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Evaluation of the Influence of Nitrogen and Phosphorus Nutrients in the Culture and Production ofbiosurfactants by MicroalgaS*pirulina*

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ABSTRACT
The aim of this study was to verify the influence of phosphorus and nitrogen nutrients in the culture and production of biosurfactants by *Spirulina platensis* LEB 52,*Spirulina platensis*Paracas and *Spirulina* sp. LEB 18. For this, experiments were performed using Full Factorial Design 2^2 to evaluate the influence of phosphorus and nitrogen nutrients on the maximum biomass concentration and maximum productivity in the cultures, as well as in production biosurfactant by extracts derived from microalgae through surface tension measurements. The *Spirulina platensis* LEB 52 provided higher biomass production when compared with the *Spirulina platensis* LEB 52 and*Spirulina platensis*Paracas, but the phosphorus and nitrogen variables showed no significant influence on the maximum biomass concentration and maximum productivity. The resultds showed that *Spirulina* sp. LEB 18 was more favorable for the production of biosurfactants in relation to the others strains, because it presented an extract with a surface tension measurement of 31.2 $mN.m^{-1}$ in a culture performed with higher concentrations of nitrogen (412 $mg.L^{-1}$) and without the addition of phosphorus.
Keywords- biomass, cyanobacterium, surfactant.

I. INTRODUCTION

Microalgal biotechnology has recently been used as a source of food, pharmaceutical, biochemical and fertilizerproducts and as an energy source[1], [2], [3] and [4].The manipulation of culture conditions, the presence, absence or concentration of certain nutrients tend to stimulate the biosynthesis of compounds ranging from food to pharmaceutical products [5], [6] and [7], as well as influencing the results of cell growth such as the components of biomass, pigments, proteins and lipids, among others [2].

Spirulina microalgae is widely studied and used for various purposes. This microalga has the advantage of being safe for use (Generally Recognized as Safe - GRAS) thus its use is permitted in the food industry without risk to health [8] and [9].

The *Spirulina*, depending on growing conditions, can presentin its constitution 38-70% protein; 13-25% carbohydrates; 6-15% lipids and 6-9% minerals [10], [11] and [12]. This microalga is producer of compounds such as glycolipids, phospholipids and neutral lipids[13] and[14], which are classified asbiosurfactant [15].

Surfactants synthesized by microorganisms by biological processes are called biosurfactants. Thesesubstances are consisting of a hydrophilic portion and a hydrophobic portion which act as surface active agents capable of reducing the free energy of the system and consequently reduce the surface and interfacial tension [16].

This behavior makes the biosurfactants suitable for various applications, such as detergents, emulsifiers, foaming agents and others. Because of these applicability options, the biosurfactants are present in various industrial sectors such as textiles, cosmetics, pharmaceutical, food and polymers [17],[18] and [19].

Biosurfactants have several advantages when compared with surfactants of petrochemical origin, these include high biodegradability, low toxicity, lower critical micelle concentration, antimicrobial activity, being produced in a milder form from fermentation processes, and having the possibility of production from renewable resources [20] and [21], with this mode being potential replacements for synthetic surfactants.

The objective of this study wasto verify the influence of phosphorus and nitrogen nutrients in the culturing and production of biosurfactantsby*Spirulina platensis*Paracas,*Spirulina platensis* LEB 52 and *Spirulina* sp. LEB 18 microalgae.

II. MATERIALS AND METHODS

2.1 Microorganisms and culture conditions

In this study were used the microalgae *Spirulina platensis*Paracas,*Spirulina platensis* LEB 52 [22]and *Spirulina* sp. LEB 18 [23] belonging to the collection of the Laboratory of Biochemical Engineering, Federal University of Rio Grande (FURG), maintained in Zarroukmedium [24].

The culturing was carried out in a tubular photobioreactors (2 L) and performed according to a full factorial design $2^2$with triplicate at the center point,withthe lower level being represented by the culture medium which had no addition of phosphorusand nitrogen, the central level by 50% of the concentration of phosphorus and nitrogencontained in Zarroukmedium [24] and the top level represented by a concentration of 100% phosphorus and nitrogen contained in Zarroukmedium [24] (Table 1).

From the experimental design, were assessed the influence of the concentration of phosphorus and nitrogen components present in the culture medium on the maximum biomass concentration and maximum productivity in the cultures, as well as in extracts derived from the biomass of microalgae by measuring the surface tension.

Table 1.Real and coded levels of concentration of phosphorus (P) and nitrogen (N)variables for the culturing of microalgae.

Variable($mg.L^{-1}$)	CodedLevels		
	-1	**0**	**+1**
P	0	57	114
N	0	206	412

The assays were performed at 30°C with an illuminance of 41.6 $\mu mol.m^{-2}s^{-1}$(12 h light/dark photoperiod)and with continuous stirring by means of injection of sterile air at 0.07 vvmby diaphragm pumps. The initial biomass concentration of the cultures was 0.2 $g\ L^{-1}$.

At the end of the culturing the microalgal biomass was separated by centrifugation (9205 g for 10 min) (CR 22GIII, Hitachi), and frozen for 24 h at 70°C and lyophilized for 48 h.

2.2 Cell Growth

The concentration of the microalgae biomass was determined every 24 h by measuring optical density at 670 nm in a spectrophotometer (Q798DRM,Quimis)[25]. The pH of the cultures was determined daily in a digital pH meter (Q400HM,Quimis).

Daily were evaluated the maximum biomass concentration and maximum productivity, with the productivity obtained by the Equation 1.

$$\text{Prod} = \frac{X_t - X_0}{t - t_0} \quad (1)$$

Where,X_t is the biomass concentration ($g.L^{-1}$) at time t (d), and X_0 the biomass concentration ($g.L^{-1}$) at time t_0 (d) [26].

2.3 Determination of nitrogen and phosphorus

Concentrations of phosphorus and nitrogen in the culture medium of different experiments were evaluated every 48 h. The phosphorus concentration was determined by colorimetric analysis (PhosVer 3,Hach, USA) and the nitrogen concentration was determined using the method proposed by Cataldo et al. [27].

2.4 Extraction of the microalgae biosurfactants

The extraction of biosurfactantswas conducted using methanol as a solvent with the dry biomass in a tubes shaker (10 min) containing glass beads, followed by ultrasonic bath (20 min), both procedures were performed three times.Subsequently, the samples were subjected to constant agitation in a rotary shaker at 160 rpm for 5 h with 50 ml of methanol, and then centrifuged (9205 g for 10 min)(CR 22GIII, Hitachi).

The evaporation of the methanol was performed on rota-vaporator at 60°C(Q344B, Quimis). After removal of methanol,was performed resuspension in water (0.1 g of dry biomass to 7.5 mL of water) and centrifugation (9205g for 10 min)(CR 22GIII, Hitachi), with the extract obtained from the supernatant fraction [28].

2.5Biosurfactant activity

Thebiosurfactant activity of the extract obtained was performed by measuring the surface tension with the sample in contact with the air, being performed in a tensiometer(K-6,Kruss) using the ring method[29].The initial surface tension measurement was carried out using distilled water, which has value of 72.0 $mN.m^{-1}$ [16].

III. RESULTS AND DISCUSSION

In all culture conditions performed for different microalgae no cellular adaptation phase was observed(Fig. 1).Lourenço[30] reports that the adaptation phase can not be observed when the inoculated cells immediately begin their growth in a

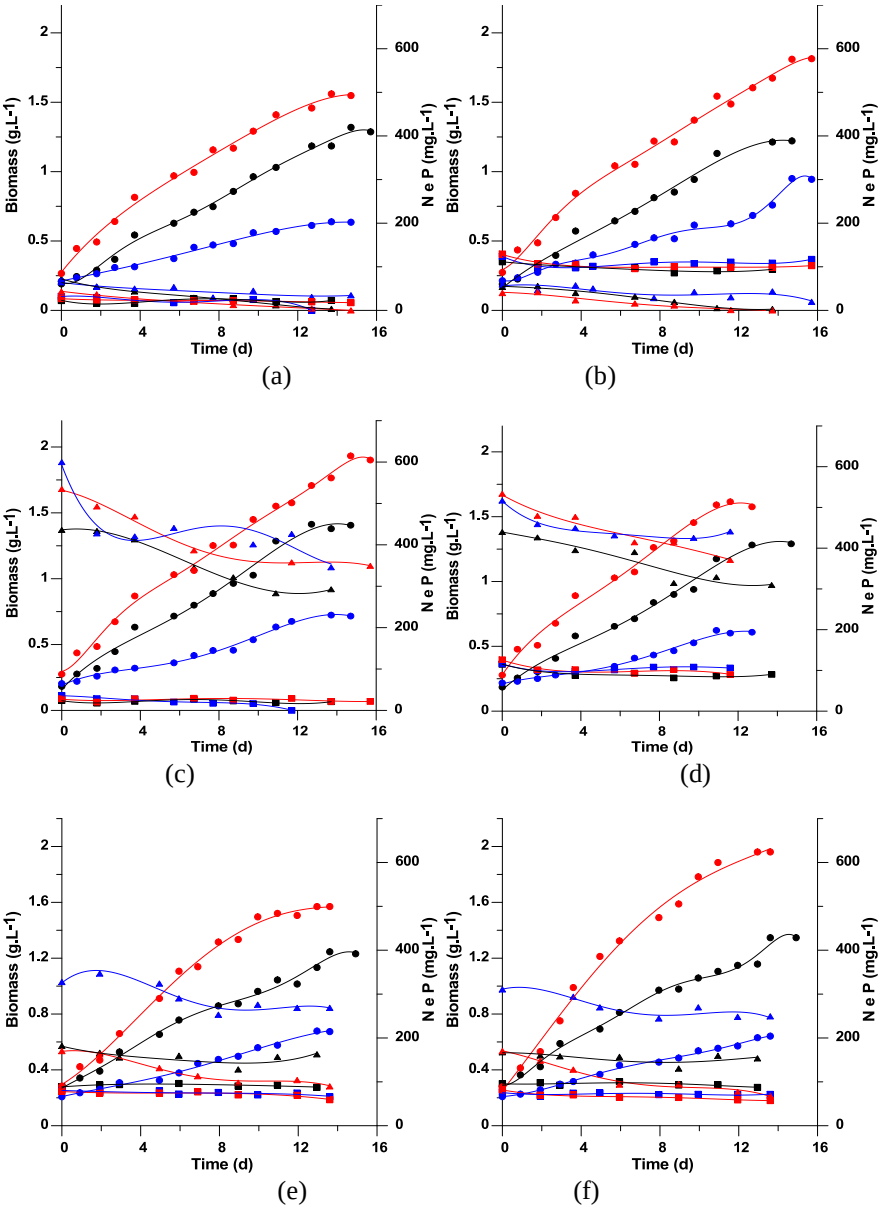
fresh culture medium, this situation can occur if the differences between the culture conditions and the inoculum are not too prominent. Besides these factors, Schmidell et al. [26] reported that the duration of this phase will also depend on the concentration of the inoculum and its physiological state.

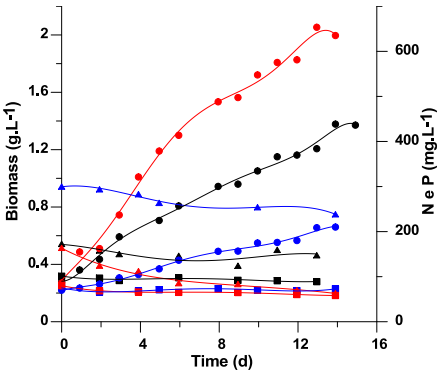
As shown in Fig. 1, the microalgae presented growth since inoculation, indicating that they adapted to the new culture conditions These is probably due to the fact that the inoculum was kept at the same temperature and luminosity used in the experiments and also by the change in the culture medium having been performed only in phosphorus and nitrogen components.

The cultures were finished between 13 and 16 d, when the microalgae reached the end of their exponential phase of growth.

The cultures performed with *Spirulina platensis*Paracas showed pH values between 9.51 and 10.94, thus being in the range of optimum pH values for this microalga, which according to Vonshak [31] is between 9.5 and 10.5. The monitoring of pH is important since the uptake of atmospheric CO_2 by *Spirulina* depends on the pH of the medium [32].

The *Spirulina platensis* LEB 52 showed pH range between 9.66 and 11.43 throughout the culture period and *Spirulina* sp. LEB 18 between 9.67 and 11.41. At the end of the culturing both microalgae had pH values above 11, according to Binaghi et al. [32] at this moment the growth of *Spirulina* becomes limited due to the large effect of alkalinity on metabolic processes besides the possibility of the occurrence of precipitation of carbonate, which follows by flocculation or sedimentation of the microalgae.





(g)

Figure 1. Biomass concentration (✂), Nitrogen (N)(▲) and Phosphorus (P)(♙) for assays(a) without addition of P and N,(b) 114mg.L^{-1} P and without adding N, (c) without the addition of P and 412mg.L^{-1} N,(d) 114mg.L^{-1}P and 412mg.L^{-1} N (e) 57mg.L^{-1}P and 206mg.L^{-1} N,(f) 57mg.L^{-1} P and 206mg.$L^{-1}$and N (g) 57mg.L^{-1} P and 206mg.L^{-1}N for microalgae *Spirulina* sp. LEB 18 (black),*Spirulina platensis* LEB 52 (red) *Spirulina platensis*Paracas(blue).

Regarding the use of phosphorus, in cultures that a phosphorus source has not been added (Fig. 1a and 1c) the *Spirulina platensis*Paracas completely consumed the remaining phosphorus from the inoculum used.While in the otherscultures, the phosphorus decreased from 8.5% to 5.7% compared to the initial concentration.

Microalgae *Spirulina platensis* LEB 52 and *Spirulina* sp. LEB 18, in the cultures without addition of phosphorus consumed 34.9% and 12.5% of this nutrient, respectively, in the culture performed without the addition of nitrogen source (Fig. 1a). In the culture conducted with 100% source of nitrogen (Fig. 1c), the phosphorus intake was lower, being 23.3% by *Spirulina platensis* LEB 52 and 11.3% by *Spirulina* sp. LEB 18.

The maximum consumption of phosphorus observed in the cultures carried out with 100% and 50% of nitrogen and phosphorus source (Fig. 1d, 1e, 1f and 1g) was 21.4% for*Spirulina* sp. LEB 18 and 29.9% for*Spirulina platensis* LEB 52, respectively.

According to the results obtained in relation to the consumption of phosphorus by the microalgae in different cultures,one can see that only *Spirulina platensis*Paracas consumed all available phosphorus in the medium carried out without addition of this nutrient. In the otherscultures,the microalgae consumed values below 50% relative to the initial concentration.

This fact indicates that the availability of phosphorus present in the media of different cultures was higher than what the microalgae need for their growth. According to Kaplan et al. [33] the amount of phosphorus present intracellularly,pH and cultivation temperature can affect the consumption of phosphorus by the microalgae.

The phosphorus present is stored intracellularly in the form of polyphosphate granules or as metaphosphateinside the cell.The microalgae can use this phosphorus reservefor growth and/or maintenance of the microbial population [34].

As for the nitrogen, in the *Spirulina platensis* LEB 52 and *Spirulina* sp. LEB 18 cultures where this nutrient was not added (Fig. 1a and 1b), both microalgae consumed all nitrogen which came from the inoculum.While in relation to the consumption of this nutrient by *Spirulina platensis*Paracasthere was a reduction between 67.0% and 46.2% in relation to the initial concentration.

In the cultures performed with addition of 100% of the nitrogen source (Fig. 1c and 1d) the consumption levels of this nutrient for all microalgae was less than 43% and in cultures performed with 50% of the nitrogen source (Fig. 1e, 1f and 1g)its maximum consumption was 61.5% by*Spirulina platensis* LEB 52.

When the nitrogen source is present in the form of nitrate, the microalgae spends cell energy to reduce this ion to nitrite through nitrate-reductase enzyme. After, occurs other reduction by nitrite-reductase generating ammonia, being the form of nitrogen used by the alga in its metabolism [35].

Von Ruckert and Giani[36] performed experiments with the cyanobacterium*Microcystisviridis*Lemmermannusing a medium with ammonium and nitrateas nitrogen source. The results show that the ammonium ion is completely removed at the tenth day, while the nitrate ion remained still available.

Given the above and as in Zarrouk medium [24]the source of nitrogen is added in the form of nitrate,could be observed in this study that the nitrate uptake by the algae occurred slowly. But even with low nitrogen consumption the microalgae continued to grow, indicating that a culture medium with lower concentrations of this nutrient can be used.

Table 2 shows the results of maximum biomass concentration, maximum productivity of cultures and

surface tension of extracts obtained in cultures of the microalgae.As can be seen microalga*Spirulina platensis* LEB 52 provided higher biomass production when compared with *Spirulina platensis*Paracas and *Spirulina* sp. LEB 18.

The concentrations of phosphorus and nitrogen used had no significant effect ($p>0.10$) in the maximum biomass concentration and maximum productivity of the microalgae *Spirulina platensis* LEB 52 and *Spirulina* sp. LEB 18, indicating that both microalgae adapted to different nutritional conditions in the cultures. According to Lourenço[30], cyanobacteria tolerate large fluctuations in temperature, salinity, pH and nutrient availability. Possibly, their tolerance to environmental variations is related to the prokaryote condition.

Among the cultures performed with*Spirulina platensis*Paracas, this showed higher values ofmaximum biomass concentration(0.95 $g.L^{-1}$) and maximum productivity(0.191 $g.L^{-1}.d^{-1}$)(Table 2), when the culturing was carried out without addition of nitrogen and 100% of phosphorus (114$mg.L^{-1}$).It can be observed that even without the addition of nitrogen in the culture medium,*Spirulina Paracas* showed higher maximum biomass concentration and maximum productivity, thus this nutrient was not a limiting factor for its growth.

According to Sassano et al. [47], in minimum conditions of nitrogen in the culture medium, the microalgae degrade phycocyanin, which is used as a nitrogen source.Colla et al. [10] in a study of *Spirulina platensis* also found that the reduction of sodium nitrate in the Zarroukculture medium did not influence the biomass productivity of microalgae.

Table 2. Maximum biomass concentration (X_{max}), maximum productivity (P_{max}) of cultures and surface tension (ST) of the extracts obtained in cultures performed according to Full Factorial Design 2^2.

Microalga	**Assay**	**Variable**		$X_{máx}$	$P_{máx}$	**ST**
		Phosphorus ($mg.L^{-1}$)	**Nitrogen ($mg.L^{-1}$)**	**($g.L^{-1}$)**	**($g.L^{-1}.d^{-1}$)**	**($mN.m^{-1}$)**
*Spirulina platensis*Paracas	1	0 (-1)	0 (-1)	0.67	0.081	58.4
	2	114 (+1)	0 (-1)	0.95	0.191	58.0
	3	0 (-1)	412 (+1)	0.72	0.097	58.5
	4	114 (+1)	412 (+1)	0.62	0.096	56.3
	5	57 (0)	206 (0)	0.67	0.102	55.8
	6	57 (0)	206 (0)	0.64	0.100	55.5
	7	57 (0)	206 (0)	0.66	0.089	56.6
Spirulina platensis LEB 52	8	0 (-1)	0 (-1)	1.56	0.180	53.5
	9	114 (+1)	0 (-1)	1.81	0.198	51.8
	10	0 (-1)	412 (+1)	1.93	0.225	53.7
	11	114 (+1)	412 (+1)	1.61	0.213	52.6
	12	57 (0)	206 (0)	1.56	0.252	52.3
	13	57 (0)	206 (0)	1.96	0.239	52.0
	14	57 (0)	206 (0)	2.05	0.265	51.8
Spirulina sp. LEB 18	15	0 (-1)	0 (-1)	1.31	0.175	53.7
	16	114 (+1)	0 (-1)	1.22	0.188	54.3
	17	0 (-1)	412 (+1)	1.41	0.259	31.2
	18	114 (+1)	412 (+1)	1.29	0.235	31.6
	19	57 (0)	206 (0)	1.24	0.137	54.0
	20	57 (0)	206 (0)	1.34	0.189	55.1
	21	57 (0)	206 (0)	1.37	0.172	54.3

On the responses of surface tension, the extracts derived from *Spirulina* sp. LEB 18 presented the lowest values, obtaining an extract with surface tension values of until to 31.2 $mN.m^{-1}$ in culturing performed without addition of phosphorus and with 412$mg.L^{-1}$of nitrogen (Table 2). According to this result,the potential of this microalga for productionbiosurfactants can be verified, because according to Batista et al.[38], the criterion used to select biosurfactantproducer microorganisms is the ability to reduce surface tension of water below 40 $mN.m^{-1}$.

For the surface tension analysis of the extracts obtained from the microalgae *Spirulina platensis*Paracasand *Spirulina platensis* LEB 52, the phosphorus and nitrogen concentration variables had no significant influence ($p>0.10$) on this response, even when the interaction between the two variables was studied.

From the results obtained through the Full Factorial Design 2^2 with triplicate at the central point for *Spirulinaplatensis*Paracaswas performed analysis of variance(ANOVA) for the responsesmaximum

biomass concentration and maximum productivity (Table 3).

Table 3.Analysis of variance (ANOVA) for maximum biomass concentration (X_{max}) and maximum productivity (P_{max}) of the cultures of *Spirulina platensis*Paracas.

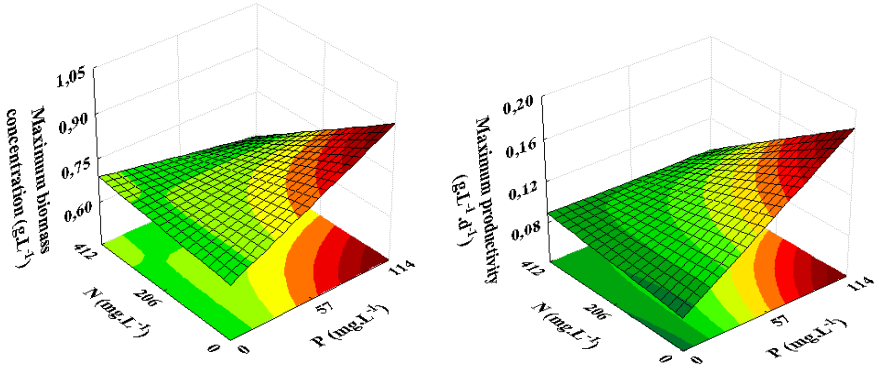
Responses	SourceofVariation	SumofSquares	DegreesofFreedom	Mean Square	$F_{calculated}$	F_{table}
X_{max}	Regression	0.0355	1	0.0355	4.55	4.06
	Residues	0.0392	5	0.0078		
	Total	0.0747	6			
P_{max}	Regression	0.0076	3	0.0025	12.50	5.39
	Residues	0.0007	3	0.0002		
	Total	0.0083	6			

According to the analysis of variance it was observed that the experimental data fitted to the models represented by variables which showed a significant effect ($p<0.10$) (Table 4), since the $F_{calculated}$was higher than F_{table}(Table 3). According to Bruns,Neto and Scarminio[39]when the $F_{calculated}$ obtained isgreater than or equal to the $F_{table}$the model is statistically valid.

Table 4. Equations and coefficients of determination (R^2) obtained from models for maximum biomass concentration (X_{max}) and maximum productivity (P_{max}) responses.

Response	Equation	R^2
X_{max}	0.70 – 0.09P*N	0.8376
P_{max}	0.11 + 0.03P – 0.02N – 0.03P*N	0.9000

Considering that the models were valid for the maximum biomass concentration and maximum productivity variable responses, the same were used to generate the response surfaces to establish the best concentration conditions of phosphorus and nitrogen(Fig. 2a and 2b).



(a) (b)

Figure 2. Response surfaces for maximum biomass concentration (a) and maximum productivity (b)of the microalga*Spirulina platensis*Paracas.

Through Fig. 2 we can observe that when the microalga*Spirulina platensis*Paracas is cultured with the highest concentration of phosphorus(114 $mg.L^{-1}$) and without addition of nitrogenit reaches higher maximum biomass concentration values (Fig. 2a) and maximum productivity (Fig. 2b).

The fact of obtaining higher biomass in the culture with 114$mg.L^{-1}$of phosphorus was also observed by other researchers [25] and [40] who reported that the production of biomass by *Spirulina platensis* was favored when this concentration of phosphorus is used.

Higher concentrations of phosphorus in the medium favors the growth of microalgae, since according to Lourenço[30] phosphorus is associated with the realization of cellular metabolic processes, forming many structural components that are necessary for the growth of microalgae.

Although nitrogenwas not added to this culture, the amount of this nutrient coming from the inoculum used must have been sufficient for the growth of microalgae, because there was a reduction of 67% of nitrogen compared to the initial concentration contained in the culture medium.

Furthermore, according to Miller et al. [41], when grown in low nitrogen concentrations the cyanobacteria can maintain their growth through the

degradation of their phycobiliproteins, obtaining nitrogen for biosynthesis.

According to the surface tension of extracts derived from *Spirulina* sp. LEB 18 obtained in the cultures, only the varying concentration of nitrogen influenced significantly ($p < 0.10$), with the increase in concentration of nitrogen causing a decrease in surface tension of 22.6 $mN.m^{-1}$(Fig. 3).

Thus, the production of biosurfactants under these conditions was not related to limited nitrogen supply, differentiating microorganisms, such as *Pseudomonas aeruginosa* and *Rhodococcus* sp. that leadtheir metabolism to the production of biosurfactants after total consumption of nitrogen [42] and[43].

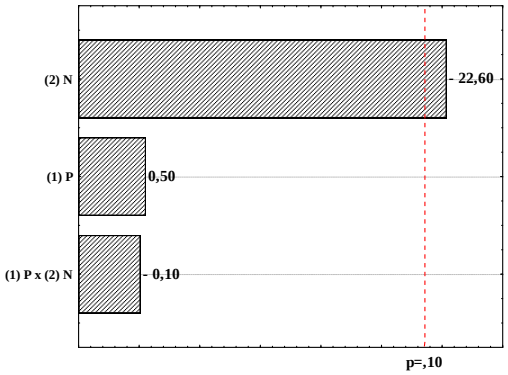


Figure 3. Pareto diagram of the variables phosphorus (P) and nitrogen (N)concentration on the surface tension of extracts obtained from *Spirulina* sp. LEB 18.

Radmann[28] in a study with *Spirulina* sp.LEB 18 verified a reduction of the surface tension in the culture medium from 70 to 43 $mN.m^{-1}$, indicating the production of biosurfactantsbythe microalga studied using glucose as a carbon source.

The biosurfactants are produced extra or intracellularly by micro-organisms [16]. In this study, such as the extraction of biosurfactants were made from biomass of *Spirulina*, one can verify intracellular production of these compounds from *Spirulina* sp. LEB 18, which present extracts with surface tension until to 31.2 $mN.m^{-1}$. This microalga was more favorable for the production of biosurfactants in relation to strains of *Spirulina platensis*, because according to Mulligan [16], a good surfactant presents ability to decrease the surface tension of water from 72.0 to 35.0 $mN.m^{-1}$.

IV. CONCLUSIONS

In this study, the variables phosphorus and nitrogen showed significant influence on the maximum biomass concentration and maximum productivity of *Spirulina platensis*Paracas, but had no significant effect of these parameters for *Spirulina platensis* LEB 52 and *Spirulina* sp. LEB 18.

The production of biosurfactant was achieved by*Spirulina* sp. LEB 18, which showed the lowest surface tension, detected in extracts obtained from the culturing performed with higher concentrations of nitrogen and without addition of phosphorus. As a result,the potential use of *Spirulina* sp. LEB 18 in the production of biosurfactantswasverified, motivating further studies in the application of extracts derived from this microalga in environment and food industry.

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