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CELLULASE AND XYLANASE IMMOBILIZED ON CHITOSAN MAGNETIC PARTICLES FOR APPLICATION IN COCONUT HUSK HYDROLYSIS

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ABSTRACT

Cellulase and xylanase were covalently immobilized on chitosan magnetic particles. They were employed as catalysts to produce reducing sugar from coconut husk individually and simultaneously. Fourier Transform Infrared Spectroscopy (FT-IR) and Bradford's protein analysis confirmed that the enzymes were covalently immobilized on the support. Cellulase from *Aspergillus niger* and *Trichoderma reesei* immobilized on chitosan magnetic microparticles yielded 0.352 g/L and 0.316 g/L of reducing sugar respectively, while immobilized xylanase from *Trichoderma longibachterium* yielded 0.432 g/L. The simultaneous use of cellulase and xylanase produced 0.8034 g/L of sugar, and the addition of glutaraldehyde as a cross-linking agent increased the amount of reducing sugar. Enzyme could maintain its activity at 91% for up to five cycles. Using nanosized particles resulted in a sugar yield of 0.49 g/l and 54.7 % of its activity maintained after five cycles.

Keywords: Cellulase; Chitosan magnetic particles; Coconut husk; Immobilization; Xylanase

1. INTRODUCTION

Lignocellulose can be converted to reducing sugar for biofuels since it consists of cellulose, hemicellulose and lignin (Alftrén & Hobley, 2014). Coconut husk, which is an abundant lignocellulosic substrate in Indonesia, is a valuable substance as a future source of energy. However, the complex structure of lignocellulosic substrate is a major obstacle in its degradation process. The most widely known method to degrade lignocellulose is by chemical or physical pre-treatment, followed by hydrolysis. Enzymatic hydrolysis has been proven to improve the yields of sugar produced, has high selectivity, low energy costs and mild operating conditions (Zang et al., 2014). However, the expense of the enzyme and its specific activity for particular substrates are drawbacks in the application on a complex substrate such as lignocellulose (Han et al., 2018; Song et al., 2016).

The combination of xylanase and cellulase has numerous advantages; for example, xylanase can degrade the xylan which is attached to the cellulose surface and block the access of cellulase to cellulose (Mardawati et al., 2018). Since xylose is generated from xylan hydrolysis, the addition

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of xylanase not only makes the cellulose accessible to cellulase but at the same time also generates more sugar (Jia et al., 2015). The use of multiple enzymes simultaneously can also be achieved by utilizing crude enzyme. Crude enzyme from *A. niger* contains numerous enzymes, including cellulases and xylanases. Furthermore, utilizing crude enzyme will make the overall process more economical. The other strategy for employing the enzymes more economically is by reusing them in the hydrolysis reaction through immobilization. This also improves their stability and adaptability in various conditions, such as temperature and pH (Han et al., 2018).

Covalent binding is commonly used as an immobilization technique, since it restricts leaching of the enzyme because of the stable covalent bond with the support (Cheng-Kang & Au-Duong, 2018). Cellulase has been shown to be able to covalently immobilize on the surface of carrageenan (Yuan et al., 2016); polystyrene, polypropylene and polyethylene (Ahirwar et al., 2017); and chitosan (El-Ghaffar & Hashem, 2010; Manrich et al., 2010). In addition, xylanase has been shown to covalently immobilize on chitosan (Manrich et al., 2010); alginate beads (Jampala et al., 2017); and magnetic nanoparticles (Soozanipour et al., 2015; Shahrestani et al., 2016). As support for enzyme immobilization, chitosan, a natural polymeric support, has previously been reported to have the ability to support cellulase and xylanase since it has various functional group, and is inexpensive, inert, hydrophilic and biocompatible (Osuna et al., 2012). Micro-sized and nanosized chitosan magnetic particles were used since their larger surface area will resolve the mass transfer resistance between the immobilized enzyme and the substrate. Chitosan will act as a coating on the magnetic particles as it delivers a functional group for covalent binding. To enhance the binding, a cross-linking agent, glutaral dialdehyde (GDA), was also supplemented (Sojitra et al., 2017). Magnetic separation from the complex samples can be performed easily using an external magnetic field (Chen et al., 2014).

The objective of this work is to study the utilization of cellulase and xylanase immobilized on chitosan magnetic particles in coconut husk hydrolysis and the simultaneous effect of using a mixture of enzymes.

2. METHODS

2.1. Materials

Coconut husk, which was obtained from North Sulawesi, Indonesia, was dried and screened until it reached 100−120 mesh. It was then pre-treated using NaOH 1% (w/v) at 80°C for 16 h. Xylanase from *T longibrachiatum* cellulase from *T reesei*, cellulase from *A niger*, magnetic particles (Fe_3O_4) ±5 μm diameter size, bovine serum albumin (BSA), chitosan low molecular weight, Iron (II) chloride tetrahydrate, Iron (III) chloride hexahydrate, ammonium hydroxide (NH_4OH, 28 wt%), acetic acid and 3,5-dinitrosalicylic acid were purchased from Sigma-Aldrich. Glutaral dialdehyde and other chemicals were purchased from Merck.

2.2. Preparation of Chitosan Magnetic Microparticles

Chitosan magnetic microparticles were prepared using the method employed by Biró et al. (2008) and Safarik et al. (2012) with some modification. Four grams of chitosan and 8 grams of magnetite particles were dissolved in 200 mL of 0.2 M acetic acid. After thorough mixing, an excess of 1 M sodium hydroxide was added to convert the solubilized chitosan into insoluble form. The chitosan containing entrapped magnetite microparticles was freeze-dried and washed with water several times.

2.3. Preparation of Chitosan Magnetic Nanoparticles

Chitosan magnetic nanoparticles were prepared using the method developed by Zang et al. (2014). Iron (II) chloride tetrahydrate and Iron (III) chloride hexahydrate was reacted together in 50 mL of deionized water, followed by the addition of NH_4OH to form magnetic nanoparticles. The reaction was carried out at 80°C for 2 h with N_2 protection. The magnetic

nanoparticles were supplemented by chitosan solution and NaOH was added to complete their preparation.

2.4. Immobilization

The immobilization process used the method of El-Ghaffar & Hashem (2010), with some modification. Magnetic chitosan 0.1 g was added to the enzyme solution (6 mg protein). The immobilization reaction was carried out for 24 h at 25°C in a shaking incubator. The precipitates and the unbound enzyme were eliminated by washing them with a phosphate buffer and by magnetic separation. The liquid was then analyzed to determine the concentration of the unbound protein. The immobilized enzymes were stored at 4°C until their use. For immobilization on chitosan with a glutaric dialdehyde supporter, chitosan magnetic was added to a glutaric dialdehyde solution 2.5% (v/v) and kept in a shaking incubator for 4 h at 25°C before the enzyme was added.

2.5. Enzymatic Hydrolysis

0.1 g immobilized enzyme was added to 1 g pre-treated coconut husk in a 20 mL pH 7 phosphate buffer, and the hydrolysis process was carried out for 48 h at 125 rpm and 60°C. The mixture was centrifuged (10,000 rpm, 4°C) and the resulting liquid was analyzed for its reducing sugar content.

2.6. Reusability Study

0.1 g immobilized enzyme was utilized to hydrolyze 1 g pre-treated coconut husk in 20 mL pH 7 phosphate buffer at 60°C for 48 h. After separation by a magnet, the immobilized cellulase was washed with the buffer, and then suspended again in a fresh reaction mixture. The reusability study was repeated for five cycles. The glucose productivity during the 48 h was used to evaluate the reusability of the enzyme.

2.7. Analytical Method

The morphology and size of the chitosan microparticles and nanoparticles were examined using SEM (Scanning Electron Microscopy) (inspect s50, Netherlands) and TEM (Transmission Electron Microscopy) (JEOL JEM 1400), respectively. The FT-IR spectra were measured using an FT-IR spectrometer (Thermo Scientific, US), while the protein content was analyzed by the Bradford method using bovine serum albumin as standard (Bradford, 1976). The reducing sugar was analyzed by the DNS (Dinitro salicylic acid) method to ascertain its concentration (Miller, 1959).

2.8. Statistical Analysis

Analysis of Variance (ANOVA) was employed to verify the significance of temperature, mixing speed and enzyme concentration in immobilization yield and to study the significance of enzyme combination in reducing sugar yield, using Minitab 16 (Minitab Inc., ITS Surabaya, Indonesia).

3. RESULTS AND DISCUSSION

3.1. Morphology and Size of Support

Three kinds of support were prepared for the immobilization, namely chitosan macroparticles, chitosan magnetic microparticles and chitosan magnetic nanoparticles. The chitosan macroparticle had a >1000 µm diameter size, and no special preparation was involved in creating them. The chitosan magnetic microparticles were prepared by utilizing the chitosan characteristic of being soluble in acidic solution. The commercial magnetic particles were added to this solution. After vigorous stirring, NaOH was added to transform the chitosan into insoluble magnetic particles entrapped within it, as shown schematically in Figure 1 (Pospiskova & Safarik, 2013). From the SEM micrograph in Figure 2a, the chitosan magnetic microparticles had an irregular shape and were <500 µm in diameter size. The synthesis of the

chitosan nanoparticles followed almost the same method and principle as that of the microparticles. The only difference was that the magnetic Fe_3O_4 particles were made using $FeCl_3$ and $FeCl_2$, and amount of chitosan added was less than that of the microparticles (Zang et al., 2014). Figure 2b shows a TEM micrograph of the nanoparticles. The chitosan magnetic nanoparticles had a uniform spherical shape and were <50 nm in size.

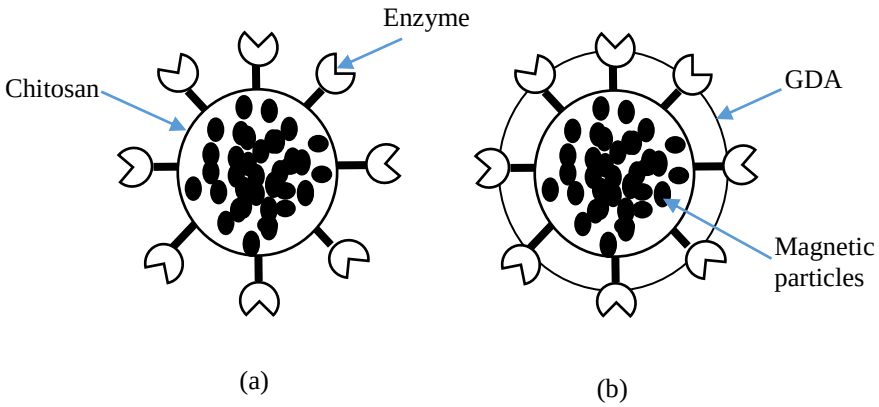

Figure 1 Illustration of: (a) enzyme covalently immobilized on chitosan magnetic particles; and (b) enzyme covalently bonded and crosslinked to chitosan magnetic particles + glutaral dialdehyde (GDA)

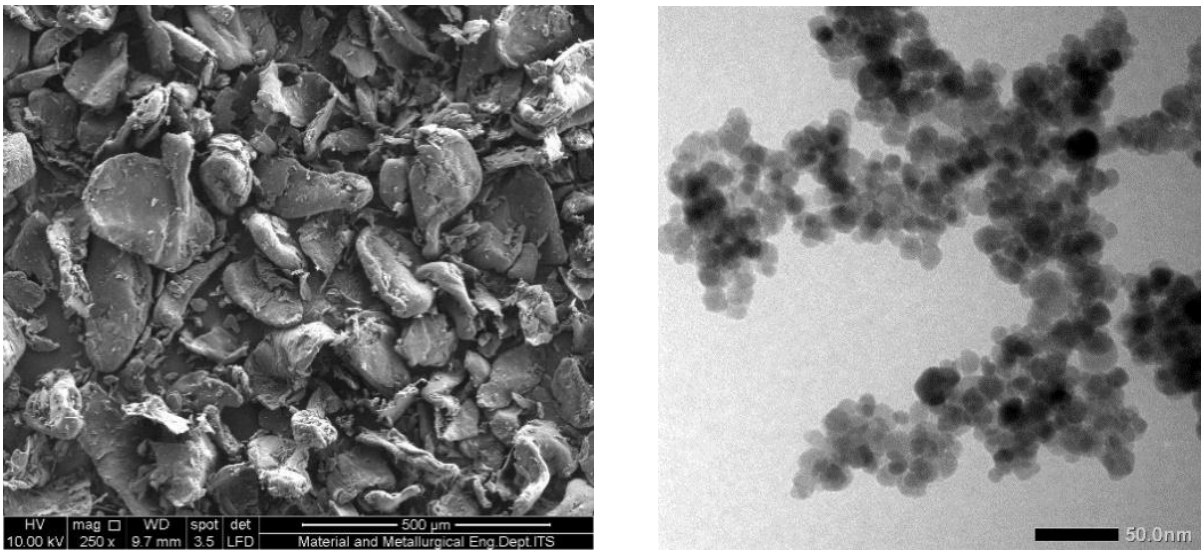

Figure 2 (a) SEM micrograph of chitosan magnetic microparticles with 500µm scale bar; and (b)TEM micrograph of chitosan magnetic nanoparticles with 50 nm scale bar

3.2. Immobilization

Immobilization yield was affected by temperature, mixing speed and enzyme concentration; the effect of these factors was also studied. Neither temperature nor mixing speed had a significant effect ($p > 0.05$) on the yield, but the concentration of added enzyme solution had a significant effect ($p < 0.05$) (data not shown). It was determined that 6 mg of protein was the optimum concentration of enzyme to add to the support. Cellulase from *A niger* and *T reesei* was able to retain a 100% immobilization yield, while xylanase from *T longibrachiatum* retained 88%, as determined by the Bradford analysis and shown in Table 1. The immobilization yield of xylanase was lower than that of cellulose, which may be due to the lack of amino groups in chitosan, and which led to incomplete immobilization (Sánchez-Ramírez et al., 2017; Xu et al.,

2011). The amino groups could be calculated by the Ninhydrin assay and NMR (Mahon et al., 2015).

Table 1 Immobilization enzyme yield

Enzyme	Initial protein (mg)	Immobilized (mg)	% immobilization yield
Cellulase from *T reesei*	6	6	100%
Cellulase from *A niger*	6	6	100%
Xylanase from *T longibrachiatum*	6	5.28	88%
Cellulase from *T reesei* with GDA	6	6	100%
Cellulase from *A niger* with GDA	6	6	100%
Xylanase from *T longibrachiatum* with GDA	6	6	100%

However, the procedure was not performed because all the enzymes reached 100% coupling with the addition of GDA, which was shown to provide more functional groups available for enzyme binding (Yuan et al., 2016). The other possible reason was the incompatibility of the pH solution and the solubility of xylanase and chitosan, which meant the isoelectric point of the immobilization process was not optimal (Jia et al., 2015).

From previous research conducted by El-Ghaffar & Hashem (2010), the possible binding between enzymes and support is illustrated in Figure 1a. Figure 1b shows a combination between a covalent bond and the cross-linking method using GDA. It can be seen from the figure that amino groups of chitosan were bonded directly with the carboxylic terminal residue in the enzymes. This reaction was also confirmed by Fourier Transform Infrared (FT-IR) spectra, as shown in Figure 3. As seen in the figures, there were significant changes in the peak in wavelength 3272.53 cm^{-1}, that was the characteristic of the amino group (N-H); wavelength 1632.67 cm^{-1}, which was the characteristic of C=O; and wavelength 1080 $cm^{-1,}$ that was the characteristic of aliphatic amide C-N (Mistry, 2009). This indicates that enzyme has been immobilized on the chitosan. The FT-IR spectra of enzyme immobilized on chitosan-GDA can be seen in Figure 3. This figure shows that there are also some significant changes in the peak in wavelength 3267.54 cm^{-1} (N-H); 1629.15 cm^{-1} (C=O); 1027.66 cm^{-1} (C-N), which is same as cellulase-chitosan; and wavelength 1376.0 cm^{-1} (C-O), indicating that GDA functioned as a cross-linking agent and spacer arm in the covalent binding.

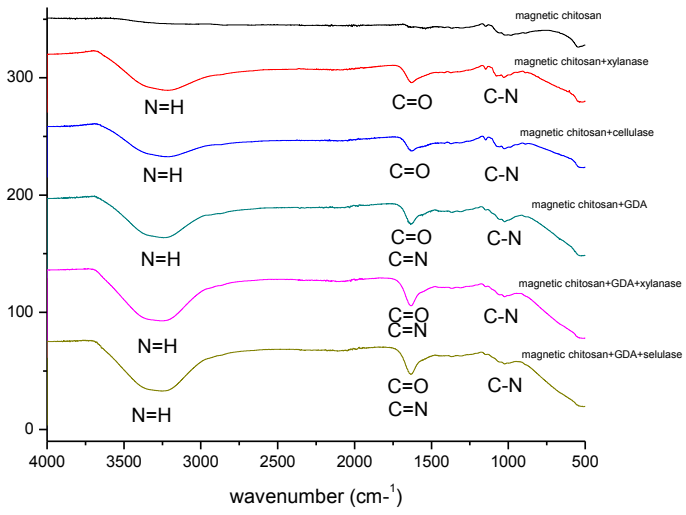

Figure 3 FT-IR spectra of support and immobilized enzymes

3.3. Effect of Support Particle Size

Table 2 summarizes the performance comparison between different-sized chitosan. Although the chitosan microparticles and nanoparticles had fewer coupled enzymes than the macroparticles, they yielded almost the same amount of sugar.

Table 2 Yield of reducing sugar from different sizes of chitosan

Immobilized enzyme	Enzyme coupling (mg)	Specific activity	Yield*
Chitosan macro+ crude enzyme	11.84	0.21	0.035
Chitosan macro+ cellulase	38.35	1.94	0.040
Chitosan micro+ cellulase	5.50	1.94	0.040
Chitosan nano+ cellulase	4	1.94	0.037

*Yield: gr reducing sugar/gr (cellulose+hemicellulose)

Furthermore, the nanoparticles had less coupled enzymes than the microparticles, but yielded nearly the same amount of sugar. The data revealed that mass transfer resistance was reduced by utilizing smaller support particles. These had a larger surface area and numerous active sites available for the enzyme molecules to be fixed (Biró et al., 2008).

3.4. Utilization of Crude Enzyme

Prior to determining the use of the combination of commercial cellulase and xylanase, crude enzyme from *A. niger* was applied in the hydrolysis of the coconut husk in its free and immobilized form. Crude enzyme has unique characteristics as it contains several enzymes, including cellulase and xylanase, as evidenced from the enzyme assay for both the enzymes (data not shown). Table 3 shows the yield of reducing sugar produced from coconut husk using various types of enzyme. The yield produced by either free crude enzyme or free commercial cellulase was higher than that of the immobilized form. This is because the solid coconut husk and chitosan support restricted the mass transfer of enzyme to the substrate. However, the decreasing yield of free crude and free commercial enzyme after immobilization was almost the same, at around 80%. This shows that the crude enzyme produced in our laboratory also has good potential to be used in immobilized enzyme. Table 3 also shows the yield using enzyme immobilized on chitosan GDA, at around 0.057 for immobilized crude enzyme and 0.089 for the immobilized commercial type. These data also confirm the previous discussion, that either crude or commercial enzyme immobilized on chitosan GDA produced a larger yield than immobilization using chitosan only. Although the yield of sugar using immobilized enzyme is still under the free enzymes immobilization will be advantageous if it is used many times in a continuous process, so that productivity will be increased. The utilization of crude enzyme immobilized on chitosan had many deficiencies. Besides its low sugar yield production, the type of enzyme which was immobilized on the support could not be controlled; the covalent binding process was not able to be confirmed due to the numerous enzymes contained within it; and the inhibitor contained in the crude enzyme also hindered the immobilization process.

Table 3 Yield of reducing sugar from free enzyme and immobilized enzyme on the macroparticles

Enzyme Type	Yield*
Free crude enzyme	0.176
Crude enzyme immobilized on chitosan	0.035
Crude enzyme immobilized on chitosan-GDA	0.057
Commercial cellulase	0.320
Commercial cellulase immobilized on chitosan	0.040
Commercial cellulase immobilized on chitosan-GDA	0.089

*Yield: gr reducing sugar/gr (cellulose+hemicellulose)

3.5. Hydrolysis of Immobilized Enzyme on Microparticles and Nanoparticles

The immobilized enzymes were applied to the coconut husk hydrolysis individually and simultaneously at different ratio. 6 mg of protein was determined as the amount of enzyme to be applied to the hydrolysis individually and simultaneously. The w/w ratio of the two types of cellulase was determined to be 1:2, as this showed the best performance (data not shown). It has been previously shown that combining two types of cellulase from different sources enhances sugar yield, since the cellulases complement each other in terms of hydrolase (Hamzah et al., 2018). Cellulase from *T reesei* yielded more exoglucanase and endoglucanase and lacked β-glucosidase. This shortage was complemented by cellulase from *A niger* (Stockton et al., 1991). Table 4 shows the code and ratio of enzymes applied.

Table 4 Ratio of immobilized enzyme on chitosan magnetic microparticles based on the amount of protein

Code	Immobilized Cellulase from *T reesei* (mg)	Immobilized Cellulase from *A niger* (mg)	Immobilized Xylanase from *T longibrachiatum* (mg)	Ratio Cellulase: Xylanase (w/w)
A	6	0	0	-
B	0	6	0	-
C	0	0	6	-
D	2.676	1.338	1.986	2:1
E	2	1	3	1:1
F	1.332	0.668	4	1:2

Coconut husk containing 26.72% cellulose and 17.73% hemicellulose (Sangian et al., 2015) was converted to reducing sugar through enzymatic hydrolysis using several types of immobilized enzyme, as shown in Table 4. The hydrolysis was performed at 60°C for 48 h. Figure 4 shows the time taken to produce reducing sugar from the hydrolysis process. After 48 h of hydrolysis reaction, cellulase from *A niger* and *T reesei* yielded 0.352 g/L and 0.316 g/L of sugar, respectively, while immobilized xylanase from *T longibachterium* yielded 0.432 g/L. Simultaneous use of different enzymes with codes D, E and F significantly improved the yield of reducing sugar to 0.751, 0.8045, and 0.8034 g/L, respectively. The simultaneous reaction by three kinds of enzyme had a significant effect on reducing sugar yield ($p < 0.05$). Xylanase not only enhanced the quantity of sugar produced by its hemicellulose degradation to xylose, but also opened the access of cellulase to cellulose, which is blocked by hemicellulose, meaning sugar production was greatly improved (Song et al., 2016). As previously reported, in the application of the free form of cellulase and xylanase the combination of the two enzymes enhances sugar production (Farinas et al., 2010; Gonçalves et al., 2015; Pengilly et al., 2015). This study confirms that this was also true for immobilized enzyme. Code F, which had the best performance, was applied again to investigate the effect of GDA as the cross-linking agent, and it was shown to enhance the quantity of reducing sugar to 1.039 g/L0. GDA, which was able to escalate the available amino groups, leading to an increase in and stabilization of the enzyme coupling, not only improved the sugar yield at the end of the process, but also every hour of the reaction. Without adding GDA, the active enzyme sites tend to overlap each other, and furthermore the solid form of both lignocellulose and immobilized enzymes may give higher resistance of the mass transfer between enzymes and substrates. GDA could decrease steric hindrance between enzyme and support, since it acts as a spacer between the enzyme and chitosan (El-Ghaffar & Hashem, 2010). The F combination was also applied to nano-sized particles, but with only 4 mg of protein, generating a sugar yield of 0.49 g/L.

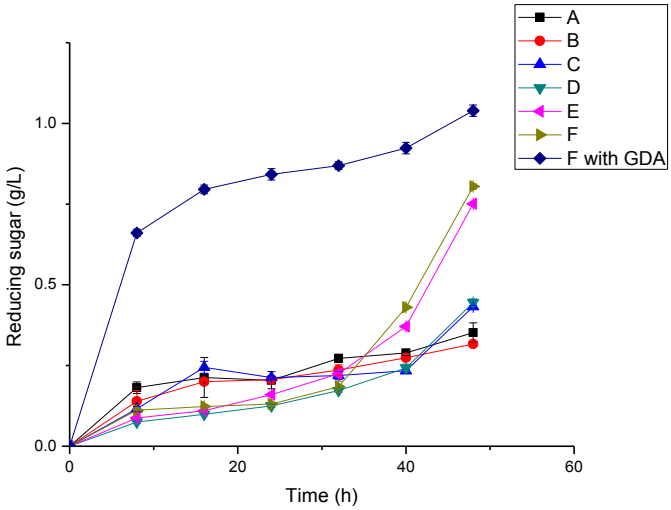

Figure 4 Timing of the hydrolysis of coconut husk by immobilized enzymes on chitosan magnetic microparticles as a biocatalyst. Codes A-F are the codes of the ratios of the enzyme combinations shown in Table 4

3.6. Reusability of Immobilized Enzyme

The reusability of immobilized enzyme was investigated with the F combination, with and without GDA. Figure 5 shows that cellulase immobilized directly on chitosan magnetic microparticles and on those cross-linked with GDA, retaining 91% and 75% of their initial activity respectively. The addition of the cross-linking agent enhanced the amount of reducing sugar produced. However, although it had less consistency in retained activity, it still produced more sugar than without GDA. This inconsistency may be due to the loss of enzyme activity caused by GDA cross-linked to important active enzyme sites (Sojitra et al., 2017). Nano-sized particles retained 54.7% remaining activity after five cycles. Several factors were responsible for the better performance of microparticles in reusability than of the nanoparticles, but the major reason was the incomplete recycling of immobilized enzyme due to its smaller size, making it easy to stick to the coconut husk.

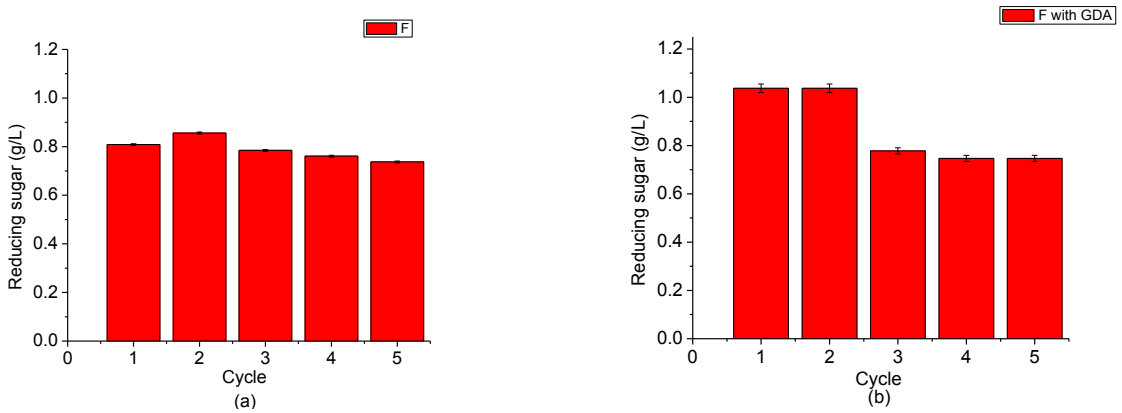

Figure 5 Reusability of: (a) chitosan magnetic microparticles; and (b) chitosan magnetic microparticles +GDA

4. CONCLUSION

Cellulase and xylanase were successfully immobilized covalently on chitosan magnetic particles. The FT-IR spectra convinced the covalent bond and the existence of GDA. The use of cellulase and xylanase collectively in the optimum ratio significantly enhanced the yield of

sugar, and the immobilized cellulase and xylanase could be utilized several times without loss of activity. This simultaneous use of three enzymes immobilized on chitosan magnetic particles has economic potential for use as a biocatalyst in lignocellulose hydrolysis.

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