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Microfluid on Chip Transesterification Reaction and Real-Time Monitor by PN Diode Photodetector

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Biodiesel is a renewable fuel made by a chemical reaction of alcohol and vegetable or animal oils, fats, or greases. Through a refinery process called transesterification. Biodiesel has been demonstrated produced by transesterification reaction by the addition of enzyme, i.e. Candida rugosa lipase [1]. In this study, we self-assemble lipase on the gold film through surface modifications. Under the gold film, a PN photodetector is pre-fabricated, and polydimethylsiloxane (PDMS) is used as a fluid channel and reaction chamber of transesterification process. Since PDMS reactor is optically transmittance, a specific wavelength of light can be used to detect transesterification reaction. The monitor for biodiesel production can be detected by means of transmittance (T %) taken as an indicator to estimate the conversion yield of the transesterification reaction. In this study, the on-chip PN diode is used as a photo detector to detect the conversion of biodiesel. As a result, the lipase-catalyzed immobilized on the Au/PN diode and a micro-fluidic device as a transesterification reaction chamber is proposed for real-time monitoring through transmittance characterization.

Keywords: biodiesel; microfluid; transesterification reaction; PN diode photodetector; real-time monitoring.

1. INTRODUCTION

Transesterification of triglycerides (vegetable oils and animal fats) to generate esters and glycerol to obtain a new engine fuel (i.e. biodiesel) is a well-prove process. Renewable biomass has also been considered as potential feedstock for vegetable oils to produce biodiesel. The biggest advantages of biodiesel as diesel fuel are liquid nature portability, ready availability, renewability, higher combustion efficiency, lower sulfur and aromatic content, and higher biodegradability [2]. However, the process catalyzes transesterification reaction often utilizes chemical methods, which produce biodiesel and glycerol in the presence of a strong acid or base catalyst, in which normally be

H_2SO_4 and NaOH or KOH respectively [3]. Hence, this procedure uses too many chemicals and is not environmental friendly.

As the energy is short and contamination problem around the world recently, the regeneration, biocompatibility, biodegradability, and environmental acceptability of biomass become noteworthiness theme. The enzymatic one is lipase-catalyzed which has the advantage of reaction at room temperature, no water waster and environmental friendly [1, 4]. However, there are some challenges for biodiesel fuel production, with immobilized lipase, from the following reasons: (i) reaction efficiency is poor and (ii) a large amount of enzyme is necessary. In the study, we design and fabricate a microfluidic system owing to its higher surface-to-volume ratios and lower reagents consumption in order to enhance the throughput of synthesis and chemical production [5]. We also use the immobilized lipase because the lipase catalyst could be reused in the immobilization technology. Our goal is to develop a cheap and portable microreactor for production of biodiesel with real-time measurement. The microfluidic reactor needs much smaller sample volumes in analysis systems technology. This system demonstrates the capability to facile measurement, which can be very importance for future real-time and portable applications [6].

2. EXPERIMENTAL

2.1 General information

All reagent and solvent were of reagent-grade quality, purchased commercially, and used without further purification unless otherwise noted.

2.2 Experimental procedure of lipase-immobilized PN diode chip

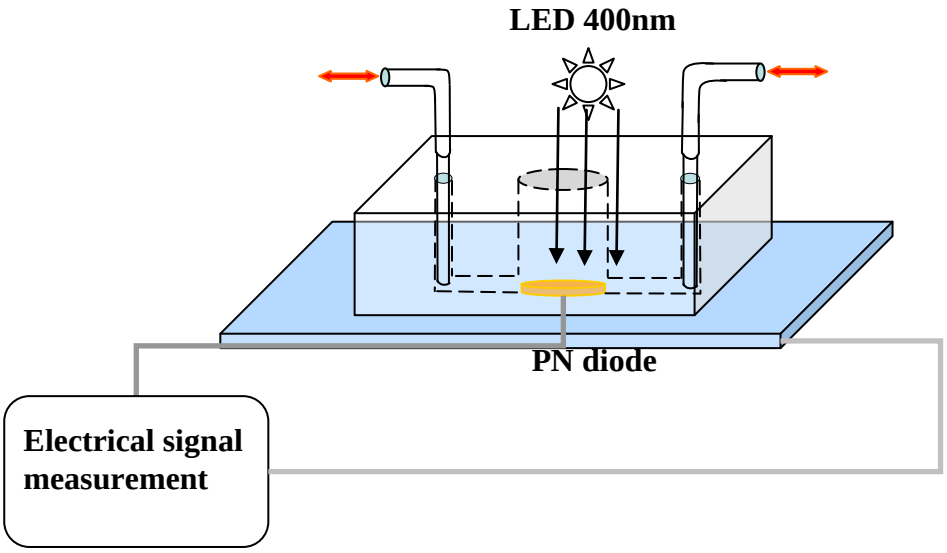


Figure 1. Concept of photoelectrical approach of on chip transesterification reaction detection technique.

The analytical concept based on transmittance assay and electrical detection is shown in Figure 1. To start with this study, PN textured diode is fabricated. At first, KOH/IPA solution is used to etch P-type silicon surface as texture morphology. Subsequently, the phosphorus (P) implantation is used for PN junction formation. Then, backside surface is metalized with Ag and front side also with Ag finger printing. In order to lipase immobilization, the result chip is sputtered 5 nm thin gold layer in front side. The purpose of gold with textured surface is to improve the immobilization sites and strength for further immobilized steps.

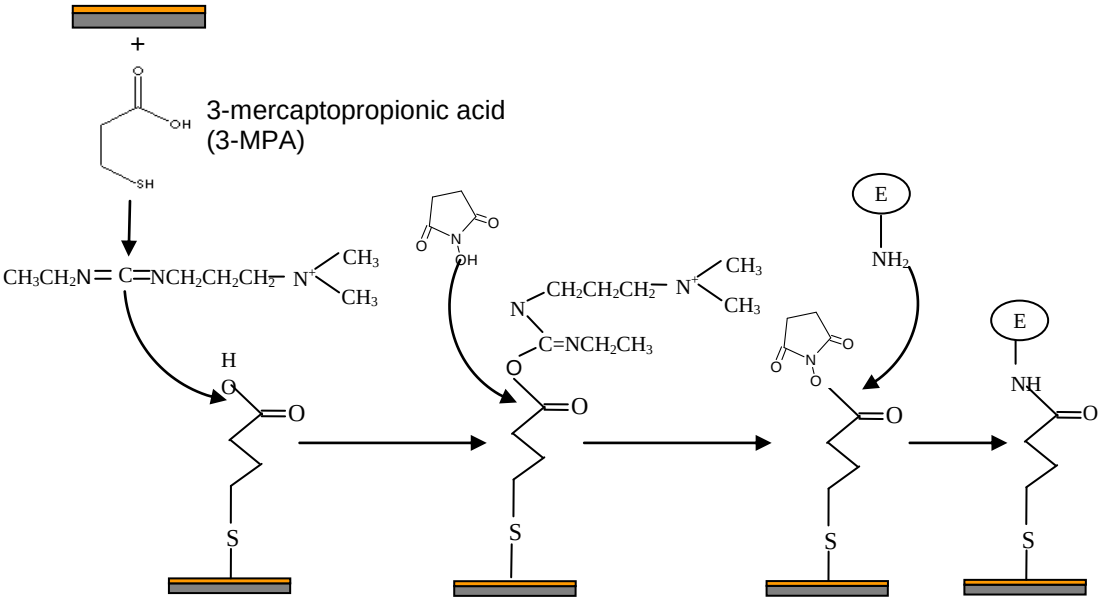
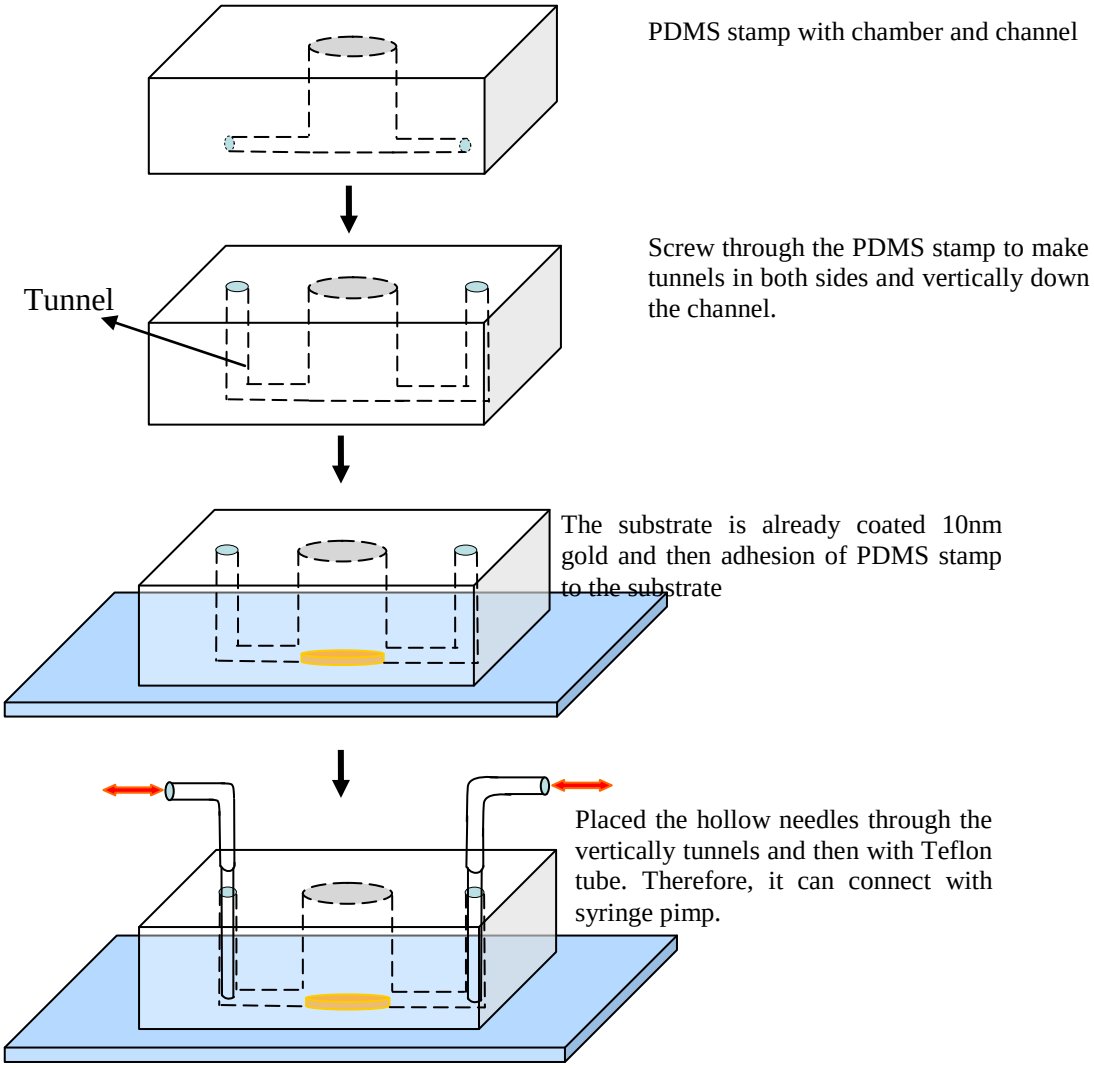


Figure 2. The immobilization sequences of the 3-MPA, EDC, NHS and lipase.

In this study, lipases-immobilized on the PN diode is self-assembled through covalent bonding. The immobilization procedure and the process is shown in figure 2, Firstly, 3-mercaptopropionic acid (3-MPA) acted as an anchor layer is applied onto gold substrate through covalent bonding. This design is because the thiol group (-SH) of 3-MPA is a strong bonding with gold surface conjugated S-Au linkage[7]. The terminal carboxylic acid groups of self-assembled 3-MPA are activated to the *N*-hydroxysuccinimide (NHS) ester, followed by reaction of this 3-MPA-NHS ester monolayer with the amino groups of lipases to create multiple amide bond linkages to the surface. This is accomplished by mixing the NHS with a carboxyl containing molecule and a carbodiimide coupling agent such as 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC). A "zero-length" cross-linking reagent has been routinely used to form stable and covalent protein/protein complexes through the formation of amide bonds between complementary amino and carboxyl groups [8, 9].

2.3 Fabrication of PDMS transesterification reaction chamber



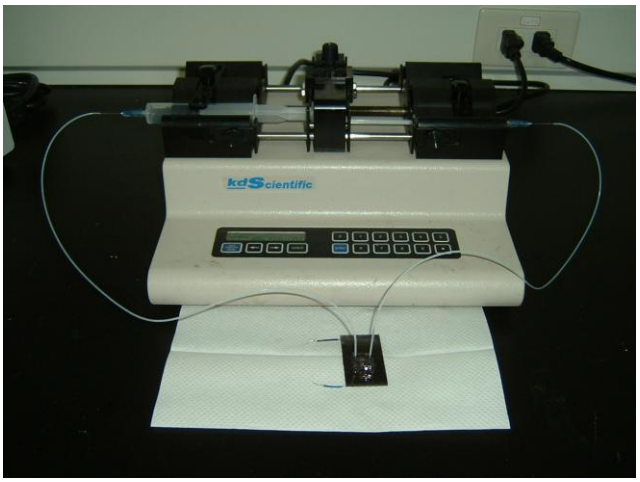
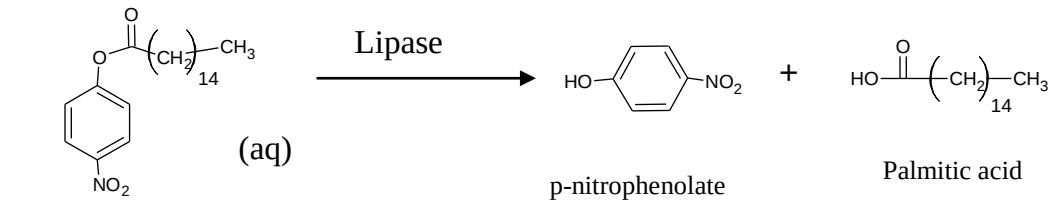


Figure 3. (a) Fabrication process of a PDMS micro-reactor, (b) The scheme of microfluidic system and syringe pump.

PDMS is the choice of material for optical transparency, elasticity, durability, flexibility, and bio-compatibility for microfluidic reaction chamber with biological sample [10]. The details of the whole fabrication process and integration the microfluidic fabrication process are introduced. The fabrication process for PDMS reaction chamber is showed in figure 3(a). Firstly, the PDMS template is designed and fabricated. Then, screw through the PDMS stamp to make tunnels in both sides and vertically down the channel. The adhesion procedure of PDMS stamp to substrate is conducted. Finally, putting hollow needles through the vertically tunnels and then with Teflon tube assembly. The needle is 0.6 mm with outside diameter (O.D.) and Teflon tube is 0.56 mm I.D. and 1.16 mm O.D. The scheme of microfluidic system and syringe pump (Model 270 Series, KD Scientific Inc., Holliston, USA) is showed in figure 3(b). The volume of rector chamber is 0.0981 cm^3, and we set the parameters of syringe pump is 0.06 ml of volume and 0.15 min/ml for fast and repeat pumping forward and reverse, in order to well mixing the reactant.

2.4 Measurement of lipase activity and transesterification reaction

The enzymatic activity of lipase can be measured by nitrophenyl derivatives. Many enzymatic activities, involving glycosidase and phosphates, are carried out with nitrophenyl derivatives as substrates. The nitrophenol (*o*- or *p*-) released by hydrolysis of these substrates contains a variable amounts of the nitrophenoxide (*o*- or *p*-) anion which absorbs at a conveniently accessible wavelength (400–420 nm) [11]. In this study, the lipase activity is measured by a colorimetric assay in which the cleavage of p-nitrophenyl palmitate (p-NPP), and a yellow water-soluble product, p-nitrophenolate (p-NP), is formed by the hydrolysis of pNPP. The general chemical reaction is demonstrated in the following equation:



The lipase is incubated for a while, and then the substrate (p-NPP) is added to this medium to quantify the activity of lipase. One unit of lipase activity is defined as 1.0 nmole of p-NP released/min. Percentage of relative specific activity (RSA) is calculated as the ratio of specific activity of free or immobilized enzyme to the specific activity at the optimum conditions shown in equation.

$$\text{Relative specific activity (\%)} = \frac{\text{Specific activity of immobilized enzyme}}{\text{Specific activity of free enzyme}} \times 100$$

As mentioned, this study is focused on microfluidic reactor with textured PN diode. Once optimized with immobilization of *lipase*, the microfluidic reactor is applied to detect transesterification reaction of pumping through a channel. Transmission measurement is done through the wavelength from 700 nm to 300 nm. Since the transmission increases with the transesterification reaction, we can detect optical responses with time progress. Figure 4 shows the detection of transmission with different incubation time. Obviously, the transmission increases with reaction time.

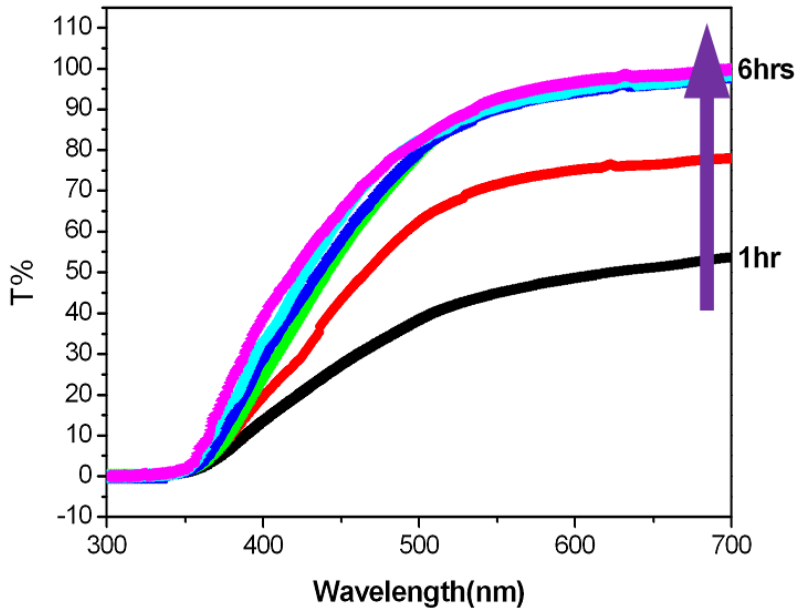


Figure 4. Transmittance characteristics of each reaction time (1 to 6 hours).

In addition to the UV-Vis transmission characterization, the nuclear magnetic resonance (NMR) spectroscopy is given to exploit the magnetic properties of certain nuclei and identify the carbon-hydrogen framework of the compound during the transesterification reaction. All of the ^{1}H-NMR experiments are performed on a Varian Unityinova 500 NMR spectrometer with description 5 mm, 7" length tubes, and the solvent is d-Chloroform ($CDCl_3$). The typical frequency shift might be 500 Hz, and the chemical shift is generally expressed in parts per million (ppm). That means the percentage yield (weight conversion) is defined as ("ppm of biodiesel" ÷ "ppm of initial peanut oil")×100% and the percentage yield is estimated using peak area integrated by NMR spectrum [12].

3. RESULT AND DISCUSSION

3.1 Optimization of immobilized lipase

Enzymes are biomolecules that catalyze the chemical reactions. Enzymes may be unstable and may not have the optimal activity. Since enzymes may become inactivated when localized to a

different environment, higher temperature or extreme pH value, during immobilization process. Furthermore, an optimized enzymatic process can have the effect of higher enzyme activity and stability for biodiesel manufacture to improve the conversion yield.

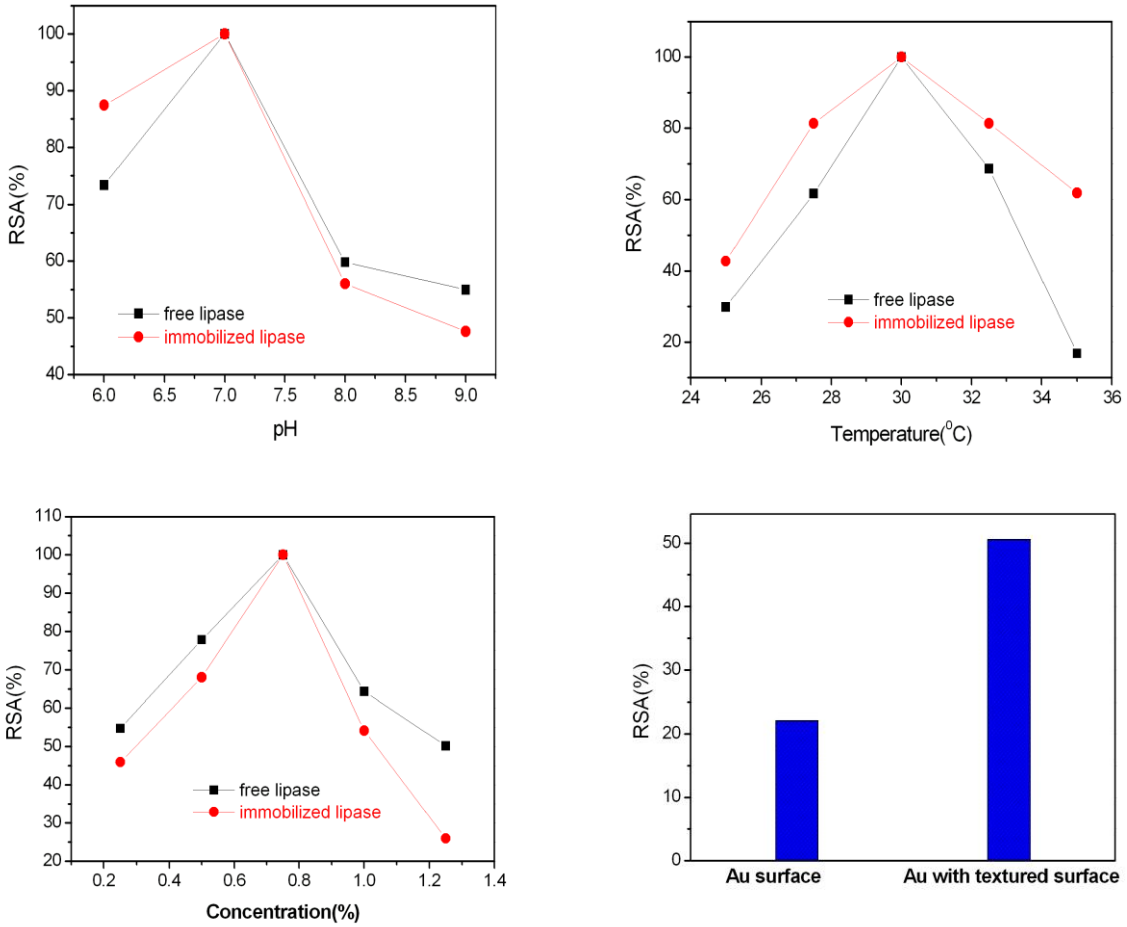


Figure 5. Relative specific activity (RSA) of comparison characteristics of free and immobilized lipase (a) with different pH values, (b) with different temperatures, (c) with different lipase concentrations, and (d) different substrates.

Firstly, pH value optimization is studied. The pH stabilities of free lipase and immobilized lipase are compared in the range of pH 6~9 shown in figure 5(a). They are incubated for 1 hr at 25°C then determining the activity at its optimum pH. The pH-stability profiles of the two enzyme preparations are stable at pH 7. The RSA is calculated considering the lipase activity at highest specific activity as 100 %. At the condition of pH 6, acidic environment, the amino groups of lipase may be protonated (NH_3 to NH_4^+) [13], and decreasing the lipase activity. However, when pH value is higher than 7, lipase activity will decrease because of the lower stability in basic environment.

As for temperature optimization, measurements of enzyme activity are carried out in standard reaction mixture at different temperatures covering the range of 25–35°C. Thermal stability is investigated by incubating the free lipase and lipase immobilized at pH 7 for 1 hr and then determining the activity at its optimum reaction temperature. As shows in figure 5(b), with the temperature increasing, the lipase activity will raise until 30°C. It is attributed to the collision frequency enhance of

lipase molecule in the reaction. However, when temperature raises over 30°C, thermal energy is enough to destroy the protein conformation and denature the protein which itself is temperature sensitive. This suggests that the temperature increase may cause the change in lipase binding mechanism.

Interestingly, in figure 5(b), the immobilized lipase has better stability than free lipase under various temperatures. This may base on the assumption that there is microenvironment between the lipase and substrate. Microenvironment surrounds the active site of lipases is the one mainly involved in the solid surfaces. Thus, lipases recognize the functional surfaces to those of their chemical environment and they suffer interfacial activation during immobilization [14].

In order to study the effect of the water content in the reaction mixture, we investigate the efficiency of immobilization at a range of enzyme concentrations to determine the immobilization capacity of the lipase. The immobilization lipase is discussed for enzyme concentrations from 0.25 % to 1.25 % under pH 7 and 30°C for 1 hr shown in figure 5(c). As the concentration increasing, the lipase activity will increase until 0.75 %, and the activity of free lipase and immobilized lipase are 4.938 U/ml and 0.361 U/ml, respectively. The lower concentration will diminish the collision frequency of lipase molecule in the reaction compared to higher concentration. After the concentration of 0.75 %, the lipase activity will decrease with increasing concentration. This means the quantification of lipase-immobilized on the substrate may be achieved the saturation situation, and not advance the lipase activity. According to figure 5(c), we determine that the lipase concentration of 0.75 % is suitable for this experiment.

In this experiment, the surface roughness of PN diode is also studied. The best condition of anisotropic texturization is etching under 1.19 % tetramethylammonium hydroxide (TMAH) and 50 % of isopropanol (IPA) solution for 120 min. There is a quantitative analysis of lipase-immobilized on the substrate with and without anisotropic texturization shown in figure 5(d). This experiment is repeated operational for 10 cycles and measured the effect of immobilized-lipase activity. The reusability of lipase activity immobilized on the textured surface is found and the activity of immobilized-lipase improves with 28.48 %. With the enhancement of lipase-immobilized and lipase activity under the optimal immobilization process, it improves the conversion yield of transesterification reaction as well.

3.2 Calibration of transesterification conversion yield

We have known that the ^{1}H-NMR spectrometers can be used to detect the transesterification reaction. In this study, the reaction is discussed on the microfluidic reactors. Here, the microfluidic reactor with texturized substrate is pumped continuous, and the reaction solution is collected each hour for preparing ^{1}H-NMR sample as shown in figure 6. The figure shows the conversion increase with transesterifacation reaction progress. The experiments processed in microfluidic reactor and the conversion is much higher compared with other lipase biocatalysis. [63] Duo to the optimal texturization process for increasing the surface and activity of lipase-immobilized and microfluidic platform for increasing the surface-to-volume ratio, the resultant conversion yield of transesterification reaction is expected to improve.

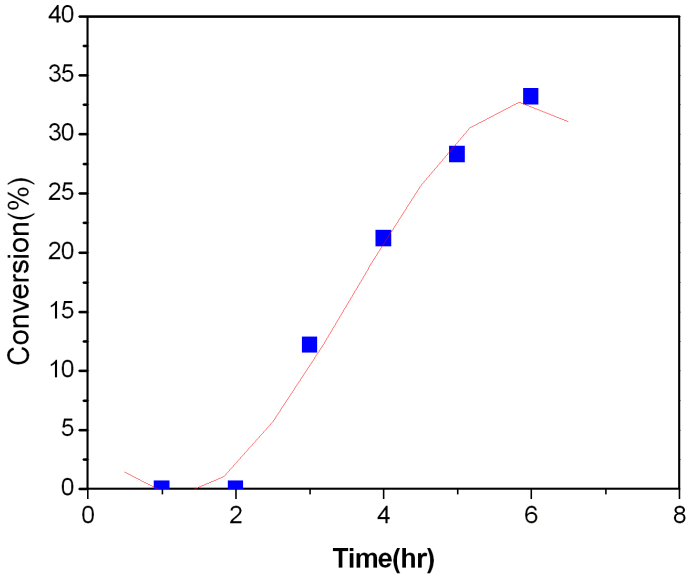


Figure 6. Conversion yield–time characteristics of 1H-NMR measurement with transesterification react from 1 to 6 hours.

Application of enzymatic microreactors usually allows for continuous real-time monitoring of reaction progress. Here we develop an optical biosensor to monitor the biocatalysis in the microfluidic system. The optical biosensor is composed of the microfluidic reactor which is exposed to 400 nm light source and the output transducer is measured the photo-electrical signal. When the light is passing through the PDMS mold, the catalysis is evaluated in the exposed regions. The incident light of 400 nm is amethyst LED with InGaN material, 80 mcd of brightness, and operation at 20 mA of the current and 3.5 V of driving voltage.

The system utilizes a photo-electric reaction to detect the transesterifiaction reaction catalyzed by immobilized lipase on the PN diode. In figure 7, the current of the experimental steps and reaction time is collected. Once the lipase is immobilized on the substrate and exposed to the 400 nm, photo current of the PN diode was decrease from 75 μA to 5 μA, due to that lipase attachment of the device. With time increasing, the current increases from 5 μA to 50 μA.

After pumping the feedstock into the microfluidic system, the photo signals decrease attributed to the less transparent characteristic caused by samples mixing. This observation also is revealed in UV-Vis transmission spectrum at the first two hour in figure 4. After mixing, the photo current signals increase gradually with time. The photo current here represents the amount of 400 nm light passed through product of transesterification reaction. Therefore, the gradually increasing photo current indicates the increasing amount of biodiesel, the product of transesterification. This method allows photodetector to real-time monitoring amount of biodiesel by the electric signal. From the figure 7 curve, we can found the microfluidic platform has the sensitivity to detect the transesterification by lipase-catalysis. The results also present in the detecting and analysis by UV-Vis spectroscopy and ^{1}H-NMR spectrum which discussed early.

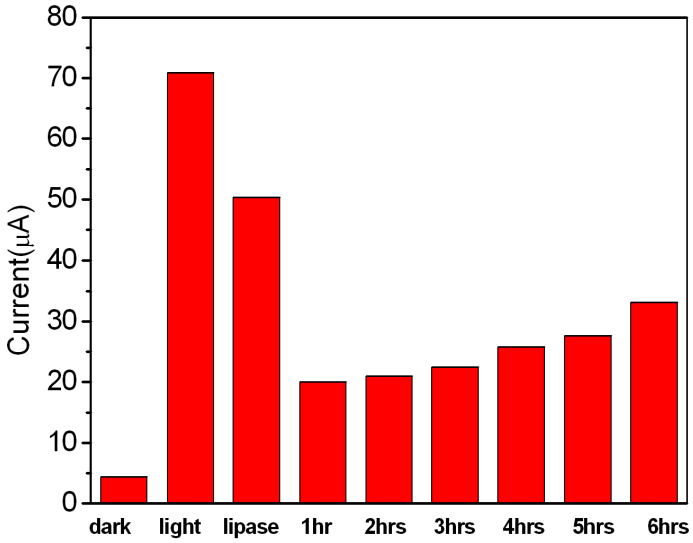


Figure 7. PN diode current characteristics plot of dark, light, lipase immobilized and transesterification react from 1 to 6 hours.

As mentioned before, the activity of the lipases after surface immobilization can be evaluated by 400 nm wavelength. This method can detect the different of transmission by the UV–Vis spectrophotometer and collect the electrical signal of solar cell for 6 hours. As the results of reaction catalysis, the detection of current increases with the transmission changes with time and enhance the conversion of reaction. The connection between the three detecting method is studied and shown in the figure 8.

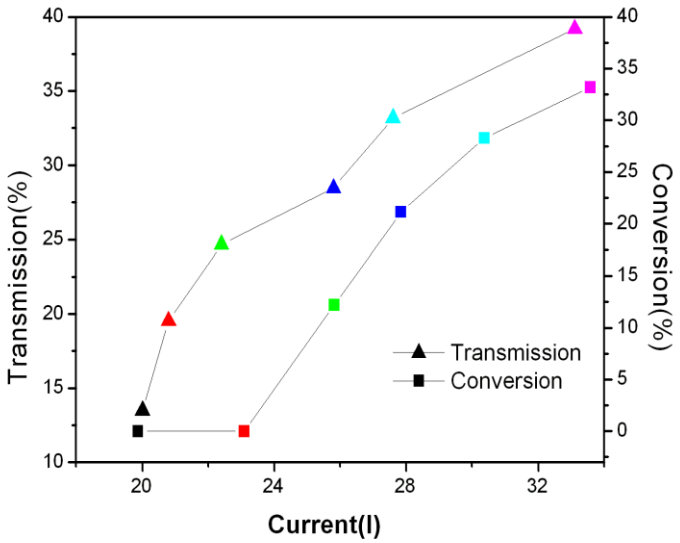


Figure 8. The relationship of the transesterification reaction compared with the current by PN diode, transmission by UV-Vis and conversion yield by NMR. The curves with (▲) and (■) represent the relationship with transmission v.s. current and conversion yield v.s. current respectively. The symbols represent transesterification react from 1 to 6 hours.

There is no obvious change of conversion at first two hours by NMR measurement, due to there is no reaction during solution emulsifying and mixing. Interestingly, UV-Vis and PN diode both get the signals of transesterification reaction. The analysis methods of UV-Vis and PN diode are based on incident optical light passing through reaction reagent that scattering or adsorption light. Therefore, the transparent changes by emulsifying and mixing affect the signal greatly. We can conclude that real-time and UV-Vis detection method have an analysis dead time of 2 hours caused by solution emulsifying and mixing. After that, the transesterification reaction can well analyzed by UV-Vis, real-time PN diode, and NMR. The real-time photodetector system can meet the UV-Vis and NMR data well and more powerful and easy operation for on-line monitoring transesterification reaction.

4. CONCLUSIONS

In this study, a novel photoelectrical approach of on chip transesterification reaction detection technique has been successfully developed and tested comparing UV-Vis and NMR assays. The feasibility of photoelectrical detection can efficiently identify the transesterification conversion yield on the issue of rapid and real time. Besides, the lipase immobilization is studied and optimized. The transesterification reaction conversion yield is much higher compared with other lipase biocatalysis [15]. Furthermore the photoelectrical device data is well match with the UV-Vis and NMR observation.

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References

1. M. Iso, B. Chen, M. Eguchi, T. Kudo and S. Shrestha, *J. Mol. Catal. B-Enzym.*, 16 (2001) 53.
2. W. Korbitz, *Renew. Energy*, 16 (1999) 1078.
3. M. Cetinkaya and F. Karaosmanoglu, *Energy Fuels*, 18 (2004) 1888.
4. K. Nie, F. Xie, F. Wang and T. Tan, *J. Mol. Catal. B-Enzym.*, 43 (2006) 142 (2006).
5. Y. Asanomi, H, Yamaguchi, M, Miyazaki and H. Maede, *Molecules*, 16 (2011) 6041.
6. S. Speccchia, F.W.A. Tillemans, P.F. van den Oosterkamp and G. Saracco, *J. Power Sources*, 145 (2005) 683.
7. F. K. Liu, Y. C. Chang, F. H. Ko, T. C. Chu, and B. T. Dai, *Microelectro. Eng*., 67 (2003) 702.
8. M. Collinson, E.F. Bowden and M.J. Tarlov, *Langmuir*, 8 (1992) 1247.
9. F. Drepper, P. Dorlet and P. Mathis, *Biochemistry*, 36 (1997) 1418.
10. J. C. McDonald and G. M. Whitesides, *Accounts Chem. Res.*, 35 (2002) 491.
11. J. Cabal, K. Kuca and J. Micova, *J. Appl. Biomed.*, 5 (2007) 167.
12. S. Collins, A.M. Kenwright, C. Pawson, S.K. Peace and R.W. Richards, *Macromolecules*, 33 (2000) 2974.
13. M. B. Stark and K. Holmberg, *Biotechnol. Bioeng.*, 34 (1989) 942.
14. J. M Palomo, G. Murioz, G. Femandez-Lorente, C. Mateo, M. Fuentes, J. M. Guisan and R.

Femandez-Lorente, *J. Mol. Catal. B-Enzym.*, 21 (2003) 201.
15. P. T. Vasudevan and M. Briggs, *J. Ind. Microbiol. Biotechnol.*, 35 (2008) 421.