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Isolation, Screening, Characterization and Biological Studies of Chitinase Producing Bacteria from Godavari Riverbank Soil Marathwada, Maharashtra

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Abstract
Chitinase-producing bacteria have garnered significant interest due to their potential applications in agriculture, biotechnology, and waste management. This study focused on the isolation, screening, characterization and biological studies of chitinolytic bacteria from soil samples collected along riverbanks. Bacteria morphologically distinct bacterial isolates were obtained and screened for chitinase activity using colloidal chitin as a substrate. Optimal chitinase production was observed at 45 °C, neutral pH, and 96 hours of incubation. Chitinase bacteria contains Gram staining found that gram positive bacteria. Chitinase bacteria producing bacterial culture showed positive results for chitinase, catalase, and gelatin hydrolysis tests, confirming its strong enzymatic potential. The chitinase activity demonstrated the bacterium's ability to degrade chitin, while catalase activity indicated its capacity to detoxify reactive oxygen species. Gelatin hydrolysis confirmed the presence of proteolytic enzymes, suggesting its capability to degrade protein substrates. Antioxidant analysis of the culture supernatant showed significant ferric-reducing activity in the FRAP assay and free radical scavenging in the DPPH assay. Both assays indicated the presence of bioactive compounds with strong antioxidant properties. These results highlight the strain's multifunctional potential for applications in biocontrol, waste degradation, and antioxidant bio-product development.
Keywords :- *Chitinase Producing Bacteria, Chitinase, Grahm Staining, Antioxidant, Catalase*

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I. Introduction:

Chitin is a tough and major biomolecular polymer, second only to cellulose in abundance. It is commonly found in the cell walls of algae and fungi. Chitin is also a key structural component in the exoskeletons of arthropods, such as crabs, lobsters, shrimp, and insects [1,2]. This natural polymer is composed of β-1,4-linked N-acetylglucosamine units. Its robust structure makes it resistant to degradation under normal conditions. However, chitin can be broken down by a specific group of enzymes known as chitinases. These enzymes are widely distributed across different organisms, including plants, bacteria, fungi, and actinomycetes. In plants, chitinases serve a crucial role in defense against pathogenic attacks. They break down the chitin in fungal cell walls, preventing infection and disease spread [3]. In the environment, chitinases help degrade chitinous waste. This is particularly important in managing the large volumes of waste produced by the seafood industry [3]. Shrimp shells are considered a major source of industrial chitin. Degrading this waste helps maintain the carbon-nitrogen balance in ecosystems. The presence of chitinolytic microorganisms in soil indicates the availability of chitin. These microbes naturally recycle chitin and contribute to soil health [4]. Chitinases have several important biotechnological applications. They are used in mosquito control, acting against chitin-containing insect pests. They also serve as biocontrol agents against fungal pathogens in agriculture. Another application is in the isolation of fungal protoplasts for genetic studies. Chitinases are useful in producing single-cell proteins for food and feed industries. They also help generate bioactive growth factors with therapeutic potential. As environmental concerns rise, the demand for microbial chitinase production has grown. This production not only reduces environmental pollution but also creates value-added products [5,6]. It represents a sustainable solution to industrial waste problems. Many microbial genomes have been found to encode chitinolytic enzymes. Despite this, further research is necessary [7,8]. Especially, studies are lacking on microbes that can utilize insoluble chitin directly as a carbon source. Expanding research in this area could open new frontiers in biotechnology. Chitin and its degrading enzymes thus hold significant promise for both environmental and industrial advancements.

II. Materials And Methods:

1.Soil sample collection and processing:

The riverbank soil samples were collected from Ramsgoan, Maharashtra India. It has a Lat 19.38543° Long 75.923622°. The riverbank soil samples were collected in clean polyethylene bags and transported to the Biochemistry laboratory, department of Biochemistry, Model collage of Ghansawangi and the soil samples were serially diluted from 10^{-1} to 10^{-7}.

2.Media preparation, inoculation, and incubation:

The growth media for isolation of chitinase producing bacteria was prepared as per above mentioned, containing gram/Litre: colloidal chitin 1%(W/V): K_2HPO_4 6.0, Na_2HPO_4 1, KH_2PO_4 3, NH_4Cl, 0.5, $MgSO_4.7H_2O$ 0.12, yeast extract 0.05 ,NaCl, 0.5, agar 15 and at (pH 7) and autoclaved at 121 ^{0}C for 15 min and added with Fluconazole at a conc. of 250 mg/mL to controlled fungal contamination The autoclaved medium was poured

into sterilized plates and cooled to room temperature and inoculated with 100 µ L of the 10^{-4} to 10^{-7} dilutions of soil sample by using pour plating techniques. The plates was incubated at 37^{0}C for four days. The chitin-degrading bacteria were selected based on clean diameter after 96 h of incubation and further screening of chitinase producing bacteria was done for maximum enzyme activity in chitin broth media [9].

3. Colloidal chitin preparation:

Colloidal Chitin 1 g of it was taken in mortar and pestle, with addition of 5 mL of acetone mixture was ground for 10 min. Then concentrated HCl 40 mL was added to the ground chitin and agitated for 4 h to dissolve the chitin, and placed at 4 ^{0}C 12 hour. Chitin solution was filtered and the filtrate was made up to 250 mL by using 50 % ethanol with constant stirring. The solution was centrifuged at 10,000 rpm for 25 min. The ppt of chitin obtained were several washed distilled waters until neutral pH. Distilled water was added to form 2 % colloidal chitin and this stock solution was stored at 4 ^{0}C until use [10].

4.Chitinase enzyme assay:

Chitinolytic activity was estimated by 3,5 dinitrosalicylic acid (DNS) method using colloidal chitin as a substrate according to the method described by Divarta with some modification. Enzyme solution (1.0 mL) was allowed to react with 1.0 mL of 0.5 % colloidal chitin in (1 mL) of 0.1 M citrate buffer (pH 7.0) for 30 min, the mixture was incubated at 37 ^{0}C in a shaking water bath. After incubation, the reaction was stopped by adding 2 mL of DNS reagent and heating it for 10 min in a boiling water bath. After cooling, the colour solution was centrifuged at room temperature for 10 min at a speed of 10,000 rpm and the supernatant's absorbance was measured at 540 nm in comparison to the control. One unit of chitinase activity was defined as the amount of enzyme that liberates of reducing sugar per min per ml [11].

5.Microorganism Gram staining:

Using a sterile inoculating loop, transfer a small quantity of the bacterial sample onto a clean glass slide. If the bacteria are grown on solid media, add a drop of water to the slide and mix the bacteria to form a thin, even smear. Allow the smear to air dry completely. Fix the bacteria to the slide by gently passing it through a flame 2 to 3 times, keeping the smear side facing up. Completely cover the dried smear with crystal violet stain and let it stand for approximately one minute. Rinse the slide carefully with distilled water. Flood the smear with iodine, which helps the primary stain bind more firmly to the bacterial cell wall, and leave it on for one minute. Gently rinse the slide again with distilled water. Hold the slide at an angle and apply a few drops of alcohol or acetone-alcohol to wash off the excess stain. Continue this step until the purple dye no longer runs off (typically 10–30 seconds). Immediately rinse the slide with distilled water to stop the decolorization. Cover the smear with safranin, the secondary stain, and allow it to sit for 30 to 60 seconds. Rinse thoroughly with distilled water, carefully blot dry with bibulous paper, and observe under a microscope [12].

6.Reducing Sugar test:

Estimation of reducing sugar by DNSA method. Take different volumes of glucose standard and dilute each to 1 mL with distilled water in separate test tubes, supernatant of centrifuged culture. Label one tube as blank (with 1 mL distilled water only). Add DNSA reagent: Add 3 mL of DNSA reagent to each test tube (including the blank and sample tubes). Heat the mixture: Place all tubes in a boiling water bath for 5–10 minutes. The solution will develop a reddish-orange color depending on the sugar concentration. Cool and stabilize: Cool the tubes to room temperature [13].

7.Gelatin hydrolysis test:

To prepare gelatin medium for 100 mL, mix 0.5 g of peptone, 0.3 g of beef extract, and 12–15 g of gelatin in 100 mL of distilled or deionized water, heating gently until fully dissolved. Adjust the pH to 6.8–7.0 and sterilize the medium by autoclaving at 121^{0}C (15 psi) for 15 minutes. Dispense 2–3 mL of the medium into 13 × 100 mm culture tubes, or 5–7 mL per tube if preparing stab tubes, and allow it to solidify vertically. After cooling in an upright position, store the medium at 2–8^{0}C and use before its expiration date. Discard any tubes showing contamination, discoloration, drying, or deterioration. For testing, inoculate the medium using a sterile inoculating needle by stabbing the center of the tube about two-thirds deep, or streak the organism on the surface if using gelatin plates. Incubate the inoculated tubes at 20–25^{0}C for 5–7 days, avoiding 370C to prevent gelatin melting and false positives. After incubation, refrigerate or place the tubes in an ice bath for 15–30 minutes, then tilt the tubes to check for liquefaction. Since gelatin melts at 28^{0}C and above, true gelatinase activity is confirmed if the medium remains liquid after chilling, indicating hydrolysis by gelatinase. In contrast, uninoculated control tubes should remain solid under the same conditions [14].

8.Catalase test:

For routine testing of aerobes, commercially available 3% hydrogen peroxide is used and should be stored refrigerated in a dark bottle. For identifying anaerobic bacteria, a 15% H O solution is necessary. In this context, the catalase test is used to differentiate aerotolerant strains of Clostridium, which are catalase-negative, from Bacillus species, which are catalase-positive. The superoxol catalase test, used for the presumptive speciation of certain Neisseria organisms, requires a different concentration of H O . There are many applications and variations of the catalase test, including the slide or drop method, the tube method, the semiquantitative catalase test for identifying Mycobacterium tuberculosis, the heat-stable catalase test for differentiating Mycobacterium species, and the capillary tube and cover slip method, which are primarily used for differentiating Staphylococci and Streptococci. In the tube method, add 4 to 5 drops of 3% H O to a 12 × 75 mm test tube. Using a wooden applicator stick, collect a small amount of the organism from a well-isolated 18 to 24 hour colony and place it into the test tube, avoiding the pickup of agar. This is particularly important if the colony was grown on agar containing red blood cells, as the carryover of red blood cells may cause a false-positive reaction. Place the tube against a dark background and observe for immediate bubble formation, indicating the release of oxygen and water. A positive reaction is characterized by immediate effervescence (bubble formation) at the end of the wooden applicator stick [15].

9. Antioxidant study by DPPH assay:

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a widely used method to evaluate the antioxidant potential of various substances. The principle of the assay is based on the reduction of DPPH, a stable free radical that exhibits a deep violet color in solution, which turns pale yellow when reduced by antioxidants such as Gallic acid or Quercetin. The decrease in color corresponds directly to the antioxidant capacity of the tested sample. For the assay, a fresh DPPH reagent is prepared by dissolving 8 mg of DPPH in 100 mL of methanol to obtain a 0.008% (w/v) solution. Standard stock solutions of Gallic acid and Quercetin are prepared by dissolving 10 mg of each compound in 10 mL of methanol to obtain a concentration of 10 mg/mL, and working standard solutions are prepared by appropriate dilutions. For the test, 0.1 mL of the sample supernatant or 1.0 mL of the standard solution (Gallic acid or Quercetin) is mixed with 0.5 mL of the DPPH reagent, while DPPH solution alone is used as the control. The mixture is gently vortexed and incubated at room temperature for 30 minutes to allow the antioxidant compounds to react with and reduce the DPPH radicals. After incubation, the color change is observed, indicating the level of antioxidant activity [16].

10. Antioxidant study by FRAP assay:

The Ferric-Reducing Antioxidant Power (FRAP) assay is a widely used method for evaluating the antioxidant potential of various substances, including plant extracts. It is based on the principle that antioxidants reduce ferric ions (Fe^{3}) to ferrous ions (Fe^{2}) in the presence of a complexing agent, resulting in the formation of a colored product. Specifically, the reduction of the Fe^{3} –TPTZ (2,4,6-tripyridyl-s-triazine) complex produces a measurable color change, with absorbance read at 593 nm or color change, which directly correlates with the reducing power of the sample. The FRAP reagent is prepared using an acetate buffer (pH 3.6), 10 mmol of TPTZ in 40 mmol HCl, and 20 mmol of iron (III) chloride, mixed in a 10:1:1 (v/v) ratio to create a stable and reactive complex. The freshness of the reagent is crucial, as its stability affects the accuracy of the results. For the assay, the supernatant sample is diluted in distilled water to concentrations ranging between 0.5 and 2 mg/mL. Then, 5 µL of the diluted sample is added to 150 µL of the FRAP reagent and incubated for approximately 15 minutes at room temperature. During this time, antioxidants in the sample donate electrons to reduce Fe^{3} to Fe^{2} , forming a colored complex that indicates the sample's antioxidant capacity [17].

III. Result And Disscussion:

Reducing sugar test:

The chitinase-producing bacterial culture also tested positive for reducing sugars, indicating the presence of simple sugars released as a result of chitin degradation. This positive result suggests that the bacterium effectively hydrolyzes chitin into its monomeric components, primarily N-acetylglucosamine and other reducing sugars. The accumulation of reducing sugars in the culture supernatant further supports the active enzymatic breakdown of chitin by bacterial chitinases. This outcome not only confirms the functional activity of the chitinase enzyme but also highlights the metabolic potential of the bacterium to utilize chitin as a carbon source. The presence of reducing sugars is also a valuable indicator for evaluating the efficiency of chitin hydrolysis in bioconversion processes.

Assay	**Results**
Chitinase enzyme assay	Positive
Microorganism Gram staining	Positive - Rod shape
Reducing Sugar Assay	Positive

Gelatin hydrolysis Assay	Positive
Catalase Assay	Positive
Antioxidant study by DPPH assay	Positive
Antioxidant study by FRAP assay	Positive
Antibiotic Assay for penicillin	Positive

Table 1 Different test for chitinase producing bacteria

Chitinase enzyme , Gelatin hydrolysis, Catalase Assay :

The positive results for chitinase, catalase, and gelatin hydrolysis tests in the chitinase-producing bacterial culture indicate the multifunctional enzymatic potential of the isolate[2,4]. The chitinase activity confirms the bacterium's ability to degrade chitin, a major structural polysaccharide found in fungal cell walls and insect exoskeletons, highlighting its potential role in biocontrol and bioconversion of chitinous waste[7,8]. The catalase-positive reaction, characterized by the rapid release of oxygen bubbles upon exposure to hydrogen peroxide, suggests that the bacterium can efficiently detoxify reactive oxygen species, enhancing its survival in oxidative environments. This trait is particularly beneficial for industrial or environmental applications where oxidative stress is common. The gelatin hydrolysis test also yielded a positive result, indicating the production of proteolytic enzymes capable of breaking down gelatin into peptides and amino acids. This protease activity suggests a broader capability of the bacterium to degrade proteinaceous substrates, which may contribute to its ecological competitiveness and nutrient acquisition. Together, these enzymatic properties suggest that the bacterial strain possesses a robust enzymatic profile, making it suitable for various biotechnological applications such as waste degradation, enzyme production, and agricultural biocontrol [6]. The combined activities also imply potential synergistic roles in microbial interactions and environmental adaptability. These results support further exploration of the isolate for industrial enzyme production and eco-friendly biotechnological processes.

Antioxidant Activity

The present study aimed to evaluate the antioxidant potential of the supernatant derived from a chitinase-producing bacterial culture using two well-established assays: Ferric Reducing Antioxidant Power (FRAP) and DPPH radical scavenging activity. The FRAP assay revealed a significant metal-reducing capacity of the bacterial supernatant, indicating its potential to donate electrons and reduce Fe^{3} to Fe^{2} [16,17]. This activity suggests the presence of redox-active metabolites in the culture supernatant. Similarly, the DPPH assay demonstrated notable free radical scavenging ability, further supporting the antioxidant nature of the bacterial metabolites [18,19]. The reduction in DPPH absorbance was dose-dependent, indicating that higher concentrations of supernatant enhanced radical neutralization. These findings imply that the chitinase-producing bacteria not only degrade chitin but also release bioactive compounds with antioxidant properties. The presence of such antioxidant activity could be due to phenolic compounds, peptides, or other secondary metabolites produced during chitin metabolism [18.19.20]. The dual antioxidant action observed through FRAP and DPPH assays suggests that the supernatant has potential for application in nutraceutical or pharmaceutical industries [18]. Overall, the study highlights the biotechnological value of chitinolytic bacteria beyond chitin degradation, emphasizing their role in generating antioxidant-rich bio-products.

IV. Conclusion

This study successfully isolated and characterized chitinase-producing bacteria from soil samples collected along riverbanks. The isolates displayed distinct morphological characteristics and demonstrated the ability to utilize colloidal chitin as a substrate, confirming their chitinolytic potential. Optimal chitinase activity was observed at 45 ^{0}C, neutral pH, and 96 hours of incubation, indicating thermotolerance and stability under neutral conditions. These findings suggest the bacteria could be applied in industrial processes that require moderate temperatures and pH levels.

Gram staining results confirmed that the effective isolates were Gram-positive, a characteristic often associated with high enzyme productivity and stability. Biochemical tests revealed that the isolates could hydrolyze gelatin and starch and ferment various carbohydrates, reflecting their diverse metabolic capabilities. The presence of catalase activity indicates their potential to survive oxidative environments, enhancing their industrial viability. Furthermore, the antioxidant properties of the isolates, demonstrated through DPPH and FRAP assays, highlight their potential use in health-related applications and food preservation. The antimicrobial activity observed suggests that these bacteria may serve as biocontrol agents against pathogenic microorganisms, offering environmentally friendly alternatives to chemical pesticides. These multifunctional properties make the isolates strong candidates for use in agriculture, such as in the bioconversion of chitin-containing waste, soil health improvement, and pest management. In biotechnology, the enzymes produced by these bacteria could play a role in the production of bioactive compounds, pharmaceuticals, and functional food ingredients.

Additionally, chitinase-producing bacteria could contribute to waste management by degrading crustacean shell waste, thus aiding in environmental sustainability. Their ability to survive in natural soil environments suggests their resilience and adaptability, making them suitable for field applications. The findings of this study open new avenues for the use of native microbial resources in eco-friendly and cost-effective biotechnological processes. However, to fully exploit their potential, further studies are necessary to purify and characterize the specific chitinase enzymes involved. Enzyme kinetics, molecular structure analysis, and genetic sequencing will provide deeper insight into their mechanisms of action.

Moreover, scaling up enzyme production and testing its efficiency in real-world applications will be essential steps toward commercialization. Understanding the regulation of chitinase gene expression could also facilitate genetic engineering approaches for enhanced production. Overall, the study lays a strong foundation for the development of microbial solutions to industrial and environmental challenges.

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