Original Research Article Website : www.ijbasr.org ISSN : 2349-1965

International Journal of Basic & Applied Science Research

[2015; 2 (2): pp 155-161]

16S rRNA PHYLOGENETIC ANALYSIS OF ACTINOMYCETES ISOLATED FROM FRUIT ORCHARD ASSOCIATED WITH LIGNOCELLULOSE DEGRADATION ACTIVITIES.

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Swati Priya, P. K. Roychoudhury and Santosh Kumar : 2014; "16S rRNA PHYLOGENETIC ANALYSIS OF ACTINOMYCETES ISOLATED FROM FRUIT ORCHARD ASSOCIATED WITH LIGNOCELLULOSE DEGRADATION ACTIVITIES". *IJBASR;* 2(2): 155-161.

ABSTRACT:

Lignocellulose has a complex structure composed mainly of lignin, hemicellulose and cellulose. Several enzymes are needed for the degradation of lignocellulose into simple sugars to produce bioethanol and it have economically valuable (Lynd, *et al.,* 1991). Novel enzymes can be discovered from environmental isolates that are known to produce a diversity of enzymes. Soil samples were collected from the rizosphere zone of litchi garden of BRA, Bihar University Muzaffarpur, Bihar, India and 20 different actinomycetes stains were isolated and it was observed that extracellular enzymes can degrade different lignocellulolosic substrates such as CMC, Xylan, Lignin and Mannan. It was also observed that out of total isolates most of the isolated strains belong to the genus *i.e.*, 70% *Streptomyces*, followed by 25% *Actinobacterium* and 5% *Nocardia*. Analysis of the 16S rRNA gene sequences and phylogenetic tree construction of the strains showed that strain SGIITD05 and SGITD02 had the highest sequence similarity *i.e.*, 99% to *Streptomyces* spLD48 and *Streptomyces* spA515Ydz-FQ respectively.

Key Words: Ligocellulolytic activity, *Actinomycetes*, 16S rRNA sequence, Phylogenetic tree.

INTRODUCTION

Actinomycetes are group of free living prokaryotic microorganisms belonging to gram positive bacteria and play an important role in the degradation of various organic materials to recycle the substances in nature. These organisms represent a ubiquitous group of microbes widely distributed in natural ecosystems around the world and subjected to produce bioactive principles.

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Date of Acceptance : 25.09.2015

Date of Publication : 30.10.2015

Actinomycetes are widely distributed in soil and they play an important role in degrading lignocelluloses components of plant cell walls (Lacey, 1973). Most of the studies were largely attributed to fungi and the ability of actinomycetes in degrading the lignocelluloses was neglected (Li, 2000). This group of bacteria is still being explored for new bioactive substances with diverse chemical structure and biological activities (Watve *et al.*, 2001; Jeffrey, *et al.*, 2007b). Alam, *et al.*, (2004) reported that isolates of *Streptomyces omiyaensis* are able to produce ligocellulolytic enzymes

Website : www.ijbasr.org ISSN : 2349-1965
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Bioconversions of lignocellulosic materials to desirable products involve complex processes which require a number of different enzymes (Alam, *et al.*, 2004). Lignocellulose is the major structural component of woody plants and non-woody plants such as grass. It is a major source of renewable organic matter. Lignocellulose consists of lignin, hemicelluloses and cellulose, (Fengel and Wegener, 1989; Eaton and Hale, 1993). Lignocellulose is the world's most abundant naturally occurring organic compounds which accumulate every year in large quantities in the form of agricultural, industrial, forest and residential waste. The potential of cellulose being a renewable source of energy was only recognized after the identification of cellulose degrading enzymes such as cellulase. In India, lignocellulosic materials such as agricultural and industrial residues are produced everyday by farmers and utilization of these residues may help in the production of animal feed, food and other organic products.

The conversion of lignocellulosic mass to fermentable sugars through biocatalyst extracellular enzyme derived from ligocellulolytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution. The aim of this study was to perform a pre-screening ligocellulose degradation by actinomycetes and similarity between different isolated strains.

MATERIALS AND METHODS:

Soil Samples Collection and Isolation of Actinobacteria:

Soil samples were collected from approximately 20cm below the soil surface from rizosphere zone of Litchi garden at B.R.A Bihar University, Muzaffarpur. The soil samples were kept in plastic bags and air dried at room temperature for 10 days. Air-dried soil was crush with mortar and pestle and dissolved in phenol solution (1.5%, 30 min at 30^0C) or wet heat in sterilized water for 15 min at 50^0C (Y. Takahashi, *et al.*, 1996 and Singh, *et al.*, 2014). It was diluted in 1:10 v/v with sterile 25% Ringer's solution and serial dilution to 10^4. 100 L of the 10^1, 10^2, 10^3 and 10^4 suspensions were spread in triplicate onto isolation media. Dilutions of soil suspensions were spread onto 6 different types of isolation media: ISP 2 (Yeast malt agar), ISP 7 (Tyrosine agar) (E. B. Shirling, *et al.*, 1966), Starch casein agar (SCA), Streptomyces agar (SA) (E. K¨uster, *et al.*, 1964), Actinomycetes isolation agar (AIA) (R. M. Atlas, 1993) and nutrient agar (J. F. Mac Faddin, 2000). All media were supplemented with cycloheximide (50mg/L), nystatin (50mg/L) and nalidixic acid (20mg/L) (S. T. Williams, *et al.*, 1965) and incubated at 30^0C 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 80^0C for

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long-term storage. Actinomycetes were isolated and confirmed through microscopic and molecular analyses by using 16S rRNA sequencing.

Qualitative Screening of ligninolytic Actinomycetes:

Lignocelluloses producing bacteria were screened on modified selective agar medium containing 0.2% KNO_3, 0.1% $KHPO_4$, 0.05% $MgSO_4.7H_2O$, 0.05% NaCl, 0.001% $FeSO_4$, 0.03% $CaCO_3$, 1.8% agar, CMC, Xylan, Mannan and Tanat acid. Plates were spot inoculated with spore suspension of pure cultures and incubated at 37^0C. After 48 hrs, plates were flooded with 1% Congo red solution for 15 mins. The diameter of zone of decolorization around each colony was measured (Khokhar, *et al.*, 2012). Bacterial identification was performed by colony morphology, microscopic observation reaction, biochemical tests and the Polymerase Chain Reactions (PCR), Sequencing (Cappuccino and Sherman, *et al.*, 1987). Colony morphology observation includes observation on the shape and colour colonies (Pelczar and Chan. 1988). All experiments were carried out in triplicate.

Enzymatic Screening:

All the isolates were screened for their cellulase producing ability using minimal medium agar (MMA) containing AZO-CM-Cellulose as substrate (Peptone -1.0gm; Yeast extract-1.0gm; $MgSO_4.7H_2O$ - 0.5gm; KH_2PO_4 - 0.5gm; $(NH_4)2$ - 1.0gm; Substrate (Megazyme) - 1.0gm; Agar-15.0gm and Distilled water - 1000ml) at pH 7. Gelatin hydrolysis assay as described by Frazier (1926) was used in the screening of protease activity. Lipase activity was screened for determination of esterastic activity (Sierra, 1957) with little modification. Formation of halo zone indicates positive reaction for the entire test conducted. Measurement of the halo zones were taken after 48 hrs of incubation (Frazier WC, 1926 and Sierra G, 1957).

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 with the available nucleotide sequences retrieved from the NCBI database by using BLAST and phylogenetic tree were constructed.

RESULTS AND DISCUSSION:

Culture of Isolated Bacteria:

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

Enzymatic Screening:

On the basis of test results, qualitative analysis of four isolates lignocellulose activity degrades ligno-

celluloses in a modified selective media (Fig. 1). A clear zone was observed around colonies of bacteria it means hydrolysis into peptides of lignocellulose is going on in this culture media. A wide clear zone width i.e., 18.00-30.00mm with a hydrolysis index ranges i.e., 1.9-3.9 (Table 1). In isolates SGIITD 05 have most extensive clear zone *i.e.,* 3.9mm as compared to other isolates and it can be expressed as a potent enzyme in hydrolysis index. Clear zone in Bacillus amyloliquefaciens is 35.85mm that is higher than that of Bacillus coagulants (15.40mm) in the CMC medium (Wizna, 2007). Observations demonstrate the ability to degrade Xylane, Mannan and

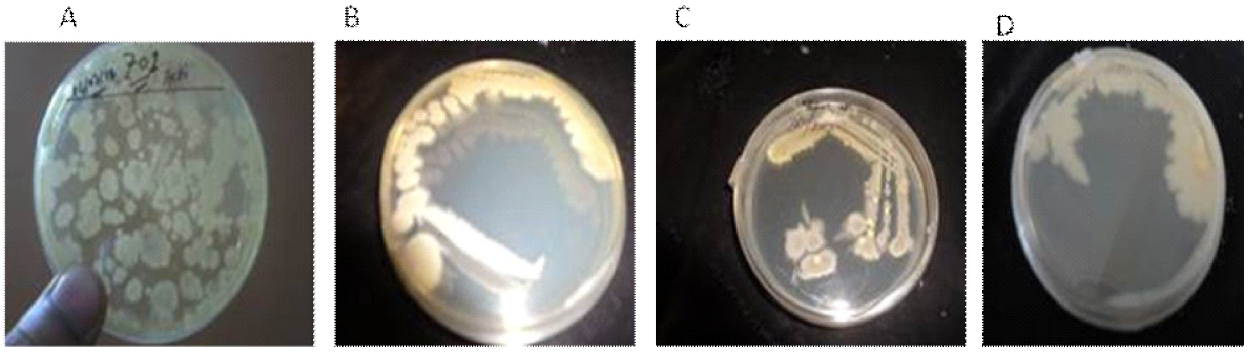

Fig-1: A to D Shows different Actinomycete strains, isolated from rizosphere zone of Litchi garden.

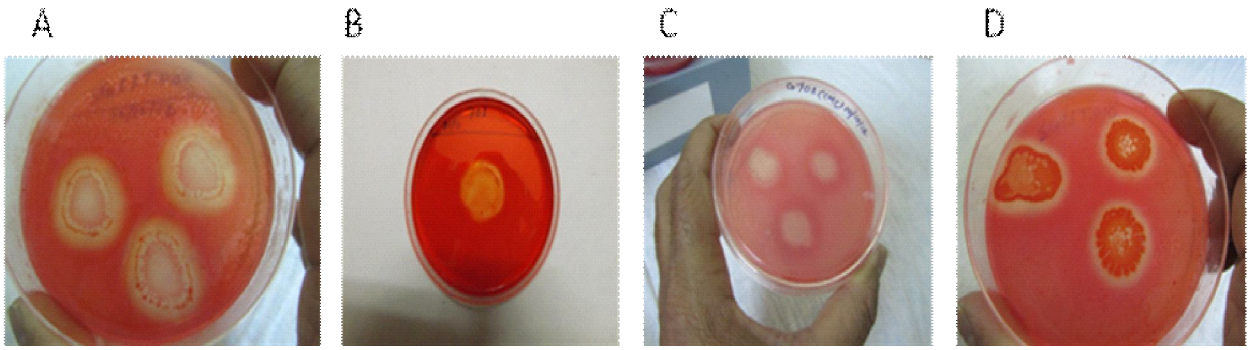

Fig- 2: Qualitative activity of ligninolytic Bacterial isolates on CMC, Xylan, Lignin and Mannan medium

Table: 1 Zone of the clearance and hydrolysis index using medium CMC, Xylan, Lignin and Mannan

No.	Isolates	Clear zone(mm)	Hydrolysis Index (HI)
1.	SGIITD 02	18.00	1.9
3.	SGIITD 05	30.00	3.9
4.	SGIITD 09	27.60	3.1
5.	SGIITD 10	24.25	2.7

Hydrolysis index = Ratio between the clear zone/Colony diameter

Cellulose is more powerfully but very weak ability to degrade lignin (Table 2). These results seen that consistent statement of xylan can be degraded by xylanase enzyme but very difficult to degrade the lignin and also reported by Bayer, et al., 1994. Tests conducted are not sufficient to determine the species of these isolates, due to variation in test results between species. To determine the species identification of SGIITD05 isolates was analysed by 16S rRNA gene sequences in bacterial taxonomic analysis.

Table: 2 Extracellular Enzyme Activity of Isolates SGIITD 05 showed zone of clearance in medium containing substrates CMC, Xylan, Lignin and Mannan Qualitative

Substrates	Clear zone
Cellulose (CMC)	++++
Xylan (from beech wood)	+++
Mannan (bean gum)	+++
Lignin (tanat acid)	+

+ = Enzyme activityTable:

Table:3 Three different species of Actinomycete strains of 16S rRNA gene sequence submitted at NCBI

Strain code	Details of Similar Species	Accession No.	Identity (%)
SGIITD-02	Streptomyces sp. LD48	AY641538	99
SGIITD-05	Streptomyces sp. A515Ydz-FQ	EU384279	99
SGIITD-09	Streptomyces sp. A515Ydz-FQ	EU384279	98

PHYLOGENETIC TREE:

The 16 S rRNA sequencing analysis of the isolate SGIITD05 yielded 1463 base pairs and NCBI BLAST search analysis showed that the sequence was 99% similar to the sequence of Streptomyces sp. A515Ydz-FQ strain. A Fast Minimum Evolution Method (Figure-6) for tree construction based on 16 S rRNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Streptomyces f*amily.

CONCLUSION:

In this study, the selected strains of actinomycetes were found to be potential degradation for activities of lignocellulolytic. Lignocelluloses degrading enzymes, especially from actinomycetes, requires a large number of isolates in order to discover novel bioactive extracellular enzymes in pharmaceutical interest. On the basis of above results of qualitative test, chemical test, molecular and phylogenetic analysis of the 16S rRNA of SGIITD05 isolates (Stretomyses) isolated from rhizosphere zone of litchi garden of Muzaffarpur might be suggested potentially degradation of lignocellulose.

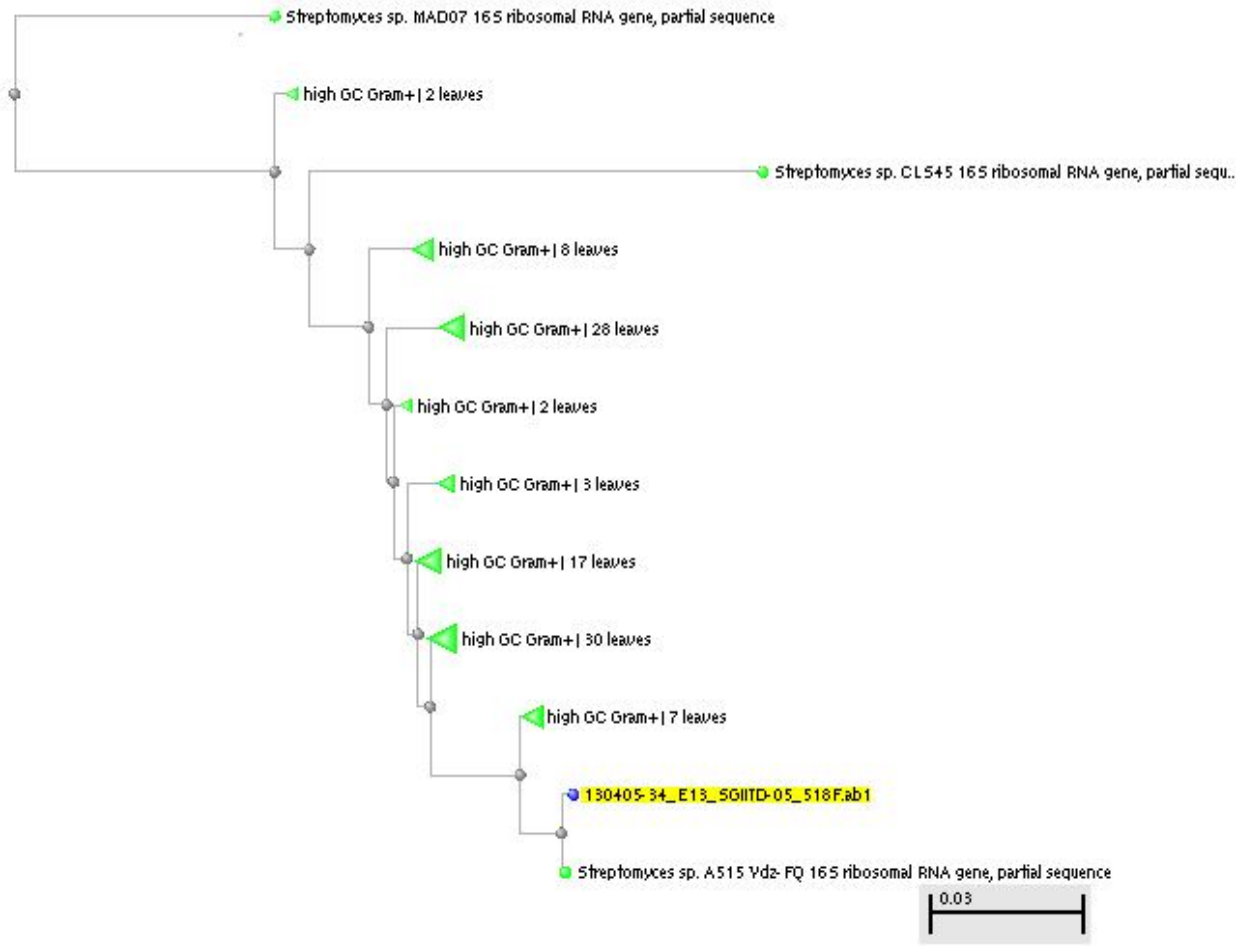

Fig: 3 Construction of phylogenetic tree of Actinomycete strain SGIITD-05.

ACKNOWLEDGMENT:

Authors are thankful to Head, Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Hauz Khas, New Delhi - 110016, (India) for providing laboratory facility and Dept. of Science and Technology (Govt. of India) for financial support.

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