Profiles of Oligosaccharides Synthesized from Under-Explored Tuber Starches Using *Aspergillus oryzae* Amylase

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ABSTRACT
Oligosaccharides are beneficial compounds for human health that are widely used in the food, cosmetic, and pharmaceutical industries. Our knowledge on the synthesis of oligosaccharides from tropical plant sources using amylases of indigenous microorganisms are still limited. This study aims to determine the profiles of oligosaccharides synthesized from under-explored tuber starches by *Aspergillus oryzae* amylase. Submerged fermentation was applied to obtain the crude enzyme. Starches used as substrate in the enzymatic reaction were from four tubers, including *Tacca leontopetaloides* (L.) Kuntze, *Canna indica* L., *Xanthosoma sagittifolium* (L.) Schott, and *Dahlia pinnata* Cav. Thin layer chromatography (TLC) showed typical oligosaccharide patterns depending on the tuber source. High performance liquid chromatography (HPLC) confirmed the presence of three types of oligosaccharides, including maltotriose, maltotetraose, and maltoheptaose in the *Tacca* starch-enzyme reaction. Higher maltoheptaose was observed when the substrate was starch from *Xanthosoma*. The oligosaccharide proportion at about 93% of total detected saccharides was obtained in the enzymatic reaction of *Xanthosoma* starch.

Keywords: *Amylase, Oligosaccharides, Aspergillus oryzae, Canna, Dahlia, Oligosaccharides, Tacca, Xanthosoma*

1. INTRODUCTION

Functional gastrointestinal disorders (FGIDs) that affect more than 40% of persons worldwide decrease the quality of human life [1]. The common symptoms that appeared in FGID include functional dyspepsia, irritable bowel syndrome, and functional constipation [2]. To overcome this problem, probiotics, prebiotics, and dietary fiber have been proposed as a treatment. Prebiotic is considered by the action in selective growth stimulation of beneficial intestinal microbiota to inhibit pathogens, to change the stool and gas composition, to stabilize intestinal environment, and to control the motoric function of intestine [3].

Obtaining novel type of functional oligosaccharides from various sources that have potential applications in food and health industries is still promising. Some physicochemical aspects including relatively low osmolality, high water-holding capacity, suitable viscosity, and mild sweetness are added-values for oligosaccharide-based functional food products. Most prebiotic oligosaccharides are obtained by acid hydrolysis or enzymatic processes of several natural sources such as starch. Starch, a tasteless, odorless white polysaccharide that contains in the roots, tubers and seeds of many plants [4], is a main source for production of maltooligosaccharides. Hydrolysis of starch into smaller oligosaccharides by amylase is one of the most important commercial enzyme processes [5]. The competitive uses of starch for many industrial purposes lead to the exploration of under-explored plant materials as substrate for enzyme-based product development.

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Hydrolysis of polysaccharides is considered to be a simple technique, lower cost, and more reproducible for oligosaccharide industry [6], Thus, α-amylase is one of never-ending exploration to obtain the unique structure and activity. Attention to the maltooligosaccharides is increased regarding new potential functions as a laxative for constipation therapy, bifidogenic, short chain fatty acid (SCFA) stimulator, and pathogens inhibitor [7,8].

Several studies of enzymatic production of maltooligosaccharides have been conducted using starch of cassava (*Manihot esculenta*) [9], black potato (*Coleus tuberosus*) [10]. Our previous study also demonstrated the successful hydrolysis of various starches by *Bacillus licheniformis* amylase [11]. However, the knowledge is still limited regarding the synthesis of oligosaccharides from tropical plant sources using amylases of indigenous microorganisms. This study aims to determine the profiles of oligosaccharides synthesized from under-explored tuber starches by *A. oryzae* amylase.

2. MATERIALS AND METHOD

2.1. Materials, microorganism and preparation

Tubers of *Tacca leontopetaloides* (L.) Kuntze, *Canna indica* L., *Xanthosoma sagittifolium* (L.) Schott, and *Dahlia pinnata* Cav. obtained from Balitkabi were used as starch sources in this study. Microbial strain used in this study was *Aspergillus oryzae* strain K1A, a working collection of the Research Center for Biology, LIPI Cibinong. Microbial culture was maintained on potato dextrose agar (PDA) regularly.

2.2. Strach extraction from tubers

The starch was extracted through serial processes, including stripping, washing, grating extraction, filtration, precipitation, drying, and sieving. The tuber-water ratio applied in the extraction of starch was 2:1 (w/v). After precipitation, the liquid is removed until only the wet starch remains. Starch was collected and dried in the oven at 35-40°C. Dried starch was crushed with a mortar and then separated using a 50 mesh stainless sieve.

2.3. Crude enzyme production and enzyme activity determination

Production of the amylase was carried out by submerged fermentation. The medium consisted of 3.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 0.1 g $CaCl_2$, 0,01 g $MnSO_4$, 1.0 g $FeSO_4$, 3.0 g malt extract, 0.5 g peptone (adjusted to pH 5.5). Starch (1% w/v) of each tuber was used as the enzyme inducer. Fermentation was performed for one day at 120 rpm, 30°C (Bioshaking incubator). Centrifugation at 9,000 rpm 10 for minutes, 4°C was conducted to obtain the supernatant as crude amylase enzyme.

2.4. Enzyme activity determination

The amylase activity was assayed by incubating 0.5 mL of the crude enzyme with 0.5 mL of 1% (w/v) starch solution (from each source Tacca, Xanthosoma, Canna, and Dahlia) in citric buffer solution 0.05 M, pH 5.5 at 30ºC for 30 min. Inactivation of reaction was by immersing in boiling water 100º C for 3 min and then cooling in tap water. After adding the 2 mL DNS reagent, the suspension was boiled at 100º C for 5 min. Color formation was measured in a spectrophotometer at λ 540 nm. One unit α-amylase is defined as the production of 1 µmol maltooligosaccharide per min under the assay condition.

2.5. Oligosaccharides production

Oligosaccharide production was through enzymatic hydrolysis according to Rahmani et al. (2013) with modification of substrate concentration. Hydrolysis starch carried out under substrate concentration of 5% (w/v). The reaction was carried out in a 100 mL Erlenmeyer flask containing 50 mL of reaction mixture in a rotary shaker at 30º C. Samples were taken and the reaction was stopped by heating the samples in boiling water 100º C for 5 min.

2.6. Determination of oligosaccharide profiles

The products of enzymatic reaction were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). TLC was carried out by the ascending method (four-time developments) on Silica f gel 60 F 254 plates (Merck Art 20-20cm, Darmstadt, Germany). All samples were applied in equal quantities (20 µL) and then resolved by a run with a solvent mixture of n-butanol: 2-propanol: water: acetic acid (7:5:4:2 by volume). Subsequent heating was conducted at 100°C for 1 hour. Spots were visualized by spraying the sugar color (4-methoxy-benzene:sulphuric acid:ethanol = 1:1:18) and subsequent heating was conducted at 150°C for 5-10 minutes. The retention factor (R_f) value is equal to the distance travelled by the compound divided by the distance travelled by the solvent.

For quantitative analysis, the oligosaccharide products were freeze-dried and analyzed by HPLC using the Agilent system (Agilent Technology 1290 Infinity, United States). The column used was Aminex @HPX 87 H, 300 mm x 7,8 mm, and the mobile phase was sulfuric acid 0,008 N. The temperature was kept at 35°C with a flow rate of 1 mL per minute, a sample volume of 20 µL, and back pressure at 1176 Psi. The effluent from the column was monitored by a refractive index detector (RFI).

2.7. Data Analysis

Data of enzyme activity, R_f, and saccharide concentration were analyzed based on descriptive method.

3. RESULTS AND DISCUSSION

Crude α-amylase enzymes of *A. oryzae* K1A were produced using four different substrates. The enzyme activities were determined as much as 0.59, 0.54, 0.30, and 0.28 U/ml when starch of *Tacca leontopetaloides* (L.) *Kuntze, Canna indica* L., *Xanthosoma sagittifolium* (L.) Schott and *Dahlia pinnata Cav*., respectively, is used as an inducer in the enzymatic reaction (Figure 1).

The crude enzyme was successfully hydrolyzing the substrate from each type of starch. TLC demonstrated the spots that are useful in comparison amongst the samples. TLC spots of *Xanthosoma* sample showed the R_f of 0.35, 0.42, 0.47, 0.56, and 0.65. In the *Tacca* sample, the spots were at the R_f of 0.31, 0.38, 0.47, 0.54, and 0.64. Only a slight spot detected in the *Canna* sample with the R_f of 0.69 (Figure 2). In general, oligosaccharides were detected in the sample of *Tacca*, *Xanthosoma*, and *Dahlia*. Even though TLC provided the compound patterns in the mixture of enzymatic products, the concentration of each substrate could not be determined precisely.

Although we did not analyze specifically each starch obtained in this study, it suggested that starch composition in tuber is associated with the oligosaccharides profiles obtained. Different profiles of oligosaccharides produced could be related to the type of starch extracted from tubers. Starch has consisted of amylose and amylopectin with various ratios depending on the plant. In the reaction, degradation of amylose by α-amylase, β-amylase or amyloglucosidase yield α-(1→4) linked maltodextrins, maltose, and glucose, respectively. Whereas, the hydrolysis of amylopectin produces α-D-Glu- α-(1→6)-D-Glu (isomaltose) and oligosaccharides with mixed α-(1→4) and α-(1→6) linkages [12].

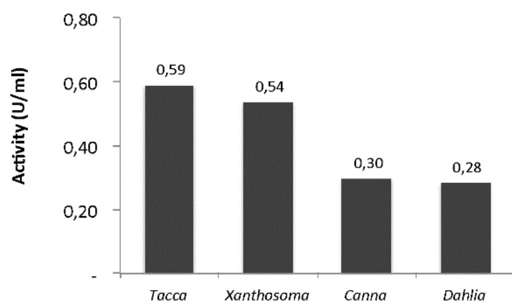

Figure 1 Alpha-amylase activities of *A. oryzae* K1A of several starches.

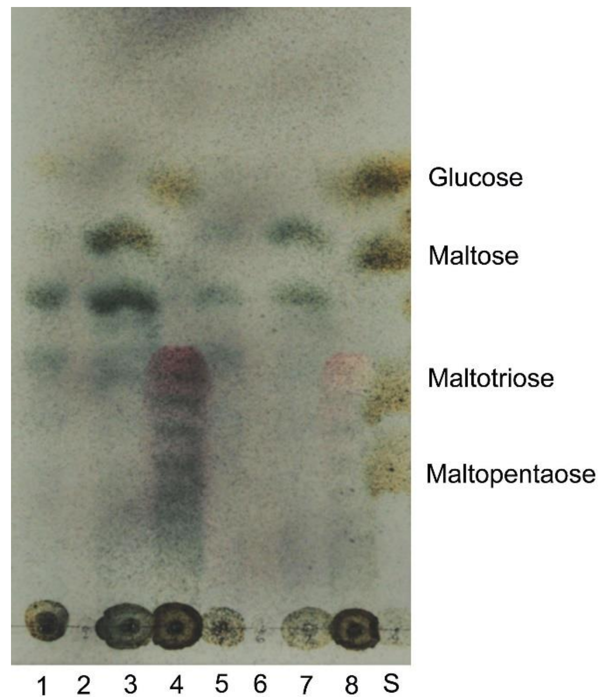

Figure 2 TLC patterns of the products of enzymatic reaction using *A. oryzae* 〈-amylase. The 25x diluted solution: *Tacca* (1), *Canna* (2), *Dahlia* (3), and *Xanthosoma* (4); the 50x diluted solution: *Tacca* (5), *Canna* (6), *Dahlia* (7), and *Xanthosoma* (8); and standard solution consisting of glucose, maltose, maltotetraose, and maltopentaose (S).

The profiles of oligosaccharides observed in this study were demonstrated by HPLC. Several HPLC peaks were detected in the retention time of 3.950 to 6.336 minutes. The *Tacca* sample provided more detected peaks than others. In this sample, three peaks were identified as maltoheptaose, maltotetraose, and maltotriose with the retention time (RT) of 3.950, 4.238, 4.539 min, respectively. Maltoheptaose is recognized as the most commonly found oligosaccharide in the samples, except the *Canna* sample. Maltotriose was the enzymatic product found in *Tacca* and *Xanthosoma* samples. Whereas, only the *Tacca* sample produced maltotetraose was observed in this study (Figure 3).

In the previous study, the use of commercial α-amylase to the rice powder suspension yields the mixture of maltotriose, maltotetraose, and maltopentaose [8]. Enzymatic reaction of starch using Brevibacterium sp. α-amylase produces the mixture of maltotriose, maltotetraose, and maltopentaose from cassava starch (Manihot esculenta Crantz) [9]. The amylase of B. licheniformis was reported also more suitable for degrading starch from starches of Canna sp. and Dioscorea sp. [11]. Indeed, various oligosaccharides are dependent on substrate and microbial strains as enzyme producer.

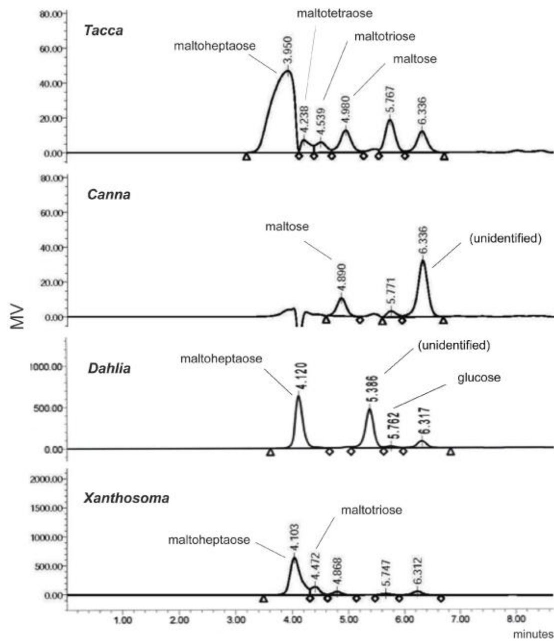

Figure 3 Electropherogram of HPLC analysis representing the oligosaccharide profiles

Maltoheptaose was produced as a dominant oligosaccharide using Xanthosoma starch. In addition, the highest concentration in the product of enzymatic reaction was achieved when *Xanthosoma* starch is used. In the comparison, maltotriose was obtained only one fourth of maltoheptaose. Total of oligosaccharides (maltoheptaose and maltotriose) reached 48.89 g/L (Table 1). The proportion of oligosaccharides produced in Xanthosoma was 93.3% of total detected saccharides (Figure 4). No oligosaccharide was produced in the sample of *Canna*. The products were mostly glucose and maltose in *Canna* starch-enzyme reaction. We also observed a significant amount of unidentified peak (RT = 6.336 min) in *Canna* starch as well as in the *Tacca* sample (Figure 3). Another unidentified peak with RT of 5.767 min was detected in all samples. Peak with the RT of 5.386 in the *Dahlia* sample showed a significant amount was unidentified. The compound was in between RT of maltose and glucose (Figure 3).

Table 1 Saccharides obtained from enzymatic reaction of several tuber starches as revealed by HPLC

	Concentration (g/L)			
	Tacca	*Canna*	*Dahlia*	*Xanthosoma*
Glucose	1,53	0,31	5,02	1,87
Maltose	0,49	0,37	ND	1,62
Maltotriose	0,47	ND	ND	8,49
Maltotetraose	0,49	ND	ND	ND
Maltopentaose	ND	ND	ND	ND
Maltoheptaose	6,62	ND	0,99	40,40
Total	9,60	0,68	6,01	52,38

ND, not detected

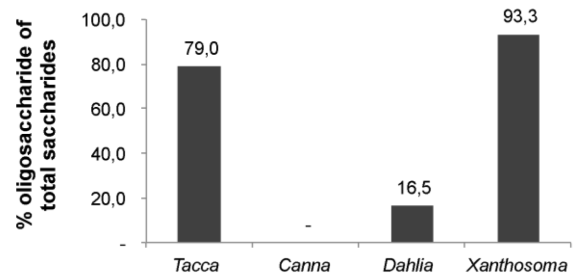

Figure 4 Proportion of oligosaccharides obtained through enzymatic reaction

In this study, products of enzymatic reaction were mostly recognized as maltooligosaccharides. Basically, maltooligosaccharides are composed of 2 to 10 α-D-glucopyranosyl units linked solely by α-1,4 glycosidic linkages. The enzyme associated with maltooligosaccharide production using starch is α-amylase (1,4-α-D-glucan glucanohydrolase) that randomly cleaves the α-1,4 glycosidic linkages. Glucose, maltodextrin, or maltooligosaccharides are produced by this enzyme. Specifically, maltotriose-forming amylase (EC 3.2.1.116), maltotetraose-forming amylase (EC 3.2.1.60), maltopentaose-forming amylase (EC 3.2.1.-), maltohexaose-forming amylase (EC 3.2.1.98) are responsible to produce various type maltooligosaccharides [13]. Altogether, this study may challenge the future prospect of characterizing the specific enzymes of *A. oryzae* in the correspond to specific substrate of tuberous starches.

4. CONCLUSION

The α-amylase of *A. oryzae* was able to produce oligosaccharides from *Tacca, Dahlia*, and *Xanthosom*a starch. Meanwhile, the oligosaccharide was not observed from the hydrolysis of *Canna* starch. More types of oligosaccharides were produced by *A. oryzae* amylase when *Tacca* starch was used as a substrate. They included maltoheptaose, maltotetraose, and maltotriose. The highest proportion of oligosaccharides (± 93% of total detected saccharides) produced by the enzyme from the *Xanthosoma* starch.

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REFERENCES

[1] A.D. Sperber, S.I. Bangdiwala, D.A. Drossman, U.C. Ghoshal, M. Simren, J. Tack, et al., Worldwide prevalence and burden of functional gastrointestinal disorders, results of Rome Foundation Global Study, Gastroenterology, 2021, 160(1):99–114. DOI: https://doi.org/10.1053/j.gastro.2020.04.014

[2] C.J. Black, D.A. Drossman, N.J. Talley, J. Ruddy, A.C. Ford, Functional gastrointestinal disorders: Advances in understanding and management, Lancet, 2020, 396(10263):1664–74. DOI: https://doi.org/10.1016/S0140-6736(20)32115-2

[3] A. Horvath, H. Szajewska, Probiotics, prebiotics, and dietary fiber in the management of functional gastrointestinal disorders, World Rev Nutr Diet, 2013, 108:40–8. DOI: https://10.1159/000351483

[4] K. Bashir, M. Aggarwal, Physicochemical, structural and functional properties of native and irradiated starch: A review, J Food Sci Technol, 2019, 56(2):513–23. DOI: https://doi.org/10.1007/s13197-018-3530-2

[5] P.M. de Souza, P.O. e Magalhães, Application of microbial α-amylase in industry – A review, Brazilian J Microbiol, 2010, 41(4):850–61. DOI: https://10.1590/S1517-83822010000400004

[6] F.A. de Moura, F.T. Macagnan, L.P. da Silva, Oligosaccharide production by hydrolysis of polysaccharides: A review, Int J Food Sci Technol. 2014, 46(5):982. DOI: https://doi.org/10.1111/ijfs.12681

[7] E.Y. Jang, Y. Ahn, H.J. Suh, K.B. Hong, K. Jo K, Amylase-producing maltooligosaccharide provides potential relief in rats with loperamide-Induced constipation, Evidence-based Complement Altern Med. 2020, 2020:5470268, DOI: https://doi.org/10.1155/2020/5470268

[8] E.Y. Jang, K.B. Hong, Y.B. Chang, J. Shin, E.Y. Jung, K. Jo, et al., In vitro prebiotic effects of malto-oligosaccharides containing water-soluble dietary fiber, Molecules, 2020, 25:5201. DOI: https://doi.org/10.3390/molecules25215201

[9] N. Rahmani, A. Andriani, Yopi, S. Hartati, Production of malto-oligosaccharides from cassava cultivar kuning, J Penelit Pascapanen Pertan, 2015, 12(3):147–55.

[10] N. Rahmani, A.D.E. Andriani, Production of maltooligosaccharides from black potato (*Coleus tuberosus*) starch by α-amylase from a marine bacterium (*Brevibacterium* sp.), Microbiol Indones, 2013, 7(3):129–36. DOI: https://doi.org/10.5454/mi.7.3.6

[11] R.D. Rahayu, J. Sulistyo, A. Dinoto, Produksi maltodekstrin berbasis pati secara enzimatik dan uji reaksi transglikosilasi, Berk Penelit Hayati., 2009, 3A:95–9.

[12] M.G. Gänzle, R. Follador, Metabolism of oligosaccharides and starch in lactobacilli: A review, 2012, 3(September):1–15. DOI: https://doi.org/10.3389/fmicb.2012.00340

[13] S. Pan, N. Ding, J. Ren, Z. Gu, C. Li, Maltooligosaccharide-forming amylase: Characteristics, preparation, and application, Biotechnol Adv, 2017, 35(5):619–32. DOI: https://doi.org/10.1016/j.biotechadv.2017.04.004