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PRODUÇÃO ENZIMÁTICA DO ESTER OLEATO DE ETILA UTILIZANDO LIPASE A PARTIR DE *Candida antarctica* B

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RESUMO

Lipases são biocatalisadores de grande importância em diferentes áreas, sendo capazes de catalisar reações em meios aquosos ou orgânicos. Além disso, estas enzimas são capazes de utilizar vários substratos sendo estáveis numa vasta gama de pH e temperatura. Lipases promovem a esterificação entre ácidos graxos e etanol produzindo ésteres oleatos. O objetivo deste trabalho é produzir o éster oleato de etila por esterificação enzimática do ácido oleico com etanol. Uma lipase de *Candida antarctica* tipo B foi utilizada a uma temperatura de 55 °C. A reação foi realizada utilizando o ácido oleico, sulfato de sódio anidro, lipase e etanol, na proporção de ácido oleico (0.03 mol ou 10 ml), lipase (0.1 mol ou 0.01 g), sulfato de sódio anidro (5 g) e etanol 99 % (100 ml). Diversos tempos de reação foram estudados, nomeadamente, 48, 72, 96 e 120 horas. Ressonância Magnética Nuclear (^{1}H e ^{13}C) e espectros de Infravermelho confirmaram a produção do éster oleato de etila para as condições estudadas. O maior rendimento da produção do oleato de etila foi obtido no tempo de reação de 96 horas. Os ésteres oleato de etila foram reportados por possuirem aplicações interessantes em vários campos industriais, tais como, alimentos, produtos aromáticos, cosméticos, detergentes, saborizantes e produtos farmacêuticos.

PALAVRAS-CHAVE: Enzima, esterificação, ester.

ENZYMATIC PRODUCTION OF ETHYL OLEATE ESTER USING A LIPASE FROM *Candida antarctica* B

ABSTRACT

Lipases are biocatalysts of great importance in different areas, being able to catalyze reactions in aqueous or organic media. Furthermore, these enzymes are capable of using several substrates being stable in a wide range of pH and temperatures. Lipases promote the esterification between fatty acids and ethanol producing oleate esters. The aim of this work is to produce ethyl oleate ester by enzymatic esterification of oleic acid with ethanol. A lipase from *Candida antarctica* type B was used at a temperature of 55 °C. The reaction was conducted using oleic acid, sodium sulfate anhydrous, lipase and ethanol, with a ratio of oleic acid (0.03 mol or 10 ml), lipase (0.1 mol or 0.01 g), sodium sulfate anhydrous (5 g) and ethanol 99 % (100 ml). Several reaction times were studied, namely 48, 72, 96 and 120 hours. Nuclear Magnetic Resonance (^{1}H and ^{13}C) and Infrared spectra confirmed the production of ethyl oleate ester for the studied conditions. The highest ethyl oleate production yield was obtained for 96 hours reaction time. Ethyl oleate esters have been reported to possess interesting applications in several industrial fields, such as food, aromatics, cosmetics, detergents, flavors and pharmaceuticals.

KEY-WORDS: enzyme, esterification, ester.

ENZYMATIC PRODUCTION OF ETHYL OLEATE ESTER USING A LIPASE FROM *Candida antarctica* B

1 INTRODUCTION

All the esterification and transesterification organic reactions can be conducted using a chemical or biochemical catalyst. Their use in organic synthesis, represent major improvements in the process, mainly on the operational costs such as reaction time, consumption of energy and manpower. Therefore, interest in the application of biochemical catalysts in organic synthesis has been growing (Faber 1997; Foresti *et al*. 2008; Hazarika *et al*. 2002).

Special attention has been devoted to lipolytic enzymes or lipases, according to their reported high catalytic activity (Kazlauskas and Bornscheuer 1998). They are used both on industrial (food, cosmetics and perfumes, biomedical, pesticides, detergents, among others) and academic levels (Jaeger and Eggert 2002; Pandey *et al*. 1999). Several advantages of enzymatic catalysis, such as the use of lipases isolated from *Mucor miehei, Candida rugosa, Rhizomucor miehei* and *Pseudomonas fluorescens*, for the synthesis of ethyl oleate and esters of oleic acid, have been reported (Hazarika *et al*. 2002).

Esters are important organic compounds with increasing number of commercial applications (Foresti *et al*. 2005). These compounds are largely used in fragrances, cosmetics detergents, flavors and pharmaceuticals. Esters (ethyl oleates) may also be used as plasticizers and lubrificants; biological additives and hydraulic fluids (Hazarika *et al*. 2002). The use of ethyl oleate in commercial applications has been hampered due to the low amounts that can be recovered from natural sources (Martinez-Ruiz *et al*. 2008; Radzi *et al*. 2006). Therefore, most of the available esters are produced by chemical or enzymatic synthesis. The biotechnological production of ethyl oleate using lipases has received particular attention due to the mild reaction conditions involved, the high degree of purity achieved, and the acceptance of these products in food industry (Martinez-Ruiz *et al*. 2008; Trubiano *et al*. 2007).

Currently, the industries have a large number of techniques to conserve the food through the addition of ethyl esters. However, most of these products are obtained by chemical via. Thus, suggests the incorporation of novel techniques for the production to biodegradable compounds, biocompatible and essentially non-toxic (Naoe *et al.* 2001; Torres and Otero 2001). In summary, these compounds have several advantages over synthetic esters such as degradability; can be synthesized from renewable substrates as carbohydrates and fatty acids, and low toxicity (Neta *et al.* 2011).

In the contribution for food industry, the aim of the present work is to develop an environmental friendly technology for the production of ethyl oleate ester, by esterification of oleic acid with ethanol, using a triacylglycerol lipase isolated from *Candida antarctica* type B.

2 MATERIALS AND METHODS

2.1 Materials

Oleic acid was purchased from Vetec Fine Chemistry Ltd.; triacylglycerol lipase purified from *Candida antartica* B was purchased from Sigma-Aldrich; sodium sulfate anhydrous was purchased from Labsynth Products for Laboratories Ltd.; and ethanol was obtained from Dynamics of Analytical Reagents, Ltd.

2.2 Methods

2.2.1 Methodology

The synthesis experiments (esterification reactions) were conducted in flasks adding oleic acid (0.03 mol or 10 ml), lipase (0.1 mol or 0.01 g), sodium sulfate anhydrous (5 g) and ethanol 99 % (100 ml). For the reaction to occur the flasks were incubated at 55 °C and 200 rpm. Four different time reactions were studied, i.e. 48, 72, 96 and 120 hours. The procedure previously described by Khaled and co-workers (1992) for product purification was adapted as described in the sections below.

2.2.1.1 Product purification

At the end of the esterification reaction, the lipase, together with the sodium sulfate anhydrous (non reactive specie), were removed by filtration using filter paper with a pore-size of 60-µm (Macherey-Nagel Inc.). Afterwards, the ethanol was evaporated in a rotoevaporator. The obtained product was analyzed and identified by thin layer chromatography (TLC), using a chloroform/ hexane (1:1, v/ v) mixture for elution, according to Ducret and collaborators' work (1995). Subsequently, the product was analyzed using a column liquid chromatography using Silica Gel 60 as stationary phase, and a mixture of chloroform/ hexane (1:1, v/ v) as mobile phase.

2.2.2 Characterization of the product

After the purification procedure described above, the product obtained was characterized according to the following techniques:

2.2.2.1 Infrared spectroscopy (IR)

The solid product (obtained using the procedure described in 2.2.1.1) to be examined by IR was crushed with a mulling agent, namely the mineral oil nujol. Subsequently, a thin film of the mull was applied onto salt plates and measured. Infrared absorption spectra were recorded on a Bio-Rad model FTS 165 spectrophotometer with a spectral band between 450 and 4000 cm^{-1}.

2.2.2.2 Nuclear Magnetic Resonance (^{1}H and ^{13}C) (NMR)

NMR spectra were recorded on a BRUKER, model Advance DPX-500, CENAUREMN spectrometer, operating at frequencies of 500 MHz for the ^{1}H, and 125 MHz for the ^{13}C. Samples were dissolved in deuterated chloroform ($CDCl_3$). The chemical shifts (δ) were expressed in mg L^{-1} using as references the residual peak of chloroform (hydrogen (δ 7.27) and the deuterated carbon (δ 77.23)).

2.2.2.3 Mass Spectrometry (MS)

Mass spectrometry was carried out using a QP5050A (Shimadzu) spectrometer, at the Department of Inorganic and Organic Chemistry, at the Federal University of Ceará (Brazil). For the analysis, an electron impact of 70 eV was used, and/ or coupled to a GC-chromatograph (model GC 17A) using a capillary column DB-1 (30 m length; 0.25 mm outer diameter; 0.25 µm film) and temperature increases of 4 °C/ min from 100 to 300 °C. The injector temperature was set to 280 °C.

2.2.2.4 Surface tension

The surface tension was determined at room temperature (25±1 °C) by the Ring method as described elsewhere (Rodrigues *et al*. 2006 a,b). A KRUSS Tensiometer (Kruss model K10) equipped with a 1.9 cm Du Nouy platinum ring was used. Measurements were done in triplicate. The concentration of final product used in these measurements was 30 % (v/ v) and the samples were prepared at 55 °C.

2.2.2.5 Solubility

The pure product solubility in several organic solvents was tested, namely in ethanol, dichloromethane, methanol, hexane, chloroform, acetone and water. All the solvents were used at 28 °C, except the water that was evaluated at two different temperatures (28 °C and 55 °C).

2.2.2.6 Quantification of the amount of final product obtained

As described above, four different reaction times were studied, i.e. 48, 72, 96 and 120 h. Three experiments were conducted for each reaction time and afterwards the reaction product was purified as described in 2.2.1.1. The final volume of reaction product obtained after the purification procedure was measured for each assay, and an average volume was determined.

3 RESULTS

3.1 Infrared (IR)

The infrared spectra of the final product (Figure 1) confirmed the presence of an ester. The most important adsorption bands obtained were 1738 cm^{-1} (C=O, ester); 2925 cm^{-1} (CH); 2855 cm^{-1} (CH); 1462 cm^{-1} (CH_2); 1373 cm^{-1} (CH_3); 1179 cm^{-1} (C=C); 1034 cm^{-1} (CO, ester) and 723 cm^{-1} (CH, CH_2 deformation).

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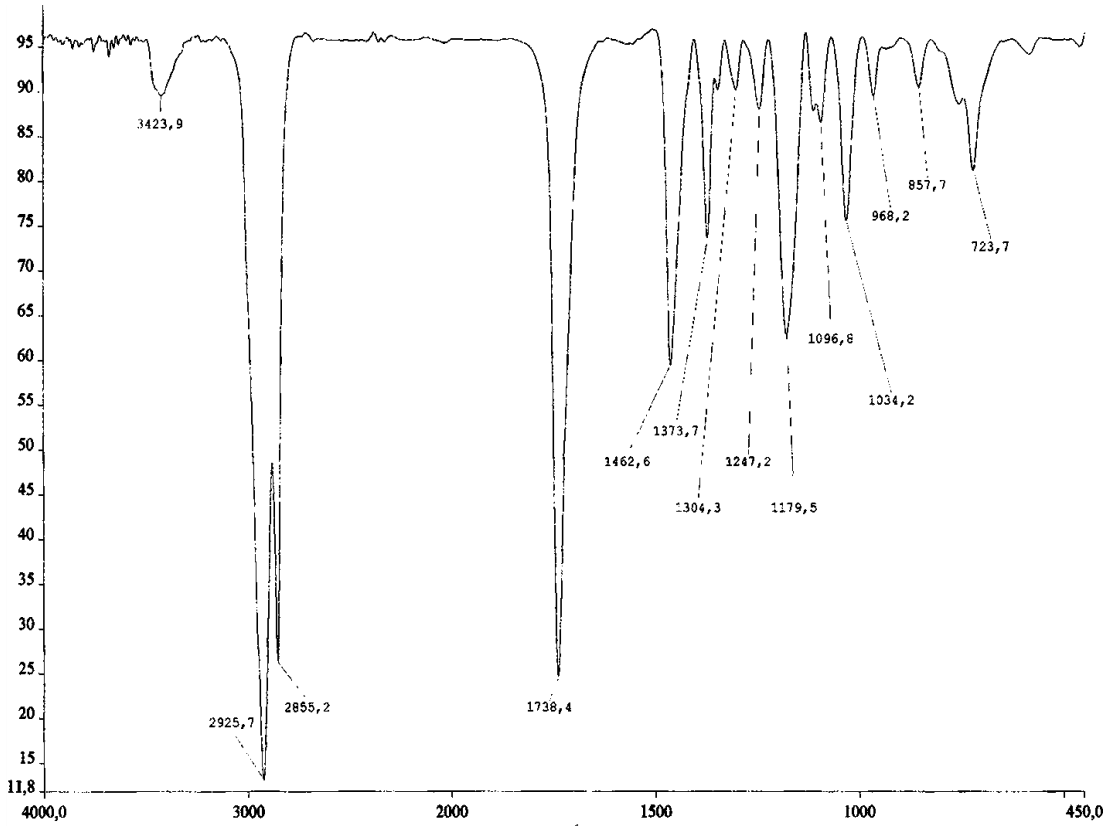

Figure 1 - Infrared absorption spectra of the final product obtained in the optimized conditions for enzymatic synthesis.

3.2 Nuclear Magnetic Resonance (^{1}H and ^{13}C) (NMR)

Based on the NMR spectra (Figures 2 and 3), it is possible to confirm that the final product is the ethyl ester of the oleic acid. From the ^{1}H NMR (Figure 2) it is possible to identify the following peaks at δ (mg L^{-1}): 5.36 (2H, 2×C=*H*); 4.14 (2H, OCH_2CH_3); 2.28 (2H, CH_2C=O); 2.02 (4H, 2×CH_2CH=); 1.62 (2H, CH_2-CH_2C=O); 1.28 (23H, OCH_2CH_3 + 10H, CH_2); 0.90 (3H, CH_3-$(CH2)_6$).

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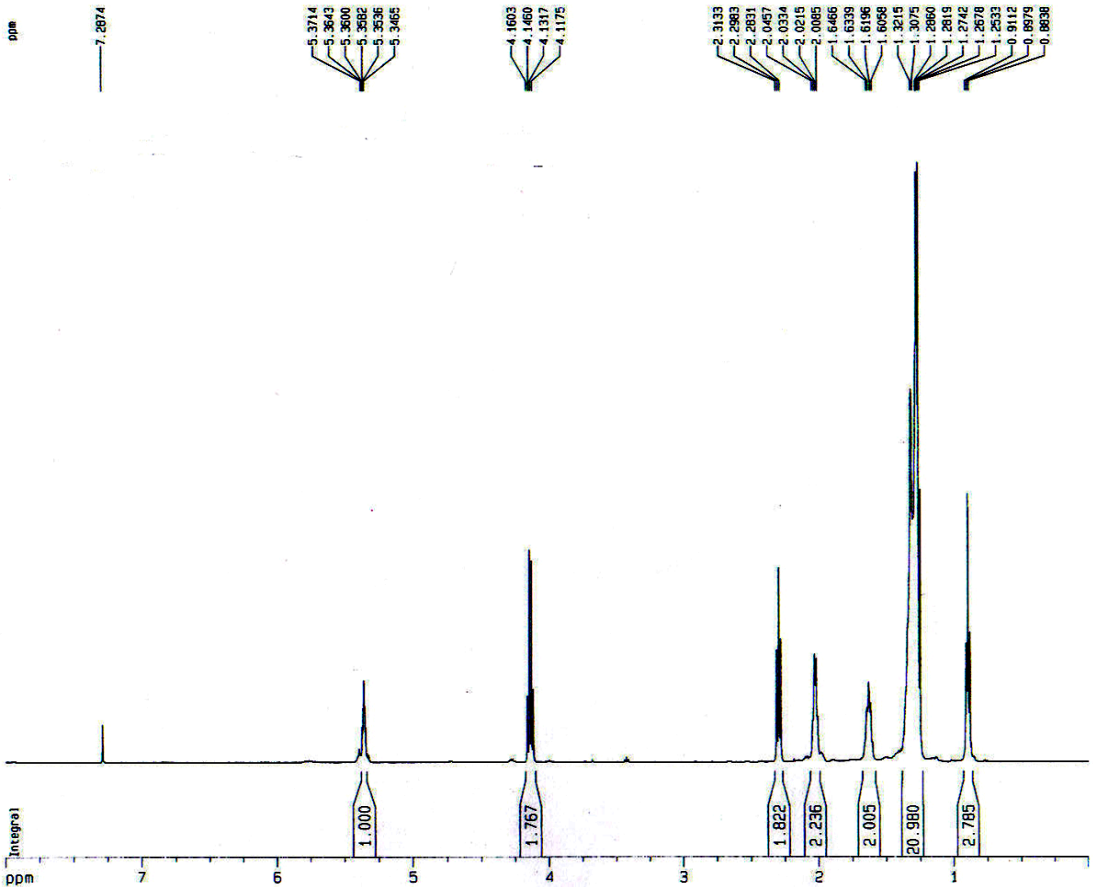

Figure 2 - Nuclear magnetic resonance spectra of the final product - Hydrogen (^{1}H) spectra.

Similarly, from the ^{13}C NMR (Figure 3) it is possible to identify the following peaks at δ (mg L^{-1}): 173.9 (*C*=O); 129.9 (*C*=C); 60.1 (O*C*H_2); 34.4-22.7 (*C*H_2); 14.1 (*C*H_3).

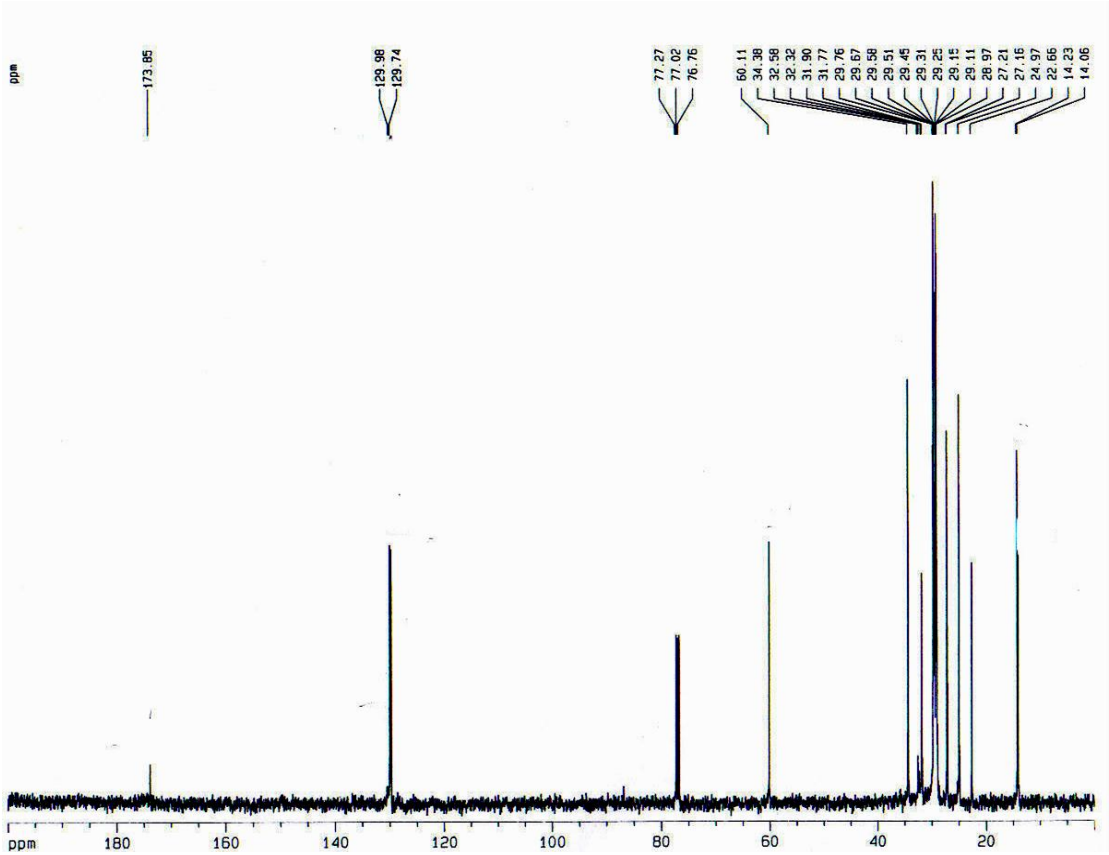

Figure 3 - Nuclear magnetic resonance spectra of the final product - Carbon (^{13}C) spectra.

These results are in accordance with the simulation performed using the software Chemdraw Ultra 9.0 (licensed) (*data not shown*). Based on the above results, the structure of the final product obtained can be established (Figure 4).

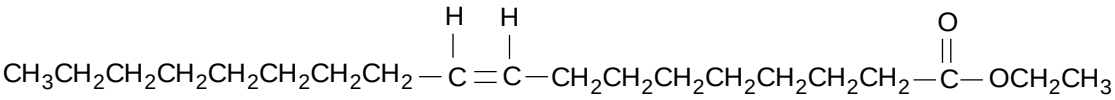

Figure 4 - Structure of the ethyl oleate ester.

3.3 Mass spectrometry (MS)

According to the structure shown in Figure 4, the calculated molecular weight was 310. This was confirmed by mass spectrometry as shown in Figure 5.

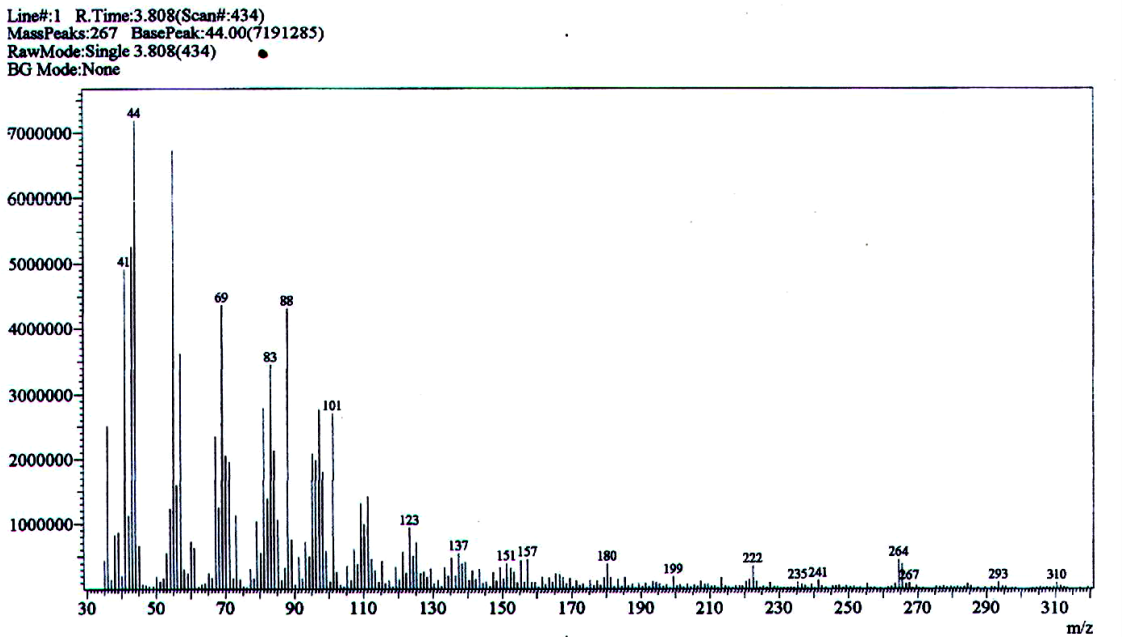

Figure 5 - Mass spectrum of the final product.

3.4. Surface tension

The final product obtained (ester) was found to reduce the surface tension of water from 72.5 to 49 (±0.87) dynes cm^{-1}.

3.5. Solubility

The solubility of the final product was evaluated for several organic solvents (Table 1). All the tested solvents, except for water, were found to be adequate to dissolve the ethyl oleate ester.

Table 1: Solubility of ethyl oleate ester. All the organic solvents were used at 28 °C.

SOLVENTS	SOLUBILITY
Ethanol (99 %)	Soluble
Dichloromethane	Soluble
Methanol	Soluble
Hexane	Soluble
Chloroform	Soluble
Acetone	Soluble
Water (28 °C)	Insoluble
Water (55 °C)	Partially soluble*

* Partially soluble - the product was not fully dissolved.

3.6 Quantification of the amount of final product obtained

The amount of final product obtained after the purification procedure was determined as described in the materials and methods section and the results are presented in Table 2.

Table 2: Amounts of ethyl oleate ester obtained after the purification procedure for different reaction times. Results are the average of three independent assays.

TIME (hours)	PRODUCT % (v/ v)
48	4.03 (±0.01)
72	5.08 (±0.14)
96	7.82 (±0.67)
120	2.18 (±0.12)

4 DISCUSSION AND CONCLUSIONS

In general, for the production of ethyl oleate ester, the presence of ethanol and an acid (for example, oleic acid) is required. As shown by the TLC, IR, NMR and MS results was obtained the ethyl oleate ester from the synthesis reaction of ethanol with oleic acid.

The product obtained presents physically as a clear and colorless liquid with tensoactive characteristics, which enables its use in several interesting applications. In this study, we showed that this ester is a potent surfactant reducing the surface tension of water from 72.5 to 49 dynes cm^{-1}. These results are in accordance with the work from Zhang and co-workers (2004) that reported a reduction of surface tension to 38.7 dynes cm^{-1}. The lower reduction in the surface tension achieved in our study is probably related with the purification procedure used.

One of the most important features of a surfactant is its ability to reduce the surface tension at interfaces (Mukherjee *et al.* 2006). Moreover, for food applications there is an increased interest in using potent surfactants, namely as emulsifiers. In view of this and according to results obtained, we can conclude that the ethyl oleate ester obtained has a great potential as an emulsifier (Feng *et al*. 2009; Ferreira *et al.* 2010).

Based on this interest, further characterization of the compound was performed. The solubility of ethyl oleate in several solvents (Table 1) is in accordance with the results previously published by Hazarika *et al.* (2002) and Zhang *et al.* (2009). It is important to notice that there is an important relation between solubility and biodegradability. The larger the diversity of solvents that can be used to dissolve a particular compound, the better its biodegradability. Our results showed that the ethyl oleate ester has a good biodegradability as compared to other commercially available esters produced by chemical synthesis (Foresti and Ferreira 2007; Ghesti *et al.* 2009; Hazarika *et al.* 2002).

From the amounts of final product obtained after the purification procedure, it is possible to infer the yield of the synthesis reaction. Results gathered on Table 2 showed that higher amounts of ethyl oleate ester were obtained for 96 h of reaction. After 120 h, the

amounts were lower, probably due to the presence of water that interferes with the reaction moving its direction towards the reagents. In summary, the reaction becomes unviable after 96 hours, because the presence of water is increased due the synthesis reaction occurring in the direction of reagents and not the products (Foresti *et al.* 2007; Zhang *et al.* 2009). According to Bucalá *et al*. (2006), water plays an important role in the liquid-liquid and chemical reaction equilibrium. Nevertheless it is extremely important report that, even for 96 h, the amounts of final product obtained after the purification procedure was very low (7.8 %). A low lipase activity might explain these results, although there are still unsolved questions that warrant further research.

From the data obtained, it is possible to observe that, according to characteristics the final product, ethyl oleate ester, has several interesting features and potential applications for example in the food industry (Foresti *et al.* 2005; Foresti and Ferreira 2005; López *et al*. 2008). Present as a compound obtained by reagents biodegradable and essentially non-toxic, different the products found widely in the literature (Naoe *et al.* 2001; Torres and Otero 2001). Ethyl oleate esters can be used for the osmotic dehydration of tomatoes and peppers, promoting the loss of water, and consequently inducing an increase of the sugar content, as well as conferring them a luminous colour (Doymaz 2004; Doymaz and Pala 2002; Lewicki 2006; Saravacos *et al*. 1988).

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