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Esterification Activity of Novel Fungal and Yeast Lipases

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Abstract The main objective of this work was the isolation and screening of microorganisms with potential for producing lipases for the synthesis of fatty esters as well as evaluating the specificity of the enzymes produced, using different alcohols (methanol, ethanol, *n*-propanol, and butanol) and fatty acids (oleic and lauric acids) as substrates. Promising biocatalysts for organic synthesis were obtained in this work. The isolated strains 69F and 161Y showed ability to efficiently catalyze the reaction for production of *n*-propyl oleate. Other strains can also be considered of potential interest, as 74F, 111Y, and 186Y. The future development of production using different substrates could result in cheap crude lipase of high importance to industrial applicability.

Keywords Lipase · Microorganism screening · Synthesis activity · Esterification

Introduction

Lipases (E.C. 3.1.1.3) can be used in modification of fat and oils by hydrolysis, esterification, and/or interesterification. These can be considered important catalysts in the production of specific fatty acids or glycerides from vegetable oils, being already successfully used as catalyst for synthesis of esters, which are produced from short-chain

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fatty acids with many industrial applications, as flavoring agents and long-chain acids used to enrich diesel fuels [1].

The ability of lipases to catalyze reactions in nonaqueous solvents has been extensively studied because when these biocatalysts are used in organic solvents with low-water content, synthetic reactions are favored and the enzymes have their stability increased [2].

These important biocatalysts can be secreted by several microorganisms. Thus, the exploration of biodiversity for searching new microorganisms that are able to produce enzymes is of great interest since most of the lipases used as catalysts in organic chemistry are of microbial origin [3]. Furthermore, the finding of promising newly isolated microorganisms can open promising scientific and commercial perspectives. Among several lipases described in the literature, only those obtained from a few species have presented biosynthetic ability for application as catalysts in non- and/or microaqueous media [4].

In this sense, the literature points out several methods for microorganisms screening [5]. However, some substrates may not be adequate for lipases detection, increasing the importance of determining the enzyme specificity. Both lipases and esterases can catalyze the hydrolysis and synthesis of an ester bond. Esterases hydrolyze esters with short-chain carboxylic acids, while lipases prefer long-chain fatty acids [6].

Solid-state fermentation (SSF) is an interesting alternative for the production of microbial enzymes due to the possibility of using agro-industrial residues and by-products as nutrient sources and support to microorganism development [7]. Fungal species are easily cultivated by solid-state fermentation. As an example, *Rhizopus* sp. [2], *Mucor hiemalis* [8], and *Aspergillus niger* [9] proved to be effective producers of biocatalysts to organic reaction medium. The strain of *Burkholderia cepacia* also showed the feasibility of producing lipase by SSF. The fermented solid could be directly used to catalyze esterification and transesterification reactions [10]. The major advantages of SSF for lipase production involve the possibility of using the catalyst in biosynthetic processes without any extraction and immobilization prior to its use. Enzymes produced in this way consist in an inexpensive and naturally immobilized biocatalyst, which can be applied for synthesis with organic solvents, since the gently dried SSF preparations do not require costly downstream process [8, 10].

Based on the previous mentioned information, the main objective of this work was to present the results obtained in the isolation and screening by solid-state fermentation of potential fungal and yeast species able to produce lipases with esterification activities.

Materials and Methods

Isolation of Microorganisms

The microorganisms were isolated from several contaminated sources as olive oil, cheese, tomato extract, soybean oil, milk cream, meat, soybean meal, and culture medium. Microorganism isolation and cultivation was carried out in Petri dishes using potato dextrose agar (PDA) medium at 30 °C. All stock cultures have been stored at −10 °C.

Screening of Microorganisms for Synthesis Reactions by Solid-State Fermentation

All previously isolated microorganisms were screened using SSF with soybean meal as substrate. The substrate was obtained from a same batch from a local industry (Olfar

SA-Erechim-RS-Brazil). The meal is the residue of soybean oil extraction after cold press and solvent extraction. The substrate was sieved (Tyler 1-115) and the major fraction (Tyler 9-16) was collected and stored at −18 °C until the moment of utilization. The soybean meal composition was (g kg^{-1}): moisture 30.0, protein 425.0, lipids 85.0, carbohydrate 300, fiber 100, ashes 60.0, nitrogen 68.0, and carbon 454.0 [11].

The fungus inoculum was grown in PDA medium at 30 °C for 7 days. Spores were collected in saline solution and used as inoculum for the SSF, standardizing the concentration at 10^8 spores/g of dry soybean meal [12]. The yeasts were grown in PC medium (% *w*/*v* in distilled water of 0.5% triptone; 0.1% glucose; 0.25% yeast extract) and LB medium (% *w*/*v* in distilled water of 1.0% triptone; 0.5% sodium chloride; 0.5% yeast extract) and incubated at 30 °C and 150 rpm. The yeasts strains 111Y, 161Y, 185Y, and 197Y were added to the fermentation matrix following inoculum standardization in terms of optical density of about 1.45.

Fermentation runs were carried out using 10 g of meal previously dried in cylindrical reactors covered with hydrophobic fabric. The moisture content of the meal was adjusted to 55% with distilled water. After sterilization (121 °C, 15 min), each reactor was inoculated and incubated at 27 °C, with humid air injection. After 48 h of fermentation, phosphate buffer (100 mM, pH 7.0), at a proportion of 4:1 (*v*/*w* of fermented cake), was added, and the enzyme extraction was carried out in rotary shaker at 150 rpm, 35 °C for 30 min [11]. After filtration of the solids, the filtrate was used for analytical assays. All experimental conditions were established based on previous works recently presented in the literature [13, 14].

Crude enzymatic extract was immediately frozen at −80 °C and then freeze-dried for 24 h [13]. Water content of the lipase extract was determined by a Karl Fisher system (model DL50 Mettler Toledo). The freeze-dried crude extracts were stored in glass flasks at ambient temperature.

Determination of Synthesis Activity

The synthesis of acid esters catalyzed by the dried crude enzymatic extracts was followed by titrimetric assays [15]. The esterification activity of lyophilized crude enzymatic extracts was measured by the consumption of acid in the esterification reaction with alcohols (1:1 equimolar ratio) with 100 mg of the enzymatic extract. In all experimental runs, a control assay (blank) was always carried out.

After incubation in shaker for 40 min at 40 °C and 160 rpm, the content of fatty acids remaining on the aliquot were extracted by the addition of 20 mL of an acetone/ethanol solution (1:1 *v*/*v*). The amount of fatty acids was then titrated with 0.01 M NaOH until pH 11. One unit of lipase activity was defined as the amount of dry enzyme preparation necessary to the consumption of 1 μmol min^{-1} of acid under assay conditions. All assays were performed in duplicate, and the results are expressed in terms of units per gram of dry substrate and calculated by Eq. 1.

$$\mathrm{EA} = \frac{(V_\mathrm{b} - V_\mathrm{a}) \times M \times 1000 \times V_\mathrm{f}}{t \cdot \mathrm{DCM} \times V_\mathrm{c}} \tag{1}$$

Where: EA=esterification activity (U/g); V_a=volume of NaOH used after 40 min (mL); V_b=volume of NaOH used at zero time of reaction (mL); M=molarity of NaOH solution; V_f=final reactional volume (mL); t=time (min); DCM=dry cake mass (g); and V_c=volume withdraw for titulation (mL).

Determination of Hydrolytic Activity

Lipase hydrolysis was assayed using an emulsion of olive oil (10%) in arabic gum (5%) in sodium phosphate buffer 0.1 mol/L pH 7.0. After incubation for 15 min at 37 °C and 160 rpm, the reaction was stopped by addition of a solution of acetone/ethanol (1:1 *v*/*v*). The fatty acids produced due to the hydrolysis were titrated with NaOH 0.05 mol/L. Control assays (blanks) were carried out adding the acetone/ethanol solution right after the enzyme addition. One unit of hydrolytic lipase activity is the amount of enzyme preparation necessary to produce 1 μmol min^{-1} of acid in the assay conditions. The results are expressed in terms of units per gram of dry substrate and calculated by Eq. 2 [16].

$$\mathrm{HA} = \frac{(V_a - V_b) \times M \times 1000}{t \times V_c} \times \frac{V_d}{m} \tag{2}$$

Where: HA=hydrolytic activity (U/g); V_a=volume of NaOH used after reaction (mL); V_b=volume of NaOH used at zero time of reaction (mL); M=molarity of NaOH solution; t=reactional time (min); V_c=volume of enzymatic extract used (mL); V_d=volume of solvent used for extraction (mL); and m=mass of solid used for extraction.

Alcohol Specificity of the Crude Enzymatic Extracts

The lyophilized crude enzymatic extracts obtained from each isolated microorganism by solid-state fermentation of soybean meal were tested as catalysts in synthesis reactions using oleic acid and different alcohols as substrates. Methanol, ethanol, *n*-propanol, and *n*-butanol were tested in this step.

Acid Specificity of the Crude Enzymatic Extracts

The lyophilized crude enzymatic extracts obtained from each isolated microorganism by solid-state fermentation of soybean meal were tested as catalysts in synthesis reactions using *n*-propanol and two different fatty acids (oleic and lauric) as substrates.

Results and Discussion

Screening of Microorganisms by Solid-State Fermentation

A total of 203 microorganisms (96 filamentous fungi, 3 bacteria, and 104 yeasts) were isolated from several sources, screened by solid-state fermentation of soybean meal, and tested as potential producers of lipases with synthesis activity. Among the 203 isolated strains, yeasts generally presented higher potential for lipases production than bacteria and fungi, considering the synthesis activity of *n*-propyl oleate.

Table 1 presents the 17 isolated strains able to produce lipases with esterification activity using oleic acid and *n*-propanol as substrates. The selected strains were obtained from samples of contaminated cream cheese, culture medium, and soybean meal. Several microorganisms were able to produce lipases with high esterification activities, ranging from 100 to 115 U/g of dry substrate, for strain 161Y and 69F, respectively. Only two filamentous fungi could be screened as potential lipase producers (69F and 74F). Another important finding was that some strains were able to catalyze reactions of synthesis (in organic

Table 1 Esterification activities of the lyophilized crude enzymatic extracts obtained from screened microorganisms using oleic acid and *n*-propanol as substrates.

Microorganism code	Source	Esterification activity (U/g dry substrate)
69F	Soybean meal	114.96±2.99
74F	Cream cheese	60.08±0.49
111Y	Soybean meal	64.80±0.67
116Y	Soybean meal	5.47±0.69
119Y	Soybean meal	8.94±1.83
134Y	Soybean meal	21.11±0.58
135Y	Soybean meal	44.50±1.85
137Y	Culture medium	9.52±1.19
141Y	Soybean meal	20.21±1.86
145Y	Cream cheese	18.20±0.41
146Y	Soybean meal	12.95±0.17
161Y	Soybean meal	100.42±4.23
182Y	Soybean meal	28.25±8.84
185Y	Soybean meal	2.59±0.25
186Y	Soybean meal	65.02±4.35
197Y	Cream cheese	19.39±0.05
208Y	Soybean meal	2.08±0.27

medium) and hydrolysis (in aqueous medium; data not shown; strains 69F, 74F, and 186Y). Similar behavior was observed by Cardenas et al. [5], which observed that all tested catalysts were active in both systems. However, the most active in one system did not necessarily showed the same behavior in the other one, demonstrating the particularities of each crude extract produced from different cultures.

Microorganisms isolated from soybean meal presented esterification activities ranging from 2.1 (208Y) to 115.0 (69F)U/g of substrate. Here, high enzyme production and easy adaptation of the substrate were observed probably due to the origin of the strains, isolated from soybean meal.

The mean water content of the crude enzymatic extracts obtained in this work, after 12 h of lyophilization, determined in Karl Fisher, was 12% (±1.3). High water contents (45%) in butyl oleate synthesis led to a reduction in enzymatic activity [17]. This is probably caused by an enzyme denaturation and the occurrence of the inverse reaction. In this way, we can verify that water content of reactants and enzyme can influence the reaction rate since lipases need low-water content to keep their structure, stability, and catalytic activity in organic medium [2, 18].

Alcohol Specificity of the Crude Enzymatic Extracts

The rate of the synthesis reactions and the extent of conversion are controlled by different variables, such as source and catalytic activity of the enzyme, type of reaction, temperature, and in a higher extent, on the substrates and the solvents involved in the process [2]. Stability and activity of enzymes in organic solvents depend not only on the properties and concentration of the organic solvents, but also on the nature of the substrates. In this sense, the control of the parameters becomes of essential importance for achieving maximum reaction rates [19].

Microbial lipases produced in substrates containing organic solvents, oils, or fat can present high stability in organic medium [20]. In this work, the effect of different alcohols on catalytic activity of lipases produced by solid-state fermentation with the previous preselected 17 strains was evaluated. Results obtained in this step are presented in Table 2. The experimental data were evaluated by Tukey's test.

The use of methanol demonstrated that the strains 141Y and 186Y (101.03 and 82.25 U/g substrate) were able to produce lipases able to catalyze the synthesis of methyl oleate. Higher esterification activities using ethanol were obtained for lipases produced by the strains 134Y, 69F, and 111Y (107.94, 104.97, and 101.67 U/g substrate, respectively). On the other hand, the use of *n*-butanol led, in general, to lower enzyme activities, indicating that the screened microorganisms were able to produce lipases with potential to catalyze reactions evolving short-chain alcohols. Similar behavior was observed by Romero et al. [21].

The differential catalytic behavior of crude enzymatic extracts in organic medium, using oleic acid and methanol, ethanol, *n*-propanol, and *n*-butanol, indicates that lipase activities vary according to the solvent. These results are in agreement with data reported to lipase from *A. niger* MYA135, in reactions using methanol, *n*-propanol [21] and also, to lipases from *Pseudomonas aeruginosa* that present lower activities in long-chain alcohols as *n*-butanol and isopropanol (log *P* of 0.8 and 0.28, respectively).

A promising value for the synthesis of *n*-propyl oleate was obtained using the isolated strain 69F, resulting in an activity of 114.96 U/g of substrate, followed by the microorganisms 161Y, 186Y, 74F and 111Y, with activities varying from 64.80 to 100.42 U/g of substrate. The activities in *n*-butanol were lower compared to the other tested alcohols. The residual activity

Table 2 Esterification activity using oleic acid and different alcohols (methanol, ethanol, *n*-propanol, and *n*-butanol) as substrates.

Microorganism code	Esterification activity (U/g dry substrate)			
	Methanol	Ethanol	*n*-Propanol	*n*-Butanol
69F	1.57±0.09a	104.97±0.20a,b	114.96±2.99a	32.56±1.35a
74F	29.88±2.25b	76.08±1.91c	60.08±0.49b	32.84±0.5a
111Y	38.63±0.82c	101.67±0.60a,b	64.80±0.67b	13.84±0.68b
116Y	1.52±0.17a	81.85±1.76c	5.47±0.69c	10.38±1.06b,c
119Y	16.53±2.18d	45.69±2.43d	8.94±1.83c,d	12.75±0.39b,d
134Y	2.40±0.19a	107.94±0.32a,e	21.11±0.58e	19.57±0.35e
135Y	42.83±2.06c	53.72±1.19f	44.50±1.85f	27.80±1.32f
137Y	28.77±1.40b	27.42±2.54g,i,j	9.52±1.19c,g	9.58±0.80c,d,g
141Y	101.03±1.06f	12.28±1.45h	20.21±1.86d,e,g	17.43±1.42b,e
145Y	53.55±2.23e	20.43±2.18i	18.20±0.41d,e,g	23.57±0.23h
146Y	14.44±0.91d	1.42l±0.13	12.95±0.17c,e	8.59±0.08c,i
161Y	18.44±0.49d	13.64±1.35h	100.42±4.23h	18.32±1.22e
182Y	42.82±2.17c	13.54±1.33h	28.25±8.84e	8.50±1.03c,j
185Y	18.89±1.58d	55.18±1.20f	2.59±0.25c	11.99±0.86b,g,i,j
186Y	82.25±0.56g	21.99±2.50g,i	65.02±4.35b	27.25±0.96f,h
197Y	26.62±1.49b	12.27±0.24h	19.39±0.05d,e,g	28.71±1.44a,f
208Y	49.05±0.08e	33.03±0.12g,j	2.08±0.27c	29.01±1.36a,f

Means ± standard error followed by different letters in the same column are significantly different ($p<0.05$) by Tukey's test

determined in methanol and ethanol, of 80% and 90%, respectively, after 24 h of exposition, corroborated the above-mentioned statement.

At this point, it is worth to mention that lipases stable to methanol and ethanol can be extremely useful for biodiesel production [22, 23], justifying researches in this field of knowledge. In this sense, just a few works are presented in the literature related to the esterification activities of lipases produced by solid-state fermentation. One can cite, as example, the results obtained by Ruiz et al. [2] in the production of ethyl oleate using a lipase from *Rhizopus* sp., with an enzyme activity of 1.5 U/mL. Dutra et al. [9] achieved a lipase activity of 120 U/g in the production of butyl oleate. Based on the literature, the results obtained in the present work can be considered good since activities of about 108 U/g were obtained in nonoptimized experimental conditions.

Acid Specificity of the Crude Enzymatic Extracts

The esterification of long-chain acids represents a good way to discriminate lipases from other hydrolases probably present in the fermented material [24]. The specificity of the lipases produced by solid-state fermentation of the 17 screened strains was carried out using *n*-propanol and oleic and lauric acids as substrates. Table 3 presents the esterification activity of the lipases in the different tested acids. By the variance analysis and Tukey's test, one could verify that higher esterification activities in lauric acid were obtained for strains 111Y and 182Y (80.56 and 81.51 U/g of substrate, respectively).

For the strain 69F, the activity in lauric acid was 66% lower (39.44 U/g of substrate) from that obtained for oleic acid. For the strain 111Y, a higher activity (80.56 U/g of substrate) was obtained in lauric acid compared to the use of oleic acid as substrate.

Promising biocatalysts for organic synthesis were obtained in this work by the screening of microorganisms. The isolated strains 69F and 161Y showed ability to efficiently catalyze

Table 3 Esterification activity using *n*-propanol and different acids (oleic and lauric) as substrates.

Microorganism code	Esterification activity (U/g)	
	Oleic acid	Lauric acid
69F	114.96±2.99a	39.44±0.22a
74F	60.08±0.49b	5.40±0.64b
111Y	64.80±0.67b	80.56±5.85c
116Y	5.47±0.69c	10.56±0.04b,d
119Y	8.94±1.83c,d	39.51±0.73a
134Y	21.11±0.58e	13.73±0.86d,e
135Y	44.50±1.85f	80.05±7.04b,e,h
137Y	9.52±1.19c,g	10.08±0.87b
141Y	20.21±1.86d,e,g	1.02±0.18b,e,f
145Y	18.20±0.41d,e,g	11.98±0.74b,e,g
146Y	12.95±0.17c,e	6.28±2.40b,e
161Y	100.42±4.23h	15.87±2.72d,f,g,h
182Y	28.25±8.84e	81.51±2.75c
185Y	2.59±0.25c	52.81±0.11i
186Y	65.02±4.35b	8.58±1.35b,e,h
197Y	19.39±0.05d,e,g	9.58±0.77b,e,h
208Y	2.08±0.27c	9.41±0.06b,e,h

Means ± standard error followed by different letters in the same column are significantly different ($p<0.05$) by Tukey's test

the reaction for production of *n*-propyl oleate. Other strains can also be considered of potential interest as 74F, 111Y, and 186Y. Future development of production using different substrates could result in cheap crude lipase of high importance to industrial applicability.

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