PRODUCTION OF MANNO-OLIGOSACCHARIDES (MOS) BY ENZYME TECHNOLOGY

SUTTIPONG SAK-UBOL

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การผลิตแมนโนอิลิโกแซคคาไรด์ (มอซ) ด้วยเทคโนโลยีเอนไซม์

นายสุทธิพงศ์ ศักดิ์อุบล

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PRODUCTION OF MANNO-OLIGOSACCHARIDES (MOS) BY ENZYME TECHNOLOGY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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กากมะพร้าว ซึ่งเป็นผลพลอยได้จากอุตสาหกรรมผลิตน้ำมันมะพร้าวสกัดเย็น อุดมไปด้วยกาแล็คโตแมนแนน และแมนแนน สามารถใช้เป็นสารตั้งต้นในการผลิตแมนโนโอลิโกแซคคาไรด์ (มอซ) ด้วยเอนไซม์เบต้าแมนแนนเนส ในการศึกษานี้เป็นการหาสภาวะที่เหมาะสมในการผลิตเอนไซม์ดังกล่าวจากเชื้อบาซิลลัส ไลเคนนิฟอมิส มาแสดงออกในเชื้อแล็คโตบาซิลลัส แพลนทารั่ม แล้วตรวจหาหาสภาวะที่เหมาะสมในการผลิตผลิตภัณฑ์ที่ได้จากการย่อยกากมะพร้าวที่กำจัดไขมันแล้วด้วยเอนไซม์ที่ผลิตขึ้นมา โดยใช้วิธีโครมาโตกราฟีแบบผิวบาง (TLC) และ เครื่องโครมาโตกราฟีแลกเปลี่ยนประจุความดันสูง (HPAEC-PAD) จากนั้นนำมอซไปทดสอบฤทธิ์ทางชีวภาพ โดยนำไปเลี้ยงเชื้อแบคทีเรียชนิดต่าง ๆ ผลการทดลองพบว่า สามารถกระตุ้นการเจริญของจุลินทรีย์กลุ่มแบคทีเรียผลิตกรดแล็คติคได้ดี ภายใต้สภาวะไม่ใช้ออกซิเจน จากนั้นจึงทำการทดสอบคุณสมบัติทางชีวภาพของมอซต่อเซลล์ผิวหนังของมนุษย์เป็นเวลา 24 ชั่วโมง พบว่าไม่มีอันตรายต่อเซลล์ดังกล่าวโดยเซลล์ดังกล่าวสามารถอยู่รอดได้ร้อยละร้อย และเมื่อนำไปศึกษาคุณสมบัติป้องกันการอักเสบกับเซลล์แมคโครเฟจของมนุษย์ที่กระตุ้นด้วยลิโพพอลิแซคคาไรด์ (LPS) พบว่ามีคุณสมบัติป้องกันการอักเสบอีกด้วยจะเห็นว่าสามารถผลิตมอซจากกากมะพร้าวโดยใช้เอนไซม์ที่ผลิตขึ้นได้เองซึ่งสามารถพัฒนาให้เป็นผลิตภัณฑ์อาหาร อาหารสัตว์และเครื่องสำอางในอนาคตได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

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Copra meal is a by-product from the coconut oil extract process. It is typically rich in galactomannans and linear mannans, which can be used as a substrate for the production of manno-oligosaccharides (MOS) by enzymatic hydrolysis using 1,4-β-D-mannanase. In this research, we reported the optimized conditions for the hydrolysis of defatted copra meal using recombinant 1,4-β-D-mannanase from *Bacillus licheniformis* strain DSM13 expressed in *Lactobacillus plantarum* WCSF1. The products were analyzed by thin layer chromatography (TLC) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The copra MOS products were used as carbon sources for culturing of various bacteria and the results showed that it can enhancee the growth of probiotic, lactic acid bacteria under anaerobic condition and pathogenic bacteria under aerobic conditions. *In vitro* toxicity assay of MOS in Human Dermal Fibroblast demonstrated 100% survival of the cells after 24 h of treatment with copra MOS. Moreover, copra MOS also showed anti-inflammatory activity against LPS-induced inflammation of human macrophage. These results indicated that MOS can be further developed to be used as valued-added health promoting additives for food, feed, and cosmeceuticals in the future.

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*Bl*ManB	=	GH family 26 β-mannanase from *Bacillus licheniformis*
DNA	=	Deoxyribinucleic acid
DNS	=	3,5-Dinitrosalicylic acid
GH	=	Glycosyl hydrolysate
IMAC	=	Immobilized metal affinity chromatography
LB	=	Luria-Bertani
MRS	=	De Man, Rogosa and Sharpe
OD	=	Optical density
OmpA	=	Outer membrane protein A
PAGE	=	Polyacrylamide gel electrophoresis
SDS	=	Sodium dodesyl sulphate

CHAPTER 1
INTRODUCTION

1.1 Significance of this study

The coconut is known for its great versatility as seen in the many uses of its different parts and found throughout the tropical area including Thailand (Hossain et al., 1996). Coconuts are part of the daily diets of many people and are grown industrially for the edible. The highly saturated oil contained in the flesh of its fruits (Canapi et al., 2005). Copra meal is the by product from the coconut oil extraction. It contains 61% of galactomannan and 26% of linear mannan (Balasubramaniam, 1976; Saittagaroon et al., 1983). The abundance of galacto mannan and linear mannan are the good source for production of manno-oligosaccharides.

For an efficient bioconversion of lignocellulose biomass to fermentable sugars, thermostable enzymes are essential during the pre-treatment step, followed by simultaneous saccharification and fermentation (SSF) processes (Turner et al., 2007; Viikari et al., 2007). Several wild-type and recombinant β-mannanases that are stable and active at high temperature from thermophilic bacterial, eubacterial (Duffaud et al., 1997; Jiang et al., 2006; Luthi et al., 1991; Talbot and Sygusch, 1990), actinomycetes (Hilge et al., 1998) and fungal sources (Do et al., 2009; Kote et al., 2009; Luo et al., 2009; Sachslehner et al., 2000; Turner et al., 2007) have been characterized. CBMs such as CBM1, naturally fused to β-mannanases, may enhance the hydrolytic efficiency on complex lignocellulosic substrates (Hagglund et al., 2003). Moreover, a successful attempt to improve the hydrolytic capacity of β-mannanase by genetically engineering an additional carbohydrate-binding module (CBM1) to the C-terminus of the enzyme (Pham et al., 2010) or by a directed evolutionary approach (Couturier et al., 2013a) have been reported.

In addition to the pre-treatment of lignocellulosic biomass to generate biofuels, another interesting application of β-mannanase is for the random hydrolysis of mannans into MOS (Songsiriritthigul et al., 2010), which have several possible beneficial

effects on health and well-being as described in the next section.

MOS can be classified unequivocally as prebiotic. Since mannans can be obtained from abundant and inexpensive agricultural wastes, bioconversion of mannan into various types of bioactive MOS and investigation of their actual biological activities are highly attractive areas of research and development.

1.2 Research objectives

1.2.1 Main objective

To produce manno-oligasaccharides (MOS) using coconut waste from virgin coconut oil processing factory.

1.2.2 Specific objectives

To express mannanase in food grade expression system.

A) To optimize the condition for the production of MOS from copra meal.

B) To investigate the physical properties of MOS.

C) To investigate the biological properties of MOS.

1.3 Scope of this study

This study was to develop a *Lactobacillus plantarum* expression system based on the pSIP-vectors for the production of β-mannanase. Copra Meal from Thai Pure Company were used as a substrate to produce Manno-oligosaccharide (MOS). The MOS production only evaluated in lab scale. The biological assay was done *in* Vitro.

CHAPTER 2

MANNAN BIOTECHNOLOGY

This work has been published in Critical Review in Biotechnology in 2014.

2.1 Abstract

Mannans of different structure and composition are renewable bioresources that can be widely found as components of lignocellulosic biomass in softwood and agricultural wastes, as nonstarch reserve polysaccharides in endosperms and vacuoles of a wide variety of plants, as well as a major component of yeast cell walls. Enzymatic hydrolysis of mannans using mannanases is essential in the pre-treatment step during the production of second-generation biofuels and for the production of potentially health-promoting manno-oligosaccharides (MOS). In addition, mannan-degrading enzymes can be employed in various biotechnological applications, such as cleansing and food industries. In this review, fundamental knowledge of mannan structures, sources and functions will be summarized. An update on various aspects of mannan-degrading enzymes as well as the current status of their production, and a critical analysis of the potential application of MOS in food and feed industries will be given. Finally, emerging areas of research on mannan biotechnology will be highlighted.

2.2 Mannan polysaccharides

2.2.1 Sources and functions

Mannans and heteromannans are polysaccharides that are widely distributed in nature as part of hemicelluloses in plant tissue (Capek et al., 2000) as well as a constituent of glycoproteins in yeast cell walls (Sandin, 1987). In plants, mannans and heteromannans are components of hemicellulose, a term that is used for a part of lignocellulose – a renewable bioresource that comprises lignin, cellulose and hemicellulose (Scheller and Ulvskov, 2010; Van Dyk and Pletschke, 2012). Lignocellulose is widely available in the form of biological wastes from forest industries,

energy crops and components of agricultural residues such as straw and grass, coffee bean extracts, palm kernel (Moreira and Filho, 2008) or copra meal (Saittagaroon et al., 1983), to name a few. The term hemicellulose comprises a group of different structural polymers of the plant cell wall consisting of various sugars such as D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose and 4-O-methyl-Dglucuronic acid (Braidwood et al., 2014; Scheller and Ulvskov, 2010; Wolf et al., 2012). It constitutes 25–30% of total dry wood weight, and its distribution and constituents vary in softwood (gymnosperms) and hardwood (angiosperms); Moreira and Filho, 2008; Popper et al., 2011). While xylans constitute the predominant hemicellulose of hardwoods and straw, galactomannans represent the largest hemicellulose fraction in softwoods (Puls, 1997). Hemicelluloses are abundant biological wastes from the production of mechanical pulps and wood-containing papers, and hence are available in huge amounts (Scheller and Ulvskov, 2010).

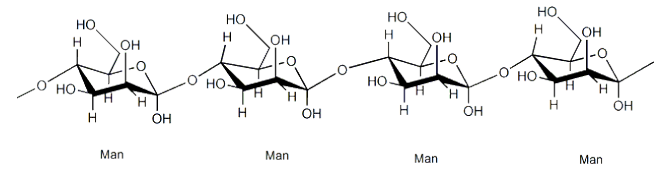
A polysaccharide diversification (including mannans) occurred during the evolution of land plants, which resulted in the structural changes in the cell wall (Lee et al., 2011). Mannans and heteromannans both serve structural elements in plant cell walls and carbohydrate reserves (Rodrı´guez-Gacio Mdel et al., 2012). Mannan-based polysaccharides exhibit a storage function as non-starch carbohydrate reserve in the endosperm wall of seeds such as coconut (*Cocos nucife*ra; Saittagaroon et al., 1983), coffee bean (*Coffea* spp.), locust (carob) bean (*Ceratonia siliqua*) or the vacuole in vegetative tissue of plants such as konjac (*Amorphophallus konjac*), ivory nut (*Phytelephas* spp.), guar (*Cyamopsis tetragonoloba*), Aloe vera, etc. (Moreira and Filho, 2008). In addition, they also help provide resistance to mechanical damage and retain resistance after exposure to water, due to their water insolubility (Reid and Edwards, 1995). Some of these mannans are well known and widely used as thickening, stabilizing and gelling agents in the food industry, e.g. galactomannan from locust bean gum or glucomannan from konjac (Cyber Colloids, 2014). In addition to structural and storage functions, mannans have also been suggested to play a role in metabolic networks devoted to various cellular processes (Liepman et al., 2007).

2.2.2 Structures

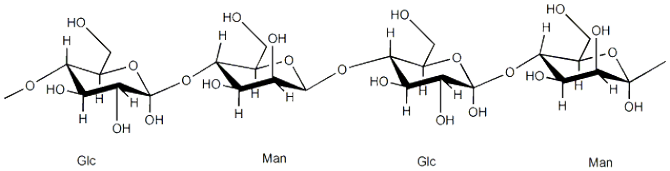
Mannans differ significantly in their structure according to their origin. Hemicellulosic mannans consist of b-1,4-linked D-mannose (and D-glucose) in the backbone and a-1,6-linked D-galactose as side chains, allowing the formation of various types of linear or branched polysaccharides (Fig. 1). These polysaccharides can be classified into four subfamilies, namely, linear mannan, glucomannan, galactomannan and galactoglucomannan (Moreira and Filho, 2008). The mannose residues on galactoglucomannans can be acetylated at the C-2 and C-3 positions to various degrees, depending on the source of the polysaccharide, resulting in acetylated galactoglucomannans (Lundqvist et al., 2002). However, it is important to note that the extraction methods can strongly influence the yield, molecular weight and structure of mannans, as observed from studies comparing different methods for preparing galactoglucomannan from spruce (*Picea abies*) (Lundqvist et al., 2003). Moreover, it has been shown that acetylation can hinder the detection of mannan by antibodies or CBM (Marcus et al., 2010). A summary of various types of mannans from plants, together with their sources, structures, common and potential applications are shown in Fig. 2.1. and Table 2.1.

In addition to the β-mannans described above, another type of mannan comes from yeast cell walls, which consists of three groups of polysaccharides, i.e. β-glucan, chitin and mannan, the latter being part of the phosphoprotein mannan complex (Nakajima and Ballou, 1974a, b). In *Saccharomyces cerevisiae*, the cell wall comprises 50 and 40% of β -1,3- glucan and mannoprotein, respectively (Lipke and Ovalle, 1998). Two important minor components of yeast cell walls are β -1,6-glucan and chitin, which make up 10% and 1–3% of the total mass of cell walls, respectively (Lipke and Ovalle, 1998). Mannoproteins are highly antigenic and glycosylated polypeptides that carry both N-linked and O-linked glycans. In many yeasts, N-glycans consist of 50–200 additional D-mannose units, while O-glycans contain only1–5 mannosyl units. The N-linked yeast mannans consist mainly of a long linear polymer of α-1,6-linked D-mannose with short side chains of D-mannosyl units attached to the backbone mainly by α -1,2-linkages, and to each other by both α -1,2 and α -1,3-linkages (Kocourek and Ballou, 1969; Kollar et al., 1997). However, in the yeast Candida

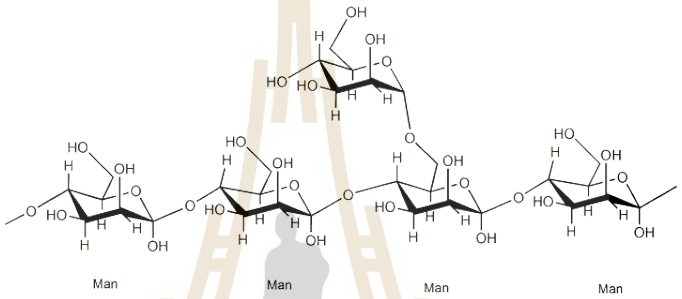
albicans, in addition to α -1,6-linked branching mannose units, β-1,2-linked mannose residues have also been identified (Shibata et al., 2007). Moreover, an unusual form of mannan, i.e. sulphated (1,3)- linked α -D-mannan, has also been reported in seaweed (Perez Recalde et al., 2009). Sulfated polysaccharides are receiving growing interest for biomedical application, especially for tissue engineering and drug delivery approaches (Silva et al., 2012).



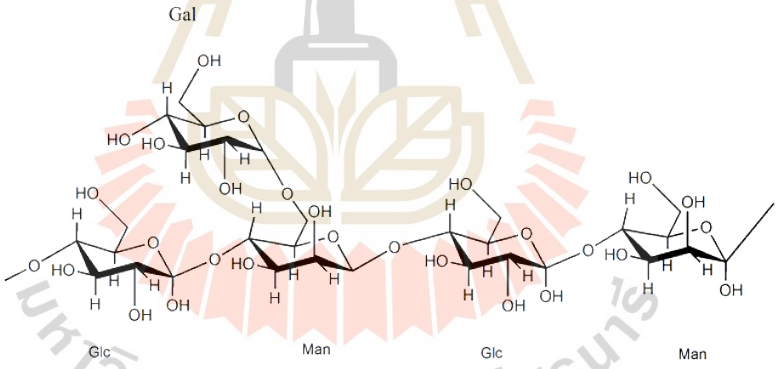
Linear mannanse



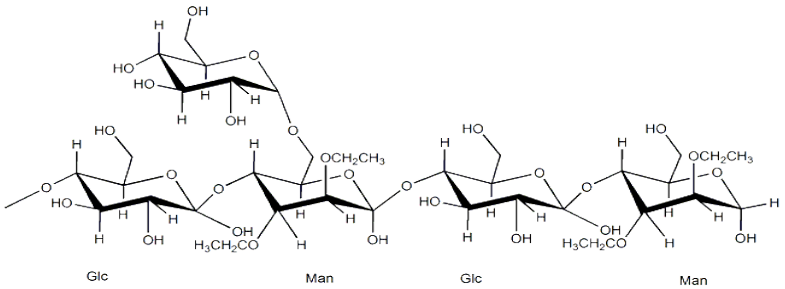
Glucomannas



Galactomannans



Galactoglucomannans



O-acetyl-galactoglucomannans

Figure 2.1 Structure of various type of Mannan.

Table 2.1 Summary of various types of mannans from plants, together with their sources, structures, common and potential applications.

Organism	Molecular Weight (kDa)	Optimum pH	Optimum temperature (°C)	Substrate/PI	Reference
Bacillus subtilis WL-3	38	6.0	60	LBG	(Yoon et al., 2008)
Bispora antennata CBS 126.38	45	6.0	70	LBG	(Liu et al., 2012)
Bispora sp. MEY-1	80	1.0-1.5	65	LBG	(Luo et al., 2009)
Caldibacillus cellulovorans	30.7	6.0	85	-	(Sunna et al., 2000)
Caldicellulosiruptor Rt8B.4	-	5.5	75	LBG-Galman	(Sunna, 2010)
Caldocellum saccharolyticum	34	6.0	80	LBG	(Lüthi et al., 1991)
Caldocellum saccharolyticum	-	6.0	80	-	(Morris et al., 1995)
Cellulomonas fimi ATCC 484.	100	5.5	42	LBG	(Stoll et al., 1999)
Cellulosimicrobium sp. HY-13	44	6.0	50	LBG	(Ham et al., 2011)
Ceriporiopsis subvermispora CS-1	-	4.5	60	LBG	(Heidorne et al., 2006)
Chaetomium sp. CQ31	50	5.0	65	LBG	(Katrolia et al., 2012)

Table 2.1 (continued).

Organism	Molecular Weight (kDa)	Optimum pH	Optimum temperature (°C)	Substrate/PI	Reference
Clostridium cellulolyticum ATCC 35319	45	-	-	-	(Perret et al., 2004)
Clostridium cellulovorans ATCC 35296	70	7.0	40	polysaccharide	(Jeon et al., 2011)
Clostridium josui	-	6.5	50	KGM,CGM	(Sakka et al., 2010)
Clostridium thermocellumd YS	70	6.5	65	-	(Halstead et al., 1999)
Clostridium thermocellumd F1	55	7.0	75	KGM	(Kurokawa et al., 2001)
Dictyoglomus thermophilum Rt46B.1	40	5.0	80	LBG	(Gibbs et al., 1999)
Erwinia carotovora CXJZ95-198	42	7.5	55	KGM	(Zhang et al., 2007)
Humicola insolens Y1.	47	5.5	70	LBG	(Luo et al., 2012)
Paenibacillus sp. BME-14,	50	4.5	60	LBG	(Fu et al., 2010)
Paenibacillus polymyxa GS01	-	5.0	50	-	(Cho et al., 2006)

Table 2.1 (continued).

Organism	Molecular Weight (kDa)	Optimum pH	Optimum temperature (°C)	Substrate/PI	Reference
Pantoea agglomerans A021	38.5	6.0	55	LBG	(J. Wang et al., 2010)
Penicillium freii F63	72	4.5	60	LBG	(Y. Wang et al., 2012)
Clostridium thermocellumd F1	55	7.0	75	KGM	(Kurokawa et al., 2001)
Dictyoglomus thermophilum Rt46B.1	40	5.0	80	LBG	(Gibbs et al., 1999)
Penicillium pinophilum C1	65	4.0	70	LBG	(Cai et al., 2011)
Penicillium sp. C6	39	4.5	70	LBG	(Cai et al., 2011)
Phanerochaete chrysosporium RP78	65	4.0-6.0	60	LBG	(Benech et al., 2007)
Phialophora sp. P13	45	1.5	60	LBG/4.5	(Zhao et al., 2010)
Piromyces sp.	68	-	-	-	(Fanutti et al., 1995)
Pseudomonas fluorescens subsp. *cellulosa*	46	7.0	-	plant structural polysaccharide	(Braithwaite et al., 1995)

Table 2.2 Sources and application of mannan.

Type of Mannan	DP	Source	Ratio (man:Glc:Gal)	Applications	References
glucomannan	More than 200	Hard wood, konjac	3:1:0	Preventive of chronic disease and weight control agent	(Popa, 1998; Ishurd et al., 2006; Vuksan et al., 1999)
galactomannan	n/a	Fenugreek gum	1:0:1	Food	(Moreira, 2008; de O. Petkowicz, 2001; Picout et al., 2002; Prest, 1991; Tuohy, 2001; Schwartz, 1983)
		Gaur gum	2:0:1	Stabilizer	
		Tara gum	3:0:1	Gel setting	
		Locus bean gum	4:0:1	Thickener	
galactoglucomannan	15-100	Endosperm	3:1:1	n/a	(Yunqiao, 2008; Van et al., 2010; Chow et al., 2005)
		Aloe vera bulk water soluble extract (BSW)	15:1:1	Immunomodulation	
	-				
arabinomannan	n/a	algae	n/a	n/a	(Pieper, 2012)

2.3 Mannandegrading enzyme

2.3.1 β-mannanases and other mannan-degrading enzyme

The two major plant mannan-degrading enzymes are mannan endo-1,4-β-mannosidase or 1,4- β -D-mannan mannanohydrolase (EC 3.2.1.78), commonly known as b-mannanase and β -D-mannoside mannohydrolase or β-mannosidase (EC 3.2.1.25). According to the Carbohydrate Active Enzyme database (www. cazy.org; Cantarel et al., 2009), β-mannanases are classified into various families of glycoside hydrolases (GH; Dhawan and Kaur, 2007), i.e. mostly for GH families 5, 26 and a few to family GH 113 (Zhang et al., 2008); whereas b-mannosidases belong to GH families 1, 2 and 5.

β- Mannanase is an endo- acting enzyme that catalyzes the random hydrolysis of the (1,4)-β-D-mannosidic linkages in mannans, galactomannans and glucomannans via a retaining double displacement mechanism as recently reviewed (Gilbert et al., 2013). The enzymes in this group belong to clan GH-A, comprising the (b/a)8 TIM barrel protein fold (Vocadlo & Davies, 2008) and non-catalytic CBMs in certain GH26 mannanases (Zhang et al., 2013). Some β -mannanases from fungi and archaea can perform transglycosylation reactions, in which a carbohydrate hydroxyl group acts as the acceptor molecule instead of water, resulting in the formation of MOS or other mannosides (Dilokpimol et al. , 2011; Park et al. , 2011) . The transglycosylation activity has been found only in GH5 and GH113, but not in GH26 mannanases. Recently, the function of endo-transglycosylase activity of β-mannanases in remodeling of the plant cell wall has been reinterpreted (Schroder et al., 2009). In addition to the catalytic domain, many β-mannanases also contain one or more non-catalytic, CBMs in the family' s CBM1, CBM6, CBM10, CBM31 and CBM35 [reviewed by Boraston et al. (2004)]. These CBM modules have been demonstrated to promote the interaction of the enzyme with the substrate, of which the linker between the catalytic and CBM modules is highly flexible, allowing maximum accessibility to structural and storage substrates (Couturier et al., 2013b).

To date, a number of three-dimensional structures of β-mannanases have been reported. These include GH26 β-mannanases from *Cellulomonas fimi* (PDB# 2BVT; Le Nours et al., 2005); *Cellvibrio japonicus* (PDB#1GVY and 2VX4; Cartmell et al., 2008); *Bacillus subtilis* (PDB# 2WHK and 2QHA; Tailford et al. , 2008; Yan et al.,;

Pseudomonas cellulosa (PDB# 1J9Y; Hogg et al., 2001); *Podospora anserina* (PDB# 3ZIZ; Couturier et al., 2013b) and GH5 β-mannanases from *Trichoderma reesei* (PDB# 1QNO; Sabini et al., 2000), blue mussel *Mytilus edulis* (PDB# 2C0H; Larsson et al., 2006), tomato fruit *Solanum lycopersicum* (PDB# 1RH9; Bourgault et al., 2005) and *Podospora anserine* (PDB# 3ZM8; Couturier et al., 2013b). These structures indicate a typical active site-cleft containing at least four subsites with the strictly conserved catalytic glutamates on β-strands 4 (nucleophile) and 7 (acid/base; Gilbert et al., 2008). The conserved Trp in subsite-1 contributes to the aromatic platform that interacts with the hydrophobic a-face of the substrate sugar ring. Recently, a GH26 β-mannanase (PaMan26A) from *Podospora anserina*, containing CMB35, with an exceptionally strong-4 subsite has been reported (Couturier et al., 2013b). This enzyme unusually releases mannotetraose and mannose from mannopentaose, instead of mannotriose and mannobiose. These results indicated that differences in subsite affinities and the presence of CBM may contribute to the differences in catalytic mode of various enzymes, and that many enzymes can act in synergy to degrade mannan-rich polysaccharides. This knowledge is essential for the selection of enzymes to obtain the desired products or to further engineer the enzymes to suit various purposes.

β-Mannosidase is an exo-acting enzyme that catalyzes the hydrolysis of the glycosidic bond of the terminal, non-reducing β-D-mannose residues in β-D-mannosides (Dhawan and Kaur, 2007). This enzyme is used for downstream hydrolysis of manno-oligosaccharides. β-Mannosidases also belong to clan GH-A. Several threedimensional structures of these enzymes have been reported, indicating diverse structural organization (Dias et al., 2004; Tailford et al., 2007).

Based on the complex structures of various mannans as depicted in Table 1, it can be inferred that other accessory enzymes such as a-galactosidases and acetyl mannan esterases are required to cleave the side chains in order to obtain fermentable sugars as well (Chauhan et al., 2012; Gilbert et al., 2008).

2.3.2 Production of mannan degrading enzyme

In the past decade, an increasing number of articles have been published on the production and characterization of native and recombinant mannanases from various sources, due to their emerging importance in biotechnological applications. Even though mannanases are found in a wide variety of organisms ranging from

bacteria, archaea (Cantarel et al., 2009), thermophiles (Klippel and Antranikian, 2011), fungi (Do et al., 2009; Jagtap et al., 2012; Kote et al., 2009; Liu et al., 2012a; Luo et al., 2009; Saeki et al., 2000), higher plants (Bourgault et al., 2005) and animals (Larsson et al., 2006; Yamaura and Matsumoto, 1993; Yamaura et al., 1996), the main focus of these studies has been on microbial β-mannanases as they can be easily adapted for large- scale production. Most native microbial β- mannanases are extracellular enzymes, and their expression can be induced by various β- mannan- containing substrates such as locust bean gum (LBG), guar gum or copra meal (Kote et al., 2009). However, some β- mannanases have been shown to be intracellular and their expression is constitutive (Dhawan and Kaur, 2007). Despite relatively high yields and interesting properties of several native β- mannanases, the high viscosity of the induction media is troublesome to the biofermentation process, limiting the production of this enzyme on an industrial scale (Do et al., 2009). Therefore, the implementation of genetic engineering techniques for the overexpression of β-mannanases, mainly in *Escherichia coli* (Songsiriritthigul et al., 2010) and Pichia pastoris (Do et al., 2009; Mellitzer et al., 2012) expression systems have been reported. The latter systems, in particular, allow high-level secretion of the recombinant enzymes into the culture media and facilitate the affinity purification of both bacterial and fungal β-mannanases for different applications (table 2). From these tables, it can be concluded that most of the fungal β-mannanases show their optimum activity at a pH in an acidic range, whereas those of bacteria are in the neutral range. In addition, a number of β-mannanases from alkalophilic Bacillus spp. show pH optima between 9 and 10. In general, the optimal temperature of β-mannanase activity varies from 30 to 90 °C, depending on the sources of the enzymes. Both wild-type and recombinant β-mannanases that are active at different pHs and temperatures are appropriate for a wide range of applications. For example, alkalophilic β-mannanases are advantageous for applications in the pulp and paper industry as well as components of detergents (Dhawan & Kaur, 2007), whereas neutral β-mannanases are suitable for bioconversion of b- mannan into MOS (Yamabhai et al., 2011). Highly acidic β- mannanase can be useful for the pretreatment of lignocellulosic biomass for the production of second-generation biofuel (Do et al., 2009).

2.3.3 Biological application of β-mannanase

The application of mannan-degrading enzymes in biotechnology has gained significant interests during the past decade based on an increasing demand for efficient utilization of renewable bio-resources for sustainable development (Do et al., 2009). In addition to cellulases and xylanases, which are the key enzymes for the hydrolysis of the polysaccharide fraction of lignocellulose, β-mannanases are also required for the production of second-generation biofuels (Van Dyk & Pletschke, 2012). For an efficient bioconversion of lignocellulose biomass to fermentable sugars, thermostable enzymes are essential during the pre-treatment step, followed by simultaneous saccharification and fermentation (SSF) processes (Turner et al., 2007; Viikari et al., 2007). Several wild-type and recombinant β-mannanases that are stable and active at high temperature from thermophilic bacterial, eubacterial (Duffaud et al., 1997; Jiang et al., 2006; Luthi et al., 1991; Talbot & Sygusch, 1990), actinomycetes (Hilge et al., 1998) and fungal sources (Do et al., 2009; Kote et al., 2009; Luo et al., 2009; Sachslehner et al., 2000; Turner et al., 2007) have been characterized. CBMs such as CBM1, naturally fused to β-mannanases, may enhance the hydrolytic efficiency on complex lignocellulosic substrates (Ha¨gglund et al., 2003). Moreover, a successful attempt to improve the hydrolytic capacity of β-mannanase by genetically engineering an additional carbohydrate-binding module (CBM1) to the C-terminus of the enzyme (Pham et al., 2010) or by a directed evolutionary approach (Couturier et al., 2013a) have been reported.

In addition to the pre-treatment of lignocellulosic biomass to generate biofuels, another interesting application of β-mannanase is for the random hydrolysis of mannans into MOS (Songsiriritthigul et al., 2010), which have several possible beneficial effects on health and well-being as described in the next section.

Other applications of β-mannanases include biobleaching of pulp and paper (Gubitz et al., 1997), textile and cellulosic fiber processing (Dhawan & Kaur, 2007), processing of instant coffee by reducing the viscosity of coffee extracts (Sachslehner et al., 2000), extraction of oil from palm kernel (Jorgensen et al., 2010), cleaning composition in laundry detergent and other cleansing reagents (Bettiol et al., 2002; Kirk et al., 2002), improvement of animal feed (Jackson et al., 2003; Wu et al., 2005; Zou et al., 2006), facilitating gas and oil drilling (Gu¨bitz et al., 2001), reduction of the

viscosity of thickening agents, clarification of fruits and vegetables and removal and inhibition of biofilm formation (Chauhan et al., 2012; Dhawan and Kaur, 2007; Moreira and Filho, 2008).

2.4 Manno oligosaccharide

Mannooligosaccharides (MOS), including both α-MOS and β-MOS, are a rather new class of oligosaccharides that have gained significant interest as a pre-biotic (Gibson et al., 2004). A prebiotic is a ‘‘selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health’’ (Roberfroid, 2007). According to this definition, a non-digestible oligosaccharide can only be classified as pre-biotic if it fulfills three criteria (1) resistance to the digestion process of the host, including gastric acids, host hydrolytic enzymes and gastrointestinal adsorption; (2) fermentation by intestinal microflora; and (3) stimulation of the growth and/or activity of selected intestinal bacteria that can potentially contribute to health and well-being (Roberfroid, 2007). These intestinal bacteria include specific groups such as *Lactobacillus* spp. *Enterococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp. and *Eubacterium* (Roberfroid et al., 2010). Only a few established pre-biotics have been described according to these above-mentioned criteria, i.e. inulin, fructo-oligosaccharides (FOS) and galacto oligosaccharides (GOS). These oligosaccharides all show pre-biotic effects as deduced from in vivo studies (Roberfroid, 2007). Despite a lack of in vivo evidence, a number of reports suggest pre-biotic effects of both α-MOS and β-MOS, derived from yeast cell wall a-mannan and plant β-mannans, respectively (Charalampopoulos & Rastall, 2012). In addition, accumulating data have suggested that α-MOS can have various other health-promoting effects on both humans and livestock (Heinrichs et al., 2003; Kim et al., 2011; St-Onge et al., 2012; Tester et al., 2012). However, these data are not entirely clear and convincing; therefore, more systematic experimental studies are needed to determine whether α-MOS or β-MOS can be classified unequivocally as prebiotic. Since mannans can be obtained from abundant and inexpensive agricultural wastes, bioconversion of mannan into various types of bioactive MOS and investigation of their actual biological activities are highly attractive areas of research and development.

2.4.1 Preparation of MOS

Mannan polysaccharides can be obtained from two main types of raw materials, i.e. from cell walls of yeast and plants, respectively. Since the cell wall components of plants and yeast are different, it can be expected that the physical and biological properties of their MOS are c6onsiderably different. This review will deal with both types of MOS.

2.4.1.1 α-MOS from *S. cerevisiae* cell walls

To prepare a-mannan, yeast cell walls are first extracted from *S. cerevisiae* by heating in an autoclave, followed by precipitation with Fehling reagent (Kocourek and Ballou, 1969). In more recent reports on the isolation of cell wall polysaccharides, the cell walls were first separated from the internal components by mechanical cell disruption. The purified polysaccharides were then treated with proteases, which caused protein lysis in the outer cell wall and the release of soluble mannan, which could be separated from insoluble glucans (Kath and Kulicke, 1999). Similar methods using a combination of enzyme and mechanical treatment to improve the extraction yield have been reported recently (Bychkov et al., 2010). Yeast cell walls can also be prepared by autolysis after incubation at pH 5.0 at 50 ˚C for 24 h (Ganner et al., 2010). In addition to published protocols, several proprietary methods for the preparation of α-MOS, based on the extraction of α-mannan-rich yeast cell walls obtained from yeast cells by autolysis, heating or hydrolysis with alkali or acid, have been reported (Yu et al., 2011). It is important to note that in the yeast Candida albicans, a third type of β-1,2-linked mannose units have been identified (Shibata et al., 2007). Since the cell wall architecture of yeast is a defined covalent complex of β-glucans, mannoprotein and chitin (Lipke & Ovalle, 1998), the general yeast cell wall preparations are likely to include both α- and β-MOS as well as other yeast cell wall components.

2.4.1.2 β-MOS from plant cell wall

β-Mannans are commonly prepared from various parts of mannan-rich plants by extraction with hot water or alkaline solutions, followed by precipitation with ethanol. Various methods for the extraction of mannan from well-known sources such as carob and locust bean, guar seeds, konjac, etc., can be found in the cyber colloid website (www.cybercolloids.net/). Coffee mannans, which are comprised

mainly of galactomannan and acetylated arabinogalactomannans, can be extracted from roasted coffee beans with hot water (coffee infusions; Nunes and Coimbra, 2001). The amount of extracting galactomannan depends on the origin of coffee and the degree of roasting (Nunes & Coimbra, 2002). Alternatively, mannan can be isolated from green defatted coffee beans by delignification, acid wash and subsequent alkali extraction (Sachslehner et al., 2000). Coconut mannan can be extracted by sequential removal of lipid, carbohydrate, lignin and protein (Saittagaroon et al., 1983).

Biological and physicochemical methods can be used to generate b-MOS from mannans that are extracted from plants. Biological treatments involve enzymatic hydrolysis using a suitable b-mannanase, which randomly cleaves β-1,4-glycosidic linkages in diverse b-mannan substrates. Enzymes that are suitable for bioconversion of mannan into β-MOS, using crude substrates, should display negligible to low activity towards other plant cell wall polysaccharides. Bacillus licheniformis ManB has been shown to be appropriate for the bioconversion of soluble and low-substituted mannan substrates such as konjac glucomannan, locust bean gum galactomannan and β-D-mannan, prepared by controlled hydrolysis of carob galactomannan (Songsiriritthigul et al., 2010). Recently, a physicochemical method for the hydrolysis of mannan to produce β-MOS of various sizes, ranging from mannobiose (M2) to mannoheptaose (M7) has been used (Otieno and Ahring, 2012b). However, β-MOS were only found in small amounts when compared with xylo-oligosaccharides (XOS), which were the main products of this process. Since both enzymatic and non-enzymatic processes have advantages and disadvantages (Otieno and Ahring, 2012a), depending on the raw material, combining both physiochemical and enzymatic methods could be an interesting approach for the preparation of β-MOS from different sources. More research and development of effective methods for the production of MOS from various mannan-rich bioresources are required.

2.4.2 Potential application of action of MOS

Several mechanisms of action have been proposed for the health-promoting effects of α- and β-MOS. The most well known is the potential pre-biotic effect since MOS are nondigestible oligosaccharides. The microbiota or a collection of microbial cells and viruses that reside inside and ouside the host body has been shown to play a fundamental role in health and disease in their hosts (O'Hara and Shanahan,

2006). The host microbiota, especially in the gut, is essential in stimulating the maturation of immune cells as well as promoting the normal development of immune functions (Clemente et al., 2012). The second mechanism of health-promoting effects of α- and β-MOS is related to competitive exclusion of pathogenic bacteria (Callaway et al., 2008; Fernandez et al., 2000; Gaggia et al., 2010). The protection mechanism was thought to be the result of the ability of MOS to bind to mannose-specific lectins of Gram-negative pathogens that express Type-1 fimbriae such as Salmonella and E. coli, resulting in their excretion from the intestine (Baurhoo et al., 2007).

2.5 Conclusion and future perspective

Mannans are components of lignocellulosic biomass, an abundant renewable resource for the productions of biofuel and high value biomaterials. Pre-treatment of lignocellulosic materials to remove lignin and hemicellulose has been shown to improve the hydrolysis of cellulose and other polysaccharides to produce mixed reducing sugars for fermentation into bioethanol (Sun and Cheng, 2002). β-Mannanases are one of the important polysaccharide hydrolases that act on galactoglucomannans and other mannan-rich hemicelluloses during the pre-treatment process (Gilbert et al., 2008). Despite some progress in cloning and characterization of various microbial β-mannanases that show attractive properties for application in bio-refinery, more understanding of the structure–function relationship and substrate recognition through the analysis of three-dimensional structures and mutational analysis remains to be achieved.

In addition to pre-treatment of lignocellulosic biomass, biotechnological applications of β-mannanases in the food, feed and detergent industries are well established by now. Nevertheless, the costs of these enzymes are still too high and, therefore, more research on the cost-effective production of highly efficient enzymes suitable for diverse applications is still in demand.

CHAPTER 3
PRODUCTION OF A BETA MANNANASE USING A *LACTOBACILLUS PLANTARUM* EXPRESSION SYSTEM

3.1 Abstract

Heterologous production of hydrolytic enzymes is important for green and white biotechnology since these enzymes serve as efficient biocatalysts for the conversion of a wide variety of raw materials into value-added products. Lactic acid bacteria are interesting cell factories for the expression of hydrolytic enzymes as many of them are generally recognized as safe and require only a simple cultivation process. We are studying a potentially food-grade expression system for secretion of hydrolytic enzymes into the culture medium, since this enables easy harvesting and purification, while allowing direct use of the enzymes in food applications. We studied overexpression of a β-mannanase (ManB), from *Bacillus licheniformis*, in *Lactobacillus plantarum*, using the pSIP system for inducible expression. The enzymes were over-expressed in three forms: without a signal peptide, with their natural signal peptide and with the well-known OmpA signal peptide from *Escherichia coli.* The total production levels and secretion efficiencies of ManB were highest when using the native signal peptides, and both were reduced considerably when using the OmpA signal. At 20 h after induction with 12.5 ng/mL of inducing peptide in MRS media containing 20 g/L glucose, the yields and secretion efficiencies of the proteins with their native signal peptides were 50 kU/L and 84 % In addition, to avoid using antibiotics, the erythromycin resistance gene was replaced on the expression plasmid with the alanine racemase (*alr*) gene, which led to comparable levels of protein production and secretion efficiency in a suitable, *alr*-deficient *L. plantarum*host.ManB were efficiently produced and secreted in *L. plantarum* using pSIP-based expression vectors containing either an erythromycin resistance or the *alr* gene as selection marker.

3.2 Introduction

Heterologous production of hydrolytic enzymes is important for green and white biotechnology since such enzymes serve as green industrial biocatalysts for the conversion of biomass into value-added products (Choi et al., 2015) . Lactic acid bacteria (LAB) are interesting hosts for the production of such enzymes because many of these bacteria are generally recognized as safe (Rungrassamee et al.), carry the qualified presumption of safety (QPS) status, and are easy to cultivate (Feord, 2002). While LAB may not be the most efficient cell factories, their safety and food-grade status make them particularly attractive for producing enzymes that are to be used in e.g. food processing. One attractive host is *Lactobacillus plantarum*, as it had been widely used for foods, and hence is food-grade and even considered a probiotic (Diep et al., 2009) with potential benefits to human health (Vijaya et al., 2005). To facilitate downstream processing in large-scale biotechnological applications, secretion of the overexpressed enzymes into the culture medium is desirable (Karlskas et al., 2014). Therefore, lactobacillal expression systems based on the so-called pSIP vectors (Sørvig et al., 2003; Sorvig et al., 2005) have been developed recently for the efficient secretion of heterologous proteins in *L. plantarum* (Mathiesen et al., 2008).

3.3 Materials and methods

3.3.1 Bacterial strains and plasmids

Table 3.1 Bacterial strains.

Strain	Relevant characteristics/Genotypes	Source of reference
Escherichia coli DH5αF'	Cloning host/ *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
Escherichia coli MB2159	Cloning host/ D-alanine auxotroph	(Strych et al., 2001)
Escherichia coli TOP10	Cloning host/ F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen
Lactobacillus plantarum WCFS1	Expression host/ wild type	(Kleerebezem et al., 2003)
Lactobacillus plantarum TLG02	Expression host/ Δ*alr* (D -alanine auxotroph)	(Nguyen et al., 2011)

Table 3.2 Plasmids used in this study.

Plasmid	Description	Source of reference
pSIP409gusA	*erm*, pSIP401 derivative, *gusA* controlled by P*sppQ*	(Sorvig et al., 2005)
pSIP609gusA	pSIP409 derivative, *alr* replaced with *erm*	(Nguyen et al., 2011)
manBOmpA-pMY202	*manB_ompA* controlled by tac	(Songsiriritthigul et al., 2010)
pSIP409manB_nt	*erm*, pSIP409 derivative, *manB_nt* controlled by P*sppQ*	This work
pSIP409manB_OmpA	*erm*, pSIP409 derivative, *manB_OmpA* controlled by P*sppQ*	This work
pSIP409manB_Mature	*erm*, pSIP409 derivative, *manB* controlled by P*sppQ*	This work
pSIP609manB_nt	pSIP409 derivative, *manB_nt* controlled by P*sppQ* ,*alr* replaced with *erm*	This work

Table 3.3 Oligonucleotide primers used in this study

Name	Sequence (5'-3')	Target gene
FlagNcoIfwlong	CATGAAAAAGACAGCTATCGCGATTG	ManB_OmpA
FlagNcoIfwshort	AAAAAGACAGCTATCGCGATTG	
6HisXhoIrvlong	TCGAGTCAATGGTGATGGTGATGGTG	
6HisXhoIrvshort	GTCAATGGTGATGGTGATGGTG	
B. liManBfwNcoIlong	CTAGAAAAAAAACATCGTTTGTTCAATCT	*manB*_nt
B. liManBfwNcoIshort	AAAAAAAACATCGTTTGTTCAATCTTCG	
B. liManB6HisXhoIlong	TCGAGTCAATGGTGATGGTGTTCCACGA CAGGCGTCA	
B. liManB6HisXhoIshort	GTCAATGGTGATGGTGTTCCACGACAGG CGTCA	
B. LimanBMatfwNcoIlong	CATGGCACACACCGTTTCTCCGGTG	Mature *manB*
B. LimanBMatfwNcoIshort	GCACACACCGTTTCTCCGGTG	
B. liManB6HisXhoIlong	TCGAGTCAATGGTGATGGTGTTCCACGA	
B. liManB6HisXhoIshort	CAGGCGTCA	
	GTCAATGGTGATGGTGTTCCACGACAGG	
	CGTCA	

3.3.1.1 Culture of Bacterial strains

Escherichia coli Top10 and *E. coli* DH5α were used as cloning hosts. They were grown in Luria- Bertani (LB) medium either on solid agar plates or in liquid medium, and incubated for overnight at 37°C cabinet (for plates) or in a shaker incubator (for liquid cultures). The 800 μg/mL of erythromycin concentration was added to the medium when growing *E. coli* Top10 strains containing the plasmids.

E. coli MB2159 (D-alanine auxotroph) was used as cloning host for food-grade expression vectors. The bacterium was grown in LB medium supplemented with 200 μg/mL of D-alanine and incubated for overnight at 37°C cabinet (for plates) or in a shaker incubator (for liquid cultures).

Lactobacillus plantarum WCFS1 (wild type) was grown either in MRS medium (Man-Rogosa-Sharpe) or on solid MRS-agar plates. *L. plantarum* cultures were

incubated under facultative aerobic condition at 37 °C without agitation for 18-24 h. *L. plantarum* WCFS1 cells containing plasmids harboring an antibiotic resistance gene were grown in agar plates and liquid medium containing 5 μg/mL of erythromycin concentration.

L. plantarum TLG02 (Δ*alr*, D-alanine auxotroph) as used as expression host for food grade expression vectors. The cells were grown in MRS medium supple-mented with 200 μg/mL D-alanine and incubated for overnight at 37ºC cabinet (for plates) or in a shaker incubator (for liquid cultures).

3.3.1.2 Molecular cloning of Bacillus licheniformis β-mannanase gene

The plasmid manBOmpApMY202 containing the mannanase (*manB*) gene with OmpA signal peptide (Songsiriritthigul et al., 2010) was used as templates for amplification of *manB* gene containing OmpA signal peptide. Furthermore, the primer of the gene of the native mannan endo-1,4-β-mannosidase was designed using the published sequence from the genomic database of *B. licheniformis* DSM13 (NCBI accession number NC006322.1) for amplification of *manB* gene containing native signal peptide. The oligonucleotides used for *manB* gene amplification were designed for cloning by a sticky PCR-based method (Yamabhai, 2009). The two forward primers (FlagNcoIfwlong and FlagNcoIfwshort) and two reverse primers (6HisXhoIrvlong and 6HisXhoIrvshort) were used to amplify the *manB* gene containing the *E. coli* outer membrane protein (OmpA) signal peptide. Two forward primers (B. liManBfwNcoIlong and B liManBfwNcoIshort) and two reverse primers (B. liManB6HisXhoIlong and B. liManB6HisXhoIshort) were used to amplified *manB* gene containing *B. licheniformis manB* native signal peptide. Two forward primers (B. LimanBMatfwNcoIlong and B. LimanBMatfwNcoIshort) and two reverse primers (B. liManB6HisXhoIlong and B. liManB6HisXhoIshort) were used to amplify the mature *manB* gene. The lists of primers in this study are shown in Table 3.3 and were compatible with the *NcoI* and *XhoI* restriction sites. The vector pSIP409gusA, which is used for gene expression in *L. plantarum* WCFS1, as double digested with *NcoI* and *XhoI* restriction enzymes for cloning.

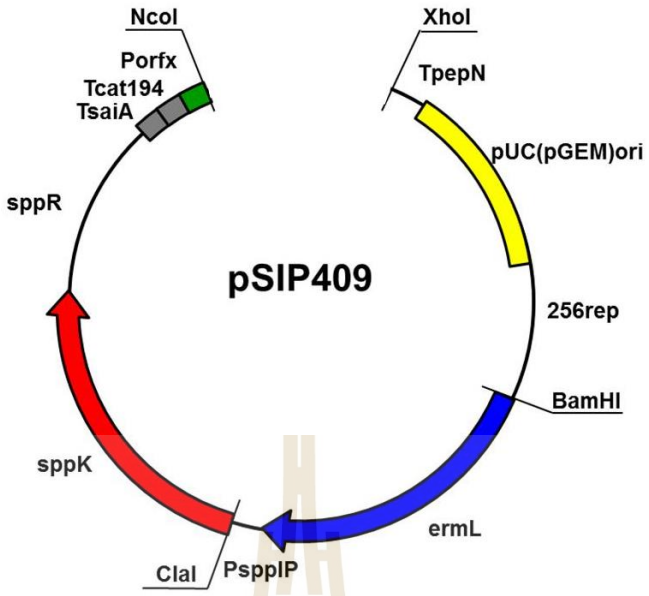


Figure 3.1 Map of vector pSIP.

3.3.2 Expression of β-mannanase

Batch fermentations with pH control were carried out in 3-L MRS medium using a BIOSTAT B plus bioreactor (Sartorius, Germany). Recombinant *L. plantarum* strains were taken from a glycerol stock stored at −80 °C, re-streaked on appropriate MRS plates (with or without antibiotic, depending on the *L. plantarum* strain; see above) and grown overnight at 37 °C. Five to ten colonies were picked and grown in 5 mL MRS broth overnight, then sub-cultured into two flasks of 100 mL of MRS, and cultivated at 37 °C without shaking for 18–24 h. The two overnight cultures were pooled together, mixed well and after measuring the cell density at 600 nm (Ultrospec 2000, Pharmacia biotech, UK) they were used to inoculate 3 L of MRS medium to an OD600 of ~0.1. After incubation at 30 °C with 100 rpm agitation under anaerobic condition to an OD600 of ~0.3, the cultures were induced with 12.5 ng/mL of IP-673 (amino acid sequence of IP-673 is Met-Ala-Gly-Asn-Ser– Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg; [34]). During further cultivation (30 °C with 100 rpm), the pH was controlled at pH 6.5 using 3.0 M sodium hydroxide. To monitor enzyme production, 40–50 mL of culture broth were harvested at 0, 3, 6, 9, 12, 18, 20, and 24 h after induction. The cells and culture supernatant were separated by centrifugation at 4000 rpm for 15 min at 4 °C (swing angle rotor, Centrifuge 5804, Eppendorf, Belgium), after which the cells were washed twice with lysis buffer (20 mM

Tris– HCl, 150 mM NaCl, pH 8.0), and re-suspended in 3–4 mL of the same buffer. The cells were broken using a sonicator (Vibra-Cell Sonicator, Sonics & Materials, Inc, USA) at 25 % amplitude, pulse 5 s, 3 min for 2 rounds on ice. The cell lysate fraction was collected by centrifugation at 13,000 rpm, 4 °C for 45 min (Thermo Scientific, USA). To measure the enzyme activity in culture supernatants, 3–5 mL of culture supernatant containing secreted enzymes were dialyzed with 10 mM Tris–HCl buffer, pH 8.0 with stirring at 250 rpm, at 4 °C for 8–12 h, using the snake skin dialysis tubing, 10 kDa kit (Thermo scientific, USA). The dialyzed fraction of approx. 4–7 mL was collected and kept on ice for no longer than 6 h before the enzyme activity was determined.

3.3.3 Enzymatic activity assay

An appropriately diluted enzyme solution (0.1 mL) was incubated with 0.9 mL of pre-heated 0.5 % (w/v) locust bean gum (dissolved in 50 mM sodium citrate buffer, pH 6.0) at 50 °C for exactly 5 min, with mixing at 800 rpm. The amount of reducing sugars liberated in the enzyme reaction was assayed by mixing 100 µL of the reaction mixture with 100 µL DNS solution, followed by heating at 100 °C for 20 min, cooling on ice, and dilution with 300 µL of de-ionized water, before measuring the absorbance at 540 nm, using 1–5 µmol/mL of d-mannose as standards. The reactions were done in triplicate and we report mean values together with their standard deviation. The substrate solution was prepared by suspending 0.5 % (w/v) locust bean in 50 mM sodium citrate buffer, pH 6.0. The suspension was then dissolved at 80 °C, using hot plate stirrer at 200 rpm. (RCT CL, IKA Laboratory, Germany), followed by heating to the boiling point, cooled and stored overnight with continuous stirring. After that insoluble material was removed by centrifugation.

3.3.4 Gel electrohoresis

Denaturing sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli [35] with 12 % (w/v) polyacryamide gels. The protein samples were briefly heated (3 min) in the loading buffer at 100 °C using a heat block (Eppendorf), and then cooled on ice before loading. Protein bands were visualized by staining with Coomassie brilliant blue R-250

3.3.5 Protein determination

Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

3.3.6 N-terminal protein sequencing

Proteins in culture supernatants were separated by SDS- PAGE and electroblotted onto a PVDF membrane (Bio-Rad) in 50 mM borate buffer containing 10 % (v/v) methanol, pH 9. After blotting, the membrane was stained with Coomassie blue for 3 min, followed by destaining with 40 % (v/v) methanol, 10 % (v/v) acetic acid. Bands were cut out of the membrane and analyzed by a commercial provider using Edman degradation on an Applied Biosystems Procise 492 protein sequencer (Protein Micro-Analysis Facility, Medical University of Innsbruck, Austria).

3.4 Results

3.4.1 Construction of expression vector

Genes encoding a mannan endo-1,4-β-mannosidase or 1,4-β-D-mannanase (EC 3.2.1.78), commonly named β-mannanase (ManB), from *B. licheniformis* strain DSM13 [11] was initially cloned into pSIP409 [7]. Subsequently, the erythromycin resistance gene (ermR) in the pSIP409-based constructs was replaced with the alanine racemase gene (alr) [13, 14] to generate food-grade pSIP609 expression vectors, as shown in Fig. 3.1. Each gene was cloned in three forms: with no signal peptide (BlManB_noSP), with their native signal peptides (BlManB_nt) or with the *E. coli* OmpA signal peptide (BlManB_OmpA). The ability of the signal peptides to direct the secretion of these enzymes in *E. coli* has previously been reported [12, 15].

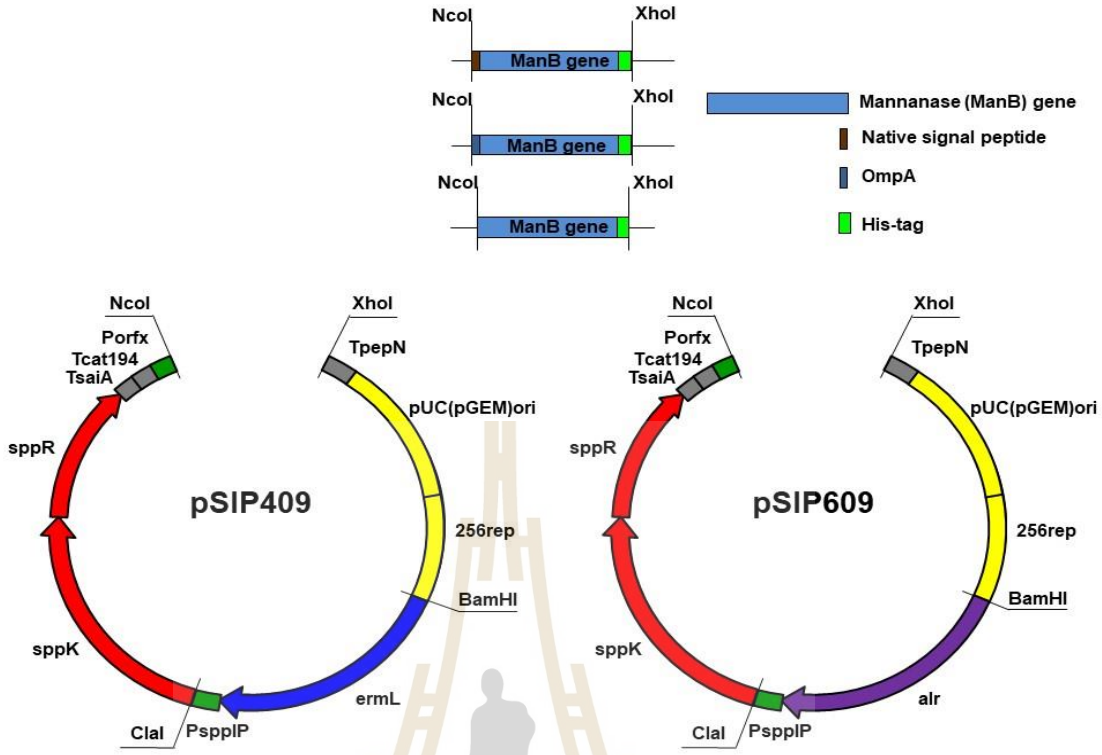


Figure 3.2 Vector construction. Both pSIP409 and pSIP609 vectors were used for expression of *B. licheniformis* β-mannanase (*Bl*ManB). Enzyme expression was under the control of the Porfx promoter (also known as PsppQ) [32], which can be induced by the 19-residue peptide pheromone IP-673. The vectors contain an erythromycin resistance (ermR) or an alanine racemase (alr) gene as selection marker, for pSIP409 and pSIP609, respectively. Polyhistidine tags were incorporated C-terminally to facilitate one-step affinity purification. The 256rep replicon allows DNA replication in *L. plantarum*. Each enzyme was cloned in three forms, two of which contain a signal peptide for secretion (native or OmpA). The genes marked in red constitute the two-component system needed for peptide-pheromone driven induction; the grey areas marked with a T are terminator sequences.

3.4.2 Expression and secretion of β-mannanase in *L. plantarum*

To demonstrate the applicability of the secretory production of recombinant ManB in the food biotechnology industry, the antibiotic selection marker in the pSIP409BlManB expression vectors was replaced with the alanine racemase (alr) gene [13, 18]. Based on the results of the experiments described above, only

constructs with native signal peptides were used. The resulting expression plasmids, pSIP609BlManB (Fig. 3.1) were transformed into *L. plantarum* strain TLG02, which is an d-alanine auxotroph [14]. The cultivation conditions were similar to those used for strains harboring pSIP409- derived vectors, except that no antibiotic was added in the culture media. Fig. 3.2 shows a comparison of the volumetric activities of BlManB using either ermR or alr as selection marker, at various time points after induction. For both enzymes, production levels were higher for the constructs with the ermR selection marker, and this was almost exclusively due to higher levels of secreted enzymes. The level of intracellular enzyme activities was hardly affected by the change in the resistance marker, and, consequently, the calculated secretion efficiencies were lower when using the alr-based vectors. A summary of total volumetric activity and secretion efficiency obtained using alr selection is provided in Table 3.2. The expression and secretion of recombinant BlManB with the food-grade L. plantarum expression system could also be detected by SDS-PAGE analysis of culture supernatants, showing strong enzyme bands.

Table 3.4 Yields and secretion efficiency of various constructs of recombinant *Bacillus* manB expressed from *L. plantarum* WCFS1, using sakacin P- based expression vectors, pSIP409.

Enzyme	Type of SP	Volumetric Activity (U/L)			% Secretion efficiency
		Broth	Cell extract	Total	
ManB	Native SP	41,600 ± 1294	8,120 ± 160	49,720 ± 1454	83.7
	OmpA SP	16,450 ± 555	6,540 ± 306	22,990 ± 861	71.6
	Mature enzyme	4,690 ± 230	20,000 ± 930	24,690 ± 1160	19.0

Table 3.5 Comparison of productivity of ManB constructs containing different signal peptide.

Condition : 3L-fermenter, 20g/L glucose, 12.5 ng/ml IP-673, 30 °C, 100 rpm, pH 6.5, 20 hr.

Type of SP	Fraction	Type of protein	Volume (ml)	Protein conc. (mg/ml)	Total Protein (mg/L)	Activity (U/mL)	Total activity (U/L)	Specific activity (U/mg)	Purity (Capek et al.)	%Yield
native SP	Supernatant	Crude	1000	0.30	300	41.6	41600	139	1	100
		Pure	6.2	1.48	9.2	2860	17730	1927	13.3	42.6
	Cell extract	Crude	60	2.89	173	136	8120	174.0	1	100
		Pure	2.4	1.24	2.9	1901	4562	1735	9.8	56.1
OmpA SP	Supernatant	Crude	1000	0.22	220	18.5	19500	183.9	1	100
		Pure	4.6	1.48	6.6	1471	11371	1723	9.4	58.3
	Cell extract	Crude	60	2.83	169	109	8540	138	1	100
		Pure	2.3	1.18	2.7	2077	4779	1770	12.8	55.8
Without SP	Supernatant	Crude	-	-	-	4.69	4690	-	-	-
		Pure	-	-	-	-	-	-	-	-
	Cell extract	Crude	125	2.19	273	201	25125	92.0	1	100
		Pure	4.7	0.15	0.7	262	1235	1765	19.1	20.3

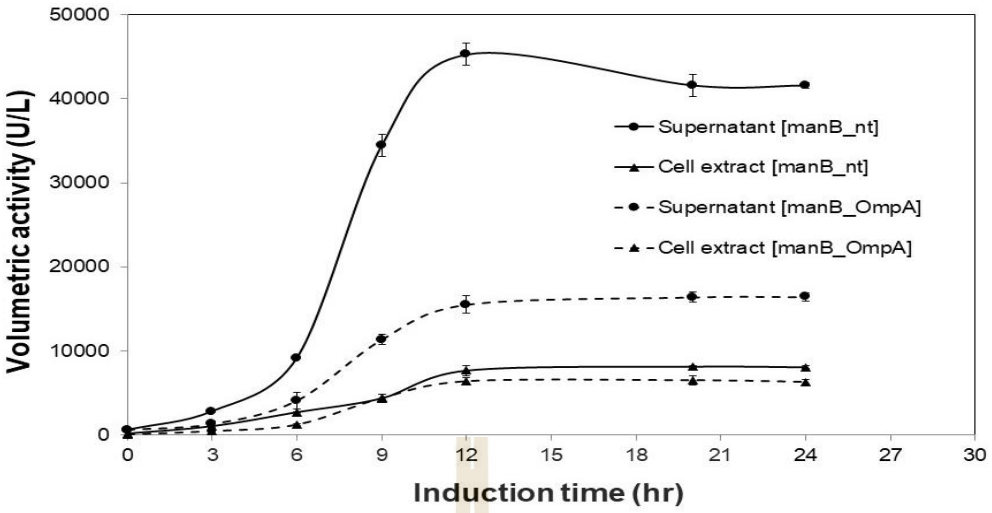


Figure 3.3 Production and secretion of *B. licheniformis* β-mannanase (*Bl*ManB,) using pSIP409-type constructs containing the native or the OmpA signal peptide. The recombinant *L. plantarum* strains were batch-cultured in 3-L vessels with pH control at pH 6.5. After harvesting at various time points, volumetric enzyme activities in both the culture supernatant and the cell lysate were determined as described in the "Methods" section. Data given are the average of two independent experiments ± their standard deviation. The lines connecting the points are drawn for illustration purposes only. Key data, supplemented with data for the constructs without signal peptide are summarized in Table 3.5.

3.4.3 Purification and analysis of secreted β-mannanase

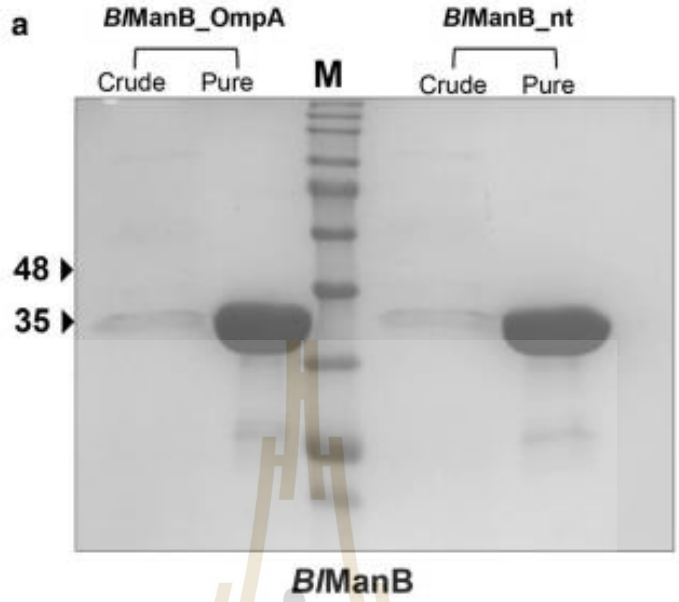


Figure 3.4 SDS-PAGE analysis of culture supernatants and purified *Bl*ManB. The coomassie-stained gels illustrate the purification of B. licheniformis ManB (and from culture supernatants. For crude culture supernatants, 20 µL of sample was loaded, whereas for the purified enzymes a total of 20 and 5 µg protein of ManB were loaded. M indicates the Kaleidoscope protein standard (Bio-Rad). Detailed information on the protein contents and enzyme activities in these samples are provided in Table 3.5.

3.4.4 N-Terminal sequencing

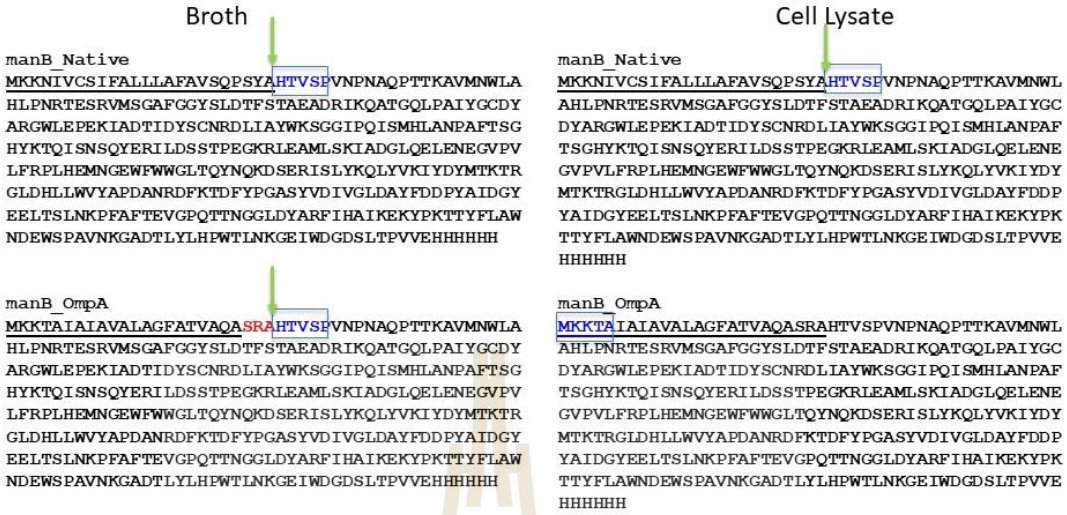


Figure 3.5 Signal peptide cleavage sites. N-terminal sequence analysis of purified secreted proteins was performed by Edman degradation. The sequences of the native Bacillus signal peptides and the *E. coli* OmpA signal peptide are underlined. Arrows indicate the cleavage sites as deduced from sequence analysis. The first five amino acids obtained by Edman degradation are colored and boxed. Amino acids in red indicate extra amino acids that were introduced during genetic engineering of the fusions between the Bacillus enzymes and the *E. coli* OmpA signal peptide.

3.5 Discussions

This is the first report on the secretory productions of two bacillus hydrolytic enzymes, namely β-mannanase (ManB) from *L. plantarum* expression system. This enzymes could be expressed and secreted at high level when compared to the level obtained from using *E. coli* expression systems. The enzymes could be efficiently expressed using pSIP-based expression vector containing either antibiotic resistant gene as well as alanine racemase gene, for food-grade applications. The success of using pSIP vector for both intracellular and secretion has been previously reported for model enzymes namely nuclease (NucA) and amylase (AmyA) as well as other enzymes such as cellulose (CelB), oxalate decarboxylase, xylanase and β-galactosidase. While most of the expressions were from antibiotic marker, only the expression of intracellular β-galactosidase using food-grade alr marker has been

reported. These results confirm the advantage of pSIP vector for *L. plantarum* expression and the benefits of alr selection marker for food-grade application. Since pSIP vector is a modular plasmid, it could be modified easily to suit desired purposes. For example the replicon could be exchanged to allow broader host range for specific neutraceutical property, such as probiotic activity or ability to produce interested acid or antimicrobial compounds. Nevertheless, since alr is a selection marker that this based on complementation, which has an advantage of being antibiotic-free and the low cost of media used, the drawback of this selection marker is the need of specific host strain that must be alanine auxotroph. These limitations must be taken into accounts for applications in other enzymes and applications.

The signal peptides that were used successfully to direct the secretion the two bacillus hydrolytic enzymes were their native bacillus signal peptides. These signal peptides were found to be equally or more efficient than previous reports on the functionality of signal peptides for the secretion of heterologous proteins in *L. plantarum*. Since Bacillus is also a Gram (+ve) bacterium, it is not surprising that their signal peptides could be efficiently used to direct the secretion of heterologous protein in *L. plantarum*, which is also a Gram (+ve) bacteria. The fact that ManB signal peptide, which was only 24 amino acid long, could direct the secretion of ManB at 80% secretion efficiently made it highly attractive to direct the secretion of other heterologous protein in *L. plantarum* system. Nevertheless, the success in secretory production of ManB and was not only the result of the choice of signal peptide used but also the nature of the secretion target, which is naturally extracelluar enzymes, which are prone to get export out of the cells. These observations have been reported in both Gram-positive and Gram-negative bacteria. The interesting information that came from this study was the fact that the OmpA signal peptide of *E. coli*, which is a Gram-negative bacterium, could be efficiently used to direct the secretion of heterologous enzyme in Gram-positive *L. plantarum* as well, despite the fact that the structure of the cell walls are greatly different. This opposite situation has also been observed as well, when Gram (+ve) signal peptide could lead to the expression of herologous proteins in Gram (-ve) E. coli. Therefore, these data confirmed previous studies indicating that the Sec-dependent secretion machineries in Gram (-ve) and (+ve) are highly similar.

In addition to secretion, this study indicated that the signal peptide could greatly affect the expression level of heterologous enzyme to more than two-folds. The high protein expression level could then determine the secretion efficiency as it is likely that if the protein is express in a very high amount, it could saturate the secretion machinery on the bacterial cell wall. The high-level expression of the recombinant ManB as well as the specific enzyme activities are equivalent to those obtained from *E. coli* expression system. When the selection marker was changed from ermR to alr, the expression level was slightly lower when using the same condition as that was obtimized for ermR. However, after further optimization by adjusting glucose concentration, inducing peptides, cultivation temperature, etc., it is likely that the yield of the recombinant enzymes could be improved for fed-batch large-scale cultivation. However, for real food-grade use, it might be more beneficial to replace the inducible promoter with constitutive or cheap-inducer promoter for cost-effective food industry application in the future.

3.6 Conclusions

An efficient expression system for food-grade production of β-mannanase has been established. The effective used of signal peptides, modular pSIP vector and alr selection markers from this system could be further applied for the expression of other heterologous proteins in *L. plantarum* in the future.

CHAPTER 4
PRODUCTION OF MANNO OLIGOSACCHARIDE (MOS) FROM COPRA MEAL BY BETA MANNANASE AND ITS BIOLOGICAL ACTIVITY

4.1 Abstract

Coconut is known for its great versatility as seen in the many uses of its different parts and it is found throughout the tropical area including Thailand. Coconut is part of the daily diet of many people and is grown industrially for the edible. Copra meal is a by-product from coconut oil extract process, and Thailand produced copra meal feed wastes around 20,000 Metric Tons annually during 2009-2018. Copra meal is typically rich in galactomannans and linear mannans, which can be used as a substrate for the production of manno-oligosaccharides (MOS) by enzymatic hydrolysis using 1,4-β-D-mannanase. In this research, we reported the optimized conditions for the hydrolysis of defatted copra meal using crude recombinant 1,4-β-D-mannanase from *Bacillus licheniformis* expressed in *Lactobacillus plantarum*. The bioconversion reactions containing defatted copra meal equivalent to 1, 2, and 4 % of total mannans were incubated with 1, 5 and 10 U/ml of enzyme at 50 °C. The products were analyzed by thin layer chromatography (TLC) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Our results indicated that the reaction containing defatted copra meal equivalent to 2% total mannan and 5 U/ml of 1,4-β-D-mannanase is the best condition for the production of MOS with approximately 10% conversion yield. The *in vitro* growth promoting effects of β-MOS, in comparison to other oligosaccharides, on various probiotic bacteria were investigated under anaerobic condition. The results showed that copra MOS could enhanced the growth of these bacteria more than those of commercial inulin and fructo-oligosaccharides (FOS). *In vitro* toxicity assay of β-MOS on human dermal fibroblast showed no cytotoxic effect. Interestingly, β-MOS at appropriate concentration also demonstrated anti-inflammatory activity against LPS-induced

inflammation of human macrophage THP-1. These results indicated that β-MOS has potential to be used as valued-added health promoting neutraceuticals or feed additives after careful studies.

4.2 Introduction

4.2.1 Coconut and copra meal

The coconut is known for its great versatility as seen in the many uses of its different parts and found throughout the tropical area including Thailand (Hossain et al., 1996). Coconuts are part of the daily diets of many people and are grown industrially for the edible. The highly saturated oil contained in the flesh of its fruits (Canapi et al., 2005).

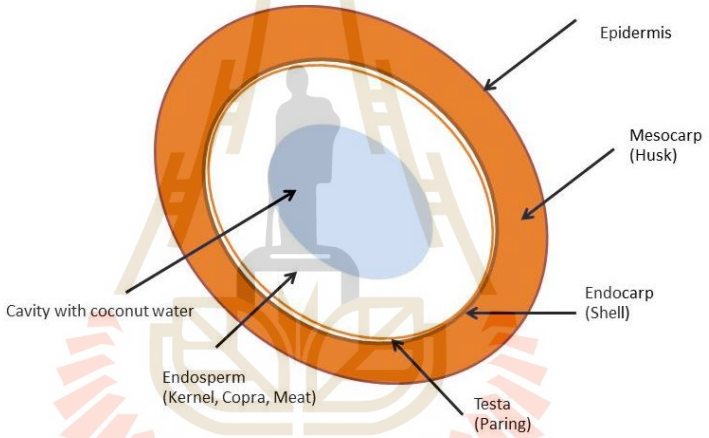


Figure 4.1 Structure of coconut.

It has a smooth epidermis cover the fibrous mesocarp (husk) which covers the hard endocarp (shell). A thin brown layer (testa) separates the hardest part (shell) from the endosperm (kernel). A cavity contains the coconut water (Heuzé et al., 2005).

Since 20017-2018, Thailand produced the copra meal feed wastes around 17,000 metric ton per year. The by-products were created from coconut oil extract process. For coconut oil extraction, the coconut was dehusked, peeled the testa out, and divided in a half. The white copra meal was ground, flaked and centrifuged called “cold process extraction”. The main by-product from this process is copra meal.

Table 4.1 Thailand copra meal feed waste domestic consumption annual growth rate.

Market Year	Production	Unit of Measure	Growth Rate
2000	25	(1000) MT	-19.35%
2001	24	(1000) MT	-4.00%
2002	24	(1000) MT	0.00%
2003	25	(1000) MT	4.17%
2004	25	(1000) MT	0.00%
2005	26	(1000) MT	4.00%
2006	25	(1000) MT	-3.85%
2007	25	(1000) MT	0.00%
2008	25	(1000) MT	0.00%
2009	25	(1000) MT	0.00%
2010	25	(1000) MT	0.00%
2011	25	(1000) MT	0.00%
2012	25	(1000) MT	0.00%
2013	25	(1000) MT	0.00%
2014	25	(1000) MT	0.00%
2015	20	(1000) MT	-20.00%
2016	13	(1000) MT	-35.00%
2017	17	(1000) MT	30.77%
2018	17	(1000) MT	0.00%

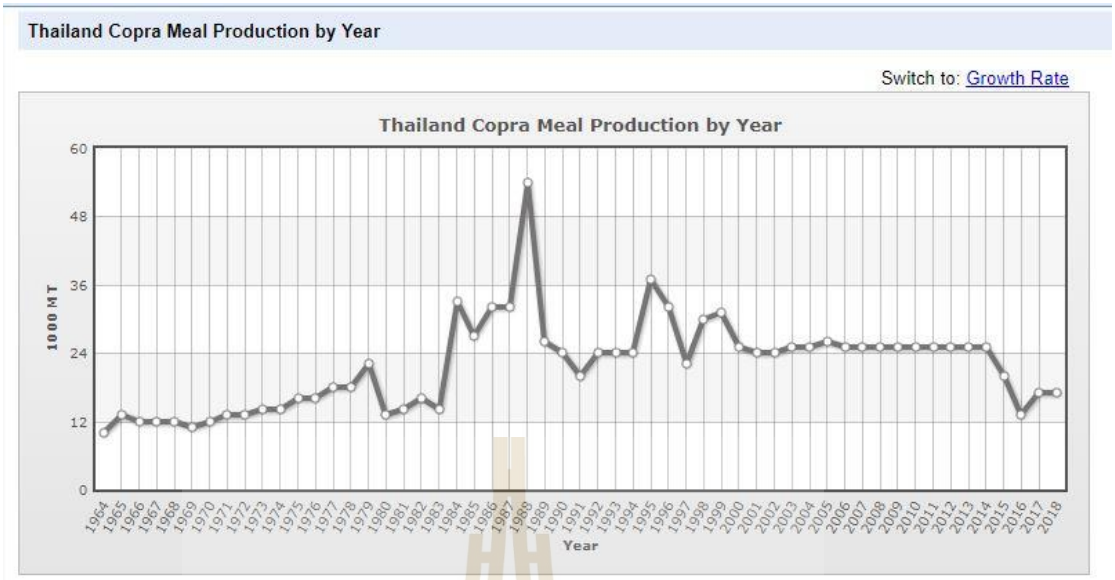


Figure 4.2 Thailand copra meal feed waste domestic consumption annual growth rate.
Source: United State Department of Agriculture

Copra meal is the by product from the coconut oil extraction. It contains 61% of galactomannan and 26% of linear mannan (Balasubramaniam, 1976; Saittagaroon et al., 1983). The abundance of galacto mannan and linear mannan are the good source for production of manno-oligosaccharides.

4.2.2 MOS and its applications

A study on the application of MOS as part of nutraceuticals or functional food is a highly attractive area of research. While the utilization of β-MOS from yeast cell walls for health benefits to animals has been fully commercialized, the production of β-MOS from mannan-rich plants is still at the beginning. Various reports on health-promoting effects of α-MOS in animals indicated that they could have benefits for humans as well. Nevertheless, more systematic research on the mechanisms of these beneficial effects as well as their safety for human uses remains to be carried out. Since plants are rich and abundant sources of β-mannans of various structures, β-MOS that are prepared from different species of plants can be expected to have different biological activities. So far, there have been only a few studies on the production β-MOS from different plants, not to mention investigations on their various biological activities. Therefore, a vast area of research on plant β-MOS remains to be explored, ranging from extraction, structure elucidation, bioconversion and systematic analysis of their various biological activities.

Another proposed mechanism of action is only specific to α-MOS from *S. cerevisiae* cell walls, which showed the direct interaction with the host immune system (Che et al., 2012). However, this association is not easy to confirm, because it could also be the result of an indirect pre-biotic effect via the alteration of the host microbiota. Recent work in mice suggested that the terminal high-mannose oligosaccharides in secretory IgA (sIgA) are the key responsible agent for innate immune defense against pathogenic bacteria. The addition of free mannose across a wide dose range could significantly inhibit biofilm formation by Vibrio cholerae (Murthy et al., 2011). Therefore, α-MOS may prevent the invasion of pathogens in a similar manner. The role of MOS on the host immune system is likely to be valid only for *S. Cerevisiae* mannan because the major bonds in high-mannose proteoglycans are α-linked. Nevertheless, this remains to be established. An experiment that directly compares the function of α- and β- MOS on the host immune system will be useful to clarify this issue.

Most of the scientific literature on the biological activities of MOS reports the effects of a-MOS from yeast cell walls, especially *S. cerevisiae*, as feed additive. These results propose certain beneficial effects of yeast α-MOS on a wide variety of domestic and farm animals, including dogs (Middelbos et al., 2007), broilers (Iji et al., 2001; Kim et al., 2011), male turkeys (Parks et al., 2001), pigs (Rosen, 2006), calves (Franklin et al., 2005; Heinrichs et al., 2003), lobsters (Sang and Fotedar, 2010) and sea bass (Torrecillas et al., 2007, 2011, 2012). Meta-analysis of the worldwide literature on the studies of the effects of *S. cerevisiae* cell wall mannan on the performance of broilers has suggested beneficial responses in terms of feed intake, live-weight gain, feed conversion ratio and mortality response (Rosen, 2007). These results came from studies of commercially available MOS. In contrast, the effect of feeding diets supplemented with α-MOS at 0.1–0.2% did not have significant effects on broiler chickens (Corrigan et al., 2011) or turkeys (Corrigan et al., 2012), except that the composition of the bacterial community in the bird cecal contents changed significantly. More research is needed, therefore, to confirm the beneficial effects of α-MOS from yeast cell walls and α-MOS from a wider group of yeast sources.

Following a ban by the EU on the feeding of all antibiotics and related drugs to livestock in January of 2006, the demand for antibiotic-free meat has been

increasing worldwide (Sofos, 2008). Alpha-MOS from yeast cell walls as well as other prebiotic oligosaccharides has been suggested as an alternative replacement for antibiotic growth promoters (Heinrichs et al., 2003). Since agricultural wastes are rich sources of raw materials for the production of β-MOS, more research on exploring the possibility of using plant β-MOS as feed additives should be performed. Successful application of plantderived β-MOS from agricultural wastes as feed additive will not only promote the meat industry, but will also support sustainable development of the agricultural sector as well.

In addition to the beneficial effects of yeast a-mannan on livestock, health-promoting effects of β-mannan and β-MOS from other sources have been reported. Coffee β-MOS, prepared from coffee extract by thermal hydrolysis (Asano et al., 2003), have been shown to reduce weight, total body volume, and adipose tissue in men but not women, when consumed as a β-MOS-containing beverage (Salinardi et al., 2010; St-Onge et al., 2012). In animals, coffee β-MOS have been shown to significantly lower the blood pressure of hypertensive rat models (Hoshino-Takao et al., 2008). Coffee mannan prepared with hot water followed by alkali extraction has also been shown to stimulate the expression of the surface lymphocyte activation marker CD69 on B-lymphocytes (Simoes et al., 2009).

Apart from coffee mannan, beneficial effects of mannanrich plants have been reported. *Aloe vera* L. extracts from inner leaves, prepared by ethanol precipitation, which is rich in galactoglucomannan (Rodrı´guez Rodrı´guez et al., 2010), has been shown to promote oral wound healing in rats by stimulating gingival fibroblast proliferation (Jettanacheawchankit et al., 2009), as well as activate proliferation, differentiation, extracellular matrix formation and mineralization of primary human dental pulp cells (Jittapiromsak et al., 2010). Konjac glucomannan has been shown to improve vaginal health recovery after antifungal treatment for Candida infection in 14 female patients (Tester et al., 2012). However, the MOS preparations that were used in most of these studies were not a pure mixture with a defined structure, and therefore it is difficult to determine the actual biological activities of MOS. This is because other active ingredients in the preparation, especially from yeast cell walls or aloe extract, could have potent pharmacological effects even in low

concentrations. Well-defined MOS products must be obtained to precisely evaluate their biological effects.

Moreover, MOS can also directly inhibit pathogenic bacteria besides promoting the growth of probiotic bacteria. The inhibitory effects of oligosaccharides on pathogens are due to the direct interaction with human mucosal receptors and hence blocking of the bacterial binding sites. Therefore, this process improved the elimination of these bacteria during excretion rather than their binding to mucosal receptors (Ishihara et al., 2000)

In this study, we present the effects of MOS from copra meal on probiotic and pathogenic bacteria, the effects on Human Dermal Fibroblast cells and THP-1 macrophages.

The objective of this study is to optimize the condition for the production of MOS from copra meal.

4.3 Materials and Methods

4.3.1 Copra meal pre-treatment method

The copra meal from Thai Pure Coconut company, Samut Sakhon province, was dried in a hot air oven at 60 ºC for 12 h (Titapoka et al., 2008). The moisture content was measured before and after drying using moisture balance (Precisa HA 300, Switzerland). The dried products were packed in a vacuum bag and stored at 4 ºC until further usage. In the next step, the dried copra was ground and the oil was removed following previously published method with some modification (Lin and Chen, 2004). Briefly, the copra meal 1 kg was boiled in approx. 2 L of DI water for 2 hr and kept at 4 ˚C to let the oil solidified before removing the fat. This process was repeated 3 times. The sample was then dried in a hot air oven at 60˚C for 12 hr. Then, the sample was suspended in 1 L n-hexane and stirred for overnight. The suspension was filtered by vacuum filter and dried in a hot air oven at 60˚ C for 12 hr. The defatted copra meal was then ground and sieved through 0.2 mm and 0.5 mesh for further analysis. The defatted copra meal was packed and kept in 4 ˚C.

4.3.2 Recombinant enzyme expression and purification

Recombinant *Bl*ManB expression from *E. coli* were performed as previously described (Songsiriritthigul et al., 2010). Briefly, the overnight culture of the transform *E. coli* TOP10 harboring recombinant plasmid containing *Bl*ManB was inoculation into

5 ml of LB broth containing 100 μg/ml of ampicillin at 37℃ for 16 h. After that, 1 ml of overnight culture was inoculated into 250-1000 ml of LB broth containing 100 μg/ml ampicillin and grown at 37℃ until the optical density at 600 nm reached ~1.0 - 1.5. Then, IPTG was added into the culture broth to a final concentration of 0.1 mM. The culture was subsequently incubated with vigorous shaking (250 rpm) at 26-28℃ (room temperature) for 3 - 4 h. The culture was collected and chilled in an icebox for 5 min and centrifuged at 2000xg for 10 min at 4℃ to separate cells and supernatant.

Immobilized metal affinity chromatography (IMAC) was used for purification of 6 × His-tagged recombinant β-mannanase by gravity-flow chromatography, using Ni-NTA Agarose according to the manufacturer protocol (Qiagen). The periplasmic extract was loaded onto a column and washed three times with increasing concentrations of imidazole of 5, 10 and 20 mM. The enzyme was then eluted by elution buffer containing 250 mM imidazole, and dialyzed using a dialysis membrane (Pierce Biotechnology, 10-kDa molecular-weight cutoff) to remove imidazole.

4.3.3 Generation of copra MOS by enzymatic hydrolysis

The bioconversion reactions containing defatted copra meal from step 4.3.1 equivalent to 1, 2 and 4% of total mannans in DI water were incubated with 5, 10 and 15 U/ml in a 10 ml reaction and incubated at 40 ℃ with 250 rpm shaker. Then, 850 uL of reaction was taken at 1, 2, 3 ,6, 9, 12, 16, 20 and 24 h and heated inactivated at 100˚C for 20 min. Then, 0.25 uL of samples and standard MOS (M1-M6) were applied on the TLC silica sheet and kept in developing chamber with mobile phase consist of ethanol : n-propanol : water (4 : 14 : 2). The hydrolytic product was visualized by sprayed with a solution of 0.5 g thymol in 95ml ethanol mixed with 5 ml of 97% sulfuric acid and heat 15-20 mins at 120℃. Manno-oligosaccharides (M2-M6) and mannose from Megazyme were used as standard. Silicagel 60 F254 aluminum sheet, n-propanol and ethanol were purchased from Merck (Damstadt, Germany). The remaining reaction was collected,heat inactivated, filtered and centrifuged at 4000 rpm for 10 min. Then, the solution was kept in -20˚C freezer overnight, before freeze dring using LSCbasic (Christ, German).

4.3.4 Product analysis by HPLC

The quantitative analysis of produced MOS was performed by high pressure anion exchange chromatography with pulse field anionic detection (HPAEC-PAD). The hydrolysis reaction 50 µL was diluted to 100 µL by adding ultra-pure water and filter through a 0.2 µm syringe filter. The products were analyzed using CarboPac ® PA1 Analytical Column (4 × 250 mm DIONEX™) with Electrochemical Detection ED40 (DIONEX™). The system was run in constant temperature 30°C with mobile phase consist of 150 mM NaOH for isocratic eluent, and the flow rate was maintained at 0.5 mL/min. Manno-oligosaccharides and galacto-oligosacchrides (Megazyme, Ireland) were used as standards.

4.3.5 Biological activities

The growth of probiotic bacteria *Lb. gasseri, Lb. acidophilus, B. longum. S. thermophilus* APC151, *S. salivarius* DSM2059, *Lb. delbrueckii* DSM20054, *Lb. amylovorous* DSM20552 were grown in MRS basal media in aerobic condition overnight. The cell were collected by centrifugation at 4 ˚C, 8000g for 10 mins. The cell pellet were resuspended in 0.85% normal saline containing to OD600 = 0.05. 20 microliter of resuspended cell were added into 180 MRS with 2% (w/v) MOS, inulin, GOS or FOS and incubated at 37°C under anaerobic condition (Titapoka et al., 2013). The pathogenic bacteria *Salmonella enterica* Serotype Thyphimurium was grown in M9 minimal media containing 2% (w/v) MOS, inulin, GOS, FOS, CHOS, lactose and glucose incubated at 37 °C and 150 rpm shaker. The turbidity of the cells was measured using microplate reader every 3 h for 24 h.

4.3.6 Human dermal fibroblast toxicity assay

The effect of MOS on human dermal fibroblast (HDF) cell (ATCC, Manassas, VA, USA) viability was determined by resazurin assay according to manufacturer's protocol (Invitrogen, California, USA). HDF cells were seeded at 5×10^4 cells/well in 96 well plate and incubated at 37 °C and 5% CO2 overnight. The cells were treated with 62.5, 125, 250, 500, 1000, and 2,000 µg/ml of MOS or 0 – 8 µg/mL puromycin (Sigma-Aldrich, Darmstadt, Germany) (control) in 10% FBS DMEM (Gibco, California, USA) for 24 h. After incubation, the culture media was removed and 100 µl of 2.5 ug/mL resazurin solution in 10% FBS DMEM was added and the cells were further incubated for 3 h, during this time, the bright pink color was developed from the

conversion of resazurin to resarufin. The absorbance was measured at 560 nm excitation and 590 nm emission filter set, using a microplate reader (BMG Labtech, Ortenberg, Germany). The data was recorded as mean ± standard deviation (SD) and were analyzed by one-way and two-way analysis of variance (Simões et al., 2009). Kruskal-Wallis test and Dunn's multiple comparisons test were applied for analyses of non-parametric data, after indicated normal distribution. Statistical testing was performed using GraphPad Prism 8 software (GraphPad Software Inc., California, USA) with the statistical tests as shown in the figure legends.

4.3.7 Anti-inflammatory assay on THP-1

The anti-inflammation effect of MOS on human mature monocyte was done accordingly to the protocol recently developed in our laboratory(Lavoie, Beauchet, Berberi, & Chornet, 2011). Briefly human THP-1monocytes (CSL, Australia). 5×10^5 cells were differentiated to mature monocyte by exposing to 0.2 µM vitamin D3 (Calbiochem, Darmstadt, Germany) for 48 h (Jitprasertwong et al., 2021). Then, the cells were pretreated with 10, 50, 100, 200, 500 and 1000 µg/ml of MOS, 0.1 ug/ml dexamethasone (Sigma-Aldrich) and 10 µM curcumin (Sigma-Aldrich) for 24 h. Then 100 ng/ml of LPS (Invitrogen, CA, USA) were added into the well and incubated for another 6 h to stimulate inflammatory reaction. Then, the media were collected and centrifuged (2000 xg for 5 mins) to collect the supernatant, which was then kept in -20 ºC. ELISA-based methods were used to determine the concentration of cytokines secreted into culture supernatant after LPS stimulations of differentiated-THP-1 cells at various experimental conditions. The analysis was done according to the manufacturer's protocols (R & D Systems; Minneapolis, USA), for Interleukin (IL)-1β (Human IL-1β DuoSet ELISA, Cat.DY201, Lot.P218283). The data was recorded as mean ± standard deviation (SD) and were analyzed by one-way and two-way analysis of variance (Simões et al.). Kruskal-Wallis test and Dunn's multiple comparisons test were applied for analyses of non-parametric data, after indicated normal distribution. Statistical testing was performed using GraphPad Prism 8 software (GraphPad Software Inc., California, USA) with the statistical tests as shown in the figure legends.

4.4 Results

4.4.1 Pretreatment results

The bioconversion of biomass to sugars, including oligosaccharides and monosaccharides have been applied in several industry. Pretreatment is important to produce the purified polysaccharides substrates. Defatting process have been used to investigate the composition. This process followed by several boiling, filtrating and drying steps to reduce the fat composition. After solidified fat for several time, the copra was mixed with hexane. Moreover, ether and hexane could remove around 85% copra oil (Lin and Chen, 2004).

Pretreatment using the boiling and refrigerating processes could remove 80% of copra oil (table 4.2). The defatted copra meals were measured moisture and lipid content according to AOAC methods (AOAC., 1990). Moisture content and lipid content before pretreatment were 48.6 ± 1.2 and 29.4 ± 1.2 while after pretreatment were 5.7 ± 0.2 and 4.2 ± 0.2, respectively which were comparable to the previous study (Ghosh et al., 2014; Lin and Chen, 2004). This process was showed the efficient in enhance enzyme accessibility production of copra MOS since there are more porous and destroyed structure of copra meal (Ghosh et al., 2014).

Table 4.2 Moisture and Lipid content after refrigeration and solvent extraction.

Parameter	Before pretreatment	After pretreatment
Moisture content (%)	48.6 ± 1.2	5.7 ± 0.2
Lipid Content (%)	29.4 ± 1.2	4.2 ± 0.2

4.4.2 Production of MOS using enzyme from food grade *Lactobacillus* expression system

Product analysis by TLC after hydrolysis of various substrates confirmed that the recombinant enzyme is indeed an endo-β-mannanase. Various manno-oligosaccharide products (M2 - M6) as well as mannose were found after enzymatic hydrolysis of locust bean gum and mannan. The enzyme production and activity of *Bl*ManB specific to mannan was studied in our previous report (Sak-Ubol et al., 2016; Songsiriritthigul et al., 2010). The enzyme activity of *Bl*ManB could up to 50K U/L. Therefore, our *Bl*ManB 5, 10 and 15 U against 1, 2 and 4 % of total mannan could be investigated to produce MOS from copra meal (Fig. 4.3A).

Products of copra meal hydrolysis by *Bl*ManB at various time point using TLC is displayed in Fig. 4.3A. The hydrolysis after 1 h to 24 h of enzymatic reaction showed the releasing of manobiose (M2), mannotriose (M3), mannotetrose (M4), mannopentose (M5) and mannohexaose (M6). The standards were run in parallel to compare the produced sugars.

Moreover, the quantitative analysis of *Bl*ManB released sugar from copra meal were measured by HPAEC-PAD. The peak of standard galacto-mannooligosaccharides (Fig. 4.3B) and standard mannooligosacchrides (Fig. 4.3C) were used to quantify the hydrolysis products of copra meal by *Bl*ManB that were presented in Fig. 4.3D The protuberant peak of mannobiose at 11.67 min, mannotriose at 14.65 min, mannotretrose at 19.35 min, and mannohexaose at 36.42 min were observed. The peak intensity was used to calculate the yield conversion from mannan substrate to sugar products (table 2). The highest yield was 8.6 ± 0.2 % in 5 U enzyme reaction. Since, *Bl*ManB was able to hydrolyzed copra meal. Therefore, we used purified *Bl*ManB to produce more purified products (Fig. 4.4A) and the products were lyophilized (Fig. 4.4B).

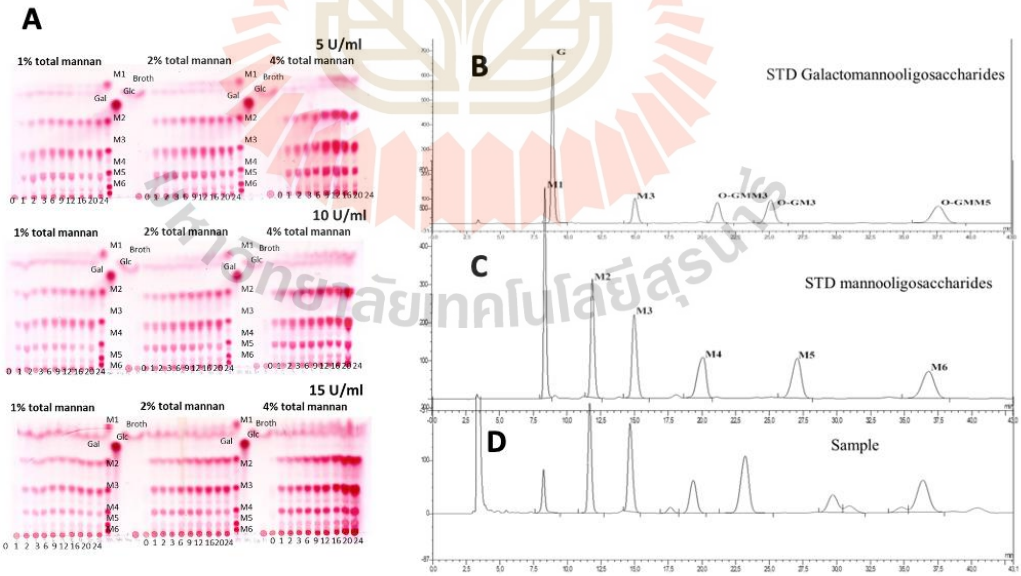


Figure 4.3 Optimization of MOS production from Copra meal by crude enzymatic methods and the chromatogram of MOS products.

Optimal condition of MOS production by β-mannanase from food grade culture. Reaction mixtures were incubated at 40˚C with 250 rpm agitation. TLC analysis displayed the release of manno-oligosaccharides products after incubation at 0, 1, 2, 3, 6, 9, 12, 16, 20 and 24 hr, respectively (A). HPAEC-PAD analysis of hydrolysis products of Copra meal by β-mannanase from food grade expression. The protuberant peak of mannobiose at 11. 67 min, mannotriose at 14. 65 min, mannotretrose at 19. 35 min, and mannohexaose at 36. 42 min were observed. The standard galactomannooligosaccharides (B), and standard mannooligosaccharides (C) were used to calculate the quantitative analysis of MOS products (D).

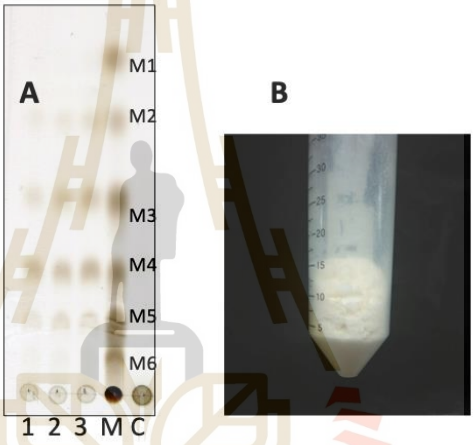


Figure 4.4 Production of MOS using purified enzyme.

The bioconversion reactions containing defatted copra meal equivalent to 2 % of total mannans were incubated with 5 U/ml of purified enzyme at 40 ˚C. The reactions (A) were carried out in a 100 ml reaction triplicated mixture, containing DI water (1, 2, 3 and control). The reaction mixture was filter and freeze dried (B).

4.4.3 Biological activities

4.4.3.1 Bacterial growth assay

The growth of probiotic bacteria in MRS basal media containing 2% (w/v) MOS, inulin, GOS or FOS and incubated at 37ºC under anaerobic condition in aerobic condition overnight are shown in Fig 4.5. It was found that GOS stimulated the highest growth of tested probiotics followed by MOS and inulin. However, commercial FOS did not support the growth of tested probiotics in this experiment. The turbidity of the cells was measured using microplate reader every 3 h for 24 h.

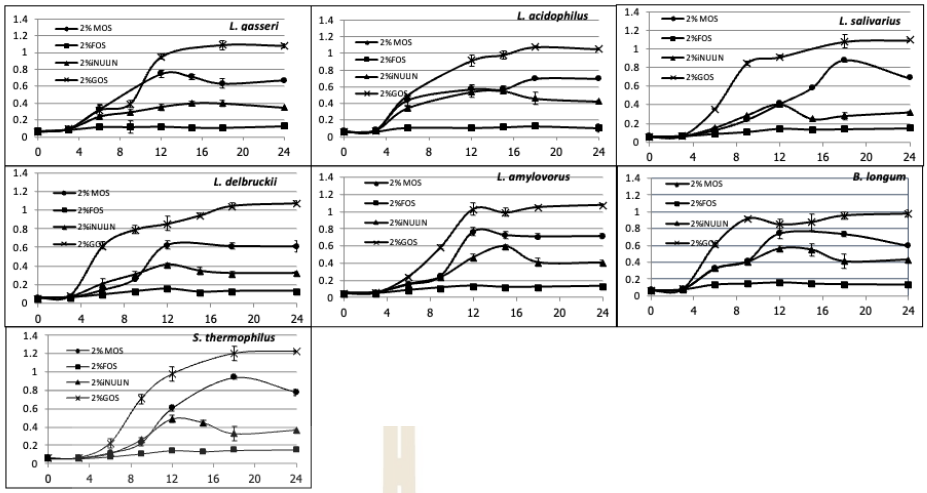


Figure 4.5 Prebiotic activity assay.

The growth of probiotic bacteria *Lb. gasseri, Lb. acidophilus, B. longum. S. thermophilus* APC151*, S. salivarius* DSM2059, *Lb. delbrueckii* DSM20054*, Lb. amylovorous* DSM20552 were grown in MRS basal media in aerobic condition overnight. The cell were collected by centrifugation at 4 °C, 8000g for 10 mins. The cell pellet were resuspended in 0.85% normal saline containing to OD600 = 0.05. 20 microliter of resuspended cell were added into 180 MRS with 2% (w/v) MOS (circle), inulin (triangle), GOS (cross) or FOS (square) and incubated at 37°C under anaerobic condition. The turbidity of the cells was measured using microplate reader every 3 h for 24 h. The data were represented as mean ± SD (n=3).

4.4.3.2 Human Dermal Fibroblast toxicity assay

The cytotoxicity assay was performed to evaluate the effects of MOS on the viability of HDF cells. Cells were exposed to various concentration of MOS (0–2000 μg/mL). As shown in Fig. 4.6 These results indicate that MOS at the concentrations used in this study have no direct effect on HDF ($p \leq 0.05$) and were not due to changes in cell viability or proliferation. While puromycin as positive control showed the reduction of viability of HDF cells.

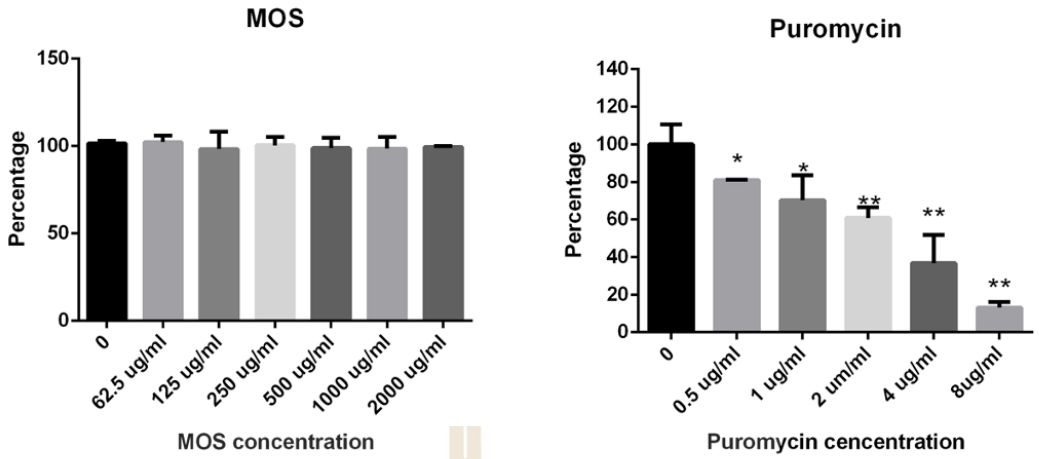


Figure 4.6 Cytotoxicity of MOS on HDF.

Effect of MOS and puromycin on Human Dermal Fibroblast. MOS (left) were used 62.5 – 2000 ug/ml for 24 h. Puromycin (right) were used 0.5 – 8 ug/ml. Cell viability was detected by resazurin assay. The data were represented as mean ± SD (n=3). $p \leq 0.05$ versus untreated. The absorbance of untreated cells was taken as 100% viability.

4.4.3.3 Anti-Inflammatory assay on THP-1

The THP-1 cells 5×10^5 cells were treated by 0.2 μM vitamin D3 for 48 h. Then, the cells were pretreated with 10, 50, 100, 200, 500 and 1000 μg/ml of MOS while dexamethasone 0.1 ug/ml and curcumin 10 μM were used as control for 24 h. The LPS100 ng/ml were added into the well and incubated for 6 h (left) and without LPS (right). The media were collected and centrifuged (2000 rpm 5 mins) to separate the cell and supernatant was kept in -20 ℃ for detection of cytokine production by ELISA. The data were represented as mean ± SD (n=3). $p \leq 0.05$ versus untreated. The absorbance of untreated cells was taken as 100% viability

In this study, VitD3-differentiated THP-1 cells were treated with MOS (0–1000 μg/mL) for 24 h and then exposed to LPS for 6 h (Jitprasertwong et al., 2021). Dexamethasone-treated cells were used as a positive control. The levels of pro-inflammatory cytokine IL-1β in the supernatants were analyzed by ELISA. Fig 4.7 shows that LPS significantly increased the secretion of pro-inflammatory cytokine IL-1β from THP-1 monocytes ($p < 0.05$). Surprisingly, MOS significantly decreased LPS-induced pro-inflammatory cytokine IL-1β from THP-1 monocytes ($p < 0.001$) in the

concentration between 100 – 200 μg/mL. Anti-inflammatory activity of MOS could be shown when MOS concentrations were as low as 10 μg/mL and the effect reasonably improved upon increasing the dose of MOS. Nevertheless, with maximum inhibition being reached at a dosage range of 100 - 200 μg/mL. Most astonishingly, the levels of anti-inflammatory activity of MOS could be reduced upon increasing the dose of MOS. The greatest inhibitory effect obtained with these soluble MOS is commensurate to the effect of 0.1 μg/mL dexamethasone.

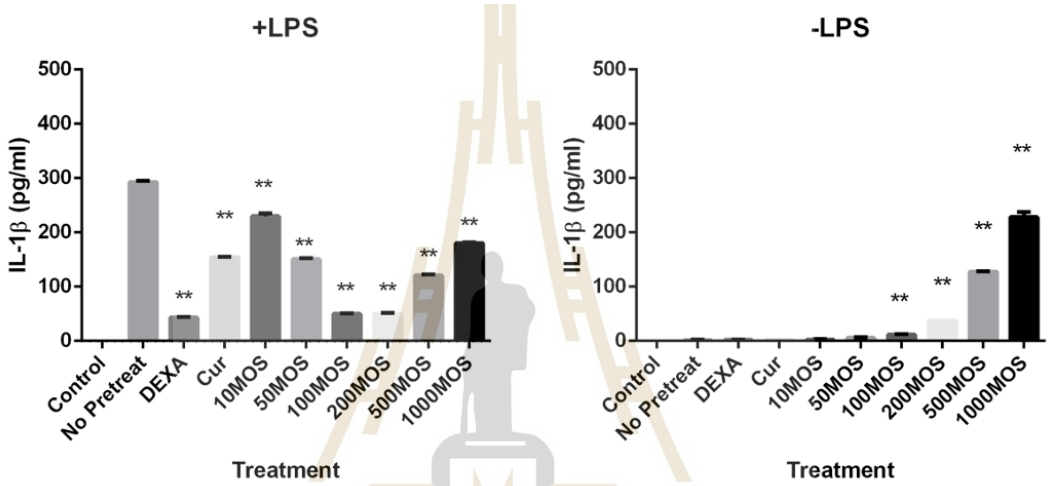


Figure 4.7 The anti-inflammation effect.

4.5 Discussion

Since the important hemicellulosic materials such as mannan and galactomannan from copra meal is low. It contained only 61 % galactomannan from previously report (BALASUBRAMANIAM, 1976. The ratio of mannose and galactomannan is 14: 1 (Regalado et al., 2000). Consequently, the pretreatment could be destabized the formation of copra meal complex for enhance enzyme approachability to increase the production of MOS (Ghosh et al., 2015) Although, Our previous publication found that partial hydrolysis of copra meal after incubation occurred after incubation of this substrate with the enzyme for 2 to 3 days (Yamabhai et al., 2011). In this study, the hydrolysis product could be presented from these defatted copra meal since first hour of reaction (Fig. 4.3A). The dominant products were MOS that different sizes, from two to six mannose units after analysis by HPAEC-PAD (Fig. 4.3D). This results showed that *Bl*ManB hydrolyzed the mannan backbone

structure in a randomly (Rungruangsaphakun et al., 2018; Yang et al., 2017). Furthermore, another studies found that the MOS from copra meal could be stimulated the growth of probiotics (Pangsri et al., 2015; Thongsook et al., 2018; Ghosh et al., 2015). Thereby, our MOS was investigated the growth of probiotics bacteria compare to commercial inulin, GOS or FOS and incubated at 37°C under anaerobic condition in aerobic condition overnight are shown in Fig. 4.5 The growth of *Lactobacillus, Bifidobacterium* and *Streptococcus* were shown promisingly in MOS and it had been reported that MOS is one of the important prebiotic substance that promotes the growth of probiotics (Mano et al., 2018; Rolim et al., 2015; Woranuch et al, 2021; Yamabhai et al, 2016)

Prebiotics have been implicated in modulating intestinal immunity, influencing the production of pro- and anti-inflammatory cytokines, and reducing inflammation (Jitprasertwong et al., 2021; Duranti et al., 2019; Wu et al., 2020; Yazbeck et al., 2019). Firstly, MOS were evaluated the effects on viability of HDF by resazurin assay. These results indicate that MOS at the concentrations used in this study have no direct effect on HDF ($p \leq 0.05$) (Fig. 4.6). Similar effects were also observed on jejunal tissue of female rat (Yazbeck et al., 2019). MOS has been related with positive modify to the intestinal microbiota, improved intestinal growth, and reductions in pro-inflammatory markers (Geier et al., 2009; Wang et al., 2016). Therefore, our study showed the increasing of anti – inflammatory activity with the optimal concentration (Fig. 4.7). Similar results were also found in *in vitro* studies, where MOS from yeast treatment reduced the expressions of pro-inflammatory cytokines and increased the expressions of anti-inflammatory cytokines of porcine intestinal epithelial cells following bacterial invasion (Wang et al., 2016; Zanello et al., 2011)

4.6 Conclusion

Our study has indicated that enzymatic hydrolysis of hemicellulose complex copra meal by β-mannanase from *Bacillus licheniformis* (*Bl*ManB) to the small water-soluble products. Importantly, the results also demonstrated that MOS has promising property. The MOS products will be further study for medical, pharmaceutical and clinical trials of anti – inflammatory effect in the next step.

REFERENCES

REFERENCES

AOAC. (1990). Official methods of analysis of the association of official analytical chemists. *AOAC Inc press,* 15th edition Gaithersburg, MD.

Balasubramaniam (1976). Polysaccharides of the kernel of maturing and matured coconuts. *Journal of Food Science,* 41(6), 1370-1373. doi:doi:10.1111/j.1365-2621.1976.tb01174.x

Benech, R.O., Li, X., Patton, D., Powlowski, J., Storms, R., Bourbonnais, R., Tsang, A. (2007). Recombinant expression, characterization, and pulp prebleaching property of a Phanerochaete chrysosporium endo-β-1, 4-mannanase. *Enzyme and Microbial Technology,* 41 (6-7), 740-747.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*. doi:10.1016/0003-2697(76)90527-3

Braithwaite, K. L., Black, G., Hazlewood, G., Ali, B., and Gilbert, H. (1995). A non-modular endo-β-1, 4-mannanase from Pseudomonas fluorescens subspecies cellulosa. *Biochemical Journal, 305* (3), 1005-1010.

Cai, H., Shi, P., Bai, Y., Huang, H., Yuan, T., Yang, P., Yao, B. (2011). A novel thermoacidophilic family 10 xylanase from Penicillium pinophilum C1. *Process Biochemistry, 46* (12), 2341-2346.

Cai, H., Shi, P., Huang, H., Luo, H., Bai, Y., Yang, P., Yao, B. (2011). An acidic β-mannanase from Penicillium sp. C6: gene cloning and over-expression in Pichia pastoris. *World Journal of Microbiology and Biotechnology, 27* (12), 2813-2819.

Cai, H., Shi, P., Luo, H., Bai, Y., Huang, H., Yang, P., Yao, B. (2011). Acidic β-mannanase from Penicillium pinophilum C1: cloning, characterization and assessment of its potential for animal feed application. *Journal of bioscience and bioengineering, 112*(6), 551-557.

Canapi, E. C., Agustin, Y. T., Moro, E. A., Pedrosa Jr, E., Bendaño, M. L. J. (2005). Coconut oil. *Bailey's industrial oil and fat products*.

Capek, P., Kubacková, M., Alföldi, J., Bilisics, L., Lišková, D., Kákoniová, D. (2000). Galactoglucomannan from the secondary cell wall of Picea abies L. Karst. *Carbohydrate Research,* 329(3), 635-645.

Cho, K. M., Hong, S. Y., Lee, S. M., Kim, Y. H., Kahng, G. G., Kim, H., Yun, H. D. (2006). A cel44C-man26A gene of endophytic Paenibacillus polymyxa GS01 has multi-glycosyl hydrolases in two catalytic domains. *Applied microbiology and biotechnology,* 73(3), 618-630.

Choi, J.M., Han, S.-S., Kim, H. S. (2015). Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnology advances,* 33 (7), 1443-1454.

Chow, J.T.N., Williamson, D. A., Yates, K. M., Goux, W. J. (2005). Chemical characterization of the immunomodulating polysaccharide of Aloe vera L. *Carbohydrate Research,* 340(6), 1131-1142.

Diep, D. B., Mathiesen, G., Eijsink, V. G., Nes, I. F. (2009). Use of lactobacilli and their pheromone-based regulatory mechanism in gene expression and drug delivery. *Curr Pharm Biotechnol, 10*. doi:10.2174/138920109787048571

Duranti, S., Lugli, G. A., Milani, C., James, K., Mancabelli, L., Turroni, F., Ossiprandi, M. C. (2019). Bifidobacterium bifidum and the infant gut microbiota: an intriguing case of microbe-host co-evolution. *Environmental microbiology,* 21(10), 3683-3695.

Fanutti, C., Ponyi, T., Black, G. W., Hazlewood, G. P., Gilbert, H. J. (1995). The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *Journal of Biological Chemistry,* 270(49), 29314-29322.

Feord, J. (2002). Lactic acid bacteria in a changing legislative environment. *Antonie Van Leeuwenhoek,* 82(1), 353-360.

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

Geier, M., Torok, V., Allison, G., Ophel-Keller, K., Hughes, R. (2009). Indigestible carbohydrates alter the intestinal microbiota but do not influence the

performance of broiler chickens. *Journal of Applied Microbiology,* 106(5), 1540-1548.

Ghosh, A., Verma, A., Tingirikari, J., Shukla, R., Goyal, A. (2014). Recovery and Purification of Oligosaccharides from Copra Meal by Recombinant Endo-β-mannanase and Deciphering Molecular Mechanism Involved and Its Role as Potent Therapeutic Agent. *Molecular Biotechnology, 57*. doi:10.1007/s12033-014-9807-4

Ghosh, A., Verma, A. K., Tingirikari, J. R., Shukla, R., Goyal, A. (2015). Recovery and purification of oligosaccharides from copra meal by recombinant endo-β-mannanase and deciphering molecular mechanism involved and its role as potent therapeutic agent. *Molecular biotechnology,* 57(2), 111-127.

Gibbs, M. D., Reeves, R. A., Sunna, A., Bergquist, P. L. (1999). Sequencing and expression of a β-mannanase gene from the extreme thermophile Dictyoglomus thermophilum Rt46B. 1, and characteristics of the recombinant enzyme. *Current Microbiology,* 39(6), 351-357.

Halstead, J. R., Vercoe, P. E., Gilbert, H. J., Davidson, K., Hazlewood, G. P. (1999). A family 26 mannanase produced by Clostridium thermocellum as a component of the cellulosome contains a domain which is conserved in mannanases from anaerobic fungiThe GenBank/EMBL accession number for the sequence reported in this paper is AJ242666. *Microbiology,* 145(11), 3101-3108.

Ham, S.-J., Lee, H. J., Kim, Y.-J., Shin, D.-H., Rhee, Y. H., Son, K.-H., Park, H.-Y. (2011). A highly active endo-β-1, 4-mannanase produced by Cellulosimicrobium sp. strain HY-13, a hemicellulolytic bacterium in the gut of Eisenia fetida. *Enzyme and Microbial Technology,* 48(4-5), 365-370.

Heidorne, F. O., Magalhaes, P. O., Ferraz, A. L., Milagres, A. M. (2006). Characterization of hemicellulases and cellulases produced by Ceriporiopsis subvermispora grown on wood under biopulping conditions. *Enzyme and Microbial Technology,* 38(3-4), 436-442.

Heuzé, V., Tran, G., Sauvant, D., Bastianelli, D. (2015). Copra meal and coconut by-products.

Hossain, M. Z., Abe, J.-i., Hizukuri, S. (1996). Multiple forms of β-mannanase from Bacillus sp. KK01. *Enzyme and Microbial Technology,* 18(2), 95-98. doi:http://dx.doi.org/10.1016/0141-0229(95)00071-2

Ishurd, O., Kermagi, A., Elghazoun, M., Kennedy, J. F. (2006). Structural of a glucomannan from Lupinus varius seed. *Carbohydrate Polymers,* 65(4), 410-413.

Jeon, S. D., Yu, K. O., Kim, S. W., Han, S. O. (2011). A celluloytic complex from Clostridium cellulovorans consisting of mannanase B and endoglucanase E has synergistic effects on galactomannan degradation. *Applied microbiology and biotechnology,* 90(2), 565-572.

Jitprasertwong, P., Khamphio, M., Petsrichuang, P., Eijsink, V. G., Poolsri, W., Muanprasat, C., Yamabhai, M. (2021). Anti-inflammatory activity of soluble chito-oligosaccharides (CHOS) on VitD3-induced human THP-1 monocytes. *PloS one,* 16(2), e0246381.

Karlskas, I. L., Maudal, K., Axelsson, L., Rud, I., Eijsink, V. G., Mathiesen, G. (2014). Heterologous protein secretion in lactobacilli with modified pSIP vectors. *PLoS ONE, 9*. doi:10.1371/journal.pone.0091125

Katrolia, P., Zhou, P., Zhang, P., Yan, Q., Li, Y., Jiang, Z., Xu, H. (2012). High level expression of a novel β-mannanase from Chaetomium sp. exhibiting efficient mannan hydrolysis. *Carbohydrate Polymers, 87*(1), 480-490.

Kleerebezem, M., Boekhorst, J., Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., Fiers, M. W. (2003). Complete genome sequence of Lactobacillus plantarum WCFS1. *Proc Natl Acad Sci USA, 100*. doi:10.1073/pnas.0337704100

Kurokawa, J., Hemjinda, E., Arai, T., Karita, S., Kimura, T., Sakka, K., Ohmiya, K. (2001). Sequence of the Clostridium thermocellum mannanase gene man26B and characterization of the translated product. *Bioscience, biotechnology, and biochemistry,* 65(3), 548-554.

Lavoie, J.-M., Beauchet, R., Berberi, V., Chornet, M. (2011). Biorefining lignocellulosic biomass via the feedstock impregnation rapid and sequential steam treatment. In *Biofuel's Engineering Process Technology*: IntechOpen.

Lin, T.C., Chen, C. (2004). Enhanced mannanase production by submerged culture of Aspergillus niger NCH-189 using defatted copra based media. *Process Biochemistry,* 39(9), 1103-1109. doi:https://doi.org/10.1016/S0032-99592(03)00 0218-8.

Liu, S., Lu, H., Hu, R., Shupe, A., Lin, L., Liang, B. (2012). A sustainable woody biomass biorefinery. *Biotechnology advances,* 30(4), 785-810.

Luo, H., Wang, K., Huang, H., Shi, P., Yang, P., Yao, B. (2012). Gene cloning, expression, and biochemical characterization of an alkali-tolerant β-mannanase from Humicola insolens Y1. *Journal of Industrial Microbiology and Biotechnology,* 39(4), 547-555.

Luo, H., Wang, Y., Wang, H., Yang, J., Yang, Y., Huang, H., Fan, Y. (2009). A novel highly acidic β-mannanase from the acidophilic fungus Bispora sp. MEY-1: gene cloning and overexpression in Pichia pastoris. *Applied microbiology and biotechnology,* 82(3), 453-461.

Lüthi, E., Jasmat, N. B., Grayling, R. A., Love, D. R., Bergquist, P. L. (1991). Cloning, sequence analysis, and expression in Escherichia coli of a gene coding for a beta-mannanase from the extremely thermophilic bacterium" Caldocellum saccharolyticum". *Applied and environmental microbiology,* 57(3), 694-700.

Mano, M. C. R., Neri-Numa, I. A., da Silva, J. B., Paulino, B. N., Pessoa, M. G., Pastore, G. M. (2018). Oligosaccharide biotechnology: an approach of prebiotic revolution on the industry. *Applied microbiology and biotechnology,* 102(1), 17-37.

Mathiesen, G., Sveen, A., Piard, J. C., Axelsson, L., Eijsink, V. (2008). Heterologous protein secretion by Lactobacillus plantarum using homologous signal peptides. *Journal of applied microbiology,* 105(1), 215-226.

Moreira, L. (2008). An overview of mannan structure and mannan-degrading enzyme systems. *Applied microbiology and biotechnology,* 79(2), 165-178.

Morris, D. D., Reeves, R. A., Gibbs, M. D., Saul, D. J., Bergquist, P. L. (1995). Correction of the beta-mannanase domain of the celC pseudogene from Caldocellulosiruptor saccharolyticus and activity of the gene product on kraft pulp. *Applied and environmental microbiology,* 61(6), 2262-2269.

Nguyen, T.-T., Mathiesen, G., Fredriksen, L., Kittl, R., Nguyen, T.H., Eijsink, V. G., Peterbauer, C. K. (2011). A food-grade system for inducible gene expression in Lactobacillus plantarum using an alanine racemase-encoding selection marker. *Journal of agricultural and food chemistry, 59*(10), 5617-5624.

Northcote, D. (1972). Chemistry of the plant cell wall. *Annual Review of Plant Physiology,* 23(1), 113-132.

Pangsri, P., Piwpankaew, Y., Ingkakul, A., Nitisinprasert, S., Keawsompong, S. (2015). Characterization of mannanase from Bacillus circulans NT 6.7 and its application in mannooligosaccharides preparation as prebiotic. *Springerplus,* 4(1), 1-11.

Perret, S., Bélaich, A., Fierobe, H.-P., Bélaich, J.-P., Tardif, C. (2004). Towards designer cellulosomes in Clostridia: mannanase enrichment of the cellulosomes produced by Clostridium cellulolyticum. *Journal of bacteriology,* 186(19), 6544-6552.

Picout, D. R., Ross-Murphy, S. B., Jumel, K., Harding, S. E. (2002). Pressure cell assisted solution characterization of polysaccharides. 2. Locust bean gum and tara gum. *Biomacromolecules,* 3(4), 761-767.

Puls, J. (1997). *Chemistry and biochemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis.* Paper presented at the Macromolecular symposia.

Roberfroid, M. (2007). Prebiotics: the concept revisited. *The journal of nutrition,* 137(3), 830S-837S.

Rolim, P. M. (2015). Development of prebiotic food products and health benefits. *Food Science and Technology,* 35(1), 3-10.

Rosen, G. (2006). Holo-analysis of the efficacy of Bio-Mos® in pig nutrition. *Animal Science,* 82(5), 683-689.

Rungrassamee, W., Kingcha, Y., Srimarut, Y., Maibunkaew, S., Karoonuthaisiri, N., Visessanguan, W. (2014). Mannooligosaccharides from copra meal improves survival of the Pacific white shrimp (Litopenaeus vannamei) after exposure to Vibrio harveyi. *Aquaculture,* 434, 403-410.

Rungruangsaphakun, J., Keawsompong, S. (2018). Optimization of hydrolysis conditions for the mannooligosaccharides copra meal hydrolysate production. *3 Biotech,* 8(3), 1-9.

Saittagaroon, S., Kawakishi, S., Namiki, M. (1983). Characterisation of polysaccharides of copra meal. *Journal of the Science of Food and Agriculture,* 34(8), 855-860. doi:10.1002/jsfa.2740340813

Sak-Ubol, S., Namvijitr, P., Pechsrichuang, P., Haltrich, D., Nguyen, T.H., Mathiesen, G., Yamabhai, M. (2016). Secretory production of a beta-mannanase and a chitosanase using a Lactobacillus plantarum expression system. *Microbial Cell Factories,* 15, 81. doi:10.1186/s12934-016-0481-z

Sakka, M., Goto, M., Fujino, T., Fujino, E., Karita, S., Kimura, T., Sakka, K. (2010). Analysis of a Clostridium josui cellulase gene cluster containing the man5A gene and characterization of recombinant Man5A. *Bioscience, biotechnology, and biochemistry,* 74(10), 2077-2082.

Sandin, R. (1987). Studies on cell adhesion and concanavalin A-induced agglutination of Candida albicans after mannan extraction. *Journal of medical microbiology,* 24(2), 145-150.

Simões, J., Madureira, P., Nunes, F. M., do Rosário Domingues, M., Vilanova, M., Coimbra, M. A. (2009). Immunostimulatory properties of coffee mannans. *Molecular nutrition & food research,* 53(8), 1036-1043.

Songsiriritthigul, C., Buranabanyat, B., Haltrich, D., Yamabhai, M. (2010). Efficient recombinant expression and secretion of a thermostable GH26 mannan endo-1,4-β-mannosidase from Bacillus licheniformis in Escherichia coli. *Microbial Cell Factories, 9*, 20-20. doi:10.1186/1475-2859-9-20

Sørvig, E., Grönqvist, S., Naterstad, K., Mathiesen, G., Eijsink, V. G., Axelsson, L. (2003). Construction of vectors for inducible gene expression in Lactobacillus sakei and L. plantarum. *FEMS microbiology letters,* 229(1), 119-126.

Sorvig, E., Mathiesen, G., Naterstad, K., Eijsink, V. G., Axelsson, L. (2005). High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum*

using versatile expression vectors. *Microbiology,* 151. doi:10.1099/mic.0.28084-0.

Stoll, D., Stålbrand, H., & Warren, R. A. J. (1999). Mannan-degrading enzymes from Cellulomonas fimi. *Applied and environmental microbiology,* 65(6), 2598-2605.

Strych, U., Penland, R. L., Jimenez, M., Krause, K. L., Benedik, M. J. (2001). Characterization of the alanine racemases from two mycobacteria. *FEMS microbiology letters,* 196(2), 93-98.

Sunna, A. (2010). Modular organisation and functional analysis of dissected modular β-mannanase CsMan26 from Caldicellulosiruptor Rt8B. 4. *Applied microbiology and biotechnology,* 86(1), 189-200.

Sunna, A., Gibbs, M. D., Bergquist, P. L. (2000). A novel thermostable multidomain 1, 4-β-xylanase from 'Caldibacillus cellulovorans' and effect of its xylan-binding domain on enzyme activity. *Microbiology,* 146(11), 2947-2955.

Thongsook, T., & Chaijamrus, S. (2018). Optimization of enzymatic hydrolysis of copra meal: compositions and properties of the hydrolysate. *Journal of Food Science and Technology,* 55(9), 3721-3730. doi:10.1007/s13197-018-3302-z

Timell, T. (1967). Recent progress in the chemistry of wood hemicelluloses. *Wood Science and Technology,* 1(1), 45-70.

Titapoka, S., Keawsompong, S., Haltrich, D., Nitisinprasert, S. (2008). Selection and characterization of mannanase-producing bacteria useful for the formation of prebiotic manno-oligosaccharides from copra meal. *World Journal of Microbiology and Biotechnology,* 24(8), 1425-1433. doi:10.1007/s11274-007-9627-9

Van Zyl, W. H., Rose, S. H., Trollope, K., Görgens, J. F. (2010). Fungal β-mannanases: mannan hydrolysis, heterologous production and biotechnological applications. *Process Biochemistry,* 45(8), 1203-1213.

Vijaya Kumar, S., Singh, S., Goyal, P., Dilbaghi, N., Mishra, D. (2005). Beneficial effects of probiotics and prebiotics on human health. *Die Pharmazie-An International Journal of Pharmaceutical Sciences,* 60(3), 163-171.

Vuksan, V., Jenkins, D., Spadafora, P., Sievenpiper, J. L., Owen, R., Vidgen, E., Bruce-Thompson, C. (1999). Konjac-mannan (glucomannan) improves glycemia and other associated risk factors for coronary heart disease in type 2 diabetes. A randomized controlled metabolic trial. *Diabetes care,* 22(6), 913-919.

Wang, J., Shao, Z., Hong, Y., Li, C., Fu, X., Liu, Z. (2010). A novel β-mannanase from Pantoea agglomerans A021: gene cloning, expression, purification and characterization. *World Journal of Microbiology and Biotechnology, 26*(10), 1777-1784.

Wang, W., Li, Z., Han, Q., Guo, Y., Zhang, B., D'inca, R. (2016). Dietary live yeast and mannan-oligosaccharide supplementation attenuate intestinal inflammation and barrier dysfunction induced by Escherichia coli in broilers. *British Journal of Nutrition,* 116(11), 1878-1888.

Wang, Y., Shi, P., Luo, H., Bai, Y., Huang, H., Yang, P., Yao, B. (2012). Cloning, over-expression and characterization of an alkali-tolerant endo-β-1, 4-mannanase from Penicillium freii F63. *Journal of bioscience and bioengineering,* 113(6), 710-714.

Woranuch, K., Sankasa, N., Sakamoto, T., Sukhumsirichart, W. (2021). Evaluation of mannooligosaccharide production from konjac by using recombinant mannanase of Bacillus sp. SWU60 as a prebiotic in vitro and in vivo. *Science, Engineering and Health Studies*, 21030010-21030010.

Wu, Y., Chen, Y., Lu, Y., Hao, H., Liu, J., Huang, R. (2020). Structural features, interaction with the gut microbiota and anti-tumor activity of oligosaccharides. *RSC Advances,* 10(28), 16339-16348.

Yamabhai, M. (2009). Sticky PCR: a PCR-based protocol for targeted protein engineering. *Biotechnol J, 4*. doi:10.1002/biot.200800198

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

Yamabhai, M., Sak-Ubol, S., Srila, W., & Haltrich, D. (2016). Mannan biotechnology: from biofuels to health. *Critical reviews in biotechnology,* 36(1), 32-42.

Yang, D., Yuan, Y., Wang, L., Wang, X., Mu, R., Pang, J., Zheng, Y. (2017). A review on konjac glucomannan gels: microstructure and application. *International journal of molecular sciences,* 18(11), 2250.

Yazbeck, R., Lindsay, R. J., Geier, M. S., Butler, R. N., Howarth, G. S. (2019). Prebiotics Fructo-, Galacto-, and Mannan-Oligosaccharide Do Not Protect against 5-Fluorouracil–Induced Intestinal Mucositis in Rats. *The Journal of nutrition,* 149(12), 2164-2173.

VITAE

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