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BACTERIA ISOLATED FROM GANGA RIVER SOIL EXHIBITING KERATINOLYTIC AND ANTIMICROBIAL ACTIVITY

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Abstract:
The present study focuses on the isolation and characterization of keratinase-producing bacteria from Ganga River soil exhibiting keratinolytic and antimicrobial activity. Keratin, a highly stable fibrous structural protein found in feathers, hair, and other keratinous wastes, is resistant to degradation due to strong disulfide bonds and hydrophobic interactions. Accumulation of keratin-rich wastes, particularly poultry feathers, poses significant environmental challenges. Soil samples collected from the Ganga River region were processed through serial dilution and cultured on nutrient agar for microbial isolation. Keratinase production was induced using feather-based fermentation media under optimized conditions of pH and temperature. Enzyme extraction was carried out through filtration and centrifugation, and keratin degradation was confirmed by Ninhydrin and Nessler's reagent tests indicating amino acid release and ammonia production. Proteolytic activity was further validated using skim milk agar assay. The gradual increase in pH during incubation confirmed active keratin degradation. The findings suggest that bacteria isolated from Ganga River soil possess efficient keratinolytic potential and may serve as promising candidates for sustainable bioconversion of keratinous waste and potential antimicrobial applications.

Keywords: *Keratinase, Ganga River Soil, Keratin Degradation, Antimicrobial Activity,skim milk activity*

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INTRODUCTION:

Bacteria are a type of prokaryotic microorganism that reproduces by binary fission and does not possess essentially no nuclear membrane. Their earliest life forms were found in a range of environments, including soils, water sources and air, food sources within the human body as well as in the land and sea. The majority of bacteria are advantageous and have significant impacts on nutrient cycling and nitrogen fixation, contributing to ecological sustainability and agricultural productivity. The nitrogenous bases of bacterial DNA are adenine, thymine (cis), cytosine (5C/Hg) and guanine (dimethyl cellulose). While many bacteria are innate and do not cause any disease, some are pathogenic and can cause diseases such as cholera, tuberculosis, typhoid, anthrax, measles, and leprosy. These microorganisms cannot be killed by their environment. A significant global health issue remains the respiratory infections caused by bacteria.?... Although antibiotics are effective against bacteria, antimicrobial resistance has risen due to their misuse. The development of bacterial disease is associated with infectivity, pathogenicity, and virulence, as well as host factors like immunity, age, nutrition, or environmental exposure. Antibiotics are also important. Contaminated food, water, air or direct contact, or vectors) to spread. The asexual reproduction of bacteria involves binary fission, leading to the formation of two identical daughter cells. Certain species develop resistant endospores that endure harsh heat, chemicals, and desiccation under unfavorable conditions. Whenever conditions become conducive, spores establish and grow into active cells.[1]

Keratin:

Feathers, hair, wool, nails and other hard tissues are coated with keratin-like fibrous structural protein that protects against environmental damage. Strong disulfide, hydrogen, and hydrophobic interactions make it unbreakable in water and impossible to decompose. Because of this structure, it is difficult for common proteolytic enzymes like trypsin and peppsent to break down keratin. Among the abundant renewable biopolymers in nature, keratin is second only to cellulose, but it is also among the most abundant after lignin, which are all polysaccharides. Industries produce a significant amount of waste products, particularly poultry feathers, which are high in keratin levels. These wastes have the potential to harm soil, water and air quality if left untreated. Nevertheless, they offer an efficient and inexpensive supply of carbon, nitrogen, sulfur, amino acids, animal feed ,and fertilizers. The degradation and extraction of keratin are more difficult than other natural polymers. Physical and chemical methods demand significant energy and have the potential to deplete crucial amino acids. Or. On the other hand, bacterial and fungal degradation is an efficient and environmentally friendly means of organisms degrading their environment. The conversion of keratin into value-added products through microorganisms is the primary reason for considering bioconversion as a sustainable waste management approach.-keratin and tar, which are found in mammals and birds and reptilian tissues respectively, are two subcategories of keratogenesis. Cyanophore bonds, hydrophobic interactions, and hydrogen bonding are responsible for its structural stability.[2]

Isolation of Keratinase-Producing Microorganisms:

There are numerous microorganisms in nature that produce keratinase and can be eliminated from soil, water or even waste with high levels of the enzyme. Keratinases can be produced by bacterial, fungal, and actinobacteria bacteria, which also use it as a carbon and nitrogen source. Keratinase is typically stimulated by the presence of keratin or other substrate-containing molecules in culture media, and it can be produced through an inducible pathway.The process of isolation usually involves collecting samples, analyzing them, classifying them into strains, and conducting their characterization. The collection of environmental samples involves the use of keratin-rich substances, such as soil or water contaminated with poultry waste. Feathers serve as a screening substrate, while degradation is monitored through structural changes and protein release. The appropriate culture media must be selected based on the type of bacteria or fungi that are being enriched. The study objective requires the optimization of screening conditions, including incubation time and temperature. Isolating the thermally stable keratinase producers at higher temperatures is a typical application. To maintain microbial diversity, it is possible to decrease enrichment processes when dealing with diverse bacterial groups.[3]

MATERIALS AND METHODS:

Soil sample:

Fig1:Ganga soil

To capture the best soil, start with the uppermost 5-10 cm of the soil as this is typically where microbial life thrives. Avoid contamination by

using sterile equipment (spatula). Apply the sample to the motor and pestle to triturate until smooth, then transfer the resulting soil into sterile bags or containers and transport in aseptic conditions. To collect soil samples from various locations and capture microbial diversity.

Preparation of Soil Suspension; Weigh 1g of soil and transfer it into a sterile container (e g; conical flask). Add 9 mL of sterile saline Solution (0.85% NACI) or distilled water to the soil. Shake or vortex the mixture vigorously for 5-10 minutes to dislodge microorganisms from the soil particles. Allow the suspension to settle for a few minutes before further processing. To release microorganisms from the soil particles into a liquid medium for easier enumeration and isolation.

Preparation of agar media :

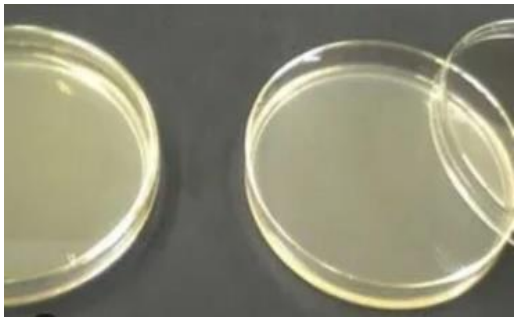

Fig 2:Nutrient agar plates

Procedure:

Determine the exact weight of this: 0.5 grams of peptone, 0.3 grams beef extract; 0.5% sodium chloride; 2 grams agar; and 100 milliliters of distilled water. Add 1 liter of pure, distilled water to the ingredients and pour it into a beaker. Mix the ingredients and dissolve them completely by stirring. Alternatively, warm up the mixture to help dissolve (but not boil). Keep stirring to prevent the agar from sticking. Repeat. 2. Use a pH scale or pH paper to determine the chemical equilibrium of the solution. Agar for nutrient agar has ph of about (7.0, 0.2).". To elevate the pH when it is acidic, introduce a solution of sodium hydroxide (NaOH) in diluted form. Add a small amount of hydrochloric acid (HCl) solution to lower the pH level if it is too high (alkaline). After adjusting the pH, stir thoroughly. Autoclaving at 121°C and 15 psi for 15-20 minutes is the recommended method for sterilizing the medium. All bacterial species are eradicated from the medium, making it suitable for inoculation of bacteria. Without access to an autoclave, it is feasible to use a pressure cooker for cooking at 121°C and 15-psi for approximately 15-20 minutes. Following autoclaving, the substance should be left to cool down until it reaches temperatures of 45-50°C, which is acceptable but still liquid. Be careful not to let the agar cool too much, as this will result in it solidifying inside. Put the nutrient agar in sterile dishes and cool it. Refill each petri dish with a third of its full size. Let the agar cool and keep it in an unchanging state. This usually takes between 30 and 1 hour. Serial dilution of the soil sample was performed under aseptic conditions to reduce microbial load. A series of sterile test tubes containing 9 mL of sterile distilled water were prepared. One millilitre of the soil suspension was transferred into the first tube to obtain a 1:10 dilution. Subsequently, 1 mL from the first dilution was transferred into the second tube to achieve a 1:100 dilution. This stepwise transfer of 1 mL into fresh tubes containing 9 mL sterile water was continued sequentially to obtain dilutions of 1:1000, 1:10,000, and 1:100,000, ensuring thorough mixing at each stage. Following serial dilution, 100 µL from each dilution was aseptically spread onto the surface of sterile agar plates using a sterile spreader to ensure uniform distribution. The plates containing garden soil samples were labelled as GS-1, GS-2, GS-3, GS-4, and GS-5. Different selective and differential media were used depending on the target organisms, including Nutrient agar for general bacterial growth, MacConkey agar for Gram-negative bacteria, Mannitol Salt Agar (MSA) for Staphylococcus species, and Actinomycete Isolation Agar (AIA) for isolating soil actinomycetes. The inoculated plates were incubated at 25–37°C for 24–48 hours to allow colony development. [4]

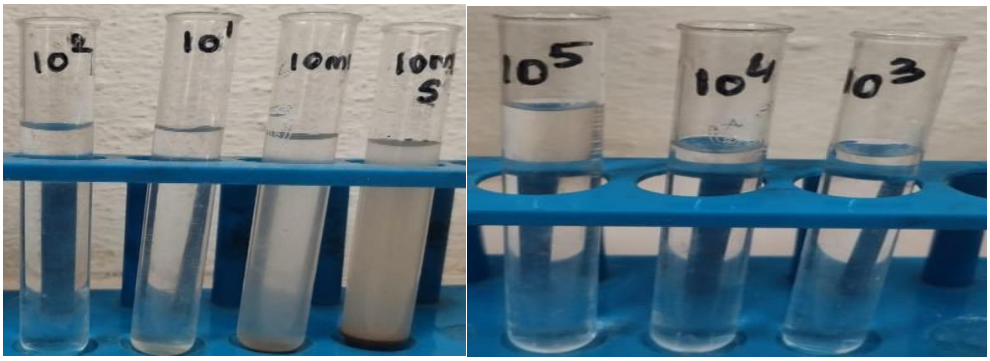

Fig 3: Serial Dilution of Soil Sample (10 to 10^{-5})

Isolation and identification : Chicken feathers, feather meal, or hair waste are typically used as the primary carbon and nitrogen source for submerged fermentation to produce keratinase. The basal medium is typically chosen from Bacillus species, with a pH of pH 7.0–7.5 and an incubation period of 30–37°C for 48–72 hours. The production medium is usually composed of chicken feathers, either raw or powdered, with mineral salts like K2HPO4 (0.3-1.4 g), KH2PO4, (0.4-0.7 ng), MgCl26H 2O or MmgSO4 (0.15 g), and NaCl (0.5 g) added. A growth supplement of 0.1 g is made up of yeast extract, which can be used to add vitamins and other nutrients. Prior to inoculation, the medium is thoroughly mixed, pH adjusted, and sterilized for contamination-free disposal. After cooling, the sterile medium is inoculated with a keratinase-producing microorganism under aseptic conditions and then incubated with appropriate agitation and fervent heating to ensure optimal nutrient distribution and oxygen transfer. The degradation of keratin is done by microorganisms during active growth, which leads to the secretion of an extracellular enzyme called kerase. After incubation, the culture broth is filtered and centrifuged to eliminate any leftover substrates and cells, producing a transparent supernatant containing crude keratinase. The enzyme can either be purified for enhanced stability or used in industrial settings, and its activity is measured using specific enzymatic methods. [5]

Fig 5: Keratin Sample (Feather)

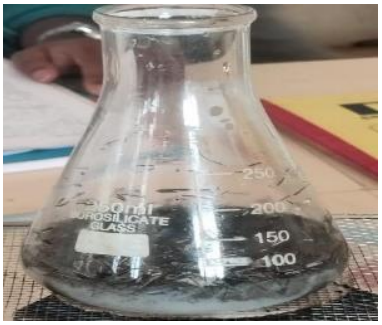

Fig 6: Preparation Of Culture Medium

Following incubation for the creation of keratinase, the fermentation broth comprising of microorganism cells and partially degraded keratin substrates was collected to recover enzymes. The broth was filtered using sterile muslin cloth or Whatman filter paper to eliminate large insoluble particles and obtain a homogeneous liquid sample. The filter was centrifuged at 5,000–10,000 g for 10–30 minutes at 4 °C to avoid the loss of enzyme denaturation.

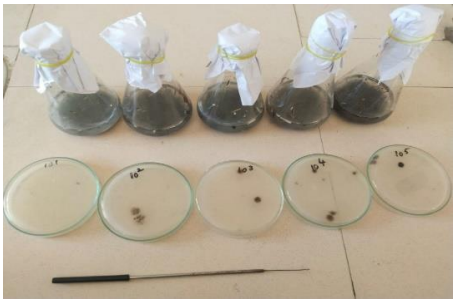

Fig 7: Inoculation of Culture into Broth Medium

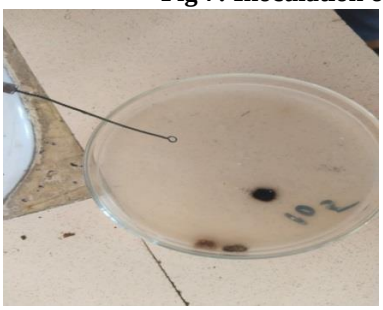

Fig 8: Loop Full of Culture

Fig 9: Conical Flasks in Orbital Shaker

Centrifugation resulted in the formation of a pellet at the bottom, which contained residual keratin materials and cells. The clear supernatant that contained extracellular kerase was collected with caution as the crude enzyme extract for additional analysis. [6]

Fig 10 : Rotor Centrifuge

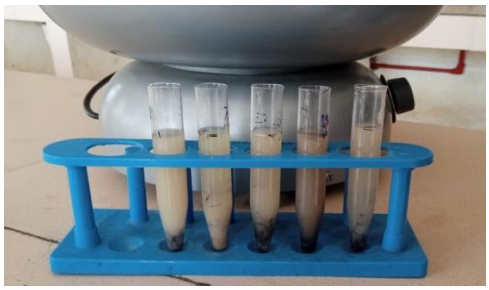

Fig 11:Centrifuged Samples

Identification tests:

1.Ninhydrintest:

The ninhydrin method was employed to determine the amino acid content of the feather hydrolysate. The hydrolysate was mixed with 150 L of phosphate citrate buffer (pH 5.0) in a test tube, and then the mixture was vortexed for 1 min. A solution rich in 0.3% ninhydrin (1:1) was added to it, followed by ice-cream (15 min). The development of colour was followed by the cooling of the tubes, which stopped the reaction. Repeated for all concentrations. Using samples of amino acids, it was shown in FIG how the ninhydrin reactivity leads to the color Ruhemann's
Purple. [7]

Fig 12:Interpretation Ninhydrin Test

Fig 13: Clear Purple Colour Ring Formation

2.Nesslersreagent:

Principle:

The reaction between ammonia (NH3) or ammonum ions (NA) and Nessler's reagent (K2HI4) produces a complex that changes from yellow to brown in an alkaline solution of potassium tetraiodolarity. The more ammonia I add, the darker color it produces.

Reagents & Equipment

Nessler's reagent (potassium tetraiodomercurate(II) + strong base),Distilled water, Clean test tubes, Pipettes or micropipettes.pH adjustment reagents (such as NaOH) can be used. Safety gear, gloves, and lab coat (containing mercury in Nessler's reagent) are required.

Test Procedure;

1. Sample Preparation:

Obtain the test solution in a clean tube, measuring from 5 to 10 milliliters.

2. Alkaline Adjustment (optional but recommended):

To enhance reaction sensitivity, a few drops of 5% NaOH in non-alkaline solution can be added to achieve optimum pH (>10).

3. Adding Reagent:

Apply Nessler's reagent to the test solution with a mixture of 2-3 Ml [8]

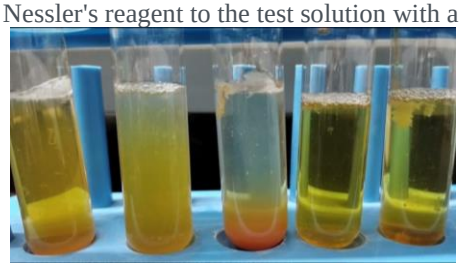

Fig 14: interpretation of Nessler's test Change

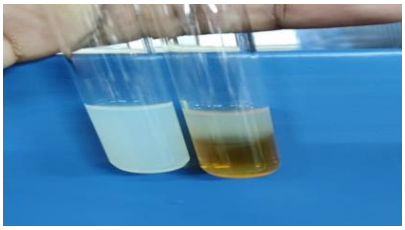

Fig 15: Interpretation of Nessler's Test Showing Colour

3.P^{H} TEST: changes were measured during incubation at room temperature for 0, 24, 48, and 72 hours to track the degradation of proteins. Moreover, following every interval, the culture was centrifuged and then a pH value was measured using an appropriate pH indicator mounted on ice. The absence of microbial inoculum did not affect the control of the substrate, as non-biological changes were excluded. The test samples showed a gradual rise in pH relative to the control, which indicated active substrate utilization. Ammonia release was suggested by the increase in alkalinity, which indicated the degradation of amino acids and confirmed protein (keratin ability). [9]

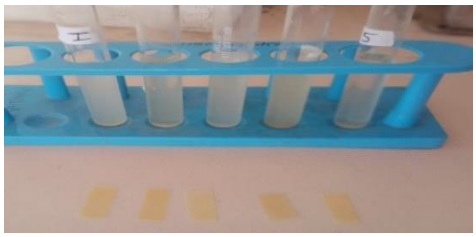

Fig 16:Assessment of Alkalinity/Acidity Using pH

4.Skim Milk Agar Test for Proteolytic Activity

Medium Preparation:Peptone (5 g), beef/yeast extract (3 g), and agar (15 g) were dissolved in 900 mL distilled water. The pH was adjusted to 7.0 and sterilized at 121°C for 15 minutes. After cooling to 50–55°C, sterile 10% skim milk suspension was aseptically added, mixed, poured into Petri plates, allowed to solidify, and stored at 4°C.

Inoculation and Incubation: Test organisms were streaked or spot inoculated on the plates. Bacillus subtilis or Proteus spp. served as positive controls, while Escherichia coli or sterile media served as negative controls. Plates were incubated for 24–48 hours at suitable temperature.

Fig 17:Nutriant Media for Skim Milk Test

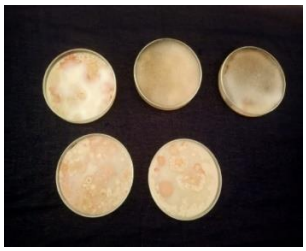

Fig 18: Proteolytic Activity Detection

Observation and Interpretation: A clear halo zone around colonies indicates casein hydrolysis (positive proteolytic activity), whereas absence of a clear zone indicates negative proteolytic activity [10].

Detection of Antibiotic Activity :

To assess their susceptibility to antibiotics, the disk diffusion (Kirby-Bauer) method was employed to evaluate certain keratinolytic isolates. A standardized bacteria inoculum was applied to Mueller–Hinton agar plates, which were then subjected to aseptic procedures to be prepared with commercial antibiotic discs. For a period of 18-24 hours, the plates were incubated at the appropriate temperature. Incubation-based testing was performed to measure the diameter of the inhibition zones surrounding each disc. A zone that was not exposed to light displayed sensitivity, while an empty zone showed no resistance. To determine the safety of the isolates for industrial and environmental use, they interpreted their results in accordance with standard antibiotic susceptibility guidelines. [11, 12, 13]

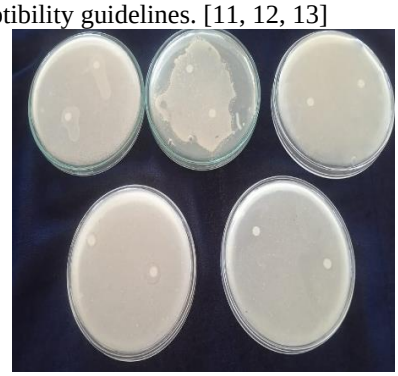

Fig 19: Disk Method for Antibiotic Susceptibility Testing

Fig 20: Zone of inhibition

RESULTS AND DISCUSSION:

ANTIBOTIC ACTIVITY:

S. NO	ISOLATES	CONC OF ANTIBIOTIC [µg]	ZONE OF INHIBITION [mm]
1.	GS-1	20	0
2.	GS-2	20	1.25
3.	GS-3	20	0
4.	GS-4	20	1.5
5.	GS-5	20	1.5

KERATINIZATION IDENTIFICATION TESTS:

CONCLUSION:

S.NO	METHOD	Gs-1	Gs-2	Gs-3	Gs-4	Gs-5
1.	Ninhydrin test	-	+++	++	++++	+
2.	Nessler's reagent test	+	+++	+++	+++	-
3.	P^H change monitoring	+	++	++	+	-
4.	Skim-milk agar test	+	+++	+	++++	-

The study successfully demonstrated the isolation and identification of keratinolytic bacteria from soil using standard microbiological techniques, including serial dilution, plating, Gram staining, and biochemical screening. Soil was confirmed as a rich source of enzyme-producing microorganisms, particularly species of Bacillus and Streptomyces, which are well known for their ability to secrete extracellular enzymes. Keratin is a highly stable and resistant structural protein that requires specialized enzymes such as keratinases for effective degradation. The isolated bacterial strains exhibited keratinolytic activity, verified through qualitative assays including skim milk agar, ninhydrin test, Nessler's reagent test, and pH change monitoring. The appearance of clear zones around bacterial colonies confirmed extracellular keratinase production and the ability to hydrolyze keratin substrates.

Antibiotic susceptibility testing further revealed variations in resistance patterns among the isolates, as indicated by differences in zones of inhibition. This assessment highlights the importance of evaluating biosafety and resistance characteristics before considering industrial applications. The findings underline the environmental and industrial importance of keratinase-producing bacteria. Microbial degradation of keratin-rich wastes such as feathers, hair, and wool provides an eco-friendly and sustainable alternative to chemical treatments. Keratinases have potential applications in waste management, animal feed production, leather processing, textile treatment, pharmaceuticals and cosmetics. In conclusion, soil-derived keratinolytic bacteria represent valuable biological resources. Future research should focus on molecular characterization, enzyme purification, optimization of fermentation conditions, and large-scale production to enhance their industrial applicability and support sustainable biotechnological advancement.

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