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

Methodology

Construction of recombinant plasmids

The mannanase gene of 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 µg/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 µl of 1% locust bean gum (LBG) in 50 mM potassium phosphate buffer pH6.0 and 100 µl of enzyme solution. The reaction mixture was incubated at 50°C for 60 min. The amount of reducing sugar released was determined by the dinitrosalicyclic acids (DNS) method using D-mannose as the standard. One unit of mannanase activity was defined as the amount of enzyme producing 1 µmol 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. subtillis* (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/SignalIP).

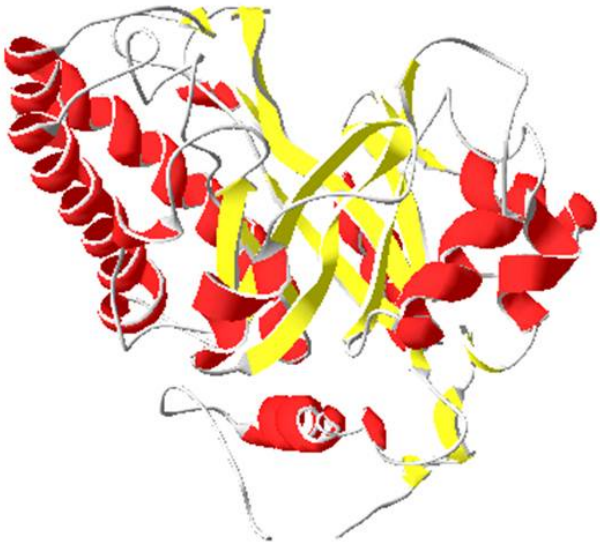

Figure 1: Tertiary structure of β-mannanase from *B. circulans* NT 6.7

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 Δ*alr* system, recombinant β-mannanse was produced only in cell. The recombiant 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.

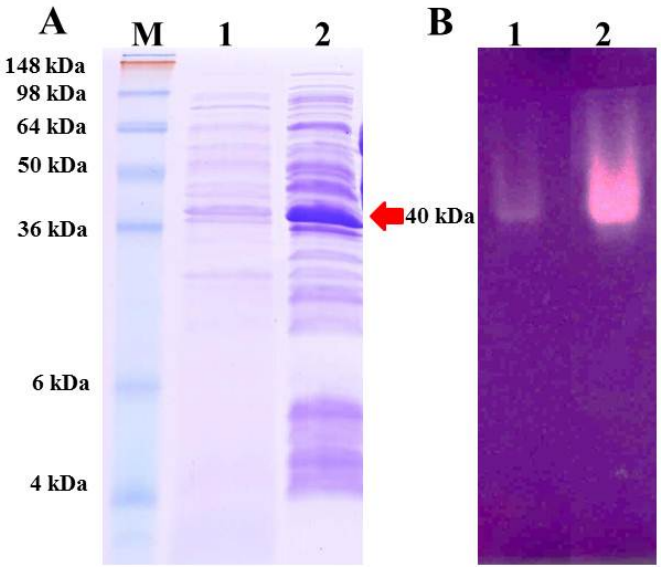

Figure 2: SDS-PAGE (A) and zymogram analysis (B) of recombinant β-mannanase

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. circuans* 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.

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