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Organic Phase Synthesis of Ethyl Oleate Using Lipases Produced by Solid-state Fermentation

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Abstract This paper reports a study of the enzymatic esterification of oleic acid and ethanol. The reaction was catalyzed by lipases produced by solid-state fermentation with *Rhizopus* sp. Olive oil and perlite were used as an inducer and inert support, respectively. Synthesis of ethyl oleate was carried out in a 10-mL batch reactor with magnetic stirring. The effects of substrate ratios, biocatalyst concentration, and temperature on the reaction rate and conversion efficiency were evaluated. The highest reaction rate (1.64 mmol/L min) was reached with an oleic acid/ethanol mol ratio of 1:5 (oleic acid 50 mM:ethanol 250 mM) and 1 g of biocatalyst. Conversions approaching 100% were obtained after 60 min of reaction at 45 °C with *n*-hexane as a solvent. The initial reaction rate increased proportionally with respect to biocatalyst concentration, which suggests that the reaction rate was not controlled by mass transfer. The biocatalyst retained more than 80% of its catalytic activity after 7 months of storage at 4 °C. The results demonstrate that the biocatalyst produced by *Rhizopus* sp. in solid-state fermentation can be successfully used for ethyl oleate synthesis over short reaction periods under conditions when ethanol is in excess.

Keywords Lipases · Solid-state fermentation · Biosynthesis · Organic solvents

Introduction

Esters are important organic compounds with an increasing number of commercial applications [1]. These compounds are widely used for the preparation of aromatics, cosmetics, detergents, flavors, and pharmaceuticals [2]. Esters may also be used as

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plasticizers and lubricants [3]. Ethyl oleate is used as a biological additive, as a plasticizer, and as a hydraulic fluid [4]. Esters from natural sources are not available in large amounts, and they are usually too expensive to be used for commercial applications [5]. Therefore, many of the esters available are produced by either chemical or enzymatic synthesis [6]. Biotechnological production of esters using lipases has received great attention because of the mild reaction conditions (temperature, pH, and pressure) involved, the high degree of purity achieved, and the acceptance of these products in the food industry [7]. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) naturally catalyze the hydrolysis of triacylglycerides to glycerol and fatty acids [8]. Nevertheless, when these biocatalysts are used in organic solvents with low water content (low a_w), synthetic reactions are favored [9], which offers some important advantages [10]. Lipases are mainly produced by submerged fermentation [11]; however, the use of solid-state fermentation (SSF) for lipase production offers biotechnological advantages [12]. One of the major advantages of SSF for lipase production involves the possibility of utilizing them in biosynthetic processes without any extraction and immobilization prior to their use. This approach involves the use of the fermented solids as biocatalysts in bioreactions under non-conventional conditions (organic solvents or gas phase). In this study, the synthesis of ethyl oleate by a biocatalyst produced by SSF was evaluated. In order to improve the synthesis reaction rate and the conversion efficiency, substrate ratios, temperature, and biocatalyst concentration were evaluated.

Materials and Methods

Microorganism

The fungus *Rhizopus* sp. (strain IRD43aIV) belonging to a collection of thermotolerant strains and isolated at 50 °C from coconut pulp at the state of Colima on the Mexican west coast was used. A preliminary identification of the isolated fungi, based on morphological observations, revealed that this strain could be identified as *Rhizopus* sp. [13]. The strain was maintained in Potato-dextrose-agar (PDA) slants (Bioxon).

Preparation of Inoculum

The spores used to inoculate the SSF medium were produced in 250-mL conical flasks containing 60 mL of PDA medium. After 6 days of incubation at 30 °C, 50 mL of a 0.01% Tween 80 solution was added to the flask, and the suspension was stirred with a magnetic stirrer for 10 min. The spore concentration was estimated by using a Neubauer hematocytometer.

Culture Medium Composition

The composition of the culture medium for lipase production by SSF was [14] (in g/L): urea 4, lactose 5, olive oil 40, K_2HPO_4 5, $MgSO_4 \cdot 7H_2O$ 1, polyvinyl alcohol 1,6, oligoelement solution 4 mL/L. The pH value was adjusted to 6.5 with 10% HCl (*v*/*v*). The composition of the oligoelement solution was (g/L): EDTA 10, $MnCl_2 \cdot 4H_2O$ 1.98, $CoSO_4 \cdot 7H_2O$ 2.81, $CaCl_2 \cdot 2H_2O$ 1.47, $CuCl_2 \cdot 2H_2O$ 0.17, $ZnSO_4 \cdot 7H_2O$ 0.29. In order to dissolve the constituents, the pH of this solution was adjusted to pH 4 with 10% HCl. All reagents were J. T. Baker. The culture medium was used without sterilization. The fast

growth rate of this strain, in addition to a heavy inoculum and a low water content (see below), allowed to carry out the culture without microbial contamination.

Lipase Production by SSF

Perlite (Dicalite, México) was used as inert support (IS). The material was milled to obtain a particle size of 0.8–1.19 mm, then, perlite was washed twice with hot tap water and twice with cold distilled water, then, it was dried at 60 °C, and stored under a dry atmosphere. The inoculated culture medium (3×10^7 spores/g IS) was used to impregnate perlite to attain a moisture content of 60%. Twenty grams of this mixture was placed into glass columns (2.0 cm in diameter and 20 cm in length) and incubated at 45 °C in a water bath. An aeration rate of 40 mL/min per column was used.

Production of the Biocatalyst

Two methods were used to prepare the biocatalyst (fermented dry solids): (1) Air-drying: dry air at a flow rate of 500 mL/min at 45 °C was passed through the fermented solids into the fermentation columns over a 24-h period. Moisture content after air-drying was lower than 1% *w*/*w*. (2) Freeze-drying: The fermented solids were placed in a laboratory freeze drier (Labconco LYPH-LOCK 6). After 6 h of lyophilization at 5–10 μm Hg and −50 °C, the fermented solids reached a moisture content lower than 1% *w*/*w*. After drying, the biocatalyst was stored at 4 °C away from light.

Esterification of Oleic Acid and Ethanol

The esterification reactions were carried out using *n*-hexane (Baker) as a solvent. Oleic acid (Baker) and anhydrous ethyl alcohol (Técnica Química, México) previously dissolved in *n*-hexane were used as substrates for the esterification reaction. The *n*-hexane dissolves long-chain fatty acids and has been previously used in this type of reaction [15]. The substrates and solvent were previously dehydrated using molecular sieves (Type 4A, Fluka).

The esterification reaction was carried out in a 10-mL reactor with magnetic agitation at 45 °C for 1 h. The reaction mixture (9 mL) containing oleic acid and ethanol was preheated at 45 °C. The reaction was started by the adding 1 g of the biocatalyst. Samples (0.5 mL) were withdrawn at regular intervals. The reaction was stopped by addition of 0.1 mL of 6 M HCl. The residual oleic acid was analyzed with the copper-pyridine reagent, as described above. The substrate conversion (%) was calculated from the oleic acid concentration at the beginning and at the end of the synthesis reaction. All the results were obtained from independent reactions. In some cases, experiments were carried out in triplicate, obtaining a variation coefficient lower than 9.8%.

Analytical Methods

Hydrolytic Lipase Activity

The olive oil emulsion for the enzymatic reaction was prepared as follows: 20 g of commercial olive oil (Carbonell, Spain) was added to 100 mL of a 100 mM Tris–HCl (Bio-Rad) buffer pH 8, containing 10 mM $CaCl_2$ (Baker), 0.25% polyvinyl alcohol (SIGMA), and 0.3% gum arabic (SIGMA). It was homogenized in an Ultra-Turrax (IKA-WERK) homogenizer at 10,000 rpm for 10 min. The copper acetate-pyridine reagent was

made by dissolving 50 g of copper acetate (Baker) in 1 L distilled water, then the mixture was homogenized for 30 min, and the pH was adjusted to pH 6.1 with pyridine (Baker). To measure the hydrolytic activity of the lipase preparation, 3 mL of preheated (45 °C) olive oil emulsion was mixed with 20 mg of the fermented dried material in 15-mL conical glass tubes (Corning), and the mixture was incubated at 45 °C for 15 min. The reaction was stopped by addition of 0.6 mL of 6 M HCl to each tube. After addition of 3 mL of *n*-hexane, the mixture was thoroughly mixed for 1 min and centrifuged at 7,000 rpm for 10 min at 4 °C. From each tube, 2.5 mL of surnageant was added to 0.5 mL of the copper acetate-pyridine reagent. This mixture was homogenized in a vortex for 1 min, and then the absorbance was measured at 715 nm with a UV/Vis spectrophotometer (Perkin-Elmer Lambda 25). The hydrolytic activity was calculated from the slope of the curve absorbance versus concentration of a standard emulsion containing olive oil emulsified in the Tris–HCl buffer (see above). One activity unit (U) was defined as the amount of enzyme releasing 1 mmol of oleic acid per minute under the above conditions.

Carbon Dioxide Analysis

Carbon dioxide (CO_2) production was used as an indirect measurement of *Rhizopus* sp. growth. CO_2 concentration in the dry air stream outlet from the fermentation column was monitored using an on-line gas chromatograph (Gow-Mac 580, Gow-Mac Instrumentation Co., Bethlehem, PA, USA) equipped with a thermal conductivity detector and an automatic injector, a gas-separating concentric column (outer column: 6 ft for 1/4 in. packed with activated molecular sieves, inner column: 6 ft for 1/8 in. packed with porous polymer mixture; CTRI, Alltech), and a software integrator program (Chroma Biosystemes, France) [16].

Moisture Content and Water Activity Analysis

The moisture content of the solid medium and the biocatalyst was gravimetrically determined using a moisture analyzer (OHAUS MB45; repeatability SD of 0.015–0.05%). An AQUA-LAB (CX-2) was used to determine the water activity of the fermented solids and the biocatalysts.

Results and Discussion

Production of Lipases

The CO_2 production rate was determined and used as a criterion to monitor and stop the fermentation process. In all cases, the incubation time for lipase production was stopped after the maximum rate of CO_2 production was reached. This procedure allowed the preparation of lipolytic extracts devoid of proteases, which are usually produced at the final stages of fermentation. The production rate of CO_2 reached its maximum value after 20 h of culture, reaching the maximum CO_2 production after 20 h of culture. This time is considerably shorter than the time reported (72 h) for lipase production with *Burkholderia cepacia* [17]. The hydrolytic lipase activity after 20 h of incubation was 75 IU/g IS. This activity represents ~65% of the activity measured in the commercial lipase preparation of Lipozyme RM IM® (Novozymes). Lipases from *Rhizopus* species have gained more attention because some of them exhibit thermostability and good stability in organic

solvents, which, therefore, have a great potential in biocatalysis [18]. For the esterification reactions, the biocatalyst was produced under the above conditions. The residual oleic acid (from olive oil) concentration in the biocatalyst was smaller than 0.14 mmol/g of inert support. This amount of residual oleic acid modifies the ratio of oleic acid/ethanol less than 5%; under these conditions, the esterification reaction modifies neither the substrate conversion nor the esterification rate.

Production of the Biocatalyst

The amount of water contained in reagents and the biocatalyst itself has a great influence on the reaction rate and on substrate conversion in reactions carried out in organic solvents. Enzymes require a small amount of water to maintain their structure, flexibility, and, therefore, their catalytic activity [19–21]. It has been calculated that the moisture content in enzyme-catalyzed reactions in non-polar solvents should range from 0.05% to 1% [22]. The drying methods and storage conditions described in the "Materials and Methods" section allowed us to obtain preparations with moisture content below 1% and water activity (a_w) values from 0.33 to 0.68. At a_w values above 0.68, the esterification rate was strongly (more than 80%) inhibited (data not shown). The hydrolytic activity was 62.3 and 61.5 UI/g IS for products obtained with air- and freeze-drying, respectively. The enzymatic activity losses were 17% and 18% when the fermented solids were air-dried or lyophilized, respectively, than the activity present in the solid fermented material before drying.

Effect of the Substrate Ratio on the Esterification Reaction

To evaluate the effect of the oleic acid/ethanol ratio on the esterification reaction, the oleic acid concentration was kept constant at 50 mM, while the ethanol concentration ranged from 50 to 450 mM. The reaction was started after addition of 1 g of biocatalyst. The highest esterification rate (Fig. 1) was obtained when the oleic acid/ethanol molar ratio was 1:5, which was more than 3.5 times greater that the esterification rate obtained with equimolar concentrations of oleic acid and ethanol. The highest (near 100%) substrate conversion was measured at an oleic acid/ethanol ratio of 1:5 (see Fig. 1). The positive effect of an excess of alcohol on esterification or transesterification reactions has been previously reported [16, 17, 23–27]. Table 1 shows values of conversion for esterification [17, 24] and transesterification [16, 25] reactions in different solvents, using similar

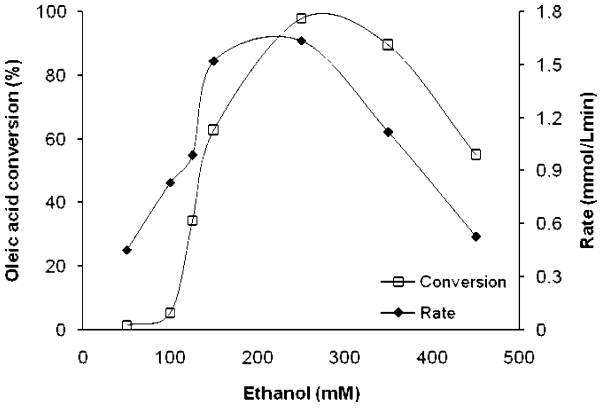

Fig. 1 Effect of the oleic acid/ethanol molar ratio on the initial esterification rate and the substrate conversion. Reaction conditions: oleic acid 50 mM, 60-min reaction at 45 °C

Table 1 Substrate conversion (%) for esterification and transesterification reactions

Substrates	Concentration (mM)	Relation molar	Temperature (°C)	Conversion (%)	Reaction time (h)	Biocatalyst	Reference
Lactic acid/ dodecanol	30	1:5	50	95	48	Novozym 435	[24][a]
Oleic acid/ethanol	70	1:5	37	94	18	Lipases by SSF	[17][b]
Anhydride acetic/ isoamyl alcohol	800	1:3	40	100	2	Novozym 435	[16][c]
Vinyl acetate/*n*-octanol	30	1:1	30	82	1.5	Novozym 435	[25][b]
Oleic acid/ethanol	50	1:5	45	97.8	1	Lipases by SSF	This study[c]

[a] In acetonitrile

[b] In *n*-heptane

[c] In *n*-hexane

catalysts and lipases produced by SSF and commercial enzymes (Novozym 435). This is compared with the data for the formation of ethyl oleate (shown in this work), which are also included in Table 1. The conversion was greater than 80% in all cases; nevertheless, the reaction time can vary from 60 min up to 48 h, depending on the reaction conditions. It has been suggested that the reaction rate and the extent of conversion are controlled by different variables, such as the source and catalytic activity of the enzyme, the type of reaction, the substrates, and the solvents used, as well as the temperature.

Effect of Substrate Concentration on the Esterification Reaction

In order to evaluate the effect of excess ethanol on the synthesis of ethyl oleate, a series of experiments were performed, keeping the oleic acid/ethanol ratio constant at 1:5, using 0.5 g of the biocatalyst. The studies were carried out in the same reactor at 45 °C. The initial esterification rates as a function of substrate concentration are shown in Fig. 2. The highest rate (1.79 mmol/L min) was obtained with oleic acid and ethanol concentrations of 70 and 350 mM, respectively. Although the highest substrate conversion (95.5%) was

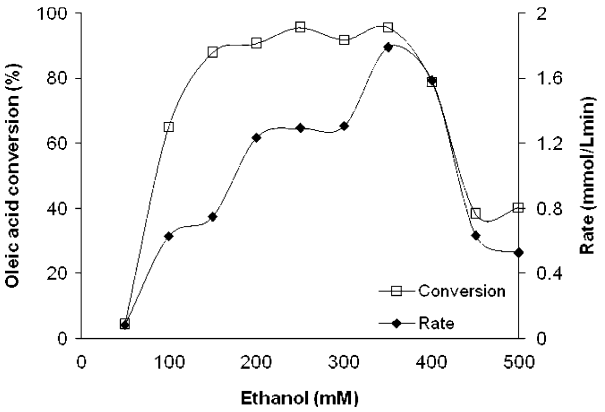

Fig. 2 Effect of substrate concentration on conversion and esterification rate. Reaction conditions: oleic acid/ethanol molar ratio 1:5, 60-min reaction at 45 °C

obtained at the same oleic acid–ethanol concentrations (70–350 mM), substrate conversions greater than 80% were obtained with an oleic acid concentration up to 80 mM (oleic acid/ethanol 80:400; Fig. 2). When ethanol was used at initial concentration greater than 400 mM, the reaction rate and the substrate conversion efficiency diminished more than 60%. Similar results were obtained by Oliveira et al. [28] for the enzymatic esterification of ethanol and oleic acid. The reduction in the esterification rate and substrate conversion at high oleic acid–ethanol concentration may be associated with the high ethanol concentration. Due to its highly hydrophilic nature, ethanol, like acetone, can affect the catalytic activity by causing protein precipitation [29]. Furthermore, it can also act as a competitive inhibitor by blocking the site that is normally destined for acylation [10].

Effect of Biocatalyst Concentration

The effect of biocatalyst concentration on the esterification rates and substrate conversion was evaluated (Fig. 3); biocatalyst concentrations from 0.2 to 1.6 g in 10 mL of the substrate solution were tested. Esterification reactions were carried out as described in "Analytical Methods". When biocatalyst concentrations above 0.02 g/mL were used, the substrate conversion efficiency was greater than 80% after 60 min of reaction at 45 °C. High conversions (near 100%), even at high biocatalyst concentrations, have been reported in previous studies [1, 3, 23]. These results may be interpreted by considering that the active sites of enzymes used in excess may not be exposed to substrates without contributing significantly to the reaction [3].

Figure 3 also shows the esterification reaction rate as a function of biocatalyst concentration. The nearly linear relationship (linear regression coefficient of 0.93) between these two variables means that the reaction depends upon the concentration of catalyst, as in the basic Michaelis–Menten model, suggesting that the reaction is not controlled by mass transfer. Similar results have been reported in another study [23] and indicate that the reaction may be kinetically controlled.

Effect of Temperature

The effect of the reaction temperature on the substrate conversion was evaluated over a range of 30 °C to 60 °C (Fig. 4). The highest esterification rate was measured when the

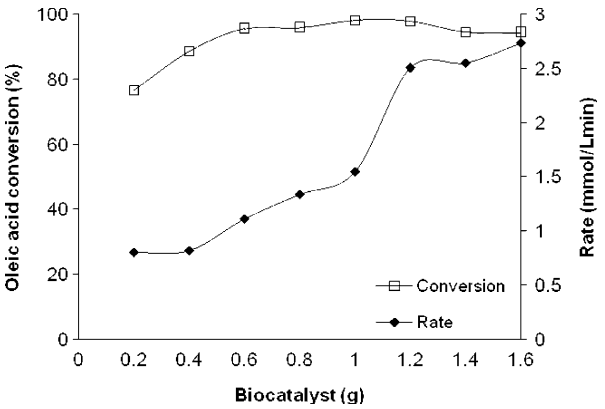

Fig. 3 Effect of biocatalyst concentration on esterification rate and substrate conversion efficiency. Reaction conditions: oleic acid 50 mM, ethanol 250 mM, 60-min reaction at 45 °C

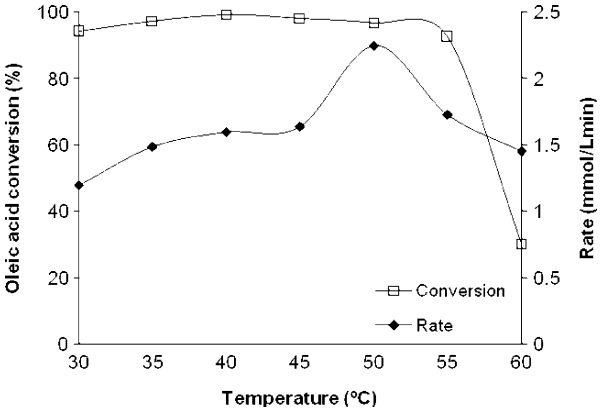

Fig. 4 Effect of temperature on the synthesis of ethyl oleate in hexane. Reaction conditions: 50 mM of oleic acid, 250 mM of ethanol, 1 g of biocatalyst, and an incubation period of 60 min

reaction mixture was incubated at 50 °C. Substrate conversion at temperatures from 30 °C to 55 °C was near to 100%. Incubation at 60 °C had a significant negative effect on substrate conversion efficiency.

The esterification rates obtained at the different temperatures assayed (30 °C to 55 °C) and the equation described by Nielsen et al. [30] were used to estimate the activation energy (37.3 KJ/mol). This activation energy is similar to the reported (38 KJ/mol) for the esterification of oleic acid with oleic alcohol and is typical of biocatalytic processes limited by the reaction rate [10]. Similar studies carried out with a mixture of oleic acid ant ethanol demonstrated that the esterification rate was kinetically controlled rather than controlled by mass transfer [7].

Biocatalyst Stability

The stability of the biocatalyst stored in the dark at 4 °C was evaluated. After 7 months, the biocatalyst retained more than 80% of its activity. This fact is important since the biocatalyst produced by SSF was air-dried without any additional treatment to improve its stability. In contrast, the lipase produced by *Rhizopus oryzae* maintained near to 100% of its activity after 235 days of storage once that it was extracted and immobilized on $CaCO_3$ [31].

Conclusions

The biocatalyst produced by *Rhizopus* sp. in solid-state fermentation was directly used in esterification reactions to synthesize ethyl oleate with high esterification rates and substrate conversion. This approach offers at least two advantages: (a) the use of air-dried solid-state fermented solids, without any additional extraction, purification, or immobilization processes, may allow the development of economically advantageous processes and (b) the biocatalyst obtained by SSF can be used in the sustainable production of products such as biodiesel from vegetable oils. The use of these types of biocatalysts has great potential for non-conventional reactions such as those in organic solvents or in the gas phase. More research will be necessary to explore aspects leading to possible applications that may be used to prepare products of commercial interest.

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