SCIENTIFIC PAPER

Effect of temperature and pH on the biomass production of Azospirillum brasilense *C16 isolated from Guinea grass*

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ABSTRACT: The objective of the study was to evaluate the effect of temperature and pH on the cell viability of the strain *Azospirillum brasilense* C16. Five temperatures (between 28 and 36 ºC) and five pH values (from 4,5 to 8,5) were studied. The effect of temperature was evaluated by measuring the radial growth (mm) of the strain, on a filter paper in sweet potato agar. In the case of pH to quantify the growth rate, biomass production and time of duplication of the strain a Rushton-type turbine-agitated fermenter was used; the fermentation conditions were: 400 rpm, 1 Lpm and 30 ºC, during 24 hours. The pH and temperature significantly influenced ($p< 0,05$) the production of *A. brasilense* C16. The temperature of 30 ºC was the most favorable for the strain to be multiplied, with 23,21 mm of radial growth; while the temperature higher than or equal to 34 ºC inhibited its growth. The best results were obtained with a pH value of 6,8, with significant differences ($p< 0,05$) compared with the others. Under this condition the highest growth rate values (1,79 h^{-1}) and biomass production (8,65 log_{10} CFU mL^{-1}), and the lowest value of the duplication time (1,09 h^{-1}), were obtained. These results have biotechnological applicability, and are very important at the moment of defining and controlling the conditions of massive biomass production of *A. brasilense* C16 for future formulations in diverse crops of interest in Colombia.

Keywords: bacterium, grass, inoculation

INTRODUCTION

The use of plant growth promoting bacteria (PGPB) for the formulation of biofertilizers has become one of the most promising clean technologies for the development of sustainable agriculture (Bashan *et al.*, 2013). Among the most outstanding PGPB are those of the genus *Azospirillum*, which has the capacity of fixing nitrogen; solubilizing phosphorus; producing cytokinins, gibberellins and indoles; reducing nitrates; as well as for forming different classes of associations with plants to improve root growth and increase the water and mineral absorption rate (Fibach-Paldi *et al.*, 2012). Because of these capacities *Azospirillum* sp. has been applied worldwide in corn, rice and wheat crops, (Díaz-Zorita and Fernández-Canigia, 2009; Bashan and De Bashan, 2010). Cárdenas *et al.* (2014) proved the efficiency of the strain *Azospirillum brasilense* C16 to promote the growth of Guinea grass (*Megathyrsus maximus* (Jacq.) B. K. Simon & S. W. L. Jacobs var. Tanzania), in the Agustín Codazzi municipality (Cesar, Colombia).

In order to enhance the microbial activity of biofertilizers it is necessary to define the cell growth parameters of the strains that form them, especially for a specific purpose such as the production of enzymes or biomass (Bhattacharyya and Jha, 2012). Although research has been conducted related to the optimization of the conditions for the synthesis of gluconic acid and polyhydroxyalkanoates by species of the genus *Azospirillum*, there are few studies about the effect of the physical and chemical conditions on its massive multiplication (Rodríguez *et al.*, 2004; Moreno-Galván *et al.*, 2012). Thus, the objective of this study was to evaluate the effect of physical and chemical factors, such as temperature and pH, on the growth of the strain *A. brasilense* C16.

MATERIALS AND METHODS

Microorganism and cultivation conditions. The strain C16, molecularly identified as *A. brasilense* (Cárdenas *et al.*, 2010), was used. It was isolated from soils cultivated with forage grasses in Codazzi (Cesar, Colombia), and selected for its potential as biofertilizer of Guinea grass (*M. maximus* var. Tanzania), according to the report by Cárdenas *et al.* (2014). *A. brasilense* C16 was reactivated in sweet potato medium (Döbereiner *et al.*, 1995), at 30 ± 2ºC, during 48 hours.

Effect of temperature. The effect of five temperatures (28, 30, 32, 34 and 36 ºC) on the growth of *A. brasilense* C16 was evaluated, with a completely randomized design in triplicate. A bacterial suspension

was prepared in a solution of NaCl at 0,85 %, from which 20 µl were taken to inoculate a filter paper disc, which was placed on the sweet potato culture medium contained in a Petri dish, to quantify the growth of the bacterium (mm) during five days.

Influence of pH. A Rushton-type turbine-agitated fermenter (MINIFORS, model INF-30174), with 3,5 L of effective workload, was used. The standardization of the strain inoculant was made in Azosp-1 medium (Rivera *et al*., 2012), using 10 % (v/v) of a bacterial suspension. Five pH values (4,5; 5,5; 6,8; 7,5 and 8,5) were evaluated and the design was completely randomized, with three repetitions. The pH values were controlled with NaOH (1N) and HCl (1N). The fermentation conditions were: 400 rpm, 1 Lpm, at 30 ºC –temperature which was the most favorable for the strain growth–, during 24 hours. The response variable was cell viability, which was expressed as $\log_{10}$ CFU mL^{-1}, in sweet potato medium, through the microdrop technique and seriated dilutions (Doyle *et al*., 2001). The viability was quantified every two hours, and the growth curve for each pH value was defined; the maximum growth rate (µx) and the duplication time of each curve were determined.

Statistical analysis. The normality and variance homogeneity of the results were determined through the Shapiro Wilk and Bartlett tests, respectively. Once these principles were demonstrated, an ANOVA and Tukey's mean comparison test were applied. The data processing was carried out with the statistical pack SPSS 17 (Analytical Software, Florida, U.S.A.), with 95 % of confidence level.

RESULTS AND DISCUSSION

The pH and temperature significantly influenced ($p< 0,05$) the production of *A. brasilense* C16. The temperature of 30 ºC was the most favorable for the growth of the strain (fig. 1), and halos of 23,21 mm were found after five days of incubation. Afterwards, smaller halos were found at 32 and 28 ºC (10 and 25 % below that value, respectively). Meanwhile, at 34 and 36 ºC the growth of C16 was totally inhibited since the 24 hours. Thus, the optimum production of C16 occurred between 30 and 32 ºC. In this sense, Kaushik *et al*. (2002) stated that suboptimum temperatures, such as 22 ºC, generated decrease in the biomass and in the production of plant growth promoting substances in *A. brasilense* CDJA and A40. Similar results were found by García *et al*. (2007) and Molina-Favero *et al*. (2008), who suggested the use of temperatures between 29 and 32 ºC for the production of biomass, auxins and nitrous oxide in bacteria belonging to the genus *Azospirillum*.

On the other hand, drastic decreases of the biomass were observed in the five evaluated pH values. The value 6,8 was the most favorable for the biomass production of the strain, and differed significantly ($p< 0,05$) from the others; with this pH value 8,65 $\log_{10}$ CFU mL^{-1} were quantified at 24 hours of growth (fig. 2).

In addition, the highest growth rate value and the lowest duplication time value were found: 1,79 h^{-1} and 1,09 h^{-1}, respectively (fig. 3). Steenhoudt and Vanderleyden (2000) stated that the ideal pH range for the production of biofertilizers based on *Azospirillum* varies between 6,5 and 7,0; which is corroborated with the result obtained in this study.

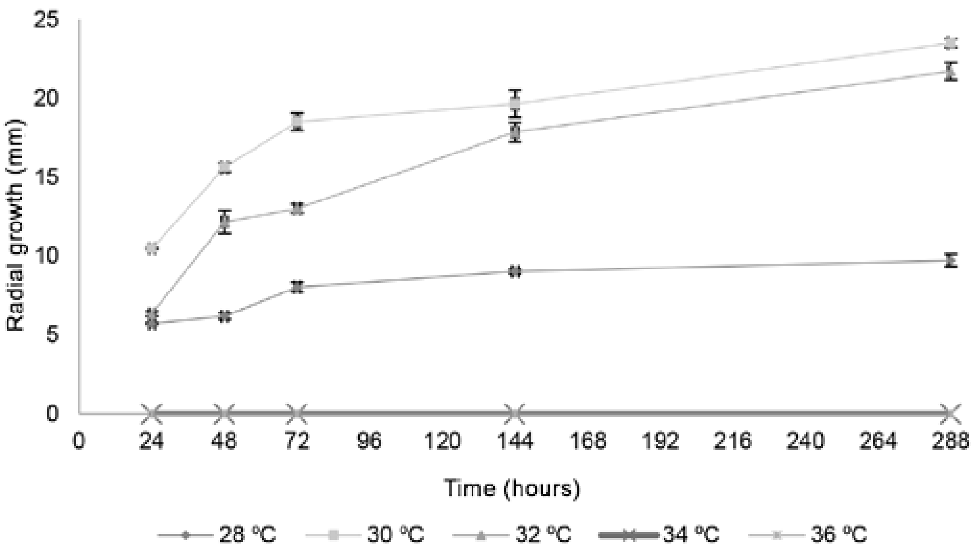


Figure 1. Radial growth of A. brasilense C16.

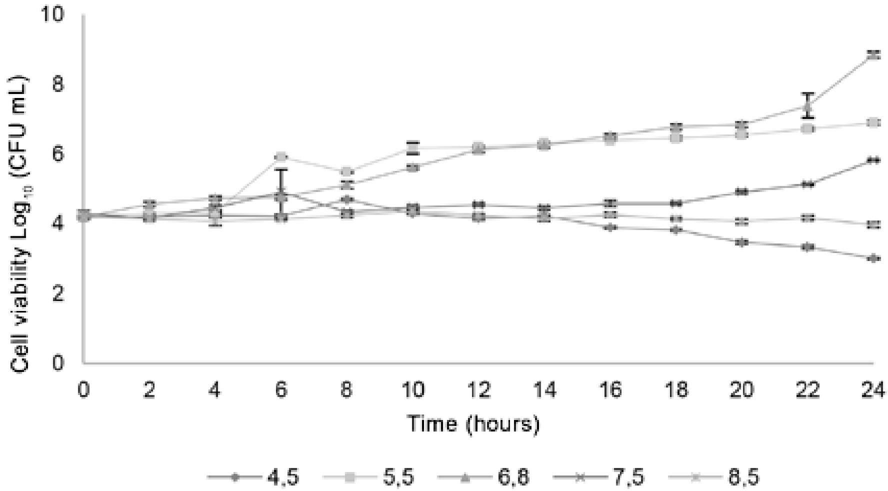
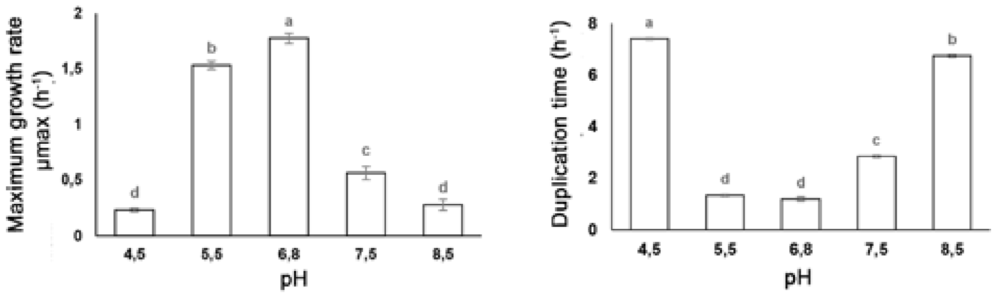


Figure 2. Growth curve of A. brasilense C16 at different pH values.



The different letters represent statistically significant differences according to Tukey's test (p < 0,05).

Figure 3. Kinetic parameters of the strain *A. brasilense* C16 under different pH conditions.

Compared with pH 6,8, with 5,5 and 7,5 biomass production values below one and three logarithmic units were quantified, at 24 hours of growth. Thus, the values of the two parameters of the growth kinetics showed statistically significant differences ($p < 0,05$). It should be stated that 5,5 favored the production more than 7,5. These results indicate that slightly acid pH values, close to neutrality, are more favorable for the biomass production of *A. brasilense* C16 than the alkaline ones.

Sivasakthivelan and Saranraj (2013) reported that in the genus *Azospirillum* the pH values higher than neutrality have more applicability for the production of compounds and metabolites of industrial interest than for the production of cell biomass. In this sense, Dahm *et al*. (1993) suggested the use of pH values higher than 7,5 to enhance the synthesis of B-group vitamins such as riboflavin.

With regards to the two most extreme pH values to neutrality (4,5 and 8,5), it was inferred that they affect the metabolism of *A. brasilense* C16. Figure 2 shows that since hour 6 there was a progressive decrease of viability through time, to the point that there is no start of exponential phase. Only a small adaptation phase, with an increase from hour 4 to hour 6, was observed in pH 4,5. Thus, the growth rate in these two pH values was lower, and the duplication time was two times higher with regards to the other pH values (fig. 3).

CONCLUSIONS

Temperature and pH significantly influenced ($p < 0,05$) the viability of *A. brasilense* C16. The maximum production was obtained at 30 ºC and a pH of 6,8. The results of this study have biotechnological applicability, and are very important at the moment of defining and controlling the conditions of massive biomass production of *A. brasilense* C16 for future formulations as biofertilizer in diverse crops of interest in Colombia.

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