# ISOLATION, SCREENING AND PRODUCTION OF XYLANASE PRODUCING BACTERIA FROM SOIL

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**Abstract**
Xylanases are a class of hydrolytic enzymes that degrade xylan which is present in lignocelluloses biomass. In this study 65 samples containing 32 soil samples were collected for isolation and screening purposes. Preliminary screening was done using Birchwood xylan by serial dilution and spread plate method. A total of four positive xylanase producing bacteria were recorded from the zone. Secondary screening by extracellular enzyme assay using DNS method showed highest enzyme activity for MV1 isolate which is 92U/ml. Morphological identification showed that the bacterial isolate as small, circular, slightly yellow, irregular, opaque , smooth, flat, shiny and gram positive rods. The positive strains were confirmed by 16SrRNA analysis. Blast analysis revealed that the sequence of strain MV1 found to be 99.84% similar to *B. Substilis* strain and the sequence length was 1340bp.

**Keywords**: Xylanase, Preliminary screening, Birchwood, *B. Substilis*

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## INTRODUCTION

Xylans, the hemicelluloses are the second most abundant polysaccharide**.** Xylanases (EC 3.2.1.8) catalyze the hydrolysis of xylan, the major constituent of hemicelluloses found in plant cell wall. (Kulkarni *et al.*, 1999). Xylanases are produced by diverse group of organisms. Xylanase is used in the pulp and paper industry, poultry feed additive, flour processing, flour, coffee, vegetable oil and starch extraction to improve the quality of baked goods and improve the nutritional properties of food and feed. Sources of plant fibers such as flax and hemp, jute and lamb, including pectinase and cellulase, are used to refine fruit juices and extract oils, and are used in conjunction with them.

Many microorganisms, including fungi and bacteria, produce 1, 4-β-D-endoxylanases and β-xylosidases (Kulkarni *et al.,* 1999; Subramaniyan and Prema, 2002). Microbial xylanases are divided into two groups according to their physical and chemical properties, such as molecular weight and photoelectric spots (Wong *et al.,* 1988). The main group consists of high molecular weight xylanases with low pI values and the second group consists of relatively low molecular weight xylanases with high pI values (Kuno *et al* ., 2000)

Bacterial xylanase produces more xylanase than most fungal enzymes. *Bacillus subtilis* Organelle produces high xylanic acid activity at alkaline pH and high temperature. (Subramaniyan and Prima 2000; Subramaniyan *et al* ., 2001). Streptococcus Several types of filamentous fungi, including: NS. injection, *S. Cuspidosporus* is also a potent producer of indoxylanase, xylanase, and polygalacturonic (Maheshwari and Chandra, 2000). *Caldocellum saccharoticum* sp. Anaerobic heat resistance. Used in the production of xylanase and endoxylanase.

## MATERIAL AND METHODS

### Sample collection

The samples for the study were collected from Wayanad District, Kerala [Muthanga, Tholpetty, Thrikaipatta, Padinjarethara, Kidanganad, Padinjarathara, Mananthavady, Sultan Bathery, Vythiri, Pulpally, Kalpetta, Ambalavayal]. The samples were collected in sterile plastic bags and were sealed and brought to the lab aseptically for further processing.

### Identification of efficient bacterial isolates

For isolation, one gram of soil sample was weighed and diluted in 10 mL of sterile distilled water and vortexed for a few minutes. The serial dilution was done up to five dilutions. 0.1 mL from the dilutions $10^{-3}$, $10^{-4}$, $10^{-5}$ was taken and spread evenly all over the surface on nutrient agar plates using an L-rod by spread plate method. These plates were incubated *at 37o C* for 24 hours. The Petri plates were observed for the

appearance of bacterial colonies. The bacterial isolates were then transferred and cultured on birch wood xylan agar plates as part of the screening process.

**Preliminary screening**

The screening for xylanase producing bacteria was done on birch wood xylan agar plates. The media for bacteria consisted of (g/L): Birchwood xylan 2.5g, Yeast extract 5g, Peptone extract 5g,$MgSO_4$ 0.2g, $K_2HPO_4$ 1g and Agar-Agar 15g. The selected bacterial isolates were grown on birch wood xylan agar media as a part of screening by selective media. The plates were incubated at room temperature for 24 hours. After the incubation period, growth was observed and then 1% of Congo red solution was added to the plates and waited for 10- 15 minutes. The Congo red staining was washed off with 1N NaCl solution, which is distaining, was done. The formation of clear zones around the colonies indicates bacterial degradation. Bacterial colonies that showed clear zones were selected and streaked on xylan agar plates and maintained as a pure culture for further studies.

**Secondary screening**

Xylanase Production the selected isolates were tested for their ability to produce extracellular xylanase enzymes. The cultures with zones of hydrolysis were selected and subjected to submerged fermentation to analyse their ability to produce xylanase enzymes as a part of quantitative screening. The selected isolates were cultured in liquid xylan media with the same composition as birch wood xylan medium, except agar is not added. The fermentation media consists of (g/L): birch wood xylan 2.5g, yeast extract 5g, peptone extract 5g, $MgSO_4$ 0.2g, and $K_2HPO_4$ 1g. The enzyme production was carried out in 100 mL Erlenmeyer flasks containing 20 mL liquid Birch wood xylan media and incubated overnight in a shaking incubator for 24h at 37˚C at 150 rpm. To determine the xylanase activity, it is important to obtain the cell free extract. The xylanase extraction was done to determine the activity of enzymes. About 2 mL of sample from the culture media was taken and centrifuged at 10000 g for 10 minutes at 4 ˚C (Cooling centrifuge) then, the supernatant was collected and pellet was discarded. The collected supernatant was then used for extracellular enzyme assay for testing xylanase activity.

**Xylanase Activity Assay**

Xylanase assay was done to determine the activity of the enzyme xylanase. The xylanase enzyme activity was done by measuring the reducing sugar released by the reaction on the birch wood xylan. Thus, the xylanase assay was done according to 3, 5 – dinitro salicylic acid (DNS) method. The amount of enzyme produced by each isolate in liquid xylan medium was found. 1.0% of the birch wood xylan was dissolved in 50 mM Glycine-NaOH buffer (pH – 9.2) and this was used as substrate. 0.5 mL of the buffered substrate (1.0% birch wood xylan and 50mM Glycine NaOH buffer) was reacted with 0.5 mL of crude xylanase enzyme at a temperature of 55℃. The reaction was stopped after 10 minutes by adding 3 mL DNS reagent and then kept in a boiling water bath for 5 minutes. After cooling for a few minutes, the released xylose was quantified at 540 nm against a reagent blank. A reagent blank was made in the same manner except the crude enzyme was not added and 0.5 mL buffer was added. An enzyme blank was also made in which the reagent was added before the addition of enzyme so that only the reducing sugar is estimated. A standard of xylose (reducing sugar) was prepared using stock concentration 1 mg/mL. One unit of xylanase enzyme activity is defined as 1 µmole of xylose liberated per minute per mL of enzyme preparation under standard assay conditions.

Standard Curve: ΔA540nm Std = A540nm Std - A540nm Std Blank

Prepare a standard curve by plotting the ΔA540nm Standard vs the µmoles of Xylose.

Sample Concentration Determination:

ΔA540nm Sample = A540nm Test - A540nm Blank

Determine the µmoles of xylose using the Standard Curve (µmoles of xylose liberated) x (df)

$U/ml$ = ($\mu molesofxyloseliber$) $x$ ($d$ ) / (10) $x$ (0.5)

df = Dilution factor 10 =Time of assay (in minutes) as per Unit Definition

0.5 = Volume (in millilitres) of enzyme used units

**Morphological and microscopical identification**

Morphological characterization of microbes was commonly performed to distinguish the microbes based on colony and cellular morphologies. Morphological analysis was performed for colonies of strains on solid media. Size, shape, and staining by gram staining was analysed using a confocal microscope.

**Gram staining**

A thin smear bacterial suspension was prepared on a clean, grease-free slide which was allowed to air dry. The smear was heat fixed on a flame. After fixation, the smear was flooded with crystal violet stain and allowed to stand for one min. The excess stain was drained off and smear washed gently

with tap water. Gram's iodine solution was then added to the smear and allowed to act for one min. This was followed by washing under tap water until excess of the iodine solution was removed. The smear was then decolorized by adding acetone alcohol for a brief period of 5 to 10 sec. It was again washed gently with tap water and the slide was flooded with Safranin, which is a counterstain. This stain was allowed to act for 20 to 30 sec. The smear was again washed gently with tap water followed by blot drying. Finally, the stained smear was examined under the oil immersion lens at 100X of the compound microscope. The cells were differentiated on the basis of stain taken up by the bacterial cells. Gram-positive bacteria were stained either blue or purple, while Gram-negative bacteria were stained either pink or red by this procedure.

**Molecular identification of efficient bacterial strains using 16S PCR**

Genomic DNA isolation by PCI method Isolates were cultured overnight (new) was centrifuged at 13,000 rpm for 10 minutes, and the supernatant was disposed of deliberately. 200 µl of cell lysis buffer was included and vortexed, at that point, 20 µl of proteinase K (50 µg/ml) was included and marginally vortexed. The tubes were stored at 55°C for an hour and a half in a water bath (microtubes were fixed with parafilm). 220 µl of PCI solution (saturated Phenol: Chloroform: Isoamyl alcohol; 25:24:1) was included and after that blended by rolling the tubes between the palms. The tubes were centrifuged at 13,000 rpm for 15 minutes and the upper layer was taken in new microtubes. Again 220 µl of PCI solution was included and centrifugation was rehashed for a moment time at 13,000 rpm for 15 min. The upper layer was taken in a new microtube. A parallel measure of chloroform was added to the upper layer. The tubes were centrifuged at 13,000 rpm for 15 minutes and the fluid stage was gathered and exchanged to new microtube. A two-fold volume of chilled absolute alcohol was included and left overnight in the -20°C fridge (kept for 1-2 hours). The tubes were centrifuged at 13,000 rpm for 20 minutes to get a DNA pellet, and the supernatant was disposed of. 300 µl of super cold 70% alcohol was included for washing the pellet taken after by centrifugation at 13,000 rpm for 10 minutes. The ethanol was painstakingly poured off and was permitted to dry for 30-45 minutes at 37°C (in an incubator). 50 µl of hydration buffer (1x MilliQ TE) was added and permitted to rehydrate at room temperature for 10 minutes and afterwards put away in a 4°C cooler. Electrophoresis was performed on a 1% agarose gel for checking the quality and quantity of DNA.

**Agarose Gel Electrophoresis**

Agarose gel at a concentration appropriate for separating the particular size fragments expected in the DNA sample was prepared in 1X TBE. The slurry was heated in a microwave oven till the agarose got dissolved. When the molten gel was cooled to around 50 °C, ethidium bromide was added to a final concentration of 1µg/ml. The slurry was mixed by gentle swirling. A comb of desired well size was placed in a gel caster and the agarose slurry was poured in the gel tank and allowed to solidify. The comb was removed carefully and the gel was put into the tank filled with a running buffer (1X TBE). The samples mixed with DNA loading dye were loaded in the wells. The gel was run at appropriate volts and thereafter, the DNA was visualized under UV light using the gel documentation system.

**Identification of potential strains by 16s rDNA-**

PCR the positive strains were confirmed by 16S ribosomal RNA (rRNA) analysis. PCR amplification was performed using the 16S rDNA. Two primers namely forward primer 5´-AGAGTTTGATCMTGG-3´and the bacterial specific reverse primer 5'ACCTTGTTACGACTT-3' were selected for PCR amplification experiments. PCR amplification was performed with initial denaturation at 95°C for 5 min, followed by 30 cycles each at 94°C for 45 s, at 52°C annealing for 45 s and at 72°C extension for 45 s followed with final elongation at 72°C 8 min. The amplified DNA was resolved on 1% agarose gel which was prepared in a 1x TBE buffer with ethidium bromide (EtBr) lµg/ml

**Phylogenetic Analysis**

Blast analysis the sequences were then used for the similarity check using Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) to identify the organism.

**Phylogenetic tree construction**

Representative sequences of similar neighbors in BLAST analysis were retrieved and aligned using multiple alignment programs. The multiple alignment file was used to create a neighbor-joining tree using MEGA-X

**RESULTS**

**Sample Collection**

The isolation and screening for search of thermo stable alkalophilic xylanase producing bacteria

was initiated in three stages. In the preliminary stage of isolation, various probable samples like soil, decaying wood, dung and decomposed lingo cellulosic material were collected from Wayanad District from different zones. Each zone consisted of specific sampling sites.

**Identification of efficient bacterial isolates**

A total of 65 samples comprising 32 soil samples were collected for isolation and screening purposes. The samples were diluted appropriately and the highest dilutions were spread plated on nutrient agar. In general soil derived from forest areas or dense plantation areas proved to be a better sampling site for isolation of bacteria possessing hydrolytic activity.

**Preliminary Screening**

Enzyme activity was observed from the zone of hydrolysis on birch wood xylan medium for xylanase enzyme. Based on the zone of hydrolysis we confirmed that the isolated organism produces xylanase enzymes.

In this study, the samples were collected from wood decaying soil for isolating producing bacteria. Six bacterial isolates were obtained on nutrient agar plates from one region of soil (A) and 3 bacterial isolates were obtained on nutrient agar from other region of soil (B) and by quantitative screening on beech wood xylan agar medium using Congo red assay method only 3 bacterial isolates from A and 1 bacterial isolates from B were selected on the basis of clear zone of hydrolysis. The Congo red dye is known to interact with (1,3- and 1,4-) β-D-glucans. The clear zones formed after staining with Congo red dye indicate that produced by the bacterial isolates hydrolyses the xylan backbone resulting in a mixture of xylo-oligosaccharides. The formation of zones on xylan media is due to high xylan content in media, hence degraded by. The bacterial isolates producing clear zones were maintained on nutrient agar plates.

In the second stage of screening process, all the isolates obtained from the preliminary screening step were allowed to grow on xylan agar medium wherein the carbon source. A total of 4 positive xylanase producing bacteria out of 65 were recorded from the zone. Maximum number of isolates was isolated from soil as a sampling source. It was observed that a maximum of 4 bacterial isolates having the ability to grow on xylan agar medium were recorded from soil samples obtained from Forest soil with isolated identities as follows: PT3, PT6, MV2, &MV1.(Fig.1)

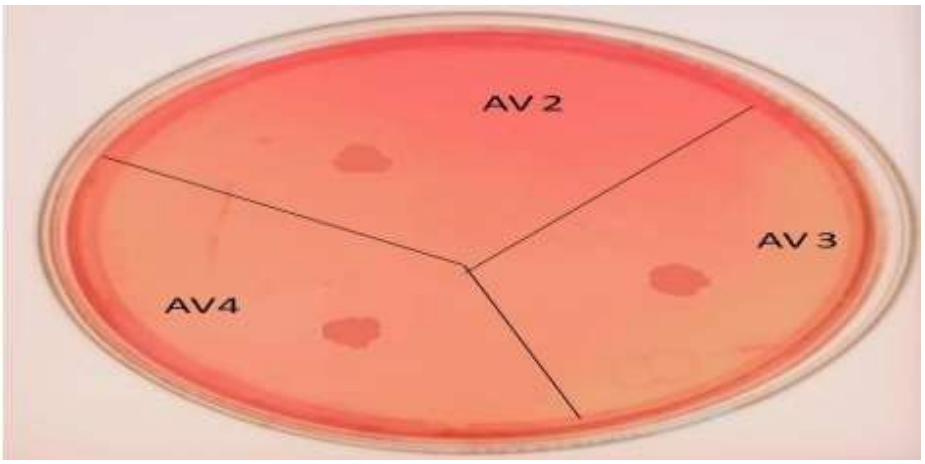


**Fig. 1.** Primary Screening of Xylanase using Congo red

Forest soil samples from Padinjarathara and Mananthavady soils contributed four xylanase producing bacteria. No positive isolates were obtained from other soil samples. Soil samples collected from wood degraded soil proved to be better sampling locations as attributed by the large number of xylanase producing bacteria isolated from these locations.

**Secondary Screening**

The collected supernatant was then used for extracellular enzyme assay for testing xylanase activity (Fig.2).

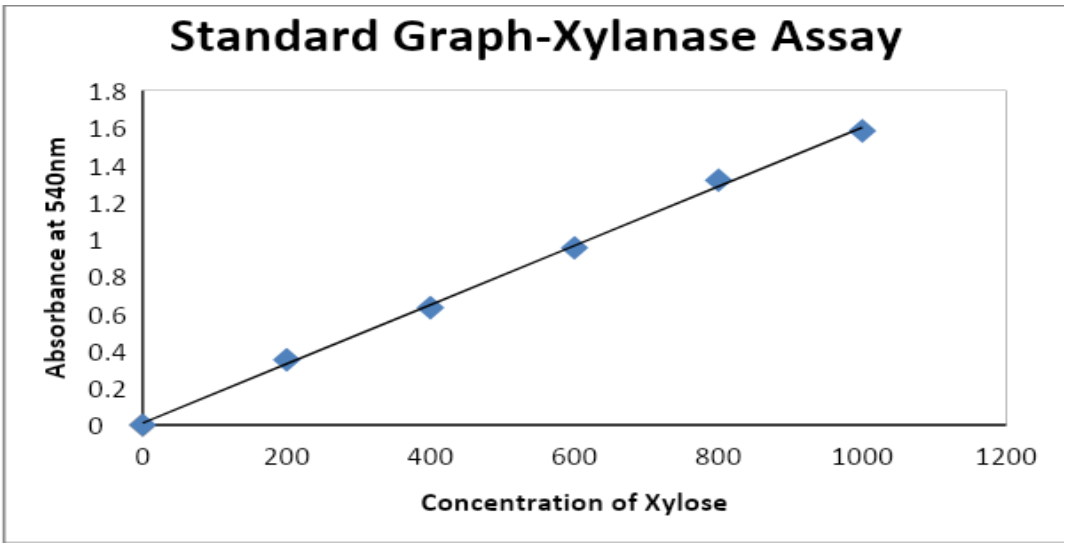


**Fig. 2.** Secondary Screening of Standard graph Xylanase assay

**Xylanase Activity Assay**

The DNS method was adopted for determining the activity. The isolate that showed highest enzyme activity was selected and further studies were carried out. The bacterial isolate that showed highest activity was a wood decay sample.

The enzyme activity was calculated from the slope of the graph with the help of the equation given below:

**Enzyme Activity = (µmoles of xylose liberated) (dilution factor) / (time of assay) (volume of enzyme used.**

MV1 bacterial isolate showed highest enzyme activity and that was 92U/ml. Further, molecular characterization and 16sRNA gene sequencing of the isolate was done to identify the organism.(Fig.3).

**Fig. 3.** Pure culture of producing bacteria (MV1)

**Morphological and microscopical identification**

Morphological and micros copical identification showed that the bacteria are small, circular, slightly yellow, gram positive rods (Table. 1).

**Table.1.** Identification and morphology of MV1

| **Characteristics** | **MV1** |
|---|---|
| Size | Small |
| Shape | Circular |
| Colour | Slightly yellow |
| Margin | Irregular |
| Opacity | Opaque |
| Consistency | Smooth |
| Elevation | Flat |
| Texture | Shiny |

| | |
|---|---|
| Gram nature | Gram Positive Rods |

**Molecular identification of efficient bacterial strains using 16SPCR**

The positive strains were confirmed by 16S ribosomal RNA (rRNA) analysis. PCR amplification was performed using the 16S rDNA gene. The genomic DNA was resolved on 1% agarose gel which was prepared in a 1x TBE buffer with ethidium bromide (EtBr) lµg/ml. The MV1 isolate was identified by PCR using primers. Primers amplified on 1500bp PCR product band. The band was visualized using Ethidium Bromide staining of agarose gel (Fig. 4,5,6).

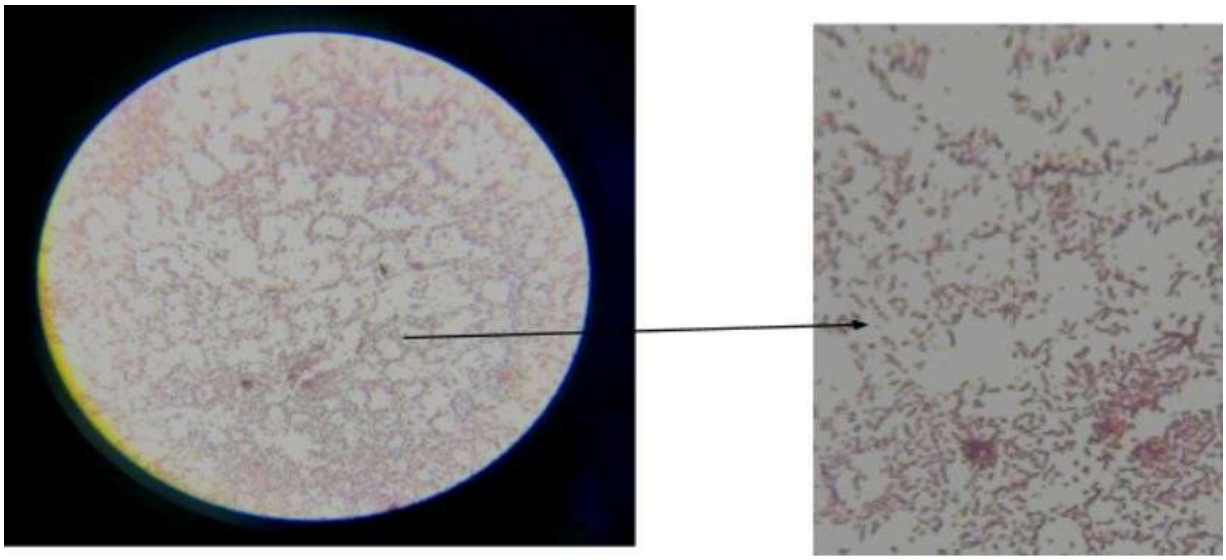

**Fig. 4.** Gram Staining of MV1

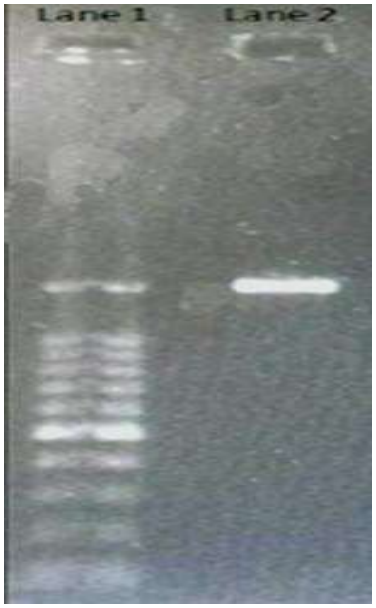


**Fig. 5.** Lane 1: Genomic DNA

**Fig. 6.** Lane 1: 100bp Ladder, Lane 2: PCR amplification of 16S rDNA

**Phylogenetic Analysis & Blast analysis**

Blast analysis revealed that the sequence of strain MV1 found to be 99.84% similar to *B. subtilis* strain and the sequence length was 1340bp. The sequence was submitted to GenBank NCBI and the accession number is OM992316 (Fig.7).

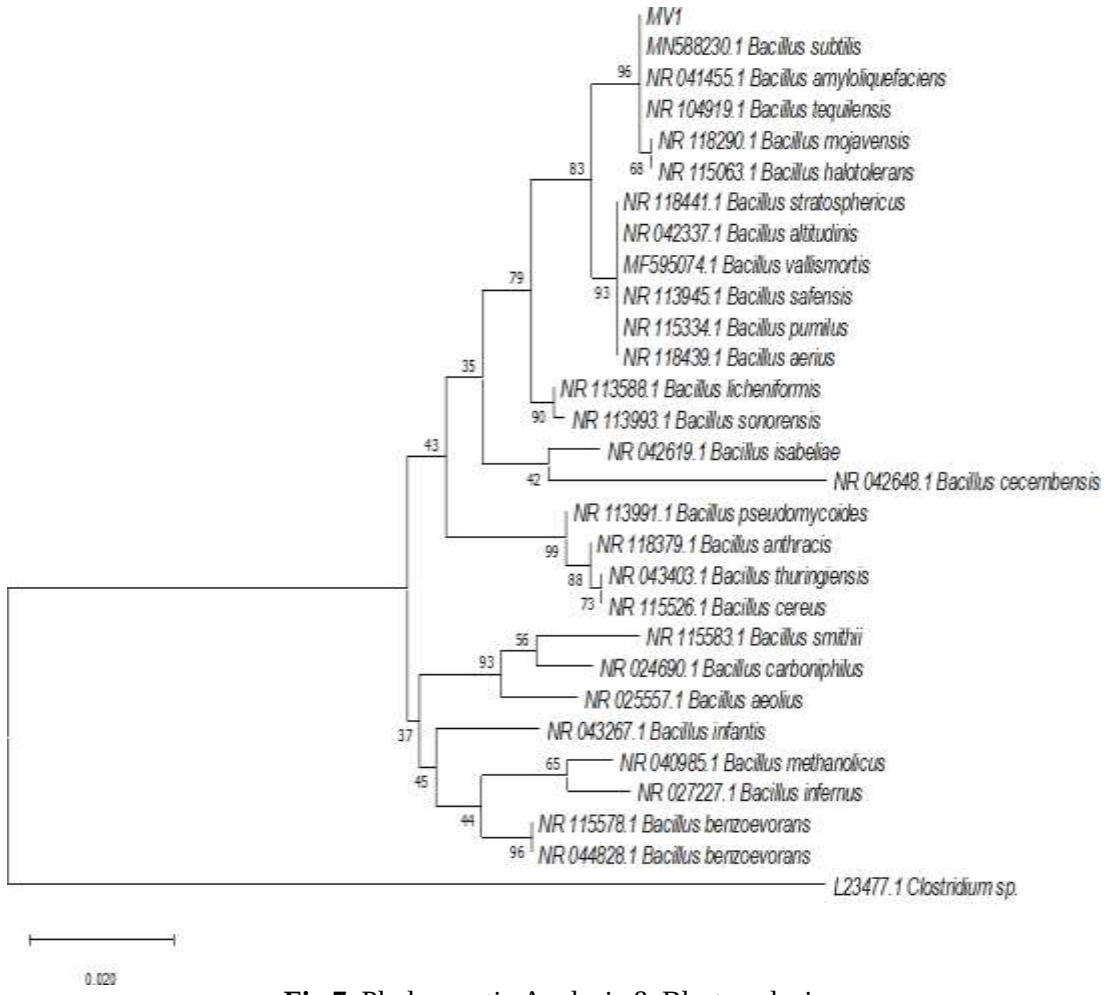


**Fig.7.** Phylogenetic Analysis & Blast analysis

**Evolutionary analysis by Maximum Likelihood method**

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-2090.79) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 661 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Fig.8).

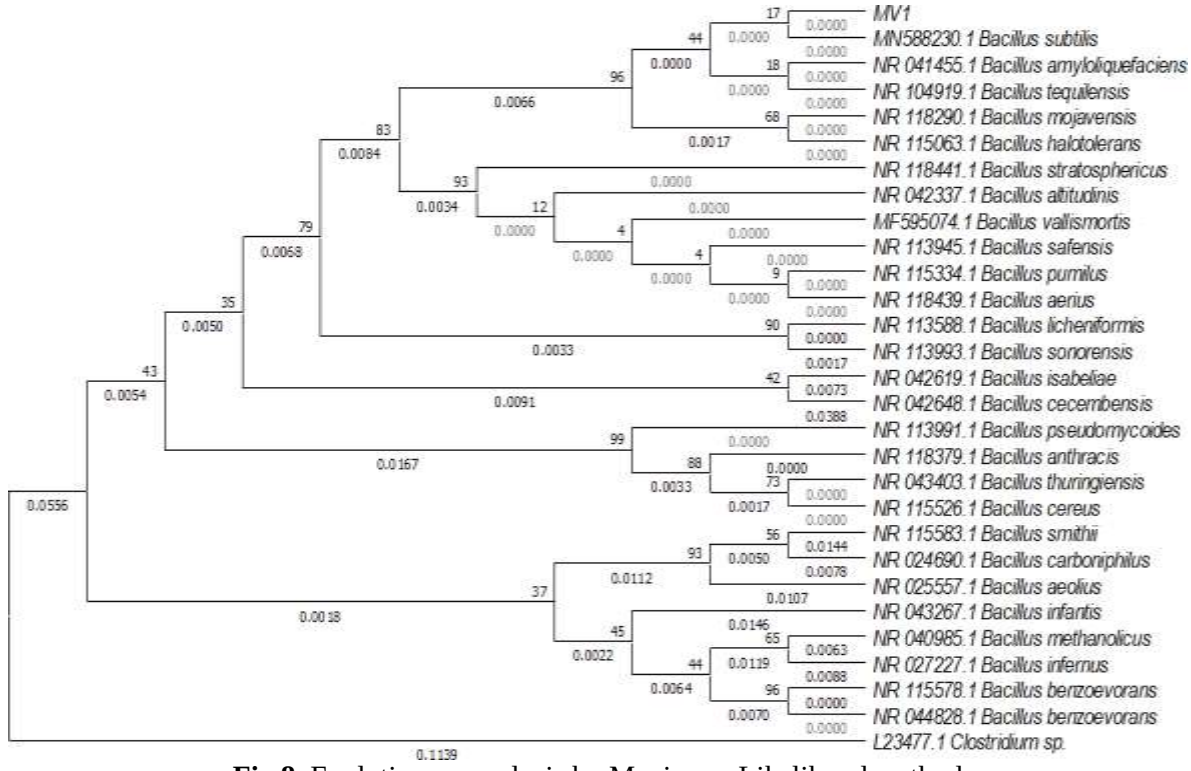


**Fig.8.** Evolutionary analysis by Maximum Likelihood method

## CONCLUSION

From the present study it can be concluded that a potent Xylanase producing Bacterial isolate was MV1 .soil derived from forest areas or dense plantation areas proved to be a better sampling site for isolation of bacteria possessing hydrolytic activity and screened on Birchwood xylan Agar media using Congo red assay method. Bacterial isolate was identified as *Bacillus substilis*based on conventional and 16S rRna sequencing method. Potent isolate produced 92 U/mlof enzyme. Blast analysis revealed that the sequence of strain MV1 found to be 99.84% similar to *B. subtilis* strain and the sequence length was 1340bp.

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## CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest

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