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Optimization of biosurfactant production from *Candida guiliermondii* using a Rotate Central Composed Design

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Accidents in petroleum platforms happen because of the absence of an effective fluid control inside the pipes, as occurred in 2010, when the scientists were not prepared to treat the oil spill in the Deepwater Horizon platform. In such environmental accident, non-biodegradable chemical dispersants were applied. As a safe alternative for the environment, the combination of the biotechnological potential of the microorganisms and the low cost waste materials results in the production of biosurfactants, known as the petroleum bioremediators. Thus, the optimization of operational parameters for biosurfactant production by *Candida guilliermondii* UCP 0992 grown in a low-cost medium and formulated with 4.0 % of corn steep, 2.5 % of molasses and 2.5 % of soybean residual oil was carried out in a 1.2 L bioreactor using response-surface methodology. The application of a Rotate Central Composed Design (RCCD) led to the identification of agitation speed, aeration, time and inoculum size as significant variables affecting the fermentation process. The optimal levels of the aforementioned variables were 250 rpm agitation speed, 132 h of cultivation time, 0.5 L/min of filtrated air and 4 % inoculum size. The experimental verifications allowed a maximum relative surface tension reduction to 31.45 mN/m and interface tension reduction to 9.04 mN/m, which was found to be equivalent to about 30.2 g/L isolated biosurfactant as estimated gravimetrically, thereby resulting in an improved production. Besides the optimization of operational parameters, the economic cost of € 22.37 was estimated to the biosurfactant produced according to the local price of the kWh. This work, therefore, showed that the fermentation time spent in flasks (144 h), could be reduced in 12 hours, increasing 3.6 times the yield and keeping the surface and interface tensions at the lowest level. Moreover, the biosurfactant produced by *C. guilliermondii* shows potential to be applied in oil spills.

1. Introduction

Each year, over 5 million t petroleum are transported through the oceans, promoting the exchange desired by the capitalists. However, the soaring gain with the mixture of hydrocarbons and oils becomes a true loss when the control technologies do not act as predicted, spilling the oil into the sea and causing economic and environmental disasters during a long time (Al-Majed et al., 2012).
Among the most severe accidents, the oil spill from the Deepwater Horizon platform cost at least U$ 40 billion for the company, which was managing the giant structure of more than 1.5 km of depth. Besides, U$ 20 billion were destined to a victim compensation fund. All this money was spent because the block valve control mechanisms did not work inside the oil pipes, being considered a project mistake. Therefore, 997 birds, 400 marine turtles and 47 mammals died simply because a group of engineers did not planned the correct control of the petroleum (Bozeman, 2011).
After 20 days from the disaster, the scientists spilled 7,000,000 L of chemical dispersants to control the oil. On the other hand, these compounds are derivated from the petroleum, raising the toxicity in the lethal zone and killing more animals. The solution to substitute the chemical surfactants is to utilize microorganisms with a

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biotechnological potential and substrates arising from waste materials. This combination will synthesize the biosurfactants: the petroleum bioremediators (Mascarelli, 2011).
The biosurfactants include glycolipids, lipopeptydes, phospholipids, and other organic compounds which form two segments in the biomolecule: one hydrophobic and other hydrophilic. The hydrophilic moiety shows affinity for polar compounds, while the hydrophobic portion can be aggregated with non-polar substances, so the microbial surfactant can be also called as an amphiphilic compound (Marchant et al., 2012).
The properties of surfactants are measured mainly by the surface and interfacial tensions. The surface tension can evince the level of humectation from any liquid according with the kind of the intermolecular bonds, while the interfacial tension expresses the intensity of aggregation between hydrophilic and hydrophobic phases. Furthermore, these features can predict other properties from the biosurfactants as detergence, emulsion, dispersion, whatever (Daltin, 2011).
Any product should be profitable to be marketed, so the microbial surfactants should have the same level of extractions from the chemical surfactants. Therefore, this is a feature to be considered when the researcher will analyze a statistical optimization from a bioproduct (Rocha e Silva et al., 2013).
In this way, the work covers a statistical study in a 1.2 L bioreactor based on the independent variables of agitation speed, aeration, time and inoculum size to produce the best *C. guilliermondii* surfactant properties with the higher extraction.

2. Material and Methods

2.1 Microorganism

The yeast *Candida guilliermondii* (UCP 0992) was kindly supplied by the *Banco de Culturas do Núcleo de Pesquisas Ambientais* from the Catholic University of Pernambuco was the biosurfactant producer. The yeast was maintained at 5 °C on Yeast Mold Agar (YMA) slants containing (w/v) yeast extract (0.3 %), malt extract (0.3 %), tryptone (0.5 %), D-glucose (1.0 %), and agar (5.0 %). In the absence of agar, the medium was called Yeast Mold Broth (YMB). Transfers were done to fresh agar slants each month to maintain viability.

2.2 Fermentation Medium

The *C. guilliermondii* was grown in solid medium at 27 °C for 48-72 h, then, a loopful of the cells were transferred to Erlenmeyer flasks of 250 mL containing 50 mL of the Yeast Mold Broth (YMB) and incubated aerobically for one day at 28 °C on a rotary shaker (200 rpm).
The production medium was composed by the industrial residues: molasses (2.5 %), corn steep liquor (4.0 %) and soybean oil (2.5 %) supplemented with water. The pH was adjusted to pH 5.5 with 1 M HCl solution.

2.3 Bioreactor

The yeast was cultivated in submerged culture with shaking in a bioreactor (1.2 L) TECNAL, illustrated in the figure 1. This machine was filled with 600 mL of distilled water with the percentages of waste materials and sterilized at 121 °C for 20 min. The inoculum was introduced in the amount of 10^4 cells/mL of the 24 h culture grown on YMB and the microorganism was incubated at 28 °C with shaking according to a Rotate Central Composed Design (RCCD).

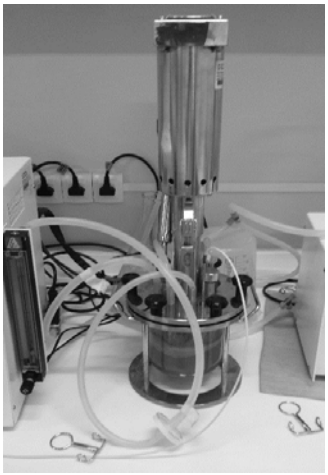

Figure 1: Bioreactor 1.2 l in a middle of a fermentation linked with the controllers

2.4 Rotate Central Composed Design

Rodrigues et al. (2008) have explained the utilization of a method to reduce the number of experiments denominated Rotate Central Composed Design, knowing a range of each variable. In this case, the surfactant properties and the extraction could be explained by the results of all variations from the table 1 that are expressed in the table 2.

Table 1 – Variables values in the levels -2, -1, 0, +1 and +2

Level	Agitation Speed (rpm)	Aeration (L/min)	Inocolum Size (%)	Time (h)
-2	175	0.0	2	96
-1	200	0.5	4	108
0	225	1.0	6	120
+1	250	1.5	8	132
+2	275	2.0	10	144

Table 2 – Matrix of planning

Experiment	Agitation Speed (rpm)	Aeration (L/min)	Inocolum Size (%)	Time (h)
1	200	0.5	4	108
2	250	0.5	4	108
3	200	1.5	4	108
4	250	1.5	4	108
5	200	0.5	4	132
6	250	0.5	4	132
7	200	1.5	4	132
8	250	1.5	4	132
9	200	0.5	8	108
10	250	0.5	8	108
11	200	1.5	8	108
12	250	1.5	8	108
13	200	0.5	8	132
14	250	0.5	8	132
15	200	1.5	8	132
16	250	1.5	8	132
17	175	1.0	6	120
18	275	1.0	6	120
19	225	0.0	6	120
20	225	2.0	6	120
21	225	1.0	6	96
22	225	1.0	6	144
23	225	1.0	2	120
24	225	1.0	10	120
25	225	1.0	6	120
26	225	1.0	6	120
27	225	1.0	6	120
28	225	1.0	6	120

The number of experiments was equal to the sum of: 2^k, indicating all combinations of the levels -1 and +1, where k consists in the number of variables; $2 \cdot k$, representing the variation between the extreme levels -2 or +2 with 0 and 4 experiments with the central values.

The results were based on the independent variables of agitation speed (AS), aeration (A), time (T) and inoculum size (IS) to evaluate the surface (ST) and interface (IT) tensions and the extraction (E) of the biosurfactant. Besides, the RCCD could reduce the number of 625 total experiments to 28 fermentations.

2.5 Surfactants Properties

The surface and interfacial tensions were measured by the DU NUOY ring method in the KSV Sigma 700 tensiometer (Finland). These methods consist in verify the tension (mN/m) through the ascension of the ring until the imminence of the breaking from two phases: air/biosurfactant for surface and n-hexadecane/biosurfactant for interfacial.

2.6 Extraction

Each culture media was centrifuged at 4500 rpm for 15 min to remove the cells. Then, a volume of biosurfactant had the pH adjusted to 2.0 with a 6 M solution of HCl and a double volume of methanol wad added. This mixture was cooled to -15 °C during 24 h, dried in an oven at 37 °C for 48 h and kept in a desiccator until a constant weight (Brasileiro, 2014).

2.7 Economic Estimative from Required Energy in the Bioreactor

The production cost is important to verify if a variation of a volume from a bioproduct increases an additional value against the total energy. Therefore, the control modules of the compressor, the thermostatic bath and the agitator were evaluated.
The price of 1 kWh was 1.2812 €, according to the resolution homologatory from the Electric Energy National Agency (ANEEL) of the group NEOENERGIA, number 1519 from the day 23 of April of 2013 (Celpe, 2013).

3. Results and discussion

3.1 Analyze from the surfactant properties and the extraction

The surfactant properties and extraction equations were made by the Statistica 11.0 and all experimental results can be seen in the figure 2.

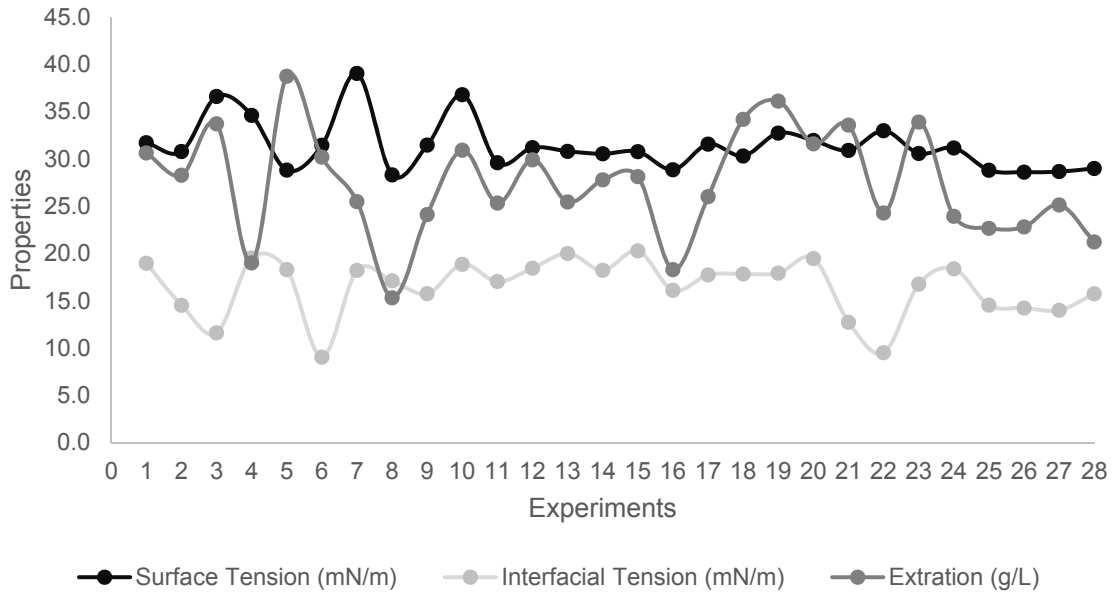

Figure 2: Results from the Surface and Interfacial Tensions and the extraction of the biosurfactant

The surface tension was influenced mainly by relation between the aeration and the inoculum size as demonstrated by the Pareto's Chart in the figure 3. This analogy can be proved when the experiments 5, 8 and 16 are compared: when the quotient between the aeration and the inoculum size was bigger, the mixture could be more homogeneous, resulting in a biosurfactant with a lower surface tension. Furthermore, the formula that represents the behavior of the surface tension is the equation 1.

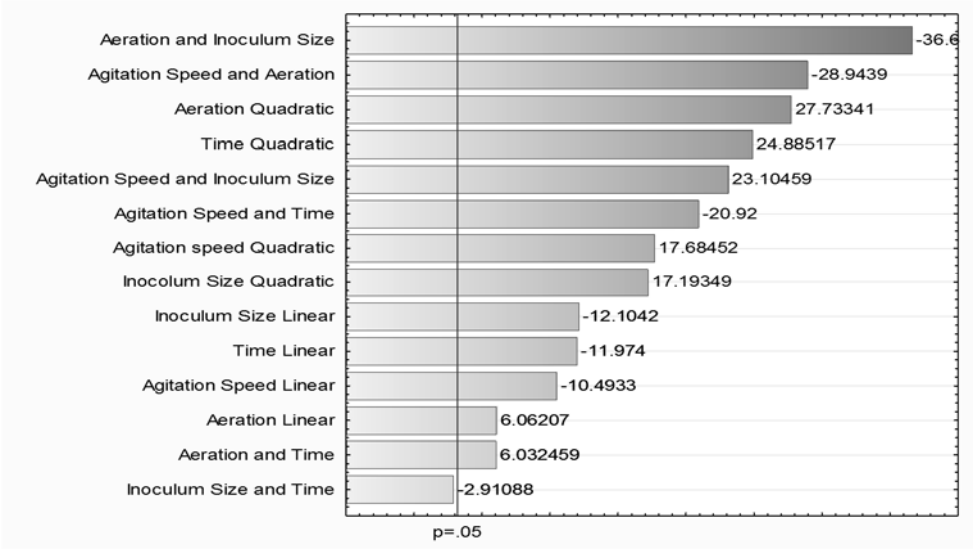

Figure 3: Pareto's Chart of the interfacial tension

$$ST = 99.114 - 0.121 \cdot AS + 0.001 \cdot AS2 + 19.168 \cdot A + 3.869 \cdot A2 - 4.265 \cdot IS + 10.150 \cdot IS2 - 0.823 \cdot T + 0.006 \cdot T2 - 0.099 \cdot AS \cdot A + 0.020 \cdot AS \cdot IS - 0.003 \cdot AS \cdot T - 1.564 \cdot A \cdot IS + 0.043 \cdot A \cdot T - 0.005 \cdot IS \cdot T \quad (1)$$

The interfacial tension was modified mainly by the relation between the agitation speed and the time as illustrated by figure 4. This influence can be ratified by the experiments 6 with 9.04 mN/m, because when an agitation of 250 rpm mix during 132 h gave a minimum interfacial tension. Besides, the formula vas illustrated in the equation 2.

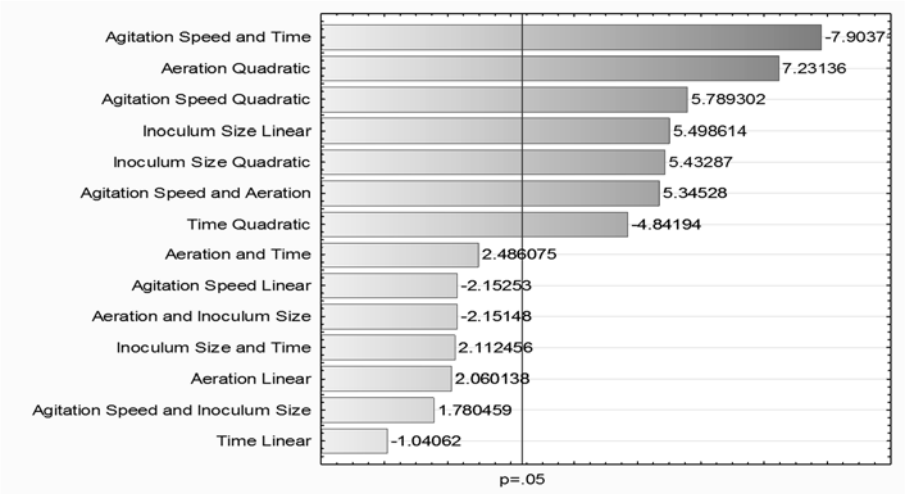

Figure 4: Pareto's Chart of the interfacial tension

$$IT = - 63.842 - 0.183 \cdot AS + 0.002 \cdot AS2 - 34.001 \cdot A + 4.540 \cdot A2 - 5.283 \cdot IS + 0.213 \cdot IS2 + 2.211 \cdot T - 0.005 \cdot T2 + 0.082 \cdot AS \cdot A + 0.007 \cdot AS \cdot IS - 0.005 \cdot AS \cdot T - 0.414 \cdot A \cdot IS + 0.080 \cdot A \cdot T + 0.017 \cdot IS \cdot T \quad (2)$$

As last factor the aeration is the most important variable, as illustrated in the figure 5, to determine the extraction, that should be proved in experiments 3, 5 and 6 with higher extractions and normal aerations. The last extraction with 30.2 g/L represented by the equation 3.

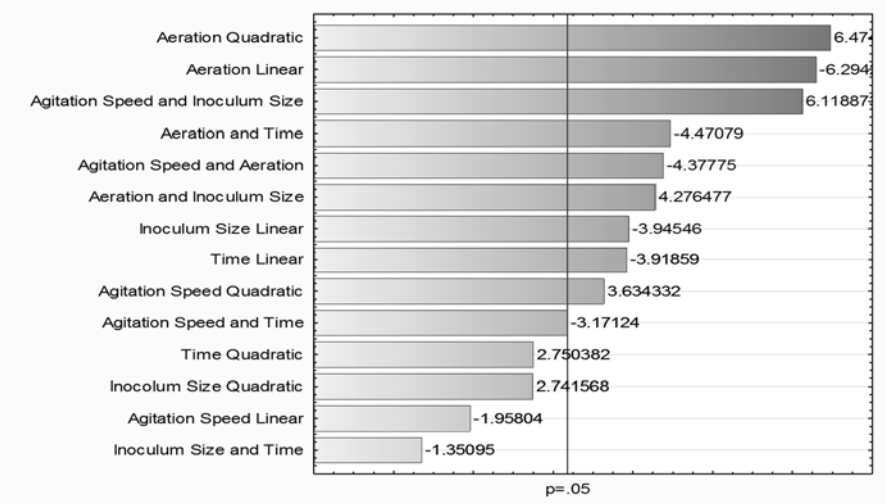

Figure 5: Pareto's Chart of the extraction

$$E = 132.095 - 0.533 \cdot AS + 0.002 \cdot AS2 + 36.469 \cdot A + 8.570 \cdot A2 - 13.529 \cdot IS + 0.227 \cdot IS2 - 0.222 \cdot T + 0.006 \cdot T2 - 0.142 \cdot AS \cdot A + 0.050 \cdot AS \cdot IS - 0.004 \cdot AS \cdot T + 1.733 \cdot A \cdot IS - 0.302 \cdot A \cdot T - 0.023 \cdot IS \cdot T \quad (3)$$

4. Conclusion

The work with biotechnology requires an elevated number of experiments for the ideal statistic, although the time of fermentation is big and it needs practical results. Furthermore, any variation of the process can modify the surfactant properties and the extraction of the biosurfactant. Because of these features, the p-values from the graphs were elevated.

The optimization occurred in the experiment number 6, when the surface tension was 31.45, spreading the surfactant in a normal level; the interface tension was the less of 9.04, raising the contact between the hydrocarbon and the biosurfactant and an elevated extraction of 30.2 g/L. The total cost of each fermentation was € 22.37.

More research in biosurfactant area should be developed to improve the application of this biomolecule.

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