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NITROGEN SOURCES ON TPOMW VALORIZATION THROUGH SOLID STATE FERMENTATION PERFORMED BY *Yarrowia lipolytica*

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Abstract - This manuscript reports the valorization of two-phase olive mill waste (TPOMW) as raw material and carbon source for solid state fermentation using *Yarrowia lipolytica* as biocatalyst. Due to its chemical characteristics, a combination of different raw materials (TPOMW and wheat bran, WB) was evaluated and two distinct nitrogen sources were applied as supplementation for lipase production. A TPOMW/WB ratio of 1:1 and supplementation with ammonium sulfate was chosen as the best condition. The productivity in 24 h reached 7.8 U/g*h and, after four days of process, only decreased about 35%. Process pH ranged from 5.5 - 5.9, remaining in an acid range. Thus, the successful use of TPOMW, a watery solid by-product with high content of lipids, as raw material for *Yarrowia lipolytica* growth and lipase production provided an environmental friendly alternative to valorize such waste.
Keywords: *Yarrowia lipolytica*; Solid state fermentation; TPOMW valorization; Nitrogen.

INTRODUCTION

The olive oil industry is characterized by its great environmental impact due to the production of a highly polluted wastewater and/or a solid residue, olive skin and stone (olive husk), depending on the extraction process. The two-phase centrifugation system for olive oil production, developed to drastically reduce the water consumption, produces olive oil plus a semi-solid residue, known as two-phase olive mill waste (TPOMW). The resulting solid waste corresponds to 800 kg per ton of processed olives, containing 2.5–3.5% of residual oil and about 60% of water (Darvishi, 2012). Thus, more research is needed on the development of new bioremediation technologies and strategies for olive mill wastes, as well as its valorization by microbial biotechnology.

Yarrowia lipolytica is a non-conventional yeast, which has been considered an industrial workhorse because of its ability to produce important metabolites, intense secretory activity and efficient system for genetic engineering transformation (Coelho *et al*., 2010). Strictly aerobic, this yeast is often isolated from environments containing hydrophobic substrates, leading to the efficient use of alkane, fatty acids and triglycerides. Moreover, *Y. lipolytica* grows as a mixture of budding yeasts and long hyphae depending on the environmental conditions, being a useful model for dimorphism studies (Cervantes-Chávez and Ruiz-Herrera, 2007). *Y. lipolytica* is used

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now as a model organism for several academic studies, making this dimorphic yeast significant for biotechnological applications. Properties like intracellular accumulation of oil, secretion of large amounts of organic acids, high capacity for enzyme secretion and its acceptance as generally recognized as safe (GRAS) focus the interest on this yeast as a potential producer of basic commodities (Barth and Gaillardin, 1997; Papanikolaou and Aggelis, 2010).

Lipases (E.C. 3.1.1.3), defined as triacylglycerol acylhydrolases, are ubiquitous enzymes that can catalyze the hydrolysis of the ester bond of long-chain fatty acids into fatty acids and glycerol and also the reverse reaction when in organic solvents with low-water content. Besides hydrolysis, lipases can catalyze a wide range of reactions including inter-esterification, alcoholysis, acidolysis, esterification and aminolysis (Bora *et al*., 2013). These are one of the most important classes of enzymes with industrial applications such as in fine chemistry, detergent, textile, and food additives (Fickers *et al*., 2011). Lipases are naturally produced by plants, animals and microorganisms; however, enzymes produced by microorganisms have received increasing attention. Microbial lipases present a great variety of catalytic activities, possible high yields, easy genetic manipulation, a regular supply due to the absence of seasonal fluctuations and the rapid microorganism growth rate (Hasan *et al*., 2006).

Lipases are one the most important biocatalysts secreted by *Y. lipolytica*. There are several papers reporting three extracellular lipases that present different substrate specificities, Lip2, Lip7, and Lip8 (Pignède *et al*., 2000; Fickers *et al*., 2011; Najjar *et al*., 2011). Among these, Lip2 attracts more attention because it is the main secreted extracellular lipase and presents high hydrolysis, transesterification and esterification activities (Yan *et al*., 2013). Lipase production is induced by fatty acids derived from fat breakdown, like oleic, elaidic and linoleic acids. Inductors of lipase secretion when used as carbon source were also reported in the literature (Najjar *et al*., 2011). Although it is well known that the production of lipolytic enzymes is modulated by carbon and nitrogen sources, there is a lack of information about the role of specific substrates in lipase synthesis (Almeida *et al*., 2012). Microbial lipases are usually produced in submerged fermentation processes. However, solid state fermentation (SSF) seems to be an interesting alternative to microbial enzyme production due to the possibility of using agro-industrial residues as the source of carbon and other nutrients, as well as support for microorganism growth (Rigo *et al*., 2010). Industrial enzymes have been successfully produced at the commercial level by SSF. Nevertheless efforts to obtain higher production with lower costs have to be continued (Thomas *et al*., 2013).

The choice of nitrogen source, organic or inorganic, has proven to be an important parameter for the improvement of lipase production. Playing a different role in enzyme synthesis, organic sources can supply cells with growth factors and amino acids, while inorganic sources can be quickly used (Almeida *et al*., 2012). Ammonia for example can be used not only as nitrogen source, but also as pH regulator during the induction stage. The ammonium ion released into the fermentation broth has a deep impact on cell growth, expression and degradation of foreign protein (Yu *et al*., 2013). Yeast extract and hydrolyzed casein are usually great nitrogen sources for lipase stimulation (Fickers *et al*., 2004; Moftah *et al*., 2012; Veerabhadrappa *et al*., 2013). Turki *et al*. (2010) reported valuable production of extracellular lipases by the yeast *Yarrowia lipolytica* LgX64.81, using a defined medium supplemented with tryptone. According to the authors the lipase production was higher using the medium enriched with tryptone than that using a complex medium, commonly employed for yeast cell growth.

The agro-industrial wastes used in SSF can differ in many properties, like the chemical nature of the constituents and water retention capacity, affecting directly the microorganism growth. TPOMW is a watery solid by-product of the olive oil industry that may represent an environmental problem due to its highly polluting organic load (Lama-Muñoz *et al*., 2013). At the same time, it represents an inexpensive and unexploited source of nutrients, rich in lipids that can act as inducers and make this waste a great alternative for lipase production in SSF. Although the presence of aromatic compounds, such as tannins and polyphenols, and the low nitrogen content in TPOMW generally inhibit microorganism growth (Muktadirul *et al*., 2013; Reina *et al*., 2013), the combination with other wastes can reduce the concentration of phenolic compounds and counteract the lack of nitrogen, improving the production of biocatalysts (Salgado *et al*., 2013).

WB is one of the most popular agro-industrial residues and is well accepted for microbial production of important enzymes applying SSF. Since it is a by-product of the milling of wheat into white flour, WB usually accounts for 14–19% of the grains' weight (Maes and Delcour, 2002; Demir and Tari, 2014). This residue is also an inexpensive carbon source that has already been reported as a good inductor for lipase activity (Romdhane *et al*., 2010).

In this study, the effect of different solid state media for lipase production by *Yarrowia lipolytica* IMUFRJ 50862 was investigated. Two-phase olive

mill waste (TPOMW) and wheat bran (WB) were used as raw material and carbon source and two distinct nitrogen sources were applied as supplementation. The best ratio between the two raw materials for lipase production was also tested.

MATERIALS AND METHODS

Microorganism

A wild-type strain of *Yarrowia lipolytica* (IMUFRJ 50682), isolated from an estuary of Guanabara Bay in Rio de Janeiro, Brazil (Haegler *et al*., 1981), was used in this work. The yeast was maintained and propagated in YPD medium containing (w/v): 1% yeast extract, 2% peptone and 2% glucose. For lipase production, cells previously grown for 72 hours under orbital agitation (160 rpm, 28 °C) were inoculated in SSF medium.

Raw Materials

Two agro-industrial residues, TPOMW and WB, were tested as substrate. TPOMW was collected from olive oil mill industries in the northern region of Portugal. This residue usually has high moisture content, in this case 73%, so it was necessary to dry it prior to the fermentations. After the required drying, the solid matrix was milled to a particle size lower than 2 mm. WB, collected from industries in the area (Braga, Portugal), was also dried, despite its low moisture content, due to the necessity of moisture standardization.

Solid State Fermentation

The fermentation was carried out in 250 mL Erlenmeyer flasks with 10 g of dry solid substrate with different ratios of the two residues. For lipase production using a mixture of raw materials, solutions of urea and ammonium sulfate were tested as nitrogen supplementation; they were also used to adjust the fermentation moisture level to 53% (wet basis). Solutions and substrates were sterilized separately at 121 °C, for 15 min. The sterilized solid substrate was inoculated with 1 mL (10^8 cells) of cell suspension and the necessary volume of nitrogen solution was added. After mixing, the fermentation flasks were incubated at 28 °C for 96 hours.

Crude Lipase Extraction

The crude lipase was extracted with a solution composed of 1% NaCl and 0.5%Triton X-100 at 4 °C in a solid/liquid ratio of 1:5. After 2 hours of agitation in a rotary shaker (200 rpm, 28 °C), the raw extract was obtained by pressing the mixture and subsequent filtration through Whatman No. 1 filter paper.

Lipase Assay

Extracellular lipase was measured by a colorimetric method using p-nitrophenyl-laurate (pNPL) as substrate. The pNPL was prior dissolved in dimethyl sulfoxide (DMSO) and potassium phosphate buffer, 50 mmol L^{-1} (pH 7.0). After incubation for 15 min at 37 °C the reaction was stopped by adding 2 mL of acetone, and the p-nitrophenol released was detected at 410 nm in a spectrophotometer (Gomes *et al*., 2011). One unit of activity was defined as the amount of enzyme that produces 1 µmol of p-nitrophenol per minute under the assay conditions.

Statistical Analysis

The results are presented on a mean and standard error basis, using Tukey’s test ($p < 0.05$) with the aid of the program Statistica® 7.0.

RESULTS AND DISCUSSION

Raw Materials Characterization

TPOMW and WB have distinct characteristics that make each of them suitable for SSF applications (Table 1). Considering TPOMW characteristics like high water content (≈75%) and aromatic compounds (expressed as total phenols), the use of such a raw material as the sole carbon source in SSF may be unfeasible. Unlike TPOMW, WB has a high nitrogen content that results in a low C/N ratio. Relatively high nitrogen concentrations and lower C/N ratios are better conditions for yeast growth, and typically required for lipase production by fungi (Lima *et al*., 2003; Cheirsilp and Louhasakul, 2013).

Table 1: Composition of TPOMW and WB.

Parameters	TPOMW[a]	WB[b]
Moisture (%)	75.31±0.14	20.0±3.4[1]
C (%)	51.66±1.54	54.3±4.2[1]
N (%)	0.86±0.19	2.6±0.3[1]
C/N	60.06	17.3-24.9[1]
Total phenols (mg/g)	2.57±0.04	-
Protein (mg/g)	0.30±0.03	15.3±0.8[2]
Lipids (mg/g)	102.46±0.04	0.3±0.0[1]
Klason lignin (g/100g)	58.16±0.41	5.0[3]

[a] Adopted from Salgado *et al*., 2013.
[b1] Adopted from Jang *et al*., 2000.
[b2] Adopted from Seguin *et al*., 2012.
[b3] Adopted from Palmarola-Adrados *et al*., 2005.

Thus, a mixture of both solids may represent a new strategy for application of non-useful waste such as TPOMW to obtain bioproducts with high added value.

Solid Matrix Composition

For the choice of the culture medium, both WB and TPOMW were tested as raw materials and the lipase production was evaluated after 24 hours of fermentation (Table 2). As expected, when used singly, TPOMW did not permit a suitable growth of *Y. lipolytica* (data not shown), which directly affected the lipase production by the yeast. This result is probably a consequence of the high amount of aromatic compounds, which are toxic for the cell. In addition, this residue has small particles that result in substrate stickiness, leading to poor water retention capacity. With a low capacity for moisture content, any high level can cause a porosity decrease, oxygen transfer limitation and poor growth (Moftah *et al*., 2012).

Table 2: Lipase activity in different solid matrix compositions.

Culture medium	Activity (U/g)
TPOMW	0.79 ± 0.15
WB	14.08 ± 0.78
TPOMW and WB (1:1)	9.69 ± 0.45

On the other hand, the combination between TPOMW and WB proved to be a good alternative for lipase production by the strain *Y. lipolytica* 50682. When used as sole carbon source, WB also showed potential for microorganism growth and the lipase activity was slightly superior (around 30%).

Considering that TPOMW is produced in large amounts and that a proper disposal of this material is not commonly realized, the utilization of viable management strategies is imperative (López-Piñeiro *et al*., 2008). The olive oil industry is very important in the Mediterranean, with a cultivated area of about 8.2 Mha and production of 0.8 ton of solid waste per ton of processed olives. Numerous investigations concerning the treatment of TPOMW have been made, but the solution of an adequate disposal has not yet been found (Federico *et al*., 2010).

To bypass the growth inhibition problem, the use of a second residue proved to be a good alternative. WB characteristics, such as high water retention capacity and high nitrogen level, ensured its potential for SSF (Javed *et al*., 2012). At the same time, the lipid content of TPOMW is higher and makes this residue interesting for lipolytic enzyme production. There are many researches in the SSF area showing the great potential of integrated use of wastes. Different residues can supply several nutrients for the fermentation, causing a synergic effect on enzyme production (Cordova *et al*., 1998; Babu and Rao, 2007; Santis-Navarro *et al*., 2011; Salgado *et al*., 2013). In the results reported herein, wastes combination showed potential for lipase production, since the limitation caused by TPOMW was apparently overcome, providing a new possibility for TPOMW utilization, adding value to the olive mill waste.

Relevant to this new strategy, some works deal with different materials, like Cordova *et al*. (1998) that increased the lipase activity produced by two species of *Rhizomucor* genus by using an equal mixture of olive oil cake and sugarcane bagasse. The authors first tested only sugarcane bagasse for lipase production and the maximal activity obtained was 4.99 U/g. But when the olive oil cake was added to SSF, lipase production reached 79.60 U/g. The authors believed that the olive oil cake had the appropriate precursors necessary for lipase production.

Sun and Xu (2008) evaluated various agro-products including rice, corn flour, wheat flour, barley, oat, wheat bran, rice bran, wheat husk and soybean powder for lipase production by *R. chinensis*. Among all residues tested, wheat bran and wheat flour gave the highest activity. Since wheat flour and wheat bran both favored lipase production, the authors decided to experiment different residue combinations, and after some tests the ratio 3/2 (w/w) of wheat flour and wheat bran was chosen as the base medium for subsequent experiments. Coradi *et al*. (2012) also perceived that the combination of two residues was the most suitable for lipase production by *T. harzianum*. The highest enzyme activity of 4.0 U/g was obtained after 96 h of fermentation, in a medium containing castor bean and sugarcane bagasse. When the authors used both residues singly, the activities decreased despite the same cultivation conditions. For them, more important for lipase production than lipid content and nitrogen concentration, is the interaction between these variables.

It is important to note that, until now, no process optimization was carried out, this being a preliminary experiment to demonstrate the viability of using a mixture of TPOMW and WB. Thus, lipase production in SSF using these raw materials has to be studied to increase its productivity.

Lipase Production in SSF

Since the mixed composition was chosen as the solid matrix for SSF, the influence of different proportions of TPOMW and WB on lipase production was evaluated. For this initial test, a 0.5% (w/v) urea

solution was used as nitrogen supplementation and for the adjustment of moisture content. A total of 10 g of solid matrix were used with the following waste quantities (TPOMW/WB): 1:1; 1:3 and 3:1. The fermentations were carried out for 96 hours and the lipase production was evaluated daily.

As shown in Figure 1, lipase production was higher in the medium with equal proportions of each raw material, reaching 94 U/g of lipase activity after 24 hours of fermentation. The medium with a minimum of TPOMW also showed its best result in the first 24 hours of fermentation (15.5 U/g), different from the medium with maximum of TPOMW that had its best lipase production only after 72 hours of fermentation (30 U/g). This result demonstrates how lipase activity is affected by lipid-rich substrates; even with the presence of phenolic compounds that could inhibit fungal growth, the medium with 75% of TPOMW also produced an expressive lipase activity. Several extracellular elements that affect the expression of lipase are described in the literature, but the carbon source seems to be a critical one. These enzymes are usually produced in the presence of lipid, such as plant seed-oils (triacylglycerols), free fatty acids, surfactants, bile salts, and glycerol, that are included in the nutrient medium as inducers (Kebabci and Cihangir, 2012). Lip2, for example, is strongly affected by oleic acid, the end product of the hydrolysis of its preferred substrate (Fickers *et al*., 2011).

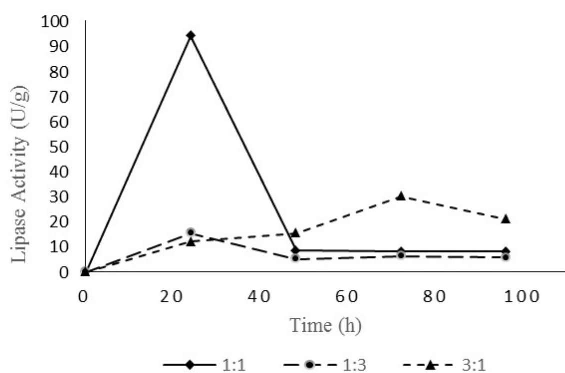

Figure 1: Lipase activity of three different raw materials ratios.

However, it is possible to observe a delay in lipase production, probably due to the high C/N ratio. It is known that the lipase produced by yeasts usually stays internally bound to the cell wall and it is only secreted to the culture medium when the carbon source becomes scarce, i.e., in the transition to diauxie (when more than one substrate is used) or to the stationary phase. In a medium with a high C/N ratio there is a surplus of carbon sources that can retard the lipase secretion (Pereira-Meireles *et al*., 2000). Similarly, a strain of *Yarrowia lipolytica* showed a delay in the transition between the two phases when an oleic acid rich medium was used. At the early stage of the culture when oleic acid was abundant, lipases remained cell-bonded, which were sufficient to support yeast growth. The enzymes were only released into the culture medium as the oleic acid concentrations decreased (Fickers *et al*., 2004).

Effect of Nitrogen Source on Lipase Production

The nitrogen source, organic or inorganic, also plays an important role in lipase production. For this reason, the effect of ammonium sulfate on lipase production was evaluated and compared to urea as nitrogen supplementation. The fermentations were performed for 96 hours with a ratio of 1:1 (TPOMW/WB). Lipase activity and pH were evaluated daily.

Ammonium sulfate showed a different production profile when compared to urea. The presence of this mineral source not only increased lipase activity, but also kept levels rising throughout most of the fermentation process, reaching 486 U/g at the end of the process (Figure 2). Similar to previous experiments, urea supplementation promoted a positive effect on lipase production with a peak at 24 hours of fermentation. However, the maximal lipase activity was 145 U/g (Figure 2), almost three fold lower than that obtained with ammonium sulfate. Nitrogen compounds also modulate the synthesis of Lip2 and, according to several authors, organic sources are better for this purpose. While the presence of nitrogen compounds such as casein tryptone, urea and yeast extract usually yields significant lipase synthesis, the opposite occurs when a mineral source is used (Pereira-Meireles *et al*., 1997; Fickers *et al*., 2004). Turki *et al*. (2009) studied the extracellular lipase production by *Yarrowia lipolytica* overproducing mutant under the effect of several organic nitrogen sources. They concluded that enzymatic hydrolyzed casein (tryptone and peptone) had a relevant stimulatory effect on lipase production, suggesting that the enhancement relies most probably on the presence of some specific bioactive peptides that act as lipase inducers. Urea is also reported as a better nitrogen supplementation than ammonium sulfate for lipase production by *Yarrowia lipolytica*. Usually, when this mineral nitrogen is used the lipase production is inhibited (Corzo and Revah, 1999; Imandi *et al*., 2010).

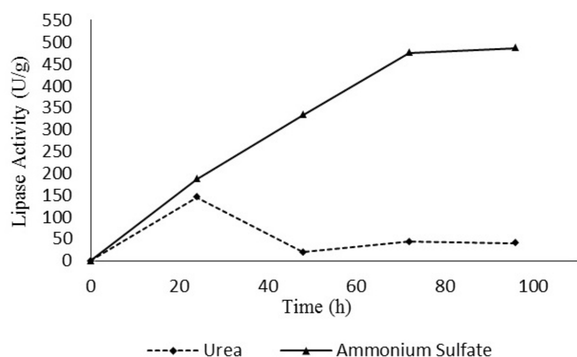

Figure 2: Influence of nitrogen supplementation on lipase activity.

Our results, in disagreement with other researchers, showed the great potential of ammonium sulfate for lipase production by the yeast *Y. lipolytica*. In some level, the conditions offered by the raw materials and ammonium sulfate allowed an improvement of the productivity (Figure 3). The fermentations with ammonium sulfate supplementation not only had higher lipase production than urea, but also presented higher productivity. In the first 24 hours, the productivity reached its best range with 7.8 U/g*h, and after four days of the process only decreased about 35%. This promising result confirms the potential of our experiments since the lipase productivity was higher than most of the productivities reported in the literature (Imandi *et al*., 2010; Moftah *et al*., 2012; Coradi *et al*., 2012; Vaseghi *et al*., 2013; Salgado *et al*., 2013), which is very important for an economically viable process. Although the organic nitrogen sources are generally cited for lipase production, the use of inorganic ones has been studied due to their low cost and to facilitate the subsequent stages of enzyme purification.

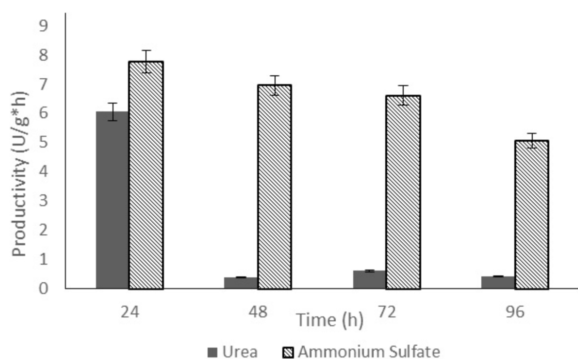

Figure 3: Influence of urea and ammonium sulfate on lipase productivity.

In recent research, the impact of NH_4^+ nitrogen source on lipase production by *Pichia pastoris* was investigated, and the results demonstrated the positive effect of this ion. Ammonium sulfate was added in order to provide the ammonium ion and keep it constant at three levels, 400 mmol/L, 440 mmol/L and 500 mmol/L. The highest lipase activity was obtained at a concentration of 440 mmol after 52 hours, with an increase of 71% considering the control system. The presence of NH_4^+ in the broth also prevented protease production (Yu *et al*., 2013), a fact that can explain the higher maintenance of lipase production all along the fermentation with this nitrogen source.

The use of ammonium sulfate showed a linear increase in lipase production with bath time that provided an almost constant productivity up to 72 h (Figure 3), meaning that no decrease in enzyme activity was observed. On the other hand, the use of urea showed a decreased in lipase activity after 24 h, which led to a low productivity. These results permit one to plan the process strategy to obtain higher productivities.

Kebabci and Cihangir (2012) studied lipase production by three different *Y. lipolytica* strains, evaluating different nitrogen supplementation (proteose-peptone, peptone, yeast extract, casein, urea, ammonium sulfate, ammonium oxalate, ammonium nitrate and ammonium carbonate) with and without olive oil. The authors concluded that addition of ammonium compounds led to the best increase in lipase production. In their case, the combination between ammonium sulfate and olive oil permitted the best condition for enzyme production in an acid pH range.

In the study reported herein, the pH was also evaluated for both nitrogen sources. Similarly, the best pH for lipase production with ammonium sulfate varied in an acid range, between 5.5-5.9 (Figure 4B). When urea was used, the pH profile was different, ranging between 7.0-7.8 (Figure 4A). The optimal pH reported for Lip2 hydrolytic activity was found to be slightly acid, although it can vary depending on the substrates and the experimental conditions (Fickers *et al*., 2011). In addition, the optimum hydrolytic activity of lipases from *Y. lipolytica* is usually obtained between pH 6.0 and 10.0, depending on the strain used (Brígida *et al*., 2013). According to Dominguez *et al*. (2003), low pH in the fermentation broth can be attributed to the release of fatty acids in the presence of lipase, which can explain the better productivity in acid pH.

On the other hand, a pH increase during SSF fermentation can be attributed to the increase in the levels of protease that leads to release of amino acids from degraded proteins (Rigo *et al*., 2010). The yeast

Y. lipolytica can produce large amounts of proteolytic enzymes; among them, alkaline protease (AEP) and acid protease (AXP) are the most common. Alkaline proteases, however, are the major protein secreted, reaching several grams per litre under optimized conditions (Nicaud *et al*., 2002). The secretion of this enzyme can occur in parallel to lipase and it is induced at neutral/basic pH. Although in this work no proteolytic data was available, the pH verified in our experiments can give us some information about the fermentations. In addition, other work in the group using the same strain in SSF demonstrated that, up to 14h of the process there is no detectable protease activity (data not shown).

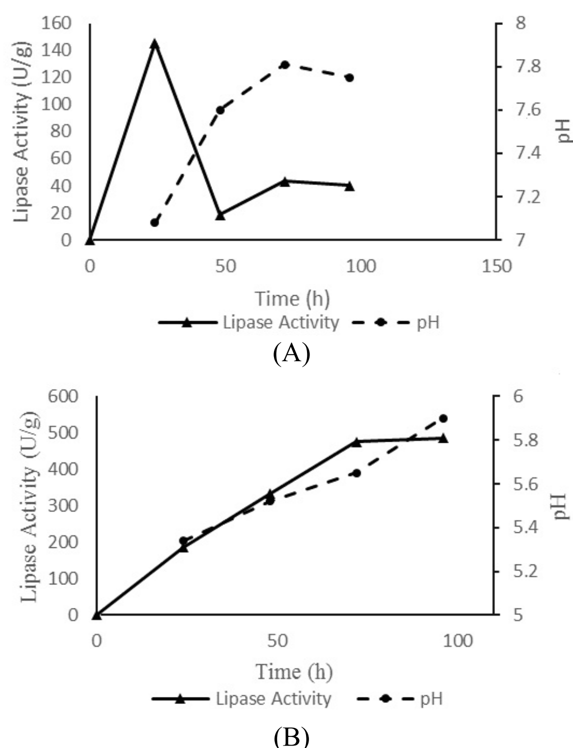

Figure 4: Comparison of lipase activity and pH in the medium with urea supplementation (A) and ammonium sulfate supplementation (B).

Nevertheless, Najjar *et al*. (2011) demonstrated that, independent of the culture medium, the lipase activity was always lost in the late stage of culture when the pH reached alkaline values, suggesting a proteolytic degradation by AEP. Similarly, our results show that, in the medium with neutral/ basic pH, the lipase activity reached its lowest value. In the medium with urea, a greater pH increase (pH 7.0 to pH 7.6) was followed by an expressive decrease in lipase production (Figure 4A). In the medium containing ammonium sulfate, that had higher lipase activity, the pH was not optimum for AEP (Figure 4B). Moreover, protease production is tightly controlled by a combination of environmental stimuli, which includes nutrient availability such as carbon, nitrogen, and sulfur starvation (Gonzalez-Lopez *et al*., 2002). The presence of nitrogen (ammonium ions, amino acids) and sulfur can have a repressive effect on AEP and AXP production (Young *et al*., 1996).

CONCLUSIONS

The yeast *Yarrowia lipolytica* IMUFRJ 50682 showed great potential for lipase production in solid state fermentation using agro-industrial residues. The raw materials TPOMW and WB had complementary effects, offering suitable conditions for SSF when used in equal proportions due to their characteristics concerning toxic compounds, moisture content and C/N ratio. In addition, the supplementation with a mineral nitrogen source enhanced lipolytic activity, reaching the best result of 486 U/g after 96 hours. Ammonium sulfate improved lipase production and the productivity of the process, whereas urea had a less significant effect.

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