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Secretion of recombinant thermo-alkali-stable endoxylanase of polyextremophilic *Bacillus halodurans* TSEV1 and its utility in generating xylooligosaccharides from renewable agro-residues

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ABSTRACT

The recombinant thermo-alkali-stable endoxylanase (Bh-xyl) of polyextremophilic bacterium *Bacillus halodurans* TSEV1 has been produced extracellularly using a combination of cloning strategies and physico-chemical treatment of recombinant *Escherichia coli* cells. Sixty percent higher secretion of recombinant xylanase has been achieved by cloning *Bh-xyl* in pET28a(+) and expression followed by optimization of the cultural variables (EDTA, lysozyme and temperature). The pure recombinant endoxylanase is of 42 kDa, which is active in the broad pH and temperature ranges between 7.0 and 12.0, and 30 and 100 °C, with optima at 9.0 and 70 °C. The K_m, V_{max} and k_{cat} values of the Bh-xyl (birchwood xylan) are 2.6 mg ml^{-1}, 252.3 μmol mg^{-1} min^{-1} and 3.36 × 10^3 min^{-1}, respectively. The enzyme has higher affinity for soft wood xylan than most of the xylanases from extremophilic microbes. The endoxylanase efficiently liberated xylooligosaccharides from the renewable agro-residues, which find application in functional foods as prebiotics.

1. Introduction

Xylan is the second most common naturally degradable material found in plant cell walls after cellulose. The xylan is a complex heteropolysaccharide comprising a backbone chain of β-1,4-D-xylopyranose units with diverse side groups (acetyl, arabinofuranose, ferulic acid, methyl glucuronic acid and others). A mixture of a group of enzymes is needed for complete breakdown of xylan. Among them, endo-1, 4-β-xylanases (E.C.3.2.1.8) depolymerises xylan by random hydrolysis of the backbone, and several specific hydrolases are needed to cleave side-groups attached to the xylan backbone [1]. The potential uses of microbial xylanolytic enzymes have garnered significant attention in the last couple of decades.

The enzymatic conversion of abundantly available lignocellulosic materials (LCMs) to value-added products will be an important alternative for human welfare. The key issue in this field is the production of useful xylanases for economic production of xylooligosaccharides (XOs) from agro-residues. The concept and beneficial effects of prebiotics received recognition in 1990s [2]. The xylan containing agro-residues can be used to generate XOs containing 2–6 xylose units linked *via* β-(1–4)-xylosidic linkages. The production of value added XOs from LCMs would be an interesting and emerging alternative because these raw materials are renewable and do not compete with the food crops and are also less expensive than the conventional agricultural feed stocks [3]. The problem faced by the industries is cost and availability of suitable enzymes that would suit extreme industrial processes conditions. Developing high productive recombinant strains, efficient fermentation and recovery systems are some of the important factors that need attention for the cost effective production of enzymes [4,5].

Pulp and paper industries are the major consumers of lignocellulosic materials on a large scale and generate a lot of pollutants. Environmental issues are forcing governments to develop more stringent laws against pollution caused by chlorinated dioxins and other chlorinated compounds formed during pulp bleaching. Thus in order to reduce the chlorine consumption, enzymatic pulp bleaching stage has been introduced [6]. Most of the commercial enzymes from fungal origin are optimally active at slightly neutral or acidic pH. For instance, Pulpzyme HA (Novozymes, Denmark) and Cartazyme (Clariant, Switzerland) are known to help in biobleaching under moderate conditions [7,8]. Besides low efficiency and high cost of the commercially available xylanases, the high temperature and alkaline conditions associated with pulp

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processing further demand xylanases having alkalistability as well as thermostability [9].

Escherichia coli is one of the most widely used hosts for the production of recombinant proteins of interest in both research and biotechnology industry due to several advantages such as fast growth, favourable economics, ease of genetic manipulation and simple fermentation [10,11]. The recovery of substantial yields of correctly folded proteins is, however, a problem. Most of the proteins synthesized in the cytoplasm of *E. coli* are not secreted into the culture medium because the cell envelope of this gram-negative bacterium has both cytoplasmic and outer membranes, which is a barrier to permeability for cytoplasmic proteins [10,12]. One approach to resolve these problems is to have recombinant proteins secreted into culture medium by membrane permeabilization. Some strategies for the permeabilization of the outer membrane of *E. coli* have been developed to enhance the secretion of periplasmic proteins into the medium. These approaches mainly include the use of *E. coli* mutants with a defect in the cell envelope components (*lky*- and *exc*-mutants) [13,14] and the addition of chemicals such as glycine and Triton X-100 [12,15]. Most of these approaches are, however, non-selective, and thus, result in the release of other periplasmic proteins into the medium along with the protein of interest. The reports on the selective extracellular production of thermo-alkali-stable xylanases are very scanty, and therefore, an attempt has been made to enhance the secretion of active recombinant xylanase from *E. coli* cells into the medium.

Bacillus halodurans TSEV1, a polyextremophilic bacterium, is capable of biomass degradation and a good source of endoxylanase [16,17]. The bacterium produces a high titre of cellulase-free and thermo-alkali-stable endoxylanase that finds application in generating XOs from agro-residues and in pre-bleaching of kraft pulp [16,18]. In this investigation, the endoxylanase of *B. halodurans* TSEV1 has been cloned and expressed in *E. coli*. The secretion of recombinant xylanase (Bh-xyl) into medium has been optimized in order to simplify the enzyme recovery. The purified recombinant enzyme was characterized and its applicability has been tested in generating XOs from renewable agro-residues. To the best of our knowledge, this is the first report on extracellular secretion of any thermo-alkali-stable bacterial xylanase by the recombinant *E. coli*.

2. Materials and methods

2.1. Strains and plasmids for DNA manipulations

The polyextremophilic bacterium, *Bacillus halodurans* TSEV1 was isolated from the effluent sample collected from the Century Pulp and Paper Industry, Lalkuan (Uttarakhand State, India) [12]. The genomic DNA of *B. halodurans* TSEV1 was used as the source of xylanase gene. The pGEM-T Easy vector (Promega, USA), pET28a(+) and pET22b(+) of Novagen (Germany) were used for sequencing and expression of the xylanase gene, respectively. *E. coli* DH5α and *E. coli* BL21 (DE3) were used for the propagation of the plasmid and expression of xylanase respectively. The restriction enzymes, DNA markers and T4 DNA ligase were purchased from New England Biolabs (UK).

2.2. Bioinformatics analysis and construction of expression plasmids pET28a-Bh-xyl as well as pET22b-Bh-xyl

The complete open reading frame (ORF) of *B. halodurans* TSEV1 xylanase was pulled out using specific primers (For_Bam_xyl 5′ CCCGGATCCATGTTTAACGGCACCATGATG 3′; Rev_Hin_xyl 5′ CCCCTCGAGTCTAGGAACCCAAACCGAAACCG 3′), having sites for *Bam*HI and *HindIII* endonucleases and cloned into pGEMT easy vector. The gene sequence was obtained after sequencing with T_7 forward and SP_6 reverse primers. The gene sequence as well as translated protein sequences were analysed using BLASTN and BLASTP server of NCBI. Multiple alignments of the amino acids from thermo-alkali-stable xylanase from related bacteria were carried out using the CLUSTALW programme (http://www.ebi.ac.uk/clustalW). The gene fragment encoding mature Bh-xyl has been obtained by double digestion of pGEMT-xyl recombinant plasmid with *Bam*HI and *HindIII* restriction enzymes and sub-cloned into pET-28a(+) and pET-22b(+) vectors at compatible sites and then transformed into *E. coli* DH5α according to Sambrook et al. [19]. The presence of *Bh.xyl* in the clones growing on LB-kanamycin (50 μg/ml) plates was confirmed by colony PCR and double digestion with respective endonucleases. The gene was amplified under the defined PCR conditions (initial denaturation 3 min at 95 °C followed by 35 cycles of 10 s at 98 °C, 30 s at 53 °C and 75 s at 72 °C with a final extension step at 72 °C for 8 min). The positive clones were processed for sequencing.

2.3. Expression of xylanase (Bh-xyl) using pET28a-Bh-xyl and pET 22b-Bh-xyl

The recombinant plasmid having the accurate sequence was then transformed into *E. coli* BL21 (DE3) competent cells for expression of the recombinant protein. The transformants were grown in kanamycin (50 μg/ml)-LB medium at 37 °C with 200 rev min^{-1} in an incubator shaker to A_{600} of 0.8–1.0. The expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to 1 mM and the culture was further cultivated at 30 °C. The samples were collected at 1 h intervals for determining the enzyme titres in soluble fraction after ultrasonication (10 cycles of 1 min pulse, 1 s on/off; 20 kHz; 50 W).

2.4. Analysis and localization of recombinant xylanase (Bh-xyl)

Localization of recombinant xylanase into the cytoplasmic, periplasmic or extracellular pools was studied by measuring xylanase activity in these fractions. The induced culture was harvested after 5 h of induction. The supernatant was collected as extracellular fraction, while the pellet was resuspended in 20 mM glycine–NaOH buffer (pH 9.0) having 25% (w/v) sucrose and 1 mM EDTA. The cell suspension was mixed in an incubator shaker at 200 rev min^{-1} for 15 min and later sedimented at 4 °C; the osmotic shock was given for 10 min by suspending the pellet into 5 mM chilled $MgSO_4$ solution to release the periplasmic fraction of the cells which was collected as supernatant after centrifugation. Finally the intracellular fraction was obtained by homogenizing the cells by sonication in chilled glycine–NaOH buffer (pH 9.0). The supernatant was separated from the cell debris by centrifugation and the xylanase activities were determined in the fractions.

2.5. Extracellular production of recombinant xylanase from E. coli BL21(DE3)

Two methods were employed for making the intracellular Bh-xyl extracellular.

2.5.1. Expression profile of pET22b-Bh-xyl in E. coli BL21(DE3)

The recombinant *E. coli* having pET22b-Bh-xyl plasmids were grown at 37 °C, 200 rev min^{-1} and induced after reaching A_{600nm} of 1.00. The induced *E. coli* BL21(DE3) cells were grown for varied time intervals and harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. The recombinant xylanase activities in various fractions at the desired intervals were calculated individually and presented as the percentage of the total xylanase activity.