II. MATERIALS AND METHODS

I. MATERIALS

The sources of the materials used in this investigation are listed below

S. No.	Product (s)	Source
1.	Agarose, polyacrylamide, ammonium persulphate (APS), N',N´-Methylene-bis-acrylamide, bovine serum albumin (BSA), chloroform, β-mercaptoethanol, phenol:chloroform (1:1), protein molecular weight markers (SDS), N-bromosuccinimide (N-BS), Woodwards reagent K (W-RK), dithiothreitol (DTT), iodoacetic acid (IAA), EDTA, EGTA, amylose, amylopectin pullulan, rice starch, sodium dodecylsulphate (SDS), N',N',N',N'- tetramethyl ethylenediamine (TEMED), Xylan, 4-O-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB-xylan), xylose, xylobiose, xylotriose, xylotetraose and xylopentaose, *p*-nitrophenylxylopyranoside, *p*-nitrophenylacetate and *p*-nitrophenylarabinofuranoside, oligonucleotides synthesis (primers)	**Sigma Aldrich Pvt. Ltd., USA**
2.	Dinitrosalicylic acid (DNS), malachite green sodium sulphite and Congo red, Poly activated charcoal (PAC),	**Central Drug House, India**
4.	Agar, ammonium chloride, ammonium nitrate, ammonium sulphate, citric acid, diethyl ether, diammonium hydrogen orthophosphate, ethanol, formaldehyde, glutaraldehyde, glycerol, hydrochloric acid, hydrogen peroxide, manganese chloride, perchloric acid, potassium iodide, phenol, sodium chloride, sodium citrate and sodium acetate, X-gal (5 -bromo-4-chloro-indolyl-β-D-galactopyranoside), IPTG (isopropyl-β-D-1-thiogalactopyranoside), Imidazole	**Merck, India**
6.	Acetic acid, Coomassie brilliant blue, calcium chloride, copper sulphate, dipotassium hydrogen phosphate,	**Qualigens, India**

	disodium hydrogen phosphate, glacial acetic acid, glucose, glycine, iodine, magnesium sulphate, potassium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium iodide, potassium nitrate, silver chloride, silver nitrate, sodium bicarbonate, sodium carbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, sodium acetate, sodium molybdate, sodium nitrate, potassium sodium tartarate, and sulphuric acid, yeast extract, beef extract, peptone	
7.	Kanamycin, ampicillin, tetracycline, chloramphenicol tryptone, urea, guanidine HCl, Luria Bertani medium, Polyvinylpolypyrrolidone (PVPP)	**HiMedia, India**
8.	Restriction enzymes, T4 DNA ligase and buffers used in cloning experiments, Taq polymerase, dNTP solution, PCR buffers, PCR grade water, 1 kb and 100 bp DNA ladders, pGEM-Teasy cloning kit	**New England Biolabs and Promega**
9.	Isoamyl alcohol, methanol, trichloroacetic acid and Tris, lysozyme, acetone, acetyl-methylcarbinol, anthrone's reagent, Folin & Ciocalteu's phenol reagent	**SRL, India**
10.	Wheat bran, corncob, rice straw, *Prosopis juliflora*, *Lantana camara* and sugarcane bagasse	**Local market, New Delhi**
11.	Plasmid isolation, Gel extraction and PCR cleanup kits	**Machery Nagel, Germany**

II METHODS

2.1. COLLECTION OF SAMPLES

Soil, sediment, effluent, and water samples have been collected from various hot and alkaline regions of India and Japan in sterile polyethylene bags/bottles. The samples were transported to the laboratory and preserved at 4 °C. Temperature and pH of the samples was recorded.

2.2. BACTERIAL STRAINS

Various strains of *Escherchia coli* (DH5α, XL1Blue, DH10B) were used as hosts for the propagation of recombinant vectors. In addition, *Bacillus subtilis* was used as a host for the expression of xylanase gene from the recombinant vector *pWHMxyl*. Different vectors used in this investigation are listed in **Table 2.1**.

2.3. PROTOCOL FOR OPTIMIZATION OF HUMIC ACID-FREE DNA FROM ALKALINE SOILS

Soil (1 gm) was suspended with 0.4 gm (w/w) polyactivated charcoal (Datta and Madamwar, 2006) and 20 μL proteinase K (10 mg mL^{-1}) in 2 mL of modified extraction buffer [N,N,N,N cetyltrimethylammonium bromide (CTAB) 1% w/v, polyvinylpolypyrrolidone (PVPP) 2% w/v, 1.5 M NaCl, 100mM EDTA, 0.1 M TE buffer (pH 8.0), 0.1M sodium phosphate buffer (pH 8.0) and 100 μL RNaseA] [Zhou *et al*., 1996] in 20 mL centrifuge tubes to homogenize the sample and incubated at 37 °C for 15 min in an incubator shaker at 200 rpm. Subsequently, 200 μL of 10% SDS was added to the homogenate and kept at 60 °C for 2 h with intermittent shaking. DNA was precipitated by adding 0.5 V PEG 8000 (30 % in 1.6 M NaCl) and left at room temperature for an hour (Yeates *et al*., 1998). The precipitated DNA was collected by centrifugation at 8000 x g at 4 °C. The supernatant was discarded and pellet was dissolved in 1 mL of TE buffer (pH 8.0) and then100 μL of 5 M potassium acetate (pH 4.5) was added and incubated at 4 °C for 15 min. The supernatant was collected after centrifugation at 8000 x g and treated with equal volumes of phenol: chloroform (1:1) followed by chloroform: isoamylalcohol (24:1) at 8000 x g for 15 min.

Table 2.1. Details of various hosts and vectors used in present study

Strains/plasmids	Characteristics	Reference/Source
E. coli DH10B	F⁻mcrAΔ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara*,*leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ⁻	
E. coli DH5α	*F2 D(lac-argF)U169 recA1 endA1 hsdR (rK2 mK 1) supE44 gyrA1 relA1 deoR thi-1 (F80dlacZDM15)*	Invitrogen, CA,USA
E. coli BL21(*DE3*)	E. coli B F⁻ dcm ompT hsdS(r^-_B m^-_B) gal λ(DE3)	Stratagene
E. coli XL1blue	Tet^R	Invitrogen, USA
Bacillus subtilis	Strain WB980, Kan^R	Calgary
pGEM-Teasy	Amp^R, LacZ	Promega,
p18GFP	Amp^R, LacZ, pUC18	Constructed
pET28a (+)	Kan^R, T7 promoter	Novagen
pET22b (+)	Amp^R ,T7 promoter	Novagen
pWH1520	Amp^R, Tet^R,	Mi-Bio

The aqueous layer was transferred to another Eppendorf tube (1.5 mL) and treated with 0.7V of isopropanol for 1h at room temperature. DNA was sedimented by centrifugation at 8000 x g for 20 min at 4 °C. The pellet was washed with 1.0 mL of 70 % v/v ethanol and dried at room temperature. The dried pellet was dissolved in 100 µL of sterile Milli Q water. DNA was also extracted without the addition of activated charcoal as control. Finally, metagenomic DNA was quantitated, visualized and analyzed by restriction digestion and PCR amplification.

2.3.1. Commercial kits

Alternatively metagenomic DNA was extracted from the alkaline soil samples by using different commercial kits (UltraClean™, PowerSoil™ [Mo Bio Laboratories Inc., Carlsbad, CA, USA], Nucleospin kit [Macherey-Nagal, Germany] and Zymo soil DNA isolation kit [CA, USA]). The DNA was finally suspended in 100 µL of sterile Milli Q water for further analysis.

2.3.2. Comparison of yield and purity of crude DNA

The soil DNA from Pantnagar and Lonar soil samples were also extracted by various manual (Desai and Madamwar, 2007; Agarwal *et al*., 2001; Yamamoto *et al*., 1998)

as well as commercial methods (MN kit, Germany; Mo-Bio kit, CA, USA; Zymo soil DNA kit, CA, USA) according to the manufacturer's protocols and compared in terms of DNA yield and purity.

2.3.3. Determination of DNA quantity and purity

The isolated DNA was diluted (1:100) with MQ. The concentration (mg mL^{-1}) of the DNA [N] was determined spectrophotometrically by recording absorbance at 260 nm (A_{260}) as:

$$A_{260} = \varepsilon_{260}[N]$$

where ε_{260} is the extinction coefficient of DNA (50 for ds DNA)

[N] = concentration (mg mL^{-1}) of DNA

The concentration of ds DNA [N] was calculated as

$$[DNA]\ (mg\ mL^{-1}) = A_{260}/\varepsilon_{260}$$
$$[DNA]\ (\mu g\ mL^{-1}) = A_{260} \times 50 \times \text{dilution factor}$$

Purity of DNA was checked by measuring absorbance at 260 and 280 nm and calculating the A_{260}/A_{280} ratio (Sambrook *et al*., 1989). A DNA sample was considered pure when A_{260}/A_{280} ranged between 1.8-1.9. An $A_{260}/A_{280} < 1.7$ indicated contamination of the DNA preparation with protein or aromatic substances such as phenol, while an $A_{260}/A_{230} < 2.0$ indicated possible contamination of high molecular weight polyphenolic compounds like humic substances.

2.4. VALIDATION OF METAGENOME OBTAINED BY THE PROTOCOL DEVELOPED IN THIS INVESTIGATION

2.4.1. Restriction digestion

Purity of the DNA extracted from various environmental samples was confirmed by subjecting the extracted DNA to restriction digestion. DNA was digested with Sau3AI (New England Biolabs). One μg of metagenomic DNA in 20 μL reaction mixture was treated with 0.5 U of Sau3AI and incubated at 37 °C for 10 min. The reaction was terminated at 80 °C for 20 min and the digested DNA was fractionated on 1.2 % (w/v) agarose gel.

2.4.2. PCR amplification of microbial population

Attempts have been made to amplify the signature sequences of bacterial, archaeal and fungal specific regions by using respective sets of primers shown in **Table 2.2**. The reactions were carried out in 50 µL reaction mixtures in a Thermal Cycler (Bio-Rad, USA) using respective primers (**Table 2.2**). The PCR conditions were optimized as follows: for Bacterial 16S rDNA, initial denaturation of 3 min at 94 ^{o}C followed by 30 cycles of 30 sec at 93 ^{o}C, 60 sec at 55 ^{o}C and 90 sec at 72 ^{o}C; Archaeal 16S rDNA, 5 min at 95 ^{o}C, 35 cycles of 50 sec at 94 ^{o}C, 60 sec at 62 ^{o}C and 60 sec at 72 ^{o}C; fungal specific ITS regions, 3 min at 95 °C, 30 cycles of 60 sec at 94 °C, 56 °C at 45 sec and 50 sec at 72 °C. Final extension time was 7 min at 72 °C in all PCR runs. Amplifications were visualized on 1.2 % w/v agarose gels.

2.4.3. Effect of storage on soil/sediment DNA extracts

An attempt was made to study the effect of storage of DNA extracts on DNA yield and purity. The DNA extracts were centrifuged and the supernatants were dispensed into 2.0 mL Eppendorf tubes and stored at -20 ^{o}C for a month. DNA precipitation and its quantification were carried out at a week intervals.

2.5. CONSTRUCTION OF METAGENOMIC LIBRARY

2.5.1. Insert DNA preparation

The metagenomic DNA extracted from above defined protocol was digested with Sau3A1 at conditions optimized to generate maximum fragment in the size range of 2-6 kb. Different concentration (0.05 to 1 unit) of enzyme was used to optimize the digestion of 1 µg of DNA. Reactions were carried out in a final volume of 30 µl each in an Eppendorf of 1.5 mL. Reaction mixture (1 µg DNA having 3 µL NEB buffer 3 and 0.3 µL of 10X BSA) were kept at 37 °C for 10 min and stopped by heat inactivation at 80 °C for 20 min. Different digested reactions were checked for the desired fragments using 0.8 % (w/v) agarose gel electrophoresis. After optimization of DNA fragments for the appropriate size, a large scale digestion was carried out and the fragments (2-8 kb) were purified from low melting agarose gel using gel extraction method according to the manufacturer's protocol (Qiagen gel extraction kit, Germany).

2.5.2. Plasmid isolation from miniprep method

The cells of *E. coli* DH10B having p18GFP vector were cultivated for overnight at 37 °C in LB medium containing ampicillin (100 μg mL^{-1}).

- The *E. coli* culture having p18 GFP vector (~1.5 mL) was taken in Eppendorf tubes and centrifuged at 10, 000 x *g* for 5 min.
- The pellet was homogenized by vortex mixing in 100 μL of homogenizing solution.

Table 2.2. Details of primers used in the present study

Primer name	Oligonucleotide sequence (5'-3')	Primers used for
DX-INT1	CATCACCATCTAATTCAACAAGAA	Sequencing of the insert
DX-INT2	CGGCCGCACGCAAACGCAA	
DX-INT3	GTGAGCGCAACGCAATTAA	
DX-INT4	GCAATTCGTTCGCGCGCTTTT	
DX-INT5	CTTTTGATGGCGAAAAGCGTAT	
DX-INT6	ACGAATCGCCAGCGGACTT	
DX-INT7	CCGCTTTCGACGCCGTCGAA	
DX-INT8	AGCGAGTTTGAGGAAGATCGCGTT	
DX-INT9	GAAAGCGAGCGTCGAGAGATAATT	
DX-INT10	AGCAGCAGCAGCAGCAGTA	
M13 For	GTAAAACGACGGCCAGT	
M13 Rev	AACAGCTATGACCATG	
DxylNheI	CCCGCTAGCATGACAGCGAGTTTGAGGAAGA	*pET28Mxyl* construct
DxylXhoI	CCCCTCGAGTTACGGCGTGTTTCCGTAGC	
DxylSpeI	CCCACTAGTATGACAGCGAGTTTGAGGAAGA	*pET22Mxyl* construct
DxylBam	CCCGGATCCTTACGGCGTGTTTCCGTAGC	
BactF	AGAGTTTGTCCTGGCTCA	Bacterial 16S rDNA
BactR	GCTCGTTGCGGGACTTAACC	
ArchF	GGCCCTAYGGGGYGCASCAGG	Archaeal 16S rDNA
ArchR	GTGTGTGCAAGGAGCAGGGAC	
FungF	TCCGTAGGTGAACCTGCGG	Fungal specific ITS
FungR	TCCTCCGCTTATTGATATGC	
Dxyl-T68F	TGCAGAGCGGCGGCCGATACCGCTCTCAGTGGAG	Primers used for SDM
Dxyl-T68R	GTATCGGCCGCCGCTCTGCAAGCAGAACGTG	
Dxyl-T90F	CCCGGCGGACGACGCCGCGTCACCTACTCCGGC	
Vxyl-T90R	GCCGGAGTAGGTGACGCGGCGTCGTCCGCCGGG	
Dxyl-T92F	GGACGACGCCGCGTCCGCTACTCCGGCACGTTC	
Dxyl-T92R	GAACGTGCCGGAGTAGCGGACGCGGCGTCGTCC	
Dxyl-T69R	GGCGGCCGATACCGCCGCCAGTGGAGCAATATC	
Dxyl-T69R	GATATTGCTCCACTGGCGGCGGTATCGGCCGCC	
DxylE117DF	ACGAATCCGCTCGTCGATTACTACATCGTCGAT3	
DxylE117DR	ATCGACGATGTAGTAATCGACGAGCGGATTCGT	
DxylE209DF	ATGGTGATGGCGACCGATGGTTATCAGAGCAGC	
DxylE209DR	GCTGCTCTGATAACCATCGGTCGCCATCACCAT	

- Two hundred µL of alkaline-SDS solution was added to the above suspension, mixed by inverting the tubes up and down 3 times and incubated for 5 min at room temperature.
- To the above mixture, 250 µL of 3 M Na-acetate (pH 4.8) was added, mixed by inverting the tubes up and down 3 times, and centrifuged at 12,000 x *g* for 10 min.
- The supernatant was collected in another micro centrifuge tube (MCT), 200 µL of phenol:chloroform solution was added, inverted two times and centrifuged at 12, 000 x *g* for 8 min at room temperature.
- The aqueous phase was transferred to new tubes and 500 µL of chilled (-20 °C) ethanol (96 %) was added.
- The tubes were centrifuged at 13,000 x *g* for 25 min at 4 °C, supernatant discarded and pellet dried for 15 min at room temperature.
- The pellet was washed with 500 µL of chilled 70 % (v/v) ethanol and centrifuged at 13, 000 rpm for 4 min at 4 °C.
- The pellet was dried at room temperature and dissolved in 50 µL of 1X TE buffer (pH 8.0) containing RNase and stored at -20 °C till further use.

2.5.3. Restriction digestion of plasmid DNA

The vector isolated as above was digested with BamHI to generate the cohesive ends. The reaction was performed in 1.5 mL Eppendorf tubes as described below.

Composition of restriction mixture (100 µL)	
Plasmid DNA	X µL (20 µg)
Bam HI	8 µL (10 U μL^{-1})
NEB buffer 4	10.0 µL
BSA (100X)	1 µL
MQ water	Y µL

The reaction mixture was incubated at 37 °C for 3 h. The digestion was stopped by heat inactivation at 65 °C for 20 min. The digestion of plasmid was checked using 1.2 % (w/v) agarose gel electrophoresis for linearization of the plasmid. The digested plasmid was purified from low melting agarose gel using gel extraction method according to the manufacturer's protocol (Qiagen gel extraction kit, Germany).

2.5.4. Dephosphorylation of the restricted plasmid

In order to minimize self ligation of vector during cloning experiments, the digested DNA was subsequently treated with calf intestinal phosphatase (CIP) [NEB, UK]. The reaction conditions and amount of CIP were optimized and varied from (0.06-1) unit/picomole DNA termini. The dephosphorylation reaction was carried out in 50 µL reaction as follows. Reaction mixture containing no restriction enzyme was treated as control. Reaction was incubated for 1 h at 37 °C and stopped by heat inactivation at 65 °C for 20 min.

2.5.5. Composition of restriction mixture (50 µL)

Linearized Plasmid DNA	X µL (1 µg)
CIP	1 µL (0.06-1 U μL^{-1})
Reaction buffer (10X)	5.0 µL
Distilled water	Y µL
Total volume	50 µL

Linearized and dephosphorylated plasmids from each reaction were purified from low melting agarose gel using gel extraction method according to the manufacturer's protocol (Qiagen gel extraction kit, Germany). 100 ng DNA from each reaction was then ligated in15 µL reaction volume containing 1.5 µL of 10X ligation buffer (NEB, England) and 0.2 µL of T4 DNA ligase to check the efficiency of self ligation after dephosphoryaltion. The ligation mixture was incubated at 16 °C for overnight and transformed into *E. coli* DH5α competent cells.

2.5.6. Ligation of insert DNA with dephosphorylated vector

Purified DNA fragments of size 2-8 kb were ligated to the treated vector using a 1:3::vector :insert ratio in a volume of 10 µL. The total amount of DNA was about 0.5 µg. Vector and insert DNA was heated to 45 °C for 10 min and the immediately chilled on ice for 5 min prior to addition of ligase and buffer. T4 DNA ligase (NEB, England) was added to a final concentration of 0.125 UμL^{-1} and reactions were incubated at 16 °C for overnight in a ligation chamber. Reaction mixture incubated under same condition without addition of the enzyme was used as control. A ligation reaction was also set up under condition with linear plasmid DNA containing the

same amount of the enzyme. The reaction served as a negative control. The ligation mixture was subjected to transformation.

2.6. BACTERIAL TRANSFORMATION

Bacterial cells were made either electrocompetent or 'calcium' competent using standard procedures (Ausubel *et al*., 2000), and then transformed with plasmid DNA as described below.

2.6.1. Electrotransformation

2.6.1.1. Preparation of electrocompetent cells (*E. coli* cells)

A protocol was employed. The procedure was carried out in cold under sterile conditions as follows:

- A single colony of *E. coli* DH10B/ DH5α/XL1blue was inoculated in 20 mL of LB medium and grown overnight at 30 °C.
- 500 mL LB medium was inoculated with 5mL of this overnight grown culture of the *E. coli* and incubated with vigorous shaking (250 rpm) at 30 °C until an A_{600} of 0.5 - 0.8 was achieved.
- The cells were chilled in ice for 10-15 min and transferred to prechilled Sorvall® centrifuge tubes and sedimented at 4,000 rpm for 20 min at 4 °C.
- The supernatant was decanted and cells were resuspended in 500 mL of sterile ice-cold water, mixed well and centrifuged as described above.
- The washing of the cells described above was repeated with 250 mL of sterile ice-cold water, following which cells were washed with 40 mL of ice-cold 10 % (v/v) glycerol and centrifuged at 4,000 rpm for 10 min.
- The glycerol solution was decanted and the cell volume was recorded. The cells were resuspended in an equal volume of ice-cold 10 % glycerol.
- Cells were then dispensed in 40 µL volumes and stored at -80 °C until required.

2.6.1.2. Preparation of calcium-competent cells

Calcium-competent cells for heat shock transformation were produced according to a simplified method from Current Protocols (Ausubel *et al*., 2000) as follows:

- 2 mL of an overnight culture of *E. coli* cells was inoculated into 100 mL LB medium and incubated with vigorous shaking at 30 °C until A_{600} of 0.8 was reached.
- Cells were collected in 50 mL plastic (Falcon) tubes, cooled for 15 min on ice and centrifuged in a pre-cooled centrifuge (4,000 rpm for 10 min at 4 °C).
- The pellet was suspended in 20 mL of ice-cold 50 mM $CaCl_2$-15% glycerol solution, maintained on ice for 15 min and centrifuged again at 4,000 rpm for 10 min at 4 °C.
- Pellet was resuspended in 2 mL of ice-cold 50 mM $CaCl_2$-15 % glycerol solution, kept on ice for 30 min and aliquoted in 400 µL in microcentrifuge tubes. These were stored at -80 °C until required.

2.6.1.3. Transformation procedure

Transformation of calcium-competent cells was carried out by the procedure detailed below:

- The competent bacterial cells were thawed briefly and 200 µL of cells was mixed rapidly with plasmid DNA (10-50 ng) in fresh, sterile microcentrifuge tubes and maintained on ice for 30 min. A negative control with competent cells only (no added DNA) was also included.
- Cell membranes were disrupted by subjecting cells to heat-pulse (42 °C) for 90 sec.
- After heat shock, cells were incubated on ice for 5 min.
- Cells were then mixed with 1 mL LB medium and incubated with shaking at 37 °C for 1 h.
- For blue/white screening 40 µL of X-gal solution (20 mg mL^{-1} in dimethylformamide) and 4 µL of the IPTG (200 mg mL^{-1}) was spread on LB-ampicillin (LB-amp) plates with a sterile glass rod. The plate was allowed to dry for 1h at 37 °C prior to spreading of bacterial cells.
- Bacterial cells (100-200 µL) were spread and the plate was incubated at 37 °C for overnight.
- White colonies were picked from the plates and suspended into LB-amp broth and cultivated to OD_{600}=0.5.

- Glycerol stocks were made to preserve the metagenomic libraries by mixing equal volume of culture broth and glycerol (80 % v/v).

2.7. SCREENING OF THE TRANSFORMANTS FOR XYLANASE ACTIVITY

Metagenomic library obtained from various extracted DNA was screened by replica plating method on 0.3 % w/v RBB xylan containing LB-amp plates. The cells were allowed to grow for overnight at 37 °C and thereafter incubated at 4 °C till the appearance of zone of hydrolysis.

A total of 36,400 clones from various environmental samples were screened.

2.8. MAINTENANCE OF THE RECOMBINANT STRAIN

Overnight grown cultures of *E. coli* DH5α, *E. coli* BL21 (DE3), *E. coli* XL1blue cells with and without constructs were preserved in 80 % v/v glycerol.

2.9. DETECTION OF XYLANASE ACTIVITY

2.9.1. Qualitative detection of xylanolytic activity by plate assay

Sonicated cells of *E. coli* having recombinant vector was centrifuged. Supernatant was dispensed into 0.2 % v/v xylan agar plate and incubated for 4 h. The plates were then flooded with Congo red solution (0.2 % w/v) for 30 min and destained with 1M NaCl solution till a clear zone of xylan hydrolysis was visible. The plates were gently shaken on a shaker to accelerate the process of staining/destaining.

2.9.2. Quantitative screening for determination of xylanase in shake flask

The clear cell-free supernatants were used as the source of crude recombinant xylanase.

2.9.3. Preparation of standard curve of xylose

A stock solution of xylose (1 mg mL^{-1}) was prepared in distilled water. A dilution series ranging from 100-1000 μg mL^{-1} was prepared from the stock solution. To 1 mL of solution, 1mL of DNSA was added and kept in a boiling water bath for 10 min and then 400 μL of sodium potassium tartrate solution was added and kept it for cooling. The absorbance was recorded in a spectrophotometer (Shimadzu, UV-VIS) at 540 nm.

2.9.4. Enzyme Assays

Xylanolytic activity was determined according to Archana and Satyanarayana (1997). The reaction mixture containing 0.5 mL of 1% birchwood xylan in glycine NaOH buffer (0.1 M, pH 9.0) and 0.5 mL of cell free sonicated supernatant was incubated at 80 °C in a water bath for 10 min. After incubation, 1 mL DNSA reagent (Miller, 1959) was added to the reaction mixture and the tubes were incubated in a boiling water bath for 10 min, followed by the addition of 400 µL of 33% w/v sodium potassium tartrate. The absorbance values were recorded at 540 nm in a spectrophotometer (Shimadzu, Japan). The liberated reducing sugars were determined by comparing the absorbance values of these with a standard curve drawn with different concentrations of xylose. One unit (IU) of xylanase is defined as the amount of enzyme required for liberating one µmol of reducing sugar as xylose mL^{-1} min^{-1} under the assay conditions.

Composition of Dinitrosalicylic acid (DNSA) reagent

NaOH	-	10.0 g
Phenol	-	2.0 g
DNSA	-	2.0 g
Distilled Water	-	1000 mL

DNSA reagent was stored in an amber bottle at 4 °C till further use. Sodium sulphite (0.05 % v/v) was added just before the use of the reagent.

2.9.5. Assays for β-Xylosidase, acetylesterase and arbinofuranosidase

The activities of β-xylosidase, xylan acetylesterase and arbinofuranosidase were measured using 1 mM *p*-nitrophenylxylopyranoside, *p*-nitrophenylacetate and *p*-nitrophenylarabinofuranoside, respectively prepared in sodium citrate buffer (0.1 M, pH 7.0). One mL of reaction mixture containing 0.2 mL of crude enzyme solution, 0.3 mL of sodium citrate buffer (0.1 M, pH 7.0) and 0.5 mL of substrate was incubated at 80 °C for 30 min. The reaction was terminated by adding 2 mL sodium carbonate-bicarbonate buffer (1.0 M, pH 10.0). The activities were determined using *p*-nitrophenol standard curve (1-10 µg mL^{-1}) drawn using absorbance values measured in spectrophotometer at 400 nm.

One unit of the enzyme is defined as the amount of enzyme that liberates 1µmole of *p*-nitrophenol $mL^{-1}min^{-1}$ under assay conditions.

2.9.6. β-Glucosidase

The reaction mixture contained 0.2 mL of enzyme sample, 0.3 mL of buffer and 0.5 mL of *p*-nitrophenyl-β-D-glucopyranoside (1.0 mM) prepared in 100 mM buffer as the substrate. The reaction was terminated after 30 min of incubation at 70 °C by adding 2 mL of sodium carbonate-bicarbonate buffer (0.1 M, pH 10.0). The liberation of *p*-nitrophenol was measured at 400 nm and its yield was determined using a standard curve of *p*-nitrophenol (1-10 μg mL^{-1}) prepared in sodium carbonate-bicarbonate buffer.

2.10. PROTEIN DETERMINATION

Soluble protein was estimated according to Lowry *et al*. (1951), using bovine serum albumin (BSA) as the standard.

2.10.1. Reagents

Solution A: 2.0 % Na_2CO_3 in 0.1N NaOH

Solution B: 0.5 % $CuSO_4.5H_20$

Solution C: 1.0 % K-Na tartrate

Solution D: 0.5 mL of solution B + 0.5 mL of Solution C + 49 mL of freshly prepared solution A

2.10.2. Procedure

Five mL of solution D was added to 1 mL of enzyme sample and incubated at room temperature for 10 min. Then 0.5 mL of (1:1 diluted) Folin & Ciocalteu's Folin Phenol reagent was added, vortexed, and the resultant mixture was incubated in dark for 30 min. The absorbance was recorded at 660 nm, and the protein content was computed from a standard curve drawn using 30-300 μg mL^{-1} bovine serum albumin.

2.10.3. Specific enzyme activity

The specific xylanase activity was calculated as follows:

$$\text{Specific enzyme activity (U mg}^{-1}\text{ protein)} = \frac{\text{Enzyme activity (U mL}^{-1}\text{)}}{\text{Soluble protein (mg mL}^{-1}\text{)}}$$

2.11. SEQUENCING AND ANALYSIS OF INSERT FROM XYLANASE PRODUCING CLONE

DNA sequencing was performed at the sequencing facility of UDSC. Ten internal primers were designed for accessing the full stretch of the insert beside M13 forward and M13 reverse primers (**Table 2.2**). The full sequence was deduced by primer walking. The complete sequence was obtained by aligning the overlapping regions. Furthermore, the ORFs have been recognized by NCBI ORF finder. The alignments of DNA and protein sequences were conducted with BLASTN and BLASTP programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed with ClustalW.

2.11.1. ORF finder program

The open reading frame (ORF) finder program available at NCBI web page (http://www.ncbi.nl.nih.gov/gorf/gorf.html) was used to determine the possible open reading frames. For translation to protein sequences, code 11 (bacterial code) was selected which allowed selection of alternative initiation codons. ORFs were translated and queried using BLAST against the NCBI non-redundant database.

2.12. SUBCLONING OF THE INSERT IN pET28a(+)/pET22b(+)/ pWH1520 VECTORS

2.12.1. Cloning of the xylanase gene from the metagenomic clone

2.12.1.1. Standard PCR amplification of xylanase gene

The xylanase gene was amplified using the forward and reverse primers having respective restriction sites (**Table 2.2**). The PCR reaction (25 µL) was performed in a Thermal cycler (Bio-Rad, USA).

Reaction components	Stock solution concentration	Desired concentration	Volume
DNA (50 ng/µL)		100 ng	2.0 µL
Primer (forward)	10 or 25 pmol/µL	10 or 25 pmol	1.0 µL
Primer (reverse)	10 or 25 pmol/µL	10 or 25 pmol	1.0 µL
dNTPS	2 mM	200 µM	2.5 µL
10 X *Taq*-reaction buffer	10 X	1 X	2.5 µL
Sterile MilliQ			add to 25 µl

The following PCR conditions were used

Step	Temperature (°C)	Time
Initial Denaturation	94-95	5 min
Denaturation*	94-95	1 min
Annealing*	50-60^{x}	30 s
Elongation*	72	1min ^{Y}
Final extension	72	10 min
Hold	4	forever

* 25 - 30 cycles
x: Annealing temperature was dependent on the T_m (melting temperature) of primers
y: Elongation was usually 1 min per kb of amplified DNA

The amplification of xylanase gene was checked on 1.2 % (w/v) agarose gel under UV Transilluminator.

2.12.1.2. Construction of the expression vectors

Xylanse gene from the metagenomic clone was subcloned in various vectors (Fig. 1A, 1B, 1C) to obtain following defined constructs.

Construct in pET28(+) vector : *pET28Mxyl*

Construct in pET22b(+) vector : *pET22Mxyl*

Construct in pWH1520 vector : *pWHMxyl*

Amplified xylanase gene was purified from gel by gel extraction method and double digested with respective restriction enzymes in 30 µL reaction mixture (20 µL PCR product, 3 µL NEB buffer 4, 0.3 µL BSA, 0.2 µL of each enzyme and 6.3 µL sterile MQ) to obtain the cohesive ends. The digested PCR product was ligated with respective vectors [already treated with same endonucleases] in 10 µL reaction (2 µL digested PCR product (100 ng), 5 µL ligase buffer, 4 µL digested plasmid (300 ng), 0.2 µL ligase enzyme and 0.8 µL sterile MQ). The ligated product was transformed into chemically competent *E. coli* DH5α cells by heat shock method. The clones were selected from appropriate antibiotic containing LB agar plate by growing overnight at 37 ^{o}C.

2.13. CONFIRMATION OF THE CLONE

2.13.1. Colony PCR

Various clones were replica plated on LB agar plate having appropriate antibiotic and analyzed for the desired recombinant vector. For confirmation of the clones, colony PCR was carried out. Colonies from the master plate were picked and swirled in 25

µL sterile MQ. The cells were lysed by boiling in water bath for 7 min. The lysate was used as DNA template for the colony PCR. The PCR was carried out in 25 µL reaction under same conditions as described in section 2.12.1.1.

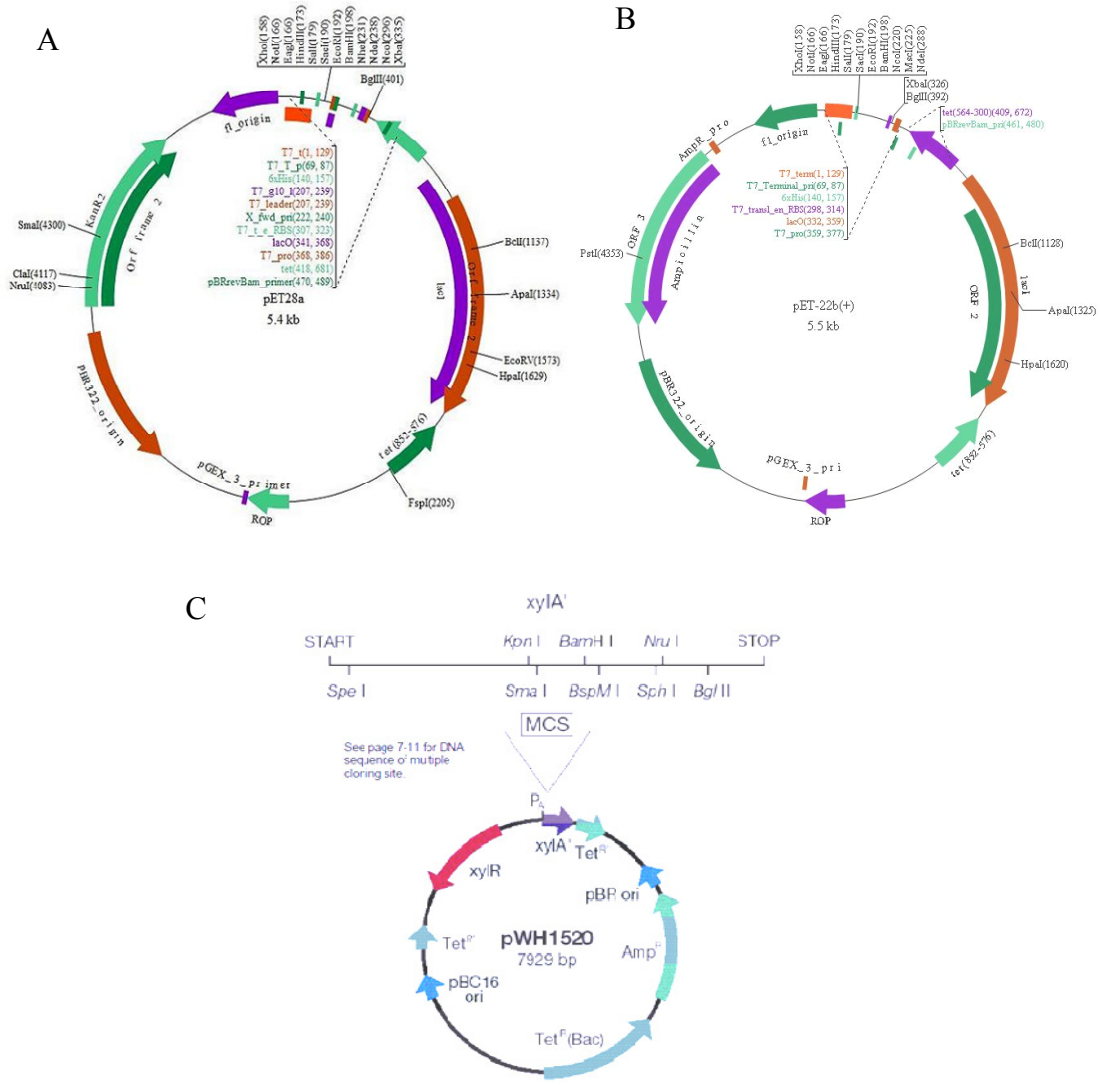

Fig. 2.1. Various expression vectors: pET28a(+) (**A**), pET22b(+) (**B**) and Shuttle vector pWH1520 (**C**) for *E. coli*/*B. subtilis*/*B. megaterium*

2.13.2. Clone confirmation by restriction digestion

In addition, the positive clones obtained from colony PCR were confirmed by double digestion of the plasmid. The positive clones from colony PCR were inoculated in 10 mL LB-Kanamycin (LB-Kan) medium and grown for overnight at 37 °C for plasmid isolation. Plasmid were isolated from each clone and digested with respective restriction enzymes for the release of the insert. The reaction for double digestion of

the construct was carried out in 25 µL as given below. The reaction mixture was incubated at 37 °C for 1h. The fall out was determined by 1.2 % (w/v) agarose gel electrophoresis.

Plasmid (500 ng)	X µL
Buffer 4 (10X)	2.5 µL
BSA (100 X)	0.25 µL
R. Enzyme I	0.2 µL
R. Enzyme II	0.2 µL
Sterile MQ	Y µL
Total volume	25 µL

2.13.3. Sequencing of the insert

After confirmation by double digestion of different constructs, the respective plasmids were sequenced.

2.14. TRANSFORMATION IN *E. coli* BL21(DE3)/ *Bacillus subtilis*

2.14.1. Transformation of *E. coli* BL21(DE3)

Plasmids having the exact sequence of xylanase gene were processed for transformation of *E. coli* BL21(DE3) for the expression of recombinant xylanase. Transformation was performed by heat shock method defined in section 2.7.1.3.

2.14.2. Transformation of *Bacillus subtilis*

The proptoplast of *B. subtilis* was generated according to McCool and Cannon (2001). Overnight grown culture was used to inoculate (1% v/v) 20 mL of fresh RHAF medium in 250 mL flasks and incubated at 35 ºC and 250 rpm to an absorbance of 0.4. RHAF medium (gL^{-1}): NH_4Cl – 1.0; Tris base-12.0; KCl-0.035; NaCl-0.058; Na_2SO4-0.30; KH_2PO_4- 0.14; $MgCl_2.5H_2O$- 4.26; yeast extract-5; tryptone-5; sucrose-68.46; and 10 mL of 200 g/L glucose. The pH was adjusted to 7.5 with HCl before addition of $MgCl_2.5H_2O$. Cells were harvested and resuspended into 2 mL of RHAF to which 600 µg/mL of lysozyme was added and incubated for 15 min. The protoplasts formed were sedimented by centrifugation at 1000 x g for 5 min and

washed gently in 2 mL of RHAF. Protoplast suspension of 200 µL with 10 µL of recombinant plasmid DNA (0.5 µg/µL) and 200 µL of 35% polyethylene glycol (MW, 8000) were mixed gently and incubated at 37 ºC for 3 min, followed by immediate dilution with 3.0 mL of RHAF and pelleting at 1000 x g for 5 min. For protoplast generation, the pellet was suspended in 1.0 mL RHAF plates with 1 % w/v agar and incubated overnight at 30 ºC. The colonies appeared on the plates were recovered with 5 mL of LB medium and then plated on selective RBB-xylan containing LB agar plates to score transformants for overnight at 37 ºC. A large number of transformants of *Bacillus subtilis* were obtained on LB agar plates. The clones were picked from LB agar plate and transferred to LB-tetracycline (LB-Tet) plate for the antibiotic selection. The selected plasmids were processed for the expression of xylanase.

2.15. EXPRESSION OF THE RECOMBINANT XYLANASE

2.15.1. Expression of *pET28Mxyl* and *pET22bMxyl* vectors in *E. coli* BL21 (DE3)

The recombinant vectors isolated from various *E. coli* DH5α cells were transformed into *E. coli* BL21(DE3) for the expression of the recombinant protein. Different clones of *E. coli* BL21 (DE3) were picked from the selection plates and grown overnight at 37 ºC as a seed culture. 50 mL of fresh LB-Kan medium was inoculated with 1% (v/v) seed culture and induced with 1 mM IPTG at the OD_{600} value of 0.5. The culture was harvested after 4h at 10000 rpm for 10 min, supernatant was kept for determining extracellular expression of xylanase from the respective hosts, while sedimented cells were washed with normal saline and suspended in lysis buffer (100 mM NaCl, 25 mM Tris HCl, 10 mM $MgCl_2$, 1 mg mL^{-1} lysozyme) and sonicated (2 sec on/off) for 5 min. Sonicated cells were separated by centrifugation and the supernatant was processed for determining the induction of the intracellular xylanase on 15 % SDS-PAGE.

2.15.1.1. Effect of IPTG induction on growth profile of *E. coli* BL21 (DE3)

The expression of the enzyme was induced by the addition of 1 mM IPTG when OD_{600} of the culture was about 0.5-0.6. Samples of various time intervals were collected to determine the OD and respective xylanase activity.

2.15.2. Expression of *pWHMxyl* in *Bacillus subtilis*

Bacillus subtilis strain carrying the recombinant plasmid was cultivated for overnight at 37 °C in LB broth having tetracycline (10 mg mL^{-1}). Fifty mL of fresh LB-tet medium was inoculated with 2 % v/v seed culture and grown to OD_{600} = 0.3. The expression of xylanase was induced by addition of 0.5 % w/v xylose. Samples were collected for varying time intervals and supernatant was processed for xylanase assay. Residual xylose sugar at each interval was determined by DNS assay and considered as base line activity that was subtracted before calculation of the xylanase activity.

2.16. OPTIMIZATION OF DIFFERENT VARIABLES FOR MAXIMUM XYLANASE PRODUCTION FROM *pET28Mxyl* AND *pET22Mxyl*

2.16.1. IPTG concentration

Different concentrations of IPTG (0.05, 0.1, 0.5, 1, 2 and 3) mM were attempted for enhancing the production of recombinant xylanase in 250 mL Erlenmeyer flask.

2.16.2. Effect of incubation time

Erlenmeyer flasks (250 mL) containing 50 mL LB-Kan medium was inoculated with 1% (v/v) overnight grown culture at 37 ºC to achieve the OD_{600} of 0.5. The cells were induced by 1 mM IPTG. Samples of various time intervals (0, 1, 2, 3, 4, 5, 6, 7) h were collected and kept at 4 ºC. The cells were sonicated and processed for protein profiling on 15 % w/v SDS-PAGE.

2.16.3. Effect of temperature

Effect of various temperatures (25, 30 and 37 ºC) was studied for the production of recombinant xylanase from *E. coli* BL21 (DE3) cells. After induction, the cells were cultivated at various temperatures (25 ºC, 30 ºC and 37 ºC) for 4 h. The cells were harvested, sonicated and assayed for xylanase activity.

2.16.4. Effect of various lysis solutions

Different lysis solution were tried to sonicate the cells. Induced cells from 50 mL LB broth were harvested and suspended in 5 mL of various lysis solutions, sonicated and processed for xylanase assay. Composition of various lysis buffers is summarized in **Table 2.3**.

Table 2.3. Composition of different lysis solution used for sonicating the cells

Lysis solution A: 100 mM NaCl, 25 mM Tris HCl,, 10 mM $MgCl_2$, 1 mg mL^{-1} lysozyme
Lysis solution B: Lysis solution A+ 0.1 % (v/v) Triton X
Lysis solution C: Lysis solution A + 6.0 M Urea
Lysis solution D: Lysis solution A + 10 mM β-ME

2.17. LOCALIZATION OF RECOMBINANT XYLANASE FROM *E. coli* (*pET28Mxyl*)

The induced culture was harvested by centrifugation after 5 h of induction. The supernatant was collected as extracellular fraction while the pellet was suspended in 20 mM TE buffer (pH 8.0) with 25 % (w/v) sucrose and 1 mM EDTA. The suspension was kept on moderate shaking for 15 min and cells were sedimented by centrifugation at 4 ºC. Thereafter, osmotic treatment was applied for 10 min by suspending the pellet into 5.0 mM chilled $MgSO_4$ to release the periplasmic fraction of the cells which was collected as supernatant on centrifugation. Finally the intracellular fraction was collected on homogenizing the cells in chilled sonication buffer (25 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 6M Urea and 1mg/mL lysozyme). The intracellular protein was released on sonication using Ultrasonic sonicator (Sonics, Vibra-cell, Connecticut, USA) with 10 cycles of 1 min pulse (2 sec on/off). The supernatant was separated from the cell debris after centrifugation and stored at -20 ºC for assessing the intracellular fraction.

2.18. PROTEIN PROFILING FROM *E. coli* (*pET22Mxyl*)

Induced *E. coli* BL21(DE3) cells having *pET22Mxyl* recombinant vector were harvested by centrifugation at 10,000 rpm for 10 min at 4 ºC. Supernatant was used as a source of extracellular xylanase while pellets were subjected to osmotic lysis in presence of 1 mL chilled 5M $MgSO_4$ to release the periplasmic fraction of recombinant xylanase. Therefore, cells were centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatant thus obtained was treated as periplasmic fraction of recombinant xylanase. Furthermore, the pellets were sonicated in presence of 1 mL lysis solution A (100 mM NaCl, 25 mM Tris HCl, 10 mM $MgCl_2$, 1 mg mL^{-1} lysozyme). Cell debris was separated after sonication to collect the intracellular

fraction of recombinant xylanase. Samples of varying time interval were collected and various fractions of recombinant xylanase were obtained as described above. Recombinant xylanase activity from various fractions was calculated individually and fractionated in terms of percentage.

2.19. EXPRESSION OF XYLANASE GENE IN *BACILLUS SUBTILIS*

The xylanase gene was amplified as described in section (2.12.1.1) with primers DxylSpeF and DxylBamHR (**Table 2.2**) and cloned into the *Spe*I and *Bam*HI site of vector pWH1520 and recombinant strain was confirmed by colony PCR and restriction digestion. The recombinant plasmid *pWHMxyl* was successfully transformed into *B. subtilis* by protoplast transformation described in section (2.14.2).

2.19.1. Xylanase production in shake flask

Recombinant *B. subtilis* (*pWHMxyl*) was grown in LB medium in Erlenmeyer flask (250 mL) to an OD_{600nm} of 0.3 at 37 ^{o}C and expression of xylanase was induced by xylose (0.5 % w/v). Different parameters like xylanase production, was determined by collecting the sample of varying time interval. The induced culture produced detectable amount of xylanase after 6 h of induction.

2.19.2. Optimization of *pWHMxyl* by one variable at a time approach

2.19.2.1. Xylose concentration

Different concentration of xylose (0.1, 0.3, 0.5, 0.7 and 1 % w/v) was used for enhancing the production of recombinant xylanase in 250 mL Erlenmeyer flask. The cells were induced at OD_{600nm} = 0.5 and xylanase activity were determined after 12 h of induction.

2.19.2.2. Inoculum density

The recombinant strain *B. subtilis* (*pWHMxyl*) was grown to different (OD_{600}=0.3, 0.6, 0.9, 1.0 and 2.0) cell density and induced with 0.5 % w/v xylose. Xylanase activity was determined after 12 h of induction.

2.19.2.3. Incubation time

After optimization of xylose concentration and inoculum density Erlenmeyer flasks (250 mL) containing 50 mL LB-Tet medium was inoculated with 2 % (v/v) overnight

grown culture and incubated at 37 °C to achieve the OD_{600} of 0.5. The cells were induced by 0.5 % w/v filter sterilized xylose. Samples of various time intervals (0-24 h) were collected and kept at 4 °C. The cells were centrifuged and supernatant was processed for xylanase assay.

2.20. CENTRAL COMPOSITE DESIGN (CCD)

The CCD approach was used for determining the optimum levels of critical variables identified by 'one-variable-at-a-time' approach: (A) xylose, (B) inoculum density and (C) incubation time for improving xylanase production and understanding their interactions. A set of 20 experiments were performed with each independent variable in the design studied at five different levels (-2, -1, 0, +1, +2). A 2^4 factorial design with eight axial points and six replicates at the center point was employed. Further an average of xylanase production done in triplicates obtained was taken as the dependent variable or response (Y). The xylanase production was then analyzed by using second order polynomial equation and the behavior of the system was explained.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC$$

Where, Y is predicted response, β_0 is intercept, β_1, β_2 and β_3 are linear coefficients. $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ are squared coefficients. $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ are interaction coefficients and *A, B, C,* A^2, B^2, C^2, *AB, AC, and BC* are independent variables. Statistical analysis of the model equation was determined by Fisher's t-value and the proportion of variance explained by the model was given by the multiple coefficient of determination, R squared (R^2) value. With the help of Design Expert Software version 6.0, contour plots were generated.

Table 2.4. Range of variables for response surface methodology

Factor code	Factor	Levels				
		-α	-1	0	+1	+α
A	Xylose (%)	0.3	0.4	0.5	0.6	0.7
B	Inoculum density (%)	0.2	0.4	0.6	0.8	1.0
C	Incubation time (h)	8.0	10	12	14	16

2.21. ELECTROPHORETIC ANALYSIS OF THE ENZYME

The purity of the enzyme was ascertained by SDS-PAGE, while its activity was demonstrated by native PAGE using 15 % gels (**Table 2.5**). The electrophoretic separation was carried out for 3-4 h at a constant voltage (70 V) at 4 °C. After the run, the gel was laid on 0.2 % xylan agar plate for 2-3 h and stained overnight with Congo red staining method, and then destained for several hours till the band for hydrolysis were clearly visible. The reagents for native/ SDS-PAGE (15 %) was prepared according to Laemmli, (1970) as described below:

2.21.1. Stock solutions

- **Acrylamide solution:** 30 g acrylamide and 0.8 g bis-acrylamide were dissolved in 100 mL of distilled water and filtered. Stable at 4 °C for months.
- **Separating gel buffer:** 1.5 M Tris-HCI, pH 8.8.
- **Stacking gel buffer:** 0.5 M Tris-HCI, pH 6.8.
- **10 % APS in water**
- **TEMED**
- **Sample buffer (5X)** : Mix the following:
    a. 15.5 mL of 1 M Tris-HCI pH 6.8
    b. 2.5 mL of a 1 % solution of bromophenol blue
    c. 7 mL of water
    d. 25 mL of glycerol

Note: Samples were mixed with the sample buffer in 4:1 ratio. The use of protein solutions in a strong buffer and with their pH not close to 6.8 was avoided. Such samples were dialyzed against diluted (1X) sample buffer.

- **Electrophoresis buffer:** 3.0 g of Tris base and 14.4 g of glycine were dissolved in water and the volume was adjusted to 1 L. The solution has a final pH of 8.3.
- **Destaining solution:** contains 100 mL methanol, 100 mL glacial acetic acid, and 800 mL water.
- **SDS gel loading buffer:** 50 mM Tris (pH 6.8), 100 mM Dithiothreitol, 2 % w/v SDS, 0.1 % w/v Bromophenol blue and 10 % v/v glycerol.

- The reagents of native PAGE used were the same as that of SDS-PAGE except that SDS was substituted with equal volume of distilled water.

2.21.2. Silver staining of protein gel

Reagents

Gel fixation solution: Trichloroacetic acid (TCA) solution, 20% (w/v).

Sensitisation solution: Glutaraldehyde solution 10% (w/v).

Silver diamine solution: 21 mL of 0.36% (w/v) NaOH was added to 1.4 mL of 35% (w/v) ammonia followed by drop wise addition of 4 mL of 20% (w/v) silver nitrate with stirring.

Table 2.5. Composition of 15 % native PAGE*/SDS-PAGE

Component	Resolving gel (15%)	Stacking gel (5%)
30 % Acrylamide	5.0 mL	0.85 mL
Tris (pH 8.8/ 6.8)	2.5 mL	0.625 mL
Water	2.3 mL	3.4 mL
10 % SDS	100 µL	50 µL
10 % APS	100 µL	50 µL
TEMED	2.0 µL	3.0 µL
Total volume	10 mL	5 mL

The final volume of the solution was made up to 100 mL with distilled water. **[**Note: When the mixture was not clear due to the formation of a brown precipitate, further addition of minimum amount of ammonia resulted in the dissolution of the precipitate. Since, silver diamine solution is unstable the solution was used within 5 min].

Developing solution: 2.5 mL of 1% (w/v) citric acid, 0.26 mL of 36% (w/v) formaldehyde made up to 500 mL with water.

Reaction termination solution: 40 % (v/v) ethanol, 10% (v/v) acetic acid in water.

Farmer's reducer: 0.3% (w/v) potassium ferricyanide 0.6% (w/v) sodium thiosulphate, 0.1% (w/v) sodium carbonate.

[Note: All solutions were prepared fresh in clean glassware using deionised/distilled water].

Procedure

Fixation

After electrophoresis, the gel was immediately fixed in TCA (20% w/v) solution and kept overnight at room temperature. Next morning, the gel was placed in reaction termination solution for 2 x 30 min and washed in the excess water for 2 x 20 min facilitating the rehydration of the gel and the removal of methanol. The loss of hydrophobic nature of the gel indicated rehydration.

Sensitisation

The gel was immersed in 10% (v/v) glutaraldehyde solution for 30 min at room temperature followed by washing with water for 3 x 20 min to remove excess glutaraldehyde.

Staining

The gel was soaked in the silver diamine solution for 30 min, followed by 3 x 5 min washings with water.

Development

The gel was placed in developing solution to detect the appearance of dark brown protein bands. The reaction was immediately terminated by immersing the gel in the reaction termination solution and then stored in distilled water.

Destaining

The over stained gels were placed in Farmer's reducing reagent for removing the excess black background. Once the individual bands appeared against clear background, the reaction was terminated by placing the gel in the reaction termination solution.

2.22. PURIFICATION OF RECOMBINANT XYLANASE FROM *pET28Mxyl* VECTOR

Recombinant xylanase was purified by affinity chromatography using Ni-NTA agarose (Novagen, Germany). Five mL Ni-NTA resins were washed with Milli Q water and equilibrated with 5 column volumes of buffer A (6.0 M urea in 100 mM phosphate buffer, pH 8.0, containing 5 mM β-mercaptoethanol) and buffer B (buffer A containing 10 mM Tris HCl, pH 8.0) successively. The supernatant obtained after sonication of the induced cells were incubated at 65 °C for 30 min and centrifuged at

12000 x g for 20 min at 4 °C. The supernatant was loaded to the column and placed on a rocker for 1 h at room temperature. The column was then washed with buffer C (buffer B containing 20 mM imidazole) to remove the unbounded proteins. The column was further washed by passing buffer C and buffer D [100 mM phosphate buffer, pH 8, 10 mM Tris-HCl, pH 8.0, containing 5 mM β-mercaptoethanol, 20 mM imidazole, and 10 % (v/v) glycerol] in gradient manner followed by final washing with buffer E (buffer D having 300 mM NaCl). The various fractions (each of 2 mL) were collected from the column by gradually increasing imidazole concentration (100-500 mM). The samples were analyzed on 15 % w/v SDS-PAGE. The fractions containing the pure recombinant xylanase were pooled and dialyzed against 100 mM phosphate buffer (pH 8.0) and stored at 4.0 °C.

2.23. ZYMOGRAM ANALYSIS

2.23.1. Native polyacrylamide gel electrophoresis (PAGE)

The activity of the purified enzyme was demonstrated on native polyacrylamide gel. The 15 % native gel was laid over an agar plate containing 0.2 % xylan buffered with glycine NaOH at pH 9.0, for 3-4 h at 70 V, and 4 °C. The gel was taken out after an incubation of 2 h and flooded with Congo red solution (0.2 % w/v) for 30 min followed by destaining with 1 M NaCl, each for a time interval of 10 min.

2.24. BIOINFORMATIC ANALYSIS

2.24.1. ProtParam's tool

ProtParam is a proteomics tool available from ExPASy (http://expasy.org/tools/) [Gasteiger *et al*., 2005]. The ExPASy (Expert Protein Analysis System) is a proteomics server of the Swiss Institute of Bioinformatics (SIB), which is dedicated to the analysis of protein sequences and structures. PROTPARAM allowed the computation of various physical and chemical parameters for the recombinant xylanase protein based on its amino acid sequence. The computed parameters included the molecular weight, theoretical pI and amino acid composition.

2.24.2. Proposed 3D structure of recombinant xylanase

The amino acid sequence of recombinant xylanase was submitted to ESyPred3D online server (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/).

The ESyPred3D made the protein model in PDB file format on the basis of its closest template (glycosyl hydrolase family 11of an uncultured bacterium) showing 74 % identity with the query. The PDB file, generated by ESyPred3D software was viewed using PyMOL software and structures were generated (Lambert, 2002).

2.24.3. Evaluation of the 3D model of recombinant xylanase

The stereochemical properties of the predicted 3D structure of xylanase were assessed by Ramachandran plot using the server RAMPAGE (Lovell *et al*. 2002), while that of residue environment was evaluated by Verify3D (Bowie *et al*., 1991; Luthy *et al*., 1992).

2.24.4. *In silico* analysis of α and β sheets in recombinant xylanase

The percentage α-helix, β-sheet and random coil content of recombinant xylanase was determined by using ESPript software as follows:

α-Helix content (%) = Amino acids encoding α-helix/Total no. of amino acids

β-Sheet content (%) = Amino acids encoding β-sheet/Total no. of amino acids

Random coils (%) = 100-[α-helix content (%) + β-Sheet content (%)]

2.24.5. Prosite

Various signature sequences of recombinant xylanase were indentified using PROSITE software of ExPASY. Catalytically important residues of glycosyl hydrolase family 11 and their respective conservation were identified by the generated logos.

2.25. CHARACTERIZATION OF RECOMBINANT XYLANASE

2.25.1. Effect of temperature

Xylanase activity was determined at various temperatures (20-100 °C) by incubating the reaction mixtures at these temperatures.

2.25.2. Effect of pH

The effect of pH on enzyme activity was assessed by using buffers (0.1 M) of desired pH in the reaction mixture where the substrate (1 % xylan) and the purified enzyme were suspended in the buffer. The buffers used were: glycine-HCl buffer (pH 2.0,

3.0), sodium acetate buffer (pH 4.0, 4.5, 5.0), citrate buffer (pH 5.5, 6.0), Tris-HCl (pH 7.0), phosphate buffer (8.0) and glycine-NaOH buffer (pH 9.0, 10.0, 11.0, 12.0).

2.25.3. Thermostability The recombinant xylanase in glycine-NaOH buffer (0.1 M, pH 9.0) was exposed to different temperatures (60, 70, 80 and 90 °C) for varying time intervals and then assayed at 80°C and half life of the enzyme was determined.

2.25.4. pH stability

Purified recombinant xylanase was also suspended in various alkaline buffers (8.0, 9.0, 10.0 and 11.0) at room temperature for varying time intervals and then assayed at 80 °C to study its pH stability.

2.25.5. Shelf life of enzyme

The shelf life of the recombinant xylanase was determined by exposing it to room temperature and 4 °C, and checking its activity at desired time intervals. The enzyme was also lyophilized and its activity was checked at different time intervals.

2.26. BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT XYLANASE

2.26.1. Effect of divalent cations on xylanase activity

The effect of divalent cations (Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+} either as sulphate/chloride salts) were evaluated by incorporating them in the reaction mixtures (1, 5 and 10 mM) and recombinant xylanase activities were determined.

2.26.2. Effect of detergents on xylanase activity

Effects of different detergents: Ionic [sodium dodecylsulphate (SDS)] and non-ionic detergents (Tween 20, Tween 80 and Triton X-100) were included in the reaction mixtures and assayed for xylanase.

2.26.3. Effect of inhibitors on xylanase activity

Different concentrations (1, 5 and 10 mM) of inhibitors [β-mercaptoethanol (βME), dithiothreitol (DTT), iodoacetamide (IAA) and ethylenediaminetetraacetic acid (EDTA)] were included in the reaction mixtures and assayed for xylanase activity.

2.27. ENZYME KINETICS

2.27.1. Effect of substrate concentration on enzyme activity and determination of K_m and V_{max} of different substrates

Xylan (Sigma) solution of various concentrations (0.5-7 mg mL^{-1}) were prepared and used in reaction mixture. The enzyme was assayed at 80 ºC and pH 9.0 and determined the K_m and V_{max}. The efficiency of xylan hydrolysis was determined as V_{max}/K_m ratio.

2.27.2. The catalytic turnover number (K_{cat}) of purified recombinant xylanase

For determining the catalytic turnover number (K_{cat}) of purified xylanase, the assays were carried out at various concentrations of enzyme (0.5-1.5 mg mL^{-1}), while keeping the substrate concentration (xylan) constant.

2.28. STRUCTURE–FUNCTION RELATIONSHIP OF RECOMBINANT XYLANASE

Purified recombinant xylanase by affinity chromatography using Ni^{2+}-NTA resins was dialyzed against 20 mM glycine-NaOH buffer (pH 9.0) and processed for the study of structure function relationship.

2.28.1. Secondary structure of xylanase

The secondary structure of the purified xylanase (0.1 mg mL^{-1}) [thermal activated at 100 ^{o}C] was determined by circular dichroism (CD) spectroscopy carried out on a JASCO-810 Spectropolarimeter equipped with in-built Peltier controlled thermostat cell holder (PTC-423S). The path length of cuvettes used was 0.2 cm. Temperature scans were performed by exposing the xylanase to different temperatures (60-90 ^{o}C) and the changes in the structural conformation scans were recorded at scanning rate of 20 nm min^{-1} from 190 to 260 nm. Each CD spectrum was always an average of six scans and data acquisition and analysis was performed on a computer, which is interfaced to the spectropolarimeter. Nitrogen was flushed continuously through the machine at the rate of 5.0 mL min^{-1}. The CD instrument was routinely calibrated with D-10-camphor-sulphonic acid.

2.28.2. Effect of temperature and pH on the secondary structure of xylanase

The changes in the structural conformation of the protein were recorded. Effect of pH was assessed by using different pH buffers [Phosphate K_2HPO_4- KH_2PO_4- 8.0, glycine-NaOH, pH 9.0 to 11.0] and changes in the secondary structure conformation of the protein were recorded. Similarly a fixed amount of xylanase (0.1 mg mL^{-1}) was incubated at various temperatures and changes in structure conformation were recorded.

2.28.3. Fluorescence measurements

Steady-state fluorescence titrations were performed in a Hitachi F-4500 fluorescence spectrofluorometer at room temperature using a slit width of 5/5 nm (exc / emi) with a slow scan of 15 nm/min and quartz cells of 1 cm path length. Fluorescence emission spectra were recorded between 300 to 400 and 315 to 400 nm using two different excitation wavelengths of 280 and 305 nm, respectively. Fluorescence spectra of purified recombinant xylanase excited at 280 nm reflected higher sensitivity as compared to that at 305 nm. All the spectra were corrected for light scattering by subtracting the data for an appropriate control. All the fluorescence spectra reported were an average of six scans and the error in the fluorescence intensity was always less than 5%.

2.28.4. Quenching of fluorescence

Relative fluorescence intensity of the protein (0.1 mg mL^{-1}) in 20 mM Glycine-NaOH buffer (pH 9.0) was recorded in the absence and presence of different concentrations of anionic/nonionic/cationic quenchers KI, acrylamide, urea and Gdn-HCl, respectively.

2.28.5. Chemical modification with N-BS

The effect of N-BS on the fluorescence of xylanase was determined by incubating the enzyme with different aliquots of N-BS at room temperature for 15 min.

2.28.6. Stability of xylanase against chemical denaturant

The stability of xylanase against different concentrations of guanidium hydrochloride (Gdn-HCl) and Woodward's reagent K (WR-K) was studied by monitoring emission of florescence intensity at 280 nm.

2.28.7. Analysis of fluorescence quenching data

The fluorescence titration (quenching /stability) data were analysed using Stern-Volmer equation

$$F_0/F = 1 + K_{sv}\,[Q] \qquad (1)$$

where, F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in the presence of quencher, [Q] is the quencher concentration, K_{sv} is the Stern-Volmer constant.

f_a is the fraction of total fluorescence available to the quencher and it was calculated from the following equation:

$$F_0/\Delta F = 1([Q]\,K_{sv} \times f_a) + 1/f_a \qquad (2)$$

Where, F_0 is the fluorescence intensity in the absence of quencher, ΔF is the fluorescence intensity in the presence and the absence of the quencher, [Q] is the quencher concentration, K_{sv} is the Stern-Volmer constant.

2.29. PROTEIN ENGINEERING

2.29.1. Directed evolution using error prone PCR using GeneMorph II mutagenesis kit

Attempts have been made to enhance the thermostability of xylanase using GeneMorphII mutagenesis kit (Stratagene, USA). The xylanase gene was mutated under low and medium mutation conditions using DxylNheF and DxylXhoI primers in 50 µL reaction mixture in a Thermal cycler (Bio-Rad, USA): 5 min at 95 ^{o}C followed by 30 cycles of 50 sec at 94 ^{o}C, 30 sec at 59 ^{o}C and 1 min at 72 ^{o}C with final extension for 5 min at 72 ^{o}C (**Table 2.6**). The amplified mutated plasmids were digested and ligated into pET28a(+) vector. The clones having recombinant plasmid were confirmed by colony PCR followed by restriction digestion. Clones having mutated gene were sequenced and transformed into *E. coli* BL21 (DE3) for the expression of the muteins.

2.29.2. SITE DIRECTED MUTAGENESIS

2.29.2.1. For enhancing thermostability

An attempt was made to substitute the arginines residues at position 68th, 90th, 92nd and 69th replacing threonine/serine using GENEART site directed mutagenesis kit

(Invitrogen, CA). The construct *pET28Mxyl* was methylated prior to amplify the whole plasmid using overlapping primers specific for the desired mutations.

Table 2.6. Reaction mixture for random mutagenesis

Reaction components	**Low error**	**Medium error**
DNA (50 ng μL^{-1})	500 ng	100 ng
Primer (forward)	25 pmol μL^{-1}	25 pmol μL^{-1}
Primer (reverse)	25 pmol μL^{-1}	25 pmol μL^{-1}
dNTPs	2 mM	2 mM
10 X Taq-reaction buffer	5 µL	5 µL
Mutazyme II	1µL	1µL
Sterile MilliQ	add to 25 µL	add to 25 µL

(Dxyl-T68F/R; Dxyl-T69F/R; Dxyl-T90F/R and Dxyl-T92F/R, **Table 2.2**). Reaction mixture for PCR was constituted as described in **Table 2.7** and **2.8**. Amplified plasmid having the mutated xylanase gene was transformed in *E. coli* DH5αTM-$T1^R$ competent cells by heat shock method. 100 µL of transformed cells were spreaded on LB-Kan plate and incubated for overnight at 37 ºC. The clones were randomly selected and respective plasmids were isolated and sequenced for each mutation. For every next mutation, the plasmid of previous mutation was used to generate other successive mutations.

Table 2.7. Details of reaction components used for PCR reaction

Reaction components	**Volume (µL)**
2X Hifi buffer	10
10X Enhancer	5
Primer mix (10 µM each)	1.5
dNTPs mix (10 mM)	1.5
Plasmid DNA (20 ng μL^{-1})	X
DNA methylase (4U μL^{-1})	1
25X SAM	2
Hifi Taq pol	0.5
Sterile MQ (Add to vol.)	50 µL

The clones were picked from the plate and sequenced for each mutation. A total of four mutations were generated at 68^{th}, 90^{th} and 92^{th} and 69^{th} positions of parental xylanase successively. All the substitutions were done by arginine amino acid.

Table 2.8. PCR conditions for site directed mutagenesis

Temperature (°C)	Duration	No. of cycle
37	20 min	1
95	5 min	1
98	20 sec	18 cycles
60	15 sec	
72	4 min.	
72	5 min	1

Plasmid from each mutant was isolated and transformed into *E. coli* BL21 (DE3) for the expression of the muteins. Mutated xylanases from each successive mutation were purified by Ni^{2+}-NTA resins using affinity chromatography.

2.29.2.1.1. Characterization of mutated xylanase

Muteins were purified by affinity chromatography and characterized for various temperature, pH, thermostability and pH stability. Enzyme kinetics was studied to determine K_m, V_{max} and K_{cat} of muteins.

3.29.2.1.2. Determination of thermostability

For determination of thermal stability, purified enzyme was incubated in glycine-NaOH buffer (pH 9.0) for varying time interval, at the corresponding temperature, and the activity was determined by DNS method. Reducing sugars produced during pre-incubation in the presence of substrate was determined as base line activity and subtracted before the calculation of the activity. The enzyme half-life ($T_{1/2}$) was determined at the pH 9.0 at 80 ºC and 90 ºC in absence /presence of substrate (1% w/v xylan).

2.29.2.2. For catalytically active site

Multiple sequence alignment of recombinant xylanase with those of known xylanases revealed Glu_{117} and Glu_{209} as catalytically important residues. Experimentally it has

been proved by site directed mutagenesis using GENEART site directed mutagenesis kit (Invitrogen, Carsband, USA). Two point mutations ($Glu_{117}Asp$ and $Glu_{209}Asp$) were created in the native xylanase gene to construct two different mutants $Mxyl_{E117D}$ and $Mxyl_{E209D}$ using DxylE117DF/ DxylE117DR and DxylE209DF/DxylE209DR primers for creating ($Glu_{117}Asp$) and ($Glu_{209}Asp$), respectively in parental xylanase gene. Plasmids were isolated from $Mxyl_{E117D}$ and $Mxyl_{E209D}$ and transformed in *E. coli* BL21(DE3) for the expression of mutated xylanase enzyme. The directed mutations were confirmed by sequencing. Mutated xylanase was processed to check the activity by DNSA assay (Archana and Satyanarayana, 2001) and plate diffusion method.

2.30. APPLICATIONS OF RECOMBINANT XYLANASE

2.30.1. Saccharification of lignocellulosic substrates

Saccharification of the natural lignocellulosic materials was performed by the method of Okele and Obi (1995). Sugarcane bagasse, wheat bran, corncob, rice straw, *Prosopis juliflora* and *Lantana camara* were taken as a suspension (0.1 g) in glycine-NaOH buffer (0.1 M, pH 9.0) and 20 U of recombinant xylanase was added to make a total volume of 10 mL of the reaction mixture. Sodium azide (0.03%) was added to each substrate suspension. Saccharification was performed at 80 °C for various time intervals. The resultant supernatants following centrifugation (10,000 rpm, 4 °C, 15 min) were assayed for total reducing sugars using DNSA reagent (Miller, 1959).

2.30.1.1. Identification of the end products of xylanase action on agroresidues

2.30.1.1.1. Thin layer chromatography

Thin layer chromatographic analysis was carried out on pre-activated silica gel plates (Merck, Germany). The xylanase reaction mixture was incubated in a water bath at 80 ºC and pH 9.0, respectively for varying time intervals 5 min, 15 min, 30 min and 1 h and the reaction was stopped by transferring the reaction tubes at 4 ºC. One µL each from the standard xylooligosaccharides (XOs) and respective hydrolysates from the mixture were spotted on TLC plates and fixed by drying using a hair dryer. Running solution [butanol: ethanol: water (5: 3: 2)] was used for solvent phase. The plates were air dried at room temperature and sprayed with aniline-diphenylamine reagent (aniline 4 mL, diphenylamine 4 g, acetone 200 mL and 85% H_3PO_4 30 mL) [Hansen, 1975] followed by incubation of the plates in an oven at 100

ºC for 2 h for the clear appearance of individual XOs as blue/black spots on a white background of TLC plates.

2.30.1.1.2. High performance liquid chromatography

The identification of sugars released by the action of recombinant xylanase were analyzed by HPLC using carbohydrate column 3.9 x 300mm (Waters) with acetonitrile-water (70:30, v/v) as the mobile phase at a flow rate of 0.6 mL min^{-1}. HPLC column was run at 30 ºC. Sugars were detected by a differential refractometer and analyzed by Empower 2154 software (Waters).

2.30.1.1.3. Scanning electron microscopy

Hydrolysis of wheat bran granules were followed by scanning electron microscopy (SEM) to see the physical action of xylanase on the surface of the bran. The SEM analysis of wheat bran hydrolysis was carried out by mounting the samples on stubs with silver glue for conduct, and the starch particles were examined under SEM-50B, scanning electron microscope (Philips, Amsterdam, Netherlands) at Electron Microscope Facility, All India Institute of Medical Sciences, New Delhi.

2.30.1.1.4. MALDI-TOF analysis

The masses of hydrolysis products obtained from wheat bran hydrolysis were detected by MALDI-TOF-MS spectra were calculated at central instrumentation facility (CIF), UDSC, New Delhi.

2.30.2. Biobleaching of pulp using recombinant xylanase

2.30.2.1. Pulp samples

Pulp samples made from wheat straw were collected from ABC Papers Mills Shaila Kurdh, Punjab, India in sealed plastic bags and biobleaching tests were performed in the Research and Development Laboratory of this industry. Initial pH of the pulp sample was measured. The pulp samples were washed repeatedly and neutralized using 1N HCl. Pulp sample was later dried at 45-50 °C for 3-4 days to a constant weight. These samples were stored in desiccators and used whenever required for further analysis.

2.30.2.2. Enzymatic treatment of pulp

The concentrated enzyme (20, 40 and 60 Ugm^{-1} dry pulp) was added to pulp to a final consistency of 7 %. The enzyme treated pulp was incubated in a water bath shaker at different temperatures (60-80 °C) for different time intervals at pH 9.0 with continuous shaking. Autoclaved enzyme, as control was added to pulp. At the end of enzymatic treatment, the pulp was dried under vacuum. The reducing sugars released [mg/g oven dried [OD] pulp] in the filtrate was measured by using DNS reagent (Miller, 1959). Lignin derived compounds (LDC), and chromophore released from pulp samples after enzymatic treatment of the pulp were determined by monitoring A_{280} and A_{465} of the filtrate, respectively (Patel *et al.,* 1993).

2.30.2.3. Optimization of conditions for enzymatic treatment of pulp

Conditions optimal for pulp solubilization were standardized and expressed in terms of reducing sugar released (mg/g) from pulp and A_{280} and A_{465} of the filtrate.

2.30.2.4. Effect of temperature on pulp solubilization

Enzymatic treatment of pulp was carried out at various temperatures (60, 70 and 80 °C) to find the best temperature for pulp solubilization.

2.30.2.5. Effect of pH on pulp solubilization

Pulp was enzymatically treated at various pH (8.0-10.0) to study the effect of pH on pulp solubilization.

2.30.2.6. Effect of enzyme dose on pulp solubilization

Enzyme dose (20, 40, 60 U gm^{-1} oven dried pulp) was altered and its effect on pulp solubilization was evaluated.

2.30.2.7. Effect of incubation time on pulp solubilization

Pulp was enzymatically treated for various time intervals (0-3 h) and pulp solubilization was monitored.

2.30.2.8. Kappa number studies on enzyme treated pulp

Pulp sample at a consistency of 6-7 % was treated with xylanase (40 U/g oven dried pulp for different time intervals (0-3 h) at 80 °C and pH 9.0. After enzymatic

treatment the contents were filtered and the filtrate was used for subsequent investigation. The pulp obtained was dried at 50 °C to a constant weight. Similar controls were run using inactivated enzyme. Kappa number was determined as described below.

2.30.2.9. Kappa number

Kappa number of the pulp applies to the determination of the relative hardness, bleachability, or degree of delignification of pulp. The kappa number was estimated according to the standard test methods (TAPPI, 1992-1993) (T-236 cm 85). Moisture free pulp (2 g) was disintegrated in 800 mL of distilled water until it became free of fiber clots and placed in a water bath maintained at 25 °C. One hundred milliliter of 0.1 N potassium permanganate and 100 mL of 4 N sulfuric acid solutions were taken in a beaker. This mixture was added to the pulp suspension with stirring. At the end of 10 minutes, the reaction was stopped by adding 20 mL of 1N KI solution. Immediately after mixing, the free iodine liberated was titrated with 0.2 N sodium thiosulphate solution, adding a few drops of starch indicator towards the end of titration. Blank determination was carried omitting the pulp. One unit of kappa number is defined as the amount of 0.1 N $KMnO_4$ solution consumed by one gram of moisture free pulp. The Kappa number of the pulp was calculated by the formula.

$$\mathbf{K} = p \times f/w$$

and,

$$\mathbf{p} = (b-a)\ N/0.1$$

Where,

K= Kappa number

f = factor for correction to a 50% $KMnO_4$ consumption dependent on value of p

w = weight of moisture- free pulp samples in grams

p = amount of 0.1N $KMnO_4$ actually consumed by test specimen in mL

b = amount of thiosulphate consumed in blank determination in mL

a = amount of thiosulphate consumed in test specimen in mL

N = normality of thiosulphate

2.30.2.10. Determination of the brightness of pulp

Brightness is a commonly used industrial term for the numerical value of the reflectance factor of a sample with respect to blue light of specific spectral and

geometric characteristics. The brightness of the pulp is measured by its ability to reflect monochromatic light in comparison to a known standard (usually magnesium oxide). The determination of the brightness of the pulp was carried out according to standard test methods (TAPPI 1992-1993) (T452 cm - 92). The preparation of the sample for the determination of the pulp brightness was prepared in accordance with TAPPI T-400. The brightness scale was based on the reflectance of magnesium oxide of 100 %.

The enzyme treated pulp was subjected to bleaching sequence. The pre-bleached pulp was subjected to chlorination and alkali treatment sequence. The pulp at various stages was analyzed using standard methods of Technical Association of Pulp and Paper Industry (TAPPI, 1996), and the observations have been recorded.