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Strategy of Using Waste for Biosurfactant Production Through Fermentation by *Bacillus Subtilis*

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Recently, the biosurfactant production has been growing up, because this substance has amphiphilic nature with high emulsifying and surface interaction. They are produced through metabolism of microorganisms and many applications are mentioned in industry (chemical, food, pharmaceutical etc.). However, the production is compromised by the use of expensive synthetic substrates. A viable alternative is resort to substrates of waste sources. This study aims to identify optimally biosurfactant production through fermentation by *Bacillus subtilis* using alternative substrates based waste (Glycerin from biodiesel production process, beet peel and corn steep liquor). To set the waste concentration were used design of experiments and process optimization strategies, aided by *STATISTICA 7* software. It was decided use in a Central Composite Rotational Delineation 2^3, evaluating the responses of Emulsifying Index after 24 h (E_{24}) and crude biosurfactant (CB). The optimum point was validated in triplicated. Experiments in scale up were performed to confirm the selected conditions. With these results, it was possible to determine the concentration of waste able to provide a good E_{24} and BC values.

1. Introduction

Surfactants are wide used in several industries around the world, due the molecular characteristic: polar and nonpolar. This particularity ensures the surfactant interfacial properties, in other words, wettability agents that lower the surface tension of a liquid. A surfactant molecule is capable of forming stable emulsions and is present in formulations of food, pharmaceutical products, petroleum compounds, cosmetic, water and soil remediation, textile and some processes. The bulk of surfactant manufacturing is dedicated to those materials that are blended into commercial detergent formulations (Schramm et al. 2003). So many applications come together disadvantages, such as high toxicity and low degradability.
In this context, alternatively arises natural surfactant or biosurfactant, produced by metabolism of microorganism (bacteria, yeast and mold). The biosurfactants have the same characteristics of the surfactants by chemical sources. For instance, biosurfactant produced by *Candida lipolytica* was applied in the remediation of heavy metals (have removed around 96 % of Zn and Cu and decreased the concentration of Pb, Cd, Fe) (Rufino et al., 2012).
The biosurfactants are more effectives than surfactants, what means that lower amount of biosurfactant may be able to have same performance. The use of biosurfactant is not widely encouraged yet, because of the cost involved in production and purification. Many researches choose use waste like medium of fermentation to biosurfactant production. Fontes et al. (2012) used crude glycerine and clarified cashew apple juice were applied as feedstocks for the microbial surfactant synthesis by *Yarrowia lipolytica*. It was studied two waste: corn steep liquor and ground–nut oil refinery as low cost nutrients for the production of a biosurfactant by Candida sphaerica (UCP 0995) (Luna et al., 2012). Cassava flour wastewater were tested such as culture media for biosurfactant production by *Bacillus sp.* And results showed a surface tension of 59 to 26 mN/m (Nitschke and Pastore, 2004).

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A good strategy for attempt to find a culture media composition (synthetic or waste compounds) to produce biosurfactant is design and optimization experimental analysis. This methodology investigates the best operating points from ranges and directs the researcher to make the best decision. In study of Samket et al. (2007), the medium components identified by the initial screening method of Plackett-Burman, were H_3PO_4, $CaCl_2$, H_3BO_3, and Na-EDTA, using *Bacillus licheniformis* K51 like producer microorganism and the response was critical micelle dilution (CMD). Results showed the relative biosurfactant yield as CMD was increased from 10x to 105x. The effects of carbon (glycerol, hexadecane, olive oil, and glucose) and of nitrogen sources (urea, ammonium sulfate, yeast extract, and peptone) were evaluated on factorial design, where the analyzed responses were maximum variation of surface tension and emulsification index, achieving 19.5 mN/m and 81.3 %, respectively (Fontes et al., 2010).
This study has aimed to investigate biosurfactant production by *Bacillus subtilis* using statistical model to determine a culture medium based just in waste: glycerin from biodiesel process, beet peel and corn steep liquor (without synthetic medium).

2. Materials and Methods

2.1 Inoculum Preparation and Standardization

The microorganism used in fermentation for biosurfactant production was the Bacillus subtilis, available in microorganism bank of the Microbiology Division at Research Center for Chemistry, Biology and Agriculture (CPQBA / Unicamp).
The medium used for inoculum preparation was Nutrient Broth (1 % glucose, 0.46 % peptone, 0.3 % meat extract and 0.06 % sodium chloride). Pre-inoculum was prepared 15 mL (same composition the medium) in a 50-mL Erlemeyer flask, which received the microorganism (for adaptation) and incubated in an orbital shaker for 24 h at 37 °C. And inoculum (150 mL of sterile nutrient broth into a 250-mL Erlemeyer flask) received the pre-inoculum and incubated in shaker for another 24 h at 37 °C.
The standardization of the inoculum was performed using a nutrient broth (same composition used above), adjusted in a spectrophotometer (625-nm-wavelength), GENESYS 10S UV-VIS – Thermo Scientific, to a range from 0.08 to 0.1 in absorbance, according to the method of McFarland (cell concentration around).

2.2 Biosurfactant production investigation

The biosurfactant production was investigated using waste substrates (glycerine from biodiesel production (X_1), peeling from beet processing (X_2) and corn steep liquor (X_3)). An experimental design tool was used for investigating the relationship between concentrations and to indicate the more conducive medium for the biosurfactant production.
Initially, it was carried central composite rotatable design (CCRD) (2^3) as an assessment way to analyze the parameters on the desired response, the percentage emulsification index with toluene (E_{24}) and concentration of crude biosurfactant (CB) produced. Table 1 shows the experimental domain levels.

Table 1. Values used in the CCRD

	Experimental domain (%v/v)				
Variables	-1.68	-1	0	+1	+1.68
X_1	0.0	2.0	5.0	8.0	10.0
X_2	0.0	1.2	3.5	5.4	7.0
X_3	0.0	1.0	2.5	4.0	5.0

All designs were developed and analyzed with the help of software *STATISTICA 7*.
The experiments were performed in 250-mL Erlemeyer flasks and a fermentation volume of 100 mL with the proportions determined by the combination of the factorial design for each variable and the pH was adjusted with NaOH or HCl until close 7.0. The flasks were autoclaved, inoculated and brought to an orbital shaker with shaking at 100 rpm at 37 º C for 96 h.

2.3 Emulsification Index (E_{24})

To analyze the emulsification index (E_{24}), the fermented medium was centrifuged (8000 rpm, 15 min, 2 ºC) to obtain cell-free supernatant. 2 mL were collected from the supernatant to mix with 2 mL of Toluene in test tubes. It was stirred by vortexing for 2 min and the mixture was allowed to stand for 24 h. The EI24 was calculated by Eq (1):

$$E_{24} = \frac{HE_{24h}}{HE_t} x100 \quad (1)$$

Where HE_{24h} is the height of the emulsion formed in 24 h and HE_t is the height of the solution. Experiments were performed in triplicate.

2.4 Recover the crude biosurfactant

To recover the biosurfactants for measure yield, cell-free supernatants from fermentation were subjected to an acid precipitation. Briefly, the supernatants were acidified (pH~2.0) with HCl 1 M and left overnight at 7 ºC. Afterwards, it was centrifuged (8,000 rpm, 15 min, 2 ºC), the supernatant was discarded and the precipitate was washed with acidified water and saved. All assays were performed in duplicate.

2.5 Batch fermentation conditions

Biosurfactant production was carried out in lab scale batch device, consisting one Kitassato flask 1.5 L containing 1 L medium optimum (determined by experimental design methodology), one heater blanket (kept ~37 ºC), one airline (flow ~0.6 L/min), one digital temperature indicator and one dissolved oxygen concentration indicator. The batch device was adapted with a collection vessel in the air-exhaust line to trap the foam overflow. The fermentation time was 96 h with experimental design.

3. Results

The assays with range selected in Table 1 were performed and showed responses zero less the run number 13 that showed a value 55.53 ± 0.99 % to Emulsification Index (E24) and 389±50 mg/L to crude biosurfactant concentration (CB). This point represents the axial level of the variable X_3 -1.68 (0) (where X_1 is 0 (5.0), X_2 is 0 (3.5), the only condition on matrix that corn steep liquor does not take on value. All this results suggest that presence of X_3 has negative effect over fermentation, reason that justifies its removal.
The range of variable values X_1 e X_2 was kept the same (Table 2) and new experiments were performed. Strategically, new CCRD was done with 2^2 =4, 3 central points and 4 axial points, totalling 11 runs (Table 3).
From these results the matrix was evaluated enabling the calculation of regression coefficient with p-value limit to 0.10. It was assessed 2 statistical models (Equation 2 and 3) reparametrizated from regression coefficient which describes the behaviour of E_{24} and CB in function of the independent variables.

$$E_{24} = 57{,}70 + 11{,}24X_1 - 23{,}36X_1^2 + 13{,}73X_2 - 18{,}18X_2^2 \quad (2)$$

$$\mathrm{CB} = 306{,}17 + 97{,}85X_1 - 129{,}11X_1^2 + 143{,}56X_2 \quad (3)$$

Where X_1 and X_2 are coded variable of the waste concentration. ANOVA results (Table 4) of the quadratic model revealed that the models equation derived by CCRD could be adequately used to describe the biosurfactant production (from E_{24} and CB) under range of operating conditions. The model resulted in a F_{cal} of 13.82 and 8.42 for regression (values upper than F_{tab}, 4.53 and 4.35) and R^2 of 90.02 and 78.30 %, respectively. Statistical model of E_{24} and CB was highly significant and adequate for the response variables that were tested.

Table 2. Values used in the full factorial design

	Experimental domain (%v/v)				
Variables	-1.41	-1	0	+1	+1.41
X_1	0.0	1.45	5.0	8.5	10.0
X_2	0.0	1.0	3.5	6.0	7.0

Table 3 shows new scenario of results achieved. There are many variations in 2 responses inherent in the process, without compromising the correlation between them.

Table 3. Combinations of factors

Run	X_1	X_2	E_{24} (%)	CB (mg/L)
1	-	-	0.0±0.0	0.0±0
2	+	-	27±5.0	100±40
3	-	+	10.4±2.1	0.0±0
4	+	+	38.08±1.05	400±105
5	-	-	0.0±0.0	0.0±0
6	+	-	24±7.07	200±0
7	-	+	0.0±0.0	0.0±0
8	+	+	62.5±0.0	600±30
9	0	0	57.16±1.16	420±80
10	0	0	55.08±0.92	310±5
11	0	0	60.87±0.0	300±10

Table 4. Analysis of variance (ANOVA) for the $EI_{24,}$ Biosurfactant Concentration (BC)

Source of variation	DF		SS		MS		F_{cal}	
	E24	CB	E24	CB	E24	CB	E24	CB
Regression	4	3	5,914.84	344,554.3	1,478.72	114,851.43	13.82	8.42
Residual	6	7	641.96	95,413.9	106.99	13,630.55		
Lack of fit	4	5	624.75	86,863.9	156.2	17,372.8	18.15	4.06
Pure error	2	2	17.20	8,550.0	8.6	4,275.0		
Total	10	10	6,556.84	439,968.2				

E_{24} (R^2=90.20 % ; $F_{4;6;0.10}$=4.53);- CB (R^2=78.30% ; $F_{3;7;0.10}$=4.35)

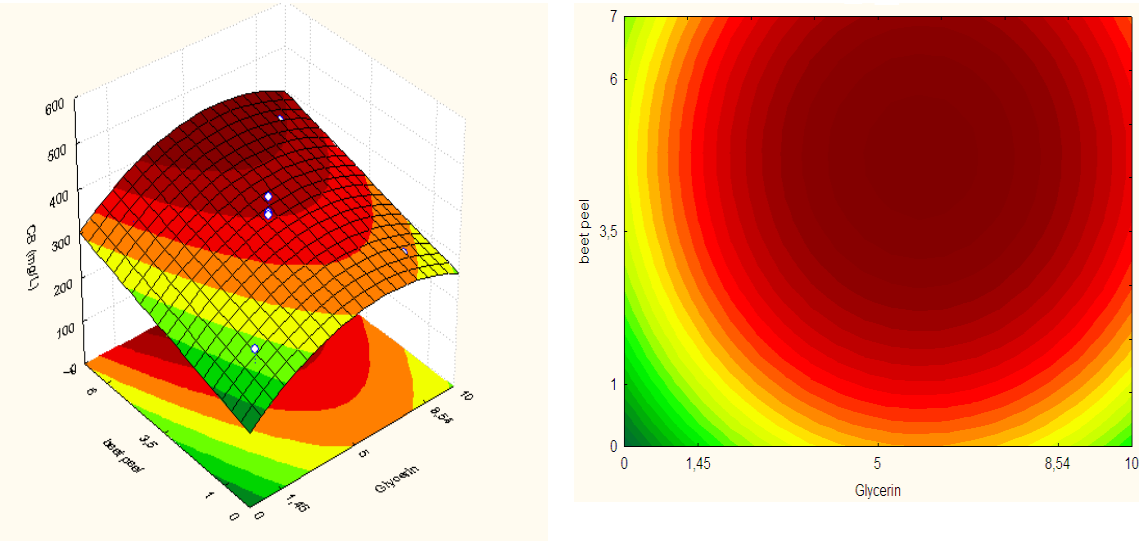

Figure 1: Surface response and contour curve from E_{24} model

Analysis from these models through contour curve and surface response (Figure 1 and 2) suggest optimum is reached if X_1 (Glycerin) is in central point (5.0) and X_2 (beet peel) is in level +1(6.0). New experiments with these conditions were made to prove model validation. The results obtained show good agreement between observed (E_{24} got 60.5±0.5 % and CB got 480±20 mg/L) and predicted (E_{24} got 53.25 % and CB got 449.73 mg/L) responses.

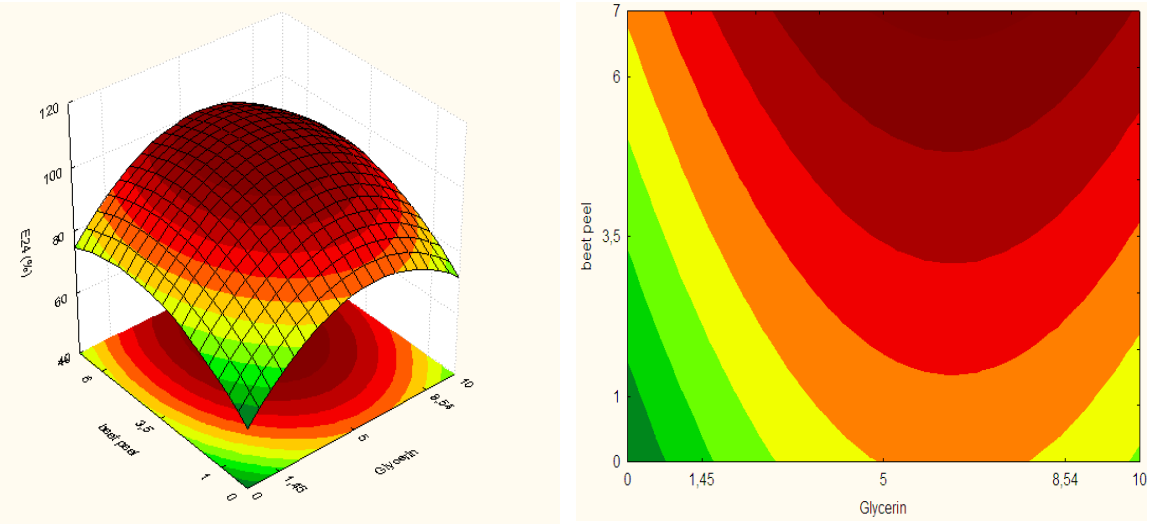

Figure 2: Surface response and contour curve from CB model

It is good highlight that experimental values obtained in experiments were of the same order found in literature. Pereira et al. (2013) isolated three *Bacillus subtilis* from Brazil crude oils and investigated biosurfactant production potential with different carbon sources, between of them, glycerin (basal medium supplemented) that reached values of 151.1±71.3, 150.3±70.5 and 965±89.3 mg/L for recovery biosurfactant and 33.5±2.7, 26.2±2.3 and 48.4±2.2 for E_{24} each microorganism respectively.

Batch fermentation

The assays previously tested were performed in control volume of 100 mL. The experiments held parameters defined by CCRD (X_1 and X_2 optimum), but the operating conditions were changed. As conditions were scale up to 1000 mL of control volume and oxygen rate was provided to systems, assessing effects of changes. Table 5 shows the variables analysed in two times (0 and 96 h).

Table 5 New fermentation condition

Fermentation: control volume of 1L	
Time 0h	Time 96h
Oxygen dissolved-6.82±0.23mg/L	Oxygen dissolved-1.81±0.08mg/L
pH-6.51±0.05	pH-6.09±0.01
Biomass-0.01±0g/L	Biomass-1.01±0,60g/L
Biosurfactant-0±0mg/L	Biosurfactant-550±85mg/L
E_{24}-0±0%	E_{24}-58±2%

The some results (Biosurfactant crude and E_{24}) reached with this experiment were similar to those developed before. Others variables were evaluated and showed peculiar behaviour (as value of oxygen dissolved), which encourage future studies focussed in increase crude biosurfactant concentration based into optimization of process variables, such as oxygen dissolved, agitation, pH or temperature.

4. Conclusions

The use of waste in biosurfactant production come increase due strong appeal from their characteristic and applications, beside low cost. In current work, strategies were investigated to use only waste (no synthetic medium) to propose a culture medium for biosurfacyant production.

Assays showed that corn steep liquor, in these experiments, does not work like medium composition, because inhibits totally biosurfactant production. The study using other waste (glycerine from biodiesel and

beet peel) were satisfactory through CCRD assessment, could generate statistical model to E_{24} and CB. Based in ANOVA results (R^2= 90 and 78 %; and F_{cal} values), the determination coefficient were considered reasonable for fermentation process, due the high difficult in variation.

The validation using optimal point was success, providing results close the value predicted from mathematical model. When, the conditions were changed (scale up and new operating parameters), the values predicted also were near observed values. This way, it could be safely define a culture medium to produce biosurfactant.

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