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Screening and Molecular Characterization of Rhizospheric Actinomycetes for Industrially Significant Cellulase Enzymes

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Abstract:

Actinomycetes, a group of Gram-positive bacteria characterized by a high G, C content and play a vital role in nutrient cycling and decomposition of organic matter in soil. This study describes the screening of Actinmycetes to produce industrially important enzymes and their molecular characterization which are crucial for breaking down complex lignocellulosic compounds. The isolates were screened for all the three components of cellulase complex (endoglucanase, exoglucanase and β-glucosidase). These enzymes have significant industrial applications in particularly biofuel production, paper, pulp industries and agriculture. However, potential of actinomycetes from different environmental niches, such as the rhizospheric zone of litchi plants, remains underexplored. Methods : A total of 25 actinomycete isolates were obtained from soil samples and collected from 8 different litchi gardens of Muzaffarpur District of North Bihar. These isolates were screened on their morphological characteristics, followed by physiological, biochemical characteristics and 16S rRNA Phylogenetic analysis including assessments of cellulase and xylanase. The screening involved culturing the isolates on carboxymethyl cellulose (CMC) agar and observing the formation of clear zones, which indicate enzyme activity.

Out of 30 actinomycete isolates, 10 showed cellulase activity, five exhibited amylase activity and two produced both enzymes and isolates identified as *Streptomyces sp.* SGIITD9, Streptomyces sp. SGIITD10. These cellulolytic strains demonstrated strong enzyme activity under submerged fermentation and shown their potential for low-cost industrial applications. Further isolation from diverse substrates is needed, as the number of enzyme-producing isolates was relatively low. Among the 10 isolates, 2 promising strains (SGIITD09 and SGIITD10) were selected and identified through their 16S rRNA sequence as *Streptomyces spp.,* exhibiting excellent enzyme activity.

Keywords : Actinomycetes, Lignocelluloses, Cellulase, 16S rRNA Sequencing

Introduction:

Lignocellulose is the most abundant renewable biomass derived from plants, composed primarily of cellulose, hemicellulose, and lignin. Cellulose, making up about 35-50% of lignocellulose, is the most prevalent component, followed by hemicellulose, which accounts for 20-35%, with xylan being the major component of hemicellulose and the second most abundant after cellulose (Senesi *et al.,* 1987; Yonebayashi *et al.,* 1985). The

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hydrolysis of these lignocellulosic materials into monosaccharides is catalyzed by hydrolytic enzymes such as cellulases, hemicellulases and ligninolytic enzymes (Abdel *et al.,* 2013; Deswal *et al.,* 2012 ; Gupta *et al.,* 2012). Cellulase enzymes, including endoglucanases, β-glucosidases, cellobiohydrolases, and exoglycanases, catalyze the breakdown of cellulose (Zhang *et al.,* 2013; Limayem *et al.,* 2012; Motta *et al.,* 2013; Soni *et al.,* 2013). The application of these enzymes in industries, particularly in pulp and paper, helps reduce chemical pollution and allows processes to occur under milder conditions. In recent years, microbial enzymes have gained significant attention as eco-friendly alternatives to chemical catalysts in various industries, including pharmaceuticals, textiles, paper, and food production (Sukumaran *et al.,* 2005 ; Tan *et al.,* 1984). Certain groups of bacteria, including both Gram-positive and Gram-negative have been reported to produce cellulase enzymes. Actinomycetes, a group of Gram-positive bacteria with high guanine and cytosine (GC) content are particularly noted for producing these enzymes, especially within the Streptomyces genus. These bacteria are primarily aerobic, forming mycelia and spores are found in diverse environment such as soil, fresh and marine water. Actinomycetes play a crucial role in nutrient and the decomposition of substrates like leaf litter and wood. They are also known for producing antibiotics and various enzymes. This study centered on the isolation, screening, and identification of actinomycetes from soil samples in the rhizospheric zone of litchi plants, with a focus on their potential to produce cellulolytic enzymes (Ruttiman *et al.,* 1987; Saha *et al.,* 2003). On various parameters confirms the presence of cellulase-producing actinobacteria in the litchi orchard, capable of converting cellulose into fermentable sugars.

Materials and Methods :

Fruit orchard as collection Source:

Soil samples were collected from approximately 5-8 cm below the soil surface from rizosphere zone of Litchi plant. In present study we have isolated actinomycetes from 8 different litchi gardens (Bhagwanpur, Susta, Babhanbigha, Atardah, Sherpur, Mushahari, Ahiapur, Shahbajpur) of Muzaffarpur District areas of North Bihar (Table-1). A total of 30 strains of Actinomycetes were isolated and purified by regular subculture and routine examination was carried out with respect to their growth morphology, number of colonies per plate, colour of colonies on solid as well as liquid media .

Isolation of Actinomycete Strains :

The actinomycete strains were isolated from soil samples collected from the rhizosphere zone of litchi orchards in various areas of Muzaffarpur District (Swati *et al.,* 2015). The soil samples were initially stored in plastic bags and air-dried at room temperature for 10 days. Once dried, the soil was crushed using a mortar and pestle and then treated with either a 1.5% phenol solution for 30 minutes at 30°C or subjected to wet heat in sterilized water for 15 minutes at 50°C, following protocols by Y. Takahashi *et al.,* (1996). The treated soil was then diluted in a 1:10 ratio with sterile 25% Ringer's solution and further serially diluted to 10-4. From these dilutions, 100 µL of the 10-1, 10-2, 10-3, and

10-4 suspensions were spread in triplicate onto various isolation media. The media used included ISP 1 to ISP 7 (Tyrosine agar) as per E. B. Swati *et al.,* 2016. Starch Casein Agar (SCA), Streptomyces Agar (SA), Actinomycetes Isolation Agar (AIA) and Nutrient Agar following J.F. MacFaddin (2000). Additional media used were Bennett's Medium, SOC Medium, SOB Agar Medium, Czapek-Dox Medium, YEME Medium and R2A Medium. To inhibit the growth of unwanted microbes, all media were supplemented with cycloheximide (50 mg/L), nystatin (50 mg/L) and nalidixic acid (20 mg/L). The plates were incubated at 30°C for 1 to 5 weeks. The isolates were cultured on ISP 2 medium and Sabouraud's agar medium. Once isolated, the purified cultures were maintained on ISP 2 medium slants at room temperature for short-term storage, and in glycerol suspensions (20% v/v) at -80°C for long term preservation. To ensure the viability of the strains, they were subcultured every fortnight under laboratory conditions. Various cultural characteristics were recorded, including colony shape, margin, elevation, surface appearance, color changes in the medium due to pigment production and odor. Additionally, the morphological features of the cells and spores were examined microscopically. The growing mycelia possessed short compact chains of 15-25 conidia on aerial branches. These morphological and biochemical characteristics (Table 2), followed by their verification by Bergey's Manual, categorically allowed the strains to be placed under actinobacteria (Whitman *et al.,* 2012). Microscopic examination of the plates (Fig. 1) revealed the presence of different types of actinomycetes. The number of viable colonies per gram of soil was calculated using conventional methods and strains isolated from different sites were documented. A total of 30 actinomycete strains were successfully isolated from the rhizosphere zone of litchi plants. Out of these, 10 strains were arbitrarily selected for further research based on their potential for lignocellulose degradation.

Screening Methods for Enzyme Production:

Primary Screening for Cellulase Production:

Cellulase production was initially assessed using a plate assay with 1% Carboxymethyl Cellulose (CMC) in a basal salt medium, following the procedure of Hankin *et al,.* (1977). After inoculating the plates and allowing growth, the cellulase activity was detected by staining with 0.1% Congo Red Solution. The plates were then counterstained with 1 M NaCl for 20 minutes. The presence of an opaque zone around the colonies indicated the hydrolysis of cellulose, signifying positive cellulase activity.

Quantitative Screening for Cellulase Enzyme:

Total cellulase activity, including both filter paper cellulase and Carboxymethyl cellulase were quantitatively measured using the following methods:

a. **Carboxymethyl Cellulase Activity:** To determine Carboxymethyl cellulase activity, 0.5 ml of crude enzyme supernatant was mixed with 0.5 ml of 2% Carboxymethyl Cellulose in 0.05 M sodium citrate buffer (pH 4.8). This mixture was incubated at 55°C for 30 minutes. Amount of reducing sugar to determine phylogenetic relationships with closely related with *Sreptomyces species*. PCR products were analyzed at Macrogen Korea, Delhi. Sequence data were compared to the NCBI non-redundant data

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Table 1: Isolates of actinomycetes from different Litchi (L) orchrd of different regions

Isolated Strains	BhL	SuL	BbL	AtL	SrL	MuL	AhL	ShL
1	+	-	-	-	-	-	-	-
2	-	+	-	-	-	-	-	-
4	-	+	-	-	-	-	-	-
5	-	-	+	-	-	-	-	-
6	-	-	-	-	+	-	-	-
7	+	-	-	-	-	-	-	-
8	-	-	-	+	-	-	-	-
9	-	+	-	-	-	-	-	-
10	-	-	-	-	-	+	-	-

Abbr : BhL=Bhagwanpur, SuL = Susta, BbL= Babhanbigha, AtL=Atardah, SrL=Sherpur, MuL= Mushahari, AhL=Ahiapur, ShL=Shahbaipur and L=Litchi.

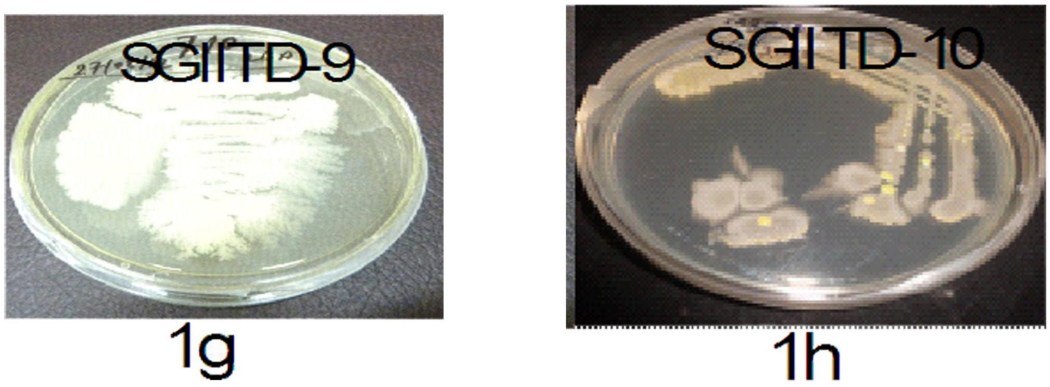

Fig 1. Different isolated Actinomycete strains (1g) and (1h) from rhizosphere zone of Litchi plants.

leased from substrate was then measured.

Filter Paper Cellulase Activity:

Filter paper cellulase activity was measured by incubating 1 ml of 0.05 M sodium citrate buffer (pH 4.8) containing 50 mg of Whatman No.1 filter paper with 0.5 ml of crude enzyme supernatant at 55°C for one hour. The reaction was stopped by adding 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent to 1 ml of the reaction mixture. The amount of reducing sugars was then quantified spectrophotometrically. The DNS reagent reacts with the reducing sugars, producing a color change that was measured at a specific wavelength. Glucose was used as a standard for calibration (Ghose, 1987). One unit of cellulase activity is defined as the amount of enzyme that releases 1 µmol of reducing sugars (expressed as glucose) per milliliter per minute.

Sequence analysis of the 16S rRNA gene sequences and phylogenetic analysis:

The 16S rRNA gene sequences of isolated strains SGIITD09 and SGIITD10 were sequenced to identify the actinomycetes at a molecular level and

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Table 2. Biochemical test of the bacterial isolate (SGIITD09) and (SGIITD10)

Sl. No.	Test	SGIITD09	SGIITD10
1	Citrate test	+	+
2	Indole Test	+	+
3	Methyl Red test	+	-
4	VP test	+	-
5	Catalase test	+	+
6	Decarboxylase	-	+
7	Urease test	+	-
8	H2S test	+	+
9	Starch hydrolysis	+	+
10	Casein hydrolysis	-	+
11	Gelatin hydrolysis	+	+
12	Cellulase hydrolysis	+	+
13	CHO fermentation	+	+
14	Fluorescent property	-	+

Note: (+ ve) indicates positive reaction and (- ve) indicates a negative reaction

Figure 2. Comparison of cellulase activity and hydrolysis index between isolates SGIITD09 and SGIITD010. The graph illustrates differences in clear zone diameter, hydrolysis index, endoglucanase activity and FPCase activity.

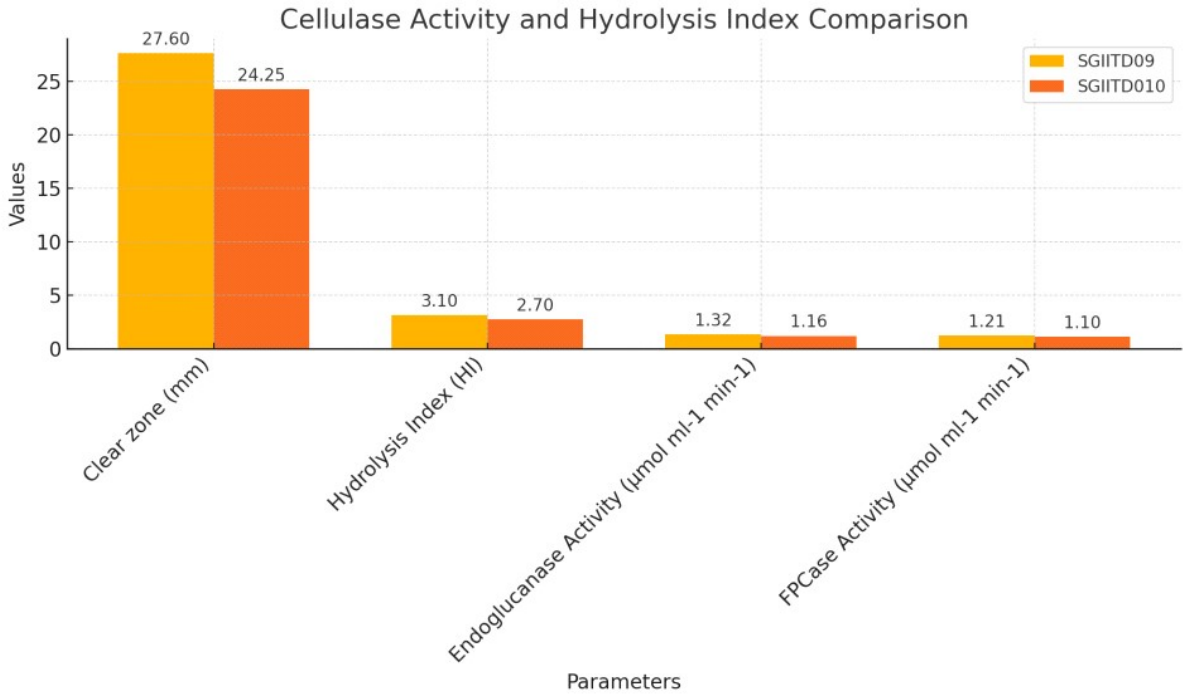

Table 3: Identification of Bacterial Culture: Strain SGIITD09 and SGIITD10

Strain code	Details of similar species	Accession number	Identity (%)
SGIITD-09	Streptomyces sp.LD48	AY641538	99
SGIITD-10	Streptomyces sp.LD48	AY641538	98

the 16S rRNA gene sequences from both isolates exhibited the highest similarity to various *Streptomyces species*. Subsequent analysis of 16S rRNA gene sequences from ten putative strains confirmed that strains SGIITD09 and SGIITD10 exhibited the highest sequence similarity (99%) to *Streptomyces sp*. LD48 and *Streptomyces sp*. A515Ydz-FQ respectively.

Molecular Identification of Selected Strains:

The two most putative isolated strains, SGIITD09 and SGIITD10 were preserved for genetic analysis and identified through 16S rRNA gene sequencing, revealing that they belong to the actinobacteria group (Table 3). The strain labeled SGIITD09 showed a 99% nucleotide similarity to *Streptomyces sp*. LD48 (Accession No. AY641538), while SGIITD10 was 99% similar to *Streptomyces sp.* A515Ydz-FQ, according to nucleotide homology and phylogenetic analysis.

Discussion:

The isolation and characterization of 30 actinomycete strains from the rhizosphere of litchi orchards in Muzaffarpur District, North Bihar, underscore the potential of these microorganisms in biotechnological applications, particularly in the production of industrially relevant enzymes. This study successfully demonstrated that the rhizosphere zone of litchi plants is a promising ecological niche for discovering actinomycetes capable of degrading lignocellulosic materials, which is critical for industries such as biofuels, paper, and pulp.

Among the isolated strains, 10 exhibited cellulase activity, 5 showed xylanase activity and 3 were capable of producing both enzymes. These findings are significant, as they highlight the potential of rhizospheric actinomycetes, particularly from the genera Nocardia and Streptomyces, as sources of enzymes that play a crucial role in the breakdown of complex organic materials. The adaptation of these microorganisms to the organic-rich environment of the rhizosphere likely contributed to their ability to produce robust enzyme systems, which are essential for efficient lignocellulose degradation. The study also utilized 16S rRNA gene sequencing to identify the most promising enzyme-producing strains. The molecular analysis revealed that strains SGIITD09 and SGIITD10 had a high nucleotide similarity with known *Streptomyces species*, further validating the rhizosphere of litchi plants as a valuable source of novel actinomycetes with potential industrial applications. However, the relatively low number of enzyme-producing isolates raises important considerations. It suggests that the screening conditions used in the study may not have been fully

optimized for the expression of cellulase and many other enzymes. Alternatively, the actinomycetes present in these specific soils might inherently have lower enzymatic activity, or the isolation methods and media employed could have influenced the diversity and activity of the isolates obtained. These factors indicate the need for further optimization of isolation and screening techniques to enhance the recovery of actinomycetes with high enzymatic potential. Consequently, this study not only adds to the growing body of knowledge on the diversity of actinomycetes in the rhizosphere but also opens avenues for exploring their biotechnological applications. The discovery of cellulase and several other industrially important enzymes producing strains from litchi orchards offers promising prospects for the development of new biocatalysts for industrial processes; though further research is needed to optimize conditions for enzyme production and to fully explore the potential of these microorganisms.

Conclusion:

This study successfully isolated and identified actinomycetes from the rhizosphere of litchi plants, with a focus on their ability to produce cellulase and several other industrially important enzymes. Although only a limited number of isolates demonstrated significant enzymatic activity, the findings suggest that the rhizosphere of litchi plants harbors actinomycetes with potential industrial applications. Molecular characterization confirmed the identity of two promising strains, SGIITD09 and SGIITD10, as closely related to Streptomyces species, known for their enzyme-producing capabilities.

Future Prospects:

Future studies should prioritize optimizing isolation and screening techniques to enhance the yield of enzyme-producing actinomycetes. This can be achieved by experimenting with different media, environmental conditions, and substrates to boost the expression of cellulase and other industrially significant enzymes. Additionally, expanding research to include various plant rhizospheres and other environmental niches could uncover novel actinomycetes with superior or unique enzymatic activities. Detailed characterization of the enzymes produced by these isolates, such as their stability, pH, and temperature optima, is crucial for their potential application in industrial processes.

Furthermore, genetic manipulation of these strains could be explored to enhance enzyme yield and efficiency. Employing metagenomic approaches could offer a more comprehensive understanding of the microbial diversity in the rhizosphere, revealing genes associated with cellulase and other enzyme production that may not be expressed under traditional isolation techniques.

Limitations:

The study was confined to 8 litchi gardens in Muzaffarpur, which may have limited the diversity of actinomycetes isolated. Expanding the geographical scope of sample collection could yield a greater variety of enzyme-producing strains. Suboptimal Screening Conditions: The screening methods employed might not have been fully conducive to detecting the full enzymatic potential of the isolates. Alternative screening strategies and conditions could reveal a higher number of enzyme

producers. While the study identified promising strains, scaling up the production of these enzymes for industrial use requires further research, including optimizing fermentation conditions.

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