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Avocado oil as an inducer of the extracellular lipase activity of *Kluyveromyces marxianus* L-2029

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Abstract

Avocado represents one of the most nutritious and consumed foods in Mexico, and its export generates significant profits; because of its fatty acids content, the avocado oil was used as an inducer for the synthesis of extracellular lipases from the non-conventional yeast *Kluyveromyces marxianus* L-2029. Lipases are able to catalyze acyl glycerides, hydrolyzing them into fatty acids and glycerol; they have several applications in various industries. In order to determine the ideal initial avocado oil concentration in the culture medium for the yeast, the induction was carried out at different concentrations in incubation for 24 h. The yeast presented the greater extracellular lipase productivity at 3.5% v/v avocado oil concentration, with a 3.47 μmol PNF/mg prot * min (U/mL) maximum activity. The enzymatic extract obtained at this condition had an optimum lipase activity temperature of 36 °C and pH 6. The *pYJR107W* lipase was detected in the enzymatic extract from avocado oil induction, and is therefore responsible for the extracellular lipase activity of *K. marxianus*.

Keywords: avocado oil; extracellular lipases; *Kluyveromyces marxianus*; lipase activity.

Practical Application: Avocado oil can be used to induce the synthesis of extracellular lipases from *K. marxianus* L-2029, being an alternative for the production of these enzymes. In addition, said strain presents a good lipase activity; the lipase properties of this yeast are attractive to be proposed in several applications in the future.

1 Introduction

Lipases are enzymes that catalyze a wide variety of reactions, such as the partial or total hydrolysis of triacylglycerides, and synthesis reactions, which are classified into two main types of reactions: esterification and transesterification (Colla et al., 2010). The lipases are serine enzymes defined as triacylglycerol acyl hydrolases (EC 3.1.1.3), capable of hydrolyzing long chain acylglycerol carboxylic esters, with chains greater than 10 carbon atoms, which distinguishes them from esterases (Casas-Godoy et al., 2009).

This type of enzymes has a high importance in the industry thanks to its multiple applications in the degradation of substrates with high fat content, as well as in esterification reactions carried out in the food, paper, pharmaceutical, energy and cosmetic industries (Diez & Sandoval, 2012; Sharma et al., 2001). In the food industry, lipases are used in the production of dairy products or with specific properties, such as cheese, cocoa butter, milk substitutes, among others. Another common application of lipases is the modification of the content of fats and oils in some foods, which allows improving the nutritional content of some of them, or improve the organoleptic characteristics of others, through the modification of compounds that provide specific flavors or fragrances (Andualema & Gessesse, 2012; Casas-Godoy et al., 2009; Hasan et al., 2006).

Microorganisms with a high capacity to produce lipases can be found in different habitats, mainly in the waste or the residues of vegetable oils used in the preparation of fried foods, in dairy products industries, in contaminated soils with oils and in deteriorated foods. The lipase production for industrial purposes has focused on those obtained only by some species, which in many cases present a lack of certain desirable characteristics for specific processes (Vakhlu & Kour, 2006). Lipase production alternatives have been continuously searched, in order to identify enzymes with potential characteristics for various biotechnological applications.

K. marxianus is a non-conventional homothallic and hemiascomycete yeast, phylogenetically related to *Saccharomyces cerevisiae* (Lane & Morrissey, 2010; Lane et al., 2011). *K. marxianus* has shown lipase activity (Deive et al., 2003; Stergiou et al., 2012) and possess desirable characteristics for its application in various biotechnological processes. For instance, it assimilates a variety of sugars, it has a rapid growth rate (with a doubling time of approximately 70 min), it is thermotolerant (with the ability to grow up to 52 °C), and it has an extraordinary secretory capacity (Jeong et al., 2012; Lee et al., 2013; Lertwattanasakul et al., 2015). The strain *Kluyveromyces marxianus* L-2029, used in the present study, was isolated from spontaneous fermentations involved in the production of Mezcal, during fermentation of *Agave cupreata*

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juice, which is elaborated in the state of Michoacán, México, (González-Hernández et al., 2012); said strain has shown a high growth capacity and ethanol production (Pérez et al., 2013), and could possess a high capacity for the production of lipases under a suitable inducer.

The synthesis of extracellular lipases is strongly induced by the presence of triglycerides in the growth medium of the microorganism (Hsu et al., 2008). Avocado is one of the most important products in agriculture and in the Mexican diet; the amount of lipids in the avocado pulp is very high (21 to 33%) (Moreno et al., 2003). For instance, the amount of saturated fatty acids in avocado oil is similar to sunflower, soybean, peanut and olive oils. Regarding the presence of monounsaturated fatty acids, the nutritional properties are similar to those of olive oil, and the main one is oleic acid, reaching concentrations of up to 60%. Concerning the polyunsaturated fatty acids ratio, the linoleic acid has a lower proportion in avocado oil (approximately 14%) than in canola oil (Ortiz et al., 2004). The high-quality requirements for the avocado exportation cause that a large part of the production is not commercialized; a fraction is used for the production of avocado oil currently by some regional companies. Avocado oil could therefore become a good substrate for the induction of lipase synthesis.

The main objective of this work was to determine the best concentration of avocado oil for the induction of the synthesis of the extracellular lipase from *K. marxianus* L-2029, as well as their subsequent identification and partial characterization.

2 Materials and methods

2.1 Strain

The strain used in the study, *K. marxianus* L-2029, was obtained from the Biochemistry Laboratory of the Instituto Tecnológico de Morelia (Morelia, Michoacán, México). The *K. marxianus* strain was previously isolated from spontaneous fermentations in the production of Mezcal (González-Hernández et al., 2012) and it was deposited in the National Collection of Microbial Strains and Cultures (CDBB) from CINVESTAV with registration number L-2029. For the maintenance and growth of the strain, YPD agar (20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 20 g/L agar) culture medium was used.

2.2 Statistical design and culture conditions

In order to determine the initial concentration of avocado oil that promoted the greater lipase activity, a comparative analysis was carried out at the flask level, in a randomized single-factor statistical design. The factor, the initial concentration of avocado oil, was varied in 7 levels: 1, 2, 3, 3.5, 4 and 4.5% v/v; as a control, the culture medium without oil was used. Each of the treatments was carried out in triplicate. For the preparation of the different treatments, commercial avocado oil Avocare® (*Persea gratissima*) was used.

The base of the culture medium was YPD liquid medium added with avocado oil at different concentrations. Culture media was inoculated with an initial concentration of $3x10^6$ cel/mL and then was incubated at 200 rpm, 30 °C and 6.5 initial pH. Samples were taken every 2 h for the determination of the kinetic parameters described below.

The evaluated response variables were the doubling time and the lipase activity (U/mL), which were compared by analysis of variance (ANOVA) at a level of significance α of 0.01 in the JMP® Software. The treatments were completely randomized performed, in triplicate.

2.3 Innoculum preparation

Flasks with a capacity of 250 mL and working volume of 100 mL of liquid YPD culture medium (20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone), supplemented with avocado oil at each concentration, were prepared for the preadaptation of the yeast. Five volumes of the yeast were transferred, from the Petri dish where it was stored, to the inoculum flask and incubated for 12 h, at 30 °C and 200 rpm.

The microbial load of the inoculum was determined after 12 h by direct counting in the Neubauer chamber; the pertinent calculations were made to begin with an initial concentration of $3x10^6$ cel/mL.

2.4 Determination of kinetic parameters

During the fermentation kinetics, the following factors were monitored:

- Cell growth: It was determined by viable cell counting in Neubauer chamber, staining with methylene blue. The count was performed in a microscope, by an average of the sum of five quadrants, considering the relevant dilution. The result was expressed in cel/mL and the generated data allow the calculation of the doubling time response variable.
- Glucose consumption: It was measured with the DNS oxide-reduction technique; reducing sugars, such as glucose, have the capacity to reduce 3,5 dinitro salicylic acid, causing a color change, which can be quantified in a spectrophotometer, at a wavelength of 540 nm. For the technique, 100 µL (or its dilution with distilled water) was added to 100 µL of DNS reagent; to promote the reaction, heating was carried out at 90 °C for 5 min, after which it was stopped by cooling in an ice bath for 5 min. A 1 mL of distilled water was added and quantified at the wavelength mentioned above. To calculate the concentration, a calibration curve was previously prepared at known concentrations with a glucose stock at a concentration of 10 g/L.

2.5 Total protein

For the quantification of total protein in the extracellular extracts, the Bradford method (Bradford, 1976) was used, which is based on the color change of Coomassie blue at different protein concentrations. Coomassie blue interacts with basic and aromatic amino acids, causing the color change from red to blue, at a wavelength of 595 nm. To a solution with 2.3 mL of distilled water and 100 µL of enzyme extracellular extract, 600 µL of Bradford reagent (5 mL of Coomassie blue G-250, 2.5 mL

of ethanol and 5 mL of phosphoric acid, for a total volume of 50 mL) were added. The mixture was stirred and measured in a spectrophotometer at the mentioned wavelength. To calculate the protein concentration in the extracts, a calibration curve was previously prepared at known concentrations using a stock of bovine serum albumin with a concentration of 1 mg/mL.

2.6 Esterase activity

In order to compare the activity of the enzymatic extract on short chain acids with respect to long chain acids, the colorimetric technique of the hydrolysis of p-nitrophenyl butyrate (colorless) to butyric acid and p-nitrophenol (yellowish color, measured at 400 nm) was used. A volume of 5 mL of phosphate buffer (100 mM KH_2PO_4, pH 7.25) was incubated with 100 µL of enzymatic extract in a water bath at 30 °C for 10 min; subsequently, 50 µL of a 100 mM p-nitrophenyl butyrate solution was added and reacted for 10 min at the same temperature. The solutions were placed in an ice bath for 10 min and were centrifuged at 2000 rpm for 5 min. The supernatant was used to determine its absorption at the aforementioned wavelength. A standard curve was made using a stock of p-nitrophenyl 100 µg/mL. A unit of esterase activity is defined as the amount of enzyme released per 1 mmol of fatty acid per minute (Casas-Godoy et al., 2009).

2.7 Lipase activity

To determine the lipase activity the hydrolysis method of p-nitrophenyl palmitate, which is converted into a quantifiable long-chain fatty acid substrate, was used (Pencreac'h & Baratti, 1996). The technique allows determining the amount of p-nitrophenyl (p-NF) released, which causes a change in the absorbance of a sample at a wavelength of 410 nm. 50 µL of 16.5 mM p-nitrophenyl palmitate solution (diluted in isopropanol), 3 mL of 50 mM Tris HCl buffer solution (Triton X100 0.4%, gum arabic 0.1%, pH 8) and 50 µL of Triton X100 were mixed and pre-incubated. at 37 °C in a water bath for 5 min; after that time, 55 µL of enzyme extract was added and then was incubated for 5 min. Finally, the absorbance was determined in a spectrophotometer at the aforementioned wavelength. To calculate the amount of hydrolyzed p-nitrophenyl, a standard curve was made at known concentrations, from a p-nitrophenyl 100 µg/mL standard. One unit of lipase activity is defined as the amount of enzyme released per 1 mmol of fatty acid per minute (Casas-Godoy et al., 2009).

2.8 Ideal pH and temperature of the enzyme extract

The ideal conditions of the enzymatic extract obtained from each treatment was deduced using the hydrolysis technique of p-nitrophenyl palmitate, described above. In order to determine the optimum pH, the pH of the solution buffer was varied from 4.5 to 9. The reaction temperature in this case was maintained at 37 °C and the absorbance of the reaction was measured every 5 min at 410 nm in a spectrophotometer. On the other hand, to determine the optimal temperature of the enzymatic extract, the pH was kept constant at 8 while the incubation temperature was varying from 30° to 50 °C. The absorbance of the reaction was measured every 5 min at 410 nm in a spectrophotometer. In both cases the results were expressed in terms of units of lipase activity.

2.9 Enzymatic stability at ideal conditions

The stability of the enzymatic extract was analyzed at the previously determined pH and temperature conditions, using the p-nitrophenyl palmitate hydrolysis technique, and monitoring the lipase activity every 5 min, for an approximate time of 1.5 h.

Identification of lipases in the enzymatic extract

The protein content of the extracellular extract from the fermentation of the treatment that showed to have a greater lipase activity was first concentrated: 200 µL of the extract were added to 800 µL of cold acetone. The mixed was then incubated at -20 °C for 1 h. Later, the sample was centrifuged at 13,000 rpm for 10 min, and the pellet was recovered as the total concentrated protein. The excess of acetone was allowed to evaporate before preparing the sample for electrophoresis.

Denaturing electrophoresis in polyacrylamide gel (SDS-PAGE) was carried out, using a 4% stacking gel and a 12.5% resolution gel (Singh et al., 2006). 5 µL of lysis buffer (0.5 M Tris HCl, 10% SDS, 0.5% bromophenol blue, 0.005% 2-mercaptoethanol and 25% glycerol), 20 µL of distilled water and 3 µL of a blue Coomassie solution were added to the concentrated protein from the extracellular extract. These preparation samples were incubated in a water bath at 90 °C for 10 mins to hydrolyze all the proteins. After that, 15 µL of the stained samples were and loaded into the previously prepared acrylamide gel; electrophoresis was run at 90 V for 1.75 h on charged 1X buffer (25 mM tris base, 192 mM glycine and 3.46 mM SDS). Once the electrophoresis was complete, the gel was separated and stained with a Coomassie blue staining solution for 30 min; the gel was then decolorized using a 5% methanol, 85% distilled water and 10% glacial acetic acid solution, 24 h. Finally, the gel was washed twice with distilled water and was revealed.

3 Results

3.1 Yeast growth in medium added with avocado oil

To determine the best initial concentration of the avocado oil inducer, fermentation kinetics were performed at different concentrations, from 1 to 4.5% v/v (6 total treatments), plus the control treatment at 0% inductor concentration. Each of the experiments was carried out in triplicate, and each kinetic had a total time of 24 h.

The presence of the inducer in the culture medium did not affect the *K. marxianus* L-2029 cell growth in any of the treatments (Figure 1A). In all cases, an exponential phase time of 8 h and a cellular concentration during the stationary phase of approximately $3x10^8$ cel/mL was observed. Despite the stress conditions generated by the absence of sugar as an energy source (which was totally consumed after the exponential phase), and the presence of the inducing agent, no phase of death was observed in any of the treatments, after 24 h of incubation.

From the cell concentration data, the response doubling time variable was calculated, which can offer more information about the influence of the inducer on the *K. marxianus* L-2029 growth on the different treatments and its adaptation to them (Table 1).

3.2 Lipase activity of the extracellular extract

In order to determine the influence of the avocado oil added to the culture medium, the extracellular protein was determined in for each treatment. In general, a slight increment in the extracellular protein was observed from the 8th hour of fermentation, at which point most of the glucose was consumed. The increase was approximately 10 mg/mL in the best case though (Figure 1C). The protein concentration in the control treatment and in the treatments with a lower concentration of avocado oil (up to 3% v/v), was approximately 20 mg/mL lower than that detected for the treatments with a higher avocado oil concentration. Indeed, the highest concentration of extracellular protein detected was in the treatment with a 4.5% v/v of avocado oil concentration, being approximately 100 mg/mL at the end of the fermentation time, significantly greater than the 60 mg/mL detected in the control treatment.

Considering the concentration of protein obtained in the different treatments, the esterase and lipase activities were calculated. Esterase activity, expressed in micrograms of p-nitrophenyl released per milligram of extracellular protein per minute (U/mL), was only detected in the treatment with the lowest avocado oil concentration, as well as in the control treatment; from a concentration up to 2% v/v avocado oil, the esterase activity was imperceptible (data not shown). Avocado oil is mainly composed of long chain fatty acids, which would promote the synthesis of lipases instead of esterases (Casas-Godoy et al., 2009).

The maximum lipase activity was detected at 8 h of fermentation in all the treatments with added avocado oil. This response variable was statistically compared using a Tukey-Kramer test at a significance level of 0.01 (Table 1). The treatments with the lowest avocado oil concentration (1 to 3% v/v) did not differ statistically from the control treatment, having a lipase activity close to 1 U/mL. In contrast, the treatments with a higher avocado oil concentration (4 and 4.5% v/v) presented a higher activity in reference to the control and the other three treatments mentioned above, practically doubling the values of the same.

Table 1. Statistical comparison by Tukey-Kramer test for doubling time and lipase activity.

Treatment	Avocado oil concentration (% v/v)	Doubling time (h)	Lipase activity (U/mL)
1	0	1.25 ± 0.23^{A}	0.75 ± 0.05^{D}
2	1	1.23 ± 0.27^{A}	1.17 ± 0.37^{CD}
3	2	1.03 ± 0.01^{A}	0.94 ± 0.22^{CD}
4	3	1.00 ± 0.04^{A}	1.16 ± 0.08^{CD}
5	3.5	1.27 ± 0.02^{A}	4.69 ± 0.23^{A}
6	4	0.97 ± 0.02^{A}	2.22 ± 0.45^{B}
7	4.5	0.97 ± 0.10^{A}	1.90 ± 0.15^{BC}

Different letters mean statistical difference for α = 0.01, n = 3.

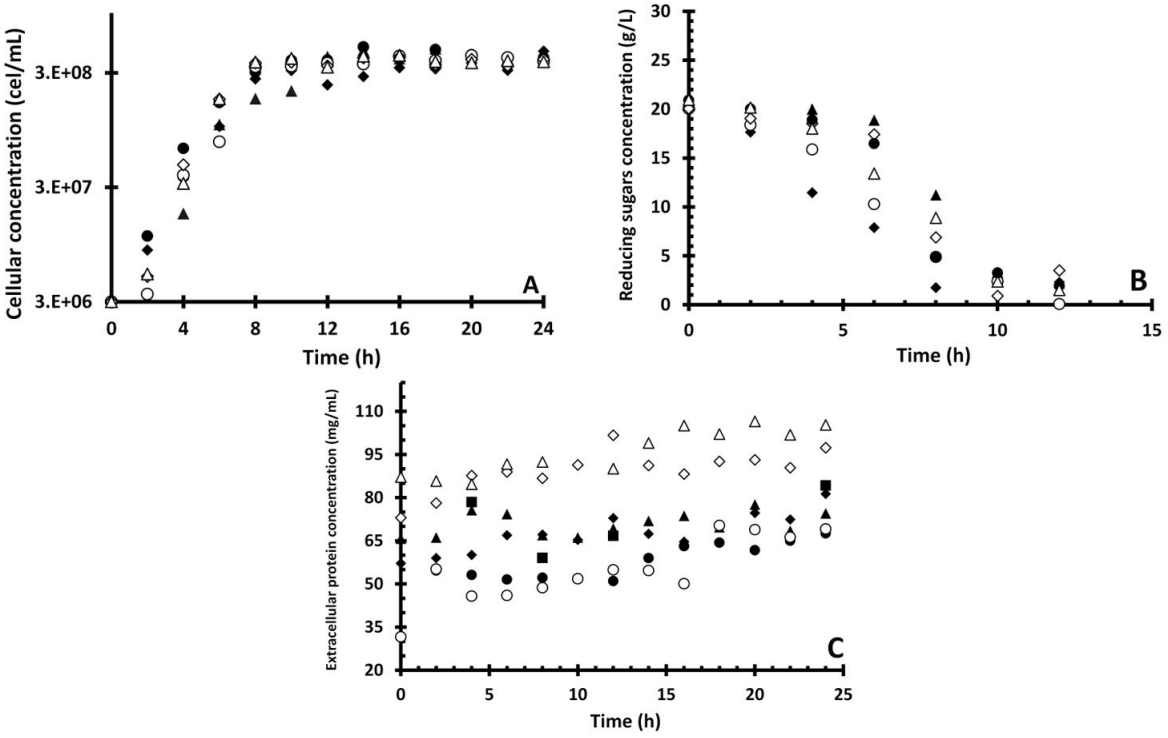

Figure 1. Parameters determined during induction with avocado oil for the synthesis of extracellular *K. marxianus* L-2029 lipases: (A) Cell growth; (B) Reducing sugar uptake and; (C) Extracellular protein concentration. Concentration of avocado oil for the different treatments: (●) 0%, (♦) 1%, (▲) 2%, (▪) 3%, (○) 3.5%, (◊) 4% and (Δ) 4.5%.

The influence of avocado oil promoting the lipase synthesis in *K. marxianus* L-2029 and their activity is evident. The treatment that showed greater activity, at a confidence level of 90%, was the treatment with an avocado oil concentration of 3.5% v/v, and an activity of 4.69 U/mL.

3.3 Optimum temperature and pH, and extracellular extract stability

The extracellular extract from the treatment with an avocado oil concentration of 3.5% v/v, was used to determine the optimum temperature and pH of the same.

The temperature profile was evaluated from 30° to 50 °C, at pH 8 (Figure 2A). The maximum activity detected in this range was at 36 °C. The lipase activity of the extract decreased considerably at temperatures above 38 °C, and it was practically nullified at 46 °C.

The enzymatic activity profile to evaluate the optimum pH of the enzymatic extract of the treatment at 3.5% v/v avocado oil, was determined in a range from 4 to 9, using a temperature of 37 °C (Figure 2B). The maximum activity point was detected at a pH 6 for the extract obtained from *K. marxianus* L-2029; in addition, a slight increase in activity was observed at pH 8.

In order to determine the stability of the enzymatic extract, the lipase activity was evaluated over time at a temperature of 36 °C was used, as well as the two pH values at which a high activity was obtained, 6 and 8 (Figure 3). At a pH 6, the activity of the enzyme remained constant for approximately 60 min, after which there was an abrupt increase in the activity detected. This increase is attributed to the stability of the reagent used in the technique and not properly to an increase in the activity of the enzyme. Using a pH of 8, the trend was similar, maintaining activity close to 2 U/mL for a total time of 90 min.

3.4 Identification of lipases in the extracellular extract

For the identification of the probable extracellular lipases from *K. marxianus* L-2029, an enzyme extract sample was obtained at the best conditions of temperature, pH, time and avocado oil concentration previously determined. Due to the low concentration in the extracellular extract, the total protein was concentrated by treatment with acetone. This was applied to four different samples obtained under the conditions already described.

After the acetone treatment, the protein concentration increased from 137.68 mg/mL, obtained directly from the fermentation extracts, to 256.45 mg/mL. In the gel obtained from the SDS-PAGE, a band was detected in each of the extracts from the induction of lipase synthesis from *K. marxianus* L-2029 using avocado oil (lanes 2 to 5, Figure 4); this band had an approximate molecular size of 40 KDa, if it is compared with

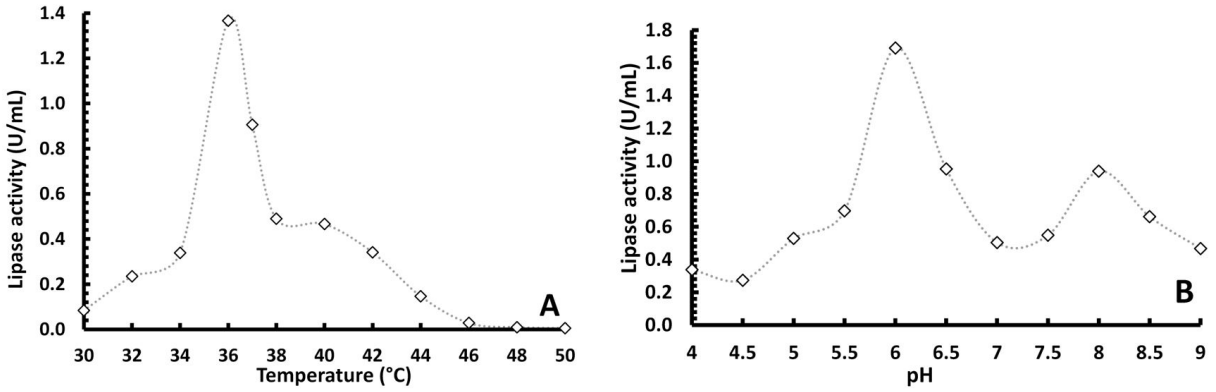

Figure 2. Influence of temperature and pH on the lipase activity of the extracellular extract obtained from *K. marxianus* L-2029 with 3.5% avocado oil: (A) Lipase activity at different temperatures and; (B) Activity lipase at different pH.

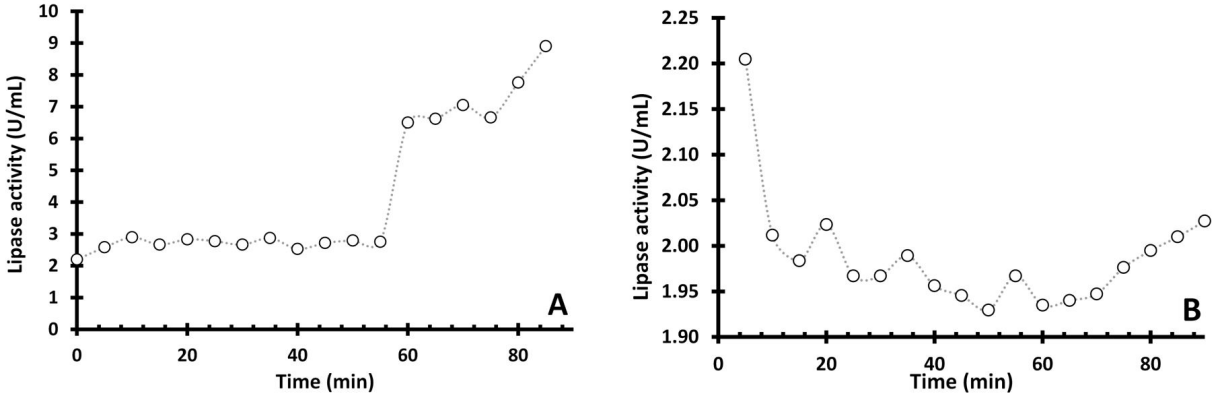

Figure 3. Stability of the extracellular extract obtained from *K. marxianus* L-2029 induced with 3.5% avocado oil at 36 °C: (A) pH 6 and; (B) pH 8.

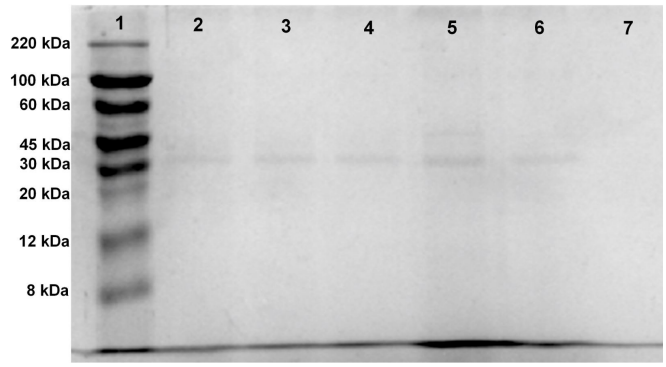

Figure 4. Identification of extracellular *K. marxianus* L-2029 lipases by SDS-PAGE electrophoresis. Lane 1: Molecular size marker; Lane 2 to 5: Samples of lipase extract obtained at ideal 3.5% oil concentration; Lane 6: Positive control: Purified lipase from *C. rugosa*; Lane 7: Negative control.

the standard used (lane 1). In addition, the LIP2 lipase from *C. rugosa*, whose molecular size is approximately 40 KDa, was used as a positive control (lane 6, Figure 4).

4 Discussion

K. marxianus shows an accelerated growth when glucose is its only carbon source, with an average doubling time of 70 min (Groeneveld et al., 2009). In spite of slight variations observed in the values of doubling time at different concentrations of avocado oil (Table 1), it remained between 0.97 and 1.25 h; in addition, no significant statistical difference was detected in any of the cases. Therefore, the avocado oil did not have a negative effect on the adaptation and growth of the yeast to the culture medium, which took advantage of the available sugar to multiply.

Reports indicate that lipases are inducible enzymes, and in the case of the LIP3, LIP4 and LIP5 lipase promoters from *Candida rugosa*, the presence of ORE (elements of response to oleate) and UAS1 motifs has been detected, which regulate their synthesis (Hsu et al., 2008). In *S. cerevisiae*, highly related to *K. marxianus*, most of the genes whose transcription is activated in the presence of oleic acid are under ORE regulation; the ORE motif consists of two CGG triplets spaced by 15 to 18 nucleotides (5'-CGGN3TNAN9-12CCG-3'), which is regulated by the Pip2p-Oaf1 factor, also subjected to catabolic repression (Gurvitz & Rottensteiner, 2006). In addition, it was reported that oleic acid is a strong inducer of lipase activity in *K. marxianus* (Deive et al., 2003; Stergiou et al., 2012) and is one of the main components of avocado oil (Pacetti et al., 2007).

Thus, the glucose present in the culture medium could have promoted the growth of *K. marxianus* L-2029, without affecting the doubling time of the yeast, despite the presence of avocado oil. In fact, the initial glucose concentration (20 g/L) was strongly diminished after 8 h of fermentation and practically consumed after 12 h (Figure 1B), the same time that lasted the exponential phase in all the treatments used in this study.

Few studies report the production of lipases by *K. marxianus*. Deive et al. (2003) informed the lipase activity of *K. marxianus* induced with different substrates: oleic acid, palmitic acid, olive oil, corn oil and glycerol. The synthesis of lipases was principally benefited by induction with oleic acid, and the enzyme extract showed stability at acidic pH and elevated temperatures. Very low activities were detected for all inducers used in this study, except for the culture media supplemented with 10 g/L of tributyrin, in which the maximum activity detected was 40 U/mL, after 9 days of incubation. The highest activity determined when olive oil was used as the inducing agent, was 4.5 U/mL, after 7 days of incubation. The higher lipase activity obtained in this study is greater than the maximum obtained using avocado oil as inducer; however, the total time in which both values were obtained, and the technique used for the determination of said parameter, must be taken into account.

On the other hand, Stergiou et al. (2012) reported the optimization of some parameters involved in the production of lipases using *K. marxianus*: initial pH, temperature and incubation time; in addition, different lipase-inducing agents were tested, such as olive oil, corn oil, meat fat and cooking oil. The optimization proposed an initial pH of 6.4 and a temperature of 32.5 °C, as suitable conditions for the production of lipases (with olive oil as inducer), which showed a lipase activity of 0.175 U/mL. In this case, the yield obtained for the induction with avocado oil (4.69 U/mL) was higher.

Several studies have recently focused on the use of vegetable oils or residues with a high fat content for the induction of lipase synthesis. The most used inducer for the synthesis of lipases has been olive oil; the maximum lipase activity detected using this inducing agent has been achieved with *Aspergillus niger* and *Aspergillus fumigatus*, that exceeds values of 60 U/mL (Coca et al., 2001).

The *Yarrowia lipolityca* yeast has been used for the production of lipases induced by agro-industrial waste, such as barley bran, crushed walnut and sunflower oil (Domínguez et al., 2003). Sunflower oil has also been used for the production of extracellular lipases from *Aspergillus carneus* (Kaushik et al., 2006). Kempka et al. (2008) reported soybean meal as the best inducer among diverse substrates, including sugarcane molasses, hydrolyzed yeast, fermented corn liquor, yeast extract, sodium chloride, soybean oil, castor oil, corn oil, olive oil and peptone. Other reported inducers used for the production of lipases are bagasse from sugarcane, olive oil, wheat bran and rice bran, as well as residual mustard, peanut and coconut oils, wastewater, corn liquor, banana residues, melon, watermelon, lentil peel, sesame oil, peptone, tributyrin, citric acid and glucose (Bhosale et al., 2012; Treichel et al., 2010). Pabline et al. (2014) optimized the lipase production in submerged fermentation under different nutrient substrates, such as corn liquor, yeast extract, peptone and soybean oil, as well as different sources of sodium, magnesium, potassium and nitrate; the authors identified four strains with high lipase activity, including *Penicillium lanosum* and three bacterial strains. The use of walnut cake for the synthesis of lipases from *A. niger* AS-02 (Salihu et al., 2016) and soy molasses, from *C. rugosa* and *Geotrichum candidum* has also been proposed (Morais et al., 2016).

Recently, Ilmi et al. (2017) proposed the use of the oil obtained by mechanical pressure of the tropical plant *Jatropha curcas* L.; they achieved the induction of lipases from *A. niger*

6516 and *Rhizomucor miehei* CBS 260.62. Oliveira et al. (2017), on the other hand, used several agro-industrial residues as lipase inducer from *Aspergillus ibericus* MUM03.49: andiroba oil cake, cupuasu oil cake, canola oil cake, macauba oil cake, palm kernel oil cake, soybean meal, cake of green coffee oil and sesame oil cake. Xiaoyan et al. (2017) proposed the use of residual cooking oil to evaluate the co-production of erythrol and lipases using *Y. lipolytica* M53, obtaining a highest 12.7 U/mL lipase activity.

In the case of *K. marxianus*, the use of olive oil, corn oil, meat fat and cooking oil has been reported (Stergiou et al., 2012). Thus, avocado oil is proposed as a good lipase-inducing agent, showing a good action on the synthesis of lipases from the non-conventional yeast *K. marxianus* L-2029.

The maximum activity detected in this range was at 36 °C, despite the fact that *K. marxianus* is a thermotolerant yeast (Lane & Morrissey, 2010; Lane et al., 2011). The optimal temperatures, reported for fungal microorganisms, are commonly between 30 and 40 °C, such as *Y. lipolytica* (30 °C) and *P. verrucosum* (37 °C) (Menoncin et al., 2010; Sathish-Yadav et al., 2011), although some of them (for example those from *Candida antarctica* ZJB09193) have reached maximum activity above 50 °C (Liu et al., 2012). However, Deive et al. (2003) detected a maximum activity of 80 U/mL at a temperature of 50 °C, for the extracellular lipases from *K. marxianus* CECT 1018 produced in medium added with tributyrin, for which there is a variation between the strains used in each study.

Despite the extracellular enzymatic extract showed a higher lipase activity at pH 6 and 8, it has been reported that the *K. marxianus* CECT 1018 extracellular lipase is sensitive to pH values close to neutrality and alkalinity; on the other hand, a high stability of said enzyme has been reported at acidic pH 4 (Deive et al., 2003).

To determine the identity of the protein detected in the extracellular extract by SDS-PAGE, a screening was done in the NCBI database (National Center for Biotechnology Information), since the *K. marxianus* DMKU3-1042 genome was recently sequenced (Lertwattanasakul et al., 2015). The BlastP analysis allowed us to recognize 8 nucleotide sequences with probable coding for lipases in the genome of the yeast; these genes are distributed in 8 of the chromosomes that structure the *K. marxianus* genome. Thus, the genes encoding the LIP2, LIP3 and LIP5 lipases are located on chromosome 1; the genes of the ATG15 and YOR059C lipases are located on chromosome 3, while the YDR444W gene is situated on chromosome 4; finally, the YJR107W and ROG1 genes of the putative lipases of *K. marxianus* are located on chromosome 6.

The two putative lipases with molecular sizes approximating those detected in the SDS-PAGE gel of the extracellular extracts from *K. marxianus* L-2029, are the *pYJR107W* and *pLIP2* lipases (44,526 and 44,682 KDa, respectively). The general objective of our working group is to study each of the putative lipases from L-2029 strain, and we have determined that the only lipases with peptide signal are the *pLIP3* and *pYJR107W* putative lipases (data to be published). Thus, we deduced that the lipase responsible for the enzymatic activity detected from avocado oil induction was the protein encoded by the YJR107W gene.

There is little reported information related to the characteristics of the *pYJR107W* lipase, although homologous proteins to the putative lipases found in the database from *K. marxianus* have been described in other yeasts. There is evidence of the existence of the *pYJR107W* lipase in *S. cerevisiae*, named *Lih1* (Galibert et al., 1996); this lipase has a 26% identity and a 44% similarity with the *Lip2* enzyme from *Y. lipolytica*, which suggests that all three lipases could be derived from a common ancestor (Meunchan et al., 2015).

5 Conclusion

In this work, we confirmed that avocado oil is a good inducer for the synthesis of lipases, derived from its high content of long chain fatty acids, mainly oleic acid, which promoted the synthesis of the *pYJR107W* lipase from *K. marxianus* L-2029, which in turn is the responsible enzyme for the extracellular lipase activity for this yeast. We will be focusing our studies on the potential applications of this lipase in the future.

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