Dual bioimprinting of *Thermomyces lanuginosus* lipase for synthesis of biodiesel

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ABSTRACT

Use of biodiesel as an alternative to non-renewable sources of energy has become an attractive option in recent years. The enzymatic synthesis of biodiesel by transesterification of fats/oils with an alcohol is a much more sustainable route than the chemical method. However, cost effectiveness of the enzymatic route is a major barrier in its commercialization. In this work, a high activity biocatalyst design of *Thermomyces lanuginosus* lipase is made by dually bioimprinting it with substrate and a surfactant (which is believed to open up the lid covering the active site of the lipase) during precipitation of the lipase in organic solvent. When the lipase was bioimprinted with only the surfactants, 28 U of the enzyme/g of oil could yield 99% biodiesel from soybean oil in about 4 h. However, when dually bioimprinted even very low enzyme load 1.4 U/g of oil, yielded 99% biodiesel within 48 h.

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1. Introduction

As a short term measure, use of biodiesel blends with diesel appears to be a good option among encouraging the switch over to alternative fuels in general and biofuels in particular [1–4]. One major worry has been that without tax breaks, replacing diesel with biodiesel blends is not possible [5,6]. At present, biodiesel blends sold at petrol pumps in various countries is made by chemical catalyst route. There is a huge gap between the cost of chemical catalysts and lipases which can be utilized as the greener choice as a catalyst [2]. Nevertheless, from the view point of development of a more sustainable process, switch over to the lipase catalysed route is very attractive. Hence, it is not surprising that interest in these enzyme catalysed processes has continued over the last few decades. A pilot plant for production of biodiesel in China which uses a lipase has been running for several years [2]. Some more are being established [7,8]. Obviously, any innovation which can reduce the cost of enzyme in this production process would facilitate further adoption of this green route at a commercial level.

The enzyme catalysed transesterification for obtaining biodiesel from fats/oils takes place in non-aqueous media [9–13]. Unfortunately, enzymes, including lipases show considerably lower catalytic efficiency than they show in aqueous buffers [14,15]. That results in lipases being used in non catalytic amounts in such cases [11].

In the present work, we describe a simple dual bioimprinting strategy to increase the catalytic efficiency of *Thermomyces lanuginosus* lipase (TLL). TLL happens to be among the least expensive commercially available lipases. We have reported earlier that precipitation rather than lyophilization is a better strategy for "drying" (removal of bulk water) enzymes before these are placed in organic solvents [16–20]. Such precipitates have been called enzyme precipitated and rinsed with propanol (EPRP) or more generally enzyme precipitated and rinsed with organic solvents (EPROS). The use of surfactants is known to activate lipases by opening of the 'lid' on the active site [21,22]. Our approach is similar to the one described by earlier workers [23–25] wherein the enzyme was dried by lyophilization. In the current work the drying has been done by precipitation. In both approaches the dried enzyme has a highly rigid conformation which results in freezing the "induced conformation". Recently, we have shown that EPROS of subtilisin prepared by precipitation with a substrate alcohol bioimprints the enzyme and increases its rate [20]. The dual bioimprinting by incorporating an ester substrate could

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enhance the reaction rates even further [20]. The dual bioimprinting strategy in the present instance consists of precipitating the lipase with a substrate alcohol in the presence of a surfactant. This led to a preparation imprinted with a substrate but in the open lid form. We also use a solvent free medium (the oil itself works as a reaction media and no additional organic solvent is added). This further reduces the cost and removes another "non green" component from the process.

2. Materials and methods

2.1. Materials

Thermomyces lanuginosus lipase (TLL) [Lipozyme TL 100L] was a kind gift from Novozyme, Denmark. Solvents like *n*-propanol and surfactant Bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). *tert*-Butanol and Triton X-100 were obtained from Fischer Scientific, Mumbai, India. Hexadecyl trimethyl ammonium bromide (CTAB) and ethanol were procured from Merck, Hohenbrunn, Germany. *N*-octyl-β-D-glucopyranoside (OG) was obtained from Calbiochem, Massachusetts, USA. Refined soybean oil was purchased from the grocery store. All other reagents and solvents were obtained from Fisher Scientific and were of the highest grade commercially available.

2.2. Methods

2.2.1. Preparation of the EPROS of lipases

The enzyme precipitated and rinsed with organic solvents (EPROS) were prepared as follows: The lipase (TLL: 100 μL) was dissolved in the 200 mM Tris HCl buffer, pH 7.5 (1 mL) containing various surfactants wherever mentioned. They were vortexed for two minutes and then precipitated in the suitable organic solvents (4 mL) at 4 °C and with shaking at 200 rpm for 30 min. The precipitates were then centrifuged at 1000*g* for 5 min. The supernatant was discarded and the precipitate was washed twice with the chilled precipitating solvent followed by three washes with chilled *n*-propanol to remove the surfactant wherever used as all the surfactants used had high solubility in alcohol. A final wash with chilled ethanol was also given. The EPROS were then used for further studies.

The unit of activity of the lipase used is the hydrolytic activity of the lipase as monitored by following the rate of hydrolysis of *p*-nitrophenyl palmitate at 37 °C spectrophotometrically at 410 nm [26]. One unit of enzyme activity was defined as the amount of lipase required to release 1 μmol of the *p*-nitrophenol per min.

2.2.2. Synthesis of Biodiesel using different biocatalyst preparations of lipase

Soybean oil (0.5 g) and ethanol were taken in the molar ratio of 1:4 in a vial. Optimum amount of water and silica (w/w of the oil) was added to the reaction mixture. The lipase preparation was added to this and incubated at the optimum temperature (40 °C) with a constant shaking at 200 rpm. Reactions were carried out in duplicate, and the yields between duplicates were found to be within 3%. The progress of reaction was monitored by taking aliquots (40 μL) from the reaction mixture at different time intervals and analysed by gas chromatography.

2.2.3. GC Analysis of alkyl esters

The fatty acid ethyl esters (biodiesel) formed were analyzed with methyl heptadecanoate as internal standard by GC on an Agilent 6890N system fitted with a capillary column EQUITY TM-5 (30 m X 0.32 mm X 0.25 μm film thickness) from Supelco (Bellefonte, USA) with flame ionization detection. The programme used was: initial oven temperature 100 °C, ramp at 15 °C/min up to 380 °C. The detector temperature was maintained at 300 °C. Peak areas of fatty acid esters and internal standard were obtained. Result for the fatty acid ester content was expressed as a mass fraction in percent using methyl heptadecanoate C17 as the internal standard by using the following formula:

$$c = \frac{\sum A - A'}{A'} \times \frac{C' \times V'}{m} \times 100\%$$

where: $\sum A$ = total peak area C14:0–C24:1; A' = internal standard peak area (methyl heptadecanoate) C' = concentration of internal standard solution in mg mL^{-1} V' = volume of internal standard solution used in mL; m = mass of the sample in mg

Reactions were carried out in duplicate, and the conversions between the sets were found to be within ±3%.

3. Results and discussion

Many sources of fats and oils have been used for obtaining biodiesel [9,11,27–31]. Lipases have been shown to work well with oils with high free fatty acid (FFA) content [9] and crude oils like oil extracted from spent coffee grounds [30]. Among the plant oils, soybean oil has been preferred in the U.S. [32]. The first pilot plant producing biodiesel has been operating in China using lipase and
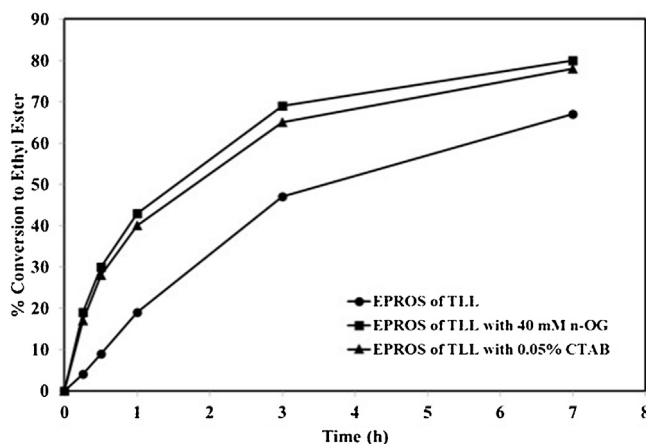
Fig. 1. Synthesis of biodiesel from soybean oil and ethanol using different preparations of *Thermomyces lanuginosus* lipase (28 U/ g of oil) in solvent free medium. 0.5 g oil was used for the reaction and 140 μL ethanol was added so that oil: ethanol ratio is 1:4. The reaction was carried out at 40 °C at 200 rpm. Aliquots were taken at different intervals and analysed by GC. The reaction was carried out in duplicates and the error within each set was within ±3%.
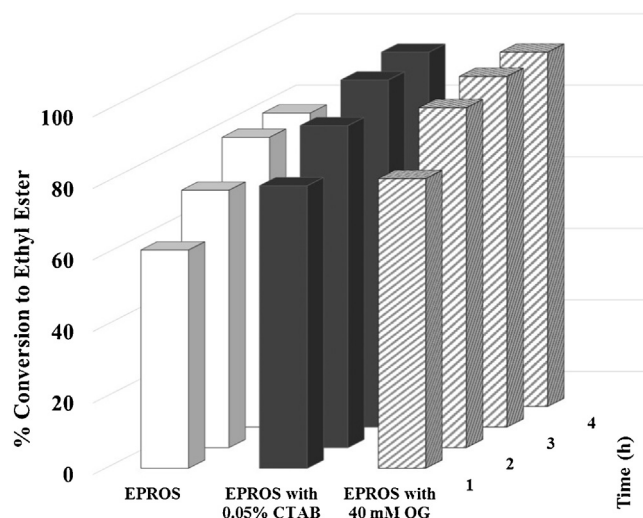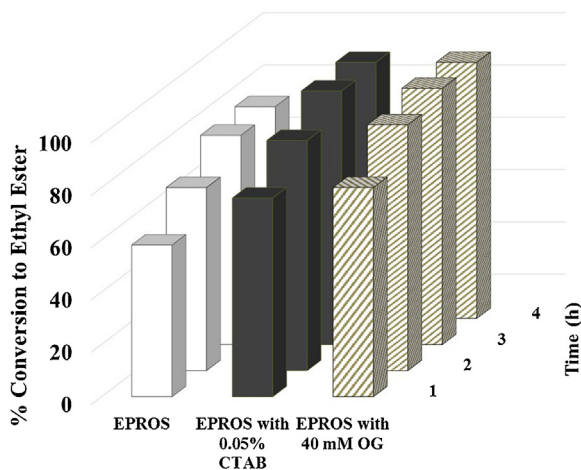
Fig. 2. Synthesis of biodiesel from soybean oil and ethanol using different preparations of *Thermomyces lanuginosus* lipase (28 U/g of oil) in the presence of silica and added water in solvent free medium. 0.5 g oil was used for the reaction and 140 μL ethanol was added so that oil: ethanol ratio is 1:4. 20% (w/w) silica and 2% (w/w) water was added. The reaction was carried out at 40 °C at 200 rpm. Aliquots were taken at different intervals and analysed by GC. The reaction was carried out in duplicates and the error within each set was within ±3%.

utilizes soybean oil as the renewable raw material [2]. The synthesis of biodiesel from soybean oil and ethanol using EPROS of TLL (28 U/g of oil) and EPROS prepared in the presence of Hexadecyl trimethyl ammonium bromide (CTAB) and N-octyl-β-D-glucopyranoside (OG) is shown in Fig. 1. A 67% conversion to ethyl esters was obtained in 7 h when EPROS was used as the biocatalyst. TLL is reported to be a 1,3 specific enzyme. Hence, transesterification of only 2 out of the 3 fatty acids on the glycerol backbone is expected. Hence, 67% conversion represents complete conversion in terms of theoretically expected conversion. It has however been reported that acyl migration from position 2–1 or 3 on the glycerol chain can take place [33]. This acyl migration can be spontaneous and is also facilitated under certain conditions. Hence, in many cases, transesterification reactions catalysed by 1,3 specific lipases have been reported to exceed their expected conversion [34]. Also, as pointed out by Schneider [35], one should view these specificities as relative specificities and not as absolute specificities. *Candida antarctica* lipase B, in fact, also has been sometime reported as a 1,3 specific lipase [36,37] but often has been observed to function like a non specific lipase [38,39]. When

the enzyme precipitates bioimprinted with CTAB (0.05%) or OG (40 mM) were used, 78% and 80% conversions to biodiesel were obtained respectively (Fig. 1).

Silica is known to facilitate acyl migration [40,41]. It is inexpensive and an abundantly available material. It is also considered as a green material [42]. Hence 20% (w/w of oil) silica was added to the reaction mixture. Also under anhydrous conditions the enzyme requires some minimum amount of water for its optimum activity. Thus, 2% (w/w of oil) water was also added to the reaction mixture. Under these optimized conditions, EPROS of TLL (28 U/g of oil) bioimprinted with OG or CTAB was able to give a 99% conversion to biodiesel within 4 h (Fig. 2) whereas non-imprinted EPROS yielded only 82% of the fatty acid methyl esters.

In the present work, to make the process more economical, even lower enzyme load was tried (Fig. 3 and 4). Even 14 U of TLL/g of oil bioimprinted with OG or CTAB could yield 98% biodiesel in 4 h (Fig. 3) and 7 U/g of oil yielded ~85% in the same time (Fig. 4). The corresponding EPROS could give 81% and 72% respectively. Bioimprinted TLL proved to be a better catalyst than the simple enzyme precipitate for biodiesel synthesis.

Fig. 3. Synthesis of biodiesel from soybean oil and ethanol using different preparations of *Thermomyces lanuginosus* lipase (14 U/g of oil) in the presence of silica and added water in solvent free medium. 0.5 g oil was used for the reaction and 140 μL ethanol was added so that oil: ethanol ratio is 1:4. 20% (w/w) silica and 2% (w/w) water was added. The reaction was carried out at 40 °C at 200 rpm. Aliquots were taken at different intervals and analysed by GC. The reaction was carried out in duplicates and the error within each set was within ±3%.
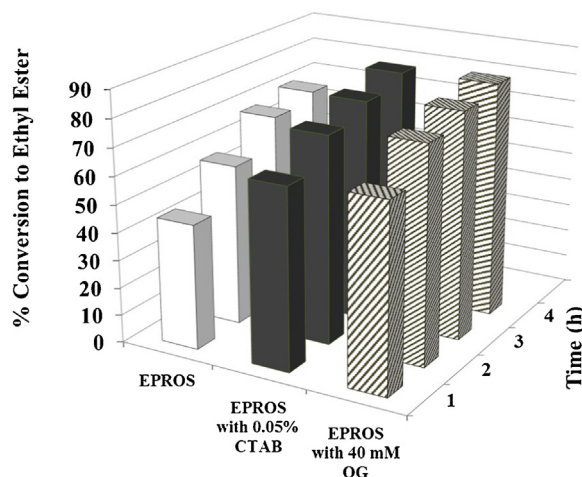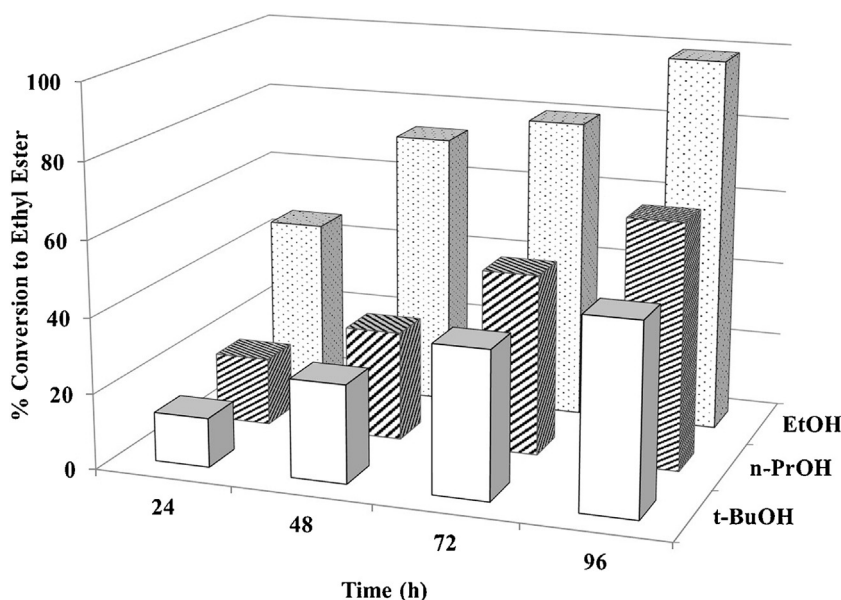
Fig. 4. Synthesis of biodiesel from soybean oil and ethanol using different preparations of *Thermomyces lanuginosus* lipase (7 U/g of oil) in the presence of silica and added water in solvent free medium. 0.5 g oil was used for the reaction and 140 μL ethanol was added so that oil: ethanol ratio is 1:4. 20% (w/w) silica and 2% (w/w) water was added. The reaction was carried out at 40 °C at 200 rpm. Aliquots were taken at different intervals and analysed by GC. The reaction was carried out in duplicates and the error within each set was within ±3%.

Recently, dual bioimprinting of subtilisin by the ester as well as the alcohol substrate used in the transesterification has been shown [20]. Biodiesel is synthesized by the transesterification of oil and an alcohol, in this case, ethanol. Thus ethanol as a substrate was decided to be tried as an *in situ* bioimprinting agent for TLL. In this case precipitation of TLL (1.4 U/g of oil) was carried out using three different short chain alcohols, propanol, *tert*-butanol and ethanol (in the absence of the surfactant OG/CTAB). The EPROS was then used for the transesterification reaction. It is to be noted that the enzyme load here was very low (1.4 U/g of oil) to see the effect of bioimprinting. At higher enzyme load, all preparations would give high conversions very fast and the effect of bioimprinting with alcohols would be masked. When EPROS was made using ethanol, which was also the alcohol substrate in this reaction, a 99% conversion in 96 h was obtained whereas the EPROS with propanol

and *tert*-butanol gave only 65% and 50% conversion respectively (Fig. 5).

Presumably the *in situ* bioimprinting of the alcohol substrate made the EPROS prepared with ethanol more efficient in the synthesis of biodiesel. Further, dual imprinting using a surfactant to open up the lid prior to precipitation was also tried. In all the three cases, bioimprinting with the surfactant made a marked difference in the conversions. However, the dually bioimprinted TLL made by precipitation in the substrate alcohol (in the presence of 40 mM OG) was the best catalyst giving 98% conversion to biodiesel in 48 h whereas the others with propanol and *tert*-butanol gave 68% and 59% respectively (Table 1). Thus a very economical process, using very low amount of enzyme (only 2% w/w of the oil), could be developed for the synthesis of biodiesel by using the dual bioimprinting procedure in case of lipases.

Fig. 5. Synthesis of biodiesel from soybean oil and ethanol in solvent free medium using different preparations of *Thermomyces lanuginosus* lipase (1.4 U/g of oil) in the presence of silica and added water. The enzyme was dissolved in Tris-HCl buffer, pH 7.5 and then precipitated in different organic solvents (chilled, 4 mL) at 4 °C and 200 rpm and shaken for 30 min. The precipitate obtained was washed twice with the chilled precipitant and then a final wash with chilled ethanol in each case (1 mL each time). 0.5 g oil was used for the reaction and 140 μL ethanol was added so that oil: ethanol ratio is 1:4. 20% (w/w) silica and 2% (w/w) water was added. The reaction was carried out at 40 °C at 200 rpm. Aliquots were taken at different intervals and analysed by GC. The reaction was carried out in duplicates and the error within each set was within ±4%.

Table 1
Effect of dual bioimprinting with the precipitant as well as surfactant. The enzyme (1.4 U/ g of oil) was dissolved in Tris-HCl buffer, pH 7.5 (with or without 40 mM OG) and then precipitated in different organic solvents (chilled, 4 mL) at 4 °C and 200 rpm and shaken for 30 min. The precipitate obtained was washed twice with the chilled precipitant and then a final wash with chilled ethanol in each case (1 mL each time). 0.5 mg oil was used for the reaction and 140 μL ethanol was added so that oil: ethanol ratio is 1:4. 20% (w/w) silica and 2% (w/w) water was added. The reaction was carried out at 40 °C at 200 rpm. Aliquots were taken at different intervals and analysed by GC. The reaction was carried out in duplicates and the error within each set was within ±4%.

	% Conversion in			
	24 h	48 h	72 h	96 h
TLL in buffer precipitated in EtOH	46	73	80	99
TLL in buffer containing 40 mM OG precipitated in EtOH	75	98	99	99
TLL in buffer precipitated in PrOH	18	29	48	65
TLL in buffer containing 40 mM OG precipitated in PrOH	39	68	88	93
TLL in buffer precipitated in *tert*-BuOH	13	26	39	50
TLL in buffer containing 40 mM OG precipitated in *tert*-BuOH	35	59	75	87

As shown in the earlier works [23–25,43] it has been shown that once the enzyme/protein is exposed to 5% (v/v) water, the induced conformation ceases to exist and the enzyme reverts to its original conformation. Hence, these bioimprinted proteins can be used in low water media only.

While in the current work, the recovery and re-solubilisation of the bioimprinted enzyme after the reaction was not attempted; in principle, its reuse should be possible by bioimprinting it all over again.

Recently Nordblad et al. [44] have carried out identification of critical parameters in the biodiesel production from rapeseed oil using another liquid formulation of TLL called Callera Trans L. Although that study has been done using a biphasic system with methanol, it provides a valuable approach for optimizing enzyme catalysed biodiesel production. In their studies, the enzyme load varied from 0.2–0.5% (w/w oil) and typical reaction times were 24 h.

We have already shown that non edible oils from Karanja (Latin name is *Pongamia milletia*, generally known as Pongamia) and spent coffee grounds can be converted to biodiesel by enzymatic route [30]. Also, esters for other applications such as biolubricants [45] and fragrances have been made using the enzymatic catalysis. Hence, the strategy used here should be useful in diverse applications where oils/fats can be converted to various value added products.

4. Conclusions

Oils/fats are renewable resources and hence ideally suited to obtain a biofuel. Hence, the biodiesel or biodiesel blends have evoked considerable interest in the last few decades [1,4,40]. In the present work, we have shown that an inexpensive lipase from *Thermomyces lanuginosus* could be converted into a biocatalyst preparation with highly enhanced catalytic efficiency by a simple inexpensive strategy of dual bioimprinting. We also use the solvent free approach, that is, no organic solvent was used and oil itself was used as the reaction medium. The strategy should apparently work equally well with any oil/fat as a feedstock.

Acknowledgements

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