



Ultrasound intensification suppresses the need of methanol excess during the biodiesel production with Lipozyme TL-IM



Preeti B. Subhedar^a, Claudia Botelho^b, Artur Ribeiro^b, Rita Castro^b, Maria Alcina Pereira^b, Parag R. Gogate^a, Artur Cavaco-Paulo^{b,*}

^aChemical Engineering Department, Institute of Chemical Technology, Matunga, Mumbai 400 019, India

^bCentre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, 4710 057 Braga, Portugal

ARTICLE INFO

Article history:

Received 2 January 2015

Received in revised form 27 February 2015

Accepted 3 April 2015

Available online 7 April 2015

Keywords:

Ultrasound

Intensification

Biodiesel

Immobilized lipase

Transesterification

ABSTRACT

The synthesis of biodiesel from sunflower oil and methanol based on transesterification using the immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL-IM) has been investigated under silent conditions and under an ultrasound field. Ultrasound assisted process led to reduced processing time and requirement of lower enzyme dosage. We found for the first time that oil to methanol ratio of 1:3 was favored for the ultrasound assisted enzymatic process which is lower than that observed for the case of conventional stirring based approach (ratio of 1.4). Our results indicate that intensification provided by ultrasound suppresses the need of the excess of the methanol reactant during the enzymatic biodiesel production. Ultrasound assisted enzymatic biodiesel production is therefore a faster and a cleaner processes.

© 2015 Elsevier B.V. All rights reserved.

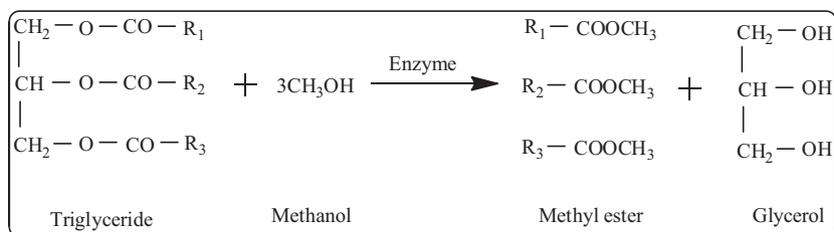
1. Introduction

Lipases are amongst the most important enzymes catalyzing the hydrolysis of oils and fats [1]. Lipases are also able to catalyze the esterification, transesterification, and interesterification reactions under ambient conditions [2]. Though lipase catalyzed approach has many advantages, the rate of reaction is very slow compared with the chemical approach especially for the case of biodiesel production [3]. Thus, it is essential to exploit the use of process intensification approaches to increase the reaction rate such that the enzyme catalyzed biodiesel production can be feasible at commercial scale. Also, apart from the enzyme related problems, process of production of biodiesel faces various problems related to the immiscible nature of the reactants causing poor mass transfer rate. Various starting materials such as soybean oil, rapeseed oil, palm oil, cotton seed oil, and waste cooking oil have been evaluated for the enzymatic biodiesel production [4]. Sunflower oil has been chosen for the present study for biodiesel production as not much work has been reported in the recent past and also based on the abundant availability of this type of oil especially in the European countries and India. The general reaction for the enzyme catalyzed methyl ester production from triglyceride has been given in Scheme 1.

* Corresponding author. Tel.: +351 253510271; fax: +351 253510293.

E-mail address: artur@deb.uminho.pt (A. Cavaco-Paulo).

Application of ultrasound can intensify the process due to the reduction in the mass transfer limitations prevalent in the case of heterogeneous enzymatic reactions [5–6]. Ultrasound irradiation can eliminate the problem of low immiscibility between reactants also possibly reducing the requirement of catalyst loading and reaction time. Recently, a review on lipase-catalyzed reactions intensified by the use of ultrasound has been published by Lerin et al. [7]. Also, there have been some reports related to the use of ultrasound of lipase catalyzed biodiesel production. Trentin et al. [8] reported the transesterification of soybean oil with ethanol using a commercial immobilized lipase, Novozym 435, under the influence of ultrasound irradiation, in a solvent-free system. Also, the production of fatty acid ethyl esters (FAEE) by means of Macauba (*Acrocomiaaculeata*) coconut oil (MCO) in solvent-free enzymatic transesterification reactions using a commercial immobilized lipase (Novozym 435) under the influence of ultrasound irradiation has been reported by Michelin et al. [9]. The analysis of the studies related to ultrasound induced intensification have revealed that the increased mass transfer effects due to the turbulence generated by ultrasound is at micro scale as compared to the conventional stirring and hence is the dominating factor in intensifying the extent of biodiesel production [10,11]. The earlier works of Yu et al. [10] and Kumar et al. [11] reported that excess of methanol/oil molar ratio is required which is around 6:1 for optimal biodiesel production yield. The work of Yu et al. [10] also mentions that minimal water content is required for the reaction



Scheme 1. General enzymatic transesterification reaction for production of methyl esters.

to take place (around 0.5%). In other independent study, the water sensitivity of immobilized lipases Lipozyme TL-IM with Novozym 435 has been compared in transesterifications [12]. In this study, it has been observed that Novozym 435 lipase is very sensitive to the amount of water and that Lipozyme TLIM is a good catalyst under the anhydrous conditions. This result suggests that, biodiesel production using Lipozyme TLIM enzyme might be carried out under no water content.

In the present study, transesterification has been carried out under the influence of ultrasound using commercial immobilized form of *Thermomyces lanuginosus* (Lipozyme TL-IM). Earlier literature dealing with biodiesel production mentions the use of lipases from *Candida antarctica*, *Enterobacter aerogenes*, *Rhizomucor miehei*, *Candida lipolytica* [11–14]. Water or other solvents have not been added in the system and the effect of the important reaction parameters such as enzyme loading, oil to alcohol molar ratio and the operating temperature has been investigated.

2. Materials and methods

2.1. Materials

Refined sunflower oil was obtained from the Pingo Doce, Braga, Portugal. The typical composition of oil consists of 25% saturated acid (stearic and palmitic) and 72.0% unsaturated acid (oleic and linoleic acid). Table 1 shows the properties of refined oil used in the work. Methanol (99%) used in the experiments and the chloroform (AR) solvent for the gas chromatography (GC) standards, i.e., methyl linoleate, methyl oleate, methyl palmitate and methyl stearate were obtained from Sigma Aldrich. Lipase enzyme Lipozyme TL IM immobilized on silica granules was kindly provided as a gift sample by Brenntag India Pvt. Ltd., Mumbai, India.

2.2. Experimental methodology

2.2.1. Enzymatic transesterification reaction

The transesterification reaction was performed in a round bottom glass flask of 250 ml capacity. The flask was placed in a constant temperature water bath and was also equipped with a condenser to reduce the loss of methanol due to evaporation. The agitation was provided using a magnetic stirrer. The mixture of sunflower oil and methanol (1:4 M ratio of oil to methanol) was

first fed into the reactor located in the water bath to attain the desired reaction temperature. The enzyme was then added and the reaction mixture was stirred. Samples were drawn from the reaction mixture at 4 h intervals and centrifuged at 6000 rpm to obtain the upper layer. The upper layer was diluted suitably in chloroform for the gas chromatography analysis. The various parameters that were studied include enzyme loading, oil to methanol molar ratio and the operating temperature. Initial experiments were performed with immobilized enzyme pre-soaked overnight in phosphate buffer of pH 7.0. Under the experimental conditions, buffer pre-soaking did not yield any significant benefit on the activity of the immobilized enzyme, therefore for the remaining experiments no buffer was used and no water was added in reaction mixture.

2.2.2. Ultrasound assisted enzymatic transesterification reaction

Ultrasonic USC600THbath (power rating of 120 W and frequency of irradiation as 45 kHz) was used for the ultrasound assisted enzymatic transesterification reaction. The actual power dissipated into the system was calculated using the calorimetric study and found to be 8.33 W. The ultrasonic transducer had a surface area of $9.8 \times 10^{-3} \text{ m}^2$ and thus, the acoustic intensity is calculated as 8503.4 W/m^2 . The acoustic pressure amplitude under these operating conditions was 1.6 bar.

The transesterification reaction was performed in a round bottom flask of 250 ml capacity. The round bottom flask was placed in an ultrasonic bath at the centre and was also equipped with a condenser to reduce the loss of methanol due to evaporation. The temperature of the reaction mixture was monitored and kept constant at $40 \text{ }^\circ\text{C}$ ($\pm 2 \text{ }^\circ\text{C}$) during the reaction. The experimental setup for ultrasound assisted enzymatic transesterification reaction is shown in Fig. 1. The procedure followed for the analysis of transesterification in the presence of ultrasound was similar to that mentioned in the case of conventional enzymatic reaction.

Table 1
Properties of refined sunflower oil.

Properties	Refined sunflower oil
Linoleic acid (%)	39
Oleic acid (%)	33
Palmitic acid (%)	21
Stearic acid (%)	4
Other acids (%)	3
Saponification value (mg KOH/g of oil)	190
Acid value (mg KOH/g oil)	0.54

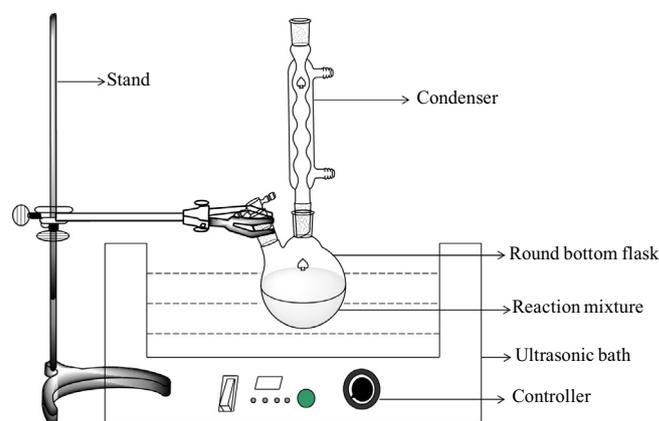


Fig. 1. Schematic representation of experimental setup.

Integral method of analysis was followed in the present work to calculate the rate constants and it has been observed that first order kinetics is able to explain the biodiesel production process well. All the experiments were repeated two times to check the reproducibility and average values have been reported in the figures. Error bars have also been shown to illustrate the obtained variation in the data.

2.3. Enzyme assay

The enzyme assay was performed with tributyrin as a substrate. Tributyrin (0.2 ml) was incubated with the enzyme (200 mg) in the presence of phosphate buffer (pH 7) for 5 min. At the end of the incubation, the reaction was terminated by the addition of 20 ml methanol and the contents were titrated against alcoholic NaOH (0.1 M) using phenolphthalein as an indicator. Immobilized enzyme activity can be expressed as μmoles of ester formed per minute and is defined by the following equation:

$$\text{Enzyme activity (TBU/g)} = \frac{V \times M \times 1000}{W \times T}$$

where V = volume in ml of NaOH which is a measure of tributyrin consumed during reaction, M = molarity of NaOH, E = amount of enzyme employed in mg and T = time of reaction in min. One unit of lipase activity was defined as the amount of enzyme required to hydrolyze 1 mol of ester bond per minute under assay conditions.

2.4. Analysis of methyl ester

Fatty acid methyl esters were quantified using gas chromatography (Varian 3800) equipped with a flame ionization detector (GC-FID). FAMES were separated on Eq. CP-Sil 52 CB 30 m \times 0.32 mm \times 0.25 μm capillary column (Teknokroma, TR-WAX). One microliter portion of the organic phase was analyzed using a split less mode. Helium was used as carrier gas at a flow rate of 1.0 ml/min. A temperature program was established for an efficient separation of the methyl esters. Initial oven temperature was set at 50 $^{\circ}\text{C}$ for 2 min with an increase of 10 $^{\circ}\text{C min}^{-1}$ to a final temperature of 225 $^{\circ}\text{C}$. Injector and detector temperatures were set at 220 and 250 $^{\circ}\text{C}$, respectively. The fatty acids were identified by comparison of the respective retention factor values (R_f) of standard fatty acid methyl esters.

3. Results and discussion

3.1. Comparison of conventional and ultrasound-assisted biodiesel production

The results obtained under conventional stirring method and ultrasound-assisted reaction were compared. Experiments were carried out under same enzyme loading (5% w/v) and temperature of 40 $^{\circ}\text{C}$ for conventional transesterification and ultrasound assisted approach. The obtained results are shown in Figs. 2a and 2b. Under ultrasonic irradiation, the reaction yield was over 95% in 4 h while under silent conditions (without ultrasound), the yield could only reach 60% after 24 h. Under silent conditions the reactions times were too long which might lead to enzyme deactivation (Fig. 2b; the expected yield based on model fitted using initial data is much higher as compared to the experimentally obtained value). The more biodiesel yield obtained in less reaction time in ultrasound-assisted reaction is attributed to enhanced mass transfer effects occurred due to the application of ultrasound. The results obtained are in accordance with Yu et al. [10].

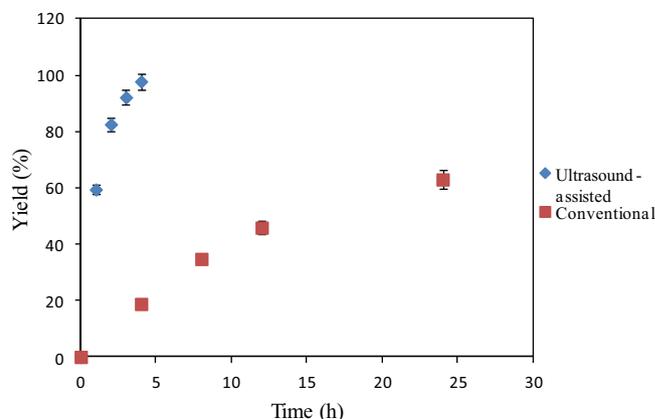


Fig. 2a. Comparison of conventional and ultrasound-assisted enzymatic transesterification.

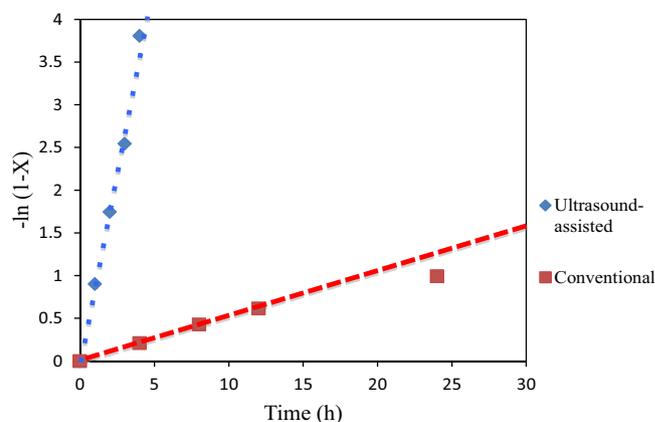


Fig. 2b. Comparison of conventional and ultrasound-assisted enzymatic transesterification in terms of reaction estimation.

3.2. Effect of molar ratio of oil to methanol

As shown in Scheme 1, the stoichiometry of the transesterification reaction for biodiesel production is 1:3. This reaction is expected to be enhanced under low water and with excess methanol. Thus experiments were performed using 1:3, 1:4 and 1:5 oil to alcohol molar ratios to investigate the effect of molar ratio in methanolysis of sunflower oil (40 $^{\circ}\text{C}$). The results are shown in Table 2. The half times of conversation data show clearly that ratio 1:4 is favored under silent condition and stoichiometric ratio (1:3) is favored for the process under ultrasound. The ultrasound assisted process seems to favor stoichiometric conditions and lower levels of methanol in the process. Our data indicate that enzyme tolerates and operates well in the presence of methanol

Table 2
Effect of molar ratio on biodiesel production.

Molar ratio	Rate constant k (hr^{-1})	R^2	Half life ($t_{1/2}$)
<i>Conventional approach</i>			
1:3	0.0486	0.9756	14.25
1:4	0.1344	1	5.15
1:5	0.0791	0.9707	8.76
<i>Ultrasound-assisted approach</i>			
1:3	0.7099	0.9998	0.97
1:4	0.4129	0.9813	1.67
1:5	0.2539	0.9421	2.72

as expected from the state of art. The fact that the enzyme is immobilized increases their tolerance to methanol. Lipases have been designed by nature to tolerate extreme hydrophilic and extreme hydrophobic environments commonly found in their substrates and reaction products. Therefore we cannot assume that an excess of methanol would greatly affect the enzyme folding and their performance despite the results reported by other researchers [15–17]. Under silent conditions, the optimum oil to alcohol molar ratio of 1:4 is in accordance with the results reported by Shimada et al. [18].

Ultrasound enhances mass transport effects and facilitates the mixing of reactants near the enzyme. Since the enzyme is immobilized and the fact that reactants are in two distinct phases, enhancement in the mass transport is crucial in such process. Ultrasound promotes this mixing so efficiently [19,20] that the process only requires the exact stoichiometric amount of oil and methanol for optimal enzymatic turn over. Ultrasonic irradiation in liquid system produces the phenomenon of cavitation. Generation of micro scale turbulence by the cavitation bubbles generated in the vicinity of the interface between oil and methanol results in production of fine emulsion of the two liquids. However, the dispersion of methanol in oil depends on the intensity of micro scale turbulence produced by the cavitation bubbles in methanol and vice versa. The intensity of micro scale turbulence generated by cavitation bubbles depends on physical properties of the liquid medium such as viscosity, density, and surface tension and also on the amplitude of the acoustic wave driving the bubble motion. Due to differences in the physical properties of oil and methanol, the intensity of micro scale turbulence in the two media is different. Both micro scale streaming (due to ultrasound) as well as micro scale convection (due to cavitation bubbles) in methanol is higher than that of oil due to high viscosity of oil. The results have firmly established that the physical mechanism responsible for the beneficial action of ultrasound is the formation of fine emulsion between oil and methanol that enhances the interfacial area for the reaction [21,22]. We are the first ones to report this for ultrasound enzymatic transesterification system, whereas some report can be observed for the lipase induced hydrolysis of oils in low water systems [14]. The requirement of excess quantum of reactants can be a significant problem in the downstream processing. Yu et al. [10] reported optimum molar ratio of oil to methanol as 1:6 giving 96% biodiesel yield in the presence of ultrasound whereas Kumar et al. [23] reported the requirement of 1:4 M ratio for 84.5% biodiesel yield. The present work using new species of lipase in the presence of ultrasound resulted in 96% biodiesel yield using 1:3 M ratio which is lower as compared to the other molar ratios. The discussion allows us to establish that the use of ultrasound to enhance mass transport and lipase from *T. lanuginosus* resulted in lowering the excess requirement of methanol which can significantly reduce the energy requirements in the downstream separations.

3.3. Effect of enzyme loading

The quantity of enzyme required for the reaction is a very important factor for successful industrial application because of the high cost of enzymes. Thus, in order to optimize the enzyme loading, it was varied from 3% to 9% (w/v). When the enzyme loading increased from 3% to 7%, biodiesel yield increased from 19.25% to 93.3%. But further increase in enzyme loading did not result in any significant increase in biodiesel yield. Since lipase-catalyzed reactions occur at the interface, the quantity of enzyme present at the interface plays a significant role during the reaction. In the present system, it is seems that after 7% enzyme loading the interface is saturated with enzyme, i.e., any further increase in the quantum of enzyme does not show a change in the rate of the

transesterification [24]. Under these conditions, the external mass transfer resistance becomes the limiting step for the transesterification of oil. The observed trend is in accordance with the results reported by other researchers [25,26].

One of the major objectives of the present work was to develop a process that could require the least quantity of enzyme and lower processing time to produce the possible maximum amount of biodiesel. For ultrasound assisted approach, the enzyme loading was varied over the range of 0.5–5%. It was observed that on increasing the enzyme loading from 0.5% to 3.0% (w/v), the yield of methyl esters increased proportionately. But further increase in the enzyme loading to 5.0% (w/v) did not result in any significant increase in the biodiesel yield. Hence, 3% (w/v) enzyme loading was considered to be the optimum for the ultrasound assisted approach with 96% biodiesel yield. It is also important to note that the obtained yield under optimum conditions in the case of ultrasound assisted approach is higher as compared to that obtained under the optimum conditions for the conventional approach. It is also important to note here that the enzyme saturation at the interface is obtained at lower enzyme loading in the presence of ultrasound which can be attributed to better mixing effects in the presence of ultrasound.

The kinetic representation of the obtained data in the Fig. 3 also confirms the significant degree of process intensification obtained due to the use of ultrasound. Under silent conditions (no ultrasound) the kinetics seems to be the same for all the enzyme dosages. Under ultrasound, the mass transport effects seem to be less effective for higher enzyme dosage especially higher than 3%, where a limit of conversion can be obtained and a different mechanism could be present. Sonication significantly influences the heterogeneous enzyme catalyzed reactions by creating uniform mixing patterns or dispersions [27]. The application of ultrasound results in a more homogeneous reaction mixture and facilitates dispersion of lipase enzyme in substrates [14]. Hence, in the case of ultrasound-assisted transesterification, to produce an equivalent amount of methyl esters, lower enzyme loading was required as compared to that required in the absence of ultrasound. Yu et al. [10] reported an optimum lipase enzyme loading as 6% to achieve 96% yield of methyl esters for ultrasound assisted enzymatic biodiesel production. The required enzyme loading in the present work is 2 times lower as compared to the work of Yu et al. [10] which clearly establishes the significant process intensification aspects.

Additional experiments were also performed to check the reusability of the catalyst in both conventional and ultrasound assisted approach. It was observed that the enzyme activity

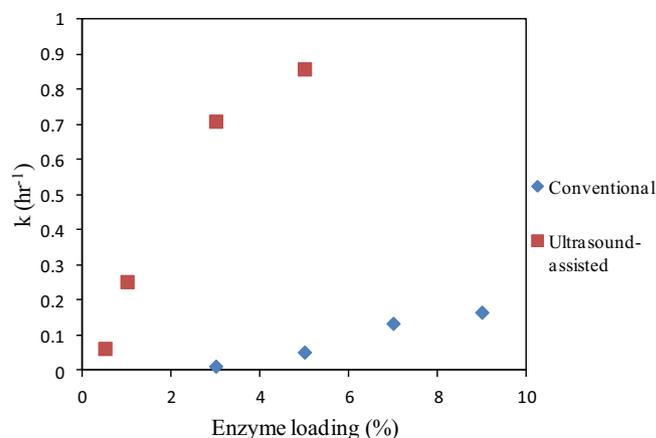


Fig. 3. Effect of enzyme loading on rate constants for silent and ultrasound assisted approach.

reduced by ~8% for both the conventional as well as ultrasound assisted approach. This indicates that there was no significant reduction in the enzyme activity due to the application of ultrasound during enzyme catalyzed transesterification reaction.

3.4. Effect of temperature and pH

Reaction temperature is an important parameter in deciding the progress of enzyme catalyzed reactions. The effect of reaction temperature on conventional enzymatic transesterification was investigated over the temperature range of 30–50 °C. It was observed that the biodiesel yield increased as temperature increased from 30 to 40 °C, followed by a decrease at higher temperature. As the reaction temperature increased, the collision frequency between enzyme and substrate molecules increased which might help to form enzyme–substrate complexes and consequently led to the increase in the biodiesel yield. The maximal biodiesel yield was achieved at reaction temperature of 40 °C. The observed decrease in the biodiesel at higher temperature is most likely because of the denaturation (alteration) of protein structure originated from the heat-induced destruction of noncovalent interactions i.e. the breakage of the weak ionic and hydrogen bonding that stabilizes the three dimensional structure of the enzyme [28]. The optimum temperature of 40 °C as obtained in the present study is in agreement with the results reported by Sim et al. [29] for *T. lanuginosus* lipase. Also, the changes in the cavitation intensity at different reaction temperatures is another contributing factor deciding the magnitude of convection produced in the system. There would be a reduction in the cavitation intensity due to the cushioned collapse of cavities as the temperature of reaction increases from 40 to 50 °C. The cavitation bubble formed due to the application of ultrasound gets supersaturated with methanol molecules due to the increasing extent of entrapment of methanol molecules in it. Consequently, the net heat capacity of the bubble increases, leading to reduction in the peak temperature at collapse. The methanol vapor causes cushioning of collapse and hence the pressure reached inside the bubble at collapse also reduces significantly giving lesser yield at 50 °C [30].

pH of the reaction mixture is the another important variable affecting the yield of biodiesel. The present system is non aqueous and hence the pH of the reaction mixture was not varied, but effect of soaking lipase in phosphate buffer of pH 7.0 was studied as pH 7.0 is the optimum pH for *T. lanuginosus* lipase. It has been observed that there was no significant effect in the yield of biodiesel after soaking enzyme overnight in buffer solution. The observed result is in well agreement with the reported results by Devanesan et al. [31] who got maximum yield of biodiesel at pH 7.0 using immobilized *Pseudomonas fluorescens*.

We found no significant increase in the conversion ratio when Lipozyme TL-IM was pre-soaked with an aqueous buffer when compared with the process with no water added. This is contrast of the reported results for the use of [10] Novozym 435. This difference can be attributed to the different water sensitivity of both immobilized enzymes [12].

4. Conclusions

The performance of Lipozyme TL-IM for transesterification reaction was greatly improved by the ultrasound assisted approach as compared to the conventional enzymatic transesterification. The ultrasonic assisted reaction resulted in higher yield of biodiesel in significantly less time compared to the conventional method. Time of reaction reduced from 24 to 4 h due to the use of ultrasonic irradiation. Under the optimum conditions (oil to methanol molar ratio of 1:3, 3% Lipozyme TL IM, 40 °C, 120 W power at 40 kHz

frequency), 96% yield of biodiesel could be achieved. Overall, the significant decrease in the reaction time and enzyme loading with enhanced effectiveness are the most attractive features of ultrasound-assisted enzymatic transesterification reaction.

Acknowledgment

The authors acknowledge Brenntag India Pvt. Ltd. for kindly providing gift sample of lipase enzyme Lipozyme TL IM to carry out the research work. All authors acknowledge the funding of Department of Science and Technology and Portuguese Science Foundation under the Indo-Portuguese collaborative program.

References

- [1] S. Nagarajan, New tools for exploring old friends-microbial lipases, *Appl. Biochem. Biotechnol.* 168 (2012) 1163–1196.
- [2] A. Rajendran, A. Palanisamy, V. Thangavelu, Lipase catalyzed ester synthesis for food processing industries, *Braz. Arch. Biol. Technol.* 52 (2009) 207–219.
- [3] C.C. Akoh, S.W. Chang, G.C. Lee, J.F. Shaw, Enzymatic approach to biodiesel production, *J. Agric. Food Chem.* 55 (2007) 8995–9005.
- [4] A. Kumari, P. Mahapatra, V.K. Garlapati, R. Banerjee, Enzymatic transesterification of Jatropha oil, *Biotechnol. Biofuels* 2 (2009) 1–7.
- [5] E.N. Vulfsion, D.B. Sarney, B.A. Law, Enhancement of subtilisin catalyzed interesterification in organic solvents by ultrasound irradiation, *Enzyme Microb. Technol.* 13 (1991) 123–126.
- [6] V.G. Yachmenev, E.J. Blanchard, A.H. Lambert, Use of ultrasonic energy for intensification of the bio-preparation of greige cotton, *Ultrasonics* 42 (2004) 87–91.
- [7] L.A. Lerin, R.A. Loss, D. Remonato, M.C. Zenevicz, M. Balen, V.O. Netto, J.L. Ninow, C.M. Trentin, J.V. Oliveira, D. Oliveira, A review on lipase-catalyzed reactions in ultrasound-assisted systems, *Bioprocess Biosyst. Eng.* 37 (2014) 2381–2394.
- [8] C.M. Trentin, A.S. Popiolki, L. Batistella, C.D. Rosa, H. Treichel, D. de Oliveira, J.V. Oliveira, Enzyme-catalyzed production of biodiesel by ultrasound-assisted ethanolsolysis of soybean oil in solvent-free system, *Bioprocess Biosyst. Eng.* (2015), <http://dx.doi.org/10.1007/s00449-014-1316-0>.
- [9] S. Michelin, F.M. Penha, M.M. Sychoski, R.P. Scherer, H. Treichel, A. Valerio, M.D. Luccio, D. de Oliveira, J.V. Oliveira, Kinetics of ultrasound-assisted enzymatic biodiesel production from Macauba coconut oil, *Renewable Energy* 76 (2015) 388–393.
- [10] D. Yu, L. Tian, H. Wu, S. Wang, Y. Wang, D. Ma, X. Fang, Ultrasonic irradiation with vibration for biodiesel production from soybean oil by Novozym 435, *Process Biochem.* 45 (2010) 519–525.
- [11] G. Kumar, D. Kumar, R. Poonam, C.P. Johari, Singh, Enzymatic transesterification of Jatropha curcas oil assisted by ultrasonication, *Ultrason. Sonochem.* 18 (2011) 923–927.
- [12] D. Zhang, Y. Li, C. Li, Y. Lv, Y. Li, Kinetics of enzymatic synthesis of L-ascorbyl acetate by Lipozyme TLIM and Novozym 435, *Biotechnol. Bioprocess Eng.* 17 (2012) 60–66.
- [13] L. Batistella, L. Lerin, P. Brugnerotto, A. Danielli, C. Trentin, A. Popiolki, H. Treichel, J. Oliveira, D. Oliveira, Ultrasound-assisted lipase-catalyzed transesterification of soybean oil in organic solvent system, *Ultrason. Sonochem.* 19 (2012) 452–458.
- [14] Y. Liu, Q. Jin, L. Shan, Y. Liu, W. Shen, X. Wang, The effect of ultrasound on lipase-catalyzed hydrolysis of soy oil in solvent-free system, *Ultrason. Sonochem.* 15 (2008) 402–407.
- [15] J. Lu, L. Deng, R. Zhao, R. Zhang, F. Wang, T. Tan, Pretreatment of immobilized *Candida* sp. 99–125 lipase to improve its methanoltolerance for biodiesel production, *J. Mol. Catal. B Enzym.* 62 (2010) 5–18.
- [16] M.P. Bousquet-Dubouch, M. Graber, N. Sousa, S. Lamare, M.D. Legoy, Alcoholysis catalyzed by *Candida antarctica* lipase B in a gas/solid system obeys a Ping Pong Bi Bi mechanism with competitive inhibition by the alcohol substrate and water, *BBA-Protein Struct. M.* 1550 (2001) 90–99.
- [17] L. Li, W. Du, D. Liu, L. Wang, Z. Li, Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium, *Mol. Catal. B: Enzym.* 43 (2006) 58–62.
- [18] Y. Shimada, Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, H. Fukuda, Y. Tominaga, Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase, *J. Am. Oil Chem. Soc.* 76 (1999) 789–793.
- [19] J. Ji, J. Wang, Y. Li, Y. Yu, Z. Xu, Preparation of biodiesel with the help of ultrasonic and hydrodynamic cavitation, *Ultrasonics* 44 (2006) 411–414.
- [20] D. Kumar, G. Kumar, R. Johari, P. Kumar, Fast, easy ethanolsolysis of Jatropha curcas oil for biodiesel production due to the better solubility of oil with ethanol in reaction mixture assisted by ultrasonication, *Ultrason. Sonochem.* 19 (2012) 816–822.
- [21] A. Kalva, T. Sivasankar, V.S. Moholkar, Physical mechanism of ultrasound-assisted synthesis of biodiesel, *Ind. Eng. Chem. Res.* 48 (2009) 534–544.
- [22] H.A. Choudhury, R.S. Malani, V.S. Moholkar, Acid catalyzed biodiesel synthesis from Jatropha oil: mechanistic aspects of ultrasonic intensification, *Chem. Eng. J.* 231 (2013) 262–272.

- [23] G. Kumar, D. Kumar, R. Poonam, C.P. Johari, Enzymatic transesterification of *Jatropha curcas* oil assisted by ultrasonication, *Ultrason. Sonochem.* 18 (2011) 923–927.
- [24] T.W. Charpe, V.K. Rathod, Biodiesel production using waste frying oil, *Waste Manage.* 31 (2011) 85–90.
- [25] E. Hernandez-Martin, E.C. Otero, Different enzyme requirements for the synthesis of biodiesel: Novozym 435 and Lipzyme TL IM, *Bioresour. Technol.* 99 (2008) 277–286.
- [26] S. Tamalampudi, M.R. Talukder, S. Hamad, T. Numatab, A. Kondo, H. Fukuda, Enzymatic production of biodiesel from *Jatropha* oil: a comparative study of immobilized-whole cell and commercial lipases as a biocatalyst, *Biochem. Eng. J.* 39 (2008) 185–189.
- [27] K.S. Suslick, G.J. Price, Applications of ultrasound to materials chemistry, *Annu. Rev. Mater. Sci.* 29 (1999) 295–326.
- [28] G.D. Yadav, P.S. Lathi, Intensification of enzymatic synthesis of propylene glycol monolaurate from 1,2-propanediol and lauric acid under microwave irradiation: kinetics of forward and reverse reactions, *Enzyme Microb. Technol.* 38 (2006) 814–820.
- [29] J.H. Sim, G.K. Khor, A.H. Kamaruddin, S. Bhatia, Thermodynamic studies on activity and stability of immobilized *Thermomyces lanuginosus* in producing fatty acid methyl ester (FAME), *Int. J. Sci. Res. Publ.* 3 (2013) 1–4.
- [30] H.A. Choudhury, S. Chakma, V.S. Moholkar, Mechanistic insight into sonochemical biodiesel synthesis using heterogeneous base catalyst, *Ultrason. Sonochem.* 21 (2014) 169–181.
- [31] M.G. Devanesan, T. Viruthagiri, N. Sugumar, Transesterification of *Jatropha* oil using immobilized *Pseudomonas fluorescens*, *Afr. J. Biotechnol.* 21 (2007) 2497–2501.