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Studies on Immobilization and Partial Characterization of Lipases from Wheat Seeds (*Triticum aestivum*)

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Abstract The objective of this study was to provide some features on immobilization and partial characterization of lipases from wheat seeds. The optimum pH and temperature were found to be 5.5 and 32–37 °C, respectively. The stability of the concentrated enzymatic extract to high temperatures (25, 35, 45, and 55 °C) showed that the incubation of the extract at 55 °C led to its complete inactivation. The concentrated enzymatic extract kept 90% of its hydrolytic and esterification activities until 70 and 40 days of storage at 4 °C, respectively. The extract presented higher hydrolytic specificity to substrates of medium and long chains and higher esterification affinity to fatty acids of short and medium chains and alcohols with two and three carbon atoms. After the immobilization process using activated coal and sodium alginate as supports, an enhancement of about threefold in lipase activity was observed. The development of the present work permitted us to point out some characteristics of lipases from wheat seeds necessary for the proposition of new future industrial applications for this important biocatalyst.

Keywords *Triticum aestivum* · Lipases · Partial characterization · Immobilization

Introduction

Lipases, triacylglycerol hydrolases, have a number of unique characteristics, including substrate specificity, stereospecificity, and the ability to catalyze a heterogeneous reaction at the interface of water-soluble to hydrolysis reactions [1].

These enzymes can be obtained from animals (pancreatic, hepatic, and gastric), microorganisms (fungi and bacteria), and plants (almond, seeds, leaves, and stems), with

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differences in the catalytic properties as a function of the source. A great interest in studies concerning the extraction of plant lipases, aiming at reducing the production costs and thus making intense their use in industrial applications, has been observed [2]. Several applications are related to this class of enzymes, and they represent nowadays about 35% of enzymes employed in biochemical processes [3]. Another important aspect is that related to the enzyme application to food (natural, Kosher and/or Halal restrictions, etc.) or pharmaceutical industries. In this case, regulatory restrictions about the use of microbial and animal enzymes can be found.

Plant lipases have been isolated from leaves, stems, latex, oils, and seeds of oleaginous and, in a lower scale, from cereal seeds, as rice and wheat (*Triticum* sp.). Wheat seeds are considered the most important cereal in human food, presenting an annual cycle of 90–180 days, depending on the ambient and genotype, cultivated during winter and spring since some varieties are dependent on cold and light intensity [4]. However, despite the huge importance of such cereal in human life, surprisingly, no work was found in the available literature regarding the immobilization and partial characterization of lipases from wheat seeds. Studies from the literature revealed that lipases from seeds present, in general, type selectivity by fatty acid [5]. However, the specialized literature in general is restricted to the hydrolytic activities of lipases from these seeds. The knowledge of some characteristics of this biocatalyst, as optimum temperature and pH, stability to pH and temperature, stability to low temperatures, and specificity to different substrates are of extreme importance for determining possible industrial applications [6–9], both in terms of hydrolytic and esterification activities.

Another important feature concerning the industrial application of lipases is related to their stability in organic solvents [10]. Therefore, the enzymes can be subject to inactivation by chemical, physical, and biological factors. In these cases, to provide an efficient model for the process, enzyme immobilization is of primary importance to provide stability to the enzymes, facilitate its recuperation, and make possible its reuse [5].

Based on these aspects, the objective of the present study was to provide some features on immobilization and partial characterization of lipases from wheat seeds (*Triticum aestivum*).

Material and Methods

Extraction of Lipase from Wheat Seeds

The experimental procedure for the extraction of lipases from wheat seeds followed the methodology proposed by Cavalcanti et al. [11], with some modifications. The choice of this raw material was based on a previous work by our research group [12] where we have evaluated the hydrolytic lipase activity using wheat germ and wheat seed as lipase sources. The results obtained permitted us to observe that the hydrolytic activities for lipases obtained from wheat germ were, in general, lower than that obtained when using wheat seed as the enzyme source.

The crude enzymatic extract was concentrated by precipitation with ammonium sulfate. The higher hydrolytic activity was 5.9 U/g with a particle size of 425 μm, solid/solvent ratio of 30:60 (*w*/*v*), and reaction time of 15 h, with maximum hydrolytic activity of 14.47 U/g after 24 h of precipitation. For esterification activity, the best result was 57.88 U/g with a particle size of 425 μm and solid/solvent ratio of 30:120 (*w*/*v*) for 5 h, with a maximum value after 10 h of precipitation reaching 208.20 U/g [12].

Analytical Methods

Lipase Hydrolytic Activity

Lipase hydrolytic activity was assayed by reaction using olive oil as substrate followed by alkali titration. Olive oil (10%, *w*/*v*) was emulsified with arabic gum (5%, *w*/*v*) in 100 mM sodium phosphate buffer, pH 7.0, for approximately 2 min and 3,000 rpm using a domestic blender. A 2-mL sample of crude enzyme was added to 18 mL of this emulsion. After incubation in a shaker for 15 min at 37 °C and 150 rpm, the reaction was interrupted by the addition of 20 mL of an acetone/ethanol solution (1:1, *v*/*v*). The amount of fatty acids liberated was then titrated with 0.05 M NaOH until pH 11. Reaction blanks were run in the same way, but adding the sample after the addition of acetone/ethanol solution. Lipase activity measurements were carried out in duplicate. One unit of hydrolytic lipase activity (HA) was defined as the amount of enzyme that yields 1 μmol of fatty acids per minute in the assay conditions [13].

Lipase Esterification Activity

The lipase esterification activity was determined as the initial rates in esterification reactions between lauric acid and *n*-propanol at a molar ratio of 3:1, temperature of 60 °C, and enzyme concentration of 5 wt.% in relation to the substrates. At the beginning of the reaction, samples containing the mixture of lauric acid and *n*-propanol were collected and the lauric acid content determined by titration with 0.04 N NaOH. After the addition of the enzyme to the substrates, the mixture was kept at 60 °C for 15 min. Then, lauric acid consumption was determined. One esterification lipase activity unit was defined as the amount of enzyme necessary to consume 1 μmol of lauric acid per minute at the established experimental conditions presented previously [14]. All enzyme activity determinations were replicated at least three times.

Partial Characterization of Concentrated Enzymatic Extract

The partial characterization of the concentrated enzymatic extract was carried out in terms of optimum temperature and pH, stability to low and high temperatures, specificity to different substrates of hydrolysis and esterification, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique.

Optimum Temperature and pH

For the determination of optimum values of temperature and pH of the concentrated enzymatic extract, a 2^2 full experimental design with triplicate of the central point was carried out. The ranges of the studied pH and temperature were 5.5 (acetate buffer), 7.0 (sodium phosphate buffer), and 8.5 (tris-amino methane buffer) and 32–42 °C, respectively. Samples were incubated for 15 min and 150 rpm. All analyses were carried out in triplicate.

Stability to High Temperatures

The stability of the concentrated enzymatic extract to high temperatures was performed by incubation of the lyophilized extracts at 25, 35, 45, and 55 °C. The extracts were dissolved in sodium phosphate buffer (100 mM, pH 7.0) and the hydrolytic and esterification activities monitored periodically until the total loss of activity.

To predict the inactivation profiles under isobaric/isothermal and constant pH conditions, the decrease in the enzyme activity as a function of treatment time was described by:

$$\frac{1}{A} = \frac{1}{A_0} + k \cdot t \tag{1}$$

where A and A_0 are the residual and initial activities (U/g), respectively, t is the exposure time (min), and k is the rate constant of inactivation (g U^{-1} min^{-1}). The slopes of these lines were determined by a linear regression analysis ($1/A$ versus time), where k is the inclination and $1/A_0$ is the intersection. For the case of temperature, the deactivation energy was calculated from the slopes of these Arrhenius plots according to Eq. 2:

$$\ln(k) = c - \frac{E_a}{R \cdot T} \tag{2}$$

where R is the gas constant (8.314 $Jmol^{-1}$ K^{-1}), c is a constant, E_a denotes the activation energy, and T is the temperature (K). The thermal stability can also be evaluated in terms of half-life time, defined as the time required for the enzyme to lose 50% of its initial activity, A/A_0=0.5. The half-life time ($t_{1/2}$) was calculated using Eq. 3:

$$t_{\frac{1}{2}} = \frac{-\ln(0,5)}{K_d} \tag{3}$$

where $t_{1/2}$ is the half-life time and K_d is the deactivation constant.

Stability to Low Temperatures

The concentrated enzymatic extracts were stored (samples of 2 g) at 4 and −10 °C, being the hydrolytic and esterification activities measured until the total loss of activity.

Specificity of the Concentrated Enzymatic Extract

Hydrolysis Substrates Different triglycerides were used for evaluating the hydrolysis activity of the crude and concentrated enzymatic extracts: olive oil, coconut oil, and tributyrin. Enzyme activity was measured as described before.

Esterification Substrates The lyophilized crude and concentrated enzymatic extracts were tested as catalysts in esterification reactions using different alcohols and fatty acids as substrates. Methanol, ethanol, and *n*-propanol and butyric, lauric, and oleic acids were used in this step. Enzymatic esterification activities were measured as described before.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The analyses of PAGE with SDS were carried out in a vertical electrophoresis system mini-VE (Amersham Biosciences) using gels with final concentrations of acrylamide monomers of 12.5% and 15% in a batch system of buffers and pH and a EPS 300 font (Pharmacia Biotech) [15, 16].

Protein samples were diluted with a volume of buffer concentrated two times (187.5 mM Tris, 5% of β-mercaptoethanol, 10% of glycerol, 1.75 mM SDS, and 1% of bromophenol blue, pH 6.8), heated for 5 min at 100 °C, and kept at 4 °C until use.

Ten microliters of each sample and 5 μL of the standard of molecular mass (unstained protein molecular weight marker, Fermentas) were applied to the gel, and the separation was carried out at 30 mA for 70 min at room temperature.

The gel was then immersed in a dye solution (ethanol/acetic acid/distilled water, 45:10:45, *v*/*v*/*v*) added to 0.2% of Coomassie brilliant blue for 18 h. Then, the gel was immersed in a decolorization solution (acetic acid/ethanol/and water, 10:30:70, *v*/*v*/*v*) under low agitation.

The zymogram was carried out under the no-reducer SDS-PAGE. After the electrophoresis, the gel was immersed in a 2.5% solution (*p*/*v*) of Triton X-100 under constant agitation for 30 min. Then, it was washed with phosphate buffer (0.1 M, pH 7.0) and put down under a transluminator UV and added 100 μL of diluted MUF solution. After the reaction, the fluorescent bands, referred to the lipases/esterases, were observed.

Immobilization of the Concentrated Enzymatic Extract

The concentrated enzymatic extract from wheat seeds was immobilized using a gel solution (16.5 g of distilled water and 0.75 g of sodium alginate), followed by the addition of 12.5 g of sucrose. After solution cooling, 2 g of the enzymatic extract, 3.5 g of glutaraldehyde, and 0.75 g of activated coal were added to the gel solution [17]. The gel solution was pumped to a calcium chloride solution (0.2 M in acetate buffer 0.1 M, pH 4.8, and 3.5% of glutaraldehyde) at 10 °C under agitation. After the adsorption process, samples were filtered under vacuum and kept in a desiccator for 48 h. Samples of the immobilized lipases were also kept at 5 °C for 24 h and then washed with distilled water and immersed in calcium chloride solution (0.05 M in acetate buffer 0.1 M, pH 4.8). Then, the hydrolytic and esterification activities were measured following the methodology presented previously. The immobilized enzymatic extracts were stored at 4 °C and the lipase activities were measured each 6 days, until 56 days of storage.

Statistical Analysis

The results obtained along the work were statistically treated by analysis of variance followed by Tukey's test ($p<0.05$) with the use of software Statistica version 5.0 (Statsoft Inc., USA).

Results and Discussion

Optimum Temperature and pH

From the best conditions found for the crude enzyme extract concentration, an experimental design was carried out so as to evaluate the effect of temperature and pH on the hydrolytic activity of the extract. The hydrolytic activities varied from 3 to 10 U/g. Data were statistically treated and the calculated F value of 33.93, higher than the listed F (6.94), permitted us to validate ($p<0.05$) an empirical coded model ($R=0.97$), presented in Eq. 4, for HA in terms of optimum temperature and pH.

$$\mathrm{HA} = 7.35 - 2.45.\mathrm{pH} + 0.89.\mathrm{pH}.\mathit{Temperature} \tag{4}$$

The model validation permitted to build the response surface (shown in Fig. 1) of hydrolytic activity in terms of the interaction between pH and temperature. From this

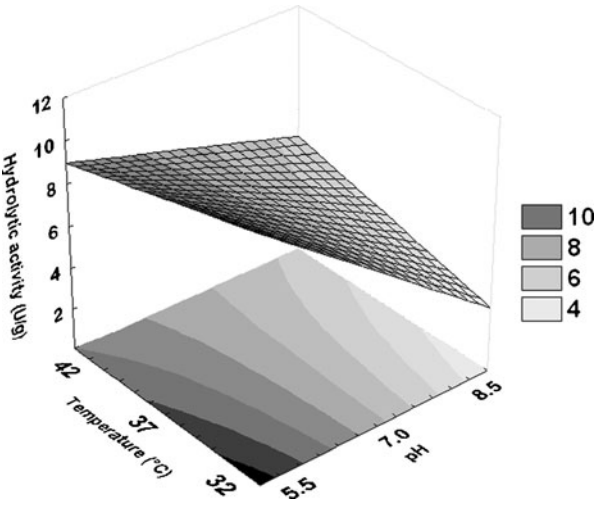

Fig. 1 Response surface for the interaction between pH and temperature for the hydrolytic activity of the concentrated enzyme extract from wheat seeds

figure, one can observe that higher hydrolytic activities were obtained at lower pH values for temperatures from 32 to 37 °C.

The literature points out optimum temperature values in the range from 45 to 55 °C and pH from 7.5 to 8.0 for lipases extracted from plant source. Cavalcanti et al. [11], for example, obtained higher hydrolytic activities using castor seeds in acid pH (about 4.0) and temperature of 30 °C. The analysis of the data presented in the literature permit us to observe that most microbial and plant lipases present optimum pH ranging from 7.0 to 9.0. This behavior was not observed for lipases from wheat seeds, making it possible to propose new applications for the enzyme obtained in this work, mainly when acid conditions are required.

Stability to High Temperatures

The stability of the concentrated lipase extract from wheat seeds to high temperatures was evaluated in the range of 25–55 °C using sodium phosphate buffer 100 mM, pH 7.0. The incubation of the enzymatic extract at 55 °C showed complete inactivation both in terms of hydrolytic and esterification activities. The data experimentally obtained were plotted as $1/A$ versus time for obtaining the K_d (thermal deactivation constant) for each evaluated temperature. The plot of ln K_d as a function of $1/T$ (K) made it possible to obtain the values of E_d (activation energy). Table 1 presents the results obtained in this step for the enzyme activities of lipases from wheat seeds. From the inclination of the curve, the K_d values were calculated as 215.5 and 239.08 kJ mol^{-1} for hydrolytic and esterification activities, respectively. The half-life times were calculated using Eq. 3, and one can observe that for both enzymatic activities, the value of this parameter enhances as the temperature increases.

The value of K_d reduces with temperature, indicating that temperature does not present a deactivation effect on the enzymatic activity as a function of the exposure time. The plot of ln K_d as a function of $1/T$ (K) was used to calculate the deactivation energy (E_d), that is, the energy necessary to overcome the energetic barrier to deactivate the enzyme. The higher the E_d, the more thermostable is the enzyme. The concentrated lipase extract presented an E_d value of 147.8 kJ mol^{-1}.

Table 1 Values of K_d and $t_{1/2}$ for hydrolytic and esterification activities of concentrated enzymatic extract from wheat seeds

T (°C)	T (K)	K_d exp.	$t_{1/2}$ (h) exp.
Hydrolytic activity			
25	298.15	0.0012	577.58
35	308.15	0.0011	630.09
45	318.15	0.0007	990.14
Esterification activity			
25	298.15	0.0012	577.58
35	308.15	0.0011	630.09
45	318.15	0.0007	990.14

Stability to Low Temperatures

The stability of the concentrated extract to low temperatures was evaluated submitting the samples to cooling (4 °C) and freezing (−10 °C) temperatures until the total loss of enzyme activity. The concentrated enzymatic extract kept 90% of its hydrolytic activity until 70 and 50 days of storage at 4 and −10 °C, respectively. The stability of the extract in terms of esterification activity showed a similar behavior, keeping 90% of its activity after 40 and 30 days of storage at 4 and −10 °C, respectively.

From the data presented above, one can verify that the storage of the concentrated enzymatic extract at cooling temperatures was more effective than that at freezing ones. Besides presenting different behaviors from most works presented in the literature, the results obtained here can be considered interesting since storage at 4 °C was effective in keeping the hydrolytic and esterification activities for a considerable period of time.

Specificity of the Concentrated Enzymatic Extract

The specificity of an enzymatic extract under several substrates, mainly related to the chain length and number of instaurations, is of primary importance for industrial applications of the enzyme [18]. Here, the concentrated enzymatic extract from wheat seeds was evaluated under several substrates of hydrolysis and esterification.

Hydrolysis Substrates

Figure 2 presents the hydrolytic activity of the crude and concentrated enzymatic extracts from wheat seeds using tributyrin, coconut oil, and olive oil as substrates. From this figure, one can observe that in general, the enzymatic extract showed higher affinity to substrates of medium and long chains (coconut and olive oils, respectively) compared with that of short chain (in this case, tributyrin). It was also verified that an enhancement in lipase activity was obtained for the concentrated extract compared with the crude one when coconut and olive oils were used as substrates. On the other hand, a reduction in enzymatic activity from concentrated (5.9 U/mL) compared with the crude (7.6 U/mL) extract was observed for tributyrin.

With some exceptions, lipases are generally more active in triglycerides of short chain length. Lipases from seeds show selectivity to the major fatty acids present in the seeds; however, some lipases can hydrolyze a high variety of fatty acids, such as those from rapeseed and pinus seeds [19].

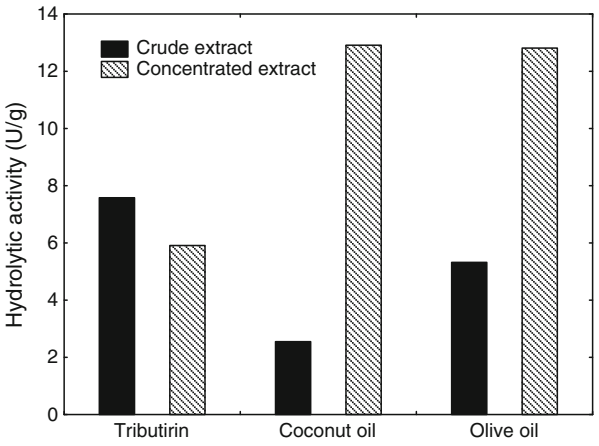

Fig. 2 Hydrolytic activity of the crude and concentrated enzymatic extracts from wheat seeds using tributyrin, coconut oil, and olive oil as substrates

As presented before, just a few works are presented in the open literature concerning the specificity of lipases from vegetable sources. Most works are related to microbial lipases where one can verify that the specificity of the enzyme to the substrate varies as a function of the source and production process (fermentation and/or extraction). Tributyrin is a short-chain triglyceride and is easily hydrolyzable by lipases and esterases [20, 21]. Lipase from *Pseudomonas fluorescens* HU380 showed higher activity in tributyrin (C4:0) [22], the same behavior observed for crude enzymatic extract from *Colletotrichum gloesporioides* [23]. Concentrated enzymatic extract from *Bacillus* sp. A30-1 showed higher specificity to medium-chain triglycerides, as trilaurin (C12:0) and tricaprilin (C10:0) [24], the same behavior observed for lipase for *Rhizopus oryzae*, which showed a more accentuated specificity for tricaprilin [25]. Lipase from *Aspergillus carneus* presented good activities using triglycerides from 4 to 18 atoms of carbon [26].

Esterification Substrates

The esterification activity of the crude and concentrated enzymatic extracts from wheat seeds using butyric, lauric, and oleic acids as substrates and different alcohols (methanol, ethanol, and 1-propanol) is presented in Fig. 3a–c, respectively.

From Fig. 3a, it is possible to observe that higher esterification activities using butyric acid and 1-propanol as substrates were achieved for the concentrated enzymatic extract (330.0 U/g, 20 times higher than that for the crude enzymatic extract). For ethanol and methanol, increases of about 1.9 and 1.3 times were observed for the concentrated extract compared to the crude one.

Using lauric acid as substrate (Fig. 3b), higher activities were observed for 1-propanol (195.1 U/g) related to the concentrated enzymatic extract. A different behavior was obtained when ethanol was used as substrate; higher activities (31.6 U/g) were observed for the crude extract. Esterification activity was not detected when methanol was used, both for crude and concentrated extracts.

Figure 3c presents the esterification activities for the substrate oleic acid. Higher activities were observed for ethanol (14.9 and 13.1 U/g for concentrated and crude extracts, respectively). Activities of about 15 U/g were observed for the crude enzymatic extract using *n*-propanol and oleic acid as substrates. A similar behavior was obtained for methanol (32.6 and 4.49 U/g for crude and concentrated extracts, respectively).

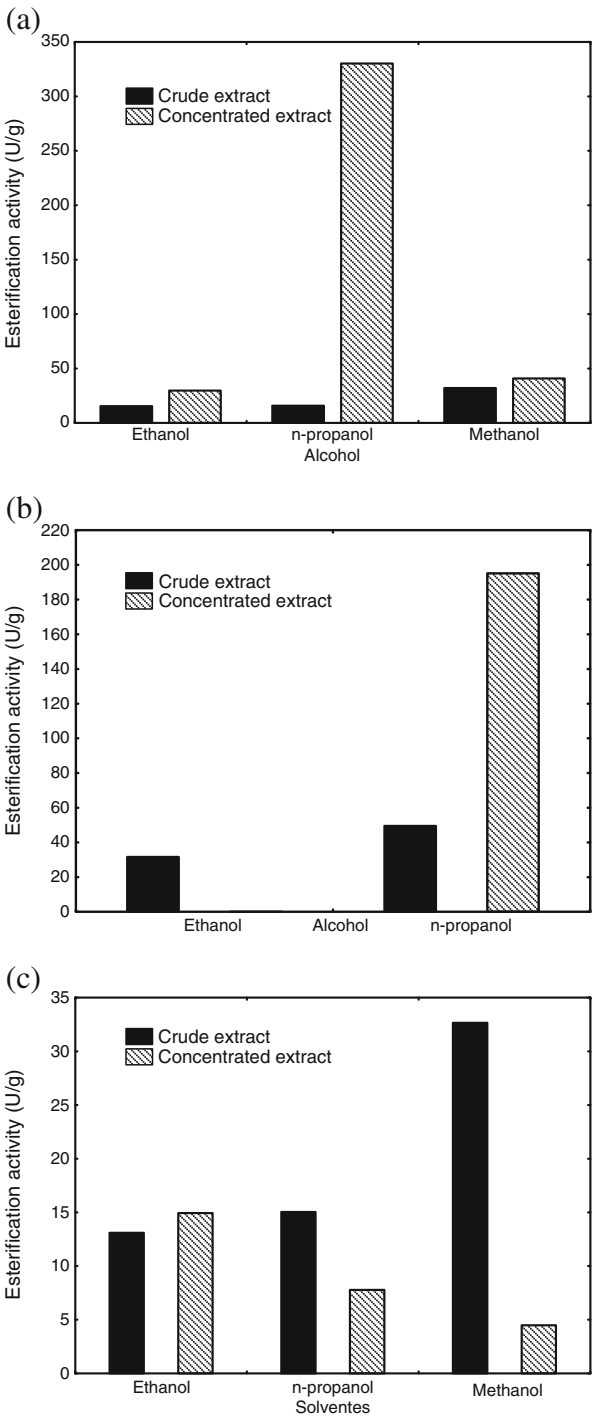

Fig. 3 Esterification activity of the crude and concentrated enzymatic extracts from wheat seeds using butyric, lauric, and oleic acids and different alcohols as substrates

Comparing the present results with those from the literature, one finds that the specificity to short chain fatty acids is in agreement with that found by Peter and Preda [7] when they

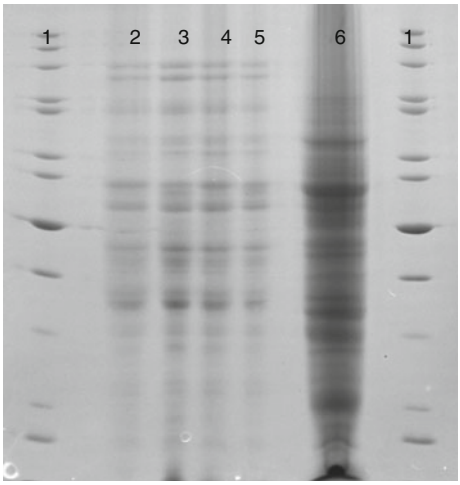

Fig. 4 SDS-PAGE of commercial standard of molecular weight (*1*), concentrated enzymatic extract (esterification activity) (*2*), concentrated enzymatic extract (hydrolysis activity) (*3*), crude enzymatic extract (esterification activity) (*4*), and crude enzymatic extract (hydrolysis activity) (*5*) commercial lipase (type I) from wheat germ (Sigma)

evaluated a pancreatic lipase. The enzyme extracted by these authors presented specificity to valeric and butyric acids and lower esterification activities using acids with long chain. Abbas and Comeau [27] evaluated the specificity of an immobilized lipase from *Mucor* sp. to short-chain fatty acids (propionic, butyric, and caproic acids). These acids were previously cited in the literature as inhibitors of the catalytic properties of the lipases; however, all of them were able to catalyze esterification reaction using different alcohols. It is important to mention that higher yields were observed using long-chain fatty acids (caproic acid).

Other works available in the literature that evaluated the lipase specificity to fatty acids with different chains cite that this group of enzymes commonly presents higher specificity to fatty acids with 8 to 16 carbons. Some examples are the works by Sun and Xu [8] using synthetic lipases and Langrand et al. [28] and Wang et al. [29] using ester hydrolytic lipases.

Related to the specificity to different alcohols, the lipase evaluated by Abbas and Comeau [27] presented a particular affinity to short-chain alcohols as methanol, ethanol, 2-propen-1-ol, and 1-butanol. These results are in disagreement with the works by Langrand et al. [28] and Sun and Xu [8] where the yields decreased with the increase of the number of carbons. According to Ghandi [30] and Abbas and Comeau [27], differences in lipase affinities to different alcohols can be explained in terms of the binding energy that is liberated when the substrate binds to the active site.

The results presented in this work showed that the enzymatic extracts from wheat seeds, in a general way, presented higher esterification activities for fatty acids of short and medium chains and alcohols with two and three atoms of carbon. The enzymatic extracts with this specificity can be applied in a wide range of reactions for producing esters, an important group of compounds widely employed in food and beverage industries [30]. These esters are usually obtained by chemical reactions, and the market value is lower than the ones obtained by natural paths.

Table 2 Enzymatic activities of the immobilized lipase from wheat seeds

Activity	Before immobilization (U/g)	After immobilization (U/g)
Hydrolysis	11.30	26.17
Esterification	98.20	269.63

SDS-PAGE of the Concentrated Enzymatic Extract

SDS-PAGE was used to identify the band that corresponded to the lipase in the enzymatic extracts. Figure 4 presents the SDS-PAGE of commercial standard of molecular weight (1), concentrated enzymatic extract (esterification activity) (2), concentrated enzymatic extract (hydrolysis activity) (3), crude enzymatic extract (esterification activity) (4), crude enzymatic extract (hydrolysis activity), and commercial lipase (type I) from wheat germ (5) (Sigma). From this figure, one can verify that all extracts obtained in this work presented a similar profile of fatty acids.

Immobilization of the Concentrated Enzymatic Extract

The concentrated enzymatic extract from wheat seeds were immobilized following the methodology described before. Table 2 presents the hydrolytic and the esterification activities of the free and immobilized enzymes. From this table, one can observe an enhancement in lipase activity after the immobilization process. This result can be associated to some characteristics of the supports used, such as the high specific surface area, good adsorption capacity, and favorable chemical interactions with the enzyme. The literature points out that the choice of an appropriate support can enhance the half-time of the enzyme and also increase the global performance of the process [31].

The stability of the immobilized enzyme to low temperatures (4 °C) was evaluated, and one could verify that both hydrolytic and esterification activities kept their values after 21 days of storage. Based on the promising results obtained in the immobilization step and considering the low cost and high availability of supports, the methodology proposed here can be considered a potential route for lipase immobilization.

The development of the present work permitted us to point out some characteristics of lipases from wheat seeds necessary for the proposition of new future industrial applications for this important biocatalyst.

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