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In situ visualization and effect of glycerol in lipase-catalyzed ethanolysis of rapeseed oil

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ABSTRACT

Immobilized lipases can be used in biodiesel production to overcome many disadvantages of the conventional base-catalyzed process. However, the glycerol by-product poses a potential problem for the biocatalytic process as it is known to inhibit immobilized lipases, most likely by clogging of the catalyst particles. In this paper, this negative effect was further investigated and confirmed in ethanolysis of rapeseed oil. A dyeing method was developed for *in situ* visualization of glycerol in order to study its partitioning and accumulation during the ethanolysis reaction. The method was used to illustrate the interaction of glycerol with immobilized lipases and thus provided an aid for screening supports for lipase immobilization according to their interaction with glycerol. Glycerol was found to have great affinity for silica, less for polystyrene and no affinity for supports made from polymethylmethacrylate and polypropylene. It was also found that the immobilization of enzyme on the support influenced the adsorption of glycerol to the surface of the enzyme carrier.

1. Introduction

The depletion of fossil fuels makes it increasingly necessary to develop renewable energy alternatives, preferably having a smaller environmental impact than fossil fuels [1]. Biodiesel shows potential as such a renewable liquid fuel for the transport sector, being non-toxic and reducing the emission of most environmentally aggressive components in comparison to petrodiesel [2–4]. Biodiesel in the form of fatty acid methyl ester (FAME) is produced on a multi-million tonne-scale annually by a base-catalyzed transesterification process, in which vegetable oils are reacted with methanol, forming FAME and the by-product glycerol [3].

In the scientific literature, an alternative lipase-catalyzed process has been suggested offering low energy consumption, reduced by-product formation and less waste [5]. Immobilizing the lipases on a support simplifies recycling of the catalyst, which is necessary to make the process economically feasible. Biodiesel produced by a lipase-catalyzed process can be considered "green" since such a process conforms to several of the principles of green chemistry, including catalytic conversion, reduced energy use and high product yield. Replacing methanol with ethanol makes the biodiesel even better from an environmental perspective since, in contrast to methanol, ethanol can be obtained from renewable agricultural products via fermentation [6]. Further, due to the higher molecular weight of ethanol compared to methanol, less oil is used to produce one litre of ethyl ester (FAEE) compared to producing one litre of methyl ester (FAME) [7]. This translates into both economic and sustainability advantages.

As a by-product of biodiesel, glycerol accounts for 10 wt% of the final product. It has become common knowledge that glycerol has a negative effect on lipase activity and stability likely by being adsorbed onto the support of the immobilized lipases and reducing the diffusion of the hydrophobic substrate to the active site of the lipase [8]. This undesirable effect of glycerol will greatly shorten the operational stability of the expensive catalyst and consequently influence the economic viability of the process. In addition, the recovery of glycerol is also made more difficult by its affinity for immobilized lipase. Since the glycerol issue could increase the production cost and affect the process design, it needs to be taken into account when immobilized lipases are used for large scale biodiesel production. This is particularly the case with ethanol-containing azeotropic water concentrations, since it has been shown that the effects of glycerol are further exacerbated if a certain amount of water and glycerol co-exist in the reaction system [9].

Many researchers have reported the inhibitory effect of glycerol on transesterifications catalyzed by immobilized lipases [8,10], but only a few systematic studies have been published on investigating the inhibitory mechanism. Furthermore, thus far little has been reported on the impact of glycerol in solvent-free lipase-catalyzed

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transesterification of a triglyceride feedstock, its interaction with different catalysts and the implications of these issues for the operation of the biodiesel processes. In this paper we address these issues by investigating the effect of glycerol on both the activity and the stability of the biocatalyst in a solvent-free transesterification system catalyzed by *Thermomyces lanuginosus* lipase immobilized on a hydrophobic resin. To assist the investigation, a simple and straightforward dyeing method was developed.

The negative effect of glycerol makes it desirable to trace it in the reaction system. Although glycerol is immiscible with oil and biodiesel and has a higher density than any other component in the liquid phase of the reaction system, it is difficult to observe the separate glycerol phase in laboratory-scale apparatus because the glycerol-rich phase is relatively small and colorless. A dyeing method is therefore introduced to indicate the glycerol partitioning *in situ*. This method was previously used by Zhou and Boocock to stain the polar phase in base-catalyzed methanolysis, ethanolysis and butanolysis to visualize phase behavior [11]. In the transesterification catalyzed by immobilized lipase, the catalyst adds a solid phase to the system. To the best of our knowledge, this is the first time that this dyeing method has been implemented in this type of multiphasic system. The dyeing method is used here to visualize the glycerol in the reaction system and the interaction between glycerol and several different catalysts and support materials.

2. Materials and methods

2.1. Materials

Rapeseed oil and rapeseed derived biodiesel (fatty acid methyl esters) were kindly donated by Emmelev A/S (Otterup, Denmark). Absolute ethanol (≥99.9%) was purchased from Fluka (Buchs, Switzerland). n-Hexane (≥97.0%), cyclohexane (≥ 99.7%) and tert-butyl methyl ether (≥99.8%) were purchased from Sigma–Aldrich (Steinheim, Germany) as HPLC grade. Amaranth, a polar food-grade pigment was also purchased from Sigma–Aldrich (Steinheim, Germany).

The silica, polystyrene, polymethylmethacrylate (PMMA) and polypropylene supports were supplied by Novozymes A/S. All of the catalysts used in this study were kindly donated by Novozymes A/S (Bagsværd, Denmark): Novozym 435 (N435), which is *Candida antarctica* lipase B (CALB) immobilized on a macroporous divinylbenzene-crosslinked polymethylmethacrylate (PMMA); Lipozyme TL IM, which is *Thermomyces lanuginosus* lipase (TLL) immobilized on silica; Lipozyme TL HC, which is TLL immobilized on a polymeric resin (an experimental catalyst).

2.2. Methods

2.2.1. Ethanolysis of rapeseed oil without agitation

The reaction mixture was composed of 10 g rapeseed oil, 5% catalyst (Lipozyme TL IM, N435 or Lipozyme TL HC) and 1.8 mL absolute ethanol, which is 1.0 molar equivalent (eq.) to the total fatty acids in the oil. Reactions were performed in 25 mL glass tubes without any agitation maintained at 35 °C. Samples (30 μL) were taken by a pipette for HPLC analysis.

2.2.2. Ethanolysis of rapeseed oil with agitation

The reaction mixture was composed of 2 g rapeseed oil, 5% catalyst (Lipozyme TL HC) and 0.36 mL absolute ethanol, i.e. 1.0 molar equivalent (eq.) to the total fatty acids in the oil. Reactions were performed in 4 mL vials at 35 °C in a thermo-mixer at a mixing speed of 1000 rpm. Samples (30 μL) were taken by a pipette for HPLC analysis.

2.2.3. Effect of glycerol on catalysts

Varying amounts of glycerol (0–1.0 g) were added into mixtures of 10 g rapeseed oil and 0.5 eq. EtOH in a 25 mL glass tube. The tubes were shaken until the glycerol was dispersed as fine droplets in the oil. Finally, 5 wt% Lipozyme TL HC was added to start the reactions. A further 0.5 eq. EtOH was added after 2 h.

2.2.4. Stability test of catalysts

The ethanolysis reaction was catalyzed by 5 wt% Lipozyme TL HC and was repeated for three batches. The oil phase was decanted after each batch (24 h) and then fresh oil and 1.0 eq. EtOH was added to start a new batch.

2.2.5. Solubility test of dye

Mixtures were prepared in 25 mL glass tubes. Mixture (a) was composed of 10 g rapeseed oil, 1.0 eq. EtOH, 5 mg dye and 5 wt% support, immobilized catalyst or glycerol. Mixture (b) was composed of 10 g biodiesel, 1.0 eq. EtOH, 5 mg dye and 5 wt% support, immobilized catalyst or glycerol.

2.2.6. Affinity test

The catalyst or support (0.5 g) was incubated with 1.0 g glycerol and 5 mg dye for 3 h in a 25 mL glass tube before 10 g rapeseed oil or rapeseed derived biodiesel was added and then mixed by shaking. The mixture was subsequently photographed. Alternatively, 5 mg dye was added to the reaction mixture described above (ethanolysis of rapeseed oil). The mixture was photographed every 2 h for the first 12 h and also after 24 h reaction.

2.2.7. Photography

The glass tubes were laid horizontally on the table with a white background. A normal digital camera (Coolpix S5, Nikon, Japan) was used to take tube photos. Some samples were transferred from the glass tube to a Petri dish for microscope photography (Leica MZ12 equipped with PixeLINK digital camera, Germany).

2.2.8. HPLC analysis of reaction mixtures

Samples (30 μL) were dissolved and diluted in cyclohexane to 1.0 mL and further diluted 100-fold in cyclohexane to achieve concentrations of around 0.25 mg/mL. 10 μL of the solution was injected on an HPLC (Dionex A/S, Hvidovre, Denmark) for analysis of the composition of FAEE, TAG, FFA, DAG and MAG. The HPLC was equipped with a U3000 autosampler, TCC-3000SD column oven and U3400A quaternary pump modules. A Varian ELSD (380-LC) was used for detection with nebulizer and evaporator temperatures of 35 °C and 40 °C, respectively, and a nitrogen flow rate of 1.6 standard liters per minute. The separation was done on a 250 mm × 4.0 mm cyanopropyl column from Sigma–Aldrich (Discovery® Cyano) with an accompanying guard column (20 mm × 4.0 mm) of the same stationary phase and a flow rate of 0.75 mL/min. Program control, data acquisition, and analysis were carried out using Chromeleon 6.8 software. A binary gradient program was applied using phase A: 99.6% hexane, 0.4% acetic acid; and phase B: 99.6% methyl-tert-butyl ether, 0.4% acetic acid.

3. Results and discussion

3.1. Effect of glycerol on immobilized lipase

3.1.1. Effect of glycerol on the activity of immobilized lipase

The effect of glycerol on the activity of immobilized lipase in the ethanolysis of rapeseed oil was studied by addition of external glycerol to the reaction mixture, with the results shown in Fig. 1A. In this case the catalyst (Lipozyme TL HC) lost most of its activity with added glycerol between 2 wt% and 10 wt% compared to the control reaction. Hence, the glycerol greatly inhibited the catalyst. Even a

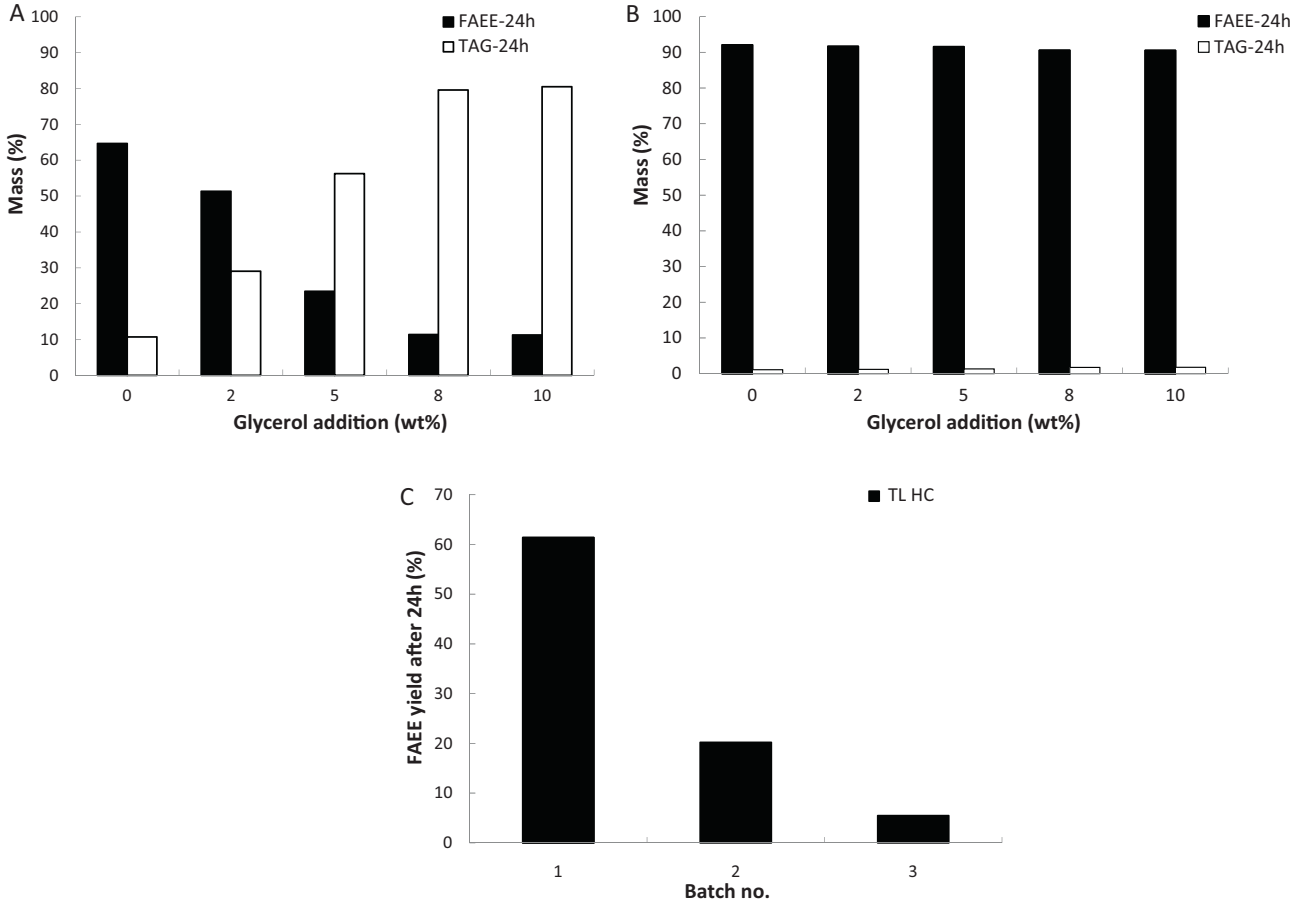

Fig. 1. Effect of glycerol co-existence on the activity and operational stability of Lipozyme TL HC in reactions. Activity without agitation (A), activity with agitation (B) and stability without agitation (C). Amount of glycerol is calculated as percent to the oil (w/w).

small amount of glycerol (2%) reduced the final conversion by 21% in the absence of agitation. It was observed that the immobilized catalyst was aggregated by the viscous glycerol and the liquid phase was opaque throughout the 24-h reaction.

It is clear that the catalyst deactivation is associated with glycerol encapsulating the particles of immobilized TLL, but there are at least three possible mechanisms that might explain the dramatic activity drop of the immobilized lipase. First the diffusion of the substrate to the lipase could be limited by a glycerol layer blocking the pores of the support. A second explanation might be that the hygroscopic nature of glycerol causes a reduced water activity in the system, affecting the performance of the enzyme. These two hypotheses have previously been proposed and studied [8]. A third possibility raised in this study is that glycerol competes with the alcohol substrate to be bound to the active site. In the study by Dossat et al., the inhibition effect was investigated in an esterification reaction system using n-hexane as co-solvent. Conditions were employed that simulated an immobilized catalyst (lipase *Rhizomucor miehei* on Duolite) covered by either glycerol; water; or glycerol maintained with optimal water activity, respectively, and it was concluded that the inhibitory effect on lipase was mainly due to mass transfer limitation caused by glycerol clogging.

To determine the dominant mechanism of glycerol inhibition in solvent-free transesterification where glycerol is also formed as a by-product, an experiment with added glycerol was repeated in a well-mixed reaction system. When sufficient agitation was used, the glycerol could be stripped from the catalyst instantaneously, resulting in no bound glycerol and therefore no catalyst clogging. The result in Fig. 1B shows that when the catalyst clogging by glycerol is avoided, the added glycerol had little effect on the activity of the catalyst and a conversion of 90% was achieved with varying additions of external glycerol. Likewise this indicates that the impact of glycerol as a competing substrate is negligible when glycerol and ethanol coexist. Furthermore, the dehydrating effect of glycerol on lipase on account of its hygroscopic nature is apparently also insignificant. This experiment thus verifies that the mechanism of inhibition suggested by Dossat et al., namely that the inhibition is caused by glycerol clogging the pores of the catalyst, is dominating in solvent-free transesterification as well. This phenomenon is visualized and confirmed by the dyeing method introduced in the following sections.

3.1.2. *Repeated use of Lipozyme TL HC in non-agitated reactions*

The clogging effect of glycerol on Lipozyme TL HC was further investigated by reusing the catalyst for transesterification in a non-agitated reaction system. The activity dropped dramatically from batch to batch and only 5.5% was left by the third batch, as shown in Fig. 1C. Analysis of the reaction data from the first batch indicate that the glycerol produced in the first batch corresponds to 4.7 wt% of the reaction mixture. The yield of FAEE in the subsequent batch dropped to 21%, which is very close to the 23% yield that was achieved in a standard, non-agitated reaction when 5 wt% external glycerol was added (Fig. 1A). This experiment illustrates that a process using Lipozyme TL HC for transesterification of glycerides must be designed to ensure removal of glycerol from the catalyst, for example by applying agitation, if operational stability is to be

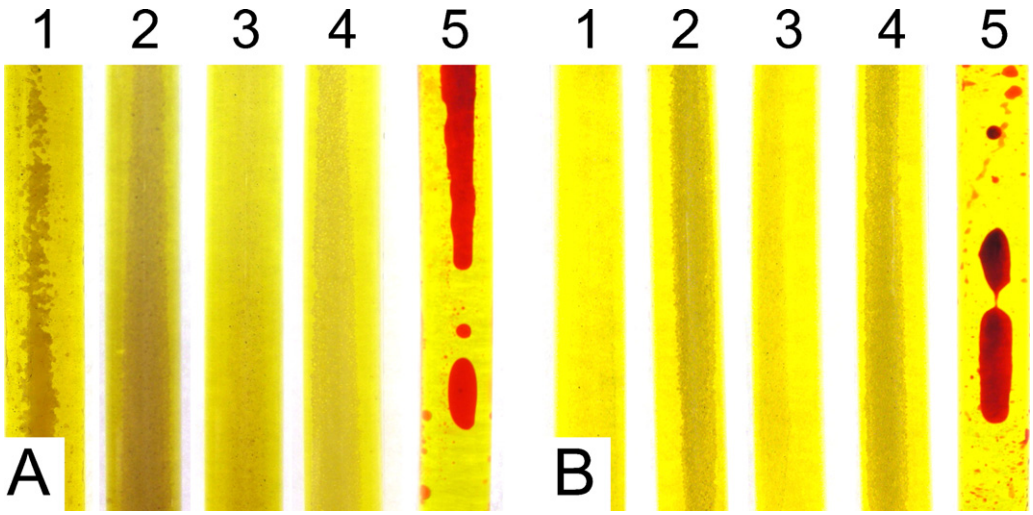

Fig. 2. The solubility of dye in rapeseed oil based mixture (oil, 1.0 eq. EtOH and dye) (A) and biodiesel-based mixture (biodiesel, 1.0 eq. EtOH and dye) (B). The solids in tube 1 – silica support, tube 2 – N435, tube 3 – Lipozyme TL IM, tube 4 – Lipozyme TL HC, tube 5 – glycerol.

achieved. Indeed, repeated use of catalyst in the agitated reaction system (used for the results shown in Fig. 1B) consistently resulted in final conversions above 90% (data not shown).

3.2. Dyeing method to indicate the effect of glycerol

3.2.1. The solubility of the dye in simulated reaction mixtures

Before the dye was introduced to indicate glycerol partitioning in the biodiesel system, its physical chemistry was assessed by simple experiments. The dye was purchased in the form of a dry powder. It is polar and consequently has no solubility in rapeseed oil or biodiesel. Instead, it exhibits great solubility in glycerol and limited solubility in anhydrous ethanol. Fig. 2A shows pictures of the dye in a solution of oil and 1.0 molar equivalent EtOH. To these mixtures were added either solid particles (hydrophilic silica support or one of three immobilized lipases: N435, Lipozyme TL IM or Lipozyme TL HC) or glycerol (10 wt%). Dye was added in an amount sufficient to stain all of the glycerol that would be produced if the oil was fully converted into biodiesel. Fig. 2B shows pictures of similar dye mixtures where the oil has been replaced with biodiesel. Both figures demonstrate that the dye could not be dissolved in either oil or biodiesel mixtures and that the dye also has no influence on silica or immobilized lipases. On the other hand, the same amount of dye powder was easily dissolved in glycerol, which was then stained red. The presence of 1.0 molar equivalent ethanol in the oil did not affect the ability of the dye to work as an indicator in these systems.

3.2.2. Affinity of the glycerol byproduct for immobilized lipases in ethanolysis

3.2.2.1. Immobilized lipases in ethanolysis. The glycerol affinity for immobilized lipase was investigated in enzyme-catalyzed ethanolysis reactions. Reactions took place at 35 °C with 5% catalyst loading, 1.0 molar equivalent of anhydrous ethanol for 24 h without any agitation. The photos in Fig. 3 show the partitioning of glycerol during the ethanolysis in this multiphasic system. As can be seen in Fig. 3A, glycerol formed a layer on the surface of Lipozyme TL IM particles. The liquid phase was clear with no free glycerol being released from the catalyst to the bulk solution, suggesting that the formed glycerol was fully adsorbed on/in the Lipozyme TL IM particles.

The affinities of N435 and Lipozyme TL HC for glycerol are less than that of Lipozyme TL IM, as can be seen in Fig. 3B and C. Part of the produced glycerol remained on the surfaces of both catalysts and some free glycerol droplets are noticeable in the oil phase of each reaction. It was also observed that the aggregation of Lipozyme TL HC was more severe than that of N435 (photo not shown), which can probably be explained by more glycerol being produced by the former catalyst. In order to test this hypothesis, the activities of N435 and Lipozyme TL HC were compared (see Fig. 4A). Under the given conditions N435 had a lower activity than Lipozyme TL HC (determined by HPLC analysis) and consequently produced less glycerol.

The difference in substrate specificity between CALB (the lipase on N435) and TLL (the lipase on Lipozyme TL IM and Lipozyme TL HC) is illustrated by comparing the consumption of TAG as a

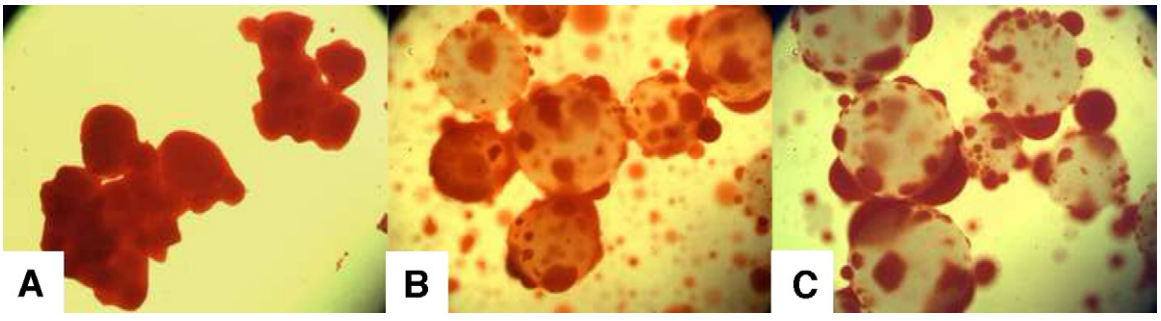

Fig. 3. Glycerol partitioning in ethanolysis of rapeseed oil, catalyzed by different immobilized catalysts. Lipozyme TL IM (A), N435 (B) and Lipozyme TL HC (C).

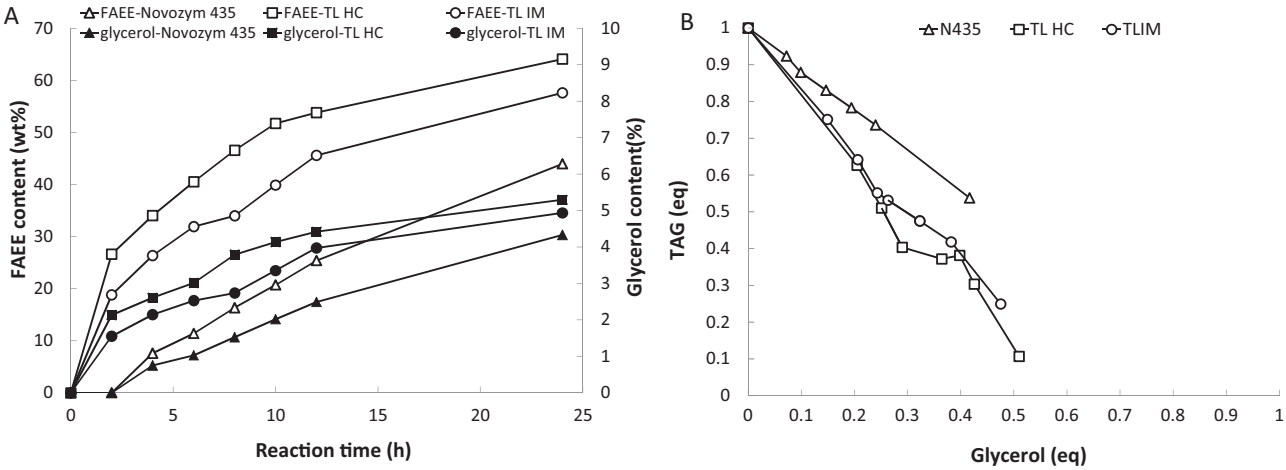

Fig. 4. Reaction profiles of N435, Lipozyme TL HC and Lipozyme TL IM. FAEE and glycerol production as function of time (A); TAG consumption as a function of glycerol production (B).

function of the production of glycerol from the three catalysts, shown in Fig. 4B. The TAG consumption curves of Lipozyme TL IM and Lipozyme TL HC are steeper than that of N435, indicating that TLL consumed more TAG than CALB to produce the same amount of glycerol. In other words, TLL worked better with TAG than with DAG and MAG, which is most likely related to its 1,3-positional specificity reported by many researchers [12,13]. DAG and MAG turned out to be the favorite substrates for CALB which is less regiospecific. This substrate specificity is likely due to the different structures of active sites. The elliptical, steep funnel-like binding site of CALB has limited space for hosting substrates [14]. TLL, structurally homologous to lipase from *Rhizomucor miehei*, has a hydrophobic crevice-like binding site with a larger space available to bulkier substrates [14,15].

3.2.2.2. Glycerol production and accumulation on the catalysts. Finally, Fig. 5A and B shows the glycerol accumulation on Lipozyme TL IM and N435, respectively, as a function of the reaction time.

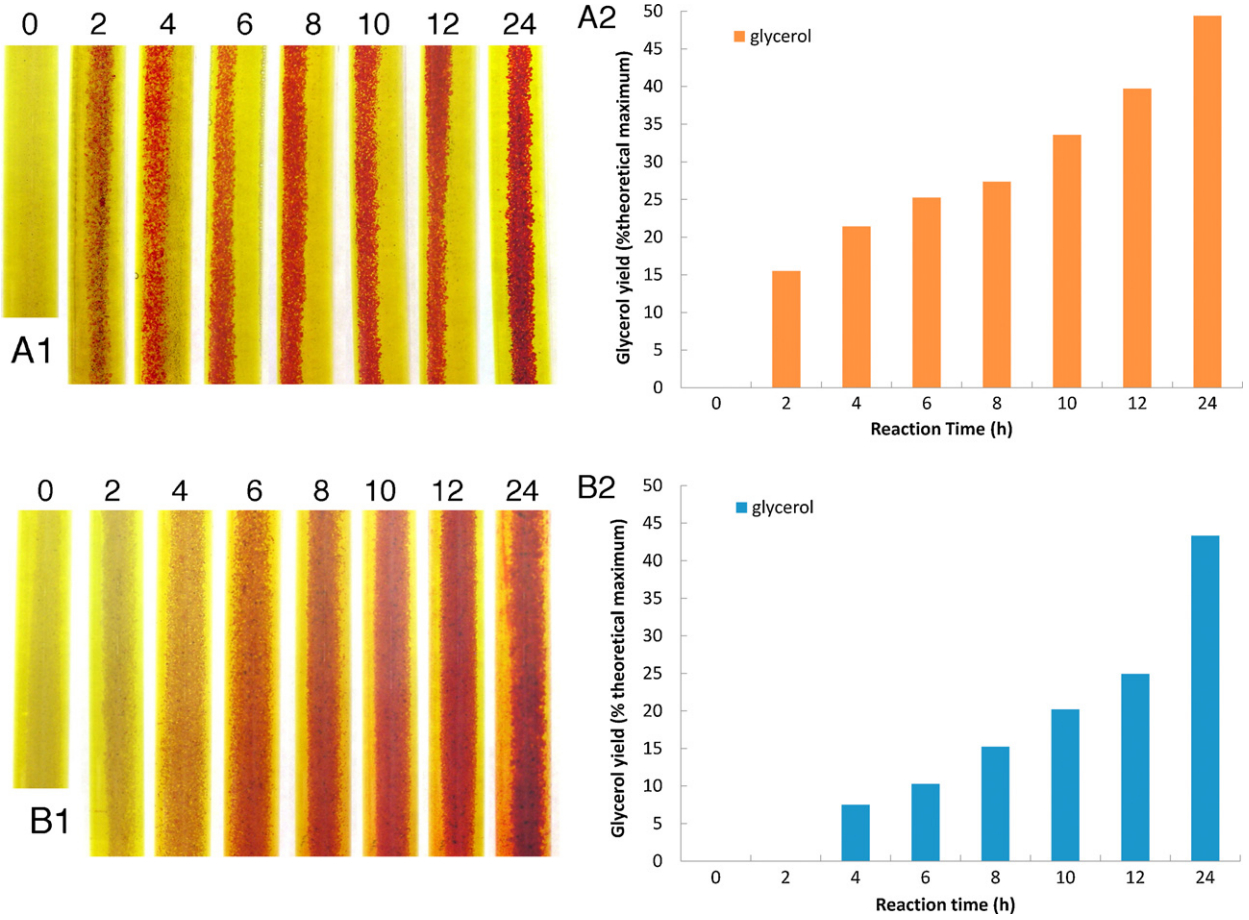

Fig. 5. Glycerol accumulation on catalysts as a function of reaction time. Lipozyme TL IM (A) and N435 (B).

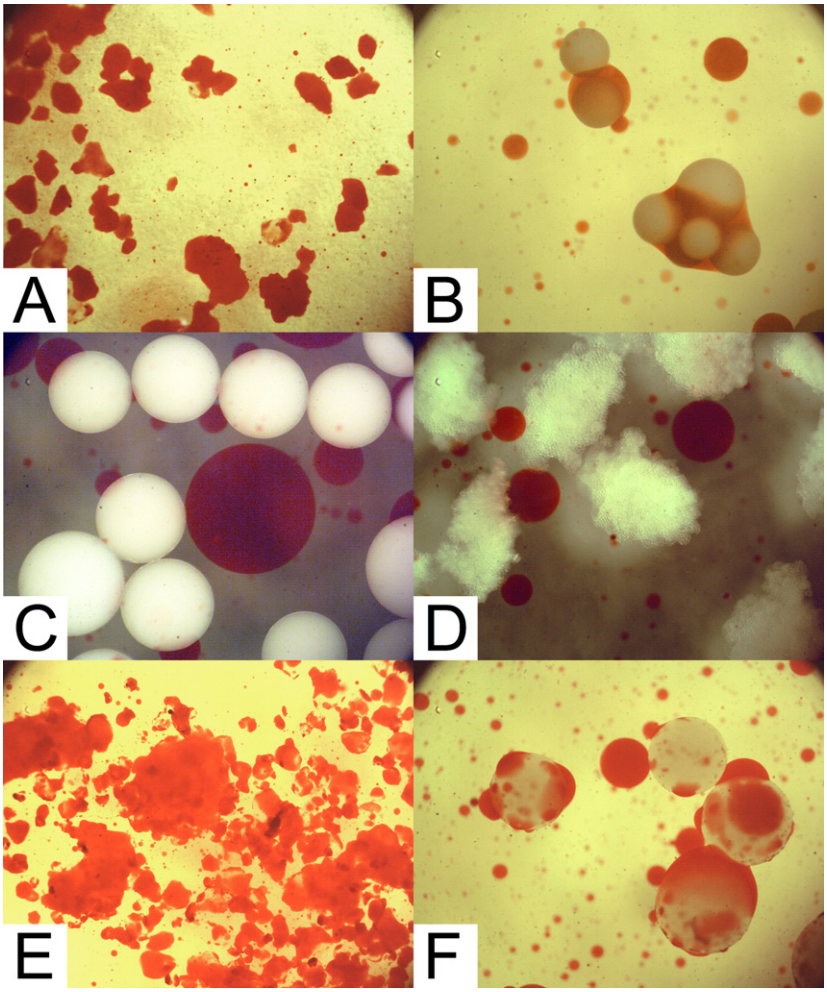

Fig. 6. Affinity of glycerol for supports and immobilized lipases. The supports shown in the photos are: silica (A), polystyrene (B), polymethylmethacrylate (PMMA) (C) and polypropylene (D). The immobilized lipases are: Lipozyme TL IM (E) and N435 (F). In each photo, the particles of supports and immobilized lipases are in white and the glycerol droplets are in red (in the printed version of this article, white and red are shown as white and black, respectively).

As the reaction proceeded from 0 h to 24 h, glycerol was produced and most of it adhered to the catalyst, particularly in the case of Lipozyme TL IM. With dye present in the reaction, the accumulation of glycerol could be followed by the intensity of the red color and correlated with the glycerol production curve (the red color appears as darker/black areas in black and white print). Each data point on the glycerol production curve was calculated from the compositions of FAEE, FFA, TAG, DAG and MAG at the corresponding time point, as measured by HPLC.

The color of the catalyst (N435 or Lipozyme TL IM) became more intense as more glycerol was produced, suggesting that the dyeing method could be developed into a tool for measuring the glycerol production by quantifying the color intensity. It was observed that glycerol began to form earlier in reactions catalyzed by Lipozyme TL IM than those by N435. The color could be detected after only 2 h in the TL IM-catalyzed reactions, whereas the color was not obvious until after 4 h in N435-catalyzed reactions as seen from the photos in Fig. 5A-1 and B-1. This correlates well with the data presented in Fig. 4A.

3.2.3. *Affinity of glycerol for immobilized lipases and supports in simulated mixtures*

Four supports were tested individually for their affinity for glycerol in a simulated system based on a synthetic mixture of glycerol, dye and oil. The results are shown in Fig. 6. The silica (Fig. 6A) was immediately covered by the glycerol, indicating that it has a great affinity for glycerol due to the hydrophilic properties of the material. No free glycerol droplets could be seen in the oil, implying that 0.5 g silica could adsorb at least 1.0 g glycerol, in agreement with the observation in silica-based TL IM-catalyzed-reaction. This was further verified in a separate experiment, where it was observed that 1.0 g silica could adsorb as much as 2.0–3.0 g glycerol (data not shown). The polystyrene support (Fig. 6B) also exhibited some affinity for glycerol in that the particles were aggregated by the glycerol, forming clumps. On the other hand, the polymethylmethacrylate (PMMA) support and the polypropylene support, shown in Fig. 6C and D, respectively, both appear to be very hydrophobic in that no glycerol was adsorbed on these materials. In these cases the free glycerol droplets were dispersed in the oil.

Immobilized lipases (Lipozyme TL IM and N435) were also tested for their affinity for glycerol using the same method. As expected, Lipozyme TL IM was immediately covered by the glycerol (Fig. 6F) just as in transesterifications carried out by this catalyst. Similar results were obtained with silica without enzyme. The effect of the enzyme on the affinity for glycerol was evident when the support of PMMA and its immobilized lipase (N435) were compared (Fig. 6C and F). The PMMA support is hydrophobic enough to avoid being covered by the glycerol even after incubation with added glycerol for 3 h at room temperature, whereas N435

attracted some glycerol onto its surface during incubation. This implies that the immobilization of lipase onto the support makes it less hydrophobic, possibly due to polar groups on the protein. More importantly, it also shows that it is not sufficient to test the support alone when investigating the glycerol affinity of an immobilized catalyst.

4. Conclusions

The glycerol formed in biodiesel synthesis by immobilized lipases can severely reduce the reaction rate by surrounding the catalyst in a hydrophilic layer, thereby limiting the mass transfer of substrate to the enzyme.

The developed dyeing method successfully visualizes this interaction between glycerol and lipase catalysts, showing extensive interaction between glycerol and a hydrophilic catalyst. Conversely, hydrophobic catalysts are able to release most of the glycerol produced, especially when agitation is applied.

Similar results can be obtained with simulated reaction mixtures, suggesting that the dyeing method could have great potential when screening enzyme supports for materials applicable to biodiesel production.

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