product of glucose fermentation in bacterial broth culture.

Results and Discussion

Isolation of Feather Degrading Bacteria

Soil samples were collected from two different places of feather enriched sites in Bengaluru *viz*. poultry farms, feather dumps, etc. The specifications of the samples were provided with the respective codes illustrating the names of the sites from where the sampling was carried out (Table 1). A total of 54 morphologically distinct and separate colonies were isolated (Plate 1) from feather enriched soil (FES), feather waste decomposing soil (FWD), poultry waste decomposing soil (PWDS) and partially degraded feather soil (PDFS) samples collected from veterinary college, Bangalore and central poultry development organization, Hesaraghatta Bengaluru. Among 54 isolates, 32 isolates were from Veterinary College Bengaluru and 22 isolates from CPDO Hesaraghatta Bengaluru (Fig. 1). Results obtained in the present study are in confirmity with the findings of Almahasheer *et al*., 2022 who isolated 42 poultry feather degrading bacterial isolates from different poultry farm waste samples. Vidyalakshmi, 2020 isolated 40 feather degrading bacterial isolates from 10 different places of feather enriched sites in and around Hoskote (tq), Bengaluru *viz*. poultry farms, feather dumps and agriculture fields. The isolates

Plate 1 : Isolated bacterial colonies on nutrient agar medium

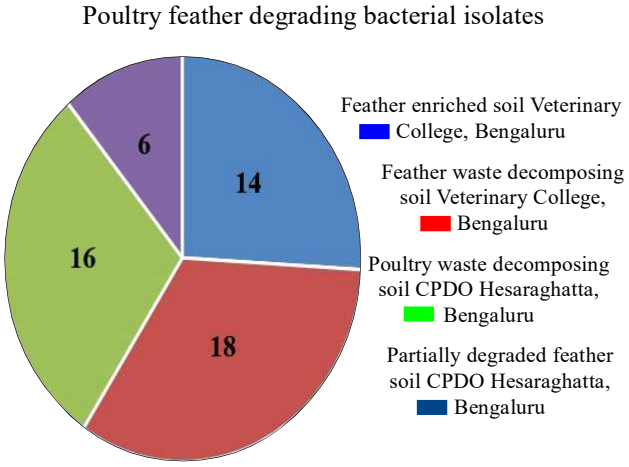

Fig. 1 : Number of feather degrading bacteria from different sites

Table 1

Sample collection for isolation of feather degrading bacteria

Sample	Location	Co-ordinates
Feather enriched soil (FES)	Poultry Science, Department Veterinary College, Bengaluru	13.0352° N, 77.583994° E
Feather waste decomposing soil (FWD)		
Poultry waste decomposing soil (PWDS)	CPDO, Hesaraghatta, Bengaluru	13.150953° N, 77.474649° E
Partially degraded feather soil (PDFS)		

obtained were further morphologically and biochemically analyzed.

Primary Screening of Poultry Feather Degrading Bacteria

Isolates were subjected to preliminary screening on skim milk agar medium (SMA). Among 54 bacterial isolates, only 16 isolates were positive for protease production by forming a clear zone around their colony on skim milk agar (SMA) medium (Plate 2), indicating the proteolytic nature of isolates. Among 16 isolates, FES8 (52.38±0.26mm), FES13 (49.72±0.29mm), PWDS11 (43.06±0.08mm), FES3 (42.42±0.25mm), FWD5 (38.75±0.23mm), FES16 (37.96±0.38mm) and FES5 (35.56±0.29mm) showed maximum clearance index. The lowest clearance index was recorded in FWD3 (25.02±0.41mm) and FES2 (25.55±0.2mm) (Fig. 2).

Plate 2 : Zone of clearance by feather degrading bacterial isolates on SMA

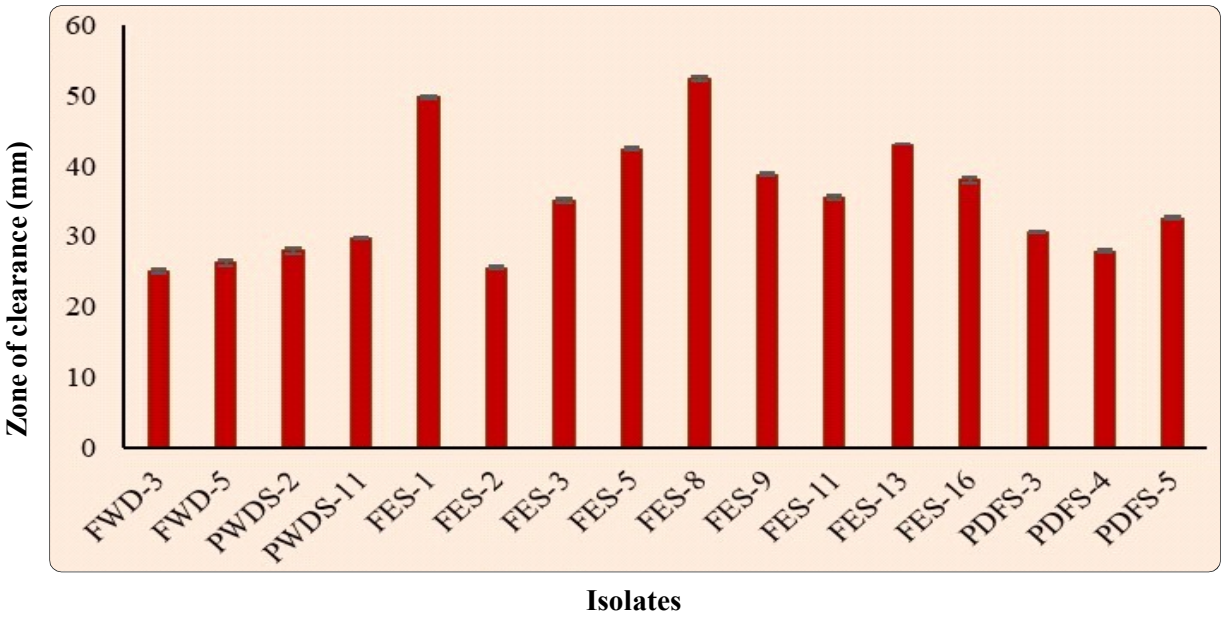

Fig. 2 : Clearance index by feather degrading bacterial isolates on SMA

Proteolytic activity is closely related to keratinase production because both enzymes belong to the broader category of proteases, which break down proteins. Proteolytic enzymes, such as those observed on skim milk agar (SMA) degrade various protein substrates. Keratinase is a specific type of protease that can degrade keratin, a tough, insoluble protein found in poultry feathers a keratin-rich material. It indicates that the bacteria can produce protease then particular bacteria can produce keratinase in keratin rich environment (Gopinath *et al*., 2015). Similarly, Vidhyalakshmi *et al,* 2022 mentioned, that out of 40 bacterial isolates, 15 isolates showed proteolytic activity by forming clear zones around their colonies on a skim milk agar (SMA) medium. Among these, isolates KDB2, KDB6 and KDB16 exhibited the highest clearance indices, indicating superior protease production. Similarly, fifteen isolates were screened for extracellular proteases on milk agar plates, seven isolates showed positive responses (De Oliviera *et al*., 2016).

Morphological Characterization

Morphological identification of the screened isolates showed that the colony morphology varied from circular to irregular/convex, white to pale yellow, surface smooth to rough, with entire, lobate and undulate margins, flat, raised and umbonate elevation. All are gram positive, rod shaped and motile. Further cell arrangement varied from single to pairs and chains and cell size varied from 0.3-3.6 µm (Table 2). It indicates that the cell walls of gram-positive microorganisms have a higher peptidoglycan and lower lipid content than gram-negative bacteria. Present results are supported by similar results obtained by Reyes *et al*. (2018), wherein poultry feather degrading bacteria colonies were white, flat to raised elevation, circular/irregular shape and gram positive. Lakshmi *et al*. (2013) identified two efficient keratinase producing isolates (BF11 and BF21) based on morphological, cultural and biochemical characteristics. BF11 and BF21 isolates were motile, aerobic, rod shaped cells and in biochemical characters, both organisms were positive for catalase activity and H_2S production.

Biochemical Characterization

All sixteen isolates tested positive for the catalase test, as evidenced by the formation of gas bubbles (Plate 3B). This reaction confirms the presence of the catalase enzyme, it decomposes hydrogen peroxide to water and oxygen, produced by bacteria that rely on oxygen for respiration. Consequently, all ten isolates possess the ability to respire (Swetlana and Jain, 2010).

All sixteen isolates were positive for casein hydrolysis by forming a clear zone around their colonies after incubation, indicating a positive result for the casein hydrolysis test. This demonstrates that seven isolates can degrade casein protein by protease activity. Proteases are enzymes that break down proteins into smaller peptides or amino acids and casein, being a protein, is one of the substrates that can be degraded by proteases (Swetlana and Jain, 2010).

The screened sixteen poultry feather degrading isolates were subjected to biochemical tests, the results are depicted in Table 3. From the sixteen isolates, eleven isolates (PWDS-2, PWDS-11, FWD-3, FWD-5, FES-1, FES-2, FES-3, FES-5, FES-8, FES-13 and FES-16) formed a clear zone around their colonies after the addition of Lugol's iodine solution, indicating their ability to hydrolyze starch (amylose and amylopectin) using the enzymes α-amylase and oligo-1, 6-glucosidase suggests metabolic versatility, as the bacterium can utilize both proteins (keratin) and carbohydrates (starch) as nutrient sources. (Plate 3A). In contrast, the remaining five isolates tested negative for starch hydrolysis (Swetlana and Jain, 2010) means the isolates preference or specialization for proteinaceous substrates (e.g., proteins like keratin or simpler sugars). Among sixteen screened poultry feather degrading isolates in this study, five isolates were positive namely FWD-3, PDFS-3, PDFS-4, PDFS-5 and FES-1 and ten isolates were negative for the urease test. Among sixteen isolates, five isolates changed broth color from orange to pink indicating that five bacteria were capable of hydrolyzing urea to produce ammonia and carbon dioxide (Swetlana and Jain, 2010).

TABLE 2

Morphological characteristics poultry feather degrading bacterial isolates

Isolate code	Gram reaction	Cell shape	Cell size	Cell arrangement	Colony	Form	Margin	Surface	Elevation	Motility
PWDS-2	+	Rod	0.3 × 2.1	Single	White	Circular	Entire	Smooth	Flat	Motile
PWDS-11	+	Rod	0.5 × 2	Chains	Pale Yellow	Circular	Entire	Smooth	Raised	Motile
FWD-3	+	Rod	0.3 × 2.4	Single	White	Irregular	Lobate	Rough	Umbonate	Motile
FWD-5	+	Rod	0.7 × 3	Single	Pale Yellow	Circular	Entire	Rough	Raised	Motile
PDFS-3	+	Rod	0.6 × 2.7	Pairs	White	Circular	Undulate	Smooth	Flat	Motile
PDFS-4	+	Rod	0.5 × 2.7	Chains	White	Circular	Raised	Smooth	Entire	Motile
PDFS-5	+	Rod	0.8 × 2.8	Single	White	Irregular	Lobate	Rough	Umbonate	Motile
FES-1	+	Rod	0.7 × 3.1	Pairs	White	Circular	Entire	Smooth	Raised	Motile
FES-2	+	Rod	0.5 × 2.2	Pairs	White	Circular	Entire	Smooth	Flat	Motile
FES-3	+	Rod	0.8 × 3.6	Chains	Pale Yellow	Circular/Convex	Entire	Rough	Raised	Motile
FES-5	+	Rod	0.62 × 3.5	Chains	White	Irregular	Undulate	Rough	Raised	Motile
FES-8	+	Rod	0.6 × 4	Chains	White	Circular/Convex	Entire	Rough	Raised	Motile
FES-9	+	Rod	0.5 × 2.9	Single	Pale Yellow	Circular	Entire	Smooth	Raised	Motile
FES-11	+	Rod	0.5 × 3	Single	Pale Yellow	Circular	Entire	Smooth	Raised	Motile
FES-13	+	Rod	0.8 × 3.5	Pairs	Off White	Circular	Entire	Smooth	Raised	Motile
FES-16	+	Rod	0.7 × 4	Pairs	White	Circular	Entire	Smooth	Raised	Motile

TABLE 3

Biochemical characteristics of poultry feather degrading bacterial isolates

Isolate code	Biochemical test								
	1	2	3	4	5	6	7	8	Probable genus
PWDS-2	+	+	+	-	-	+	+	-	*Bacillus* sp
PWDS-11	+	+	+	-	+	+	-	+	*Bacillus* sp
FWD-3	+	+	+	+	+	-	+	-	*Bacillus* sp
FWD-5	+	+	+	-	+	+	-	+	*Bacillus* sp
PDFS-3	+	+	-	+	+	-	+	-	*Bacillus* sp
PDFS-4	+	+	-	+	-	-	-	+	*Bacillus* sp
PDFS-5	+	+	-	+	-	-	-	+	*Bacillus* sp
FES-1	+	+	+	+	+	+	-	+	*Bacillus* sp
FES-2	+	+	+	-	+	+	+	-	*Bacillus* sp
FES-3	+	+	+	-	-	+	-	+	*Bacillus* sp
FES-5	+	+	+	-	+	+	-	+	*Bacillus* sp
FES-8	+	+	+	-	+	+	-	+	*Bacillus* sp
FES-9	+	+	-	-	+	-	-	+	*Bacillus* sp
FES-11	+	+	-	-	+	-	-	+	*Bacillus* sp
FES-13	+	+	+	-	+	+	-	+	*Bacillus* sp
FES-16	+	+	+	-	+	+	-	+	*Bacillus* sp

Catalase test, Casein hydrolysis, Starch hydrolysis, Urease test, Citrate utilization, Gelatin hydrolysis test, Methyl red test and Vogerproskauer test

Out of the sixteen screened isolates, twelve isolates showed a color change from green to blue on Simmons citrate agar after incubation namely PWDS-11, FWD-3, FWD-5, PDFS-3, PDFS-4, FES-1, FES-2, FES-5, FES-8, FES-9, FES-11, FES-13 and FES-16, indicating a positive result for the citrate utilization test (Plate 3C). This confirms their ability to use citrate as a sole source of energy along with keratin indicates adaptability to nutrient-diverse or nutrient-limited environments, such as soils or waste systems and also indicates potential ecological significance in both protein recycling (keratin) and organic acid utilization (citrate). In contrast, the remaining four isolates tested negative for citrate utilization because the isolates specialized in protein degradation, focus on keratin and potentially other proteinaceous substrates (Manirujjaman *et al*., 2016).

The screened sixteen poultry feather degrading isolates were subjected to a gelatin liquefaction test. Ten isolates tested positive for gelatin liquefaction (PWDS-2, PWDS-11, FWD-5, FES-1, FES-2, FES-3, FES-5, FES-8, FES-13 and FES-16), while six were negative. The positive results indicated that these ten isolates produce the enzyme gelatinase and it demonstrates a broad proteolytic capability, able to degrade both keratin and gelatin, thrive in environments rich in a variety of proteins and has potential applications in industries requiring enzymes for breaking down diverse proteins (Swetlana and Jain, 2010), enabling them to liquefy gelatin (Plate 3E). The six negative isolates do not produce gelatinase or produce it in insufficient amounts, resulting in no liquefaction of the gelatin medium.Among sixteen isolates, four isolates namely PWDS-2, FWD-3,

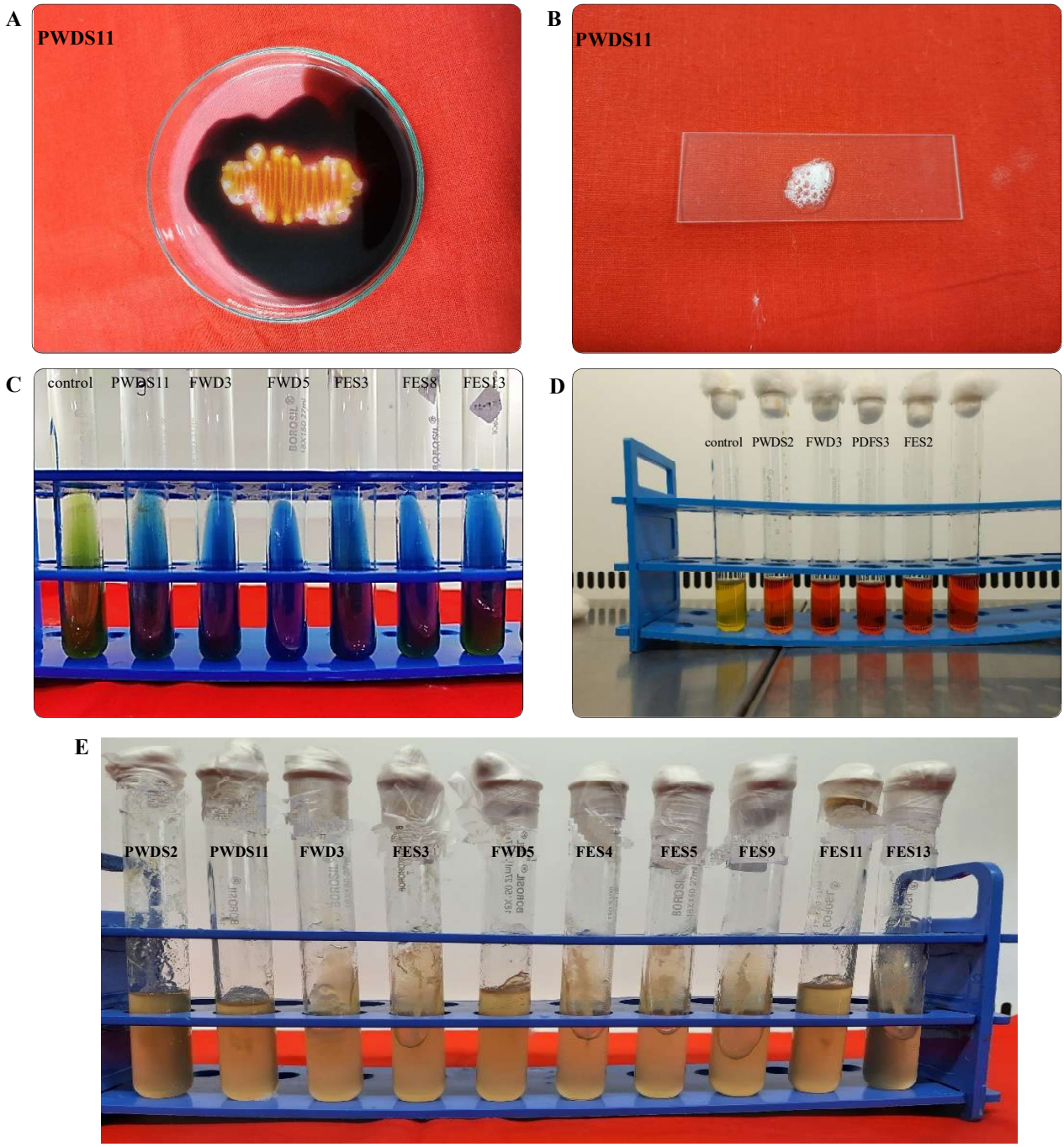

Plate 3 : Biochemical characterization of poultry feather degrading organisms; A) Starch hydrolysis test; B) Catalase test; C) Citrate utilization test; D) Gelatin hydrolysis test and E) Methyl red test

PDFS-3 and FES-2 produced a red color after adding the methyl red indicator which indicates positive for the methyl red test and can produce and maintain stable acid end products from glucose fermentation (Manirujjaman *et al*., 2016), whereas the remaining twelve isolates were negative for the methyl red test indicating that isolates relies on neutral to alkaline environments (Plate 3D).

Out of sixteen screened isolates, twelve isolates were positive for the Voger Proskauer test namely PWDS-11, FWD-5, PDFS-4, PDFS-5, FES-1,

FES-3, FES-5, FES-8, FES-9, FES-11, FES-13 and FES-16 that indicates that the bacteria are capable of producing acetoin from glucose by neutral fermentation, which is then converted into 2, 3-butanediol (Manirujjaman *et al*., 2016). The remaining 4 isolates were negative, suggesting that they either do not produce acetoin or follow a different fermentation pathway, such as mixed-acid fermentation, which does not lead to acetoin production, possibly favoring acidic fermentation pathways or other metabolic routes.

Based on the morphological (Table 2) and biochemical results (Table 3) together with the reference of 'Berger's Manual of Systematic Bacteriology, the strain were preliminarily confirmed as *Bacillus* sp. Similarly, Sun *et al*. (2021) screened for poultry feather degrading bacteria among 15 isolates JYL isolate was Gram-positive, slender rod shaped with central spore and positive to catalase, oxidase, protease, Voges-Proskauer reaction, methyl red test and gelatin liquefaction and negative for lipase, nitrate reduction, indol production, identified as *Bacillus pumilus* JYL according to its morphology, biochemical characteristics and 16S rRNA.

The results are in confirmation with the results obtained by Manirujjaman *et al*. (2016) where they isolated feather degrading soil from chicken farms. The isolate SAR1 showed higher keratinase production and it was positive for citrate utilization and gelatin liquefaction and negative for starch hydrolysis, methyl red and Voger Proskauer test. Similarly, Vidhyalakshmi *et al*. (2020) isolated and screened ten feather degrading bacteria from feather enriched soil samples. These isolates were biochemically characterized and were positive for methyl red test, voges-proskauer test, citrate utilization, catalase and protease production.

The objective of this study was to isolate and characterize poultry feather-degrading bacteria. A total of fifty-four distinct bacteria were isolated and screened for proteolytic activity among fifty-four, sixteen isolates showed proteolytic activity on SMA, primary screening results indicated that sixteen isolates have the ability to degrade protein and they have the ability to degrade keratin protein. The morphological and biochemical analysis confirmed screened sixteen isolates were Gram-positive, catalase-positive and capable of gelatin liquefaction and casein hydrolysis, with varying results for other biochemical tests. Further analysis of keratinase production by these isolates is essential for advancing sustainable keratin waste management.This research highlights the potential of these bacterial strains for biotechnological applications in feather degradation and waste management, which is crucial due to the environmental pollution caused by keratin-rich wastes from poultry industries.

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