Fouling-induced Enzyme Immobilization of Alcohol Dehydrogenase (ADH) using Graphene Oxide (GO) Based Polymer Membrane

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***Abstract* - The use of Carbon dioxide (CO_2) to produce another beneficial product is a great advantage to humankind because CO_2 is harmful if the amount in the environment is not balance since it can cause greenhouse effect. Enzyme immobilization is use to produce methanol with alcohol dehydrogenase (ADH) as model enzyme in addition of nicotinamide adenine dinucleotide (NADH) as cofactor. The objectives of this research are to immobilize ADH in graphene oxide based polymer membrane by using fouling induced enzyme immobilization technique and to examine biocatalytic productivity of the enzymatic membrane reactor system. The conversion will be achieved using the optimum value of pressure (2bar), pH (7) and enzyme concentration (0.1 gL^{-1}) to see how many cycle that the membrane can do to achieve the high yield of methanol. Based on the results, the amount of enzyme immobilize, permeate, retentate and washing are 1.36 mg, 0.7126 mg, 0.3114 mg, and 0.6171 mg respectively. For enzymatic reaction, conversion rate are 60% and 14% for reaction one and two respectively.**

Keywords— enzyme immobilization, membrane, graphene oxide, alcohol dehydrogenase, membrane fouling.

I. INTRODUCTION

Enzyme is a natural biocatalys that can increase the rate of reaction without being consume itself [1]. The use of enzymatic technology in industrial process has increased because of the current demands of the sustainable green methodologies [1]. One of enzyme technology is enzyme immobilization. The enzyme immobilization is widely used because it can increase the efficiency, selectivity, environmentally friendly and moreover it is less costing [2]. According to Mohamad et al., (2015), immobilized enzyme is 'enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously.' However, as stated by Garcia-Galan et al., (2011), to reuse the enzyme it must meet some requirement. It can be accomplished by using enzymatic membrane reactor [3].

In recent study, membrane fouling enzyme immobilization is used as strategy for enzyme immobilization using membranes [3]. Even though membrane fouling is the major limitation if involving bioreactors since it can reduce membranes performance [4], it still can help in enzyme immobilization since they have a number of features [3]. In order to optimize the enzyme activity in the membrane, the variable and parameters such as pH, enzyme concentrations and pressure need to be consider. Besides, the orientation of the membranes also affect the enzyme immobilization. The example for types of orientation are skin layer facing feed and support layer facing feed [3]. The orientations will determine permeate flux.

One of the uses of enzyme immobilization in or on membranes is apply in multi-enzymatic conversion of carbon dioxide, CO_2 to methanol, CH_3OH. The sequential reduction of CO_2 to methanol can be carried out with three types of enzymes which are formate, formaldehyde and alcohol dehydrogenase [5]. But, as studies by El-Zahab et al., (2007), they use four types of enzymes which is glutamate dehydrogenase as addition from the previous three enzymes. Glutamate dehydrogenase was used to regenerate nicotinamide adenine dinucleotide, NADH [3]. In this reduction, NADH was used as terminal electron donor or cofactor [6]. Cofactor is a non-protein chemical compound or metallic ion that is required for a protein's biological reaction to happen ZZ. Enzyme and cofactor are immobilized separately.

From previous study, process of CO_2 to methanol also can be carried out by partial hydrogenation. Unfortunately, this process however limited because of high price of the renewable hydrogen [7]. At the same time, the use of enzyme is more interesting and more appealing since it provide facile route [6]. It is also specific and have no side reaction. Alcohol dehydrogenase was used as the model enzyme with NADH as the cofactor in the production of methanol. It is known that more than two hundred types of enzymes can be used [8]. But, ADH is choose to see the efficiency of the enzyme by using fouling induced enzyme immobilization technique. Next, to get the high yield of methanol, it must meet some requirements. The parameters and other variables need to be consider. Enzyme also work the same. The most important thing to observe is enzyme stability so it can withstand sudden different in temperature, pH or solution composition [8]. The conversion will be achieved using enzyme immobilization and the optimum value of pressure (2bar), pH (7) and enzyme concentration (0.1 g/L) to see how many cycle that the membrane can do to achieve the high yield. The objectives of the research are to immobilize ADH in graphene oxide based polymer membrane by using fouling induced enzyme immobilization technique. The next objective is to examine biocatalytic productivity of the enzymatic membrane reactor system.

In this experiment, the membrane used is graphene oxide (GO). GO has two dimensional (2D) shape and derived from graphene [9]. It carries a huge number of oxygenated functional groups, capable to be a useful antifouling material because of its high hydrophilicity, charge properties, and antimicrobial properties. For example, due to its hydrophilicity, the adhesion

forces between organic foulants and membrane surfaces can be weaken. GO also create many negative charges to the membranes resulting the electrostatic repulsion against microorganism deposition therefore the membrane fouling can be inhibit [10].

Due to its large specific surface area and abundant functional groups, GO is considered as ideal substrate for enzyme immobilization. As indicated by Arivalagan et al., (2011), even without using any cross-linking reagents and additional surface modification, the enzyme immobilization on GO sheets still can occur. The observation can be done with the help of atomic force microscopy (AFM). Other than that, the interaction of enzyme molecules with the surface functional groups of the substrate can determine the catalytic performance of the immobilized enzymes and using AFM image, the conformation of immobilized enzyme is found to be the major point to improve its catalytic performance [11].

II. METHODOLOGY

A. Chemicals and membrane

The chemicals used are Alcohol dehydrogenase (ADH EC 1.1.1.1) from Saccharomyces cerevisiae, L-glutamate dehydrogenase from bovine liver, b-nicotinamide adenine dinucleotide reduced form (NADH), b-nicotinamide adenine dinucleotide hydrate (NAD+), formaldehyde, L-glutamic acid, monopotassium phosphate (KH_2PO_4) and dipotassium phosphate (K_2HPO_4). All these are purchase from Sigma-Aldrich (St. Louis, MO, USA). The enzyme and substrate solutions are prepare using 0.1M phosphate buffer at pH 7.0. The isoelectric point (IEP) of ADH enzyme is 5.4–5 (manufacturer's information). The molecular weights of ADH, GDH, NADH/NAD+, formaldehyde and glutamic acid are 141, 300, 0.7, 0.03 and 0.15 kDa, respectively. The membrane used is Graphene Oxide membrane [4].

B. Experimental Set-Up and Procedures

The dead-end batch filtrations are performed in a commercial stirred cell (Amicon 8050, Millipore, USA). The stirring speed is at 100 rpm and the impeller is driven by a magnetic agitator. The working volume of the cell is 50 mL, the diameter of membrane disc that can be fit in the device is 44.5 mm within the module and the effective membrane surface area is 14.2 cm^2. The nitrogen gas is fill into the cell in order to keep pressure constant and a beaker or a precise cylinder place on electronic scale (PJ300, Mettler, USA) is use to collect permeate to monitor the permeate flux. The experiments are conduct at temperature of 23°C. The membrane is place in reverse mode at the bottom of the stirred cell. First, the membranes are clean by using ultrapure water. Next, rinse it in ultrapure water to check the water permeability at pressure of 2 bar. Then, ADH enzyme solution having a concentration of 0.1 g L^{-1} and 50 mL volume at pH 7 are inject into the cell for subsequent immobilization operations. The experiments are repeat about three times [3].

C. Enzyme ImmobilizaTion

The enzyme immobilization is perform using dead-end filtration at pressure of 2 bar, pH 7 and 0.1 g/L enzyme concentration. Vials or tube is use to collect permeate and replace manually for every 4 mL. Towards the end of filtration, the fouled membrane is rinse three times with 5 mL of buffer in the absence of pressure. Then, final retentate and the rinsing residuals are collect in order to calculate the amount of enzyme immobilized by the mass balance. Lastly, the permeability of the enzyme-fouled membrane is measure at 2 bar using a buffer solution [3].

D. Enzymatic Reactions

At the pH of 7, 50 mL of the substrate mixture (100 mM HCOH + 100 µM NADH) are pour into the stirred cell equipped with the "fouled" membrane. To make sure the conversion rate is independent of the substrate concentration, formaldehyde, HCOH concentration is intentionally high and in excess of the cofactor level (NADH). Collect permeate until it reach 48 m with the pressure set to 2 bar (collect in aliquots of 4 mL). Towards the end of reaction, the retentate (2 mL) is collect for analysis to make sure that no reaction happened in the bulk solution [4].

E. Analytical Methods

By using spectrophotometer at 280 nm (Perkin Elmer lambda20 UV/Vis, UiTM Shah Alam), the enzyme concentration is compute whereas at 340 nm the cofactor NADH concentration is monitor by absorbance. Based on preliminary experiments, the absorbance stability of the enzyme and the NADH during the filtration are confirm [3].

F. Calculated Parameter

The amount of immobilized enzyme (m_i) is calculate by the mass balance equation:

$$m_i = m_t - C_p V_p - C_R V_R - C_r V_r$$

The immobilization efficiency is express as enzyme loading per unit area of membrane (mg cm^{-2}) or loading rate (%):

$$Loading\ rate\ (\%) = \frac{m_i}{m_t} X100$$

The enzyme activity is evaluate from the conversion rate (X, %) of NADH/NAD+:

$$Conversion\ rate\ (\%) = \frac{C_i - C_p}{C_i} x100$$

III. RESULTS AND DISCUSSION

A. Enzyme Immobilization

The amount of immobilized enzyme, m_i is

$$m_i = m_t - C_p V_p - C_r V_r - C_w V_w$$

$$m_i = 3 - (0.0254)(28) - (0.0195)(16) - (0.0220)(28)$$

$$m_i = 1.36\ mg$$

The loading rate is,

$$Loading\ rate\ (\%) = \frac{m_i}{m_t} X100$$

$$Loading\ rate\ (\%) = \frac{1.36}{3} X100$$

$$Loading\ rate\ (\%) = 45\%$$

Table 1 : Concentration and Mass of Enzyme

	Volume (mL)	Concentration (mg/mL)	Enzyme amount (mg)
Feed	30	0.1000	3.0000
Permeate	28	0.0254	0.7126
Retentate	16	0.0195	0.3114
Washing	28	0.0220	0.6171

B. Enzymatic Reaction

The conversion rate for reaction one is :

$$Conversion\ rate\ (\%) = \frac{C_i - C_p}{C_i} x100$$

$$Conversion\ rate\ (\%) = \frac{0.07 - 0.0270}{0.07} x100$$

$$Conversion\ rate\ (\%) = 61\%$$

For reaction 2 is, the conversion rate is 14.80%.

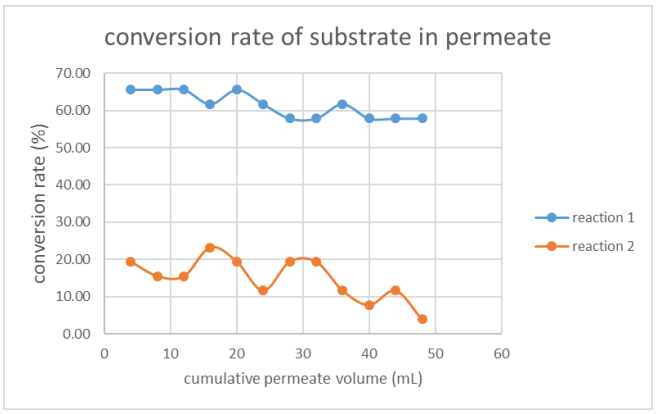

Fig 1 : Conversion Rate of Substrate In Permeate

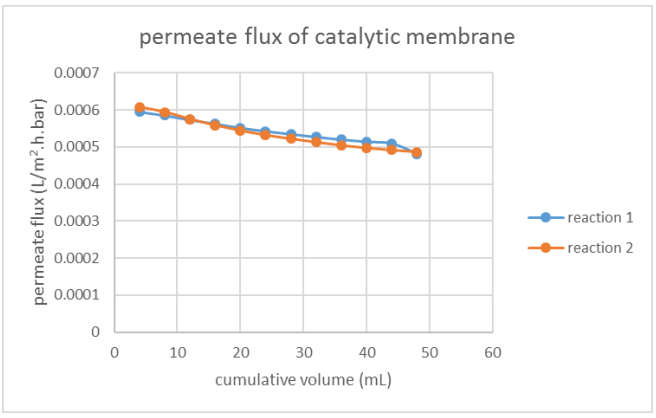

Fig 2 : Permeate Flux of Catalytic Membrane

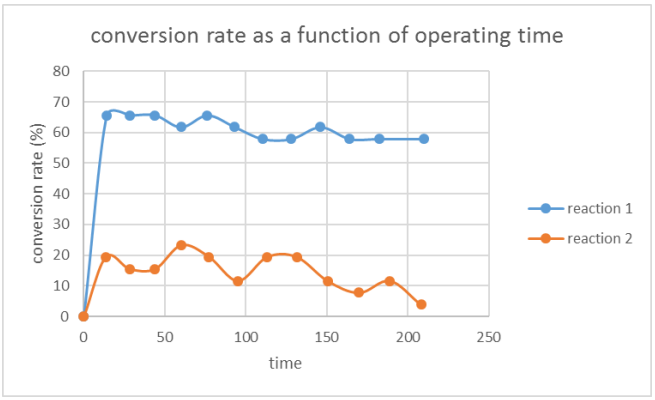

Fig 3 : Conversion Rate as a Function of Operating Time for Catalytic Membrane

Tables and graphs above are the results for the experiments. For enzyme immobilization, the mass of enzyme immobilize was 1.36 mg from total mass which was 3 mg and give loading rate at 45%. Since the experiments were conducted at optimum pressure, concentration and pH, the loading rate is quite lower compared to other research. According to Luo et al., (2014), at optimum parameter, the loading rate was 50% but with different type of membrane. The loading rate is lower may be due to the membrane used is already foul since the material of support used plays important role in the process of immobilization due to the strong effect of these materials on the properties of the produced catalytic system (Jakub Zdarta, 2018). But this is not the major factor because graphene oxide (GO) membrane is known as a good membrane for enzyme immobilization process. The surface of GO membrane rich with functional groups and make the immobilization process occur quickly by electrostatic interaction without using any cross-linking reagents [12]. The concentration and mass of enzyme in the feed, permeate, retentate and washing are shown in the table 1.

In enzymatic reaction, for the first reaction, the conversion rate was 61% and for the second reaction was 14%. According to Zdarta et al., (2018), the interaction of enzyme molecule with the surface functional groups of the substrate will determine the catalytic performance. Zdarta et al,. (2018) also state that enzyme loading will not control the enzyme specific activity as long as substrate surface was not fully closed by the enzyme. So, that is why the conversion rate is higher at reaction 1 because there is higher interaction between enzyme and substrate. At second reaction, the percentage of conversion is lower because the amount of enzyme immobilize in the membrane are getting lower. The immobilize enzyme might be diffuse back into the bulk solution during the first reaction causing the next reaction having lower conversion rate [3]. This is illustrate in the fig. 3 where the first cycle have higher conversion rate compared to second cycle.

Next, based on fig. 1, conversion rate against cumulative volume, the conversion rate is uniform between 60% - 70% at reaction 1 and also uniform at reaction 2 which is between 0% - 20%. This is because the concentration of product produce is constant. For fig. 2, the permeate flux are decreasing with increasing time. This is due to concentration polarization and adsorption and precipitation of solutes on the surface of the membranes [13]. According to Chen et al., (1992), concentration polarization occur when solute concentration next to membrane surface is greater than in the bulk solution. As for adsorption and precipitation of solutes on the surface of the membranes, this may be cause of blocking of membrane pores by solute molecules due to their average diameter is same as membranes holes. That is why, the graph show slow flux decline because of the formation of the gel layer on the surface.

IV. CONCLUSION

In conclusion, the amount of enzyme immobilize, permeate, retentate and washing are 1.36 mg, 0.7126 mg, 0.3114 mg, and 0.6171 mg respectively. For enzymatic reaction, conversion rate are 60% and 14% for reaction one and two respectively. The objective of the experiment to immobilize alcohol dehydrogenase (ADH) enzyme on graphene oxide membrane is achieved since 1.38mg of enzyme being immobilized. Second objective is the productivity of enzymatic membrane. To achieve the objective, two mixture of substrate are used for the enzymatic reaction and second cycle show a lower conversion rate since the enzyme already used in first substrate mixture. Overall, the experiment was a success considering all the objectives were achieved despite the results are quite lower compared to other research.

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