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ORIGINAL ARTICLE

Study of immobilized candida rugosa lipase for biodiesel fuel production from palm oil by flow microcalorimetry

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Candida Rugosa;
Immersion calorimetry

Abstract Enzymatic transesterification of palm oil with methanol and ethanol was studied. Of the four lipases that were tested in the initial screening, lipase Candida Rugosa (CR) resulted in the highest yield of mono alkyl esters. Lipase CR was further investigated in immobilized form within an activated carbon as support. The activated carbon was prepared by activation physical. Using the immobilized lipase CR, the effects of water and alcohol concentration, enzyme loading and enzyme thermal stability in the transesterification reaction were investigated. The optimal conditions for processing 50 g of palm oil were: 37 °C, 1:14.5 oil/methanol molar ratio, 1.0 g water and 500 mg lipase for the reactions with methanol, 35 °C, 1:15.0 oil/ethanol molar ratio, 1.0 g water, 500 mg lipase for the reactions with ethanol, and 35 °C, 1:10.0 oil/*n*-butanol molar ratio, 1.0 g water, 500 mg lipase for the reactions with ethanol. Subject to the optimal conditions, methyl and ethyl esters formation of 70 and 85 mol% in 1 h of reaction were obtained for the immobilized enzyme reactions. The flow microcalorimetry is an important and novel techniques is used in evaluation of biodiesel production.

1. Introduction

Vegetable oils have attracted attention as a potential renewable source for the production of an alternative source of fuel for petroleum based diesel due to continuous huge world-wide consumption of petroleum reserves and environmental consequences of exhaust gases from diesel engines. Methyl and ethyl esters of fatty acids, also known as biodiesel, are considered nontoxic, biodegradable, and an excellent replacement for petroleum diesel. Biodiesel's cetane number, energy content, viscosity and other diesel characteristics are also similar to those of petroleum-based diesel fuel (Mittelback and Tritthart,

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1988). Moreover, biodiesel is essentially sulfur free and is considered ultra-sulfur fuel quality and the diesel engines fueled by biodiesel emit significantly fewer particulates, hydrocarbons, and carbon monoxide, carbon dioxide than those operating on conventional fossil fuel diesel. Emissions of NOx, however, are slightly higher than those of diesel engines operating on conventional diesel fuels (Schumacher et al., 1996; Ali et al., 1995). Transesterification of vegetable oils and animal fats with significant free FFA (free fatty acids) contents are regarded a well established industrial process for the production of fatty acid alkyl esters also known as biodiesel. Biodiesel is well miscible with diesel and reduces significantly air pollution emission including sulfur and currently produces commercially in significant amounts in Europe, USA and some Asian countries all over the world using renewable oil feedstock sources. For the product and process development of biodiesel, enzymatic transesterification has been suggested to produce a high purity product with an economic, environment friendly process at mild reaction conditions. The enzyme cost being the main hurdle can be overcome by immobilization. Immobilized enzyme, which has been successfully used in various fields over the soluble counterpart, could be employed in biodiesel production with the aim of reducing the production cost by reusing the enzyme. The conventional biodiesel technology involves the use of an inorganic base or acid catalyst at or near the boiling temperatures of the triglyceride/alcohol mixture. The removal of catalyst is through neutralization and eventual separation of salt from the product esters, which is difficult to achieve. Moreover, the physiochemical synthesis schemes often result in poor reaction selectivity and may lead to undesirable side reactions. Enzymatic conversion of triglycerides has been suggested as a realistic alternative to the conventional physiochemical methods (give the relative reference). Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional process. However, the high cost of the enzymes often makes the enzymatic processes economically unattractive. The key step in enzymatic process lies in successful immobilization of the enzyme, which will allow for its recovery and reuse (Balcao et al., 1996). Immobilization is the most widely used method for achieving stability in lipases and to make them more attractive for industrial use (Cowan, 1996; Clark, 1994; Kawakami and Yoshida, 1996). Common immobilization techniques include physical adsorption onto a solid support (e.g. Bosley and Pielow, 1997), covalent bonding to a solid support (e.g. Walt and Agayn, 1994) and physical entrapment within a polymer matrix support (e.g. Pizarro et al.,1997). Entrapment of lipase entails capture of the lipase within a matrix of a polymer (Hartmeier, 1985). The immobilized lipase by entrapment is much more stable than physically adsorbed lipase and unlike the covalent bonding method, this method uses a relatively simple procedure and at the same time the immobilized lipase maintains its activity and stability (Kennedy and Melo, 1990). A variety of methods have been used for trapping lipases in a polymer matrix (Bickerstaff, 1997). Entrapment of enzymes in an inorganic polymer matrix is one method that has received a considerable attention in recent years. This method which was pioneered by Avnir et al. (1994) is based on sol–gel process. The application of the sol–gel material in the immobilization of lipases is well documented (e.g. Reetz, 1997). A substantial collection of research on the enzymatic transesterification of triglycerides has focused on free enzyme reactions with and without organic solvents. Nelson et al. (1996) studied the lipase-catalyzed transesterification of triglycerides in hexane, using different lipases and a variety of triglycerides and alcohols. The lipase from Mucor miehi (Lipozyme IM-20) was found to be the most effective in converting tallow oil to their respective mono alkyl esters with primary alcohols, whereas the lipase from Candida antarctica was found to be most suitable for reacting with secondary alcohols, giving branched alkyl esters. Wu et al. (1999) employed response surface methodology (RSM) to optimize reaction parameters such as temperature, time, level of lipase, and molar ratio of reactants in the Pseudomonas cepacia lipase and Candida antarctica lipase catalyzed transesterification reaction of restaurant grease to mono alkyl ethylesters using 95% of ethanol. Results showed a synergistic effect when the two lipases were used in sequence which surpassed the RSM predictions. Abigor et al. (2000) studied the lipase-catalyzed transesterification of two Nigerian lauric oils, palm kernel oil and coconut oil, by transesterification of oils with different alcohols using PS-30 lipase as catalyst. In the conversion of palm kernel oil to mono alkyl esters, ethanol resulted in the highest conversion of 72%. Other alcohols tested and their corresponding transesterification conversions included: *t*-butanol 62%, 1-butanol 42%, *n*-propanol 42%, and iso-propanol 24%, while only 15% methylesters was observed using methanol. Kaieda et al. (2001) studied the effect of methanol and water concentrations on the methanolysis of soybean oil using different lipases in a solvent free system. Lipase from Pseudomonas cepacia showed the greatest methanol resistance among the tested lipases. This lipase also exhibited the highest catalytic activity toward the transesterification reaction, even in the absence of water, methylester content reached 32% after 50 h of reaction. Cao et al. (1992) reported the esterification and transesterification reactions catalyzed by Porcine pancreatic lipase adsorbed on glass, acetone precipitated on porous glass, kieselghur, aluminum oxide and agar beads in organic solvents. Results showed the lipase adsorbed on kieselguhr and agar beads with the highest activity. Shimada et al. (1999) and Watanabe et al. (2000) used immobilized Candida antarctica (Novozyme 435) for the conversion of vegetable oil to biodiesel. Results showed incomplete methanolysis of vegetable oil which was attributed to the inactivation of the enzyme. Stepwise addition of methanol prevented this inactivation and conversions in excess of 98% were obtained. Samukawa et al. (2000) investigated the effect of the preincubation of immobilized Candida antarctica lipase (Novozyme 435) in methyloleate and soybean oil prior to the biodiesel production. Results indicated a much faster rate of methanolysis for the preincubated lipase. Hsu et al. (2001) developed a novel procedure for the immobilization of lipase from Pseudomonas cepacia within a phyllosilicate sol–gel matrix. In this process phyllosilicate clay saturated with sodium ions was suspended in water and then exchanged with alkylammonium ions by the addition of cetyltrimethyl ammonium chloride. This mixture was then used in the entrapment of Pseudomonas cepacia with tetramethoxysilane (TMOS) as the polymerization precursor. The immobilized enzyme so prepared was then used in the transesterification of tallow and grease where conversions in excess of 95% were reached. In the present study, lipase PS from candida rugosa was entrapped within an activated carbon matrix, prepared by lignocellulosic waste. The immobilized enzyme (Candida Rugosa) was used in the transesterification of palm oil with

methanol and ethanol. In addition to this, we proposed a study of microcalorimetric as a technique alternative for analysis of process of transesterification.

2. Methods

2.1. Materials

All lipases were purchased by Amano Enzyme (Nagoya, Japan), and Meito Sangyo (Nagoya, Japan). Palm oil was purchased to Federación Nacional de Cultivadores de Aceite de Palma (Colombia). Methanol, ethanol and *n*-butanol were purchased from Fisher Scientific (Pittsburgh, PA). Bis-(trimethylsilyl) trifluroacetamide (BSTFA, derivative grade), 1,2,3-tridecanoylglycerol (tricaprine, 95%) and pyridine. Hexane (GC grade) was purchased from EM Science (Gibbstown, NJ). Tetramethoxysilane (TMOS, 95%), iso-butyltrimethoxysilane (iso-BTMS, 97%), and sodium fluoride (NaF) were purchased from the Aldrich Chemical Company (Milwaukee, WI).

2.2. Lipase immobilization

A specified amount of lipase Candida Rugosa (optimally 1 g) was measured into a flask and 10 ml of water was added. The mixture was stirred at 200 rpm using a magnetic stirrer for about 15 min. To this mixture, 0.5 ml of a 0.5 M NaF solution and the activated carbon was added. The flask was removed from the stirrer and left sealed at room temperature for 24 h. The flask was then uncapped and was incubated in a water bath at 37 °C for about 48 h. The powder was washed with 100 ml of distilled water in a 250 ml flask for 1 h at a mixing speed of 500 rpm. The mixture was then filtered. The activated carbon with the lipases was dried again at 37 °C for 48 h. The granular carbon was stored at 4 °C until use. Based on the degree of immobilization tests, about 95% of the enzyme was immobilized in this procedure. The actual enzyme loading was determined at 500 mg of lipase Candida Rugosa 10 g of activated carbon.

2.3. Reaction setup and optimization

The reactions were carried out in a constant temperature water bath, under which a Barnstead Thermolyne Haake DC10-W46/B was placed to agitate the reaction mixture. Water was circulated into the bath/circulator which, via an external probe, was able to control the temperature of the bath to within ±0.01 °C. A standard set of conditions was used as the baseline in the optimization studies. These conditions were recommended by Noureddini et al. (2002) in the literature. The initial conditions were 10 g palm oil, 1 g methanol (methanol to oil molar ratio was 5.0), 1.0 g water, 5 g immobilized lipase Candida Rugosa, 37 °C, 800 rpm, and 1 h reaction time. In the reactions with ethanol an *n*-butanol, too, 1.0 g of water and 1 g of ethanol and/or *n*-butanol (ethanol and *n*-butanol to oil molar ratio too 5.0) were used under otherwise identical conditions. In the optimization studies, also the reaction parameters were maintained at the same time. For example, when the effect of molar ratio was investigated, the rest of the reaction conditions were maintained at: 10 g palm oil, 1.0 g methanol, 1.0 g water, 5 g immobilized lipase Candida Rugosa I (CR), 800 rpm, and 1 h reaction time. Lipase Candida Rugosa was used in all of the optimization studies.

2.4. Degree of immobilization tests

The immobilized enzyme was washed with water and after filtration; about 100 ± 5 ml of supernatant was collected. This supernatant may potentially contain free enzyme, partially hydrolyzed precursors, alcohols, and soluble oligomers. To quantify the amount of enzyme in the supernatant, a calibration curve relating the formation of free fatty acids as a function of free enzyme loading was constructed, which was based on the hydrolysis of soybean oil. Details about this work may be found elsewhere (Noureddini et al., 2002, 2005). By comparing the supernatant from the immobilization wash procedure with this calibration, the amount of enzyme in the supernatant and the degree of immobilization was determined. In order to model the exact reaction conditions, in the calibration studies, a blank gel with no enzyme was prepared. The supernatant, which was collected from washing this gel, was used as the reaction medium in the calibration experiments. Otherwise, the reaction procedure for the calibration experiments was identical to the free enzyme hydrolysis reactions. The enzyme loading was varied in the range of 0.3–30 mg lipase per 1 g of palm oil for this calibration. The calibration curve showed a monotonically increasing activity as the enzyme loading was increased. The activity of the enzyme leveled off beyond the upper limit of 40 mg of enzyme per 1 g of palm oil.

2.5. Sampling and analysis

Samples were 0.8–2.0 ml in volume and were collected in 5 ml sample vials. Samples from the reactions were initially heated to ensure enzyme denaturation, freeze dried to remove excess water and alcohol, and finally derivatized with BSTFA. The silylating agent reacts with the carboxyl groups of fatty acids and results in trimethylsilylated fatty acids which are readily separated and quantified. The derivatized samples were then analyzed by a gas chromatograph (GC) to determine the concentration of fatty acid esters, free fatty acids, mono, di, and triglycerides. A Shimadzu (United States) 2010 Series GC system was used. This equipment was equipped with a column suitable that was designed specially for this work. Sample volumes were 1.5 μL, the carrier gas was helium, and the GC was operated in constant flow mode with a flow rate of 10.0 ml/min. A split injector was used with a split ratio of 10:1 and a temperature of 350 °C. The FID detector was operated at 370 °C and used a helium makeup flow to maintain a constant detector flow of 30.0 ml/min. The oven was initially held at 90.0 °C and was then elevate to 200 °C at 20.0 °C/min, to 300 °C at 2.0 °C/min, and finally to 350 °C at 10.0 °C/min. The oven was held at this temperature for 32.65 min before returning to 90.0 °C. Total run time for this method was 64.0 min. Calibration of the GC method was carried out by analyzing standard solutions of mixed glycerol, fatty acid esters, free fatty acids, mono-, di-, and triglycerides. The standards were derivatized in the same fashion as the reaction samples. More details about the sample preparations and analysis procedures are explained elsewhere (Wagner, 1999).

2.6. Data analysis

Experimental results are presented in Figs. 1–6. In these figures, the activity of the lipases is presented by the formation of esters and other reaction products in terms of molar percent of the components. The data presented in Figs. 1–6 were replicated at least five times. The mean values for the replicated data not are presented in the graphs. In Figs. 1–6, the standard deviations for fatty acids were approximately ±2% (this not show in the graphics) of the mean values and are not shown in the figures. The data was analyzed by Statistica Software®.

2.7. Determination of production biodiesel with flow microcalorimetry

For these measurement a micromacalorimeter was employed which was constructed in our Laboratory (Giraldo et al., 1998). Fig. 7 shows a general scheme of the flow microcalorimeter that has been built. The apparatus consists of:

1. An outer heat sink (A) which is an aluminum cylinder (150 mm diameter by 220 mm long) with an inner hole (75 mm diameter) to accommodate the calorimetric units. The cylinder has been cut horizontally so that one half serves as lid, as shown in the figure.
2. Two inner heat sinks (B), each of which consists of two aluminum cylinders (75 mm diameter by 25 mm long) that contain two thermopiles and the reaction vessel. The whole set is held in place by three stainless steel screws.
3. The thermopiles (Tellurex Corporation, 42 mm by 42 mm by 3.8 mm) (C) work at a maximum intensity of 6 A and a maximum potential of 15.5 V.

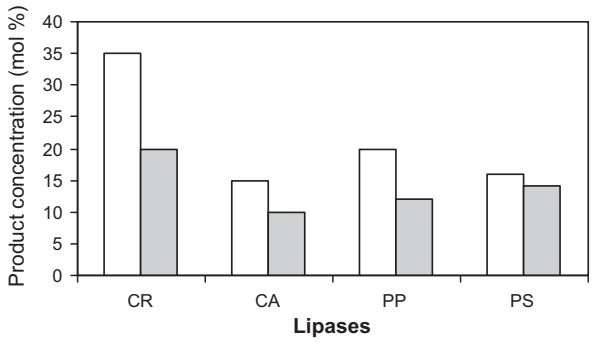

Figure 1 Lipase screening on transesterification of palm oil, a loading 300 mg free lipase, 1.0 g methanol, 1.0 g water, 10 g palm oil, a stirring rate of 800 rpm and a 1-h reaction at 37 °C. (□) Methylesters and (■) free fatty acids.

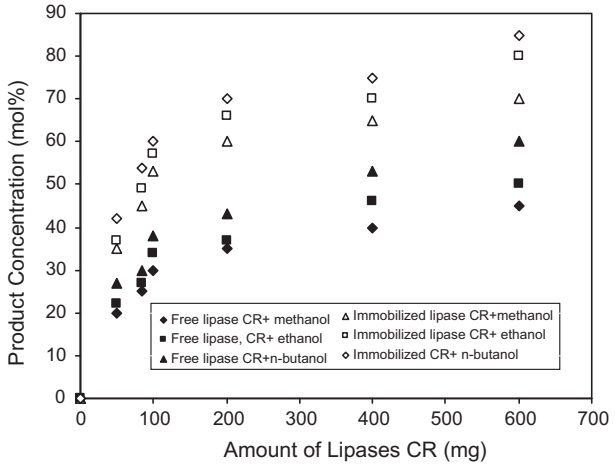

Figure 2 Effect of enzyme loading on transesterification of palm oil 1.0 g water, 10 g palm oil, a stirring rate of 800 rpm and a 1 h reaction at 37 °C. (♦) Free lipase CR, 1 g of methanol. (Δ) Immobilized lipase CR, 1.0 g of methanol. (■) Free lipase CR, 1 g of ethanol (□) Immobilized lipase CR, 1 g of ethanol. (▲) Free lipase CR, 1 g of *n*-butanol (◇) Immobilized lipase CR, 1 g of *n*-butanol.

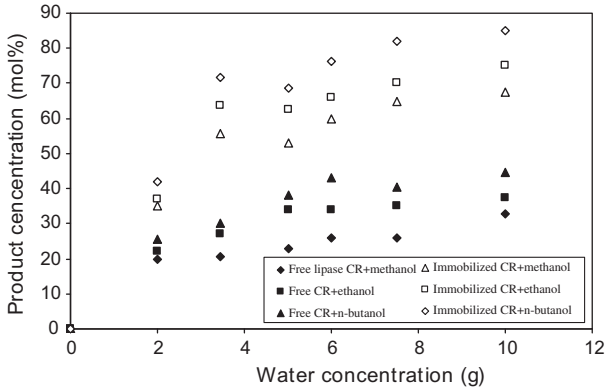

Figure 3 Effect of water concentration on immobilized lipase catalyzed transesterification of palm oil, subject to a loading of 10 of activated carbon and palm oil, a stirring rate of 800 rpm and a 1 h reaction at 37 °C. (♦) Free lipase CR, 1 g of methanol. (Δ) Immobilized lipase CR, 1.0 g of methanol. (■) Free lipase CR, 1 g of ethanol (□) Immobilized lipase CR, 1 g of ethanol. (▲) Free lipase CR, 1 g of *n*-butanol (◇) Immobilized lipase CR, 1 g of *n*-butanol.

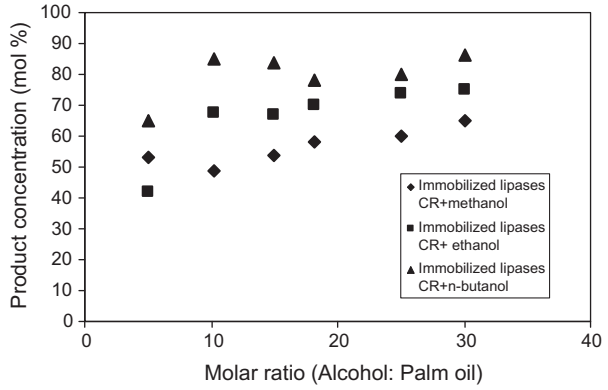

Figure 4 Effect of alcohol concentration on immobilized lipase catalyzed transesterification of palm oil, subject to a loading of 10 g activated of palm oil, a stirring rate of 800 rpm and a 1 h reaction at 37 °C. (♦) Immobilized lipase CR, 1.0 g of methanol. (■) Immobilized lipase CR, 1 g of ethanol. (▲) Immobilized lipase CR, 1 g of *n*-butanol.

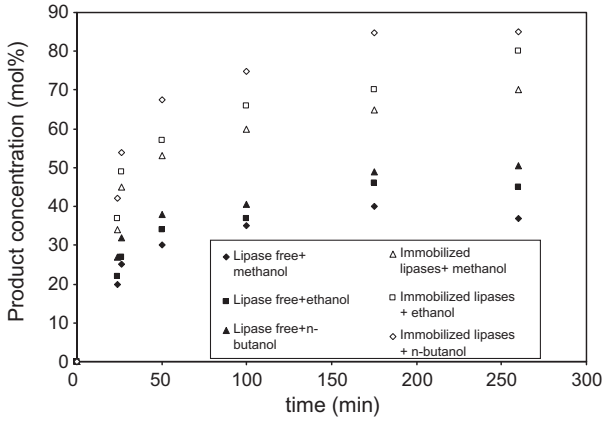

Figure 5 Time course of the transesterification of palm oil, subject to a loading of 10 g of activated carbon, a stirring rate of 800 rpm for at 37 °C. (♦) Free lipase CR, 1 g of methanol. (Δ) Immobilized lipase CR, 1.0 g of methanol. (■) Free lipase CR, 1 g of ethanol (□) Immobilized lipase CR, 1 g of ethanol. (▲) Free lipase CR, 1 g of *n*-butanol (◇) Immobilized lipase CR, 1 g of *n*-butanol.

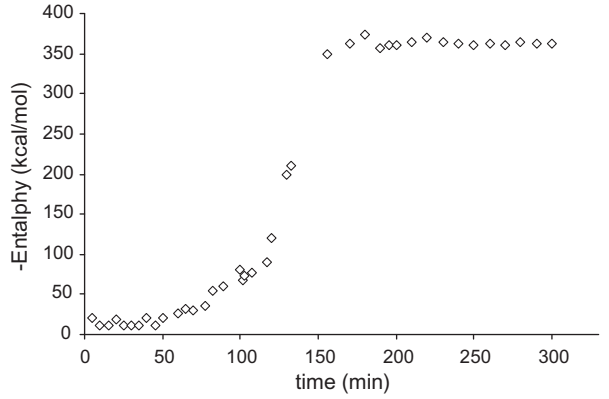

Figure 6 Enthalpy development in the production of biodiesel.

4. The reference and reaction calorimetric cells (D) are accommodated in aluminum containers that allow a good thermal contact with the thermopiles. Two different reaction vessels were constructed; one is a gold spiral tube (0.2 mm inner diameter) placed inside aluminum parallel plates; a very good thermal contact is assured by filling the empty space with aluminum powder. The other is a silver plate (43 mm by 43 mm by 1.8 mm) with an engraved channel that accommodates a silver tube (0.2 mm diameter). The reference cell differs with the reaction cell in that the former has only one tube for the entrance of liquids. The silver reference cell is identical to the reaction cell. The calibration resistance (99.9 Ω and 0.125 W) resides within the body of each reaction cell. The inner heat sink, the thermopiles, and the flow reaction cell constitute a calorimetric unit. In order to eliminate external heat effects, a similar calorimetric unit (with the reference cell) is connected opposite to the previously described. This twin arrangement is frequently used in microcalorimetry. The heat exchanger (E) is a tin cylinder (75 mm diameter by 60 mm long) that contains two concentric stainless steel spirals through which the calorimetric liquids are driven. The function of the heat exchanger is to minimize the temperature gradients between the solutions, as well as to equilibrate their temperature with that of the reaction cell. The equipment is placed in a chrome steel box hermetically sealed immersed in a water thermostat (75 L capacity) maintained at 25.000 ± 0.005 °C. The whole arrangement is located in a room kept at 25.0 ± 0.2 °C by means of an air thermostat. The potential signal generated by the thermopiles is read by a digital precision multimeter (Hewlett–Packard 3478A) with sensitivity of 0.1 μV that is connected to a personal computer by a GPIB NI-488.2 interface (National Instrument). Data storage and processing are performed by the computer program CETAC developed in this laboratory. The calorimetric liquids are conducted to the reaction cells by means of a peristaltic pump (Master Flex 7520-25) with variable flow rate between 0.2 mL and 42 mL min^{-1}. The system they place in the cells and there is fixed a constant flow of the palm oil and is begun to register the respective signals. Then, is evaluated enthalpic value in function of time.

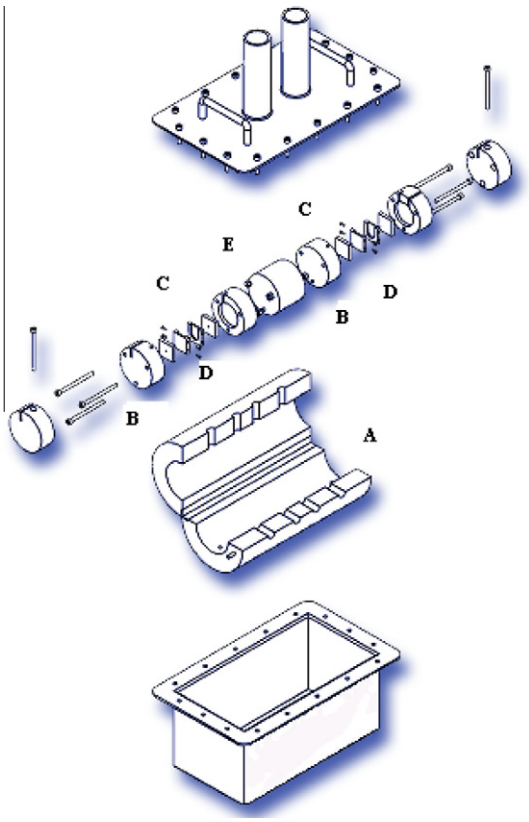

Figure 7 Flow microcalorimeter used for the transesterification of biodiesel study.

3. Results and discussion

3.1. Lipase screening

Although lipases generally catalyze the hydrolysis of carboxylic esters, they bring about a range of bioconversion reactions

Table 1 Lipases tested in the transesterification screening.

Lipase	Source organism
CR	Candida rugosa
PP	Porcine pancreatic
PS	Pseudomonas cepacia
SP435	Candida antarctica

such as esterification, transesterification, acidolysis, and aminolysis. Lipase screening was performed to find the lipase with the best catalytic activity in the transesterification of soybean oil. The most active lipase was then used in the imobilization studies. Four lipases, as listed in Table 1, were screened for their transesterification activity. As the screening experiments were intended for an initial evaluation of the activity of the lipases, they were conducted under a preliminary set of reaction conditions which may not have been the optimum set for all the lipases. In a typical reaction, 300 mg of dry enzyme was added to the mixture of 1 g methanol (5.0 molar ratio of methanol to soybean oil), 1.0 g of water and 10 g of palm oil. The reactions were carried out at 37 °C and according to the reaction setup which was described earlier. The screening results for the tested lipase are presented in Fig. 1. Reaction products are presented as mol% of methyl esters of fatty acids in the reaction mixture.

The formation of free fatty acids is also included in this figure since the presence of water in the reaction medium naturally promotes the competing hydrolysis reaction. As this figure shows, among the tested lipases, lipase Candida Rugosa showed the highest activity toward the transesterification of palm oil with alcohols, specially with the *n*-butanol. Other lipases showed very little or no activity toward the transesterification reaction. After 1 h of reaction with lipase Candida Rugosa, the product contained 37 mol% methyl esters, 5 mol% of fatty acids, 4 mol% of monoglycerides, 52 mol% of diglyceride and 2 mol% of triglycerides.

3.2. Enzyme loading

Experiments were performed to determine the effect of enzyme loading on the extent of the transesterification reaction. Enzyme loading in the range of 0–800 mg of free enzyme and 0–5.0 g of the immobilized enzyme were examined in the transesterification of palm oil with methanol, ethanol and *n*-butanol. One gram of immobilized enzyme corresponds to 170 mg free enzyme in these reactions. Based on the concentration of palm oil, the alcohol concentration was at 5.0 molar equivalents for methanol, ethanol and *n*-butanol (1.0 g methanol, 1.0 ethanol and 1.0 g *n*-butanol). Other reaction parameters were as was stated earlier in the reaction setup and optimization section. Reaction results for the formation of methyl and ethyl esters of fatty acids are presented in Fig. 2. For all cases studied, as the enzyme loading was increased there was a sudden surge in the formation of alkyl esters, followed by a slower rate at higher enzyme loadings. This surge was steeper and the formation of alkyl esters was significantly higher for the immobilized enzymes compared with the free enzymes. For the reactions with methanol, the concentration of methylesters reached 45 and 70 mol% for the free and the immobilized lipase, respectively. At the end of the reaction period, the concentration of triglyceride in both system reached negligible levels, while, the formation of fatty acid, mono- and di-glyceride were 5, 7, and 48 mol% for the free enzyme reaction and 5, 20, and 10 mol% for the immobilized enzyme. Similarly, for the reaction with ethanol, the level of ethylesters reached 50 and 80 mol% for the free and the immobilized lipase. At the end of the reaction period, the concentration of triglyceride in both system reached low levels, while, the formation of alkyl-esters were 6, 3, 49 mol% for the free enzyme reaction and 6, 19, and 12 mol% for the immobilized. Also, for the reaction with *n*-butanol, the level of alkyl-esters reached 60 and 85 mol% for the free and the immobilized lipase. At the end of the reaction period, the concentration of triglyceride in both system reached negligible levels, while, the formation of alkyl-esters were 2, 2, 54 mol% for the free enzyme reaction and 4, 16, and 17 mol% for the immobilized This behavior of the immobilized lipase was not consistent with those of other researchers (e.g. Reetz, 1997) and has attributed not only to lipophilic nature of the alkyl group but to activated carbon that for high superficial area allows that a number great of lipases settle and the process is more efficiently. Free alkyl groups in the support-lipase create a lipophilic microenvironment that subsequently interacts with the lipase, triggering a phenomenon similar to a classical interfacial interaction. However, unlike the interfacial activation, which is an interactive process, the alkyl effect is believed to be due to a more favorable lipase conformation caused during the carbon activated-lipse-oil interaction. The lipophilic environments are also believed to facilitate the transport of the organic substrate to the biocatalyst sites in the outer surface of the support and possibly in and out of the matrix.

3.3. Effect of water concentration

Lipases possess the unique feature of acting at the interface between an aqueous and an organic phase. Lipase interfacial action is due to the fact that their catalytic activity generally depends on the aggregation of the substrates. Activation of the enzyme involves unmasking and restructuring of the active site through conformational changes of the lipase molecule, which requires the presence of oil–water interface. Lipase activity generally depends on the available interfacial area. With the increased addition of water, the amount of water available for oil to form oil–water droplets increases, thereby, increasing the available interfacial area. However, since lipases usually catalyze hydrolysis in aqueous media, excess water may also stimulate the competing hydrolysis reaction. The optimum water content is a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction. The effect of water concentration in the range of 0.02–5.0 g and at constant alcohol concentrations of 5.0, 15.2 and 17.5 molar ratios of methanol, ethanol and *n*-butanol with respect to palm oil were examined (3.9 g methanol, 8.0 g ethanol and 10.0 *n*-butanol). The reactions were carried out according to the reaction setup and optimization section described earlier. Results presented in Fig. 3 indicate very little enzyme activity at low water concentrations which supports the fact that a minimum amount of water is required to activate the enzyme. With the increased addition of water there was a considerable increase in the ester production showing the enhancement in the activity of the enzyme. In the case with methanol, the concentration of methylesters reached 32.7

and 62.4 mol% for the free and the immobilized lipase, respectively. The production maximum is reached with the *n*-butanol 45 and 85 mol% for the free and the immobilized lipase, respectively of about 65 mol% at 0.3 g water.

3.4. Effect of alcohol concentration

In the immobilized enzyme transesterification reaction, the reactants initially form a three-phase system (triglyceride/alcohol/support). The reaction is diffusion controlled and poor diffusion between the phases exists. As alkyl esters are formed, they act as a mutual solvent for the reactants and a two phase liquid/solid system results (Noureddini and Zhu, 1997; Noureddini et al., 2005). However, as the reaction progresses toward completion and the glycerol concentration is increased, a mainly alcohol and glycerol phase separates from the rich alkyl ester phase and a three-phase system forms again. This is more likely at lower initial alcohol concentrations and higher extent of reaction and in the range of alcohol concentration that was investigated this separation did not occur. Alcohol in excess of the stoichiometric molar ratio of 3:1, was used to: (1) ensure higher reaction rates as the transesterification of triglycerides with alcohols consist of three stepwise and reversible reactions and (2) minimize the diffusion limitations specially in this case by the use of activated carbon as support. However, excess alcohol levels may inhibit the enzyme activity and thereby decrease its catalytic activity toward the transesterification reaction. Experiments were performed to optimize the amount of ester production by varying the alcohol concentration. Optimum alcohol requirements were determined for both methanol and ethanol. The amount of alcohol added was varied from 2.7 to 13.7 molar equivalents for methanol and from 5.7 to 26.7 molar equivalents for ethanol, based on the moles of triglycerides. Water concentration was kept constant at the optimum level of 0.5 g for the methanol reactions and at 0.3 g for the ethanol reactions. Results are summarized in Fig. 4. As was expected, an increase in the number of moles of alcohols with respect to the triglycerides resulted in an increase in the production of esters. Ultimately, the formation of esters reached a maximum level and further increases in the alcohol concentrations resulted in a decrease in the formation of esters. The optimum alcohol concentration was determined at 10.4 molar ratio for alls alcohols to palm oil where about 85 mol% of methyl esters was formed with *n*-butanol.

3.5. Determination of biodiesel with flow microcalorimetry

This one is a very versatile technology that in our laboratory we use for several decades to characterize solid porous as the coal activated with excellent results. In this research it has been implemented to evaluate the production of biodiesel under the experimental conditions of this work. For it before I realize a study of production of the biodiesel with regard to the time as one shows in the Fig. 5. These results show an ideal production to 150 min for each of the studied systems.

In these conditions we take to study the respective results we represent time in function enthalpy. The results show themselves in the Fig. 6. This show appears clearly that there is a change abrupt in the value of enthalpy about 150 min.

This shows that the microcalorimetry of flow is an effective and best tool to do the follow-up of the reaction of transesterificación of the oil of palm in the production of the biodiesel. This type of study has not been reported by the literature.

4. Conclusions

Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional physiochemical process. The key step in enzymatic processes lies in successful immobilization of the enzyme which will allow for its recovery and reuse. In this study the immobilized enzyme transesterification of soybean oil with methanol and ethanol was investigated. Lipase Candida Rugosa was immobilized by entrapment within a activated carbon which was prepared by activation physical. The immobilized lipase so prepared was consistently more active than the free lipase toward the transesterification of palm oil. The immobilized lipase also proved to be stable and lost little activity when was subjected to repeated uses. The flow microcalorimetry is a technical novel for followed of transesterification of oil palm process.

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