DEFATTED COPRA MEAL HYDROLYSATE AS A NOVEL CANDIDATE FOR PREBIOTIC

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Mannooligosaccharides (MOS) can be produced from mannan, which is found in many plants such as konjac, ivory nut, locust bean, guar gum, palm kernel, coffee bean and copra meal. In this study, we focused on mannooligosaccharide (MOS) preparation from defatted copra meal by mannanase enzyme from an effective mananase producer, *Bacillus circulans* NT 6.7. Results showed that MOS could be produced under optimal hydrolysis condition (20 Units/mL of concentrated enzyme and 50 min of reaction time). Various sizes of sugar: mannose mannobiose mannotriose mannotetaose mannopentaose and mannohexaose were detected in the Defatted copra meal (DCM) hydrolysate. Moreover, this hydrolysate could promote probiotic bacteria growth as effectively as commercial mannooligosaccharides. Therefore, the results here suggested that the DCM-hydrolysate could be used as prebiotic to improve human and animal health.

Key words : Mannanase, defatted copra meal hydrolysate, Prebiotic, Mannooligosaccharides

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Introduction

A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health (Gibson *et al*., 2004). Therefore, a prebiotic substrate must be particularly readily available to some groups of bacteria (of which lactobacilli and bifidobacteria are considered indicator organisms) that are beneficial to intestinal health but less available to potentially pathogenic bacteria, such as toxin-producing Clostridia, proteolytic *Bacteroides* and toxogenic *Escherichia coli* (Gibson *et al*., 2004).

The examples of prebiotics are inulin, galactooligosaccharides (GOS), fructooligosaccharides (FOS), xylooligosaccharides (XOS), and mannooligosaccharides (MOS). Mannooligosaccharides can be produced from mannan, the major polysaccharides of hemicellulose which is composed of glucomannan and galactomannan. Mannans, the major constituent of many plants such as ivory nut, locust bean, guar gum, konjac, palm kernel, coffee seed and copra meal. Copra meal is a by-product from the coconut oil process and contains a large amount of mannose in the form of mannan. Mannan consists of repeating beta-1,4 mannose units and a few alpha-1,6-galactose units attached to a beta-1,4 mannose backbone (Mohammad *et al*.,1999). Therefore, beta-mannanase is the most important enzyme for the hydrolysis of copra meal into mannooligosaccharide, which the copra mannan hydrolysates are stimulating the growth of the beneficial bacteria and limiting the pathogenic bacteria in the colon (Titapoka *et al*.,2008).

Objectives

This research regards information about mannooligosaccharides from defatted copra meal (DCM-hydrolysate) as a nutrient for growth of probiotic bacteria. Thus, the aims of this work were preparing mannooligosaccharides from defatted copra meal and testing the prebiotic properties on probiotic and pathogenic bacteria.

Materials and Methods

Preparation of copra meal

The copra meal was collected from Pratunum Pra-in market, Pathumthani, Thailand. The copra meal was dried at 70 °C for 2-4 hours. After that, the copra meal was blended and milled by hammer mill to 0.5 mm in size. Then, fat in copra meal was removed by the Soxhlet apparatus (Gerhardt soxtherm MULTISTAT/SX PC) (AOAC,1990). Defatted copra meal was used for mannanase production and mannooligosaccharide preparation.

Microorganisms and culture conditions for mannanase production

1% (v/v) inoculum of *Bacillus circulans* NT. 6.7 (Phothichitt *et al*., 2006) was added to a 5-liter fermenter (Biostat-B) containing 3.5 liters of an enzyme producing medium (PM), consisting of 1% defatted copra meal, 1.5% $KH2PO_4$, 3% polypeptone, 0.06% $MgSO_4$, and 2.5% corn steep liquor (Mohammad *et al*.,1999). The cultivation was conducted at agitation speeds of 600 rpm, aeration 0.75 vvm (Feng *et al*.,2003), at 45°C (Phothichitto *et al*., 2006) for 6 hours. Cells were removed by centrifugation at 8,000 rpm at 4°C for 15 min. The crude enzyme was concentrated ten-fold by ultrafiltration with 10 kDa cut-off membrane (Minimate TFF System, PALL, USA) and mannanase activity was determined.

Determination of mannanase activity

Mannanase activity was assayed in the reaction mixture containing 0.5 ml of 1% locust bean gum (LBG,Sigma aldrich) in 50 mM phosphate buffer (pH 6.0) with 0.5 mL of supernatant. Incubation was at 50°C for 60 min. Reducing sugar was determined by the Dinitrosalicyclic Acid (DNS) Method (Miller,1959).

One unit (U) of mannanase activity is defined as the amount of enzyme produced per 1 μmol of mannose per minute under assay conditions.

Determination of optimal conditions for DCM-hydrolysate preparation

Optimal enzyme concentration

Various crude enzyme concentrations at 1, 5, 10, 15, and 20 Unit/mL were used. The reaction mixture contained 0.5 ml of concentrated enzyme and 0.5 ml of 1.0% defatted copra meal in 50 mM potassium phosphate buffer pH 6.0. Incubation at 50°C for 30 min was used, and the reaction mixture was stopped at 100°C for 5 min. The DCM-hydrolysate supernatant was filtered through a 0.22 μm membrane and used for probiotic enhancement.

Optimal reaction time

Optimal enzyme concentrates were used in this experiment for various times (0, 5, 10, 20, and 50 min) at 50°C incubation, and the reaction mixture was stopped at 100°C for 5 min. The DCM-hydrolysate supernatant was filtered through a 0.22 μm membrane and used for probiotic enhancement.

Sugar analysis

Sugar analysis of DCM-hydrolysate was performed by HPLC (Water2414) under the following conditions: column, Aminex-HPX42C (Bio-rad); mobile phase, DI water; column temperature, 75°C; flow rate, 0.4 ml/min; and refractive index detector. Standard sugars are mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5), and mannohexaose (M6) (Megazyme).

Enhancement/inhibition properties of DCM-hydrolysate on beneficial and pathogenic bacteria

DCM-hydrolysate was prepared under optimal conditions. Twelve strains of beneficial bacteria and five strains of pathogenic bacteria were cultivated in 5 ml of MRS broth (Merck), brain heart infusion (BHI) broth (Himedia) , or NB medium (Merck)with 1% DCM-hydrolysate or commercial mannooligosaccharides. Lactobacillus and Pediococcus were cultivated in 5 ml of MRS broth and BHI broth (Agnes *et al*., 2005) respectively, at 37°C for 4 hours under anaerobic conditions. *Enterococcus* and pathogenic bacteria were cultivated in 5 ml of BHI broth and NB medium, respectively, at 37°C under aerobic conditions for 4 hours. Cell growth was determined by measuring optical density at 600 nm. The enhancement and inhibition activities were calculated by the following equations (Phothichitto *et al*.,2006) :

$$\text{Enhancement activity (\%)} = \frac{(SB-CB)}{CB} \times 100$$

$$\text{Inhibition activity (\%)} = \frac{(CB-SB)}{CB} \times 100$$

SB is the optical density of cell in medium with DCM-hydrolysate product

CB is the optical density of cell in medium without DCM-hydrolysate product

Results and discussion

Mannanase enzyme production

B. circulans NT. 6.7 showed the highest mannanase activity (27.66 Units/ml) at 6 hours as shown in Figure 1. In addition, we found that defatted copra meal had higher activity than copra meal, when used as carbon source. Defatted copra meal was a good carbon source for mannanase enzyme production because the high oil content in copra meal could inhibit the growth of microorganisms (Lin and Chen,2004). Moreover, the enzyme production time in the fermenter was shorter than flask. Optimum condition (in terms of agitation, aeration, and temperature) created high activity enzymes, was similar to Feng *et al* (2003). They reported that the maximum beta-mannanase activity of 212 Units/ml was achived in 36 hours of cultivation under optimal conditions (0.75 vvm, 600 rpm, and 30°C).

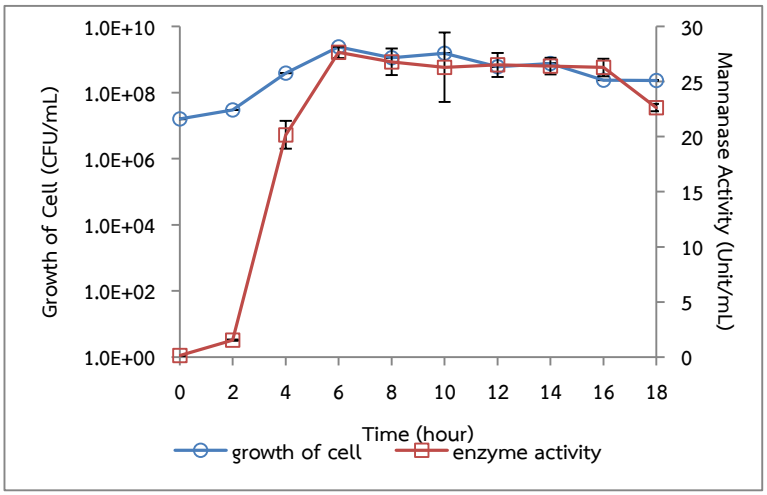

Figure 1 Mannanase production in 5 litre fermenter using defatted copra meal as carbon source

Determination of optimal conditions for DCM-hydrolysate preparation

DCM-hydrolysate, prepared from all enzyme concentrations (1, 5, 10, 15, and 20 Units/ml) had enhanced activity for *Lactobacillus reuteri* KUB-AC5, a probiotic bacteria, and the highest enhanced activity was 49.01% at 20 units/ml of enzyme concentration (Table 1). Moreover, The reaction time of defatted copra meal hydrolysis (0, 5, 10, 20 and 50 min) was observed at 20 Units/ml concentrated enzyme. The result showed that DCM-hydrolysate incubated for 50 min could enhance *Lactobacillus reuteri* KUB-AC5 growth up to 18.37%, as shown in Table 3. This result has shown that DCM-hydrolysate at 20 Units/ml at 50 min is the first point for creating various sizes of mannoligosaccharides (mannose, mannobiose, mannotriose, mannotetraose, mannopentaose, and mannohexose), as shown in Table 2 and 4. This result was similar to the report by Phothichitto *et al*. (2006), the culture filtrate of *B. circulans* NT. 6.7 grown on copra meal medium could promote *L. reuteri* AC-5 up to 49.593% and could not inhibit pathogenic bacteria. Moreover, the copra meal hydrolysate of isolate CW2-3 (*Klebsiella oxytoca*) showed a higher enhanced activity on growth of *L. reuteri* KUB-AC5 (Titapoka *et al*.,2008). This result showed that the enzyme concentration used in DCM-hydrolysate preparation was an important factor affecting the enhanced inhibition activity of target bacteria and mannooligosaccharides obtained. Therefore, the enzyme concentration at 20 Units/ml at 50 min was selected for studying optimal reaction time in DCM-hydrolysate preparation.

Table 1 Effect of enzyme concentration on DCM-hydrolysate preparation

Enzyme concentration (Units/mL)	Enhanced activity (%) of *L. reuteri* AC-5
1	2.88±1.23
5	10.76±2.65
10	19.33±6.32
15	29.14±5.34
20	49.01±9.65

Sugar analysis of DCM-hydrolysate

Sugar in DCM-hydrolysate was analyzed. The results showed that mannooligosaccharides (mannobiose, mannotriose, mannotetaose, and mannopentaose,M2-M5) were products from enzymes concentrated at 1, 5, and 10 Units/ml, while mannobiose, mannotriose, mannotetaose, mannopentose, and mannohexose (M2-M6) were products from enzymes concentrated at 15 Units/ml. Whereas, products from the enzyme concentrated at 20 Units/ml were mannooligosaccharides (mannose, mannobiose, mannotriose, mannotetaose, mannopentose, and mannohexose,(M1-M6). These results showed that mannanase at 20 Units/ml efficiently cleavaged the mannan in copra meal into mannooligosaccharides (M1-M6). However, product hydrolysis of 20 Units/ml with 50 min reaction time resulted in mannose, mannobiose, mannotriose,mannotetaose, mannopentaose, and mannohexaose. Therefore, the optimal condition for mannooligosacchrides preparation is 20 Units/ml, 50 min reaction time. Under optimum conditions, DCM-hydrolysate has mannobiose and mannotriose as the main products, as shown in Tables 2 and 4. These results correlated with Titapoka *et al*. (2008), the products hydrolyzed from copra meal by the S1 enzyme were mannotriose and mannotetraose (Titapoka *et al*.,2008). Moreover, when copra meal was incubated with the enzyme *Bacillus subtilis* WY34, mannnotetraose, mannotriose, and mannobiose were produced (Jiang *et al*., 2006).

Also, mannanase from *Bacillus* sp. KK01 hydrolyzed copra mannan into mainly mannobiose mannotriose and mannotetraose (Mohammad *et al*., 1996) While hydrolysis of coffee mannan with crude mannanases from *S. rolfsii* was detected mannobiose during the initial phase of the hydrolysis experiment, whereas mannobiose and mannotriose were the main reaction products in the latter phase of the hydrolysis experiment (Sachslehner *et al*., 2000). Thus, mannooligosaccharides produced by mannanase enzyme, could be used as prebiotic for growth promotion of beneficial intestine microorganism.

Table 2 Sugar analysis of DCM-hydrolysate in all enzyme concentrations

Enzyme concentrated (Unit/ml)	Mannose (mg/ml)	Manno biose (mg/ml)	Manno triose (mg/ml)	Manno tetraose (mg/ml)	Manno pentaose (mg/ml)	Manno hexaose (mg/ml)
1		0.161	0.194	0.100	0.036	
5		0.226	0.267	0.122	0.025	
10		0.244	0.274	0.101	0.047	
15		0.270	0.310	0.096	0.114	0.126
20	0.267	0.221	0.248	0.093	0.096	0.125

Table 3 Effect of reaction time on DCM-hydrolysate preparation

Time (min)	Enhanced activity (%) of *L. reuteri* AC-5
0	7.63±7.53
5	6.24±13.41
10	6.54±18.18
20	16.46±23.29
50	18.37±14.93

Table 4 Sugar analysis of DCM-hydrolysate at all reaction times

Time (min)	Mannose (mg/ml)	Manno biose (mg/ml)	Manno triose (mg/ml)	Manno tetraose (mg/ml)	Manno pentaose (mg/ml)	Manno hexaose (mg/ml)
0					0.129	
5		0.289	0.256	0.087	0.201	
10		0.241	0.259	0.089	0.097	
20		0.237	0.258	0.089	0.093	
50	0.253	0.306	0.350	0.097	0.119	0.146

Enhancement/inhibition properties of DCM-hydrolysate on probiotic/pathogenic bacteria

Twelve strains of probiotic bacteria and five strains of pathogenic bacteria were challenged. DCM-hydrolysate could stimulate growth of probiotic bacteria and DCM-hydrolysate could inhibit only *Shigella dysenteria* DMST 1511 as shown in Table 5. The result demonstrated that DCM-hydrolysate could support the growth of probiotic bacteria. This showed that probiotic bacteria could use the sugar in DCM-hydrolysate to support growth, as there were many reports of probiotic sugar utilization. *Lactobacillus* sp. could grow in glucose maltose or mannose. *Enterococcus* sp. used maltose, raffinose, arabinose, and malezitose in its carbohydrate fermentation (Macfaddin,2000). *Pediococcus* sp. could ferment maltose, manitol, sorbitol, xylose, ribose, lactose, rhamnose, and melezitose (Macfaddin,2000). From these results, it is suggested that mannooligosaccharides have the potential to promote intestinal microflora (such as prebiotics) and inhibit pathogenic bacteria, resulting in better animal and human health. Defatted copra meal-hydrolysate has prebiotic properties similar to commercial mannooligosaccharides. Also, defatted copra meal-hydrolysate could be a novel candidate for a prebiotic.

Table 5 Effect of DCM- hydrolysate on probiotic bacteria and pathogenic bacteria

Genus – species	Code	Enhancement (%)
Probiotic bacteria		
Lactobacillus plantarum	TISTR 541	15.98±16.39
Lactobacillus plantarum	ATCC 14917	11.84±7.53
Lactobacillus sake	TISTR 840	16.53±22.71
Lactobacillus sake	TISTR 890	16.52±12.40
Lactobacillus sake	JCM 1157	7.19±7.13
Lactobacillus sake	TISTR 912	11.84±6.40
Lactobacillus lactis	ATCCC 19435	12.91±4.04
Lactobacillus reuteri	KUB-AC5*	9.39±1.00
Pediococcus pentosacens	JCM 5885	3.44±2.00
Pediococcus acidilactici	TISTR 953	3.04±1.81
Enterococcus faecalis	JCM 5805	5.54±3.36
Enterococcus faecalis	TISTR 927	1.82±1.31
Pathogenic bacteria	**Code**	
Shigella dysenteria	DMST 1511	4.52±0.88
Staphylococcus aureus	TISTR 029	-
Salmonella entevica sevovar	DMST 17368	-
Escherichia coli	E010*	-
Salmonella serovar Enteritidis	S003*	-

*Isolated from chicken intestines by Nitisinprasert, *et al*. (2000)

Conclusion

Defatted copra meal could be used as substrate for mannanase production and mannooligosaccharide preparation. The optimal condition of DCM-hydrolysis by *Bacillus circulans* NT 6.7 mannase was 20 Units/mL of concentrated enzyme and 50 min of reaction time. The DCM-hydrolysate consisted of mannose, mannobiose, mannotriose, mannotetraose, mannopentaose, and mannohexaose, recognised as prebiotic mannoligosaccharides. The hydrolysate could effectively promote the growth of probiotic bacteria. Therefore, these results could provide preliminary data, regarding the capability of the DCM-hydrolysate, as a novel candidate for prebiotic.

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