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Modulation of cancer cell proliferation by unusually produced β(1-6) linked Mannan-oligosaccharides and β(1-6) linked Galacto-oligosaccharides using β–galactosidase from *Aspergillus oryzae*

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ABSTRACT

This study introduced an alternative way to produce NDOs, utilizing the recently reported unusual β-galactosynthase-β-mannosynthase dual activity of this enzyme, using free monosaccharide substrates. The enzyme successfully converted galactose and mannose monomer sugars efficiently to NDOs. Glycoblotting and MALDI-TOFMS analyses confirmed that the enzyme produced a maximum of 46.37% galacto-oligosaccharides (36.88% galactobiose, 8.52 % galactotriose, and 0.9746 % galactotetraose) from galactose monosaccharides and 32.62 % mannan-oligosaccharides (30.95 % mannobiose and 1.68 % mannotriose) from mannose monosaccharides. Both MOS and GOS modulated cell growth of different human cancer cell lines, with apparent antiproliferative effect on prostate adenocarcinoma (PC-3) cells and mitogenic effects on A549, HepG2, and HT-29. Mitochondrial membrane potential and careful morphological evaluation of PC-3 cells treated with GOS and MOS suggested that MOS treated cells showed possible inhibition of cellular adhesion that might have caused apoptosis to advance. The high-yielding NDOs synthase character of this cheaply available β–galactosidase makes this new strategy a good potential alternative in producing prebiotic NDOs.

1. Introduction

The β-galactosidase from *Aspergillus oryzae* (*Ao*-β-gal) belongs to the GH-35 family of β-galactosidases or lactases, enzymes known to catalyze hydrolysis of β(1-3) and β(1-4) galactosyl bonds in general acid hydrolysis (Maksimainen et al., 2011), and is often used to produce non-digestible oligosaccharide (NDO) of galactose economically in good yield (Neri et al., 2009). These enzymes catalyze enzymatic condensation, reverse hydrolysis and transglycosylation reactions for its particular substrates (Fujimoto et al., 1998; Pocedicova et al., 2010; Maksimainen et al., 2012). Rare reports have been published on the observed activities and specificities of these enzymes on different substrate analogs (Yamamoto and Davis, 2012; Nishio et al., 2002).

Recently, we reported a novel β–galactosynthase – β–mannosynthase dual-activity of *Ao*- β-gal, which was uncovered for the first time using free monosaccharide substrates (Tan et al., 2016). The enzyme was found to have the capacity to synthesize galactobiose and mannobiose from monosaccharides galactose and mannose, respectively, which were both linked in a β(1-6) fashion. The synthesis was efficient enough that it produced 41.45 % galactobiose and 16.80 % mannobiose after 24 hours. Both monosaccharides were thought to have been accommodated by the enzyme's catalytic site due to the believed enzyme active site's open-close conformation, which was previously observed in the same glycosyl hydrolase family of *Ao*- β-gal (Rojas et al., 2004). This β–galactosynthase – β–mannosynthase dual-activity of this enzyme could potentially revolutionize NDOs syntheses for prebiotic production, and it is in this light that this study was conducted.

In this study, we took advantage of the β–galactosynthase – β–mannosynthase dual-activity of *Ao-β–gal* to produce NDOs. NDOs have been gaining more and more popularity not only in food and pharmaceutical industries but also in probiotics,

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cosmetics (Adachi and Vallee, 2002; Minoru and Yoshio, 2002), animal feed (Mahious et al., 2006), and agricultural sectors due to its broad applications and uses (Patel and Goyal, 2011). These OS, also known as non-digestible oligosaccharides (NDOs), contain monosaccharides with glycosidic bond configuration not susceptible to hydrolytic activity of the digestive enzymes found in humans (Pokusaeva et al., 2011). NDOs from various origins have been used as food ingredients, dietary fiber (Qiang et al., 2009), weight controlling agents, humectants in confectioneries, and breweries. They are also effective regulators of blood glucose in diabetics (Thakur and Dixit, 2008) and serum lipids in hyperlipidemics (Villamiel et al., 2002), promotes gastrointestinal normal flora proliferation and pathogen suppression (Sánchez et al., 2008; Chen et al., 2000), facilitates mineral absorption, good source of antioxidant (Chen et al., 2000), dental caries prevention, enhances immunity (Tuohy et al., 2005; Moure et al., 2006), and useful for enhancing resistance to bacterial colonization (Hopkins and Macfarlane, 2003). Pharmaceutically, they are used in drug delivery (Gambarin et al., 1993; Du et al., 2009; Johnstone et al., 2010) and some are being modified to be heparin sulphate mimetics as antiangiogenic and anti-cancer (Johnstone et al., 2010).

Among the classified NDOs that have great significance in human health are manno-oligosaccharides (MOS) and galacto-oligosaccharides (GOS). They both are prebiotics that pass through the small intestine (Bosscher et al., 2009; Torrecillas et al., 2007; Burr et al., 2008; Macfarlane et al., 2008) and maintaining human health by selective stimulation of bifidobacteria that create an acid medium by converting soluble NDOs to organic acids (Tuohy et al., 2005; Van Munster and Nagengast, 1993), which results to an inhibition in the growth of harmful and undesirable bacteria (Sako et al., 1999). MOS are generally produced by extraction from the yeast's cell wall and via reverse hydrolysis using mannosynthases. It is a very common additive in animal foods as alternative to antibiotics. They were reported to suppress enteric pathogens and improving the integrity of animal intestinal mucosa, and modulate immune response (Waldroup et al., 2003; Spring et al., 2000). They bind to various bacteria and blocks bacterial attachment and colonization thus reduce the pathogen colonization in intestinal tract (Stahlhut et al., 2009). GOS, on the other hand, had gained its recognition mostly in foods and pharmaceutical industries. To date, high yield production of GOS relies solely on the optimized hydrolysis condition of lactose and immobilization of the enzyme for increase stability and recoverability of the biocatalyst for efficient use (Gaur et al., 2006; Albayrak and Yang, 2002a; 2002b; Pan et al., 2009; Freitas et al., 2011). Although immobilization may solve the existing problems regarding enzyme stability and recoverability, the GOS yield observed from substrate conversion has been no significant increase, some reported yields were even less than the free enzyme product. Furthermore, in terms of product purity, the existing method does not produce pure product, which causes industries to spend more money and effort in product purification. Hydrolysis of lactose does not only produce GOS but also lactose, glucose and galactose, which constitutes a significant fraction and complicates the products (Albayrak and Yang, 2002a; 2002b; Zheng et al., 2006). These monosaccharide products also limit the production of GOS by inhibiting the β-galactosidase (Coker et al., 2003; Kim et al., 2004a; 2004b; Chockchaisawasdee et al., 2005; Nguyen et al., 2006; Vera et al., 2011), with glucose inhibition as more effective in GOS production than galactose inhibition (Kim et al., 2004b).

This study introduces an alternative way to produce NDOs, taking advantage of the unusual β-galactosynthase-β-mannosynthase dual activity of the *Ao-β-gal*, using free monosaccharide substrates. In this study, NDOs produced by the enzyme were determined and purified. The produced NDOs were also tested for their biological activities against human cancer cell line.

2. Materials and methods

2.1. Materials

The β-galactosidase from *Aspergillus oryzae* (EC 3.2.1.23) used in this study is a powdered form (11.2 units/mg solid) and was purchased from Sigma Chemical Co. *Tri-N*-acetylchitotriose was purchased from SEIKAGAKU CORPORATION (Tokyo, Japan). Ultrafiltration membranes were supplied from Milipore, Carrigtwohill, Co. Cork, Ireland. Phosphate buffer was prepared from 0.05 M KH_2PO_4, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 0.1 M NaOH was used to adjust the pH. D-Galactose, D-mannose, 2,5-dihydroxybenzoic acid (DHB) and *O*-benzylhydroxylamine hydrochloride (BOA) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Glycoblotting beads (BlotglycoH™) was purchased from Sumitomo Bakelite, Co. (Tokyo, Japan). Unless otherwise noted; solvents and other reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Tokyo Chemical Industry (Tokyo, Japan), and Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Cell Counting Kit 8 was secured from Dojindo Molecular Technologies, Inc (Dojindo, Kumamoto, JAPAN). Human lung adenocarcinoma (A549) and Human prostate adenocarcinoma (PC-3) cell lines were secured from Health Science Research Resource Bank, Human hepatoma (HepG2) cell line was purchased from RIKEN cell bank, and Human epithelial colon cancer cells (HT-29) was from American Type Culture Collection. Cell culture media (DMEM and Ham's F-12K) were purchased from Gibco, Carlsbad, CA and supplemented with 10% (v/v) FBS (Gibco, Carlsbad, CA) and 1% Penicillin G (500 units/mL)-Streptomycin (500 units/mL) (Gibco, Carlsbad, CA). Cell culture wells were from Thermo Scientific Rochester, NY. MALDI-TOFMS

analysis was performed using Ultrflex I (Bruker Daltonics, Bremen, Germany) in a reflector, positive ion mode, typically totalling 200 x15 shoots, and controlled and analyzed by the Flexcontrol 3.0 software package (Bruker Daltonics, Bremen, Germany).

2.2. Methods

2.2.1. Enzyme purification

The crude enzyme (1.0 mg) was dissolved completely in 1.0 mL of mili Q water and 500 µL was loaded to each ultrafiltration membrane with nominal molecular weight limit of 30,000 (YM-30). The solutions were centrifuged at 13,000g and 4°C for 10 minutes, added with 400 µL water and re-centrifuged; the process was repeated until all the extenders were removed. A 1.0 µL enzyme concentrate was analyzed using MALDI-TOF to verify the removal of the extenders.

2.2.2. Oligosaccharide preparation and monitoring

Free monosaccharides (200 mg) were dissolved in buffer (50 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol; pH 4.7) and were added with β-galactosidase from *A. oryzae* (100 U) to a total volume of 1.0 mL. A 5 µL aliquot, in each time interval, were withdrawn from the mixture incubated at 40 °C and 45 °C for galactose and mannose, respectively. The aliquots were deactivated at 90°C for 20 minutes and lyophilized. The solids were dissolved in miliQ water to make 5 µg/µL solution and the samples were used for glycoblotting analysis. The remaining solutions were deactivated at 90°C for 20 minutes, centrifuged at 100, 000 rpm for 30 minutes, the supernatant was lyophilized and acetylated. Briefly, the lyophilized supernatants that contain OS were added with anhydrous sodium acetate and dissolved in acetic anhydride; the solutions were stirred overnight at 80°C, cooled at room temperature, pounded to cold saturated sodium bicarbonate solution and stirred for 1.5 hours. The solutions were extracted with CH_2Cl_2 and dried over anhydrous Na_2SO_4, concentrated under reduced pressure and subjected to flash column chromatography using ethyl acetate and hexane (2:1) as eluants to isolate the OS.

2.2.3. NDOs hydrolysis

To determine if the enzyme could utilize the OS as the substrate for hydrolysis, free galactose and free mannose were incubated for 24 hours at 40°C and 45°C, respectively. The crude mixtures were deactivated at 90°C, centrifuged and the supernatants were lyophilized. The solids were separately acetylated and the sugar monomers were partially removed to clearly see the effects of hydrolysis reaction on the oligosaccharides. The samples were then deacetylated using sodium methoxide. A 2.0 mg solid samples were dissolved in phosphate buffer (pH 4.7), incubated with β-galactosidase, and the formation of monosaccharides were monitored, at 0, 24, and 36 hours using glycoblotting analysis.

2.2.4. Glycoblotting analysis

The quantitation of OS was performed using a very sensitive and efficient glycoblotting-based protocol as shown in Fig. 1. A 100 µL aliquot of BlotglycoH beads in a 10 mg/mL suspension in water was placed into a MultiScreen Solvinert filter plate well (Milipore, Billerica, MA) and the water was drained by a vacuum. A 10 µL of sample solution and 10 µL of 0.225 mM of *tri-N*-acetylchitotriose, as the internal standard, were impregnated into the well followed by addition of 180 µL of 2% acetic acid in acetonitrile.

The plate was incubated at 80°C for 45 minutes to dryness in a thermostat to capture the whole oligosaccharides in the sample mixture onto the beads. The plate was washed twice with 200 µL of miliQ water and 1% triethylamine in methanol, added with 100 µL of 10% acetic acid in methanol and incubated at 25°C for 30 minutes to cap the unreacted hydrazide functional groups on the beads. After incubation, the solution was removed by vacuum and then washed twice successively with 200 µL of 10 mM HCl, methanol, and dioxane. Trans-iminization reaction on the oligosaccharides captured on beads was performed by addition of 20 µL of 50 mM BOA followed by treatment with 180 µL of 2% acetic acid in acetonitrile. The plate was incubated for 45 minutes at 80°C. The labelled oligosaccharides were eluted with 100 µL of water. The enrichment analysis was performed in three trials per sample per time interval.

2.2.5. MALDI-TOFMS analysis

The recovered BOA-tagged oligosachharides (1.0 µL) was mixed with the same volume of the matrix DHBNa:DHB (1:9) consisting 10mg/mL in 30% acetonitrile on a target plate MYP 384 (polished steel TF, Bruker Daltonics, Bremen, Germany) and dried to afford crystals of the sample-matrix. The crystals were analyzed using MALDI-TOFMS. The quantitation analysis was performed in three trials per sample and the data were reported as the average (±SD) of the trials.

2.2.6. Cell culture

HepG2, HT-29, and A549 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS and 1% Penicillin G (500 units/mL)-Streptomycin (500 units/mL) at 37°C with 5% CO_2. PC-3 cells were grown in Ham's F-12K Nutrient mixture supplemented with 10% (v/v) FBS, Penicillin G (500 units/mL) and Streptomycin

(500 unit/mL) at 37°C with 5% CO_2. All cell lines were incubated to sub-confluence.

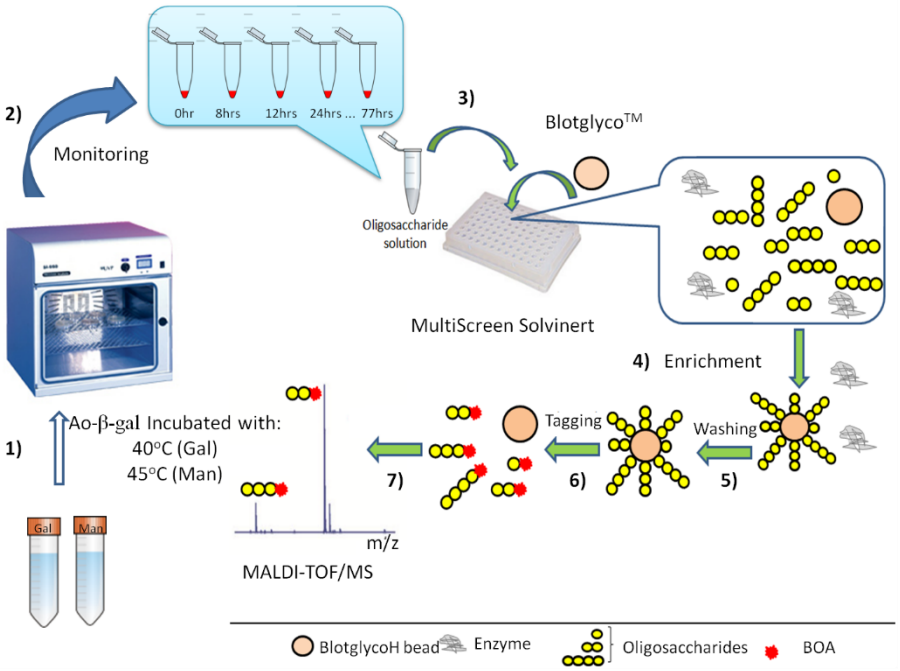


Fig. 1: General protocol for NDOs production using Ao-β-gal and glycoblotting-based high throughput analysis of oligosaccharides

2.2.7. Cell growth assay

HepG2, HT-29, A549, and PC-3 cells were seeded in a 96-well plate (Thermo Scientific), at an initial cell concentration of $5.0x10^3$ per wells, and incubated at 37°C with 5% CO_2 for 24 hours. The cells were treated with GOS and MOS for 48 hours, 72 hours, and 120 hours. Measurement of the cell growth was determined by adding 10 µL of cell counting kit 8, incubate for 2-4 hours and optical density was measured using a model 550 Microplate reader (BioRad, Hercules, CA, USA). Data were expressed as mean (±SD) and reported in terms of percent viability (±SD) of the trials (n = 3).

2.2.8. Measurement of mitochondrial membrane potential (ΔΨm)

The quantification of ΔΨm of cells stained with MitoTracker Deep Red was done by microscopic examination and confirmed by Laser Scanning Confocal Microscopy using LSM510 META Confocor 2 (Carl Zeiss Inc., Germany). To quantify the effects of GOS and MOS on the mitochondrial function in the prostate adenocarcinoma, cells were cultured in an 8-well Nunc Lab-Tek II Chamber Slide System (Thermo Scientific™) with GOS and MOS. After the treatment, the culture media with OS were replaced with fresh media, added with MitoTracker Deep Red 633 and incubated for 30-60 minutes for mitochondrial staining. Cells were inspected and detected for their relative fluorescence unit (RFU) using confocal microscopy. The images were analyzed using ImageJ software. Each assay was carried out in three (3) trials and the results were expressed as mean (±SD) of RFU and reported as percent RFU with respect to the control.

3. Results and discussion

3.1. GOS production and quantitation

The acceptability of the free galactose as substrate of Ao-β-gal has been reported recently (Tan et al., 2016). Ao-β-gal has been observed to synthesized galactobiose in Gal-β(1-6)-Gal glycosydic linkage after 24 hours of incubation. These facts were the basis to explore a likely possibility that the enzyme can synthesize longer chain of GOS using monosaccharide galactose. After prolonged incubation, at 40 °C for 8, 12, 24, 36, 48, and 77 hours, and product enrichment using glycoblotting analysis revealed only three varying compositions of the product namely galactobiose, galactotriose, and galactotetraose (Fig. 2).

Galactobiose was observed increasingly from 8-36 hours and a decrease after 36 hours, while galactotriose and galactotetraose were only detected from 8-77 hours and 36-77 hours, respectively. Although galactotriose was observed at the onset of the reaction, its concentration is barely noticeable until 36 hours. The highest concentration of GOS reached up to 46.37 % (92.74 g/L in 200 g/L galactose) of the total sugar at 36 hours as revealed by glycoblotting analysis (Fig. 3), which is made up of 79.53% galactobiose, 18.37% galactotriose, and 2.10% galactotetraose, and dramatically decreased down to 26.92% at 48 hours with a gradual increase at 77 hours to 35.75 %. The decrease in GOS was compensated by the increase amount of galactose

monomer and a slight increase in galactotriose and galactotetraose components (Fig. 3).

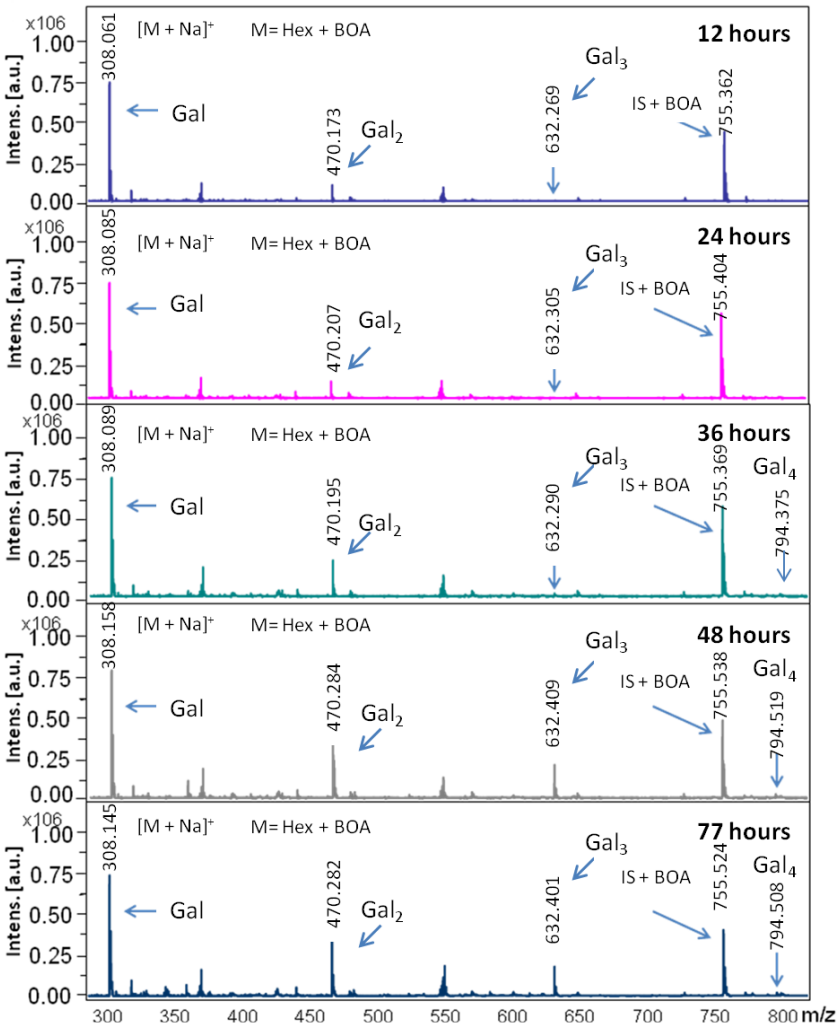


Fig. 2: Representative MALDI-TOFMS spectra of GOS products enriched by glycoblotting method. IS means the signal due to BOA-labelled *tri-N*-acetylchitotriose used as an internal standard for the quantitation. Gal means galactose; Gal_2 means galactobiose; Gal_3 means galactotriose; Gal_4 means galactotetraose

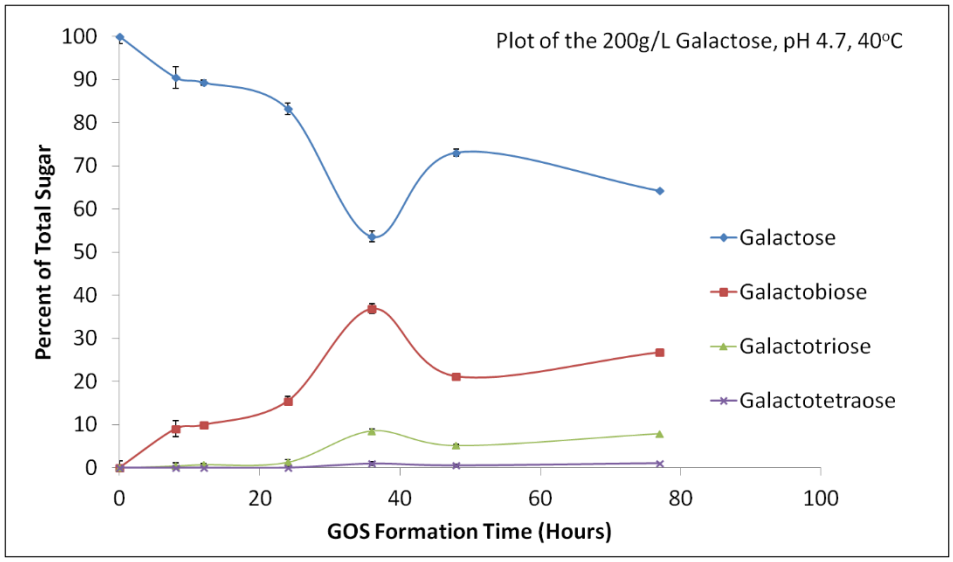


Fig. 3: Product profile after Glycoblotting analysis of GOS. The values presented are the average of all MALDI-TOFMS data. Error bar means standard deviation of the trials (n=3)

This decrease might be likely due to the shift in the reaction equilibrium that converted the GOS formed back to its monomer component through the hydrolysing action of the enzyme as shown in Fig. 4. Result showed that an increase in galactose monomer and the decreased amount in galactobiose

after incubation of the GOS for 24 hours and 36 hours signify the hydrolysing ability of the enzyme upon equilibrium shift.

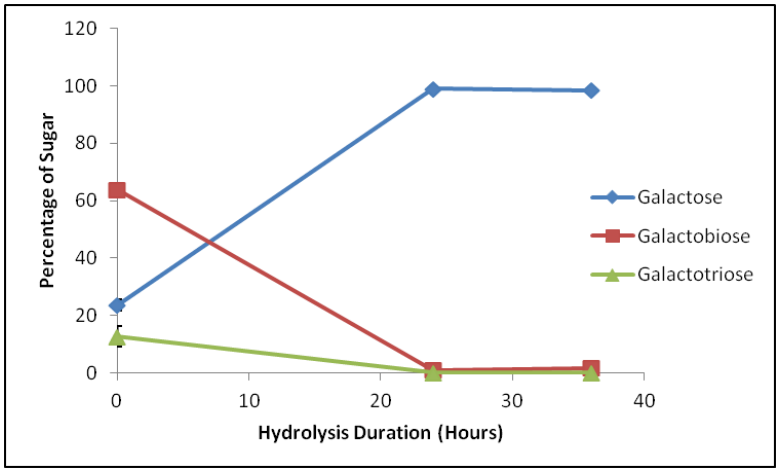


Fig. 4: Evidence of hydrolysis reaction of GOS back to its component sugar monomers by β-galactosidase (*A. oryzae*). Error bar means standard deviation of the trials (n=3)

Production of GOS as prebiotics has long been commercialized utilizing lactose as starting material. However, the current method of production has not been eluded with drawbacks such as product purity and product limitation due to enzyme inhibition (Vera et al., 2011; Boon et al., 1999). This study could potentially eliminate those problems by using an alternative substrate that enables production of pure and high yield GOS. This study has proven that galactose, aside from lactose, can be a good substrate of β-galactosidase in GOS formation in a thermodynamically favoured equilibrium shift towards condensation or reverse hydrolysis upon prolonged incubations. Therefore, this formation of GOS could be accounted from a favourable reverse hydrolysis reaction.

The ability of galactose in inhibiting lactose hydrolysis and GOS formation was thoroughly delved in some reported studies (Kim et al., 2004a). However, studies on its fate when incubated with β-galactosidase alone remain unravelled. Albayrak and Yang (2002a, 2002b) observed no appreciable amount of oligosaccharides formed when they used 50g/L of galactose as substrate of *A. oryzae* lactase for 5 hours, which led them to conclude that galactose is not good for GOS formation. The same observation was observed in this study. However, drastic increased has been observed after 12 hours of incubation.

Kim et al. (2004b) confirmed that in lactose hydrolysis and GOS production, high concentration of galactose did not cause inhibition on β-galactosidase. Further, their observation explained that galactose can bind to the free enzyme to make enzyme-galactose intermediate complex and undergoes transgalactosylation reaction with glucose or lactose as an acceptor, but it does not bind to an enzyme-galactose complex. On the contrary, this study succeeded to yield GOS using only galactose as the sole substrate and thus suggested that galactose does bind to the enzyme-galactose complex and act as an acceptor. The stronger effect of galactose in hydrolysis reaction due to its strong affinity to the enzyme, as compared to lactose (competitive inhibition) (Vera et al., 2011), might explains the observation that hydrolysis reaction dominates at high lactose concentration but shifted to reverse hydrolysis, after increasing the galactose concentration exogenously, causing increased GOS production but decreased rate of glucose release (Kim et al., 2004b). The reported and observed inhibitory effect of galactose could be due to the transglycosylation reaction that's competing with the hydrolysis reaction in the presence of either exogenous or by-product galactose in the reaction mixture. However, since galactose is the only substrate used for GOS production in this study, inhibition might not be a problem in this method.

3.2. MOS production and quantitation

Another novel activity of Ao-β-gal is its reported β-mannosynthase (Tan et al., 2016). In a reported reaction of the enzyme with the monosaccharide mannose, as the substrate, Ao-β-gal was able to synthesize mannobiose in Man-β(1-6)-Man glycosydic linkage after 24 hours of incubation. Unlike its β-galactosynthase activity, which was frequently reported in a bunch of literatures, the mannosynthase activity was discovered in the quest of finding wide range of possible acceptable substrates of this enzyme. The search for different acceptable substrates was initiated due to the reported open-close conformation of some enzymes belonging to the same family. These facts were the basis to explore a likely possibility that the enzyme can synthesize longer MOS chain using monosaccharide mannose. After prolonged incubation with free mannose, at 45 °C for 8, 12, 24, 36, 48, and 77 hours, and product enrichment using glycoblotting analysis revealed only mannobiose and mannotriose products as shown by its product profile in Fig. 5.

This difference in incubation temperature compared to GOS was due to the observed higher amount of MOS produced at 45 °C than at 40 °C (data not shown). An increase in concentration of the product is observed as the reaction progressed. Interestingly, the MOS formation is slowly increased all throughout the experiment, despite the slight decrease in the amount of mannotriose. The highest total MOS concentration was observed at 77 hours with 32.62 % (composed of 94.88 % mannobiose and 5.12 % mannotriose), although the % MOS in 77 hours is significantly closer to the amount in 36 hours and 48 hours with 30.84 % and 31.30 %, respectively (Fig. 6). The mannobiose produced during this time range is also very slow compared to the drastic increase during 8 hours to 36 hours incubation period. Interestingly, however, the slow formation of mannobiose was compensated by the increasing amount of mannotriose from 1.22 % to 1.68 %.

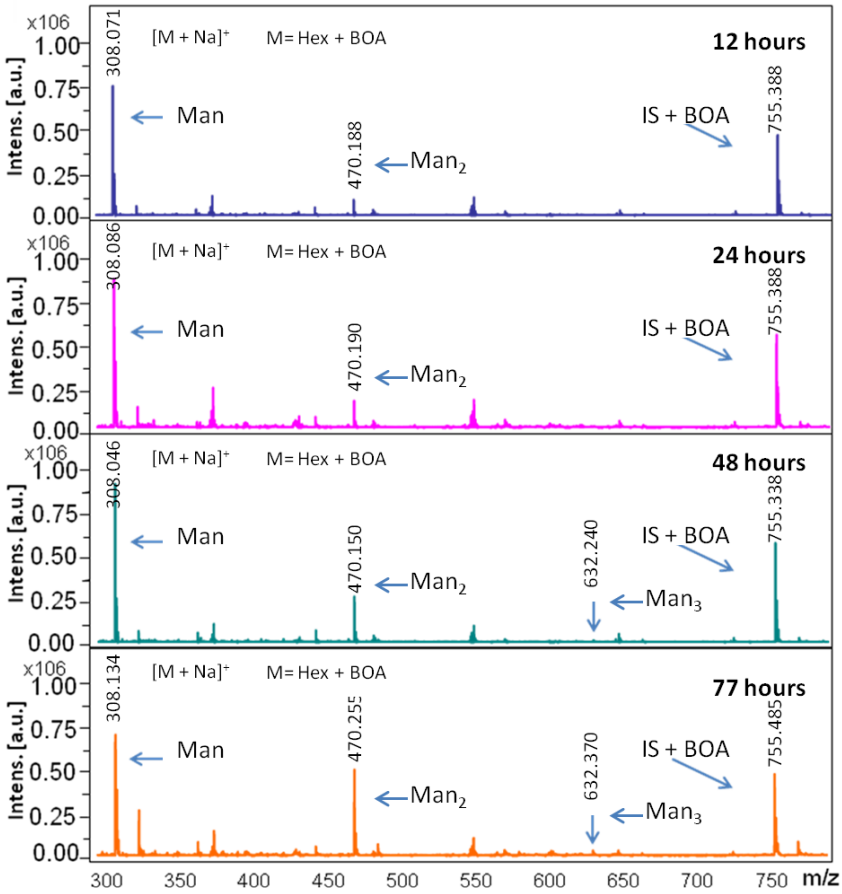


Fig. 5: Representative MALDI-TOFMS spectra of MOS products enriched by glycoblotting method. IS means the signal due to BOA-labelled *tri-N*-acetylchitotriose used as an internal standard for the quantitation. Man means mannose; Man_2 means mannobiose; Man_3 means mannotriose

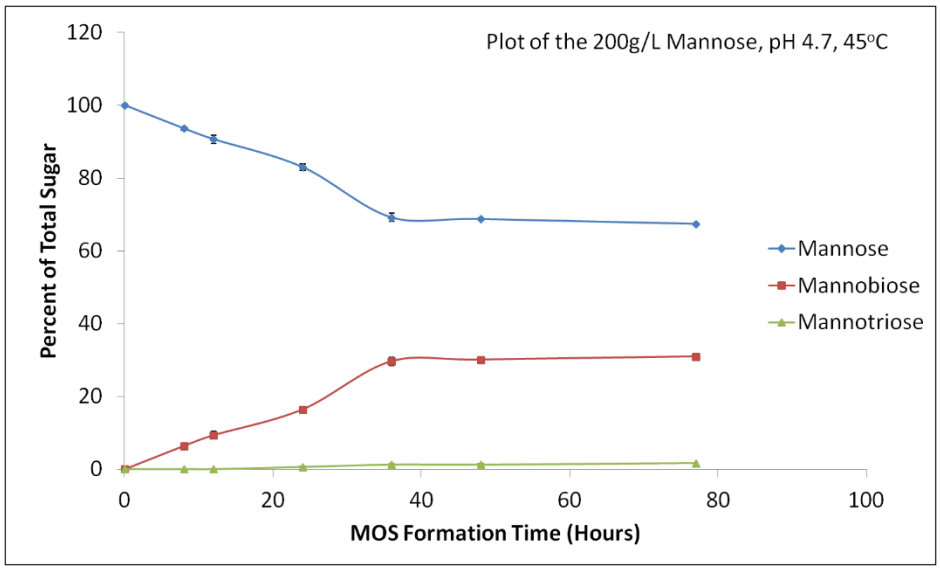


Fig. 6: Product profile after Glycoblotting analysis of MOS. The values presented are the average of all MALDI-TOFMS data. Error bar means standard deviation of the trials (n=3)

Mannose is a well-known substrate in mannosidases. Mannosidases are also known to produce monno-oligosaccharides by transglycosylation or reverse hydrolysis (Bubb, 2003; Pestlin et al., 1997). Unlike GOS, the formation of MOS in this reaction is relatively slower, as shown in their relative rates of formation in Fig. 7A and Fig. 7B, producing 1.726 g $L^{-1}hr^{-1}$ of MOS (0.8631 % MOS per hour) compared to 2.616 g $L^{-1}hr^{-1}$ GOS (1. 308 % GOS per hour) shown in Table 1.

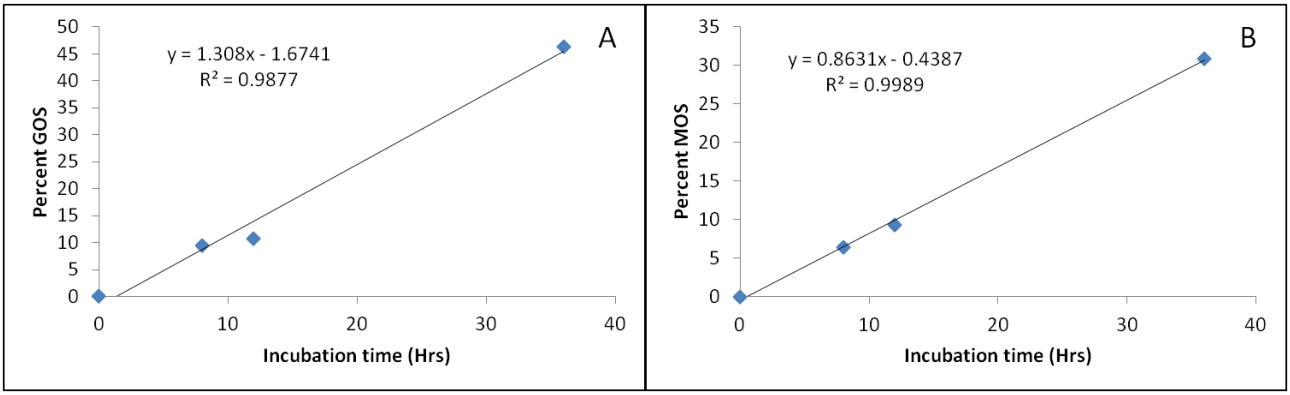


Fig. 7: Rate of formation of (A) GOS and (B) MOS

This slow formation of mannobiose could also be due to the competing transglycosylation reaction with the hydrolysis reaction, since MOS can also be hydrolyzed by the enzyme upon MOS build-up, as shown in Fig. 8.

3.3. Effects of GOS and MOS on cancer cell proliferation

After the investigation of their effects on different cancer cell lines, it was found out that GOS and MOS modulated the growth of different cancer cell lines after co-incubation, as shown in Fig. 9. Different responses were noticed in different cell lines after incubation. GOS inhibited the growth of A549 and PC-3 cells at higher concentration (Fig. 9a), with higher anti-proliferative effect on PC-3, which is evident in a significant decrease in cell viability in all treatment groups. Generally, GOS exhibited mitogenicity, at lower GOS concentration, towards A549, HepG2, and HT-29. The MOS, on the other hand, also has a different response to different cell lines at different exposure periods (Fig. 9b). Generally, MOS promoted proliferation of A549, HepG2, and HT-29 in all treatment groups but not PC-3. Like GOS, MOS inhibited the PC-3 proliferation in all treatment groups. It seems likely that PC-3 cells are the ones severely affected by both GOS and MOS.

Table 1: Summary of the rate of oligosaccharide formation

Substrate	Initial conc.(g/L)	Product	Rate of formation (%)/hr	Total $C_{Product}$ (g $L^{-1}hr^{-1}$)
Mannose	200	MOS	0. 8631	1.726
Galactose	200	GOS	1.308	2.616

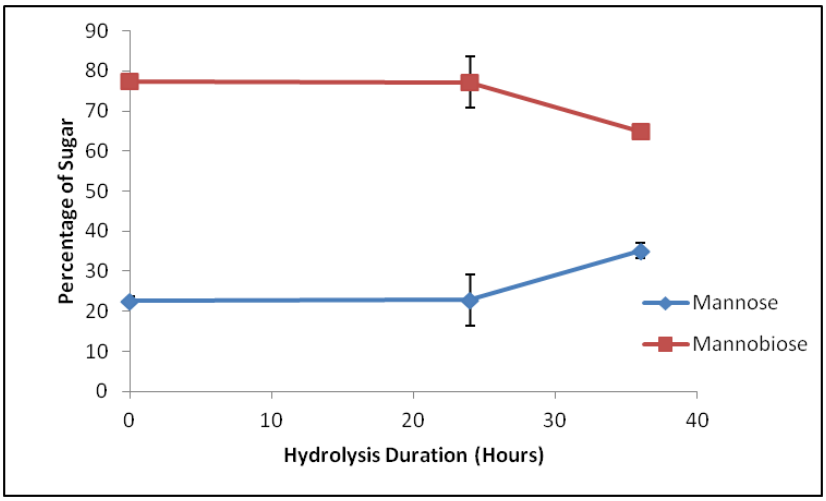
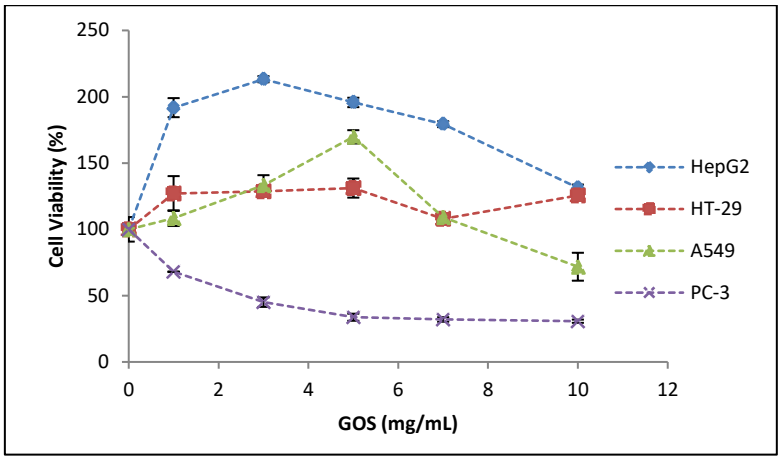
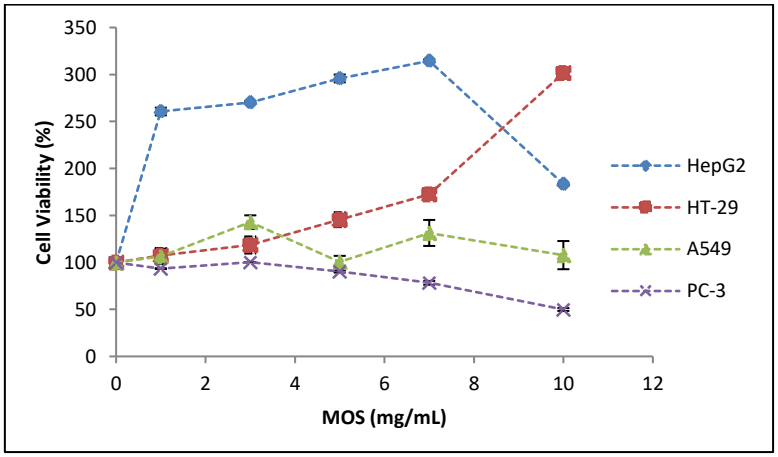


Fig. 8: Evidence of hydrolysis reaction of MOS back to its component sugar monomers by β-galactosidase (*A. oryzae*). Error bar means standard deviation of the trials (n=3)



a



b

Fig. 9: Effects of NDOs in human cancer cell growth. Effect of GOS on cancer cell growth (A) and the effect of MOS on cancer cells (B). Human hepatoma cell (HepG2), Human epithelial colon cancer cells (HT-29), Human lung adenocarcinoma (A549), and Human prostate adenocarcinoma (PC-3). Data shown were mean values with error bars indicating ±SD of n = 3

3.4. Cytotoxicity and cytostaticity

To determine if GOS and MOS are cytotoxic or cytostatic to PC-3 cells that caused anti-proliferation, we cultured PC-3 cells and treated with GOS and MOS for 72 hours. Cells were then washed, supplemented with fresh medium and incubated for 48 hours to observe cell growth recovery. Results showed (Fig. 10) that GOS and MOS are cytotoxic to PC-3 cells, as indicated by the strong cell growth inhibition and inability of the cells to recover after 72 hours of oligosaccharides exposure.

3.5. Effects of GOS and MOS on mitochondrial Function in prostate cancer cells

Mitochondrial membrane potential can be used to detect mitochondrial dysfunction as caused by membrane polarization, inhibition of ATP synthesis, which could ultimately lead to apoptosis or programmed cell death (Montaigne et al., 2010).

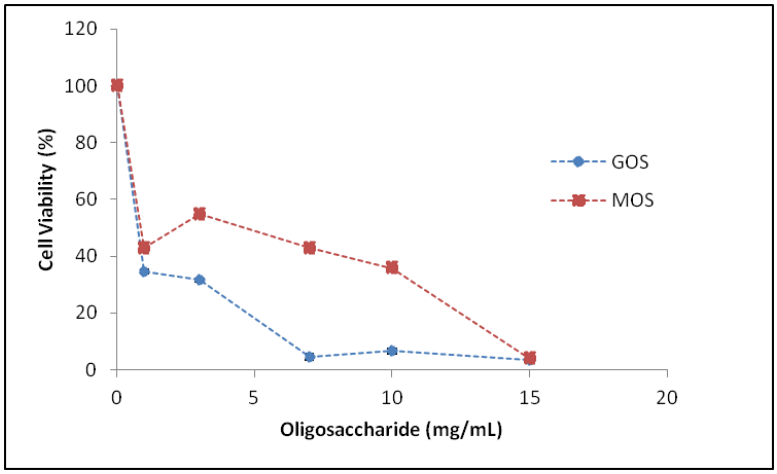


Fig. 10: Cell viability of Human prostate adenocarcinoma after 48 hours of recovery. Data shown were mean values with error bars indicating ±SD of n = 3

To investigate further the specific anti-proliferative effect of both GOS and MOS on PC-3 cells, mitochondrial membrane potential (ΔΨm) of PC-3 cells was analyzed. This could also determine if apoptosis is due to intrinsic apoptosis pathway or not. As shown in Fig. 11A, intensity of fluorescence was determined and found to have no significant decrease with respect to the control. This finding may suggest that the apoptosis may not be due to intrinsic apoptosis pathway, some even improved mitochondrial membrane potential of PC-3 cells. Confocal microscopy also showed that between those cells treated with GOS and MOS, the cells treated with MOS showed absence of adhesion and were readily undergoing apoptosis, as evident by the circular-shaped cells morphologically resembling non-adherent cells shown in Fig. 11B. Results suggested that MOS inhibited cell adhesion that might have led to early apoptosis compared to those treated with GOS.

4. Conclusion

This study demonstrated the efficiency of the β-galactosidase (*A. oryzae*) enzyme to produce NDOs (GOS and MOS), efficiently in higher yield, from free galactose and mannose, respectively. This method of producing NDOs has great advantage in terms of yield, purity of the product, and ease in product purification. The enzyme can also hydrolyze NDOs back to its monomer component. The rate of MOS formation is relatively slower than GOS formation at the optimum temperature, as quantitatively determined by glycoblotting analysis. Both MOS and GOS modulated the cell growth of different human cancer cell lines with apparent antiproliferative effect on PC-3 and mitogenic effects on A549, HepG2, and HT-29. This high yield NDOs production makes this new strategy a potential alternative in producing GOS and MOS as NDOs with cancer cell growth modulating capacity.

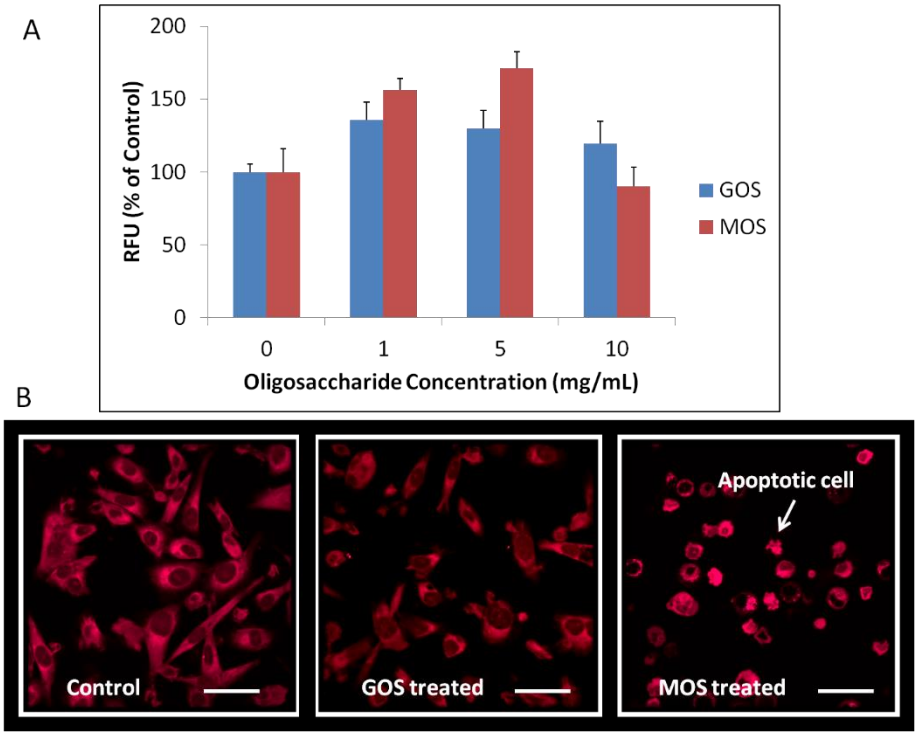


Fig. 11: Effects of GOS and MOS on PC-3 cells. Mitochondrial membrane potential of cells after treatment (A). Morphology of PC-3 cells in confocal microscopy. Error bars indicating ±SD of n = 3. White arrow pointing an apoptotic cell. Cells were stained with MitoTracker Deep Red. Scale bar, 50 µm

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