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A monolithic lipase reactor for biodiesel production by transesterification of triacylglycerides into fatty acid methyl esters

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Abstract

An enzymatic reactor with lipase immobilized on a monolithic polymer support has been prepared and used to catalyze the transesterification of triacylglycerides into the fatty acid methyl esters commonly used for biodiesel. A design of experiments procedure was used to optimize the monolithic reactor with variables including control of the surface polarity of the monolith via variations in the length of the hydrocarbon chain in alkyl methacrylate monomer, time of grafting of 1-vinyl-4,4-dimethylazlactone used to activate the monolith, and time used for the immobilization of porcine lipase. Optimal conditions involved the use of a poly(stearyl methacrylate-*co*-ethylene dimethacrylate) monolith, grafted first with vinylazlactone, then treated with lipase for 2 h to carry out the immobilization of the enzyme. Best conditions for the transesterification of glyceryl tributyrate included a temperature of 37°C and a 10 min residence time of the substrate in the bioreactor. The reactor did not lose its activity even after pumping through it a solution of substrate equaling 1,000 reactor volumes. This enzymatic reactor was also used for the transesterification of triacylglycerides from soybean oil to fatty acid methyl esters thus demonstrating the ability of the reactor to produce biodiesel.

Keywords

biodiesel; transesterification; lipase; soybean oil; fatty acid methyl esters; polymer monolith; design of experiments; response surface methodology; immobilization

Introduction

Biodiesel is typically prepared by transesterification of triglycerides with an alcohol in the presence of a chemical or biological catalyst. Enzymatic transesterification using lipases offers advantages such as easy product separation, minimal needs for wastewater treatment, easy glycerol recovery, and the absence of side reactions (Jegannathan et al. 2008; Xiao et al. 2009; Vyas et al. 2010). On the other hand, the practical use of lipase in homogenous reaction systems can be costly and fraught with technical difficulties such as contamination of the product with residual active enzyme (Al-Zuhair, 2007). While various acyl acceptors have been tested, methanol is most often used in the enzyme catalyzed synthesis of biodiesel, (Halim and Harun Kamaruddin, 2008; Kaieda et al. 1999; Soumanou and

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Bornscheuer, 2003). Addition of 2-methylpropanol to the reaction mixture solves the issue of poor solubility of methanol in a fatty organic system as this alcohol solubilizes both methanol and the reaction byproduct - glycerol - while largely eliminating the inhibiting effect of methanol and glycerol on the catalytic activity of lipase (Halim and Harun Kamaruddin, 2008; Royon et al. 2007). The high cost of lipases is currently the major roadblock to its wide application in industrial transesterifications.

Immobilization of an enzyme onto a solid support, represents an attractive way to reduce the consumption of lipase. The current literature describes several immobilization techniques including physical adsorption (Vidinha et al. 2006), entrapment (Reddy and Vadgama, 2002), electrostatic interaction (Rahman et al. 2004), and covalent binding (Tumturk et al. 2000). The last method is preferred since it is most effective in avoiding the loss of enzyme during operation. Supports for enzyme immobilization are generally selected on the basis of their mechanical strength, microbial resistance, thermal and chemical stability, polarity, ease of regeneration, loading capacity, and cost, with the latter being particularly important in industrial applications (Karube et al. 1977). In general, the supports that are selected have good stability and a wide variety of chemistries are available (Zhao et al. 2011). They are also available in various formats such as porous beads and monoliths with the latter excelling in facilitated mass transfer due to their highly permeable porous structure.

Porous polymer-based monolithic stationary phases were introduced about 20 years ago and first used in liquid chromatography for the rapid separations of large molecules including proteins, nucleic acids, and synthetic polymers (Hjerten et al. 1989; Tennikova et al. 1990; Svec and Frechet, 1992). These monoliths are typically prepared *in situ* within the confines of a container such as the type of tubes used for chromatographic columns, or fused silica capillaries using thermally or UV initiated polymerization of a mixture of monomers and porogenic solvents. The simplicity of this process makes the monolithic polymeric media attractive for use in narrow bore capillaries and microfluidic devices for which packing with particulate materials is a very challenging task (Svec, 2004; Urban and Jandera, 2008). An additional benefit of monoliths prepared in capillaries is the ease with which their pore surface can be functionalized via photografting (Rohr et al. 2003; Stachowiak et al. 2006a; Logan et al. 2007; Krenkova et al. 2009a). Typically, a generic monolith with optimized porous properties is first prepared, then modified by the photografting of functional monomers. This approach enables the independent optimization of both the porous properties and the final chemistry of the monolith.

Polymer monoliths have also proven to be excellent supports for the immobilization of enzymes and protein ligands for affinity chromatography (Petro et al. 1996; Xie et al. 1999; Josic and Buchacher, 2001; Vlakh et al. 2004; Bartolini et al. 2004; Palm and Novotny, 2005; Svec, 2006; Mallik and Hage, 2006; Logan et al. 2007; Bencina et al. 2007; Ma et al. 2008; Krenkova and Svec, 2009). However, it should be noted that all immobilized enzyme reactors reported to date have been used to process substrates dissolved in aqueous media. We now demonstrate for the first time the use of an enzyme immobilized on a polymer-based monolithic support, which operates in both organic solvents and their biphasic mixtures with water. Applying response surface methodology (RSM) (Halim et al. 2009; Azocar et al. 2010; Jeong and Park, 2010; Akbari et al. 2010), we have optimized the immobilization of lipase on monoliths of various polarities. The optimized capillary reactors were then used in pure organic solvent for the transesterification of fatty acid esters.

Materials and Methods

Chemicals

Butyl methacrylate (BMA, 99%), lauryl methacrylate (LMA, 99%), stearyl methacrylate (SMA, 65%), ethylene dimethacrylate (EDMA, 98%), 2,2‘-azobisisobutyronitrile (98%, AIBN), benzophenone, 3-(trimethoxysilyl)-propyl methacrylate (99%), methanol (99%), basic alumina, 1,4-butanediol, 1-propanol, hexane, methanol (both HPLC grade), and glyceryl tributyrate (tributyrin, >98.5%) were purchased from Aldrich (Milwaukee, WI, USA). 2-Vinyl-4,4-dimethylazlactone (vinyl azlactone) was purchased from TCI America (Portland, OR, USA). n-Dodecane was purchased from MB Biomedicals (Santa Ana, CA, USA). Crisco Soybean kitchen oil was a commercial product (The J.M. Smucker Company, Orrville, OH, USA). Porcine lyophilized lipase (powder, >65 U/mg at 37 °C) was obtained from Lee Bio (St. Luis, MO, USA). All monomers (BMA, LMA, SMA, and EDMA) were passed over activated basic alumina prior to use. All other reagents were used as received. Phosphate buffer (250 mmol/L, pH 7.2) was prepared by dissolving potassium dihydrogen monophosphate and potassium hydrogen phosphate (Aldrich, Milwaukee, WI, USA) in water and adjusting the pH with HCl. PTFE-coated UV transparent 100 μm i.d. fused silica capillary tubing was obtained from Polymicro Technologies (Phoenix, AZ, USA).

Preparation of Monoliths in Capillary

The inner surface of UV-transparent fused-silica capillaries was first activated with 3-(trimethoxysilyl)propyl methacrylate. The capillary was quickly rinsed with acetone and water, then flushed with 0.2 mol/L sodium hydroxide solution for 30 min at a flow rate of 0.25 μL/min using a syringe pump (KdScientific, Holliston, MA, USA), and quickly rinsed with water. Next, 0.2 mol/L hydrochloric acid was pumped through the capillary for 30 min at a flow rate of 0.25 μL/min, followed by water and ethanol. A 20% w/w solution of 3-(trimethoxysilyl)propyl methacrylate prepared in 95% ethanol with an apparent pH adjusted to 5 using acetic acid was pumped through the capillary at a flow rate of 0.25 μL/min for 90 min. The capillary was then washed with acetone, dried in a stream of nitrogen, and left at room temperature for 24 h.

Typical polymerization mixtures based on our previous work (Eeltink et al. 2007) comprised 24% monovinyl monomer (BMA, LMA, or SMA), 16% crosslinker (EDMA), and 60% porogens (1,4-butanediol and 1-propanol in different ratios) (all percentages are w/w). AIBN was used as a radical initiator to start the polymerization reaction (1 wt% with respect to monomers). After purging the mixture with nitrogen for 10 min, the surface-modified capillaries were filled with the polymerization mixture and then sealed with a septum. The polymerization reaction was then carried out for 20 h by heating in a water bath held at 70°C. The monolith was then washed with methanol at a flow rate of 0.5 μL/min for 30 min using a syringe pump.

Activated Supports

A two-step activation reaction develop earlier (Stachowiak et al. 2007) was used to activate the monolithic support. Benzophenone initiator was first grafted to the surface of the monolith by rinsing the monolith in capillary with a 5 wt% benzophenone solution in methanol at a flow rate of 0.5 μL/min for 30 min and then exposing to UV irradiation for 4 min. After photografting of the benzophenone, the monolith was rinsed with methanol at a flow rate of 0.5 μL/min for 30 min to remove the unbound compounds.

A mixture consisting of 15% vinyl azlactone in 75:25% *tert*-butanol-water mixture (all percentages are w/w) was pumped through the benzophenone-functionalized monolith at a flow rate of 0.5 μL/min for 30 min. Vinyl azlactone was photografted onto the surface of the

monoliths using UV light with exposure times of 1, 10, and 20 min. The monolith was then washed with *tert*-butyl alcohol-water mixture followed by potassium phosphate buffer at a flow rate of 0.5 μL/min for 1 h to remove the excess reagents.

The intensity of the UV light of the Spectroline microprocessor-controlled UV source (Spectronics Corp., Westbury, NY, USA) used in both grafting steps was adjusted to 4.2 mW/cm^2 using a 254-nm probe head.

Immobilization of Lipase

A solution of lipase in 250 mmol/L potassium phosphate buffer pH 7.2 (5 mg/mL) was pumped through the vinyl azlactone-modified monolith at a flow rate of 0.25 μL/min for 2, 4 and 8 h. The monolith was then rinsed with 250 mmol/L potassium phosphate buffer for 0.5 h. Finally, the reactor was washed with hexane and used for transesterification.

Assay of Lipase Activity

Typically, 100 μL tributyrin solution in hexane (0.26 mg/mL) with dodecane added as an internal standard was pumped through the reactor at 0.1 μL/min. The effluent leaving the reactor was then analyzed using GC (*vide infra*) and the yield of butyric acid was calculated with respect to the total tributyrin concentration in the initial solution. In kinetic experiments, the enzyme activity was calculated from the difference in tributyrin concentration in the initial solutions (5–700 nmol/L) and in the reactor effluent as determined using GC. All of these solutions were pumped through the reactor at a flow rate of 0.1 μL/min while the reactor was heated to 37°C. The lipase activity is expressed in nanomoles of converted substrate in 1 min normalized to 1 mL of reactor volume. Experiments with free lipase in solution were carried out in a glass vial with a mixture of 100 μL tributyrin solutions and 30 μL lipase solution (5 mg/mL) for 4 hours at 37°C.

Design of Experiments and Statistical Analysis

Since we did not have readily available commercial software, we developed home-written routine in MATLAB (MathWorks, Natick, MA, USA) enabling us to minimize the number of experiments by applying the design of experiments and to evaluate non-linear regression of a mathematical model. Using this routine, Box-Behnken design of experiments for three factors and three levels has been applied to optimize both support and conditions for lipase immobilization affording the highest activity in organic solvents. The three factors included in the design were the type of the monomer used in the preparation of monolithic support (BMA, LMA, and SMA), the grafting time of vinyl azlactone (1, 10, and 20 min), and the time used for lipase immobilization (2, 4, and 8 h). The yield of reaction product (butyric acid) was used as a response factor. Table 1 shows the design space covered with 15 experimental runs. The center point was run three times to allow for a more uniform estimate of the prediction variance over the entire design space.

Experimental data were analyzed using the response surface methodology to fit the second-order polynomial equation. A different home-written routine in MATLAB was then used for the non-linear evaluation of multivariate data. Insignificant variables were removed and a model describing the effects of the experimental parameters on the yield of butyric acid was improved and its predictive power increased. The number of parameters used in the initial model was gradually reduced, depending on the value of the calculated probability factors, *f*, which are a measure of the relevance of the experimental variables on the response. Probability factors higher than 0.05 mean that the parameter does not affect significantly the system response. The terms related to these parameters are then removed from the model in several subsequent steps. Only a single parameter with the highest calculated *f* value is removed in each step (Scheffe, 1963; Snee, 1979).

Gas Chromatography

A gas chromatograph HP 6890 GC (Agilent, Palo Alto, CA) equipped with a 30.0 m × 250 μm × 0.25 μm J&W DB-XLB column and FID and MS detectors was used for analyses. The chromatographic separations were carried out using a temperature ramp 50-200 °C in 15 min with inlet temperature set at 250 °C. Injection volume was always 0.2 μL.

Results and Discussion

The individual steps used for the preparation of monolithic supports, their activation, and use for immobilization of lipase are shown schematically in Figure 1.

Preparation of Polymer Supports

In the first set of experiments, we prepared monolithic columns from polymerization mixtures in which only the length of the alkyl chain of the monovinyl monomer was changed from C_4 to C_{12} to C_{18} while keeping all other components and percentages constant. The polymerization mixtures comprised 24% monovinyl monomer, 16% crosslinker (EDMA), 20% 1,4-butanediol and 40% 1-propanol (both porogens). Under these conditions, the porosity of all monolithic supports is 60% reflecting the percentage of porogens in the polymerization mixture. Changing the nature of the methacrylate monomer has a significant effect on the permeability of the monolithic supports. For example, the monoliths prepared from LMA and SMA using these conditions were permeable for washing with solvents under the low pressure exerted by a syringe pump. In contrast, the monolith prepared using BMA was no longer permeable. However, if the percentages of porogenic solvents in the BMA polymerization mixture were adjusted to 26% butanediol and 36% propanol, a permeable BMA monolith was obtained. We found in an unrelated study (Eeltink et al. 2007) using direct measurement of porous properties that BMA monolith prepared from the same polymerization mixture via polymerization in bulk exhibited a mean pore size of 1970 nm (mercury porosimetry) and a surface area of 47 m^2/g (nitrogen adsorption). Since this monolithic support placed in a capillary exhibited resistance to flow very similar to that observed for capillaries including LMA and SMA monoliths, we infer from this result that the pore size in monoliths prepared from all three monovinyl monomers is comparable. Unfortunately, the quantity of the authentic monoliths in capillaries is not sufficient for direct measurements.

While their permeability is excellent, the poly(alkyl methacrylate-*co*-ethylene dimethacrylate) monoliths cannot be used directly in the attachment of an enzyme as they do not possess any reactive functionality. A survey of the literature shows that the azlactone functionality has often been used to immobilize enzymes on polymer-based supports (Coleman et al. 1990; Heilmann et al. 2004; Drtina et al. 2005). Therefore, the reactive monomer 2-vinyl-4,4-dimethylazlactone can be copolymerized during the preparation of the monolith (Xie et al. 1997; Xie et al. 1999; Eeltink et al. 2007) or grafted on the pore surface of previously prepared monoliths (Tripp et al. 2001; Stachowiak et al. 2006b; Connolly et al. 2007; Krenkova et al. 2009b). In our early work, a single-step photografting process, in which a solution containing both vinylazlactone and photoinitiator was used to fill the pores of the monolith prior to UV initiation of the grafting process, was used to activate the monolith (Rohr et al. 2003). A disadvantage of this approach is that ungrafted polymer formed in the polymerization mixture also fills the pores and may be difficult to wash out due to its high viscosity. Therefore, we introduced a two-step grafting-from technique (Stachowiak et al. 2007) in which the initiator is first attached to the pore surface of the monolith by UV irradiation, and photografting of the reactive monomer is then carried out from the activated surface. We have used this two-step approach in the present study as it also enables the use of different solvents for each of the two steps.

Optimization of the Enzymatic Reactor

We applied design of experiments to optimize the activity of the enzyme reactor. Primarily, we focused on the extent of immobilization expressed in terms of lipase activity varying the length of the alkyl group of the methacrylate monomer, the grafting time of vinyl azlactone and the length of time allowed for lipase immobilization. All other variables, such as percentage of vinyl azlactone in the grafting solution and concentration of lipase in the immobilization mixture were kept constant. The parameter range used in the design was selected based on our previous studies of photografting and enzyme immobilization via azlactone chemistry (Stachowiak et al. 2006; Logan et al. 2007).

Table 1 lists the final set of fifteen experiments. A quadratic model was selected to fit the experimental data in order to analyze and determine the effects of factors that are varied. The initial model equation correlates the effects of the three experimental factors x_1 through x_3 representing the type of monomer x_1, grafting time of vinyl azlactone x_2, and enzyme immobilization time x_3, respectively, with the amount of formed butyric acid, y, as response:

$$y=p_0+p_1\cdot x_1+p_2\cdot x_2+p_3\cdot x_3+p_4\cdot x_1\cdot x_2+p_5\cdot x_1\cdot x_3+p_6\cdot x_2\cdot x_3+p_7\cdot x_1^2+p_8\cdot x_2^2+p_9\cdot x_3^2 \quad (1)$$

Parameters $p_1 - p_3$ represent the extent of effects of individual factors, while the cross-term interaction parameters $p_4 - p_6$ account for the combined effects, and parameters $p_7 - p_9$ correspond to quadratic terms of the model.

The significance of the effects of the parameters $p_1 - p_9$ in Eq. (1) on immobilized lipase activity was evaluated in seven subsequent steps as illustrated in Table 2. The insignificant experimental factors were removed based on the value of probability factors f_i, which are a measure of the effect of the experimental factors on the response. Probability factors larger than 0.05 do not affect significantly the response at a probability level of 95% and the term with these parameters was removed from the model. Table 2 shows that only a single parameter with the highest f value was removed in each step (Scheffe, 1963; Snee, 1979).

After cancelling the insignificant factors, we obtained the optimized model for reactor activity expressed in terms of yield of butyric acid as a function of all three factors varied:

$$y=4.38\cdot x_3+0.86\cdot x_1\cdot x_2-0.44\cdot x_1\cdot x_3+0.12\cdot x_2^2 \quad (2)$$

Table 3 compares experimental values of yields of butyric acid with those calculated using the optimized model presented in Equation (2). The average absolute deviation was only 5.3% with only two columns exhibiting a somewhat higher absolute deviation of 9%. The linear correlation confirms a good match of the predicted and measured values with correlation coefficients R of 0.76.

According to their absolute value, the final model parameters may be either synergistic (increasing response) or antagonistic (decreasing response). Experimental factors with high absolute values of the regression parameter p_i affect the targeted property most significantly. Equation (2) clearly confirms that the activity is mostly affected by the time of lipase immobilization. Figure 2 presents a graphical expression of the activity of immobilized lipase in coordinates of experimental factors with a fill of the whole experimental space with data calculated using Equation (2). The black points in the Figure correspond to the original 15 experiments. Figure 2 also shows that the activity of immobilized lipase increases with the alkyl length of the monomer used to prepare the monolithic support and with the time of lipase immobilization. Interestingly, the grafting time of vinyl azlactone only has a minor

effect on the final enzymatic activity. The disparity between graphical expression of lipase activity in Figure 2 and the high value of parameter p_3 for immobilization time can be explained by the steric crowding of the lipase immobilized at the pore surface together with the finite number of grafted vinylazlactone units. An increase in grafting time of vinyl azlactone to 60 min did not lead to any improvement in reactor activity thus confirming the small significance of this variable.

Using the optimized model of Equation (2) and Figure 2, we derived the optimal conditions affording the highest lipase activity: (i) use of a stearyl methacrylate monolithic support, (ii) grafting with vinyl azlactone for 20 min, and (iii) 2 h duration of the lipase immobilization. These results also demonstrate that our initial conditions included in the design of experiments were well selected. Reactors prepared using these conditions were then utilized in the following experiments, which focused on optimizing the conditions of the enzymatic reaction.

Reaction Catalyzed with Immobilized Lipase

The transesterification of tributyrin with methanol using our immobilized lipase reactor is also shown schematically in Figure 1. Gas chromatography/mass spectrometry analysis uncovered three main compounds of tributyrin transesterification: butyric acid, butyric acid methyl ester, and butyric acid dihydroxypropyl ester. Figure 3 shows the effect of reaction temperature on the percentages of these compounds in the effluent mixture leaving the reactor. The highest percentages of butyric acid and butyric acid methyl ester are formed at the physiological temperature of 37°C, while they decrease as the reaction temperature is increased further. In contrast, the percentage of butyric acid dihydroxypropyl ester rapidly increases as the temperature increases (Figure 3). Interestingly, the sum of the percentages of both esters in the product does not depend significantly on the reaction temperature. These results suggest that reaction temperature can be used as a simple tool to control the synthesis of the desired ester.

Transesterification using free lipase as a catalyst is slow and the overall reaction rate reaches a maximum after 4 – 24 h (Jegannathan et al. 2008; Vyas et al. 2010). Figure 4 demonstrates that immobilization of the enzyme on the porous polymer monolith enables a dramatic reduction in the reaction time as defined by the residence time of the substrate in the reactor, which is controlled by its flow rate. An increase in residence time from 2 to 10 min has a profound effect on the percentages of products formed in the reactor. Once again, the convective mass transport of substrate to the enzyme catalyst contributes to an increase in reaction rate (Petro et al. 1996; Xie et al. 1999; Svec, 2006; Krenkova and Svec, 2009). However, increasing the residence time does not lead to any further improvement. It is likely that other factors such as inhibition of activity by the products prevail, thus preventing a further increase in conversion.

The viability of an immobilized enzyme reactor is defined by the stability of its activity. Figure 5 shows the effect of the total volume of the tributyrin substrate solution continuously pumped through the reactor on percentages of the products as determined by GC-FID. Clearly, no significant decrease in composition of products is observed until about 1,000 μL of substrate is converted. At this point, the activity of immobilized lipase decreases leaving more tributyrin substrate unconverted. Since the pore volume of our 200 mm long capillary reactor is about 1 μL, the activity is kept unchanged for about 1,000 reactor volumes. It is worth noting that no decrease in lipase activity is observed after storing the reactor filled with hexane for several weeks.

Kinetics of Transesterification Reaction

Optimal conditions for the transesterification reaction catalyzed with immobilized lipase include a temperature of 37 °C and a substrate residence time of 10 min. Therefore, we used these conditions to determine the kinetic parameters of the transesterification reaction using our reactor in order to compare them with those found with lipase used in solution. The activity of the immobilized enzyme was assayed by pumping 100 μL of solutions differing in concentration of tributyrin through the reactor at a flow rate assuring a residence time of 10 min. Using the Lineweaver–Burke linearization approach shown in Figure 6 the value of Michaelis–Menten constant, K_m, as well as maximum reaction rate, V_{max}, were calculated. We determined K_m = 12.4 mmol/L for immobilized lipase and 1.8 mmol/L for lipase in solution. These values suggest that immobilized lipase operates six times faster than lipase in solution. Similarly, the maximum reaction rate for immobilized lipase is 18.6 μmol/L·min, almost four times faster than the 5.0 μmol/L·min observed in solution. These results clearly confirm the advantage derived from immobilization of the enzyme on the monolithic support.

Production of Fatty Acids Methyl Esters

Finally, we used our immobilized enzyme reactor for the preparation of fatty acid methyl esters from kitchen grade soybean oil dissolved in a mixture of methanol and 2-methyl-2-propanol. Figure 7 compares the chromatograms of the initial reaction mixture with that obtained after passing through the reactor, which confirms the successful preparation of several fatty acids methyl esters: while no methyl esters are present in the original oil, GC-MS confirms that significant amounts of these esters are present in the effluent.

Conclusions

This report clearly demonstrates the advantages of using porous polymer monoliths as supports for the immobilization of enzymes and the fabrication of highly active enzymatic reactors capable of operating in a non-aqueous environment. In contrast to previous studies, the transesterification of triacylglycerides using lipase is carried out in an organic solvent mixture. The comprehensive optimization based on mathematical design of experiments including the hydrophobic monomer used to prepare the precursor monolith, grafting time of monomer comprising the reactive azlactone functionalities, and time of the enzyme immobilization led to an equation that facilitated the calculation of conditions which should afford a reactor with maximum activity. Final tuning involved the selection of reaction temperature and residence time of the substrate in the reactor. Under optimum operating conditions, the kinetic parameters of immobilized lipase were superior to those for lipase in solution. While the specific features of immobilized enzyme have been explored using tributyrin as a model substrate, we have also confirmed the suitability of the reactor in the transformation of a commercial kitchen oil to methyl esters thus demonstrating the potential use of our approach in the production of biodiesel following suitable scale-up. Obviously, production of biodiesel on a large scale will require large monolithic units. For example, BIA Separations (biaseparations.com) manufactures monoliths with a volume of 8 L and radial flow pattern. Even larger units are likely to emerge soon. Multiplicity of units like this could be then used in series. Also, UV initiated grafting demonstrated in this study will have to be replaced by thermally initiated grafting of vinyl azlactone using an azo initiator attached to the pore surface, a process that we have developed in our earlier studies (Tripp et al. 2001).

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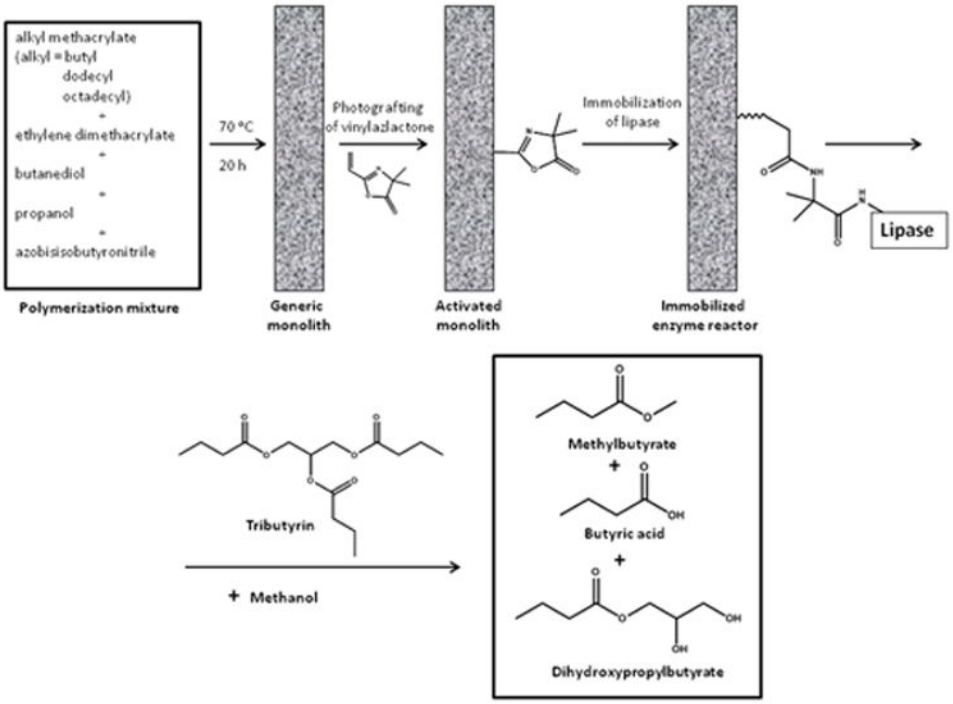

Figure 1.

Scheme showing the preparation of the monoliths using methacrylates with varying length of the alkyl chain, activation with azlactone, and enzyme immobilization and the transesterification of tributyrin with methanol using immobilized lipase reactor.

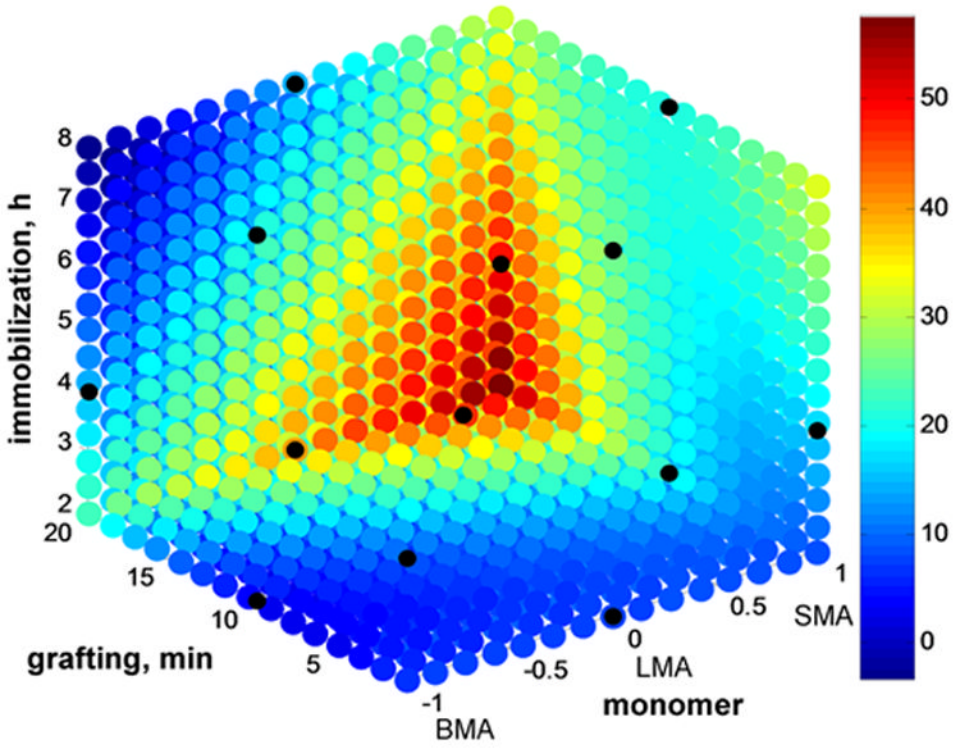

Figure 2.
Graphical expression of optimized model describing the effect of monomer chemistry (butyl methacrylate BMA, lauryl methacrylate LMA, and stearyl methacrylate SMA), vinylazlactone grafting time and lipase immobilization time on formation of butyric acid in the immobilized enzyme reactor as a response. The black points are original data measured due to the design of experiments. The bar corresponds to the percentage of butyric acid formed.

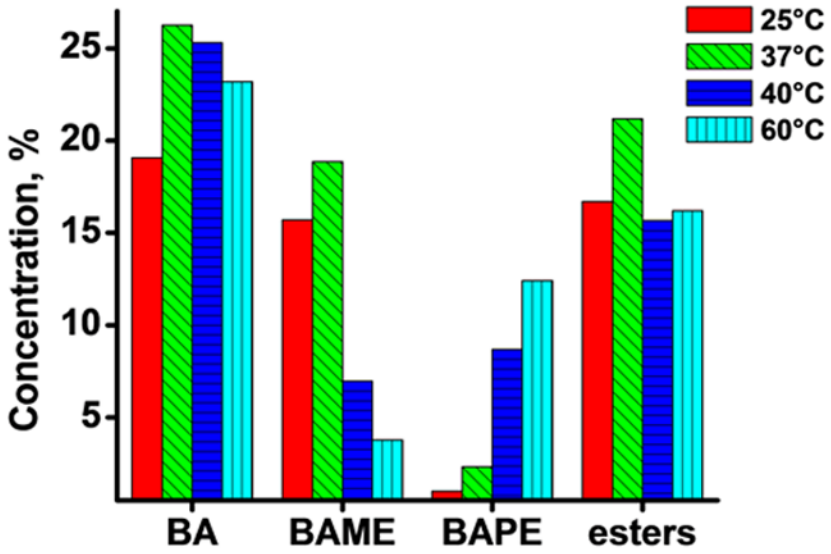

Figure 3.
Percentage of butyric acid (BA), butyric acid methyl ester (BAME), butyric acid dihydroxypropyl ester (BAPE), and a sum of both esters (esters) formed from tributyrin at various temperatures.

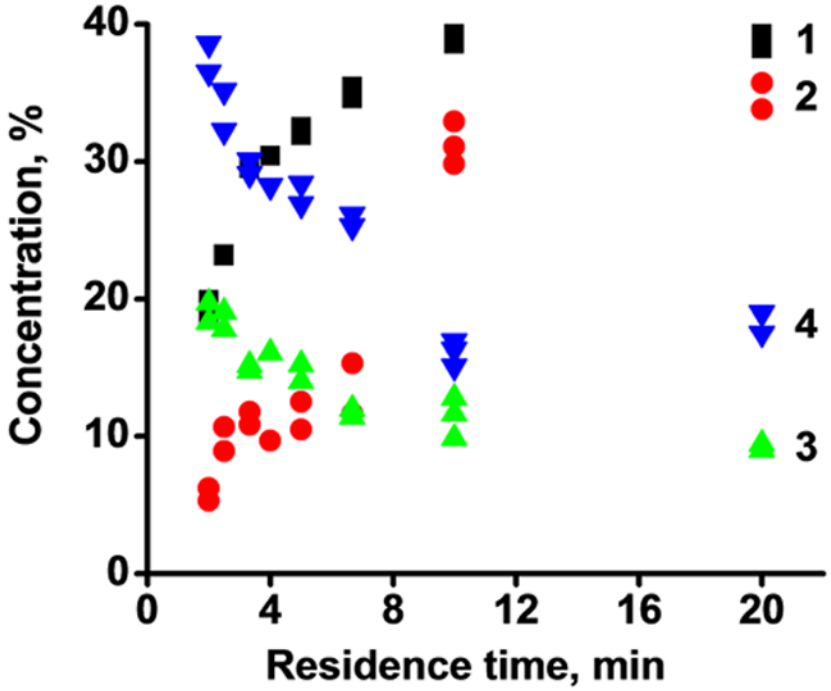

Figure 4.
Effect of residence time of substrate in the capillary reactor with immobilized lipase on the percentage of the unreacted tributyrin (line 4) and butyric acid (1), butyric acid methyl ester (2), and butyric acid dihydroxypropyl ester (3) as products of transesterification reaction carried out at a temperature of 37°C.

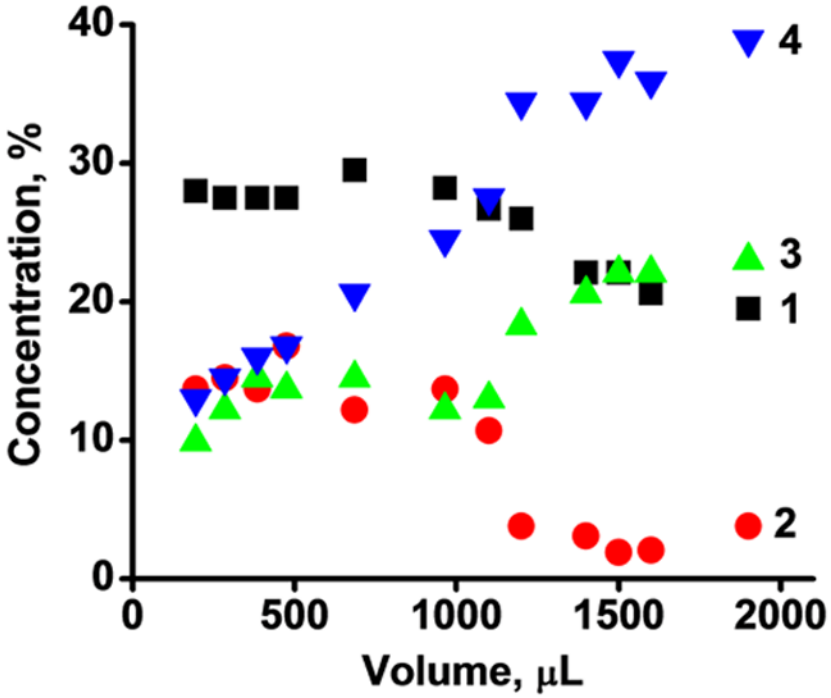

Figure 5.
Stability of the immobilized lipase activity expressed as a percentage of transesterification reaction products with respect to the overall volume of substrate pumped through the reactor. Reaction temperature 37°C, residence time 10 min. 1 – butyric acid, 2 – butyric acid methyl ester, 3 – butyric acid dihydroxypropyl ester, 4 - tributyrin.

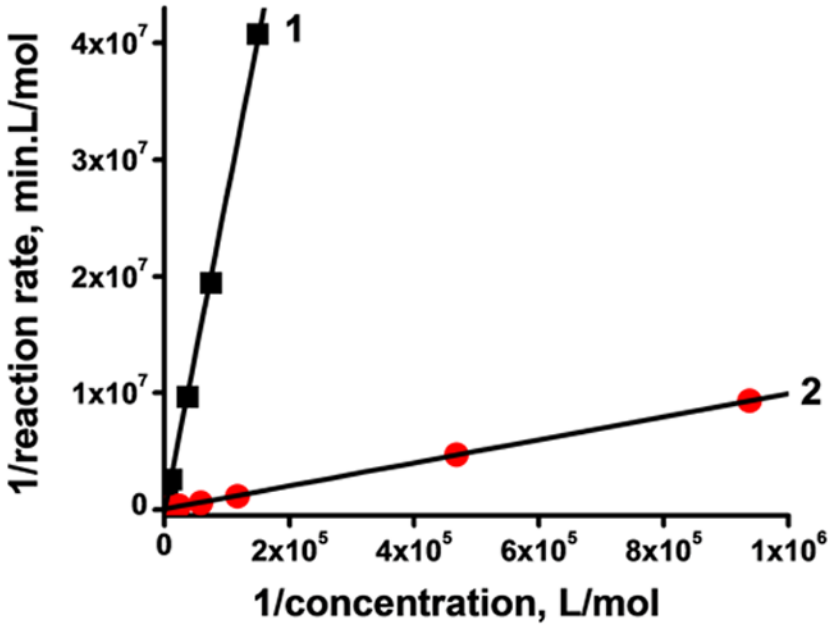

Figure 6.
Lineweaver-Burke plots for catalysis using free lipase in the solution (1) and lipase immobilized on organic polymer monolith (2). Conditions: 5 mg/mL lipase solution in 250 mmol/L phosphate buffer, 5 – 700 nmol/L tributyrin in hexane, reaction temperature 37°C.

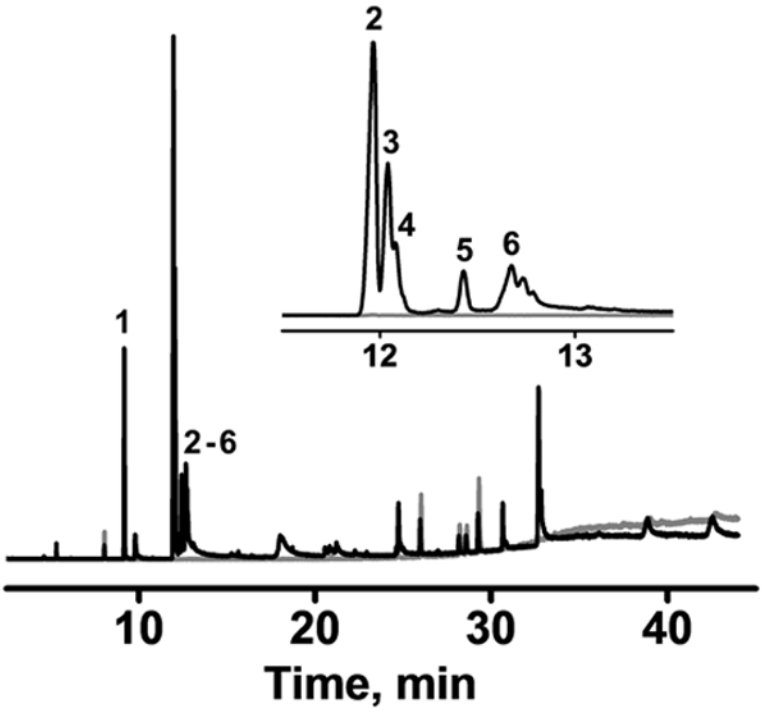

Figure 7.

GC-MS chromatograms of reaction mixture containing 100 μL kitchen vegetable oil, 300 μL methanol, 500 μL hexane, and 500 μL *tert*-butanol before (a) and after (b) pumping through the optimized reactor with immobilized lipase. Insert shows enlarged part of the chromatogram (b). Reaction temperature 37°C, residence time 10 min. Peaks: (1) pentadecanoic acid methyl ester, (2) 8,11-octadecadienoic acid methyl ester, (3) 9-octadecenoic acid methyl ester, (4) 9,12,15-octadecatrienoic acid methyl ester, (5) octadecanoic acid methyl ester, (6) 9,12-octadecadienoic acid methyl ester.

Table 1

Box-Behnken design of experiments calculated for space with three variables: x_1 – type of monomer; x_2 – grafting time of vinylazlactone, min; x_3 – lipase immobilization time, h.

Run	x_1[a]	x_2	x_3
1	BMA	1	4
2	BMA	20	4
3	SMA	1	4
4	SMA	20	4
5	BMA	10	2
6	BMA	10	8
7	SMA	10	2
8	SMA	10	8
9	LMA	1	2
10	LMA	1	8
11	LMA	20	2
12	LMA	20	8
13	LMA	10	4
14	LMA	10	4
15	LMA	10	4

[a] BMA – butyl methacrylate, LMA – lauryl methacrylate, SMA – stearyl methacrylate.

Table 2

The optimization of the original model of Equation 1 for production of butyric acid as calculated from results of gas chromatography analysis using the probability factors *f*. x_1 is the type of monomer used, x_2 is the vinylazlactone monomer grafting time, and x_3 is the lipase immobilization time; p_i are the regression coefficients of Equation 1, R is the correlation coefficient.

	***f*, probability factors**						
	step 1	**step 2**	**step 3**	**step 4**	**step 5**	**step 6**	**step 7**
constant	0.820	0.730	0.720	-	-	-	-
x_1	0.744	0.718	0.695	0.677	-	-	-
x_2	0.706	0.674	0.652	0.294	0.269	-	-
x_3	0.643	0.090	0.065	0.001	0.001	0.000	0.000
$x_1 \cdot x_2$	0.055	0.034	0.022	0.014	0.002	0.002	0.002
$x_1 \cdot x_3$	0.299	0.252	0.213	0.185	0.107	0.110	-
$x_2 \cdot x_3$	0.045	0.027	0.017	0.001	0.001	0.000	0.001
x_1^2	0.927	0.916	-	-	-	-	-
x_2^2	0.144	0.106	0.078	0.061	0.049	0.000	0.000
x_3^2	0.967	-	-	-	-	-	-
	***p_i*, regression coefficients**						
	step 1	**step 2**	**step 3**	**step 4**	**step 5**	**step 6**	**step 7**
p_0	3.91	3.35	3.13	-	-	-	-
p_1	3.00	3.00	3.00	3.00	-	-	-
p_2	0.53	0.54	0.53	0.83	0.83	-	-
p_3	3.17	3.44	3.44	3.94	3.94	4.38	4.38
p_4	1.16	1.16	1.16	1.16	1.25	1.25	0.86
p_5	-1.67	-1.67	-1.67	-1.67	-1.30	-1.30	-
p_6	-0.40	-0.40	-0.40	-0.44	-0.44	-0.44	-0.44
p_7	-0.45	-0.46	-	-	-	-	-
p_8	0.09	0.09	0.09	0.08	0.08	0.12	0.12
p_9	0.03	-	-	-	-	-	-
R	0.8445	0.8444	0.8441	0.841	0.8373	0.8122	0.7545

Table 3

Comparison of yields of butyric acids predicted using Eq. 2 and found in experiment.

Run	Predicted, %	Experiment, %	Δ, % [a]
1	15.0	18.3	3.3
2	14.7	8.4	6.4
3	16.8	15.9	0.8
4	49.0	49.9	0.9
5	3.8	12.6	8.8
6	4.0	6.0	2.0
7	20.9	31.9	11.0
8	21.1	6.8	14.3
9	8.0	1.6	6.4
10	31.7	33.8	2.1
11	40.5	36.9	3.7
12	14.6	19.7	5.2
13	12.4	16.4	3.9
14	12.4	19.8	7.4
15	12.4	9.3	3.1

[a] Absolute deviation between calculated and experimental values.