IV. DISCUSSION
Lignocellulosic materials comprise approximately 50% of the total biomass in the world, and its annual production by plants through photosynthesis is around $10^{12} - 10^{12}$ tons (Claassen et al., 1999). For technical, environmental and economical reasons, the demand for enzymes suitable for use in industrial processes has increased immensely in the last decade. Therefore, constant attempts are being made to get ideal enzymes having improved properties. Xylan, a homo/heteropolysaccharide is extensively dispersed in plant cell walls, and in nature, its degradation is carried out by several bacteria and fungi that secrete a wide range of xylanolytic enzyme for the proper breakdown of lignocellulosic compounds. Xylanases have received attention due to their intrinsic properties like high specificity, high reaction yield, high catalytic efficiency and enantioselectivity. Several potential applications of xylanases as in bread making, clarification of juices, feed industry and textile industry motivated substantial research in order to achieve higher xylanases. The degradation of lignocellulosic materials and retrieval of xylan and their lower xylooligosaccharides is necessary for feed/food based industries and also to maintain the microflora and fauna of soil. Xylanases, particularly glycosyl hydrolase family 11, have particular application in the paper processing industry, since xylanase action can decrease the consumption of chlorine, chlorine dioxide and ozone used in pulp biobleaching. Glycosyl hydrolase family 11 xylanases have received more attention than the GH10 xylanases because of features like low molecular weight, free from cellulases and high specificity on the substrates. Viikari et al. (1986) first reported the application of xylanases as biobleaching agents, and substantial reduction in chlorine consumption by 20-35% in comparison with the traditional approach. The current emphasis on employing clean technologies gives a boost to the use of xylanases further. The encouraging results recorded in pre-bleaching of pulps using xylanases in the mill trials have further provided a stimulus in employing xylanases in pulp bleaching. With more competent enzyme producers, the cost of xylanase production is coming down, and thus, this technology can become a commercial reality. The direct application of thermostable and alkalistable xylanases in paper industry after kraft cooking has attracted immense attention. The process that employs thermo-alkali-stable xylanases will result in savings in energy and cost of chemicals used for lowering the pH. Other problems reported in paper based mills like corrosion by sulphuric acid used for lowering the pH and extremes of pH encountered due to improper mixing can also be avoided using alkalistable xylanases. Xylanases that can
withstand higher temperature and pH are, therefore, needed to make the process economical and practical. Furthermore, it is essential for xylanase preparations to be free from cellulase activity, because the presence of cellulase in the xylanase preparation will lead to the loss in the strength of cellulosic fibers and reduction in paper quality. The applicability of xylanases in paper and pulp industries stimulated search on finding potent xylanase producing microbes suitable for use in the paper pulp industry. The global research efforts on microbial xylanases have indicated that it is difficult to get a thermostable and alkalistable xylanases from the culturable microbes. Despite the fact that a good beginning has been made in improving the properties of xylanase by protein engineering, it is far from the requirement. There is a long way to go before it can be called a sure shot tool for developing tailor made enzymes to suit industrial processes.

A Pandora’s box has just been opened that deals with the retrieval of genes from the environmental DNA encoding the desirable enzymes. This approach opens the door for obtaining novel genes from the inaccessible 99% of the unculturable microorganisms present in the environment. The metagenomic approach has been successfully attempted to access novel xylanase genes from the unculturable microbes. Very few xylanases have been recovered by the metagenomic approaches, and therefore, further research efforts are needed in this direction. In this effort, the first and foremost requirement is to obtain a good quality and high molecular weight DNA from the environmental samples. This becomes more complicated with soil and sediment metagenomics. Water, skin and gut metagenomics are quite simpler than soil metagenomics because of less humic substances that co-purify with DNA and interfere with basic steps involved in restriction and amplification of community DNA for the construction of metagenomic libraries (McGregor et al., 1996). Humic substances chelate Mg$^{2+}$ ions needed for DNA polymerases during PCR, and thus, indirectly affect the activity of enzyme (Tsai and Olson, 1992). The presence of minute quantities of humic compounds can also affect restriction digestion, amplification and transformation efficiencies by binding to respective enzymes used at various steps in molecular biology (Porteous and Armstrong, 1991; Gabor et al., 2003). The extraction of humic acid free DNA from environmental samples is, therefore, the first step in downstream processing of metagenomes for various molecular studies. The purification of DNA is a very decisive step in the isolation of
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metagenomes, particularly from soils and sediments. There are a number of methods, which can aid in this task to a varied extent. The purification of DNA based on gel filtration has been attempted with direct application of slurry to various matrices like Sephadex 100 (Leff et al., 1995) and Sephadex G75 (Cullen and Hirsch, 1998) through which the precipitated and dissolved DNA has to be passed and eluted for obtaining pure DNA. Other methods based on adsorption (Purdy et al., 1996), ion exchange (Desai and Madamwar, 2007) or sometimes the combined approaches have also been tried in order to remove the humic substances.

These methods are optimized with specific soil and do not fit for every type of soil. UltraClean™, PowerSoil™, PowerMax™ (Mo Bio Laboratories Inc., Carlsbad, CA, USA), SoilMaster™ (Epicentre Biotechnologies, Madison, WI, USA) and FastDNA® Spin kit for soil (MP Biomedicals, solon, Oh, USA) are commonly used kits for metagenomic DNA isolation. These kits work to some extent, but the quality and yield get compromised. The majority of the available protocols for soil DNA isolation insist on two steps, extraction of DNA (first step) followed by purification (second step). Purification of DNA from any environmental sample is mandatory for downstream processing. Conventionally impure DNA is purified either by silica based columns or by cation/anion exchangers, which leads to significant loss of DNA (Miller 2001; Schneegurt et al., 2003; Kauffmann et al., 2004; Leff et al., 2005; Desai and Madamwar 2006; Sharma et al., 2007). Three different protocols have been used in developing an appropriate method. The very first strategy was adapted from Zhou et al. (Zhou et al., 1996), which was based on direct lysis of microbiota present in a soil sample; this allows recovery of ~ 92-99% of DNA from an environmental sample. Most of the methods reported in the literature (Tsai and Olson, 1992; Grey and Herwig, 1996; Ogram, 2000; Sharma et al., 2007) for metagenomic DNA isolation revolve around this protocol. The protocol developed by Zhou et al. (1996) does not meet the purity requisites; the purity ratios $[A_{260}/A_{230}$ (for humic substances; $>2.0$) and $A_{260}/A_{280}$ (for proteins; $>1.7$)] do not suit downstream processing (Corapcioglu and Huang, 1987). The protocol was thus modified to overcome the purity constraints of the metagenomic DNA by incorporating Powdered Activated Charcoal (PAC) and PolyVinylPolyPyrrolidone (PVPP) that remove humic substances significantly (Perez-Candela et al., 1995). Activated charcoal, bone char and PAC have been extensively used for the removal of humic acid, lignin sulfonate,
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tannic acid, arabic gum polyphenolic compounds, many biodegradable/ non-biodegradable colored compounds and heavy metals (Seco et al., 1997; Logan et al., 1997; Seo et al., 2001; Rondon et al., 2001; Martin-Laurent et al., 2001). As a second strategy, concentration of PAC was optimized to lower the intense brown colour of slurry. PAC, known as activated carbon or activated coal, is a form of carbon that has been processed to make it extremely porous and thus has a vast surface area and pore volume that allows adsorption of various humic substances (Seco et al., 1997, Rondon et al., 2005). The use of 0.4 and 0.2 gm PAC gm⁻¹ of soil resulted in high purity ratios as compared to the control (no PAC); this suggested its significant role in the removal of humic substances. Although, Desai and Madamwar Desai and Madamwar, (2007) used PAC to extract the inhibitor-free metagenome from polluted sediments, the extracted DNA needed further treatment with amberlite IRA-400 resins (anion exchanger) to obtain metal ion and organic inhibitor free metagenome. The precipitation of metagenomic DNA using 30 % (w/v) PEG 8000 was the third approach. PEG has been used for the precipitation of soil metagenome (Yeates et al., 1997, La Montagne et al., 2002), since isopropanol or ethanol favors precipitation of DNA along with humic substances. PEG does not co-precipitate humic substances (Steffen et al., 1998; Wechter et al., 2003). Similarly, La Montagne et al., (2002) reported the use of 10 % PEG 8000 instead of isopropanol that resulted in a four-fold reduction in humic substances without decreasing DNA yields. At this stage, metagenomic DNA becomes almost free from humic substances, which was finally precipitated with isopropanol after routine phenol: chloroform: isoamyl alcohol (PCI) treatment. As PEG is supposed to be interfering agent in PCR reactions, a second phase of DNA precipitation was carried out. DNA was quantitated after PEG as well as isopropanol precipitations. There were no significant differences in the level of DNA yield in both precipitation methods. The metagenome obtained through the combined strategy was of high molecular weight and non-sheared with high purity ratios (A₂₆₀/A₂₈₀ = 1.72) and (A₂₆₀/A₂₃₀ = 1.66), and (A₂₆₀/A₂₈₀ = 1.82) and (A₂₆₀/A₂₃₀ = 1.96) for protein and humic acid free DNA from Pantnagar and Lonar soil samples, respectively (Verma and Satyanarayana, 2011). The method was further used for extraction of metagenomic DNA from various alkaline soil and sediment samples. All the DNA samples exhibited purity with acceptable absorbance ratios, and thus, confirmed the reproducibility of the protocol.
The absorbance ratios of the extracted DNA have been compared with other manual as well as commercial methods. The protocol of Wechter et al. (2003) relies on indirect lysis, where the cells were first separated from the soil samples and then exposed to cell lysis. This is the approach to avoid and cope with the humic substances by separating the cells from any environmental samples, but the extracted DNA is merely 25 to 30 % of total number of bacteria present in the soil (Van Elsas, 1997). Therefore, a large number of different bacterial colonies that strongly adhere to soil particles do not come in extracted metagenome. The protocols developed by Agarwal et al., (2001) and Desai and Madamwar, (2007) are modified versions of Zhou et al., (1997) and are based on DNA extraction followed by purification either by sephadex G 200 or cation exchanger like amberlite resins. Both these methods have led to a significant loss of DNA. Soil metagenome obtained according to Yamamoto et al., (1998) was based on repeated freezing and thawing of the soil suspension. In this method, cells were exposed to physical shock, which does not assure the complete lysis of all the cells present in an environmental sample. The kits are based on bead beating method followed by DNA elution from spin columns. Yeates et al., (1997) had also achieved acceptable purity ratios through bead beating method, but this method is prone to cause shearing of DNA and the metagenome obtained is not the ideal representative of the community DNA (Brosius et al., 1978; Yeates et al., 1998). Moreover, DNA yield is proportional to the beating time that enhances shearing, and therefore, one has to compromise with getting the sheared stretches of metagenome. The commercial kits did not result in a satisfactory DNA yield and absorbance ratios of protein as well as for humic substances. DNA yields were not also satisfactory as compared to other manual methods and those using commercial kits. The protocol developed by Verma and Satyanarayana, (2011) allowed extraction of protein and humic acid free DNA.

The satisfactory quality of metagenomic DNA extracted from various soil samples was further confirmed by restriction digestion, amplification of specific regions of the genomes of various microorganisms as well as by constructing metagenomic library. Metagenomic DNA isolated from different soil samples could be digested with Sau3AI within 5 min. In addition, bacterial and archaeal specific16S rDNA as well as fungal specific ITS regions were nicely amplified from different metagenomic DNA samples. Restriction digestion and PCR amplification have been
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used to validate the purity of metagenomic DNA extracted from the environmental samples (White et al., 1990; Sharma et al., 2007). The extracted DNA represents the microbial community of the environment, since it contained signature sequences of bacteria, archaea and eukarya. Since the extraction of metagenome is meant for recovering xylanase encoding genes in this investigation, the metagenomic library was plated out on LB-RBB xylan-amp plate. The screening of metagenomic library further permitted selection of a positive clone for xylanase. The clone has been shown to produce xylanase extracellularly as well as intracellularly, which was confirmed by using quantitative xylanase assay (White et al., 1990). The DNA extracts could be preserved for a month at -20 °C prior to PEG precipitation and used for accessing DNA whenever required. The purity of DNA remained unchanged, although a slight reduction in DNA yield from soil/sediment was recorded. Although several xylanases have been reported from diverse microbiota using traditional culture dependent approaches (McGregor et al., 1996; Sunna and Bergquist, 2003; Ranjan et al., 2005; Sharma et al., 2007), majority of them do not endure the extreme temperature and alkaline conditions prevailing in industrial processes. Therefore, an alternate strategy was adapted to retrieve a thermo-alkali-stable xylanase gene by culture-independent metagenomic approach. The metegenomic library constructed with DNA extracted from the compost-soil samples yielded a clone that produced xylanase. Although the compost soils are in the acidic pH range, an alkali-stable and thermostable endoglucanase had been reported from rice straw compost (Tsai and Olson, 1992). The culture-independent approach has been shown to yield useful biocatalysts from the hidden Pandora’s Box (Porteous and Armstrong, 1991; Cullen and Hirsch, 1998; Gabor et al., 2003). A considerable success has also been achieved in obtaining xylanases with diverse attributes by using metagenomic approaches (Miller, 2001; Purdy et al., 1996; Kauffmann et al., 2004; Leff et al., 2005; Sharma et al., 2007).

Five micrograms of 20-30 kb of high molecular weight metagenomic DNA was digested with Sau3AI and collected 5 to 8 kbp fragments and ligated into p18GFP vector with an efficiency of $3.6 \times 10^4$ clones per microgram of DNA in constructing the library with insert sizes of 3 to 8 kb having an average size of 5.5 kb. A clone having xylanase gene was detected on RBB xylan containing LB-amp plate. The full sequence of the insert showed the size of 6232 bp that revealed its prokaryotic origin on blast analysis. The complete insert showed a total of nine transcriptional units in an
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operon having complete ORF of 1077 bp long xylanase gene. The sequence showed 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, respectively. The xylanase gene encoded protein comprising 358 amino acids, of which 16 are acidic and 21 basic. The predicted molecular weight, pI and instability index of recombinant xylanase are ~40 kDa, 8.8 and 33.44, respectively. Predicted xylanase showed 43 amino acids long leader sequence at the N terminal region followed by a catalytic domain (44th-212th) of GH11 family interrupted by a short stretch of arginine and threonine rich non catalytic region (WSVRQ2R2TG2TIT2). In addition, serine rich Q linker region (S2GS2DITVG2TS2G2TS2G2S2S2S2G3G4) has also been detected from amino acid 213th to 248th just after catalytic domain. Such repeated amino acids make linker regions that usually discriminate catalytic domain from carbohydrate binding domain (Gilke et al., 1991; Brennan et al., 2004; Li et al., 2009). Moreover linkers have also been reported as integral part of various xylanases that connect thermostabilizing domains, surface layer homology domains and dockerin domains which play a role in stabilizing the protein (Fontes et al., 1995; Winterhalter and Liebl, 1995; Li et al., 2009; Shibuya et al., 2000; Notenboom et al., 2002). The xylanase has been classified as GH11 xylanase based on the deduced amino acid homology with other xylanases available in database with a high molecular mass (>30 kDa). This is not exceptional because xylanases of Clostridium stercorarium also had a high molecular mass (56 kDa), and still has been classified as GH11 xylanase (Sakka et al., 1993). The xylanase showed five conserved regions (I-V) of GH11 xylanase having two catalytically important residues (Glu109 and Glu217) in signature sequence II and V. Amino acid homology showed maximum identity of 79 % with the xylanase gene of an uncultured bacterium and Actinoiplanesis sp. SE50/110 followed by a metagenomic GH 11 xylanase (71%). It shares 63-75 % homology with xylanases of Streptomyces. The xylanase retrieved in this investigation shows 75, 67 and 64 % similarity with the endo-1,4 β-xylanases of Cellulomonas fimii, Micromonospora aurantiaca 27029 and Amycolatopsis mediterranei U32, respectively. It, however, has lower homology with the xylanases of Microbulbifer hydrolyticus (63%), Pseudomonas sp. ND137 (62%), uncultured Cellvibrio sp. (58%), Cellvibrio mixtus (57%) and Aspergillus fumigatus AF293 (52%). The distinct characteristics, lower homology and unexpected higher molecular weight (>30 kDa) make this a novel xylanase.
Xylanase gene without its signal peptides was successfully cloned into pET28a(+) vector. It was confirmed by the release of insert from the vector on double digestion with NheI and XhoI. The sequencing results further confirmed the insert. The recombinant plasmid was expressed in *E. coli* BL21 (DE3), when induced with 1.0 mM IPTG at A600 of 0.6-0.7 and 30 ºC. At higher level of expression, it led to the formation of inclusion bodies, which could be solubilized using 6.0 M urea. The highest titre of the recombinant enzyme was achieved in 4 - 6 h.

The construct *pET28Mxyl* showed a high proportion of the expressed xylanase in cytoplasmic fraction (83%), followed by periplasmic (9%) and extracellular (8%) fractions after 4-5 h of induction. Recombinant xylanases from *Glacieola mesophila* KMM241 and *Geobacillus thermoleovorans* exhibited a similar profile of recombinant protein localization (Guo *et al*., 2009; Verma and Satyanarayana, 2012). When the xylanase gene was cloned into pET22b(+) vector and expressed, a high proportion of intracellular enzyme (>60%) was produced in the initial 3 h of induction, and thereafter, it declined. The periplasmic xylanase was optimum at 12 h. The extracellular fraction gradually increased and it reached a peak (29 %) in 24 h; this pattern is similar to that in *B. halodurans* (Mamo *et al*., 2006). Integrated N-terminal pelb signal sequence in pET22b(+) directed the enzyme to periplasm that further led to secretion into the extracellular environment.

Muteins having Glu117Asp and Glu209Asp exhibited drastically lower enzyme activity. These two glutamates are highly conserved residues in the signature sequences LVEYYYIVDN and MATEGY, and these are responsible for catalytic activity of GH11 xylanase (Shi *et al*., 2011).

In order to take the advantage of thermostability of the recombinant xylanase, it was subjected to high temperature prior to purification by Ni-NTA agarose resins. This step reduced the extra load of non-His tagged, less thermostable and contaminant host proteins. The recombinant xylanase was purified by Ni-NTA resins using affinity chromatography and the purified recombinant protein could be eluted using imidazole (100 - 400 mM). The protein appeared as a single band of 40 kDa against the protein marker on 15 % SDS-PAGE and the recombinant xylanase revealed as a clear band of xylan hydrolysis by zymogram analysis.
When the effects of various physical and chemical parameters on the recombinant xylanase have been assessed, it exhibited broad range of pH (6.0 -12.0) for activity with optimum at 9.0, and the enzyme showed ~55% residual activity at pH 10.0. The recombinant xylanase is active in the temperature range between 40 and 100 °C with optimum at 80 °C, and retains more than 90-95 % activity at 60 and 70 °C after 3 h. The enzyme has a $T_{1/2}$ of 2.0 h at 80 °C and 20 min at 90 °C. *Dictyoglomus thermolacticum, Thermotoga maritima, Bacillus acidicaldarius* and *Geobacillus thermoleovorans* are known to produce xylanases with optima at or above 80 °C (Uchino and Fukuda, 1983; Mathrani and Ahring, 1992; Yoon *et al*., 2004; Verma and Satyanarayana, 2012). The activity and stability of xylanases at higher pH are the crucial properties of xylanases for their utility in paper processing industry. Xylanases of *G. thermoleovorans, Bacillus firmus, B. stereothermophilus T-6* exhibited optimum at pH at 8.0 or above (Khasin *et al*., 1993; Chang *et al*., 2004; Verma and Satyanarayana, 2012). The recombinant enzyme does not lose the activity after 3 h exposure to pH 8.0 and 9.0, and thereafter, it declines (50 % residual activity after 4 h). Approximately 20-45 % loss in the activity was recorded on either side of the pH optimum after 1 h incubation.

The shelf life of recombinant xylanase is more than 3 months at 4 °C with more than 90 % retention of activity. The recombinant xylanase is optimally active at 80 °C and pH 9.0 that distinguishes it from already reported xylanases. The xylanase of *Thermotoga maritima* has $T_{\text{opt}}$ of 90 °C, but it gets inactivated fast in acidic pH 6.0 (Yoon *et al*., 2004). The highly thermostable xylanase with optimum temperature in the range between 80 °C and 105 °C are available, but these xylanases exhibit their maxima either at acidic or neutral pH (Mathrani and Ahring, 1992; Yoon *et al*., 2004). Similarly the alkalistability at higher pH is reported in many xylanase but are active at lower temperatures (Khasin *et al*., 1993; Chang *et al*., 2004). The recombinant xylanase of GH10 family from *Bacillus halodurans* showed both properties together having optima at 75 °C and pH 9.0, but it losses 50 % activity at 65 °C after 4 h and gets inactivated very fast at 80 °C (Mamo *et al*., 2006). The xylanase, on the other hand, has good thermostability at higher temperatures (60, 70 and 80 °C) with only 20-30 % loss after 3 h. The most significant aspect of this investigation is procuring a highly alkalistable ($pH_{\text{opt}}$ 9.0) and thermostable ($T_{\text{opt}}$ 80 °C) xylanase from environmental samples by a metagenomic approach.
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The cations Mg$^{2+}$, Sn$^{2+}$ and Fe$^{2+}$ stimulated the xylanase activity like that of *B. subtilis* AMX-4 and an uncultured microbe (Yoon *et al.*, 2009; Zhao *et al.*, 2011). Even at low concentration (1.0 mM), Hg$^{2+}$ and Mn$^{2+}$ significantly inhibited the activity. The inhibition of xylanase by Hg$^{2+}$ suggests the presence of tryptophan residues that oxidize the indole ring, thereby inhibiting the xylanase activity (Maalej *et al.*, 2009; Zhang *et al.*, 2009; Liu *et al.*, 2011; Zhao *et al.*, 2011). The other metal ions exerted a slight inhibitory action (residual xylanase activity was between 54 and 81 %) More than 70 % activity was lost in the presence of Cu$^{2+}$ and Mn$^{2+}$. Xylanases from *Streptomyces olivaceoviridis* A1, *Streptomyces* sp. S27 and *B. subtilis* strain R5 had been shown to be stimulated in the presence of Fe$^{2+}$ and Mg$^{2+}$ (Wang *et al.*, 2007; Li *et al.*, 2008; Hu *et al.*, 2009; Jalal *et al.*, 2009; Li *et al.*, 2009), and total loss of enzyme in the presence of Hg$^{2+}$ and Mn$^{2+}$ was reported in the xylanases from an uncultured microbe (Hu *et al.*, 2008; Li *et al.*, 2009), *Penicillium* sp. (Liu *et al.*, 2010) and *Plectosphaerella cucumerina* (Zhang *et al.*, 2007). The effect of Cu$^{2+}$ was inhibitory (70 %) like majority of the xylanases (Li *et al.*, 2008; Son-Ng *et al.*, 2009; Zhao *et al.*, 2011; Matteotti, *et al.*, 2012). EDTA exhibited a stimulatory effect on xylanase, which could be due to a different catalytic mechanism as reported in xylanases of *Aspergillus niger* and *Fusarium proliferatum* (Irena *et al.*, 2006; Hang *et al.*, 2007). In *Glaciecola mesophila* KMM 241, EDTA caused ~25% enhancement in activity (Guo *et al.*, 2009). N-BS and PMSF inhibited the activity to a significant extent at 1 mM concentration. N-BS inhibition suggests the involvement of tryptophan in catalytic activity (Vieira and Degreve, 2009; Yang *et al.*, 2010;). β-ME and DTT completely inhibited activity as reported in several xylanases; this suggests the distortion of disulfide linkages present between cystein residues (Maalej *et al.*, 2009; Matteotti *et al.*, 2012). Detergents showed slight stimulatory effects on the recombinant xylanase which is a common feature of the other reported xylanases. SDS exhibited inhibitory action on the recombinant xylanase as reported in several other xylanases (Li *et al.*, 2009; Zhao *et al.*, 2011; Matteotti *et al.*, 2012).

The xylanase hydrolyzed xylan from various sources. A high specificity was observed on birchwood xylan (100%) followed by xylan from beech wood (97%) and arabinoxylan (80%). The xylanase has no activity on CMC (carboxy methyl cellulose) and other non-xylan polysaccharides (starch, pullulan and chitin). The $K_m$ and $V_{max}$ values on birchwood xylan are $5 \pm 1.21$ mg mL$^{-1}$ and $300 \pm 0.92$ μmol min$^{-1}$ mg$^{-1}$. 
The structural similarity of beechwood and birchwood xylans may be the reason for this proximity (Guo et al., 2009). The enzyme exhibited similar specificities on oat spelt and arabinoxylan. Oat spelt xylan is a type of arabinoxylan very rich in arabinose (xylose/arabinose=66:34) (Gruppen et al., 1992; Kormelink and Voragen, 1993).

The codon usage is the problem in heterologous cloning, while homologous cloning reads the same amino acids for the respective codons. An interesting example of homologous cloning was reported in Bacillus strain 168, whose xylanase encoding gene was reintroduced in various strains of B. subtilis. Two of these strains BS1 and BS2 produced xylanases similar to the xylanase A of wild type B. subtilis strain 168, while the third strain BS3 produced a xylanase that was different from the wild type counterpart by two amino acids. Although this xylanase was resistant to xylanase inhibitor present in the flour, it was not suitable for paper and pulp industries due to its instability at 50 °C (Zofia Olempska-Beer, 2004). A fragment of 9.4 kbp obtained from the genomic library of Bacteroides succinogenes encoding xylanase activity was subcloned into pBR322 through a series of subcloning steps, resulting in a trimmed fragment of 3.0 kbp, whose translation product actually retained the xylanase activity. The cloning and subcloning manipulations, however, reduced the actual activities ~10 fold as compared to the enzyme activity exhibited from the long 9.2 kbp strech. This may be attributed to the deletion of a structural or regulatory region from the native stretch (Sipat et al., 1987). A 60-fold increase in enzyme activity was attained from homologous cloning of xylanase gene from Streptomyces lividans 1326 to xyn- mutant of Streptomyces lividans. Therefore, homologous cloning is suitable for codon usage as well as secretion of the desired protein extracellularly in the medium (Iwasaki et al., 1986). Although efforts have been made to produce xylanase in the periplasmic and extracellular fractions using pET22Mxyl using the advantage of pelb leader sequence for directing the xylanase into periplasmic fraction followed by extracellular in the medium. The extracellular fraction reached to a maximum of 29 % after 24 h of induction. Therefore, xylanase gene was cloned into shuttle vector pWH1520 and expressed into B. subtilis. When the recombinant strain of B.subtilis was induced by 0.3 % (w/v) xylose at OD_{600nm} of 0.5, 40 U mL^{-1} of extracellular recombinant xylanase was attained after 6 h of induction. The xylanase production by the recombinant B. subtilis was initially optimized using conventional methods.
Several physical and chemical parameters like carbon and nitrogen source, temperature, agitation, inoculum density, and aeration significantly improves xylanase production by *Bacillus* spp. or *Bacillus* based hosts (Park *et al*., 2002; Kumar and Satyanarayana, 2012). The medium components were not optimized for enhancing the production of xylanase. One-variable-at-a-time approach confirmed that xylose concentration, incubation time and inoculum density to significantly affect xylanase production. Similar optimization has been reported for the production of recombinant keritanase from *Bacillus* based host; higher level of extracellular recombinant keritinase was obtained from recombinant *B. megaterium* using pWH150 shuttle vector (Radha and Gunasekhran, 2007). The effect of each factor was further assessed by the use of perturbation plots to show the response changes as each factor moves from the chosen reference point, with all other factors held constant at reference values (Oh *et al*., 1995). The one-variable-at-time-approach produced 75 UmL⁻¹ of recombinant xylanase in the medium by the recombinant strain *B. subtilis* (pWHMxyl).

The ‘one-variable-at-a-time’ strategy is one dimensional approach and does not allow understanding the interactions between variables or conditions actually responsible for higher production of an enzyme. Therefore, an alternate strategy has been adapted to understand the interactions between variables of factors. The response surface methodology (RSM) is an efficient statistical approach that has been used to analyse the overall interactions among all the possible combinations of considered variables. The methodology deals with complete factorial search by analysing simultaneous, systematic and efficient variation of all components. RSM is useful for less number of variables (3-5), and feasible for understanding the interactions among large number of variables due to increasing number of experimental runs. For such cases, Plackett and Burman design was recommended followed by RSM. This statistical approach has been widely used to optimize the production of enzymes (Gigras *et al*., 2002; Hashemi *et al*., 2010; Kumar and Satyanarayana, 2012). Central Composite Design (CCD) of RSM suggested that the interactions between these variables (xylose concentration, incubation time and inoculum density) are significant for recombinant xylanase production. The R squared value provides a measure of variability in the observed response and can be explained by experimental factors and their interactions. For a good model, R squared value should lie in the range of 0.75-1.0 however, R-squared value greater than 0.90 is supposed to be highly
significant. The coefficient of determination ($R^2$) was 0.9743 for recombinant xylanase production, which is in good agreement with experimental and predicted values. The predicted $R^2$ (0.8506) and adjusted $R^2$ (0.9512) are in reasonable agreement between experimental and predicted values for recombinant xylanase production. The adjusted $R^2$ corrects the $R^2$ value for the sample size and for the number of terms in the model. Adequate precision is a measure of the signal to noise ratio and a value greater than 4.0 is desirable. The adequate precision value of 22.14 indicated an adequate signal and suggested that the model can be used to navigate the design space. The F-value of the model for recombinant xylanase was 42.13 and the ‘Prob>F’ value was <0.0001, which suggests that the model is very significant. Model terms A and C of the 3 linear A, B and C and squared model terms $A^2$, $B^2$, $C^2$ are significant. In addition, interaction between AB, AC and BC are also noteworthy.

The effect of interaction of various physiochemical parameters on xylanase production (z axis) was studied by plotting 3-dimensional response surface curves against any two independent variables. The positive interaction was observed between inoculum density, xylose concentration and induction time. With simultaneous increase in xylose and inoculum density, enzyme yield increased indicating synergistic effect of both variables. A similar interaction was reported between inoculum density and temperature on recombinant keratinase production in *B. licheniformis* MKU3 (Radha and Gunasekran, 2007). Statistical designs used for the selection of critical variables that affect enzyme production and understanding the interactions between the selected variables is a good and useful technique (Kumar and Satyanarayana, 2007; Sivaramakrishnan et al., 2008; Hashemi et al., 2010), and thus, has been used successfully for optimizing the xylanase production by the recombinant *B. subtilis*. The RSM suggested the importance of various factors at different levels and interactions among them. A 2.9-fold improvement in extracellular xylanase production by recombinant strain *B. subtilis* was attained in shake flasks. By using statistical approach, 24 % and 5.4- fold enhancement in $\alpha$-amylase and xylanase production was reported in *G. thermoleovorans* (Rao and Satyanarayana, 2003), *Aspergillus niger* DFR-5 (Gigras et al., 2002), respectively; this has been attributed to right balance of nutrients and favourable conditions that support good growth and enzyme production. The comparable predicted and experimental enzyme yields reflect the accuracy and applicability of RSM for optimizing the process for enzyme
production. The application of response surface methodology has gained attention for optimizing media components and process variables (Pham, 1998; Puri et al., 2001; Bocchini et al., 2002; Dey et al., 2002; Wejse, 2003; Heck et al., 2005; Sharma and Satyanarayana, 2006). The xylanase production by *G. thermoleovorans* optimized by employing one variable at a time approach was low. By applying the statistical methods Plackett-Burman design and RSM, the optimal levels for supporting maximum level of enzyme production have been determined. A 2.85-fold increase in xylanase production was attained when *G. thermoleovorans* was grown in the statistically optimized medium. Similar findings have been reported by Bocchini et al. 2002, where a total of 315 % higher xylanase production was attained by RSM. Xylanase production by novel halophilic bacterium has also been increased 20-fold by RSM (Wejse et al., 2003), and a 2.6- fold increase in protease production was achieved in *Bacillus* sp. (Puri et al., 2001). A marked enhancement in xylanase production by *B. circulans* has also been reported (Heck et al., 2005).

Circular Dichroism (CD) is a versatile technique as it can explore protein structure under a broad range of experimental conditions and can be used to measure the rates of structural changes. In contrast, X-ray crystallography or NMR provides structural information of proteins at atomic level resolution. The technique is also a valuable spectroscopic technique for examining the structure of proteins in solution (Kelly et al. 2005). CD signals arise due to absorption of radiations, and hence, special bands are easily assigned to distinct structural features of a molecule. In proteins, the chromophores of interest include peptide bond (absorption below 240 nm), aromatic amino acid side chains (absorption in the range 260-320 nm) and disulphide bonds (weak broad absorption band centered around 260 nm). CD measurements in the far UV can give quantitative estimates of secondary structure, which can be compared with those from X-ray crystallography or NMR. The recombinant xylanase contained 4.57 % α-helix and 46.99 % β-sheets and 48.44 % random coils at pH 9.0 and 80 ºC. As the pH was changed to values below or beyond the optimum, a decrease in α-helices and increase in β-sheets and random coils was recorded. These changes suggest loss of ordered secondary structure, which manifests as decrease in the enzyme activity. On increasing the temperature beyond optimum (80 ºC), there was decrease in α-helices while β-sheets and random coils increased
indicating that at high temperature the protein gets denatured and structural conformation of the protein is disturbed.

The structural investigation is 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 (Gao et al., 2005). Anionic quencher (KI) has also significantly affected the fluorescence intensity and enzyme activity and it shows significant quenching of xylanase with KI, revealing the uncharged state of local environment around Trp fluorophores. Charged quenchers are known to probe only the surface exposed Trps, while polar and uncharged acrylamide can diffuse into the protein matrix and quench the fluorescence of the surface as well as buried Trp residues (Eftink and Ghiron, 1981). Quenching was further analyzed by the Stern-Volmer equation. A decrease in fluorescence intensity and activity was observed on adding acrylamide, implying the more solvent exposed towards Trp residues.

Although Gdn-HCl is believed to have a similar mode of action (Pace, 1990), Gdn-HCl is a monovalent salt that has both ionic and chaotropic effects (Monera et al., 1994). It has been reported that Gdn-HCl reacts reversibly with the carboxyl groups of amino acids (Ghatge and Deshpande, 1993). Complete loss of conformational structure was observed on increasing the concentration of Gdn-HCl.

The role of Trp residues in recombinant xylanase was investigated by analyzing the changes in fluorescence of the N-BS treated enzyme at λ280. N-BS treatment led to the loss of both activity and fluorescence intensity, which is considered to be due to the oxidation of Trp to oxi-indole. The experimental $K_{sv}$ and $f_a$ values at 280 nm were 28.73 and 0.99, respectively. Higher $K_{sv}$ value indicates that all trp residues are in solution. In the presence of N-BS, α-helix content decreased drastically with increase in β-sheets and random coils. A decrease in xylanase activity was observed at very low concentration of N-BS (0.1 mM). The complete inhibition of activity by N-BS suggests the presence of at least one essential tryptophan residue, most likely located at the active site in a substrate binding or catalytic role (Macarron et al., 1995).
Discussion

On extensive comparisons of mesophilic and thermophilic xylanases it has been revealed that thermophilic xylanases have, on an average, higher arginine contents on the surface of xylanase and are more stable than mesophilic proteins at higher temperatures. Threonine and serine residues were selected for the substitution because these are present on the surface and very far from the catalytic groove. Therefore, such substitutions will not alter the conformation as well as catalytic behavior of the xylanase. All the substitutions were made by replacing ser/thr residues with arginine. The substitution of threonine and serine with arginine on the enzyme surface is expected to improve the thermostability. MXMUT1 and MXMUT2 showed very similar profile of thermostability compared to that of nonmutated xylanase. However, MXMUT3 was better in compared to control. While MXMUT4 showed significant improvement in thermostability having $T_{1/2}$ of 150 min and 20 min at 80 °C and 90 °C, respectively. The Mutated xylanases exhibited higher thermostability in the presence of substrate than without it. The higher stability of xylanase is due to several reasons like arginine is a bulky and basic amino acid and provides positive charge at the surfaces and allows charge to charge interactions for the formation of multiple non-covalent interactions. Moreover, arginine guanidino moiety has a high pKa value of 12, and therefore, it shows low dissociative behavior towards chemicals and the solution. This way it provides hydrophobicity to the protein and makes the structure more compact and improves the thermostability. Higher pKa value of arginine maintains net positive charge at elevated temperature (Argos et al., 1979; Menendez-Arias and Argos, 1989; Mrabet et al., 1992; Vogt et al., 1997; Kumar et al., 2000). An increase in the thermostability of Trichoderma reesei and Aspergillus niger xylanase II has been reported, when five arginine residues had been engineered into the Ser/Thr surface (Turunen et al., 2002; Sriprang et al., 2007). Substitution of four arginine residues led to a slight, but insignificant reduction of specific xylanase activity, $V_{\text{max}}$ and $K_{\text{cat}}$ and slight increase in $K_m$ values. These effects may occur because arginine residues contain large guanidium group (Mrabet et al., 1992; Borders et al., 1994). Consequently, the presence of four arginine residues might have led to a bulkier surface of the enzyme as compared to the Mxyl (without substitutions).

The saccharification of the agro-residues such as wheat straw, rice straw, sugarcane bagasse, corn cob, corn stover, Prosopis juliflora and Lantana camara by
Discussion

The recombinant xylanase resulted in the release of reducing sugars. The reducing sugars liberated by the action of xylanase on wheat bran were higher than in others. A high level of saccharification was achieved in wheat bran followed by corn cobs and sugarcane bagasse. Various xylo-oligosaccharides were detected in the hydrolysates by TLC. Interestingly the recombinant xylanase liberated xylooligosaccharides from xylan in just 5 min and it was sustainable on prolonging incubation. Several xylanases have been reported from various microorganisms that liberate xylooligosaccharides following xylan hydrolysis. Alkaline xylanases show better action on agro-residues by lowering the steric hindrance caused by cellulose and enhance the solubility of hemicellulosic materials (Gruppen et al., 1992). Xylanases from *G. thermoleovorans* (Verma and Satyanarayana, 2012), *Bacillus halodurans* (Mamo et al., 2007) *Thermomonospora fusca* (Sun et al., 2007) have been reported to generate a similar profile of XOs. The metagenomic xylanase finds application in food industry for the production of xylooligosaccharides as prebiotics (Vazquez et al., 2000). For the enhancement of brightness of the pulp, treatment of cellulosic fiber with chemicals is necessary in order to remove the residual lignin after kraft cooking process.

The pulp and paper processing industries are one of the prevalent users of plant biomass now-a-days and therefore major polluters of the environment, due to the established chemical pulp processes are relatively unproductive and environmentally expensive (Raghukumar et al., 2004). Formation of chlorinated dioxins and other compounds are the chief concern that provoked a technological revolution to reduce the chlorine/chlorine dioxide from the bleaching sequences. Therefore, one of the new technologies that are making fast evolution in pulp bleaching process is biological bleaching using xylanases (Roncero et al., 2005). A number of investigations have been carried out on the application of xylanases in pre-bleaching of pulps from different wood and non-wood raw materials (Turner et al., 1992; Viikari et al., 1986; Shah et al., 2000; Dhillon et al., 2000; Techapun et al., 2002; Choudhary et al., 2006).

Softening of chips from wood involves the usage of sodium sulphide and sodium hydroxide to partially removing lignin and the hemicellulosic compounds. After the removal of hemicellulosic layer, lignin layer becomes easily available for the degradative action of ligninolytic enzymes (Eriksson, 1985). In addition, during chlorine bleaching, the exposed lignin layer requires less chlorine to achieve the
required brightness. As a result, reduced amounts of chlorinated compounds of lignin are discharged as effluent, causing less environmental pollution and damage (Raghukumar, 2004). Low molecular weight xylanases are usually preferred during pretreatment, because the average pore size in kraft pulp would be about 5-10 µm allowing the proper diffusion of xylanase with a molecular mass of 40 kDa or less, assuming a spherical conformation (Stone and Scalan, 1968). Different factors that have been reported to affect the hydrolysis of hemicellulose include cellulose fiber crystallinity, porosity, hemicellulose and lignin content (McMillan, 1994). Pretreatment of lignocellulosic materials prior to xylanase treatment considered as helping step to reduce the cellulose crystallinity and enhancement in porosity that improves the hydrolysis and removes lignin and hemicellulosic compounds (McMillan, 1994). The purpose of all these treatments is to modify or remove the obstacles realted to structural and compositional profiles of hemicellulosic compounds in order to enhance the hydrolysis rate of xylanase (Mosier, 2005). It has been observed that diluted alkali treatment (NaOH) of lignocellulosic compounds tremendously enhances the solubilization step. Treatment of lignocellulosic materials improves swelling, enhancement in surface area, reduce the degree of polymerization, breaks the covalent bonds and disintegrate the integrity of wood chips and and make it suitable for making the wood into thin quality papers (Fan et al., 1987). Recombinant xylanases of this investigation had the all the requisite properties for the successful application in pre-bleaching of pulps. The xylanase has optimum activity at 80 °C and pH of 9.0, and it is cellulase free, and thus, make it a suitable biocatalyst for prebleaching. The extent of hydrolysis clearly indicates the release of lignin derived compounds, chromophores and reducing sugars. Absorbance at 280 nm and 465 nm indicates the total lignin/lignin related components and chromophore. The reducing sugars, chromophore release and kappa number have been extensively followed to ensure pulp solubilization (Ratto et al., 1992; Jefferies, 1996; Shah et al., 1999). The recombinant xylanase is best at its optimum temperature for maximum release of the reducing sugars, lignin derived compounds and chromophores. Several xylanases from thermophilic microorganisms have been used as pre-bleaching agents (Shah et al., 1999; Kumar and Satynarayana, 2012; Verma and Satynarayana, 2012).

In this investigation, 40 U gm⁻¹ of OD pulp at 80 °C after 2h of reaction time led to higher pulp solubilization. Under optimum conditions, pretreatment of pulp
with recombinant xylanase resulted in the liberation of reducing sugars and reduction in kappa number. Similar observations have been reported using the xylanase of *B. halodurans* TSEV1 (Kumar and Satyanarayana, 2012). 10 U and 12 U of xylanases per gram of oven dried pulp were optimum for achieving the liberation of sugars and other related components (Shah *et al*., 1999; Kulkarni and Rao, 1996). The pretreatment of pulp with enoxylanase of *Streptomyces lividans* exerted a significant effect on bleaching (Ragauskas *et al*., 1994). Merely 3.57 U gm⁻¹ of OD pulp from *Streptomyces* sp. QG-11-3 was able to bleach the xylanase at 50 °C and pH 8.5 in 2 h (Beg *et al*., 2000). It is well known that chlorine can only be reduced by 20-35% using xylanase at prebleaching stage, therefore, it is necessary to understand the effect of xylanase on cellulosic fibers after sequential CEH bleaching stages. Therefore, it is important to know various physical parameters (breaking length, tear factor, burst factor and others). In the present investigation, 24 % reduction in chlorine consumption was achieved. A significant decrease in kappa number has been reported by using the xylanases of *B. coagulans* (Damiano *et al*., 2003) and *B. licheniformis* 77-2 (Choudhury *et al*., 2006). An enhancement in brightness (81.94) was attained using recombinant xylanase in this investigation as reported by Kaur *et al*. (2010), where an increase of 5.3 % in brightness was achieved. Scanning electron micrographs clearly indicated the action of recombinant xylanase on the fibrillation of pulp. It has been observed that with longer treatment of cellulosic fibers with xylanase, smooth surface of fiber changes to rough surface that clearly indicates the enhancement in surface area and porosity of the fiber for proper penetration of bleaching agent as reported with xylanases of *B. halodurans* TSEV1 and *Thermotoga maritima* (Jiang *et al*., 2006; Kumar and Satyanarayana, 2012).

The xylanase, retrieved by the metagenomic approach in this investigation, has all the characteristics required for its application in generating xylo-oligosaccharides from agro-residues and in pre-bleaching of paper pulps. Further efforts are, therefore, called for cloning and expression of *Mxyl* in *Pichia pastoris* for enhancing titres in high cell density cultivation in order to bring down the cost of enzyme production.