PRODUCTION AND CHARACTERIZATION OF MANNANASE FROM *Aureobasidium* sp. FOR MANNOOLIGOSACCHARIDE PREPARATION FROM SPENT COFFEE GROUND

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การผลิตและลักษณะสมบัติของแมนแนเนสจาก *Aureobasidium* sp. เพื่อการเตรียมแมนโนออลิโกแซกคาไรด์จากบดกาแฟที่ใช้แล้ว

นายซาเรีย นูห์ มัวลานา มาลิค อิบราฮิม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PRODUCTION AND CHARACTERIZATION OF MANNANASE FROM *Aureobasidium* sp. FOR MANNOOLIGOSACCHARIDE PREPARATION FROM SPENT COFFEE GROUND
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Field of Study	Biotechnology
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ซาเรีย นูห์ มัวลานา มาลิค อิบราฮิม : การผลิตและลักษณะสมบัติของแมนแนเนสจาก *Aureobasidium* sp. เพื่อการเตรียมแมนโนออลิโกแซกคาไรด์จากบดกาแฟที่ใช้แล้ว. (PRODUCTION AND CHARACTERIZATION OF MANNANASE FROM *Aureobasidium* sp. FOR MANNOOLIGOSACCHARIDE PREPARATION FROM SPENT COFFEE GROUND) อ.ที่ปรึกษาหลัก : พงศ์ธาริน โล่ห์ตระกูล, อ.ที่ปรึกษาร่วม : วิชาณี แบนคีรี

วัตถุประสงค์ของการศึกษานี้คือเพื่อผลิตและศึกษาลักษณะสมบัติของแมนแนเนสจาก *Aureobasidium* สายพันธุ์ไทยที่คัดเลือก และใช้เอนไซม์ในการย่อยสารสกัดจากกากกาแฟบด (SCGE) เพื่อเตรียมแมนโนออลิโกแซกคาไรด์ (mannooligosaccharides, MOs) เมื่อสกัด SCGE ด้วยน้ำร้อนโดยใช้ภาวะที่เหมาะสม (อัตราส่วนของแข็งต่อของเหลว 1:30 เวลาบ่ม 60 นาที ที่ 121 องศาเซลเซียส ความดัน 15 ปอนด์ต่อตารางนิ้ว) ได้สารสกัดปริมาณ 9.5 กรัมต่อ 100 กรัมกากกาแฟบด SCGE มีเฮมิเซลลูโลสเป็นองค์ประกอบมากอันดับที่ 2 ที่ 28 เปอร์เซ็นต์ จากการคัดเลือก *Aureobasidium pullulans* NRRL 58524 ผลิตแมนแนเนสได้มากที่สุดที่ 8.07 ยูนิตต่อมิลลิลิตร อาหารที่ใช้ผลิตแมนแนเนสถูกปรับโดยใช้ SCGE และแอมโมเนียมซัลเฟต ในอัตราส่วนคาร์บอนต่อไนโตรเจน 1.15 โดยไม่เติม แอล-แอสพาราจีน ให้ผลผลิตแมนแนเนสสูงสุดเมื่อเลี้ยงไป 72 ชั่วโมง ภาวะที่เหมาะสมในการทำงานของแมนแนเนสคือที่ 55 องศาเซลเซียส ใน 50 มิลลิโมลาร์ ซิเทรตบฟเฟอร์ ที่ความเป็นกรดด่าง 4.0 เอนไซม์มีความทนต่ออุณหภูมิสูงที่ 50 และ 55 องศาเซลเซียสนานมากกว่า 6 ชั่วโมงโดยแทบไม่สูญเสียแอกทิวิตี และมีความทนต่อ 50 มิลลิโมลาร์ ซิเทรตบฟเฟอร์ ที่ความเป็นกรดด่าง 4.0 ได้นานกว่า 6 ชั่วโมงโดยยังคงมีแอกติวิทีมากกว่า 80 เปอร์เซ็นต์จากเริ่มต้น แมนแนเนสมีความจำเพาะต่อซับเสตรตสูงต่อกลูโคแมนแนน และกาแลกโทแมนแนน และถูกกระตุ้นแอกทิวิตีได้ด้วย Cu^{2+} Ca^{2+} และ Mg^{2+} เอนไซม์ถูกยับยั้งด้วยแมนโนส และกาแลกโทส ที่ความเข้มข้น 40 และ 30 มิลลิโมลาร์ ตามลำดับ ในการเตรียม MOs มีภาวะที่เหมาะสมคือใช้เอนไซม์ 65.7 ยูนิตต่อกรัม SCGE เวลาบ่ม 5 วัน 9 ชั่วโมง 36 นาที ได้ผลผลิตน้ำตาลรีดิวซ์ 0.66 กรัมต่อกรัม SCG MOs ที่ผลิตได้ไม่สามารถกระตุ้นการเติบโตของแบคทีเรียโพรไบโอติกที่ทดสอบได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2564 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6272007223 : MAJOR BIOTECHNOLOGY

KEYWORD: Spent coffee ground, Aureobasidium, mannanase, production and characterization, mannooligosaccharides

Syahriar Nur Maulana Malik Ibrahim : PRODUCTION AND CHARACTERIZATION OF MANNANASE FROM *Aureobasidium* sp. FOR MANNOOLIGOSACCHARIDE PREPARATION FROM SPENT COFFEE GROUND. Advisor: Assoc. Prof. PONGTHARIN LOTRAKUL, Ph.D. Co-advisor: WICHANEE BANKEEREE, Ph.D.

The aims of this study are to produce and characterize mannanase from the selected Thai *Aureobasidium* strain and use the enzyme for hydrolysis of spent coffee ground extract (SCGE) to prepare mannooligosaccharide (MOs). The optimum condition for SCGE extraction by hot water was 1:30 solid to liquid ratio with 60 min incubation time at 121℃ 15 lbs per in2. The maximum yield was 9.5 g/100 g substrate. The second dominant composition of SCGE was hemicellulose at 28 %. *Aureobasidium pullulans* NRRL 58524 was selected as the best mannanase producer with 8.07 U/mL. The mannanase production medium was optimized by using SCGE and ammonium sulphate without L-asparagine at a C/N ratio of 1.15. The highest enzyme yield was obtained after 72 hours. The optimal condition for mannanase was at 55 ℃ in 50 mM citrate buffer (pH 4.0). The enzyme was highly thermostable at 50 and 55 ℃, retaining most of its activity after 6 h incubation. It was also stable in 50 mM citrate buffer (pH 4.0) with more than 80 % of initial activity remained after 6 h. The mannanase was highly specific to glucomannan and galactomannan, and its activity was enhanced by Cu^{2+}, Ca^{2+} and Mg^{2+}. The enzyme was inhibited by mannose and galactose at 40 mM and 30 mM, respectively. The optimal MOs preparation was by using the enzyme at 66.7 U/g substrate and incubating for 5 days 9 h and 36 min which yielded the maximal reducing sugars at 0.66 g/g substrate. The produced SCG MOs failed to enhance the growth of all probiotic bacteria tested.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....

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Syahriar Nur Maulana Malik Ibrahim

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CHAPTER I
INTRODUCTION

1.1 Rationale

Mannan is a group of hemicellulosic polymers of which backbone chains consisting of mannose and other sugars linking by β-1,4-glycosidic bonds. It can be found in yeast and plant cell walls (White et al., 2002). Structures of hemicellulosic mannan vary widely among different plant families and products, for instance the linear mannan from copra meal and ivory nut, the glucomannan from hardwoods and konjac, the galactomannan from guar gum and locus bean gum, the galactoglucomannan from softwoods and aloe bulk and the acetylated galactoglucomannan from both softwoods and hardwoods (Yamabhai et al., 2016). The β-1,4-linkages in mannan can be hydrolyzed by mannanase, a glycoside hydrolase, yielding mannooligosaccharides (MOs), mannose, and other monosaccharides. MOs have many interesting physical and biological properties and have been applied in food and feed industries. They can be used as prebiotics in animal feeds and health supplements in human. The supplementary of MOs for livestock increases feed intake, live-weight gain, and ratio of feed conversion, while the beneficial effects of MO supplements in human include weight reduction in men, B-lymphocyte stimulation, primary dental pulp cell activation and vaginal health recovery (Rosen, 2007; Simoes et al., 2009; Jittapiromsak et al., 2010; St-Onge et al., 2012; Tester et al., 2012).

Mannanases are a group of mannan-hydrolyzing enzymes belonging to the glycoside hydrolase families of GH5 or GH26 that can be further categorized into two types of (i) β-mannanase (1,4-β-D-mannan mannohydrolase; EC 3.2.1.78) and (ii) β-mannosidase (β-D-mannoside mannohydrolase; EC 3.2.1.25) based on the hydrolytic cleavage site at the mainchain polysaccharide (Hagglund et al., 2003). The β-mannanase randomly cleaves the intramolecular bonds within the backbone whereas the β-mannosidase hydrolyzes the terminal linkages (Chauhan et al., 2012). In addition to mannanase, there are three enzymes that can cleave the backbone or

side chains, depending on the type of mannan, including β-glucosidase (EC 3.2.1.21) specific to β-glucoside of glucomannan and galactoglucomannan, α-galactosidase (EC 3.2.1.22) specific to α-galactoside of galactomannan and galactoglucomannan, and acetyl-mannan-esterase (EC 3.1.1.6) specific to O-acetyl groups of galactoglucomannan (Malgas, van Dyk, and Pletschke, 2015). β-mannanase can generally be produced by many microorganisms, both bacteria and fungi, such as Gram-positive *Bacillus subtilis*, actinomycete *Kitasatospora*, filamentous fungus *Penicillium*, and yeast-like *Aureobasidium* (Akino, Nakamura, and Horikoshi, 1987; Kremnický et al., 1996; Maitin, Athanasopoulos, and Rastall, 2004; Rahmani et al., 2017).

Members of the genus *Aureobasidium* are yeast-like fungi in the ascomycetous order Dothideales. The best-known species of this genus is *Aureobasidium pullulans*, which produces many valuable compounds and enzymes that can be used in a variety of industries (Chi et al., 2009; Prasongsuk et al., 2018). *Aureobasidium pullulans* used to be a species complex, and recently its previously reported strains have been classified into four different species, *A. pullulans*, *A. melanogenum*, *A. namibiae* and *A. subglaciale* (Barnet and Hunter, 1998; Yurlova, de Hoog, and van den Ende, 1999; de Hoog et al., 1999, Zalar et al., 2008, Gostinčar et al., 2014). In Thailand, several strains of *A. pullulans, A. melanogenum* and the novel *A. thailandense* have been isolated from different habitats in Thailand (Punnapayak et al., 2003; Prasongsuk et al., 2007; Lotrakul et al., 2009; Manitchotpisit et al., 2009; Peterson, Manitchotpisit, and Leathers, 2013; Yanwisetpakdee et al., 2016). These *Aureobasidium* strains are genetically diverse and have many prospects in production of various biopolymers, hydrolytic enzymes, and sugar alcohols such as pullulan, β-glucan, polymalate, xylanase, amylase, pullulanase, and etc. (Manitchotpisit et al., 2011; Manitchotpisit et al., 2012; Lotrakul et al., 2013; Prasongsuk et al., 2013; Bankeeree et al., 2014; Yanwisetpakdee et al., 2016; Piemthongkham et al., 2019; Patipong et al., 2019). According to Chi et al. (2009), some species of *Aureobasidium* can produce other hydrolytic enzymes including lipase, protease, β-fructofuranosidase, maltosyltransferase, siderophore, and mannanase. Kremnický and

Biely (1996 and 1998), reported that *A. pullulans* produced mannanase up to 41.5 mU/mg at 30 ℃ and pH 4.8 and these have been the only reports on optimization of mannannase production by *Aureobasidium*. Curiously, the researchers did not report the enzyme properties. Therefore, it is interesting to study *Aureobasidium* mannanase properties and optimize its production by Thai *Aureobasidium* strains and further investigate its potential in mannan hydrolysis.

Coffee (*Coffea* sp., Rubiaceae) is a flowering plant of which seed has a commodity for food and beverage. The coffee seeds, called beans, can be processed by roasting, grinding, and brewing into different types of beverages. Due to its ever-increasing popularity, coffee becomes one of the most drunk plant-base beverages in the world with approximately 10 billion kg of beans consumed in 2020 (Gökcen and Şanlier, 2019; Cornelis, 2019; International Coffee Organization, 2020: Online). As a result of the widespread coffee consumption, the spent coffee ground has become one of the most abundant plants wastes in any city with only limited utilization as compost (Banu et al., 2020). Therefore, it is of interest to explore other application of the spent coffee ground, especially in the production of value-added compounds. Since the dominant hemicellulose composition in spent coffee ground is galactomannan (Favaro et al., 2020; Redgwell et al., 2003; Nunes and Coimbra, 2002), it has a potential to be quantitatively extracted and hydrolyzed to produce MOs which reportedly shows prebiotic property (Li et al., 2020). Therefore, the purpose of this study is to optimally produce and characterize mannanase from a selected Thai *Aureobasidium* strain and use it for the hydrolysis of galactomannan from spent coffee grounds. The resulting MO prebiotic properties are also tested. The results of this study can be used for future development of a commercial MO manufacturing process from spent coffee ground mannan.

1.2 Objective of this study

1. To produce and characterize mannanase from a selected Thai *Aureobasidium* strain and use for spent coffee ground mannan hydrolysis and mannooligosaccharides preparation.

2. To test prebiotic activity of the mannooligosaccharide prepared from spent coffee ground.

1.3 Expected beneficial outcome

Results from this study will be used for mannanase production in industrial scale by using spent coffee ground extract.

1.4 Research framework

The research framework of this study displayed in the scheme (Figure 1).

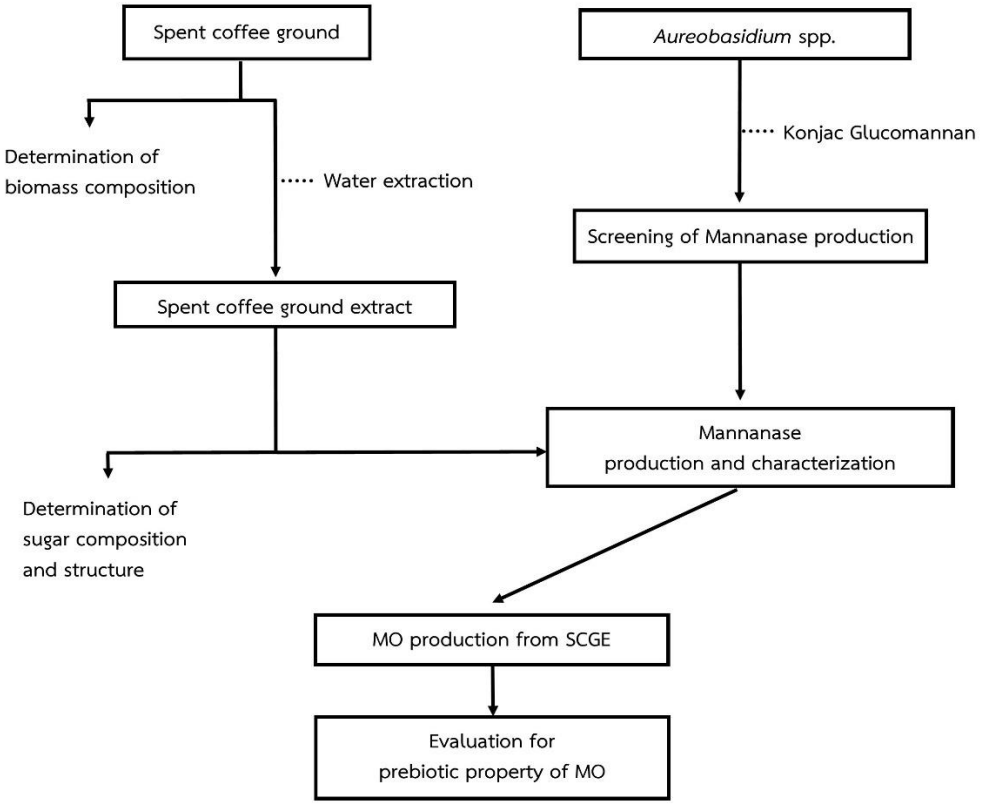

Figure 1. Research scheme of mannanase production and characterization for preparing MOs by Thai *Aureobasidium* strain.

CHAPTER II
LITERATURE REVIEW

2.1 Mannan

Mannans and heteromannans are polysaccharides which can be extensively found in nature as a part of hemicelluloses in plant tissues (Scheller and Ulvskov, 2010), and as additional constituent of glycoproteins in yeast cell walls (Sandin, 1987). In many plants, mannan is one of the major compounds of lignocellulosic biomass. While xylans represent the essential hemicellulose of hardwoods and straw, galactomannans constitute the largest hemicellulose fraction in softwoods (Puls, 1997). Although lignocellulosic biomass is the most abundant renewable organic compound on earth (Van Dyk and Pletschke, 2012), most of them are considered as biological wastes, especially those from forestry and agricultural residues, and received little interest (Moreira and Filho, 2008; Saittagaroon et al., 1983).

2.2.1 Functional and structural variations

Plant mannans and heteromannans both serve as structural components in cell wall and non-starch carbohydrate reserves inside the seeds (Rodríguez-Gacio Mdel et al., 2012) such as those of coconut (*Cocos nucifera*; Saittagaroon et al., 1983), espresso bean (*Coffea* spp.), locust (carob) bean (*Ceratonia siliqua*), and inside the vegetative tissue of konjac (*Amorphophallus konjac*), ivory nut (*Phytelephas* spp.), guar (*Cyamopsis tetragonoloba*), *Aloe vera*, etc. (Moreira and Filho, 2008).

Mannan structures are diverse depending on their origin. Hemicellulosic mannan is composed of the main chain β-1,4-linked mannose (and D-glucose) molecules and the side chain α-1,6-linked D-galactose. The differences in degree of side chain and functional group substitution result in the various types of linear or branched mannans that can be classified into four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Figure 2) (Moreira and Filho, 2008). The mannose residues of galactoglucomannan can be acetylated at C-2 and C-3 positions varying degrees, depending on the source of the polysaccharide, resulting in acetylated galactoglucomannan (Lundqvist et al., 2002).

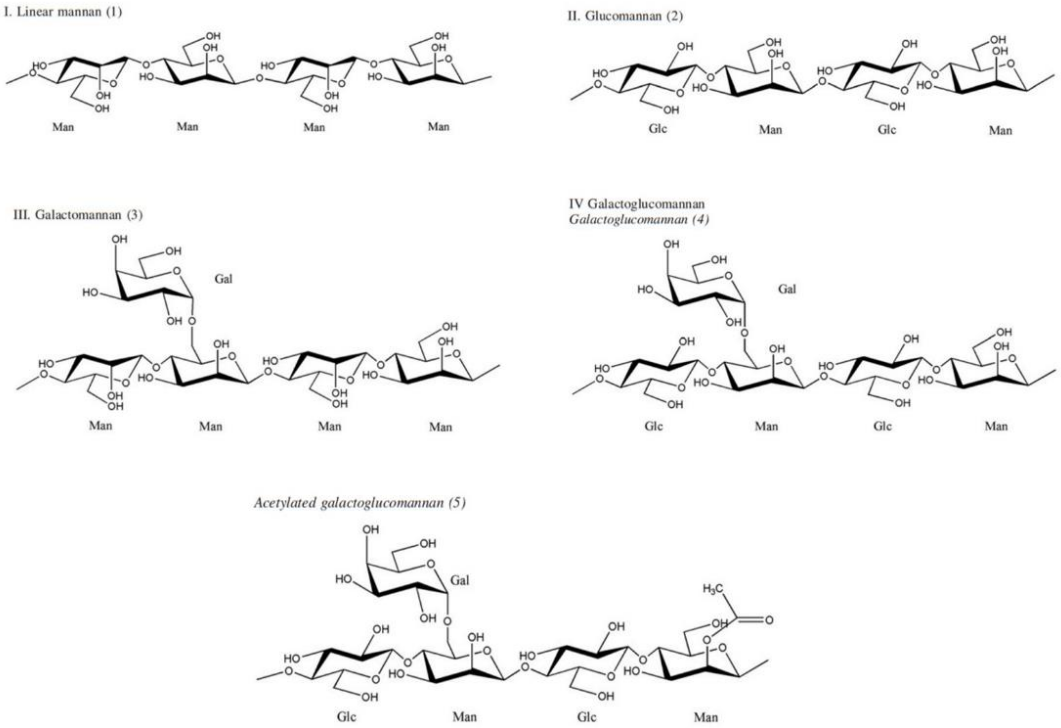

Figure 2. Various mannan structures as hemicellulose in plant (Yamabhai et al., 2016).

2.2 Mannanase

The mannan-degrading enzymes are composed of β-mannanase (1,4-β-D-mannan mannohydrolase, EC 3.2.178), β-mannosidase (1,4-β-D-mannopyranoside hydrolase, EC 3.2.1.25), and β-glucosidase (1,4-β-D-glucoside glucohydrolase, EC 3.2.1.21). Additional enzymes including acetyl mannan esterase (EC 3.1.1.6) and α-galactosidase (1,6-α-D-galactoside galactohydrolase, EC 3.2.1.22) are required to eliminate side-chain substituents connecting at numerous positions in the mannan structure (Filho, 1998). β-Mannanase, an endozyme, randomly cleaves β-1,4-connected inner linkages of the mannan backbone, providing new chain ends (Figure 3). The degradation of galactomannan and galactoglucomannan by β-mannanase is significantly hindered by the presence of side chains and substitutions of the backbone. β-Mannosidase, an exozyme, cleaves β-1,4-connected mannosides, liberating mannose from the nonreducing ends of mannans and

mannooligosaccharides (Coughlan et al., 1993; Wong and Saddler, 1993). β-Glucosidase, an exozyme, hydrolyze 1,4-β-D-glucopyranose on the nonreducing ends of the oligosaccharides released from glucomannan and galactoglucomannan by β-mannanase. α-Galactosidase, a debranching enzyme, catalyzes the hydrolysis of α-1,6-linked D-galactopyranosyl chains of galactomannan and galactoglucomannan. Acetyl mannan esterase releases acetyl companies from galactoglucomannan.

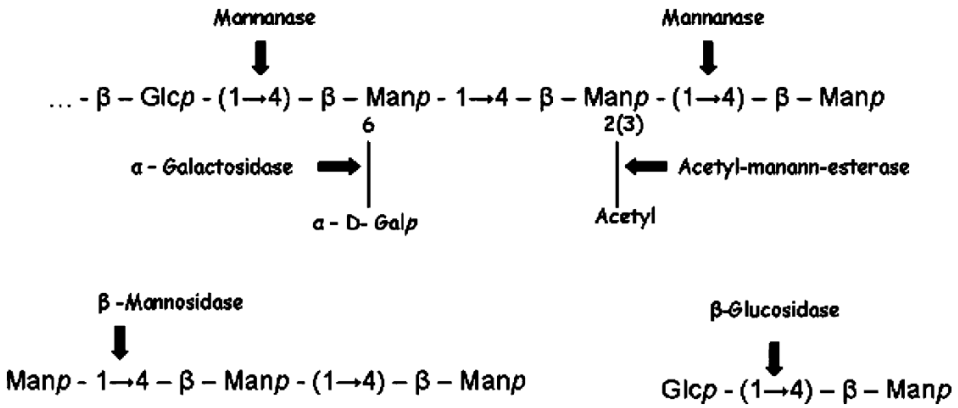

Figure 3. Enzymatic mechanism at galactoglucomannan structures (Puls, 1997).

such as, bacteria, yeast, mold, and mushroom produce mannanases. These microbial mannanases exhibit different properties, such as optimal pH and temperature and stability. The most studied prokaryotic mannanases are from *Bacillus* spp. One of them was alkaliphilic (pH 9.0) and was produced at temperature as high as 70 °C (Ma et al., 2004). There also have been a number of reports on mannanase production by filamentous fungi such as *Penicillium occitanis* Pol6 which was optimally produced at 40 °C and pH 4.0 (Blibech et al., 2010), and *Ceriporiopsis subvermispora* growing on pine wood chips at acidic pH and 50 °C (Zhu et al., 2016). In addition, Kremnický et al. (1996) reported mannanase production from a black yeast, *Aureobasidium pullulans* NRRL Y-2311-1 in acidic environments.

2.3 *Aureobasidium* spp.

The genus *Aureobasidium* (Ascomycota: Dothideales) comprises the yeast-like fungi commonly found in various habitats worldwide. The yeasts are generally

referred as black yeasts due to melanin pigment accumulation that causes the dark coloration of the cells/colonies (de Hoog, 1993). The most prominent characters usually related to *Aureobasidium* yeasts are the elongate, branching, septate filaments, massive chlamydospores and smaller elliptical yeast-like cells. The colony colorization, starting as yellow, cream, light purple or light brown during early growth stage, characteristically turns black or dark olivaceous green due to chlamydospore formation when aged (Chi et al., 2009).

Specific and subspecific classification of the genus *Aureobasidium* are traditionally based on morphology, physiology, and metabolic products. According to these characters, four varieties of *A. pullulans*, the most well-known member of the genus, had been recognized previously, namely, *A. pullulans* var. *pullulans* (Viala and Boyer, 1891), *A. pullulans* var. *melanogenum* (Hermanides-Nijhof, 1977), *A. pullulans* var. namibiae (Zalar et al., 2008), and *A. pullulans* var. *subglaciale* (Zalar et al., 2008). In 2014, genome sequence analysis of these varieties revealed significant and sufficient differences enough to separated them into four different species, namely, *A. pullulans*, *A. melanogenum*, *A. subglaciale*, and *A. namibiae*, respectively (Gostinčar et al., 2014). Additional species of *Aureobasidium* were also proposed following multilocus analyses using different regions of the rDNA gene clusters, including *A. leucospermi*, *A. proteae* (Crous et al., 2011), and *A. thailandense* (Peterson et al., 2013).

Different strains of *Aureobasidium pullulans* exhibit distinctive biochemical characteristics. Since they are ubiquitous and can be found in various habitats and environmental situations, the yeasts have been considered as a great resource for biotechnological exploitation (Table 1). Many reports have shown their high potential in various agricultural and industrial applications (Prasongsuk et al., 2017).

Table 1. *Aureobasidium* spp. products (Prasongsuk et al., 2017; Bozoudi and Tsaltas, 2018).

Products	Specific products	Reported studies
Anti-microbial	Aureobasidins	Zain et al. 2009; Prasongsuk et al. 2013

Table 1. *Aureobasidium* spp. products (Prasongsuk et al., 2017; Bozoudi and Tsaltas, 2018) (continued).

Products	Specific products	Reported studies
Enzymes	Amylase	Manitchotpisit et al. 2011
	Cellulase	Leite et al. 2008
	Lipase	Leathers et al. 2013
	Xylanase	Ohta et al. 2010
	Protease	Xiumei et al. 2008
	Laccase	Rich et al. 2013
	Mannanase	Chi et al. 2009
Polysaccharides	Pullulan	Cheng et al. 2011
	β-glucan	Li et al., 2015
	Aubasidan-like β-glucan	Lotrakul et al. 2013
Others	Poly (β-L-malic acid)	Manitchotpisit et al. 2012
	Liamocins	Manitchotpisit et al. 2014
	Siderophores	Bozoudi and Tsaltas, 2018

2.4 Spent coffee ground

Coffee is the second most valuable commodity in the world after oil and its derivatives (Tucker, 2011). It is traded mainly as coffee beans and coffee grounds. Coffee grounds are made from coffee beans and used to make drinks popular worldwide. In 2015, global coffee consumption reached approximately 9.1 billion kilograms (International Coffee Organization, 2020). Considering such massive consumption quantity, the processing of coffee cherries, collectively with the milling of dried beans, the roasting of green coffee beans and coffee brewing, produces annually an enormous amount of biowaste, which contributes to environmental pollution. The global manufacturing of instant espresso and coffee brewing generates approximately 6 million tons of spent coffee grounds (SCG) yearly (Getachew and Chun, 2017).

SCG comprises hemicellulose (32–42 % w/w), cellulose (7–13 % w/w), lignin (0–26 % w/w), protein (10–18 % w/w), lipids (2–24 % w/w), CGAs (1–3 % w/w), caffeine (0–2 % w/w) and ashes (1–2 % w/w), (Table 2). The inorganic part consists of plentiful microelements including potassium, magnesium, calcium, iron and phosphorous (Mussatto et al., 2011). The major portions of bioactive chemical substances in SCG are worthy enough for recovery and valorization for nutraceutical, pharmaceutical, meals or quality chemical industries. On the other hand, the ecotoxicity of SCG, in which it is reportedly poisonous to aquatic organisms and has mutagenic and genotoxic activities, provides a similarly motivation to utilize it instead of discarding in landfill (Fernandes et al., 2017).

Table 2. Components (wt (%)) of green, roast coffee and spent coffee ground (Massaya et al. 2019).

Component	Green coffee	Roasted coffee	Spent coffee ground
Hemicellulose	3–10	Not determined	32–42
Cellulose	32– 43	Not determined	7–13
Lignin	1– 3	3	0–26
Lipids	8–18	10–16	2–24
Proteins	9–15	8–17	10–18
Ash	3–5	4–6	1–2
Caffeine	1– 3	1–3	0–0.4
Chlorogenic Acids	1–12	2–9	1– 3
Moisture	9–10	1	50– 60
Pectins	2	2	0
Total sugars	Not determined	Not determined	7–14
Total Amino Acids	7–11	5–6	Not determined
Total dietary fibre	Not determined	47–50	21–59

2.4.1 SCG Mannan

Hemicellulose is the major carbohydrate in SCG, consisting of galactomannan and arabinan (Massaya et al., 2019), which has potential for bioproduct production (Table 3). SCG mannan can be used for production of mannanase by with *Bacillus subtilis* GA2 and prebiotic compounds which enhance growth of lactic acid bacteria while inhibit gut pathogens (Wongsiridetchai et al., 2021). SCG mannan hydrolysate can be used for synthesis of polyols, such as bioethanol and mannitol (Campos-Vega et al., 2015; Arya and Rao, 2007), and carotenoid and polyhydroxyalkanoates (Obruca et al., 2014 and 2015).

Table 3. Utilization of SCG mannan.

Utilization	Reference
Prebiotics Anti-pathogenic compounds	Wongsiridetchai et al., 2021
Anti-cancer compounds	Hernández-Arriaga, Oomah and Campos-Vega, 2017
D-Mannose	Cho et al., 2022
Polyol products	Cho et al., 2022; Arya and Rao, 2007
Carotenoid	Obruca et al., 2015
Polyhydroxyalkanoates	Obruca et al., 2014

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Equipment

- Autoclave: Model 7SX, Foundry Co., Inc, USA
- Autopipette: Dragon-Lab, China
- Bright-field microscope: Model CH30, Olympus, Japan
- Digital laboratory shaker: Model VS-37S, Vision Scientific, Korea
- Centrifuge: Hettich, Germany
- Microcentrifuge: Model Denville 260D, Denville Scientific Inc., USA
- Fourier-transform infrared spectrophotometer: Model 6700, Thermo Nicolet Corp, USA
- Hot air oven: Model B30, Memmert, Germany
- Hot plate magnetic stirrer: C-MAG HS10, Sigma Aldrich, USA
- Laminar flow: Model BV 123, ISSOC, Thailand
- Membrane filter: Whatman No.1, GE Healthcare Bio-Sciences, Sweden
- pH meter: Model PP-50, Sartorius, Germany
- Spectrophotometer: Model V-570, Jasco Corp., Japan
- Vortex: Charan Associates Co., Ltd, Thailand
- Weight balance, 2 digits: Model BL610, Sartorius, Germany
- Weight balance, 4 digits: Model AB204-S/FACT, Mettler Toledo, USA

3.2 Chemicals

- 3, 5-dinitrosalicylic acid (DNS): Sigma-Aldrich Inc., USA
- Acetic acid: Ajex Finechem, New Zealand
- Ammonium sulphate: Ajex Finechem, New Zealand
- Ammonium nitrate: Ajex Finechem, New Zealand
- Arabic gum: Krungthepchemi, Thailand
- Bacto peptone: Difco, USA
- Beechwood xylan: Fluka, Switzerland
- Bovine serum albumin (George et al.): Ajex Finechem, New Zealand
- Calcium chloride dihydrate: Ajex Finechem, New Zealand

- Copper sulfate: Carlo Erba, Italy
- Congo red: Ajex Finechem, New Zealand
- Ethylene diamine tetra-acetic acid (EDTA): Ajex Finechem, New Zealand
- Ferrous sulfate: Fluka, Switzerland
- Guar gum: Krungthepchemi, Thailand
- Konjac glucomannan: Xi'an Pincredit Biotech, China
- Potassium hydrogen sulphate: Ajex Finechem, New Zealand
- Magnesium chloride: Scharlau, Spain
- Magnesium sulfate heptahydrate: Scharlau, Spain
- Manganese sulfate heptahydrate: Scharlau, Spain
- Methanol: Merck, Germany
- Sodium carbonate: Scharlau, Spain
- Sodium chloride: Scharlau, Spain
- Sodium hydroxide: Ajex Finechem, New Zealand
- Sodium nitrate: Ajex Finechem, New Zealand
- Xanthan gum: Krungthepchemi, Thailand
- Zinc sulfate heptahydrate: Scharlau, Spain

3.2 Procedures

3.3.1 Spent coffee ground preparation, composition determination, and mannan extraction

3.3.1.1 Preparation of spent coffee ground

Spent coffee ground (SCG from *Coffea arabica* roasted beans) was collected from a commercial coffee shop in Bangkok, Thailand, oven dried at 60° C for three days or until constant weight was reached and sieved to the particle size of <1 mm before further used.

3.3.1.2 Determination of biomass composition

Biomass composition of SCG including cellulose, hemicellulose, lignin, and ash was measured according to protocol from Goering and Van Soest (1970).

3.3.1.3 Extraction of hemicellulose

The extraction of hemicellulose, with a focus on mannan, was carried out with water extraction according to Nunes and Coimbra (2002) at 121 °C and 15

lbs/inch^2. The extraction was optimized using the factorial design with the combinations of incubation time (30, 60 and 90 min) and solid : liquid ratio (1:10, 1:20, 1:30 and 1:40). The extract yield (true recovery) was calculated as the percentage of total hemicellulose in SCG.

3.3.1.4 Determination of sugar composition and structure of mannan

Sugar composition of the extracted mannan was determined after acid hydrolysis by thin layer chromatography (TLC) (Ovodov et al., 1966) and high-performance liquid chromatography (HPLC) (Sluiter et al., 2010) with mannose, glucose, and galactose as standards. The mannan structure was analyzed using Fourier-transform infrared spectroscopy (FTIR) (Liu et al., 2020).

3.3.2 Mannanase screening of *Aureobasidium* spp.

3.3.2.1 Aureobasidium spp. cultivation

Thirty-nine lyophilized stocks of *Aureobasidium* spp. were obtained from Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand (Table 4). The reference strain, *A. pullulans* Y-2311-1, was obtained from the ARS Culture Collection, USDA, Peoria, IL, USA. The yeasts were re-cultured on yeast malt (YM) agar and incubated for 3 days at room temperature (28 ± 2 ºC). The cultures were stored at 4 ºC for the short-term storage and freeze dried before stored at -20 ºC for the long-term storage (Bankeeree et al., 2014).

Table 4. Isolate collection that used in this experiment.

Isolate (Strain)	Reference	Isolate (Strain)	Reference
A. pullulans	Manitchotpisit	NRRL58551	Manitchotpisit
NRRL Y-2311-1*	et al., 2007	NRRL58552	et al., 2007
NRRL58516		NRRL58554	
NRRL58519		NRRL58557	
NRRL58522		NRRL58558	

Table 4. Isolate collection that used in this experiment (continued).

Isolate (Strain)	Reference	Isolate (Strain)	Reference
NRRL58523		NRRL58559	
NRRL58524		*A. melanogenum*	Yansiwetpakdee
NRRL58525		AP13	et al., 2016
NRRL58526		AP22	
NRRL58527		AP34	
NRRL58528		AP44	
NRRL58536		AP46	
NRRL58538		AP50	
NRRL58539		AP55	
NRRL58540		AP58	
NRRL58541		AP63	
NRRL58542		AP71	
NRRL58543		AP73	
NRRL58544		AP75	
NRRL58547		*A thailandense*	
NRRL58549		AP77	
NRRL58550			

3.3.2.2 Primary screening

The selected *Aureobasidium* strains was screened for mannanase activity on minimal medium agar (Leather, 1986) containing 3% (w/v) konjac glucomannan (KG) as the sole carbon source. After 3-day incubation at room temperature (28 ± 2 ºC), medium plates were stained with 0.1% (w/v) Congo Red for 10 minutes and washed with 1 M NaCl solution. The presence of a clear halo was considered as positive result. The mannanase activity index was determined as the ratio between the halo zone and the colony diameter (Ibrahim et al., 2018).

3.3.2.3 Secondary screening

The mannanase positive strains were inoculated in YM broth and incubated for 3 days at room temperature with agitation at 150 rpm. The cultures were used as the inoculums after adjusted to 1.0×10^7 cells/mL (Prasongsuk et al., 2005) and sub-cultured (0.1 %, v/v) in mannanase production medium containing 0.5% (w/v) KG (Leathers, 1986) for 3-day incubation under the same condition. After incubation, cells were removed by centrifugation (6,000 × g, 10 minutes) and the supernatants were used as the crude enzyme. Mannanase activity of each crude enzyme was quantitatively assayed in a reaction mixture containing 0.1 % (w/v) KG as substrate in 50 mM acetate buffer (pH 4.7) at 50 °C. The reaction mixture was incubated for 30 minutes and the amounts of reducing sugars were determined using the 3,5-di-nitro salicylic acid (DNS) assay (Miller, 1959; Yang et al., 2019). Mannose was used to construct the standard curve. One unit (U) of mannanase was defined as the amount required to release 1 μmole mannose equivalent per minute. Protein content was determined by Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard. The experiment was performed in triplicate and the result were presented as mean ± one standard deviation. The strain with the highest mannanase activity was selected for further study.

3.3.3 Mannanase production and characterization

3.3.3.1 Optimization of mannanase production

Two carbon sources (konjac glucomannan and SCG mannan) were used at 0.5 % (w/v) whereas five nitrogen sources (yeast nitrogen base, peptone, ammonium nitrate, ammonium sulphate, and sodium nitrate) were used at 0.67 % (w/v) in the production medium for the mannanase production optimization using one-factor-at-a-time approach. After the combination of the carbon and nitrogen sources that yielded the highest activity was selected, the optimal concentration of those selected carbon and nitrogen was analyzed by using Response Surface Methodology (RSM). The experiment was designed based on Central Composite Design (CCD) with 5 levels for each variable and the relationships within and between variables were analyzed from the responses (mannanase activity) using Analysis of Variance (ANOVA)

to obtain the optimum point. The design was constructed by Design Expert 7 (Stat-Ease Inc., Minneapolis, U.S.A.). The effect of L-asparagine supplementation was determined using the optimized medium. The enzyme production curve was constructed to determine the suitable harvest time by observing the enzyme activity in the optimized production medium every 24 hours for five days.

3.3.3.2 Mannanase characterization

3.3.3.2.1 Optimal temperature and pH

The optimal condition for the mannanase activity was determined based on factorial design. The reaction mixtures (section 2.3) with different combinations of pH (4.0-9.0) and temperature (45 to 75 °C) were incubated for 30 minutes prior to reducing sugar determination by DNS assay. Four different buffers were used including 50 mM citrate buffer (pH 3.0-4.0), 50 mM acetate buffer (pH 4.0-5.0), 50 mM phosphate buffer (pH 5.0-8.0), and 50 mM Tris-HCl buffer (8.0-9.0) (Bankeeree et al., 2014). The experiment was performed in triplicate and the results were presented as mean ± one standard deviation.

3.3.3.2.2 Thermostability and pH stability

The thermostability was determined by incubating the crude enzyme in sterile distilled water at 50, 55, 60, 65, and 70 °C without substrate. The incubated enzyme was collected every hour up to 5 hours. The residual activity was assayed under the optimal condition. The activity of crude enzyme collected at 0 minute and kept at 4 °C was used as the control and defined as 100 % residual activity (Bankeeree et al., 2014). The pH stability was conducted by incubated the enzyme in different pH (4.0-7.0) without substrate at 4 °C and collected every hour up to 5 hours. The residual activity was assayed under the optimal condition, and the activity of the enzyme collected at 0 minute was considered as 100 %.

3.3.3.2.3 Substrate specificity

The crude enzyme was incubated with nine selected substrates including KG, SCG mannan, guar gum (GG), Arabic gum (AG), xanthan gum (XG), β-glucan, beechwood xylan, cellulose, and carboxymethyl cellulose (CMC) at the final concentration of 0.5 % (w/v) (Bankeeree et al., 2014). Enzyme activity was assayed

under the optimal condition and the activity with KG as the substrate was considered as 100 %.

3.3.3.2.4 The effects of ions and chelator on the mannanase activity

Nine ions, calcium ($CaCl_2$), cobalt ($CoCl_2$), copper ($CuCl_2$), potassium (KCl), iron ($FeSO_4$), magnesium ($MgSO_4$), manganese ($MnSO_4$), sodium (NaCl) and zinc ($ZnSO_4$) and an ion chelator, Ethylenediaminetetraacetic acid (EDTA), were individually added to the reaction mixture at the final concentration of 1 mM, and the crude enzyme was assayed under the optimal condition (Bankeeree et al., 2014). The relative activity was calculated as the percentage of that without metal ion or chelator added.

3.3.3.2.5 Feedback inhibition

Mannose and galactose were separately added to the reaction mixtures (KGM as the substrate) at the final concentration ranging from 10 to 60 mM. Enzyme activity was assayed under the optimum condition and the relative activity was calculated as the percentage of that with neither mannose nor galactose added (Bankeere et al., 2014).

3.3.4 MO production from SCG mannan

The extracted mannan 1 % (w/v) was hydrolyzed by the crude mannanase under the optimal condition with 150 rpm agitation. Hydrolysis reaction was terminated by boiling the mixture for 15 minutes. The total reducing sugar was determined by the DNS assay and the content of mannose and mannooligosaccharide was analyzed using TLC and HPLC (Patipong et al., 2019; Sluiter et al., 2010). The optimal enzyme unit and incubation time was determined by using RSM. The experiment was designed based on CCD with 5 levels in each variable and the relationship within each variable was analyzed from the response of total content of MO using ANOVA to obtain the optimum point, the design was constructed by Design Expert 7 (Stat-Ease Inc., Minneapolis, U.S.A.). The obtained MO was lyophilized and used for further study.

3.3.5 Evaluation for prebiotic property of MO

Seven probiotic bacteria; *Lactobacillus casei* TISTR 390, *L. brevis* TISTR 868, *L. acidophilus* TISTR 2365, *L. casei* subsp. *rhamnosus* TISTR 047, *Bifidobacterium longum* subsp. *longum* TISTR 2195, *B. breve* TISTR 2130 and *B. animalis* subsp. *animalis* TISTR 2194 were used for prebiotic activity test. The bacterial stocks were maintained on Man Rogosa Sharpe (MRS) agar at 37 °C. The seed culture was prepared by inoculating the bacterial stocks to MRS broth (20 mL) and incubated for 16 hours at room temperature with agitation at 150 rpm. The bacterial seed culture was transferred at 1 % (v/v) in MRS broth supplemented with either MOs or commercial yeast β-glucan at 2 mg carbon ml^{-1}. Glucose was used as the negative control and the medium without carbon source added as used as blank. The cultures were incubated at 37 °C with static condition in the anaerobic jar for 18 hours. Plate count technique was used for assessing the bacterial cell number (Patipong et al., 2019; Zhou et al., 2019).

3.3.6 Statistical analysis

Statistical differences between average values were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) using SPSS 22.0 software package (SPSS Inc., Chicago, U.S.A.). The differences at $P<0.05$ were considered significant.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Spent coffee ground extraction and characterization

4.1.1 Biomass composition

The major chemical composition of SCG comprised of extractive (34%), hemicellulose (28%), cellulose (23%), and lignin (13%) whereas only a trace amount of ash was present (Table 5) which was similar to the previously reported coffee ground by Girotto et al. (2018). However, the hemicellulose content was much lower than that profiled by Gu et al. (2020) (49.9%) which was possibly caused by the different of coffee bean and analysis chemical composition method.

Table 5. Biomass composition of untreated SCG.

Composition	This study	Gu et al. (2020)	Girotto et al. (2018)
Extractive	34.3[a]	ND[b]	ND[b]
Hemicellulose	28.5[a]	49.9[a]	24.8[a]
Cellulose	23.1[a]	30.1[a]	24.3[a]
Lignin	13.4[a]	27.5[a]	13.5[a]
Ash	0.4[a]	ND[b]	ND[b]

[a] Content percentage

[b] ND = Not determined

4.1.2 Mannan extraction and structural analysis

The major sugar monomers found in the extracted SCG hemicellulose were mannose and galactose (Fig. 4A). Similar sugar compositions were reported earlier from green and roasted coffee grounds although a small amount of arabinose and glucose was also detected (Navarini et al., 1999; Nunes et al., 2005; Passos et al., 2019; Moreira et al., 2019). FTIR spectra of the SCG hemicellulose extracted by hot water and NaOH (Fig. 1B) showed peaks typical of galactomannan previously reported (Nascimento et al., 2019), including those at 3600-3100 cm^{-1} (-OH), 1742 cm^{-}

^{1} (C=O), 1156 cm^{-1} (C-O), 1000 cm^{-1} (C-O-H) 815 cm^{-1} (α-configuration) and 872 cm^{-1} (β-configuration) (Ballesteros et al., 2014; Soares et al., 2014; Nascimento et al.). Although the FTIR spectra were almost identical (Fig. 4B), the ratio of mannose to galactose was higher in the SCG mannan extracted by hot water than that of the alkaline extraction (Fig. 4A). Radgwell et al. (2003) reported a higher amount of galactose obtained when the coffee bean was extracted by KOH compared to water, possibly due to the solubilization of arabinogalactan in alkaline condition. Together, these results indicated that the hemicellulose extracted from SCG by hot water was galactomannan and, therefore, the hot water extraction was opted for the subsequent SCG mannan extraction.

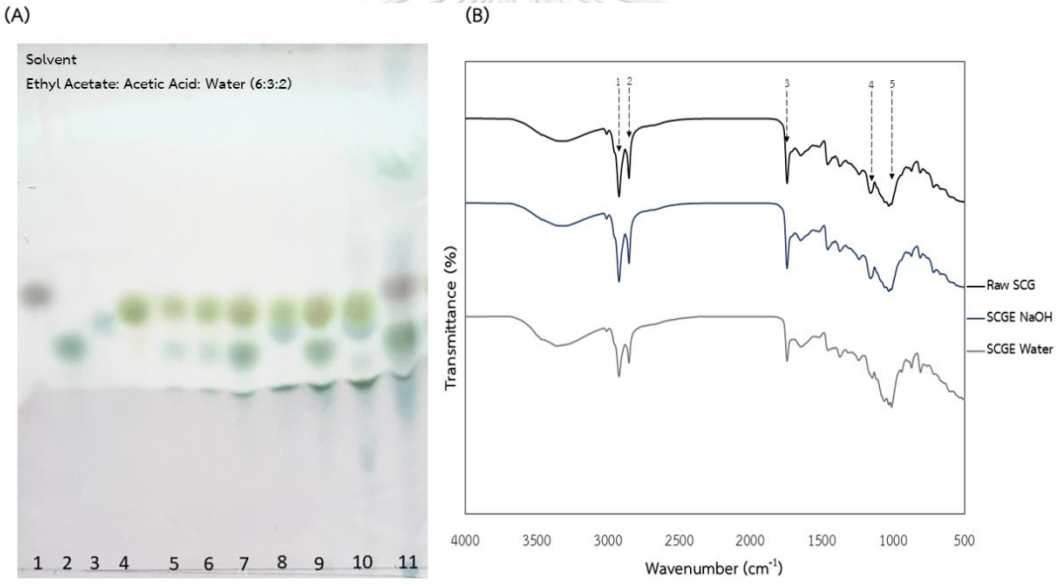

Figure 4. TLC of sugars obtained from the complete acid hydrolysis of SCG mannan (A); lane 1-4: arabinose, glucose, galactose, and mannose standards, lane 5: untreated SCG, lane 6: hot water extracted SCG mannan, lane 7: NaOH extracted SCG mannan, lane 8: KGM, lane 9: GG, lane 10: XG, and lane 11: AG; FTIR spectra (B) of the untreated SCG, NaOH extracted SCG mannan and hot water extracted SCG mannan. Arrows indicate peaks typical of galactomannan.

When the hot water extraction was further optimized, the highest mannan yield (9.5 g/100 g SCG, ~33 % recovery) was obtained at 1:30 solid to liquid ratio and

60-minute extraction period (Table 6). The optimal solid to liquid ratio here was lower than those reported earlier at 1 : 10 using microwave (Passos and Coimbra, 2013) and bomb vessel on hot oil bath (Gu et al., 2020). However, using autoclave as in this study was simple to manage and allowed a larger scale extraction. Its lower temperature (121°C compared to ≥ 140 °C) might gave a slightly lower yield, but also a lower risk of mannan structural degradation and contamination of arabinogalactan (Passos et al., 2019; Lopes et al. 2020). The mannan yield at obtained in this study was comparable to that reported earlier by Passos et al. (2019) at 8.9 g/ 100 g SCG (140 °C) and Gu et al. (2020) at 14.2 g/ 100g SCG (140 °C).

Table 6. SCG mannan yields obtained from various conditions of hot water extraction.

Ratio (w/v)	Hemicellulose yield (g/100 g substrate)†		
	30 min	60 min	90 min
1:10	6.5 ± 0.07 ^{ef}	6.3 ± 0.40 ^{f}	7.2 ± 0.60 $^{d-f}$
1:20	8.6 ± 0.80 ^{abc}	8.7 ± 0.85 ^{abc}	9.1 ± 0.36 ^{ab}
1:30	9.3 ± 0.45 ^{ab}	9.5 ± 0.64 ^{a}	9.0 ± 0.35 ^{abc}
1:40	7.9 ± 0.70 ^{cde}	8.1 ± 0.23 ^{bcd}	7.0 ± 0.85 ^{ef}

† Mean ± SD derived from three replicates. The different superscript letters indicated significant differences at $P < 0.05$ (ANOVA and DMRT).

4.2 Mannanase screening of *Aureobasidium* spp.

Based on primary screening, the positive strains covered all three species tested, *A. pullulans*, *A. melanogenum*, and *A. thailandense*, and the mannanase activities were produced ranging from 0.22 to 8.07 U/mL. Among 40 strains screened, the best mannanase producer was *A. pullulans* NRRL 58524, with the highest index activity (+++++) and enzyme activity at 8.07 U/mL (Table 6). Although the primary plate screening was efficient for identifying mannanase positive strains, the activity index was not always correlated with the enzyme activity quantitatively determined by the mannanase assay. Such discrepancies were apparent in the high mannanase-

producing *A. pullulans* NRRL58542 (6.40 U/mL) and NRRL58538 (7.27 U/mL) that exhibited low activity index (++) and the low mannanase-producing NRRL58543 (2.40 U/mL) and NRRL58559 (1.08 U/mL) that showed high activity index (++++ and +++, respectively). The only *A. pullulans* strain previously reported for mannanase, NRRL Y-2311-1 (Kremnický et al. 1996), produced the enzyme at a much lower yield, 6.39 U/mL under the same condition. In previous report by Kremnický et al. (1996), the *A. pullulans* NRRL Y-2311-1 produced mannanase at 1,000-fold lower (0.633 U/mL) than this study, which might be caused by different production medium and sugar determination method used. In addition, this study was the first report of mannanase production in *A. melanogenum* and *A. thailandense*. The highest mannanase production in these two species were observed in *A. melanogenum* AP13 and *A. thailandense* AP77 with 3.74 U/mL and 0.38 U/mL, respectively. Therefore, *A. pullulans* NRRL 58524 was selected for the following mannanase production optimization and characterization.

Table 7. Mannanase activity of *Aureobasidium* spp. screened in this study.

Isolate (Species / Strain)	Index Activity	Enzyme Activity (U/mL)†	Isolate (Species / Strain)	Index Activity	Enzyme Activity (U/mL)†
A, pullulans			NRRL58551	+++++	7.04 ± 0.26 ^{cd}
NRRL Y-2311-1*	+++++	6.39 ± 0.42 ^{ef}	NRRL58552	+++++	7.22 ± 0.16 ^{bc}
NRRL58516	++	2.44 ± 0.22 ^{h}	NRRL58554	++++	6.30 ± 0.28 ^{ef}
NRRL58519	+++	0.95 ± 0.34 $^{j-l}$	NRRL58557	+++	7.57 ± 0.17 ^{ab}
NRRL58522	++	0.69 ± 0.27 $^{l-n}$	NRRL58558	++++	6.58 ± 0.30 $^{d-f}$
NRRL58523	+++	6.02 ± 0.28 ^{f}	NRRL58559	+++	1.08 ± 0.32 $^{i-l}$
NRRL58524	+++++	8.07 ± 0.12 ^{a}	*A. melanogenum*		
NRRL58525	ND	-	AP13	+++	3.74 ± 0.30 ^{g}
NRRL58526	++	6.62 ± 0.45 ^{de}	AP22	+	0.89 ± 0.10 $^{k-m}$
NRRL58527	++	6.43 ± 0.57 ^{ef}	AP34	++	0.66 ± 0.14 $^{l-n}$
NRRL58528	+	2.32 ± 0.03 ^{h}	AP44	++	2.71 ± 0.33 ^{h}
NRRL58536	+++	5.66 ± 0.43 $^{d-f}$	AP46	+++	3.28 ± 0.30 ^{g}

Table 7. Mannanase activity of *Aureobasidium* spp. screened in this study (continued).

Isolate (Species / Strain)	Index Activity	Enzyme Activity (U/mL)†	Isolate (Species / Strain)	Index Activity	Enzyme Activity (U/mL)†
NRRL58538	++	7.27 ± 0.03 ^{bc}	AP50	++++	1.28 ± 0.20 $^{i-k}$
NRRL58539	++	1.03 ± 0.07 $^{i-l}$	AP55	+	0.64 ± 0.17 $^{l-n}$
NRRL58540	+++	6.26 ± 0.51 ^{ef}	AP58	++	0.22 ± 0.06 ^{n}
NRRL58541	+++	6.41 ± 0.36 ^{ef}	AP63	+	1.17 ± 0.21 $^{i-l}$
NRRL58542	++	6.40 ± 0.19 ^{ef}	AP71	+	1.47 ± 0.34 ^{ij}
NRRL58543	++++	2.40 ± 0.06 ^{h}	AP73	+	1.57 ± 0.13 ^{i}
NRRL58544	+++	6.77 ± 0.50 ^{de}	AP75	++	2.67 ± 0.14 ^{h}
NRRL58547	+++	3.51 ± 0.24 ^{g}	*A. thailandense*		
NRRL58549	ND	-	AP77	+	0.38 ± 0.10 ^{mn}
NRRL58550	++	1.26 ± 0.21 $^{i-k}$			

† Mean ± SD derived from three replicates. The different superscript letters indicated significant differences at $P < 0.05$ (ANOVA and DMRT).

*Reference isolate

Index activity:

ND (Not Detectable) = 1.0

\+ = 1.01—1.10

++ = 1.11—1.20

+++ = 1.21—1.30

++++ = 1.31—1.40

+++++ = ≥1.4

4.3 Mannanase production and characterization

4.3.1 Optimization of mannanase production

One-factor-at-a-time design was employed to select the optimal medium composition, especially the carbon and nitrogen sources and amino acid supplementation. The carbon sources tested were KGM and SCG mannan (Fig. 5A) with yeast nitrogen base as the nitrogen source. The mannanase activity in the medium containing KGM was significantly higher than that containing SCG mannan at 8.1 and 6.9 U/mL, respectively. Among five nitrogen sources selected (with SCG mannan as the carbon source), ammonium sulphate gave the highest activity at 7.94 U/mL (Fig 5B). When compared with the screening medium containing KGM and yeast nitrogen base (7.97 U/mL) (Fig 5C), there was no significant difference in the mannanase activity produced in the medium containing SCGE and ammonium sulphate (7.73 U/mL). Since the substrate cost for mannanase production (0.03$ per 100 U) was much lower (up to 30-fold) when SCG mannan and ammonium sulphate were used compared with that containing KGM and yeast nitrogen base (1.13$ per 100 U), they were selected as the carbon and nitrogen sources for the following medium optimization. Since previous reports have also showed that the supplementation of L-asparagine could enhance the enzyme production (Leather et al., 1986), L-asparagine was added to the optimized medium at 2 g/L. However, no significant difference was observed between medium without and with L-asparagine supplementation (Fig. 5D). Therefore, L-asparagine supplementation was omitted due its high cost.

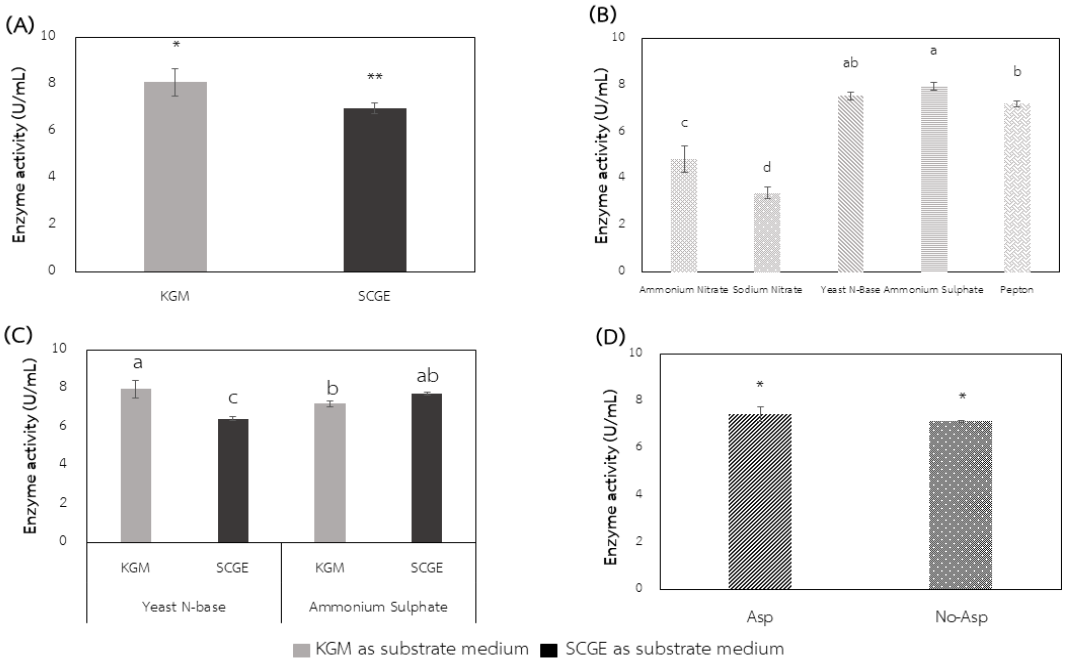

Figure 5. Medium optimization for mannanase production. (A) carbon source selection, (B) nitrogen source selection, (C) comparison of carbon and nitrogen combinations, and (D) effects of L-asparagine supplementation. Mannanase activity is shown as the mean ± SD, derived from triplicate. Different letter and asterisk above each bar indicate a significantly different value (ANOVA and DMRT and student's t-test where applicable, $P < 0.05$).

To further optimize the carbon and nitrogen concentrations, CCD with two variables and 5 levels and RSM were employed (Table 8 and Figure 6). The response (mannanase activity, Y) could be expressed by the equation:

$$Y = +7.16 + 0.33X_1 + 0.11X_2 + 0.11X_1X_2 - 0.20{X_1}^2 - 0.17{X_2}^2,$$

where *Y* is the mannanase activity (U/mL), X_1 is the SCGE concentration (%, w/v), and X_2 is the ammonium sulphate concentration (%, w/v).

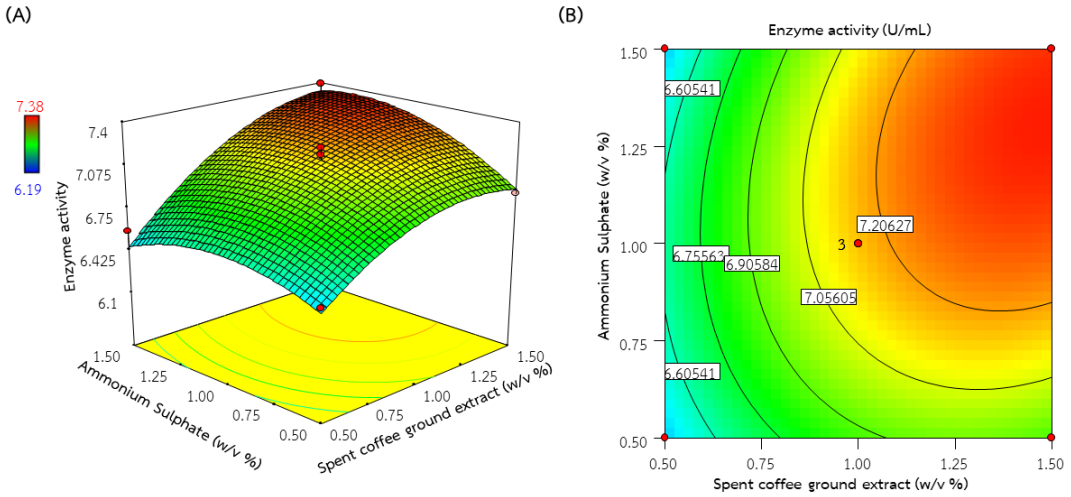

Figure 6. Response surface methodology for mannanase production optimization. (A) the three-dimensional response surface plot and (B) the contour plot showing the effect of SCGE and ammonium sulphate on mannanase activity produced by *A. pullulans* NRRL 58524.

According to R^2 value (0.97) and insignificant lack of fit (P = 0.17), the model equation was sufficient for predicting the mannanase production across the specified ranges of variables employed (Table 9). The RSM analysis revealed that the maximum mannanase yield (7.38 U/mL) could be achieved when adjusting the concentrations of SCG mannan and ammonium sulphate to 1.5 % and 1.32 % (w/v), respectively. A separated experiment was conducted to validate the prediction and the mannanase activity obtained was 7.51 ± 0.39 U/mL, which was not significantly different from the predicted value. Even though not significantly different, by using the optimized medium, the mannanase production yield was 1.05-fold higher than the unoptimized medium. Thus, this optimized medium composition was used to further experiment.

Table 8. Central composite design matrix with experimental and predicted values of mannanase production by *A. pullulans* NRRL 58524.

Run	Code Level		Actual Level		Enzyme activity (U/mL)	
	X_1	X_2	SCGE (%, w/v)	$(NH_4)_2SO_4$ (%, w/v)	Predicted	Actual
1	-1	-1	0.50	0.50	6.46	6.49 ± 0.08
2	+1	-1	1.50	0.50	6.90	6.87 ± 0.14
3	-1	+1	0.50	1.50	6.46	6.58 ± 0.04
4	+1	+1	1.50	1.50	7.33	7.39 ± 0.17
5	-1.414	0	0.29	1.00	6.29	6.20 ± 0.20
6	+1.414	0	1.71	1.00	7.22	7.22 ± 0.43
7	0	-1.414	1.00	0.29	6.66	6.67 ± 0.20
8	0	+1.414	1.00	1.71	6.97	6.86 ± 0.26
9	0	0	1.00	1.00	7.16	7.11 ± 0.07
10	0	0	1.00	1.00	7.16	7.16 ± 0.23
11	0	0	1.00	1.00	7.16	7.21 ± 0.20

Table 9. Analysis of variance (ANOVA) of the central composite design model developed for mannanase production by *A. pullulans* NRRL 58524.

Source	Sum of Squares	Df	Mean Square	F-Value	P-value (Prob > F)
Model	1.32	5	0.26	28.38	0.0011*
A-SCG Extract	0.87	1	0.87	93.71	0.0002
B-Ammonium Sulphate	0.095	1	0.095	10.26	0.0239
AB	0.047	1	0.047	5.07	0.0740
A^2	0.23	1	0.23	24.46	0.0043
B^2	0.17	1	0.17	17.84	0.0083
Residual	0.046	5	9.300E-003		
Lack of Fit	10.36	3	0.014	4.90	0.1741
Pure Error	5.567E-003	2	2.783E-003		
Cor Total	1.37	10			

R^2 = 0.97 Adj R^2 = 0.93; Coefficient of variance = 1.40%; *Significant at $P < 0.05$.

To find the suitable harvest time, the growth and enzyme production curves were constructed by incubating the strain in optimized medium for five days at room temperature with 150-rpm agitation (Figure 7). The peak of mannanase production was reach at 72 h (three days). Although the enzyme activity continued to slightly rise at 100 and 120 h, they were not significantly different. After three days, the cell density was decreased from 2.14×10^{-8} to 1.38×10^{-8} which was likely caused by nutrient depletion (Yatmaz et al., 2020). The results were like the previous study of mannanase production by *A. pullulans* NRRLY-2311-1 which was harvested at 3 days after incubation (Kremnický et al. 1996). Therefore, for the subsequent experiments, the mannanase enzyme was harvested at day 3 for this optimized medium.

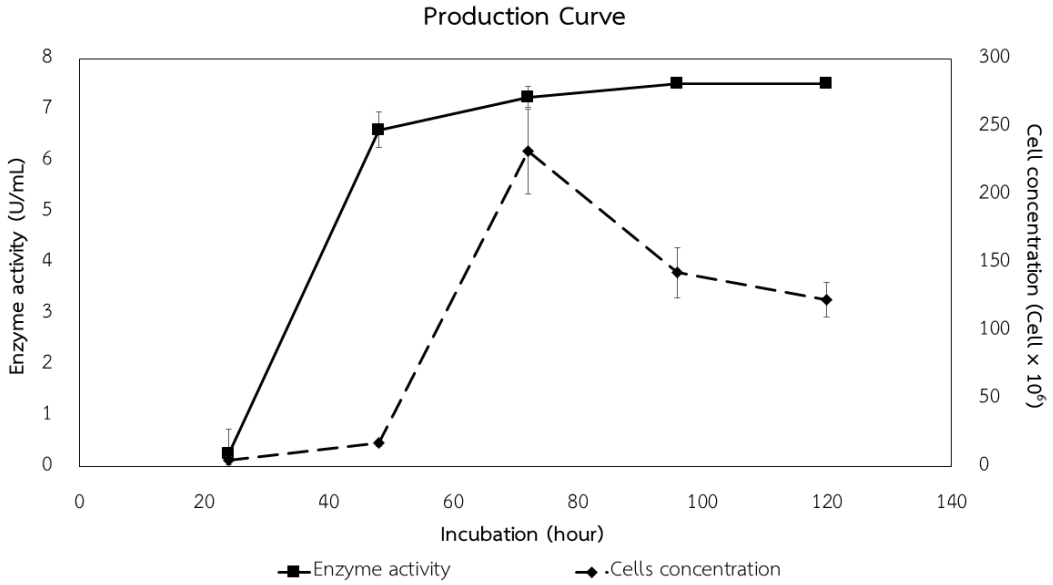

Figure 7. Mannanase production curve after optimization. Mannanase activity and cell concentration are shown as the mean ± SD, derived from triplicate. Different letters above each bar indicates a significantly different values (ANOVA and DMRT, $P < 0.05$).

4.3.2 Mannanase characterization

The crude mannanase was characterized for the optimal catalytic condition, and stability. The substrate specificity, effects of metal ion, and feedback inhibition were also determined. Mannanase activity from *A. pullulans* NRRL 58524 was the highest in 50 mM citrate buffer (pH 4.0) at 55 ℃ for 30 minutes (Figure 8). The preference for acidic pH seems to be common for fungal mannanases since similar results were reported for *Yarrowia lipolytica* (pH 4.8 for recombinant ManA/HmA) (Chiyanzu et al., 2014), *Aspergillus niger* (pH 4.8) (Favaro et al., 2020), *Aspergillus oryzae* (pH 5.0) (Jana et al., 2018) and recombinant *Pichia pastoris* (pH 6.0) (Chen et al., 2021). The optimal temperature at 50 ℃ was identical with that from *P. pastoris* (Chen et al., 2020), but it was still relatively low compared to other fungi, including 80 ℃ for *Y. lypolytica* (Chiyanzu et al., 2014), 69 ℃ for *A. niger* (Favaro et al., 2020) and 60 ℃ for *A. oryzae* (Jana et al., 2018). However, the lower temperature means the lower energy input required for the hydrolysis.

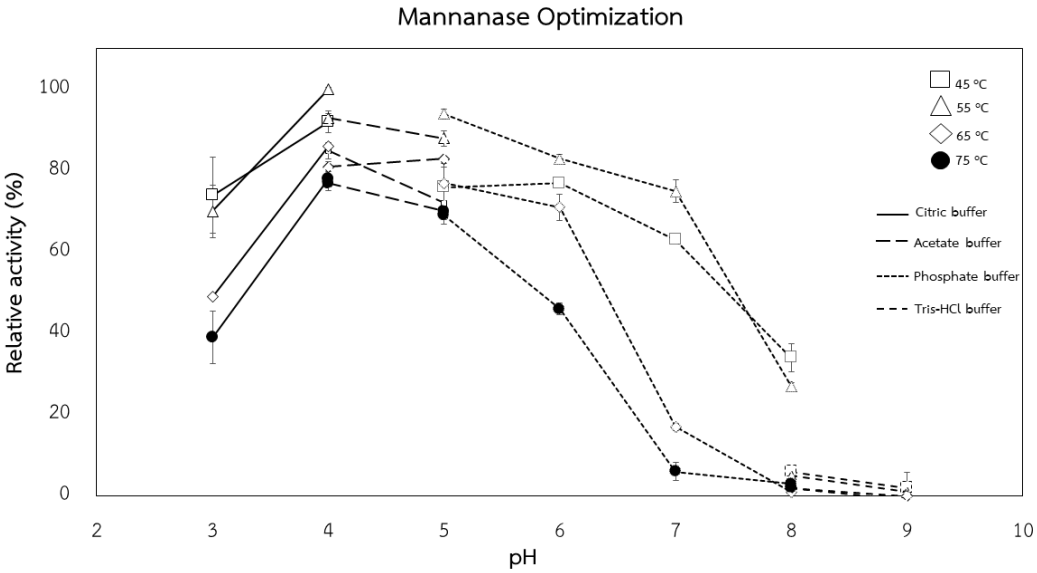

Figure 8. The effects of temperature and pH on *A. pullulans* NRRL 58524 crude mannanase activity. Relative activity is calculated as the percentage of the maximal activity and shown as the mean ± SD, derived from triplicate.

The crude mannanase from *A. pullulans* NRRL 58524 was highly stable at 50 and 55 ℃ with residual activity at 88 and 86 %, respectively after 6 h (Figure 9). The enzyme was relatively stable at 60 and 65 ℃ with more than half of the initial activity remained after 6 h. The enzyme rapidly lost its activity at 70 ℃, with entire activity depleted after 2 h. The crude mannanase from *A. pullulans* NRRL 58524 was more stable than those from other fungi such as those from recombinant *P. pastoris* (half of activity remained after 2.5 h at 60 ℃) (Chen et al., 2021), and *Lichtheimia ramosa* (40 to 60 ℃ for 30 min, above 80 % of residual activity) (Xie et al. 2020). Interestingly, for an enzyme that catalyzed well at relatively low temperature (55 ℃), it was even more stable than thermophilic mannanases such as that from *A. oryzae* (residual activity around 80% at 60 ℃ for 90 min) (Jana et al., 2018) and *Aspergillus calidoustus* (60 % residual activity after 3 h at 60 ℃) (Sun et al., 2021). Fungal mannanase with similar thermostability was reported in *A. niger* (more than 80 % residual activity after 24 h at 50 ℃) (Favaro et al., 2020).

For pH stability, the crude enzyme was highly stable in pH ranging from moderately acidic to neutral (pH 4.0 to 7.0) incubated at 4 ℃, with more than 70% residual activity after 6 h. Crude mannanase of *A. pullulans* NRRL 58524 was more stable than *A. calidoustus* (80 % residual activity for 2 h at 25 ℃ in pH 2.0-7.0) (Sun et al., 2021), recombinant *P. pastoris* (residual activity lower than 70 % in pH 6.0-8.0 after 30 minutes at 50 ℃) (Chen et al., 2021), and *A. oryzae* (in pH 5 at room temperature for 4 h the residual activity above 80 %) (Jana et al., 2018). Xie et al. (2020), was reported *L. ramosa* mannanase pH stability with residual activity more than 80 % for 24 h at 50℃ in pH 3.0-8.0 that alike crude *A. pullulans* NRRL 58524.

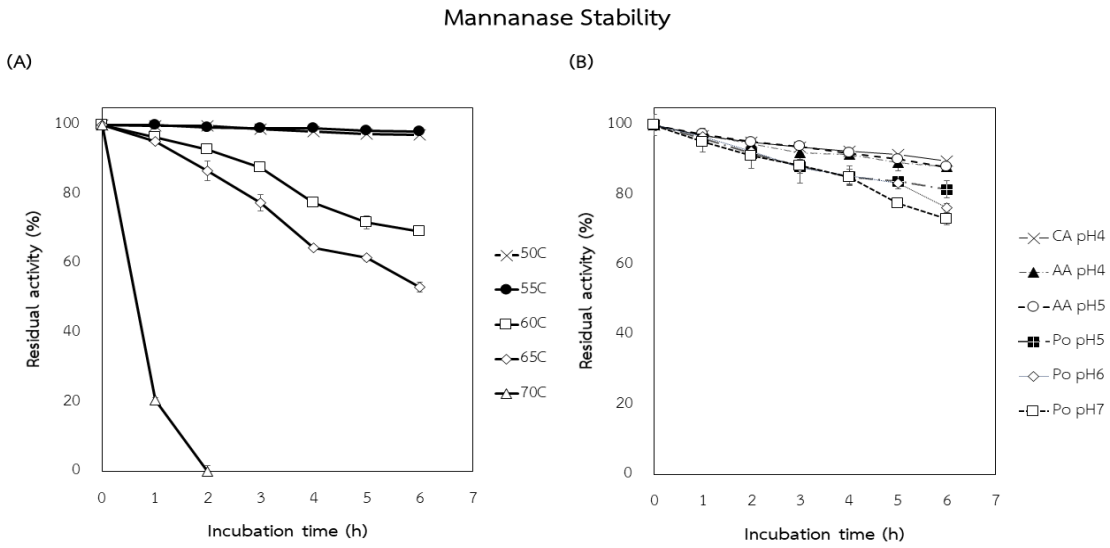

Figure 9. Temperature and pH stability of *A. pullulans* NRRL 58524 crude mannanase. Residual activity is calculated as the percentage of the initial activity and shown as the mean ± SD, derived from triplicate.

The substrate specificity was conducted by using selected β-linked plant polysaccharides with different structures and sugar compositions, including β-linked between glucose and mannose (KGM), β-linked between mannoses (SCG mannan and GG), β-linked between glucoses (β-glucan, XG, cellulose and CMC), β-linked between various hexoses (AG) and β-linked between xyloses (a pentose) (xylan) (Table 10). The results showed that the crude mannanase from *A. pullulans* NRRL 58524 was highly specific to glucomannan from konjac (Table 10). It could also digest

galactomannan from SCG and guar gum although with much lower rate. Limited digestion was observed with β-glucan, AG, XG, cellulose and CMC. Xylan, the polysaccharide containing pentoses could not be digested by the enzyme. Overall, the enzyme is highly specific to β-linked aldohexoses, especially between glucose and mannose and, to a lesser extent, between mannoses. Highly specific enzyme such as this has an advantage in a selective digestion of mixed polysaccharide substrates in which purification step can be omitted to reduce the production cost.

Table 10. Enzyme specifity of *A. pullulans* NRRL 58524 mannanase at nine difference substrates.

Mannan sources	Relative activity (%)[†]
KGM	100.0 ± 2.1[a]
SCGE	31.6 ± 2.5[b]
GG	26.9 ± 3.1[c]
β-glucan	5.2 ± 0.4[d]
AG	1.2 ± 0.2[de]
XG	2.9 ± 0.5[ef]
Cellulose	1.0 ± 0.4[ef]
CMC	0.9 ± 0.4[ef]
Beechwood xylan	-0.3 ± 0.4[f]

[†] Percentage of the maximum activity expressed as mean ± SD derived from three replicates. The different superscript letter indicated significant differences at $P < 0.05$ (ANOVA and DMRT).

The effects of metal ions and a chelator were observed at 1 mM final concentration (Table 11). Cu^{2+}, Mg^{2+}, and Ca^{2+} significantly enhanced the mannanase activity at 1.12, 1.10 and 1.09-fold, respectively. In contrast, the enzyme was strongly inhibited by Mn^{2+}. Co^{2+}, K^{+}, Fe^{2+}, Na^{+}, and Zn^{2+} did not significantly affect this enzyme. Since the enzyme activity was not inhibited by EDTA, it indicated that this mannanase is not metalloenzyme. The enhancing effect of EDTA might be contributed by the removal

of inhibiting ions. Similar enhancement by copper and manganese ions was reported in the mannanase produced by *A. oryzae* (ManAo) and *L. ramosa* (LrMan5B) (Jana et al. 2018; Xie et al. 2020). Calcium ion enhancement was also reported in *A. niger* β-mannanase, although this enzyme was inhibited by Cu^{2+} (Naganagouda et al. 2009).

Table 11. Effect of ions and chelator (1 mM) on mannanase activity from *A. pullulans* NRRL 58524.

Additive	Relative activity (%)[†]
None	100.0 ± 1.3^{de}
$CaCl_2$	109.5 ± 5.4^{abc}
$CoCl_2$	98.2 ± 2.8^{de}
$CuCl_2$	112.6 ± 2.3^{a}
KCl	101.6 ± 1.7^{cde}
$FeSO_4$	94.2 ± 5.2^{de}
$MgSO_4$	110.8 ± 1.6^{ab}
$MnSO_4$	51.5 ± 0.3^{f}
NaCl	103.5 ± 0.7^{bcd}
$ZnSO_4$	103.6 ± 2.3^{bcd}
EDTA	110.1 ± 1.9^{ab}

[†]The relative activity 100 % corresponded to mannanase activity under the optimum assay condition without additives and expressed as mean ± SD derived from three replicates. The different superscript letter indicated significant differences at $P < 0.05$ (ANOVA and DMRT).

In many polysaccharide digesting enzymes, monosaccharide products can act as feedback inhibitors (Sharma et al., 2021). Therefore, the inhibition by monosaccharide (Table 12) was conducted with mannose and galactose to observe feedback inhibition of galactomannan digestion. Significant inhibition by both monosaccharides was apparent at concentration higher than 20 mM (0.36 g/L). Previous reports suggested that mannoses can inhibit mannanase activity as a

competitive inhibitor, as in *Penicillium occitanis* pol6 and *Sclerotium rolfsii* (at 4 mM and 150 mM, respectively) (Blibech et al., 2010; Sachslehner and Haltrich, 1999). There was no reported of mannanase activity inhibition by galactose before and it was predicted by Lisboa et al. (2006) if galactose might be affected to mannanase activity.

Table 12. Feedback inhibition of *A. pullulans* NRRL 58524 crude mannanase by mannose and galactose.

Monosaccharides	Relative activity (%)*					
	10 mM	20 mM	30 mM	40 mM	50 mM	60 mM
Mannose	185.3 ± 34.9[a]	134.8 ± 24.1[b]	84.8 ± 13.9[c]	29.9 ± 5.5[d]	15.1 ± 8[d]	13.5 ± 9.6[d]
Galactose	112.7 ± 55.3[a]	100.4 ± 22.7[a]	31.5 ± 4.6[b]	ND†	ND†	ND†

*Relative activity is calculated as the percentage of reaction without mannose and galactose addition and shown as the mean ± SD, derived from triplicate.

†ND = Not detectable.

4.4 SCG mannan hydrolysis

SCG mannan was hydrolyzed by *A. pullulans* NRRL 58524 crude mannanase under the optimum condition, 55 °C in 50 mM citric buffer (pH 4.0), with 150-rpm agitation. RSM using CCD was performed with two variables (enzyme unit and incubation time). The variation levels used were based on the preliminary study (data not shown) and the reducing sugar content was observed as the response value (Table 12).

The response surface plot and contour plot are shown in Figure 10 (A) and (B), respectively. The response Y (reducing sugar) could be expressed by the equation:

$$Y = +0.63 + 0.082X_1 + 0.055X_2 + 0.017X_1X_2 - 0.068{X_1}^2 - 0.080{X_2}^2,$$

Y is the reducing sugar (g/g substrate), X_1 is the enzyme load (U/g substrate), and X_2 is the incubation time (days).

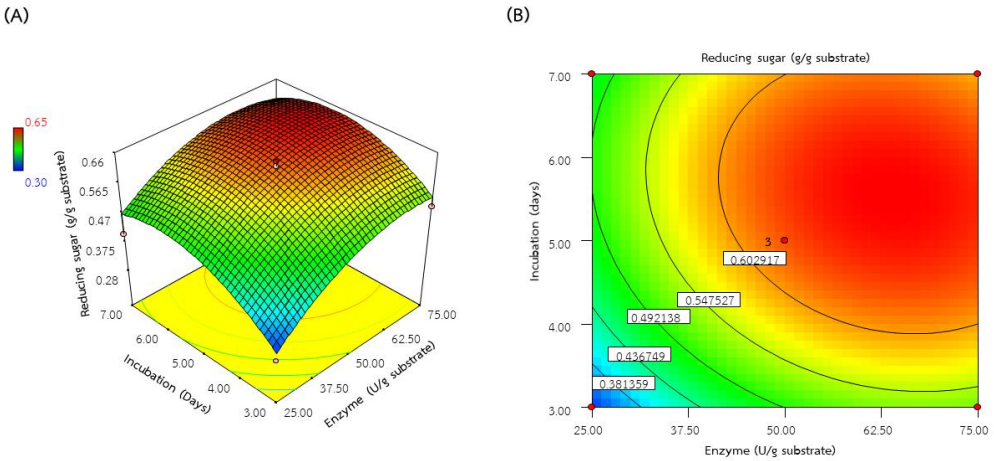

Figure 10. Response surface methodology for the SCGE hydrolysis by *A. pullulans* NRRL 58524 crude mannanase. (A) the three-dimensional response surface plot and (B) the contour plot was showing the effect of mannanase enzyme unit and incubation time with the reducing sugar content as the response.

Based on the total reducing sugar content, the crude mannanase efficiently hydrolyzed SCG mannan up to more than 60 % (Table 13). The hydrolytic products comprised M1, M2, M3 and longer oligosaccharides, with M2 as the major products among MOs. The predicted maximum yield of reducing sugar was at 0.66 g/g substrate when the crude enzyme was used at 66.7 U/g substrate and the reaction was incubated for 5 days 9 h and 36 min. However, when the hydrolysis was repeated under the suggested condition to validate the predicted yield, a significantly lower yield was obtained (0.61 ± 0.01 g/g substrate). This indicated that the regression model derived by the program was poor (Table 14). The P value of the model was significant, but the value was relatively high (0.036). The P value of lack of fit was significant with a very low value at 0.0068, indicating significant error. The R^2 value was relatively low at 0.86 showing that the model could not completely explain the responses. Therefore, the model could not accurately predict the response of the variables. To receive a better model, a repeated experiment must be carried out with new central values for both variables (Carley, Kamneva, and Reminga, 2004).

Table 13. Central composite design matrix with experimental and predicted values of enzymatic of SCG mannan by *A. pullulans* NRRL 58524 crude mannanase.

Run	Code Level		Actual Level		Reducing sugar (g/g subtrate)		Sugar Composition (g/100g substrate)		
	X_1	X_2	Enzyme (U/g substrate)	Incubation (Days)	Actual	Predicted	Mannose (M1)	Mannobise (M2)	Manntriose (M3)
1	-1	-1	25.0	3.0	0.30 ± 0.01	0.33	0.04	2.55	0.31
2	+1	-1	75.0	3.0	0.49 ± 0.02	0.52	0.07	4.07	1.52
3	-1	+1	25.0	7.0	0.40 ± 0.01	0.47	0.05	4.36	0.39
4	+1	+1	75.0	7.0	0.53 ± 0.04	0.60	0.05	6.51	1.77
5	-1.414	0	14.6	5.0	0.42 ± 0.01	0.38	0.03	3.61	1.34
6	+1.414	0	85.4	5.0	0.66 ± 0.01	0.61	0.16	10.05	2.65
7	0	-1.414	50.0	2.2	0.41 ± 0.01	0.39	0.04	3.97	1.09
8	0	+1.414	50.0	7.8	0.62 ± 0.02	0.55	0.06	5.72	1.48

Table 13. Central composite design matrix with experimental and predicted values of enzymatic of SCG mannan by *A. pullulans* NRRL 58524 crude mannanase (continued).

Run	Code Level		Actual Level		Reducing sugar (g/g subtrate)		Sugar Composition (g/100g substrate)		
	X_1	X_2	Enzyme (U/g substrate)	Incubation (Days)	Actual	Predicted	Mannose (M1)	Mannobise (M2)	Manntriose (M3)
9	0	0	50.0	5.0	0.63 ± 0.02	0.63	0.10	3.77	1.07
10	0	0	50.0	5.0	0.63 ± 0.04	0.63	0.09	4.71	1.28
11	0	0	50.0	5.0	0.62 ± 0.05	0.63	0.09	4.02	1.04

Table 14. Analysis of variance (ANOVA) of the central composite design model developed for enzymatic hydrolysis of SCG mannan by *A. pullulans* NRRL 58524 crude mannanase.

Source	Sum of Squares	Df	Mean Square	*F*-Value	*P*-value Prob > F
Model	0.127	5	0.025	5.976	0.0360*
A-Enzyme	0.053	1	0.053	12.554	0.0165
B-Incubation	0.024	1	0.024	5.676	0.0630
AB	1.19E-03	1	0.001	0.280	0.6196
A^2	0.026	1	0.026	6.133	0.0561
B^2	0.036	1	0.036	8.503	0.0332
Residual	0.021	5	4.25E-03		
Lack of Fit	0.021	3	7.04E-03	146.3474	0.0068*
Pure Error	9.62E-05	2	4.81E-05		
Cor Total	0.148	10			

R^2 = 0.86; Adj R^2 = 0.71; Coefficient of variance = 12.53%; *Significant at $P < 0.05$.

The mannanase of each fungus and incubation condition was specifically different for the reducing sugar result of SCG, for instance, *Streptomyces* sp. (Bhaturiwala and Modi, 2020) was used 353.4 U/mL of mannanase to degrade SCG for 20 hours at 45 °C with 60 rpm gained 0.089 g/g SCG and *A. niger* mannanase (Favaro et al., 2020) unspecified unit enzyme can produce reducing sugar ~0.009 g/g SCG under 50 °C for 24 hours with 30 rpm. *A. pullulans* NRRL 58524 crude mannanase (66.7 U/mL) was more attractive because it produced reducing sugar 0.61 g/g SCG at hot temperatures (55 °C) for 5 days 9 h. Besides, the substrate condition was affected to get optimum enzymatic hydrolysis, for example, the pretreatment of SCG by hot

temperature. That showed by Gu et al. (2020), pretreated autohydrolysis SCG at 140 °C combined with *Trichoderma reesei* mannanase for 60 minutes at 50 °C got almost 0.075 g/g of substrate and Chiyanzu et al. (2014) was achieved 0.12 g/g of substrate by pretreated SCG using steam explosion at 200 °C mixed with *Y. lipolytica* mannanase for 5 hours at 60 °C. It was supported by Chiyanzy et al. (2015), that reported the combination of steam explosion pretreatment and enzymatic hydrolysis was effective to solubilize SCG. This study proved the pretreatment of SCG by hot temperature gained the optimum result of enzymatic hydrolysis (0.61 g/g substrate). Moreover, the mannanase capability for each fungus was different and *A. pullulans* NRRL 58524 crude mannanase shown was stable at high temperature (55 °C) for 7 days and yielded more reducing sugar and MOs.

4.5 Prebiotic evaluation

Based on the test (Table 15), from 4 strains of *Lactobacilli*, *L. acidophillus* TISTR 2365 showed the highest than others with 5×10^{-6} CFU/mL and *B. breve* TISTR 2130 was the best from other *Bifidobacterium* strains, that has 3.4×10^{-8} CFU/mL. Wongsiridetchai et al. (2021) reported the prebiotic activity of MOs from SCGE at *L. acidophilus* TISTR 1338 and *L. casei* TISTR 1463 with the result 7×10^{6} and 9×10^{6} CFU/mL, respectively. FOS (Fructooligosaccharides) used as a commercial prebiotic and MRS supplemented with mineral salt broth and 50 mM citrate-phosphate buffer (pH 6.0) as a control. Mannooligosaccharides (DP 3-7) has potency as prebiotic that consumed by *L. acidophilus* NIT 200 (Pan et al., 2009), even though the FOS and xylooligosaccharides (XOS) showed higher than MOs. In this study, MOs not shown the prebiotic activity because the log CFU of MOs is lower than glucose. It could be happened by the specific strain bacteria that used.

Table 15. Prebiotic effect to *Lactobacillus* spp. and *Bifidobacterium* spp. by some of carbon sources.

No	Species	Carbon sources	Log CFU
1	*Lactobacillus casei* TISTR 390	No Carbon	5.47 ± 0.058
		Glucose	8.77 ± 0.022
		β-Glucan	5.61 ± 0.054
		MOs	6.46 ± 0.119
2	*L. casei* subsp. *rhamnosus* TISTR 047	No Carbon	5.39 ± 0.077
		Glucose	8.68 ± 0.036
		β-Glucan	5.54 ± 0.063
		MOs	6.63 ± 0.031
3	*L. brevis* TISTR 868	No Carbon	5.42 ± 0.049
		Glucose	8.63 ± 0.043
		β-Glucan	5.96 ± 0.102
		MOs	6.12 ± 0.105
4	*L. acidophilus* TISTR 2365	No Carbon	5.50 ± 0.070
		Glucose	8.26 ± 0.103
		β-Glucan	5.64 ± 0.041
		MOs	6.69 ± 0.080
5	*Bifidobacterium animalis* subsp. *animalis* TISTR 2194	No Carbon	5.41 ± 0.051
		Glucose	8.53 ± 0.073
		β-Glucan	5.60 ± 0.028
		MOs	6.65 ± 0.021

Table 15. Prebiotic effect to *Lactobacillus* spp. and *Bifidobacterium* spp. by some of carbon sources (continued).

No	Species	Carbon sources	Log CFU
6	*B. breve* TISTR 2130	No Carbon	5.45 ± 0.042
		Glucose	8.60 ± 0.034
		β-Glucan	5.59 ± 0.030
		MOs	7.54 ± 0.044
7	*B. longum* subsp. *longum* TISTR 2195	No Carbon	5.36 ± 0.078
		Glucose	8.55 ± 0.038
		β-Glucan	5.57 ± 0.038
		MOs	6.61 ± 0.035

CHAPTER V
CONCLUSION

This study clearly showed that SCG had high potential to produce galactomannan which could be used for mannanase and MO production. The hot water extraction procedure was very simple and effective. SCG has been an under-utilized waste with no value, thus by using it could reduce the production cost of value-added products like mannanase and MOs. This study was also the first report of mannanase production in *A. melanogenum* and *A. thailandense* strains. The *A. pullulans* NRRL 58524 crude manananase was highly specific to glucomanan and galactomannan which suggested a high potential in selective hydrolysis of these mannans in mixed biomass. The major products of SCG mannan hydrolysis by this enzyme were M2 and M3 which have been reported as prebiotics and could be further developed as functional food supplementation.

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APPENDIX

APPENDIX A

CULTURE MEDIA

1. Yeast Malt Agar (YMA), (Atlas 1993)

Yeast extract	5.0 g
Malt extract	5.0 g
Bacto-peptone	5.0 g
Glucose	20.0 g
Agar	15.0 g

Dissolved in distilled water to final volume 1 liter.

2. Mannanase Screening Agar (Modified from Leathers, 1986)

Konjac Glucomannan	30.0 g
Yeast extract	5.0 g
Agar	15.0 g

Dissolved in distilled water to final volume 1 liter.

2. Mannanase Production Medium (Modified from Leathers, 1986)

Konjac Glucomannan	5.0 g
Yeast extract	5.0 g
L-Asparagine	2.0 g
Potassium dihydrogen phosphate	5.0 g

Dissolved in distilled water to final volume 1 liter.

APPENDIX B

BIOMASS CHEMICAL COMPOSITION ASSAY

1. Chemical reagent

1.1. Neutral detergent solution

Sodium lauryl sulfate	30.00 g
Disodium ethylenediaminetetraacetate (EDTA)	18.61 g
Sodium borate decahydrate	6.81 g
Disodium hydrogen phosphate	4.56 g
2-ethoxyethanol (ethylene glycol monoethyl ether)	10.00 mL

Dissolved EDTA and sodium borate decahydrate in distilled water and add sodium lauryl sulfate and 2-ethoxyethanol to solution. Dissolved disodium hydrogen phosphate in distilled water and add solution containing other ingredients. Check pH range 6.9 to 7.1.

1.2 Acid detergent solution

Concentrated sulfuric acid	26.20 mL
Cetyl trimehylammonium bromide (CTAB)	20.00 g

Dissolved CTAB in sulfuric acid and make up to final volume 1 liter with distilled water.

1.3 Saturated potassium permanganate

Potassium manganate	50.00 g
Silver sulfate	0.05 g

Dissolved potassium manganate and silver sulfate make up to final volume 1 liter with distilled water. Keep out of direct sunlight.

1.4 Lignin buffer solution

Ferric nitrate	6.00 g
Silver nitrate	0.15 g
Acetic acid	500 mL
Potassium acetate	5.00 g
Tertiary butyl alcohol	400 mL
Distilled water	100 mL

Dissolved ferric nitrate and silver nitrate in distilled water. Combine acetic acid and potassium acetate. Add tertiary butyl alcohol and mix.

1.5 Demineralizing solution

Oxalic acid dihydrate	50.00 g
95% Ethanol	700 mL
12 N Hydrochloric acid	50 mL
Distilled water	250 mL

Dissolved oxalic acid dihydrate in 95% ethanol and add concentrated hydrochloric acid. Adjust volume with distilled water.

2. Method (Goering and Van Soest, 1970)

2.1 Neutral detergent

Dry crucible at 100 °C for 12 h and weight. Weight 1 g of dry sample into a beaker of refluxing apparatus. Add in order, 100 mL neutral detergent solution, 2 mL decahydronaphthalene and 0.5 g sodium sulfate. Adjust boiling to 80 °C and reflux for 1 hour. Remove suspend solid into crucible and rinse sample with hot water then wash twice with acetone. Dry the crucible at 100 °C for 12 h and weigh.

2.2 Acid detergent fiber

Remove residual sample into beaker of refluxing apparatus and add 100 mL acid detergent solution and 2 mL decahydronaphthalene. Reflux the sample for 60 minutes at 80 °C then filter the suspend solid in crucible and wash twice by hot water. Repeat wash by 80 % ethanol until the color remove totally. Dry crucible at 100 °C for 12 h and weigh.

2.3 Permanganate lignin, cellulose, insoluble ash, and silica

Add 25 mL of combined saturated potassium permanganate and lignin buffer solution (2:1 by volume) to the crucible containing previous sample and incubate on ice for 90 minutes. Remove crucible to filtering apparatus and suck to dry. Fill half of crucible by demineralizing solution for 5 minutes, then suck until dry and repeat the step until the fiber becomes white. Total time required is not over 20 minutes. Rinse the crucible by 80% ethanol and wash twice by acetone. Dry the crucible at 100 °C for 16 h and weigh.

2.4 Ash content

The residual sample in crucible burn to ash in furnace at 500 °C. After the crucible cool, weigh to calculate the lignin content as loss in weight from the original tare.

3. Calculation

$$\%\, NDF = \frac{(W_{NDF} - W_T)}{S} \times 100 \quad (1)$$

$$\%\, ADF = \frac{(W_{ADF} - W_T)}{S} \times 100 \quad (2)$$

$$\%\, Hemicellulose = \%\, NDF - \%\, ADF \quad (3)$$

$$\%\, Lignin = \frac{(W_{ADF} - W_l)}{S} \times 100 \quad (4)$$

$$\%\, Cellulose = \frac{(W_L - W_A)}{S} \times 100 \quad (5)$$

$$\%\, Ash = \frac{(W_A - W_T)}{S} \times 100 \quad (6)$$

W_{NDF} = Weight of oven-dry crucible including natural detergent fiber

W_{ADF} = Weight of oven-dry crucible including acid detergent fiber

W_L = Weight of oven-dry crucible including lignin extracted fiber

W_A = Weight of oven-dry crucible including ash

W_T = Tared weight of oven-dry crucible

S = oven-dry sample

VITA

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AWARD RECEIVED	ASEAN/Non-ASEAN Scholarship