



Synthesis of alkyl esters by cutinase in miniemulsion and organic solvent media

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Keywords:	alkyl esters, miniemulsion, organic solvent, cutinase



Research Article**Synthesis of alkyl esters by cutinase in miniemulsion and organic solvent media**

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Abstract

The main objective of this work was to study and test the nature and influence of reaction media (organic solvent vs. miniemulsion system) on the synthesis of alkyl esters catalyzed by *Fusarium solani pisi* cutinase. Ester synthesis and cutinase selectivity for different chain length of acids and alcohols (ethyl and hexyl) were evaluated.

In *iso*-octane cutinase exhibits rates of esterification after one hour between 0.24 $\mu\text{molmg}^{-1}\text{min}^{-1}$ for ethyl oleate and 1.15 $\mu\text{molmg}^{-1}\text{min}^{-1}$ for ethyl butyrate, while in miniemulsion system were from 0.05 $\mu\text{molmg}^{-1}\text{min}^{-1}$ for ethyl heptanoate to 0.76 $\mu\text{molmg}^{-1}\text{min}^{-1}$ for ethyl decanoate. The reaction rate for the synthesis of hexyl esters in miniemulsion system was from 0.19 $\mu\text{molmg}^{-1}\text{min}^{-1}$ for hexyl heptanoate to 1.07 $\mu\text{molmg}^{-1}\text{min}^{-1}$ for hexyl decanoate.

High conversion yields of 95 % at equilibrium after 8h of reaction in *iso*-octane for pentanoic acid (C_5) with ethanol at equimolar concentration (0.1M) was achieved.

Additionally, this work also shows that a significant and unexpected change in cutinase selectivity occurred for longer chain length carboxylic acids ($\text{C}_8 - \text{C}_{10}$) in miniemulsion system comparing with organic solvent (*iso*-octane) and previous studies in reverse micellar systems where a selectivity towards short chain fatty acids ($\text{C}_4 - \text{C}_6$) was observed. The possibility to work with higher concentration of substrates, without inhibitory effect on enzyme, was another advantage of miniemulsion system.

1. Introduction

Alkyl esters belong to the group of flavor compounds that are very important parts of aromas, applied in food, beverage, cosmetic and pharmaceutical industries. The esters of short chain acids (C_6 - C_{10}) and alcohols (C_2 - C_6) are well-known flavor and fragrance compounds. These compounds have odors of different fruits and are parts of complex mixtures providing specific aromas. Longer chain acids esters (C_{18}) are used in cosmetic industry as emollient for personal care applications [1].

Enzymatic synthesis of flavor esters is an interesting and innovative process for aroma production for current and future industrial applications.

Until now the aroma esters were generally produced via the chemical catalytic or by extraction from natural sources. The products chemically synthesized are characterized by low purity and they are not classified as natural products. On the other hand natural aromas obtained by the extraction are very expensive and their quality very often depends on climate and geographic conditions.

Specificity and selectivity of biocatalysts are providing the possibility of working at mild reaction conditions, without undesirable side reactions, generating the product with high degree of purity. As a further benefit the products are no racemic mixtures but usually only one stereoisomer. These are the main advantages of using enzymes in new industrial flavor manufacture processes [2-4].

Moreover, the increasing sensitivity today for environmentally friendly process

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4 and preference for “green” technology and “natural” products favor the synthesis
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6 of flavor compounds via biotechnology route [5-6].
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9 Lipases are mostly used as biocatalyst for the reactions of hydrolysis,
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11 esterification and transesterification. Lipases are characterized by their catalytic
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13 action preferably at the interface between water and an insoluble substrates or
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15 organic solvent containing the reactatns. Another interesting group of hydrolytic
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17 enzymes are cutinases, also which belong to the class of serine esterase as most of
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19 the lipases do [7-8] but in comparison to the lipase they are not characterized by
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21 the phenomenon of interfacial activation.
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24 Industrially, lipases were first used in hydrolytic processes in aqueous media, but
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26 increasing interest focuses on their potential use as biocatalysts for new processes
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28 in various reaction media due to their great importance and role for esterification
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30 reactions. Thus, the esterification reactions were performed in non-conventional
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32 media such as organic solvents (biphasic systems, reverse micellar, and
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34 monophasic system) [7], supercritical fluids [9], ionic liquids [10], aqueous two-
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36 phase systems [11], or solid-gaseous media [12]. There are several advantages in
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38 using organic solvent as reaction media such as increased solubility of nonpolar
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40 substrates and products, shifting thermodynamic equilibrium to favor the ester
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42 synthesis in relation to hydrolysis, and the minimization of enzyme inhibition by
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44 substrates or products, etc. Still, there are disadvantages of using organic media,
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46 which can lead to denaturation or inhibition of the enzyme, separation of solvent
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48 from the product, lack of *green* solvent, and their compatibility with the demands
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50 and regulations of FDA and other official regulatory agencies [5]. Supercritical
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4 fluids have been used in reaction of enzymatic synthesis regarding their physical
5 properties [13-14]. Recently, ionic liquids have been explored as alternative for
6 organic solvent, but this investigation still has to be approved in future [15-16].
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10 As in esterification reactions two products, ester and water are generated, the
11 influence and accumulation of water in the reaction medium has significant
12 influence on the chemical equilibrium. For successful high-yield esterification the
13 water has to be removed from the site of the reaction. Different techniques were
14 used for water removal to shift the equilibrium from reactants to products [17-18].
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17 Irrespective of the limiting aspect of water in esterification reactions, successful
18 efforts have been put in using water as a (green) reaction medium for enzyme
19 transformations [19-20].
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23 Miniemulsions are heterophase systems consisting of uniform, small droplets in a
24 continuous phase characterized by high stability. They are obtained using
25 intensive shearing force. A typical miniemulsion system used for an esterification
26 reaction is composed of an oil phase (as substrates) containing a hydrophobic
27 agent (e.g. hexadecane), an emulsifier (usually non-ionic surfactant) stabilizing
28 the oil reactant droplets, and water. The mixture is homogenized by high shear
29 (e.g. ultrasonification) to obtain homogenous and monodisperse droplets in the
30 size range of about 200 nm dispersed in water [21-22]. Esterification is performed
31 by the addition of an enzyme as a biocatalyst dissolved in the aqueous phase Fig 1.
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33 Lipase catalyzed esterification is well established [28] and was shown to be highly
34 efficient in the production of long chain phenylalkanoates at mild reaction
35 conditions..
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4 The objectives of the presented studies are to investigate and compare the
5 conditions of the enzymatic synthesis of flavor esters by *Fusarium solani pisi*
6 cutinase produced by *Sacharomyces cerevisiae* SU50 in two different media: first,
7 organic media, in particular *iso*-octane, an organic solvent recognized as safe
8 ingredient in food and beverage industrial processes by FDA, and second in
9 miniemulsion system allowing to use water as reaction medium for ester synthesis.
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17 **2 MATERIALS AND METHODS**

18 **2.1 Materials**

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24 Cutinase from *Fusarium solani pisi* was produced as described previously [23-24]
25 and used for enzymatic esterification. Reagents including acetic (96%, ACROS,
26 Belgium), butyric (99.5%, FLUKA, Germany), pentanoic (99.5%, FLUKA,
27 Germany) , hexanoic (98%, FLUKA, Germany), heptanoic, octanoic (99%,
28 SIGMA, Germany), decanoic and oleic acid (FLUKA, Germany), and ethanol abs.
29 (Merck, Germany) and hexanol (Merck, Germany) were used for ester synthesis
30 while hexadecane 99% (SIGMA, Germany) and LutensolAT-50 powder (donation
31 from BASF, Germany) were used to obtain miniemulsion system. All other
32 chemicals used were analytical grade. NaOH (Merck, Germany) as reagent and
33 tetrahydrofuran (THF) (Merck, Germany) as solvent for titration were used.
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45 *Iso*-octane (Sigma-Aldrich, Germany) was used in the reaction medium and *n*-
46 decane (VWR, Germany) was used as internal standard for Gas Chromatography
47 (GC) analysis. Anhydrous sodium sulfate, in powder form (Sigma-Aldrich,
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Germany), was used to dry samples from organic solvent after esterification and before GC analysis.

2.2. Production of cutinase

The *F. solani pisi* cutinase wild-type was produced by recombinant *S. cerevisiae* SU50 strain. The cutinase producing *S. cerevisiae* SU50 strain (Mata, leu2-3, ura3, gall:URA3, ML-S, 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) glycerol (Merck, Germany) [23].

2.3 Purification of cutinase and production of lyophilized enzyme preparation

The isolation and purification of cutinase excreted by recombinant *S. cerevisiae* SU50 strain was carried out by expanded bed adsorption (EBA) [24]. 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 from Amersham Pharmacia Biotech, Sweden was used to isolate the cutinase directly from the fermentation broth. The cutinase activity and protein concentration of the collected effluent fractions from the column were determined after each run. The pool of elution fractions exhibiting highest cutinase activity were previously dialyzed against 20mM 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 deep-frozen at -20°C before used in esterification reactions.

2.4 Characterization of the cutinase preparations

The cutinase estereolytic activity and protein content were assayed and lyophilized cutinase preparations characterized [25-27].

The protein concentration was determined by the method of PEARCE (BCA assay). Protein concentrations were determined with reference to a standard, the Bovine Serum Albumin (BSA) (Merck) [25].

The cutinase estereolytic activity was assayed using a spectrophotometric method, based on monitoring the hydrolysis of p-nitrophenylbutyrate (p-NPB) to nitrophenol (p-NP), a yellow compound easily identify and quantify by the absorbance at 400 nm. An aliquot (20 µl) was added to a reaction mixture composed of 980 µl of a solution in 50 mM potassium phosphate buffer pH = 7.0 also containing 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 controlled adjusted 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) [26].

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4 Specific activity of lyophilized cutinase preparations was determined as 170 U/mg
5 and samples of these enzyme preparations when analyzed with gel electrophoresis
6 showed only one band of 22kDa, i.e., pure cutinase [27].
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10 11 12 13 **2.5 Enzymatic esterification reaction in miniemulsion system**

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15 Unless otherwise stated a typical esterification reaction was carried out in 30 ml
16 capped flasks used as reactor with 20 ml of working volume with equimolar
17 substrate concentration (Table 1). Alcohol, acid, hexadecane, water and Lutensol,
18 (relative amounts see Table 2) are homogenized by magnetic stirring for 1 h. The
19 emulsion was ultrasonicated for 120 s with pulses of 5 s and pauses of 10 s at
20 70 % amplitude (Branson sonifier W450 digital, tip size 6.5 mm) with ice cooling.
21 20 ml of the miniemulsion was added into the reaction vessel containing the
22 appropriate amount of enzyme (5 mg/ml of reaction mixture). The reaction was
23 performed in thermostated incubator (Advanced ChemTec PLS 4x4, 400 rpm) at
24 40 °C unless otherwise stated. This setup enabled parallel experiments by
25 simultaneous use of 5 reactors against at least one blank without enzyme.
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38 Samples were withdrawn periodically (20µl) using a needle (as to preserve the
39 rubber cap) and then mixed with NMR solvent (DMSO-d₆) (5ml) prior to NMR
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Analysis of reaction mixture was determined by liquid ¹H NMR spectroscopy
(Bruker 400 MHz) using DMSO-d₆ (Deutero GmbH, Germany) as solvent.

The esterification yield has been calculated from the ratio of the area of the methylene protons adjacent to the carboxyl-group ($^{\alpha'}\text{CH}_2$) at 2.26 ppm to the sum of the areas of ester ($^{\alpha'}\text{CH}_2$) at 2.26 ppm and acid protons ($^2\text{CH}_2$) at 2.18 ppm defined as in Eq. (1). The sum of ester and acid concentrations was equal to the initial acid concentration.

Eq. (1):

$$\% \text{ esterification} = \frac{[\text{RCOOR}]}{[\text{RCOOH}]_0} = \frac{[\text{integration area of ester}]}{[\text{integration area of ester}] + [\text{integration area of acid}]}$$

An example for ^1H NMR spectra for synthesis of ethyl decanoate is shown in Fig. 2.

Titration of free acids in reaction media (Schott Titronic, Germany) was used as control method for monitoring the progress of esterification. At given incubation periods, aliquots of 100 μl of the reaction mixture were withdrawn using a syringe and were immediately diluted in 300 μl THF in order to stop the enzyme action. The degree of esterification was monitored by determination of residual acid content by titration against sodium hydroxide (0.1M NaOH) using phenolphthalein as an indicator.

2.6. Esterification catalyzed by lyophilized cutinase in iso-octane

The esterification of acid and alcohol at equimolar concentration (0.1M) catalyzed by cutinase was also carried out in *iso*-octane, as reaction medium because it was observed that the enzyme is inhibited by substrate concentrations higher than

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4 0.2M. Unless stated otherwise, a typical reaction for ethyl ester synthesis was
5 carried out in 7 ml working volume inside of 10 ml capped flasks with rubber
6 stoppers (EPDM stoppers, black, Sigma-Aldrich, Germany), as to minimize
7 evaporation or loss of volatile compounds. Alcohol, acid and n-decane were
8 mixed thoroughly in *iso*-octane before addition into a new reactor containing the
9 enzyme (lyophilized form). Initial conditions were set as to minimize enzyme
10 inhibition by the substrates. The first sample (zero point of the reaction) was
11 collected before enzyme addition. The enzymatic ester synthesis was performed at
12 30°C in a multi magnetic stirrer at 300 rpm (S.B.S. Instruments A-23). This device
13 enables operation of multiple parallel experiments by simultaneous use of 6 to 12
14 reactors. At least one blank without enzyme running in parallel for each ester
15 synthesis reaction was performed. Experiments were carried on in duplicate
16 leading to highly reproducible conversion results with errors lower than 5%.
17 Samples were withdrawn periodically using a needle (as to preserve the rubber
18 cap) and then dried prior to GC analysis with anhydrous sodium sulfate. The
19 conversion yield was calculated according to the molar ratio between ethyl ester
20 and respective acid.
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42 **2.6.1. Monitoring and determination of esterification degree in *iso*-octane**

43 The concentration of ethanol, carboxylic acids (acetic acid, pentanoic acid,
44 hexanoic acid, octanoic acid, decanoic acid, oleic acid) and ethyl esters (ethyl
45 acetate, ethyl pentanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl
46 oleate) was determined by a Hewlett-Packard model 5890 gas chromatography
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4 (GC) equipped with a flame ionization detector (FID). The WCOT Fused Silica
5 coating CP Chirasil- Dex CB column, 25m x 0.25mm, DF=0.25 (Varian Inc.) was
6 used. N-decane was used as the internal standard and to calculate ethyl esters and
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(GC) equipped with a flame ionization detector (FID). The WCOT Fused Silica coating CP Chirasil- Dex CB column, 25m x 0.25mm, DF=0.25 (Varian Inc.) was used. N-decane was used as the internal standard and to calculate ethyl esters and respective substrates concentrations in the reaction media based on the results of GC analysis according the following specific conditions applied for each particular ethyl ester and respective substrates:

Ethyl acetate: oven temperature was held at 50°C for 4 min before being elevated to 150°C for 3.67 min at 30°C/min; injector temperature was set at 200 °C; detector temperature was set at 250 °C; carrier gas was nitrogen.

Ethyl butyrate: oven temperature was held at 50°C for 4 min before being elevated to 150°C for 2.34 min at 15°C/min; injector temperature was set at 200 °C; detector temperature was set at 250 °C; carrier gas was nitrogen.

Ethyl pentanoate: oven temperature was held at 50°C for 4 min before being elevated to 170°C for 2 min at 30°C/min; injector temperature was set at 200 °C and detector temperature is set at 250 °C; carrier gas was nitrogen.

Ethyl hexanoate: oven temperature was held at 50°C for 4 min before being elevated to 160°C for 1.67 min at 15°C/min; injector temperature was set at 200 °C; detector temperature was set at 250 °C; carrier gas was nitrogen.

Ethyl octanoate: oven temperature was held at 50°C for 4 min before being elevated to 180°C for 3.67 min at 30°C/min; injector temperature was set at 200 °C; detector temperature was set at 250 °C; carrier gas was nitrogen.

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5 *Ethyl decanoate*: oven temperature was held at 50°C for 4 min before being
6 elevated to 190°C for 4.34 min at 30°C/min; injector temperature was set at 200
7 °C; detector temperature was set at 250 °C; carrier gas was nitrogen.
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11 *Ethyl oleate*: oven temperature was held at 50°C for 4 min before being elevated
12 to 190°C for 5.34 min at 30°C/min; injector temperature was set at 200 °C;
13 detector temperature was set at 250 °C; carrier gas was nitrogen.
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17 18 **3. RESULTS AND DISCUSSION**

19 20 **3.1. Influence of chain length of acids and alcohols on enzymatic** 21 **esterification** 22 23

24 25 **3.1.1. Esterification in miniemulsion system** 26 27

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29 This studies on the synthesis of alkyl esters using cutinase were based on previous
30 work carried out by Aschenbrenner et al. [28]. The authors successfully
31 established a miniemulsion system for the synthesis of monoesters of linear
32 carboxylic acids with ω -phenyl alkanols using various lipases (Chirazyme L-5
33 from *Candida antartica*, Lipase G from *Penicillium cammemberti*, Lipase PS from
34 *Pseudomonas cepacia*). As this system has shown to yield high conversions in
35 very short reactions times at mild conditions, the range of substrates was extended
36 to linear alcohols. Various carboxylic acids including hexanoic (C₆), heptanoic
37 (C₇), octanoic (C₈), decanoic (C₁₀) and oleic acid (C₁₈), and two short chain
38 alcohols ethanol (C₂) and hexanol (C₆) were used for enzymatic esterification.
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With other acids with chain length lower than C₆ it was not possible to obtain

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stable miniemulsion systems, while hexanoic acid (C₆) is the shortest acid that allows to obtain a miniemulsion system. Nevertheless the system is rather unstable as few minutes after addition of the enzyme and start of the esterification reaction, phase separation occurred and the reaction stopped. The water solubility of the short chain acids is too high, so the acid will diffuse from the droplet into the aqueous phase. This observation is consistent with the results obtained by Aschenbrenner et al., who also observed phase separation in systems with acid substrates shorter than seven C-atoms.

This is probably due to the fact that acids with lower chain lengths can act as co-surfactants at the oil/water interface and lower the interfacial tension so that the droplet size decreases or do not allow the formation of stable emulsion droplets, in spite of using a concentration of Lutensol of 1.62% (w/w), the surfactant in the present miniemulsion system (Table 2). Additionally, these short acids are also characterized by low viscosity and hydrophobicity that allows diffusion from the emulsion droplets into the water phase [29].

The acids with longer chain length than C₆ and the two alcohols tested in the miniemulsion systems have different solubility in water and, consequently, different capacity to be encapsulated into the emulsion droplets. Ethanol is completely miscible with water, whereas hexanol is only slightly soluble in water (590 mg/100g at 25°C) [33] while the solubility of the acids decreases with increasing chain length (Table 3). According to these values of solubility it is possible to conclude that almost all of the acids and hexanol were located inside

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4 the emulsion droplets while ethanol can be found in the water phase as well as in
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6 the droplets (Table 3).

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8 Furthermore cutinase presented an extraordinary synthetic capacity in the
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10 miniemulsion system with optimized composition (Table 2) which was carried out
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12 previously by other enzyme [28].

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14 The main goal of this work was thus to explore the esterification potential of
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16 cutinase for these types of substrates (as named previously), and to assess the
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18 possibility to work with high substrates concentrations (Table 1). The influence
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20 of different concentration of substrates on reaction rate and mass transport
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22 phenomena will be studied and published in following work.

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24 The conversion rates of ester synthesis by cutinase in miniemulsion show a direct
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26 relationship to increasing acid chain length with a maximum for decanoic acid
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28 (C_{10}). This could be observed for both alcohols applied (ethyl esters and hexyl
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30 esters) with rates of 0.76 and 1.07 $\mu\text{molmg}^{-1} \text{min}^{-1}$ for ethanol and hexanol,
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32 respectively obtained in the first hour of reaction (Fig 3).

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34 These results show an interesting and unexpected shift of cutinase specificity to
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36 higher chain length of acids (C_{10}) when compared with results obtained for
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38 reactions of esterification and transesterification performed in reverse micellar
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40 system. Here the short chain acids (C_4 - C_6) were esterified with higher specificity
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42 [30-32]. On other hand, cutinase showed higher esterification reaction specificity
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44 for hexanol than ethanol confirming the results obtained already in reverse
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46 micellar systems [30-32] The only exception was found for ethyl oleate and hexyl
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48 oleate where rate of ester synthesis was almost equal (0.33 and 0.29 μmolmg^{-1}
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4 min⁻¹ for ethyl and hexyl esters respectively). Thus, it is possible to conclude that
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6 miniemulsion system forms a different microenvironment for the enzyme that
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8 changes slightly the cutinase specificity in favor of the longer chain of acids (C₈-
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10 C₁₀) and not for specific type of alcohol.
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13 The esterification reactions in miniemulsion system were monitored during 24h
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15 showing that the equilibrium was definitively achieved for all esters after 8h. The
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17 specific time, for which maximum conversion could be detected, depends on the
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19 chain length of acid and alcohol. For instance, for ethyl esters the equilibrium for
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21 ethyl decanoate and ethyl oleate was achieved after 2h, and with smaller chain
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23 length of ethyl esters, the maximum of conversion was at 1h. The reaction
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25 equilibrium for hexyl octanoate was reached after 2h, and for hexyl decanoate and
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27 hexyl oleate after 8h of reaction. However it is possible that low conversions for
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29 shorter chain ethyl esters can be attributed to the instability of the respective
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31 miniemulsion system. Phase separation and destabilization of the miniemulsion
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33 during the synthesis of hexyl heptanoate was observed leading to the low
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35 conversion yield termination of the reaction after 1h.
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38 The profile of conversion yields at the equilibrium was very similar for ethyl and
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40 hexyl esters but the latter generally shows higher conversion yields in compared
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42 to ethyl esters (Fig. 4). The maximum conversion yields were found for decanoic
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44 acid (C₁₀) for both ethyl (59%) and hexyl (76%) esters. The conversion yields for
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46 ethyl heptanoate (C₇) and octanoate (C₈) were very low 1% and 5%, respectively
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48 and the synthesis of ethyl hexanoate (C₆) was not detectable. Conversely, for oleic
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50 acid (unsaturated C₁₈), the conversion at equilibrium was 40% (Fig. 4). Regarding
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4 hexyl esters, shorter chain acids also gave lower conversion yield not detectable
5 for hexyl hexanoate, and of 10, 52 and 76% for heptanoate, octanoate and
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14 decanoate, respectively. Also the esterification of hexanol with oleic acid (C₁₈) by
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These results confirm the observations by Manabe et al. [29] of chemical synthesis *dehydrative esterification*, that hydrophobic and lipophobic substrates favor esterification reactions in miniemulsion systems, probably due to the formation of very stable hydrophobic droplets. Additionally, due to their nano-size that allow a huge reaction surface and the water, which is formed by esterification inside of the droplets, is directly expelled out of the hydrophobic droplets even in presence of a large amount of water used as continuous phase [29]. Thus, compared to completely water soluble reactants as acids of short chain length, the equilibrium of the reaction with longer and high lipophilic acids is displaced in favor of ester formation.

Cutinase used in a similar type of miniemulsion system shows potential not only for hydrophobic substrates (carboxylic acids, C₁₀ and C₁₈) but also for enzymatic synthesis of hydrophilic (ethanol) substrates, and partly hydrophilic substrates (hexanol, carboxylic acids from C₆ to C₈). In more detailed studies it will be necessary to optimize the miniemulsion composition especially the amount of hydrophobic agent and surfactant.

The low conversion yield and the low initial conversion rate for ethyl esters may be the result of the high solubility of ethanol in the continuous phase water and a

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4 strong inhibition effect of ethanol on cutinase activity. This is due to the fact that
5 ethanol is more polar than hexanol, and its concentration in the aqueous phase is
6 high. This could induce stripping of essential water from the enzyme environment,
7 resulting in an inhibition effect.
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12 13 14 **3.1.2. Esterification in *iso*-octane**

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16 *Iso*-octane as an adequate esterification reaction medium for ethyl ester formation
17 from different carboxylic acids with different chain lengths as acetic (C₂), butyric
18 (C₄), pentanoic (C₅), hexanoic (C₆), octanoic (C₈), decanoic (C₁₀) and oleic acid
19 (unsaturated, C₁₈) was also investigated. Reactions were performed with
20 equimolar concentration of the substrates (0.1M) at 30°C. The higher
21 concentration of substrates showed inhibition effect on cutinase activity in the
22 esterification in *iso*-octane (data not shown).
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32 A maximal rate of ethyl ester formation (V , $\mu\text{molmg}^{-1}\text{min}^{-1}$) catalyzed by cutinase
33 was obtained for butyric acid (C₄) and the minimal values was found for acetic
34 (C₂) and oleic (C₁₈) acid, with the extreme of chain lengths tested in this work.
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38 The results are in partial agreement with those reported by Sebastião et al. in
39 reversed micellar system [23] (Fig. 5). The exception is that in reversed micellar
40 systems, cutinase does not present a significant activity on oleic acid. Although,
41 esterification reactions performed by cutinase in *iso*-octane presents a “lag” phase
42 which is the time that cutinase needs before starting to be detected and quantified,
43 the formation and accumulation of ester in the reaction media (Figure 5) and this
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time increases with the chain length of acids and they were found to be 10, 20 and 40 min for octanoic, decanoic and oleic acids, respectively (Figure 5).

The esterification reactions for different chain length of acids at these experimental conditions reaches their equilibria after 8h with a maximum value for ethyl pentanoate (95%) and high yields for almost all the other ethyl esters, such as acetate (88%), butyrate (86%), hexanoate (64%), octanoate (91%), decanoate (82%) and lowest value for oleate (43%). The rates of esterification after 1h for ethyl hexanoate and ethyl octanoate were not significantly different (Fig. 5), but the final conversion yield for ethyl hexanoate was slightly lower maybe due to some inhibitory or deactivation effect of hexanoic acid on cutinase activity (Fig. 6). Hexanoic acid inhibitory effect at concentration of 0.1M and cutinase specificity for short chain acids was observed by Cunnah et al. [32] in CTAB reverse micellar system. Significant drop in conversion yield (Figure 6) between butyric acid and hexanoic acid may be results of cutinase specificity, was shown by previous work too [32]. At this substrate concentration octanoic acid shown less inhibitory effect on cutinase. Still, these results confirm the selectivity of cutinase for shorter chain acids ($C_4 - C_6$) and *iso*-octane shows to be an adequate organic solvent to perform enzymatic esterifications.

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4. Concluding remarks:

The enzymatic esterification of ethyl- and hexyl esters by cutinase in two different reaction media, a miniemulsion system and *iso*-octane, was demonstrated.

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Cutinase shows a distinct selectivity for short chain ethyl esters in *iso*-octane confirming the results obtain by previous authors [30-32] as the maximum of cutinase activity was exhibited for ethyl butyrate (C₄) and pentanoate (C₅). Enzymatic synthesis in an organic solvent (*iso*-octane) still favors the synthesis of short chain alkyl esters and good esterification yields were obtained (ethyl butanoate 86%, ethyl pentanoate 95%).

Miniemulsion systems also showed to have a great potential as a reaction media for synthesis of ethyl- and hexyl esters catalyzed by cutinase. The cutinase was more tolerant for high substrate concentration, comparing with the *iso*-octane as reaction medium. Interestingly the syntheses are characterized by a shift of the enzyme selectivity towards carboxylic acids with longer chain length especially decanoic acid (C₁₀) and additionally surprisingly good activity on oleic acid (C₁₈).

It was interesting to verify and prove with this work that cutinase catalyzed esterification reaction occurred in a miniemulsion system and surprisingly led to excellent conversion yields of 59 and 76% after 8h for decanoic acid (C₁₀) with ethanol and hexanol, respectively, applied with the 4 to 10 fold concentration substrates than in *iso*-octane. This is only possible because of the accumulation of substrates in miniemulsion droplets prevents direct contact with the enzyme as when used in *iso*-octane as reaction media. This prevents the equilibrium to be shifted back to the educts by hydrolysis of the ester.

These results underline that the miniemulsion system has perspective for the synthesis of longer chain alkyl esters (C₁₈), and as the reaction occur mainly in water ("clean solvent"), it is a very interesting approach for the application for

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4 products of green chemistry, furthermore miniemulsions show to be an excellent
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6 reaction medium for esterification reaction without enzyme inhibition.
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22 Conflict of interest statement

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24 The authors have declared no conflict of interest
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Figure Captions

Fig. 1. Principle of esterification in a miniemulsion system.

Fig. 2. ^1H NMR spectrum for synthesis of ethyl decanoate

Fig. 3. Rate of synthesis after 1h of ethyl- and hexyl esters for carboxylic acids from C_6 to C_{18} in miniemulsion system. Reaction condition are shown in Table 1.

Fig. 4. Conversion yields at equilibrium (8h) for the synthesis of ethyl- and hexyl esters for carboxylic acids from C_6 to C_{18} in miniemulsion system.

Fig. 5. The influence of the acid chain length on the synthesis parameters v and lag time of ethyl esters by cutinase in *iso*-octane.

Fig 6. Conversion yields (8h) for the synthesis of ethyl esters by cutinase in *iso*-octane.

Table 1. Equimolar concentration of substrates in miniemulsion system for the synthesis of ethyl- and hexyl esters

Acids	For ethyl esters (M)	For hexyl esters (M)
Hexanoic	1,0	0.75
Heptanoic	0.9	0.70
Octanoic	0.8	0.65
Decanoic	0.7	0.6
Oleic	0.5	0.4

Table 2. The composition of the miniemulsion systems with 80% of amount of water as esterification reaction medium.

Ingredients	% (w/w)
Substrates	16.56
Hexadecane	0.66
H ₂ O	81.16
Lutensol	1.62
Σ	100

Table 3. Solubility of carboxylic acids in water at 20 °C. [33]

Acids	Solubility, g/100ml
Hexanoic acid	1.1
Heptanoic acid	0.24
Octanoic acid	0.068
Decanoic acid	very low
Oleic acid	very low

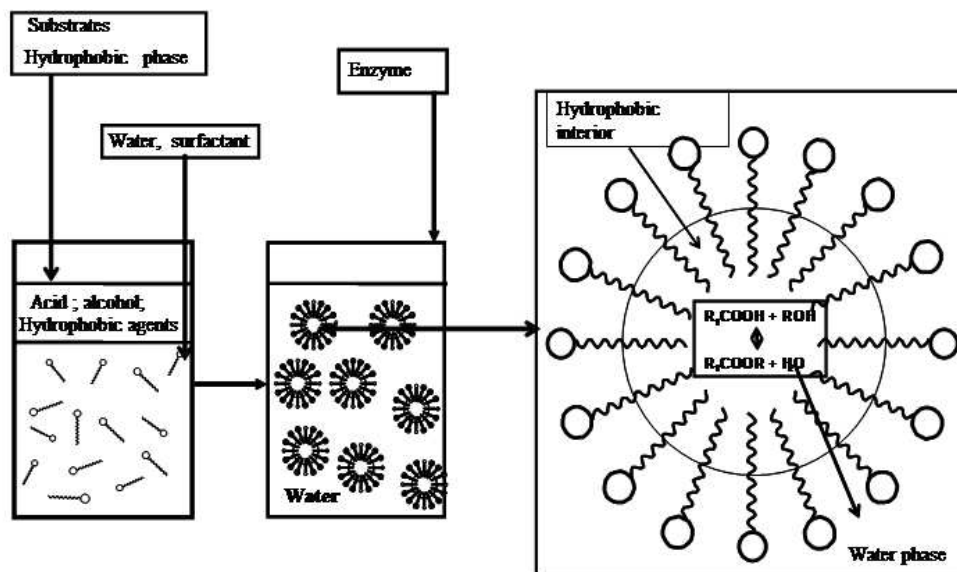


Fig. 1.
Barros et al.

254x190mm (72 x 72 DPI)

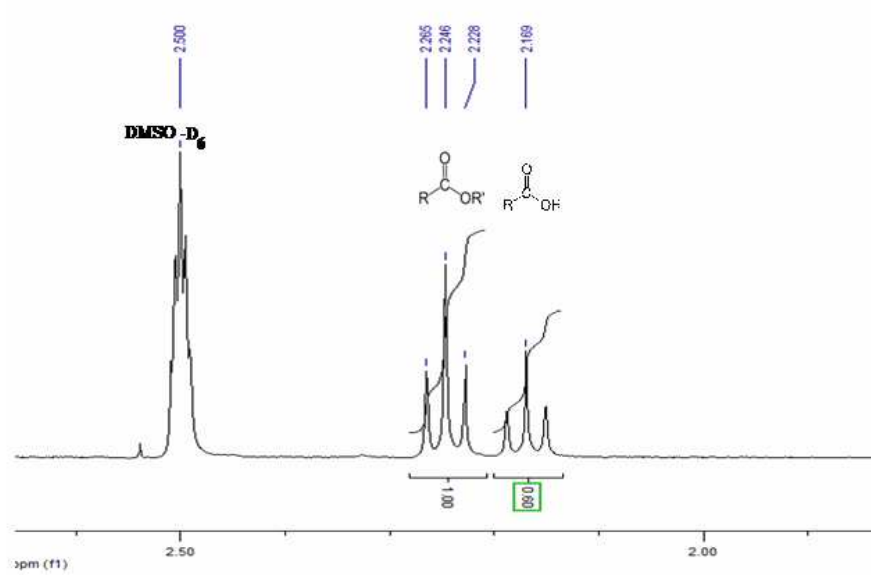


Fig. 2

Banos et al.

254x190mm (72 x 72 DPI)

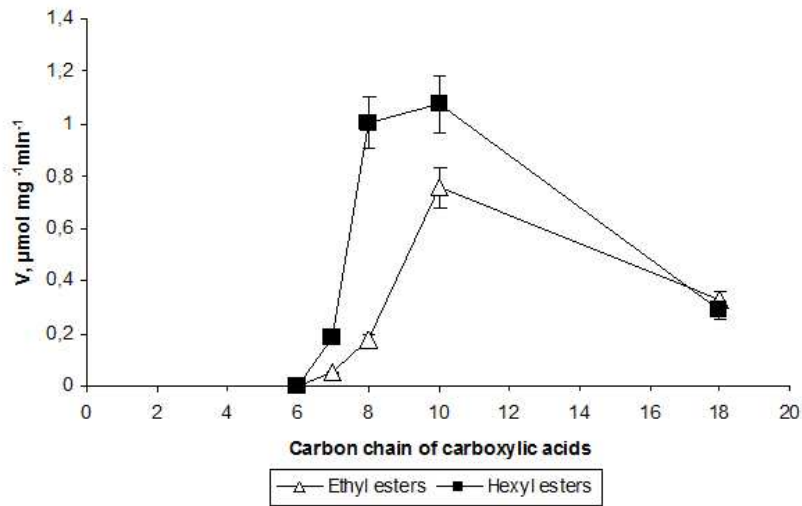


Fig. 3.
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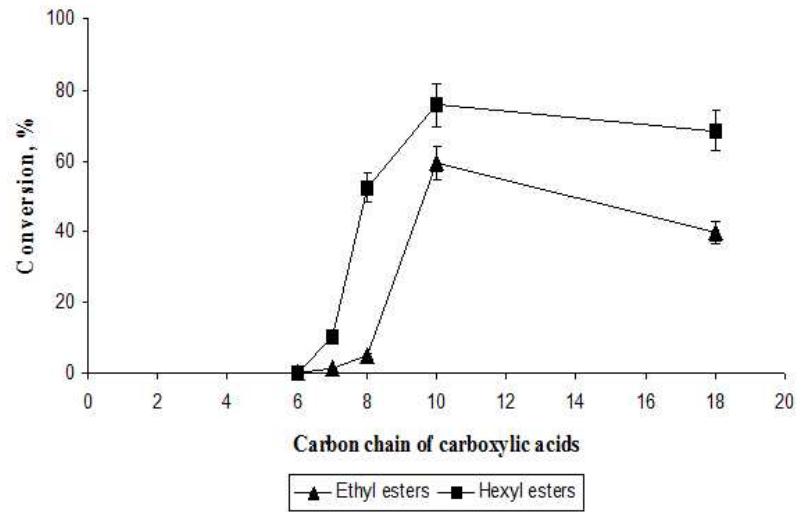


Fig. 4
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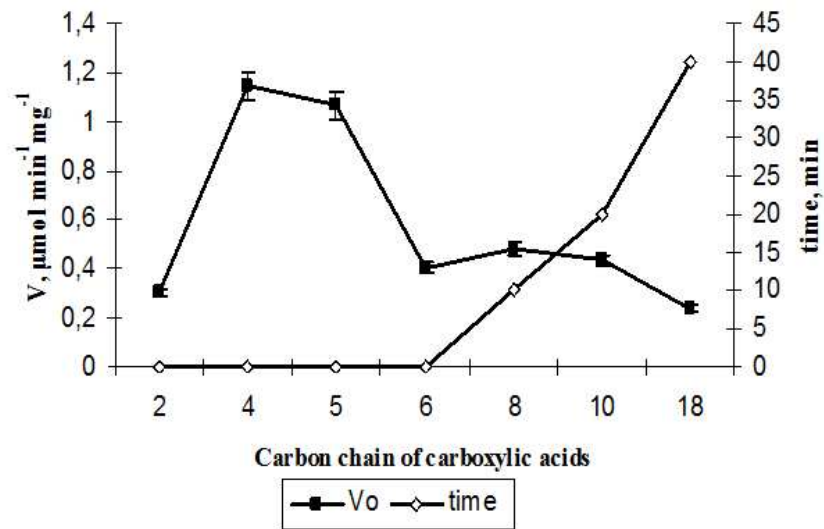


Fig. 5.

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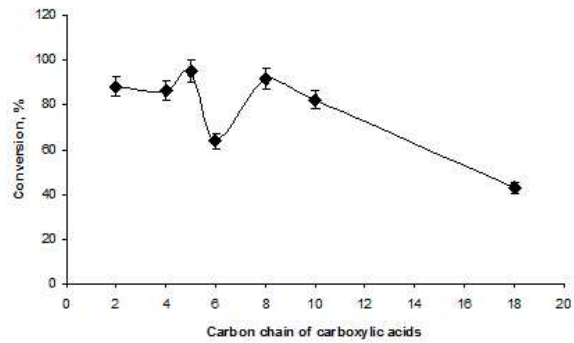


Fig. 6.

Barros et al.

Fig 6. Conversion yields for the synthesis of ethyl esters by cutinase in iso-octane.
254x190mm (72 x 72 DPI)