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Research article

Heterologous expression and enhanced production of β-1,4-glucanase of *Bacillus halodurans* C-125 in *Escherichia coli*

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ABSTRACT

Background: Recombinant DNA technology enables us to produce proteins with desired properties and insubstantial amount for industrial applications. Endo-1, 4-β-glucanases (Egl) is one of the major enzyme involved in degradation of cellulose, an important component of plant cell wall. The present study was aimed at enhancing the production of endo-1, 4-β-glucanases (Egl) of *Bacillus halodurans* in *Escherichia coli.*
Results: A putative Egl gene of *Bacillus Halodurans* was expressed in *E. coli* by cloning in pET 22b (+). On induction with isopropyl-b-D-1-thiogalactopyranoside, the enzyme expression reached upto ~20% of the cell protein producing 29.2 mg/liter culture. An increase in cell density to 12 in auto-inducing LB medium (absorbance at 600 nm) enhanced β-glucanase production up to 5.4 fold. The molecular mass of the enzyme was determined to be 39 KDa, which is nearly the same as the calculated value. Protein sequence was analyzed by CDD, Pfam, I TASSER, COACH, PROCHECK Servers and putative amino acids involved in the formation of catalytic, substrate and metal binding domains were identified. Phylogenetic analysis of the β-glucanases of *B. halodurans* was performed and position of Egl among other members of the genus *Bacillus* producing endo-glucanases was determined. Temperature and pH optima of the enzyme were found to be 60°C and 8.0, respectively, under the assay conditions.
Conclusion: Production of endo-1, 4 β-glucanase enzymes from *B. halodurans* increased several folds when cloned in pET vector and expressed in *E. coli*. To our knowledge, this is the first report of high-level expression and characterization of an endo-1, 4 β-glucanases from *B. halodurans*.
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1. Introduction

Endo-1,4-β-glucanases (EC 3.2.1.4) play a significant role for hydrolyzing large and complex cellulose chains into oligosaccharides [1]. These enzymes act randomly on the interior β 1,4 linkages of the less crystalline region, resulting in a rapid change in the degree of polymerization, which is favorable for further processing of cellulose to glucose [2].

Cellulases are produced by several types of fungi, actinomycetes, and bacteria including actinomycetes [3,4]. For commercial scale production of cellulases (particularly endoglucanases), cost effectiveness is desirable. Recombinant expression of endo-1,4-β-glucanases in *E. coli* enables robust and cost effective production and ultimately better saccharification of cellulosic substrates.

Several researchers previously reported the production of endo-1,4-β-glucanases in bacteria [5,6,7]. Genes encoding the enzymes from different sources, including *cel C* of *Clostridium thermocellulum* [8], *Cel5A* of *Eubacterium cellulosolvens* [9] and *celA* of *Thermobifida fusca* [10], *Cel9A* of *Cytophaga hutchinsonii* [11] have been cloned and expressed in *E. coli*. Facultative alkalophilic bacterium *Bacillus halodurans* C-125 grows well at pH 7 to 10.5. *Bacillus halodurans* C-125 genome (NCBI accession no. NC_002570.2) contains 4076 genes (85), of which several are putative endo-1, 4 β-glucanase genes (*Egl*). One of the genes, designated as BH_RS15755, found in position 324, 8335 to 324, 9420 in the genome sequence, encodes a putative endo-1,4 β-glucanase (*Egl*) of 361 amino acids and belongs to peptidase M42 family (UniProt ID: wp_010899276) [12]. The function of the protein of this gene is predicted *in silico* using gene prediction method by homology modeling. Here we reported for the first time

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the functional analysis of this gene product by cloning, expression, and characterization of this endo-1,4 β-glucanase (*Egl*) in *E. coli*.

2. Materials and methods

2.1. PCR Amplification and Cloning of Egl

B. halodurans C-125 was grown aerobically at 37°C in 50 ml LB broth (Sigma-Aldrich, USA). Genomic DNA was extracted as reported elsewhere [13] with some modifications. Endo-1,4 β-glucanase gene was amplified by PCR using genomic DNA as a template with gene specific forward FEgl (5'-*CATATG*GCACAATTAGACGAACGATTG-3′) and reverse primer REgl (5'-*GGATCC*TTAATCAAATGTAATGCGGTTCACG-3′) such that *Nde*I and *Bam*HI recognition sequences (shown respectively as underline and italicized) were placed at 5′ and 3′ ends of the coding region. The PCR reactions were set in 50 μl reaction volume and conditions were as follows; one cycle of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 30 s, extension at 72°C for 60 s and a cycle of the final extension at 72°C for 20 min [14].

The amplified product was purified using Invitrogen gel extraction kit, T/A cloned in pTZ57R/T vector, and then subcloned between *Nde*I and *Bam*HI sites of the pET-22b (+) vector to generate chimeric pET-Egl expression plasmid, which was maintained in *E. coli* DH5α, selected on LB medium containing 100 μg/ml ampicillin. The junction sequences of the recombinant plasmid were confirmed by Sanger's sequencing.

2.2. Expression analysis of Egl

Competent *E. coli* BL21(DE3) CodonPlus were transformed with the recombinant plasmid pET-Egl for expression analysis. Transformants (containing recombinant vector) were confirmed by restriction digestion and colony PCR. For expression analysis, one of the positive colony was grown overnight in 25 ml LB-ampicillin medium at 37°C. One percent of this overnight culture was used to inoculate 200-ml fresh medium followed by incubation at 37°C in an orbital incubator shaker at 150 rpm. Protein expression was induced with either 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at culture density A_{600} of 0.5–0.6. Cells were allowed to grow till the maximum growth was attained (~12 h) and then harvested by centrifugation at 4500 rpm for 15 min. During cultivation, 1-ml aliquots were collected after every 2 h to monitor the growth (A_{600}) and analyze protein expression by 12% SDS-PAGE [15]. Percentage of Egl was determined by densitometric scanning of the Coomassie stained gel.

2.3. Purification of endo-1,4 β-glucanase

Culture was grown in 250 ml LB media was harvested by centrifugation (6500 rpm, 10 min), resuspended in 50 ml of 50 mM glycine-NaOH buffer (pH 8.0) and disrupted by sonication (10 × 30 s bursts with intervals of 1 min between successive pulses) in a UP 400s Ultrasonicator (Dr. Hielscher GmbH, Germany). Lysate thus obtained was again centrifuged as described previously, and soluble proteins in the supernatant were purified for the enzyme activity assay. For purification supernatant were precipitated by adding ammonium sulfate slowly to 75% saturation at 4°C and incubated for 6 h. The precipitates were collected by centrifugation at 6500 rpm for 30 min at 4°C. The precipitates were dissolved in 25 ml of 50 mM glycine-NaOH buffer (pH 8.0) and dialyzed overnight against three changes of the same buffer. The solution was filtered through 0.45 μm Millipore filter and loaded on the Sephadex G-75 column (1.6 × 40) for gel filtration chromatography. After equilibration with 50 mM glycine-NaOH buffer (pH 8.0), the sample was loaded and the proteins were eluted with the same buffer at a flow rate of 0.5 ml per min. Collected fractions were analyzed for Egl activity.

2.4. Enzyme (Endo-1,4 β-glucanase) assay

Endo-1, 4 β-glucanase activity of the supernatant after sonication and purified fractions was determined by dinitrosalicylic acid (DNS) method [16]. For the assay, 0.5 ml of the appropriately diluted enzyme in 0.05 M Tris-Cl buffer (pH 8.0) along with 1% Carboxymethylcellulose (Sigma Chemicals Co. USA) was incubated at 60°C for 20 min in a shaking water bath. After incubation, the reaction was stopped by adding 3 ml of DNS reagent and heating in boiling water for 10 min. The liberated reducing sugars in the supernatant were quantified at 590 nm and activity was calculated from glucose standard graph. One unit of enzyme activity is defined as the amount of enzyme which released 1 μmol of reducing sugars equivalent to glucose per minute under the standard assay conditions.

2.5. Endo-1,4 β-glucanase characterization

Effect of pH on enzyme activity at 60°C was determined by maintaining a different pH of assay mixtures with 0.05 M potassium phosphate buffer (pH 6.0–7.0), 0.05 M Tris-Cl buffer (pH 7.5–9.0) and glycine-NaOH buffer (pH 8.0–10.0).

Stability was expressed as a percentage of residual activity. Stability at different pH (6.0–10.0) was determined by incubating suitably diluted enzyme in buffers of various pH at room temperature for 1 h and determining the residual activity at 60°C under the standard assay conditions. Thermal stability was determined by pre-incubating enzyme in 0.05 M glycine-NaOH buffer pH 8.5 at various temperatures ranging from 30–70°C. The enzyme samples were drawn after 1 h, and residual enzyme activity was determined as below:

$$\text{Residual activity (percent)} = (\text{enzyme activity before incubation} - \text{enzyme activity after incubation}) \times 100$$

2.6. In Silico protein characterization and phylogenetic analysis

Protein sequences of endo-1,4 β-glucanase from 20 different species were retrieved using the BLAST program available at the NCBI server. Multiple sequence alignments were performed using the Clustal W program with default parameters using the alignment explorer option of the MEGA 5.2.1 software package [17]. The distance was calculated by the maximum parsimony model [18] and a phylogenetic tree was constructed [19]. The 3-D structure of the protein was predicted by I-TASSER [20], and different domains were predicted by PROCHECK [21], COACH [22] server, and family was checked by Pfam database [23].

3. Results and discussion

3.1. Construction of recombinant expression plasmid

About 1.086-nucleotide long fragment of Egl containing *Nde*I and *Bam*HI sites at the 5′ and 3′ termini, respectively, was PCR amplified with different $MgCl_2$ concentrations (1.5, 2.0, 2.5 mM was used) to optimize the PCR conditions (Fig. 1). Two millimolars of $MgCl_2$ was found to be the best concentration for amplification (Fig. 1). Variations of the Mg^{2+} concentration below 4 mM, can improve the performance of PCR by affecting the specificity (lower concentrations raise specificity, higher concentrations lower the specificity) [24]. The amplified PCR product was cloned in T7/*lac* promoter based pET-22b (+) vector at *Nde*I/*Bam*HI sites to generate pET-Egl expression plasmid (Fig. 2). The recombinant plasmid was first maintained in *E. coli* DH5α for vector propagation and then transformed into *E. coli* BL21 (DE3) CodonPlus for expression studies. The presence of the insert in positive transformants was confirmed by colony PCR (Fig. S1)

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