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β-Mannanase from *Bifidobacterium adolescentis* DSM 20083: Molecular Cloning, Expression, and Biochemical Characterization for Manno-Oligosaccharides Production Using Makapuno

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ABSTRACT

β-Mannanase has gained increasing interest recently due to its ability to degrade mannan polymers and produce high-value products such as manno-oligosaccharides (MOS). In this work, the tendency of β-mannanase from a human isolate, *Bifidobacterium adolescentis* DSM 20083, to produce potentially prebiotic MOS by hydrolyzing mannan-rich agricultural substrates such as makapuno (*Cocos nucifera* L.), a naturally occurring coconut variant, was investigated. A truncated variant of the β-mannanase gene was successfully cloned and heterologously expressed in *Escherichia coli* BL21(DE3). In SDS-PAGE, the recombinant β-mannanase was apparently homogeneous and had a molecular mass of about 110 kDa. The purified enzyme was found to have a specific activity of 66.1 U_{man}/mg when locust bean gum was used as substrate. The optimum temperature and pH of activity for the enzyme were obtained at 37 °C and pH 5.3, respectively. Among the cations tested, Co^{2+} was found to increase enzyme activity by 63%. Kinetic measurements showed K_m, v_{max}, k_{cat} and k_{cat}/K_m values of 0.32 ± 0.03 mg/mL, 42.4 ± 1.2 μmol/min-mg, 71.3 ± 1.9 /s and 221 ± 30 mL/mg-s, respectively. Analysis using HPAEC-PAD indicated that using makapuno, the major hydrolysis products of the enzyme are 6^1-α-D-galactopyranosyl-β-1,4-mannobiose (107 mg/g) and 6^1-α-D-galactopyranosyl-β-1,4-mannotriose (68.1 mg/g) amounting to 88% of total MOS produced. This study has shown that the β-mannanase from *B. adolescentis* could be used to produce MOS which could be of interest to the food industry.

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KEYWORDS

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INTRODUCTION

The human gut microbiota has gained more interest because of its perceived importance in health (Wang et al. 2017). Several strategies have been suggested to modulate gut microbiota such as prebiotics, probiotics, and synbiotics (Markowiak and Slizewska, 2017; Umu et al. 2017). Probiotics are live microbes that provide health benefits on the host when their numbers are sufficient in the gut (Hill et al. 2014). Several *Lactobacillus* and *Bifidobacterium* strains are known as probiotics and are well-studied because they are generally recognized as safe (Picard et al. 2005; Belicová et al. 2013). Meanwhile, prebiotics are substrates that selectively stimulate the growth of beneficial bacteria resulting in improved health (Gibson et al. 2017). Some of the well-established prebiotics include fructo-oligosaccharides, lactulose, and galacto-oligosaccharides. Recent reports considered manno-oligosaccharides or MOS as emerging prebiotics based on their bioactive properties (Jana et al. 2021).

MOS are products of the hydrolysis reaction catalyzed by β-mannanase (EC 3.2.1.78) using mannan or heteromannan as substrate (Chauhan et al. 2012). Mannans cannot be hydrolyzed by human digestive enzymes however in previous reports, intake of partial hydrolysate of galactomannan from guar gum was shown to stimulate the proliferation of several bifidobacterial species in the human gut (Tomlin et al. 1986).

The Philippines is considered one of the world's largest coconut producers and the existence of a naturally occurring coconut mutant with abnormal endosperm (locally known as makapuno) has been reported as early as 1914 (Gonzales, 1914). Unlike normal coconuts, makapuno endosperm develops into a spongy and amorphous soft mass, which almost fills the cavity of the shell (Angeles et al. 2018). This characteristic texture of makapuno can be attributed to its high galactomannan content (Mendoza et al. 1985). Compared to a matured normal coconut kernel with 1:12 galactose:mannose ratio, makapuno cell wall was reported to have mannose:galactose ratio of about 2.5:1 (Lado et al. 2019). As such, it is our aim to utilize makapuno for production of MOS making it more interesting to the food industry. To our knowledge, this is the first study on using makapuno as the substrate for the enzymatic production of MOS.

MATERIALS AND METHODS

Cloning of *B. adolescentis* β-Mannanase

Bifidobacterium adolescentis DSM 20083 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). The genomic DNA of *B. adolescentis* was used as a template for the amplification of a truncated variant of the β-mannanase gene (BaMan101K). Using proofreading Phusion High-Fidelity DNA Polymerase, amplification was performed with the primer pairs BaMan_Fw_NoS (5'-ATACCATGGCAGAAGGAAAATCGGCATCC-3') and BaMan_Rv_101K (5'-AATCTCGAGTGAACCGGTGCGGGACAG-3'). The forward primer and reverse primer contain *NcoI* and *XhoI* recognition sites (underlined), respectively. The amplified genes were digested, inserted into the pET-21d(+) vector, and transformed into *E. coli* BL21(DE3) from the New England Biolabs (Frankfurt am Main, Germany) by electroporation. The amplified sequences were confirmed by Sanger sequencing (Microsynth, Vienna, Austria).

Enzyme Purification

E. coli BL21(DE3) carrying the β-mannanase expression plasmid was grown in 600 mL Luria-Bertani medium with 100 μg/mL ampicillin. The culture was incubated at 37 °C to reach the early exponential phase of bacterial growth (OD_{600nm} 0.6). Isopropyl β-D-thiogalactopyranoside (0.5 mM) was then mixed into the culture and incubated further for 20 h at 25 °C with shaking. Cells were separated by centrifugation (4000 *x g*, 4 °C, 10 min), washed with sodium citrate buffer (50 mM, pH 5.3) and then ruptured using a French pressure cell press (AMINCO, Maryland, USA). The cell-free extract was collected by centrifugation and purified following the procedure of Arreola and co-workers (2014). Purified enzyme was kept at 4 °C in 50 mM, pH 5.3 sodium citrate buffer until further use.

Enzyme Characterization

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to determine the molecular mass of β-mannanase. Protein samples were added with Laemmli buffer (1:1 v/v) and then the mixture was heated for 3 min at 100 °C prior to loading into Bio-Rad Mini-PROTEAN TGX pre-cast gel. Coomassie brilliant blue R-250 was used to stain the gel. Unstained Bio-Rad Precision Plus Protein™ molecular mass standard was used as the ladder.

Standard Enzyme Assays

Mannanase activity was determined using 3,5-dinitrosalicylic acid (DNS) assay as previously described (Sak-Ubol et al. 2016). A 100 μL of appropriately diluted enzyme solution was incubated with 900 μL 0.5% locust bean gum (LBG) solution at 50 °C for 5 min with shaking at 800 rpm. The mixture was then heated at 99 °C for 5 min to stop the reaction. DNS method was used to quantify the released reducing sugar. One unit (U_{man}) of β-mannanase activity is equal to the amount of enzyme that produces 1 μmol of D-mannose per minute under experimental conditions.

Protein concentration was determined using bovine serum albumin as standard (Bradford, 1976). A 600 μL of Bradford reagent was incubated for 15 min with 15 μL of appropriately diluted enzyme and its absorbance was read at 595 nm. All measurements were done in triplicates.

pH and Temperature Dependency of β-Mannanase

The influence of pH on enzyme activity was assessed following the standard β-mannanase assay in different buffer solutions (pH 4.0-8.0). For pH 4.0-6.0, LBG was dissolved in sodium citrate buffer (50 mM) while for pH 7.0-8.0, LBG was dissolved in sodium phosphate buffer (50 mM). To determine the optimum temperature for enzyme activity, the standard β-mannanase assay was performed at varying temperatures (20, 30, 37, 50, 60 °C). To determine the temperature stability of BaMan101K, the purified enzyme was incubated at various temperatures (30, 37, 50, 60 °C) and the remaining enzyme activity was determined at certain time intervals using the standard assay.

Effect of Cations on the Activity of the Enzyme

Standard β-mannanase assay was performed to determine the effect of cations ion enzyme activity. Assay was conducted in the presence of various cations (1 mM), such as Li^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+}, and Zn^{2+}.

Kinetic Measurement

Kinetic measurements were performed at 30 °C using various concentrations of LBG. The observed data were fitted into the Michaelis-Menten equation and the kinetic parameters K_m, v_{max}, k_{cat}, and k_{cat}/K_m were calculated using SigmaPlot (Illinois, USA).

Manno-oligosaccharide Production

Preparation of Makapuno

Mature makapuno fruits, collected from the local market of Los Baños, Laguna, Philippines, were homogenized using 70% ethanol. The homogenized endosperm was dried (20 mm Hg) at 40 °C, ground further using a coffee grinder, and then defatted

using petroleum ether for 10 h in a Soxhlet apparatus. The defatted endosperms were subsequently extracted thrice for 2 h using distilled water at 40 °C with constant stirring and washed twice with 95% ethanol. The defatted makapuno was dried in a vacuum oven and stored at ambient temperature for further use.

Enzymatic Hydrolysis

Using the purified *β*-mannanase (5 U_{man}), conversion of 0.5% each of LBG, guar gum, konjac glucomannan, and makapuno was carried out on a 2-mL scale for 24 h at 30 °C with 300 rpm agitation. Samples were then drawn for analysis by HPAEC-PAD (high-performance anion exchange chromatography equipped with a pulsed amperometric detector).

The time course of the hydrolysis of LBG and makapuno by *β*-mannanase was also studied. The substrates were incubated with 5 U_{man} at 30 °C for 24 h. At various time intervals, samples were obtained and analyzed using HPAEC-PAD.

HPAEC−PAD equipped with CarboPac PA-1 column (4 mm × 250 mm) and CarboPac PA-1 guard column was used to carry out MOS analysis using Dionex DX-500 system (Dionex Corp., California, USA). MOS separations were performed with an isocratic run with 150 mM NaOH for 40 min at 30 °C. The external standards used were mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5), mannohexaose (M6), 6^1-α-D-galactopyranosyl-β-1,4-mannobiose (GM2), 6^1-α-D-galactopyranosyl-β-1,4-mannotriose (GM3) and 6^3,6^4-α-D-galactopyranosyl-β-1,4-mannopentaose (GGM5). M1 was obtained from Sigma (Missouri, USA) while M2-M6, GM2, GM3 and GGM5 were obtained from Megazyme (Wicklow, Ireland).

RESULTS AND DISCUSSIONS

Cloning and Overexpression

The β-mannanase enzyme belongs to glycoside hydrolase (GH) families namely GH5, GH26, GH44, GH45, GH113, and GH134 (www.cazy.org). In addition, they can show a modular architecture, with the catalytic domain linked to other domains such as carbohydrate-binding modules.

The strain *B. adolescentis* DSM 20083 used in this study is the same as the one used in the study of Kulcinskaja and co-workers (2013), which is *B. adolescentis* ATCC 15703. The gene predicted to encode the full-length β-mannanase was obtained from the genome sequence of *B. adolescentis* ATCC 15703 (GenBank accession no. NC_008618.1) and had the locus tag BAD_1030. When the full β-mannanase gene was expressed, the obtained enzyme activity was very low (data not shown). Thus, a truncated variant was similarly cloned and expressed. The predicted modular organization of the full-length β-mannanase gene consists of N-terminal secretion signal peptide followed by a GH26 catalytic module and Ig-like module. This is followed by two carbohydrate-binding modules of family 23 (CBM23), a 64 amino acid linker and finally, a transmembrane helix in the C-terminus (Kulcinskaja et al. 2013). The truncated variant (BaMan101K) contains four modules: the two CBM23 modules, catalytic, Ig-like, and a putative 64 amino acid linker. The truncated variant of the mannanase gene was used in this study. The truncated variant was successfully cloned into pET-21d(+) vector resulting in the pET21d(+)-BaMan101K plasmid. BaMan101K was intracellularly overexpressed in *E. coli* BL21(DE3) and the highest enzyme activity obtained was 58.0 ± 2.0 U/mL of fermentation broth.

Enzyme Purification and Biochemical Characterization

A single-step purification protocol was used to purify BaMan101K with Ni-immobilized metal ion affinity chromatography column (IMAC). The purified recombinant enzyme has a specific activity of 66.1 U_{man}/mg protein. The obtained purification fold was 3.4 with a yield of 4.8% (Table 1). Based on SDS-PAGE analysis, the purified enzyme had a molecular mass of approximately 110 kDa. This obtained molecular mass is in agreement with its molecular mass calculated according to the amino acid sequence, which is 101 kDa. This result is comparable to the β-mannanase from *Thermoanaerobacterium polysaccharolyticum* which has a molecular mass of 116 kDa (Cann et al. 1999). Most β-mannanase have a molecular weight in the range of 30-80 kDa however, some modular β-mannanase have a molecular weight of about 100 kDa or higher such as in this study.

Table 1: Purification of BaMan101K.

Enzyme Fraction	Crude	Pure
Total activity (U_{man})	9530	459
Total protein (mg)	485	6.90
Specific activity (U_{man}/mg)	19.6	66.1
Purification fold	1.0	3.4
Recovery (%)	100.0	4.8

Effect of Temperature and pH on Enzyme Activity

Enzyme activity is highly dependent on temperature and pH thus, it is important to determine its optimum temperature and pH. Enzyme activity was optimal at 37 °C (Figure 1A) and at pH 5.3 (Figure 1B). The obtained optimum temperature is similar to β-mannanases obtained from *Flavobacterium* sp., *Lactobacillus plantarum* (ATCC® 14917™) and *Bacillus subtilis* Bs5, which are within the range of 35 – 40 °C (Zakaria et al. 1998; Huang et al. 2012; Nadaroglu and Dikbas, 2018). Huang and co-workers (2012) also reported that 35 °C is the lowest optimum temperature of β-mannanase obtained from bacteria. It was also observed that the purified enzyme has a narrow pH optimum. The enzyme activity dropped significantly, i.e. ~60%, when the pH was changed to either pH 5.0 or pH 5.5 from pH 5.3. This obtained optimum pH is within the range of pH 3.0 – 8.0 of β-mannanases from various sources (Dhawan and Kaur, 2007). Some reported β-mannanase with comparable optimum pH are *Paenibacillus polymyxa* GS01, *Dictyoglomus thermophilum* Rt46B.1 and *Bacillus* sp. MSJ-5 (Zhang et al. 2009; Gibbs et al. 1999; Cho et al. 2006). Temperature stability is another important factor in determining the successful industrial applications of β-mannanase. After incubation at 30 °C and 37 °C, the remaining enzyme activity was 76.0 ± 1.0% and 48.0 ± 1.0%, respectively (Figure 1C). The enzyme rapidly lost of the enzyme.

Effect of Cations on Enzyme Activity

Certain metal ions have been reported to enhance or inhibit activities of several β-mannanases (Yamabhai et al. 2016). Cations such as Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+} Mn^{2+} and Zn^{2+} enhanced enzyme activity, with Co^{2+} showing the highest activating effect on enzyme activity with a relative activity of 163.0 ± 4.0% (Table 2). On the other hand, the addition of Ca^{2+} and Li^{+} does not affect the enzyme activity. Similar results were obtained for β-mannanases from *Bacillus circulans* CGMCC 1416, *Streptomyces tendae* and *Lactobacillus plantarum* (ATCC® 14917™) (Yoo et al. 2015; Li et al. 2008; Nadaroglu and Dikbas, 2018). None of the cations tested showed an inhibitory effect. These results suggest that the use of BaMan101K is convenient for industrial applications.

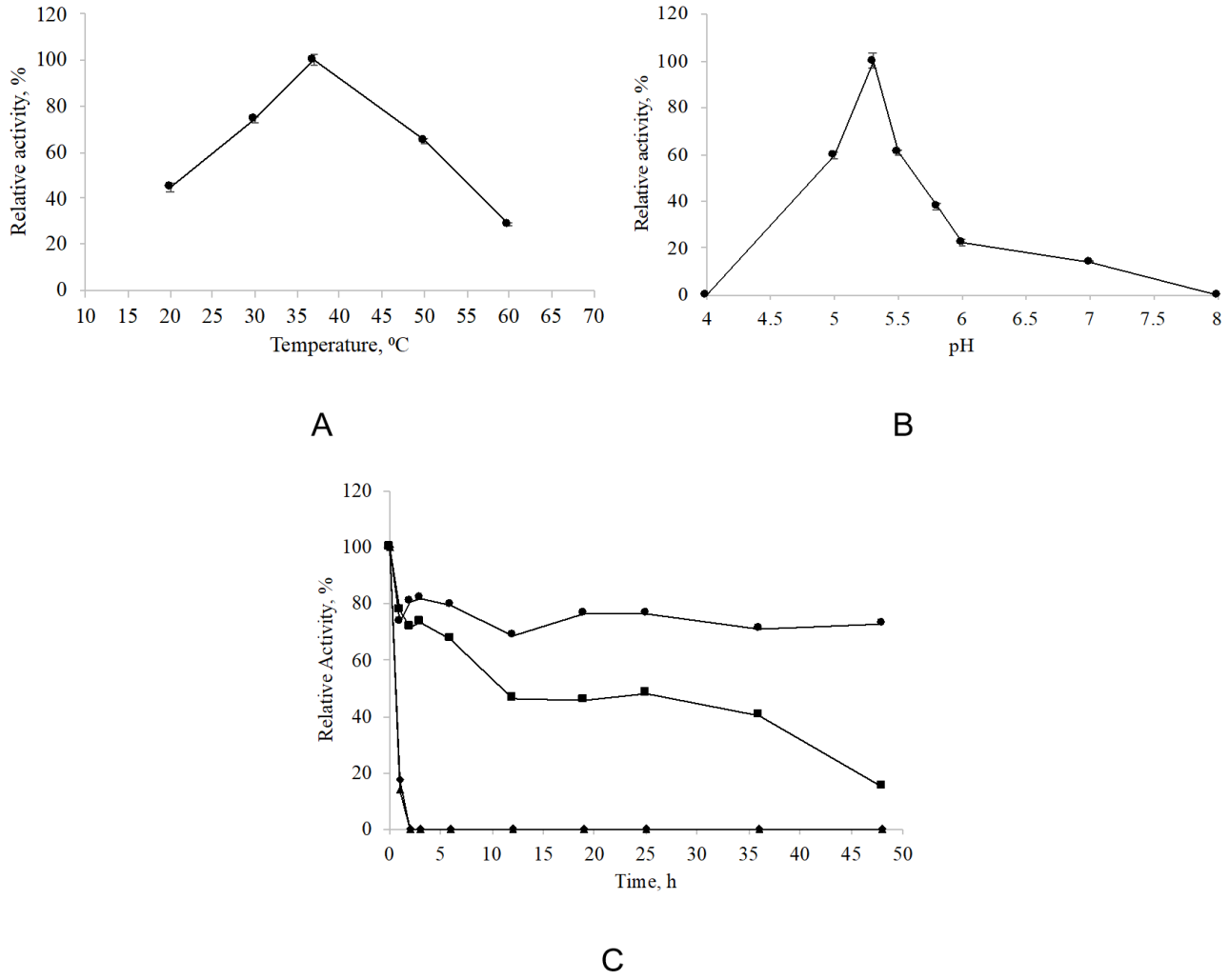

Figure 1: Effects of (A) temperature, (B) pH, and (C) temperature stability of BaMan101K. For C, (●) 30 °C, (■) 37 °C, (◆) 50 °C and (▲) 60 °C.

Table 2: Effect of various cations on the enzyme activity of BaMan101K. Final concentration of each cation in the reaction mixture is 1 mM.

CATION	RELATIVE ACTIVITY*, %
Control	100 ± 0
Li^{+}	100 ± 3
Mg^{2+}	104 ± 2
Ca^{2+}	100 ± 2
Mn^{2+}	156 ± 3
Fe^{3+}	104 ± 1
Co^{2+}	163 ± 4
Cu^{2+}	107 ± 2
Zn^{2+}	114 ± 2

* mean ± standard deviation of three replicates

Kinetic Parameters

The measured v_{max} and K_m values for LBG were 42.4 ± 1.2 μmol/min-mg and 0.32 ± 0.03 mg/mL, respectively. The k_{cat} and k_{cat}/K_m values were 71.3 ± 1.9 /s and 221 ± 30 mL/mg-s, respectively. The obtained K_m is lower than the K_m of β-mannanase reported from *Bacillus licheniformis* DSM13 (17.5 mg/mL), *Caldicellulosiruptor* Rt8B.4 (2.5 mg/mL), and *Cellulomonas fimi* (2.3 mg/mL) (Le Nours et al. 2005; Sunna, 2010; Songsiriritthigul et al. 2010). Meanwhile, the obtained K_m value is similar to K_m of β-mannanase from *Aspergillus aculeatus* MRC11624 (0.3 mg/mL), and *Klebsiella oxytoca* KUB-CW2-3 (1.06 mg/mL) (Chantorn et al. 2013; Setati et al. 2001). The relatively low K_m value obtained can be an advantage since it will form hydrolysis products even at low substrate concentration. Moreover, it is an important advantage especially inside the gut where numerous microorganisms are present, and β-mannanase from *B. adolescentis* can readily compete for the substrates since it has a high affinity for galactomannan substrates.

Manno-oligosaccharide Production

The purified recombinant β-mannanase can successfully convert LBG, guar gum, makapuno galactomannan, and konjac glucomannan into manno-oligosaccharides at varying degree of polymerization. Separation of M6 and GGM5 using HPAEC−PAD was not possible since these two sugars have close retention times, therefore the quantification of M6 and GGM5 is reported as the sum of both sugars. The substrate specificity of the recombinant enzyme to hydrolyze galactomannan and glucomannan is presented in Table 3. Hydrolysis of 0.5% each of LBG, guar gum and konjac glucomannan for 24 hours resulted in broad spectrum of MOS products. The sugars M3 (218 mg/g) and GM2 (136 mg/g) had the highest concentrations when LBG was used as the substrate. The concentrations of the sugars M4 and M6 were rather low, probably because the enzyme also hydrolyzed these larger oligosaccharides into shorter MOS. This product spectrum is quite similar to the products formed from the hydrolysis reactions using the β-mannanases of *Bacillus subtilis* YH12, *Bacillus pumilus* GBSW19, and *Bifidobacterium animalis* subsp. lactis Bl-04 (Morrill et al. 2015; Zang et al. 2015; Liu et al. 2015). The same product spectrum of MOS was obtained

using guar gum as substrate, however at significantly lower amounts: M3 (34.4 mg/g) and GM2 (66.7 mg/g). This might be due to the highly substituted structure of guar gum. Guar gum resembles LBG in being composed of D-galactose and D-mannose units combined through glycosidic linkages but with different proportions of these two sugars (Figure 2A). While LBG has an average ratio of 1:4 D-galactose to D-mannose units, guar gum has a higher ratio of D-galactose to D-mannose (1:2), giving it different properties. Hydrolysis of konjac glucomannan, on the other hand, resulted in the production of M3 (151.8 mg/g) followed by M2 (43.4 mg/g). We detected no substituted MOS formed, which suggests that its structure consists of linear D-mannose units in the main chain with randomly distributed D-glucose units (Figure 2B).

Table 3: Hydrolysis products from 0.5% each of locust bean gum, guar gum, and konjac glucomannan after 24 h of hydrolysis at 30 °C using 5 U_{man} BaMan101K.

MOS	AMOUNT OF HYDROLYSIS PRODUCTS (mg/g)*		
	Locust bean gum	Guar Gum	Konjac Gum
Mannobiose	66.41	28.01	43.43
Mannotriose	217.93	34.39	151.75
Mannotetraose	2.95	2.13	1.91
Mannopentaose	ND	1.84	ND
Mannohexaose and 6^3, 6^4-α-D-galactopyranosyl-β-1,4-mannopentaose	23.62	2.18	ND
6^1-α-D-galactopyranosyl-β-1,4-mannobiose	136.08	66.65	ND
6^1-α-D-galactopyranosyl-β-1,4-mannotriose	99.04	21.34	ND
TOTAL	**546.03**	**156.54**	**197.09**

*ND = none detected

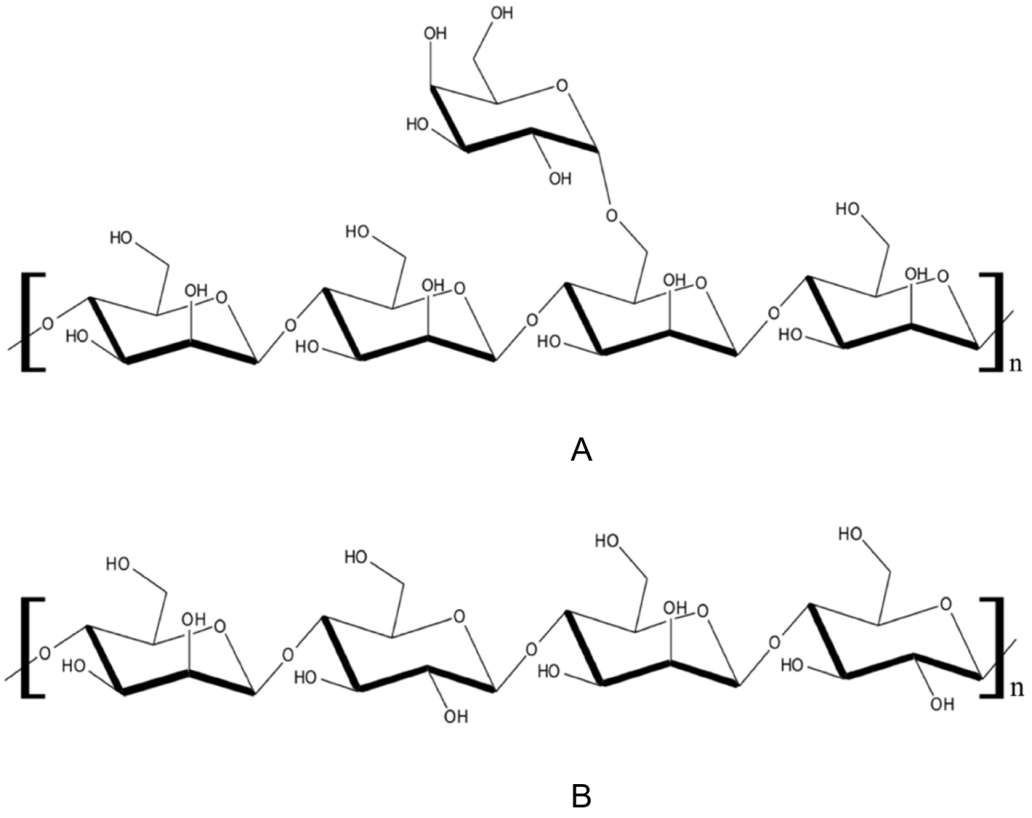

Figure 2: Chemical structures of (A) galactomannan, and (B) glucomannan (Yamabhai et al., 2016).

The ability of BaMan101K to convert makapuno to MOS was subsequently investigated. Makapuno galactomannan was isolated from mature makapuno endosperm, and the obtained yield was 27.7 ± 1.3%. MOS products formed from the conversion of LBG and makapuno were studied by analyzing the reaction mixture at 30 °C and at different time points up to 24 h. The enzyme activity remained higher than 50% after 24 h of hydrolysis. The time course of the production of MOS is very useful especially when one decides to produce one type of MOS over the other. When LBG was used as substrate (Figure 3A), it was observed that after 1 h of hydrolysis reaction, the major product is M3 (120.6 mg/g) followed by GM3 (75.8 mg/g) and GM2 (49.7 mg/g). While after 24 h of reaction the major product is M3 (195.7 mg/g) followed by GM2 (126.5 mg/g) and GM3 (102.1 mg/g). Similar MOS are the major products but at varying proportions. M2, M4 and M6 + GGM5 were also formed but to a lower extent. When makapuno was used as substrate (Figure 3B), GM2 increased from 61.45 mg/g (1 h) to 107.13 mg/g (24 h) making it the major hydrolysis product throughout the reaction time. GM3 was found to be the second major product and its concentration increased from 26.85 mg/g (1 h) to 68.10 mg/g (24 h). Throughout the reaction time M2, M3, M4 and M6+GGM5 were also formed but to a lower extent. These observations suggest that makapuno might have a structure

similar to that of guar gum, which is a highly substituted galactomannan. If the production of substituted MOS is preferred, makapuno could be used as substrate.

Comparison of the MOS produced from LBG and makapuno after 24 h of reaction time at 30 °C showed that they have similar product spectra (Figure 4). MOS yield from the conversion of LBG was 54.6% while for makapuno it was 20.0%. The present study confirms that β-mannanase from *B. adolescentis* can be a potential candidate for applications in MOS formation processes.

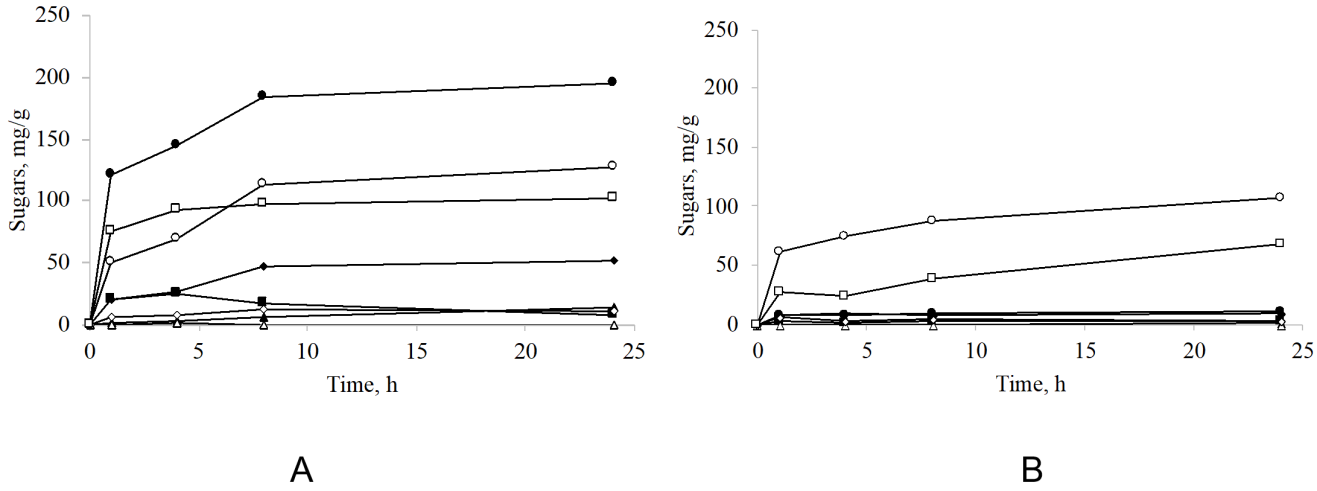

Figure 3: Time course of the formation of different MOS from (A) LBG and (B) makapuno using 5 U_{man} BaMan101K at 30 °C. (▲) M1, (◆) M2, (●) M3, (■) M4, (△) M5, (◇) M6 and GGM5, (○) GM2, (□) GM3.

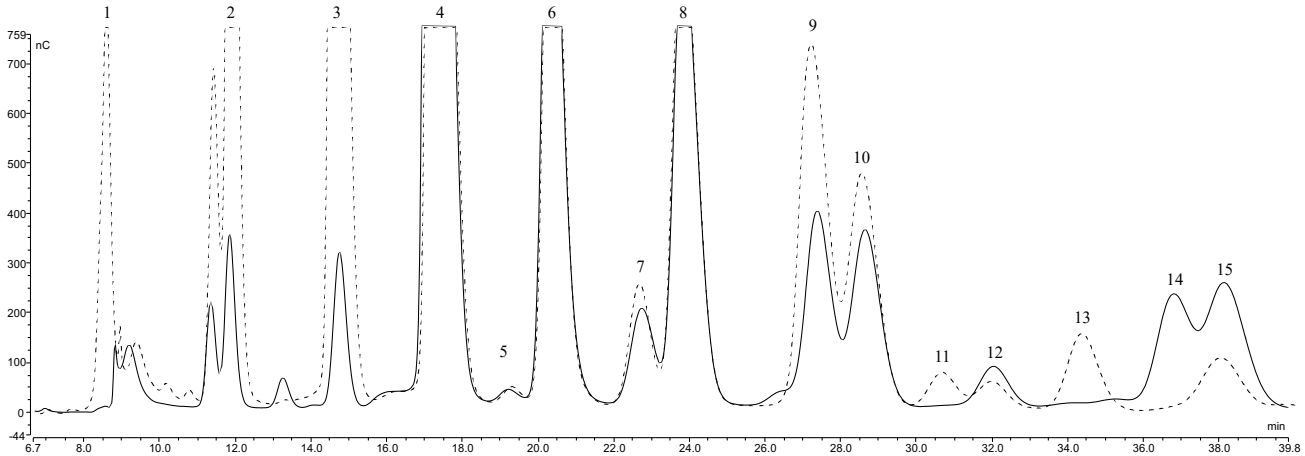

Figure 4: HPAEC-PAD chromatograms for the MOS production from LBG (- - -) and makapuno galactomannan (—) using 5 U_{man} BaMan101K after 24 h of reaction at 30 °C. The identified compounds are (1) M1, (2) M2, (3) M3, (4) GM, (5) M4, (6) GM2 and (8) GM3. Peaks 7, 9-15 were not yet identified.

CONCLUSIONS

This study presents the cloning, expression and biochemical characterization of BaMan101K. The recombinant enzyme was purified and used for the conversion of different galacto- and glucomannans to form manno-oligosaccharides. The enzyme successfully hydrolyzed mannan-rich substrates producing MOS mixtures with varying lengths. When a substrate with lesser substitution was used such as LBG, the major products were M2, M3 and GM2. On the other hand, when a highly galactosyl-substituted substrate was used such as makapuno, the major products were GM2 and GM3. Application of BaMan101K to produce potentially prebiotic manno-oligosaccharides using mannan-rich agricultural by-products, e.g., copra meal, spent coffee ground, and palm kernel cake, is highly recommended.

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CONFLICT OF INTEREST

There are no known conflicts of interest in this study.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

All authors contributed to the conception and design of the study. AAY collected and analyzed the data. All authors interpreted the results. AAY wrote the initial draft. LSGA, SLBA, THN, and DH reviewed and edited the manuscript. All authors read and approved the final manuscript.

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