

**Selection and isolation of fungi strains and the use of banana peel a suport for lipases production****Isolamento e seleção de linhagens fúngicas e o uso da casca de banana como suporte para produção de lipases**

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**ABSTRACT**

Lipases are important enzymes in biotechnological processes, mainly due to their ability to catalyze a wide range of reactions of interest to the food and pharmaceutical industry, including anti-cholesterol, anti-inflammatory and thrombolytic agents, in the chemical industry, among others. These enzymes can modify the properties of lipids either by changing the position of the fatty acid chain in the molecule, or by exchanging one or more acids for different ones. Lipases are obtained from animal, vegetable or microbial cells, however, those of microbial origin are currently the most used industrially. This work aimed to isolate and select fungi that produce lipolytic enzymes, as well as the study of the production of these enzymes by semi-solid fermentation using banana peel and using residues from the agribusiness, wheat bran and bagasse of sugar cane. After the selection process, using baits soaked in soy oil, olive oil and coconut milk, the isolates with the highest enzyme index were used to study the production of lipase using residues in fermentation. Among the isolates selected as lipase producers, maximum production was achieved by the MO 56 strain from baits soaked in soybean oil, when 40% of wheat bran was used as a substrate and banana peel as a support, resulting in 1.46  $\mu\text{mol.g}^{-1}$  of enzymatic activity. The choice of banana peel as a support proved to be adequate and its granulometry provided an appropriate aeration for the supports, preventing their compaction, in addition to maintaining their homogeneous humidity without showing exudation throughout the fermentation process.

**Keywords:** microbial enzymes, substrates, semi-solid fermentation.

## RESUMO

As lipases são enzimas importantes nos processos biotecnológicos, principalmente devido a sua capacidade em catalisar uma grande gama de reações de interesse para a indústria de alimentos, produtos farmacêuticos, incluindo anticolesterolêmicos, anti-inflamatórios e trombolíticos, na indústria química, entre outras. Essas enzimas podem modificar as propriedades dos lipídeos seja alterando a posição da cadeia dos ácidos graxos na molécula, seja trocando um ou mais ácidos por outros diferentes. Essas enzimas são produzidas tanto por organismos animais e vegetais, quanto por microrganismos, porém as de origem microbiana são as mais utilizadas industrialmente. Este trabalho teve como objetivo o isolamento e seleção de fungos produtores de lipases, e o estudo da obtenção da enzima usando resíduos provenientes da agroindústria, farelo de trigo e bagaço de cana-de-açúcar, e o uso de casca de banana como suporte para a fermentação usada neste trabalho. Após o processo de seleção, os isolados que apresentaram maior índice enzimático foram utilizados para o estudo da produção de lipases. Dentre os isolados selecionados como produtores de lipases, a máxima produção foi atingida pela linhagem MO 56 proveniente das iscas embebidas em óleo de soja, quando utilizado 40% de farelo de trigo como substrato e a casca de banana como suporte, resultando em 1,46  $\mu\text{mol.g}^{-1}$  de atividade enzimática. A escolha da casca de banana como suporte mostrou ser adequada e sua granulometria propiciou uma aeração apropriada para os suportes impedindo a compactação dos mesmos, além de manter sua umidade homogeneia sem apresentar exsudação durante todo o processo fermentativo.

**Palavras-chave:** enzimas microbianas, substratos, fermentação semissólida.

## 1 INTRODUCTION

Lipases (glycerol ester hydrolases E.C.3.1.1.3) are lipolytic enzymes that act in the glycerol esters chain of long-chain fatty acids without needing a cofactor. The main biological function of lipases is to accelerate the hydrolysis of triglycerides, however, when there is little water available in the medium, these enzymes are able to catalyze reverse reactions such as esterification and transesterification. Therefore, these enzymes are widely used in biotechnological applications in the food, chemical, pharmaceutical industry, among others (BON et al., 2008; SHING et al., 2012).

Lipolytic enzymes can be obtained from animal, vegetable or microbial cells, however, those of microbial origin are currently the most used industrially. In the biotechnological area, microbial lipases are the most produced and applied, where most of these enzymes are isolated from fungi (ADRIO; DEMAIN, 2014). In general, enzymes are extracellular products found in microbial culture and fungal mycelia. In addition, they are used naturally making them a low-cost and profitable product, since biomass can be used directly, without the need for isolation, purification or immobilization of the enzyme, and yet, minimizing the loss of enzymatic activity (TORRES et al., 2003).

In the industry, microbial enzymes stand out due to the easy process of obtaining them from fermentative broth, in addition to presenting low production costs. Enzymes of microbial origin have

many advantages over animal and vegetable equivalents, such as the possibility of large-scale production in industrial fermenters, they are also generally more stable and with more diverse properties (GANDRA et al., 2008). The diversity of existing microorganisms justifies the search for new enzyme producers (CARVALHO et al., 2005).

The culture media used for the production of microbial lipases must contain nutrients necessary for the development of the microorganism and the production of sufficient metabolites to supply the energy of biosynthesis and cellular maintenance (LIN; WANG; SUNG, 2006). However, the medium is one of the most relevant components in the cost of production, therefore, the interest in the use of agro-industrial and forestry lignocellulosic waste as raw materials increasingly increases in biotechnological processes. The residues that stand out the most are sugarcane bagasse, rice and wheat residues, such as husks and bran, castor cake, among others. In addition, the use of these residues contributes to the disposal of these tailings (DA SILVA, 2016; TREICHEL et al., 2016). Taking into account, the amount of agro-industrial waste generated in the environment by banana peels is of great importance to seek ways to allocate and use them, one of the possibilities, for example, is the use of this residue as a support for semi-solid state fermentation, resulting in an improvement in environmental quality, preventing the shells from being disposed of in landfills, causing no harm to society (MOURA et al., 2020).

Some organic residues can also assist fermentation for the production of enzymes, such as supports, which act as a physical barrier that prevents the free mobility of enzyme molecules and is the main responsible for the good performance or not, of the immobilized system (GUPTA et al., 2013; SIRISHA et al., 2016). Organic supports also offer microbial resistance, since inorganic supports do not serve as a substrate for the growth of microorganisms. In addition, they ensure proper aeration by preventing the compaction of the medium (SIRISHA et al., 2016).

The discovery of new microbial sources, mainly non-toxic to the human organism, is of great industrial interest because, in addition to ensuring the supply of enzymes in different processes, they make it possible to develop new enzymatic systems that cannot be carried out from enzymes plants or animals (OLIVEIRA et al., 2006).

From this, the objective of this work was to isolate and select filamentous fungi producing lipases by using banana peel as a support for microbial growth, in a semi-solid state fermentation.

## 2 MATERIALS AND METHODS

### 2.1 ISOLATION OF MICROORGANISMS

The fungi collection was obtained from different points of the Universidade Estadual Paulista - UNESP, in São José do Rio Preto, where baits (pieces of 10x10 cm<sup>2</sup>) of cotton cloths were spread, embedded in three different substrates: olive oil, soy and coconut milk, purchased from local stores.

After one month of exposure, the microorganisms that grew on each bait were isolated in sterile petri dishes containing PDA (potato dextrosado agar) by the streak depletion technique and incubated at 25°C for 48 hours.

The different strains of each plate were picked in tubes containing PDA, duly identified with a number from 01 to 62. After an incubation period of 72 hours at 25°C, the tubes were stored in BOD at 0°C, and they were picked monthly in new tubes containing PDA.

### 2.2 IDENTIFICATION OF THE BEST PRODUCING STRAIN

Primary selection was performed according to the methodology described by Hankin and Anagnostakis (1975), using a culture medium composed of peptone (1%); sodium chloride (0.5%) and agar (2%). To this medium, 1% Tween-20 (sorbitan monolaurate) was added to observe the production of lipase.

The isolated strains were inoculated, in triplicate, in the center of the Petri dishes with the aid of a sterile platinum loop. The plates were kept in a growth oven at 28°C for 12 days. The best strain was chosen by determining the enzyme index (IE), by the ratio: diameter of the halo formed by the crystals of calcium salts of lauric acid by the diameter of the colony, measured with a caliper, multiplied by 100.

The lipolytic activity was evidenced by the presence of crystals of calcium salts of lauric acid, released around the colonies forming a halo. Thus, the isolates that exhibited the highest EI in the growth media are those that have the greatest extracellular enzyme activity.

### 2.3 FERMENTATION FOR THE PREPARATION OF CRUDE ENZYME EXTRACT

The production of crude lipase extract for each selected fungus was obtained by semi-solid fermentation. Inoculating 1 mL of spore suspension in a 500 mL Erlenmeyer containing banana peel as a support added with wheat bran or sugarcane bagasse, in different concentrations (70, 60, 50, 40 and 30%), with a content 50% humidity, previously sterilized in an autoclave at 121 °C, at a pressure of 1 atm. for 15 minutes. Each of the erlemeyers contained 30 g of fermentation medium (sum of the masses of the water, the support and the substrate). Following that, they were placed for fermentation for 240 hours at 37 °C. This incubation time was chosen based on three previous tests. At the end, the

medium received 200 mL of distilled water and was gently stirred for 30 minutes (45 rpm), to avoid inactivating the enzyme. After stirring, the extract was vacuum filtered on Whatman n. 1 and used as an enzyme source.

#### 2.4 DETERMINATION OF ENZYME ACTIVITY

The determination of protein concentration in the enzymatic extracts obtained was carried out by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

### 3 RESULTS AND DISCUSSION

The selection of lipase-producing fungi consisted of isolating these microorganisms from the environment. For this, baits embedded in three different substrates were placed: soy oil, olive oil and coconut milk in predetermined locations. Since, the natural substrates for lipases are oils and fats that contain triacylglycerols, consisting of glycerol esters and long-chain fatty acids, that is, triple ester bonds (BROCKMAN, 1984; DIAZ et al., 2006).

The oils that were used as substrates proved to be efficient, since at the end of one month of exposure there was growth and a great variety of microorganisms present in the baits. 62 different microorganisms were collected and isolated in Petri dishes containing PDA, which received an identification code and the name of the substrate used to stimulate growth, soybean oil, olive oil or coconut milk (Table 1).

**Table 1** - Isolated microorganisms and the related substrates used in the baits for their development.

Isolated microorganisms				
Soy oil (SO)	Olive oil (OO)	Coconut milk (CM)		
MO 02	MO 01	MO 43	MO 06	MO 30
MO 09	MO 03	MO 48	MO 07	MO 31
MO 22	MO 04	MO 49	MO 08	MO 59
MO 23	MO 05	MO 50	MO 10	MO 60
MO 32	MO 13	MO 55	MO 11	MO 61
MO 37	MO 14	MO 56	MO 12	MO 62
MO 38	MO 19	MO 57	MO 15	
MO 39	MO 20	MO 58	MO 16	
MO 44	MO 21		MO 17	
MO 45	MO 33		MO 18	
MO 46	MO 34		MO 24	
MO 47	MO 35		MO 25	
MO 51	MO 36		MO 26	
MO 52	MO 40		MO 27	
MO 53	MO 41		MO 28	
MO 54	MO 42		MO 29	

**Legend:** MO – microorganism.

After the isolation stage, the pre-selection phase of the lipase-producing microorganisms was carried out. For this, each of the 62 microorganisms were inoculated again in sterile Petri dishes

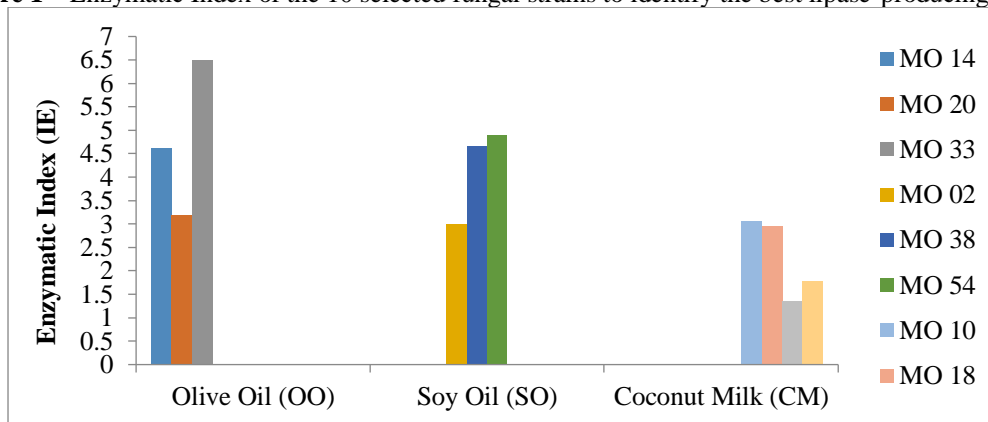
containing the medium proposed by Hankin; Anagnostakis (1975) and incubated at 28 ° C for 12 days.

Amongst the 62 cultured microorganisms, 28 showed lipase production, of which 11 yeasts, which were not used, and 17 filamentous fungi. The 10 fungi that showed the highest lipolytic activities, that is, the largest halos of lauric acid calcium salts were chosen for the evaluation of the enzyme index (IE).

The fungi selected to verify the IE Registry of the baits embedded in the soy oil substrate were MO 02, MO 38; and MO 54; the ones chosen from the baits soaked in coconut milk were MO 10, MO 18, MO 31 and MO 62; and the ones originating from the baits soaked in olive oil MO 14, MO 20 and MO 33.

The enzyme index (IE) was determined by the ratio between the diameters (mm) of the halo formed by the crystals of calcium salts divided by the diameter (mm) of the colonies that produced them. The 10 selected strains were inoculated using the medium proposed by Hankin; Anagnostakis (1975) at 28 ° C for another 12 days. At the end, the IE were calculated and their results are shown in Figure 1.

**Figure 1** – Enzymatic Index of the 10 selected fungal strains to identify the best lipase-producing strains



The highest enzyme indices observed in Figure 1 are of the fungus MO 33, resulting in 6.50, coming from the baits embedded in the substrate of olive oil, and MO 54 and MO 38, coming from soy oil, with 4.9 and 4.66, respectively. For the coconut milk substrate, the highest EI was obtained with MO 10, 3.07 and MO 18 with 2.96.

Thus for the production of crude enzymatic extract, the microorganisms MO 18 (baits soaked in LC), MO 33 (baits soaked in AO), and MO 54 (baits soaked in OS) were chosen, as they had a high IE, and also for presenting low standard deviation and large enzyme halos.

Authors such as Mahadik et al. (2002) and Castilho et al. (2000) used olive oil as an inducer for the production of lipases. However, it is clear that the cost of adding olive oil as an inducer is high

in the production of lipases on an industrial scale. Thus, it is necessary to use other inducers, such as soy oil from fried foods, which would be easily obtained, as it is a residue from restaurants and industry (ROVEDA et al., 2010).

The production of crude enzymatic extract was carried out by semi-solid fermentation, for 240 hours at 37° C, using 5 different concentrations of the substrates of sugarcane bagasse and wheat bran (70%, 60%, 50%, 40% and 30%), thus having 10 different combinations for each microorganism studied. The banana peel was used as a support to complement the concentration of 100% of the medium (substrate added with banana peel) to carry out the semi-solid fermentation.

Banana peel is an organic residue rich in carbohydrates and basic nutrients that can act as a support for microbial development. In addition to being a low-cost and easily acquired material, the use of this residue as a source of medium for microbiological growth, and as substrates for the production of fungal biomass can be very advantageous (KARTHIKEYAN; SIVAKUMAR, 2010).

After the fermentation was completed, the crude enzyme extract was prepared. Table 2 presents the average enzymatic activities, of the crude enzymatic extract, measures of three repetitions and their standard deviation for each of the determinations of the concentrations of the substrates used. A unit of enzymatic activity was defined as the amount of enzyme required to release 1  $\mu\text{mol}$  of fatty acid, per minute of reaction under the pre-determined test conditions.

**Table 2** – Enzymatic activity of crude enzymatic extract ( $\mu\text{mol.mL}^{-1}$ ) obtained with different combinations and concentrations of substrates using banana peel as a support

	MO 18 (Coconut Milk)		MO 33 (Olive Oil)		MO 54 (Soy Oil)	
	Bagasse of cane	Wheat bran	Bagasse of cane	Wheat bran	Bagasse of cane	Wheat bran
70%	0,97 $\pm$ 0,025	1,20 $\pm$ 0,008	0,86 $\pm$ 0,025	1,11 $\pm$ 0,025	1,04 $\pm$ 0,025	1,18 $\pm$ 0,025
60%	1,00 $\pm$ 0,051	1,15 $\pm$ 0,101	0,73 $\pm$ 0,025	1,22 $\pm$ 0,025	1,17 $\pm$ 0,025	1,34 $\pm$ 0,025
50%	1,07 $\pm$ 0,008	1,20 $\pm$ 0,006	1,01 $\pm$ 0,025	1,31 $\pm$ 0,025	1,18 $\pm$ 0,025	1,32 $\pm$ 0,025
40%	1,03 $\pm$ 0,034	1,23 $\pm$ 0,101	1,01 $\pm$ 0,025	1,31 $\pm$ 0,025	1,07 $\pm$ 0,025	1,46 $\pm$ 0,025
30%	1,01 $\pm$ 0,025	1,22 $\pm$ 0,017	0,90 $\pm$ 0,025	1,31 $\pm$ 0,025	1,09 $\pm$ 0,025	1,41 $\pm$ 0,025

From Table 2, it is possible to verify that the values of enzyme activity were close between the substrates and for the different concentrations used, varying between 0.73 and 1.46  $\mu\text{mol.g}^{-1}$ . Among the microorganisms tested, MO 54 showed greater activities for the two substrates used, however, the activity values that stood out the most were of those obtained in culture media containing FT than in those containing BC.



Among the other microorganisms MO 18 and MO 33, it was also possible to verify that the FT had greater effectiveness. This is probably due to the fact that FT is a richer and more diversified source of nutrients for microbial growth than crushed BC, which, despite having a higher concentration of simple carbohydrates than in FT, may have less or slowed growth of microorganisms (RODRÍGUEZ-ZUÑIGA et al., 2011).

Among the concentrations of substrates tested, it is possible to see in Table 2, that the lowest concentrations, such as 40%, obtained better activities for FT. As well as for BC, which had its peak activity in concentrations of 50% in dry mass.

Another factor that may have influenced the difference between enzyme activity is the granulometry between the substrates and the support. The banana peel (granulometry of 5 mesh units) interacted efficiently in all cases, avoiding the compaction of the substrates and kept the humidity of the culture medium homogenous without syneresis in these mediums. The mixtures of banana peel and BC (9 mesh units) showed greater compaction than those mixtures containing FT (5 mesh units). Therefore, the granulometry used provided less aeration in the substrates containing BC allowing the compaction of the same, besides decreasing the humidity of the substrate.

The pore size, the morphology and the distribution of the support are properties that can directly influence the diffusion effects of the structure and production of enzymes, and also directly interfere in the yield. Thus, porosity is an important factor to be taken into account when choosing the support (CARVALHO; LIMA; SOARES, 2015).

The fungus MO 54 that used wheat bran as substrate showed the best results of enzymatic activity reaching the highest value in the concentration of 40% of substrate in dry mass,  $1.46 \mu\text{mol}\cdot\text{mL}^{-1}$ . However, this value was lower than those found in similar studies, such as the study by Coimbra et al. (2012), where fungi from the environment were also selected and wheat bran (50%) was used as substrate, in a semi-solid fermentation of 120 h at  $35^\circ\text{C}$ , the enzymatic activity was also obtained by trituration resulting in  $8.96 \mu\text{mol}\cdot\text{mL}^{-1}$  with soybean oil as a substrate.

The authors Marotti et al. (2017) evaluated ten species of the genus *Penicillium*. isolated from different environments using olive oil as inducer, the highest enzymatic activity of the filtered crude extract was  $27.1 \text{ U}\cdot\text{g}^{-1}$ , a value above that found in this work, of  $1.46 \mu\text{mol}\cdot\text{mL}^{-1}$ .

It is interesting to remember that the differences in activity between the strains isolated in this work are also due to the fact that they are lipases from different sources. In addition to being used to hydrolyze different substrates, since, for the reactions to determine enzymatic activity, the original substrates of the “baits” were used from which each of the fungi was isolated, that is, for the MO 18 microorganisms, MO 33 and MO 54 were respectively used coconut milk, olive oil and soy oil for



the quantification of lipolytic activities. However, this comparison is valid, since it provides a good estimate of the enzymatic activity of the enzymes presented by these fungi.

According to Carvalho et al. (2005), the enzyme activity values in the hydrolysis of olive oil can vary significantly, but they should be used for comparison and selection of lipase-producing strains. Since, depending on the type of fermentation, composition of the culture medium and also other fermentation variables, such as pH, temperature and inductors such as vegetable oils, these values will be very different, therefore, they serve as a reference for the progress of the production process.

#### 4 PROTEIN CONTENT OF CRUDE EXTRACT

The protein content measured in the crude extract is shown in Table 3.

**Table 3** – Protein content of crude extract (%)

	MO 18 (Coconut Milk)		MO 33 (Olive Oil)		MO 54 (Soy Oil)	
	Bagasse of cane	Wheat bran	Bagasse of cane	Wheat bran	Bagasse of cane	Wheat bran
70%	1,09 ± 0,006	1,40 ± 0,008	0,45 ± 0,001	0,82 ± 0,001	0,51 ± 0,008	1,35 ± 0,003
60%	1,61 ± 0,005	0,70 ± 0,004	1,37 ± 0,010	1,26 ± 0,010	1,00 ± 0,003	1,06 ± 0,007
50%	1,61 ± 0,006	0,89 ± 0,011	1,18 ± 0,005	0,69 ± 0,005	1,37 ± 0,002	0,83 ± 0,002
40%	1,20 ± 0,001	0,73 ± 0,005	1,14 ± 0,008	1,68 ± 0,008	0,88 ± 0,006	0,76 ± 0,003
30%	1,16 ± 0,002	0,96 ± 0,008	1,62 ± 0,004	1,39 ± 0,004	1,38 ± 0,015	1,94 ± 0,000

In table 3, it can be seen that there was little difference in protein levels between the three strains chosen, ranging from 0.70 to 1.9%. However, there were large variations between the different concentrations within the same series, from 0.70 to 1.40%, for MO 18; 0.69 to 1.68% for MO 33 and 0.76 to 1.94% for MO 54, with FT as substrate. For BC, the crude protein values were closer between the different concentrations.

These differences may be due to the presence of other proteins that are not enzymes, as well as the detachment of biological material from the fungi, or proteins from the substrates or support dissolved in the extracts.

Coimbra et al. (2012), also selected fungi from the environment and used wheat bran (50%) as substrate, in a semi-solid fermentation of 120 h at 35 °C, obtained 1.31 to 1.90% of crude protein in their analyzes, values according to those found in this research, which varied from 0.70 to 1.94%, the highest content determined in MO 54, with 30% of wheat bran as a substrate.

**5 CONCLUSION**

The technique used to isolate the microorganisms, resulted in 62 strains, of these 28 showed lipolytic activity.

The selection and production of the enzymes showed activity, where the strain that showed the highest performance was MO 54, from baits soaked in soybean oil, resulting in 1.46  $\mu\text{mol.g}^{-1}$  of enzymatic activity.

The use of banana peel as a support for fermentation in a semi-solid state proved to be adequate, as well as the granulometry used, as it provided homogeneous aeration of the culture media, preventing their compaction while maintaining its moisture without exudation.

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