

## ARTÍCULO DE INVESTIGACIÓN

## Desarrollo y evaluación de un medio de cultivo alternativo para la multiplicación de *Azospirillum brasilense* C16 mediante diseños estadísticos secuenciados

## Development and evaluation of an alternative culture medium for mass cultivation of *Azospirillum brasilense* C16 using sequential statistical designs

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## RESUMEN

Para la producción masiva de inoculantes basados en bacterias promotoras de crecimiento vegetal (PGPR), es fundamental un medio de cultivo de alto rendimiento.

La aplicación secuenciada de diseños estadísticos fue usada para optimizar la producción de biomasa de *Azospirillum brasilense* C16, seis fuentes nutricionales (glicerol, glutamato, manitol, ácido cítrico, extracto de levadura y  $K_2HPO_4 \cdot 3H_2O$ ) y tres fuentes minerales ( $MgSO_4 \cdot 7H_2O$ ,  $FeCl_3$  y  $NaCl$ ) fueron evaluadas mediante cinco experimentos estadísticos - Placket-Burman, factorial fraccionado, diseño de paso ascendente, análisis de superficie de respuesta y screening mineral, para tal efecto. La composición optimizada del medio ( $g L^{-1}$ ) fue: 28,33 glutamato, 2,92 extracto de levadura, 1,34  $K_2HPO_4 \cdot 3H_2O$ , 0,5  $MgSO_4 \cdot 7H_2O$  y 0,02  $FeCl_3$ , la cual luego de 24 h de incubación permitió producir una cantidad de proteína (32,04  $\mu g$ ) y biomasa seca (1,51  $g L^{-1}$ ) del 1,72 y 1,68 veces más alta, respectivamente, en relación al medio de cultivo convencional.

**Palabras clave:** optimización de medio de cultivo, diseños factoriales, diseño de medio de cultivo, producción de bioinoculantes

## ABSTRACT

For mass plant growth-promoting inoculant production, a high-yield culture medium is fundamental. Sequential application of statistical designs was used to optimize *Azospirillum brasilense* C16 biomass production. Six nutritional (glycerol, glutamate, mannitol, citric acid, yeast extract and  $K_2HPO_4 \cdot 3H_2O$ ) and three mineral sources ( $MgSO_4 \cdot 7H_2O$ ,  $FeCl_3$  and  $NaCl$ ) were evaluated using five statistical experiments Placket-Burman, factorial design, steepest ascent, response surface analysis, and mineral screening. The optimum medium composition ( $g L^{-1}$ ) was as follows: 28.33 glutamate, 2.92 yeast extract, 1.34  $K_2HPO_4 \cdot 3H_2O$ , 0.5  $MgSO_4 \cdot 7H_2O$  and 0.02  $FeCl_3$ . After 24 hours of incubation, protein (32.04  $\mu g$ ) and dry biomass (1.51  $g L^{-1}$ ) were 1.72 and 1.68 times higher than in conventional growth medium.

**Key words:** culture medium optimization, factorial designs, culture medium design, bioinoculant production

## INTRODUCTION

The *Azospirillum* genus has been widely studied in recent years. This microorganism has usually been associated with the rhizosphere of both cereals and grasses, being recognized as plant growth-promoting bacteria (PGPB) (Bashan and de-Bashan, 2010; Arzanesh *et al.*, 2011; Bashan *et al.*, 2011). Bacterial synthesis of growth-promoting substances -cytokinins, gibberellins, and indole- has demonstrated the ability to improve root development and enhance water and mineral uptake (Dobbelaere and Okon, 2007; Spaepen *et al.*, 2007). These capabilities have made this PGPB suitable for application to corn, rice, and wheat crops (Díaz-Zorita and Fernández-Canigia, 2009; Hartmann and Bashan, 2009; Bashan *et al.*, 2011).

The goal of PGPB inoculant design is their application by growers (Bashan *et al.*, 2011). Inoculants are based on high numbers of viable cells in standardized formulations. For mass production of microorganisms -in flasks as well as bioreactors-, an optimized, defined, and yet cheap culture medium is necessary. Different strategies for optimization of culture media have been applied (Mendes *et al.*, 2001; Liu

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*et al.*, 2003; Wang *et al.*, 2007), but as Ren (2008) described, very few papers have applied all experimental designs well. An alternative culture medium *de novo* design and its optimization require an integrative application of experimental designs. Screening of nutritional factors, optimization of a subset of components (steepest ascent analysis), and application of a response surface methodology are steps to obtain the maximum response (Liu *et al.*, 2003; Ren *et al.*, 2008).

Although Bashan *et al.* (2011) reported two new growth media based on substitution of nutritional sources, to our knowledge, no studies have focused on the *de novo* design of a culture medium for *Azospirillum brasilense* mass production. Hence, our goal was to establish the most suitable nutritional conditions to optimize the biomass yield for inoculant production based on the use of this PGPB, using a sequential statistical approach.

## MATERIALS AND METHODS

### Bacterial strain and culture conditions

*Azospirillum brasilense* C16 was provided by the Banco de Germoplasma de Microorganismos con Potencial Biofertilizante – Corpoica, Mosquera, Colombia. Strain C16 was isolated from the grass *Panicum maximum* Jacq. in a sylvopastoral system in Cesar, Colombia, and selected based on its potential to be used as an inoculant for grasses (Cárdenas *et al.*, 2010). The strain was grown and maintained on a DYGS culture medium (composition g L<sup>-1</sup>: 2.0 glucose; 2.0 malic acid; 2.0 yeast extract; 1.5 peptone; 0.5 K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O; 0.5 MgSO<sub>4</sub> 7H<sub>2</sub>O; 1.5 L glutamic acid; pH 6.0). The cultures were incubated for 48 h at 120 rpm and 28±2°C.

### Experimental strategy

Sequential statistical designs were used to investigate the effect of nutritional sources on biomass production. First, a Plackett-Burman (P-B) design was used to identify the sources with positive effects on the response variable (Plackett and Burman, 1946). Applying a factorial design, we focused on the critical subset of positive effect sources, identifying positive interactions. Then, the optimal region was found using the steepest ascent method (Ren *et al.*, 2008). Finally, the selected sources were optimized by employing a response surface analysis "Box-Behnken design" (Box and Behnken, 1960). Additionally, mineral screening was performed to identify C16 minor growth needs.

### Experimental conditions

The experiments were carried out in 50 mL flasks at 28°C, 150 rpm, and incubation for 24 h standard conditions. The pH was not controlled during the time of the experiment;

to let the microorganism select nutritional sources with the best physicochemical effect with the statistical model. Each flask was inoculated with 200 µL of a twice-washed bacterial suspension adjusted to DO<sub>600</sub> = 0,500. The total protein content of washed cells was measured as a response variable as reported by Bradford (1976).

### Biomass production trial

A comparative trial between both the alternative and DYGS media was carried out. Both media were compared by their protein (µg) and weight biomass (dry weight in g L<sup>-1</sup>) production under the conditions described above for the statistical designs.

### Experimental design and statistical analysis

Each statistical design was tested in three replicates (blocks), where a single Erlenmeyer flask was set as the experimental unit. Statistical experimental designs used in this paper were generated and analyzed using the statistical packages: Statgraphics Centurion XV (version 15, Statgraphics, USA) and SPSS 19 (SPSS®, IBM).

## RESULTS AND DISCUSSION

### Screening stage: Plackett–Burman design

Due to the large number of media components assessed in this studywork, running experiments would cost time and money, so a P-B design was chosen to screen important sources. Six nutritional sources, including glycerol, monosodium glutamate, mannitol, citric acid, yeast extract, and K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O, were selected based on the metabolic characteristics previously described by Baldani *et al.* (2005). Two levels were established for each factor: low (-1) and high (+1) (Table 1). Levels for nutritional sources were set by adjusting:

*High levels* (+1) for C, N, and P were considered at the same concentrations as DYGS. The amount of total carbon was adjusted to 2.1 g L<sup>-1</sup> carbon. When more than one C source was present, their amounts were equally divided, resulting in the same total carbon concentration. Yeast extract and monosodium glutamate were though just as nitrogen sources, ignoring additional minor nutritional contributions.

*Low levels* (-1) for glycerol, glutamate, mannitol, and citric acid were set to 0 g L<sup>-1</sup>. For yeast extract and K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O, low levels were set at 10% of the high level.

P-B design resolution is III, this means that the main effects are not confused. However, one or more than

two-way interactions may be confounded; therefore, it must be assumed to be zero for main effects are meaningful. Results showed that media components having a positive effect were: monosodium glutamate, yeast extract, and  $K_2HPO_4 \cdot 3H_2O$  ( $P \leq 0.05$ ). Citric acid and glycerol were removed due to their negative and non-significant effects, respectively ( $P \geq 0.05$ ) (Table 2). Mannitol was kept despite its significance, in order to maintain two carbon sources mannitol and monosodium glutamate for the next design. Interestingly, we did not find a positive response when glycerol was assessed, in contrast to the results obtained by Bashan *et al.* (2010). We found that glutamate and yeast extract were notable available growth sources.

**Table 1.** Level of the variables and statistical analysis of Plackett–Burman design

Source	Low level (-1)	High level (+1)	Effect	F-ratio	P-value
	Source concentration (g L <sup>-1</sup> )				
A: Glycerol	0	5.37	0.871	3.37	0.0776
B: Monosodium glutamate	0	5.82	1.443	9.24	0.0052
C: Mannitol	0	3.86	0.937	3.98	0.0588
D: Citric acid	0	5.61	-3.856	65.94	0
E: Yeast extract	0.0025	0.025	3.283	47.82	0
F: $K_2HPO_4 \cdot 3H_2O$	0.0025	0.025	1.508	10.10	0.0037

R-sq = 83.86% R-sq (Adjusted) = 80.53%

**Table 2.** Plackett–Burman experiment and results with factor levels

Run	Coded nutritional sources <sup>a</sup>						Protein (µg)
	Glycerol	Glutamate	Mannitol	Citric acid	Yeast extract	$K_2HPO_4 \cdot 3H_2O$	
1	+1	+1	-1	+1	-1	-1	< 0.01
2	+1	-1	+1	+1	-1	+1	< 0.01
3	-1	-1	+1	+1	+1	-1	< 0.01
4	-1	+1	-1	-1	-1	+1	2.085 ± 0.23
5	-1	+1	+1	+1	-1	+1	< 0.01
6	+1	+1	-1	+1	+1	-1	< 0.01
7	-1	-1	-1	-1	-1	-1	< 0.01
8	-1	-1	-1	+1	+1	+1	< 0.01
9	+1	-1	-1	-1	+1	+1	7.405 ± 0.80
10	-1	+1	+1	-1	+1	-1	7.040 ± 0.69
11	+1	+1	+1	-1	+1	+1	7.405 ± 0.76
12	+1	-1	+1	-1	-1	-1	< 0.01

<sup>a</sup>The coded nutritional sources correspond to: for glycerol: -1 (0 g L<sup>-1</sup>), +1 (5.37 g L<sup>-1</sup>). For glutamate: -1 (0 g L<sup>-1</sup>), +1 (5.82 g L<sup>-1</sup>). For mannitol: -1 (0 g L<sup>-1</sup>), +1 (3.86 g L<sup>-1</sup>). For citric acid: -1 (0 g L<sup>-1</sup>), +1 (5.61 g L<sup>-1</sup>). For yeast extract: -1 (0.0025 g L<sup>-1</sup>), +1 (0.025 g L<sup>-1</sup>). For  $K_2HPO_4 \cdot 3H_2O$ : -1 (0.0025 g L<sup>-1</sup>), +1 (0.025 g L<sup>-1</sup>). ± indicates standard deviation. Each value is the mean of three replicates.

### Primary optimal stage: factorial design

The resolution of the factorial design used was IV; meaning that both the main and two-way effects are not confounded. Concentrations of the selected sources: monosodium glutamate, mannitol, yeast extract, and  $K_2HPO_4 \cdot 3H_2O$  were evaluated with a 2<sup>4-1</sup> factorial design -in an effort to reposition the experimental region- seeking a more optimal region. For data analysis, the mid-level (for the center points) was coded as zero. Substrate high levels were doubled from the center point concentrations, and low levels were kept as in the screening design. The model could explain 98.09% of the variation (R-sq) present in the system. Observed and predicted protein values were close, showing the model's goodness of fit and the lack-of-fit value determined that the current model adequately represents the observed data (Table 3). Mannitol had a negative effect on protein production and therefore was removed (Table 4).

**Table 3.** Factorial experiment - 2<sup>4-1</sup> - and results with factor levels

Nutritional sources (g L <sup>-1</sup> )								Protein (µg)	
Mono-sodium glutamate		Yeast extract		Mannitol		$K_2HPO_4 \cdot 3H_2O$		Observed	Predicted
C	UC	C	UC	C	UC	C	UC		
-1	0	+1	2	+1	9.88	-1	0.1	16.49±0.68	17.15
0	2.91	0	1	0	2.47	0	1	22.07±1.38	20.50
+1	11.65	+1	2	-1	0	-1	0.1	34.33±0.37	34.98
+1	11.65	-1	0.1	-1	0	+1	2	19.63±0.45	20.28
-1	0	-1	0.1	-1	0	-1	0.1	3.36±0.30	4.01
0	2.91	0	1	0	2.47	0	1	24.18±0.37	20.50
+1	5.82	+1	2	+1	4.94	+1	2	36.24±1.70	36.89
+1	5.82	-1	0.1	+1	4.94	-1	0.1	12.81±0.99	13.46
-1	0	+1	2	-1	0	+1	2	33.96±2.49	34.61
-1	0	-1	0.1	+1	9.88	+1	2	1.94± 0.37	2.59

± indicates standard deviation. Each value is the mean of three replicates

C: Coded; UC: Uncoded

### Confirmation stage: steepest ascent method

A necessary condition for a unique maximum is the presence of negative quadratic effects; if the optimal region for running the process has been identified, a response surface design is appropriated. The factorial design could explain second-order interactions but not quadratic effects. Therefore, additional experiments should be performed to seek the optimal region. Directional search methods - steepest ascent and ridge analysis- use the magnitude and sign of the linear effects

**Table 4.** Level of the variables and statistical analysis of factorial 2<sup>4-1</sup> design

Source	Sum of squares	Df	Mean square	F-Ratio	P-Value	Effect
A: Monosodium glutamate	837.68	1	837.68	216.18	0	11.81
B: Yeast extract	2601.04	1	2601.04	671.25	0	20.82
C: Mannitol	212.08	1	212.08	54.73	0	-5.94
D: K <sub>2</sub> HPO <sub>4</sub> 3H <sub>2</sub> O	230.19	1	230.19	59.41	0	6.19
AB+CD	18.45	1	18.45	4.76	0.0412	-1.75
AC+BD	73.29	1	73.29	18.91	0.0003	3.49
AD+BC	20.04	1	20.04	5.17	0.0341	-1.82
blocks	7.09	2	3.54	0.91	0.4167	
Lack-of-fit	68.75	17	4.04	1.39	0.4466	
Pure error	8.74	3	2.91			
Total (corr.)	4077.39	29				

R-sq = 98,10% R-sq (Adjusted) = 97,49%

to determine the direction toward a higher response. The steepest ascent path begins at the center of the current design space - optimal levels from previous design- and stretches well outside the design space. A sequence of equally spaced locations along the path is then selected which form a set of experiments (Ren *et al.*, 2008).

Based on the optimal source concentrations determined from the factorial design, three nutritional sources -monosodium glutamate, yeast extract and K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O- were identified, selected, and applied in the steepest ascent method. A four step path was designed from the optimal nutrient levels obtained from the factorial design -named as Δ step-, and the concentrations were increased by twice, three-, and four-times, respectively. From the results, it was possible to identify that step 2Δ was the most suitable for biomass production (Table 5).

**Table 5.** Steepest ascent experiment

Step	Nutritional source (g L <sup>-1</sup> )			Protein (μg)*
	Monosodium glutamate	Yeast extract	K <sub>2</sub> HPO <sub>4</sub> 3H <sub>2</sub> O	
Δ	11.80	1.80	0.81	15.69 ± 1.00 c
2Δ	23.60	3.60	1.62	19.46 ± 0.15 a
3Δ	35.40	5.40	2.43	18.54 ± 0.15 ab
4Δ	47.20	7.20	3.24	18.62 ± 0.38 ab

\* HSD Tukey homogeneous sub-grouping with 95% of confidence of three replicates. ± indicates standard deviation. Each value is the mean of three replicates.

### Optimization stage: Box-Behnken design

On the basis of step 2Δ, a response surface analysis was executed. Concentrations of the selected sources: glutamate, yeast extract, and K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O were adjusted up (+1) and down (-1) from the center point with a 50% variation (Table 6). The multiple determination coefficient (R-sq) indicated that the model could explain 86.19% of

the variation present in the system (Table 7). A non-linear expression was deduced from the obtained results, which explained the biomass production (Equation 1).

$$Y = 36.9713 + 2.19754 \times A - 1.50746 \times B - 1.36342 \times C - 7.12367 \times A^2 - 4.43717 \times AB - 5.21258 \times AC - 4.5335 \times B^2 + 0.45825 \times BC - 5.17225 \times C^2 \quad (1)$$

Where Y is μg of protein produced in 24 h; A, B and C are the values of monosodium glutamate, yeast extract and K<sub>2</sub>HPO<sub>4</sub> 3H<sub>2</sub>O, respectively.

**Table 6.** Response surface analysis and results with factor levels

Run	Nutritional sources (g L <sup>-1</sup> )						Protein (μg)	
	Monosodium glutamate		Yeast extract		K <sub>2</sub> HPO <sub>4</sub> 3H <sub>2</sub> O		Observed	Predicted
	C	UC	C	UC	C	UC		
1	0	23.6	0	3.6	0	1.62	36.92 ± 0.02	36.97
2	-1	11.8	-1	1.8	0	1.62	15.82 ± 0.11	20.19
3	+1	35.4	0	3.6	+1	2.43	18.28 ± 0.11	20.30
4	0	23.6	+1	5.4	-1	0.81	24.29 ± 0.22	26.66
5	-1	11.8	0	3.6	-1	0.81	20.64 ± 0.76	18.63
6	-1	11.8	0	3.6	+1	2.43	28.32 ± 0.30	26.33
7	0	23.6	0	3.6	0	1.62	35.90 ± 0.19	36.97
8	0	23.6	-1	1.8	+1	2.43	29.32 ± 0.38	26.95
9	0	23.6	-1	1.8	-1	0.81	32.95 ± 0.52	30.59
10	+1	35.4	0	3.6	-1	0.81	31.46 ± 1.12	33.45
11	+1	35.4	-1	1.8	0	1.62	33.10 ± 0.07	33.46
12	+1	35.4	+1	5.4	0	1.62	25.93 ± 3.36	21.57
13	-1	11.8	+1	5.4	0	1.62	26.41 ± 2.70	26.05
14	0	23.6	0	3.6	0	1.62	38.10 ± 0.13	36.97
15	0	23.6	+1	5.4	+1	2.43	22.50 ± 0.22	24.85

± indicates standard deviation. Each value is the mean of three replicates. C: Coded; UC: Uncoded.

**Table 7.** Statistics of response surface analysis

Source	Sum of squares	Df	Mean square	F-Ratio	P-Value	Effect
A: Monosodium glutamate + block	115.90	1	115.90	13.78	0.0008	4.39
B: Yeast extract + block	54.53	1	54.53	6.48	0.0157	-3.01
C: K <sub>2</sub> HPO <sub>4</sub> 3H <sub>2</sub> O + block	44.61	1	44.61	5.30	0.0277	-2.72
A <sup>2</sup>	562.12	1	562.11	66.84	0	-14.24
AB	236.26	1	236.26	28.09	0	-8.87
AC	326.05	1	326.05	38.77	0	-10.42
B <sup>2</sup>	227.66	1	227.66	27.07	0	-9.06
BC	2.52	1	2.52	0.30	0.5878	0.91
C <sup>2</sup>	296.33	1	296.33	35.24	0	-10.34
blocks	2.24	2	1.12	0.13	0.8753	
Total error	277.52	33	8.40			
Total (corr.)	2009.70	44				

R-sq = 86,19% R-sq (adjusted) = 81,58%

The predicted protein values were very close to the experimentally obtained ones, indicating the model's goodness of fit. The response surfaces and their contours determined each optimum variable level for maximum productivity (Figure 1A, B and C). The model predicted a maximum of 37.94  $\mu\text{g}$  protein in 24 h, with the optimum concentrations of 28.33  $\text{g L}^{-1}$  of monosodium glutamate; 2.92  $\text{g L}^{-1}$  yeast extract, and 1.34  $\text{g L}^{-1}$   $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ . Three second-order polynomial equations were found to explain protein production (Equations 2, 3, and 4).

$$Y = 34.1815 + 2.19754 \times A - 1.36342 \times C - 6.77494 \times A^2 - 5.21258 \times AC - 4.82352 \times C^2 \quad (2)$$

$$Y = 33.7884 + 2.19754 \times A - 1.50746 \times B - 6.7258 \times A^2 - 4.43717 \times AB - 4.13563 \times B^2 \quad (3)$$

$$Y = 32.5875 - 1.50746 \times B - 1.36342 \times C - 3.98553 \times B^2 + 0.45825 \times BC - 4.62428 \times C^2 \quad (4)$$

Where Y is  $\mu\text{g}$  of protein produced in 24 h; A, B and C are the values of monosodium glutamate, yeast extract and  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , respectively.

Selected sources for growth of *A. brasilense* C16 were glutamate, yeast extract, and dibasic potassium phosphate. Under aerobic conditions, glutamate is reported to allow both growth and nitrogen fixation in *A. brasilense* and *A. halopraeferens* (Baldani *et al.*, 2005). Glutamate and yeast extract interaction may be based on: 1) yeast extract effect has been reported to decrease lag phase and stimulate vigorous growth (Peña *et al.*, 1997; Bashan *et al.*, 2011), and 2) glutamate is usually assimilated at a low rate (Baldani *et al.*, 2005). Hence, yeast extract may be used as a main nutrient source allowing later expression of the glutamate metabolizing enzymes: glutamate dehydrogenase (GDH), glutamate-oxaloacetate-aminotransferase (GOT), and glutamate-pyruvate-aminotransferase (GTP). Interestingly,

glutamate exhibited the most significant effect in all the statistical designs, being used as the main growth source by *A. brasilense* C16.

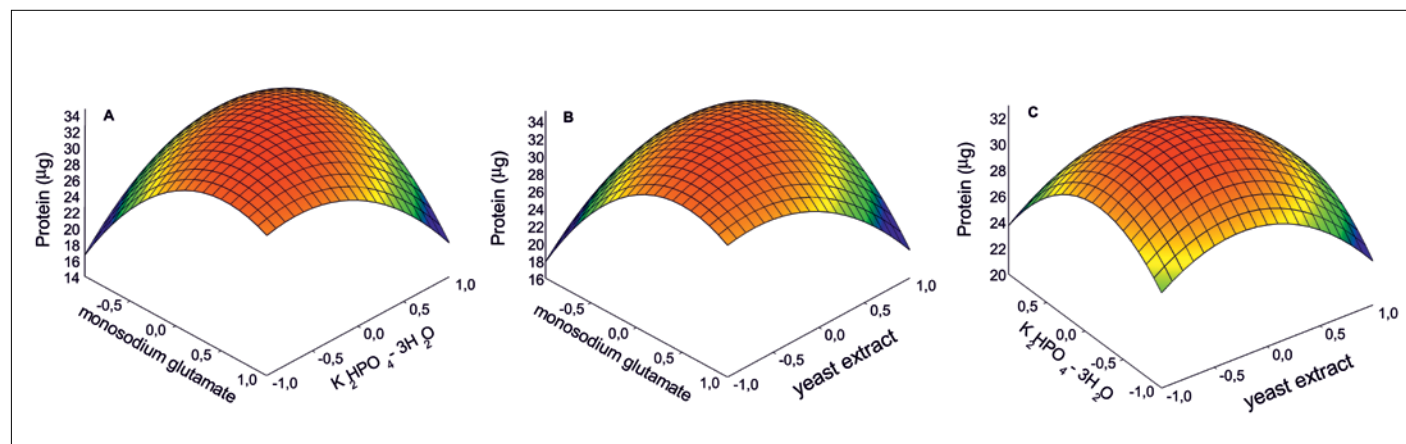
### Mineral screening design

Based on optimal source concentrations for *A. brasilense* C16 -from response surface analysis-, a quick screening design for minerals was applied. Minor nutritional needs from  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , NaCl and  $\text{FeCl}_3$  salts were identified (Table 8). The Mg and Fe salts used exhibited a positive effect on bacterial growth, while NaCl had a negative one. Interestingly, divalent metals optimized biomass production, even taking into account that yeast extract might cover minor nutritional requirements (Table 9). Mg and Fe have been described as playing a role as protein cofactors and being associated with important metabolic responses, such as nitrogen fixation. So, the optimal source concentrations that maximized biomass production were: 28.33  $\text{g L}^{-1}$  monosodium glutamate, 2.92  $\text{g L}^{-1}$  yeast extract, 1.34  $\text{g L}^{-1}$   $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 0.5  $\text{g L}^{-1}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.02  $\text{g L}^{-1}$   $\text{FeCl}_3$ .

**Table 8.** Mineral screening design media and results with factor levels

Run	Nutritional sources ( $\text{g L}^{-1}$ )						Protein ( $\mu\text{g}$ )
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$		NaCl		$\text{FeCl}_3$		
	C	UC	C	UC	C	UC	
1	+1	0.5	+1	0.1	+1	0.02	18.88 $\pm$ 1.20
2	-1	0	-1	0	+1	0.02	27.69 $\pm$ 2.13
3	+1	0.5	-1	0	-1	0	17.99 $\pm$ 1.41
4	+1	0.5	-1	0	+1	0.02	22.08 $\pm$ 1.68
5	-1	0	+1	0.1	+1	0.02	9.51 $\pm$ 0.54
6	+1	0.5	+1	0.1	-1	0	19.70 $\pm$ 1.63
7	-1	0	-1	0	-1	0	14.89 $\pm$ 0.90
8	-1	0	+1	0.1	-1	0	18.73 $\pm$ 1.60

$\pm$  indicates standard deviation. Each value is the mean of three replicates.  
C: Coded; UC: Uncoded



**Figure 1.** Response surfaces and their contours for protein production. (a) glutamate and  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  interaction. (b) glutamate and yeast extract interaction. (c)  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  and yeast extract interaction

**Table 9.** Level of the variables and statistical analysis of the mineral screening design

Nutritional sources	Mean square	F-Ratio	P-Value	Effect
A: MgSO <sub>4</sub> 7H <sub>2</sub> O	13.89	6.23	0,026	1.52
B: NaCl	92.72	41.58	0	-3.93
C: FeCl <sub>3</sub>	19.61	8.79	0.010	1.80
AB	59.25	26.57	0	3.14
AC	0.03	0.01	0.905	-0.07
BC	261.85	117.42	0	-6.60
ABC	111.46	49.98	0	4.31

R-sq = 94.61% R-sq (Adjusted) = 92.40%

### Biomass production trial

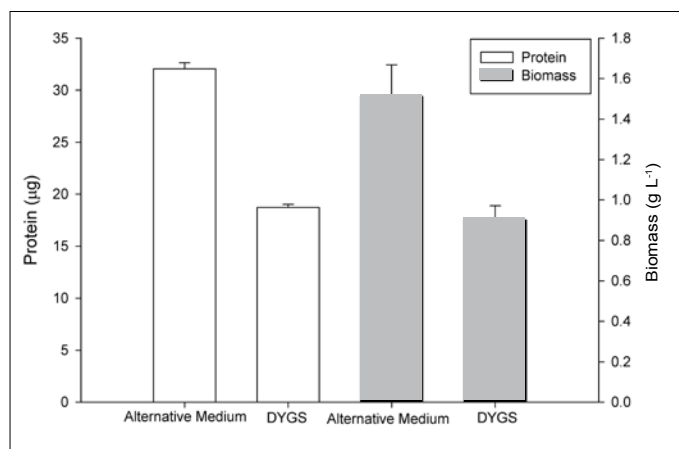
Our alternative media produced 1.71-times more protein than DYGS in 24h (Figure 2). Similarly, biomass production exhibited 1.68 times more production than DYGS (0.9 g L<sup>-1</sup>). The results evidenced that this alternative medium is suitable for producing higher amounts of *Azospirillum brasilense* C16 protein and dry biomass in 24 h, allowing for a biological inoculant design with higher cell amounts that ensure plant colonization, as Bashan *et al.* (2011) reported.

### CONCLUSION

When high yields of biomass are required, the substitution of either carbon or nitrogen sources may be not enough to optimize production. Our optimization strategy proved to be

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**Figure 2.** Protein and biomass production comparing DYGS and alternative growth medium. Error bars indicate standard deviation. Each value is the mean of three replicates

an interesting tool to set levels and combinations of sources that maximize biomass production. This alternative culture medium, designed *de novo* with a sequential statistical strategy, selected and optimized carbon, nitrogen, and minor nutrient sources, allowing the increasing of both protein and dry weight biomass amounts when compared to DYGS. Our alternative medium proved to be efficient for bioinoculant production based on *A. brasilense* C16. To our knowledge, this is the first study to report the use of sequential statistical designs to obtain a suitable culture medium for mass multiplication of this PGPB.

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