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Scale-up from shake flasks to pilot-scale production of the plant growth-promoting bacterium *Azospirillum brasilense* for preparing a liquid inoculant formulation

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Abstract *Azospirillum brasilense* has industrial significance as a growth promoter in plants of commercial interest. However, there is no report in the literature disclosing a liquid product produced in pilot-scale bioreactors and is able to be stored at room temperature for more than 2 years. The aim of this work was to scale up a process from a shake flask to a 10-L lab-scale and 1,000-L pilot-scale bioreactor for the production of plant growth-promoting bacterium *A. brasilense* for a liquid inoculant formulation. Furthermore, this work aimed to determine the shelf life of the liquid formulation stored at room temperature and to increase maize crops yield in greenhouses. Under a constant oxygen mass transfer coefficient ($K_L a$), a fermentation process was successfully scaled up from shake flasks to 10- and 1,000-L bioreactors. A concentration ranging from 3.5 to 7.5×10^8 CFU/mL was obtained in shake flasks and bioreactors, and after 2 years stored at room temperature, the liquid formulation showed one order of magnitude decrease. Applications of the cultured bacteria in maize yields resulted in increases of up to 95 % in corncobs and 70 % in aboveground biomass.

Keywords Scale-up · Oxygen mass transfer · *Azospirillum brasilense* · Rhizosphere · Corn

This study is dedicated to the memory of Professor Jesus Caballero-Mellado, Ph.D. (1953–2010) of the Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, the pioneer of *Azospirillum* research in Mexico.

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Introduction

Azospirillum brasilense is the best well-known plant growth-promoting rhizobacteria (PGPR) for its capacity to fix nitrogen and also for the ability to produce key signals and components of plant growth promotion (as auxins, cytokinins, and gibberlellins, as well as nitric oxide) (Bashan and de-Bashan 2010; Herschkovitz et al. 2005; Nezarat and Gholami 2009; Tapia-Hernandez et al. 1990). *Azospirillum* spp. are diazotrophs associated with several plants, mainly including wheat, sorghum, barley, rice, and maize (Fibach-Paldi et al. 2012; Paredes-Cardona et al. 1988; Rodriguez-Salazar et al. 2009; Venieraki et al. 2011). The *Azospirillum* genus belongs to the Alphaproteobacteria class and comprises free-living, nitrogen-fixing bacteria, which produce polar and peritrichous

flagella (Lerner et al. 2009). Data about the large-scale production of *A. brasilense* are scare, and few studies reported the production of this as a biofertilizer in bioreactors (Cappuyns et al. 2007; Didonet and Magalhaes 1997; Fallik and Okon 1996; Okon and Itzigsohn 1995; Ona et al. 2005).

Different reports regarding factors affecting growth and production of key auxins in *A. brasilense* have been reported (Bashan et al. 2011; Didonet and Magalhaes 1997; Fallik and Okon 1996; Ona et al. 2005; Vande et al. 1999; Vendan and Thangaraju 2007). Most published literatures are based on the effect of culture media components on growth and shelf life of inoculants based on *A. brasilense* (Bashan 1998; Bashan et al. 2011; Joe et al. 2010; Kefalogianni and Aggelis 2002). However, few studies are based on the environmental critical parameters on growth such as oxygen, pH, or temperature. In the case of dissolved oxygen tension (DOT) Didonet and Magalhaes (1997) suggest that semi-anaerobic oxygen conditions (around 50 % DOT at the start of shake flasks cultures) are a determinant factor in nitrite production and in the increment of growth rate in different *Azospirillum* spp. (Didonet and Magalhaes 1997). Furthermore, Ona et al. (2005) propose that the growth rate of *A. brasilense*, culture time, and production of auxins (mainly indole-3-acetic acid) are highly related to low oxygen concentrations in the bioreactor (near to 3 % DOT during *A. brasilense* exponential growth). However, *A. brasilense* cultures near 30 % DOT produce similar biomass concentration comparing with those cultures carried out at 3 % DOT. Moreover, the presence of high oxygen (up to 70 % DOT, associated with some carbon nitrogen sources) increases the phenomena of flocculation and floc formation by *A. brasilense* (Burdman et al. 1998; Joe et al. 2010; Sadasivan and Neyra 1985). Additionally, highly dissolved oxygen tensions (>85 % DOT) causes an initial long lag phase (12 h) and a decrease in specific growth rate ($\mu = 0.17\ h^{-1}$) in *Azospirillum lipoferum*-submerged cultures, while in cultures performed at lower DOT (near 30 %), a short lag phase (8 h) and higher specific growth rate ($\mu = 0.26\ h^{-1}$) were observed (Paul et al. 1990).

By scaling up the fermentation stage, the aim is to obtain larger cell/product quantities at a large scale (industrial or pilot plant) with at least the same "viability/quality" as obtained at a laboratory scale. However, this is not an easy task due to the changes in vessel geometries, a reduction in mixing quality, and the occurrence of nutrients/oxygen gradients inside the bioreactors (Garcia-Ochoa and Gomez 2009; Schmidt 2005). For successful scaling up, a key parameter (or a combination of some) has to be selected among those affecting heat, momentum, and mass transfer, typically agitation, aeration, mixing time, power input, tip speed, and oxygen mass transfer coefficient ($K_L a$), among others (Marques et al. 2010; Seletzky et al. 2007). Moreover, some physical parameters have to be combined in order to obtain dimensionless numbers intended to be kept constant during the scaling up (or scaling down) processes. The most commonly used are the power number and dimensionless mixing number (Schmidt 2005). The little knowledge concerning the influence of the operating conditions on mass transfer and hydromechanics on shake flasks hampered scaling up to stirred tanks. However, in recent years, the properties of shake flasks have been described with experimental and empirical models (Buchs et al. 2000a, b; Klockner and Buchs 2012; Peter et al. 2006), and some attempts have been reported to try to scale up from flasks to stirred tanks (Gamboa-Suasnavart et al. 2013; Patino-Vera et al. 2005; Reyes et al. 2003; Seletzky et al. 2007; Silberbach et al. 2003).

In aerobic cultures, the oxygen uptake rate (OUR) has to be lower than the oxygen transfer rate (OTR) in order to assure non-oxygen-limited culture conditions. The OUR depends on the specific growth rate, the biomass oxygen yield, and the biomass concentration (Seletzky et al. 2007). On the other side, OTR is the rate of oxygen transferred into the bulk liquid through the gas–liquid interphase (liquid surface in shake flasks and bubbles and liquid surface in bioreactors) and can be described as follows:

$$OTR = \frac{dC_L}{dt} = k_L a\left(C_L^* - C_L\right) \quad (1)$$

where k_L is the mass transfer coefficient, a is the volume-specific transfer area, and $C_L^* - C_L$ is the driving force causing the mass transfer; C_L^* and C_L refer to the liquid phase oxygen concentration at saturation and oxygen concentration in the liquid bulk at any time, respectively (Klockner and Buchs 2012). To evaluate if a given culture vessel would be able to supply oxygen at a non-limiting rate, it is essential to have a good estimate of the oxygen transfer capacity of the vessel (Özbek and Gayik 2001). This can be measured in terms of the oxygen mass transfer coefficient ($K_L a$). The $K_L a$ often serves to compare the efficiency of bioreactors and their mixing devices and, as previously said, is an important scaling up criterion in bioprocesses.

An optimized culture medium for the production of a liquid inoculant formulation based on the plant growth-promoting bacterium *A. brasilense* was developed in shaking flasks for a company in Mexico (Biofábrica Siglo XXI S.A. de C.V.) by our research group. Although the inoculant is already being sold in México since 2003, the new optimized culture medium (produced in higher cell concentrations and longer shelf life) was needed to be scaled up to large bioreactors. In addition, it was necessary to test the characteristics of the inoculant and growth promoter in plants, when compared to chemical fertilizers. In this work, a practical strategy of a four-order magnitude scaling up is presented, based on empirical and experimental volumetric mass transfer coefficient ($K_L a$) data. The success of the scaling-up strategy is demonstrated by the *A. brasilense* final biomass concentration, the shelf life, and the

greenhouses inoculation experiments carried out with the final liquid inoculant formulation.

Materials and methods

A. brasilense strains and culture media Two native Mexican strains of *A. brasilense* (namely, *Start* and *Calf*) were kindly provided for this study by Eng. Agustin de Leonardo (from the company Biofábrica Siglo XXI S.A. de C.V., http://www.biofabrica.com.mx/). These strains are in the company's culture collection, and an additional cell bank is in the culture collection of the Center for Genomic Sciences, UNAM. These strains were previously isolated by Professor Jesus Caballero-Mellado, Ph.D. (Centro de Ciencias Genómicas de la Universidad Nacional Autónoma de México). The only difference between both strains is the ability to adapt to different types of soils within the country. This is the reason why the final inoculant formulation is composed of a blend of these two strains.

Pre-cultures were performed in 500-mL Erlenmeyer flasks at 30 °C, 150 rpm, with 100 mL of modified NFb, which contains in grams per liter the following: 5.0 malic acid, 0.5 K_2HPO_4, 0.2 $MgSO_47H_2O$, 0.1 NaCl, 0.02 $CaCl_2$, 0.012 $FeSO_4$, 0.0015 Na_2MoO_4, 0.01 $MnSO_4$, 4.8 KOH, 0.3 NH_4Cl, 0.01 H_3BO_4, and yeast extract 0.3.

Growth was determined by optical density at 600 nm (Beckman DU730 spectrophotometer). Moreover, the colony-forming units (CFU) per milliliter were determined by 1:10 serial dilutions with NaCl 0.85 %, cultured in NFb medium with Congo red dye (2.5 mL of 1 %*w*/*v* of dye for 1,000 mL of NFb medium) used to differentiate *Azospirillum* from the other genres (Rodríguez-Cáceres 1982) at 30±2 °C for 48 h. Final CFU per milliliter were determined by multiplying the number of colonies in the Petri dish by the dilution factor.

The colony-forming unit protocol was also used for monitoring the shelf life of the liquid pre-formulated bacterial cultures at room temperature (22–26 °C). The shelf formulation is a proprietary formulation of the Biofábrica Siglo XXI S.A. de C.V. Trials were made monthly by triplicate. The pH of the pre-formulated bacterial cultures was measured in the same samples used for shelf life, with a lab pH meter (540 Pinnacle, Corning, Inc., USA).

Culture conditions, bioreactors, and control *A. brasilense Start* and *Calf* were grown in individual 500-mL Erlenmeyer shake flasks containing 100 mL of a new modified enriched non-defined culture media at 30 °C and 220 rpm (C25I, New Brunswick–Eppendorf Co., USA). This newly modified non-defined culture medium was optimized previously by a factorial design (data not shown) in order to increase the biomass growth. This optimization was based on the NFb medium, varying the carbon/nitrogen ratio.

Cultures in 10- and 1,000-L bioreactors were also grown in a newly modified enriched non-defined culture media. Submerged cultures were carried out in batch, in 14-L stirred tanks (Labroferm FS305, New Brunswick–Eppendorf Co., USA; working volume of 10 L), tank diameter of 21 cm, equipped with three Rushton impellers (ratio of impeller/tank diameter=1:3), and four baffles of 1/10 of tank diameter. The pH was controlled within 7.0±0.2 by an on-off control adding H_3PO_4 (40 %). All fermentations were conducted at 30 °C using an agitation speed of 205 rpm and a total gas flow rate of 5.0 L/min (0.5 vol per vol per min (vvm)). Dissolved oxygen tension, temperature, agitation, and pH were displayed online and stored in a hard drive for further analysis using a homemade data acquisition control system based on LabView® (Peña et al. 2000; Trujillo-Roldán et al. 2001).

Pilot-scale submerged cultures were carried out in batch, in a homemade SS316L 1,400 L stirred tank (maximum working volume of 1,000 L) of the torispherical bottom and head. The basic design is based on standard dimensions: tank diameter 0.94 m and tank height 1.88 m, equipped with three Rushton impellers of 0.31 m (impeller/tank diameter ratio=1:3). Rushton impellers are discs of a diameter of 0.19 m and blade height and width of 0.06 and 0.07 m, respectively. The tank was equipped with four baffles of 1/10 of tank diameter. All fermentations were conducted at 30 °C using an agitation speed of 52 rpm. Air was sparged on the bottom of the tank by a ring sparger at 500 L/min (0.5 vvm), and the head pressure was controlled at 0.2 kg/cm^2. The pH control at 7.0 ±0.2 was made by an on-off control, adding concentrated H_3PO_4. Filling volume, dissolved oxygen tension, temperature (culture and sterilization), agitation, and pH were displayed online and stored in a hard drive for further analysis using a homemade data acquisition control system based on LabView® (Peña et al. 2000; Trujillo-Roldán et al. 2001).

Determination of the K_La for shake flasks The empirical correlation reported by Klöckner and Büchs (2012) was used for the evaluation of K_La. This correlation was made using the chemical (sulfite) oxygen consumption method (Ruchti et al. 1985). In this correlation, the following operating parameters are considered in SI units: maximum inner shake flask diameter (d) (in meter), shaking frequency (n) (one per second), shaking diameter (d_0) (in centimeter), and filling volume (V) (in cubic meter):

$$k_La_{Sulf} = 3.212\cdot10^{-4}\cdot d^{1.92}\cdot n^{1.16}\cdot d_0^{0.38}\cdot V_L^{-0.83}. \quad (2)$$

This empirical correlation (Klockner and Buchs 2012) shows the same results as previously reported (Maier 2002;

Seletzky et al. 2007) for standard glass Erlenmeyer flasks (DIN 12380) with hydrophilic walls, shaking frequencies of 50–500 rpm, relative filling volumes of 4–20 % (defined as the filling volume divided by the flask nominal volume), shaking diameters of 1.25 to 10 cm, and flask nominal volume between 50 and 1,000 mL. In this work, regular 500-mL shake flasks were used with a maximum internal diameter of 9.8 cm, filled with 100 mL of culture medium, and agitated at 220 rpm in a 2.54-cm standard shaker under controlled temperature (C25I, New Brunswick–Eppendorf Co., USA).

Experimental determination of the K_La for 10- and 1,000-L bioreactors In 10- and 1,000-L bioreactors, the experimental chemical (sulfite) oxygen consumption method was used. This method is based on the reaction of a reducing agent (sodium sulfite) with the dissolved oxygen in water to produce sulfate, in the presence of a catalyst (usually Cu^{++} or Co^{++}) (Imai et al. 1987; Ruchti et al. 1985). K_La was determined using tap water in 10- and 1,000-L bioreactors. However, some experiments were done to determine K_La (by the same method) using the modified enriched non-defined culture media, and no significant differences were found. The culture vessels were first filled up to the working volume with water/medium. The water/medium was then oxygen purged by adding an enough amount of sodium sulfite until the medium was oxygen free. Finally, the system was sparged with air, and the resulting dissolved oxygen tension was measured online by the InPro dissolved oxygen sensor (Mettler Toledo, USA) and displayed online and stored in a hard drive for further analysis using a homemade data acquisition control system based on LabView® (Peña et al. 2000; Trujillo-Roldán et al. 2001). Mass transfer coefficient (K_La) was determined by integrating Eq. 1 (as presented in Eq. 3), resulting in the Eq. 4. The value of K_La was the resulting linear slope, plotting the logarithmic expression against time.

$$\int_{C_{L1}}^{C_{L2}} \frac{1}{C_L^* - C_L} dC_L = k_La \int_0^t dt \tag{3}$$

$$\ln\left(\frac{C_L^* - C_{L1}}{C_L^* - C_{L2}}\right) = k_La \times t \tag{4}$$

Plant growth promotion experiments Seeds of corn (*Zea mays*) were surface sterilized by soaking them for 5 min in sodium hypochlorite (5 %) followed by five washes with sterile distilled water. Four treatments were carried out at the same time, and for each one, five seeds were sown in five pots. All pots were filled with 5 kg of sterile soil and irrigated with a nutrient solution (Jensen, 50 % of the nitrogen source) once a week, alternating with tap water twice a week. Jensen medium composition is in gram per liter as follows: sucrose 20, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, NaCl 0.5, $FeSO_4$ 0.1, $Na_2MoO_4{\cdot}4H_2O$ 0.005, and $CaCO_3$ 2.0. All pots were maintained in a greenhouse at 23–28 °C and a 12–13-h light period. The first test was the control, where just chemical fertilizer (1,000 mL of Jensen solution once a week) was added. In the second test, *A. brasilense* from the 1,000-L bioreactor of 8 months of shelf life was added in a ratio of 1×10^4CFU/seed. The third experiment was similar to the second one, but 1×10^4 CFU/seed was added to another 1,000 L culture of a 2-month shelf life. Finally, the fourth experiment was similar to the second and third ones, but this treatment was considered as a positive control by adding the conventional product of Biofábrica Siglo XXI S.A. de C.V. (a shelf life of 8 months) in a ratio of 1×10^4 CFU/seed. After germination, all plants were grown up to 60 days. Harvesting was done by cutting the stem from the ground and removing the cobs to weigh them separately (the leaf surrounding the cob is weighed together with the above-ground biomass). The dry biomass was determined (dried for 48 h at 70 °C in oven Binder model FD53).

Statistical analysis All K_La measurements and the shake flasks and bioreactor-submerged cultures were made at least in triplicate. Plant growth promotion experiments were done by quintuplicate. One-way ANOVA for independent samples and pair-wise comparisons using Tukey HSD (test for post-ANOVA) was carried out to calculate the statistical differences, unless otherwise stated. The analyses were done using Excel® (2007), and the "VassarStats: website for statistical computation" is available online from the Vassar College, NY, USA (http://faculty.vassar.edu/lowry/anova1u.html).

Results

The volumetric mass transfer coefficient (K_La) was the criterion used for scaling up the culture process from shaking flasks to 10- and 1,000-L bioreactor. Figure 1 shows the estimation of the K_La occurring in a shaking flask using the equation proposed by Klöckner and Büchs (2012) and K_La occurring in 10- and 1,000-L bioreactors determined experimentally. For a K_La of 31 h^{-1}, an agitation of 220 rpm is required in conventional Erlenmeyer flasks of 500 mL (containing 100 mL of culture medium). A study of the effect of agitation rate in shake flasks was done, and the best *A. brasilense* growth was obtained at 220 rpm, evaluating 100, 150, 200, 220, and 250 rpm (data not shown).

To obtain a similar value of K_La (31 h^{-1}), the 10-L bioreactor needs to be agitated at 205 rpm (maintaining an aeration of 0.5 vvm) by using an interpolation of experimental K_La measured by chemical (sulfite) oxygen consumption (Fig. 1). Finally, in order to scale up this culture to 1,000-L bioreactor

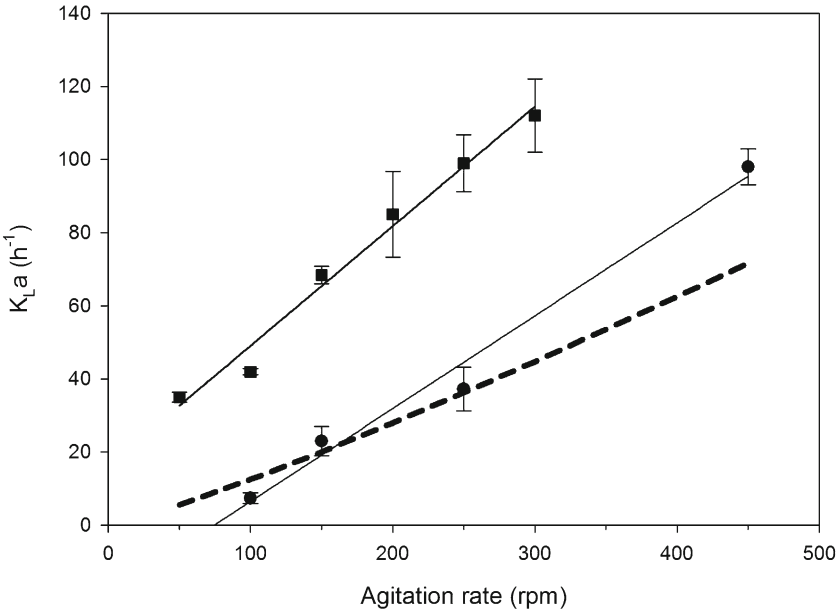

Fig. 1 Volumetric mass transfer coefficient (K_La) as a function of the shaking frequency. The *short dash line* represents data using the empirical correlation for shake flasks reported by Klöckner and Büchs (2012). *Circles and squares* represent experimental data of K_La in 10- and 1,000-L bioreactors, determined by the sulfite oxidation reaction

using the same K_La (31 h^{-1}), an agitation of 52 rpm is required, maintaining an air flow rate of 0.5 vvm (Fig. 1).

The effect of scaling up, based on K_La, over the growth of two different strains of *A. brasilense* is shown in Fig. 2. As observed, the growth was followed in all cultures by measuring the optical density in at least three independent experiments (per scale) and by measuring the colony-forming units per milliliter at the end of the cultures. With the intention to provide uniformity in comparing the growth of *A. brasilense*, it was decided to initiate all shake flasks and bioreactors at an optical density of 0.1±0.04 absorbance unit (A.U.) by using the corresponding volume of inoculum and completing the reaction

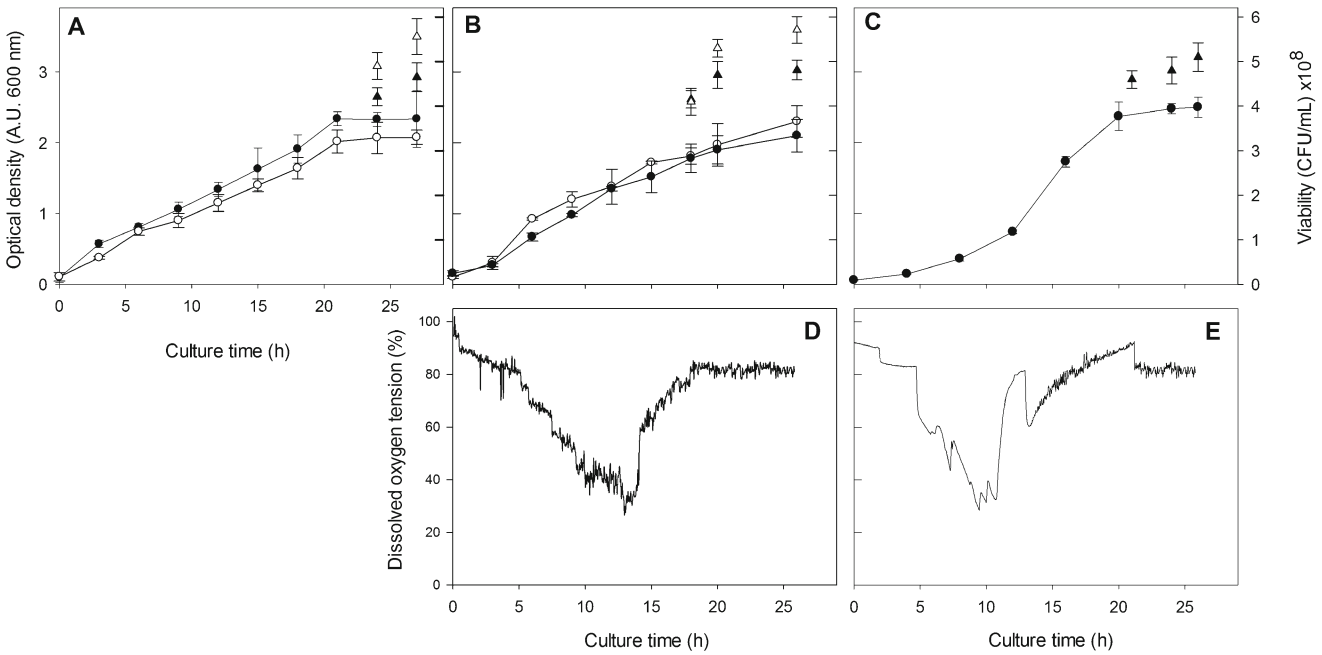

Fig. 2 Biomass growth measured as optical density (*dots*) and colony-forming units (*triangles*) for *A. brasilense Start* (*filled symbols*) and *Calf* (*open symbols*) strains cultured in shake flasks (*a*), 10-L bioreactor (*b*), and 1,000-L bioreactor (*c*), scaling up at the same volumetric mass transfer coefficient (K_La, determined by the sulfite oxidation reaction). Dissolved oxygen tension behavior during culture for 10-L bioreactor (*d*) and 1,000-L bioreactor (*e*). Operation conditions: shake flasks: 100 mL filling volume (500-mL nominal volume flasks), 30 °C, 220 rpm shaking frequency, and 2.54 cm shaking diameter; 10 L fermenter: at 30 °C, 205 rpm, and 0.5 vvm; 1,000 L fermenter: 30 °C temperature, 52 rpm agitation rate, and 0.5 vvm

volume to the size of the scale. Figure 2(a) shows the growth kinetics of the cultures in shake flasks, and no significant differences were observed between both *A. brasilense* strains (2.33±0.4 and 2.08±0.1 A.U. for *Calf* and *Start*, respectively). A similar behavior was found in cultures carried out in 10-L bioreactors (Fig. 2(b)), where 2.12±0.23 and 2.31±0.22 A.U. were found at the end of *Start* and *Calf* strain cultures, respectively, without significant differences. For 1,000-L bioreactor cultures, a decision was made to merge both strains in the same inoculum, in view of the fact that for field testing and product shelf life, both strains are used in the final formulation. Moreover, it had been shown that there was no competition in the growth of both strains, and the only difference between these strains is the ability to adapt to different types of soils in México (data not shown). Upon scaling up this culture up to 1,000 L, a maximum biomass of 2.52±0.14 A.U. was observed (Fig. 2(c)), and no significant differences were found between shake flasks and 10-L bioreactor cultures. The data reported in this work are in the same range as those reported for batch cultures by Ona et al. (2005), who reported a maximum growth of 1.596±0.06, 1.338±0.06, 0.824±0.07, and 0.682±0.06 A.U. for different carbon sources of L-malate, D-gluconate, D-fructose, and DL-lactate, respectively. Each of this carbon sources was used at a concentration of 0.02 M in a minimal medium (MMAB) (Vanstockem et al. 1987), supplemented with tetracycline (25 μg/mL). Using 5.0 g/L of L-malate, Ona et al. (2005) reported a maximum of 2.045±0.05 A.U. However, a fed-batch strategy did not increase the biomass growth (1.839±0.08 A.U.).

The successful scaling up strategy was also corroborated by measuring the colony-forming units at the end of each culture (Fig. 2). Upon comparing the final viability of *A. brasilense Calf* strain in shake flasks ($5.56\pm0.40\times10^8$ CFU/mL) and 10-L bioreactor cultures ($5.71\pm0.22\times10^8$ CFU/mL), no significant differences were obtained. The same result was obtained for *A. brasilense Start* strain with no significant difference between shake flasks ($4.65\pm0.32\times10^8$ CFU/mL) and 10-L bioreactor cultures ($4.81\pm0.22\times10^8$ CFU/mL). However, significant differences were found between strains using the same growth vessel. This is probably due to an increased capacity in the accumulation of intracellular polyhydroxyalkanoates (mainly polyhydroxybutyrate or PHB) for strain *A. brasilense Start*. In microscopic observations, *A. brasilense Start* seems to have higher PHB granules than in *A. brasilense Calf*. It would be interesting to measure the amount of PHB accumulated by the bacteria and to determine the relation between the amount of this polysaccharide and the product shelf life.

The specific growth rates of shake flask cultures were 0.25±0.02 and 0.23±0.02 h^{-1} for *Start* and *Calf* strains, respectively. No significant differences were found with cultures carried out in 10-L bioreactor (0.25±0.02 and 0.26±0.03 h^{-1} for *Start* and *Calf* strains, respectively) and 1,000-L bioreactor (0.21±0.03 h^{-1}). Comparing the specific growth rate for both *A. brasilense* strains at the three scales, they were similar to those reported by Ona et al. (2005) (0.20±0.03 h^{-1}). However, Ona et al. (2005) reported that two distinct growth patterns were observed in the growing phase: an initial fast-growing phase (μ_1) followed by a second slow-growing phase (μ_2). The transition was observed when the carbon source was exhausted. An average of 0.20±0.03 and 0.09±0.07 h^{-1} was reported for μ_1 and μ_2, respectively. In this work, no deceleration phase was observed at any scale (Fig. 2). This may be due to differences between the culture medium formulae used by Ona et al. (2005): a L-malate minimal medium and the modified enriched non-defined culture media.

By modifying the agitation to 400 rpm in a 10-L bioreactor, K_La increased in 264 % (83 h^{-1}), and the final biomass concentration (2.09±0.35 A.U.) was not affected as compared to that obtained at a K_La of 31 h^{-1}, but the growth rate was reduced by almost 35 % (0.16±0.02 h^{-1}), and a short lag phase was observed in the log-graphed growth (data not shown). In those cultures (carried out at a K_La of 83 h^{-1}), the DOT did not decrease significantly, and the values above 90 % DOT (with respect to air saturation) were always present in the culture, but in cultures carried out at a K_La of 31 h^{-1} (either in 10- or 1,000-L bioreactors), DOT decreased to values near 30 % DOT in the exponential growing phase (Fig. 2(d, e)). Those data are consistent with those previously reported by Paul et al. (1990), who reported, in cultures carried out at K_La between 48 and 65 h^{-1} (lower oxygen concentration, <30 % DOT), a short lag phase (8 h) and a faster growth rate ($\mu=0.26$ h^{-1}), while an initial long lag phase (12 h) and a decrease in specific growth rate ($\mu=0.17$ h^{-1}) were obtained at K_La between 90 and 120 h^{-1} (high oxygen concentrations during culture, >85 % DOT) (Paul et al. 1990).

It is clear that the growth promotion depends on the presence of live bacteria, and the effect on roots is directly related to the bacterial concentration of *A. brasilense* (Fallik et al. 1988; Fallik and Okon 1996). To produce a beneficial effect on maize, the optimal bacterial concentration has been reported to be 1.0×10^7 CFU/plant (Fallik et al. 1988, 1989). In Mexico, the inoculants available in the market generally have a titer between 1×10^7 and 1×10^8 CFU/mL, and 250 mL of pre-formulated product is an adequate amount for 1.0 ha (2.47 ac). Enough samples of each culture were stored at room temperature for monitoring the shelf life of the liquid pre-formulated bacterial cultures from shake flasks, 10- and 1,000-L bioreactor cultures (Fig. 3a), and at least three samples were sacrificed each time a viability reading was performed. No significant differences were obtained in shelf life during the first 8 months between cultures at each scale. Moreover, one order of magnitude was lost after a 2-year shelf life period for those cultures carried out in a 1,000-L bioreactor. These data improve the data reported by Fallik and Okon (1996), where in fed-batch cultures of *A. brasilense* $1–3\times10^{10}$ CFU/mL were

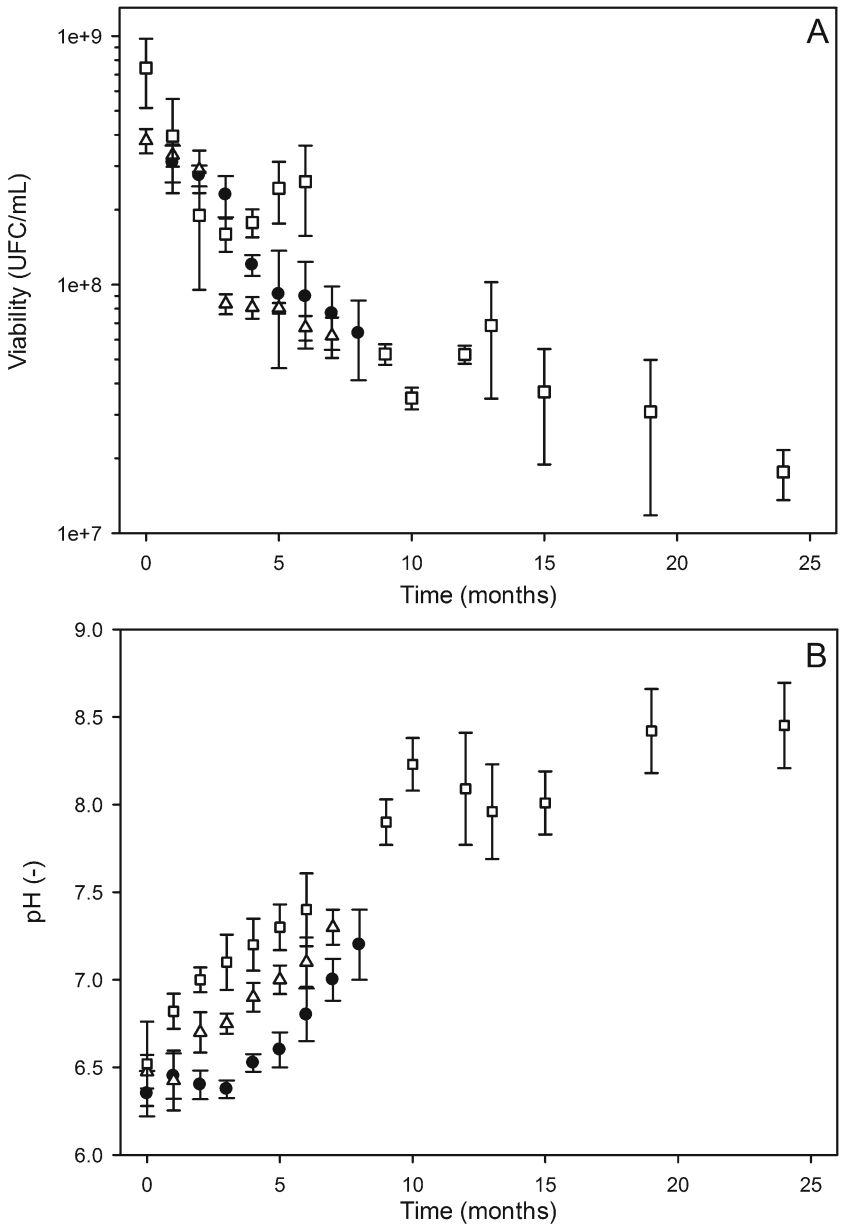

Fig. 3 **a** Viability of the liquid inoculant formulation of *A. brasilense* (mixture of *Start* and *Calf* strains) maintained at room temperature (circa 22–26 °C) and measured as colony-forming units for cultures cultured in shake flasks (*filled dots*), 10-L bioreactor (*open triangles*), and 1,000-L bioreactor (*open squares*). **b** Evolution of the pH of the liquid inoculant formulation during shelf life

obtained, and two magnitude orders of viability were lost after 6 months of storage on different solid carriers (ground or granular peat, vermiculite, talcum powder, basalt granules, and bentonite) (Fallik and Okon 1996). A formulation based on cyst *Azospirillum* cells in minimal salts medium was reported (Vendan and Thangaraju 2007) where the survival of cyst cells was observed up to 1 year and 2 months, and 1×10^8 CFU/mL was maintained. As shown in Fig. 3b, as the viability decreases over time storage, the pH is increased from values close to 6.2 to 8.5 in 2 years. Although, there are no reports (to our knowledge) of the direct relationship between pH and loss of viability in *A. brasilense*, we believe that this increase should be due to lysis allowing an intracellular material outlet.

Figure 4 shows four plant growth promotion experiments. As seen in the said figure, in the control experiment using only chemical fertilizer nutrient solution (Jensen, 50 % of the nitrogen source) once a week, alternating with tap water twice a week, an average of 21.4±3.6 g of aboveground biomass and a cob average of 16.8±7.7 g were obtained. Significant differences were found comparing the first experiment, where chemical fertilizer was used alone, with all other experiments where biofertilizer was used. However, no significant differences were obtained between the experiments using *A. brasilense* from 1,000-L culture with a shelf life of 8 or 2 months and the positive control with a shelf life of 8 months. In average, in biofertilizer trials, 36.5±7.3 and 32.7±6.5 g of aboveground biomass and cobs were obtained, respectively.

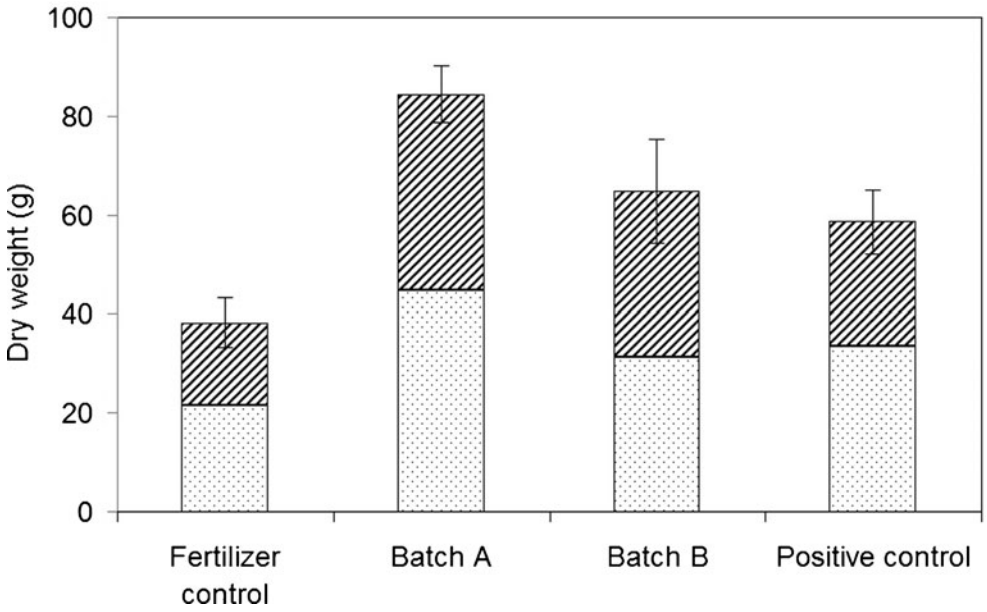

Fig. 4 *Z. mays* aboveground biomass (*dotted*) and cobs (*striped*) dry weight in plant growth promotion experiments. Each experiment was done by quintuplicate, and the standard deviations of complete biomass (aboveground) and cobs are presented. The first test was the control, where a nutrient solution (Jensen, 50 % of the nitrogen source) was added once a week, alternating with tap water twice a week. In the second test, half of the chemical fertilizer was added to each pot, and 1×10^4 CFU/seed was added (from the 1,000-L culture), with a shelf life of 8 months. The third experiment was similar to the second one, but 1×10^4 CFU/seed was added (from another 1,000-L culture), with a shelf life of 2 months. The fourth experiment was similar to the second and third ones, but 1×10^4 CFU/seed was added from peat moss solid formulation, with a shelf life of 8 months. After germination, all plants were grown up to 60 days after sowing

Discussion

In this paper, using an oxygen transfer scaling criterion, a new way to obtain large amounts of inoculants based on *A. brasilense* was presented. In addition, under this scaling up process, high cell concentrations are achieved, measured as colony-forming units. It can be assumed that the use of low $K_L a$ values to scale up this bioprocess is a parameter to be used in scaling up similar PGPR (*A. lipoferum* or *Azotobacter vinelandii*), due to its sensitivity to highly dissolved oxygen in the bioreactors (Barak et al. 1982; Ona et al. 2005; Zhulin et al. 1997). It would be interesting to evaluate lower values of $K_L a$ in shake flasks or in bioreactors. For example, in shake flasks, the oxygen is transferred from the headspace to the liquid bulk, and the $K_L a$ may be modified by just increasing the filling volume or decreasing the agitation speed (Klockner and Buchs 2012; Maier 2002).

Moreover, under a liquid formulation suitable for conservation at room temperature, a product shelf life of up to 2 years can be reached. This was obtained by the design of a suitable liquid formulation. As seen in Fig. 3, after 24 months, viability of circa 10^8 CFU/mL was found using viable cells in preformulated bacterial cultures. Storage and utilization of polybetahydroxyalkanoate polymers (mainly PHB) are important to the shelf life of the bacteria in the production of inoculants, and some reports present *A. brasilense* as an excellent producer, comprising up to 70 % of the cell dry weight (Okon and Itzigsohn 1995). Although the aim of this paper is not to determine the production of PHB, it would be interesting to evaluate the relationship between the production of this polysaccharide and the shelf life of *A. brasilense*. Also, it will be valuable to evaluate the effect of ambient culture conditions (as pH and dissolved oxygen tension) on the formation of PHB in *A. brasilense* to develop new inoculant formulations.

Even subtle differences in pH formulations were found (Fig. 3b), no effect on shelf life from the three types of bioreactors was observed. These differences in pH may be due to variations in formulation of inoculants, as also to some dissimilarity in bacterial aggregation for each culture (Burdman et al. 1998). The pH increases observed in Fig. 3b might be associated with the release of an intracellular material (involved in cell death) and/or the appearance of by-products in the consumption of PHB.

Furthermore, the final product can significantly increase the performance of aboveground biomass and corncobs in plants in greenhouse experiments. Compared to crops using just chemical fertilizer, an increase of at least 70 % of the weight of aboveground biomass and 95 % in cobs was obtained in biofertilized plants. An increase in the productivity of maize using *Azospirillum* has been reported repeatedly in both greenhouses and experimental fields (Arruda et al. 2013; Fulchieri and Frioni 1994; Shawky 1990; Stancheva and Dinev 1992; Tilak et al. 1982). In general, it has been mentioned that inoculation with *Azospirillum* sp. resulted in a significant increase of

10 to 30 % (Bashan et al. 2004). Although the experimental strategies are different, few reports can be mentioned, such as those measuring the aerial biomass; an increase of up to 70 % has been reported in Argentina in season 2007/2008 (Puente et al. 2009). Moreover, 15 days after the seeds were sown, a 22 to 60 % increase of maize aerial sprouts was observed by inoculation using *A. brasilense*, and the mature dry weight increased from 27 to 34 % (Lin et al. 1983).

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Conflict of Interest The authors declare that they have no conflict of interest.

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