Assessing Enzyme Immobilization on Reverse Asymmetric Membranes and Biocatalytic Reactor Performance

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Assessing Enzyme Immobilization on Reverse Asymmetric Membranes and Biocatalytic Reactor Performance

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Abstract

Integration of membrane filtration and biocatalysis has appealing benefits in terms of simultaneous substrate conversion and product separation in one reactor. Nevertheless, the interaction between enzymes and membrane is complex and the mechanism of enzyme docking on membrane is similar to membrane fouling. In this study, focus is given on the assessment of enzyme immobilization mechanism on reverse asymmetric polymer membrane based on the permeate flux data during the procedure. Evaluation of membrane performance in terms of its permeability, fouling mechanisms, enzyme loading, enzyme reusability and biocatalytic productivity were also conducted. Alcohol Dehydrogenase (EC 1.1.1.1), able to catalyze formaldehyde to methanol with subsequent oxidation of NADH to NAD was selected as the model enzyme. Two commercial, asymmetric, flat sheet polymer membranes (PES and PVDF) were immobilized with the enzyme in the reverse mode. Combination of concentration polarization phenomenon and pressure driven filtration successfully immobilized almost 100% of the enzymes in the feed solutions. The biocatalytic membrane reactor recorded more than 90% conversion, stable permeate flux with no enzyme leaching even after 5 cycles. The technique showing promising results to be expanded to continuous membrane separation setup for repeated use of enzymes.

Keywords: Enzyme membrane reactor, Enzyme immobilization, Membrane fouling, Ultrafiltration, Biocatalytic productivity, Concentration polarization

1. Introduction

Enzyme is a useful biocatalyst that widely use in the industry due its green technology (Wohlgemuth 2010). Enzyme catalyst can increase the rate of reaction up to 10^{20} compared to traditional chemical catalyst that only increase 10^2 to 10^4 (Campbell and Farrel 2015). Furthermore, enzyme is more preferrable because it have selectivity to produce higher preferred product compared to chemical catalyst (Zhang and Xing 2011). Even though enzyme can provide better greener sustainability and very helpful in the industry, the drawback of the enzyme is it is expensive and it is hard to remove from the reaction medium (Donato et al. 2014). Therefore, it need special requirement to use it such as reusability and immobilization to increase the efficiency of enzyme performance and commercialization potential (Luo et al. 2013).

Meanwhile, membrane is a material that consist of semi permeable polymeric material that use for separation of particle from the fluid. The particle that can pass through is based on different pore size and the construction of the membrane such as ultrafiltration, microfiltration and nanofiltration (Schaschke 2014). Ultrafiltration membrane is widely use in industry in drinking water and wastewater treatment process under filtration system (Gao et al. 2016). Recent researches show that membrane work together with enzyme to become biocatalytic membrane (Luo et al. 2020).

Enzymatic membrane reactor (EMR) is one type of membrane technology that integrate the membrane filtration and biocatalysis in one system which the enzyme will immobilize on the membrane (membrane work as support for enzyme). EMR widely use in wastewater treatment and it is a eco-friendly technology because it use mild condition in term of pH, temperature and pressure (Zdarta et al. 2019). It help enzyme to enhance the biocatalysis and remove the product after achieve reaction (Zdarta et al. 2019).

There are a few techniques in immobilizing the enzyme such as adsorption, entrapment, cross-linking, covalent bonding and affinity. Every technique of immobilizing the enzyme has its advantages and disadvantages (Cao 2011; Zhang and Xing 2011; Datta et al. 2013; Nguyen and Kim 2017; Cen et al. 2019). Immobilizing the enzyme can be the solution to control the enzyme drawbacks of purchasing

cost and hard to remove from reaction (Cao 2011). Immobilizing the enzyme have more advantages compared to the free enzyme which it is more stable compared to the free enzyme (Konovalova et al. 2016)(Vitola et al. 2016). It also has more enzyme loading and enzymatic activities compared to the free enzyme (Xu et al. 2017). Plus, immobilizing enzyme on the membrane also can help enzyme improves its reusability. Enzyme reusability is useful in industry because it will be reducing the cost of enzyme purchasing (Amaly et al., 2018). Certain enzymes can withstand its activity in certain reaction cycle. For example, the lipase's enzyme activities decrease slowly and 69% of its activities remain after 10 cycles on nanofibrous membrane (J. Zhu & Sun, 2012).

However, enzyme docking on the membrane causing membrane fouling therefore reducing the membrane performance in term of separation efficiency and permeate flux (Zdarta et al. 2019). There are a few types of membrane fouling such as pore blocking, physical adsorption, gel or cake formation or biofouling (Luo et al. 2014b) (Luo et al. 2014a). Even though fouling reducing the membrane performance, it also a strategy to use membrane weakness to fully utilize the enzyme by improvise the membrane function (work as enzyme support) since the enzyme immobilization and membrane fouling have similarities (Luo et al. 2013).

Since the enzyme can be immobilized on the membrane, we hypothesized that the different type of membranes (on reverse mode) will give different degree of membrane and biocatalytic performance and reusability of enzyme. Reverse mode is when support layer of the membrane facing feed in dead end set up. In this study, the assessment is conducted to investigate the enzyme immobilization mechanism on reverse asymmetric polymer membrane. The set-up of the enzymatic membrane reactor and the enzyme used in the research will be controlled so the membrane performance, biocatalytic performance and enzyme reusability can be evaluate. Enzyme immobilization of ADH is conducted on the propylene support layer of 2 commercial polymer membranes (PES and PVDF) for the development of enzymatic membrane reactor. Mechanism of enzyme adsorption on the membrane fibrous structure is discussed. The performance of the enzymatic membrane reactor is evaluated in terms of the membrane permeability, membrane fouling and enzyme reusability.

2. Methodology

2.1 Chemicals and membranes

Alcohol dehydrogenase from *Saccharomyces cerevisiae* (ADH, EC 1.1.1.1), β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), formaldehyde (37% w/w), potassium phosphate monobasic (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and ethanol (98% v/v) were purchased from Sigma Aldrich (St. Louis, MO, USA). The molecular weights of ADH, NADH, and formaldehyde are 141000, 700, and 30 Da, respectively. KH_2PO_4 and K_2HPO_4 are diluted together in ultrapure water to make 0.1M phosphate buffer solution of pH 7. All enzyme and substrate solutions are prepared by using this buffer solution unless otherwise stated. Commercial membranes used in this experiment are ultrafiltration membranes from Synder Filtration (Vacaville, California, USA). The detail properties of both membranes are summarized in Table 1.

Table 1: Properties of commercial flat sheet polymer membranes.

Characteristics	Information	
Membrane	PES	PVDF
Manufacturer	Synder	Synder
pH range operation	1-11	1-11
Molecular weight cut off (kDa)	50	30
Membrane surface area (cm^2)	13.4	13.4
Skin material	Poly(ether)sulfone (PES)	Polyvinylidene fluoride (PVDF)
Support material	Polypropylene	Polypropylene
Isoelectric point (IEP)	pH 4.9[α]	pH3.5[β]
Contact angle		
Thickness (mm)[γ]	0.16	0.17
Permeability ($L/m^2 \cdot h \cdot bar$)[γ]	72.3	100.0

[α](Zhao et al. 2003)
[β](Schulze et al. 2016)
[γ]Own measurement

2.2 Experiment procedures

2.2.1 Cell setup

The membrane was cut and fixed, reverse mode in a 50 ml dead-end Amicon stirred cell (Milipore, USA). Reverse mode is when the support layer is facing the feed and the skin layer is supported by another polypropylene layer. The membrane was soaked in a mixture of ethanol (50% v/v) for 10 minutes following the manufacturer's instruction. The cleaned membrane will be filtered with ultrapure water at a pressure of 3 bar and 100 rpm in stirred cell in normal orientation (skin layer facing feed). After compression, 50 ml of ultrapure water is introduced in the stirred cell and the membrane is set up in reverse mode to determine the permeability with applied pressure of 1 bar and 100 rpm of stirring.

2.2.2 Enzyme immobilization

The feed for immobilize reaction contained 0.1 g/L of ADH in a 100 mM phosphate buffer solution pH 7. The condition of the stirred cell is in 1 bar pressure and 100 rpm. The time taken for 4 ml aliquot of permeate was recorded and the sample was collected. The samples are then assayed with Bradford Reagent to determine ADH rejection (immobilization) by the membrane using UV-VIS spectrophotometer (Cary60, Agilent, USA) at a wavelength of 340 nm.

2.2.3 Biocatalysis

The reaction feed contained 30 ml of 100 μM of formaldehyde and 100 μM of NADH. The substrates were diluted in 100 mM phosphate buffer solution pH 7. A pressure of 2 bar and 100 rpm stirring was applied. 4 ml aliquot of permeate was collected and the time taken was recorded. The sample was then analysed for the remaining NADH available with UV-VIS spectrophotometer (Cary60, Agilent, USA) at a wavelength of 340 nm. The sample was then assayed with Bradford reagent to analyse for any enzyme leakage in the permeate. The feed was re-introduced for 5 cycles to observe the enzymatic reusability and biocatalytic productivity.

2.2.4 *Contact angle measurement*

The surface hydrophilicity of PES & PVDF membrane is measured by using contact angle goniometer instrument (AST/VCA-3000s). The angle of the water and both membranes is measured. The measurement is taken 3 times for both membranes and the results are compared.

2.3 *Determination of various parameters*

The percentage of enzyme loading is the calculation to determine the efficiency of enzyme immobilizing on/in the membrane. The formula of the percentage of enzyme loading is:

$$Enzyme\ Loading\ (\%) = \frac{m_i}{m_f} \times 100 \qquad (1)$$

where m_i is the amount of the enzyme that immobilized and m_f is the amount of enzyme in the feed solution.

Flux recovery ratio (FRR) is a calculation to identify the type of fouling resistance that forms on the membrane surface by taking the flux of the permeate. The formula for FRR is:

$$FRR(\%) = \left(\frac{J_{w2}}{J_{w1}}\right) \times 100 \qquad (2)$$

Total fouling ratio (R_t), reversible resistance (R_r) and irreversible resistance (R_{ir}) are calculated as follow:

$$R_t\ (\%) = \left(1 - \frac{J_p}{J_{w1}}\right) \qquad (3)$$

$$R_r\ (\%) = \left(\frac{J_{w2} - J_p}{J_{w1}}\right) \qquad (4)$$

$$R_{ir}\ (\%) = \left(\frac{J_{w1} - J_{w2}}{J_{w1}}\right) \qquad (5)$$

Where J_p = permeate flux; J_{w1} = initial pure water flux and J_{w2} = final pure water flux

The conversion rate of reaction is calculated to determine the conversion of NADH by the enzyme.

166 The formula of the conversion rate is:

167 $$Conversion\ rate\ (\%) = \frac{C_f - C_p}{C_f} \times 100 \qquad (6)$$

168 where C_f is the concentration of NADH in the feed solution and C_p is the concentration of NADH in
169 permeate solution.

170 Biocatalytic productivity is a calculation to calculate the efficiency of the enzyme converting substrate
171 to product. The formula for the biocatalytic productivity is:

172 $$Biocatalytic\ productivity = \frac{m_p}{m_e} \qquad (7)$$

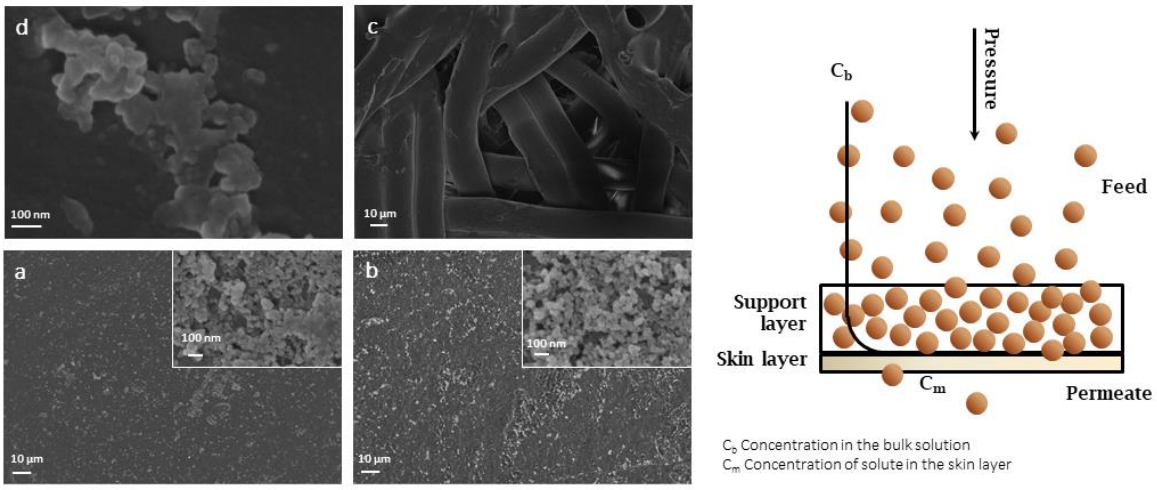
173 where m_p is the mass of the production and m_e is the mass of the enzyme.

174

175 **3. Results and Discussion**

176 *3.1 Biocatalytic membrane reactor configuration and mechanism of enzyme immobilization*

177 Two types of commercial membranes, PES and PVDF were used in this work. The polymer membranes
178 are asymmetric, characterized by its anisotropic structure, comprised of two main layers with different
179 properties in morphology and permeability. The active layer with the MWCO specification is denoted
180 as the skin layer (Figure 1a&b), whereas the fibrous structure (Figure 1c) is the support layer.
181 Experiment is designed to immobilize ADH in the support layer of the membrane. Utilising reverse
182 asymmetric membrane will cause concentration polarization and subsequently leads to membrane
183 fouling (Guerra et al. 1997). This is advantageous in ensuring maximum enzymes can be immobilized
184 and preventing enzyme from leaching out because of the dense feature of the skin layer (Figure 1)
185 (Marpani et al. 2015). As shown in Table 2, both membranes successfully immobilized almost 100%
186 of enzymes in the feed solution. There was no leaching out of enzymes observed throughout the
187 experiment.



188

189 Figure 1: Illustration of concentration profile for feed stream at the surface of the support layer. Inserted
190 SEM images of the polymer membrane, (a) PES skin layer; (b) PVDF skin layer; (c) the membrane
191 support layer; (d) closed-up image of the membrane support layer.

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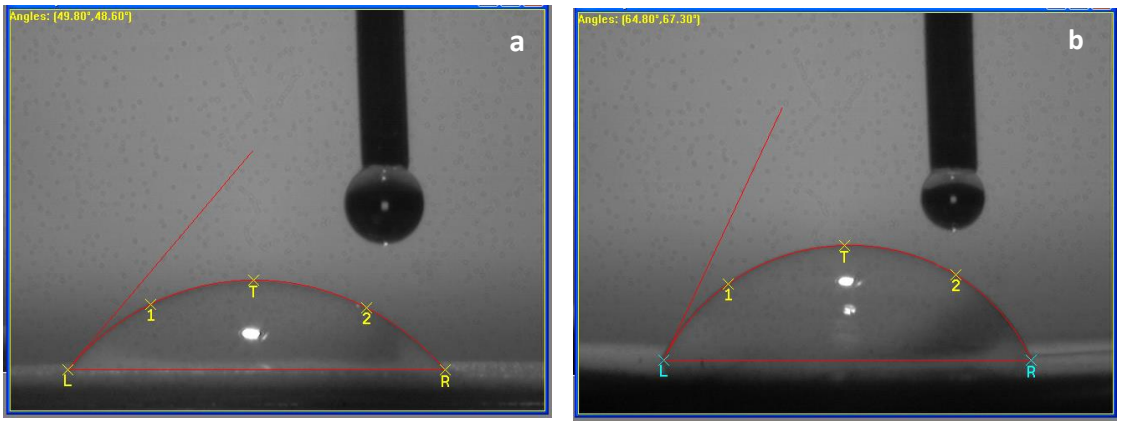

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194 Figure 2: Contact angle measurement for a) PVDF and b) PES membranes.

195

196 Table 2: Enzyme loading on PES and PVDF membrane.

Membrane	Amount of Enzyme (mg)					Percent Loading (%)
	Feed	Permeate	Retentate	Washing	Loading	
PES	3	0.0028	0.0174	0.0058	2.9741	99.14
PVDF	3	0.0007	0.0780	0.0455	2.9667	98.89

During the filtration of ADH, a high local concentration of enzyme will arise near the support layer-solution interface, due to a balance between the convective drag force towards and through the membrane and back transport away from the membrane (Figure 1). This phenomenon is called concentration polarization (CP). CP will result in flux decline due to the increment of the osmotic pressure near the surface of the membrane. Hence, the effectiveness of pressure-driven filtration is reduced. Figure 3 shows the flux profile during enzyme immobilization which shows 3 phases of flux decline related to enzyme deposition mechanism in/on the membrane. The initial phase (Phase 1) includes macromolecular sorption and particle deposition. Fresh membrane is exposed to the ADH and adsorb onto the polypropylene fibrous strand (Figure 1c&d). Part of the ADH may aggregate because of electrostatic forces (from ionizable chemical species in the buffer) and build up in between the support and skin layer interface. These aggregates then served as nucleation sites for the continued deposition of other enzymes. As can be observed in Figure 1a&b, the skin layer is dense, and the pores are too small for the ADH to pass through. In Phase 2, the first sublayer developed into multi-sublayers and increase the osmotic pressure which in turn compresses the sublayers. Permeate flux decline is more prominent at the end of this phase. In the final phase (Phase 3), the ADH in the sublayer rearrange themselves and finally stabilized. All enzymes are supposed to be immobilized at this stage.

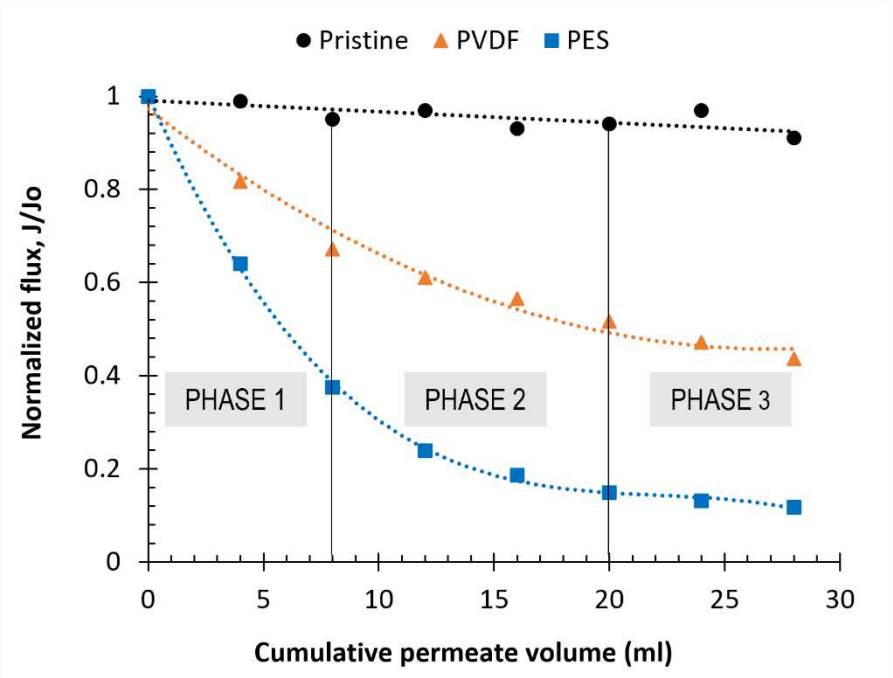

Figure 3: Flux profile during enzyme immobilization showing 5 different phases.

Another mechanism responsible for the docking of ADH on the surface of the membrane is charge interaction. Membrane charge is significant separating factor for ultrafiltration, nanofiltration and reverse osmosis membranes (Oatley-Radcliffe et al. 2017) driving the attachment of ADH enzymes in the membrane support layer. Membrane is composed of polymer repeating units (Scheme 1). PES for example, composed of the SO_3H functional group, a strong acidic group, which dissociates over a very wide pH range (Oatley-Radcliffe et al. 2017). Membrane develop charge by the adsorption of charged elements from dissociation of functional groups of the membrane (polyelectrolytes), ions in the buffer solution and macromolecules (ADH and respective substrates). These charged species physically adsorb to the surface of the membrane through van de Waals forces (Childress and Elimelech 2000). The buffer solution used in this experiment is at pH 7. The isoelectric point (pI) of ADH, and propylene support layer, PES and PVDF skin layer are 5.4-5.8 (Luo et al. 2014a), 3.3 (Smole et al. 2009), 4.9 (Zhao et al. 2003) and 3.5 (Schulze et al. 2016) respectively. The ADH and the membrane elements will be negatively charged and should cause repulsion between the membrane and the charged solute of feed solution. Nevertheless, the membrane skin layers are both hydrophilic. Hydrophilic surfaces reduce

electrostatic charge accumulation (Omastova 2016) and combined with the pressure driven dead-end filtration aid in the docking of enzyme in/on the membrane.

The permeate flux of PES and PVDF membranes decreases as the cumulative volume of permeate increasing. This is because of the increasing of concentration polarization that happens on the surface of the membrane. The concentration polarization is increase when the fouling on/in the membrane increase (Giacobbo et al. 2018). In our study, this is desirable because it indicates that the enzyme is successfully adsorbed and fouled the membrane.

3.2 Evaluation of membrane fouling

As described in the previous section, the governing mechanism of membrane fouling due to enzyme immobilization procedure in this study is macromolecular adsorption. Two models were applied to evaluate membrane fouling in this study, namely the flux recovery ratio (FRR) and Hermia's model. Hermia's model is used because it is the most comprehensive prediction models presenting membrane fouling scenario which includes complete blocking, standard blocking, intermediate blocking, or cake layer (Chang et al. 2011; Wang et al. 2012). Furthermore, it suitable to use for the dead-end membrane separation (Ismail et al. 2021). FRR measures antifouling properties of the membrane. Generally, a lower FRR indicates serious membrane fouling. PES obtained higher percentage FRR with 88.6%, compared to PVDF with 66.1% (Figure 4). This could indicate that the fouling in PES is more critical than PVDF, even though both membranes load almost the same amount of enzymes (Table 2).

As shown in Figure 4, PES and PVDF membranes have a higher percentage ratio of reversible resistance with 39.24% and 12.25% respectively, compared to the irreversible resistance with 33.92% and 11.45% respectively. Reversible resistance is where the fouling agents (ADH) that are loosely attached to the membrane and possible to be desorbed to the bulk solution. On the other hand, irreversible fouling indicates that the fouling agents are tightly bound to the membrane and no possibility of desorption to the bulk solution. A lower total resistance ratio (R_t) will cause a higher FRR resulting in a lower total flux loss as compared to the pristine membrane.

From the regression data summarized in Table 3, intermediate fouling (Figure 4b) is predicted using Hermia's model for both membranes suggesting that each foulant has a probability to either deposit on an unobstructed area of the membrane or deposit onto a previously deposited foulant particle (Kirschner et al. 2019). This is in line with the estimation of total resistance where it was found that reversible fouling is higher than irreversible fouling in PES and PVDF membrane.

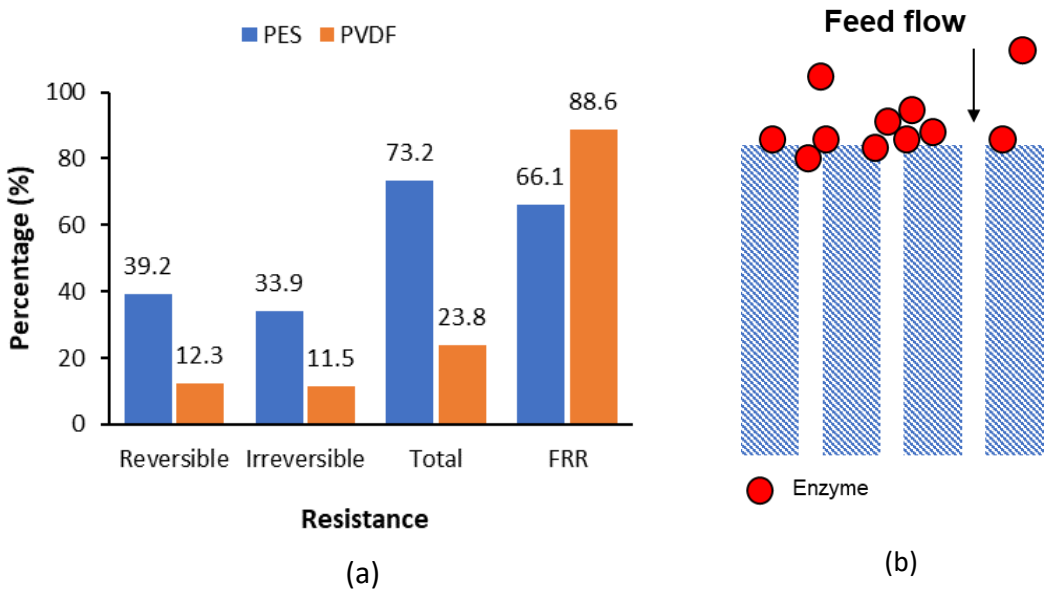

Figure 4: (a) Membrane resistance and flux recovery ratio (FRR) of PES and PVDF membranes and (b) Illustration of intermediate fouling by Hermia's model.

Table 3: Regression data to determine membrane fouling mechanism according to Hermia's model.

Membrane	Membrane Fouling Mechanism			
	Complete	Standard	Intermediate	Cake
PES	0.9597	0.9901	1.0000	0.9685
PVDF	0.9974	0.9994	1.0000	0.9974

3.3 *Enzymatic membrane reactor performance*

The permeate flux of PVDF membrane is higher compared to the permeate flux of PES membrane during reaction (Figure 5). Both permeate flux profiles are stable at and have the same value as permeate flux at the end of Phase 3 during enzyme immobilization procedure (Figure 2). Even though the MWCO of PES is larger than PVDF (Table 1), the permeate flux profiles are contradicted due to the enzyme immobilization mechanism as discussed in section 3.1.

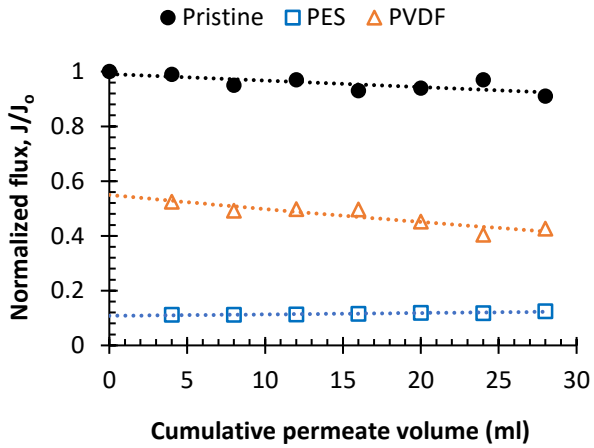

Figure 5: Flux profile during the reaction.

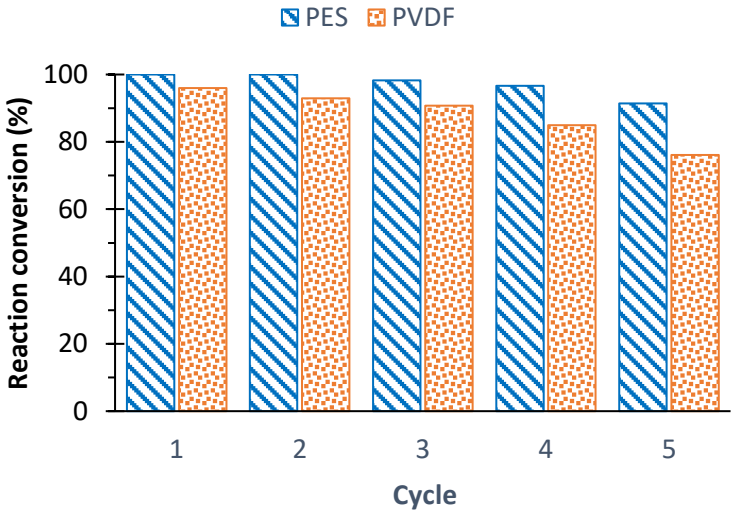

Figure 6: Percentage conversion of formaldehyde to methanol for 5 cycles.

281 Figure 6 shows the percentage reaction conversion of formaldehyde to methanol for 5 cycles. The
282 average conversion for PES membrane is higher than PVDF membrane. ADH enzyme on the PES
283 membrane can retain its activity above 80% within five cycles while the ADH enzyme on the PVDF
284 membrane can retain its enzyme activity above 50%. In previous research, ADH retain its activity at
285 20.1% and 79.6% of its original activity on MNP-ADH and MGO-ADH membrane respectively (Liu
286 et al. 2015). Despite the amount of enzyme immobilized is almost the same for both membranes (Table
287 2), the productivity is not corresponded to it. Biocatalytic productivity for PES and PVDF membrane
288 is 5.2 and 4.7 mg methanol/mg ADH respectively. This is very much related to the arrangement of
289 enzymes in/on the membrane due to the enzyme-enzyme and enzyme-membrane interactions. Protein
290 molecules are surrounded by a hydration shell in solution (Chen and Sun 2003). In the presence of salt
291 (buffer) and ionizable chemical species, the hydration shell will be stripped off from the protein
292 molecule due to the hydration effect of the salt molecules of the protein environment (Lin et al. 2000).
293 This will result with an exposed area of hydrophobic zones of the enzymes making the hydrophobic
294 interactions between the ADH and the adsorbent surface (polypropylene) become stronger. The
295 hydrophobic interactions will induce conformational changes of the enzyme structure thus altering its
296 activity. It is also believed that high permeate flux during reaction by a more hydrophilic PVDF
297 membrane, thus some of the substrate not in contact with enzyme while passing through the membrane.
298 Slower permeate of PES during immobilization would distribute enzyme evenly on the polypropylene
299 fibre strands and skin-support interface, hence slower permeate flux during reaction ensuring optimum
300 contact time with substrate.

301

302 **4. Conclusion**

303 The fibrous structure of asymmetric membrane support layer has the potential to be exploited as the
304 enzyme immobilization matrix. Almost 100% of enzymes in the feed were successfully immobilized
305 following adsorption mechanism and charge interaction. A combination of concentration polarization
306 effect which is critical in dead end filtration method and convective transport, drive the mass transfer
307 of the enzymes and further, aided in the docking. There are three phases of permeate flux decline which

308 are strongly related to the process of enzyme adsorption mechanism in the membrane, following this
309 pattern: molecular sorption and particle deposition – development of multi-sublayers – sublayers
310 rearrangement and stabilization. Ionizable chemical components and membrane surface, van der Waals
311 force, isoelectric point are consolidated factors, responsible for the membrane charges interaction which
312 further aid in the enzyme's entrapment. Membrane fouling is described via Hermia's model and flux
313 recovery ratio. Both models conclude that intermediate fouling dominates. Reversible fouling is higher
314 than irreversible for both membranes, but the intensity of irreversible fouling is more prominent in PES,
315 indicating fouling is severe in PES. The system showed stable, high enzyme conversion of more than
316 80% of formaldehyde to methanol in five cycles. This promotes a good biocatalytic productivity data
317 for the enzymes proving that enzyme immobilization in the reverse asymmetric membrane feasible to
318 be applied in other biocatalytic reactions.

319

320 **Declarations**

321 *Ethics approval and consent to participate:* Not applicable

322 *Consent for publication:* Not Applicable

323 *Availability of data and materials:* Not applicable

324 *Competing interests:* The authors declare that they have no competing interests.

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329 *Authors' contributions:* ASZ conduct the experiments, collecting data for analysis writing the
330 manuscript. FM secured the grants, supervising ASZ, research framework and manuscript writing. SMP
331 and ANR partly writing the manuscript Section 3.2. HC responsible on polishing the introduction
332 section. NHO and NHA advise on the structure of the manuscript and proof reading.

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