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Unlocking the Biotechnological Potential of Actinomycetes for various industrial application: A Comprehensive Investigation

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Abstract :

Actinomycetes species is widespread distribution and genetic manipulability. It plays a significant role in this microbial community. aime of the study is to investigate the potential of Actinomycetes, especially *Streptomyces spp.,* isolated from fruit orchard in Muzaffarpur District, North Bihar, India. It focused on their ability to produce cellulase, xylanase, and amylase enzymes. This versatility makes them crucial contributors to the environmental processes of lignocellulose degradation, illustrates their potential. Out of 30 isolates, 15 exhibited significant enzymatic activity, particularly in cellulase production. These isolates, which displayed various colony colors ranging from dark brown to dark grey, were selected for further analysis. Following purification, enzymatic screening revealed that 15 isolates demonstrated the ability to secrete cellulase, lipase, and protease. From these, 2 (SGIITD04 and SGIITD07) of the most promising isolates were identified as *Streptomyces spp.* through 16S rRNA sequencing. This study underscores the enzymatic potential of Streptomyces spp., particularly in cellulase production, exhibit their importance in lignocellulose degradation.

Introduction:

Actinomycetes are prokaryotic organisms classified as bacteria, yet they possess distinctive features that merit separate consideration. Typically, actinomycetes constitute a smaller proportion of the total bacterial population, generally one to two orders of magnitude less. They play a vital role within the bacterial community, particularly under extreame conditions of high pH, elevated temperatures, or water stress. Morphologically, actinomycetes are often compared to fungi due to their elongated cells that branch into filaments or hyphae. However, these hyphae are significantly smaller than those of fungi. Actinomycetes, a group of filamentous gram-positive bacteria, are renowned for their diverse metabolic capabilities, including the production of bioactive compounds (Pelczar *et al.,* 1988; Priya *et al.,* 2015). Among these compounds, enzymes play a crucial role in various biotechnological processes, including the degradation of lignocellulosic biomass (Pan *et al.,* 2006; Zhao *et al.,* 2012a). A key feature of actinomycetes is their ability to degrade a wide array of substrates in soil, including difficult-to-decompose insect and plant polymers like chitin, cellulose and hemicellulose. Although they were

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originally identified as soil microorganisms, recent studies have illustrated the importance of rhizophoric zone of several fruit plants such as guava, banana and litchi plants actinomycetes. These rizosphoric variants have been found to produce novel secondary metabolites, introducing a new dimension to the diversity of microbial natural products (Jensen *et al.,* 2005). Lignocellulosic biomass, comprising cellulose, hemicellulose, and lignin, represents the most abundant source of renewable organic material on Earth, with significant potential for biofuel production and the synthesis of value-added products. The complex structure of lignocellulosic biomass, particularly the recalcitrant nature of lignin, poses challenges for its efficient utilization (Crawford *et al.,* 1978; Paterson *et al.,* 1984). Microbial degradation of lignin offers advantages over traditional chemical methods due to its energy efficiency and environmental sustainability . While wood-degrading fungi have been extensively studied for their ligninolytic enzymes, actinomycetes also play a significant role in lignocellulosic degradation (Fortman *et al.,* 2008; Frazier *et al.,* 1926). Actinomycetes produce a variety of enzymes, including cellulases and xylanases, which are essential for the breakdown of cellulose and hemicellulose, respectively (Frazier *et.al.,* 1926; Lynd *et al.,* 2002). These enzymes hold promise for lignocellulose conversion and have potential applications in industries such as biofuel production, enzyme manufacturing, and bioremediation (Zhu *et al.,* 2008). Despite advancements, our understanding of the biochemistry of lignin degradation by actinomycetes remains limited, and the enzymes involved require further elucidation (Zhao *et al.,* 2012b). Additionally, the complex interplay between different groups of enzymes in lignocellulose solubilization warrants further investigation (Fortman *et al.,* 2008 ; Sannigrahi *et al.,* 2010; Foust *et al.,* 2008; Jensen *et al.,* 2005; Rodriguez *et al.,* 1996). Therefore, there is a pressing need to explore the potential of actinomycetes in lignocellulose degradation and to develop innovative bioprocessing strategies for sustainable biomass utilization. In light of these considerations, this study aims to identify and characterize soil actinomycetes capable of producing potent extracellular enzymes, such as cellulases and xylanases and other industrially applicable enzymes for lignocellulose degradation. Furthermore, the study seeks to optimize enzyme production parameters and explore the production of other bioactive molecules with industrial significance. Identification and characterization of genes involved in enzyme production will also be a focus of this research.

Materials and Methods :

Study Area, Soil Sampling, and Actinobacteria **Isolation Process :**

For this study, soil samples were collected from the Muzaffarpur district, situated at 26° 07' N and 85° 24' E in North Bihar, India. The region is renowned for its diverse fruit cultivation, including mango, litchi, banana, and guava, owing to the soil's rich nutrient content. Actinobacteria-rich soil samples were obtained from litchi orchards approximately 20 cm below the soil surface, focusing on the rhizosphere

zone. The collected soil samples were air-dried at room temperature for 10 days. Subsequently, the dried soil was crushed using a mortar and pestle and subjected to either phenol solution treatment (1.5%, 30 min at 30°C) or wet heat sterilization in water (15 min at 50°C). Following sterilization, the soil was diluted in a 1:10 v/v ratio with sterile 25% Ringer's solution and serially diluted to obtain suspensions of 10-1 to 10-4 dilutions. These suspensions were then spread in triplicate onto various isolation media, including ISP 2 (Yeast malt agar), ISP 7 (Tyrosine agar), Starch casein agar (SCA), Streptomyces agar (SA), Actinobacteria isolation agar (AIA), and nutrient agar. All media were supplemented with Cycloheximide (50mg/L), Nystatin (50mg/L), and Nalidixic acid (20mg/L), and incubated at 30°C for 1-5 weeks. Purified cultures were maintained on ISP medium, with two slants stored at room temperature for short-term storage, and glycerol suspensions (20% v/v) stored at -80°C for long term preservation. The isolation of Actinobacteria was confirmed through microscopic examination and molecular analysis, utilizing Identification of Actinomycetes by Cover slip

Method :

Microbes isolated from the rizosphere zone of Litchi, guava and banana plants were confirmed as actinomycetes by observing their morphology under microscope. The starch casein agar (SCA) was poured on sterile slide and allows solidifying. Then the organisms were streaked onto media and incubate at 37 for 48 hrs. After that 2 drops of methylene blue dye was added and allows it for a minute. Then the slide was covered with cover slip and observed their morphology under microscope to assess their cultural characteristics, including shape, margin, elevation, surface appearance of colonies, pigment formation causing color changes in the medium, and odor of of cells and spores were observed under a microscope. Actinomycetes were identified by their earthy smell and the distinctive color of their hyphae. Their morphological characteristics, such as aerial and substrate mycelium color, colony branching, and texture, were studied according to guidelines from the Bergey's Systematic Bacteriology Manual and the International Streptomyces Project (ISP). The isolates were grown on ISP-2 medium for 7-14 days to observe cultural features, including spore arrangement and hyphal structure, under a microscope. These characteristics were compared with those described in Bergey's Manual for species-level identification. Additionally, Gram staining was used to confirm the nature of the isolates. This process ensured accurate identification and characterization of the *Actinomycete species*.

Qualitative Screening of Ligninolytic Actinobacteria :In the pursuit of identifying lignocellulose-producing bacteria, a tailored selective agar medium was employed (Chun *et al.,* 1995). This medium consisted of 0.2% KNO3, 0.1% KHPO4, 0.05% MgSO4.7H2O, 0.05% NaCl, 0.001% FeSO4, 0.03% CaCO3, and 1.8% agar, supplemented with CMC, xylan, mannan, and tannic acid. Following inoculation with pure culture spore suspensions, plates were incubated at 37°C for 48 hours.

Subsequently, Congo red solution (1%) was flooded onto the plates for a 15-minute duration to visualize the diameter of the decolorized zone surrounding each colony . Identification of the isolated bacteria was meticulously conducted using various methods, including colony morphology assessment, microscopic observation, biochemical tests, Polymerase Chain Reaction (PCR), and sequencing as reported earlier (Zhao et al., 2012). The morphology of colonies, encompassing shape and color characteristics, was scrutinized according to the protocol established by Pelczar and Chan (1988). To ensure robustness, all experiments were executed in triplicate. cultures. Additionally, cultural morphological features

Enzymatic Screening:

To assess cellulase production, all isolates underwent screening using minimal medium agar (MMA) containing AZO-CM-Cellulose as the substrate (Priya et al., 2015). The composition of the MMA medium included peptone (1.0gm), yeast extract (1.0gm), MgSO4.7H2O (0.5gm), KH2PO4 (0.5gm), (NH4) 2SO4 (1.0gm), substrate (Megazyme) (1.0gm), agar (15.0gm) and distilled water (1000ml) adjusted to pH 7. Gelatin hydrolysis assay, described by Frazier (1926), was employed to screen for protease activity. For lipase activity determination by Sierra (1957) was utilized with slight modifications. The formation of halo zones around colonies indicated a positive reaction for the respective tests. Measurement of the halo zones was conducted after 48 hours of incubation (Frazier *et al.,* 1926; Sierra *et al.,* 1957). Screening of Cellulose and Lignin **Degrading Actinomycetes:** All isolated Actinomycetes underwent screening for their cellulase-producing ability using minimal medium agar (MMA) containing AZO-CM-Cellulose as a substrate. The MMA composition included peptone (1.0 g), yeast extract (1.0 g), MgSO4.7H2O (0.5 g), KH2PO4 (0.5 g), (NH4)2 (1.0 g), substrate (Megazyme) (1.0 g), agar (15.0 g) and distilled water (1000 ml) at pH 7. The lignin hydrolysis assay was utilized to screen for protease activity. Screening for lignin degradation was conducted on modified ISP medium No. 2, consisting of malt extract (10 g per liter), glucose (4g per liter) and 0.01% guaiacol as a lignin model compound, supplemented with agar (18 g per liter). Guaiacol oxidation was assessed after 25 days of inoculation, evident by the appearance of a clear red-brown zone around the colony. Lipase activity was screened using a method for the determination of esterase activity with slight modifications. Actinomycetes isolates were spot-inoculated onto the media and incubated at 30°C for 72 hours to facilitate cellulose depolymerization and cellulase production into the surrounding medium. All plates were flooded with 0.15% Congo red solution followed by destaining with 1M NaCl.

DNA Extraction:

Total DNA isolation was performed (Pitcher *et al.,* 1989). Actinomycete strains were cultured in 100 ml LB (Luria-Bertani) broth, Miller, with agitation at 30°C for 6-7 days. Cells were harvested by centrifugation (8,000 rpm for 5 min) and the pellet

was resuspended in 500 µl of 10 mM Tris-HCl/1mM EDTA (TE) buffer (pH 8), supplemented with lysozyme (20 mg/ml). Following a 30-minute incubation at 37°C, 100 µl of 10% SDS and 20 µl of proteinase K were added, and the mixture was incubated at 55°C for 30 min.

After cooling, the lysate was centrifuged at 13,000 rpm for 10 min, and the supernatant was collected and extracted with an equal volume of phenol solution (v/v, 1:1) at 13,000 rpm for 10 minutes. The aqueous phase was carefully transferred to a fresh tube, and DNA was precipitated by adding 90% ethanol and incubating at -20°C for 30 minutes. The resulting pellet was formed by centrifugation at 13,000 rpm for 10 minutes, washed twice with 90% ethanol and dissolved in TE buffer (20 µg/ml), followed by incubation at 37°C for 1 hr. DNA was analyzed by 1.0% agarose gel electrophoresis using TBE buffer.

PCR Amplification:

PCR amplification reactions were conducted in a total volume of 25 µl. Each reaction mixture contained the following components: 1.5 µl genomic DNA, 0.5 µl of 10 µM forward 16S rDNA universal primer 27f (5'-AGAGTTTGATCMTGGCTCAG-3'), 0.5 µl of 10 µM reverse 16S rDNA primer 1525r (5'-AGAAAGGAGGTGWTCCARCC-3'), 0.5 µl of 10 mM deoxyribonucleoside 5'-triphosphate (dNTP's), 2.5 µl of 10× PCR buffer, and 0.5 µl Taq polymerase (5U/µl) (Thermo Scientific). Water was added to make up the final volume to 25 µl. The thermal cycler (Bio-Red) was programmed as follows: an initial denaturation step at 94°C for 4 minutes, followed by 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 68°C for 1.3 minutes, with a final extension at 68°C for 5 minutes. PCR-amplified products were analyzed by 1.0% agarose gel electrophoresis using TBE buffer. The resulting DNA patterns were visualized under UV light using a transilluminator, photographed, and analyzed using a gel documentation system. Partial 16S rRNA sequencing and analysis of sequence data:

The amplification product underwent partial 16S rRNA sequencing at Macrogen Korea. Subsequently, the obtained 16S rRNA sequences were aligned with nucleotide sequences retrieved from the NCBI database using BLAST, followed by the construction of a phylogenetic tree.

Results :

Cultural and Morphological Characterization of Isolates All Actinomycete strains were isolated from soil samples and maintained under laboratory conditions, with subculturing performed twice a month. The colonies were observed to be dry, whittish, and relatively large, often emitting a characteristic earthy odor of an isolated strain SGIITD4 (Figure 1b). For the cultural and morphological characterization of the selected Strain 1, the strain was inoculated into sterile ISP media 1, 2 and 4. Key cultural properties, including colony morphology, type of aerial hyphae, growth of vegetative hyphae, pigmentation and spore formation were observed following guidelines established by the International Streptomyces Project (ISP) [Shirling

& Gottlieb, 1966; Nanjwade *et al.,* 2010; Chhaya & Modi, 2013a]. Bennet's agar was utilized to promote sporulation. Spore arrangement pattern was examined using slide culture technique as described by Williams *et al,.* (1989). Average number of colonies of actinobacteria isolated from soil after 3, 5, 8 & 10 weeks on 8 different isolation media Table 1 and Fig 1a.

Cellulose Degrading Actinomycetes Screening: Out of the 30 isolated actinomycetes, 5 isolates (SGIITD04, SGIITD07, SGIITD09 and SGIITD10) were selected for assessing their cellulase enzyme production and cellulose degrading potential. Enzymatic activity was quantified with one unit of activity defined as amount of enzyme releasing 1 µmol of reducing sugars (measured as glucose) per mL per minute. Endoglucanase activity ranged from 0.810 ± 0.014 to 1.672 ± 0.024 IU/mL, while FPCase activity ranged from 0.810 ± 0.001 to 1.672 ± 0.001 IU/mL (refer to Table 2 and Fig 1c). Notably, actinomycetes isolates SGIITD04 and SGIITD07 exhibited highest cellulase activity compared to other isolates.Table 2 displays the clear zone diameter, hydrolysis index (HI), and enzymatic activities of endoglucanase and FPCase for two different isolates. Measurements illustrate the varying efficiency of these isolates in cellulose degradation with isolate SGIITD04 showing highest hydrolysis index and enzymatic activities compare to SGIITD07 (Fig 1 c). Clear Zone is measured in millimeters (mm), Hydrolysis Index (HI) is a dimensionless ratio of Endoglucanase and FPCase Activities are expressed in µmol ml^{-1} min^{-1}. This graph illustrates variations in these metrics among the different isolates.

Phylogenetic Analysis:

16S rRNA sequencing analysis of the isolate SGIITD07 yielded 1563 base pairs (Fig 1d). An NCBI BLAST search revealed that the sequence shared 99% similarity with *Streptomyces sp*. Streptomycessp. A515Ydz-FQ strain. A phylogenetic

Table 1 ; Average number of colonies of actinobacteria isolated from soil after 3, 5, 8 & 10 weeks on 8 different isolation media

Medium	3 weeks	5 weeks	8 weeks	10 weeks
ISP 1 Medium	25	35	42	65
ISP 2 Medium	15	30	40	50
ISP 3 Medium	10	25	36	38
LB Medium	12	21	28	37
SCA Medium	10	21	25	31
SOB Medium	8	15	21	30
SOC Medium	17	26	30	35
Bennett's medium	11	15	20	30

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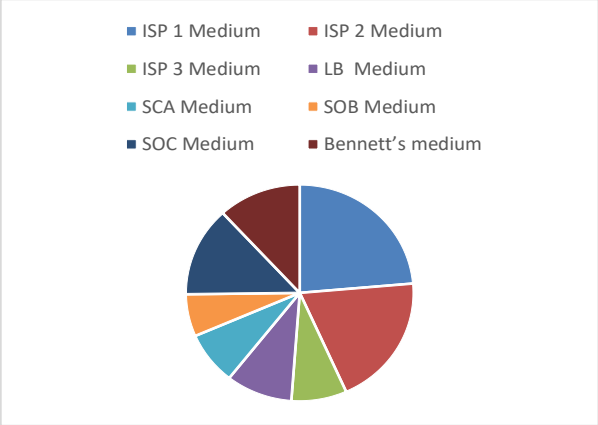

Fig 1a. Average number of colonies of actinobacteria isolated from soil after 3, 5, 8 & 10 weeks on 8 different isolation media

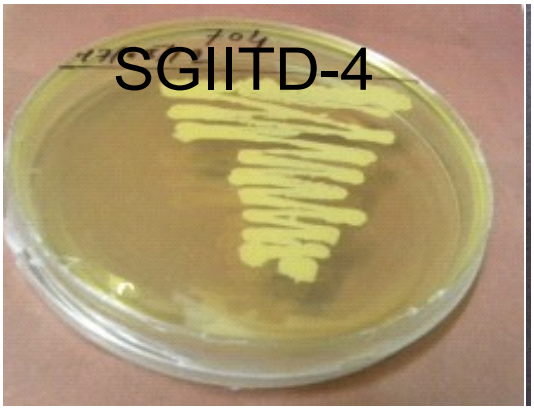

Fig. 1 b : Isolated strain (SGIITD04) Actinomycetes from Litchi orchard Fruit Orchard : Location- Muzaffarpur (Lat : 26007N', Lon : 85027'E)

Table 2 : Enzymatic Activities and Hydrolysis Indices of two different Isolates

Isolate	Clear Zone (mm)	Hydrolysis Index (HI)	Endoglucanase Activity (µmol ml-1 min-1)	FPCase Activity (µmol ml-1 min-1)
SGIITD07	20.00	1.8	0.710	0.615
SGIITD04	25.00	2.8	1.150	1.420

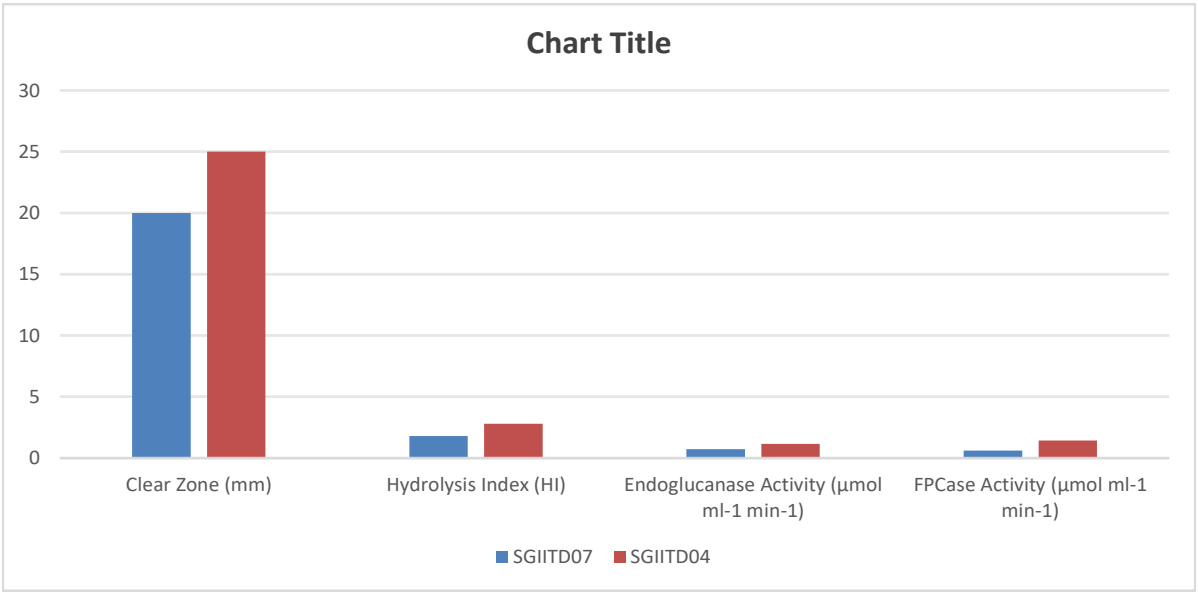

Fig. 1c : Comparison of Clear Zone, Hydrolysis Index, Endoglucanase Activity and FPCase Activity across different isolates (SGIITD04 and SGIITD07).

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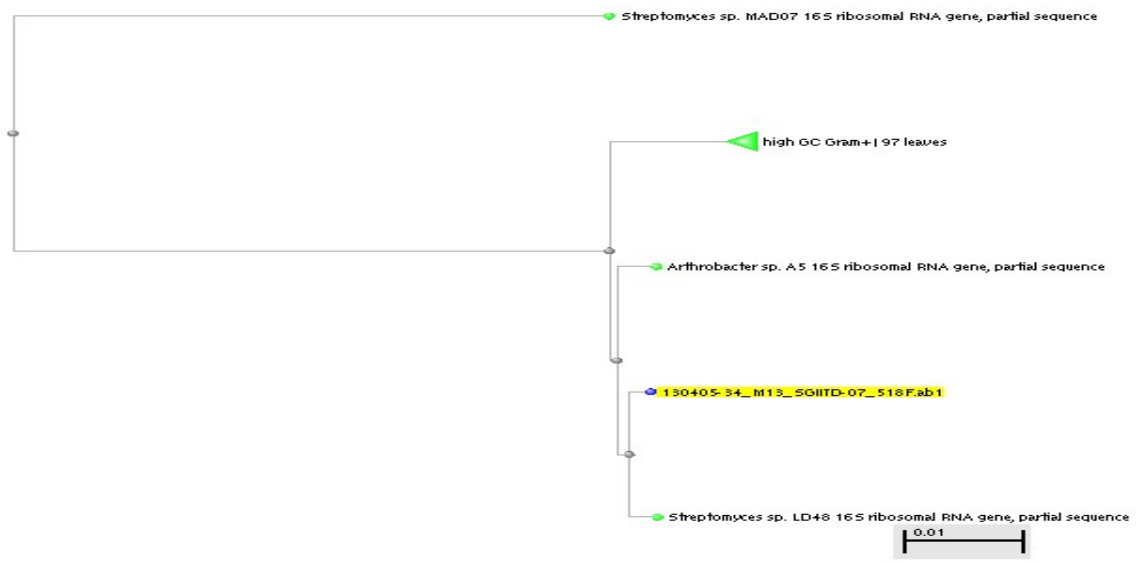

Fig 1d: Phylogenetic Tree Construction Using Fast Minimum Evolution Method Based on 16S rRNA Sequences

tree was constructed using the Fast Minimum Evolution method, which demonstrated that the isolate occupies a distinct phylogenetic position within the radiation, including representatives of the Streptomyces family.

Discussion:

The study demonstrates that actinomycetes isolated from the rhizosphere of litchi orchards in Muzaffarpur, Bihar, possess significant potential for lignocellulose degradation. Specifically, isolates such as SGIITD04, and SGIITD07 showed robust enzymatic activities, including cellulase, lipase, and protease production. These findings are in line with previous research indicating the high lignocellulolytic potential of actinomycetes (Chun et al., 1995 ;Crawford et al.,1978) . The 16S rRNA sequencing confirmed that the isolates belong to the Streptomyces genus, with a high similarity to known Streptomyces strains, corroborating the role of this genus in producing bioactive extracellular enzymes. The degradation of lignocellulose is critical for bioethanol production, as it involves breaking down complex polymers into fermentable sugars. This process is often hindered by the recalcitrance of lignocellulose, making the discovery of efficient enzymes a key area of research (Shallom *et al.,* 2003 ; Kansoh *et al.,*2004; Kumar et al., 2009). The actinomycetes isolated in this study exhibited promising enzyme activities, suggesting their potential application in biorefineries to produce biofuels and other value-added products (Himmel *et al.,* 2007 Stackebrandt *et al.,* 1981) .

Conclusion:

The isolated actinomycetes from the rhizosphere of litchi gardens in Muzaffarpur show promising potential for lignocellulose degradation. The isolates, particularly SGIITD04 and SGIITD07 demonstrated high cellulase activity, making them suitable candidates for biotechnological applications in bioethanol production and other industrial processes. The molecular and phylogenetic analyses confirmed the identity of these isolates as members of the Streptomyces genus, which are well-known for their enzymatic capabilities. Further studies to optimize the conditions for maximum enzyme production from the isolated actinomycetes. Scale-Up Studies: Conducting pilot-scale experiments to evaluate the feasibility of using these enzymes in industrial applications. Metabolic engineering pathways of these actinomycetes to enhance their enzyme production and activity.

Diversity of actinomycetes in various regions and effectiveness of the enzymes produced. It has not been tested in vivo. Stability and activity of the enzymes under various industrial conditions need to be evaluated.

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