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THESIS

CLONING AND EXPRESSION OF MANNANASE FROM *BACILLUS CIRCULANS* NT 6.7

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Yotthachai Piwpankaew 2014: Cloning and Expression of Mannanase from *Bacillus circulans* NT 6.7. Doctor of Philosophy (Genetic Engineering), Major Field: Genetic Engineering, Interdisciplinary Graduate Program. Thesis Advisor: Assistant Professor Suttipun Keawsompong, Ph.D. 106 pages.

The full-length mannanase gene of *B. circulans* NT 6.7 including its native signal sequence was cloned and expressed in *Escerichia coli and Lactobacillus plantarum* expression systems. The *B. circulans* NT 6.7 mannanase gene consisted of 1,083 nucleotides encoding 360 amino acid residue long polypeptide which belong to glycosyl hydrolase family 26. In *E. coli* system, the gene was cloned into pET21d and expressed in *E. coli* BL21* (DE3). The recombinant β-mannanase was successfully produced and also secreted. β-Mannanase activities in the culture supernatant and crude cell extract were 37.1 and 515 U/ml, respectively. The mannanase gene was cloned into pSIP403 and expressed in *Lactobacillus plantarum* WCFS1 Δ*alr*. β-Mannanase activity was detected in cell, 0.82 u/ml. Therefore this *E. coli* expression system was very efficient for the secretory production of recombinant β-mannanase from *B. circulans* NT 6.7. The optimum temperature of recombinant β-mannanase activity was 50°C and the optimum pH was 6.0 with high stability at this condition. The enzyme was very specific for β-mannan substrates with a preference for galactomannan. The recombinant β-mannanase showed the random manner with required at least 4 mannose monomers for degradation. It hydrolyzed mannan substrates consisted of locust bean gum, konjac glucomannan and defatted copra meal into various mannooligosaccharides including mannohexaose, mannopentaose, mannotetraose, mannotriose and mannobiose, while mannose could not be detected. The recombinant β-mannanase from *B. circulans* NT 6.7 showed the good enzyme characteristic including optimum temperature and pH with high specificity that can be used for several applications.

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Student's signature Thesis Advisor's signature

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Yotthachai Piwpankaew

February, 2014

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INTRODUCTION

β-Mannanases (endo-1,4-β-D-mannanase) is endohydrolase that catalyze the random hydrolysis of the β-1,4-D mannopyranosyl linkage within the main chain of various mannan-based polysaccharides to yield manno-oligosaccharides products (Puls, 1997). β-Mannanase is very useful enzyme that has been used in several industrial applications including food, feed, pulp and paper industries. This enzyme can be used to improve the bleaching of pulp by facilitating the release of lignin from paper pulp leading to the reduction of chemical reagents (Suurnakki *et al.*, 1997; Buchert *et al.*, 1993). It can be used to reduce the viscosity of instant coffee and to clarify fruit juices and wines in food industry (Wong and Saddle, 1993). β-Mannanase also have been used as animal feed additive enzyme to increase the nutritional value of animal feed components (Lee *et al.*, 2003). Moreover, there is increasing interest in using β-mannanase to produce mannooligosaccharides (MOS) which have the prebiotic properties from natural mannan-based substrates (Gibson *et al.*, 2005; Rastall *et al.*, 2005; Biggs and Parsons, 2007; Smith *et al.*, 2010).

β-Mannanase have been isolated and characterized from different sources including bacteria, fungi, higher plants and animals (Puchar *et al.*, 2004; Dutta *et al.*, 1997; Araujo and Ward, 1990). However, microbial mannanases are wildly used in the industrial application. β-Mannanases was classified based on the amino acid sequence similarity into glycoside hydrolase (GH) families 5 and 26 and a few member of family 113 (Henrissat and Bairoch, 1993; Dhawan and Kaur, 2007). These enzymes from different organisms have different properties such as enzyme activity, optimal pH and optimal temperature. So, β-mannanases with high specific activity and remarkable enzymatic properties are required for the application.

Bacillus circulans NT 6.7 was isolated from soil of coconut factory located at Nakornpathom province, Thailand and β-mannanase from this organism was previously shown to have interesting properties for industrial applications including the high specific activity and stability. However, the production levels in wild type organism are not sufficient for the applications and the presence of several extracellular enzymes and proteins cause the difficulty for purification and specific applications. So, molecular cloning and expression which can produce large amounts of an individual enzyme in a suitable host strains is very interest for large scale production and applications

This research aims on cloning and expression of mannanase gene from *B. circulans* NT 6.7 in a suitable host which can produce the efficient recombinant β-mannanase. Moreover, the recombinant β-mannanase from the effective expression system was also characterized in this study.

OBJECTIVES

1. To clone the full-length mannanase gene of *Bacillus circulans* NT 6.7

2. To express the mannanase gene of *Bacillus circulans* NT 6.7

3. To characterize the recombinant β-mannanase from *Bacillus circulans* NT 6.7

LITERATURE REVIEW

1. Mannan

Hemicelluloses, the second most abundant polysaccharide in nature, are found in plant cell walls as linkers between lignin and cellulose constituents. Mannan-type polysaccharides are one of the major components of the hemicelluloses of hardwoods and softwoods and also in leguminous seeds, beans and some marine algea (Kuhad, 1997; Moreira and Filho, 2008). Mannan plays a major role as binding molecule that covalently linked with lignin and non-covalently linked with cellulose. This interaction is important in cellulose integrity (Puls and Schuseil, 1993). Mannan also plays a storage function in endosperm walls and vacuoles of seeds (Meier and Reid, 1982). In addition, it also has the function as a signaling molecule in plant growth and development (Liepman *et al.*, 2007).

Mannan polysaccharides consisted of β-1,4-linked backbone of mannose or a combination of glucose and mannose residues with can be substituted with side chains of α-1,6-linked galactose residues. Mannan can be classified into four types depending on their structures consisted of linear mannan, glucomannan, galactomannan, and galactoglucomanan (Moreira and Filho, 2008).

Linear mannan is homopolysaccharides composed of linear main chains of mannose residues. It is the major structural units in many plant seeds such as ivory nuts and green coffee beans (Aspinall, 1959).

Glucomannan is a main component of hemicellulose found in softwoods which has a heterogeneous backbone with randomly arranged of β-1,4-linked mannose and glucose residues as shown in Figure 1A. The ratios of mannose to glucose unit are 3:1 and 1.5-2:1 in softwood and hardwood glucomannan, respectively (Timell, 1967; Northcote, 1972; Popa and Spiridon, 1998; Hongshu *et al.*, 2002).

Galactomannan is a component in seeds of leguminous plants. It consisted of a homogeneous backbone of β-1,4-linked mannose residues with side chain of single α-1,6-linked galactose residue that attached along the backbone chain as shown in Figure 1B. There is the difference in the distribution of galactose residue along the mannose backbone in the galactomannan from different source (Bresolin *et al.*, 1997). The ratios of mannose to galactose unit of galactomannan from guar gum, tara gum and locust bean gum are 2:1, 3:1 and 4:1, respectively (Duffaud *et al.*, 1997; Sittikijyothin *et al.*, 2005). Copra meal is one of the natural sources that contain high amount of non-starch polysaccharides especially mannan polysaccharide. About 60% of total polysaccharide in copra meal is galactomannan (Balasubramaniam, 1976) with the ratio of mannose to galactose unit is 14:1 (Regalado *et al.*, 2000).

Galactoglucomannan, a predominant hemicellulose in wood of gymnosperms, is polysaccharides that contain β-1,4-linked mannose and glucose backbone with α-1,6-linked galactose residues attached to both mannose and glucose units as terminal branches (Aspinall *et al.*, 1962; Popa and Spiridon, 1998). The ratio of mannose, glucose, and galactose residues is 3:1:1 (Puls and Schuseil, 1993). Some mannose residues are partially substituted by O-acetyl groups, called acetylated galactoglucomannan (Popa and Spiridon, 1998).

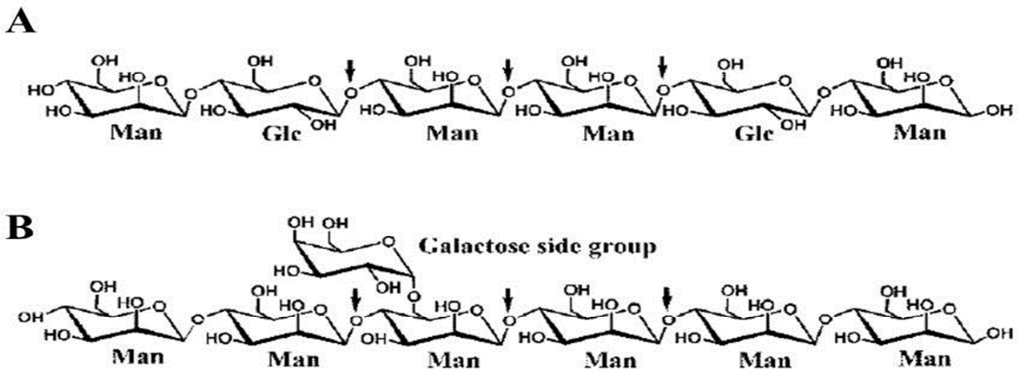

Figure 1 Structure of glucomannan (A) and galactomannan (B).

Source: Zhang *et al.* (2008)

2. Mannan-degrading enzymes

The mannan-degrading enzymes, a variety of hydrolytic enzymes involves in the degradation of mannan polysaccharides, are consisted of β-mannanase, β-mannosidase, β-glucosidase and other addition enzymes such as acetyl mannan esterase and α-galactosidase (Moreira and Filho, 2008). The overall mechanism of mannan-degrading enzymes is shown in Figure 2. β-Mannanase, endo acting enzyme, randomly hydrolyzes the β-1,4 linked internal linkage of the mannan backbone to produce mannooligosacharides and new chain ends. β-Mannosidase, exo acting enzyme, cleaves β-1,4 linked mannosides and releases mannose from the nonreducing ends of mannan and mannooligosacharides. β-Glucosidases, exo acting enzyme, hydrolyzes β-1,4-glucopyranose at the nonreducing ends of oligosaccharides that released from glucomannan and galactoglucomannan degradation. In addition, the side chain sugars that attached at various points on mannan are removed by α-galactosidase and acetyl mannan esterase. α-Galactosidase hydrolyzes α-1,6-linked D-galactopyranose at side chain of galactomannan and galactoglucomannan. Acetyl mannan esterase releases acetyl groups from galactoglucomannan.

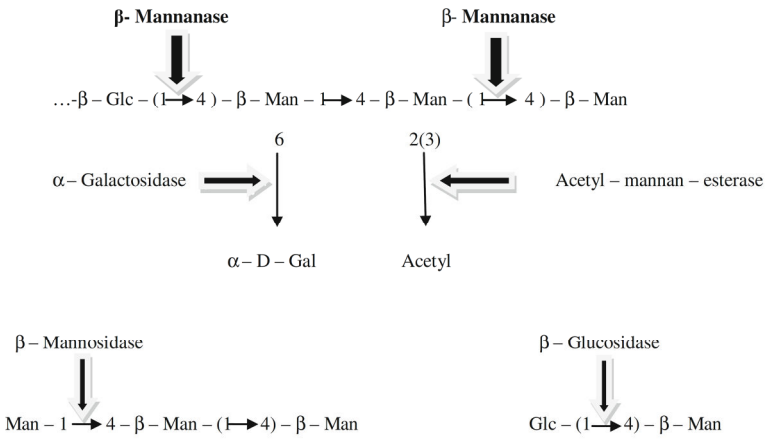

Figure 2 Mechanism of mannan-degrading enzymes in the galactoglucomannan degradation

Source: Puls and Schuseil (1993)

However, mannan degradation by these enzymes is affected by the degree and pattern of substitution of glucose and glucose residues in backbone of these polysaccharides (Van Zyl *et al.*, 2010). In addition, pattern of distribution of O-acetyl group in glucomannan also affect the hydrolysis by the enzymes.

2.1 β-Mannanase

β-Mannanase or endo-1,4-β-D-mannanases (EC 3.2.1.78) is the endo hydrolase enzyme that catalyze the random hydrolysis of internal β-1,4-mannosidic linkages in backbone chain of mannan polysaccharides, producing various size of mannooligosacharides (Puls, 1997). β-Mannanase requires at least 4 sugar residues of backbone chain for enzyme binding and efficient hydrolysis (Davies *et al.*, 1997). β-Mannanase hydrolyzes mannooligosaccharides up to a degree of polymerization (DP) of 4 (Chauhan *et al.*, 2012).

β-Mannanase is classified into glycoside hydrolases (GH) base on the sequence similarity. **Glycoside hydrolase** is a group of enzymes that hydrolyze the glycosidic bond between molecules in polysaccharides. Based on the amino acid sequence similarity, glycoside hydrolases are classified into 131 families. This classification is available on The Carbohydrate-Active Enzymes database (CAZy). Moreover, each glycoside hydrolase families can be classified into clan base on the catalytic domains and the protein structure. The different glycoside hydrolase families have been found to have different folds but, some enzymes from different families have related folds. Based on these criterias, β-mannanase is classified into glycoside hydrolase family 5, 26 and a few members in 113 (Dhawan and Kaur, 2007; Zhang *et al.*, 2008). Both of these families are classified in to clan A which have a typical structure of $(\beta/\alpha)_8$-barrel fold (TIM barrel fold). The crystal structure of β-mannanases from both of GH family 5 and 26 showed that glutamic acid is conserved catalytic modules (nucleophiles and acid/base) that are positioned on the C-terminal of β4 and β7 strands, respectively. In addition, β-mannanase also has an open cleft with at least 4 subsites on the active site (Hogg *et al.*, 2003; Zhang *et al.*, 2008; Chauhan *et al.*, 2012).

Glycosyl hydrolases consist of 2 hydrolysis mechanisms, retaining and inverting, leading to either overall retention or inversion of the anomeric configuration at the hydrolysis site (Henrissat *et al.*, 1995). β-Mannanase hydrolyzes the mannan substrates by retaining mechanism by double displacement reaction (Figure 3). For the retaining mechanism, 2 carboxylic acid residues on the active site of enzyme involve in the hydrolysis as nucleophiles and acid/base catalytic module. In the double displacement reaction, β-mannanase enzyme attacks by a nucleophilic carboxylate on the anomeric carbon with concomitant releases of the aglycon, resulting in a covalent intermediate. Then, the covelent intermediate is attacked by a nucleophilic water lead to releasing of the glycoside from the enzyme (Chauhan *et al.*, 2012).

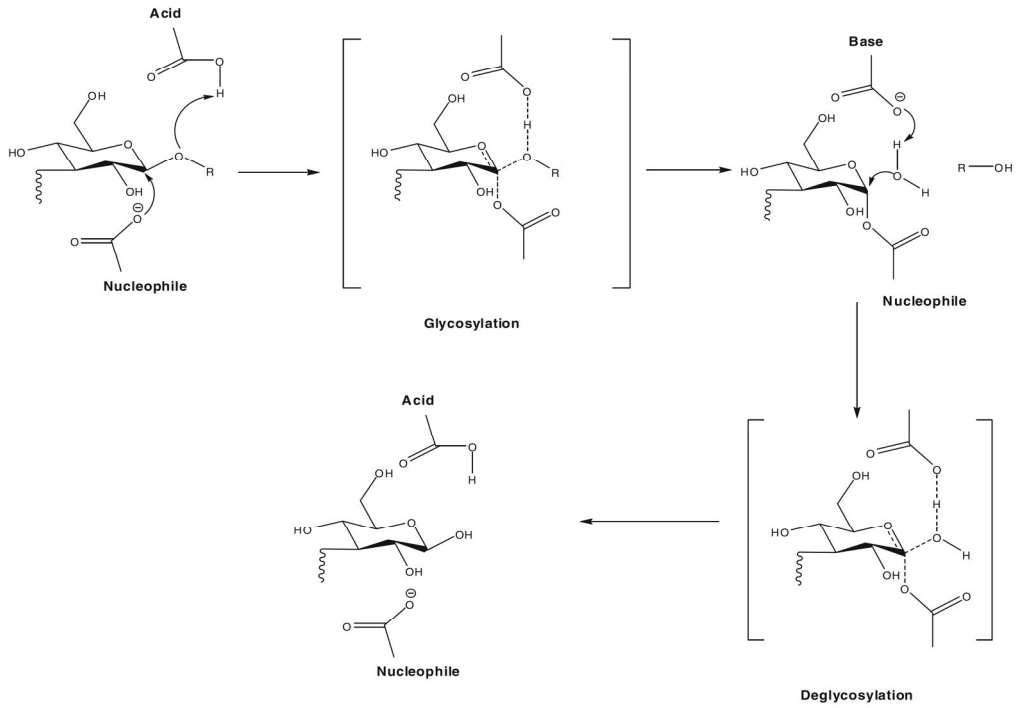

Figure 3 The retaining mechanism of β-mannanase

Source: Chauhan *et al.* (2012)

Although, β-mannanases have the similar structure including active site, but there are the different in the presence and position of catalytic carbohydrate binding modules (CBMs) that may contain in the structure (Ximens *et al.*, 2005). CBMs is use for enhance β-mannanase activity for degrading of cellulose-conjugated mannan polysaccharide (Benech *et al.*, 2007). β-mannanases from GH family 5 contain CBMs that bind crystalline polysaccharides. For example, β-mannanase from *Cellulomonas fimi*, GH family 5, has a mannan-binding module that binds in a reversible way to soluble mannan, cellulose, chitin, and xylan (Stoll *et al.*, 2000). Moreover, overexpression of mannanase gene that contains CBMs in *Escherichia coli* showed the activity of enzyme for several cellulose substrates (Ximens *et al.*, 2005). But, β-mannanases from GH family 26 lack of CBMs so there no enzyme activity in crystalline polysaccharides substrate (Hogg *et al*., 2003). So, this refers to the different substrate target of β-mannanase in GH family 5 and 26 in natural.

β-Mannanse enzymes have been isolated and characterized from several different sources such as bacteria, fungi, plants and animals. Although the β-mannanases are widely distributed in microorganisms, higher plants, and animals but the microbial mannanases are regarded to be useful for their applications. Numerous bacterial and fungal β-mannanases have been identified and characterized from various strains in 20 different genuses. Among bacteria, most of β-mannanases producing strains are gram positive bacteria such as *Bacillus* species and *Clostridia* species (Dhawan and Kaur, 2007). However, some gram negative bacterial strains have been reported to produce β-mannanases such as *Klebiella* specie (Titapoka *et al.*, 2008) and *Vibrio* specie (Tamamura *et al.*, 1995). The most of β-mannanases producing strains among fungi are in the genus *Aspergillus* while *Penicillium* species and *Trichoderma* species have also been reported (Chauhan *et al.*, 2012). Moreover, some actinomycetes such as *Streptomyces* specie and Cellulomonas species have been reported to be β-mannanases producing strains (Stoll *et al.*, 2000; Bhoria *et al.*, 2009).

Most of microbial β-mannanases are extracellular enzyme and the production of β-mannanase by bacteria and fungi is the inducible with have the mannan polysaccharides as induce (Moreira and Filho, 2008; Chauhan *et al.*, 2012).

Many nutritional and physiochemical factors involve and influence in the β-mannanase production such as carbon and nitrogen sources, inorganic salts, temperature, pH, time and dissolved oxygen concentration (Moreira and Filho, 2008; Chauhan *et al.*, 2012). There are the differences in the incubation time and optimum temperature and pH for microbial β-mannanase production. For the incubation time, it ranges from 24 h to 96 h in bacteria and 3 days to 11 days in fungi. The optimum temperature for β-mannanase production is in the mesophilic range that corresponds with temperature for microbial growth. For the optimum pH, neutral to alkaline pH is the best condition for bacterial growth and production and acidic pH for fungi (Chauhan *et al.*, 2012). However, bacterial β-mannanases are more thermostable than fungal β-mannanases that important for industrial application such as β-mannanases from *Bacillus* species have the advantages of high activity and convenient isolation and thus have been used in research and industry (Araujo and Ward, 1990).

2.1.1 β-Mannanase from *Bacillus circulans* NT 6.7

The use of microbial β-mannanases in the industrial application significantly increases because of their functions and properties. So, the new β-mannanases production strains with high enzyme activity and proper properties have been isolated and characterized.

In 2006, Photichitto *et al.* isolated β-mannanase producing strains from soil sample of coconut factory from Nakornpathom province, Thailand consisted of 19 bacterial strains and 4 fungal strains. The result showed that β-mannanase from *B. circulans* NT 6.7 had the high enzyme activity with optimum temperature and pH at 50°C and 6.0-9.0, respectively. It also showed the broad inhibition to pathogens, *Salmonella* serovar Enteritidis S003 and *Escherichia coli* E010, and could promote growth of probiotic *Lactobacillus reuteri* AC5. These results indicated that β-mannanase from *B. circulans* NT 6.7 had interesting properties and could be applied in prebiotic production (Photichitto *et al*., 2006)

β-Mannanase from *B. circulans* NT 6.7 was also produced, purified and characterized (Pangsri, 2014). β-Mannanase was produced in 5-litre fermenter with defatted copra meal as carbon source. The highest β-mannanase activity in this production was 27.66 u/ml. Purified β-mannanase consisted of 2 forms which had the same characteristics such as optimum temperature and pH, stability and substrate specificity. In addition, these enzymes can hydrolyzed various mannan substrates into mannooligosaccharides including copra meal. Mannooligosaccharides from copra meal hydrolysis by the purified β-mannanase from *B. circulans* NT 6.7 can promoted probiotic bacteria and inhibited pathogenic bacteria.

From these results indicated the efficiency of β-mannanase from *B. circulans* NT 6.7 that can applied in several applications especially in the mannooligosaccharides production from the natural sources such as copra meal.

3. Application of β-mannanase

β-Mannanase has been used in several industrial application because of its board substrate specificity.

3.1 Pulp and paper industry

β-Mannanase can be used in the enzymatic bleaching of softwood pulps to digest the mannan component without affecting the cellulose component. The use of β-mannanase with other enzymes is the alternative method that can equally facilitate lignin removal in pulp bleaching and give results comparable to alkaline pretreatment without the environmental pollution problems (Dhawan and Kaur, 2007).

3.2 Detergent industry

Alkaline β-mannanase with stable in detergents has been used in laundry segments as stain removal boosters. Mannans are generally used as thickening agents in several products such as hair-gel, shampoo, conditioner and toothpaste. The stains

containing mannan are difficult to remove so this enzyme can cleave into smaller carbohydrate fragments that can reduce the stain cleaning process and remove during the washing. Moreover, β-mannanase can be formulated as sanitization products, contact lens cleanser and hard surface cleansers (Chauhan *et al.*, 2012).

3.3 Food industry

β-Mannanase has been used in the viscosity reduction of coffee bean extracts by hydrolyzed the mannan component in the coffee extract (Chauhan *et al.*, 2012). It has been used in the maceration of fruit and vegetable materials and clarification of fruit juices (Moreira and Filho, 2008). In addition, β-mannanase can be used in enzymatic oil extraction of coconut. The enzymatic process can eliminate the problems of alfatoxin contamination and oxidative rancidity of products (Chauhan *et al.*, 2012).

3.4 Feed industry

β-Mannanase can be used to improve the nutritional value of animal feed especially poultry. Mannan polysaccharides commonly found in feed ingredients such as soyabean meal, guar meal, copra meal, palm kernel meal and sesame meal. All these meals have some common properties such as high fiber content, low palatability, lack of several essential amino acids and high viscosity coupled with several anti-nutritional factors such as mannan, galactomannan, xylan and arabinoxylan that limit the utilization in the animal intestine (Chauhan *et al.*, 2012). Moreover, they have been found to be highly deleterious to animal performance, severely compromising weight gain and feed conversion as well as glucose and water absorption (Dhawan and Kaur, 2007). Therefore, incorporation of β-mannanase in feed can help to cleave mannan and release of nutrients results in increased villus height in duodenum and jejunum that leads to increase in surface area and adsorption and decreased intestinal viscosity. So, it can improve both the weight gain and feed conversion efficiency (Adibmoradi and Mehri, 2007). Hemicell supplied by

ChemGen, USA is a fermentation product of *B. lentus* containing high amount of β mannanases that degrade mannan in feed (Daskiran *et al.*, 2004).

3.5 Mannooligosaccharide production

Mannooligosaccharide (MOS) is non-oligosaccharides digestibles (NODs) that can be produced from mannan polysaccharides. MOS is widely used in nutrition as a natural additive which can improve gastrointestinal health and also overall health by supporting the microflora in the digestive system.

MOS affects bacterial attachment in the intestinal tract lead to the reduction in the prevalence and concentration of different strains of *Salmonella*, as well as *E. coli*. Moreover, it effects on promoting beneficial bacteria, such as *Lactobacillus and Bifidobacteria* (Spring *et al.*, 2000). MOS help to limit of the pathogenic microorganisms bacteria by blocking the colonization on the intestinal mucosa lead so they cannot physically reach or adhere to the intestinal cells and are eliminated from body.

Large surface area is a key for optimal digestive function of small intestine so the surface of the small intestine should be covered with long healthy villi. Several studies of MOS in poultry have looked at the intestinal structure and discovered longer villi and a more shallow crypt (Yang *et al.*, 2008; Baurhoo *et al.*, 2009). From these studies showed the results that MOS could increase the energy of digestion and production of digestive enzymes such as alkaline phosphatase, maltase and leucine aminopeptidase. Moreover, MOS could increase the goblet cells, mucus producing cells so the villi and intestinal surface could be more protected.

3.6 Pharmaceutical applications

Mannose has been used as a component of medicine because of its properties such as fast dissolving and structure forming (Chauhan *et al.*, 2012). Mannose also has been used as a remedy for urinary tract infection (Van Zyl *et al.*,

2010). There is a significantly increase in using of this sugar so β-mannanase and other enzymes can be used for the economical production of mannose from low cost mannan substrates such as palm kernel cake and copra meal. Guar gum has the positive effects on some physiological functions like reducing plasma cholesterol and body fat without reducing protein utilization and increase fecal excretion volume (Takeno *et al*., 1990). Therefore, a partially hydrolyzed guar gum (PHGG) with β-mannanase is used in beverage form for treatment of several diseases such as irritable bowel syndrome (IBS). PHGG can increase stool weight and decrease colon transit time by providing non-digestible bulk, retaining water, and serving as a substrate for microbial growth in the colon (Parisi *et al.*, 2002). In addition, PHGG supplemented with oral rehydration solution is also used for the treatment of acute diarrhea in children by providing short chain fatty acids in large intestine and maintaining the balance of salt and water (Alam *et al.*, 2000).

3.7 Other applications

β-Mannanase can use as slime control agent in water purification system, waste water treatment and cooling water treatment system. It has been used in the enzymatic hydrolysis of galactomannan to enhance the flow of oil and gas in drilling operation in the oil and gas industries. This enzyme is also used for the pretreatment of biomass in the bioethanol production (Chauhan *et al.*, 2012).

4. The expression systems

Mannanase gene from various microorganisms has been isolated, cloned and expressed in various heterologous hosts (Chauhan *et al.*, 2012). Most of bacterial β-mannanases have been expressed in *E. coli* and some studies also expressed in other hosts such as *Bacillus, Brevibacillus and Pichia* species. For fungal β-mannanase, *Pichia* and *Aspergillus* species have been used as expression hosts.

4.1 *Escherichia coli* expression system

E. coli is one of most successful expression system which has many advantages for the recombinant protein production. This is a low cost and easy operation system which also gives high yield of protein product and the purification is simple. Numerous genes form bacteria, eubacteria and archaea have been successfully expressed in *E. coli* using different expression vectors. Moreover, eukaryotic proteins that do not need the posttranslational modification have been produced from this system.

There are many different vectors and host strains that were developed. In 1986, Studier *et al.* developed the standard protocol for in *E. coli* by using RNA polymerase gene of T7 bacteriophage. RNA polymerase gene was incorporated into *E. coli* genome which could control the transcription of exogenous DNA. The inserted RNA polymerase gene was under the control of the lac repressor (Studier and Moffatt, 1986). This system was further developed to become very famous system known as pET system. The pET vectors, derived from pBR322 vector, are medium copy-number plasmids (15-20 copies) (Novagen®, 2003). In pET vectors, gene of interest is cloned under the control of T7 bacteriophage transcription and translation signals. Gene expression is induced by providing a source of T7 RNA polymerase in the genetically modified *E. coli* host. The *E. coli* was modified by incorporate the T7 RNA polymerase gene, lac promoter and lac operator into genome such as *E. coli* BL21* (DE3). Under the control of lac operator in host genome, the expression of gene of interested can be induced by lactose or isopropyl β-D-thiogalactoside (IPTG). Nowadays, more than 40 types of pET vectors and 15 different hosts have developed to use in the different purposes of gene expression (Yamabhai *et al.*, 2011).

The pET21 series vector is designed for expression of target genes that already carry their own prokaryotic ribosome binding site and AUG start codon. It carry an N-terminal T7•Tag® sequence and C-terminal His•Tag® sequence. This vector contains ampicillin resistance gene as a selectable marker. The sequence of this

plasmid is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region and other unique sites are shown in Figure 4 (Novagen®, 2003).

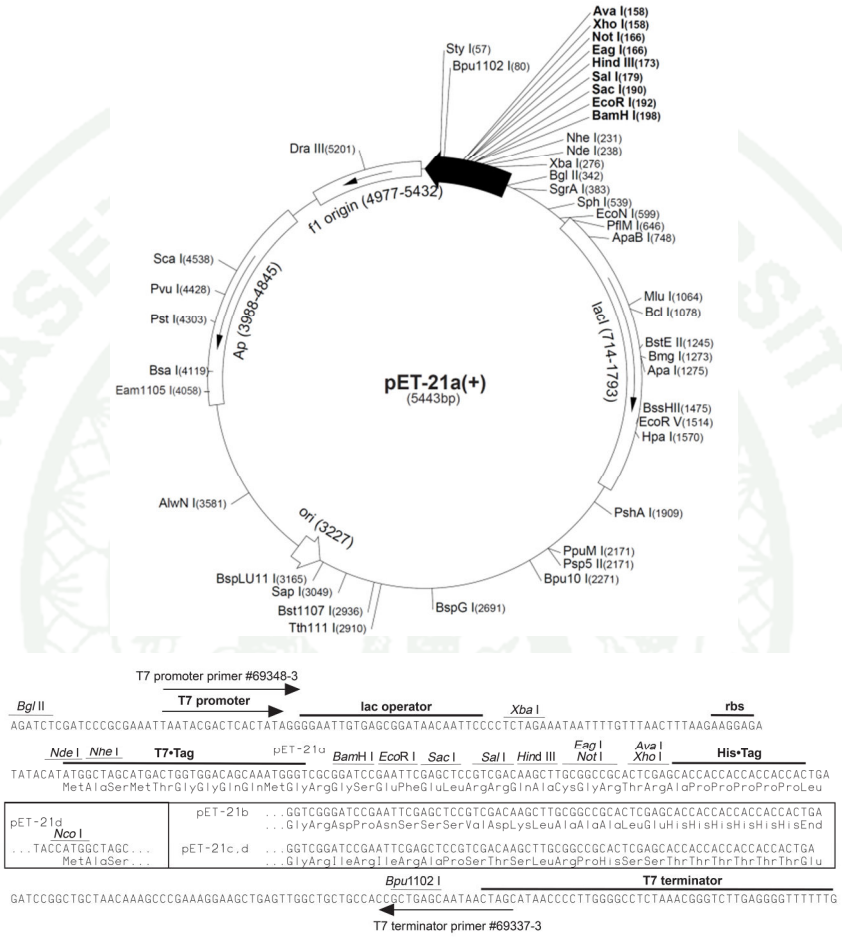

Figure 4 Circular map of pET21a-d(+) vector.

Source: Novagen®, 2003

E. coli BL21* (DE3) is a strain that is designed for applications that require high-level expression of non-toxic recombinant proteins from low-copy number, T7 promoter based expression system. Genotype of this strain is F- *ompT hsdS*B (rB-mB-) *gal dcm rne131* (DE3). It carries a mutated *rne* gene (*rne131*) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA,

resulting in an increase in mRNA stability. It not contains the *lon* protease and also deficient in the outer membrane protease, OmpT. The lack of these proteases reduces the degradation of heterologous proteins expressed in this strain. This strain contains the DE3 lysogen that carries the gene for T7 RNA polymerase under control of the *lac*UV5 promoter. IPTG is required to induce expression of the T7 RNA polymerase (Invitrogen™, 2010).

4.2 Food grade expression system

Although *E. coli* is the effective prokaryotic expression system but the antibiotic resistance markers are considered for using in food-related applications. Thus, food-grade expression systems such as lactic acid bacteria (LAB) including lactobacilli and lactococci were developed for the production of recombinant proteins or enzymes.

For food grade production of recombinant proteins, the food grade selection markers were used instead of the antibiotic resistance markers. Food grade selection markers can be classified as the dominant markers that provide the new ability to the host strains by the complementation of genes that lack or have been deleted which are necessary for growth under certain conditions (Nguyen *et al.*, 2011). Bacteriocin resistance (Takala and Saris, 2002), heat-shock resistance (El Demerdash *et al.*, 2003) and sugar utilization (Boucher *et al.*, 2002) are the ability that can be used as food grade selection markers. Although, these complementation base selection markers on do not require the supplements in the cultivation medium but the expression hosts require the specific modifications.

LAB consists of a very heterogeneous group of gram positive bacteria that has the similar metabolic pathways. LAB has the generally recognized as safe (GRAS) status which is generally harmless to human beings and has been used in food and feed industries in a wide variety of processes and products (Peterbauer *et al.*, 2011). From these properties, LAB was developed to be the alternative, safe and food grade production hosts for the recombinant protein production in the food related

application. Based on the quorum sensing mechanisms involved in the regulation of bacteriocin production in LAB, 2 effective inducible expression systems were developed consisted of nisin controlled gene expression system that derived from *L. lactis* and sakasin base expression system that derived from genes involved in the sakacin A and P production of *L. sakei* (Nguyen *et al.*, 2011)

4.2.1 Nisin controlled gene expression system

Nisin controlled gene expression or NICE is the system that was developed for recombinant protein production in LAB. In this system is controlled by nisin, the antimicrobial peptide produced by LAB. Nisin is the food grade compound and is used for the induction of gene expression by regulating a protein cassette. For the NICE system, nisin bind to the membrane-bound histidine protein kinase (NisK). The phosphate group from activated NisK is transferred to the intracellular response regulator (NisR). Then, the activated NisR induces the nisin operon at the nisin A promoter (PnisA) lead to controlling of the expression of the genes of interest (Maischberger *et al.*, 2010). In this system, nisin is the effective inducer that completely activates and regulates the PnisA promotor at nanogram levels. This expression system has been used to produce both of homologous and heterologous proteins and proteins can be produced in the intracellular, extracellular and also cell wall of host (Maischberger *et al.*, 2010).

The NICE system was used to produce the food related recombinant enzyme in *L. lactis* host such as peptidase (Wegmann *et al.*, 1999 and Hickey *et al.*, 2004) and β-galactosidase (Maischberger *et al.*, 2010).

4.2.2 Sakasin base expression system

This system was also developed based on the the secretion of peptide pheromones with no or little bacteriocin activity via the quorum-sensing mechanisms of bacteriocins production regulation. In this regulation system, the peptides pheromone activates a two-component regulatory system consisted of the

membrane-bound histidine kinase and the intracellular response regulator. Then, the activated intracellular response regulator that was activated by activated histidine kinase induces the promoters of the operons involved in bacteriocin production (Halbmayr *et al.*, 2008). From these mechanisms, efficient gene expression and high level recombinant protein production were developed for expression in LAB such as *L. sakei* and *L plantarum* (Sorvig *et al.*, 2003; Mathiesen, *et al.*, 2004; Sorvig *et al.*, 2005).

pSIP expression vectors are the vectors of the expression system for *L. sakei* and *L. plantarum* that were developed based on promoters and regulatory genes involved in the production of the class II bacteriocins, sakacin A (s*ap* gene cluster) or sakacin P (*spp* gene cluster). For these vectors, gene of interest is controlled by a strong inducible bacteriocin promoter and gene expression is induced by peptide pheromone, the external inducer (Halbmayr *et al.*, 2008). The pSIP expression system was used to produce efficient recombinant enzymes such as β-galactosidase (Halbmayr *et al.*, 2008; Nguyen *et al.*, 2012) and also food grade β-galactosidase (Nguyen *et al.*, 2011).

4.3 Heterologous production of β-mannanase

Cloning and expression provides the possibility to express individual enzyme in host strain. Various protein expression systems have been explored for the expression of heterologous proteins. The efficient cloning and expression system can produce the enzymes with less purification steps than enzyme from original source which have benefit for the application. Moreover, strong expression system can produce the enzyme with high activity. In addition to the recombinant enzyme overproduction, gene cloning and expression also have the benefits for protein structure determination and protein engineering for alteration of enzyme properties and functions.

High production level of enzymes is required for the industrial applications. Heterologous expression for high level production of β-mannanase was

increased in recent year. The β-mannanase activity was high as 500 and 3,795 u/ml in the recombinant production from *Biospora* sp. MEY-1 in *P. pastoris* (Luo *et al.*, 2009) and *Bacillus* sp. N16-5 in *Kluyveromyces cicerisporous* (Pan *et al*., 2011), respectively. The specific activity of recombinant β-mannanases was increased in the expression of gene from *B. circulans* CGMCC 1554 (Yang *et al.*, 2009) and *B. circulans* CGMCC 1416 (Li *et al.*, 2008) in *E. coli* BL21. In addition to the high level production, structural analysis and protein engineering of β-mannanases were study from the recombinant proteins from *E. coli* expression. Catalytic residues of β-mannanase from *A. sulphureus* were study and mutant β-mannanase showed the development of enzyme stability (Chen *et al*., 2008).

For *B. circulans* NT 6.7, mannanase gene was cloned and sequenced. The results showed the partial sequence of *B. circulans* NT 6.7 mannanase gene consisted of 771 bp (Sakulsirirat, 2008). This partial sequence was also cloned into into pHT43 and expressed in *E. coli* DH5α and the results showed that the highest relative β-mannanases activity was 0.304 unit/mg (Haemin, 2008). These results lead to the study of fullength *B. circulans* NT 6.7 mannanase gene and the heterologous production for their applications.

5. Homology modeling

Although the development of techniques in molecular biology that allow rapid identification, isolation, and sequencing of genes and proteins but it is still a time-consuming task to obtain the three-dimensional structures of these proteins from X-ray diffraction or NMR. One method that can be applied to generate reasonable models of protein structures is homology modeling.

Homology modeling is construction of atomic-resolution model of the target protein from the amino acid sequence and determination three-dimensional structure of a related homologous protein. Homology modeling relies on the identification of one or more known protein structures likely to resemble the structure of the query

sequence, and on the production of an alignment that maps residues in the query sequence to residues in the template sequence (Krieger *et al.*, 2003).

Homology modeling procedure consists of 4 steps: template selection, target-template alignment, model construction, and model assessment. Homologous proteins are identified and determined from their sequence similarity with one another and the unknown by alignment of the amino acid sequences. For the aligment, structurally conserved and structurally variable regions are identified and generate coordinates for core (structurally conserved) residues of the unknown structure from those of the known structure. Then conformations for the loops (structurally variable) in the unknown structure are generated and build the side-chain conformations. Finally, the unknown structure is refined and evaluated.

The purpose of protein modeling is to predict a structure from its sequence with an accuracy that is comparable to the best results achieved experimentally. This would allow users to safely use rapidly generated *in silico* protein models in all the contexts where today only experimental structures provide a solid basis: structure-based drug design, analysis of protein function, interactions, antigenic behavior, and rational design of proteins with increased stability or novel functions. In addition, protein modeling is the only way to obtain structural information if experimental techniques fail. Many proteins are simply too large for NMR analysis and cannot be crystallized for X-ray diffraction.

MATERIALS AND METHODS

1. Cloning and sequencing of mannanase gene of *Bacillus circulans* NT 6.7

1.1 Chromosomal DNA extraction

Chromosomal DNA of *B. circulans* NT 6.7 was extracted from cell pellet by using illustra™ bacteria genomicPrep Mini Spin Kit (GE Healthcare, UK) according to the manufacture's instruction. *B. circulans* NT 6.7 was cultured in Luria-Bertani (LB) broth and incubated in 200 rpm shaking incubator at 37°C for 16 h. Bacterial culture was added into a 1.5 ml microcentrifuge tube then centrifuged at 13,000 rpm for 1 min at room temperature. After discarding the supernatant, cell pellet was resuspended in 40 µl of lysozyme buffer and 10 µl of 10 mg/ml lysozyme. The sample mixture was completely mixed and incubated at room temperature for 10 min. Then, 10 µl of 20 mg/ml Proteinase K was added and incubated at 55°C for 15 min. After enzymes digestion, cell was continuously digested with 500 µl of Lysis solution 2 and incubated at room temperature for 10 min. Then, the sample mixture was applied into the illustra bacteria mini column and centrifuged at 13,000 rpm at 4°C for 1 min. The flow-through was discarded and column was placed back in the same collection tube. The column was washed with 500 µl of Lysis solution 2 and centrifuged at 13,000 rpm at 4°C for 1 min. Then DNA in the column was washed with 500 µl of Wash buffer and centrifuged at 13,000 rpm at 4°C for 1 min. After washing, column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the column by adding 50 µl of pre-heat Elution buffer. The column was equilibrated for 1 minute at room temperature and centrifuged at 13,000 rpm at 4°C for 1 min. Chromosomal DNA was stored at -20°C until used.

1.2 Mannanase gene amplification

Chromosomal DNA of *B. circulans* NT 6.7 was used as template for polymerase chain reaction (PCR) amplification. Fullenegth mannanase gene of *B. circulans* NT 6.7 was amplified with the primers ManFw1 and ManRv1 that were

designed based on the sequence of *Bacillus* mannanase gene in GH26 family from NCBI database (Table 1). The PCR reaction mixture consisted of 5 µl of 10X DreamTaq™ buffer, 5 µl 10 mM of dNTP mix (2.5 mM each), 4 µl of 10 pmol/µl primers mix (ManFW1 and ManRV1), 0.5 µl of DreamTaq™ DNA polymerase, 0.5 µl of DNA template and the final volume of reaction mixture was adjusted to 50 µl with water. The PCR reaction cycle consisted of initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min and the final extension at 72°C for 5 min. PCR amplification product was visualized by 1% agarose gel electrophoresis with ethidium bromide staining and cloned into the pGEM-T Easy vector for sequencing.

The PCR product was purified from agarose gel by using The E.Z.N.A.® Gel Extraction Kit (Biotek, USA) according to the manufacture's instruction. The expected positive DNA band was excised and determined the volume of gel. Then, gel was dissolved in the equal volume of Binding buffer at 60°C until gel completely dissolve. The mixture was applied to HiBind® extraction column and centrifuged at 13,000 rpm at 4°C for 1 min. The column was washed with 300 µl Binding buffer and twice with 700 µl of SPW buffer. After washing, column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the column by adding 50 µl of Elution buffer. The column was equilibrated for 1 min at room temperature and centrifuged at 13,000 rpm at 4°C for 1 min. The purified PCR product was stored at -20°C until used.

1.3 Construction of cloning plasmid

The purified PCR product was cloned into pGEM-T Easy vector (Promega, USA). Ligation reaction mixture consisted of 7 µl of purified PCR product, 1 µl of pGEM-T easy vector, 1 µl of 10X ligation buffer and 1 µl of T4 DNA ligase. The reaction mixture was incubated overnight at 4°C. Then, ligation products were transformed into *E. coli* DH5α using heat shock method. For transformation process, 5 µl of ligation products were added into 100 µl of ice-thawed *E. coli* DH5α competent cells and incubated on ice for 30 min. The competent cells were heat

shocked at 42°C for 30 s then incubated on ice for 3 min. After heat shock process, 1 ml of SOC medium was added into cells and incubated with shaking at 37°C for 1 h. Cells were centrifuged at 13,000 rpm at 4°C for 1 min and discarded 1 ml of supernatant. Cell pellet was resuspended and 50 µl of cell suspension was spreaded on LB/100 µg/ml ampicillin/IPTG/X-Gal/ agar plates and incubated overnight at 37°C.

The positive clones were firstly screened by blue-white colony selection on agar plates and the recombinant plasmids were examined the presence of mannanase gene by colony PCR. White colonies were used as template with ManFw1 and ManRv1 primers.

1.4 Recombianant plasmid extraction

The recombinant plasmid with mannanase gene was extracted by using QiAprep® Spin Miniprep Kit (QIAGEN, USA) according to the manufacturer's instructions. A single positive colony was inoculated into 5 ml of LB media with 100 µg/ml ampicillin and incubated in shaking incubator at 37°C for 16 h. Cell pellet was resuspended in 250 µl of P1 buffer. Then, 250 µl of P2 buffer was added into cell suspension and mixed by inverting. Homogenized cells was incubated for 2 min at room temperature and mixed with 350 µl of N3 buffer. After centrifugation at 13,000 rpm at 4°C for 10 min, aqueous phase of sample was transferred to QIAprep spin column and centrifuged at 13,000 rpm at 4°C for 1 min. The flow-through was discarded and QIAprep spin column was placed back in the same collection tube. Then, the column was washed with 500 µl of PB and 750 µl of PE buffer. DNA will be eluted from the spin column by adding 50 µl of EB buffer. The purified recombinant plasmids with mannanase gene were sequenced.

1.5 Mannanase gene sequencing analysis

The nucleotide sequencing of mannanase gene from *B. circulans* NT 6.7 was performed by 1st BASE Laboratory, Malaysia. The mananase gene sequencing results were compared with nucleotide from GenBank database using BLAST

analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Signal sequence was predicted by using SignalP4.0 analysis (http://www.cbs.dtu.dk/services/SignalIP). The tertiary structural of *B. circulans* NT 6.7 β-mannanase was predicted by using SWISS-MODEL homology modeling (http://swissmodel.expasy.org). The binding of β-mannanase with mannooligosaccharides was predicted from the tertiary structure model by using SwissDock molecular docking (http://www.swissdock.ch). The molecular weight of recombinant β-mannanase was calculated by using Compute pI/Mw tool of ExPASy (http://web.expasy.org/compute_pi/).

2. Expression of mannanase gene in *Escherichia coli* BL21* (DE3)

2.1 Mannanase gene amplification

After sequencing analysis, mannanase gene was re-amplified from chromosomal DNA of the *B. circulans* NT 6.7 using specific primers, ManFW2 and ManRV2 (Table 1), that were designed from nucleotide sequence of *B. circulans* NT 6.7 mannanase gene. ManFw2 and ManRv2 contain *Xho*I and *Nco*I recognition site, respectively. The PCR reaction mixture consisted of 12.5 µl of Phusion master mix (5X Phusion HF buffer with $MgCl_2$, 10 mM of dNTP mix (2.5 mM each) and 1 U of Phusion DNA polymerase), 4 µl of 10 pmol/µl primers mix (ManFW2 and ManRV2), 1.0 µl of DNA template and the final volume of reaction mixture was adjusted to 25 µl with water. The PCR reaction cycle consisted of initial denaturation at 98°C for 3 min followed by 30 cycles of 98°C for 30 s, 60°C for 30 s and 72°C for 45 s and the final extension at 72°C for 5 min. PCR amplification products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining.

The PCR product were purified from agarose gel by using illustra™ GFT™ PCR DNA and gel band purification kit (GE Healthcare, Germany) according to the manufacture's instruction. The expected DNA band was excised from the gel and determined the volume of gel. Then, gel was dissolved in the equal volume of Capture buffer type 3 at 60°C until gel completely dissolved. The mixture was applied to MicroSpin™ column and incubated at room temperature for 1 min then centrifuged

at 13,000 rpm at 4°C for 1 min. The column was washed with 500 µl Wash buffer type 1. After washing, column was transferred into a clean 1.5 ml microcentrifuge tube. DNA was eluted from the column by adding 30 µl of Elution buffer type 6. The column was equilibrated for 1 min at room temperature and centrifuged at 13,000 rpm at 4°C for 1 minute. The purified PCR product was stored at -20°C until used.

2.2 Construction of expression plasmid

For the expression of mannanase gene in *E. coli* BL21* (DE3), pET21d (Novagen, Germany) was used as expression vector. The purified mannanase gene and pET21d vector were digested with *Xho*I and *Nco*I restriction enzyme (Fermentas, USA). The reaction mixture consisted of 30 µl of each PCR product and pET21d vector, 20 µl of 10X Tango buffer, 1 µl of 10 U/µl *Xho*I restriction enzyme, 1 µl of 10 U/µl *Nco*I restriction enzyme and the final volume of reaction mixture was adjusted to 100 µl with water. The reaction mixture was incubated overnight at 37°C. The double digestion of both mannanase gene and pET21d vector were purified by using illustra^{TM} GFX^{TM} PCR DNA and gel band purification kit (GE Healthcare, Germany) Mannanase gene was ligated into *Xho*I-*Nco*I digestion site of pET21d. Ligation reaction consisted of 5 µl of *Xho*I-*Nco*I cut mannanase gene, 1 µl of *Xho*I-*Nco*I cut pET21d, 1 µl of 10X T4 DNA ligase buffer, 1 µl of T4 DNA ligase (Promega, USA) and the final volume of reaction mixture was adjusted to 10 µl with water. Ligation reaction was incubated overnight at 4°C. Then, ligation products were transformed into *E. coli* BL21* (DE3) using heat shock transformation method. Transformants were cultured on LB agar with 100 µg/ml ampicillin and incubated overnight at 37°C. The positive clones with mannanase gene of *B. circulans* NT 6.7 were determined by colony PCR with ManFw2 and ManRv2 primers.

2.3 Expression of mannanase gene

For the expression of mannanase gene 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 1.0 then induced with IPTG to a final concentration of 0.1, 0.5 and 1.0 mM. After IPTG induction, the cultivation was incubated at 18°C with 150 rpm shaking for 16, 18 and 20 h. Both of culture supernatant and cell were collected. Cell was harvested by centrifugation at 8,000 rpm at 4°C for 30 min. The cell pellet was washed twice with 50 mM potassium phosphate buffer pH 6.0 then resuspended in the same buffer. Cell was disrupted 3 times by glass bead stirring with 5,000 rpm for 30 seconds. Cell-free extracts were collected by centrifugation at 13,000 rpm for 30 min and used for subsequent analysis.

3. Expression of mannanase gene in *Lactobacillus plantarum* WCFS1 Δ*alr*

3.1 Construction of expression plasmid

For the expression of mannanase gene in *L. plantarum* WCFS1 Δ*alr*, pSIP403 was used as expression vector. The purified mannanase gene and pSIP403 vector were digested with *Xho*I and *Nco*I restriction enzyme (Fermentas, USA) and mannanase gene was ligated into *Xho*I-*Nco*I digestion site of pSIP403. Ligation products were transformed into *L. plantarum* WCFS1 Δ*alr* using electroporation with 1.5 kV of voltage, 25 μF of capacitance and 400 Ω of resistance. Transformants were cultured on MRS agar and incubated at 37°C for 24-36 hours. The positive clones with mannanase gene of *B. circulans* NT 6.7 were determined by colony PCR with ManFw2 and ManRv2 primers.

3.2. Expression of mannanase gene

For the expression of mannanase gene in this system, *L. plantarum* WCFS1 Δ*alr* containing the recombinant mannanase gene was cultured in MRS medium and induced with peptide pheromone IP-673, 19 amino acid residues with the sequence MAGNSSNFIHKIKQIFTHR (Brurberg *et al.*, 1997 and Nguyen *et al.*, 2011). The expression conditions of recombinant mannanase were optimized including of initiated cell concentrations, concentrations of inducer and incubation

periods. The culture was incubated at 37°C until the OD600 reached 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 then induced with peptide pheromone IP-673 to a final concentration of 10-100 ng/ml. After induction, the cultivation was incubated until OD600 reached about 7.0. Both of culture supernatant and cell were harvested by centrifugation at 8,000 rpm at 4°C for 30 min. The cell pellet was washed twice with 50 mM potassium phosphate buffer pH 6.0 then resuspended in the same buffer. Cell was disrupted 3 times by glass bead stirring with 5,000 rpm for 30 seconds. Cell-free extracts were collected by centrifugation at 13,000 rpm for 30 min and used for subsequent analysis.

Table 1 Primers for amplification of mannanase gene from *Bacillus circulans* NT 6.7

Primer name	Sequence	Restriction site
ManFw1	ATGCTTAAAAAGTTAGCAGTCTGYCT	-
ManRv1	TTATTCCGCGATCGGCGTCAA	-
ManFw2	GCGGCCATGGCTATGCTTAAAAAGTTAGCA	*Xho*I
ManRv2	CCGGCTCGAGTTCCGCGATCGGCGT	*Nco*I

4. Plasmid stability

4.1 Plasmid stability in the recombinant enzyme production

E. coli BL21* (DE3) harboring pET21d with mannanase gene from *B. circulans* NT 6.7 was cultivated in 10 ml of LB medium with 100 µg/ml of ampicillin at 37°C with 200 rpm shaking and expressed with the selected condition. After 16 h of induction, the culture was inoculated into fresh medium, cultivated and expressed. It was sub-cultivated and expressed for 10 passages. The amount of bacterial cells in each passage was measured by total plate count method. Both of culture supernatant and cell were collected at every passage for measuring the enzyme activity using standard assay and also for plasmid extraction from cell.

4.2 Plasmid stability in the storage condition

E. coli BL21* (DE3) harboring pET21d with mannanase gene from *B. circulans* NT 6.7 was cultivated in 10 ml of LB medium with 100 µg/ml of ampicillin at 37°C with 200 rpm shaking. At these conditions, about 12 generations of growth passed in 6 h. Bacterial culture was diluted into fresh medium every 6 h for a total cultivation time of 84 h (7 transfers). The culture in each passage was induced and expressed with the selected condition after 6 h of cultivation and collection 16 h of induction. The amount of bacterial cells in each passage was measured by total plate count method. Both of culture supernatant and cell were collected at every passage for measuring the enzyme activity using standard assay and also for plasmid extraction from cell.

4.3 Plasmid copy number measurement by real-time PCR

Recombinant plasmids were extracted by using QiAprep® Spin Miniprep Kit (QIAGEN, USA) according to the manufacturer's instructions. The plasmid copy number of each passage was measured by using LightCycler® 480 (Roche, Germany). The reaction mixture consisted of 10 µl of 2X SYBR Green I master mix (Roche, Germany), 0.8 µl of 5 pmol/µl primers mix (ManFW2 and ManRV2), 2 µl of plasmid DNA and the final volume of reaction mixture was adjusted to 20 µl with water. The amplification cycle consisted of initial denaturation at 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 45 s and the final extension at 72°C for 5-18 s. The plasmid copy number in each passage was measured according to the manufacturer's instructions with the serial dilution of recombinant plasmid as the standard.

5. Determination of β-mannanase activity

The standard β-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 (Miller, 1959).

One unit (U) of mannanase activity was defined as the amount of enzyme producing 1 μmol of mannose per minute under the assay conditions.

6. Determination of protein concentration

Protein concentration was determined by Bradford method using Protein Assay Reagent (BioRad, Austria) with bovine serum albumin as the standard.

7. Protein localization and protein electrophoresis

For localization of recombinant β-mannanase within cell, periplasmic fraction and cell lysate fraction were separated following the study of Songsiriritthigul *et al.*, 2010. For extraction of periplasmic content, cell was resuspended in spheroplast buffer. After incubation for 5 min on ice, cell was collected by centrifugation at 8,000 rpm for 15 min and re-suspended in water with 1 mM $MgCl_2$. The supernatant was collected by centrifugation at 8,000 rpm for 15 min as the periplasmic fraction. Then, cell was washed once with lysis buffer, resuspended with lysis buffer and sonicated on ice for 2 min. After centrifugation at 8,000 rpm for 15 min, the supernatant was collected as the cell lysate fraction. Protein in each fraction was analyzed by protein electrophoresis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify the size of the recombinant β-mannanase using 15% (w/v) polyacrylamide in separating gel and 4% (w/v) polyacrylamide in stacking gel with SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, USA) as a protein standard. Protein bands were visualized by Coomassie brilliant blue staining.

8. Zymogram analysis

Zymogram of recombinant β-mannanase was performed by gel activity assay using LBG as substrate (Haemin, 2008). Both of extracellular and intracellular recombinant β-mannanases were run on 15% (w/v) native polyacrylamide gel. After electrophoresis, native gel was placed on substrate gel and incubated at 50°C for 1 h. Then, the 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 as clear zone against blue background.

9. Characterization of the recombinant β-mannanase enzymes

9.1 Effect of temperature on recombinant β-mannanase activity

The optimum temperature of the recombinant β-mannanase was determined using the standard assay with 1% LBG in 50 mM potassium phosphate buffer pH 6.0 as substrate at the temperature range of 30-60°C.

The temperature stability of the recombinant β-mannanase was determined by incubating the enzyme in 50 mM potassium phosphate buffer pH 6.0 at various temperatures. The enzyme samples were withdrawn and measured the residual enzyme activity using standard assay condition at certain intervals.

9.2 Effect of pH on recombinant β-mannanase activity

The optimum pH of recombinant β-mannanase was determined by the standard assay with 1% LBG in the pH range of 4.0-11.0 as substrate using 50 mM citrate buffer pH 3.0-5.0, 50 mM potassium phosphate buffer pH 6.0-8.0 and glycine-NaOH buffer pH 9.0-10.0.

The pH stability was determined by measuring the remaining enzyme activity under the standard condition after the enzyme was incubated at various pH and 50°C for 1 h.

9.3 Effect of various metal ions and chemical on recombinant β-mannanase activity

The effect of metal ions on β-mannanase activity was determined by measuring the activity of enzyme in the presence of various metal ions consisted of EDTA, Li^{+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+} and Co^{+} with final concentration of 1 mM using standard assay condition.

9.4 Substrate specificity

The substrate specificity of recombinant β-mannanase was examined by measuring the enzyme activity on 1% of various substrates in 50 mM potassium phosphate buffer pH 6.0 consisted of LBG, konjac glucomannan, ivory nut mannan, copra meal, defatted copra meal, guar gum, alpha-mannan (yeast cell wall), xylan from oat spelt, carboxymethylcellulose (CMC) and Avicel.

9.5 Analysis of hydrolysis products of recombinant β-mannanase

For the hydrolysis study, LBG, konjac glucomannan, defatted copra meal and mannooligosaccharides consisted of mannohexaose, mannopentaose, mannotetraose, mannotriose and mannobiose were used as substrates. One percent of substrates were incubated with 10 units of recombinant β-mannanase for 0, 0.5, 1, 3, 6, 12 and 24 h. The reactions were stopped by boiling for 5 min. The hydrolysis products were analyzed by high pressure liquid chromatography (HPLC) using Water2414 (Water, USA) and Aminex-HPX42C column (Bio-rad, USA) with water as mobile phase and the standard mannooligosaccharides consisted of mannohexaose (M6), mannopentaose (M5), mannotetraose (M4), mannotriose (M3), mannobiose (M2), and mannose (M1).

RESULTS AND DISCUSSION

1. Sequence analysis of mannanase gene of *Bacillus circulans* NT 6.7

Mannanase gene of *B. circulans* NT 6.7 was obtained by using PCR cloning. Partial sequencing and structural analysis showed the results that *B. circulans* NT 6.7 mannanase gene was classified into GH26 family (Sakulsirirat, 2008). So, specific primers for fullength mannanase gene of *B. circulans* NT 6.7 (ManFw1 and ManRv1) were designed based on the multiple sequences alignment result of *Bacillus* mannanase gene in GH26 family. PCR amplification product of mannannase gene of *B. circulans* NT 6.7 with this primer pair was predicted to have about 1,100 bp in length (Figure 5).

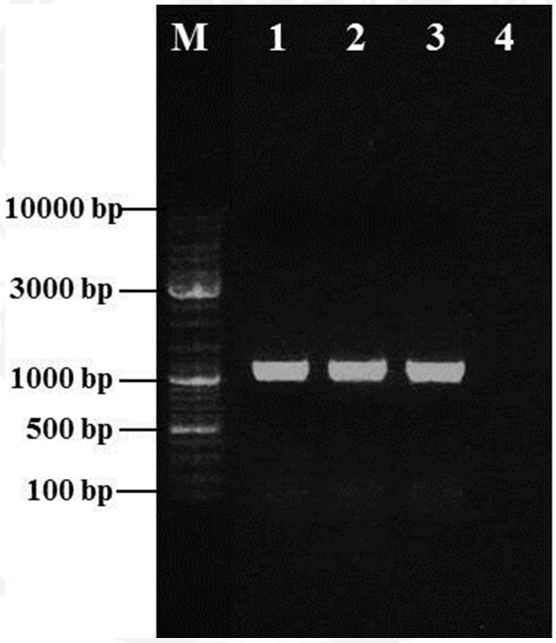

Figure 5 PCR amplification of mannanase gene of *B. circulans* NT 6.7

Lane M : GeneRuler^{TM} 1 kb DNA (Fermentas, USA)

Lane 1-3 : PCR product of chromosomal DNA of *B. circulans* NT 6.7 as template

Lane 4 : Negative control

After purification from agarose gel, PCR product was cloned into pGEM-T-Easy vector at multiple cloning sites adjacent to T7 promoter. Positive clones were screened by blue white colony selection from the property of vector and confirmed by colony PCR. PCR product was sequenced by using specific primers for T7 promoter and SP6 promoter of pGEM-T-Easy. Nucleotide sequence sequencing showed the result that mannanase gene of *B. circulans* NT 6.7 consist of a total 1083 bp which encode 360 amino acid residues (Figure 6). Mannanase gene sequence of *B. circulans* NT 6.7 was submitted into GenBank of NCBI database with Accession No. JF724077.

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>gi|345846856|gb|JF724077.1| Bacillus circulans strain NT6.7
beta-mannanase gene, complete cds

 1     ATG CTT AAA AAG TTA GCA GTC TGT CTG TCT ATC GTT TTA CTA CTC TTA GGA GCC GCC AGT CCG ATA TCG GCT   72
 1     Met Leu Lys Lys Leu Ala Val Cys Leu Ser Ile Val Leu Leu Leu Gly Ala Ala Ser Pro Ile Ser Ala   24

 73    CAC ACC GTT TAT CCC GTC AAC CCA AAT GCC CAG CAG ACG ACA AAA GAT ATC ATG AAC TGG CTG GCC CAC CTG   144
 25    His Thr Val Tyr Pro Val Asn Pro Asn Ala Gln Gln Thr Thr Lys Asp Ile Met Asn Trp Leu Ala His Leu   48

 145   CCC AAC CGT TCA GAA AAC AGG GTC ATG TCC GGA GCG TTC GGC GGG TAC AGC GAT GTC ACT TTT TCA ATG ACA   216
 49    Pro Asn Arg Ser Glu Asn Arg Val Met Ser Gly Ala Phe Gly Gly Tyr Ser Asp Val Thr Phe Ser Met Thr   72

 217   GAG GAA AAC CGC TTG AAA AAC ACG ACG GGA CAG TCT CCC GCC ATC TAC GGC TGT GAC TAT GGG AGA GGG TGG   288
 73    Glu Glu Asn Arg Leu Lys Asn Thr Thr Gly Gln Ser Pro Ala Ile Tyr Gly Cys Asp Tyr Gly Arg Gly Trp   96

 289   CTG GAA ACA GCG GAG ATC ACC GAT ACT ATC GAT TAC AGC TGC AAC AGC AGC TTA ATC TCA TAC TGG AAA AGC   360
 97    Leu Glu Thr Ala Glu Ile Thr Asp Thr Ile Asp Tyr Ser Cys Asn Ser Ser Leu Ile Ser Tyr Trp Lys Ser   120

 361   GGC GGC CTC CCT CAG GTC AGC CTG CAT CTC GCA AAT CCG GCC TTT CCA TCC GGA AAC TAT AAA ACG GCC ATC   432
 121   Gly Gly Leu Pro Gln Val Ser Leu His Leu Ala Asn Pro Ala Phe Pro Ser Gly Asn Tyr Lys Thr Ala Ile   144

 433   TCA AAC AGC CAG TAC AAA AAC ATC CTT GAC CCT TCA ACT GTG GAA GGA AAA CGG CTT GAG GCG CTG CTC AGC   504
 147   Ser Asn Ser Gln Tyr Lys Asn Ile Leu Asp Pro Ser Thr Val Glu Gly Lys Arg Leu Glu Ala Leu Leu Ser   168

 505   AAA ATT GCC GAC GGC CTT ACT CAG CTG AAA AAT CAA GGC GTC ACC GTT CTG TTC AGA CCG CTG CAT GAA ATG   576
 169   Lys Ile Ala Asp Gly Leu Thr Gln Leu Lys Asn Gln Gly Val Thr Val Leu Phe Arg Pro Leu His Glu Met   192

 577   AAC GGC GAG TGG TTC TGG TGG GGG CTG ACA GGC TAC AAC CAA AAA GAC AAT GAG AGA ATC TCG CTG TAC AAA   648
 193   Asn Gly Glu Trp Phe Trp Trp Gly Leu Thr Gly Tyr Asn Gln Lys Asp Asn Glu Arg Ile Ser Leu Tyr Lys   216

 649   GAG CTT TAC AAG AAG ATA TAC CGC TAT ATG ACA GAG ACA AGA GGA TTG GAT AAC CTT TTG TGG GTG TAT TCG   720
 217   Glu Leu Tyr Lys Lys Ile Tyr Arg Tyr Met Thr Glu Thr Arg Gly Leu Asp Asn Leu Leu Trp Val Tyr Ser   240

 721   CCG GAT GCC AAC AGA GAC TTT AAA ACA GAC TTC TAC CCA GGC TCA TCT TAT GTG GAT ATT ACC GGT CTG GAC   792
 241   Pro Asp Ala Asn Arg Asp Phe Lys Thr Asp Phe Tyr Pro Gly Ser Ser Tyr Val Asp Ile Thr Gly Leu Asp   264

 793   GCT TAC TTC ACT GAT CCG TAT GCG ATA TCA GGC TAT GAT GAA ATG CTG TCT CCG AAA AAA CCG TTT GCC TTT   864
 265   Ala Tyr Phe Thr Asp Pro Tyr Ala Ile Ser Gly Tyr Asp Glu Met Leu Ser Pro Lys Lys Pro Phe Ala Phe   288

 865   GCC GAA ACC GGT CCG TCC GGC AAT ATC GGA AGC TTT GAT TAT GCT GCT TTT ATT AAT GCG ATC AGG CAA AAA   936
 289   Ala Glu Thr Gly Pro Ser Gly Asn Ile Gly Ser Phe Asp Tyr Ala Ala Phe Ile Asn Ala Ile Arg Gln Lys   312

 937   TAC CCT CAG ACC GCG TAC TTT TTG ACA TGG GAT GAA CAA TTA AGT CCG GCG GCC AAG CAA GGC GCG CAA AGC   1008
 313   Tyr Pro Gln Thr Ala Tyr Phe Leu Thr Trp Asp Glu Gln Leu Ser Pro Ala Ala Lys Gln Gly Ala Gln Ser   336

 1009  CTT TAT CAA AAC AGC TGG ACG CTG AAC AAG GGC GAA ATA TGG AAC GGC GGG TCC TTG ACG CCG ATC GCG GAA   1080
 337   Leu Tyr Gln Asn Ser Trp Thr Leu Asn Lys Gly Glu Ile Trp Asn Gly Gly Ser Leu Thr Pro Ile Ala Glu   360

 1081  TAA   1083
 361   End
```

Figure 6 Nucleotide sequence of mannanase gene and amino acid sequence of β-mannanase of *B. circulans* NT 6.7. Signal sequence was shown in grey.

B. circulans NT 6.7 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* NT 6.7 β-mannanase showed 99% identity with β-mannanase from *B. subtillis* (ABB91433.1). All of mannanase gene and β-mannanase were in the GH26 family. From amino acid sequence analysis also showed that *B. circulans* NT 6.7 β-mannanase was classified into GH26 family based on the sequence identity (Altschul *et al.*, 1997). The N-terminal signal sequence of *B. circulans* NT 6.7 mannanase gene was predicted from amino acid sequence by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalIP at at Febuary 18th, 2012) and the result showed that 24 amino acid residues (residue number 1-24, Figure 6) were predicted as the signal sequence of *B. circulans* NT 6.7 mannanase gene as shown in figure 7 (Petersen, 2011). SignalP 4.0 predicts the presence and location of signal peptide cleavage sites in the nucleotide and amino acid sequences from different prokaryotic and eukaryotic organisms. The method incorporates a prediction of cleavage site, signal peptide and non-signal peptide base on a combination of several artificial neural networks. The neural networks in SignalP produce 3 output scores for each position in the input sequence consisted of predicted cleavage site value (C-score), predicted signal peptide value (S-score) and combination of C- and S-scores (Y-score). In addition, it also showed the discrimination score (D-score) that used to discriminate signal peptide form non-signal peptide (Petersen, 2011).

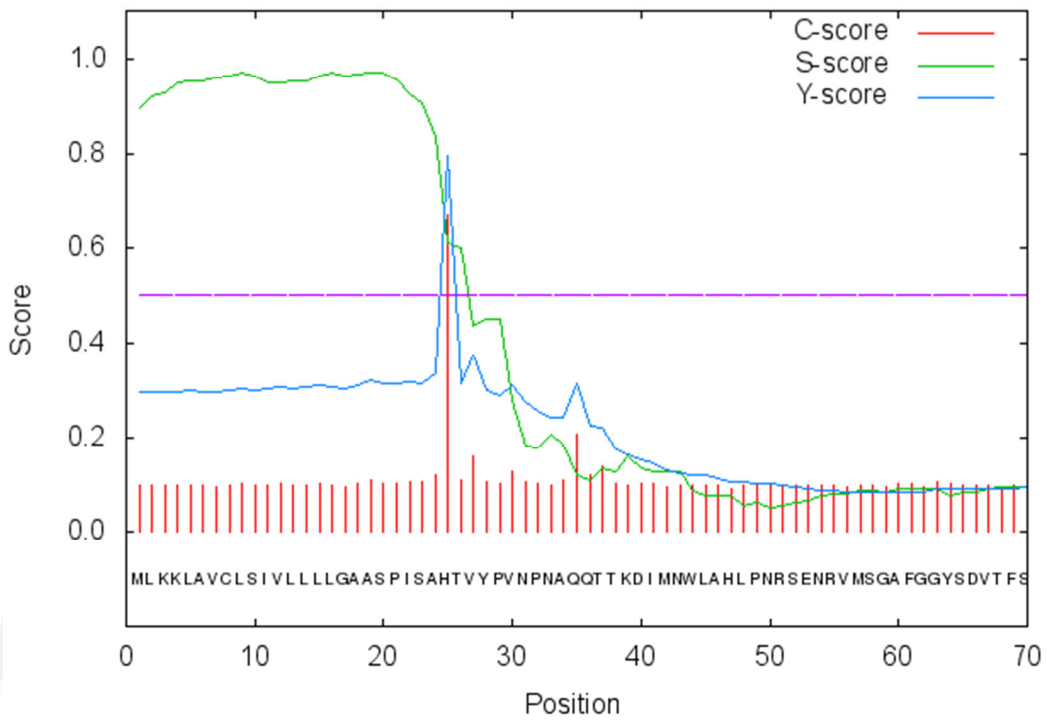


Figure 7 The N-terminal signal sequence prediction of *B. circulans* NT 6.7 mannanase gene by SignalP 4.0

The tertiary structure of *B. circulans* NT 6.7 β-mannanase was predicted by using SWISS-MODEL (http://swissmodel.expasy.org at Febuary 18th, 2012). Tertiary structure model of *B. circulans* NT 6.7 β-mannanase with 360 amino acid residues was constructed base on the structure of β-1,4-mannanase from *B. subtillis* Z-2, BCman (2QHA) template (Yan *et al.*, 2008) with 75% sequence identity. The model of *B. circulans* NT 6.7 β-mannanase structure from BCman template showed the $(\beta/\alpha)_8$ TIM barrel folding type of the GH-A glycoside hydrolase family which contains a hydrophobic core by the folding of 8 β-strands and 8 α-helices that are exposed to the solvent around the exterior (Figure 8A). Three-dimensional structure showed the shallow-dish-shaped active center of β-mannanase (Figure 8B). The open ends of the active site allows the enzyme to ride on the chain of mannan based substrates then internally cleave and generate mannooligosaccharide products with various degrees of polymerization (Yan *et al.*, 2008).

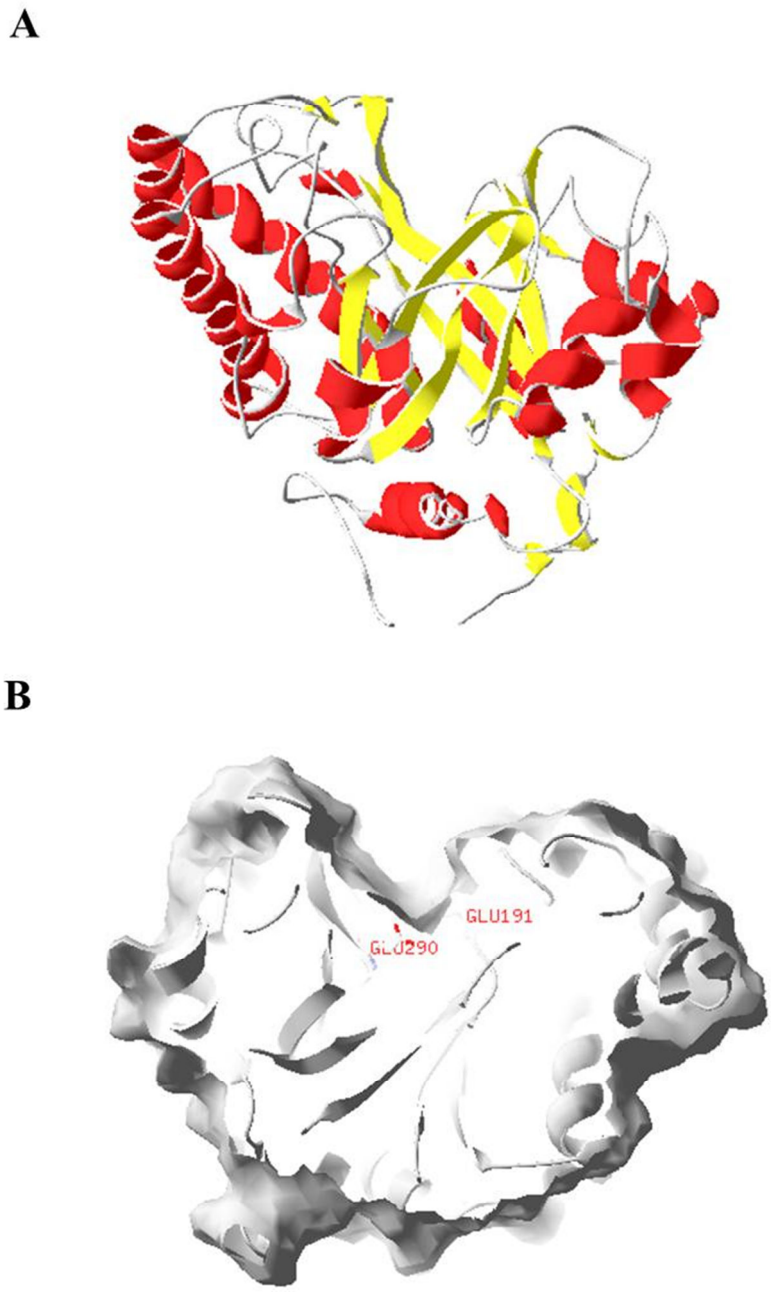


Figure 8 Tertiary structure of *B. circulans* NT 6.7 β-mannanase from homology modeling by using *B. subtillis* Z-2 β-mannanase (2QHA) as a template in ribbon (A) and ribbon with molecular surface (B) model

Amino acid sequence alignment between β-mannanase from *B. circulans* NT 6.7 and *B. subtillis* Z-2, BCman, showed the results that *B. circulans* NT 6.7 β-mannanase contained the same amino acid residues in active sites with the template (Figure 9). From the alignment result indicated that the catalytic acid/base residue and nucleophile residue of *B. circulans* NT 6.7 β-mannanase were Glu191 and Glu290, respectively. Moreover, the analysis of the amino acid residues around the BCman active site according to the expanding width of galactomannan showed 15 amino acid residues in loops around the active center which created potential hydrogen bonds with the substrate (Yan *et al.*, 2008). The amino acid sequence alignment also showed the consistence amino acid residues between these 2 β-mannanases so this indicated the substrate binding residues of β-mannanase from *B. circulans* NT 6.7 that might consisted of Phe61, Val67, Arg94, Trp96, Trp140, Trp196, Phe197, Asp242, Try266, Ser294, Trp322, Asp323, Glu324, Try325 and Trp326. The Trp196 and Trp322 residues were the conserved solvent-exposed tryptophan rings throughout family GH26 and the Trp196 residue was demonstrated to be involved in binding and catalysis of mannooligosaccharides and mannan polysaccharides (Yan *et al.*, 2008). In addition to the containing of solvent-exposed aromatic rings of the Trp residues, the binding site also contained several hydrophilic and charged amino acid residues that interacted with substrate by hydrogen bonding. These residues played a definite role in the binding between enzyme and mannan substrates and also influenced the catalytic function (Yan *et al.*, 2008). From the results of catalytic acid/base residue, nucleophile residue and substrate binding residues can suggested the catalytic process of β-mannanase from *B. circulans* NT 6.7. After mannan substrates stabilization through the hydrogen bonds from the binding residues, the glycosidic bond was cleaved through a combination of nucleophilic attack of the anomeric carbon by the nucleophile Glu290 and protonation of the glycosidic oxygen by the catalytic acid/base Glu191, generating a glycosyl–enzyme intermediate then it was hydrolyzed by water attacking the anomeric carbon of the glycosyl group, resulting in the degradation of mannan polysaccharides to mannooligosaccharides (Yan *et al.*, 2008).

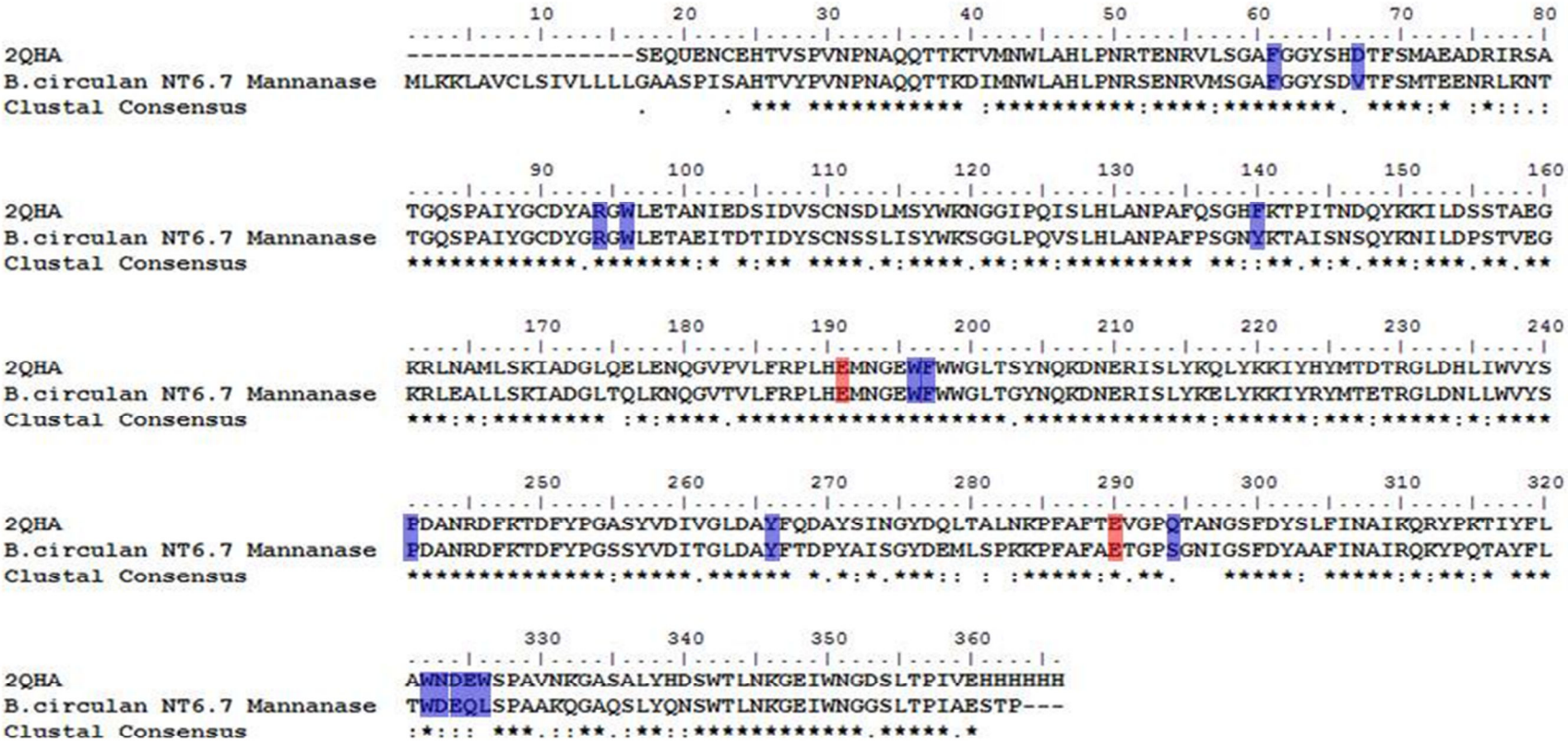


Figure 9 Amino acid sequence alignment between β-mannanase of *B. subtillis* Z-2, BCman (2QHA) and *B. circulans* NT 6.7.

The catalytic acid/base and nucleophile residues were shown in red and the substrate binding residues were shown in blue.

The binding of β-mannanase enzyme with mannobiose (ZINC13543975), mannotriose (ZINC87528098) and mannotetraose (ZINC85427430) was predicted by using SwissDock (http://www.swissdock.ch at March 4th, 2014). The model of *B. circulans* NT 6.7 β-mannanase was use as protein target for molecular docking. The molecular docking results of β-mannanase model and 3 mannooligosaccharide ligands showed that the binding of β-mannanase with mannotetraose showed the lowest estimated free energy of binding (-8.72 kcal/mol) when compared with mannotriose (-7.52 kcal/mol) and mannobiose (-7.18 kcal/mol) (Grosdidier *et al.*, 2007). These results indicated that the affinity of *B. circulans* NT 6.7 β-mannanase with mannotetraose was higher than the other 2 substrates (Grosdidier *et al.*, 2007). In the process of molecular docking, the structure of β-mannanase was kept rigid, while all the flexible bonds of mannooligosaccharide ligands were set to be rotatable so that all possible binding modes could be considered and performed the interacting energy calculations (Lengauer and Rarey, 1996; Kitchen *et al.*, 2004; Grosdidier *et al.*, 2007). The lowest estimated free energy of binding indicated the highest affinity between protein and ligand so the estimated free energy was used to select the best proteins, ligands or protein-ligand interaction from the molecular docking (Lengauer and Rarey, 1996; Feig *et al.*, 2004; Kitchen *et al.*, 2004; Grosdidier *et al.*, 2007; Da Costa *et al.*, 2014). Moreover, the binding energy was decreased when the β-mannanase from *B. circulans* NT 6.7 interacted with the longer mannooligosaccharides indicated that the binding affinity was increased when interacted with mannan polysaccharides substrates. In addition to binding affinity, the function of β-mannanase from *B. circulans* NT 6.7 might increase and mannotetraose might be the smallest mannooligosaccharides substrate for this enzyme.

2. Cloning and expression of mannanase gene from *Bacillus circulans* NT 6.7

For the efficiently production of recombinant β-mannanase from *B. circulans* NT 6.7, two expression systems consisted of *E. coli* BL21* (DE3) and *L. plantarum* WCFS1 *Δalr* were used for expression of *B. circulans* NT 6.7 mannanase gene and the recombinant enzyme production was compared between these systems for further production and characterization.

Based on the nucleotide sequence of *B. circulans* NT 6.7 mannanase gene, ManFw2 and ManRv2 primers were designed for construction of recombinant plasmids for expression. PCR amplification of mannanase gene with these primers was about 1,100 bp in length. After purification and digestion with *Xho*I and *Nco*I restriction enzyme, mannanase gene was ligated into *Xho*I – *Nco*I cut pET21d and *Xho*I – *Nco*I cut pSIP403 for expression in *E. coli* BL21* (DE3) and *L. plantarum* WCFS1 *Δalr*, respectively. For expression in *E. coli* system, ligation products were transformed into *E. coli* BL21* (DE3). For expression in *L. plantarum* system, ligation products were transformed into *E. coli* MB2159 and screened for the positive recombinant plasmids. The recombiant plasmid pSIP403 with mannanase gene, were extracted from *E. coli* MB2159 then transformed into *L. plantarum* WCFS1 *Δalr* for expression. The positive clones of both hosts were confirmed by colony PCR and both of transformants were collected for expression.

2.1 Expression of mannanase gene in *Escherichia coli* BL21*(DE3)

The mannanase gene of *B. circulans* NT 6.7 was successfully cloned into pET21d and the protein could be expressed in *E. coli* BL21* (DE3) driven by the T7 RNA polymerase promoter. In this system, *E. coli* BL21* (DE3) harboring pET21d with mannanase gene was culture until OD OD600 reached 1.0 then induced with IPTG for stimulation of T7 promoter then protein production under T7 promoter was performed at 18°C. For the effective production of recombinant β-mannanase by this system, expression conditions were optimized including IPTG concentration and incubation time after induction.

For optimization of IPTG concentration, 5 ml of *E. coli* BL21* (DE3) with mannanase gene of *B. circulans* NT 6.7 was induced with difference concentration of IPTG consist of 0.1, 0.5 and 1.0 mM. After incubation for 16 hours at 18°C, β-mannanase activity was measured in both culture supernatant and cell lysate fractions. β-mannanase activity was difference between each IPTG concentration (Figure 11). This expression system produced the recombinant β-mannanase and also secreted into the culture medium. Highest β-mannanase activity of both extracellular and intracellular fractions was found in cell culture that was induced with 1.0 mM IPTG. Although, there was the similar level of β-mannanase activity in cell lysate fraction in all IPTG concentration but there was the difference in the extracellular β-mannanase activity. These results indicated that 1.0 mM IPTG was the appropriate concentration for recombinant β-mannanase production and secretion with highest activity.

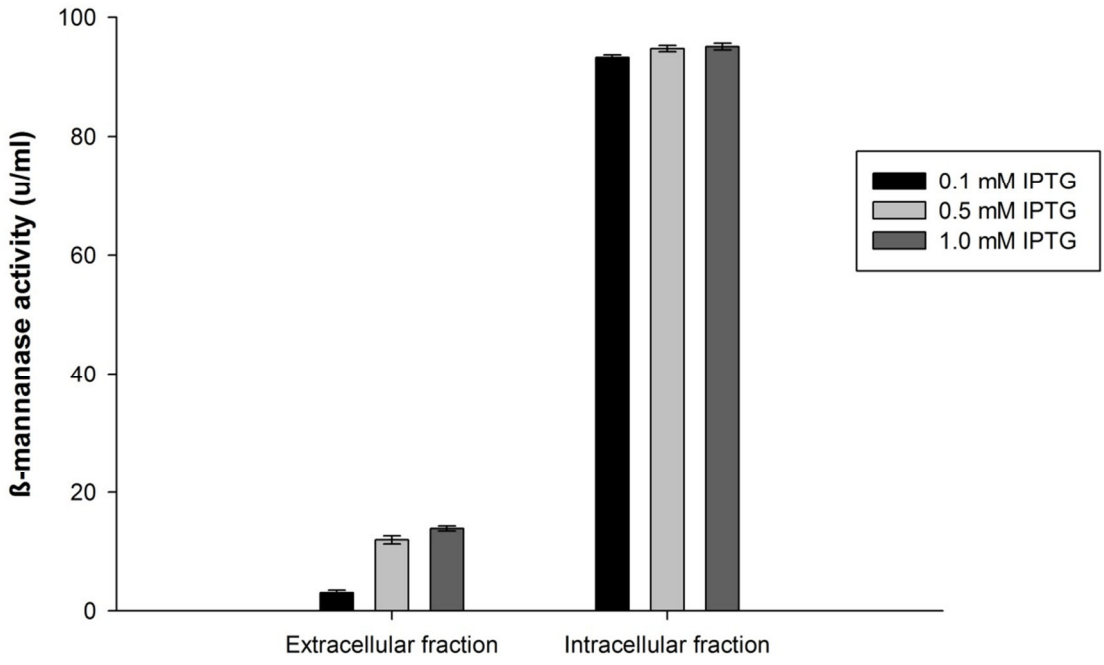


Figure 10 β-mannanase activity in extracellular and intracellular fractions from the different concentration of IPTG induction

Incubation period after induction was optimized by 5 ml cultivation of *E. coli* BL21* (DE3) with mannanase gene of *B. circulans* NT 6.7 and induction with 1.0 mM IPTG. Then, cell cultures were incubated at 18°C for difference times consisted of 16, 18 and 20 hours and β-mannanase activity was measured in both culture supernatant and cell lysate fractions. At the difference time of incubation, there was the similar β-mannanase activity in culture supernatant. However, intracellular mannanase activity decreased after incubation more than 16 h (Figure 12). The intracellular activity was decreased after longer incubation because of the dead-cell lysis in the stationary phase of *E. coli* (Nitta et al., 2000). So, the intracellular enzyme was degraded by protease from cell lysis after the cells were in stationary phase for longer period. These results indicated that the appropriate incubation time after induction for production of recombinant β-mannanase was 16 h.

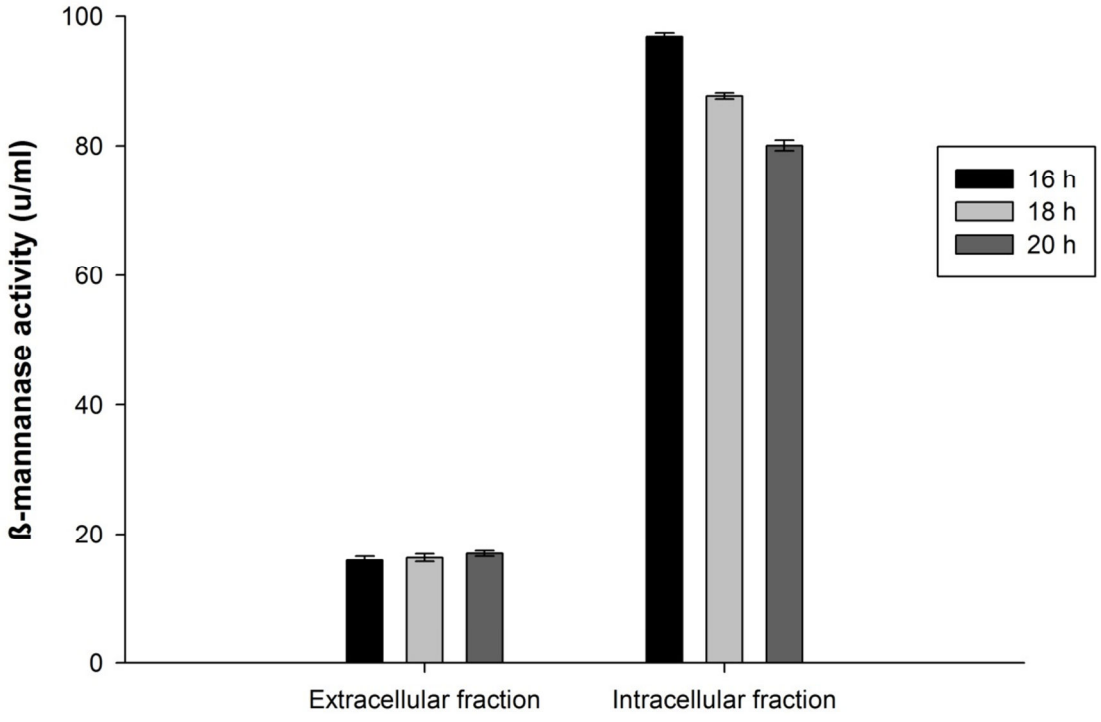


Figure 11 β-mannanase activity in extracellular and intracellular fractions from the different incubation time after IPTG induction

So, the selected condition for the efficient expression and secretion of recombinant β-mannanase in *E. coli* BL21* (DE3) consisted of 1.0 mM IPTG for expression induction and 16 h incubation after induction. At this condition, 100 ml of *E. coli* BL21*(DE3) harboring pET21d with mannanase gene were collected and measured the β-mannanase activity. Extracellular and intracellular and β-mannanase activities were 37.14 and 515.40 u/ml, respectively (Table 2).

Mannanase gene was successfully cloned into pET21d expression vector and expressed in *E. coli* BL21* (DE3), a commercially standard and successful expression system (Nguyen *et al.*, 2012). The fullength mannanase gene was cloned into the vector under T7 promoter without additional signal sequence. However, recombinant β-mannanase was secreted into the culture media by this system when induce with appropriate IPTG concentration. Because of the wild type β-mannanase from *B. circulans* NT 6.7 was the extracellular enzyme so this enzyme may contain the effective signal sequence for secretion. The N-terminal signal sequence prediction by SignalP 4.0 showed that 24 amino acid residues were predicted as the signal sequence of mannanase gene. The signal sequence of residues 1 to 24 (**MLKK**LAVCLSIVLLLLGAASPISA) predicted by SignalP 4.0 shows structural features that have been found as common for other signal sequences as well in that it is composed of a hydrophobic H-domain rich in Ala, Val and Leu, preceeded by a short, positively charged N-domain, which is here shown in bold (Choi and Lee, 2004). Moreover, mannanase gene without signal sequence was also cloned and expressed using the same system to examine the function of this signal sequence. *B. circulans* NT 6.7 mannanase gene without signal sequence was also cloned into pET21d and expressed with the same condition. The results showed that recombinant β-mannanase without signal peptide was not secreted at all IPTG concentration and β-mannanase activity in cell was also decreased about 50 times. The intracellular β-mannanase activity was only 9.72 u/ml. These results indicated that predicted signal sequence involved in the secretion of recombinant β-mannanase in this system and also in the function of recombinant β-mannanase.

The mannanase gene was cloned into the pET21d vector and expressed in *E. coli* BL21* (DE3), a successful standard and commercial expression system (Nguyen *et al.*, 2012). The full-length mannanase gene was cloned into the vector under the T7 promoter without additional signal sequences that are often used in *E. coli* for secretion to the periplasm or extracellular environment (Choi and Lee, 2004; Mergulhão *et al.*, 2005). Interestingly, recombinant β-mannanase was secreted into the culture media by this system when induce with appropriate IPTG concentrations, and most of the activity in the fraction termed intracellular was localized in the periplasm, suggesting that the natve *Bacillus* signal peptide was recognized by the translocation system of *E. coli* and the recombinant was transported out of the cytosol. We also cloned and expressed the mature mannanase gene without its signal sequence using the same system. The enzyme without signal peptide was not secreted at any IPTG concentration used. In addition, intracellular β-mannanase activity was decreased by more than 50 times, indicating that the efficiency of expression was much lower for this construct.

This *E. coli* expression system (*E. coli* BL21* (DE3) with pET21d plasmid) is the effective expression system that can produce the recombinant β-mannanase from *B. circulans* NT 6.7 with efficient secretion, high activity and high specific activity. Although, most of the recombinant β-mannanase was produced within the cell especially in periplasmic space but the enzyme activity of secreted recombinant β-mannanase had almost 20 times higher than wild type β-mannanase from *B. circulans* NT 6.7 at the same production scale. Furthermore, overall enzyme activity of this recombinant β-mannanase was about 10 times higher than recombinant β-mannanase from *B. licheniformis* DSM13, β-mannanase in the same GH26 family, which was produced from the different *E. coli* expression system (Songsiriritthigul *et al.*, 2010). This also showed the efficient secretion when compared with the recombinant β-mannanase *B. circulans* CGMCC 1416 and *B. circulans* CGMCC 1554, recombinant enzymes from GH5 family that were produced from same expression system (Li *et al.*, 2008; Yang *et al.*, 2009). These results presented the very successful expression system for production of high efficient recombinant β-mannanase from *B. circulans* NT 6.7 that can applied in the industrial application.

Table 2 β-mannanase activity from *Escherichia coli* expression system (from 100 ml culture)

Sample	Volume (ml)	Activity (u/ml)	Protein concentration (mg/ml)	Specific activity (u/mg)
Crude culture supernatant	100	37.14	0.03	1,238.00
Crude cell lysate	100	515.40	0.08	6,442.50

2.2 Expression of mannanase gene in *Lactobacillus plantarum* WCFS1 Δ*alr*

The *B. circulans* NT 6.7 mannanase gene was cloned into pSIP403 under P_{sppA} promoter and the expression was induced with peptide pheromone. In this system, *L. plantarum* WCFS1 Δ*alr* harboring pSIP403 with mannanase gene was culture and induced with IP-673 for stimulation of sakasin P promoter for protein production. For the effective production by this system, concentration of cell culture before induction and IP inducer were optimized for the highest production of recombinant β-mannanase by this system.

For optimization of concentration of cell culture before induction, recombinant *L. plantarum* WCFS1 Δ*alr* was induced at difference concentration. The results showed that β-mannanase activity was found only in cell at the difference concentration of cell before induction. Induction at OD600 reached 2.0 showed the highest β-mannanase activity (Figure 13). Moreover, there was no difference of β-mannanase activity when induced with more than 25 ng/ml of IP-673.

So, the condition for mannanase gene expression in *L. plantarum* WCFS1 Δ*alr* consisted of induction with 25 ng/ml of IP at culture OD600 reached 2.0. However, there was no secretion of recombinant β-mannanase from this system. *L. plantarum* WCFS1 Δ*alr* that contain mannanase gene were cultured in 100 ml of MRS. After expression with this condition, β-mannanase activity in cell lysate was 0.82 u/ml (Table 3).

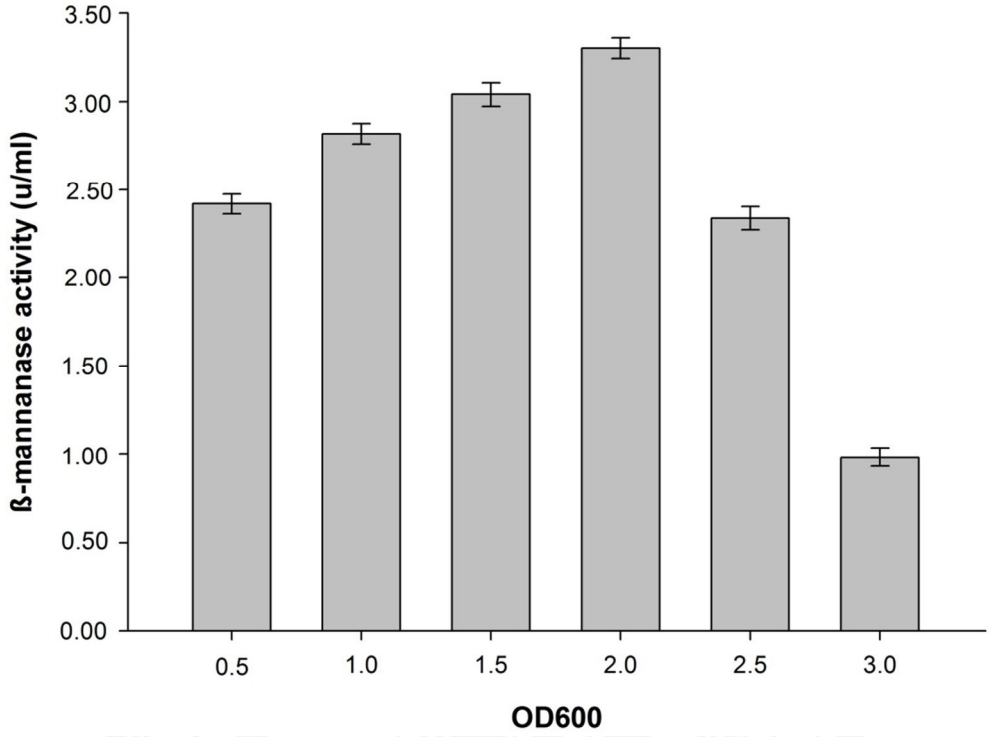


Figure 12 Optimization of cell concentration before induction in *L. plantarum* expression system

Table 3 β-mannanase activity from *Lactobacillus plantarum* expression system (from 100 ml culture)

Sample	Volume (ml)	Activity (u/ml)	Protein concentration (mg/ml)	Specific activity (u/mg)
Crude culture supernatant	100	ND*	-	-
Crude cell lysate	100	0.82	0.22	3.73

* ND = Not detected

The LAB expression system was selected for expressed the *B. circulans* NT 6.7 mannanase gene because of LAB are generally harmless and severally used in numerous food applications. Moreover, they have been developed for used as the expression system especially food-grade expression systems for using in food-related applications (Peterbauer *et al.*, 2011). 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 (Nguyen *et al.*, 2011 and Nguyen *et al.*, 2012). The system is the nisin-controlled gene expression system. Because of *Bacillus* and *Lactobacillus* are the gram positive bacteria so the native signal sequence of *B. circulans* NT 6.7 mannanase gene could lead to the extracellular production. 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. In addition, the overall production of the recombinant β-mannanase was very low, so β-mannanase will not be detected in the culture supernatant with the inefficient secretion system (Nguyen *et al.*, 2012). 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 (Nguyen *et al.*, 2012). However, this is the first report of food grade recombinant β-mannanase production.

2.3 Analysis of recombinant β-mannanase from *Escherichia coli* expression system

Comparison of *E. coli* and *L. plantarum* expression system for expression of mannanase gene from *B. circulans* NT 6.7 showed the results that *E. coli* expression system was the effective system for production of from *B. circulans* NT 6.7. The overall recombinant β-mannanase production from *E. coli* BL21* (DE3) was 750 times higher than *L. plantarum* WCFS1 *Δalr* expression system with high

specific activity. Another important property of *E. coli* expression system in this study was the secretion of the recombinant β-mannanase into culture media which high activity and specificity when compared with the wild type enzyme. So, both of extracellular and intracellular recombinant β-mannanase from *E. coli* BL21* (DE3) expression was used for further study.

The theoretical molecular weight of recombinant β-mannanase with C-terminal hexa-histidine tag was about 40 kDa (Gasteiger *et al.*, 2005). Protein electrophoresis showed that both of extracellular and intracellular recombinant β-mannanase had the same size about 40 kDa which consistence with the theoretical calculation (Figure 14). The extracellular and intracellular protein bands in Figure 14 were consistence with the β-mannanase activities at different time after induction with 1.0 mM IPTG (Table 4). In addition, recombinant β-mannanase in both fractions showed the activity on LBG substrate gel (Figure 15). These results indicated that recombinant β-mannanase in both fractions was the same and could confirm the secretion in this system. However, size of recombinant β-mannanase was smaller than the purified wild type β-mannanase from *B. circulans* NT 6.7. The wild type β-mannanase was about 60 kDa in weight (Pangsri, 2014). Previous study on proteins from *Bacillus* showed that several proteins need posttranslational modification such as tyrosine phosphorylation in biofilm formation (Kiley and Stanley-Wall, 2010), cytochrome *c-551* (Kai *et al.*, 1997) and subtilin (Izaguirre and Hansen, 1997) in *B. subtilis.* So, posttranslational modification in wild type β-mannanase from *B. circulans* NT 6.7 might cause the difference in protein molecular weight that larger than recombinant enzyme. Moreover, posttranslational modification prediction from amino acid sequence of β-mannanase from *B. circulans* NT 6.7 using FindMod (http://web.expasy.org/findmod/) showed the methylation and glycosylation sites in the sequence of β-mannanase. These results also indicated the posttranslational modification that can occurred in the wild type β-mannanase from *B. circulans* NT 6.7 (Gasteiger *et al.*, 2005)

From the previous results showed that most of recombinant β-mannanase was in the cell so position of this enzyme in cell was also localized. In this study, cell was extracted and separated into 2 fractions consisted of periplasmic fraction and cell lysate fraction. Protein electrophoresis showed that most of the intracellular recombinant β-mannanase solubilized in the periplasmic space of cell (Figure 16). This result indicated that most of recombinant β-mannanase was accumulated in the periplasmic space of the cell and some was secreted outside after IPTG induction and incubation with the selected conditions. Soluble recombinant β-mannanase in cell was benefit for using the intracellular enzyme with high activity in the applications.

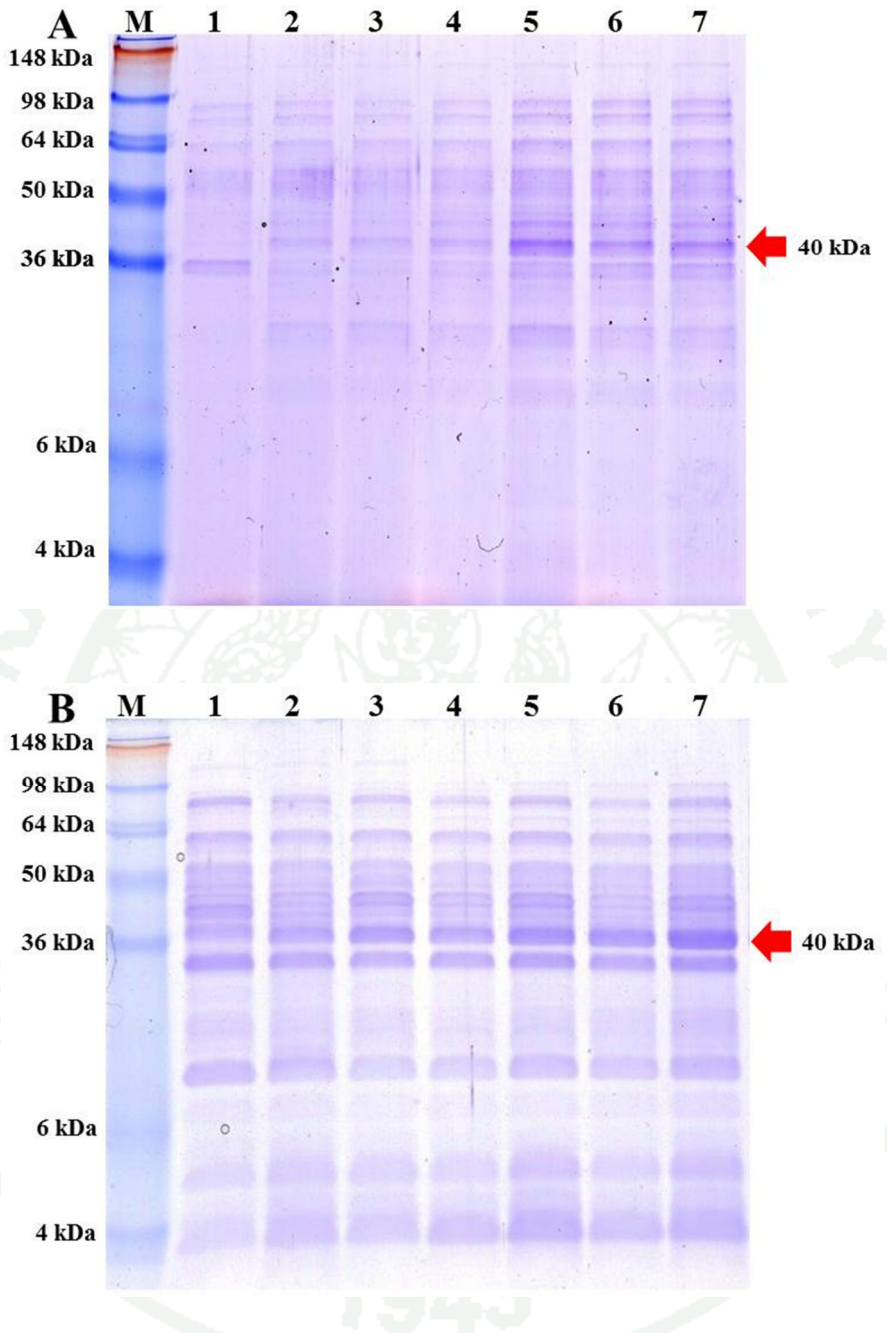


Figure 13 SDS-PAGE of extracellular (A) and intracellular (B) recombinant β-mannanase at different time of IPTG induction

Lane M : SeeBlue® plus2 pre-stained standard (invitrogen)

Lane 1 : Negative control (*E. coli* BL21* (DE3) with pET21d)

Lane 2-7 : Crude enzyme at 0, 1, 3, 6, 12 and 16 h after IPTG induction, respectively.

Table 4 β-mannanase activity at different time after induction with 1 M IPTG in *Escherichia coli* expression system

Enzyme fraction	β-mannanase activity (u/ml)					
	0h	1h	3h	6h	12h	16h
Extracellular	8.34	15.62	22.58	30.70	38.64	40.85
Intracellular	122.35	214.78	302.64	387.56	412.63	445.04

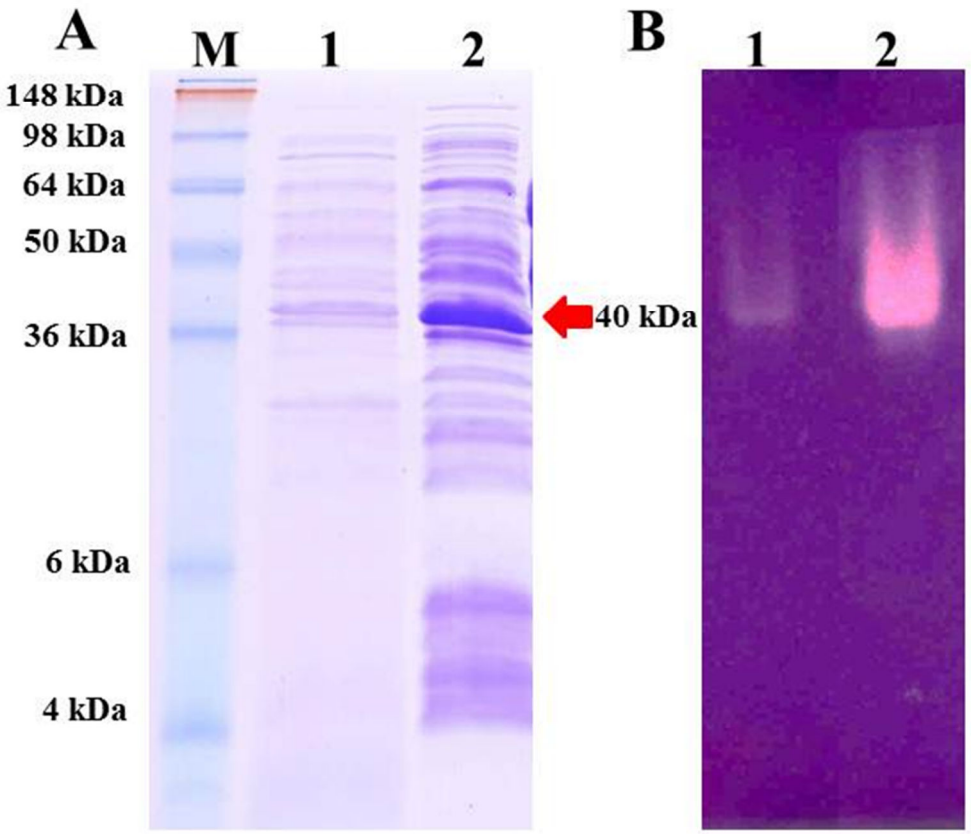


Figure 14 SDS-PAGE (A) and zymogram analysis (B) of recombinant β-mannanase
Lane M : SeeBlue® plus2 pre-stained standard (invitrogen)
Lane 1 : Crude culture supernatant
Lane 2 : Crude cell lysate

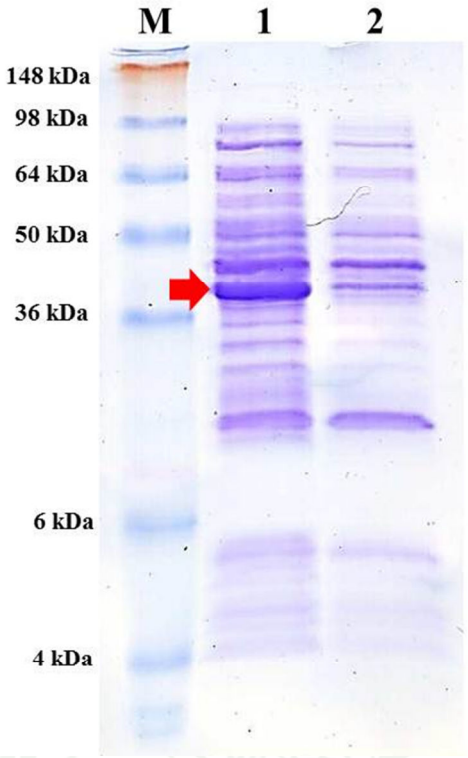


Figure 15 Localization of recombinant β-mannanase in *E. coli* BL21* (DE3) cell
Lane M : SeeBlue® plus2 pre-stained standard (invitrogen)
Lane 1 : Periplasmic space fraction
Lane 2 : Cell lysate fraction

2.4 Plasmid stability in *Escherichia coli* expression system

The stability of pET21d plasmid with mannanase gene from *B. circulans* NT 6.7 in the recombinant β-mannanase expression in the *E. coli* BL21* (DE3) was examined in two conditions consisted of continuous expression and storage by sub-culturing, expression and measuring the enzyme activity, cell concentration and plasmid copy number in each culture passage.

For the continuous expression, the recombinant strain was cub-cultured in to fresh media after 16 h of induction. The results showed that β-mannanase activity was decreased more than 50% at the second passage and continuously decreased until the sixth passage (Figure 17). The decreasing of β-mannanase activity was consistent with the result of plasmid copy number of cell in each culture passage that was decreased in each culture passage (Figure 18). However, cell concentration in each sub-culturing passage was increased from 10^9-10^{12} cfu/ml after ten times sub-culturing. The continuous sub-culturing and expression of *E. coli* BL21* (DE3) with mannanase gene from *B. circulans* NT 6.7 led to the instability of recombinant plasmid. As shown in Figure 18, amount of the recombinant plasmid was decreased and contrasted with the amount of cells indicated that cell grown better after the plasmid was lost. This result was also consistent with the results of β-mannanase activity that was lost as same as the amount of the plasmid. The pET21d plasmid was the medium copy number plasmid which could lose or not functioned when cell was in the toxic conditions or the target gene generated the cell-toxic products (Novagen®, 2003). However, loosing of enzyme activity after continuous expression is the benefit for commercial recombinant enzyme production. The customer cannot produce the effective recombinant enzyme from the strain that can be contained in the commercial enzyme.

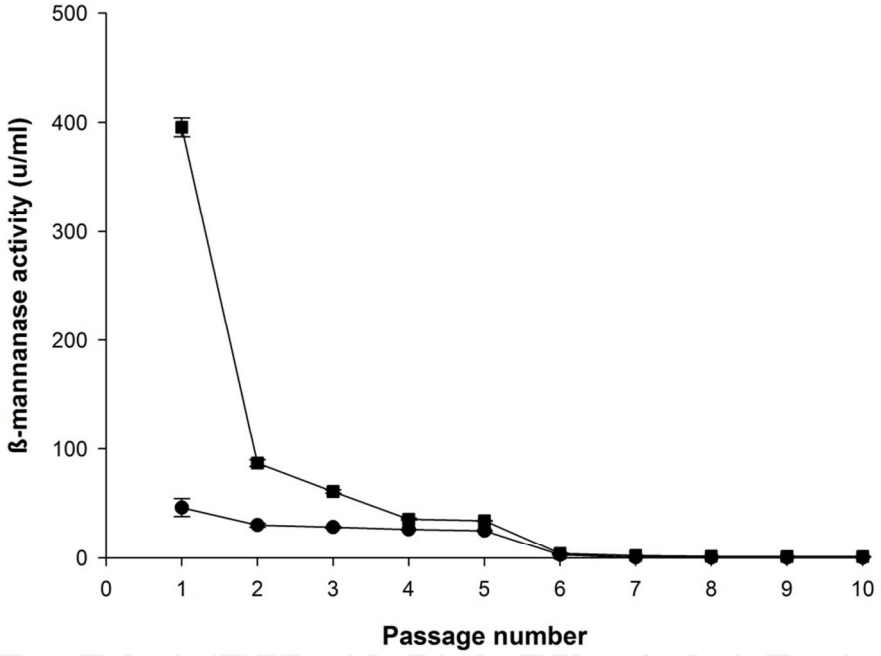


Figure 16 Extracellular (—●—) and intracellular (—■—) β-mannanase activity in each sub-culturing passage of continuous expression

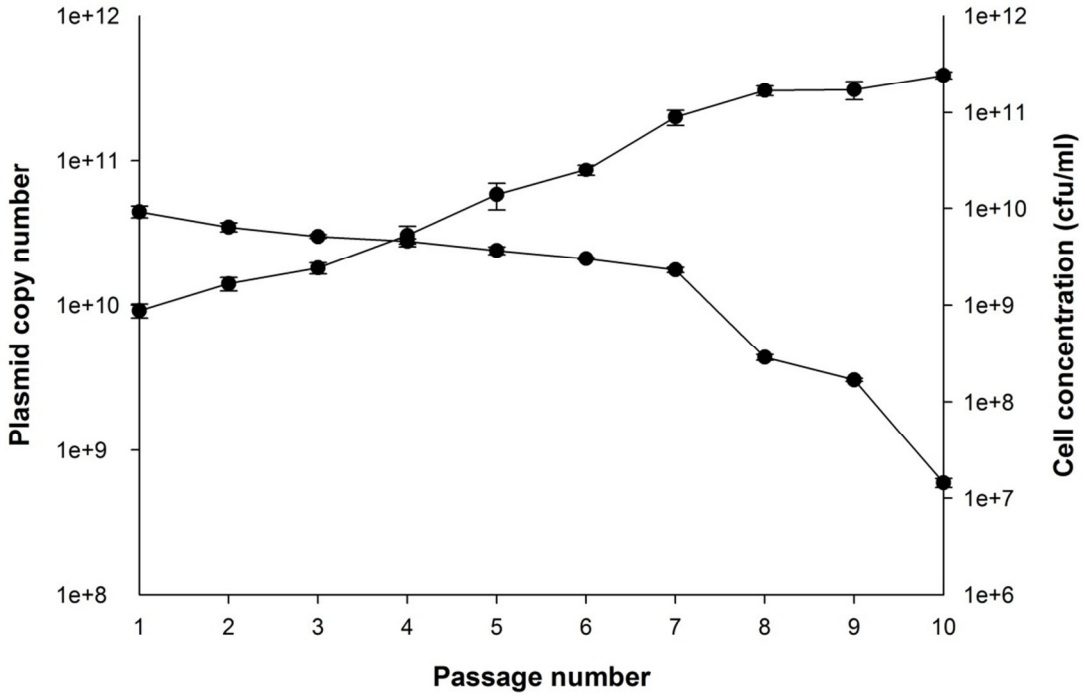


Figure 17 Plasmid copy number (—●—) and cell concentration (—■—) in each sub-culturing passage of continuous expression

For the storage condition, the recombinant strain was cultured for about 12 generations (6 h) then it was sub-cultured into fresh media and induced. The results showed that both of extracellular and intracellular β-mannanase activities were in the same level of 7 transfers of cultivation (Figure 19). In addition, cell concentration and plasmid copy number of cell in each passage were also in the same level (Figure 20). Cell concentration was about 10^9 cfu/ml in each passasge while plasmid copy number was about 10^10 copy per passage. These results were consistent with the study of the stability of pET21 with protective antigen of *B. anthracis* that the recombinant plasmid was completely stable after 15^{th} generation and the yield of protein production was less decreased after 50 generations (Vahedi *et al.*, 2011). These results showed the stability of this plasmid and expression system that can be storage for long time without decreasing of the amount of recombinant plasmid and protein production. These also would be useful for the further production of recombinant β-mannanase and also other recombinant protein especially in the industrial production.

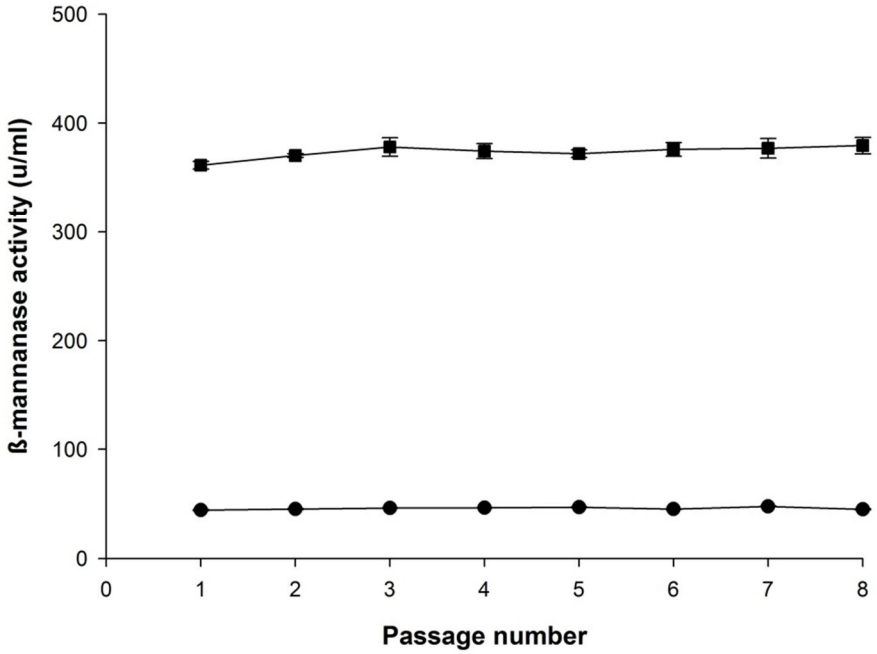


Figure 18 Extracellular (—●—) and intracellular (—■—) β-mannanase activity in each sub-culturing passage of storage condition

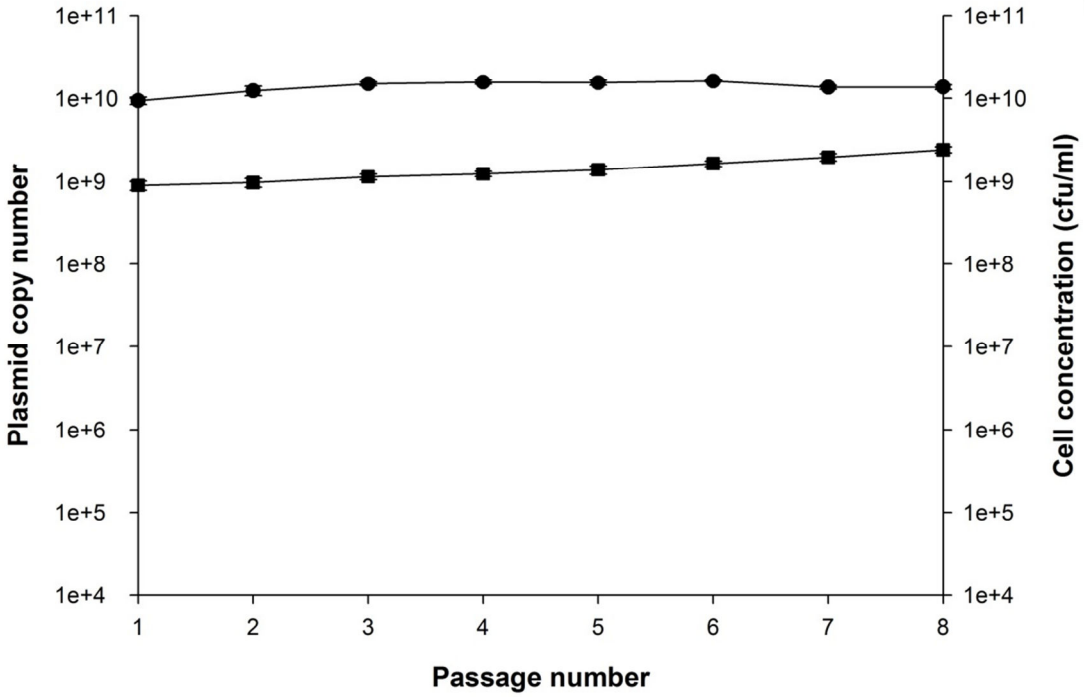


Figure 19 Plasmid copy number (—●—) and cell concentration (—■—) in each sub-culturing passage of storage condition

3. Characterization of recombinant β-mannanase

3.1 The optimum temperature and temperature stability

The effects of temperature on enzyme activity were determined in both of extracellular and intracellular recombinant β-mannanase. The optimum temperature was measured with the standard assay at the temperature range of 30 - 80°C. The optimum temperature of both recombinant β-mannanases was 50°C (Figure 21A). The optimum temperature of recombinant β-mannanase was similar with wild type. Optimum temperature of crude and purified wild type β-mannanase *B. circulans* NT 6.7 was 50°C (Pangsri, 2014). The enzyme was stable at 50°C. After 24 hours of incubation at 50°C, the remaining enzyme activity of extracellular and intracellular recombinant β-mannanase was 70% and 90%, respectively (Figure 21B). However, β-mannanase activity extremely decreased at the temperature above 50°C. In the standard assay at the 60°C, the relative activity was only 50% when compared with highest activity at the optimum temperature, 50°C (Figure 21A). Moreover, the remaining enzyme activity was only 10% in both of extracellular and intracellular recombinant β-mannanase after incubation for 30 minutes at 60°C.

3.2 The optimum pH and pH stability

The effects of pH on β-mannanase activity were determined in both of extracellular and intracellular recombinant enzyme with the standard assay at the pH range of 3.0-10.0. The optimum pH of both extracellular and intracellular recombinant β-mannanase was 6.0. These reults were also same as wild type β-mannanase. Recombinant β-mannanase was very active in pH range of 5.0-7.0 (Figure 22A). Recombinant β-mannanase was stable at pH 6.0-8.0 after 1 h. The remaining activity of extracellular and intracellular recombinant β-mannanase at pH 6.0 was about 70% and 85%, respectively while at pH 7.0 and 8.0 were about 60% (Figure 22B).

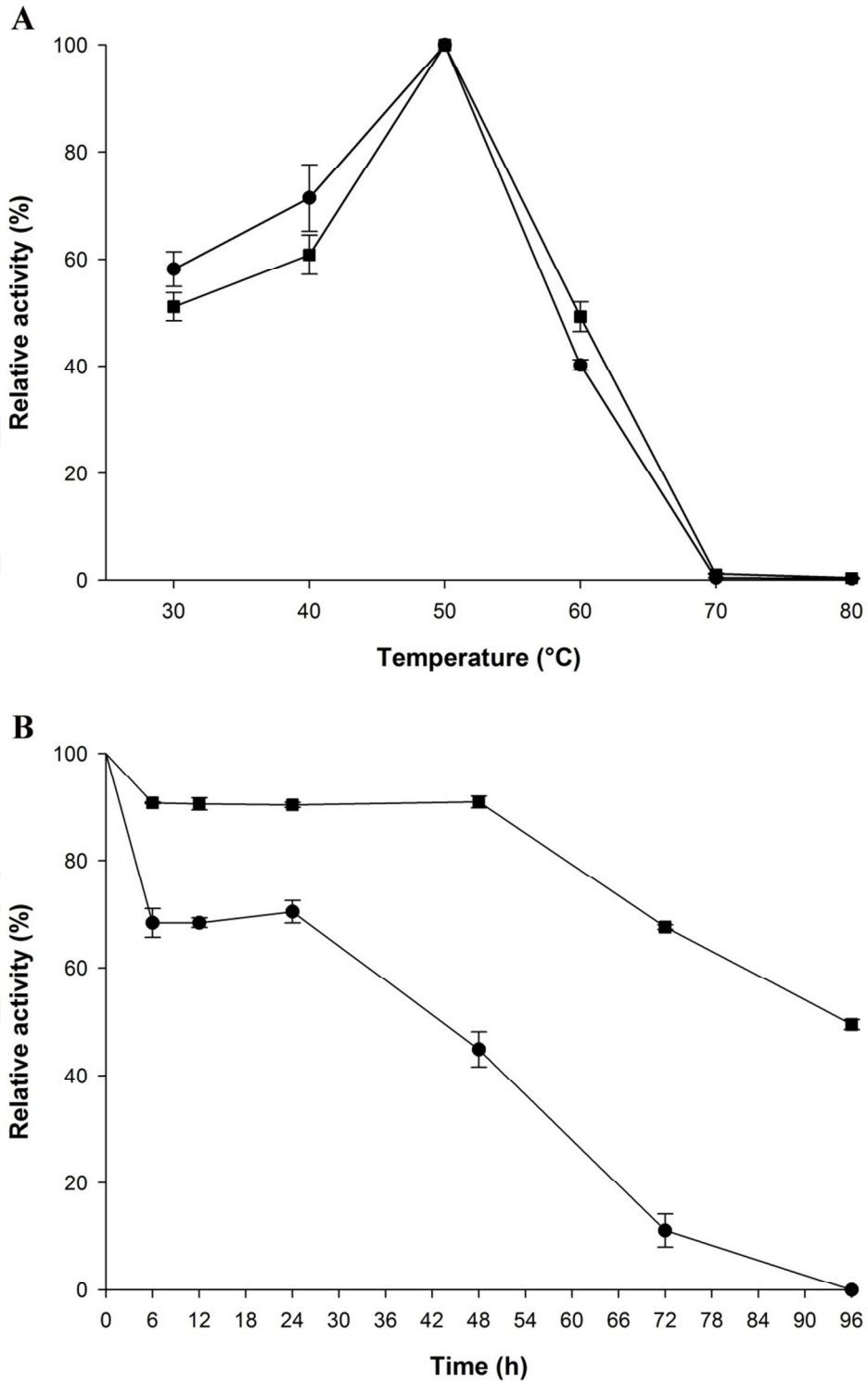


Figure 20 Effect of temperature on recombinant β-mannanase activity (A) and stability (B). Extracellular (—●—) and intracellular (—■—) were determined and expressed as relative activity.

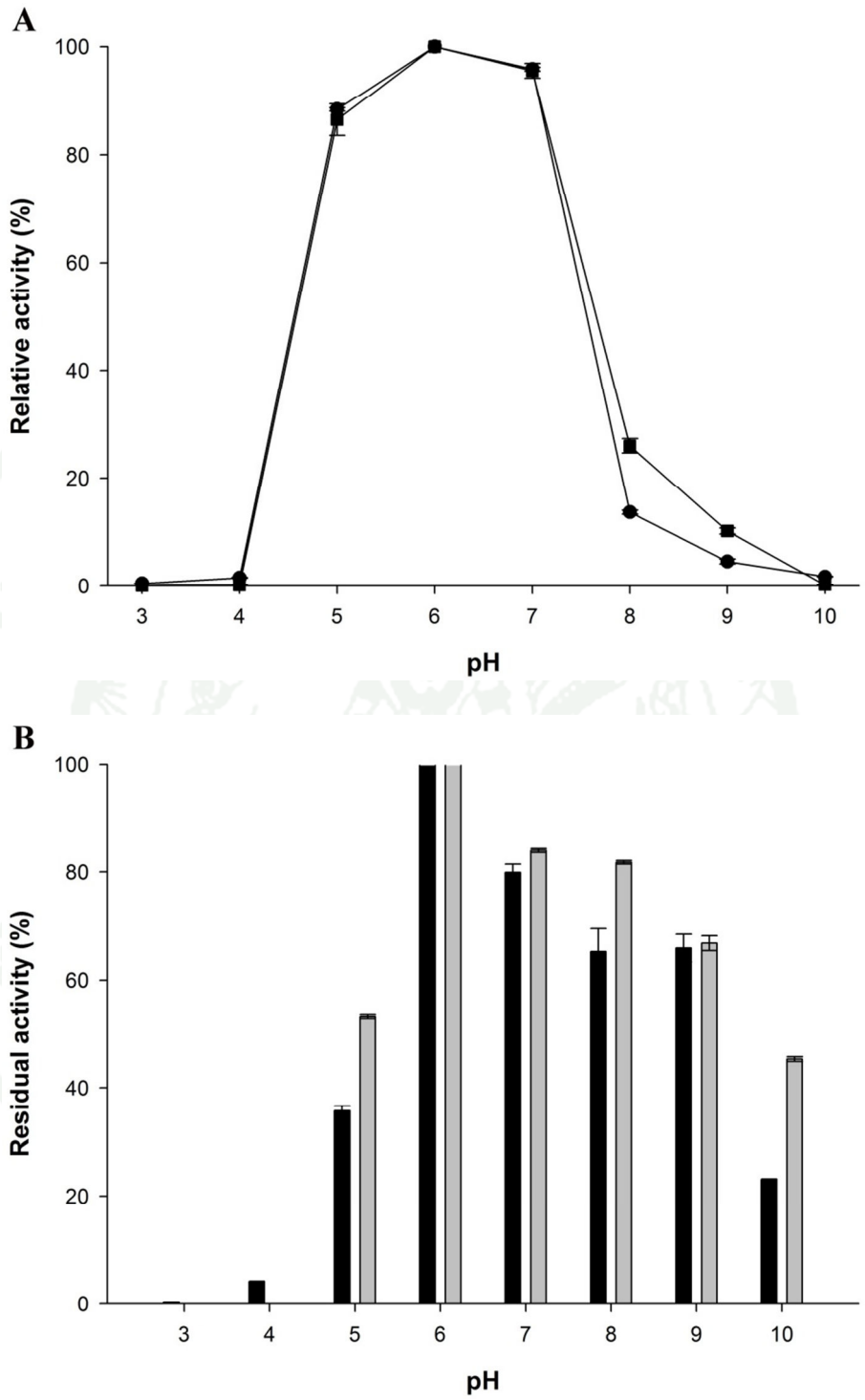


Figure 21 Effect of pH on recombinant β-mannanase activity (A) and stability (B). For optimum pH extracellular (—●—) and intracellular (—■—) were determined and expressed as relative activity. For the pH stability of extracellular (black) and intracellular (grey) enzyme were determined at 1 h as residual activity.

3.3 Effect of various metal ions and chemical on recombinant β-mannanase activity

The effect of metal ions and chemicals on recombinant β-mannanase activity was tested by adding 1mM of selected metal ions and chemical to the enzyme reactions. The effect of metal ions and chemical on both extracellular and intracellular recombinant β-mannanase activity was shown in Table 5. Co^{+}, Li^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions had slightly effect on enzyme activity of both extracellular and intracellular recombinant β-mannanase. There were only about 5% changes in β-mannanase activity of both fractions in the presence these ions. Cu^{2+} ion had a relatively moderate effect on the enzyme activity. Fe^{2+} ion and EDTA strongly inhibited the β-mannanase activity by 60% and 65%, respectively. Both of wild type β-mannanases from *B. circulans* NT 6.7 and recombinant β-mannanases were inhibited by EDTA, the strong chelating agent that interacted with the metal ion cofactors of this enzyme (Yoon *et al.*, 2008 and Pangsri, 2014). The enzyme activity was loss in the presence of Fe^{2+} ion because the Fe^{2+} was oxidized to Fe^{3+} in the ambient conditions. It was expected that the enzyme activity was lost by Fe^{3+} (Yoon *et al.*, 2008). However, there were some differences in the effect of metal ions on wild type and recombinant β-mannanases. Although Cu^{2+} and Fe^{2+} ions inhibited recombinant β-mannanases activity but wild type β-mannanases activity was not inhibited by these ions. Wild type β-mannanase activity was highly increased in the presence of Mg^{2+}, Co^{+}, Mn^{2+} and Cu^{2+} ions (Pangsri, 2014). The effect of these metal ions was similar with previous studies on both of wild type and recombinant β-mannanase from several sources which had less effect or enhance the enzyme activity (Ma *et al.*, 2004, Li *et al.*, 2006, Yoon and Lim, 2007, Titapoka, 2008, Yan *et al.*, 2008, Yoon *et al.*, 2008, Yang *et al.*, 2009 and Fu *et al.*, 2010). These results indicated that there were some differences in the metal ions biding sites between wild type and recombinant β-mannanases that might come from the different host and protein processing. However, the effects of various metal ions and also reagents were different in each microbial β-mannanases depended on the source and sequence of enzyme.

Table 5 Effect of various metal ions and chemical on the recombinant β-mannanase activity

Metal ions / Chemical	Relative activity (%)	
	Extracellular enzyme	Intracellular enzyme
Control	100.00	100.00
Co^{+}	104.74	106.32
Ca^{+}	95.45	97.29
Zn^{2+}	97.50	92.24
Mn^{2+}	95.26	95.65
Mg^{2+}	94.32	95.46
Li^{+}	96.82	97.70
Cu^{2+}	79.05	64.08
Fe^{2+}	40.12	39.18
EDTA	32.00	37.95

3.4 Substrate specificity

The recombinant β-mannanase showed the highest activity on LBG followed by konjac glucomannan and ivory nut mannan. This recombinant enzyme also hydrolyzed copra meal and defatted copra meal, mannan based substrates from natural product. There no activity on alpha-mannan, xylan and cellulose substrates. These results indicated that recombinant β-mannanase was specific only on β-mannan substrates with the preference on galactomannan (Table 6). This recombinant β-mannanase was very active on mannan based polysaccharide substrates especially LBG, a high molecular mass galactomannan as same as wild type enzyme. However, the recombinant β-mannanase hydrolyzed konjac glucomannan less than wild type enzyme that can hydrolyzed in the same level as LBG (Pangsri, 2014). The enzyme activity on other galactomannan substrates with the different mannose/galactose ratio was reduced more than 90% indicated that the recombinant β-mannanase was limited by a number of branched galactose residues. The results of substrate specificity indicated that the recombinant β-mannanase was specific for hydrolysis of β-1,4-linkages of mannan based polysaccharides without other activity.

Table 6 Substrate specificity of recombinant β-mannanase

Substrate	Substrate type	Relative activity (%)	
		Extracellular enzyme	Intracellular enzyme
Locust bean gum	Galactomannan	100.00	100.00
Konjac glucomannan	Glucomannan	61.89	68.35
Ivory nut mannan	Linear mannan	50.50	53.83
Defatted copra meal	Galactomannan	3.26	3.60
Guar gum	Galactomannan	0.42	0.48
Copra meal	Galactomannan	0.36	0.15
Yeast cell wall	α-Mannan	0.00	0.00
Xylan from oat spelts	Xylan	0.00	0.00
Carboxymethylcellulose	Cellulose	0.00	0.00
Avicel	Cellulose	0.00	0.00

3.5 Hydrolysis products of recombinant β-mannanase

The hydrolysis of LBG, konjac glucomannan and defatted copra meal was examined by both of extracellular and intracellular recombinant β-mannanase. Both of enzyme fractions showed the same hydrolysis pattern in each substrate (Table 7 and Appendix Figure 6). After 6 h of hydrolysis, about 80% of LBG was hydrolyzed to be various mannooligosaccharide products consisted of mannohexaose, mannopentaose, mannotretaose, mannotriose and mannobiose without mannose (Appendix Figure 6A). In addition, mannose was also not detected in the hydrolysis of konjac glucomannan and defatted copra meal substrates. Konjac glucomannan was hydrolyzed into mannohexaose, mannopentaose, mannotriose, mannobiose and the product which had size between mannotetraose and mannobiose (Appendix Figure 6B). The hydrolysis products of deffated copra meal substrate consisted of mannotretaose, mannotriose and mannobiose (Appendix Figure 6C). Amount of mannooligosaccharide products of all substrates at 6 h of hydrolysis was shown in Table 7. Although the incubation time was extended for 24 h but mannose was still not detected in the hydrolysis products of LBG, konjac glucomannan and defatted copra meal.

Table 7 Mannooligosaccharide products of the hydrolysis of LBG, konjac glucomannan and defatted copra meal with extracellular and intracellular recombinant β-mannanase at 6 h of hydrolysis.

Mannooligosaccharide product	Amount of hydrolysis product (mg)					
	LBG		Konjac glucomannan		Defatted copra meal	
	Extracellular enzyme	Intracellular enzyme	Extracellular enzyme	Intracellular enzyme	Extracellular enzyme	Intracellular enzyme
M6 and M5	0.063	0.051	0.042	0.035	-	-
M4	0.109	0.149	-	-	0.134	0.148
M3	0.346	0.376	0.050	0.048	0.116	0.124
M2	0.237	0.288	0.284	0.205	0.053	0.057

HPLC analysis of hydrolysis products of mannooligosaccharide substrates showed the results that the recombinant β-mannanase hydrolyzed mannohexaose, mannopentaose and mannotretaose into small molecules but mannotriose and mannobiose were not hydrolyzed (Table 8 and Appendix Figure 7). After 6 h of hydrolysis, the amount of hydrolysis products of each substrate was shown in Table 8. Mannohexaose and mannopentaose were hydrolyzed to be mannotretaose, mannotriose, mannobiose after 0.5 h of incubation and also mannose after an extended incubation for 6 h. At 6 h of incubation, almost 90% of mannohexaose and 70% of mannopentaose were hydrolyzed. Moreover, the recombinant enzyme hydrolyzed mannotretaose into smaller oligomers including mannose after 6 h of incubation and the products were increased after 24 h of incubation.

HPLC analysis of hydrolysis products confirmed the results that recombinant β-mannanase from *B. circulans* NT 6.7 can efficiently and randomly hydrolyzed mannan backbone into oligosaccharides with different size. The recombinant β-mannanase can hydrolyzed LBG, konjac glucomannan and defatted copra meal into mannooligosaccharides with 6 to 2 mannose monomers. The hydrolysis products from recombinant enzyme were consistent with the hydrolysis study of wild type β-mannanase from *B. circulans* NT 6.7 (Pangsri, 2014). These results indicated that mannooligosaccharide products from the recombinant enzyme also had the prebiotic property as same as products from wild type enzyme especially from defatted copra meal. So, recombinant β-mannanase from *B. circulans* NT 6.7 can be used for prebiotic mannooligosaccharides production from natural resources with cheap and high galactomannan content such as defatted copra meal.

Table 8 Mannooligosaccharide products of the hydrolysis of mannooligosaccharide substrates with recombinant β-mannanase at 6 h of hydrolysis.

Substrates	Amount of hydrolysis product (mg)					
	M1	M2	M3	M4	M5	M6
M6	0.048	0.995	1.207	1.517	0.000	-
M5	0.068	1.148	1.475	0.532	-	-
M4	0.040	0.074	0.089	-	-	-
M3	-	-	-	-	-	-
M2	-	-	-	-	-	-

The results of the hydrolysis of various mannooligosaccharide substrates showed the random manner of this recombinant β-mannanase which randomly hydrolyzed mannooligosaccharide based on the length of substrate. Although this enzyme can produced mannose from the hydrolysis of mannotetraose substrate but there was the different in the hydrolysis products from the hydrolysis of longer substrates, mannopentaose and mannohexaose. It preferred to produce mannobiose with mannotriose and mannotetraose in the hydrolysis of mannopentaose and mannohexaose, respectively. These results indicated that recombinant β-mannanase required at least 4 mannose monomers for degradation and the main products were mannobiose, mannotriose and mannotetraose depended on the substrate length. In addition, this also consistent with the results from molecular docking that the binding of enzyme was increased in mannotetraose substrate compared with mannobiose and mannotriose and mannotetraose was the smallest substrates for binding. HPLC is the most effective and accurate technique for the detection of hydrolysis products from this recombinant β-mannanase. In this study, the less amount of mannose product from the hydrolysis of mannotetraose can be detected by HPLC.

The characterization of recombinant β-mannanase from *B. circulans* NT 6.7 showed that both of extracellular and intracellular recombinant β-mannanase produced from *E. coli* expression system had the same characteristics consisting of optimum temperature and pH, stability, substrate specificity, effect of metal ions and

also the hydrolysis patterns. These results also confirmed that both fractions were the same enzyme that was produced in the cells and secreted. Moreover, the recombinant β-mannanase also had the same enzyme properties with the wild type enzyme (Pangsri, 2014). The optimum temperature and pH of both of wild type and recombinant β-mannanase from *B. circulans* NT 6.7 were 50°C and 6.0 and they also showed the high stability at these conditions. This recombinant enzyme was very active on mannan substrates especially LBG, a high molecular mass galactomannan as same as the wild type enzyme. So, this efficient recombinant β-mannanase can be used in several applications such as prebiotic production from copra meal as same as wild type β-mannanase from *B. circulans* NT 6.7.

CONCLUSION

Mannanase gene of *B. circulans* NT 6.7 consisted of 1,083 nucleotides which encode 360 amino acid residues. This gene was submitted into GenBank of NCBI database with Accession No. JF724077. β-Mannanase of *B. circulans* NT 6.7 was classified into glycosyl hydrolase family 26 (GH26) base on the sequence identity with 24 amino acid residues (residue number 1-24) were predicted as the signal sequence.

Mannanase gene of *B. circulans* NT 6.7 was cloned and expressed in *E. coli* and *L. plantarum* expression system. In *L. plantarum* expression system, mannanase gene was cloned into pSIP403 and expressed in *L. plantarum* WCFS1 *Δalr*. However, β-mannanase was produced only in cell without secretion and the β-mannanase activity in cell lysate was only 0.82 u/ml. In *E. coli* expression system, mannanase gene was cloned into pET21d and successfully expressed in *E. coli* BL21* (DE3). In this system, the recombinant β-mannanase was also secreted into the culture media. β-Mannanase activity in culture supernatant and cell were 37.14 and 515.40 u/ml, respectively. So, recombinant β-mannanase from *E. coli* BL21* (DE3) with high enzyme activity and secretion was used for characterization.

Both of extracellular and intracellular recombinant β-mannanase showed the same characteristics. The optimum temperature and pH of the recombinant β-mannanase was 50°C and 6.0, respectively with high stability at this condition. The recombinant enzyme had the same optimum temperature and pH as wild type β-mannanase from *B. circulans* NT 6.7. This recombinant enzyme was very specific on β-mannan substrates indicating that the recombinant β-mannanase was specific for hydrolysis of β-1,4-linkages without other activity. This recombinant β-mannanase hydrolyzed LBG, konjac glucomannan and defatted copra meal substrates into mannooligosaccharides with 6 to 2 mannose monomers without mannose detected which was similar to the wild type β-mannanase. Hydrolysis of various mannooligosaccharide substrates showed the random manner of this recombinant enzyme which randomly hydrolyzed mannooligosaccharide based on the length of

substrate. Recombinant β-mannanase required at least 4 mannose monomers for degradation and the main products were mannobiose, mannotriose and mannotetraose depended on the substrate length.

This study showed the efficient expression system for production of β-mannanase from *B. circuans* NT 6.7 which have high enzyme activity and secretion. The recombinant β-mannanase also has the good enzyme characteristics including optimum temperature and pH with high specificity, high stability and the effective hydrolysis of mannan substrates. This recombinant β-mannanase can be used for several applications especially prebiotic mannooligosaccharides production from natural resources with cheap and high galactomannan content such as copra meal.

LITERATURE CITED

Adibmoradi, M. and M. Mehri. 2007. Effects of β-mannanase on broiler performance and gut morphology, pp 471–47. *In* **16th European Symposium on Poultry Nutrition**, Stasburg, France

Alam, N. H., R. Meier, H. Schneider, S. A. Sarker, P. K. Bardhan, D. Mahalanabis, G. J. Fuchs and N. Gyr. 2000. Partially hydrolyzed guar gum supplemented oral rehydration solution in the treatment of acute diarrhea in children. **J Ped Gas Nut.** 31: 503–507.

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

Araujo, A. and O. P. Ward. 1990. Hemicellulases of Bacillus species: Preliminary comparative studies on production and properties of mannanases and galactanases. **J Appl Bacteriol.** 8: 253-261**.**

Aspinall, G. O. 1959. Structural chemistry of the hemicelluloses. **Adv Carbohydr Chem.** 14: 429–468.

Aspinall, G. O., R. Begbie and J. E. McKay. 1962. The hemicelluloses of European larch (*Larix decidua*). Part II. **J Chem Soc.** 214: 219.

Balasubramaniam, K. 1976. Polysaccharides of the kernel of maturing and mature coconuts. **J Food Sci.** 41: 1370-1373.

Baurhoo, B., P. R. Ferket and X. Zhao. 2009. Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. **Poult. Sci.** 88(11): 2262–72.

Benech, R. O., X. Li, D. Patton, J. Powlowski, R. Storms, R. Bourbonnais, M. Paice and A. Tsang. 2007. Recombinant expression, characterization, and pulp prebleaching property of a *Phanerochaete chrysosporium* endo-β-1,4-mannanase. **Enzyme Microb Technol.** 41: 740–747.

Bhoria, P., G. Singh and G. S. Hoondal. 2009. Optimization of mannanase production from *Streptomyces* sp. PG-08-03 in submerged fermentation. **Bioresources.** 4(3): 1130–1138.

Biggs, P. and C. M. Parsons. 2007. The effects of several oligosaccharides on true amino acid digestibility and true metabolizable energy in cecectomized and conventional roosters**. Poult Sci**. 86(6): 1161-1165.

Boucher, I., M. Parrot, H. Gaudreau, C. P. Champagne, C. Vadeboncoeur and S. Moineau. 2002. Novel food-grade plasmid vector based on melibiose fermentation for the genetic engineering of *Lactococcus lactis*. **Appl Environ Microbiol.** 68(12): 6152–61.

Bresolin, T. M., P. C. Sandler, F. Reicher, M. R. Sierakowski, M. Rinaudo and J. L. M. Ganter. 1997. Viscometric studies on xanthan and galactomannan systems. **Carbohydr Polym.** 33: 131–138.

Brurberg, M. B., I. F. Nes and V. G. H. Eijsink. 1997. Pheromone-induced production of antimicrobial peptide in *Lactobacillus*. **Mol Microbiol.** 26(2): 347-360.

Buchert, J., J. Salminen, M. Sika-aho, M. Ranua and L. Viikari. 1993. The role of *Trichoderma reesei* xylanase and mannanase in the treatment of softwood kraft pulp prior to bleaching. **Holzforschung.** 47: 473-478.

Chauhan, P. S., N. Puri, P. Sharma and N. Gupta. 2012. Mannanases: microbial sources, production, properties and potential biotechnological applications. **Appl Microbiol Biotechnol.** 93: 1817–1830.

Chen, X., W. Lu, Y. Cao and D. Li. 2008. Prokaryotic expression, purification and characterization of *Aspergillus sulphureus β*-mannanase and site-directed mutagenesis of the catalytic residues. **Appl Biochem Biotechnol**. 149: 139–144.

Choi, J. H., and S. Y. Lee. 2004. Secretory and extracellular production of recombinant proteins using Escherichia coli. **Appl. Microbiol. Biotechnol.** 64: 625-623.

Da Costa, K. S., E. Leal, A. M. Dos Santos, A. H. Lima E Lima, C. N. Alves and J. Lameira. 2014. Structural analysis of viral infectivity factor of HIV type 1 and its interaction with A3G, EloC and EloB. PLos One. 9(2): 1-9.

Daskiran, M. R. G., D. W. Teeter, D. Fodge and H. Y. Hsiao. 2004. An evaluation of endo-ß-D-mannanase (Hemicell) effects on broiler performance and energy use in diets varying in β-mannan content. **Poult Sci.** 83: 662–668.

Davies, G. J. K. S. Wilson and B. Henrissat. 1997. Nomenclature for sugar binding subsites in glycosyl hydrolases. **Biochemistry.** 321: 557–559.

Dhawan, S. and J. Kaur. 2007. Microbial mannanases: an overview of production and applications. **Crit Rev Biotechnol.** 27(4): 197-216.

Duffaud, G. D., C. M. McCutchen, P. Leduc, K. L. Parker and R. M. Kelly. 1997. Purification and characterization of extremely thermostable β-mannanase, β-mannosidase, and α-galactosidase from the hyperthermophilic Eubacterium *Thermotoga neapolitana* 5068. **Appl Environ Microbiol.** 63: 169–177.

Dutta, S., J. K. Bradford and J. D. Nevins. 1997. Endo-betamannanase activity present in cell wall extracts of lettuce endosperm prior to radicle emergence. **Plant Physiol.** 113: 155-161.

El Demerdash, H. A., K. J. Heller and A. Geis. 2003. Application of the *shsp* gene, encoding a small heat shock protein, as a food-grade selection marker for lactic acid bacteria. **Appl Environ Microbiol.** 69(8): 4408–12.

Feig, M., A. Onufriev, M. S. Lee, W. Im , D. A. Case and C. L.Brooks. 2004. Performance comparison of generalized born and Poisson methods in the calculation of electrostatic solvation energies for protein structures. **J. Comput. Chem.** 25(2): 265–284.

Fu, X., X. Huang, P. Liu, L. Lin, G. Wu, C. Li, C. Feng and Y. Hong. 2010. Cloning and characterization of a novel mannanase from *Paenibacillus* sp. BME-14. **J Microbiol Biotechnol.** 20(3): 518–524.

Gasteiger, E., C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel and A. Bairoch. 2005. *Protein Identification and Analysis Tools on the ExPASy Server*. *In* J. M. Walker, eds. **The Proteomics Protocols Handbook.** Humana Press, New York.

Gibson, G. R., A. L. McCartney and R. A. Rastall. 2005. Prebiotics and resistance to gastrointestinal infections**. Br J Nutr.** 93(Suppl 1): 31-34.

Grosdidier, A., V. Zoete and O. Michielin. 2007. EADock: Docking of small molecules into protein active sites with a multiobjective evolutionary optimization. **Proteins.** 67(4): 1010-1025.

Haemin, P. 2008. **Cloning and Expression of Mannanase Gene from *Bacillus amyloliquefaciens* NT 6.3 and *Bacillus circulans* NT 6.7.** M.Sc. Thesis, Kasetsart University.

Halbmayr, E., G. Mathiesen, T. H. Nguyen, T. Maischberger, C. K. Peterbauer, V. G. H. Eijsink and D. Haltrich. 2008. High-level expression of recombinant β-galactosidases in *lactobacillus plantarum* and *lactobacillus sakei* using a sakacin p-based expression system. **J Agric Food Chem.** 56: 4710–4719.

Henrissat, B. and A. Bairoch. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. **J Biochem**. 293: 781–788.

Henrissat. B, I. Callebaut, S. Fabrega, P. Lehn. J. Mornon and G. Davie. 1995. Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. **Proc Natl Acad Sci.** 92: 7090-7094.

Hickey, R. M., R. P. Ross and C. Hill. 2004. Controlled autolysis and enzyme release in a recombinant lactococcal strain expressing the metalloendopeptidase enterolysin A. **Appl Environ Microbiol.** 70: 1744–1748.

Hogg, D., G. Pell, P. Dupree, F. Goubet, S. M. Martin-oure, S. Armand and H. J. Gilbert. 2003. The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation. **Biochem J.** 371: 1027-1043

Hongshu, Z., Y. Jinggan and Z. Yan. 2002. The glucomannan from ramie. **Carbohydr Polym.** 47: 83–86.

Invitrogen™. 2010. **BL21 Star™(DE3) One Shot®, BL21 Star™(DE3)pLysS One Shot® chemically competent cells.** Life Technology Corporation, California.

Izaguirre, G. and J. N. Hansen. 1997. Use of alkaline Phosphatase as a reporter polypeptide to study the role of subtilin leader segment and the SpaT transporter in the posttranslational modifications and secretion of subtilin in *Bacillus subtilis* 168. Appl Environ Microbiol. 63(10): 3965-3971.

Kai, K., S. Noguchi and N. Sone. 1997. Over-expression and post-translational modification of thermophilic *Bacillus* cytochrome *c*-551 in *Bacillus subtilis*. **J Ferment Bioeng.** 84(3): 190-194.

Kiley, T. B. and N. R. Stanley-Wall. 2010. Post-translational control of *Bacillus subtilis* biofilm formation mediated by tyrosine phosphorylation. **Mol Microbiol.** 78(4): 947-963.

Kitchen, D. B., H. Decornez, J. R. Furr and J. Bajorath. 2004. Docking and scoring in virtual screening for drug discovery: methods and applications. **Nat. Rev. Drug Discov.** 3(11): 935–949.

Krieger, E., S. B. Nabuurs and G. Vriend. 2003. Homology modeling, pp 507-521. *In* P. E. Bourne and H. Weissig, eds. **Structural Bioinformatic.** John Wiley & Sons, New Jersey.

Lee, J. T., C. A. Bailey and A. L. Cartwright. 2003. Beta-mannanase ameliorates viscosity-associated depression of growth in broiler chickens fed guar germ and hull fractions. **Poult. Sci.** 82: 1925-1931.

Lengauer, T. and M. Rarey. 1996. Computational methods for biomolecular docking. **Curr. Opin. Struct. Biol.** 6(3): 402-406.

Li, Y. N., K. Meng, Y. R. Wang and B. Yao. 2006. A β-mannanase from *Bacillus subtilis* B36: purification, properties, sequencing, gene cloning and expression in *Escherichia coli*. **Z Naturforsch. 61c:** 840-846.

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

Liepman, A. H., C. J. Nairn, W. G. T. Willats, I. Sorensen, A. W. Roberts and K. Keegstra. 2007. Functional genomic analysis supports conservation of function among cellulose synthase-like A gene family members and suggest diverse roles of mannans in plants. **Plant Physiol.** 143:1881–1893.

Luo, H., Y, Wang, H. Wang, J. Yang, Y. Yang, H. Huang, P. Yang, Y. Bai, P. Shi, Y. Fan and B. Yao. 2009. A novel highly acidic β-mannanase from the acidophilic fungus *Bispora* sp. MEY-1: gene cloning and overexpression in *Pichia pastoris*. **Appl Microbiol Biotechnol.** 82: 453–461.

Ma, Y., Y. Xue, D. Yuetan, X. Zhenghong, T. Wenyi and Z. Peijin. 2004. Characterization and gene cloning of a novel β-mannanase from alkaliphilic *Bacillus* sp. N16-5. **Extremophiles.** 8:447–454.

Maischberger, T., I. Mierau, C. K. Peterbauer, J. Hugenholtz and D. Haltrich. 2010. High-level expression of lactobacillus β-galactosidases in lactococcus lactis using the food-grade, nisin-controlled expression system NICE. **J Agric Food Chem.** 58: 2279–2287.

Mathiesen, G., E. Sorvig, J. Blatny, K. Naterstad, L. Axelsson and V. G. H. Eijsink. 2004. High-level gene expression in *Lactobacillus plantarum* using a pheromone-regulated bacteriocin promoter. **Lett Appl Microbiol.** 39(2): 137–143.

Meier, H. and J. S. G. Reid. 1982. Reserve Polysaccharides other than starch in higher plants, pp 418–471. *In* F. A. Loewus and W. Tanner, eds. **Encyclopedia of Plant.** vol. 13A. Springer, Berlin**.**

Mergulhão, F. J. M., D. K. Summers and G. A. Monteiroa. 2005. Recombinant protein secretion in *Escherichia coli*. **Biotechnol Adv.** 23: 177-202.

Miller, G. L. 1959. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. **Anal Chem.** 31: 426-428.

Moreira, L. R. S. and E. X. F. Filho. 2008. An overview of mannan structure and mannan-degrading enzyme systems. **Appl Microbiol Biotechnol.** 79: 165–178.

Nguyen, H. A., T. H. Nguyen, V. Křen, V. G. H. Eijsink, D. Haltrich, and C. K. Peterbauer. 2012. Heterologous expression and characterization of an *N*-acetyl-*β*-D-hexosaminidase from *Lactococcus lactis* ssp. lactis IL1403. **J Agric Food Chem.** 60: 3275−3281.

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

Nguyen T. T., G. Mathiesen, L. Fredriksen, R. Kittl, T. H. Nguyen, V. G. H. Eijsink, D. Haltrich and C. K. Peterbauer. 2011. A food-grade system for inducible gene expression in *Lactobacillus plantarum* using an alanine racemase-encoding selection marker. **J Agric Food Chem.** 59: 5617–5624.

Nguyen T. T., H. A. Nguyen, S. L. Arreola, G. Mlynek, K. Djinovic-Carugo, G. Mathiesen, T. H. Nguyen and D. Haltrich. 2012. Homodimeric β-galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081: expression in *Lactobacillus plantarum* and biochemical characterization. **J Agric Food Chem.** 60: 1713−1721.

Nitta, T., H. Nagamitsu, M. Murata, H. Izu and M. Yamada. 2000. Function of the σ^E regulon in dead-cell lysis in stationary-phase *Escherichia coli*. **J Bacteriol.** 182(18): 5231-5237.

Northcote, D. H. 1972. Chemistry of the plant cell wall. **Annu Rev Plant Physiol.** 23: 113–132.

Novagen®. 2003. **pET system manual.** 10th ed. EMD Biosciences, USA.

Parisi, G. C., M. Zilli, M. P. Miani, M. Carrara, E. Bottona, G. Verdianelli, G. Battaglia, S. Desideri, A. Faedo, C. Marzolino, A. Tonon, M. Ermani and G. Leandro. 2002. High-fibre diet supplementation in patients with irritable bowel syndrome (IBS): a multicenter, randomized, open trial comparison between wheat bran diet and partially hydrolyzed guar gum (PHGG) **Diges Disea Sci.** 47(8): 1697–1704.

Pan, X., J. Zhou, A. Tian, K. Le, H. Yuan, Y. Xue, Y. Ma and H. Lu. 2011. High level expression of a truncated β-mannanase from alkalophilic *Bacillus* sp. N16-5 in *Kluyveromyces cicerisporus*. **Biotechnol Lett.** 33: 565–570.

Pangsri, P. 2014. **Purification and characterization of mannanase from *Bacillus circulans* NT 6.7 and its application in mannooligosaccharides preparation.** Ph.D. Thesis, Kasetsart University.

Peterbauer, C. K., T. Maischberger and D. Haltrich. 2011. Food-grade gene expression in lactic acid bacteria. **Biotechnol. J.** 6: 1147–1161.

Petersen, T. N., S. Brunak , G. von Heijne, and H. Nielsen. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. **Nat Methods.** 8: 785-786.

Petkowicz, C. L. O., F. Reicher, H. Chanzy, F. R. Taravel and R. Vuong. 2001. Linear mannan in the endosperm of *Schizolobium amazonicum*. **Carbohydr Polym.** 44:107–112.

Phothichitto, K. 2006. **Isolation and Characterization of mannanase producing microorganism.** M.Sc. Thesis, Kasetsart University.

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

Popa, V. I. and J. Spiridon. 1998. Hemicelluloses: structure and properties, pp 297–311. *In* S. Dumitriu, eds. **Polysaccharides: structural diversity and functional versatility.** Marcel Dekker, New York.

Puchar, V., M. Vrsanska, P. Svoboda, J. Pohl, B. Z. Ogel and P. Biely. 2004. Purification and characterization of two forms of endo-beta-1,4-mannanase from a thermotolerant fungus, Aspergillus fumigatus IMI 385708 (formerly Thermomyces lanuginosus IMI158749). **Biochim Biophys Acta.** 1674: 239-250.

Puls, J. 1997. Chemistry and biochemistry of hemicelluloses-relationship between hemicellulose structure and enzymes required for hydrolysis. **Macromol Symp.** 120: 183-196.

Puls, J. and J. Schuseil. 1993. Chemistry of hemicellulose: relationship between hemicellulose structure and enzyme required for hydrolysis, pp. 1–27. *In* M. P. Coughlan and G. P. Hazlewood, eds. **Hemicellulose and hemicellulases.** Portland Press Ltd., London

Rastall, R. A., G. R. Gibson, H. S. Gill, F. Guarner, T. R. Klaenhammer, B. Pot, G. Reid, I. R. Rowland and M. E. Sanders. 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications**. FEMS Microbiol Ecol.** 52(2): 145-152.

Regalado, C., B. E. Garcia-Almendarez, L. M. Venegas-Barrera, A. Tellez-Jurado, G. Rodriguez-Serrano, S. Huerta-Qchoa, J. R. Whitaker. 2000. Production, partial purification and properties of β-mannanase obtained by solid substrate fermentation of spent soluble coffee wastes and copra paste using *Aspergillus oryzae* and *Aspergillus niger*. **J Sci Food Agric.** 80: 1343–50.

Sakulsirirat, S. 2008. **Cloning and Structural Analysis of Genes Encoding Mannanase from *Bacillus amyloliquefaciens* NT6.3 and *Bacillus circulans* NT6.7.** M.Sc. Thesis, Kasetsart University.

Sittikijyothin, W., D. Torres and M. P. Goncalves. 2005. Modelling the rheological behaviour of galactomannan aqueous solutions. **Carbohydr Polym.** 59: 339–350.

Smith, D. L. Jr., T. R. Nagy, L. S. Wilson, S. Dong, S. Barnes and D. B. Allison. 2010. The Effect of mannan oligosaccharide supplementation on body weight gain and fat accrual in C57Bl/6J Mice**. Obesity (Silver Spring).** 18(5): 995–999.

Songsiriritthigul, C., B. Buranabanyat, D. Haltrich, and M. Yamabhai. 2010. Efficient recombinant expression and secretion of a thermostable GH26 mannan endo -1,4-β-mannosidase from *Bacillus licheniformis* in *Escherichia coli*. **Microb Cell Fac.** 9(20): 1-13.

Sorvig, E., G. Mathiesen, K. Naterstad, V. G. H. Eijsink and L. Axelsson. 2005. High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. **Microbiology.** 151(Pt7): 2439–2449.

Sorvig, E., S. Gronqvist, K. Naterstad, G. Mathiesen, V. G. H. Eijsink and L. Axelsson. 2003. Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *L. plantarum*. **FEMS Microbiol Lett.** 229(1): 119–126.

Spring, P. C. Wenk, K. A. Dawson and K. E. Newman. 2000. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. **Poult Sci.** 79(2): 205–211.

Stoll, D., A. Boraston, H. Stålbrand, B. W. McLean, D. G. Kilburn and A. J. Warren. 2000. Mannanase Man26A from *Cellulomonas fimi* has a mannan-binding module. **FEMS Microbiol Lett.** 183: 265–269.

Studier, F.W. and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high- level expression of cloned genes. **J Mol Biol.** 189: 113-130.

Suurnakki, A., M. Tenkanen, J. Buchert and L. Viikari. 1997. Hemicellulases in the bleaching of chemical pulps. **Adv Biochem Eng Biotechnol.** 57: 261-287.

Takala, T. M. and P. E. Saris. 2002. A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene nisI. **Appl Microbiol Biotechnol.** 59 (4-5): 467–71.

Takeno, F., H. Yamada, K. Sekiya, B. Fujitani and K. Ohtsu. 1990. Effect of partially decomposed guar gum on high-cholesterol-fed rats and non-dietary fiber-fed rats. **J Jpn Soc Nutr Food Sci.** 43: 421–425.

Tamamura, Y., T. Araki, H. Amagoi, H. Mori and T. Morishita. 1995. Purification and characterization of an extracellular beta-1,4-mannanase from a marine bacterium, *Vibrio* sp. strain MA-138. Appl Environ Microbiol. 61(12): 4454-4458.

Timell, T. E. 1967. Recent progress in the chemistry of wood hemicelluloses. **Wood Sci Technol.** 1: 45–70.

Titapoka, S., S. Keawsompong, D. Haltrich and S. Nitisinprasert. 2008. Selection and characterization of mannanase producing bacteria useful for the formation of pre biotic manno oligosaccharides from copra meal. **World J Microbiol Biotechnol.** 24: 1425–1433.

Vahedi, F., F. Khatami and A. Es-haghi. 2011. The stability of pET21 + PA, a pET derived plasmid, under different culture conditions. **World J Microbiol Biotechnol.** 27: 253–257.

Van Zyl, W. H., S. H. Rose, K. Trollope and J. F. Gorgens. 2010. Fungal β-mannanases: mannan hydrolysis, heterologous production and biotechnological applications. **Pro Biochem.** 45: 203–1213.

Wegmann, U., J. R. Klein, I. Drumm, O. P. Kuipers and B. Henrich. 1999. Introduction of peptidase genes from *Lactobacillus delbrueckii* subsp. *lactis* into *Lactococcus lactis* and controlled expression. **Appl Environ Microbiol.** 65: 4729–4733.

Wong, K. K. Y. and J. N. Saddler. 1993. Applications of hemicellulases in the food, feed, and pulp and paper industries, pp. 127-143. *In* M. P. Coughlan and G. P. Hazlewood, eds. **Hemicellulose and Hemicellulases.** Portland Press Ltd., London

Ximenes, E. A., H. Chen, I. A. Kataeva, M. A. Cotta, C. R. Felix, L. G. Ljungdahl and X. L. Li. 2005. A mannanase, ManA, of the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2 has carbohydrate binding and docking modules. **Can J Microbiol.** 51: 559–568.

Yan, X. X., X. M. An, L. L. Gui and D. C. Liang. 2008. From structure to function: insights into the catalytic substrate specificity and thermostability displayed by *Bacillus subtilis* mannanase BCman. **J Mol Biol.** 379: 535–544.

Yamabhai, M., B. Buranabanyat, N. Jaruseranee and C. Songsiriritthigu. 2011. Efficient *Escherichia coli* expression systems for the production of recombinant β-mannanases and other extracellular enzymes. **Bioengineered Bugs.** 2(1): 45-49.

Yang, P., Y. Li, Y. Wang, K. Meng, H. Luo, T. Yuan, Y. Bai, Z. Zhan and B. Yao. 2009. A novel *β*-mannanase with high specific activity from *Bacillus circulans* CGMCC1554: Gene cloning, expression and enzymatic characterization. **Appl Biochem Biotechnol.** 159: 85–94.

Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen and M. Choct. 2008. Effects of mannanoligosaccharide and fructooligosaccharide on the response of broilers to pathogenic Escherichia coli challenge. **Br Poult. Sci.** 49(5): 550–9.

Yoon, K. H. and B. L. Lim**.** 2007. Cloning and strong expression of a *Bacillus subtilis* WL-3 mannanase gene in *B. subtilis*. **Microbiol. Biotechnol.** 17(10): 1688–1694.

Yoon, K. H., S. Chung and B. L. Lim. 2008. Characterization of the *Bacillus subtillis* WL-3 mannanase from a recombinant *Escherichia coli*. **J Microbiol.** 46(3): 344-349.

Zhang, Y., J. Ju, H. Peng, F. Gao, C. Zhou, Y. Zeng, Y. Xue, Y. Li, B. Henrissat, G. F. Gao and Y. Ma. 2008. Biochemical and structural characterization of the intracellular mannanase AaManA of *Alicyclobacillus acidocaldarius* reveals a novel glycoside hydrolase family belonging to clan GH-A. **J Biol Chem.** 283 (46): 31551–31558.

APPENDIX

APPENDIX

1. Media for bacteria cultivation

1.1 Lauria Bertani (LB) media

Bacto tryptone	10	g
Yeast extract	5	g
NaCl	10	g
Bacteria agar (for agar plate)	15	g

All components (except agar) were dissolved in 1 L of distilled water and sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes.

1.2 MRS media

MRS	55	g
Bacteria agar (for agar plate)	15	g

All components (except agar) were dissolved in 1 L of distilled water and sterilize by autoclaving at 121°C under pressure of 15 lb/square inch for 15 minutes.

2. Determination of reducing sugar by DNS method

2.1 Reagent

2.1.1 DNS reagent

3,5-Dinitrosalicylic acid	10	g
NaOH	16	g
$NaKC_4H_4O_6$ (Rochelle salt)	300	g

3,5-Dinitrosalicylic acid was dissolved in distilled water then NaOH was added and allowed to dissolved. The solution was warmed to the temperature of 45°C then slowly added $NaKC_4H_4O_6$ into the solution. After completely dissolved, the volume was adjusted to 1L by distilled water. Then, the solution was filtered and kept in dark.

2.1.2 Mannose standard (2 mg/ml)

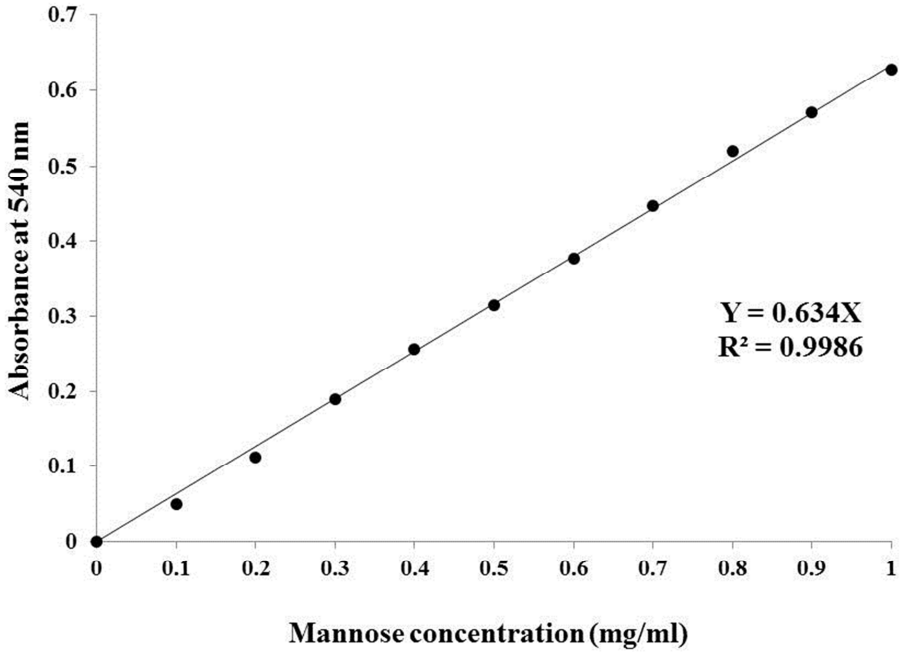
20 mg of Mannose was dissolved in distilled water and the volume was adjusted to 10 ml.

2.2 Method

200 µl of sample was mixed with 200 µl of DNS solution in test tube. The reaction mixture was incubated in the boiling water for 5 min then stopped the reaction by immediately cool down in ice-bath. Two ml of distilled water was added and mixed well. Absorbance at 540 nm was measured by spectrophotometer.

2.3 Mannose standard curve

Mannose solution was prepared in the concentration of 0.1-1.0 mg/ml. The reactions were performed with the method as described above. The standard curve was plotted as absorbance at 540 nm against mannose concentration as in Appendix Figure 1.



Appendix Figure 1 Standard curve of mannose from DNS method

3. Determination of protein concentration by Bradford assay

3.1 Reagent

3.1.1 Dye Reagent Concentrate (Bio-Rad)

Dye reagent for Bradford method was prepared by diluting 1 part of Dye Reagent Concentrate in 4 part of distilled, deionized water. Then, the solution was filtered and kept in dark at 4°C.

3.1.2 Bovine serum albumin (BSA)

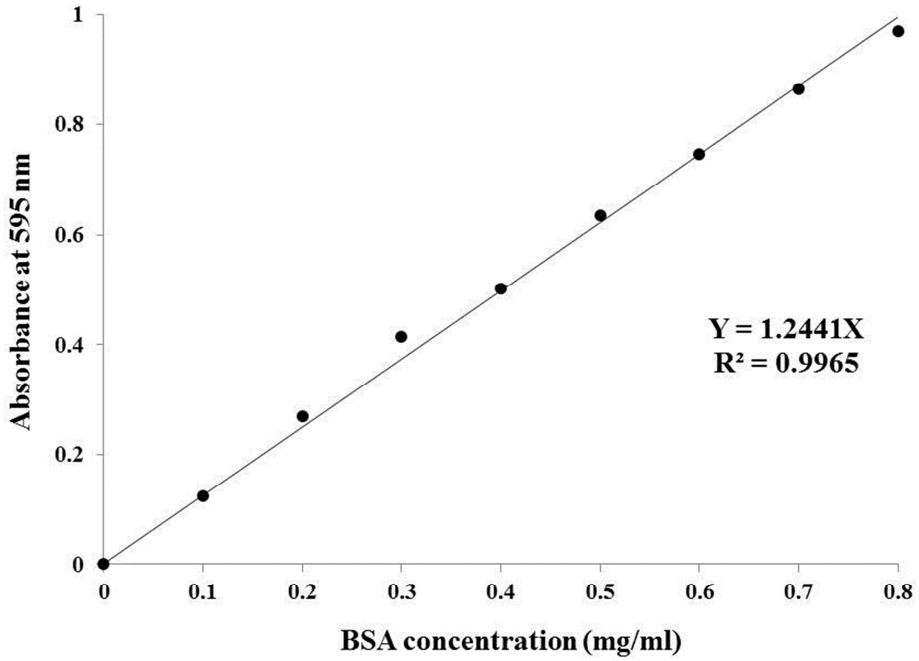
20 mg of BSA was dissolved in distilled, deionized water and the volume was adjusted to 10 ml.

3.2 Method

100 µl of sample was mixed with 5 ml of diluted dye reagent. The reaction mixture was incubated at room temperature for 5 min. Absorbance at 595 nm was measured by spectrophotometer.

3.3 BSA standard curve

BSA solution was prepared in the concentration of 0.1-1.0 mg/ml. The reactions were performed with the method as described above. The standard curve was plotted as absorbance at 595 nm against BSA concentration as in Appendix Figure 2.



Appendix Figure 2 Standard curve of protein concentration from Bradford assay

4. Buffer preparation

4.1 Citrate buffer (0.1 M)

Citrate buffer with different pH was prepared by mixing of 0.2 M citric acid and 0.2 M sodium citrate at different volume as shown in Appendix Table 1. The volume was adjusted to 100 ml by distilled, deionized water.

Appendix Table 1 Citrate buffer preparation

0.2 M Citric acid (ml)	0.2 M Sodium citrate (ml)	pH
46.5	3.5	3
33	17.0	4
20.5	29.5	5

4.2 Potassium phosphate buffer

Potassium phosphate buffer with different pH was prepared by mixing of 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 at different volume as shown in Appendix Table 2. The volume was adjusted to 100 ml by distilled, deionized water.

Appendix Table 2 Potassium phosphate buffer preparation

0.2 M KH_2PO_4 (ml)	0.2 M K_2HPO_4 (ml)	pH
43.9	6.1	6
19.5	30.5	7
2.7	47.3	8

4.3 Glycine-NaOH buffer

Glycine-NaOH buffer with different pH was prepared by mixing of 0.2 M glycine and 0.2 M NaOH at different volume as shown in Appendix Table 3. The volume was adjusted to 100 ml by distilled, deionized water.

Appendix Table 3 Glycine-NaOH buffer preparation

0.2 M glycine (ml)	0.2 M NaOH (ml)	pH
50	4.4	9
50	16.0	10

5. Molecular docking

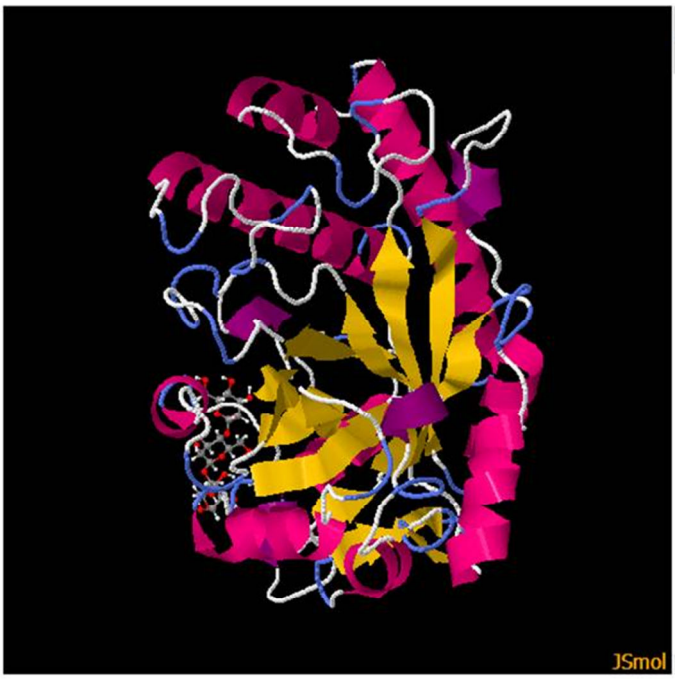
Molecular docking results of β-mannanase from *B. circulans* NT 6.7 with mannobiose, mannotriose and mannotetraose was shown in Appendix Figure 3.

A

Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	0	0	-1315.01	-7.18
○	0	1	-1314.32	-7.67
○	0	2	-1310.86	-7.06
○	0	3	-1309.96	-7.57
○	0	4	-1308.17	-7.35
○	0	5	-1304.96	-6.55
○	1	0	-1312.35	-7.11
○	1	1	-1312.24	-7.01
○	1	2	-1312.13	-7.03
○	1	3	-1310.15	-6.78
○	1	4	-1306.92	-6.42
○	1	5	-1304.18	-6.99
○	1	6	-1300.27	-7.37
○	1	7	-1294.95	-6.30
○	2	0	-1311.54	-6.81
○	2	1	-1311.51	-6.80
○	2	2	-1308.90	-7.16
○	2	3	-1307.69	-6.64
○	2	4	-1298.98	-6.77
○	3	0	-1311.38	-7.00
○	3	1	-1310.95	-7.30

Appendix Figure 3 Molecular docking of β-mannanase from *B. circulans* NT 6.7 with mannobiose (A), mannotriose (B) and mannotetraose (C).

B



Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	0	0	-1243.10	-7.52
○	0	1	-1242.44	-7.64
○	0	2	-1241.16	-7.64
○	0	3	-1230.78	-7.01
○	1	0	-1242.72	-7.49
○	1	1	-1242.21	-7.43
○	1	2	-1242.21	-7.43
○	1	3	-1242.21	-7.43
○	1	4	-1240.24	-7.23
○	1	5	-1228.03	-7.07
○	1	6	-1223.29	-6.80
○	1	7	-1222.85	-6.89
○	2	0	-1242.37	-7.46
○	2	1	-1242.30	-7.46
○	2	2	-1241.29	-7.19
○	3	0	-1242.34	-8.25
○	3	1	-1238.58	-8.12
○	3	2	-1238.58	-8.12
○	3	3	-1238.58	-8.12
○	3	4	-1238.58	-8.12
○	3	5	-1236.46	-8.08

C

Show	Cluster	Element	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
◉	0	0	-1172.93	-8.72
○	0	1	-1168.95	-8.32
○	0	2	-1160.63	-7.49
○	0	3	-1159.02	-7.03
○	0	4	-1156.45	-7.92
○	0	5	-1153.97	-8.39
○	0	6	-1153.76	-8.38
○	0	7	-1151.66	-8.13
○	1	0	-1169.30	-8.00
○	1	1	-1166.22	-7.65
○	1	2	-1166.22	-7.65
○	1	3	-1166.22	-7.65
○	1	4	-1166.22	-7.65
○	1	5	-1166.22	-7.65
○	1	6	-1164.43	-7.45
○	1	7	-1164.43	-7.45
○	2	0	-1168.61	-8.20
○	2	1	-1168.61	-8.20
○	2	2	-1164.72	-8.12
○	2	3	-1164.72	-8.12
○	2	4	-1164.72	-8.12

Appendix Figure 3 (Continued)

6. Effect of temperature on β-mannanase activity

Appendix Table 4 β-Mannanase activity at different temperature (100 ml of cultivation)

Temperature	β-Mannanase activity (u/ml)	
	Extracellular fraction	Intracellular fraction
30	21.57	185.80
40	26.48	221.06
50	37.14	363.82
60	14.94	178.99
70	0.15	4.17
80	0.09	1.35

Appendix Table 5 β-mannanase activity after incubation for different time at 50°C, pH 6.0 (100 ml of cultivation)

Time of incubation (h)	β-Mannanase activity (u/ml)	
	Extracellular fraction	Intracellular fraction
0	36.92	363.82
6	25.23	330.21
12	25.24	329.53
24	26.00	328.80
48	16.55	330.74
72	4.10	245.87
96	0.00	179.96

7. Effect of pH on β-mannanase activity

Appendix Table 6 β-Mannanase activity at different pH (100 ml of cultivation)

Temperature	β-Mannanase activity (u/ml)	
	Extracellular fraction	Intracellular fraction
3	0.19	0.11
4	0.57	0.45
5	32.84	314.69
6	37.14	363.82
7	35.60	347.52
8	5.08	94.55
9	1.69	37.01
10	0.65	0.74

Appendix Table 7 β-mannanase activity after incubation in different pH at 50°C for 1 h (100 ml of cultivation)

Time of incubation (h)	β-Mannanase activity (u/ml)	
	Extracellular fraction	Intracellular fraction
3	0.08	0.000
4	1.18	0.000
5	10.12	169.99
6	28.31	319.56
7	22.62	268.48
8	18.46	261.43
9	18.64	213.52
10	6.52	144.94

8. Effect of various metal ions and chemical on β-mannanase activity

Appendix Table 8 β-mannanase activity after incubation with different metal ions and chemical at 50°C, pH 6.0 for 1 h (100 ml of cultivation)

Time of incubation (h)	β-Mannanase activity (u/ml)	
	Extracellular fraction	Intracellular fraction
Control	42.75	445.04
Co^{+}	44.78	473.17
Ca^{+}	40.80	432.98
Zn^{2+}	41.68	410.51
Mn^{2+}	40.72	425.86
Mg^{2+}	40.32	424.84
Li^{+}	41.39	434.81
Cu^{2+}	33.79	285.18
Fe^{2+}	17.15	174.37
EDTA	13.68	168.89

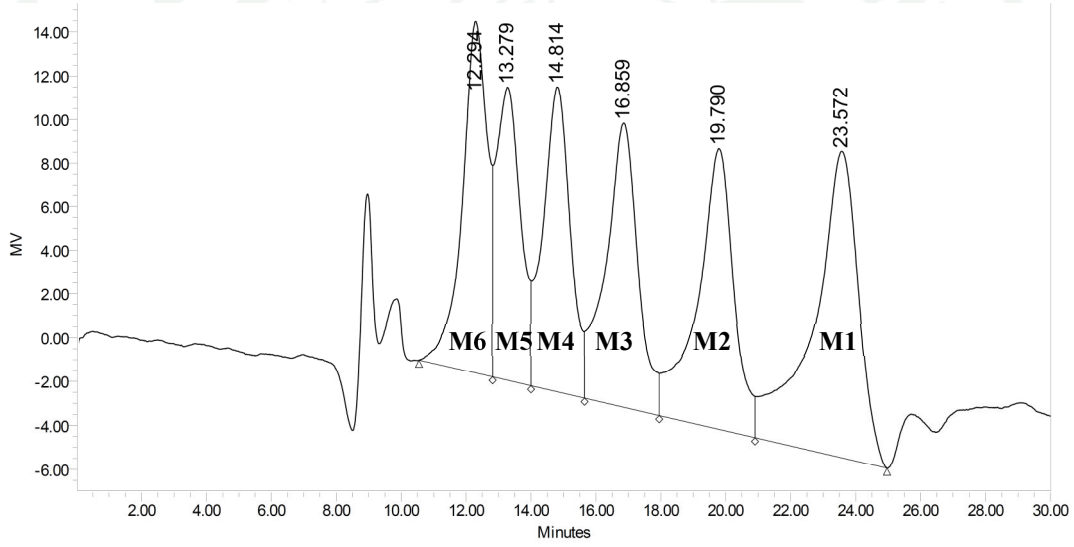
9. Effect of various substrates on β-mannanase activity

Appendix Table 9 β-mannanase activity on the different substrates (100 ml of cultivation)

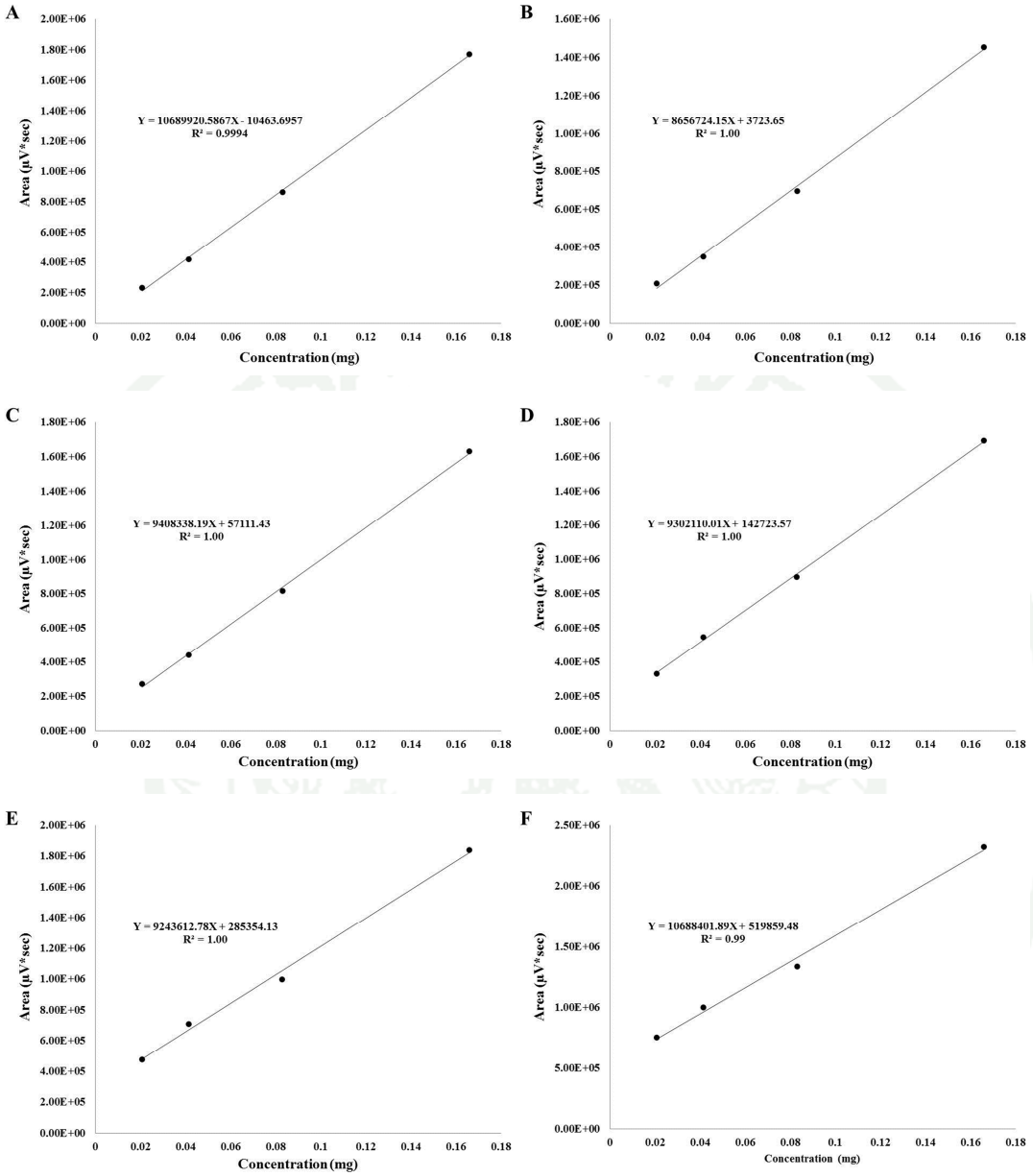
Substrate	β-Mannanase activity (u/ml)	
	Extracellular fraction	Intracellular fraction
Locust bean gum (LBG)	37.14	361.87
Konjac glucomannan	18.75	194.80
Ivory nut mannan	22.98	247.33
Defatted copra meal	1.21	2.16
Gour gum	0.42	1.74
Copra meal	0.13	0.53
α-mannan (yeast cell wall)	0.00	0.00
Xylan from oat spelts	0.00	0.00
Carboxymethylcellulose (CMC)	0.00	0.00
Avicel	0.00	0.00

10. Determination of the amount of mannooligosaccharides in hydrolysis product

Standard mannooligosaccharides (M6-M1) were mixed together and diluted by 2 fold to get the final concentration of 0.166, 0.083, 0.0415 and 0.02075 mg/each mannooligosaccharides. The standard mannooligosaccharide mixture in each concentration was analyzed by HPLC using water as mobile phase with flow rate of 0.4 ml/min as in Figure A3. The standard curve was plotted as peak area against mannooligosaccharide concentration as in Figure A4. The amount of each mannooligosaccharide was calculated from the equation from each standard curve.

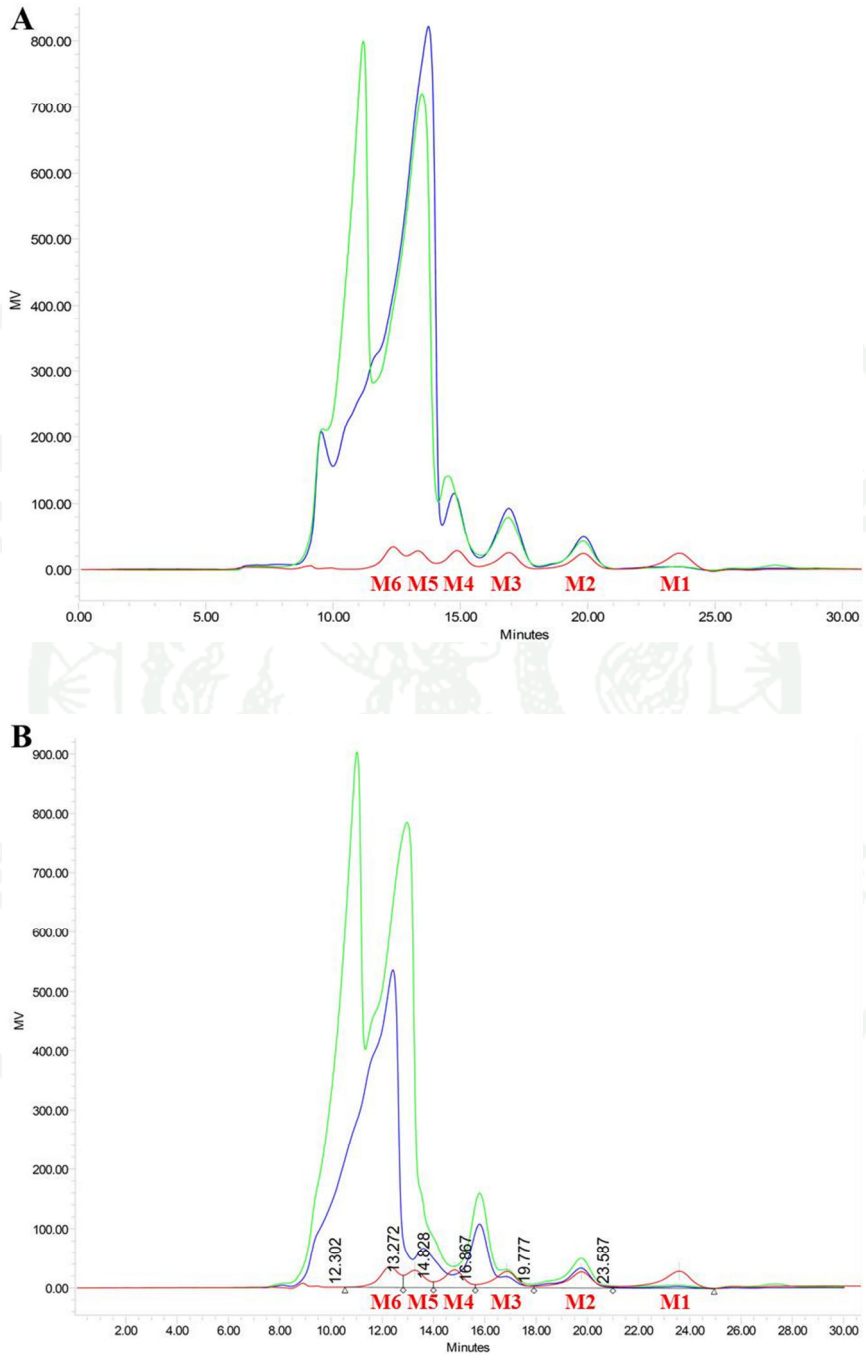


Appendix Figure 4 Chromatogram of standard mannooligosaccharides

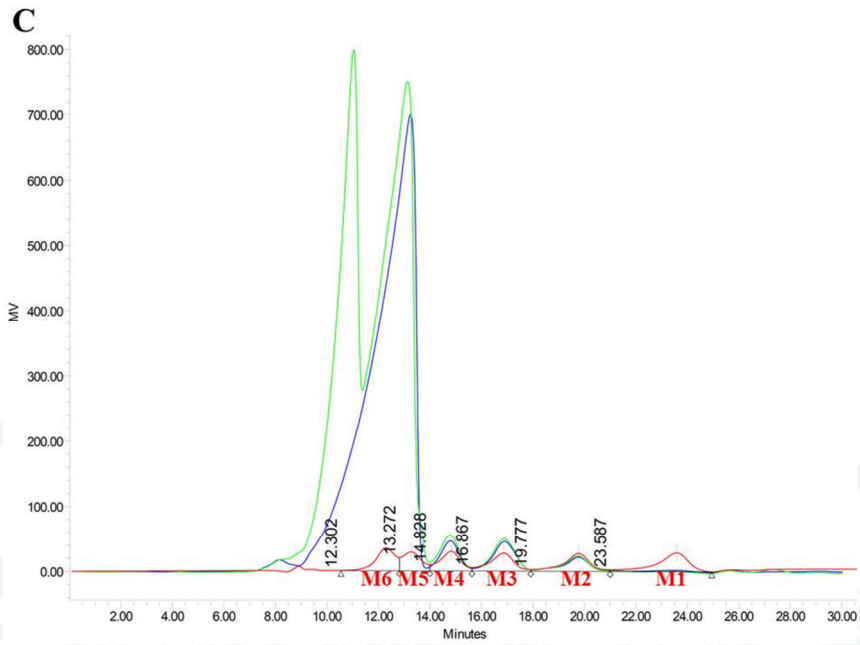


Appendix Figure 5 Standard curve of M6 (A), M5 (B), M4 (C), M3 (D), M2 (E) and M1 (F)

11. Hydrolysis products of various mannan substrates

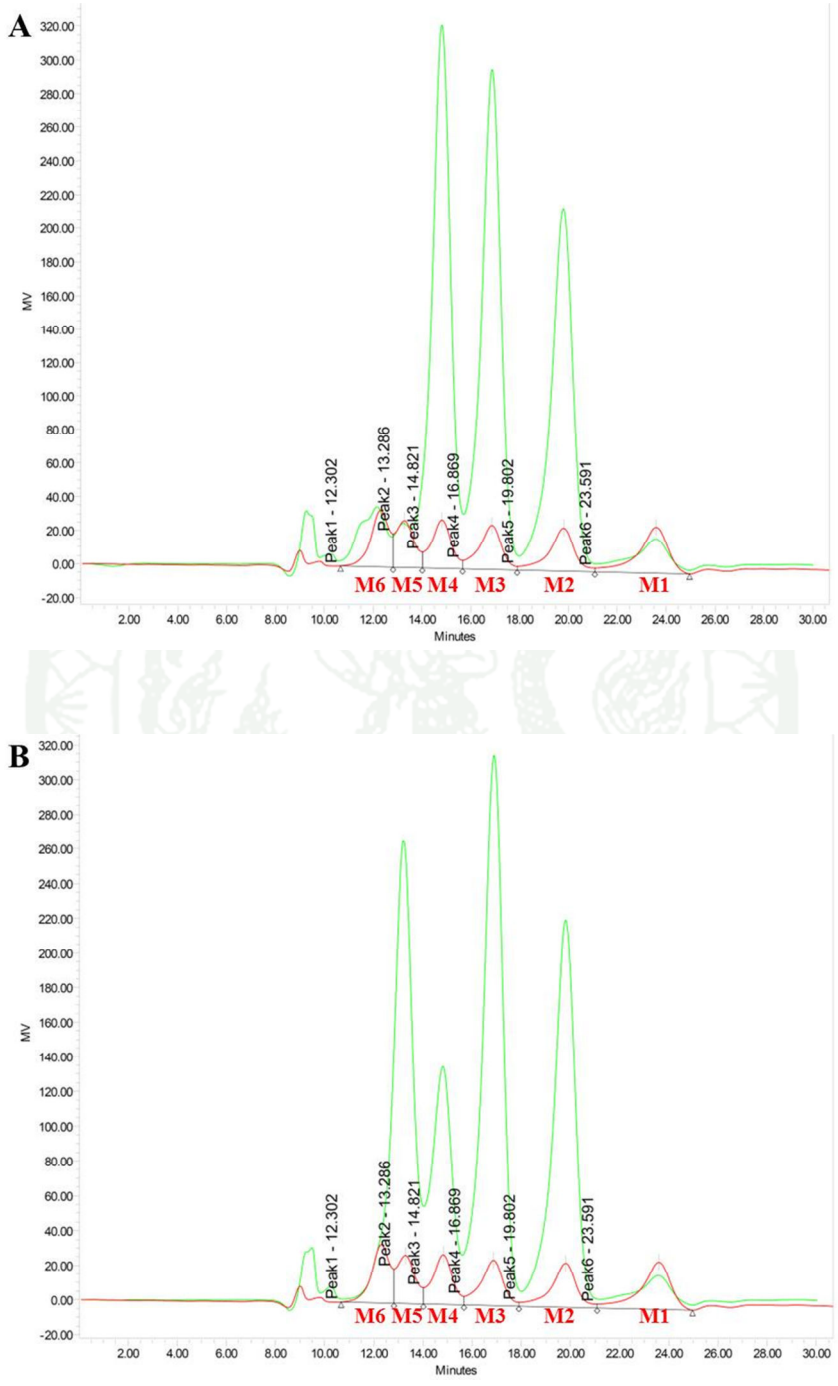


Appendix Figure 6 Hydrolysis products of LBG (A), konjac glucomannan (B) and defatted copra meal (C) substrates by extracellular (——) and intracellular (——) recombinant β-mannanase with standard mannooligosaccharides (——).

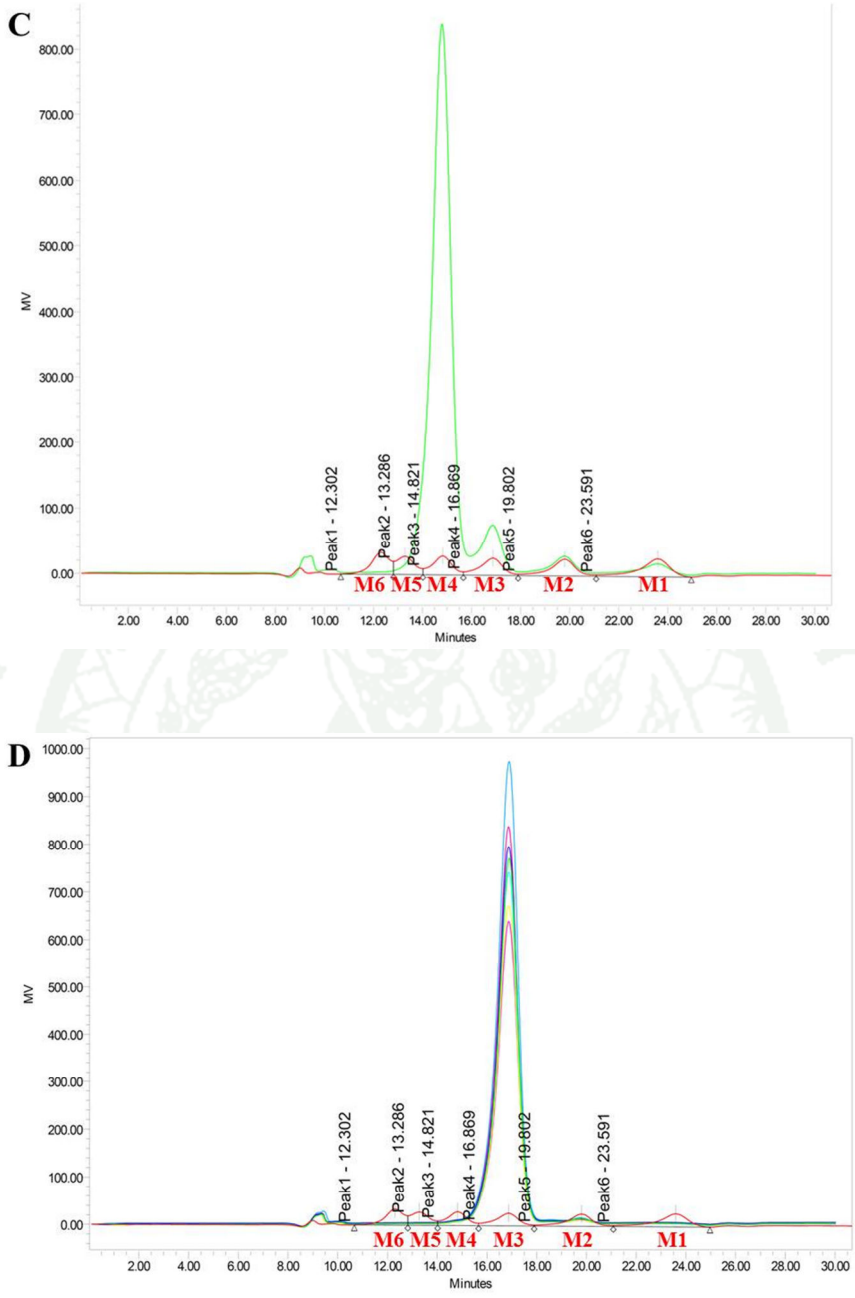


Appendix Figure 6 (Continued)

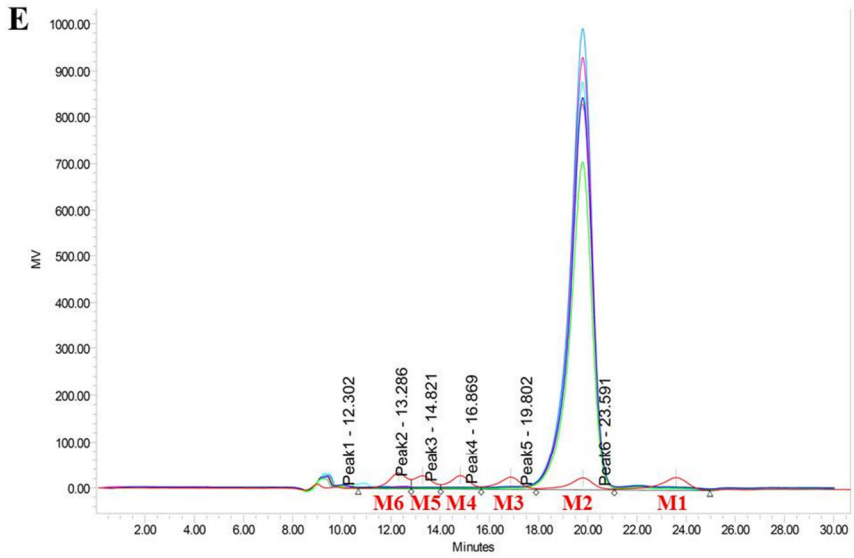
12. Hydrolysis products of various mannooligosaccharide substrates



Appendix Figure 7 Hydrolysis products of mannohexaose (A), mannopentaose (B), mannotretaose (C), mannotriose (D) and mannobiose (E) substrates with standard mannooligosaccharides.



Appendix Figure 7 (Continued)



Appendix Figure 7 (Continued)

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