The effect of biomass particle size and chemical structure on the enzymatic hydrolysis reaction of galactomannan from sugar palm fruit by β-mannanase from Kitasatospora sp. KY576672

To cite this article: R Pangestu et al 2019 IOP Conf. Ser.: Earth Environ. Sci. 251 012008

View the article online for updates and enhancements.

You may also like

- The effect of combination of sugar palm fruit, carrageenan, and citric acid on mechanical properties of biodegradable film
  S A Rinanda, M Nastabiq, S H Raharjo et al.

- Morphological characteristics and productivity of sugar palm saps at several levels of tapping age
  D K Wardani, A Juniaedi, S Yahya et al.

- Thermal and physicochemical properties of sugar palm fibre treated with borax
  I Mukhtar, Z Leman, M R Ishak et al.
The effect of biomass particle size and chemical structure on the enzymatic hydrolysis reaction of galactomannan from sugar palm fruit by β-mannanase from *Kitasatospora* sp. KY576672

R Pangestu¹, N Rahmani¹, R Palar¹, P Lisdiyanti¹ and Yopi¹

¹Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong, West Java, Indonesia

Email: pangestu.radityo@gmail.com

Abstract. Mannooligosaccharides is essential to support gut health. Unfortunately, obtaining these compounds by chemical synthesis is considered labor extensive. In contrast, production using enzymatic reaction is simpler. Mannan, one of lignocellulose fractions, is a suitable raw material for this purpose. Sugar palm fruit (*kolang-kaling*) contains a high level of galactomannan, which makes it potential to be used as a resource for MOS bio-production. Our previous work revealed that strain which showed highest mannan-degrading activity was *Kitasatospora* sp. KY576672. In this study, the use of β-mannanase enzyme produced by this strain to degrade galactomannan from sugar palm fruit was examined. The effect of biomass particle size and chemical structure of polymer towards reaction were studied by conducting reaction using biomass with three different particle sizes and comparing the hydrolysis profile of sugar palm fruit and locust bean gum. Reaction was performed at 40°C and 190 rpm in sodium phosphate buffer pH 6. Results suggested that reaction rate did not significantly change by altering biomass particle size from 150 μm to 75 μm, but it decreased by the use of 150-300 μm particle-size biomass. In addition, locust bean gum produced a more balanced ratio of hydrolysis products (M1-M6) compared to sugar palm fruit. Mannohaxaose (M6) was the main hydrolysis products from sugar palm fruit and locust bean gum.

1. Introduction

Lignocellulose is a versatile material. Due to its abundance and its potential value as raw material for producing biofuel, chemicals, and food products, lignocellulose is very appealing for industry [1]. Sadly, most of lignocellulose sources are wasted and even burnt. Lignocellulose includes carbohydrate polymer (cellulose and hemicellulose) and non-carbohydrate polymer (lignin) [2]. Agricultural, industrial and forest residuals are prominent biomass sources of lignocellulose [3].

However, about a third of the total components in plants is hemicellulose [4]. In softwoods, such as fruits and plant seeds, hemicellulose is prominently available as mannan [5]. Mannan is able to be hydrolyzed to produce high-value compounds, including mannose and mannooligosaccharides (MOS). Mannose is potentially used as an alternative sweetener, and MOS is known for its prebiotic effect [6,7]. Production of MOS using this approach is beneficial as chemical synthesis of oligosaccharides involves complicated steps, gives poor stereoselectivity and low yields, and faces a problem with scale-up production [8–10].
As one of mega-biodiverse country, Indonesia has myriad of hemicellulose resources which are potential to be explored; sugar palm (*Arenga pinnata*) fruit, or widely known in Indonesia as *kolang-kaling*, is no exception. The cooked endosperm of nut from this fruit is frequently consumed as an additional ingredient for certain drinks [11]. Proximate analysis showed that sugar palm fruit contained about 92% water, 3.42% carbohydrate (mainly galactomannan), 1.52% crude fiber, 1.42% protein and minor constituents [12]. Despite its high content of mannan, the utilization of sugar palm fruit in hemicellulose processing is still limited.

There are enormous mannane-producing microorganisms which had been studied; however, the exploration of Actinomycetes as microbial source is still provoking interest owing to its diversity [13,14]. Several Actinomycetes strains were known to have mannolytic activity [15–18]. Our previous study revealed that various mannan-containing polymers could be hydrolyzed to produce MOS using β-mannanase enzyme from *Kitasatospora* sp. KY576672. This strain exhibited highest mannolytic activity compared to other isolates screened [17]. Present study aimed to investigate the enzymatic hydrolysis of galactomannan from sugar palm fruit using β-mannanase from this strain by comparing its reaction pattern to locust bean gum’s, another galactomannan source which had widely been studied, and also consider the influence of physical parameter, focusing on the particle size, which may affect the reaction rate. The information about particle size effect is essential for industry since reducing particle size consumes more energy.

2. Materials and Methods

2.1. Chemicals, reagents, and materials

Cooked endosperm of nut from *A. pinnata* was collected from Caringin, Bogor, West Java, Indonesia. *Kitasatospora* sp. KY576672 was obtained from Research Center for Biotechnology, Indonesian Institute of Sciences. Peptone, malt extract, yeast extract, and agar were purchased from Becton Dickinson & Co (USA). Locust bean gum (LBG) and D-(+)-mannose (M1) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). β-D-mannobiose (M2), β-D-mannotriose (M3), β-D-mannotetrose (M4), β-D-mannopentose (M5) and β-D-mannohexaose (M6) were purchased from Megazyme (Wicklow, Ireland). Other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Production of mannanase enzyme

*Kitasatospora* sp. (frozen stock) was maintained on ISP2 agar with the following composition (%): yeast extract, 0.4; malt extract, 1.0; glucose, 0.4; and agar, 1.7. It was incubated at 28 °C for 3 days. A single colony was cultivated in ISP2 medium (composition mentioned, without addition of agar) at 28°C and 190 rpm for 3 days. An aliquot of pre-culture was inoculated into fermentation medium with a ratio of 1:9 (v/v). Fermentation medium contained the following compositions (g per 100 mL): locust bean gum (LBG), 1.25; peptone, 0.075; yeast extract, 0.05; (NH₄)₂SO₄, 0.14; KH₂PO₄, 0.20; MgSO₄.7H₂O, 0.03; CO(NH₂)₂, 0.03; CaCl₂, 0.039625; FeSO₄.7H₂O, 0.0005; MnSO₄.7H₂O, 0.0976125; ZnSO₄.7H₂O, 0.00014; CoCl₂, 0.00036625; and buffer pH 8. Fermentation was conducted in a shaker flask for 4 days at 28 °C and 190 rpm. The enzyme was harvested from culture supernatant by centrifugation (22,540 × g, 4 °C for 30 min.) and filtration (0.2 μm).

2.3. Enzyme activity assay

Mannolytic activity of enzyme obtained was assayed using DNS method [19]. LBG (200 μL, 1%, w/v) was mixed with 100 μL of distilled water and 100 μL of phosphate buffer pH 6 (50 mM). Diluted enzyme (50 μL) was added and the mixture was incubated at 60 °C for 15 min. After reaction, 500 μL of DNS reagent was added. The mixture was heated to 90 °C for 15 min. and, then, cooled by ice. Sugar formed was measured by spectrophotometer at 540 nm (SpectraMax Paradigm, Molecular Devices). The same procedure was also carried out to measure control (without addition of enzyme) and standards (mannose with a concentration of 50, 100, 200 and 300 ppm). Enzyme activity was expressed as unit per mL (U/mL). Unit of enzyme activity was calculated from the amount of sugar released (in μmol) per min.

2.4. Enzymatic hydrolysis reaction
Sugar palm fruit (biomass) was sliced, dried (40-50 °C) and crushed using food processor. The powder was subsequently separated using several sieves (300 μm, 150 μm and 75 μm). Each fraction was diluted by 200 mL of phosphate buffer pH 6 (50 mM) and mixed to 200 mL of crude enzyme. The reaction was conducted in a shaker flask at 40 °C and 190 rpm. Products were sampled at 2, 4, 8, 24 and 32 h. Qualitative physical change of solution was observed during the reaction. Reaction was stopped by heating treatment at 100 °C for 15 min. The products were collected from supernatant after centrifugation at 15,660 × g for 15 min. Similar procedure was applied to locust bean gum (particle size ≤ 75μm).

2.5. Analysis of hydrolysis products

Products were analysed by TLC (qualitative) and HPLC (quantitative). Standards used were mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5) and mannohexaose (M6). Qualitative analysis was carried out by spotting samples onto TLC silica gel G60 F254 (Merck, Germany). Samples were developed with a mixture of n-butanol, glacial acetic acid, and water (2:1:1, v/v/v). Visualization was obtained after the plate was sprayed by DAP reagent (mixture of diphenylamine, aniline, acetone and phosphoric acid, 0.2:0.2:10:1.5, v/w/v/v) and heated at 120°C for 15 min [20,21]. Quantitative analysis was performed using an Agilent Technology 1200 Infinity Series HPLC with refractive index detector (RID) and Hi-PlexCa (Duo) column (300 x 6.5 mm, 8 μm). Before injection, sample was centrifuged (9,700 × g for 15 min.) and filtered (0.2 μm) to remove impurities. Volume of sample injected was 10 μL. Ultrapure water (HPLC grade) was used as mobile phase. Eluent flow rate was 0.60 mL/min. Temperature of column and detector were set at 80°C and 40°C, respectively. Running time of each sample was 30 min. This procedure was the optimization of manufacturer’s guideline [22].

3. Results and Discussions

3.1. Production of enzyme and analysis of its activity

Mannooligosaccharides can be generated by degradation of mannan. This process involves breaking β-1,4-linkage of mannose-containing backbone side by hydrolase enzyme, named mannanase [7]. According to our previous study, *Kitasatospora* sp. KY576672 was a prospective microbial source for producing this enzyme [17].

The production of extracellular β-mannanase enzyme was carried out in a 2L Erlenmeyer flask. Locust bean gum was used as a carbon source. Other nutrients, such as nitrogen, water, and salt, were fulfilled by the addition of ingredients mentioned. Concentration of pre-culture inoculated to fermentation media was 10% (v/v) for optimum growth [23]. Considering the mechanical limit of shaker flask, the ratio volume of culture to flask was 1:5. The extracellular enzyme was separated from cell by centrifugation and filtration.

The result of enzyme activity assay is shown in Table 1. The activity of enzyme was lower than that of recombinant enzyme from our previous study because the purification process was not conducted [17]. Instead, the crude enzyme was used to figure out its application for industrial process.

### Table 1. Activity of crude β-mannanase enzyme produced from *Kitasatospora* sp. KY576672

| Sample (S) | Absorbance Control (C) | S-C | Concentration of sugar released (ppm) | Activity (U/mL) |
|-----------|------------------------|-----|--------------------------------------|-----------------|
| 1         | 1.2490                 | 0.7776 | 184.14                              | 68.20           |
| 2         | 1.3851                 | 0.8410 | 199.61                              | 73.93           |
| 3         | 1.5120                 | 0.7562 | 178.92                              | 66.27           |

|       | 69.47 ± 3.98b |

*Values represent the means of duplicate measurements.*

*Average ± SD.*

3.2. Preparation of biomass and enzymatic hydrolysis reaction
Sugar palm fruit (SPF) was collected from *A. pinnata* fruit and prepared in fresh condition to minimize degradation action of microorganism. According to Torio [12], SPF contained about 4% carbohydrate (mainly galactomannan). In other words, the content of galactomannan was about 42% of dried weight of biomass. Thus, SPF was expected to yield a high concentration of MOS. Drying process was an essential key to obtain desired biomass size [24]. Biomass was heated below 60 °C to avoid thermal degradation. Separation process by sieves yielded three different fractions of biomass: SPF-I (particle size < 75 μm), SPF-II (particle size: 75-150 μm) and SPF-III (particle size: 150-300 μm).

The reaction was performed at 40 °C and pH 6 in accordance with the optimization condition of reaction and stability of Actinomycetes mannanase studied by Takahashi *et al.* [25]. Shaking treatment was applied to homogenize the mixture and enhance the reaction rate. The reaction was stopped by high-temperature treatment to denaturalize enzyme. Similar procedure was also conducted to locust bean gum. Hydrolysis products analysis (by TLC) is presented in Figure 1. Generally, all samples produced M1, M2, M3, M4, M5, and M6. Longer unidentified oligosaccharides were also produced since there were several spots with lower Rf values present in all samples. These oligosaccharides might be homo-oligomers containing mannose more than six units and/or hetero-oligomers whose structure possesses galactose unit(s) linked to mannose unit(s) via α-(1→6) linkage.

![Figure 1. TLC analysis of enzymatic hydrolysis products](image)

Solubility of SPF in sodium phosphate buffer was lower than that of LBG (Figure 2). This was due to the high degree of branching of galactomannan in SPF [12]. However, as enzymatic reaction progressed, the solubility and homogeneity of both SPF and LBG increased. The mixtures became less viscous at 32 h. It seemed that SPF-I and SPF-II became completely soluble and only dirt floated, yet SPF-III was still rather viscous at 32 h. LBG is completely soluble without any dirt left as we use pure galactomannan for this experiment. The observation proved that the enzyme actively hydrolyzed galactomannan (long chain polymer) from biomass to shorter chain of mannooligosaccharides as simpler sugars are more soluble in water.
3.3. The effect of biomass particle size

The effect of biomass particle size was studied through the hydrolysis profile of three different particle size fractions of SPF. Figure 3(a)-(c) shows the chromatograms of biomass hydrolysis products at the various time and Figure 4(a)-(c) depicts the concentration change of each MOS. Generally, M6 was the predominant product from all samples while other MOS were only minors. During reaction, concentration of M6 was 10 times higher than other MOS in all three samples. Concentration of these oligosaccharides remained below 1,000 mg/L, except for M4 in SPF-I at 8 h and 32 h. Further degradation of unidentified oligosaccharides to M6 was observed as its peak area became smaller but the opposite trend occurred to M6. Peak splitting of unidentified oligosaccharides was observed (all samples) at 8 h indicating that there might be more than one unidentified compounds. The concentration of M5 could not be calculated due to peak overlapping with M6, but the spots on TLC analysis (Figure 1) gave more convincing interpretation that M5 was produced.

Meanwhile, the concentration of M1, M2, M3, and M4 showed downward trends after 4 h and 8 h. This interpretation observed in M1 whose peak even disappeared at 4 h and afterward. These drops were probably caused by trans-glycosylation since some of β-mannanase in GH5, and GH113 exhibited this activity [26]. This prediction needs further confirmation. However, M1 peak appeared again in SPF-I at 32 h (observed both in TLC and HPLC analysis), and small increases in M2 and M4 were possibly the products of further-hydrolysis reaction of longer oligosaccharides.

![Figure 2. Physical characteristic change of biomass solution during enzymatic hydrolysis reaction. Picture was taken from above flask.](image-url)
Figure 3. HPLC analysis of hydrolysis products of (a) SPF-I; (b) SPF-II; (c) SPF-III; (d) LBG; by β-mannanase enzyme (U = 69.47 ± 3.98). See text for details.

The effect of biomass particle size was figured out by comparing the concentration of each MOS. Based on theory of reaction kinetics, reduction of particle size can increase the reaction rate. However, the treatment did not affect SPF-I (particle size < 75 μm) and SPF-II (particle size: 75-150 μm) as the concentration of MOS from those two did not differ significantly. Nonetheless, the effect was obviously observable in SPF-III (particle size: 150-300 μm). In general, concentrations of hydrolysis products from SPF-III were lower than SPF-I and SPF-II indicating a decrease in reaction rate.
Figure 4. Comparison of concentration of several mannooligosaccharides produced from enzymatic hydrolysis of (a) SPF-I; (b) SPF-II; (c) SPF-III; (d) LBG; by β-mannanase enzyme ($U = 69.47 \pm 3.98$). See text for details.

3.4. The effect of galactomannan chemical structure

The chemical structure of galactomannan varies depending on its source [7]. This diversity may affect the enzymatic hydrolysis pattern of particular biomass. Hydrolysis profile of SPF and LBG could be studied from their chromatograms (Figure 3(a) and (d)) and the comparison of their hydrolysis products (Figure 4(a) and (d)).

In LBG, M6 was also the major hydrolysis product. Interestingly, the quantity of M6 produced from SPF almost doubled and even tripled (at 4 h and 8 h) the concentration of M6 from LBG. Conversely, the concentration of M1, M2, M3, M4, and M5 produced from LBG were considerably higher than that from SPF. Concentration of M2 (at 2 h – 24 h), M3 (at 24 h and 32 h), M4 (at 4h – 32 h) and M5 (at 2h – 32 h) in LBG were higher than 1,000 mg/mL. This could be explained by the difference of mannose/galactose (M/G) ratio of galactomannan. M/G ratio of SPG and LBG were 2:1 and 4:1, respectively. The presence of galactose as branch unit hinder the enzymatic cleavage of backbone chain [27]. Because chemical structure of SPF contained more galactose units, longer-chain hydrolysis products were more favorable.

4. Conclusion

Galactomannan from sugar palm fruit could be hydrolyzed by β-mannanase from *Kitasatospora* sp. KYS76672 to produce simpler sugars including mannose, mannobiose, mannotriose, mannotetraose, mannopentaose, mannohexaoe, and longer oligosaccharides. Sugar palm fruits with a particle size ≤ 75 μm and 75-150 μm were predicted to have relatively similar reaction rate and that with particle size
150-300 μm exhibited a lower reaction rate. Also, in SFP, shorter chain manno-oligosaccharides, such as M1, M2, M3, and M4, were produced in a low concentration due to higher concentration of galactose present in its polymer structure. M6 is the major biodegradation product of both SPF (all biomass particle sizes) and LBG.

5. Acknowledgment

The authors gratefully acknowledge the financial from DIPA (National Priority for Health) - Research Center for Biotechnology LIPI and Incubation Program of PUSINOV LIPI.

6. References

[1] Kuhad R C and Singh A 1993 Crit. Rev. Biotechnol. 13 151–72
[2] Anwar Z, Gulfraz M and Irshad M 2014 J. Radiat. Res. Appl. Sci. 7 163–73
[3] Kumar R, Singh S and Singh O V 2008 J. Ind. Microbiol. Biotechnol. 35 377–91
[4] Chauhan P S, Puri N, Sharma P and Gupta N 2012 Appl. Microbiol. Biotechnol. 93 1817–30
[5] Scheller H V and Ulvskov P 2010 Annu. Rev. Plant Biol. 61 263–89
[6] Hu X, Shi Y, Zhang P, Miao M, Zhang T and Jiang B 2016 Comprehensive Reviews in Food Science and Food Safety 15 773–85
[7] Soni H and Naveen K 2013 Advances in Enzyme Biotechnology ed. Shukla P and Pletschke (New Delhi: Springer) 41–56
[8] Rastall R A and Bucke C 1992 Biotechnol. Genet. Eng. Rev. ISSN 10 253–82
[9] Monsan P and Paul F 1995 FEMS Microbiol. Rev. 16 187–92
[10] Muthana S, Cao H and Chen X 2011 Curr. Opin. Chem Biol. 13 573–81
[11] Sayuti K, Yenrina R and Anggraini T 2017 Pakistan J. Nutr. 16 69–76
[12] Torio M A O, Saez J and Merca F F 2006 Philipp. J. Sci. 135 19–30
[13] Priyadharshini P and Dhanasekaran D 2015 J. Saudi Soc. Agric. Sci. 14 54–60
[14] Saini A, Aggarwal N K, Sharma A and Yadav A 2015 Enzyme Research. 279381 15 pages
[15] Montiel M D, Rodriguez J, Pérez-Leblic M I, Hernández M, Arias M E and Copa-Patiño J L 1999 Appl. Microbiol. Biotechnol. 52 240–45
[16] Ratnakomala S, Suhartono M T, Meryandini A and Prasetya B 2014 Annales Bogorienses 18 1–12
[17] Rahmani N, Kashiwagi N, Lee J, Nakamura N, Matsumoto H, Kahar P, Lisdiyanti P, Yopi, Prasetya B, Ogino M and Kondo A 2017 AMB Express 7 2–11
[18] Arcand N, Kluypfel D, Paradis F W, Morosoli R and Shareck F 1993 Biochem. J. 290 857–63
[19] Miller G L 1959 Anal. Chem. 31 426–8
[20] Hansen S A 1975 J. Chromatogr. 107 224–6
[21] Humbel R and Collart M 1975 Clin. Chim. Acta 60 143–5
[22] Ball S, Bullock S, Lloyd L and Mapp K 2011 Analysis of Carbohydrates, Alcohols, and Organic Acids by Ion-Exchange Chromatography - Agilent Hi-Plex Columns Applications Compendium ed. A Ewen (Pirmasens, Germany: Agilent Technologies) 3
[23] Abulencia J P and Theodore L 2015 Open-Ended Problems - A Future Chemical Engineering Education Approach. (Massachusetts: Scrivener Publishing LLC) 394
[24] Jung H, Lee Y J and Yoon W B 2018 Processes 6 1–16
[25] Takahashi R, Kusakabe I, Maekawa A, Suzuki T and Murakami K 1983 Japan. J. Trop. Agr., 27 140–8
[26] Rosengren A, Reddy S K, Sjöberg J S, Aurelius O, Logan D T, Kolenová K and Stålbrand H 2014 Appl. Microbiol. Biotechnol. 98 10091–104
[27] Mahammad S A, Conford S, Kelly D A and Khan R M 2007 Biomacromolecules 8 949–56