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Castor Oil Transesterification Catalysed by Liquid Enzymes: Feasibility of Reuse under Various Reaction Conditions

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In the present work, biodiesel production by reaction of non-edible castor oil with methanol under enzymatic catalysis is investigated. Two liquid enzymes were tested: Eversa Transform and Resinase HT. Reactions were performed at 35 °C and with a molar ratio of methanol to oil of 6:1. The reaction time was 8 hours. Stepwise addition of methanol was necessary to avoid enzyme inhibition by methanol. In order to minimize the enzyme costs, the influence of enzyme activity loss during reuse of both enzymes was evaluated under two distinct conditions. In the former, the enzymes were recovered and fully reused; in the latter, a mixture of 50 % reused and 50 % fresh enzymes was tested. In the case of total reuse after three cycles, both enzymes achieved only low conversions. The biodiesel content in the oil-phase using Eversa Transform was 94.21 % for the first cycle, 68.39 % in the second, and 33.35 % in the third cycle. Resinase HT gave 88.05, 86.60 and 58.24 %, respectively. For the partial enzyme reuse, after three cycles, the biodiesel content was 88.36 and 88.05 % respectively for the Eversa Transform and Resinase HT, showing that partial reuse of enzymes could be beneficial for enzymatic transesterification reactions.

1. Introduction

Biodiesel is considered a promising bio-derived fuel able to reduce the dependence on limited and non-renewable energy resources. It has the potential of reducing the level of pollutants and the level of potential carcinogenic compounds, in contrast to mineral diesel. Additionally, it is biodegradable, non-toxic, derived from a renewable feedstock, and in the case of castor oil, is grown in arid regions. Furthermore, biodiesel is an excellent lubricant, which extends the useful life of diesel engines (Gomes et al., 2010).
Biodiesel is a mono-alkyl ester of long chain typically produced by transesterification of triglycerides derived from vegetable oils or animal fats, with low molecular weight alcohols such as methanol or ethanol (Al-Zuhair, 2007). Used or virgin vegetable oils are the most attractive raw materials because of their environmental benefits and renewability (Balat and Balat, 2010). This reaction produces glycerol as a byproduct, as well as di- and monoglycerides as intermediate products. Figure 1 shows the stepwise reactions for biodiesel production, in which three molecules of alcohol are required per molecule of triglyceride.

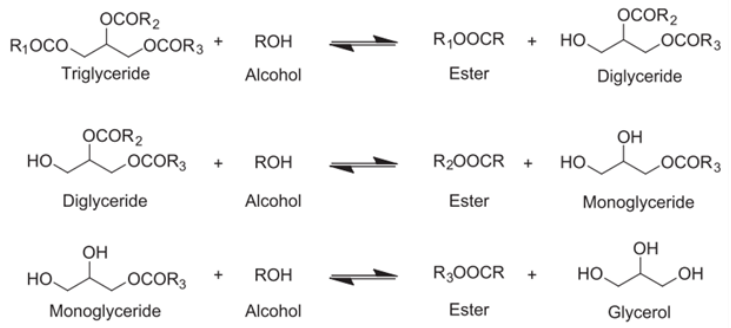

Figure 1: Stepwise reactions for biodiesel production (R is an alkyl; R_1, R_2 and R_3 are fatty acid chains).

Transesterification reactions are conventionally acid or alkali catalyzed, leading to high conversion rates achieved in a short reaction time at low process costs. However, several drawbacks are associated with the chemical production of biodiesel. Saponification reactions and hydrolysis affect biodiesel purity, due to its sensibility to both water and the free fatty acids contents in the feedstock. Recovery and purification of catalysts and glycerol are expensive. In addition, neutralization and waste water treatment are required. Therefore, those processes are energy intensive (Christopher et al., 2014).

Enzymatic catalysis overcomes these problems offering a biological route for biodiesel production with several advantages. Enzymes can operate under mild reaction conditions and have a high selectivity towards substrates (Poppe et al. 2015). They are able to effectively catalyze the transesterification of triglycerides in either aqueous or non-aqueous systems, catalyzing both triglycerides and free fatty acids in one step.

Enzymes can be found at liquid or immobilized conditions. Immobilized enzymes have been used in most of the recent research on biocatalytic transesterification reactions (Kovács and Hancsók, 2009; Mata et al., 2012; Adewale et al., 2015). Some researchers include the reuse of immobilized enzymes. In this case, Kalantari et al. (2013) obtained 55 % conversion after five times of reuse of Pseudomonas cepacia. Aguieiras et al. (2016) investigated the reuse of three commercial immobilized lipases (Novozym 435, Lipozyme RM IM, and Lipozyme TL IM) without solvent washing as well as the use of three solvents (ethanol, butanol, and hexane), proving that washing the enzymes before reuse can increase their efficiency since the solvents (ethanol and butanol) remove the glycerol produced.

Liquid enzymes are within an aqueous solution where stabilizers are usually added to prevent enzyme denaturation. The use of liquid enzymes avoids the addition of an extra solid phase to the reaction system that can result in slower reactions. However, some disadvantages, including the activity loss and mainly their high cost compared to inorganic acids and bases, make the industrial implementation limited.

Enzyme stability, recoverability and reusability are some of the advantages of immobilized enzymes as opposed to liquid enzymes (Goswami et al., 2012). However, even though liquid enzymes are considerably more expensive than chemical catalysts, they are still cheaper than the immobilized ones. Consequently, the enzyme reuse appears to be a required step to improve the economy of the process. Nevertheless, that reuse is limited (Nielsen et al., 2008).

Liquid enzymes and their reuse to produce biodiesel have been studied to a limited extent. Therefore, this work aims to investigate the reuse of two different liquid enzymes (Eversa Transform and Resinase HT) in the transesterification reaction of castor oil with methanol to produce fatty acid methyl esters (biodiesel).

2. Materials and Methods

2.1 Materials

Castor oil was purchased from Urtegaarden ApS (Denmark). Liquid enzymes Eversa® Transform and Resinase® HT were kindly donated by Novozymes A/S (Denmark). The enzyme mixtures contained 75 % water, according to information from the manufacturer. Methanol, acetonitrile, hexane and isopropanol of HPLC-grade were purchased from Sigma-Aldrich. The following HPLC calibration standards were also purchased from Sigma-Aldrich: methyl esters (methyl ricinoleate, linolenate, linoleate and oleate), fatty acids (ricinoleic, linoleic and oleic acid) in stated grades of 99% of purity. Standards of tri-, di- and monoglycerides were prepared by transesterification and separation on a preparative HPLC.

2.2 Experimental procedure

The reactions were carried out in a 100 mL round-bottom flask equipped with a stirrer and immersed in a thermostat oil bath at 35°C. The reaction mixture was castor oil-to-methanol in a 6:1 alcohol-to-oil molar ratio and the liquid enzyme (10 wt% of the castor oil sample). The reactions were performed for 8 hours at 750 rpm. To avoid inhibition of the liquid enzymes, methanol was added into the reactor in four stepwise additions at two hour intervals. After the reaction, enzymes were recovered by means of centrifugation at 4000 rpm for 15 min. For each enzyme, two different procedures were used. In the first procedure, recovered enzymes were fully reused in a new batch reaction. In the second one, a mixture of 50 % of recovered and 50 % of fresh enzymes was used for each new batch reaction. The experiments were carried out in duplicate.

2.3 Sample preparation and High-Performance Liquid Chromatography analysis

Samples of 0.2 mL were collected from the reaction mixture every two hours and centrifuged for 5 min to separate oil, glycerol and enzymatic phases. Samples from the oil phase were diluted in a 4:5 w/w hexane to isopropanol solution, mixed thoroughly in vortex mixer and filtered into small vials. These samples were injected into the HPLC. To quantify the production of biodiesel, an Agilent 1200 Series HPLC system with UV detection at 205 nm equipped with a Phenomenex Luna C18, 3 µm, 150 x 4.60 mm column was used. A volume of 10 µL was injected for analysis. A three-gradient system (acetonitrile, water and 4:5 w/w hexane to

isopropanol solution) was used as solvents in a flow rate of 0.8 mL/min. The runtime for each analysis was 35 minutes. The composition of fatty acid methyl esters (FAME), triglycerides (TAG), diglycerides (DAG), monoglycerides (MAG) and free fatty acids (FFA) in the oil phase was done based on the generated calibration curves.

3. Results and Discussion

Castor oil is mainly composed by ricinoleic acid. Because of the hydrogen bonding of its hydroxyl group, castor oil has a good solubility with alcohols, reducing the mass transfer limitations during the transesterification, without the need of solvent addition.

The results obtained for the two enzymes were compared in terms of FAME and FFA content, both expressed as percentage of the total oil-phase. FAME was produced by transesterification of castor oil with methanol. FFA was produced due to the presence of water in the liquid enzymes leading to the hydrolysis of castor oil. Figure 2 summarizes the FAME content for each condition. Similarly, the content of FFA is shown in Figure 3.

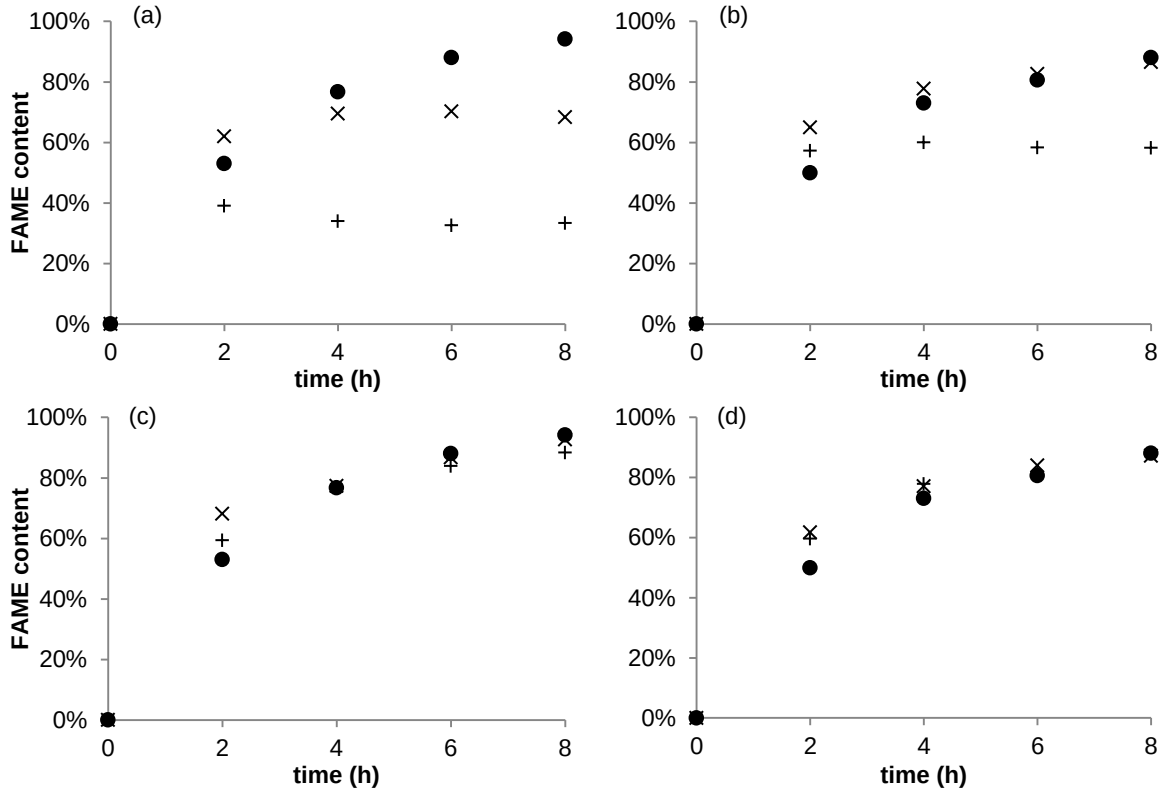

Figure 2: FAME content in oil-phase for the first batch run (●), for the second batch run with enzyme reused (×) and for the third batch run with enzyme (+). (a) Total reuse of Eversa Transform. (b)Total reuse of Resinase HT. (c) Partial reuse of Eversa Transform. (d) Partial reuse of Resinase HT.

3.1 Total enzyme reuse

When the first batch is considered, Eversa Transform (ET) was identified as the most effective enzyme compared to Resinase HT (RHT). Indeed, comparing Figure 2(a) and 2(b), at the completion of the reaction time, 94.21 % and 88.05 % of the oil-phase was composed by FAME when ET and RHT was used as catalyst respectively. The FFA content is shown in Figure 3(a) and 3(b). It is noticed that when RT is used as a catalyst, the FFA composition is 4.49 %. This value reaches 7.57 % for the RHT enzyme.

This means that under the defined operating conditions and at temperature of 35 °C, the highest FAME yield is achieved using ET, while the hydrolysis of triglycerides is more significant using RHT. The predominance of hydrolysis with RHT compared to ET can be observed at two hours of reaction, when the oil-phase is composed by 18.02 % of FFA in the reaction with ET (Figure 3(a)) and 23.36 % in the reaction with RHT (Figure 3(b)).

The loss of the enzyme activity is seen in Figure 2(a) for the ET. With this enzyme, the second batch provided 68.39 % of biodiesel, decreasing around 27 % with respect to the first batch. Conversely, examining Figure 3(b), the full reuse of RHT showed a lower loss in enzyme activity for biodiesel production as compared to ET. The FAME content after the second and the third batches reached respectively 86.60 % (2 % decrease) and 58.24 % (34 % decrease). The different behaviour of the two enzymes can be explained by examining the oil phase composition reported in Table 1. For the ET, the reduction of the FAME production in the second batch is due to the incomplete conversion of triglycerides, resulting in 20.56 % of MAG. In the third batch, the drop in the conversion was even higher, reaching 65 % in relation to the first batch, resulting in only 33.35 % of FAME in the oil-phase composition. Due to the loss of activity of the enzymes, 20.91 % of the oil-phase was composed by unreacted triglycerides and 34.32 % of MAG, after the third batch. For the RHT, after the third batch the TAG content was lower (9.00 %) when compared to the RT.

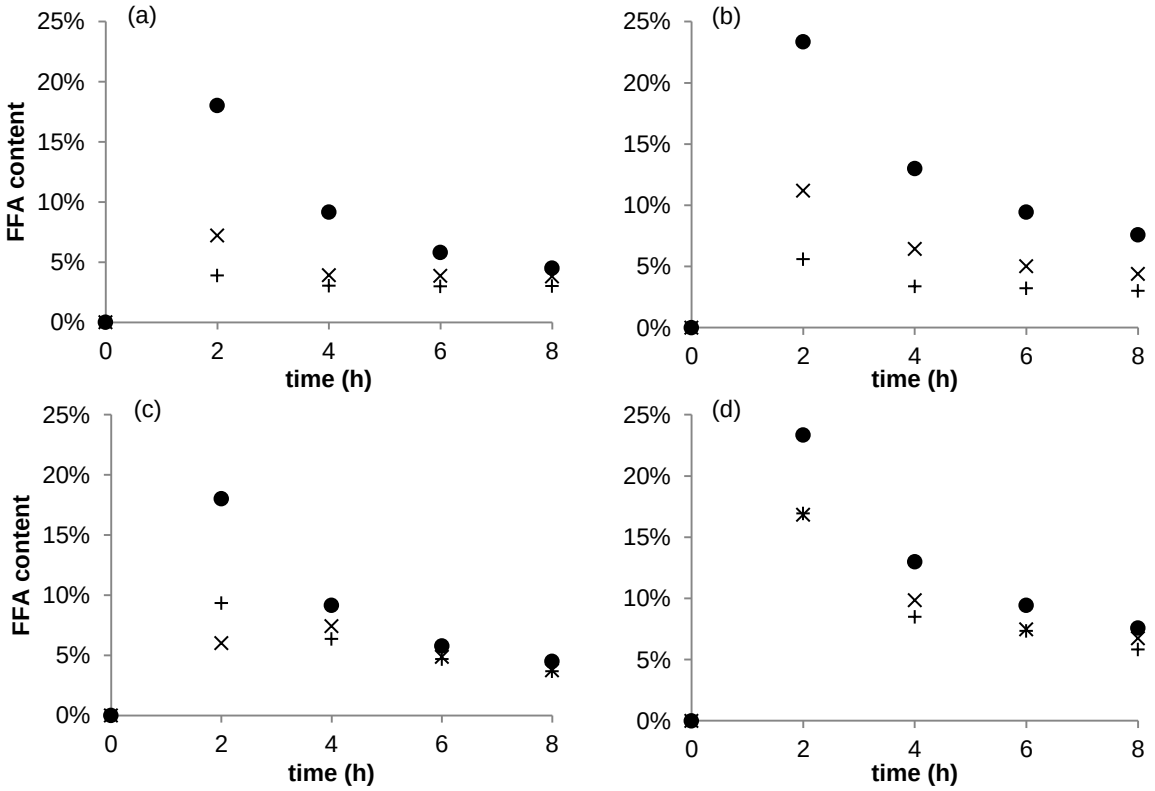

Figure 3: FAME content in oil-phase for the first batch run (●), for the second batch run with enzyme reused (×) and for the third batch run with enzyme (+). (a) Total reuse of Eversa Transform. (b)Total reuse of Resinase HT. (c) Partial reuse of Eversa Transform. (d) Partial reuse of Resinase HT.

From Figures 3(a) and 3(b), a reduction of FFA production was noticed for both enzymes along the batches. This reduction in production probably happened since part of the water present in the liquid enzymes reacted with triglycerides in the previous batches producing FFA. In this case, the water concentration is reduced for each following batch. However, this reduction in FFA production is more pronounced with Resinase HT.

Table 1: Oil-phase composition after 8 hours of reaction (in percentage)

Enzyme reuse	Batch	Eversa Transform					Resinase HT				
		TAG	DAG	MAG	FAME	FFA	TAG	DAG	MAG	FAME	FFA
Total	1	0.00	0.00	1.30	94.21	4.49	0.00	0.00	4.38	88.05	7.57
	2	2.78	4.47	20.56	68.39	3.80	0.00	2.01	7.01	86.60	4.38
	3	20.91	8.41	34.32	33.35	3.01	9.00	5.94	23.82	58.24	3.00
Partial	1	0.00	0.00	1.30	94.21	4.49	0.00	0.00	4.38	88.05	7.57
	2	0.00	0.00	3.51	92.73	3.76	0.00	1.55	4.54	87.17	6.74
	3	0.00	1.54	6.41	88.36	3.68	0.00	0.90	5.22	88.05	5.82

The behaviour of the FAME content for the second and third batches of both enzymes, differently from the first batch, is virtually linear after two hours of reaction, showing that the reaction slows down after a certain time, even after the consecutive additions of methanol (see Figure 2(a) and (b)). This might be caused by loss of enzymatic activity, which would then be directly related to the presence of glycerol. Part of this by-product probably remained in the enzymatic phase after the centrifugation and could cause enzyme inhibition.

3.2 Partial enzyme reuse

Aiming to reduce the high process costs of enzymatic transesterification reactions, but keeping the process efficiency at the same time, the partial reuse of enzymes was tested.
From Figure 2(b) and 2(c) it is possible to notice that, using a mixture of 50 % reused and 50 % fresh enzymes, the FAME content suffered lower reduction in the second and third batches. In particular, for ET as seen in Figure 2(c) 92.73 % FAME content was obtained in the second batch and 88.36 % in the third batch, resulting in only 6 % reduction with respect to the first batch. Concerning the use of RHT reported in Figure 2(d), the FAME content was 87.17 and 88.05 % after the second and third batches, respectively. For this specific enzyme, the FAME content in the oil-phase is nearly constant.
After each consecutive reaction, the content of free fatty acids also decreased, as seen from Figures 3(c) and 3(d). Nevertheless, due to partial addition of liquid enzymes, the depletion is smaller compared to the reduction observed for the total enzyme reuse.
The production of FAME in the three batches for the partial reuse of Eversa Transform obtained higher results than the consecutive batches using Resinase HT. However, RHT has shown greater stability since its production of FAME stayed virtually constant proving that RHT is a more resistant enzyme. In contrast, the MAG content, reported in Table 1, kept increasing when ET was used as a catalyst, indicating that the drop of FAME content will continue decreasing for the next batches, followed by the increasing of DAG and MAG content. In that case, partial reuse of ET should not be continued after the third batch.

3.3 Enzyme recovery

After each transesterification reaction, the centrifugation of the products resulted in a three-phase system. The upper layer composed by the oil-phase, the intermediate-phase was constituted mainly by glycerol, and the bottom-phase concerned the enzymes. For both enzymes, after the first batch, about 90-95 % of their volume was recovered. Regarding the centrifugation after the second batch, the recovery percentage was reduced to a value between 80-90 %. Consecutive centrifugations seemed to continuously reduce the enzyme recovery, confirming that the reuse is not viable after the third batch. Likewise, the deposition of glycerol in the enzyme-phase after the centrifugations appears to be one of the main causes for the lower conversion when reusing the enzymes.
The reuse of liquid enzymes was studied under specific reaction conditions. A more thorough evaluation of their reuse should be performed using different reaction conditions such as different temperatures and alcohol-to-oil molar ratios. The influence of varying the enzyme content should also be evaluated, even though the decrease of enzymes concentration directly affects the FAME production. The reduction of the enzymes amount is followed by the reduction of the total quantity of water, which needs to be compensated by its direct addition. In this case, higher amount of water added in the system counterbalances the volume of water present in the liquid enzymes. The presence of water in the system leads to the hydrolysis of TAG, producing free fatty acids. These reactions are followed by the esterification of FFA with methanol. This operational condition could minimize the biodiesel production cost by reducing the enzyme consumption. However, the increase in FFA concentration must be investigated.
Different recovery methods for the enzymes also ought to be tested. Centrifugation followed by extraction of glycerol in the enzymatic phase or the use of membranes to recover the enzymes should be considered. Nevertheless, the viability of these methods needs to be taken into consideration since they increase the production cost.

4. Conclusions

Several advantages can justify the choice of using liquid enzymes as a catalyst in transesterification reactions. High cost of the enzymes is the major drawback that makes this use questionable. In this sense, reuse of liquid enzymes was proposed for castor oil transesterification with methanol at 35 °C under two different conditions.
The total reuse of liquid enzymes has shown that the chosen enzymes lose their activity in the third batch reaction, especially when using Eversa Transform. This loss of enzyme activity can be caused by the enzyme deterioration during the reaction batches, and also the presence of glycerol in the enzymatic phase. Meanwhile, the partial reuse of enzymes gave more consistent high biodiesel production. This indicates that

this configuration can be adopted to keep a higher production rate. Resinase HT seems a more resistant enzyme, since the FAME content obtained for the batches is more consistent, pointing to a lower loss of activity. However, Eversa Transform still gave higher FAME production in the three batches, even though the results point to a significant reduction of FAME production after the third batch.

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