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Production of Manooligomannan from Palm Kernel Cake by Mannanase Produced from Streptomyces Cyaenus

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Abstract

The increase of public attention to health has prompted researchers to look for new sources of functional food. Palm Cake Kernel (PKC) waste was abundant in Indonesia, Oligosaccharide has an important benefit for human health. Recently oligosaccharide is not only important as an artificial sweetener, but also as a functional food component. This study was aimed to produce oligo-mannan enzymatically from PKC waste using mannanase derived from of Streptomyces cyaenus isolates of indigenous Indonesia. The enzyme concentration was determined by enzyme activity assay while oligo-mannan content in the PKC was analyzed using TLC and HPLC. Mannanase enzyme activity of 1706 U/ml on the second day of agitation 200 rpm at a temperature of 30°C Hydrolysis of mannooligomannan by using mannanase produced by streptomyces cyaenus. The optimum mannanase enzyme activity obtained on day 2 with the value of the activity as much of 0.702 U/mL. The protein content of the 2nd day at an agitation speed of 150 rpm, 200 rpm, and 250 rpm, respectively, were 1783, 1950 and 2283 ppm. *Streptomyces cyaenus* is Indonesian original isolates potentially producing mannanase that can produce mannooligomannan.

How to Cite

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INTRODUCTION

The increase of public attention to health has prompted researchers to look for new sources of functional food. Functional food is a natural or processed food containing bioactive components which can give a positive impact on the human body's metabolic functions. According to the "Scientific Concepts of Functional Foods in Europe" issued by the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE). Indonesia can also be used for the production of oligosaccharides. Among the candidates, a new type of oligosaccharides are the oligosaccharides derived from mannan, a kind of hemicellulose. The production of oligosaccharides from materials other than starchy carbohydrates is still in a small number. Non-starch carbohydrates such as mannan can be used as a basic material in the production of these oligosaccharides. Mannan biomass is widely available in palm kernel cake (PKC), coconut cake, coffee Mannan biomass is widely available in palm kernel cake (PKC), coconut cake, coffee beans and others. In addition, there is also mannan on the type of bulb porang (*Amorphophallus muelleriblume*), similar to the root of konjac (*Amorphophallus konjac*).

PKC is abundant as a by product in the extraction of palm kernel obtained through the chemical and mechanical processes. PKC is the residue from the extraction of palm oil, which contains carbohydrates and mannan total as much of 48.5% and 35.2% (Cervero et al., 2010), while in porang tuber that grows in the highland area of forestry, it was reported about 70% mannan of dry weight. Mannan is a specific polysaccharide widely available in Indonesia, especially from the side of the palm oil industry. There are about 2 million tons of palm kernel cake produced per year. There is also a porang tuber not consumed by our society. Due to the importance and the availability of mannan materials and the collection of local potential microbial isolates producing enzymes mannanase, it is expected become the early stages of mano-oligosaccharide (Yopi et al., 2007).

Galactomannan is a neutral polysaccharide that is found as material deposits in the endosperm of certain leguminous plant seeds and palm kernel cake (Jorgensen et al., 2010). Palm kernel cake contains 78% hemicellulose fiber in the form of mannan and 12% in the form of cellulose. In contrast to substituted mannan, galactomannan soluble in water and can absorb water, thus providing the function of holding water for seed. Galactomannan chemical structure consists of the main structure of the linear β-(1→4) D-mannopyranosyl, and the side chain α-(1→6) galactopyranosyl.

The palm oil industry is one the important industries in Indonesia. Facing the global market competition, it is expected that secondary effluent from palm oil industry could be processed to something that has added value and good for the environment. The utilization of Palm kernel cake, currently, only limited as animal feed with a low level of efficiency. Whereas Mannan content of the PKC can be utilized to produce Manno-oligosaccharide which potentially used as prebiotic, a component of functional food and animal feed, as well as high economic value. Galactomannan can be recognized based on the ratio of mannose to galactose, galactose, and its molecular weight distribution. The solubility and viscosity of galactomannan were influenced by the ratio of mannose to galactose which various at 1 to 5. Further distributions may vary substituents which also affects the physical properties of galactomannan. Two examples of galactomannan already well-characterized and commercially produced are galactomannan from seed endosperm *of Ceratonia siliqua* and *Cyamopsis tetragonolo*, respectively known as locust bean gum and guar gum. Galactomannan of locust bean gum has a ratio of mannose: galactose is about 5:1 with a molecular weight of 310000, while guar gum galactomannan has a ratio of mannose: galactose is about 2:1 with a molecular weight of 220000. Galactomannan has strong gelling properties, so often used as a thickener, stabilizer and coatings in the food and feed, cosmetics, medicine and paper (Hagglund, 2002).

Mannanase is an enzyme that is capable of degrading a polysaccharide mannan and heteromannan (galactomannan, glucomannan, and galactoglucomannan) by cutting β-1,4 glycoside bond. There are two types of mannanase enzymes involved in the decomposition of mannan, ie. exo, and endo-mannanase-β-mannanase (Sae-Lee, 2007). Exo-enzymes mannanase or β-mannosidase (1,4-β-D-manopyranoside hydrolase) is the enzyme that cuts β-1,4 bond mannosidase and releases the mannose from which the non-reduced end portion of a mannan, heteromannan, or mano-oligosaccharide. In contrast, endo-b-mannanase (1,4-β-D-mannanmanohydrolase) is an enzyme that cuts at random in the middle of the glycoside bond mannan to produce mano-oligosaccharide or monosaccharide (Figure 2) (Moreira & Filho, 2008). Enzymes mannanase found them on *Aspergillus*

niger, Aspergillus Awamori, Aspergillus fumigatus, Aspergillus aculatus, Clostridium butyricum, Sclerotiumrolfsii, Saccharomyces cerevisiae, Streptomyces lividans, Bacillus subtilis, Bacteroidesovatus, Trichoderma reesei, Pichia pastoris (Dhawan & Kaur, 2007; Sae-Lee, 2007; Moreira & Filho, 2008). Mannan decomposition is strongly influenced that the level and pattern of substitution and the main chain mannanase types of enzymes are used.

Utilization of mannanase enzyme producing bacteria are able to decipher the complex polysaccharides of plant tissue into simple molecules such as mannose mano-oligosaccharide and has an important role in the pulp and paper industry, animal feed, food and detergent industries (Dhawan & Kaur, 2007). Mannanase enzymes produced from different sources have different characteristics and specificity (Moreira & Filho, 2008). In general, mannanase enzyme showed high activity at pH 3.0-7.5 and temperature 45-92^{o}C. The thermal stability varies between 10 minutes to 8 hours at different temperatures. Mannanase enzyme is also different in terms of kinetic parameters and isoelectric point.

Oligosaccharide was originally classified as an anti-nutrient because it can cause flatulence. Some are raffinose, stakiosa and verbascosa which are contained in vegetable foodstuffs such as beans (eg, soybeans) and some types of tubers (eg sweet potato). In Japan, many industries produce oligosaccharides to be sold as functional food ingredients. Those oligosaccharides will be fermented by bacteria in the colon, and will further change the composition of the gut bacteria. Beneficial bacteria Bifidobacterium (bifidus bacteria) and lactobacillus will increase in number, while harmful bacteria such as clostridium, coliform and enterococci will decrease in number (Muchtadi, 1996). The amount of polysaccharide/oligosaccharide krestin which is able to mop up free radicals would decrease because a number of free radicals exceed the capacity of polysaccharide krestin (Wahyuningsih et al., 2016).

PKC from Lampung was used as a raw material for the manufacture of the oligosaccharide. Mannan carbohydrate hydrolysis process is done by using special enzymes mannanase which serves to cut the chain of mannose of carbohydrates to produce oligosaccharides. The aims of this study are to produce mannanase enzyme from *Streptomyces cyaenus*, to analyze oligosaccharide mixture product of biomass hydrolysis with mannanase enzymes, and to get oligosaccharide mixture products by hydrolyzing the mannan biomass with mannanase enzymes. Basic research on microbes and enzymes related to the degradation of polysaccharides mannan has been done, however, no information on the industrial scale production. Therefore, this study emphasized on bioprocess strategies that will be used so that a pilot plan can be designed for the production of oligosaccharides from oil palm biomass waste. The mannan of the PKC can be a good source of Manno-oligosaccharides that function as probiotics, a component of functional food and animal feed, as well as providing high economic value added.

METHODS

Research site

The research was conducted in the Fermentation and Biocatalyst Laboratory, Research Center for Biotechnology, Indonesian Institue of sciences. Jl. Raya Bogor km 46 Cibinong Bogor. West Java Indonesia.

Material

Streptomyces cyaenus mannanase is a collection of Biotechnology Culture Collection (BTCC) Research Center for Biotechnology LIPI. PKC originated from Lampung was used as a biomass to produce oligosaccharides. The optimation of mannanase enzyme production in 2 Liter stirred fermentor and 100 mL scale was conducted by growing the Streptomyces cyaenus in a sterilized pre-culture medium containing 0.3% locust bean gum (LBG) as substrate supplemented with peptone, yeast extracts, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$, $CO(NH_4)_2$, $CaCl_2$, $FeSO_4.7H_20$, $ZnSO_4.7H_2O$, $CoCl_2$ in distilled water, in a 500 ml of scouth bottle and pH 7.0. The preculture was then incubated in an incubator shaker at a speed of 150 rpm, temperature 37°C during 4 days. The activity of mannanase enzyme was measured in every 24 hours.

Mannanase enzyme production

Mannanase enzyme production in a scale of 2 L in stirred fermentors (Winpact water lift) was conducted by growing the Streptomyces cyaenus in an 1800 ml of sterile medium with the same composition as preculture medium, pH 7.0. As much as 200 mL of pre-culture suspension was transferred into the fermentation reactor aseptically. The fermentation was conducted with the agitation speed of 150 rpm and a temperature of 30°C. The sample was taken every 24 hours for 4 days. The enzyme was obtained by centrifuging the culture at a speed of 10,000 rpm for 10 minutes at a temperature of 4°C. The supernatant was collected and stored at 4°C. Mannanase en-

zyme activity was analyzed using a spectrophotometer at a wavelength of 540 nm (Miller 1959).

Reaction Enzyme-Substrate

Mannanase enzyme solution was reacted with 0.5% PKC substrate (1:1) then incubated in the shaker incubator for 24 hours at a temperature of 37°C, 150 rpm. The sample was taken on hour-2, 4, 6, 8, and 24. PKC with a concentration of 0.5% was used as the substrate, dissolved in water and homogenized using a vortex. Mannanase enzyme solution used is the result of fermentation of Streptomyces cyaenus. Products were analyzed using TLC (Thin Layer Chromatography) method, reducing sugar (Miller method), total sugar content (phenol method) and oligosaccharides content with HPLC instruments.

Analysis mannooligosaccharides

The saccharides in the mixture (galactose, glucose, mannose, mannobiose, mannotriose, mannotetraose and mannohexaose) were separated by High-Performance Liquid Chromatography (HPLC), column packing system was Zorbax carbohydrates X150 mm 4.6 mm ID. Separation was performed at a temperature of 30°C, 1 mL/min of flow and mobile phase (acetonitrile: water = 60:40) for 15 minutes. Detection was carried out using HP1100 detector RID. The sample solution was filtered by using filter paper 0.2 µm before injected into the instrument (Cervero et al., 2010).

RESULTS AND DISCUSSION

Mannanase production

Optimization of the production of mannanase in a scale of 100 mL using the medium of 0.3% locust bean gum (LBG) as a substrate yielded the enzyme activity on day 0 as much of 0.153 U / mL, day 1 as much of 0.205 U / mL, the day 2 as much of 0.702 U / mL, day 3 as much of 0.659 U / mL and day 4 has an activity as much of 0.208 U/mL. The optimum activity of mannanase was obtained on day 2 with the value of the activity as much of 0.702 U/mL.

Figure 1 shows the growth of *Streptomyces Cyaenus* in the 2 L stirred fermentor for 4 days with the agitation speed of 150 rpm, 200 rpm, and 250 rpm at a temperature of 30 ° C. At the agitation speed of 150 rpm shows bacterial cell growth increased until day 3, while on day 4 decreased. On day 3 the value of optical density (OD) at a wavelength of 660 nm was 0.650. At the agitation speed of 200 rpm, bacterial cell growth increased until day 2, while the 3rd and 4th decreased. The bacterial cell growth reached the maximum value on day 2 with the OD at the wavelength of 660 nm was 0.700. The maximum bacterial cell growth at a speed of 250 rpm agitation occurred on day 2 OD at a wavelength of 660 nm as much of 0.650. The smallest value of bacterial cell growth compared to the other variation of speed was occurred at a speed of 200 rpm on day 2 with a value of bacterial cell growth as much of 0.700.

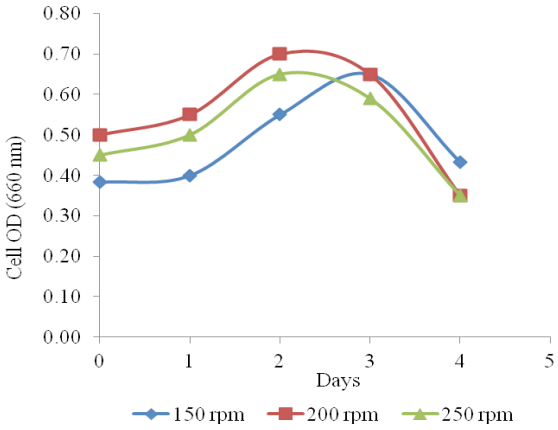

Figure 1. The cell growth of *Streptomyces cyaenus* in 2L stirred fermenter at a speed of agitation: 150 rpm, 200 rpm, and 250 rpm for 4 days at a temperature of 30°C.

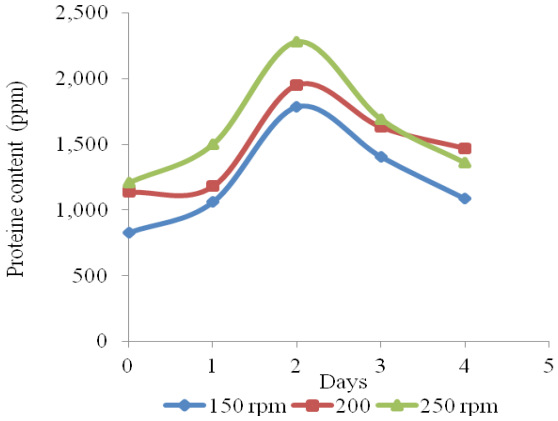

Figure 2. The content of the cell protein of *Streptomyces cyaenus* in 2 L fermenter stirrer at a speed of agitation: 150 rpm, 200 rpm, and 250 rpm for 4 days at a temperature of 30°C

The fermentation process by *Streptomyces cyaenus* for 4 days with agitation speed of 150 rpm, 200 rpm, 250 rpm, at temperature of 30°C and wavelength of 280 nm resulted the data as follows: the protein content with the agitation speed of 150 rpm was increased on day 0 until day 2 whereas on day 3 and 4 the protein content was decreasing. It might be caused by the lysis of cells that consume media. The highest protein content

of the three different kinds of agitation (150, 200, 250 rpm) was occurred on the day 2. The protein content on day 2 at the agitation speed of 150 rpm, 200 rpm, and 250 rpm, respectively, were 1783, 1950 and 2283 ppm. The relation between the content of protein in bacterial cells of Streptomyces cyaenus with the agitation speed in a 2 L scale stirred fermenter can be seen in Figure 2.

Streptomyces cyaenus cultures were grown in liquid media containing LBG substrate. Mannanase enzyme activity increased until the 2nd day, then decreased in the 3rd and 4th. The decreasing of activity was caused by the formation of a product in the form of mannose or mannooligosaccharide. Mannanase activity will increases when the product in the form of mannose or mannooligosaccharide decreases. The total of mannose or mannooligosaccharide decreased because this product was consumed by bacteria as a carbon source for nutrients and reproduction. The activity of mannanase increased from day 0 and optimum on the 2nd day with the highest values obtained as much of 1.619 U / mL at a speed of agitation of 150 rpm, at the speed of agitation of 200 rpm obtained the activation value of 1.706 U/mL while on the agitation speed of 250 rpm the activation values of 1.637 U/mL was observed (Figure 3).

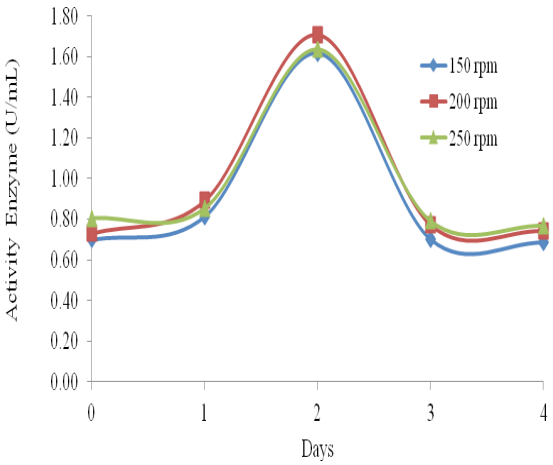

Figure 3. mannanase enzyme activity at a speed of agitation: 150, 200, and 250 rpm at a temperature of 30°C for 4 days.

The reaction of the enzyme with substrate mannanase biomass mannan

The oligosaccharide composition of PKC sample is determined by calculating the degree of polymerization (DP). The degree of polymerization of oligosaccharides is the division of the total sugar content with a reducing sugar content. A total of 6 mL of 0.5% PKC was reacted directly with 6 mL mannanase enzyme (an activity of 1.706 U / mL) in a test tube with a lid for 24 hours. After 24 hours of reaction, the product was measured and analyzed for the total sugar content, reducing sugar and TLC. The degradation of mannan from PKC by mannanase enzyme from Streptomyces cyaenus yielded mannooligosaccharides, such as mannotriose, mannotetraose, mannopentaose, mannohexaose, mannose, and mannobiose. The analysis of the degradation of mannan by using TLC can be seen in Figure 4.

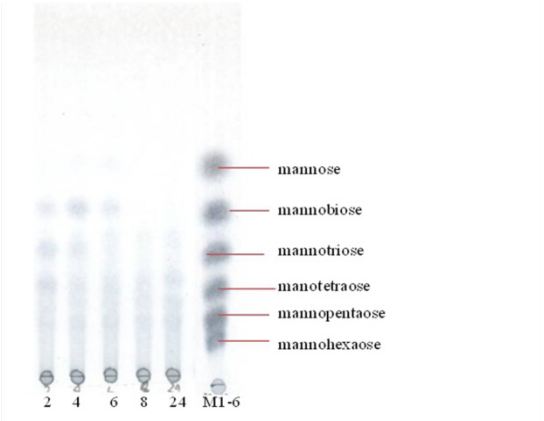

Figure 4. Profile TLC, the hydrolysis of biomass PKC, by enzyme mannanase. Note: M_{1-6} = standard mannose, mannobiose, mannotriose, mannotetraose, mannopentaosa, mannohexaose; 2, 4, 6, 8, 24 hours is the time of the hydrolysis reaction

Although the profile of oligosaccharide is not very seen clearly, these could result indicate that the mannanase enzyme from Streptomyces cyaenus suitable to be used for degrading mannan of PKC. TLC profile of hydrolysis in the reaction 2, 4, 6, 8, 24 hours looks less clearly compared to the standard spot of mannooligosaccharide (M1-6 = mannobiose, mannotriose, mannotetraose, mannopentaose, mannohexaose). This is due to the impurity in biomass PKC because it still contains a lot of protein, fat, and crude fiber with significant levels. Crude fiber bonds firmly to lignin forming the lignocellulosic structure, so that the mannanase enzyme is difficult to penetrate the cell wall of PKC. The saccharification of the lignocellulosic biomass by the enzymes and the subsequent fermentation of the polysaccharide to oligo mannan by yeast take place in a single reactor indirect saccharification and fermentation (DSF) process. The DSF gives higher yield and requires lower amounts of enzyme because end product inhibition from cellobiose and glucose which are formed during enzymatic hydrolysis is

relieved by the yeast fermentation (Juanssilfero et al., 2015)

Total sugar content, reducing sugar and the degree of polymerization of the hydrolysis product at 2, 4, 6, 8, and 24 hours are shown in Table 1. The degree of polymerization of oligo mannan at 2, 4, 6, 8 and 24 hours were 4.1; 7.3; 2.5; 12.0; 4.0; and 2.7. This result respectively suggested that the reaction for 2 hours generates mannotetraose, 4 hours generates mannoheptulose, generating mannotriose at 6 hours, 8 hours generates mannooligosaccharide, and 24 hours produces mannotriose. There are two types of enzymes involved in the decomposition of mannan ie. exo and endo-mannanase-β-mannanase (Sae-Lee 2007). The reaction of PKC substrate with the mannanase enzyme yielded various oligosaccharides.

Oligosaccharide solution was analyzed using HPLC. PKC hydrolysis reaction for 2 hours yielded 6 peaks namely: Peak I was mannobiose having a retention time of 5.7 minutes at a concentration of 530 ppm. Peak II is mannotriose, having a retention time of 6.67 minutes with a concentration of 290 ppm. Peak III is mannohexaose, having a retention time of 11.31 minutes with a concentration of 1310 ppm, the peak IV is predicted as manotetraose with a retention time of 7.88 minutes, peak V is predicted as mannopentaose with 9.49 minutes retention time and peak 6 is predicted as mannohepulose with the retention time of 11.82 minutes. PKC saccharide hydrolysis at 2 hours can be seen in Figure 5.

PKC hydrolysis reaction for 4 hours yielded six peaks as follows: Peak I was mannobiose at a retention time of 5.7 minutes and a concentration of 1040 ppm. Peak II was mannotriose at a retention time of 6.67 minutes and a concentration of 170 ppm. Peak III was mannohexaose at a retention time of 11.31 minutes and concentration of 1330 ppm, the peak IV was predicted as mannotetraose at a retention time of 7.88 minutes, peak V was predicted as mannopentaose at retention time of 9.49 minutes and peak VI is predicted as maltoheptaose at retention time of 11.82 minutes (Figure 6).

PKC hydrolysis reaction for 6 hours yielded 5 peaks, namely: Peak I was mannobiose, having a retention time of 5.7 minutes and a concentration of 1090 ppm. Peak II was mannotriose, having a retention time of 6.67 minutes with a concentration of 190 ppm. Peak III was mannohexaose, having a retention time of 11.31 minutes and a concentration of 1630 ppm. Peak IV mannoheptulose as mannotetraose, and a retention time of 7.88 minutes, peak V was predicted as mannopentaose, at the retention time of 9.49 minutes (Figure 7).

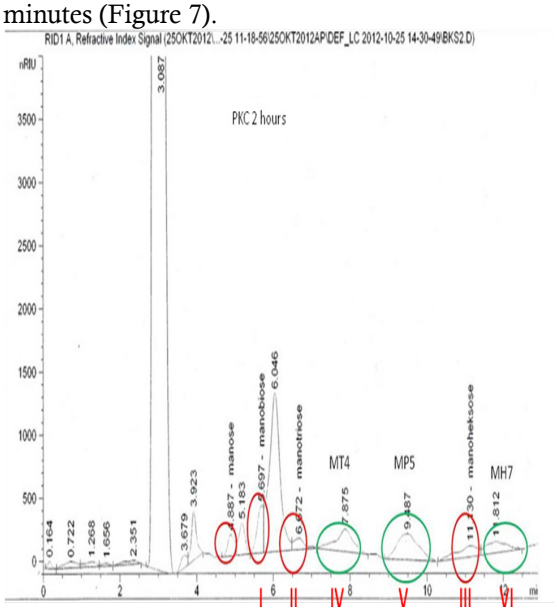

Figure 5. HPLC chromatograms of reaction between 0.5% PKC substrate with mannanase enzyme for 2 hours.

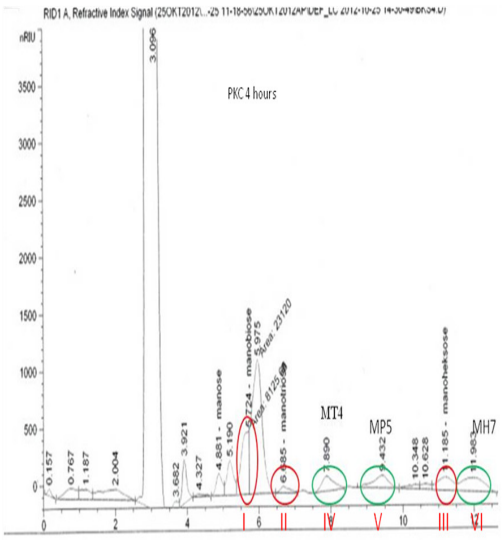

Figure 6. HPLC chromatogram of the reaction between 0.5% PKC substrate with mannanase enzyme for 4 hours.

PKC hydrolysis reaction for 8 hours yielded 5 peaks as follows: Peak I was mannobiose, having a retention time of 5.7 minutes at a concentration of 880 ppm. Peak II was mannotriose, having a retention time of 6.67 minutes and a concentration of 190 ppm. Peak III was mannohexaose, having a retention time of 11.31 minutes and a concentration of 1850 ppm. Peak IV was predicted as mannotetraose, at a retention time of 7.88 minutes, peak V was predicted as

mannopentaose, at a retention time of 9.49 minutes (Figure 8).

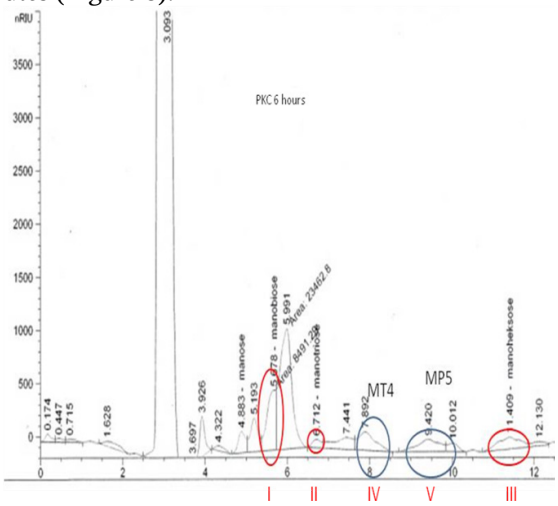

Figure 7. HPLC chromatograms of the reaction between 0.5% PKC substrate with mannanase enzyme for 6 hours.

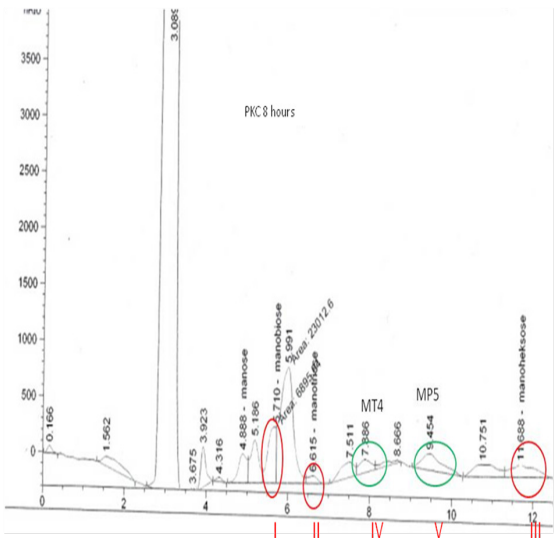

Figure 8. HPLC chromatograms of the reaction between 0.5% PKC substrate with mannanase enzyme for 8 hours.

The analysis of oligosaccharides products at 2, 4, 6, 8 hours (Figure 5,6,7,8) yielded mannobiose, mannotriose, and mannohexaose. PKC chromatograms generated from the hydrolysis reaction for 2 and 4 hours, in addition to oligosaccharides (mannobiose, mannotriose, and mannohexaose) also predicted the production of saccharides: mannotetraose, mannopentaose and mannoheptulose with a retention time of 7.88; 9.40; and 11.82 minutes. Respectively In the hydrolysis reaction for 6 and 8 hours it is predicted to yield oligosaccharides mannotetraose and mannopentaose at retention time of 7.88 and 9:42 minutes. Respectively All results are calculated based on standard production oligo mannan. The resolution of these sugars also depended very strongly on the composition of the mobile phase. Although the isocratic regime could resolve the monomeric sugars, it was impossible to resolve dimeric sugars. The dimmers were retained on the column but when the mobile phase was made more polar by increasing the water content, the dimers were eluted and resolved (Agblevor et al., 2004). *Streptomyces cyaenus* is native to Indonesia that can produce mannanase enzyme. Oligomannan hydrolysis of carbohydrates mannan can serve as a functional food which can prevent the growth of pathogenic bacteria in the human gut.

CONCLUSION

TLC analysis results showed that the mannanase enzyme from Streptomyces cyaenus could hydrolyze PKC mannan into oligosaccharides, that are disaccharide to heptasaccharide. HPLC analysis showed that hydrolysis mannan from PKC with mannanase enzymes Streptomyces cyaenus yielded oligosaccharides which were predicted as mannobiose, mannotriose, mannotetraose, mannopentaose, mannohexaose, and mannoheptulose. The optimum production of the enzyme manannase *Streotomyces cyaenu*s in Stirrer fermentor 2 liters was reached on the second days with the value of the enzyme activity of 1.7 mL / mL.

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