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Feasible Novozym 435-Catalyzed Process to Fatty Acid Methyl Ester Production from Waste Frying Oil: Role of Lipase Inhibition

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1. Introduction

Fatty acid methyl ester (FAME) or biodiesel is a biofuel conventionally produced from edible oil and methanol, using an alkaline catalyst, through a transesterification reaction. As FAME is mostly produced from edible vegetable oils, crop soils are used for its production, increasing deforestation and producing a fuel more expensive than diesel. In addition, between 70 and 80% of the total FAME production costs correspond to the vegetable oils. Therefore, the use of waste lipids such as waste frying oils (WFO), waste fats and soapstock has been proposed as low-cost alternative to feedstock. Non-edible oils such as jatropha, pongamia and rubber seed oil are also economically attractive. In addition, microalgae, bacteria, yeast and fungi with 20% or higher lipid content are oleaginous microorganisms known as single cell oil and have been proposed as feedstock for FAME production. Alternative feedstocks are characterized by their elevated acid value due to the high level of free fatty acid (FFA) content, causing undesirable saponification reactions when an alkaline catalyst is used in the transesterification reaction. The production of soap consumes the conventional catalyst, diminishing FAME production yield and simultaneously preventing the effective separation of the produced FAME from the glycerin phase. These problems could be solved using biological catalysts, such as lipases or whole cell catalysts, avoiding soap production since the FFAs are esterified to FAME. In addition, by-product glycerol can be easily recovered and the purification of FAME is simplified using biological catalysts.

Lipase-catalyzed processes have been widely investigated for FAME production from alternatives raw material. Although interesting results have been reached up to date, the enzymatic catalysis has not become competitive compared to the conventional chemical process. The main reasons explaining this issue are the long reaction time (until 48 h), the loss of enzymatic activity due to methanol use in the reaction and the high operational costs because the lipases cannot be reused. The present chapter described an investigation to a get a feasible lipase-catalyzed process to FAME production from WFO, avoiding lipase inhibition.

2. Lipase catalyzed process to FAME production from waste frying oil: Improving the yield

In spite of that some investigations have been carried using WFO instead of edible oils in FAME production using lipases, there is not clear the effect in the process of replace the raw material. Using *Rhizopus oryzae* as the biocatalyst, FFA from a synthetic WFO were esterified to produce FAME with an improved reaction yield (Li et al., 2007). In addition, using *Thermomyces lanuginosus* lipases immobilized on a microporous polymer, 97% FAME content from edible sunflower oil was reached, while only 90.2% FAME content was obtained from WFO (Dizge et al., 2009). Watanabe et al. (2001) tested the immobilized the *Candida antartica* lipase immobilized on acrylic resin (Novozym 435) and obtained a 5.5% reduction in FAME conversion yield when using WFO compared to edible oil as the feedstock. They concluded that the oxidized fatty acid compounds in WFO may be responsible for this decrease.

As the effects of using WFO instead of edible oil in lipase-catalyzed processes are not clear, the aim of this study was to elucidate the effect of WFO incorporation in feedstock mixed with rapeseed oil on FAME production yield using Novozym 435 as the catalyst by means of the response surface methodology (RSM). In addition, specific WFO and rapeseed oil chemical characteristics were investigated to identify the components that were responsible for these results. Finally, a preliminary study to establish the optimal time for methanol addition during the reaction was proposed.

Both filtered WFO collected from restaurants and crude rapeseed oil from a local factory from Southern Chile were used as the feedstocks. Novozym 435 from Sigma-Aldrich was used as the catalyst. Methyl heptadecanoate, 1,2,3-butanetriol and 1,2,3-tricaprinoylglycerol were used as internal standards and were chromatographically pure.

RSM was used to analyze and optimize the interaction effects of four variables on FAME production yield (Table 1): the WFO content in the feedstock mixture (% wt), the final methanol-to-oil ratio (mol/mol), temperature (°C) and Novozym 435 dosage (% wt based on oil weight). A central composite matrix with 5 levels was used and 30 runs were carried out in a random order. Each run was performed in triplicate.

All reactions were incubated in flasks containing 1 mL of oil at 200 rpm. The volume of the flasks was selected to maintain perfect agitation of the samples when using both the highest dosage of catalyst and the lowest methanol-to-oil molar ratio. Under these conditions, different combinations of feedstock mixture, dosage of catalyst, methanol-to-oil molar ratio and temperature were used. Methanol was added in two steps to avoid lipase inhibition (Shimada et al., 2002). In the first step, one-third of the total molar ratio was added according to Table 1, while in the second step, the remaining two-thirds of the total molar ratio was added to generate the final methanol-to-oil molar ratio.

To establish the reaction and second methanol addition times, a preliminary study was performed. This experiment was carried out for 48 h using the RSM central point, with 50% (wt) WFO in a mixed feedstock, a methanol/oil molar ratio of 3:1 and 9% (wt) Novozym 435 at 45°C and stirring 200 rpm.

Samples were immediately stored at 4°C to stop the reaction. The upper layer was analyzed by gas chromatography for FAME quantification.

Independent variables	Symbols	Levels				
		-2	-1	0	1	2
WFO in feedstock [wt %]	X_1	0	25	50	75	100
Methanol to oil ratio [mol/mol]	X_2	1.50	2.25	3.00	3.75	4.50
Temperature [°C]	X_3	35	40	45	50	55
Novozym 435 [wt %]	X_4	3	6	9	12	15

Table 1. Variable and levels used in the response surface methodology

The experimental data obtained were fitted to a second-order polynomial equation. This equation describes the relationship between the predicted response variable (FAME production yield) and the independent variables (Table 1). The polynomial model for FAME yield may be written as follows (Eq. 1):

$$\text{FAME yield} = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ij} X_i X_j \quad (1)$$

Where β (0 = intercept, i = linear, ii = quadratic and ij = interaction) and Xi, Xj (i = 1, 4; j = 1, 4; i ≠ j represent the coded independent variables) are the model coefficients. With the fitted quadratic polynomial equation, contour plots were developed to analyze the interaction between terms and their effects on FAME production yield.

To identify and quantify the fatty acids in the feedstock a Clarus 600 chromatograph coupled to a Clarus 500T mass spectrometer of Perkin Elmer (GC-MS) was utilized. An Elite-5ms capillary column with a length of 30 m, thickness of 0.1 μm and internal diameter of 0.25 mm was used. The vials were prepared by adding 3 μg of sample to 100 μL methyl heptadecanoate as an internal standard (initial concentration of 1300 mg/L). The following temperature program was used: 50°C for 1 min and then increasing temperature at a rate of 1.1°C/min up to 187°C. Both the injector and detector temperatures were 250°C and He was used as the carrier gas. Before injection into the GC-MS equipment, the WFO and rapeseed oil were methylated according to Araújo (1995). Specific gravity was measured at 20°C using a manual densimeter. Kinematic viscosity was measured at 40°C using a capillary viscosimeter. The acid value was determined by titration with KOH using phenolphthalein as an indicator. The peroxide value was determined by titration with $Na_2S_2O_3$, and the iodine value was determined by the Wijs method (Araújo 1995). The following parameters were measured according to ASTM Standard Methods: water and sediments (ASTM Standard D 1976-97), pour point (ASTM Standard D 97-04), sulfur (ASTM Standard D 7039-04) and flash point (ASTM Standard D 56-02a).

To establish the degree of oil conversion, monoacylglycerols (MG), diacylglycerols (DG), triacylglycerides (TG) and FFA in the oil feedstock were quantified using an HP 6890 series gas chromatography system (GC-MS) with an adaptation of the EN-14214 (The)methodology. TG was determined by mass balance. A 50-m long BPX-5 column with a thickness of 0.5 μm and an internal diameter of 0.32 mm was used. The vials were prepared by combining 10 mg of sample with 0.8 μL of 1,2,3-butanetriol and 10 μL of 1,2,3-tricaprinoylglycerol dissolved in pyridine as internal standards. N-methyl-N-

trimethylsilyltrifluoroacetamide (MSTFA) was added to the vials, which were then shaken and incubated for 15 min to transform the sample into more volatile siliade components. Subsequently, the preparation was dissolved in 0.8 mL of n-heptane. The following temperature program was used: 15ºC for 1 min and three consecutive ramps of 15ºC/min to 180ºC, 7ºC/min to 230ºC and 10ºC/min to 320ºC and 320ºC for 15 min. The detector temperature was 380ºC and a split rate 10 was used for injection of 1 µL of sample.

Methanol addition. Methanol solubility is less than 1.5:1 methanol/oil (mol/mol), but a molar ratio of 3:1 methanol/oil is necessary to complete the transesterification reaction for FAME production (Shimada et al., 2002). However, very high initial concentrations of methanol could lead to the inhibition of the lipase used in this study due to its low solubility in oil (Du et al., 2004; Shimada et al., 2002). Therefore, the experiment was designed so that the methanol was added in two steps (Fig. 1).

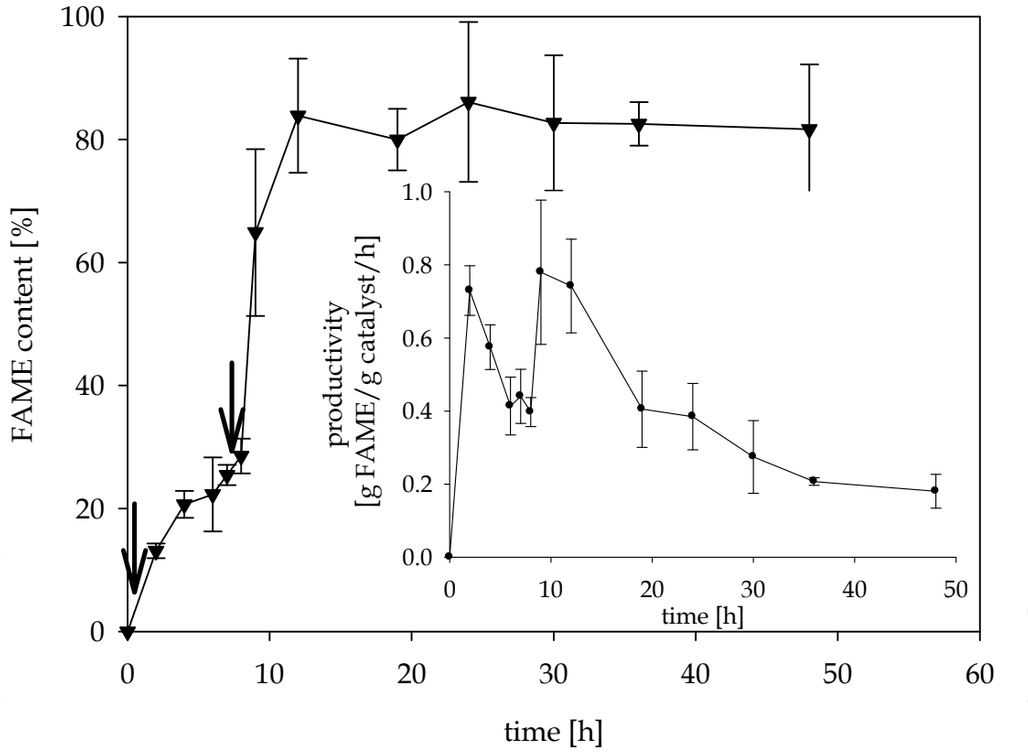


Fig. 1. FAME content and productivity during the reaction time. Operational conditions: 50 wt% WFO in the mixed feedstock, methanol to oil molar ratio of 3:1, 9 wt % Novozym 435 and 45°C, at 200 rpm. Arrows indicate methanol addition.

The experiment was started with an initial concentration of one-third of the necessary moles of methanol. When about one-third of the oil was converted to FAME after 8 hours, the remaining two-thirds of the methanol were added (Fig. 1). The addition of the higher concentration of methanol in the second step was determined based on Shimada et al. (2002), who established that methanol is more soluble in FAME than in TG.

This two-step technique of methanol addition was shown to diminish the reaction time by obviously preventing enzyme inhibition. Li et al. (2009) showed that in spite of the high yield reached using stepwise methanol addition, both the reaction time and rate of FAME conversion were slower. In experiments using a three-step methanol addition and 4% (wt) Novozym 435 as the catalyst, high FAME production yields were reached but only after 50 hours of reaction (Du et al., 2004; Watanabe et al., 2001). Similarly, in another recent study, an reaction time of 50 hours was also used (Ognjanovic et al., 2009). Usually, the second methanol addition starts after one-third (33.3%) of the FAME content has been generated and the initially added methanol has been completely consumed (Du et al., 2004; Shimada et al., 2002). This methodology produces a stationary phase of FAME production yield, diminishing the reaction productivity.

As Novozym 435 was shown to be a robust and stable catalyst compared to other lipases in the presence of short chain alcohols (Hernandez-Martin et al., 2008), all of the experiments in this study were performed using this commercially available immobilized enzyme. When approximately 28.6% of the FAME content was reached after 8 hours of reaction, the second aliquot of methanol was added. This stepwise addition of methanol increased the reaction productivity by avoiding the stationary production phase and led to a plateau in FAME production at 12 hours. Similar reductions in the reaction time by shortening the stationary phase of FAME production have already been reported (Li et al., 2009). Therefore, in the RSM experiments, the first amount of methanol (one-third of the molar ratio shown in Table 1) was added at the start of the reaction, while the second aliquot of methanol (two-thirds of the molar ratio shown in Table 1) was added after 8 hours, and the total reaction time was 12 hours.

Optimization of methanolysis conditions. Four variables previously shown to have significant effects on FAME production yield were investigated. The methanol-to-oil ratio, temperature and Novozym 435 dosage were established based on previous studies in the literature (Du et al., 2004; Fukuda et al., 2001; Kose et al., 2002). According to previous experiments (data not shown), the effect of the WFO content in the feedstock mixture was also tested in the lipase-catalyzed process. The experimental central composite design matrix is presented in Table 1.

To understand and optimize the relationship between the tested variables, the obtained experimental data were analyzed by second-order polynomial equations by means of the RSM. The analysis of variance (ANOVA) of the quadratic polynomial model showed low p-values (0.125) and both high determination coefficients (R^2=95.3%) and high adjustment of the determination coefficients (Adj. R^2=94.5%). The low p-value obtained indicates that the model accurately represented the relationship between response and the variables. The R^2 value obtained indicates that the variation in FAME production yield correlated with 95.3% of the independent variables and the obtained Adj. R^2 value indicates a 94.5% correlation between the independent variables. The lack of fit refers to the fact that a simple linear regression model may not adequately fit the experimental data. The obtained p-value not indicate a significant lack of fit and, therefore, gave no reason to evaluate a more complex model.

The p-values obtained from the regression analysis results showed that all of the coefficients of the linear, interaction and quadratic terms had a significant effect on FAME production yield. All of the linear and quadratic terms, as well as the interaction terms X_1X_3, X_2X_4 and X_3X_4, were significant at the 1% level. The interaction terms X_1X_2, X_1X_4 and X_2X_3 were

significant at the 5% level. Therefore, the final response model equation in terms of the variable factors can be written as follows (Eq. 2):

$$\begin{aligned} FAME_{yield} = & -896.52 + 1.90 \cdot X_1 + 91.40 \cdot X_2 + 28.06 \cdot X_3 + 20.00 \cdot X_4 - 0.01 \cdot X_1^2 \\ & - 14.46 \cdot X_2^2 - 0.24 \cdot X_3^2 - 0.85 \cdot X_4^2 - 0.12 \cdot X_1 \cdot X_2 - 0.02 \cdot X_1 \cdot X_3 \\ & - 0.03 \cdot X_1 \cdot X_4 - 0.59 \cdot X_2 \cdot X_3 + 4.13 \cdot X_2 \cdot X_4 - 0.26 \cdot X_3 \cdot X_4 \end{aligned} \quad (2)$$

The main factors that affect the FAME production yield were the linear terms X_1, X_2, X_3 and X_4, the quadratic term X_2^2 and the interaction term X_2X_4. The linear terms with positive coefficients indicate an increase in FAME production yield. As X_1 is a positive term, it is possible to establish that WFO incorporation could increase FAME production yield.

To validate the model obtained, an experimental point was compared to the model prediction (Eq. 2), and an error of less than 8% was obtained. In addition, the optimal methanolysis conditions were obtained through the regression model (Eq. 2) according to the limit criterion of the maximum response of FAME production yield. The obtained optimal conditions were 100% (wt) WFO, a methanol-to-oil ratio of 3.8:1 (mol/mol), 15% (wt) Novozym 435 and 44.5°C, which generated 100% FAME production yield. These predicted conditions show that WFO incorporation increases FAME production yield.

Moreover, using this model, it was also possible to predict several optimal conditions to reach the highest FAME production yields, while simultaneously reducing production costs and enzyme use. For instance, 96% FAME production yield could be reached using 75% (wt) WFO in the mixed feedstock, with a methanol-to-oil ratio of 3.75:1 (mol/mol), 12% (wt) Novozym 435 and 40°C.

To illustrate that several optimal combinations are able to produce the highest FAME production yields, contour plots were generated. Figure 2 and 3 show the effect of variable interaction on FAME production yield in contour plots predicted by the model. The contour plots were generated to show the effect of two variables on the response, while the other two independent variables were held constant.

Figure 2 shows the interaction between two independent variables (WFO in the mixed feedstock and Novozym 435 dosage) and their effects on the response variable FAME production yield, while the other two variables were held at zero. At these conditions, an increase in Novozym 435 dosage led to an enhancement in FAME production yield. This change is additionally increased when WFO is incorporated at up to 80% in the mixed feedstock. WFO seems to be a more available substrate compared to rapeseed oil for Novozym 435 catalysis under the conditions investigated.

FAME production yield was more sensitive to Novozym 435 dosage, since high doses were necessary to obtain high FAME production yields. In this sense, in order to decrease the production costs for future economically sound industrial applications, further experiments should be conducted to investigate the effect of WFO incorporation using another inexpensive biologic catalyst instead of Novozym 435. In addition, because Novozym 435 has shown high residual activity in successive applications (Hernandez-Martin et al., 2008), further experiments with a subsequent recovery protocol and reuse of the immobilized catalyst should be conducted under the optimal conditions established in this work.

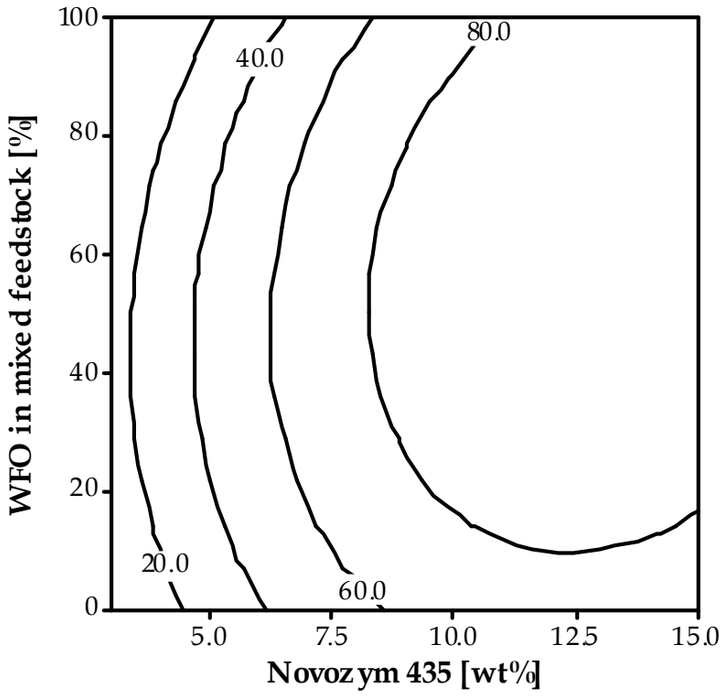


Fig. 2. Contour plot of FAME production yield predicted from the model at 45ºC and molar methanol to oil ratio 3:1 mol/mol.

Recently, Issariyakul et al. (2008) investigated the use of mixed WFO and rapeseed oil using an alkaline catalyst. However, lower ester production yields were observed when WFO was incorporated in the reaction mixtures. These results agreed with Fukuda et al. (2001), who established that the FFA in WFO can be completely converted to FAME using lipases as catalysts, whereas soap is produced when an alkaline catalyst is used, which diminishes the production yield. Although the cost of lipases is significantly higher than that of alkaline catalysts, their use may partially solve the main drawbacks of the conventional biodiesel production process: the use of pure vegetable oils (which account for around 80% of the cost of the total process) and the competition with food products (Gui et al., 2008). We have shown that the incorporation of WFO in the feedstock to partially replace rapeseed oil in processes catalyzed by Novozym 435 may diminish the production costs while simultaneously increasing the production yield, which makes it a potential alternative method for FAME production on an industrial scale.

FAME production yield was more sensitive to both the methanol-to-oil ratio and Novozym 435 dosage, compared to temperature (Fig. 3). FAME production yield was enhanced when the temperature increased to 45-50ºC; however, the opposite tendency was observed for temperatures higher than 50ºC. These results agree with the results obtained by Kose et al. (2002), who established that at about 50ºC, FAME production yield decreases as a result of enzyme deactivation at high temperatures (Fig. 3A). An enhancement in FAME production yield was observed when both the methanol-to-oil ratio and Novozym 435 dosage increased (Fig. 3B). When using a high immobilized biocatalyst load, large amounts of alcohol were

needed to provide sufficient liquid to maintain a uniform suspension of the biocatalyst (Hernandez-Martin et al., 2008). In addition, as Novozym 435 is a robust biocatalyst in the presence of short chain alcohols, the excess alcohol kept the glycerol in solution, which prevented deactivation of Novozym 435 by glycerol blockage of the entrance to catalyst pores (Hernandez-Martin et al., 2008). Therefore, the levels of Novozym 435 used and the stepwise methanol addition seem to avoid the possible diffusion limitations, which resulted in high production yields.

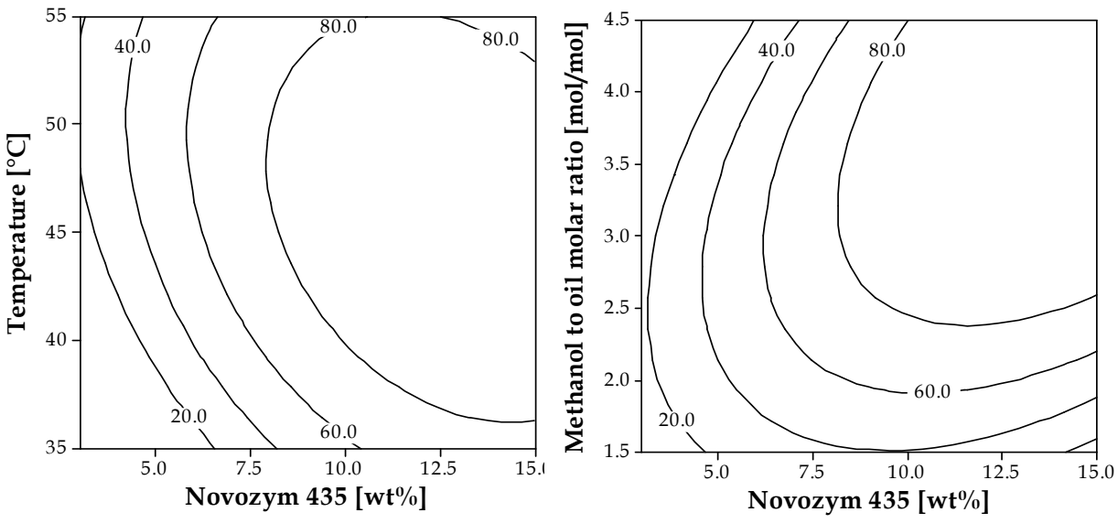


Fig. 3. Contour plots of FAME production yield predicted from the model for 50% (wt) WFO at (A) a methanol-to-oil ratio of 3:1 (mol/mol) and (B) 45ºC.

Properties of feedstock that affect methanolysis optimization. The feedstock characteristics were investigated to determine the components responsible for the results obtained in the RSM, particularly the increase in the FAME yield when WFO was incorporated in the process (Table 2).

Differences were found in most of the physical properties of the oil feedstock measured (Table 2). The food frying process produced an increase in both acid and peroxide values from WFO due to the hydrolysis and oxidation reactions, respectively (Araújo 1995). The high peroxide value of the WFO could positively affect the FAME characteristics by increasing the oxygen content, which enhances its burning efficiency (Lin et al., 2007). However, a higher oxygen content in oils may also promote a higher nitrogen oxide concentrations during the burning process (Lin et al., 2007).

The analyzed WFO was originally an edible soybean oil and sunflower oil mixture, which is characterized by a typical iodine value of about 130 g I_2/100 g oil. As the iodine value is diminished by polymerization reactions during the frying process, a similar iodine value compared to rapeseed oil was found for WFO (Table 2). A lower iodine value increases the pour point and may also improve the oxidative stability of the oil (Canakci 2007). However, a high pour point can negatively affect FAME performance in an engine, when used at low

temperatures. The kinematic viscosity of WFO was also measured and was slightly higher compared to rapeseed oil, probably as a result of polymerization reactions. As WFO is refined oil, the sulfur content was lower in WFO compared to rapeseed oil, which is an unrefined oil. In addition, a higher FFA content in WFO compared to rapeseed oil was observed, which may enhance FAME production yield using a biological catalyst, such as Novozym 435, through direct FFA esterification (Li et al., 2007).

Physicochemical properties	Feedstock	
	WFO	Rapeseed oil
Specific gravity [kg/m^3 at 20°C]	925	927
Kinematic viscosity at 40°C [cst]	37	34
Acid value [mg KOH/g]	4.5	0.8
Iodine value [g I_2/100 g oil]	111	105
Peroxide value [meq/kg]	15.2	4.1
Water and sediments [%v/v]	1.3	< 0.01
S [mg/L]	2.0	7.0
Pour point [°C]	-6.7	-23.3
Flash point [°C]	>100	>100
Fatty acid composition [wt%]		
Palmitic [C16:0]	12.2	4.2
Stearic [C18:0]	4.1	1.3
Oleic [C18:1]	28.5	66.6
Linoleic [C18:2]	49.5	18.7
Linolenic [C18:3]	3.5	7.7
Arachidic [C20:0]	0.2	0.4
Eicosenic [C20:1]	0.1	1.0
Eicosapentaenoic [C20:5]	0.2	0.1
Erucic [C22:1]	0.0	0.1
Others	1.7	0.0
Acylglycerols and FFA composition [wt%]		
Monoacylglycerol	0.1	0.0
Diacylglycerol	3.3	0.5
Triacylglycerol	93.5	98.7
Free fatty acid	3.1	0.8

Table 2. Properties and composition of oil feedstocks (samples were filtered before to the analysis).

The FFA esterification reaction produces 1 mol of water per 1 mol of FAME produced. In this work, water and sediments were quantified and low levels were found in both rapeseed oil and WFO, with a slightly higher water content for WFO (Table 2). However, as WFO has a high FFA content, incorporation of WFO in the mixed feedstock may increase the water content through FFA esterification reactions. Although lipases need an optimal amount of water to maintain their activity in organic media, a recent work established that Novozym 435 itself appears to contain sufficient water to preserve its catalytic activity without requiring the presence of an oil/water interface (Ognjanovic et al., 2009). It has also been shown that Novozym 435 needs a nearly anhydrous reaction medium to be effective (Salis et al., 2005). According to these previous studies, when WFO was incorporated in a mixed feedstock, the reaction yield diminished as a result of the high water content. Therefore, an improvement in FAME production yield when WFO is incorporated into a feedstock should not be produced due to the presence of water in the transesterification reaction.

Differences between the fatty acid compositions of WFO and rapeseed oil were also detected (Table 2). In order to analyze the role of fatty acids in FAME production, the input and output fatty acid compositions were compared. However, no significant differences between input and output fatty acid composition were detected. (data not shown). Products from oil conversion, i.e., MG, DG, TG and FFA, in the used feedstock were compared using GC-MS. MG, DG and FFA account for about 6.5% of the WFO composition, compared to only 1.3% in the case of rapeseed oil (Table 2). Turkan & Kalay (2006) established that in Novozym 435-catalyzed transesterification, the first step (i.e., conversion of TG to DG) is the rate-determining step, since TG is converted to FAME without significant accumulation of MG and DG. This fact indicates that MG, DG and FFA can be more easily converted to FAME compared to TG. Therefore, WFO is a more available substrate in the process investigated, compared to rapeseed oil, and incorporation of WFO in feedstock increased the FAME production yield.

Finally, is possible to establish that according to the RSM, the optimized predicted combination of conditions to obtain 100% FAME production yield was the use of 100% (wt) WFO, a methanol-to-oil ratio of 3.8:1 (mol/mol), 15% (wt) Novozym 435 and 44.5°C at 200 rpm. Methanol addition in two steps was previously optimized. An earlier second addition of methanol after 8 hours of reaction time was an effective technique to decrease the total reaction time to 12 hours, while preventing enzyme inhibition. The model obtained from the RSM predicted that several optimal conditions were able to reach the highest FAME production yield. According to this model, the addition of WFO increased the FAME production yield, and this effect was mainly attributed to the higher contents of MG, DG and FFA of WFO compared to rapeseed oil, which are more available substrates for enzymatic catalysis. Therefore, a partial replacement of rapeseed oil by WFO in processes catalyzed by Novozym 435 may diminish the cost of biodiesel production by using a less expensive feedstock that simultaneously increases the production yield.

3. Enzymatic biodiesel production in an anhydrous medium with lipase reutilization

A major problem in lipase-catalyzed processes to FAME production is the high acidity of the final product, mainly caused by water presence. In fact, water favors the hydrolysis reaction

producing FAME with a high acid value. Therefore, to accomplish with international biofuel standards, additional treatment are needed (Fukuda et al., 2008). In addition, this problem could be enhanced by using alternative feedstock characterized by high acid value, such WFO, jatropha, microalgae and crude palm oil (Azócar et al., 2010b).

To solve these drawbacks the addition of water adsorbents to the lipase-catalyzed reaction has been recently investigated (Li et al., 2009; Wang et al., 2006). Some reported absorbents are blue silica gel, maceo-pored silica gel, fine-pored silica gel, and molecular sieves of 3 Å, 4 Å and 5 Å (Li et al., 2009; Wang et al., 2006). Among the adsorbents examined, blue silica gel has been reported as the best one, increasing FAME yield when immobilized lipase from *Penicillium expansum* was used as catalyst (Li et al., 2009). However, high dosage of silica gel could provoke low biodiesel yield. In fact, as the pore size of silica gel is much larger than the methanol molecule size, silica adsorbs methanol negatively affecting the reaction performance (Wang et al., 2006).

The possible advantages of using water adsorbents in lipase-catalyzed processes to improve FAME production yield may be in contradiction with reports showing that lipases activation may need water presence. In fact, lipases activation involves the active site restructuration, which requires the presence of an oil-water interface (Yu et al., 2010). As a result, small dosages of water in the reaction have been proposed to achieve an effective process (Yu et al., 2010). Novozym 435 has been shown to contain enough water in its support medium to preserve its catalytic activity in an anhydrous medium (Ognjanovic et al., 2009; Tamalampudi et al., 2008). In a response surface methodology study, Organovic et al. (2009) correlated Novozym 435 concentration with water concentration, achieving the highest biodiesel yield of 92% using the medium with the lowest water content level (0%) and the highest enzyme concentration (5% based on oil weight). In another study, Novozym 435 showed low activity in the presence of water and an anhydrous medium for efficient catalyzing was proposed (Tamalampudi et al., 2008).

Novozym 435 has been widely reported as an effective biocatalyst for promoting high FAME production yields (Azócar et al., 2010b). Optimal conditions to reach 100% FAME yield using WFO as feedstock were determined to be the following: Methanol-to-oil molar ratio 3.8:1 (wt), 15% (wt) Novozym 435 and incubation at 44.5°C for 12 h with agitation at 200 rpm (Azócar et al., 2010a). Novozym 435 activity loss and regeneration have been also investigated. It has been established that the immobilization material (acrylic resin) could adsorb polar components such as methanol and glycerol, provoking enzyme inactivation. Therefore, Novozym 435 reutilization by means of washing processes using acetone, soybean oil, *tert*-butanol, isopropanol and 2-butanol has been investigated (Chen et al., 2003; Samukawa et al., 2000). In addition, the incorporation of a co-solvent in the reaction has been recently proposed (Royon et al., 2007; Yu et al., 2010).

According to previous research, Novozym 435 has been proved to be an effective catalyst in FAME production and has been shown to preserve its catalytic activity in an anhydrous medium. However, Novozym 435 has not been investigated yet regarding catalyzed-processes when adsorbent materials are added to the reaction in order to obtain high quality biodiesel with low acid value. In this sense, it is necessary to evaluate the behavior of the enzymatic reaction in anhydrous medium. In addition, it is necessary to determine the effect

of water absence in the enzyme activity during consecutive reactions and to evaluate alternatives for enzyme recovery during successive reactions.

The aim of this work was to test an anhydrous medium in Novozym 435 catalyzed-process to produce FAME with a low acid value. In addition, the behavior of Novozym 435 in the anhydrous medium was discussed and enzyme recovery alternatives were proposed.

Filtered WFO collected from restaurants and crude rapeseed oil from a local factory in Southern Chile were used as feedstock. The WFO was characterized by an acid value of 5.61 mg KOH/g oil, 2.8% FFA, 1.3% (v/v) water content, 0.1% (wt) MG, 3.3% (wt) DG and 925 Kg/m^3 of specific gravity. The rapeseed oil was characterized by an acid value of 0.8 mg KOH/g oil, 1.6% FFA and 927 Kg/m^3 of specific gravity. Novozym 435 donated by Novo Industries (Denmark) was used as catalyst. 3 Å molecular sieves (Sigma-Aldrich) were used to generate the anhydrous medium. Chromatographically pure methyl heptadecanoate, 1,2,3-butanetriol and 1,2,3-tricaprinoylglycerol were used as internal standards.

FAME production using Novozym 435 in an anhydrous medium was studied by adding 3 Å molecular sieves to the reaction. All reactions were carried out in flasks containing 1 mL of WFO (0.925 g) and 15% Novozym 435 (% wt based on oil weight) as the biocatalyst. A methanol-to-oil molar ratio of 4:1 was used. Methanol was added in two steps in order to avoid lipase inhibition (according to previous experiments in section 2). The flasks were incubated in a shaker at 35 °C and stirred at 200 rpm. Each flask was managed as a destructive sample at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 hours of reaction time and each condition was carried out in triplicate. Controls were carried out under the same conditions but without molecular sieves.

To calculate the dosage of molecular sieves, first the maximal theoretical water content in the reaction was estimated. This value was established by taking the initial water content in the WFO and the amount of water produced during the reaction by esterification of FFA contained in the WFO. The water produced by esterification of FFA was estimated by using the mass balance, considering that 1 mol of esterified FFA produces 1 mol of water. With an FFA content of 2.8% and water content of 1.3% in the WFO described above, a total water content of 4.1% was used to estimate the molecular sieve dosage in the reaction, as shown in Eq. 3.

$$Molecular\ sieves = \frac{V\ oil \bullet \rho\ oil \bullet H_2O}{WAC} \approx \frac{1 \bullet 0.925 \bullet 4.1}{20} = 0.19\ g \quad (3)$$

Where V_{oil} (mL) is the volume of oil added, ρ_{oil} (g/mL) is the density of the oil, H_2O is the total water content during the reaction (water content in the oil more water produced by esterification of FFA) (% v/v) and WAC (%) is the water absorption capacity of the sieves. At the end of the reaction period, the samples were stored at 4 °C to stop the reaction. The samples were centrifuged and the upper layer was extracted for the analysis of FAME yield and acid value.

Different treatments to recover enzyme activity for further reutilization were investigated. Prior to the enzyme recovery treatments, reactions of FAME production were carried out in flasks which were incubated in a shaker under the same operating conditions. The

operational conditions were the following: Methanol-to-oil ratio of 3.8:1 (mol/mol); 15% Novozym 435 (% wt based on oil weight); 44.5 ºC; stirring at 200 rpm; and 12 hours of reaction time. Methanol was added in two steps as described previously.

For each reaction, 8 mL of WFO (7.4 g) with an acid value of 5.61 mg KOH/g oil were used. To maintain an anhydrous medium, 0.95 g of 3 Å molecular sieves were added to each reaction flask, which were calculated according to Eq. (3). Each reaction was carried out in triplicate. Samples of 70 μL were taken consecutively at 3, 6, 9 and 12 hours of reaction time. The samples were centrifuged and the upper layer was extracted to analyze the FAME yield.

For enzyme recovery, the molecular sieves were separated with a strainer after the transesterification reaction. A paper filter over a vacuum system was placed underneath the strainer containing the molecular sieves to retain the enzymes. The product of the reaction was passed through the two filters: The molecular sieves captured in the strainer were eliminated, whereas the enzymes captured in the paper filter were placed in a new flask for the recovery treatment.

Three treatments to reutilize the enzymes were performed according to the reference studies (Chen et al., 2003; Samukawa et al., 2000; Yu et al., 2010) and a previous experiments (data not shown). A control experiment reusing the enzymes without treatment was also carried out. The treatments were the following: (A) Acetone washing: The enzymes were washed by adding a small dosage of acetone to the flask containing the enzymes. The flask was shaken and the liquid residue was eliminated. This was repeated successively until the liquid was clear. The total volume used in this process was about 10 mL of acetone per gram of enzyme. Subsequently, a wash with 10 mL of WFO per gram of enzyme was carried out to eliminate residual acetone in the enzymes. (B) Waste frying oil washing: The enzymes were washed in a sufficient quantity of WFO to maintain the enzymes submerged in the flask. The flask was shaken and the liquid residue was eliminated. The washing was repeated 3 times. (C) *tert*-Butanol washing: The washing was carried out by adding a specific dosage of *tert*-butanol to the flask containing the enzymes. Then, the mixture was shaken and the residual liquid was eliminated. This sequence was repeated consecutively until the liquid was clear. The total volume used in this process was about 10 mL of *tert*-butanol per gram of enzyme. After this, a wash with about 10 mL of WFO per gram of enzyme was carried out to eliminate residual *tert*-butanol in the enzymes.

After each washing step, a known dosage of WFO was added to the flasks containing the enzymes, which were incubated in oil overnight at ambient temperature. After about 10 hours, both methanol and 3 Å molecular sieves were added to carry out a new FAME production reaction in the same incubation medium. The control was carried out by transferring the enzymes directly to a new reaction for FAME production. Cycles that consisted of both a reaction for FAME production and a treatment for enzyme recovery were repeated 4 times.

As an alternative to enzyme reuse for FAME production using an anhydrous medium, the addition of *tert*-butanol as a co-solvent in the reaction was investigated. To carry out the experiments, consecutive cycles of enzymatic FAME production using the same enzymes (reutilized) were carried out in an anhydrous medium and in a *tert*-butanol system. Each

experiment was carried out in flasks by adding 5 mL of WFO (4.6 g), 15% of Novozym 435 (% wt based on oil weight) and a methanol-to-oil ratio of 3.8:1 (mol/mol). Methanol was added to the flask in two steps, similar to the previous experiment. In addition, both the selected dosage of *tert*-butanol and an amount of 3 Å molecular sieves (estimated according to Eq. 1) were added to the flasks. The *tert*-butanol dosage was established according to previous experiments at 0.75% (V/V) (data not show). Under these conditions, the flasks were incubated at 44.5 °C and 200 rpm during 12 hours of reaction time. All the experiments were carried out in triplicate and samples were taken throughout the reaction time. The upper layer of the centrifuged samples was analyzed to determine the FAME yield, according to the analytical procedures.

The acid value and FFA content in both the feedstock and throughout the reaction time were determined by titration with a KOH solution of known concentration and using phenolphthalein as indicator. Water and sediments were measured according to ASTM Standard Method D 1976-97(American). FAME, MG and DG in feedstock and reaction products were quantified according to methodology previously described in section 2.

Biocatalysis in an anhydrous medium. In order to reduce the acid value of the produced FAME, the alternative of carrying out FAME production reaction in an anhydrous medium was studied. Adsorbent materials such as blue silica gel and molecular sieves of different sizes have been shown to be effective in water removal during biodiesel production (Li et al., 2009). However, in this study, blue silica gel was discarded due to results from previous experiments showing possible methanol adsorption, negatively interfering with the reaction (data not shown). The adsorption of methanol could also occur when molecular sieves with large pore size are used. Thus, 3 Å molecular sieves were chosen to produce an anhydrous medium through water absorption.

The acid value and FAME yield results obtained using an anhydrous medium and a control run are shown in Fig. 4. The acid value obtained from the reaction using the anhydrous medium was maintained in about 1 mg KOH/g oil throughout the entire reaction time, whereas the control showed a value higher than 3 mg KOH/g oil by the end of the reaction (Fig. 4A). The values obtained using the anhydrous medium are close to those established by the biodiesel norm. As FFA present in WFO were esterified producing water and FAME at the start to the reaction (Fig. 4A), water was removed from the reaction by adsorption onto the molecular sieves, avoiding hydrolysis reactions and further FFA production. Therefore, the biocatalysis in an anhydrous medium produces a higher quality product compared to a typical standard reaction using Novozym 435.

FAME yield was also analyzed in an anhydrous medium (Fig. 4B). The results obtained show that water removal during FAME production generated a significant increase in FAME yield using Novozym 435 as the catalyst. These results are in agreement with those obtained by Li et al. (2007) who established that using 3 Å molecular sieves as adsorbent to remove excessive water could significantly increase the FAME yield when 100% FFA is used as raw material for biodiesel production. In this study, WFO containing only 2.8% FFA and 1.3% water was employed as the raw material, but FAME yield increased drastically. In addition, the control run only using molecular sieves indicates that this material did not catalyze the reaction (data not shown). Therefore, the increment in FAME yield is only related to the anhydrous medium. This corroborates the results obtained by Tamalampudi

et al. (2008) who suggest that Novozym 435 transesterification activity is inhibited by the presence of added water and that it needs a nearly anhydrous medium for an efficient performance.

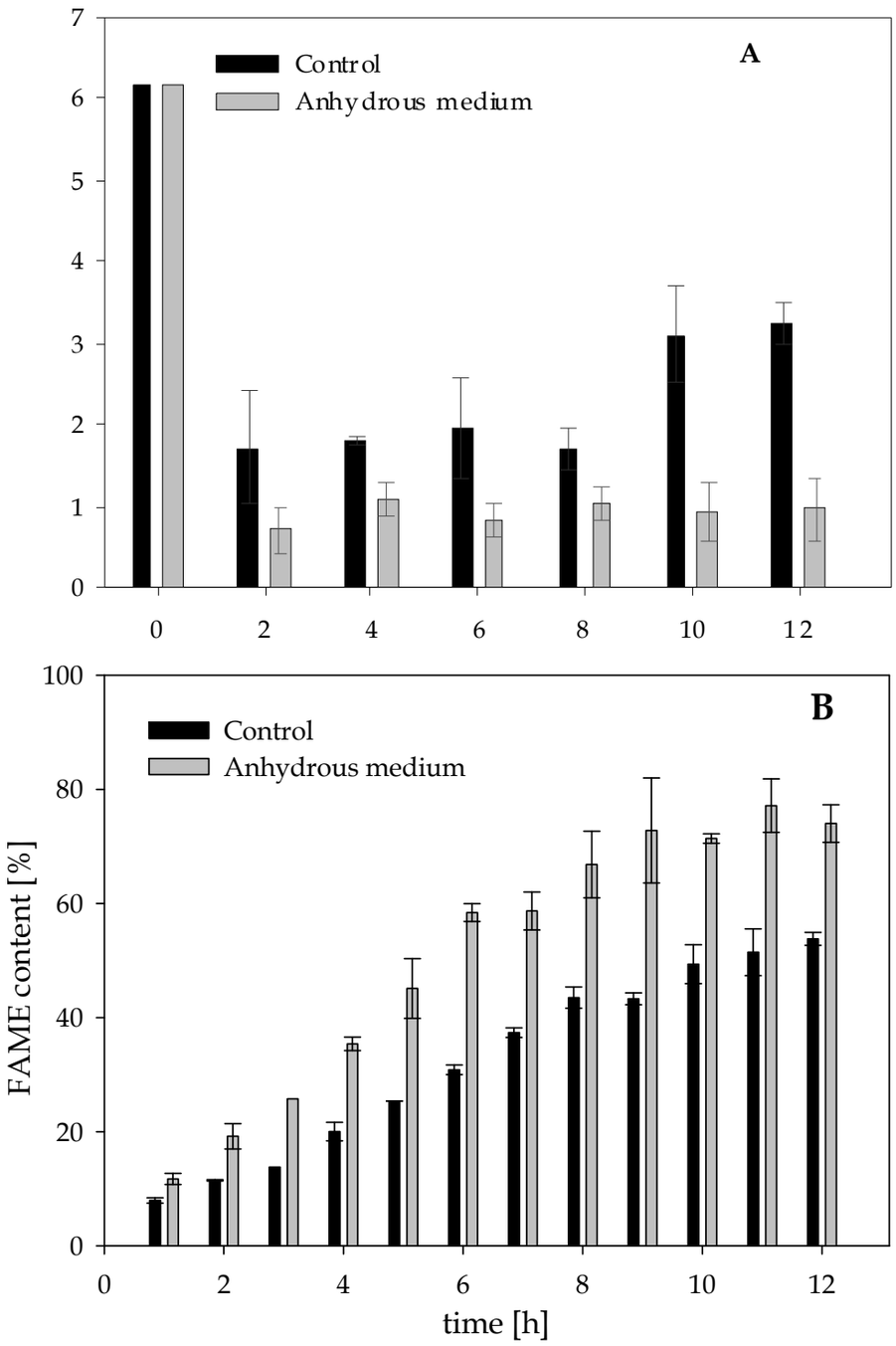


Fig. 4 Acid values (A) and FAME yield (B) during the reaction in an anhydrous medium with a methanol-to-oil ratio of 4:1 (mol/mol), 15% (wt) Novozym 435 (based on the weight of oil), 35 ^{o}C, 200 rpm and 12 hours of reaction time.

A reasonable explanation for the high FAME yield obtained may be related to the work of Cabrera et al. (2009). They suggest that lipase may exist in two different structural forms; in one form, the site of the lipase is isolated from the medium whereas in the other form, the active site is exposed to the reaction medium. In a homogeneous aqueous medium the lipase is in equilibrium between these two structures. In the case of Novozym 435, it is immobilized in acrylic resin with hydrophilic properties. Therefore, the higher yield in the reaction may occur because of interactions with the hydrophobic medium, where the lipase shifts towards the open structure form, increasing its activity. These conformational changes enable lipases to be greatly altered by controlled immobilization of the support material properties. This is in accordance with the results obtained by Samukawa et al. (2000) who established that preincubation of the enzyme in oil prior to the reaction improves the yield. This is because water adsorbed during the reaction prevented oil penetration, whereas this did not occur with the preincubated enzyme.

Treatments for enzyme reutilization in an anhydrous medium. The main advantage of using immobilized lipases is that the enzyme can be used repeatedly in a semi-continuous process. However, this objective is not always reached under the optimized conditions as short chain acyl acceptors can produce a loss of enzyme activity in successive reactions (Ognjanovic et al., 2009). In this study, the stability of the enzyme and treatments for its reutilization in an anhydrous medium were examined. Fig. 5 shows the results obtained by different treatments to maintain lipase activity after each FAME production reaction in an anhydrous medium. Fig. 5A shows the reutilization of Novozym 435 without treatment after reaction. According to the results obtained, FAME yield decreased considerably in the second cycle. This tendency was maintained in successive cycles, with values close to 0% of FAME yield in the fourth cycle. These results are in accordance with the findings of Ognjanovic et al. (2009), who reported 0% FAME yield in the fourth cycle using Novozym 435 and methanol as the acyl acceptor in an organic medium. Thus, although the activity of the enzyme increased in the first cycle using an anhydrous medium compared to the control (Fig. 4), the loss of activity seems to be similar in both types of medium when successive cycles are carried out (Fig. 5A). Lipases have shown high synthesis activity and stability in hydrophobic solvents, but alcohol and glycerol are immiscible in those solvents (Halim et al., 2008). This situation could produce poor solubilization of polar compounds in the medium, leading to their adsorption onto the lipases hydrophilic support and therefore provoking a low transesterification rate (Halim et al., 2008). So far, an alternative process is needed to allow the reuse of the enzyme achieving a low-cost process which can feasibly be implemented at an industrial scale.

The results of the first treatment studied for reusing the enzyme are shown in Fig. 5B. Applying an acetone washing step a higher FAME yield was achieved in the second cycle compared to the control (Fig. 5B and 5A, respectively). This result is caused by the fact that acetone is a hydrophilic solvent that could remove the glycerol and the methanol adsorbed onto the hydrophilic support material of the enzyme. However, in the third cycle the FAME yield diminished drastically (Fig. 5B). Acetone is a hydrophilic solvent with a very low log P (partition coefficient in a standard octanol-water two-phase system) value of -0.24. According to Yu et al. (2010) water has higher affinity to these hydrophilic solvents rather than to the enzyme. It has been reported that Novozym 435 can contain a

sufficient quantity of water to preserve its catalytic activity in an anhydrous medium (Ognjanovic et al., 2009; Tamalampudi et al., 2008). Therefore, the enzyme activity may be reduced when using an acetone washing step, because the enzyme might lose its flexibility conformation due to the lack of bound water (Yu et al., 2010). The enzyme washing step using WFO followed by overnight incubation in WFO was also studied (Fig. 5C). The activity of Novozym 435 was higher in the third cycle in comparison to the two previous alternatives. Samukawa et al. (2000) reported that Novozym 435 preincubated in methyl oleate and subsequently in soybean oil could enhance the rate of FAME production through impregnation of these compounds into the enzyme support material. In this study, it was assumed that the enzyme could contain methyl ester residues when incubated in WFO and therefore, the treatment should be similar to that carried out by Samukawa et al. (2000).

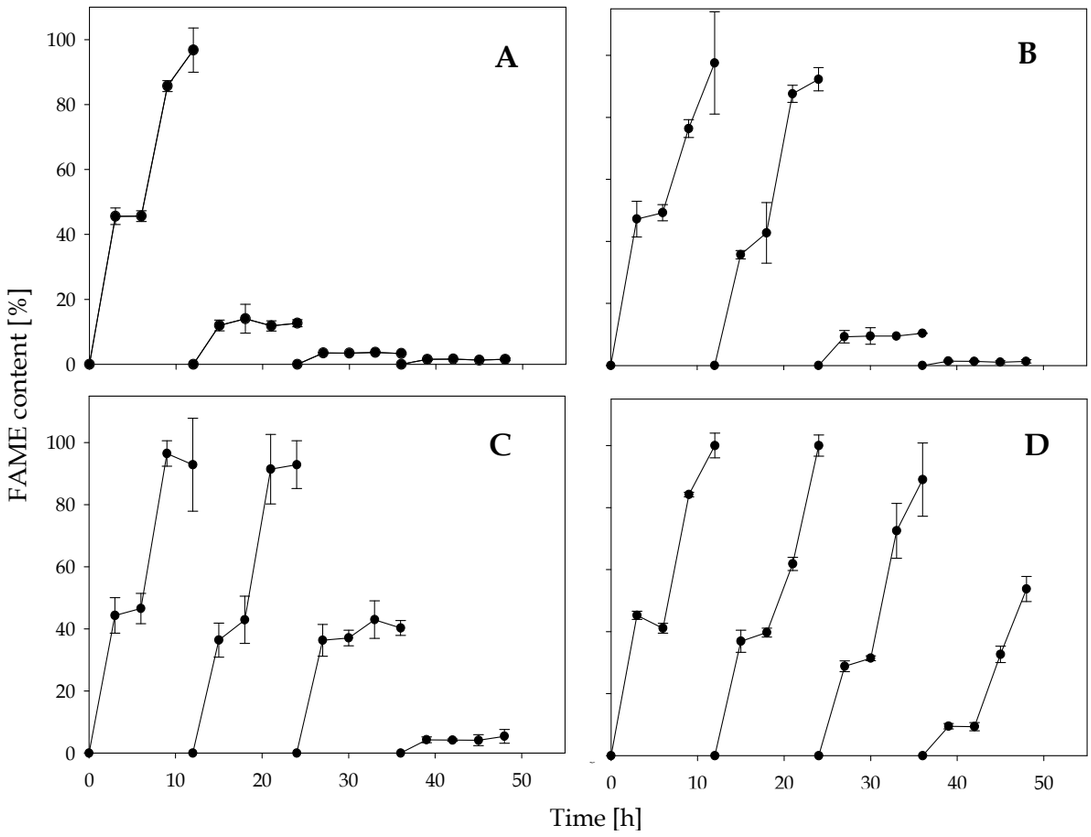


Fig. 5. Comparison of reutilization treatments of Novozym 435 after the reactions to FAME production with a methanol-to-oil ratio of 3.8:1 (mol/mol), 15% (wt) Novozym 435 (based on the weight of oil), 44.5 ºC, 200 rpm and 12 hours of reaction time. A) Control, B) Acetone washing, C) Waste frying oil washing D) *tert*-Butanol washing.

This treatment using WFO is advantageous compared to the acetone washing process because it is a cheaper and more environmental friendly process. In addition, the same WFO that was used for the incubation was used subsequently for the reaction of FAME

production, and therefore less equipment is needed to carry out this process. In another study, Chen and Wu (2003) achieved a FAME yield five times higher when the enzyme was incubated overnight in soybean oil. However, when they used the same oil in incubation experiments to recover the enzyme after the reaction, the activity began to decay after the fourth reutilization. Although the advantages of using WFO in enzyme recovery, FAME yield also declined in this study, as reported by Chen and Wu (2003). The reason may be the low solubility of alcohol and glycerol in the oil (hydrophobic wash), which impedes the efficient washing of the enzyme by oil.

As the washing process with hydrophobic and hydrophilic solvents did not allow an efficient recovery of the enzyme activity by more than three successive reactions in an anhydrous medium, a moderate polar solvent was also investigated. *Tert*-butanol, a tertiary alcohol with a log P value of 0.35 has been shown to improve enzyme activity more than linear alcohols. This fact could be attributed to the differences in miscibility with triglycerides, as compared to alcohols with the same carbon numbers, branched ones have better miscibility with triglycerides compared to linear isomers (Yu et al., 2010). Therefore, a washing treatment of the enzymes with *tert*-butanol, followed by incubation in WFO, was conducted. The results showed that this enzyme pretreatment achieved the best results, maintaining a higher activity over the time compared to the previous experiments (Fig. 5D). It is likely that *tert*-butanol recovered the enzyme activity because it has the advantages of both hydrophilic and hydrophobic solvents but none of the drawbacks. Therefore, *tert*-butanol should promote the removal of both glycerol and methanol from the lipase support material because of its hydrophilic properties, while its hydrophobic properties should help maintain a high level of lipase activity. This is in accordance to Chen and Wu (2003) who found that *tert*-butanol can be even used to regenerate a deactivated, immobilized enzyme such as Novozym 435. Therefore, *tert*-butanol is a promising alternative for enzyme recovery after reaction in an anhydrous medium, and is also highly stable and less reactive than other butanol isomers. Based on these results, a new experiment was carried out which included *tert*-butanol as a co-solvent in the reaction in an anhydrous medium.

Successive reactions to FAME production in an anhydrous medium using tert-butanol as a co-solvent. A previous study found that *tert*-butanol is inert in the methanolysis system, whereas it is also a potential co-solvent that could maintain enzymatic activity (Li et al., 2006). According to this, the performance of *tert*-butanol as a co-solvent was studied in an anhydrous medium to determine its ability to maintain Novozym 435 activity in successive reactions. In Fig. 6 is shown the successive reactions for FAME production that were carried out in an anhydrous medium under previously optimized operational conditions, using a previously selected dosage of *tert*-butanol (0.75% v/v, data not show). A control without *tert*-butanol as co-solvent was also carried out, showing that FAME yield was drastically reduced in the second cycle (< 20% FAME yield). The best results were obtained in the system using the co-solvent, where FAME yield was maintained over 50% after 17 cycles (Fig. 6). According to similar findings from other studies, the enzyme is inhibited throughout the reaction (Royon et al., 2007). The inhibitory effect at the beginning of the reaction is due to the presence of methanol which has poor miscibility in oil. Subsequently, once methanol concentration decreases, inhibition is caused by a glycerol layer coating the

catalyst. Therefore, the positive effect of *tert*-butanol in the reaction could be first related to the increment in the oil-methanol miscibility, preventing direct contact between enzyme and alcohol at the beginning of the reaction and improving the reaction yield. Secondly, due to *tert*-butanol's hydrophilic properties, it is able to dissolve both methanol and glycerol during the reaction, preventing enzyme inhibition throughout the reaction. Thirdly, *tert*-butanol's hydrophobic properties maintained the high lipase activity through the successive reactions.

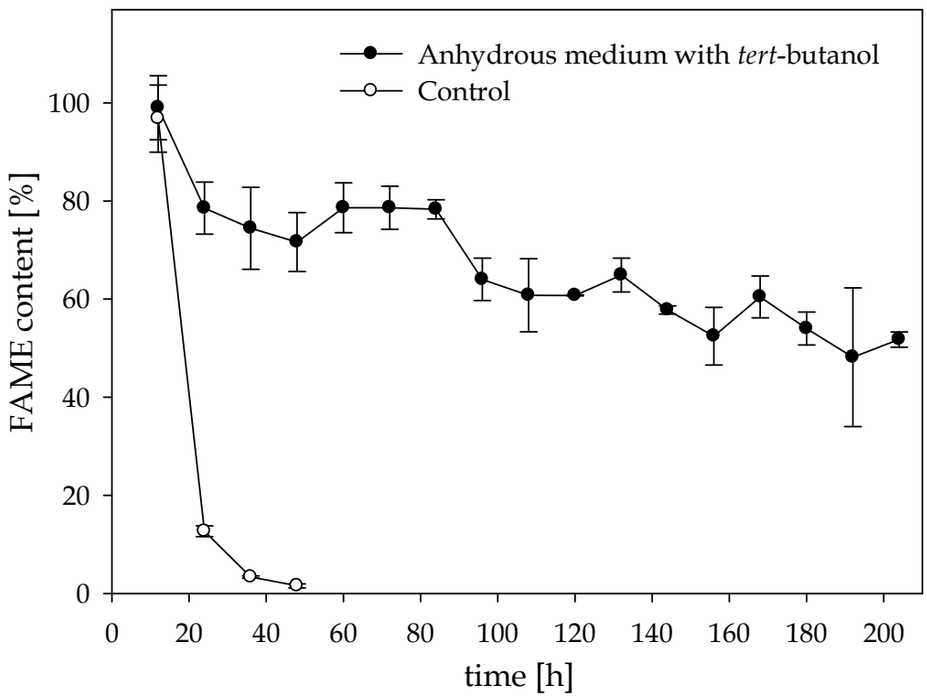


Fig. 6. Time course of FAME yield during 17 batch reaction cycles with a methanol-to-oil ratio of 3.8:1 (mol/mol), 15% (wt) Novozym 435 (based on the weight of oil), 44.5 ºC, 0.75% v/v of *tert*-butanol, 0.9 g of molecular sieves, 200 rpm and 12 hours of reaction time. Symbols: in *tert*- butanol system (closed circles), control (open circles).

According to Yu et al. (2010), different properties such as viscosity, dielectric constant, solubility parameters and log P should be considered when choosing a co-solvent to improve enzyme performance in biodiesel production. Another aspect which should be considered is the feasibility of recovering the co-solvent through distillation, which is related to the boiling point. In this sense, *tert*-butanol has advantages compared to others co-solvents reported, such as amyl alcohol, which has very similar properties compared to the *tert*-butanol but a higher boiling point (102 °C) close to the boiling point of the water. As the boiling point of *tert*-butanol is 82 °C and the distillation of methanol occurs at 65 °C, the recovery of *tert*-butanol should not increase the amount of energy spent that much in comparison to the advantages of using this co-solvent. Therefore, employing *tert*-butanol as a co-solvent could allow the use of an anhydrous medium as an industrial alternative for FAME production using Novozym 435 as biocatalyst.

The results of this section showed that the use of an anhydrous medium (resulting from water extraction by using molecular sieves), FAME yield was improved by avoiding hydrolysis and esterification reactions, producing FAME mainly through transesterification of TG. The enzyme activity cannot be recovered successively neither using a hydrophilic washing step with acetone nor by hydrophobic washing with WFO. However, 17 successive cycles of FAME production using *tert*-butanol as a moderate polar co-solvent, show that Novozym 435 can be reused in anhydrous medium. These results also show that the anhydrous medium could enable the implementation of lipase-catalyzed processes on an industrial scale for biodiesel production mainly by transesterification reaction. Different raw materials could be used for this, achieving properties close to the norm and potentially avoiding post-treatments to refine the produced biodiesel.

4. Enzymatic transesterification reaction kinetic in a *tert*-butanol system for biodiesel production

For the design of suitable reactors to FAME production using lipases, kinetic information of the rate of product formation and the effects of changes in system conditions is needed. Investigation about kinetic of FAME production using different lipases has been recently reported. The first researches were focused in the esterification of FFA, where the Ping Pong model with competitive inhibition by methanol was used to describe the reaction (Krishna et al., 2001). After, Al-Zuhair et al. (2007) proposed a kinetic model based in the transesterification of TG. The aim of this work was study the kinetic of the transesterification to FAME production catalyzed by Novozym 435. The investigation was carried out in anhydrous medium, using WFO as raw material, methanol as acyl acceptor and *tert*-butanol as co-solvent. In addition, the set-up of a semi-continuous enzymatic bioreactor was carried out. To realize the study Ping Pong model with competitive inhibition by methanol was used (Eq. 4).

$$\upsilon = \frac{V_{\max}}{1 + \frac{K_W}{[W]}\left[1 + \frac{[M]}{K_{IM}}\right] + \frac{K_M}{[M]}} \tag{4}$$

Where υ (mol/L/min) is the initial reaction rate, $V_{máx}$ is the maximum rate of reaction (mol/L/min), K_W and K_M are the binding constants for the WFO (W) and the methanol (M) (mol/L), [W] is WFO concentration (mol/L), [M] is methanol concentration (mol/L) and K_{IM} is the inhibition constant for the methanol (mol/L).

To determinate the sole effect of methanol in the transesterification, the experiments was run using methanol concentrations in the range of 100-3000 mol/L, equivalent at methanol to oil molar ratio 0.6-15 (mol/mol), at a constant WFO concentration of 300 mol/L. The WFO concentration was chosen to ensure operating outside WFO limitation or inhibition region. To determinate the sole effect of WFO in the transesterification , the experiments was run using WFO concentrations in the range of 200-350 mol/L, at a constant methanol concentration of 1400 mol/L. The methanol concentration was chosen to ensure operating outside alcohol limitation or inhibition region. Michaelis-menten kinetic was used to estimate the kinetic constants Vmáx, K_W y K_M. Subsequently, the values were optimized and

the kinetic constant K_{IM} was obtained using Excel solver to find the minimum objective function that compares the measured rate of the reaction with those predicted by the proposed kinetic equation. Each assay were performed in quadruplicate in Erlenmeyer flask of 25mL and incubated in an orbital shaker during 4 hr under the same operational condition: 15% (wt) of Novozym 435, 44°C, 0.75% (V/V) of *tert*-butanol, 0.5 g of molecular sieves and 200 rpm. In order to remove the *tert*-butanol, the samples were centrifuged and heated at 85°C during 30 minutes. The supernatant was used to quantify FAME yield using GC-MS methods.

Using the experimental results the kinetic parameters estimated for the model were: $V_{máx}$= 0.018 mol/L/min, $K_{M,\ metanol}$ = 1030 mol/L, $K_{W,\ WFO}$= 397 mol/L y $K_{IM,\ metanol}$= 1,815 mol/L. The Fig. 7 shows a comparison between the model predictions and the experimental data of the initial rate of reaction at different methanol concentration using Novozym 435.

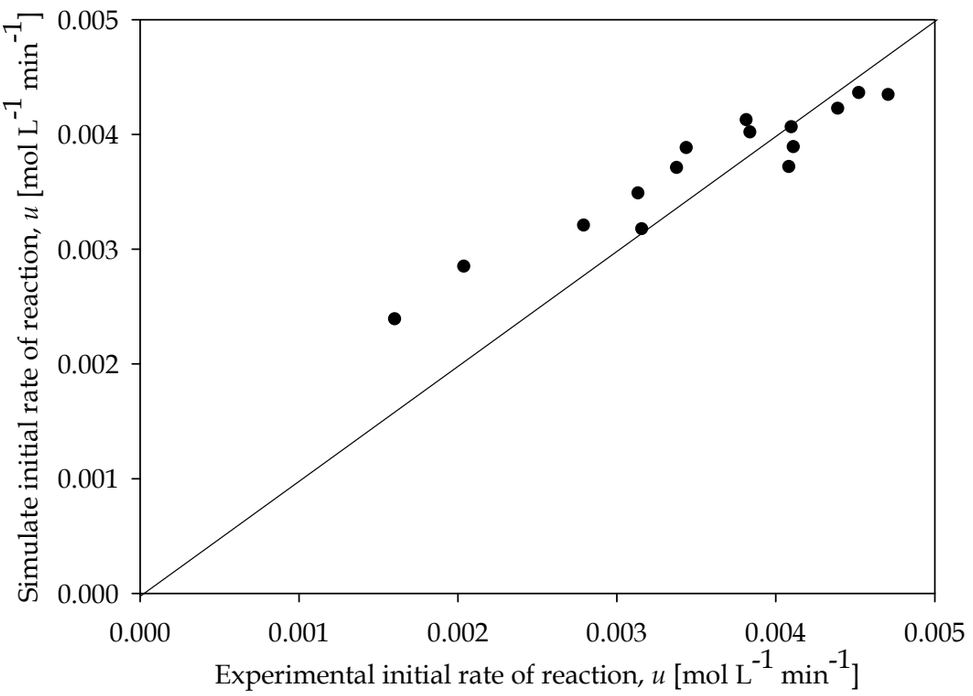


Fig. 7. Comparison between the experimental results and the Ping-Pong kinetic model equation with the estimated constants to different initial methanol concentration and initial WFO concentration of 300 mol L^{-1}.

According to Fig.7 the kinetic model is suitable to predict the behavior of the reaction, indicating that the use Michaelis-Mentel kinectic could simplify the calculation of kinectic constant of complex model such as Ping Pong kinetic model. The model and empirical data showed that Novozym 435 presented a low inhibition by methanol, even at higher concentration maintaining a high initial reaction rate until a methanol to WFO molar ratio of 8/1 (mol/mol). The model predicts a moderate decrease in the initial reaction rate for higher concentration of methanol. This behavior could be associated to the incorporation of the co-solvent in the reaction due its capacity to improve the miscibility of reaction mix. This is an advantage because higher concentration of methanol favor the production formation since the transesterification is an equilibrium reaction, decreasing the operation time of the FAME production using enzymatic catalysts.

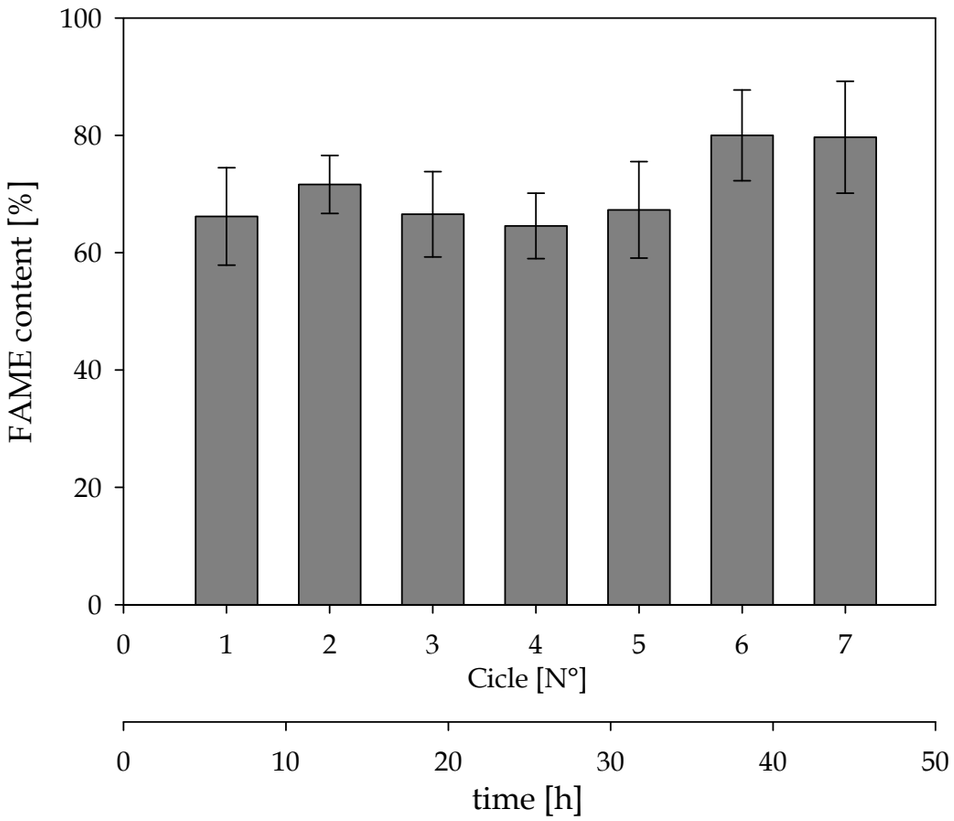


Fig. 8. FAME yield during the set-up of a semi-continuous enzymatic reactor in anhydrous medium with enzymes reutilization. Operational conditions: methanol-to-oil ratio of 8/1 (mol/mol), 15% (wt) Novozym 435 (based on the weight of oil), 44.5 ºC, 0.75% v/v of *tert*-butanol, 200 rpm and 4 hours of reaction time.

The results of the kinetic study were applied in the operation of a semi-continuous reactor to enzymatic FAME production. The bioreactor was made of glass with a volume of 0.5 L. The temperature was controller by a thermostat. The agitation of the bioreactor was supply by a magnetic stirrer. In order to extract water constantly from media, a glass column filled with molecular sieves was connected to the bioreactor, where the media was recycling using a peristaltic pump. The glycerol was removed from bottom by a settler connected to the bioreactor. The operational conditions to each reaction cycle were: methanol to oil molar ratio 8/1 (mol/mol), 15 % (wt) Novozym 435, 0.75% (v/v) of *tert*-butanol, 44.5 °C, 200 rpm and 4 hours of reaction time. The enzymes were reused successively remaining into the reactor during all the cycles. In Fig. 9 are showed the results obtained.

The bioreactor was operated several cycles of charge and discharge during 30 h, adding only raw material and changing the molecular sieves to maintain an anhydrous media. Under these conditions was possible to maintain FAME yields over 80% during 7 reaction cycles (Fig. 8). Therefore, the use of both an anhydrous media and *tert*-butanol as co-solvent are effective strategies for the implementation of a semi-continuous enzymatic process that can become economically competitive with traditional chemical catalysts to produce FAME.

5. Conclusions

The short reaction time of the proposed process and the reuse of the enzymes generate a feasible alternative to be implemented at industrial scale. This process has several advantages compared to the chemical process. Although the chemical process requires only 1 hour of reaction time, after the process a washing step to remove catalyst residues is necessary. This washing step is not necessary in the enzymatic process because Novozym 435 is a solid catalyst. In addition, the enzymatic process has the advantage to be flexible allowing the use of alternative and low cost raw materials. Therefore, the results obtained in this work have generated an enzymatic process that could become not only environmentally friendly but also economically competitive with the chemical-catalyzed process.

The kinetic study and the reactor operation showed that Novozym 435 was not inhibited to high methanol concentrations and could be reused without significant loss of activity. These results shown that under the conditions investigated enzymatic FAME production could be a competitive process to be implemented to industrial scale. To reach this objective is necessary to carry on the bioreactor operation in the long time, in order to establish the lifespan of the enzymes. In addition is necessary to establish a molecular sieves recuperation protocol and to looking for inexpensive material such as zeolites to decrease the operational cost.

6. Acknowledgement

Research described was supported by Desert Bioenergy S.A., Chilean project “Inserción de Capital Humano Avanzado en el Sector Productivo Chileno 78110106”, Chilean Fondecyt project 3120171, Chilean CONICYT Project 79090009 and FONDECYT iniciación project 11110282.

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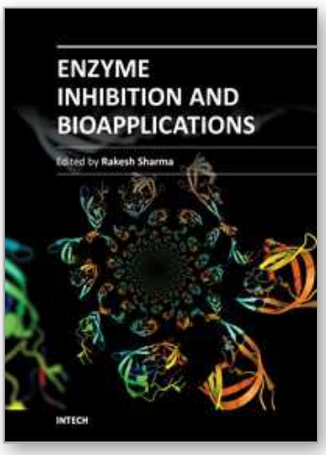
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Enzyme Inhibition and Bioapplications

Edited by Prof. Rakesh Sharma

ISBN 978-953-51-0585-5
Hard cover, 314 pages
Publisher InTech
Published online 09, May, 2012
Published in print edition May, 2012

Enzyme Inhibition and Bioapplications is a concise book on applied methods of enzymes used in drug testing. The present volume will serve the purpose of applied drug evaluation methods in research projects, as well as relatively experienced enzyme scientists who might wish to develop their experiments further. Chapters are arranged in the order of basic concepts of enzyme inhibition and physiological basis of cytochromes followed by new concepts of applied drug therapy; reliability analysis; and new enzyme applications from mechanistic point of view.

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Laura Azócar, Gustavo Ciudad, Robinson Muñoz, David Jeison, Claudio Toro and Rodrigo Navia (2012). Feasible Novozym 435-Catalyzed Process to Fatty Acid Methyl Ester Production from Waste Frying Oil: Role of Lipase Inhibition, Enzyme Inhibition and Bioapplications, Prof. Rakesh Sharma (Ed.), ISBN: 978-953-51-0585-5, InTech, Available from: http://www.intechopen.com/books/enzyme-inhibition-and-bioapplications/feasible-novozym-435-catalyzed-process-to-fatty-acid-methyl-ester-production-from-waste-frying-oil-b

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