VI. SUMMARY AND CONCLUSIONS
Xylans are hetero- and homo-polysaccharides which are the main constituents of hemicellullosic biomass of terrestrial plants and second most abundant polysaccharide on the Earth. Its core comprises $\beta-1, 4$ linked xylosyl residues, and the attachment of this core chain with various groups (4-O-methyl-D-glucuronosyl, a-arabinofuranosyl residues and others) makes it a heteropolysaccharide. Among xylanolytic enzymes, xylanases have attracted considerable attention because their catalytic activity releases lower xylooligosaccharides, and their potential application in textile, feed and biofuel industries. Xylanases attracted attention after Viikari et al., (1986) reported their applicability in bleaching of pulp in the paper industry. The inclusion of xylanase treatment step in pulp bleaching process could reduce chlorine consumption up to 25-35 %, and therefore, the process will become environment-friendly.

Although several xylanases have been reported from a number of microorganisms, most of them do not have adequate thermostability and alkali-stability for their application in paper and pulp industries. Majority of xylanases have been obtained from the cultivable 0.1 to 1.0 % of total microbial diversity existing in natural environments. The xylanases produced by cultured microbes are either thermostable or alkali-stable. The culture-independent metagenomic approaches may permit retrieval of genes encoding xylanases with alkali-stability and thermostability, and therefore, this approach was used in order to obtain the genes from environmental samples without involving the rigours of cultivation of microbes. The constant increase in the demand for alkali-stable and thermostable xylanases encouraged us to employ metagenomic strategy for the retrieval of genes that encode thermo-alkal-stable xylanases. The significant findings recorded in this investigation are summarized below:

I. Samples were collected from various hot and alkaline environments (Centuary Paper Mill, Uttrakhand; Lonar lake, Buldhana, Maharstra; Pachapdra and Sambhar Salt Lake, Rajasthan; Unapdev temple hot water spring, Maharstra; Manikaran hot water spring, Himanchal Pradesh; High temperature compost plant, Fukuoka, Japan) of India and Japan to retrieve the genes encoding alkali-stable and thermostable xylanase.

II. An improved single-step protocol has been developed for extracting pure community humic acid-free DNA from alkaline and hot soils and sediments.
Summary and Conclusions

The method is based on direct cell lysis in the presence of powdered activated charcoal and polyvinylpolypyrrolidone followed by precipitation with polyethyleneglycol and isopropanol. The strategy allows simultaneous isolation and purification of DNA, while minimizing the loss of DNA with respect to other available protocols for the DNA extraction. Moreover, the purity levels are significant, which are difficult to attain with any of the methods reported in the literature for DNA extraction from alkaline environmental samples. The DNA thus extracted was free from humic substances, and therefore, could be processed for restriction digestion, PCR-amplification as well as for the construction of metagenomic libraries.

III. A total of 36,400 transformants containing metagenomic inserts were screened for the xylanase activity. One clone (Mxyl) exhibited a clear zone of hydrolysis on RBB xylan agar plate from compost-soil based metagenomic library. The sequencing of the insert confirmed the size of the insert to be of 6232 bp. The open reading frame (ORF) finder program available at NCBI revealed a total of 10 genes having complete ORF of phosphoglucomutase and xylanase encoding genes along with some hypothetical protein encoding genes.

IV. When the nucleotide sequence of the ORF was aligned with the available sequences from NCBI database, it showed maximum identity of 78 % with a xyl23 xylanase of an uncultured bacterium followed by 76 % with acetyl-xylan esterase of Streptomyces lividans and xylanase Streptomyces coelicolor A3(2). The nucleotide sequence has putative sequences of -35 (CACGCCA), -10 (TAAAAA) and ribosomal binding sites (AGGGG) at the upstream of xylanase gene followed by complete ORF having ATG and TAA as start and stop codons. Xylanase was interrupted by a short stretch of arginine and threonine rich non catalytic region (WSVRQ2R2TG2TIT2). In addition, serine rich Q linker region (S2GS2DITVG2TS2G2TS2G2S3G2S10G4) has also been observed.

V. On multiple sequence alignment, five signature sequences were identified having two catalytically important residues of glutamate at position 117 and 209. The logos retrieved from PROSITE analysis further validated the conserved residues of glutamate in the signature sequences.
VI. Amino acid homology analysis showed maximum identity of 71% with the glycosyl hydrolase family 11 xylanase of an uncultured bacterium followed by *Microbulbifer hydrolyticus* (63%) and *Saccharophagus degradans* (62%). Furthermore, it shared 57-58% homology with xylanases produced by various species of *Cellvibrio*. Phylogenetic analysis further showed homology of this xylanase with other GH11 xylanases.

VII. A 1077 bp xylanase gene encoding 358 amino acids was successfully cloned in pET28a(+) and pET22b(+) expression vectors and successfully expressed in *E. coli* BL21 (DE3). The purified recombinant xylanase was optimally active at pH 9.0 and 80 °C. The purified recombinant xylanase (Mxyl) retained >80% of its activity after exposure to 60 °C and 70 °C for 3 h, and the T_{1/2} at 80 °C was 120 min, and 15 min at 90 °C. When the pH stability of the enzyme was studied by exposing the recombinant xylanase (Mxyl) to different pH values (4.0 to 12.0) over a period of 3 h and subsequently assayed at 80 °C, it retained more than 90% activity for 24 h at pH 8.0 and 9.0. While ~20-45% decline in enzyme activity was observed at pH 10.0 and 11.0 after 16 h of incubation. The K_{m}, V_{max} and K_{cat} of the recombinant xylanase are 8.01 mg ml^{-1}, 333.0 µmol^{-1} mg^{-1} min and 2220 s^{-1}, respectively.

VIII. Most of the metal ions did not affect the activity at 1 mM. The xylanase activity was, however, significantly inhibited at higher concentration by Pb^{2+}, Ag^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+}, Cd^{2+} and Co^{2+}. The cations Fe^{2+}, Mg^{2+} and Sn^{2+} showed slight stimulatory effect on xylanase, while Hg^{2+} completely inhibited enzyme at 1 mM.

IX. The maximum intracellular xylanase fraction from the recombinant (pET22Mxyl) was attained after 3 h of induction. While the optimum xylanase production (45%) from periplasmic fraction was attained after 15 h and extracellular recombinant xylanase was maximum (29%) after 24 h of induction.

X. The PyMOL software was used to propose a 3D model based on the comparison with the available template of xylanase in NCBI database. The GH11 xylanase of an uncultured bacterium (PDB ID: 2VUL) showed
highest similarity (74 %) with this xylanase. The proposed structure shows all the signature sequences of GH11 xylanases. Structure mainly comprises of β sheets and one α-helix. The β-jelly shape structure of xylanase reveals fingers, thumb, catalytic cleft and serine/threonine rich surfaces. Two catalytically important residues of glutamate are detected in the cleft at the positions 117 and 209.

XI. A drastic loss in xylanase activity was observed by site directed mutagenesis of the catalytic residues, confirming the role of these residues in catalysis. To understand structure function relationships, different techniques including fluorescence and circular dichroism (CD) spectroscopy were employed. The recombinant xylanase contained 4.57 % α-helix and 46.99 % β-sheets at pH 9.0 and 80 ºC. When the pH and temperature were changed to values below or beyond the optimum, α-helices declined and β-sheets and random coils increased.

XII. The investigation for understanding the structure of the enzyme was followed in different quenchers like neutral (acrylamide) and anionic (I-) denaturants. The structural investigation followed under different concentrations of quenchers, such as neutral (acrylamide) and anionic (I-) denaturants. A drastic decrease in fluorescence intensity was observed on adding acrylamide upto 10.0 mM. The activity of enzyme was not drastically lost, which indicated that acrylamide interacts by simple collision process and does not apparently interfere with the substrate binding site. Significant loss of conformational structure was observed on increasing the concentration of Gdn-HCl. Drastic inhibition in fluorescence intensity and xylanase activity at very low concentration of N-BS (0.1 mM) signifies the role of tryptophan in maintaining the conformation and integrity. Reduction in fluorescence intensity confirms the participation of acidic residues in catalysis.

XIII. Xylanase gene was successfully cloned into shuttle vector pWH1520 and expressed in the heterologous host Bacillus subtilis. When the recombinant Bacillus subtilis was induced by 0.3 % w/v xylose at the OD₆₀₀nm of 0.5, extracellular recombinant xylanase production was detected after 6h of induction. By optimization using one-variable-at-time-approach, 75 U ml⁻¹ of recombinant xylanase was attained. The factors were further optimized by
RSM, which resulted in 2.9-fold improvement in extracellular xylanase production (119 U mL⁻¹) in shake flasks.

XIV. There was no significant change in thermostability of mutant enzymes MXMUT1 and MXMUT2. The mutant MXMUT3 showed slight improvement in thermostability. MXMUT4 exhibited improved thermostability at 80 and 90 ºC. Therefore, mutant xylanase MXMUT4 was studied further. Thermostability of MXMUT4 was improved by 20 min with T₁/₂ at 80 ºC of 150 min, which is higher than that of the parent xylanase (120 min).

XV. Recombinant metagenomic xylanase saccharified lignocellulosic agro-residues, and the saccharification was maximum (~15 %) in wheat bran, which further enhanced on extending incubation.

XVI. When the pulp was treated with xylanase, an overall 24 % reduction in chlorine consumption was recorded in comparison with the control (pulp samples without pretreatment with xylanase). Furthermore, kappa number of the pulp was reduced with concomitant increase in brightness to ~82 %. Handmade paper from the enzyme- treated and bleached pulp showed a clear difference in brightness against the control.

**CONCLUSIONS**

Most of the xylanases retrieved either by culture-dependent or culture-independent approaches exhibit their optimum pH and temperature in the range of 6.0-8.0 and 40-60 ºC, respectively. The xylanase retrieved in this investigation by metagenomic approach is not only optimally active at pH 9.0 and 80 ºC, but it has a high thermostability (T₁/₂ of 2 h at 80 ºC). This is the first report of xylanase with twin stabilities obtained using culture-independent approach. Low similarity in amino acid sequence of the enzyme with other known xylanases makes it a novel xylanase. The possibility of obtaining thermo-alkali-stable xylanase from composts may lead to an intense search for similar enzymes in this niche.