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Contributed Paper

Bioconversion of Agricultural Wastes to Mannooligosaccharides and Their Prebiotic Potential

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ABSTRACT

The hydrolysate from the action of crude *Penicillium oxalicum* KUB-SN2-1 mannanase was determined by Thin Layer Chromatography (TLC). The main products that were obtained from copra meal and coffee residue after hydrolyzed by crude mannanase were mannotetraose, mannotriose, mannobiose and mannose while the product of potato peel and sugarcane bagasse was glucose. For soybean meal, the main products were glucose and galactose. Interestingly, the hydrolysate from the degradation of agricultural wastes possessed prebiotic properties in that they promoted growth of 10 strains of Lactic Acid Bacteria (LAB) including *Lactobacillus plantarum* TISTR 541, *Lactobacillus sake* TISTR 840, *Lactobacillus sake* TISTR 890, *Lactobacillus sake* TISTR 912, *Lactobacillus reuteri* KUB-AC-5, *Lactococcus lactis* ATCC 19435, *Pediococcus pentosacens* JCM 5885, *Pediococcus acidilactici* TISTR 953, *Enterococcus faeclis* TISTR 927 and *Enterococcus faeclis* JCM 5805. In addition, the same hydrolysate could inhibit the growth of pathogenic bacteria including *Staphylococcus aureus* TISTR 029 and *Escherichia coli* E010, but not *Shigella* DMST 1511. The hydrolysate from coffee residue effectively inhibited the growth of *Staphylococcus aureus* TISTR 029 at the level 76.190 ± 3.88% while the soybean meal hydrolysate moderately inhibited the growth of *Staphylococcus aureus* TISTR 029 as well as *E. coli* E010 at the level of 34.78 ± 4.35 % and 20.0 ± 9.0%, respectively. For the aspects of health benefits and value added agricultural wastes, these mannooligosaccharide products are an attractive candidate as an environmental friendly feed supplement.

Keywords: agricultural wastes, mannanase, lactic acid bacteria, mannooligosaccharides, prebiotics

1. INTRODUCTION

Thailand is an agricultural country with an estimate of 38.2% total cultivated area as equal to 122 million hectares [1]. After the postharvest handling and the multi-process in agricultural industries, vast amounts of agricultural wastes (AWs) have been generated and the major component of AWs is lignocellulose. Lignocellulose is a major component of plant cell walls and is mainly composed of cellulose, hemicellulose and lignin. They can be broken down into simple sugars or oligosaccharides by a synergistic

action of lignocellulases. Oligosaccharides are sugars consisting of approximately 2-20 saccharide units. Various types of oligomers that are nondigestible and have beneficial effects on hosts can confer prebiotic properties. These include fructooligosaccharides (FOS), galactooligosaccharides (GOS), soybean oligosaccharide, isomaltooligosaccharides (IMO), glucooligosaccharides (GOS), xylooligosaccharides (XOS), and mannooligosaccharides (MOS) which have been previously documented to potentially promote microorganisms such as bifidobacteria and lactobacilli in the colon [2]. Chaiongkarn *et al*. [3] isolated *Penicillium oxalicum* KUB-SN2-1 from soil and indicated that this fungus had mannanase activity of 19.31 Unit/ml using copra meal as a carbon source. In addition, Chantorn *et al*. 2013 [4] reported that *P. oxalicum* KUB-SN2-1 cultured in modified medium with initial medium pH of 5.0 at 30°C gave the highest mannanase activity of 53.77 Unit/ml on day 3 of incubation period. The hydrolytic activity of crude mannanase from *P. oxalicum* KUB-SN2-1 on various AWs hydrolysate and its degradation products with their potential prebiotic properties have been investigated in this study.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals, media, and media supplements were of analytical grade obtained from Sigma-Aldrich Chemical Ltd., USA; Carlo Erba reagent, France; and HiMedia Laboratories Ltd. Standard sugars were obtained from Megazyme, Ireland.

2.2 Preparation of Agricultural Wastes (AWs)

Five agricultural wastes including potato peel, soybean meal, coffee residue, sugarcane bagasse, and copra meal were used as carbon sources, inducers, and substrates for mannanase production. AWs were dried at 60°C for 48 h, blended, milled by a hammer mill (IKA Labortechnik; Janke & Kunkel, Germany), and then sieved to obtain products with an average particle size of 30 mesh. All samples were kept in a desiccator until used.

2.3 Mannanase Production

2.3.1 Inoculum cultivation

Penicillium oxalicum KUB-SN2-1 was obtained from the laboratory stock culture at the Specialized Research Unit: Prebiotics and Probiotics for Health, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand. *P. oxalicum* KUB-SN2-1 was maintained on Potato Dextose Agar (PDA) plates. After 7 days of the cultivation at 30°C, spore concentration was adjusted to 10^6 spore/ml with sterile 0.2% (v/v) tween 80 and used as a starting inoculum.

2.3.2 Enzyme production

The enzyme production medium (EPM) (modified from Chantorn *et al*. 2013 [4]) consisted of (%w/v) 0.4 bacto-peptone, 0.3 KH_2PO_4, 0.2 K_2HPO_4, 0.05 $MgSO_4 \cdot 7H_2O$, 0.0002 $CoCl_2 \cdot 6H_2O$, 0.0005 $FeSO_4 \cdot 7H_2O$, 0.0002 $MnSO_4 \cdot H_2O$, 0.0016 $ZnCl_2$, 0.5 yeast extract, 1.0 guar gum, and 0.25 coffee residues were adjusted to pH 5.0-5.5 using 0.5% tartaric acid. The 10% (v/v) of inoculum was transferred to 500 ml of EPM and incubated at 30°C with shaking at 200 rpm for 4 days. Samples were collected by centrifugation at 11,000Xg, 4°C for 15 min. Supernatant was collected at -20°C and used as crude mannanase. All experiments were performed in triplicate and results represented the mean values of the activities.

2.3.3 Enzyme assay

The mannanase activity was measured

by incubating the reaction mixture of 0.5 ml of sample and 0.5 ml of 10 mM pH 4.0 citrate-phosphate buffer with 1% locust bean gum at 60°C for 15 min. Amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method [5].

One unit of mannanase activity was defined as the amount of enzyme producing 1 micromole mannose per minute under the experimental condition.

2.4 Hydrolysis Study

Various AWs including potato peel, soybean meal, coffee residue, sugarcane bagasse, and copra meal were hydrolyzed by crude mannanase from *P. oxalicum* KUB-SN2-1. The highest of reducing sugar contents were achieved from 10% of coffee residue, copra meal, potato peel, and sugarcane bagasse and 8% of soybean meal [6]. In this study, crude mannanase of 36.01±0.13 Unit/ml was performed in the reaction mixture of 80 ml of crude extracellular enzyme sample and 80 ml of each AWs in 10 mM pH4.0 citrate-phosphate buffer and carried out at 60°C for 48h. The reactions, after 3, 6, 9, 24, 27, 30, and 48 h of incubation period, were stopped by placing the samples in boiling water for 10 min. The AWs hydrolysate products were collected for further analysis.

2.5 Analysis of AWs Hydrolysate Products

2.5.1 Proximate analysis

The amount of reducing sugar of AWs hydrolysate products were analyzed by DNS methods.

2.5.2 Thin layer chromatography

The hydrolysate products pattern were determined by TLC modified from the method of Apiraksakorn *et al.* 2008 [7]. Glucose, maltose, galactose, mannose, and mannooligosaccharide M2-M7 were used as standards. The solvent used as a mobile phase was composed of butanol: isopropanol: ethanol: deionized water in the ratio of 2:3:3:2 respectively. Two microlitres of each degradation product was applied on 60-plate aluminum silica gel (Merck, Germany) and developed for 90 min in developing solvent. The brown spots of sugars were developed by dipping in 0.2% (w/v) orcinol in 10% (v/v) sulfuric acid in ethanol and incubated at 100°C for 15 min.

2.6 Assessing Prebiotic Potential of AWs Hydrolysates

Effect of AWs hydrolysates on bacterial growth were used to determine the prebiotic properties using modified methods of Chantorn *et al.* [8].

2.6.1 Bacterial strains

All bacteria used in this study were obtained from the laboratory stock culture at the Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand and were shown in Table 1. Ten strains of Lactic Acid Bacteria (LAB) were routinely cultured in MRS broth at 37°C for 4h, and three strains of pathogens were cultured in nutrient broth (NB) at 37°C for 18h.

Table 1. Bacterial strains used in this study.

	Target strains
Pathogens	
1	*Shigella* DMST 1511
2	*Staphylococcus aureus* TISTR 029
3	*Escherichia coli* E010
Lactic acid bacteria	
1	*Lactobacillus plantarum* TISTR 541
2	*Lactobacillus sake* TISTR 840
3	*Lactobacillus sake* TISTR 890
4	*Lactobacillus sake* TISTR 912
5	*Lactococcus lactis* ATCC 19435
6	*Lactobacillus reuteri* KUB-AC5
7	*Pediococcus pentosacens* JCM 5885
8	*Pediococcus acidilactici* TISTR 953
9	*Enterococcus faecalis* JCM 5805
10	*Enterococcus faecalis* TISTR 927

2.6.2 Effects of AWs hydrolysate on lactic acid bacteria growth

Ten LAB as shown in Table 1 were used as the target strains to determine possible growth effects of AWs hydrolysate. Each culture solution of the target strains was adjusted to the absorbance of 0.5 at 600 nm. The culture solution of each target strain (1%) and 1% of each AWs hydrolysate were transferred into 5 ml of MRS medium. The culture of each target strain (1%) and 1% (v/v) of EPM (without AWs hydrolysate solution) were used as the control. The mixture was incubated at 37°C for 4h and then the number of colonies were measured by the spread plate technique at suitable dilutions. The prebiotic properties of AWs hydrolysate were defined as an enhancing activity of LAB growth according to the following equations:

$$\text{Enhancing activity on growth of LAB (\%)} = \frac{[SF - CF]}{CF} \quad (1)$$

where SF and CF were the number of colonies obtained in the growth experiments containing AWs hydrolysate and the number of colonies in control experiments (log cfu/ml), respectively.

2.6.3 Effects of AWs hydrolysate on the pathogenic growth

Three pathogenic strains as shown in Table 1 were used as the target strains to determine possible growth effects of AWs hydrolysate. Each culture solution of the target strains was adjusted to the absorbance of 0.5 at 600 nm. The culture solution of each target strain (1%) and 1% of each AWs hydrolysate were transferred into 5 ml of NB medium. The culture of each target strain (1%) and 1% (v/v) of EPM (without AWs hydrolysate solution) were used as the control. The mixture was incubated at 37°C for 5h and then the number of colonies were measured by spread plate technique at suitable dilutions. The prebiotic properties of AWs hydrolysate were defined as an inhibition activity on growth of pathogen according to the following equations:

$$\text{Inhibition activity on growth of pathogen (\%)} = \frac{[CF - SF]}{CF} \times 100 \quad (2)$$

where SF and CF were the number of colonies obtained in the growth experiments

containing AWs hydrolysate and the number of colonies in control experiments (log cfu/ml), respectively.

2.7 Data Analysis

The data collected were subjected to the multivariate analysis of variance (ANOVA) as well as the descriptive analysis. Data sets were analyzed using Statistical Package for Social Scientists (SPSS) version 17. Levels of significance, means, and standard deviations were obtained from various data sets. The differences were accepted as significance at $p<0.05$ and the separation of means was done using the Duncan multiple range with three replicates.

3. RESULTS

3.1 The Analysis of Oligosaccharide Mixtures

Five AWs were hydrolyzed by crude mannanase of 36.01±0.13 Unit/ml at 60°C, pH 4.0 for 48h. The amount of reducing sugar from AWs hydrolysate were evaluated by DNS methods. The reducing sugar contents of potato peel, soybean meal, coffee residue, sugarcane bagasse, and copra meal hydrolysates are 3.31±1.43, 2.32±0.15, 3.35±0.16, 4.10±0.40, and 7.51±0.52 mg/ml, respectively. To determine the degradation products from the action of crude mannanase from *P. oxalicum* KUB-SN2-1 on various AWs, each reaction was performed for 60 h as shown in Figure 1. The degradation products from AWs hydrolysis were considered to be galactose, triose, tetraose and the oligosaccharides larger than mannotetraose. The compositions of copra meal and coffee residue hydrolysates were mannose, mannobiose, mannotetraose, and mannohexose. While potato peel hydrolysates contained only glucose, sugarcane bagasse hydrolysates had glucose and mannobiose and soybean meal hydrolysate compositions were glucose and galactose.

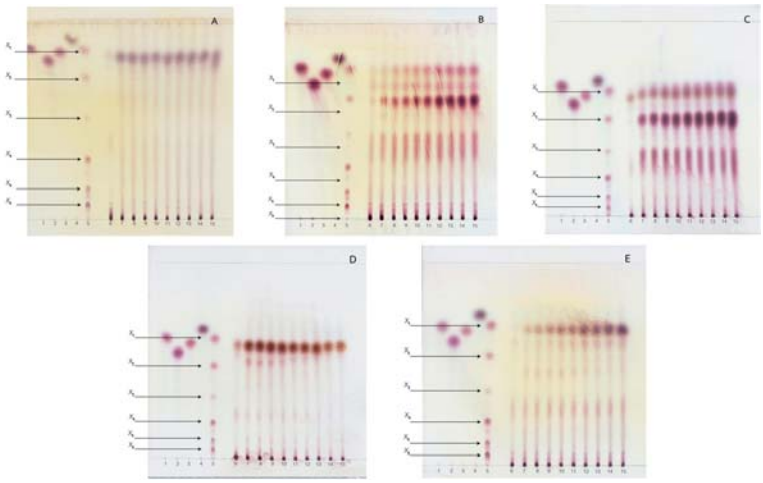

Figure 1. Oligosaccharide patterns from AWs hydrolysates by crude mannanase from *P. oxalicum* KUB-SN2-1 for 60h. Ten percent (w/v); potato peel hydrolysate (A), coffee residue hydrolysate (B), copra meal hydrolysate (c), sugarcane baggasse hydrolysate (D), and 8% (w/v) of soybean meal hydrolysate (E). Lane 1: mannose, Lane 2: galactose, Lane 3: glucose, Lane 4: xylose, Lane 5: manno-oligosacharide (X1: mannose, X2: mannobiose, X3: mannotriose, X4: mannotetraose, X5: mannopentose, X6: mannohexose), Lane 6: 0 h , Lane 7: 2 h, Lane 8: 4 h, Lane 9: 6 h, Lane 10: 9 h, Lane 11: 12 h, Lane 12: 24 h, Lane 13: 36 h, Lane 14: 48 h, Lane 15: 60 h.

3.2 Effects of Mannooligosaccharides on the Growth of Lactic Acid Bacteria

The effects of mannooligosaccharides (MOS) derived from AWs hydrolysate were investigated using 10 strains of LAB. The growth on MOS of these organisms was evaluated by calculating the enhancing activity on growth of LAB (%). Figure 2 showed that MOS from coffee residue and copra meal hydrolysate were able to promote the growth of almost every strain of LAB tested but copra meal hydrolysate could not promote the growth of *En.faeclis* JCM 5805. Although MOS were not shown to be presented in oligosaccharide mixtures from potato peel, soybean meal, as well as sugarcane bagasse hydrolysates (Figure 1), they also were able to promote most LAB tested with exceptions of *L. sake* TISTR840, *L. lactis* ATCC 19435 and *En.faeclis* JCM 5805 which could not be inhibited by potato peel, soybean meal, and sugarcane bagasse hydrolysates, respectively.

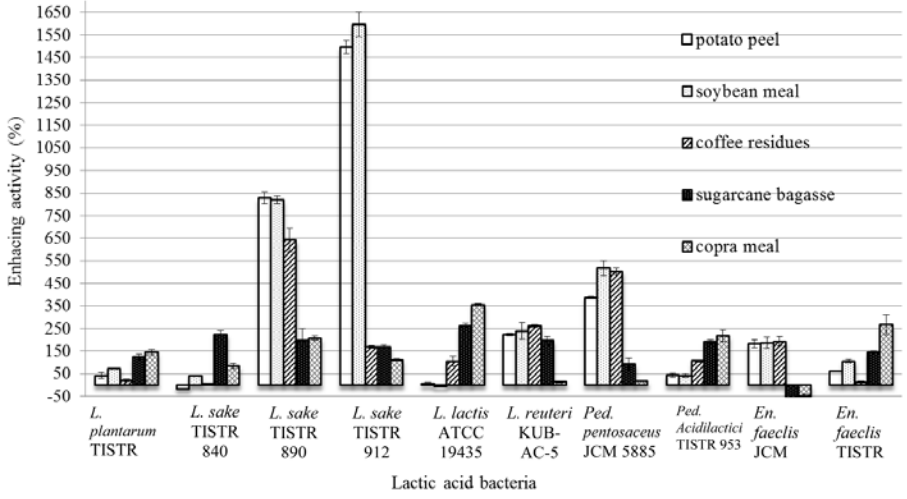

Figure 2. Effect of agricultural waste hydrolysates on the growth of Lactic Acid Bacteria.

3.3 Effects of Oligosaccharide Mixtures on Pathogenic Growth

Shigella DMST 1511, *Escherichia coli* E010, and *Staphylococcus aureus* TISTR 029 were selected for the growth assessment in different oligosaccharide mixtures. Interestingly, *S. aureus* TISTR 029 was prone to be inhibited by the MOS from both coffee residue and copra meal hydrolysates at 76.19±3.88% and 11.64±2.15%, respectively. Additionally, the same effect was observed from oligosaccharide mixtures from potato peel and soybean meal but sugarcane bagasse had no effect on *S. aureus* TISTR 029 growth. Only oligosaccharide mixtures from soybean meal hydrolysates could inhibit the growth of *Escherichia coli* E010 at 20±9.0% and no inhibitory effect was observed on the growth of *Shigella* DMST 1511 from any of the AWs hydrolysates (Figure 3).

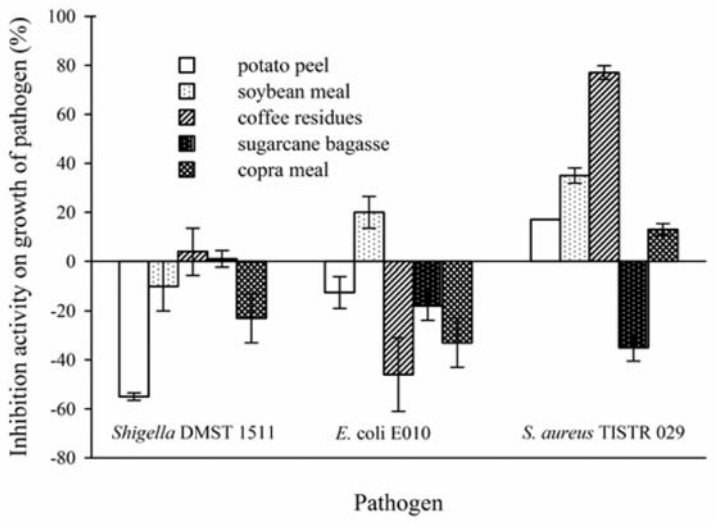

Figure 3. Effects of agricultural waste hydrolysates on the inhibition activity of pathogenic bacteria.

4. DISCUSSIONS

Large amounts of agricultural wastes have been abandoned not only because of no value but also because of an increase in environmental pollution. Bioconversions of these wastes into beneficial products could be a wise and profitable alternative. Potato peel, soybean meal, coffee residue, sugarcane bagasse, and copra meal are hemicelluloses in which copra meal and coffee residue have high contents of mannans [9]. The enzymatic hydrolysis of mannans by the action of mannanases result in MOS. When compared to FOS and GOS, there have not been as many reports of the effects of MOS as those of prebiotics [10]. Although the MOS from yeast cell walls were mainly documented for their prebiotic properties, recently, there have been increasing data on the production of MOS from the enzymatic hydrolysis of AWs. Pangsri *et al.* (2015) [11] showed that the MOS produced by *Bacillus circulans* NT 6.7 mannanase using copra meal as a substrate could promote the growth of LAB as well as inhibited pathogenic bacteria when compared to the commercially available MOS obtained from yeast cell walls. Pongsapipatana *et al.* (2016) [12] investigated the MOS containing copra meal hydrolysates produced by *Klebsiella oxytoca* KUB-CW2-3 mannanase as a recombinant enzyme and found that it could enhance the growth of LAB as well as inhibit the pathogenic growth. Dhawan *et al.* (2015) [13] studied the action of a beta-mannanase from thermotolerant *Paenibacillus thiaminolyticus* and showed that the MOS containing hydrolysate positively influenced the growth of probiotics, *Bifidobacterium* and *Lactobacillus* sp. Despite the mechanisms of the action of MOS remained to be elucidated, various evidences to date clearly convinced us that MOS would be a potential candidate for health benefits of both human and livestock. For the future aspect, the purified MOS will be a promising substance to help understanding the mechanism of prebiotic actions.

5. CONCLUSIONS

In this study, five agricultural wastes including potato peel, soybean meal, coffee residue, sugarcane bagasse, and copra meal have been investigated for their potentials to be sources of prebiotic production. Once the

crude mannanase from *P. oxalicum* KUB-SN2-1 catalyzed these lignocellulosic compounds, it resulted in various types of MOS as shown in Figure 1. Interestingly, these MOS, apparently shown to possess the prebiotic properties, of which they could promote the growth of LAB tested in this study suggesting that LAB were able to effectively use MOS as carbon sources for their growths. Moreover, coffee residue and copra meal hydrolysate clearly had inhibitory effects on the growth of *S. aureus* TISTR 029 which was one of the problematic pathogens. This study suggested the value added version of low-cost agricultural wastes and potential roles of MOS as prebiotics.

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