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SYNTHESIS OF BIOMOLECULES BY SOLID FERMENTATION USING BY-PRODUCTS FROM THE TIMBER INDUSTRIES

Maria Vitória de Miranda Rodrigues
Instituto Federal do Paraná - Campus Jaguariaíva, Jaguariaíva
http://lattes.cnpq.br/9467107181242572

Milena Caroline Dalanora
Instituto Federal do Paraná - Campus Jaguariaíva, Jaguariaíva
http://lattes.cnpq.br/0998247553866219

Isadora Campos Dresch
Instituto Federal do Paraná - Campus Jaguariaíva, Jaguariaíva
http://lattes.cnpq.br/6942564650109111

Juliana Guerra de Oliveira
Instituto Federal do Paraná - Campus Jaguariaíva, Jaguariaíva
http://lattes.cnpq.br/6060311017743656

Danielle Hiromi Nakagawa
Instituto Federal do Paraná - Campus Jaguariaíva, Jaguariaíva
http://lattes.cnpq.br/4827583401397889

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Abstract: Biosurfactants and biodemulsifiers are compounds of great interest produced by bacteria, yeasts or filamentous fungi. The main objective of the present work is to verify the production of biomolecules with potential biosurfactant and biodemulsifier activity by the bacterium Bacillus subtilis, in a solid state fermentation regime, using by-products from wood industries. Sawdust was used as a support for the fermentation process, and sugarcane molasses as a carbon source. The fermentation was incubated for 120 hours at 35°C ± 2°C. Analyzes of pH, electrical conductivity, humidity and cell count were performed. The relative surface tension of the cell-free broth obtained from the fermented compound, and emulsification indices were verified using toluene and soybean oil. The biodemulsifier test was carried out with an emulsion composed of kerosene, water and the surfactant Tween 80. A breakdown of 11% of the emulsion was verified, verifying the biodemulsifying potential of the biomolecule obtained. It can be noted that there was production of biosurfactant at different times of cultivation, with emulsification rates of up to 54%. Thus, sawdust showed potential to act as a substrate/support in the fermentation process in the production of biomolecules.
Keywords: Biosurfactants, Biodemulsifiers, Sawdust.

INTRODUCTION

Biosurfactants and biodemulsifiers are compounds produced by bacteria, yeasts or filamentous fungi, which can help in industrial and environmental areas. Specifically, biosurfactants can act in detergency, emulsification, lubrication, foaming capacity, solubilization and phase dispersion (NITSCHKE; PASTORE, 2002). Biodemulsifiers are potential products for many commercial applications in petroleum and industrial effluents, food processing, biomedicine, and pharmaceutical industries (RODRIGUES, 2013).

The timber industry produces a large amount of waste daily, which can bring impacts to the environment that are directly linked to logging and the amount of wasted or burned sawdust (NASCIMENTO; DUTRA; NUMAZAWA, 2006). The bacterial fermentation process is seen as an alternative for using this type of waste; can be performed in solid state using sawdust as a support.

Demulsifiers and chemical surfactants are polymers of high molecular weight, when compared to biological ones, they have disadvantages such as production cost, since they are chemically synthesized from petroleum and therefore their cost fluctuates according to the international price for exploration and production of the oil. oil (RODRIGUES, 2013). A variety of biomolecules have been produced from bacteria isolated from the environment contaminated by hydrocarbons, which are less toxic, have a high biodegradability when compared to chemicals, in addition to being quite efficient (WEN et al., 2010).

Thus, the present work aims to develop a process that induces the production of biomolecules with potential biosurfactant and biodemulsifying activity by the bacteria: *Bacillus subtilis natto*, in a solid fermentation regime, characterized by presenting potential cost reduction, since it uses by-products from the wood industries, which are in abundance in the country and have great representation in the Brazilian economy.

MATERIALS AND METHODS

OBTAINING AND PREPARING THE SAWDUST

Sawdust from Pinus sp, from a sawmill in the municipality of Jaguariaíva-PR - Brazil, was used as a support/carbon source for the fermentation. The sawdust was standardized, using an electromechanical stirrer and sieves with a granulometry of 1.2 mm.

PREPARATION OF FERMENTATION

Fermentation took place with the addition of sawdust that had an absorptivity of 4mL/g, molasses at a concentration of 3%, according to the methodology of Bezerra (2006), and yeast extract at a concentration of 1%, as described by Decesaro et al. (2013). The experiment was carried out in 1000 mL erlenmeyer flasks in triplicate, properly sterilized. Fermentation conditions are described in table 1.

Identification	Amount of sawdus (g)	Bed height (cm)	Incubation period
F1	100	6,2	120 h
F2	50	4,5	96 h

Table 1. Parameters of the fermentation process.

Source: Personal Collection.

PREPARATION AND REALIZATION OF THE INOCULUM

Bacillus subtilis natto was the microorganism selected in this study. The starter culture was grown in Tryptic Soy Broth (TSB) for 24 hours at 35±2°C. Subsequently, 1.8 mL of this culture broth was added to each fermentation Erlenmeyer flask at a count of 1.8 x 108 CFU/ml. These were incubated at 35°C±2°C.

CELL COUNT

Microbial counting was performed using the serial dilution method, up to 10-10 dilution. The dilutions were plated on Tryptic Soy Agar (TSA) by the pour plate method and the plates were incubated for 24 hours in a bacteriological oven at 35°C±2°C.

DETERMINATION OF PH, ELECTRICAL CONDUCTIVITY AND HUMIDITY

To measure the physical-chemical conditions such as pH and electrical conductivity of a solid substance, it is necessary to submit the material to be analyzed to a stirring process using a magnetic bar, after the equipment is stopped, it starts its decantation, being possible to analyze the pH index with the aid of a digital pH meter and the electrical conductivity using a conductivity meter (TEDESCO et al., 1995). In addition, with the aid of a hygrometer, the fermentation humidity was determined.

OBTAINING THE BIOMOLECULES

After each fermentation period, water was added to the fermented compound in a proportion of 1:1, remaining for approximately 12 hours in contact at room temperature. After this period, the liquid was filtered and taken to a centrifuge process for 15 minutes at 8000 RPM.

RELATIVE SURFACE TENSION

The verification of the surface tension reduction in the cell-free liquid is an important measure for the detection of the presence of biosurfactant and biodemulsifier. The supernatant broth was dripped onto a coin until extravasation and the same procedure was performed with water to measure the relative surface tension.

EMULSIFICATION INDEX

Emulsification indices were determined with the hydrocarbon toluene and with soybean oil. The emulsification index was evaluated by adding 2 ml of toluene/soybean oil in 100 x 15 mm test tubes with 2 ml of the cell-free culture broth obtained after centrifugation. The tubes were vortexed for 2 minutes. The reading was then performed after 24 hours of rest. The emulsification index was calculated according to IQBAL et al. (1995): IE24= (emulsion layer height (mm) / total column height (mm)) x 100.

BIODEMULSIFICATION RATE

The biodeemulsification test was based on the methodology of Rodrigues (2013), with modifications. The methodology consists of preparing a water/kerosene (W/Q) emulsion, with 100 ml of kerosene, 50 ml of water and 6.34 ml of Tween 80.

The emulsion was used in the A/Q emulsion breakdown test, where 2mL of the centrifuged liquid from the fermentation was added to 18mL of emulsion, remaining in a bacteriological oven at 35°±2°C for 120 hours.

The demulsification rate was calculated using the equation shown in Figure 1.

$$\text{demulsification rate} = \frac{\text{Separated Kerosene Volume} \times 100\%}{\text{Volume of Kerosene in Original Emulsion}}$$

Figure 1. Equation to calculate the demulsification rate.

Source: Huang et al. (2012).

RESULTS AND DISCUSSION

EVALUATION OF PH, ELECTRICAL CONDUCTIVITY AND HUMIDITY

The initial physical-chemical characterization of the sawdust was: pH of 6.3 and electrical conductivity of 96.73 µS/cm. Studies by Camargo (2013) point out that the pellets (an alternative for using sawdust) of Pinus ssp, usually have an acid pH that is equivalent to that found for wood. As for electrical conductivity, samples tend to have low conductivity, which means low ion content in samples, since conductivity measures the mobility of ions in solution and high values of conductivity in samples can contribute to possible corrosion of equipment (CAMARGO, 2013).

The molasses solution obtained a pH of 7.6. Marim (2014) observed in their work that sugarcane molasses supplemented with yeast extract and peptone, when adjusted to pH around 7, tends to be influenced by the increase in biosurfactant synthesis. In addition, it must be noted that molasses favors cell growth and the production of biomolecules, probably because the by-product of the sugar industry contains some elements (salts and amino acids) that favor cell growth (MARIM, 2014).

Identification	Initial PH	Final pH	Moisture	Electric conductivity
F1	5,91	12,61	68%	322,8 µS/cma
F2	5,34	6	65,5%	403,1 µS/cma

Table 1. Characterization of the fermentation process.

Source: Personal Collection.

Table 1 shows the results obtained in the fermentation process, it is observed that the F1 fermentation presented a pH of 12.61 after 120 hours. Studies indicate that the synthesis of biomolecules tends to occur at pH close to neutrality, so basicity is not a favorable environment for production. According to Ferreira (2007), the decline in pH in some concentrations of substrates may be associated with the catabolism of carbohydrates that results in the production of acids.

While the F2 fermentation presented an initial pH of 5.34 and electrical conductivity of 541.65 µs/cm at 25°C and pH and electrical conductivity after 96 hours of 6 and 403.1 µs/cm at 25°C, respectively. It was obtained with final process humidity 65.5%. Bueno et al. (2010), assures that the highest percentages of emulsification indexes and, also, better surface tension reductions occurred with the procedure being performed with pH around 5 to 7.

Liu et al. (2009), certifies that favorable conditions of pH, oxygenation and temperature are essential for the production of biodemulsifiers. As for the biosurfactant, Calvo et al. (2009) states that physical parameters such as temperature, aeration and pH influence the type of polymer produced.

BED HEIGHT

Since the experiment was carried out using different amounts of sawdust, the possible interference of oxygenation in the process can be evaluated. Veenanadig et al., (2000) studied the effect of air supply on semi-solid fermentation in fixed bed bioreactors using: *Bacillus subtilis*. They found better results in experiments with higher air rates (VEENANADIG; GOWTHAMAN; KARANTH, 2000). The same occurred in the experiment carried out, the biomolecules produced under higher air rates (F2) were superior to the others in the tests that proposed the verification of productivity.

CELL COUNT

Cell counts were performed at 0 hours, 96 hours and 120 hours. The growth of 1.5 x 1010 CFU/g in 120 hours was observed in the process identified as F1, when compared to the initial inoculum of 1.8 x 108 CFU/g. In the F2 fermentation, it can be seen that the initial inoculum corresponded to 4.9 x 107 CFU/g and the final inoculum to 1.6 x 108 CFU/g in 96 hours. Massi (2013) states that when the microbial growth exceeds the concentration of 108, it means that this microorganism was able to use the carbon and nitrogen sources contained in the residues.

EMULSIFICATION TEST

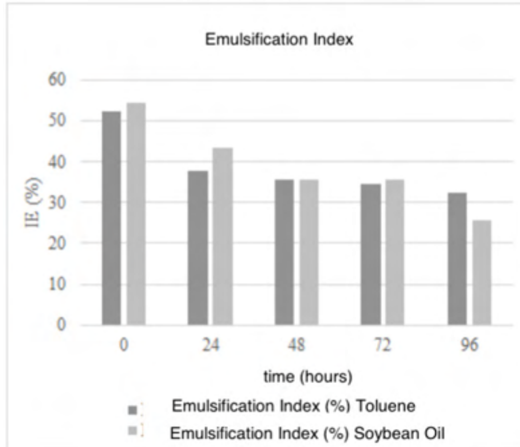

Figure 3. Graph of the emulsification index of the synthesis obtained in F1.

Source: Personal Collection.

Figure 3 demonstrates emulsification results for toluene and soybean oil up to 96 hours. Emulsification was observed in all experiments. It was possible to observe that soybean oil was more efficient in relation to emulsification, however, in the test with toluene there was greater stability up to 96 hours of rest.

The results obtained by Barros et al. (2008) state that all tested emulsions showed high emulsification rates, except those made with toluene, which justifies the low rate presented.

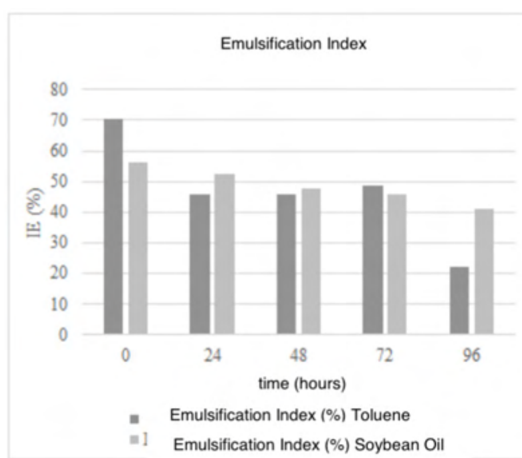

Figure 4. Graph of the emulsification index of the synthesis obtained in F2.

Source: Personal Collection.

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Figure 4 demonstrates that the products obtained from the fermentation that had higher air rates presented better results than the previous one. The emulsification index using soybean oil in 96 hours was 40.95%, while previously it was 25.5%, noting that the bed height of the procedure directly influences the process.

The studies by Pinto et al. (2015) reported an IE of 24% for soybean oil. Rovina (2018), presented as an emulsification index, values lower than 30% for soybean oil.

RELATIVE SURFACE TENSION TEST

An important property of biosurfactants is their ability to reduce the surface tension of aqueous solutions (NITSCHKE et al., 2004; YOUSSEF et al., 2004). When potentially demulsifying bacteria strains are able to greatly reduce surface tension, the emulsion breakage rate is usually greater than 70% (RODRIGUES, 2013). Such statements intensify the need for analysis to verify the synthesis of biomolecules.

The verification of the relative surface tension of the cell-free culture broth in relation to water, verified the presence of biosurfactant and biodemulsifier in the supernatant. The results obtained for the relative surface tension were a reduction of up to 40% in relation to the surface tension of the water, against the F2 fermentation. While the F1, showed a decrease of 67% of the surface tension of the supernatant in relation to the water. Bugay (2009) obtained a reduction of about 29% for solid fermentation by: *B. subtilis*, reinforcing the synthesis of the biomolecules targeted in the present work.

The bacteria: *Bacillus subtilis* is one of the most cited in the literature for producing the biosurfactant known as surfactin and considerably reducing surface tension (PINTO; MARTINS; COSTA, 2009). Among the most effective biosurfactants are the lipopeptides produced by bacteria of the genus: *Bacillus*, especially those produced by *Bacillus subtilis* (BOGNOLO, 1999).

Bueno (2010), states that bacteria belonging to the families *Pseudomonadaceae and Bacillaceae* are capable of producing an efficient biosurfactant in the removal of oil and its polluting derivatives from water.

BIODEMULSIFICATION TEST

The cell-free broth obtained from the fermented compound F2 showed the ability of 11% to perform the demulsification process in W/Q emulsions. However, the cell-free broth from the F1 fermentation process did not demonstrate the ability to break the emulsion.

The study by Rodrigues (2013) states that a lower surface tension implies a greater production of bio-demulsifier. Since the cell-free broth showed a 67% decrease in tension in relation to that of water, we can say that the biomolecule obtained has potential biodemulsifying activity.

CONCLUSION

We concluded that sawdust has the potential to act as a substrate/support in the fermentation process, proving to be an alternative by-product for cost reduction in the production of biomolecules.

It can be observed that there was the production of both biomolecules in the fermented compound, corroborated by the experiments carried out. However, it is necessary that adaptations are made to enhance the production of biodemulsifier.

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