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Improved production of alkaline polygalacturonate lyase by homologous overexpression *pelA* in *Bacillus subtilis*

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ABSTRACT

A polygalacturonate lyase (PGL), PelA, was purified from the culture broth of *Bacillus subtilis* 7-3-3, with a molecular weight, optimal temperature, and pH of approximately 45 kDa, 55 °C, and 9.4, respectively. The PGL gene (*pelA*) was homologously overexpressed in *B. subtilis* 7-3-3 to increase the gene copies and enhance the PGL production. The resulting PGL activity was 2138 U mL^{-1} at 44 h, and the productivity reached 48.58 U (mL h)$^{-1}$ through the homologous overexpression of strain B-pN-pelA in a 7.5 L fermentor, the highest PGL production compared to those reported in literature to the best of our knowledge. Crude enzyme has high PGL and PGase activity, which can remove 50.58% of pectin in unpretreatment ramie fibers at 50 °C for 4 h. Meanwhile, the enzyme system with a low level hemicellulase and almost no cellulase will further help in enhancing the efficiency of degumming besides maintaining tenacity of plant fiber. The *B. subtilis* B-pN-pelA shows high genetic stability and has great potential in the textile industry.

1. Introduction

Polygalacturonate lyases (PGLs, EC 4.2.2.2) catalyze the trans-eliminative cleavage of polygalacturonate, which generates Δ4:5 unsaturated oligogalacturonates [1]. PGLs are produced by various bacteria and some pathogenic fungi with more abundant endo-PGLs than exo-PGLs. Alkaline pectinase is an important kind of industrial enzyme that is widely applied in current biotechnological fields such as textile processing, degumming of plant bast fibers, treatment of pectic wastewaters, paper making, and coffee and tea fermentation [2]. Alkaline PGLs are the indispensable components of alkaline pectinase. Genetic engineering has been used in the improvement of PGL production to obtain high productivity and low cost. In 1997, three PGL genes, namely, *pelA*, *pel*B, and *pel*C were found in *Bacillus subtilis* 168 genome when it was sequenced [3]. PelA was first cloned and expressed [4,5] and its three-dimensional (3-D) structure was analyzed [6]. PelA from *Bacillus* was widely cloned and heterogenously expressed in *Escherichia coli* [7,8], *B. subtilis* WB800 [9], and *Pichia pastoris* [10]. To date, the highest PGL activity reported in literature is 1631 U mL^{-1} with a productivity of 16.3 U mL^{-1} h^{-1}, which is from a gene engineering of the strain *P. pastoris*. The productivity and activity of PGL need to be further increased to lower production cost and to enhance enzyme application in various industries.

Besides heterogenous expression, homologous overexpression is also a common way to construct engineering strains to improve the production of metabolin [11,12] or of protein [13]. Two main methods to obtain gene overexpression are known: screening and modifying a promoter, and increasing copies of the goal gene by transforming a plasmid. However, few studies feature the homologous overexpression of enzymes in *Bacillus* genus, especially the industrial production strain from *B. subtilis*.

B. subtilis 7-3-3, a strain-producing alkaline PGL, was isolated from soil and saved in our laboratory. The strain rapidly grows in low-cost substrates. The media components and culture conditions were optimized by shake flask cultivation and a 7.5 L fermentor in our laboratory. The highest PGL activity recorded was 743.5 U mL^{-1} with the average productivity of 19.6 U (mL h)$^{-1}$ at 38 h. However, the protein concentration of the fermentation broth was only 2.0 mg mL^{-1}, which meant that the production of PGL in *B. subtilis* 7-3-3 could still be improved. In this study, we purified and characterized PelA, an alkaline PGL, and conducted homologous overexpression of PelA in *B. subtilis* 7-3-3 by increasing the copy of *pelA* expression cassette from *B. subtilis* 7-3-3 to further improve the production of alkaline PGL. The enzyme system of the overexpression crude enzyme was measured and the effect on degumming of ramie was studied.

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2. Materials and methods

2.1. Strains and plasmids

B. subtilis 7-3-3 was isolated from soil in Shandong University and preserved in the China Center for Type Culture Collection (NO: M200038). The *pelA* from *B. subtilis* 7-3-3 was cloned and propagated in *E. coli* DH5α by pMD18-T (Takara, Japan) and expressed in *B. subtilis* 7-3-3. The plasmid pNW33N (*Bacillus* Genetic Stock Center, BGSC, USA), which did not contain a promoter, a signal peptide sequence, and a terminator for protein expression, was used as a *B. subtilis–E. coli* shuttle vector for PGL expression.

2.2. Media recipe and fermentation conditions

2.2.1. Fermentation in shake flask

The fermentation inoculum of *B. subtilis* 7-3-3 was prepared by cultivating the cells at 37 °C and 200 rpm for 7 h in a 300 mL shake flask containing 50 mL seed culture medium (glucose, 10 g L^{-1}; peptone, 5 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 5 g L^{-1}; K_2HPO_4, 10 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 0.5 g L^{-1}; pH 8.0) using shaker QYC-2102C/KYC-1102C (Shanghai Fuma Laboratory Instrument Co. Ltd., China). Next, 10% (v/v) of the inoculum was inoculated into a 500 mL shake flask with 90 mL fermentation medium [wheat bran, 54 g L^{-1}; $(NH_4)_2SO_4$, 3 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 2 g L^{-1}; Na_2CO_3, 1 g L^{-1}; Tween-80, 1 g L^{-1}; pH 8.0], and then fermented at 34 °C and 200 rpm using shaker QYC-2102C/KYC-1102C.

LB media (Luria-Bertani broth, pH 7.0), which was used for the cultivation of *E. coli* and *B. subtilis*, contained 10 g L^{-1} peptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl.

2.2.2. Fed-batch fermentation in 7.5 fermentor

A total 10% (v/v) of the inoculum was inoculated into a 7.5 L fermentor (Bioflo® & Celligen® 310, New Brunswick Scientific, USA) with a 4 L initial fermentation media [wheat bran, 47.3 g L^{-1}; $(NH_4)_2SO_4$, 1.5 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 1.0 g L^{-1}; Na_2CO_3, 0.5 g L^{-1}; Tween-80, 0.5 g L^{-1}]. The pH value of the media was adjusted and maintained at 6.5 with ammonia and 30% phosphoric acid. The temperature was controlled at 34 °C, and the dissolved oxygen level was maintained at over 5% of air saturation by a cascaded control of agitation rate (350–700 rpm) and aeration rate (2–5 L min^{-1}) (Silent Air Compressor, Shanghai Dynamic Industry Co., Ltd., China). After 15 h, 1 L feeding media [wheat bran, 81.0 g L^{-1}; $(NH_4)_2SO_4$, 9.0 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 6.0 g L^{-1}; Na_2CO_3, 3.0 g L^{-1}; Tween-80, 3.0 g L^{-1}] was fed to the fermentor. The pH level was adjusted to 6.0 after feeding.

2.3. Purification of PelA

Crude enzyme preparations were taken after definite intervals and then centrifuged to remove microbial cells and undigested solid substrates. Thereafter, the supernatant was saturated with $(NH_4)_2SO_4$ to two cut-offs (0% to 40%, 40% to 80% saturation). The precipitates were dissolved in a minimum amount of Gly–NaOH buffer (containing 200 mM glycine, pH 8.5) and dialyzed against the same buffer. The sample was loaded onto a Q Fast Flow column (1.6 cm × 20 cm; GE Healthcare, USA), equilibrated with 20 mM phosphate buffer (pH 8.0). The column was washed with 20 mM phosphate buffer and then eluted with a linear gradient of 0 M to 1 M NaCl in the equilibration buffer. The PGL activity of the fractions was assayed.

The thermal stability of PelA was measured through the incubation of the purified enzyme at 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C for 8 h in pH 7.0 buffer, respectively. The PGL activity of the samples was determined at 45 °C. The pH stability of PelA was measured through similar steps with the exception of temperature, which was replaced by pH, and incubation at 4 °C. The pH gradient was 7.0, 8.0, 9.0, 9.4, 10.0, and 10.6.

2.4. Sequencing and modeling of pelA

The *pelA* gene was amplified from *B. subtilis* 7-3-3, cloned to pMD18-T and sequenced in BGI. The *pelA* gene structure was predicted based on a previously published *B. subtilis* PelA structure [6]. The 3-D structure of the PelA was modeled using the automated mode of the SWISS-MODEL.

2.5. Construction of expression plasmids and electroporation of B. subtilis 7-3-3

The *B. subtilis* 7-3-3 PGL gene with the promoter, signal peptide sequence, and terminator sequence was amplified by polymerase chain reaction (PCR) in 50 μL reaction mixture containing 30 ng DNA template (the genome of *B. subtilis* 7-3-3), 25 pmol of each primer, 100 μM dNTP, 1 mM $MgCl_2$, and 2.5 U Primer STAR DNA polymerase (Takara, Japan), using the following program: 94 °C, 45 s; 55 °C, 45 s; 72 °C, 1 min 20 s; 30 cycles. The primer pair (q-pelA1: 5′-GAAGCTAGGGCATAAAGCAAGG-3′ and q-pelA2: 5′-GCTTTACTGCTGACTGTTTTTCTG-3′) was designed based on *B. subtilis* SO113 *pelA* sequences from GenBank (Accession No. X74880) [4]. The primer sequence, q-pelA1, differed from the DNA sequence in *B. subtilis* SO113 *pelA* by one nucleotide. The PCR product was separated by electrophoresis and the 1.6 kb fragment was purified from gel using the TIANgel Midi Purification Kit [Tiangen Biotech (Beijing) Co., Ltd., China]. The purified fragment was ligated into pMD18-T with T_4 DAN ligase (Beijing TransGen Biotech Co., Ltd., China) and transformed into *E. coli* DH5α. The recombination plasmid was extracted and digested for 2 h at 37 °C with FastDigest® *Pst*I (Fermentas, Canada) and FastDigest® *Xba*I (Fermentas, Canada). The 1.6 kb fragment was purified from gel using the TIANgel Midi Purification Kit. The purified fragment was ligated into *Pst*I- and *Xba*I-digested pNW33N. The resulting plasmid, pNW33N-pelA, was transformed into *E. coli* DH5α.

The electroporation of *B. subtilis* 7-3-3 was carried out according to the previous transformation protocol of Xue et al. [14] with minor modifications.

2.6. Enzyme degumming of ramie

The process of enzyme degumming of ramie is as follows: 2 g ramie was bathed in 26 mL Gly–NaOH buffer (pH 8.5) with PGL activity of 320 U (equivalently 12.3 U mL^{-1}), and then incubated at 50 °C with 150 rpm for 4 h. After treatment, ramie was washed with water to cleanse the pectin and then dried to constant weight at 105 °C. The degumming rate was the ratio of ramie weight loss and pectin content of ramie, and the weight loss rate was the ratio of ramie weight loss and dry ramie weight. Pectin content of ramie was measured according to the following method: 1 g ramie was bathed in boiled 30 mL 0.5 M NaOH solution for 1 h. Thereafter, NaOH solution was renewed for boiling for 2 h and finally dried to constant weight at 105 °C. The percent of weight loss in dry weight of ramie corresponded to the pectin content of ramie.

2.7. Analysis

2.7.1. Assay of enzyme activities

For mannase, xylanase, carboxymethyl cellulase (CMCase), and filter paper activity (FPA), one unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of reducing sugar per min under the assay conditions. The enzyme activity was measured in Gly–NaOH buffer (pH 8.5).