



Expression of mannanase gene from *Bacillus circulans* NT 6.7 in *Escherichia coli* and *Lactobacillus plantarum*

Yotthachai Piwpankaew^{1,3}, Supa Sakulsirirat², Sunee Nitisinprasert^{1,2,3}, Thu-Ha Nguyen⁴, Dietmar Haltrich⁴, Suttipun Keawsompong^{1,2,3*}

¹Interdisciplinary Program in Genetic Engineering, Graduate School

²Department of Biotechnology, Faculty of Agro-Industry,

³Center for Advanced Studies in Agriculture and Food, Institute for Advanced Studies, Kasetsart University, Bangkok, 10900, Thailand

⁴Food Biotechnology Laboratory, Department of Food Sciences and Technology, University of Natural Resources and Life Sciences, Vienna, 1180, Austria

*e-mail: fagisuk@ku.ac.th

Abstract

The mannanase gene of *B. circulans* NT 6.7 was cloned and expressed in *Escherichia coli* and food-grade *Lactobacillus plantarum* expression system. *B. circulans* NT 6.7 mannanase gene consisted of 1,083 nucleotides encoding 360 amino acid residues which belonging to glycosyl hydrolase family 26. In *E. coli* system, mannanase gene was cloned into pET21d and expressed in *E. coli* BL21* (DE3). Beta-mannanase activities in culture supernatant and cell were 37.14 and 515.40 u/ml, respectively. The secreted recombinant β -mannanase was 20 times higher than native enzyme at the same production scale and the enzyme activity of this recombinant β -mannanase was 30 times higher than the recombinant β -mannanase from *B. circulans* CGMCC 1416 and *B. circulans* CGMCC 1554 which were performed by the same expression system. So, this expression system was suitable for production of recombinant β -mannanase from *B. circulans* NT6.7. For food grade expression system, mannanase gene was cloned into pSIP403 and expressed in *L. plantarum* WCFS1 Δ alr. The recombinant β -mannanase was produced only in the cells with relatively low activity at 0.82 u/ml. This study showed the expression system to produce recombinant β -mannanase from *B. circulans* NT 6.7 which suitable for industrial applications and also presented the food grade recombinant β -mannanase production for food and feed application.

Keywords: β -mannanase, *Bacillus circulans* NT 6.7, *Escherichia coli*, *Lactobacillus plantarum*

Introduction

Beta-mannanase (Endo-1,4- β -D-mannanase) is an endohydrolase enzyme that catalyze the random hydrolysis of the β -1,4-D mannopyranosyl linkage in the main chain of mannan-based polysaccharides. This enzyme is produced by several living organisms and it has been widely used in the industrial application. *Bacillus circulans* NT 6.7 was isolated from soil of coconut industry from Nakornpathom province. Beta-mannanases from *B. circulans* NT 6.7 has high enzyme activity for locust bean gum (LBG), 0.306 unit/ml with appropriate optimum temperature and pH for industrial applications (Phothichitto et al. 2006). However, there is increasing interested in β -mannanase applications especially production of manno oligosaccharides (MOS) as prebiotic properties. *Escherichia coli* has been well established and developed for the recombinant protein production. *Lactobacillus plantarum*, food-grade expression systems, are considerable interested in producing of food-grade recombinant enzymes for food and feed application. So, this study focused on the high level expression and secretion of recombinant β -mannanase from *B. circulans* NT 6.7 using *E. coli*

BL21 expression system and food-grade recombinant β -mannanase in *L. plantarum* expression system.

Methodology

Construction of recombinant plasmids

The mannanase gene of *B. circulans* NT 6.7 was amplified from genomic DNA by using the specific primers, Man6.7F (GCGGCCATGGCTATGCTTAAAAAGTTAGCA) and Man6.7R (CCGGCTCGAGTTCCGCGATCGGCGT), that were designed based on the sequencing analysis. The amplification product was cloned into pET-21d and pSIP403(alr) then transformed into *E. coli* BL21* (DE3) and *L. plantarum* WCFS1 Δ alr, respectively. Positive clones were confirmed by colony-PCR and sequence analysis.

Expression of mannanase

For the expression of mannanase in *E. coli* BL21* (DE3), overnight culture of *E. coli* BL21* (DE3) containing the recombinant expression plasmid was inoculated into 100 ml of LB medium with 100 μ g/ml of ampicillin. The culture was incubated at 37°C with 200 rpm shaking until the OD600 reached 0.6 then induced with 1.0 mM IPTG. After IPTG induction, the cultivation was incubated at 18°C with 200 rpm shaking for 16 h. For the expression of mannanase in food grade system, *L. plantarum* WCFS1 Δ alr containing the recombinant mannanase gene was cultured in MRS medium until OD600 reached 2.0 then induced with 25 ng/ml peptide pheromone IP-673 (IP). After induction, the cultivation was incubated until OD600 reached about 7.0. The culture supernatant and cell pellet of both expression hosts were collected for enzyme activity study.

Enzyme assay and protein determination

The standard mannanase assay consisted of 100 μ l of 1% locust bean gum (LBG) in 50 mM potassium phosphate buffer pH6.0 and 100 μ l of enzyme solution. The reaction mixture was incubated at 50°C for 60 min. The amount of reducing sugar released was determined by the dinitrosalicylic acids (DNS) method using D-mannose as the standard. One unit of mannanase activity was defined as the amount of enzyme producing 1 μ mol of mannose per minute under the assay conditions. Protein concentration was determined by Bradford method with bovine serum albumin as the standard.

Protein electrophoresis and zymogram analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify the size of the recombinant β -mannanase using 15% (w/v) polyacrylamide gel. Protein bands were visualized by Coomassie brilliant blue staining. Zymogram of recombinant β -mannanase was performed by gel activity assay using 10% (w/v) polyacrylamide gel with locust bean gum as substrate. After electrophoresis, substrate gel was stained with Congo red solution, destained with 1M sodium chloride and background stained with 5% acetic acid. Enzyme activity on substrate gel was detected by clear zone against blue background.

Results

Cloning and sequence analysis of mannanase gene from *Bacillus circulans* NT 6.7

Mannanase gene of *B. circulans* NT 6.7 consist of a total 1,083 bp which encode 360 amino acid residues. It was submitted into GenBank of NCBI database with Accession No. JF724077. Nucleotide sequence of *B. circulans* NT6.7 mannanase gene showed the highest identity (99%) with β -mannanase gene from *B. amyloliquefaciens* strain CICC 23260 (GQ589479.1) and *B. subtilis* strain A33 (DQ269473.1). Amino acid sequence of *B. circulans* NT6.7 mannanase showed 99% identity with β -mannanase from *B. subtilis* (ABB91433.1) and this enzyme was classified into glycoside hydrolase family 26 (GH26) base on the sequence identity (Altschul et al. 1997). The tertiary structure of *B. circulans* NT 6.7 β -mannanase was constructed by using SWISS-MODEL (<http://swissmodel.expasy.org>) and showed the $(\beta/\alpha)_8$ TIM barrel folding type of the GH-A glycoside hydrolase family with the shallow-dish-shaped active center of β -mannanase (Figure 1). Twenty four amino acid residues (residue number 1-24) were predicted as the signal sequence of *B. circulans* NT 6.7 by using SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP>).

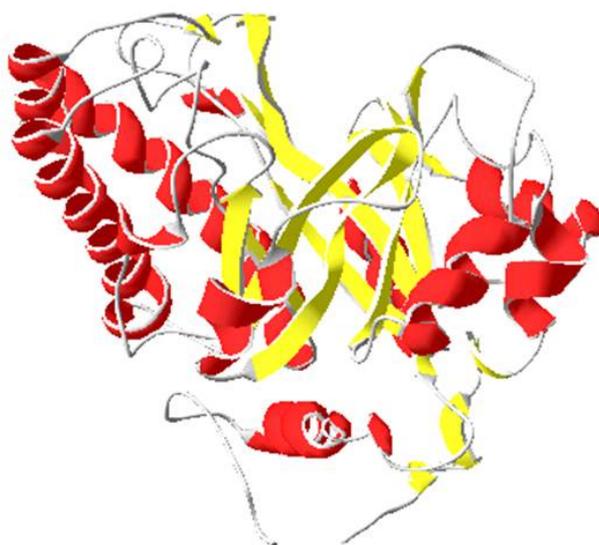


Figure 1: Tertiary structure of β -mannanase from *B. circulans* NT 6.7

Expression of mannanase gene from *Bacillus circulans* NT 6.7

E. coli BL21*(DE3) harboring pET21d with mannanase gene produced the recombinant β -mannanase and also secreted into the culture medium. Extracellular and intracellular β -mannanase activities were 37.14 and 515.40 u/ml, respectively. Both intracellular and extracellular β -mannanase had the same size of protein band and also showed the activity on substrate gel on zymogram (Figure 2). In *L. plantarum* WCFS1 Δalr system, recombinant β -mannanase was produced only in cell. The recombinant β -mannanase activity in cell lysate was only 0.82 u/ml. Because of a few amount of enzyme activity of recombinant β -mannanase from this expression system so there was no activity band appears on the substrate gel on zymogram.

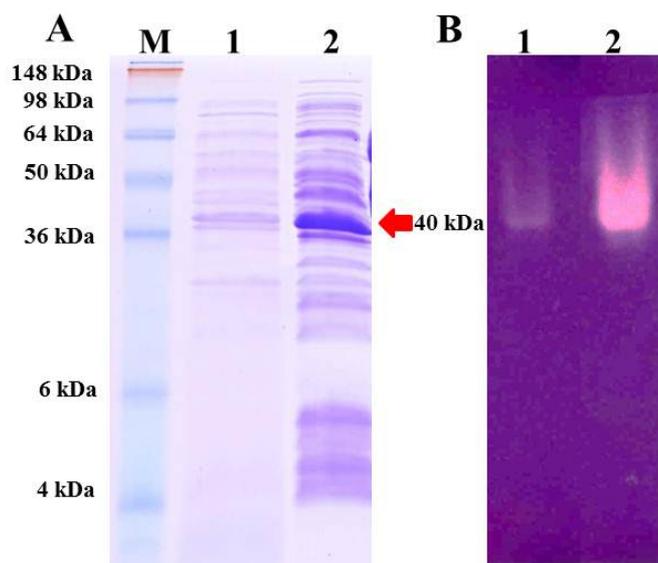


Figure 2: SDS-PAGE (A) and zymogram analysis (B) of recombinant β -mannanase

Discussion

Mannanase gene was cloned into pET21d expression vector and expressed in *E. coli* BL21* (DE3), a commercially standard and successful expression system. The full length mannanase gene was clone into the vector under T7 promoter without additional signal sequence. However, recombinant β -mannanase was secreted into the culture media by this system. This system can produce the efficient recombinant β -mannanase from *B. circulans* NT6.7 with efficient secretion and activity. Although, most of the recombinant β -mannanase was produced within the cell but the enzyme activity of secreted recombinant β -mannanase had almost 20 times higher than native enzyme at the same production scale. Furthermore, this also showed the efficient secretion when compared with the recombinant β -mannanase *B. circulans* CGMCC 1416 and *B. circulans* CGMCC 1554, recombinant that were produced from same expression system (Li et al. 2008). Although, *E. coli* expression systems can produce large amount of recombinant enzyme but the antibiotic resistance marker are considered for using in food-related applications. Thus, food-grade expression was developed for the production of recombinant proteins or enzymes. In this study, we used pSIP403 that contain the alanine racemase (*alr*) gene as an expression vector and *L. plantarum* WCFS1 Δalr , D-alanine auxotroph strain, as an expression host. Although, recombinant β -mannanase was extracellular produced in *E. coli* system but secretion of β -mannanase was not occurred in this system. β -mannanase activity was detected only in cell at the very low level when compared with the recombinant β -mannanase from *E. coli*. So, these results indicated that native signal sequence was not efficient in this system. The low level production of recombinant β -mannanase in this expression system was similar with the expression of recombinant chitinase from *B. licheniformis* DSM13 which was produced from the same expression system (Nguyen et al. 2012). This may be caused by several factors such as codon usage, gene of interest and unsuitable combination between gene and expression promoter. However, this is the first report of food grade recombinant β -mannanase production for food and feed applications.

Conclusion

E. coli BL21* (DE3) with pET21d was the efficient expression system for production of β -mannanase from *B. circulans* NT6.7 which have high enzyme activity and secretion. This study demonstrated the food grade β -mannanase production from *L. plantarum* expression system for food-related applications.

Acknowledgement

This research was funded by Center for Advanced Studies in Agriculture and Food, Institute for Advanced Studies, Kasetsart University, Thailand. It was also supported by Graduate school, Kasetsart University, Thailand.

References

Altschul S.F., Madden T. L., Schaffer A. A., Zhang J., Zhang Z., Miller W., Lipman D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389-3402.

Li Y., Yang P., Meng K., Wang Y., Luo H., Wu N., Fan Y., Yao B. (2008) Gene cloning, expression, and characterization of a novel β -mannanase from *Bacillus circulans* CGMCC 1416. *J. Microbiol. Biotechnol* 18(1):160–166.

Nguyen H.A., Nguyen T.H., Nguyen T.T., Peterbauer C.K., Mathiesen G., Haltrich D. (2012) Chitinase from *Bacillus licheniformis* DSM13: expression in *Lactobacillus plantarum* WCFS1 and biochemical characterization. *Protein Expr Purif* 81:166–174.

Phothichitto K., Nitisinprasert S., Keawsompong S. (2006) Isolation, screening and identification of mannanase producing microorganisms. *Kasetsart J (Nat. Sci.)* 40(Suppl.):26-38.