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Synthetic application and activity of cutinase in an aqueous, miniemulsion model system: Hexyl octanoate synthesis

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ABSTRACT

Cutinase has been shown to be a promising biocatalyst for applications in processes targeted for industrial scale. This work aims to further contribute for highlighting such role, by bestowing detailed insight on the application of cutinase for the synthesis of value added compounds in miniemulsion environments (oil-in-water). The synthesis of hexyl octanoate was used as model system, due to the relevance of this compound for industrial applications as flavor and fragrance agent. The nature, specificity and selectivity of the catalyst enable operation under mild reaction conditions, without undesirable side reactions, leading to a very pure product. A high conversion yield, about 86%, for an enzyme concentration of 5 mg ml^{-1}, was achieved.

Hexyl octanoate synthesis and cutinase activity for different acid: alcohol molar ratio (R), under different pH environments, were evaluated. A maximum cutinase activity of 2.20 μmol mg^{-1} min^{-1} was observed for $R = 0.5$, which was tentatively ascribed to the stabilizing effect of hexanol on the miniemulsion. It is thus considered that the accumulation of the hydrophobic hexyl octanoate inside the droplets stabilize the miniemulsion system.

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1. Introduction

Low- and medium-chain molecular esters constitute a significant part of aroma compounds. Many of such esters contribute to the fruity nature of flavors or are used as emollients in the cosmetic industry [1].

Biocatalysis may provide advantageous alternatives to purely chemical routes for industrial processes, among them those involving synthesis of esters [2–4]. The use of enzymatic processes for ester synthesis have cost-effective, industrially appealing features, such as the mild reaction conditions, along with the high selectivity and specificity, which enable the synthesis of flavor molecules with high quality and purity. Moreover, and although from the chemist point of view, there is no difference between a compound synthesized in nature and the identical molecule produced by chemical synthesis, there is a consistently growing trend towards the introduction of biological and environmentally friendly processes for the production of a wide plethora of molecules. Together with strict US and Europeans regulation, that define that "natural" flavor substances can only be prepared either by physical, enzymatic or microbial processes, the whole clearly favors the use of biocatalysts [5,6]. Furthermore, biological production processes have a better public acceptance, particularly when targeted for products used as ingredients or in formulations related to personal- and healthcare and food and feed sectors [7]. Major applications of enzymes are in medicine, textile industry, and food and beverage industry. Recently enzyme technology has also become prominent in the development of analytic aids [8]. Esterases and lipases, particularly the later, are among the enzymes that are widely used in industrially relevant processes, namely when esterification reactions are aimed at [9–12]. The use of cutinases for such applications presents an interesting option, since they do not require interfacial activation, unlike classical lipases [13]. Moreover, cutinase may display activity over a substrate irrespectively of its being in highly aggregated or soluble states, which are otherwise typical behaviors of lipases and esterases, respectively, cutinase being therefore able to hydrolyze soluble esters as well as emulsified triacylglycerols [14,15]. Cutinases can thus be considered an intermediate between those other two enzymes [16]. Besides, cutinases have been shown to display stability and activity under adverse conditions, viz. presence of organic solvents, ionic liquids, detergents, proteases and oxidizing agents, and their activity in hydrolysis, esterification and transesterification has been highlighted in recent years. Recently, the thermal stability of cutinases in aqueous solution has actually been considered higher than that of any lipase [17,18]. When considering ester synthesis, cutinases present a sound alternative to lipases, since the use of the later can be hampered by their

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relatively large size and relatively low stability under industrial process conditions [16]. Given these features, it is therefore understandable that cutinases find application in several areas, among them fine chemicals and pharmaceuticals, laundry, detergents and personal care products, food industry, textiles, polymer chemistry and in pesticide and insecticide degradation [16,18]. The catalytic triad Ser-120, Asp-175, His-188, is accessible to the solvent and presents catalytic activity with different substrates [16]. Particular attention has been recently given to the use of cutinases in (trans)esterification reactions of fat or oils in low water activity environments [17–19].

Several reports on the use of cutinases in different reaction media, often dissolved in aqueous solution, but also suspended as a powder or in immobilized form, have been published [17–20]. Immobilization has been commonly achieved by adsorption onto solid supports [21,22]; and encapsulation in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) [23–26], phosphatidylcholine [27], or cetyltrimethylammonium bromide (CTAB) [28]. Lyophilized cutinase was used in fundamental studies on the hydrolysis of triglycerides [29]; transesterification reactions [30–32], esterification reactions [33–35]; and in the elucidation of the reaction mechanisms of cutinase related to the stereoselectivity and specificity of this enzyme [36,37].

Water-in-oil microemulsions, or reverse micelles, are often used for bioconversion processes, involving either cutinases or lipases [38]. The application of microemulsions in several chemical and industrial processes is due to key properties of these systems, such as the very low interfacial tension, the large interfacial area and the thermodynamic stability [39]. When organic synthesis is considered, organic solvents used as reaction media account for about 80% of mass utilization in typical pharmaceutical and fine chemical processes [40]. Many organic solvents used within such scope are toxic and/or volatile. In the scale-up of industrial processes, the use of organic solvents may therefore raise safety and environmental concerns.

Water as reaction medium is, on the other hand, an environmentally compatible solvent and since most biological processes and enzymatic reactions take place in water, implementation of synthetic biocatalytic processes in aqueous media would be clearly advantageous [41–43]. Oil-in-water emulsions, with droplets in the size range of about 200 nm, often referred to in the literature as miniemulsions, have been investigated, especially as nanoreactors for polymerization [44]. Recently aqueous miniemulsion systems have been shown to provide promising media for enzymatic reactions catalyzed by lipase such as enzymatic polymerization [45,46], preparation of optical active organic compound [47,48], sterification [49], and by cutinase [50], for the synthesis of alkyl esters.

Aqueous miniemulsions (oil-in-water) are two phase systems where small droplets (organic phase) with high stability are dispersed in a continuous phase (water), by using high shear forces originated by ultrasonication. The water phase contributes with about 80% (w/w). The oil phase is composed mostly of the substrates (16.5%, w/w), a hydrophobic agent for osmotic stabilization of the droplets, and a surfactant for colloidal stability [44]. The droplets are of uniform size and stabilized against coagulation by the presence of ionic or nonionic surfactant, and against diffusion degradation (Ostwald–Ripening) by the addition of a co-stabilizing, extremely hydrophobic, agent (ex. hexadecane). The water produced in the dehydrative esterification [51] is expelled from the continuous aqueous phase, favoring product formation. The other very important factors that favor the formation of esters are the enormous interfacial area, readily available for interfacial catalysis, and the stability of the miniemulsion throughout the reaction, which is increased with increasing hydrophobicity of the oil phase [49,50].

The catalytic activity of cutinase for ester synthesis in a miniemulsion system was uncovered in a previous work [50]. This prior study shows the high potential and advantages of using water-based miniemulsions as a green reaction environment for the biosynthesis of ethyl- and specially hexyl acid esters (acid chain length from C_6 to C_{18}) by lipases and *Fusarium solani pisi* cutinase.

On the aftermath of these promising results, the present work aims to evaluate the effect of the concentration of the substrates on enzyme activity and stability of the reaction media. The focus was on structural and catalytic aspects of cutinase in oil-in-water miniemulsion. Given the conformational change of cutinase during the time course of the reaction, it was possible to establish the effect of the reaction media on enzyme activity.

The synthesis of hexyl octanoate was used as model system, since this ester is a flavor compound incorporated in a wide range of aromas such as apple, banana, cider, grape and melon [52].

2. Experimental

2.1. Catalyst

2.1.1. Production of cutinase

F. solani pisi cutinase wild-type was biosynthesized by recombinant *Saccharomyces cerevisiae* SU50 strain as described by Calado et al. [53]. The cutinase producing *S. cerevisiae* SU50 strain (Mata, leu2–3, ura3, gal1: URA3, MAL-8, MAL3, SUC3) contains the expression vector pUR7320 constructed and provided by the Unilever Research Laboratory, Vlaardingen, The Netherlands. The strain was stored at −80 °C in frozen tubes containing 50% (v/v) selective medium and 50% (v/v) of glycerol (Merck, Dannstadt, Germany). The inoculum (0.4 l) was cultivated in cotton-stopped shake flasks (4 × 1 l) at 30 °C and 200 rpm in an orbital shaker (Agitorb 160E; Aralab, Lisboa, Portugal) in a selective medium (medium lacking leucine) composed of 20 $g\,l^{-1}$ D(+)-glucose anhydrous (Merck, Germany), 6.7 $g\,l^{-1}$ of yeast nitrogen base without amino acids (Difco, Sparks, MD, USA) and 20 $mg\,l^{-1}$ L-histidine (Merck) until a biomass concentration between 1.1 and 1.8 $g_{dcw}\,l^{-1}$ was attained. This concentration was established based on a correlation curve relating optical density at 600 nm and cell dry weight, determined previously. This inoculum was transferred into the cultivation media for enzyme biosynthesis, and corresponded to 10% (v/v) of the working volume. These cultivations were performed in a 5 l bioreactor (Biostat MD; B. Braun, Melsungen, Germany) containing 4 l working volume adjusted at pH 6.0 (by automatic addition of NaOH or HCl solutions, both at 2 N). The fermentation for biocatalyst production was performed at 30 °C, with a minimum dissolved oxygen tension of 30%. A constant air flow rate of 4.4 l min^{-1} (equivalent to 1.1 vvm) was established during the cultivation.

2.1.2. Purification and lyophilization of cutinase

The isolation and purification of cutinase excreted by the recombinant *S. cerevisiae* SU50 strain was carried out by expanded bed adsorption (EBA) [54]. Frontal adsorption experiments were carried out in an EBA column Streamline 25, with a settled bed adsorbent height of 15 cm and the column top adjusted to 45 cm. The cation adsorbent Streamline SP XL (Amersham Pharmacia Biotech, Sweden) was used to isolate cutinase directly from the fermentation broth. The adsorbent was washed and equilibrated previously with 20 mM citrate buffer pH 4.5. During the expanded bed operation, and in order to stabilize bed expansion, a volumetric flux of 270 $cm\,h^{-1}$ was maintained. Once the bed expansion was stabilized and the adsorbent equilibrated with the buffer, the fermentation broth (previously diluted with water 4:1), was loaded through the EBA column. After frontal feedstock adsorption, the bed was washed until the effluent was devoided of yeast cells. The elution was carried out in packed bed mode in downward flow, using a

Table 1
Relative amounts of the compounds in miniemulsion systems.

Miniemulsion (ME)	Water (%)	Substrates (%)	Hexadecane (%)	Lutensol (%)
ME 80% (w/v)	81.2	16.5	0.7	1.6

constant volumetric flux of 122 cm h^{-1} with the starting buffer containing 150 mM NaCl. The pH of all effluent fractions was corrected with NaOH (1 M) to pH 6.0–7.0, the optimal values for enzyme stability. Cutinase activity and protein concentration of the collected effluent fractions from the column were determined after each run, as described elsewhere [55].

The pool of elution fractions exhibiting the highest cutinase activity were firstly dialyzed against 20 mM phosphate buffer pH 7.0 and then frozen at −80 °C and lyophilized overnight (Christ Alpha 2-4 lyophilizer, B.BRAUN Biotech, Melsungen, Germany). Lyophilized pure cutinase was stored at −20 °C before use in the esterification reactions.

2.1.3. Characterization of the cutinase preparations

The esterolytic activity of cutinase and the protein content were established, thus allowing the characterization of the lyophilized cutinase preparations [55,56].

The esterolytic activity of cutinase was assayed using a spectrophotometric method based on monitoring the hydrolysis of p-nitrophenylbutyrate (p-NPB) to nitrophenol (p-NP), a yellow compound easily quantified by absorbance at 400 nm [55]. Briefly, 20 μl samples of cutinase solution were added to 980 μl of a reaction mixture containing 0.56 mM p-NPB, 11.3 mM sodium cholate and 0.43 M tetrahydrofuran, in 50 mM potassium phosphate buffer pH 7.0.

One unit of cutinase estereolytic activity was defined as the amount of enzyme required to convert 1 μmol of p-NPB to p-NP per minute, at 30 °C and pH 7.

The protein concentration was determined by the method of Pierce (BCA, bicinchoninic acid, assay) from Thermo Scientific (Rockford, USA) with reference to Bovine Serum Albumin (BSA) (Merck) as standard solution [56].

Specific activities of lyophilized cutinase preparations were calculated as the ratio between estereolytic activity and protein concentration (170 U mg^{-1}).

2.2. Enzymatic esterification reaction in miniemulsion system

Substrates, hexadecane, water and Lutensol, that make up the esterification reaction media, were mixed in different relative amounts, w/w (Table 1), and thoroughly mixed by magnetic stirring for 1 h. The two phase system was ultrasonicated for 120 s (total sonication time) in pulses of 10 s and pauses of 5 s, at 65% amplitude (SONOPULS, Bandelin, Berlin, Germany) with ice cooling. Unless otherwise stated, a typical esterification reaction (Fig. 1) was carried out in 20 ml rubber-capped flasks with 10 ml of working volume, using a solution with an equimolar concentration of the substrates (0.650 M), and taking into account the phase relationship as depicted in Table 1. 10 ml of the miniemulsion were added into the reaction vessel containing the appropriate amount of enzyme.

The esterification reactions were performed in a thermostated incubator (Advanced ChemTec PLS 4 × 4, Louisville, Kentucky, USA, 400 rpm magnetic stirring) at 40 °C unless otherwise stated. This setup enabled parallel experiments by the simultaneous use of 5 reactors against at least one blank without enzyme.

Samples were withdrawn periodically (100 μl) using a needle (as to preserve the rubber cap) and then mixed with methanol (quenching agent) prior to HPLC analysis.

The miniemulsion systems and experiments were performed in duplicate or triplicate and the results are the respective average values.

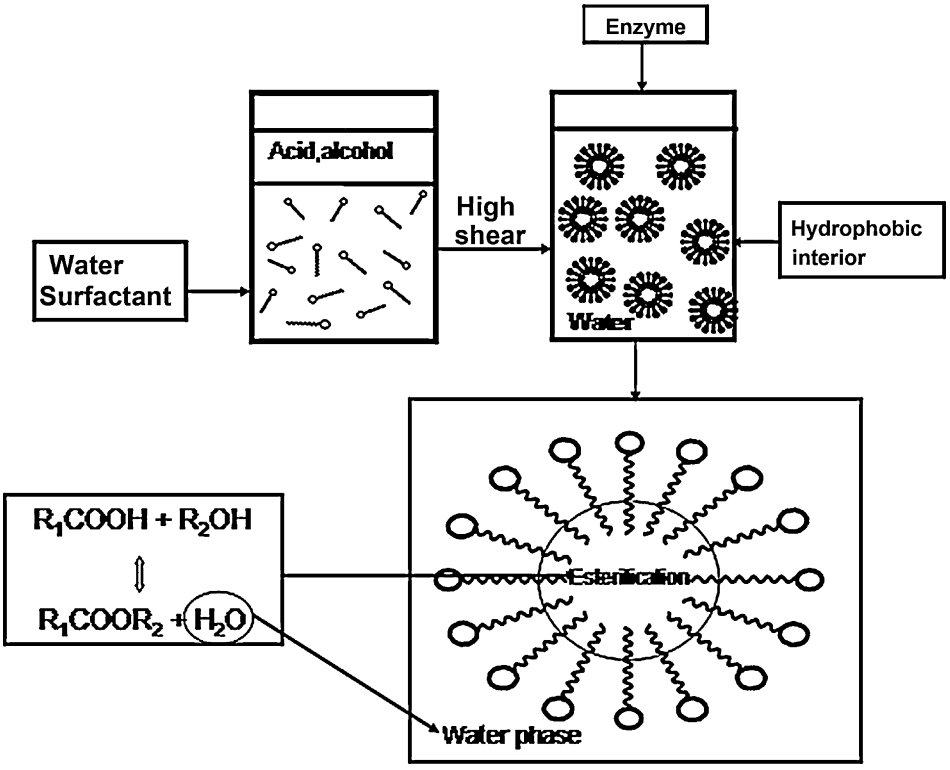

Fig. 1. Principle of an esterification reaction in miniemulsion.

2.3. Determination of the esterification yield in the miniemulsion system

The reaction mixture was analyzed by reverse-phase liquid chromatography (HLPC) using LiChroCART® 250-4 (Purospher® RP-18 column, VWR, Germany). The mobile phase was composed of acetonitrile/water (90:10, v/v) at a flow rate of 0.8 ml min^{-1}. Detection was performed with a UV detector at 220 nm. The concentrations of octanoic acid and hexyl octanoate were established using as reference a calibration curve.

The concentration of hexanol, was determined by GC, in a Hewlett–Packard model 5890 gas chromatograph, equipped with a flame ionization detector (FID) and a WCOT Fused Silica coating CP Chirasil-Dex CB column, 25 m × 0.25 mm, DF = 0.25 (Varian Inc.).

The reaction yield was calculated according to the molar ratio between the hexyl ester and respective limiting substrate, alcohol or acid.

The rate of ester synthesis, *V*, was calculated as the amount of ester formed per mg of protein and per min from aliquots collected in the first hour of reaction (μmol of ester mg $prot^{-1}$ min^{-1}).

2.4. Fluorescence measurements

Samples (1 ml) were collected periodically from the reaction mixture using a needle, and centrifuged for 60 min at 14,000 rpm at 4 °C (Centrifuge 5417R, Eppendorf) for phase separation. The water phase was used for fluorescence analysis. 100 μl of a sample was added to 900 μl of distilled water and fluorescence measurement was done using fluorescence cuvettes.

Fluorescence measurements were performed by excitation at 280 nm (excitation of both tryptophan and tyrosine residues), and emission was collected within 290–450 nm, with excitation end emission slits set at 5 nm. Readings were performed on a Cary Eclipse fluorescence spectrophotometer (Varian Inc.) with 90° geometry, equipped with a thermostated cuvette holder set up at 30 °C and an agitation device. The instrumental response at each wavelength was automatically corrected using in-built procedures, according to the manufacturer.

Cutinase spectra were established by subtracting a baseline obtained with a miniemulsion solution prepared in the same conditions without the enzyme. The spectrum obtained of both Trp and Tyr residues was normalized relatively to the fluorescence intensity at the λ_{max} (wavelength where the maximum intensity occurs).

2.5. Dynamic light scattering (DLS)

The micellar size was measured by DLS (Nano-Zetasizer, Malvern Instruments) at 20 °C under a scattering angle of 173° at a wavelength of 633 nm. The samples were added to a glass cuvette before the measurement. Particle sizes and PDIs are given as the average of three measurements. PDI (dimensionless) is a measure of the particle size distribution, displaying the heterogeneity of the sample. As such, it can range from 0 (monodisperse) to 1 (polydisperse). The *Z*-average diameter is the mean diameter based on the intensity of scattered light.

3. Results and discussion

3.1. Effect of the lyophilized enzyme concentration on ester synthesis

To establish the optimal amount of catalyst for carrying out the targeted esterification, different quantities of lyophilized cutinase were used. The lyophilized cutinase was added to the reaction media so that enzyme concentrations ranging from 1 to 10 mg ml^{-1} were obtained, for equimolar substrates concentration of 0.65 M.

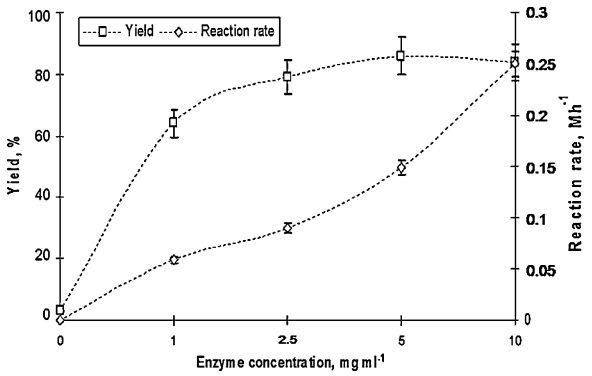

Fig. 2. The effect of the lyophilized cutinase concentration on the synthesis of hexyl octanoate. Reaction conditions: acid/alcohol molar ratio $R = 1$; $T = 40$ °C.

The amount of enzyme present in the bioconversion medium clearly influenced the esterification yield and the reaction rate (Fig. 2). The esterification yield increased from 64%, for an enzyme concentration of 1 mg ml^{-1}, to 86%, for an enzyme concentration of 5 mg ml^{-1}. A further increase of enzyme concentration showed no influence on ester yield (85% for 10 mg ml^{-1}). On the other hand, the reaction rate increased consistently within the range of enzyme concentrations evaluated, up to 0.250 Mh^{-1} for 10 mg ml^{-1}, in a roughly linear manner.

Given that above 2.5 mg ml^{-1} enzyme concentration there is no significant increase in the esterification yield, and considering that the amount of enzyme required is often a critical feature when overall production process costs are considered, further experiments were performed with 2.5 mg ml^{-1} enzyme concentration.

3.2. Effect of substrate molar ratio on ester synthesis

As it is well known, in order to shift the equilibrium of a reaction towards the product side, one of the reactants can be applied in excess.

However, increasing the amount of alcohol or acid above a certain concentration could result in an inhibitory effect on the enzyme. As an outcome, lower reaction rates/final product yields are foreseen.

Without changing the relative amounts (Table 1), acid/alcohol molar ratio (*R*) was increased from 0.1 to 10 (Fig. 3). The individual effect of the acid and alcohol concentration on enzyme activity was studied starting with a small concentration of the acid ($R = 0.1$). By increasing *R* from 0.1 to 0.5, the initial rate of reaction increases and reaches a maximum value of 2.20 μmol min^{-1} mg^{-1}

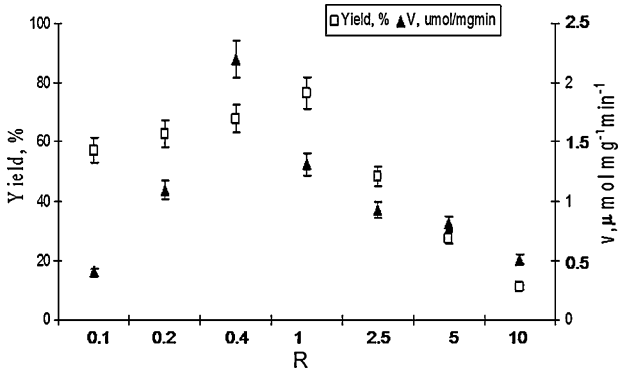

Fig. 3. The effect of the acid/alcohol molar ratio (*R*) on the synthesis of hexyl octanoate by cutinase.

at $R = 0.5$. For $R = 1$ (equimolar concentration of substrates) the initial rate of reaction decreases to 1.31 μmol min^{-1} mg^{-1}. Further increase of R showed further deleterious effect on the enzyme activity.

When the final ester yield is addressed, a slight but steady increase is observed from $R = 0.1$ to $R = 1$, where a 76.4% yield is achieved.

With an excess of alcohol ($R = 0.4$), the initial rate of the reaction was the highest, however a lower final ester yield was obtained. Nevertheless the increasing acid concentration from $R = 1$ up to $R = 10$ decreases the activity and ester yield. This inhibition effect of acids was previously reported for CTAB/reverse micelles as well as for organic solvents [28,57], and was ascribed to the polar acid microenvironment surrounding the enzyme surface. The inhibition effect of the alcohol is smaller, however it could be observed, (Fig. 3), until $R = 1$. When addressing the esterification of nonanoic acid with 3-phenylpropanol, Aschenbrenner et al. [49] also observed a similar profile, since reactions with either an excess of acid or an equimolar substrate ratio showed lower reaction rates than reactions with an excess of alcohol. One reason for the enhanced activity in the presence of an excess of hexanol may be a stabilizing effect of hexanol on cutinase, reported before for reverse micellar systems [28].

3.3. Fluorescence studies of enzyme activity

A steady state fluorescence study was performed to evaluate and gain some insight in changes in cutinase conformation during the time course of the esterification, in a miniemulsion system for different acid/alcohol molar ratios.

Fluorescence emission of cutinase is originated from the aromatic amino acids, one tryptophan (Trp) and six tyrosines (Tyr) [58].

The intensity of the tryptophan fluorescence emission is weak when exciting native cutinase and it could be due to the single tryptophan residue of cutinase at position 69, which is strongly quenched by an adjacent disulfide bridge between cysteines 31 and 109 [58–60]. Therefore the emission of native cutinase is dominated by the six tyrosines [60] and its maximum intensity is at $\lambda_{max} = 303$ nm [51,52].

The denaturation of cutinase can be followed by an increase in tryptophyl emission, because this residue is released from the vicinity of the disulfide bridge when cutinase unfolds. It was shown that with the unfolding of cutinase, a shift in the emission maximum is also observed towards a higher wavelength [59]. For unfolding protein, the spectra became dominated by tryptophyl emission with the maximum occurring at 335 nm [60].

For an acid/alcohol molar ratio $R = 1$ (Fig. 4), a gradual red shift was observed, characteristic of a spectra representative of a denaturation process involving Trp residues along time. Data suggest that under the experimental conditions used, the unfolding of cutinase sets off after 1 h, given the red shift from 303 nm, for $t = 10$ min up to 1 h (considered native cutinase) to 319 nm after 2 h of reaction.

The trend towards a red shift, characteristic of denaturation, under exposure to a miniemulsion environment was further evaluated, for the remaining values of R used (Fig. 5).

In the case of hexanol excess (Fig. 5a), the red shift is noticeable after 70, 120 and 240 min for acid/alcohol molar ratios of $R = 0.4$, $R = 0.2$ and $R = 0.1$, respectively. Data suggest that with the increase of the alcohol concentration denaturation of cutinase evolves at a slower pace along the reaction time even if the ester yields decrease slightly (around 10%). The reason could be due to a stabilizing effect of hexanol, previously described in AOT reversed micellar system [24,59,60]. Hexanol, as a medium chain alcohol, acts as a co-surfactant, increasing the interface flexibility and interdroplet interaction [60].

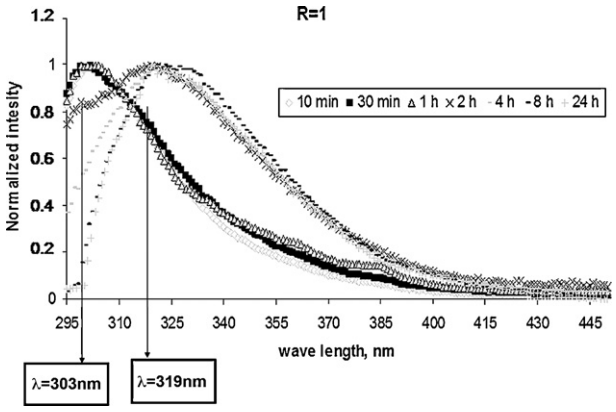

Fig. 4. Normalized spectra of cutinase in miniemulsion at $R = 1$ (acid/alcohol molar ratio).

On the other hand, with the increase of octanoic acid concentration ($R = 2.5$, 5 and 10), the red shift was almost immediate, suggesting a faster denaturation of the cutinase.

In both cases the fluorescence results are in good agreement with the time course of the reactions, since the conversion of the substrates roughly stops coincidently with the occurrence of the red shift (data not shown). For $R = 2.5$, the red shift is noticeable at $t = 30$ min, but for higher concentration of octanoic acid, the enzyme no longer presents its active form after 10 min. These correlate neatly with the very low esterification yield and activity of cutinase observed for R values from 2.5 to 10 (Fig. 3), and could be due to the low pH of the system, since the optimum pH for cutinase was found to be 7–8 [25]. Hence further work was performed to assess the effect of the variation of pH (ΔpH), resulting from different acid/alcohol molar ratio, on enzyme activity and on the stability of the miniemulsion.

By previous work in reverse micelles system [28,60] it was found that cutinase deactivation and denaturation is a reversible process, the next step would be to study reutilization of the cutinase in miniemulsion system.

3.4. Stability and Influence of pH of the system

Enzyme activity depends on the pH of the system, which in the case of a miniemulsion system is determined by the continuous phase [49]. The higher esterification yields and activities were obtained for $R = 0.4$ and $R = 1$, that correspond to initial pH of 4.22 and 4.06, respectively (Table 2). Maximum ΔpH (change of pH from $t = 0$ min to $t = 24$ h) observed in these cases can be related with the higher amount of ester formed, which results in an increase in the pH of the reaction medium. On the other hand, low enzyme activity with relatively low esterification yield (Fig. 3) was observed

Table 2
pH profile of the bioconversion system for different acid/alcohol ratios and a 24 h incubation period.

Molar ratio	pH		ΔpH
	$t = 0$ min	$t = 24$ h	
0.1	5.10	5.74	0.64
0.2	4.63	5.30	0.67
0.4	4.22	5.27	1.05
1	4.06	5.01	0.95
2.5	4.23	4.39	0.16
5	3.72	4.18	0.46
10	3.78	4.26	0.48

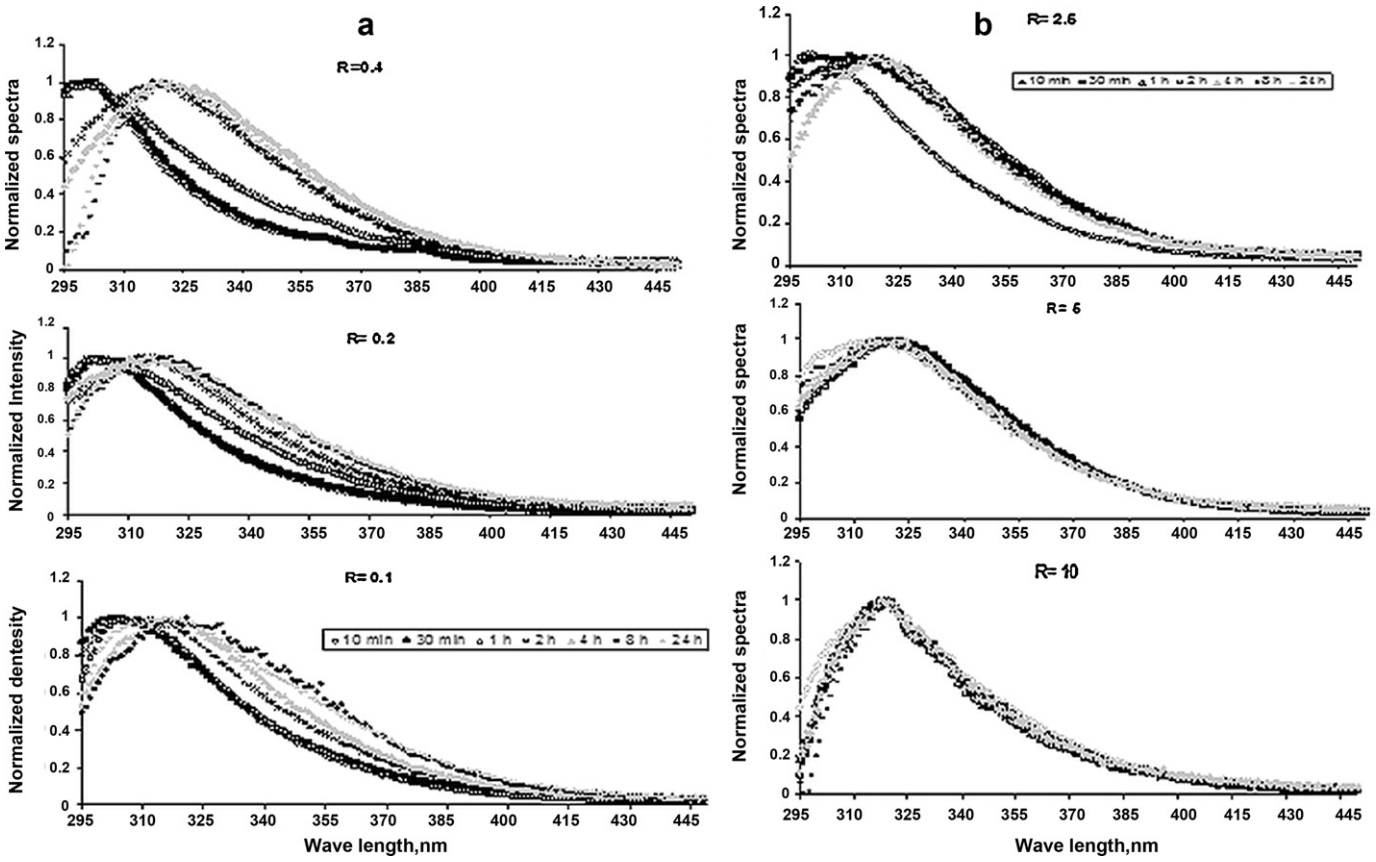

Fig. 5. Fluorescence spectra for different acid/alcohol molar ratio (a) excess of hexanol and (b) excess of octanoic acid.

for $R = 0.1$, corresponding to an initial pH of the system of 5.10 (Table 2). The lower yield with initial pH higher than 5, was reported by other authors [49], for the esterification of nonanoic acid with 3-phenylpropanol in miniemulsion (pH = 6.8) using Amano lipase PS. These authors showed that with a higher pH of the system, the presence of non-ionic surfactant could tamper with enzyme activity. This could take place by interaction with the active site of the enzyme, adsorption on the enzyme or modification of the conformation of the active site. At lower pH, the carboxylic acid is in protonated form and cannot behave as a surfactant. With an excess of the acids, the pH of the system decreases and an inhibitory effect takes place, a pattern also observed in organic solvent [57] and reverse micellar [28] systems, too.

A possibility to explain the difference in ester yields and cutinase activity alongside the range of acid/alcohol molar ratio (Fig. 3) could be a change in the reaction microenvironment, induced by phase separation in the different miniemulsion system. Previous works [49,50] suggested that the stability of miniemulsion systems is related to the hydrophobicity of substrates. Esterification reactions in miniemulsion favor hydrophobic substrates due to the formation of very stable hydrophobic droplets [50].

The stability of the miniemulsion is also influenced by changes of the R (Fig. 6). The miniemulsion systems with R above 2.5 clearly show phase separation after a 24 h incubation period. This could be due to the low pH of the system and inhibition effect on the catalyst already referred (Table 2).

Apparently miniemulsions with lower acid/alcohol molar ratio (due to excess of hexanol) are more stable and phase separation was not observed after 24 h. It was confirmed that the esterification yield in these cases is higher than with the system with higher acid/alcohol molar ratio (excess of acid). The stabilizing effect of hexanol already referred for the reverse micellar systems [28] was confirmed in fluorescence studies (Fig. 5). In the stable miniemulsion the reaction surface is larger and ester yield is higher.

From the Z-average diameter (nm) of the miniemulsion, it was possible to observe the evolution in the size of droplets during time course of the reaction.

Before the addition of the enzyme the Z-average size of droplets was 220 ± 20 nm for all molar ratios (Fig. 7). By increasing the time

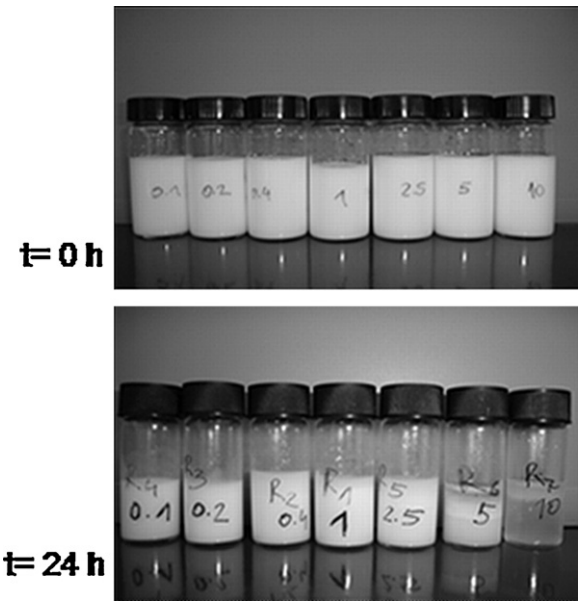

Fig. 6. Stability of miniemulsion.

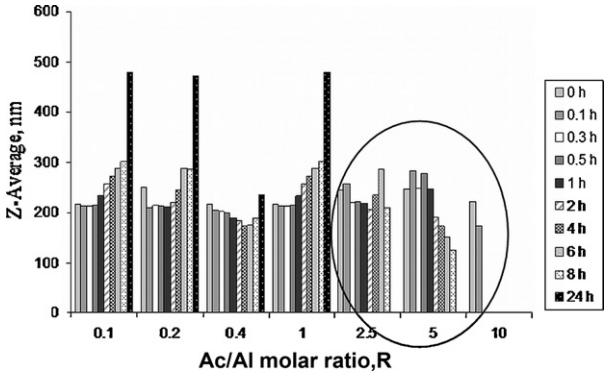

Fig. 7. *Z*-Average diameter of the miniemulsion droplets during the time course of the reaction.

of the reaction the size of droplets slightly increased in almost all cases, except for molar ratio $R = 0.4$. For $R = 0.4$ the size of droplets seems uniform, but for 24 h, probably because the miniemulsion is stable, which again could contribute to the higher ester yield observed in this case.

For acid/alcohol molar ratio higher than 2.5 destabilization of miniemulsion was noticed, resulting in phase separation. This was particularly perceptible for $R = 10$, where phase separation was observed in first 30 min.

After 24 h an increase of *Z*-average diameter was observed for $R = 0.1$, 0.2, 0.4 and 1 molar ratio (Fig. 7). This behavior could be ascribed to the accumulation of hexyl octanoate inside the droplets, which increase their diameter and stabilize the miniemulsion system by its hydrophobicity (Fig. 7).

4. Conclusions

The present work shows that *F. solani pisi* cutinase from recombinant *S. cerevisiae* could be an efficient biocatalyst for the synthesis of hexyl octanoate in a miniemulsion system. However, the results obtained indicate that the variation in molar ratio of substrates has a significant influence on enzyme activity and reaction yield due to inhibitory effects on cutinase activity.

Maximum ester yield (86%) for hexyl octanoate was obtained with alcohol:acid molar ratio $R = 1$ and enzyme concentration of 5 mg ml^{-1}.

Cutinase activity achieved a maximum value (2.20 μmol min^{-1} mg^{-1}) at $R = 0.5$, due to the stabilizing effect of hexanol on cutinase. This is in agreement with previous results carried out in reversed micellar systems. A strong inhibition effect above $R = 1$ acid:alcohol molar ratio was observed.

A steady state fluorescence study showed changes in cutinase conformation. By appearance of the red shift during time curse of the reaction, it is noticeable that denaturation of cutinase depended of different acid/alcohol molar ratios.

The stability of the miniemulsion was influenced by changes of the pH and molar ratio. Accumulations of hydrophobic hexyl octanoate inside the droplets, confirmed by the increase in their diameter, stabilize the miniemulsion.

As cutinase deactivation and denaturation in reverse micelles (microemulsion) is a reversible process, the study of the cutinase reversibility in miniemulsion is a key issue namely when for the feasibility for industrial application is assessed.

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