การโคลนและลักษณะสมบัติของแมนแนนเนสจากแบคทีเรียที่แยกจากตัวอ่อนด้วงเฮอร์คิวลิส

Dynastes hercules

นายสิทธิพล ลีระวัฒนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2560
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CLONING AND CHARACTERIZATION OF BACTERIAL MANNANASE ISOLATED FROM

Dynastes hercules LARVAE

Mr. Sitipon Leerawatthanakun

จุฬาลงกรณ์มหาวิทยาลัย
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By	Mr. Sitipon Leerawatthanakun
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Thesis Advisor	Assistant Professor Rath Pichyangkura, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

--Dean of the Faculty of Science

(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

--Chairman

(Assistant Professor Kanoktip Packdibamrung, Ph.D.)

--Thesis Advisor

(Assistant Professor Rath Pichyangkura, Ph.D.)

--Examiner

(Associate Professor Teerapong Buaboocha, Ph.D.)

--External Examiner

(Wonnop Vissessanguan, Ph.D.)

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เมื่อวิเคราะห์ยีน 16S rRNA ของแบคทีเรียสายพันธุ์ HM7 ที่แยกได้จากของเสียของตัวอ่อนด้วงแล้ว พบว่าเป็นแบคทีเรียกลุ่ม Bacillus แมนแนนเนสที่ได้จากการเลี้ยงเชื้อในอาหารเลี้ยงเชื้อที่มีการเติมผงบุกมีแอกติวิตีจำเพาะสูงสุด 138 ยูนิตต่อมิลลิกรัมโปรตีนที่เวลา 36 ชั่วโมง และที่มีการเติมกากมะพร้าวมีแอกติวิตีจำเพาะสูงสุด 114 ยูนิตต่อมิลลิกรัมโปรตีนที่เวลา 48 ชั่วโมง จากนั้นทำการโคลนและแสดงออกยีนแมนแนนเนส (*Man26HM7*) ที่มีขนาด 1089 คู่เบสใน *Escherichia coli* BL21 (DE3) โดยใช้ pET21-b และศึกษาลักษณะสมบัติ พบว่ามีภาวะที่เหมาะสมในการเร่งปฏิกิริยาของแมนแนนเนสจากสายพันธุ์ HM7 (MAN26HM7) คือ 50 °C ที่ พีเอช 6.0 จากการเปรียบเทียบความทนทานต่อ SDS ของเอนไซม์ MAN26HM7 และ แมนแนนเนส จาก *Bacillus subtilis* CAe24 (MAN26CAe24) ต่อ SDS พบว่า เมื่อทำการบ่มเอนไซม์ในสภาวะที่มี SDS ความเข้มข้น 1% (w/v) ที่อุณหภูมิ 37 °C เป็นเวลา 180 นาที MAN26HM7 มีแอกติวิตีเหลือมากกว่า 90% ขณะที่แอกติวิตีของ MAN26CAe24 เหลือมากกว่า 60% และในสภาวะที่มี SDS เท่ากับ 2% (w/v) ที่อุณหภูมิ 50 และ 60 °C MAN26HM7 และ MAN26CAe24 มีแอกติวิตีเหลือ 60% และ 40% ตามลำดับ และเมื่อบ่มแมนแนนเนสในสภาวะที่มีสารซักฟอกแบบผง ที่ความเข้มข้น 0.5% (w/v) ลดแอกติวิตีของเอนไซม์ MAN26HM7 และ MAN26CAe24 เหลือ 55% และ 51% ตามลำดับ ได้มากกว่าสารซักฟอกแบบน้ำที่ความเข้มข้น 5% (w/v) MAN26HM7และ MAN26CAe24 มีแอกติวิตีเหลือ 95% และ 90% ตามลำดับ เมื่อเปรียบเทียบลำดับกรดอะมิโนของ MAN26HM7 และ MAN26CAe24 พบว่ามีกรดอะมิโนที่ต่างกันทั้งหมด 7 ตำแหน่ง โดยมี 1 ตำแหน่งที่อยู่ด้านในของเอนไซม์ คือ ลิวซีนที่ตำแหน่ง 238 ใน MAN26HM7 ที่ต่างจากMAN26CAe24 เมื่อทำการกลายพันธุ์เฉพาะจุดของกรดอะมิโนในตำแหน่ง ไอโซลิวซีน 238 ของ MAN26CAe24 โดยเปลี่ยนจาก ไอโซลิวซีน เป็น ลิวซีน พบว่าสามารถเพิ่มความทนทานของเอนไซม์ MAN26CAe24 ต่อ SDS มากขึ้น แมนแนนเนสที่มีสมบัติทนทานต่อ SDS สามารถนำไปประยุกต์ใช้ในอุตสาหกรรมสารซักฟอกได้

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต	-----------------------------------
สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อ อ.ที่ปรึกษาหลัก	-----------------------------
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SITIPON LEERAWATTHANAKUN: CLONING AND CHARACTERIZATION OF BACTERIAL MANNANASE ISOLATED FROM *Dynastes hercules* LARVAE. ADVISOR: ASST. PROF. RATH PICHYANGKURA, Ph.D., 125 pp.

Bacterial isolate HM7 was isolated from *Dynastes hercules* larvae excrement. It was identified as *Bacillus* sp. by 16S rRNA gene analysis. When the bacteria were cultured for β-mannanase production in medium containing konjac flour it had the highest specific activity of 138 U/mg at 36 h, and when cultured in medium containing coconut meal it had the highest specific of 114 U/mg at 48 h. The 1089 base pairs long of a β-mannanase encoding gene (*Man26*HM7) was successfully cloned and expressed in *Escherichia coli* BL21 (DE3), using pET21-b expression system. The enzyme was purified and characterized. The optimal condition MAN26HM7catalysis was 50 °C and pH 6.0. MAN26HM7 showed surfactant-tolerant ability when compared to *Bacillus subtilis* CAe24 mannanase (MAN26CAe24). The residual activity of MAN26HM7 and MAN26CAe24 was 90% and 60% after incubated with 1.0 % (w/v) SDS at 37 °C for 180 min, and retaining 60 % and 40 % of MAN26HM7 and MAN26CAe24 activity at 2.0% (w/v) SDS at 50 and 60 °C, respectively. Powder detergent at 0.5 % (w/v) decreased β-mannanase activity, retaining more than 55 % and 51 % of MAN26HM7 and MAN26CAe24) activity, respectively, more than 5 % (v/v) liquid detergent, retaining more than 95 % and 90 % of MAN26HM7 and MAN26CAe2424) activity. Amino acid sequence alignment of MAN26HM7 and MAN26CAe24 showed amino acid changes at 7 positions, one of which was found in the interior of the protein, leucine 238. Leucine at this position in MAN26HM7 was replaced with Isoleucine in MAN26CAe24. Sited-directed mutagenesis of amino acid at this position in MAN26CAe24 from I to L improved SDS-tolerant of the enzyme. Surfactant-tolerant β -mannanase can be applied in detergent industry.

Department:	Biochemistry	Student's Signature	-------------------------------------
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CHAPTER I

INTRODUCTION

Carbohydrate polymers such as cellulose, hemicellulose and starch have been rigorously studied, and have been used in various applications. Because the main structure of carbohydrate polymers is composed of carbon that can be infinitely produce from nature or agriculture, it has sustainability and renewability. These materials were used in various manufacturing processes and industry especially in agricultural country. Many agricultural by products from cultivation and industry are produced, which can be converted to higher value products such as oligosaccharides.

1.1 Mannan

Polysaccharide are abundant in the nature, we can find them in the cell wall of plants and microorganisms. The study of the plant cell wall structure showed that cell wall consists of three major components cellulose, hemicellulose and lignin (Dekker, 1985). Hemicellulose can be found in both primary and secondary cell wall (Puls, 1997). Hemicellulose is not soluble in water, but soluble in aqueous alkali solution (O'Neill and Selvendran, 1985). Hemicellulose is composed of mannan, heteromannan, xylan and arabinan (O'Neill and York, 2003), as shown in Table 1. Mannan and heteromannan can be found in hard wood, soft wood (Capek et al., 2000) seeds(Handford et al., 2003), (Buckeridge et al., 2000) and beans. Mannan links

between lignin and cellulose, covalently. Mannan bounded lignin produced a coat around cellulose using hydrogen bonding. This linkage protects and maintains the integrity of the cell wall structure against degradation by cellulose (Lundqvist et al., 2002).

Table 1   Hemicellulose composition

Carbohydrate	Application
Xylan	• biopolymer ethers and esters (Mackie and Preston, 1974) • functional food due to prebiotic properties (Petzold-Welcke et al., 2014) • inhibitory action on mutagenicity activity (Broekaert et al., 2011) • Fermentative process of xylan for production of biofuels (Ebringerová et al., 1995)
Glucuronoxylan	• Glucuronoxylan-mediated silver nanoparticles for antimicrobial and wound healing (Pauly and Keegstra, 2010) • Application as oxygen barrier films (Muhammad et al., 2017) • The antiviral potency of 4OMe-glucuronoxylan sulfates (Gröndahl et al., 2004)
Arabinoxylan	• Reduction of serum cholesterol and improved adsorption of calcium and magnesium by production of short-chain fatty acids from cecal fermentation. (Pujol et al., 2016), (Hopkins et al., 2003), (Lopez et al., 1999) • Antioxidant activity (Izydorczyk and Dexter, 2008) , (Rao and Muralikrishna, 2006) • Formation of films without any addition of plasticizers (Höije et al., 2005)
Mannan	• Decrease overweight, diabetes and cardiovascular disease (Livieri et al., 1992)

	• Inhibition of bacterial cell adhesion and recognition (Wellens et al., 2008) • Improvement animal feed (Chandrasekariah et al., 2001)
Xyloglucan	• Thickening, gelling and stabilizing agents in <u>food</u> (Nitta and Nishinari, 2005), (Mishra and Malhotra, 2009) • Sizing agents for textiles (Zhou et al., 2007) • Inhibition of UV-induced immune suppression and interleukin-10 production (Strickland et al., 1999)

Mannans are divided into mannan, glucomannan and galactomannan. Mannan is β -mannopyranose linked together with β -1,4 glycosidic bond. Glucomannan is β -mannopyranose and glucopyranose residues linked together with β -1,4 glycosidic bond (Puls and Schuseil, 1993). Galactomannan is mannose residues are linked at the O-6 position of α-galactose as shown in Figure 1. Leguminous seeds use galactomannan as main carbohydrate fuel source, 20% of total dry weight (Maeda et al., 1980). *Saccharomyces cerevisiae* produce mannan as the major component of its cell wall (McCleary, 1988). Rigidity and osmotic pressure protection of cell wall were caused by the presence of mannan(Liu et al., 2009) In green algae, mannan was produced as the major component of the cell wall, backbone, and mantle structure. They have been described as non-cellulosic organism (Inoue et al., 1995).
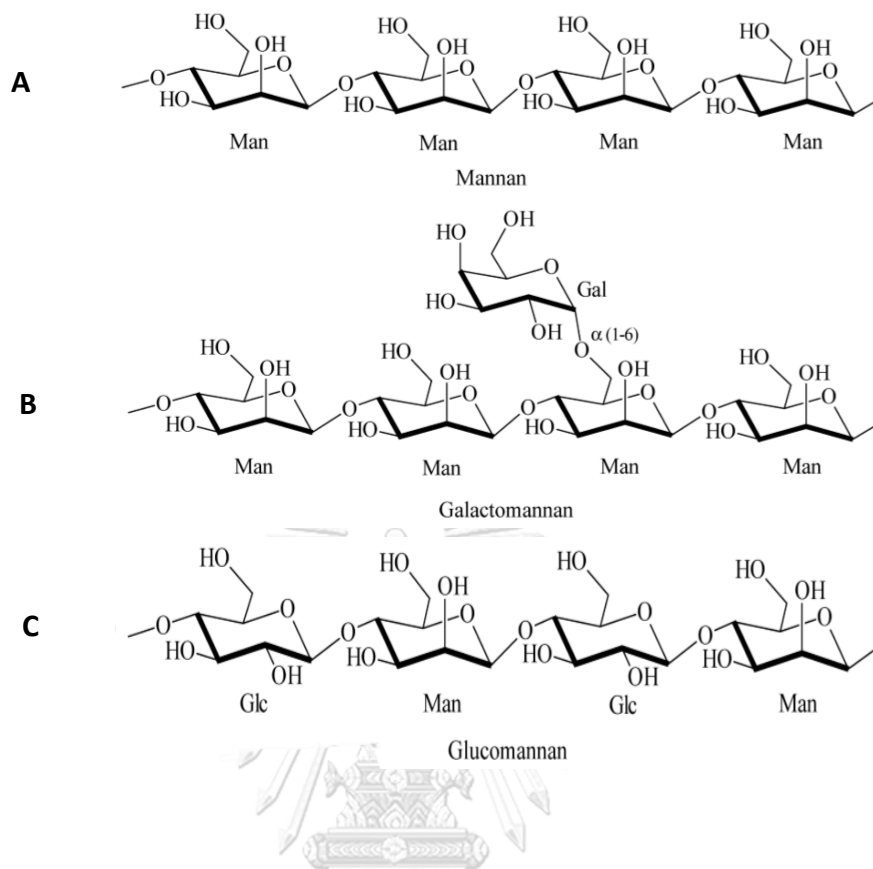
Figure 1   **Structure of mannan and heteromannan**

A) β-1,4 linked mannose residues, mannan, B) β-1,4 linked mannose residues with α-1,6 linked galactose residues, galactomannan and C) β-1,4 linked mannose and glucose residues, glucomannan (Dekker and Richards, 1976)

1.2   Application of mannan and manno-oligosaccharide

1.2.1   Application of mannan in medicine

Mannan, manno-oligosaccharide, and derivatives of mannose have been used for medical applications. Mannan was used as dietary fiber in patient. Overweight, diabetes and cardiovascular disease can be treating by consumption of fiber food. It has been reported that glucomannan can reduce body weight (Walsh et al., 1984), (Birketvedt et al., 2005). Glucomannan are soluble in water and high viscosity. Previous study, nutrient absorption from viscous fiber supplement digestion is difficult (Schneeman, 1987)

Manno-oligosaccharide was used as prebiotic, these oligosaccharides increase colon intact by inflammatory and pathogen infection reduction (Drakoularakou et al., 2011). Manno-oligosaccharide reduces weight and visceral fat by interruption of fat absorption (Kumao and Fujii, 2006).

Derivatives of mannose were used in bacterial infection protection. Urinary tract infection is commonly occurred by uropathogenic E. coli adhesion (Klein et al., 2010). Bacterial adhesion was caused by FimH in bacterial surface. Synthesis of inhibitor for FimH, α-D-mannoside derivatives such as SAMan, AzoMan, methyl α-D-mannoside (MeMan) and p-nitrophenyl α-D-mannoside (pNPMan) (Wellens et al., 2008) protected bacterial adhesion in animal model (Hartmann and Lindhorst, 2011).

1.2.2   Application of manno-oligosaccharide in feed

Manno-oligosaccharide has benefit in poultry, swine and cattle. In poultry, manno-oligosaccharide enhance intestinal integrity and microbial populations in the Ceca.(Baurhoo et al., 2007) and increase digestive enzyme activity such as maltase in

jejunum (Iji et al., 2001). Manno-oligosaccharides can function as prebiotics, these oligosaccharides are *Bifido* bacteria carbon source in the caeca that promote the cell proliferation(Baurhoo et al., 2009). Manno-oligosaccharide promote immunoglobulins production and quality in sow colostrum to decrease piglet's disease (Rosen, 2007). *Manno-oligosaccharide has higher effect in younger pig than older* ones (Castillo et al., 2008). Manno-oligosaccharide increase weight, immune response, milk protein and fat in cattle (Tassinari et al., 2007), (Bagheri et al., 2009). Antibiotic usage in cattle can be reduced in animal consuming manno-oligosaccharide supplemented feed(Kegley and Kafka, 2003). For this reason, Modified feed by supplementing manno-oligosaccharide is very appealing for feed improvement.

1.3   Mannanase

1.3.1   Classification of Mannases

Endo-1,4-β-mannanases (E.C 3.2.1.78, mannan endo-1,4-β-mannosidase) breakdown mannan to manno-oligodaccharides and sugars. β-1,4 glycosidic bond in mannan backbone is hydrolyzed internally by endo-1,4-β-mannanases(McCleary and Matheson, 1986). Mannanases are classified into two glyucosyl hydrolase (GH) family, GH family 5 and 26 (Henrissat, 1991). Hydrolysis property can be found both families but transglycosylation activity can be found only in family 5 (Schröder et al., 2006).

Mannan cannot be hydrolyzed completely by mannanase. A mixture of mono-saccharides and oligo-saccharides are normally the result of enzymatic hydrolysis. Furthermore, additional of galactosidases (EC3.2.1.22) (Craven et al., 1965) are necessary for removing galactosyl residue from the mannan backbone (Clarke et al., 2000). The mechanism of endo-1,4- β -mannanases was reported to be a retaining mechanism, which has two catalytic acidic amino acid residues involved in catalysis. The first acidic amino acid residue, Glu, serves as a nucleophile and the

second Glu serves as acid/base. This mechanism is an acid/base catalysis, as shown in Figure 2.
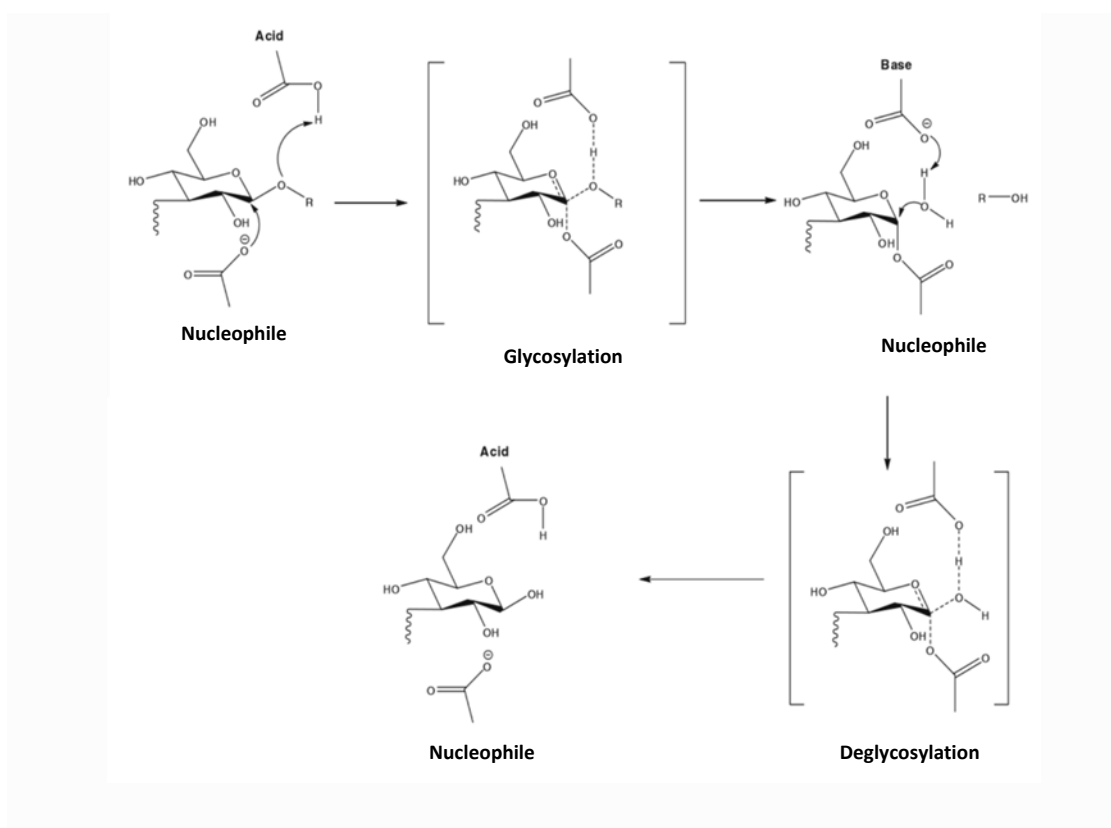
Figure 2   General mechanism for retaining glycosyl hydrolase. The acid/base catalytic residues are shown (Withers, 2001)

Mannanase can be found in many microorganisms such as yeasts, fungi and bacteria (Puchart et al., 2004). *Bispora* sp., *Aspergillus aculeatus* and *Agaricus bisporus* are mannan degrading fungi (Luo et al., 2009), (Pham et al., 2010), (Tang et al., 2001) *Bacillus* species (Akino et al., 1989) and *Clostridia* (Perret et al., 2004) species are gram positive bacteria, while *Vibrio* (Tamaru et al., 1995) and *Pseudomonas* (Yamaura et al., 1990) are gram negative bacteria that can produce mannanase. Mannanase can also be isolated from plants and mollusk (Xu et al., 2002).

Some β -D-mannanases has transglycosylation property. Tomato (*Solanum lycopersicum* L.) in ripening phase and kiwifruit flower (*Actinidia*) was reported to have high level of transglycosylation activity (Schröder et al., 2004).

1.3.2   Applications of mannanase

Mannnase was used and applied in many industries, such as pulp and paper, food and food-processing and household products.

Mannanase was used in pulp production to increase the brightness of the product. Pulp brightness process produces massive of chemical wastes, so mannanase usage reduces the use of these chemical reagent (Bajpai, 1999). Pulp is used as the starting material for lyocell and viscose (Schild and Sixta, 2011), which can be applied in the textile industry (Nicolai et al., 1996). High concentration of mannan decreases the strength of these products (Gübitz et al., 1997).

Moreover, mannanase was applied in instant coffee production. green coffee bean mainly consists of water-insoluble mannan (Bradbury and Halliday, 1990). High concentration of mannan in roasted coffee extract cause high viscosity which interferes the freeze-drying process (Harding et al., 2017). Mannanase hydrolysis can simplify the production of instant coffee in the freeze-drying process (Sagara and Ichiba, 1994).

Furthermore, mannanase was added into many detergents. Sticky stabilizers containing mannan are popular and widely used in many household products, cosmetic and food such as glucomannan and guar gum (Mudgil et al., 2014), (Bourriot et al., 1999). Elimination of stabilizers on cloths by mannanase hydrolysis produces water-soluble and non-binding fabrics polysaccharide can help remove stains or soil produced by these additives. Surfactant tolerant mannanase have been studied for potentiality application in commercial detergent (Zhang et al., 2016).

1.4   Objective of this project

Mannanase producing bacterial isolate HM7, from Dynastes hercules larvae excrement was identified by 16S rRNA gene analysis. Manno-oligosaccharide pattern produced from the hydrolysis of konjac flour and coconut meal by mannanase from isolated HM7 was studied. Mannanase gene from isolate HM7 was cloned and expressed in *E. coli.* Mannanase was purified by column chromatography. Purified mannanase from *Bacillus* sp. HM7 was characterized and the stability of mannanase from *Bacillus* sp. HM7 in ionic surfactant was studied, comparing with the previously characterized *B. subtilis* CAe24 mannanase.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

All chemical and reagents used in this study were analytical grade chemicals purchased from the following companies : Ajax Finechem (Australia), BDH (England), Becton Dickinson (USA), Biobasic (Canada), Biorad (USA), Carlo Erba (French), Criterion (USA), Daejung (KOREA), Fischer chemical (USA), Genplus (USA), Merck (Germany), RCI Labscan (THAILAND), Scharlua (Spain), Sigma-Aldrich (USA), Vivantis (Malaysia).

2.2 Restriction enzymes and DNA modification enzyme

Table 2   List of restriction enzymes and DNA modification enzyme

Enzyme	Supplier
*Bgl*II	New England Biolabs, Ipswich, MA, USA
Lysozyme	Sigma, USA
*Nde*I	New England Biolabs, Ipswich, MA, USA
Proteinase K	GIBCOBRL, USA
PrimeStar HS DNA polymerase	TAKARA Bioscience, Japan
RNase A	Sigma, USA
T4 DNA ligase	Biotechrabbit, Germany
*Xho*I	New England Biolabs, Ipswich, MA, USA

2.3   Plasmid vectors

Table 3   List of plasmid vectors

Plasmid Vector	Supplier
pET 21-b	Novagen, Madison, WI, USA
pJET blunt end	Thermo Fischer, Waltham, MA, USA

2.4   Microorganism

Table 4   List of microorganisms

Bacterial Strain	Bacterial Genotypes	Supplier or Sources
E. coli TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Novagen, Madison, WI, USA
E. coli BL21(DE3)	F- ompT hsdSB (rBmB-) gal dcm (DE3)	Novagen, Madison, WI, USA
Bacillus sp. HM7	Man$^+$	Isolated from *Dynastes hercules* excrement
B. subtilis CAe 24	Man$^+$	Isolated from soil in Thailand

2.5 Computer Programs

This work used following programs in Table 5

Table 5 Lists of Computer Programs

Program	Purpose of use	Web server/Owner and reference
Blast (Altschul et al., 1990)	Finding similarity in sequence	https://blast.ncbi.nlm.nih.gov/
ClustalW (Thompson et al., 1994)	Nucleotide and amino acid sequence alignment	http://www.ebi.ac.uk/Tools/msa/clustalo/
SignalP (Petersen et al., 2011)	Prediction of cleavage sites	http://www.cbs.dtu.dk/services/SignalP/
SWISS MODEL (Biasini et al., 2014)	Prediction of protein 3D structure	https://swissmodel.expasy.org/
ExPaSy (Bjellqvist et al., 1993)	Amino acid sequence analysis	https://www.expasy.org/
GenBank (Benson et al., 2012)	Genetic sequence database	https://www.ncbi.nlm.nih.gov/

Oligo Calc (Kibbe, 2007)	Check PCR primer usage and properties	http://biotools.nubic.northwestern.edu/
Protein DATA Bank (Berman et al., 2006)	Retrieving the 3D structure of protein	https://www.rcsb.org/
Primer3Plus (Untergasser et al., 2012)	Design oligonucleotide primer	http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
CAZy (Cantarel et al., 2008)	Carbohydrate-Active enzymes Database	http://www.cazy.org/

2.6   Oigonucleotide primers

This work used following primers in Table 6

Table 6   Lists of primer

Primer Name	Sequence	Length	Purpose of use
NdeIFwd.	5' GGGGAGATGCATATGTTTAAGA 3'	22	Amplification of *Man26HM7*
ManRwd.	5' GCTCTAATCAGACGTTCCGC 3'	20	Amplification of *Man26HM7*
pAFwd.	5' AGAGTTTGATCCTGGCTCAG 3'	20	Amplification of 16S rRNA gene
pHRwd.	5' AAGGAGGTGATCCAGCCGCA 3'	20	Amplification of 16S rRNA gene
I238LFwd.	5' CTTGATCATTTGCTGTGGGTTTACTC 3'	26	Amplification of *I238L Man26CAe24*
I238LRwd.	5' GAGTAAACCCACAGCAAATGATCAAG 3'	26	Amplification of *I238L Man26CAe24*

2.7   Media

2.7.1   Luria-Bertani (LB medium)

LB medium;1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl. For LB plate, 1.5% (w/v) agar was added. For cloning and expression, ampicillin was added to LB medium to the final concentration of 100 µg/ml.

2.7.2   Mannan minimum medium

Mannan minimal medium; 1.5% (w/v) glucomannan, 0.05% (w/v) yeast extract, 0.03% (w/v), magnesium sulfate, 0.1% (w/v) ammonium sulfate, 0.6% (w/v) potassium dihydrogen phosphate, 1% (w/v) hydrogen phosphate di-potassium. To induce the β-mannanase production, 1.5% (w/v) konjac flour (glucomannan) or coconut meal (galactomannan) was used as carbon source. Konjac flour was purchased from Monkey King Food (BKK, Thailand). De-oiled coconut meal was a gift from Dr.Wannop Visessanguan, BIOTEC (Pathum Thani, Thailand).

2.8   Agarose electrophoresis

For agarose electrophoresis, agarose gel concentration of 0.7-1.5% (w/v) was used. The gels were run in TAE buffer (20 mM acetate, 40 mM Tris-HCl and 2 mM EDTA, pH8.0) at 100 volts and stained with ethidium bromide solution. UV transluminator was used to detect DNA bands in the gel.

2.9   DNA extraction from agarose gel

The gel extraction kit (Geneaid, Taiwan) was used according to the manual protocol for DNA extraction from agarose gel.

2.10   Amplification of DNA by PCR technic

Genes were amplified from the chromosomal DNA or recombinant plasmid by PCR(Marchesi et al., 1998). Standard protocol for PCR was as follows; The reaction total volume of 50 µl includes PrimeSTAR HS DNA Polymerase 1 U, 50 ng of chromosomal DNA or 20 ng of plasmid DNA as template, 10 µl of 5x PrimeStar buffer, 0.2 mM each of dNTPs, 0.25 pmole each of forward and reverse primer. The condition for PCR was as follows; firstly, pre-denaturation at 98 $^{\circ}$C for 30 seconds and denaturation at 98 $^{\circ}$C for 10 seconds. secondly, annealing at 55 $^{\circ}$C for 10 seconds, thirdly, extension at 72 $^{\circ}$C for 2 minutes and finally, post-extension 72 $^{\circ}$C for 10 minutes. These conditions were used for amplifying 16S rRNA and mannanase gene

2.11   Identifying bacterial strains by 16S rRNA gene sequence analysis

A 16S rRNA gene was amplified by PCR method using *Bacillus* sp. HM7 genome and primers (pAFwd. and pHRwd.) (Edwards et al., 1989). The PCR product was cloned into pJET cloning vector by T4 DNA ligase. The recombinant plasmid was transformed into *E. coli* Top10 competent cell. Plasmid extraction was done by Presto ™Mini Plasmid Kit (Geneaid, New Taipei City, Taiwan). Nucleotide sequence was analyzed by BLASTn programs of the National Center for Biotechnology Information (NCBI).

2.12 Production of β-mannanase from isolate bacteria HM7 from *Dynastes hercules* larvae excrement

A single colony of HM7isolate was picked an inoculated into mannan minimum medium broth and cultivated at 30 °C for 120 h with shaking at 250 rpm. To induce β-mannanase production, 1.5% (w/v) konjac flour or coconut meal were added in the medium. The cultured medium was collected every 12 h to measure the enzymatic activity and specific activity. β -mannanase was collected by centrifugation at 8000 xg for 10 min.

2.13 β-mannanase activity assay by DNS method

β-mannanase enzyme activity was assayed using 0.8% (w/v) konjac flour as substrate, in 50 mM citrate buffer pH 6.0. The reaction mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding DNS reagent (Miller, 1959). The amount of enzyme yielded 1 µmol of reducing sugars per minute was defined as one unit of mannanase.

2.14 Protein concentration measurement

Modified Bradford method was used for protein concentration measurement (Bradford, 1976). The protein and dye were incubated for 10 min and measured OD_{595}.

2.15   Analysis of mannanase by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Stacking and separating gel had 5% (w/v) and 10% (w/v) of acrylamide, respctively. The electrophoresis was conducted at a constant current of 20 mA. Staining solution (1% (w/v) Coomassie Brilliant Blue R250, 10% (v/v) glacial acetic acid and 45% (v/v) methanol) was added to the gels and stained for at least 30 min. The stained gels were soaked in de-staining solution (10% (v/v) glacial acetic acid and 10% (v/v) methanol) overnight. Standard molecular weight proteins and sample protein migration distances were compared for molecular weight calculation.

2.16   Analysis of manno-oligosaccharide product pattern from konjac flour and coconut meal

Crude mannanase from bacterial isolated HM7, cultured in medium containing konjac flour or coconut meal, was incubated with konjac flour or coconut meal at the ratio of 10 U, 1 U and 0.1 U of enzyme per 1 gram of konjac flour or coconut meal at 37 °C for overnight with agitation 100 rpm. Products were analyzed by TLC.

2.17   Purification of β -mannanase by column chromatography

Crude enzyme was purified by DEAE Toyopearl 650 in 25 mM Tris-HCl buffer pH 7.5 and eluted by Sodium chloride gradient from 0 to 1 M. Then the fractions containing mannanase activity were pooled and applied onto Phenyl Toyopearl 650 column chromatography and eluted by ammonium sulfate gradient from 1-0 M, at 4 °C. The DEAE and Phenyl Toyopearl bead was purchased from TaKaRa Bioscience,

Japan. The purity and molecular weight of β-mannanase were analyzed by using SDS-PAGE with 10% polyacrylamide gel.

2.18   Cloning of β-mannanase gene MAN26HM7

β-mannanase gene was amplified by PCR method using *Bacillus* sp. HM7 genome as template. NdeIFwd and ManARwd primers were designed using *Bacillus subtilis* BEST7613 (Accession Number: AP012495) mannanase gene sequence. The PCR product was ligated into *Xho*I and *Nde*I site of pET-21b by T4 DNA ligase. The recombinant plasmid (pET-21b-MAN26HM7) was transformed into *E. coli* Top10 competent cell. The pET-21b-MAN26HM7 extraction was done, and the nucleotide and deduced amino acid sequence were analyzed by BLASTn and BLASTp, National Center for Biotechnology Information (NCBI), respectively. The signal peptide of MAN26HM7 was predicted by SignalP. Multiple alignment of amino acid sequence was achieved by Clustal Omega.

2.19   Expression and purification of recombinant β-mannanase from *Bacillus* sp. HM7 and *B. subtilis* CAe24

The recombinant β-mannanase plasmid, pET-21b-MAN26HM7 was transformed into *E. coli* BL21 (DE3) competent cells. Transformant was picked and cultured and mannanase gene was induced with 1.0 mM IPTG, then cultured at 16, 30 and 37 °C to observe the optimum expression temperature. To determine the optimum IPTG concentration, the transformant was cultured at 37 °C and induced mannanase production by adding IPTG to the final concentration of 0.2 to 1.0 mM. Supernatant and cell pellet were separated by centrifugation at 10000 xg at 4 °C for 10 min. To break the cells, cell pellet was re-suspended in 300 µl of 50 mM citrate

buffer pH 6.0, then the cell suspension was frozen at -80 $^{\circ}$C and thawed at room temperature, in sonicator bath, to get intracellular protein fraction. Measurements of supernatant and intracellular enzymatic activity and protein concentration were done. MAN26CAe24 was expressed and purified by the same method as mannanase from HM7 isolated. The molecular mass of the purified enzyme was determined using 10% SDS-PAGE.

2.20   Biochemical characterization

The activity of purified MAN26HM7 and MAN26CAe24 was measured in 50 mM citrate buffer pH 6.0 for 10 min at a temperature range of 30 to 70 $^{\circ}$C to determine the optimal temperature of the enzyme. Thermal stability of the purified MAN26HM7 and MAN26CAe24 was obtained by pre-incubation protein with 50 mM citrate buffer pH 6 at 40, 50, 60 and 70 $^{\circ}$C for 360 minutes and measured the residual activity of the enzyme at 50 $^{\circ}$C in citrate buffer pH 6.0.

The purified MAN26HM7 activity was measured at 50 $^{\circ}$C for 10 min in buffers pH 3.0 to 10.0 in order to determine the optimal pH of the enzyme. pH stability was evaluated by measuring the residual enzymatic activity after incubation of the enzyme solution in 50 mM various buffers pH ranging from 3.0 to10.0 (citrate for pH 3.0 to 6.0, phosphate for pH 6.0 to 8.0 glycine-NaOH for pH 8.0 to 10.0) at 4 $^{\circ}$C for 24 h.

The effects of metal ions and chemical reagents on the activity of purified MAN26HM7 were examined in 50 mM acetate buffer pH 6 supplemented with various metal ions and chemicals. Metal ions; $CaCl_2$, $CuSO_4$, $MgCl_2$, $FeCl_2$, $CoCl_2$, $ZnCl_2$, and $MnCl_2$, at the final concentration of 5 mM, and. EDTA at the final concentration of 5

mM was added to the reaction. One percent (v/v) SDS and TritonX-100 were also used as chemical reagents.

The effects of SDS at various temperature on purified MAN26HM7 and MAN26CAe24 was observed by measuring the activity of purified enzymes in 50 mM citrate buffer pH 6.0 with 1% (w/v) SDS at 30, 40, 50, 60 and 70 $^{\circ}$C respectively. Measurement of the purified MAN26HM7 and MAN26CAe24 activity in the presence of SDS concentration ranging from 0.5 to 2.0 % (w/v) at 50 $^{\circ}$C and 60 $^{\circ}$C in 50 mM citrate buffer pH 6.0 was done. The stability of the purified MAN26HM7 and MAN26CAe24 in SDS was studied. Purified MAN26HM7 and MAN26CAe24 were pre-incubated in 1.0% (w/v) SDS for 180 min at 37 $^{\circ}$C, then the remaining activity was measured at 37 $^{\circ}$C in 50 mM citrate buffer pH 6.0 with 1.0% (w/v) SDS.

The effect of local detergents on the purified MAN26HM7 and MAN26CAe24 was studied. Various detergent namely, OMO (liquid detergent), BREEZE (liquid detergent), PAO (washing powder), Attack (washing powder) were used to examined the stability of the enzyme in detergent. The purified enzyme was incubated with 0.5 % (w/v) powder detergent or 5.0 % (v/v) liquid detergent at 37 $^{\circ}$C for 180 min then measured the remaining activity at 37 $^{\circ}$C.

2.21   Mutagenesis of mannanase

PCR driven overlap was used for sited-directed mutagenesis (Heckman and Pease, 2007). The PCR reaction was done using plasmid from *B. subtilis* CAe24 as template. Pair of primers (I238LRwd. and I238LFwd.) was designed for mismatch mutation. First amplification was done by two pairs of primer (NdeIFwd.-I238LRwd and I238LFwd.-T7promoter). Full-length gene was constructed by second amplification using the DNA fragments as template. Mutated gene was directly cloned into pET-21b and expressed in *E. coli* BL21 (DE3). Nucleotide sequencing was done.

2.22   *In silico* analysis

A structural modeling of MAN26HM7, MAN26CAe24 and I238L MAN26CAe24 were generated by I-TASSER (Iterative Threading ASSEmbly Refinement) (Zhang et al., 2008), (Roy et al., 2010), (Yang et al., 2015), using the crystal structure of mannanases (PDB ID-2QHA and 3CBW) (Yan et al., 2008) as template. Interactions were predicted using Arpeggio (Jubb et al., 2017).

CHAPTER III

RESULTS

3.1   Identification of bacterial isolate HM7 by 16S rRNA genes sequence analysis

16S rRNA gene was amplified for the identification of the bacterial strain. The 16S rRNA gene was inserted into pJET. Positive clones were identified by *Bgl*II digestion of the recombinant plasmids (Fig. 3). The 1540 bp 16S rRNA gene was sequenced was BLAST against GenBank database. The result showed 99% identity with *B. amyloliquefaciens* G341 (Accession Number: CP011686), *B. methylotrophicus* JJ-D34 (Accession Number: CP011346.1) and *B. subtilis* strain Bs-916 (Accession Number: CP009611.1). Due to the high degree of homology of the *Bacillus* 16S rRNA gene, we were unable to designate the exact species for HM7 isolate. Therefore, the strain was designated as *Bacillus* sp. HM7 (Fig. 4).
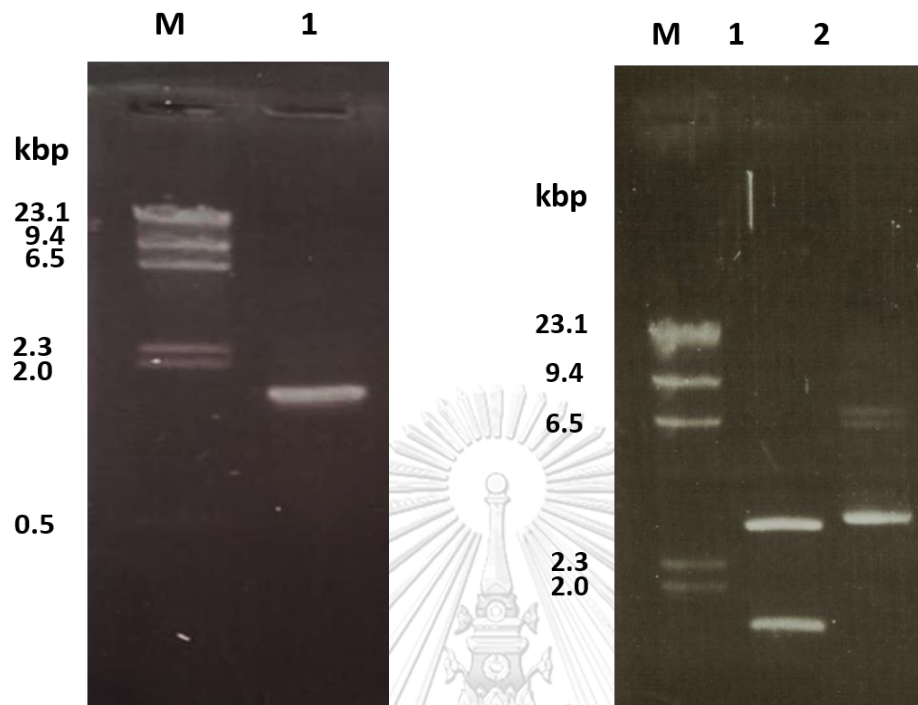
Figure 3   PCR amplification and cloning of 16S rRNA gene from isolate HM7

Left panel: 16S rRNA was amplified using chromosomal DNA as template then analyzed on 1.5% (w/v) agarose gel electrophoresis

Lane M : λ/*Hind*III Marker

Lane 1 : 16S rRNA gene of isolated HM7

Right panel: Restriction enzyme digestion of positive recombinant plasmid analyzed on 1% (w/v) agarose gel electrophoresis

Lane M: λ/*Hind*III Marker

Lane 1: *Bgl*II digested recombinant plasmid

Lane 2: Un-digested recombinant plasmid

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HM7                CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGA 180

amyloliquefaciens  CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGA 180

subtilis           CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGA 180

methylotrophicus   CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGA 180

                   ************************************************************

HM7                ACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCG 240

amyloliquefaciens  ACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCG 240

subtilis           ACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCG 240

methylotrophicus   ACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCG 240

                   ************************************************************

HM7                CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAG 300

amyloliquefaciens  CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG 300

subtilis           CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG 300

methylotrophicus   CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG 300

                   *******************************************  ***************

HM7                ACAAGGTAGCCGTATCGGAAGGTGCGGCTGCATCACCTCCTT 1540

amyloliquefaciens  ACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTT 1540

subtilis           ACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTT 1540

methylotrophicus   ACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTT 1540

                   ******************************* **********
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Figure 4　　　Sequence alignment of 16s rRNA gene of *Bacillus* sp. HM7, B. amyloliquefaciens, B. subtilis and B. methylotrophicus

　　　　B. amyloliquefaciens (AC number; CP011686.1), *B. subtilis* (AC number; CP009611.1) and *B. methylotrophicus* (AC number; CP011937.1) sequences were from GenBank. The non-conserved nucleotides were labeled in red.

3.2   Production of β-mannanase from bacteria HM7 from *Dynastes hercules* larvae feces

      Bacillus sp. HM7 was cultured in mannan minimum agar plate. Interestingly, black pigment was secreted from *Bacillus* sp. HM7 within 2 days. (Fig. 5). The bacteria-cultivation showed extracellular β-mannanase activity. β-mannanase was produced when the bacteria were grown in medium containing 1.5% (w/v) konjac flour or coconut meal. The maximum β-mannanase activity from medium containing coconut meal was higher than konjac flour, 19 U/ml at 96 h and 11U/ml at 36 h, respectively (Fig. 6). On the other hand, the maximum β-mannanase specific activity in konjac flour containing medium was higher than that of the coconut meal containing medium, 138 U/mg at 36 h and 114 U/mg at 48 h, respectively (Fig. 7).
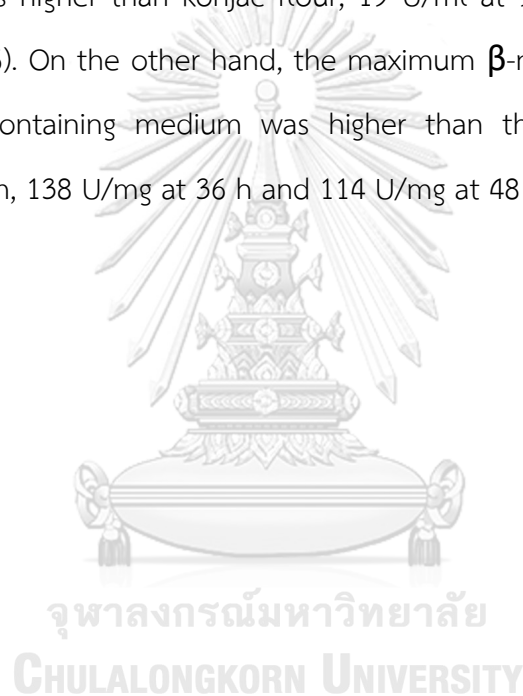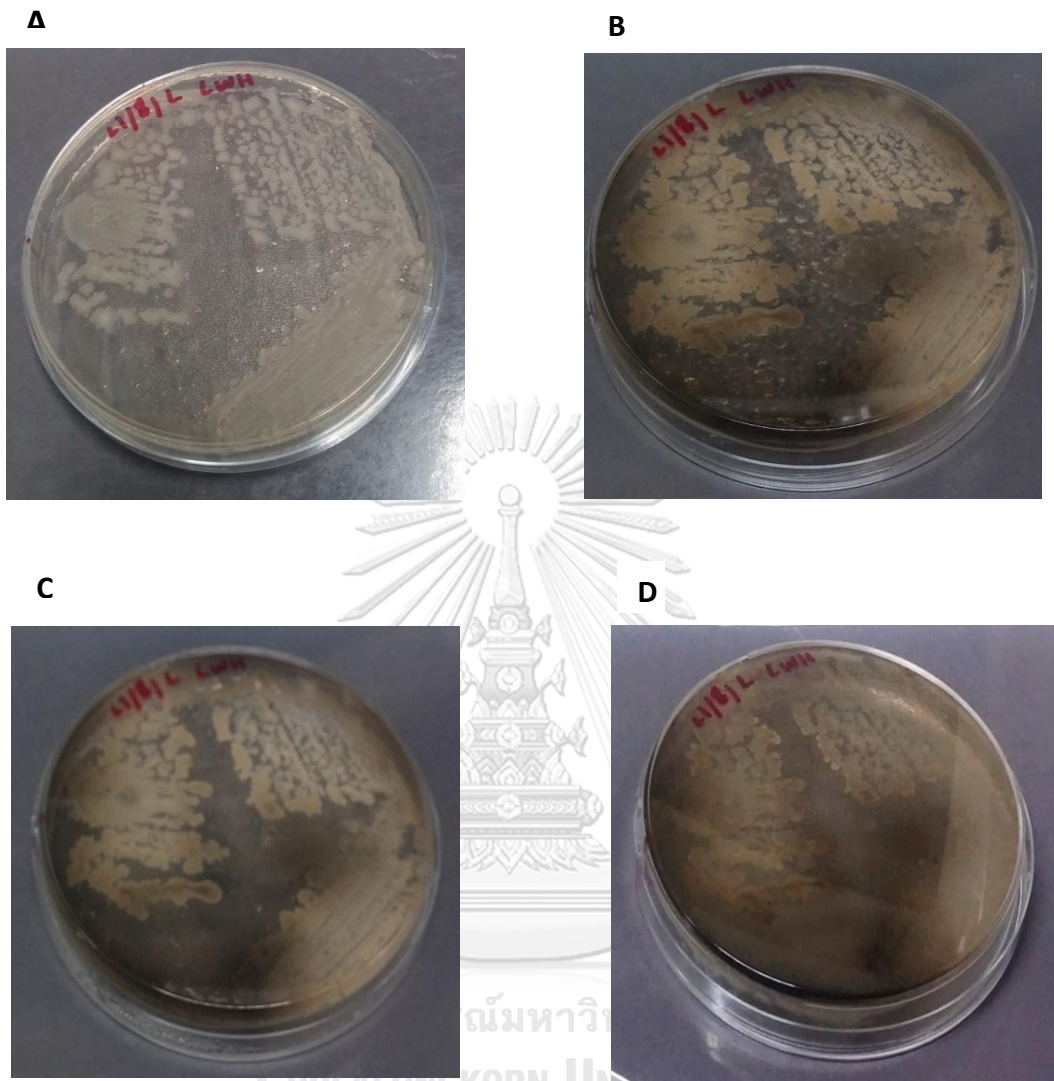
Figure 5   Morphology of *Bacillus* sp. HM7cultivated on 1% (w/v) konjac flour plate

A: The bacteria cultivated for 1 day   C: The bacteria cultivated for 3 days

B: The bacteria cultivated for 2 days   D: The bacteria cultivated for 4 days
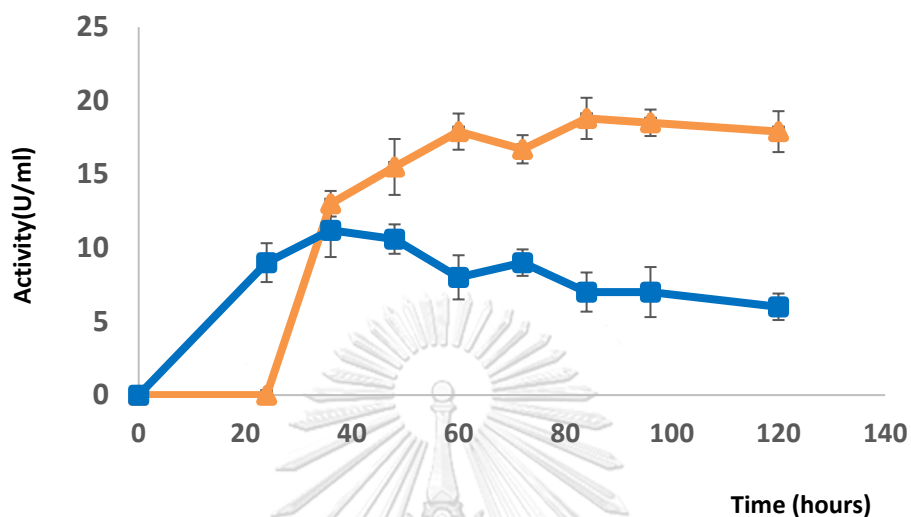
Figure 6 **β-Mannanase production of *Bacillus* sp. HM7 grown in medium containing different carbon source**

    Bacillus sp. HM7 was grown in minimal medium containing coconut meal or konjac flour and cultured at 37 °C for 120 h. β-mannanase activity assay condition was in citrate buffer pH 6.0 at 37 °C, using konjac flour as substrate. The data values represent means ± SD (bars). Each experiment was performed in triplicates

(▮, konjac flour, ▲ ; coconut meal).
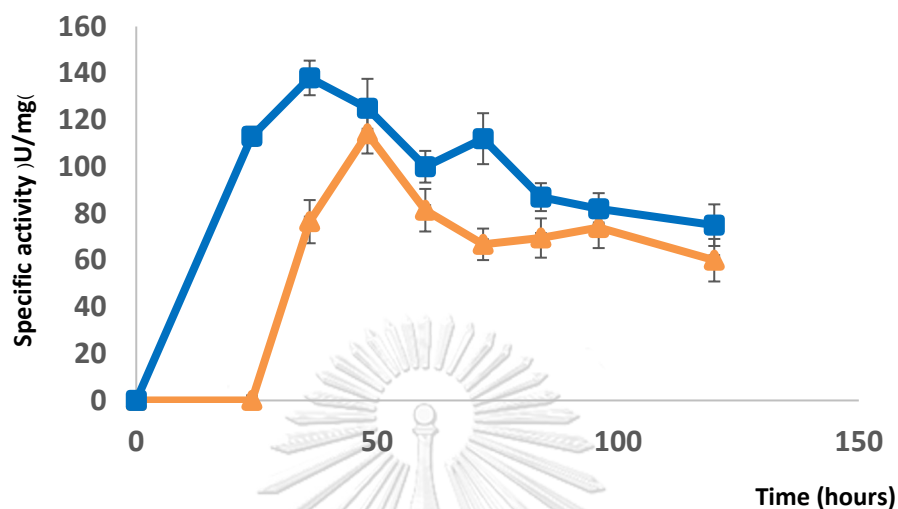
Figure 7   Specific activity of **β**-Mannanase from *Bacillus* sp. HM7 in medium containing different carbon sources

Bacillus sp. HM7 was grown in minimal medium containing coconut meal or konjac flour and cultured at 37 °C for 120 h. **β**-mannanase assay condition was in citrate buffer pH 6.0 at 37 °C, using konjac flour as substrate. The total protein concentration was determined by Modified Bradford method. The data values represent means ± SD (bars). Each experiment was performed in triplicates.

(■, konjac flour, ▲ ; coconut meal).

3.3   Analysis of manno-oligosaccharide product pattern from konjac flour and coconut meal

Crude **β**-mannanase from *Bacillus* sp. HM7 was used to hydrolyze konjac flour and coconut meal. The enzyme substrate ratio was increased and the hydrolytic products were analyzed. The reaction contains 10U, 1U and 0.1U of **β**-mannanase and 0.015 g/ml final concentration substrate, either coconut meal or konjac flour. The reaction was in citrate buffer pH 6.0, at 37 °C, for 18 hours. The hydrolytic products were analyzed by TLC.

Broad ranges of product size were produced in the reaction. When konjac flour was used as substrate more products were produced, which can be observed from the TLC pattern, than when coconut meal was used as substrate. The konjac flour hydrolysis products were detected when 10U and 1U was used. **β**-mannanase produced from coconut meal containing medium can hydrolyzed coconut meal better than the enzyme produced from konjac flour containing medium, Since, we can start to see hydrolytic products when only 0.1U of the enzyme was used (Fig. 8-11).

Figure 8   Analysis of manno-oligosaccharide from konjac flour digestion by crude mannanase from *Bacillus* sp. HM7 cultured in coconut meal

Crude **β**-mannanase from *Bacillus* sp. HM7 was used to hydrolyze konjac flour**.** The reaction contains 10U, 1U and 0.1U of **β**-mannanase and 0.015 g/ml final concentration substrate in citrate buffer pH 6.0. The reaction was incubated at 37 °C for 18 hours. The hydrolytic products were analyzed by TLC.

Lane 1: Konjac flour

Lane 2: Standard glucose marker

Lane 3, 5 and 7: Crude mannanase

Lane 4: Reaction product using 10 U of enzyme per 1 gram of konjac flour

Lane 6: Reaction product using 1 U of enzyme per 1 gram of konjac flour

Lane 8: Reaction product using 0.1 U of enzyme per 1 gram of konjac flour

Figure 9 **Analysis of manno-oligosacchariade from coconut meal digestion by crude mannanase from *Bacillus* sp. HM7 cultured in coconut meal**

Crude β-mannanase from *Bacillus* sp. HM7 was used to hydrolyze coconut meal. The reaction contains 10U, 1U and 0.1U of β-mannanase and 0.015 g/ml final concentration substrate in citrate buffer pH 6.0. The reaction was incubated at 37 °C for 18 hours. The hydrolytic products were analyzed by TLC.

Lane 1: Coconut meal

Lane 2: Standard glucose marker

Lane 3, 5 and 7: Crude mannanase

Lane 4: Reaction product using 10 U of enzyme per 1 gram of coconut meal

Lane 6: Reaction product using 1 U of enzyme per 1 gram of coconut meal

Lane 8: Reaction product using 0.1 U of enzyme per 1 gram of coconut meal

Figure 10   **Analysis of manno-oligosaccharide from konjac flour digestion by crude mannanase from *Bacillus* sp. HM7 cultured in konjac flour**

Crude β-mannanase from *Bacillus* sp. HM7 was used to hydrolyze konjac flour. The reaction contains 10U, 1U and 0.1U of β-mannanase and 0.015 g/ml final concentration substrate in citrate buffer pH 6.0. The reaction was incubated at 37 °C for 18 hours. The hydrolytic products were analyzed by TLC.

Lane 1: Konjac flour

Lane 2: Standard glucose marker

Lane 3, 5 and 7: Crude mannanase

Lane 4: Reaction product using 10 U of enzyme per 1 gram of konjac flour

Lane 6: Reaction product using 1 U of enzyme per 1 gram of konjac flour

Lane 8: Reaction product using 0.1 U of enzyme per 1 gram of konjac flour

Figure 11   Analysis of manno-oligosaccharide from coconut meal digestion
by crude mannanase from *Bacillus* sp. HM7 cultured in konjac flour

Crude β-mannanase from *Bacillus* sp. HM7 was used to hydrolyze coconut meal. The reaction contains 10U, 1U and 0.1U of β-mannanase and 0.015 g/ml final concentration substrate in citrate buffer pH 6.0. The reaction was incubated at 37 °C for 18 hours. The hydrolytic products were analyzed by TLC.

Lane 1 : Coconut meal

Lane 2 : Standard glucose marker

Lane 3, 5 and 7 : Crude mannanase

Lane 4 : Reaction product using 10 U of enzyme per 1 gram of coconut meal

Lane 6 : Reaction product using 1 U of enzyme per 1 gram of coconut meal

Lane 8 : Reaction product using 0.1 U of enzyme per 1 gram of coconut meal

3.4   Purification of β-mannanase from *Bacillus* sp. HM7by column chromatography

β-mannanase from *Bacillus* sp. HM7 grown in medium containing konjac flour could not be purified because there are left over konjac flour polymers interfering with the chromatographic separation step (Data not shown). Therefore, β-mannanase from *Bacillus* sp. HM7 was purified from the medium containing coconut meal instead. The cell suspension was centrifuged at 8000 xg for 10 min. The specific activity of the crude mannanase was 80 U/mg. The extracellular enzyme was ultra-filtrated through 10kDa MW cut-off membrane. The ultra-filtrated crude enzyme was then subjected to DEAE Toyopearl-650M column chromatography. The chromatographic profile of mannanase from isolated HM7 on DEAE Toyopearl-650M was shown (Fig 12). The enzymatic specific activity of the enzyme increased to 1,588 U/mg, with the remaining yield of 7 % after purified by Phenyl Toyopearl 650M column (Table 7) (Fig 13). The purified β-mannanase revealed a single band on SDS PAGE with a molecular mass of 40 kDa (Fig 14).

Table 7   Purification of mannanase from *Bacillus* sp. HM7

Purification Steps	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold
Crude	340	4080	51	80	100	1
DEAE Toyopearl	70	1732	15.71	110.3	42.5	1.4
Phenyl Toyopearl	160	254	0.16	1587.5	6.23	19.8
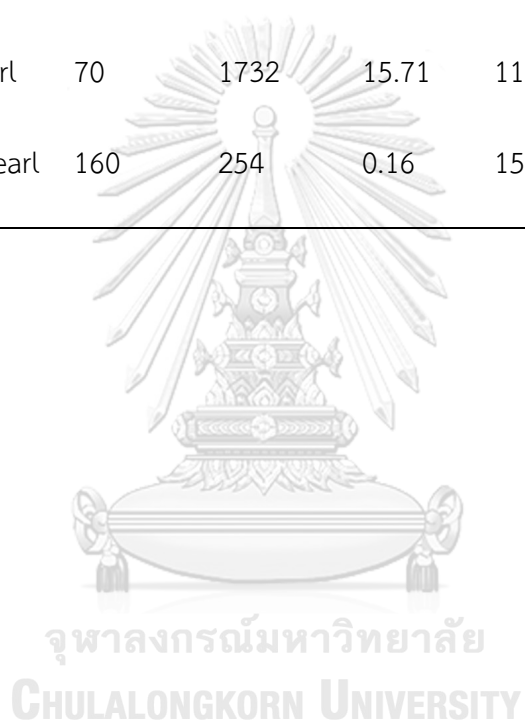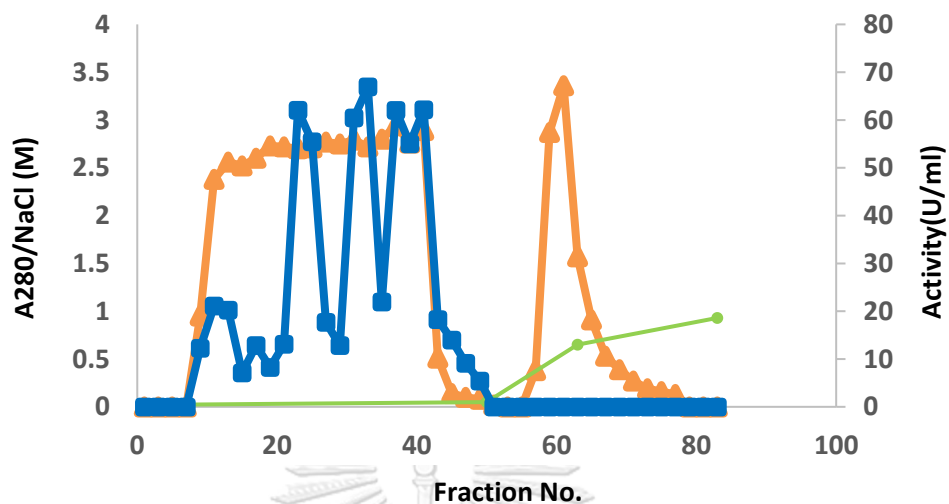
Figure 12   DEAE Toyopearl-650M Chromatographic profile of mannanase purification from *Bacillus* sp. HM7

DEAE-Toyopearl-650M column was equilibrated with 25 mM Tris-HCl buffer pH 7.5. Then mannanase was loaded to DEAE-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 0 - 1 M NaCl was used to eluted bound proteins. The elution started at fraction 51. Fractions 21-42 with mannanase activity were pooled.

(■ : MAN Activity, ▲ : A280 and ● : Concentration of NaCl)
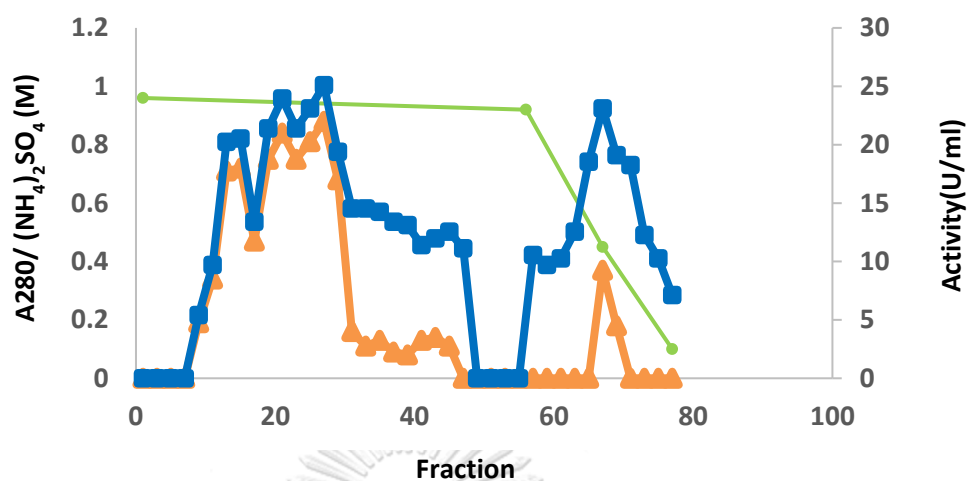
Figure 13 Phenyl Toyopearl-650M Chromatographic profile of mannanase purification from *Bacillus* sp. HM7

Phenyl-Toyopearl-650M column was equilibrated with 1 M $(NH_4)_2SO_4$ in 25 mM Tris-HCl buffer pH 7.5. Then mannanase was loaded to Phenyl-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 1 - 0 M $(NH_4)_2SO_4$ was used to eluted bound proteins. The elution started at fraction 56. Fractions 65-69 with mannanase activity were pooled.

(■ : MAN Activity, ▲ : A280 and ● : Concentration of $(NH_4)_2SO_4$)
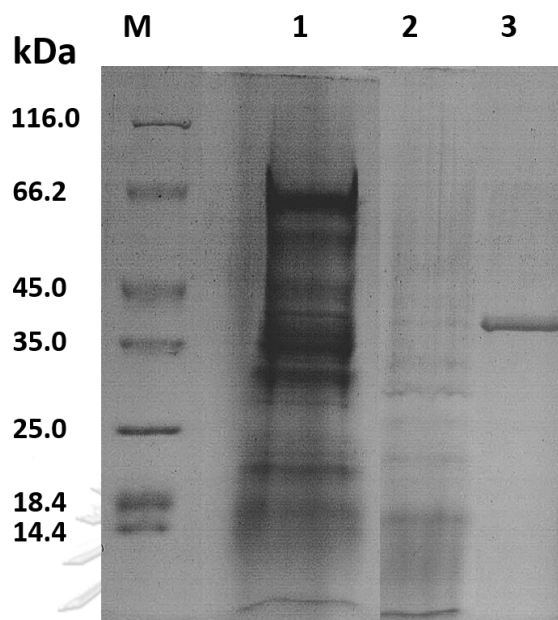
Figure 14   SDS-PAGE analysis of purified mannanase from *Bacillus* sp. HM7

Lane M: Standard molecular weight protein

Lane 1: Crude mannanase

Lane 2: DEAE-Toyopearl-650M pooled fractions with mannanase activity

Lane 3: Phenyl-Toyopearl-650M pooled fractions with mannanase activity

3.5 Cloning of β-mannanase gene

β-mannanase gene was amplified from the genomic DNA of *Bacillus* sp. HM7 (Fig. 15). The recombinant plasmid was digested by *Nde*I and *Xho*I in order to check for the correct size of the inserted DNA fragment (Fig. 16*).

Bacillus sp. HM7 β-mannanase (*Man26HM7*) has an open reading frame of 1,089 base pairs *Man26HM7* encodes for MAN26HM7 of 362 aminio acid residues. Residues 1 to 21 at the N-terminus of the protein was predicted by SignalP program to be a putative N-terminal signal peptide of MAN26HM7. The full-length ORF of *Man26HM7* was deposited to GenBank (AC number: KY930891.1).

Nucleotide and amino acid sequence alignment of *Man26HM7* and MAN26HM7 showed similarities with GH26 endo-β-mannanase, without carbohydrate-binding module. The catalytic residue E193 and E292 were found in MAN26HM7. When the nucleotide and amino acid sequence of MAN26HM7 was compared with β-mannanase of *B. subtilis* (AC Number: GQ859462.1 and ACX94024.1) they exhibit 99% sequence identity (Fig. 17) (Fig. 18).
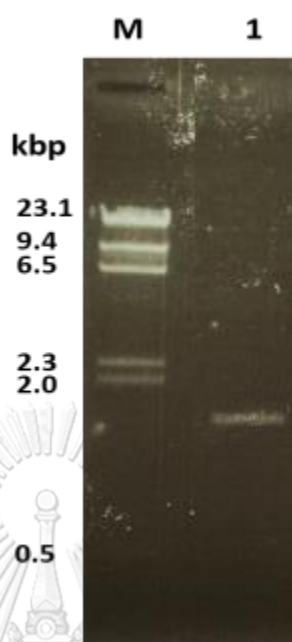
Figure 15 **Amplification of *Man26HM7* from *Bacillus* sp. HM7**

Lane M : λ/*Hind*III Marker

Lane 1 : PCR product of *Man26HM7* from *Bacillus* sp. HM7
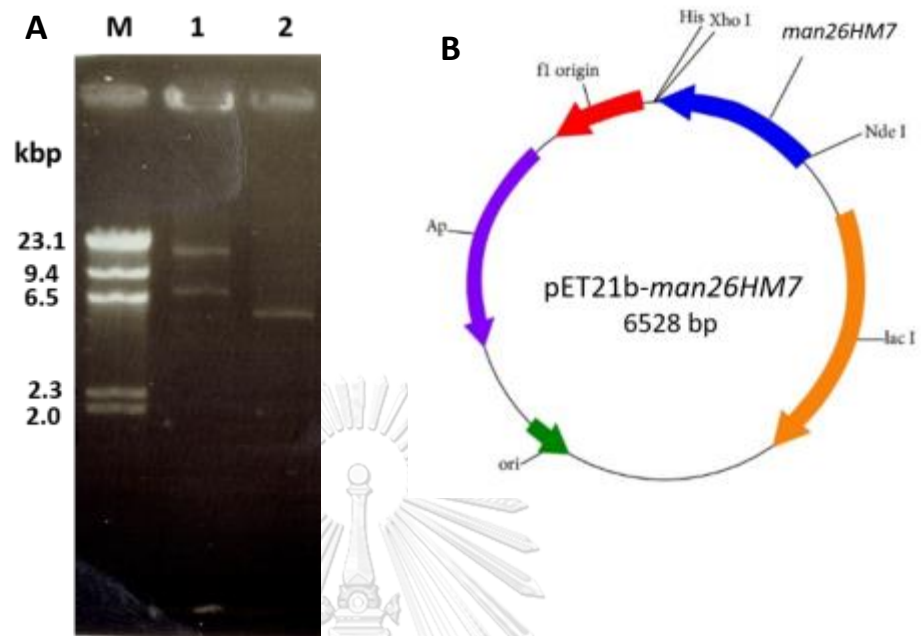
Figure 16 **Cloning of *Man26HM7* from *Bacillus* sp. HM7**

(A) Digestion of recombinant plasmid analyzed by agarose gel electrophoresis

Lane M : Standard marker protein

Lane 1 : pET21b- *Man26HM7* digested with *Nde*I and *Xho*I

Lane 2 : Non digested plasmid

(B) Plasmid map of pET21b- *Man26HM7*

```
subtilis   TTGTTTAAGAAACATACGATCTCTTTGCTCATTATATTTTTACTTGCGTCTGCTGTTTTA 60
HM7        ATGTTTAAGAAACATACGATCTCTTTGCTCATTTTATTTTTACTTGCGTCTGCTGTTTTA 60
           :**********************************:*************************

subtilis   GCAAAACCAATTGAAGCGCATACTGTGTCGCCTGTGAATCCTAATGCCCAGCAGACAACA 120
HM7        GCAAAACCAATTGAAGCGCATACTGTTTCGCCTGTGAATCCTAATGCACAGCAGACAACA 120
           ************************** ********************.************

subtilis   AAAACAGTGATGAACTGGCTTGCGCACCTGCCGAACCGAACGGAAAACAGAGTCCTTTCC 180
HM7        AAAGCAGTGATGAACTGGCTTGCGCACCTGCCGAACCGAACGGAAAACAGAGTCCTTTCC 180
           ***.********************************************************

subtilis   GGAGCGTTCGGAGGTTACAGTCATGACACATTTTCTATGGCTGAGGCTGATAGAATCCGA 240
HM7        GGAGCGTTCGGAGGTTACAGTCATGACACATTTTCTATGGCTGAGGCTGATAGAATCCGA 240
           ************************************************************

subtilis   AGCGCCACCGGGCAATCGCCTGCTATTTATGGCTGCGATTATGCCAGAGGATGGCTTGAA 300
HM7        AGCGTCACCGGGCAATCGCCTGCTATTTACGGCTGCGATTATGCCAGAGGATGGCTTGAA 300
           **** ************************ ******************************

subtilis   ACAGCAAATATTGAAGATTCAATAGATGTAAGCTGCAACAGCGATTTAATGTCGTATTGG 360
HM7        ACAGCAAATATTGAAGATTCAATAGATGTAAGCTGCAACAGCGATTTAATGTCGTATTGG 360
           ************************************************************

subtilis   AAAAATGGTGGAATCCCTCAAATCAGCTTGCACCTGGCGAATCCTGCTTTTCAGTCAGGG 420
HM7        AAAAATGGTGGAATCCCTCAAATCAGCTTGCACCTGGCGAATCCTGCTTTTCAGTCAGGG 420
           ************************************************************

subtilis   CATTTTAAAACACCGATTACAAACGATCAGTATAAAAAAATACTAGATTCTTCAACAGCA 480
HM7        CATTTTAAAACACCGATTACAAACGATCAGTATAAAAAAATACTAGATTCTTCAACAGCA 480
           ************************************************************

subtilis   GAAGGGAAGCGGCTGAATGCCATGCTCAGCAAAATTGCTGACGGACTTCAAGAGCTGGAG 540
HM7        GAAGGGAAGCGGCTGAATGCCATGCTCAGCAAAATTGCTGACGGACTTCAAGAGCTGGAG 540
           ************************************************************

subtilis   AACCAAGGTGTGCCTGTTTTGTTCAGGCCGCTGCATGAAATGAACGGTGAATGGTTTTGG 600
HM7        AACCAAGGTGTGCCTGTTTTGTTCAGGCCGCTGCATGAAATGAACGGTGAATGGTTTTGG 600
           ************************************************************
```

```
subtilis    TGGGGACTTACATCATATAACCAAAAGGATAATGAAAGAATCTCTCTATATAAACAGCTC 660

HM7         TGGGGACTTACATCATATAACCAAAAGGATAATGAAAGAATCTCTCTATATAAACAGCTC 660

            ************************************************************

subtilis    TACAAGAAAATCTATCATTATATGACCGACACAAGAGGACTTGATCATTTGCTTTGGGTT 720

HM7         TACAAGAAAATCTATCATTATATGACCGACACAAGAGGACTTGATCATTTGCTTTGGGTT 720

            ************************************************************

subtilis    TACTCTCCCGACGCCAACCGAGATTTTAAAACTGATTTTTACCCGGGCGCGTCTTACGTG 780

HM7         TACTCTCCCGACGCCAACCGAGATTTTAAAACTGATTTTTACCCGGGCGCGTCTTACGTG 780

            ************************************************************

subtilis    GATATTGTCGGATTAGATGCGTATTTTCAAGATGCCTACTCGATCAATGGATATGATCAG 840

HM7         GATATTGTCGGATTAGATGCGTATTTTCAAGATGCCTACTCGATCAATGGATATGATCAG 840

            ************************************************************

subtilis    CTAACAGCGCTTAATAAACCATTTGCTTTTACAGAAGTCGGCCCGCAAACAGCAAACGGC 900

HM7         CTAACAGCGCTTAATAAACCATTTGCTTTTACAGAAGTCGGCCCGCAAACAGCAAACGGC 900

            ************************************************************

subtilis    AGCTTCGATTACAGCCTATTTATCAATGCAATAAAACAAAGATATCCTAAAACCATTTAC 960

HM7         AGCTTCGATTACAGCCTATTTATCAATGCAATAAAACAAAGATATCCTAAAACCATTTAC 960

            ************************************************************


subtilis    TTTCTGGCATGGAATGATGAATGGAGCCCAGCTGTAAACAAGGGGGCTTCAGCTTTATAT 1020

HM7         TTTCTGGCATGGAATGATGAATGGAGCCCAGCTGTAAACAAGGGTGCTTCAGCTTTATAT 1020

            ********************************************* ***************

subtilis    CATGACAGCTGGACACTCAATAAGGGAGAAATATGGAATGGCGATTCTTTAACGCCAATC 1080

HM7         CATGACAGCTGGACACTCAACAAGGGAGAAATATGGAGTGGCGATTCTTTAACGCCAATC 1080

            ********************* ****************.********************
```

```
subtilis      GTTGAGTGA 1089

HM7           GTTGAGTGA 1089

              *********
```

Figure 17   Comparison of mannanase gene nucleotide sequences from *Bacillus* **sp. HM7**

The nucleotide sequence of mannanase gene from *B. subtilis* (Accession number: GQ859462.1) GenBank. The transcription start site was highlighted in yellow. The non-conserved nucleotides are labeled in red.

```
subtilis    MFKKHTISLLIIFLLASAVLAKPIEAHTVSPVNPNAQQTTKTVMNWLAHLPNRTENRVLS 60

HM7         MFKKHTISLLILFLLASAVLAKPIEAHTVSPVNPNAQQTTKAVMNWLAHLPNRTENRVLS 60

            ***********:*****************************:*******************

subtilis    GAFGGYSHDTFSMAEADRIRSATGQSPAIYGCDYARGWLETANIEDSIDVSCNSDLMSYW 120

HM7         GAFGGYSHDTFSMAEADRIRSVTGQSPAIYGCDYARGWLETANIEDSIDVSCNSDLMSYW 120

            ********************.***************************************

subtilis    KNGGIPQISLHLANPAFQSGHFKTPITNDQYKKILDSSTAEGKRLNAMLSKIADGLQELE 180

HM7         KNGGIPQISLHLANPAFQSGHFKTPITNDQYKKILDSSTAEGKRLNAMLSKIADGLQELE 180

            ***********************************************************

subtilis    NQGVPVLFRPLHEMNGEWFWWGLTSYNQKDNERISLYKQLYKKIYHYMTDTRGLDHLLWV 240

HM7         NQGVPVLFRPLHEMNGEWFWWGLTSYNQKDNERISLYKQLYKKIYHYMTDTRGLDHLLWV 240

            ***********************************************************

subtilis    YSPDANRDFKTDFYPGASYVDIVGLDAYFQDAYSINGYDQLTALNKPFAFTEVGPQTANG 300

HM7         YSPDANRDFKTDFYPGASYVDIVGLDAYFQDAYSINGYDQLTALNKPFAFTEVGPQTANG 300

            ***********************************************************

subtilis    SFDYSLFINAIKQRYPKTIYFLAWNDEWSPAVNKGASALYHDSWTLNKGEIWNGDSLTPI 360

HM7         SFDYSLFINAIKQRYPKTIYFLAWNDEWSPAVNKGASALYHDSWTLNKGEIWSGDSLTPI 360

            ***************************************************.*******

subtilis    VE 362

HM7         VE 362

            **
```

Figure 18   **Comparison of amino acid sequence of MAN26HM7**

Amino acid sequence alignment of Man26HM7 was done by Clustal Omega. *B. subtilis* β-mannosidase (AC number: ACX94024.1) amino acid sequence was retrieved from BLASTp program in GenBank database. The non-conserved amino acid are labeled in red

3.6   Expression of recombinant β-mannanase from *Bacillus* sp. HM7

We successfully expressed *Man26HM7* encoding for MAN26HM7 of 362 amino acid residues (with the predicted signal peptide from residues 1 to 21) in *E. coli* BL21 (DE3). The enzyme was secreted and the activity was found in the supernatant, when cultivated above 30 °C. Intracellular fractions had activity at every temperature. The highest activity was observed when the cells were cultured at 37 °C. The highest activity of extracellular MAN26HM7 was 61.3 U/ml, when induced MAN26HM7 production with 1 mM IPTG at 37 °C (Fig. 19), (Fig. 20). SDS-PAGE analysis showed that MAN26HM7 was in both the extracellular and intracellular fractions (Fig. 21), (Fig. 22). The extracellular enzyme was selected for further purification due to its less contaminant proteins than the intracellular fraction.
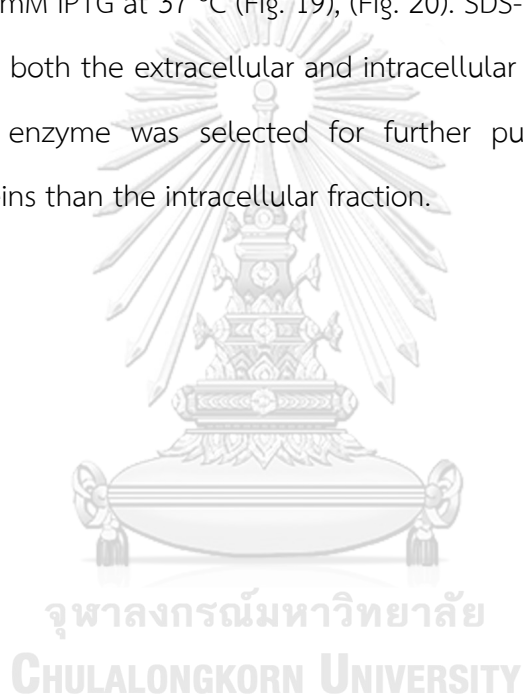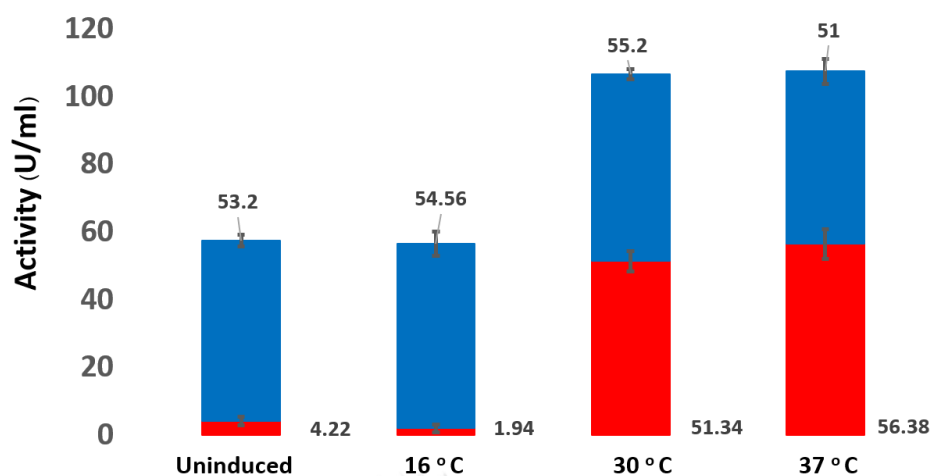
Figure 19   Effect of temperature on the expression of *Man26HM7*

The expression of *Man26HM7* was done by 1 mM IPTG induction at various temperatures. The enzymatic activity of the extracellular fraction was measured at 37 °C in 50 mM citrate buffer pH 6.0 with 0.8% (w/v) konjac flour as a substrate.

(■ Extracellular fraction and ■ Intracellular fraction)
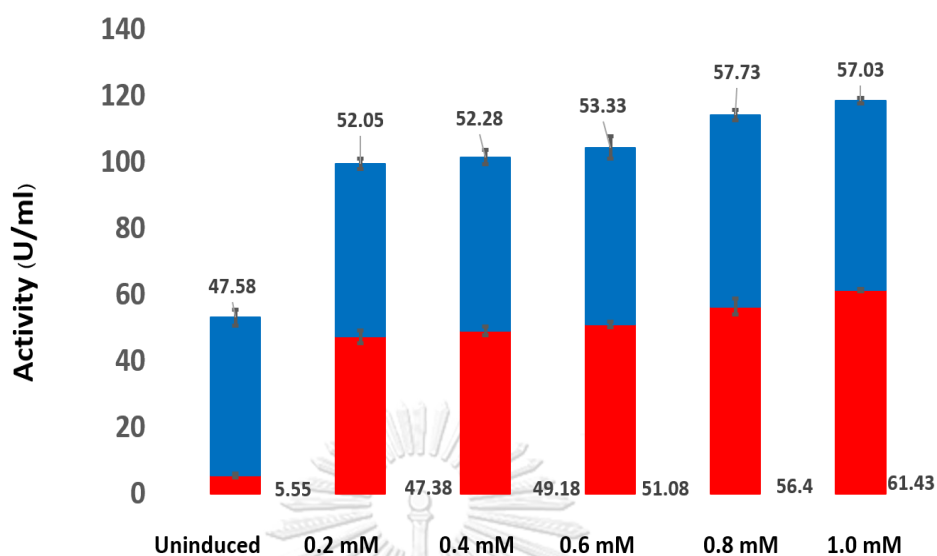
Figure 20   Effect of IPTG concentration on the expression of *Man26HM7*

The expression of *Man26HM7* induced by 0-1 mM IPTG concentration. The enzymatic activity of the extracellular fraction was measured at 37 °C in 50 mM citrate buffer pH 6.0 with 0.8% (w/v) konjac flour as a substrate. (■ Extracellular fraction and ■ Intracellular fraction)
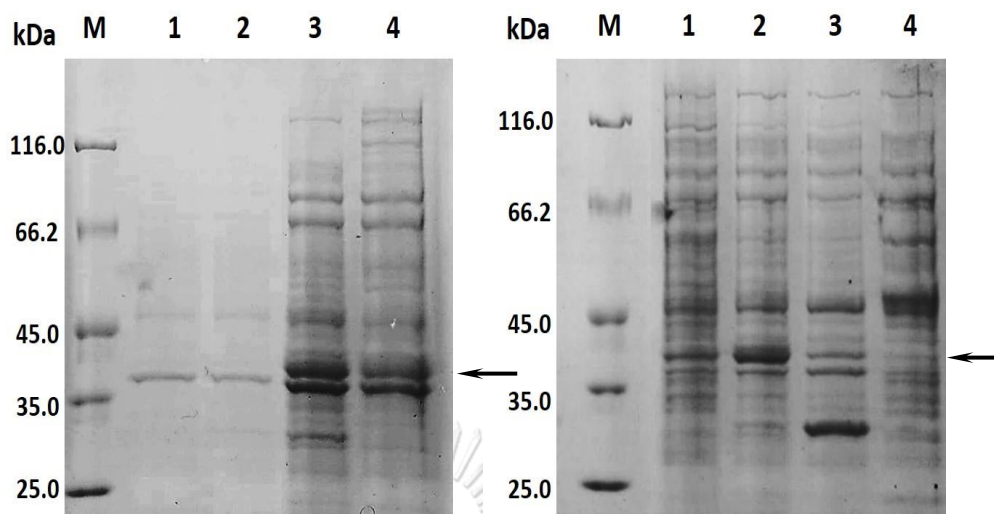
Figure 21   Expression of *Man26HM7* in pET 21-b at various temperature

Analysis of the expression of *Man26HM7* in *E. coli* BL21 (DE3) at various temperatures was done by SDS-PAGE. The arrow identified 40 kDa of mannanase.

Lane M: Standard molecular weight protein

Lane 1: Non-induced

Lane 2: Induction of *Man26HM7* at 16 °C

Lane 3: Induction of *Man26HM7* at 30 °C

Lane 4: Induction of *Man26HM7* at 37 °C

Left panel: extracellular fraction, Right panel: intracellular fraction
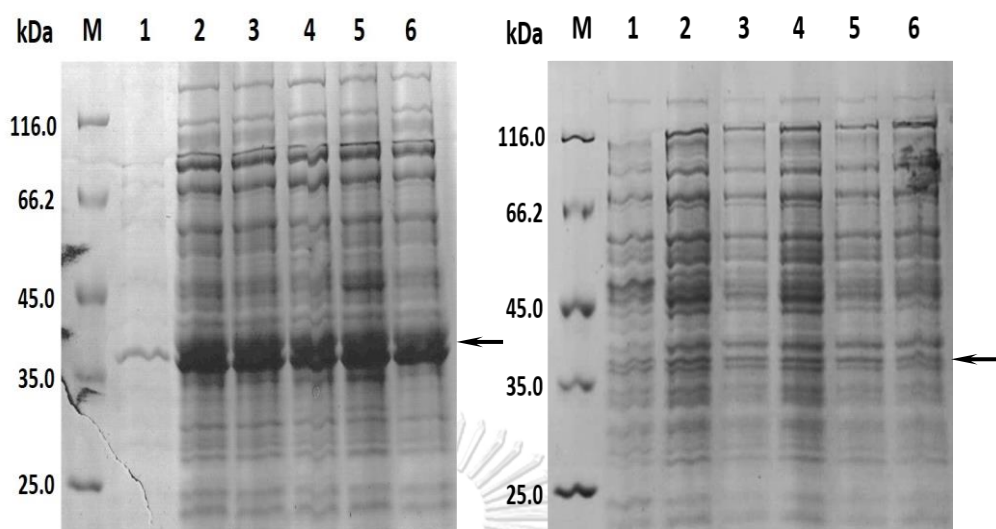
Figure 22   **Expression of Man26HM7 in pET 21-b vary concentration of IPTG**

Analysis of the expression of *Man26HM7* in *E. coli* BL21 (DE3) at various IPTG concentrations was done by SDS-PAGE. The arrow identified 40 kDa of mannanase.

Lane M: Standard molecular weight protein

Lane 1: Non-induced

Lane 2: Induction of *Man26HM7* at 0.2 mM IPTG

Lane 3: Induction of *Man26HM7* at 0.4 mM IPTG

Lane 4: Induction of *Man26HM7* at 0.6 mM IPTG

Lane 5: Induction of *Man26HM7* at 0.8 mM IPTG

Lane 6: Induction of *Man26HM7* at 1.0 mM IPTG

Left panel: extracellular fraction, Right panel: intracellular fraction

3.7 Purification of MAN26HM7

The chromatographic profile of MAN26HM7 on DEAE Toyopearl-650M was shown (Fig 23). The enzymatic specific activity of the enzyme increased to 34,500 U/mg, with the remaining yield of 17% after purified by Phenyl Toyopearl 650M column (Fig 24) (Table 8). The purified MAN26HM7 revealed a single band on SDS PAGE with a molecular mass of 40 kDa (Fig 25).
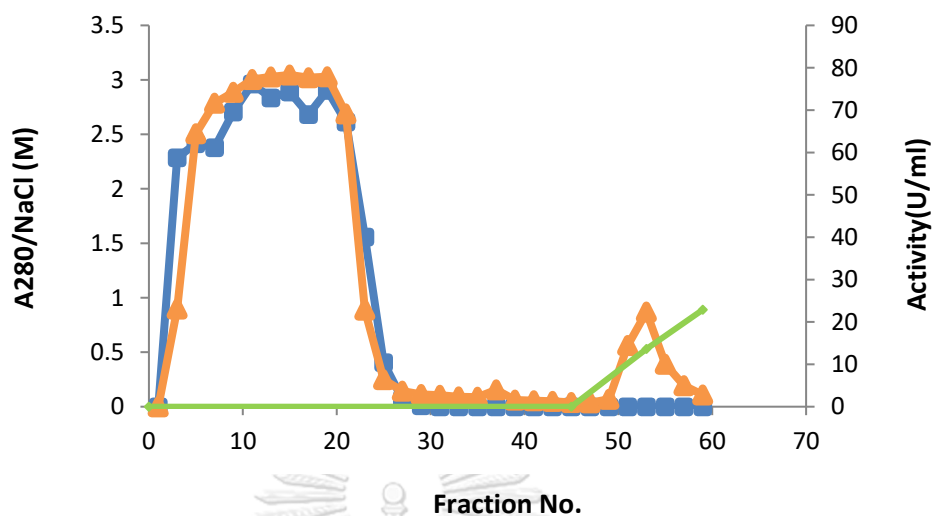
Figure 23   DEAE Toyopearl-650M Chromatographic profile of MAN26HM7 purification

DEAE-Toyopearl-650M column was equilibrated with 25 mM Tris-HCl buffer pH 7.5. Then mannanase was loaded to DEAE-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 0 - 1 M NaCl was used to eluted bound proteins. The elution started at fraction 45. Fractions 4-21 with mannanase activity were pooled.

(■ : MAN Activity, ▲ : A280 and ● : Concentration of NaCl)

Figure 24 **Phenyl Toyopearl-650M Chromatographic profile of MAN26HM7 purification**

Phenyl-Toyopearl-650M column was equilibrated with 1 M $(NH_4)_2SO_4$ in 25 mM Tris-HCl buffer pH 7.5. Then mannanase was loaded to Phenyl-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 1-0 M $(NH_4)_2SO_4$ was used to eluted bound proteins. The elution started at fraction 33. Fractions 43-47 with mannanase activity were pooled.

(■ : MAN Activity, ▲ : A280 and ● : Concentration of $(NH_4)_2SO_4$)

Table 8   Purification of MAN26HM7

Purification Steps	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold
Crude	60	3660	1.65	2218	100	1
DEAE Toyopearl	45	3130	1.35	2319	85.5	1.05
Phenyl Toyopearl	13	620	0.018	34500	17	15.5
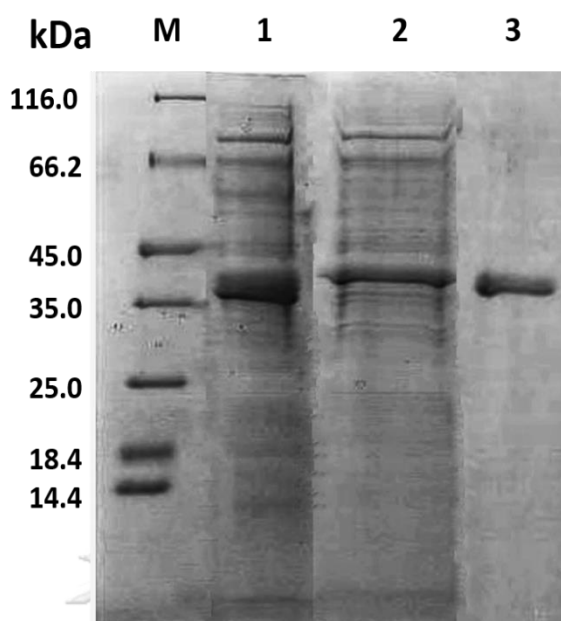
Figure 25   SDS-PAGE analysis of purified MAN26HM7

Lane M: Standard molecular weight protein

Lane 1: Crude mannanase

Lane 2: DEAE-Toyopearl-650M fraction pooled fractions with mannanase activity

Lane 3: Phenyl-Toyopearl-650M fraction pooled fractions with mannanase activity

3.8   Biochemical characterization

The optimal condition of MAN26HM7 was pH 6.0 at 50 $^{\circ}$C (Fig. 26), (Fig.27). Purified MAN26HM7 was stable at pH ranging from 4.0 to 10.0, after pre-incubated the enzyme at 4 $^{\circ}$C for 24 h (Fig. 28). Man26HM7 was stable at 60 $^{\circ}$C for 360 min (Fig. 29). The effects of various metal ions and chemical reagents on purified MAN26HM7 were observed (Table 9). Divalent ion of Co^{2+} and Mn^{2+} at 5.0 mM increased the activity of the enzyme approximately 20%, on the other hand, the presence of 5.0 mM Fe^{3+} and Cu^{2+} inhibits the activity of the enzyme over 90%. The activity of the enzyme remained close to 100% when non-ionic detergents, Triton X-100 and Tween 80 were added to the reaction. Interestingly, 65 % of Man26HM7activity remained in the presence of 1.0% SDS.
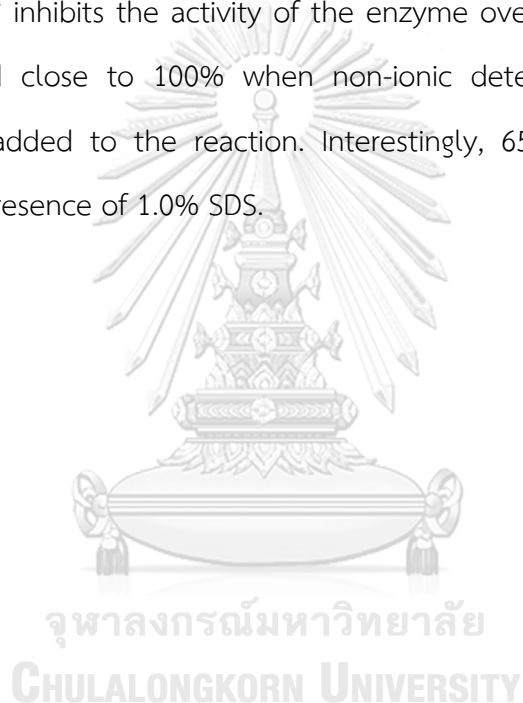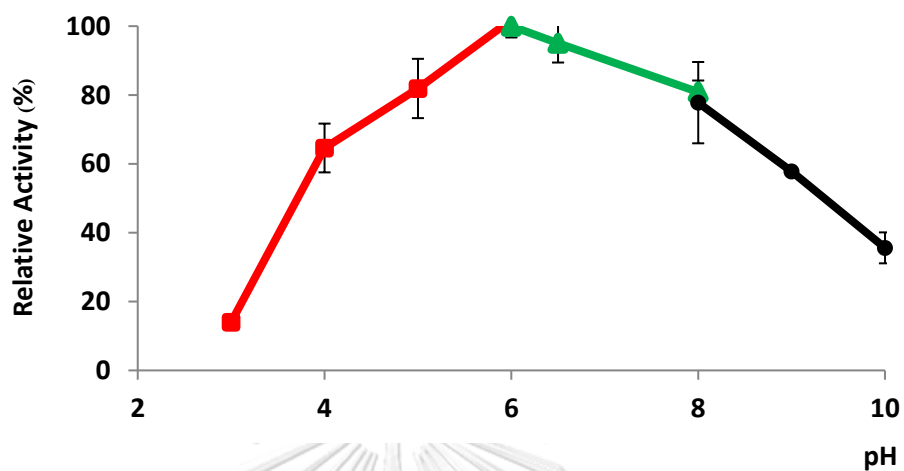
Figure 26   Optimal pH of MAN26HM7

The reaction was incubated at 50 $^{\circ}$C in 50 mM of different buffer with various

pH with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. ■, ▲ and

● represented citrate, phosphate and glycine-NaOH buffer respectively.
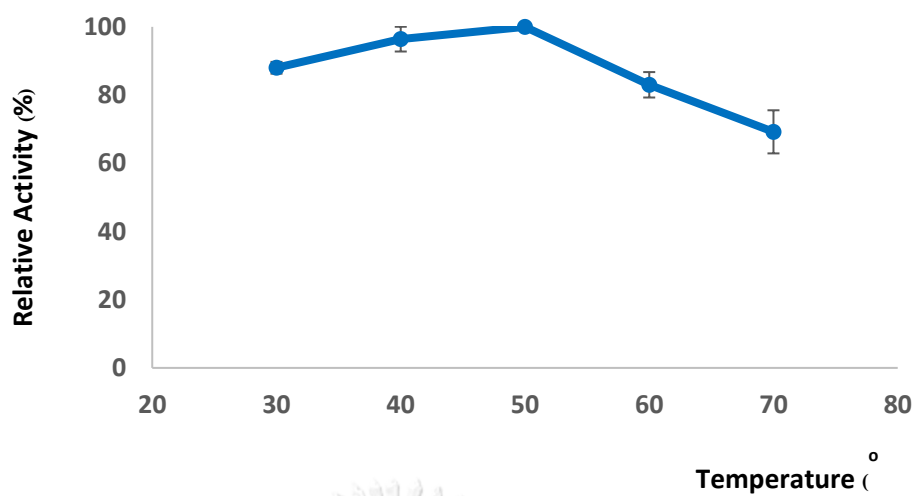
Figure 27   Optimal temperature of MAN26HM7

The reaction was incubated in 50 mM citrate buffer pH 6.0 at various temperatures from 30 to 70 °C with 0.5% (w/v) konjac flour as substrate, then incubated for 10 min.
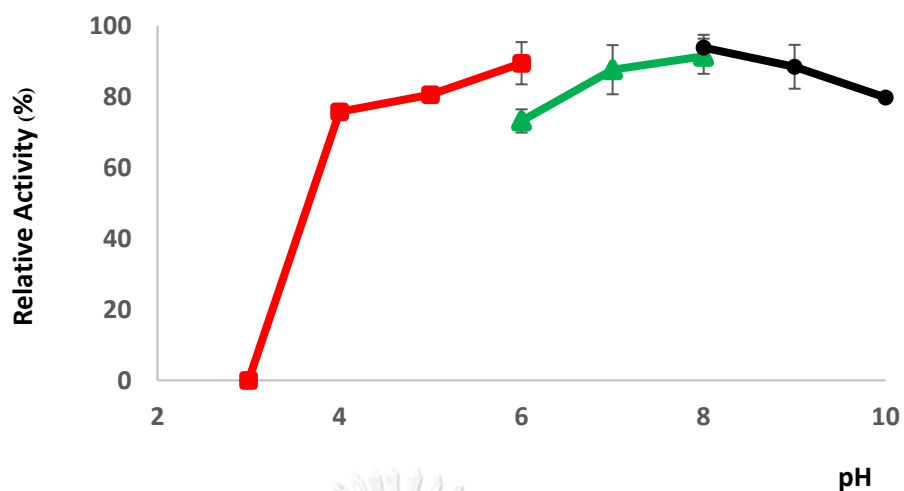
Figure 28   **Effect of pH on MAN26HM7 stability**

The purified enzyme was pre-incubated in 50 mM different buffer at various pH for 24 h at 4 °C. Incubation of reaction at 50 °C in 50 mM citrate buffer pH 6.0 with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. The initial enzymatic activity was defined as 100%. ■, ▲ and ● represented citrate, phosphate and glycine-NaOH buffer respectively.
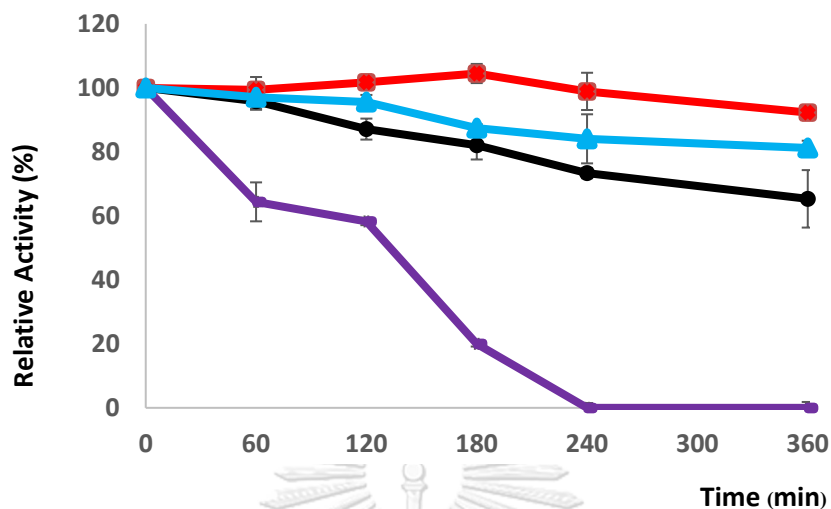
Figure 29   **Thermal effects on MAN26HM7 stability**

The purified enzyme was pre-incubated in 50 mM citrate buffer at various temperatures for 360 min and measured the enzymatic activity every 60 min of pre-incubation. The reaction was incubated at 50 $^{\circ}$C in 50 mM citrate buffer pH 6.0 with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. The initial enzymatic activity was defined as 100%. ■, ▲, ● and ━ represented 40, 50, 60 and 70 $^{\circ}$C respectively.

Table 9   The effects of various metal ions and chemical reagents on purified MAN26HM7

Substance	Relative activity (%)	Substance	Relative activity (%)
None	100 ± 1.1	$ZnCl_2$	86.7 ± 4.4
$FeCl_3$	12.8 ± 0.7	$CoCl_2$	122.5 ± 1.3
$MgCl_2$	91.8 ± 7.1	EDTA	93 ± 3.9
$MnCl_2$	126 ± 8.2	SDS	65.6 ± 2.4
$CuSO_4$	5.2 ± 0.4	Triton X-100	96 ± 3.0
$CaCl_2$	91.7 ± 2.6	Tween 80	94.6 ± 2.5

The reaction was incubated at 50 °C for 10 min in 50 mM acetate buffer pH 6.0 using 0.5% (w/v) konjac flour as substrate with 5 mM various metal ions or 1% chemical reagents.

3.9 Expression, purification and biochemical characterization of recombinant β-mannanase from _B. subtilis CAe24_

Expression and purification of MAN26CAe24 used the same method as MAN26HM7. The chromatographic profile of MAN26CAe24 on DEAE Toyopearl-650M was shown (Fig 30). The enzymatic specific activity of the enzyme increased to 9000U/mg, with the remaining yield of 30.8 % after purified by Phenyl Toyopearl 650M column (Fig 31) (Table 10). The purified MAN26CAe24 revealed a single band on SDS PAGE with a molecular mass of 40 kDa (Fig 32).

The optimal condition of MAN26CAe24 was 60 °C at pH 6.0 (Fig. 33), (Fig.34). Purified MAN26CAe24 was stable at pH ranging from 8.0 to 10.0, after pre-incubated the enzyme at 4 °C for 24 h (Fig. 35). MAN26CAe24 was stable at 60 °C for 360 min (Fig. 36). The effects of various metal ions and chemical reagents on purified MAN26CAe24 were observed (Table 11). Divalent ion of Mg^{2+} and Mn^{2+} at 5.0 mM increased the activity of the enzyme approximately 10%, on the other hand, the presence of 5.0 mM Fe^{3+}, Cu^{2+}, Zn^{2+} and EDTA inhibits the activity of the enzyme over 80%. The activity of the enzyme remained close to 51% when 1.0% SDS was added to the reaction.
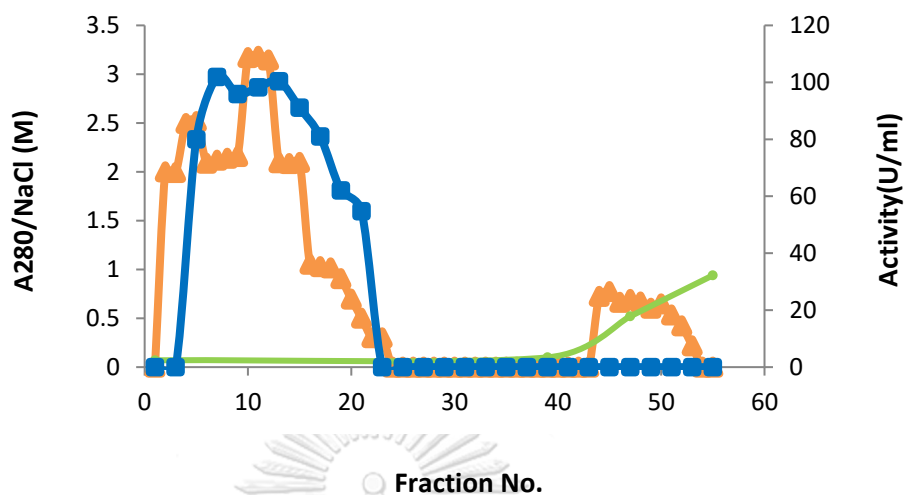
Figure 30   DEAE Toyopearl-650M Chromatographic profile of MAN26CAe24 purification

     DEAE-Toyopearl-650M column was equilibrated with 25 mM Tris-HCl buffer, pH 7.5. Then mannanase was loaded onto DEAE-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 0-1 M NaCl was used to eluted bound proteins. The elution started at fraction 39. Fractions 5-17 with mannanase activity were pooled.
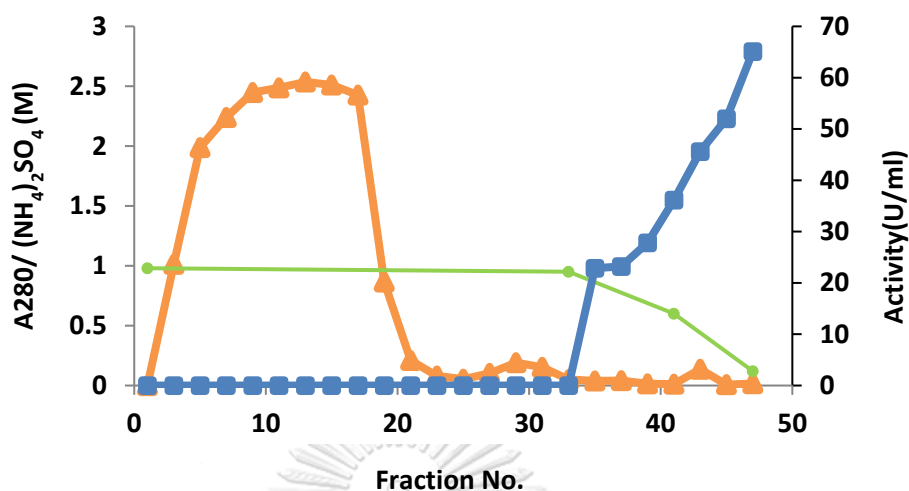
(■ : MAN Activity, ▲ : A280 and ● : Concentration of NaCl)
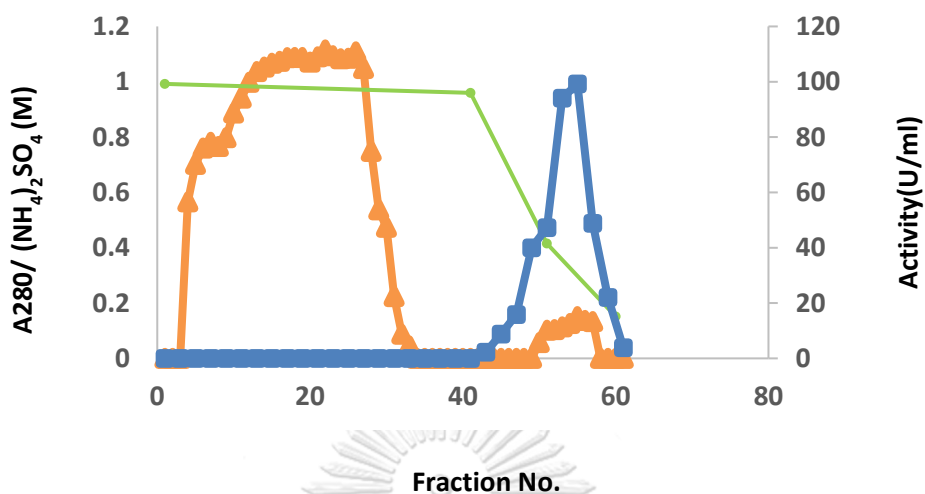
Figure 31   **Phenyl Toyopearl-650M Chromatographic profile of MAN26CAe24 purification**

Phenyl-Toyopearl-650M column was equilibrated with 1 M $(NH_4)_2SO_4$ in 25 mM Tris-HCl buffer pH 7.5. Then mannanase was loaded onto Phenyl-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 1-0 M $(NH_4)_2SO_4$ was used to eluted bound proteins. The elution started at fraction 41. Fractions 51-57 with mannanase activity were pooled.

(■ : MAN Activity, ▲ : A280 and ● : Concentration of $(NH_4)_2SO_4$)

Table 10   Purification of MAN26CAe24

Purification Steps	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Fold
Crude	60	4680	**4**	1170	100	1
DEAE Toyopearl	40	3360	2	1680	71.8	1.4
Phenyl Toyopearl	20	1440	0.12	12000	30.8	10.3
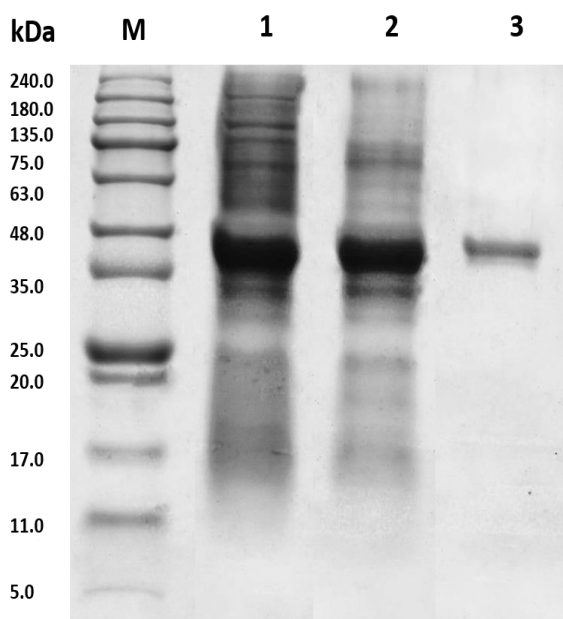
Figure 32 SDS-PAGE analysis of purified MAN26CAe24

Lane M: Standard molecular weight protein

Lane 1: Crude mannanase

Lane 2: DEAE -Toyopearl-650M fraction pooled fractions with mannanase activity

Lane 3: Phenyl-Toyopearl-650M fraction pooled fractions with mannanase activity
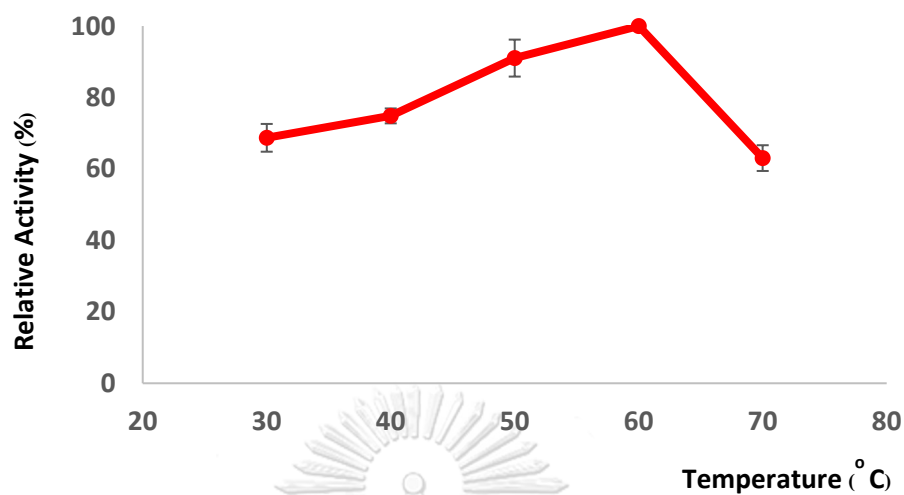
Figure 33   Optimal temperature of MAN26CAe24

The reaction was incubated in 50 mM citrate buffer pH 6.0 at various temperatures from 30 to 70 °C with 0.5% (w/v) konjac flour as substrate, then incubated for 10 min.
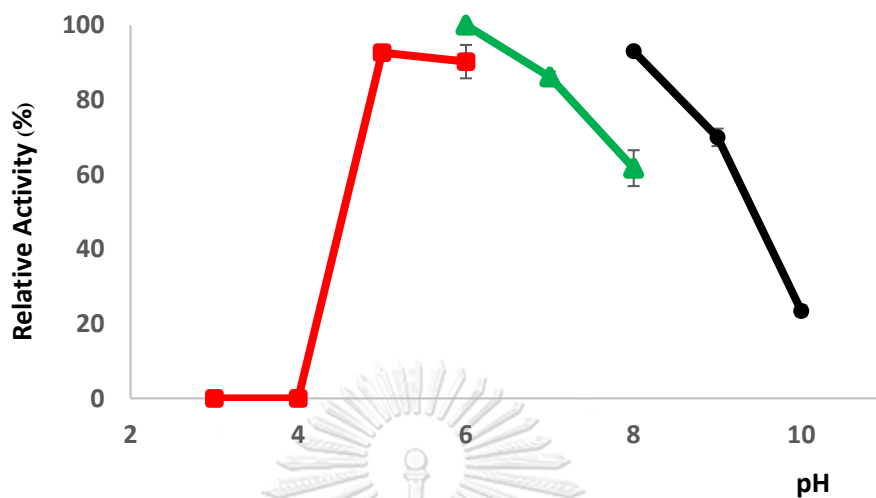
Figure 34   Optimal pH of MAN26CAe24

The reaction was incubated at 60 °C in 50 mM of different buffer with various pH with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. ■, ▲ and ● represented citrate, phosphate and glycine-NaOH buffer respectively.
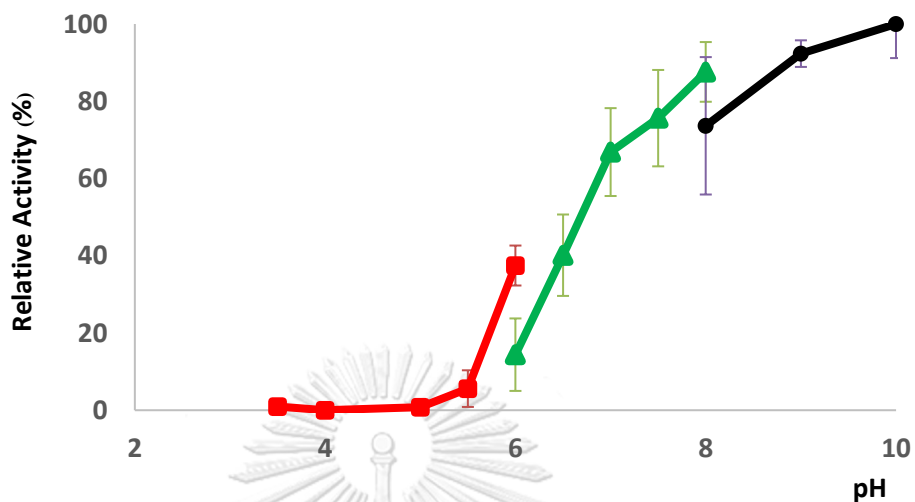
Figure 35　　**Effect of pH on MAN26CAe24 stability**

　　　　The purified enzyme was pre-incubated in 50 mM different buffer at various pH for 24 h at 4 °C. Incubation of reaction at 60 °C in 50 mM citrate buffer pH 6.0 with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. The initial enzymatic activity was defined as 100%. ▮, ▲ and ● represented citrate, phosphate and glycine-NaOH buffer respectively.
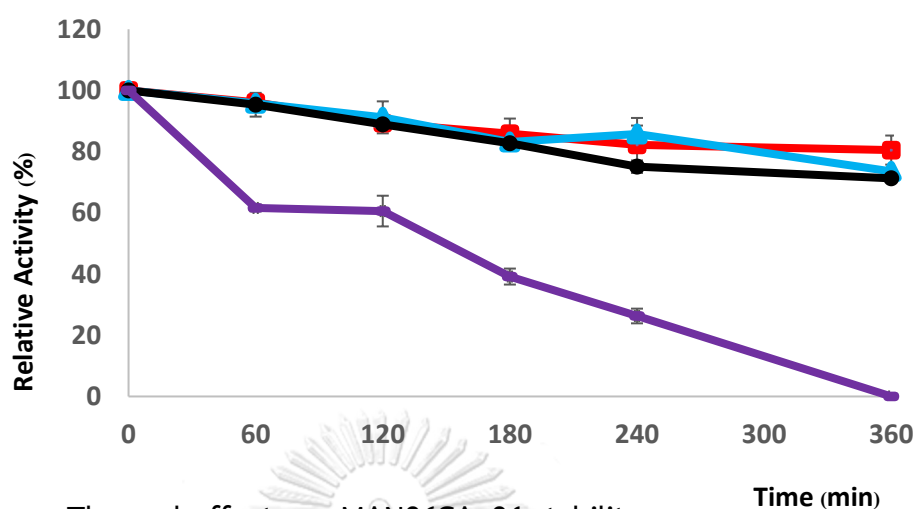
Figure 36   **Thermal effects on MAN26CAe24 stability**

The purified enzyme was pre-incubated in 50 mM citrate buffer at various temperatures for 360 min and measured the enzymatic activity every 60 min of pre-incubation. The reaction was incubated at 60 °C in 50 mM citrate buffer pH 6.0 with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. The initial enzymatic activity was defined as 100%. ■ , ▲ , ● and ▬ represented 40, 50, 60 and 70 °C respectively.

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Table 11 **The effects of various metal ions and chemical reagents on purified MAN26CAe24**

Substance	Relative activity (%)	Substance	Relative activity (%)
None	100 ± 3.1	$ZnCl_2$	18.5 ± 1.1
$FeCl_3$	11.5 0.4	$CoCl_2$	85.8 ± 2.7
$MgCl_2$	107 ± 4.1	EDTA	24.7 ± 8.5
$MnCl_2$	108.9 ± 2.7	SDS	50.9 ± 2.2
$CuSO_4$	0		
$CaCl_2$	85.2 ± 5.7		

The reaction was incubated at 60 °C for 10 min in 50 mM acetate buffer pH 6.0 using 0.5% (w/v) konjac flour as substrate with 5 mM various metal ions or 1% chemical reagents.

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3.10 Effect of local detergents and SDS on enzyme activity of purified MAN26HM7 and MAN26CAe24

Effect of SDS on mannanase activity was studied. When SDS was added to the reaction buffer to the finial concentration of 1%, the residual activity of purified MAN26HM7 and MAN26CAe24 were 70% and 51%, respectively, at its optimum (Fig. 37A). Furthermore, when SDS was added to the reaction buffer to the finial concentration of 2%, the residual activity of purified MAN26HM7 and MAN26CAe24 were 57% and 38%, respectively. (Fig. 37B).

The stability of the enzymes in SDS was studied. The enzymes were pre-incubated in 1% SDS for 180 min at 37 °C. After pre-incubation of both enzymes, the enzymes were assayed in a reaction mixture containing 1% SDS at 37 °C, respectively. MAN26HM7 and MAN26CAe24 retained 90 % and 60 % of its initial activity, respectively (Fig.37C). After pre-incubation of both enzymes, the enzymes were assayed in a reaction mixture without 1% SDS at 37 °C. MAN26HM7 and MAN26CAe24 retained 87 % and 22 % of its initial activity, respectively (Fig.37D).

Effect of detergents on mannanase activity was studied. When powder detergent was added in the reaction, the activity MAN26HM7 and MAN26CAe24 was reduced approximately 51-56%. On the other hand, when liquid detergent was added in the reaction, only a slight decrease in the activity was observed. The remaining activity of the MAN26HM7 and MAN26CAe24 was 97 % and 90 %, respectively.

The stability of the enzymes in detergents was studied. Pre-incubation MAN26HM7 and MAN26CAe24 with liquid detergents at 37 °C for 180 min, did not significantly reduced the activity of MAN26HM7 in both Breeze and OMO, nor the activity of MAN26CAe24 in OMO liquid detergent. However, Breeze liquid detergent reduced the activity of MAN26CAe24 from 97.5% to 88%. Pre-incubation MAN26HM7

and MAN26CAe24 with powder detergents at 37 °C for 180 min, reduce the activity of

MAN26HM7 to 82-85% and MAN26CAe24 to 75-77 %, respectively (Table 12).
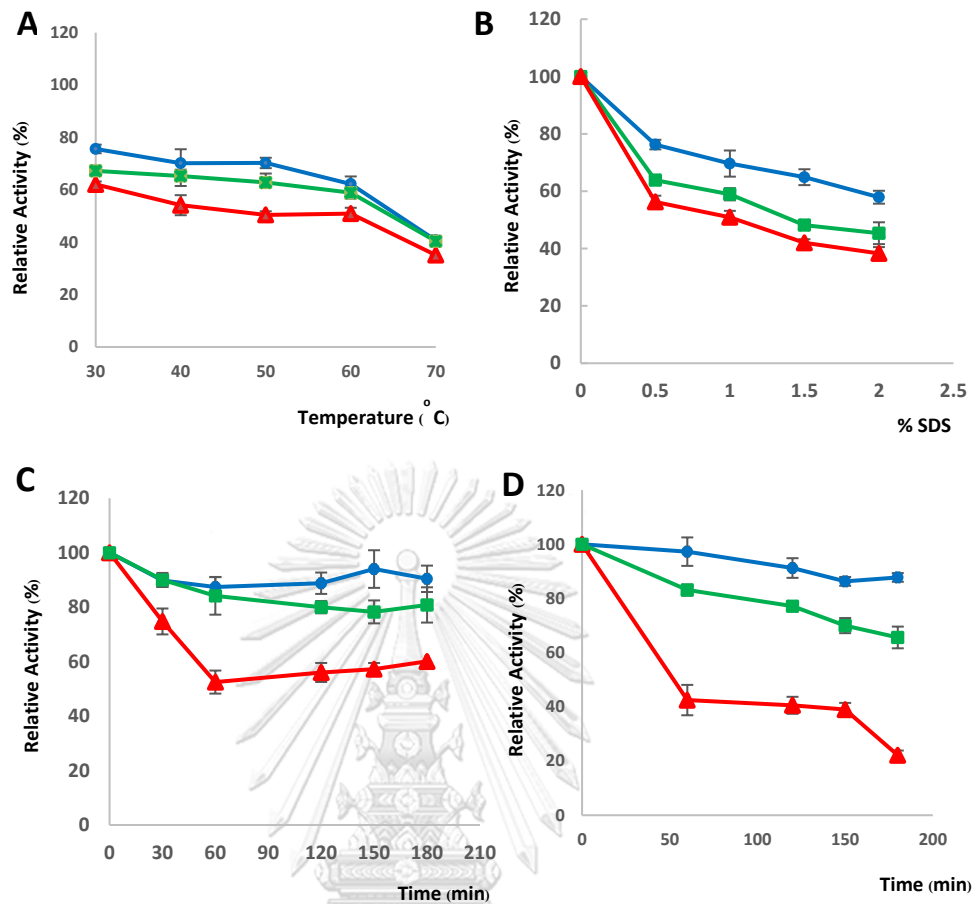
Figure 37   **The effect of SDS on the activity of MAN26HM7 compares with MAN26CAe24 and I238L MAN26CAe24**

(A) Effect of SDS at different temperatures on enzymatic activities. The enzymatic activities measured at various temperatures with 1 % (w/v) SDS in the reaction. (B) Influence of SDS concentration on activities of enzymes was determined by assay activity in various concentration of SDS at 50 °C and 60 °C respectively. (C) and (D) Stability of enzymes in the presence of 1 % (w/v) SDS, enzymes were pre-incubated in SDS at 37 °C for 180 min. Assay the residual activities of recombinant enzymes every 30 min for 180 min at 37 °C and pH 6.0 in the presence and absence of 1% SDS, respectively. The data that shown, represents an average of relative activity conducted in triplicates. ●, ▲ and ■ represented MAN26HM7, MAN26CAe24 and I238L MAN26CAe24, respectively.

Table 12   The effects of various local detergents on the purified MAN26HM7 and MAN26CAe24 activity

Local detergent	Initial relative activity (%)[a]		Remaining activity after 180 min incubation time (%)[a, b]	
	MAN26HM7	MAN26CAe24	MAN26HM7	MAN26CAe24
None	100 ± 0.5	100 ± 3.2	100 ± 1	100 ± 0.97
5 % (v/v) Breeze	98.3 ± 2.1	97.5 ± 1.3	96.8 ± 2	88 ± 1.2
5 % (v/v) OMO	95.2 ± 6.4	90.2 ± 1.5	96.7 ± 2.7	91.5 ± 2.1
0.5 % (w/v) PAO	56 ± 1.1	53.2 ± 1.3	85.6 ± 2.4	77 ± 3.42
0.5 % (w/v) Attack	55.4 ± 1.8	51.2 ± 1.4	82 ± 3.3	75.5 ± 3.5

[a] Values represent the means ± SD (n=3) relative to the untreated control samples

[b] Stability assay

3.11   Rational mutagenesis of *Man26CAe24*

Amino acid sequence alignment and homology modeling of MAN26HM7 and MAN26CAe24 revealed that these two proteins have different in amino acid at 7 positions (Zhang 2008, Roy et al. 2010, and Yang et al. 2015), (Fig. 38). (Fig. 39).

3.11.1 Site-directed mutagenesis of L238 *Man26CAe24*

Site-directed mutagenesis of L238 *Man26CAe24* was done by PCR-driven overlap method. Two fragments of PCR products were constructed by two pairs of primers (Fig. 40). Amplification of total gene was done (Fig. 41). The recombinant plasmid was digested by *Nde*I and *Xho*I in order to check for the correct size of the inserted DNA fragment (Fig. 42).

3.11.2 Purification of I238L MAN26CAe24

Expression and purification of I238L MAN26CAe24 used the same methods as MAN26CAe24. The chromatographic profile of I238L MAN26CAe24 on DEAE Toyopearl-650M was shown (Fig. 43). The enzymatic specific activity of the enzyme increased to 15400 U/mg, with the remaining yield of 45.8 % after purified by Phenyl Toyopearl 650M column (Fig 44) (Table 13). The purified I238L MAN26CAe24 revealed a single band on SDS PAGE with a molecular mass of 40 kDa (Fig. 45).

3.11.3 Biochemical characterization of I238L MAN26CAe24

The optimal condition of I238LMAN26CAe24 was 60 °C at pH 6.0 (Fig. 46), (Fig. 47). Purified I238L MAN26CAe24 was stable at pH ranging from 8.0 to 10.0, after pre-incubated the enzyme at 4 °C for 24 h (Fig. 48). I238L MAN26CAe24 was stable at 60 °C for 360 min (Fig. 49). The effects of various metal ions and chemical reagents on

purified I238L MAN26CAe24 were observed (Table 14). Divalent ion of Mg^{2+} and Mn^{2+} at 5.0 mM increased the activity of the enzyme approximately 10%, on the other hand, the presence of 5.0 mM Fe^{3+}, Cu^{2+}, Zn^{2+} and EDTA inhibits the activity of the enzyme over 80%. The activity of the enzyme remained close to 59% when 1.0% SDS was added to the reaction.

```
HM7      MFKKHTISLLILFLLASAVLAKPIEAHTVSPVNPNAQQTTKAVMNWLAHLPNRTENRVLS

CAe24    MFKKHTISLLIIFLLASAVLAKPIEAHTVSPVNPNAQQTTKTVMNWLAHLPNRTENRVLS

         **********:***************************:*******************

HM7      GAFGGYSHDTFSMAEADRIRSVTGQSPAIYGCDYARGWLETANIEDSIDVSCNSDLMSYW

CAe24    GAFGGYSHDTFSMAEADRIRSATGQSPAIYGCDYARGWLETANIEDSIDVSCNGDLMSYW

         ********************.****************************.******

HM7      KNGGIPQISLHLANPAFQSGHFKTPITNDQYKKILDSSTAEGKRLNAMLSKIADGLQELE

CAe24    KNGGIPQISLHLANPAFQSGHFKTPITNDQYKKILDSSTVEGKRLNAMLSKIADGLQELE

         **************************************.*******************

HM7      NQGVPVLFRPLHEMNGEWFWWGLTSYNQKDNERISLYKQLYKKIYHYMTDTRGLDHLLWV

CAe24    NQGVPVLFRPLHEMNGEWFWWGLTSYNQKDNERISLYKQLYKKIYHYMTDTRGLDHLIWV

         *******************************************************:**

HM7      YSPDANRDFKTDFYPGASYVDIVGLDAYFQDAYSINGYDQLTALNKPFAFTEVGPQTANG

CAe24    YSPDANRDFKTDFYPGASYVDIVGLDAYFQDAYSINGYDQLTALNKPFAFTEVGPQTANG

         ************************************************************

HM7      SFDYSLFINAIKQRYPKTIYFLAWNDEWSPAVNKGASALYHDSWTLNKGEIWSGDSLTPI

CAe24    SFDYSLFINAIKQKYPKTIYFLAWNDEWSPAVNKGASALYHDSWTLNKGEIWNGDSLTPI

         *************:**************************************.*******

HM7      VE

CAe24    VE

         **
```

Figure 38   **Amino acid sequence comparison of MAN26HM7 and MAN26CAe24**

Clustal Omega was used for amino acid sequence alignment of mannanase gene from *Bacillus* sp. HM7 and *B. subtilis* CAe24. The non-conserved amino acids are labeled in red.
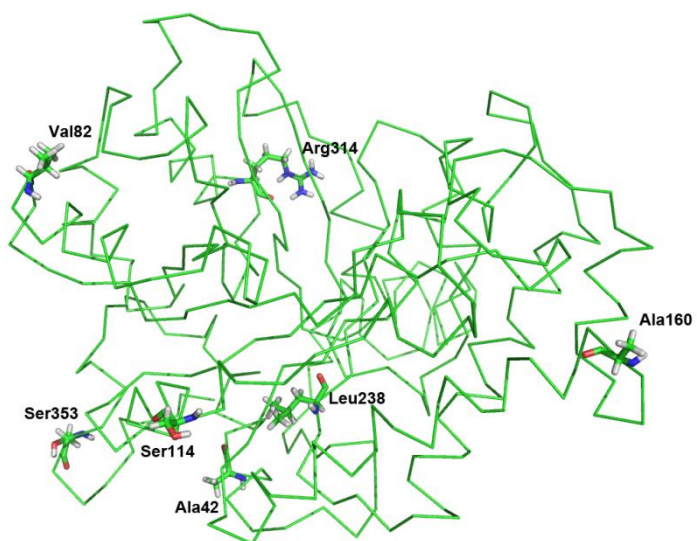
Figure 39   Structural protein of MAN26HM7

Backbone diagram of MAN26HM7 homology model generated by I-TASSER
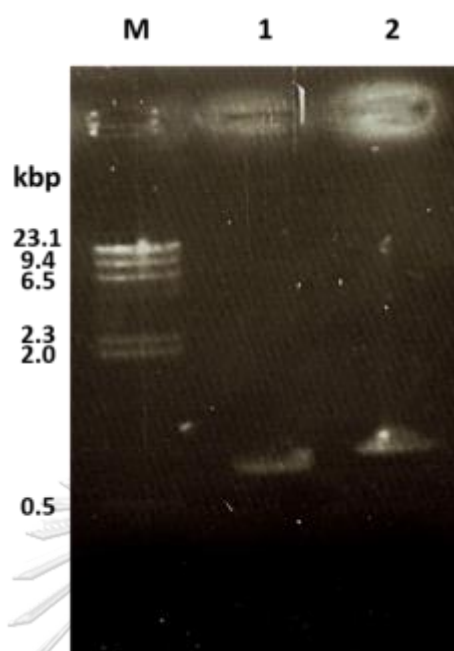
Figure 40   **PCR mutagenesis of *Man26CAe24* by PCR-driven overlap extension method**

Lane M : λ/*Hind*III Marker

Lane 1 : PCR product of *Man26CAe24* using *Nde*I Fwd. and I238L Rwd. primer

Lane 2 : PCR product of *Man26CAe24* using I238L Fwd.and T7 terminator primer
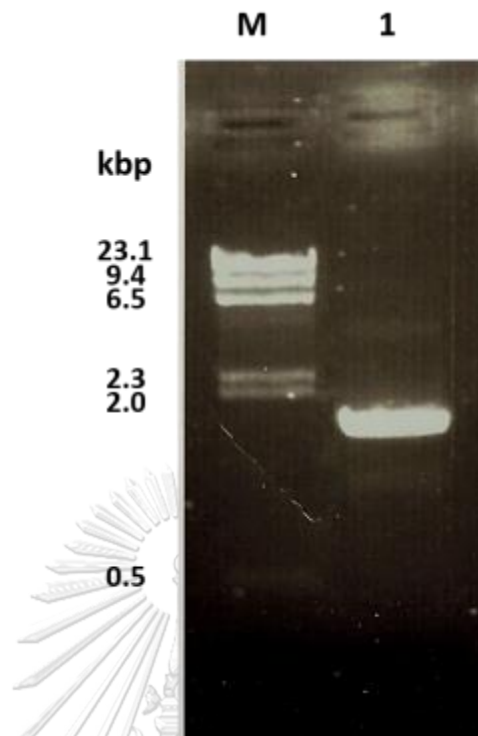
Figure 41   **PCR-driven overlap extension mutagenesis product** *I238L*

Man26CAe24

Lane M : λ/*Hin*dIII Marker

Lane 1 : PCR product of *I238L Man26CAe24*
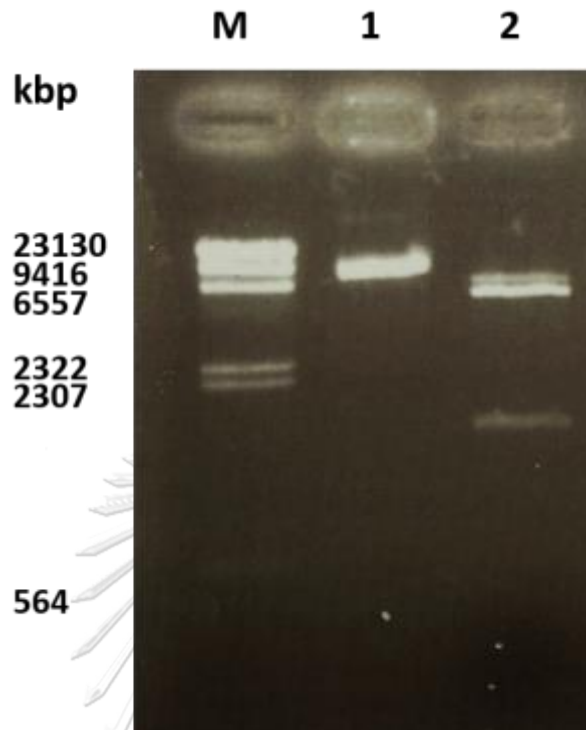
Figure 42 **Cloning of *I238L Man26CAe24***

Digestion of recombinant plasmid analyzed by agarose gel electrophoresis

Lane M : Standard marker protein

Lane 1 : Non digested plasmid

Lane 2 : *pET21b- I238L Man26CAe24* digested with *Nde*I and *Xho*I
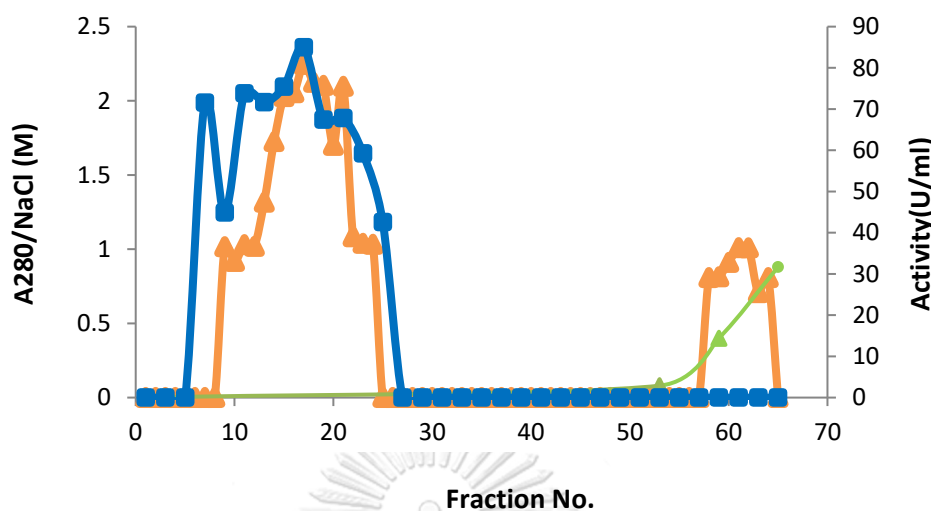
Figure 43   **DEAE Toyopearl-650M Chromatographic profile of I238L MAN26CAe24 purification**

DEAE-Toyopearl-650M column was equilibrated with 25 mM Tris-HCl buffer pH 7.5. Then mannanase was loaded to DEAE-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 0-1 M NaCl was used to eluted bound proteins. The elution started at fraction 53. Fractions 7-24 with mannanase activity were pooled.

(■ : MAN Activity, ▲ : A280 and ● : Concentration of NaCl)
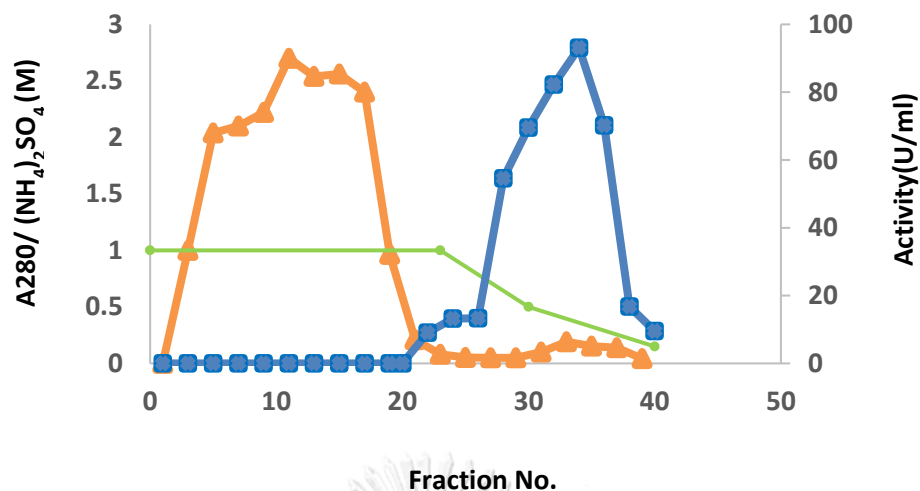
Figure 44   **Phenyl Toyopearl-650M Chromatographic profile of I238L MAN26CAe24 purification**

Phenyl-Toyopearl-650M column was equilibrated with 1 M $(NH_4)_2SO_4$ in 25 mM Tris-HCl buffer pH 7.5. Then mannanase was loaded to Phenyl-Toyopearl-650M column using flow rate 0.6 ml/min. Linear gradient of 1-0 M $(NH_4)_2SO_4$ was used to eluted bound proteins. The elution started at fraction 22. Fractions 28-37 with mannanase activity were pooled.

(▇ : MAN Activity, ▲ : A280 and ● : Concentration of $(NH_4)_2SO_4$)

Table 13   Purification of I238L MAN26CAe24

Purification Steps	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg	Yield (%)	Fold
Crude	60	5040	1.8	2800	100	1
DEAE Toyopearl	55	3850	0.55	7000	76.4	2.5
Phenyl Toyopearl	30	2310	0.15	15400	45.8	5.5
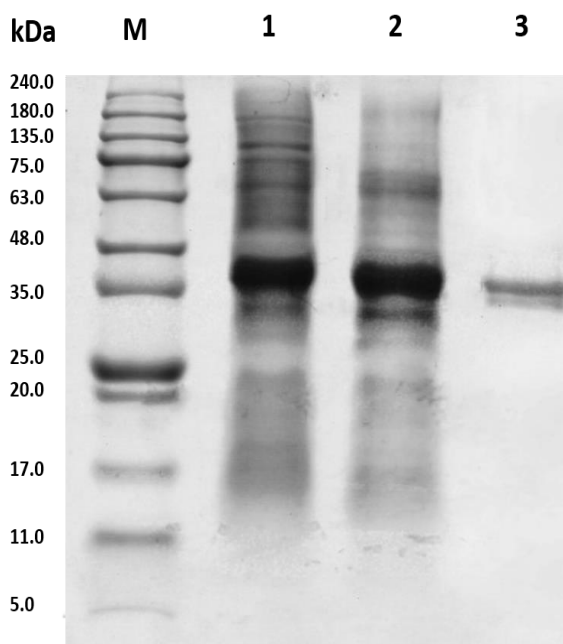
Figure 45   SDS-PAGE analysis of purified I238L MAN26CAe24

Lane M: Standard molecular weight protein

Lane 1: Crude mannanase

Lane 2: DEAE Toyopearl-650M fraction pooled fractions with mannanase activity

Lane 3: Phenyl Toyopearl-650M fraction pooled fractions with mannanase activity
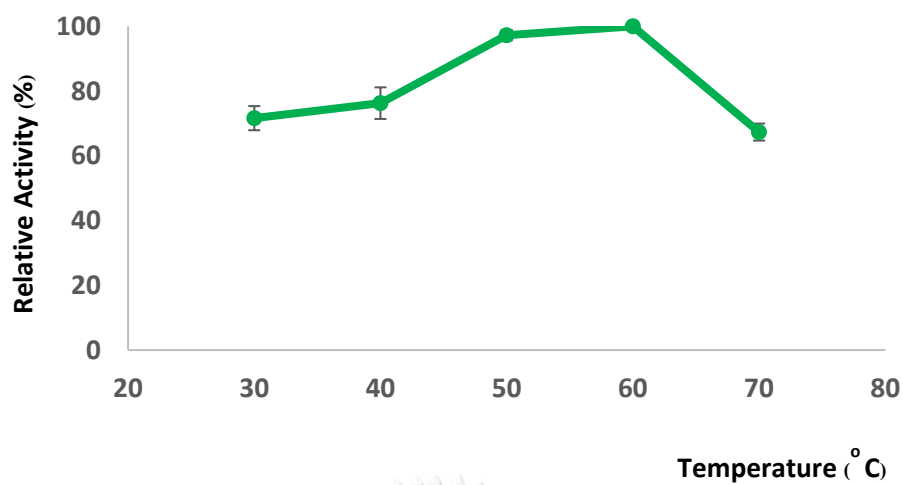
Figure 46   Optimal temperature of I238L MAN26CAe24

The reaction was incubated in 50 mM citrate buffer pH 6.0 at various temperatures from 30 to 70 °C with 0.5% (w/v) konjac flour as substrate, then incubated for 10 min.
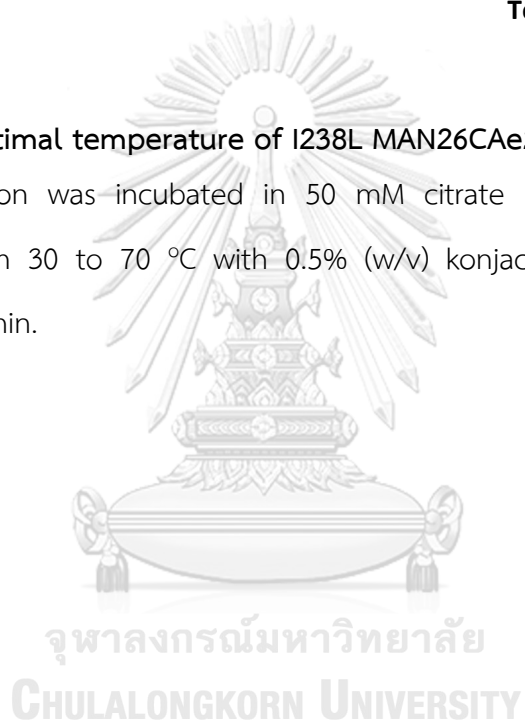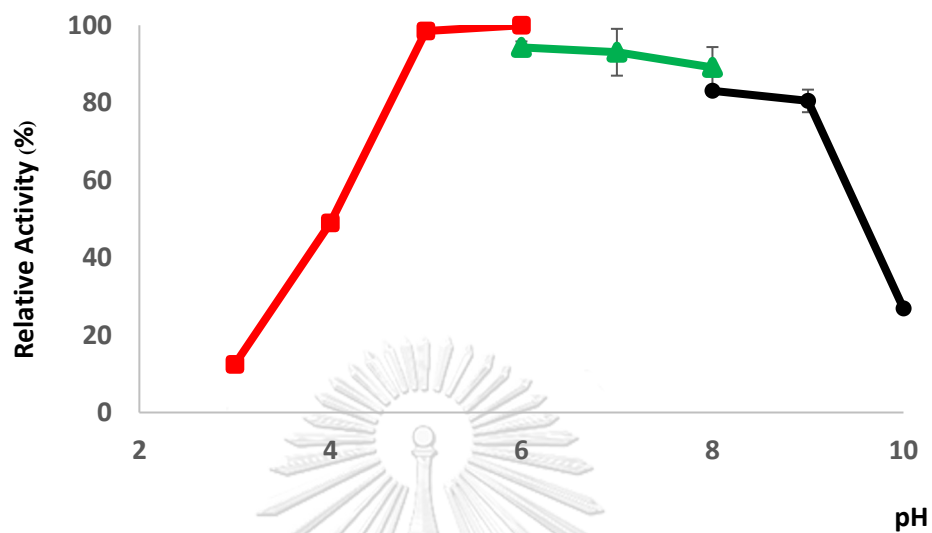
Figure 47   **Optimal pH of I238L MAN26CAe24**

The reaction was incubated at 60 °C in 50 mM of different buffer with various pH with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. ■, ▲ and ● represented citrate, phosphate and glycine-NaOH buffer respectively.
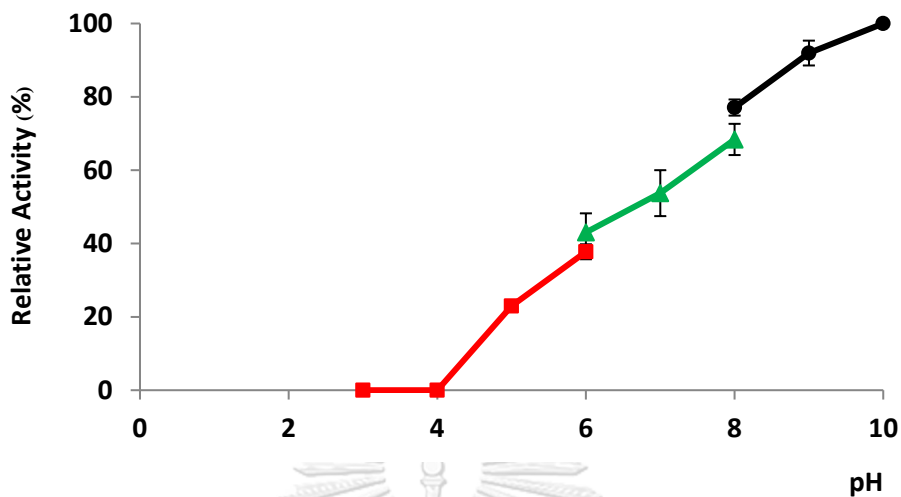
Figure 48   **Effect of pH on I238L MAN26CAe24 stability**

The purified enzyme was pre-incubated in 50 mM different buffer at various pH for 24 h at 4 °C. Incubation of reaction at 60 °C in 50 mM citrate buffer pH 6.0 with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. The initial enzymatic activity was defined as 100%. ■, ▲ and ● represented citrate, phosphate and glycine-NaOH buffer respectively.
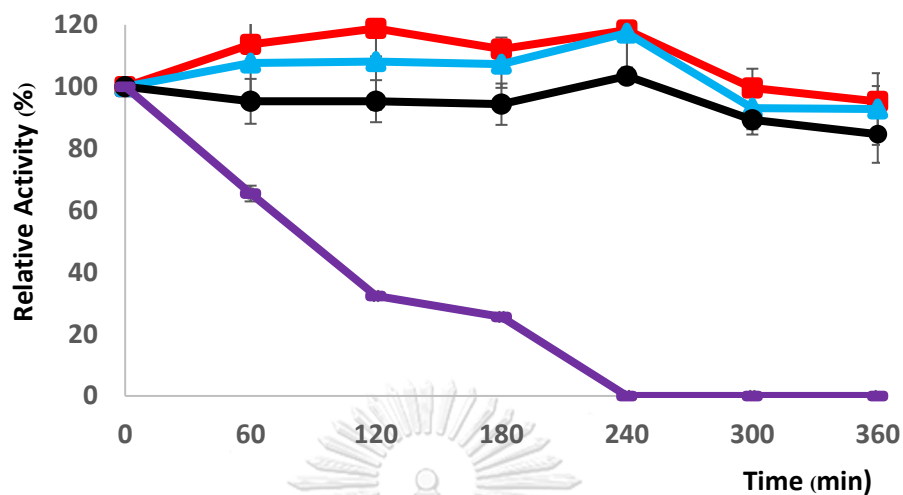
Figure 49   **Thermal effects on I238L MAN26CAe24 stability**

The purified enzyme was pre-incubated in 50 mM citrate buffer at various temperatures for 360 min and measured the enzymatic activity every 60 min of pre-incubation. The reaction was incubated at 60 °C in 50 mM citrate buffer pH 6.0 with 0.5% (w/v) konjac flour as substrate then incubated for 10 min. The initial enzymatic activity was defined as 100%. ■ , ▲ , ● and ▬ represented 40, 50, 60 and 70 °C respectively.

Table 14   The effects of various metal ions and chemical reagents on purified I238L MAN26CAe24

Substance	Relative activity (%)	Substance	Relative activity (%)
None	100 ± 0.7	$ZnCl_2$	20.7 ± 3.3
$FeCl_3$	0	$CoCl_2$	76.8 ± 10.7
$MgCl_2$	113.6 ± 3.4	EDTA	16.6 ± 1.7
$MnCl_2$	115.2 ± 6.4	SDS	58.9 ± 2.2
$CuSO_4$	0		
$CaCl_2$	74.6 ± 4.2		

The reaction was incubated at 60 °C for 10 min in 50 mM acetate buffer pH 6.0 using 0.5% (w/v) konjac flour as substrate with 5 mM various metal ions or 1% chemical reagents.

3.12 Effect of mutagenesis on SDS-tolerant property of the enzyme

The results of SDS-tolerant property study of I238L MAN26CAe24 comparing to MAN26HM7 and MAN26CAe24 is shown in Fig. 37. The result indicated that I238 is involved in SDS-tolerant property of mannanase enzyme due to the structural stabilization of the N-terminus of the protein. The interactions of residue 238 with other residues in the protein was analyzed by Arpeggio The analysis revealed that the mutation of isoleucine to leucine of MAN26CAe24 increased the interactions of this residue with other residues present in the α-helical structures found at bottom of TIM-barrel structure of the protein, increasing the stability of the structure at the N-terminus of the protein (Fig. 50). Table 15 shows the interactions found at this position in I238L MAN26CAe24 and MAN26CAe24. A total of 10 Vander Waal interactions were found in MAN26CAe24 involving residues 40, 43, 44, 185, 261 and 262. However, a total of 14 Vander Waal interactions were found in I238L MAN26CAe24 involving residues 31, 40, 43, 47, 185, 186, 187, 261 and 262.

Table 15   Amino acid residue and distance of Vander Waal interactions involving residue 283 of MAN26CAe24 and I283L MAN26CAe24

Ile238 interaction	Distance	Leu238 interaction	Distance
Pro 31	-	Pro 31	4.09
Pro 31	-	Pro 31	4.46
Thr 40	3.85	Thr 40	3.40
Thr 40	3.51	Thr 40	3.90
Val 43	3.52	Val 43	3.74
Met 44	4.32	Met 44	3.94
Met 44	3.57	Met 44	3.81
Met 44	-	Met 44	4.46
Met 44	-	Met 44	4.23
Leu 47	-	Leu 47	4.47
Pro 185	4.39	Pro 185	4.18
Val 186	-	Val 186	3.33
Leu 187	-	Leu 187	4.11
Leu 187	-	Leu 187	4.30
Asp 261	3.66	Asp 261	-
Asp 261	3.36	Asp 261	-
Ile 262	3.82	Ile 262	-
Ile 262	4.17	Ile 262	-
Total interactions	**10**	**Total interactions**	**14**
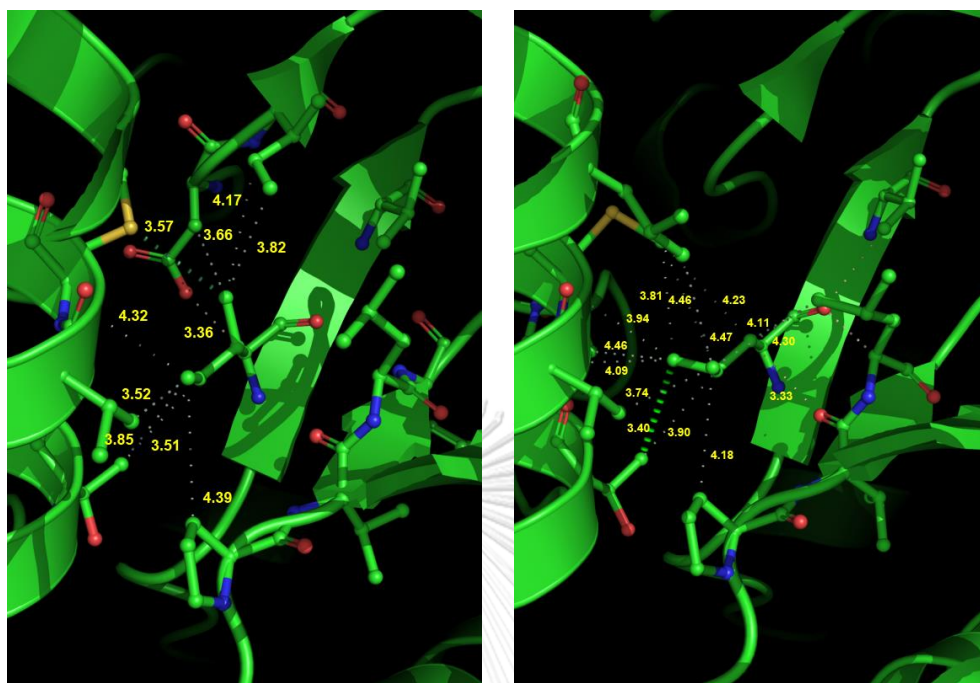
Figure 50   Position and amount of Vander Waal interactions involving residue 283 of MAN26CAe24 and I283L MAN26CAe24

CHAPTER IV

DISCUSSION

4.1 Isolation of bacteria and production of mannanase

Dynastes hercules larvae ingest wood and other organic matter in the soil, which contains mannan (Karácsonyi et al., 1975). Bacterial isolated HM7 from *Dynastes hercules* larvae intestinal tract was found to produce mannanase. Mannanase from isolated HM7 was secreted into the medium when cultured in media containing 1.5% (w/v) konjac flour (glucomannan) or coconut meal (galactomannan). Specific activity of mannanase from isolated HM7 was 114 and 138 U/mg protein in the cultivation of medium containing coconut meal and konjac flour for 36 and 48h, respectively. Konjac flour and coconut meal was also reported to be an inducer for *Vibrio* sp. Strain MA-138 and *Klebsiella oxytoca* CW-23 mannanase production, respectively (Tamaru et al., 1995) (Titapoka et al., 2008). In *Vibrio* sp. Strain MA-138 a specific activity of 0.082 U/mg can be induced by 0.5% konjac flour, while in *Klebsiella oxytoca* CW-23 a specific activity of 0.185 U/mg protein can be induced by 1% coconut meal, respectively. The specific activity of mannanase from both reports was lower than the specific activity of mannanase from HM7 Isolate. Isolated HM7 produced black pigment after long term culturing time. Previous studies have reported some *Bacillus* spp. produce pigmentation in their spore, such as carotenoids and melanin that increased the resistance of spore to UV radiation and environmental stress (Moeller et al., 2005). Melanin biosynthetic pathway in microorganisms studies revealed that melanin production is a result of enzymatic reaction such as tyrosinase and tyrosine hydroxylase (Plonka and Grabacka, 2006) (Singh et al., 2013). These enzymes conversed L-tyrosine via L-DOPA (L-3,4-dihydroxyphenylalanine) to DOPA-quinone, a precursor of melanin.

4.2   Identification of bacteria isolated HM7

The 16S rRNA gene sequence alignment was used for identification. The alignment of nucleotide sequence from 16S rRNA gene showed 99% identity with three *Bacillus* sp, including *B. amyloliquefaciens* G341 (Accession Number: CP011686), *B. methylotrophicus* JJ-D34 (Accession Number: CP011346.1) and *B. subtilis* strain Bs-916 (Accession Number: CP009611.1). The identity indicates that these bacteria were closely related. Therefore, we assigned this bacterium isolated HM7 as *Bacillus* sp. HM7.

4.3   Manno-oligosaccharide product pattern

Manno-oligosaccharide was produced from konjac flour and coconut meal by mannanase hydrolysis. *Bacillus* sp. HM7 crude mannanase cultured in different carbon sources exhibited the same cleavage pattern on konjac flour and coconut meal hydrolysis products. Hydrolysis of konjac flour by crude mannanase from *Reinekea* sp. KIT-YO10 (Hakamada et al., 2014) also produced many product size similarly to *Bacillus* sp. HM7. On the other hand, hydrolysis of coconut meal by crude mannanase from *Bacillus subtilis* WY34 mannanase mainly produced mannobiose, mannotriose and mannotetraose (Jiang et al., 2006). Comparison of manno-oligosaccharide product pattern from konjac flour and coconut meal hydrolysis on TLC showed that konjac flour hydrolitic product had more product species than coconut meal. Konjac flour is soluble and more accessible for the enzyme than coconut meal (Chen et al., 2013), (Jeon et al., 2011).

4.4   Cloning and analysis of *Man26HM7* gene

β-mannanase gene from *Bacillus* sp. HM7 was cloned successfully. We have successfully used primers designed from *Bacillus subtilis* BEST7613 genome sequence (Accession Number: AP012495) to amplify mannanase gene from *Bacillus subtilis* CAe24, therefore the same primer set was used successfully for *Man26HM7*. *Man26HM7* has an open reading frame of 1086 base pairs which encode to 362 amino acids. *Bacillus subtilis* WL-3 mannanase gene has an open reading frame of 1080 base pairs which close to *Man26HM 7* (Yoon and Lim, 2007)*,* β-mannanases are grouped int*o* glycoside hydrolase family GH 5 and GH 26 (Henrissat, 1991). The nucleotide sequence alignment of *Man26HM7* puts it in the GH 26 family, showing high homology with other mannanase in the GH26 family (Dhawan and Kaur, 2007). GH26 family mannanase is found in bacteria such as *Bacillus* sp. (Hatada et al., 2005) and *Cellumonas* (Le Nours et al., 2005).

4.5   Expression of MAN26HM7mannanase

We have successfully expressed *Man26HM7* using pET expression system under the regulation of T7 promoter. MAN26HM7 had maximum activity 61.3 and 57 U/ml in extracellular and intracellular fraction, respectively. The enzyme activity found in the extracellular and intracellular was similar. Previous report of the expression of *Bacillus circulans* NT 6.7 mannanase in *E. coli* BL21 (DE3) under T7 promoter exhibit similar result, where the enzymatic activity was found equally distributed in the extracellular and intracellular fraction. *Bacillus circulans* NT 6.7 had maximum activity 37 U.ml in extracellular and intracellular fraction (Piwpankaew et al., 2014).   Moreover, when mannanase gene from *Bacillus subtilis* WL-3 was expressed under *B. subtilis* promoter higher activity was observed, 300 U/ml in extracellular fraction (Yoon and Lim, 2007).

4.6   Purification of mannanase

MAN26HM7 was purified by DEAE and Phenyl Toyopearl column. Purified MAN26HM7 had 15.5 purification fold with 17% recovery. MAN26CAe24 was purified by the same methods as MAN26HM7. Purified MAN26CAe24 had 10.3 purification fold with 31% recovery. The same chromatographic steps were used in *Bacillus* sp. *CFR1601* mannanase purification. Purified mannanase from *Bacillus* sp. *CFR1601* had 50 purification fold with 21.3% recovery (Srivastava and Kapoor, 2016). Recently, Ni-charged affinity was usually used in mannanase purification. Intracellular mannanase from *Penicillium oxalicum* GZ-2. A single step purification by nickel-charged affinity chromatography column can gain a 4.5 fold purification with 56% recovery (Liao et al., 2014). Such tag could be used to improve our purification yield.

4.7   Characterization of MAN26HM7

MAN26HM7 has activity in a broad range of pH with the optimum pH of 6.0. The optimum pH of MAN26HM7 was similar to other bacterial mannanase which has the optimum pH in the range of 6.0 to 8.0 (Mabrouk and El Ahwany, 2008). *Bacillus subtilis* WY34 mannanase had the same optimum pH as MAN26HM7 (Jiang et al., 2006). However, mannanase from alkalophilic bacteria exhibited higher optimal pH than MAN26HM7. *Bacilus* sp. N16-5 and *Bacillus* sp. JAMB-750 mannanase had highest activity at pH 9.5 (Ma et al., 2004) and 10.0, respectively. Interestingly, most fungal mannanases had the optimum pH in the acidic range (Blibech et al., 2010) such as *A. aculeatus* VN, *Sclerotium rolfsii* and *Bispora* sp. mannanase the highest activity at pH 3,3.0 and 1 respectively (Christgau et al., 1994) (Gübitz et al., 1996) (Luo et al., 2009). (Hatada et al., 2005).

The optimum temperature of MAN26HM7 was 50 °C. *Cellulosimicrobium* sp. strain HY-13 had reported the same optimum temperature as MAN26HM7 (Ham et

al., 2011). Mannanae with higher optimum temperature was found in *Dictyoglomus thermophilum* and *Eubacterium Thermotoga neapolitana*. These thermophilic mannanase from *Dictyoglomus thermophilum* and *Eubacterium Thermotoga neapolitana* had an optimum temperature of 80 and 90 °C, respectively. (Gibbs et al., 1999), (McCutchen et al., 1996). Mannanase from *Cryptopygus antarcticus*, *Paenibacillus* sp.MSL–9 and *Sphingomonas* sp*.* showed the highest activity in lower temperature, 30 to 40 °C (Song et al., 2008), (Manjula et al., 2010), (Zhou et al., 2012) which was lower than MAN26HM7.

MAN26HM7 was stable at 60 °C for 360 min, which was higher than the previous reports, *Bacillus* sp. N16-5 mannanase , at 60 °C for 150 min (Ma et al., 2004). The molecular weight of MAN26HM7 from SDS-PAGE was 40kDa. *Klebsiella oxytoca* KUB-CW2-3 mannanase had molecular mass 43.2 kDa which close to MAN26HM7 (Pongsapipatana et al., 2016). $CoCl_2$ and $MnCl_2$ activated MAN26HM7. The presence of Co^{2+} has been reported for enhanced *Pantoea agglomerans* mannanase activity (Wang et al., 2010). MAN26HM7 was significantly inhibited by $FeCl_2$ and $CuCl_2$. The inhibition of Cu^{2+} and Fe^{2+} were also found in *Vibrio* sp. strain MA-138 mannanase (Tanaka et al., 2009).

4.8   Effect of SDS and detergent on MAN26HM7 and MAN26CAe

β-mannanase from different origins express different SDS-tolerant property, *Bacillus circulans* CGMCC1554 (Li et al., 2008) and. *B. subtilis* WY34 (Jiang et al., 2006) β-mannanase had the 0% and 55% residual activity in the presence of 0.03% SDS in the reaction. The toleration of Endo-mannanase to SDS is important for the compatibility in detergent and made it possible to be applied in the detergent industries. *Bacillus* sp. N16-5 (Ma et al., 2004) produced SDS-tolerant alkalophilic mannanase while *Bacillus* sp. HJ14 (Zhang et al., 2016) produced SDS-tolerant neutral mannanase. In this study, characterization of Man26HM7 reveals that the

enzyme is a SDS-tolerant neutral β-mannanase. When SDS was added to the reaction buffer to the finial concentration of 0.1%, the residual activity of purified *Bacillus* sp. N16-5 was 115% at its optimum. While *Bacillus* sp. HJ14 mannanase activity was 82% at its optimum when SDS was added in to the reaction to the final concentration 0.03%. The residual activity of purified MAN26HM7 was 70% when SDS was added to the reaction buffer to the finial concentration of 1%.

Some mannanase are stable in detergent such as MAN26HM7 and *Bacillus* sp. HJ14 mannanase. MAN26HM7 was stable in liquid detergent more than *Bacillus* sp. HJ14 mannanase. *Bacillus* sp. HJ14 mannanase activity was 73.5% when incubated the enzyme in 1% liquid detergent at 37 °C for 60 min and liquid detergent was added in to the assay reaction to the final concentration of 1% (Zhang et al., 2016). The residual activity of purified MAN26HM7 was 97% when incubated enzyme in 5% liquid detergent at 37 °C for 180 min and liquid detergent was added to the assay reaction to the final concentration of 5%.

We found that MAN26HM7 activity was retained up to 97% when liquid detergent was added to the final concentration of 5% and 55% when powder detergent was added to the reaction to the final concentration of 0.5%. pH comparison of commercial laundry detergent from local market showed powder laundry detergent had pH more than liquid detergent (Boonchai and Iamtharachai, 2010). Therefore, the alkaline properties of powder detergent might have decrease the activity of β-mannanase more than liquid detergent.

4.9   Homology modeling

From the homology modeling reveals that MAN26HM7 had TIM barrel structure with E193 and E292 as the conservative catalytic residue. All of GH-family 26 enzymes have the TIM barrel structure (Zhao et al., 2009) and conserved catalytic glutamates (Hogg et al., 2001).

4.10   Rational mutagenesis of MAN26CAe24

From homology modeling, MAN26HM7 had seven amino acid residues that differs from MAN26CAe24. These six amino acid residues were located on the surface which is unlikely to result in the higher stability to SDS of MAN26HM7, furthermore, most of them were quite conserved. Leu238 was found in the interia of the protein and may be involed in intramolecular interctions that may enhance MAN26HM7 stability in SDS.

We discovered that the mutation of isoleucine to leucine in MAN26CAe24 increased Vander Waal interactions of this residue with neighboring amino acid residues. A total of 10 Vander Waal interactions at this position, Leu238, were found in MAN26CAe24 involving residues 40, 43, 44, 185, 261 and 262. However, a total of 14 Vander Waal interactions were found in I238L MAN26CAe24 involving residues 31, 40, 43, 47, 185, 186, 187, 261 and 262. Previous report, of site-directed mutagenesis of methyl parathion hydrolase from *Ochrobactrum* sp. M231 at residues 194 from Gly to Pro also increased the number of internal van der Waals interactions surrounding the mutated site, which also improved the stability of enzyme (Tian et al., 2010).

CHAPTER V

CONCLUSION

Mannanase activity was found in cell culture when *Bacillus* sp HM7 was cultured in medium containing konjac flour or coconut meal. Identification of isolated bacteria by 16S rRNA analysis revealed that they were classified into *Bacillus* group. Manno-oligosaccharide can produce by hydrolysis of konjac flour and coconut meal. Mannanase gene *Man26HM7* was clones and expressed. It had the deduced amino acid sequence of 362 amino acid residues with 21 amino acid residues signal peptide. Purified Man26HM7 resulted in a 15.5-fold purification with 16.9 % yield. When analyzed on SDS-PAGE, MAN26HM7 had molecular mass 40 kDa. The optimum condition for enzymatic catalysis was 50 $^{\circ}$C and pH 6.0. Man26HM7 was stable at pH 4.0 to 10.0, 4 $^{\circ}$C for 24h and 60 $^{\circ}$C for 360min. Man26HM7 was activated by $CoCl_2$ and $MnCl_2$ but inhibited by $CuCl_2$ and $FeCl_2$. MAN26HM7 had SDS-tolerant property more than MAN26CAe24. MAN26HM7 had relative activity 70% and 57% in the reaction containing 1% and 2%(w/v) SDS at its optimum, respectively. After pre-incubation MAN26HM7 in 1% SDS for 180 min at 37 $^{\circ}$C, MAN26HM7 was assayed in a reaction mixture containing 1% SDS at 37 $^{\circ}$C. MAN26HM7 retained 90 % of its initial activity. Amino acid substitution of MAN26CAe24 at position I238 with leucine increased SDS-tolerant of enzyme, indicating that this residue was important for SDS tolerant property. Further analysis demonstrates that the substitution increases intramolecular interaction of the protein.

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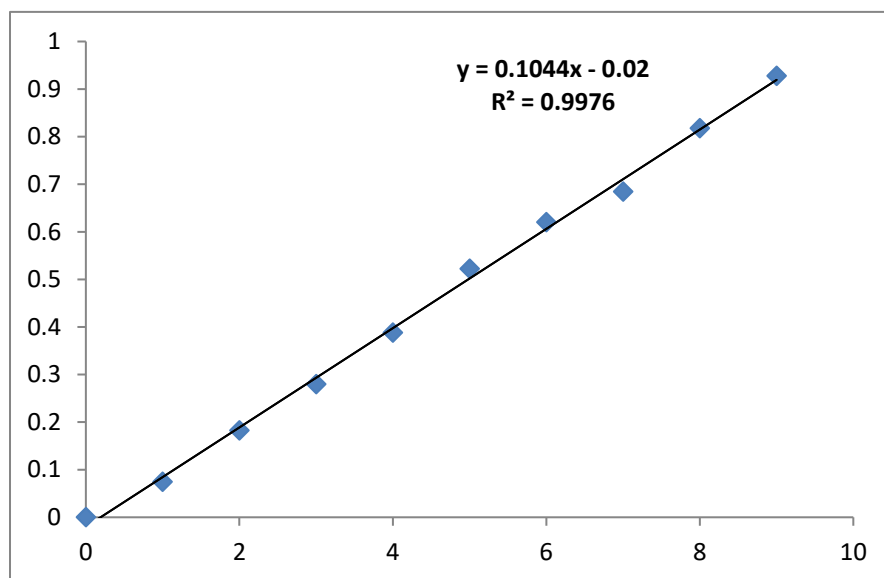
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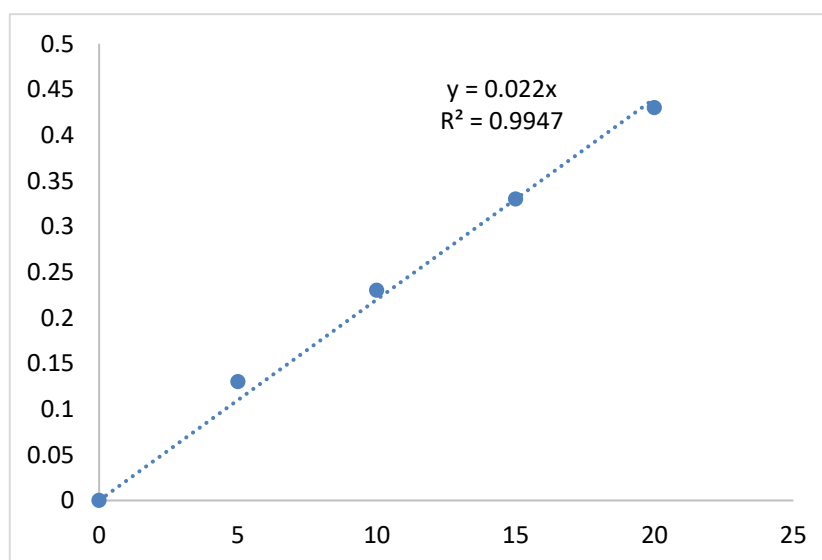
APPENDIX

Appendix A

Standard curve of DNS reagent

Appendix B

Standard curve of Bradford reagent

$$y = 0.022x$$
$$R^2 = 0.9947$$

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Appendix C

Nucleotide sequence of 16S rRNA gene from isolated bacterial HM7

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGG
ACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
CCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACCGCA
TGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCT
CTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAG
AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCAC
GTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTC
TGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACG
CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTG
CATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT
GGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTT
CGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA
CACCCGAAGTCGGTGAGGTAACCTTTATGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGG
GGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGCATCACCTCCTT

VITA

My name is Mr. Sitipon Leerawatthanakun. I was born on 14th April 1993. I live in Bangkok. I graduated a Bachelor Degree of Science in Biochemistry at Chulalongkorn University in 2015. In 2015, I received the Scholarship from the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibol Aduladej and the 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) to study in Biochemistry and Molecular Biology, Department of Biochemistry, Faculty of Science.

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