Cloning, purification and characterization of *Bacillus* mannanases

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การโคลน การทำให้บริสุทธิ์ และการศึกษาคุณสมบัติของเอนไซม์มานาเนส จากบาซิลัส

นายบัญชา บูรณะบัญญัติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Mannan endo-1,4-β-mannosidase หรือ 1,4-β-D-mannan mannohydrolase (EC 3.2.1.78) หรือรู้จักกันทั่วไปว่า มานาเนส สามารถย่อยพันธะ β-1,4-glycoside ของแมนแนน กลูโคแมนแนน และกาแลคโตกลูโคแมนแนน ได้เป็นแมนโนโอลิโกแซคคาไรด์ มานาเนสได้ถูกประยุกต์ใช้อย่างกว้างขวาง เช่น ในอาหาร ยารักษาโรค อาหารสัตว์ การทำกระดาษ สื่อสิ่งพิมพ์ สีย้อม เส้นใย น้ำมันสกัด และ งานวิจัยทางชีววิทยา ในงานวิจัยนี้ได้นำยีนของมานาเนสจากบาซิลัสสองสายพันธุ์ ได้แก่ *Bacillus subtilis* สายพันธุ์ 168 และ*B. licheniformis* สายพันธุ์ DSM13 มาโคลนและแสดงออกในระบบของ *Escherichia coli* เอนไซม์มานาเนสจาก *B. subtilis* ลูกผสม (pETManBsub) ที่มีส่วนของสัญญาณเปปไทด์ดั้งเดิมได้ถูกโคลนเข้าสู่ pET 21d(+) vector ภายใต้การควบคุมการแสดงออกของ T7 lac promoter และถูกแสดงออกโดย *E. coli* สายพันธุ์ BL21(DE3) ในส่วนของเอนไซม์มานาเนสจาก *B. licheniformis* ลูกผสม (pFManBli13) ได้ถูกโคลนเข้าไปใน pFLAG-CTS vector ภายใต้การควบคุมของ *tac* promoter ซึ่งเอนไซม์ลูกผสมนี้ได้มีส่วนของสัญญาณเปปไทด์ OmpA ของ *E. coli* และถูกแสดงออกโดย *E. coli* สายพันธุ์ TOP10 เอนไซม์มานาเนสลูกผสมทั้งสองชนิดได้มีส่วนของกรดอะมิโนฮิสติดิน หกอะมิโนที่ส่วนปลายด้านคาร์บอกซิล และสามารถใช้เพื่อทำให้บริสุทธิ์โดยวิธี immobilized metal affinity chromatography (IMAC) ได้ในขั้นตอนเดียว จากการศึกษาคุณสมบัติทางชีวเคมีของเอนไซม์ลูกผสมบริสุทธิ์ได้ดังนี้ ที่อุณหภูมิ 50 องศาเซลเซียลเป็นอุณหภูมิที่เหมาะสมของเอนไซม์มานาเนสทั้งสองสายพันธุ์ เอนไซม์มานาเนสจาก *B. licheniformis* สามารถทนต่ออุณหภูมิได้ถึง 55 องศาเซลเซียสหลังจากที่บ่มเอนไซม์ที่ค่าความเป็นกรด-ด่างที่ 6 เป็นเวลา 30 นาที ค่าความเป็นกรด-ด่างที่เหมาะสมในการทำงานของเอนไซม์อยู่ในช่วง 6 ถึง 7 และสามารถทนต่อความเป็นกรด-ด่างได้ในช่วง 5 ถึง 12 หลังจากบ่มเอนไซม์ที่ปราศจากสารตั้งต้นที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 30 นาที และ ในช่วงความเป็นกรด-ด่าง 6 ถึง 9 หลังจากบ่มเอนไซม์เป็นเวลานาน 24 ชั่วโมง และ มีค่าความจำเพาะต่อสารตั้งต้นเท่ากับ 6832±11 ยูนิตต่อมิลลิกรัม โดยใช้โลคัสบีนกัม (locust bean gum) เป็นสารตั้งต้น ในส่วนของเอนไซม์มานาเนสจาก *B. subtilis* สามารถทนต่ออุณหภูมิได้ถึง 50 องศาเซลเซียสหลังจากที่บ่มเอนไซม์ที่ความเป็นกรด-ด่าง 6 เป็นเวลา 30 นาที ค่าความเป็นกรด-ด่างที่เหมาะสมในการทำงานของเอนไซม์อยู่ในช่วง 6 ถึง 7 และสามารถทนต่อความเป็นกรด-ด่างได้ในช่วง 5 ถึง 12 หลังจากบ่มเอนไซม์ที่ปราศจากสารตั้งต้นที่อุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 30 นาที และ ในช่วง ความเป็นกรด-ด่าง 6 ถึง 9 หลังจากบ่ม

เอนไซม์เป็นเวลานาน 24 ชั่วโมง และ มีค่าความจำเพาะต่อสารตั้งต้นเท่ากับ 1672±96 ยูนิตต่อมิลลิกรัม โดยใช้โลคัสบีนกัม (locust bean gum) เป็นสารตั้งต้น วิธีโครมาโตกราฟฟีแบบแผ่นบาง (thin-layer chromatography) ได้ถูกนำมาใช้วิเคราะห์ผลิตภัณฑ์ที่ได้และวิธีนี้ยังใช้ยืนยันว่าเอนไซม์มานาเนสลูกผสม เป็นชนิดเอนไซม์เอนโดมานาเนส (endo-mannanase) ดังนั้น เอนไซม์นี้เหมาะสมที่จะใช้งานในหลากหลายอุตสาหกรรม

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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BANCHA BURANABANYAT : CLONING, PURIFICATION AND CHARACTERIZATION OF *BACILLUS* MANNANASES. THESIS ADVISOR : ASSOC. PROF. MONTAROP YAMABHAI, Ph.D., 107 PP.

MANNANASE/ *Bacillus licheniformis*/ *B. subtilis*/ EXPRESSION

Mannan endo-1,4-β-mannosidase or 1,4-β-D-mannan mannohydrolase (EC 3.2.1.78), commonly known as mannanase, randomly hydrolyzes β-1,4-glycosidic linkage of mannan, glucomannan, galactomannan and galactoglucomannan, to yield mannooligosaccharides. The β-mannanase has been widely applied in such areas as food, drug, feed, papermaking, printing and dyeing, textile, oil exploitation, and biological research. In this thesis, genes encoding β-mannanase from two species of *Bacillus* i.e., *B. subtilis* 168 and *B. licheniformis* DSM13 were cloned and over expressed in *Escherichia coli* expression system. Recombinant *B. subtilis* mannanase (pETManBsub) containing native signal peptides was cloned into pET21d(+) expression vector (Novagen) under the control of T7 lac promoter and expressed by *E. coil* BL21(DE3), whereas recombinant *B. lichenisformis* mannanase (pFManBli13) was cloned into pLFAG-CTS expression vector under the control of *tac* promoter so that the mature enzyme was fused with the *E. coli* OmpA signal sequence and expressed by *E. coli* TOP10. Both of the recombinant mannanases were tagged with hexa-histidine at the Carboxy terminal and could be purified to apparent homogeneity by one-step immobilized metal affinity chromatography (IMAC). Biochemical characterization of the purified enzymes was performed. For *B. licheniformis*, optimal temperature and pH values for activity were 50 ^{o}C and 6.0-7.0, respectively. The enzyme was stable up to 55 ^{o}C after incubation for 30 min at pH 6.0. After incubation

for 30 min and 24 hr at 50 ^{o}C without substrate, the enzyme was stable within pH 5.0-12.0 and 6.0-9.0, respectively. Substrate specificity was 6832±11 U/mg with locust bean gum as a substrate. For *B. subtilis*, optimal temperature and pH values for activity were 50 ^{o}C and 6.0, respectively. The enzyme was stable up to 50 ^{o}C after incubation for 30 min at pH 6.0. After incubation for 30 min and 24 hr at 50 ^{o}C without substrate, the enzyme was stable within pH 2.0-10.0 and at pH 6.0, respectively. Substrate specificity was 1672±96 U/mg with locust bean gum as a substrate. The product analysis by thin-layer chromatography confirmed that the recombinant enzymes were an endo-mannanase. These results suggested that, these enzymes could be suitable for various industrial applications.

School of Biotechnology

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Student's Signature_____

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c=negative contril;M=marker; p=periplasmic extraction; ft=flow though; w1=wash1; w2=wash2; w3=wash3; e1=purified recombinant mannanase1; e2= purified recombinant mannanase2. Panel D shown as a zymogram analysis of pFManBli13……..….28

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LIST OF ABBREVIATIONS

°C	degree celcius
µg	microgram
µl	microlitre
bp	base pair
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5’ triphosphate
et al.	Et alia (and other)
g	gram
hr	hour
l	liter
M	morality
mg	milligram
min	minute
ml	milliliter
mM	millimolar
N	normality
ng	nanogram
PCR	polymerase chain reaction
pmol	picomol
rpm	revolution per minute
UV	ultraviolet

CHAPTER I

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) grown in tropical regions around the world is one of the most valuable plants to mankind since every part of the tree can be used. In 2005, the oilseed produced from copra meal in South-East Asia was estimated to be 4.39 million metric tons, with approximately 1.67 million metric tons obtained from Thailand (Titapoka et al., 2008). Huge amounts of coconut residual cake are discharged as a by-product in the process of oil extraction from copra because the main objective of the coconut industry is to extract as much oil as possible and to use it as a raw material for various goods such as soap, edible oil, wax, detergent, biofuel, etc. Because coconut residual cake contains a large amount of mannose in the form of 1,4-β-D-mannan, usually called copra mannan, there is a need to use the coconut residual cake effectively, not only to increase the commercial value of coconut products but also to minimize pollution (Hossain et al., 1996). One of the attractive strategies to increase the value of copra mannan is bioconversion into value-added mannooligosacharide (MOS) using enzyme mannanase. MOS is useful as one of the best growth factors for *Bifidobacterium* sp. and *Lactobacillis* sp. which is important in maintaining human normal intestinal condition (Hill, 1983).

The mannan, an important component of the hemicelluloses family, can be classified into four sub families: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Petkowicz et al., 2001). Each of these polysaccharides comprises a β-1,4-linked backbone containing mannose or a combination of glucose

and mannose residues (Hossain et al., 1996; Moreira and Filho, 2008). They may be present as reserve polysaccharides in the endosperm of palm nuts such as ivory nut (*Phytelephas macrocarpa*), in the seeds of leguminous plants such as Lucerne (*Medicago sativa*), locust bean gum (*Ceratonia siliqua*), in green coffee beans (*Coffea arabica*) and in roots and tubers of many plant species (Chhabra et al., 2001).

Mannan endo-1,4-β-mannosidase or 1,4-β-D-mannan mannohydrolase (EC 3.2.1.78), commonly known as β-mannanase, randomly hydrolyzes of β-1,4-glycosidic linkage of mannan, glucomannan, galactomannan and galactogluco-mannan, to yield mannooligosaccharide (Stalbrand et al., 1995). The β-mannanase has been widely applied in such areas as food, drug, feed, paper making, printing and dyeing, textile, oil exploitation, and biological research (Clarke et al., 2000; Howard et al., 2003).

β-mannanase can be found in many organisms such as fungus *Aspergillus niger* (Bien-Cuong et al., 2009), *A. tamari* (Civas et al., 1984), *Penicillium purpurogenum* (Park et al., 1987), *Trichoderma reesei* (Stalbrand et al., 1993), bacteria (Ethier et al., 1998; Hossain et al., 1996; Sysusch, 1990) and animal *Helix lucorum* L. (Villie et al., 1995).

In this study mannanase gene from *Bacillus subtilis*168 and *B. licheniformis* DSM13 were cloned and over expressed in *Escherichia coli*. Purification of recombinant β-mannanases were done on the basis of immobilized metal affinity chromatography (IMAC) (QAIGEN). The biochemical characteristics of these enzymes were analyzed.

CHAPTER II

REVIEW AND LITERATURE

2.1 Mannan

Mannan and heteromannans are widely distributed in nature as part of the hemicellulose fraction in softwood and plant tissues (Capoe et al., 2000). In plants, they present a structure role, acting as hemicelluloses that bind cellulose (Petkowicz et al., 2001). In addition, they also display a storage function as nonstarch carbohydrate reserves in endosperm walls and vacuoles of seeds and vacuoles in vegetative tissues. Mannan can be classified in four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Moreira and Filho, 2008).

Linear mannans are homopolysaccharides composed of linear main chains of 1,4-linked β-D-mannopyranosyl residues and contain less than 5% of galactose. Some of these mannans, especially from aloe vera, show immunopharmacological and therapeutic properties. They are the major structural units in woods and in seeds of many plants, such as ivory nuts (*Phytelephas macrocarpa*), date (*Phoenix dactylifera*) and green coffee beans (*Coffea arabica*) (Aspinall, 1959; Petkowicz et al., 2001). Mannans can be also found in the red algae *Porphyra umbilicalis* and in various species of the green algae *Codium* (Painter, 1983). In some algae species, linear mannan seems to replace cellulose as the main cell wall glycan. In most cases, these polysaccharides are highly insoluble in water. Accordingly, it has been suggested that mannan forms the molecular basis for the hardness which is characteristic for palm

kernels, such as the ivory nut. In the cell wall of the seed endosperm of ivory nut, mannan is the major component and it has been characterized in some detail. Based on their solubility in alkali, two different fractions of mannan have been isolated from the ivory nut. These fractions differ mainly in their DP and morphology (Hagglund, 2002; Moreira and Filho, 2008). In the coconut palm contains a large amount of mannose in the form of 1,4-β-D-mannan, usually called copra mannan (Hossain et al., 1996).

Galactomannans consist of water-soluble 1,4-linked β-D-mannopyranosyl residues with side chains of single 1,6-linked α-D-galactopyranosyl groups attached along the chain. Differences in the distribution of D-galactosyl units along the mannan structure are found in galactomannans from different sources. The galactomannans are reserve polysaccharides in the seed endosperm of leguminous plants (*Leguminosae*) (Dey, 1978). They retain water by salvation, and their presence in seeds from regions with high atmospheric temperatures is very important in preventing the complete drying of the seeds that would lead to protein denaturation, especially of those enzymes essential for seed germination. The D-galactosyl side branches of the polymer are the hydrophilic parts of the molecule, and the solubility in water increases when the galactose yield increases (Dea and Morrison, 1975). Both the solubility and the viscosity of the galactomannans are influenced by the mannose/galactose ratio, which can vary from 1 to 5. Two of the most well characterized galactomannans are those found in locust bean gum and guar gum, isolated from the seeds of *Ceratonia siliqua* and *Cyanaposis tetragonolobus*, respectively. Locust bean gum galactomannan comprises linear main chain of β-1,4-linked mannose units and side chains of the α-1,6-galactose unit, they have a

mannose/galactose ratio of approximately 5/1 and molecular weight of 310,000. Guar gum galactomannan comprises D-galactopyranose residues as non-reducing end groups that terminate side chains, they have a mannose/galactose ratio of 2/1 and a molecular weight of 220,000. Galactomannans have also been isolated from several lichen species. As galactomannans have strong gelling properties they are used as thickeners in the food and feed industries. Galactomannans were also used in paper making, mining and in the textile industry (Hagglund, 2002; Moreira and Filho, 2008; Pollard et al., 2008).

Glucomannans are found as storage polysaccharides in the seeds of certain annual plants, for example some lilies (*Liliaceae*) and irises (*Iridaceae*) (Meier and Reid, 1982). Furthermore, glucomannans are found in the bulbs, roots and tubers of several other types of plants. Many of these glucomannans are water soluble and have the same general structure as glucomannans found in wood: they composed of a β-1,4-linked mannan chain with interspersed glucose residues in the main chain and are often acetylated. The mannose.glucose ratio ranges from 4/1 to below 1/1. One of the most thoroughly characterized of these glucomannans is the konjac mannan, isolated from the tubers of Amorphophallus konjac. This polysaccharide has a mannose/glucose ration of 1.6/1 and a degree of polymerization above 6,000 (Nishinari et al., 1992).

Galactoglucomannans are polysaccharides containing D-galactose residues attached to both d-glucosyl and d-mannosyl units as α-1,6-linked terminal branches (Aspinall, 1959). They are preponderant hemicelluloses in the wood of gymnosperms (Timell, 1965). Mannose, glucose, and galactose residues are reported to be in the

molar ratio of 3:1:1. The backbone consists of β-(1→4)-D-mannopyranosyl and β-(1→4)-D-glucopyranosyl residues with a α-(1→6)-D-galactopyranosyl and *O*-acetyl groups (Timell, 1965). In softwoods galactoglucomannan, acetyl groups have been reported to be attached at the C-2 and C-3 positions of some mannose residues (Lundqvist et al., 2002). O-acetyl galactoglucomannan can be divided in two fractions, which are soluble in water or aqueous alkali, and have a galactose/glucose/mannose ratio of 1:1:3 or 0.1:1:3, respectively (Timell, 1965).

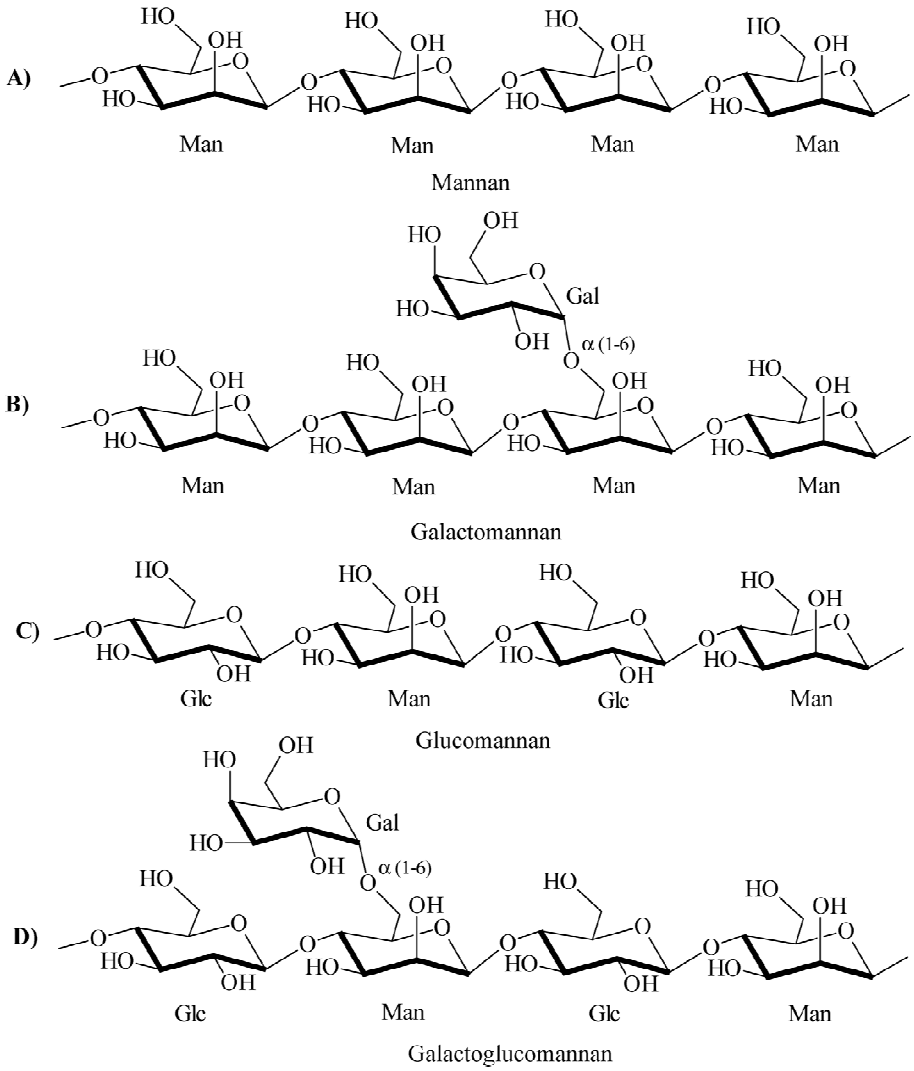

Figure 1. General structure of mannan and heteromannans. A) A typical mannan structure, a main chain of $\beta-1,4$ linked mannose (Man) residues; B) A typical galactomannan structure, a main chain of $\beta-1,4$ linked mannose residues with $\alpha-1,6$ linked galactose (Gal) residues attached to some (Man) residues; C) A typical glucomannan structure, a main chain of $\beta-1,4$ linked mannose (Man) and glucose (Glc) residues; D) A typical galactoglucomannan structure, a main chain of $\beta-1,4$ linked mannose

(Man) and glucose (Glc) residues, with α−1,6 linked galactose (Gal) residues attached to some (Man) residues (Samriti and Jagdeep, 2007).

2.2 Mannan endo-1,4-β-mannosidase (β-mannanase)

Mannan endo-1,4-β-mannosidase or 1,4-β-D-mannan mannohydrolase (EC 3.2.1.78), commonly known as β-mannanase, randomly hydrolyzes of β-1,4-glycosidic linkage of mannan, glucomannan, galactomannan and galactoglucomannan, to yield mannooligosaccharide (Stalbrand et al., 1995). This enzymes belong to glycosyl hydrolase (GH) families 5 and 26 according to the Carbohydrate Active Enzymes database, http://www.cazy.org (Cantarel et al., 2009). The β-mannanase has been widely applied in such areas as food, drug, feed, paper making, printing and dyeing, textile, oil exploitation, and biological research (Clarke et al., 2000; Howard et al., 2003). In paper industry, β-mannanase has been used to degrade hemicelluloses in the pretreatment of pulp to improve lignin extraction, significantly reducing the amount of chemicals used and hazardous waste (Khanongnuch et al., 1998). For coffee industry, the mannan present causes high viscosity of the coffee extract when it is concentrated before drying. β-mannanase is used to decrease viscosity of coffee extracts in instant coffee preparation. In addition, it helps improved volatile aroma, taste and visual appearance of instant coffee (Sachslehner et al., 2000). Moreover β-mannanase is used to clarify fruit juice and wine. It is also used in preparation of manooligosaccharide, which can be used as non-nutritional food additive for selective growth of human beneficial intestinal microflora (*Bifidobacterium* sp. and *Lactobacillus* sp.) (Vladimir et al., 2004). β-mannanase found in ripening tomato

could have a role in its softening (Sozzi et al., 1996). *Bacillus* mannanases used in this study were compared with the other *Bacillus* mannanases was shown in Table 1.

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2.3 Research objectives

In this research, the molecular cloning was used to improve the property of enzyme mannanase. The expression and purification were optimized for recombinant enzyme production. The characteristics of recombinant mannanase were studied.

The objectives of this research are listed below.

1. To clone β-mannanase gene from *B. subtilis* 168 and *B. lichenisformis* DSM13.
2. To express the recombinant β-mannanase in *E. coli*.
3. To purify and characterize the properties of recombinant β-mannanase.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

Bacteria	**Strain**	**Genotype**
Escherichia coli	BL21 (DE3)	F- *ompT hsdS*$_B$ (r_{B-}, m_{B-}) *gal dcm* (DE3)
Escherichia coli	DH5α F’	F'/*end*A1 *hsdR17* (r_K^- mK $^+$) *supE44 thi-1 recA1 gyrA* (*Nal*^{r}) *relA1* Δ(*lacZYA-argF*)*U169 deoR* (ϕ80 *dlac*Δ*(lacZ)M15)*

Bacillus subtilis strain 168 (American Type Culture Collections).

Bacillus licheniformis DSM13 (ATCC 14580)

3.1.2 Instruments

Autoclave: Hiclave HA-3000MIV, Hirayama, Japan

Balance: Precisa 205A, Precisa Instruments, Switzerland

Precisa 3000C, Precisa Instruments, Switzerland

Centrifuge machine: Sorvall RC5C plus, Kendro laboratory Products, USA

Eppendrof centrifuge 5810 R, Eppendrof, US

Deep freezer -70 ^oC: Heto, Ultra Freeze, Denmark.

ELISA reader:	Sunrise, TECAN, Austria
Electroporator :	Eppendrof 2510, Eppendrof, USA
Freezer -20 ^{o}C:	Heto, HLLF 370, Denmark.
	MyBio LFT420, DAIREI, Denmark
Gel Document set:	White/Ultraviolet Transilluminator GDS7500, UVP, USA
	Digital Graphic Printer UP-D890, Sony, Japan.
Gel dryer:	Drygel sr. SLAB GEL Dryer model SE1160,
	Hoefer Scientific Instruments, USA
Gel electrophoresis apparatus:	Mini Protean® 3 cell, BioRad, USA
Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator:	Memmert, BE 500, WTB Binder BD115,
	Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood:	Holten LaminAir HBB 2448, Denmark.
	BH2000 Series ClassII Biological Safety Cabinets,
	BHA120 & BHA180, Clyde-Apac,
Membrane transfer machine:	Semi Phor, Hoefer Scientific instruments, USA
Microcentrifuge:	Mini spin plus, Eppendrof, USA
	Eppendorf 54154, Eppendorf, Germany

pH meter:	Ultra Basic pH meter UB-10, Denver Instruments, Germany
PCR machine:	DNA Engine PTC 200 peltier Thermal cycler, MJ Research, USA
Rotator:	Certomat TCC, B. Braun Biotech International, Germany Rotator AG, Fine PCR, Korea
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK Certomat TC2, B. Braun Biotech International, Germany
Sonicator:	Waken GE100 Ultrasonic processor, Japan
Spectrophotometer:	Ultrospec 2000, Pharmacia biotech, UK
Stirrer:	Variomag Electronicrührer Poly 15, Germany Magnetic stirrer MSH300, USA Hot plate stirrer Labtech, Korea
Thermomixer:	Thermomixer compact, Eppendrof, USA

3.2 Methods

3.2.1 Cloning and expression

3.2.1.1 Cloning of mannanase from *Bacillus subtilis* 168

Gene of the *B. subtilis* mannanases were cloned by a PCR-based method. The primers; B.subManfw: 5′CTG TGC CCA TGG GGT TTA AGA AAC ATA CGA TCT CTT TGC TC3′ and B.subManrv: 5′CTG TGC TCG AGC TCA ACG ATT GGC GTT AAA GAA TCA CC3′ were used for PCR to amplify of the mannanase genes. These primers were designed from the published genomic database of *B. subtilis* str. 168 (NCBI accession number Z99107), and were compatible with the *Nco*I and *Xho*I

restriction sites respectively. DNA encoding hexahistidine tag was incorporated into the reverse primers to generated 6xHis tagged recombinant enzymes for further purification step. The vector pET-21d (+) (Novagen) was selected to express mannanase in *E. coli* strain BL21(DE3). The DE3 contain the λDE3 lysogen was carried the gene for T7 RNA polymerase under control of the *lac*UV5 promoter. IPTG was used to induce expression of the T7 RNA polymerase. The single colony of *B. subtilis* was grown on M1 medium at 37^{o}C overnight and single colony was picking for the PCR reaction in total volume 50µl. Each reaction contained 0.5 µmole of each primer, 200 µM of dNTP, 3 units of *pfu* DNA polymerase and 5 µl of 10x buffer for *pfu* DNA polymerase. The condition of PCR reaction was carried out as follows, denaturation at 95^{o}C 2 min., after that repeated 30 cycles of denaturation at 95^{o}C 45 sec., annealing at 50^{o}C 30 sec. and primer extension at 72^{o}C 2.2 min., then end with extension at 72^{o}C 10 min. The PCR products were separated on 1% agarose gel containing ethidium bromide and visualized under a UV transilluminator. PCR products were purified using PCR purification kits (Qiagen). The PCR product and vector were digested with the restriction enzymes *Nco*I and *Xho*I before ligation. The ligation reaction contained 3:1 mole of mannanase gene insert and vector which was dephosphorylated by CIP enzyme in a reaction containing 2 µl of 10x buffer of T4 DNA ligase, 200 units of T4 DNA ligase. The ligation was done at 16^{o}C for 16 hours. The ligated vector was transformed into *E. coli* DH5α by electroporation (pulse 1,800 V) and selection on LB agar plate containing 100 µg/ml ampicillin. The positive clones were selected to grow on LB broth containing 100 µg/ml ampicillin overnight and plasmid was extracted using QIAGEN™ plasmid preparation kit. Then plasmids were digested with *NcoI* and *XhoI* to analyze the restriction pattern by agarose gel

electrophoresis. The DNA sequence and the integrity of the constructs were confirmed by automated DNA sequencing (Macrogen, Korea). The recombinant plasmid (pETManBsub) was transformed into *E. coli* BL21 (DE3) for enzyme expression.

3.2.1.2 Cloning of mannanase from *Bacillus licheniformis* DSM13

B. licheniformis DSM13 (ATCC 14580) was obtained from DSMZ; German Culture Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were grown at 37°C and kept in M1 medium. The gene of the mature β-mannanase from *B. licheniformis* was cloned by a PCR-based method. The primers B.liManfwXhoI: 5′CTG TGC CTC GAG CAC ACA CCG TTT CTC CGG TG3′, and B.liManrv6HiBgl2: 5′CTG TGC AGA TCT TCA ATG GTG ATG GTG ATG GTG TTC CAC GAC AGG CGT CAA AGA ATC GCC3′ were used for PCR amplification of the mannanase gene. These primers were designed using the published sequence from the genomic database of *B. licheniformis* DSM13 (NCBI accession number NC_006322), and were compatible with the *Xho*I and *Bgl*II cloning sites of pFLAG-CTS expression vectors (Sigma). The DNA encoding native signal peptides were omitted, and the gene of the mature enzyme was fused with the *E. coli* OmpA signal peptide instead in order to enable efficient secretion into the periplasm and culture media. In addition, DNA encoding a hexahistidine tag was incorporated into the reverse primers to generate 6xHis tagged recombinant enzymes for further purification. PCR reactions were performed according to the recommendations from the manufacture in a thermal cycler. Templates were prepared by boiling a single colony of *B. licheniformis* in 100 µl DI for 5 min, and 50 µl of that solution were directly used in the PCR reaction. The PCR reaction (total volume of 100 µl)

consisted of 0.5 μM of primers, 0.2 mM dNTP, 3 units of *Pfu* DNA polymerase (Promega), and 10× reaction buffer, provided by the manufacturer. The amplifications were done as follows: initial DNA denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 45 sec, annealing at 58°C for 1 min, extension at 72°C for 2.5 min, and a final extension at 72°C for 10 min. The PCR products were separated on 1% agarose gels containing ethidium bromide and visualized under a UV transilluminator. PCR products were purified using PCR purification kits (Qiagen, Germany). The PCR products were then cut with *Xho*I and *Bgl*II and ligated into the pFLAG-CTS expression vector that has been cut with corresponding enzymes. The ligation reactions were transformed into *E. coli* DH5α. The DNA sequence and the integrity of the constructs were determined by automated DNA sequencing (Macrogen, Korea). The recombinant plasmid (pFManBli13) was transformed into *E. coli* TOP10 for enzyme expression.

3.2.2 Optimization

Freshly transformed *E. coli* Top 10 harboring the pFManBli13 and *E. coli* BL21 (DE3) containing pETManBsub were incubated in LB broth contained ampicillin 100 μg/ml and grew at 37°C with shaking at 200 rpm, until OD_{600} reached 0.6. Induction of the expression of mannanase was done by adding Isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 1mM and incubated with shaking at 28°C overnight. Fifty ml of culture medium were sampling at 0, 1, 3, 6 h, and overnight to optimize the induction time. Crude enzymes were separated by centrifugation at 4,000 rpm for 30 min, at 4°C to separate supernatant and cell pellet. The crude enzymes in supernatants were collected at 4°C. The cell pellets were resuspened in lysis buffer (50 mM Tris-HCl + 0.5 mM EDTA) and lysed by sonication

(Ultrasonic Processor; 60 amplitude, pulser 6 sec, for 2 minutes) on ice. The lysate were centrifuged for 30 min at 8,000 rpm to eliminate cellular debris. The supernatant with internal mannanase enzyme was collected at 4^{o}C. The recombinant enzymes were assayed by SDS-PAGE and zymogram.

3.2.3 Expression of recombinant enzymes

Expression of recombinant *Bacillus* mannanase was done as previously published for this expression system (Yamabhai et al., 2008). Freshly transformed *E. coli* TOP 10 harboring the pFManBli13 and *E. coli* BL21 (DE3) containing pETManBsub were incubated into 5 ml of LB broth containing 100 µg/ml of ampicillin at 37^{o}C for 16 hr. After that, 1% of overnight culture was inoculated into 250 ml of LB broth containing 100 µg/ml ampicillin and grown at 37^{o}C until the OD_{600nm} reached 0.6. Then, IPTG was added into the culture broth to a final concentration of 1 mM. The culture was then incubated with vigorous shaking (200 rpm) at 30^{o}C for 3 hr. Then, the culture was collected and chilled in an ice box for 5 min. and centrifuged at 4,000 rpm for 30 min at 4^{o}C to collected cells and supernatants. For the preparation of 6xHis-tagged periplasmic proteins from *E. coli*, the cells were resuspended in 2.5 ml of cold (4^{o}C) spheroplast buffer [100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 µg/ml phenylmethylsulfonyl fluoride (PMSF)]. After incubation for 5 min on ice, bacterial cells were collected by centrifugation at 4,000 rpm for 30 min at 4^{o}C and re-suspended in 2 ml of ice-cold sterile water supplemented with 1 mM $MgCl_2$ and incubated on ice for 5 min with frequent shaking. The supernatant of nearly 2 ml was then collected by centrifugation at 8,000 rpm at 4^{o}C for 30 min is the osmotic shock fluid containing periplasmic proteins. To extract the cell lysate, the precipitated cells from the previous step were

washed once with lysis buffer (50 mM Tris-HCl + 0.5 mM EDTA), resuspended in 2 ml of lysis buffer, and sonicated (Ultrasonic Processor; 60 amplitude, pulser 6 sec, for 2 minutes) on ice. The cell debris was then spinning down at 8,000 rpm and the supernatant was collected as the cell lysate.

3.2.4 Purification of recombinant mannanase

Immobilized metal affinity chromatography (IMAC) was used for purification of 6xHis-tagged recombinant mannanases by gravity-flow chromatography, using the nickel-nitrilotriacetic acid (Ni-NTA) resins according to QIAGEN's protocol. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag (Figure 2). Recombinant mannanases were purified from the osmotic shock fluid containing periplasmic proteins. The slurry of Ni-NTA resins were packed into column and remove liquid phase out by gravity and then the resins were equilibrated with lysis buffer pH 8.0 (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 10 mM imidazole) for 10 min. After that the supernatant was removed by gravity. The osmotic fluid fractions were added into the Ni-NTA resins column and rotated for 2 hr at 4^{o}C and then the flow though solution was collected. Fifteen ml of washing buffer pH 8.0 (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 20 mM imidazole) were added and the column was rotated for 15 min. at 4^{o}C. After, the washing buffers were removed by gravity, the washing step was repeated for 2 times and the washes samples were kept for SDS-PAGE analysis later. One ml of elution buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 250 mM imidazole) was added into purified column and rotated for 15 min. The supernatant containing soluble recombinant mannanases were removed by gravity and dialyzed using Nanosep centrifugal filter (MW cut off 10kD, Pall Life Sciences) to remove

immidazole. Fifty mM citrate buffer pH 6.0 was used as washing buffer and the fraction of 1 ml was collected and glycerol was added to final concentration of 15% and stored at -20^{o}C. Pure recombinant mannanases, crude enzymes and sample from purification step were subjected to SDS-PAGE analysis.

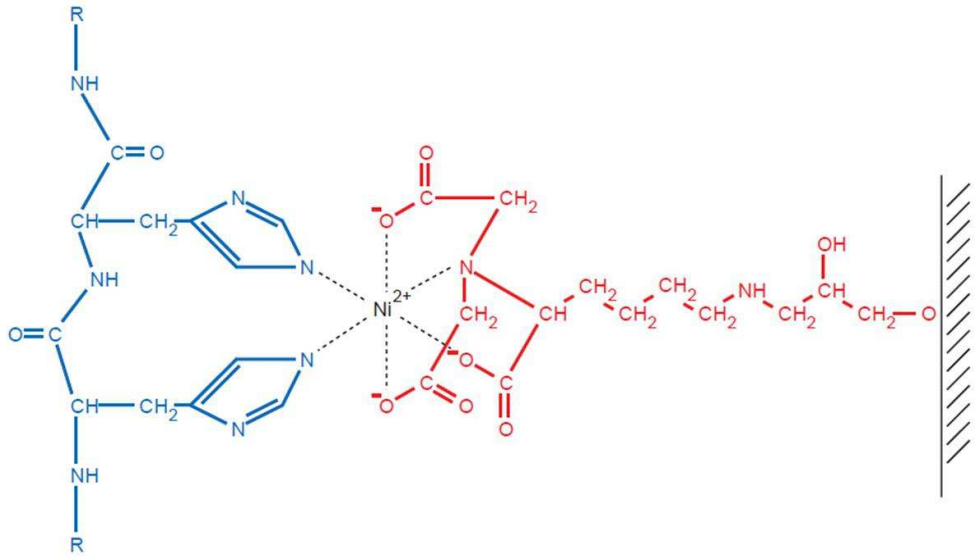

Figure 2. Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix (QAIGEN).

3.2.5 Gel electrophoresis and zymogram analysis

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (Laemmli 1970), in a 12% (w/v) polyacrylamide. The protein samples were briefly heat at 100^{o}C in a heat block (Eppendorf) for 3 min in the loading buffer. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The molecular weight markers were from Biorad.

A Zymogram of mannanases activities was generated by in-gel activity assay using 0.25% locust bean gum as substrate copolymerized with 10% (w/v) polyacrylamide. The enzyme samples were mixed with the loading buffer in the absence of reducing agent, and then applied on a polyacrylamide gel. After electrophoresis, the gel was soaked in 2.5% Triton X-100 for 30 min at 4^{o}C, wash with DI water for remove Titon X-100 and incubated in citrate buffer pH 6.0 at 50^{o}C for 1 hr. The gel was then rinsed with DI water, and then stained with 0.1% Congo red solution with gentle shaking for 20 min prior to destain with 1 M NaCl for 20-30 min, and thereafter was placed in 5% acetic acid for 30 min (optional). Mannanase activity was detected as clear zones against red (after staining with Congo red) or blue background (after soaking in 5% acetic acid)

3.2.6 Protein determination

Proteins concentration was determined by Quick start^{TM} Bradford Protein Assay (Bio-Rad) using bovine serum albumin as standard. The dye reagent was mixed 1 to 4 with DI water and filtrated. The properly diluted samples (60 µl) were mixed with 200 µl of dye reagent and stand at room temperature for 10 min. The absorbance at 595 nm was measured.

3.2.7 Characterization

Recombinant mannanases activity were assayed using the DNS method (Miller, 1959). The substrate, 0.5% locus bean gum (Sigma), was dissolved in 50 mM sodium citrate buffer, pH 6.0 by homogenizing at 80^{o}C, heating to the boiling point, cooled and stored overnight with continuous stirring, after that insoluble were removed by centrifugation. The substrate (900 µl) was preincubated at 50^{o}C for 30 min. After that 100 µl of the enzyme solution was added and incubated for 5 min.

The enzyme reaction was heat at 100^{o}C for 10 min. The reducing sugar liberated in the enzyme reaction was assayed by mixing 100 µl of the enzyme reaction with 100 µl DNS solution, heating at 100^{o}C for 20 min, cooling on ice, and measured the absorbance at 540 nm. One unit of mannanase activity is defined as the amount of enzyme which liberates 1 µmol reducing sugar (using D-Mannose as a standard) per minute under the experimental conditions.

3.2.8 Effect of temperature and pH on enzyme activity

The optimal temperature of mannanases activity was measured by incubating the enzyme samples with the substrate at temperature ranging from 4-100^{o}C in 50 mM citrate buffer pH 6.0. Thermal stability of the enzyme was determined by incubating the enzyme samples in 50 mM citrate buffer, pH 6.0 at various temperatures ranging from 4-80^{o}C for 30 min, then the remaining enzyme activity were measured under standard assay condition. In addition, the residual enzyme activity after incubation in 50 mM citrate buffer, pH 6.0 at 50 and 60^{o}C at certain time intervals were also assayed under standard condition.

The optimal pH of the mannanases activity was measured between pH 2.0-12.0 under standard assay condition, using 50 mM of three buffer systems: sodium acetate (pH 2.0-6.0), potassium phosphate (pH 6.0-9.0), and glycine (pH 9.0-12.0). To determine the pH stability of mannanases, the enzyme samples were incubate at various pH values using the same buffer system at 50^{o}C for 30 min or 24 hr, and then the remaining enzyme activity was measured under standard assay condition.

3.2.9 Substrate specificity and kinetic parameters

The relative activity of the mannanases was determined by preincubating 5 mg/ml of each substrates in 0.1 M phosphate buffer pH 7.0 at 50^{o}C for 30 min with

constant agitating in a Thermomixer comfort (Eppendorf AG). After adding the purified enzyme, the reaction was incubated at 50^{o}C with shaking for 5 min, and then terminated by boiling for 10 min. A release of reducing sugars was detected by DNS method. Specific hydrolyzing activity against various substrates was calculated by converting A540 to µmoles of reducing sugars using a standard calibration curve constructed with varying mannose from 0 to 1,200 mg/ml.

The purified enzymes demonstrated kinetic parameters with low viscosity LBG, konjac glucomannan and 1,4-β-D-Mannan. The km and Vmax values were calculated from a non-linear regression function, using the SigmaPlot 2000 software. The reactions were done under the standard assay conditions by varying the substrate concentration rang 0-20 mg/ml.

Low viscosity locust bean gum was prepared according to previously published protocol (Kremnicky et al. 1996). Thirty grams of LBG was dissolved in 900 ml of water and boiled. After cooling, the solution was acidified to pH 2.6 using concentrate HCl and autoclaved for 15 min at 121 kPa. The insoluble particles were separated by centrifugation and the supernatant was precipitated with 2 volumes of 95% ethanol (1.7 l) and kept overnight at 4^{o}C. The precipitate was collected by centrifugation at 4,000 rpm for 15 min, suspended in 1.2 l of water warmed to 70^{o}C and dissolved by shaking. The homogeneous solution was lyophilized.

3.2.10 Thin-layer Chromatography

Hydrolysis of 15 mM mannohexaose (M6) and 0.1 mg LBG (high viscosity) by recombinant mannanases was carried out in a 30 µl reaction mixture, containing 0.1 M citrate buffer, pH 6.0, and purified enzymes. The reaction was incubated at 50^{o}C with shaking for 5, 10, 30, 60 min and overnight prior to termination by boiling

for 5 min. Each reaction mixture was applied five times (one µl each) to a Silicagel 60 F_{254} aluminum sheet (Merck, Germany) (6.0x10.0 cm), and then chromatographed two times (2hr each) in a mobile phase containing n-propanol: ethanol: water (7:1:2) (v/v), followed by spraying with 5% sulphuric acid in ethanol and baking at 180^{o}C for 3 min. A mixture of M1-M6 (5 nmol each) were used as standard.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Cloning and expression of *Bacillus* mannanase

B. subtilis and *B. licheniformis* were used as the source for the isolation of the β-mananase gene since these strains have been extensively for large-scale production of various industrial enzymes including serine protease (subtilisin)(Schallmey et al., 2004). The genomes of both strains have recently been sequenced, and a number of new genes of potential biotechnological application have been identified(Veith et al., 2004). PCR cloning was used to generate the recombinant mannanase containing either their native signal peptides or signal peptide from *E. coli* outer membrane protein, OmpA. Genes encoding precursor enzymes containing native signal peptides were cloned into the pET21d(+) expression vector under the control of T7 promoter; whereas gene for mature mannanase fused with the *E. coli* OmpA signal sequence was cloned into the pFLAG-CTS expression vector under the control of *tac* promoter. Both of the recombinant mannanases in either pET21d(+) or pFLAG-CTS vector could be induced for overexpression by 1 mM IPTG in *E.coli* BL21 (DE3) or Top10, respectively. The mannanase gene from *B. subtilis*168 was cloned into pET-21d(+) as shown in Figure 3A. The constructs was designated as pETManBsub. The mannanase gene from *B. licheniformis* DSM13 was cloned into pFLAG-CTS as shown in the Figure 3B. The construct was designated as pFManBli13. Hexahistidine was incorporated into the constructed vector to create his-tagged fusion enzyme for further

purification step. The recombinant mannanases could be secreted into periplasmic space and culture broth (Yamabhai et al., 2008).

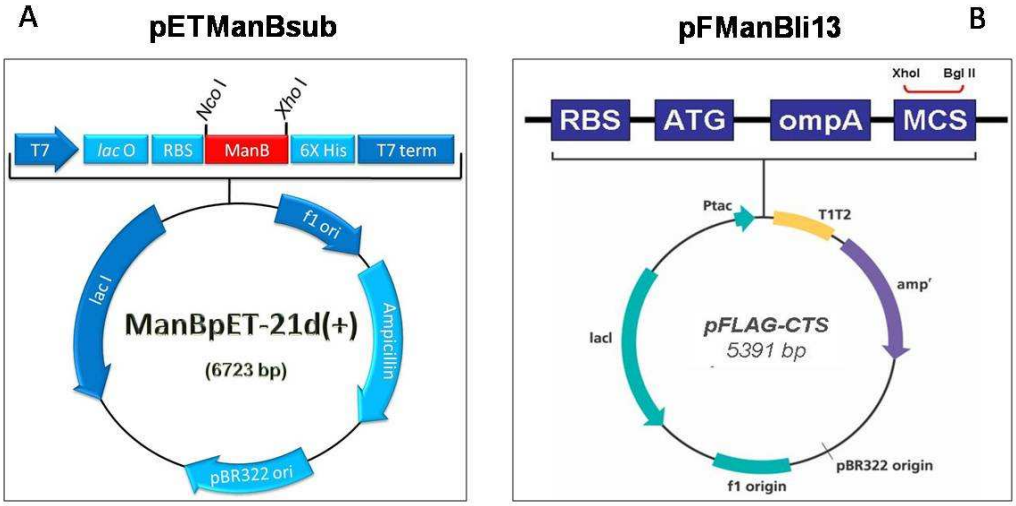

Figure 3. Map of recombinant *Bacillus* mannanases. The gene of *B. subtilis* mannanase containing native signal peptides was cloned into pET21d(+) under the control of T7 lac promoter (Panel A). The mannanase gene from *B. licheniformis* was cloned into pFlag-CTS vector with E. coli OmpA signal peptide replacement and expressed under *tac* promoter (Panel B).

The recombinant mannanases could be induced for over-expression and secretion into periplasmic space and culture media (Yamabhai et al., 2008). Figure 4 shows the production and secretion of recombinant mannanase after induction with IPTG. At 4 hr after induction, a large amount of the enzymes were accumulated in periplasmic space, and after inducing overnight, the enzymes could be found in both culture media and periplasmic space. To prepare the enzyme for affinity purification and characterization, we preferred to prepare the enzyme from periplasmic extract

after induced at 3 hr, as the enzyme was highly concentrated, facilitating the next affinity purification step.

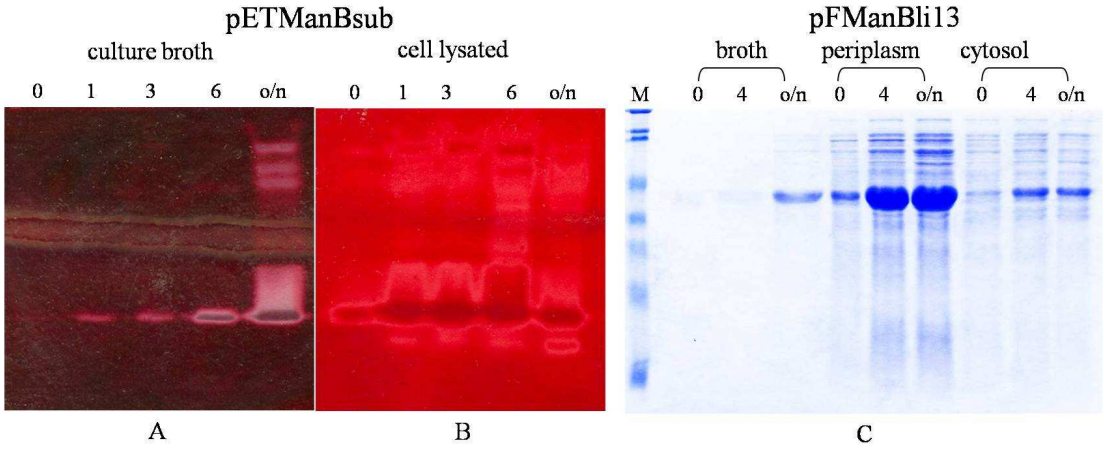

Figure 4. Zymogram analysis and SDS-PAGE of cell lysated and culture broth of recombinant Bacillus mannanase at various time of induction. Panel A and B; zymogram analysis of cell lysated and culture broth of pETManBsub. 0=before time induction; 1=time induction at 1 h; 3=time induction at 3 h; 6=time induction at 6 h; o/n=time induction at overnight. C; SDS-PAGE of pFManBli13 was prepared from culture broth, periplasmic space and cytosol.

4.2 Purification of recombinant mannanase

Immobilized-metal affinity chromatography (IMAC) was used for purification of recombianant mannanase by gravity-flow chromatography, using the Ni-NTA Agarose.

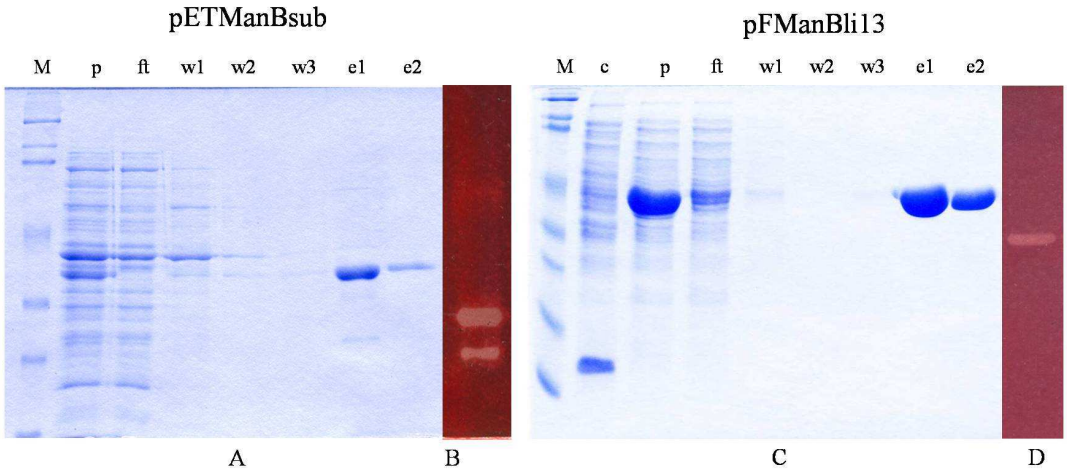

Figure 5. Coomassie staining and Zymogram analysis of purified recombinant mannanases were used in this study. SDS-PAGE analysis of purified pETManBsub is shown in panel A. M=marker; p=periplasmic extraction; ft=flow though; w1=wash1; w2=wash2; w3=wash3; e1=purified recombinant mannanase1; e2= purified recombinant mannanase2. Panel B shown as a zymogram analysis of pETManBsub. SDS-PAGE analysis of purified pFManBli13 is shown in panel C. c=negative contril; M=marker; p=periplasmic extraction; ft=flow though; w1=wash1; w2=wash2; w3=wash3; e1=purified recombinant mannanase1; e2= purified recombinant mannanase2. Panel D shown as a zymogram analysis of pFManBli13.

We found that approximately 5 mg and 25 mg of pETManBsub and pFManBli13 could be obtained from a 1-liter culture, respectively. The molecular weight of pETManBsub and pFManBli13 were 41 kDa, confirming the theoretical mass of both recombinant enzymes 41 kDa (www.currentprotocols.com).

4.3 Secondary structure prediction

The amino acid sequence of pETManBsub and pFManBli13 were analysed by automated DNA sequencing (Macrogen, Korea) and the two enzymes were highly similar at 78% identity. The alignment was done by using Clustal W and Espript program was used to predicted secondary structure of recombinant mannanase compared with BCman (PDB code 2QHA) (Xiao-Xue Yan, 2008).

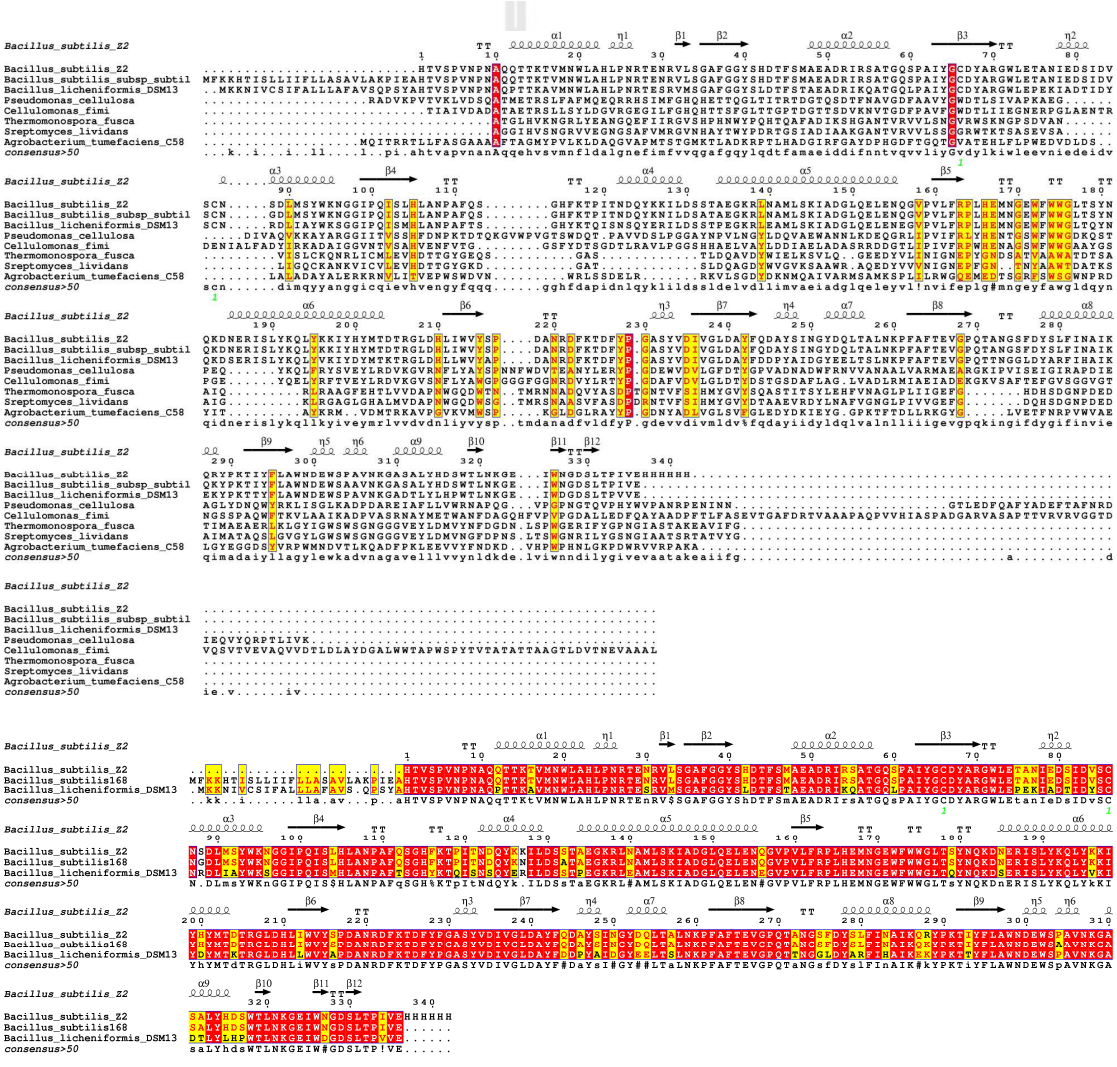

Figure 6. Secondary structure prediced of recombinant mannanases.

4.4 Tertiary structure prediction of pETManBsub

The 3-D structure of pETManBsub was predicted by compared with ManBC PDB file. The 3D structure is shows a TIM-barrel compose with 8-β-sheet and 8-α-helix stand. This enzymes belong to glycosyl hydrolase family 26 (GH26) according to the Carbohydrate Active Enzymes database, http://www.cazy.org. The catalytic domains of GH26 members are located at the C-terminus (Cantarel et al., 2009).

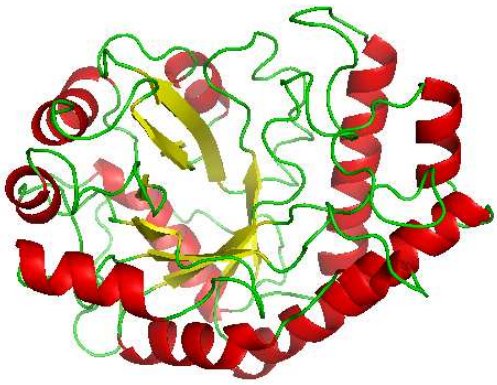

Figure 7. Tertairy structure predicted of pETManBsub.

4.5 Enzymatic assay

4.5.1. Temperature profile

The optimal temperature of pETManBsub and pFManBli13 were at 60^{o}C. The activity of pETManBsub was higher than that of pFManBli13 in rang of 40-50 ^{o}C, although the activity of pFManBli13 was better than that of pETManBsub in rang 50-60 ^{o}C, as shows in Figure 8.

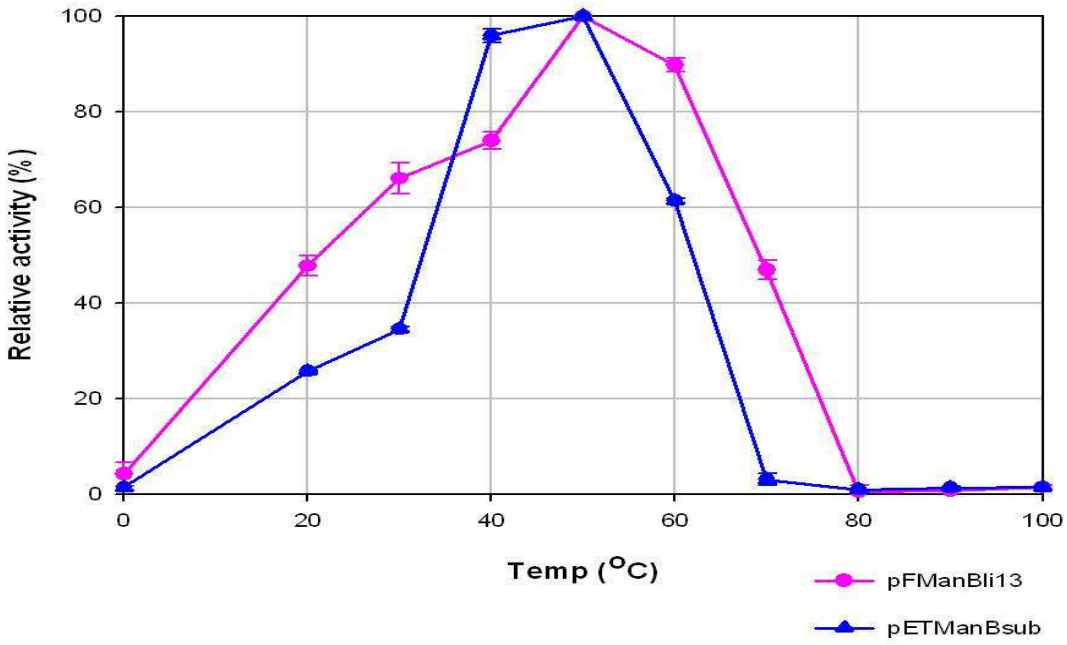

Figure 8. Optimal temperature of pETManBsub and pFManBli13 activity. Mannanase activity was measured by the DNS method assay using LBG as substrate for 5 min at designated temperatures.

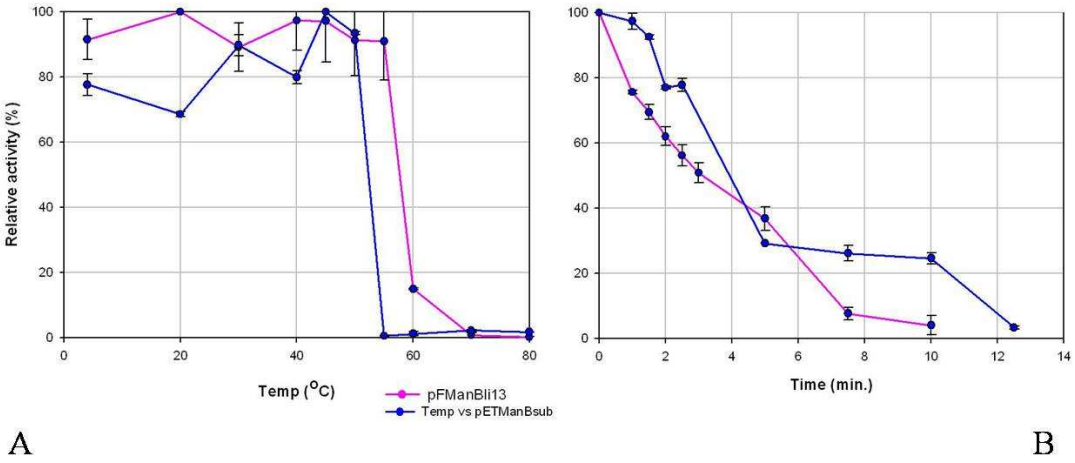

Figure 9. Thermosatability of recombinant mannanases. Panel A; The temperature stability was determined by measureing the remaining activity after incubation without substrate at various temperatures at pH 6.0 for 30 min. and measuring the residual activity using the standard assay. Panel B; illustrates the remaining enzyme activity after incubation at 55 ^{o}C (blue) and 60 ^{o}C (pink) at various time points.

The enzyme was stable up to 55 ^{o}C after incubation for 30 min at pH 6.0 (Figure 9A) whereas 50% of activity was remained after incubation at 60 ^{o}C for 30 min.

4.5.2. pH profile

The activity of pETManBsub was optimal at pH 6.0 as shown Figure 10. For the pH optima of pFManBli13 was between optimal pH 6-7.

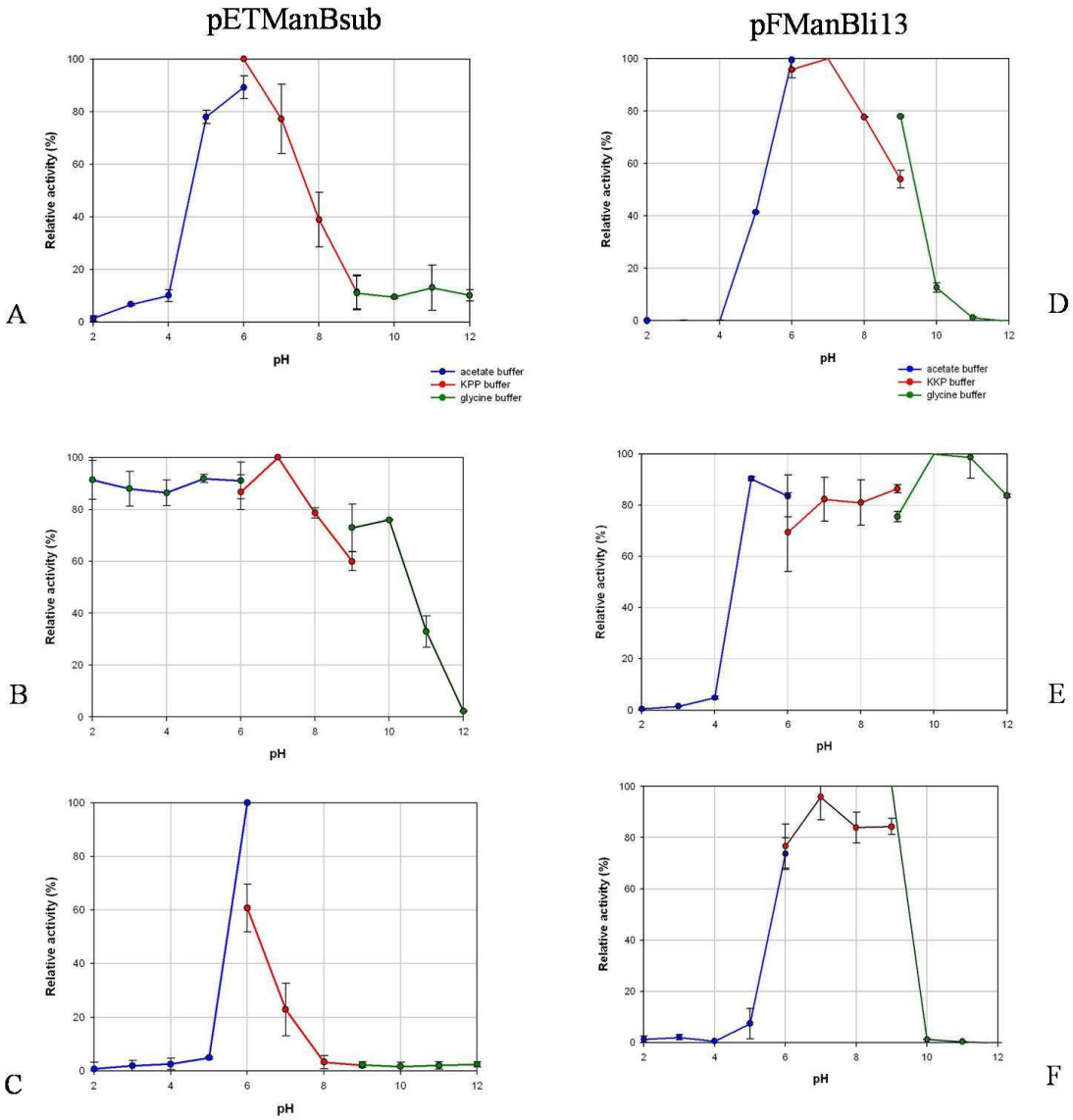

Figure 10. Effect of pH on the recombinant mannanases activity. Panel A and D; Optimum pH of recombinant mannanases from *B. subtilis* and *B. licheniformis*. Panel B and E; The pH stability of *Bacillus* mannanases were determined by measuring the remaining activity after incubation at various pH values at 50 ^{o}C for 30 min. and 24 hr (C, F).

pH stability of pFManBli13 was within pH 5.0-12.0 after incubation for 30 min at 50 ^{o}C, and within pH 6.0-9.0 after incubation for 24 hrs, without substrate.

The pETManBsub was stable within pH 2.0-10.0 after incubation for 30 min at 50 ^{o}C, and pH 6.0 after incubation for 24 hrs at 50 ^{o}C.

4.6 Substrate specificity and kinetic parameter

The relative activities of *Bacillus* mannanases for various substrates were determined as submitted in Table 2. The enzymes exhibited the highest activity on glucomannan prepared from konjac followed by pure 1,4-β-D-mannan and the galactomannan locust bean gum (LBG). The activity of the enzyme with highly substituted galactomannan from guar gum and copra meal was negligible when using the standard assay. However, we found that partial hydrolysis of copra meal after incubation occurred after incubation of this substrate with the enzyme for 2 to 3 days (data not shown).

Table 2. Substrate specificity of *Bacillus* mannanases.

Substrate	**Relative activity (%)**	
	pETManBsub	**pFManBli13**
Glucomannan (Konjac)	128	219
1,4-b-D-Mannan	295	166
Locust bean gum	100	100
Guar gum	nd	nd
Copra meal	nd	nd

Furthermore, the kinetic constants for the hydrolysis of selected substrates were determined. Because of the extremely high viscosity of LBG solutions especially at higher concentrations necessary for the determination of the kinetic

constants, low-viscosity LBG was prepared by partial hydrolysis (Kremnicky et al., 1996) and used as a substrate in addition to glucomannan from konjac and pure 1,4-β-D-mannan. When present in saturating concentrations, low-viscosity LBG was the preferred substrate as judged both from the highest turnover number kcat and specificity constant k_{cat}/K_m (Table 3).

Table 3. Kinetic parameters of *Bacillus* mannanases with various substrate.

Kinetics		**Substrate**		
		Glucomannan	**LBG low viscosity**	**β-D-mannan**
Vmax ($\mu molmin^{-1}mg^{-1}$)	pETManBsub	17,000	45,000	14,600
	pFManBli13	30,400	45,300	26,400
Km ($mgml^{-1}$)	pETManBsub	5.3	18.1	11.8
	pFManBli13	14.9	17.5	15.2
***k**cat* (s^{-1})	pETManBsub	12,400	32,700	10,600
	pFManBli13	21,000	31,200	18,200
***k**cat*/**Km** ($mg^{-1}s^{-1}ml$)	pETManBsub	2,400	1,800	900
	pFManBli13	1,400	1,800	1,200

4.7 Thin layer chromatography

Product of the recombinant enzymes were analyzed by TLC method. When the locust bean gum was used as a substrate, the main products were M2, M3, and M4. Recombinant mannanases degradation pattern was shown in Figure by TLC method.

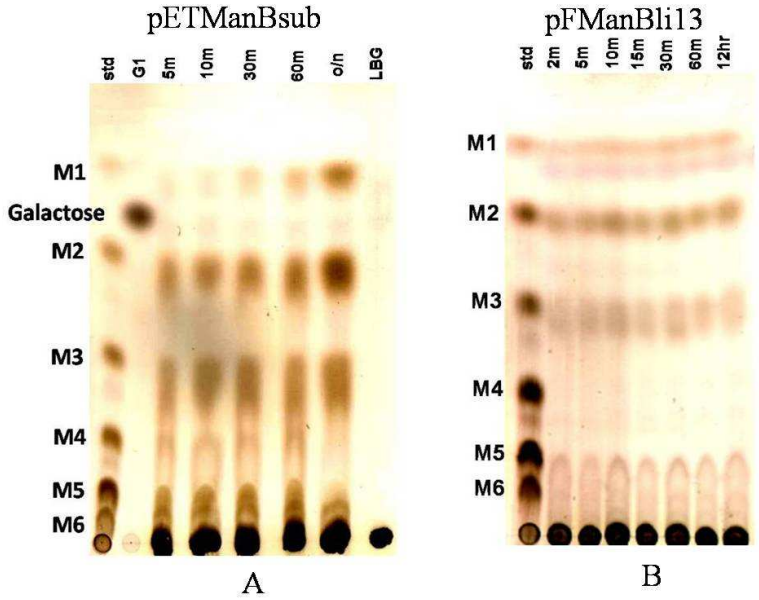

Figure 11. Thin layer chromatography analysis of hydrolysis products using locust bean gum as a substrate. pETManBsub and pFManBli13 hydrolized locust bean gum were shown in Panel A and Panel B respectively. Std: a standard mixture of M1-M6; G1: galactose; 2 to o/n are the reaction products after incubation at various time at 2 min to overnight.

The products of recombinant mannanase were mannobiose, mannotriose, and mannotretaose. TLC analysis of hydrolysis products confirmed that recombinant *Bacillus* mannanse is an endo-mannanase, which can efficiently and randomly cleave higher molecular weight mannans containing more than six mannose monomers. This property suggests that this enzyme could be applicable for the generation of prebiotic manno-oligosaccharides (MOS).

CHAPTER VI

CONCLUSIONS

In summary, the *Bacillus* mannanases were cloned into *E. coli* system under the control of T7 and tac promoter for compared native signal peptides and signal peptide from E. coli outer membrane protein (OmpA). The recombinant *Bacillus* mannanases could be induced by IPTG for over-expression and secretion into periplasmic space. Immobilized-metal affinity chromatography (IMAC) was used for purification of recombianant mannanase by gravity-flow chromatography, using the Ni-NTA Agarose. Optimum temperature of mannanases from both strain were 50 ^{o}C, the recombinant *B. licheniformis*DSM13 was stable up to 55 ^{o}C and recombinant *B. subtilis*168 was stable up to 50 ^{o}C after incubated at pH 6.0. The optimal pH of mannanase from *B. licheniformis* DSM13 was pH 6.0-7.0, stable within pH 5.0-12.0 after incubation for 30 min at 50 ^{o}C and, within pH 6.0-9.0 after incubation for 24 hr. The optimal pH of mannanase from *B. subtilis* 168 was pH 6.0, stable within pH 2.0-10.0 after incubation for 30 min at 50 ^{o}C and pH 6.0 after incubation for 24 hr. The mannanase from *B.lichenisformis* and *B. subtilis* belonged to glycosyl hydrolase family 26 (GH26). The recombinant Bacillus mannanases shows the highest relative activity for glucomannan prepared from konjac. Based on the kinetic characterization, the galactomannan locust bean gum (low viscosity) is the preferred substrate. TLC analysis of hydrolysis products confirmed that recombinant *Bacillus* mannanse is an endo-mannanase.

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APPENDIX I

MEDIA PREPARATION

1. REAGENT

1.1 LB medium (Luria-Bertani medium)

tryptone, 10 g

yeast extract, 5 g

NaCl, 10 g

deionized H2O, to 950 ml

Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume to 1 liter with H2O.

Sterilize by autoclaving

1.2 0.5% of LBG

The substrate, 0.5% locus bean gum (Sigma), was dissolved in 50 mM sodium citrate buffer, pH 6.0 by homogenizing at 80^{o}C, heating to the boiling point, cooled and stored overnight with continuous stirring, after that insoluble were removed by centrifugation.

1.3 LBG low viscosity

Low viscosity locust bean gum was prepared according to previously published protocol (Kremnicky et al. 1996). 30 g of LBG was dissolved in 900 ml of water and boiled. After cooling, the solution was acidified to pH 2.6 using concentrate HCl and autoclaved for 15 min at 121 KPa. The insoluble particles were separated by centrifugation and the supernatant was precipitated with 2 volumes of 95% ethanol

(1.7 l) and kept overnight at 4^{o}C. The precipitate was collected by centrifugation at 4,000 rpm for 15 min, suspended in 1.2 l of water warmed to 70^{o}C and dissolved by shaking. The homogeneous solution was lyophilized.

APPENDIX II

RAW DATA

2.1 STANDARD CURVE

2.2.1 Standard curve for DNS method

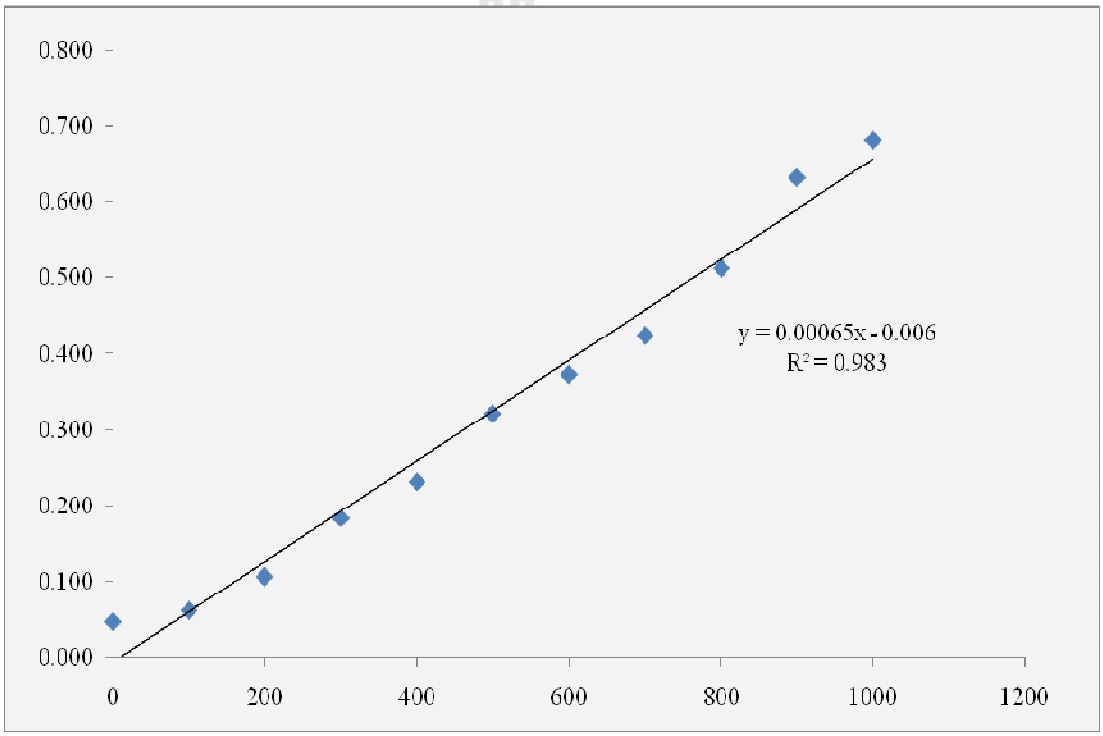

Figure 1. Standard curve of mannose for DNS method.

2.2.2 Standard curve for Bradford method

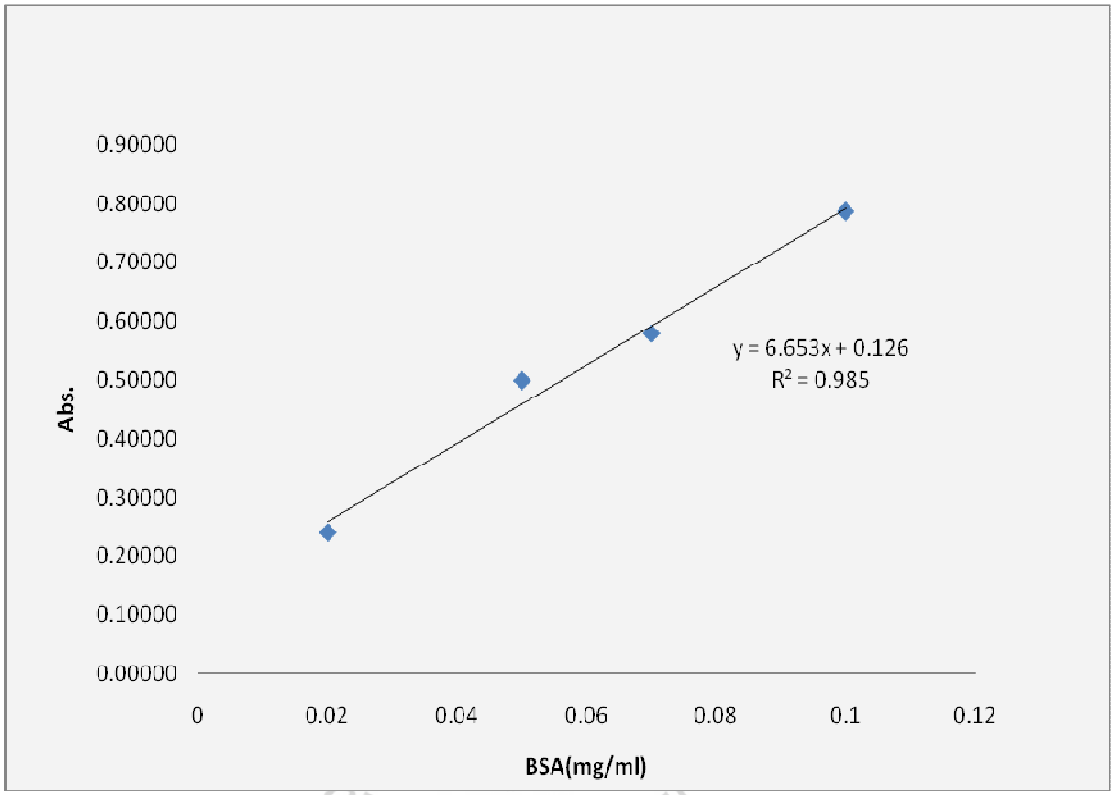

Figure 2. Standard curve BSA for protein determination.

2.2 Result data

2.2.1 Optimum temperature

Tabel 1. Optimum temperature of *Bacillus* mannanase.

Temperature (°C)	pFManBli13				pETManBsub			
	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV
0	0.602	1.435	4.15	2.42	0.046	0.000	1.45	0
20	11.389	12.037	47.74	2.12	4.630	4.676	25.69	0.21
30	15.694	16.713	66.04	3.29	6.296	6.389	34.5	0.4
40	17.917	18.380	73.96	1.73	18.333	17.917	95.9	1.37
50	24.630	24.444	100	0	18.935	18.889	100	0
60	21.852	22.176	89.72	1.41	11.574	11.435	61.4	0.41
70	11.204	11.806	46.89	1.98	1.111	0.463	3.02	1.2
80	0.000	0.417	0.57	1.21	0.000	0.000	0.97	0
90	0.046	0.324	0.76	0.8	0.046	0.000	1.33	0.17
100	0.417	0.278	1.42	0.39	0.093	0.000	1.45	0

2.2.2 Thermostability

Table 2. Thermostability of *Bacillus* mannanase after incubation for 30 min at pH 6.0.

Temperature (°C)	pFManBli13				pETManBsub			
	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV
4	416.67	429.63	91.49	6.26	389.40	409.89	77.61	3.36
20	477.78	447.22	100.00	0.00	351.82	353.53	68.49	0.72
30	401.85	423.15	89.19	7.43	452.59	471.38	89.72	3.21
40	435.19	464.81	97.30	9.09	406.48	416.72	79.93	1.97
45	423.15	475.93	97.20	12.62	517.49	512.37	100.00	0.00
50	400.00	443.52	91.19	10.93	485.04	476.50	93.37	0.52
55	395.37	446.30	90.99	12.05	1.71	3.42	0.50	0.24
60	70.37	67.59	14.91	0.27	3.42	8.54	1.16	0.71
70	0.93	5.56	0.70	0.74	10.25	11.96	2.16	0.25
80	0.93	0.00	0.10	0.14	8.54	8.54	1.66	0.01

Table 3. Thermostability of *Bacillus* mannanase after incubation at 60 °C, pH 6.0.

Time (min)	pFManBli13				pETManBsub			
	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV
0	550.93	567.59	100.00	0.00	423.70	420.00	100.00	0.00
1	418.52	426.85	75.58	0.54	420.00	401.49	97.37	2.50
1.5	391.67	385.19	69.45	2.28	394.09	386.69	92.54	0.67
2	352.78	340.74	62.00	2.83	327.49	321.94	76.97	0.45
2.5	322.22	305.56	56.13	3.29	323.79	333.04	77.85	2.03
3	291.67	276.85	50.83	2.94	-	-	-	-
5	216.67	194.44	36.76	3.59	116.56	129.51	29.17	0.00
7.5	34.26	50.93	7.62	1.95	105.46	114.71	26.10	2.35
10	10.19	34.26	3.97	2.96	105.46	101.76	24.56	1.71
12.5	-	-	-	-	18.50	9.25	3.29	0.47

2.2.3 Optimum pH

Table 4. Optimum pH of *Bacillus* mannanases

pH	pFManBli13				pETManBsub			
	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV
Acetate buffer pH2	-0.93	0.93	1.46	1.03	22.20	6.83	0.00	0.26
Acetate buffer pH3	-3.70	0.00	6.82	0.38	66.61	66.61	-0.36	0.51
Acetate buffer pH4	0.00	-3.70	10.16	2.28	119.55	80.27	-0.38	0.53
Acetate buffer pH5	212.04	202.78	77.98	2.60	773.67	749.76	41.40	0.33
Acetate buffer pH6	482.41	513.89	89.23	4.30	937.63	809.54	99.53	6.79
KPP buffer pH6	488.89	471.30	100.00	0.00	1016.19	939.34	95.84	0.23
KPP buffer pH7	509.26	492.59	77.22	13.18	689.98	812.95	100.00	0.00
KPP buffer pH8	395.37	383.33	38.98	10.44	321.08	435.51	77.73	0.13
KPP buffer pH9	262.96	277.78	11.45	6.44	70.02	150.29	54.01	3.36
Glycine buffer pH9	396.30	385.19	11.10	6.42	66.61	146.88	78.01	0.27
Glycine buffer pH10	58.33	68.52	9.62	0.53	93.93	93.93	12.68	1.74
Glycine buffer pH11	4.63	6.48	13.13	8.53	194.70	66.61	1.11	0.29
Glycine buffer pH12	-1.85	-0.93	10.25	2.15	119.55	81.98	-0.28	0.12

2.2.4 pH stability

Table 5. pH stability of *Bacillus* mannanases after incubation at 50 ^{o}C for 30 min.

pH	pFManBli13				pETManBsub			
	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV
Acetate buffer pH2	4.17	2.78	0.44	0.12	1175.02	1086.21	91.35	7.47
Acetate buffer pH3	14.17	8.33	1.50	0.12	1127.20	1050.35	87.96	6.70
Acetate buffer pH4	45.83	23.15	4.86	0.64	1093.05	1045.22	86.35	5.00
Acetate buffer pH5	853.33	478.70	90.38	0.91	1130.62	1145.99	91.89	1.54
Acetate buffer pH6	789.17	409.26	83.60	8.18	1168.19	1086.21	91.06	7.07
KPP buffer pH6	655.83	422.22	69.43	15.34	995.70	1152.82	86.61	6.69
KPP buffer pH7	777.50	464.81	82.33	8.55	1216.01	1262.13	100.00	0.00
KPP buffer pH8	765.00	393.52	81.04	8.79	939.34	1009.36	78.61	1.93
KPP buffer pH9	815.83	460.19	86.40	1.55	760.01	724.14	59.94	3.62
Glycine buffer pH9	713.33	389.81	75.56	2.03	964.95	838.57	72.90	9.13
Glycine buffer pH10	944.17	525.93	100.00	0.00	922.26	959.83	75.95	0.15
Glycine buffer pH11	932.50	550.00	98.75	8.24	348.41	469.67	32.93	6.05
Glycine buffer pH12	790.00	442.59	83.67	0.69	29.03	25.62	2.21	0.25

Table 6. Thermostability of *Bacillus* mannanase after incubation at 50 ^{o}C for 24 hr.

pH	pFManBli13				pETManBsub			
	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV	Activity (U) 1	Activity (U) 2	Relative activity (%)	STDEV
Acetate buffer pH2	7.41	1.85	1.37	1.18	17.08	-6.83	0.74	2.48
Acetate buffer pH3	9.26	4.63	2.04	1.00	3.42	22.20	1.90	1.97
Acetate buffer pH4	2.78	0.93	0.55	0.39	27.33	6.83	2.51	2.11
Acetate buffer pH5	38.89	11.11	7.37	5.89	35.87	30.74	4.90	0.49
Acetate buffer pH6	262.96	239.81	73.64	6.20	683.15	674.61	100.00	0.00
KPP buffer pH6	278.70	244.44	76.64	8.56	372.32	452.59	60.79	8.90
KPP buffer pH7	344.44	310.19	95.89	8.93	203.24	107.60	22.85	9.76
KPP buffer pH8	297.22	275.93	83.93	6.02	34.16	10.25	3.26	2.46
KPP buffer pH9	291.67	284.26	84.31	3.15	10.25	17.08	2.02	0.73
Glycine buffer pH9	337.04	346.30	100.00	0.00	20.49	10.25	2.26	1.05
Glycine buffer pH10	4.63	3.70	1.22	0.22	3.42	18.79	1.64	1.62
Glycine buffer pH11	1.85	0.93	0.41	0.20	20.49	6.83	2.01	1.41
Glycine buffer pH12	-0.93	0.00	-0.14	0.19	11.96	20.49	2.39	0.91

2.2.5 Kinetic parameters

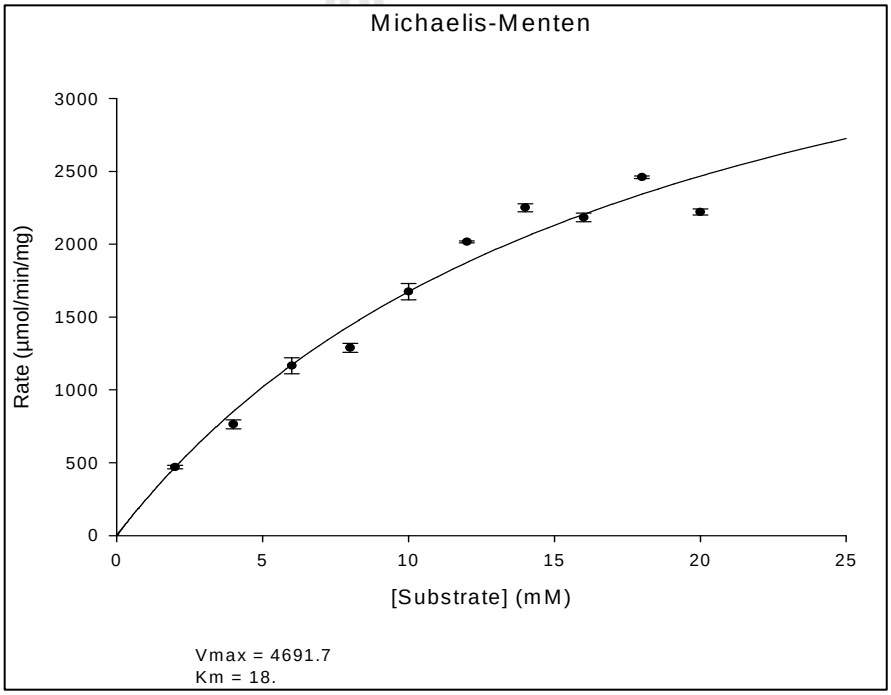

Figure 3. Kinetic parameter of *Bacillus* mannanase used β-D-mannan as a substrate.

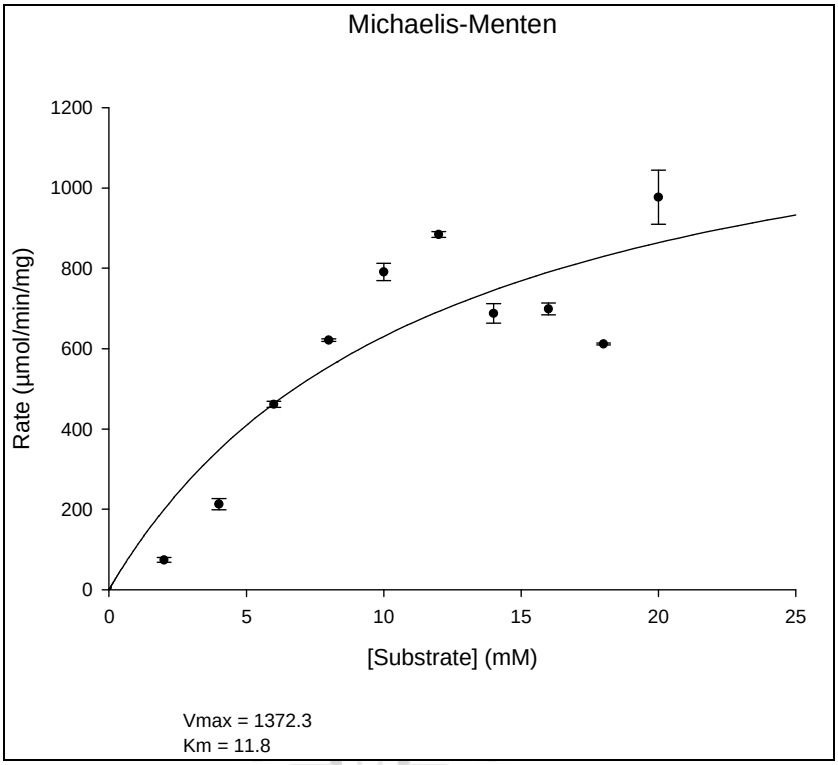

Figure 4. Kinetic parameter of *Bacillus* mannanase used LBG low viscosity as a substrate.

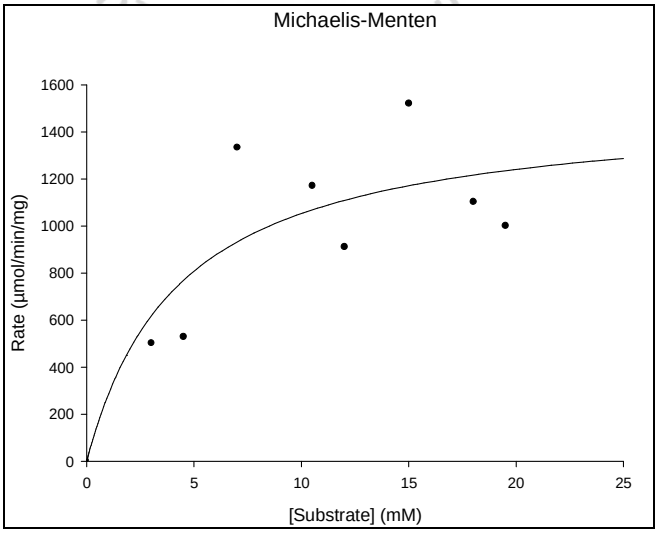

Figure 5. Kinetic parameter of *Bacillus* mannanase used glucomannan as a substrate.

APPENDIX III

PUBLICATION

Chomphunuch Songsiriritthigul, **Bancha Buranabanyat**, Dietmar Haltrich, and Montarop Yamabhai. Efficient Recombinant Expression and Secretion of a Thermostable β-Mannanase from *Bacillus licheniformis* in *Escherichia coli*. (Manuscript in preparation)

Yamabhai, M., Emrat, S., Sukasem, S., Pesatcha, P., Jaruseranee, N., **Buranabanyat, B.**: Secretion of recombinant *Bacillus* hydrolytic enzymes using *Escherichia coli* expression systems. J Biotechnol 2008, 133: 50-57.

BIOGRAPHY

Bancha Buranabanyat was born on May 3, 1977 in Nakhon Ratchasima, Thailand. He graduated with Bachelor degree from Department of Biotechnology, Faculty of Agricultural, Rajamangara Institute of Technology, in 1999. After graduation, He has opportunity to study master degree in school of Biotechnology, Institute of Agricultural at Suranaree University of Technology. He conducts to research in the topic of cloning, purification and characterization of *Bacillus* mannanases as his thesis work.

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