Entrapping Immobilisation of Lipase on Biocomposite Hydrogels toward for Biodiesel Production from Waste Frying Acid Oil

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Abstract: A new application of biocomposite hydrogels named gelatin-alginate (GA) and pectin alginate (PA) enables the use of the hydrogels as carriers for lipase entrapment during biodiesel production. Waste frying acid oil (WFAO), a raw material, was converted to biodiesel via an esterification reaction catalysed by two different immobilised biocatalysts: gelatin-alginate lipase (GAL) and pectin-alginate lipase (PAL). The highest immobilisation yield of GAL and PAL beads was achieved at 97.61% and 98.30%, respectively. Both of them gave biodiesel yields in the range of 75–78.33%. Furthermore, capability and reusability of biocatalysts were improved such that they could be reused up to 7 cycles. Moreover, the predicted biodiesel properties met the European biodiesel standard (EN14214). Interestingly, entrapped lipase on composite hydrogels can be used as an alternative catalyst choice for replacing the chemical catalyst during the biodiesel production.

Keywords: biocomposite hydrogel; waste frying acid oil; esterification reaction; immobilised lipase; biodiesel

1. Introduction

Typically, vegetable/animal oils, are widely used by restaurants, household kitchens, hotels and food processing plants [1]. Waste frying oil (WFO), discharged from them, causes serious water pollution environmental problems. Recently, several strategies have been reported for WFO recycling into value added products such as biodiesel, biolubricant, alkyl resin and washing products. Among these applications, biodiesel production from WFO has been defined as the most effective recycling process [2]. Furthermore, biodiesel also has lower energy consumption and greenhouse gas emission during its life cycle assessment [3]. Accordingly, biodiesel production from WFO can meet cost effective and environmentally friendly waste management.

Currently, biodiesel can be produced via three different processes: (i) alkaline-transesterification, (ii) acid-esterification, and (iii) biocatalysis. Transesterification of triglycerides (TGs) with short chain alcohol (methanol or ethanol) in the presence of basic catalysts such as sodium hydroxide, potassium hydroxide, or a methoxide process is generally applied for industrial scale production. The major drawback of this process is the sensitivity of alkaline catalysts to free fatty acid (FFA) in oil, which results in soap formation, reduction of biodiesel yield and complications with the separation process [4,5]. In case of oil with high FFA content, it can be converted to biodiesel via esterification catalysed by acid catalysts like sulfuric acid (H2SO4), hydrochloric acid (HCl), and phosphoric acid (H3PO4).
acid (HPO₃). However, there are some disadvantages of the process such as long reaction time, lower reaction rate, the presence of impurity glycerol and a large amount of wastewater from the purification step [6,7]. Enzymatic biodiesel production can be considered as an environmentally friendly process as well.

Lipases (EC.3.1.1.3), which are biocatalysts, can simultaneously catalyse transesterification of TGs and esterification of FFA in high acid oil into biodiesel. However, enzymatic biodiesel production still has hindering aspects such as high cost, low stability and long reaction time. Therefore, lipase immobilisation on hydrophilic support is the key parameter to achieve enhanced stability, reusability and alcohol resistance during biodiesel production. Such immobilisation has advantages as it can prevent: lipase from reaction with media, variable pH, variable temperature, and shear stress impact; it serves to preserve enzyme catalytic activity and allows substrates to easily access to the active site [8,9]. Typically, four immobilisation techniques are widely used (i) entrapment, whereby an enzyme is encapsulated within the polymeric network, (ii) physical adsorption, whereby the enzyme is attached on the solid support surface by weak force, (iii) a covalent binding of the enzyme as it attaches to the support material by covalent bonding, and (iv) activation of cross-linking of the enzyme to form intermolecular cross-linkage with other enzyme molecules. Among these techniques, the entrapment technique provides benefits in terms of being a low-cost approach, a simple procedure, a technique that can be conducted under mild conditions, high activity recovery, providing easy diffusion of substrates through the matrix pore and providing stable enzyme conformation for a long period of time because it is not destroyed by physical force and chemical reaction [10,11]. In addition, immobilised enzyme on hydrogels provides many advantages such as its swelling behaviour, holding a large amount of water and maintaining the three dimensional structure of enzyme. Presently, carbohydrate and peptide biopolymers are classified as hydrogel, for examples, alginate, gelatin, and pectin.

Alginate, a kind of bio-macro polymer, is popular for enzyme immobilisation via the entrapment technique due to its characteristics: it is biocompatible, biodegradable and non-toxic [12]. It was reported that entrapment lipase within an alginate bead could retain enzyme activity and also gave high immobilisation yield up to 80% [13]. On the other hand, alginate has some limitations regarding its properties, such as high solubility in water and degradation of alginate, which can easily occur under high temperature and harsh chemical environments [14]. An effective approach to address this problem is blending alginate with other biopolymers [12] such as pectin, gelatin, chitosan, K-carrageenan, xanthan, maltodextrin and polyvinyl alcohol (PVA). A natural protein of gelatin, derived from collagen, presented good biocompatible and biodegradable. It has been widely used as hydrogel for medical and pharmaceutical applications [15]. Pectin, one of the potential materials for entrapment, showed non-toxicity, high hydrophilicity, high gelling capability and can serve as an emulsion stabiliser. Typically, pectin is used as an effective wall material for encapsulation of bioactive compounds [16].

Accordingly, there are many research works that attempted to improve stability of alginate via a combination with pectin and gelatin. Both biopolymers are capable of forming gel in the presence of divalent cations such as calcium, zinc, manganese, etc. [17]. Previously, some researchers reported that the addition of gelatin mixed with alginate that can make an immobilised bead with hydrogel properties [18]. Gelatin based hydrogel was also used to entrap lipase from Canida rugosa for hydrolysis of p-nitrophenyl palmitate (p-NPP). After 10 consecutive cycles, it retained 68% of its original activity [19]. Pectin, an inexpensive anion heteropolysaccharide, is widely used as a co-biopolymer of alginate in food applications such as edible film [20]. Alginate-pectin bead, also used for entrapment of bitter gourd peroxidase (BGP), was employed for the treatment of disperse dyes [21]. In addition, Batista et al. [22] reported that a lipase immobilised on pectin (PECp-lipase) maintained 100% of initial activity for 35 days of storage in room temperature. The thermal stability of immobilised lipase compared to a free form and also tridimensional structure was preserved. Currently, immobilised lipase on pectin-alginate and gelatin-alginate beads have not been applied for biodiesel production. It is
interesting that entrapping lipase in these biopolymers could be used as alternative choices during the production of biodiesel.

Therefore, in this work two kinds of composite hydrogels were prepared with combinations of pectin and alginate (PA)/gelatin and alginate (GA). Hereafter they were called pectin-alginate (PAL) and gelatin-alginate (GAL) lipase beads that were further used as carriers for biodiesel production. Their potentials as biocatalysts for biodiesel production from waste frying acid oil (WFAO) were investigated. The optimal condition for production was considered for various effects of reaction time, methanol to FFA molar ratio, temperature and agitation speed. In addition, water content, swelling behaviour, temperature sensitivity, reusability, retention of activity affecting the PAL/GAL beads during production, as well as biodiesel properties were evaluated and discussed. This work demonstrated production of biodiesel from WFAO using immobilised lipase on hydrogels as an alternative catalyst. Their potential for replacing chemical catalysts in biodiesel application were also considered.

2. Results

2.1. Characterisation of Hydrogels

2.1.1. Water Content of Hydrogels

The water content contained in hydrogels is an important criteria for determining its capability to absorb and retain water. Both PA and GA beads clearly showed capability to absorb and retain water. To absorb water, the water content of PA beads was 97.14 ± 0.07%, while GA beads had a similar water content of about 96.13 ± 0.03%. It was due to blending alginate with gelatin at pH 7 that resulted in a lower degree of swelling of hydrogel that can be observed in a previous study [14]. The water barrier property of polysaccharide/gelatin film was effective at pH 5 [23].

2.1.2. Swelling Behaviour

Hydrogel swelling behaviour is a key parameter for testing its ability to absorb water and can be used to prove how well it serves for enzyme conformation. It was found that two different hydrogels started to adsorb water after they were dropped into deionised water (See Figure 1). Their swelling degree gradually increased as time increased and reached an equilibrium state at 360 min. Similarly, Feki et al. [24] prepared a composite polysaccharides hydrogel from blue crab chitosan (Cs) and red marine macroalga Falkenbergia rufolanosa polysaccharide (FRP). The hydrogel was immersed in deionised water to determine the swelling kinetic at 25 °C. The results showed that the hydrogel could adsorb a larger amount of water as retention time increased until an equilibrium condition was reached (3 h).

![Figure 1](image_url)  
**Figure 1.** Swelling degree of hydrogels as a function of retention time: (▲) Gelatin-Alginate (GA), (●) Pectin-Alginate (PA).
2.1.3. Test of Temperature Sensitivity

Generally, polysaccharide is known as a thermo-sensitive compound. In Figure 2, the equilibrium water uptake of hydrogels in deionised water at different temperatures in the range of 25 to 80 °C is shown. The results show the swelling status of both hydrogels did not change until 40 °C. After 50 °C, the water content of pectin-alginate (PA) bead slightly decreased, while the gelatin-alginate (GA) bead presented sharp reduction of its swelling degree. This is in agreement with previous work [25] which reported that a combination of pectin with other biopolymers could improve thermal and structure properties. On the other hand, previously another group also reported that an alginate-gelatin bead degraded rapidly due to the gelatin network, which showed dissociation at 37 °C [26].

![Swelling degree of hydrogels at different temperatures](image)

**Figure 2.** Swelling degree of hydrogels at different temperatures: (▲) Gelatin-Alginate, (●) Pectin-Alginate.

2.2. Lipase Activity and Immobilisation Yield

Before immobilisation on the hydrophilic support, initial specific lipase activity was measured and showed 0.0032 U/mg proteins. It should be noted that specific activity of immobilised GAL/PAL beads were increased up to 0.48 and 0.46 U/g support. Their conformations of lipase attaching on hydrophilic support became highly stable. Thus, immobilised lipase showed higher enzyme activity than when it was used in the free form [27]. Based on specific activity, it was implied that the GAL bead could be entrapped lipase at about 0.48 U/g support, while 0.46 U of lipase could be absorbed within PAL 1 g. Furthermore, the highest immobilisation efficiency of GAL (97.61%) and PAL (98.30%) beads were observed. As noted in Table 1, immobilisation of lipase via the entrapment technique in this study gave higher immobilisation yield than other previous works. This was due to the lipase entrapping on hydrogels, which can be conducted under mild conditions. Therefore, enzyme conformation showed more stability, since it was not destroyed by physical force, nor by chemical reaction [10,11].
Table 1. Comparison of immobilised yield of lipase via different supporting materials and techniques.

| Enzymes                  | Carriers                  | Immobilisation Techniques | Immobilisation Yields * | References |
|--------------------------|---------------------------|---------------------------|-------------------------|------------|
| Lipase from *R. oryzae* (rROL) | Gelatin-alginate          | Entrapment                | 97.61                   | This work  |
| Lipase from *R. oryzae* (rROL) | Pectin-alginate           | Entrapment                | 98.30                   | This work  |
| Lipase from *R. oryzae* (rROL) | Lewatit VPOC 1600         | Adsorption                | 77.40                   | [28]       |
| Lipase from *R. oryzae* (rROL) | Amberlite IRA-96          | Covalent binding          | 58.80                   | [28]       |
| Lipase from *R. oryzae* (rROL) | Lifetech ECR1030M         | Adsorption                | 79.50                   | [28]       |
| Lipase from *R. oryzae* (rROL) | Lifetech ECR8285M         | Covalent binding          | 78.40                   | [28]       |
| Lipase from *R. oryzae* (rROL) | Lifetech AP1090M          | Adsorption                | 70.10                   | [28]       |
| Lipase from *Aspergillus niger* | Sodium titanate nanotubes | Adsorption                | 80.00                   | [29]       |
| Lipase from *R. miehei* | X-shaped zeolitic imidazolate frameworks (ZIF-8) | Encapsulation            | 90.00                   | [30]       |
| Porcine pancreatic lipase | PW@MIL-100(Fe)            | Encapsulation            | 91.00                   | [31]       |
| Lipase from *Candida antarctica* | Fe₃O₄@SiO₂ core-shell magnetic nanoparticles | Covalent binding | 84.00                   | [32]       |

* Note: immobilisation yields were determined based on the protein content.

2.3. Effect of Time on Biodiesel Production

Reaction time is one of the main variables for the esterification process. An increasing reaction time leads to higher biodiesel yield. Both biocatalysts showed high catalytic efficiency and gave high FFA conversion rates between 4 and 8 h. However, after a reaction time of 8 h, biodiesel yield significantly decreased (see Figure 3). An explanation could be that lipase activity was inhibited by methanol after encountering a long reaction time. It was resulted in a backward esterification reaction. [33]. The highest biodiesel yields of immobilised PAL/GAL bead were 62.09 and 64.17%. Meanwhile, the lowest FFA in the reaction medium of GAL (4.36 mg KOH/g oil) and PAL (5.09 mg KOH/g oil) were achieved at 8 h. Thus, reaction time of 8 h was considered as the optimal time for the reaction. These results were similar to the previous work [34] which also reported the optimal time of 7 h for biodiesel production from WFO catalysed by immobilised *Rhizopus oryzae*. Another case explained by Zhang et al. [35], the esterification process of acidified waste cooking oil (AWCO) using lipase immobilised on epoxychloropropane-modified Fe₃O₄ sub-microspheres, was completed within 9 h.

2.4. Effect of Methanol to Fatty Acids Molar Ratio on Biodiesel Production

Methanol to fatty acids molar ratio, one of the most important parameters in enzymatic esterification, could give a higher ester yields under an excess alcohol concentration. It widely called as a reversible reaction. It was observed that as methanol to fatty acids molar ratio continually increased, a higher conversion of fatty acids was obtained (see Figure 4). It could be explained that the esterification reaction is reversible; an increase in the alcohol concentration led to a higher product formation rate and became a forward esterification reaction However, biodiesel yield did not increase considerably when the ratios of methanol to FFA were higher than 4:1 and 5:1 for immobilised GAL/PAL beads. The reason was the excessive amount of methanol presented in the reaction medium led to enzyme deactivation and reversible esterification reaction [36]. Therefore, methanol to fatty acids molar ratios of 4:1 and 5:1 were selected as optimal methanol to fatty acids molar ratio for the GAL/PAL beads, respectively. The biodiesel yield (65.87%) and FFA (3.55 mg KOH/g oil) were obtained at 4:1 methanol to fatty acids molar ratio for the immobilised GAL bead, while in case of the PAL bead, a methanol to fatty acids molar ratio of 5:1 gave a biodiesel yield of 66.76% and a FFA of 3.7310 mg KOH/g oil. Furthermore, the results obtained
were similar to previous studies [37,38]. They found the optimal methanol to fatty acids molar ratio for biodiesel production via esterification of fatty acids in hydrolysed waste frying oil catalyzed by R. oryzae lipase was in the range of 5:1 to 7:1. Chen et al. [5] also reported on biodiesel production from WCO using Pseudomonas mendocina cells immobilised on magnetic microspheres as the biocatalyst. They found that the maximum biodiesel yield was achieved at a methanol to FFA ratio of 4:1. This is in agreement with previous studies of Wang et al. [39] and Karimi et al. [40]. They found that the optimal methanol to FFA ratio for enzymatic biodiesel production from WCO were 5:1 and 6:1, respectively.

![Figure 3](image)

**Figure 3.** Percentage of biodiesel yield and acid number as functions of reaction time: (▲) gelatin-alginate lipase (GAL): acid number, (●) pectin-alginate lipase (PAL): acid number. Reaction conditions: Acid waste frying oil (AWFO), 50 mL; GAL or PAL beads, 2% w/w; time, 4–24 h; methanol to fatty acids molar ratio, 3:1; temperature, 40 °C; agitation rate, 200 rpm. The figure indicates the average corresponding to at least three independent experiments. Error bars represent the standard deviation (SD) and significant differences were considered at \( p < 0.05 \).

![Figure 4](image)

**Figure 4.** Percentage of biodiesel yield and acid number as a function of the methanol to free fatty acid (FFA) molar ratio. (▲) GAL: acid number; (●) PAL: acid number. Reaction conditions: Acid waste frying oil (AWFO), 50 mL; GAL or PAL beads, 2% w/w; time, 8 h; methanol to fatty acids molar ratio, 1:1 to 7:1; temperature, 40 °C; agitation rate, 200 rpm. The figure was revealed that the average results corresponded to at least three independent experiments. Error bars show the SD; differences were considered significant at \( p < 0.05 \).
2.5. Effect of Temperature on Biodiesel Production

Typically, it is also known that temperature is an important parameter affecting the reaction rate, enzyme activity and chemical equilibrium. With an increase in reaction temperature from 30 to 50 °C, the biodiesel yield increased remarkably, while the FFA formation showed a downward trend for both biocatalysts (see Figure 5). Elevated temperature can increase lipase activity and decrease the viscosity of the substrate, which promotes mobility of substrate, leading to a higher reaction rate. However, lower biodiesel yield was observed with further increasing reaction temperature up to 60 °C. This probably was due to the deactivation of lipase at high temperature (>60 °C) [41]. In addition, esterification of carboxylic compound into ester compound is an exothermic reaction. As a result, high temperature causes the equilibrium constant to decrease [42]. According to the highest biodiesel yield of GAL (75.00%) 2.66 mg KOH/g oil and PAL (77.83%), 2.36 mg KOH/g oil were achieved at 50 °C. Therefore, the reaction temperature was fixed at 50 °C in further experiments. Similarly, these results agreed with the previous study [43] which concluded the optimal temperature for biodiesel production from waste sunflower frying oil catalysed by Rhizomucor miehei was 45 °C. Ashjari et al. [44] also reported that the optimal temperature for biodiesel production from WCO catalysed by immobilised lipase from Thermomyces lanuginosa lipase (TLL) was 50 °C. In addition, the results obtained from previous work [45] indicated that lipase has high catalytic efficiency for temperatures in the range of 35 to 50 °C.

![Figure 5](image_url)

**Figure 5.** Percentage of biodiesel yield and acid number as functions of reaction temperature: (▲) GAL: acid number, (●) PAL: acid number. Reaction conditions: acid waste frying oil (AWFO), 50 mL; GAL or PAL beads, 2% w/v; time, 8 h; methanol to fatty acids molar ratio, 4:1 for GAL and 5:1 for PAL; temperature, 30 to 60 °C; agitation rate, 200 rpm. The figure indicates the average corresponding to at least three independent experiments. Error bars represent the SD and differences are considered significant at p value < 0.05.

2.6. Effect of Agitation Rate on Biodiesel Production

Agitation rate plays an important role in the formation of biodiesel, since the oil agitation, methanol and catalyst mixture could enhance the reaction rate. As shown in Figure 6, the efficiency of two biocatalysts increases continuously as the agitation rate increases from 100 to 200 rpm, since lipase catalyses the reaction between two layers of hydrolysed oil and methanol. As a result, agitation can increase the interfacial area between WFAO, methanol and biocatalyst and minimise mass transfer...
limitation, leading to higher biodiesel yield. However, increasing the agitation rate higher than 200 rpm could not increase biodiesel yield. The high agitation rate caused enzyme denaturation. The enzyme was damaged by mechanical collision and shear stress [33]. Maximum biodiesel yield of immobilised GAL (75%) and PAL (78.33%) beads and minimum FFA of GAL (2.66 mg KOH/g oil) and PAL (2.31 mg KOH/g oil) were obtained at 200 rpm. Thus, an agitation rate of 200 rpm was selected as the optimal agitation rate for the esterification reaction. The results obtained were similar to previous studies [13], which reported that the optimal agitation rate for esterification of fatty acids from WCO catalysed by Novozyme 435 was 200 rpm.

In Figure 8, GAL could protect 86.19% of the activity, while in the case of PAL a 90% activity level was due to the gelatin network dissociating at 37 °C. Because alginate and pectin show a crystalline nature whereas gelatin determined the semi-crystalline nature [18]. Previously, another group also reported that the gelatin–alginate bead degraded rapidly because alginate and pectin show a crystalline nature whereas gelatin determined the semi-crystalline nature [26].

The residual activity of immobilised lipase was a key factor to describe deactivation kinetics [28]. In Figure 8, GAL could protect 86.19% of the activity, while in the case of PAL a 90% activity level was displayed after 7 reuses. The results illustrated that both immobilised GAL/PAL beads showed higher reusability than previous studies (See Table 2). Although, only 2%wt biocatalyst loading was
used for biodiesel production from WFO, biodiesel yield for both catalysts were in agreement with the literature works which stated that under short reaction time and no water adsorbent, the biocomposite hydrogels provided high catalytic efficiency for biodiesel production.

**Figure 7.** Percentage of biodiesel yield and acid number as functions of cycle number: (▲) GAL: acid number; (●) PAL: acid number. Reaction conditions: acid waste frying oil (AWFO), 50 mL; GAL or PAL beads, 2% w/v; time, 8 h; methanol to fatty acids molar ratio, 4:1 for GAL and 5:1 for PAL; temperature, 50 °C; agitation rate, 200 rpm. Figure 7 indicates the average corresponding to at least three independent experiments. Error bars represent the SD and differences were considered significant at \( p < 0.05 \).

**Figure 8.** Residual activities of immobilised rROL on hydrogels as functions of the cycle number: ▲ GAL bead, ○ PAL bead. Reaction conditions: acid waste frying oil (AWFO), 50 mL; GAL or PAL beads, 2% w/v; time, 8 h; methanol to fatty acids molar ratio, 4:1 for GAL and 5:1 for PAL; temperature, 50 °C; agitation rate, 200 rpm. Figure 8 indicates the average corresponding to at least three independent experiments. Error bars represent the SD and differences were considered significant at \( p < 0.05 \).
Table 2. Comparative biodiesel yield from waste frying oil (WFO) via different processes.

| References | Processes and Conditions | Catalysts | Biodiesel Yield (%) | Reusability (Cycles) |
|------------|--------------------------|-----------|---------------------|----------------------|
| This study | Esterification: 2% w/v biocatalyst, 50 °C, 4:1 methanol to fatty acids molar ratio and agitation speed 200 rpm for 8 h. Esterification: 2% w/v biocatalyst, 50 °C, 5:1 methanol to fatty acids molar ratio and agitation speed 200 rpm for 8 h. | GAL bead, PAL bead | 75.00, 78.33 | 7, 7 |
| [43]      | Transesterification: 5% biocatalyst, 45 °C, 3:1 methanol to oil ratio for 24 h. | Lipase from *P. fluorescens* | 63.84 | - |
| [46]      | Transesterification: 3% w/w biocatalyst, 37 °C, 3:1 methanol to oil ratio and agitation speed 200 rpm for 24 h. | Immobilised *P. cepacia* lipase on epoxy-acrylic resin | 46.32 | 6 |
| [32]      | Transesterification: 5% w/w biocatalyst, 37 °C, 3:1 methanol to oil ratio for 96 h. | Immobilised *C. antractica* lipase on core-shell magnetic nanoparticles | 100 | 6 |
| [47]      | Transesterification: 10% w/w biocatalyst, 30 °C, 3:1 methanol to oil ratio, 0.25% w/w water and agitation speed 350 rpm for 24 h. | Immobilised *T. lanuginosus* lipase on Octadecyl methacrylate | 80 | - |
| [44]      | Transesterification: 15.2% w/w biocatalyst, 40 °C, 29.2% w/w t-butanol to oil ratio and 40.5% w/w water adsorbent for 48 h. | Immobilised lipase from *R. miehei* on silica core-shell magnetic nanoparticles (Fe₃O₄@SiO₂) | 57.5 | 5 |
2.8. Biodiesel Composition

The FAME’s composition was analysed by gas chromatography-mass spectroscopy (GC-MS). The results show that the produced biodiesel mainly contains oleic acid methyl ester (31.52%), palmitoleic acid methyl ester (29.43%), stearic acid methyl ester (26.95%) and other components as shown in Figure 9. The results obtained were in line with previous studies [48,49], which reported that biodiesel from WFO was mainly composed of stearic-, oleic-, palmitic- and palmitoleic acids. The results also revealed that the proportion of monounsaturated fatty acids found in biodiesel was higher than the proportion of saturated fatty acids. There are some advantages of saturated fatty acids such as stearic acid (C18:0) present in biodiesel, giving high cetane number and a reduction of nitrogen oxides (NOx) exhaust emissions. It was also reported that the presence of a high proportion of monounsaturated fatty acids such as palmitoleic acid (C16:1) and oleic acid (C18:1) could decrease oxidative stability and cold flow problems of biodiesel [50,51]. In addition, the fatty composition of biodiesel in this work was further used to predict biodiesel via calculation as described in Section 2.9.

![Figure 9. Composition of fatty acids in biodiesel (%).](image)

2.9. Prediction of Biodiesel Properties

Table 3 shows some biodiesel properties tested by using standard ASTM methods together with the regression models that were explained in previous work [52]. It is interesting to note that the predicted biodiesel properties obtained from WFAO meet the European biodiesel standard.
Table 3. Predicted properties of biodiesel.

| Parameters                           | Test Methods | Predicted Values * | European Standard |
|--------------------------------------|--------------|--------------------|-------------------|
| Kinematic viscosity (mm²/s)          | ASTM D445    | 5.09               | 3.5–5.0           |
| Flash point (℃)                      | ASTM D93     | 132.89             | >101              |
| Cloud point (K)                      | ASTM D2500   | 286.52             | No specification  |
| Pour point (K)                       | ASTM D97     | 280.35             | No specification  |
| Cold filter plugging point (K)       | ASTM D6371   | 273.13             | No specification  |
| Cetane number                        | ASTM D613    | 54.80              | >51               |
| Iodine number                        | EN14111      | 72.47              | <120              |

* The predicted values were calculated from the regression models explained by Alviso et al., 2020.

3. Materials and Methods

3.1. Raw Materials

Two kinds of carbohydrate of alginate and pectin, and another protein of gelatin were used for hydrogels preparation. Lipase powder of Rhizopus oryzae lipase (rROL), p-nitrophenyl laurate (p-NPL), p-nitrophenol and methyl heptadecanoate were purchased from Sigma Co., Ltd., Company, Bangkok, Thailand. Waste frying acid oil (WFAO) was collected from food court centers, within the Khon Kaen University area in Khon Kaen, Thailand. It was then pretreated by heating under 80 ℃ for 30 min and filtered by cotton sheet prior to use as feedstock for biodiesel production. It was then characterised; saponification and acid value of WFAO were 190 and 19.50 mg KOH/g oil, while the fatty acids profile were 29.43% of palmitoleic acid (C16:1), 26.95% of stearic acid (C18:0), 31.52% of oleic acid (C18:1) and others (12.10%). In addition, most of chemical reagents used were analytical grade such as calcium chloride, sodium hydroxide, monobasic sodium phosphate, phenolphthalein, bovine serum albumin, copper sulfate, sodium potassium tartrate, ethanol, methanol, etc.

3.2. Preparation of Hydrogels

Both the pectin-alginate (PA) and gelatin-alginate (GA) beads were separately prepared in each 100 mL phosphate buffer (pH 7.0). The gelatin-alginate/pectin-alginate solutions contained 2% in each alginate-gelatin/pectin-gelatin (w/v). All of them were heated to 40 ℃ and stirred at 1000 rpm in order to obtain a homogeneous solution within 20 min. After that, they were cooled down at room temperature. Finally, the mixture solutions were dropped into 0.1 M CaCl₂ and stored at 4 ℃ for 12 h in order to form hydrogels with dimension 3 mm.

3.3. Characterisation of Hydrogels

3.3.1. Water Content of Hydrogels

The water content was calculated according to AOAC (2000) methods. The water content of each hydrogel was measured before and after drying at 105 ℃ using Equation (1).

\[ WC = \frac{W_0 - W_1}{W_0} \times 100 \]  

where \( W_0, W_1 \) mean the weight of hydrogel before and after drying at 105 ℃, respectively.

3.3.2. Swelling Behaviour of Hydrogels

The swelling behaviour of each hydrogel was determined by a gravimetric method following the previous work [24]. The hydrogel was immersed into deionized water to determine the swelling
kinetics at 25 °C. The samples were weighed after wiping out the excess water. Then, they were dried in a hot air oven at 105 °C. The water uptake (WU) of hydrogel can be calculated as in Equation (2).

\[
WU = \frac{Ws - Wd}{Wd} \times 100
\]  

(2)

where, \(W_s\) and \(W_d\) are the mass of the swelling sample at time \(t\) and the mass of the dried sample, respectively.

### 3.3.3. Temperature Sensitivity Test

For the temperature sensitivity test, raw hydrogels were able to swell to equilibrium in deionised water at different temperatures in the range of 25 to 80 °C. After the equilibrium state, the samples were removed from water and dried in a hot air oven (105 °C). Equilibrium water uptake (EWU) was calculated as shown in Equation (3).

\[
EWU = \frac{We - Wd}{Wd} \times 100
\]  

(3)

where, \(W_e\) and \(W_d\) are the mass of swollen hydrogel at the equilibrium state and the mass of dried sample, respectively.

### 3.4. Immobilisation of Rhizopus Oryzae Lipase (rROL) on Hydrogels

The lipase (rROL) was immobilised using the entrapment technique on two different carriers: (i) a gelatin-alginate mixed bead and (ii) a pectin-alginate mixed bead. It should be noted that the procedure for preparation of supporting materials was described in Section 2.2. In brief, the lipase powder (2 g) was mixed in pectin-alginate/gelatin-alginate solutions. Subsequently, the mixtures were dropped into 0.1 M CaCl\(_2\) by sterile syringe in order to form immobilised lipase beads. The beads were stored in 0.1 M CaCl\(_2\) at 4 °C overnight. Then, two immobilised GAL/PAL beads were washed with distilled water twice. Finally, they were kept in distilled water at 4 °C before they were used as biocatalysts in the esterification reaction for biodiesel production.

### 3.5. Esterification of WFAO for Biodiesel Production

Free fatty acids (FFA) in WFAO (50 mL), GAL/PAL (2% w/v) and methanol were added together in a 250 mL Erlenmeyer flask, using an incubator shaker to perform the esterification reaction. The reaction was optimised by varying reaction time (4 to 24 h), methanol: oil molar ratio (1:1 to 7:1), reaction temperature (30 to 60 °C) and agitation rate (100 to 300 rpm). After reaction, immobilised lipase was recovered and further used by filtration and washing with distilled water before storing in distilled water at 4 °C. The oil sample was analysed as described in the section of analytical methods.

### 3.6. Analytical Techniques

#### 3.6.1. Lipase Activity

Lipase activity was determined by colorimetric method using p-nitrophenyl laurate (p-NPL) as the substrate as described by Khaskheli et al. [53]. The substrate solution was prepared in a ratio of solution A (1 mM p-NPL in propanol) to solution B (phosphate buffer solution pH 7.0) of 1:9. Immobilised lipase or free lipase was then mixed with the substrate solution and incubated at 37 °C for 30 min. The amount of released p-nitrophenol was detected by a UV-vis spectrophotometer at a wavelength of 410 nm. One unit of lipase activity was defined as the release of 1 µmol p-nitrophenol per minute under the assay conditions. Specific activity was defined as the number of activity units per milligram protein or milligram supports.
3.6.2. Percentage of Immobilisation Yield

The protein content was used as an indicator for the efficiency of immobilisation as described in the previous work [54]. Protein in solution was measured by Lowry’s method and immobilisation yield can be calculated as shown in Equation (4).

\[
\text{Immobilisation (\%)} = \frac{P_i - P_f}{P_i} \times 100
\]  

(4)

where \(P_i\) and \(P_f\) were initial protein without support (mg/mL) and protein in solution after immobilisation (mg/mL), respectively.

3.6.3. Biodiesel Analysis

Conversion of FFA in WFAO was calculated based on the official methods for acid number (Cd 3d-63) of AOCS (American Oil Chemist’s Society). Samples were analysed by a standard acid–base titration procedure for the residual FFA determination. The acid number was expressed as mg KOH/g oil. The biodiesel yield was calculated as the FFA reduction as shown in Equation (5). The composition of biodiesel was analysed using gas chromatography-mass spectroscopy (GC-MS) as described in the previous study [55].

\[
\text{Biodiesel yield (\%)} = \frac{AN_1 - AN_2}{AN_2} \times 100
\]  

(5)

where \(AN_1\) and \(AN_2\) represent acid number of the original WFAO and acid number of the esterified WFAO, respectively.

3.6.4. Reusability Test

Reusability of immobilised lipase on GAL/PAL was evaluated during consecutive 10 h batches and carried out under the same reaction condition for biodiesel production. After the reaction, each biocatalyst was removed from the reaction mixture by the filtration technique. Then, it was washed with distilled water three times before reutilisation in the next batch. The residual activity of each biocatalyst was calculated as shown in Equation (6), in order to describe deactivation of biocatalyst [28].

\[
\text{Residual activity (\%)} = \frac{BN - B_0}{BN} \times 100
\]  

(6)

where \(BN\) and \(B_0\) were biodiesel yield in each batch and the first batch, respectively.

3.6.5. Statistical Analysis

All experiments were carried out in triplication and the data obtained was reported as mean ± SD. The data was analysed using single factor ANOVA with Microsoft Excel 2010. The result was considered statistically significant for \(p\) value less than 0.05.

4. Conclusions

Successfully, entrapment of rROL on GAL/PAL beads was firstly applied to produce biodiesel from WFAO. Two different carriers of GAL/PAL improved catalytic efficiency, achieved high conversion rate, high biodiesel yield (75–78.33%) and lipase stability for 7 cycles under optimal conditions. Its predicted properties included kinematic viscosity, flash point, cloud point, pour point, cold filter plugging point, cetane number and iodine number. All of them met the European standard. The WFAO showed a high potential to use as a low cost substrate as well as reusability of the immobilised catalyst. Furthermore, the process also demonstrated broad prospect as an alternative to the traditional biodiesel process by enzymatic/chemical transesterification for high purity and impurity oils.
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