DESENVOLVIMENTO BIOCATALISADOR DO FUNGO ASPERGILLUS PARA APLICAÇÃO NA OLÉO VEGETAL DE SOJA PARA A PRODUÇÃO DE BIODIESEL

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ABSTRACT

The biodiesel production process uses extracellular lipases, generally processed, which undergo several purification processes (extraction, adsorption, chromatography, crystallization). These processes add value to the lipase that is passed on to the final product, biodiesel. Seeking an alternative to reduce costs is the application of whole cells of lipase-producing filamentous fungi, isolated wild Aspergillus from industrial effluents. The formulation of a solution of suspended macrocells with the cells developing certain metabolic functions is the fundamental discovery for application in the biodiesel process. This development of the cells' metabolic capabilities was identified by the excretion of extracellular lipase, which used the analysis of relative lipase activity (U/mg) as a metabolic indicator. Determining the submerged fermentation time of 40h for the preparation of the fungal macrocell solution, called primary inoculum. The thermostability of this primary inoculum showed that it can work in the range of 30ºC to 51ºC without affecting the relative lipase activities (U/mg). Based on this information, a primary inoculum was developed that can be applied to biodiesel production in soybean vegetable oils.

Keywords: Lipase1 enzyme. fungus 2, Biodiesel 3. Keyword 4. innovation 5.

1 INTRODUCTION

A Lipase used as a biocatalyst is more attractive compared to chemical catalysts as there is better product quality and the reaction occurs under milder temperature conditions. Other factors to be considered are: the ease of isolating glycerin without additional purification, there is no formation of chemical residues, no complex operations are necessary to recover the glycerol and eliminate the catalyst (Ban, Kaieda, Matsumoto, Kondo, & Fukuda, 2001) (Harding, Dennis, von Blottnitz, & Harrison, 2008) (Karmee, Patria, & Lin, 2015).

The use of purified extracellular enzymes has a high market value, as it goes through several purification processes (extraction, adsorption, chromatography, crystallization) which adds the cost of these processes to the final value of the lipase enzyme(Christopher, Hemanathan Kumar, & Zambare, 2014). Consequently, when using lipase in the production of biodiesel, the cost will be transferred to the final product, biodiesel. Karmee (2015), made a comparison between chemical catalysts and the biocatalyst, lipase, in all aspects raised the costs were similar or lower. However, the cost of lipase (Novozym-435) significantly influenced the value of biodiesel produced by 15% (Karmee et al., 2015). Trying to avoid these lipase enzyme purification processes, an alternative would be to apply whole cells of lipase-producing microorganisms in the biodiesel process.

2 MATERIAL & METHODS

2.1. Isolation, Selection, Identification and Production of Free Cells (whole-cell)

2.1.1 Collection of microorganisms:
The selection of microorganisms with potential for lipase production was carried out in the industrial effluent habitat. The Palmeira dos Índios Dairy Industry ILPISA (Vale Dourado), located in the city of Itapetinga in southwestern Bahia, allowed the collection of some sewage samples in the grease separation box part of the Effluent Treatment Station. Other companies such as Cooleite and Calçados Azaléia Nordeste S/A also contributed to collecting samples in their industrial effluents, but the microorganism with the greatest potential used in this research originated from Vale Dourado.

2.1.2 Isolation of microorganisms:
Isolation was carried out through a sequence of platings, with the medium on the plates containing 10% or 2% extra virgin olive oil and antibiotics. Cultivation for qualitative identification was done by plating using Rhodamine B 0.001%. However, it was in submerged fermentation that produced the primary inocula with the spores of the selected fungi. Through the analysis of Hydrolysis Activity that defined the largest producer of lipase.

2.1.3 Genetic Identification:

The fungal spores were developed into macrocells in the primary inoculum for 40h, 30ºC and 180 rpm. Total DNA was extracted by collecting one ml of cell culture by centrifugation and suspending them in 600 l of lysis buffer (0.2 MTris - HCl; 25 mM EDTA; 1% SDS; 25 mM NaCl pH8). After incubation at 65°C for 30 min with occasional shaking, the lysate was extracted once with phenol:chloroform (1:1), centrifuged at 13,000 rpm for 15 min, and the aqueous phase was extracted once with chloroform:isoamyl alcohol (24:1) and centrifuged as above. DNA from the aqueous phase was precipitated for 2h at 20ºC with two volumes of cold ethanol, washed with 70% ethanol, air-dried and suspended in 100 ll, TE buffer (10 mM tris-HCl, 1mM EDTA, pH 8) silva son 2005 The PCR was performed in a 24µL reaction. The PCR contains 3mM MgCl2, 1 x buffer, 0.2 mM of each dNTP (Invitrogen), 5mol of each primer, 0.05U/µL of Taq DNA polymerase (Invitrogen) and 1.5 µL of template DNA.
Sequencing and amplifications were carried out at ACTGene/Ludwig Biotec (UFRGS Biotechnology Center, Porto Alegre) using the ABI-PRISM 3100 Genetic Analyzer Armed automated sequencer with 50 cm capillaries and POP6 polymer (Aplicado Biossistemas). The identification of the microorganism isolated in the dairy industry through genetic sequence was analyzed using the programs Phrap/Phred (Erwin&Green, 1998) and Blastn (Altschul,S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, 1990).

2.2. Preparation of Fungal Primary Inoculum

Working with fungal spores directly in soybean oil did not yield viable results in the production of esters. It was necessary to prepare a base medium composed of: 3% refined soybean oil (m/v), 7% yeast extract (m/v), 0.05% MgSO4.7H2O (m/v), 0.1% KNO_2 (m/v), 0.1% KH2PO4 (m/v) and top up with water (He, et al. 2016). 100mL of sterile base medium was used in a 250mL Erlenmeyer flask with 108 spores of isolated wild Aspergillus to investigate the development of biomass and relative lipase activity (U/mg).

This medium was inoculated at 30ºC and 180rpm to promote the growth of macrocells. The development of biomass (macrocells) was quantified through weight, after the filtering process. The production of relative lipase activity (U/mg) was also quantified. The times monitored for the development of macrocells in the primary inoculum were: 24h, 40h, 48h, 72h, 92h and 120h. The reactors were in three replicates for each time studied.

2.3 Influence of Temperature on the Relative Lipase Activity (U/mg) of the Fungal Primary Inoculum

Having defined the preparation time for the primary inoculum, it was necessary to test the thermostability of the enzyme, lipase. The primary inoculum produced was placed in hermetically sealed test tubes and placed in thermal baths with temperatures of 10ºC, 30ºC, 40ºC, 51ºC, 60ºC, 70ºC, 80ºC, 90ºC and 100ºC, which remained for 15 minutes. Two to three replicates were performed, but the lipase quantification analysis was performed in triplicate.

2.4 Lipase Production

2.4.1. Optimized Lipase Production in Soybean Oil Pretreatment with Primary Inoculum

The amount of 1mL of primary inoculum adapted to the new environment it was exposed to and produced the enzyme, lipase. After this period called conditioning, alcohol (methanol) was added in installments, 1 mol of alcohol every 6 hours, totaling 3 mol. After adding the alcohol, 72 hours were allowed for the reaction to occur.
Then, a completely randomized design-DIC statistical experimental design was set up. This basic design involves two principles: repetition and randomization. Mainly because it works with the growth of fungal microorganisms, which requires homogeneity in experimental conditions and greater variation in treatments and replications (Table 1). 3 independent reactor replicates were used for each condition and the quantification of the specific lipase activity was carried out in triplicate, according to the Hydrolysis Activity methodology adapted from Leal, 2000.

Table 1: Experimental Planning Completely Randomized Design-DIC of lipase production regarding the amount of water and conditioning time with 10 units of SPB

Água/Tempo	**12h**	**48h**	**84h**
0%	**100% de óleo (10 g óleo)**	**100% de óleo (10 g óleo)**	**100% de óleo (10 g óleo)**
10%	10% água e 90% óleo (1mL água e 9 g óleo)	10% água e 90% óleo (1mL água e 9 g óleo)	10% água e 90% óleo (1mL água e 9 g óleo)
20%	**20% água e 80% óleo (2mL água e 8 g óleo)**	**20% água e 80% óleo (2mL água e 8 g óleo)**	**20% água e 80% óleo (2mL água e 8 g óleo)**
30%	30% água e 70% óleo (3mL água e 7 g óleo)	30% água e 70% óleo (3mL água e 7 g óleo)	30% água e 70% óleo (3mL água e 7 g óleo)

2.4.2. Quantification of lipase activity

Lipase-specific lipase activity was measured by hydrolysis activity, which is quantified by titration according to the methodology: using an emulsion prepared with 5% (m/v) pure gum arabic powder from the brand Synth, 10% (m/v) of Galo brand extra virgin olive oil and complete the volume with sodium phosphate buffer (0.1 mol/L and pH 7) and stir for 5 minutes. In a 125mL Erlemney

flask (triplicate), prepare the reaction by adding 5mL of the emulsion and 0.5mL of the sample. In another 125mL Erlemney flask (triplicate) prepare the blank with 5mL of the emulsion. Incubate at 35º C with 200rpm for 20 minutes. Stop the reaction with 5mL of an ethanol/acetone solution (1:1) and add 0.5mL of the sample to the blank (Leal, 2000). Relative lipase lipase activity is defined as the specific lipase activity divided by the protein content determined by the Bradford methodology.

3 RESULTS & DISCUSSION

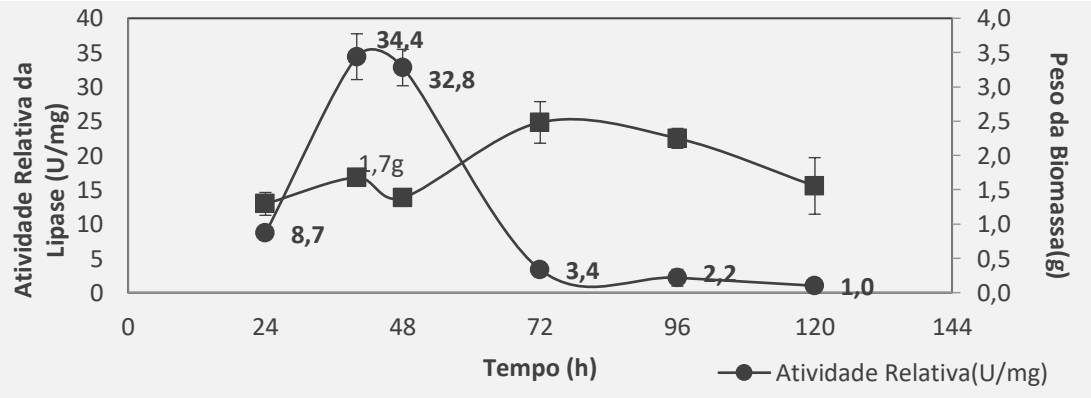
3.1. Selection of Fungus with Potential for Lipase Production

The microorganism isolated in industrial effluent was selected for the research based on preliminary tests on the production of lipase activity. The sample originated from the sewage of the Palmeira dos Índios Dairy Industry ILPISA (Vale Dourado).
The genetic sequence was in the 26S rDNA region, which presented the genus Aspergillus, but there was homology for several species.

3.2. Preparation of Fungal Primary Inoculum

In this case, refined soybean oil was used in the medium, which stimulated the production of the lipase enzyme. The development of the metabolism of fungal macrocells is fundamental, as it defines when there is the greatest production of secondary metabolites. Lipase activity in the primary inoculum in relation to incubation time is indicative of the metabolic development of fungal macromolecules and the amount of biomass contained. According to Figure 1(a), the incubation time of the primary inoculum was 40h, which obtained the highest productivity in relative lipase activity, 34.4 U/mg. The biomass produced was 1.7g.

Figure 1: Study of Relative Lipase Activity (U/mg) in the primary inoculum. and Fungal biomass (g) in relation to cultivation time Experimental conditions: 30ºC and 180rpm.



3.3 The Influence of Temperature on the Relative Lipase Activity (U/mg) of the Fungal Primary Inoculum

Another parameter studied of the fungal primary inoculum was the relative lipase activity (U/mg) in relation to temperature. Then various temperature ranges were tested to find out how increasing the temperature affected the thermostatic stability of the enzyme. One advantage of using enzymatic catalysts is the activation of the enzyme at lower temperatures than chemical catalysts.
The temperature to be used in the transesterification reaction catalyzed by biocatalysts must be the optimum lipase temperature, as with a greater increase in the reaction temperature, the initial reaction rate also increases, reducing the conversion time. However, if the reaction temperature is higher than the optimum lipase temperature, the enzyme denaturates, thus decreasing its activity and its conversion efficiency decreases (Guldhe, Singh, Mutanda, Permaul, & Bux, 2015).
The efficiency of lipase enzymatic activity (U/mg) in primary inoculum samples was tested by exposing it to different temperatures, immediately after analyzing the activity. Figure 2 shows the temperatures to which the primary inoculum was exposed and the relative lipase activity (U/mg).

Figure 2: Study of the Relative Lipase Activity (U/mg) in the primary inoculum to investigate the influence of temperature

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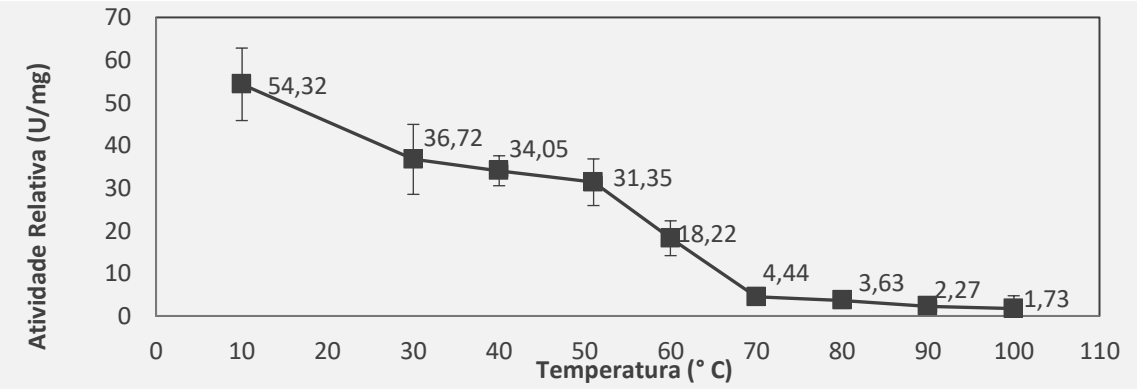

4 CONCLUSION

▪ Quantification of the relative activity of extracellular lipase (U/mg) was used as an indicator of the metabolic development of whole cells in submerged fermentation that lasted 40h for the preparation of the primary inoculum.

▪ The temperature that can be used in the biodiesel production process is 30ºC to 51°C without reducing the catalytic capacity of the lipase.

▪ The primary inoculum of isolated wild Aspergillus was used in soybean oil as a pre-treatment stimulating the production of extracellular lipase. The optimal excretion conditions for this lipase were 84 hours of conditioning with 20% water.

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