



Use of agro-industrial residues for lipase production by *Candida viswanathii* in a solid-state cultivation system

Utilização de resíduos agroindustriais para a produção de lipase por *Candida viswanathii* em sistema de cultivo em estado sólido

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Lipases are an important group of enzymes that catalyze the hydrolysis of triacylglycerol and there are many industrial applications. The aim of this work was to produce the lipase by the yeast *Candida viswanathii* using solid state culture with agro-industrial wastes (barley bagasse, corn husk, corncob, soybean seed coat and soybean husk). The biomass pretreatment methods were evaluated, as well as the media supplementation with nitrogen and mixing substrates. Also, the efficiency of olive oil and poultry fat was evaluated on the induction of lipase production, followed by the scale-up from 20 g to 100 g. The enzyme activities in the cultures without pretreatment were higher when soybean seed coat supplemented with both olive oil (7.06 U/gss) and poultry fat (8.40 U/gss) were used. However, the pretreated substrates did not demonstrate a satisfying induction of lipolytic activity. From the nitrogen sources, yeast extract showed an increase of approximately twice the original production with both olive oil (18.12 U/gss) and poultry fat (15.98 U/gss) supplementation. On the scale-up step, the results demonstrated that, for the 20 g culture, the best lipase production was observed on the 7th day (33.52 U/gss), while for the 100 g culture the highest lipase activity was after 5 days (17.88 U/gss). The cultivation of ground soybean skin without pretreatment supplemented with yeast extract as a source of nitrogen, with fresh barley bagasse and poultry fat was the best combination.

Keyword: supplementation, scaling up, poultry fat.

Lipases são um grupo importante de enzimas catalisadoras da hidrólise de triacilglicerol e possuem diversas aplicações industriais. Com o objetivo de produzir a enzima lipase, utilizou-se a levedura *Candida viswanathii* por meio de cultivos em substratos sólidos empregando resíduos agroindustriais (bagaço de cevada, palha de milho, sabugo de milho, tegumento de soja e casca de soja). Foram avaliados pré-tratamentos da biomassa, a suplementação do meio de cultivo com fontes de nitrogênio e com mistura de substratos. Também foram avaliados a eficiência de azeite de oliva e gordura de frango na indução da produção de lipase e posterior escalonamento de 20 g para 100 g. As atividades enzimáticas dos cultivos sem pré-tratamento foram maiores utilizando tegumento de soja suplementado tanto com azeite de oliva (7,06 U/gss) quanto com gordura de frango (8,40 U/gss). Porém os substratos pré-tratados não foram propícios para indução da atividade lipolítica. A adição de extrato de levedura aumentou a produção em aproximadamente 2 vezes, tanto suplementando-se com azeite de oliva (18,12 U/gss) quanto com gordura de frango (15,98 U/gss). No escalonamento, no cultivo de 20 g a melhor produção de lipase foi após 7 dias de cultivo (33,52 U/gss), já no cultivo com 100 g a máxima atividade de lipase foi obtida no quinto dia (17,88 U/gss). O cultivo de tegumento de soja moído sem pré-tratamento suplementado com extrato de levedura como fonte de nitrogênio, com bagaço de cevada *in natura* e gordura de frango foi a melhor combinação.

Palavras-chave: suplementação, escalonamento, gordura de frango.

1. INTRODUCTION

Biotechnology is one of the most extraordinary technological tools today. Its applications have contributed to the structuring of new economic and social models. Among the various applications of biotechnology, some enzymes act directly in the fermentation processes to obtainment a range of products [1].

Enzymes are proteins produced by all living organisms, acting as a catalyst for numerous biochemical reactions. In addition to being *in vivo* catalysts, enzymes can also be *in vitro* catalysts for various reactions, especially in the industry [2]. Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3), are an important group of catalyst enzymes and have diverse applications in the food, detergent, and pharmaceutical industries [3].

In Brazil, agricultural waste is produced in large quantities, but its main use has been limited to animal feed or simply sent to landfills. However, agricultural residues are ideal for use in biotechnological processes, mainly due to their low cost, accessibility, and nutritional compositions, as they contain carbon, nitrogen, and minerals. These have been used in biotechnological processes to produce compounds with high added value [4-6]. In addition, it has been a practice to perform pre-treatments on substrates before producing the enzyme, to make the lignocellulosic complex nutrients more accessible to the microorganism, thus favoring its growth [7-9].

To use these residues as a whole, solid-state fermentation or cultivation is a tool to be used. This process refers to the cultivation of microorganisms on or within particles in a solid matrix, where the liquid content is at a water level that ensures cell growth and metabolism but does not exceed the maximum binding capacity of water with the matrix solid. It is also possible to add nutrient solutions to the substrate for better adaptation of the microorganism to the nutritional conditions of the medium [10-12].

As part of the industrial production cost of enzymes is due to the fermentation medium [13-15], the present work had as objective the production of lipase by *Candida viswanathii* through cultivation in solid substrates using agro-industrial residues.

2. MATERIALS AND METHODS

2.1 Substrates and lineage maintenance

Corn husk and corn cob were provided by Pamonharia da Dona Júlia, Gurupi-TO. The barley bagasse was provided by Cervejaria Serra Beer, Porangatu-GO and the soybean husk and soybean tegument were provided by Fazendão, Gurupi-TO. Rice husk was offered by Beneficiadora Gargetins, Gurupi-TO. The olive oil used in the crops was from the Galo brand and the poultry fat was provided by Frigorífico Fricok, Rio Claro-SP. The other reagents used were of analytical grade.

Candida viswanathii strain is being maintained at the Laboratory of Biotechnology, Analysis of Food and Product Purification (LABAP), Federal University of Tocantins, Campus de Gurupi-TO. The strain is maintained by the method of Castelani (1967) [16] at 4 °C and periodic cultures were carried out in dextrose potato agar medium at 30 °C, for 3 days.

2.2 Solid-state cultivation

2.2.1 Pretreatments of lignocellulosic biomass

The substrates corn husk, corn cob, soybean husk, soybean husk, barley bagasse were previously subjected to a thermal and chemical pre-treatment using sulfuric acid. The substrates were ground (particles between 1 mm – 2 mm) in a knife mill. One part has undergone pre-treatment and another part has not been treated. Heat treatment was performed with superheated steam for 15 min at 121 °C. Subsequently, the acid treatment was carried out with a solution of sulfuric acid (H₂SO₄) 1.09% with a 1:5 ratio (w/v) for a period of 27 min at 121 °C. After treatment, vacuum filtration and successive washings were carried out until the pH stabilized.

2.2.2 Preparation of the inoculum

Candida viswanathii strain was previously cultivated in potato dextrose agar medium in a slanted tube for three days at 30 °C. The pre-inoculum was prepared in Erlenmeyer flasks (125 mL) containing 50 mL of modified Vogel liquid medium [17] supplemented with 1.5% (m/v) of olive oil and 0.2% (m/v) of yeast extract, pH 6.0 and sterilized at 121 °C, for 15 min. The inoculation of the culture medium was carried out with 1 mL of a cell suspension (10^7 cells/mL) removed from the tube in 0.85% saline solution (NaCl) and kept under agitation at 210 rpm, at 28 °C for 24 hours [10]. Five milliliters of this pre-inoculum were inoculated into substrates used for solid substrate cultivation.

2.2.3 Preparation of solid substrates

The solid-state cultivation were prepared in Erlenmeyer flasks (250 mL) containing 10 g of each substrate ground in nature or pre-treated: corn straw, corn cob, barley bagasse, soybean husk, and soybean husk. The substrates were added with 10 mL of a Vogel salt solution [17] without nitrogen to provide an initial moisture content of 50%. Cultivations were supplemented with olive oil or poultry fat (40%, m/v) to induce lipase production. Culture flasks were sterilized at 121 °C for 20 min. Then, they were inoculated with the pre-inoculum and incubated at 30 °C for 5 days. Cultivations were carried out in duplicate.

2.3 Enzyme extraction and determination of enzyme activity

After cultivation, to proceed with the extraction of the enzyme, ice-cold distilled water was added and kept under orbital agitation of 140 rpm, at 28 °C for 60 min. For the cultivation of 10 g, 20 g, and 100 g, 100 ml, 200 ml, and 1000 ml of water were added, respectively. Subsequently, vacuum filtration was performed in an ice bath, with 3 to 4 layers of gauze, to promote the separation of solids, and the supernatant was centrifuged at 9000 rpm, 4 °C for 20 min. The supernatant resulting from the centrifugation was used to determine the lipase activity.

Lipase activity was determined with p-nitrophenyl palmitate (pNPP) as a substrate. pNPP was solubilized in 0.5 ml dimethylsulfoxide (DMSO) and then diluted to 0.5 mM with McIlvaine pH 4.0 buffer containing 0.5% Triton X-100. In test tubes, 900 µl of reaction medium were added and the hydrolysis of pNPP was determined discontinuously at 40 °C by the release of p-nitrophenol. After pre-incubation of the substrate in a water bath for 5 minutes, the reaction was started by adding 0.1 mL of the properly diluted sample. The reaction was stopped at different intervals with thermal shock (90 °C, 1 min), followed by the addition of 1mL of saturated sodium tetraborate solution. Absorbance reading was performed at 410 nm. Controls were prepared without the addition of enzymes [18]. A unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of pNP per min per mL.

The enzymatic activity was determined through the relationship between absorbance (Abs), molar absorptivity coefficient (ϵ), used volume of protein extract (V_i), final reaction volume (V_f), reaction time (t), and dilution factor (d), given by Equation (1).

$$A = \frac{Abs}{\epsilon} \cdot \frac{1}{V_i} \cdot 10^3 \cdot V_f \cdot \frac{1}{t} \cdot d$$

Eq. (1)

2.4 Supplementation with nitrogen sources

Crops with ground soybean tegument were supplemented with the addition of different nitrogen sources at a concentration of 5% (m/m) [19]: casein, ammonium chloride, yeast extract, and urea.

2.5 Supplementation within natura substrates

The ground soybean seed coat substrate was supplemented with other substrates in nature 1:1 (m/m): rice husk, soybean seed coat, and barley bagasse to promote less compaction and better use of the substrate. Yeast extract was added to the cultures as a source of nitrogen, at a concentration of 5% (m/m) and inoculated with 8 mL of pre-inoculum. Cultivation was carried out in duplicate.

2.6 Scaling up

The ground soybean tegument substrate was supplemented with fresh barley bagasse and was cultivated in two scales, 20 g, and 100 g, using the ratio 1:1 (m/m). Yeast extract (5%), poultry fat (40%) and pre-inoculum in the proportion 2:1 (w/v) were added to the cultivation, in addition to Vogel in the proportion 1:1 (w/v). The cultures were kept at 30 °C and samples were collected at intervals of 3, 5, and 7 days. The 20 g cultivation was carried out in Erlenmeyer flasks and the 100g was carried out in autoclavable polypropylene bags (20cm x 25 x 0.06).

To extract the enzyme from the 100 g culture, 1000 mL of ice-cold distilled water was added to the culture in a beaker, after homogenization with a glass stick, the volume was divided into 250 mL Erlenmeyer flasks and the same conditions were used. from previous extractions.

3. RESULTS AND DISCUSSION

3.1 Selection of lignocellulosic substrates for lipase production

In this first stage, the productive capacity of *Candida viswanathii* to secrete lipase in different lignocellulosic substrates, which received physicochemical pre-treatments and supplements, was evaluated (Table 1).

Table 1: Lipase production using different agro-industrial residues as substrate.

Substrate	No Pretreatment (U/gss)		With Pretreatment (U/gss)	
	Olive Oil	Poultry Fat	Olive Oil	Poultry Fat
Barley bagasse	2.16	2.47	2.07	1.66
Corn Straw	1.54	1.40	0.97	1.32
Corn cob	4.07	1.44	0.49	0.37
Soybean Integument	7.06	8.40	0.95	1.18
Soybean hulls	-	-	1.42	0.82

*Cultures were carried out for 120 hours at 30 °C. The inoculum was prepared in submerged fermentation for 24 hours at 28 °C. The substrates were supplemented with 10 mL of Vogel salts solution without nitrogen source and 40% of olive oil or poultry fat.

The enzymatic activities of the crops without pretreatment were higher using soybean tegument as substrate, reaching 7.06 and 8.40 U/gss when supplemented with olive oil and poultry fat, respectively. With barley bagasse and corn husk, the lowest levels of production were obtained, using olive oil or poultry fat as a supplement. The differences observed in production levels among agro-industrial residues may be related to the different chemical compositions of biomass, which in turn influence the growth and accessibility of substrates by microorganisms and, as a result, the secretion of enzymes. [20, 21].

Subsequently, the yeast was cultivated on substrates that underwent physicochemical pretreatment and even supplementing with olive oil or poultry fat, there was no induction in the production of lipase when compared to lignocellulosic material without pretreatment. The lipolytic activity in this composition was maximum using barley bagasse supplemented with olive

oil (2.07 U/gss). A decrease in activity was noted when the substrates corn straw, soybean coat, and soybean husk were used and supplemented with olive oil or poultry fat. Corn on the cob was shown to be inefficient for lipase production supplemented with both olive oil and poultry fat.

Although the goals of pretreatment are to make the lignocellulosic complex nutrients more accessible to microorganisms [22, 23], depending on operating conditions, hemicellulosic sugars solubilized as a result of pretreatment can follow reaction pathways that lead to degradation products, such as furfural, hydroxymethylfurfural (HMF), and various organic acids that derive from lignin degradation, and have been described as potential inhibitors of microorganism growth. This is probably why the pretreated substrates were not efficient for lipase production [21, 24].

From the different substrates evaluated, the soybean tegument was selected for the next step, due to its ability to induce the production of the lipase enzyme, in addition to being easy to obtain and its low cost.

3.2 Supplementation with nitrogen sources

Some nitrogen sources were analyzed in the production of lipase by *C. viswanathii*. The results suggest that among the nitrogen sources, yeast extract increased production by approximately 2-fold, both supplemented with olive oil (18.124 U/gss) and with poultry fat (15.998 U/gss) (Table 2). Inorganic nitrogen sources such as ammonium chloride (NH₄Cl) caused a decrease in lipase production when compared to the substrate without supplementation.

Table 2: Lipase production using soybean tegument supplemented with a nitrogen source.

Nitrogen source	Lipase activity (U/gss)	
	Olive oil	Poultry fat
Casein	5.62	6.38
Yeast extract	18.12	15.98
Ammonium chloride	1.08	0.26
Urea	7.81	10.66

*Cultures were carried out for 120 hours at 30 °C. The inoculum was prepared in submerged fermentation for 24 hours at 28 °C. The substrates were supplemented with 10 mL of Vogel salts solution without nitrogen source and 40% of olive oil or poultry fat.

Almeida et al. (2016) [19] observed that supplementing wheat bran with 5% (w/w) yeast extract resulted in a 3.8-fold increase in lipase production when compared to cultivation without the addition of the source. of nitrogen. Moftah et al. (2012) [21] used cake from the manufacture of olive oil to produce lipase using *Candida utilis* supplemented with 3% (m/m) of yeast extract and achieved an activity of 25.0 U/gss and by combining the production with alkaline treatment achieved a 39% increase. The author also suggests that the increase in lipase production when using nitrogen sources in cultivation is probably based on the presence of some specific bioactive peptides that act as inducers of lipase production.

Thus, yeast extract was selected for supplementation with other substrates and subsequent scaling.

3.3 Supplementation with in natura substrates

The use of solid substrate can affect the microorganism's accessibility due to porosity and particle sizes of the substrate, as it hinders the extent and rate of microbial colonization, air penetration, CO₂ removal, and enzyme extraction [25]. The size of the substrate particle should provide greater spacing between them, facilitating gas exchange and, consequently, microbial growth. Exceedingly small particles agglomerate and impair aeration, resulting in low enzyme production [26, 27]. To reduce compaction, the cultivation was supplemented with other natural substrates, such as rice husk, soybean seed coat, and barley bagasse.

The cultivation of *C. viswanathii* in-ground soybean tegument combined with fresh barley bagasse, supplemented with poultry fat increased the level of lipase production from 15.98 to 20.04 U/gss (Tables 2 and 3). The opposite occurred with the presence of olive oil in which the lipase production decreased by 7.00 U/gss.

Poultry fat is a by-product of poultry slaughter and contains high contents of the fatty acids palmitic, oleic, and linoleic [28, 29]. Oleic acid is indicated as an inducer of lipase production due to the stimulation of microbial metabolism and the lipase biosynthesis pathway [30].

Table 3: Lipase production using soybean tegument supplemented within natural substrates.

Substrates	Lipase activity (U/gss)	
	Olive oil	Poultry fat
Rice husk	9.68	11.50
Soy Integument	4.18	8.73
Barley bagasse	11.06	20.04

*Cultures were carried out for 120 hours at 30 °C. The inoculum was prepared in submerged fermentation for 24 hours at 28 °C. The substrates were supplemented with 20 mL of Vogel salts solution without nitrogen source and 40% of olive oil or poultry fat.

Regarding the use of barley bagasse, the polysaccharide and protein content make it particularly susceptible to microbial growth and degradation. Thus, the use of this readily available, low-cost residue as a substrate for enzyme production can be one of the ways to substantially reduce the cost of enzyme production [31]. Thus, the mixture of soybean tegument with barley bagasse supplemented with poultry fat was selected for scaling.

3.4 Scaling up

To scale up, two types of cultures were carried out: one containing 20 g of solid substrate and another containing 100 g of solid substrate, and the lipase activity was evaluated at times 3, 5, and 7 days (Table 4). The results showed that in the 20 g culture the best lipase production was observed on the seventh day (33.52), while in the 100 g culture the maximum lipase activity was obtained on the fifth day (17.88). On the third day of 100 g culture, there was a decrease in lipase production, probably due to the difficulty of growing the biomass in a solid medium, as the increase in lipase activity is associated with cell growth [32].

The seventh day of 100 g culture was not efficient, possibly due to nutrient limitation that decreased microbial growth [33], thus disfavoring lipase activity. Therefore, the scaling to 100 g did not obtain good results regarding the cultivation in 20 g of substrate.

Table 4: Scaling of lipase production using soybean tegument supplemented with barley bagasse and poultry fat at different times (days).

Time (days)	20 g	100g
	Lipase Activity (U/gss)	Lipase Activity (U/gss)
3	17.17	1.55
5	32.37	17.88
7	33.52	7.59

In solid-state cultivation, oxygen transfer and the complexity of temperature and water content control are limiting factors [34, 35]. The substrates used in solid cultivation have low thermal conductivities, consequently, they increase the heat accumulation inside the bioreactor. Therefore, heat removal is a fundamental step in several studies [36, 37]. With the use of larger size particles, heat removal can be achieved with forced air circulation, through larger particles that have more space between them [34].

4. CONCLUSION

The solid-state cultivation of *C. viswanathii* using different agro-industrial residues, aiming to increase the scale of lipase production, allows us to conclude that the cultivation of ground soybean tegument without pretreatment supplemented with yeast extract as a nitrogen source, with barley bagasse in nature to provide aeration and poultry fat was the best combination.

There are still points to be explored regarding the production of the lipase enzyme, such as improving the technique of cultivation and extraction of the enzyme and improving the heat exchange in the medium. In addition, it is valid to perform a characterization of the enzyme in question for further escalation.

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