SUMMARY

Alkaline thermostable xylanases are currently gaining importance as they can replace the conventional method, using chlorine, for the bleaching of pulp which is undesirable due to the environmental pollution it causes. In this regard we have reported earlier that thermostable alkalistable xylanase(s) from isolate NG-27 is a well-suited candidate. In the present study, on the basis of detailed morphological, biochemical, fatty acid and nucleic acid characterization, this isolate was classified as a new alkalophilic species of the genus Bacillus and was designated as alkalophilic *Bacillus* sp. NG-27. Synthesis of xylanases(s) from this isloate was found to be subject to 30 fold induction by xylan while a complete repression in the presence of glucose was observed. Further, *Bacillus* sp. NG-27 was found to produce multiple xylanases, as indicated by the presence of multiple bands of xylanolytic activities in a zymographic analysis on xylan. However, a zymograph on carboxy methyl xylan showed that only 42 kDa protein was the true endoxylanase.

Complete nucleotide sequence of the cloned 2.6 kb *Hind* III-*Hpa* I fragment was determined and an ORF capable of encoding a protein of about 44.5 kDa (which corresponded well with the molecular weight of NG-27 xylanase, as determined by SDS-PAGE) was located. On the basis of homology in the upstream region, two promoter like elements, a typical ribosome binding site and a 14 mer sequence similar to the catabolite responsive element of Bacillus were located. In the downstream region, ORF terminated at the end of the cloned fragment, indicating that the gene did not carry a transciptional terminator of its own. Twenty eight amino acids at the N-terminal of the enzyme were

found to be representing a typical bacterial signal peptide. Active site for family F was located at amino acid positions 303 to 313 with an active glutamate at 310. On comparison, NG-27 showed homology with the thermostable as well as alkaline xylanase. An interesting feature of our analysis was that despite being highly thermostable, NG-27 xylanase was not having any thermostabilising cysteine residue. Instead, N-terminal sequences of NG-27 xylanase had high homology with the sequences which are known to provide thermostability to xylanases from other organisms. These N-terminal sequences might be responsible for conferring thermostability to the NG-27 xylanase.

To clone NG-27 xylanase, 4.3kb *Hin*d III fragment of NG-27 DNA was cloned into vector pBR322 (recombinant designated as pGNG17) and expressed in *E.coli*. Expression of NG-27 xylanase in *E.coli* was confirmed by the presence of 42 kDa plasmid-coded protein in maxicells. Further, these results were confirmed by the presence of a 42 kDa protein showing xylanase activity in a zymograph using xylan as substrate. Restriction analysis of the 4.3 kb cloned fragment revealed unique restriction sites for *Sal* I, K*pn* I, *Hpa* I and *Bst* E II. On deletion analysis, smallest fragment able to give active xylanase was found to be 2.6 kb *Hin*d III - *Hpa* I fragment. *E.coli* containing NG-27 xylanase gene produced 1.6 times more xylanase than the native strain out of which about 25-50% of the xylanase was secreted outside the cell. In contrast to *Bacillus* sp. NG-27, expression of cloned xylanase was found to be refractory to the presence of inducers like xylan.

Due to low levels of improvement in the synthesis and inefficient secretion of NG-27 xylanase, when cloned into *E.coli*, it was considered worthwhile to clone this

enzyme in a xylanase negative strain of *B. subtilis* (*B subtilis* A8). A structurally stable construct in Bacillus (pGNG 24) was generated by cloning 2.6 kb *Hin*d III-*Hpa* I fragments of pGNG17 in shuttle vector pBR373 in such an orientation that ORF ended with T0 transcriptional terminator of the vector since the cloned fragment did not carry a transcriptional terminator of its own. Although structurally stable, pGNG24 was segregationally unstable in *B.subtilis*. Recombinant Bacillus carrying pGNG24 produced 200 times more xylanase than the native strain. As has been observed in the native strain, xylanase in recombinant Bacillus could be induced in the presence of xylan. When xylanases from recombinant and native strains were characterised and compred, both were found to be similar in most of the properties, except that recombined strain produced only 42 kDa endoxylanase while the native strain produced small quantities of other xylanolytic enzymes as well.

Since the recombinant strain of Bacillus carrying the NG-27 xylanase gene could produce high levels of alkali stable thermostable xylanases, it increases the possibility of exploitation of this enzyme at the commercial scale.