# BIOPROSPECTING XYLANASE ENZYMES FROM ENVIRONMENTAL SAMPLES

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## CERTIFICATE

This is to certify that the thesis entitled "**BIOPROSPECTING XYLANASE ENZYMES FROM ENVIRONMENTAL SAMPLES**" which is being submitted by **Ms. Arati Nayak**, Roll No. **412LS2045**, for the degree of Masters of Science in Life Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

R. Nayak 11/05/2014

**R. Jayabalan.**

## DECLARATION

I hereby declare that the thesis entitled "**Bioprospecting xylanase enzymes from environmental samples**" submitted to the Department of LIFE SCIENCE, National Institute of Technology, Rourkela for the partial fulfilment of the Master of Science in Life Science is a faithful record of original research work carried out by me under the guidance and supervision of Dr. R. Jayabalan, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date: 11·05·2014

Place: Rourkela

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## List of Figures


| Fig no. | Particulars | Page no. |
|---|---|---|
| 1. | Distribution of xylan in plant cell wall | 1 |
| 2. | Enzymatic hydrolysis of xylan | 3 |
| 3. | Members of xylanase family | 5 |
| 4. | Xylanase producing microorganisms screened using Congo red staining on Xylan agar plate | 16 |
| 5. | Effect of Temperature on enzyme action | 17 |
| 6. | Effect of pH on enzyme action | 18 |
| 7. | Km and Vmax of JS4(7) | 19 |
| 8. | Gram stained image and electron micrograph of JS3(4) and JS4(7) | 20 |


## List of Tables


| Table no. | Particulars | Page no. |
|---|---|---|
| 1. | Covalent linkages between xylan and non-carbohydrate cell wall constituents | 2 |
| 2. | Properties of some endoxylanase from fungi on oat spelt xylan | 9 |
| 3. | Characteristics of some xylanase producing enzymes, their optimum pH, optimum temperature and molecular weight | 10 |
| 4. | Results of gram staining | 20 |

# List of Abbreviations

| | |
|---|---|
| **G** | **Gram** |
| **mg** | **Milligram** |
| **L** | **Litre** |
| **ml** | **Millilitre** |
| **°** | **Degree** |
| **C** | **Centigrade** |
| **H** | **Hour** |
| **Min** | **Minute** |
| **Psi** | **Pressure per sq. inch** |
| **%** | **Percentage** |
| **TSA** | **Trypticase soy agar** |
| **M** | **Molar** |
| **mM** | **Millimolar** |
| **FE-SEM** | **Field Emission Scanning Electron Microscopy** |

## List of Contents


| Sl.No. | Contents | Page |
|---|---|---|
| **1.** | Introduction | 1-6 |
| **2**. | Review of Literature | 7-12 |
| **3.** | Aims and Objectives | 13 |
| **4**. | Materials and Methods | 14-15 |
| **5.** | Results and discussion | 16-21 |
| 6. | Conclusion | 22 |
| **7**. | References | 23-26 |

**Abstract**

Hemicelluloses are a group of heteropolysaccharides which accounts for 33% by dry weight of the total lignocellulosic biomass. Xylan is one of the most important and abundantly found hemicellulose which has diverse structures according to the source from which it is extracted. Due to its structural diversity, xylan is hydrolysed by a class of xylanase enzymes into its monomeric xylose subunits. These xylose residues when treated with yeasts get converted into ethanol which can be used as a supplement along with natural fuels. Ethanol production by biological means not only reduces the cost of production but also decreases the formation of inhibitors which are formed during chemical pretreatment of xylan that hampers the process of fermentation. Besides bioethanol production xylanases have also got many other productive uses in various industries such as paper pulp bleaching, oil extraction, food additives, bakeries, detergents, fodder industries, etc. Twelve strains were isolated from environmental samples like cow dung, leaf litter, waste water, soil, termites, etc. The isolated strains were confirmed for xylanase production on xylan agar plates and Congo red assay. Activity of endoxylanases (1-4 β-D xylanases) was determined by measuring the amount of reducing sugar from xylan which was estimated spectrophotometrically with 3,5 dinitrosalycilic acid using xylose as standard. The isolated strains produced maximum xylanase at 37$^{o}$C at a pH of 6.8 to 7.0 after 120 h of incubation. Xylanases extracted from different strains showed maximum activity at an optimal pH of 7.0 and optimal temperature 40$^{o}$C. The value of km and Vmax recorded for highest xylanase producing strain JS4(7) was recorded to be 0.25mg/ml and 83µg/ml/min. The morphology of the two highest xylanase producing strains JS3(4) and JS4(7) was found to be bacillus and coccus respectively under SEM imaging.

# 1. Introduction

Carbohydrates are polyhydroxy aldehydes or polyhydroxy ketones and compounds that can be hydrolyzed to them. Depending upon whether they hydrolyze or not and the product of their hydrolysis they are classified into three types as monosaccharides, oligosaccharides and polysaccharides. Polysaccharides are also called as glycans. Polysaccharides are again of two types such as homo-poly saccharides and hetero-poly saccharides depending upon the type(s) of monomeric units present. Plant cell wall consists of lignocellulosic substrates including both homo-polysaccharides and hetero-polysaccharides. The three main lignocellulosic constituents of plant cell wall are cellulose, hemicelluloses and lignin. As a whole plant biomass constitutes 40% cellulose, 33% hemicelluloses and 23% lignin by dry weight. For the first time a scientist, named Schulz introduced the term hemicelluloses for the fraction of plant material that was extracted by dilute alkali or hot water. Hemicelluloses include heteropolymers like xylan, mannan, glucomannan, xyloglucan, arabinogalactan, galactoglucomannan, etc. (Corral and Ortega, 2006; Motta et al., 2013)

## 1.1 Xylan

Xylan is one of the most important hemicelluloses and one of the major constituents of polysaccharides, which accounts for one third of the total renewable carbon on the Earth. It includes polymers like xylan, xyloglucan, glucomannan, galactoglucomannan and arabinogalactan. Plant cell wall comprises of three layers, which include primary cell wall, middle lamella and secondary cell wall. Xylan is present in secondary cell wall. It is present at the interface between lignin and cellulose via covalent and non covalent bonds to provide cell wall integrity and fiber cohesion (Motta et al., 2013; Corral and Ortega, 2006; Butt et al., 2008).

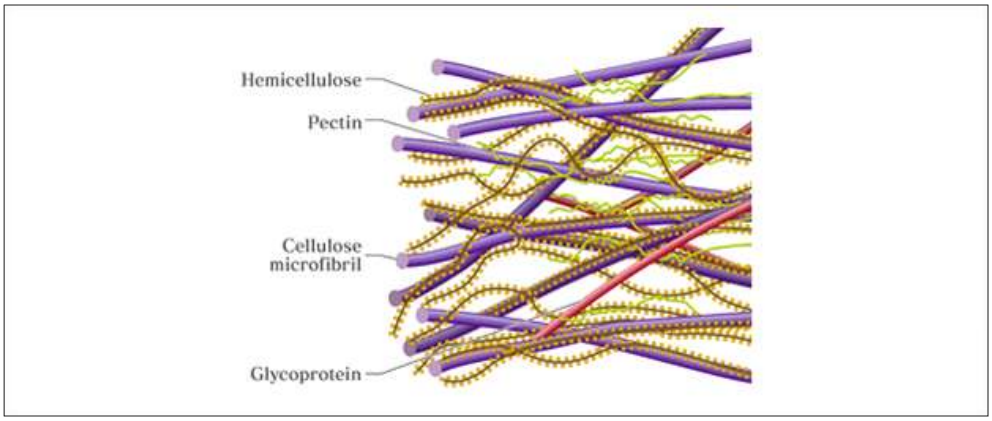


Fig1: Distribution of xylan in plant cell wall

## 1.2 Structure and distribution

Xylan is generally found as a highly branched structure of heteropolysaccharide with a homomopolymeric backbone chain of 1, 4- β-D-xylopyranosyl units with different side chains like glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl or p-coumaroyl. Xylan is largely present in angiospermic hardwoods in the β form of O-acetyl-4-O-methylglucurono xylan by 15-30% of cell wall content, in gymnospermic soft woods as arabino-4-O-methylglucurono xylans by 7-10% and <30% in annual plant as arabinoxylans. The degree of polymerization in hardwoods ranges from 150-200 β -xylopyranosyl units whereas in soft woods it is 70-130 respectively. Xylan in its homopolymeric form are found with a linear backbone of β 1-4 xylosyl residues which are rarely found in certain grasses like esparto, tobacco stalk, certain algae and husk of guar seed. They are linked either with only 1-4Bxylopyranosyl residues or with both 1-3, and 1-4 B xylopyranosyl residues. However, xylan is abundantly found in heteropolymeric form in its complex substituted forms. The presences of side chains present in the substituted form determine the degree of solubility, physical configuration, mode of enzymatic action and reactivity with other hemicellulosic components (Motta et al., 2013; Corral and Ortega, 2006).

## 1.3 Family of xylans

Due to heterogeneity in its structure xylan is classified into four main families:

a) Arabinoxylans-with side chains of single terminal units of α-L-arabinofuranosyl substituents.
b) Glucuronoxylan - with α-D-glucuronic acid or its 4-O-methyl ether derivative as substituent.
c) Glucuronoarabinoxylan-with α-D-glucuronic (and 4-O-methyl-α-D-glucuronic) acid and α-L-arabinose as substituents.
d) Galactoglucuronoarabinoxylans-with terminal β-D-galactopyranosyl residues on complex oligosaccharide side chains of xylans (Motta et al., 2013; Corral and Ortega, 2006).

Table1: Covalent linkages between xylan and non-carbohydrate cell wall constituents (Corral and Ortega, 2006)

| **Linkage** | **Sugar carbon atom involved** | **Sugar moiety of xylan involved** | **Non carbohydrate moiety** |
|---|---|---|---|
| Glycosidic | C1 | Xylopyronose | *p*-Coumaric acid |
| Ether | C2,C3<br>C5 | Xylopyronose<br>Arabinofuronose | Cα-Lignin<br>Cα-Lignin |

| | | | |
|---|---|---|---|
| Ester | C6<br>C6<br>C5<br>C5<br>C5 | 4-O-methyl glucuronic acid<br>Glucuronic acid<br>Arabinofuronose<br>Arabinofuronose<br>Arabinofuronose | Cα-Lignin<br>Ferulic acid<br>*p*-Coumaric acid<br>Cγ-Lignin |

### 1.4 Enzymatic hydrolysis of xylan:

The complex heteropolymer of xylan can be cleared by breaking the glycosidic bonds to form xylooligosaccharides and xylosyl residues by several xylanolytic enzymes such as endoxylanase, β-xylosidase, α-glucuronidase, α -arabinofuranosidase and acetylxylan esterase, which come under the super class of hydrolytic enzymes.

Xylans are cleared by the combined action of a group of xylanolytic enzymes. Endoxylanase cleave the xylan into xylooligosaccharides, B-xylosidase release xyloses from the non-reducing ends of xylooligosaccharides and acetyl eaterases remove acetyl substituents from the B-1,4 D-xylose backbone of long chains of xylan (Motta et al., 2013;Corral and Ortega, 2006; Butt et al., 2008).

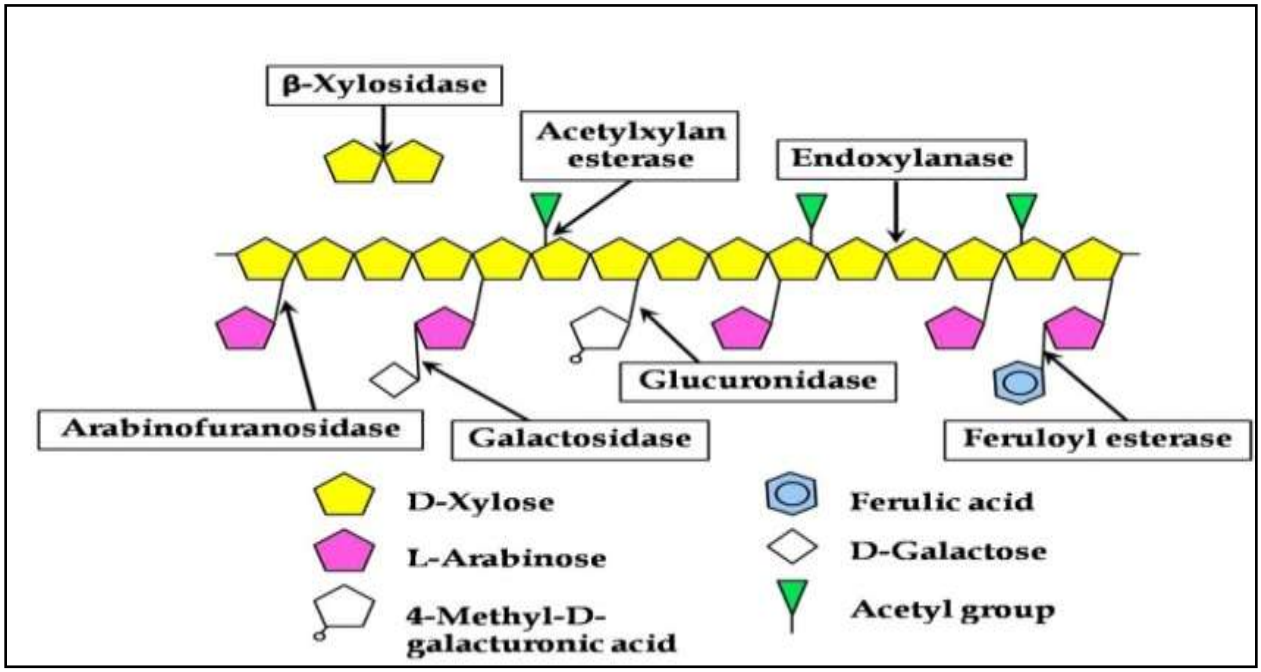


Fig2: Enzymatic hydrolysis of xylan

## 1.5 Sources of Xylanases:

Xylanases are the xylosidic hydrolase enzymes commonly found in microorganisms like marine algae, intestine of termites, insects, seeds, protozoons, crustaceans, snails, bacteria, fungi and actinomycetes, which cleave the glycoside bond of xylans forming hemi acetyls and glycans (Motta et al., 2013; Corral and Ortega, 2006;Butt et al., 2008;Sharma and Kumar, 2013).

## 1.6 Classification:

As per Carbohydrate Active Enzyme Database, xylanases have been classified to 14 glycosidic hydrolase families based on molecular weight, isoelectric property, crystal structure, kinetic property, substrate specificity and product profile (Motta et al., 2013). Those 14 different families includes 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62, among these families 5, 7, 8, 10, 11 and 43 have distinct catalytic domains to cleave the endo-1,4-B-glycosidic linkage. Families 16, 51 and 62 are bifunctional with two catalytic domains and lastly families 9, 12, 26, 30 and 44 have secondary xylanase activity. From among the families of 5,7,8,10,11and 43, hydrolysis action of families 5,7,10,11 result in the retention of anomeric center of the sugar monomer of the carbohydrate whereas the hydrolysis action of families 8 and 43 lead to the inversion of the anomeric centers (Motta et al., 2013;Butt et al., 2008). Family 10 have low molecular mass and high pI ranging between 8-9.5 in contrast to family 11 having high molecular mass and low pI values ranging 35-36. They possess a (α/β) 8-barrel fold structure. It includes enzymes like endo-1, 4-β xylanases and endo-1, 3-β-xylanases that can hydrolyze the aryl β-glycosidases of xylobioses and xylotrioses at the aglyconic bond. These enzymes have characteristics of 4 to 5 short substrate-binding sites to bind only short xylooligosaccharides. These have more catalytic versatility and lower substrate specificity (Motta et al., 2013; Corral and Ortega, 2006). On the other hand, enzymes of family 11 are called as true xylanases as they act only on D-xylose containing substrates. They have a small size, high substrate specificity, high catalytic activity with various optimum temperatures and pH values making them suitable to act in various conditions and with diverse applications. These enzymes have low molecular mass and high pI with a β-jelly roll fold structure. These enzymes have around seven larger substrate- binding clefts to act on long chains of xylooligosaccharides. These enzymes can also hydrolyze the aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond (Motta et al., 2013).

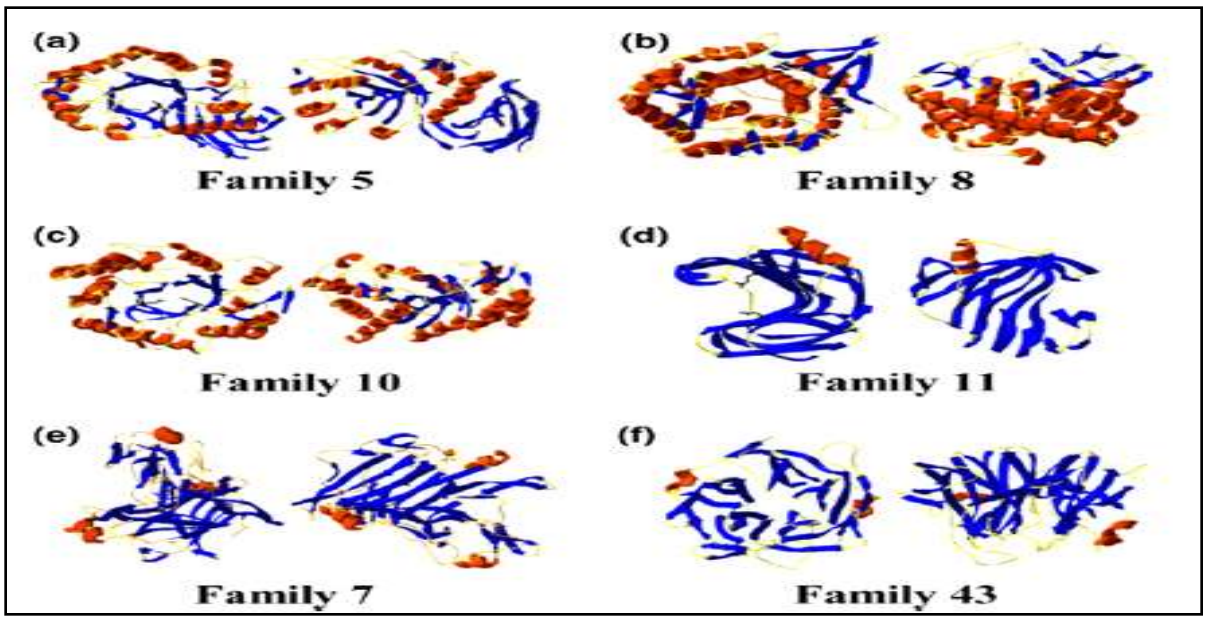


Fig3: Members of xylanase family

### 1.7 Applications of xylanases:

Xylanases have the potentialities to hydrolyze xylan, lignocellulosic materials and agro wastes into useful products for industrial applications and of commercial importance. They are used in various purposes like baking industries, bio ethanol production, paper pulp bleaching, fruit juice clarifications, beer manufacturing industries, bioprocessing of textiles like rayon, cellophane, manufacture of certain chemicals such as cellulose ethers and cellulose esters, animal fodder industries, etc.

1. **Bio bleaching paper pulp:** Bio bleaching property of xylanase was reported in 1980 (Viikari l et al,1986) Bio bleaching paper pulp with xylanase hydrolyze the hemicellulosic chain between the cellulose and lignin thus removing the loosened lignin from the required cellulose. In this manner, it reduces the release of organo chlorine pollutants like dioxin leading to a chlorine free bleaching without adversely affecting the strength of the paper.
2. **Improving animal feed:** Addition of xylanase to the animal feed reduces the viscosity of the fodder, which makes the fodder easily digestible by the animal gut. It increases the diffusion of pancreatic enzymes into the food and simultaneously improves the absorption of the nutrients. When used in the animal feed it reduces the unwanted wastes thus lowering the environmental pollutions.
3. **Bakery:** It improves the quality of bakery products by improving the strength of gluten networks and improves the dough characteristics by hydrolyzing the arabinoxylan and starch thus separating and isolating the gluten from the starch in the wheat flour, making it more flexible, machinable, and stable with a more loaf volume and crumb structure. Thus, it increases the durability of bread storage and keeps it fresh for a long period.

4. **Fruit juice production and clarification:** Imparting xylanase to the fruits increases the increased production of fruit juices and help in maceration. Xylanase helps in liquefaction of fruits and vegetables, stabilization of fruit pulp, recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc., reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate. Hence, it helps in juice filterability and clarification.
5. **Beer manufacturing industries:** Xylanase enable to hydrolyze the hemicelluloses present in the barley thus helping in production of more reducing sugars which for making beer. It also reduces the viscosity of the fermented liquid making it more clear and easy to filter. It also processes the spent barley into animal fodders.
6. **Miscellaneous:** Xylanases also improves the silage, which acts as a good manure in agricultures. They also produce nutrients, which are beneficial for the ruminal microflora. Xylanases also enhance the cleaning ability of washing detergents to clean different types of stains. Xylanases help in degradability of disposable plant organic wastes. These enzymes are also used in extraction of oil from plant materials like corn oil from corn embryos. Xylanases improves the process of retting of flax fibers before they are processed to form linen. Xylooligosaccharides formed from the hydrolysis of xylan have got much periodic effects as they cannot be either hydrolysed further or absorbed by the upper GIT thus stimulating the growth of some beneficial microbes in the colon. They also help in reduction of cholesterol, maintenance of health of GIT and improved availability of calcium to our body. They also inhibit the reterogradation of starch. Another important product is xylitol, which is used to sweeten food products such as chewing gum, candy, soft drinks and ice creams. It acts as a natural sweetener in toothpaste and various pharmaceutical products (Motta et al., 2013; Corral and Ortega, 2006; Butt et al., 2008;Sharma and Kumar, 2013).

## 2 Review literature:-

### 2.1 Xylanase:

Xylan hydrolyzing enzymes are called as xylanases (Motta et al., 2013; Corral and Ortega, 2006; Butt et al., 2008; Sharma and Kumar, 2013). These are single chain glycoproteins ranging from 60-80 KDa generally active at an optimal temperature of 40-60° C and pH of 4.5-6.5 (Butt et al., 2008). However, the optimum temperatures and pH varies according to the substrates used as xylan does have diversity in its structure (Butt et al., 2008; Latif et al., 2006; Cesar and Mrsa, 1996; Hazlewood and Gilbert, 1993). The main enzyme involved in cleaving the back bone of xylan is endo-1,4xylanases which convert the complex xylan into xylooligosaccharides. The debranching of side chains is done by another variety of enzymes called as β-xylosidases which include (α-L arabinofuranosidases and D-glucuronidases) and esterases which liberate acetyl, coumaroyl and feruloyl respectively (Butt et al., 2008; Corral and Ortega, 2006; Motta et al., 2013; Bieley et al., 1997; Subramanian and Prema, 1998; Jeffries, 1996).

### 2.2 Mode of action:

#### 2.2.1 Endo-1, 4-ß-xylanases:

Endo-1, 4-ß-xylanases the so called 1, 4-β-D-xylan xylohydrolases are known to cleave the glycosidic linkages of the hetero xylan back bone, resulting in monomeric units and thus decreasing the degree of polymerization of the substrate. It cleaves according to the length and degree of branching of the substrate and the presence of the substituents (Coughlan,1992). Some endoxylanase cleave at the uninterrupted sequences whereas some cleave at the vicinity of the substituted region which might be required for proper orientation of the substrate in the active site (Coughlan et al., 1993). There are some xylanases which act after the liberation of the arabinoses only. In that case xylanases are classified as per the end product released after hydrolysis (Corral and Ortega, 2006). In that respect they are classified into two types such as non-debranching and debranching enzymes. Most of the endoxylanase extracted from bacteria and fungi are glycosylated , single subunit proteins with a molecular weight of 8.5 to 85kDa , optimal temperature between 45 and 75ºC (Coughlan et al., 1993; Corral and Ortega, 2006;Polizeli et al., 2005) and few thermophilic bacteria like *Thermogota* with 80ºC (Winterhalter and Lible,1995) and isoelectric point value (pI)of 4.0 and 10.3 respectively. These are classified into two main classes based on their molecular weight, such as less than 30kD are grouped into category of basic proteins and those with greater than 30kD are grouped as acidic proteins (Butt et al., 2008).

#### 2.2.2 β -xylosidases:

Thermogota β -xylosidases the so-called 1, 4- β-D-xyloside xylohydrolases exist in either of the forms such as monomeric, dimeric or as trimeric form with Mw ranging from 26 to 360 KDa. They are produced by both fungi and bacteria and are supposed to cleave artificial β-xylosides and unsubstituted β

-1, 4-linked xylooligosaccharides (Coughlan et al., 1993; Polizeli et al., 2005; Corral and Ortega, 2006). Generally, it does not affect xylan or affect only 30% of what it affects the xylobioses. Their action on xylooligomers starts from the non-reducing ends and substrates with low degree of polymerization (Corral and Ortega, 2006).

**2.2.3 Exo-xylohydrolases:**

These enzymes preferably act on the non-reducing ends of xylan and xylooligosaccharides hence they have affinity to act on substrates with higher degree of polymerization (Corral and Ortega, 2006).

**2.2.4 α - arabinofuranosidases:**

Arabinofuranosidases usually exist as monomers, but sometimes dimeric, tetrameric and octameric forms have also been found with Mw ranging from 53 to 495 KDa ,optimum pH of 2.5 to 6.9 and a pI of 3.6 to 9.3 respectively.

There are thus two types of arabinofuranosidases:

a) **Endo-1, 5-α-L-arabinofuranosidases:** active only toward linear arabinan, and are not able to hydrolyze *p*-nitrophenyl-α-L-arabinofuranoside or arabic gum.
b) **Exo-acting α-L-arabinofuranosidases:** act on branched xylan and are active against *p*-nitrophenyl-α-L-arabinofuranoside (Corral and Ortega, 2006).

Most of these enzymews are exoacting. (Polizeli et al., 2005) Some α-L-arabinofuranosidases can cleave both 1,3- and 1,5- α-L-arabinofuranosyl linkages as seen in case of Aspergillus niger which first cleave α-L-1,3- linked arabinofuranosyl residues then cleave the α-L-1,5-arabinan to arabinose residues.(Kaji and Tagawa,1970) The enzyme 1,4-β-D-arabinoxylan arabinofuranohydrolase exclusively act on hydrolyzing arabinoxylans to arabinoses.(Kormelink et al., 1991; Corral and Ortega, 2006)

**2.2.5 α-glucuronidases:**

α-D-Glucuronidases enzyme is supposed to cleave α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan which has been reported in digestive juice of snails, bacteria and fungi. It enzyme is highly substrate specific.

**2.2.6 Acetylxylan esterases:**

Some acetyxylan esterases act in the native state of substituted xylans while some act after alkali extraction which remove the O-acetyl substituent at the C2 and C3 position of xylose residues (Corral and Ortega, 2006; Tenkanan and Poutanan,1992).

There are several families of xylanase producing enzymes and third of which are polyspecific. Enzymes of a particular family have similar three-dimensional structures and molecular mechanism. They may have similar actions on small, soluble synthetic substrates. There are about 14 glycosidic hydrolase (GH) clans starting from GH-A-GH-N each with two to three families except GH A with 17 families (Butt et al., 2008).

Table2: Properties of some endoxylanase from fungi on oat spelt xylan (Corral and Ortega, 2006)

| Sources | MW(KDa) | Optimal pH | Optimal Temperature | pI | Km |
|---|---|---|---|---|---|
| *Aspergillus kawachii* | | | | | |
| *A* | 35 | 5.5 | 60 | 6.1 | |
| *B* | 26 | 4.5 | 55 | 4.4 | |
| *C* | 29 | 2.0 | 50 | 3.5 | |
| *Aspergillus niger* | | | | | |
| *XynAI* | 36 | 5.0 | 50 | 7.3 | 4.5 |
| *XynAII* | 73 | 4.5 | 45-50 | 4.05 | 1.3 |
| *Aspergillus oryzae XynF1* | 35 | 5.0 | 60 | | |
| *Fusarium oxysporum* | 80 | 5.0 | 50 | | |
| *Hymenoscyphus ericae* | 58.4 | 4.5 | 55-60 | 4.8-5.2 | |
| *Humicola insolens3* | | | | | |
| *Xyl1* | 6 | 6-6.5 | 55-60 | 9.0 | |
| *Xyl2* | 21 | 6-6.5 | 48 | 7.7 | |
| *Penicillium capsulatum* | | | | | |
| *XynA* | 28.5 | 4.0 | 48 | 5-5.2 | 348 |
| *Xyn B* | 29.5 | 4.0 | 40 | 5-5.2 | 53 |
| *Penicillium chysogenum* | 35 | 6.0 | 45 | 4.2 | 4.1 |
| *Myrotecium verrucaria* | 16 | 5.5 | | 4.35 | 33 |
| *Trichoderma reesei1* | | | | | |
| *pI 5.5* | 19 | 4-4.5 | | 5.5 | |
| *pI 9.0* | 20 | 5-5.5 | | 9.0 | |
| *Trichoderma reesei1* | | | | | |
| *I* | 32 | 4-5 | | 4.1-4.2 | |
| *II* | 23 | 4-5 | | 6.4-6.5 | |
| *Talaromyces emersonii* | 35.7 | 4.7 | 75 | 4.25 | |
| *Xyn I* | 59 | 4.3 | 77 | 4.2 | |
| *Xyn II* | 30.1 | 4.2 | 73 | 3.98 | |
| *Xyn III* | 58.5 | 4.3 | 77 | 4.05 | 2.6 |
| *Xyn IV* | 45 | 4.2 | 80 | 3.87 | 13.3 |
| *Xyn V* | 48.6 | 4.4 | 78 | 4.06 | |
| *Xyn VI* | 54.3 | 4.7 | 78 | 4.32 | 2.7 |
| *Xyn VII* | 62.2 | 4.0 | 79 | 4.25 | 2.0 |
| *Xyn VIII* | 36,6 | 4.4 | 79 | 4.02 | 1.7 |
| *Xyn IX* | 47.9 | 4.4 | 75 | 4.38 | 1.3 |
| *Xyn X* | 52.8 | 4.1 | 73 | 4.42 | 1.4 |
| *Xyn XI* | | | | | |

Table3: Characteristics of some xylanase producing enzymes, their optimum pH, optimum temperature and molecular weight (Butt et al, 2008).

| Microbes | MW/KDa | Optimal temp | Optimal pH |
|---|---|---|---|
| *Acrophialophora nainiana* | 22 | 55 | 7.0 |
| *Aspergillus awamori* | 39 | 40-55 | 5.5-6 |
| *Aspergillus nidulans* | 34 | 56 | 6 |
| *Aspergillus nidulansKK-99* | | 55 | 8 |
| *Aspergillus oryzae* | 35 | 60 | 5 |
| *Aspergillus sojae* | 32.7 | 50-60 | 5-5.5 |
| *Aspergillus terreus* | | 50 | 7 |
| *Aspergillus terreus* | | 45 | 4.5 |
| *Myceliophthora sp.* | 53 | 75 | 6 |
| *Penicillium capsulatum* | 22 | 48 | 3.48 |
| *Streptomyces sp.* | 24.5,38,37.5 | 55-60 | 6-6.8 |
| *Thermomyces lanuginosus* | 24.7 | 70 | 6-6.5 |
| *Trichoderma harzianum* | 20 | 50 | 5 |
| *Trichoderma longibrachiatum* | 37.7 | 45 | 5-6 |
| *Trichoderma viride* | 22 | 53 | 5 |

Xylanases are of varying specificities, folds and primary sequences.

**2.3 Xylanase sources:**

Every type of xylanases has extracted from a wide variety of living organisms basically the microorganisms. They include bacteria both existing in marine and fresh water habitats as well as in terrestrial habitats. Bacteria from rumen have also been found to show xylanolytic activities. The main ecological niches of xylanase producing microbes are accumulated deteriorated plant materials.

Fungi, actinomycetes, marine algae, protozoans, snails, crustaceans, insects, terrestrial plants and seeds, intestine of termites also secrete xylanase enzymes. Among these organisms filamentous fungi have been reported to produce xylanases to a larger extent. Fungal xylanases are of much industrial interest because they can be extracted without disruption of the cell prior to purification. Fungi also produce some auxiliary enzymes which can degrade the substituted xylan (Sunna and Antranikian, 2007), (Ploizeli, 2005). *Trichoderma*, *Aspergillus*, *Fusarium* and *Pichia* are certain fungal species which produce much of xylanases. (Adsul M.G. et al, 2005) The white-rot fungus are widely used in food industries, manufacturing pharmaceutical products and cosmetics. (Qinnghe C et al, 2004)White rot basidiomycetes degrade lignocellulosic materials by sectring a large amount of xylanase enxymaes. *Phanerochaete chrysosporium* produce high amounts of α-glucuronidases (Castanares A et al.1995) and Coriolus versicolor produces a combination of various xylanases.(Abd El-Nasser et al,1997) Cuninghamella

subvermispora also secrete much xylanases when subjected on wood chips and polysaccharides of plant cell wall. (de Souza-Cruz PB et al 2004) Bacteria are generally used in research and industrial works because of their alkaline thermostable nature. Generally the optimal pH of bacteria is more than that of fungus thus making them suitable to be used in paper pulp industries. Rhodothermus marinus is an extreme thermoplile which produces α-arabinofuranosidases ,so also Bacillus polymyxa which produces two polypeptides along with α-arabinofuranosidases.

There are some microbes which show optimal activity at higher temperatures are *Geobacillus thermoleovorans, Streptomyces sp.* S27, *Bacillus firmus*, *Actinomadura sp.* strain Cpt20 and *Saccharopolyspora pathunthaniensis* which are active at temperatures 65-90°C (Verma D and Satyanarayan T ,2012) and another is Thermotogoa which show maximum activity at 100-105°C (Yoon H et al , 2004).

Particularly microbes such as *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridia* and *Bacillus* high potentials to produce xylanase enzymes (Kulkarni, 1999; Qinnghe et al., 2004; Wubah et al., 1993; Matte et al., 1992). Microorganisms cleave xylans by secreting 1,4-β-D endoxylanases and β-xylosidases (Esteban et al., 1982).

**2.4 Manufacture of xylanases:**

Xylanases are generally produced by two types of procedures such as submerged fermentation n(SmF) and solid state fermentation(SSF). For large scale production submerged fermentation is an acceptable method which yields about 90% of enzyme and is also cost effective. Yet solid state fermentation technique is also in practice for purified substrates like xylans having low molecular weights which act as good xylanase inducers. Lignocellulosic substrates like husk of barley, corn cobs, straw and bran of wheat are some of the best substrates along with leaf litter, cow dung ,rotten vegetable products, agricultural wastes, food processing wastes, forestry wastes, etc.(Corral and Ortega, 2006).

In case of SmF we can extract a large amount of purified enzymes but in case of SSF we can achive a diverse group of xylan degrading enzymes.

Enzyme properties such as pH and thermostability are also improved in case of SSF technique (Corral and Ortega, 2006).

For a lumpsum amount of enzyme production optimization s of conditions like temperatures, pH, substrate conditions, amount of substrate, effect of metal ions, oxygen saturation and the type of media used. Besides optimization of the culture conditions maximum enzyme production can be formed from isolation of mutant strains (Corral and Ortega, 2006). *Fusarium oxysporium* is a mutant strain which can release both xylanase and β-xylosidase three times more than the parental strains when it acts mainly on wheat bran.(Singh et al.1995) It has been found that aspergilus strains produce much enzymes when they

are subjected to parasexual recombinations between over producing strains( Loera et al.,2003). Recombinant DNA technology has also proved important in making xylanase overproducing strains. Commercially two microbial strains, *Trichoderma* spp. and *Aspergillus* spp. have immence importance in industries due to their beneficial properties such as thermostability, pH stability and no cellulose activity (Corral and Ortega, 2006).

**2.5 Xylanase assay:**

Xylanase assay is usually done by DNSA assay or Nelson Somogyi assay in order to analyse the presence of reducing sugars (Motta et al, 2013). It can also be tested by using 4-O methylglucuronoxylans covalently dyed with RBB –Remazol brilliant blue as a substrate where xylanase is assayed as per the release of dye fragments.In some cases xylanase is assayed using fluorescence based method that is EnzChek, ultra xylanase kits are also available along with some xylanase tablets which uses auzurine-crosslinked arabinoxylans as substrates which on hydrolysis produces water soluble dye fragments.

**2.6 Uses of xylanases in bioethanol production:**

Due to rapid increase in crisis of fossil fuels, ethanol is considered as a substituent of fossil fuels, which can compensate the loss of fossil fuels. Second-generation ethanol produced by bioconversion of lignocellulosic materials is considered as most demanding fuels in terms of market value and volume. It is also considered as eco friendly as compared to first generation ethanol production and the chemically produced ethanol.

## 3. Objectives

**3.1** To isolate and screen xylanolytic microorganisms from environmental samples.

**3.2** To identify the structural morphologies of the isolated strains by Gram's staining and SEM analysis.

**3.3** To produce xylanase enzymes from isolated microbes.

**3.4** To study the characteristics (optimum pH and temperature, Km and Vmax) of the crude enzyme produced by the isolated microbes.

## 4. Materials and Method:

### 4.1 Isolation of xylanase producing bacteria

Soil and water samples were collected from Dhenkanal and Sambalpur. Samples were serially diluted and inoculated in Trypticase soy agar (TSA) media (15 g Tryptone, 5 g Soytone, 5 g Sodium Chloride, 15 g agar/ 1 L Distilled water) and incubated at 37°C for 24 hours. Individual colonies were obtained by streaking the culture on same media.

### 4.2 Screening of xylanase producing bacteria

Confirmation of xylan-degrading ability of bacterial isolates was performed by streaking on the Xylan agar media (5 g Xylan, 1 g $NaNO_3$, 1 g K2$HPO_4$, 1 g KCl, 0.5 g $MgSO_4$, 0.5 g yeast extract, 1 g glucose, 17 g agar/ 1L Distilled water). The use of Congo red (1 mg/ml) as an indicator for xylan degradation in an agar medium and 1 M NaCl as destaining solution provides the basis for a rapid and sensitive screening test for Xylanolytic bacteria (XB). Colonies showing discoloration of Congo red was taken as positive Xylan-degrading bacterial colonies and only these were taken for further study.

### 4.3 Xylanase enzyme production

The selected xylanolytic bacterial isolates were cultured at 37°C at 150 rpm in 100 ml of enzyme production media composed of 0.1 g $NaNO_3$, 0.1 g $KH_2PO_4$, 0.0.1 g KCl, 0.5 $MgSO_4$, 0.5 g yeast extract , 0.1 g glucose, 0.5 g xylan at pH 6.8–7.2. Broth culture after 120 h of incubation period was subjected to centrifugation at 5000 rpm for 15 min at 4°C. Supernatant was collected and stored as crude enzyme preparation at 4°C for further enzyme assays.

### 4.4 Xylanase assay

Xylanase activity was assayed by measuring the amount of reducing sugar formed from xylan. Determination of enzyme activity is measured using methods suggested by International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). In these tests, 0.5 ml of crude enzyme extract is incubated with 0.5 ml of 1mg/ml concentration of xylan in 10 mM citrate buffer at different temperatures and pH and the amount of reducing sugars formed were estimated spectrophotometrically with 3, 5-dinitrosalicylic acid at 540 nm using xylose as standard. Then enzymatic activities of total endoxylanase were defined in units. One unit of enzymatic activity is defined as the amount of enzyme that releases one micromole reducing sugars (measured as xylose) per minute.

### 4.4.1 Effect of temperature on xylanase activity

The crude enzyme extract along with the substrate (1mg/ml xylan in 10 mM citrate buffer) was incubated for 15 min at different temperatures such as 30, 40, 50, 60 and 70°C and assayed spectrophotometrically for the effect of temperature on enzyme activity.

### 4.4.2 Effect of pH on xylanase activity

The pH of the test solution was adjusted to different pH range of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with phosphate buffer (10 mM $Na_2HPO_4.2H_2O$, 1.8 mM $KH_2PO_4$) at their respective optimal temperature for 15 minutes. The test samples were assayed to study the effect of pH on enzyme activity.

### 4.4.3 Determination of Km and Vmax of the xylanase with highest activity

The values of the Michaelis constant (*K*m) and the maximum velocity (*V*max) were obtained by measuring the rate of xylan hydrolysis under optimal temperature and pH conditions. Reaction mixture was determined for endoxylanase by incubating in phosphate buffer pH 7.0 at 50 $^{o}$C with xylan at concentrations ranging from 0.2 to 4 mg/ml. Values for *K*m and *V*max were determined from Michaelis-Menten kinetics.

# 5. Result and Discussion

## 5.1 Isolation of xylanase producing microorganisms

Isolated colonies of seventy-four strains of microorganisms were obtained on cultured Soybean casein digest agar plates otherwise called Trypticase soya xylan agar. Trypticase soya xylan agar being a basal media nurtures different types of microorganisms such as bacteria and fungi, and enables them to grow, multiply and proliferate by utilizing the mineral nutrients present in the media. These isolated strains were preserved in slants of the same media and stored at 4$^{o}$C for future use and screening.

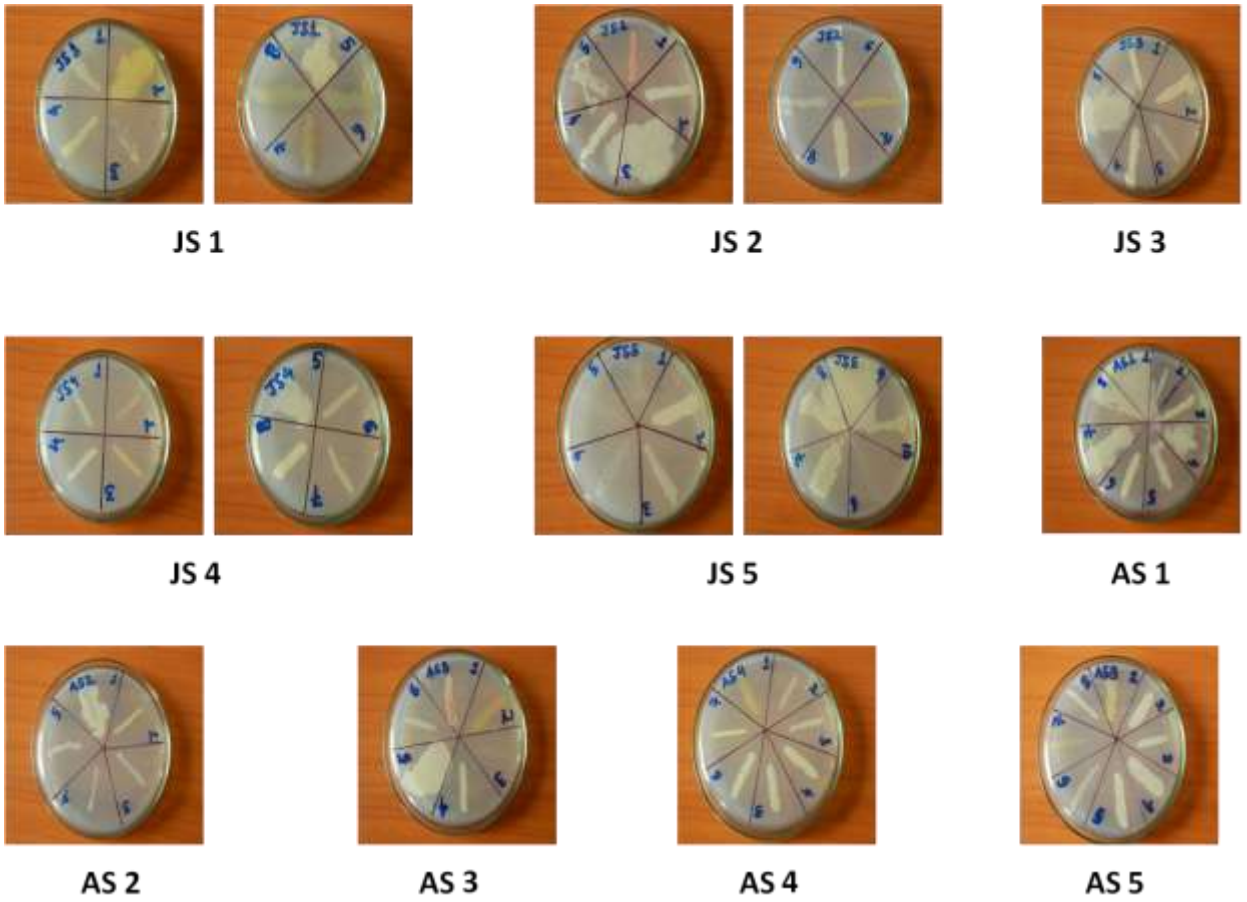


Fig4: Isolated colonies of strains streaked on trypticase soya agar plates

## 5.2 Screening of xylanase producing microorganisms

Twelve microbial strains, which formed clear halo zones around their colonies on Congo red stained xylan agar plates were, tested positive for xylanase activity. It is the property of Congo

red to bind with the complex polymer xylan but not to its reduced sugars monomeric subunits such as xylose. Therefore, the portions of the xylan agar plates where Congo red had bind to xylans, which looked red except around those colonies, which had produced xylanase enzymes and hydrolysed the complex xylan to xylose residues, there by forming a clear halo zone around the colony by unabling Congo red to bind to the xylose residues.

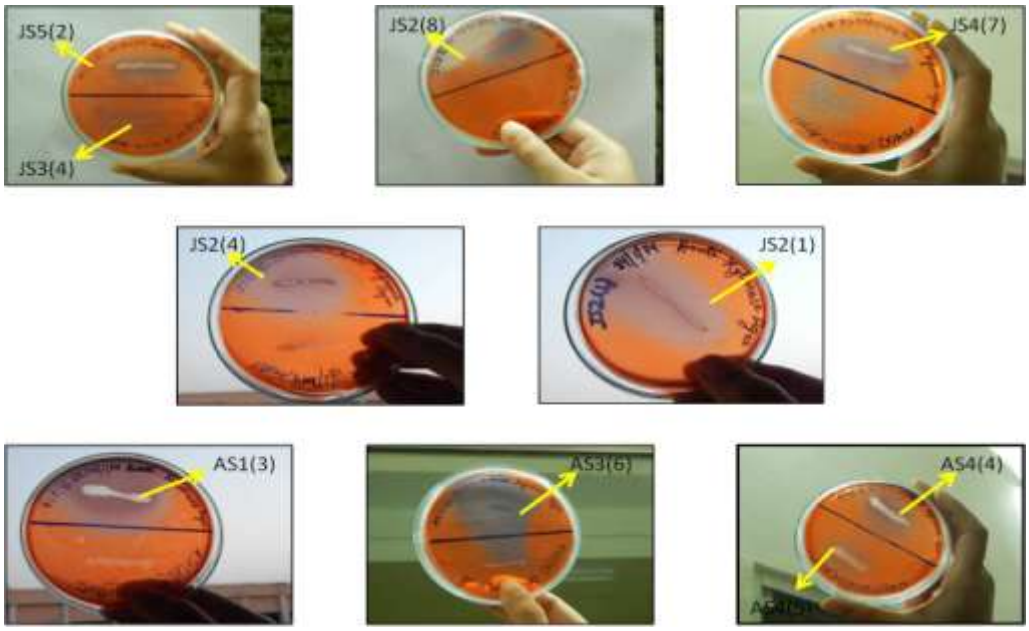


Fig5: Xylanase producing microorganisms screened using Congo red staining on Xylan agar plate

### 5.3 Xylanase enzyme production

Xylanase enzyme was produced after 120 h of incubation of the twelve xylanolytic strains in enzyme production media constituting birch wood xylan, yeast extract, $KH_2PO_4$, $MgSO_4$, and peptone of pH ranging between 6.8 to 7.2 at 37°C and 150 rpm. The crude enzyme was extracted as the supernatant by centrifuging the broth culture at 5000 rpm for 15minutes at 4°C and discarding the pellet containing the cellular sediments. This enzyme was stored at 4°C refrigeration condition for assays and characterization. Proper temperature and revolutions were maintained for production, extraction and storage of the enzyme as extreme temperature and revolutions would degrade the protein.

### 5.4.1 Effect of temperature on xylanase activity

Enzyme activity recorded at different temperatures from 30°C to 70°C revealed that the optimum temperature for all the xylanolytic strains is 40°C where maximum hydrolyzing activity of the enzymes was found. Among the twelve isolated strains, JS4(7) strain showed a maximum enzymatic activity of 513.3333U/ml at 40°C. Every enzyme has got its optimum temperature at which it shows maximum activity. This temperature lies between the melting point of water (0$^{o}$C) and the boiling point of water(100$^{o}$C). Above or below the optimum temperature the enzyme becomes inactive. It happens because at lower temperature the enzyme is in dormant state to act on the substrate as there is less free energy to start up the reaction and at higher temperature the enzyme gets degraded as excess energy provided tend to break some of the intramolecular attractions between polar groups and as well as the hydrophobic forces between the non polar groups within the protein structure. The change and distribution in these forces changes the three-dimensional structure of the protein and so the conformation of its active site due to which the enzyme is unable to catalyze the substrate.

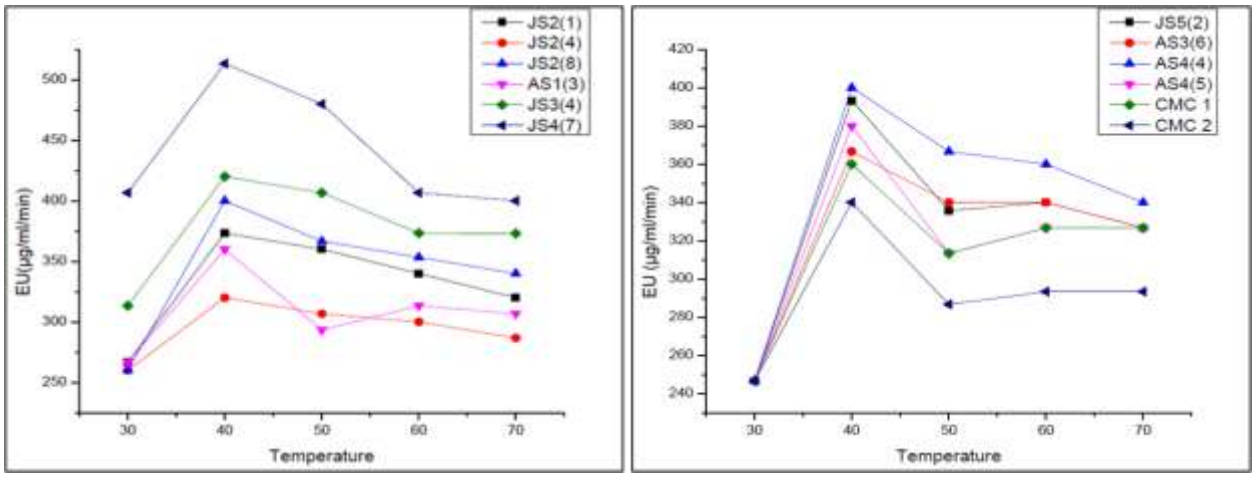


Fig6: Effect of Temperature on enzyme action

### 5.4.2 Effect of pH on xylanase activity

Enzyme activity recorded at different pH ranging from 3 to 9 revealed that the optimum pH for all xylanolytic strains is pH 7 where maximum hydrolyzing activity of the enzymes was found. Among the twelve isolated strains, JS4(7) strain showed a maximum activity of 566.6666U/ml at pH 7. Every enzyme has its own optimal pH. An optimal pH is required for an enzyme to show its maximum activity, because change in pH affects the ionization of the acidic and basic

aminoacids of an enzyme. Any change in the ionization of the aminoacids of an enzyme lead to an alteration in the ionic bonds that helps to determine the 3-D structure of the enzyme. This can lead to an altered enzyme recognition or even make an enzyme inactive. Therefore, an optimal temperature is required for maintaining the ionization state of the aminoacids at the catalytic site of the enzyme as well as that of the substrate for proper binding of the substrate to the enzyme.

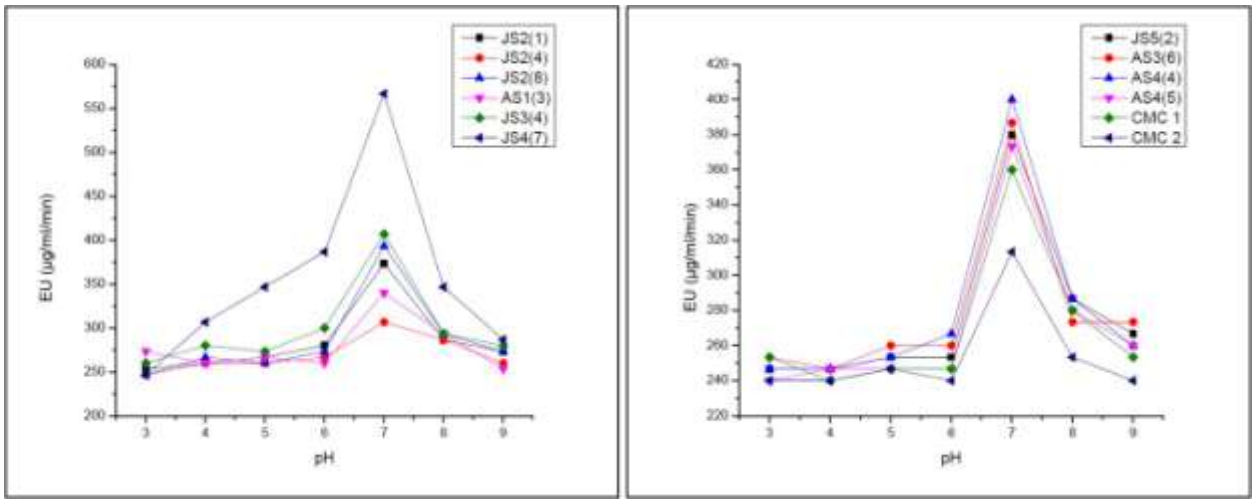


Fig7: Effect of pH on enzyme action

### 5.4.3 Determination of Km and Vmax of the xylanase with highest activity

The value of Km and Vmax recorded for JS4(7) with highest xylanase activity are 0.25mg/ml and 83 µg/ml/min respectively for a range of substrate concentration taken from 0.2mg to 4mg/ml. The recorded substrate concentration at which the velocity of the enzyme has reached half of its maximum velocity indicates that the substrate has higher affinity towards the enzyme. A small concentration of the substrate is enough for the enzyme to become saturated hence at a low substrate concentration the maximum velocity of the enzyme can be reached. The Vmax indicates that at this velocity all the substrate has been converted into enzyme substrate complex.

| Substrate (mg/ml) concentration | Measured velocity (µg/ml/min) |
|---|---|
| 0 | 0 |
| 0.2 | 35.25 |
| 0.4 | 48.713 |
| 0.6 | 55.466 |
| 0.8 | 58.873 |
| 1 | 64.7 |
| 1.2 | 64.91 |
| 1.4 | 71.733 |
| 1.6 | 85.733 |
| 1.8 | 84.1 |
| 2 | 85.446 |
| 2.2 | 83.81 |
| 2.4 | 85.506 |
| 2.6 | 87.793 |
| 2.8 | 89.713 |
| 3 | 100.3 |
| 3.2 | 85.806 |
| 3.4 | 84.453 |
| 3.6 | 86.586 |
| 3.8 | 88.006 |
| 4 | 90.13 |

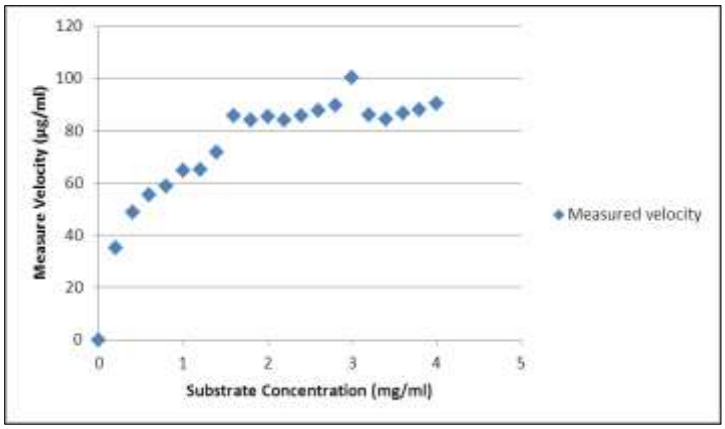


Fig8: Km and Vmax of JS4(7)

## 5.5 Morphological study of isolated organisms using SEM and Gram staining

The results of the gram staining concluded that all the strains responded to grams staining,which indicated that all the isolated strains might be bacterial strains . Out of the twelve strains, three strains were found to be gram positive whereas the rest nine were supposed to gram negative. Most of these strains were bacillus and of coccus shapes with varying sizes. From the SEM images of the two high enzyme producing strains it was observed that at 200,000X resolutions the morphology of the strain JS4(7) was coccus in nature whereas at 20,000X resolution the morphology of the strain JS3(4) was found to be bacillus in nature.

**Table4: Results of Gram's staining on twelve isolated stains**

| SL. NO | STRAIN | RESULT OF GRAM STAINING | MORPHOLOGY |
|---|---|---|---|
| 1 | JS2(1) | +ve | Bacillus |
| 2 | JS2(4) | -v e | Coccus |
| 3 | JS2(8) | -ve | Streptobacillus |
| 4 | JS3(4) | -ve | Bacillus |
| 5 | JS4(7) | +ve | Coccus |
| 6 | JS5(2) | -ve | Bacillus |
| 7 | AS1(3) | -ve | Coccus |
| 8 | AS3(6) | -ve | Coccus |
| 9 | AS4(4) | -ve | Bacillus |
| 10 | AS4(5) | -ve | Bacillus |
| 11 | CMC1(WET) | +ve | Coccus |
| 12 | CMC2(DRY) | -ve | Spiral |

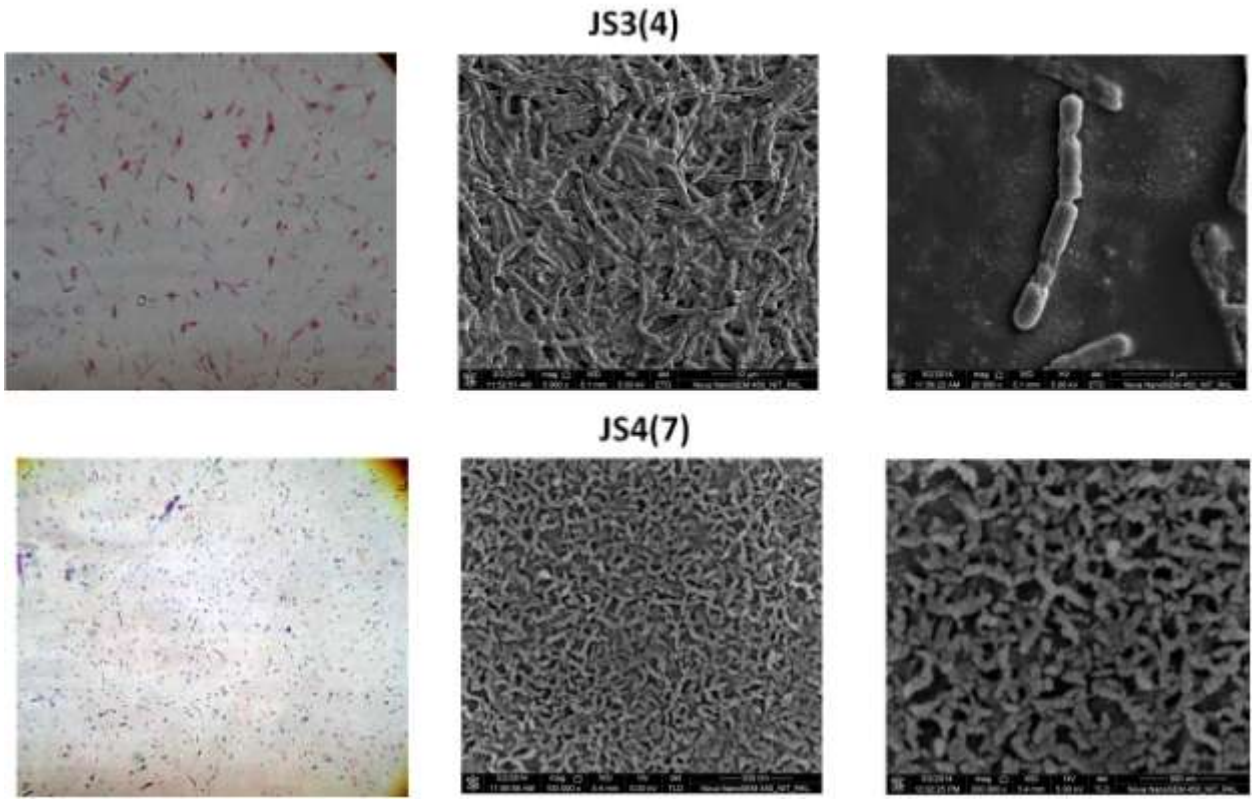


Fig9: Gram stained image and electron micrograph of JS3(4) and JS4(7)

## 6. Conclusion

Xylan being a hemicellulose with diverse structures due to the presence of many side chains and bonds need a complex enzyme called as xylanase for its hydrolysis to form monomeric subunits. Xylanase is abundantly found in fungi. It is also found in other microorganisms like bacteria, gut of termites, algae, gut of rumens,etc. Xylan on hydrolysis with the action of xylanases gets converted into xylose residues which on fermentation with yeasts gets converted into ethanol. Not only in the production of bioethanol but also in many other industries xylanase has got its fruitful applications, such as in bakeries, paper bleaching, oil extraction, juice clarification, etc. Bioethanol production by microbes reduces the cost of production of pretreatment and also reduces the production of inhibitors as found in case of chemical pretreatments. For xylanase enzyme production seventy four isolated strains were screened on xylan agar plates out of which twelve strains showed xylananolytic positive due to the formation of halo zones around their colonies. From these twelve colonies isolated three were gram positive and the rest nine were gram negative, all with different structures. The SEM imaging of the two highest xylanase producing strains JS4(7) AND JS3(4), taken at 200,000X and 20,000X resolutions clearly showed that one was bacillus and the other was coccus in nature. Xylanase enzyme was produced in a selective media containing birchwood xylan, yeast extract, peptone, $KH_2PO_4$, $MgSO_4$, and water. This was considered as the crude enzyme extract which was further used for studying the enzyme characteristics. The endo-β-D1-4xylanase activity of the extracted enzyme was tested with 3, 5 dinitrosalysilic acid using xylose as the standard which showed that enzyme of JS4(7) was having the highest activity. All the enzymes showed maximum activity at an optimal pH 7 and optimal temperature 40°C. This clearly indicates that these strains are neutrophilic and mesophilic in nature. From the Km and Vmax study, it was revealed that the enzyme was having more affinity towards the substrate as the value of the km was found to be 0.25mg/ml at an enzyme velocity of 42.5 µg/ml/min and that of the value of Vmax was 83µg/ml/min respectively. Hence, it can be concluded that if these enzymes are further purified then these may show far better results as per the hydrolyzing activity is concerned.

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