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Lipase Production by *Yarrowia lipolytica* in Solid State Fermentation Using Different Agro Industrial Residues

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Lipases (triacylglycerol ester hydrolases, E.C. 3.1.1.3) are very important enzymes, mainly in an underexploited lipid technology and bioindustry, and have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion worldwide enzyme market. On the other side, taking in advance the fact that Brazil generates, annually, thousands of tons of agricultural and agro industrial residues, the bioconversion of these residues for lipase production, as well as other value added products, would point out Brazil a prominent position in the future biotechnology developments. Oil cakes of various residues, obtained from extraction of oils, have been used as support and substrate for fermentative production of lipases, mainly in solid-state fermentation (SSF) technique. This is because their residual oil content serving as inducer for lipase production. Several agricultural residues have been reported to be effective for lipase production and these include brans, oil cakes, bagasse and soybean sludge (to be used as a supplement in SSF). The cottonseed and soybean cakes have attracted increasing attention as abundant and cheap renewable feedstocks and, additionally, it could reduce the human impact on the environment. Thus, the use of cottonseed and soybean cakes as substrates to produce microbial lipase, beyond being a cost-effective process, seems to be technically promising, once these residues have essential nutrient composition for microorganism growth. Regarding microbial lipase production, *Yarrowia lipolytica* is well-known producer of a large variety of bioproducts, but lipase is the most relevant biomolecule produced by this yeast. In this way, the soybean cake and its sludge were used in synergy for lipase production by *Y. lipolytica* in SSF and presented promising results like139±3 U/g in 14h of fermentation, reaching 9.9 U/g*h of productivity. The cottonseed cake was also used as substrate in SSF and reached a very good level of enzyme activity, 102 ± 6 U/g in 28h of fermentation, without use of any supplement.

1. Introduction

Lipases (triacylglycerol ester hydrolases E.C.3.1.1.3.) are one of the most important classes of industrial enzymes .They can catalyze both the hydrolysis and the synthesis of esters from glycerol and long-chain fatty acids. The last reaction occurs only in the presence of water traces. These enzymes, under specific conditions, are also capable to catalyze reversible reactions: interesterification, aminolysis and transesterification reactions. (Jaerger and Reetz 1998). This enzyme has demonstrated a large applicability, mainly in food, cleaning, cosmetic, pharmaceutical and paper processing industries (Long et al., 2009), oil chemical industry and biosurfactant synthesis.

Over the recent years, research on the selection of suitable substrates for fermentative process has mainly been focused on agroindustrial residues, due to their potential advantages. Utilization of agroindustrial wastes provides alternative substrates and may help solving pollution problems, which otherwise might be caused by their disposal (Treichelet al., 2010). The biotechnological process that can add value to agroindustrials residues, converting them into bioproducts by microorganisms, is recognized as solid state fermentation (SSF). This technique consists in using the solid medium as nutrient source and as a

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microorganism growing support. The microorganism growth can occur inside or in the superficial area of the solid. SSF is defined as the fermentation of solids in the absence of free water, however, the substrate must possess enough moisture to support the growth and metabolism of microorganism (Babu and Rao, 2007). The microorganisms, such as bacterias, yeasts and moulds, are recognized as potential extracellular lipase producers (Treichel et al., 2010). Recent researches reported the use of yeasts in SSF as a new area to be discovered and elucidated. *Y. lipolytica* is a very interesting yeast to be used in this type of fermentation. This yeast, besides degrading hydrophobic substrates in a very efficient manner (Coelho et al., 2010), also presents dimorphism and, in this way, they could fix their hyphaes in a solid medium, allowing minimum conditions of survivor. According to *Y. lipolytica* characteristics, the strategy of using this yeast as a lipase producer using SSF and, as consequence, agroindustrial residues as substrates, appears to be a very interesting process to be exploited.

2. Materials and Methods

2.1 Substrate

The cottonseed cake, soybean bran and its sludge were used as substrates, provided by PETROBRAS. These materials were stored at 5ºC and were used without any chemical pretreatment. The cottonseed cake was separated according to particle size separation process, obtaining particles smaller than 1.18 mm.

2.2 Microorganisms and maintenance of culture

The yeast *Y. lipolytica* IMUFRJ 50682 was used in the presented work. It was stored at 4ºC on YPD-agar medium.

2.2.1 Inoculum preparation

For pre-inoculum, cells were cultivated at 28ºC in a rotator shaker at 160 rpm, in 500 mLflasks containing 200 mL of YPD medium (w/v: Yeast extract, 1%; Peptone, 2%; Glucose, 2%).The cells were cultivated in this medium under followed conditions: 28ºC on a shaker at 160 rpm for 70 h. The concentration of inoculum used was 6.7 mgd.w./mL.

2.3 Solid-state fermentation (SSF)

To prepare the culture medium, the solid cake was separated in a sieve shaker obtaining size particles smaller than 1.18 mm. The system was sterilized at 121ºC, by autoclaving, for 20 minutes. The cottonseed cake was used without any supplementation and the soybean bran was supplemented with soybean sludge (4%w/w). Fermentations were carried out in lab-scale tray-type bioreactors, containing 10 g of cake, forming a 1 cm deep layer. Fermentations were incubated in fermentation chambers with temperature and moisture control, set to 28 °C and 99% water saturation, respectively. Each fermentation was inoculated with 0,71 mg of cells/g of solid in the medium. The moisture of medium, using cotton seed cake as substrate was set to 63% and 58% for soybean residues medium. The fermentation was carried during 48 h. Samples (whole trays) were taken every 10h, 21h, 24h, 28h, 32h, 36h and 48h for SSF using cottonseed cake and, 7h,10h,14h, 21h, 24h, 28h, 32h, 36h and 48h using soybean sludge and bran.

2.3.1 Enzyme extraction

Phosphate buffer (50mmol L^{-1}, pH 7.0, 25 mL) was added to each becker containing the fermented solids, and enzyme extraction was done in a rotary shaker at 35 °C and 200 rpm for 20 min. Afterwards solid–liquid separation was achieved with pressing followed by centrifugation at 2000*g* for 2 min. The supernatant was stored at − 4 °C.

2.4. Hydrolysis activity assay

The hydrolysis of p-nitrophenyl laurate was defined as standard method to determine lipase hydrolysis activity from crude extract of *Y. lipolytica*. The reaction occur at 37ºC by the addition of 0.1 mL of enzyme solution to 1.9 mL of 560µM p-nitrophenyl laurate (pNP-laurate) dissolved in 50mM potassium-phosphate buffer (pH 7.0), containing 1% (v/v) of dimethyl sulfoxide (DMSO). The reaction was conducted along 100 seconds in a spectrophotometer (Shimadzu UV-1800) at λ=410nm. One enzyme unit is defined as the amount of enzyme which releases 1µM of p-nitrophenol per minute at pH 7.0 and 37ºC (Amaral, 2007). The hydrolysis activity per gram (U/g) was calculated by multiplication of U/L and buffer volume used in extraction step divided by dry weight of cake used.

2.5. Lipolytic activity assay

The enzyme extract was added to an emulsion of olive oil and arabic gum, and incubated for 20 min at 37 °C and 200 rpm. Lipase activity was determined by titration of the free fatty acids released by enzyme action,

according to Freire (1996).One lipase unit was defined as the enzyme amount that causes the release of 1 µmole of fatty acids per minute, under the assay conditions. Enzyme activity was expressed as units per gram of initial dry solid medium.

2.6. Proteolytic activity assay

A colorimetric method for the determination of the proteolytic activity used in this work was described by Charneyand Tomarelli (1947). This method is based on formation of colored substances derived from azocasein, once this molecule is used as a protease substrate. The reaction was carried at 37°C for 40 minutes.

3. Results and Discussion

In this item, it will be presented the results obtained from lipase production by *Y. lipolytica* through solid state fermentation, using cottonseed cake without any supplementation and bran and sludge of soybean as substrates. The Table 1 shows the medium composition used for lipase production and, Table 2 displays the elemental analysis of cottonseed and soybean cakes.

Table 1: Medium composition for lipase production by Y. lipolytica in SSF using cottonseed cake, bran and sludge of soybean as substrates.

	Cottonseed	Soybean
Cake (g)	9.5	-
Bran (g)	-	9.3
Sludge (%)	-	4.0
Moisture (%w/v)	63	58
Inoculum (mg/g)	0.71	0.72

Table 2: Elemental analysis of cottonseed and soybean cakes made by central laboratory analyzes of CENPES / Petrobras.

Elemental analysis (%)	Cottonseed cake	Soybean bran
Carbon	**44.9±0.5**	**43.4±0.2**
Hydrogen	**7.4±0.3**	**7.1±0.0**
Nitrogen	**4.6±0.2**	**7.5±0.1**
Sulfur	**3.0±0.2**	**3.0±0.2**

The results and discussion pertinent to lipase production will be presented segregated by each residue:

3.1 Cottonseed cake:

The cottonseed cake was tested without any supplementation. The Figure 1 shows the hydrolytic activity profile during 48 h of fermentation.

According to Figure 1, it is possible to perceive that *Y. lipolytica* produced lipase, showing increasing values of hydrolytic activity until 28h and, subsequently, the activity decreased, reaching null value in 48h of fermentation. In this sense, the maximum activity reached was 102±6 U/g*h in 28h of fermentation and, as consequence, the productivity was 3.7 U/g*h. According to proteolytic activity profile, it is possible to observe a slight increment of activity until 32h and it became constant after 48h, reaching values equal to 53±1U/g. The Figure 1 shows the kinetics along 48h of fermentation, through spectrophotometric method using p-nitrophenyl laurate as substrate for enzymatic reaction, in order to determine the hydrolytic activity. Afterwards this evaluation, the best result was collected aiming at analyzing this sample using titrimetric method with olive oil as substrate. In order to understand the differences between these two methods, it is important to resort the knowledge of these ones and the substrates used to determine the lipolytic activity. Thus, firstly, the spectrophotometric method was used to evaluate the potential of this enzyme to hydrolyze p-nitrophenyl laurate (Figure 1). However, this method was not able to distinguish between esterases and lipases, once the last were capable to efficiently hydrolyze the p-nitrophenol esthers (Freire, 1996). In this sense, it was decided to use titrimetric method to determine lipase and it consists in measurement of free fat acids from olive oil hydrolysis. This is the oldest method used to determine the quantification of “true” lipases (Jarger et al*.,* 1999). Thus, the result for lypolytic activity was found to be 50,1U/g in 28h of fermentation.

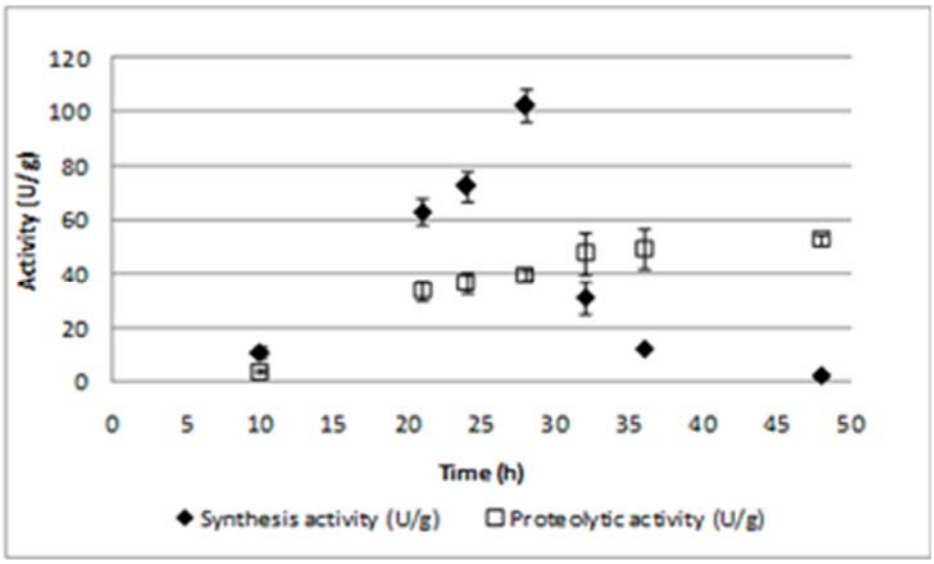

Figure 1: Hydrolytic and proteolytic activities profiles produced by Y. lipolytica in the course of fermentation, using cottonseed as substrate.

It is important to point out that this fermentation occurred without any supplementation and, undoubtedly, it becomes very attractive economically. In order to elucidate the characteristics of cottonseed cake that promote this significant result, from Table 2 is possible to determine the C/N ratio of 9.8. This value is very similar to others cakes as babassu (13.81), and canola (8.36) (Castro, 2010), that were used to produce lipase by yeast and filamentous fungi in SSF (Gutarra et al., 2009; Souza et al., 2013).
Another point to be discussed is that *Y. lipolytica* probably used the remaining cotton oil, present in the cake, as carbon source since the determined oil concentration in the cake was 6.60%.Many works reported that the most relevant factor in lipase production is the carbon source, once lipases were considered to be an enzyme easily induced. In fungi, lipidic substrates and fatty acids generally act as inducers(Dalmau et al., 2000) and hence, the lipidic carbon source seems to be essential to increase the lipase production by microorganisms.

3.2 Soybean bran and sludge

The soybean bran and sludge were also tested as substrates in SSF using *Y. lipolytica*. The Table 3 displays the soybean sludge composition.
In table 3, it can be noticed the sludge composition that consists in triacylglycerol and fat acids. This composition meets the results presented by lipase production in SSF using sludge as supplement. In fact, the first experiment was done without any supplementation, only using soybean bran as substrate and, in the same condition, *Y. lipolytica* did not exhibit any lipase production. It could be elucidated by the low remaining oil concentration in the bran, at about 0.45%.

Table 3: Composition of soybean sludge made by Central laboratory analyzes of CENPES / Petrobras.

Composition (%)	Soybean sludge
Fat acids	**47.8**
Triacylglycerol	**52.2**
Total Glycerin(*)	**5.5**

(*)Part of tryacylglycerol composition.

Then, the soybean sludge was used as oil supplementation and performed as an inducer for lipase production. In the Figure 2, it is possible to observe hydrolytic activity along 48h of fermentation. Additionally, the C/N ratio of soybean bran equal to 5.8 (Table 2), showed to be appropriated to yeast growth and lipase production.

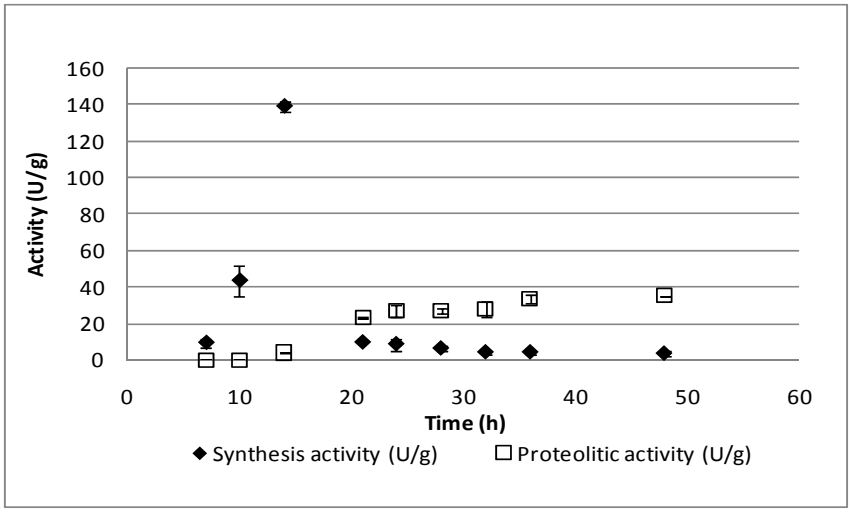

Figure 2: Hydrolytic activity profile produced by Y. lipolytica *along 48h of solid state fermentation, using sludge and bran of soybean as substrate.*

According to results presented in the Figure 2, it can be observed the maximum enzyme production in 14h, reaching activity equal to 139±4 U/g and, subsequently, it is possible to see a sharp fall in enzyme synthesis. In the same interval, it was observed the increasing of proteolytic activity and, probably, the proteases were able to hydrolyze the enzymes, decreasing its activity after 14h of fermentation. Another advantage presented by this fermentation was the productivity, reaching results like 9.9 U/g*h. Additionally, the lipolytic activity was also evaluated and reached 46±1 U/g. In this way, the high lipase productivity obtained in SSF of soybean bran and sludge may be related to the specificity of this lipase for substrates containing medium chain fatty acids and, probably, soybean slugde behaved as a inducer. In this sense, it is possible to conclude that depending on the raw material used in SSF, it is possible to obtain maximum lipase production in different times of fermentation.

Another variable that is essential for SSF is the free water presented in the medium. Moisture content of substrate plays a vital role for the microbial growth and for effecting biochemical activities in SSF. Then, it is relevant to set the ideal moisture for each residue. Lipase production can decrease at very higher moisture content, which may be attributed to the decrease in porosity and hence decrease in gaseous exchange, leading to suboptimal growth conditions and less enzyme production. Lower moisture content can promote less lipase activity due to reduction in the solubility of nutrients, lowers the degree of swelling and, it can create higher water tension (Imandi et. al., 2010a). In this work, the moisture set for soybean bran and cottonseed cake was 58% and 63% respectively. Mahanta et al. (2008) reported that the ideal moisture was equal to 50% (w/w), using *Jatropha curcas* as substrate. Others works reported 70% (w/w) (Silva et al., 2010; Imandi et al., 2010a), 60% (w/w) (Imandi et al., 2010b) and 51% (w/w) (Moftah et al., 2012). Therefore, it can be concluded the ideal moisture will depend on the water concentration that each solid matrix will trap (Balajiand Ebenezer, 2008), as soon as each microorganism adaptation.

4. Conclusions

The yeast *Y.lipolytica* was capable to grow and produce lipase in SSF, using cottonseed cake, without any supplementation, reaching 102±6 U/g and 50±1 U/g in 28h of fermentation, for hydrolytic and lipolytic activities, respectively. In this sense, cottonseed cake showed to be an excellent carbon and nitrogen sources for *Y. lipolytica*, minimizing the medium supplementation and, as consequence, the costs related to production.

The SSF using soybean cake and sludge, as substrates, presented a great potential of lipase production, reaching 139±4 U/g for hydrolytic activity and 46±1 U/g for lipolytic activity. Besides having high productivity, this result becomes interesting by using two agroindustrial residues from soybean industry.

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