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Maximization of Biosurfactant Production by *Bacillus Invictae* Using Agroindustrial Residues for Application in the Removal of Hydrophobic Pollutants

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Surfactants are amphipathic molecules that reduce surface and interfacial tension, conferring properties such as detergency, emulsification and phase dispersion, making them versatile chemicals. Most of the surfactants in use are derived from petroleum; however, interest in microbiological surfactants has increased because of its biodegradability and reduced toxicity. The objective of this work was to evaluate the production of a biosurfactant by the bacterium *Bacillus invictae* UCP 1617 using a complete 2^3 factorial design as a tool to optimize the variables agitation, temperature and inoculum size in 5 L bioreactor in mineral medium containing 2 % unconventional raw material. In the factorial tests, the surface tension and the yield in isolated biosurfactant were evaluated. The best condition of the experimental design was transferred to a 50 L bioreactor, being evaluated the kinetics of biosurfactant production. The biosurfactant formulation was carried out with the addition of 0.2 % of potassium sorbate. The formulation was then subjected to stability evaluation under different environmental conditions. The oil surface wash test with the biosurfactant was performed with motor oil. The best result obtained from the 2³ complete factorial design was the cultivation condition test number 5 (175 rpm at 28 ° C and 2 % inoculum), considering the best yield (1 g/L) in 72 hours of cultivation. The transfer of biosurfactant production to the 50 L bioreactor showed a surfactant productivity corresponding to 2.42 ± 1.1 g/L in 72 hours. The surface tension of the medium was reduced from 69.5 to 30.2 mN/m after 60 hours of fermentation, demonstrating the presence of surfactants. The chemical composition of the biosurfactant suggested the presence of 65 % of lipids and 32 % of carbohydrates, meaning its glycolipidic nature. The formulation produced with the addition of potassium sorbate proved to be a promising product because of its stability at various pH, temperature and NaCl concentrations. The biosurfactant removed 95.42 % of the oil adhered to the glass surface. Therefore, the biosurfactant presents potential application in remediation processes for the reduction of environmental impacts on ecosystems.

1. Introduction

Oil spills cause environmental contamination, leading to disastrous consequences for living organisms. It is estimated that 0.08% - 0.4% of world oil production eventually reaches the oceans. In Brazil, accidents involving petroleum-derived hydrocarbons such as gasoline and fuel oil have caused serious environmental problems (Rocha E Silva et al., 2019). And one way to reduce the impacts of these accidents is by using remediation processes, such as the use of microbial surfactants or biosurfactants that are metabolites produced primarily by bacteria and yeast, although some fungi also produce them. Because of their biodegradability and environmental compatibility, these compounds, unlike similar (synthetic) petrochemicals, have been increasingly studied (Pereira et al., 2017). They also have numerous advantages over chemical surfactants, such as low toxicity, stability over a wide pH range and high temperatures, as well as resistance to high salt concentrations (Sarubbo et al., 2015). They are differentiated by their biochemical nature and by

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microbial producing species. The major classes include glycolipids, lipopeptides, lipoproteins, polymeric biosurfactants, phospholipids, and some fatty acids (Irorere et al., 2017; Satpute et al., 2017).
Oily dregs, waste oils, sugarcane molasses, cheese, corn, potato and cassava processing residues are some examples of residues and by-products with potential for biotensive production (Sarubbo et al., 2015). Substrate selection, however, depends on the choice of a residue that has in its composition nutrients necessary for microbial growth and biosurfactant production (Huiqing e Qingxin, 2017; Perfumo et al., 2018). In this sense, the objective of this Work Plan was to maximize the production of *Bacillus invictae* UCP 1617 biosurfactant grown in low cost medium and to perform stability tests in order to evaluate the potential application of the biomolecule as an aid in the decontamination processes of hydrophobic pollutants generated in Petroleum industry.

2. Material and methods

2.1 Organism

The bacterium *Bacillus invictae* UCP 1617 isolated from the sludge residue of the Muribeca landfill, Jaboatão dos Guararapes / PE and deposited in the Bank of Cultures of the Nucleus of Research in Environmental Sciences (NPCIAMB) of the Catholic University of Pernambuco will be tested as a producer of the biosurfactant. Maintained in Nutrient agar (AN) g/L, beef extract, 5 g, 10 g peptone, 5 g NaCl, 17 g agar, pH 7.0, temperature 5 °C, and peaked every three months.

2.2. Cultural conditions and biosurfactant production

The fermentation process of the complete Factorial Design 2^3 was carried out in a mineral medium described by Dubey and Juwarkar (2001), supplemented with 1.5 % of millhocin, with its pH adjusted to 7.0 for the production of biosurfactant at the temperature of 28 °C in 96 hours. The medium components were solubilized and sterilized in an autoclave for 20 minutes at 121 °C.

2.3 Maximizing Biosurfactant Production Using Factorial Design

Fermentations for biosurfactant production were performed in a 5 L bioreactor. The production medium was subjected to variation of cultivation conditions (rotation speed, cultivation temperature and inoculum size) according to a complete Factorial Design 2³ presented in tables 01 and 02. At the end of cultivation, which will last 72 hours, samples were centrifuged and filtered to determine surface tension and yield as parameters used to select the best production condition.

Table 1: Independent variable values at levels -1 and +1 and at center point

Level	Agitation (rpm)	Cultivation Temperature (°C)	Inoculum Size (%)
-1	175	28,0	2
0	200	33,0	3
+1	225	38,0	4

Table 2: Planning matrix

	Level		
Experiments	Agitation(rpm)	Cultivation Temperature (°C)	Inoculum Size (%)
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0

2.4 Determination of surface tension and Emulsification index (E_{24})

The surface tension of the biosurfactant was measured on a KSV Sigma 700 (Finland) tensiometer using the NUOY ring. The surface tension was measured by immersing the platinum ring in the liquid and registering the force necessary to pull it through the air-liquid interface. And the emulsification index was measured using the method described by Cooper and Goldenberg (1987).

2.5 Biosurfactant production in 50L bioreactor

The production scale-up was performed in a 50 L bioreactor under the same conditions selected from the Factorial Design at 28 ºC. Samples were taken every 12 hours throughout cultivation to determine growth kinetics, surface tension and yield in isolated biosurfactant.

2.6 Isolation and chemical characterization of biosurfactant

The cell-free metabolic fluid of the condition had its pH reduced to 2 using hydrochloric acid and kept overnight at 5 ºC, according to the methodology described by Nitschke and Pastore (2002). Isolated biosurfactant was characterized by total protein (Bradford, 1976), Carbohydrates (Dubois et al., 1956) and lipids (Manocha et al., 1980).

2.7 Biosurfactant Formulation

Cell-free metabolic fluid obtained after centrifugation of the biomass was added with 0.2 % potassium sorbate as a preservative and stored under sterile conditions in hermetically sealed vials at room temperature for 30 days.

2.8 Evaluation of the stability of the formulated biosurfactant (effects of pH, NaCl addition, time under heating and temperature)

The effects of different temperatures (5 ° C, 70 ° C, 100 ° C and 120 ° C), different NaCl concentrations (2.0, 4.0, 6.0, 8.0 and 10.0 %) , and different pHs (2.0, 4.0, 6.0, 8.0, 10.0 and 12.0) on biosurfactant activity were evaluated in cell free metabolic fluid to determine surface tension. All analyzes were performed in triplicate.

2.9 Oily Surface Wash Test

A known sheet of dough had part of its surface uniformly contaminated with 100 µl of petroleum derivatives. The contaminated section of the slide was submerged in the biosurfactant solution formulated under constant agitation for 10 minutes. The slide was immersed in distilled water to remove any excess formulated biosurfactant. The slide was then oven dried at 40 ° C for 30 minutes and its weight noted. The removal index was calculated by the formula:

$$I = 100 \times \frac{(Mc - Ml)(Mc - Ml)}{(Mc - Mi)(Mc - Mi)} \quad (1)$$

Where *Mc* represents the weight of the contaminated slide, *Ml* the weight of the post-wash slide and *Mi* the initial weight of the slide.

2.10 Statistical analysis of data obtained during the experiments

The data collected were expressed as the mean ± standard deviation of the triplicate tests. Statistical analysis of variance of ANOVA was applied to determine significance, where p values <0.05 will be considered significant.

3. Results and Discussion

In this study, a complete Factorial Design 2^3 was used, where the independent variables analyzed were agitation, temperature, inoculum size and the response variables were surface tension and biosurfactant yield alone. The complete factorial design 2^3 involved 12 trials with eight variables and 4 central points. Analyzes of the results were determined using the Statistica 8.0 StatSoft software. Table 3 presents the variables (factors) with the levels studied and the values of the response variables, where the values of the results obtained for surface tension were not significant for the established mathematical model, however, the biosurfactant yield values are correlated to optimization. Therefore, to obtain a significant mathematical model of the surface tension response variable, it will be necessary to perform a new Factorial Design.

Table 3: Result of Complete Factorial Design 2^3 for surface tension and yield.

Experiments	Level			Results	
	Agitation (rpm)	Cultivation Temperature (°C)	Inoculum size (%)	Surface tension (mN/m)	Extraction (g/L)
1	175	28,0	2	43,3	0,46
2	225	28,0	2	45,5	0,51
3	175	38,0	2	47,5	0,71
4	225	38,0	2	49,8	0,70
5	175	28,0	4	42,5	1,00
6	225	28,0	4	46,3	0,69
7	175	38,0	4	48,7	0,87
8	225	38,0	4	49,1	0,88
9	200	33,0	3	56,7	0,66
10	200	33,0	3	56,9	0,67
11	200	33,0	3	57,0	0,59
12	200	33,0	3	56,8	0,69

In the Pareto diagram (Figure 1) it can be observed that, for a 95 % confidence level, only the interaction of the independent variables temperature and inoculum produced a negative and statistically significant effect on the increase of the isolated biosurfactant yield, however, others the variables and their interactions had positive and statistically significant effects, contributing to the increase of the biosurfactant production.

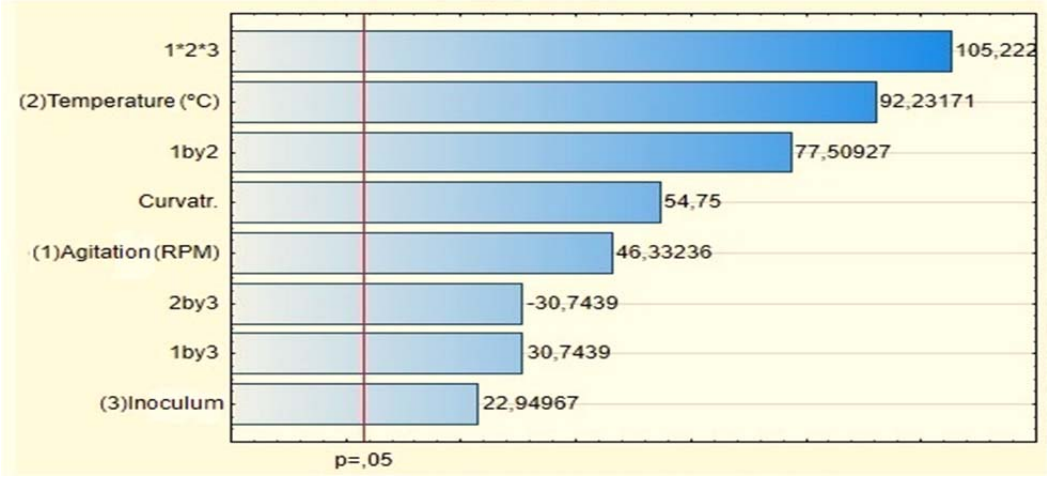

Figure 1: Pareto diagram to estimate the effects on yield of biosurfactant produced by Bacillus invictae UCP 1617 using a complete factorial design 2^3.

The obtained result was satisfactory, since in the fermentation a surface tension was obtained around 42.50 mN/m, which means a reduction of 40.98 % in relation to the water tension (72 mN/m), considering the best yield 1 g/L. The biosurfactant produced by Bacillus invictae UCP 1617 showed stable emulsion over 24 hours showing 93.75 % motor oil emulsification. Production of biosurfactant in 50 L bioreactor was performed from the conditions of test 5 (175 rpm Agitation, Temperature 28 ° C and Inoculum 2 %) of factorial design, adding aeration (0.5 vvm). The maximum growth rate occurred between 24 and 36 hours with a maximum of 0.24 / h. The maximum log 10 (CFU/ml) reached was 8.63 ± 0.12 at 48 h of fermentation (Figure 2). The surfactant productivity corresponding to the maximum surfactant yield was 2.42 ± 1.1 g/L in 72 hours. The surface tension of the culture medium at the beginning of the fermentation was 69.5 ± 1.2 mN/m, and after 60 h of fermentation, the tension increased to 30.20 ± 0.7 mN/m, demonstrating the presence of surfactants.

Analysis of the chemical composition of the biosurfactant produced by Bacillus invictae UCP 1617 suggested the presence of 65 % lipids and 32 % carbohydrates, possibly meaning a glycolipid nature of the compound. Biosurfactants have application in various industrial processes due to various structures and properties (Perfumo et al., 2018).

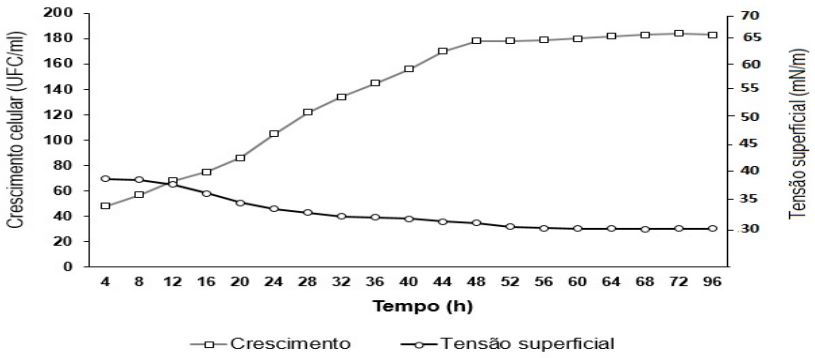

Figure 2: Cell growth and surface tension of Bacillus invictae UCP 1617 biosurfactant in 50 L bioreactor over 72h at 175 rpm and 28ºC.

Preservatives are chemicals whose function is to preserve the initial condition of the product in order to avoid significant changes in its properties over time. The addition of preservatives, such as potassium sorbate ($C_6H_7KO_2$) in biosurfactants, is a potential alternative for use in microbiological products, preserving key characteristics for immediate application. The formulation of the biosurfactant produced proved to be a promising product to be applied in different cleaning processes, as it has stability at various pHs, temperatures and NaCl concentrations, its surface tension suffered few changes, as described in Table 4.

Table 4: Stability of formulated Bacillus invictae UCP 1617 biosurfactant evaluated under different pH, temperature and NaCl addition by determining surface tension.

pH	Surface Tension(mN/m)	NaCl concentration (%)	Surface Tension(mN/m)	Temperature(ºC)	Surface Tension(mN/m)
2	34,45 ± 0,40	2	32,47 ± 0,30	5	33,25 ± 0,90
4	33,74 ± 0,20	4	33,24 ± 0,26	70	32,58 ± 0,60
6	32,17 ± 0,41	6	33,65 ± 0,02	100	33,85 ± 0,40
8	32,40 ± 0,60	8	33,14 ± 0,70	120	34,40 ± 0,20
10	33,45 ± 0,43	10	34,37 ± 0,40	-	-
12	33,62 ± 0,52	-	-	-	-

Biosurfactants may increase the removal of hydrophobic pollutants through solubilization and mobilization processes. Solubilization capacity depends on the ability of the surfactant to increase the solubility of hydrophobic constituents in the aqueous phase (SARUBBO et al., 2015). The results of the removal of oily compounds adsorbed on solid surfaces by the potassium sorbate formulated biodetergent, biosurfactant (1x CMC), stabilizer and a commercial degreaser showed that the surfactant has the ability to solubilize motor oil as it has a 98.42 % removal as we can see in Figure 3.

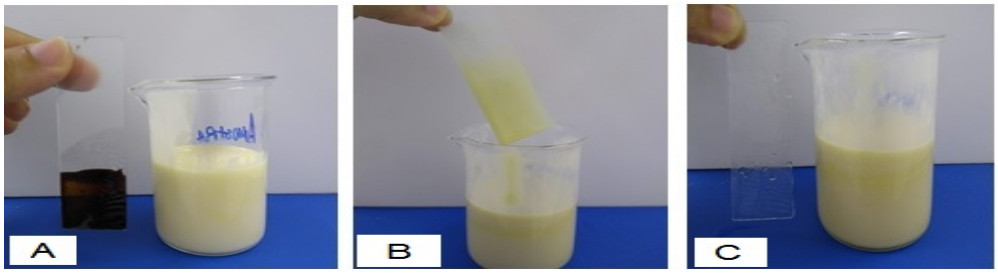

Figure 3: Illustration of engine oil removal on solid surface by formulated biosurfactant obtained from Bacillus invictae UCP 1617. (A) Contaminated slide and biosurfactant solution, (B) Washing process, (C) Washed slide and post-wash biosurfactant solution.

4. Conclusions

The results obtained from the factorial design, the appropriate interactions between the response variables and the different production conditions are significant and demonstrate the best condition for maximizing biosurfactant production. It was found that the model presented is close to optimization, with more than 90% of statistical confidence in relation to the real data, being feasible a new planning in order to adjust the model with even greater precision. In addition, the biosurfactant showed a very stable behavior under adverse conditions, being able to maintain its physicochemical properties, besides presenting efficient removal of oily compounds.

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