OBTAINING OF FUNGAL BIOCOMPOUNDS BY SOLID STATE FERMENTATION

OBTENÇÃO DE BIOCOMPOSTOS FÚNGICOS POR FERMENTAÇÃO EM ESTADO SÓLIDO

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ABSTRACT

The cultivation of microorganisms to obtain biosurfactants and lipases using solid-state fermentation (SSF) is considered a sustainable and low-cost form. The simultaneous production of these biocompounds is still little explored, especially when using solid matrices as a substrate aiming at reducing residues and environmental impacts and the potential environmental application of the produced biocompounds, in bioremediation processes and effluent treatment. The aim was the simultaneous production of lipases and biosurfactants via SSF from the fungus *Aspergillus niger*. Screening of cultivation media and conditions was carried out, with the studied variables being the proportions of soybean meal (SM) and soybean waste (SW), moisture, concentrations of carbon source (molasses), oily inducer and nitrogen source, through experimental designs. The media were fermented for 6 days, evaluating the lipase and emulsifying activities and the reduction in surface tension obtained every 2 days. In the culture medium composed of SM + SW (70/30), production of lipases (31.13 ± 0.55 U) and biosurfactants (11,92 % of reduction of surface tension and emulsifying activity below 2.00 EU) was observed under conditions of 50% of moisture, 1.5% of nitrogen source and 1% of oily inducer.
Keywords: lipases, biosurfactants, *Aspergillus niger*, surface tension, emulsifying activity

RESUMO

O cultivo de microrganismos para obtenção de biossurfactantes e lipases utilizando fermentação em estado sólido (FES) é considerado uma forma sustentável e de custo reduzido. A produção simultânea desses biocompostos ainda é pouco explorada, principalmente quando utilizado matrizes sólidas como substrato visando a redução de resíduos e impactos ambientais e a potencial aplicação ambiental dos biocompostos produzidos, em processos de biorremediação e tratamento de efluentes. Objetivou-se a produção simultânea de lipases e biossurfactantes via FES a partir do fungo *Aspergilus niger*. Realizou-se o screening de meios e condições de cultivo, sendo as variáveis estudadas as proporções de farelo de soja (FS) e resíduo de soja (RS), a umidade, as concentrações de fonte de carbono (melaço), indutor oleoso e da fonte de nitrogênio, através de delineamentos experimentais. Os meios foram fermentados por 6 dias, avaliando-se as atividades lipásica e emulsificante e a redução da tensão superficial obtidas a cada 2 dias. No meio de cultivo composto por FS+RS (70/30) foi observada produção de lipases (31.13 ± 0.55 U) e biossurfactantes (redução da tensão superficial de 11.92% e atividade emulsificante inferior a 2.00 UE) nas condições de 50% de umidade, 1.5% de fonte de nitrogênio e 1% de indutor oleoso.
Palavras-chave: lipases, biosurfactants, *A. niger*, surface tension, agroindustrial waste, emulsifying activity

1. INTRODUCTION

Solid-state fermentation (SSF) can be used as a cultivation method to obtain fungi biocompounds, being considered a sustainable and low-cost method, due to the low consumption of water and energy, due to the absence of free water in the system, absence of agitation of the medium and use of residues as a substrate for microbial growth (PANDEY et al., 2000; SHARMA and ARORA, 2010; CHEN, 2013). Among the possible residues to be used in the SSF, agro-industrial residues, such as shells, bran, cob, bagasse, and seeds stand out for promoting microbial growth acting as a nutritional source (PANDEY, 2003; SINGHANIA et al., 2016; SADH et al., 2018).

Several microorganisms are studied for the production of biocompounds via SSF, among them fungi, yeasts and bacteria, however, filamentous fungi stand out for their ability to grow in solid or semi-solid matrices with low moisture (SHUSTER et al., 2002; COLLA et al, 2010; CAVKA et al, 2014). The *Aspergillus niger* fungus is characterized by its potential producer of lipolytic enzymes and extracellular biosurfactants (THOMAS et al, 2013, SALIHU et al., 2016; COSTA et al., 2017; ASGHER et al., 2020).

Biosurfactants and lipases are widely used in the pharmaceutical, chemical, and food industries, with a still limited environmental application (FAKRUDDIN, 2012; VERMA et al., 2012; AKBARI et al., 2018). Among the potential environmental uses of these biocompounds, there are the degradation processes of oily contaminants in soils (DECESARO et al., 2017. MACHADO et al., 2020) and water (PRASAD and MANJUNATH, 2011; OSTENDORF et al., 2019), which promotes this study, given the potential they have to act in the degradation of compounds of oily origin (MARTINS et al., 2008; KUMAR - KANWAR, 2012; SINGH et al, 2018).

The aim of this study was to evaluate the influence of cultivation conditions and carbon sources on the release of lipases and extracellular biosurfactants produced in SSF by the fungus Aspergillus niger using agro-industrial waste as a substrate.

2. METHODOLOGY

2.1 Characterization of waste used as substrate

The substrates used in the preparation of the culture media were soybean waste (SW) and soybean meal (SM) (BSBios, Passo Fundo / RS, Brazil). The SM is obtained from the process of extracting soybean oil with hexane, and the SW is the result of the soybean cleaning process, consisting of stalks, pods, soy husks, and a small percentage of broken grains and soy straw. The sugar cane molasses (SCM) (Alisul Alimentos S.A., Carazinho / RS, Brazil), was collected in a storage tank for the animal feed production process.

The substrates were characterized in terms of protein, lipid content, fixed mineral residue (ash), moisture, total carbohydrates, and particle size, according to standard methodology (AOAC, 1995). The concentration of glucose present in the molasses residue was also quantified by the determination of total reducing sugars (ART) using 3,5 dinitrosalicylic acid (DNS), after pre hydrolysis of sucrose in an acid medium (HCl 2 N) in a water bath (67.5 ºC, 12 minutes) and the determination of ART from a standard anhydrous glucose curve, according to Miller (1959).

2.2 Microorganism, preparation of inoculum and culture media

The microorganism used was the fungus *Aspergillus niger* strain O-4, GenBank number KC545858.1 (REINEHR et al., 2017), belonging to the strain bank of the Biochemistry and Bioprocess Laboratory of the University of Passo Fundo, maintained in test tubes with Potato Dextrose Agar (PDA) under refrigeration at 4ºC.

The inoculum was prepared by adding 10 mL of a 0.01% (v / v) solution of Tween 80 in a test tube containing the isolated strain. The spore suspension (5 mL) was added to a 1 L conical flask containing 100 mL of PDA previously sterilized in an autoclave for 20 min at 121ºC, followed by incubation for 5 days at 30ºC for growth and hyphae formation. After this period, 50 mL of 0.01% Tween 80 solution and 3 sterile glass beads were added to the flask to obtain a spore solution, used later for inoculation of the media.

The culture media were prepared from 40 g of dry material containing varying proportions of SM/SW (70/30, 90/10, 80/20). The percentages of inductors, moisture, and SM were added to the medium according to the variations proposed in the Fractional Factor Design (item 2.3) and the Full Factorial Design (item 2.4). Then, 30 ml of a saline solution composed of 2 g /L of potassium phosphate (KH2PO4), 1g/L of magnesium sulfate (MgSO4) and 10ml/L of trace solution, composed of 0.63 mg/L were added of iron sulfate (FeSO4.7H2O), 0.01 mg/L of manganese sulfate (MnSO4) and 0.62 mg/L of zinc sulfate (ZnSO4) as a source of micronutrients. The experiments were carried out in 250 mL beakers, with the culture media sterilized at 121ºC for 20 minutes. Inoculation was performed using 2 ml of spore suspension for each 40 g of the prepared medium, making an initial spore concentration of 2,106 spores/g. Erlenmeyer's were incubated in an oven at 30 ºC for 6 days, samples being taken every 2 days to evaluate the production of biosurfactants by determining the water-in-oil emulsifying activity and surface tension, and production of lipolytic enzymes, through lipase activity.

2.3 Experimental strategy

To screen the significant variables on the simultaneous production of lipases and biosurfactants, a Fractional Experimental Design 2^{5-1} IV presented in Table 2 was used, with the addition of central points, totaling 20 experiments. The procedures for media preparation, inoculation, and incubation were performed according to item 2.2.

From the variables that showed significant effects ($p < 0.10$) (MONTGOMERY and RUNGER, 2016) in the first experimental design, a second Factorial Experimental Design with the addition of central points was proposed. For this Design, the proportion of SM/SW in 70/30 and the addition of 1% SCM were kept as fixed variables. The varied percentage of glycerol used as an inducer was 1% (-1), 5% (+1) and 3% (0), moisture at 50% (-1), 60% (+ 1) and 55% (0) and sodium nitrate by 1.5% (-1), 4.5% (+1) and 3% (0). The procedures for media preparation, inoculation, and incubation were performed according to item 2.2.

2.4 Analytical determinations

After the SSF process, the bran fermented in the crops was subjected to methods of extracting the biocompounds produced to identify the emulsifying and lipolytic activities and to reduce surface tension (COLLA et al, 2010). To obtain the extract and to determine the emulsifying activity, in 250 ml Erlenmeyer, 5 g of fermented bran and 30 ml of distilled water at 90 ° C was added, is then placed in a water bath for 30 min for extraction and after the filtration of the solids was carried out. with cotton to obtain the supernatant for use in the analyzes. In the extraction to determine the lipolytic activity in 250 ml Erlenmeyer, 1 g of fermented bran was added with the addition of 10 ml of 2 M phosphate buffer solution, pH 7.0, which was stirred at 160 rpm for 30 minutes at 37ºC, and the solids were subsequently filtered in cotton.

2.4.1 Determination of emulsifying activity and surface tension

The determination of the water-in-oil ($EA_{W/O}$) emulsifying activity was carried out using the method adapted from the emulsification index, as previously proposed by Cooper and Goldenberg (1987), where 3.5 mL of the extract previously obtained and 2mL of biodiesel respectively, were added

in test tubes and then stirred for 1 min on a Vortex shaker at 700 rpm. After 24 hours of rest, the total emulsion height (water/oil emulsion plus the remaining height of the oil layer) and the water/oil emulsion formed was measured with a digital caliper. From Equations 1 and 2, the water-in-oil emulsifying activity produced was calculated. Blanks will be made using distilled water instead of the extract.

$$E = (\frac{h_{emulsion}}{h_{total}}) * 100 \quad (1)$$

$$EA_{W/O} = (E_{sample} - E_{blank}) \quad (2)$$

Where: $EA_{w/o}$ = water-in-oil (EU) emulsifying activity, $H_{emulsion}$ = height of the emulsion layer, H_{total} = height of the total layer, E = centesimal relationship between the height of the water/oil emulsion and the total height.

The surface tension was assessed using the ring method (Du-Nuoy's ring method). In this method, a volume of 30 mL of the extract used for the determination of the water-in-oil emulsifying activity was added to a Biolin Scientific tensiometer, model Sigma 702. The reduction of the surface tension of the media concerning the time of the beginning of the culture was calculated according to Equation 3.

$$STR\ (\%) = \frac{TS_{initial} - TS_{final}}{TS_{initial}} * 100 \quad (3)$$

Where: $TS_{initial}$ = Surface tension obtained in the initial cultivation time (mN/m) and TS_{final} = Surface tension obtained in the final cultivation time (mN/m).

2.4.2 Determination of lipase activity

To determine lipase activity, the methodology described by Burkert et al. (2004). To 75 ml of 7% solution (w/v) of gum arabic was added 25 ml of olive oil. This mixture was stirred at 500 rpm on a vortex shaker for 5 min to form an emulsion. For the enzymatic reaction, 5 ml of the prepared emulsion, 1 ml of the enzyme extract, and 2 ml of 2 M phosphate buffer solution pH 7.0 were added in a 250 ml conical flask. The reaction occurred for 30 min at 160 rpm on a shaking table at 37 ºC and was subsequently paralyzed with 15 mL of 1:1 alcohol: acetone solution. Then, the obtained solution was titrated with 0.01 mol / L NaOH. A unit of lipase activity was defined as the amount of enzyme that releases 1 µmol of fatty acid per minute per gram of moist fermented bran (1 U = 1 µmol $min^{-1}g^{-1}$), according to Equation 4.

$$LA = \frac{v*M*f*11000}{t*m} \quad (4)$$

Where: LA = A unit of lipase activity (U), v = NaOH volume spent on titration (mL); M = molar mass of NaOH used for the titration (mmol / mL), f = NaOH correction factor, t = Time spent in the reaction of 1 mL of enzyme extract (min), m = Mass of moist fermented bran (g).

2.5 Treatment of the data obtained

The fractional experimental design was analyzed using analysis of variance (ANOVA) for a 90% confidence level, and the complete experimental design for a 95% confidence level.

3. RESULTS AND DISCUSSION

3.1 Characterization of waste used as a substrate

Table 1 and Figure 1 show the results of the chemical characterization and particle size of the substrates used in the bioprocesses. The soybean waste presented results similar to those demonstrated by Silva et al. (2006) concerning the content of lipids (1.67%) and ash (5.20%). The protein and carbohydrate content of SW obtained in our study were high (18.31% and 57.21%, respectively), indicating the potential of this residue to be used as a macro nutrient source. Karr-Lilienthal et al. (2006) evaluated the composition of the SM obtaining protein content between 45.1% and 52.6%, similar to that obtained in this study (38.21%). The lipid content obtained by the authors was low, varying between 4.1% and 9.0%, which was also verified in the present study (2.14%). García-Rebollar et al. (2016) evaluated the influence of the origin of soybean meal on its chemical composition, observing ash content (between 7.18% and 7.57%) close to that obtained in our study (5.87%). Regarding the particle size of the residues, both have a particle diameter greater than 14 mm (82.65% for the SM and 83.36% for the SR), while the SM is still 16.93% composed of particles smaller than 20 mm.

The high content of carbohydrates (57.21%) present in SW is mainly due to the structure of this residue, formed by lignocellulosic materials already characteristic of vegetables, these materials being characterized by the presence of cellulose, hemicellulose and lignin, where cellulose is generally the structural polysaccharide dominant plant cell walls (35 – 50%), followed by hemicellulose (20 – 35%) and lignin (10 – 25%) (SAHA, 2003; MUSSATTO and TEIXEIRA, 2010). According to nee'Nigam et al. (2009) residues from soy have a lignocellulosic composition of 34.5% cellulose, 24.8% hemicellulose and 19.8% lignin. Lignocellulosic materials, mainly because they have a complex structure, need to be subjected to the hydrolysis process to convert the polysaccharides into fermentable sugars, hydrolysis can be carried out using acid or enzymatic catalysts, where enzymatic hydrolysis stands out because it requires less energy input and greater conversion efficiency, however, enzymatic hydrolysis may be hampered by the high recalcitrant structure of lignocellulosic biomass that inhibits them from decomposing into favorable by-products for later use (BALLESTEROS, 2010; HU et al., 2016; PRASAD et al. 2019).

The molasses showed a high concentration of total reducing sugars (325.30 g / L) as well as a high percentage of total carbohydrates (70.07%) according to the previous characterization performed by Kreling et al. (2020), characterizing molasses as a simple and easily assimilated carbon source for microorganisms contributing to their growth and also helping to form the hydrophilic portion of biosurfactants (DESAI; BANAT, 1997; CHAPRÃO et al., 2015). In their study Costa et al. (2000) evaluated, for use in animal feed, the composition of sugar cane molasses, where 54.3% of the composition is total carbohydrates, 20.89% moisture, 8.5% ash, 2.6% protein and 0.70% lipids. In addition to being a carbohydrate source, sugarcane molasses has high concentrations of calcium (1.0 – 1.1%), magnesium (0.4 – 0.5%), potassium (3 – 4%), chlorine (2 – 3%) and sulfur (0.45 – 0.60%), but it is low in phosphorus (0.1%) and also has trace metal concentrations such as aluminum, iron and potassium (DE OLIVEIRA et al., 2007).

Table 1 – Chemical characterization of residues used as a culture medium for solid state fermentation

Characteristics	**Residues**	
	Soybean meal (SM)	**Soybean waste (SW)**
Moisture (%)	13.54 ± 0.04	16.28 ± 0.13
Lipids (%)	2.14 ± 0.06	2.15 ± 0.15
Proteins (%)	38.21 ± 0.02	18.31 ± 0.04
Ashes (%)	5.78 ± 0.02	6.05 ± 0.08
Total carbohydrates (%)	40.25 ± 0.06	57.21 ± 0.02

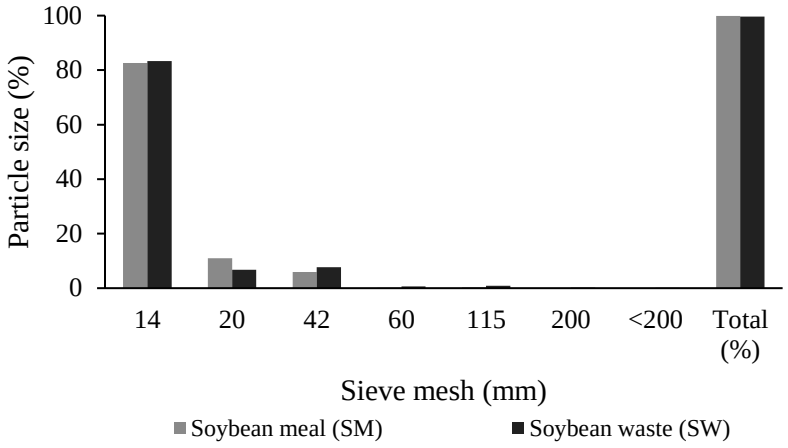


Figure 1 – Particle size of residues used as a culture medium for solid state fermentation

3.2 Results of the screening of variables from the Fractional Factorial Design

Table 2 shows the maximum productions of biosurfactants and lipases and the times when the maximum productions occurred for the 20 tests performed in the Fractional Experimental Design 2^{5-1} IV. Only 8 tests showed emulsion production in 6 days of cultivation. The greatest emulsifying activities were verified for experiments E1 (70/30 SM/SW, 0% SCM, 55% moisture, 1% inducer and 3% nitrogen) and E11 (70/30 SM/SW, 1% SCM, 55% moisture, 5% inducer and 3% nitrogen), of 6.36 ± 0.83 EU and 4.88 ± 0.13 EU, respectively, in 6d of cultivation, the results of this cultivation time being used to perform the statistical analysis of this response variable.

Only a considerable reduction in surface tension was observed in the final time of the bioprocess (6d), which was defined as the best cultivation time for the analysis of the effects performed. The greatest reductions were seen in the E9 tests (70/30 SM/SW, 0% SCM, 55% moisture, 5% inductor, 0% nitrogen), from 37.53 ± 0.45 to 34.10 ± 0.00 Mn/m (9.13%), and in the E16 test (90/10 SM/SW, 1% SCM, 65% moisture, 5% inductor, 3% nitrogen), from 37.26 ± 0.15 to 34.15 ± 0.70 Mn/m (8.35%), in the 6d periods and 4d of cultivation, respectively. Regarding the production of lipolytic enzymes, the highest yields of 39.28 ± 1.77 U and 35.15 ± 0.28 U were verified in the E1 tests (70/30 SM/SW, 0% SCM, 55% moisture, 1% inducer and 3% nitrogen) and E11 (70/30 SM/SW, 1% SCM, 55% moisture, 1% inducer and 0% nitrogen), both in 4 days of cultivation. For this reason, and verifying that 9 of the 20 experiments evaluated showed high enzymatic production in the 4 d of cultivation, this is selected as the best production time for the analysis of the subsequent effects.

The analysis of effects performed at a 90% confidence level indicated, for the production of $EA_{W/O}$, that the variables humidity (negative effect -2.3778 and $p_{umidity}$ = 0.0285) and sodium nitrate as a source of nitrogen (positive effect) were significant. +1.9768 and p = 0.0495), thus, lower percentages of humidity and higher nitrogen concentration favored the increase in the production of water-in-oil emulsions. Regarding the reduction of the surface tension of the culture medium, the addition of the nitrogen source had a significant and negative effect (-2.1591 and p = 0.0934), indicating that lower concentrations of this promote a reduction in the surface tension of the culture medium. For the production of lipases, moisture had a significant negative effect (-17.8701; p = 0.0213), indicating that it should be reduced to increase enzyme production.

Moisture was an important variable for the production of both biocompounds, with the best results obtained at lower levels of humidity. This variable directly affects the oxygenation of the culture medium, influencing microbial growth, and the consequent production of biocompounds of interest (SALIHU et al., 2012).

Table 2 – Results of production of biosurfactants and lipases and times of greatest production for the Fractional Factor Design

Experiments	Dependent variables					Response variables					
	Proportion SM/SR (%)	**Molasses (%)**	**Moisture (%)**	**Inducer* (%)**	**Nitrogen** (%)**	**EA W/O (EU)**	**Time (d)**	**STR (%)*** **	**Time (d)**	**Lipase activity (U)**	**Tempo (d)**
E1	70/30 (-1)	0 (-1)	55 (-1)	1 (-1)	3 (+1)	6.36 ± 0.83	6	10.44	2	39.28 ± 1.77	4
E2	90/10 (+1)	0 (-1)	55 (-1)	1 (-1)	0 (-1)	3.02 ± 0.15	6	9.14	6	5.07 ± 0.29	2
E3	70/30 (-1)	1 (+1)	55 (-1)	1 (-1)	0 (-1)	ND	-	1.96	6	31.55 ± 1.83	4
E4	90/10 (+1)	1 (+1)	55 (-1)	1 (-1)	3 (+1)	2.83 ± 0.10	6	6.11	6	27.99 ± 0.26	4
E5	70/30 (-1)	0 (-1)	65 (+1)	1 (-1)	0 (-1)	ND	-	3.11	6	6.40 ± 0.89	2
E6	90/10 (+1)	0 (-1)	65 (+1)	1 (-1)	3 (+1)	ND	-	2.75	6	12.86 ± 0.28	4
E7	70/30 (-1)	1 (+1)	65 (+1)	1 (-1)	3 (+1)	3.19 ± 0.49	6	0.01	6	8.32 ± 0.25	4
E8	90/10 (+1)	1 (+1)	65 (+1)	1 (-1)	0 (-1)	ND	-	2.47	6	24.73 ± 1.03	4
E9	70/30 (-1)	0 (-1)	55 (-1)	5 (+1)	0 (-1)	0.25 ± 0.02	4	9.13	6	4.99 ± 0.22	2
E10	90/10 (+1)	0 (-1)	55 (-1)	5 (+1)	3 (+1)	3.36 ± 0.19	6	10.38	2	30.63 ± 0.81	4
E11	70/30 (-1)	1 (+1)	55 (-1)	5 (+1)	3 (+1)	4.88 ± 0.13	6	ND	-	35.15 ± 0.28	4
E12	90/10 (+1)	1 (+1)	55 (-1)	5 (+1)	0 (-1)	1.52 ± 0.45	6	4.01	-	20.84 ± 1.13	4
E13	70/30 (-1)	0 (-1)	65 (+1)	5 (+1)	3 (+1)	ND	-	ND	-	6.44 ± 0.00	2
E14	90/10 (+1)	0 (-1)	65 (+1)	5 (+1)	0 (-1)	ND	-	1.50	6	11.61 ± 1.13	2
E15	70/30 (-1)	1 (+1)	65 (+1)	5 (+1)	0 (-1)	ND	-	3.54	6	11.06 ± 0.56	2
E16	90/10 (+1)	1 (+1)	65 (+1)	5 (+1)	3 (+1)	ND	-	8.35	4	9.61 ± 0.54	2
E17	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	6.91	6	14.25 ± 0.57	2
E18	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	5.53	2	10.63 ± 1.38	2
E19	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	8.66	6	27.1 ± 2.85	2
E20	80/20 (0)	0,5 (0)	60 (0)	3 (0)	1.5 (0)	ND	-	9.73	6	7.64 ± 0.00	2

*Inducer: Glycerol; **Nitrogen source: Sodium nitrate; ***STR: Surface tension reduction; ND: Not detected by method

Greater reductions in surface tension were obtained with lower concentrations of nitrogen in the culture medium. Cooper and Paddock (1984) indicate that the effective production of biosurfactants with surfactant properties demonstrate surface tensions below 35 Mn/m, which was not verified in this study, since the tensions presented for the experiments reach values very close to 35 Mn/m, remaining between 34 and 33 Mn/m. This indicates that, regardless of the culture medium used for the production of both biocompounds, the fungus *Aspergillus niger* is not capable of producing biosurfactants with the capacity to reduce surface tension (UZOIGWE et al., 2015), making the use of this variable unviable response to the indicative of the production of biosurfactants.

The nitrogen source was significant for the formation of water-in-oil emulsions, indicating the need to increase its concentration for greater production of biosurfactants. This nutritional source is necessary for protein synthesis, leading to cell growth and the consequent production of biocompounds (FONTES et al., 2008; SALIHU et al., 2011). The concentration of the glycerol inducer to produce biosurfactants and lipases was not significant ($p = 0.3962$). It appears that the formation of emulsions was less and that they occurred in a longer cultivation time. The low productivity of water-in-oil emulsions and the consequent production of biosurfactants when using the glycerol inducer can be justified by the solubility of this inducer in aqueous media. Thus, it is used for the production of the hydrophilic portion of the biosurfactant molecule, the formation of the hydrophobic portion of the molecule being compromised due to the absence of an oil-insoluble inducer in water, making it difficult to produce biosurfactants, which does not occur when using soybean oil (ACCORSINI et al., 2012).

For the subsequent complete factorial design, the percentage of moisture was reduced (50, 55 and 60%), the percentage of the nitrogen source added in the form of sodium nitrate was increased (1.5, 3 and 4.5%) and the variation of glycerol concentration added to the culture medium was maintained at 1, 3 and 5%. The other variables, which did not show significance in the production of the biocompounds, were maintained in the following conditions: the proportion of SM/SW in 70/30 to guarantee the greater oxygenation of the culture medium since the soybean residue has greater granulometry, and consequent greater porosity to the culture medium; and adding 1% SCM. Aiming at increasing the production of biosurfactants, since this is considered an easily assimilated carbon source.

3.3 Complete Factorial Design

Figure 2a shows the production of biosurfactants over 6 days of cultivation. Regarding $EA_{W/O}$, the low production also verified in the Fractional Factor Design is confirmed. The production of emulsions was not observed for any experiment in the 2d of culture. In the 4d and 6d of cultivation, low yields were verified for the tests, the highest observed for the E28 tests (60% moisture, 4.5% nitrogen and 5% inductor), from 2.55 ± 0.01 EU in the 6d culture, and 1.84 ± 0.32 UE in the E31 experiment (55% moisture, 3% nitrogen and 3% inductor), in the 4d of cultivation.

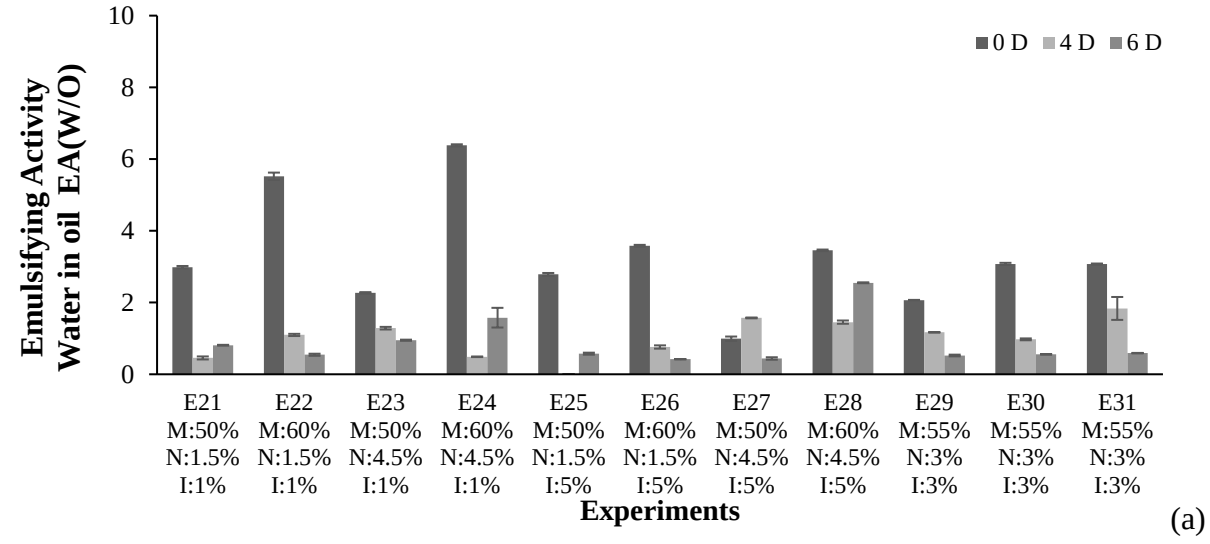
The difficulty in the production of biosurfactants may be related to the use of bran and soybean residue as a base medium for cultivation, since both are composed of lignocellulosic materials, consisting of highly complex carbonic bonds, as stated by Cassales et al. (2001), who indicate that compounds from soybeans may have a high composition (74.3%) of cellulose, hemicellulose, and lignin, which may compromise its use as a carbon source for the microorganism used, due to the need for hydrolysis of this residue for conversion to glucose, hindering the metabolization of this nutritional source as a hydrophilic carbon source, necessary for the formation of the polar fraction of the biosurfactant molecule (NITSCKE; PASTORE, 2002; ARAUJO et al., 2013), thus compromising the formation of this biocompounds. Still,

Utami et al. (2017) indicate that high concentrations of inducers can be toxic to the culture medium, negatively influencing the transfer of oxygen and absorption of nutrients, reducing microbial growth, and the production of biocompounds.

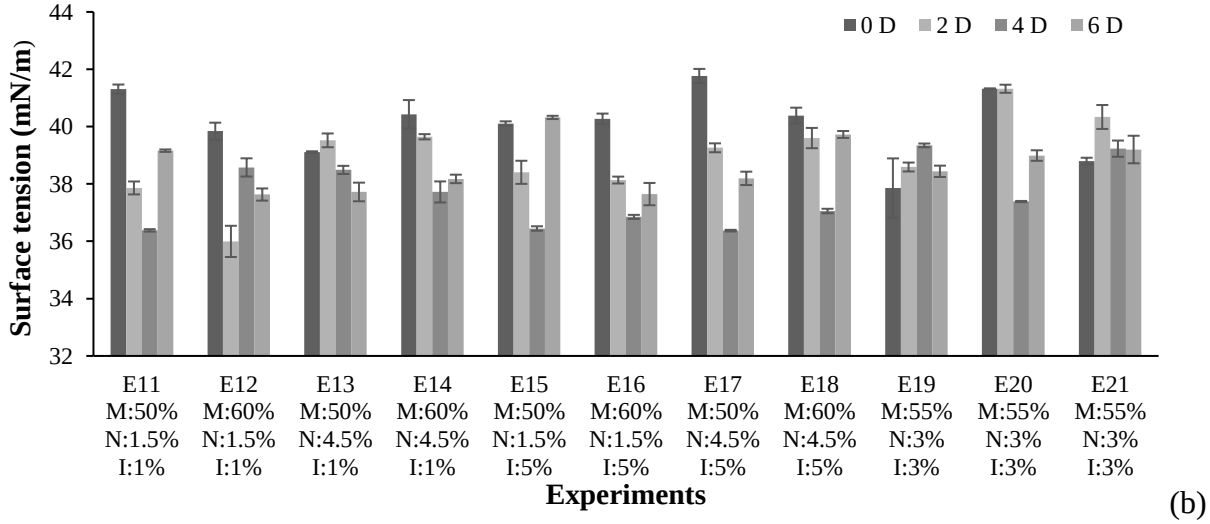
Figure 2b shows the results of the reduction of surface tension evaluated over 6d of cultivation. The greatest reductions were seen in the E27 tests (50% moisture, 5% inductor, 4.5% nitrogen) with a reduction from 41.77 ± 0.25 to 36.37 ± 0.13 Mn / m (12.92%) and E21 (50% moisture, 1% inductor, 1.5% nitrogen) showing a reduction from 41.31 ± 0.16 to 36.38 ± 0.04 Mn / m (11.92%) both results were obtained in the 4d of culture. The reduction in surface tension is directly related to the production of biosurfactants observed in the present study, since the reduction in surface tension is proportional to the concentration of biosurfactants in the medium, as assessed by SILVA et al. (2017) who in their study evaluated the production of biosurfactants from *Pseudomonas cepacia*, obtained a maximum production of biosurfactants of 8g / L in submerged fermentation in 0.1 L medium at the same time obtained a reduction of surface tension of 70, 00 to 27.00 Mn / m.

For the formation of lipolytic enzymes, high production was observed in all experiments performed (Figure 2c), with the highest yields observed in 6d of cultivation time, being in the E21 experiment (50% moisture, 1.5% nitrogen and 1% inductor) verified the highest enzymatic production for all tests performed (31.13 ± 0.55 U). The high production in this context can be justified using the glycerol inducer, requiring high production of lipases for the use of this source as a nutrient. The synthesis of the lipase enzyme occurs from the need for lipid degradation, and as the fermentation process occurs, the availability of the substrate decreases over time, requiring the production of extracellular enzymes to promote the degradation of the substrate, ensuring cell survival. The release of enzymes into the culture medium increases the contact of the enzyme-substrate complex, and consequently increases the assimilation of nutrients, obtaining the maximum activity of extracellular lipases (CONTESINI et al., 2010, SPERB et al., 2015).

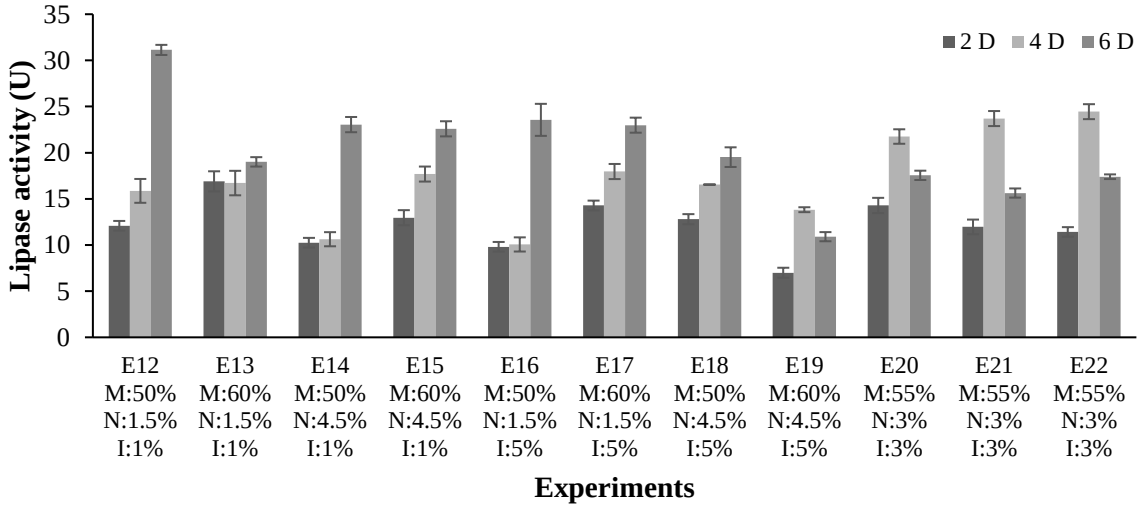
The effects analysis was performed at a 95% confidence level for the 4d of cultivation for $EA_{W/O}$ and reduction of surface tension and in the 6d of cultivation for LA, where the highest yields were observed, respectively. For the formation of emulsions, the addition of higher concentrations of nitrogen in the culture medium was significant (p = 0.0003) and positive (0.6211). For the reduction of surface tension, there was no statistically significant difference between the variables analyzed (p> 0.05). For the lipase activity, significant ($p_{umidity}$ = 0.0016, $p_{nitrate}$ = 0.0024, $p_{inducer}$ = 0.0046) and negative ($efeito_{umidity}$ = -5.4433, effectotnitrate = -5.1571, inducing effect = -4.7019) were analyzed. This, taking into account the above, the E21 experiment (50% moisture, 1.5% nitrogen, and 1% inducer) is selected as the best composition of culture medium, as it has the lowest added percentages of moisture, nitrogen, and inductor. The choice is justified by the high enzyme production verified in this assay (31.13 ± 0.55 U), corroborating what was demonstrated in the analysis of effects to produce lipases.



(a)



(b)



I

Figure 2 – Production of biosurfactants (a), the reduction of surface tension (b) and the production of lipolytic enzymes (c) of the Complete Factorial Design *

*M: moisture (%); N: nitrogen (%), I: inducer (%)

Rigo et al. (2010) verified the production of lipases in a culture medium composed of 10 g of soybean meal, 55% moisture, 1% urea, and 0.3% soybean oil as an inducer. Using the microorganism *Penicillium* P58, the authors obtained an approximate production of 200 U / g

of enzymatic activity, indicating that soybean meal associated with low moisture and inducer concentrations promote high enzymatic production.

The type of substrate used in the SSF was evaluated by Prabaningtyas et al. (2018), who cultivated Aspergillus niger in different solid matrices (soybean meal, palm oil meal, and coconut flour) and 2% olive oil inducer. The authors verified the highest production of lipases for the 7 d of cultivation (110.83 U/g) with soybean meal as a substrate, stating that high concentrations of inducer can interfere with enzyme production, an observation also verified in our study, where lower concentrations inducer promoted greater lipase activity.

Souza et al. (2014) evaluated the use of 2% glycerol as an inducer in the production of biosurfactants for the microorganism *Bacillus subtilis* ATCC 6633. The verified emulsifying activities were below 2.2 EU, proving that even in low concentrations the use of the glycerol inducer can be inefficient for the production of biosurfactants, which was also verified in our study. The study confirms the high potential not yet fully explored of the application of biosurfactants and lipases in several areas, mainly in the environmental area, in which it can be applied in processes of bioremediation of oily contaminants as a low-cost alternative compared to other treatment processes of contaminants (KRELING et al, 2019).

4. CONCLUSIONS

Among the tests carried out for the Fractional Factor Design, the best results to produce biosurfactants were 6.36 ± 0.83 EU of emulsifying activity for the E1 experiment and the greatest reduction in surface tension was 9.13% reduction in E9, both in the 6d time. For lipolytic activity, the maximum production was observed in E1 as 39.28 ± 1.77 U in 4d time. For the Complete Factorial Design, the best production of lipases occurred for the E12 test (50% moisture, 1.5% nitrogen, and 1% inductor), of 31.13 ± 0.55 U. For the same experiment, the reduction in surface tension was with a reduction of 11.92% and the production of biosurfactants was less than 2.00 EU, indicating that when using lignocellulosic materials and water-soluble inducers in the composition of the culture medium, the production of biocompounds is compromised.

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