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POTENTIAL FOR XYLOOLIGOSACCHARIDE (XOS) AND MANNOOLIGOSACCHARIDE (MOS) PRODUCTION FROM A HEMICELLULOSE FRACTION OF BIOMASS AND APPLICATION AS PREBIOTICS AND ANTIOXIDANTS

KANOKNAT WORANUCH

Graduate School Srinakharinwirot University

2019

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กนกณัฐ วรนุช

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
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ปีการศึกษา 2562

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KANOKNAT WORANUCH

A Dissertation Submitted in partial Fulfillment of Requirements

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BY

KANOKNAT WORANUCH

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AT SRINAKHARINWIROT UNIVERSITY

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Title	POTENTIAL FOR XYLOOLIGOSACCHARIDE (XOS) AND MANNOOLIGOSACCHARIDE (MOS) PRODUCTION FROM A HEMICELLULOSE FRACTION OF BIOMASS AND APPLICATION AS PREBIOTICS AND ANTIOXIDANTS
Author	KANOKNAT WORANUCH
Degree	DOCTOR OF PHILOSOPHY
Academic Year	2019
Thesis Advisor	Associate Professor Dr. WASANA SUKHUMSIRICHART , Ph.D.

Most prebiotics are substances in the group of short carbohydrates or oligosaccharides, obtained from lignocellulosic biomass. In this study, two types of oligosaccharides were produced, including xylooligosaccharides (XOS) from alkaline-pretreated rice straw using rXynSW3 and manooligosaccharides (MOS) from Konjac by using rManS2 and assessed for their potential as prebiotics and antioxidants. The antioxidant property was evaluated using DPPH assay. The results showed that the antioxidant value (IC_{50}) were 542.27±0.59 µg/ml and 643.06±0.05 µg/ml for XOS and MOS, respectively, compared to 494.72±0.15 µg/ml of ascorbic acid as standard. The prebiotic property was performed by *in vitro* and *in vivo* fermentation in probiotics. For the *in vitro* experiment, XOS was fermented with probiotic strains, *Lactobacillus plantarum* TISTR543, *L. casei* TISTR390, and *Bifidobacterium bifidum* TISTR2129, whereas MOS was fermented with *L. plantarum* N25. The *in vitro* study of prebiotic effect on probiotics found that XOS and MOS were capable of stimulating the growth of all of the probiotics tested. For the *in vivo* study, the XOS and MOS were tested in mice (BALB/cAJcl) for the valuation of prebiotic property and safety. The results indicated that all mice treated with XOS and MOS were normal compared to the control group and there were no deaths. In the MOS experiment, the amount of probiotics significantly increased in mice-treated with a synbiotic (*L. plantarum* and MOS) compared to probiotic and control groups ($P<0.001$). Furthermore, short chain fatty acids (SCFAs) production by MOS were also investigated both *in vitro* and *in vivo* by using HPLC. The results of *in vitro* SCFAs production found lactic acid and acetic acid, whereas the *in vivo* study found more SCFAs in the feces samples of mice, including lactic acid, acetic acid, propionic acid and butyric acid. All of the results from this study demonstrated that XOS from alkaline-pretreated rice straw and MOS from Konjac mannan have a potential for use as a prebiotic and an antioxidant.

Keyword : xylooligosaccharide, mannooligosaccharide, prebiotics, probiotics

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CHAPTER I
INTRODUCTION

Lignocellulosic waste material for example straw, bagasse, corn stover, wheat stover etc.[1, 2] are produced from many agro-industrial each year. Therefore, conversation of this wastes to high-valuable compounds or renewable energy will promote an income and protect environment. For these reasons, technology and methods that develop for agricultural and agro-industrial wastes to obtained value-added products are important.[3, 4] In the recent years, researchers focused on producing prebiotics[5] from new sources included natural products which save for used, low-cost, and have potential of prebiotic etc.[6] The definition of prebiotic following to FAO that ‘‘Prebiotics are ingredients of food that non-digested by host but correlated with modulation of the microbiota to promotes good health for host” which not all fibre are prebiotic.[7] Prebiotics are important for encourage the sustainability of healthy good bacteria therefore have benefit on the host (probiotics).[8, 9] The prebiotic that have been known such as sorbitol, isomalt, xylitol, inulin, oligosaccharides etc. The oligosaccharides that act as prebiotic included fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), and mannooligosaccharides (MOS).(10, 11).[10, 11]

Xylooligosaccharides (XOS), a type of oligosaccharide, established from xylose monomer with β-1,4-glycosidic linkage which are calorie-free and non-digested by mammals and contain in vegetables, fruits, milk, honey and other foods.[12] Recently, the prebiotics have been increased roles and are interested in many industries including foods and medicals.[13] They have important properties such as antioxidant and stimulated growth of probiotics, especially, effect on probiotics which beneficial to host. *Bifidobacterium* and *Lactobacillus* spp. are beneficial intestinal microflora.[14] Many researches showed prebiotic potential of XOS such as the experiment in rat that used XOS additive which the result demonstrated that XOS-fed rat group have variety of *Bifidobacterium* and *Lactobacillus* spp. increased in feacal sample as compared to control group.[15] The XOS derived from corn cobs combined with *Lactobacillus*

plantarum showed antioxidant potential better than either XOS or probiotic alone.[16] The XOS syrup producing from biomass by endoxylanase of *Bacillus pumilus* B20 stimulated growth of *L. brevis*.[17] Furthermore, the XOS can be modulated metabolic pathway of *B. adolescentis* 15703 for highly promoted the growth.[18] In addition to its antioxidant and promoted growth of probiotics, prebiotics also have the ability to prevent diseases from pathogens in gastrointestinal tract including *Escherichia coli*,[19-21] *Clostridium difficile*,[22, 23] Rotavirus,[24-26] *Salmonella* sp.,[27-29] *Bacteriodes* sp.[30]

Mannooligosaccharides (MOS) able to produce from agri-waste by digestion with endo-β-(1,4)-mannanase. The MOS has significant stability against human enzyme and stimulated good bacteria in the gastrointestinal tract which is an essential parameter for oligosaccharides to serve as a prebiotic.[31] The MOS can be promoted the proliferation of normal bacterial flora such as *L. acidophilus* and *B. infantis* were promoted host health[32] and inhibited pathogenic microorganisms which have been reported to stimulate the immune system to against pathogen such as *Vibrio harveyi* in Pacific white shrimp.[33] Furthermore, MOS can be also inhibited the growth of pathogenic strains using primary mode in fermentation.[34] Therefore, the objectives of this study are as following;

Objectives

- To produced xylooligosaccharide (XOS) from rice straw by using rXynSW3 of *Streptomyces* sp. SWU10
- To produced mannooligosaccharide (MOS) from Konjac by using rManS2 of *Bacillus* sp. SWU60
- To evaluate antioxidant activity of enzymatic products
- To assess prebiotic effect on probiotics *in vitro* and *in vivo*
- To study the safety of MOS and XOS *in vivo*
- To analyze short chain fatty acids (SCFAs) production by MOS *in vitro* and *in vivo*

CHAPTER II
LITERATURE REVIEW

1. The biomass of plant cell wall

Plant cell biomass refers to lignocelluloses or lignocellulosic biomass include cellulose, hemicelluloses (carbohydrate polymers) and lignin (aromatic polymer) (Fig. 1). The relative composition of lignocellulosic biomass correlated to the type, species, and the source of biomass.[2] The lignocellulosic biomass are derived from agriculture residues, waste and byproduct of several industrials for example corn stover, rice husk, cane bagasse, wheat straw etc. Generally, cereal residues consist a large fraction of lignocellulose matter more than grasses, fruit and vegetable wastes.[35] Table 1 shows composition of the main complements of cell wall from a variety sources of plant cells.[36] The lignocellulosic biomass as potential source of energy and raw materials for biological products.[37, 38]

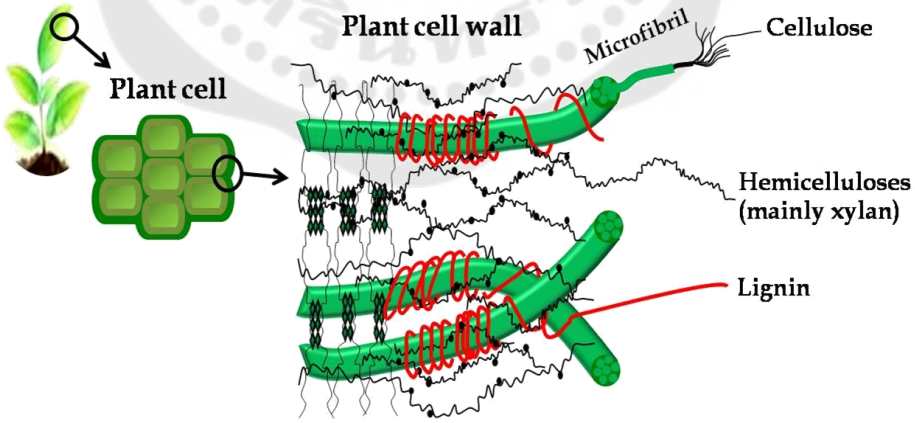


Figure 1. Compose of lignocellulosic biomass

Ratanakhanokchai et al. (2013). *Paenibacillus curdlanolyticus* Strain B-6 Multienzyme Complex: A Novel System for Biomass Utilization.

Table 1. Percentage of components of lignocellulosic biomass from different source.

Biomass type	Cellulose	Hemicellulose	Lignin
Softwoods	27-30	35-40	25-35
Hardwoods	38-52	16-33	20-25
Rice straw	23-28	28-36	12-14
Corn stover	38-40	24-26	7-19
Dicots	45-50	20-30	7-10
Grasses	25-40	25-50	10-30

1.1 Polymer of cellulose

Cellulose $(C_6H_{10}O_5)n$ is an unbranched polysaccharide established from a linear chain of D-glucose monomer (Fig. 2).[39, 40]

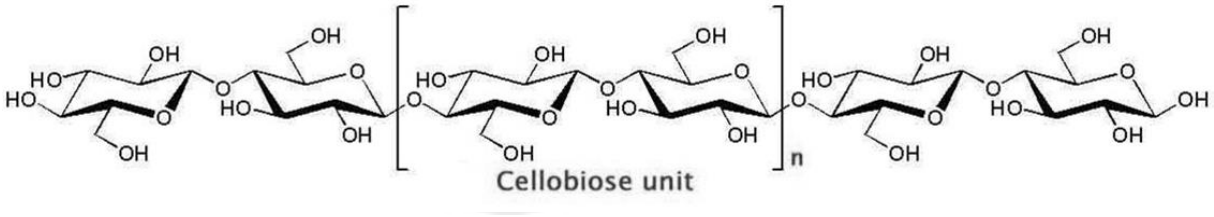


Figure 2. Cellulose polymer structure

Klemm D et al. (2005). Cellulose: fascinating biopolymer and sustainable raw material p. 3358.

1.2 Polymer of hemicellulose

Hemicellulose are heterogeneous polysaccharide contain of various sugar units (monomers) in shorter chains than cellulose such as xylan, mannan. Hemicelluloses

consisting of hexoses, pentoses, small amounts of L-sugars and many urgonic acids as side chain for example ferulic acid, rhamnose, arabinose (Fig. 3).[41, 42]

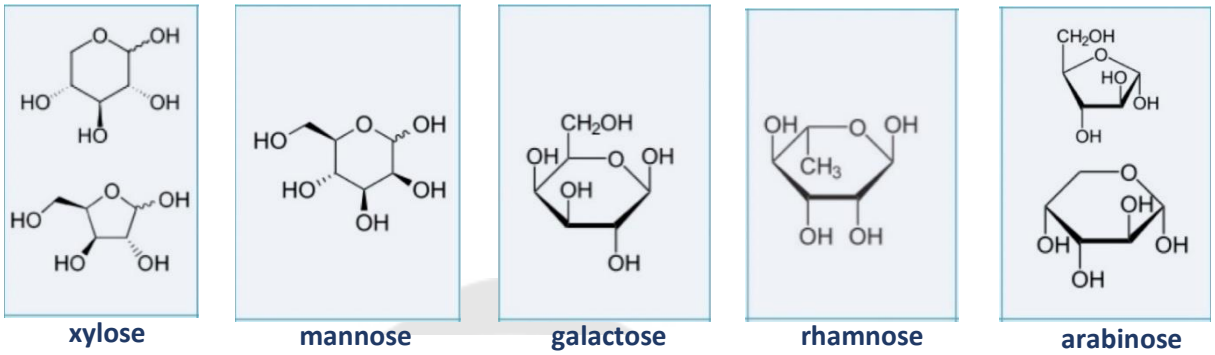


Figure 3. The type of sugar monomers found in hemicellulose

Available from: https://www.e-education.psu.edu/egee439/node/664

1.3 Polymer of lignin

Lignin are complex polymer of aromatic residues known as phenolic monomers (monolignols).[43, 44] Lignin play role in the mechanical strength to wood including stems, leaves and rigidity of plant walls (Fig. 4).[45, 46]

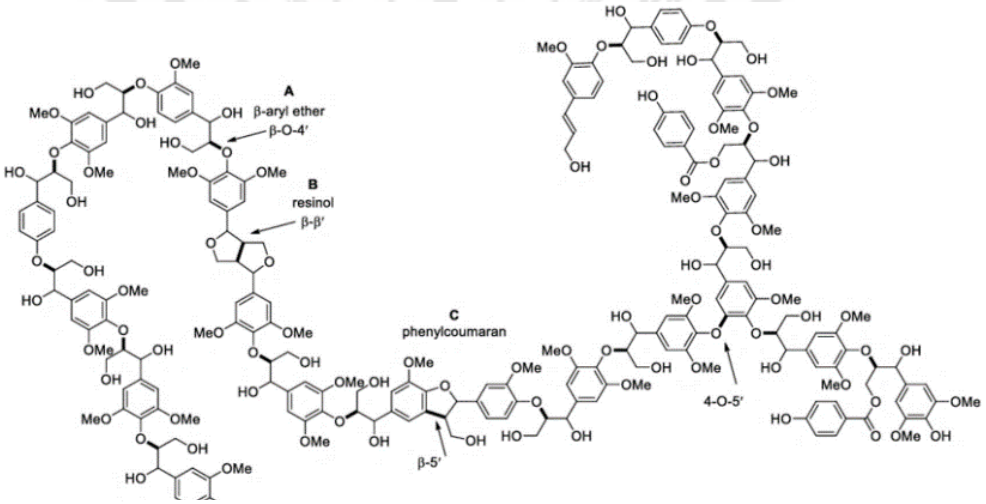


Figure 4. Lignin polymer structure

Nimz H. (2013). Beech Lignin—Proposal of a Constitutional Scheme.

2. Xylan

Xylan is main group of hemicelluloses fraction that found in plants cell wall of grasses. Xylan is a complex polysaccharide, consisting of β-D-(1-4)-glycosidically bonds between xylosyl residues with linear structure. Typically, account for 20%–30% of total raw mass that allows for used of feedstock materials for many industries.[47] The xylan has variably substituted by side chains of acetyl, methylglucuronyl, L-arabinofuranosyl, glucuronyl, feruloyl and p-coumaroyl residues (Fig. 5).[48, 49] Normally, 10-20% of backbone have substituted with arabinose which attached directly at the C2/C3 positions.

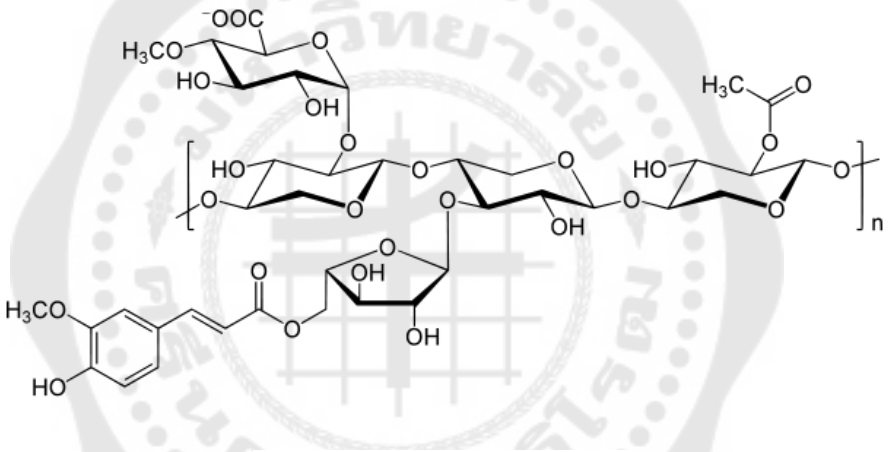


Figure 5. Xylan structure

Available from: http://en.wikipedia.org/wiki/Glucomannan

2.1 Xylanolytic enzymes

The structure of a typical xylans have various substituted at side chain. In order to completely digest the structure must be synergistic action of enzymes. in the xylanolytic enzymes group (Fig. 6).[50, 51]

Endo-xylanase (EC 3.2.1.8) responsible for randomly inside xylan backbone.

β-Xylosidases (EC 3.2.1.37) hydrolyze xylooligosaccharides at non-reducing end to xylose.

Acetyl xylan esterases (EC 3.1.1.6) remove the O-acetyl groups from C2/C3 positions.

α-Glucuronidases (EC 3.2.1.131) hydrolyze of D-xylose backbone at **α**-1,2-glycosidic linkage to remove D-glucuronic acids.

α-L-Arabinofuranosidases (EC 3.2.1.55) break linkage between arabinofuranose residues.

p-Coumaric acid esterases (EC 3.1.1.-) digest ester bond between arabinose and coumaric acid residues.

Ferulic acid esterases (EC 3.1.1.73) digest ester linked of L-arabinose and ferulic acid.

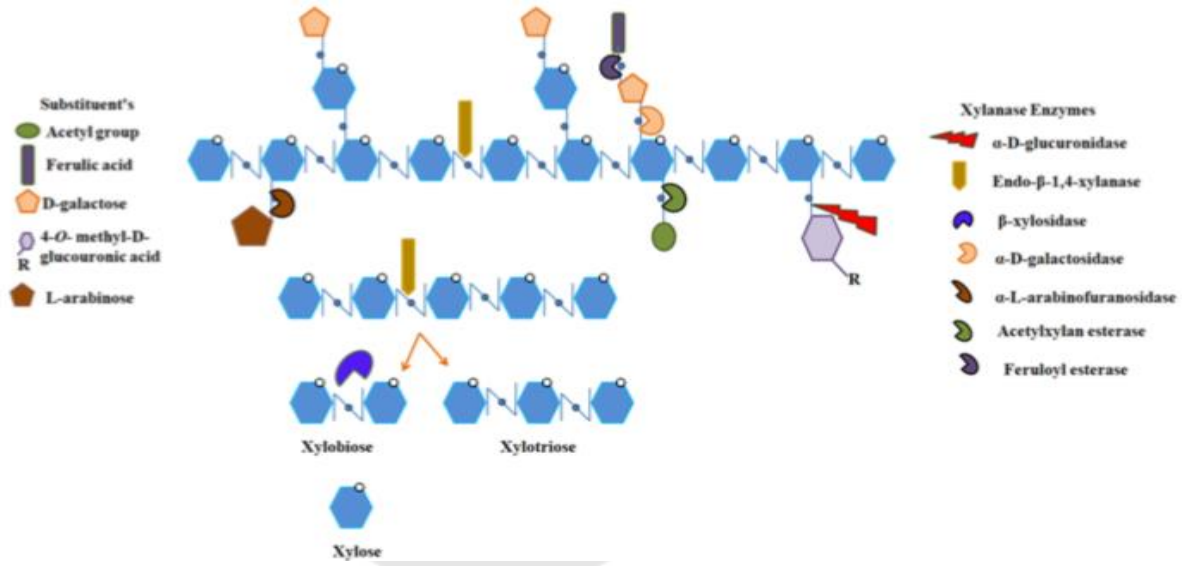


Figure 6. Xylanolytic enzyme

Bhardwaj N et al. (2019). A detailed overview of xylanases: an emerging biomolecule for current and future prospective p.40.

2.2 The recombinant xylanase (rXynSW3) production

Xylanases (EC 3.2.1.8) are used in a variety plant-based industries for example paper and pulp bleaching, food processing, textile manufacturing, vegetable oil and starch, production of bioethanol, cotton, solvents etc.[52] These enzymes produced from many organisms but can not by mammals which microorganisms have high activity

(Fig. 7).[53] *Streptomyces* sp. SWU10 in previous research demonstrated that endo-xylanase from this strain, termed *XynSW3* gene was overexpressed in *Escherichia coli* DH5α. The recombinant enzyme (rXynSW3) was endo-β-1,4-xylanase in classified of GH11. The rXynSW3 was used for the xylooligosaccharides production.[54]

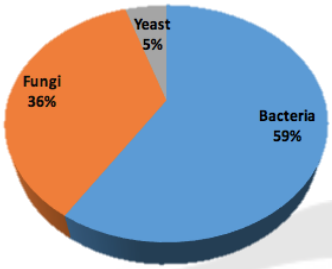


Figure 7. Pie chart of isolation of xylanase in different organism

2.3 Degradation of xylan by rXynSW3

The rXynSW3 has substrate specificity towards xylans with different degrees of substitution in which the substrate with low residue substitutions can be degrade by rXynSW3 more specific than the high residue substitutions. The main product of enzymatic degradation is xylooligosaccharides (Fig. 8), which have value and potential applications in many industries.[54]

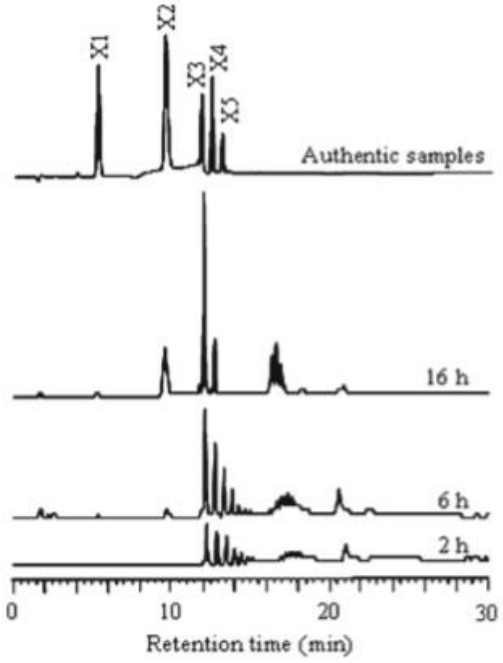


Figure 8. HPAEC assay of degradation products. Authentic samples X1 to X5 represent xylose to xylopentose, respectively

Sukhumsirichart et al. (2014). Expression and Characterization of Recombinant GH11 Xylanase from Thermotolerant *Streptomyces* sp. SWU10 p. 436.

3. Mannan

Mannan is a type of the hemicellulose which comprised from β-(1,4)-linked D-mannopyranosyl backbone. Mannan have four distinct groups depends on the relative amounts of the chemical structure (Fig. 9).[55, 56] In some type of plants found as storage polysaccharides such as the glucomannan from the konjac plant.[57, 58]

3.1 Classification of mannan

3.1.1 Linear mannan

It's homopolysaccharides (>95%) found in plant seed and endosperms of certain plant species such as vegetable ivory (ex. taqua plum seed).

3.1.2 Glucomannan

These types of mannose formed by β-linked between mannose and glucose.[49]

3.1.3 Galactomannan

The backbone comprised of a mannose with side groups is galactose.[59]

3.1.4 Galactoglucomannan

In galactomannan consist of D-mannose linked D-glucose backbone have side chains of D-galactose residues attached with α-1,6-linked.[60]

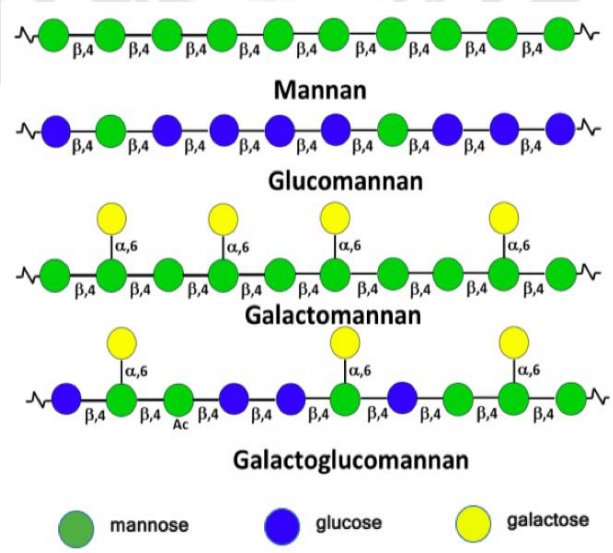


Figure 9. Mannan structures

Moreira LR et al. (2008). An overview of mannan structure and mannan-degrading enzyme systems p.165.

3.2 Mannan-degradation enzymes

The variety of hydrolytic enzymes of mannan polysaccharide structures are glycosyl hydrolases (EC 3.2.1.-).[61] The complication of the hemicellulose chemical of cell wall structure, wall structure, action of synergistic enzymes are required for complete hydrolyzation (Fig. 10).[62] The enzymes related in the degradation of mannans including β-mannanases (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), β-glucosidase (EC 3.2.1.21), acetyl mannan esterase (EC 3.2.1.6) and α-galactosidase (EC 3.2.1.22) are necessary for removing of the side chain of the mannans structure.[57, 63, 64]

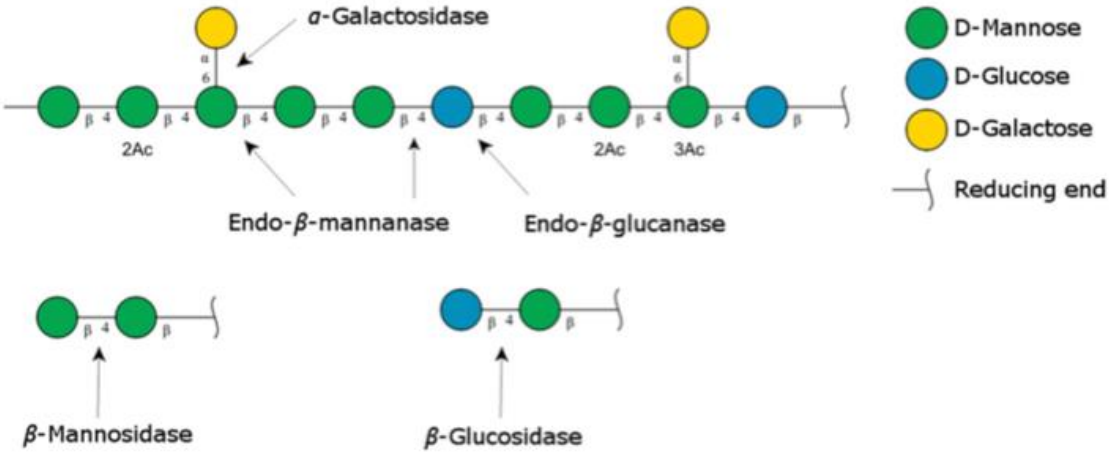


Figure 10. Mannan gedradation enzymes

Freiesleben P et al. (2018). Boosting of enzymatic softwood saccharification by fungal GH5 and GH26 endomannanases.

3.3 Mannanase production from *Bacillus* sp. SWU60

β-1,4-mannanases (EC 3.2.1.78) are necessary for facilitation dissolving and releasing of mannan from the substrate biomass, hydrolyses by clevage the internal β-1,4-glycosidic linked of mannan backbone.[65, 66] Generally, the range of the optimal temperature and pH of mannanase are 35-70 °C and 3.0-7.5 respectively. The molecular mass of mannanase ranges from 28-80 kDa.[67] The β-mannanases are classifed of depend on amino acid sequence into four groups of glycosyl hydrolase (GH) families were GH5, GH26, GH113 and GH134 based on CAZy.[65] *Bacillus* sp. SWU60 is bacteria that can produce mannanase enzyme, termed ManS2. The optimal pH and temperature

are 6.0 and 60°C, respectively. Overexpresstion of *ManS2* gene in *Escherichia coli* BL21 resulting the recombinant enzyme mannanase (rManS2). This enzyme is an endo-β-1,4-mannanase use Konjac glucomannan as substrate and able to released galactomannan from locust bean gum which demonstrated that rManS2 have potential for glucomanno- and galactomanno-oligosaccharides production.[68]

3.4 Degradation of Konjac glucomannan (KGM) by rManS2

The rManS2 is a recombinant enzyme of mannanase that showed the highest activity toward Konjac glucomannan (KGM). It can be hydrolyzed the linkages between mannose and glucose units same as those between mannose units. The main products of KGM degradation by mannanase are large amounts of oligosaccharides including glucosyl mannobiose, cellobiosyl mannobiose by HPAEC analysis (Fig. 11).[68] Therefore, it can be used in food industries especially for prebiotic production.

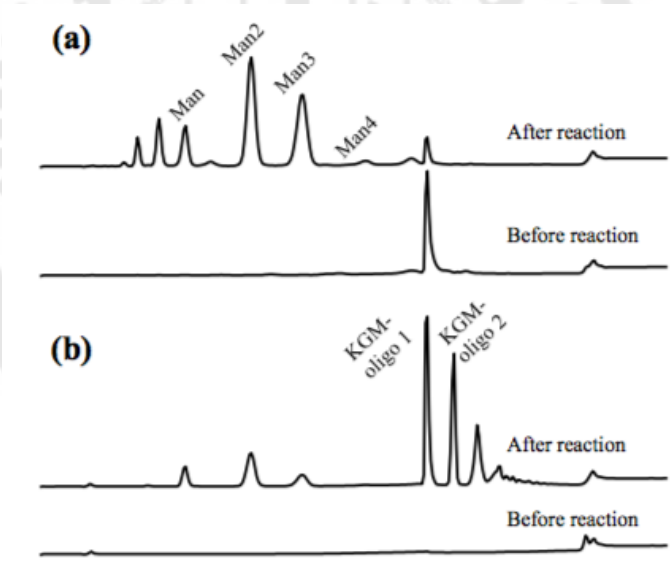


Figure 11. HPAEC analyses of substrate degraded by rManS2, (a) Linear mannan and (b) Konjac glucomannan (KGM), KGM-1, glucosyl mannobiose; KGM-2, cellobiosyl mannobiose.

Seesom et al. (2017). Purification, characterization, and overexpression of an endo-1,4-β-mannanase from thermotolerant *Bacillus* sp. SWU60 p.53.

4. Prebiotic

The prebiotic was dietary food that non-digestible by gastrointestinal system but digestible by selectively of microorganism (probiotics) in the gastrointestinal, and

promoting health. It is the first idea of prebiotics definition from Glenn Gibson and Marcel Roberfroid in 1995 and unchanged for long time.[69] Then, the definition of prebiotics changed but it was still the same: that selectively elements in food which the specific changes of probiotics bacteria and promoting the host health.[70] Although, meaning of prebiotics have revised in the scientific literature for many time but the above-mentioned difinition can be accepted.[71]

According to this definition, prebiotics as a group of functional foods such as fructooligosaccharides (FOS), galactooligosaccharide (GOS), inulin that can be degraded by intestinal microorganism and related with human overall health (Fig. 12). The main of metabolic products like as short chain fatty acids (SCAFs) that are benefit to the body, especially the gastrointestinal system.[72]

4.1 The basis for classification of prebiotic compounds

- Non-digestible by mammal enzymes
- Unable to be absorbed in a gastrointestinal system
- Can be degraded by probiotics
- The growth and/or activity of the gastrointestinal microflora (probiotics) be able to promoted host's health

4.2 Types of Prebiotics

4.2.1 Disaccharide

Generally, refers to disaccharide sugar alcohol including lactitol and xylitol which badly absorbed into the bloodstream from the small intestine, they have lower calories than sugars. So, they are reduced-calorie and small change in blood sugar in a diabetic diet.[73] Furthermore, synthetic disaccharide composed of galactose and glucose called ''lactulose'' as non-absorbable sugar found that used in the treatment of some disease (ex. constipation, hepatic encephalopathy). [74, 75]

4.2.2 Oligosaccharide

This type is non-digestible oligosaccharide consists of fructooligosaccharide (FOS)[76], galactooligosaccharide (GOS)[77, 78] and inulin. They are

commercialized as food ingredients. Previously, many studies showing about gut microbiota can ferment them and have benefits for human health in the process of balancing a disturbed colon microbiota, so inhibiting many undesirable diseases.[79]

4.2.3 Resistant starch

There are carbohydrate that resistant to digested in the gut called as ''resistant starch (RS)''. RS occurs naturally in foods for example oats, grains, legumes, raw potato starch, firm bananas etc.[49] Intestinal microbiota can digested RS and increases the production of SCFAs able to improved the function of digestive system.[80, 81]

4.2.4 Non-carbohydrate oligosaccharide

The some biological substances that are not classified as carbohydrates but classified as prebiotics, such as cocoa-derived flavonols.[82, 83]

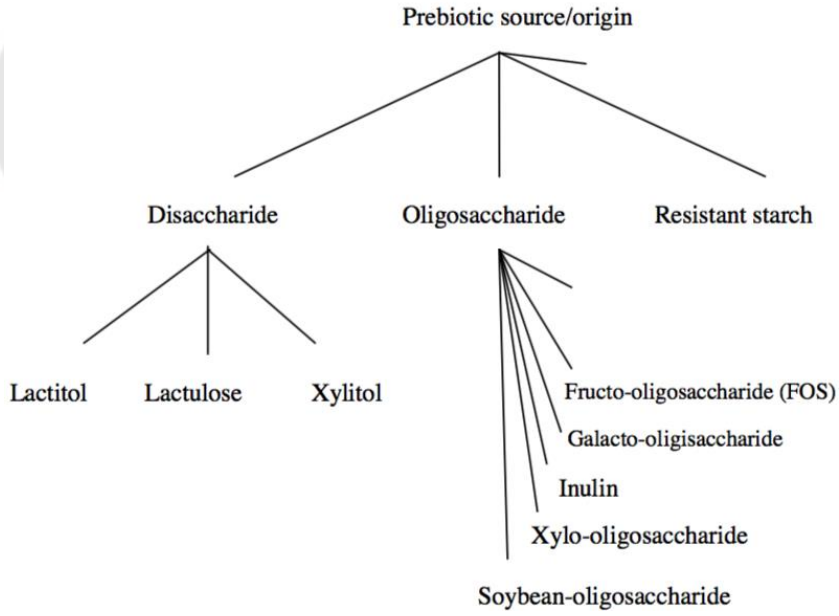


Figure 12. Prebiotic origin. Adapted from Magalhaes et al.

Tzounis X et al. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study.

4.3 Prebiotic effect on probiotics

Over the past of many years, the idea of probiotics defined from a variety of sources, including Food and Agriculture Organization (FAO), International Life Science Institute (ILSI), European Food and Feed Cultures Association (EFFCA), and World Health Organization (WHO) which has the overall meaning that the consumption of living microbes in sufficient amounts will affect consumers' health benefits. [84]

So, probiotics as live microorganisms need to provide advantage to the host via mode of metabolism in the gastrointestinal tract of host. An ideal characterization for probiotic organism showed in Figure 13.[85] The popularly used probiotic microorganisms are *Bifidobacterium* and *Lactobacillus* sp. such as *B. adolescentis*[86], *B. bifidum*[87], *L. plantarum*[88, 89], *L. casei*[85] etc.

As described above, prebiotic can be degrade and/or ferment by probiotics. Products from fermentation used as energy source of probiotics activity for promote growth and produce beneficial products for host's health. The action together of probiotics and prebiotics are called ''synbiotics''.[90]

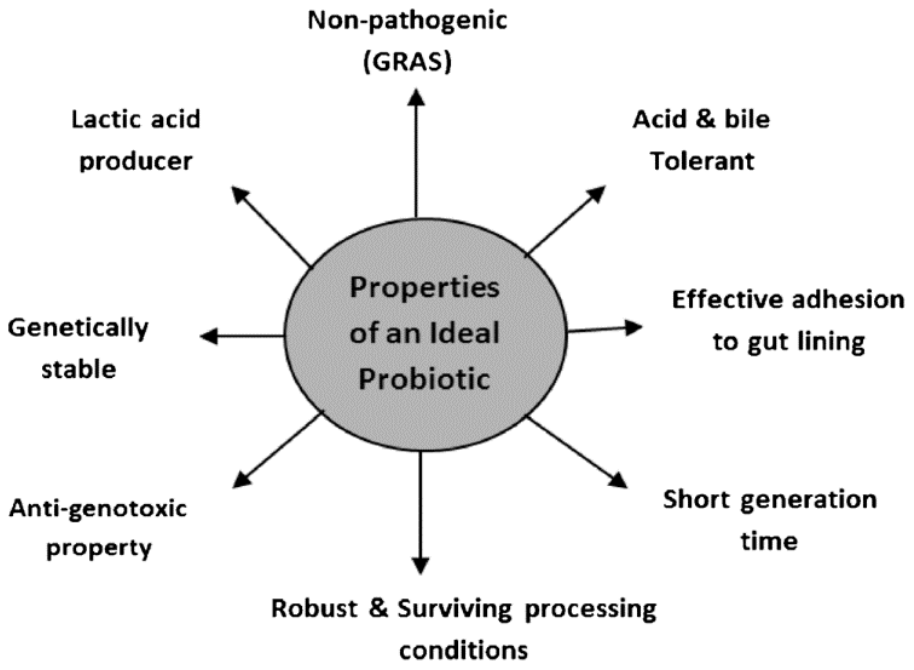


Figure 13. Ideal for probiotic strain

Pandey KR et al. (2015). Probiotics, prebiotics and synbiotics- a review.

4.4 Prebiotic effect on pathogens

Intestinal pathogens are infection caused by viruses, bacteria, or parasites that causes inflammation of the gastrointestinal tract (GI).[91] The characteristic symptoms such as excessive, watery diarrhea, stomach pain, bloating, constipation, nausea and vomiting, swallowing problems, weight gain or loss etc.[92] Many microbiota have reported that gut pathogens include Adenovirus, Astrovirus, Campylobacter, *Clostridium* sp., *Escherichia coli*, *Helicobacter pylori*, Rotavirus, Norovirus, *Salmonella enterica*, *Staphylococcus aureus*. For example, pathogenic strains of *E. coli* can absorb a unique sugars of intestinal tract.[93] *Salmonella*-induced inflammation of intestine cause to the tetrathionate produced by intestinal epithelial cells.[94] Prebiotics have not effect on gut pathogens.[95] But, the combination of probiotics and prebiotics can be change intestinal environment which unsuitable for growth of pathogens. For example most of prebiotics products are acidic which can be reduce the intestinal tract pH.[96, 97]

5. Application of prebiotics

In recent years, many research show beneficial of prebiotics for widely application. Especially, about prebiotics used for health benefits. Prebiotics plays role in intestinal health, that specific modified in complement and/or activity of gasrointestinal bacteria, leads to benefits on host health. The health outcome indicated that prebiotic consume may be able to decrease the inflammation or symptoms of intestinal included pathogens infection, prevent obesity, colon cancer, and increase absorption of minerals.[69]

5.1 Infectious diarrhea

In 2006, Konikoff et al; found neutrophil in both plasma and stool in patients with bowel disease after comsume of mixture of FOS-inulin which confirmed that prebiotics can be induce pro-inflammatory immune.[98] The mixture of 2'-fucosyllactose and GOS/FOS have potential to prevent Rotavirus-induced darrhea in children.[99] GOS have effect to adapt the gastrointestinal bacteria and enhanced the growth of

bifidobacteria improvements in patients are chronic gastrointestinal disorder (IBS).[100] Probiotic may be helpful in relieving symptoms of inflammation.[101] The galacto-oligosaccharide have potential for decrease the risk of diarrhea within 1 day and protection show after a week of treatment.[102]

5.2 Immunomodulation

The mechanism about the benefits of prebiotics on the immune system response in the digestion system has unclear but some evidence that possibility:[85, 103]

- Increasing of SCFAs product from prebiotic digested be able to control hepatic lipogenic enzymes production
- SCFAs production has been modulated of histone tail acetylation which increases genes expressed
- The fructooligosaccharides and some other prebiotics have shown increased white blood cell numbers
- Enhanced IgA secretion

Prebiotics may be improving immune function through by SCFAs production ex. acetate, propionate, butyrate.[104] In some study, SCFAs able to promoted natural killer cells function.[105]

5.3 Lipid metabolism

Many studies reported that prebiotics enhance the hypocholesteromic activity which control levels of choresterol in the blood.[106, 107] One study using inulin in hamsters result showed that 29% of total choresterol and 63% of triglycerides are decrease.[108] Chicory inulin can decreased serum triglycerides in humans with hyperchoresterolemia.[109] Other study demonstrated a 27% of triglycerides can decrease after using XOS in Sprague-Dawley rats.[110]

5.4 Other benefits

Typically, prebiotics as fibers have high energy and low fat which prevent the risk of overweight by improving satiety and weight.[111] In 2012, demonstrated that experimental study in mice between lean and obese groups about energy balance.[112] Recent study showed that prebiotic have potential to enhanced of

satiety, and suppress of hunger feel.[113] The macronutrients as Ca^{2+}, Mg^{2+}, $Fe^{2+/3+}$, K^{+} etc. are necessary for normal function of the body. Studies indicated that higher absorption of Ca^{2+} after fed rats treated inulin and FOS.[114] GOS/FOS mixture can be increase Ca^{2+}, P^{3+} and Mg^{2+} absorptions in a model of growing rats.[115]

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Microorganisms

Probiotic strains including *Lactobacillus plantarum* N25, *L. plantarum* TISTR 543, *L. casei* TISTR390 and *Bifidobacterium bifidum* TISTR2129 and pathogenic strains including *Escherichia coli* TISTR073, *Salmonella enterica* subs.*enterica* TISTR2519 and *Clostridium butyricum* TISTR1032 were purchased from Thailand Institute Scientific and Technological Research, Bangkok, Thailand.

Probiotic strains *L. plantarum* F33 and *B. adolescentis* JCM1275, pathogenic strains *Bacteroides vulgatus* JCM5826 and *C. hiranonis* JCM10541 were obtained from Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan.

1.2 Chemicals and reagents

The pCold-xynSW3 in *E. coli* DH5α were prepared from gene of *Streptomyces* sp. SWU10[54]. The pET32a-mans2 in *E. coli* BL21 (DE3) were prepared from gene of *Bacillus* sp. SWU60[71]. The HisTrap HP affinity chromatography column (5 mL) was obtained from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, UK). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

1.3 Animal

Male mice (BALB/cAJcl) as model study, all mice ages 5 weeks was purchased from National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom, Thailand. At animal housing in Faculty of Medicine, Srinakharinwirot university, all the mice were checked to prepare for the test in the cage about one week before treatment. All mice were cage (two, three mice per housed) in the room with 20-25^{o}C, and 12 h light/dark cycles.

2. Methods

2.1 Preparation and assay for prebiotic properties of xylooligosaccharide (XOS)

Flowchart for preparation and assay for prebiotic properties of XOS is shown in Figure 14.

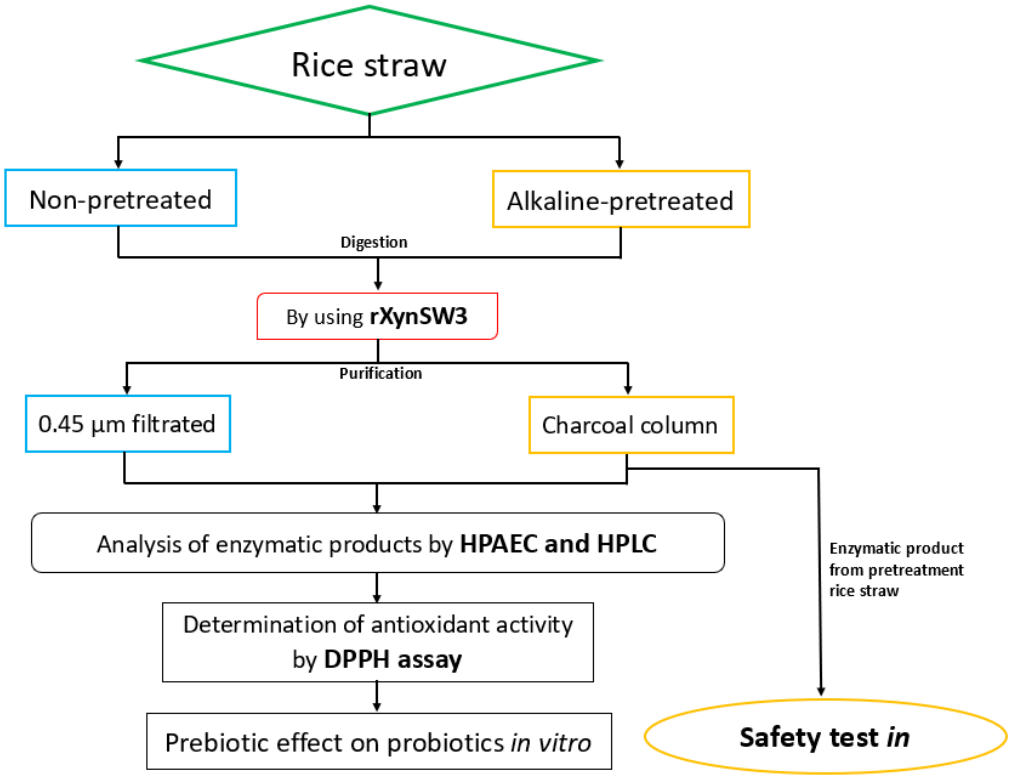


Figure 14. Flowchart of xylooligosaccharide (XOS) study

2.1.1 Rice straw preparation

Rice straw of *Oryza sativa* were collected from north-eastern part of Thailand. The straw was cut into 2-3 cm and extensively rinsed with water, then autoclaved and dried in an incubator at 60°C overnight, then grind to powder with size about 3-8 mm. Rice straw powder (3.5 mg) was pretreatment by incubating in 100 ml of 0.1 M KOH at 70 °C for 3 h, then washed using trap water, dried at 60°C for 4 h. The pretreatment rice straw was weight and used as substrate for xylanase enzyme.

2.1.2 The preparation of rXynSW3 enzyme

The plasmid comprising xylanase gene, pCold-xynSW3, in host *E. coli* DH5α[54] in glycerol stock (10 µl) was pre-activated in 10 ml of LB medium containing

ampicillin (50 µg/ml). Then, 10% of pre-activated was cultured in LB broth containing ampicillin (50 µg/ml) at 37°C for 16 h. The 10 ml of activated culture was added in 1000 ml LB broth containing ampicillin at 37°C and shaking at 250 rpm for 1 h. After that, 1 mM isopropyl β-thiogalactopyranoside (IPTG) added when the OD600 of the culture 0.5-0.6 nm, and continued incubation at 15 °C for 4 days. The cells were collected by centrifugation for 10 min at 5000 rpm, washed twice with 20mM KPB pH 6.0, dissolved the 1 g of pellet in 10 ml of 20mM KPB pH 6.0. The xylanase enzyme in the bacterial cell was extracted by sonication at 35% amplitude for 5 min then immediately on ice, repeated the steps in triplicate, the cell lysate was centrifuged for 10 min at 5000 rpm to collected the supernatant. The supernatant containing crude enzyme was further purified using His tagged bacterial protein purification kit in which the column had been equilibrated with 10 mM imidazole. Two milliliter of crude enzyme was added into the column incubated at 4oC for 45-60 min washed the unbound protein with 25 mM imidazole and eluted the enzyme that bound in column by using 5 column volume of 40 mM imidazone and passed the eluent through 30 kDa membrane centrifuge cut-off to get rid of imidazone. The purified enzyme was the dissolved in 20mM KPB pH 6.0.

2.1.3 Rice xylan degrading rXynSW3 and purification of XOS by charcoal column

One litter of pretreatment rice straw powder (2%) in 0.02 M KPB, pH 6.0 was incubation with rXynSW3 (7.8 U) at 45°C with shaking at 120 rpm for 24 h and boiled to inactive enzyme. Then, supernatant from enzyme degradation of rice straw (or hydrolysate) was filtrated through 0.45 µm membrane filter to get rid of the undigested rice straw. Then, the XOS in hydrolysate was purified by using activated charcoal column that had been equilibrated with 3 column volumes with double distilled water, the adsorbed XOS on charcoal was eluted by using 1-60% ethanol. Thereafter, the ethanol was removed by evaporation and freeze-dried. The purified XOS product was analyzed by using high performance anion exchange chromatography (HPAEC) with the conditions showed in Table 2.

Table 2. HPAEC condition for enzymatic products analysis.

Detection		Pulsed ampermetic detection
Injection volumn		10 μl
Column		CarboPac PA-1(4*250mm; Dionex)
Flow rate		1.0 ml/min
Solvent	0 - 5 min	0.1 M NaOH
	5 - 35 min	0.1 M NaOH, 0-0.45M sodium acetrate
	35 - 40 min	0.1 M NaOH, 0-0.45M sodium acetrate
	40 - 55 min	0.1 M NaOH

2.1.4 Determination of antioxidant activity by DPPH assay

Two groups of xylooligosaccharides was examined for antioxidant properties, XOS of pretreated rice straw and XOS combination with feruloyl-polysaccharide of non-pretreated rice straw groups. The pretreated group was the XOS that purified by charcoal column, and the XOS with degree polymerization 2-5 were collected for analysis. The non-pretreated group was the XOS that only passed through 0.45 μm membrane which contained tiny amount of XOS and large amount of feruloyl-polysaccharide. The solution of XOS of the pretreated groups was performed a serial dilution from 0 to 1000 μg of XOS in 1 ml of double distilled water, similarly the non-pretreated group was performed a serial dilution from 0 to 1000 μg of all products (XOS and feruloyl-polysaccharide) in 1 ml of double distilled water. One milliliter of each XOS solution was added into 1 ml of 0.1 mM DPPH solution (ratio 1:1), then incubated the solution at room temperature for 30 min in a dark. After that the absorbance of the solution was measured at a wavelength 517 nm using double distilled water with 0.1 mM DPPH as a blank. In addition, ascorbic acid was performed a serial dilution from 0 to 1000 μg in 1 ml double distilled water and was used as standard antioxidant. The experiment was done triplicate.

The measurement of the DPPH radical scavenging assay for the analytical sample at each concentration was performed by the IC_{50} calculation with ascorbic acid as a positive control and repeated in triplicate. The IC_{50} calculated equation was as followed; %Antioxidant activity = 1- (Abs of Sample / Abs of Blank) *100

2.1.5 Determination of reducing sugar in enzymatic products

The dinitrosalisylic acid (DNS) method used for the quantitative determination of reducing sugars in degradation products related to enzyme activity when the enzyme hydrolyse the bond between sugar molecules in backbone of polysaccharides and/or oligosaccharides.

This study the measurement of reducing sugar content in enzymatic products is widely used by in many researches because it is easy, fast, and safe. This method is a color measurement technique consisting of redox reactions between DNS and reducing sugar in samples which the reducing power come from carbonyl group (C=O). The redox reaction causing the DNS (yellow) when dissolved change to the 3-amino-5-nitrosalicylic acid (orange-brown) in an alkaline condition. The color intensity is proportional to the concentration of reducing sugars in the hydrolysate which measure by spectrophotometer at 570 nm. In addition, each type of reducing sugars at the same concentration affects the color of the reaction unequal. Therefore, it must have a standard graph of each type of sugar.

The sample analysis was performed by centrifugation the hydrolysate at 12,000 rpm for 5 minutes and pipetted 1 ml the clear portion mixed of DNS solution (ratio 1:1), then boil for 10 min, put in the ice immediately for 5 min, measured the absorbance at 570 nm (Table 3 and Fig.15).

Table 3. Preparation of each xylose concentration.

Tube no.	mg/ml	Distilled water (µl)	Final conc. (g/ml)
1	0	1000	0
2	50	950	50
3	100	900	100
4	200	800	200
5	300	700	300
6	400	600	400
7	500	500	500

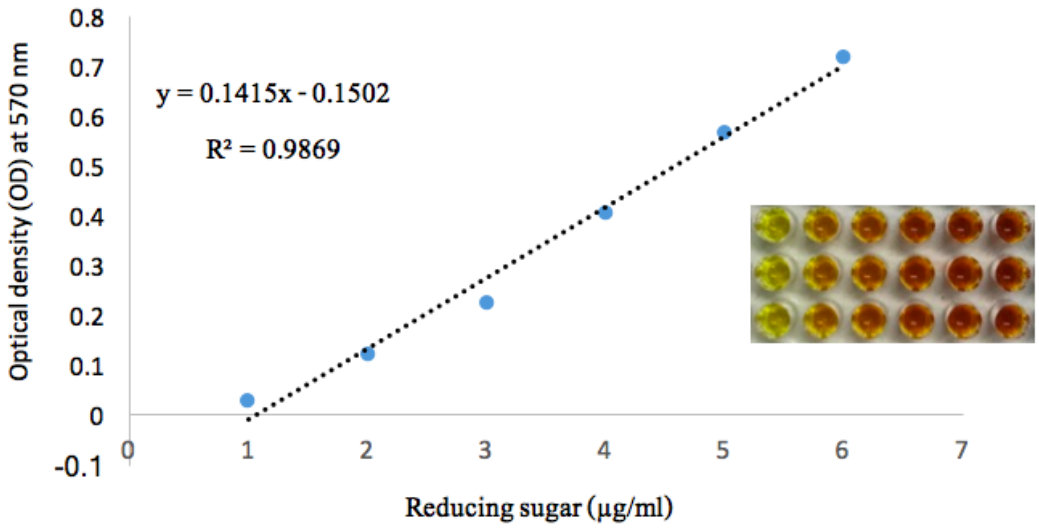


Figure 15. Calibration curve of each xylose concentration at 570 nm by using dinitrosalicylic acid (DNS) assay.

2.1.6 Prebiotic effect on probiotics

2.1.6.1 Preparation of microorganisms

One hundred microliter of each bacteria (probiotic and pathogen bacteria) in glycerol stock was added into 5 ml MRS broth, incubated at 37°C for 48 hours. These activate culture were streaked on plates and incubated at the conditions mentioned above, inoculated of single colony into 5 ml MRS broth for used as the initial stock for study.

2.1.6.2 Preparation culture for products of non-pretreatment rice straw tested

To study the prebiotic activity of the enzymatic products from non-pretreatment rice straw on probiotics, 50 µL of overnight cultures of both probiotic strains, *L. plantarum* F33 and *B. adolescentis* JCM1275, and both pathogenic strains, *B. vulgatus* JCM5826 and *C. hiranonis* JCM10541, were cultivated added into 50 mL of MRS media (probiotic) and LB (pathogen) and incubated at 37°C for 12 h in an anaerobic condition. The growth and condition of culture media was assessed by determining the OD600 and pH values at 2 h intervals

2.1.6.3 Preparation culture for products of pretreatment rice straw tested

To study the prebiotic activity, culture media was basal medium [peptone 2.0 g, yeast extract 2.0 g, K_2HPO_4 0.04 g, KH_2PO_4 0.04 g, $MgSO_4$.7H2O 0.01 g, NaCl 0.1 g, Tween 80 0.5 g and L-cysteine HCl 0.5 g dissolved in 1 L of water, adjusted to pH 7.2] supplemented with xylooligosaccharide (with degree of polymerization range of 2 - 5 molecules) as a carbon source (final conc. 10 mg/ml). Then 50 µL of overnight cultures of all probiotic strains, *L. plantarum* TISTR 543, *L. casei* TISTR390 and *B. bifidum* TISTR2129, all pathogenic bacteria, *E. coli* TISTR073, *S. enterica* subs.*enterica* TISTR2519 and *C. butyricum*, were added into 5ml of culture media and cultivated anaerobically at 37°C for 48 h. Growth was assessed by determining the OD_{600} values at 0, 4, 8, 16, 24 and 48 h.

2.1.7 Safety test *in vivo*

-Toxicity study

For acute toxicity study, 20 male BALB/cAJcl mice were used for a study. All mice aged 5 weeks were detained to investigate abnormality and adaptation at the laboratory for 1 week (Animal housing, Faculty of Medicine, Srinakharinwirot university), with feeding formula 082G and drinking water all the time (Ab lib). The mice were divided into 3 experimental and 1 control groups, or 5 mice per group (n = 5). The concentration of the substance used in the experiment was referenced from the study data of lethal dose 50% (LD50) of fructooligosaccharide which is a substance that cannot digested by enzymes in the body as XOS. The previous toxicity studies found that the maximum tolerance dose (MTD) was 9,000 mg/kg per day.[116] Therefore, this experiment was conducted by division the mice into 4 groups with different dose of XOS as follows,

Group 1 (Control): Double distilled water

Group 2 (Low dose): XOS 2000 mg/kg per day

Group 3 (Mid dose): XOS 5000 mg/kg per day

Group 4 (High dose): XOS 9000 mg/kg per day

Each group was administered by oral (gavage), once a day for 14 days. The solution was prepared by dissolving the xylooligosaccharide of each concentration (2000, 5000, 9000 mg/kg per day) with 0.5 ml double distilled water. All mice were observed the symptoms including sick or death of mice. At 15th day of the study, the mice were euthanized by isoflurane inhaled anesthesia and the blood was obtained from the coronary arteries. The parameters of hematology, and clinical chemistry for blood analysis are shown in table 4.

Table 4. Parameters measured for hematology and clinical chemistry analysis.

Parameters	Test	
Hematology	Hematocrit (Hct)	
	Hemoglobin (Hb)	
	Mean corpuscular hemoglobin concentration (MCHC)	
	Mean corpuscular hemoglobin (MCH)	
	Mean corpuscular volume (MCV)	
	Red blood cell distribution width (RDW)	
	Platelet count	
	Red blood cell (RBC)	
	White blood cell (WBC)	
	Neutrophil	
	Lymphocyte	
	Monocyte	
	Eosinophil	
	Basophil	
Clinical chemistry	Evaluation of kidney function	BUN
		Creatinine
	Evaluation of liver function	Total protein
		Albumin
		Globulin
		A/G ratio

2.1.8 Statistical analysis

The results were performed as mean ± S.D. (S.E.) for at least three times experiments. Data were analyzed using SPSS for Windows (version 26.0).

2.2 Preparation and prebiotic properties assay of mannooligosaccharide (MOS)

Flowchart for preparation and prebiotic properties assay of MOS is shown in Figure 16.

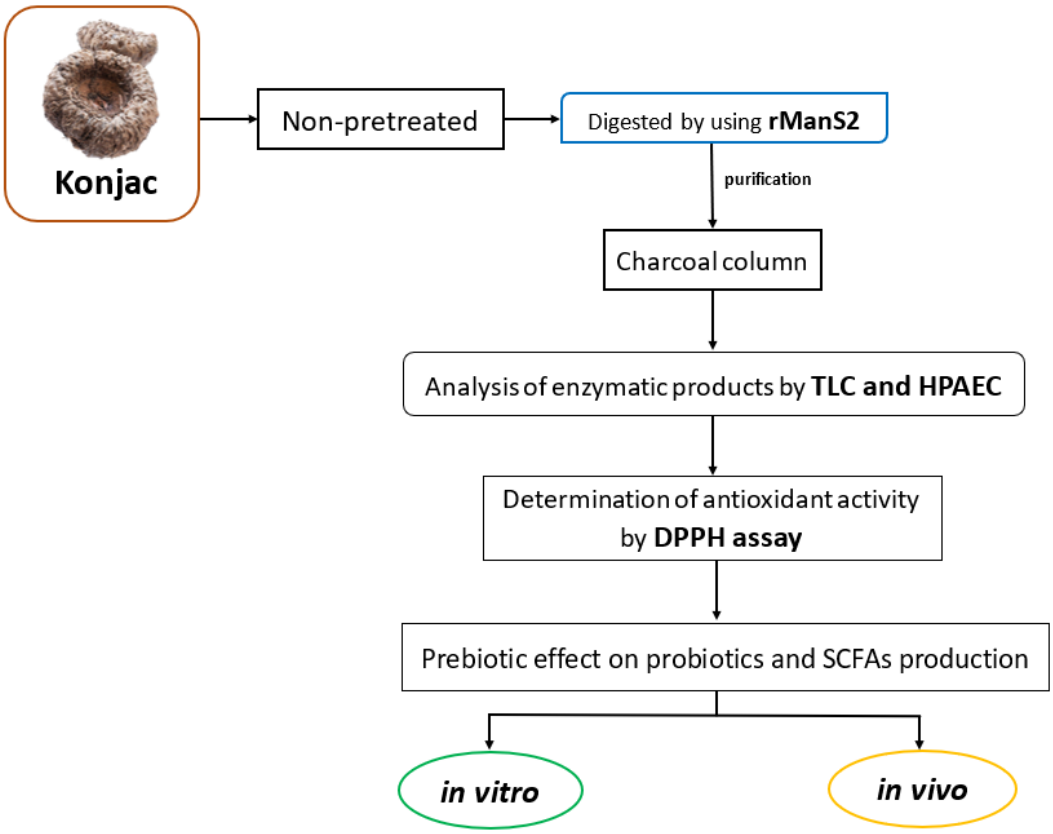


Figure 16. Flowchart of mannooligosaccharide (MOS) study

2.2.1 Production and purification of 1,4-β-mannanase enzyme

The pET32a-manS2 constructed in *E. coli* BL21 (DE3) was inoculated into 10 ml of LB medium containing antibiotic (ampicillin, 50 µg/ml), incubated at 37°C with shaking at 220 rpm for 16 h. Then, added pre-culture into 1 L of LB medium containing ampicillin, and incubated in same conditions for 1.30 h or OD_{600} between 0.6-0.8. After that, the gene expression was induced by using 100 ml IPTG (0.1 mM as final concentration) and continued incubation for 3 h. The pellet was harvested by centrifugation at 5000 rpm for 20 min, rinsed with 20 mM potassium phosphate, pH 6.0 for 2 times. Then, the enzyme was extracted from the cells by sonification. The mannanase activity was examined by using 0.02% AZCL-galactomannan, specific

synthetic substrate, that dissolved in 20 mM KPB pH 6.0, then incubated at 60°C for 30 min. The solution with mannanase activity, after reaction the solution changed to blue color.

2.2.2 Preparation of glucomannan from Konjac and digestion with rManS2

Konjac was cleaned with water and peeled. After that, Konjac was slice and rinsed with distilled water. Then, dried in incubator at 60°C for 2-3 days or until absolutely dry. Then, grind to powder using blender and kept the Konjac powder in dry cabinet. The Konjac powder was dissolved in 0.02 M potassium phosphate buffer pH 6.0, the concentration 1.0 % and autoclave at 121°C for 15 min as substrate solution. Enzyme digestion was performed by using 1 L of 1% Konjac substrate solution, added rManS2 (7 U) and incubated at 40°C for 24 h. The reaction was stopped by boiling for 10 min and filtrated through 0.45 µm membrane filter.

2.2.3 Purification of mannooligosaccharides

- **Purification of Konjac-oligosaccharide with activated charcoal column**

Seven grams of activated charcoal was rinsed using 20% ethanol followed by 10% ethanol and then with distilled water for 3 times. Then, packed the activated charcoal into the column sized 3 cm diameter, equilibrated with distilled water 2-3fold of column volume with flow rate of 1 ml/min. Then, add the hydrolysate of enzyme digestion into the column and change the flow rate to 2 ml/min. After that, wash with distilled water, eluted with linear gradient of ethanol from 1% to 60%, and10 ml of eluent per fraction was collected. Total sugar content in each fraction was examined by using phenol-sulfuric acid method and the solution from positive fractions were combined. Then, the ethanol was removed by evaporation and the Konjac-oligosaccharide was dried using freeze-dry

- **Analysis for degree polymerization of Konjac-oligosaccharide**

The degree polymerization of Konjac-oligosaccharide was examined by using thin layer chromatography (TLC) that separated the digested products by size when compared with standard sugar by using water: acetic acid: butanol at the ratio of 1:1:3 as a mobile phase. The standard sugar for comparison were mannose,

mannobiose, mannotriose, and mannotetraose. The separated products on the TLC plate were detected by dipping the plate in the solution containing methanol and sulfuric (90:10), then air-dried and heat until the brown spots appeared on the TLC plate. The quantity and sugar composition of the enzymatic products were analyzed by using High Performance Anion Exchange Chromatography (HPAEC) with pulsed amperometric detection (PAD). The HPAEC condition was showed in Table 5.

Table 5. HPAEC condition of enzymatic products analysis.

Detection		Pulsed ampermetic detection
Injection volumn		10 µl
Column		CarboPac PA-1(4*250mm; Dionex)
Flow rate		1.0 ml/min
Solvent	0 - 5 min	0.1M NaOH
	5 - 35 min	0.1M NaOH, 0-0.45M sodium acetrate
	35 - 40 min	0.1M NaOH, 0-0.45M sodium acetrate
	40 - 55 min	0.1M NaOH

2.2.4 Assay of antioxidant activity by DPPH assay

Mannooligosaccharide sample was performed a serial dilution from 0 to 1000 µg of MOS in 1 ml double distilled-water. Thereafter, the 0.2 mL of each dilution of mannooligosaccharide sample was mixed with 0.8 ml of 100 mM Tris-HCl (pH7.4) in test tube and then 1 ml of the DPPH solution was added, immediately mixed with a vortex for 10 s and left at room temperature for 30 min in the dark. After 30 min, the absorbance of each solution was measured at 517 nm. A mixture of ethanol (1.2 ml) and 100 mM Tris-HCl, pH 7.4 (0.8 ml) was used as the blank.

The measurement of the DPPH radical scavenging assay for the mannooligosaccharide sample at each concentration was performed by calculated the IC_{50} using equation when ascorbic acid was used as a positive control and the experiment was carried out triplicate.

2.2.5 Study of prebiotic properties *in vitro*

2.2.5.1 Preparation of culture media

The media used to study of SCFAs production and growth promotion of probiotics was basal medium. The 1 L of basal medium was prepared from peptone (2.0 g), yeast extract (2.0 g), K_2HPO_4 (0.04 g), KH_2PO_4 (0.04 g), $MgSO_4.7\ H_2O$ (0.01 g), NaCl (0.1 g), Tween 80 (0.5 g), and L-cysteine HCl (0.5 g), adjusted to pH 7.2 and added MOS (concentration is 10 mg/ml) or FOS (concentration is 10 mg/ml) as carbon source, then autoclave at 121°C for 15 min.

2.2.5.2 Preparation of probiotics

The probiotics used was *L. plantarum* N25, 50 µl of glycerol stock was activated in 5 ml MRS broth in an anaerobic condition at 37 °C for 48 h. Then, the seedling culture of probiotic bacteria was prepared by culture in the MRS broth at the same conditions for 24 h, then centrifuged at 13,000 rpm for 10 min to eliminate culture media. The bacteria cells were washed and adjusted volume by adding 0.85 % NaCl to obtain 1 OD of absorbance at 600 nm.

2.2.5.3 Study of the promotion of probiotics growth *in vitro*

One milliliter of seedling cultured from 2.2.5.2 was transferred to 5 ml of culture medium to obtain the starter culture of probiotic at 0.2 OD, then incubated at 37 °C for 24 h. The culture was sampling at 0, 6, 12, 24 h, and measured OD at 600 nm to compare the growth of probiotic. The supernatant at 0, 24, 48 h of culture were analyzed for SCAFs production by using HPLC.

2.2.5.4 Production of short chain fatty acids (SCFAs) *in vitro*

The culture of probiotic at 0, 24, 48 h were collected for analysis of SCAFs including acetic, propionic, butyric and lactic acid. The culture was centrifuged at 13000 rpm for 5 min, the supernatant was filtrates through 0.22 µm paper filter. The analysis of SCAFs was achieved by high performance liquid chromatography, using a

300*8.0 mm Shodex SUGAR SH1011 column (Showa denko, Tokyo, Japan) and used 5 mM H_2SO_4 as eluent solvent (1 ml/min as flow rate).

2.2.6 Properties of prebiotic in animal experiment

In this study, 20 male BALB/cAJcl mice were used in the experiment. All mice aged 5 weeks were detained to investigate abnormality and adaptation at the laboratory (Animal housing, Faculty of Medicine, Srinakharinwirot university) for 1 week, with feeding formula 082G and drinking water all the time (Ab lib). The 20 mice were divided into 3 experimental and 1 control groups, or 5 mice per group (n = 5) follows,

Group 1 (Control): PBS buffer pH 7.0

Group 2 (Prebiotic): Mannooligosaccharide in PBS pH 7.0

Group 3 (Probiotic): *L. plantarum* N25 in PBS pH 7.0

Group 4 (Synbiotic): *L. plantarum* N25 and Mannooligosaccharide in PBS pH 7.0

Each group treated by oral gavage administration, once a day for 14 days. The MOS solution was prepared for feeding dose at 1000 mg/kg per day, and *L. plantarum* N25 was prepared at 10^8-10^9 cells or the OD_{600} range 0.8-1.0 and dissolved in phosphate pH 7.2. The volume that mice can accept was 0.5 ml. All experiments were based on the National Research Council Guidelines of Laboratory Animals.[123] All mice were observed the symptoms including body weight and death of mice and they were euthanasia on 15th day using isoflurane and feces was collected from the caecum for analysis of SCFAs production and the number of probiotics *in vivo*.

2.2.6.1 The study of the increase of probiotics in experimental animals

The feces from the caecum (0.1 g) were diluted from 10^{-1} to 10^{-3} by using PBS buffer pH 7.2. Then, 0.1 ml of diluted samples were spreaded on MRS plates, and incubated at 37 ^{o}C for 48 h. Each dilution was performed triplicate.

2.2.6.2 Extraction for analyzed of SCAFs production from feces

The experiment was performed by adding 0.5 mM H_2SO_4 into feces sample with ratio of 2 ml:5 mg. Then, mixed solution by using pipette and sonicated for 30 sec, and centrifuged at 13000 rpm for 20 min. After that filtrated through 0.22 mm paper filter and the supernatant was used for analysis of SCFAs included acetate, propionate, lactate and butyrate products by using HPLC.

2.2.7 Statistical analysis

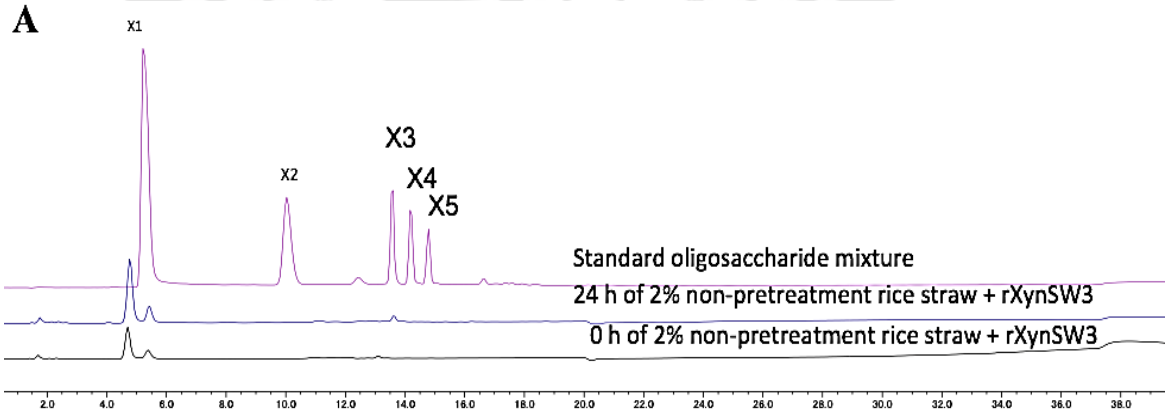
The results were performed as mean $\pm$ S.D. (S.E.) for at least three times experiments. Data were analyzed using SPSS for Windows (version 26.0). Differences were considered significant at $P<0.001$.

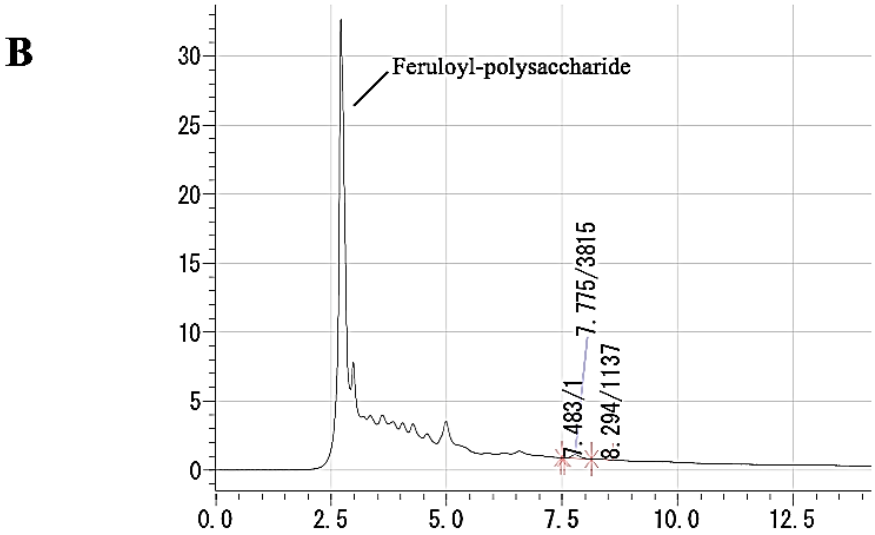
CHAPTER IV
RESULTS

Part 1: Xylooligosaccharide (XOS)

Degradation of rice xylan by rXynSW3

HPAEC analysis of enzymatic products of non-pretreatment rice straw after digestion by rXynSW3 for 24 h showed that there was small amount of xylooligosaccharides (Fig. 17A). However, analysis found that the product contained ferulic acid indicated that they are feruloyl-polysaccharides (Fig. 17B). The enzymatic products of alkaline-pretreatment rice straw after degradation by rXynSW3 for 24 h showed that the products were xylooligosaccharides included xylobiose, xylotriose, xylotetraose, and xylopentose (Fig. 17C).





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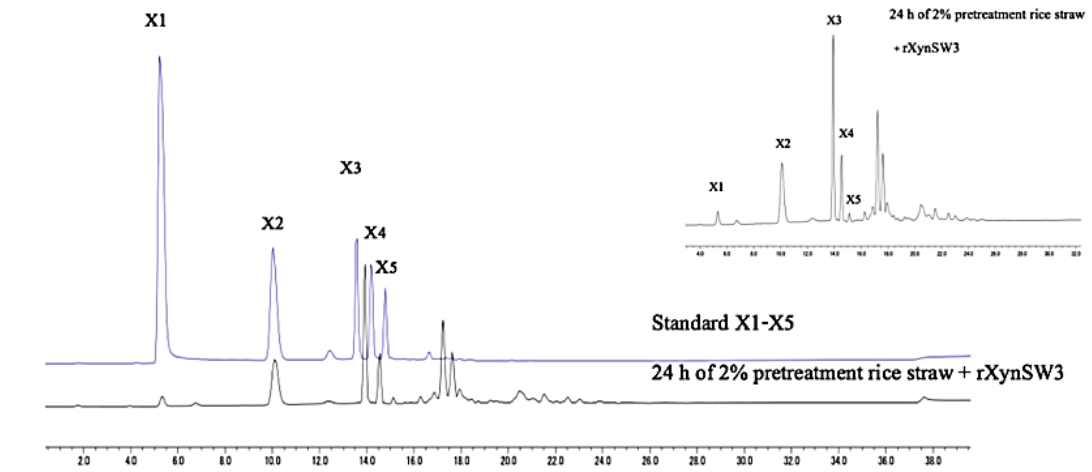

Figure 17. HPAEC analysis of enzymatic products (A) non-pretreatment rice straw with rXynSW3 for 24 h and (B) HPLC analyzed of the enzymatic product from non-pretreatment rice straw (C) HPAEC analysis of enzymatic products from alkaline-pretreatment rice straw with rXynSW3 for 24 h. The standard xylose and xylooligosaccharides are X1, X2, X3, X4; xylose, xylobiose, xylotriose, and xylotetraose, respectively.

Antioxidant property of xylooligosaccharides

The results showed that the IC_{50} values of non-pretreated and alkaline-pretreated rice straw was 611.10 ± 0.52 µg/ml and 542.27 ± 0.59 µg/ml, respectively, whereas the antioxidant activity of the standard ascorbic was a 494.72 ± 0.15 µg/ml (Fig. 18).

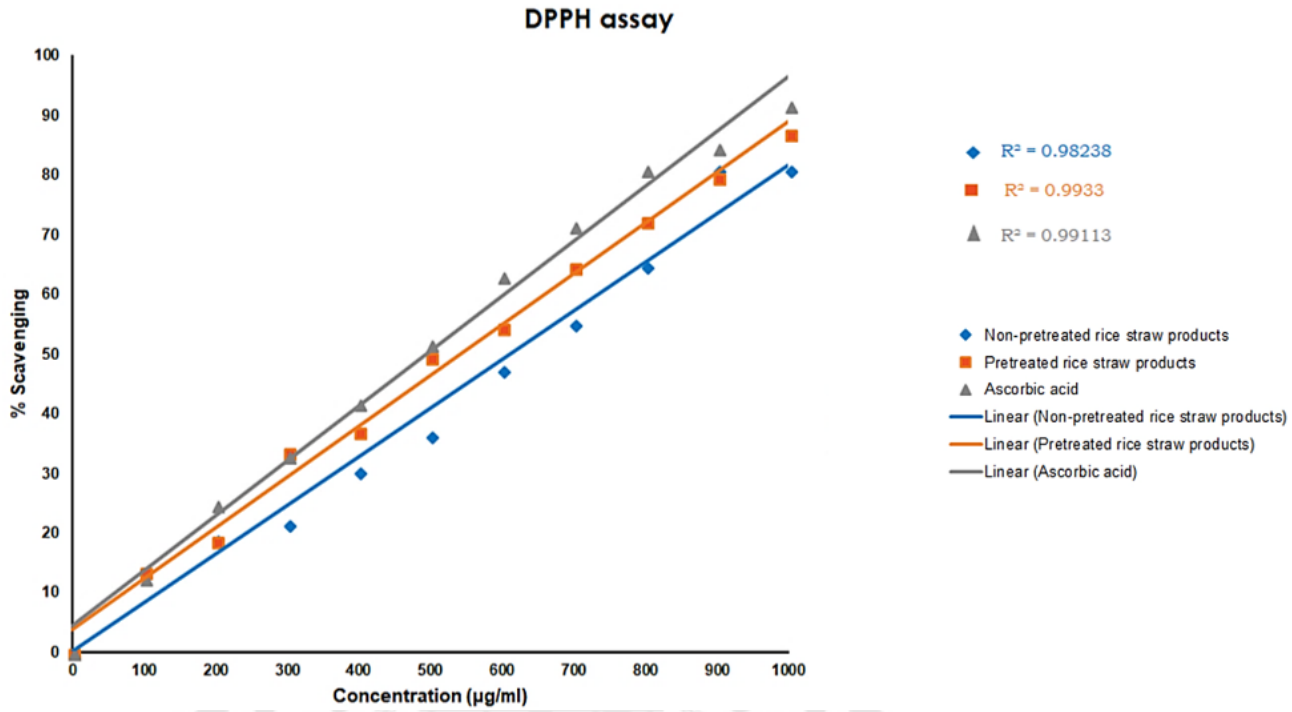

Figure 18. Percent scavenging by DPPH assay; (◆) Non-pretreated rice straw products, (■) Pretreated rice straw products, and (▲) Ascorbic acid.

Prebiotic effect of enzymatic products on probiotics

-Non-pretreated rice straw on probiotics

After incubation of the enzymatic products from non-pretreated rice straw which contained majority of feruloyl-polysaccharides and tiny amount of XOS with probiotics and pathogenic bacteria including *L. plantarum* F33, *B. adolescentis* JCM1275, *B. vulgatus* JCM5826, and *C. hiranonis* JCM10541, the results showed that the enzymatic products promoted growth of *L. plantarum* F33 (Fig. 19 A) and *B. adolescentis* JCM1275 (Fig. 19 C) and lowered the pH values of the culture media of both probiotic strains (Fig. 19 B, D, respectively). However, the enzymatic products have no affect on growth rate and pH values of pathogenic bacteria comprising *B. vulgatus* JCM5826 (Fig. 20 A, B) and *C. hiranonis* JCM10541 (Fig. 20 C, D).

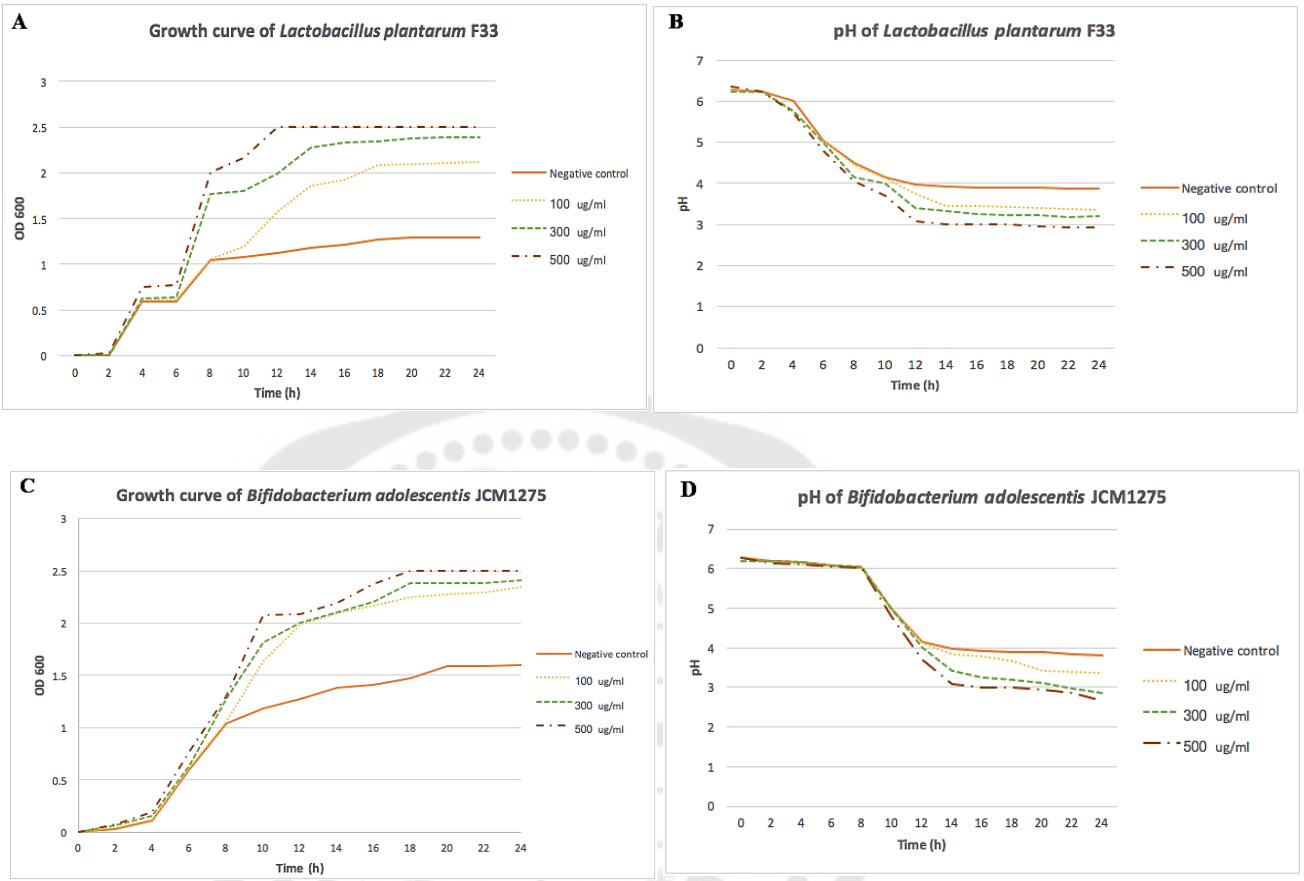

Figure 19. Growth curve of probiotics and pH values of MRS media with the enzymatic products from non- pretreated rice straw; *L. plantarum* F33 (A, B), and *B. adolescentis* JCM1275 (C, D), the MRS medium without enzymatic products was used as negative control.

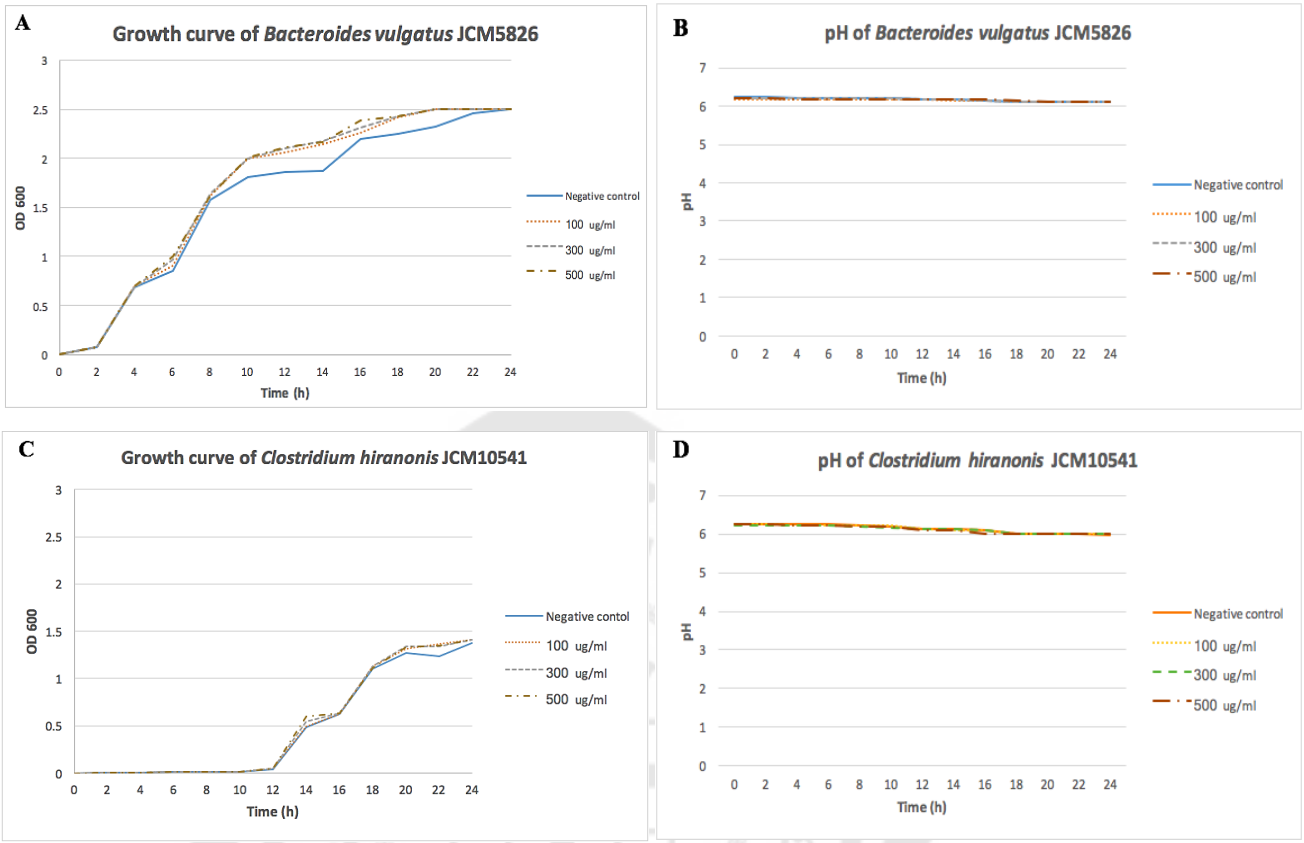

Figure 20. Growth curve of pathogens and pH values of MRS media with the enzymatic products of non-pretreated rice straw; *B. vulgatus JCM5826* (A, B), and *C. hiranonis* JCM10541 (C, D), the MRS medium without enzymatic products was used as negative control.

-Alkaline-pretreated rice straw on probiotics

The XOS products with degree polymerization 2-5 from degradation of pretreated-rice straw with rXynSW3 were incubated with probiotic including *L. plantarum* TISTR543, *L. casei* TISTR390, *B. bifidum* TISTR2129, and pathogenic bacteria comprising *E. coli* TISTR073, *S. enterica* subs. enterica TISTR2519, *and C. typhimurium* TISTR1032. The results showed that the XOS promoted growth of all probiotic strains (Fig. 21 A, B, C) but have no affect on growth of all pathogenic strains (Fig. 22 A, B, C).

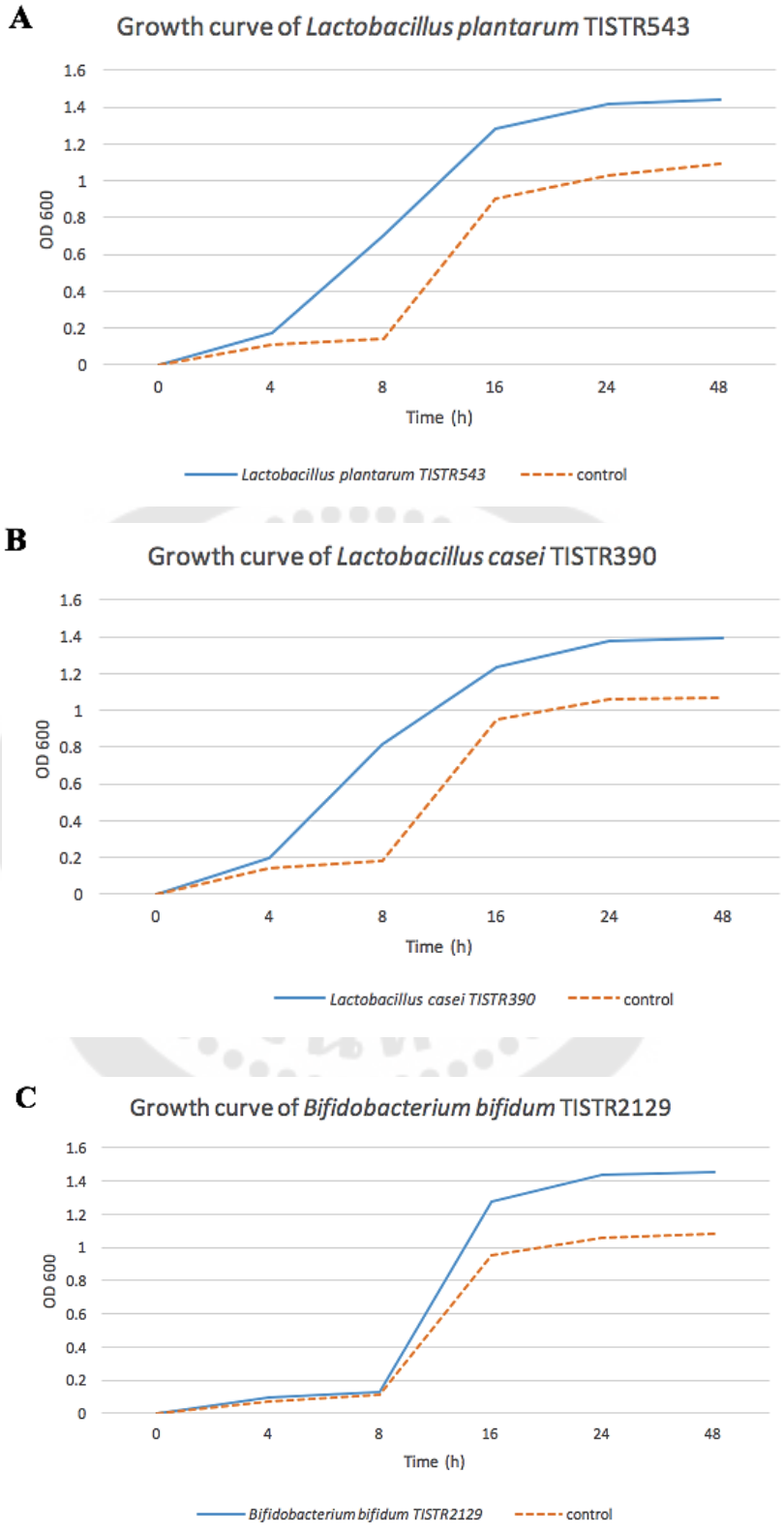

Figure 21. Effect of XOS on probiotics (A) Growth curve of *L. plantarum* TISTR543, (B) Growth curve of *L. casei* TISTR390, and (C) Growth curve of *B. bifidum* TISTR2129, the basal medium without XOS sample was used as control.

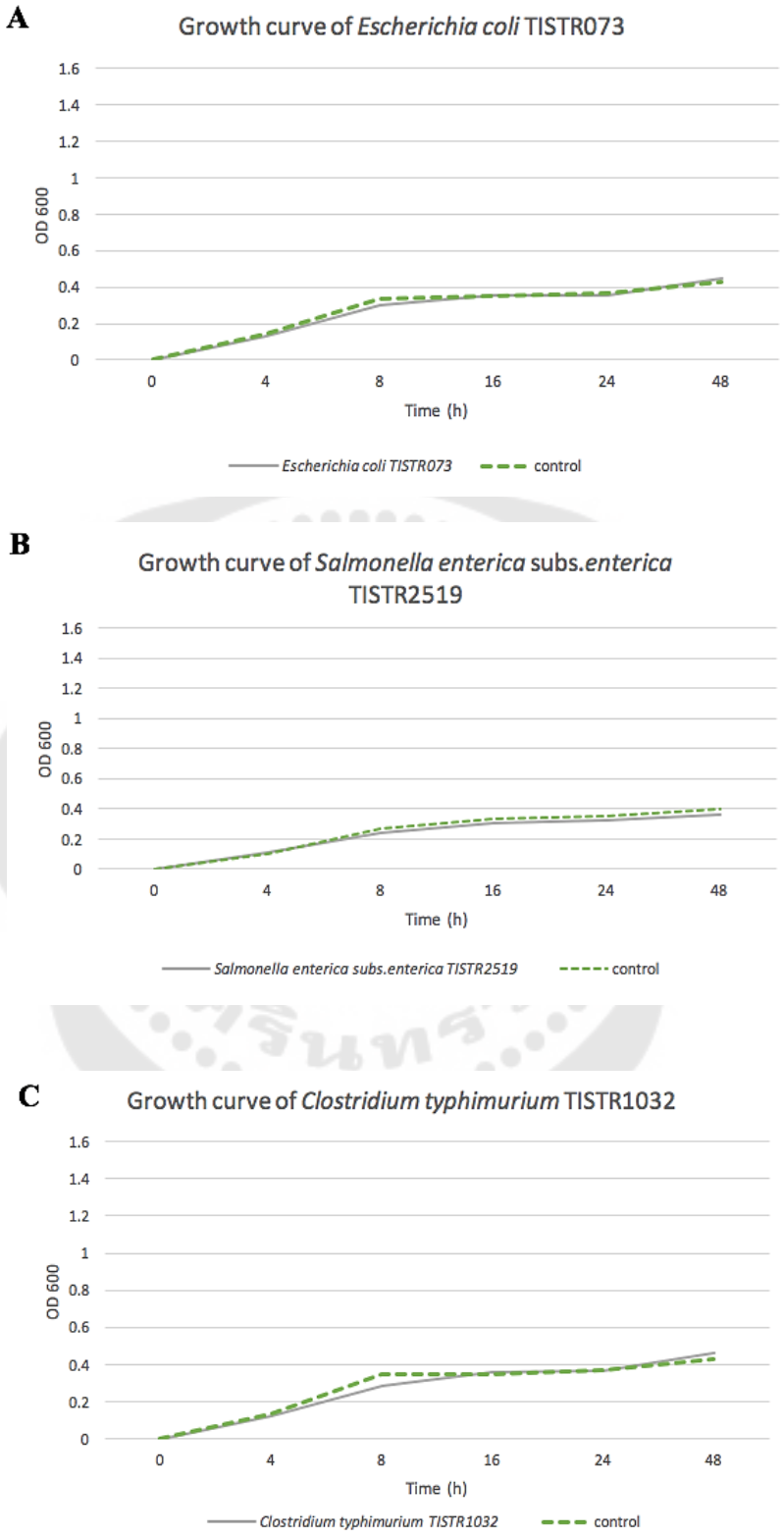

Figure 22. Effect of XOS on pathogenic bacteria (A) Growth curve of *E. coli* TISTR073, (B) Growth curve of *S. enterica* subs.*enterica* TISTR2519, and (C) Growth curve of *C. typhimurium* TISTR1032, the basal medium without XOS sample was used as control.

In vivo testing for safety of the XOS from pretreated-rice straw

-Body weight of experimental mice

In the safety experiment, the mice in each group consumed XOS samples with the following dose; 2000, 5000, and 9000 mg/kg per day by oral administration. The mean body weight values of mice are shown in Table 6 and growth curves of mice from 0 to 14 days of experiment are presented in Figure 23. The results demonstrated that all doses of XOS were unaffected on body weight of mice compared with the control group.

Table 6. Mean of body weight (g) values of mice treated with the XOS from rice straw.

Group	Days		
	0	7	14
Control (double-distilled water)	24.58 ± 1.11	27.19 ± 2.00	27.91 ± 1.24
Low dose (2000 mg/kg per day)	24.56 ± 0.89	27.85 ± 0.84	28.07 ± 1.23
Mid dose (5000 mg/kg per day)	24.32 ± 0.69	27.25 ± 0.77	27.41 ± 0.96
High dose (9000 mg/kg per day)	24.20 ± 0.96	27.54 ± 2.09	27.86 ± 1.68

*Mean of body weight (g) = mean ± S.D.

**Experiments were performed in triplicate.

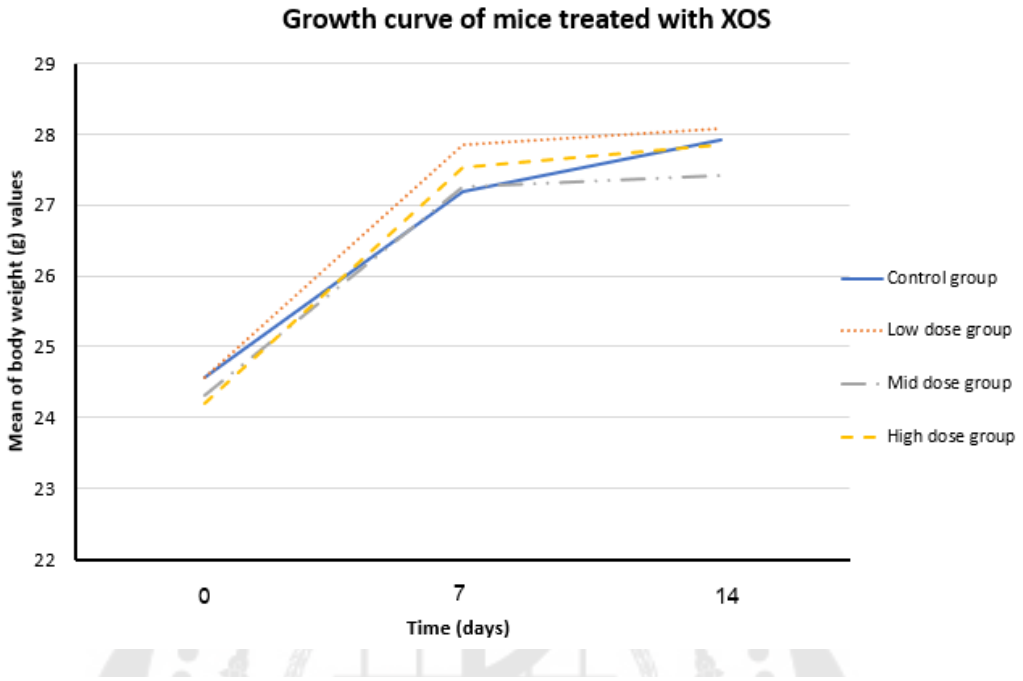

Figure 23. Growth curve of mice daily feeding with XOS (mgXOS/kg per day); control (double-distilled water), low dose (2000) , mid dose (5000), and high dose (9000) group. XOS: xylooligosaccharide.

-Blood testing

The mean value of each parameter of mice blood from hematological and clinical chemistry test are demonstrated in Table 7. The results showed that mice-treated high dose group showed normal of test-related effects on hematology and clinical chemistry values in both the kidney and liver during the experimental period compared to the control group.

Table 7. Mean values of hematology and clinical chemistry.

	Group		Control	High dose
	Dose mg XOS/kg per day		0	9000
	N		5	5
Hematology	Hematocrit, Hct (g/dl)		52.50 ± 2.12	51.83 ± 1.94
	Hemoglobin, Hbg (%)		15.45 ± 0.21	15.00 ± 0.68
	Mean corpuscular hemoglobin concentration, MCHC (g/dl)		29.60 ± 0.71	30.13 ± 0.69
	Mean corpuscular hemoglobin, MCH (pg)		15.35 ± 0.21	13.12 ± 0.60
	Mean corpuscular volume, MCV (fl)		52.30 ± 0.14	51.17 ± 0.33
	Red blood cell distribution width, RDW (%)		19.60 ± 0.00	19.25 ± 0.54
	Platelet count		104.25 ± 0.01	101.83 ± 0.51
	Red blood cell, RBC		10.07 ± 0.29	9 9.70 ± 0.39
	White blood cell, WBC		4.55 ± 0.09	3.30 ± 0.48
	Neutrophil (%)		13.50 ± 0.54	12.33 ± 0.94
	Lymphocyte (%)		85.00 ± 2.83	84.67 ± 0.44
	Monocyte (%)		1.50 ± 0.71	1.83 ± 0.75
	Eosinophil (%)		0.00	1.17 ± 0.98
	Basophil (%)		0.00	0.00
Clinical chemistry	Evaluation of kidney function	BUN (mg/dl)	19.5 ± 0.71	20.11 ± 0.75
		Creatinine (mg/dl)	0.22 ± 0.10	0.19 ± 0.02
	Evaluation of liver function	Total protein (g/dl)	4.95 ± 0.07	4.68 ± 0.15
		Albumin (g/dl)	3.15 ± 0.07	3.12 ± 0.18
		Globulin (g/dl)	1.80 ± 0.14	1.54 ± 0.18
		A/G ratio	1.75 ± 0.13	2.03 ± 0.33

* Clinical chemistry values = mean ± S.D.

**Experiments were performed in triplicate.

Part 2: Mannooligosaccharide (MOS)

MOS preparation from Konjac degrading by rManS2 enzyme

Figure 24A showed the enzymatic products of Konjac with rManS2 at 0 h and 24 h analyzed by using HPAEC. The MOS products from Konjac at 24 h of degradation were mannose, mannobiose, mannotriose, mannotetraose, and mannopentose gluco-mannotetraose in which mannobiose was the highest amount, follow by gluco-mannotetraose (Fig. 24B and Table 8).

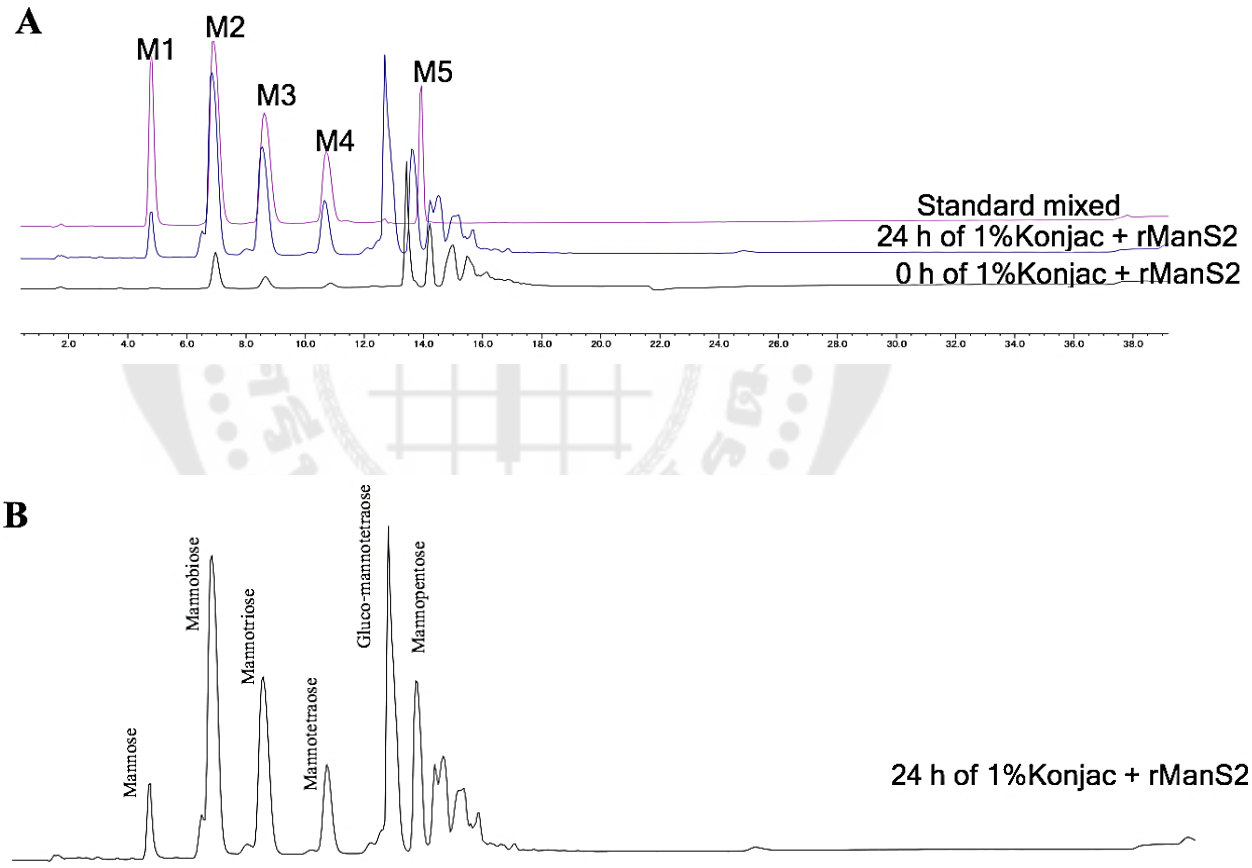

Figure 24. HPAEC analysis of enzymatic products (A) 1%Konjac with rManS2 at 0, 24 h and (B) 1%Konjac with rManS2 at 24 h. M1, M2, M3, M4, and M5; mannose, mannobiose, mannotriose, mannotetraose, and mannopentose, respectively.

Table 8. Percent yield of MOS from degradation Konjac by rManS2 enzyme.

Products	(%) Yield of product
Mannose	4.32
Mannobiose	32.01
Mannotriose	17.70
Mannotetraose	7.50
Gluco-mannotetraose	25.63
Mannopentose	12.84
Total MOS from Konjac	**100**

Assay of antioxidant activity by DPPH method

DPPH radical scavenging assay of MOS products from Konjac degradation found that the IC_{50} of MOS was 643.06 ± 0.05 µg/ml whereas the antioxidant activity of the standard ascorbic was a 494.72 ± 0.15 µg/ml. *IC_{50} value is the concentration causing 50% free radical inhibition (Fig. 25).

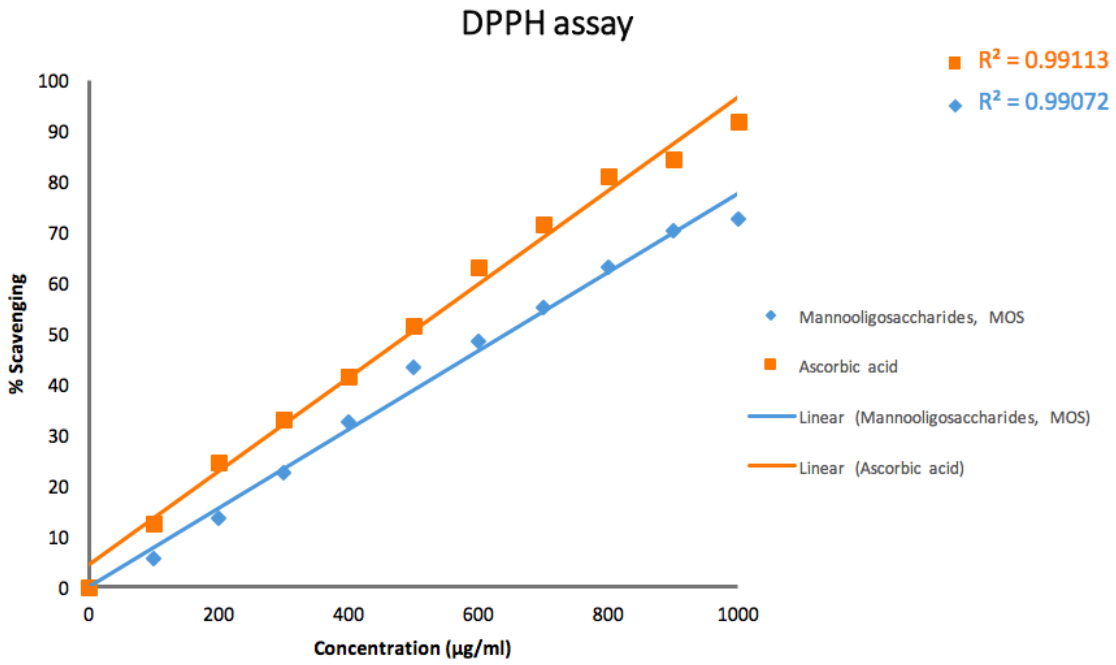

Figure 25. Percent scavenging by DPPH assay; (◆) MOS from Konjac, and (■) Ascorbic acid.

Prebiotic effect of MOS from Konjac on probiotics

After investigating the prebiotic efficiency of MOS from Konjac on *L. plantarum* N25 by focusing on the growth-promoting. The results showed that the growth of *L. plantarum* N25 in basal medium with MOS significantly higher than basal medium without MOS ($P<0.001$). However, it was significantly lower than the basal medium with FOS ($P<0.001$), which indicated that the MOS can promote the growth of *L. plantarum* N25 but lower than FOS (Fig. 26).

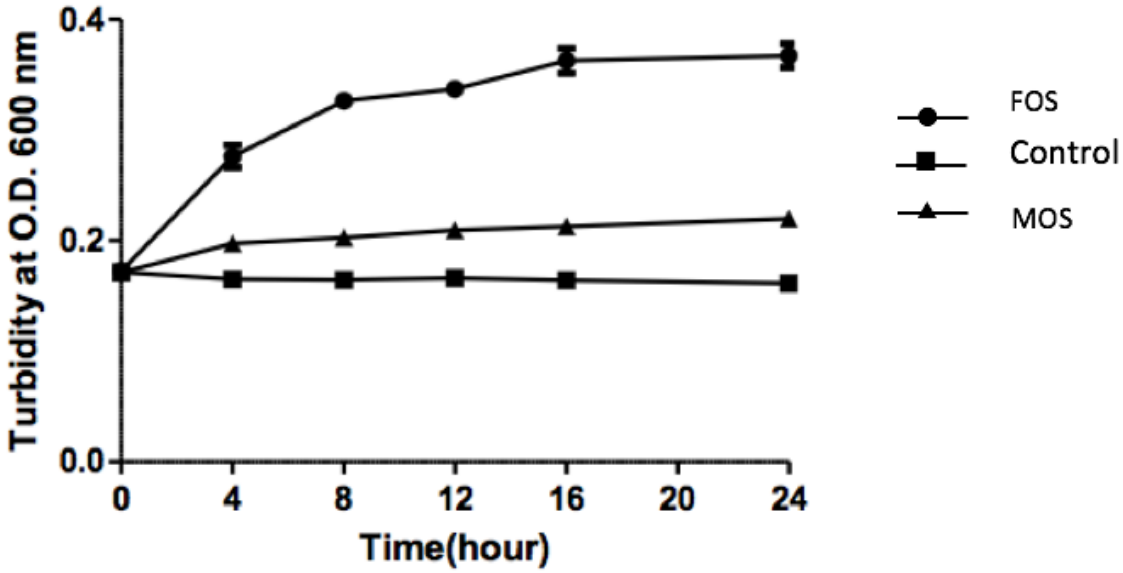

Figure 26. Growth curve of *L. plantarum* N25 at 0 to 24 h after incubation with various carbon sources in basal medium; (■) No carbon source as control, (▲) MOS as carbon source, and (●)FOS as carbon source. Data are shown in mean ± SD analyzed by Two-way ANOVA.

Detection of SCFAs *in vitro* of MOS tested

The results of SCFAs production *in vitro* showed that when *L. plantarum* N25 used MOS as a carbon source. The fermented product was acetic acid which increased to 2.6- and 4.8-folds at 24 h and 48 h, respectively. It also found that

L. plantarum N25 that used MOS as carbon source cannot produce propionic and butyric acid. In addition, the amount of lactic acid was reduced while the amount of acetic acid increased inferred that lactic acid was used as the primary product for synthesis acetic acid as secondary product (Fig. 27). Furthermore, it was found that *L. plantarum* N25 that used MOS as carbon source did not produce propionic and butyric acid *in vitro* (Table 9).

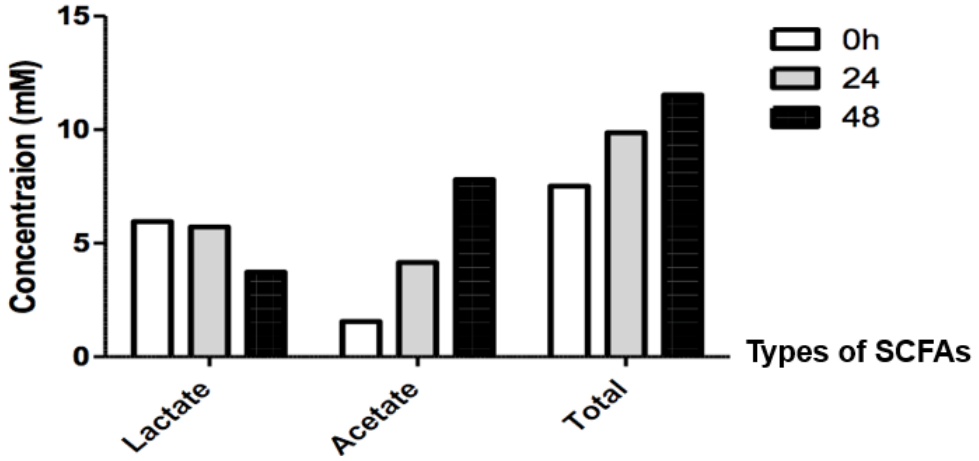

Figure 27. The graph of SCFAs concentration after incubation MOS from Konjac with *L. plantarum* N25 *in vitro*.

Table 9. The SCFAs products concentration *in vitro* after incubation MOS from Konjac with *L. plantarum* N25.

Time	Concentration (mM)			
	Lactic acid	Acetic acid	Propionic acid	Butyric acid
0 h	5.96	1.56	0	0
24 h	5.15	4.01	0	0
48 h	3.37	7.53	0	0

Properties of prebiotic in animal experiment

To study properties of prebiotic, oral gavage administration of sample in mice was performed by measuring the body weight of control, prebiotic, probiotic, and symbiotic groups on 15th day. The mean of body weight and growth curves are presented in Table 10 and Fig. 28, respectively. The results indicated that the body weights of mice from prebiotic, probiotic, and symbiotic were significantly unaffected at all the doses of MOS-treated when compared to the control group (P<0.001).

Table 10. Mean of body weights (g) values of mice treated with MOS from Konjac.

Group	Days		
	0	7	14
Control	22.91 ± 0.51	25.53 ± 2.00	27.41 ± 0.74
Prebiotic	22.90 ± 0.39	26.52 ± 0.91	27.74 ± 0.67
Probiotic	23.32 ± 0.34	26.92 ± 0.48	27.41 ± 0.96
Synbiotic	23.04 ± 0.51	26.37 ± 0.89	27.53 ± 1.24

*Mean of body weight (g) = mean ± S.D.

**Experiments were performed in triplicate.

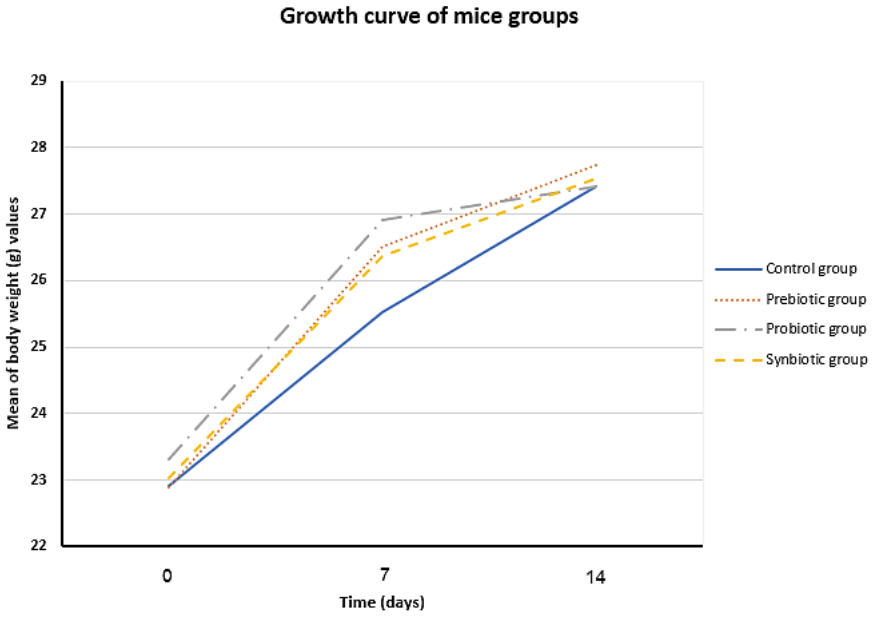

Figure 28. Graph showed growth curve each mice group.

The study of the increase of probiotics in experimental animals

The results from plate count of *L. plantarum* N25 from the initial amount of 0.1 grams of feces sample. The synbiotic group showed the highest number of *L. plantarum* N25 which significantly different from results of the probiotic and control groups (P<0.001). Therefore, MOS has an ability to stimulated the growth of probiotic strain, *L. plantarum* N25 *in vivo* (Table 11 and Fig. 29).

Table 11. Mean of number of *L. plantarum* N25 from 100 mg feces sample by plate count method.

	CFU ✕ 10^2/ 100 mg of feces				
N	1	2	3	4	5
Control	0	0.64	0	0	0
Prebiotic	0	0	0	0	0
Probiotic	15.00 ± 4.00	10.67 ± 4.51	5.00 ± 3.00	13.00 ± 2.00	8.33 ± 9.45
Synbiotic	22.00 ± 5.57	17.00 ± 9.00	20.33 ± 8.08	14.00 ± 2.00	19.00 ± 8.00

*Data as mean ± S.D.

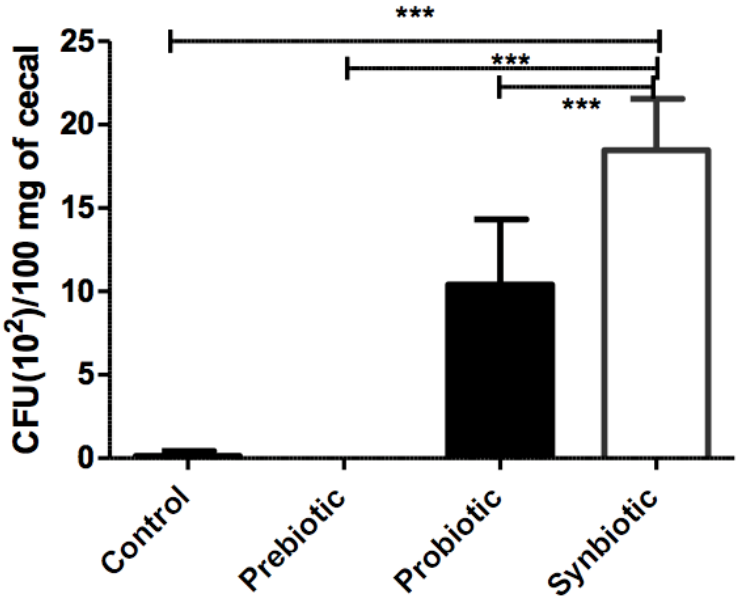

Figure 29. The number of *L. plantarum* N25 per 100 mg of feces sample from each mice group including control, prebiotic, probiotic, and symbiotic groups. The statistics used in these tests were mean ± SD, and One-way ANOVA for the comparison between groups.

The analysis of SCFAs in feces in MOS-treated mice

The results of SCFAs analysis from the supernatant obtained from feces extraction from each mice group found four SCFAs including lactate, acetate, propionate and butyrate. The concentration of acetate and total SCFAs of synbiotic group were significant higher than the control group (P<0.001) (Fig. 30). The results also revealed that the increasing of acetic acid concentration had no effect on the concentration of lactic acid when compared with control of all mice groups (Table 12).

Table 12. The concentration of SCFAs production found in each mice group.

SCFAs (mM)	Group			
	Control	Probiotic	Prebiotic	Synbiotic
Lactic acid	2.73 ± 0.44	2.76 ± 0.35	3.09 ± 0.47	1.85 ± 0.28
Acetic acid	10.33 ± 1.32	9.57 ± 0.93	8.28 ± 1.41	18.60 ± 0.44
Propionic acid	1.81 ± 0.35	1.62 ± 0.26	1.59 ± 0.36	3.57 ± 1.21
Butyric acid	4.12 ± 0.46	4.14 ± 1.20	2.67 ± 0.69	4.22 ± 1.31
Total	18.99 ± 3.33	18.29 ± 3.05	15.63 ± 2.58	28.24 ± 6.72

*Data as mean ± S.D.

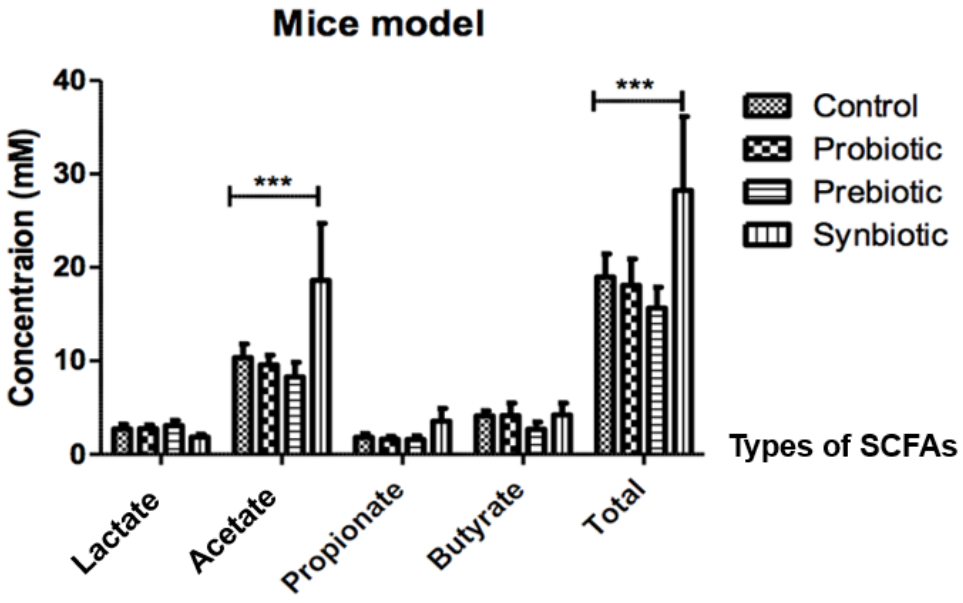

Figure 30. Concentration of short chain fatty acids (SCFAs) production in each mice group. The statistics used for the analysis were mean ± SD, compared between groups using Two-way ANOVA.

CHAPTER V
Discussion

Part 1: Xylooligosaccharide (XOS)

Rice xylan degrading by rXynSW3 enzyme

The recombinant enzyme rXynSW3 was xylanase of *Streptomyces* sp. SWU10 constructed in bacteria derived from previous studied by Sukhumsirichart et al.[54] Its molecular weight was 48 kDa with the optimum pH and temperature of 6.0 and 50°C, respectively. In this study, the rXynSW3 was used to degrade alkaline-pretreated rice straw at 45°C for 24 h and the main products were xylooligosaccharides (XOS) including xylobiose, xylotriose, xylotetraose, and small amount of xylopentose. In contrast, the rXynSW3 degraded non-pretreatment rice straw in same conditions yielding a tiny amount of XOS, the major product was feruloyl-polysaccharides. Normally, the structure of rice xylan contains a higher substitute, which less activity of enzyme degradation with complex structure of substrate.[117] Therefore, pretreated rice straw with alkaline methods are needed before degradation with xylanase enzyme in order to get high yield of XOS. The advantages of alkaline-pretreatment rice straw were changed or eliminated the component in structure of rice straw, then enhance the enzymatic degradation and increase yield of products which this process is an easy and cost-effective method.

Assay of antioxidant activity of XOS from rice straw

In this study, the DPPH method was used for measurement free radical scavenging property of XOS from 2 rice straw samples including XOS from pretreatment rice straw, and XOS and feruloyl-polysaccharide from non-pretreatment rice straw. This method is the most widely used by various researches as it is easy and provides standardized results *in vitro*. The results of DPPH assay is shown in terms of the IC_{50}. The IC_{50} means that concentration of compound required to inhibit 50% of free radicals *in vitro* calculated following formula: A% = % inhibition = $[1-(A_0-A_t)/A_0-A_t)]\times100$, where A_0 and A_t were the optical density at time zero of sample and the control, respectively,

whereas A_0, and A_t, were the optical density of the sample and the control at 30 minutes, respectively. Normally, the lowest value of IC_{50} correlated to the highest antioxidant activity. Ascorbic acid as standard substance for comparison.[118] This study, the IC_{50} of the composition between XOS (tiny) and feruloyl-polysaccharide (major product) from non-pretreated products rice straw was 611.10 $\pm$ 0.52 μg/ml and XOS (X2-X5) from pretreated rice straw was 542.27 $\pm$ 0.59 μg/ml, while the IC_{50} of ascorbic acid was 494.89 $\pm$ 0.16 μg/ml. The antioxidant activity of oligosaccharides derived from a hydroxyl group (R-OH) of monosaccharide sugar in the structure and xylose is a classified in aldopentose type.[119] It can be seen that the XOS from pretreatment rice straw have ability to be antioxidant as well as the ascorbic acid because they have a high antioxidant value of 90% compared to the percent of antioxidant activity of ascorbic acid. For these results demonstrated that, the XOS product from alkaline-pretreatment rice straw can be taken advantage for preparation of functional foods.

Prebiotic effect of XOS from rice straw on probiotics

For prebiotic effect of XOS and feruloyl-polysaccharides products from non-pretreatment rice straw by rXynSW3 showed that the enzymatic products promoted growth of probiotics both *L. plantarum* F33 and *B. adolescentis* JCM1275 and have no affect on growth rate and pH values of pathogenic bacteria including *B. vulgatus* JCM5826 and *C. hiranonis* JCM10541. The results of this study are in the same direction as the results of prebiotic effect of XOS from pretreatment rice straw by rXynSW3. The *L. plantarum* TISTR543, *L. casei* TISTR390, and *B. bifidum* TISTR2129 are probiotics strains in gastrointestinal tract that are beneficial to host's health both produced vitamin or energy source of host and reduce the inflammation, symptoms, and can also prevent diseases of intestinal pathogens infection including *E. coli* TISTR073, *S. enterica* TISTR2519, *C. typhimurium* TISTR1032 etc.[120] According to this study, it was found that the XOS product from pretreatment rice straw can promote the growth of the probiotics. The prebiotic is effective in enhancing growth of probiotic of specific strain, for example, some prebiotic promote growth of *Bifidobacterium* sp. better than *Lactobacillus* sp.[121] In this study, the XOS products can be stimulated both strains

(*Bifidobacterium* sp. and *Lactobacillus* sp.) and have no effect in stimulating the growth of intestinal pathogens. In addition, some prebiotics can be able to prevented or inhibited growth of pathogenic strains, such as competition with pathogens to adhesion host cell.[122] Furthermore, fermentation of *Bifidobacterium* sp. or *Lactobacillus* sp. can produce great amount of acidic organic compounds for example formic, lactic, acetic, propionic, and butyric acid etc.[123] Thus, the pH values of environment have reduced to acidic pH and unsuitable for pathogens growth.[124]

Safety of XOS by *in vivo* study

The toxicity study performed by oral gavage to mice for 15 days. The doses of XOS tested mice groups were 0, 2000, 5000, and 9000 mg.XOS/kg per day for control, low dose, mid dose, and high dose of mice groups, respectively. These results of body weight and growth curve of mice showed that the XOS-treatment have no affected on weight and growth of all mice groups for all doses tests. There were no mice deaths or abnormal during start until the end the experiment with a total of 15-days. In previous study, the body weight changed when mice treated with 9000 mg/kg per day with fructooligosaccharide (FOS).[125]

All mice groups showed normal of test-related effects on hematology and clinical chemistry values both in the kidney and liver throughout the study. The BUN and creatinine are nitrogenous end products of metabolism and evaluated functional of the kidney. The normal range of BUN and creatinine in human are 10-20 and 0.6-1.2 mg/dl, respectively.[126] A total protein included albumin and globulin in blood tested are evaluated functional of the liver. The normal range of total protein, albumin, and globulin are 6.0-8.0, 3.0-5.0, and 2.0-3.5 (g/dl), respectively.[127] The results showed that high dose group and control group mice-tested compared to standard, indicated that these mice were normal function of both kidney and liver of mice tested.

Part 2: Mannooligosaccharide (MOS)

Konjac degrading by rManS2 enzyme

In previous study, the recombinant enzyme rManS2 of *Bacillus* sp. SWU60 was constructed in bacteria by Seesom et al.[68] Its molecular weight was 38 kDa. The optimal temperature and pH were 60°C and 6.0, respectively. This study, Konjac mannan was used as substrate for rManS2 digestion at 45°C for 24 h. The main products were mannooligosaccharides (MOS) included mannobiose, gluco-mannotetraose, mannotriose, mannopentose, and mannotetraose. In general, the structure of Konjac mannan consisting residues of glucose and mannose molecules linked by β-1,4-glycosidic bonds. The rManS2 is an endo-mannanase enzyme that random digest within the backbone structure of the mannan polysaccharides and generate the products with different sizes of structures.[128]

Assay of antioxidant activity of MOS from Konjac mannan

One interesting prebiotic properties of the substance is the ability to be antioxidant. This study used DPPH assay method for evaluation antioxidant activity of MOS, the enzymatic products from Konjac mannan. The result showed that the MOS have an antioxidant property compared to ascorbic acid which used as standard when the result displayed in the form of an IC_{50} value. This value is inversely proportional to the free radical scavenging activity or antioxidant property of sample tested *in vitro*. The IC_{50} of MOS and ascorbic acid were 643.06 $\pm$ 0.05 and 494.89 $\pm$ 0.16 μg/ml, respectively. Normally, the lowest IC_{50} corresponds to the highest of DPPH scavenging activity.[129] Although the value of antioxidants of MOS was less than the standard, but the MOS has an antioxidant value up to 70% compared with ascorbic acid.

Prebiotic effect of MOS from Konjac mannan on probiotics

The prebiotic properties of MOS products from Konjac mannan were tested by cultivating the MOS with probiotic strain *L. plantarum* N25 and the results indicated that the MOS from Konjac mannan was a suitable carbon source for promoting the growth of *L. plantarum* N25 *in vitro* when compared to the control. But, its was slightly promoted the growth of probiotic when compared with fructooligosaccharides (FOS). Although, MOS can be stimulated growth of *L. plantarum* N25 less than FOS but it had

reported that MOS has considered one of the important qualities of a prebiotic substance that promotes growth of probiotics.[130] Ability of prebiotic to stimulate the growth of probiotics are different, because of the different prebiotic structures and/or specificity of each probiotic species. In addition, there are a few research studies reported about the prebiotic properties of MOS for example MOS from copra meal.[131] Therefore, the results of this study informed that MOS from Konjac can be produced as prebiotics which is a natural sources.

Short chain fatty acids (SCFAs) detected *in vitro* of MOS tested

This results about prebiotic effect on probiotics, the MOS from Konjac mannan can be promoted the growth of *L. plantarum* N25 *in vitro*. It can be seen that the MOS had some prebiotic properties. Therefore, the production of SCFAs *in vitro* was investigated by using supernatant of cultured media to detect SCFAs products including lactic, acetic, propionic, and butyric acids by HPLC. The amount of acetic acid was significant increased after incubation at 24 h and 48 h whereas, the propionate and butyrate cannot detect. Furthermore, the amount of lactic acid decreased while the amount of acetic acid increased. It can be explained that lactic acid was used as the primary product for synthesis acetic acid as secondary product in the fermentation process of the lactic acid bacteria (LAB).[132] The propionic acid and butyric acid were not detected at cultured media both incubated at 24 h and 48 h. This may be due to the limitations of each strain in which different types of SCFAs are produced *in vitro* and the type of prebiotics that have different effects on probiotics. For example, *B. bifidum* and *L. gasseri* can express propionic acid in significant amount but not produce butyric acid, *Bifidobacterium sp.* can be produced butyrate from inulin whereas produced acetate and lactate from FOS .[133, 134]

Properties of MOS as prebiotic in experiment mice

To study properties of prebiotic *in vivo*, oral gavage administration of samples to mice for a period of 15 days. All mice groups including control, prebiotic, probiotic, and synbiotic groups were weighted on 15th day of study. It was found that the body weights all mice groups were unaffected by MOS supplement in the food compared to control group. Furthermore, they were not any symptoms and no deaths

during the experiment period. Therefore, it indicated that MOS from Konjac mannan at dose-tested is considered as a safe prebiotic in mice.

The increasing of probiotics *in vivo* was performed by plate count method for evaluate amount of *L. plantarum* N25 from mice feces. The study demonstrated that, the increased amount of *L. plantarum* N25 in synbiotic group (probiotic and prebiotic) was higher than the probiotic group which treated mice with *L. plantarum* N25 in the same amount. Therefore, it can be seen MOS product from Konjac mannan has prebiotic property to promoted growth of probiotic *in vivo*.

The SCFAs production *in vivo* was detected from feces of mice-tested by HPLC. The SCFAs products included acetate, lactate, propionate, and butyrate whereas, propionate and butyrate didnot detect by *in vitro* study. This may be due to the limited of growth conditions of probiotics such as carbohydrate sources and different environment compared to *in vivo*.[135] In animal model, the synbiotic group (*L. plantarum* N25 and MOS) produced higher amount of SCFAs, such as acetic acid and propionic acid, and total SCFAs which differed from the mice-control group while the concentration of lactate and butyrate were similarity of all mice groups. In addition, the amount of SCFAs such as acetic, lactic, propionic, and butyric acids were different from *in vitro* because butyric acid and propionic acid were found *in vivo*, which may be due to in the gastrointestinal tract (GIT) of mammalian composition of the several gastrointestinal microflora. Normally, SCFAs are obtained from bacteria fermentation. In this study, MOS was fermented by anaerobic pathway of probiotic bacteria. In the case of MOS hydrolysis, the bacteria producing SCFAs through the anaerobic glycolysis, also known as lactic acid system.[136] Generally, the bacteria produce acetic, propionic and butyric acid released at a ratio of 60:20:20. These products are a source of energy for the metabolism in the body. In addition, it was found that the SCFAs have also been shown to provide other benefits for health such as a protective barrier to protect the intestines from attaching and growing of pathogens, change gut environment to acidic that unsuitable for the growth of pathogens etc.[137] Especially, the butyric acid is an important source of energy for the colonocytes which has a positive effect on preventing

gastrointestinal disorders, reducing the risk of inflammation bowel disease, or prevent the occurrence of certain cancers such as colon cancer.[138] Therefore, it can be seen that SCFAs are useful and necessary for health to maintain a good balance of the body.[139]

All of the results in this study showed that XOS from xylan of pretreatment rice straw and MOS from Konjac mannan have ability to be antioxidants *in vitro*. They also have prebiotic properties which can be stimulated the growth of all the probiotic strains used in this study. Furthermore, MOS can be produced SCFAs *in vitro* and *in vivo* whereas butyric acid was detected only *in vivo*. Both XOS and MOS were safety when used in animal. Therefore, the XOS and MOS can be used as prebiotics which can be applied in various industries including the pet food and livestock, segment of food industries, infant formula, pharmaceutical as well as in the other medical fields.

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