Mass Transfer and Reaction in Hydrolysis of Coconut Husk Using Immobilized Enzyme on Chitosan Magnetic Nanoparticle

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Abstract. A more sustainable energy source is needed to fulfill the increasing demand for energy. In Indonesia, biomass waste utilization as biofuel source shows high potential. Coconut husk is a biomass comprised of cellulose and hemicellulose that can be converted to reducing sugar through enzymatic hydrolysis and can be further converted into biofuel. However, enzymatic hydrolysis is expensive, as enzyme is soluble and cannot be separated after use. Immobilization is a technique where an enzyme is trapped onto an insoluble inert support material so that the enzyme can be recovered and reused. Chitosan magnetic nanoparticles (CMNP) are used in this experiment as the support as it is cheap and readily available. Since coconut husk is insoluble, mass transfer phenomena remains a major bottleneck in hydrolysis due to limitations in the substrate to enzyme accessibility, especially when the enzyme is immobilized on an insoluble material like CMNP. If only mass transfer resistance is accounted, hydrolysis only occurs on the enzyme particles’ surface. The mass transfer coefficients obtained are 0.0000723, 0.0368, 0.0295 in respect of A. niger cellulase, T. reesei cellulase, and T. longibrachiatum xylanase. Higher coefficients values contribute proportionally to higher yield. If the reaction is assumed to occur uniformly within the spherical immobilized enzyme, then the substrate will diffuse molecularly through the enzyme, where internal mass transfer is no longer negligible. Thiele’s Modulus obtained are 2.197, 0.0368, 0.0795 in respect of A. niger cellulase, T. reesei cellulase, and T. longibrachiatum xylanase. A smaller value in Thiele’s Modulus (ϕ ≤ 1) shows that diffusion occurs at a faster rate than the enzymatic reaction.

1. Introduction
Indonesia ranks at top five of energy consumption in Asia Pacific, behind China, India, Japan, and South Korea. It is predicted that demand will reach up to 4.469 billion BOE by 2050 compared to 2016’s 795 billion BOE [1]. As fossil fuels reserves deplete, it is imperative that a more sustainable source of energy is utilized to meet this demand. As an Asia’s largest producer of coconuts, biomass can potentially be largely used as a new source of energy. When lignocellulosic feedstock like coconut husk is converted through enzymatic hydrolysis, it yields reducing sugar which can be further fermented into biofuels like bioethanol or biohydrogen [2].

This method can often be expensive as enzymes are soluble in water, making it hard to recover after hydrolysis process. Enzymes can possibly be separated for reuse by immobilizing it to an inert, insoluble material, which makes the process considerably cheaper. Covalent attachment employed as the bond between enzymes and supporting materials are stronger than physical attachment [3]. The existence of amino groups in chitosan allows covalent bonds to form between chitosan and enzymes. The existence of magnetic materials in the immobilized enzymes ensure that the enzymes can be recycled magnetically. Previously, chitosan had been applied in enzyme immobilization and could retain its activity up to five cycles [4]. Since enzymes can only work on specific substrates, a
combination of cellulase and xylanase is employed to increase the yield of reducing sugar, which consists of glucose, xylose, and other reducing sugars.

Due to the insoluble nature of the substrate and immobilized enzymes, mass transfer remains a major bottleneck in hydrolysis since it can limit the accessibility of the enzymes to the substrate [5]. Figure 1 illustrates the process of mass transfer between immobilized enzyme and soluble substrate.

![Figure 1. Schematic of mass transfer in immobilized enzyme with a soluble substrate: (1) displacement from bulk liquid to an unmixed liquid layer around the immobilized enzyme, (2) diffusion on the unmixed liquid layer, (3) diffusion from the surface of particle to the active site of an enzyme [3]](image-url)

Steps 1 and 2 are external mass transfer resistance, and step 3 is the intraparticle mass transfer resistance. Previous experiments reported that the hydrolysis conversion is higher in free enzymes compared to immobilized enzymes [6] [7]. Enzyme immobilization affects the stability of an enzyme that can lead to enzyme inactivation under specific conditions (high temperature/pH). Kinetic constants (Km, Vmax) of an enzyme was different in its free and immobilized form. It was due to an internal structure changes which influences the substrate’s ability to access the active site of enzyme [3]. The value of these apparent kinetic parameters (immobilized enzyme) can be different from the intrinsic value of the free enzyme. This can be caused by property changes of the solution in the area of immobilized enzyme, or because of the molecular diffusion in internal environment.

This is not always consistent, as other experiments have shown that hydrolysis conversion with immobilized enzymes is higher [5] [8]. Thus, the contribution of mass transfer to the enzymatic hydrolysis reaction must be studied further to explain this phenomenon better.

2. Materials and methods

2.1. Materials

The materials used in this experiment are coconut husk from North Sulawesi, Indonesia, dinitrosaliclyc acid, carboxymethyl cellulose, xilan, xylose, Coomassie Briliant Blue, T. reesei cellulase with 700 U/g activity, solid A. niger cellulase with ≥0.3 U/mg activity, solid T. longibrachiatum xylanase 1 U/mg all purchased from Sigma-Aldrich, Germany, sodium potassium tartrate, glucose, H2SO4, NaCl, ethanol 95%, distilled water, NaOH, Na2HPO4, iron (II) chloride tetrahydrate, Iron (III) chloride hexahydrate, phosphoric acid 85%, glacial acetic acid, glutaraldehyde nitrogen gas purchased from Merck, Germany, sodium metabisulfite (Sigma-Aldrich, Italy), Serum Bovine Albumin (Sigma-Aldrich, Switzerland), low molecular weight chitosan (Sigma-Aldrich, Iceland).

2.2. Method

2.2.1. Pre-treatment of coconut husk. 25 g of sundried and ground coconut husk was mixed with 500 ml of 1% w/v NaOH in a boiling flask. The solution was heated at 80°C while being constantly stirred. After 16 hours, the solution was cooled and sieved, and washed with distilled water until the pH reached 7. The remaining solids were put in the oven at 60°C for 24 hours and ground to powder.

2.2.2. Chitosan magnetic nanoparticles. Coprecipitation was used to synthesize Fe3O4 nanoparticles. 28% w/w NH4OH was added to 5.4 g of FeCl3·6H2O and 1.99 g FeCl2·4H2O and diluted in 50 mL distilled water while being shaken vigorously. Reaction was carried out at 80°C for 2 hours. Nitrogen gas was injected to ensure oxygen was able to escape. Fe3O4 nanoparticles were then washed with
distilled water until the pH reaches 7, and was added to 0.25 g of chitosan and diluted in 50 mL of acetic acid (1&, v/v). With constant stirring for 30 minutes, Fe₃O₄ nanoparticles would disperse homogenously in the chitosan solution. 50 mL of NaOH (1 M) was added to the solution and the Fe₃O₄ nanoparticles would be coated with chitosan. The resulting particles were washed with distilled water until the pH reached 7 and is stored at 4°C for immobilization.

2.2.3. Enzyme immobilization. 0.1 g of supporting material chitosan-GDA 1% was added into the enzyme solution in an incubator shaker for 4 hours. The mixture was then filtrated and the enzyme that didn’t react was separated by washing three times with phosphate buffer (pH 7). Immobilized enzyme was stored at 4°C.

2.2.4. Enzymatic hydrolysis. 1 g of pretreated, delignified coconut husk was mixed with 0.1 g of immobilized enzyme in an Erlenmeyer. 20 mL of phosphate buffer (pH 7) was added into the solution, and the hydrolysis was carried out at an incubator shaker at a speed of 125 rpm and a constant temperature of 60°C. The resulting mixture was further analyzed for glucose concentration using DNS method.

3. Results and discussion

3.1. Immobilization

Immobilization experiment was performed by (Hakim et al, 2019), with chitosan magnetic particles alongside cellulase from *Aspergillus niger* and *Trichoderma reesei* also xylanase from *Trichoderma longibrachiatum*. The same immobilization was also conducted by (Hamzah, 2019) with the addition of glutaral dialdehyde (GDA). From previous research conducted by El-Ghaffar & Hashem (2010), it was illustrated in Figure 2a the possibility of binding between enzyme and support. A combination between a covalent bond and cross-linking using GDA was also illustrated in Figure 1b. From the figure, in can be inferred that there is a direct bond between amino groups of chitosan with carboxylic terminal residue in the enzymes.

![Figure 2. Illustration of: (a) covalent immobilization of enzyme on chitosan magnetic particles; and (b) covalent bond and crosslink of enzyme to chitosan magnetic particles with (GDA)](image)

These reactions were confirmed by using Fourier Transform Infrared (FT-IR) spectra performed by [4], as shown in Figure 3. As show in the figure, there was a significant peak change in the wavelength 3272.53 cm⁻¹ which showed the characteristic of amino group (N-H), wavelength 1632.67 cm⁻¹ showed the characteristic of C=O, and wavelength 1080 cm⁻¹ which showed the characteristic of aliphatic amide C-N (Mistry, 2009). These three significant change in the peak of the wavelengths indicated the success of the immobilization methods.
3.2. Enzyme kinetic parameter

3.2.1. Mass transfer coefficient. Hydrolysis process by immobilized enzyme on an insoluble particle is greatly influenced by external mass transfer resistance, the rate of mass transfer is proportional to the driving force and the concentration difference [3]. Therefore, the rate of reaction of the enzyme can be expressed by the Michaelis-Menten equation.

A study was performed by Hakim et al [9], in using immobilized enzyme on chitosan magnetic nanoparticles to hydrolyse carboxymethyl cellulose (CMC) and xylan. The research resulted the kinetic parameters as shown in table 1.

| Enzyme                              | Km (mg/mL) | Vm (M/min) |
|-------------------------------------|------------|------------|
| Cellulase from free *A. niger*      | 2.17       | 0.8        |
| Cellulase from immobilized *A. niger* | 115.08     | 0.77       |
| Cellulase from free *T. reesei*     | 53.17      | 1.01       |
| Cellulase from immobilized *T. reesei* | 83.89      | 1.26       |
| Xylanase from free *T. longibrachiatum* | 14.73      | 2.27       |
| Xylanase from immobilized *T. longibrachiatum* | 25.22      | 1.87       |

In order to determine the effects of mass transfer resistance caused by enzyme immobilization in enzymatic hydrolysis, effectiveness factor must first be calculated. Steady-state assumption was used and free enzyme is used as the basis. The calculation resulted the data in table 2.

| Enzyme                              | Km (mg/mL) | Vm (M/min) |
|-------------------------------------|------------|------------|
enzymes

| Enzyme                             | Ksa (10^{-5}) |
|------------------------------------|---------------|
| Cellulase from A. niger            | 0.00723       |
| Cellulase from T. reesei           | 3.68          |
| Xylanase from T. longibrachiatum   | 2.95          |

It can be seen from Table 2, cellulase enzyme from T. reesei had a higher mass transfer coefficient value than cellulase enzyme from A. niger. This values are confirmed by calculating the hydrolysis yield, which are shown in Table 3. It shows that immobilized cellulase from T. reesei had a higher yield than immobilized cellulase from A. niger.

**Table 3. Yield of CMC/Xilan hydrolysis using immobilized enzymes**

| Enzyme                             | Yield (%) |
|------------------------------------|-----------|
| Cellulase from A. niger            | 6.65      |
| Cellulase from T. reesei           | 23.17     |
| Xylanase from T. longibrachiatum   | 49.05     |

A previous research conducted by Hamzah (2019), produced data that contradicted the value found in Table 2. The data in Table 4 shows that immobilized cellulase from A. niger had a higher yield than immobilized cellulase from T. reesei. Further study must be conducted to find the kinetic parameter values from Hamzah (2019) [4] study in order to confirm the effects of mass transfer coefficient in enzymatic hydrolysis.

**Table 4. Yield of coconut husk hydrolysis using immobilized enzymes**

| Enzyme                             | Yield (%) |
|------------------------------------|-----------|
| Cellulase from A. niger            | 1.02      |
| Cellulase from T. reesei           | 0.85      |
| Xylanase from T. longibrachiatum   | 1.19      |

If we take into account the internal mass transfer resistance, a series of assumption must be made. The reaction is to occur not only on the surface, but on every side within the immobilized enzyme through molecular diffusion [3]. Assuming the particle is spherical, using a cylindrical coordinates model, the domain where diffusion and enzyme take place is denoted below in Figure 4. The variable \( r \) measures the radial distance from the \( z \)-axis.

Following a first-order kinetic reaction, the Thiele’s modulus, which relates the reaction rate with the diffusion rate inside the immobilized enzyme [10], the diffusivity of the substrate in an immobilization matrix can be obtained through the following equation.

\[
x_s = \frac{\sinh(3\theta r)}{r\sinh(3\theta)}
\]

where,
For simplicity, $r$ refers to the radius of the chitosan magnet nanoparticle used in immobilized enzyme, making $r$ a constant value. In this case, the radius used is 20 nm.

\[ \chi_s = \frac{C_s}{C_{2b}} \]  
\[ \phi = \frac{r}{3} \sqrt{\frac{k}{D_s}} \]  

For simplicity, $r$ refers to the radius of the chitosan magnet nanoparticle used in immobilized enzyme, making $r$ a constant value. In this case, the radius used is 20 nm.

Figure 4. Shell balance of a substrate in immobilized enzyme (Al-Mayah, 2012)

Table 5 Internal mass transfer resistance parameters

| Enzym Terimobilisasi       | $D_s$     | $\phi$  | $\chi_s$ |
|---------------------------|-----------|---------|---------|
| Aspergillus niger         | $6.7 \times 10^{-22}$ | 2.197   | 0.018   |
| Tricoderma reesei         | $1.2 \times 10^{-17}$ | 0.0368  | 0.822   |
| Tricodherma longibrachiatum | $2.1 \times 10^{-18}$ | 0.0795  | 0.443   |

A lower value of Thiele’s modulus ($\phi \leq 1$) indicates that the enzyme reaction is slow compared to the diffusion rate. For T. reesei and T. longibrachiatum, this means that the substrates diffuse into the core of particle. Throughout the radial location of a parameter, the concentration distribution would be fairly flat [3]. At a constant initial substrate concentration, the higher the value of the Thiele’s modulus, the greater reaction time to achieve a certain conversion value. At an increased initial substrate concentration, the differences in efficiency between the immobilized and free enzyme will be diminished. This can be attributed to the increase in mass transfer rate [11].

3.3. Mass Transfer Inhibition

3.3.1. Coconut Husk Insolubility. Insolubility of coconut husk might also have played a part in its difficulty to be hydrolyzed by an immobilized enzyme. This is caused by the addition of mass transfer resistance from the coconut husk surface to the bulk liquid. Whereas a soluble substrate would be easily mixed homogeneously into a bulk liquid. Figure 5 shows the illustration of a possible coconut husk mass transfer to an immobilized enzyme in a bulk liquid.
The mechanism described that the coconut husk transferred to the bulk liquid were not hydrolyzed completely by the immobilized enzyme. These occurrences would result in a low yield of coconut husk hydrolysis. Due to its insolubility, the solution appeared slurry-like and high in viscosity, which could pose another bottleneck to the hydrolysis process. The speed of the stirring, for example, would have to be specific as to minimize the existence of dead-zones and insufficient contact between product and reactant [12].

3.3.2. Shear Stress. A solution with high concentration of solid substrate would have a high viscosity. A slurry, which have a high amount of solid, showed a characteristic of high viscosity and non-Newtonian fluid which caused shear-thinning in lignocellulosic enzymatic hydrolysis which gave stress to hydrolysis yields. According to a research performed by Hodge et al [13], 12-15% (w/w) is the maximum concentration of solid allowed to allow mixing in a CSTR, which proved to be a possible limitation in lignocellulosic hydrolysis.

Viscosity of a Newtonian fluid will be lowered when the molecular structure became irregular, in accordance to shear-thinning effects. Previous research conducted by Ingebrigtsen & Tanaka [14], stated that shear effects which applied in the same direction as the flow of fluid would create space for molecules to move and assemble while the fluid expand. This event would not occur to a non-Newtonian fluid. Illustration of what would happen was illustrated in Figure 6.

Hydrogen bonding and interaction between particles resulted a highly viscous fluid. A study performed by Du et al [12] stated that decreased distance between particles in slurry caused shear force resistance in a high flowrate and viscosity fluid which had shear-thinning dan non-Newtonian characteristic. The possible cause of the occurrence is the opening and reconstruction of fiber structure in lignocellulose and mixing velocity. Mixing velocity would minimalize the creation of dead zone and improper contact between reactants and products.
4. Conclusion

The mass transfer coefficients obtained for when only external mass resistance is considered are 0.0000723, 0.0368, 0.0295 in respect of *A. niger*, *T. reesei*, and *T. longibrachiatum*. Higher coefficients values contribute proportionally to higher yield. If the reaction is assumed to occur uniformly within the spherical immobilized enzyme, then the substrate will diffuse molecularly through the enzyme, where internal mass transfer is no longer negligible. Thiele’s Modulus obtained are 2.197, 0.0368, 0.0795 in respect of *A. niger*, *T. reesei*, and *T. longibrachiatum*. A smaller value in Thiele’s Modulus (ϕ ≤ 1) shows that diffusion occurs at a faster rate than the enzymatic reaction, that is, reaction-controlled regime. Similarly, bigger values in Thiele’s Modulus shows that intraparticle diffusion is more significant, that is, diffusion-controlled regime.

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