Biocatalytic Productivity of Alcohol Dehydrogenase (ADH) In Membrane Bioreator: Effect of pH

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***Abstract* — in the present study, immobilization enzymes are necessary for increasing the biocatalytic efficiency. At low pH during immobilization, the enzyme was liable to accumulate on the membrane surface due to the electrostatic adsorption and reduced the stability on enzyme. While when the solution of enzyme at high pH value (away from IEP) during immobilization, the membrane and enzyme was negatively charged and lead to much lesser accumulation on the membrane surface due to the strongest of electrostatic repulsion. Therefore, it will induce to lower fouling on the membrane. At the neutral pH (near IEP), the hydrogen bonding between enzyme and membrane was stabilized and lead to maintain the enzyme activity.**

***Keywords*— Enzyme immobilization, Membrane fouling, Alcohol dehydrogenase and Biocatalytic**

I. INTRODUCTION

The Global anthropogenic emissions of carbon dioxide recently reached a high record level of 35.7 billion tons per year and still increasing. Carbon capture has been extensively recognized as an efficient option for reducing atmospheric CO_2 concentration. In general, it can be regarded as the process of capturing waste CO_2 from specific sources, such as fossil-fuel power plants and CO_2 utilization can be regarded as the process of directly using CO_2 as a reaction medium or transforming renewable CO_2 into useful chemicals, materials or fuels [2].

In addition, one of the most promising options for CO_2 conversion in nature is via sequential enzymatic. According Obert and Dave, the sequential enzymatic conversion of CO_2 to CH_3OH can be accomplished in solution in a cascade system by three different of dehydrogenases which were encapsulated in silica sol-gel matrices in the presence nicotinamide adenine dinucleotide, NADH as a terminal electron donor for each dehydrogenase-catalyzed reduction [3]. The overall process consist three steps; initialy reduction of CO_2 to formate catalyzed by formate dehydrogenase, reduction of formate to formaldehyde catalyzed by formaldehyde dehydrogenase and reduction of formaldehyde to methanol catalyzed by alcohol dehydrogenase [3]. The enzymatic conversion of CO_2 to CH_3OH was carried out at low temperatures and low pressures [4]. The overall reaction process is shown in Figure 1.

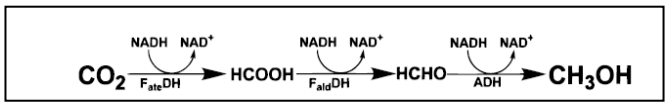

Figure 1: Overall reaction of sequential enzymatic reduction of CO_2 to CH_3OH

Immobilization enzymes are required for increasing the biocatalytic efficiency, especially for the manufacturing of low-medium value compounds. Immobilizing enzymes on membranes is beneficial as a level of compound separation can be accomplished concurrently with the biocatalytic reactions in enzyme membrane reactors (EMRs) [5]. Enzyme immobilization is an essential for many uses of the enzymes as industrial biocatalyst because the primary objective of the immobilization is to facilitate enzyme reuse. Many researchers have focused their research on how enzyme properties like stability, activity or selectivity can be improved by immobilization [6]. Immobilization of enzymes in/on membranes can be accomplished via adsorption, covalent bonding, cross linking or entrapment [5]. The porous membrane performance as a selective barrier as well as a support for enzyme immobilization allowing an enzyme re-use which can help an enzyme stability, eliminate inhibition and allow for continuous processing [7]. [8] also stated that, the most important beneficial of immobilization of enzymes in terms of improving the process economics by enhancing overall productivity and enabling re-usability of enzymes, that can be accomplished by using enzyme membrane reactor (EMR) in which enzymes are retained and separated from products [9]. Furthermore, it has been reported that in some cases enzyme immobilization results in significant increase the stability of enzyme and activity [10]. Generally, the amount of immobilized protein can be determined by mass balance between the initial solution (feed) and the solutions after immobilization process which is either retentate or permeate [11].

However, the membrane fouling commonly, compromise the performance of the membrane [12] in terms of separation efficiency and permeate flux [13]. Membrane fouling is the permeability of membrane loss due to adsorption or precipitation solute on/in the membrane. The main mechanisms for membrane fouling are surface adsorption, pore blockage, inorganic precipitation, gel or cake formation and biological fouling [7] which are caused by complex interactions between membranes and foulants such as hydrophobic/electrostatic adsorption, aggregation, hydrogen bonding and bio-affinity [13]. Fouling is the main issues in/on membrane reactors as fouling result in dramatic permeate flux decline and changes in the membrane selectivity [10].

Since membrane fouling and enzyme immobilization share a number of characteristics (Figure 2), we can conclude that, deliberate promotion of fouling might be used as strategy for immobilization enzymes in membranes. Forced membrane fouling then can be employed directly as a strategy of immobilization for adsorption and entrapment of enzymes in a membrane. If a functional reagent is added into enzyme solution, the enzyme may covalent bound to the membrane by cross-linking and is called as “combined fouling” [7].

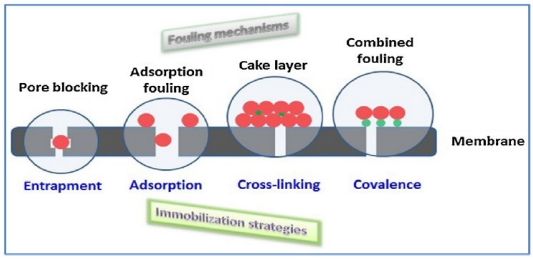

Figure 2: Schematic of connection between fouling mechanisms and immobilization strategies

A large number of studies about the effects of process parameter and membrane properties on membrane fouling have been reported mainly for protein fouling in ultrafiltration [13]. [14] found that fouling by adsorption was higher at lower pH values and by increasing salt concentration can reduces electrostatic repulsion between the positively charged protein molecules on regenerated cellulose membranes. Besides that, [15] also stated that, the existence of salts can adjust electrostatic protein-protein interactions and produce the effect of shielding charge and dampening out of intermolecular protein interactions.

The purpose of the present study is to evaluate the effect of modifying pH value on the fouling-induced enzyme immobilization and to examine the possible significance of the membrane orientation for the enzymes immobilization. Alcohol dehydrogenase (ADH) has been selected as a model enzyme. This because [13] alcohol dehydrogenase (ADH) capable to catalyze the conversion of formaldehyde (HCOH) to methanol (CH_3OH) with oxidation of NADH to NAD^+ which is the third step of multi-enzymatic catalysis of CO_2 to methanol.

II. METHODOLOGY

A. Materials

Alcohol dehydrogenase (ADH, EC 1.1.1.1) from *Saccharomyces cerevisiae*, β-nicotinamide adenine dinucleotide reduced form (NADH), formaldehyde, monopotassium phosphate (KH_2PO_4) and dipotassium phosphate (K_2HPO_4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All enzyme and substrate solutions are prepared using 0.1 M phosphate buffer at pH of 5.7, 7 and 8. The isoelectric point (IEP) of ADH enzyme is 5.4-5.8 (manufacturer's information). The molecular weight of ADH, NADH and formaldehyde are 141, 0.7 and 0.03 kDa respectively. The commercial UF membrane (MK, Synder) had a polysulphone as a skin layer and polypropylene as a support layer and a molecular weight cut-off of 30 kDa, are used in this experiment. The isoelectric point (IEP) of MK membrane is 4-5 [13]. Therefore, the membranes will be place in reversed mode which is support layer facing feed.

B. Experimental set-up and procedure

The dead-end batch filtrations were conducted in a stirred cell (Amicon 8050, Millipore, USA). The stirring speed was fixed at 100 rpm and the effective membrane surface area was 13.4 cm^2. The working volume of the cell was 50 mL. Constant pressure was generated by filling nitrogen gas into the cell and permeate was collected in centrifuge tube in order to monitor the permeate flux (permeate density was defined as 1000 kg/m3). All experiments were performed at a controlled temperature of 23 ± 1˚C and new membrane was used for each experiments. A sketch of the experimental set-up apparatus is shown in Figure 3.

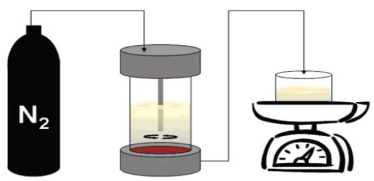

Figure 3: Sketch of the experimental set-up apparatus

The membrane was placed at the bottom of the stirred cell in reverse mode which support layer as a facing feed. MK membrane was first immersed with 0.1 M NaOH solution for 1 hour. Afterward, water permeability of membranes was measured at 2 bar with phosphate buffer at pH 7 for 10-30 minutes. Then, 30 mL of ADH enzyme solution with concentration 0.1gL^{-1} and different pH (5.7, 7 and 8) were injected into the cell for subsequent immobilization operations. All the experiments were repeated at least three times.

C. Enzyme immobilization

Enzyme immobilization was conducted at different pH values (5.7, 7 and 8) with pressure 2 bar and 0.1 gL^{-1} enzyme concentration. Then, permeate was collected by using centrifuge tubes for analysis and the centrifuge tubes were replaced manually for every 4 mL until 28 mL permeate was obtained. At the end of filtration, the fouled membrane was rinsed for 3 times with 5 mL of buffer (pH=7) without applying any pressure. The combined final retentate and the rinsing residuals were collected in order to calculate the amount of enzyme immobilization from mass balance. Therefore, the fouled membrane was washed by buffer (pH=7) at pressure 2 bar and permeate was collected for analysis until enzyme concentration in permeate was close to zero.

D. Enzymatic reaction

50 mL of the substrate mixture (1000mM HCOH + 100µM NADH) at constant pH 7 was poured into stirred cell equipped with the "fouled" membrane in the "sandwich" configuration. The HCOH concentration was purposely high and in excess of the cofactor level (NADH) to ensure conversion rate was independent of the substrate concentration and that reverse reaction are negligible. A pressure of 2 bar was set immediately until 48 mL permeate was obtained (collected in every of 4 mL). At the end of reaction, the retentate (2 mL) was collected for analysis to ensure no reaction occur in the bulk solution.

E. Analytical method

ADH enzyme concentration during immobilization and reaction was determined as protein concentration by using spectrophotometer (DR 5000, UV/VIS) at 280 nm or using Bradford protein assay. The cofactor NADH concentration was monitored by absorbance at 340 nm. The stability of enzyme and the NADH during filtration was established in preliminary experiments.

F. Calculated parameter

The observed rejection of the enzyme was defined as

$$R_{obs}(\%) = \left(1 - \frac{C_P}{C_0}\right) \times 100 \quad (1)$$

Where; Cp is the enzyme concentration in permeate and Co is the feed (initially) during immbolization.

The amount of enzyme immobilization was calculated from mass balance equation:

$$m_i = m_t - C_p V_p - C_r V_r - C_w V_w \quad (2)$$

Where; m_i and m_t are the immobilized enzyme and the total enzyme amounts respectively; Cr is the enzyme concentration in the mixture of retentate and rinsing residual, and Cw is the average enzyme concentration obtained in the pressure-driven washing; Vp, Vr and Vw are the volumes of the permeate during immobilization, the mixture of retentate and rinsing residual and the washing permeate respectively.

The immobilization efficiency was expressed as enzyme loading rate (%):

$$Loading\ rate\ (\%) = \frac{m_i}{m_t} \times 100 \quad (3)$$

The enzyme activity was evaluated from the conversion rate of NADH:

$$Conversion\ rate\ (\%) = \frac{C_i - C_p}{C_i} \times 100 \quad (4)$$

Where; C_i and C_p are the NADH concentration in feed and permeate, respectively.

The bio-catalytic productivity of reactions was relating to the effective use of enzyme catalyst and defined as

$$Biocatalytic\ productivity = \frac{m_p}{m_e} \quad (5)$$

Where; m_p and m_e are the molar mass of product and enzyme, respectively.

Resistance in series model is used to analyze fouling mechanisms at different process parameters and can be described as:

$$J_p = \frac{\Delta P}{\mu R_t} = \frac{\Delta P}{\mu(R_m + R_{cp} + R_{rf} + R_{if})} \quad (6)$$

Where; ΔP is the transmembrane pressure (Pa); μ is the solvent viscosity (Pa s); R_t, Rm, Rcp, R_{rf}, and R_{if} are intrinsic membrane resistance (m^{-1}), resistance of concentration polarization (CP) layer, resistance resulting from reversible fouling (e.g: particle deposit) (m^{-1}) and irreversible fouling including pore blocking or cake formation (m^{-1}), respectively.

For dead-end filtration process at constant pressure, the laws of filtration can be written as

$$\frac{d^2t}{dv^2} = K\left(\frac{dt}{dv}\right)^n \quad (7)$$

Where; t is filtration time (s); V is the volume of permeate (m^3); K is constant value and n can have different values depending on different types of fouling: n = 2 indicates complete blocking model, n = 1.5 indicates standard blocking model, n = 1 indicates intermediate blocking model and n = 0 indicates cake layer model. By integrating Equation (7), four linear equations can be obtained when fixed the values of n:

when n = 2, $$ln\, J_p = ln\, J_0 - K_c t \quad (8)$$

when n = 1.5, $$\frac{1}{J_p^{0.5}} = \frac{1}{J_0^{0.5}} + K_s t \quad (9)$$

when n = 1, $$\frac{1}{J_p} = \frac{1}{J_0} + K_i t \quad (10)$$

when n = 0, $$\frac{1}{J_p^2} = \frac{1}{J_0^2} + K_{cl} t \quad (11)$$

Where; Jo is the certain permeate flux when t = 0; K_c, K_s, K_i and K_{cl} are the constant for complete blocking model, standard blocking model, intermediate blocking model and cake layer model, respectively. However, the value of n will change during filtration due to the evolution of fouling with time [13].

III. RESULTS AND DISCUSSION

A. *The effects of pH on enzyme immobilization*

The solution of pH is the main key in the charge of characteristics of molecules and affected the electrostatic interaction between the membrane surface and ADH enzyme. Then, it will cause foulant deposition on the membrane. [16] reported, the electrostatic interaction is a main factor in dominate behaviour of protein adsorption on membrane surfaces. Figure 4.1 shows the effect of pH on permeate flux in the dead-end stirred ultrafiltartion with 2 bar. The effect was observed at varies of pH values (5.7, 7 and 8). During the first 4 mL of filtration, the permeate flux was 102.34 $L/m^{-2}h^{-1}$ for pH 5.7, 143.28 $L/m^{-2}h^{-1}$ at pH 7 and 153.5181 $L/m^{-2}h^{-1}$. After the next 8 mL, the permeate flux was decreased at each pH values and resulting of formation fouling membrane. Furthermore, the isoelectric point (IEP) of ADH enzyme are of pH 5.4-5.8 [13]. [16] stated if the pH of protein is less than isoelectric point, the net charge of electrostatic interactions is positive whereas if pH more than isoelectric point, then the net charge is negative. When the solution of enzyme at pH 5.7 (near with IEP) the membrane was approximately neutral and enzyme was positively charged and lead too much of accumulation on the membrane surface (severely flux).

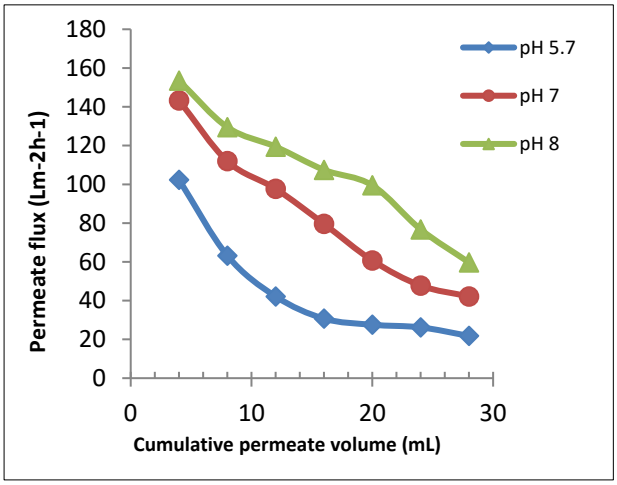

Figure 4: Effect of pH on permeate flux

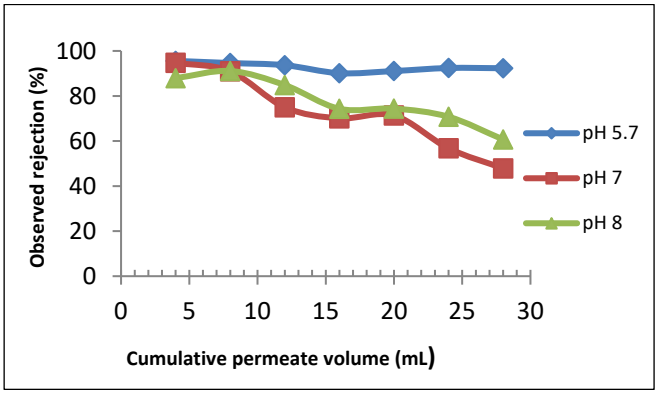

Figure 5: Effect of pH on observed rejection

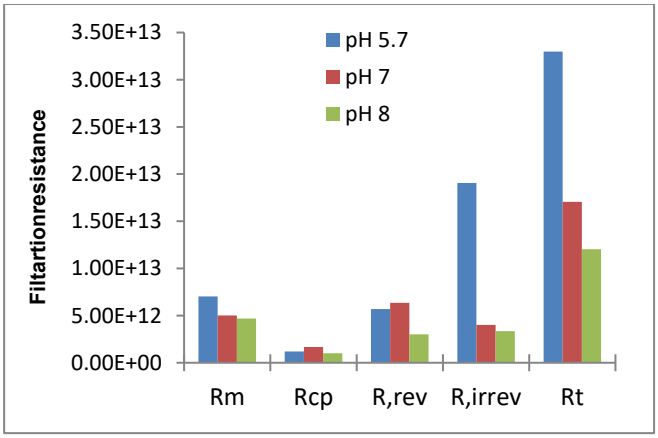

Figure 6: Effect of pH on Filtration Resistance

While for pH 8 (away from IEP), the accumulation is lesser than. Besides that, permeate flux for pH 5.7 was rapidly declined and the

irreversible fouling resistance was much higher due to the gel layer formation on the membrane surface and it indicating the amount of immobilized enzyme on the membrane higher other that two pH values. The observed rejection of enzyme (Figure 4.2) at pH 5.7 is steady but for pH 7 and 8, observed rejection of enzyme is decreased. At the same time, the higher of loading rate indicate the

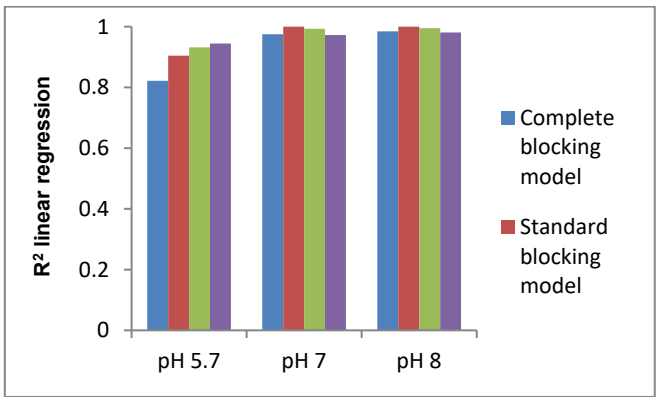

Figure 7: Effect of pH on Fouling Mechanism

too much molecules were deposited and aggregation on the membrane surface At the same time, the higher of loading rate indicate the too much molecules were deposited and aggregation on the membrane surface. The fouling mechanisms were analyzed by Hermia's model. [13] reported, at the beginning step of filtration, the membrane had a higher flux and more enzymes were loaded in the membrane. The decreased of permeation flux was dominated increased of fouling and the loading rate decreased. This is because the membrane surface was engaged by possible adsorption and entrapment site. Based on the Figure 4.1, cake formation was mainly at pH 5.7. For pH 7 and 8 were dominant by blocking mechanisms. At pH 5.7, more enzymes accumulated on the membrane and easiest to the aggregation of enzyme occur at pH nearest its IEP and consequently, cake layer interface of skin and support layer of membrane

B. The effects of pH on reactive membrane performance

After the enzyme was immobilized at varies pH values, these fouled membranes was used to performance the reaction at constant pH 7 and NADH was used as a cofactor for the reaction. As shown in Figure 4.5, during the first filtration, the permeate flux were 34.67 $Lm^{-2}h^{-1}$ for pH 5.7, 52.17 $Lm^{-2}h^{-1}$ for pH 7 and 64.73 $Lm^{-2}h^{-1}$ for pH 8 and slightly steady for each pH after the next filtration. The lowest permeate flux of the membrane during reaction was enzyme immobilization on the membrane surface at pH 5.7; due to largest of irreversible fouling resistance (Figure 4.3) and formation of cake layer. Therefore, the higher of enzyme was assembling on the membrane surface. Even though the enzyme was immobilized on the membrane surface at pH 8 is quietly high but for conversion rate was decreasing during the reaction (Figure 4.6). The previous study [17], effect of pH on the reaction during conversion of Phenazine-1-Carboxylic acid to 2-Hydroxyphenazine at pH 3 to 11, the optimal pH was for conversion was 7. Since the reactive performance was changed the pH to 7, the electrostatic repulsion is weak at lower pH and might be some of enzyme passing through the membrane. Due to the lowest enzyme loading, immobilization at neutral pH produced highest conversion rate.

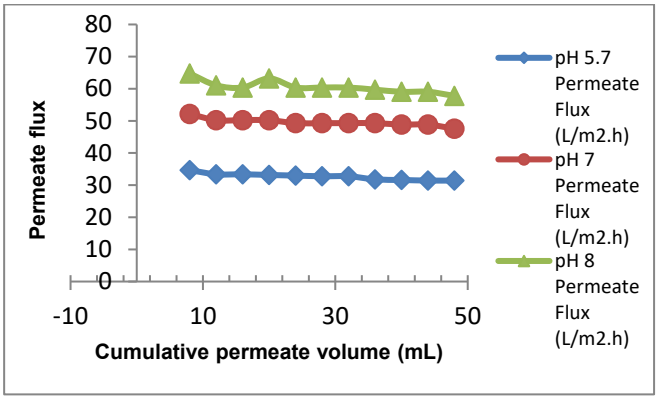

Figure 8: Effect of pH for immobilization on permeate flux

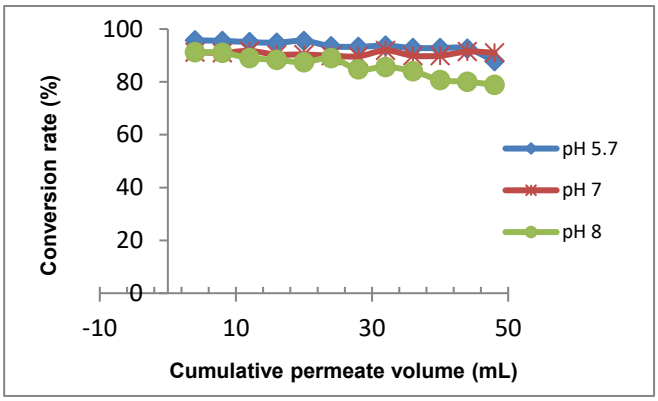

Figure 9: Effect of pH on Conversion Rate

IV. CONCLUSION

According the results presented, at low pH during immobilization, the enzyme was liable to accumulate on the membrane surface due to the electrostatic adsorption. It also produces the high enzyme loading rate but declined flux permeation caused by deposition of enzyme on membrane caused a While when the solution of enzyme at high pH value (away from IEP), the membrane and enzyme was negatively charged and lead to much lesser accumulation on the membrane surface due to the strongest of electrostatic repulsion. Therefore, it will induce to lower fouling on the membrane. At the neutral pH (near IEP), the hydrogen bonding between enzyme and membrane was stabilized and might be lead to maintain the enzyme activity

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