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Research Paper

Enzymatic Hydrolysis of Crude Oil and Isolated Acylglycerides from Andiroba's Seed.

Biotechnology

KEYWORDS : *Carapa guianensis*, enzymatic hydrolysis, lipases, P*enicillium citrinum.*

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ABSTRACT

The andirobeira's seed oil (Carapa guianensis Aublet.) presents remarkable interest in the pharmaceutical and cosmetics industries, because of the palmitoil, oleil and stearil glycerois present in this oil. The application of the enzymatic hydrolysis to the crude oil and to a fraction enriched with acylglycerides by count current chromatography but without inhibitor were hydrolyzed by a concentrate of lipase produced by Penicillium citrinum in submerged culture at 35 °C and 150 rpm. The hydrolysis were carried out at 25 °C and pH 5.6, monitored by TLC and GC-MS analysis. The net yields obtained from crude oil were 5.4% (w/w), 11.75% (w/w) and 1.47% (w/w) of palmitic, stearic and oleic acids, respectively, expressing a potential hydrolysis of this enzyme in the presence of natural inhibiters.

Introduction

The andiroba tree, called *andirobeira* (*Carapa guianensis* Aublet) is an Amazonian oleaginous plant that arouses great interest in the pharmaceutical and cosmetics industries, and science. The andirobeira is an arboreal species of upland forest and wetlands, easy to spread, mainly exploited by its seeds, from which extracted a high commercial value oil with medicinal uses, insecticide, antibacterial, anti-parasitic and as an anti-cancer remedy (Ambrozin et al., 2006) and anti-inflammatory (Penido et al., 2006).

The lipid composition of this oil is of interest in the cosmetic industry, because of the emollient potential products, for topical uses from the incorporation of assets. It is also used in the preparation of facial creams, shampoos, soaps, bath oils, perfume's fixative and moisturizers; also acting as soothing the skin and surface stains bleaching: impinges (*Tinea corporis*) and rashes (Ferreira et al, 2010) and emulsions.

Several, andiroba's oil methods extraction are known; from the artisanal to the industrial. In this work, the process known as "virgin oil or sunshine oil" has led to the extracted oil. The industrial process stands out for fewer steps and less time extraction. (Shanley; Serra; Medina, 2010).

Fatty acids produced from the hydrolysis of natural fats and oils are important products in the economic exploitation of renewable raw materials for manufacturing zinc and magnesium stearate, facial powders, soaps calcium or aluminum and triethanolamine oleate (Machado, 1998).

The obtaining of free fatty acids, mainly palmitic, oleic, stearic and lauric, have difficulties presented with chemical pathways, since chemical hydrolysis has great environmental impact and product separation is laborious. The alternative is to use enzymes such as lipases, classified as glycerol ester hydrolases (EC 3.1.1.3) (Jaeger, 2002), which act under aqueous conditions on the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol. The natural substrates of lipases are long-chain triacylglycerols, which have very low solubility in water; and the reaction is catalyzed at the lipid–water interface (Gupta et al., 2004). Hydrolysis of emulsified triglycerides by lipases has great practical importance, as it represents a more attractive alternative for fatty acid production than the traditional chemical process (Jurado et al., 2008).

The main routes, currently used for hydrolysis of fats and oils to produce fatty acids, consist of the application of heat and high pressure (splitting), alkaline and/or enzymatic hydrolysis (Murty et al., 2002). The high temperature and pressure (typically 100-280 °C, 7 psi) (Gioielle et al., 1991), may promote thermal degradation, polymerization and dehydration of oils and fats. Alkaline hydrolysis has high costs of energy and generates chemical waste harmful to the environment (Linfield et al. 1984).

The enzymatic hydrolysis, in addition to minimizing power consumption, have other advantages over conventional chemical processes for generating products with more and better qualities. Have fewer steps, high catalytic efficiency as a function of downstream process control, act under mild conditions of temperature and atmospheric pressure, they do not accelerate oxidative and thermal degradation (Castro et al, 2004). However, the lipase is unable to promote the total hydrolysis due to the phenomenon of inhibition caused by the products generated as fatty acids that are compounds and surfactants, which reduce the interfacial tension, interfering on the action of lipases (Graille et al., 1988).

This work was carried out with a lipolytic concentrated enzyme produced by *P. citrinum* according to (Barbosa-Junior, 2014), and applied to the hydrolysis of glycerides obtained from crude andiroba's oil.

Experimental Section

The andiroba oil used in this work was obtained by seed fermentation process arising from the Marajó Island, city of Salvaterra-Pará-Brazil, collected in 2013. The sample solution was prepared by vigorous mixing in separating funnel for 5 minutes:

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50.5 g of oil, 70.0 mL of hexane, 25 mL of methanol (MeOH) and 25 ml of acetonitrile (ACN). Two separated phases by decantation yielded the upper phase (UP), constituted of neutral lipid compounds and low polarity dissolved in hexane; and the lower phase (LP) constituted of medium and high polarity compounds dissolved in the mixture of ACN/MeOH. We used an enzyme concentrate with lipase activity produced in submerged culture of *P. citrinum*, development on Systematic Research Laboratories in Biotechnology and Molecular Biodiversity-UFPA, according to (Barbosa-Júnior, 2014). All reagents used were of analytical grade: ethyl ether (Tedia); hexane (Tedia), methanol (synth), glacial acetic acid (Synth), acetonitrile (synth), methanol (synth), ethanol (synth), fosfomolibidic acid (Cromoline), N, O-bis (trimethylsilyl) - trifluoroacetamide (BSTFA) were of analytical grade (>99%). HPTLC (High Performance Thin Layer Chromatography) aluminum plates (20x20cm) coated with silica gel 60 with fluorescent indicator were used (Macherey-Nagel). A differential counter-current chromatography (DCCC) instrument QUATTRO CCC (Quick PREP™, UK) equipped with a stainless steel coil 358.0 ml was used coupled to a flash liquid chromatograph ISOLERA model (LC-Biotage Flash-, Sweden) with an automatic sample collector and four pumps for solvent mixture. The fractionation performed without detector. Concentration of the fractions obtained was made into a Turbovap concentrator; model LV Evaporator Isolera (Biotage) at a 5 psi air pressure and 40 °C for 25 minutes. The compounds separation in the DCCC was developed at the normal phase mode ("tail-to-head") with the mobile phase. The solvent system employed was hexane:acetonitrile:methanol (2:1:1, v/v/v), that were shaken and the phases separated yielding the nonpolar mobile phase (hexane) and the polar stationary phase (ACN/MeOH). The coil was feed with the lower phase in the mode tail to head; pumping 1.5 CV (1CV= 358mL), executed by LC-Flash-Biotage, according to the predetermined solvent gradient. The volume of mobile phase used was 1,5 VC during separation. The pump flow rate was 10 $mL.min^{-1}$ with a rotation speed of 868 rpm at 40 °C. The sample solution injected in the coil through a 15 ml loop were 5g of oil dissolved in 20mL of up phase. The fractions were collected in LC-Flash, on grid containing 35 tubes (ø 15 mm x 120 mm), with 14.0 ml each.

The enzymatic hydrolysis was carried out in 1,5x14 mL cylindrical glass tubes, which were added 100.0 µL of andiroba oil, 800.0 µL of ultrapure water, 350.0 µL of hexane and 10.0 mg of enzyme concentrate . The reaction was performed in a orbitalar shaker at 40 °C and 300 rpm for 3h. The extraction (2 x 500.0 µl) was performed with a solution of hexane: ether (1:1 v/v). The control was prepared without enzyme addition under the same experimental conditions of the sample.

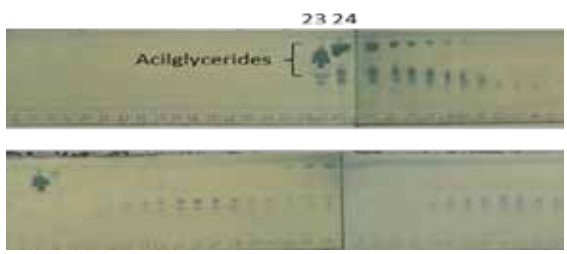
The samples were transesterifyed: 10mg of sample added of 250µL of n-hexane, 100 µL NaOH in MeOH (2N), hited at 50ºC for 5 min with agitation of 600 rpm. After this procedure the solvent was evaporated and sililated with 50 µL of BSTFA at 45ºC for 15 min. !mL was analysed in a GC/MS. Analyses was performed on Thermo Scientific trace 1300 gas chromatograph equipped with an electronically controlled split/splitless injection port and interfaced to a Thermo Scientific mass spectrometer MS-ISQ Single Quadrupole auto-sampler AI 1310 detector. The GC was equipped with a RTX-65 TG capillary column (15m x 0,25mm x 0,1µm film thickness; Restek, USA) coupled directly to the mass detector. The injection operated at 250 °C in the splitless mode. The carrier gas was He with a constant flow rate of 1 ml/min. The following oven temperature program was used: initial temperature of 40 ºC ramped at 6ºC min^{-1} to 200 ºC, ramped at 15ºC min^{-1} to 300 ºC, held for 5.0 min, ramped at 15ºC min^{-1} to 340 ºC. A solvent delay time of 4 min was used to protect the filament and ion multiplier of the MS instrument from saturation. Ion source operated at 280ºC and the transfer line temperature was 280 °C. Registered mass spectra obtained by Electron impact at acceleration energy of 70 eV. Performed Data acquisition were in the full-can mode during the optimization of the method (mass range 40–1000), with 2scan/min. Quantification was carried out in Wilei2009 library software. The measured concentration of free fatty acids (FFA) was calculated by the peak area normalization using the following equation:

Where, is the analyte peak area and is the sum of areas of all peaks in the chromatogram obtained.

Results and Discussion

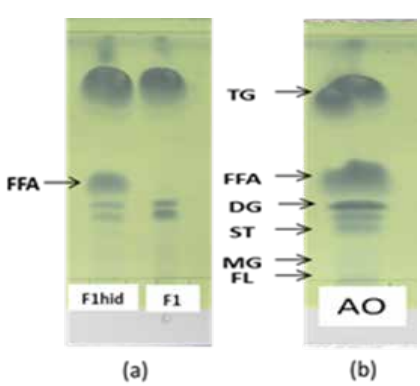
The fractionation of oil on DCCC, resulted in 156 fractions which were analyzed by TLC, and the Rf is in accordance with lipid classes described by Gunstone et al. (2007). The triacilglycerides (Rf = 0.83 ± 0.02), fractions 23 and 24 from UP, were pooled yielding 19,50g (F1) of lipids without enzyme inhibiter substances like phospholipids, steroids and principally, free fatty acids (figure 1).

Figure 1. Fractions 23 and 24 of andiroba's oil from UP separated by DCCC and analyzed by TLC: acylglycerides presence.



This material (F1) was subjected to enzymatic hydrolysis to obtain the FFA, characterized by the appearance of a spot at Rf = 0.45 in hydrolyzed fraction (F1hid), comparing with the analysis of control under the same conditions; in this way it was clear to prove the enzyme hydrolysis, but it is not easy to quantify by TLC (Figure 2a).

Figure 2. Lipid profile of *F1hid* fraction after enzymatic hydrolysis and acylglicerides fraction from andiroba's oil *F1* as a blank (a); the lipid profile in crude vegetable oil (b). Triglycerides (TG), diglycerides (DG), monoglycerides (MG), steroids (ST) and phospholipids (FL) present in andiroba's oil.



In addition, we observe that for the enzyme hydrolysis of crude oil, it was similar efficient, even in the presence of inhibiters. The Figure 2b shows the TLC's lipid profile of the crude oil, and the high amount of free fatty acids in the sample was not an impeachment to this specific enzyme action.

The crude oil was hydrolyzed by lipase concentrate and the free fatty acids were silylated. The comparison between the GC/MS

analysis of enzymatic hydrolysis of the crude oil hydrolyzed (***AOhid***) and the integral crude oil (***AO***) was important to observe how robust the enzyme was. In this work was used the theory that only acylglycerides can be esterified and free fatty acids are silylated; although, each amount of esterified and silylated compound has its own chromatographic factor and its peak cannot be expressed with real area in a chromatogram. In this work, the comparison of chemical compositions of lipids present in ***AO*** and ***AOhid*** shows a decreasing in the concentration of glycerides present in the crude oil: diacylglycerols as 1,2-palmitin reduced from 17.83% to 4,8% and monoglycerides from 0.74% to 0.34%, Table 1.

Tabela 1. Comparison of Chemical profile enzymatic hydrolyzed and crude andiroba's oil.

Retention time (min) Rt	Substances identified based on Rt and mass spectra	Content of the substances (%) AO	AOhid	Reverse Fit of identification from data base#. Comparison of the mass spectra. AO	AOhid
15,75	Miristic acid sililated	0,52	0,23	947	931
17,44	Palmitic acid metil ester	0,58	0,18	928	784
18,68	Palmitic acid sililated **	26,78	32,18	917	915
20,24	Oleic acid metil ester	1,31	Nd*	956	Nd*
20,45	Linoleic acid metil ester	0,73	Nd*	925	Nd*
21,41	Oleic acid sililated**	32,56	44,31	947	952
21,48	Stearic acid sililated**	2,89	4,36	935	958
21,53	Linoleic acid sililated	2,14	3,45	925	925
23,75	Araquidic acid sililated	0,93	2,02	929	940
25,56	Monopalmitin sililated	Nd*	0,20	Nd*	856
27,47	Monolinolein sililated	0,32	1,77	784	756
27,99	Monoolein sililated	0,74	0,34	868	726
32,89	1,2-di-palmitin sililated***	3,39	0,36	761	749
33,59	1,2-di-palmitin sililated***	17,83	4,58	781	777
34,45	2-hidroxy,1-oleic, 3-palmitin***	6,23	4,85	787	775
35,72	1,3-diolein***	1,07	0,32	820	823

*Nd-not detected; ** increased concentration; *** decreased concentration, # percentage probability of similarity of the peak of the mass spectrum detected with the WILEY2009 library.

It was observed high release of fatty acids from acylglycerides; they were seen as silylated palmitic, oleic and stearic acids with net yield of 5.4%, 11.75% and 1.47%, respectively. Silveira and Carioca (2003) performed hydrolysis of andiroba oil with commercial immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM). They observed a releasing of 1.18% for palmitic acid. These results showed an important hydrolytic action of the enzyme concentrate produced by the fungus *Penicillium citrinum* with high potential for hydrolyzing crude oil in a technological process.

CASE STUDY The present study investigated the potential of the lipase concentrate in the hydrolyze of crude oil and acylglycerides produced by DCCC. In this case, the enzyme present high potential to hydrolyze of crude oil with high acidity with out be inhibited. This kind of enzyme is required by the industry due the robustness and can be an alternative to hydrolyze of crude oil to be applied in cosmetic industry.

Conclusions

The andiroba's oil is an excellent source of palmitic, oleic and stearic acids enabling the use of enzymatic hydrolysis as a technological alternative to obtain these fatty acids with green technology for cosmetic use. In this work, the increasing of the concentration of fatty acids after enzyme hydrolysis showed that the enzyme concentrate acted with high potential without inhibition in the generation of fatty acids from andiroba's crude oil with high acidity.

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