ศักยภาพการเป็นพรีไบโอติกและสารต้านอนุมูลอิสระของเฟอรูโลอิลโพลีแซคคาไรด์ที่ได้จากการย่อยฟางข้าวด้วยรีคอมบิแนนท์เอนไซม์ไซลาเนสของเชื้อ *Streptomyces* sp. SWU10

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บทคัดย่อ

พรีไบโอติกและสารต้านอนุมูลอิสระนั้นมีความสำคัญต่อการส่งเสริมสุขภาพ ในการศึกษาครั้งนี้ผลิตภัณฑ์จากการย่อยฟางข้าวที่ไม่ผ่านการปรับสภาพด้วยรีคอมบิแนนท์เอนไซม์ไซลาเนสจาก *Streptomyces* sp. SWU10 (rXynSW3) ถูกนำมาทดสอบเพื่อหาคุณสมบัติการเป็นพรีไบโอติกและสารต้านอนุมูลอิสระ โดยนำฟางข้าวไปบดให้เป็นผงแล้วละลายในบัฟเฟอร์ฟอสเฟตที่มีความเป็นกรด-ด่าง เท่ากับ 6.0 จากนั้นทำการย่อยผงฟางข้าวด้วยเอนไซม์ rXynSW3 แล้ววิเคราะห์ผลิตภัณฑ์ที่ได้โดยใช้เครื่อง HPLC และ HPAEC คุณสมบัติการเป็นพรีไบโอติกถูกประเมินในหลอดทดลอง ด้วยการหมักผลิตภัณฑ์จากการย่อยกับโปรไบโอติก สายพันธุ์ *Lactobacillus plantarum* F33 และ *Bifidobacterium adolescentis* JCM1275 และแบคทีเรียก่อโรค สายพันธุ์ *Bacteroides vulgatus* JCM5826 และ *Clostridium hiranonis* JCM10541 ความสามารถในการเป็นสารต้านอนุมูลอิสระของผลิตภัณฑ์ที่ได้วิเคราะห์โดยใช้วิธี DPPH assay ผลการวิเคราะห์โดยเทคนิค HPLC และ HPAEC พบว่าผลิตภัณฑ์หลักที่ได้จากการย่อยคือ เฟอรูโลอิลโพลีแซคคาไรด์ (feruloyl-polysaccharide) ผลการต้านอนุมูลอิสระของผลิตภัณฑ์ที่ได้ทั้งหมดโดยวิธี DPPH assay ปรากฏว่าได้ค่า IC_{50} เท่ากับ 611.10 µg/mL เมื่อเทียบกับ 494.72 µg/mL ของวิตามินซีที่ใช้เป็นสารมาตรฐาน จากการศึกษาคุณสมบัติการเป็นพรีไบโอติกพบว่าผลิตภัณฑ์ที่ได้จากการย่อยสามารถส่งเสริมการเจริญเติบโตของโปรไบโอติกทั้งสองสายพันธุ์ แต่ไม่มีผลต่อการเจริญเติบโตของแบคทีเรียก่อโรค โดยสรุป จากการศึกษาในหลอดทดลองพบว่า เฟอรูโลอิลโพลีแซคคาไรด์ผลิตภัณฑ์หลักที่ได้จากการย่อยฟางข้าวที่ไม่ผ่านการปรับสภาพด้วยรีคอมบิแนนท์เอนไซม์ rXynSW3 มีศักยภาพเป็นสารต้านอนุมูลอิสระและพรีไบโอติก

คำสำคัญ: พรีไบโอติก สารต้านอนุมูลอิสระ เฟอรูโลอิลโพลีแซคคาไรด์ ฟางข้าว *Streptomyces* sp. SWU10 ไซลาเนส

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Prebiotic and antioxidant potential of feruloyl-polysaccharide from rice straw digested by recombinant xylanase enzyme of *Streptomyces* sp. SWU10

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Abstract

Prebiotics and antioxidants are important substances for promoting health. In the present study, the products from degradation of non-pretreatment rice straw by recombinant xylanase of *Streptomyces* sp. SWU10 (rXynSW3) was investigated for their prebiotic and antioxidant activities. The rice straw was ground to powder, dissolved in phosphate buffer solution pH 6.0 before digesting by rXynSW3. The enzymatic products were analyzed by HPLC and HPAEC. The prebiotics property was evaluated by fermentation of the enzymatic products with probiotic strains including *Lactobacillus plantarum* F33 and *Bifidobacterium adolescentis* JCM1275 and pathogenic bacterial strains such as *Bacteroides vulgatus* JCM5826 and *Clostridium hiranonis* JCM10541. *In vitro* antioxidant activity of the enzymatic products was determined by DPPH assay. The HPLC and HPAEC analysis revealed that the major product was feruloyl-polysaccharide. The antioxidant activity of the enzymatic products determined by DPPH assay showed the IC_{50} at 611.10 µg/mL compared to 494.72 µg/mL of ascorbic acid which was used as standard. The enzymatic products promoted growth of both probiotic strains but no effect on growth of pathogenic bacteria. In conclusion, the *in vitro* studies revealed that the feruloyl-polysaccharide the main product from non-pretreatment rice straw digested by rXynSW3 have potential of antioxidant and prebiotic activities.

Keywords: prebiotics, antioxidant, feruloyl-polysaccharide, rice straw, *Streptomyces* sp. SWU10, xylanase

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Introduction

Lignocellulosic waste material such as rice straw, wheat straw, bagasse, etc are produced from several agro-industrial each year.[1,2] The conversion of these wastes to high-valuable compounds or renewable energy can increase an income and protect environment. Therefore, technology and methods to improve the use of agricultural and agro-industrial residues to obtain food and/or biofuel are important.[3,4] In the recent years, there has been a focus on producing prebiotics from new natural products which are safe for use and low-cost.[5,6] Xylan is a hemicellulose comprising of a xylose backbone and branched by other monosaccharides depending on the xylan sources.[7] Xylooligosaccharides (XOS) are oligomers of xylose residues which have no calories and cannot be digested by the human enzymes.[8] The XOS has received much attention for being used as prebiotics with health benefit to humans.[9] Many studies showed prebiotic potential of XOS such as some experiment in rat that used XOS as additive and the result demonstrated that the XOS-fed rat group have increased number of *Bifidobacterium* and *Lactobacillus* spp. in feacal sample. [10] The XOS derived from corn cobs combined with *L. plantarum* showed antioxidant potential better than either XOS or probiotic alone.[11] The XOS syrup producing from biomass by endoxylanase of *Bacillus pumilus* B20 can stimulate growth of *L. brevis.*[12] Furthermore, the XOS can modulate metabolic pathways of *B. adolescentis* 15703, and increasing its growth.[13] In addition to its antioxidant property and growth promoting effect on probiotics, the XOS also has the ability to prevent infection by gastrointestinal tract pathogens including *Escherichia coli*,[14-16] *Clostridium difficile*,[17,18] Rotavirus,[19-21] *Salmonella* sp.,[22-24] *Bacteroides* sp.[25]

Therefore, in this study, the enzymatic products from non-pretreatment rice straw digested by xylanolytic recombinant enzyme of thermoterant *Streptomyces* sp. SWU10 (rXynSW3) were evaluated for their antioxidant activity as well as prebiotic effect on probiotics.

Materials and Methods

Chemicals and reagents

The recombinant plasmid, pCold-xynSW3, in *Escherichia coli* DH5α[26], HisTrap HP column (QIAGEN, Hilden, Germany), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution (Sigma-Aldrich, Germany).

Microorganisms

Probiotic strain including *L. plantarum* F33 and *B. adolescentis* JCM1275. Pathogenic strain *Bacteroides vulgatus* JCM5826 and *Clostridium hiranonis* JCM10541. All microorganism were obtained from Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan.

Rice straw preparation

Rice straw of *Oryza sativa* was collected from north-eastern part of Thailand. The straw was cut and extensively washed with water, ground to powder and dried in an

incubator at 60°C, and then dissolved in PBS, pH 6.0.

Production and purification of rXynSW3 enzyme

Ten microliters of glycerol stock solution of recombinant plasmid containing xylanase gene, pCold-xynSW3 in host *E. coli* DH5α^{26} was activated in 10 mL of LB medium containing ampicillin (50 µg/mL) and incubated at 37°C for 16 h. Then 10 milliliters of activated culture were transferred into 1 liter of LB medium containing ampicillin (50 µg/mL) and cultured at 37°C for 1 h. Then, isopropyl β-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to the culture, and was further continued at 15°C for 4 days. Then the cells in culture broth were harvested by centrifugation and lysed in BugBuster Master Mix. After centrifugation, the cell-free extract was diluted with distilled-water and loaded onto a HisTrap HP equilibrated with a buffer consisting of 20 mM KPB buffer (pH 6.0), 500 mM NaCl, and 20 mM imidazole. The rXynSW3 was eluted with a linear gradient of imidazole (from 0 to 300 mM) in the same buffer. The purified enzyme was kept at 4°C for further study of enzyme activity.

Degradation of rice xylan by rXynSW3

Three grams of rice straw powder in 150 mL phosphate buffer solution pH 6.0 (2%) was incubated with 1 mL of rXynSW3 solution at 50°C for 24 h and then boiled for 5 min to inactive enzyme. Subsequently, the enzymatic products were analyzed by using HPLC and HPAEC.

Assay of antioxidant activity by DPPH assay

Ten milliliters of enzymatic products were dissolved in autoclaved distilled-water to a concentration of 1 mg/mL and further performed a serial dilution from 0 to 1,000 µg/mL. Then, 0.2 mL of each diluted sample was mixed with 0.8 mL of 0.1 M Tris-HCl buffer (pH 7.4) in a test tube, and 1 mL of the DPPH solution was added. The sample solution was vigorously mixed for 10 sec. and left in the dark at room temperature for 30 min. After 30 min, absorbance at a wavelength of 517 nm of the solution was measured by using a mixture of 1.2 mL of ethanol and 0.8 mL of 0.1M Tris-HCl buffer (pH 7.4) as the blank. The measurement of the DPPH radical scavenger at each concentration was performed by calculation the IC_{50} using the following formula:

$$A\% = [1-(A_0-A_t)/(A_{0'}-A_{t'})]\times 100,$$

where A_0 and $A_{0'}$ are the absorbance at time zero of the sample and the control, respectively, whereas A_t and $A_{t'}$ are the absorbance of the sample and the control at 30 minutes, respectively, using ascorbic acid as a positive control. The experiment was performed triplicate.

Prebiotic effect on probiotics

To study the prebiotic activity of the enzymatic products 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 in 5 mL of MRS media containing each concentration of enzymatic products (100, 300 and 500 μg/mL) at 37°C for 12 h in the anaerobic condition. Growth of the bacteria was assessed by determining the OD_{600} and pH value was measured, both were performed at 2 h intervals.

Statistical analysis

The results were performed as mean ± standard deviation (SD) for at least three independent experiments.

Results

Degradation of rice xylan by rXynSW3

The HPLC analysis of rice straw powder in phosphate buffer solution without treatment with enzyme was used as a control (Fig. 1A). The enzymatic products of non-pretreatment rice straw with rXynSW3 indicated that the major product was the feruloyl-polysaccharide (Fig. 1B). The 24 h enzymatic products analyzed by HPAEC showed that there was small amount of xylooligosaccharides (Fig. 2).

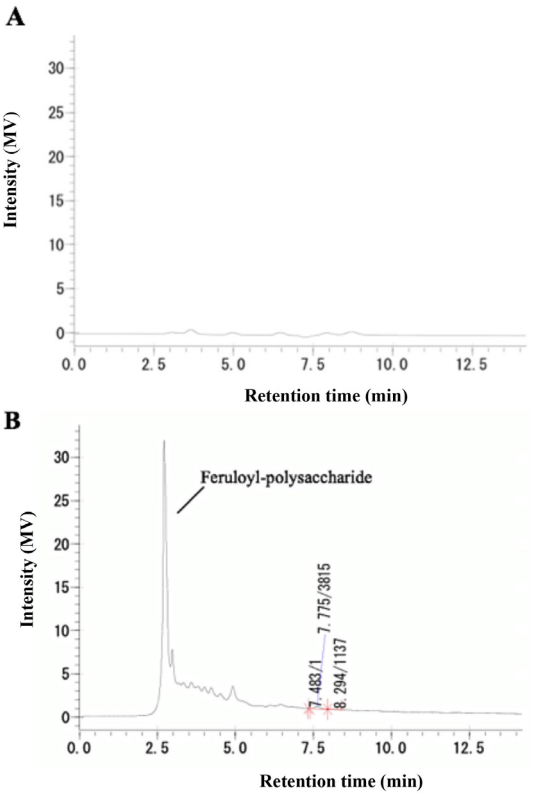

Figure 1 HPLC analysis of the enzymatic products from non-pretreatment rice straw; (A) non-pretreatment rice straw in phosphate buffer solution without rXynSW3 digestion (B) non-pretreatment rice straw digested by rXynSW3 for 24 h.

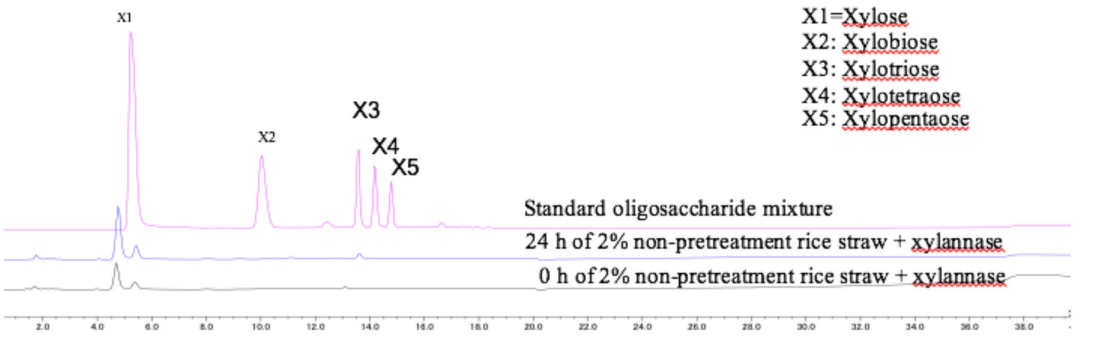

Figure 2 HPAEC analysis of the enzymatic products from non-pretreatment rice straw digested by rXynSW3.

Assay of antioxidant activity

The DPPH radical scavenging assay showed that the IC_{50} of the enzymatic products was 611.10 μg/mL when the IC_{50} was calculated from the equation, y = 0.0816x + 0.134, R2 = 0.98313. The antioxidant activity of the standard ascorbic was a 494.72 μg/mL (Fig. 3).

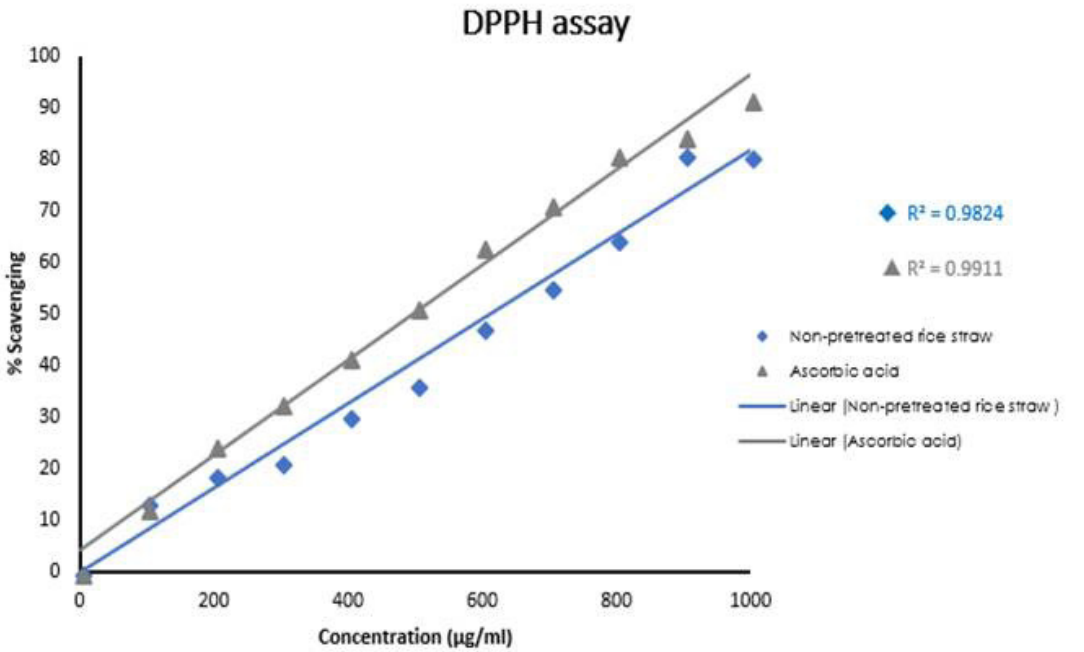

Figure 3 DPPH free radical scavenging curve of the enzymatic products.
Data were obtained from triplicate results of three independent experiments and shown as mean±SD.

Prebiotic effect on probiotics

After incubation of the enzymatic products with probiotic bacteria including *L. plantarum* F33, *B. adolescentis* JCM1275, and pathogenic bacteria such as *B. vulgatus* JCM5826, and *C. hiranonis* JCM10541, the results showed that the enzymatic products promoted growth of *L. plantarum* F33 (Fig. 4A) and *B. adolescentis* JCM1275 (Fig. 4C), and lowered the pH values of the culture media of both probiotic strain (Fig. 4B, and D, respectively). However, the products did not affect growth rate and pH values of pathogenic bacteria including *B. vulgatus* JCM5826 (Fig. 5 A, B) and *C. hiranonis* JCM10541 (Fig. 5C, D), respectively.

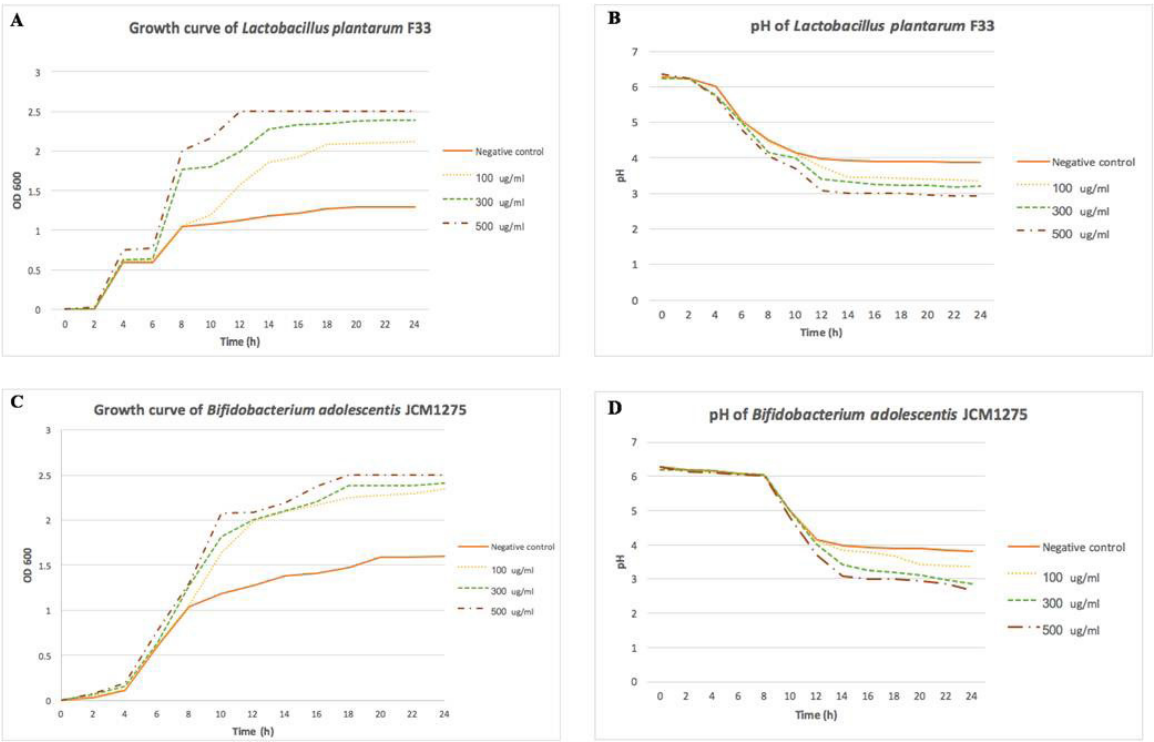

Figure 4 Growth curve and pH value of probiotics in MRS media with the enzymatic products; *L. plantarum* F33 (A, B), and *B. adolescentis* JCM1275 (C, D), the MRS medium without enzymatic products were used as negative control.

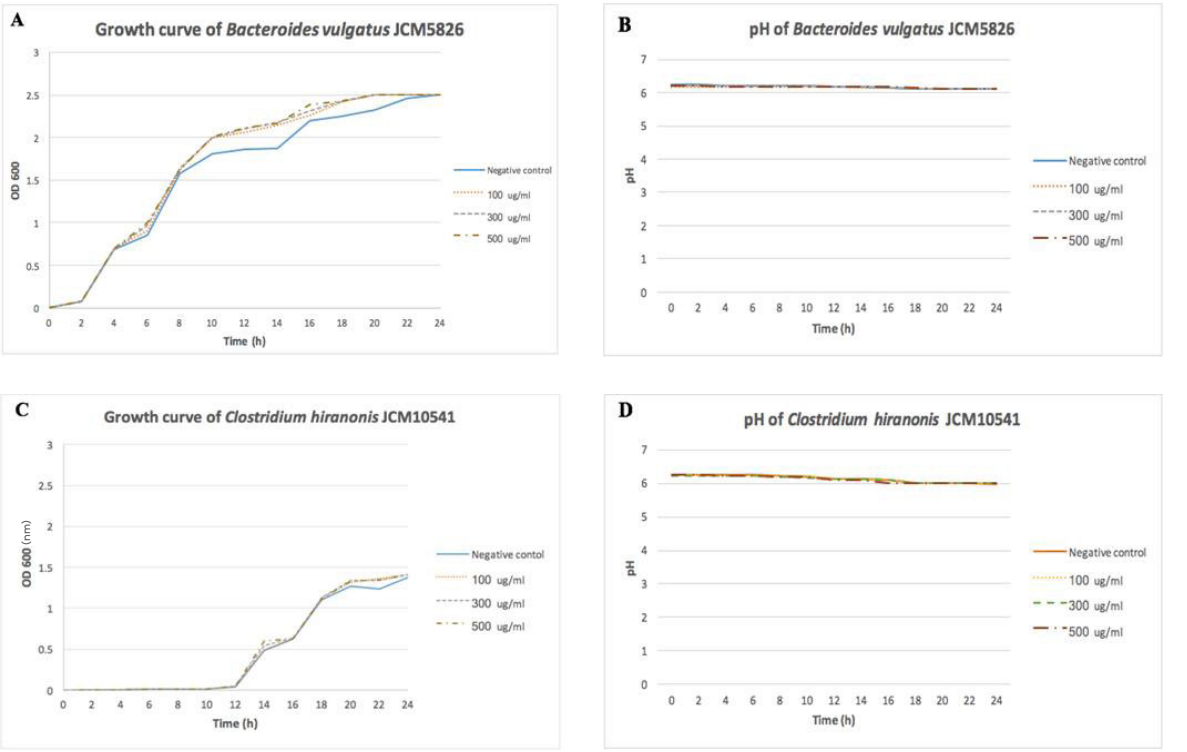

Figure 5 Growth curve and pH value of pathogens in MRS media with the enzymatic products; *B. vulgatus* JCM5826 (A, B), and *C. hiranonis* JCM10541 (C, D), the MRS medium without the enzymatic products were used as negative control.

Discussion

In previous study, the recombinant enzyme rXynSW3 of *Streptomyces* sp. SWU10 was constructed in bacteria by Sukhumsirichart et al.[26] Its molecular weight was 48 kDa with optimum temperature and pH at 50°C and 6.0, respectively. Substrate specific of rXynSW3 was wheat xylan, and the main products were xylooligosaccharides (XOS) including xylobiose, xylotriose and xylotetraose.[26] In this study, the rXynSW3 degraded non-pretreatment rice xylan yielding tiny amount of XOS, the main product was feruloyl-polysaccharide. This implies that the rice straw requires pretreatment with alkaline or acid or others methods before digestion with xylanase enzyme in order to get high yield of XOS. Typically, the structure of the rice xylan contains a high amount of substitute, which is arabinose attached at the xylose backbone[27], and the arabinose mostly contains ferulic acid as side chain. In this study, ferulic acid was detected from the enzymatic products by HPLC indicating that the main products contained ferulic acid as feruloyl-polysaccharide. Confirmation experiment was carried out by adding ferulic acid into the

solution of enzymatic products and the result verified that the main products contain ferulic acid (data not shown). The ferulic acid has previously been shown to have several good benefits such as antioxidants which can neutralize free radicals (superoxide, nitric oxide and hydroxyl radical). Therefore, it can protect cells damage by ultraviolet light, oxidative stress, mutation, and reduce risk of several diseases.[28] In this study, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used for measurement antioxidant activity of the enzymatic products from rice xylan. This method is the widely used by various researches as it is easy and provides standardized results *in vitro*. By measuring the results of DPPH assay that is shown in terms of the IC_{50}. In general, the lowest IC_{50} corresponds to the highest DPPH scavenging activity. Ascorbic acid is the standard substance for comparison. This study, the IC_{50} of the enzymatic products (feruloyl-polysaccharide) was 611.10 µg/mL, while the IC_{50} of ascorbic acid was 494.72 µg/mL. Although the feruloyl-polysaccharide have lesser ability to be antioxidant than the ascorbic acid but it have a high antioxidant value of 76% compared to the percent of antioxidant activity of ascorbic acid. The above results indicate that the enzymatic products from non-pretreated rice straw can be potentially used as nutritional supplement. The *L. plantarum* and *B. adolescentis* are probiotics strains which promote health by reducing the inflammation and symptoms, and they also prevent intestinal pathogens infection including *E. coli*, *Salmonella* sp., *Bacteroides vulgatus*, *Clostridium hiranonis*, etc.[29] According to this study, it was found that the enzymatic products from non-treatment rice straw can promote the growth of the probiotics (*L. plantarum*, *B. adolescentis*). In other studies, the prebiotic can enhance growth of specific strain of probiotic such as *Bifidobacterium* sp. and *Lactobacillus* sp.[30] However, in this study, the feruloyl-polysaccharide can stimulate growth of both strains but did not promote growth of intestinal pathogens. In addition, some prebiotics may also have ability to prevent or inhibit growth of pathogens, such as reduction of pathogenic bacterial adhesion and competition for host cell binding sites.[31] Furthermore, fermentation of *Lactobacillus* and *Bifidobacterium* spp. can produce a large amount of organic acids including acetic acid, lactic acid, formic acid, butyric acid, and propionic acid, etc.[32] Thus, lowered pH values of cultured media might not be suitable for growth of pathogens.[33]

Conclusion

The feruloyl-polysaccharide, the major product obtained from degradation of non-pretreatment rice straw by rXynSW3 of *Streptomyces* sp. SWU10, showed prebiotic property by promoting growth of the probiotics, *L. plantarum* and *B. adolescentis* and also have antioxidant activity with IC_{50} of 611.10 µg/ml in *in vitro* experiments. Therefore, the feruloyl-polysaccharide can be further studied *in vivo* for medical applications.

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