Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 34 (2005) 25–32
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

A comparative study of the synthesis of *n*-butyl-oleate using a crude lipolytic extract of *Penicillum coryophilum* in water-restricted environments

Alessandra M. Baron [a], Maria Inez M. Sarquis [b], Mario Baigori [c], David A. Mitchell [d], Nadia Krieger [a,*]

[a] *CEPESQ (Centro de Pesquisa em Química Aplicada), Departamento de Química, Universidade Federal do Paraná, Cx.P. 19081, Centro Politécnico, Jardim das Américas, Curitiba 81531-990, Paraná, Brazil*
[b] *FIOCRUZ (Coleção de Cultura de Fungos, IOC), Av. Brasil, 4365, Manguinhos, 21045-900, Rio de Janeiro, RJ, Brazil*
[c] *PROIMI (Planta de Procesos Industriales Microbiologicos), Avenida Belgrano y Pasaje Caseros 4000, San Miguel del Tucumán, Argentina*
[d] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Cx.P. 19046, Centro Politécnico, Jardim das Américas, Curitiba 81531-990, Paraná, Brazil*

Received 24 September 2004; received in revised form 6 April 2005; accepted 13 April 2005
Available online 10 May 2005

Abstract

We evaluated the potential of a lipolytic extract from *Penicillium corylophilum* for use in biocatalysis. Lipolytic activity was stable when the extract was incubated in the presence of hydrophobic solvents. In fact, when the extract was incubated in *n*-heptane with an initial water activity of 0.95, the activity increased by 30%. Synthesis of *n*-butyl-oleate in different systems was compared. In AOT/*n*-heptane reverse micelles, a 100% yield of ester was obtained within 12 h, with a water content (W_0, $[H_2O]/[AOT]$) of 10. Several macro-heterogeneous systems involving *n*-heptane as the solvent were also compared. Direct addition of lyophilised enzymatic preparation to this system gave a 100% yield in 48 h, with an initial water activity of 0.11. Performance was poorer with direct addition of enzymatic preparation co-lyophilised with β-cyclodextrin and with addition of enzymatic preparation immobilized on a hydrophobic gel, with yields significantly less than 100%. We conclude that the lipolytic extract of *P. corylophilum* can be used in synthesis reactions in low-water systems, which justifies further efforts to purify lipolytic enzymes from the extract and to undertake an in-depth evaluation of their potential for use in biocatalysis, for the production of fine chemicals.

Keywords: Lipases; *Penicillium corylophilum*; Biocatalysis; Low-water systems; Reverse micelles

1. Introduction

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) have an important position amongst the biocatalysts used in synthesis in water-restricted environments due to their stability and the wide range of reactions that they can catalyse within these systems (esterification, transesterification, interesterification and aminolysis of esters), with applications in the pharmaceutical, food, environmental, and biotechnological industries [1–5].

The use of biocatalysts in non-aqueous reaction media is advantageous because the water content can be controlled to favour synthesis reactions and, in the case of lipases, these systems provide better solubility of hydrophobic substrates. Beyond this, in comparison to aqueous systems, non-aqueous reaction media may activate the lipolytic enzyme and confer greater stability [6–8]. The water-restricted organic systems used in biocatalysis can be classified as liquid–liquid macro-heterogeneous systems, in which water represents 1–5% of the reaction medium, liquid–solid macro-

* Corresponding author. Tel.: +55 41 361 3470; fax: +55 41 361 3186.
E-mail address: nkrieger@ufpr.br (N. Krieger).

1381-1177/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2005.04.002

heterogeneous systems, and micro-heterogeneous systems. In macro-heterogeneous systems there is a visible separation of phases, while in micro-heterogeneous systems the separation of phases can only be observed microscopically, such as is the case with reverse micelles. In reverse micelles, the amount of water in the system is expressed in terms of the parameter W_0 ([H_2O]/[AOT]), while in other systems it is expressed in terms of the water activity (a_w) [9–12].

With the upsurge in the development of applications of lipases, recently there has been much interest in the production of fungal lipases, which are typically extracellular and therefore relatively easy to recover after the fermentation. Many species of *Penicillium* produce lipases with desirable properties, these lipases having potential applications in a number of different areas [13]. Recently, we have isolated and optimised the production of a lipase from a strain of *Penicillium corylophilum* [14]. The strain produced the enzyme in an inexpensive medium (mineral salts, glucose at 0.1 g/L and olive oil at 2% (v/v)) and, after optimisation of the culture conditions and medium composition, a volumetric activity of 7.1 U/mL was achieved, with a very high specific activity (165 U/mg).

These results suggest the possibility of the development of a low cost system for the production of lipases by *P. corylophilum*. However, such production would only be justified if the enzyme had characteristics that suited it to various applications. The aim of the current work was to evaluate the suitability of a crude lipolytic extract of *P. corylophilum* for possible applications in biocatalysis, using different water-restricted media. As such, we evaluated the stability of the extract in organic solvents and also compared the results of synthesis reactions in various organic systems.

2. Materials and methods

2.1. Microorganism

P. corylophilum was isolated locally and tested for lipase production on agar plates that contained, per litre of distilled water, 15 g agar, 10 mL olive oil, 0.01 g Rhodamine B and 0.001% (w/v) Tween 80. After 7 days of incubation at 29 °C, the plate was irradiated with UV light at 350 nm and produced a fluorescent halo indicating lipolytic activity. Lipase production by this strain was further confirmed in a liquid medium (data not shown). The strain was deposited as IOC 4211 in the Fungal Culture Collection, FIOCRUZ (Oswaldo Cruz Foundation, Rio de Janeiro, Brazil).

The strain was maintained on potato dextrose agar (PDA). It was incubated for 7 days at 29 °C, after which a cube of mycelium and medium was cut from the plate and added to 5 mL of sterile distilled water and maintained at 4 °C. Before each experiment, one such cube was inoculated into a PDA plate and incubated at 29 °C for 7 days. A spore solution (10^8 spore/mL) was then prepared by adding 5 mL of distilled water containing 0.1% Tween 80.

2.2. Growth conditions

The medium used contained, per litre, 2.0 g NH_4NO_3; 1.1 g $MgSO_4{\cdot}7H_2O$; 1.0 g KH_2PO_4; 0.15 g $ZnSO_4{\cdot}7H_2O$; 0.01 g $FeSO_4{\cdot}7H_2O$; 1.0 g glucose and 2% (v/v) olive oil. The pH of the medium was corrected to 7.0 with 0.5 M NaOH. For the seed culture, 1 mL of spore solution was inoculated into a 500 mL Erlenmeyer flask with 100 mL medium, without the olive oil, and incubated at 29 °C and 120 rpm. After 24 h, the whole volume was transferred into another 500 mL Erlenmeyer flask containing 50 mL of water and 2% (v/v) olive oil. Cultures were again incubated at 29 °C and 120 rpm.

2.3. Preparation of the crude lipolytic extract

After 144 h, when the lipolytic activity was maximal, the contents of the flask were filtered through gauze to remove the mycelium. Ammonium sulphate was added to the filtrate to 80% of saturation, with mild agitation in an ice bath. The solution was then maintained under gentle stirring at 4 °C for 12 h, then centrifuged at 12,000 × *g* for 10 min. The supernatant was removed and the precipitate re-suspended in 1 mL of 50 mM phosphate buffer pH 7.0. To produce sufficient enzymatic preparation for the studies, re-suspended precipitates from several flasks, each treated as described above, were pooled. This suspension was dialyzed against the same buffer, at 4 °C, with two exchanges. The molecular weight cut-off of the dialysis membrane was 14,000 Da. The dialysed fraction retained within the bag was used. This concentrated crude extract had a volume of 120 mL (after dialysis) and a protein content of 1.0 mg mL^{-1}. Its activity was determined in aqueous media by the *p*-nitrophenylpalmitate (*p*NPP) method [15] (203 U/mg) and by the titrimetric method [16] using triolein as the substrate to confirm lipase activity (102 U/mg).

2.3.1. Lyophilisation of the crude extract

The crude extract prepared as described above was lyophilised for 12 h at −45 °C in a Jouan LP3 Lyophilizer and was stored at 4 °C. The loss of activity due to the lyophilisation was determined as being 9.2% (based on *p*NPP-hydrolysing activity, in aqueous medium, before and after the lyophilisation). This material was used during this work, either directly or re-suspended in pH 7.0 50 mM phosphate buffer.

The effect of co-lyophilisation of the crude extract with β-cyclodextrin was also investigated. This compound can protect enzymes against denaturation during lyophilisation [17]. In this case, 1.0 mL of the crude extract and 25 mg of β-cyclodextrin were added to 10 mL of 50 mM phosphate buffer at pH 7.0 and then lyophilised.

2.3.2. Immobilization of the crude extract

The enzymatic preparation was immobilized by absorption on the hydrophobic gel Octyl Sepharose 4 Fast Flow (Amersham-Pharmacia Biotech). The gel, which is supplied in a 20% (v/v) alcoholic solution, was washed twice with pH

7.0, 50 mM phosphate buffer. Immobilization was achieved by diluting the lyophilised crude extract in the same buffer as that used in the washing step, to give 14 μg of protein per milligram of gel, which had been determined previously as the maximal adsorption capacity for the gel. After 20 min, the liquid phase was removed by centrifugation at 12,000 × *g* for 3 min, and the gel with immobilized enzymatic preparation was washed three times with 50 mM pH 7.0 phosphate buffer. The washings were assayed using *p*NPP in aqueous solution and showed no lipolytic activity.

2.3.3. Preparation of reversed micellar solution

Reversed micellar solutions with the required values of W_0 ([H_2O]/[AOT]) were prepared using the injection method [18] with 100 mM AOT (dioctyl sodium sulphosuccinate) in *n*-heptane, by adding the appropriate amount of enzymatic preparation (72 μL for a W_0 of 5; 144 μL for a W_0 of 10; and 216 μL for a W_0 of 15), prepared from the lyophilised powder dissolved in 50 mM phosphate buffer at pH 7.0, to 8 mL of reaction medium. The mixture was strongly agitated in a vortex mixer to give a clear micellar solution [19]. The amount of protein was maintained constant at 0.38 mg of protein per milliliter of micellar medium.

2.3.4. Stability of the crude lipolytic extract in organic solvents

For water-soluble solvents, the enzymatic preparation was incubated directly with different proportions (10, 20, 40, 60 and 75%, v/v) of the following solvents: ethanol, acetone, isopropanol and butanol in water. The log *P* values for these solvents are, respectively, −0.24, −0.23, 0.29 and 0.80. After 1 h of incubation at 20 °C, samples of 100 μL were taken and their residual activities were measured using the standard *p*NPP aqueous assay system.

For tests with the hydrophobic solvents toluene, hexane, *n*-heptane and isooctane (log *P* values of 2.50, 3.50, 4.00 and 4.51, respectively) the enzymatic preparation was previously immobilized (see Section 2.3.2). In these tests, the immobilized enzymatic preparation was incubated at 20 °C during 16 h in closed environments in the presence of different saturated salt solutions to ensure the desired a_w [20] prior to the stability tests (LiCl for an a_w of 0.11, $Mg(NO_3)_2$ for an a_w of 0.5 and KNO_3 for an a_w 0.95). After this, 3 mg of the carrier with immobilized enzymatic preparation (containing 1.5 μg protein/mg carrier) was incubated in the presence of the solvents (100 μL solvent, 30 min, 37 °C). After the incubation, the material was washed twice with 50 mM pH 7.0 phosphate buffer and centrifuged (11,000 × *g*, 2 min) to remove the buffer. The residual hydrolytic activity of the immobilized enzymatic preparation was determined against *p*NPP in the standard aqueous system.

2.4. Esterification reactions in different organic systems

The performance (activity and ester yields) of the *P. corylophilum* crude lipolytic extract was compared in different organic systems, using the synthesis of *n*-butyl-oleate as the model reaction. The systems studied were a micro-heterogeneous system (AOT/*n*-heptane reverse micelles) and macro-heterogeneous systems, obtained by adding lyophilised enzymatic preparation (either with or without co-lyophilisation with β-cyclodextrin) or the immobilized enzymatic preparation directly into the organic solvent *n*-heptane. In the *n*-heptane/enzyme macro-heterogeneous systems, the lyophilised enzymatic preparation did not dissolve when added to the reaction medium, but remained as visible granules. In the reverse micelles, the water content was controlled by changing W_0. In the other systems, the water content was controlled by equilibrating the biocatalyst and the solvent with different saturated salt solutions to achieve the required a_w, as described above (see Section 2.3.4).

2.4.1. Synthesis of the n-butyl-oleate

The standard assay of synthesis of *n*-butyl-oleate for all systems was carried out in 25 mL Erlenmeyer flasks, using 5 mL of organic medium (either reversed micellar solution or *n*-heptane, both containing the enzymatic preparation) at 37 °C, in a shaker at 200 rpm. The reaction was started by the addition of the substrates butanol (210 mM) and oleic acid (70 mM). These concentrations were chosen on the basis of previous experiments [19]. At fixed intervals, 100 μL samples of the mixture were collected from the reaction medium and analysed for residual free fatty acids by the Lowry–Tinsley method [21], which has proved to be reliable in previous work [19]. The yield of the reaction in terms of ester production was calculated by consumption of free fatty acids from the reaction medium.

The protein concentration (0.38 mg mL^{-1} of the reaction medium) and the molar ratio of butanol to oleic acid of three were maintained constant throughout all the experiments in the different systems. In the case of the reverse micelles, the enzymatic preparation was diluted in 50 mM phosphate buffer at pH 7.0 prior to encapsulation.

2.5. Lipase assay in aqueous solution and protein determination

The *p*NPP method was used to determine the activity in aqueous medium [15]. The coefficient of extinction of *p*-nitrophenol (*p*NP), 0.98×10^3 L mol^{-1} cm^{-1}, was determined from the absorbance at 410 nm of standard solutions of *p*NP at pH 7.0. One unit of enzymatic activity was defined as the liberation of 1 μmol of *p*-nitrophenol per minute at 37 °C and pH 7.0 (in 50 mM phosphate buffer). The titrimetric method was also used to confirm the lipolytic activity in the crude extract, using triolein (Sigma) as the substrate [16]. Protein was determined Bradford method [22].

2.6. Scanning electron microscopy (SEM)

A Hitachi S-4500 field emission scanning electron microscope (Hitachi Instruments Inc., Irvine, CA) was used to

examine the surface morphology of the dry carrier (0.5 mg) before and after the immobilization and after the synthesis experiments. The samples were sputter-coated with gold prior to analysis.

3. Results and discussion

3.1. Stability in organic solvents

The stability of a lipase in organic solvents will determine whether it can be used to catalyse synthesis reactions. It can also guide solvent selection for such reactions. We, therefore, investigated the stability of the enzymatic preparation in hydrophilic and hydrophobic solvents.

When incubated in hydrophilic solvents (i.e. $-2.5 < \log P < 0$) for 30 min at 37 °C, the lipolytic activity was stable only at a solvent concentration of 10% (v/v). At 10% (v/v), the residual activities were 100% for ethanol, 74% for acetone, 52% for isopropanol and only 1.7% for butanol. At 20% (v/v), the residual activity decreased dramatically: 31% for ethanol, 19% for acetone and 0% for isopropanol and butanol. At concentrations above 20% (v/v) there was no residual activity after incubation in any of the solvents tested.

The lipolytic activity was reasonably stable when the immobilized enzymatic preparation was incubated for 30 min at 37 °C in hydrophobic solvents (i.e. $2 < \log P < 4$), equilibrated at various water activities. It was most stable with *n*-heptane (Table 1), for which the residual activities were in fact 14% and 30% higher than the original activity, after incubation at a_w values of 0.53 and 0.95, respectively. It was also quite stable in hexane, with 100% residual activity after incubation at an a_w of 0.95. For these two solvents, the residual activity increased as the water activity was increased, whereas in the case of isooctane and toluene, the residual activity decreased as the water activity was increased.

3.2. Esterification reactions

Synthesis of *n*-butyl-oleate was studied in different organic systems. The micro-heterogeneous system (reverse micelles) was studied at W_0 values of 5, 10 and 15. For the macro-heterogeneous system we used an *n*-heptane/enzyme system at water activities of 0.11, 0.53 and 0.95, *n*-heptane being selected because the crude extract was most stable in this solvent in the previous experiments. Several enzymatic preparations were compared in the macro-heterogeneous system, namely, direct addition of lyophilised enzymatic preparation, addition of immobilized enzymatic preparation and direct addition of the enzymatic preparation co-lyophilised with β-cyclodextrin.

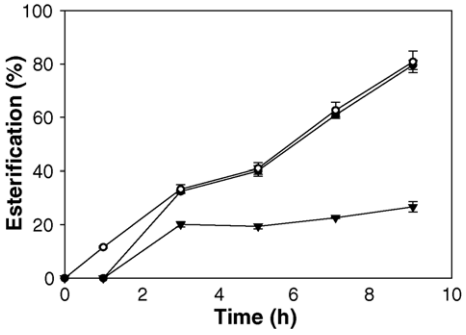


Fig. 1. Kinetics of the synthesis of *n*-butyl-oleate at 37 °C, catalysed by the lipolytic preparation from *Penicillium corylophilum* in AOT/*n*-heptane reverse micelles. Key: (◯) W_0 of 5; (●) W_0 of 10; (▼) W_0 of 15. The protein concentration was 0.38 mg mL^{-1}.

3.2.1. Esterification in reverse micelles

The W_0 affected the synthesis of *n*-butyl-oleate by the crude lipolytic extract in the reversed micellar system (Fig. 1). The best results after 9 h of reaction were obtained at W_0 values of 5 and 10, with an 80% yield. The W_0 of 15 gave a yield of only 26%.

The W_0 is one of the most important parameters for enzymatic catalysis in reverse micelles. The optimum value for enzyme activity depends on several factors, amongst which two of the most important are the dependence of the reaction rate on the water concentration and the effect of the water on the size of the micelles within the system. The type of reaction to be catalysed is crucial, with esterification reactions requiring lower W_0 values than hydrolytic reactions, in order to shift the position of the reaction equilibrium towards the production of ester [18,23]. The poor yield obtained at the W_0 of 15 is therefore not unexpected. In fact, W_0 values between 5 and 10 give optimal rates for many esterification reactions in AOT-micellar systems. For instance, the synthesis of octyl-decanoate by *Humicola lanuginosa* lipase is optimal at a W_0 of 5 while the same reaction is optimal at a W_0 of 10 for *Rhizomucor miehei* lipase [24], the synthesis of octyl-oleate is optimal at a W_0 of 7 [25], the synthesis of *n*-butyl-butyrate is optimal at a W_0 of 10 [26] and the synthesis of ethyl-

Table 1
Stability of the enzymatic preparation of *Penicillium corylophilum* in hydrophobic solvents

Organic solvent	Log *P*	Residual activity after 30 min incubation at 37 °C[a] (%)		
		a_w 0.11	a_w 0.53	a_w 0.95
Toluene	2.5	72	106	63
Hexane	3.5	22	66	100
n-Heptane	4.0	71	114	130
Isooctane	4.5	100	89	68

[a] Residual activities were determined by the hydrolysis of *p*NPP in aqueous medium.

laurate using Lipolase (Novozyme) is optimal at a W_0 of 10 [19].

The reaction rate obtained in the present work is promising. The only shorter esterification time reported in a reverse micelle system is 60 min for a 90% yield of ethyl-laurate, using Lipolase in AOT/isooctane reverse micelles [19]. Most workers have needed much longer times in order to obtain high ester yields. For instance, 24 h was required for a 95% yield of butyl-laurate, using the lipase of *C. cilindracea* in the AOT/isooctane reversed micelle system [27], and also for a 90% yield of octyl-oleate, using the lipase of *Rhizopus delemar* in a microemulsion system [26]. Even longer times have been reported. A 95% yield of ethyl caproate required 168 h, using the esterase of *Bacillus licheniformis* in *n*-heptane at 45 °C [28], while a 95% yield of isoamyl acetate required 72 h, using an immobilized lipase of *R. miehei* at 40 °C [29].

3.2.2. Macro-heterogeneous system with direct addition of lyophilised enzymatic preparation

In the *n*-heptane/enzyme system in which lyophilised enzymatic preparation was added directly, the maximum yield of 58% was obtained with an initial a_w of 0.11 (Fig. 2). With increasing a_w, the yield and activity both decreased slightly: with an initial a_w of 0.53 the yield was 50%, and with the initial a_w of 0.95 the yield was 42%.

The a_w is an important parameter in macro-heterogeneous systems and the initial a_w of the reaction has been controlled in many studies of ester synthesis. Nevertheless, its effect on the reaction rate and on enzyme activity does not always follow the same pattern. Chowdary and Prapulla [30] studied the transesterification of *n*-butanol and ethyl-butyrate over a range of initial a_w values from 0.11 to 0.95 and obtained different optimal a_w values for different lipases. A maximum yield of 92% after 24 h was obtained with the lipase of *C. rugosa* with an a_w of 0.33. Conversely, Tweddell et al. [31] synthesized ethyl-oleate, using the lipase of *R. niveus* in *n*-hexane, obtaining the highest yield (60%) with an a_w of 0.75 after 192 h.

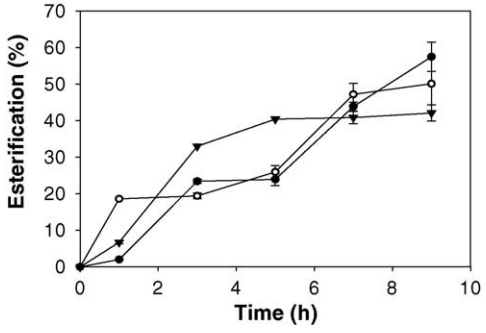


Fig. 2. Kinetics of the synthesis of *n*-butyl-oleate at 37 °C, with different water activities, by the lipolytic extract of *Penicillium corylophilum* in the macro-heterogeneous system *n*-heptane/enzyme in which lyophilised enzymatic preparation was added directly. Key: (●) a_w of 0.11; (◯) a_w of 0.53; (▼) a_w of 0.95. The protein concentration was 0.38 mg mL^{-1}.

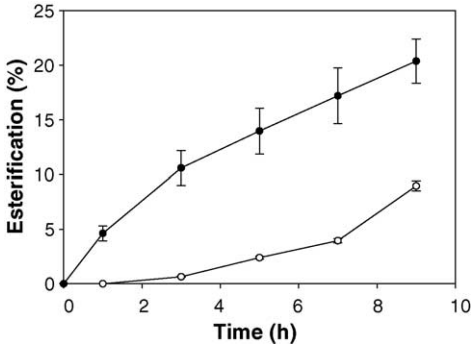


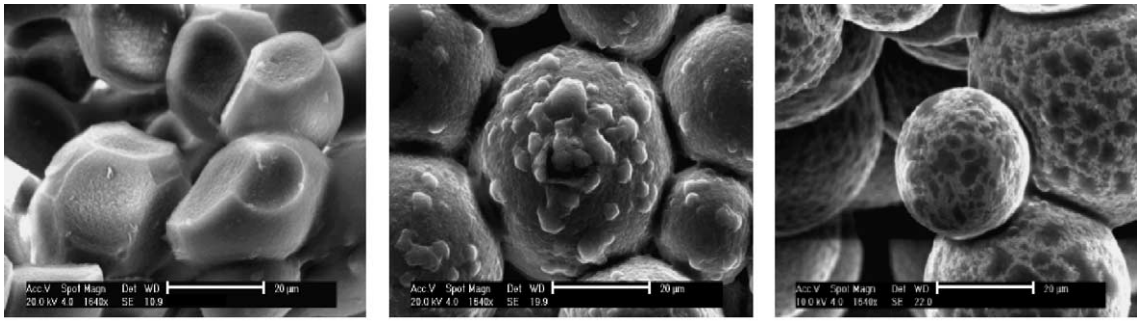
Fig. 3. Kinetics of the synthesis of *n*-butyl-oleate at 37 °C and with an initial a_w of 0.11, by the lipolytic extract of *Penicillium corylophilum* in the macro-heterogeneous *n*-heptane/enzyme systems. Key: (●) addition of enzymatic preparation co-lyophilised with β-cyclodextrin; (◯) addition of enzymatic preparation immobilized on octyl-sepharose. The protein concentration for both assays was 0.38 mg mL^{-1}.

Since the studies with direct addition of the enzymatic preparation to the *n*-heptane/enzyme system gave the highest ester yield at an initial a_w of 0.11, this a_w was used in studies involving the same system, but with addition of the enzymatic preparation co-lyophilised with β-cyclodextrin or with addition of enzymatic preparation immobilized on octyl-sepharose gel.

Addition of enzymatic preparation co-lyophilised with β-cyclodextrin gave an ester yield of only 20% at 9 h (Fig. 3). In contrast, for hydrolysis of *p*NPP in aqueous medium, the same enzymatic preparation co-lyophilised with β-cyclodextrin gave a specific activity seven times greater than that of the free lyophilised enzyme (data not shown). Little information is available about the activity of enzymes co-lyophilised with cyclodextrins. Hasegawa et al. [17] studied transesterification reactions catalysed by proteases co-lyophilised with cyclodextrins and found that the enzyme-β-cyclodextrin complex had a much higher catalytic activity than the free protease. Fluorescence spectroscopy suggested that the co-lyophilisation increased the stability of the protease in their acetonitrile–water system. In our case, we added the enzyme-β-cyclodextrin complex to the non-polar solvent *n*-heptane, so the difference in results is not surprising.

3.2.3. Macro-heterogeneous system with addition of immobilized enzymatic preparation

The synthesis of *n*-butyl-oleate was very poor when enzymatic preparation immobilized on octyl-sepharose gel was added to *n*-heptane, with a yield of 9% after 9 h (Fig. 3). To verify changes in the immobilized enzymatic preparation during this reaction, an SEM image obtained after 9 h of reaction was compared with images obtained for the support before and immediately after immobilization. After immobilization, the surface cavities of the original gel beads (Fig. 4a) were filled by rounded structures, which are presumably protein aggregates (Fig. 4b). After 9 h of reaction in the *n*-heptane system, the appearance of the bead surfaces had changed (Fig. 4c). It is possible that the hydrophilic sub-



(a) (b) (c)

Fig. 4. Photomicrographs of the octyl-sepharose support (a) before immobilization; (b) after immobilization and (c) after 9 h of esterification of *n*-butyl-oleate in *n*-heptane. The scale bars represent 10, 20 and 10 μm in (a), (b) and (c), respectively.

strate *n*-butanol, present in the reaction medium at an initial concentration of 210 mM, dehydrated the superficial layer of immobilized material, thereby causing the low activity. Note that in the results for solvent stability (Section 3.1) the residual activity was only 1.7% after incubation of the enzymatic preparation for 1 h in a 10% (v/v) *n*-butanol solution.

Although dehydration must have also occurred when lyophilised enzymatic preparation was added directly to the reaction medium, the higher activities obtained in that system can be explained by the denaturation being limited to the surface of the solid aggregates, leaving the enzymes in the interior active, as suggested by Goldberg et al. [32].

3.3. Comparison of water-restricted systems

The reversed micellar system was the best for the synthesis of *n*-butyl-oleate, but even in this system the reaction was not complete after 9 h. In order to see whether any of the systems were capable of giving yields of 100%, reactions were followed for 48 h at 37 °C, with a molar ratio of 1:3 (70 mM of acid to 210 mM of alcohol), using the best conditions identified in the previous studies: AOT/*n*-heptane reverse micelles at a W_0 of 10 (obtained with 50 mM phosphate buffer pH 7.0) and *n*-heptane macro-heterogenous systems at an initial a_w of 0.11.

A yield of 100% was obtained after 12 h in the reverse micelles (Fig. 5), confirming this system as the best for *n*-butyl-oleate synthesis. The *n*-heptane/enzyme system to which lyophilised enzymatic preparation was added directly also reached completion, but only at 48 h. For the *n*-heptane/enzyme system to which enzymatic preparation co-lyophilised with β-cyclodextrin was added, the reaction was still proceeding after 48 h, whereas for the *n*-heptane/enzyme system to which immobilized enzymatic preparation was added the reaction had stopped by 12 h, with a yield of only 14%, probably due to enzyme denaturation by dehydration as discussed above.

Table 2 compares our results with the two previous comparative studies of lipase-catalysed reactions in different organic systems. Tweddell et al. [31] compared the production

Table 2
Comparison of ester synthesis reactions catalysed by lipolytic preparations in different organic reaction systems

Source, conditions, substrates and reaction media [reference][a]	Systems[b]	Water content	Best yield and conditions
Rhizopus niger, 40 °C, enzyme 10% (m/v); oleic acid and ethanol 1:1.5, *n*-hexane [31]	MHB-LL surfactants free enzyme	5–50%	85%, 6 h with a 10% (v/v) aqueous phase
	MHB-LL free enzyme	5–50%	85%, 12 h with a 10% (v/v) aqueous phase
	MA-SL lyophilised enzyme	a_w 0.52–0.98	60%, 192 h, a_w 0.52
Mucor miehei, 40 °C, enzyme 0.7 mg mL^{-1}; butyric acid and butanol 1:1, *n*-hexane [25]	MA-SL lyophilised enzyme	0.05%, (m/m)	100%, 25 h
	RM-AOT/hexane free enzyme	W_0 5–30	70%, 30 h,W_0 5
	MHB-LL free enzyme	2.5–40%, (v/v)	90%, 25 h, 5% water (v/v)
Penicillium corylophilum, 37 °C, enzyme 0.38 mg mL^{-1}, oleic acid and *n*-butanol 1:3 *n*-heptane [This work]	RM-AOT/heptane free enzyme	W_0 5–15	100%, 12 h, W_0 10
	MHB-SL lyophilised enzyme	a_w 0.11, 0.53, 0.95	100%, 48 h, a_w 0.11
	MHB-SL enzyme co-β-cd	a_w 0.11	63%, 48 h, a_w 0.11
	MHB-SL enzyme immob	a_w 0.11	14%, 48 h, a_w 0.11

[a] The ratios represent molar ratios of acid to alcohol.

[b] Key: MHB-LL, macro-heterogeneous-biphasic liquid–liquid; MHB-SL, macro-heterogeneous-biphasic solid–liquid; MA-SL, micro-aqueous solid–liquid; RM-AOT/hexane, reverse micelles AOT/*n*-hexane; RM-AOT/heptane, reverse micelles AOT/*n*-heptane; co-β-cd, enzyme co-lyophilised with β-cyclodextrin; immob, enzyme immobilized in octyl-sepharose gel.

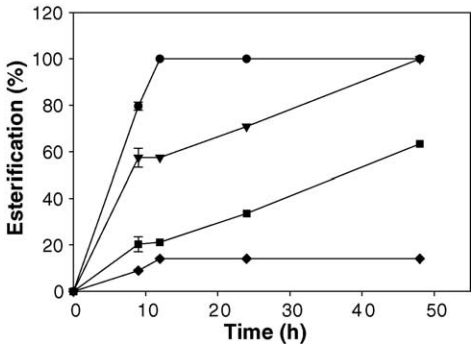


Fig. 5. Comparison of the kinetics of synthesis of *n*-butyl-oleate at 37 °C, catalysed by the enzymatic preparation obtained from *Penicillium corylophilum*. (●) Reverse micelles at a W_0 of 10; (▼) *n*-heptane/enzyme system to which lyophilised enzymatic preparation was added directly, at an a_w of 0.11; (■) *n*-heptane/enzyme system to which enzymatic preparation co-lyophilised with β-cyclodextrin was added, at an a_w of 0.11; (♦) *n*-heptane/enzyme system to which enzymatic preparation immobilized on octyl-sepharose was added, at an a_w of 0.11. The concentration of proteins in all systems was 0.38 mg mL^{-1}.

of ethyl-oleate in three organic systems, using a commercial lipase of *R. niger*. Of the systems used, the best was a biphasic system with surfactant, with a yield of 85%, obtained in 6 h with a 10% (v/v) aqueous phase. An increase in the water content to 50% (v/v) decreased the reaction yield to approximately 25%. In a biphasic system without surfactant, a yield of 85% was also obtained, but in double the time. The micro-aqueous system was least effective, with the best result being a yield of 60% after 192 h, obtained with an initial a_w of 0.52. Above this a_w, the enzymatic preparation formed agglomerates that adhered to the wall of the reaction vessel. Tweddell et al. [31] concluded that the better yields obtained with the biphasic system containing surfactants were due to the increase in interfacial area, which favours catalysis by lipases. Another comparative study of organic systems was done for the synthesis of *n*-butyl-butyrate by the commercial lipase of *Mucor miehei*, again in three different organic systems (Table 2) [25]. The best results were obtained in the micro-aqueous system, with a 100% yield after 25 h.

Our result for the reversed micellar system was the best amongst the results presented in Table 2, in terms of both the yield of ester (100%) and the time to achieve the maximum yield (12 h). Note that our results for the direct addition of the enzymatic preparation (macro-heterogeneous (SL) *n*-heptane/lyophilised enzyme) compare well with the others, in terms of yield and time for completion.

4. Conclusion

In this work, we compared different organic media for the synthesis of the ester *n*-butyl-oleate, using a crude lipolytic extract produced by *P. corylophilum*, the best systems being the reversed micelle system (a micro-heterogeneous system) and the macro-heterogeneous (SL) *n*-heptane system with the direct addition of the lyophilised enzymatic preparation. The water content of the systems, expressed either as W_0 or as a_w, was of prime importance. Although the lipolytic enzymatic preparation had poor stability in high concentrations of hydrophilic solvents, it did show itself as promising for the biocatalysis of synthesis reactions in systems containing hydrophobic solvents. The 100% yield of *n*-butyl-oleate obtained in 12 h in the AOT/*n*-heptane reversed micelle system was especially promising, as the rate and yield compared favourably to those obtained in previous studies.

Acknowledgements

We wish to thank the Araucaria Foundation (Fundação Araucária), an agency of the state government of Paraná, which supports research and CAPES/SETCIP (Brazil-Argentina International Cooperation Project) for financial support. David Mitchell and Nadia Krieger thank the Brazilian National Council for Scientific and Technological Development (CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico) for research scholarships.

References

[1] R.K. Saxena, A. Sheoran, B. Giri, W.D. Sheba, J. Microbiol. Methods 52 (2003) 1.
[2] T. Garcia, N. Sanchez, M. Martinez, J. Aracil, Enzyme Microb. Technol. 25 (1999) 584.
[3] A. Pandey, S. Benjamin, C.R. Soccol, P. Nigam, N. Krieger, V. Thomaz-Soccol, Biotechnol. Appl. Biochem. 29 (1999) 119.
[4] N. Krieger, M. Taipa, M.R. Aires-Barros, E.H.M. Melo, J.L. Lima-Filho, J.M.S. Cabral, J. Chem. Biotechnol. 69 (1997) 77.
[5] T. Watanabe, R. Matsue, Y. Honda, M. Kuwahara, Carbohyd. Res. 275 (1995) 215.
[6] Z. Knezevic, S. Bobic, A. Milutinovic, B. Obradovic, L. Mojovic, B. Bugarski, Process Biochem. 38 (2002) 313.
[7] Y.Y. Linko, M. Lämsa, A. Huhtala, O. Rantanen, J. Am. Oil Chem. Soc. 72 (1995) 1293.
[8] N.A. Turner, E.N. Vulfson, Enzyme Microb. Technol 27 (2000) 108.
[9] G.A. Sellek, J.B. Chaudhuri, Enzyme Microb. Technol. 25 (1999) 471.
[10] R. Verger, Trends Biotechnol. 15 (1997) 32.
[11] S.D. Jonathan, Enzyme Microb. Technol. 11 (1989) 194.
[12] Y.L. Khmelnitsky, A.V. Levashov, N.L. Klyachko, K. Martinek, Enzyme Microb. Technol. 10 (1988) 710.
[13] V.M.G. Lima, N. Krieger, M.I.M. Sarquis, D.A. Mitchell, L.P. Ramos, J.D. Fontana, Food Technol. Biotech. 41 (2003) 105.
[14] A.M. Baron, Masters Thesis, Federal University of Paraná, 2003, p. 113.
[15] U.K. Winkler, M. Stuckmann, J. Bacteriol. 138 (1979) 663.
[16] W. Stuer, K.E. Jaeger, U.K. Winkler, J. Bacteriol. 168 (1986) 1070.
[17] M. Hasegawa, S. Yamamoto, M. Kobayashi, H. Kise, Enzyme Microb. Technol. 32 (2003) 356.
[18] E.P. Melo, M.R. Aires-Barros, J.M.S. Cabral, Biotechnol. Annu. Rev. 7 (2001) 87.
[19] M.L.M. Fernandes, N. Krieger, A.M. Baron, P.P. Zamora, L.P. Ramos, D.A. Mitchell, J. Mol. Catal. B: Enzym. 30 (2004) 43.
[20] E. Wehtje, P. Adlercreutz, in: E.N. Vulson, P.J. Halling, L.H. Holland (Eds.), Enzymes in Nonaqueous Solvents. Methods and Protocols, Vol. 15, Part I, Humana Press, Totowa, NJ, 2001, p. 127 (Chapter 12).

[21] R.R. Lowry, J.I. Tinsley, J. Am. Oil Chem. Soc. 53 (1976) 470.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[23] N. Krieger, M.A. Taipa, E.H.M. Melo, J.L. Lima-Filho, J.M.S. Cabral, Appl. Microbiol. Biotechnol. 67 (1997) 85.
[24] G.E. Crooks, G.D. Rees, B.H. Robinson, M. Svensson, G.R. Stephenson, Biotechnol. Bioeng. 48 (1995) 78.
[25] F. Borzeix, F. Monot, J.P. Vandecasteele, Enzyme Microb. Technol. 14 (1992) 791.
[26] D.G. Hayes, E. Gulari, Biotechnol. Bioeng. 35 (1990) 793.
[27] K. Nagayama, S. Matsu-Ura, T. Doi, M. Imai, J. Mol. Catal. B: Enzym. 4 (1998) 25.
[28] E. Alvarez-Macarie, J. Baratti, J. Mol. Catal. B: Enzym. 10 (2000) 377.
[29] S.H. Krishna, B. Manohar, S. Divakar, S.G. Prapulla, N.G. Karanth, J. Biotechnol. 87 (2001) 191.
[30] G.V. Chowdary, S.G. Prapulla, Process Biochem. 38 (2002) 393.
[31] R.J. Tweddell, S. Kermasha, D. Combes, A.E. Marty, Enzyme Microb. Technol. 22 (1998) 439.
[32] M. Goldberg, D. Thomas, M.D. Legoy, Enzyme Microb. Technol. 12 (1990) 976.