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THE SYNERGY OF RECOMBINANT XYLANOLYTIC ENZYME ON XYLAN HYDROLYSIS

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Abstract

Microbial xylanases or xylanolytic enzyme have received considerable attention over the last years owing to a multitude of possible applications. These enzymes have potential in the biodegradation of lignocellulosic biomass to fuels, chemicals, fruit juice, animal feed and in improving rumen digestion. More recently, the use of xylanases as bleaching agent in the pulp and paper industry has been suggested to replace of some of the chemicals presently used for this purpose. Such applications could have an important positive impact on the environment. The purpose of this research was determining the synergy of 3 recombinant xylanolytic enzymes (β-xylosidase, exo-xylanase and α-L-arabinofuranosidase) from recombinant *Eschericia coli* BL21 (DE-star) in xylan hydrolysis by analysis the reduction sugar product. Purified of recombinant xylanolytic enzyme β-xylosidase (Xyl), exo-xylanase (Exo-Xyl) and α-L-arabinofuranosidase (Abfa) with Ni-NTA resin. Seven samples of enzyme (each and enzyme mixture) used to hydrolyze xylan substrate (*oat-spelt xylan*). Analysis of hydrolysis product was done by HPLC. The xylanolytic activities of this enzyme before and after purification were 0,91 and 9,94 U/mL (Exo-Xyl); 1,65 and 14,2 U/mL (Xyl); 0,65 and 5,6 U/mL (Abfa). The xylosidase activity were 2,37 and 14,3 U/mL (Xyl); 1,49 and 10,5 U/mL (Exo-Xyl); 2,54 and 18,6 U/mL (Abfa). The highest hydrolysis product of xylan (xylose) shown in enzyme mixture of exo-xylanase and β-xylosidase was 1,084 mg/mL.

Keywords: α-L-arabinofuranosidase, β-xylosidase, exo-xylanase, xylan, xylose

1. Introduction

Various lignocellulosic agricultural wastes that contain hemicellulose, were rich xylan in rice straw, oil palm bunches, leaves, peanut, soybean leaves, cobs, stems and leaves of maize [1]. Hemicellulose is a group of polysaccharides that function binds the cellulose microfibril in cross-links. Hemicellulose is heteropoli-saccharide with low molecular weight consisting of xylan, arabinan, glucan and mannan. In general, compilers hemicellulose sugars are D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, D-galacturonic acid, 4-o-methylglucuronic acid, and glucuronic acid [2-4].

Microbial enzyme systems that can degrade hemicellulose has improved a lot the last twenty years. Enzymes that can degrade agricultural waste is hemicellulase enzymes (hydrolytic), one of which is the group xylanolytic enzyme (xylanase), which can hydrolyze xylan. Xylan complete hydrolysis requires the synergistic activity among other groups of xylanolytic enzymes, there are endo-β-xylanase, exo xylanase, β-xylosidase, α-L-arabinofuranosidases acetyl xylan esterases α-glucuronidase, ferulic esterase, ρ-coumaroic esterase [3].

Puspaningsih [5] isolated a xylanolytic gene cluster from the thermophilic bacterium *Geobacillus thermoleovorans* IT-08 (GenBank accession No. DQ345777, DQ387046 and DQ387047). The xylanolytic genes encoding the enzyme β-xylosidase (Xyl), α-L-arabinofuranosidase (Abfa) and exo-xylanase (Exo-Xyl) was successfully cloned on *Escherichia coli* DH5a host called pTP510. These three genes were also successfully separated and subcloned into the pET systems (pET-xyl, pET-abfa and pET-exoxyl) [6]. This study aimed to determine the synergism between the three recombinant xylanolytic enzymes (Xyl, Abfa and Exo-Xyl) in hydrolyzes xylan (oat-spelt xylan) with the analysis of reducing sugar products.

2. Methods

Material. Bacteria used as a source of recombinant xylanolytic enzyme is *E. coli* BL21 (DE-star) (pET-xyl, pET-abfa and pET-exoxyl). Oat-spelt xylan (Megazyme), pNP-X and pNP-A (Merck), ρ-nitrofenol, D-xylose, L-arabinose (Sigma).

Production and purification of recombinant xylanolytic enzymes. The cells were cultured by the method of Puspaningsih [5]. The cells were harvested by centrifugation at 10,000 ×*g* for 10 min at 4 °C and the cell pellet was suspended in 50 mM sodium phosphate buffer (pH 8.0) consisted of 250 mM NaCl and 5 mM imidazole and 5 mM β-mercaptoetanol (buffer A). The cells were then lysed by sonification. The sonificated sample was centrifuged at 10,000 ×*g* for 10 min at 4 °C to remove cell debris. The supernatant containing crude enzyme was subjected to heat treatment at 70 °C (Abfa) for 30 min and centrifugation to remove the heat-denatured proteins. The recombinant xylanolytic was purified by Ni-NTA affinity chromatography. Each 5 mL of recombinant xylanolitic enzymes supernatant (Abfa, Xyl, Exo-Xyl) was mixed with 1 mL of fresh Ni-NTA resin (Qiagen, Germany) and kept on ice (4 °C) under gentle shaking for 2 hours. The enzyme-bound resin was then packed into a column and eluted the enzyme with 0.5 mL buffer B (50 mM sodium phosphate buffer (pH 8.0), 250 mM NaCl, 100 mM imidazole (for Abfa) and 50 mM imidazole (for Xyl and Exo-Xyl) and 1 mM β-mercaptoetanol). Purified determined by SDS-PAGE.

Enzyme assays. The xylanolytic activity was determined by the method described by Miller [7]. Xylanolytic assays were performed by incubating 100 µL of oat-spelt xylan (1%, w/v) suspension in 100 mM phosphate citrate buffer (PC) (pH 6.0) with 100 µL of enzyme solution at 70 °C (Abfa) and 50 °C (Xyl, Exo-xyl) for 10 min. Reactions stopped by the addition of either 600 µL of 3,5-dinitrosalicylic acid (DNS) reagent. Reducing sugars were measured at 550 nm with D-xylose and L-arabinose as a standard. The specific enzyme activity was determined by the method of Puspaningsih [5]. The β-xylosidase activity was quantitated using the chromophoric substrate ρ-nitrophenyl-β-D-xylopyranoside (pNP-X) for Xyl/Exo-Xyl and ρ-nitrophenyl-α-L-arabinofuranoside (pNP-A) for Abfa. ρ-Nitrophenol released from synthetic substrates was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme which produced reducing sugar equivalent to 1 µmol of xylose or release 1 µmol of ρ-nitrophenol per min under the conditions described above.

Xylan hydrolysis with recombinant xylanolytic enzymes. Xylan substrate samples (oat-spelt xylan) 1% (w/v) reconstituted with PC buffer pH 6.0 coupled with each enzyme xylanolytic (Abfa, Xyl, Exo-Xyl) and mixed enzyme (Abfa+Xyl, Abfa+Exo-Xyl, Xyl+Exo-Xyl and Abfa+Xyl+Exo-Xyl). All samples with a ratio of substrate : enzyme (1 : 1) v/v, hydrolyzed at a temperature of 70 °C (Abfa) and 50 °C (Xyl and Exo-Xyl) each for one hour. Each enzyme or enzyme mixture used at 5 U/mL of all samples. The supernatant of hydrolysis results were used as sample for HPLC analysis.

Synergism analysis of the hydrolysis results by HPLC. The synergism activity of the recombinant xylanolytic enzyme (Abfa, Xyl, Exo-Xyl) is shown at hydrolysis results were analyzed by HPLC (Waters systems), refractive index detector (Waters 2414), which used carbohydrate column (Biorad-Aminex HPX-87P), water solvent, flow rate 1 mL/min, injection volume 10 µL, column temperature 85 °C, pressure 700-1100 psi, analysis time 100 min. As a standard compound used in xylose and arabinose (Sigma). Each sample was injected into the HPLC, first filtered with 0.2 µm membrane filter.

3. Results and Discussion

The crude extract of the three recombinant xylanolytic enzymes (Abfa, Xyl and Exo-Xyl) purified by affinity chromatography using Ni-NTA resin. The result of enzyme activity before and after purification is shown in Table 1. The analysis of enzyme purity by SDS-PAGE. The molecular weight of Xyl and Abfa shown at 57 kDa, while Exo-Xyl at 80 kDa. These results are shown in Figure 1. Chromatogram profile of hydrolysis products with oat-spelt xylan xylanolitic recombinant enzyme shown in the Figure 2-9.

Recombinant xylanolytic enzyme can be produced by the pET-xyl, pET-abfa and pET-exoxyl. Recombinant xylanolytic enzyme produced the enzyme β-xilosidase (Xyl), α-L-arabinofuranosidase (Abfa) and Exo-xylanase (Exo-Xyl). Abfa and Xyl synergistically hydrolyze xylan (oat-spelt xylan) to xylose, as its main product in addition to arabinose and xylobiose [4]. Recombinant xylanolytic enzyme was produced and purified, the next will be tested and analyzed their activity and then proved the existence of synergistic

Table 1. Data of Recombinant Xylanolytic Enzyme Activity

Enzyme	Enzyme Assay with DNS method (U/mL)		Enzyme Assay with pNP-X/pNP-A (U/mL)	
	Before	After	Before	After
Abfa	0.65	5.6	2.54	18.6
Xyl	1.65	14.2	2.37	14.3
Exo-Xyl	0.91	9.94	1.49	10.5

mechanisms of the three recombinant xylanolytic enzymes in xylan hydrolyze.

Recombinant xylanolytic enzyme (Abfa, Xyl and Exo-Xyl) is an intracellular enzyme that is necessary for lysed by sonification of bacterial cell wall, because in previous research [8], activity was observed at the highest intracellular xylanolytic enzymes. Third recombinant xylanolytic enzyme (Abfa, Xyl and Exo-Xyl) purified by affinity chromatography with Ni-NTA resin (Ni-NTA His-Bind Resin). This resin is used for rapid purification of one stage in the protein-containing sequen His-Tag with metal chelating chromatography. Mixed of Ni-NTA resin for the aim of treating an enzyme sequen His-Tag that will be bound by the cations Ni^{2+} and trapped in the Ni-NTA resin such. After unbound protein is released, the target protein will be bound back with eluting imidazole, this happens when washing with buffer A and B. Purification with based on this resin that will occur between 6-10 histidine affinity with trapping of metal ions Ni^{2+}. Metal will chelating by covalently reactive strong groups. In the Ni-NTA resin, used nitriloacetic acid (NTA) as a chelating which has four sides that can interact with metal ions. Minimize the release of chemical NTA metal ions during the purification process, and it still works with the addition of β-mercaptoethanol to reduce disulfide bonds [9].

In the process of purification, of washing buffer B were analyzed by SDS-PAGE. The purified enzyme Abfa, Xyl and Exo-Xyl shown by the presence of one band on SDS-PAGE electroforetogram. Xyl and Abfa enzymes have similar molecular weight of approximately 57 kDa (Figure 1) that corresponds to the number of pairs of nucleotides. Rohman *et al*., [10] also reported the molecular weight of Xyl from pET-xyl for 57 kDa. This indicated that the native enzyme in the form of structured polymers are likely homopolymers. Some data of Abfa molecular weight derived from bacteria that is 59 kDa in *Geobacillus caldoxylolyticus* TK4 [11]; 65 kDa in *Bacillus subtilis* [12]; 61 kDa in *Bacteroides xylanolyticus* [13]; 60 kDa in *Streptomyces diastaicus*-C2 [14]. Exo-Xyl has a molecular weight of 80 kDa (Figure 1). Exo-Xyl and Xyl has a low homology at the Gene Bank, this shows the character of the two genes encoding novel enzymes.

Before and after purification, the three recombinant xylanolytic enzymes are able to hydrolyze oat-spelt xylan with sequentially xylanolytic activity for Abfa (0.65 and 5.6 U/mL), Xyl (1.65 and 14.2 U/mL), Exo-Xyl (0.91 and 9.94 U/mL), Increased activity of extracts of recombinant xylanolitic is roughly eight times (Abfa and Xyl) and 10 times (Exo-Xyl). On the assay of specific enzyme activity with the substrate ρ-nitrophenyl-β-D-xilopiranosida (pNP-X) is also seen increased xylosidase activity between before and after the refinery is 2.37 to 14.3 U/mL (Xyl) and 1.49 become 10.5 U/mL (Exo-Xyl). Increased arabinofuranosidase activity was also demonstrated by enzyme activity assay with specific substrate ρ-nitrophenyl-α-L-arabinofuranosidase (pNP-A) is from2.54 to 18.6 U/mL (Abfa). Increased xylosidase/

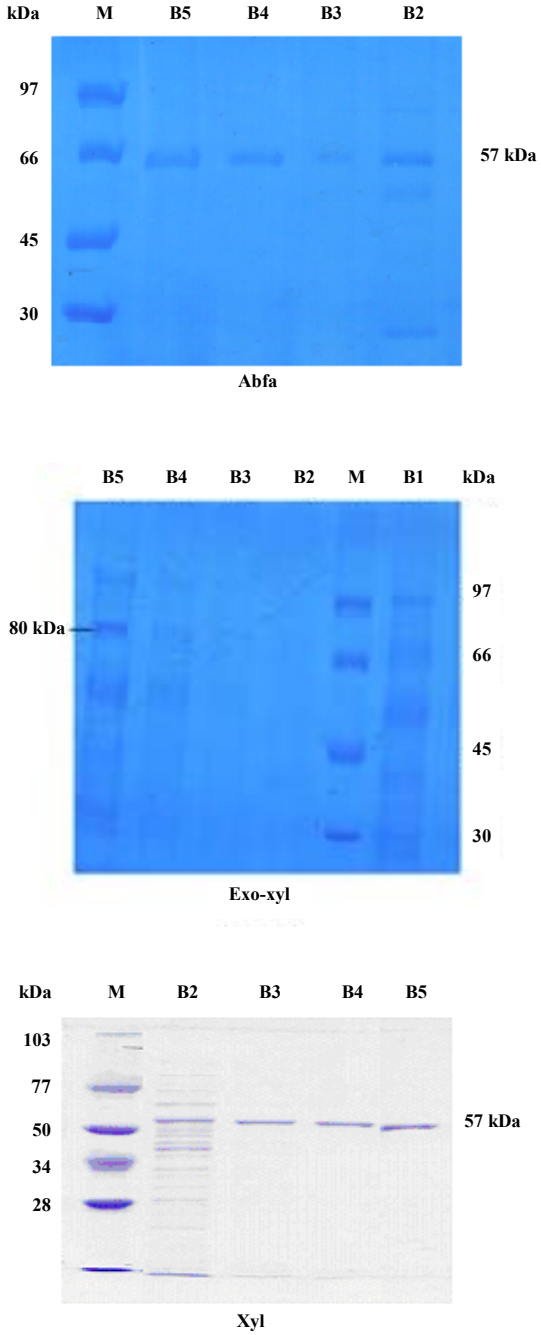

Figure 1. Electroforetogram of the Purified Enzyme by SDS-PAGE. β-xilosidase (Xyl), α-L-arabinofuranosidase (Abfa), Exo-xylanase (Exo-Xyl) : M = Marker, B1 = Leaching into the Buffer B-1, B2 = Leaching into the Buffer B-2, B3 = Leaching into the Buffer B-3, B4 = Leaching into the Buffer B-4, B5 = Leaching into the Buffer B-5

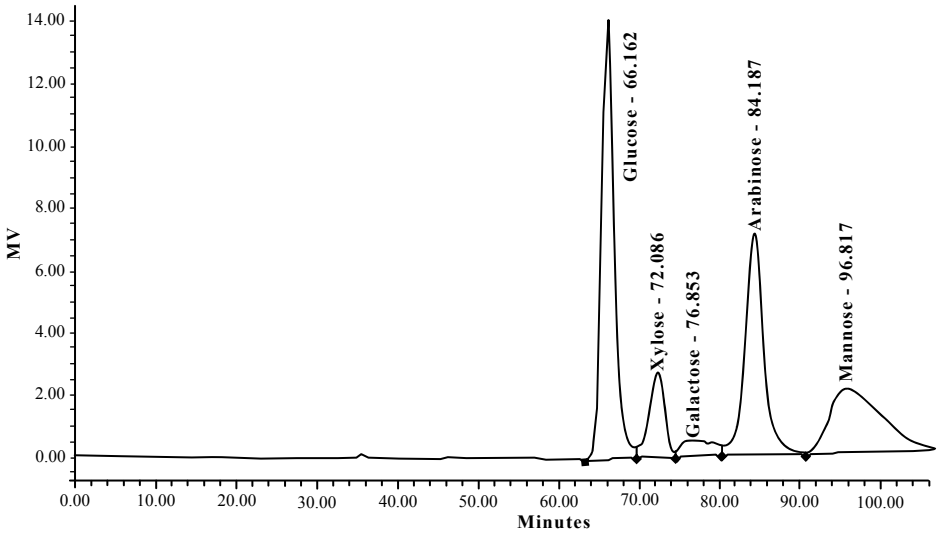

Figure 2. HPLC Chromatogram Standards : Xylose (RT = 72.086), Arabinose (RT 84.187)

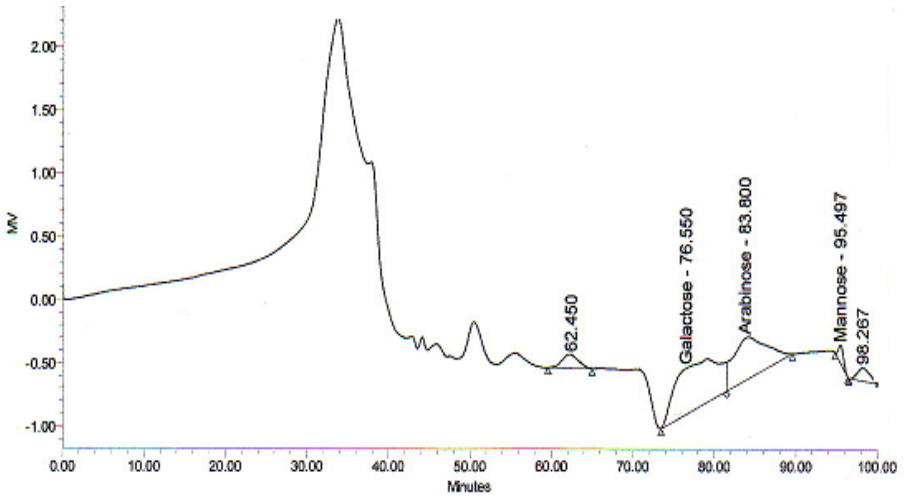

Figure 3. HPLC Chromatogram of Enzyme Hydrolysis Products Abfa : Arabinose (RT = 83.8)

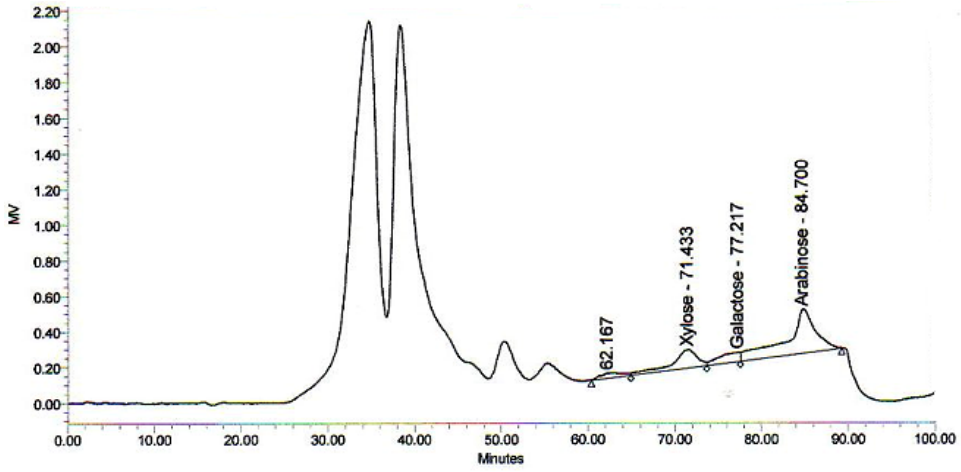

Figure 4. HPLC Chromatogram of Enzyme Hydrolysis Products Exo-xyl : Xylose (RT = 71.433), Arabinose (RT = 84.7)

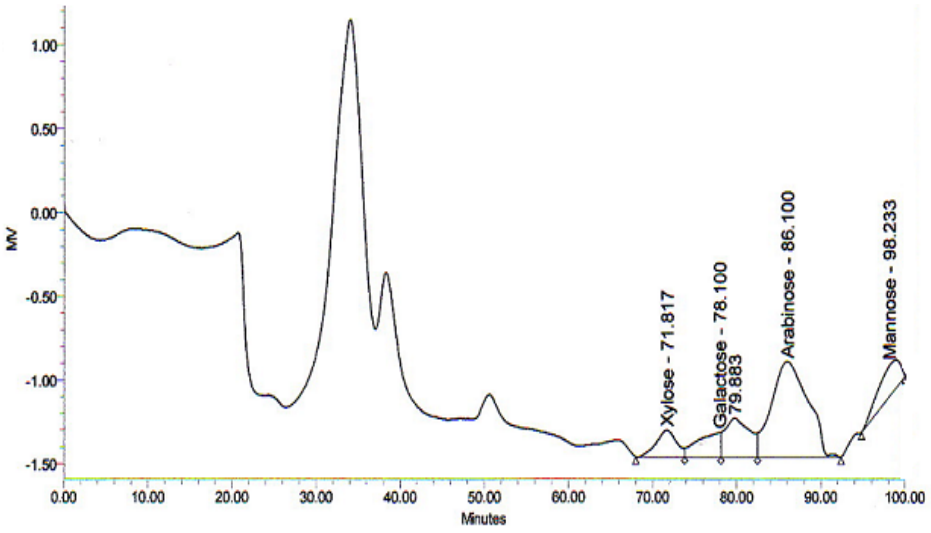

Figure 5. HPLC Chromatogram of Enzyme Hydrolysis Products Xyl : Xylose (RT = 71.817), Arabinose (86.1)

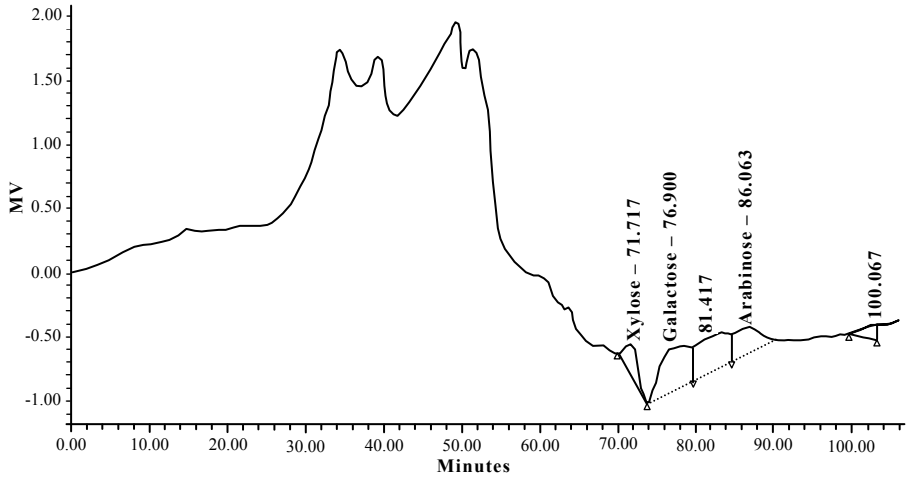

Figure 6. HPLC Chromatogram of Hydrolysis Products of Enzyme Mixture Abfa and Exo-xyl : Xylose (RT = 71.717), Arabinose (RT = 86.083)

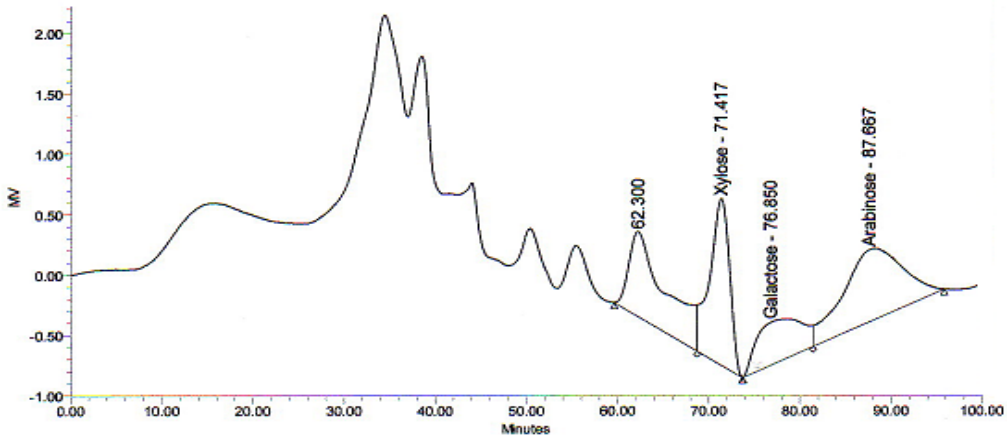

Figure 7. HPLC Chromatogram of Hydrolysis Products of Enzyme Mixture Abfa and Xyl : Xylose (RT = 71.417), Arabinose

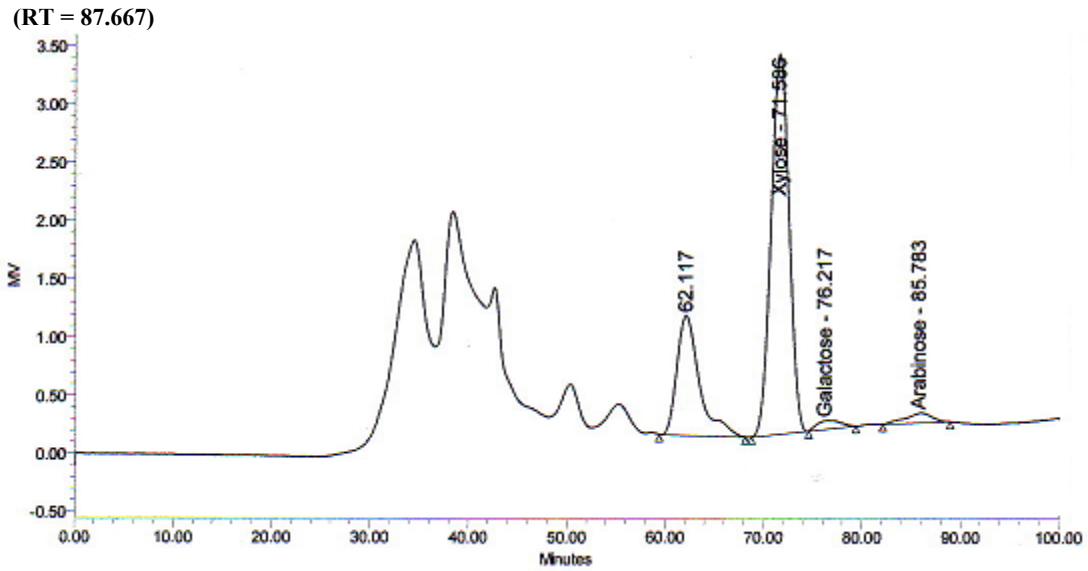

Figure 8. HPLC Chromatogram of Hydrolysis Products of Enzyme Mixture of Exo-xyl and Xyl : Xylose (RT = 71.586), Arabinose (RT = 85.783)

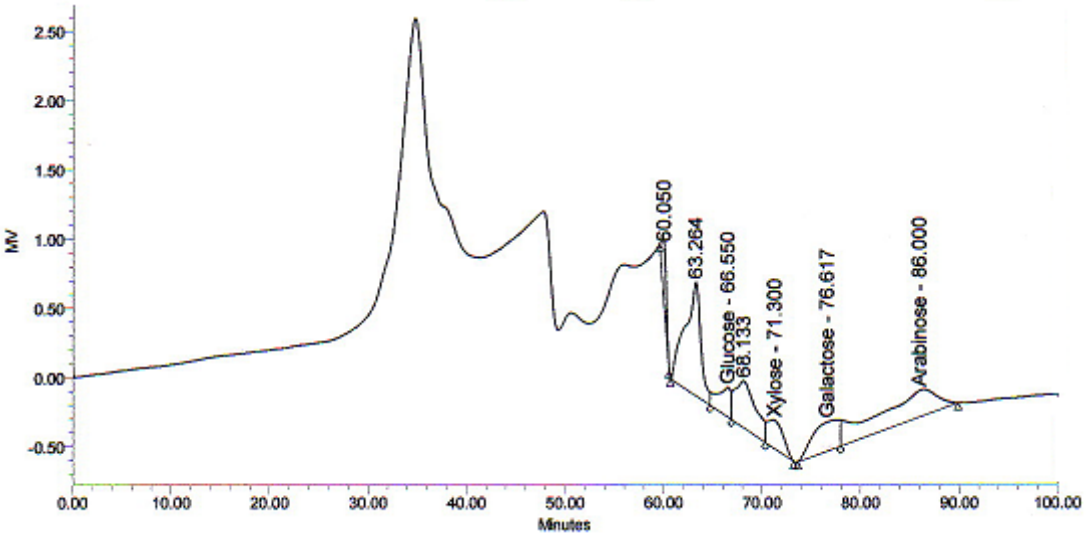

Figure 9. HPLC Chromatogram of Hydrolysis Products of Enzyme Mixture Abfa, Exo-xyl and Xyl : Xylose (RT = 71.3), Arabinose (RT = 86.0)

arabinosifuranosidase activity of Abfa, Xyl and Exo-Xyl to extract roughness that is 7 times (Abfa and Exo-Xyl) and 6 times (Xyl).

Differences third xylanolitic activity of recombinant enzymes that different due to hydrolyze ability of each enzyme to xylan substrate, so the amount of xylose produced is also different. Abfa hydrolyze non-reducing end of the branch between the bond of α-L-arabinofuranoside with various polysaccharides that contain arabinofuranose. Exo-Xyl hydrolyze xylose polymer chains (xylan) at the end of reduction, resulting in a xylose as the main product and a number of short-chain oligosaccharides. Xyl hydrolyze xylooligosaccharide from non-reducing end to produce xylose. In the HPLC chromatogram around the retention time (RT) 70 min appeared peak of xylose, arabinose, while peak appeared at around RT 80-90 minutes (Figure 2-9). Peak at RT 30-50 minutes indicated of xylooligosaccharide peak. Tuncer *et al*. [15] and Rahman *et al*. [16] also reported on the results of hydrolysis xylooligosaccharide emergence xylan.

Amount of xylose and arabinose which produced by the synergism Abfa and Xyl more than enzyme synergism of Abfa and Exo-Xyl is shown in figure 6 and 7. In

contrast, peak on the chromatogram allegedly xylooligosaccharide of synergism Abfa and Xyl smaller than synergism of Abfa and Exo-Xyl. Synergistic working of Abfa, Xyl and Exo- Xyl primary produce xylose and arabinose. This is consistent with several reports of research results in a synergistic hydrolysis of another group of xylanolytic enzymes, where contains of endo xylanase, β-xylosidase and α-L-arabinofuranosidase of *Rhizomcor pusillus* HHT1 with oat-spelt xylan substrate was produce xylose and arabinose [16]. β-Xylosidase and α-β-L-arabinofuranosidase synergistically produced by extracellular *Thermomonospora fusca* BD25 can also hydrolyze 28-58% oat-spelt xylan, where hydrolysis results mainly xylose and arabinose [14]. Arabinoxylan hydrolyzing synergistically by endo xylanase, β-xylosidase and α-L-arabinofuranosidase from *Aspergillus oryzae* HL15 also yields xylose and arabinose as main products [17]. Synergistic activity was also demonstrated by the enzyme endo xylanase, β-xylosidase and α-L-arabinofuranosidase production Novo [18].

In the analysis of hydrolysis products by HPLC seen the greatest amount of xylose was obtained from a combination of Exo-xyl and Xyl Exo-xyl and Xyl (1.084 mg/mL). This is due to the synergism both enzyme, where Exo-xyl will cut the main chain of xylan first became shorter xylooligosaccharide and less xylose chain. Xyl will cut the remainder xylooligosaccharide short chain into xylose, so that the total number of xylose to be larger (Figure 8).

4. Conclusion

The recombinant xylanolytic enzymes (Abfa, Xyl and Exo-Xyl) produced from *E.coli* BL21 (DE-star). Increase of xylanolytic activity of Abfa, Xyl and Exo-Xyl to the roughness extract that is 8x (Abfa), 8x (Xyl), 10x (Exo-Xyl). Increased xylosidase/arabinosidase activity of Abfa, Xyl and Exo-Xyl to roughness extract that is 7x (Abfa), 6x (Xyl), 7x (Exo-Xyl). Xylan hydrolysis product (xylose) is indicated by the largest enzyme mixture the Exo-xyl and Xyl amounted to 1.084 mg/mL. Suggestions for further research are needed variations and optimations concentration of recombinant xylanolytic enzymes for hydrolysis natural hemicelluloses from lignocelluloses agriculture waste to resulting big amount xylan hydrolysis product (xylose, arabinose and xylooligosaccharide).

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