# IDENTIFICATION, SCREENING AND MOLECULAR CHARACTERIZATION OF XYLANASE PRODUCING BACTERIA FROM SUGARCANE FIELD

V.PUSHPA RANI[*1], J.DELPHINE JAYAMARY[2], ALEXIE[3]
[1]Assistant Professor
[2]Ph.D Research Scholar,
[3]M.Sc,
[1,2,3]PG &Research Department of Advanced Zoology and Biotechnology,
Loyola College, Nungambakkam, Chennai-600 034. Tamil Nadu, India

**ABSTRACT**
**Background:** Enzyme xylanase belongs to the glucanase enzyme family. Xylanase 5, 7, 8, 43 has been identified and studied to a lesser extent. Its applications are very useful in various fields. Further applications for xylanases may still arrive in the future. So screening of xylanase producing microorganisms still need more studies. **Objective:** To identify, screen xylanase producing bacteria from sugarcane field and molecular characterization of the organism. **Materials and Methods:** This study was conducted through molecular characterization methods. **Result:** Through screening of xylanase producing organism out of 10 bacterial strains 1 strain produced better zone. This strain was isolated for studies. Then the colony was stained using Gram staining procedure. It was identified that it was Gram positive bacteria, spherical shaped cocci. Then DNA isolation process was done to isolate Genomic DNA and it was made to run in the Agarose gel for DNA visualization and observation. As the DNA was observed the sample was allowed to run for polymerase chain reaction. The perfect DNA band was obtained at 48° C. A visible thick band of DNA was observed it shows the presence of Xylan. Then the sample was sent to (bar coding) sequencing and the organism identified as *Bacillus pumilus* X8 16S ribosomal RNA gene with 99 % accuracy and the identified organism was placed in Blast (Bio-informatics).**Conclusion:** It is concluded that enzyme xylanase 8 producing organism is *Bacillus pumilus* strain. It is 16S ribosomal RNA gene and its partial sequences length is 1450.
**KEYWORDS:** *Bacillus pumilus*, Molecular Characterization, 16S ribosomal RAN gene, Xylanase 8 enzyme.

## INTRODUCTION

**Xylan** is one of the most abundant polysaccharide in nature. About one third of the dry weight is comprised of xylan in higher plants. It is a complex heteropolysaccharide consisting of a linear back bone made of β-1,4 linked xylose glycosidases (*O*-glycoside hydrolases, EC3.2.1.x) which catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan.

Xylanases are hydrolytic enzymes which randomly cleave the β 1, 4 backbone of the complex plant cell wall polysaccharide xylan. Xylanase is classified into different families like xylanase 5, xylanase 7, xylanase 8, xylanase 10, xylanase11 and xylanase 43. Enzyme xylanase belongs to the glucanase enzyme family. xylanase 5,7,8,43 has been identified and studied to a lesser extent **(Collins *et al.*, 2004).**

Xylanases have significant impact on an industrial scale; they will need to be consistently effective under various operating conditions. Currently research is being directed towards the discovery of enzymes that are more robust with respect to their pH and temperature kinetics. Techniques used include protein engineering by identification of active site residues through chemical modification, X-ray crystallography and site-directed mutagenesis **(Kulkarni *et al.*, 1999; Beg.Q.K *et al.*, 2001).**

**Xylanases** used in **bioremediation** for the **treatment** of **agriculture** and **food industry wastes** ,bioconversion process such as the production of **fermentable products, renewable fuel(bio ethanol) ,food industries** for baking, milling, pastry, confectionery, and in coffee processing , in starch industry the use of xylanase improves the gluten starch separation, in paper industries xylanase improves the efficiency and paper strength and also facilitates the drinking process and reduce the use of alkalie in the process etc., Further applications for xylanases may still arrive in
the future.

***Bacillus*** species are Gram-positive, endospore-forming, chemoheterotrophic rod-shaped bacteria which are usually motile with peritrichous flagella; they are aerobic or facultative and catalase positive. **(Waltes *et al.*, 2008).** Members of the *Bacillus* genus are generally found in soil and represent a wide range of physiological abilities, allowing the organism to grow in every environment and complete desirably with other organisms within the environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms (**Kuta *et al.*,2008).**

Many *Bacillus* species are of remarkable importance because they construct antibiotics. (**Waltes *et al.*, 2008).**The potential of bacillus species to synthesize a wide variety of metabolites with antimicrobial activity has been widely used in medicine and pharmaceutical industry; one of its abilities is to control various diseases in animals, humans and plants when applied as a biological control agent (**McKeen 1986*;* Silo-Suh *et al.* 1994; Leifert *et al.*, 1995).**Owing to the fact that bacillus species have constructed antibiotics in the soluble protein structure have been inexpensive and more effective in studies accomplished to date, these microorganisms are desirable for commercial production **(Priest *et al.*, 1989;Debabov *et al.*,1982).**

Xylanases produced by fungi, yeast and
bacteria, filamentous fungi are preferred for commercial production as the levels of the enzyme produced by fungal cultures are higher than those obtained from yeast or bacteria **(Haltrich *et al.*,1996).**

***Bacillus pumillus*** is a non-toxigenic, non-pathogenic naturally occurring microorganism that is especially common in soil and on dead plant tissue. It is rod shaped Gram positive, aerobic bacterium. **(Pleus *et al.*, 2014).**

## MATERIALS AND METHODS

### SAMPLE COLLECTION

The decaying plant biomass samples were randomly and aseptically collected from sugarcane field in Chennai. The sample were aseptically collected and placed in sterilized containers and immediately transported to the laboratory for processing.

### SCREENING OF XYLANASE PRODUCING ORGANISM

Xylanase producing pure strains were isolated using the screening medium that contains xylan from oats as a substrate. The pure cultures were streaked at the center of the sterile xylan agar plates and the plates were incubated at 37° C for 24 hour for isolation of pure colony. Then screening for xylanase producing organism was done for the production of extracellular organisms
**(Ten. *et al.*,2003).**

### GRAM STAINING

The smear was heat fixed and covered with crystal violet reagent on a slide for 1 minute. The slide was rinsed with water and flooded with grams iodine for 1 minute followed by rinsing with water. Then the smear was covered with safranin for 1 minute and rinsed with water. It was air dried and observed under oil immersion objective. The morphological characterization was observed by gram staining using the light microscope **(Harrigan *et al.*, 1966).**

## XYLANASE ENZYME ASSAY

### BACTERIAL CRUDE CULTURE PREPARATION

After production of culture suspension was centrifuged at 5000rpm for 15 minutes and cell free extract was subjected to enzyme assay.

### PLATE ASSAY

The plate assay was performed using agar plates amended xylan from oats the agar plates .After solidification of agar around 10mm diameter of well

was cut out aseptically with the help of corkborer. The well was filled with the culture filtrate and incubated at 37°C for overnight. The observation was made to see the hydrolytic zone around the well for better appearance of the zone around 0.1% of Congo red solution was over layered on the agar plates and kept for 15 minutes. Destaining was made using 1M NaCl to make the zone visible and clear.

**Genomic DNA Isolation CTAB method**

Culture (1.5ml) was transferred to a micro centrifuge tube and centrifuged at 10,000 rpm for 2 minutes at 4°C.The pellet was collected and to that 467 microliter of proteinase k .This was incubated for 1 hour at 37° C. After incubation equal volume of phenol: chloroform was added (12:1) and centrifuged at 12,000 rpm for 12 minutes at 4° C. The supernatant and 1/10th volume of 3M sodium acetate was added and twice the volume of ethanol was added and centrifuged at 12,000 rpm for 15 minutes at 4° C. Then the supernatant was discarded and it was air dried and pellet was dissolved in 100 microliter to TE buffer .Then 18 microliter of sample was taken and mixed with gel loading dye bromophenol blue. **(Sarvananda *et al.*,2016).**

**Qualitative Analysis - Agarose gel electrophoresis**

Ethidium bromide (5ul) was added per 25 ml of agarose gel once the temperature of the agarose solution reaches 600°C. Agarose gel was poured in the central part of the tank and kept for solidify. Then 1 x TAE buffer was poured into the gel tank till the buffer level stand at 0.5 to 0.8 cm above the gel surface. Power supply was loaded about 20 µl in the wells and the voltage was set to 50 volts. This process was done till the sample runs to ¾ of the gel for 1-2 hours

**(Sarvananda *et al.*, 2016).**

**DNA Quantification-spectrophotometric Method**

Measure absorbance of the solution at wavelengths 260 and 280nm and ratio is calculated.µ is an absorbance (A260) of 1.0 corresponds to 50µg ds DNA/ml of the solution respectively. From this, the concentration of DNA in the test sample can be calculated. **(Sarvananda *et al.*, 2016).**

**Qualitative Analysis- Agarose gel electrophoresis**

Gel loading buffer (2µl) was added to each of the PCR tubes. Pipette out 20µl of the sample and load into 0.2% agarose gel along with a 100bp DNA ladder. Run the samples at 50volts for 1-2 hours till bromophenol blue present in the gel loading dye reaches 3/4th of the length of the gel (**Sarvananda *et al.*,2016).**

**POLYMERASE CHAIN REACTION (PCR)**

Denaturation of two strands melt to form single strand DNA is carried out at temperature ranging in 92-96. Annealing of primers to each original strand for new strand synthesis is carried out between 45-55° C. Extension at 72°C the polymerase adds dNTP's complementary to the template at the 3 prime end of the primer. These three steps are repeated 20-32 times an automated thermocycler. . **(Fredricks *et al.*, 2005).**

**BLAST**

Click on BLAST NCBI home page, click to wavelengths 260 and 280nm open nucleotide BLAST. Paste the Nucleotide query sequence in the submission box. In choose search set; click Nucleotide collection (Nr/nt) for database. Click show results in new window. A request ID is given and the result page appears in few seconds (**Johnson et al., 2008).**

**RESULTS AND DISCUSSION**

Screening of xylanase producing bacteria from sugarcane field and molecular characterization of the organism has been revealed through various methods and the results are given below:

**Screening of xylanase producing organism**

The screening for xylanase producing organism was done for the production of extracellular organisms. Because most of the plant wastes are degraded by the native microbes that are growing over that waste. In such away the microbes which are isolated from the sugarcane field may have ability to produce xylanase. From the samples around 10 bacterial strains were isolated. But later during screening it was found that 1 strain produced better zone. This bacterial strain was considered for further study. The growth study of the organism is essential for the production of enzymes because most of the extracellular enzymes are produced during log phase of the organism.

**Gram staining**

Through this common staining technique gram positive bacteria spherical shaped cocci was identified. Due to the presence of a thick layer of

peptidoglycan in their cell walls, they retain the crystal violet on their cells.

**Genomic DNA Isolation**

The genomic DNA was isolated this is a basic necessity for genome characterization, gene mapping procedures and for the identification and isolation of genes for genetic engineering.

**Qualitative Analysis -Agarose gel electrophoresis**

Visualization and observation of DNA and purification were done through this valuable method.

**DNA Quantification by UV Spectroscopy**

When ethidium bromide was added to agarose gel and electrophoresis. The clear visible of DNA bands was observed under UV transilluminator. To quantify DNA, DNA quantification was done before performing DNA profiling PCR.

**Polymerase chain reaction (Visualization of Genomic DNA fragment)**

The DNA was observed when the sample was allowed to run for polymerase chain reaction at various anneling temperatures like 44°, 45°, 46°, 47° C and 48° C. The perfect DNA band was obtained at 48° C. A clear visible thick band of DNA was observed and it shows the presence of Xylan.

**BLAST (Basic Local Alignment Search Tool) Analysis**

Then the sample which was sent to (bar coding) sequencing and the organism is identified as ***Bacillus pumilus*** **X8 16S ribosomal RNA gene** with **99 % accuracy** and the identified organism was put in Blast (Bio-informatics).

This reveals the enzyme **xylanase 8** producing organism is ***Bacillus pumilus*** strain. It is 16S ribosomal RNA gene and its partial sequences length is 1450.

The isolated ***Bacillus pumilus*** shows the **maximum homology (99%)** with others from the data base. The potential xylanase producing bacteria ***Bacillus pumilus*** was curtained its **systematic position** based on **16S rRNA**.

**BLAST** homology was also carried out to compare with other 16S rRNA partial sequence available in the gene bank of NCBI and revealed that the bacterial sequence of ***Bacillus pumilus.***

**Fig: 1 Blast result of Molecular identification of xylanase producing bacteria. Sequences producing significant alignments.**

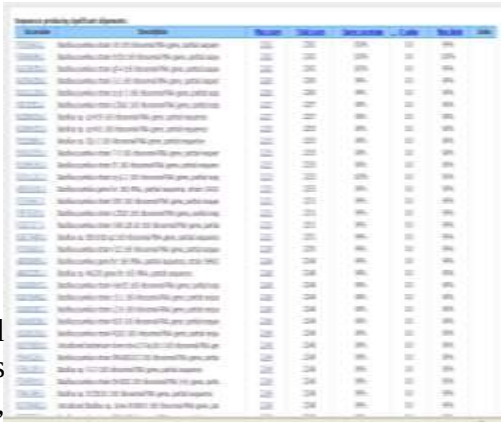
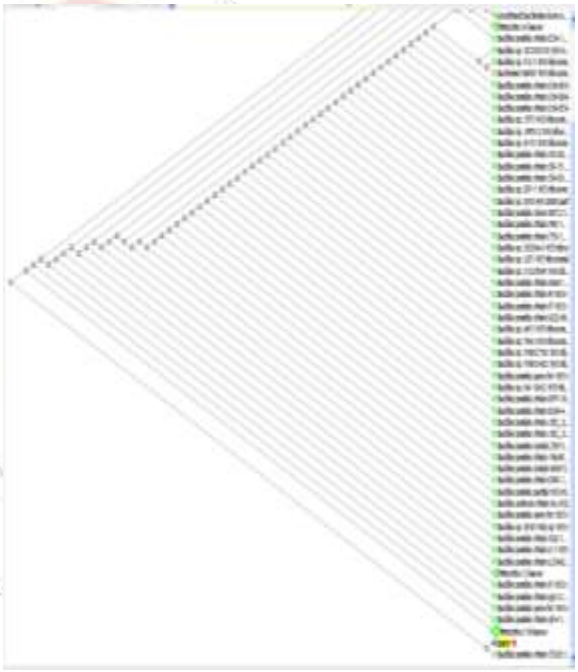


**Fig : 2 Blast Graphical Representation for sequence alignment of xylanase producing bacteria *Bacillus pumillus.***



**CONCLUSION**

Therefore this study reveals that the enzyme **xylanase 8** producing organism is ***Bacillus pumilus*** *strain.* Which are **16S ribosomal RNA gene** and its **partial sequences length** is **1450.** This identification of **Xylanase 8** producing bacteria ***Bacillus pumilus*** is very useful for further applications of xylanases which may still arrive in the future.

## REFERENCES

**Collins.T, Gerday.C, Feller.G (2004).** Xylanases, xylanase families and extremophilic xylanases.FEMS Microbiology Reviews.29:3-23.

**Debabov .V.G (1982).**The Industrial Use of Bacilli.The Molecular Biology of the Bacilli.New York:Academic Press.

**Fredricks.D.N,Smith.C,Meier.A(2005).**Comparis on of Six DNA Extraction Methods for Recovery of Fungal DNA as Assessed by Quantitative PCR. Journal of clinical Microbiology .43(10): 5122–5128.

**Harrigan.W.F and Mc Cance.M.E(1966).** Department of Bacteriology,The West of Scotland Agriculture College. Auchincruive, Ayr,Scotland.Academic press London and New York.pp:8-9.

**Halrich.D,Nidetzky.B,Kulbe.K.D,Steiner.Wand Zupancic.S.**Biores.Tecnol.58:137-161.

**Johnson.M, Zaretskaya.I, Raytselis .Y, Merezhuk.Y, McGinnis.S ,Madden.T.L, (2008).** NCBI BLAST: a better web interface. Nucleic Acids Research,Vol. 36, Web Server issue.

**Kulkarni N, Shendye A, Rao M (1999)** Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411– 456 .

**Kuta.F.A., (2008).**Antifungal effects of Calotropis Procera stem bank extract against Trichoplyton gypseun and Epiderinopiyton Flocosum.Afr J Biotechnol.7(13):2116-8.

**Leifert.C,Li.H,Chidburee.S,Hampson.S,Workm an.S,Sigee.D(1995).**Antibiotic production and biocontrol activity by Bacillus subtilis CL27 and Bacillus pumilus CL45.J Appl Bacteriol.78(2):97-108.

**McKeen.C.D,Reilly.C.C,PuseyP.L,(1986).**Product ion and partial characterization of antifungal substances antagonistic to Monilinia fructicola from Bacillus subtilis.Phytopathology.76(2):136-9.

**Pleus.R.C et al., (2014).**GRAS Notification Bacillus pumillus for use in post-harvest processing of bananas.Biowish Technologies, Inc.pp:1.

**Priest.F.G(1989).** Products and Applications.In:Harwood Cr editor. Biotechnology Hand Books, Bacillus..New York-London:Plenum Press.pp293-320.

**Saravananda.L,Abed.S.S.A,Rohini.J,Sathyamurt hy.B (2016).**Molecular Identification Of The Medicinal Plant ***Justicia gendarussa*** Using MATK Gene.European Journal of Pharmaceutical And Medical Research.3(1):259-266.

**Ten.L.N,Im.W.T,Kim.M.K,Kang.M.S,Lee.S.T(2 003).**Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates.Journal of Microbiological Methods.56(2004):375-382.

**Waites.M.J,Morgan.N.L,Rockey.J.S,Higton.G.(2 008)**. Industrial Microbiology an Introduction.London:Blackwell Publisher;