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Catalytic properties of lipase from *Ficus trichopoda* and *Euphorbia unispina* latex: Study of their typo-selectivity.

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ABSTRACT

Objective: The search for lipase with distinct features, from plant latex is of great importance for industrial applications. The catalytic properties of lipases from *Ficus trichopoda* and *Euphorbia unispina* latex were characterized.

Methodology and Results: Fresh latex from *Ficus trichopoda* and *Euphorbia unispina* were collected and dried through solar. Dried latex was taken for complete proximate analysis and their activity was analysed by thin layer chromatography. The two lipases were optimally active at pH=5 and temperature of 35°C and 50°C for *Ficus trichopoda* and *Euphorbia unispina* latex, respectively. The presence of metal ions enhances the activity of *Ficus trichopoda* latex, while no significant enhancement was observed in the case of *Euphorbia unispina* latex. Both lipases were able to hydrolyze saturated esters, and showed typo-selectivity for this group. However, the lipases are weak selective for the hydrolysis of unsaturated esters, especially for 18:2 fatty acids.

Conclusions and application of finding: The enzyme from *Ficus trichopoda* latex was able to attack specific oil to generate free fatty acids or ester as the major product. This understanding may help in devising efficient methods to produce valuable modified oils.

Key words: Latex; Lipase activity; esterification reaction; typo-selectivity; *Ficus trichopoda*; *Euphorbia unispina*.

INTRODUCTION

The African vegetable biomass is of growing interest for the research and industrial communities owing to the significant number and variety of bioactive components it contains (Villeneuve 2003). Among them, the identification and the characterization of plant lipases were little reported and therefore, could show an interest in the context of bioconversion of greasy substances like fats and oils. Lipases are among the most important class of industrial enzymes. In recent years, the growing demand of lipolytic enzymes has increased due to their potential use in various manufacturing processes for industrial goods such as detergent, food, cosmetics, flavour enhancers and also in pharmaceutical (Cancino *et al.*, 2008). It is clearly known that lipases contained in latex in some plants have catalytic properties and

numerous industrial applications (Paques and Macedo 2006; Mazou *et al*., 2016). For instance, papaya (*Carica papaya*) latex has already been described in the modification of fats and oils (Villeneuve 2003), in esterification and transesterification reactions (Caro *et al*., 2000), and more recently in the resolution of racemic mixtures (Mazou *et al.,* 2016; Cheng & Tsai 2004). The modification of fats and oils mean the use of lipases as biocatalysts indicates to modify the composition and the distribution in fatty acids of a greasy substance in order to improve the nutritional and/or rheological properties of them. These results have prompted interest in other latex plant extracts, in particular, the unripe fruit of the babaco plant (Kyndt *et al*., 2005), which contains a latex similar to that in *C. papaya*. Similarly, babaco (*Carica pentagona*) has also exhibited biocatalytic activities in lipolysis and acyl transfer reactions (Dhuique-Mayer *et al*., 2003). Latex lipases from plants in the *Euphorbiaceae* (Villeneuve *et al.*, 2005), *Asclepiadaceae* (Giordani *et al*., 1991) or *Caricaceae* (Dhuique-Mayer *et al.*, 2003) families have also been described as useful biocatalysts for several synthesis applications in the food, pharmaceutical and detergent industries. For example, in Benin, the production of local cheese called waragashi (Peulh cheese) is made from milk and *Calotropis procera* latex (Mazou *et al.*, 2012). Besides, *Carica papaya* latex presents an interesting lipolytic activity, which was previously used to produce synthetic cocoa butter, human milk analogs, terpene, biopolymers, and wax esters (Caro *et al.*, 2000; Villeneuve 2003; Tchobo 2008). However, underline that, in some cases, plant latex enzymes present advantages over animal and microbial lipases due to some quite interesting features such as specificity, low cost, availability and ease of purification, representing a great alternative for potential commercial exploitation as industrial enzymes (Mazou *et al*., 2016; Barros *et al*., 2010). In the present study, we report the partial characterization of the catalytic properties of unusual lipase from *Ficus trichopoda* (Photo 1) and *Euphorbia unispina* (Photo 2) latex for valuable applications in modified vegetable oils.

Photo 1 : *Ficus trichopoda*

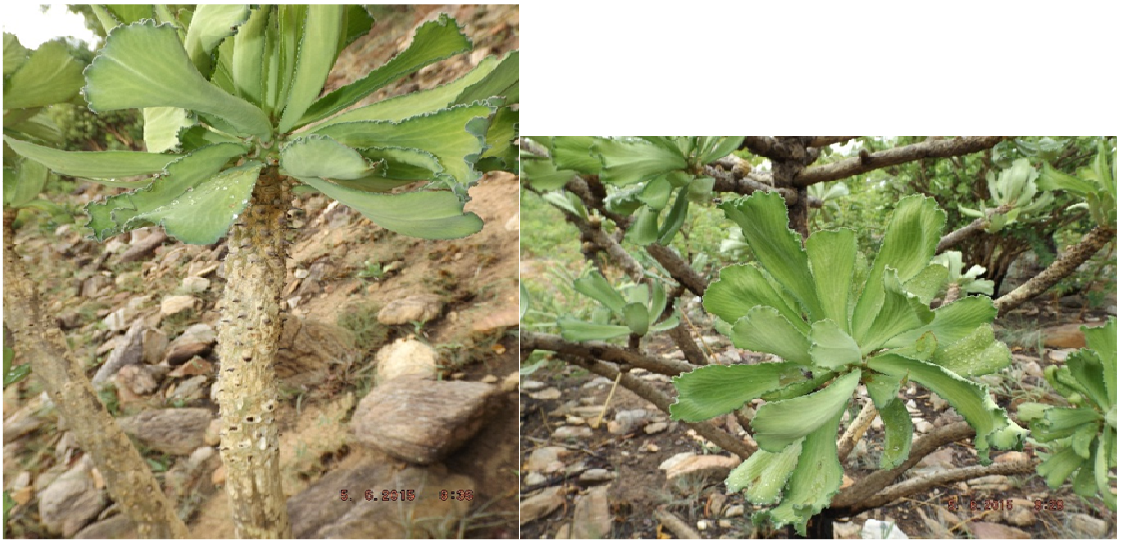

***Photo 2*:** *Euphorbia unispina*

MATERIALS AND METHODS

Materials: The latexes of *Ficus trichopoda* and *Euphorbia unispina* were sampled in Benin Republic, in the North-West, in the department of Donga, at the foot of the mountains of Tanéka-koko (commune of Copargo). Fresh latex of *Ficus trichopoda* and *Euphorbia unispina* were collected after incision of their trunk. The exuded latex was allowed to run down and drip into collecting devices. The collected latex were spread on trays and left for drying in air at 40˚C for 14 h. All trays and other materials used in the latex collection and drying were washed thoroughly with water and detergent soap and kept dried. The dried products were packed in air-tight plastic containers and stored in a cool, dry place. Plastic containers of 100 g capacity were used to pack dried latex since metal containers would result in a loss of enzyme activity (Jeana *et al.*, 2013). They were kept and stored in a freezer at −20˚C (Nitsawang *et al.,* 2006) in order to avoid reduction of its shelf life. In fact, it is generally accepted that a month's stability of an enzyme at 45˚C is roughly equal to that of one year at room temperature (Jeana *et al.*, 2013). All the chemicals, reagents and solvents used in the experiments were of analytical grade. Complete proximate analysis of dried latex samples and lipase activity were done in Benin while the determination of lipase typoselectivity was achieved in SuperAgro/INRA at Montpellier in France.

Complete proximate analysis of dried latex samples: A 100 g of crude latex was taken for complete proximate analysis. Dried latex sample of 100 g each was transferred separately to plastic containers and brought to laboratory, for complete proximate analysis. Water content, crude fibre and ash were determined according to the method described by AOAC (1990). The protein content was measured by the methodology of Biuret : samples were analyzed in spectrophotometer (Agilent Technologies 8453) at 540 nm. The lipid level was given after extraction of the total soluble lipids of latexes by the method of Clark *et al.* (1982). Solubility was determined according to the method of Corke and Li (1999).

Lipase activity: All the reactions of hydrolysis were carried out with 1g of oil diluted in 5mL of Cyclohexane. To this mixture, were added 20 µL of calcium chloride solution (0.2M), 2mL of phosphate buffer (pH = 7) and 0.1 to 0.2 g of dried latex. Thus, the mixture obtained is maintained at the ambient temperature and is homogenized with a magnetic stirrer at 300 rpm during 60 min. The reaction was stopped by adding 3.5 mL of an ethanol/acetone mixture (Basri *et al.*, 1997). Free acidity is titrated with NaOH (10^{-1} mol/L) solution using phenolphthalein (1% in ethanol) as indicator. A control test is carried out under the same condition in absence of enzyme. Herein the enzymatic activity expresses the number of mole of free fatty acids which 1 mg of enzyme releases per unit of time (min).

Expression of the results: The Enzymatic activity (EA) is obtained by the following formula:

$$EA = \frac{C(V - Vo)}{m\ t}$$

C: Concentration of NaOH (10^{-1} mol/L)

Vo: Volume of the dummy trial
V: Volume of the fall of burette
t: homogenization time
m: mass of enzyme.

Influence of pH and temperature on the hydrolytic activity of latex lipases : The catalytic properties of the crude lipase extracts, such as optimal temperature and pH, were determined as the relative activity in the range of pH between 4.0 and 8.0 (buffer sodium acetate pH 4.0–5.0, buffer sodium phosphate 5.5–8.0) and temperature varying from 30 to 90 °C. The mixture contained 1 g of oil diluted in 5 mL of Cyclohexane, 20 µL of a Calcium chloride solution (0.2M), 2 mL of phosphate buffer (pH = 7) and 0.2 g (20 % of substrate) of enzyme was incubated for 1 h with a continuous stirring, and the activity determined for each pH and temperature. A thermostated, water-jacketed reaction chamber with shaker was employed for determining the temperature dependence of lipase activity. The maximum activities of lipases from latex were defined as 100% relative activity.

Effects of ions : To determine the effects of ions, the method of Mukundan *et al*. (1985) was adapted. For the investigation, 0.02 M solutions of sodium chloride (NaCl), calcium chloride (CaCl2), mercuric chloride (HgCl2) and ethylene diamine tetra-acetic acid (EDTA) were prepared. 1 ml of each of these solutions was added to separate assay mixtures and incubated for 1h with continuous stirring. Lipase activity was determined as described above.

Determination of the catalytic activity of latex lipases toward different substrates : The catalytic activity of the lipase was determined in the hydrolysis of emulsified oils such as palm, coconut, soy and peanut in the same procedure as mentioned above

Effect of latex concentration on lipolysis : To determine the effects of latex concentration on lipolysis, 5 to 30 % of latex was used in the same procedure as mentioned above

Determination of lipase typoselectivity : Typo-selectivity was determined by multi compétitive reactions according to Rangheard *et al*., (1983). In this assay, a higher concentration (%) of a given FFA indicates a stronger lipase activity. The FFAs band on the silica plate was scraped and transferred to 50 mL round-bottom flasks for ethylation and the resulting FAMEs were analyzed by GC.

Esterification of the oleic acid using latex : In a bottle of 250 mL, the esterification of 10 g of oleic acid and 1.625 g of ethanol corresponding to a molar ratio oleic acid /Ethanol of (1:1). The reactions were initiated by addition of 1.5 g latex (either 15 % m/m substrates). The bottles hermetically closed were agitated using a magnetic bar at ambient temperature (~30°C) at 400 rpm. The aliquot of 1 ml was taken periodically of the reaction medium. The enzyme was removed by centrifugation with 2000 rpm for 5 minutes, then the supernatant was analyzed by titration with NaOH (0.1 N), by using phenolphthalein as indicator and 10 ml of ethanol as extinction agent. The conversion (%) of the ester synthesis was calculated starting from the conversion of the acid into ester after a given time. Approximately 40 µL of aliquot is carried out and analyzed qualitatively by TLC. The presence of an activity results in the appearance on the line of development of TLC plate spot characteristic of ethyl vegetable oil esters resulting from the esterification of the oleic acid with ethanol.

Expression of the results *(Conversion rate):* The conversion rate (Cr) is obtained by the following relation:

$$Cr = 100 - \frac{C\ V\ M}{10m}$$

C : NaOH concentration (10^{-1} mol/L)
M : molar mass of oleic acid (283.47 g/mol)
V : Volume of the fall of burette (mL)
m : mass of sample (g)

Analysis by thin layer chromatography (TLC) : In this work, only a qualitative analysis by TLC was carried out for the follow-up of the reactions. Samples of 44 µL of reaction medium were diluted in 4 ml of hexane. Then, the aliquot of 10 to 20 µL were analyzed by chromatography on silica plates (TLC-cards DC-Alufolien-Kieselgel). TLC plates were migrated using a mixture of hexane/ ethyl ether / acetic acid (90:10:1, v/v/v) to observe the ethyl esters of vegetable oil. The compounds of the plates are then revealed by carbonization at 180°C in a furnace during 10 minutes after immersion of the plate in a saturated solution with sulfate copper /phosphoric acid/methanol/water (10:8:5:78, v/v/v/v). TLC bands were quantified using a TLC Scanner 3 (Camag, Muttenz, Switzerland) at 325 nm. For products from FAEEs hydrolysis, 300 µL of the hexane phase was spotted on a TLC silica gel plate 60 (Merck, Darmstadt, Germany), using an automatic sampler Linomat 4 (Camag, Muttenz, Switzerland). The plate was developed with hexane : diethyl ether : formic acid (70:30:1, v/v/v). The FFAs bands from FAEEs hydrolysis were scraped from the plate and stored in 50 mL round-bottom flasks for typoselectivity analysis.

Statistical analysis : The data obtained from these studies were analyzed using SPSS Statistics 17.0. The statistical analyses carried out were averaged with standard deviation and analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Physicochemical characteristic of dried latex: Proximate analysis of dried crude latex of *Ficus trichopoda* and *Euphorbia unispina* revealed high amount of crude protein (68.48%), crude fat (7.66%) and crude fibre (1.02%) in *Ficus trichopoda* latex however, the moisture and ash were 3.74% and 2.76%, respectively (Table 1).

Table 1: Proximate analysis of dried latex of *Ficus trichopoda* and *Euphorbia unispina*

Dried latex	Solubility (%)	Crude Protein (%)	Moisture (%)	Ash (%)	Crude Fat (%)	Crude Fibre (%)
F. trichopoda	18.17±0.31	68.48±1.10	3.74±0.41	2.76±0.34	7.66±1.4	1.02±0.04
E. unispina	32.72±0.31	64.41±6.78	6.20±0.45	2.18±0.02	15.52±0.089	0.67±0.09
***C. papaya (*Macalood *et al*., 2013; Cambon, 2008; Caro, 2001)**	NA	57.24±0.69	17.76±0.09	7.00±0.01	5.21±0.13	0.67±0.09

NA = not available

Euphorbia unispina latex showed high content of fat (15.52%) than that detected in *Ficus trichopoda* latex. However, the latex of *Euphorbia unispina* was more soluble than *Ficus trichopoda* latex. Latex is basically, an aqueous emulsion with a complex mixture of constituents, like proteins, vitamins, carbohydrates, lipids, terpenes, alkaloids, and free amino acids (Macalood *et al.*, 2013). The proximal characteristic of the two latexes was similar to other latex such as *C. papaya* latex, which has high level of protein (57.24%), and crude fat (5.21%) (Macalood *et al*., 2013; Cambon, 2008; Caro, 2001). Nevertheless, the *C. papaya* latex shows the high amount mineral constituent (7%) compared to that of the both studied latex. We also observed that *Ficus trichopoda* latex is very rich on crude fat.

Effect of the pH value of the reaction on the activity of lipases from dried crude latex: Besides proximate analysis, lipase activity in both *Euphorbia unispina* and Ficus *trichopoda* latexes was evaluated. The effect of the pH value of the reaction on the activity of lipases from dried crude latex for palm oil hydrolysis was measured in the range of 4.0–8.0 at 37 ◦C. The results were shown in Fig. 1. As can be observed, enzyme activity increased with an initial increase in pH and optimum activity was noted at pH = 5 suggesting an acidic nature of the enzymes. Further increase in pH caused a rapid decrease in the enzyme activity. This observation also suggests that under such condition the ionization of the enzymes chemical groups leads to optimal biocatalytic function of the protein. Cambon *et al.* (2006) found two optimal pH values (4 and 7) for *Plumeria* latex whereas Eastmond (2004) observed maximum catalytic activity for castor bean lipase at pH 4.5.

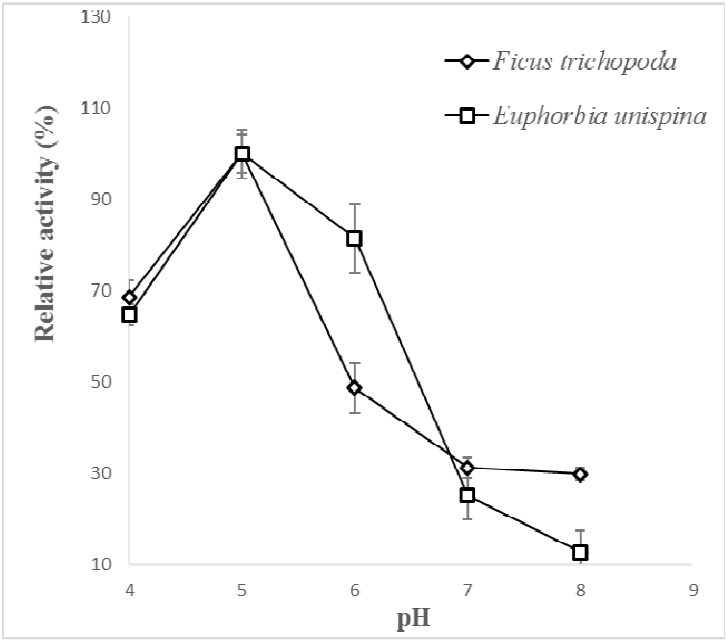

Fig. 1: Influence of pH on the catalytic activity of dried crude latex of *Ficus trichopoda* and *Euphorbia unispina*.

Effect of reaction temperature on the activity of lipases from dried crude latex : In Fig. 2, the effect of reaction temperature on the relative activity of lipases was shown within the range of 30–90°C at the pH 7. The optimum reaction temperature was 35°C for *Ficus trichopoda* latex. At 40°C, the lipase activity was reduced. However, the optimum temperature for *Euphorbia unispina* latex was 50°C. As reported, purified lipase from sunflower seeds was found to be 50°C (Sagiroglu & Arabaci, 2005), and an increase of 5°C resulted in a 40% reduction of its catalytic activity. Lipase from corn seeds presented also the optimal temperature of about 50-55°C (Santos *et al.*, 2013). In the present study, although activity declined above 35°C and 50°C for *Ficus trichopoda* and *Euphorbia unispina,* respectively, substantial lipolysis was still detected, even at 70°C, indicating a high thermo-stability of the enzyme. However, the temperature behaviour observed in this study is almost closed to that observed by Moussavou *et al.* (2016). It seems that vegetal lipase exhibits high thermo-stable than that from microbial source.

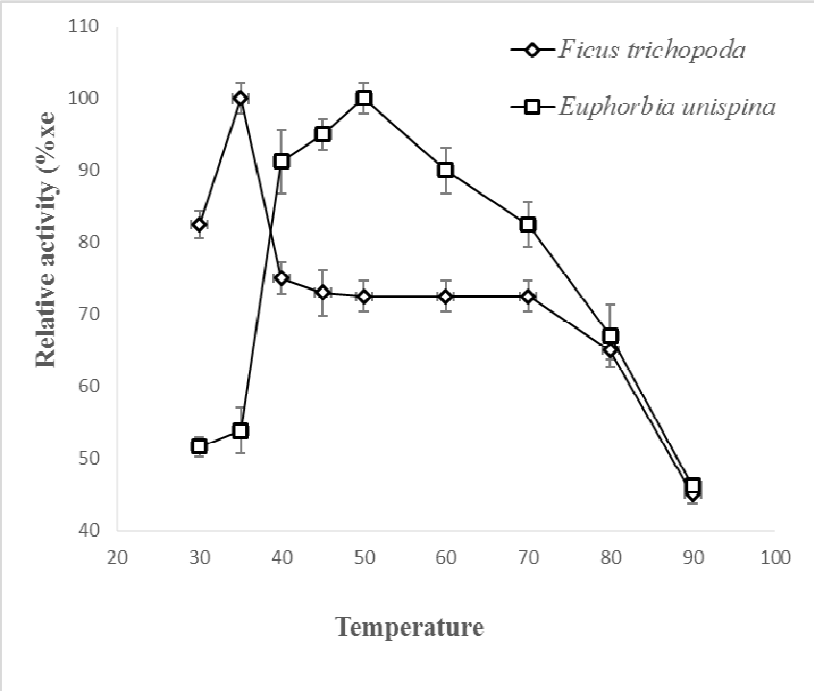

Fig. 2 : Influence of temperature on the catalytic activity of dried crude latex of *Ficus trichopoda* and *Euphorbia unispina*.

Influence of the fatty acid composition of the vegetable oils on the hydrolysis level: To examine the catalytic activity of lipases on various oils, the hydrolysis of vegetable oils with different fatty acids in their composition was tested. As shown in Fig 3, crude latex of *Ficus trichopoda* exhibited significant activity on palm oil, while *Euphorbia unispina* exhibited significant activity with peanut oil, palm oil as well as soy oil. Remarkably, the two latex did not show significant activity on coconut oil, composed mainly of medium-chain fatty acids.

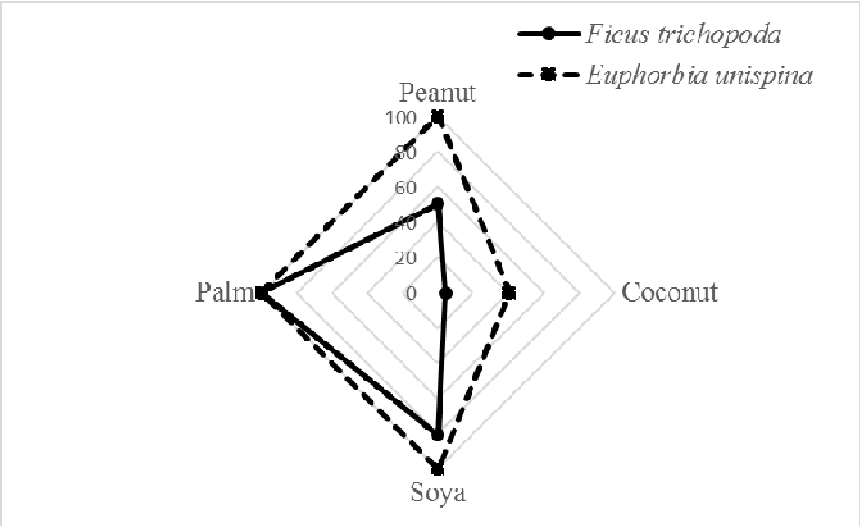

Fig. 3 : Influence of the fatty acid composition of the vegetable oils on the hydrolysis level.

The typoselectivity of lipases from dried crude latex : The typoselectivity of lipase contained in both studied plant latex was evaluated using an equimolar emulsified mixture of pure triacylglycerols. Selectivity of *E. unispina* and *F. trichopoda* lipase towards chain length (Fig. 4a and Fig. 4b) and unsaturation number (Fig. 4b) was

determined by competitive reaction and relative content (%) of FA was evaluated. With the mixture of saturated TAG, both latex extracts were not capable to hydrolyze preferentially length chain between 8:0 to 14:0 (Fig. 4a). With the mixture composed of saturated and unsaturated TAG, the latex from *E. unispina* latex catalyzed hydrolysis of all TAGs at the same rate, whereas *F. trichopoda* latex exhibited a high preference to 18:0. However, during the reaction a small decrease was observed when the unsaturation number increase (Fig. 4b). *E. unispina* latex showed a behaviour similar to that reported recently by Akil *et al* (2016) with *Yarrowia lipolytica* lipase in its free form and immobilized.

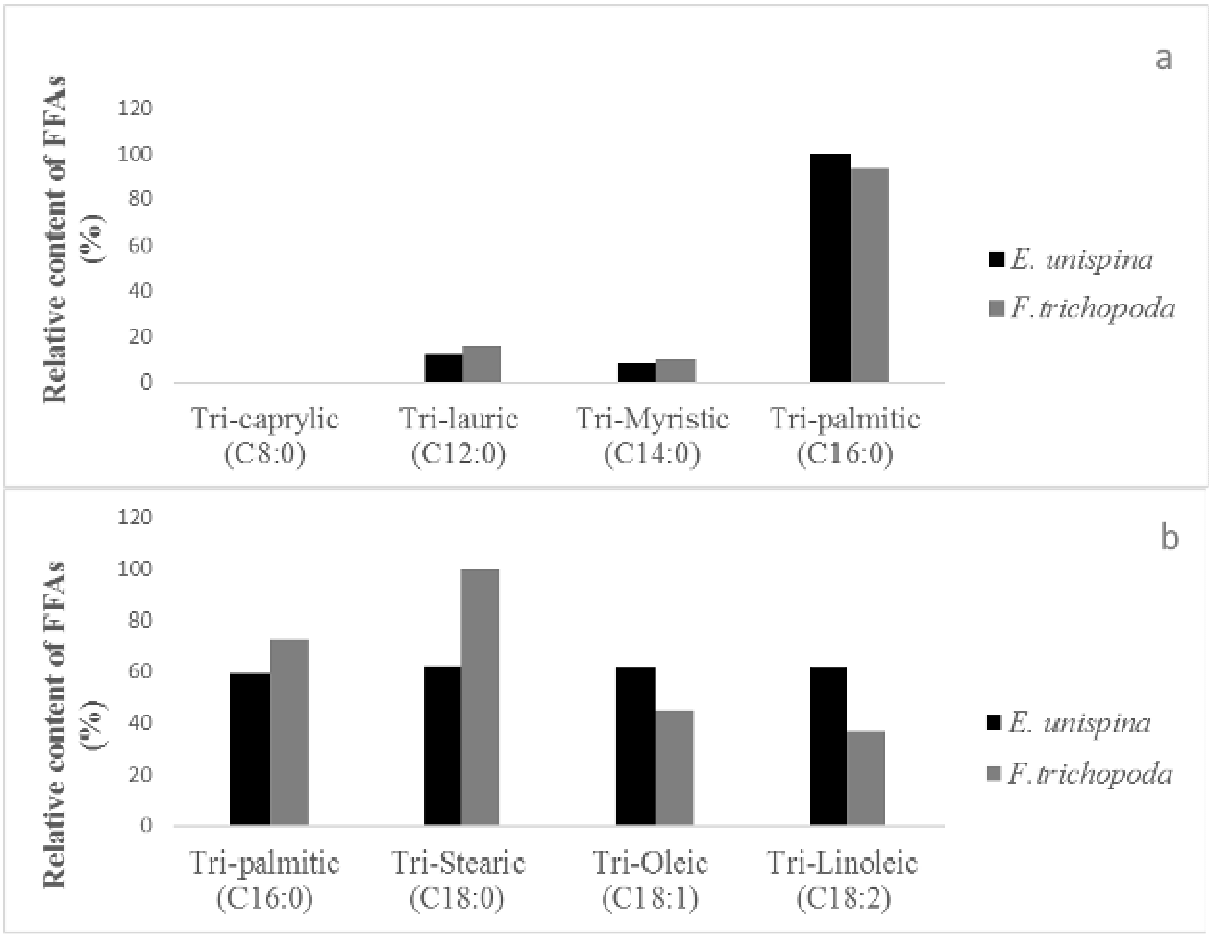

Fig. 4 : Selectivity of *E. unispina* and *F. trichopoda* lipase towards chain length (a and b) and unsaturation number (b).

Influence of the dried crude latex concentration on the hydrolysis level: As the enzyme, can be the limiting factor, an increase in the amount of enzyme is expected to enhance the reaction rate. Results showed that the hydrolysis of vegetable oils increased by increasing the latex concentration, especially from 2 to 10% m/m (Fig 5). Maximum hydrolysis percentage was attained at 15% and 20% m/m for dried latex of *Ficus trichopoda* and *Euphorbia unispina* respectively. However, the highest lipase concentration (25 to 35 % m/m) did not verify a strong increase in the hydrolysis percentage as expected because the oil droplets may encapsulate lipase molecules in its inner, leading to the reduction of their catalytic activity or a possible bad dispersion of the crude extract in the medium (Santos *et al.,* 2013).

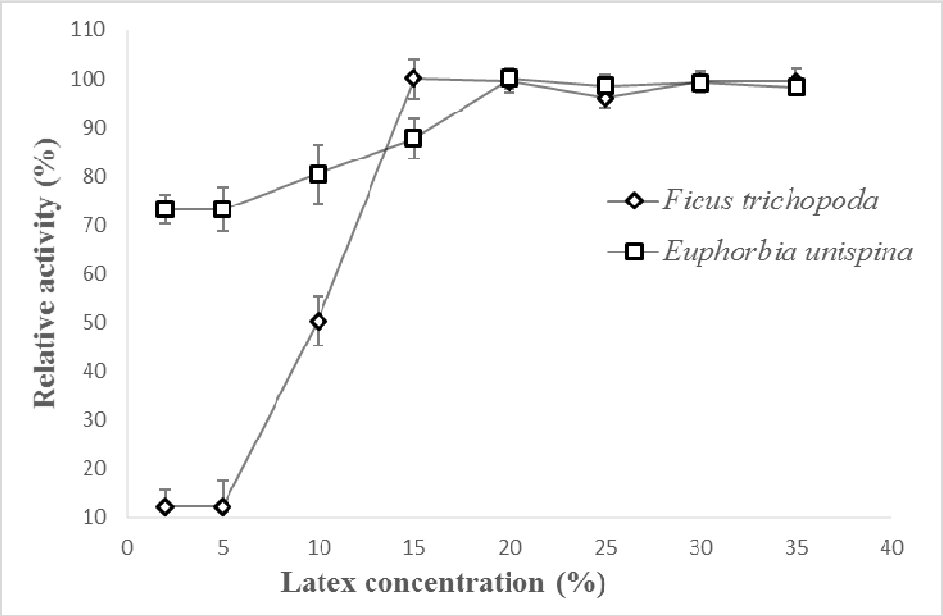

Fig. 5: Influence of the dried crude latex concentration on the hydrolysis level.

Effects of ions on the activity of lipase: Table 2 shows the effects of ions on the activity of lipase from the two latexes. As shown in table 2 neither $CaCl_2$, $HgCl_2$ nor EDTA affect significantly the activity of lipase from *Ficus trichopoda* latex, however sodium chloride inhibited the activity of the lipase from this latex. In others works from the literature, Enujiugha *et al.* (2004) found that Calcium ion in the reaction mixture brought about 64% increase in activity of lipase activity in dormant seeds. Abigor *et al.* (2002) and Haas *et al*. (1992) also observed an enhancement of lipase activity by calcium ion inclusion. From the work of Mukundan *et al*. (1985), the chloride ion did not cause any inhibitory effect but the metal ions (Na^+, Hg^{2+}) enhanced lipase decrease.

Table 2: Effects of different ions on the activity of dried crude latex of *Ficus trichopoda* or *Euphorbia unispina.*

Salt/ion	Activity (mol of released acid/g of latex/min) 10^{-05}	
	F. trichopoda	***E. unispina***
NaCl	4.18±1.02a	2.25±0.03a
$CaCl_2$	6.76±0.23b	2.76±0.02a
$HgCl_2$	6.54±0.54b	2.75±0.04a
EDTA	6.83±0.14b	2.40±0.20a

The mean values followed by same letter in the same column are not significantly different ($p< 0.05$).

To determine synthesis activity of both plant latex, reaction assay was conducted in the solvent-free synthesis of ethyl oleate in the conditions already described. The two latexes being active as catalysts for the hydrolysis of the triglycerides, it appears that they therefore contain lipases. From the results obtained, it arises that only the latex of *F. trichopoda* presents a catalytic activity during esterification (Fig. 6). Several studies emphasizing on the relation between the hydrolytic activities in aqueous medium and the activities of syntheses in nonconventional medium were undertaken. According to Klibanov (1997) and Gandolfi (2000), it arises that the data on the hydrolytic activities of the enzymes did not make it possible to predict the behaviour of their synthetic activities in organic medium. Investigations on the effect of water and alcohol on the catalytic activity of lipases of latexes during esterification need to be carried out. These parameters were known to belong to the parameters, which control the catalytic

activity of lipases and the yield of esterification reaction (Bajaj *et al*., 2010).

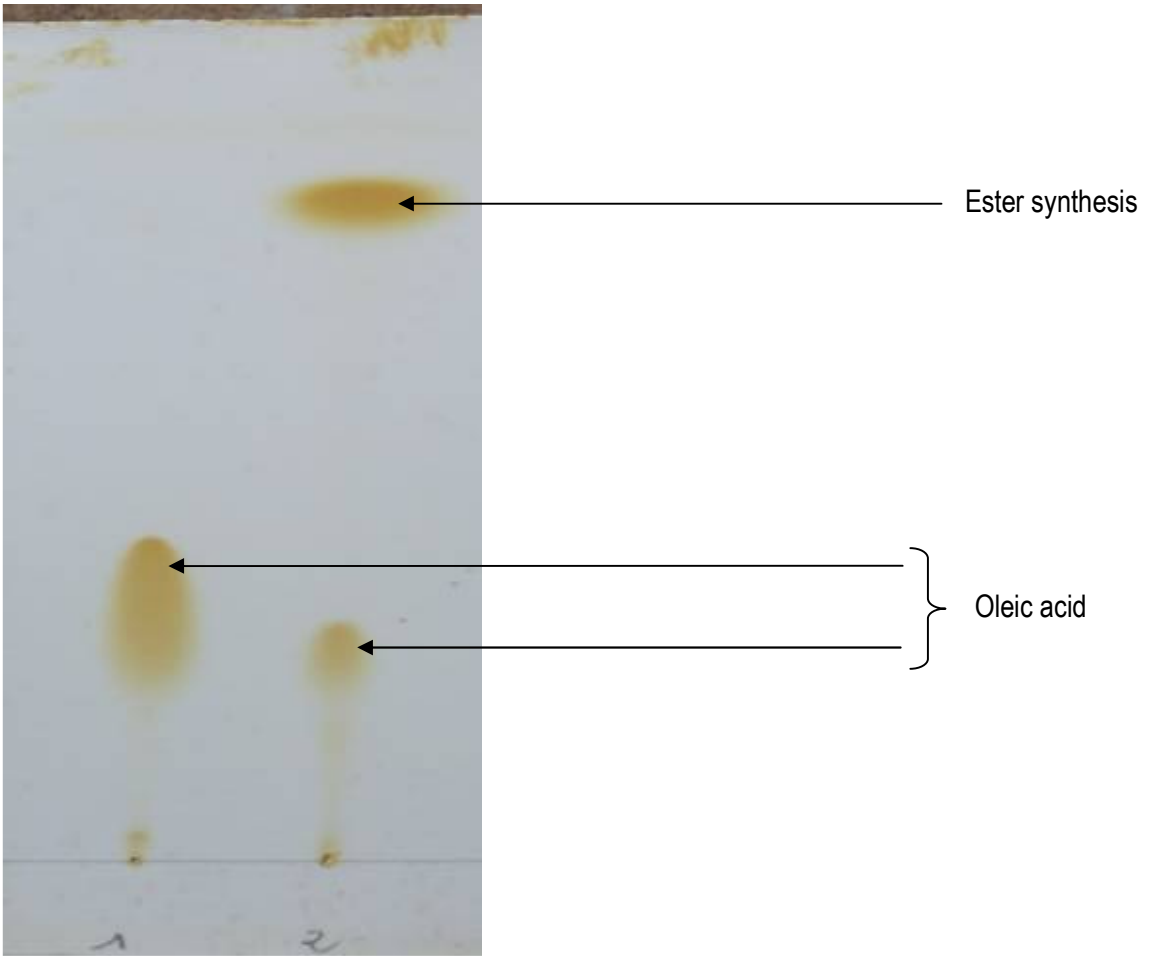

Fig. 6 : Thin layer chromatography of esterification of the oleic acid using latex of Ficus trichopoda

CONCLUSION

Chemical analysis of dried crude latex of *Ficus trichopoda* and *Euphorbia unispina* revealed the presence of crude proteins in higher amount as compared to other chemical constituents. This study suggests the potential of *Ficus trichopoda* and *Euphorbia unispina* crude latex in the production of modified oils. Formerly, the hydrolysis of oils and fats has been performed preferentially by commercial lipases. The lipases showed typo-selectivity for saturated fatty acids, however both lipases showed typo-selectivity towards long-chain unsaturated FA, especially for 18:1. However, *Ficus trichopoda* latex, showed features of a potential bio-catalyst that can be effectively used for customizing oils and fats aiming at lipid modification and synthesis of structured lipids. According to our results, plant lipase from latex proved to be a promising biocatalyst in the production of concentrated fatty acids by hydrolysis of vegetable oils from several sources than those lipases available commercially from microbial and animal sources. Furthermore, this study can serve as a reference among researchers to continue investigating more valuable information on the potentials offered by *Ficus trichopoda* and *Euphorbia unispina* to improve bio-energy and resolved issues on environmental degradation and health related problems. Moreover, this study will boost agricultural production using crude latex of *Ficus trichopoda* as biodiesel production knowing its protein contents and its lipase activity.

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