Supported Enzymes as Catalysts for Biodiesel Production

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Abstract

Biodiesel is emerging as a significant replacement for petroleum based automotive fuels. When the present level of the production of biodiesel from both edible and non-edible oil is considered, we see that it will not be able to cater for the demand required to replace conventional fuels without producing biodiesel in mass scale at a competitive price. It has been shown in the recent past that biocatalysts have tremendous potential in the production of biodiesel and, thus, enzymatic catalysis is getting more attention over the past decades. The advantages of enzymatic catalysis with reference to traditional alkaline catalysts are based on substrate and product specificity, activity and the ability of catalyzing the desired reactions efficiently in very mild conditions such as temperature, pressure, aqueous medium, and neutral pH, with reference to their native environment.

Zeolite (NaY-LZY-52 is the name of the zeolite used in this experiment) supported lipases have been shown to catalyze the transesterification of triglycerides efficiently, providing very stable catalysts. It has shown in the past, from the research by various people, that lipase itself is a promising catalyst for the production of biodiesel. This thesis work covers the study of assessing the possibility of using immobilized enzyme on zeolites as catalysts for the production of biodiesel by transesterification of triglycerides with alcohol. A set of zeolite supported immobilized enzyme catalysts has been prepared and kinetically characterized by the hydrolysis of a synthetic substrate (*p-nitrophenyl butyrate*) reaction. This allowed us to check for supported enzyme activity. The catalysts, which were found active, were used to produce biodiesel in a small scale using a triglyceride and butanol.

KEYWORDS: transesterfication, biodiesel, Zeolite supported lipases, immobilized enzyme

Introduction

Biofuels can play a vital role in transportation sector in future. Fossil based oil is depleting but yet no large scale replacement for oil-based transportation has emerged, except for a limited number of electric vehicles. Due to growing demand of fuels and high rate of increase in the number of cars, from 1 billion in 2010 to an expectable 2.5 billion on the road by 2050, it is the duty of researchers and engineers to find an alternative fuel for the vehicles. The biggest challenge is the production of biofuels in mass scale to cater for the demand. A variety of feed stocks with different technologies have been tested and tried over the past two to three decades to achieve this objective but still the task is huge. Much research is being done all over the world on biofuels to produce them in large scale as well as to improve the quality needed to replace fossil fuels. Biofuels as a renewable energy source are essential elements of the world's energy system. Biofuels are important because they help

in solving some of the most important challenges associated with energy use, the overwhelming dependency and increase in demand of fossil fuels, and the emissions associated to their use. A lot of research has already been carried out on this and it has been observed that by using a variety of biomass resources with recent technological developments it is possible to produce biofuels in mass scale. Lignocellulostic biomass derived from non-food sources such as grasses and trees can also be used to produce bioethanol, using enzyme catalysts. It can be used to produce other forms of biofuels by a variety of processes including liquefaction, pyrolysis or gasification. Non edible seeds such as *Jatropha* can also be used to produce biodiesel using transesterfication. However all these processes require an uninterrupted supply of feedstock in large quantities to produce biofuels in mass scale.

Materials and Methods

Reversed micelles for biodiesel production

Cutinase is dissolved in 400 mM phosphate or Tris buffer of pH 8.0. It is further microencapsulated in 150 mM AOT in isooctane with molar ratio of water: surfactant W_o as 2.7 by the injection method. This method consisted in adding drop wise the aqueous solution with cutinase to the AOT / isooctane solution while strong vortex mixing for 15 s. For biodiesel production in the reversed micelles solution was previously dried with molecular sieve and then a total reaction volume of 5 ml enclosed in a glass reactor of (10 ml) with magnetic stirrer. The conditions buffer molarity, pH, AOT concentration and W_o were chosen taking into account the desired optimization. The biodiesel production starts by adding the alcohol and triolein substrates to in the reversed micelles. The alcohol initial concentration is defined at 390 mM and the substrates molar ratio of alcohol to fatty acid chains of 1.6 has been used. The reaction started by adding alcohol with strong vortex mixing of 15 s.

Immobilization

50ml of cutinase has been washed and purified using phosphate buffer solution of pH 8.5, 50mM using a dialysis membrane (UHP-43, 50 ml). Two reactors; one at room temperature (RT) and the other at 4^{o}C were used. 0.1g of Zeolite NAY-LZY-52 (from Union Carbide) was added to 5 ml of free enzyme solution and kept stirring at 300 rpm over 24 hrs. After immobilization, the product has been centrifuged over 10 minutes at 5300 rpm (Eppendorf Centrifuge 5810R) to separate solid and supernatant.

Inoculum

Sterile petri dish with LB media supplemented with sterile ampicillin (150 µg ml^{-1}) in agar solid cultures were inoculated with *E. coli* WK-6 frozen cells, from the storage stocks at -80 ^{o}C and incubated at 37 ^{o}C.

Pre-fermentation

The composition of the medium for pre-fermentation was 20 g L^{-1} of LB medium (from Becton and Dickson) with initial pH of 7.5. The medium and shake flasks were previously sterilized by autoclaving at 121 ^{o}C for 20 min. 250 ml shake flask with a volume of 50 ml of the corresponding pre-fermentation media, supplemented with sterile ampicillin (150 µg ml^{-1}),

have been inoculated with cells of inoculum and have been left at 37 ^{o}C in an orbital shaker (Agitorb 200) at 250 rpm over 3 hrs.

Fermentation

The culture medium has been composed of 250 ml of TB medium by dissolving the chemical compounds: 12 gL^{-1} Bacto^{TM} Triptone and 24 gL^{-1} Bacto^{TM} Yeast Extract from Becton Dickinson, 5 ml L^{-1} Glycerol (from Acros), 3.81 gL^{-1} KH2PO4 (from Merck) and 12.51 gL^{-1} K_2HPO_4 (from Panreac), necessary to adjust and keep the pH at 7.1. The medium and shake flasks were previously sterilized by autoclaving at 121 ^{o}C, for 20 min. 1 M $MgSO_4.6H_2O$ (from Riedel-de Haen) solution was prepared and autoclaved separately to avoid the formation of precipitates, and then have been added aseptically after cooling, to obtain a concentration of 20 mM in the fermentation medium. The fermentation medium was used for the production of cutinase when the cells achieve high saturation density. In order to start fermentation with an optical density (OD at 600nm) of 0.2, a certain volume of the cells cultured in pre-fermentation medium have been transferred to 2 l shake flask with an initial volume of 250 ml of the fermentation culture medium, supplemented with ampicillin to a final concentration of 150 µg/ml. The fermentations have been performed in the orbital shaker at 25 ^{o}C and 250 rpm. At OD (600 nm) approximately of 1.0, the sterile inducer IPTG (isopropyl-β-D-thiogalactopyranoside) (from Bioline) was added to a final concentration of 0.1 mM, with a reinforcement of ampicillin (125 µl of a solution of 100 mg/ml to 250 ml of fermentation media). The sterilization of the thermolabile IPTG and ampicillin were perfomed by filtration using sterile Milex-Gp filter unit (0.22 µm pore diameter from Millipore).

Cutinase Activity assay

The activity of cutinase can be determined through a spectrophometric method followed by the hydrolysis of *p*-nitrophenylbutyrate (*p*-NPB) at 400 nm. This ester is hydrolyzed by cutinase following a Michaelis-Menten kinetics, and one unit of enzyme activity (U) corresponds to the amount of enzyme required for the production of 1 µmol of *p*-Nitrophenol (*p*-NP) per minute. As the *p*-NPB is temperature sensitive, it is necessary to keep the substrate in freezer before use, in order to prevent naturally occurring degradation. This assay is used in order to determine the activity of the cutinase obtained throughout the enzyme production process. Enzyme activity assay is determined using *p*-NPB in a 70 mM concentrated stock solution in pure acetronitrile. 15 µl of enzyme extract, previously diluted in Tris buffer, is added to reaction mixtures composed by 1470 µl of 20 mM Tris.HCl with pH 8 and 15 µl of *p*-NPB 70 mM solution inside the glass optical cell (3 ml) mixed by a small magnetic stirrer subjected to magnetic agitation of 700 rpm (Electronic Stirrer Model 300 Rank Brothers Ltd). The cell holder is kept at temperature of 30^{o}C by circulating warm water from a thermostatic bath (Thermomix MM,B.BRAUN) also at 30 ^{o}C. The reaction rate is determined by monitoring spectrophotometrically the hydrolysis of *p*-NPB and formation of *p*-NP at 400 nm (ε = 15,400$M^{-1}cm^{-1}$) at 30^{o}C for one minute. Dilutions of the enzyme samples in Tris Buffer were made in order to work in linear variation of the concentration of p-NP, i.e., intensity of its yellow colour (Hitachi U-2000

Spectrometer) using a software program (Hitachi Hyper Terminal software) to record the variation of absorption at 400 nm against time with respect to the amount of enzyme in this reaction mixture.

Transesterification procedure

The transestrification is performed in a batch stirred reactor at 35 ^{o}C maintained by an incubator from AGITOR B 160 E, ARALAB while the magnetic stirring is kept at 900 rpm using Thermo Scientific VAR 10 MAG Tele system. All biodiesel production reactions have been performed over 48hrs.

Transesterification Reactions

Reaction 1 (R1):
Control of buffer – This was prepared adding 72 µl of Tris. HCl buffer (400mM, pH8), 0.74g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex.
Biocatalyst Free Enzyme [Ext1] - This was prepared adding 72 µl of enzyme solution (Protein 4 µg/ µl, Activity of 706 U/ml), 0.77g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex.

Reaction 2 (R2):
Control of Buffer- This was prepared adding 25 µl of Tris. HCl buffer (400mM, pH8), 0.36g of Triolein
Control of buffer – This was prepared adding 72 µl of Tris. HCl buffer (400mM, pH8), 0.74g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex.
Biocatalyst Free Enzyme [Ext1] - This was prepared adding 72 µl of enzyme solution (Protein 4 µg/ µl, Activity of 706 U/ml), 0.77g of Triolein and 566 µl of n-Butanol to 10 ml of AOT / isooctane during 15 s in vortex and 283 µl of n-Butanol to 5 ml of AOT/ isooctane during 15 s in vortex.
Control of Zeolite – This was prepared adding 25 µl of Tris buffer (400mM, pH8), 0.36g of Triolein, 10mg of Zeolite(NaY,LZY,52) and 283 µl of n-Butanol to 5 ml of AOT/ isooctane during 15 s in vortex.

Biocatalyst IME1[Ext2] - This is prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein, 25 µl Immobilized suspension of enzyme at 4^{0}C (Protein 3.9 mg/ml, Activity 17.2U/ml),and 283 µl of n-Butanol to 5 ml of AOT/ isooctane during 15 s in vortex.

Reaction 3 (R3):
Control Zeolite - This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein , 20mg of Zeolite (NaY,LZY,52) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.
Biocatalyst IME2 [Ext3]– This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein , 80mg of Immobilized Enzyme Zeolite (NaY,LZY,52) support(Protein 3.6mg/ml, Activity 55.7 U/ml) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.
Biocatalyst IME3 [Ext4]– This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein , 80mg of Immobilized Enzyme Zeolite (NaY,LZY,52) support(Lyophilized enzyme of 1.6mg/ml, Activity 20.2 U/ml) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.
Reaction 4 (R4):
Biocatalyst IME4[Ext5] - This was prepared adding 25 µl of buffer (400mM, pH8), 0.36g of Triolein, 190mg of Immobilized enzyme Zeolite support (NaY,LZY,52) (Protein 9.5mg/ml, Activity 213

U/ml) and 283 µl of n-Butanol to 5 ml of AOT /isooctane during 15 s in vortex.

Results and Discussion

Calibration of High Performance Liquid Chromatography (HPLC) for analysis of alkyl glycerides and Biodiesel Production

The figure 1 shows the HPLC chromatograms obtained in the identification of substrates, alcohol, and products involved on the biodiesel production. The baseline changes present 4 characteristics plateaus, first one between 3-4 min, the second one between 5.5 and 9 min, the third one between 12 and 15 min, and fourth one between 18 and 21 min and they are in agreement with the different phases of the effluent gradient.

Figure 1 Typical chromatogram for the injection of a sample of triolein in reversed micelles reaction media.

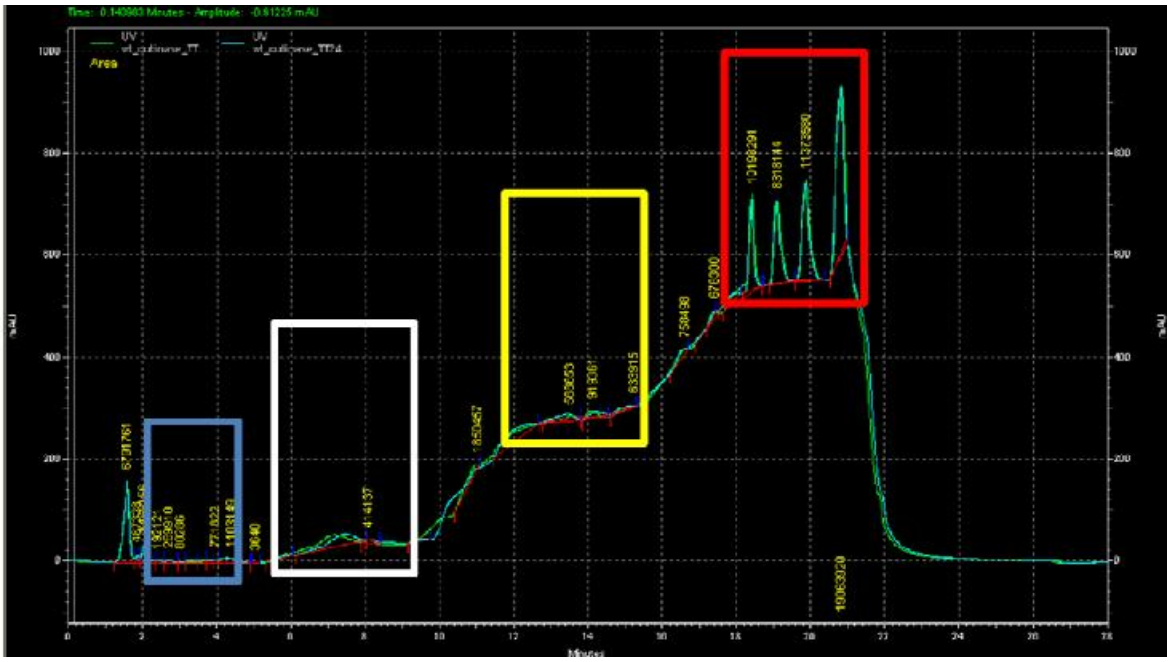

Figure 2 Resume of typical chromatogram and the time zone of substrates, intermediates and products. Blue rectangle – time zone of MG detection, White rectangle – time zone of Alkyl Esters detection, Yellow rectangle – time zone of DG detection, Red rectangle – time zone of TG detection.

The blue rectangle represents the usual retention time zone where the peaks corresponding to the monoglycerides (MG) are detected, the white rectangle time zone for alkyl esters (AE) detection, the yellow rectangle the time zone for diglycerides (DG) detection and the red rectangle the zone for triglycerides (TG) detection. It is expected that in the beginning of the reaction, (time zero) there exists a high concentration of TG, a low concentration of DG and MG and no alkyl esters present.

Biodiesel Reactions

The transesterification of triglycerides were performed in closed small scale reactors with total volume of 10 ml. Control experiments were performed in four different ways with: just buffer, pure zeolite, free enzyme, lyophilized enzyme; all experiments, including the ones with immobilized enzyme were carried-out using optimal conditions previously defined. The control experiments of R1, R2 and R3 performed with buffer and zeolite alone do not show any significant alteration of the chromatograms for zero and 48 hours reaction and consequently, indicate that no degradation occurs of the triolein neither has there been any partial hydrolysis of triolein with formation of DGs or MGs and transesterification as there is no peak corresponding to the alkyl esters resulting from the reaction of one of the triglycerides of the triolein with butanol.From the analysis of the different reactions and chromatograms it is possible to conclude the following: In R1, the production of biodiesel with

free enzyme with very high activity (Ext1) no significant consumption of the triglycerides occurred even after 48 hrs and neither significant amount of butyl esters (<10%) and diglycerides (<5%). The reason for this low transesterification yield is due to the high amount of water $W_o \geq 4$ present in the reversed micellar system. In R2, the cutinase from Ext2 was immobilized in the zeolite but also in this case there is not significant consumption of the triglycerides after 48 hours and neither butyl esters or diglyerides formation. The reason for this apparent failure on biodiesel production is due to low activity of the immobilized enzyme preparation (IME1) and the high amount of water $W_o \geq 5.4$ present in the reversed micellar system. In R3, two cutinase preparations (80 mg) previously lyophilized were used, the first one powder of free enzyme (IME 2) and the second one immobilized on zeolite (IME3) to control the amount of water in the reversed micellar system. Both enzyme preparations IME 2 and IME 3 gave an interesting transesterification yield (50 and 10%) and simultaneously formation of diglycerides (15 and 5%), respectively. The interesting results are

biodiesel production offers many advantages over the chemical catalysis that has been used over a years in practice. It has shown that it is more efficient due to enzyme specificity and selectivity. It greatly helps to consume less energy as it works under very mild conditions such as low temperature and pressure. It also helps to reduce water waste and produce more environmentally friendly product. Repeated use of the enzymes, enhancement of their thermal and operational stability, effective control of the amount of water in micellar system $W_o = 2.7$ already optimized by Badenes, 2010. These results can be improved by using cutinase extracts with higher activity. In R4, the IME4 was obtained from cutinase extract Ext5 with very high activity. After immobilization the IME4 the enzyme solution was removed by centrifugation and then completely dried at 30 ^{0}C before use in biodiesel production and kept with W_o at 2.7. By comparison of the all previous immobilized samples it is possible to conclude that the final immobilized sample IME4 transesterification yield is almost 100% as there was total consumption of triolein and more important without mono and diglycerides formation.With this work it is possible to conclude that reversed micellar system based on AOT and Isooctane and cutinase immobilized in zeolite can be an interesting biocatalyst on the biodiesel production.

Conclusions

From the results presented we can conclude that cutinase immobilized on zeolite NaY-LZY-52 is a promising catalyst for the production of biodiesel from triglycerides. Enzymatic reaction parameters, etc. are the benefits of the use of catalytic transesterification using immobilization of lipases. It also greatly helps to reduce the production cost of biodiesel. The possibility of using continuous reactors, packed and fluidized bed reactors, membrane reactors and stirred tanks with catalyst recycling are added advantages of using immobilized cutinase in biodiesel production.

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