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Biosynthesis of ethyl caproate and other short ethyl esters catalyzed by cutinase in organic solvent

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ABSTRACT

The main objective of this work was to study the enzymatic synthesis of short chain ethyl esters, a group of relevant aroma molecules, by *Fusarium solani pisi* cutinase in an organic solvent media (iso-octane), and to assess the influence of different parameters on the reaction yield.

Cutinase displayed high initial esterification rates in iso-octane, which amounted to 1.15 μmol min^{-1} mg^{-1} for ethyl butyrate (C_4 acid chain) and 1.06 μmol min^{-1} mg^{-1} for ethyl valerate (C_5 acid chain). High product yields, 84% for ethyl butyrate and 96% for ethyl valerate, were observed after 6 h of reaction, for an initial equimolar concentration of substrates (0.1 M).

The highest product yield (97%) was observed for ethyl caproate (C_6) synthesis, a compound which is a part of natural apple and pineapple flavour, for an alcohol:acid molar ratio of 2 (0.2 M ethanol concentration).

Cutinase affinity for short chain length carboxylic acids (C_4–C_6) in ester synthesis in iso-octane confirmed previous observations in reversed micellar system.

1. Introduction

Short chain acid esters, as flavour compounds, are part of a large group of natural aromas. Finding a cheaper way of producing these compounds is a relevant matter due to their high demand by industry sectors, specially food, beverage, cosmetic and pharmaceutical [1,2]. Short chain acid esters are commonly obtained either by chemical synthesis for an alcohol and an organic acid in the presence of an acid catalyst, or by extraction from natural sources. Natural aromas obtained by plant extraction consist of a mixture of different flavour compounds. These natural aromas are very expensive due to their low concentration in the natural product and the very low extraction yields. In contrast, the chemical synthesis of these fatty acid esters is cheap but these are not classified as natural products and manufacturing demands in the presence of strong acids or alkali as catalyst and high temperature and pressure [3,4].

Enzyme catalyzed esterification is an alternative to chemical synthesis for the production of fatty acid esters and other similar flavours. The enzymatic approach allows mild reaction conditions and the resulting products are classified as natural by food regulatory agencies, a feature that increased their public acceptance as ingredients for food industry, when compared to those synthesized by chemical processes [5,6].

Lipases and esterases are a particularly useful class of enzymes with great potential for the production of a wide range of ester products in non-conventional media, for instance, pure flavours (e.g. ethyl esters like caproate, valerate, octanoate, acetate, among many others) that can be blended with the goal of replacing the natural fruit aromas [7]. Lipases are the most common enzymatic systems used at the small scale for the synthesis of those flavour esters of short chain carboxylic acids [8,9].

Cutinases are a group of enzymes that can be considered as a link between esterases and lipases. As small carboxylic ester hydrolases, the sub-family of cutinases consists of 20 members based on amino-acid sequence similarity, which display hydrolytic activity on cutin polymers and efficiently hydrolyze soluble esters and emulsified triacylglycerols. Cutinase belongs to the family of serine hydrolases containing its catalytic serine center at the middle of a sharp turn between a β-strand and an α-helix [10,11]. The catalytic triad, *Ser-120*, *Asp-175* and *His-188*, is accessible to the solvent and it can possibly accommodate different substrates. The *Asp-175* hydrogen binds to histidine-188, thus promoting the interaction of the imidazole ring with *Ser-120*. The histidine acts as a base, deprotonating the serine to generate a very nucleophilic alkoxide (–O–) group (Fig. 1). The serine in the active center of the enzyme is a very strong nucleophile, which attacks the carbonyl group of the acid, forming a stable tetrahedral intermediate acyl enzyme complex. The acyl enzyme complex is stabilized by the oxyanion

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Nomenclature

d*Abs*/d*t*	change in absorbance per minute
D	dilution factor of enzyme sample
ε	absorption coefficient (=1.84 × 10^4 M^{-1} cm^{-1})
V_s	volume of cutinase sample added to the cuvette (μl)
V_c	volume of cuvette (ml)

hole [12]. Water is then released and the structure reverts to the planar carbonyl flat plane acyl enzyme intermediate. The alcohol acts afterwards as a new nucleophile and links to the tetrahedral intermediate. Subsequently, as the final step, the resolution of tetrahedral complex yields the ester and the free enzyme. This reversible reaction was shown to follow a Ping-Pong bi–bi mechanism [13,14].

Cutinases belonging the family of esterases are lipolytic enzymes, whose potential for applications in industrial products and processes has been documented. Given its versatility, cutinase has been used in dairy industry for the hydrolysis of milk fat, in the formulation of house hold detergents, in oleo-chemical industry, in the synthesis of structured triglycerides, polymers and surfactants, in the synthesis of ingredients for personal-care products and in the synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers. Taking advantage of its in vivo cutinolytic activity, cutinase preparations have been developed for enhancing the pharmacological effect of agriculture chemicals [15,16]. Some of these processes are already applied in industry, while others are still under evaluation at a research level. The esterification potential of cutinase was tentatively evaluated for the production of aroma esters, which are relevant molecules in the aroma industry [17]. The promising results obtained, namely regarding the short reaction time and high yield of product, suggested that cutinase could provide a competitive alternative when compared with other lipolytic enzymes, although further insight on the bioconversion system was required.

Cutinases have been used in reaction media often dissolved in aqueous solution but also suspended as a powder or immobilized. Immobilization has been commonly obtained by adsorption onto solid supports [18–20], or encapsulation in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT), phosphatidylcholine, or cetyltrimethylammonium bromide (CTAB) [21–23]. Up to now, lyophilized cutinase was only used in fundamental studies on the hydrolysis of triglycerides [24,25], in order to clarify its mechanism of action regarding stereo-selectivity and specificity [24], and in kinetic studies of esterification reactions [26,27].

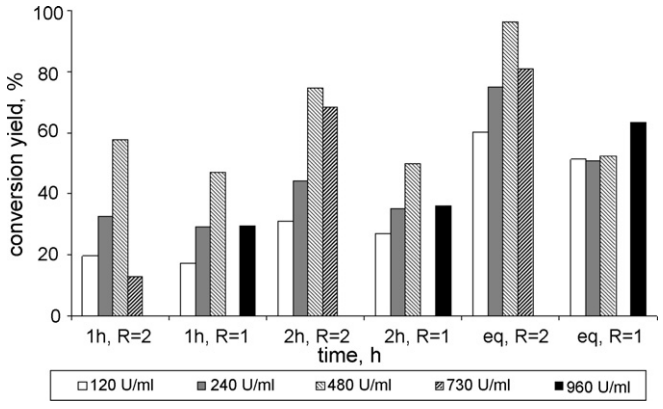

Fig. 1. The effect of the lyophilized cutinase concentration on the synthesis of ethyl caproate for alcohol/acid molar ratios of $R = 2$ ([caproic acid] = 0.1 M) and $R = 1$ ([caproic acid] = 0.2 M). eq – equilibrium. Other reaction conditions: [ethanol] = 0.2 M; stirring speed = 300 rpm; $T = 30\,^{\circ}C$; cutinase specific activity = 170 U mg^{-1}.

Cutinase was first cloned in *Escherichia coli*, although its expression in *Saccharomyces cerevisiae* is more efficient and safe for the production of ingredients for food and pharmaceutical products [28]. The goal of this study was to pinpoint and optimize key operational conditions for the synthesis of fruit flavours with cutinase from *S. cerevisiae*.

Ester synthesis was performed in iso-octane. There are several advantages of using the organic solvent media for the enzymatic ester synthesis, such as an increased solubility of non-polar substrates and products and shifting the thermodynamic equilibrium of the reaction to favour ester synthesis over hydrolysis.

Enzyme activity/stability in esterification reactions in non-conventional media and its efficiency in the synthesis of short chain alkyl esters such as ethyl acetate, butyrate, valerate, caproate, octanoate, decanoate and oleate were evaluated as well. The optimization of the bioconversion system was performed using ethyl caproate synthesis as model system, since it is a flavour compound that is incorporated in a wide range of aromas such as apple, green apple, banana, beer, butter, cognac, herbal, pineapple and wine [29].

2. Materials and methods

2.1. Materials and chemicals

Carboxylic acids: acetic (C_2) (96%, Acros, Geel, Belgium), butyric (C_4) (99.5%, Fluka, Germany), valeric (C_5) (99%, Fluka, Germany), caproic (C_6) (99%, Fluka, Germany), octanoic (C_8) (99%, Sigma, Germany), decanoic (C_{10}) and oleic (C_{18}) (Fluka, Germany), ethanol abs. (VWR, Germany) and ethanol 96% (AGA, Portugal) were used for ester synthesis, while iso-octane (99.5%, Fluka, Germany) was used as organic solvent and *n*-decane (VWR, Germany) was used as an internal standard for gas chromatography (GC). Sodium sulfate anhydride (Acros, Geel, Belgium) was used to dry iso-octane as organic media before and after esterification reactions. Salts used for water activity and all other chemicals used were of analytical grade.

2.2. Production of cutinase

Fusarium solani pisi cutinase wild-type was biosynthesized by recombinant *S. cerevisiae* SU50 strain as described by Calado et al. [30]. 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 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 Leu) 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 the cell concentration between 1.1 and 1.8 g dcw l^{-1} was attained. This inoculum was transferred at 10% (v/v) ratio into the cultivation media for enzyme biosynthesis. 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, both at 2 N) and at 30 °C with a minimum dissolved oxygen tension of 30% of air saturation. A constant air flow rate of 4.4 l min^{-1} (equivalent to 1.1 vvm) was provided during the cultivation.

2.3. Purification and lyophilization of cutinase

The isolation and purification of cutinase excreted by recombinant *S. cerevisiae* SU50 strain were carried out by expanded bed adsorption (EBA) [28]. 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 the 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 pH 4.5 (previously diluted with water 4:1) was loaded through the EBA column. After frontal feedstock adsorption, the bed was washed until the effluent was devoid of yeast cells. The elution was carried out in packed bed mode in downward flow, using a 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 [31].

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

The enzyme used for esterification was obtained from two different batches with a different specific activity due to some enzyme deactivation.

2.4. Characterization of the cutinase preparations

The cutinase esterolytic activity and the protein content were assayed and in this way lyophilized cutinase preparations were characterized [31–33].

2.4.1. Cutinase activity

The cutinase estereolytic activity was assayed using a spectrophotometric method based on monitoring the hydrolysis of p-nitrophenylbutyrate (p-NPB) to p-nitrophenol (p-NP), a yellow compound easily identified and quantified by the absorbance at 400 nm [30]. 20 μl of sample was added to a reaction mixture composed of 980 μl of a solution in 50 mM potassium phosphate buffer pH 7.0 containing also 0.56 mM p-NPB, 11.3 mM sodium cholate and 0.43 M tetrahydrofuran.

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 1 min, under specific condition. The extinction coefficient of p-NP was considered to be 1.84×10^4 (M^{-1} cm^{-1}), as indicated by the supplier (Sigma).

Activity in μmol min^{-1} ml^{-1} (=U ml^{-1}):

$$\frac{dAbs/dt \times D \times V_c \times 10^6}{\varepsilon \times V_s}$$

Specific activities of lyophilized cutinase preparations were of 170 U mg^{-1}. These enzyme samples when loaded in SDS electrophoresis gel showed basically only one band of 22 kDa [32].

2.4.2. Protein concentration

The protein concentration was determined by the method of PEARCE (BCA assay) with reference to a standard, the Bovine Serum Albumin (BSA) (Merck) [33].

2.5. Methods for monitoring substrate and ester concentrations

The concentration of ethanol, carboxylic acids (acetic, butyric, valeric, caproic, octanoic, decanoic and oleic) and ethyl esters (acetate, butyrate, valerate, caproate, octanoate, decanoate and oleate) was determined by a Hewlett-Packard model 5890 gas chromatograph, equipped with a flame ionization detector (FID). The WCOT fused silica coating CP Chirasil-Dex CB column, 25 m × 0.25 mm, DF = 0.25 (Varian Inc.) was used. *n*-Decane was used as the internal standard as to calculate ethyl esters and respective substrates concentrations in the reaction media based on the results of gas chromatography (GC) analysis. These were performed according to the following specific conditions applied for each particular ethyl ester and respective substrates: In all cases, nitrogen was used as carrier gas. The injector temperature was set at 200 °C; the detector temperature was set at 250 °C. The retention time (tR), in minutes, and the temperature program for each ester were as follows: Ethyl acetate: 50 °C (4 min) – 30 °C min^{-1} – 150 °C (3.67 min); tR 2.72. Ethyl butyrate: 50 °C (4 min) – 15 °C min^{-1} – 150 °C (2.34 min); tR 6.40. Ethyl valerate: 50 °C (4 min) – 30 °C min^{-1} – 170 °C (2 min); tR 6.65. Ethyl caproate: 50 °C (4 min) – 15 °C min^{-1} – 160 °C (1.67 min); tR 9.52. Ethyl octanoate: 50 °C (4 min) – 30 °C min^{-1} – 180 °C (3.67 min); tR 9.20. Ethyl decanoate: 50 °C (4 min) – 30 °C min^{-1} – 190 °C (1.67 min); tR 10.50. Ethyl oleate: 50 °C (4 min) – 30 °C min^{-1} – 190 °C (11.34 min); tR 18.50.

2.6. Enzymatic esterification

The esterification of acid and alcohol by cutinase was carried out in iso-octane as organic solvent. Unless otherwise stated a typical esterification reaction for ethyl ester synthesis was carried out in 7 ml of working volume inside a 10 ml flasks capped with rubber (EPDM stoppers, black, Sigma–Aldrich, Germany), as to minimize the evaporation or loss of volatile compounds. Alcohol, acid and *n*-decane were mixed thoroughly in iso-octane before the addition of enzyme (lyophilized form). The first sample (zero point of the reaction) was collected before the enzyme addition. The enzymatic ester synthesis was performed in an incubator (AGITORB 160E, Aralab, Portugal) at 30 °C (optimum temperature for the enzymatic reaction) with 0.05% (w/w) of water and cutinase presenting an activity of 240 ± 10 U ml^{-1} of reaction media (r.m.) or otherwise as stated in the text. The experimental setup enabled the operation of multiple parallel experiments using multiple magnetic stirrer (S.B.S. Instruments A-23) by simultaneous use of 6–12 reactors, as well as easy control of experimental conditions using at least a blank (without the enzyme) running in parallel for each enzymatic ester synthesis. Experiments were performed at least in duplicate with an experimental error of less than 8%. During the reaction, a magnetic stirring set at 300 rpm was performed. Samples were withdrawn periodically using a needle without destroying the rubber cap and analyzed by GC. The reaction yield was calculated according to the molar ration between the ethyl ester and respective limiting substrate, alcohol or acid.

2.7. Determination of water content

The water content in the medium was determined by Karl Fisher titration. The titration was performed with a KF coulometer (703 Ti Stand, Metrohm, Switzerland) at room temperature. Hydranal®-Coulomat AG (Riedel-de Haën, Germany) was used as analyte for coulometric Karl Fisher titration. The water content in reaction media solution was determined by injecting a 0.1 ml of the sample and titrating with reagent.

2.8. Equilibration of substrates and enzymes with saturated aqueous salt solution

The selected reaction medium and enzyme were pre-equilibrated separately at 30 °C, for 72 h, inside closed vessels containing saturated aqueous salt solution. The chosen water activities (a_w) were: KOH (a_w = 0.07); CH_3COOK (a_w = 0.23); $MgCl_2$ (a_w = 0.32); K_2CO_3 (a_w = 0.43); NaBr (a_w = 0.56); $CuCl_2$ (a_w = 0.68); NaCl (a_w = 0.75); KCl (a_w = 0.84); KNO_3 (a_w = 0.94) [44].

3. Results and discussion

3.1. Effect of the lyophilized enzyme concentration on ester synthesis

The effect of the lyophilized cutinase concentration on the synthesis of ethyl caproate in iso-octane was studied at 30 °C with enzyme concentrations ranging from 0.7 to 5.6 mg ml^{-1}, corresponding to enzymatic activity ranging from 120 to 960 U ml^{-1} r.m. (reaction mixture) at two different alcohol:acid molar ratios (R), 1 and 2, and at a constant alcohol concentration of 0.2 M.

Irrespective of the molar ratio of substrates, and up to incubation periods of 2 h, an increase in the ester yield with enzyme concentration up to 2.8 mg ml^{-1} (480 U ml^{-1} r.m.) was observed, but for higher enzyme concentrations the ester yield decreased. This negative effect in the early stages of the reaction may be due to the aggregation of lyophilized enzyme particles observed at the higher concentrations tested. Along the time course of the reaction these aggregates eventually dissolve, water released throughout the process contributing to this feature. Similar influence of enzyme concentration on ester synthesis was described by other authors [34–36]. Gandhi et al. [34] suggested that the active site of the enzymes could be hidden inside the bulk of lyophilized enzyme particles, thus not accessible for the substrates and, consequently, a significant part of the lyophilized enzyme would not be involved in the reactions.

Higher ester yields were always observed for R = 2 independently of the enzyme concentrations. This was due to the inhibition of the enzyme activity as a result of a higher concentration of caproic acid for R = 1 (0.2 M) (Fig. 1).

The ester yield of 97% after 6 h achieved in this study is quite high and comparable with other values obtained by other authors and also with other enzymes, mostly lipases in immobilized forms. An ester yield around 95% after 72 h and substrates concentration 0.06 M was reported for the synthesis of isoamyl acetate using immobilized lipase from *Rhizomucor miehei* [35] and also for ethyl hexanoate with *Rhizopus chinesis* CCTCC M201021 cells in non-aqueous phase a yield of 96.2% after 72 h (6 g l^{-1} enzyme concentration, 0.5 M substrates concentration) was reported [36].

The subsequent experiments were performed with an enzyme concentration of 1.4 mg ml^{-1} which corresponds to 240 ± 10 U ml^{-1} r.m., in order to provide reliable data regarding initial reaction rates.

3.2. Effect of temperature on ester synthesis by the cutinase

The effect of the temperature on cutinase activity was evaluated in the range of 25–50 °C, for an acid ratio (R) of 2. The ester yield increased with temperature up to 30 °C, where the highest ester yield (70%) was achieved after 10 h of incubation. For higher temperatures the conversion yield decreased (Fig. 2).

A different temperature–activity profile was observed for an equimolar alcohol:acid molar ratio especially for temperatures higher than 30 °C, although the optimal temperature remained unchanged (Fig. 3). The presence of polar substrates (ethanol is soluble in water but the solubility of caproic acid in water is just

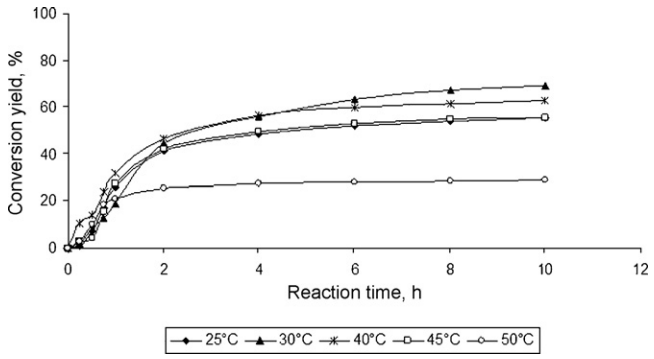

Fig. 2. The effect of temperature on the synthesis of ethyl caproate. Alcohol/acid molar ratios of R = 2 ([caproic acid] = 0.1 M); cutinase = 240 ± 10 U ml^{-1} r.m.; stirring speed = 300 rpm.

1.1 g l^{-1} (20 °C) [36]) could induce higher inhibitory or deactivation effects on enzyme activity which will be more significant at higher temperature.

3.3. Effect of water on ester synthesis by cutinase

Water content is another important parameter in esterification reactions and enzyme activity in non-conventional medium. Water molecules maintain the three-dimensional conformation of enzymes and retain their activity. However, water is a second product during the reaction of esterification and to favour the synthesis over hydrolysis the amount of water in reaction medium needs to be minimized and controlled [38].

The most convenient parameter to study the influence of the water content on enzymatic activity in the reaction in non-aqueous media is water activity, a_w. Many authors reported that a maximum in enzymatic activity is usually reached at specific a_w values [39–42]. There are different approaches to control water activity [43] and in this work enzyme and all components of the system (reactants and reaction medium) were pre-equilibrated over aqueous saturated salt solution of known a_w [44] presented in Table 1.

Fig. 4 shows the effect of the initial a_w on the initial rates of esterification at fixed alcohol:acid molar ratio R = 2. The esterification activity of cutinase increases along with water activity, up to a maximum for a_w comprehended between 0.68 and 0.75. This behaviour was also observed for different lipases and esterases [45–47]. In particular, the initial rates of esterification of ethyl caproate by cutinase from *F. solani pisi* were similar to what have been observed by *Fusarium oxysporum* esterase with a maximum value at a_w = 0.75 in the synthesis of short chain geranyl esters [45]. The final esterification

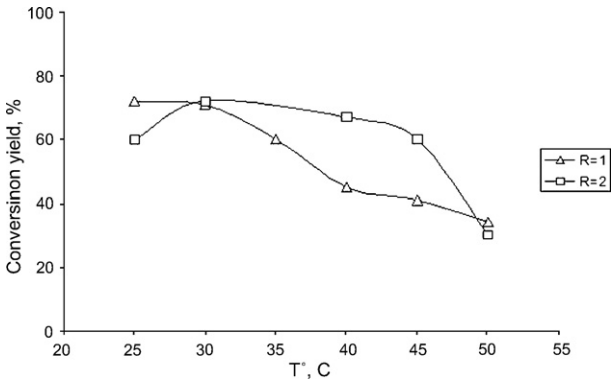

Fig. 3. The effect of temperature on the synthesis of ethyl caproate for alcohol/acid molar ratios of R = 2 ([caproic acid] = 0.1 M) and R = 1 ([caproic acid] = 0.2 M). Reaction time = 24 h; stirring speed = 300 rpm; cutinase = 240 ± 10 U ml^{-1} r.m.

Table 1
Water activity for aqueous saturated salt solution and ethyl caproate yield (%) after 6 h, obtained upon esterification under a given initial water activity.

Salt	Water activity, a_w	Conversion (%)
CH_3COOK	0.23	80
$MgCl_2$	0.32	83
K_2CO_3	0.43	85
NaBr	0.56	83
$CuCl_2$	0.68	91
NaCl	0.75	78
KCl	0.84	74
KNO_3	0.94	58

yield observed after a 6 h incubation period was also influenced by water activity (Table 1), but in a mitigated manner, when compared to the effect of the later parameter in the initial rate of reaction. The highest ester yield was observed for $a_w = 0.68$ (91% at 6 h). In the range of a_w from 0.23 to 0.56 there is almost no difference in ester yield, but for a_w above 0.84 a significant decrease in ester yield was observed (for $a_w = 0.94$ is 58% at 6 h). Still the pattern observed is roughly coherent with the pattern observed for the initial reaction rate.

The second set of experiments was carried out adding the known amount of water at the beginning of the reaction. This approach of optimizing the initial water content of the reaction is convenient for the practical application of ester synthesis in larger scale such as industrial production.

When the effect of water concentration on cutinase activity in the synthesis of ethyl caproate was studied at a water content in the reaction medium ranging from 0% to 0.45% (w/w), and for substrates ratio of $R = 2$, a trend similar to the one displayed when the effect of a_w in enzyme activity was observed. A maximum yield of ester (80% at 8 h) and a highest initial rate of the reaction (2 μmol mg^{-1} min^{-1}) were determined for 0.05% (w/w) of water in the reaction medium (Fig. 5). The concentration of water initially added above 0.05% decreased the conversion yield and the initial rate of reaction. Such higher amounts of water in the reaction medium favour the hydrolysis reaction hence significantly decreasing the esterolytic activity of cutinase. The initial reaction rate for 0.05% (w/w) matches the maximum value for the same parameter when the effect of a_w was evaluated.

If absolute ethanol was used in the reaction and iso-octane previously dried, cutinase was almost inactive during the first 20 min, probably due to the formation of the inactive form of enzyme as a result of the lack of the essential amount of water that is necessary to hydrate lyophilized cutinase preparation in an active form in iso-octane. However, after 20 min, the reaction starts rather abruptly probably due to the accumulation of minimal and essential amount of water in reaction medium that activates the cutinase.

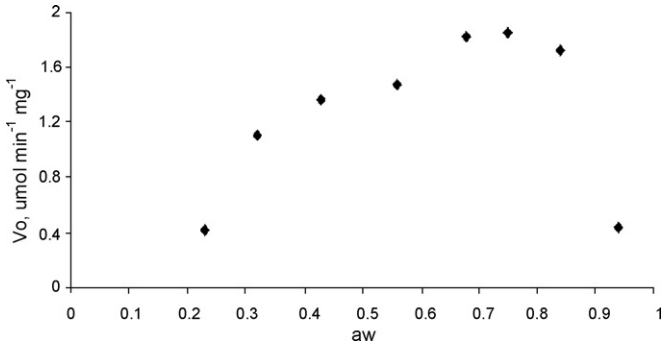

Fig. 4. The effect of water activity (a_w) on the synthesis of ethyl caproate for alcohol/acid molar ratios of $R = 2$ ([caproic acid] = 0.1 M) and $R = 1$ ([caproic acid] = 0.2 M). Reaction time = 6 h; stirring speed = 300 rpm; cutinase = 240 ± 10 U ml^{-1} r.m.

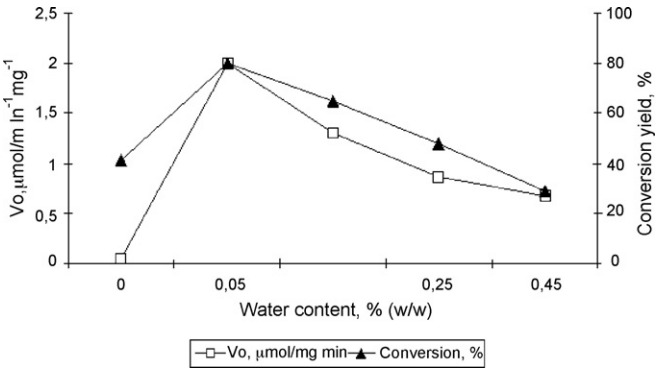

Fig. 5. The effect of water on the synthesis of ethyl caproate by the cutinase for alcohol/acid molar ratios of $R = 2$ ([caproic acid] = 0.1 M). Reaction time = 8 h; cutinase = 240 ± 10 U ml^{-1} r.m.; stirring speed = 300 rpm.

A maximum yield of ester (80% at 8 h) and a highest initial rate of the reaction (2 μmol mg^{-1} min^{-1}) were determined for 0.05% (w/w) of water in the reaction medium (Fig. 5). The concentration of water initially added above 0.05% decreased the conversion yield and the initial rate of reaction. Such higher amounts of water in the reaction medium favour the hydrolysis reaction hence significantly decreasing the esterolytic activity of cutinase.

3.4. Effect of substrate concentration and the substrate molar ratio on ester synthesis by cutinase

For equimolar concentration of substrates (0.1–0.5 M), the highest ester yield (77%) was observed for a substrate concentration of 0.2 M (Fig. 6). Further increase of the substrate concentration significantly decreased the ester yield. Similarly, Hu et al. [36] observed a substrate inhibitory effect on ethyl caproate synthesis with whole-cell lipase from *R. chinesis* but for substrate concentrations above 0.6 M.

Studies on the synthesis of short chain alkyl esters by goat pregastric lipase in the form of suspended enzyme powder have also shown that for a concentration of hexanoic acid above 0.25 M, and for a 1:1 alcohol/acid molar ratio, the initial rate of synthesis of butyl caproate decreases. This trend was ascribed to a significant change in the catalytic micro-environment [37].

3.4.1. Influence of acid concentration

The influence of acid concentration on ethyl caproate synthesis was evaluated, namely since an increase in acid concentration provides a way to shift the equilibrium of the reaction towards the synthesis. The individual effect of acid concentration was studied by decreasing the alcohol:acid molar ratio (R) from 8 to 0.8 at a

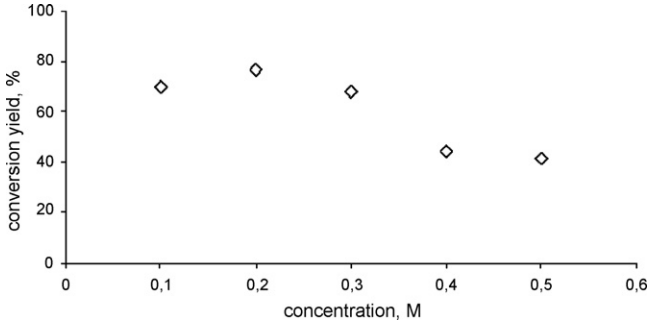

Fig. 6. Effect of the concentration of substrates on the conversion yield of ethyl caproate at equilibrium (8 h). Reaction conditions: alcohol/acid molar ratio $R = 1$; cutinase = 240 ± 10 U ml^{-1} r.m.; stirring speed = 300 rpm.

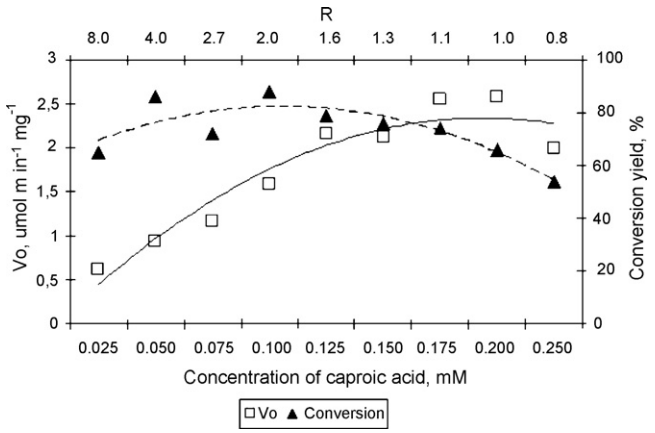

Fig. 7. The effect of acid concentration (alcohol:acid molar ratio R) on the synthesis of ethyl caproate by cutinase. Reaction conditions: [ethanol] = 0.2 M; T = 30 °C; stirring speed = 300 rpm; cutinase = 240 ± 10 U ml^{-1} r.m.

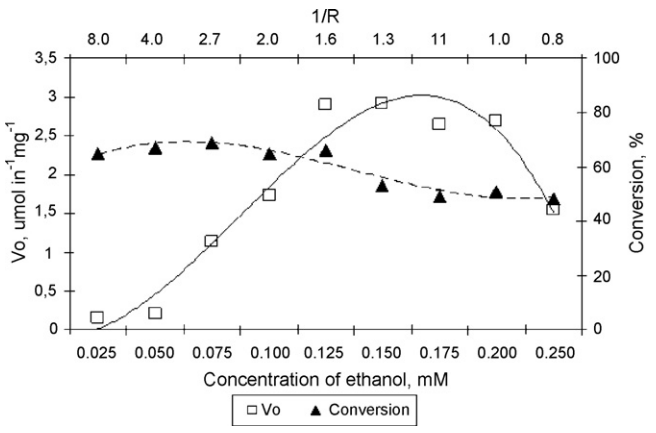

Fig. 8. The effect of alcohol concentration (alcohol:acid molar ratio 1/R) with fixed acid concentration on the synthesis of ethyl caproate by cutinase. Reaction conditions: [caproic acid] = 0.2 M; T = 30 °C; stirring speed = 300 rpm; cutinase = 240 ± 10 U ml^{-1} r.m.

fixed alcohol concentration of 0.2 M. The acid concentration ranged therefore from 0.025 to 0.25 M.

By increasing the acid concentration from 0.025 to 0.25 M, the initial rate of the reaction increases and reaches a maximum value of 2.58 μmol min^{-1} mg^{-1} at R = 1 ([acid] = 0.2 M). For R = 0.8 ([acid] = 0.25 M), the initial rate of the reaction decreases to 1.99 μmol min^{-1} mg^{-1} probably due to the inhibitory effect of acid. When the final ester yield is addressed, the best result was obtained for R = 2 (88%, 6 h). Increased deleterious effects on the ester yield were noticeable for higher concentration of caproic acid (Fig. 7). This inhibition effect on the cutinase activity was previously reported for concentrations above 0.15 M in a CTAB/reverse micellar system as a result of polar acid micro-environment surrounding the enzyme interface that decreases the pH of the reaction medium [23].

3.4.2. Influence of alcohol concentration

The equilibrium of the esterification reaction can also be shifted towards ester formation by increasing the concentration of alcohol (nucleophile). However, many authors reported an inhibitory effect of the excess of alcohol on enzyme activity for different types of lipases [34–36]. The influence of the alcohol concentration on cutinase activity used in the synthesis of ethyl caproate was evaluated by increasing alcohol concentration from 0.025 to 0.25 M (acid/alcohol molar ratios 1/R), at fixed acid concentration of 0.2 M.

The initial reaction rate increased with the increase of ethanol concentration up to 0.15 M, where a reaction rate of 2.91 μmol min^{-1} mg^{-1} was observed. A strong inhibitory effect was observed for 0.25 M ethanol, for the initial reaction rate was pretty low (1.54 μmol min^{-1} mg^{-1}). Additionally, there was no significant difference in the ester yield after 6 h of reaction, for the molar ratio ranging from 1.6 to 8, although for ethanol concentrations above 0.125 M the conversion yield decreased significantly down to 48% (Fig. 8).

Alcohol acts as a nucleophile/acyl acceptor in the reaction mechanism and an initial increase of its concentration (up to 0.125 M) enhances the ester yield, probably due to greater availability and excess of nucleophile for acyl transfer. Above the specific (critical) concentration of alcohol, the negative effect of the high alcohol concentration can be explained by the formation of dead-end complex between the alcohol and cutinase [21] or by accumulation of ethanol in the aqueous micro-environment that surrounds the enzyme causing dehydration and loss of the enzyme activity. Similar results were reported for whole-cell lipase from *R. chinesis* CCTCC M201021 [36] during the synthesis of ethyl caproate. In this case, the increase of acid:alcohol molar ratio from 1:1.3 to 1:3 decreased the yield of ethyl caproate from 98.5% to 50%.

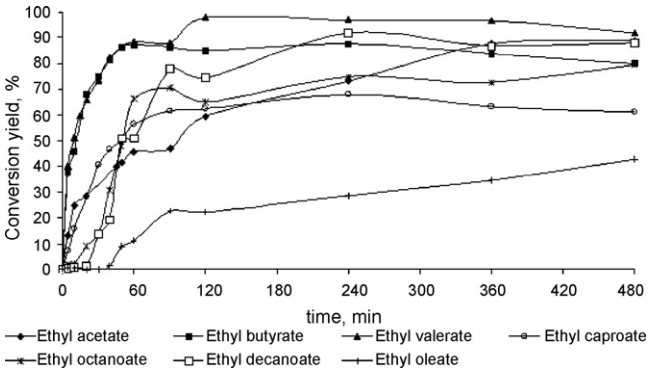

Fig. 9. Effect of acid chain length on the synthesis of ethyl esters by the cutinase. Reaction conditions: alcohol/acid molar ratio R = 1 ([ethanol] = 0.1 M); stirring speed = 300 rpm; T = 30 °C; cutinase = 240 ± 10 U ml^{-1} r.m.

3.5. Effect of acid chain length on ester synthesis by the cutinase

The effect of acid chain length on ethyl ester synthesis by cutinase was assessed by using seven different acids with increasing chain length from C_2 to C_{18} atoms (*i.e.*, acetic C_2, butyric C_4, valeric C_5, caproic C_6, octanoic C_8, decanoic C_{10} and oleic acids C_{18} acids, respectively) at equimolar reaction conditions (0.1 M concentrations of substrates).

Acid chain length showed a significant influence on the initial rate of the esterification reaction by cutinase and the reaction with octanoic and decanoic acid almost did not occur in the first 10 and 20 min, respectively (Fig. 9). A "lag" time of 40 min was observed for oleic acid (C_{18}) too (Table 2).

Table 2
Influence of chain length of acid on initial rate of reaction.

Carbon chain of acids	V_o (μmol min^{-1} mg^{-1})	"Lag" reaction time (min)
2	0.30	0
4	1.15	0
5	1.06	0
6	0.41	0
8	0.48	10
10	0.44	20
18	0.24	40

The reaction conditions are the same as in Fig. 9.

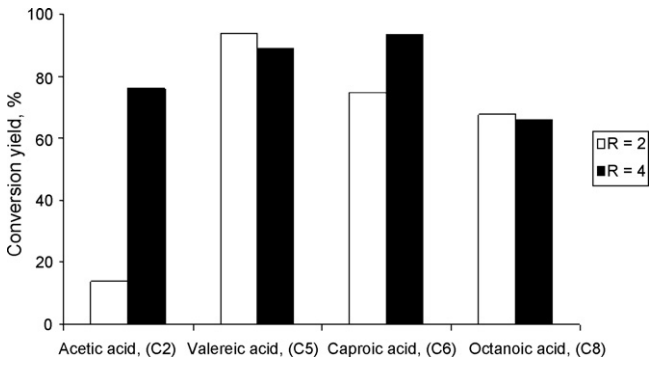

Fig. 10. Effect of acid chain length on the synthesis of ethyl esters by cutinase in iso-octane after 4 h of reaction. Reaction conditions: [ethanol] = 0.2 M; stirring speed = 300 rpm; T = 30 °C; cutinase = 240 ± 10 U ml^{-1} r.m.

The results demonstrated that cutinase showed a higher selectivity for shorter chain fatty acids mainly for butyric and valeric acids, at the experimental conditions applied. The highest initial rates of the reaction (1.15 and 1.06 μmol min^{-1} mg^{-1}) were obtained for butyric (C_4) and valeric acid (C_5), respectively.

The initial rates of esterification for larger acid chain length, e.g. C_6, C_8 and C_{10} were significantly smaller and the variation of the initial rates were also rather small (0.41–0.48 μmol min^{-1} mg^{-1}) (Table 2).

Furthermore, these results suggest that cutinase shows similar substrate selectivity towards short chain fatty acids (C_4–C_6) which is in accordance with previous studies performed in reverse micellar systems [14,21–23].

The ester yield at 6 h was higher for valeric acid (96.5%) than for butyric acid (84%) and caproic acid (62%) and a significantly lower ester yield of 43% at 8 h was obtained for oleic acid.

Enzyme selectivity for the acid chain length is usually related to the hydrolytic activity of lipases and esterases. Cutinase showed higher hydrolytic activity in aqueous media with tributyrin (glyceryl esters of butyric acid) than with triolein (triglyceride and unsaturated fat formed from oleic acid) and this is due to the fatty acids of long chain (e.g. triolein). These acids react with the active site (*Ser-120*) of cutinase, and possibly block the access of the alcohol to the intermediate acyl enzyme and, consequently, the formation of the product [48]. However, the low ethyl acetate yield in relation to other ethyl esters was due to a high acetic acid concentration used (0.1 M at R = 2) suggesting that a strong deactivation of cutinase may occur due to the very polar acid micro-environment surrounding the enzyme interface [35].

The effect of acid chain length on the esterification by the cutinase in iso-octane was assayed for another two different alcohol:acid ratios, R = 2 and R = 4 after 4 h of the reaction. The system with alcohol/acid molar ratio R = 4 ([ethanol] = 0.2 M and [acid] = 0.05 M) attained significantly better yield of ethyl acetate in relation to previous experiments of ethyl acetate probably due to smaller enzyme inhibition of acetic acid at the concentration of 0.05 M. Higher yields were observed when valeric and caproic acids were used as substrates, as compared to octanoic and acetic acids (Fig. 10). This behaviour of cutinase is similar to the previously observed in reverse micellar system [23].

4. Conclusions

The present work shows that *F. solani pisi* cutinase from recombinant *S. cerevisiae* could be an efficient biocatalyst for the synthesis of short chain alkyl esters in an organic solvent system, *i.e.*, iso-octane. 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 (97%) for ethyl caproate was obtained with alcohol:acid molar ratio R = 2 and 2.8 mg ml^{-1} concentration of cutinase at a temperature of 30 °C and with an initial water content of 0.05% (w/w). The strong inhibitory effect of acetic acid could be overcame by controlling the alcohol:acid molar ratio.

Higher ester yields and initial reaction rates were observed in the esterification of butyric (C_4) and valeric acid (C_5) as compared with the shorter or longer chain length acid. This is in agreement with previous results carried out in reversed micellar systems showing also higher selectivity for C_4–C_6 carboxylic acids.

The kinetics of ester synthesis by cutinase in iso-octane is under evaluation and a reversible reaction described by a Ping-Pong bi–bi mechanism is under construction.

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