Production of Konjac Oligo-glucomannan and Their Effect on the Gut Microbiota

Catarina Aprilia Ariestanti

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Functional Food and Nutrition

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Konjac glucomannan (KGM) is neutral polysaccharide that can be produced by extraction from the tuber of *Amorphophallus konjac*. Degradation product of konjac glucomannan is konjac oligo-glucomannan (KOG). KOG can be produced by enzymatic hydrolysis of KGM using β-mannanase. Response surface methodology (RSM) was used to optimize the hydrolysis temperature, time, pH and enzyme to substrate ratio (E/S) to obtain high yield of KOG with the variations performed with design of experimental using Box-Behnken design (BBD). The highest content of oligosaccharide from hydrolysis of KGM analyzed by HPSEC was 9.21 mg/mL under the conditions of 48˚C, 4h hydrolysis time, pH 5.5 and E/S of 0.05%. The result showed that KOG was produced successfully by β-mannanase from KGM. In order to increase the purity of KOG, purification step was performed by ultrafiltration (UF) membrane. The oligosaccharide content in KOG after purification was analyzed by high performance size exclusion chromatography (HPSEC). The final product after purification step with UF pore size of 3,000 NMWC produced two groups of KOG with different fraction from permeate and retentate. Low molecular weight fraction (LKOG) was found in permeate with the concentration of oligosaccharides was 9.54 mg/mL and high molecular weight fraction (HKOG) was found in retentate with the concentration of oligosaccharides was 8.49 mg/mL. The effect of KOG on changes in human fecal bacterial populations and short chain fatty acids (SCFAs) production were evaluated. Bacterial populations of bifidobacteria increased significantly ($P<0.05$) after 24h and lactobacilli was slightly increased after 6h during LKOG fermentation. Population numbers of bacteroides and clostridia after 24 and 6h, respectively, decreased significantly. Prebiotic index (PI) of LKOG was 0.76, lower than KGM (1.26), inulin

(0.97) and PGM (1.23). SCFAs analysis results showed that LKOG can enhance the production of butyric acid in the colon. The concentration of butyric acids was increased as the time fermentation increased with 8.24 mM was the highest concentration and found at 72h fermentation. Positive PI value of LKOG and its ability to support the growth of beneficial bacteria especially bifidobacteria are indicated that LKOG has potency as a prebiotic and it may have specific health function related with butyric acid production. Further study of LKOG might be needed to investigate its beneficial effect in human.

ACKNOWLEDGEMENT

I would like to thank my advisor, Asst. Prof. Dr. Santad Wichienchot, and my co-advisors Asst. Prof. Dr. Vatcharee Seechamnanturakit and Prof. Dr. Eni Harmayani for their suggestions and guidance during the entire period of my research.

I am also grateful to all of the members of Interdisciplinary Graduate School of Nutraceutical and Functional Food laboratory for their help and support during the course of my study.

My sincere gratitude goes to my family and my loved ones for their unwavering support during and after the period of my research, and also, I would like to extend my appreciation to my friends that has been there supporting me, cheering me up and teaching me. I love you all.

In addition, I would like to express my gratitude to Almighty God, for his kindness and mercy for availing me this wonderful opportunity in my life.

Catarina Aprilia Ariestanti

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Nowadays, healthy lifestyle and healthy diet has been increasingly gained consumers attention. Chronic diseases are caused by unhealthy lifestyle involving consumption “fast food”, food that rich in sugar, saturated fats and salt. Consumers eagerness to elevate the quality of life lead them to consume food that can provide health and wellness benefits. For these reasons, functional food consumption is prospering in the market (Kapsak *et al*., 2011). Functional food definition according to Functional Food Science in Europe (FUFOSE) is a food that can give beneficial functions in the body to boost health and / or to decrease the risk of disease beyond sufficient nutrition (Vicentini *et al*., 2016). Functional food can be natural or processed food that has been enriched by biologically active compounds such as vitamins, minerals, probiotics and prebiotics including dietary fiber (Keservani *et al*., 2010).

Dietary fiber is one of the active components that added into food to enhance its functionality in the body. More than 50% of functional foods in the market contains dietary fiber as their active component. Codex Alimentarius Commission defines dietary fiber as carbohydrates polymers consist of ten or more monomeric units that are unhydrolyzed in the small intestine by human enzyme (Macagnan *et al*., 2016). Consumption of dietary fiber related with reduce the prevalence of diseases. Dietary fiber can be found in fruits, vegetable and mainly cereal, and it is divided into water-soluble and water insoluble dietary fibers based on their solubility in water. Soluble dietary fiber improves host health by act as prebiotic for the beneficial microorganisms and as immunomodulators in the intestine (Chawla and Patil, 2010).

One of natural water soluble dietary fiber is glucomannans. Glucomannans are polysaccharides that can be found in family of Araceae, from genus of *Amorphophallus, Colocasia, Xanthosoma,* and *Alocasia*. *Amorphophallus konjac* has been known as the source of glucomannan with the high content of glucomannan in its tuber (Tester and Al-Ghazzewi, 2017). Konjac glucomannan (KGM) composed

of mannose and glucose and can be extracted from the corm of *Amorphophallus konjac*. It has been used as emulsifier, thickener, or as nutritional supplements for constipation, high cholesterol and obesity patients (Behera and Ray, 2016).

Different molecular weights between KGM and its degradation product (konjac oligo-glucomannan/ KOG) has distinct functions related with prevention and reducing the risk of some non-communicable diseases. KOG can be produced by depolymerization of KGM such as acid degradation, oxidative degradation, physical methods and enzymatic hydrolysis (Liu, *et al*., 2015). In the market, products containing KOG have not been marketed yet in terms for health benefit functions and because of its purity (Connolly *et al*., 2010).

As a dietary fiber, KGM and KOG are resists digestion and absorption in small intestine and then partially or completely fermented by colonic microflora therefore it can act as prebiotic. Prebiotics improve health benefits by enhanced short chain fatty acid (SCFA) production, reduce the number of pathogenic bacteria populations and improve the host immunity (Slavin, 2013). The effect of total intake dietary fiber has relationship with the risk of cancer in colon or rectum. Common mechanisms of probiotics are its resistance to colonization of harmful microbiota, produce short chain fatty acid (SCFA), has ability to regulate the gastrointestinal transit and competitive compare to pathogenic microorganisms. As the common probiotics, lactobacilli and bifidobacteria should be safe for human consumption because of its mechanisms (Olveira and González-Molero, 2016).

Production of KOG can be done by enzymatic hydrolysis using β-mannanase to cut the polysaccharide of KGM into oligosaccharide which may have different functions to the body (Jian *et al*. 2013). KOG can be produced by optimizing the hydrolysis conditions of production by controlling the hydrolysis time, hydrolysis temperature, pH and enzyme to substrate ratio (E/S) to obtain a high KOG yield from hydrolysis of KGM (Chen *et al*., 2013). Hypothesis of this research were optimization of hydrolysis conditions of KGM would be produced high yield of KOG and high purity of KOG may have functionality to act as prebiotic.

1.2 Review of Literature

1.2.1 Glucomannan

Glucomannans are polysaccharides that can be found in family of Araceae (Ray and Behera, 2016), from genus of *Amorphophallus*, *Colocasia*, *Xanthosoma*, and *Alocasia*. They are plentiful in nature from mannan family particularly in softwood, roots, tuber and plants bulbs. Glucomannans have been applied in several fields such for drug delivery related with chronic stomach disease (Wang and He, 2002). In Asia, glucomannans generally used as food thickener and have been traditionally used for noodles, tofu and other food products (Tien *et al.*, 2011).

1.2.1.1 Sources of glucomannan

There are several sources of glucomannan. *Amorphophallus* sp. as the source of glucomannan grows mainly in South East Asia. Available commercial glucomannan are extracted from dried tuber, particularly *A. konjac* (Yanuriati *et al.*, 2017). In Vietnam, *Amorphophallus paeoniifolius* and *Amorphophallus corrugatus* are the most abundant *Amorphophallus* species that grows in the forests. Studied by Tien *et al.* (2010) found out that glucomannan from *A. paeoniifolius* has molecular weight (MW) of 1.115 x 10^6 Da with glucomannan content about 5-9% in its tuber. The results showed that glucomannan from *A. paeoniifolius* has potential application as a food and medicine for human. In another study, glucomannan content of *A. corrugatus* was about 15% with the MW was 1.57 x 10^6 Da and it showed the promising candidate to be applied in food, pharmaceutical industry and biomedical products (Tien *et al.*, 2011). In Indonesia, one of the sources of glucomannan is *Amorphophallus onchophyllus* and it is known as porang for local. Porang chips has been exported to China and Japan. Porang flour contained 18.05% of glucomannan. As a diet supplement, porang glucomannan has the ability to inhibit *Escherichia coli* growth and enhance SCFA production. It might indicate that porang glucomannan can be used as functional food (Harmayani *et al.*, 2014).

1.2.2 Konjac

Amorphopallus konjac is a member from family of Araceae and grows in mountain or hilly areas in subtropical regions in Asia and South-East Asia, such as Thailand, Vietnam, and Indonesia (Zhang *et al.*, 2005). It is a perennial herbaceous herb growing from a large corm up to 25 cm in diameter. The single leaf is up to 1.3 m across and bipinnate type. The flowers are produced up to 55 cm long (Henriquez *et al.*, 2014). In China and Japan, it has been used as source of food and traditional medicine. Konjac is grown for its large starchy corms to create flour, jelly and for substitute for gelatin. According by World Health Organization, products of konjac are the one of "top 10 health foods". The extracted soluble fiber has been used as an appetite suppressant and as an agent to reduce serum lipids and blood sugar. The isolated, biologically-active constituents, such as glucomannans, can be used as new sources for development of pharmaceutical additives (Chua *et al.*, 2010).

1.2.3 Konjac glucomannan

Konjac glucomannan (KGM) is the primary component of *Amorphophallus konjac* tuber. *Amorphophallus konjac* has been known as the source of glucomannan with the high content of glucomannan in its tuber (Tester and Al-Ghazzewi, 2017). KGM is a high molecular weight polysaccharide (10^5-10^6Da) consist of mannose and glucose in the ratio 1.6:1 joining by β-1,4 glycosidic linkage (Fang and Wu, 2004; Chen *et al.*, 2013). Glucomannan has functions and applications in food, food additives, pharmaceutical, bio-technical and fine chemical industries (Zhang *et al.*, 2005).

1.2.3.1 Production of konjac glucomannan

Basically, previous commercial glucomannan granules are extracted by slicing, drying, milling to remove impurities which stick to the glucomannan granules, followed by pulverizing to flour, sifting and air clarifying (Zhao *et al.*, 2010). In recent decades, methods for extraction of KGM have been developed. KGM is extracted by dry processing use mechanical methods, or wet processing, use chemical methods. Dry processing methods include milling of dried konjac chips become crude konjac flour (CFK) and subsequently purified by wind shifting (Sanderson, 1993). Some of the

techniques can be applied to extract glucomannan from konjac flour, for example enzyme treatment, dialysis, washing with alcohol, or simple centrifugation process (Chua *et al.*, 2012).

Konjac flour that produced from extraction process contains impurities such as cellulose, mostly insoluble starch, few protein and lipids. To produce konjac flour with high content of glucomannan, the impurities inside need to be removed by washed with ethanol solution or by using enzymatic purification (Xu *et al.*, 2014). Currently, konjac flour is produced commercially. The content of glucomannan is a key indicator for evaluating the quality of konjac flour (Li *et al.*, 2005). Table 1.1 shows the content of konjac glucomannan in different forms per 100 grams.

Table 1.1 Content of konjac glucomannan in different forms per 100 grams

Forms	**Content (gram)**	**References**
Raw tuber	8-10	Tatirat and Charoenrein (2011)
Konjac flour	50-70	Akesowan (2002)
Purified powder	>90	Fang and Wu (2004)

1.2.3.2 Health benefit of konjac glucomannan

Konjac glucomannan (KGM) has functional benefits for human health such as for disease related with life style like colorectal cancer, coronary heart disease, diabetes, obesity, cardiovascular disease, sudden death, hypercholesterolemia, hyperlipidemia, and stroke (Behera and Ray, 2016). Intake of dietary fiber has functional benefit to decrease the risk of the life style diseases. Conventionally, KGM has been used related with its application in food as a thickening agent and emulsifier. Another health benefits of KGM include anti-obesity, anti-inflammatory, antioxidant, anti-diabetic, regulation in lipid metabolism, laxative effect/constipation mitigation, and as prebiotic (Tester and Al-Ghazzewi, 2013).

1.2.4 Konjac oligo-glucomannan

Konjac oligo-glucomannan (KOG) is the degradation products of konjac glucomannan (KGM) and one type of oligosaccharide (Liu *et al.*, 2016). It can be produced by the depolymerization of KGM such as acid degradation, oxidative degradation, physical methods and enzymatic hydrolysis (Courtois, 2009). Enzymatic hydrolysis is the best choice to produce oligosaccharides in large-scale production because its produce high yield of oligosaccharides and it is possible to apply when the end product required oligosaccharides with desired molecular weight and minimum chemical modification (Barreteau *et al.*, 2006). Because of the characteristics, safety and can be done in room temperature, enzymatic hydrolysis has been most widely used in the application of producing KOG (Jian *et al*., 2013).

1.2.4.1 Production of konjac oligo-glucomannan

Konjac oligo-glucomannan (KOG) can be produced by hydrolysis of konjac glucomannan (KGM) using β-mannanase. β-mannanase is an endo hydrolase that catalyzes the random hydrolysis of β-1,4-mannosidic linkages (Liu *et al.*, 2017) in the main chain of galactomannan, galactoglucomannan, mannan, and glucomannan by releasing various length of linear and branched manno-oligosaccharides (Chen *et al.*, 2016). Production of KOG by enzymatic hydrolysis can be done by controlling the hydrolysis conditions for temperature, time, pH and enzyme per substrate ratio during the reaction. These conditions can be varied and optimize using response surface methodology (RSM) to get the optimum condition of KOG production (Chen *et al*., 2013). Purity of KOG from enzymatic hydrolysis process was one of the reasons to improve its quality in the market related with the functionality for health. Suitable method is needed to purify the KOG and ultrafiltration is one of the method that has been applied in the separation and purification of polysaccharides because of its advantages (Xing and Li, 2009).

1.2.4.2 Purification of konjac oligo-glucomannan

Konjac oligo-glucomannan (KOG) purification is needed to improve the purity of KOG content in the product. Purification of oligosaccharides is one of the key

factors that can affect their physiological benefit. Food grade oligosaccharide products are mixtures containing different molecular weight of oligosaccharides, polysaccharides and monosaccharides. Purifying of oligosaccharides is important step to separate the prebiotic oligosaccharides from its impurities because not all of the sugars have prebiotic activity (Goulas *et al.*, 2002). There are several methods to purify oligosaccharides such as ethanol precipitation, activated charcoal adsorption, chromatographic process and membrane filtration (Córdova *et al.*, 2017). Column chromatography and ultrafiltration by membrane has been used in the purification of polysaccharides. The yield of oligosaccharides obtained by column chromatography is lower than ultrafiltration method. For this reason, ultrafiltration method is widely applied in purification step. Development and advantages in membrane based filtration techniques such as high efficiency, simple operation and equipment and low operation energy consumption made the membrane filtration become one of the important separation techniques (Li and Chase, 2010).

Membrane filtration has potential to purify oligosaccharides. General principle of the membrane filtration is based on the permeability of the membrane to allow the target substances pass through the membrane. Based on the size exclusion mechanisms, membrane techniques are classified into nanofiltration, ultrafiltration and microfiltration which are found out to be suitable for sugar separation because of their ability to separate various molecular size within in the range of the membrane pore size. The importance of membrane filtration techniques has been increased for refining the present of oligosaccharides in carbohydrates (Li and Chase, 2010).

Ultrafiltration (UF) is one of the methods to separate the oligosaccharides from polysaccharides by applying the driving force through the membrane. Generally, high molecular weight substances will be removed by UF. It has the range of pore size from 0.002 to 0.1 microns with pressure during operation start from 10 to 100 psi. UF techniques are used to purify commercial oligosaccharides by removing the higher molecular weight sugars from oligosaccharides mixtures or by separated and concentrated oligosaccharides from polysaccharides (Kim *et al.*, 2003). Studied by Jian *et al.* (2013) showed that oligosaccharides from KGM degradation with β-mannanase was effectively separated by ultrafiltration.

1.2.4.3 Gut fermentation of konjac oligo-glucomannan

Carbohydrates fermentation by bacterial in the large intestine are produced short chain fatty acids (SCFAs) as the end products, especially acetate, propionate and butyrate. This products are used by the host to improve their health benefit (Tuohy *et al.*, 2005). Konjac glucomannan (KGM) is high molecular weight polysaccharides and consist of mannose and glucose and it is a source of dietary fiber (Fang and Wu, 2004). Konjac oligo-glucomannan (KOG) is indigestible oligosaccharide that serve as dietary fibers and prebiotics (Patel and Goyal, 2011).

Probiotics can be considered as live microbial food supplements that give health benefits to consumers by maintaining or improving consumers intestinal microbial balance when taken in prescribed quantity. Lactobacilli and bifidobacteria are common probiotics in the gastrointestinal that can promote health benefit for the human body (Al-Ghazzewi *et al.*, 2007). Gut microorganism needs substrate to grow, and dietary fiber and non-digestible oligosaccharides are the main substrates for the growth of gut microorganisms (Blaut *et al.*, 2017). As prebiotics, KOG pass into the large bowel and increase the number of lactobacilli and bifidobacterial colonies (Chen *et al.*, 2013). Lactobacillus is a lactic acid bacteria (LAB) group which has low pH range and it can increase the tolerance of acidity. This may help lactobacillus to outcompete other bacteria in a natural fermentation. LAB and bifidobacteria can provide a benefit in the clinical symptoms such as inflammatory bowel disease (IBD) (Saez-lara *et al.*, 2015).

SCFAs are undegradable by human digestion (Primec *et al.*, 2017), which include acetate, propionate and butyrate as the most abundant SCFAs in the body (den Besten *et al.*, 2013). The rate and amount of SCFAs production depend on the species and amounts of microflora present in the colon, the substrate source and gut transit time. Acetate is used in lipogenesis by enters the peripheral circulation (Scott *et al.*, 2013). Propionate is used to generate ATP and it is largely taken up by liver (Gibson, 2004). Butyrate is the major energy source for colonocytes and may have ability to suppress the colonic inflammation and carcinogenesis (Sivaprakasam *et al.*, 2016).

Health promoting effects of SCFAs including reduce the risk of developing gastrointestinal disorders, diabetes, inflammatory diseases, induce apoptosis, differentiation of colon cancer cells and cardiovascular disease (Behera and Ray, 2016). SCFAs have physiological effects because they contribute for shaping the environment of gut, affected colon physiology and act as source of energy for intestinal microbiota and host cells. Increase production of SCFAs may result protective effects for the host body (Ríos-Covián *et al.*, 2016).

Gastrointestinal health and its diseases are related with organisms that inhabit in the gut. Change in environmental factors, lifestyle, diseases and infections lead to impact the compositions of bacterial communities. Diet can affected the composition of the organisms in the gut (Frank and Pace, 2008) because the intestinal environment will be affected by food daily intake and it is related with the production of SCFAs. The concentrations of SCFAs may contribute for the acidification of intestinal environment (Shimizu, 2012).

Study about konjac glucomannan hydrolysate (GMH) on bacterial composition and its activity on fecal microbiota has been done by Connolly *et al.* (2010). On their study, GMH was selectively fermented by bifidobacteria and lactobacilli and produced SCFAs rich in propionic acid. Potential use of KGM degraded product as prebiotic showed that it stimulated the growth of bifidobacteria and lactobacilli. In UHT milk, uses of konjac hydrolysate as a carbon source supported the bacterial growth better than inulin (Al-Ghazzewi *et al.*, 2007). These studies showed that degradation product of KGM has some characteristic of prebiotic inulin and it may have potential to be used in foods as prebiotic.

1.2.5 Dietary fiber

Dietary fiber is part of food derived from plants which is resistant to enzymatic digestion in the small intestine and will be complete or partially fermented in the large intestine which includes cellulose, non-cellulosic polysaccharides, and a non-carbohydrates component lignin. The benefit of dietary fiber is to promote physiological effects including laxation, control of blood cholesterol and blood glucose (Dhingra *et al.*, 2012). Generally, dietary fibers can be divided into soluble and insoluble dietary fiber based on their solubility. Soluble fiber is slow to digest and may help lower the risk of heart disease. It has ability to form gel and act as emulsifier, and it can be found in oat bran, barley, nuts, seeds, beans, lentils, peas, and some fruits and vegetables (Mudgil and Barak, 2013). Soluble fiber has the effect at the colon and it attracts water and turns into gel during digestion. Its readily fermented and may promote the growth of intestinal bacteria and their end products such as gas and short chain fatty acids (SCFAs) that may improve the health benefit to the host (Dai and Chau, 2016). Insoluble fiber, which can found in foods such as wheat bran, vegetables, and whole grain, adds bulk to the stool and help food to pass through the stomach and intestine (Dhingra *et al.*, 2012).

Non-starch polysaccharides, oligosaccharides, lignin and substances from plant are included as dietary fiber. The growth of intestinal bacteria can be improved by prebiotic activity of dietary fiber. Many studies have been focused on fructo-oligosaccharides (FOS), inulin and galacto-oligosaccharides (GOS) as prebiotics (Connolly *et al.*, 2010). Glucomannan has gained the attention amid those prebiotics because of its functionality to improve health with the common source of it are found in *Amorphophallus konjac* plant. Konjac glucomannan (KGM) in human studies that has been done by Chen *at al.* (2008) in constipated Chinese women showed that consumption of 4.5 g/d of KGM resulted in increasing significantly of defecation frequency weekly. KGM is polysaccharides that cannot be hydrolyzed by digestive enzymes in the human upper gastrointestinal tract and therefore regarded as an indigestible dietary fiber. Indigestible dietary fiber has been demonstrated to be effective in weight reduction, regulation of lipid metabolism, improvement of glucose metabolism and cholesterol reduction (Xiong *et al.*, 2009). Konjac oligo-glucomannan

as degradation product of KGM can be produced enzymatically from KGM. KOG has smaller molecular weight compared to KGM and it has ability to promote the growth of probiotics (bifidobacterial and lactobacilli) in single cultures (Al-Ghazzewi *et al.*, 2007). In the market, products containing KOG have not been marketed yet in terms for health benefit functions and because of its purity (Connolly *et al*., 2010).

1.2.6 Oligosaccharides

Oligosaccharides are low molecular weight carbohydrates. Non-digestible oligosaccharides have bifidogenic activity and another health benefit for human. Oligosaccharides with prebiotic properties have physiological benefit for the human body (Gobinath *et al.*, 2010). Purity of oligosaccharide is one of the key factor that can affect their physiological benefit (Mussatto and Mancilha, 2007). Oligosaccharides can be developed by extracted from natural sources. Most of the it are produced by enzymatic or acid hydrolysis of polysaccharides or extracted from natural sources (Du *et al.*, 2011).

Oligosaccharides can be obtained by extraction of polysaccharides from natural sources using water, ethanol, acids or enzymes. (Yang *et al.*, 2011). Production of oligosaccharides by acid hydrolysis is relative simple because of cheap and easy to stop the process (Warrand and Janssen, 2007)**.** Besides the advantages, this technique may have disadvantages, such as it will have formed the toxic substances from degradation of monosaccharides and produce low yield of oligosaccharides (Burana-osot *et al.*, 2010). Physical hydrolysis to produce oligosaccharides occurs in water under high temperature conditions (Vázquez *et al.*, 2006) may not require the chemical products, obstacle the purification process in the end products, so it will generate the environmental wastes (Rose and Inglett, 2010). Even they represent a fast and clean way to produce oligosaccharides (Burana-osot *et al.*, 2010), but it has limited application in industries, still need to find about the optimize condition to obtain the better yield of oligosaccharides (Zhao *et al.*, 2009), and the high temperature during the process may cause depolymerization, debranching, deesterification, and brown products formation (Coelho *et al.* 2014).

Enzymatic hydrolysis is the best choice to produce oligosaccharides in large-scale production because its produce high yield of oligosaccharides and it is possible to apply when the end products required oligosaccharides with desired molecular weight and minimum chemical modification (Barreteau *et al.*, 2006). Although produced high yield of oligosaccharides, this technique may require undergo concentration to minimize microbiological contaminant even though the quality of the end products is depends on the microorganisms capacity to adapt on the substrate (Cazetta *et al.*, 2005).

KOG can be produced by enzymatic hydrolysis of KGM using neutral β-mannanase. β-mannanase is an endo hydrolase that catalyzes the random hydrolysis of β-1,4-mannosidic linkages (Liu *et al.*, 2017) in the main chain of galactomannan, galactoglucomannan, mannan, and glucomannan by releasing various length of manno-oligosaccharides (Chen *et al.*, 2016). β-mannanase has been shown to degrade KGM and produced mixture of oligosaccharides as the end product such as partially hydrolyzed mannan and manno-oligsaccharides (Chen *et al.*, 2016). Studied by Cescutti *et al.* (2002) showed that mannose (M) and glucose (G) in the form of GM, M, GGM and GMM are produced during the enzymatic hydrolysis of KGM using β-mannanase. Degradation products of KGM from previous study with different molecular weight showed that KOG can act as anti-tumor and immune regulation (Liu *et al.*, 2015). KGM has been consume as an additional ingredient in food such as noodles, jelly and tofu in small amount related with its high viscosity. In food processing industry, application of KOG is more approving because its viscosity is significantly reduced and in other hand KOG has function to act as prebiotic (Jiang *et al.*, 2018).

Purity of KOG was the limitation in KOG production from degradation of KGM. Column chromatography and ultrafiltration by membrane has been used in the purification of polysaccharides (Wu *et al.*, 2012). Lower production of oligosaccharides purity in column chromatography method makes ultrafiltration is widely applied in purification step. Studied by Jian *et al.* (2013) showed that oligosaccharides from KGM degradation with β-mannanase was effectively separated by ultrafiltration.

1.2.7 Prebiotic

Prebiotics are non-digestible short chain carbohydrates but fermentable in the colon. It can selectively improve the growth or activity of limited indigenous bacteria in the colon which can give health benefits to the host. Prebiotics improve health benefits by enhancing short chain fatty acid (SCFA) production, reduce the number of pathogenic bacteria populations and improve the host immunity (Slavin, 2013). The candidate of prebiotics must resist gastric acid and unhydrolyzed by human enzyme in the upper gastrointestinal tract, can be fermented by intestinal microbiota in the colon and can selectively stimulate of growth and activity of intestinal bacteria which can contribute to health of the host. It needs to be proven by *in vivo* and *in vivo* test (De Morais, 2015). Prebiotics passed through the small intestine and utilized by probiotics to produce SCFA that are used as source of energy for the host organism to improve the host health (Al-Sheraji *et al*., 2013).

1.2.8 Probiotics

Gut microbiota is microorganisms that inhabited human intestinal lumen and consists of 100-1000 different bacterial species (Li *et al.*, 2016). The relationship between the host and most of the gut microbiota is mutualistic or symbiotic relationship. Positive effects of gut microbiota on human health include providing vitamins and energy source to the host, helping in the development of intestinal tissue and immune system, limiting inflammatory responses at local and distal organs, decreasing carcinogenesis and inhibiting colonization of gut with pathogenesis microorganisms (Bedani *et al.*, 2015).

Probiotics are defined as living microorganisms, such as yeast and lactic acid bacteria, that has beneficial effect, so it can improve the health of the host when they are ingested in adequate amounts. Probiotics must be survive during the storage, manufacturing process, in the intestinal ecosystem to give beneficially health effect to the host (Anadón *et al.*, 2016). Probiotics, commonly known as bifidobacteria and lactobacilli, should be safe for human consumption because of its mechanisms. Common mechanisms of probiotics are its resistance to colonization, produce short

chain fatty acid (SCFA), ability to regulate the gastrointestinal transit, and competitive compare to pathogenic microorganisms (Olveira and González-Molero, 2016).

1.2.9 Short chain fatty acids (SCFAs)

Short chain fatty acids (SCFAs) are a sub-group of fatty acids with aliphatic tails of two to six carbons and are the major end products of bacterial fermentation in the colon during fermentation of resistant carbohydrates. SCFAs are undegradable by human digestion (Primec *et al.*, 2017), which include acetate, propionate and butyrate as the most abundant SCFAs in the body (den Besten *et al.*, 2013). The rate and amount of SCFA production depends on the species and amounts of microflora present in the colon, the substrate source and gut transit time. Acetate is used in lipogenesis by enters the peripheral circulation (Scott *et al.* 2013). Propionate is used to generate ATP and it is largely taken up by liver (Gibson, 2004). Butyrate is the major energy source for colonocytes and may have ability to suppress the colonic inflammation and carcinogenesis (Sivaprakasam *et al.*, 2016).

Health promoting effects of SCFAs including reduce the risk of developing gastrointestinal disorders, diabetes, inflammatory diseases, induce apoptosis, differentiation of colon cancer cells and cardiovascular disease (Behera and Ray 2016). SCFAs have physiological effects because they contribute for shaping the environment of gut, affected colon physiology and act as source of energy by intestinal microbiota and host cells. Increase production of SCFAs may result protective effects for the host body (Ríos-Covián *et al.*, 2016).

1.3 Objectives

1. To optimize the conditions for production of konjac oligo-glucomannan from konjac glucomannan by enzymatic hydrolysis.
2. To purify and obtain konjac oligo-glucomannan powder by ultrafiltration membrane and spray drying.
3. To evaluate prebiotic properties of konjac oligo-glucomannan by fecal fermentation
4. To study the effect of konjac oligo-glucomannan in gut microbiota composition and its short chain fatty acids (SCFAs) profiles.

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CHAPTER 2

PRODUCTION OF KONJAC OLIGO-GLUCOMANNAN

2.1 Abstract

Konjac oligo-glucomannan (KOG) can be produced by hydrolysis of konjac glucomannan (KGM) using β-mannanase. Reaction of enzymatic hydrolysis of KGM was investigated under the conditions of reaction temperature 43-53˚C, reaction time 2-6 h, pH 5-6, and E/S ratio of 0.025-0.1%. The results from chromatography techniques showed the area of peak from polysaccharides, oligosaccharides and monosaccharides. Area from the peak of oligosaccharides in each variation was used to determine the optimum conditions of producing KOG in the bigger scale for prebiotic potency study. From the result, optimum conditions to produce KOG were at 48ºC, for 4h with pH 5.5 and E/S of 0.05% (w/w). This condition produced highest oligosaccharides content with concentration of 9.21 mg/mL. As a result, this condition was chosen to be the condition to produce KOG.

2.2 Introduction

Carbohydrates can be defined into monosaccharides, oligosaccharides, or polysaccharides due to their degree of polymerization (number of monosaccharide units combined) and molecular weight (de Moura *et al.*, 2016). Carbohydrates also can be classified into digestible and non-digestible. Non-digestible carbohydrates are not easy to hydrolyze by human enzyme digestive system and this functional property makes them deserved as dietary fibers and prebiotics. Oligosaccharides are low molecular weight carbohydrates containing 2-10 units of sugar, with degrees of polymerization (DP) between 3 to 10 (Weijers *et al.*, 2008). Functional oligosaccharides are used in nutraceutical, pharmaceutical, prebiotics, cosmetics and agriculture system for their applications in industrial field. Non-digestible oligosaccharides have bifidogenic activity and another health benefit for human (Mussatto and Mancilha, 2007).

Konjac oligo-glucomannan (KOG) is the degradation products of konjac glucomannan (KGM) (Liu *et al.*, 2016). KOG can be produced by the depolymerization of KGM such as acid degradation, oxidative degradation, physical methods and enzymatic hydrolysis (Courtois, 2009). The most widely used method to produce KOG is enzymatic hydrolysis because of its characteristics, safety and can be done in room temperature (Jian *et al.*, 2013). Enzymatic hydrolysis is the best choice to produce oligosaccharides in large-scale production because its produce high yield of oligosaccharides and it is possible to apply when the end product required oligosaccharides with desired molecular weight and minimum chemical modification (Barreteau *et al.*, 2006).

Enzymatic hydrolysis is a process that required many factors to maximize its efficiency. In order to get the optimum condition from enzymatic hydrolysis, response surface methodology (RSM) has been successfully improved the possibilities of statistical interpretation, reduced time involving during the process and number of experiments in various industrial processes (Chen *et al.*, 2013). RSM is a protocol of statistically design experimental which concurrently varied several factors. In analytical chemistry, there are several experimental designs that can be applied such as full three-level factorial designs, Box-Behnken designs (BBD), central composite designs and Doehlert designs. Each design has its own advantages and disadvantages. Among those designs, BBD are more efficient and economical compared to other designs when involved the large number of variables (Bezerra *et al*., 2008).

KOG can be produced by enzymatic hydrolysis of KGM using neutral β-mannanase. β-mannanase is an endo hydrolase that catalyzes the random hydrolysis of β-1,4-mannosidic linkages (Liu *et al.*, 2017) in the main chain of galactomannan, galactoglucomannan, mannan, and glucomannan by releasing various length of linear and branched manno-oligosaccharides (Chen *et al.*, 2016). Production of KOG by enzymatic hydrolysis can be done by controlling the hydrolysis conditions for temperature, time, pH and enzyme per substrate ratio during the reaction. These conditions can be varied and optimize using RSM to get the optimum condition of KOG production (Chen *et al*., 2013). KOG have been reported to have prebiotic functions (Chen *et al.*, 2013) because it can give stimulation to support for the growth of

Bifidobacterium and *Lactobacillus acidophilus* (Chen *et al.*, 2005). Cescutti *et al.* (2002) showed that mannose (M) and glucose (G) in the form of GM, M, GGM and GMM are produced during the enzymatic hydrolysis of KGM using β-mannanase. Degradation products of KGM from previous study with different molecular weight showed that KOG can act as anti-tumor and immune regulation (Liu *et al.*, 2015). In food processing industry, application of KOG is more approving because its viscosity is significantly reduced and in other hand KOG has function to act as prebiotic (Jiang *et al.*, 2018).

In this study, KOG produced from depolymerization of KGM by enzymatic hydrolysis using β-mannanase. RSM with application of BBD were chosen to vary the parameters of hydrolysis in order to get the optimum condition of enzymatic hydrolysis process. Parameters of hydrolysis were temperature, time, pH and E/S. The range of each parameters were chosen from previous studies (Liu *et al.*, 2017; Chen *et al.*, 2013) with modification and based on the optimum conditions of the enzyme that are given by the company.

2.3 Materials and methods

2.3.1 Materials

All chemicals and reagents used were of analytical grade. 95% purified konjac glucomannan was purchased from Pyson Co., Ltd., (Shaanxi, China). β-mannanase was purchased from Mianyang Habio Bioengineering Co., Ltd., (Sichuan, China). Sodium acetate obtained from Sigma-Aldrich Co., (St. Louis, USA), sodium hydroxide obtained from Loba Chemie, Pvt., Ltd., (Mumbai, India). Konjac glucomannan (P-GLML) was purchased from Megazyme (Wicklow, Ireland) and used as standard.

2.3.2 Design of experimental

Hydrolysis parameters were varied by response surface methodology (RSM) using four variables Box-Behnken factorial design (BBD) from Minitab 16 software to determine the optimum conditions for producing KOG. The parameters were temperature, time, pH and E/S. Table 2.1 showed the range and central point of parameters.

Table 2.1 Parameters and the range used in response surface design

Parameters	Level		
	-1	0	1
X_1: Time (h)	2	4	6
X_2: Temperature (ºC)	43	48	53
X_3: pH	5.0	5.5	6.0
X_4: E/S (%)	0.025	0.050	0.100

2.3.3 Production of konjac oligo-glucomannan

Konjac glucomannan (1% w/v) was added into 0.2 M CH_3COONa (sodium acetate) buffer and then mixed with β-mannanase (E/S ranged from 0.025-0.1% w/w) to start the reaction. The mixture stirred continuously and incubated with shaker water bath at pH variations between 5 to 6 for reaction times range from 2 to 6h. The temperature of water bath was controlled at the temperature of 43 to 53 ºC. Activity of enzyme was inactivated by heating the sample for 10 minutes and then concentrated by centrifugation at 8,000 g. The supernatant then analyzed using high performance size exclusion chromatography (HPSEC; Agilent model 1200 series, CA, USA) to analyze KOG content and molecular weight distribution. Chromatography analysis gives the results that showed the ratio of polysaccharides, oligosaccharides, and monosaccharides from enzymatic hydrolysis process. The results were compared with the standard and used to determine the optimum conditions of producing KOG in a bigger scale for prebiotic potency study. Figure 2.1 shows the schematic production of konjac oligo-glucomannan by enzymatic hydrolysis of KGM.

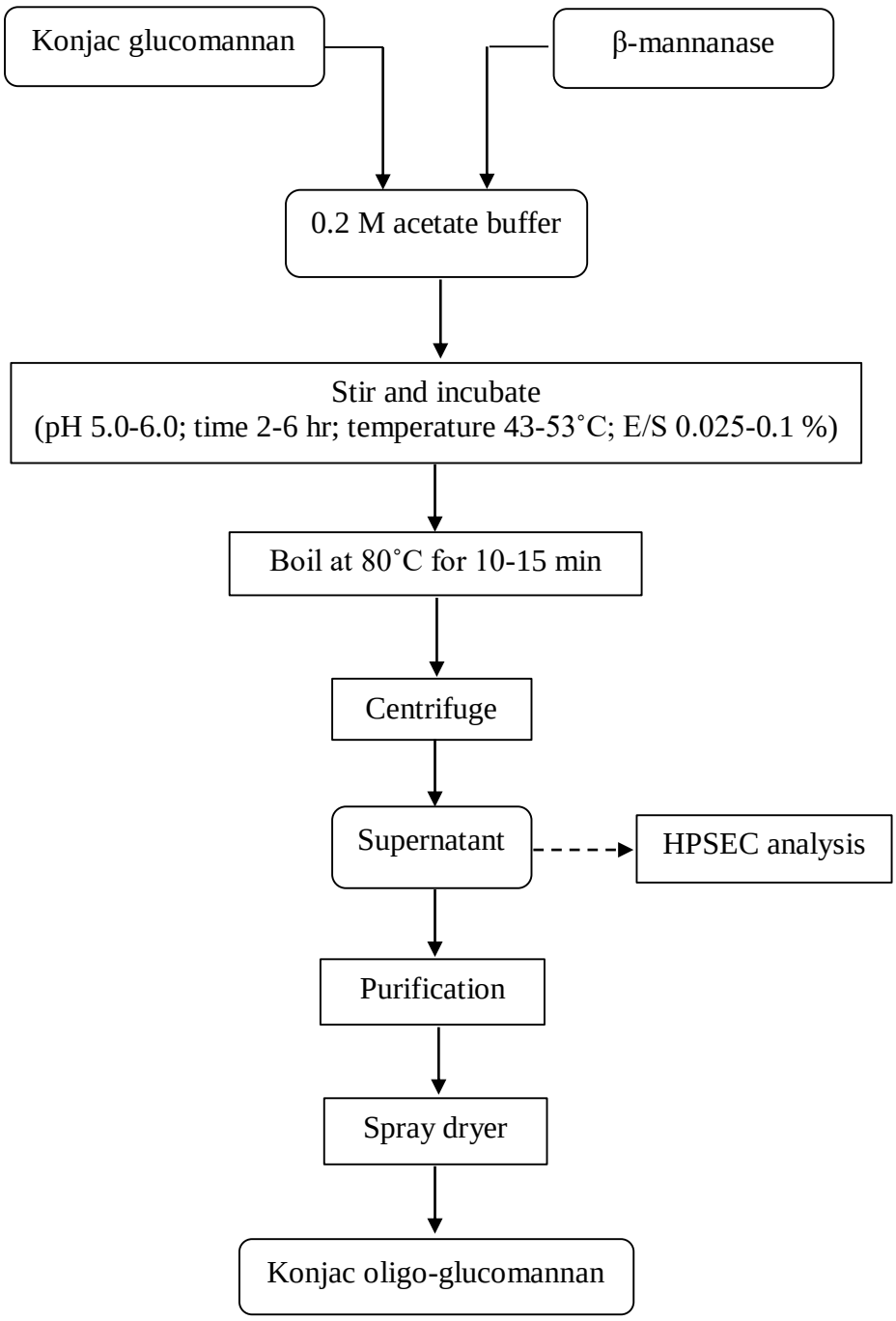

Figure 2.1 Schematic production of konjac oligo-glucomannan

2.4 Results and discussion

2.4.1 Oligosaccharides concentration from enzymatic hydrolysis of konjac glucomannan

Box-Behnken factorial design (BBD) experimental from Minitab 16 software was carried out to optimize the hydrolysis temperature, hydrolysis time, pH and enzyme to substrate ratio (E/S). The reaction temperature was set to temperature 43, 48 and 53 ºC with the pH of 5.0, 5.5 and 6.0 and E/S of 0.025, 0.05 and 0.1 %. The reaction time was set at 2, 4 and 6 h. Each variation of the factors was analyzed by high performance size exclusion chromatography (HPSEC) to determine the sugar content after hydrolysis.

Response surface methodology (RSM) were used to optimize the hydrolysis parameters with BBD to determine the combination of parameters to predict the optimum condition for KOG production. From the prediction using the RSM, the optimum condition was under time of 5.68 h, temperature of 50.37ºC, pH 6.0 with E/S 0.1%. HPSEC was conducted to analyze the oligosaccharides concentration based on the optimum condition given by RSM.

The concentration (mg/mL) of oligosaccharides from enzymatic hydrolysis process in each condition after analyzed by HPSEC are showed in Table 2.2. The results were calculated from the area of oligosaccharides peak in chromatogram using the oligosaccharides standard curve to get the concentration of oligosaccharides produced in mg/mL. From the result, the highest oligosaccharides concentration was 9.21 mg/mL and found under the conditions of 48 ºC hydrolysis temperature, pH 5.5, E/S of 0.05% (w/w) after 4h of hydrolysis time. The result under the optimum condition from prediction by RSM found out that the concentration of oligosaccharides was 7.76 mg/mL with polysaccharides concentration was 7.67 mg/mL. The concentration of oligosaccharides under prediction of optimum condition was lower compared to one of the treatments described above. This might be occurred because the pH (6.0) and temperature (50.37ºC) was higher than the recommendation (pH 5.5, temperature 48ºC) for enzyme to work optimally.

As a result, condition with 48 ºC hydrolysis temperature, pH 5.5, E/S of 0.05% (w/w) and 4h of hydrolysis time was chosen to be the condition to produce KOG

because contained highest concentration of oligosaccharides after enzymatic hydrolysis of KGM. Another study to optimize KOG production using RSM has been done by Chen *et al.* (2013). They optimized the temperature of hydrolysis, incubation time, pH and E/S ratio (w/w). The optimum temperature of the study was lower than this study with shorter incubation time. The pH was higher than this research with E/S almost ten times higher. Their study was not reported the yield of KOG produced and its molecular weight. This optimum condition to produce KOG in our research is comparable with them and it indicated that this method can be used to produce KOG in bigger scale.

Table 2.2 Concentration of oligosaccharides from enzymatic hydrolysis process

Treatment				
Time (hr)	**Temp (ºC)**	**pH**	**E/S (%)**	**Oligosaccharides concentration (mg/mL)**
4	43	5.5	0.025	8.26
6	43	5.5	0.050	8.85
2	43	5.5	0.050	8.49
4	43	5.5	0.100	9.08
4	43	5.0	0.050	8.46
4	43	6.0	0.050	8.63
2	53	5.5	0.050	8.25
6	53	5.5	0.050	8.88
4	53	5.5	0.025	8.92
4	53	5.5	0.100	8.73
4	53	5.0	0.050	8.93
4	53	6.0	0.050	8.85
2	48	5.5	0.025	8.80
2	48	5.5	0.100	6.40
6	48	5.5	0.100	8.67
4	48	5.5	0.050	8.96
4	48	5.5	0.050	8.87
6	48	5.5	0.025	8.49
6	48	5.0	0.050	8.81
4	48	5.0	0.025	8.39
4	48	5.0	0.100	8.65
2	48	5.0	0.050	8.30
4	48	6.0	0.025	7.86
4	48	6.0	0.100	8.42
6	48	6.0	0.050	8.78
4	48	5.5	0.050	9.21
2	48	6.0	0.050	8.71

2.4.2 Konjac oligo-glucomannan production

Konjac oligo-glucomannan (KOG) can be produced by hydrolysis of konjac glucomannan (KGM) using β-mannanase. In this study, KOG was produced by optimization the enzymatic hydrolysis conditions. According to the instruction that are given by the manufacturer of the enzyme, the temperature and pH for enzyme to work optimally in this study are appropriate with their instruction. Chen *et al*., (2013) found that as the time of hydrolysis increased, the activity of β-mannanase was likely decreased. It was discovered that 4h was the optimum hydrolysis time. This result is suitable to ours. E/S ratio was varied based from previous studies. The average of oligosaccharide obtained from 0.1% of E/S ratio was not showed too much different compared with E/S ratio of 0.05%. For this reason, 0.05% was set to be the center point of E/S ratio. In this study, KOG was produced successfully by hydrolysis of KGM using β-mannanase. Despite the fact that this method can be applied in production of KOG, the product still containing unhydrolyzed KGM that showed in the percentage of polysaccharide that presented in the optimum condition. The result informed that β-mannanase was partially hydrolyzed KGM within 4h of reaction time. Purity of KOG from enzymatic hydrolysis process was one of the reasons to improve its quality in the market related with the functionality for health. Suitable method is needed to purify the KOG and ultrafiltration is one of the method that has been applied in the separation and purification of polysaccharides because of its advantages (Xing and Li, 2009). For prior study, purification step is needed to purify KOG mixture to get the higher concentration of oligosaccharide in the final product.

2.4.3 Molecular weight of konjac oligo-glucomannan

Molecular weight (MW) of KOG in this study for each condition is analyzed by HPSEC. The MW of oligosaccharides produced analyzed by gel permeation chromatography using KGM as standard. The result of MW KOG showed in Table 2.3. The MW of oligosaccharides produced in this study of each condition is in the range of 1,200 – 2,700 Da. Under the optimum condition of in this study, MW of KOG was 1,552 Da ± 2.83.

Table 2.3 Molecular weight distribution of KOG analyzed by high performance size exclusion chromatography (HPSEC)

Treatment				
Time (hr)	Temp (˚C)	pH	E/S (%)	MW (Da)
4	43	5.5	0.025	1684 ± 4.95
6	43	5.5	0.050	1691 ± 2.12
2	43	5.5	0.050	1381 ± 1.41
4	43	5.5	0.100	1383 ± 2.83
4	43	5.0	0.050	1715 ± 7.07
4	43	6.0	0.050	1669 ± 1.41
2	53	5.5	0.050	1745 ± 7.78
6	53	5.5	0.050	2103 ± 6.36
4	53	5.5	0.025	2260 ± 4.24
4	53	5.5	0.100	1948 ± 2.83
4	53	5.0	0.050	2622 ± 4.95
4	53	6.0	0.050	1854 ± 4.95
2	48	5.5	0.025	2064 ± 8.49
2	48	5.5	0.100	2117 ± 6.36
6	48	5.5	0.100	1793 ± 7.07
4	48	5.5	0.050	1566 ± 5.66
4	48	5.5	0.050	1327 ± 2.12
6	48	5.5	0.025	1367 ± 4.95
6	48	5.0	0.050	1461 ± 9.19
4	48	5.0	0.025	1775 ± 6.36
4	48	5.0	0.100	1273 ± 8.49
2	48	5.0	0.050	1477 ± 4.24
4	48	6.0	0.025	1455 ± 6.36
4	48	6.0	0.100	1364 ± 5.66
6	48	6.0	0.050	1466 ± 5.66
4	48	5.5	0.050	1552 ± 2.83
2	48	6.0	0.050	1496 ± 7.78

2.5 Conclusion

The optimal conditions for production of KOG were 48˚C of hydrolysis temperature, pH 5.5, E/S of 0.05% and hydrolysis time of 4 h with the concentration of oligosaccharides produced was 9.21 mg/mL. The product still containing unhydrolyzed KGM that shown in the peak area of polysaccharide. Purification step is needed in order to get higher concentration of KOG.

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CHAPTER 3

PURIFICATION OF KONJAC OLIGO-GLUCOMANNAN

3.1 Abstract

Purification of konjac oligo-glucomannan (KOG) was performed by polysulfone (PSU) ultrafiltration (UF) hollow fiber membrane. The aim of using the membrane was to remove the polysaccharides of KGM that was not hydrolyzed by enzyme to get higher purity of KOG. Molecular weight cut off (MWCO) of the membrane that used in this study was 3,000 Da with 140 cm^2 membrane effective area After purifying by membrane, both permeate and retentate of KOG then dried using spray dryer to obtain powder of KOG. It was found that purity of KOG in permeate increased because the concentration of oligosaccharides increased up to 9.54 mg/mL after filtration by UF hollow fiber membrane for 9h of operating time. Purified KOG then dried at flow rate of 0.17 L/h with outlet temperature of 170 ºC and inlet temperature of 80 ºC. KOG powder from both permeate and retentate are used to prebiotic properties test. The result showed that UF membrane was successfully purified KOG.

3.2 Introduction

Food-grade oligosaccharide products are mixtures containing different molecular weight of oligosaccharides, polysaccharides and monosaccharides. Purifying of oligosaccharide are important step to separate the prebiotic oligosaccharides from its impurities because not all of these sugars have prebiotic activity (Goulas *et al.*, 2002). Impurities can be removed by membrane filtration or by chromatographic methods. Development and advantages in membrane based filtration techniques such as high efficiency, simple operation and equipment and low energy consumption during the operation made the membrane filtration become one of the important separation techniques in sugar purification (Li and Chase, 2010).

Membrane filtration has potential to purify oligosaccharides. General principle of the membrane filtration is based on the permeability of the membrane to

let the target substances pass through the membrane. Based on the size exclusion mechanisms, membrane techniques can be classified into nanofiltration, ultrafiltration and microfiltration which are found out to be suitable to sugar separation because of their ability to separate various molecular size within in the range of the membrane pore size. The importance of membrane filtration techniques has been increased for refining the present of oligosaccharides in carbohydrates (Li and Chase, 2010).

Ultrafiltration (UF) is a type of membrane that use the pressure to separate the substance by passing through the semipermeable membrane. Generally, high molecular weight substances will be removed by UF. It has the range of pore size from 0.002 to 0.1 microns with pressure during operation start from 10 to 100 psi. UF techniques are used to purify commercial oligosaccharides by removing the higher molecular weight sugars from oligosaccharides mixtures or by separated and concentrated oligosaccharides from polysaccharides (Kim *et al.,* 2003) There are two products from purification process using membrane. Those are permeate and retentate. Permeate is the product that pass through the membrane and contains of water and low molecular weight solutes. Retentate is the extract and contains high molecular weight of solutes. UF membrane using particle capture or size exclusion as based for substrates separation (De Jong *et al.*, 2006).

Utilization of UF membrane in food industry have been successfully developed from laboratory equipment to be industrial processing equipment. It has been used to treat substrates such as in cereal products, agricultural and dairy products (Señorans *et al.,* 2003). In separation of food macromolecules, UF membrane commonly use to recover or purify dietary fiber from small ingredients such as sugars (Toledano *et al.*, 2010). Applications of UF membrane are incorporated in the pure water production, fractionation and separation in food industries, treatment of agricultural wastewaters and in pharmaceutical and biotechnological industries (Susanto and Ulbricht, 2005).

Hollow fiber membranes are showing great commercial interest in the application of bio separations, purification of drinking water and wastewaters treatment. Mass transfer ability of hollow fiber membrane is the advantage in the several fields such as medical and water purification (Feng *et al.*, 2013). Another

advantages are including high effective membrane surface area, self-supported characteristics and can be handle effortlessly. Self-supported characteristics of hollow fiber membrane makes membrane easy to clean (Wan *et al.*, 2017).

Purification of KOG after enzymatic hydrolysis of KGM is needed to improve the final product of oligosaccharides. Column chromatography and ultrafiltration by membrane has been used in the purification of polysaccharides. Lower production of oligosaccharides purity in column chromatography method makes ultrafiltration is widely applied in purification step. Studied by Jian *et al.* (2013) showed that oligosaccharides from KGM degradation with β-mannanase was effectively separated by ultrafiltration. The purpose of purification step was to concentrate oligosaccharides in permeate by rejecting big molecules of polysaccharide back into the feed tank as retentate. In this study, KOG was filtered using polysulfone (PSU) ultrafiltration (UF) hollow fiber membrane with molecular weight cut off (MWCO) was10,000 and 3,000 Da.

3.3 Materials and methods

3.3.1 Materials

KOG produced from the enzymatic hydrolysis process in this study with concentration of oligosaccharides was 9.21 mg/mL. Sodium hypochlorite (10%) was purchased from Vidhyasom (Thailand). Sodium hydroxide was purchased from Ajax Finechem Pty Ltd. (Australia).

3.3.2 Purification of konjac oligo-glucomannan by ultrafiltration membrane

KOG was filtered using polysulfone (PSU) ultrafiltration (UF) hollow fiber membrane (GE Healthcare Pte Ltd., Singapore) with pore size of 10,000 and 3,000 NMWC (membrane effective area was 110 and 140 cm^2, respectively). Membrane was operated for 6h for 10,000 NMWC and 9 h for 3,000 NMWC and the conditions of trans-membrane pressure (TMP) was set to 1.5 bar with feed tank temperature 50 ºC. Both retentate and permeate were collected and then analyzed by HPSEC to determine the performance of the membrane in purification of KOG mixtures. Purified KOG then dried by spray dryer.

3.3.3 Oligosaccharides content analysis

Oligosaccharides content in both permeate and retentate was analyzed using high performance size exclusion chromatography (HPSEC). The concentration mg/mL of oligosaccharides was calculated from the area of oligosaccharides peak in chromatogram using the oligosaccharides standard curve to get the concentration of oligosaccharides produced in mg/mL.

3.3.4 Drying of purified konjac oligo-glucomannan

KOG powder from purification process was obtained using spray dryer (Buchi Mini Spray Dryer B-209, Switzerland). Outlet temperature was 170 ºC, inlet temperature was 80 ºC and flow rate of the sample was 0.17 L/h. Spray dryer was fed with water for 30 min before feeding with purified KOG. Powder of purified KOG the sealed and stored in -20 ºC for next study about prebiotic effect of KOG

3.4 Results and discussion

3.4.1 Purification of konjac oligo-glucomannan

Membrane filtration that used for this purification step was ultrafiltration (UF) membrane with pore size 10,000 NMWC and 3,000 NMWC. The purpose for this step was to purify the mixture of konjac oligo-glucomannan (KOG) by rejected the polysaccharides present. As the molecular weight of polysaccharides from konjac glucomannan (KGM) was 10^5-10^6 Da, this size of membrane was expected to reject the high molecular weight molecules from the product. High molecular weight of polysaccharides will be concentrated in retentate and the low molecular weight of oligosaccharides will be obtained in permeate. Both retentate and permeate then analyzed by high performance size exclusion chromatography (HPSEC) to know the performance of the membrane to purify KOG mixtures.

According to the result in the production of KOG under the optimum conditions, the KOG mixtures under hydrolysis time 4 h, temperature 48˚C, pH 5.5, and E/S 0.05% contained 12.81 and 9.21 mg/mL of polysaccharides and oligosaccharides, respectively. This KOG mixture was selected to pass through 10,000 and 3,000 NMWC

UF membrane. Concentration of polysaccharides and oligosaccharides presented after analyzed by HPSEC are shown in Table 3.1. Schematic diagram of membrane filtration is shown in Figure 3.1.

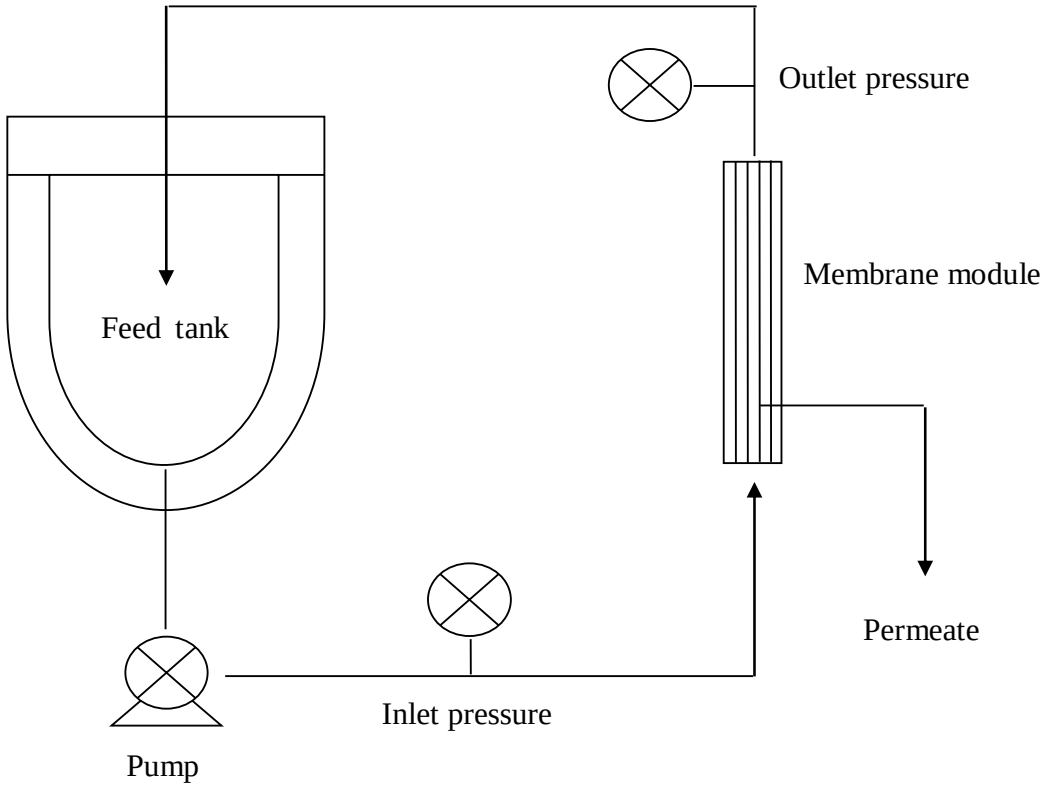

Figure 3.1 Schematic diagram of membrane filtration

Table 3.1 Concentration of KOG

Concentration (mg/ mL)				
Before UF	Polysaccharide		Oligosaccharide	
	12.81		9.21	
After UF	**10,000 NMWC**		**3,000 NMWC**	
	Polysaccharide	Oligosaccharide	Polysaccharide	Oligosaccharide
Permeate	7.93	9.24	5.65	9.54
Retentate	13.60	8.64	13.35	8.49

The result after analyzed by HPSEC showed that there was different concentration of oligosaccharides before and after purification step. Oligosaccharides content in KOG before passed through UF was 9.21 mg/mL. After passed through 10,000 NMWC UF membrane, concentration of oligosaccharides in permeate increased up to 9.24 mg/mL. In permeate from 3,000 NMWC, concentration of oligosaccharides increased up to 9.54 mg/mL. Concentration of oligosaccharides in retentate from 10,000 and 3,000 NMWC was 8.64 and 8.49 mg/mL, respectively. The concentration of high molecular weight was increased as the filtration time increased. High molecular weight was concentrated in retentate and the smaller molecules were obtained in the permeate. Molecular weight of oligosaccharides before purity was 1552 ± 2.83 Da, as shown in Table 2.3. Analysis of the molecular weight of permeate and retentate found that MW in permeate was smaller compare to retentate. Permeate with high concentration of oligosaccharides contained low molecular weight of oligosaccharides and in this research named low MW of KOG (LKOG) according to MW inside the product. Retentate with oligosaccharides concentration was 8.49 mg/mL labelled as high MW of KOG (HKOG) based on its MW that higher than LKOG. Oligosaccharides in the retentate might be presented because of the fouling happened during operating time that blocked the pore size and made the oligosaccharides flow back into the feed tank. The 10,000 NMWC membrane pore size might not effective to purify KOG in this study because the concentration of oligosaccharides in permeate was lower than the concentration obtained from permeate of 3,000 NMWC. From the results of HPSEC

analysis, it can be concluded that 3,000 NMWC obtained the highest concentration of KOG in permeate. Purity of KOG increased as the concentration of oligosaccharides increased. Study on preparation and separation of KOG that has been done by Jian *et al.* (2013) using combination of γ-irradiation and enzymatic hydrolysis was successfully produced KOG with MW bigger than 2,000 Da. They found out that 1,000 Da MWCO membrane could effectively separate and purify KOG. Our research was produced smaller MW and stated the purity that can be increased using UF membrane compared to their study.

Both permeate and retentate collected from 3,000 NMWC membrane were the dried using spray dryer with the conditions of outlet temperature was 170 ºC, inlet temperature was 80 ºC and flow rate of the sample was 0.17 L/h. The powders of KOG obtained from this process then used for prebiotic effect study of KOG.

3.5 Conclusion

The result showed that there was different concentration of oligosaccharides in the permeate and retentate of KOG compare to mixture of KOG before passed through the membrane. The 10,000 NMWC membrane pore size might not effective to obtained oligosaccharides from KOG mixtures. UF membrane with pore size of 3,000 NMWC was able to effectively purify KOG by increasing the concentration of oligosaccharides up to 9.54 mg/mL in permeate. From the results of HPSEC, it can be concluded that permeate from 3,000 NMWC obtained highest purity of KOG.

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CHAPTER 4

GUT FERMENTATION OF KONJAC OLIGO-GLUCOMANNAN

4.1 Abstract

The effect of konjac oligo-glucomannan (KOG) on changes in human fecal bacterial populations and short chain fatty acids (SCFAs) production were evaluated. The prebiotic effect KOG was performed using fecal fermentation by batch culture. Bacterial populations were analyzed using fluorescent *in situ* hybridization (FISH) technique. The result showed that LKOG with 9.54 mg/mL concentration of oligosaccharides has positive prebiotic index (PI) value with 0.76. KOG can enhance the production of butyric acid in the colon with the highest concentration (8.24 mM) was found at 72h fermentation and its fermentation was increasing the populations of beneficial bacteria (bifidobacteria and lactobacilli). Positive PI value of LKOG and its ability to support the growth of beneficial bacteria especially bifidobacteria are indicated that LKOG has potency as a prebiotic and it may have specific health function related with butyric acid production. Further study of KOG might be needed to investigate its beneficial effect in human.

4.2 Introduction

Gastrointestinal health and its disease related with organisms that inhabit in the gut. Change in environmental factors, lifestyle, diseases and infections lead to impact the compositions of bacterial communities. Composition of the gut habitat can also be affected because of the diet (Frank and Pace, 2008). Intestinal environment will be affected by food daily intake because it is related with the production of SCFAs by intestinal microbiota. The concentrations of SCFAs may contribute for the acidity of intestinal environment (Shimizu, 2012). SCFAs are undegradable by human digestion (Primec *et al.*, 2017), which include acetate, propionate and butyrate as the most abundant SCFAs in the body. The amount of microflora presence and its species in the colon, source of the substrate and gut transit time are affected SCFAs production (den Besten *et al.*, 2013).

The most frequently strain used as probiotics are *Bifidobacterium* and *Lactobacillus*, predominant and subdominant groups, respectively, of gastrointestinal microbiota. They have been used in many functional foods and in dietary supplements (Bermudez-Brito *et al.*, 2012). Carbohydrates fermentation by bacterial in the large intestine are produced SCFAs as the end products, especially acetate, propionate and butyrate. This products are used by the host to improve their health (Tuohy *et al.*, 2005). SCFAs have physiological effects because they contribute for shaping the environment of gut, affected colon physiology and act as source of energy for intestinal microbiota and host cells. Increase production of SCFAs may result protective effects for the host body (Ríos-Covián *et al.*, 2016).

Acetic acid is the principal SCFA in the colon, and it has been shown to increase cholesterol synthesis after absorption (Lin *et al.*, 2012). Acetic acids are metabolized by muscle, kidney, heart and brain in the peripheral tissues after passed through the liver. It is used by the body to gain energy. Production of acetate is widely distributed among different group of bacteria compared to propionic and butyric production (den Besten *et al.*, 2013). Lower pH in the colon will affects the gut microbiota composition. Bacteria related with production of acetic acid become dominant in the colon when pH increases to 6.5. Almost 70% of acetate is taken up by the liver and used as a substrate for other essential molecules such as cholesterol, long chain fatty acid, glutamine, and glutamate (Li *et al.*, 2016).

Propionic acid is one of primarily short chain fatty acid that metabolites by gut microbiota from dietary carbohydrates fermentation. It is mainly utilized by liver and was shown to reduce food intake (Lin *et al.*, 2012), protected against diet-induced obesity and insulin resistance and induced gut hormones (Richards *et al.*, 2016). Propionate produce by more specific groups of substrates and bacteria compared to acetate production. It can be used as a food preservation and also in food production because of its characteristics to inhibit the growth of microorganisms (Fluegge, 2017).

Butyric acids are mostly produced from resistant starch fermentation (Morrison and Preston, 2016) which supports the health and healing of cells in the small and large intestine. Butyric acids have anti-carcinogenic that are important for keeping colon cells healthy (Greer and O'Keefe, 2011). Several studies have been conducted

about the role of butyrate in nourishing the colonic mucosa and prevention of cancer of the colon (Manderson *et al.*, 2005). Daily energy in the gastrointestinal mucosa is provided by butyric acids for almost 50% (Tuohy *et al.*, 2005).

Konjac glucomannan (KGM) is high molecular weight polysaccharides and consist of mannose and glucose and it is a source of dietary fiber (Fang and Wu, 2004). Konjac oligo-glucomannan (KOG) is indigestible oligosaccharide that serve as dietary fibers and prebiotics (Patel and Goyal, 2011). Gut microorganism needs substrate to grow, and dietary fiber and non-digestible oligosaccharides are the main substrates for the growth of gut microorganisms (Blaut *et al.*, 2017). As prebiotics, KOG pass into the large bowel and increase the number of lactobacilli and bifidobacterial colonies (Chen *et al.*, 2013).

Prebiotic activities may have a contribution for health by promoting the growth of bifidobacteria and/ or lactobacilli (Connolly *et al.*, 2010). Prebiotic index (PI) value is used to characterize the prebiotic activities of a substrate. Association between changes in beneficial bacteria with unwanted bacteria related with their beginning level was described as PI (Thitiratsakul and Anprung, 2014) and it can be calculated from the formula using the number of enumeration bacteria in FISH technique. Study about konjac glucomannan hydrolysate (GMH) on bacterial composition and its activity on fecal microbiota has been done by Connolly *et al.* (2010). On their study, GMH was selectively fermented by bifidobacteria and lactobacilli and produced SCFAs rich in propionic acid. Potential use of KGM degraded product as prebiotic showed that it stimulated the growth of bifidobacteria and lactobacilli. In UHT milk, uses of konjac hydrolysate as a carbon source supported the bacterial growth better than inulin (Al-Ghazzewi *et al.*, 2007). These studies showed that degradation product of KGM has some characteristic of prebiotic inulin and it may have potential to be used in foods as prebiotic.

In this study, powder of purified KOG that obtained in permeate and retentate are used to test for its prebiotic properties by fecal fermentation using batch culture model. Samples that used for this research were low molecular weight of KOG from permeate (LKOG) and high molecular weight of KOG from retentate (HKOG),

inulin as positive control, KGM, porang glucomannan (PGM) as comparison and additional vessel without treatment.

4.3 Materials and methods

4.3.1 Materials

Purified KOG from this study with 9.54 mg/mL (LKOG) and 8.49 mg/mL (HKOG) of oligosaccharides concentration. Probes of DNA (Bif164, Lab158, Cris150, Bac303 and Eub338) used for FISH technique were purchased from Sigma-Aldrich. Konjac glucomannan (P-GLML) was purchased from Megazyme (Wicklow, Ireland) and used as standard. Porang glucomannan (PGM) used as comparation was a gift from Faculty of Agricultural Technology, Gadjah Mada University (Yogyakarta, Indonesia). Commercial inulin was purchased from BENEO-Orafti (Belgium).

4.3.2 Preparation of fecal slurry

Fecal samples were collected from six healthy male and female donors who had not consumed probiotics, prebiotics or received antibiotics treatment for at least 3 months, and had no digestive system disease. Fresh fecal samples were put into an anaerobic chamber and mixed with 0.1M phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4 and 0.24 g/l KH_2PO_4) pH 7.4 for the final concentration 10% (w/v). The slurry was homogenized by stomacher for 2 min and filtered using dialysis bag before inoculated into vessels.

4.3.3 Batch culture of konjac oligo-glucomannan

Water jacket glass vessels were used and filled with batch culture medium that consisted of (per liter) 0.9 g peptone water, 0.9 g yeast extract, 0.045 g NaCl, 0.018 g K_2HPO_4, 0.018 g KH_2PO_4, 0.0045 g $MgSO_4.7H_2O$, 0.0045 g $CaCl_2.6H_2O$, 0.9 g $NaHCO_3$, 0.225 g L-cysteine.HCl, 0.225 g bile salts, 0.9 mL Tween 80, 4.5 µL vitamin K, 0.0225 g hemin and 0.45 mL of 0.025% resazurin. The medium was dissolved in distilled water, adjusted to pH 7.0 and then sterilized by autoclave. Sterilized medium (90 mL) was placed and stirred magnetically into each water jacket

glass vessel with temperature and pH were controlled (37 ºC, pH 6.8) using circulated water bath and pH controller, respectively. The medium was maintained under an anaerobic environment by pumped in the nitrogen gas into vessel to imitate the conditions in colon (Connolly *et al.*, 2010) overnight. The batch culture vessels were inoculated with 10 mL of a 10% (w/v) fecal slurry. Samples of LKOG, HKOG, KGM, PGM and inulin were added and dissolved into five vessels for final concentration of 1% (w/v). One additional vessel was used as control without added any sample. Final volume in each vessel was 100 mL. The sample in each vessel was taken for 10 mL at 0, 6, 12, 24, 48 and 72h of incubation time for SCFA analysis and FISH technique. For enumeration of fecal bacteria analysis by FISH technique, all samples are stored overnight in 4% of paraformaldehyde before kept for further analysis. All samples for both analyses are kept in -20°C.

4.3.4 Short chain fatty acids (SCFAs) analysis

Samples from batch culture (1125 µL) were centrifuged at 13,000 g for 15 minutes 4ºC. Supernatant was obtained and filtered through 0.22 µm membrane nylon filter. The samples were analyzed for SCFA concentration for acetic, propionic and butyric acid by injecting into HPLC system. The column used was ion-exclusion Aminex HPX-87H 7.8 x 300 mm from Bio-Rad (Richmond CA, USA) and maintained at 50°C. Diode array detector (DAD) was used as the detector and the UV absorbance was set at 215.4 nm. Mobile phase was 0.005 M H_2SO_4 with flow rate 0.6 mL/min. The quantification of SCFA content was calculated from the calibration curve of the standard by comparing the peak area (mAU*s) of the samples with the standard in the similar retention time.

4.3.5 Enumeration of fecal bacteria

FISH technique is using 16S rRNA-targeted oligonucleotide and it can determine the changes in human fecal bacterial populations at six time points during fermentation. Cells were entered by the probe and it will be hybridized specifically in the ribosomes (Hugenholtz *et al.*, 2002). Fecal batch culture samples (375 µl) were mixed and fixed overnight in cold 4% paraformaldehyde (pH 7.2) at 4 °C in a ratio 1:3

of sample to 4% paraformaldehyde (v/v). These samples then centrifuged under conditions of 13,000 rpm for 20 minutes at 4 °C. Supernatant was removed and washed the pellet by resuspending in 1 mL phosphate-buffered saline (PBS, pH 7.4) and centrifuged for 13,000 rpm for 20 minutes at 4 °C. The addition of PBS and centrifugation were repeated for three times and the remaining pellet was resuspended in 150 µl PBS, added with 150 µl of 96% (v/v) ethanol and stored at -20 °C for further use up to 3 months. The sample at the optimal dilution was mixed and dropped (20 µl) onto Teflon/ Poly-L-Lysine coated six well microscope slides (Tekdon Inc., Myakka City, Florida, USA). The samples on the slide were dried using slide warmer (Digicon MD-700A, Thailand) at 46 °C for 15 min and then dehydrated using alcohol series (50, 80 and 96 %) for 3 min in each concentration. Cells targeted with Lab158 probe was a little bit modified by dropping 20 µl lysozyme into each well at room temperature for 15 min and the slides then washed in cool water (3 sec) before dehydrated in alcohol series. Allowed the ethanol to evaporate and returned to the slide warmer followed by adding 50 µl mixture of probe and hybridization buffer (1:9; v/v) to each well. Hybridization was performed in hybridization oven (Boekel Scientific InSlide Out Slide Hybridizer 241000, Pennsylvania, US) for 4h at appropriate temperature for the probes. The slides were washed with 50 mL of washing buffer for 15 min followed by briefly washed using cool water and dried with compressed air. Antifade agent (10 µl; Invitrogen, USA) was applied onto each well and covered with cover slip before stored in the dark room. The bacterial group was enumerated by fluorescence microscopy (Nikon E400 Eclipse, Nikon Instruments, Inc., New York, USA) and 15 randomized views from each well (Wichienchot *et al.*, 2017) were counted using NIS-Elements BR 3.00, SP6 software (Nikon Instruments, Inc., New York, USA) for each sample. Prebiotic candidate can be evaluated with the value of its prebiotic index. Calculation of prebiotic index (PI) (Palframan *et al.*, 2003) is described below:

$$PI = (Bif/ Total) + (Lac/ Total) – (Bac/ Total) – (Clos/ Total)$$

where Bif is number of bifidobacteria, Lac is number of lactobacilli, Bac is number of bacteroides and Clos is number of clostridia. The numerator is collected by the numbers of bacteria at sample time/ number of bacteria at inoculation. Total bacteria is obtained by number of eubacteria count at sample time/number of eubacteria count at inoculation.

4.3.6 Statistical analysis

Statistical analysis for enumeration of bacteria was performed using SPSS software version 21.0. The statistical significance level was set to $p<0.05$ and analyzed using analysis of variance (ANOVA) with Duncan's test.

4.4 Results and discussion

4.4.1 Prebiotics effect of konjac oligo-glucomannan

In the fecal batch culture, populations of fecal bacteria were counted. The microbial groups counted were bifidobacteria, lactobacilli, bacteroides, clostridia and eubacteria as total bacteria. Bifidobacteria and lactobacilli are represented the beneficial bacteria of gastrointestinal microbiota. The pathogenic bacteria are represented by clostridia and bacteroides.

Bifidobacteria number of populations in each sample showed in Figure 4.1. It shows that the growth of bifidobacteria increased in the fermentation of LKOG, KGM, PGM and inulin. The highest population of bifidobacteria was found in the fermentation of KGM at 12h (9.82 ± 0.12 log cell/mL). LKOG fermentation was significantly increased ($P<0.05$) the bacterial groups for bifidobacteria after 24h with the population number of 9.46 ± 0.06 log cell/mL. PGM fermentation showed the population number of bifidobacteria was significantly increased after 6h with the highest number of population (9.57 ± 0.07 log cell/mL) found at 24h fermentation. Inulin fermentation was significantly increased the number of bifidobacteria populations at 6h with the highest population at 12h was 9.69 ± 0.04 log cell/mL. Fermentation of HKOG was significantly increased the number of bifidobacteria after 6h of fermentation and decreased after 12h. Fermentation of LKOG at 24h supported the highest population of bifidobacteria but it was decreased in HKOG fermentation at the same time. This result might be related with the amount and MW of oligosaccharides inside the substrate. Increasing in the number of bifidobacteria populations in all the samples fermentation indicated that LKOG was be able to be used by bifidobacteria as a carbon source as well as in KGM and inulin fermentation.

Figure 4.2 showed fecal bacterial counts of lactobacilli in batch culture of all samples. LKOG fermentation was slightly increased lactobacilli populations after 6h (8.89 ± 0.10 log cell/mL) of incubation compared to 0h. The comparation growth of lactobacilli between LKOG and HKOG was not significantly different. The highest lactobacilli population was found from inulin fermentation at 48h with the population of 9.11 ± 0.15 log cell/mL. Lactobacilli population from LKOG showed better number with the highest number was 8.97 ± 0.06 log cell/mL at 48h compared to highest number of lactobacilli population in KGM (8.87 ± 0.16 log cell/mL),. Among all the samples tested for batch culture fermentation, PGM showed the lowest number of lactobacilli. It may relate with the structure of PGM that make lactobacilli difficult to ferment it.

Lactobacilli have lower amount of population compared to bifidobacteria population. As shown in Figure 4.1 and 4.2, number of bacterial populations of bifidobacteria was higher than lactobacilli in all samples. It showed that both bifidobacteria and lactobacilli were be able to use the sample as their carbon source to support their growth but the ability of bifidobacteria to ferment the samples was higher compared to lactobacilli.

Bacterial populations of bacteroides is shown in Figure 4.3 for all samples. LKOG fermentation decreased significantly in population numbers of bacteroides after 12h and kept decreasing by the time of fermentation, as well as in the fermentation of HKOG. Significantly decreased of bacteroides population showed in the fermentation of KGM and PGM after 12h. Inulin and control are given the similar trend of bacteroides population with the number decreased significantly after 24h of fermentation. Generally, the number of bacteroides is decreased for all samples at the end of fermentation compared to 0h. Previous study about prebiotic activity of PGM *in vivo* showed that PGM has prebiotic activity by suppressing the growth of *E. coli* (Harmayani *et al.*, 2014). In this study, PGM showed the ability to promote the growth of bifidobacteria and significantly reduced the number of clostridia.

Eubacteria in this study is used as the total bacteria in each sample. The population of it showed in Figure 4.5. Population number of eubacteria in LKOG

fermentation was the highest compared to another sample. It indicated that LKOG can be fermented and used by bacteria in the gut to be their substrate to support their growth.

Recent study about protective effects of degraded KGM on bifidobacterial against antibiotic damage (Mao *et al.*, 2018) showed that KGM and its degraded products almost have no influenced to the growth of five strain bifidobacterial in their study. KGM and its degraded product showed to have protective effect on bifidobacteria against penicillin and streptomycin inhibition significantly. The results indicated that KGM and its degradation product have the effects to protect gut probiotic bacteria to against the damage caused by antibiotics. LKOG in our study promoted the growth of bifidobacteria compared to their study, but its effect for the specific strain of bifidobacteria need to be further investigated. Another study by Yang *et al.* (2017) showed that KOG produced by enzymatic hydrolysis with molecular weight that has been reduced from its native was be able to be fermented by lactobacilli and bifidobacterial so that KOG have potential to be used as prebiotics. This result is comparable with ours.

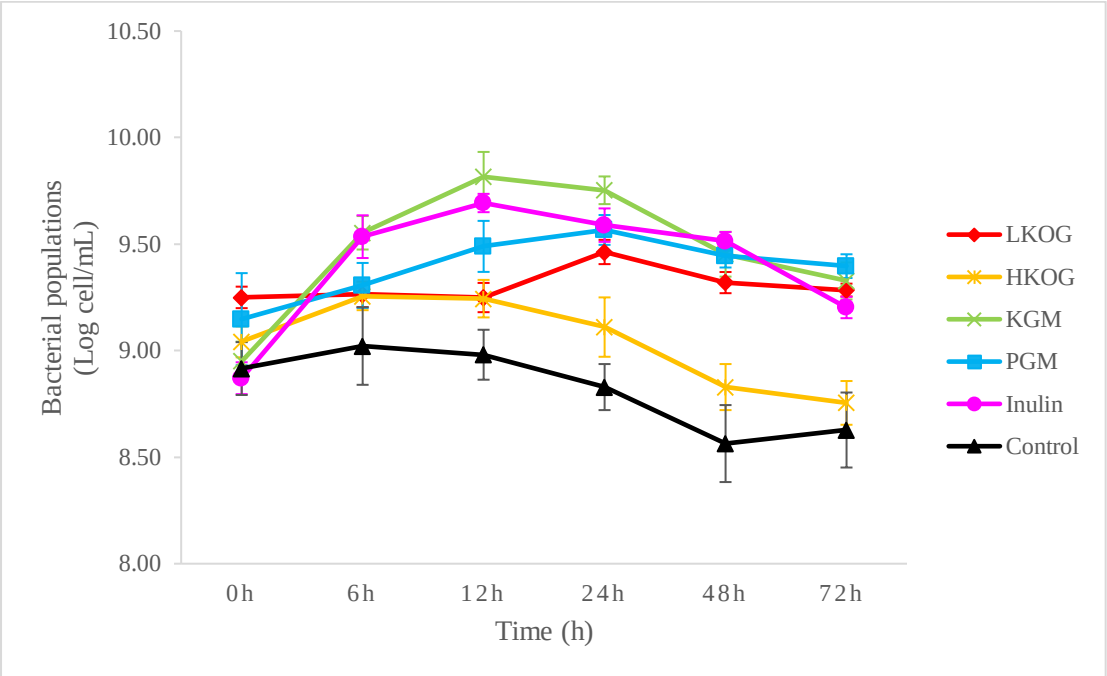

Figure 4.1 Fecal bacterial populations of bifidobacteria in batch culture fermentation

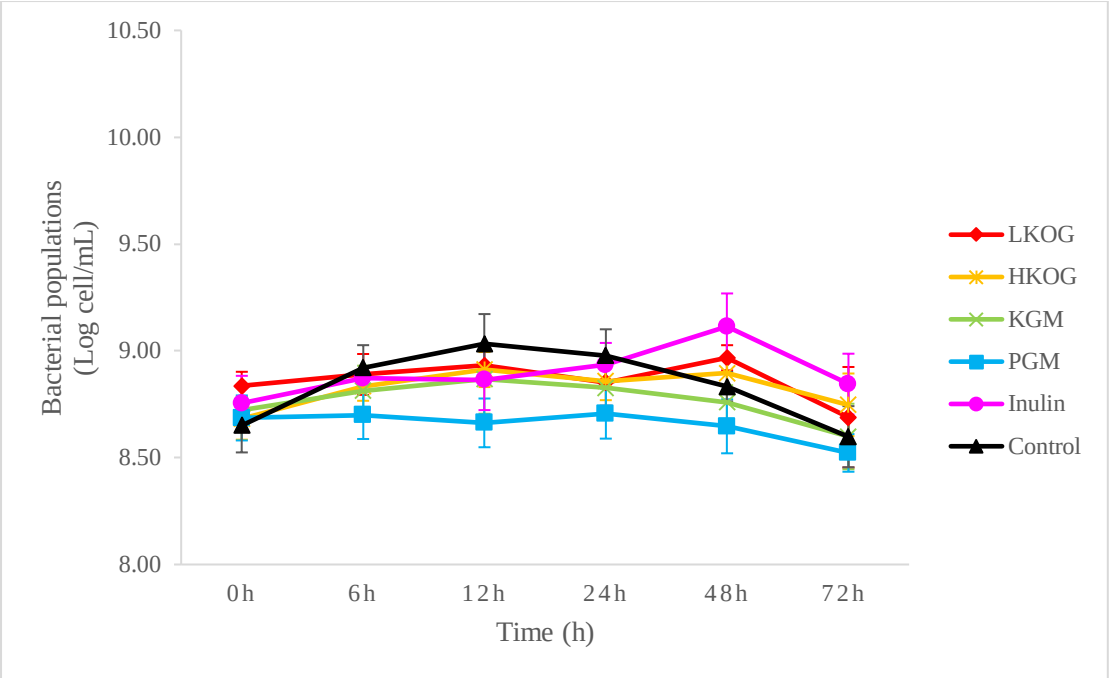

Figure 4.2 Fecal bacterial populations of lactobacilli in batch culture fermentation

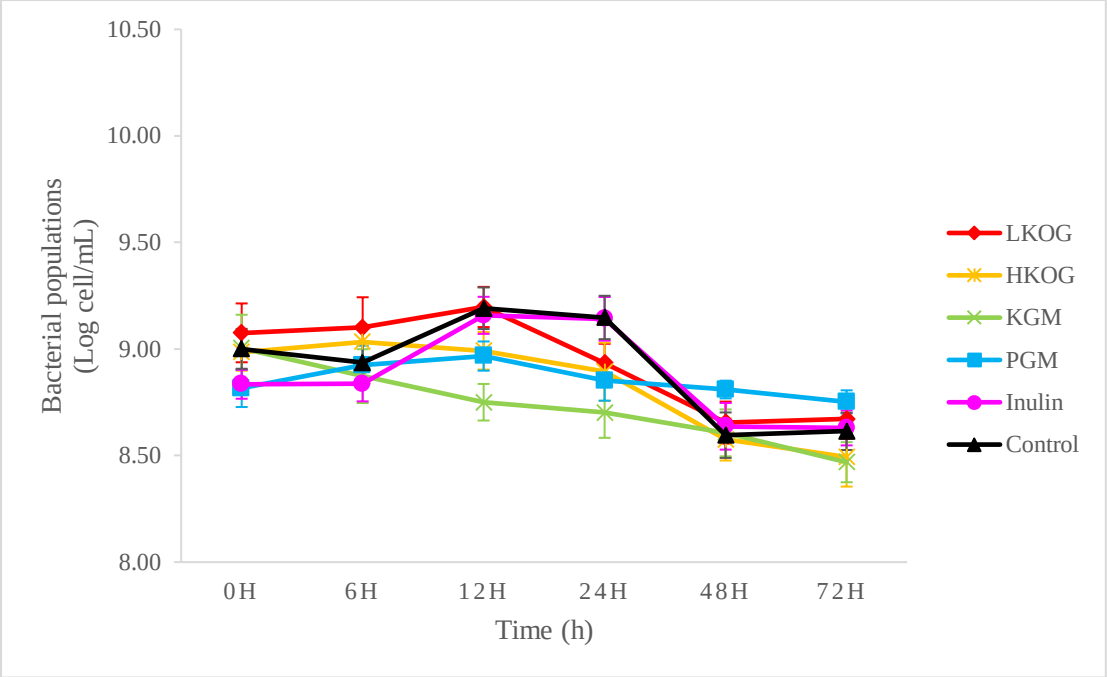

Figure 4.3 Fecal bacterial populations of bacteroides in batch culture fermentation

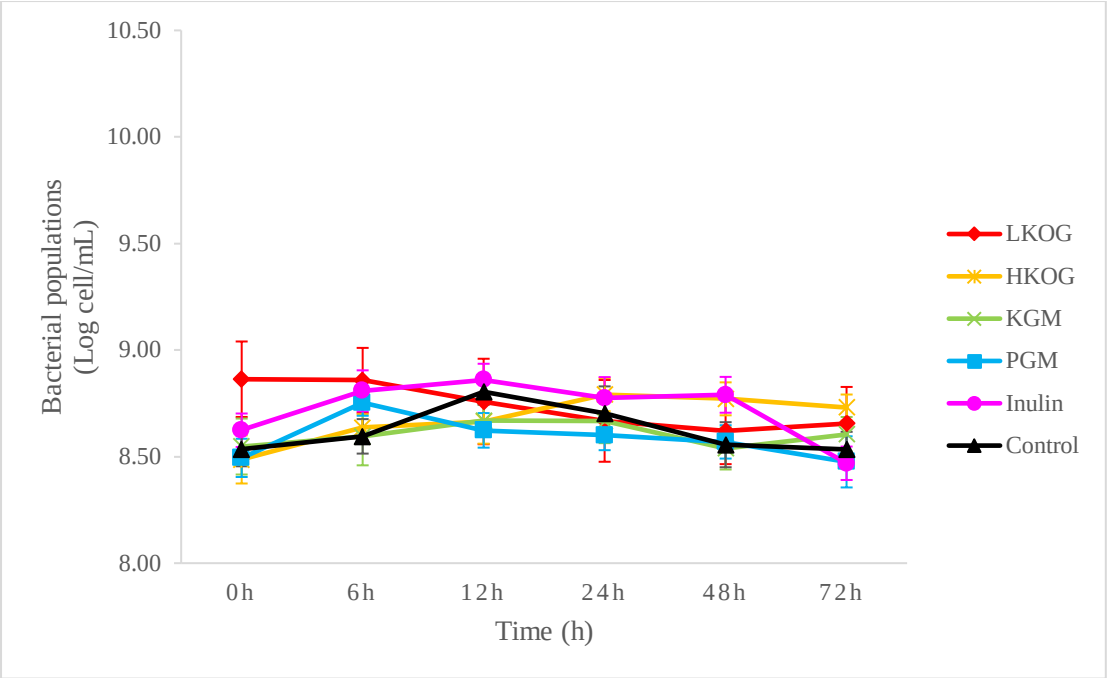

Figure 4.4 Fecal bacterial populations of clostridia in batch culture fermentation

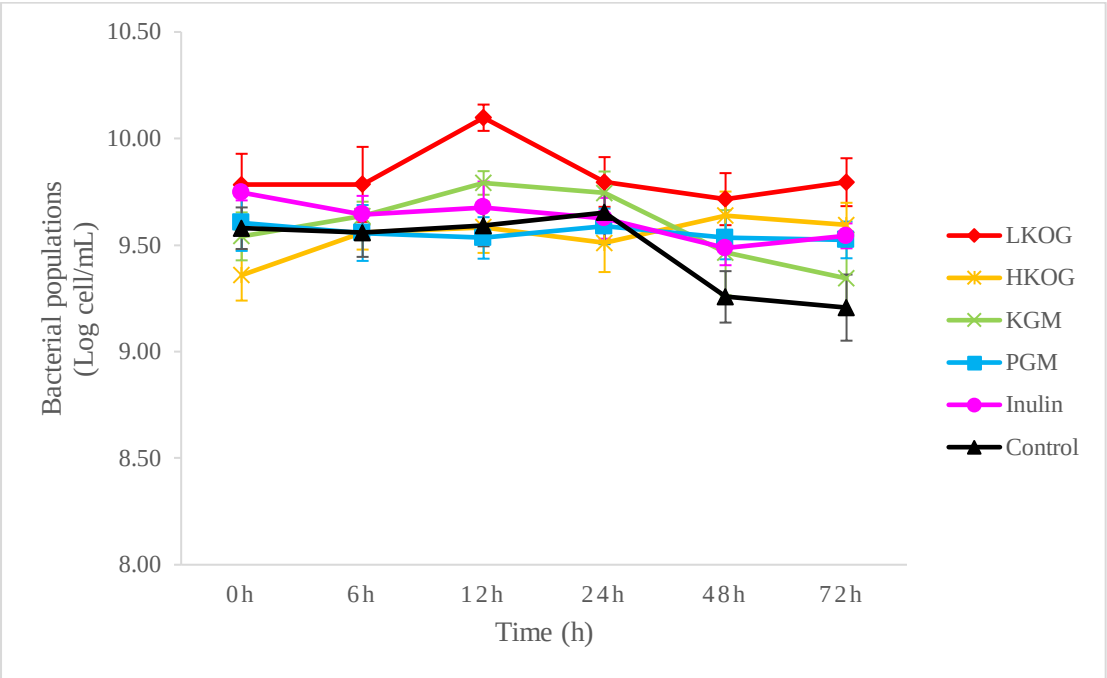

Figure 4.5 Fecal bacterial populations of total bacteria (eubacteria) in batch culture fermentation

Prebiotic activities may have a contribution for health by promoting the growth of bifidobacteria and/ or lactobacilli (Connolly *et al.*, 2010). Prebiotic index (PI) value can be used to evaluate the prebiotic activities by giving the quantitative score. Association between changes in beneficial bacteria with unwanted bacteria related with their beginning level was described as PI (Thitiratsakul and Anprung, 2014) and it can be calculated from the formula using the number of enumeration bacteria in FISH technique. The result of prebiotic index score (Table 4.1.) showed that LKOG and HKOG had positive PI of 0.76 and 0.47, respectively. KGM and PGM as the polysaccharides form of glucomannan had PI value higher than the hydrolyzed glucomannan (1.26 and 1.23, respectively). Inulin as positive control and known as the universal prebiotic had positive PI value of 0.97. LKOG showed to have the ability as prebiotic by showing positive PI value even if it is lower than KGM, PGM and inulin as can be seen in Table 4.1.

Table 4.1 Prebiotic index score of the sample from batch culture fermentation

Sample	**Prebiotic Index (PI)**
LKOG	0.76
HKOG	0.47
KGM	1.26
PGM	1.23
Inulin	0.97
Control	0.41

The fecal batch culture showed that LKOG has prebiotic potency by showing the positive value of PI and its fermentation was increasing the populations of beneficial bacteria (bifidobacteria and lactobacilli). In other side, the fermentation of LKOG had reduced the population of clostridia and bacteroides.

4.4.2 Short chain fatty acids (SCFAs) production

Short-chain fatty acids (SCFAs) are major product of bacterial fermentation process from carbohydrates in fecal batch culture. Concentration of SCFAs of LKOG, KGM and inulin are presented in Table 4.2 and concentration of SCFAs of PGM, HKOG and control are presented in Table 4.3. In each sample, SCFAs are calculated using the equation from diagram of SCFAs standard. The result showed that all the samples are produced acetic and propionic acid during fermentation. Acetic acid was produced dominantly in all fermentation and increased significantly ($P<0.05$) after 12h for KGM and HKOG, 6h for inulin and 24h for PGM. Concentration of acetic acid in HKOG (Fig. 4.3) almost two times lower compared to LKOG in each time but averagely still higher than KGM. This might have correlation with amount of low MW weight inside of the substrates with MW of KGML>HKOG>MLKOG. Acetic acid is the largest SCFAs produced in the colon (Ríos-Covián *et al*., 2016) and in this study, it was highly found in LKOG fermentation compare to KHOG, KGM, PGM and inulin. Propionic acid production required more specific substrate and bacterial group compared to acetic acid (Morrison and Preston, 2016). Increasing of propionic acid production in LKOG was found significant after 6h. KGM could produce propionic acid but it was decreased at 12h. Inulin fermentation produced propionic acid in a smaller amount than acetic acid production. Average production of propionic acid in LKOG fermentation was lower compared to inulin and PGM. But when compared to HKOG, LKOG produced higher concentration of propionic acid. Table 4.2 showed that LKOG fermentation produced butyric acid after 6h and significantly increased by time of fermentation. In HKOG fermentation, butyric acid produced during 6h up to 24h. PGM fermentation produced small concentration of butyric acid but it was not detected after 6h of fermentation. Butyric acid was not produced during inulin fermentation and it was detected at 6h from fermentation of KGM. LKOG promoted the production of butyric acid with the highest concentration (8.24 mM) found at 72h of fermentation. Butyric acid produced from LKOG fermentation are increased as the time fermentation increased. Butyric produced by LKOG fermentation in this study was higher compared to previous study by Connolly *et al*. (2010).

Table 4.2 Concentration of short-chain fatty acids in batch culture fermentation of LKOG, KGM and inulin

Time (h)	Concentration of SCFAs (mM)								
	Acetic acid			Propionic acid			Butyric acid		
	PKOG	KGM	Inulin	PKOG	KGM	Inulin	PKOG	KGM	Inulin
0	105.13 ± 0.03^{a}	10.73 ± 1.28^{b}	5.06 ± 0.05^{d}	3.52 ± 0.04^{b}	16.98 ± 0.93^{a}	3.88 ± 0.17^{c}	ND	ND	ND
6	100.89 ± 1.11^{ab}	10.46 ± 0.37^{b}	29.00 ± 0.34^{c}	7.16 ± 1.93^{a}	18.10 ± 0.32^{a}	3.14 ± 0.16^{c}	4.62 ± 1.62^{b}	3.29 ± 0.18^{a}	ND
12	95.23 ± 2.17^{b}	42.90 ± 1.33^{a}	34.83 ± 0.80^{bc}	5.46 ± 0.11^{ab}	5.36 ± 0.72^{c}	7.44 ± 0.08^{b}	5.52 ± 0.11^{ab}	ND	ND
24	97.38 ± 2.29^{b}	49.47 ± 0.30^{a}	43.13 ± 3.56^{a}	6.54 ± 0.78^{ab}	6.94 ± 0.22^{bc}	12.46 ± 1.40^{a}	7.67 ± 1.02^{a}	ND	ND
48	97.13 ± 2.34^{b}	62.28 ± 0.26^{a}	41.14 ± 1.10^{ab}	6.36 ± 0.63^{ab}	9.93 ± 1.34^{b}	11.48 ± 0.19^{a}	7.35 ± 0.13^{ab}	ND	ND
72	105.76 ± 0.80^{a}	44.30 ± 1.42^{a}	39.15 ± 3.00^{ab}	6.91 ± 0.51^{a}	5.84 ± 2.36^{bc}	10.24 ± 1.16^{a}	8.24 ± 0.12^{a}	ND	ND

Table 4.3 Concentration of short-chain fatty acids in batch culture fermentation of HKOG, PGM and control

Time (h)	Concentration of SCFAs (mM)								
	Acetic acid			Propionic acid			Butyric acid		
	RKOG	PGM	Control	RKOG	PGM	Control	RKOG	PGM	Control
0	43.74 ± 0.05^{c}	12.28 ± 1.47^{b}	3.03 ± 0.03^{d}	3.70 ± 0.21^{bc}	9.29 ± 0.12^{a}	3.15 ± 0.02^{a}	ND	3.65 ± 0.20^{a}	ND
6	46.70 ± 0.83^{c}	11.17 ± 0.00^{b}	6.83 ± 0.28^{c}	2.87 ± 0.01^{c}	11.92 ± 0.50^{a}	2.29 ± 0.12^{c}	5.44 ± 0.37^{b}	3.30 ± 0.06^{b}	ND
12	50.76 ± 0.78^{b}	28.37 ± 1.04^{ab}	9.05 ± 0.11^{b}	4.81 ± 0.06^{ab}	2.99 ± 0.01^{c}	2.52 ± 0.11^{bc}	7.53 ± 0.20^{a}	ND	ND
24	56.99 ± 1.61^{a}	38.03 ± 1.13^{a}	10.00 ± 0.58^{b}	5.59 ± 0.14^{a}	4.95 ± 0.09^{c}	2.76 ± 0.01^{ab}	2.89 ± 0.04^{c}	ND	ND
48	53.69 ± 1.28^{ab}	40.56 ± 0.42^{a}	11.65 ± 0.48^{a}	6.17 ± 0.71^{a}	5.00 ± 0.18^{c}	3.10 ± 0.23^{a}	ND	ND	ND
72	55.98 ± 1.32^{a}	51.75 ± 0.24^{a}	12.72 ± 0.05^{a}	6.29 ± 0.65^{a}	6.37 ± 2.52^{bc}	2.94 ± 0.00^{a}	ND	ND	ND

ND, not detected

Different letters of superscript in the same column mean value was significant difference (P<0.05).

Figure 4.6 showed the ratio of acetic, propionic and butyric acid production in each time for every substrate. In this study the ratio of SCFA production was 88.78:5.30:5.92, acetic:propionic:butyric (mM). Previous study by Macfarlane and Macfarlane (2003) found that human colonic microbiota produced acetic acid as their major SCFA production and propionic and butyric acid are produced in the equal concentrations. Our result of SCFA is similar to their study with propionic and butyric acid are produced almost in the same amount.

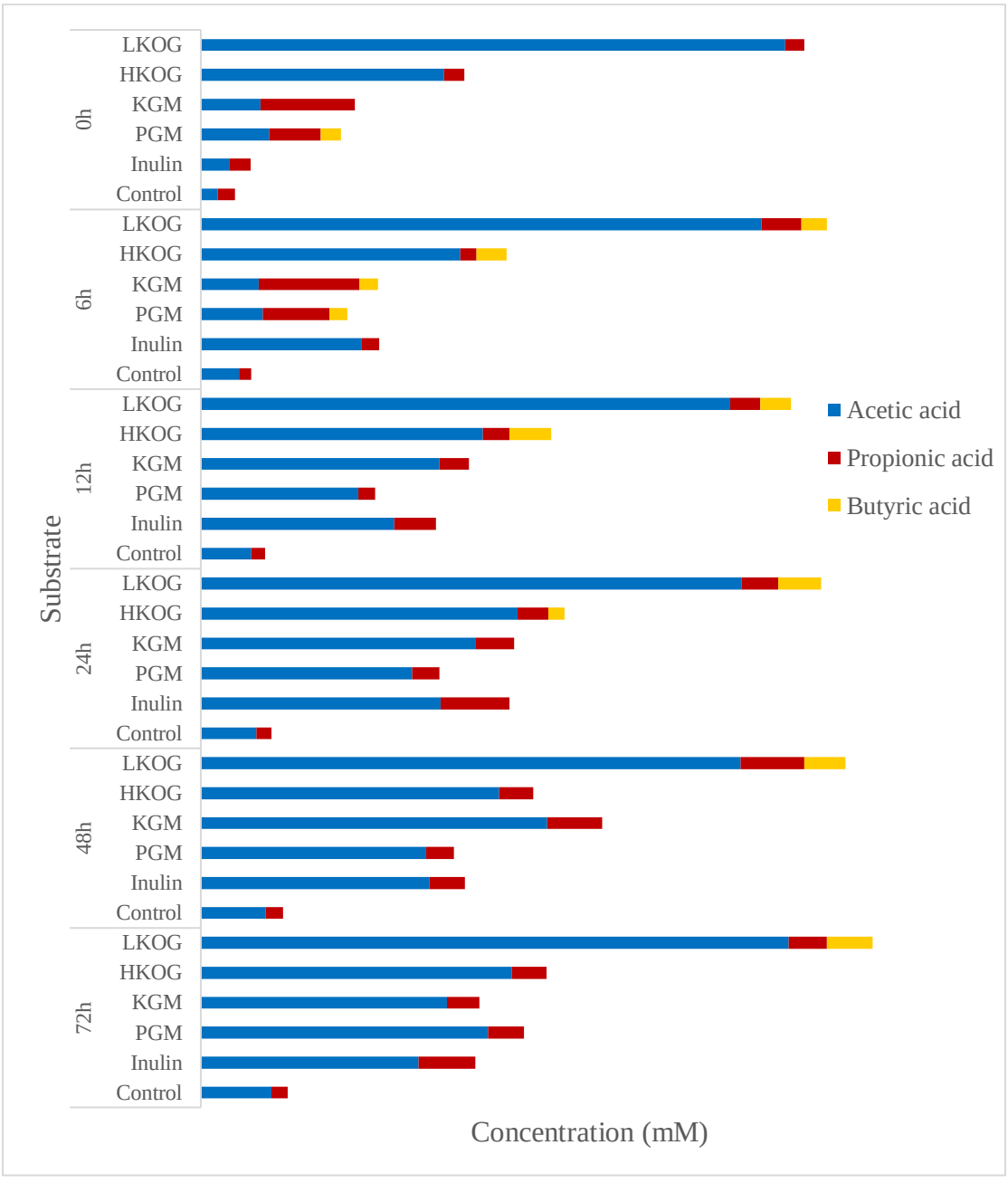

Figure 4.6 Total SCFA production during batch culture fermentation

Butyric acid is the main source of colonocytes energy and mainly produced from resistant starch fermentation. Increasing production of butyric acid in the gut has been linked for the colon cancer and colonic inflammation reduction (Al-Sheraji *et al*., 2013). For this reason, many works has been done to investigate its role (Sivaprakasam *et al*., 2016). Concentration of butyric acid from LKOG was higher than propionic acid production in this study. SCFAs production in this study was in order of acetic, butyric and propionic acid. It is suggested that LKOG has different benefits with KGM and may have specific health function related with butyric acid production.

4.5 Conclusion

LKOG that produced from this study can enhanced the production of butyric acid in the fecal fermentation compared to its native KGM, PGM and inulin. LKOG had positive prebiotic index value of 0.76 and its fermentation was increasing the populations of beneficial bacteria (bifidobacteria and lactobacilli). Further study of LKOG might be needed to investigate its beneficial effect in human.

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CHAPTER 5

CONCLUSION AND SUGGESTION

5.1 Conclusion

KOG was successfully produced by hydrolysis of KGM using β-mannanase with oligosaccharides concentration was 9.21 mg/mL. KOG purity can be improved by 3,000 NMWC UF membrane purification and the result showed its concentration increased up to 9.54 mg/mL. LKOG that produced from this study can enhanced the production of butyric acid in the fecal fermentation compared to its native KGM and inulin with the highest concentration (8.24 mM) was found at 72h fermentation. LKOG had positive prebiotic index value of 0.76. LKOG can be concluded as candidate prebiotics.

5.2 Suggestion

The yield of KOG needs to be improved along with its purity to get higher concentration of oligosaccharides in the end product in order to improve the functionality of KOG. Enzymatic membrane reactor (EMR) might be used as alternative to increase the concentration of oligosaccharides in further study. Animal study about KOG in constipated mice has been investigated in separate study. The results showed that prebiotic and laxative properties of KOG can prevent and relief constipation effectively. The study concluded that KOG could be used as an alternative management of constipation in mice. Based on the previous study, KOG was more acceptable in the food industry regarding to its lower viscosity compared to KGM and its activity as prebiotic. This may indicate that KOG with low viscosity can be apply as food ingredients in the products that does not need KOG as thickener but still need its prebiotic activity such as yoghurt and dairy products. Low molecular weight of KOG produced butyric acid during the fermentation compared to inulin. This result indicated that LKOG has potency to compete with inulin as prebiotic. Further study of LKOG might be needed to investigate its beneficial effect in human.

Appendix

Standard curve of molecular weight distribution (Mn) was established by konjac glucomannan (P-GLML) to determine the Mn of konjac oligo-glucomannan after analyzed by high performance size exclusion chromatography (HPSEC).

GPC Addon Calibration Report

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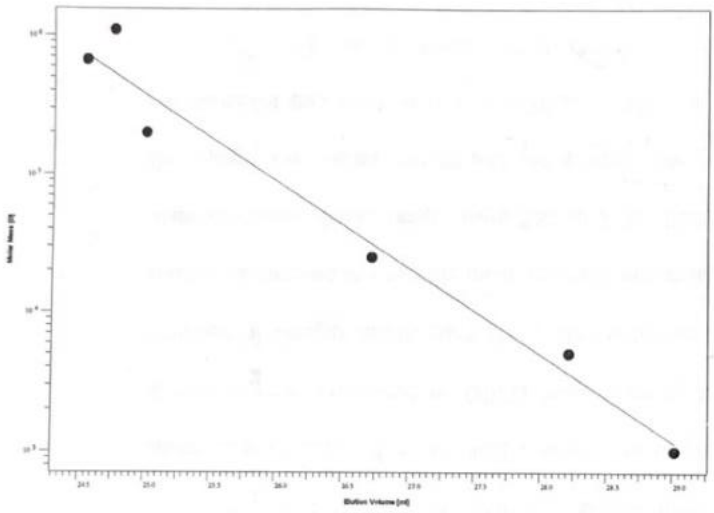
Comment:



Calibration Parameters:
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Regression coeff. R: 0.986046
Chi squared : 0.034230
Calibration by:
on column:

Mark-Houwink Coefficients:
a = 0.000 K = 1.0000

Internal standard Information:
Name: at: 50.00 ml

Regression Parameters:
Coeff. 1: 21.20840
Coeff. 2: -0.62550
Coeff. 3: 0.00000
Coeff. 4: 0.00000
Coeff. 5: 0.00000
Coeff. 6: 0.00000
Coeff. 7: 0.00000
Coeff. 8: 0.00000

Calibration Table:

Elution Volume [ml]	Molar Mass [D]	Sample Name	Slope	Deviation [%]
24.75	1100000.00	predict glucomannan 1100000	-0.63	-51.48
24.54	670000.00	predict glucomannan 670000	-0.63	7.80
25.00	200000.00	predict glucomannan 200000	-0.63	86.18
26.72	25000.00	predict glucomannan 25000	-0.63	25.07
28.22	5000.00	predict glucomannan 5000	-0.63	-27.91
29.02	1000.00	predict glucomannan 1000	-0.63	13.88

Table 1. Growth of eubacteria during batch culture fermentation in various carbon source

Incubation time (h)	Bacterial population (log cell/mL)					
	LKOG	HKOG	KGM	PGM	Inulin	Control
0	9.78 ± 0.14^{a}	9.36 ± 0.12^{c}	9.54 ± 0.11^{b}	9.60 ± 0.13^{c}	9.75 ± 0.04^{c}	9.58 ± 0.10^{c}
6	9.78 ± 0.18^{a}	9.56 ± 0.08^{b}	9.64 ± 0.08^{a}	9.56 ± 0.13^{a}	9.64 ± 0.09^{ab}	9.56 ± 0.11^{c}
12	10.10 ± 0.06^{ab}	9.58 ± 0.12^{b}	9.79 ± 0.06^{ab}	9.53 ± 0.10^{b}	9.68 ± 0.12^{a}	9.59 ± 0.10^{a}
24	9.80 ± 0.12^{b}	9.51 ± 0.14^{a}	9.74 ± 0.10^{a}	9.59 ± 0.08^{b}	9.63 ± 0.10^{b}	9.65 ± 0.14^{b}
48	9.72 ± 0.12^{b}	9.64 ± 0.11^{a}	9.64 ± 0.20^{b}	9.53 ± 0.10^{b}	9.49 ± 0.08^{b}	9.26 ± 0.12^{c}
72	9.80 ± 0.11^{b}	9.59 ± 0.10^{a}	9.34 ± 0.16^{ab}	9.52 ± 0.06^{c}	9.54 ± 0.06^{d}	9.21 ± 0.16^{c}

Table 2. Growth of bifidobacteria during batch culture fermentation in various carbon source

Incubation time (h)	Bacterial population (log cell/mL)					
	LKOG	HKOG	KGM	PGM	Inulin	Control
0	9.25 ± 0.05^{c}	9.04 ± 0.07^{b}	8.95 ± 0.09^{e}	9.15 ± 0.22^{e}	8.87 ± 0.07^{e}	8.92 ± 0.12^{ab}
6	9.26 ± 0.06^{c}	9.26 ± 0.07^{a}	9.55 ± 0.08^{b}	9.31 ± 0.11^{d}	9.53 ± 0.10^{c}	9.02 ± 0.18^{a}
12	9.25 ± 0.07^{c}	9.24 ± 0.09^{a}	9.82 ± 0.12^{a}	9.49 ± 0.12^{ab}	9.69 ± 0.04^{a}	8.98 ± 0.12^{a}
24	9.46 ± 0.06^{a}	9.11 ± 0.14^{b}	9.75 ± 0.06^{a}	9.57 ± 0.07^{a}	9.59 ± 0.08^{b}	8.83 ± 0.11^{b}
48	9.32 ± 0.05^{b}	8.83 ± 0.11^{c}	9.45 ± 0.11^{c}	9.44 ± 0.05^{bc}	9.51 ± 0.04^{c}	8.56 ± 0.18^{c}
72	9.28 ± 0.03^{c}	8.75 ± 0.10^{d}	9.33 ± 0.09^{d}	9.40 ± 0.06^{c}	9.20 ± 0.05^{d}	8.63 ± 0.18^{c}

Different letters of superscript in the same column mean value was significant difference ($P<0.05$).

Table 3. Growth of lactobacilli during batch culture fermentation in various carbon source

Incubation time (h)	Bacterial population (log cell/mL)					
	LKOG	HKOG	KGM	PGM	Inulin	Control
0	8.84 ± 0.07^{b}	8.68 ± 0.10^{c}	8.72 ± 0.06^{c}	8.69 ± 0.11^{a}	8.76 ± 0.13^{c}	8.65 ± 0.13^{d}
6	8.89 ± 0.10^{ab}	8.83 ± 0.07^{b}	8.81 ± 0.12^{ab}	8.70 ± 0.11^{a}	8.87 ± 0.04^{b}	8.92 ± 0.11^{b}
12	8.93 ± 0.08^{ab}	8.91 ± 0.08^{a}	8.87 ± 0.16^{a}	8.66 ± 0.11^{a}	8.86 ± 0.14^{b}	9.03 ± 0.14^{a}
24	8.85 ± 0.11^{b}	8.86 ± 0.09^{ab}	8.83 ± 0.13^{ab}	8.71 ± 0.12^{a}	8.93 ± 0.10^{d}	8.98 ± 0.12^{ab}
48	8.97 ± 0.06^{a}	8.90 ± 0.08^{ab}	8.76 ± 0.08^{bc}	8.65 ± 0.13^{a}	9.11 ± 0.15^{a}	8.83 ± 0.07^{c}
72	8.69 ± 0.24^{c}	8.75 ± 0.15^{c}	8.60 ± 0.15^{d}	8.52 ± 0.09^{b}	8.84 ± 0.14^{bc}	8.60 ± 0.14^{d}

Table 4. Growth of bacteroides during batch culture fermentation in various carbon source

Incubation time (h)	Bacterial population (log cell/mL)					
	LKOG	HKOG	KGM	PGM	Inulin	Control
0	9.08 ± 0.14^{b}	8.99 ± 0.09^{a}	9.00 ± 0.16^{a}	8.82 ± 0.09^{b}	8.83 ± 0.07^{b}	9.00 ± 0.09^{b}
6	9.10 ± 0.14^{b}	9.03 ± 0.08^{a}	8.87 ± 0.08^{b}	8.92 ± 0.09^{a}	8.84 ± 0.08^{b}	8.94 ± 0.10^{b}
12	9.20 ± 0.09^{a}	8.99 ± 0.09^{a}	8.75 ± 0.09^{c}	8.97 ± 0.07^{a}	9.16 ± 0.09^{a}	9.19 ± 0.10^{a}
24	8.93 ± 0.09^{c}	8.89 ± 0.14^{b}	8.70 ± 0.12^{c}	8.85 ± 0.09^{b}	9.14 ± 0.10^{a}	9.15 ± 0.10^{a}
48	8.65 ± 0.10^{d}	8.58 ± 0.10^{c}	8.61 ± 0.11^{b}	8.81 ± 0.04^{b}	8.64 ± 0.11^{c}	8.60 ± 0.11^{c}
72	8.67 ± 0.09^{d}	8.49 ± 0.14^{d}	8.47 ± 0.09^{d}	8.75 ± 0.05^{c}	8.63 ± 0.08^{c}	8.61 ± 0.09^{c}

Table 5. Growth of clostridia during batch culture fermentation in various carbon source

Incubation time (h)	Bacterial population (log cell/mL)					
	LKOG	HKOG	KGM	PGM	Inulin	Control
0	8.86 ± 0.18^{a}	8.49 ± 0.11^{c}	8.55 ± 0.13^{b}	8.49 ± 0.09^{c}	8.62 ± 0.08^{c}	8.53 ± 0.08^{c}
6	8.86 ± 0.15^{a}	8.64 ± 0.06^{b}	8.59 ± 0.13^{ab}	8.75 ± 0.06^{a}	8.81 ± 0.10^{ab}	8.59 ± 0.08^{c}
12	8.76 ± 0.20^{ab}	8.66 ± 0.10^{b}	8.67 ± 0.07^{a}	8.62 ± 0.08^{b}	8.86 ± 0.08^{a}	8.80 ± 0.06^{a}
24	8.67 ± 0.19^{b}	8.79 ± 0.08^{a}	8.67 ± 0.10^{a}	8.60 ± 0.07^{b}	8.77 ± 0.10^{b}	8.70 ± 0.13^{b}
48	8.62 ± 0.16^{b}	8.77 ± 0.08^{a}	8.54 ± 0.10^{b}	8.57 ± 0.08^{b}	8.79 ± 0.08^{b}	8.56 ± 0.11^{c}
72	8.66 ± 0.17^{b}	8.73 ± 0.06^{a}	8.61 ± 0.08^{ab}	8.48 ± 0.12^{c}	8.47 ± 0.08^{d}	8.53 ± 0.08^{c}

Different letters of superscript in the same column mean value was significant difference (P<0.05).

VITAE

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1. Thailand's Education Hub for Southern Region of ASEAN Countries (TEH-AC) scholarship
2. Graduate School Prince of Songkla University research funding.

List of Publication and Proceedings

Ariestanti, C. A., Seechamnanturakit, V., Harmayani, E. and Wichienchot, S. Optimization on production of konjac oligo-glucomannan and their effect on the gut microbiota. Food Sci Nutr. (Under revision).

Ariestanti, C. A., Seechamnanturakit, V., Harmayani, E. and Wichienchot, S. Optimization on production of konjac oligo-glucomannan by enzymatic hydrolysis. The First International Conference on Innovation of Functional Foods in Asia, January 22-24, 2018.