Protein Expression and Purification 139 (2017) 29–35

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Secretory expression of β-mannanase from *Bacillus circulans* NT 6.7 in *Lactobacillus plantarum*

Kwankanit Intaratrakul [a, b], Sunee Nitisinprasert [a, b], Thu-Ha Nguyen [c], Dietmar Haltrich [c], Suttipun Keawsompong [a, b, *]

[a] Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand
[b] Center for Advanced Studies for Agriculture and Food, Kasetsart University Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand (CASAF, NRU-KU, Thailand)
[c] Food Biotechnology Laboratory, Department of Food Sciences and Technology, BOKU-University of Natural Resources and Life Sciences, Vienna, Austria

ARTICLE INFO

Article history:
Received 29 April 2017
Received in revised form
8 July 2017
Accepted 11 July 2017
Available online 13 July 2017

Keywords:
β-mannanase
Bacillus circulans NT 6.7
pSIP403
Lactobacillus plantarum WCFS1
Manno-oligosaccharides

ABSTRACT

The β-mannanase gene of *Bacillus circulans* NT 6.7 was successfully cloned in *Lactobacillus plantarum* WCFS1 using the pSIP403 expression vector and secreted to the supernatant rather than accumulated in the cells. The highest activity was achieved by controlling the pH at 6 during cultivation. Maximum mannanase activities detected in the supernatant and cell-free extract of 200 ml MRS broth were 8.2 and 0.86 U/ml, respectively. Enzyme activity in the supernatant increased to 27 U/ml by fermentation in a 5-L bioreactor with automatic pH control. The optimum temperature of recombinant β-mannanase was 50 °C and stable between 30 and 50 °C. The optimum pH was 6 with stability in the range 5–7. Enzyme activity slightly increased with Co^{2+} but was strongly inhibited by EDTA. The enzyme exhibited high specificity to galactomannan substrates. The main products of copra meal and locust bean gum hydrolysis were manno-oligosaccharides. Therefore, recombinant β-mannanase produced from a food grade host, *L. plantarum* WCFS1, showed potential for use in manno-oligosaccharides production and other food-related applications.

1. Introduction

β-mannanase (endo-1,4-β-D-mannanase) is an endohydrolase that catalyzes the random hydrolysis of the β-1,4-D mannopyranosyl linkage within the main chain of mannans and various mannan-based polysaccharides releasing short and long chain manno-oligosaccharides as the main reaction products. These enzymes show potential for use in various industries [1,2] including the production of manno-oligosaccharides as potential prebiotics [3] that can be used in food and feed.

In previous studies we described the isolation of the β-mannanase-producing strain *Bacillus circulans* NT 6.7 from soil collected from coconut factories in Thailand, and showed that its β-mannanase can efficiently hydrolyze copra meal, a mannan-rich by-product from coconut milk and coconut oil processing, into different types of manno-oligosaccharides (MOS). These were shown to promote growth of probiotic bacteria and inhibit pathogenic bacteria [4,5]. The β-mannanase gene from *B. circulans* NT 6.7 was cloned and successfully expressed in *Escherichia coli* BL21(DE3), it belongs to glycoside hydrolase family (GH) 26, and it was shown to be specific for galactomannan [6], indicating that the potential for MOS production from copra meal, an underutilized by-product of the coconut industry that is readily available in Southeast Asia.

A number of publications have shown that *E. coli* is an effective expression system for β-mannanase overproduction [6–10]. Typically, enzyme production is intracellular in *E. coli* in these instances and the enzyme accumulates in the cytoplasm; thus cell disruption is necessary. In addition, a disadvantage of recombinant protein production in *E. coli* is the possible formation of endotoxins, which are pyrogenic in humans and other mammals [10]. Therefore, *E. coli* is not always an ideal system for the production of food-relevant enzymes and a food-grade expression system could offer a number of advantages.

Most lactic acid bacteria (LAB) are food-grade microorganisms with the generally recognized as safe (GRAS) status, and therefore

* Corresponding author. Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.
E-mail address: fagisuk@ku.ac.th (S. Keawsompong).

http://dx.doi.org/10.1016/j.pep.2017.07.005
1046-5928/

they are highly attractive as potential food-grade cell factories [11–14]. One well-known expression system for LAB is the pheromone-inducible pSIP system. It was developed based on the quorum-sensing mechanism involved in the production of the class II bacteriocins sakacin A and sakacin P [15].

Lactobacillus plantarum has previously been used as a host for heterologous gene expression, and the usefulness of the *L. plantarum*-pSIP expression system has been pointed out by several authors [12,14,16–18]. Recently, the β-mannanase gene from *B. licheniformis* DSM13 was successfully overexpressed using this system [19]. This current study shows the secretory expression of the β-mannanase gene from *B. circulans* NT 6.7 in *L. plantarum* WCFS1 using the pSIP403 expression vector, and emphasizes the need of pH-controlled cultivations on enzyme production. In addition, analyses of the hydrolysis products from defatted copra meal and locust bean gum using this recombinant β-mannanase produced by the pSIP system were performed to study a possible application of this enzyme in the production of MOS prebiotics.

2. Material and methods

2.1. Bacterial strains, plasmid and medium

Recombinant *E. coli* BL21(DE3) harboring plasmid pET-21d(+)/*man6.7* was constructed as described by Piwpankaew et al. [6]. *E. coli* NEB5α (New England Biolabs, UK) and plasmid pJET1.2/blunt (Thermo Fisher Scientific, Austria) were used in the cloning experiments. *L. plantarum* WCFS1 and plasmid pSIP403 were used for the expression of the β-mannanase gene. *E. coli* was cultivated in Luria-Bertani (LB) medium containing 100 μg/ml of ampicillin at 37 °C with shaking at 200 rpm, whereas *L. plantarum* WCFS1 was grown in deMan, Rogosa and Sharpe (MRS) medium containing 5 μg/ml of erythromycin at 37 °C under anaerobic condition (resting cultures).

2.2. Construction of the pSIP403/man6.7 expression plasmid

The β-mannanase gene (*man6.7*) was amplified by PCR using the plasmid pET harboring the β-mannanase gene from *B. circulans* NT 6.7 as a template. Primer Fman/*Nco*I (CCGGCCATGGACACCGTTTATCCCGTC) and Rman/*Xho*I (CCGGCTCGAGTTATTCCGCGATCGG) were designed based on the sequence of the mannanase gene of *B. circulans* NT 6.7 deposited in the GenBank Database (GenBank accession no. JF724077). The PCR reaction contained 100 ng of DNA template, 10 pmol of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 1x buffer, and 1 U of Phusion High-Fidelity DNA polymerase (New England Biolabs, UK) in a total reaction volume of 25 μl. The amplifications were done as follows: initial denaturation at 98 °C for 3 min; 30 cycles of denaturation at 98 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were purified using illustra™ GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK), and subsequently cloned into the *Nco*I and *Xho*I sites of the pSIP403 expression vector [15]. The obtained plasmid pSIP403/*man6.7* was then transformed into *L. plantarum* WCFS1 by electroporation. Positive clones were checked by colony PCR and sequencing (Microsynth AG, Switzerland).

2.3. Expression of β-mannanase in L. plantarum WCFS1

To express the β-mannanase gene, overnight cultures of *L. plantarum* WCFS1 harboring the recombinant plasmid pSIP403/*man6.7* were diluted in 200 ml of fresh MRS medium containing 5 μg/ml of erythromycin to obtain an OD_{600nm} of ~0.1 and incubated at 37 °C. Then, cells were induced at an OD_{600nm} ~0.3 by adding the peptide pheromone IP-673 (IP) to a final concentration of 25 ng/ml [17]. The pH was controlled and maintained manually at pH 6 by adding 5 M NaOH during cultivations. Culture supernatant and cells were collected to measure the activity of extracellular and intracellular enzyme.

Scale-up cultivations for the production of recombinant β-mannanase were performed in a 5-L fermenter containing MRS medium, and IP was added as an inducer at an OD_{600nm} ~0.3. Cells were grown at 37 °C, and the pH was automatically controlled at 6 by adding 5 M NaOH. Culture supernatant and cells were collected at time intervals for measuring the enzyme activity.

2.4. Enzyme assay

The assay to determine mannanase activity was composed of 100 μl of 1% locust bean gum (LBG) in 50 mM potassium phosphate buffer, pH 6 and 100 μl of appropriately diluted enzyme. The reaction mixtures were incubated at 50 °C for 60 min. The amount of sugar released in the reaction was determined by the dinitrosalicylic acid (DNS) method using D-mannose as the standard [20]. One unit of β-mannanase activity is defined as the amount of enzyme that liberates 1 μmol of mannose equivalents per minute under assay conditions.

2.5. Gel electrophoresis

SDS-PAGE was performed using Mini PROTEAN®TGX™ Precast gels. Protein bands were visualized by staining with Biosafe™ Coomassie G-250, and the Precision Plus™ Protein Standard kit was used as a protein marker.

2.6. Effect of temperature on enzyme activity and stability

The optimum temperature of mannanase activity was determined with temperatures ranging from 30 to 80 °C under otherwise standard assay conditions. To estimate the thermal stability, recombinant β-mannanase was incubated at various temperatures (30–80 °C, pH 6) for 1 h, and the residual activity was measured under standard assay conditions.

2.7. Effect of pH on enzyme activity and stability

The optimum pH of β-mannanase activity was measured under standard assay condition over a pH range 3–10 using 50 mM sodium citrate buffer (pH 3–5), 50 mM potassium phosphate buffer (pH 6–8), and 50 mM glycine-NaOH buffer (pH 9–10). For the analysis of pH stability, the enzyme was incubated in these buffers at difference pH values at 50 °C for 1 h, and then the residual activity of each sample was then measured under standard assay condition.

2.8. Substrate specificity

The substrate specificity of β-mannanase was determined by measuring the enzyme activity after incubating the enzyme with 1% (w/v) of substrate dissolved or dispersed in 50 mM potassium phosphate buffer, pH 6 under standard assay conditions. Substrates used in this experiment included LBG, konjac glucomannan, copra meal (obtained from a coconut factory, Thailand), defatted copra meal (prepared by soxhlet extraction using hexane as an organic solvent), yeast cell wall (α-mannan), xylan from oat spelt, and carboxymethylcellulose (CMC). All commercial substrates were obtained from Megazyme or Sigma-Aldrich.