RECOVERY AND EXPRESSION OF THERMO-ALKALI-STABLE XYLANASE GENE BY METAGENOMIC APPROACHES

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ABSTRACT

Xylans are the main constituents of hemicellulose component of terrestrial plant cell walls and second most abundant polysaccharide on the Earth. Its core comprises β-1,4-linked xylosyl residues, and to this are attached various groups (4-O-methyl-D-glucuronosyl, α-arabinofuranosyl residues and others) that makes it a heteropolysaccharide. Xylanases have attracted considerable attention due to their catalytic activity for releasing lower xylooligosaccharides and their potential application in textile, feed and biofuel industries. Viikari and her coworkers reported their applicability in bleaching of pulp in the paper industry in 1986, and this was confirmed by several workers subsequently. The inclusion of xylanase treatment step in the pulp bleaching process reduces the chlorine consumption up to 25-35 % in bleaching kraft pulp to make the process environment friendly. The majority of xylanases have been produced from the culturable 0.1 to 1 % of the total microbial diversity existing in natural environments. The culture-independent metagenomic approaches, on the other hand, permit retrieval of genes encoding useful enzymes from environmental samples without involving the rigours of cultivation of microbes. The demand for alkalistable and thermostable xylanases in pulp bleaching encouraged us to adapt this innovative strategy for retrieving genes that encode thermo-alkali-stable xylanases.

In this investigation, metagenomic libraries have been constructed from the DNA extracted from various hot and alkaline environmental samples and screened for clones with xylanase activity. The xylanase encoding gene from the DNA insert was subcloned and expressed, and the recombinant xylanase was purified, characterized and tested for its applicability.

An improved single-step protocol has been developed for extracting pure community humic acid-free DNA from alkaline soils and sediments. A total of 36,400 transformants containing metagenomic DNA inserts were screened for xylanase activity. One clone (TSDV-MX1) exhibited a clear zone of xylan hydrolysis on RBB xylan agar plate from the compost-soil based metagenomic library. On sequencing, the insert size was determined as 6232 bp. The insert contained full-length xylanase. The amino acid sequence analysis suggested a
maximum identity of 71% with the glycosyl hydrolase family 11 xylanase of an uncultured bacterium followed by *Microbulbifer hydrolyticus* (63%) and *Saccharophagus degradans* (62%). A 1077 bp xylanase gene (*Mxyl*) encoding 358 amino acids was successfully cloned in pET28a(+) and pET22b(+) expression vectors and successfully expressed in *E. coli* BL21 (DE3). The maximum intracellular xylanase fraction from the recombinant pET22Mxyl was attained after 3 h of induction. The periplasmic fraction contained 45% of the xylanase after 15 h of induction, while the extracellular fraction was maximum (29%) after 24 h.

The purified recombinant xylanase was optimally active at pH 9.0 and 80 °C. The recombinant xylanase (Mxyl) retained >80% activity after exposure to 60 °C and 70 °C for 3 h, and it has $T_{1/2}$ of 120 min at 80 °C and 15 min at 90 °C. When the pH stability of the enzyme was studied by exposing the recombinant xylanase 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 recorded at pH 10.0 and 11.0 after 16 h of incubation. The recombinant xylanase exhibited $K_m$, $V_{max}$ and $K_{cat}$ of 8.01 mg ml$^{-1}$, 333.0 μmol$^{-1}$ mg$^{-1}$ min and 2220 s$^{-1}$, respectively. Most of the metal ions tested did not affect activity at 1 mM, but the activity was significantly inhibited at higher concentrations of Pb$^{2+}$, Ag$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Ba$^{2+}$, Cd$^{2+}$ and Co$^{2+}$. The cations Fe$^{2+}$, Mg$^{2+}$ and Sn$^{2+}$ exhibited a slight stimulatory effect on xylanase activity, while Hg$^{2+}$ completely inhibited enzyme activity at 1 mM.

When xylanase production by the recombinant strain *Bacillus sibtilis* (*pWHMxyl*) was optimized using one-variable-at-time-approach, a titre of 75 UmL$^{-1}$ was attained. These factors were further optimized by RSM that resulted in 2.9-fold improvement in extracellular xylanase production in shake flasks.

In an attempt to enhance thermostability using site-directed mutagenesis, a mutant MXMUT4 was developed that exhibited 20 min of higher $T_{1/2}$ at 80 °C of 150 min than that of the parental xylanase (Mxyl) [120 min].

When agro-residues were treated with recombinant xylanase, lignocellulosic materials were saccharified and it was maximum (~15%) in wheat bran that further enhanced on increasing the reaction time.
Abstract

When the pulp was treated with metagenomic xylanase, an overall 24 \% reduction in chlorine consumption was attained as compared to the control (pulp samples without enzymatic treatment). The kappa number of the pulp was reduced to 2.2 with concomitant increase in brightness of \(~82\) \%. Handmade paper made using the treated pulp samples showed a clear difference in brightness in comparison with the control.

In this investigation, alkali-thermo-stable xylanase encoding gene was retrieved from soil-compost metagenome, cloned and expressed. The metagenomic xylanase was purified and characterized, and the thermostability of the enzyme was improved by site directed mutagenesis. It was found to be useful in generating xylooligosaccharides from agro-residues and in pulp bleaching.

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