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Selection of the main micronutrients for the production of biosurfactant by an isolated Bacillus subtilis strain

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***Abstract*— Biosurfactants possess various unique properties, such as good environmental compatibility, low toxicity, high selectivity, high biodegradability and are stable over a wide range of temperature, pH and ionic strengh. These advantages allow the substitution of chemically synthesized surfactants by those of biological origin in different industrial sectors, mainly for enhanced oil recovery. This study presents the influence of different micronutrients (B, Fe, Zn, Cu, Mn, Ca, Mo and Co) in biosurfactant production by Bacillus subtilis, LFB 732 strain, using very high polarization (VHP) raw sugar, as the one and only cost-effective carbon source. A Plackett-Burman design, with 12 tests, in replicate, was employed for screening eight mineral salts, which are regular constituents of fermentative medium, aiming to reduce the cost of the bioprocess. Batch fermentation experiments were carried out in shake-flasks at the controlled agitation speed of 150 rpm and temperature of 30°C, for 24 and 48 h. The experimental data revealed that Fe and Zn ions were the most important micronutrients in the fermentation broth for production of this biosurfactant and the absence of the others did not affected the production.**

***Index Terms*— Biosurfactant, Bacillus subtilis, VHP sugar, Plackett-Burman design, mineral salts medium.**

I. INTRODUCTION

Biosurfactants, comparatively to the chemical surfactants available in the market, are amphiphilic molecules with similar surface-active properties [1]. In contrast, the biomolecules have a larger structural diversity compared to the non-biossyntetic products due to the possibility of production by a wide variety of microorganisms, including yeast, filamentous fungi and mainly bacteria [2].

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The biomolecules present many advantages when compared to chemically synthesized surfactants, such as biodegradability, high selectivity, low toxicity and stability against environmental variations (temperature, pH, ionic strength) [3]. Thus, biosurfactants became an environment-friendly alternative to chemical surfactants. Also, the use of synthesized surfactants is not recommended due to its toxic effects on living organisms and on the environment, causing secondary pollution.

Bacillus species, in particular strains of *Bacillus subtilis* (non-pathogenic microorganism considered as GRAS - generally considered as safe) are potential producers of different biosurfactants [4]. Many studies in the literature show that the main bio-product synthesized by *B. subtilis* is surfactin, a lipopeptide with interesting surface-active properties, foaming emulsifier and wetting agent, which allow its application in several industrial areas [5], such as in microbial enhanced oil recovery (MEOR) reducing the capillary forces that prevent the movement of the oil through the pores of the rock [6].

However, microbial surfactants are not yet widely used due to the high production costs, associated with inefficient methods of product recovery and the use of expensive substrates on the fermentative process. The economic production issue of biosurfactants can be significantly reduced with the use of alternative sources of nutrients [7]. One possible strategy is to apply renewable sources in the production medium composition, which can reduce production costs by up to 30% [8,9]. Therefore, recent studies in this field aim at the use of industrial residues as nutrients in production medium and the optimization of the fermentation conditions seeking the best strategies for the production of microbial surfactants in large scale [10].

Thus, the present work emphasized the use of VHP (very high polarization) sugar as a carbon source for the production of the biosurfactant by a *B. subtilis* strain. The choice of VHP sugar as carbon source is justified because it is the raw form of refined sugar, thus representing less production costs. Also, this work evaluated the influence of reducing the addition of micronutrients in the production medium, since the composition of the culture media currently used by the industry covers a large quantity and variety of micronutrients. In this way, it is expected to obtain a low cost biosurfactant with higher economic feasibility for the application in the oil recovery.

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II. MATERIALS AND METHODS

A) *Microorganism*

The strain used was the *Bacillus subtilis* LFB 732 from the *Bacillus* Cultivation Collection and Correlated Genus/ Fiocruz maintained in Agar nutrient and stored at 4°C.

B) Quantification of the components content in VHP sugar

The chemical elements of the VHP sugar (Ca, Mg, K, Mn, Fe, Co, Cu, Zn, S and P) were analyzed by inductively coupled plasma optical emission spectrometry (ICPOES) with the equipment Horiba Jobin Yvon, model Ultima 2. The calibration was done with appropriated standard solutions for each element. The procedure was adapted from the norm ASTM-D1976 *Standard Test Method for Elements in Water by Inductively-Coupled Argon Plasma Atomic Emission Spectroscopy*, usually applied for general aqueous samples. The VHP sugar used in this study was composed by (mg/kg): Ca (34), Mg (14), K (52), Fe (0.83), Zn (0.23), Cu (< 0.5), Mn (< 0.5), Co (< 0.5), P (2), S(27).

C) *Inoculum preparation*

The *Bacillus subtilis* LFB 732 was inoculated in Petri dishes containing Agar nutrient and incubated at 30°C for 24h. Thereafter, two loops of the activated culture were transferred to 500 mL Erlenmeyer flasks, containing 100 mL of nutrient medium containind (g/L): meat peptone (5), yeast extract (3), glucose (9) and NaCl (9), at pH 7 and sterilized at 121°C for 15 minutes in the autoclave. The flasks were incubated in a rotary shaker (CIENTEC – 712 RN) at 150 rpm and 30°C. After the culture reached optical density (OD) of 0.3 at 600 nm, ten milliliters of that inoculum was transferred to 100 ml of the production media in Erlenmeyer flasks.

D) Batch Fermentation

All the assays were conducted in Erlenmeyer flasks of 500 ml on a rotary shaker at 150 rpm and 30°C, with 100 ml of production medium, sterilized at 121°C for 15 minutes in the autoclave, and 5 ml/L of micronutrients solution, previously sterilized by filtration. The fermented medium was centrifuged at 15,300 g for 10 minutes to carry out the analytical determinations.

E) Selection of the main micronutrients for the biosurfactant production

A defined production medium was firstly prepared with the following composition (g/L): NH_4NO_3 (4), KH_2PO_4 (3), Na_2HPO_4 (6) e $MgSO_4$ (0,2), and VHP sugar (10), adjusted to pH 7. A Plackett-Burman type planning (PB12) was carried out with the goal to select the micronutrient solution components added to the production medium that effect the biosurfactant production. The fermentations were carried out for 24 and 48 hours, and the biosurfactant concentration was used as the main evaluation criterion. A full first-order polynommial model was used to represent the adjustment of the response values:

$$Y(x_1, x_2, x_3) = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_6x_6 + b_7x_7 + b_8x_8$$

Where: Y= response estimation; b= model coefficients; x=variables.

F) pH

The pH measurements were performed directly on cell-free fermented medium in the digital model DMPH-1 (Digimed).

G) Surface Tension

Surface tension was determined in the cell-free fermented broth using a tensiometer SIGMA 70 Surface tension measurement report (KSV Instruments Ltd) at 25°C, according to the De Nouy ring method [11], using uninoculated media (62,5 mN/m) as abiotic negative controls.

H) Biomass

Cell growth was determined by measuring the optical density of the samples, using a Hach spectrophotometer (DR 5000) at 600 nm. Cell concentration was determined using a calibration curve that related the values of optical density and dry weight [12].

I) Biosurfactante concentration

Biosurfactant concentration was determined using a high-performance-liquid chromatographer equipped with a UV detector (LC 10A- Shimadzu), at 205 nm, and a Symmetry C18column (150 x 4.6 mm, 5 µm). The mobile phase consisted of 20% (v/v) trifluoroacetic acid (3.8 mM) and 80% (v/v) acetonitrile. The elution rate was 1.0 mL/min at 30°C and the sample size was 20 µL [13]. The identity of the purified surfactin was obtained by using the commercially available 98% pure surfactin (Sigma-Aldrich) as the reference compound.

III. RESULTS AND DISCUSSION

The Plackett-Burman experimental design was done by analysing the eight variables (x_1 to x_8) and the different levels of each variable (-1 and 1), as indicated in Table I.

Table I: Concentration values of the variables and their respective levels of the Plackett-Burman experimental design PB12.

Variables	Components	Levels (g/L)	
		-1	1
x_1	$FeSO_4.7H_2O$	0	0,1
x_2	$MnSO_4.H_2O$	0	0,23
x_3	H_3BO	0	0,4
x_4	$CoCl_2.6H_2O$	0	0,01
x_5	$CuSO_4.5H_2O$	0	0,01
x_6	$(NH_4)_6Mo_7O_{24}$	0	0,02
x_7	$ZnSO_4$	0	0,17
x_8	$CaCl_2$	0	0,2

Table II presents the PB12 experimental matrix and the results of surface tension and biosurfactant concentration obtained in the different experimental conditions after 24 and 48 hours of fermentation.

Table II: PB12 experimental matrix for each assay and their variables levels. Variables: $FeSO_4.7H_2O$ (x_1), $MnSO_4.4H_2O$ (x_2), H_3BO_3 (x_3), , $CuSO_4$ (x_4),$(NH_4)_6Mo_7O_{24}$ (x_5), $CoCl_2$ (x_6), $ZnSO_4$ (x_7), $CaCl_2$ (x_8), superficial tension (ST) and biosurfactant concentration (BC).

Assay	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	ST_{24h}(mN/m)	ST_{48h}(mN/m)	BC_{24h} (mN/m)	BC_{48h} (mN/m)
1	1	-1	1	-1	-1	-1	1	1	29,1	29,6	137,3	223,0
2	1	1	-1	-1	-1	1	-1	1	28,8	29,7	137,5	171,5
3	-1	1	1	1	-1	-1	-1	-1	29,7	30,0	88,1	125,4
4	1	-1	1	-1	1	1	-1	-1	29,0	29,8	128,6	184,5
5	1	1	-1	1	-1	1	1	-1	28,7	29,6	163,0	224,0
6	1	1	1	1	1	-1	-1	1	29,3	29,8	107,2	173,6
7	-1	1	1	-1	1	1	1	-1	28,5	29,5	117,1	132,2
8	-1	-1	1	1	-1	1	1	1	28,9	29,7	100,0	147,3
9	-1	-1	-1	1	1	1	-1	1	28,7	29,0	104,9	146,8
10	1	-1	-1	1	1	-1	1	-1	29,0	30,0	138,8	184,5
11	-1	1	-1	-1	1	-1	1	1	28,8	30,0	155,1	197,1
12	-1	-1	-1	-1	-1	-1	-1	-1	28,7	29,6	119,3	128,1

At 24 and 48 hours of cultivation, the surface tension lowering values were similar for the 12 assays tested. This can be explained, since the biosurfactants are able to lower the surface tension to values close to 30 mN/m even at low concentrations in the culture medium, [14; 15]. Recent studies in the literature report that values of surface tension in the range obtained in this work indicate that the bioproduct may have efficient surfactant activity, even in contact with a complex mixture of hydrocarbons, such as crude oil. Fernandes et al [16] presented that the injection of 600mg/L of biosurfactant resulted in approximately 69% recovery of residual oil in a glass column (Kontes Flex Column, Fisher Scientific K420401-2510) of 2.5x10 cm that remained after injection with water.

The maximum biosurfactant production in 24 hours was obtained in the assay 5 (163 mg/L), which increased 37% of the bioproduct concentration after 48 hours of fermentation (224 mg/L) with the decrease in productivity, as in all the other assays. Thus, taking into consideration the drop in productivity when using 48 hours of process, the further experimental planning were done with 24 hours of cultivation.

The model obtained for biosurfactant concentration after 24 hours of experiment is presented below:

$$Y_{BC\ 24h} = 124,7 + 21,3x_1 + 6,5x_2 - 23,4x_3 - 15,5x_4 + 1,1\ x_5 + 0,9\ x_6 + 21,0\ x_7 - 2,2\ x_8.$$

The generated model was used to design a Pareto Diagram (Fig. 1), where it is possible to determine the variables with the highest statistical significance for the biosurfactant production.

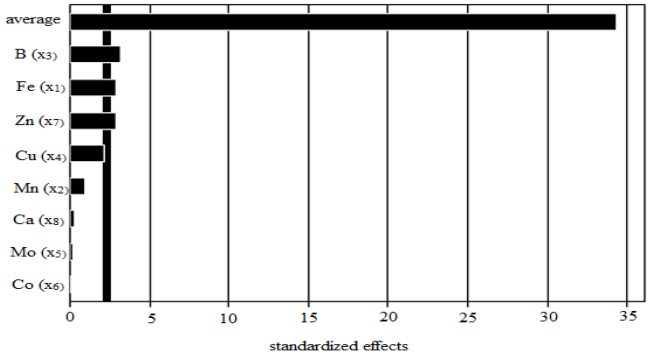

Fig. 1: Pareto Diagram for the experimental design PB12.

The obtained model indicates that the higher the coefficient value, the greater the impact of the change in the variable level, for the the evaluated response. Thus, for 24 hours fermentation, the highest coefficients for the biosurfactant concentration are obtained with the salts $FeSO_4.7H_2O$ and $ZnSO_4$ in the production medium. The values associated with the elements H_3BO_3, $CuSO_4$ and $CaCl_2$ had negative effects on the biosurfactant production, this indicates that those salts can be considered as unnecessary for the biosurfactant production by the strain used, as well as all others that had lower coefficient values.

The results obtained in this sudy are in accordance with Wei et al. [17] with Gu, et al [18]. The former studied the influence of the trace elements Mn^{2+} (0.2 mM) and Fe^{2+} (0.3 mM) in the mineral medium 4% glucose for the production of biosurfactant by *Bacillus subtilis* ATCC 21332 and concluded that the presence of one of the two metals is sufficient to allow efficient production of biosurfactant. The latter presented that $FeSO_4$ (6.7879 µM) and $ZnSO_4$ (0.0377 µM) were significant variables for lipopeptide production, iron being a common cofactor for microbial enzymes and proteins and therefore an essential mineral nutrient for microorganisms [19] and the addition of ZnSO4 to the growth of *B. subtilis* [20]. The results are also in agreement with Cooper, et al [20] and Abdel-Mawgoud, et al [21] who concluded that Cu^{+2} appears as a potent inhibitor of cell growth and biosurfactant production.

IV. CONCLUSION

The present study showed the importance of Fe and Zn ions in the micronutrient solution added to the biosurfactant production medium by *Bacillus subtilis* LFB 732 strain. The qddition of the ions Mn, Ca, Mo and Co showed no influence.

On the other hand, the B and Cu ions showed a negative effect on the production. Thus, the elimination of some micronutrients from the production medium will not affect the yield of the process, in addition to reducing the costs of the bioproduct.

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