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### Article

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## Research paper

# Effectiveness of using deep eutectic solvents as an alternative to conventional solvents in enzymatic biodiesel production from waste oils

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## HIGHLIGHTS

- Enzymatic biodiesel production from waste oils in DES was considered.
- Use of ternary DESs at different water contents was investigated.
- Enzyme-DES system was regenerated for five consecutive cycles through washing with 1-butanol.

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## ABSTRACT

Ionic liquids (ILs) have been proposed as a more benign replacement to toxic and volatile organic solvents in enzymatic biodiesel production, used to minimize methanol inhibition effect and enhance the stability of immobilized enzyme. Despite their several advantages, ILs are expensive, which renders the overall process unfeasible. In this work, the use of low-cost deep eutectic solvents (DESs) has been investigated as a new reaction medium for enzymatic biodiesel production from waste oils. A DES composed of chlorine-chloride and glycerol (ChCl:Gly) was tested and its effectiveness was compared to 1-butyl-3-methylimidazolium hexafluorophosphate, [bmim][PF<sub>6</sub>], which has shown positive results. To decrease the viscosity of ChCl:Gly DES, ternary ChCl:Gly:water DESs with different water contents were prepared and tested. Despite lower cost compared to ILs, without successful repeated reuse of the DES-immobilized lipase system, their advantages remain theoretical. Therefore, the reusability of the DES-Novozym®435 system in consecutive cycles was examined. It was shown that 34% biodiesel production yield could be achieved in ChCl:Gly at 1:2 molar ratio, compared to 23% when [bmim][PF<sub>6</sub>] was used. The yield increased further, when the ternary DES of ChCl:Gly:water (1:2) with 3 wt% water was used, reaching 44%. By removing the by-product glycerol, the reusability of the DES-immobilized system was improved, with better results achieved using 1-butanol compared to using ethylene glycol.

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## 1. Introduction

The interest in biodiesel production using immobilized lipase has increased in recent years, due to the several advantages that immobilized enzymes offer over conventional alkaline catalyzed process (Marchetti et al., 2007). Novozyme®435, which is lipase from *Candida antarctica* immobilized on acrylic resin, is the most commonly used enzyme in biodiesel production (Taher et al., 2014; Madras et al., 2004; Ha et al., 2007). Despite its advantageous features, the major obstacle facing the commercialization of

enzymatic process is the high cost of the enzyme and the methanol inhibition (Taher et al., 2014; Modi et al., 2007). Typically, the direct exposure of the lipase to excess methanol strips-off the hydration layer necessary for the enzyme to be active.

To overcome the high cost problem, the enzyme has to be used in immobilized form to allow its repeated reuse (Modi et al., 2007; Lu et al., 2007). In some cases, the enzyme was reported to be reused effectively for 50 cycles (Shimada and others., 1999). However, using immobilized enzyme has its own challenges, which are mainly the mass transfer limitations and the by-product glycerol accumulation in the pores of the porous matrix of the support preventing reaction substrates from reaching the enzyme active sites (Xu et al., 2011).

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Using an organic solvent, as a reaction medium, have been suggested to enhance the mass transfer diffusion, and to reduce the methanol inhibition and glycerol accumulation. In that regard, *n*-hexane and *tert*-butanol were used, the former is suggested to alter the substrates affinity (Yang et al., 2004) and the former was selected because of its high glycerol solubility on one hand, and low interference with the reaction on the other (Park et al., 2013). Nevertheless, from an environmental point of view the use of these volatile organic solvents is not recommended, as they are toxic and non-biodegradable. In addition, they require an additional separation unit to separate them from the final product. Therefore, the search for a more environmental benign solvent has been gaining more attention in recent years.

Ionic liquids (ILs) have been suggested as alternatives to volatile solvents (Ha et al., 2007). ILs are non-volatile compounds composed of organic cations and inorganic anions with properties that can be tailored easily by changing the anion or cation part. In addition to solubilizing the substrates and reducing the enzyme inhibition, being practically involatile, makes ILs more environmental friendly. Furthermore, the charged cations and anions in the ILs may interact electrostatically with the lipase in a favorable manner resulting in an enhanced activity and stability (Kim et al., 2014). 1-butyl-3-methylimidazolium hexafluorophosphate, [bmim][PF<sub>6</sub>], and 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, [bmim][NTf<sub>2</sub>], have been widely tested for biodiesel production (Taher et al., 2017; de Diego et al., 2014; Lai et al., 2012), with yields reaching 86% and 72% were achieved in [bmim][PF<sub>6</sub>] from microalgae lipids (Lai et al., 2012) and waste oils (Taher et al., 2017), respectively. The main disadvantage of ILs is their high cost preventing their employment at large scales. For example, it was reported that the cost of [bmim][PF<sub>6</sub>] is 10 times higher than that of *n*-hexane (Wasserscheid, 2002).

On the other hand, DESs are cheap and easily prepared by mixing an ammonium salt with a hydrogen bond donor and heating at moderate temperature for a couple of hours (Zhao et al., 2015). The DESs shares the same favorable properties of ILs, and at the same time have a low cost and their components can be chosen so that the DESs are biodegradable and non-toxic, making them more preferable than ILs for use in enzymatic biodiesel production. Choline chloride (ChCl) based DESs have been suggested in different applications (Zhao et al., 2015, 2011, 2013) because of the favorable characteristics of ChCl. For example, ChCl and glycerol (Gly) was tested, at different molar ratios, to extract the reaction by-product glycerol from biodiesel, produced by a chemical catalyst, namely potassium hydroxide (Abbott et al., 2007; Hayyan et al., 2010). The same DES was also used in the enzymatic biodiesel production using Novozym®435. High yields, reaching 97%, were obtained within 3 h from Miglyol® oil 812 (Zhao et al., 2011) and 81% from soybean oil after 24 h of reaction (Zhao et al., 2013).

Although the use of DESs with immobilized enzymes seems promising, the viscosity of such solvents is high, reaching 376 mPa s for ChCl:Gly (1:2) at 45 °C (Yadav et al., 2014; Smith et al., 2014), compared to 97.8 mPa s for [bmim][PF<sub>6</sub>], at same temperature. This could be a problem at large-scale production and in continuous flow processes. To overcome this, the addition of a third component, such as water, to form a ternary DES, has been suggested to reduce the viscosity. Yadav et al. (2014) measured the viscosity of pure ChCl:Gly (1:2) and different ChCl:Gly:W mixtures and found that the viscosity decreased with the increase of water fraction in the mixture. The viscosity of pure ChCl:Gly decreased to 70 mPa s when 3% water was added. Reducing the viscosity enhances the mass transfer of the reaction substrates to the enzyme active sites, and therefore increases the reaction rate and yield.

In this work, the effectiveness of using DESs in enzymatic biodiesel production from waste oils was examined and compared

**Table 1**  
Fatty acid composition of the waste cooking oil used in this work.

Fatty acids	Carbon chain	Composition (wt%)
Myristic	C14:0	0.27
Palmitic	C16:0	8.61
Stearic	C18:0	4.6
Oleic	C18:1	57.2
Linoleic	C18:2	27.1
Linolenic	C18:3	1.22
Arachidic	C20:0	0.6
Lignoceric	C24:0	0.4

to [bmim][PF<sub>6</sub>]. Ternary ChCl:Gly:W mixtures containing different quantities of water were prepared and tested. The work tested the activity and reusability of Novozym®435 in selected ChCl:Gly(1:2) water ternary DESs.

## 2. Materials and methods

### 2.1. Chemicals and enzyme

*n*-Hexane (99% assay), methanol (99.9% assay), 1-butanol (99% assay), Choline-chloride (98% assay), glycerol (99.5% assay) and standards solution of high purity fatty acids methyl esters (FAMEs) consisting of: 4% myristic acid (C14:0), 10% palmitic acid (C16:0), 6% stearic acid (C18:0), 35% oleic acid (C18:1), 36% linoleic acid (C18:2), 2% of arachidonic acid (C20:0) and behenic acid (C22:0) were purchased from Sigma-Aldrich, USA. 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim][PF<sub>6</sub>]) with a purity ≥ 99% was obtained from io-li-tec, Germany. High purity helium was purchased from Air Product Company, UAE. Zero air and hydrogen were produced by gas generator (F-DGSi, France). Novozym®435 with an activity of 11,900 propyl laurate (PLU) g<sup>-1</sup> with an optimum operating temperature between 30 and 50 °C was a kind gift from Novozymes A/S, Denmark (Venter and Nieuwoudt, 1998).

Waste cooking oil was obtained from Lootah Biofuels Company, UAE. Fatty acid analysis of the oil was determined using Gas Chromatography (GC) according to procedure described in Section 2.6, and the results are shown in Table 1. As per supplier's information, the free fatty acids content does not exceed 2%–3%. Anyway however, the free fatty acids content does not affect the enzymes activity.

### 2.2. Preparation of DESs

The ChCl:Gly DES was prepared by mixing the choline ChCl with Gly at a 1:2 molar ratio, as described by Abbott et al. (2003). Ternary DESs, denoted in this work as ChCl:Gly:W were prepared by mixing the pure ChCl:Gly (1:2) with specific amounts of distilled water. It should be noted that the water percentages, reported in the manuscript, are not with respect to the reaction volume, but with respect to the ternary DES used. Adding the water during the preparation of the DES makes it part of the solvent through strong hydrogen bonding and not free or available for the enzyme. In all cases, the mixtures were heated and mixed at 80 °C until a homogeneous liquid was formed without any solid precipitation. The formed DES was then cooled to room temperature. The procedure for preparing the DESs was much easier and less expensive than that needed for ILs synthesis.

### 2.3. Enzymatic biodiesel production

Transesterification reactions were performed in 5 ml screw-capped vials incubated in an orbital shaker (Stuart SI 500, UK) at a constant temperature of 45 °C and a shaking speed of 150 r min<sup>-1</sup>.

Each reaction vial contained 0.5 ml of waste oil, and 0.135 ml methanol, equivalent to 6:1 methanol to oil (M:O) molar ratio. Different amounts of Novozym®435, namely 50, 100 and 200 mg of, equivalent to 8, 17 and 35 wt% per oil were tested. The work started by showing the advantage of using DES over solvent free system. Experiments were carried for 4 h using 8% with and without the addition of ChCl:Gly (1:2) and compared to [bmim][PF<sub>6</sub>], used in our previous work (Taher et al., 2017). Glycerol was selected as the hydrogen bond donor, because it is a by-product of the every biodiesel process. Therefore, 0.375 ml of ChCl:Gly (1:2) DES, equivalent to 75% of oil volume, was used as a reaction medium, which was the optimum amount of IL reported in our previous publication (Taher et al., 2017). To reduce the viscosity of ChCl:Gly, the effectiveness of using ternary DESs containing water, denoted in this work as ChCl:Gly:W, of different water contents was examined. In this work, ternary DESs containing water in the range of 1–5 wt% were tested. The water percentage in the reaction mixture was about 0.3%–1.1%, and the effect of such a low concentration of water on enhancing hydrolysis is insignificant (Mora-Pale et al., 2007). After specified reaction times (2, 4, 6 and 8 h), 3 ml of *n*-hexane were added to the reaction mixture to extract produced FAMEs. After mixing thoroughly, the mixture was centrifuged at 4700 r min<sup>-1</sup> for 15 min in a centrifuge (Heraeus Megafuge 40R, Thermo Fisher Scientific Inc., USA), and the upper organic layer with the dissolved FAME was collected, diluted to 10 ml of *n*-hexane, and then analyzed using GC.

#### 2.4. DES-Novozym®435 system stability and reusability

Although DESs are cheaper than ILs, their uses with immobilized enzymes in biodiesel production cannot be economically feasible, unless DES-immobilized lipase system is successfully reused for several cycles. Therefore, after extracting the product with *n*-hexane, fresh oil and methanol were added to the residue to perform another reaction cycle.

The produced biodiesel has a very high solubility in *n*-hexane, and can be completely removed at the end of each cycle by the added solvent. However, the solubility of the other by-product glycerol in *n*-hexane is low, and tends to accumulate inside the reaction cell after the end of each cycle. A solvent that can dissolve the by-product, glycerol, was therefore used at the end of the reaction, after removing the main product, biodiesel. In this work, 3 ml of two different washing solvents was tested for removing glycerol and completely recover the DES-immobilized enzyme system. The first was 1-butanol, which has shown its effectiveness in regenerating Novozym®435 (Maceiras et al., 2009). It has been tested to wash the enzyme used for waste oil transesterification in four consecutive cycles. The work presented here investigated the superiority of using 1-butanol in washing enzyme-DES solvent. The other tested solvent was ethylene glycol (EG), which was selected due to its high polarity and solubility of glycerol (Corporation, 2003). All reusability tests were carried out using ChCl:Gly:W containing 3% water, in which each cycle was allowed to run for 60 min.

#### 2.5. Morphological observation of immobilized enzyme

Surface morphologies of the used immobilized enzyme, after the first cycle of reaction, with and without glycerol extraction by 1-butanol and EG were visualized by Quanta FEG 250 scanning electron microscope by FEI (Hillsboro, OR, USA). The acquisition of the scanning was at 10 kV. The samples were prepared by gold/palladium alloy and analyzed at x50 magnification.

#### 2.6. FAME analysis

The amount of produced FAME were assessed with Agilent 7890A (Agilent Technologies Co. Ltd., USA) GC equipped with a

flame-ionization detector (FID) and a 30 m × 530 μm × 1 μm column (HP 1909N-123; Agilent Technologies Co. Ltd., USA). The injection volume was 1 μl of diluted FAME sample. The oven temperature was 200 °C. Helium was used as carrier gas. The initial oven temperature was set at 75 °C and held for 1 min, then increased to 220 °C at 5 °C min<sup>-1</sup> at which it was maintained for 17 min. The injector and detector temperature was 260 °C. FAMEs yield (wt%) in each experimental run was calculated using Eq. (1) (Taher et al., 2017).

$$Y_{\text{FAMES}} = \frac{m_{\text{FAMES}}}{m_{\text{oil}}} \times 100\% \quad (1)$$

where,  $Y_{\text{FAMES}}$  is produced FAME yield (%),  $m_{\text{FAMES}}$  is the amount of FAME produced (mg) determined from the concentration of FAMEs produced and multiplied by the volume of *n*-hexane used for sample dilution, and  $m_{\text{oil}}$  is the amount of oil used at the beginning of the reaction and determined by multiplying the 0.5 ml of oil with oil density. The specific yield of FAMEs produced in all tested cycles was calculated according to Eq. (2)

$$\hat{Y}_{\text{FAMES}} = \frac{1}{E} \frac{\sum_{i=1}^{i=5} m_{\text{FAMES}}}{\sum_{i=1}^{i=5} m_{\text{oil}}} \times 100\% \quad (2)$$

where,  $\hat{Y}_{\text{FAMES}}$  is the specific yield (%),  $E$  is the amount of enzyme used in mg,  $i$  is cycle number and  $\sum_{i=1}^{i=5} m_{\text{FAMES}}$  and  $\sum_{i=1}^{i=5} m_{\text{oil}}$  are the sum of all FAMEs produced and total oil used in the five cycles, respectively.

#### 2.7. Statistical analysis

All experiments were performed in duplicate, and the average values were presented in the figures. Reproducibility of the results were confirmed by the standard deviations presented as error bars. Differences between the different solvents and the control were compared using *t*-test and ANOVA analysis. For comparisons, *p*-values were determined, with values less than 0.05 indicating significant differences/effect. The dotted lines in the figure between the experimental data were used to highlight the trend.

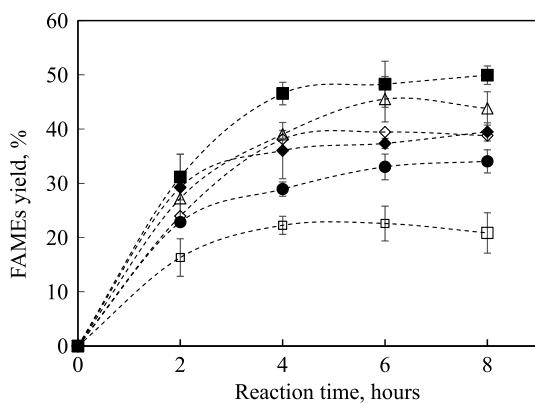
### 3. Results and discussion

#### 3.1. Using Chlorine based DESs as a replacement to [bmim][PF<sub>6</sub>]

The effectiveness of using ChCl:Gly DES at a 1:2 ratio tested and compared to solvent-free reaction system. FAMEs yield was found to increase from only 6.3% in the absence of ChCl:Gly to 30.3% when the DES was used. To prove that the DES used did not contribute to the catalytic action, and that the reaction was catalyzed solely by the enzyme, a control experiment was carried out at the same operating condition but in absence of enzyme. The production yield was only 0.14% in enzyme-free system, which confirmed that the catalytic activity of ChCl:Gly was negligible. Another experiment was carried out with same amounts of oil, ChCl:Gly and enzyme loading, but in the absence of methanol. A negligible production yield of less than 1% was achieved, which proved that the DES acted only as a reaction medium and not as a reaction substrate.

To show the superiority of DES over IL, the experiment was carried out at 45 °C using 0.5 ml oil, 6:1 M:O molar ratio, 8% enzyme loading and 0.375 ml of different solvents. Fig. 1 shows the change of biodiesel production yield with time using ChCl:Gly (1:2) DES and ChCl:Gly:W DESs of different water contents, compared to that using [bmim][PF<sub>6</sub>].

As shown in Fig. 1, by replacing [bmim][PF<sub>6</sub>] with pure ChCl:Gly (1:2), the FAME yield increased from 21 to about 34% after 8 h of reaction. The increase in the reaction rate and yield with



**Fig. 1.** FAMEs yield versus time, at 45 °C, 6:1 M:O molar ratio, 8% enzyme loading in □ [bmim][PF<sub>6</sub>], • pure ChCl:Gly and ChCl:Gly:W of ◇ 1, △ 3, ■ 4 and ♦ 5% H<sub>2</sub>O.

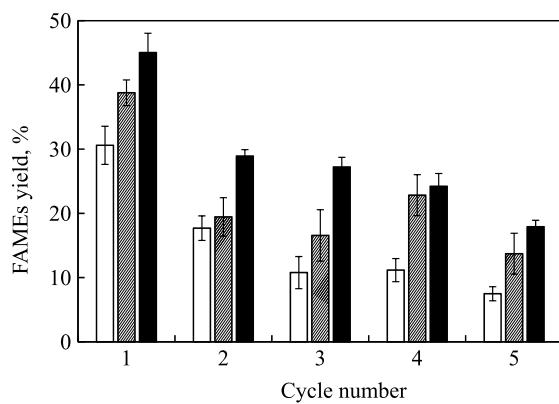
the use of DES, is mainly attributed to the favorable enzyme conformation and enhanced activity. Using the same enzyme, Novozym®435, with an organic IL, namely [bmim][PF<sub>6</sub>], higher maximum biodiesel yields were achieved from microalgae oil, which was reported to be 86% (Lai et al., 2012) and 72% (Taher et al., 2017) from waste oils. However, a much longer reaction time of 48 h and higher solvent to oil and methanol to oil ratios were applied in the former work (Lai et al., 2012), and a much higher enzyme loading of 40% was used in the latter (Taher et al., 2017).

Using the ternary ChCl:Gly:W DES, of 1% water resulted in increasing the yield to 40%. It was also found that the initial reaction rate increased from 8.2 mg h<sup>-1</sup> when [bmim][PF<sub>6</sub>] was used to 12.0 mg h<sup>-1</sup> when ChCl:Gly:W (1% H<sub>2</sub>O) was used. Among all tested reaction media, ChCl:Gly:W containing 4% water resulted in the highest initial reaction rate of 15.6 mg h<sup>-1</sup> and maximum yield of 50% after 8 h of reaction. This better performance could be attributed to the reduction in the viscosity of the DES solution with the increase in water content. However, increasing the water content beyond 4% resulted in a significant drop in the yield with a *p*-value of 0.04. The water content also changes the polarity of the solvent, due to the increase of the hydroxyl groups (Mulia, 2017), which was the main reason for the drop in reaction yield.

Transesterification of soybean oil was also tested using Novozym®435 and ChCl:Gly:W (1% H<sub>2</sub>O) (Zhao et al., 2013). A production yield of about 80% was achieved after 24 h of reaction at 50 °C using larger amounts of enzyme loading (40%) and DES (7:1 v/v solvent to oil ratio) (Zhao et al., 2013), compared to only 8% loading and 0.75:1 v/v used to generate the results shown in Fig. 1.

### 3.2. Reusability of enzyme-DES system

Fig. 2 shows the FAMEs yields at 6:1 M:O molar ratio, 45 °C, ChCl:Gly:W (3% H<sub>2</sub>O), which showed the best performance, and different enzyme loadings. As shown, the production yield was 30% at 8% loading (equivalent to 50 mg of Novozym®435), and increasing the enzyme loading to 17% enhanced the yield to 40%. Further increase in the enzyme loading to 35% resulted only in a slight increase in the yield, reaching 45%. This indicates that the enzyme saturation was approached, at which all available substrates would be attached to an active site. At that point any further increase in active sites, by increasing the loading, would have no effect on reaction rate. Similar observation was also found in the transesterification of lamb fats (Taher et al., 2011) and microalgae lipids (Taher et al., 2014) with Novozym®435 and soybean oils (Noureddini et al., 2005) with immobilized *Pseudomonas cepacia* lipase.

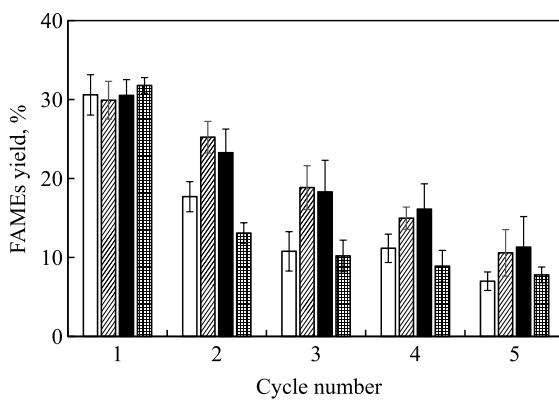


**Fig. 2.** FAMEs yield after 1 h of reaction at 45 °C, 6:1 M:O molar ratio using ChCl:Gly:W (3% H<sub>2</sub>O) at □ 8, ▨ 17 and ■ 35% enzyme loadings.

Fig. 2 also shows the reusability of the DES-immobilized enzyme system in five consecutive cycles of 60 min each, after removing only the main product, biodiesel, with *n*-hexane, at the end of each cycle. The reusability of the DES-immobilized enzyme system was found to drop sharply after the first cycle in all tested enzyme loadings, which was mainly due to the accumulation of glycerol that did not dissolve in the extraction solvent, *n*-hexane (Venter and Nieuwoudt, 1998; Dossat et al., 2002). Accumulation of glycerol inside the reaction cell, pushed the reaction backward, increased the mixture viscosity and deposited inside the supporting matrix of the immobilized enzyme, preventing the substrates from reaching the active site. This phenomenon has been documented in literature Luque and others (2016). All these effects resulted in the drop reaction rate and yield. The drop in the activity of the DES-immobilized enzyme system was most severe at the lowest enzyme loading of 8%, retaining only 22% of the original activity in the fifth cycle. As the enzyme loading increased, better reusability was achieved. The remaining activities in the fifth cycle, compared to the first, were 35% and 42% at enzyme loadings of 17% and 35%, respectively.

To overcome the problem of glycerol accumulation, after removing the main product, biodiesel, with *n*-hexane, two solvents were tested to remove the by-product, glycerol. The experiments were carried out in ChCl:Gly:W DES containing 3% water at the same conditions of 45 °C, 6:1 M:O molar ratio, and a reaction time of 60 min in each cycle. The smallest enzyme loading of 8% was used, at which the largest drop in the activity of the DES-immobilized enzyme was encountered in the previous experiment, as shown in Fig. 2. The FAMEs yields attained after the removal of both products, and compared to that with removing only the main product are shown in Fig. 3. It can be seen that the reusability of the DES-immobilized enzyme was improved with the removal of glycerol, by both solvents. By using 1-butanol, more than 88% and 47% of the activity in the first cycle, were retained in the second and fifth cycles, respectively, compared to 58% and 24% after the second and the fifth cycles without glycerol removal. The enzyme reusability was also improved using EG, but the performance of 1-butanol was better. About 73% and 31% of the activity in the first cycle, were retained in the second and fifth cycles, respectively, when EG was used. The better performance of 1-butanol could be attributed to its lower viscosity, compared to that of EG, which results in a better mass transfer within the system.

The experiment was repeated using higher enzyme loadings of 17% and 35%. The results shown in Fig. 4, shows similar results, wherein removing the glycerol with 1-butanol enhanced the reusability of the DES-immobilized enzyme system. It was found that the reusability increased with the increase in loading. At a

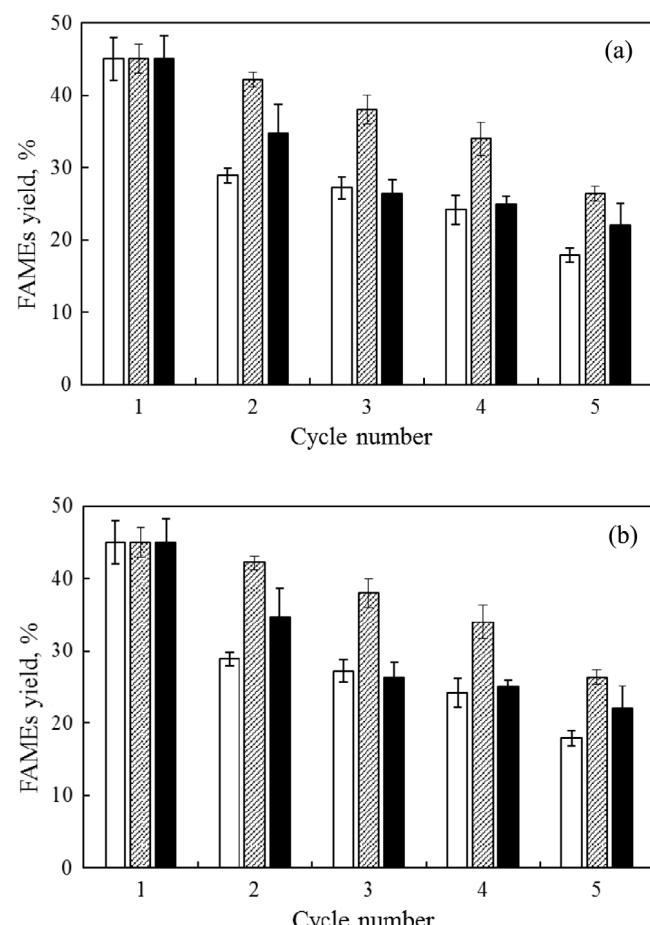


**Fig. 3.** FAMEs yield after 1 h of reaction at 45 °C, 6:1 M:O molar ratio and 8% enzyme loading, using ChCl:Gly:W (3% H<sub>2</sub>O) using □ no glycerol removal, ■ glycerol removal by EG, ■ glycerol removal by 1-butanol and ▨ washed enzyme with fresh DES in each cycle.

loading of 17%, by using 1-butanol, more than 85% and 36% of the activity in the first cycle, were retained in the second and fifth cycles, respectively, compared to only 49% and 33% after the second and the fifth cycles without glycerol removal. At a loading of 35%, by using 1-butanol, more than 77% and 49% of the activity in the first cycle, were retained in the second and fifth cycles, respectively, compared to only 64% and 40% after the second and the fifth cycles without glycerol removal.

Without removing the glycerol, the specific yield of produced FAMEs in the five cycles at enzyme loading of 8% was calculated to be 1.55 mg mg<sup>-1</sup>. With glycerol removal by 1-butanol from enzyme-DES system, the specific yield in the five cycles increased to 2.02 mg mg<sup>-1</sup> (30% higher), which would reflect positively on the overall cost of biodiesel production. Similar observation was also found at other tested enzyme loadings, but the effect was less, reaching 25% and 13% in five cycles, at enzyme loadings of 17% and 35%, respectively. If the enzyme is to be used for only one cycle, then the amount used would definitely determine the economic feasibility of the process. However, the process becomes more feasible when the same amount of enzyme is used repeatedly for several cycles. In this case, the reusability, which this work is focusing on, becomes more important than the amount, and therefore the higher amounts were considered. Then again, it was shown that the highest amount of produced biodiesel in five cycles per amount of enzyme used was indeed at 8%.

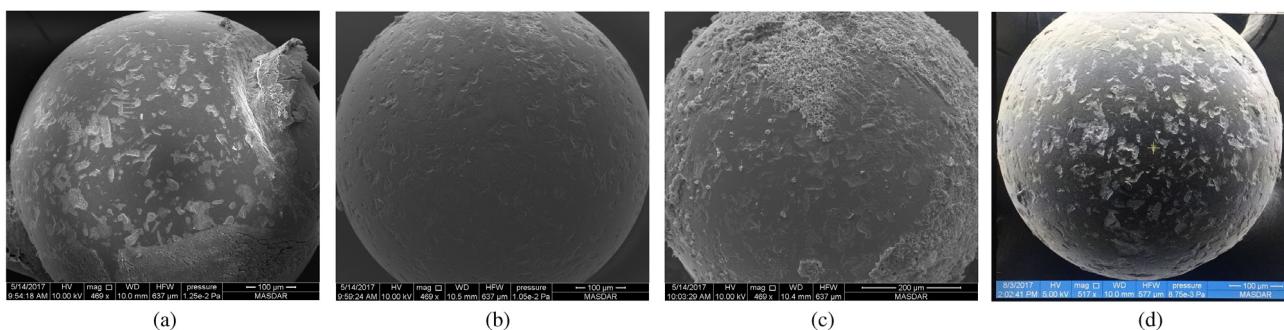
For a better understanding of the effect of the solvents on the DES-immobilized enzyme system, morphological visualization of the residue was investigated and compared to the fresh enzyme before use. Fig. 5(a)–(d) shows Scanning Electron Microscope (SEM) images for enzyme surface before and after being washed with the selected solvents, 1-butanol and ethylene-glycol, as compared to the fresh enzyme before being used for the reaction, and the enzyme after the reaction and removal of the products, but before being washed with the polar solvent. All images are for immobilized lipase at the end of the first cycle, carried out at 45 °C, 6:1 M:O molar ratio and 8% loading for 1 h reaction. When only *n*-hexane was used, large amounts of deposition, on the immobilized surface was observed (Fig. 5(b)), which is mainly glycerol. The deposition is believed to be from glycerol, which is produced throughout the reaction. Due to its hydrophilic nature, glycerol tends to separate from the hydrophobic reaction medium and deposit on the support of immobilized lipase, which is a documented phenomenon (Luque and others, 2016). Much less deposition can be seen when 1-butanol was used, and the surface of the immobilized lipase (Fig. 5(c)) was almost identical to the



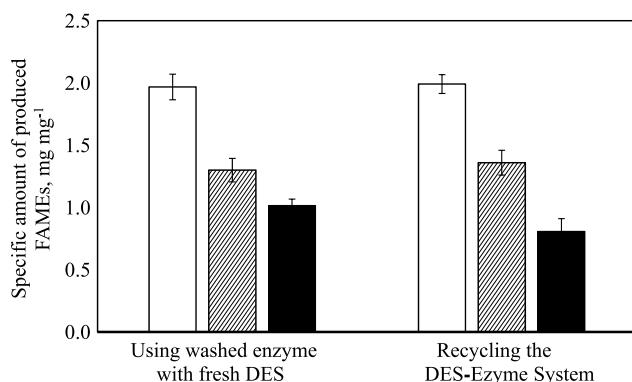
**Fig. 4.** FAMEs yield after 1 h of reaction at 45 °C, 6:1 M:O molar ratio and (a) 17% and (b) 35% in different cycles, using ■ enzyme-DES system, ▨ enzyme with fresh DES, and □ unwashed enzyme-DES system.

image of the fresh enzyme (Fig. 5(a)). Using EG resulted also in less deposition (Fig. 5(d)), but it was still higher than that when 1-butanol was used. These observations agree with the previous results, shown in Fig. 3.

It was shown that the reusability of the DES-immobilized enzyme system could be improved by removing both main and by products using *n*-hexane and 1-butanol, respectively. During the preparation stage of the DES, complex hydrogen bonding network is formed between the constituents, i.e., ChCl and Gly, making the solvent very stable and would not be affected by simple liquid-liquid extraction. A much higher temperature, than the one used in the reaction and regeneration experiments, is required for glycerol to be removed from the DES. Nevertheless, as glycerol is a main component of the ChCl:Gly (1:2), which has to be prepared in a specific molar ratio, it was important to assess the possibility of either reducing glycerol molar ratio in the DES by removing some of the bound glycerol with the direct exposure to 1-butanol, or increasing the ratio by the produced glycerol from the reaction. Therefore, the experiment was repeated, but in this case, at the end of each experiment and after removing the main product, the immobilized enzyme was separated from the reaction medium by filtration, and washed with *n*-hexane and 1-butanol, before being used in another cycle with fresh reaction substrates and freshly prepared ChCl:Gly:W (3% H<sub>2</sub>O), and the results are shown in Fig. 3. As compared to the case when the DES-Immobilized system was reused using *n*-hexane and 1-butanol, it was found that there was not a significant difference between FAMEs yields in the five cycles



**Fig. 5.** SEM images of enzyme particle after 1 h reaction (i.e., first cycle) at 45 °C, 6:1 M:O molar ratio and 8% loading: (a) after removing the product by *n*-hexane, (b) after removing the product and washing with 1-butanol and (c) after removing the product and washing with, as compared to (d) freshly used enzyme.



**Fig. 6.** The specific amount of FAMEs produced in the five consecutive cycles of 1 h each, using ChCl:Gly:W (4% H<sub>2</sub>O), at 45 °C and 6:1 methanol: oil ratio and enzyme loadings of □ 8%, ■ 17% and ■ 35%.

for the two cases, especially at the low enzyme loadings. At the enzyme loadings of 8% and 17%, the *p*-values were 0.99 and 0.18, respectively, which confirm that the used 1-butanol removed only the glycerol deposited on enzyme surface and did not affect the DES. This also proves that the procedure described in this work could be used successfully to enhance the reusability of the DES-immobilized enzyme system. The effect, however, became more significant (*p*-value = 0.032) at high enzyme loading of 35% and a higher yield was obtained when fresh DES was used in each cycle. This could be due to the larger amount of glycerol produced at the higher loading, as shown in Figs. 4(b) and 6, in which the 1-butanol used was inadequate to extract all the glycerol from the DES-immobilized enzyme system, unlike in case the immobilized enzyme was separated and washed with the 1-butanol.

Although enzymatic biodiesel production using DES has been reported in other studies, it is the first time to test the reusability of the enzyme-solvent system for several cycles, which is very important for efficient and feasible enzymatic biodiesel production process. The work also looked into the effect of removing the glycerol, and its effect on the solvent and the reusability. In this work, not only the enzyme was reused, but the enzyme-solvent system altogether. In addition, this is the first work to attempt using the ternary DES, wherein the water is added during the solvent preparation. Using the ternary DES, containing 3% water, the viscosity of the DES dropped by more than 5 folds.

#### 4. Conclusions

The effectiveness of using ChCl:Gly DES as an alternative to an IL, [bmim][PF<sub>6</sub>], in enzymatic biodiesel production from waste oil was assessed. It was shown that DESs are more efficient and

achieved higher yields than the IL. After 4 h of reaction, the yield obtained in ChCl:Gly (1:2) DES was 63% higher than that obtained in [bmim][PF<sub>6</sub>], and further increased to 71.4% when the ternary DES containing 4% water was used. The reusability of the DES-Novozym®435 system in five consecutive cycles was also examined when the main product, FAMEs, was only removed between cycles and when by-product glycerol, was also removed. The best reusability of the DES-immobilized system was achieved when 1-butanol was used, as a washing solvent.

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#### Conflict of interest

All authors have declared no conflict of interest with materials discussed in this manuscript.

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