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THESIS

ANTIOXIDANT AND PREBIOTIC PROPERTIES OF OLIGOSACCHARIDES EXTRACTS FROM COMMERCIALLY DEFATTED RICE BRAN

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A Thesis Submitted in Partial Fullfilment of
the Requirement for the Degree of
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Utilization of by-product from rice bran oil production was studied for its antioxidant and prebiotic properties. The extracts were prepared from commercially defatted rice bran with the assistance of two commercial enzymes, Viscozyme L (endo-β-glucanase) and Pentopan Mono BG (endo-β-xylanase). The enzymatically treated extracts were fractionated by gradual ethanol precipitation. Three fractions, the <60%, 60-90% and >90% fractions, were obtained from each enzymatic treatment. Each fraction was determined for its phenolic content and antioxidant capacity by reducing power and DPPH radical-scavenging methods. By both methods, the highest antioxidant activity was observed in the >90% fraction of either Viscozyme or Pentopan treatment, which was in line with its phenolic content. Fractions 60-90% were selected for *in vitro* determination of prebiotic properties due to the low amount of starch, protein and sugar. The results showed that P60-90 was not significantly different with inulin in term of digestibility. Interestingly, P60-90 also showed higher capability in supporting probiotic (*Bifidobacterium breve* TISTR 2130, *B. longum* TISTR 2194, *Lactobacillus acidophilus* ATCC 4356, *L. plantarum* BCC 39798) growth with inulin. Unfortunately, compared to inulin, the growth of *Escherichia coli* TISTR 887 in this fraction was higher. It was found that the ratio of short chain fatty acid (SCFA), such as acetate, propionate and butyrate, from each culture was varied. The highest ratio of acetate production was found in the media of *L. acidophilus* ATCC 4356 with V60-90 and P60-90 as the substrates. Feeding *L. plantarum* BCC 39798 with all subtrates and *B. breve* TISTR 2130 with inulin could produce the highest ratio of propionate and butyrate. V60-90 contains more unbranched arabinoxylooligosaccharides (AXOS) with lower DP, while P60-90 has more branched AXOS with DP>4 when identified by HPAEC-PAD. Viscozyme and Pentopan assistance are useful to release the phenolic compounds and oligosaccharides from commercially defatted rice bran, which have positive capability as the source of antioxidant and prebiotic.

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Dwita Ratih Fitriani

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LIST OF ABBREVIATIONS

α-L-Ara*f*	=	alpha-L-arabinofuranosyl
β-D-Xyl*p*	=	beta-D-xylopyranosyl
AX	=	arabinoxylan
ABTS	=	2,2’ – azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)
AUC	=	area under curve
AXOS	=	arabinoxylo-oligosaccharide
A^zX	=	A; α-L-Ara*f* + β-D-Xyl*p*, ^{z}; linkage position between α-L-Araf and β-D-Xylp, X; (β)-D-Xyl(p)
DGGE	=	denaturing gradient gel electrophoresis
DP	=	degree of polymerization
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
FOS	=	fructo-oligosaccharide
GAE	=	gallic acid equivalent
GC	=	gas chromatography
GH	=	glycosyl hydrolase
GI	=	gastro intestinal
GLP	=	glucagon like peptide
GOS	=	gluco-oligosaccharide
HPAEC	=	high performance anion exchange chromatography
IMOS	=	isomaltulo-oligosaccharide
ITF	=	inulin type fructose
ORAC	=	oxygen radical absorbing capacity
PAD	=	potential amperometric detector
PCR	=	polymer chain reaction
RT	=	retention time
SCFA	=	short chain fatty acid
SOS	=	Soybean-oligosaccharide
XOS	=	xylo-oligosaccharide

ANTIOXIDANT AND PREBIOTIC PROPERTIES OF OLIGOSACCHARIDES EXTRACTS FROM COMMERCIALLY DEFATTED RICE BRAN

INTRODUCTION

Rice (*Oryza sativa* L.) is a major source of carbohydrate for the world's population, especially in Asia. World production of rice is estimated at around 740 million tons (FAOSTAT, 2012). By-products of rice processing are rice bran and rice hulls, which protect rice seeds during growth that contribute 20% of the rice crop. Nowadays, rice bran becomes a source of widely studied rice bran oil and numerous individual bioactive compounds, which have beneficial effects in cells, animals, and humans (Friedman, 2013).

Unfortunately, the defatted rice bran is normally added to feed production despite the research showing its usefulness in nutrition and as a bioactive. As interest in value-added processing research grows, attempts are being made to increase the value of agricultural crop by-products, including rice bran, by increasing their pharmaceutical or nutraceutical potential. Rice bran oil is a good source of antioxidants such as tocopherol and γ-oryzanol, while its modified arabinoxylan has been investigated for cholesterol-lowering and anti-carcinogen (Janike *et al.,* 2005; Bang *et al.,* 2010; Cholujova *et al.,* 2013).

There were many studies about dietary fiber extraction from rice bran. Those extractions were generally using heat or chemical process which was preceded by lignin, protein and starch removal treatment. The pretreatment may cleave some important compounds and lowering the bioavailability (Rondini *et al.,* 2010). This study was motivated to utilized by-product from rice bran oil production to extract oligosaccharides with enzymatic assistant to minimized the lost of beneficial compounds. Two different enzymes were compared for their ability on extracting oligosaccharides with antioxidant and prebiotic properties.

OBJECTIVES

Overall objective

To add the value of commercially defatted rice bran, a by-product from rice bran oil production, for its potential to be the source of antioxidant and prebiotic.

Specific objectives

1. To study the capability of two commercial enzymes, Viscozyme L and Pentopan Mono BG, to extract functional compounds from commercially defatted rice bran.
2. To study the antioxidant capacity of enzymatically extract from defatted rice bran.
3. To study the prebiotic properties of extracts from enzymatic-treated rice bran using pure culture fermentation.
4. To identify the oligosaccharides derived from enzymatic-treated rice bran using HPAEC-PAD.

LITERATURE REVIEW

Functional foods are considered as food or their ingredients that can give health benefit beside their normal nutritional status. Functional food industries have rapidly grown especially in developed countries because the increasing of health awareness. Antioxidants and prebiotics are one of the promising functional food that have been studied recently due to their abilities to promote health and well-being (European Commission, 2010, Patel and Goyal, 2012). Fruits and vegetables have been largely used as the source of these compounds, but in this last 10 years the cereal brans are widely intensively investigated because these products are still underutilized for human consumption.

1. Rice Bran

Bran is manufactured from grains, using methods adequate for structurally different cereal species, resulting in bran containing diverse grain layers. The major components of cereal plant cell are about 70% polysaccharides, 15% lignin and 10% protein (Puls,1993). The main polymers in the cell wall are hemicellulose (40-50%), cellulose (25-35%) and lignin (7-10%) (Ishii, 1997).

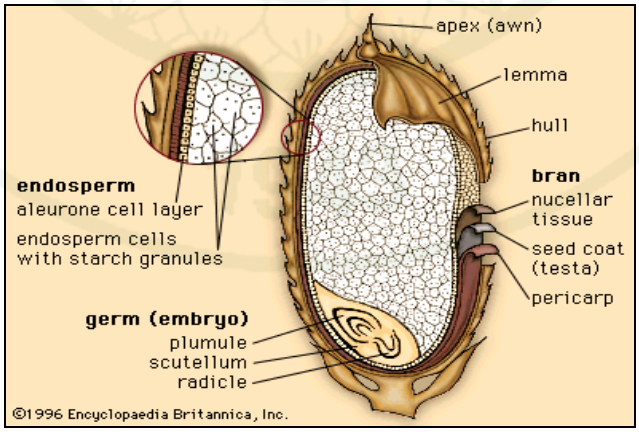


Figure 1 Rice layers

Source: Encyclopedia Britanica (1996).

Rice bran, layers between endosperm and rice hull (Figure 1), is by-product of rice milling. By milling one ton of grain, 60-80 kg rice bran, are produced depending on rice varieties and degrees of milling. Defatted rice bran contains about 17-20% lipid, 16-20% protein 28-34% carbohydrate with 20-25% total dietary fiber (20-24% insoluble fiber and 0.1-1.5% soluble fiber) (Faria *et al.*, 2012).

Cereal whole grains contain AX from 1.2 wt% (in rice (*Oryza sativa* L.)) to 8.5 wt% (in rye), whereas xylans in more lignified tissues of cereals (leaves, straws, husks) represent 25-35% of dry biomass (Aspinall, 1970; Henry, 1985; Härkönen *et al.*,1997; Moure *et al.*, 2006). Quantitatively, most of the AX is situated in the bran layers, which contribute about 25% of the dry weight of the grain (Fulcher and Duke, 2002).

The xylan group includes both alkali and water-extractable polysaccharides (Aspinall, 1970) but regardless of the extraction method, AX has different solubility in water, since water-solubility is dependent on the structure of the polymer. Without substitution, xylans are nearly water-insoluble, but arabinose side groups make the polymers more water-soluble (Sternemalm *et al.*, 2008; Pitkänen *et al.*, 2009). The amount and structural arrangement of the side chains of AX vary between cereal species and even among different parts of the same plant (Pastell *et al.*, 2010).

The most approach to isolating AX from various plant materials involves aqueous and alkali extraction of these polymers either from whole grains or from specific plant tissues. Once isolate from the cell wall matrix, AX is water soluble. However, in the intact wall, these polymers are cross-linking with other wall constituents to from a fabric structure that is not soluble in an aqueous environment. Some of the cross-linking involved are non-covalent and, while individually weak, they may confer strength and insolubility if present in large numbers (e.g. hydrogen bond). AX chains can also be covalently cross-linking to each other or to other cell wall constituents. As a consequence of these cross-linking, a certain portion of AX cannot be easily extracted from the plant materials with water and requires harsher treatments with alkali solutions to liberate them from network of covalent and non-

covalent bonds, as well as physical entanglements. Various methods and procedures for obtaining highly purified water-soluble AX from common cereals for analytical purposes have been published. Extractions are usually conducted in water or in buffer. After extraction, purification procedures usually involve inactivation of endogenous enzymes in aqueous extracts and the use of hydrolytic enzyme to eliminate contaminating proteins and starch from the preparations.

To wash the bran and to precipitate the AX, the extraction needs large amount of ethanol, which can offers cost saving by integrating the extraction process. However, AX production now still very limited especially rice-derived AX, as the result not many functions that have been studied are implemented (Mustafa *et al.*, 2007).

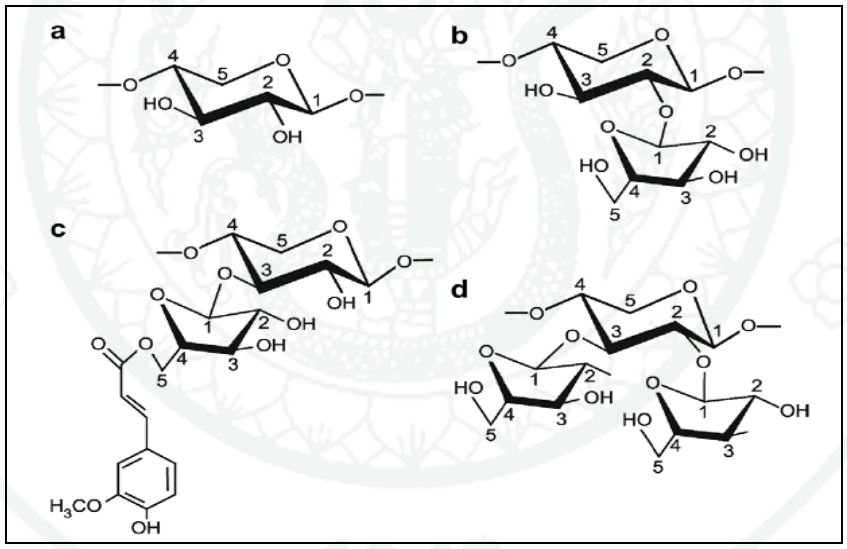


Figure 2 Structural elements present in AX: (a) unsubstituted Xylp; (b) monosubstituted Xylp at O-2; (c) monosubstituted Xylp at O-3 with ferulic acid residue esterified to Araf and (d) disubstituted Xylp at O-2,3.

Source: Izydorczyk *et al.* (2007)

Figure 2 shows that the linear main chain of arabinoxylans is composed of (1→4)-linked β-D-xylopyranosyl (β-D-Xyl*p*) residues. Cereal xylans are mainly

substituted with (1→2)- and/or (1→3)-linked α-L-arabinofuranosyl (α-L-Ara*f*) residues, thus resulting in mono- and di-substituted β-D-Xyl*p* residues. The α-L-Ara*f* side groups are usually (1→3)-linked with β-D-Xyl*p* residues in all cereals (Ebringerová *et al.*, 1990; Izydorczyk *et al.*, 2007). Mono-substituted β-D-Xyl*p* units with (1→2)-linked α-L-Ara*f* groups are usually found in alkali-extractable barley flour AX (22%) but they have been found in rice husk AX with small amounts. According to the same study, rice husk also carry β-D-glucopyranosyluronic acid (β-D-GlcA) or its 4-*O*-methyl ether (4-*O*-Me-α-DGlcA) (Pastell *et al.*, 2009). Ferulic and *p*-coumaric acids may be ester-linked to the AX structure at the O-5 position of some of the α-L-Ara*f* units (Saulnier *et al.*, 1995; Ishii, 1997). Acetyl groups, ferulic and *p*-coumaric acids are easily released in alkaline-extraction (Puls, 1993). The main substiuent of arabinoxylan in cereal are described in Figure 3. According to Ebringerová and Heinze (2000), main substituents of rice bran AX are α-L-Ara*f* (1→3) mono and (4-*O*-Me)-α-DGlcA (1→2).

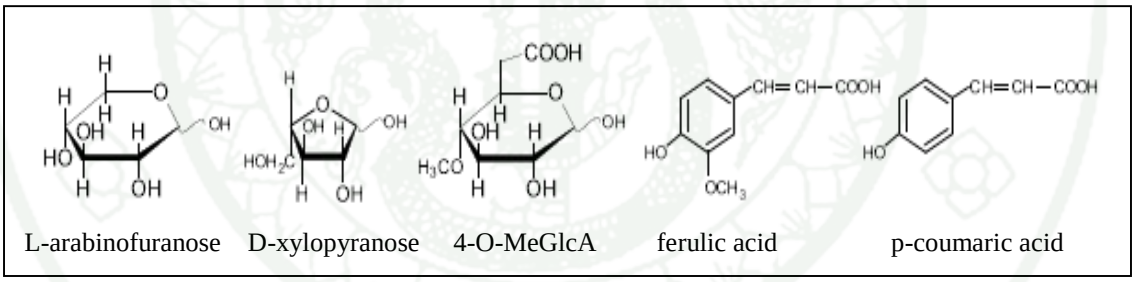


Figure 3 Structure of main substiuent of arabinoxylan in cereals.

Source: Pastell *et al.* (2009)

The arabinoxylan backbone is degraded by glycoside hydrolysis (GH) that are able to hydrolyze the glycosidic bond between ((1→4)-linked β-D-Xyl*p* units. Such enzymes are produced e.g. by various bacteria and fungi. The plant AX backbone is randomly hydrolyzed, using endo-1,4-β-D-xylanases (EC 3.2.1.8), producing a mixture of various oligosaccharides. Xylanases are currently classified based on the structural and sequence similarities in GH families 5, 7, 8, 10, 11 and 43, but

researchers mainly focused on the family GH10 and GH11 enzymes (CAZy - Carbohydrate Active enzymes) (Collins *et al.*, 2005).

Arabinoxylo-oligosaccharides (AXOS) can be produced from polymeric arabinoxylans, using restricted acid hydrolysis, hydrothermal processing (auto-hydrolysis), extensive dry ball milling or enzymatic hydrolysis (Rose and Inglet, 2009; Tenkanen, 2004). Even enzymatic hydrolysis allows more specific regulation of the end- products and it is possible to obtain AXOS having the desired degree of polymerization (DP) and substitution patterns (Pastell, *et al.*, 2009), auto-hydrolysis does not require any hard chemical and enzyme that will be costly for large scale production (Kabel *et al.*, 2002).

2. Cereal brans and health benefits

Cereal brans are rich sources of fibers, vitamins, minerals, and phytochemicals including phenolics, carotenoids, vitamin E, lignans, b-glucan, resistant starch, sterols, and phytates that may provide health benefits beyond their basic nutrition (Liu, 2007). According to Friedman (2013), rice bran contains a lot of bioactive compounds such as phenolics, flavonoids, steroidal, hemicellulose, and various proteins.

2.1. Phytochemicals

The most phytochemicals has been studied from cereal brans are phenolics, which have at least one one hydroxyl group on aromatic ring (Okarter and Liu, 2010). Included in this group of compounds are phenolic acids, alkylresorcinols, and flavonoids. Laokuldilok *et al.* (2011) reported that rice bran contained 2101 µg/g phenolic acids, 71 µg/g α-tocopherol, and 3681 µg/g γ-oryzanol. These compounds had been studied for its strong antioxidant capacity to reduce risk of cancer proliferation (Tabaraki and Natghi, 2011 and Chiou *et al.*, 2013).

An antioxidant is a substrate that prevents oxidation of other compound by neutralizing or stabilizing the damaging effects of free radicals, which are natural

by-products of cell metabolism. Free radicals could be formed during oxidation or any metabolism performed in the body or chemical species that posses an unpaired electron in the surface of the molecule (Badarinath *et al.,* 2010).

The correlation coefficient between total phenolic compounds and antioxidant activity is quiet high from 0.80 for the pericarp and testa layers, 0.96 for the whole grain, up to 0.99 for the external layers and the endosperm with the embryo (Zielinski and Kozlowska, 2000). Methanolic extracts of grains in Korea (red sorghum and black rice) were also shown to have higher antioxidant activities than white rice, brown rice, foxtail millet, proso millet and barley. The polyphenolic content of the extracts positively correlated by the ABTS radical cation scavenging activity method (Choi *et al.,* 2007). Another study reported *in vitro* antioxidant potential of whole grain from maize (181 mmol vit. C eq/g grain), followed by wheat (77 mmol vit. C eq/g grain), oats (75 mmol vit. C eq/g grain), and rice (56 mmol vit. C eq/g grain) (Adom and Liu, 2002).

Rice contains potentially antioxidant compounds, notably in the outer layers of the grain (bran). Well known antioxidants like vitamin E and γ-oryzanol can be extracted from rice bran. In rice bran, γ-oryzanol may contribute more to the reduction of cholesterol oxidation than vitamin E, which is usually considered to be the major antioxidant (γ-oryzanol content of rice bran is 10-times that of vitamin E) (Xu *et al.,* 2001). Anthocyanins, such as oligosacc-3-glucoside and peonidin 3-glucoside from colored rice varieties are also a great antioxidant (Hu *et al.,* 2003).

Although they contain many antioxidants, cereal grains have been thought to be less important sources of antioxidants compared to fruit and vegetables (Liu, 2007), despite being the major dietary components worldwide. The common methods used to measure the *in vitro* antioxidant potential of cereals and their fractions are the Oxygen Radical Absorbance Capacity (ORAC), the 2,2 0 –azinobis 3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), the 2,2-diphenyl-1- picrylhydrazyl (DPPH) and the reducing power assays. These methods are related to different mechanism of antioxidants such as donating hydrogen to radicals, reducing power, free radical

scavenging activity, metal chelating ability, inhibition of β-carotene bleaching and quenching singlet oxygen (Fardet *et al.*, 2008).

2.2. Dietary fiber

Another functional bioactive compound of rice bran is dietary fiber. The dietary fiber found in bran is mostly hemicellulose, which in rice bran consisted mainly highly branched arabinoxylan (AX) and xyloglucan (Shibuya and Iwasaki, 1985; Mod *et al.*, 1978). Rice whole grains contain AX 1.2%, where xylan is in more lignified tissues of cereals (leaves, straws, husks) represent 25-35% of dry biomass (Aspinall, 1970; Henry, 1985; Härkönen *et al.*,1997; Moure *et al.*, 2006). Quantitatively, most of the AX is situated in the bran layers, which contribute about 25% of the dry weight of the bran (Fulcher and Duke, 2002).

Ghoneum *et al.* (2011) found that MGN-3-BioBran, which composed of denatured AX that obtained by reacting rice bran AX with multiple carbohydrate hydrolyzing enzymes from *Hypomycetes mycelia*, could enhance the phagocytosis by neutrophils of *E. coli* pathogenic bacteria *in vitro* but did not affect the growth of 31 strains of bacteria so that it might be effective in the treatment of infections in elderly or immune-compromised patients. In 2013 Ghoneum *et al.* also reported that MGN-3-BioBran was a potent biological response modifier (BRM) and suggesting its possibility for infections and cancer therapy.

Hemicellulose and tocotrienol in rice bran also performed effect on lowering blood cholesterol level and improved the LDL/HDL rasio *in vivo* (Sanders and Reddy, 1992; Gerhardt and Gallo, 1998; Qureshi *et al.*, 2002). Qureshi *et al.*, 2002 study found that tocotrienols are effective in lowering serum total and LDL-cholesterol levels by inhibiting the hepatic enzyme activity of β-hydroxy-β-methylglutaryl coenzymeA (HMG-CoA) reductase through the post-transcriptional mechanism in hypercholesterolemic human subjects. Hu and Yu (2013) investigated the mechanism and found that hemicelluloses from rice bran bound and bile acid which resulted low cholesterol intake from food.

In term of fiber, hemicelluloses from cereal brans also contributed to the prebiotic properties. Pastell *et al.* (2009) reported that AX and AXOS from rice husk had ability to support the growth of Bifidobacteria. Study on mice showed that there were significant improvement of lactic acid bacteria and bifidobacteria after 1 week consumption of rice bran, and also increased resistance against Salmonella infection (Kumar *et al.*, 2012).

3. Preparation of oligosaccharides from cereal bran

There were some studies that extracted oligosaccharides from hemicelluloses of cereal brans. Many researchers focus on xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS) due to their functionality in food processing and health benefits. AXOS can be produced from polymeric arabinoxylans, using restricted acid hydrolysis, hydrothermal processing (auto-hydrolysis), extensive dry ball milling or enzymatic hydrolysis (Rose and Inglet, 2009; Tenkanen, 2004; Van Craeyveld *et al.*, 2009). Even enzymatic hydrolysis allows more specific regulation of the end- products and it is possible to obtain AXOS with the desired degree of polymerization (DP) and substitution patterns (Pastell, *et al.*, 2009).

AXOS can also be produced enzymatically from lignocellulosic material without AX extraction. In the study of Swennen *et al.* (2006), AXOS were produced on a large scale from wheat bran (500 kg). Wheat bran was first enzymatically destarched and deproteinized and later treated with *Bacillus subtilis* GH11 endo-1,4-α-D-xylanase. The obtained AXOS were fractionated with a graded ethanol precipitation, in which the ethanol concentration was increased stepwise until a final concentration of 90% (v/v) was reached. The AXOS produced had an average DP of 15 and Ara/Xyl ratio of 0.27. The yield of AXOS was 6% w/w with a purity of 72%. The α-D-Xylp units were generally monosubstituted with β-L-Araf O-3 residues, but the AXOS obtained with 40-70% ethanol precipitation contained nearly as much β-L-Araf doubly substituted α-D-Xylp units.

In the study of Maes *et al.* (2004), destarched and deproteinized wheat bran (1000 g) was incubated with endo-1,4-α-D-xylanase from *B. subtilis* (GH11; Grindamyl H640, Danisco A/S, Copenhagen, Denmark) and *Aspergillus aculeatus* (GH10; Shearzyme 500L, Novozymes A/S, Bagsvaerd, Denmark). Endo-1,4-α-D-xylanase from *B. subtilis* was able to hydrolyze 41 wt% of water-unextractable AX into AXOS with average DP of 15, while *A. aculeatus* endo-1,4-α-D-xylanase hydrolyzed only 18 wt% (average DP of 8). The same study showed that, *A. aculeatus* endo-1,4-α-D-xylanase preferred to degrade water-extractable AX, so the extracted AX and AXOS were diluted in aqueous phase.

Physical, chemical and enzyme assistance were used in order to release those compound from cell wall and hydrolyzed the long chain of hemicellulose into oligosaccharides. Kabel *et al.* (2002), Carvalheiro *et al.* (2003) Gullón *et al.* (2008), used high temperature and high pressure to extract AXOS from cereal by products. Alkaline assistance is the commonly used treatment to release hemicellulose from cereal cell wall, but this treatment usually followed by acid or enzymatic treatment to prepare oligosaccharides (Swennen *et al.*, 2006; Pastell *et al.*, 2009).

4. Role of prebiotics

Prebiotic is non digestible food or feed ingredients that selectively stimulate probiotic growth. Probiotic is microorganisms that grow in almost all part of the body, such as mouth, colon, skin, nose, eyes, lungs, vagina, etc, and give beneficial contribution to the host health. But prebiotic is only referred to those which can promote probiotic in gastro intestinal track (gut) (Patel and Goyal, 2012).

In fact, most of prebiotic health benefits come up with their cooperation with probiotics, but since probiotics are more difficult to handle during the food manufacture, storage, distribution and preparation, prebiotics application in food product grows rapidly. Some prebiotics (e.g. pectin, glucomannan, inulin) were previously used for food additive that helps emulsification or solubilization (Musatto

and Mancilha, 2007). The mechanism of health benefits of prebiotic is described below (Figure 4).

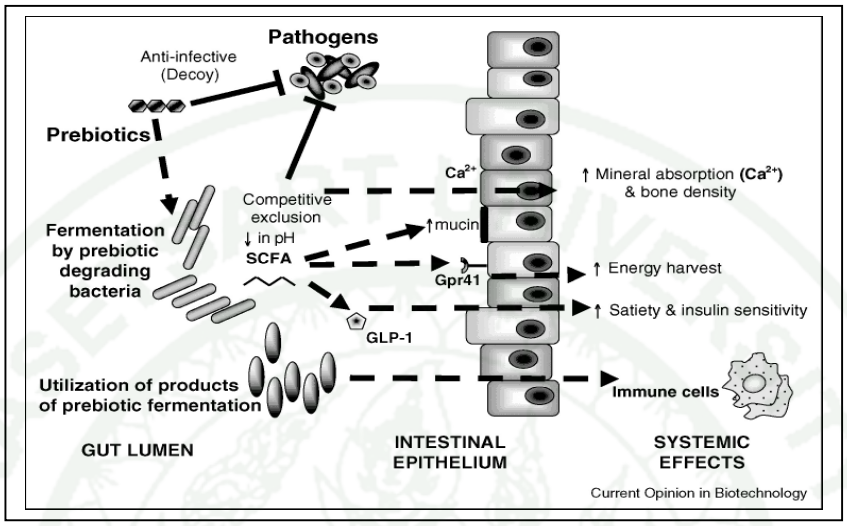


Figure 4 Beneficial roles description of prebiotics in mammalian gut.

Source: Saulnier *et al.* (2009)

4.1. Maintain gut health

One criterion that has to be fulfilled by prebiotics is the ability to improve selective beneficial bacteria in gut such as bifidobacteria and lactobacilli. Some studies related to this assumed that lactobacilli and bifidobacteria can produce specific enzymes and oligosaccharide transporters to digest the prebiotics, which not produced by pathogenic bacteria. Therefore, prebiotics can stimulate only the gut commensal microorganisms and simultaneously cause inhibition to undesirable microorganisms (Saulnier *et al.,* 2009; Barrangou *et al.,* 2006).

Study on glucooligosaccharides (GOS) showed that prebiotic can interact with pathogen-binding cellular receptors in the gut and reduce the adherence enteropathogenic E.coli. Therefore, prebiotic may be able to inhibit pathogen to colonilized in host gut (Shoaf-Sweenay and Hutkins, 2009; Shoaf *et al.,* 2006).

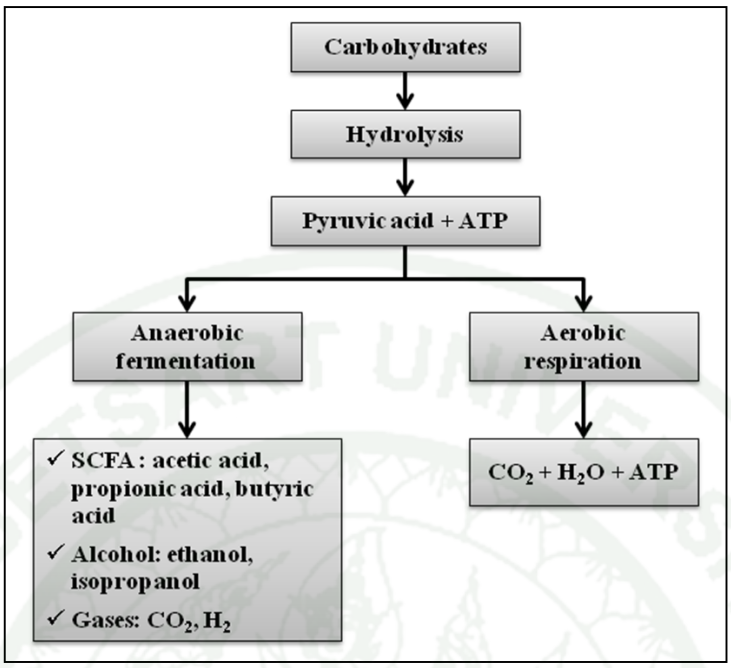


Figure 5 Carbohydrate fermentation by lactobacilli and bifidobacteria.

Source: Rautiola (2013)

Anaerobic fermentation of carbohydrates (Figure 5), including prebiotics, by lactobacilli and bifidobacteria produce short chain fatty acids (SCFA) such as acetic acid, butyric acid and propionic acid, which also play an important role in maintaining and improving gut health. SCFA production decreased the pH of colon which suppress the pathogen growth and influence the intestinal motility (Blaut, 2002 and Dass *et al.*, 2007).

Most of SCFA are rapidly absorbed by colonic mucosa and contribute to the energy required of the host. They are further metabolized by host in different organs. Acetate is metabolized in muscle, kidney, heart, and brain, which can rapidly provide energy. Propionate goes to liver, which a possible gluceogenic substrate. Most butyrate is metabolized by colonic epithelium and becomes the major energy substrate and regulates the cell growth and differentiation. Unlike acetate and lactate, butyrate and propionate are un-degradable by other bacteria allowing contribution to another indirect health benefit (Roberfroid *et al.*, 2010).

4.2. Reduce gut inflammation and colonic cancer risk

Short chain fatty acids that produced from fermentation of prebiotics, can also stimulate mucin production of gut mucosa. Study in carcinogen treated rats, AXOS supplemental on feed was able to reduce the preneoplastic lesions occurrence in colon (Femia *et al.,* 2010). Nurmi *et al.* (2005) reported that propionate showed influence on anti-inflammatory effect with respect to colon cancer. Whereas butyrate is believed to have protective effect against colon cancer by apoptosis stimulation and suppress expression of transcription factor NF-B in cancer cell (Macfarlane *et al.*, 2008; Vos *et al.,* 2010).

4.3. Increase in mineral absorption in colon

Prebiotic supplementation on food may improve host's mineral (such as calcium and magnesium) absorption. Inulin type fructans and crude fraction of chicory showed the effect of enhanced calcium absorption primarily through colonic human mucosa and have shown improvement on bone parameters (Mitamura and Hara, 2005; Raschka and Daniel, 2005).

The presence of SCFA stimulates the trans-epithelial calcium transport in caecum due to the reduction pH that raising the availability of calcium ions. Furthermore, the presence of intact inulin and trans-epithelial even in a moderately low concentration at the mucosal side was able to stimulate the trans-epithelial net calcium transport in caecum and distal colon which likely to the increasing of the paracellular permeability. The increased absorptive surface area in caecum and intracaecal calcium availability may explain the net effect of higher calcium absorption caused by dietary inulin and oligofructose *in vivo* (Scholz-Ahrens and Schrezenmeir, 2002).

4.4. Risk reduction of type II diabetes and lipid homeostasis

Study in diabetic rats found that inulin type fructose (ITF) increased glucagon like peptide-1 (GLP-1) production. An increase on GLP-1 has been shown to stimulate pancreatic β-cell differentiation, which helps to regulate glucose level in blood stream. The recent human study conducted by Cani and coworker (2009) confirmed that ITF supplementation increased GLP-1 production and mitigated postprandial glucose response after breakfast.

The glucose regulation could also influence the lipid metabolism because glucose and insulin are one of lipogenesis activator in liver. Furthermore, the production of SCFA that reach the liver is able to lower the hepatic triacylglycerides. Since propionate is cleared by the liver, it is a possible that prebiotic becomes gluceogenic precursor which suppresses cholesterol synthesis (Salazar *et al.*, 2008).

5. Sources of prebiotic

Prebiotic should be able to survive from gastro intestinal digestion, so usually they are also part of dietary fiber. Prebiotic must resist gastric acid acidity, gastrointestinal enzymatic hydrolysis, and gastrointestinal absorption. Oligosaccharides are the most common prebiotics, even there are other compound such as monosaccharide (xylitol), disaccharides (lactose) or polysaccharides (polydextrose and resistant starch) that also showed prebiotic potential.

Namely inulin, fructooligosaccharide (FOS), galactooligosaccharide (GOS), and lactulose are most recognized and established perbiotics that widely used in food products (Gibson *et al.*, 2004). The other candidates for prebiotics are lactitol, raffinose, sorbitol, soybeanoligosaccharides (SOS), isomaltulooligosaccharides (IMO), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), and some resistant starch (Patel and Goyal, 2012; Gray, 2006). IMO, XOS and AXOS were mostly found in cereal grains and concentrated in the brans.

6. Antioxidant testing method

The latest studies suggesting the involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention of scientists on the role of antioxidants in prevention and treatment of diseases. The capacity of antioxidants from various sources of foods had been assessed by different methods under different conditions. Even there are many available methods; none of them can determine antioxidant capacity accurately and quantitatively. The mechanisms and dynamics of antioxidant action in human might be different with the methods are proposed to assess the capacity of radical scavenging and inhibition of lipid peroxidation both *in vitro* and animal. (Niki, 2010)

6.1. Determination of antioxidant *in vitro*

The increasing interest in antioxidant compounds for consumers, food scientists lead to the need of a quick and easy method for determining antioxidant capacity. Antioxidant capacity is a measure of the amount (in moles) of a given free radical scavenged by a sample. Measurements of antioxidant capacity yield the amount of a heterogeneous mixture of antioxidants that react together to produce the total scavenging ability of the sample (Fardet *at al*., 2008).

An *in vitro* method should be able to measures actual compound(s) occurring in potential application(s), use biologically relevant radical source, simple, good reproducibility, adaptable to hydrophilic and lipophilic antioxidants. There are two mechanisms on *in vitro* determination of antioxidant capacity; hydrogen atom transfer (HAT) and single electron transfer (SET). Both reactions may occur in parallel, but dominant mechanism is influence by antioxidant structure and properties, solubility and partition coefficient and system solvent. (Prior *et al.,* 2005).

HAT methods measure the ability of an antioxidant candidate to quench free radicals by hydrogen donation (AH). Since the methods are rely on competition kinetics, HAT reactions are solvent and pH independent and relatively rapid. The

presence of reducing agents (including metals) could interfere the result. Some methods had been developed using this mechanism, such as oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP). (Prior *et al.,* 2005; Badarinath *et al.,* 2010)

SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals. SET methods are based mainly on deprotonation and ionization potential of the reactive functional group (Wright *et al.,* 2001). SET reactions required long times to complete, and antioxidant capacity calculations are based on percent decrease in product rather than kinetics. These methods are very sensitive to ascorbic acid, uric acid and any reducing compounds. *In vitro* methods that developed from this mechanism are ferric reducing antioxidant power (FRAP) and copper reduction assay (CUPRAC) (Prior *et al.,* 2005; Berker *et al.,* 2007).

Although some studies classified ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method as SET reactions, these two indicator radicals in fact may be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer (Jiménez *et al.,* 2004).

Reactivity patterns and mechanisms of those methods are usually difficult to interpret without detailed information about the composition and structures of antioxidants candidates. Interpretation is particularly difficult whenever small molecule of reducing agents is present in phenolic extracts. (Prior *et al.,* 2005)

Another *in vitro* method that has been used recently is cultured cells. Cultured cells have often been used explain the basic mechanisms of oxidative stress and evaluate the protective effects of antioxidants against oxidative agents. The advantage of using cultured cells is that the study can focus on certain stressors and cell types including model systems for some specific disease can be used for

evaluation of the antioxidant effects. This method was developed widely due to the difficulty found in animal experiment. (Niki, 2010)

6.2. Determination of antioxidant *in vivo*

The determination of antioxidant capacity *in vivo* was conducted to observe various factors and effects. One of the most important factors is the bioavailability of antioxidant. The antioxidant compounds should be absorbed, transported, distributed, and retained properly in its indicated site. The antioxidant candidate must be able to survive not only from digestion, but also from first phase metabolism in liver. This method is also important to study the relationship between dose and efficacy of antioxidant candidate. (Niki, 2010; Fardet *at al*., 2008)

The capacity and efficacy of antioxidants *in vivo* could be assessed from their effect on the level of oxidation in biological fluids and tissues, such as plasma, erythrocytes, urine, and cerebrospinal fluids, from humans and experimental animals (Halliwell *et al.*, 2005). The measurement of oxidation products is monitored from biomarkers of lipids (eg. aldehydes, ketone, oxysterol), protein (eg. protein carbonyl, hydroperoxide, albumin) or DNA (eg. thymine glycol, 5-hydroxyuracil, 2-,8-hydroxyadenine) oxidation (Dalle-Donne *et al.*, 2006).

7. Prebiotic testing method

Rigorous testing of candidate molecules must be performed using standardized methodologies to become a prebiotic. For each candidate, the result should demonstrate: 1) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, 2) fermentation by intestinal microflora, and 3) selective stimulation of growth and/or activity of intestinal bacteria. (Roberfroid *et al.*, 2010)

Preliminary investigation is performed to fulfill the non-digestibility property. Non-digestibility determinations of prebiotic carry out to the ability of the molecule resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal

absorption. *In vitro* method involves determining resistance to acidic and hydrolysis using salivary, pancreatic, and small intestinal enzymes which referred to the of human gastrointestinal environment conditions. This method measures monosaccharide's production after those treatments. (Roberfroid *et al.*, 2010)

The next experiment is to find the candidate ability of fermentation by intestinal microflora, and selective stimulation of growth and/or activity of intestinal bacteria. There are two main methods for these studies, which are *in vitro* and *in vivo* study. (Roberfroid *et al.*, 2010)

7.1. *In vitro* study

The aim of *in vitro* study is to observe prebiotic effects independently from their passage through the upper parts of the GI tract. These models only indicate a potential prebiotic effect and they do not prove the prebiotic attribute of a particular product such as their selectivity stimulates the growth and/or metabolic activity. *In vivo* studies must be performed to definitively demonstrate that the compound under investigation gives health benefits to the host. (Roberfroid *et al.*, 2010).

In batch culture studies, different substrates are incubated with either pure culture of selected bacteria or mixed fecal bacteria subsequently analyzed for microbial composition. This method can be used to study the selectivity of fermentation (including possible mechanism of selectivity) by Bifidobacteria or Lactobacilli of different substrates or different size of the molecules. *In vitro* study can be used to show changes in fecal microbial (e.g. increase in Bifidobacteria), to measure and to compare the evolution of pH changes, gas and SCFA production as results of the fermentation of different substrates. (Rycoft *et al.*, 2001)

Classical microbiological techniques and molecular approaches are used to analyze microbes and or metabolites composition after incubation. These techniques are only single step of procedure by culturing probiotic or mixed fecal bacteria using the potential substrate for carbohydrate source and determine the

growth of bacteria by its turbidity, optical density and level of SCFA production (Pastell *et al.,* 2009). While the molecular approaches involve the fermentation that observe and analyze metabolites and their effect on enzyme activities, the composition of microbiota was identified and quantified by PCR and DGGE. (Blaut *et al.,* 2002)

Some *in vitro* models have been used to study the resistance to upper bowel digestion, fermentation by intestinal microflora, and selective growth stimulation of intestinal bacteria by prebiotic candidate. Those are 1) single stage model that compare standard prebiotic to prebiotic candidate and analyze microbial composition, 2) continuous culture system that observe the growth and metabolite of bacteria, 3) three stage model that mimicking three parts of colon (proximal, transverse and distal) to monitor the enzyme activities and confirm the single or continuous model, 4) the model of human gut environment that consists of a series of five temperature and pH-controlled vessels which simulate the environment of stomach, small intestine, ascending, transverse and descending colons, and 5) TNO-intestinal model that regulate not only the temperature and pH but also simulate the peristaltic mixing. (Roberfroid, *et al.,* 2010)

The *In vitro* study is important not only to find the prebiotic potential but also to determine any possibility toxic metabolite(s) production during the fermentation of mixed fecal microbial. This is required if the compound going to the next step, *in vivo* study in human.

7.2. *In vivo* study

In vivo models are used to ensure the expected prebiotic effect on gut after oral administration, added to food or drinking water, to either animal or human. Usually the dose given to germ-free animal pretreated with an antibiotic to suppress the intestinal flora. The more invasive *in vivo* methods involve intubation into the gastrointestinal system of living anesthetized animal, which called *in situ* study (Gibson and Roberfroid, 1995, Gibson and Rastall, 2006).

In animals, the measurement can be done either by cecal collections or by anesthetized and killed animals at predetermined time intervals. Fecal samples and the contents of the gastrointestinal segments are collected for analysis. *In situ* study can be also used to determine the effect of SCFA production on increasing the mineral absorption. (Harmsen *et al.*, 1999)

There are two approaches that are used to study the substrates fermentation in humans. The first one is indirect method that collects breath air at regular time intervals from volunteers to measure the concentration of gas (hydrogen), which previously given an oral dose of the substrate. The other approach consists of collecting feces and measuring recovery of the tested substrate after oral administration.

Any dietary component that reaches the colon intact is a potential candidate for prebiotic, however, it is latter found that fermentation by intestinal microflora, and selective stimulation of growth and/or activity of intestinal bacteria still the most difficult to fulfill. Even if in addition to ITF, GOS, and several dietary carbohydrates (e.g isomalto-oligosaccharides, gluco-oligosaccharides, xylo-oligosaccharides, gentio-oligosaccharides, mannan-oligosaccharides, lactose, hemicelluloses, resistant starch, resistant dextrin, oat bran and some sugar alcohols) show selectivity when tested in *in vitro* study but the ultimate test for prebiotic activity in human volunteer is lacking for the majority of these compounds. Most are due to the different environment of gut and composition of microflora within human depending on the eating habit and physical activities. (Roberfroid *et al.*, 2010)

8. Characterization of oligosaccarides

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is a common method for oligosaccharide separation and analysis. HPAEC-PAD could give resolution of either neutral or charged oligosaccharide isomers with good reproducibility (Lee, 1996; Thayer *et al.*, 1998).

Carbohydrates are weak acids with pKa > 12 and separation is achieved at high pH by using strong alkaline solutions (0.1 M NaOH) in anion-exchange columns (Lee, 1990; Johnson *et al.*, 1993). High pH caused dissociation of protons from hydroxyl groups, which creates oxyanions retained in column (Thayer *et al.*, 1998). Therefore, pKa value and molecular mass of the carbohydrates will affect on retention time (RT). Retention times could be decreased by addition of acetate ions (OAc) (Johnson *et al.*, 1993).

HPAEC is commonly equipped with pulsed amperometric detector (PAD), which oxidized the carbohydrates at the gold (Au) electrode (Johnson and LaCourse, 1990). The sensitivity for carbohydrates detection by Au electrodes requires pH about 12 (Johnson *et al.*, 1993). The detector works in three steps, firstly, the carbohydrate is oxidized at the Au electrode, resulting in a specific oxidation current and the signal is collected. Secondly, a large positive potential achieve anodic formation of surface oxides to clean the electrode. The last is a reactivation step, where the oxidized electrode surface is reduced back to a reactive Au surface (Johnson and LaCourse, 1990).

PAD could not provide a uniform response to the carbohydrates with the same monosaccharide units but it differentiate structural arrangement of the residues. Therefore, this method requires a quantification standard for each oligosaccharide to be determined (Lee, 1990). Quantitative analyses can be performed by measuring area or height of chromatogram peaks. In integrating the peaks, either peak height or peak area can only be used whenever good resolution and shape of the peak are achieved. Sometimes peak height is more precise than area in measuring the quantity by this method, although area is more widely used in chromatography (Dyson, 1998; Bicking, 2006).

MATERIALS AND METHODS

Materials

1. Raw Material

Commercially defatted rice bran was received from rice bran oil company, Ruam Jai Vegetable Oil, Co. Ltd, Thailand.

2. Equipments

2.1. Electronic balance (Metler Toledo JB1203-CA-F Carat Scale, Switzerland).

2.2. Hot air oven (Binder, USA).

2.3. pH meter (Mettler-Toledo S20 SevenEasy™, USA).

2.4. Water bath (UMac Scientific Co. Ltd., Thailand).

2.5. Head stirrer (IKA® RW 20 digital, IKA-Werke GmbH & Co. KG, Germany).

2.6. Refrigerate centrifuge (Himac CR 20B2, Hitachi Co. Ltd., China).

2.7. Rotary evaporator (Buchi® Rotavapor R152, Switzerland).

2.8. Vortex mixture (Vortex-Genie 2, Scientific Industries Inc., USA).

2.9. Spectrophotometer (GENESYS 10 Scanning UV/Visible Spectrophotometer, Thermo Scientific®, USA).

2.10. Color analyzer (UltraScan, HunterLab, Reston, USA).

2.11. Anaerobic chamber (Bactron® IV, 17 Cu.Ft. Anaerobic Chamber, SHEL LAB®).

2.12. GC system (Agilent 6890N, Agilent Technology, USA) with flame ionization detector.

2.13. GC column (DB-FFAP 125-3237, J&W Scientific, Agilent Technologies Inc., USA) is a fused-silica capillary column with free fatty acid phase of 30m x 0.53mm i.d. and 0.50 µm film thickness.

2.14. HPAEC-PAD system (BioLc, Dionex, USA) equiped with an ED40 PAD and Chromoleon software 6.7 data handling.

2.15. HPAEC column (CarboPac PA200. Thermo Scientific®, USA) is consisted of a 5.5 μm diameter (6% cross-linked) ethylvinylbenzene/ divinylbenzene substrate, agglomerated with a 275 or 34 nm MicroBead quaternary amine functionalized latex.

2.16. Ultrapure water system (Nanopure, Thermo Scientific®, USA).

2.17. Filter syringe 0.45 μm (PDVF Durapore® Membrane, Merck KGaA, Germany).

3. Chemical reagent

3.1. Reagents for extraction

3.1.1. Enzymes:

3.1.1.1. Viscozyme (Major enzyme is endo-β-glucanase and side activities of enzyme xylanase, hemicellulase and cellulase are also included)

3.1.1.2. Pentopan (endo-(1,4)-beta-xylanase) from Novozymes, Denmark.

3.1.2. Hydrochloric acid 37% (HCl: Analytical grade, RCI Labscan, Dublin, Ireland).

3.1.3. Sodium hydroxide (NaOH: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.1.4. Ethanol 95% (Denatured Ethanol, Common Group Ltd., Bangkok, Thailand)

3.1.5. Absolute ethanol (CH_3CH_2OH: Analytical grade, RCI Labscan, Dublin, Ireland).

3.2. Reagents for protein determination

3.2.1. Sodium Hydroxide (NaOH: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.2.2. Sulfuric acid (H_2SO_4: Analytical grade, RCI Labscan, Dublin, Ireland).

3.2.3. Boric acid (HBO_3: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.2.4. Hydrochloric acid 37% (HCl: Analytical grade, RCI Labscan, Dublin, Ireland).

3.3. Reagents for starch determination

3.3.1. Total starch assay (Megazyme International, Ireland).

3.3.2. Glacial acetic acid (CH_3COOH: Analytical grade, RCI Labscan, Dublin, Ireland).

3.3.3. Sodium hydroxide (NaOH: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.3.4. Calcium chloride ($CaCl_2$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.3.5. Potassium hydroxide (KOH: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.4. Reagents for reducing sugar determination

3.4.1. Hydrochloric acid 37% (HCl: Analytical grade, RCI Labscan, Dublin, Ireland).

3.4.2. Sucrose (Analytical grade, Univar, Ajax Fine Chem., Australia).

3.4.3. Copper sulfate ($Cu_2SO_4.5H_2O$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.4.4. Potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6 \cdot 4H_2O$: Analytical grade, Univar, Ajax Fine Chem., Australia)

3.4.5. Sodium hydroxide (NaOH: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.5. Reagents for antioxidant determination

3.5.1. Gallic acid (reference material, TraceCERT®, Sigma Aldrich, USA).

3.5.2. Ascorbic acid (reference material, TraceCERT®, Sigma Aldrich, USA).

3.5.3. Butil hydroxyl toluene (BHT: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.5.4. Folin Ciocalteu's Phenol Reagent (Sigma Aldrich, USA).

3.5.5. Sodium carbonate (Na_2CO_3: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.5.6. Potassium ferricyanide ($K_3[Fe(CN)_6]$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.5.7. Ferric chloride ($FeCl_3$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.5.8. Trichloroacetic acid ($C_2Cl_3O_2$: Analytical grade, Fisher Chemicals, Fisher Scientific, England).

3.5.9. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH reagent) (C18H12N5O6: Sigma Aldrich, USA).

3.5.10. Di-sodium hydrogen phosphate-7 hydrate ($Na_2HPO_4.7H_2O$, Analytical grade, Univar, Ajax Fine Chem., Australia).

3.5.11. Sodium di-hydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$, Analytical grade, Univar, Ajax Fine Chem., Australia).

3.6. Reagents for digestibility

3.6.1. Inulin (HSI, Beneo™ HSI, Orafti, Tienen, Belgium).

3.6.2. Di-sodium hydrogen phosphate-7 hydrate ($Na_2HPO_4.7H_2O$, Analytical grade, Univar, Ajax Fine Chem., Australia).

3.6.3. Sodium di-hydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$, Analytical grade, Univar, Ajax Fine Chem., Australia).

3.6.4. Sodium chloride (NaCl: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.6.5. Enzymes for digestibility enzymes were human salivary α-amylase (Sigma A1031), porcine pepsin (Sigma P7012), and porcine pancreatin (Sigma P7545) from Sigma Aldrich, USA.

3.6.6. Calcium chloride ($CaCl_2$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.6.7. Hydrochloric acid 37% (HCl: Analytical grade, RCI Labscan, Dublin, Ireland).

3.6.8. Sodium hydroxide (NaOH: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.7. Reagents for fermentation

3.7.1. Inulin (HSI, Beneo™ HSI, Orafti, Tienen, Belgium).

3.7.2. Volatile acid mixed 46975-U (Supelco, Bellefonte, USA).

3.7.3. Glucose ($C_6H_{12}O_6$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.7.4. MRS Broth (Oxoid, Thermo Scientific, UK).

3.7.5. TSB Broth (Oxoid, Thermo Scientific, UK).

3.7.6. Peptone (Soymeal peptone, Merck KGaA©, Darmstadt, Germany).

3.7.7. Meat extract (Millipore, Merck KGaA©, Darmstadt, Germany).

3.7.8. Yeast extract (Millipore, Merck KGaA©, Darmstadt, Germany).

3.7.9. Dipotassium hydrogen phosphate (K_2HPO_4: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.7.10. Sodium acetate trihydrate ($CH_3COONa.3H_2O$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.7.11. Triammonium citrate ($(NH_4)_3C_6H_5O_7$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.7.12. Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.7.13. Manganous sulfate tetrahydrate ($MnSO_4.4H_2O$: Analytical grade, Univar, Ajax Fine Chem., Australia).

3.7.14. L-cystein (Himedia, Mumbai, India)

3.7.15. Phosphoric acid (H_3PO_4: Analytical grade, Merck KGaA©, Darmstadt, Germany).

3.8. Reagents for HPAEC analysis

3.8.1. Arabinose (Standard, Sigma Aldrich, USA).

3.8.2. Xylose (Standard, Sigma Aldrich, USA).

3.8.3. Xylobiose (Standard, Megazyme, Wicklow, Ireland).

3.8.4. Xylotriose (Standard, Megazyme, Wicklow, Ireland).

3.8.5. Xylotetraose (Standard, Megazyme, Wicklow, Ireland).

3.8.6. Sodium hydroxide (NaOH 10M: Electronic industry special grade, Kanto Chemical, Co. Inc., Tokyo, Japan)

3.8.7. Sodium acetate trihydrate ($CH_3COONa.3H_2O$): HPLC grade, Fisher Chemical, USA).

4. Microorganisms for fermentation

4.1. *Bifidobacterium breve* TISTR 2130 (Thailand Institute Scientific and Technological Research, Pathumthani, Thailand).

4.2. *Bifidobacterium bifidum* TISTR 2129 (Thailand Institute Scientific and Technological Research, Pathumthani, Thailand).

4.3. *Bifidobacterium animalis* TISTR 219 (Thailand Institute Scientific and Technological Research, Pathumthani, Thailand).

4.4. *Bifidobacterium longum* TISTR 2194 (Thailand Institute Scientific and Technological Research, Pathumthani, Thailand).

4.5. *Lactobacillus acidophilus* ATCC 4356 (Thermo Scientific, USA)

4.6. *Lactobacillus plantarum* BCC 39798 (Biotec Culture Collection, Pathumthani, Thailand)

4.7. *Escherichia coli* TISTR 887 (Thailand Institute Scientific and Technological Research, Pathumthani, Thailand).

Methods

The commercially defatted rice bran was extracted using enzyme assisted at 5 different time points to investigate the optimal incubation time. Rice bran extract was then fractionized with gradual ethanol precipitation in order to separate the compounds by their molecular weight. All fractions were analyzed for theprotein, starch and reducing sugar contents - and antioxidant capacity. The fractions that had promising potential to be a prebiotic were then tested with common probiotics (Lactobacillus and Bifidobacteria) and compared to the commercial prebiotic (inulin) as reference. Selected fraction was also determined for its oligosaccharide composition.

1. Rice Bran Extraction.

Defatted rice bran was thawed to room temperature and oven dried at 80 °C for 2 h. The bran was then passed through sieve 60 mesh before extraction. 100g bran was suspended in 1000 mL distilled water and pH was adjusted to 5.5 with 6N HCl. The extractions were assisted with addition of 70 unit of enzyme for 100g of bran (Pentopan Mono BG or Viscozyme L). The mixture was incubated under continues stirring at 50 °C for 1, 4, 8, 16 and 24 h. After the incubation, enzymes were inactivated by heating to 100 °C for 10 min. The soluble compound was separated by centrifugation for 30 min at 4000xg and the residue was washed with water. The centrifugation was repeated and the supernatant was collected to increase oligosaccharide yield.

The fractionation was conducted using the ethanol precipitation method by Swennen *et al.* (2005). 95% v/v ethanol was added into the supernatant to get 60% v/v

ethanol concentration and kept overnight to precipitate starch and other large molecules. Centrifugation at 4000xg for 30 min was conducted to separate the 60% precipitate. The supernatant was evaporated and concentrated to 1/6 of its volume using a rotary evaporator. Absolute ethanol was then added to reach 90% v/v ethanol concentration. The 60-90% precipitate was separated by centrifugation as previously described. The supernatant was concentrated to about 250 mL, refrigerated and named as >90% fraction. Fractions <60% and 60-90% were hot air dried (50 °C) until their moisture contents were approximately 7%, then named as V60 and V60-90 for Viscozyme treatment and P60-90 for Pentopan treatment.

The yield was calculated as the percent of dried fraction gained from DFRB. The protein and reducing sugar contents of each fraction were determined according to AOAC 2001.11 and the Lane-Eynon method (ASEAN, 2011), respectively. The color was determined on 0.5% extract solution by color analyzer, UltraScan.

2. Antioxidant capacity

Antioxidant capacity of all fractions from rice bran extracts were determined using reducing power and DPPH radical-scavenging methods. The result was then associated with the phenolic content of those fractions.

2.1. Sample preparation

500 mg of <60% and 60-90% fractions was diluted to 25 mL distilled water as a stock sample solution for further antioxidant analysis. Fraction >90% was thawed to room temperature before analysis.

2.2. Phenolic content

Total phenolic content was determined by the Folin–Ciocalteu colorimetric method (Shen *et al.*, 2009). The 2 mL sample stock solution were mixed with 1 mL 0.5 N Folin–Ciocalteu reagent, and then neutralized with 2 mL saturated

sodium carbonate (75 g/L). After 2 h incubation at room temperature, the absorbance was observed using a UV/Vis spectrophotometer at 760 nm. Gallic acid solution was used as a standard and total phenolic contents were expressed as milligrams of gallic acid equivalent (mg GAE) per 100 g of dry weight.

2.3. Reducing Power

The reducing power was determined using the method from Wang and coworker (2009). The 0.5 mL sample solution of fraction was mixed with 0.5 mL distilled water and 2.5 mL of a phosphate buffer (0.2 M, pH6.6). The mixture was incubated at 50 °C for 30 min with an additional 2.5 mL of aqueous potassium ferricyanide solution (1% m/v) and terminated by adding 2.5 mL of 10 % m/v TCA solution. The mixture was centrifuged at 3000xg for 10 min and 2.5 mL of supernatant aliquot was mixed with 2.5 mL of distilled water and 0.5 mL of aqueous ferric chloride solution (0.1 % m/v). The absorbance was observed at 700 nm by UV/Vis spectrophotometer with ascorbic acid as the reference material.

2.4. DPPH radical-scavenging

The DPPH radical scavenging activity was determined by the method of Daou and Zhang (2011) with slight modifications. As an aside, 1.0 mL DPPH reagent (0.2 mM) was added to 2 mL of the sample stock solution that had been added with 2 mL distilled water, and then mixed vigorously. The absorbance was measured at 517 nm using a UV/Vis spectrophotometer after incubation for 30 min under dark condition. Distilled water was used as a control. The equation for DPPH radicals-scavenging activity was:

DPPH radical-scavenging activity (%) = [1- (A517 of sample/A517 of control)] × 100

3. Digestibility index

The digestion method was performed according to the procedure established by Hollebeeck and coworker (2013). The batch model simulated three steps of digestion in mouth, stomach and small intestine at 37 °C. A sample of 30 mg substrate was suspended in 10 mL phosphate buffered saline (20 mM, pH 6.9). First step was substrate treatment with human salivary α-amylase to adapt the digestion of carbohydrate in mouth. The solution was added with 0.5 ml human salivary α-amylase solution (3.9 U/mL, 10 mg/ml in $CaCl_2$ 1 mM) and incubated at adjusted pH 6.9 for 5 min.

The next step was stomach digestion with porcine pepsin under strong acidic condition. The pH of stomach step normally ranged from 1-2.5 for 90 min, but pH might be changed during digestion process. The solution was adjusted to pH 2, with HCl 6 N, and vortexed 5 sec every 10 min, which adapted the physiological stomach movement. It was then added with 1.25 mL (71.2 U/mL) porcine pepsin suspension (1 mg/mL in NaCl 9 g/L) and incubated for 90 min.

The last step was included not only similar vortex treatment to substrate solution, but also anaerobic condition which created by flushing nitrogen to the tube and tightly closed it. This step was adjusted to duodenal digestion with addition of pancreatin from porcine pancreas. To the solution, 10 mL porcine pancreatin (0.5 mg/mL in $CaCl_2$ 25 mM) for later incubation. The solution was vortexed 5 sec every 10 min during incubation. Degradation product (sugar) was quantified using Lane-Eynon method.

4. Fermentation Experiment

4.1. Substrate preparation

Each tested compound (glucose, inulin, V60-90, and P60-90 fractions) was prepared by diluting 20 g substrate in 200 mL sterile water, and the suspension

was sterilized by passing through 0.20-µm syringe filter, which excluded 17.6 kDa molecules, and stored at 4 °C until use.

4.2. Microbial strain and Medium Preparation

Microbial strains were obtained from different culture suppliers and strain collections, and grown in appropriate growth media (MRS or TSB) under anaerobic conditions. Media and test tubes were prepared with boiling system to generate anaerobic growth conditions. Microbial strains were precultured from stocks (stored at -70 °C) in appropriate glucose-containing media for 24 hours at 37 °C. The precultured microbes were then inoculated in a fresh growth medium containing glucose (MRS or TSB) and incubated for another 24 hours. Cell suspension (1% v/v) was prepared in the test medium, which did not include added carbohydrates, and was used immediately for the growth assays.

4.3. Microbial growth experiment

The fermentation experiment was adapted from Pastell *et al.* (2009b). Basal MRS broth (90 mL) was supplemented with 10 mL of 10% (w/v) sterile carbon source suspension and inoculated with 1 mL inoculums of an overnight microbial culture. The inoculated media were incubated under anaerobic condition using anaerobic chamber at 37 °C. Every 4 h, the cultures were vortexed for 30 s to disperse the bacterial cells and 2 mL of the cultures were taken for determination of the cell growth of each strain by measuring the optical density (OD) at 620 nm using a spectrophotometer.

At 24 and 32 h incubation, each 2 mL of cultured media was transferred to appendorf tube, added 0.5 mL of formaldehyde solution (37%) and kept at -20 °C for further SCFA analysis. Two replications were made for the experiment.

4.4. Gas Chromatography (GC) method for SCFA analysis

Prior to quantification of SCFA, the samples were acidified to pH < 2 with 17% H_3PO_4 and then analyzed using GC (Hewlett-Packard 5890 series II; Hewlett-Packard, Palo Alto, CA). The temperatures of the injector and detector were 175 and 240 °C, respectively. The temperature gradient was as follows: the initial temperature was 110 °C, ramped at 5 °C/min to 140 °C, maintained at 140 °C for 6 min, ramped at 45 °C/min to 200 °C, and maintained at 200 °C for 3 min. The injection volume of the sample was 2 µL. The quantified SCFA were analyzed as acids: acetic, propionic, and butyric acids. The standards used in the SCFA analyses were the same acids as mentioned above with concentrations of 2-10 mM.

5. Characterisation of rice bran extract

The sample preparation for characterization was adopted from Swennen *et al.* (2005). Each 10 mg of fraction was diluted in water 100 mL. Mild acid hydrolysis was performed to observe the distribution of xylan backbone chain lengths in the supernatants. The pH was adjusted to 2.8 with freshly prepared hydrochloric acid (0.1 M). The solutions were heated in a sealed tube for 4 h at 90 °C. The solutions were cool down to room temperature and neutralized with sodium hydroxide (0.1 M).

For monosaccharides determination, each 10 mg of fraction was diluted in water 50 mL, followed with addition of 10 mL of H_2SO_4 1M. The solutions were boiled in a sealed tube for 2 h. The solutions were cooled down to room temperature and neutralized with sodium hydroxide (10 M). The solution volume was brought up to 100 mL.

Samples were then deproteinized, to avoid interference and signal diminution through trailing of amino acids and peptides on the gold electrode, by adding 1 mL of 36 g/L of $K_4Fe(CN)_6 \cdot 3H_2O$ and 1 mL 72 g/L of $ZnSO_4 \cdot 7H_2O$) to 2 mL of sample. After centrifugation (14,000 rpm, 18 °C, 15 min), the supernatant was filtered (0.45-µm syringe filter with nylon membrane) for further analysis. The injection volume

was typically 10 µl in the measurements. Chromeleon software 6.7 (Thermo Scientific) was used for system control and data analysis.

The mobile phases were filtered with 0.45-µm membrane filter. The eluents for gradient analysis of the oligosaccharides were A: deionized water, B: 200 mM NaOH, and C: 400 mM NaOAc in 100 mM NaOH and the total analysis time was 90 min, as gradient solution (table 1). The waveform had the following quadruple potential: E1 = 0.10 V (t1 = 0.00−0.40 s) with integration from 0.20 to 0.40 s; E2 = −2.00 V (t2 = 0.41−0.42 s); E3 = 0.60 V (t3 = 0.43 s); and E4 = −0.10 V (t4 = 0.44−0.50 s) at 30 °C.

Table 1 Gradient elution condition applied for HPAEC-PAD with CarboPac PA200 using a ternary gradient eluent.

flow rate (mL min−1)	time (min)	ternary gradient		
		A (%)	B (%)	C (%)
Oligosaccharides characterization		double deionized water	200 mM NaOH	100 mM NaOH with 400 mM NaOAc
0.25	0.0	90.0	10.0	0.0
0.25	10.0	90.0	10.0	0.0
0.25	10.1	50.0	50.0	0.0
0.25	15.0	50.0	50.0	0.0
0.25	55.0	34.0	34.0	32.0
0.25	70.0	0.0	0.0	100.0
0.25	75.0	0.0	0.0	100.0
0.25	75.1	90.0	10.0	0.0
0.25	80.0	90.0	10.0	0.0
Monosaccharides characterization		double deionized water	50 mM NaOH	-
0.25	0.0	97.0	3.0	-
0.25	21.0	97.0	3.0	-
0.30	22.0	97.0	3.0	-
0.30	24.0	0.0	100.0	-
0.30	30.0	0.0	100.0	-
0.30	32.0	97.0	3.0	-
0.25	33.0	97.0	3.0	-
0.25	45.0	97.0	3.0	-

A mixture of xylose, xylobiose, xylotriose and xylotetraose was used as an external standard. The standard mixture had concentration of (A) 50 µg/mL monosaccharides (arabinose and xylose) and 20 µg/mL oligosaccharides (xylobiose,

xylotriose and xylotetrose); (B) 25 μg/mL glucose and 10 μg/mL mixed arabinose, xylose, xylobiose, xylotriose and xylotetrose.

6. Statistical analysis

Experiment was conducted duplicate (at least) and averaged for statistical analysis. All statistical analysis was performed using Minitab version 16. The significance of data was determined by Analysis of variance (ANOVA) followed by Fisher's multiple range test and means were considered significantly different at $p \leq 0.05$.

RESULT AND DISCUSSION

1. Rice Bran Extraction.

The release of hemicellulose from cell wall can be done by physical treatment, such as heat, high pressure or microwave assisted chemical (alkaline or acid) extraction or enzymatic treatment. Compared to either physical or chemical treatment, enzymatic treatment is more specific, safe and environmental friendly (Moure *et al.*, 2006). Heat with high pressure or chemical treatments were conducted to produce large scale of hemicelluloses and oligosaccharides for being the source of fiber or food additives (Elleuch *et al.*, 2011). Enzymatic treatments were preferred to avoid any unexpected structural damage or by-product because these treatments were commonly conducted under low temperature and at normal pH (Aachary and Prapulla, 2011).

In this experiment, two different enzymes (Viscozyme and Pentopan) were used to extract hemicellulose from commercially defatted rice bran. According to Burton and Fincher (2012) in grasses family (Poaceae), the major non-cellulosic polysaccharide is heteroxylan which consists of (1,4)-linked backbone of β-D-xylopyranosyl (Xylp) residues and (1,3;1,4)-β-glucans, where the heteroxylan in rice is mostly arabinoxylan (AX). In order to cleave those linkages, Viscozyme that has β-endoglucanase as main activity and Pentopan with (1,4)-β-endoxylanase as main activity were chosen. Those enzymes were expected to be able to loosen up the cell wall structure, release more hemicellulose, hydrolyzed arabinoxylan. Szwajgier *et al.* (2005) used Viscozyme to produce oligosaccharides from barley malt while Makaravičius *et al.* (2011) had studied on oligosaccharides production wheat and rye arabinoxylan.

In preliminary extraction, the optimum pH and temperature conditions were conducted following the product specification sheet, while incubation time was varied to 1, 4, 8, 16, and 24 h. The enzymatic extracts were fractionated by adding ethanol to obtain 60% and 90% ethanol concentration. This fractionation method was used due

to the finding of Swennen and coworker (2005, 2006), who reported the precipitation of oligosaccharides with average DP 16 from wheat bran in 60-90% fraction.

Table 2 Percent yield of <60% and 60-90% fractions and reducing sugar content in >90% fractions after 1, 4, 8, 16, and 24 h incubation time.

Time (h)	Yield (% w/w)				Reducing Sugar (% w/v)	
	V60	P60	V60-90	P60-90	V90	P90
1	4.27 ± 0.10^{d}	4.89 ± 0.11^{d}	0.43 ± 0.01^{e}	0.36 ± 0.00^{d}	1.46 ± 0.01^{d}	1.45 ± 0.01^{e}
4	5.01 ± 0.12^{c}	5.53 ± 0.13^{c}	1.94 ± 0.03^{d}	1.93 ± 0.02^{c}	1.91 ± 0.02^{a}	1.56 ± 0.02^{d}
8	9.61 ± 0.22^{b}	10.91 ± 0.25^{b}	2.84 ± 0.03^{c}	1.92 ± 0.02^{c}	1.84 ± 0.02^{b}	1.78 ± 0.02^{b}
16	11.75 ± 0.27^{a}	12.92 ± 0.30^{a}	3.25 ± 0.04^{b}	2.41 ± 0.03^{b}	1.61 ± 0.01^{c}	1.62 ± 0.02^{c}
24	11.67 ± 0.27^{a}	12.97 ± 0.30^{a}	3.69 ± 0.05^{a}	3.63 ± 0.03^{a}	1.94 ± 0.02^{a}	1.86 ± 0.02^{a}

Note: P: Pentopan treatment; V: Viscozyme treatment; 60: residue gained from 60% ethanol precipitation; 60-90: residue gained from 90% ethanol precipitation; 90: supernatant from 90% ethanol precipitation. S: significant different of each value in the same column ($p \leq 0.05$).

Incubation time at 24 h was chosen for further study due to the highest yield of V60-90 and P60-90 fractions (Table 2). The incubation time was not prolonged more than 24 h to avoid too much sugar that released from the rice bran. It was found that reducing sugar content in both V90 and P90 fractions were increased from about 1.6% to 2% after the incubation time was changed from 16 h to 24 h. This might be because the enzyme cleavage has reached the reducing end (van den Brink and de Vries, 2011). The >90% fractions were not completely dried due to the difficulties in removing mixed solvent (water and ethanol) from the hydrolysates. These fractions became viscous materials; therefore their yields and moisture contents could not be determined . To be able to observe the releasing di- and monosaccharides to these fractions, determination of reducing sugar in was conducted.

Table 2 shows that precipitant of 60% ethanol (V60 and P60) provided the highest yield of precipitants among all fractions, where the yield of P60 was higher than that of V60. Both fractions contained high amounts of starch and protein, especially in P60 fraction (Table 3). This result was consistent with the study of

Swennen *et al.* (2005), where ethanol fractionation could gradually precipitate compounds with different molecular size. The fractionation with 60% ethanol resulted in the precipitation of polysaccharides derived from wheat bran with molar mass above 5.9 kDa and DP>30 (Swennen *et al.*, 2005). Starch and protein that have molar mass larger than 10 kDa (Zobel, 1988) will be certainly precipitated in 60% ethanol solution. On the other hand, the higher level of reducing sugar in V90 and P90 fractions indicated the precipitation with relatively lower molecular weight, compared to other fractions.

Table 3 Moisture, protein, starch and reducing sugar contents of extracts obtained after enzymatic treatment with Viscozyme (V) and Pentopan (P) followed by gradual ethanol precipitation.

	Moisture	**Protein**	**Starch**	**Reducing Sugar**
DFRB (%)	8.6 ± 0.35^{a}	17.35 ± 1.18^{b}	26.58 ± 0.26^{c}	n.d
V60 (%)	7.10 ± 0.29^{b}	11.58 ± 0.02^{c}	37.25 ± 0.59^{b}	0.208 ± 0.001^{e}
V60-90 (%)	7.08 ± 0.28^{b}	1.41 ± 0.22^{d}	1.10 ± 0.28^{d}	0.583 ± 0.004^{c}
V90 (%)	n.d	0.14 ± 0.02^{f}	n.d	1.944 ± 0.023^{a}
P60 (%)	7.10 ± 0.29^{b}	21.95 ± 0.31^{a}	45.75 ± 0.28^{a}	0.125 ± 0.001^{f}
P60-90 (%)	7.08 ± 0.28^{b}	0.37 ± 0.28^{e}	0.34 ± 0.05^{d}	0.541 ± 0.003^{d}
P90 (%)	n.d	0.00 ± 0.01^{f}	n.d	1.861 ± 0.018^{b}

Note: The protein and starch contents of defatted rice bran (DFRB), <60% fraction, and 60-90% fraction were expressed based on dry mass and >90% fraction were expressed based on wet mass. n.d: not determined.

The most different composition was found between V60 and P60, which was most likely caused by the different mechanism of those enzymes on breaking rice bran cell wall. β-endoxylanase in Pentopan belongs to glycosyl hydrolase family 11 (GH 11), which works on xylan backbone (Makaravičius *et al.*, 2011). GH 11 family showed the better capability of cleaving glycosidic linkages in the xylan main chain closer to the substituents than GH 10 (Vardakou *et al.*, 2003). Meanwhile, Viscozyme that showed mainly endo-β-(1-3(4))-glucanase activity (GH 45) could hydrolyze cellulosic-glucans, resulting in the loosening of cellulose structure (Lafond *et al.*, 2012). Since cellulose fibril is present as long un-branched fibril, it is difficult for the

enzyme to completely open the cell wall and release trapped soluble protein and starch granule (Levi *et al.*, 2002; Somerville *et al.*, 2004). Nevertheless, Viscozyme was a common pretreatment enzyme on protein extraction from cereal brans (Rosset *et al.*, 2012; Bandyopadhyay *et al.*, 2012). In this case, the breaking of xylan backbone by β-endoxylanase (Pentopan) could open the primary cell wall by breaking xylan (hemicellulose) structure and released more soluble protein and starch than did endo-β-(1-3(4))-glucanase.

The highest content of reducing sugar was found in V90 fraction, which was the supernatant of 90% ethanol precipitation. Viscozyme was reported that it had capability to cleave the xylan backbone of two or three xylo-backbone at the end terminal and also to cleave some side chains (Petersen *et al.*, 2014). Therefore, this enzyme could release more sugar than did Pentopan.

Table 4 Color measurement of rice bran extracts and high soluble inulin (HSI) solutions at 0.5% (w/v) concentration.

Samples	Color Value		
	L*	**a***	**b***
HSI	99.265 ± 0.225^{a}	-0.115 ± 0.005^{a}	0.890 ± 0.090^{f}
V60	99.245 ± 0.245^{a}	-0.525 ± 0.005^{b}	3.285 ± 0.015^{e}
V60-90	98.660 ± 0.000^{b}	-1.600 ± 0.300^{c}	6.960 ± 0.010^{c}
V90	97.475 ± 0.065^{d}	-2.350 ± 0.000^{d}	14.200 ± 0.090^{a}
P60	99.110 ± 0.010^{a}	-0.035 ± 0.005^{a}	0.985 ± 0.015^{f}
P60-90	98.530 ± 0.450^{b}	-0.720 ± 0.010^{b}	3.825 ± 0.025^{d}
P90	97.865 ± 0.055^{c}	-2.160 ± 0.000^{d}	11.700 ± 0.090^{b}

Note: HSI: high soluble inulin. a*: green (-) to red (+). b*: blue (-) to yellow (+). Different letter in a column means significantly different ($p \leq 0.05$).

After fractionation, the color of each fraction in a solution form was also determined because it is an important characteristic for further utilization of these extracts. The color of rice bran extract solution was also compared to that of high soluble inulin (HSI) solution because this inulin is commercially used as a prebiotic.

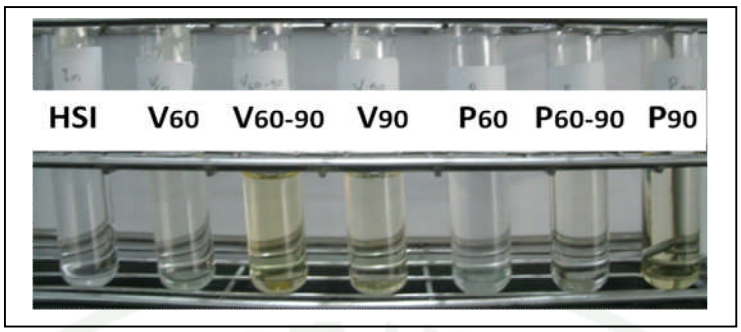


Figure 6 The appearance of rice bran extracts and inulin solutions at 0.5% (w/v) concentration.

The lightness values (L*) of V60, P60 and inulin solutions were not significantly different and were higher than those of other fractions (Table 4). The lightness of V60 and P60 solutions may be influence by the high amount of starch in both fractions, giving the most color effect on the solutions. Regarding to a* and b* values, V60 solution had slightly more green and yellow color than P60 solution.

The lowest luminance (L*) was found in V90 and P90 solution (Figure 6). These >90% fractions also showed the highest green (-a*) and yellow (b*) value. Brown color could be also caused by non enzymatic oxidation of polyphenol compound in the solution that produce quinone compounds, as usually observed in the change of fruit juice color (Robards *et al.*, 1999). Millard reaction might also accur during enzyme inactivation which conducted at 100 °C with the precence of sugar and protein and/or peptide (Martins *et al.*, 2001).

The brown color of 60-90% fractions solution might be developed during drying process and the presence of arabinoxylan (AX) or lignin that has light brown to brown color. When 60-90% fractions were drying at 60 °C for 8 h, oxidation reaction might occur on phenolic compounds. This can occur through hydrolysis of glycosides (spontaneous or metal-catalyzed air oxidation) and color changes due to pH or metal chelation, or condensation with aldehydes (Singleton *et al.*, 1972).

2. Antioxidant capacity.

Antioxidant is any substance in low concentration that has ability to delay or prevent oxidative reaction in cell. In plant kingdom, potential antioxidant compounds are coming from phenolic compounds which a largely abundant metabolites (Dai and Mumper, 2010). Rice bran is one of the by-product that contain high level of antioxidant, namely γ-oryzanol, phenolic and anthocyanin compounds. It has widely recognized that rice bran oil contain high γ-oryzanol, while the other two antioxidants are mostly bound with hemicellulose of rice bran (Friedman, 2013). This experiment was intended to observe the existence of antioxidant compounds and the ability of enzymatically treatment to extract it from commercially defatted rice bran which is a by-product from rice bran oil production.

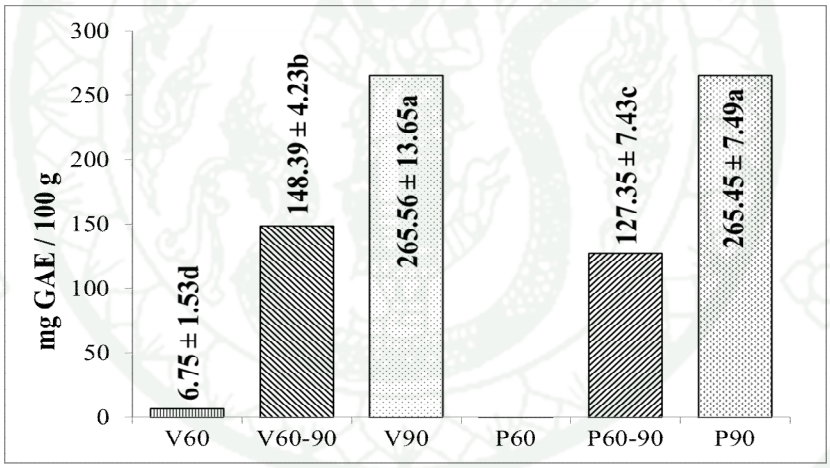


Figure 7 Total phenolic compounds of rice bran extracts with gallic acid as standard. Different letter means significantly different ($p \leq 0.05$).

The most common phenolic acids in rice and its fiber extract were ferulic acid, p-coumaric acids and sinapic acid, which was present in free, esterified, and insoluble-bound forms with mostly hemicellulose (Guo and Beta, 2012; Adom and Liu, 2002). Phenolic antioxidants depend mainly on structural features resonance delocalization of the phenol radical and steric hindrance derived from bulky groups substituting hydrogen in the aromatic ring (Wang *et al.*, 2009).

Among all fractions, the highest phenolic content was found in both the >90% fractions after Viscozyme or Pentopan treatment, followed by 60-90% fractions and <60% fractions, respectively (Figure 7). Phenolic compounds in V90 and P90, which obtained after 90% ethanol precipitation, it was speculated to be more in the free form because Ti and coworker (2014) reported that free phenolic compounds from rice bran could be extracted by semi polar solvent such as methanol and ethanol.

Compared to V90 and P90 fractions, approximately the same level of phenolic content (220-320 mg GAE/100 g) was also observed by Chotimarkorn and coworker (2008), when they extracted 5 commercial rice bran cultivars in Thailand with methanol. Adom and Liu (2002) investigated the phenolic content in whole grain rice and found that 5.56±0.17 μmol of GAE/g (approximately 94.59 mg GAE/100g) of grain mostly in bound form with the cell wall.

When Wang and coworker (2009) prepared the extract from wheat bran using similar enzymes (xylanase from *Bacillus subtilis*), the total phenolic compound was 0.3712 ± 0.0025 g FAE (ferulic acid equivalent) per gram of wheat bran enzymatic hydrolysate. In their study, starch and protein were removed using thermostable α-amylase, protease and amyloglucosidase, before xylanase treatment and using column chromatography for the purification. Lower phenolic compounds might be caused by the low content of phenolic acid in wheat bran or the degradation of this compound during heat treatment on stabilizing the bran or removal of starch (Yu, 2008).

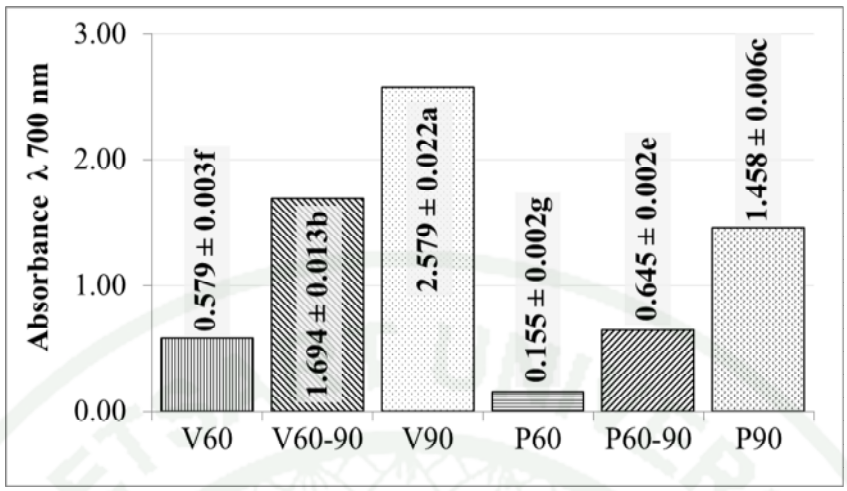


Figure 8 Antioxidant capacity of rice bran extracts determined by reducing power method. Different letter means significantly different ($p \leq 0.05$).

Figure 8 shows the reducing power activity of all fractions with higher absorbance values depicting higher reducing activity. Under the same concentration (10 mg/mL), the highest absorbance value (at 700 nm) was found in the >90% fraction that was treated with Viscozyme. Among the three fractions obtained by each enzymatic treatment, the >90% fraction provided the highest reducing power, followed by the 60-90% and <60% fractions, respectively. These reducing power activities were associated with the phenolic compounds in their fractions. Compared to the study of Adom and Liu (2002), the reducing power of V90 and P90 fractions (154.08±1.39 and 81.96±0.39 µmol of vitamin C equiv/g of DFRB) were higher than that of whole grain rice (55.77±1.62 µmol of vitamin C equiv/g of grain).

Reducing power method was based on redox reaction that measured the amounts of ferric ion that change into ferrous form as described by Berker *et al.* (2007):

Fe^{3+} + antioxidant ↔ Fe^{2+} + oxidized-antioxidant

$Fe^{2+} + Fe(CN)_6^{3-} \leftrightarrow Fe[Fe(CN)]^-$

or

$Fe(CN)_6^{3-}$ + antioxidant ↔ $Fe[Fe(CN)]^{4-}$ + oxidized-antioxidant

$Fe[Fe(CN)]^{4-} + Fe^{3+} \leftrightarrow Fe[Fe(CN)]^-$

The presence of any molecules that rapidly donate a hydrogen atom to radicals (such as reducing sugar) and chelating ion might interfere with the result (Dai and Mumper, 2010; Niki, 2010). Therefore, Viscozyme's fractions, which had higher reducing sugar content compared with Pentopan's, contributed to the greater reducing power. The higher amount of reducing sugar might be caused by endo-β-glucanase which is the main enzyme in Viscozyme. In addition, some peptides in Viscozyme's fractions might be able to donate hydrogen atoms and caused the higher reducing power, compared to Pentopan's.

A second method with a different mechanism was needed to ensure the antioxidant capacity of each fraction. The principal of the DPPH radical-scavenging method is the measurement of the ability of substrates to stabilize the electrons from free radicals (Badarinath *et al.*, 2010). Antioxidant capacity of the extract was strongly influenced by phenolic compounds which stabilize the radical by localize the singlet electron within their aromatic ring (Fardet *et al.*, 2008). The structural configuration of the phenolic compounds and steric hindrance in aromatic ring led to ability on stabilizing radicals (Wang *et al.*, 2009, Guo & Beta, 2013).

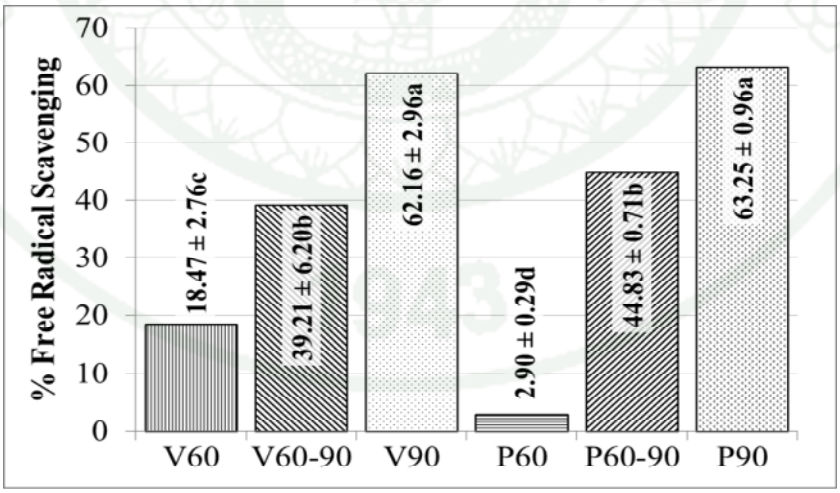


Figure 9 Antioxidant capacity of rice bran extracts determined by DPPH radical scavenging method. Different letter means significantly different ($p \leq 0.05$).

The antioxidant activity profile of each fraction from different enzyme treatment was similar (Figure 9), where the highest activity was found in either V90 (62.16±2.96% DPPH) or P90 (63.25±0.96% DPPH), and followed with 60-90% fractions (39.21±6.20% DPPH (Viscozyme) and (44.83±0.71% DPPH (Pentopan)). The lowest % DPPH was observed in <60% fractions and it was significantly different between Viscozyme (18.47±6.20% DPPH) and Pentopan treatment (2.90±0.29% DPPH). The antioxidant activities measured by DPPH radical-scavenging method were also consequent with the phenolic contents, so it can be concluded that antioxidant capacity was mainly caused by phenolic compounds. Compared to a synthetic antioxidant, the antioxidant activity of the >90% fraction (1%) was slightly lower than that of butylated hydroxyltoluene (BHT), which provided 62.64±0.26% DPPH with 0.25% concentration.

The DPPH results were agreed with Daou and Zang (2011) who studied on antioxidant capacity of defatted rice bran. They found that 1% of total dietary fiber from defatted rice bran was able to produce over 60% DPPH but soluble dietary fiber provided less than that. Szwajgier, Pielecki and Targonski (2005) studied on 5 commercial enzymes (Celluclast®, Viscozyme®, Shearzyme®, Cereflo®, and Ultraflo®) to release feruloylated arabinoxylans and free ferulic acid from brewer's spent grain. They found that Viscozyme released effectively of both ferulolyated oligosaccharides and free ferulic acid because the ferulic acid esterase activity found in this enzyme. Therefore, higher antioxidant activity in this study might be influence by this Viscozyme's composition.

The fractions in this study was gained by gradual ethanol precipitaion that separate the compouns by their molecular weight, followed from Swennen *et al.* (2005). They reported that 60-90 fraction mostly contained oligosaccharides, while >90% fractions were smaller molecule with average DP 2. In this case, the phenollic compound in >90% fractions could be in the free form and 60-90% fractions were mostly bound with oligosaccharides. Therefore 60-90% fractions could be the better source of antioxidant because Rondini and coworker (2004) found that bound phenolic compound had better bioavalibility in rat than the free form.

3. Digestibility.

Digestibility experiment was important in the screening of prebiotic because one of the requirements of prebiotic is resistance to upper gastrointestinal digestion. *In vitro* method with more physiological approaches using human and porcine enzyme preparations was chosen to determine *in vitro* starch digestibility in different cereal products and legumes. This method basically consists of the three steps mimicking digestion in the mouth, stomach and small intestine. (Lebet *et al.*, 1998)

Since this experiment was focused on the degradation of carbohydrate in rice bran extract, uncooked corn starch was used as a reference and inulin was the comparable substrate for commercial prebiotic. The digestibilities of V90 and P90 fractions were not determined because it was assumed that these fractions only contain carbohydrate with low molecular weight, mostly sugar form.

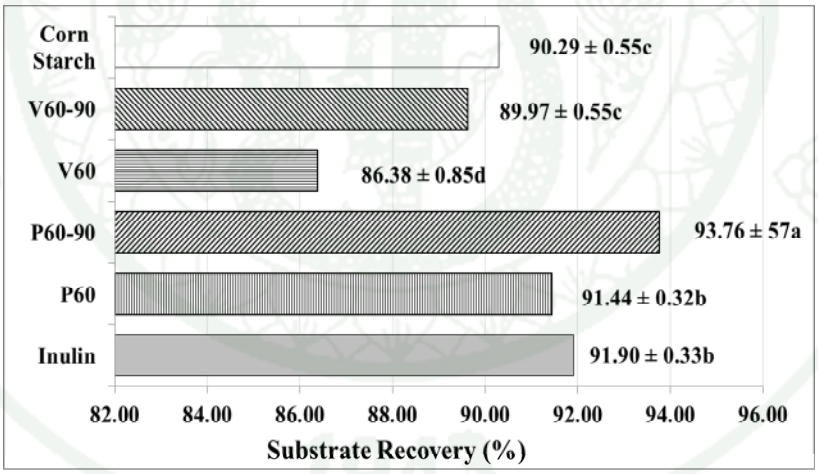


Figure 10 Substrate recovery of *in vitro* digestion of <60% fractions (V60, P60) and 60-90% fractions (V60-90, P60-90) compared with corn starch and inulin. Different letter means significantly different ($p \leq 0.05$).

The lowest digestibility was observed in P60-90 fraction followed by Inulin and P60 fraction, respectively (Figure 10). It indicated that these two fractions had better ability to survive from upper bowel digestion, compared to fractions treated by

Viscozyme. Pentopan has single enzyme activity, endo-β-xylanase, that belongs to GH 11 family. This enzyme could only work on xylan backbone to produce xylan-derivatives. Xylan-derivatives could be only hydrolyzed by xylosidase and arabinofuranosidase (GH 3, 5, 8, 10, 11, 16, 30, 39, 43, 51, 52, 54, and 62). Among all those glycosylhydrolase (GH) family, human body can only produce GH 30 (glucosylceramides) and GH 39 (α-L-iduronidase) in very limited amount (Broekaert *et al.*, 2011). P60-90 fraction may contain xylan-derivatives because Pentopan cleaved the β-(1-4) linkage of xylan backbone (Makaravičius et al, 2011). Therefore, this fraction would be a potential candidate for prebiotic in the next study.

On the other hand, the highest digestibility was found in V60 fraction, showing the ease of attack by enzymatic and/or acid digestion in human gut. Viscozyme that contain mixed enzyme activity might release different hemicelluloses with Pentopan. These compunds might have higher digestibility than inulin since purification of extract was not completely done.

4. Fermentation.

The fermentation experiment was conducted to observe the ability of probiotic such as Bifidobacteria and Lactobacillus to use the oligosaccharides from rice bran extracts. Inulin, a widely used commercial prebiotic, was used as the control to compare the fermentation profile of the extracts. P60-90 and V60-90 fractions were chosen because they had low digestibility and performed potential antioxidant activity. Fractions <60% (V60 and P60) were not chosen due to the high protein contents., The presence of protein can stimulate the growth of pathogenic bacteria and produces amine that induces inflammation in colon Gibson *et al.* (2004).

In this experiment, the prebiotic potential was assessed through monitoring bacterial growth by observing the turbidity development of media, pH change and short chain fatty acid (SCFA) production during incubation. In order to investigate the difference of bacteria growth with different carbohydrate sources, area under growth curve was used for comparison. This technique could compare not only the maximum

bacterial growth in media but also the rate of substrate consumption by bacteria (Mäkeläinen *et al.*, 2010).

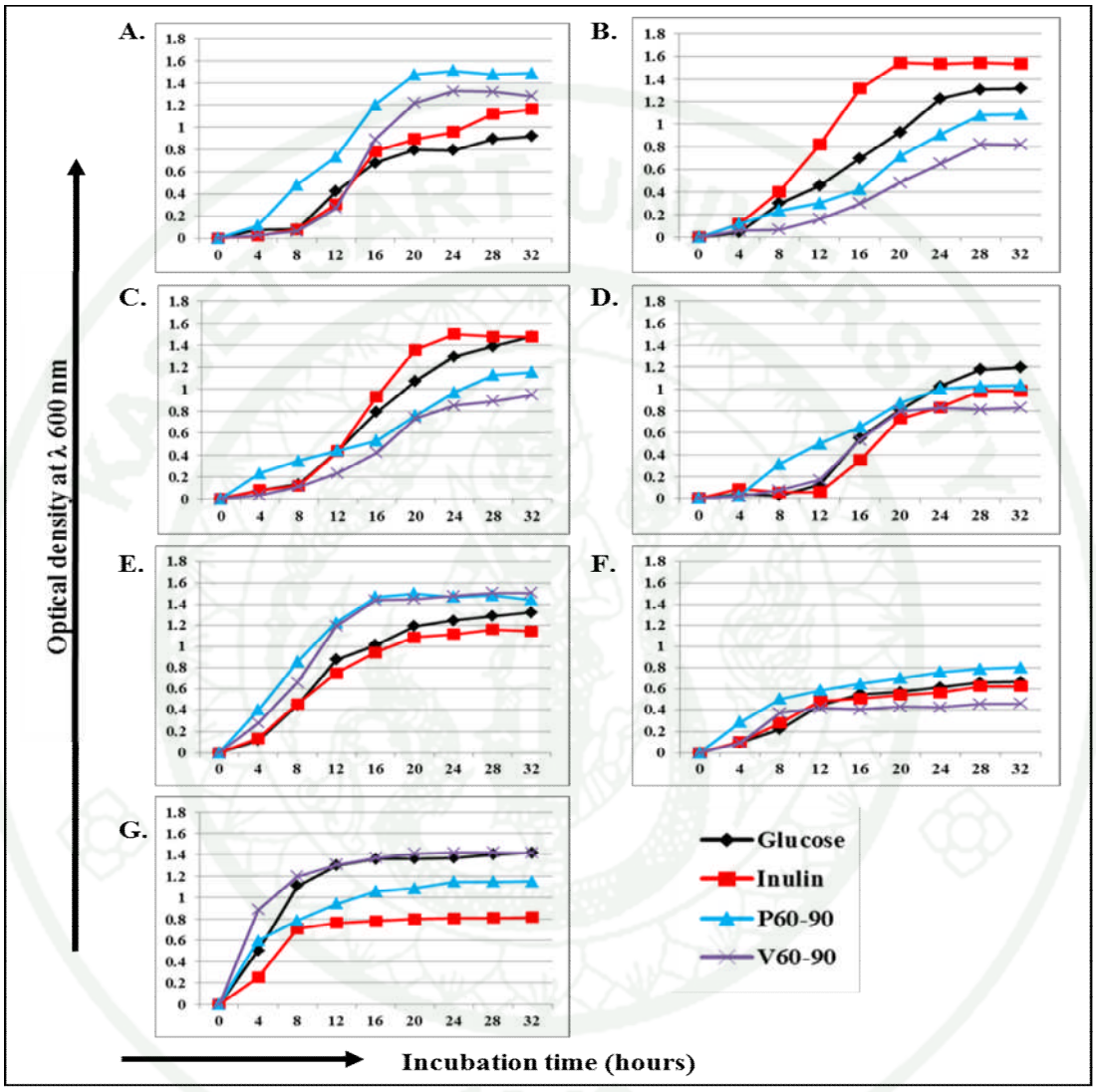


Figure 11 The growth of *Bifidobacterium breve* TISTR 2130 (A), *B. bifidum* TISTR 2129 (B), *B. lactis supp. animalis* TISTR 2195 (C), *B. longum* TISTR 2194 (D), *Lactobacillus acidophilus* ATTC 4356 (E), *L. plantarum* BCC 39798 (F), *Escherichia coli* TISTR 887 (G) under anaerobic condition at 37°C for 32 h, with glucose, inulin, P60-90 or V60-90 as a carbon source.

Each bacteria performed differently on the utilization of the substrates. *Bifidobacterium breve* TISTR 2130 could grow well in media with P60-90 fraction as

carbohydrate source (Figure 11A). This fraction could be fermented immediately by the bacteria as the turbidity of media was raised after 4 h incubation and even better than inulin and glucose. This is interesting because the result was in line with a study of Gullon *et al.* (2008) that found higher growth of *B. breve* feeding with xylooligosaccharides from rice husk compared to glucose. On the contrary, Pastell *et al.* (2009) and Mäkeläinen *et al.* (2010) found the limited growth of *B. breve* when arabinoxylooligosaccharides produced from wheat and corn bran were used as substrates. The low ability of *B. breve* to utilize xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS) was due to the limitation of produced enzyme, such as β-D-xylosidase and α-L-arabinofuranosidase. However, Shin and coworker (2003) found that some strains isolated from human intestine were able to produce both enzymes. These enzymes were important to hydrolyze xylan backbone and cleave the side chain. It seems that *B. breve* might be able to utilize more branched oligosaccharides because rice bran generally contains more branched hemicellulose than wheat and corn brans (Pitkänen *et al.*, 2009).

AXOS extraction from wheat and rye brans with Pentopan assistance mostly released arabinoxylotetraose (XA^3XX) and arabinoxylotriose ($A^{2,3}XX$), but the highest content was XOS (Makaravicius *et al.*, 2011). The mixed oligosaccharides gave beneficial bacteria an alternate mechanism on utilization. Some di-substituted molecules had low fermentability especially by Bifidobacteria, in contrast to mono-substituted molecules and low degree of polymerization (DP 2-5) (Kabel *et al.*, 2002).

The best carbon source for the growth of *Bifidobacterium bifidum* TISTR 2129 and *Bifidobacterium lactis animalis* TISTR 2195 in this study was inulin, (Figure 11B and 11C). However, those bacteria reached the stationary state after 20 h incubation which was faster than those fed with other substrates. The stationary state was reached quickly if there was no more substrate available to be digested. Feeding these two strains with both rice bran fractions showed the lower growth, compared to inulin and glucose. V60-90 was fermented very slowly by both bacteria which meant that these bacteria were lack of enzymes for digesting this substrate.

After 4 h incubation, *Bifidobacterium longum* TISTR 2194 could grow fast in media with P60-90 fraction, compared to other substrates (Figure 11D). However, it achieved stationary state after incubation for 24 h and the final optical density of P60-90 fraction was similar to inulin. Compared to inulin and V60-90, the area under growth curve (AUC) of this Bifidobacteria fed with P60-90 fraction was significantly higher, meaning that P60-90 fraction may contain compounds with different fermentability with both of them. Reviere *et al.* (2013) reported that *B. longum* could hydrolyze AXOS via two mechanisms; first step was the cleavage of arabinose side chain that caused an increase in XOS and then followed by the cleavage of xylan backbone. In addition, they also found that *B. longum* could not hydrolyze XOS with DP higher than 6 (xylohexaose).

Table 5 Area under growth curve of bacteria with different carbohydrate source after 32 h anaerobic incubation at 37 °C.

Bacteria	Area Under Curve (A.h)			
	Glucose	**Inulin**	**V60-90**	**P60-90**
B. breve	16.971 ± 0.081^{c}	19.002 ± 0.079^{c}	23.055 ± 0.459^{b}	31.017 ± 0.243^{a}
B. bifidum	22.453 ± 0.116^{b}	32.166 ± 0.479^{a}	11.794 ± 0.398^{d}	17.295 ± 0.242^{c}
B. animalis	23.783 ± 0.339^{a}	26.604 ± 0.022^{a}	15.046 ± 0.891^{c}	19.977 ± 0.528^{b}
B. longum	17.410 ± 0.161^{a}	14.334 ± 0.111^{b}	14.613 ± 0.492^{b}	19.622 ± 0.559^{a}
L. acidophilus	27.351 ± 0.157^{b}	24.873 ± 0.245^{b}	35.021 ± 0.312^{a}	36.491 ± 1.393^{a}
L. plantarum	13.986 ± 0.106^{b}	13.688 ± 0.416^{b}	11.307 ± 0.698^{b}	18.716 ± 0.919^{a}
E. coli	36.453 ± 0.756^{a}	21.360 ± 0.257^{c}	38.840 ± 1.256^{a}	29.381 ± 0.263^{b}

Note: A.h = Absorbance x hours. Different letter in the same row means significantly different ($p \leq 0.05$).

From both bacterial growth (Figure 11E) and AUC (Table 5), feeding with V60-90 and P60-90 fractions resulted in the highest growth of *Lactobacillus acidophilus* ATTC 4356, compared to the growth of other beneficial bacteria. It seemed that this bacteria can immediately utilize the substrates in these fractions, either because the substrates had small molecules or this bacterium could produce extracellular enzymes for fermenting the substrates. Unfortunately, the rapid growth of this bacteria caused quick stationary state where it started after 16 h incubation, due

to the lack of substrates and accumulation of metabolites. Charalampopoulos and coworkers (2002) reported that the main inhibitors of microbial growth were pH and nutrient limitations in media, but in gut environment SCFA will be absorbed or utilized by other bacteria.

For V60-90, the growth of *B. breve* and *L. acidophilus* might be supported by the presence of glucans which was the product of β-glucanase activity in Viscozyme. Therefore, it was presumed that the oligosaccharides released from the bran were mainly oligoglucans (Gänzle & Follador, 2012). Lactobacilli could utilize glucans very well due to its ability to produce extracellular enzymes and different strains might have alternative pathways for fermentation (Gänzle & Follador, 2012).

The growth of *E. coli* TISTR 887 in media with V60-90 fraction was not significantly different with glucose (Figure 11G and Table 5). The presence of glucans may also contribute to the growth of *E. coli* TISTR 887 in media with V60-90 fraction because Mäkeläinen *et al.* (2010) was also found the same growth of *E. coli* on glucooligosaccharides compare to glucose cultured media.

The results that shown in pure culture experiments are only the preliminary stage of prebiotic selection, which -are more focused on the observation of the candidate's ability to support the growth of probiotic (Roberfroid *et al.*, 2010). The prebiotic index (PI) should be calculated by culturing the prebiotic candidate on mix fecal bacteria *(in vitro*) or feeding to any mammalian (*in vivo*) and compare the growth of total bifidobacteria, bacteroides, lactobacilli, and clostridia (Vulevic *et al.*, 2004; Vardakou *et al.*, 2008). Those methods, could observe a low pathogenic growth compare to probiotic due to the presence of short chain fatty acids as metabolites of substrate fermentation by probiotic (Gibson and Rastall, 2006).

In contrast with *L. acidophilus* ATCC 4356 growth, *Lactobacillus plantarum* BCC 39798 showed the lowest ability to grow in all media under strictly anaerobe condition (Figure 11F). This may be caused by the nature of this bacteria which is

facultative anaerobe (Smetanková, 2012), where this strain requires a small amount of oxygen to support its growth.

Table 5 shows that *L. acidophilus* ATCC 4356, *B. breve* TISTR 2130, *B. longum* TISTR 2194 and *L. plantarum* BCC 39798 utilized P60-90 very well compared to feeding with other substrates. Nevertheless, it had lower ability than inulin on the growth of *B. bifidum* TISTR 2129 and *B. animalis* TISTR 2195. This result was similar to the study of Palframan *et.al.* (2003) who found that *B. breve* was grown better on xylooligoscharides than inulin. P60-90 may has a smaller molecular size than inulin because according to Ryan *et al.* (2005), *B. breve* UCC2003 produced fructofuranosidase intracellular, where a large molecule may take longer time to absorb into bacterial cell before fermentation.

Table 6 The end pH of media and short chain fatty acid (SCFA) production of probiotic with different substrates after 32 h incubation.

Bacteria	Substrate	pH	SCFA (mM)			
			Acetate	Propionate	Butyrate	Total
B. breve **TISTR 2130**	Glucose	4.49 ± 0.05b	32.66 ± 0.95c	19.18 ± 0.53b	6.35 ± 0.18b	58.19 ± 1.66b
	Inulin	4.33 ± 0.03b	27.19 ± 0.83d	20.69 ± 0.57a	7.63 ± 0.21a	55.51 ± 1.62b
	P60-90	4.44 ± 0.05b	50.99 ± 1.47b	21.05 ± 0.57a	4.91 ± 0.13c	76.96 ± 2.18a
	V60-90	4.78 ± 0.03a	57.84 ± 1.64a	15.64 ± 0.43b	4.40 ± 0.12d	77.87 ± 2.19a
B. bifidum **TISTR 2129**	Glucose	5.73 ± 0.04a	37.40 ± 1.05a	13.18 ± 0.36c	3.82 ± 0.10b	54.40 ± 1.52a
	Inulin	4.75 ± 0.07d	36.21 ± 1.06a	11.67 ± 0.32d	4.70 ± 0.12a	52.57 ± 1.51a
	P60-90	5.19 ± 0.02c	28.29 ± 0.88b	15.14 ± 0.41b	3.92 ± 0.10b	47.35 ± 1.40b
	V60-90	5.45 ± 0.00b	30.54 ± 0.96b	17.88 ± 0.50a	4.70 ± 0.13a	53.12 ± 1.59a
B. animalis **TISTR 2195**	Glucose	4.88 ± 0.03b	38.58 ± 1.09a	14.36 ± 0.39b	3.23 ± 0.08c	56.17 ± 1.58a
	Inulin	4.03 ± 0.03c	31.34 ± 0.94b	12.97 ± 0.35c	4.84 ± 0.13a	49.16 ± 1.43b
	P60-90	5.13 ± 0.09a	27.02 ± 0.84c	13.60 ± 0.37c	3.03 ± 0.08c	43.65 ± 1.30c
	V60-90	4.69 ± 0.12b	32.11 ± 0.99b	18.21 ± 0.50a	4.08 ± 0.11b	54.39 ± 1.60a
B. longum **TISTR 2194**	Glucose	5.05 ± 0.07b	59.19 ± 1.63a	21.23 ± 0.60c	4.60 ± 0.15c	85.02 ± 2.38a
	Inulin	5.48 ± 0.03a	46.54 ± 1.39c	23.50 ± 0.65b	3.38 ± 0.16d	73.41 ± 2.21c
	P60-90	4.60 ± 0.14c	55.67 ± 1.70b	25.55 ± 0.70a	5.88 ± 0.16b	87.10 ± 2.57a
	V60-90	4.99 ± 0.01b	49.57 ± 1.50c	22.70 ± 0.63b	6.64 ± 0.18a	78.91 ± 2.31b
L. acidophilus **ATCC 4356**	Glucose	3.84 ± 0.08a	42.28 ± 3.45b	11.76 ± 0.86b	3.40 ± 0.25b	57.45 ± 4.57b
	Inulin	3.98 ± 0.03a	42.20 ± 3.35b	15.35 ± 1.12a	7.22 ± 0.21a	64.77 ± 5.00a
	P60-90	3.63 ± 0.03b	39.38 ± 3.19c	8.11 ± 0.59c	2.29 ± 0.13c	49.77 ± 3.96c
	V60-90	3.59 ± 0.01b	48.27 ± 3.83a	11.50 ± 0.84b	2.45 ± 0.18c	62.23 ± 4.85a
L. plantarum **BCC 39798**	Glucose	5.65 ± 0.07a	21.12 ± 0.84b	17.20 ± 0.60b	5.33 ± 0.18c	43.64 ± 1.62b
	Inulin	5.60 ± 0.14a	18.22 ± 0.67c	20.17 ± 0.70a	9.34 ± 0.32a	47.72 ± 1.70a
	P60-90	5.64 ± 0.09a	23.28 ± 0.90a	19.27 ± 0.67a	5.10 ± 0.17c	47.66 ± 1.74a
	V60-90	5.78 ± 0.03a	20.26 ± 0.79b	19.59 ± 0.68a	6.38 ± 0.22b	46.22 ± 1.69a

Beside turbidity of media, the bacterial growth was also monitored by its metabolites. In this experiment, the pH of media was measured and SCFA production was determined by GC-FID. SCFA production was compared with mixed external standard that contained 10 mM of acetic, propionic, and butyric acid. By using standard curve, the concentration of acetic, propionic, and butyric acid in media was calculated as shown in Table 6.

The growth of bacteria was also monitored from pH changes during the incubation. As shown in Table 6, the lowest end pH was found in media with *L. acidophilus* after 32 h incubation. It was consistent with the bacterial growth, where the highest growth of *L. acidophilus* ATCC 4356 media was observed in most, except feeding with inulin. On the other hand, *L. plantarum* BCC 39798 showed a lowest change in pH media. All culture media was prepared at starting pH about 7 and after incubation for 24 h the pH media were dropped to lower than 6. This was a consequence of carbohydrate degradation by bacteria which produced organic acids as metabolites. Different composition of organic acids would also contribute to the pH of media as each of it had various pKa where lactate>acetate>propionate (Salminen *et al.*, 2004).

Primary metabolites of bacteria in human gut are organic acids (formate, lactate, acetate, propionate and butyrate), alcohol (ethanol), methane (CH_4), gases (CO_2, H_2, H_2S) (Louis *et al.*, 2007). The production of organic acids was influence by the availability of substrates and enzymes. Carbohydrate fermentation by gut microbiota will produce more SCFA, gases, and biomass form, while protein fermentation produces more ammonia, branched chain fatty acids (BCFA), phenol/indoles, amines and sulfides (Scott *et al.*, 2013). Furthermore, different carbohydrate sources were fermented by various pathways, where fructans (eg. FOS, inulin), starch, cellulose and galactomannans were utilized via glycolitic pathway but xylans and pectin would be digested through pentose phosphate pathway (Macfarlane, 2003). The abilities of certain bacteria in producing enzymes related to each pathway contributes to their growth and acid production (Louis *et al.*, 2007; Gänzle & Follador, 2012).

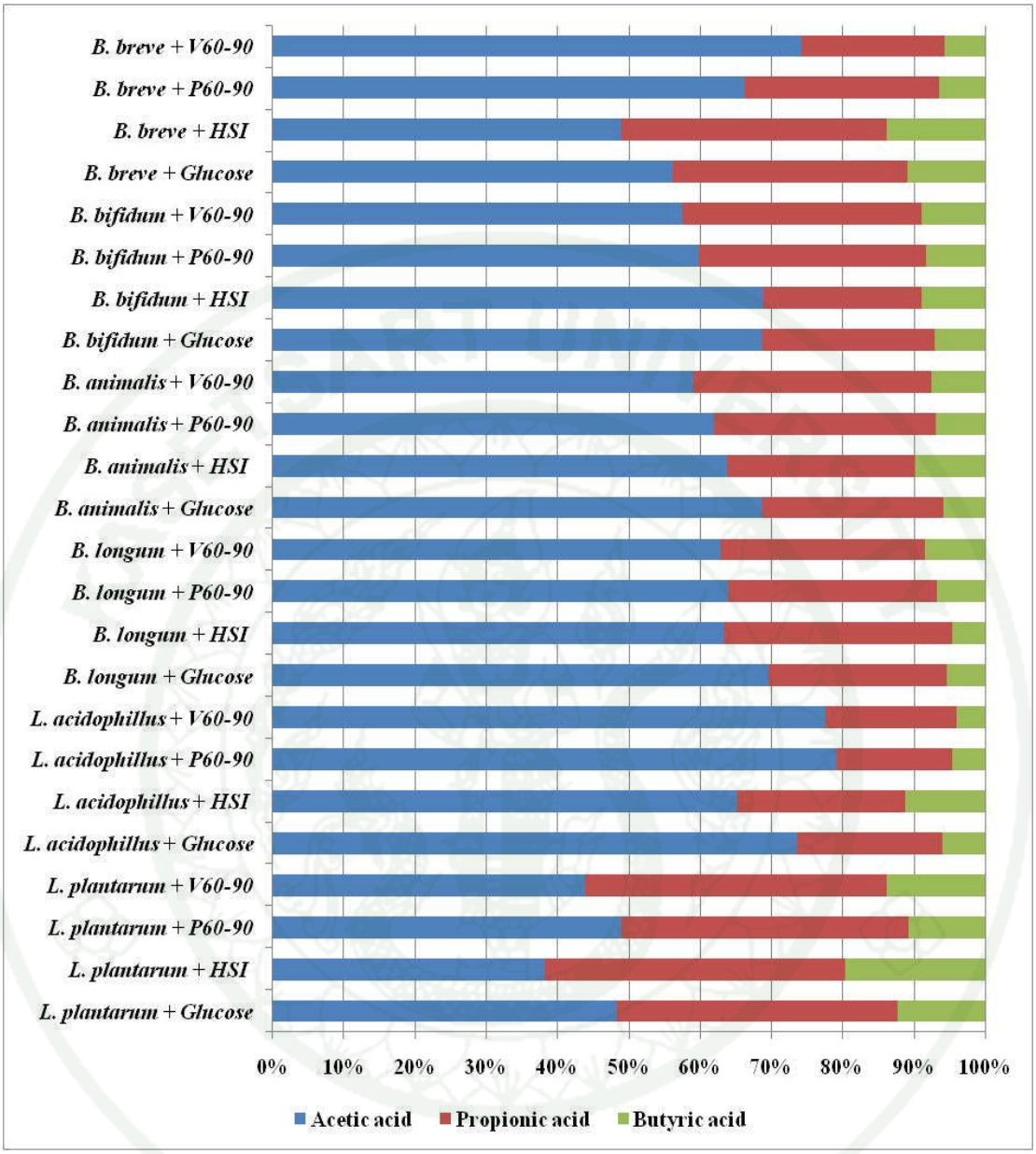


Figure 12 Short chain fatty acid proportion of probiotic with different substrates after 32 h incubation.

The highest concentration of SCFA was found in *B. longum* TISTR 2194 culture, reaching more than 80 mM (Table 6) and having higher proportion of acetate (Figure 12). According to Pokusaeva and Fitzgerald (2011), *B. longum subsp. longum* produced arabinofuranhydrolases during its growth on arabinoxylan-oligosaccharides (AXOS). Agreed with Fernando and coworker (2010), who studied on rice fiber,

acetate was also found that the most favored acid produced by Bifidobacteria spp. It was speculated that this bacteria utilized AXOS in P60-90 and V60-90 fractions, but *B. longum* and other bifidobacteria might have different preferences on utilizing carbohydrate sources, which also observed by Rycoft *et al.* (2001) and Pastell *et al.*, (2009).

Meanwhile, the same study by Rycoft *et al.*, (2001) also observed that Lactobacillus spp produces relatively equal amount of acetate and lactate. Therefore, even though *L. acidophilus* ATCC 4356 performed the highest growth in all media and generated the lowest pH in this study, but the SCFA concentration was not as much as those produced by Bifidobacteria. It could be due to the ability of Lactobacilli to produce more lactate than acetate, however, lactate was not determined in the present study.

On the other side, even *L. plantarum* BCC 39798 had the lowest growth, it produced propionate almost the same amount as acetate, especially on media supplemented with P60-90 and V60-90 fractions. Bifidobacteria that also produced high portion of propionate was *B. bifidum* TISTR 2129 and *B. breve* TISTR 2130 with Inulin and P60-90 fraction as the substrates (Figure 12). Lower ratio of acetate to propionate was contributed to hipolipidemic because propionic acid had an ability to inhibit the gluconeogenesis and hepatic cholesterogenesis, while acetic acid was transferred into acetyl-CoA in the liver and used as the precursor for lipogenesis that stimulated the gluconeogenesis (Salazar *et al.*, 2008; Remesy *et al.*, 1992).

The current *in vitro* study using pure culture bacteria was the first step of testing prebiotic potential of a candidate substrate. The experiment using mixed fecal bacteria, in situ or in vivo might provide different results. By those methods, the competition of each bacteria on utilizing the substrates and the presence of other bacteria (eg. *Eubacterium halii*, *Anerostipes caccae*) that consume lactate and acetate could change the concentration of SCFA in fecal (Scott *et al.*, 2013).

5. Characterization.

Arabinose, xylose, glucose, xylobiose, xylotriose and xylotetraose were used as standards for composition determination. The elution order of mixed standards was investigated in order to compare with the samples. According to the study of Xu and coworker (2013), PA200 column could not separate xylose and glucose, so they were eluted as one peak at the same elution time.

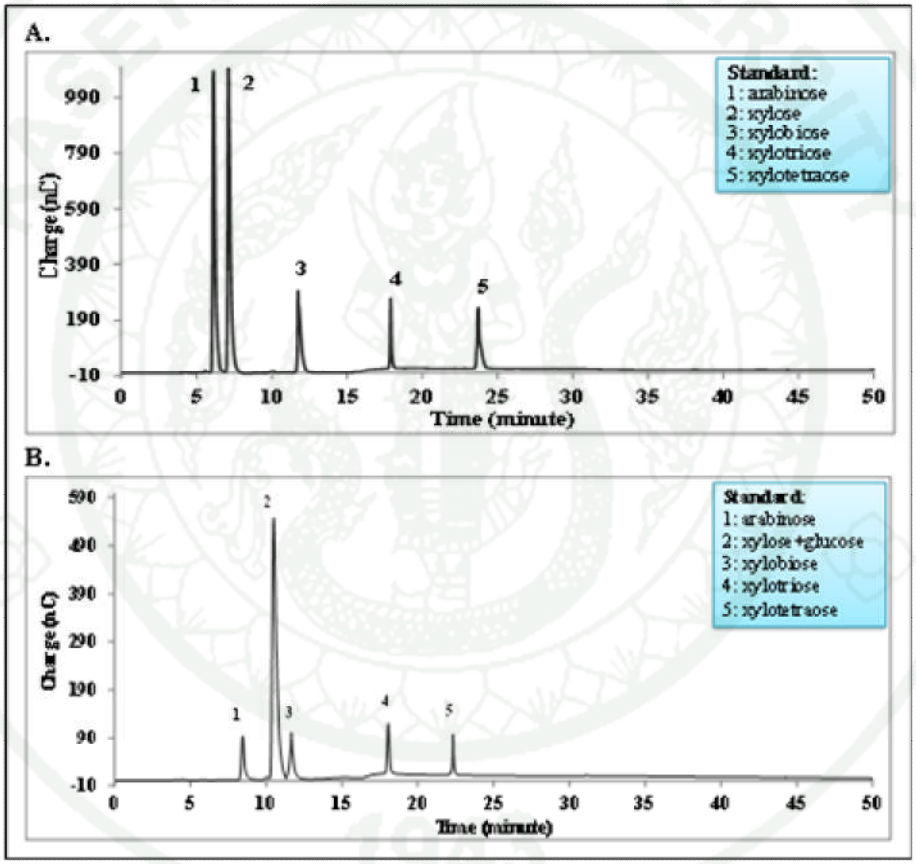


Figure 13 HPAEC-PAD chromatograms of mixed standards of arabinose, xylose, glucose, xylobiose, xylotriose, and xylotetraose. A) arabinose (50 µg/mL), xylose (50 µg/mL), xylobiose (20 µg/mL), xylotriose (20 µg/mL), and xylotetraose (20 µg/mL). B) arabinose (10 µg/mL), xylose (10 µg/mL), glucose (25 µg/mL), xylobiose (10 µg/mL), xylotriose (10 µg/mL), and xylotetraose (10 µg/mL).

Interestingly, it was found in this study that the presence of glucose caused the shift of retention time (RT) of arabinose from 6.1 min to 8.4 min and also xylose from 7.1 min to 10.4 min (Figure 13). Some weak hydrogen bonds among compounds and eluent and interaction between those molecules with the column containing amine as the active functional group may affect the elution time of these monosaccharides (Pastell *et al.*, 2009). Thus, the presence of –OH groups available for dissociation and interactions with the column material is very important in HPAEC analysis.

Meanwhile, the retention time of xylobiose, xylotriose and xylotetraose were not affected by the presence of glucose. The retention time and pKa values of carbohydrates were inversely correlated and the RT will increase with higher molecular mass, in case of linear XOS, the RT differs according to the homologous oligosaccharide series (Rivière *et al.*, 2013).

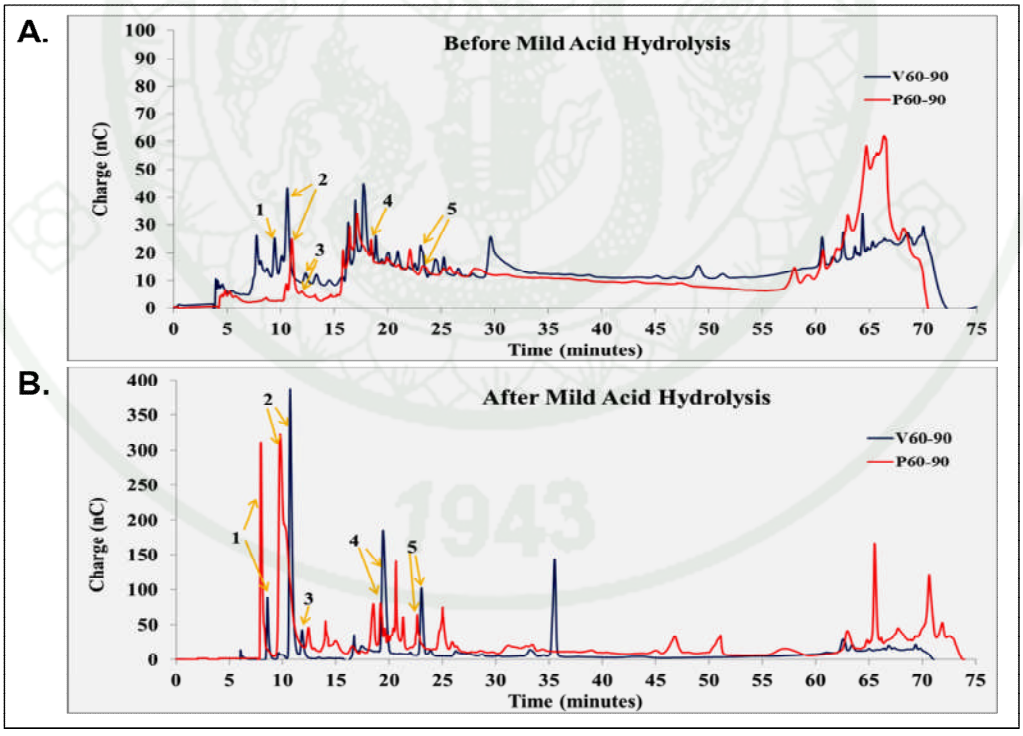


Figure 14 HPAEC-PAD chromatograms of fractions before and after mild acid hydrolysis. (1) Arabinose; (2) Xylose/Glucose; (3) Xylobiose; (4) Xylotriose; (5) Xylotetraose.

Figure 14 shows the chromatogram of 60-90% fractions, which were choosen to be characterized due to their potency as the source of antioxidant and prebiotic. Before hydrolysis with mild acid, both V60-90 and P60-90 fractions showed a low intensity of monosaccharides or xylobiose, xylotriose, and xylotetraose, less than 50 nC (Figure 13A), meaning that there were only low amounts of those compounds in the fractions. The mono- and di-saccharides were found because the fractionation was conducted by gradually ethanol precipitation without purification step. According to Swennen *et al.* (2005), a small amount of the low molecular weight compounds, including monosaccharides were still found in 60-90% fraction.

V60-90 fraction showed higher intensity of small molecules such as arabinose, xylose/glucose, xylobiose, xylotriose, and xylotetraose than P60-90 fraction. This might be caused by more various enzyme activities in Viscozyme that could produce smaller molecules. In addition, P60-90 fraction contained higher amounts of large molecules that were eluted later than 60 min (Figure 14A). Previous studies on XOS and AXOS (Pastell *et al.*, 2009; Rivière *et al.*, 2013; Rantanen *et al.*, 2007) reported that the peaks of XOS with DP>6 or AXOS with DP>4 were eluted after 30 minutes when there was a high concentration of sodium acetate in eluent.

Oligosaccharidecomponents in 60-90% fractions were investigated by comparing the retention time of peaks that appeared on the chromatogram. Mild acid hydrolysis (0.01 M HCl), adapted from Swennen and coworker (2005), was conducted to hydrolyze arabinose side chain from xylan backbone, including AXOS. Figure 14B shows that mild acid hydrolysis of V60-90 fraction resulted in high amount of monosaccharides. This agreed with the digestibility experiment that showed low resistance of this extract on acid hydrolysis (pH 2). Arabinose, xylobiose, xylotriose and xylotetraose peaks were observed at RT 8.5, 11.1, 16.6 and 23.1 min, respectively (Figure 14B), confirmed that this fraction contained AXOS. Therefore, Viscozyme has ability to release hemicellulose such as AXOS from rice bran. Nevertheless, the peak at RT 10.7 min, showing the mixture of xylose and glucose, was very intense. When it was compared with the standards, this peak seemed to have

more glucose molecules than xylose, since it was delayed quite long from the RT of xylose (7.1 min) (Johnson *et al.*, 1993; Xu *et al.*, 2013).

After mild acid hydrolysis, P60-90 fraction showed high intensity peaks of both arabinose (RT 7.9 min) and xylose (RT 9.7 min) (Figure 13B). Compared to V60-90 fraction, P60-90 fraction may contain more AXOS with arabinose side chain because the arabinose peak of P60-90 fraction was greater than that of V60-90 fraction. The more branched AXOS is beneficial to the growth of bifidobacteria and lactobacillus because arabinose can promote their growth.

Referred to the study of Swennen and coworker (2005), the mild acid hydrolysis of wheat bran-derived AXOS resulted in the release of about 90% of the arabinofyranosyl units and 8% of the xylopyranosyl units. They used the mild acid to cleave only the side chain of AXOS and retain most of the xylan backbone. The presence of peak 1 on the chromatogram (Figure 13B) might be coming from molecules with low polymerization but highly substituted.

Although the results of V60-90 and P60-90 fractions were comparable, the exact amount of each AXOS was difficult to quantify due to the limitation of AXOS standards and incomplete side chain hydrolysis. The hydrolysis of side chain may not be completed, even specific enzymatic hydrolysis by arabinofuranosidase, due to some steric hindrance or the presence of ferulic acid (Rivière *et al.*, 2013). They also observed unidentified peaks after glycosylhydrolases (GH 51 and GH 43) treatment. This study was also found many unidentified peaks on either V60-90 or P60-90 hydrolysates. Furthermore, mild acid hydrolysis could also cleave some xylan backbone as observed in high intensity of xylose (peak 2).

According to the study of Makaravičius *et al.* (2011), Pentopan produced more arabinoxylotetraose (XA^3XX) than any other xylanase enzymes. However, Figure 14B shows that P60-90 fraction contained lower amount of xylotriose and xylotetraose than V60-90 fraction, which means Viscozyme could produce lower DP

of AXOS, while Pentopan produced oligosaccharides from xylan backbone with higher xylofuranosyl chain.

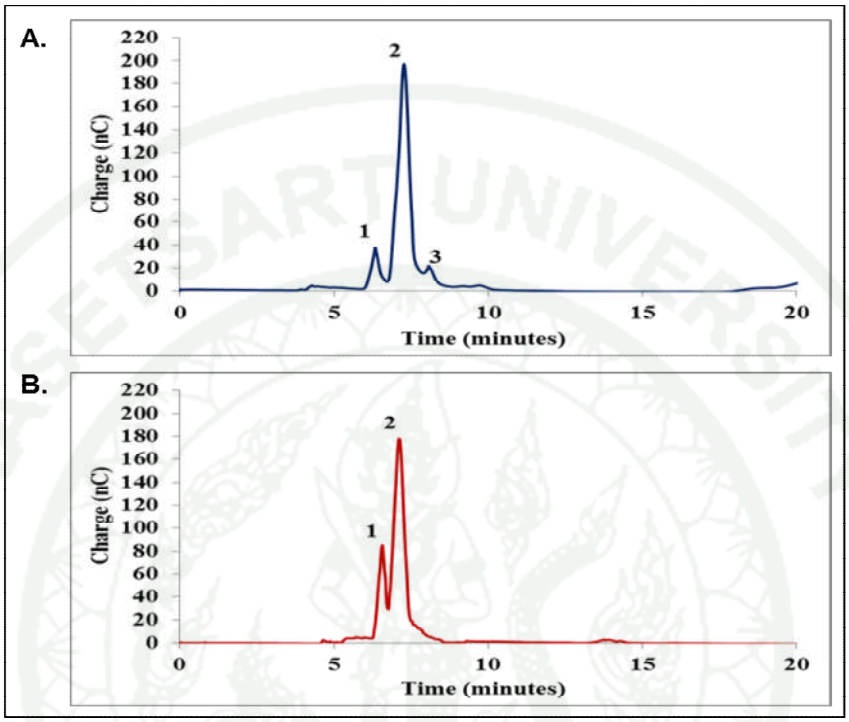


Figure 15 HPAEC-PAD chromatograms of monosaccharides of V60-90 fraction (A) and P60-90 fraction (B); (1) Arabinose and (2) Xylose (3) Glucose.

In order to calculate the degree of substitution (DS) of AXOS from V60-90 and P60-90 fractions, complete acid hydrolysis (H_2SO_4 1 M) was performed to produce monosaccharides. Degree of substitution was calculated using the ratio of peaks area of chromatogram. Figure 15 shows that P60-90 fraction had higher ratio of arabinose to xylose (0.275 ± 0.010) than did V60-90 fraction, (0.185 ± 0.016), indicating that Pentopan produced more branched AXOS than Viscozyme.

Figure 15 shows that in V60-90 contained a small amount of glucose (peak 3) that might be contribute to the shifting of xylose peak on Figure 14. The presence of glucose or glucans was agreed with the high growth of *E. coli* in fermentation experiment.

CONCLUSION AND RECOMMENDATION

Conclusion

Commercially defatted rice bran, a by-product from rice bran oil production, was studied for its potential as a source of antioxidant and prebiotic. Enzymatic extraction using Viscozyme (endo-β-glucanase) and Pentopan (endo-β-xylanase), followed by gradual ethanol precipitation (60 and 90%) could prepare the extracts with antioxidant property due to their phenolic contents. The >90% fractions contained the highest total phenolic content; hence their reducing capacity and antioxidant activity were also highest when compared to other fractions. From this study, Viscozyme seemed capable of extracting more phenolic compounds from commercially defatted rice bran than did Pentopan. Regarding to prebiotic potential of the extracts, Pentopan assistance showed the promising results, where 60-90% fraction had the strongest ability to survive from digestion and to be utilized by probiotic bacteria, especially *L. acidophilus*, *B. longum*, and *B. breve*. By analysis with HPAEC-PAD, it was found that Viscozyme treatment generated the extract (60-90% fraction) containing smaller molecules and oligosaccharides with less arabinose branch chain compared to the Pentopan treatment. In general, Pentopan seemed more potential on extracting compounds with antioxidant and prebiotic properties.

Recommendation

The further study on purification of extract is necessary to be able to investigate the compounds and their degradation process by probiotic bacteria. Combination of Viscozyme and Pentopan should be able to increase the yield of extracts and the amounts of functional compounds. *In vivo* study or mixed fecal culture bacteria should be chosen to receive more sophisticated information about prebiotic potential of these extracts.

LITERATURE CITED

Aachary, A.A. and S.G. Prapulla. 2011. Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications. **Compr. Rev. Food Sci. Food Safety**. 10: 2-16.

Adom, K.K. and R.H. Liu. 2002. Antioxidant activity of grains. **J. Agric. Food Chem.** 50(21): 6182–6187.

Badarinath, A.V., K. M. Rao, C.M.S. Chetty, S. Ramkanth, T.V.S. Rajan and K. Gnanaprakash. 2010. A review on in-vitro antioxidant methods: comparisons, correlations and considerations. **Int. J. Pharm. Tech. Res**. 2(2): 1276-1285.

Bandyopadhyay, K., C. Chakraborty and A.K. Barman. 2012. Effect of microwave and enzymatic treatment on the recovery of protein from Indian defatted rice bran meal. **J. Oleo Sci**. 61(10): 525-529.

Bandyopadhyay, K., C. Chakraborty and A.K. Barman. 2012. Effect of microwave and enzymatic treatment on the recovery of protein from Indian defatted rice bran meal. **J. Oleo Sci**. 61(10): 525-529.

Bang, M.H., T.V. Riep, N.T. Thinh, L.H. Song, T.T. Dung, L.V. Truong, L.V. Don, T.D. Ky1, D. Pan, M. Shaheen and M. Ghoneum. 2010. Arabinoxylan rice bran (MGN-3) enhances the effects of interventional therapies for the treatment of hepatocellular carcinoma: a three-year randomized clinical trial. **Anticancer Research**. 30(12): 5145-5151.

Barrangou, R., M.A. Azcarate-Peril, T. Duong, S.B. Conners, R.M. Kelly and T.R. Klaenhammer. 2006. Global analysis of carbohydrate utilization by Lactobacillus acidophilus using cDNA microarrays. **Proc. Natl. Acad. Sci.** 103: 3816-3821.

Berker, K.I., K. Güçlü, İ. Tor and R. Apak. 2007. Comparative evaluation of Fe (III) reducing power-based antioxidant capacity assays in the presence of phenanthroline, batho-phenanthroline, tripyridyltriazine (FRAP), and ferricyanide reagents. **Talanta**. 72: 1157–1165.

Blaut, M. 2002. Relationship of prebiotics and food to intestinal microflora. **Eur. J. Nutr.** 1(1): I11–I16.

Broekaert, W.F, C.M. Courtin, K. Verbeke, T.V. De Wiele, W. Verstraete and J.A. Delcour. 2011. Prebiotic and other health-related effects of cereal-derived. **Crit. Rev. Food Sci. Nutr**. 51:178–194.

Burton, R.A. and G.B. Fincher. 2012. Current challenges in cell wall biology in the cereals and grasses. **Front. Plant Sci**. 3(130): 1-6.

Charalampopoulos, D., S.S. Pandiella and C. Webb. 2002. Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. **J. Appl. Microbiol**. 92: 851–859.

Chiou, T. Y., A. Ogino, T. Kobayashi and S. Adachi. 2013. Characteristics and antioxidative ability of defatted rice bran extracts obtained using several extractants under subcritical conditions. **J. Oleo Sci**. 62: 1−8.

Cholujova, D. , J. Jakubikova, B. Czako, M. Martisova, L. Hunakova, J. Duraj, M. Mistrik and J. Sedlak. 2013. MGN-3 arabinoxylan rice bran modulates innate immunity in multiple myeloma patients. **Immunotherapy**. 62(3): 437-445.

Chotimarkorn, C., S. Benjakul and N. Silalai. 2008. Antioxidant components and properties of five long-grained rice bran extracts from commercial available cultivars in Thailand. **Food Chem**. 111: 636–641.

Dai, J. and R.J. Mumper. 2010. Review: plant phenolics: extraction, analysis and their antioxidant and anticancer properties. **Molecules**. 15: 7313-7352.

Dalle-Donne, I., R. Rossi, R. Colombo, D. Giustarini and A. Milzani. 2006. Biomarkers of oxidative damage in human disease. **Clin. Chem**. 52:601–623.

Daou, C. and H. Zang. 2011. Physico-chemical properties and antioxidant activities of dietary fiber derived from defatted rice bran. **Adv. J. Food. Sci. Technol**. 3(5): 339-347.

Dass, NB., AK. John, AK. Bassil, C.W. Crumbley, W.R. Shehee, F.P. Maurio, G.B.T. Moore, C.M. Taylor and G.J. Sanger. 2007. The relationship between the effects of short-chain fatty acids on intestinal motility *in vitro* and GPR43 receptor activation. **Neurogastroenterol Motil** 19: 66–74.

Elleuch, M., D. Bedigian, O. Roiseux, S. Besbes, C. Blecker and H. Attia. 2011. Dietary fibre and fibre-rich by-product of food processing: characterization, technological, functionality, and commercial application. **Food Chem**. 124: 411-421.

FAOSTAT. 2012. **Food and Agriculture Organization**. Available source: http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/E, 11 November, 2012.

Fardet, A., E.Rock and C. Rémésy. 2008. Review: Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? **J. Cereal Sci**. 48: 258-276.

Faria S.A.D.C., P.Z. Bassinello and M.dV.C. Penteado. 2012. Nutritional composition of rice bran submitted to different stabilization procedures. **Brazilian J. Pharm. Sci**. 48(4): 651-657.

Fernando, W.M. A. D. B., C.S. Brennan, S. Flint, K.K. D. S. Ranaweera, A. Bamunuarachchi and H.R. Morton. 2010. Enhancement of short chain fatty acid formation by pure cultures of probiotics on rice fibre. **Intl. J. Food Sci. Tech**. 45: 690–696.

Friedman, M. 2013. Rice brans, rice bran oils, and rice hulls: composition, food and industrial uses, and bioactivities in humans, animals, and cells. **J. Agric. Food Chem**. 61: 10626−10641.

Gänzle, M. G. and R. Follador. 2012. Metabolism of oligosaccharides and starch in lactobacilli: a review. **Front. Microbiol**. 3(340): 1-15.

Gerhardt, A. L. and N.B. Gallo. 1998. Full-fat rice bran and oat bran similarly reduce hypercholesterolemia in humans. **J. Nutr**. 128: 865−869.

Ghoneum, M., N.K. Badr El-Din, S. M. Abdel Fattah and L. Tolentino. 2013. Arabinoxylan rice bran (MGN-3/Biobran) provides protection against holebody γ-irradiation in mice via restoration of hematopoietic tissues. **J. Radiat. Res**. 54: 419−429.

Ghoneum, M. and S. Agrawal. 2011. Activation of human monocytederived dendritic cells *in vitro* by the biological response modifier arabinoxylan rice bran (MGN-3/Biobran). **Int. J. Immunopathol. Pharmacol**. 24: 941−948.

Gibson, G.R., H.M. Probert, J. Van Loo, R.A. Rastall and M.B. Roberfroid. 2004. Dietary modulation of human colonic microbiota: Updating the concept of prebiotic. **Nutr. Res. Rev.** 17: 259-275.

Gibson, G.R. and R.A. Rastall. 2006. **Prebiotics: Development and Application**. John Wiley & Sons Ltd., England.

Gray, J. 2006. **Dietary Fibre: Definition, Analysis, Physiology and Health**. International Life Sciences Institute Europe Concise Monograph Series. Brussels, Belgium.

Gullón, B., P. Gullón, F. Tavaria, M. Pintado, A.M. Gomes, J.L. Alonso and J.C. Parajó. 2014. Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. **J. Funct. Foods**. 6: 438-449.

Guo, W. and T. Beta, T. 2012. Phenolic acid composition and antioxidant potential of insoluble and soluble dietary fibre extracts derived from select whole-grain cereals. **Food Res. Int**. 51: 518–525.

Harmsen, H.J., P. Elfferich and P. Schut. 1999. A 16S rRNAtargetedprobe for detection of lactobacilli and enterococci in fecal samples by fluorescent in situ hybridization. **Microb. Ecol. Health Dis**. 11: 3–12.

Hernández-Salas, J.M., M.S. Villa-Ramírez, J.S. Veloz-Rendón, K.N. Rivera-Hernández, R.A. González-César, M.A. Plascencia-Espinosa and S.R. Trejo-Estrada. 2009. Comparative hydrolysis and fermentation of sugarcane and agave bagasse. **Bioresource Technol**. 100(3): 1238–1245.

Hollebeeck, S., F. Borlon, Y.J. Schneider, Y. Larondelle and H. Rogez. 2013. Development of a standardized human in vitro digestion protocol based on macronutrient digestion using response surface methodology. **Food Chem**. 138: 1936–1944.

Hu, G. and W. Yu. 2013. Binding of cholesterol and bile acid to hemicelluloses from rice bran. **Int. J. Food Sci. Nutr**. 64: 461−466.

Janicke, B., G. Onning and S.M. Oredsson. 2005. Differential effects of ferulic acid and p-coumaric acid on s-phase distribution and length of s-phase in the human colonic cell line caco-2. **J. Agric. Food Chem**. 53: 6658–6665.

Jiménez, A., A. Selga, J.L. Torres and L. Julià. 2004. Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols. **Org. Lett.** 6: 4583-4586.

Johnson, D.C., D. Dobberpuhl, R. Roberts and P. Vandeberg. 1993. Pulsed amperometric detection of carbohydrates, amines and sulfur species in ion chromatography. The current state of research. **J. Chromatogr**. 640: 79-96.

Kabel, M.A., L. Kortenoeven, H.A. Schols and A.G.J. Voragen. 2002. In vitro fermentability of differently substituted xylooligosaccharides. **J. Agric. Food Chem**. 50:6205–6210.

Kumar, A., A. Handerson, G.M. Forester, A.W. Goodyear, T.L. Weir, J.E. Leach, S.W. Dow and E.P. Ryan. 2012. Dietary rice bran promotes resistance to Salmonella enteritica serovar Typhimurium colonization in mice. **BMC Microbiology**. 12(71): 1-9.

Lafond, M., D. Navarro, M. Haon, M. Couturier and JG. Berrin. 2012. Characterization of a broad-specificity β-glucanase acting on β-(1,3)-, β-(1,4)-, and β-(1,6)-glucans that defines a new glycoside hydrolase family. **Appl. Environ. Microbiol**. 78(24): 8540.

Laokuldilok, T., C. F. Shoemaker, S. Jongkaewwattana and V. Tulyathan. 2011. Antioxidants and antioxidant activity of several pigmented rice brans. **J. Agric. Food Chem**. 59: 193−199.

Lebet, V., E. Arrigoni and R. Amado. 1998. Digestion procedure using mammalian Enzymes to obtain substrates for in vitro fermentation studies. **Lebensm.-Wiss. u.-Technol**. 31: 509-515.

Levy, I., Z. Shani and O. Shoseyov. 2002. Modification of polysaccharides and plant cell wall by endo-1,4-β-glucanase and cellulose-binding domains. **Biomol. Eng**. 19: 17-30.

Lichtenthaler, H.K. and J. Schweiger. 1998. Cell wall bound ferulic acid, the major substance of the blue-green fluorescence emission of plants. **J. Plant Physiol**. 152(2): 272–282.

Liu, R.H. 2007. Whole grain phytochemicals and health. **J. Cereal Sci**. 46: 207–219

Louis, P., K.P. Scott, S.H. Duncan and H.J. Flint. 2007. Understanding the effects of diet on bacterial metabolism in the large intestine. **J. Appl. Microbiol**. 102: 1197–1208.

Macfarlane, S. and G.T. Macfarlane. 2003. Regulation of short-chain fatty acid production. **P. Nutr. Soc**. 62:67-72.

Makaravičius T., L. Bašinskiene, G. Juodeikiene and M. Tenkanen. 2011. Preparation of oligosaccharides from wheat and rye arabinoxylans using different xylanase preparations. **Maisto Chemija ir Technologija**. 45(1): 23-30.

Mäkeläinen, H., M. Saarinen, J. Stowell, N. Rautonen and A.C. Ouwehand. 2010. Xylo-oligosaccharides and lactitol promote the growth of Bifidobacterium lactis and Lactobacillus species in pure cultures. **Beneficial Microbes**. 1(2): 139-148.

Manzocco L., S. Calligaris, D. Mastrocola, M.C. Nicoli and C.R. Lerici. 2000. Review of non-enzymatic browning and antioxidant capacity in processed foods. **Trends Food Sci. Tech**. 119(9–10): 340–346.

Martins, S.I.F.S., W.M.F. Jongen and M.A.J.S. van Boekel. 2001. A review of Maillard reaction in food and implications to kinetic modelling. **Trends Food Sci. Tech**. 11: 364–373.

Mitamura, R. and H. Hara. 2005. Prolonged feeding of difructose anhydride III increases strength and mineral concentrations of the femur in ovariectomized rats. **Brit. J. Nutr**. 94: 268–274.

Mod, R.R., E.J. Conkerton, R.L. Ory and F.L. Normand. 1978. Hemicellulose Composition of Dietary Fiber of Milled Rice and Rice Bran. **J. Agric. Food Chem**. 26 (5): 1031–1035.

Moure, A., P. Gullón, H. Domínguez and J. C. Parajó. 2006. Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. **Process Biochem**. 41: 1913–1923.

Mussatto, S. I. and I.M. Mancilha. 2007. Non-digestible oligosaccharides: a review. **Carbohyd. Polym**. 68: 587–597.

Niki, E. 2010. Review article. Assessment of antioxidant capacity in vitro and in vivo. **Free Radical Bio. Med**. 49: 503-515.

Okarter, N. and R.H. Liu. 2010. Health benefits of whole grain phytochemicals. **Crit. Rev. Food Sci. and Nutr**. 50(3): 193-208.

Palframan, R.J., G.R. Gibson and R.A. Rastall. Carbohydrate preferences of bifidobacterium species isolated from the human gut. **Curr. Issues Intest. Microbiol**. 4: 71-75.

Pastell, H., P. Tuomainen, L. Virkki and M. Tenkanen. 2008. Step-wise enzymatic preparation and structural characterization of singly and doubly substituted arabinoxylo-oligosaccharides with non-reducing end terminal branches. **Carbohyd. Res**. 343: 3049-3057

Pastell, H., P. Westermann, A.S. Meyer, P. Tuomainen and M. Tenkanen. 2009. In vitro fermentation of arabinoxylan-derived carbohydrates by bifidobacteria and mixed fecal microbiota. **J. Agric. Food Chem**. 57: 8598- 8606.

Patel, S. and A. Goyal. 2012. The current trends and future perspectives of prebiotics research: a review. **3 Biotech**. 2: 115-125.

Pitkänen, L., L. Virkki, M. Tenkanen and Tuomainen, P. 2009. Comprehensive multidetector HPSEC study on solution properties of cereal arabinoxylans in aqueous and DMSO solutions. **Biomacromolecules** 10: 1962-1969.

Pokusaeva, K., G. F. Fitzgerald and D. van Sinderen. 2011. Review. Carbohydrate metabolism in Bifidobacteria. **Genes Nutr**. 6: 285–306.

Prior, R.L., X. Wu and K. Schaich. 2005. standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. **J. Agric. Food Chem**. 53: 4290-4302.

Qureshi, A. A., S. A. Sami, W. A. Salser and F.A. Khan. 2002. Dose dependent suppression of serum cholesterol by tocotrienol-rich fraction (TRF25) of rice bran in hypercholesterolemic humans. **Atherosclerosis**. 161: 199−207.

Rantanen, H., L. Virkki, P. Tuomainen, M. Kabel, H. Schols and M. Tenkanen Preparation of arabinoxylobiose from rye xylan using family 10 *Aspergillus aculeatus* endo-1,4-β-D-xylanase. **Carbohyd. Polym**. 68: 350–359.

Raschka, L. and H. Daniel. 2005. Mechanisms underlying the effects of inulin-type fructans on calcium absorption in the large intestine of rats. **BONE**. 37: 728–735.

Rastall, R.A. and GR. Gibson. 2004. Functional Foods. Developments in colonic functional foods for improved digestive health. **Bioscience-explained.** 2(1): 1-7.

Rautiola, E. 2013. **Short chain fatty acid production by probiotic organism in the gastrointestinal tract**. Senior Honors Theses. Eastern Michigan University.

Remesy, C., S.R. Behr, M-A. Levrat and C. Demigne. 1992. Fiber fermentability in the rat cecum and its physiological consequences. **Nutr. Res**. 12: 1235–1244.

Rivière, A., S. Eeltink, C. Pierlot, T. Balzarini, F. Moens, M. Selak and L. De Vuyst. 2013. Development of an ion-exchange chromatography method for monitoring the degradation of prebiotic arabinoxylan-oligosaccharides in a complex fermentation medium. **Anal. Chem**. 85: 4982−4990.

Robards, K., P.D. Prenzler, G. Tucker, and P. Swatsitang and W. Glover. 1999. Phenolic compounds and their role in oxidative processes in fruits. **Food Chem**. 66: 401-436.

Roberfroid, M., G.R. Gibson, L. Hoyles, A. L. McCartney, R. Rastall, I. Rowland, D. Wolvers, B. Watzl, H. Szajewska, B. Stahl, F. Guarner, F. Respondek, K. Whelan, V. Coxam, MJ. Davicco, L. Léotoing, Y. Wittrant, N.M. Delzenne, P.D. Cani, A.M. Neyrinck and A. Meheust. 2010. Prebiotic effects: metabolic and health benefits. **Brit. J. Nutr.** 104: S1-S63.

Rondini, L., M.N.P Maillard, A.M. Baglieri, G. Fromentin, P. Durand, D. Tomé, M. Prost and C. Berset. 2004. Bound ferulic acid from bran is more bioavailable than the free compound in rat. **J. Agric. Food Chem**. 52(13): 4338–4343.

Rosset, M., V.R Acquaro Junior and A.D.P. Beléia. 2012. Protein Extraction from Defatted Soybean Flour with Viscozyme L Pretreatment. **J. Food Process. Pres.** 38(3): 784–790.

Ryan, S.M., G.F. Fitzgerald and D. van Sinderen. 2005. Transcriptional regulation and characterization of a novel beta-fructofuranosidase-encoding gene from *Bifidobacterium breve* UCC2003. **Appl. Environ. Microbiol.** 71: 3475–3482.

Rycroft, C.E., M.R. Jones, G.R. Gibson and R.A. Rastall. 2001. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. **J. Appl. Microbiol**. 91: 878-887.

Salazar, N., M.Gueimonde, A. M.Hernández-Barranco, P. Ruas-Madiedo and C.G. de los Reyes-Gavilán. 2008. Exopolysaccharides produced by intestinal Bifidobacterium strains act as fermentable substrates for human intestinal bacteria. **Appl. Envir. Microbiol**. 74: 4737–4745.

Salminen, JP. and M. Karonen . 2011. Chemical ecology of tannins and other phenolics: we need a change in approach. **Funct. Ecol**. 25: 325–338.

Salminen, S., A. von Wright and A. Ouwehand. 2004. **Lactic Acid Bacteria: Microbiological and Functional Aspects**. 3rd ed. CRC Press - Publisher

Sanders, T. A. B. and S. Reddy. 1992. The influence of rice bran on plasma lipids and lipoproteins in human volunteers. **Eur. J. Clin. Nutr**. 46: 167−172.

Saulnier, D.M.A., J.K. Spinler, GR. Gibson and J. Versalovic. 2009. Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. **Curr. Opin. Biotech**. 20: 135-141.

Scheller, H.V. and P. Ulvskov. 2010. Hemicelluloses. **Annu. Rev. Plant Biol**. 61: 263–289.

Scholz-Ahrens, K.E. and J. Schrezenmeir. 2002. Inulin, oligofructose and mineral metabolism – experimental data and mechanism. **Brit. J. Nutr**. 87: S179–S186.

Scott, K.P., S.W. Gratz, P.O. Sheridan, H.J. Flint and S.H. Duncan. 2013. The influence of diet on the gut microbiota. **Pharmacological Research**. 69: 52– 60

Shen, Y., L. Jin, P. Xiao, Y. Lu and J. Bao, J. 2009. Total phenolic, flavonoids, antioxidant capacity in the rice grain and their relation to grain color, size, and weight. **J Cereal Sci**. 49: 106-111.

Shibuya, N. and T. Iwasaki. 1985. Structural features of rice bran hemicellulose. **Phytochemistry**. 24(2): 285–289.

Shin, H. Y., S. Y. Park, J. H. Sung and D.H. Kim. 2003. Purification and characterization of a-L-arabinopyranosidase and a-Larabinofuranosidase from Bifidobacterium breve K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc. **Appl. Environ. Microbiol**. 69: 7116–7123.

Shoaf, K., G.L. Mulvey, G.D. Armstrong and RW. Hutkins. 2006: Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells. **Infect. Immun**. 74:6920-6928.

Shoaf-Sweeney, KD. and RW. Hutkins. 2009: Adherence, anti-adherence, and oligosaccharides preventing pathogens from sticking to the host. **Adv Food Nutr. Res**. 55:101-161.

Singleton, V.L. l972. Common Plant Phenols Other Than Anthocyanins-Contribution to Colouration and Discolouration, in "Chemistry of Plant Pigments". **Adv. Food Res.** 3: 143-191.

Smetanková, J., Z. Hladíková, F. Valach, M. Zimanová, Z. Kohajdová and G. Greif, M. Greifová. 2012. Influence of aerobic and anaerobic conditions on the growth and metabolism of selected strains of *Lactobacillus plantarum.* **Acta Chimica Slovaca**, Vol. 5(2): 204—210.

Somerville, C., S. Bauer, G. Brininstool, M. Facette, T. Hamann, J. Milne, E. Osborne, A. Paredez, S. Persson, T. Raab, S. Vorwerk and H. Youngs. Toward a systems approach to understanding plant cell walls. **Science**. 306: 2206-2211.

Swennen, K., C.M. Courtin, B.Vd. Bruggen, C. Vandecasteele and J.A. Delcour. 2005. Ultrafiltration and ethanol precipitation for isolation of arabinoxylooligosaccharides with different structures. **Carbohyd. Polym**. 62: 283–292.

Swennen, K., C.M. Courtin, G.C.J.E. Lindemans and J.A Delcour. 2006. Large-scale production and characterisation of wheat bran arabinoxylooligosaccharides. **J. Sci. Food Agric**. 86: 1722–1731.

Szwajgier, D., J. Pielecki and Z. Targonski. 2005. The release of ferulic acid and feruloylated oligosaccharides during wort and beer production. **J. Inst. Brew**. 111: 372-379.

Tabaraki, R. and A. Nateghi. 2011. Optimization of ultrasonic-assisted extraction of natural antioxidants from rice bran using response surface methodology. **Ultrason. Sonochem**. 18: 1279−1286.

Thayer, J.R., J.S. Rohrer, N. Avdalovic and R.P. Gearing. 1998. Improvements to in-line desalting of oligosaccharides separated by high-pH anion exchange chromatography with pulsed amperometric detection. **Anal. Biochem**. 256: 207-216.

Ti, H., Q. Li, R. Zhang, M. Zhang, Y. Deng, Z. Wei, J. Chi and Y. Zhang. Free and bound phenolic profiles and antioxidant activity of milled fractions of different indica rice varieties cultivated in southern China. **Food Chem**. 159: 166–174.

Topping, D.L. and P.M. Clifton. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and non starch polysaccharides. **Physiol Rev.** 81(3): 1031–1064.

van den Brink, J. and R.P. de Vries. 2011. Fungal enzyme sets for plant polysaccharide degradation. **Appl. Microbiol. Biotechnol**. 91: 1477–1492.

van den Broek, L. A. M., S.W.A. Hinz, G. Beldman, JP. Vincken and A. G. J. Voragen. 2008. Review. Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. **Mol. Nutr. Food Res**. 52: 146 – 163.

Vardakou M., P. Katapodis, M. Samiotaki, D. Kekos, G. Panayotou and P. Christakopoulos. 2003. Mode of action of family 10 and 11 endoxylanases on water-unextractable arabinoxylan. **Intl. J. Bio. Macromol**. 33: 129–134.

Vardakou, M., C.N. Palop, P. Christakopoulos, C.B. Faulds, M.A. Gasson and A. Narbad. 2008. Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction of hydrolase activity in gut microflora. **Intl. J. Food Microbiol**. 123: 166–170.

Vulevic, J., R.A. Rastall and G.R. Gibson. 2004. Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides. **FEMS Microbiol**. Letters 236: 153–159.

Wang, J., X. Yuan, B. Sun, Y. Tian and Y. Cao. Scavenging Activity of Enzymatic Hydrolysates from Wheat Bran. **Food Technol. Biotechnol**. 47 (1): 39–46.

Wright, J.S., E.R. Johnson and G.A. DiLabio, 2001. Predicting the activity of phenolic antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidants. **J. Am. Chem. Soc**. 123: 1173-1183.

Xu, Y., L. Fan, X. Wang, Q. Yong and SY. Yu. 2013. Simultaneous separation and quantification of linear xylo- and cello-oligosaccharides mixtures in lignocellulosics processing products on High Performance Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection. **BioResources**. 8(3): 3247-3259.

Yu, L. 2008. **Wheat antioxidants**. John Wiley & Sons, Inc., USA.

Zobel, H. F. 1988. Molecules to Granules: A Comprehensive Starch Review. **Starch**. 40: 44–50.

APPENDICES

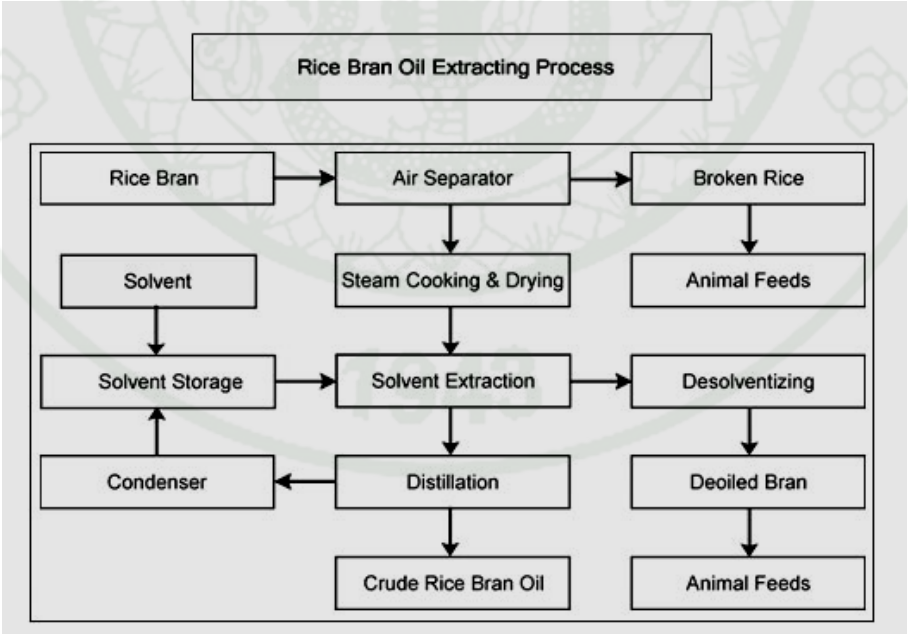
Appendix A

Material Specifications

Material specification

1. Rice bran specification

Appendix Figure A1 Defatted rice bran; (A) original package and (B) dried rice bran.



Appendix Figure A2 Rice bran oil production at Ruam Jai Vegetable Oil Co. Ltd.

Source: Thai Edible Oil Co. Ltd (2005)

Commercially defatted rice bran was brought from Ruam Jai Vegetable Oil Co. Ltd., which crude protein ≥ 15.0%, crude fiber ≥ 10.0%, moisture ≤12.0%, and crude fat ≤2.0%.

2. Enzymes for extraction

2.1. Viscozyme was declared as beta-glucanase (endo-1,3(4)-) 100 fungal beta-glucanase unit (FBG)/g with side activities of xylanase, cellulase, hemicellulose from *Aspergillus aculeatus*.

2.2. Pentopan was declared as endo-1,4-beta-xylanase 2500 fungal xylanase units (FXU)/g from from *Thermomyces lanuginosus* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism.

Appendix B

Preparation and Analysis Methods

Preparation and Analysis Methods

3. Rice bran extraction

3.1. Enzymes dose: 70 unit of enzyme was equal to 57.9 mL Viscozyme and 28 mg Pentopan.

3.2. Ethanol (95%) ratio to extract solution on 60% ethanol precipitation was 631.58 : 368.42.

3.3. Ethanol (99%) ratio to extract solution on 90% ethanol precipitation was 90.91 : 909.09.

4. Protein determination (AOAC 2001.11)

4.1. Reagents

4.1.1. Digestion catalyst was contained 7 g of K_2SO_4 and 0.8 g $CuSO_4$.

4.1.2. Sodium hydroxide solution 50%. Weigh 500 g sodium hydroxide, dissolve in water and make up to 1 L with water

4.1.3. Hydrochloric acid 0.1 N solution. Pipette 8.3 mL of concentrated hydrochloric acid to approximately 500 mL distilled water in 1 L volumetric flask soaked in ice cold water. Allow to cool and make up to volume with distilled water.

4.1.4. Methyl red/bromcresol green indicator solution. Dissolve 0.2 g methyl red and dilute to 100 mL with 95% ethanol. Dissolve 1.0 g bromcresol green and dilute to 500 mL with 95% ethanol. Mix one (1) part methyl red solution with five (5) parts bromcresol green solution.

4.1.5. Boric acid solution (4%). Dissolve 40 g boric acid in hot distilled water, cool and make up to 1 L with distilled water.

4.2. Method

4.2.1. Quantitatively 1.0 g sample was weighed, filled in digestion tube, followed with addition of catalyst and 25 mL concentrated sulfuric acid.

4.2.2. The digestion tube was transferred to the digestor and was digested initially at low temperature to prevent frothing and boil briskly until the solution was clear and free of carbon or until oxidation is complete (clear blue solution).

4.2.3. A 50 mL of warm water was added to reduce the viscosity of mixture.

4.2.4. Erlenmeyer flask containing 50 mL of 4% boric acid with indicator as was placed on the receiver distillation unit.

4.2.5. The mixture was distilled with 70 mL of 50% sodium hydroxide until all ammonia had been released (10 min).

4.2.6. The distillate was titrated with the 0.1 N hydrochloric acid until just before the appearance of the pink color.

4.2.7. Record volume of acid used to the nearest 0.05 mL.

4.2.8. Total protein content was calculated by:

$$\% \text{ Protein} = \frac{(\text{mL 0.1N HCl sample} - \text{mL 0.1N HCl blank}) \times 0.0014 \times \text{N HCl} \times 100}{\text{Weight of sample}} \times 6.31$$

5. Total sugar determination

5.1. Reagents

5.1.1. Copper sulfate solution: dissolve 34.639 g of copper sulfate in water, dilute to 500 mL and filter through filter paper.

5.1.2. Alkaline tartrate solution: dissolve 173 g potassium sodium tartrate and 50 g NaOH in distilled water, dilute to 500 mL, let stand for 2 days.

5.1.3. Fehling's solution: prepare by mixing equal volume of reagents 2.1 and 2.2.

5.1.4. Sucrose solution: sucrose was oven dried at 100 °C for 1 h. 5 g of dried sucrose was added with 2.5 mL concentrated HCl and diluted with water to approximately 100 mL. The solution was stored for 3 days at room temperature, then diluted to 500 mL.

5.2. Method

5.2.1. Sample preparation: 1 g of sample was diluted with 50 mL water and preheated to 60 °C for 30 min, then cooled down to room temperature. The solution was then brought up to 100 mL and filtered to erlenmeyer flask.

5.2.2. Accurately pipette 10 mL Fehling solution to Erlenmeyer flask and boiled for 3 minutes. 1 mL sugar solution was added at a time, with few seconds interval, until disappearance of blue color.

5.2.3. 3 drops of methylene blue indicator was added and continued adding sugar solution at intervals of 10 seconds until the indicator is completely decolorized.

5.2.4. Total sugar was calculated by:

$$\% \text{ sugar (g per 100 g)} = (F-M) \times I \times 100 \times 100 \times 100 / (W \times 50)$$

where: F = volume of standard sugar solution required to reduce 10 mL mixed Fehling's solution; M = volume of standard sugar solution used in back titration of; the sample I = gram sugar per mL working standard solution; W = weight of sample.

6. Color determination

6.1. Sample preparation: 50 mg of dried fraction was diluted to 10 mL water and vortex until completely solubilized.

6.2. The L*, a*, and b* of the solution was measured using HunterLab UltraScan Pro.

7. Cell culture preparation

The bacteria stock was thawed to room temperature. A 25 mL vial bottle was filled with 9 mL MRS Broth or TSB media and autoclaved 15 min at 118 °C. When it was cooled down, 1 mL of bacteria stock was added and incubated for 24 h. This cell culture was refresh by the same step for another 24 h before used in fermentation experiment.

8. Basal media for fermentation

The mixture of peptone 10.0g, meat extract 10.0g, yeast extract 4.0g, dipotassium hydrogen phosphate 2.0g, di-ammonium hydrogen citrate 2.0g, sodium acetate 5.0g, magnesium sulfate 0.2g and manganese sulfate 0.04g was diluted in 1 L of water. The mixture was transferred to incubation bottle (100 mL) and then autoclaved 15 min at 118 °C.

9. Eluent preparation for characterization

9.1. Deionized water was passed through ultra-purification instrument to get double deionized water.

9.2. Fill 1 L volumetric flask with double deionized water, remove quantitatively a volume that would be replaced with 10M NaOH (20 mL for eluent B and 10 mL for eluent C) and mark the level.

9.3. Weigh 54.43g sodium acetate trihydrate and transfer to the marked volumetric flask for eluent C and dilute it with double deionized water until mark.

9.4. Pass 1 L of double deionized water through 0.45 membrane filter for eluent A, the remained of double deionized water from volumetric flask for eluent B and sodium acetate solution, then transfer each solution to the instrument eluent bottle.

9.5. Sonicate each bottle for 3 h until no gas bubble was observed.

Appendix C

Statistical Analysis and Standard Curves

Statistical Analysis and Standard Curves

Appendix Table C1 ANOVA of percent yield of <60% fractions after 1, 4, 8, 16, and 24 h incubation time.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	9	0.0241856	0.0026873	540.92	0.000
Within groups	10	0.0000497	0.0000050		
Total	19	0.0242353			

Appendix Table C2 ANOVA of percent yield of 60-90% fractions after 1, 4, 8, 16, and 24 h incubation time.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	9	0.0024818	0.0002758	6056.72	0.000
Within groups	10	0.0000005	0.0000000		
Total	19	0.0024822			

Appendix Table C3 ANOVA of percent yield of >90% fractions after 1, 4, 8, 16, and 24 h incubation time.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	9	0.0001340	0.0000149	726.78	0.000
Within groups	10	0.0000002	0.0000000		
Total	19	0.0001342			

Appendix Table C4 ANOVA of moisture content of rice bran, <60% and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	6	150.8143	25.1357	726.78	0.000
Within groups	7	0.4444	0.0635		
Total	13	151.2587			

Appendix Table C5 ANOVA of protein content of rice bran, <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	6	1113.133	185.522	619.70	0.000
Within groups	7	2.096	0.299		
Total	13	1115.229			

Appendix Table C6 ANOVA of starch content of rice bran, <60%, and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	4	3430.054	857.513	15010.08	0.000
Within groups	5	0.286	0.057		
Total	9	3430.339			

Appendix Table C7 ANOVA of reducing sugar content of <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	5	6.676593	1.335319	25331.38	0.000
Within groups	6	0.000316	0.000053		
Total	11	6.676909			

Appendix Table C8 ANOVA of L* value on color determination of inulin, <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	6	8.7970	1.4662	32.02	0.000
Within groups	14	0.6410	0.0458		
Total	20	9.4380			

Appendix Table C9 ANOVA of a* value on color determination of inulin, <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	6	16.5304	2.7551	213.87	0.000
Within groups	14	0.1804	0.0129		
Total	20	16.7108			

Appendix Table C10 ANOVA of b* value on color determination of inulin, <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	6	492.0370	82.0062	22533.59	0.000
Within groups	14	0.0510	0.0036		
Total	20	492.0880			

Appendix Table C11 ANOVA of phenolic content of <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	5	233576.2	46715.2 55.5	841.53	0.000
Within groups	12	666.1			
Total	17	234242.3			

Appendix Table C12 ANOVA of reducing power method of <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	5	11.98622	2.39724	20421.39	0.000
Within groups	12	0.00141	0.00012		
Total	17	11.98763			

Appendix Table C13 ANOVA of DPPH radical scavenging method of <60%, 60-90% and >90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	5	8643.9	1728.8	74.68	0.000
Within groups	12	277.8	23.2		
Total	17	8921.7			

Appendix Table C14 ANOVA of digestibility of corn starch, inulin, <60%, and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	5	0.0098167	0.0019633	88.23	0.000
Within groups	6	0.0001335	0.0000223		
Total	11	0.0099503			

Appendix Table C15 ANOVA of area under growth curve of *B. breve* TISTR 2130 on glucose, inulin and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	3	231.293	77.098	228.00	0.000
Within groups	4	1.353	0.338		
Total	7	232.645			

Appendix Table C16 ANOVA of area under growth curve of *B. bifidum* TISTR 2129 on glucose, inulin and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	3	450.536	150.179	651.89	0.000
Within groups	4	0.921	0.230		
Total	7	451.457			

Appendix Table C17 ANOVA of area under growth curve of *B. lactis animalis* TISTR 2195 on glucose, inulin and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	3	150.295	50.098	84.37	0.000
Within groups	4	2.375	0.594		
Total	7	152.670			

Appendix Table C18 ANOVA of area under growth curve of *B. longum* TISTR 2194 on glucose, inulin and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	3	37.669	12.556	42.27	0.002
Within groups	4	1.188	0.297		
Total	7	38.857			

Appendix Table C19 ANOVA of area under growth curve of *L. acidophilus* ATTC 4356 on glucose, inulin and 60-90% fractions.

Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	3	194.30	64.77	61.00	0.001
Within groups	4	4.25	1.06		
Total	7	198.55			

Appendix Table C20 ANOVA of area under growth curve of *L. plantarum* BCC 39798 on glucose, inulin and 60-90% fractions.

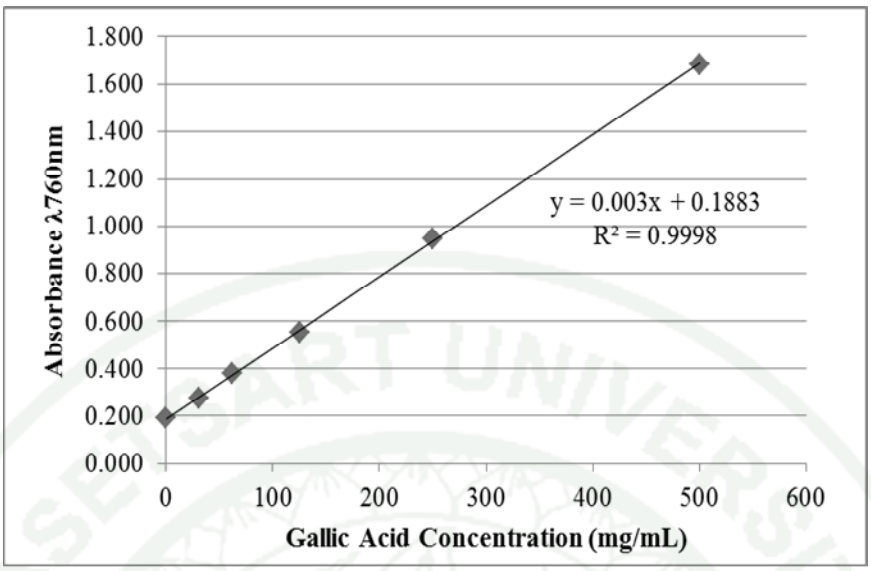
Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	3	57.743	19.248	25.36	0.005
Within groups	4	3.036	0.759		
Total	7	60.780			

Appendix Table C21 ANOVA of area under growth curve of *E. coli* TISTR 887 on glucose, inulin and 60-90% fractions.

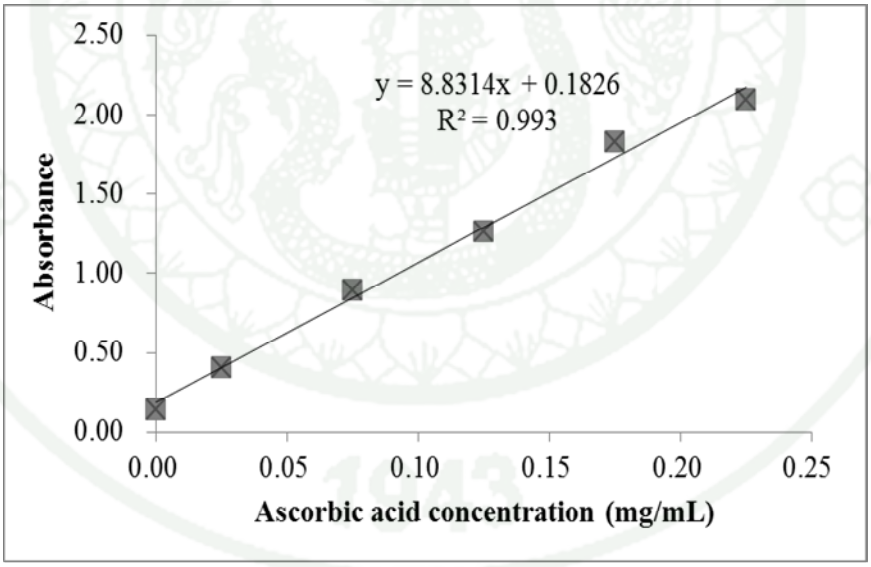
Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	3	371.42	123.81	108.35	0.000
Within groups	4	4.57	1.14		
Total	7	376.00			

Appendix Table C22 ANOVA of pH cultured media that supplemented with glucose, inulin and 60-90% fractions after 32 h incubation.

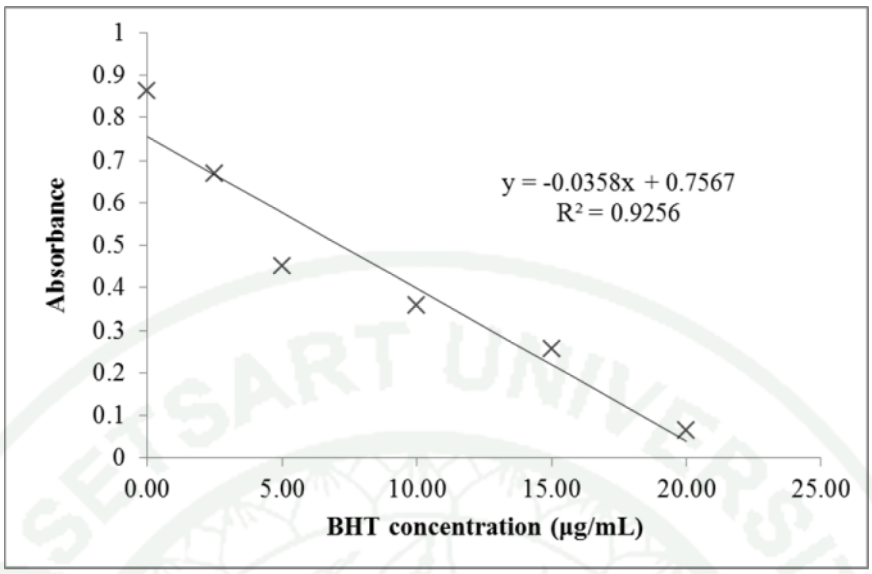
Source	df	Sum Squares	Mean Squares	F	Sig.
Between groups	27	23.63714	0.87545	179.19	0.000
Within groups	28	0.13680	0.00489		
Total	55	23.77394			



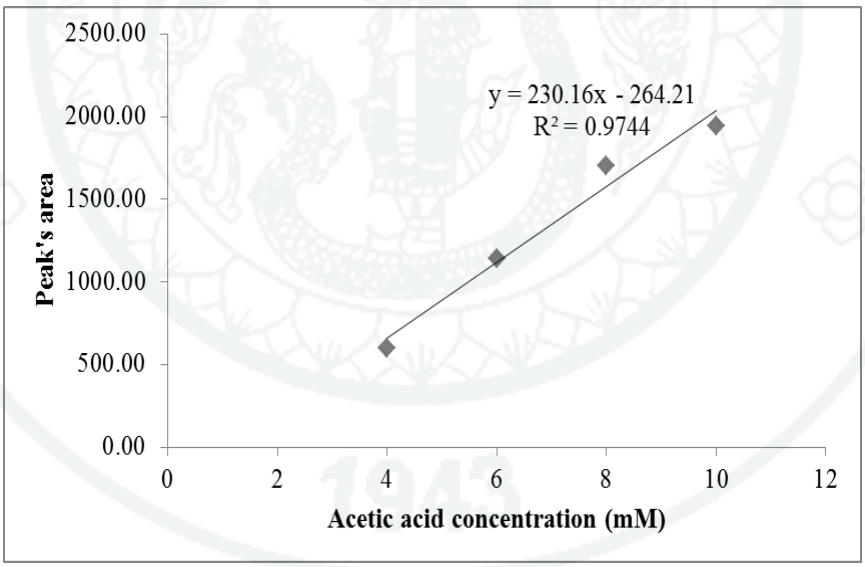
Appendix Figure C1 Standard curve for phenolic content determination using gallic acid as standard.



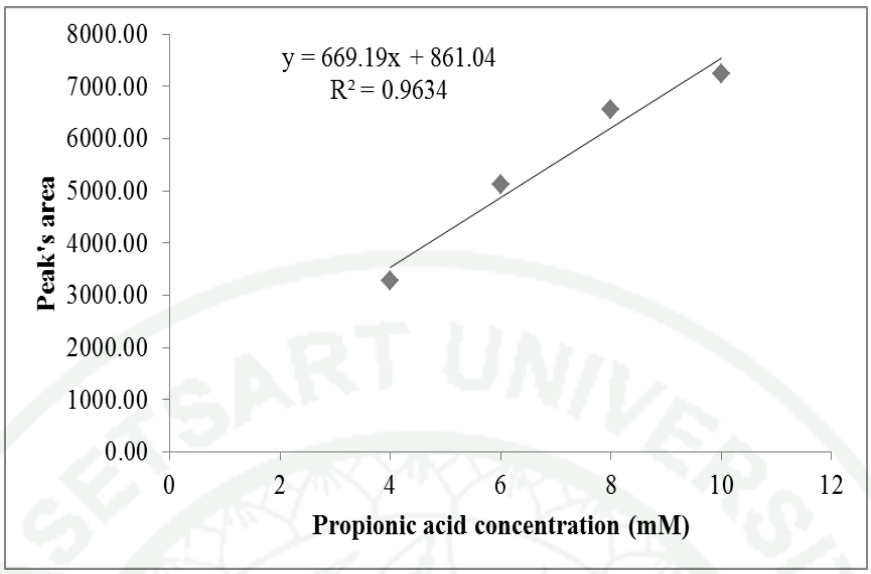
Appendix Figure C2 Standard curve for reducing power method using ascorbic acid as standard.



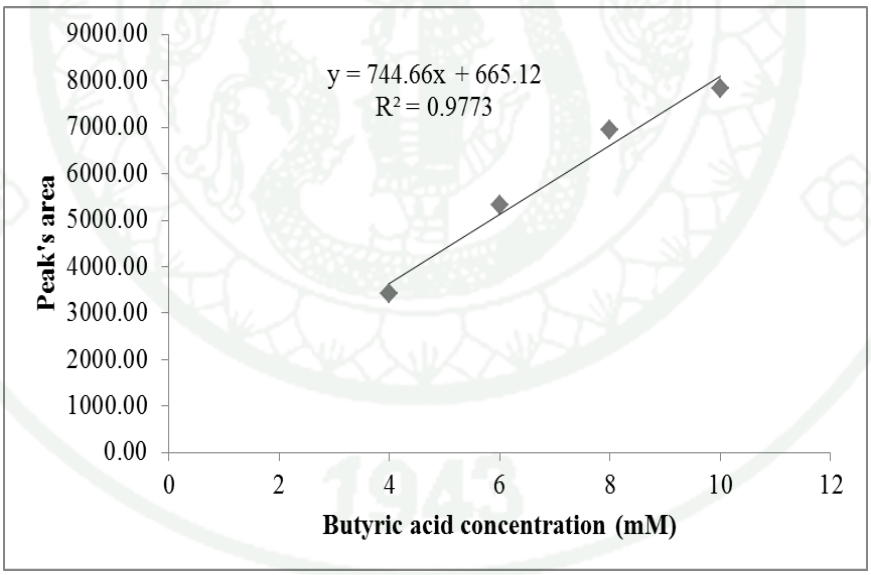
Appendix Figure C3 Standard curve for DPPH radical scavenging method using butyl hydroxyl toluene (BHT) as standard.



Appendix Figure C4 Standard curve for acetic acid determination on fermentation experiment.



Appendix Figure C5 Standard curve for propionic acid determination on fermentation experiment.



Appendix Figure C6 Standard curve for butyric acid determination on fermentation experiment.

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