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Production of Lipolytic Enzymes Using Agro-Industrial Residues

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Lipase (E.C.3.1.1.3) is a versatile and key enzyme in various bioprocesses involving the esterification and transesterification reactions for biodiesel generation. However, the production and recovery of enzymes is very expensive and often constitute an obstacle to wide use in bioprocesses. In this context, the solid state fermentation (SSF) could be an low-cost alternative, since it allows the use of agro-industrial wastes with low added value and promoting the production of more concentrated biocatalyst. The utilisation these residues in the fermentations, not only minimize the quantity off these residues in the environmental, but also add value to raw material, trough the production of economical interesting substances. The objective of this work is to verify the lipase production using pretreated sugarcane bagasse as substrate inoculated with lipolytic microorganism. The sugarcane bagasse is an agro industrial residue with high availability in Brazil and can have multiple uses, representing 25 % to 30 % of the total weight of the sugar cane. The microorganisms used were Penicillium sp. and Rhizomucor sp. combined with temperature operating conditions of 28 °C, 33 °C and 38 °C, moisture content of 60 %, 70 % and 80 % and olive oil concentration 5 %, 7.5 % and 10 % as inducer. The study was performed by using factorial design of type (3^3) with central points. The results shown that best microorganism for lipase production was the Rhizomucor sp. (0.58 IU/$g_{substrate}$), although there is very slight difference when compared to the Penicillium sp. (0.47 IU/$g_{substrate}$). These results were found in the conditions of 33 °C, 80 % moisture content and 10 % of inducer for both microorganisms. After analyses statistic was verified that the moisture content of the medium interfered in the enzyme production for Penicillium sp. For the Rhizomucor sp. the moisture content and the concentration of the inductor olive oil interfered in the enzyme production.

1. Introduction

The use of enzymes as biocatalysts in industrial processes has been widely studied and it has shown promising for the production of high value-added compounds. According to Li et al. (2012), currently are known about 4,000 enzymes and of these, around 200 are used commercially. Among the enzymes used as biocatalysts stand out lipases (E.C.3.1.1.3), which constitute the most important group of biocatalysts for biotechnological applications (Hasan et al. 2006). They catalyse the hydrolysis of fats and oils releasing fatty acids, diglycerides, monoglycerides and glycerol. These enzymes also catalyse esterification, transesterification and interesterification when the present amount of water is sufficiently low as to shift the thermodynamic equilibrium towards synthesis. These enzymes have a wide range of substrates, are stable to temperature changes and different pH and concentrations of organic solvents and also catalyse reactions showing a high enantioselectivity (Krieger et al., 2004). As lipases display a high degree of specificity in esterification and transesterification reactions, they became a principal biocatalyst for the production of several fine oleochemicals (Mustafa et al., 2016).Lipases are commonly found in nature and can be obtained from animal sources (pancreatic lipase, gastric, and liver), plant and microbial (Damaso et al., 2008). Both eukaryotic microorganisms (yeast and fungi) and prokaryotic (bacteria, including actinomycetes) are lipase producers and their properties vary according to the origin. Microbial enzymes are most often used because of the wide variety of catalytic activity, ease of

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genetic manipulation, rapid growth, are more stable, their production is more convenient and safer (Wiseman, 1995). Microorganisms of the same strain have the potential to produce enzymes with features completely or partially differentiated, and therefore the search for new microbial sources remains focus of many researchers. A potential tool of interest in obtaining enzymes is in solid state fermentation (SSF), which allows the use of waste from other industrial processes as substrate and support for the growth of microorganisms, thus reducing the cost of production of enzymes (Silva et al., 2002). The SSF is based on the growth of microorganisms on a solid substrate with low water activity and has several advantages compared to the submerged fermentation such as space savings, simplicity in fermentation media, simple equipment and easy to control, high production yields , lower energy demand, and a higher concentrated metabolite of interest (Ângelo et al., 2014). Water absorption is critical to the success of this process and for this reason fibrous substrates are employed, and these provide the nutrients required for cell growth. Several agro-industrial waste can be used as support for the SSF. Mohseni et al. (2012) studied several agricultural products for lipase production, including rice bran, sugarcane bagasse, wheat bran, barley bran and corn meal. Amin et al.(2014) also realised a screening of different agricultural wastes such as rice bran, wheat bran, canola seed oil cake, sunflower hulls and peanut shells for the production of lipase by Aspergillus Melleus. Liu et al. (2014) used a mixture of sugarcane bagasse and sunflower seed cake as di-substrates for lipase production from Burkholderia cenocepacia, The utilisation these residues in the fermentations, not only minimise the quantity off these residues in the environmental, but also add value to raw material, through the production of economical interesting substances.

Although SSF displays the benefits described above, there are some limitations such as the choice of microorganisms capable of growing under reduced moisture conditions, control and monitoring of parameters such as pH, temperature, humidity and air flow. Within this context, the objective of this study was to evaluate lipase production by solid state fermentation using Penicillium sp. and Rhizomucor sp., and sugarcane bagasse pretreated with acid-base solution as support and source of nutrients. We investigated the effect of temperature, moisture and concentration of olive oil as inducer, through the factorial design 3^3.

2. Materials and methods

2.1 Microorganisms

The Penicillium sp. and Rhizomucor sp. were isolated and provided by the research group of Department Environment Engineering of Federal University of Espírito Santo and kept agar slant (potato dextrose agar 3.9 %) and frozen in glycerol solution 20 % (- 80 °C).

2.2 Preparation of Inoculum

The fungus was grown on agar plate containing PDA 3.9 % at 28 °C. The time required to obtain inoculum was studied and therefore samples were collected every 24 h and analysed spores concentration. The spores were scraped with 10 mL twen 80 (0.1 %v/v) and counted in a Neubauer chamber.

2.3 Solid state fermentation

For enzyme production was used sugarcane bagasse with particle diameter sizes between 0.6 mm and 2 mm and pretreated with acid-base solution. The sugarcane bagasse (natural and pretreated) was characterised according to Morais et al. (2010). This substrate was dried at 55 °C for 24 h. The bagasse was then added to Erlenmeyer flasks (10 g) and moistened with Mandel's mineral salt solution to obtain the desired moisture. The flasks were autoclaved at 121 °C for 20 min and, after cooling, inoculated with 1 mL of liquid inoculum with a concentration of 10^8 spores/mL. We studied the cultivation temperature (28 °C, 33 °C and 38 °C), moisture content (60 %, 70 % and 80 %) and the concentration of inductor olive oil (5 %, 7.5 % and 10 %) for the time cultivation of 120 h (Table 1). For the experiments we used a factorial design 3^3, with three central points, totaling 29 experiments for each microorganism. Statistical analysis of the data were performed in Statistic v. 13.0 and the values were considered significant when p-value < 0.05.

Table 1: Variables and levels used in the experiments

	-1	0	+1
Temperature of cultivation (°C)	28	33	38
Moisture content (%)	60	70	80
Concentration of inductor (%)	5	7.5	10

The enzyme extraction was carried out using 100 mL of solution NaCl 2 %(m/v). The mixture of fermented solid (10 g) and the NaCl solution (100 mL) were placed in an orbital shaker for 1 h at 200 rpm and 29 °C. The mixture was first filtered and then centrifuged for 30 min at 6,000 g. The supernatant obtained was used for determination of enzyme activity.

2.4 Enzymatic activity

The determination of the hydrolytic activity of the enzyme was performed measuring the hydrolysis of p-nitrophenyl butirate (pNPB) in 2-propanol at 25 °C with the addition of 0.01 g of lipase. For this reaction, a volume of 29 mL of 100 mM phosphate buffer pH 8.0, was added to 1.0 mL of pNPB 15 mM in 2-propanol in a stirred and jacketed reactor and then the enzyme addition. The change in absorbance at a wavelength of 410 nm was monitored for 10.5 min. One unit of activity ($IU/g_{substrate}$) was defined as the amount of enzyme required to produce one µmol of p-nitrophenol (pNP) per min.

3. Results and discussion

3.1 Kinects of spores production

The spores production of Penicillium sp. and Rhizomucor sp. were studied at 28 °C, carrying out spores counting every 24 h. The results are shown in Figure 1 (Penicillium sp.) and Figure 2 (Rhizomucor sp.).

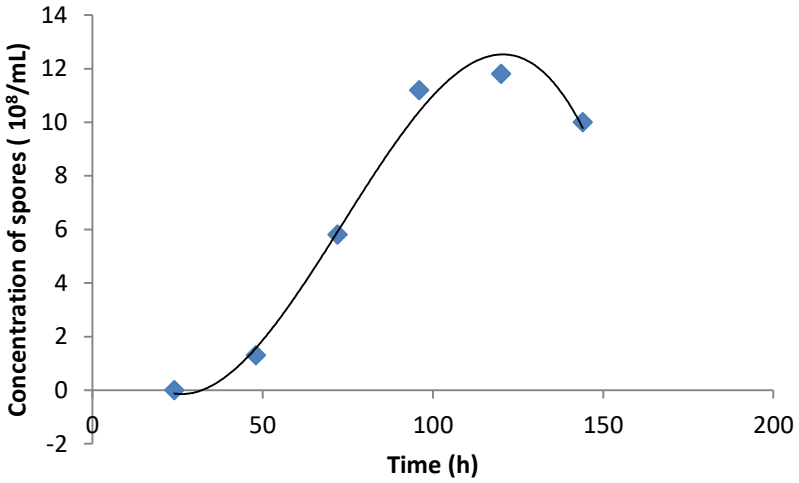

Figure 1: Results of kinetic profile of spores production of Penicillium sp.

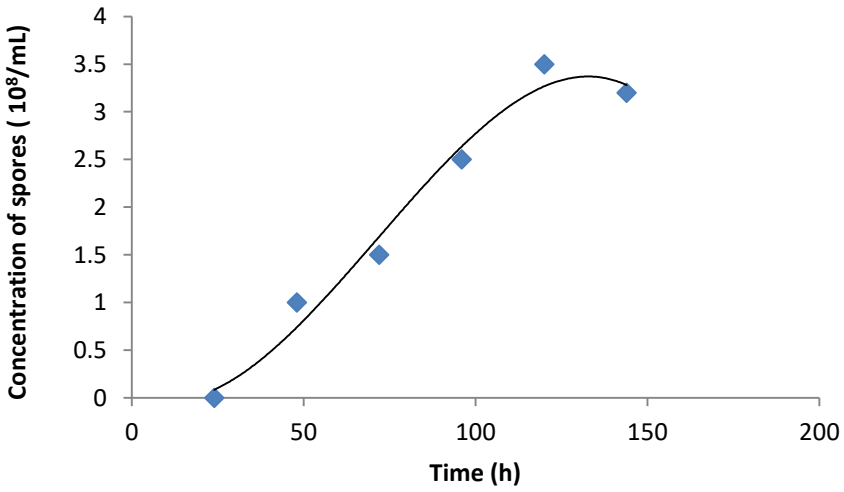

Figure 2: Results of kinetic profile of spores production of Rhizomucor sp.

It can be seen that the maximum production was approximately of 120 h, therefore we selected these time for carrying out the inoculation with the fungus.

3.2 Characterisation of lignocellulosic biomass

The results of characterisation of sugarcane bagasse (natural and pretreated with acid-base solution) are presented in Table 2.

Table 2: Composition lignocellulosic of sugarcane bagasse

Fraction	Natural Sugarcane Bagasse	Pretreated Sugarcane Bagasse
Lignin (%)	22.8	8.1
Cellulose (%)	29.7	62.6
Hemicellulose A (%)	25.2	13.6
Hemicellulose B (%)	13.5	9.6
Total	91.2	93.9

The pretreatment of sugarcane bagasse with the acid-base solution was effective in removal of lignin and hemicellulose, improving the availability of the cellulose for the microorganisms.

3.3 Solid state fermentation (SFF)

Results of enzyme production from the fungus are presented in Table 3. It can be seen that the lipase production was better in the conditions of 33 ºC, 80 % moisture content and 10 % of inducer for both microorganisms.

Table 3: Results obtained in the fermentations using Penicillium sp. and Rhizomucor sp.

Run	T (°C)	Moisture content (%)	Concentration of inductor (%)	Enzyme activity ($IU/g_{substrate}$) *Penicillium* sp.	Enzyme activity ($IU/g_{substrate}$) *Rhizomucor* sp.
1	28	60	5	0.142	0.219
2	28	70	5	0.156	0.240
3	28	80	5	0.211	0.333
4	28	60	7.5	0.144	0.227
5	28	70	7.5	0.167	0.260
6	28	80	7.5	0.243	0.345
7	28	60	10	0.189	0.289
8	28	70	10	0.199	0.298
9	28	80	10	0.301	0.412
10	33	60	5	0.144	0.222
11	33	70	5	0.166	0.257
12	33	80	5	0.234	0.320
13	33	60	7.5	0.168	0.238
14	33	70	7.5	0.186	0.259
15	33	80	7.5	0.366	0.502
16	33	60	10	0.179	0.256
17	33	70	10	0.234	0.295
18	33	80	10	0.470	0.583
19	38	60	5	0.154	0.224
20	38	70	5	0.172	0.267
21	38	80	5	0.342	0.335
22	38	60	7.5	0.135	0.247
23	38	70	7.5	0.187	0.301
24	38	80	7.5	0.198	0.321
25	38	60	10	0.135	0.239
26	38	70	10	0.177	0.288
27	38	80	10	0.187	0.335
28	33	70	7.5	0.170	0.267
29	33	70	7.5	0.188	0.271

The experimental data were statistically analysed by analysis of variance (ANOVA) and the results are shown in Table 4. After statistical analysis it was found that the moisture (L) was the variables that interfered in the enzyme production ($p < 0.05$) when using Penicillium sp., obtaining the Eq(1). Higher moisture values led to better production.

$$\mathrm{IU/g_{substrate}} = 0.204966 + 0.064556\,\mathrm{M} \quad (1)$$

For fermentations with the Rhizomucor sp. it has been found that the moisture (L + Q) and the concentration of inductor (L) influenced the results of lipase production, resulting Eq(2).

$$\mathrm{IU/g_{substrate}} = 0.300148 + 0.073611\,\mathrm{M} - 0.020361\,\mathrm{M^2} + 0.032111\,\mathrm{I} \quad (2)$$

Table 4: The results of analysis of variance (ANOVA) for enzyme production using Penicillium sp. and Rhizomucor sp.

	Sum of Squares	DF	Mean square	F-Value	P-Value
Penicillium sp.					
Temperature (ºC)(L)	0.000235	1	0.000235	0.08821	0.769246
Temperature (ºC)(Q)	0.012543	1	0.012543	4.71406	0.040979
Moisture Content (%)(L)	0.075014	1	0.075014	28.19179	0.000025
Moisture Content (%)(Q)	0.010851	1	0.010851	4.07797	0.055793
Concentration of Inductor (%)(L)	0.006806	1	0.006806	2.55768	0.124023
Concentration of Inductor (%)(Q)	0.001576	1	0.001576	0.59247	0.449652
Error	0.058538	22	0.002661		
Total SS	0.163253	28			
Rhizomucor sp.					
(1)Temperature (ºC)(L)	0.000242	1	0.000242	0.10110	0.753517
Temperature (ºC)(Q)	0.007525	1	0.007525	3.14357	0.090074
(2)Moisture content (%)(L)	0.097535	1	0.097535	40.74538	0.000002
Moisture content (%)(Q)	0.012984	1	0.012984	5.42402	0.029445
(3)Concentration of inductor (%)(L)	0.018560	1	0.018560	7.75358	0.010808
Concentration of inductor (%)(Q)	0.000168	1	0.000168	0.07012	0.793633
Error	0.052663	22	0.002394		
Total SS	0.187860	28			

4. Conclusions

It can be concluded that both microorganisms produce lipase by solid state fermentation and the sugarcane bagasse is a good substrate. The moisture content is a variable that influenced the lipase production when was used the Rhizomucor sp. as well as Penicillium sp. being therefore a variable that requires further study.

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