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High-level soluble expression of a thermostable xylanase from thermophilic fungus *Thermomyces lanuginosus* in *Escherichia coli* via fusion with OsmY protein

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ABSTRACT

A thermostable xylanase is encoded by *xynA* from fungus *Thermomyces lanuginosus*. The problem emerged from overexpression of xynA in *Escherichia coli* has been the formation of inclusion bodies. Here we describe the xynA was fused with the hyperosmotically inducible periplasmic protein of *E. coli*, OsmY. The fusion protein OsmY-xynA was expressed as almost all soluble form. The soluble expression level of fusion protein reached 98 ± 6 U/ml when cells containing pET-OsmY-xynA were expressed without IPTG induction at 37 °C. The induction is probably due to auto-induction due to lactose in the medium (Studier (2005) [21]). The cells harboring pET-OsmY-xynA expressed an activity level about 24 times higher than that expressed from pET-20b-xynA. Xylanase activity was observed in the extracellular (36 ± 1.3 U/ml) and the periplasmic (42 ± 4 U/ml) when cells containing pET-OsmY-xynA were induced without IPTG addition. After the cold osmotic shock procedure followed by nickel affinity chromatography, the purified fusion protein showed a single band on SDS–PAGE gel with a molecular mass of 44 kDa. The purified fusion enzyme exhibited the highest activity at 65 °C and pH 6.0.

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Introduction

Thermomyces lanuginosus produces a thermostable GF11 endoxylanase encoded by *xynA* gene. This xylanase is free of cellulase activity, and hydrolyses xylan to produce xylooligosaccharides with little xylose [1]. These properties make the enzyme attractive for the industrial application. *Escherichia coli* is one of the most extensively used prokaryotic organisms for the industrial production of enzyme because of its well-characterized genetics, and its ability to grow rapidly and at high density on inexpensive substrates [2].

The xylanase gene *xynA* has been sequenced and cloned into *E. coli* as a LacZ fusion protein, but efficient expression was not obtained [3]. Recently, the DNA sequence of *xynA* has been optimized, and the expression of the enzyme in *E. coli* has reached a high level by using recombinant plasmid pET-20b-*xynA*. However, the recombinant enzyme was mainly found in inclusion bodies, and only a small proportion was soluble and active [4]. It is a common problem that some recombinant proteins will aggregate to form inclusion bodies in the cytoplasm and/or periplasm [5]. Inclusion body formation of eukaryotic proteins in *E. coli* with many contributing factors: insolubility of the product at the concentrations being produced, inability to fold correctly in the bacterial environment, or lack of appropriate bacterial chaperone proteins [6]. Many attempts have been made to improve the soluble expression of recombinant proteins in *E. coli*. The formation of inclusion bodies could be decreased by changing the promoter to regulate the level of expression, controlling the growth conditions (especially the pH of the culture), controlling fermentation medium, changing the temperature of induction and enabling secretion into the periplasm, fusing the target gene to another gene [7].

OsmY has been used as a fusion partner to excrete target proteins into the medium [8–10]. When fused to OsmY, *E. coli* alkaline phosphatase, *Bacillus subtilis* a-amylase, and human leptin could be secreted into the medium at high levels [11].

Here we report the construction of vector pET-OsmY-xynA, and the overexpression of soluble fusion protein OsmY-xynA in *E. coli*.

Materials and methods

Bacterial strains, plasmids and growth media

E. coli DH5α (TaKaRa, Dalian, China) was used as hosts for gene cloning. *E. coli* BL21(DE3) (TaKaRa, Dalian, China) was used as hosts

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for the expression of fusion protein. The cells harboring expression plasmids were cultured in Luria–Bertani (LB)[1] medium supplemented with ampicillin (100 μg/ml).

Construction of fusion expression plasmid pET-OsmY-xynA

Based on the nucleotide sequences coding for the OsmY protein (GenBank accession No. NC_000913.3), the PCR amplification was carried out by using *E. coli* DH5α genome as template, with the following primers: 5′-GCCGAATTCATGACTATGACAAGACTGAAG-3′ and 5′-GGCGGATCCCTTAGTTTTCAGATCATTTTTAAC-3′. PCR was carried out using the high-fidelity *Pyrobest* DNA polymerase (TaKaRa, Dalian, China). PCR products were purified using the QIAquick PCR purification kit and followed by digestion with *Bam*H I and *Eco*R I restriction enzyme(s). The reverse PCR amplification was carried out by using plasmid pET-20b-xynA [4] as template, with primers X1 (5′-GCCGAATTCTATATCTCCTTCTTAAAGTTAAAC-3′) and X2 (5′-GGCGGATCCCAGACTACCCCGAACTCTGAAG-3′). PCR products were purified using the QIAquick PCR purification kit and followed by digestion with corresponding restriction enzyme(s). The digested PCR products were ligated to OsmY at *Bam*H I/*Eco*R I sites.

Expression and purification of fusion protein

The plasmid pET-OsmY-xynA was transformed into the *E. coli* BL21(DE3) by electroporation. The cells carrying pET-OsmY-xynA were grown at 37 °C, and induced for gene expression by addition IPTG (isopropyl-β-D-thio galactopyranoside). The recombinant enzyme was isolated from the periplasm by cold osmotic shock according to a published protocol [12]. The cells (wet weight 1.5 g) harvested by centrifugation at 6000×*g* for 5 min were re-suspended in 12 ml of 100 mM Tris–HCl containing 20% sucrose and 1 mM EDTA (pH 8.0), and then pelleted by centrifugation at 8000×*g* for 5 min followed by re-suspension in 5 ml of ice-cold water for 10 min. After the addition of $MgCl_2$ to a final concentration of 1 mM, the cell suspension was incubated on ice for a further 10 min before being pelleted by centrifugation at 8000×*g* for 10 min at 4 °C. The supernatant (5 ml) was loaded onto a 1 ml HisTrap HP columns (GE Healthcare), washed with 60 mM imidazole and 0.5 M NaCl in 20 mM Tris–HCl buffer (pH 7.9), and eluted with 1 M imidazole and 0.5 M NaCl in 20 mM Tris–HCl buffer (pH 7.9). The pooled fractions were dialyzed into storage buffer containing 1 mM EDTA, and 20% (v/v) glycerol before the enzyme was stored at −20 °C. The SDS–PAGE was performed according to standard procedures. Protein concentration was determined by the Bradford method using BSA as a standard [13].

Enzyme assays

Xylanase activity was determined by the 4-hydroxybenzoic acid hydrazide method [14]. Xylan from birch wood (Sigma Aldrich, Munich, Germany) was used as the substrate. The reaction mixture comprised of 100 μl 1% (w/v) birch wood xylan in water, 90 μl phosphate buffer (50 mM, pH 6.0) and 10 μl properly diluted enzyme. The reaction was conducted at 65 °C for 10 min, and stopped when 600 μl of 4-hydroxybenzoic acid hydrazide solution were added into the reaction mixture. The reducing sugar was determined by reading the absorbance at 410 nm after the test tubes were incubated for 10 min in boiling water bath and cooled down on ice. One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar per min.

[1] *Abbreviations used:* LB, Luria–Bertani; IPTG, isopropyl-β-D-thio galactopyranoside.

Results

Construction of expression plasmids

The gene encoding the OsmY (including the signal sequence) was amplified from the genomic DNA of *E. coli* DH5α, and inserted into the plasmid pET-20b-xynA (pelB signal sequence was deleted) [4] at *Bam*H I/*Eco*R I sites. Newly generated plasmid is designated as pET-OsmY-xynA (carried an N-terminal OsmY signal sequence). Target protein xylanase from fungus *T. lanuginosus* was linked to the C-terminus of OsmY by a *Bam*H I site sequence. The fusion protein OsmY-xynA was expressed with a C-terminal His-tag.

Expression level and solubility of the fusion protein OsmY-xynA

The recombinant plasmid pET-OsmY-xynA was isolated, which was then transformed into *E. coli* BL21(DE3) for the production of fusion protein OsmY-xynA using IPTG induction. The xylanase activity of fusion protein was obtained after induction at different IPTG concentrations (Fig. 1). Interestingly, xylanase activity produced by cells containing pET-OsmY-xynA with 0 mM, 0.1 mM, 0.3 mM, 0.5 mM IPTG, was 98 ± 6 U/ml, 6.4 ± 0.2 U/ml, 6.3 ± 0.15 U/ml, 6 ± 0.1 U/ml, respectively. There was a gradual decrease in xylanase activity upon increasing the IPTG concentration (Fig. 1). The recombinant cells induced without IPTG addition produced an activity level 16 times higher than that expressed with 0.5 mM IPTG induction.

Previously, intracellular expression of the xylanase was improved by sequence optimization by site-directed mutagenesis without changing the protein sequence [4]. But the recombinant xylanase mainly appeared as inclusion bodies [4]. In the current study, the expression levels and solubility of the fusion protein expressed from pET-OsmY-xynA were shown in Fig. 2. The fusion protein expression level is very high when the fusion gene expression was induced by the addition of IPTG at 37 °C (Fig. 2, line 3–5), and only a small proportion was soluble and active (Fig. 2 line 7–9). However, the fusion protein was expressed as almost soluble form when the fusion gene expression was induced without IPTG addition (Fig. 2 line 2 and 6).

Secretion of fusion protein in E. coli

When pET-OsmY-xynA vector was used to express fusion protein, the xylanase activities in the extracellular, periplasmic and cytoplasmic fractions were monitored (Fig. 3). Protein expression was initiated by the addition of IPTG to a final concentration of 0.5 mM when the optical density at 600 nm (OD_{600}) reached 0.8.

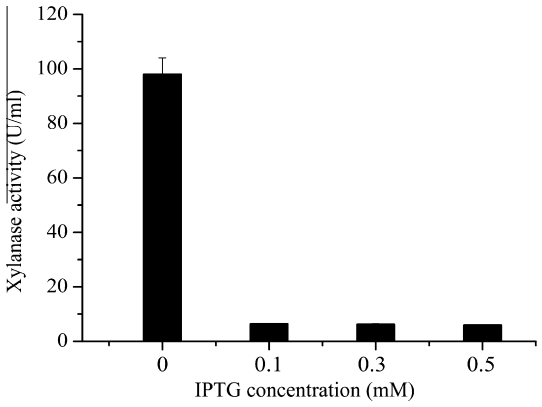

Fig. 1. Total expresion levels of fusion protein OsmY-xynA in cytoplasm, periplasm and culture medium were observed after induction at different IPTG concentrations.

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