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Isolation, characterization and identification of lignocellulose degrading actinomycetes from litchi fruit orchard of Muzaffarpur, Bihar, India

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Abstract
Background: Exploitation of plant biomass as a renewable resource has paved a momentum for research on microbial degradation of lignocellulose. The ubiquity and diversity in actinomycetes species as well as strain improvement by genetic manipulation, and large-scale cultivation easily form an important part of the microbial community responsible for lignocellulose degradation in the environment. In addition, their growth as branching hyphae is well adapted to the penetration and degradation of insoluble substrates such as lignocellulose.
Methods: A total of 28 Actinomycete isolates from 8 soil samples were collected from 3 different litchi gardens (Bhagwanpur, Susta and Ahiapur) of Muzaffarpur District area of North Bihar. Screening of isolates was based on morphological characteristics followed by physiological and biochemical analysis (like cellulase, xylanase and amylase activity).
Results: The present study was confined to 5 bioactive isolates which exhibited a range of colony colours (dark brown, brownish, whitish, yellowish white and dark grey). These isolates were later purified and subjected to a few enzymatic screening. Results indicate that 245 isolates showed the ability to secrete cellulase, lipase and protease respectively. 5 of the most promising isolates were selected and identified using their 16S rRNA sequence. All 5 isolates were identified as *Streptomyces spp*.

Keywords: actinomycetes, fruit orchard, rhizosphere, 16S rRNA sequencing

1. Introduction
Increasing energy demand and our great dependence on fossil resources are considered problematic both from environmental and societal aspects. Biofuel plays a vital part in replacing petroleum based fuels in present energy situation. Amongst all the feed stocks, Lignocellulosic biomass acquired from biomass crop and agricultural residues might be the best option in the long term (Atabani *et al*., 2012) [3]. However, the key barrier for transitioning lignocellulosic feedstock is the complex structure of the plant cell wall, predominantly the presence of lignin. The microbial degradation of lignin has potential advantages over the prevailing chemical degradation, due to energy and environmental concerns. But degrading lignocellulosic biomass by microorganisms is complicated to meet the industrial demands (Afrida *et al.*, 2009) [1]. Thus it is indispensable to isolate new strains of microorganisms for degradation of lignin. It has been well documented that some wood-degrading fungi contain ligninolytic enzymes and is observed as a colourless halo around microbial growth and thus initially screened indirectly on solid media containing different indicator compounds (Dhouib *et al.*, 2005) [8]. Although, wood decay fungi are primarily basidiomycetes, other microorganisms like actinomycetes are also involved in the lignocellulosic decaying processes (Nilsson *et al.*, 1989) [19]. The cellulolytic activity of actinomycetes has understandably received attention as Actinomycete cellulase are inducible extracellular enzymes having activity similar to fungal hydrolytic cellulases. Recent development of sensitive radiometric assay techniques permit Actinomycete xylanase activity which confirm patterns of production and activity similar to other bacteria and fungi, but they have been relatively little-studied (Pavelka, 2014) [20]. Nevertheless, their ability to solubilise grass lignins may have a role in humification and biotechnological applications of lignocellulose conversion. In addition, the available scientific evidence supports the recognition of fructooligosaccharides as dietary fibers with health beneficial properties (Bharti *et al*., 2013, 2015) [5, 4]. The biochemistry of lignin degradation by actinomycetes remains poorly understood and the enzymes involved have yet to be identified. In addition, there is more information on the enzymology of cellulose

and hemicellulose degradation, but how these different groups of enzymes and their component proteins interact to solubilise lignocellulose is largely unknown. Elucidation of the biochemistry of lignocellulose degradation and development of Actinomycete-based systems for lignocellulose conversion assisted by the application of the recombinant DNA techniques is the need of the hour.

Therefore, this study is focused on the identification and purification of soil actinomycetes that produces potent extracellular enzyme like cellulase and xylanase that degrade cellulose and xylan respectively. In addition, parametric optimization for the production of cellulose, amylase, phytase, xylanase and other bioactive molecules of industrial importance as well as identification and amplification of genes for enzyme production have been analyzed.

2. Methodology

2.1 Study area, soil sample collection and selective isolation of action bacteria

For the present study the soil samples were collected from the Muzaffarpur district situated at 26º 07' N and 85^0 24'E of North Bihar location of India. A wide range of fruits like mango, litchi, banana, guava, etc is grown over here as the soil is enriched with all kinds of essential nutrients. The soil samples rich in actinomycetes were collected in plastic bags from litchi orchard approximately 20 cm below the soil surface from rhizosphere zone and kept at room temperature for 10 days to get air dried. Then after the dried soil was crushed with mortar and pestle and dissolved in phenol solution (1.5%, 30 min at 30 ^{0}C) or wet heat in sterilized water for 15 min at 50 ^{0}C. It was diluted in 1:10 v/v with sterile 25% *Ringer's solution* and serially diluted to 10^4. About 100 L of 10^1, 10^2, 10^3 and 10^4 suspensions were spread in triplicate onto different isolation media: ISP 2 (Yeast malt agar), ISP 7 (Tyrosine agar), Starch casein agar (SCA), Streptomyces agar (SA), Actinomycetes isolation agar (AIA) and nutrient agar. All these media were supplemented with Cycloheximide (50mg/L), nystatin (50mg/L) and nalidixic acid (20mg/L) and incubated at 30 ^{0}C for 1-5 weeks. Purified cultures were maintained on ISP medium, 2 slants at room temperature for short-term storage and as glycerol suspensions (20% v/v) at 80 ^{0}C for long-term storage. Actinomycetes were isolated and confirmed through microscopic and molecular analyses by using 16S rRNA sequencing.

2.2 Cultural and morphological characteristics

The isolates were grown on xylan-agar medium. The cultural characteristics such as shape, margin, and elevation, surface appearance of the colonies, colour change in the medium due to pigment formation, and odor of the cultures were recorded. Morphological features of the cells and spores were also observed microscopically.

2.3 Enzymatic screening

Enzymatic tests were conducted to determine biodegrading ability of actinomycetes for agricultural usage. All the isolates were screened for their cellulase producing ability using minimal medium agar (MMA) containing AZO-CM-Cellulose as substrate (Peptone, 1.0 g; yeast extract, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; $(NH_4)_2$, 1.0 g; substrate (Megazyme), 1.0 g; agar, 15.0 g and distilled water, 1000 ml) at pH 7. Gelatin hydrolysis assay was used in the screening of protease activity (Kasana *et al*., 2011) [16]. Lipase activity was screened using method for determination of esterase activity (Sierra, 1957) [23] with little modification. Tween 80 used in the esterase assay test was replaced with Tween 20. Formation of halo-zone indicates positive reaction for the entire test conducted. Measurement of the halo-zones was taken after 48 h of incubation.

2.4 Screening of cellulose and lignin degrading actinomycetes

All the isolated Actinomycetes were screened for their cellulase producing ability using minimal medium agar (MMA) containing AZO-CM-Cellulose as substrate (Peptone, 1.0 g; yeast extract, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; $(NH_4)_2$, 1.0 g; substrate (Megazyme), 1.0 g; agar, 15.0 g and distilled water, 1000 ml) at pH 7. The lignin hydrolysis assay as described by (Ander and Eriksson, 1976) [2] was used in the screening of protease activity. The screening for lignin degradation was observed on modified ISP medium No. 2 containing 10 g per lit malt extract, 4g per lit glucose, 0.01% guaiacol as lignin model compound and 18 g per lit agar. The guaiacol oxidation was evaluated after 25 days of inoculation from the clear red brown zone around the colony. Lipase activity was screened using method for determination of esterase activity with little modification. Tween 80 used in the esterase assay test was replaced with Tween 20. The isolated actinomycetes were spot inoculated on to the media and incubated at 30 ^{0}C for 72 hrs to express cellulose depolymerisation through cellulose production into its surrounding medium. After incubation all plates were flooded with 0.15% Congo red followed by de-staining with 1M NaCl.

2.5 DNA extraction

For the isolation of total DNA the method of Hintermann *et al.,* (1981) [13] have been employed. Actinomycete strains were grown in 100 ml LB (Luria-Bertani) broth, Miller with agitation at 30 °C for 6-7 days. Cells (10 ml) were harvested by centrifugation (8,000 rpm for 5 min), pellet was resuspended in 500 µl of 10 mM Tris-HCl/ 1mM EDTA (TE) buffer (pH-8) supplemented with lysozyme (20 mg/ml). The tube was incubated at 37 °C for 30 minutes. Then 100 µl of 10% SDS and 20 µl of proteinase K were added and incubated at 55 °C for 30 min. The lysate was cooled down and centrifuge at 13,000 rpm for 10 min. The supernatant was then collected and extracted with equal volume of phenol: chloroform solution (v/v, 1:1) at 13,000 rpm for 10 minutes. The aqueous phase was transferred carefully to a fresh tube and DNA was precipitated by adding 90% ethanol and keeping at -20 °C for 30 minutes. The pellet was formed by centrifuging at 13,000 rpm for 10 minutes. The pellet was washed twice with 90% ethanol and dissolved the pellet in TE buffer containing (20 µg/ml) and then incubated at 37°C for 1 hr. DNA was analyzed by 1.0% agarose gel electrophoresis using TBE buffer.

2.6 PCR amplification

The PCR amplification reactions were performed in a total volume of 25 µl. Each reaction mixture contained the following solutions: 1.5 µl genomic DNA, 0.5 µl of 10 µM forward 16S rDNA universal primer 27f (5'-AGAGTTTGATCMTGGCTC AG-3'); 0.5 µl of 10µM reverse 16S rDNA primer 1525r (5'-AGAAAGGAGGTGWTCC ARCC-3'); 0.5 µl of 10mM Deoxyribonucleoside 5'-triphosphate (N= A,T,G,C) (dNTP's); 2.5 µl of 10× PCR buffer and 0.5 µl Taq polymerase (5U/µl) (Thermo scientific) and water was added up to 25 µl. The thermal cycler (Bio-Red) was programmed as

follows: 4 min initial denaturation at 94 °C, followed by 30 cycles that consisted of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension at 68 °C for 1.3 min and a final extension of 5 min at 68 °C. The PCR amplified product was analyzed by 1.0% agarose gel electrophoresis using TBE buffer. The resulting DNA patterns were examined with UV light under trans-illuminator, photographed and analyzed using gel documentation system.

2.6 Partial 16S rRNA sequencing and analysis of sequence data

The partial 16S rRNA sequencing of the amplified product was performed at, Macrogen Korea. The 16S rRNA sequences were aligned manually with the available nucleotide sequences retrieved from the NCBI database by using BLASTN. Based on maximum identity score, selected sequences were aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4. The putative strains were then isolated and analyzed.

Results

3.1 Cultural and morphological characteristics

Cultural and morphologic observations of the isolates grown on xylan-agar medium, showed typical growth characteristics of actinomycetes on solid media, *i.e.*, dry, whitish, bigger-sized colonies characterized with peculiar smell like wet soil. In liquid media, they formed ball-shaped structures. The microscopic observations showed that the isolates possessed the typical spore chain morphology of *Streptomyces*.

3.2 Screening of cellulose and lignin degrading actinomycetes

The study showed that SGIITD05, SGIITD09 and SGIITD03 isolates of actinomycetes produced cellulase, lipase and protease respectively. The plates showed positive cultures with a zone of clearance around the cell growth when treated with Congo red followed by de-staining with NaCl. Formation of halo-zone after 3 days of the test indicated positive reaction (Plate 1) for the entire test conducted.

3.3 Isolation and enumeration of microbes

Colony forming unit per gram (cfu/g) of soil, showed the density of actinomycetes isolated from the soil was highest at 8.0×10^7 from the limau purut soil and the lowest was from the ciku soil with just 9.8×10^6. From all of the samples collected, a total of 62 isolates of actinomycetes were isolated (Table 1). Each of the isolates were later categorized according to their morphology and colony colour ranging from dark grey to grey, dark brown to brownish, whitish and yellowish white (Figure 1).

3.4 Isolation of bacteria from soil and their culture

All the Actinomycete strains were obtained from soil and maintained in the laboratory and subcultured twice in a month. Figure 1 shows some isolated strains.

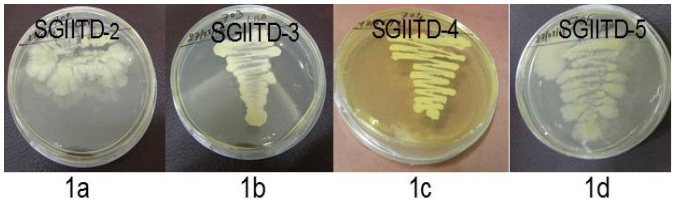

Fig 1a-1d: Different isolated Actinomycete strains from rhizosphere zone of Litchi plants.

3.5 Screening of cellulose degrading actinomycetes

Among the 20 isolated actinomycetes, 5 isolates (SGIITD02, S GIITD03, SGIITD05, SGITD09 and SGIITD10) are selected for the production of *cellulase* enzymes and their cellulose degrading potential is estimated. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per mL per minute. The total *cellulase* activity ranges from 0.810 ± 0.014 to 1.672 ± 0.024 IU/mL for *endoglucanase* while 01.672 ± 0.001 to 0.810 ± 0.001 IU/mL for *FPCase* activity (Table 1) The three actinomycetes isolate SGIITD05,SGIITD09 and SGIITD03 performed highest cellulase activity as compared to the other isolates.

Table 1: Endoglucanase activity: highest cellulase activity was performed by SGIITD05.

No.	Isolates	Clear zone(mm)	Hydrolysis Index (HI)	Cellulase activities for Endoglucanase (µmol $ml^{-1}m^{-1}$)	Cellulase activity for FPCase (µmol $ml^{-1}m^{-1}$)
1.	SGIITD02	18.00	1.9	0.810	0.415
2.	SGIITD03	26.00	2.9	1.245	1.305 (max)
3.	SGIITD05	30.00	3.9	1.672 (max)	1.200
4.	SGIITD09	27.60	3.1	1.325	1.205
5.	SGIITD010	24.25	2.7	1.164	1.104

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

The sequencing of PCR product for 16S rRNA gene of isolated strains SGIITD02 and SGIITD05 was carried out to identify the actinomycetes strains at molecular level and to establish its phylogenetic relationship with closely related *Streptomyces* for which the PCR samples were analyzed at Macrogen Korea, Delhi. Based on homology searches using BLAST against the NCBI non-redundant database (http://www.ncbi.nlm.nih.gov/) the 16S rRNA gene sequences from both isolates were found to have highest sequence similarities to *Streptomyces* species. Based on maximum identity score first ten sequences were selected and aligned using Clustal W software program. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 4. Finally, 10 putative strains were isolated and their analysis of the 16S rRNA gene sequences showed that strain SGIITD02 and SGITD05 had the highest sequence similarity (99%) to *Streptomyces* spLD48 and *Streptomyces* spA515Ydz-FQ respectively.

3.7 Molecular Identification of Selected Strains

Two putative isolated strains which were thought to be enzyme producer (SGIITD02 & SGIITD05) were maintained and preserved for genetical estimation so that their sequences are required during the research work in the area of micro biology. The strains were identified by 16S rRNA gene and it was shown to be the members of action bacteria (Table 2).

The culture, which was labeled as Sample– SGIITD02 and SGIITD05 had the highest similarity (99%) to *Streptomyces* sp.LD48, Accession No. AY641538 and *Streptomyces* sp. A515Ydz-FQ respectively based on nucleotide homology and phylogenetic analysis.

Table 2: Identification of Bacterial Culture: Strain SGIITD02 and SGIITD05

Strain code	Details of similar species	Accession number	Identity (%)
SGIITD-02	*Streptomyces* sp.LD48	AY641538	99
SGIITD-03	*Streptomyces* sp.LD48	AY641538	98

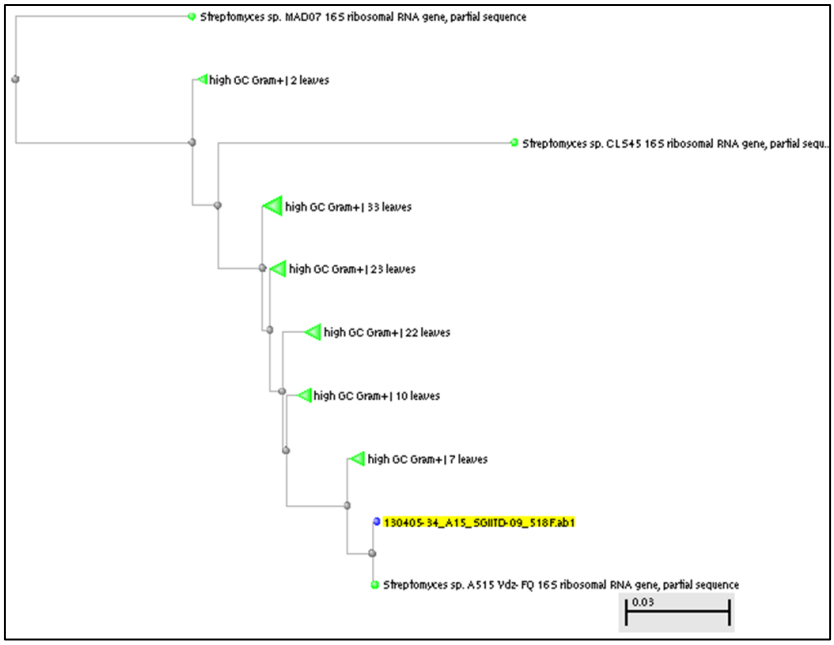

Fig 5: Construction of phylogenetic tree for soil samples of rhizosphere zone of litchi plant for Actinomycete strain SGIITD-09.

Discussion

Lignocellulose recalcitrance has hindered its utilization as a feedstock in fuel and chemical production; however, the current drivers as well as technological development have renewed interest in lignocellulose (Himmel *et al.*, 2007) [12]. Lignocellulose processing is envisioned to occur analogously to oil refining, meaning that the feedstock is efficiently utilized for the production of fuels, chemicals and energy in a concept called bio refining (Foust *et al.*, 2008) [11]. The biochemical processing route of lignocellulosic biomass aims at enzymatic depolymerisation of cellulose and hemicellulose to monomeric sugars that may be further converted to various desired chemical products, such as ethanol, butanol and alkanes by exploiting microbial metabolism (Fortman *et al.*, 2008) [10] or chemical conversion. The degradation of the complex polymer, cellulose and xylan of lignocellulose, has an application in bioethanol production through fermentation of sugars such as xylose, or glucose formed as by-products through the hydrolysis of xylan and cellulose, respectively. One limitation of bioethanol production through this means is the expense involved in the production of enzymes such as xylanase and cellulase (Lynd *et al.*, 2002) [18]. For bioethanol production to be commercially viable, the discovery of cellulase and *xylanase* with greater catalytic activity to degrade xylan and cellulose is necessary.

Researchers are trying their best to convert them into various value added products like biofuels, chemicals, and cheap energy sources for fermentation as well as animal feeds. The utility of lignin in the production of aromatics compounds like ethylene, propylene, benzene, toluene and xylene, which in turn produces ethanol to be used in various purposes. Ethanol is either used as a chemical feed stock or as an enhancer so the industrial production of ethanol is globally found to be useful. Soil microorganisms produce enzymes that degrade lignocellulose for use as an energy source for survival. Cellulose comprises the highest of the carbon sources in plants apart from lignin. Enzymes from actinomycetes act as a degrader of organic compounds when applied for agricultural usage.Some higher ascomycetes, like *Daldinia concentrica*, degraded Aspen wood (Nilsson *et al.*, 1989) [19], *Chrysonilia sitophila* could degrade rice hull and *Pinus radiata* bark products produce ligninolytic and cellulolytic enzymes (Ferraz *et al.*, 2000) [9]. Different *Penicillium* strains have been described as potential degraders of compounds with related lignin structures (Rodriguez *et al.*, 1996) [21] while the family *Xylariaceae* could produce *lac case* and peroxidase as potential ligninolytic enzymes (Liers *et al.*, 2006) [17]. The ability of actinomycetes to provide extracellular enzymes is high. Hydrolysis of lignocellulose is undoubtedly an important step toward proper utilization of abundantly available lignocellulosic material in nature. Many studies have been performed on cellulase and xylanase from bacteria and fungi, but actinomycetes have been explored to the lesser extent, especially regarding xylanase and cellulase production. (Kansoh *et al*., 2004) [14].

In the present study, an attempt has been made to isolate, identify and purify the members of microbial system, actinomycetes to confer resistance to natural hazards including antibiotics. A total of 28 strains in the present investigation were grown on different solid as well as liquid media and were thoroughly examined under microscope. A survey of strains keeping different parameters was allowed us to concentrate on 10 strains, which was done to illustrate the source of the rhizosphere zone of Litchi plant from fruit orchard. The idea to select rhizosphere of the fruit orchard of Muzaffarpur district owed land's virgin approach on the

microbial consortium. Moreover the area was fruit prone especially for Litchi, providing ample opportunity for carbon source for microbes. Thus, the aims and objectives of the investigation required isolation, identification of microbial strains and application of several parameters of PCR amplification of 16S rRNA (Chun and Good fellow, 1995) [6] and a few desirable genes with relation to enzyme production of industrial importance like lignocellulose, protease, xylanase, etc. by the strains (Dal Bosco *et.al.*, 2014) [7]. For this reason genomic DNA from bacterial strains was isolated using alkaline lysis method as described by Sambrook *et al*., (1989) [22] from actinomycetes isolate (SGIITD02 and SGIITD09). The isolates were then identified on the basis of 16S rRNA gene analysis. The analysis showed a maximum identity of 98% with *Streptomyces* sp. LD 48 strain; accession number AY641538 and 99% with *Streptomyces* sp. A515YdZ-FQ, accession number-EU384279.

Conclusion

In the present investigation the 28 strains were grown on different solid/ liquid media. Of which actinomycetes isolate (SGIITD02 and SGIITD03) strains were examined thoroughly by applying parameters of PCR amplification of 16S rRNA and a few desirable genes with relation to enzyme production of industrial importance like lignocellulose, protease, xylanase, etc. to illustrate the source of the microbial consortium of the rhizosphere zone of Litchi plant from fruit orchard. The 16S rRNA gene analysis showed a maximum identity of 98% with *Streptomyces* sp. LD 48 strain; accession number AY641538 and 99% with *Streptomyces* sp. A515YdZ-FQ, accession number-EU384279.

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