



Assessment of lipolytic activity of isolated microorganisms from the savannah of the Tocantins

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ABSTRACT. Current study assesses the biodiversity and selects lipase-producer microorganisms with industrial interest, from the savannah of the state of Tocantins, Brazil. Seventeen pequi microorganisms (*Caryocar brasiliense*) were isolated in the decomposition stage and 35 microorganisms were retrieved from the soil fraction under the collected pequi. *Yarrowia lipolytica* strain was used as positive control in all assays. The 52 strains were subjected to tests in a solid medium with Tween 20 for checking halos formed by crystals, indicating lipase production by inoculated strains. Another test to confirm lipase producers was conducted in microplates with liquid medium and enriched with p-nitrophenyl palmitate (pNPP) monitored at 410 nm. The AS16 and AP5 strains showed the highest activity for test conditions, namely, 0.072 and 0.067 U mL⁻¹ respectively. Rates were higher than the lipase activity of *Yarrowia lipolytica* (0.052 U mL⁻¹), a reference strain in current assay.

Keywords: lipase, *Caryocar brasiliense*, *Yarrowia lipolytica*, p-nitrophenyl palmitate.

Avaliação da atividade lipolítica de microrganismos isolados do Cerrado tocantinense

RESUMO. O atual estudo avalia a biodiversidade e selecionou microrganismos produtores de lipases de interesse industrial, a partir do cerrado tocantinense. O principal objetivo deste trabalho é avaliar a biodiversidade e selecionar microrganismos do cerrado tocantinense, produtores de lipases de interesse industrial. Foram isolados 17 microrganismos de Pequi (*Caryocar brasiliense*) em estágio de decomposição e também 35 microrganismos provenientes da fração do solo que se encontrava abaixo do pequi coletado. Foi utilizada uma cepa de *Yarrowia lipolytica* como controle positivo em todos os ensaios. Todas as 52 cepas foram submetidas a testes em meio sólido, utilizando Tween 20 para a verificação da presença de halos formados por cristais, indicando produção de lipase pelas linhagens inoculadas. Outro teste para confirmação dos produtores de lipase foi realizado em microplacas contendo meio líquido e enriquecido com pNPP (Palmitato de p-nitrofenila), monitorado a 410 nm. As cepas AS16 e AP5 apresentaram as maiores atividades para as condições de ensaio a que foram submetidas: 0,072 e 0,067 U mL⁻¹ respectivamente, sendo estes valores maiores que a atividade lipolítica da *Yarrowia lipolytica* (0,052 U mL⁻¹), considerada como cepa referência para este trabalho.

Palavras-chave: lipase, *Caryocar brasiliense*, *Yarrowia lipolytica*, Palmitato de p-nitrofenila.

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are industrially important enzymes due to their several applications (MAHADIK et al., 2002). Lipases catalyze a variety of reactions such as partial or complete hydrolysis of triglycerides and esterification, transesterification and interesterification of lipids (COLLA et al., 2010). Recent interest in the production of lipase is associated to their application as additives in food products (flavor modification), fine chemicals (ester synthesis), detergents (hydrolysis of fats), wastewater treatment (decomposition and removal of greasy compounds), cosmetics (removal of lipids), pharmaceuticals (digestion of fats and oils in

foods), leather processing (removal of lipids from animal hides and skins) and biomedical tests (blood triglycerides) (BURKERT et al., 2004). Further, lipases may be applied in the field of bioenergy, especially in the production of biodiesel, which is becoming a booming sector owing to increasing demands of renewable energy worldwide (COLLA et al., 2010).

Microorganisms with a potential for lipases production may be found in different habitats, including waste vegetable oils, dairy products, oil contaminated environment, seeds and spoiled food (SHARMA et al., 2001). In fact, Nature provides a remarkable potential for identifying new sources of lipases with novel properties, since lipases derive from enzyme classes widely used in biotechnological

applications and in organic chemistry (GUPTA et al., 2004). Due to their variety of habitats, microorganisms usually produce several types of lipases with different specificities according to the use of substrate and for the best pH and temperature (JAEGER; EGGERT, 2002).

Current biocatalysts are actually a small fraction of what may in fact exist. Only about 1% of the existing microbial diversity may be grown when standard methodology is used. Brazil has a territorial advantage that makes extremely attractive the screening process for new enzymes in the use of catalytic processes. The catalyst complex of a wild microorganism may be evaluated through efficient selection methodologies and/or screening for highly interesting enzymes. The term 'selection' is used for techniques that enhance growth or survival of organisms that exhibit interesting enzymatic functions. However, screening procedures involve the application of a fast, very sensitive and specific system that identifies positive variations. Further, 96-well microplates tests are quite relevant because they provide quantitative results and may be automated by using robotic systems and microplate readers for data retrieval. It is evident that screening methodology should be planned and adapted to find the enzymatic activity of interest (OLIVEIRA; MONTOVANI, 2009).

The selection of lipolytic microorganisms is justified by a climate of high temperatures in the state of Tocantins, Brazil, where microorganisms that produce thermostable enzymes that ensure great interest in industrial applications may be extant. Current assay establishes a culture collection of microorganisms applicable to biofuels and bioremediation and other processes. The method provides a confirmation of the production of lipase by microorganisms in solid medium, by growing them in a liquid medium with a specific substrate that generates a colored product by the enzyme's activity.

Material and methods

Collection site

Microorganisms were selected by collecting pequi fruits (*Caryocar brasiliense*) in a stage of decomposition, coupled to a soil fraction collected beneath the collected pequi. Pequi fruits were collected near the town of Gurupi, Tocantins States, Brazil, at 11° S and 49° W, altitude 308 m. Samples were added to 50 mL flasks containing 50 mM phosphate buffer, pH 7.0. Flasks were conditioned in thermal boxes with ice for transport and transferred to a cold room at 5°C.

Isolation of bacteria and yeasts

The isolation of bacteria and yeast was performed in a NYSM medium, with 8 g of nutrient broth; 1 g yeast extract; 1 g monobase potassium phosphate (KH_2PO_4); 10 mL of a solution of mineral salts (micro-elements); 20 g agar and pH adjusted to 7.0 for 1 L of the solution. Further, 50 μL of samples collected on the field and spread by glass beads were transferred to Petri dishes and incubated at 30°C in a bacteriological buffer. Yeasts and bacteria colonies up to 7 days of incubation were isolated after 24h. A strain of *Yarrowia lipolytica*, provided by the Laboratory for Ecology and Biotechnology of Yeast (ICB/UFMG), and frequently quoted in specialized literature as a lipase-producing substance, was used in all tests as positive control.

Screening of lipase-producing bacteria and yeast

Two methods were employed to screen lipolytic bacteria and yeast. The first method was based on a methodology which consisted in transferring isolated colonies on petri dish containing medium supplemented with 1% (v v^{-1}) Tween 20 (peptone 10.0 g L^{-1} , NaCl 5.0 g L^{-1} , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g L^{-1} , agar 15 g L^{-1} and pH adjusted to 7.4) to verify the presence of halos formed by crystals, indicating lipase-producing strains (SIERRA, 1957). The second method consisted in transferring isolated colonies in 24-well microplates containing the same liquid medium enriched with 100 μL of 1mM *p*NPP (*p*-nitrophenyl palmitate). The transferring of colonies in the two methods was with the aid of a sterile toothpick and that square grid below of the petri dishes. Both experiments were incubated at 30°C for 24h. After this period, a solution of 10% sodium bicarbonate was added to the 24-well microplates after this period.

Lipase-producing microorganisms cultivation

The selected yeasts and bacteria were grown in 250 mL Erlenmeyer containing 100 mL of medium (peptone 10.0 g L^{-1} ; NaCl 5.0 g L^{-1} ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g L^{-1} ; Gum Arabica 0.2 g L^{-1}) supplemented with 2% glucose and olive oil at 2%. The cultures were incubated at 30°C and stirred at 100 rpm for 48 hours, while cells were centrifuged at 4000 rpm for 10 minute. The sediment was recovered in a solution of 50% glycerol and stored at -80°C. The supernatant was subjected to the enzymatic activity test.

Enzymatic assay

Lipase activity was determined with substrate *p*-nitrophenylpalmitate (Sigma, St. Louis, USA) at a

final concentration of 0.3 mg mL^{-1} was dissolved in isopropanol and mixed with 9 mL of 0.05 M sodium phosphate buffer (pH 7.0) containing Gum Arabica (0.1%) and Triton X-100 (0.6%). The reaction was carried out at 30°C by adding $100 \mu\text{L}$ of enzyme extract to $900 \mu\text{L}$ of substrate solution for 10 minute. Further, $1000 \mu\text{L}$ of sodium bicarbonate 10% were added in test tubes prior to measurement of the optical density at 410 nm (WINKLER; STUCKMANN, 1979). One unit of enzyme activity was defined as the amount of enzyme that released $1 \mu\text{mol/min}$ of *p*-nitrophenol (*p*NP) under standard assay conditions for milliliter of enzyme extract.

Results and discussion

Reactions in biotechnological processes involving biocatalysts should be carried out without organic solvents, since the later may inhibit enzymes, and require a careful treatment of waste at the end of the procedure. The use of surfactants in food, such as Tween (fatty acid ester of a polyoxyalkylene derivative sorbitan) for identifying lipase-producing isolates is justified by the fact that it is a biodegradable surfactant contributing towards the development of an environmentally friendly technology (FELTES et al., 2010).

Seventeen bacterial strains and 35 yeast strains from decomposed pequi fruits (*Caryocar brasiliense*) (identified as AP1 - AP15) were isolated during the research, coupled to soil fragments beneath the pequi fruits (identified as AS1 - AS35). The strains were tested with Tween 20, resulting in the selection of lipase producing 6 bacterial strains and 20 yeast strains. Table 1 shows isolates with the most promising results (Table 1).

Table 1. Isolates with more promising results.

Isolates	Microorganism	Isolates	Microorganism
AP5	Bacterium	AS21	Yeast
AS12	Yeast	AS23	Yeast
AS16	Yeast	AS28	Yeast
AS18	Bacterium	AS31	Yeast
AS19	Bacterium	AS35	Yeast
AS20	Yeast		

The lipase-producing microorganisms could be easily identified due to the appearance of crystalline halo in plates, possibly caused by insoluble calcium salt of the fatty acid released by lipase (Figure 1).

Experiments conducted in Petri dishes enhanced a higher sensitivity to be carried out both directly and quantitative dosage, even when there is a small amount of lipase (SANDOVAL;

MARTY, 2007). The formation of an opaque area around the colonies while the rest of the medium remains clear demonstrates that the crystalline aspect is not simply due to any possible Tween interference with any other component of the medium (SIERRA, 1957).

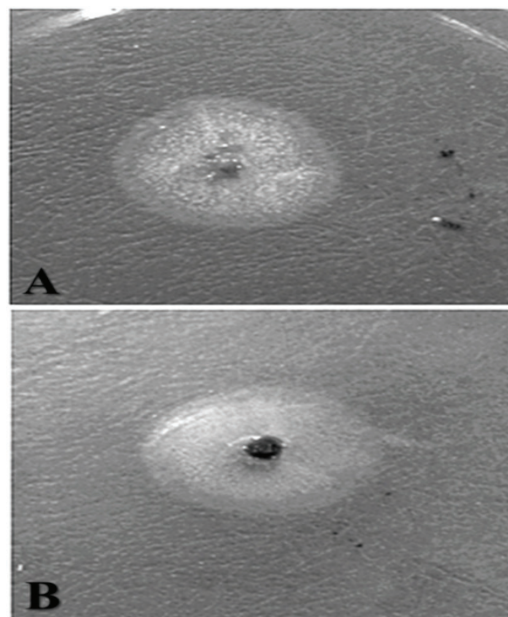


Figure 1. Crystalline halo formation by isolates inoculated (A) isolates AS21 (B) isolates AS31.

The wells with a yellowish color indicated that it was a lipase-producing microorganism due to its release of *p*-nitrophenol during the hydrolysis of *p*-nitrophenyl palmitate. We observed the formatting of free fatty acid pellicle on liquid media after 48 h of cultivation and this may be indicative of hydrolysis, besides the formation of the characteristic yellow color of *p*-nitrophenol released. Results were confirmed by the inoculated isolates on a solid medium, which showed the lipolytic halo formed by crystals due to Tween 20 hydrolysis (Figure 2).

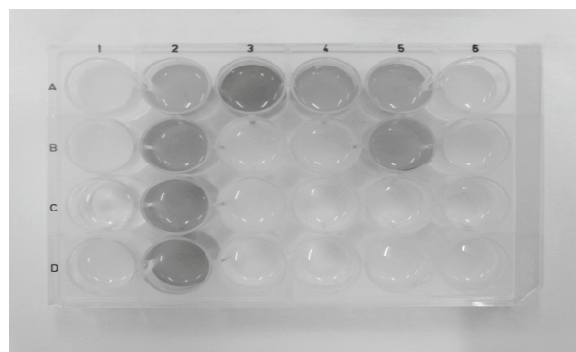


Figure 2. Microplates after release of *p*-nitrophenol indicating lipase production. Positive control (1A and 1B); negative control (1C, 1D); positive isolates (2A, 2B, 2C, 2D, 3A, 4A, 5A, 5B).

Fatty acid ester are hydrolyzed by esterases and lipases, but esterases hydrolyze the ester bonds of the water's soluble fatty acid ester with short-chain acyl groups ($\leq C_8$), whereas lipases hydrolyze long-chain acyl groups ($\geq C_{10}$) (WINKLER; STUCKMANN, 1979). The same authors did not detect any activity with *p*-nitrophenyl caprylate (C_8), *p*-nitrophenyl laurate (C_{12}) and *p*-nitrophenyl palmitate as substrate of esterase.

Quantitative analysis is highly relevant to compare the activities of various lipolytic isolates. There is no single universal method for lipase assay. The choice of a particular method depends on the user's own particular requirements. For the assay of any enzyme, sensitivity, substrate availability and easiness in the procedure have to be considered (HENDRICKSON, 1994). Speed and sensitivity for fatty acids esters tests may be increased by using colorimetric methods (LOWRY; TINSLEY, 1976). A specific substrate (*p*-nitrophenyl palmitate) was used in the method in current assay, which, after hydrolysis, may be easily converted into a final yellowish product (*p*-nitrophenol). Lipase activity in the supernatant of cultures was positive after the preliminary tests. The hydrolysis of fatty acids esters was monitored by the spectrophotometric method at 410 nm (WINKLER; STUCKMANN, 1979). Enzyme activity is expressed as μmol of *p*-nitrophenol released per minute (BECKER et al., 1997).

Although tributyrin may also be employed to select lipolytic microorganisms, vegetable oils, such as olive oil, may be emulsified and used as initiators in lipases production, since they are a substrate in the selection processes (CARDENAS et al., 2001).

A strain of *Yarrowia lipolytica*, kindly supplied by the Laboratory for Ecology and Yeast Biotechnology of ICB/UFMG was grown on a slanted tube on the same culture medium used for the cultivation of previously selected positive isolates. The strain was maintained at 30°C to ensure its survival. *Yarrowia lipolytica* was used as positive control in all assays (Figure 3).

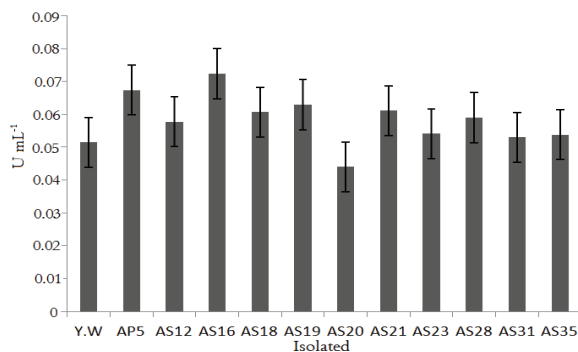


Figure 3. Comparison of enzymatic activity of 11 selected strains with increased activity in relation to *Yarrowia lipolytica* (Y.W).

Figure 3 presents lipase activities per mL of the 10 selected strains as the greatest lipolytic producers lipolytic by Tween Test and by the Microplates Test. The strains were designated according to their origin: AP from *Caryocar brasiliense* and AS from the soil fraction beneath the collected pequi fruits.

Strains AS16 and AP5 provided the highest activities for test conditions, or rather, 0.072 and 0.067 U mL⁻¹, respectively. These activities are also higher than the activity of the *Yarrowia lipolytica* strain (0.052 U mL⁻¹) taken as a reference strain in current assay. In fact, the yeast has proved to be one of the most promising strains in lipase production (CHIRVASE et al., 2010). Strains AS12, AS20, AS23, AS31 and AS35 were below average lipolytic enzyme (0.058 U mL⁻¹). The smallest lipase activity was displayed by strain AS20 (0.044 U mL⁻¹).

Lipolytic enzyme activity rates were obtained after 48 hours of incubation under the conditions described above. The highest lipase activities ever recorded were obtained with *C. cylindracea* NRRL Y-17506 and *G. candidum* NRRL Y-553 (0.46 and 0.52 U mL⁻¹, respectively) after 168h fermentation (D'ANNIBALE et al., 2005). Olive oil was used in current assay to induce the lipase production phase. According to Carvalho et al. (2005), enzyme rates in the hydrolysis of olive oil have been widely used for comparison and selection of strains producing lipase. Rates may vary significantly since they depend on the type of fermentation, the composition of the culture medium and also on other variables in the fermentation process, such as pH, temperature of incubation and presence of inducers of the lipase synthesis, such as vegetable oils (POKORNY et al., 1997).

Lipases and their broad substrate specificity, coupled to the diversity of reactions catalyzed by these enzymes, make it difficult to define a universal test for lipase activity. Since hydrolytic and synthetic activities are not always correlated, specific screening methods for synthesis activity of lipases are needed (KIM et al., 2006). In current assay, we proposed a confirmation method for the screening of lipase-producing microorganisms. Hence, we need to assay the enzyme directly from the culture broth by rapid and sensitive colorimetric methods since screening of lipases with synthetic activity in organic solvents has been proposed and validated by recent research (KIM et al., 2006; TENG; XU, 2007).

Conclusion

The enzymatic assay using *p*-nitrophenyl palmitate was positively undertaken in the selected strains by the above-mentioned tests. Rates for lipase activity were

approximately between 0.053 and 0.072 U mL⁻¹ after 48 hours. The literature mentions higher rates for lipolytic enzyme activity, but the production time is 96 hours or more. However, in current test conditions, one strain of bacteria and nine yeast strains were obtained at a higher level of enzymatic activity than that of *Yarrowia lipolytica* considered by literature as a promising lipase producer.

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