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Antioxidant Activity of Manno-oligosaccharides Derived from the Hydrolysis of Polymannan by Extracellular Carbohydrase of *Bacillus* N3

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Abstract The aim of this study is to elucidate the biochemical properties of manno-oligosaccharides (MOS) hydrolyzed by extracellular enzyme of *Bacillus* N3. We strived to characterize the biochemical properties of MOS since N3 can effectively hydrolyzed natural polymannans such as galactomannan (GM) and konjac (glucomannan, KM), respectively. The hydrolysis of GM and KM was applied by the strain N3 in terms of reducing sugars and the highest production of reducing sugars was estimated to be about 750 mg/L and 370 mg/L respectively, which were quantified after 7 days of cultivation in the presence of both substrates. Hydrolysates derived from the hydrolysis of KM showed the significant antioxidant activity based on DPPH and ABTS radical scavenging activity with increasing of tyrosinase inhibitory activity. On the other hand, hydrolysates derived from the hydrolysis of GM showed only ABTS radical scavenging activity without showing significant changes on tyrosinase inhibitory activity. Our data suggest that those biological characteristics may be depend on the primary structure and the size of MOS, which may be useful as potent additives for diet foods.

Keywords : Manno-oligosaccharides, *Bacillus*, Glucomannan, Anti-oxidants, Extracellular proteins, Algal lytic enzymes

Introduction

Bacillus spp. are Gram-positive bacteria found diversely in nature. They can establish endospores to survive in incompatible conditions such as high temperature, inappropriate light and harsh chemical reagents [1]. Therefore, seeking and screening a novel strain producing unique enzymes have been performed for several decades. Amongst, cellulase is a name of the group of enzymes which act together to catalyze the cellulose into soluble sugars [2,3]. Cellulose is most abundant, linear, crystalline homo-polymer of repeating cellobioside units in which two anhydrous D-glucose residues are connected through β -1,4-linked glucose units. In order to expand the applications of such precious material in various research fields, cellulases have been used for wide applications in pulp, paper, pharmaceutical, textile, food processing and detergent productions [4]. Microbial cellulases are chosen over animal and plant cellulases because they have lower production costs, no specific seasons, and rapid development [5]. Subsequently, purification and characterization of unique enzymes are therefore critically necessary as it plays a vital role in the development of effective applications of enzyme at in-

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dustrial scale. Thus far, enzyme characterizations have been carried out to find the best enzymatic activities for enhanced efficiency of the process.

Beside glucose based carbohydrates, manno-oligosaccharides (MOS) consisting mainly of mannose or galactose derived from the hydrolysis of galactomannans or glucomannans by endo-δ-1,4 mannanases have been focused as functional additives for human beneficial intestinal microflora as non-nutritional foods. Endo-δ-1,4 mannanases have been used in various industrial processes such as bioconversion of biomass to fermentable sugars, and reduce the viscosity of coffee extracts [6]. However, the initial interest in using MOS was to protect gastrointestinal health from the *Salmonella* infections. As earlier studies demonstrated that *Salmonella* can bind to mannose unit *via* type-1-fimbriae which is known as finger-like projections. Binding of type-1-fimbriae to mannose resulted in decreasing the risk of pathogen colonization inside the intestinal tract [7]. Not only for human beneficial intestinal microflora, but also MOS can be used in diets for various animals such as dogs, horses, cats and birds due to its benefits for their health to protect gastrointestinal health [8]. As described above, many studies on the supplementary food, diet, and pathogen colonization inside the intestinal tract were presented, less is known about the antioxidant activity of MOS and other biochemical properties. In this regard we aimed to characterize the antioxidant activity of the MOS derived from GM and PM which were consisting of mainly mannose with extracellular enzyme of the isolated strain *Bacillus* N3.

Materials and Methods

Materials

Sulfuric acid (H_2SO_4), 1,1-diphenyl-2-picryl-hy-drazyl (DPPH), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), ferric chloride, potassium persulfate, sodium hydroxide and L-ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Galactomannan (GM) and konjac (glucomannan, KM) were also purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hydroxide (NaOH) and 4-Hydroxy-benxhydrazid (PHABAH) were used for quantification of reducing sugar and was obtained from Junsei Chemical Co. (Tokyo, Japan). *p*NP-glucosae, *p*NP-mannose, *p*NP-cellobioside, LB media and tyrosinase from mushroom were purchased by Sigma Chemical Co. (St. Louis, MO, USA). TLC plates (Silica gel 60F254; Merck, Germany) were used to monitor the hydrolysates by enzyme activity. All other reagents were used of the highest grade available.

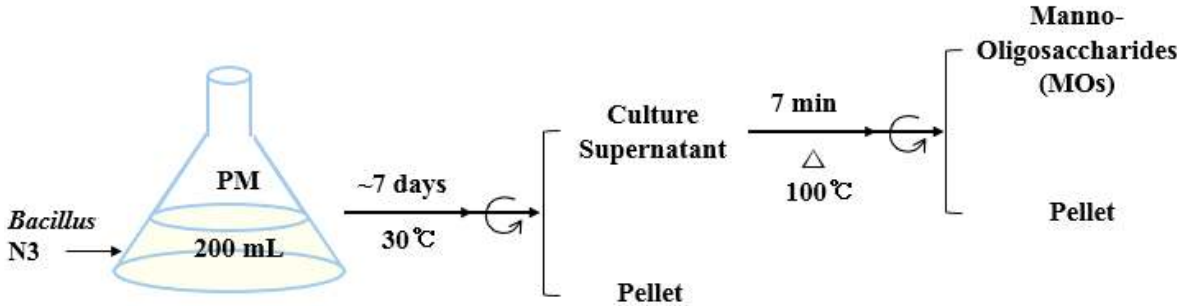
Microorganisms and culture conditions

Bacillus N3, isolated and named based on preliminary study for the screening study (unpublished data) was cultivated in LB medium at 37 °C supplemented with 20−30 μg/㎖ of kanamycin when necessary. To induce extracellular enzyme production including protease, strain N3 was cultured at 37 °C with constant agitation of 200 rpm in the seed medium containing (w/v) 1.0 % tryptone, 1.0 % beef extract and 0.5 % NaCl, at pH 7.0. After two days of cultivation, the crude broth was centrifuged at 13,000 rpm for 10 min and the supernatant was collected and used as the crude extracellular enzyme to elucidate the basal level of enzyme activity using artificial substrates such as *p*NP-glucose, *p*NP-mannose, *p*NP-cellobioside, and etc.

Manno-oligosaccharide production

Scheme 1. shows the production of MOS, briefly, the strain N3 was incubated in 200 ㎖ polymannan solution (0.5 % Polymannan in 1/20 diluted LB) for about 7 days at 30 °C. Culture medium was then centrifuged and culture supernatant was collected and heated at 100 °C for 7 min. After centrifugation of this mixture, supernatant containing active hydrolysates was collected and used as MOs for further studies. *Bacillus* N3 was incubated in 200 ㎖GM or KM (0.5% as polymannan in 1/20 LB) for 7 days at 30 oC, respectively. Culture supernatants were collected, heated at 100 oC for 7 min, and centrifuged to

remove denatured or insoluble materials. After centrifugation, supernatants of the hydrolysis of GM or KM were collected and used as GMOS or KMOS for further studies, respectively.



Scheme 1. Manno-oligosaccharides (MOS) production.

Reducing sugar contents

This method was perform with slight modifications to previous method [9], briefly, 16 mM PHABAH (4-Hydroxy-benxhydrazid) solution was used to quantify the concentration of reducing sugars in the testing samples. The prepared solution was mixed with samples in an appropriate ratio and resulting mixture was then heated at 100 °C for 5 min. Sample mixture was then centrifuged at 13,000 rpm for 5 min to remove the insoluble matters. Amount of reducing sugars of the sample was determined after normalizing with wide range of glucose, used as control. Afterwards sample's absorbance was read at 405 nm using UV-spectrophotometer (Infinite M200 Pro Nano-quant, TECAN, and Austria) and reducing sugar of sample was quantified after normalizing with control (mannose).

Tyrosinase inhibitory activity

The method used for the detection of tyrosinase inhibitory activity is briefly described as [10]; 1 mM L-tyrosine was added in 25 mM Phosphate butter (pH 6.8) and then was mixed with 24 ㎕ of enzyme solution consisting of 100 μg/㎖ tyrosinase from mushroom in the presence or absence of testing samples (30 ㎕). The mixed solution was then incubated at room temperature for 30 min, and the absorbance was monitored at 405 nm using a UV-spectrophotometer (Infinite M200 Pro Nano-quant, TECAN, and Austria).

TLC analysis

Chemical composition and basic properties of GMOS obtained from the hydrolysis of polymannans were analyzed by TLC analysis, with slight modification based on the previous study [11]. A chemical mobile solvent mixture consisting of N-propanol: NH_4OH: H_2O = 7: 1.5: 1.5 (v/v) was prepared in fresh. GMOS were separated on TLC plate were visualized under baking at 180 °C by providing sufficient time for the appearance of sugars in dark-spots, using a developing solution consisting of 3 % phosphoric acid and 8 % copper sulfate (v/v), which is reliable to observe sugar containing materials.

DPPH radicals scavenging activity

The DPPH radical scavenging assay was performed according to this procedure [12-13]: 1 mM DPPH solution was mixed with test samples in 3:1 (v/v) ratio and the reaction was carried out in dark at room temperature for about 20 min. Samples were then read at 517 nm wavelength using a UV-spectrophotometer (Infinite M200 Pro Nano-quant, TECAN, and Austria). The scavenging relative activity (%) was calculated as: RA (%) = [1-(As)/Ac] * 100, where, As = absorbance of the sample, Ac = absorbance of control.

ABTS-radicals scavenging assay

ABTS-radicals scavenging activity was determined by this method [14], briefly, ABTS (7 mM) and potassium persulfate (7.35 mM) were dissolved in distilled water, and the resulting solution was kept for 16 h at room temperature in dark. Afterward the ABTS solution was mixed in a ratio of 10:1 with testing sample. The reaction mixture was left at room

temperature for 6 min. Absorbance of samples was taken at 734 nm using a UV spectrophotometer (Infinite M200 Pro Nanoquant, TECAN, and Austria). The ABTS radical scavenging relative activity was calculated: RA (%) = [1-(As)/Ac] * 100, where, As = absorbance of the sample, Ac = absorbance of control.

Ferric reducing antioxidant power (FRAP) assay

FRAP reagent was prepared by mixing TPTZ (40 mM 2, 4, 6-tri (2-pyridyl)-s-triazine), 20 mM ferric chloride and 0.3 M acetate solution, with slight modification based on the previous study [14]. Resulting mixture was mixed with testing sample solution and water in 33:1:14 ratio, and the reaction was carried out at 37 °C for 4 min. The absorbance was taken at 595 nm using a UV spectrophotometer (Infinite M200 Pro Nanoquant, TECAN, and Austria) to calculate the reducing power percentage of WSP: RA (%) = [1-(Ac/As)] * 100, where, Ac = the absorbance of the control, and As = the absorbance of the sample.

Statistical analysis

Each sample contained three replicates and all experiments performed in this study were repeated at least three times. Results were presented as the mean value ± standard deviation (S.D.) with statistical significance ($p \leq 0.05$).

Results and Discussion

Carbohydrate analysis

Bacterial strain N3 isolated from soil with colony morphology of large, dry, white color with wavy, lobed margins was selected to sub-culture and confirmed by the Gram's stain and partial 16S rDNA gene sequencing and hence named as *Bacillus* N3 (unpublished data which will be reported in separate paper). Upon testing in preliminary study, we found that the culture supernatant of *Bacillus* N3 has shown the significant enzyme activity toward *p*NP-glucose, *p*NP-cellobioside and *p*NP-mannose, respectively (data not shown). In recent, we have focused on making hydrogels using several natural polysaccharides such as alginate, chitosan, and konjac (mainly consisting of glucomannan) as a food model. Amongst konjac is well known as less calorie diet food. Therefore, we have focused on the activity of enzyme toward *p*NP-mannose rather than other substrates, indicating that N3 may have multi-potent enzymes corresponding to the environmental conditions. Afterwards we directly applied the strain N3 on the hydrolysis of polymannans such as GM and KM to characterize the bacterial strain and its of products. As the results we found that the strain N3 was stably able to hydrolyze GM (Fig. 1) and KM to the same pattern in terms of increasing reducing sugars (data not shown). With increasing time, the concentration of reducing sugar from the hydrolysis of GM and KM increased and reached to maximum 750 mg/L and 370 mg/L, respectively, after 7 days at 30 °C. Fig. 1 shows only the results for GM because of the similar pattern observed from the hydrolysis of KM.

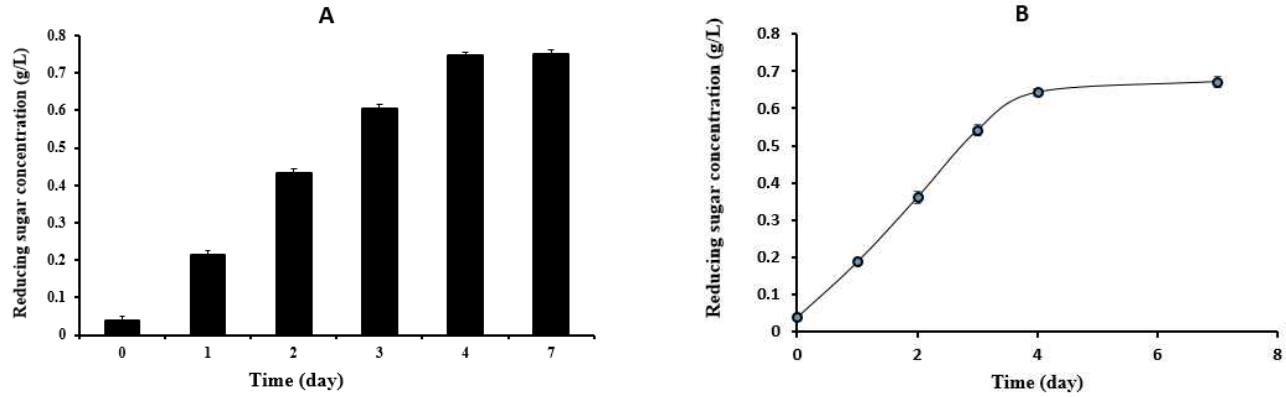


Figure 1. Reducing sugar quantification of GMOS (A) and KMOS (B). *Bacillus* N3 was incubated in 200 ㎖ GM or KM (0.5 % GM/KM in 1/20 diluted LB) for 7 days at 30 °C. The contents of hydrolysates named hereafter GMOS or KMOS in the culture supernatant were quantified by reducing sugar assay. Mannose was used as standard.

Antioxidant activity of MOs

The central goal of this study was to evaluate the antioxidant activities related to the hydrolysates designated to GMOS derived from the parental polysaccharides GM. GMOS were further characterized to investigate their antioxidant activities on DPPH radical scavenging effect. Scavenging activity of DPPH radical slightly increased with the increasing of the days of incubation when compared to control, ascorbic acid. DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers [12]. Further it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability [13]. Thus, scavenging of DPPH free radical was directly affected by the amount of oligosaccharides molecules. However, the significant DPPH free radical scavenging activity of MOS derived from the hydrolysis of GM was not observed during the test (data not shown). This results suggest that GMOS consisting of a mannose backbone with galactose side groups are neutral sugars that may not be able to a proton donor to the molecule of DPPH under the neutral pH. Besides activity toward DPPH, our results have not shown significant change in color with FRAP assay with increase in time of incubation until period of 7 days. This result also can be explained as the structural base of sugars which may not be able to reduce metal ions. Therefore, GMOS from GM are not the potent proton donor or reducing power of metal ions.

The 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS•+) can be generated by the enzymatic system formed by hydrogen peroxide and horseradish peroxidase in an organic medium [14]. In this experiment, ABTS radical scavenging activity was higher than DPPH and FRAP scavenging activities. In most cases, ABTS radical cation (ABTS•+) is reactive towards most antioxidants, and it is applicable to determine lipophilic and hydrophilic antioxidant capacities over a wide pH range [14]. Relative ABTS activity was found to be around 80 % after 1 day of incubation and remained constant up to 7 days. Whereas specific ABTS activity reduced over 7 days of time period based on the fact that decreased total carbohydrate content and increase in reducing sugar contents (Fig. 2). Based on these results we suggest that GMOS derived from the parental polysaccharide GM may interact with ABTS•+ in hydrophobic attraction together. As increasing the concentration of reducing sugars after 2-days culture, although it is not able to determine the average molecular weight of GM, probably certain size of molecules can be involved in ABTS radical scavenging activity.

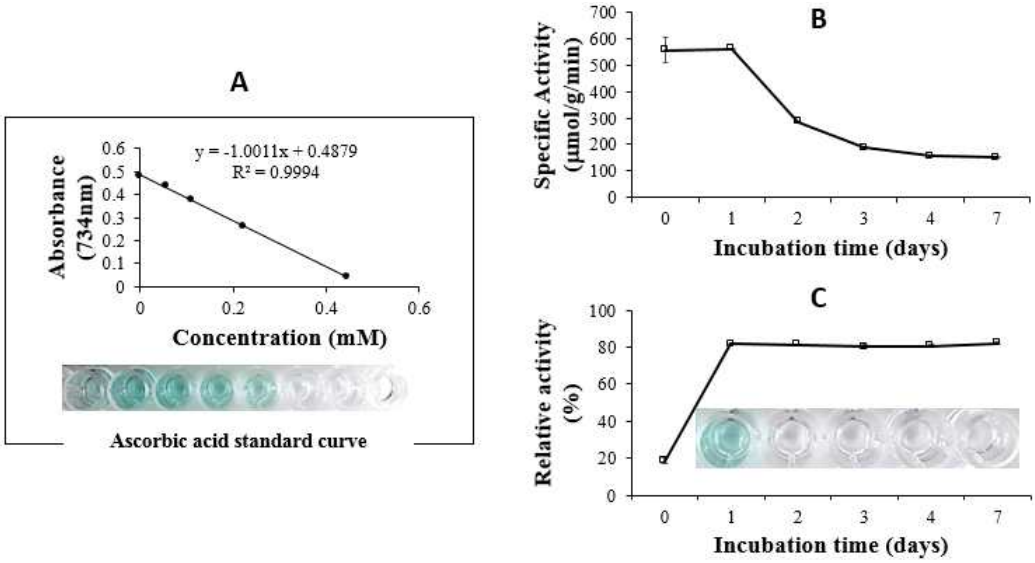


Figure 2. ABTS radical cation scavenging activity of GMOS. *Bacillus* N3 was incubated in 200 ㎖ consisting of 0.5 % GM in 1/20 diluted LB for 7 days at 30 °C. Culture supernatant was collected and heated (100 °C, 7 min) and centrifuged. After centrifugation, supernatant was collected and used as GMOS for ABTS assay. A: control, B: specific activity (❍mol/g/min), C: relative activity (%).

Tyrosinase inhibitory activity of MOs

As described in the Fig. 2, we found that GMOS showed significant ABTS radical scavenging activity, which is quite related with the whitening effect by inhibiting tyrosinase activity. Therefore, we applied these GMOS derived from the hydrolysis of GM for tyrosinase inhibitory activity. The enzyme tyrosinase is a metalloenzyme group of polyphenol oxidases that occurs in various organisms and perform some specific functions in melanogenesis [15] converted to dopaquinone (diphenolase activity is the second step of tyrosinase reaction). Furthermore, tyrosinase catalyzes the oxidation of 5,6-di-hydroxyindole to form indole-5,6-quinone of the melanin precursors [16]. Compounds, which cause inhibition of tyrosinase, could be effective as depigmenting agents. Tyrosinase is an enzyme widely distributed in nature containing copper and catalyzes key reactions in the melanin pathway. Its inhibition is the major goal in developing new whitening agents now days. However, unfortunately we cannot detect any specific tyrosinase inhibitory activity/whitening activity from GMOS from GM (data not shown). Many tyrosinase inhibitors are polyphenol derivatives of flavonoids or of trans-stilbene so this can be the reason we were not able to detect any activity [17,18]. On the other hand, all testants showed higher changes of absorbance compared to the control.

Biological applications of MOS derived from the hydrolysis of KM

We next find the biological activities of *Bacillus* N3 and in this regard we firstly focused on the ability of N3 to hydrolyze konjac as glucomannan (KM). The KM is made up of 1,4-linked β-D- mannose residues and a branched chain structure joined through C3 of the hexose residues of the main chain. KMOS has been implicated as a glucomanno-protein complex which is derived by enzymatic hydrolysis of the inner cell wall of the fungi *Aspergillus* [19]. The outer portion of the fungal cell wall contains another functional carbohydrate-glucomanno-protein, which can bind to mycotoxins [20]. Furthermore, it is worthy to note that KMOS can be phosphorylated to increase pathogenic binding capacity, it can effectively bind and adsorb various pathogens including bacteria (*Escherichia coli, Pseudomonas* spp., *Staphylococcus aureus, Salmonella* spp., *Shigella* spp. and *Vibrio* spp.) and ultimately reduce growth of these microbes [21].

Thin layer chromatography (TLC) is routinely used by synthetic organic chemists and natural products chemists working on a variety of types of molecules. Carbohydrate biochemists in the past largely relied on paper chromatography for rapid analysis. Carbohydrates are highly polar molecules and often require derivation to be analyzed by TLC. Fig. 3 shows that in all the bands remained at the bottom line corresponding to each sample from 0-6 which correspond to the incubation time of each sample. As compared to standard galactose and mannose which upon baking of TLC plate separated and segregated, however all samples stay at origin that may be due to relatively high molecular weight and this is why samples cannot be separated on TLC plate. Although there were no significant migration changes, intensity of samples taken at time difference increased that corresponds to the results of Fig. 1. Herewith we hypothesized that *Bacillus* N3 may hydrolyze GM/KM randomly and use smaller size of molecules as nutrients like mono- or dimeric glucans derived from the function of endo and exo-enzymes. Therefore, it is hard to detect sort of smaller size of molecules in the culture supernatant with microorganism. Instead of high production of enzyme, in this study we focused on the biological activity of the products GMOS and KMOS. Nevertheless, we were able to get increasing amounts of reducing sugar from the hydrolysis based on time-dependence (Fig. 1). As the results it was found that it might be mannohaxoses or higher molecular weight that it cannot travel higher along the silica gel plate of TLC as compared to control.

Figure 3. TLC development of KMOS derived from the hydro lysis of KM. *Bacillus* N3 was incubated in 200 ㎖ medium consisting of 0.5 % KM in 1/20 diluted LB for 7 days at 30 °C. Culture supernatant was collected and heated at 100 °C for 5min. Then, KMOS in the supernatant derived from the time-dependent hydrolysis were developed on TLC plate. G: Galactose; M: Mannose; 0: KMOS-0; 1: KMOS-1; 2: KM OS-2; 5: KMOS-5; 6: KMOS-6. Numbers indicate the time (day) of incubation as shown in Scheme 1, as shown in the Materials and Methods section.

Furthermore, we applied KMOS from KM to determine antioxidant activity of these samples (Table 1). The chronic exposure of human skin to ultraviolet radiation has several effects such as inflammatory response, erythema, pigmentation, immunosuppression and hyperplasia. The use of sunscreen and related products is the main purpose of cosmetics for the skin protection against the harmful effects of UV rays. Antioxidants prevent the formation of free radicals and help to repair the damage caused by them. Natural antioxidant compounds are capable of absorbing energy in the UV rays. In this regard we apply the KMOS for the for 3 different antioxidant assays that are DPPH, ABTS and FRAP antioxidants assays (Table 1). As we hypothesized that KMOS from KM will show higher antioxidant activity but unfortunately no activity was observed in case of DPPH and FRAP, whereas specifically ABTS antioxidant activity was obtained and highest relative activity was observed up to 60 % of relative activity for KO-5. The reason behind non detection of DPPH and FRAP relative activity might include the pH dependence nature of these chemicals, as described in other section. Next, we apply KMOS for tyrosinase inhibitory activity also called as whitening activity. Tyrosinase catalyzes the oxidation of 5,6-di-hydroxyindole to form indole-5,6-quinone of the melanin precursors [22]. Because tyrosinase participates in at least three stages of melanogenesis, tyrosinase inhibition would serve to block melanin biosynthesis and would be important as a base of formulations used for the treatment of skin blemishes [22]. However, all KMOS were unable to inhibit tyrosinase activity which can be linked to its inability to inhibit the production tyrosine in the melatonin pathway. The outcomes of this study elaborate that MOS produced from GM or KM by N3 has only potent antioxidant activity against ABTS radical scavenging effect, respectively.

Table 1. Detection of antioxidant activity.

	Relative activity (%)*
	ABTS
KMOS-0	32.1
KMOS-1	39.8
KMOS-2	42.8
KMOS-5	61.3
KMOS-6	57.9

*Relative activity (%): (1-Abs of sample/Abs of negative control) x 100. Glucose, Mannose and DW were used as negative control.

As described in the previous study [23], an alkalophilic ㅇ-1,3 D-mannosidase purified *Pseudomonas* species and characterized that this enzyme has shown significant algal-lytic activities against *Alexandrium tamarense, Akashiwo sanguine, Gymnodinium catenatum, Gymnodinium mikimotoi, Prorocentrum dentatum* and *Cochlodinium polykrikoides* known as harmful algal species based

on the substrate specificity of enzyme. Screening and characterization of new bacterial strains are always attracting our attention to elucidate the enzymatic function toward many different types of substrates described above. Initially, therefore, *Bacillus* N3 was isolated as one of candidates producing algal lytic enzymes against microalgal biomasses to develop biological and environmental friendly conditions for biodiesel production. Instead of finding significant hydrolysis activity toward microalgal biomasses (data not shown), we found that *Bacillus* N3 can degrade polymannans used in this as substrates to produce highly hydrophilic low molecular oligosaccharides that can be utilized as a potent dietary food additive. Further study on the purification and kinetic properties of enzyme is undergoing. In conclusion, our preliminary data demonstrate that those biological characteristics of derived from the hydrolysis of polymannans by *Bacillus* N3 probably be depending on the primary structure and the size of them.

Acknowledgments

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Conflicts of Interest

The authors report no conflicts of interest in this work.

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