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Research article

Characterization of *Bacillus subtilis* GA2(1) mannanase expressed in *Escherichia coli* Rosetta (DE3) for enzymatic production of manno-oligosaccharides from spent coffee grounds and *in vitro* assessment of their prebiotic properties

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Abstract

In modern times, prebiotics have received increasing, widespread interest as a functional food, with growing demand in the global functional food market. Most commercial prebiotics are non-digestible oligosaccharides. Recombinant mannanase was characterized and investigated for its potential in prebiotic production. The recombinant mannanase from *Bacillus subtilis* GA2(1) had maximal activity at pH 7.0 and 50°C, and was highly activated by Co^{2+} while strongly inhibited by Hg^{2+}. This enzyme effectively hydrolyzed pretreated spent coffee grounds (SCG) with final manno-oligosaccharides (MOS) products, mainly mannobiose (M2) and mannotriose (M3). Two types of lyophilized (LP) and non-lyophilized (NP) hydrolysate products had total hydrolysis levels of 25.2% and 28.2%, respectively. The de Man, Rogosa and Sharpe (MRS) broth supplemented with either LP or NP significantly ($p < 0.05$) increased the growth of the tested probiotic strains. Nutrient broth supplemented with either LP or NP strongly inhibited the growth of the tested pathogen strains. Interestingly, *Lb. plantarum* TISTR 1465 cultured in MRS-NP medium had a higher %hydrophobicity than that in the commercial prebiotic. This study developed an alternative and eco-friendly process to produce high-quality prebiotics and indicated the potential to add value to SCG as a major industrial waste.

Introduction

Numerous studies have indicated that the gut microbiome can impact on the healthy state of the host, so there is increasing interest in the potential dietary strategy that can manipulate this microbial community (Flint et al., 2012; Shreiner et al., 2015; Lordan et al., 2019. Prebiotics have been marked as a key substance that boosts the levels of gut-friendly bacteria and also restores gut flora dysbiosis (Macfarlane and Cummings, 1999). Prebiotics are defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota,

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thus conferring benefit(s) upon host health" (Gibson et al., 2010). The most popular commercial prebiotics are a group of oligosaccharides, particularly fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS) and manno-oligosaccharides (MOS). Among them, MOS is well known as an adhesion-inhibiting agent that can adhere to enteropathogenic cells, reducing their infection capability (Ghosh and Mehla, 2012).

Health-promoting MOS is built up from repeat mannose subunits linked together via the β-(1→4) glycosidic bond (Tanimoto et al., 2002. They can be produced from mannan-rich lignocellulosic wastes, including spent coffee grounds (SCG), copra meal, and palm kernel meal (Singh et al., 2018). Notably, there is a high mannan content of 20–30% weight per weigh on a dry weight basis (w/w) in SCG material, which makes it a more attractive substrate for MOS production (Bradbury and Halliday, 1990). MOS can be produced by the hydrolytic activity of beta-mannanase (EC 3.2.1.78) which randomly cleaves within the ß-1,4-glycosidic linkages of mannan (Chauhan et al., 2012). Large amounts of bacteria and fungi can produce ß-mannanase, particularly Gram-positive bacteria in the genus *Bacillus* (Dhawan and Kaur, 2007). Chantorn et al. (2015) isolated *B. subtilis* GA2(1) from soybean field soil in Khon Kaen province, Thailand and reported that this strain had mannanase activity levels of 0.35 U/mL and 0.44 U/mL at its optimum temperature and pH, respectively. Later, Piyapittayanun and Chantorn (2019) successfully isolated and cloned the gene encoding mannanase from *B. subtilis* GA2(1) (*man* gene) into the pET vector (pET28a-*man*). The current study aimed to overexpress pET28a-*man* in *Escherichia coli* Rosetta (DE3), to characterize the recombinant mannanase for its exploitation in MOS production from alkaline-treated SCG and finally to examine its prebiotic properties *in vitro*.

Materials and Methods

Materials

Chemicals and solvents were of analytical grade and were procured from Sigma-Aldrich (USA), Merk (Germany) and Carlo Erba (France). All media and media components were purchased from Himedia (India). Standard MOS was purchased from Megazyme (Ireland). Commercial fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and manno-oligosaccharides (MOS) were obtained from Sigma-Aldrich (USA), Carbosynth (UK) and Actigen (USA), respectively.

Bacterial strains

The lactic acid bacteria (LAB) and pathogen strains were obtained from laboratory stock culture at the Department of Biotechnology, Faculty of Science, Ramkhamhaeng University, Thailand. Three strains of LAB were grown in de Man, Rogosa and Sharpe (MRS) broth at 37°C for 6 hr, and three strains of pathogens were grown in nutrient broth (NB) at 37°C 16 hr. The *E. coli* Rosetta (DE3) (pET28a-*man*) was obtained from the Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Thailand. This recombinant strain was stored at -20°C until used.

Recombinant mannanase production

The pET28a-*man* transformed *E. coli* Rosetta (DE3) was used for the recombinant mannanase production by cultivating in Luria-Bertani (LB) broth containing 30 µg/mL kanamycin and 34 µg/mL chloramphenicol and incubating at 37°C with shaking at 200 revolutions per minute (rpm) until reaching 0.5–0.6 optical density at 600 nm (OD_{600}). Subsequently the cells were induced using 0.5 mM isopropyl-ß-D-thiogalactoside and incubated at 30°C with shaking at 200 rpm for 24 hr and harvested using centrifugation at 8,000 rpm and 4°C for 10 min. Then, the cell pellet was resuspended in cold lysis buffer and lysed using a microfluidizer with a z-type interaction chamber (Microfluidics; USA) at 103421.39 kPa and 4°C for 3 cycles. The soluble protein was separated from cell debris using centrifugation at 10,000 rpm and 4°C for 30 min and used as crude recombinant mannanase.

Mannanase activity assay

The mannanase activity was assessed by measuring the amount of reducing sugars released from locust bean gum (LBG) using 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959). The assay reaction mixture containing 0.5 mL of 0.5% (weight per volume; w/v) of LBG in 50 mM phosphate buffer pH 7.0 and 0.5 mL of diluted enzyme was incubated at 40ºC for 15 min. The reducing sugar content was measured based on absorbance at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes LBG to liberate reducing sugar equivalent to 1 µmol within 1 min under experimental conditions.

Characterization of recombinant mannanase

Effect of temperature on recombinant mannanase activity and stability

The optimum temperature of recombinant mannanase was determined by assaying the mannanolytic activity at pH 7.0 (50 mM phosphate buffer) over the temperature range 30–100°C, while thermal stability was evaluated based on the residual activity after holding the recombinant mannanase at 30–100°C for 3 hr.

Effect of pH on recombinant mannanase activity and stability

The optimum pH of biocatalytic activity was tested over the pH range 3.0–10.0 using the following buffering systems: 50 mM citrate buffer (pH 3.0–6.0), 50 mM phosphate buffer (pH 6.0–8.0) and 50 mM glycine-NaOH buffer (pH 8.0–10.0). For evaluating pH stability, the recombinant mannanase was incubated at 4°C using the same buffering systems in the pH range 3.0–10.0 up to 36 hr. Then, the residual activity was examined under optimum conditions.

Effect of metal ions and chemical reagents on recombinant mannanase activity

To determine the effects of metal ions (Ag^{+}, K^{+}, Li^{+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}) and chemical reagents with 10 mM concentration (ethylenediaminetetraacetic acid; EDTA, cetyl trimethyl ammonium bromide; CTAB, sodium dodecyl sulfate; SDS, dimethyl sulfoxide; DMSO, ethanol, methanol, Tween-80) on recombinant mannanase activity, the enzyme with each additive was incubated at 30°C for 30 min. The residual activity was assessed under optimum conditions.

Oligosaccharide production

The alkaline treated-SCG used in this study was provided by Wongsiridetchai et al. (2018). For MOS production, enzymatic hydrolysis was performed with an equal ratio (1:1, volume per volume; v/v) of recombinant mannanase: 1% (w/v) of treated SCG dissolved in 50 mM phosphate buffer pH 7.0 for 30 hr under optimum conditions. Then, the mixture was boiled for 15 min and centrifuged for 20 min at 10,000 rpm. The supernatant was collected and filtered successively through grade 4 and grade 3 cellulose filter paper and 0.2 µm cellulose acetate membrane (Whatman; USA). This supernatant hydrolysate was then called the non-lyophilized hydrolysate product (NP). The NP was processed in a freeze dryer (SIM FD8-3; SIM International; USA) and the obtained product was the lyophilized hydrolysate product (LP). The total sugar and reducing sugar contents of both NP and LP were assayed using the phenol-sulfuric acid method of DuBois et al. (1956) and the DNS method, respectively, and the oligosaccharides content in the treated-SCG hydrolysate was estimated using Equation1:

$$\text{Oligosaccharide content} = \text{Total sugar} - \text{Reducing sugar} \quad (1)$$

Analysis of hydrolysate sugar compositions

The hydrolysate product patterns were determined using modified thin-layer chromatography (TLC; Rosengren et al., 2019). The NP and LP were subjected to TLC using a mobile phase of butanol : ethanol : water (10 : 8 : 7, volume per volume per volume) and then visualized in visualization solution (65 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride in ethanol containing 3% sulfuric acid). Subsequently, the TLC plate was heated at 110°C until red-violet spots appeared. Commercial mannose and MOS (mannobi-, tri-, tetra-, penta- and hexaose) (Megazyme; Ireland) were used as standard sugars.

Oligosaccharide prebiotic activity

Simulation of human gastrointestinal digestion

Resistance to the oral cavity, stomach and small intestine digestion process were tested according to a standardized digestion method developed by Minekus et al. (2014) with slight modification. Commercial MOS, FOS and GOS were used as positive controls. All the simulated digestion fluids consisting of simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as specified by Minekus et al. (2014).

Artificial digestion in the mouth

To mimic oral phase digestion, the samples were thoroughly mixed with SSF at pH 7.0 at a final ratio of sample to SSF of 50:50 (v/v), followed by adding α-amylase to obtain a final activity volume of 75 U/mL. This mixture was incubated at 37°C for 5 min under constant, sufficient stirring and then was withdrawn at 0 min, 1 min, 3 min and 5 min for residues analysis of the oligosaccharides. Digestion was terminated by boiling for 10 min to inactivate the enzyme. The percentage of oligosaccharide hydrolysis during *in vitro* digestion was calculated using Equation 2:

$$\text{Hydrolysis (\%)} = 100 \times 1 - (\text{Oligosaccharide}_{\text{residues}} / \text{Oligosaccharide}_{\text{initial}}) \quad (2)$$

Artificial digestion in the stomach

The digestion started by mixing the samples and SGF in a volume ratio of 50:50; then, the pH was adjusted to 2.0 by adding 1 M HCl. Subsequently, this mixed solution was incubated in a shaking water bath at 37°C for 120 min and sampled at time intervals of 0 min, 15 min, 30 min, 60 min and 120 min for analysis of the remaining oligosaccharides. The mixture was neutralized to pH 7.0 with 1 M NaOH to terminate the acid hydrolysis reaction. The percentage of hydrolyzed oligosaccharides was calculated using Equation 2.

Artificial digestion in the small intestine

For the simulated small intestine digestion method, the samples and SIF pH 7.0 were mixed in equal amounts (50:50, v/v), and bile extract solution was added to provide 10 mM in the final digestion mixture. Then, all mixtures were shaken in a 37°C water bath for 120 min; the resulting hydrolysate samples were collected at 0 min, 15 min, 30 min, 60 min and 120 min to monitor the residual oligosaccharides concentration. The mixture was heated in boiling water for 10 min to end the hydrolysis reaction. The percentage digested was determined using Equation 2. The overall hydrolysis of oligosaccharides occurring in mimicked human gastrointestinal (GI) digestion was calculated using Equation 3:

$$\text{Total hydrolysis (\%)} = \text{Hydrolysis during the oral phase (\%)} + \text{Hydrolysis during the gastric phase (\%)} + \text{Hydrolysis during the small intestine phase (\%)} \quad (3)$$

Proliferative effect of oligosaccharide on lactic acid bacteria

The ability of beneficial bacteria to utilize an oligosaccharide was carried out according to the method of Chantorn et al. (2018) with slight modification. Three strains of LAB (*Lactobacillus acidophilus* TISTR 1338, *Lactobacillus casei* TISTR 1463, *Lactobacillus plantarum* TISTR 1465) were chosen to represent probiotic bacteria. For the inoculum preparation procedure, all lactobacilli were cultured anaerobically in MRS broth at 37°C for 48 hr and then the turbidity of the bacterial suspension was adjusted at 600 nm to 1.0. Each experiment commenced with the inoculation of 1% (v/v) inoculum into MRS broth supplemented with oligosaccharide derived from treated-SCG hydrolysate (NP and LP) at a final concentration of 5 mg/mL and subsequently incubated at 37°C under anaerobic conditions for 6 hr. Then, viable LAB cells were enumerated based on spread plating on MRS agar; the pH value was also measured. Commercial MOS, FOS and GOS were used as positive controls and the MRS medium without any prebiotic substances served as the blank control. All samples were sterilized by passage through a 0.2-micron sterile filter and transferred to sterile MRS broth at the final concentrations described above. The enhancing activity was calculated using Equation 4:

$$\text{Enhancing activity (\%)} = (SF - CF) / (CF \times 100) \quad (4)$$

where SF is the number of colonies of LAB grown in MRS medium added with oligosaccharide derived from treated-SCG hydrolysate or commercial prebiotic and CF is the number of colonies of LAB grown in the control MRS medium, with both measured in colony forming units (CFU) per milliliter.

Anti-proliferative effect of oligosaccharide on pathogenic bacteria

To study pathogenic growth in pure culture, an experimental method was performed with reference to Chantorn et al. (2018) with a few modifications. The pathogen strains selected for the current study were: *Bacillus cereus* ATCC 1178, *E. coli* ATCC 25922 and *Salmonella paratyphi* DMST 15673. They were grown in NB at 37°C with shaking at 200 rpm for 24 hr; then, the cell density was adjusted to an OD_{600} value of 1.0. At this point, the starter culture was obtained. For the anti-proliferative tests, the starter cultures (1%, v/v) were individually transferred into NB medium with 5 mg/mL (final concentration) of oligosaccharide added, derived from treated-SCG hydrolysate (NP and LP; test group) or commercial MOS, FOS or GOS (positive control group); the cultures were shaken at 200 rpm and 37°C for 5 hr. Afterward, a proper dilution was selected and spread onto the nutrient agar and immediately the pH of the culture medium was measured. The experimental results were compared to pathogenic bacteria grown in NB medium without additives (blank control group), and the inhibition activity was calculated according to Equation 5:

$$\text{Inhibition activity (\%)} = (CF - SF) / (CF \times 100) \quad (5)$$

where SF is the number of colonies of pathogens grown in NB medium supplemented with oligosaccharide derived from treated-SCG hydrolysate or commercial prebiotic and CF is the number of colonies of pathogens grown in the control NB medium, with both measured in colony forming units (CFU) per milliliter.

Cell surface hydrophobicity assay

The microbial adhesion to hydrocarbons (MATH) test was assessed following the protocol of Krausova et al. (2019), modified as follows. Lactobacilli starter cultures were prepared as mentioned above. This experiment started with the inoculation of 1% (v/v) fresh starter culture into the MRS broth supplemented with the different samples as described previously; then, anaerobic cultures were cultivated overnight at 37°C. The cell pellets were separated from the culture medium using centrifugation at 4°C and 8,000 rpm for 15 min, washed and resuspended in phosphate buffered saline pH 7.0 and the cell suspension was diluted to an absorbance at 600 nm of 0.5. Next, 0.5 mL of n-hexadecane was added to 1 mL of cell suspension and incubated at 30°C for 10 min. The mixtures were mixed on tube rotators (Stuart; UK) at 20 rpm for 2 min and then allowed to stand until an upper hydrocarbon phase had thoroughly separated. After separation, the aqueous phase was carefully withdrawn and absorbance was measured again. The percentage of cell surface hydrophobicity was computed based on Equation 6:

$$\text{Hydrophobicity (\%)} = 100 \times 1 - (OD_{600} \text{ after mixing with n-hexadecane}) / OD_{600} \text{ after mixing with n-hexadecane} \quad (6)$$

Statistical analysis

The collected data were analyzed using one-way analysis of variance and tested for differences between groups by applying Tukey's test. Significance was considered based on $p < 0.05$. All analyses were performed using the SPSS® statics software version 24 (IBM; USA).

Results

Biochemical properties of recombinant mannanase

Effect of temperature on recombinant mannanase activity and stability

The optimum temperature for mannanase activity was 50°C, with the highest activity being 43.060 ± 0.721 U/mL (Fig. 1A). The thermo stability of the recombinant mannanase is shown in Fig. 1B. After incubation, the enzyme at 60ºC for 3 hr retained more than 50% of its activity. Mannanase activity was relatively stable at 30–50°C for 3 hr; then, it decreased at the higher temperature of 70°C.

Effect of pH on recombinant mannanase activity and stability

The recombinant mannanase had maximum activity (41.8 ± 0.703 U/mL) at pH 7.0, while the catalytic activity declined suddenly at pH 4.0 and non-enzymatic activity at pH 3.0 (Fig. 2A). The recombinant mannanase was very stable for up to 1 hr over a wide pH range (3.0–10.0); enzyme activity remained at more than 80% (Fig. 2B). The enzyme was more active in an alkaline pH range than under acidic conditions as it kept more than 50% of its mannanase activity at pH 7.0–10.0 after 36 hr of incubation, whereas by contrast, its activity speedily dropped when the pH value was outside this range.

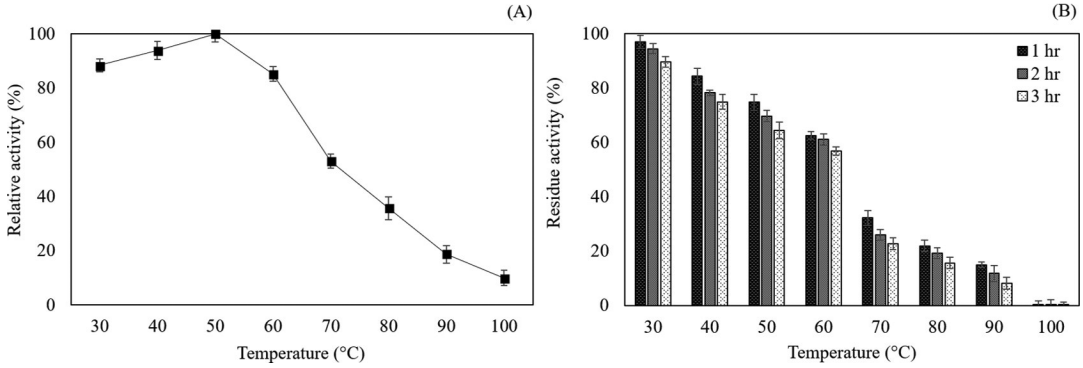

Fig. 1 Influence of temperature on *B. subtilis* GA 2(1) mannanase: (A) activity; (B) stability, where assays were conducted in the range 30–100°C, residual mannanase activities were immediately measured after pre-incubation of the enzyme at a given temperature for various time points and error bars indicate standard deviation (SD)

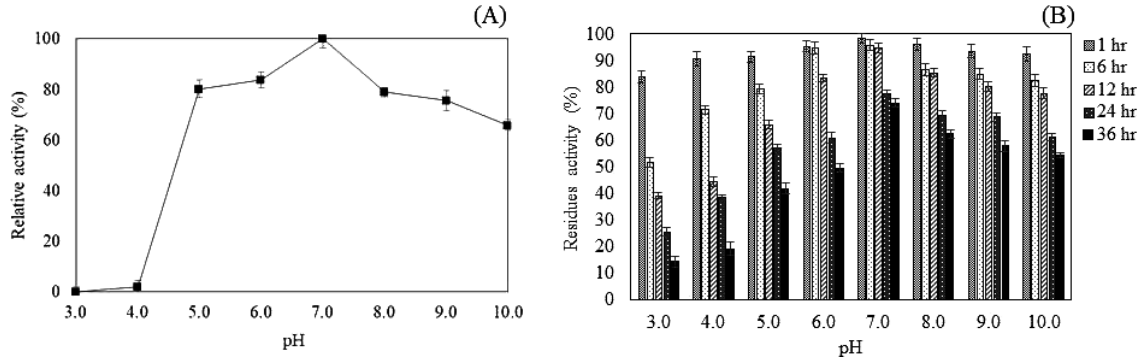

Fig. 2 Influence of pH on *B. subtilis* GA 2(1) mannanase: (A) activity; (B) stability, where activity assays were performed at 50°C for pH range 3.0–10.0 using different buffer solutions (50 mM), residual activities were assayed after incubating these enzymes alone in buffers at 4°C and error bars indicate standard deviation (SD)

Effect of metal ions and chemical reagents on recombinant mannanase activity

The effects of diverse metal ions and chemical reagents on recombinant mannanase activity are listed in Table 1. The result illustrated that Ag^{+}, K^{+}, Li^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, DMSO and Tween-80 positively altered enzyme activity. In particular, the presence of Co^{2+} raised the catalytic activity to 173.1%. In contrast, enzyme activity was inhibited by SDS, CTAB, ethanol, and methanol; in particular, EDTA and Hg^{2+}, were strong inhibitors that diminished the relative activity by 21.5% and 17.3%, respectively. However, Cu^{2+}, Ni^{2+}, Fe^{2+} and methanol did not affect the mannanolytic activity of the recombinant mannanase.

Oligosaccharide derived from enzymatic hydrolysis of treated spent coffee grounds

An experiment was conducted on mannanase activity under the optimum conditions (50°C and pH 7.0). The performance of the mannanase-catalyzed hydrolysis of treated-SCG was based on the amount of reducing sugar released from this substrate over the 30 hr reaction period. Hydrolyzed treated-SCG substrate yielded 11.7 ± 0.001 mg/mL, 45.7 ± 0.007 mg/mL, 50.5 ± 0.004 mg/mL, 137.1 ± 0.018 mg/mL, 157.6 ± 0.009 mg/mL and 167.7 ± 0.002 mg/mL of reducing sugar for incubation times of 30 min, 240 min, 300 min, 18 hr, 24 hr and 30 hr, respectively (Fig. 3A). These data showed no significant difference in the reducing sugar content between the reaction times of 24 hr and 30 hr; hence, a processing time of 24 hr was chosen. This analysis identified the main products of hydrolysis by the recombinant mannanase (both the NP and LP forms) were M2 and M3 (Fig 3B), with the oligosaccharide concentration of the NP and LP samples being 314.8 ± 1.4 mg/mL and 263.3 ± 1.8 mg/g respectively.

Table 1 Effect of various metal ions and chemical reagents on enzyme activity

Chemical	Relative activity (%)
Control	100±1.4^{a}
Ag^{+}	111.4±3.8^{b}
K^{+}	106.4±5.2^{ch}
Li^{+}	109±2.6^{c}
Ca^{2+}	150.6±5.6^{d}
Co^{2+}	173.1±6.9^{e}
Cu^{2+}	100±8.4^{a}
Fe^{2+}	97.7±3.9^{am}
Hg^{2+}	17.3±4.6^{f}
Mg^{2+}	125.3±2.2^{g}
Mn^{2+}	107.1±2.3^{h}
Ni^{2+}	99.2±12.5^{am}
Zn^{2+}	106.1±5.1^{h}
Fe^{3+}	104.5±2.4^{h}
EDTA	21.8±2.8^{i}
CTAB	31.4±5.2^{j}
SDS	70.7±3.6^{k}
DMSO (50 % (v/v) concentration)	105±2.3^{h}
Ethanol (50% (v/v) concentration)	85.9±3.5^{l}
Methanol (50% (v/v) concentration)	96.7±3^{m}
Tween-80 (0.2% (v/v) concentration)	161.1±9.8^{n}

EDTA = ethylenediaminetetraacetic acid; CTAB = cetyl trimethyl ammonium bromide; SDS = sodium dodecyl sulfate; DMSO = dimethyl sulfoxide; v/v = volume per volume.

Values (mean ± SD) with same superscript letter are not significantly ($p < 0.05$) different.

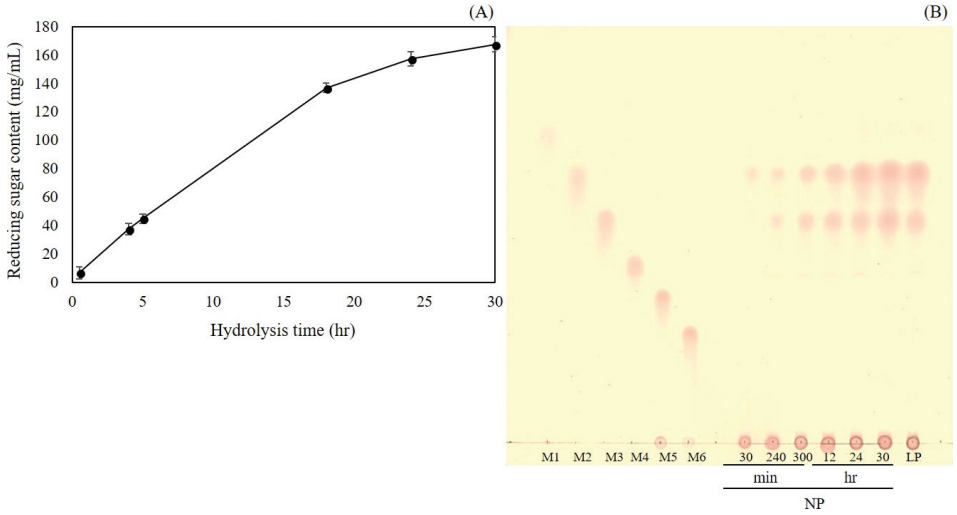

Fig. 3 (A) Reducing sugar content from enzymatic hydrolysis of treated-SCG by recombinant mannanase at different times: (B) thin-layer chromatography profile of treated-SCG hydrolyzed end products, where hydrolysis reaction occurred at 50°C and pH 7.0, with an aliquot of a mixture withdrawn at each time represented, standard sugars were M1 = mannose; M2 = mannobiose; M3 = mannotriose; M4 = mannotetraose; M5 = mannopentose; M6 = mannohexaose and error bars indicate standard deviation (SD)

Potential prebiotic activity

In vitro digestibility of oligosaccharides

The prebiotic oligosaccharides were separately digested by artificial saliva, gastric juice, and small intestine juice with bile. As shown in Fig. 4A, all samples and selected commercial oligosaccharides were highly resistant to α-amylase digestion with no significant differences among the hydrolysis values when tested from the same hydrolysis time. The lowest hydrolysis after 5 min of incubation with an α-amylase (1.5%) was observed in the NP sample. In the simulated gastric conditions, the rate of hydrolysis was increased by prolonging the incubation period. After 120 min gastric digestion, the LP product had the greatest resistance (hydrolysis degree of 16.7%) to these low pH conditions (Fig. 4B) whilst commercial MOS, FOS and GOS were significantly less tolerant to acid hydrolysis with hydrolysis degrees of 21.9%, 21.4% and 23.3%, respectively. In the small intestine digestion studies, as observed in Fig. 4C, the rate of hydrolysis of all samples slowly increased during the first hour of incubation time. At the end of the hydrolysis process, there were no significant differences in the hydrolysis percentages of the two samples (NP and LP) and commercial prebiotics (MOS, FOS, and GOS). After passage through each digestion step, total hydrolysis was also calculated. As can be seen in Table 2, the LP product had the best resistance to the entire process of simulated GI digestion (oral, gastric and small intestine digestion phases) with a minimum overall hydrolysis level of 25.2%, followed by the NP product (28.2%), FOS (31.2%) and MOS (33%), whereas the highest level of hydrolysis was observed in the GOS sample (34.4%).

Impact of oligosaccharide supplementation on lactic acid bacteria

The growth of probiotic bacteria on different prebiotic substances was studied using pure cultures of *Lb. acidophilus* TISTR 1338, *Lb. casei* TISTR 1463 and *Lb. plantarum* TISTR1465, and quantified by counting colonies, expressed as enhancing activity (%). After 6 hr of fermentation, both NP and LP promoted the growth of all tested probiotic strains (Table 3). The maximum enhancing activity (155.7%) was observed for *Lb. plantarum* TISTR 1465 grown in MRS medium added with LP, while lowest final pH of 5.4 was obtained from the same media. Commercial MOS and FOS had higher efficacy to support the population of *Lb. acidophilus* TISTR 1338 than all of the hydrolysate products at the same concentration. The *Lb. casei* TISTR 1463 strain utilized NP as a source of energy such as commercial prebiotic FOS (but not significantly so), whereas this strain had less growth than LP using GOS.

Table 2 Total amount of hydrolysis of hydrolysate product and different commercial prebiotics for simulated digestion along entire gastrointestinal tract

Sample	Total hydrolysis (%)
Non-lyophilized product	28.254
Lyophilized-product	25.254
Commercial MOS	32.956
Commercial FOS	31.204
Commercial GOS	34.434

MOS = manno-oligosaccharides; FOS = fructo-oligosaccharides; GOS = galacto-oligosaccharides.

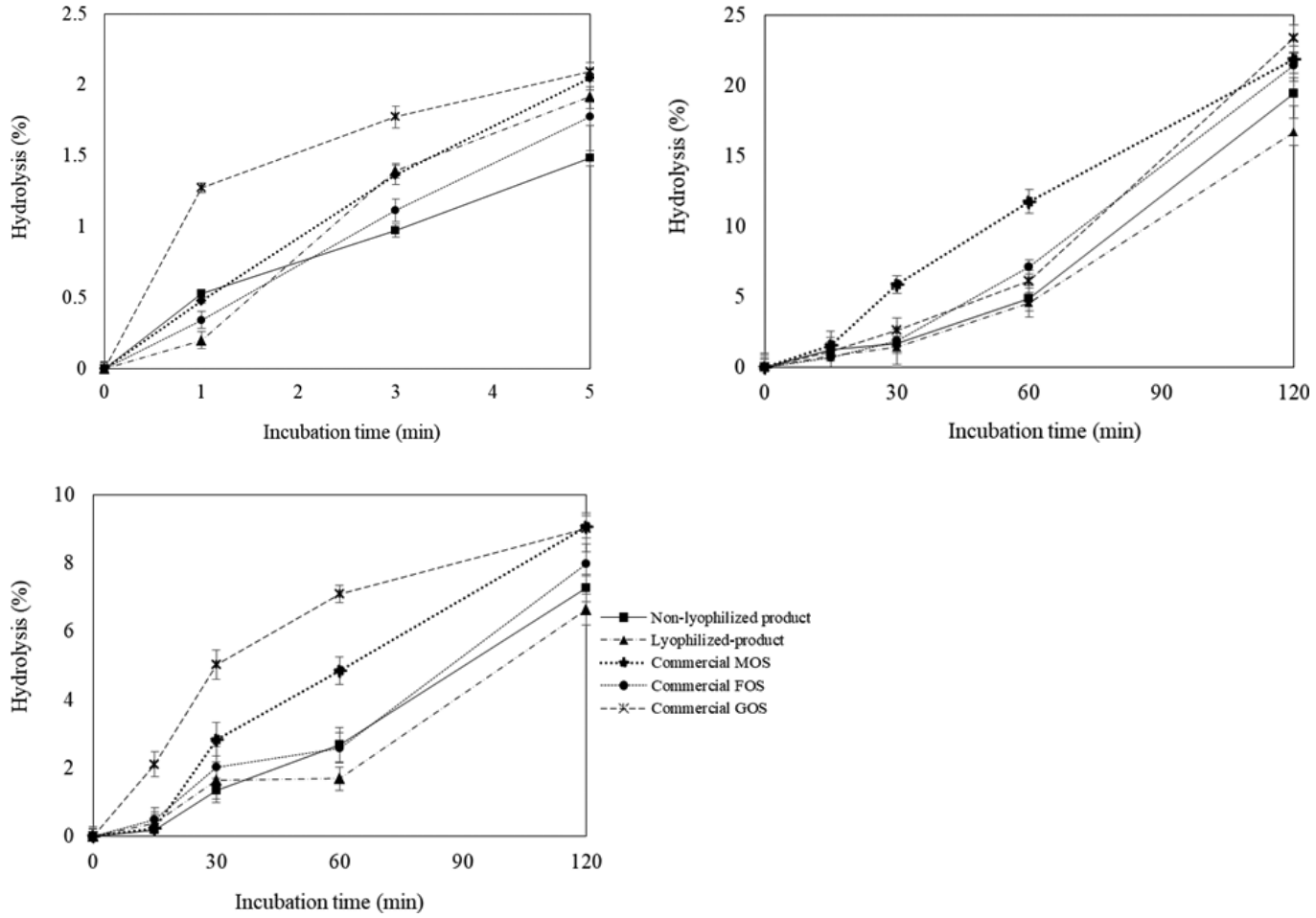

Fig. 4 *In vitro* digestibility of hydrolysate products as compared to commercial prebiotics (A) simulated α-amylase digestion for 5 min (B) simulated gastric digestion for 120 min and (C) simulated small intestine digestion for 120 min.

Impact of oligosaccharide supplementation on pathogenic bacteria

The changes in harmful pathogen populations (*B. cereus* ATCC 1178*, E. coli* ATCC 25922, *S. paratyphi* DMST 15673) on the tested prebiotic substances are presented in Table 3. The results indicated that none of the oligosaccharides promoted the growth of any of the three pathogenic strains. Noticeably, the presence of LP product produced a maximal inhibition activity on *B. cereus* ATCC 1178, *E. coli* ATCC 25922 and *S. paratyphi* DMST 15673 of 100%, 99.8%, and 95.2%, respectively. The *B. cereus* ATCC 1178 population showed very similar changes with all of the selected prebiotic substances, with a reduction in bacterial numbers after 5 hr incubation. Among the commercial prebiotics, FOS and GOS had a significantly lower capability to inhibit the growth of *S. paratyphi* DMST 15973 compared to media supplemented with the NP and LP product; likewise, both FOS and GOS had significantly poor inhibitory action on *E. coli* ATCC 25922.

Adhesion properties of lactic acid bacteria

The surface hydrophobicity of lactobacilli cells was studied using MATH assay, with n-hexadecane as a polar solvent. The three selected probiotic strains had significantly different hydrophobicity values between all prebiotic substances and the negative control, as shown in Table 4. The highest percentage of cell surface hydrophobicity (32%) was observed in *Lb. plantarum* TISTR 1465 cultured in MRS medium containing NP product and that was not significantly different with the commercial prebiotic MOS. About five-fold higher hydrophobicity values of *Lb. casei* TISTR 1463 were gained in the presence of LP product and commercial MOS. The best adhesion ability of *Lb. acidophilus* TISTR 1338 cells was with n-hexadecane ($p < 0.05$) on commercial GOS followed by commercial MOS, LP, NP and FOS, respectively.

Table 3 Effect of manno-oligosaccharides (MOS) on growth profile of lactic acid bacteria and pathogenic bacteria

Strain	Prebiotic substances									
	Non-lyophilized product		Lyophilized-product		Commercial MOS		Commercial FOS		Commercial GOS	
	Activity (%)	pH	Activity (%)	pH	Activity (%)	pH	Activity (%)	pH	Activity (%)	pH
L. acidophilus TISTR 1338	120.833	5.4	85.417	6.0	125	5.4	108.333	5.8	127.083	5.4
L. casei TISTR 1463	62.5	5.9	56.250	6.1	57.8	5.8	64.062	6.1	71.875	5.9
L. plantarum TISTR 1465	155.738	5.4	65.574	6.0	134.4	6.1	24.590	6.0	26.230	6.0
B. cereus ATCC 1178	93.22	6.6	95.25	7.0	94.83	6.9	91.23	6.3	92.60	6.3
E. coli ATCC 25922	99.80	6.9	99.84	7.0	99.80	7.0	94.22	5.5	61.60	5.0
S. paratyphi DMST 15673	99.03	6.7	100	6.9	99.30	6.8	39.23	6.0	56	6.3

FOS = fructo-oligosaccharides; GOS = galacto-oligosaccharides.

Discussion

The different factors affected enzyme activity, especially pH, temperature, and the presence of activators/inhibitors; thereby they work best under optimum conditions. At the optimum temperature, the amount of recombinant mannanase was comparable to that of the native *B. subtilis* GA2(1) mannanase (Chantorn et al., 2015) as well as other mannanases from *Bacillus* sp. (Piwpankaew et al., 2014; Kim et al., 2017). Additionally, the recombinant mannanase from *B. subtilis* GA2(1) was considered very stable at high temperatures compared to other bacterial mannanases (Liu et al., 2015; Suzuki et al., 2018). The optimum pH of the recombinant mannanase was comparable to those of mannanase from the native *B. subtilis* GA2(1) (pH 6.0) (Chantorn et al., 2015) and *Bacillus* sp. CFR1601 (pH 6.0–7.0) (Srivastava and Kapoor, 2015). Based on the pH stability result, this mannanase should have potential in a wide range of applications such as in the paper and pulp industry. In the presence of different metal ions and chemical reagents, similar results were obtained in recombinant *B. circulans* CGMCC 1416 ß-mannanase where it was activated by Co^{2+}, Ca^{2+} and Li^{2+} and inactivated by Hg^{2+} (Li et al., 2008). For enzymatic hydrolysis of treated-SCG, Wongsiridetchai et al. (2018) reported that the hydrolyzed treated-SCG products from native *B. subtilis* GA2(1) mannanase were M2 and M3; similarly, the main products of those hydrolyzed by the recombinant mannanase were M2 and M3. This result was consistent with that from the hydrolysis of coffee mannan using the *Vibrio* sp. strain MA-138 mannanase expressed in *E. coli* BL21 (DE3) (Tanaka et al., 2009).

A prebiotic substance must be able to resist digestion by the digestive enzymes and digestive juices of the host, so that it can pass through the upper GI tract into the colon where it is selectively fermented by probiotics (Davani-Davari et al., 2019). Therefore, *in vitro* digestibility study was examined as the first characteristic of a candidate prebiotic. The results of the current study showed that α-amylase could not hydrolyze the studied hydrolysate product or the selected commercial prebiotics. This result might be attributed to the fact that α-amylase can cleave the α-1,4-linkages in carbohydrate compounds (Jacobsen et al., 1972), but mostly the sugar oligomers of all tested oligosaccharides are joined by the ß-1,4-glycosidic bond. However, the hydrolysis level of all samples occurred largely during *in vitro* gastric conditions, suggesting that the glycosidic linkages are acid-sensitive covalent bonds and thus easily disrupted by acid or dilute acid (Bartnik and Facey, 2017). The two forms of hydrolysate product showed

Table 4 Surface hydrophobicity of lactic acid bacteria cells

Strain	Cell surface hydrophobicity (%)					
	Control	Non-lyophilized product	Lyophilized-product	Commercial MOS	Commercial FOS	Commercial GOS
L. acidophilus TISTR 1338	4	9.3	11.6	13	7.8	14
L. casei TISTR 1463	4.1	11.6	19.8	20	12.5	9.3
L. plantarum TISTR 1465	3.2	32	20.3	30.5	13.8	18.8

MOS = manno-oligosaccharides; FOS = fructo-oligosaccharides; GOS = galacto-oligosaccharides.

better tolerance against bile extract in artificial small intestine fluid. A similar result was recorded by Asano et al. (2003), who found that MOS derived from coffee mannan showed good resistance to human salivary α-amylase, artificial gastric juice, a pancreatic enzyme and intestinal mucous enzyme digestion. Based on these characteristics, all forms of MOS product may be considered as non-digestible oligosaccharides, and for this reason, they can reach the large intestine area and be accessible and utilized by probiotic microbes.

Among prebiotic features, a competency to selectively encourage probiotic growth is the one of most crucial factors. The current results demonstrated that different types of oligosaccharides influenced the growth characteristics of LAB. Another study indicated that the monomer sugar composition, molecular weight, degree of polymerization, linkage-type and branching also affect the fermentation selectivity of a prebiotic by probiotic microorganisms. (Ferreira-Lazarte et al., 2019). From Table 3, an increment in enhancing activity corresponded to a decreased pH in the culture medium, which was consistent with the fact that the LAB can metabolize an oligosaccharide to organic acid as a major end-product, thus causing the pH drop (Sarbini and Rastall, 2011). The current findings highlighted that the NP and LP products were comparable to commercial prebiotics (MOS, FOS, and GOS) in their probiotic growth-promoting activity. In addition, they displayed anti-proliferative activity by suppressing the level of pathogenic bacteria (Pongsapipatana et al., 2016; Chantorn et al., 2018; Thongsook and Chaijamrus, 2018). From the trial findings, the produced MOS had the important prebiotic property of being able to selectively proliferate beneficial bacterial growth and thus contribute to its host's well-being.

In probiotic bacteria, the ability of competitive adherence to intestinal epithelial cells and mucosal surfaces can block the colonization of pathogens and will reduce the risk of infection by harmful bacteria in the intestinal tract (Monteagudo-Mera et al., 2019). The current results demonstrated that the MOS mixtures from hydrolysate play a vital role in the adhesive potential of lactobacillus cells, particularly for *Lb. plantarum* TISTR 1465 cells toward n-hexadecane. These findings follow consistently for Cao et al. (2019), who reported the greatest activity of *Lb. plantarum* ATCC 14917 adhesions was obtained from cultured with MOS. Furthermore, when *Lb. plantarum* AKK30 was cultured in MRS combined with MOS, they presented maximum adhesion activity compared with inulin (Sophian et al., 2019). Notwithstanding, it would be necessary to confirm some of their prebiotic activity using *in vitro* gut fermentation models.

Consequently, the MOS prepared from hydrolyzed treated-SCG using recombinant mannanase revealed a potential prebiotic effect, opening new opportunities for further development of a commercial prebiotic and also potential agricultural waste utilization.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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