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Full Length Research Paper

Optimising carbon and nitrogen sources for *Azotobacter chroococcum* growth

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The present work deals with selecting and optimization of carbon and nitrogen sources for producing biomass from *Azotobacter chroococcum*. Four carbon sources (glucose, sucrose, manitol and sodium benzoate) and four nitrogen sources (yeast extract, meat extract, NH_4Cl and $(NH_4)_2SO_4$) were evaluated during the first stage by using a 4^2_{x3} factorial design; greater bacterial growth was obtained from sucrose and yeast extract sources (without detriment to nitrogen-fixing activity). A second factorial design (3^2_{x3}) was used for optimising the selected sources according to response surface analysis for the optimum concentrations predicted for sucrose and yeast extract (13.06 and 3.70 gL^{-1} respectively). Biomass productivity obtained (0.1117 $gL^{-1}h^{-1}$) with the optimised carbon and nitrogen sources was 1.5 times higher than that obtained with the starting culture medium.

Key words: *Azotobacter chroococcum*, medium optimization, carbon source, nitrogen source.

INTRODUCTION

Species from the *Azotobacter* genus are usually aerobic and gram-negative bacilli, being habitual inhabitants of the soil where they constitute the main percentage of free-living nitrogen-fixing microorganisms. However, some species have been found in water sediments (Revillas et al., 2000; Becking, 2006). The biological fixing of nitrogen is considered to be one of the most important microbial activities for life on earth, since it is fundamental for the homeostasis of nitrogen in the biosphere, making atmospheric nitrogen available for plants. In fact, the beneficial effect of inoculation with diazotroph micro-organisms has been widely demonstrated in many agronomically interesting cultures (Aquilanti et al., 2004; Adesemoye and Kloepper, 2009; Babaloa, 2010). *Azotobacter chroococcum* is one of the most important asymbiotic diazotroph bacteria; it is used in formulating many biofertilising products. Its agronomic impact lies in its nitrogen-fixing ability and its ability to solubilise phosphates, produce antibiotics, plant growth auxin-, gibberellin- and cytokinin-like stimulating substances and produce vitamins and aminoacids (Revillas et al., 2000; Aquilanti et al., 2004; Ahmad et al., 2005; Babaloa, 2010).

Large-scale submerged fermentation has been used for producing *A. chroococcum* biomass used in biofertilising products and the factors mostly affecting the cost of this process have been the substrates employed in the culture medium. Production medium composition is thus one of the key aspects in optimisation processes, especially for carbon and nitrogen sources since these represent about 70% of the total culture medium cost (Glazer and Nikaido, 2001). *A. chroococcum* uses a wide variety of organic acids, sugars and alcohol derivates as carbon and energy source (fructose, glucose, sucrose, manitol, ethanol, acethylmethylcarbinol, acetate, fumarate, pyruvate, oxoglutarate, gluconate, benzoate and succinate) (Tchan and New, 1984; Becking, 2006). Regarding the nitrogen source, this microorganism is able to grow in nitrogen-free mediums and use both inorganic (nitrates and ammonium) and organic sources (amino acids and peptones) (Oppenheim and Marcus, 1970; Vela and Rosenthal, 1972; Revillas et al., 2000).

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The present study was aimed at evaluating the combined effect of four carbon sources (glucose, sucrose, manitol and sodium benzoate) and four nitrogen sources (yeast extract, meat extract, NH_4Cl and $(NH_4)_2SO_4$) on *A. chroococcum* growth and *in vitro* plant growth promoter activity, expressed in nitrogen-fixing (acetylene reduction) and indole acetic acid (IAA) production. The selected carbon and nitrogen sources' concentrations were also optimised.

MATERIALS AND METHODS

Microorganism

The *A. chroococcum* isolate used in this study was supplied by the fermentations laboratory located in the Instituto de Biotecnología (IBUN), Universidad Nacional de Colombia. This was reactivated in Ashby agar (5 gL^{-1} manitol, 1 gL^{-1} KH_2PO_4, 0.005 gL^{-1} $MgSO_4.7H_20$, 0.2 gL^{-1} $FeSO_4.7H_20$, 0.2 gL^{-1} NaCl, 0.2 gL^{-1} $CaCl_2.2H_20$, 15 gL^{-1} agar, pH 7.0 ± 0.2), incubated at 30°C for 48 h.

Experimental conditions

The inoculum was prepared by sowing a microorganism cultivated in Ashby agar in liquid culture medium (10 gL^{-1} manitol, 0.2 gL^{-1} $MgSO_4$, 0.1 gL^{-1} NaCl, 0.5 gL^{-1} gL^{-1} K_2HPO_4 and 10 gL^{-1} yeast extract), incubated for 36 h at 30°C and 140 rpm. The experiments were carried out in 500 ml Erlenmeyer flask, having a 250 ml salt-based medium working volume (0.66 gL^{-1} K_2HPO_4, 0.16 gL^{-1} KH_2PO_4, 0. 2 gL^{-1} $MgSO_4.7H_2O$, 0.134 gL^{-1} $FeSO_4$, 0.2 gL^{-1} NaCl, 0.2 gL^{-1} $CaCl_2$, 0.00996 $FeSO_4$, 0.0008 $CuSO_4{\cdot}5H_2O$, 0.00024 gL^{-1} $ZnSO_4{\cdot}7H_2O$, 0.0028 gL^{-1} H_2BO_3, 0.002 $Na_2MoO_4{\cdot}2H_2O$, 0.003 gL^{-1} $MnSO_4{\cdot}H_2O$, pH 7± 0.2), supplemented with the carbon and nitrogen sources to be evaluated, according to the selected experimental design. Each Erlenmeyer flask was inoculated with 25 ml of the previously obtained inoculum, incubated at 30°C in an orbital agitator at 140 rpm for 48 h. Each experiment was kinetically followed-up every 6 h, determining biomass production, nitrogenase (EC 1.18.6.1) activity (acetylene reduction) and IAA production, according to case (see experimental design). All experiments were done in triplicate.

Experimental design and statistical analysis

A 4^2 factorial design was used to establish the combined effect of four carbon sources (glucose, sucrose, manitol and sodium benzoate) and four nitrogen sources (yeast extract, meat extract, NH_4Cl and $(NH_4)_2SO_4$) on bacterial growth, using two variables (carbon source and nitrogen source) each one having four levels, for a total of 16 experiments. Nitrogen source concentration was calculated maintaining a 8:1 (C:N) molar ratio regarding fixed concentration for carbon sources according to that reported by other authors. The resulting concentrations and combinations for the sources selected for *A. chroococcum* are shown in Table 1. Design Expert statistical software (version 7.1.6) was used for the design and analysing the results.

The four combinations selected during the previous stage were evaluated during a second phase for determining their effect on plant growth promoter activity (expressed in terms of nitrogen fixing and indole-3-acetic acid production) as well as their effect on biomass production. Variance analysis was applied on the results obtained and Tukey test was used for determining whether the levels for each factor had significant differences (SPSS 17.0 software).

Optimum concentration was then determined for the carbon and nitrogen sources selected in the previous stages. A 3^2_{x3} factorial design was used, having two variables (carbon and nitrogen sources selected from the first design). Each variable was represented by three levels {high level (+1), medium level (0) and low level (-1)} for 27 experiments (Table 3). Design Expert (7.1.6) statistical software was used for the design and analysing the results. Response surface methodology (RSM) was used for correlating the independent variables; the response variable was fitted to a second-order polynomial model whose general equation was:

$$yi = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Where, Y_i is the predicted response; p, x_i and x_j are the independent variables influencing response variable Y; β_0 is the independent coefficient; β_i is the linear coefficient; β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

Analytical determinations

Biomass concentration was expressed as total cell dry weight per unit of volume; a turbidimetric technique (600 nm Abs) and a calibration curve ($y = 1.103*x$, $R^2 = 0.98$) for comparing absorbance to dry weight were used for determining it. The cells were washed three times with saline solution (0.85% p/v) prior to measuring absorbance. The acetylene reduction technique was used for determining nitrogenase activity using gas chromatography (Dobereiner, 1997), whilst Salkowsky's technique was used for measuring IAA production (Sarwar et al., 1992; Glickmann and Dessaux, 1995).

RESULTS AND DISCUSSION

The effect of carbon and nitrogen sources on cell growth

Table 1 shows experimental results for the first factorial design and Table 2 shows the analysis of variance (ANOVA) results. It can be seen that the first-order model fit the experimental data since only 4.99% of total variation could not be explained by the model (R^2 = 0.9501) for biomass and 4.92% (R^2 = 0.9508) for productivity. Both carbon source ($p < 0.0001$) and nitrogen source ($p < 0.0001$) and their interaction ($p < 0.0001$) had a significant effect on both response variables, and carbon source having the greater influence ($F = 164.39$ for biomass and $F = 158.60$ for productivity).

Greater biomass and productivity values were presented in the experiments with sucrose or glucose as carbon source (Table 1, experiments 1, 3, 6, 9, 10, 12, 13 and 14), whilst lower values were obtained in the experiments with benzoate or manitol (Table 1, experiments 2, 4, 5, 7, 8, 11, 15 and 16). *A. chroococcum* has all the oxidative enzymes for degrading a great variety of organic carbon compounds via the tricarboxylic acid cycle, as well as its alcohol derivates such as manitol (Tchan and New, 1984; Becking, 2006). The best results were obtained when sucrose was used as carbon source.

Table 1. Average results for the first factorial design (2^4) for selecting carbon and nitrogen sources.

Run	Variables		Biomass production (gL^{-1})	Biomass productivity (g biomass $L^{-1}h^{-1}$)
	Carbon source (gL^{-1})	Nitrogen source (gL^{-1})		
1	Sucrose (10)	$(NH_4)_2SO_4$ (5.0)	1.249 ± 0.194	0.0297 ± 0.0046
2	Manitol (10)	Yeast extract (5.4)	0.668 ± 0.333	0.0191 ± 0.0095
3	Glucose (10)	$(NH_4)_2SO_4$ (4.7)	1.637 ± 0.225	0.0390 ± 0.0054
4	Sodium benzoate (5)	Yeast extract (4.0)	0.003 ± 0.002	0.0001 ± 0.0001
5	Manitol (10)	NH_4Cl (3.7)	0.054 ± 0.002	0.0013 ± 0.0000
6	Glucose (10)	Meat extract (4.7)	2.023 ± 0.214	0.0413 ± 0.0044
7	Sodium benzoate (5)	$(NH_4)_2SO_4$ (3.5)	0.100 ± 0.002	0.0044 ± 0.0001
8	Manitol (10)	Meat extract (4.6)	1.350 ± 0.053	0.0366 ± 0.0025
9	Sucrose (10)	Meat extract (4.9)	3.159 ± 0.107	0.0831 ± 0.0028
10	Glucose (10)	Yeast extract (5.4)	2.257 ± 0.350	0.0511 ± 0.0105
11	Sodium benzoate (5)	Meat extract (3.4)	0.102 ± 0.003	0.0060 ± 0.0001
12	Glucose (10)	NH_4Cl (3.7)	1.846 ± 0.396	0.0452 ± 0.0081
13	Sucrose (10)	Yeast extract (5.7)	3.374 ± 0.633	0.0888 ± 0.0166
14	Sucrose (10)	NH_4Cl (3.9)	2.930 ± 0.885	0.0698 ± 0.0211
15	Sodium benzoate (5)	NH_4Cl (2.7)	0.043 ± 0.008	0.0019 ± 0.0003
16	Manitol (10)	$(NH_4)_2SO_4$ (4.7)	0.067 ± 0.016	0.0019 ± 0.0004

Table 2. Variance analysis results from the first factorial design for selecting carbon and nitrogen source.

Effect	Sum of squares	d.f.	Mean square	F-ratio	P - value
Variable: Biomass production (gL^{-1})					
Model	65.62	15	4.37	40.62	< 0.0001*
A : Carbon source	53.12	3	17.71	164.39	< 0.0001*
B: Nitrogen source	5.99	3	2.00	18.53	< 0.0001*
AB	6.51	9	0.72	6.72	< 0.0001*
Total error	3.45	32	0.11		
Total (corr.)	69.07	47			
R^2 = 0.9501; R = 0.9267; Adequate precision = 17.787					
Variable: Biomass productivity ($gL^{-1}h^{-1}$)					
Model	0.040	15	2.667×10^{-3}	41.22	< 0.0001*
A : Carbon source	0.031	3	0.010	158.60	< 0.0001*
B: Nitrogen source	4.038×10^{-3}	3	1.346×10^{-3}	20.81	< 0.0001*
AB	5.185×10^{-3}	9	5.762×10^{-3}	8.91	< 0.0001*
Total error	2.070×10^{-3}	32	6.469×10^{-3}		
Total (corr.)	0.042	47			
R^2 = 0.9508; R = 0.9277; Adequate precision = 19.087					

*Significant at the 5% level.

It has been reported that *A. chroococcum* induces invertase enzyme (E.C. 3.2.1.26) synthesis in the presence of sucrose, this being responsible for dissociating sucrose in glucose and fructose. The synthesis of this enzyme is not inhibited by the presence of fructose in such a way that sucrose substrate degradation becomes constant (Vega et al., 1991). Probably in this case, *A. chroococcum* was thus able to metabolise both substrates thereby representing greater energetic gain than that invested in cell reproduction and maintenance and thus favouring biomass production.

On the other hand, lower biomass values were obtained with benzoate as carbon source. This source was evaluated due to the fact that there have being reports where it has been used, not just for isolating and identifying species from the Cursive genus according

Table 3. Experimental and predicted results for the second factorial design (3^2_{x3}).

Run	Sucrose (gL^{-1})	Yeast extract (gL^{-1})	Biomass productivity (g biomass $L^{-1}h^{-1}$)	
			Experimental	Predicted
1	0 (10)	-1 (0.8)	0.0879	0.090
2	-1 (5)	+1 (5.7)	0.0870	0.083
3	0 (10)	0 (2.9)	0.1076	0.110
4	-1 (5)	-1 (0.8)	0.0681	0.072
5	0 (10)	0 (2.9)	0.1099	0.110
6	-1 (5)	-1 (0.8)	0.0701	0.072
7	+1 (15)	-1 (0.8)	0.0727	0.073
8	-1 (5)	-1 (0.8)	0.0722	0.073
9	0 (10)	+1 (5.7)	0.0951	0.100
10	+1 (15)	-1 (0.8)	0.0746	0.073
11	0 (10)	-1 (0.8)	0.0953	0.090
12	0 (10)	0 (2.9)	0.1076	0.110
13	+1 (15)	+1 (5.7)	0.0873	0.084
14	-1 (5)	+1 (5.7)	0.0806	0.083
15	+1 (15)	+1 (5.7)	0.0859	0.084
16	+1 (15)	0 (2.9)	0.0824	0.089
17	+1 (15)	0 (2.9)	0.0912	0.089
18	-1 (5)	0 (2.9)	0.0821	0.088
19	-1 (5)	+1 (5.7)	0.0855	0.083
20	-1 (5)	0 (2.9)	0.0888	0.088
21	0 (10)	+1 (5.7)	0.0976	0.100
22	+1 (15)	+1 (5.7)	0.0865	0.084
23	+1 (15)	0 (2.9)	0.0847	0.089
24	0 (10)	+1 (5.7)	0.0955	0.100
25	0 (10)	-1 (0.8)	0.0927	0.090
26	-1 (5)	0 (2.9)	0.0889	0.088
27	+1 (15)	-1 (0.8)	0.0729	0.073

to the pigmentation characteristics produced by this compound's metabolism, but in evaluating its nitrogen-fixing ability using aromatic compounds as carbon and energy source where growth rates comparable with or greater than those obtained with non-aromatic substrates have been achieved (Pin et al., 1993). Other aromatic compounds such as p-hydroxybenzoic acid and protocatechuic acid have been used as carbon source for *A. chroococcum* (B-Juarez et al., 2005); however, the results obtained in the present study did not correspond to such reports, since the biomass produced was about 3 times lesser than that obtained with sucrose. Other authors have described a similar effect for *Azotobacter vinelandii* where adding sodium benzoate (1% w/v) to culture medium has resulted in partial inhibition of bacterial growth (Claus and Hempel, 1970).

Even though *A. chroococcum* is able to fix atmospheric nitrogen and thus grow in mediums lacking nitrogen, adding nitrogen from another source to culture medium has a positive influence on biomass production regarding both the quantity produced and on growth rate. Such effect has been reported for inorganic sources, such as nitrates and ammonium, and organic ones, such as amino acids and peptones (Oppenheim and Marcus, 1970; Vela and Rosenthal, 1972; Revillas et al., 2000). Two organic nitrogen sources were selected for this study (meat and yeast extract) as well as two inorganic ones (NH_4Cl and $(NH_4)_2SO_4$); it was found that greater biomass and productivity values were obtained in most cases when organic nitrogen sources were used (Table 1, experiments 3, 6, 8, 9, 10, 13 and 14). This result corresponded to what had been expected, since the advantages of using this type of complex source are well known, and in addition to providing nitrogen, they are also rich in minerals and vitamins, especially those from the B-complex, thereby representing a micronutrient and growth factor source as well as nitrogen.

The effect of carbon and nitrogen sources on vegetal growth promoting activity

Four experiments were selected from the results obtained during the first stage as they presented greater biomass

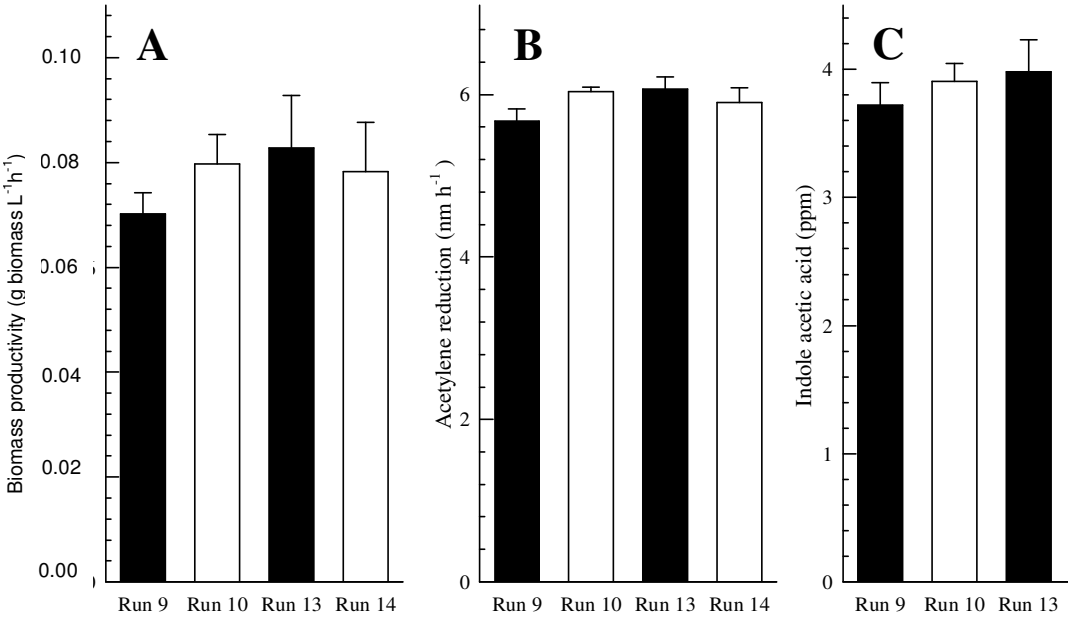

Figure 1. Growth (A), Acetylene reduction (B) and indole acetic acid production (C) from *A. chroococcum*, using different carbon and nitrogen sources. Exp 9: sucrose (10 gL^{-1}), meat extract (4.9 gL^{-1}); Exp 10: glucose (10 gL^{-1}), yeast extract (5.4 gL^{-1}); Exp 13: sucrose (10 gL^{-1}), yeast extract (5.7 gL^{-1}); Exp 14: sucrose (10 gL^{-1}), NH_4Cl_2 (3.9 gL^{-1}).

and productivity values (Table 1, experiments 9, 10, 13 and 14) for evaluating the effect of carbon and nitrogen sources on plant growth promoting activity, expressed as nitrogenase activity and IAA production (Figure 1). The productivity values achieved were very similar to those obtained during the first stage (Table 1: Exp 9: 0.0702 ± 0.0040 $gL^{-1}h^{-1}$, Exp 10: 0.0797 ± 0.0056 $gL^{-1}h^{-1}$, Exp 13: 0.0828 ± 0.0100 $gL^{-1}h^{-1}$, Exp 14: 0.0782 ± 0.0094 $gL^{-1}h^{-1}$). Even though the value obtained in experiment 13 (10 gL^{-1} sucrose and 5.7 gL^{-1} yeast extract) was slightly greater, such difference was not statistically significant ($p = 0.299$). IAA production was not seen to be affected by the carbon and/or nitrogen sources evaluated ($p = 0.617$). The values obtained (from 3.777 ± 0.081 to 3.982 ± 0.246 ppm) were within the ranges reported for this microorganism (Fiorelli et al., 1996; Torres-Rubio et al., 2000; Ahmad et al., 2005).

The effects of the presence of exogenous, organic or inorganic nitrogen on nitrogenase activity have been studied in many diazotrophs. N_2 fixing generally becomes reduced or becomes completely inhibited when alternative nitrogen sources are available; such effect could result from the inhibition of nitrogenase activity or synthesis inhibition and could be reversible or irreversible, depending on the source and concentration of nitrogen added, culture conditions and the microorganism (Helber et al., 1988; Rudnick et al., 1997; Cheng et al., 1999; Tejera et al., 2004). The acetylene reduction results obtained in the present study indicates nitrogenase activity in all the mediums evaluated (Figure 1B), without there being any significant difference for the different nitrogen sources used ($p = 0.034$), even though the values obtained (5.671 ± 0.151 to 6.071 ± 0.150 nmh^{-1}) were significantly lower than those obtained for the same strain in nitrogen-free mediums (data not shown).

Cejudo and Paneque (1986) studied the effect of adding nitrate to *A. chroococcum* nitrogen-fixing, and observed that inhibitory effect happened rapidly and ceased when the nitrogen source became exhausted in the medium. The same pattern could have occurred in these experiments since activity was determined after 42 h culture, this being the time at which maximum biomass production was obtained and the stationary stage began. Even though nitrogen concentration in the medium was not measured, it is probable that exogenous nitrogen had become exhausted in the culture in this stage and thus the inhibitory effect had ceased. Nevertheless, the results obtained indicated that the inhibition given by the nitrogen sources evaluated here was not irreversible, this being convenient for the present study's objectives.

Determining optimum carbon and nitrogen source concentration for producing biomass from *A. chroococcum*

Sucrose was selected as carbon source and yeast extract as nitrogen source according to previously described results since the greatest biomass productivity values were obtained with this combination in both stages

Table 4. Variance analysis results for the second factorial design.

Effect	Sum of squares	d.f.	Mean square	F-ratio	*P* - value
Model	2.969×10^{-3}	5	5.939×10^{-4}	45.56	< 0.0001*
A: Sucrose	3.207×10^{-6}	1	3.207×10^{-6}	0.25	0.6250
B: Yeast extract	4.965×10^{-4}	1	4.965×10^{-4}	38.09	< 0.0001*
AB	2.645×10^{-7}	1	2.645×10^{-7}	0.020	0.8881
A^2	1.780×10^{-3}	1	1.780×10^{-3}	136.58	< 0.0001*
B^2	7.835×10^{-4}	1	7.835×10^{-4}	60.11	< 0.0001*
Residual	2.737×10^{-4}	21	1.304×10^{-5}		
Total error	1.113×10^{-4}	18	6.184×10^{-6}		
Total (corr.)	3.243×10^{-3}	26			

R^2 = 0.9156; R = 0.8955; Adequate precision = 19.838. * Significant at the 5% level.

1 (Table 1) and 2 (Figure 1A). This combination also did not have any influence on plant growth promoter activity, expressed in acetylene reduction and IAA production (Figure 1B and C). A second factorial design was thus used for determining the optimum concentration for both sources. The levels evaluated and biomass productivity results are shown in Table 3 and ANOVA results in Table 4.

The experimental results were fitted to a second-order polynomial quadratic equation. A multiple correlation analysis was done and the following equation was thus obtained:

$$\text{Biomass productivity (g biomass } L^{-1}h^{-1}) = 9.2334^{-003} + 0.013904 \times A + 0.9014939 \times B - 1.20774^{-005} \times AB - 6.89034^{-004} \times A^2 - 1.95002^{-003} \times B^2 \quad (2)$$

Where, A = Sucrose concentration; B = yeast extract concentration.

The predicted productivity values shown in Table 3 were obtained when A and B values were substituted in the equation (Equation 2), such values being very close to the experimentally obtained ones, thereby indicating the model's goodness of fit. The multiple determination coefficient (R^2) was 0.9156 (Table 4), indicating that the model could explain 91.56% of variation presented in the system. On the other hand, the model's adequate precision value was 19.84 (Table 4); the adequate precision value is an index of the signal to noise ratio in a system, values greater than 4 being an essential prerequisite for considering that the model was fit to the data (Chauhan and Gupta, 2004). The significance of the model's terms evaluated by ANOVA indicated that all the model's terms, including the model itself, were significant, with the exception of A (sucrose) and AB (sucrose* yeast extract) (Table 4).

A response surface was also produced for determining each variable's optimum level for maximum productivity response (Figure 2A). The model predicted a maximum *A. chroococcum* biomass productivity value of 13.06 gL^{-1} for sucrose concentration as carbon source and 3.70 gL^{-1} for yeast extract as nitrogen source. Likewise, a response surface was produced for nitrogenase activity (acetylene reduction, Figure 2B), even though the quadratic model's fit was not adequate (R^2 = 0.5835), a similar tendency was found with that obtained for productivity, indicating that nitrogenase activity was being affected by the amount of biomass produced. It should be stressed that in no case was nitrogenase activity irreversibly inhibited, an identical pattern being observed for IAA production (data not shown). A validation experiment was carried out using the model's predicted values, a 0.1117 g biomass $L^{-1}h^{-1}$ productivity value being obtained. This value was much higher than any of the others obtained in the 27 initial experiments and 150% higher than that obtained in the growth medium usually used in the laboratory (data not shown), indicating that optimization had been satisfactory.

Conclusion

A 150% increase in *A. chroococcum* biomass productivity was achieved using a salt-based medium supplemented with 13,06 gL^{-1} of sucrose as carbon source and 3.70 gL^{-1} of yeast extract as nitrogen source, when compared to the medium usually used in production, without affecting *in vitro* plant growth promoter activity, expressed in terms of nitrogen fixing (acetylene reduction) and IAA production.

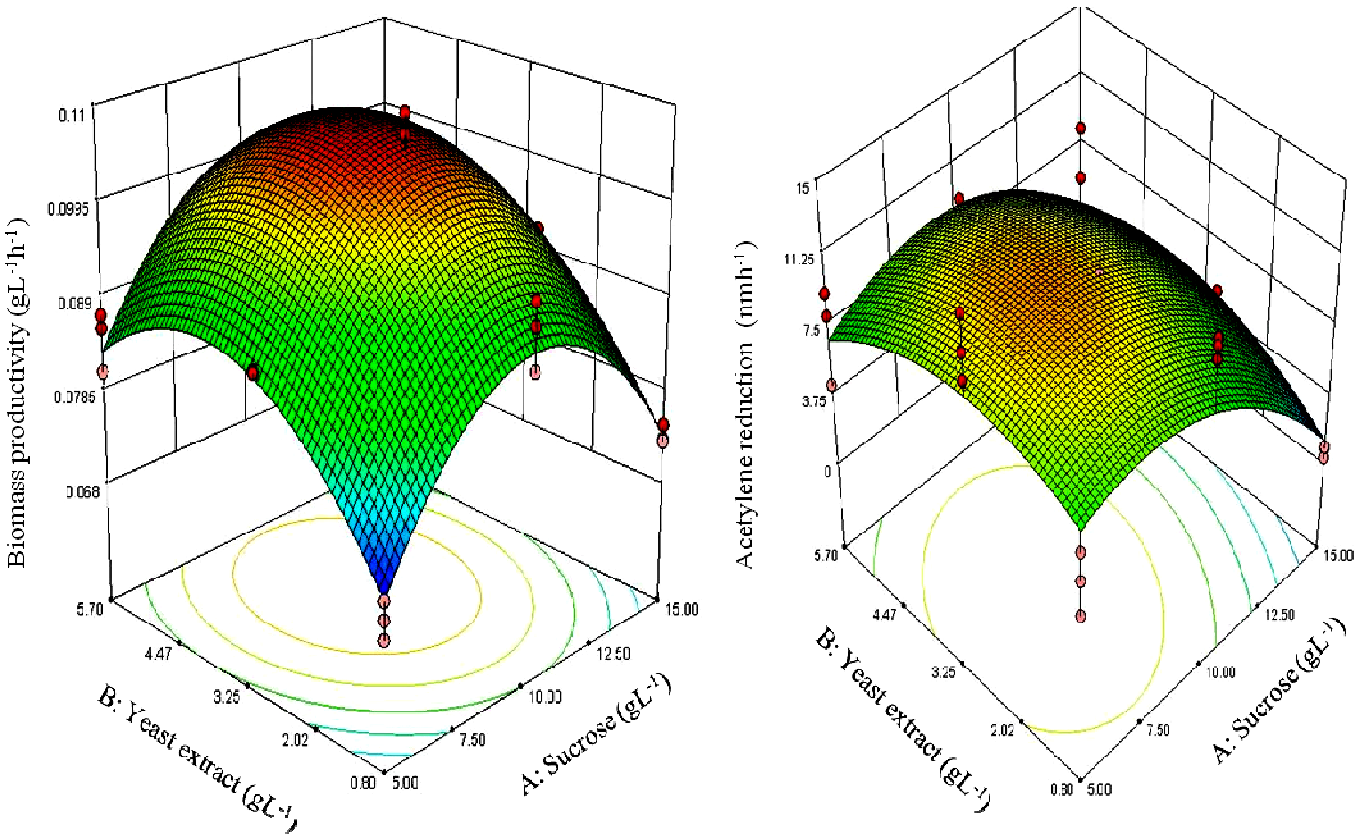

Figure 2. Response surface for biomass productivity (A) and nitrogenase activity (acetylene reduction) (B) of *A. chroococcum*, as a function of sucrose concentration as the carbon source and yeast extract as nitrogen source.

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