Vol. 20, No. 2 (2021) 1091-1109
Revista Mexicana de Ingeniería Química

Roles of culture media and oxygen transfer in the scale-up from shake flasks to pneumatic bioreactor of the plant growth-promoting bacterium *Rhizobium phaseoli*

El papel del medio de cultivo y la transferencia de oxígeno en el escalado desde matraces agitados a biorreactores neumáticos de la bacteria promotora de crecimiento vegetal *Rhizobium phaseoli*

R.I. García-Cabrera, N.A. Valdez-Cruz, A. Daniel-Vázquez, A. Blancas-Cabrera, M. A. Trujillo-Roldán*

Programa de Investigación de Producción de Biomoléculas, Unidad de Bioprocesos, Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. Ciudad de México, México.

Received: December 13, 2020; Accepted: May 05, 2021

Abstract

The success of a *Rhizobium* sp. inoculant depends on a product with a high number of live bacteria that can nodulate a leguminous plant. With the idea of developing a functional biofertilizer with a long shelf-life culture media and oxygen transfer were studied to improve the production of *R. phaseoli* in chemically defined and undefined media for scale-up from shake flasks to a pneumatic bubble column bioreactor (BCB). The effects of carbon and phosphate on *R. phaseoli* growth were evaluated, and the specific volumetric oxygen transfer coefficient (k_La) was determined as a criterion to scale-up to BCB. Six-fold more viable biomass was obtained by increasing the phosphate concentration by 7.5-fold when using glucose. However, this increase was not observed when succinate was used. In the undefined medium, glucose induced a higher production of *R. phaseoli* in comparison with other carbon sources. The k_La of 6-11 h^{-1} produced a five-fold growth of biomass in shake-flask cultures and was used to produce a biofertilizer in BCB. The formation of nitrogen-fixing nodules was confirmed in bean plants (*Phaseolus vulgaris*). The improvement of culture media with a suitable oxygen transfer demonstrates that *R. phaseoli* can be used as an inoculant that exerts beneficial effects on the initial growth of bean plants.

Keywords: Biofertilizer; bubble column bioreactor; culture medium; k_La; *Rhizobium phaseoli*; scale-up; shelf life.

Resumen

El éxito de un inoculante basado en *Rhizobium* sp. depende de que el producto tenga un alto número de células viables. Con la idea de desarrollar un biofertilizante funcional de alta vida de anaquel se estudio el efecto del medio de cultivo y la transferencia de oxígeno para mejorar la producción de *R. phaseoli* en medios definidos y no definidos quimicamente para escalar desde matraces agitados a biorreactores neumáticos de columna de burbujeo. Se evaluó el efecto de la fuente de carbono y fosfato en el crecimiento de *R. phaseoli*, además se determinó el coeficiente volumétrico de transferencia de oxígeno (k_La) en matraces y en biorreactores. Se obtuvo seis veces más biomasa viable incrementando 7.5 veces la concentración de fostato cuando se usa glucosa como fuente de carbono. Sin embargo, cuando se usa succinato no se observan aumentos en el crecimiento. En el medio no definido, la glucosa induce la mayor producción de *R. phaseoli* en comparación con las otras fuentes de carbono. Un valor de k_La de 6 a 11 h^{-1} produce un aumento de hasta cinco veces más biomasa en matraces agitados. Este k_La fue usado para producir un biofertilizante en biorreactores neúmaticos. La formación de nodulos fijadores de nitrógeno fue confirmada en plantas de frijol (*Phaseolus vulgaris*). La mejora del medio de cultivo y una apropiada transferencia de oxígeno demuestra que *R. phaseoli* puede ser usado como inoculante con efectos beneficos en el crecimiento de plantas de frijol.

Palabras clave: Biofertilizante, columna de columna de burbujeo, medio de cultivo, k_La, *Rhizobium phaseoli*, escalamiento, vida de anaquel.

* *Corresponding author. E-mail*: maurotru@biomedicas.unam.mx; maurotru@gmail.com
Tel. 55556229192
https://doi.org/10.24275/rmiq/Bio2297
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

The quality of a *Rhizobium* sp. inoculant is determined on the basis of number of live bacteria that can nodulate a leguminous plant. The cost of raw material used to prepare the growth medium for bacterial inoculants is an important issue, especially when the process is scaled-up for industrial production (Bashan *et al*., 2011; Kaminsky *et al*., 2019; Villamarín-Gallegos *et al*. 2020). Both chemically defined and undefined (complex) culture media have their advantages and disadvantages for rhizobacterial growth. For example, a defined medium is useful for metabolic, proteomic, and physiological studies and where material balances can be made with more ease (Schwinghamer, 1960). Likewise, the study of extracellular components, such as the production of organic acids and exopolysaccharides, is facilitated when the culture medium is chemically defined (Dudman, 1964). However, the advantages in rapid growth and improved shelf life of undefined complex culture media are usually greater than those of defined media (Castellane *et al*., 2015; Hernández-Forte and Nápoles-Garcia, 2018). Similarly, undefined culture media are generally cheaper than chemically defined media for performing industrial scale bioprocesses. Accordingly, agroindustrial byproducts or wastewater sludges have been used to reduce production costs of cultures, but these sources produce different yields for each batch of waste that is used (Bissonnette *et al*., 1986; Tittabutr *et al*., 2005; Ben-Rebah *et al*., 2007; Tzintzun-Camacho *et al*., 2019; González-Leos *et al*., 2019; Valenzuela-Cobos *et al*., 2020).

In both, defined and undefined culture media, several C-sources have been used, such as glucose (Mandal and Chakrabartty, 1993; Sierra *et al*., 1999), sucrose (Dudman, 1964), glycerol (Barbour *et al*., 1991), and gluconic acid (Bosworth *et al*., 1994). Additionally, dicarboxylic acids, such as succinate, have been extensively used in culture media (Beringer, 1974; Mandal and Chakrabarty, 1993; Encarnación *et al*., 1995; Sierra *et al*., 1999; Sahonero-Canavesi *et al*., 2015) due to these are the main energy source for bacteroids in the nodule (Geddes and Oresnik, 2014; Iyer *et al*., 2016). Although the quality of *Rhizobium* inoculants and legislation (official standards) differ among countries, a product carrying a high number of bacteria with the capacity to nodulate a leguminous plant is always needed (Date, 2001; Deaker *et al*., 2016). The accepted criterion is that the cell viability of the product must be in the order of 10^7-10^9 CFU mL^{-1} to be recognized as a commercial product (Brahmaprakash and Sahu, 2012; Deaker *et al*., 2016; de Souza and Ferreira, 2017). Likewise, there are a few studies on the production of liquid rhizobacterial inoculants (Okon and Itzigsohn, 1995; Trujillo-Roldán *et al*., 2013; Buntić *et al*., 2019) and scaling-up and application on an industrial scale (Okon and Itzigsohn, 1995; Trujillo-Roldán *et al*., 2013; Jagjot *et al*., 2018; Lobo *et al*., 2019) due to the industrial secrecy of most companies. Moreover, few studies are based on the definition of the critical process parameters in the production of cell biomass of rhizobacterial inoculants (e.g., oxygen, pH, and temperature). In the case of oxygen, Didonet and Magalhaes (1997) suggested that semi-anaerobic oxygen conditions play important roles in nitrite production and in the increment of growth rate in different species of *Azospirillum*. Furthermore, Ona *et al*., (2005) proposed that low oxygen levels in bioreactor cultures increase the growth rate of *A. brasilense* and the production of auxins (mainly indole-3-acetic acid). Elevated dissolved oxygen tension (DOT) was favorable to the cell growth of *Rhizobium radiobacter*, the culture broth became highly viscous when the DOT was over 40% (percentage of air saturation), as was the maximal concentration of coenzyme Q10 obtained in these conditions (Wu *et al*., 2003). In industrial production of rhizobacterial inoculants, DOT is not normally controlled, and parameters such as the volumetric oxygen transfer coefficient (k_La) or the oxygen transfer rate (OTR) may be used to meet the culture oxygen demands (Trujillo-Roldán *et al*., 2013; Jagjot *et al*., 2018; García-Cabrera *et al*., 2020).

The objective of this work was to develop a defined and undefined culture medium to produce a *R. phaseoli* biofertilizer that can be scaled-up on a commercial scale while maintaining shelf life and its nodulation characteristics. In this sense, here we present specifically the role played by carbon, potassium sources in culture media and k_La as the criteria to scale-up from shake flask to a pneumatic bioreactor.

2 Materials and methods

2.1 Bacterial strain and culture media

The *R. phaseoli* strain (Rhizofer) was provided for this study by Biofabrica Siglo XXI S.A. de C.V. (http://www.biofabrica.com.mx/), which is used and marketed as an inoculant in Mexico. This strain was previously isolated in the Genomic Sciences Center (CCG) of the National Autonomous University of Mexico (UNAM). Cell banks of this strain are maintained in the company's culture collection, in CCG's culture collection, and in the culture collection of our laboratory. *R. phaseoli* cells are cryopreserved at -72 °C in a mix of culture broth in exponential growth in complex tryptone-yeast extract medium (TY) (Beringer, 1974) and glycerol (40%).

The composition of the chemically defined medium (Bergersen, 1961) contains (g L^{-1}): NH_4Cl (0.54), $MgSO_4$ (0.10), $CaCl_2$ (0.22), $FeCl_3$ (0.005), nalidixic acid (0.02), spectinomycin (0.05), and biotin (0.001). The concentrations of K_2HPO_4 here evaluated included the original concentration (0.2 g L^{-1}) proposed by Sherwood (1970) along with 0.5, 1.0, 1.5, and 2.0 g L^{-1}. Glucose or succinate (3.0 g L^{-1}) was used as the C-source. Stock solutions of $MgSO_4$, $CaCl_2$, $FeCl_3$, and glucose were prepared at the concentration of 200, 200, 5, and 500 g L^{-1}, respectively, and were sterilized at 121 °C for 20 min. The antibiotics and biotin were sterile filtered (Millex-GV 0.22 μm, Merck-Millipore, MA, USA).

The undefined (complex) culture medium was Yeast Extract Mannitol (YEM), a traditional medium used for rhizobia growth and inoculant production (Vincent, 1970; Stowers, 1985; Hossain *et al*., 2019), and it contained (g L^{-1}): NaCl (0.1), yeast extract (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.2), and mannitol (10), representing a ratio of 74 gC/gN. Three carbon/nitrogen ratios were evaluated in this study (37, 74, and 111 gC/gN). These gC/gN ratios were achieving by modifying mannitol (YEM, 5.0, 10.0, and 15.0 g L^{-1}), glucose (YEG, 4.9, 9.9, and 14.8 g L^{-1}), fructose (YEF, 4.9, 9.9 and 14.8 g L^{-1}), or sucrose (YES, 4.7, 9.4, and 14.1 g L^{-1}) concentration in culture media. The mass amounts of C and N of the yeast extract were taken from Zapata-Velez and Trujillo-Roldán (2010).

2.2 *Analytical determination*

R. phaseoli growth was measured by direct counting of colony forming units (CFU mL^{-1}) in TY media with spectinomycin and nalidixic acid supplementation (Vincent, 1970; Beringer, 1974) using the drop-plate method (O´Hara *et al*., 2016). The biomass was also quantified spectrophotometrically (Genesys 5, Thermo Spectronic, Rochester, USA) by measuring optical density at 600 nm (O´Hara *et al*., 2016) and the cell dry weight (CDW), also exopolysaccharide concentration was determined gravimetrically (Trujillo-Roldán *et al*., 2001). Glucose was determined using a biochemical analyzer (YSI 2900, Biochemical Analyzer, Life Sciences, USA). Succinate was evaluated using high-pressure liquid chromatography (Shimadzu Corp., Kyoto, Japan) equipped with a pump system (LC-20AT, Shimadzu, Corp., Kyoto, Japan), a UV/VIS detector (SPD-20A, Shimadzu Corp., Kyoto, Japan), and an Aminex column (HPX-87H, Bio-Rad, Hercules, CA, USA). The device operating conditions were 50 °C, flow rate of 0.6 mL min^{-1} (mobile phase 4 mM H_2SO_4), and recorded wavelength of 210 nm. An organic acid standard analysis was used for succinate quantification (Bio-Rad, Laboratories Inc.). The samples were centrifuged at 10,000 g for 10 min (Eppendorf 5804R Centrifuge, Hamburg, Germany) and the supernatant was filtered using a 13 mm GHP Acrodisc (pore size, 0.45 μm, PALL Corporation, Ann Arbor, MI, USA). The sample pH was measured using a potentiometer (Corning 430, Corning NY, USA). The viscosity of the culture broths was measured in a cone/plate viscosimeter (Wells-Brookfield LVT, series 82198; Brookfield Engineering Laboratories, Stoughton, MA) at 6 and 60 rpm and using cone CP-52 that corresponds to a shear rate of 12 and 120 s-1, respectively.

2.3 *Submerged cultures in shake flasks and pneumatic bioreactor*

For all experiments, the inoculum preparation process was the same. Inoculum was prepared by transferring 1.0 mL of the cryopreserved *R. phaseoli* to 100 mL of TY medium in 500 mL Erlenmeyer flasks and incubating at 150 rpm for 24 h at 30 °C (New Brunswick Scientific C251, Eppendorf, Inc. CT, USA). Next, the cells were centrifuged at 7,000 g for 15 min at 25 ± 2 °C (Eppendorf 5804R centrifuge, Hamburg, Germany) and resuspended in 20 mL of the modified chemically defined/undefined medium. Appropriate aliquots for the concentrated medium were added to fresh modified defined/undefined media to reach an initial optical density of approximately 0.15 absorbance units (A.U.).

Shake-flask experiments were carried out in triplicates using 100 mL of the respective culture medium in 500 mL Erlenmeyer flasks (Duran® Erlenmeyer flask, narrow neck, Borosilicate Glass, USA) and incubated at 150 rpm at 30 °C (New

Brunswick Scientific C251, Eppendorf, Inc. CT, USA). The same Erlenmeyer flasks were used to evaluate the effect of the k_La in YEG culture medium at 30 °C, evaluating two orbital agitation rates (100 and 200 rpm, New Brunswick Scientific C251 shaker, Eppendorf, Inc. CT, USA) and four filling flask volumes (80, 40, 20, and 10%). It has been reported that the oxygen transfer rate is modified by altering the orbital agitation rate and/or the filling volume of shaken flasks, especially through the k_La (Maier *et al*., 2004).

The pneumatic bioreactor was a bubble column bioreactor (BCB) designed in-house with a working volume of 5.0 L (using YEG culture medium, initial pH 6.8) and a nominal volume of 6.6 L, in Pyrex glass with stainless-steel top and bottom (Ángeles-Argáiz *et al*., 2020). In order to evaluate the effect of k_La on the scale-up process in the BCB the airflow was constantly maintained during each culture, and 0.1, 0.3, and 1.0 volume of air per volume of culture medium (vvm) (0.5, 1.5, and 5.0 standard air liters per minute, L min^{-1} [slpm]) were evaluated. At least three independent cultures were carried out for each airflow evaluated. Air was sterilized through a vent filter unit (0.2 μm, Merck-Millipore, Massachusetts, USA) and diffused in small bubbles using one nozzle of 3/64" diameter (0.11 cm). BCB cultures were controlled at 30 ± 2 °C using a proportional-integral-derivative (PID) control strategy (Trujillo-Roldán *et al*., 2001) with a heating jacket. BCB cultures were instrumented with pH, DOT, and temperature sensors (Mettler Toledo, Ohio, USA and Applisens, Applikon Biotechnology, Netherlands) and on-line data was acquired in an ADI-1030 bio-controller (Applikon Biotechnology, Netherlands), using the BioXpert® Software (Applikon Biotechnology, Netherlands).

2.4 Kinetic and stoichiometric parameters

The specific bacterial growth rate (μ) is defined by Equation 1 and was calculated as the slope from the biomass (in natural logarithm)-time graph during the exponential growth phase as follows:

$$\frac{dX}{dt} = \mu x \tag{1}$$

The stoichiometric parameters were calculated as follows:
Yield ($Y_{X/S}$):

$$Y_{X/S} = \frac{X_f - X_i}{s_i - s_f} \tag{2}$$

Volumetric productivity:

$$(Q_P): \quad Q_P = \frac{X_f}{t} \tag{3}$$

The specific substrate consumption rate (q_S):

$$q_s = \frac{\mu}{Y_{x/s}} \tag{4}$$

2.5 Measurement of k_La using the gassing-out method

The oxygen availability is dictated by the oxygen transfer rate (OTR) and oxygen uptake rate (OUR) of the biomass:

$$\frac{dC_L}{dt} = OTR - OUR \tag{5}$$

The OTR is defined as the rate of oxygen transferred through the gas-liquid interphase into the bulk liquid (Quijano *et al*., 2020):

$$OTR = k_La(C_L^* - C_L) \tag{6}$$

where $(C_L^* - C_L)$ corresponds to the oxygen concentration gradient between the interfacial saturation (C_L^*) and the liquid bulk (C_L), and the k_La is the volumetric mass transfer coefficient. In the absence of cells (in gassing-out method) OUR is equal to zero, which makes:

$$\frac{dC_L}{dt} = OTR \tag{7}$$

The determination of k_La results after the integration of Equation (7) (between two different times), as follows:

$$\ln\left(\frac{C_L^* - C_{L2}}{C_L^* - C_{L1}}\right) = -k_La(t_2 - t_1) \tag{8}$$

Experimentally, shake flasks were filled with the respective culture medium (without cells), and the oxygen was removed by adding Na_2SO_3 with $CoCl_2$ as a catalyst to a final concentration below 6×10^{-3} M and 5×10^{-7} M, respectively. There are significant differences when k_La was obtained by this method using water or culture media, this probably due to different physicochemical properties of culture (Maier *et al*., 2001). The sulfite oxidation reaction displaced the dissolved oxygen in the liquid phase, and the k_La was measured when the oxidation reaction finished. The agitation/aeration was started, and DOT was recorded online (Reynoso-Cereceda *et al*., 2016). The

DOT in shake flasks was measured using a PSt3-sensor-spot attached to the bottom inside of each Erlenmeyer flask, and the probe was placed outside the flask over a coaster (Reynoso-Cereceda *et al*., 2016); the signal was recorded online with the oxygen optical meter Fibox3 (PreSens, Regensburg, Germany). Since Equation (8) is valid for a linear response, only data measured between 10 and 60% DOT were used for k_La estimation.

The k_La in the BCB was measured by bubbling nitrogen to displace the dissolved oxygen and assure an oxygen-free culture medium (Trujillo-Roldán *et al*., 2013). The DOT was measured by a sterilizable polarographic oxygen probe (Mettler-Toledo, Columbus, OH, USA) and was recorded using ADI-1030 and ADI-1010 bio-controllers (Applisens, Applikon Biotechnology, Netherlands) and stored on-line (BioXpert® Software, Applikon Biotechnology, Netherlands). In BCB the k_La was also obtained as the resulting linear slope by plotting the logarithmic expression against time (Equation 8) with DOT between 10 and 60%.

The response time of the sensors measured as the time needed to reach 63.2% of the final reading upon a step change in DOT is ~ 3 s for PSt3-sensor-spot (Reynoso-Cereceda *et al*., 2016) and ~ 20 s for the polarographic probe (Trujillo-Roldán *et al*., 2001). Assuming a first order dynamic response of the electrode, a simple criterion for the suitable selection of the electrode usually that the response time is less than the inverse of k_La (Trujillo-Roldán *et al*., 2001). Here, the response time of the electrodes was fast enough and no considerations on these measurements were needed.

2.6 Greenhouse evaluation

Black Jamapa common bean (*Phaseolus vulgaris* L.) (Aparicio-Fernandez *et al*., 2005) seeds were inoculated with *R. phaseoli* (~ $7.0 \pm 3.0 \times 10^4$ CFU per seed) grown in shake flasks in the chemically defined medium containing glucose and 1.5 g L^{-1} K_2HPO_4 (n = 28); more seeds were inoculated with *R. phaseoli* grown in shake flasks and pneumatic bioreactors using YEG media (n = 12, each). A positive control was prepared with microorganisms cultured in TY medium (n = 9) and harvested at 20 h of culture (~$4.0 \pm 2.0 \times 10^5$ CFU per seed); the negative control was non-inoculated seeds (n = 10).

The methodology used for seed germination has been previously described (López-López *et al*., 2010). The seeds were washed twice with sterile water, submerged, and stirred first in ethyl alcohol (70%) for 5 min and then in commercial sodium hypochlorite (20%) for 20 min. Finally, they were washed 10 times with sterile water. Six seeds were placed on a sterile Petri dish with agar-water medium (0.7%) and incubated at 28 °C for three days. The germinated seeds were transferred to 250 mL Erlenmeyer flasks with 200 mL sterile solidified Fahraeus medium (Fahraeus 1957), flasks were inoculated with *R. phaseoli* and coated with aluminum foil (except for the negative control). Flasks were covered with a dark plastic; when the plant grew beyond the flask mouth, the aluminum foils were replaced with soft foam caps. Plants were grown in a greenhouse (18-32 °C, relative humidity 30-60%) for 25 days and then harvested to determinate the height (H), shoot wet weight (SWW), root wet weight (RWW), and number of nodules (NN).

2.7 Statistical analysis

All cultures were carried out at least in triplicate. Independent samples and multiple-comparison tests were used to estimate significant differences in the culture parameters (two-way analysis of variance ANOVA and Tukey's post hoc test were used). A threshold significance level of 95% ($P < 0.05$) was applied.

3 Results

3.1 Dipotassium phosphate improves R. phaseoli growth in the presence of glucose but not in the presence of succinate in a chemically defined medium

R. phaseoli growth and C-source consumption in a chemically defined medium (Bergersen, 1961) with either glucose or succinate displayed different kinetic behaviors in shake flasks (Fig. 1). The increase in the salt concentration (up to 1.5 g L^{-1} of K_2HPO_4) led to one order of magnitude increase in viable cell concentration when glucose is used as C-source ($1.71 \pm 0.04 \times 10^9$CFU mL^{-1}; Fig. 1A, Table 1). This high cell concentration was not reached when the K_2HPO_4 was further increased to 2.0 g L^{-1} ($1.31 \pm 0.08 \times 10^9$CFU mL^{-1}). A similar behavior was followed when biomass growth was measured as optical density (Fig. 1B). On the same way, the specific growth rate (μ) calculated from the profiles

of viable cells increased from 0.043 ± 0.001 h^{-1} (0.2 g L^{-1} of K_2HPO_4) up to 0.109 ± 0.002 h^{-1} (1.5 g L^{-1} of K_2HPO_4) (Table 1).

A complete glucose consumption was observed when 1.0, 1.5, and 2.0 g L^{-1} of K_2HPO_4 was used, but not for the cultures containing 0.2 and 0.5 g L^{-1} of K_2HPO_4 (Fig. 1C, Table 1). The biomass over substrate yield ($Y_{X/S}$), is in concordance with the growth and substrate consumption profiles, where there was an approximate two-fold improvement in yield when K_2HPO_4 concentration was increased (Table 1). The specific substrate consumption rate (q_S) increased slightly using higher amounts of K_2HPO_4 (Table 1). Nevertheless, the benefits of increasing the salt concentration were also observed in the volumetric biomass productivity (Q_p) which enhanced from $25 \pm 6 \times 10^6$ CFU mL^{-1} h^{-1} to $71 \pm 2 \times 10^6$ CFU mL^{-1} h^{-1} for media containing 0.2 and 1.5 g L^{-1} K_2HPO_4, respectively (Table 1).

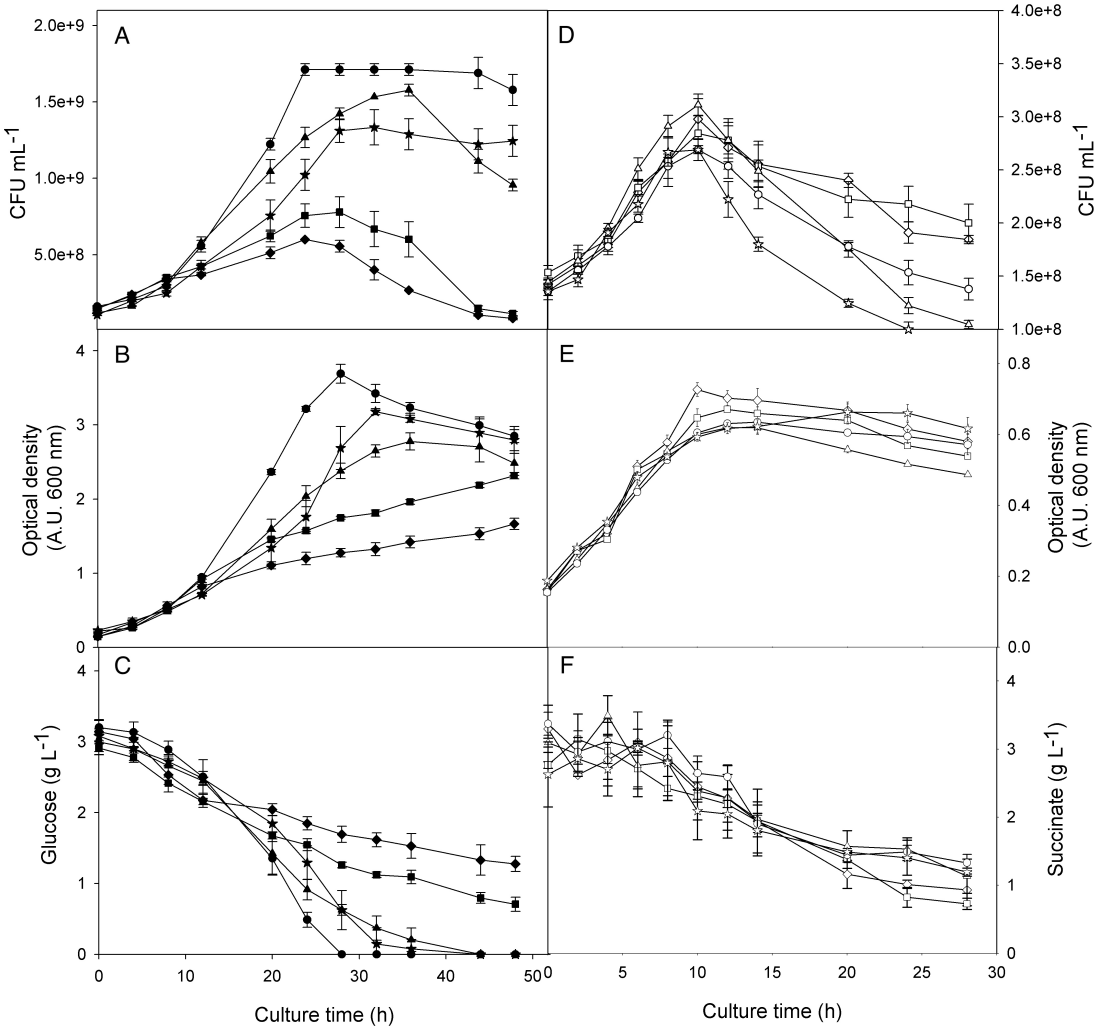

Fig. 1. Kinetics of *R. phaseoli* growth evaluated as colony forming units, CFU mL^{-1} (A, D) and optical density at 600 nm (B, E) for submerged culture of *R. phaseoli* in shake flask with defined medium at 150 rpm and 30°C. The C-source consumption for glucose (C) and succinate (F) are also shown. The K_2HPO_4 concentration evaluated for both C-sources were 0.2 (diamond), 0.5 (square), 1.0 (triangle), 1.5 (circle) and 2.0 (star) g L^{-1}, for glucose (left, close symbols) and succinate (right, open symbols). Data show the mean and standard deviation for at least three biological replicates per condition.

Table 1. Kinetic and stoichiometric parameters for shake-flasks cultures of *R. phaseoli* in defined medium with different K_2HPO_4 concentrations and glucose or succinate as carbon sources. Specific growth rate (μ), maximum biomass (Xmax), residual C-Source (RC), yield biomass-substrate ($Y_{X/S}$), specific substrate consumption (q_s), and volumetric biomass productivity (Q_p). The mean and standard deviation for at least three biological replicates are shown. a,b,c,d: Tukey multiple comparisons, The data with the same letter are not statistically different.

K_2HPO_4 (g L^{-1})		**0.2**	**0.5**	**1.0**	**1.5**	**2.0**
Glucose	X_{max} (CFU mL^{-1}) × 10^8	6.0±0.9 [a]	7.8±1.0 [b]	15.8±0.4 [c]	17.1±0.4 [c]	13.1±0.8 [d]
	X_{max} (A.U. 600 nm)	1.67±0.08	2.21±0.06	2.73±0.10	3.69±0.13	3.18±0.04
	RC (g L^{-1})	1.3±0.1	0.7±0.1	0.0	0.0	0.0
	μ (h^{-1})	0.043±0.001 [a]	0.051±0.004 [a]	0.085±0.002 [b]	0.109±0.002 [c]	0.086±0.009 [b]
	$Y_{X/S}$ (CFU pg^{-1})	0.35±0.04 [a]	0.34±0.05 [a]	0.52±0.04 [b]	0.57±0.04 [b]	0.44±0.03 [b]
	q_S (pg CFU^{-1} h^{-1})	0.12±0.02 [a]	0.15±0.03 [ab]	0.16±0.01 [b]	0.19±0.02 [c]	0.20±0.02 [c]
	Q_p (CFU $mL^{-1}h^{-1}$) x10^6	25±6 [a]	31±3 [b]	44±1 [c]	71±2 [d]	47±3 [c]
Succinate	X_{max} (CFU mL^{-1}) x 10^8	3.0±0.2 [ab]	2.8±0.2 [ab]	3.1±0.1 [b]	2.7±0.1 [a]	2.7±0.1 [a]
	X_{max} (A.U. 600 nm)	0.71±0.06 [a]	0.64±0.04 [b]	0.60±0.02 [b]	0.61±0.04 [b]	0.64±0.04 [b]
	RC (g L^{-1})	0.9± 0.2	0.7±0 .1	1.1± 0.3	1.3±0.1	1.2±0.1
	μ (h^{-1})	0.085±0.013 [a]	0.075±0.008 [a]	0.096±0.005 [a]	0.080±0.007 [a]	0.088±0.014 [a]
	$Y_{X/S}$ (CFU pg^{-1})	0.17±0.07 [a]	0.12±0.02 [a]	0.18±0.04 [a]	0.16±0.07 [a]	0.15±0.02 [a]
	q_S (pg CFU^{-1} h^{-1})	0.56±0.21 [ab]	0.62±0.05 [a]	0.55±0.12 [a]	0.50±0.07 [a]	0.58±0.26 [a]
	Q_p (CFU $mL^{-1}h^{-1}$) x10^6	30±1 [a]	28±2 [a]	34±3 [a]	27±4 [a]	30±4 [a]

However, the change in K_2HPO_4 concentration did not improve any of the parameters when succinate was used as the C-source. The viable cell concentration achieved for all the K_2HPO_4 concentrations was in the order of 3 $\times 10^8$ CFU mL^{-1} (Fig. 1D, Table 1). The optical density revealing similar growth profiles (Fig. 1E). The μ calculated from the viable cell profiles did not exhibit any significant difference between any of the conditions (Table 1). Succinate was not exhausted for any of the conditions evaluated (Fig. 1F, Table 1). $Y_{X/S}$ and Q_p were statistically similar among almost all conditions evaluated (Table 1). In summary, the results demonstrated an improvement in growth, kinetics, and stoichiometric parameters when K_4HPO_4 increases (up to 1.5 g/L) and glucose was used as the C-source.

3.2 *Glucose improves the growth of R. phaseoli over mannitol, fructose, or sucrose in undefined culture medium at different C/N ratios*

Four C-sources were evaluated in an undefined medium in shake-flask cultures, for 48 h incubation and at least in triplicate. The highest μ occurred when glucose (YEG) was used, followed by mannitol (YEM) and sucrose (YES, Table 2). Conversely, the lowest μ were found when fructose (YEF) was used at C/N ratios of 74 and 111 (Table 2). Similarly, there were no significant differences between the final viable biomasses when YEG, YEM, or YES were used (on average 2.5 $\times 10^9$UFC mL^{-1}), but there was a significant difference in the low carbon/nitrogen ratio in YEF (at a C/N ratio of 37), which was the only condition where growth was observed with fructose (Table 2). In terms of biomass measured as optical density and dry weight, the highest values were found with a C/N ratio of 74 in YEG and YEM (Table 2). Although we did not determine the consumption of the C-source during cultures initiated with 10 g L^{-1} of the C-source, the maximum growth value was 3.5 ± 0.2 $\times 10^9$CFU mL^{-1} in YEG (Table 2), almost twice than those previously reported when 3.0 g L^{-1} of glucose was used in defined medium (1.71 ± 0.04 $\times 10^9$CFU mL^{-1}, Table 1).

3.3 *Low values of the volumetric mass transfer coefficient increase growth in shake-flask cultures*

A major issue in the scale-up of bacterial inoculants bioprocesses is the oxygen mass transfer (Bissonnette *et al*., 1986; Trujillo-Roldán *et al*., 2013; Kosmachevskaya *et al*., 2020), since oxygen has

been shown to play important roles in *Rhizobium* sp. non-symbiotic growth (Rutten and Poole, 2019; Kosmachevskaya *et al*., 2020) and in the establishment of the legume-Rhizobium symbiosis (Crockford *et al*., 1995; Hérouart *et al*., 2002; Rutten and Poole, 2019). The parameter that represents the oxygen transfer phenomenon in submerged cultures is the k_La. Here, k_La was measured in conventional Erlenmeyer flasks (500 mL) with two orbital agitation rates (100 and 200 rpm) and four filling volumes (80, 40, 20, and 10 %) to evaluate six k_La values (Table 3).

Table 2. Effect of carbon source and C/N ratio on *R. phaseoli* grown in undefined media in shake flasks cultures. Specific growth rate (μ), maximum biomass was measured as colony forming units, cell dry weight and by optical density. The mean and standard deviation for at least three biological replicates are shown. Cultures were carried out in shake flaks at 100 rpm, 20 % filling volume and 30±2°C.

Carbon source	C/N (g g^{-1})	μ (h^{-1})	X_{max} (A.U.)	X_{max} CDW (g L^{-1})	X_{max} (UFC mL^{-1}) × 10^9
Glucose (YEG medium)	37	0.107±0.002 ^{a}	1.58±0.05 ^{f}	0.66±0.06 ^{h}	2.5±0.8 ^{n}
	74	0.116±0.003	2.09±0.04	0.87±0.08 ^{j}	3.3±0.2 ^{n}
	111	0.061±0.002	1.16±0.07	0.48±0.09	1.5±0.3 ^{n}
Fructose (YEF medium)	37	0.101±0.005 ^{a,d}	1.54±0.07 ^{f}	0.64±0.12 ^{h}	0.9±0.4
	74	0.015±0.007 ^{b}	0.11±0.08 ^{e}	0.05±0.01 ^{g}	N/O*
	111	0.023±0.005 ^{b}	0.10±0.02 ^{e}	0.04±0.01 ^{g}	N/O*
Sucrose (YES medium)	37	0.104±0.005 ^{a,d}	1.39±0.12 ^{f}	0.58±0.07 ^{h}	3.7±0.8 ^{n}
	74	0.088±0.003 ^{c}	1.54±0.07 ^{f}	0.64±0.04 ^{h}	3.0±0.9 ^{n}
	111	0.098±0.006 ^{d}	1.58±0.09 ^{f}	0.66±0.06 ^{h}	2.7±0.7 ^{n}
Mannitol (YEM medium)	37	0.101±0.005 ^{a,d}	1.60±0.06 ^{f}	0.66±0.12 ^{h}	2.3±0.5 ^{n}
	74	0.089±0.009 ^{c}	1.79±0.03	0.74±0.07 ^{j,h}	1.7±1.0 ^{n}
	111	0.070±0.004^{o}	0.71±0.03	0.29±0.02	2.0±1.2 ^{n}

Tukey multiple comparisons, the data with the same letter are not statistically different. N/O: Not observed.

Table 3. Effect of the volumetric oxygen mass transfer coefficient (k_La) on kinetic and stoichiometric parameters for cultures of *R. phaseoli* growth in YEG. Specific growth rate (μ), maximum biomass (Xmax), residual C-Source (RC), exopolysaccharides (EPS), yield biomass-substrate ($Y_{X/S}$) and specific substrate consumption (q_s). Cultures were carried out in shake flasks at 30 ± 2°C. The mean and standard deviation for at least three biological replicates condition are shown.

Orbital shaking speed (rpm)	**100**			**200**		
Filling volume (%)	**80**	**40**	**20**	**40**	**20**	**10**
k_La (h^{-1})	3.9±0.2	6.7±0.3	10.8±1.8	26.8±2.7	35.0±3.7	59.2±3.8
X_{max} (A.U. 600 nm)	0.56±0.06 ^{b}	2.22±0.26 ^{a}	2.43±0.16 ^{a}	0.65±0.11 ^{b}	0.60±0.09 ^{b}	0.55±0.08 ^{b}
X_{max} (CFU mL^{-1}) × 10^9	1.1±0.4	5.1±1.3 ^{b}	4.2±1.1 ^{b}	0.9±0.5 ^{c}	0.8±0.3 ^{c}	0.9±0.4 ^{c}
μ (h^{-1})	0.071±0.002	0.187±0.007	0.201±0.006	0.108±0.010 ^{d}	0.113±0.005 ^{d}	0.111±0.003 ^{d}
RC (g L^{-1})	6.0±0.1 ^{a}	5.3±0.3 ^{b}	4.7±0.2	6.1±0.1 ^{a}	5.9±0.2 ^{a}	5.5±0.2 ^{b}
$Y_{X/S}$ (CFU pg^{-1})	0.28±0.09 ^{a}	1.08±0.21 ^{b}	0.79±0.21 ^{b}	0.18±0.11 ^{a}	0.19±0.09 ^{a}	0.20±0.08 ^{a}
q_S (pg CFU^{-1} h^{-1})	0.24±0.04 ^{a}	0.19±0.03 ^{a}	0.23±0.02 ^{a}	0.61±0.04 ^{b}	0.59±0.03 ^{b}	0.57±0.02 ^{b}
EPS (g L^{-1})	0.43±0.12 ^{a}	1.05±0.31 ^{b}	0.94±0.25 ^{b}	0.39±0.08 ^{a}	0.81±0.07 ^{b}	0.46±0.08 ^{a}
Viscosity (cps)	1.2±0.2 ^{b}	3.0±0.5 ^{a}	2.7±0.9 ^{a}	1.5±0.5 ^{b}	2.1±0.6 ^{a}	1.4±0.2 ^{b}

The data with the same letter are not statistically different (Tukey multiple comparisons). Viscosity, EPS and maximum biomass (Xmax in CFU mL^{-1} ×10^8) was measured at the end of the culture (24 h). All shake-flasks cultures started with 1.0±0.8 ×10^7 UFC mL^{-1}.

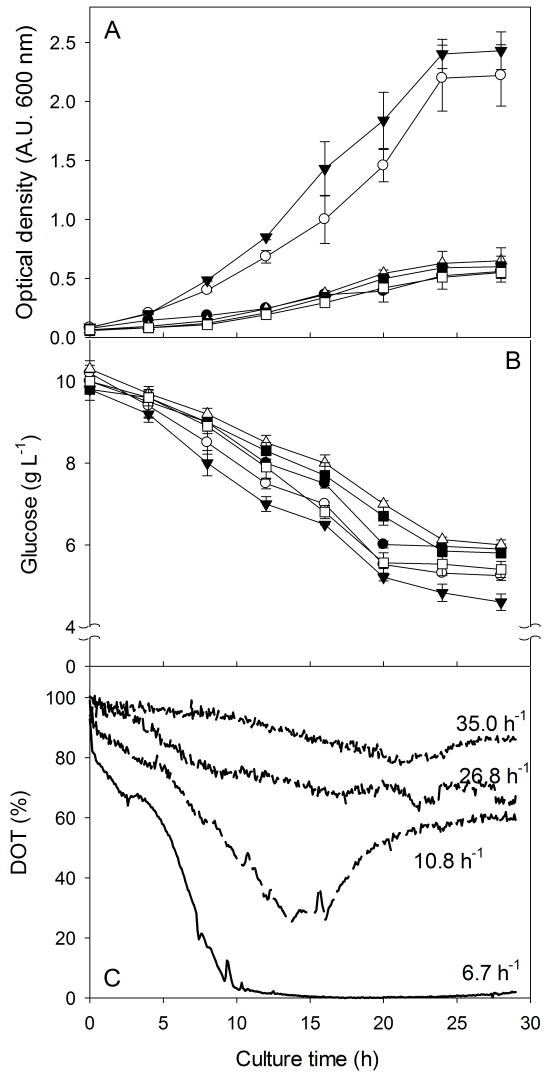

Fig. 2. Effect of the volumetric oxygen mass transfer coefficient (K_La) in shake flasks on cultures of *R. phaseoli* growth evaluated as optical density (A), glucose consumption (B) and dissolved oxygen tension, DOT (C). K_La values of 3.9±0.2 (closed circle), 6.7±0.3 (open circle), 10.8±1.8 (close downside triangle), 26.8±2.7 (open triangle), 35.0±3.7 (close square) and 59.2±3.84 (open square) h^{-1} were evaluated. Data show the mean and standard deviation for at least three biological replicates per condition. In the case of the dissolved oxygen tension, a representative trend of one culture is shown.

The experiments were carried out in the optimized YEG medium for 36 h for growth comparison. The highest biomass growth (2.22 ± 0.26 and 2.43 ± 0.16 A.U.), viability (5.1 ± 1.3 and 4.2 ± 1.1 $\times 10^9$ UFC mL^{-1}), and μ (0.187 ± 0.007 and 0.201 ± 0.006 h^{-1}) were found in those cultures grown with a k_La of 6.7 ± 0.3 and 10.8 ± 1.8 h^{-1} (Table 3, Fig. 2A). Similarly, the lowest q_S of glucose (16 ± 3 and 17 ± 1 pg CFU^{-1} h^{-1}) were found in these conditions (Table 3).

Residual glucose was observed to be approximately 5.5 g L^{-1}; however, YEG media may be limited by another nutrient differing from the C-source or by the accumulation of a by-product that limits growth (Fig. 2B). As we demonstrated in the defined medium phosphate might be the limiting nutrient since 0.5 g L^{-1} of K_2HPO_4 was used. An evaluation of the effect of phosphates on undefined media was not performed in the present study and should be considered in subsequent studies as a possible improvement to the process. Accordingly, the highest values of $Y_{X/S}$ were obtained in the intermediate k_La values (6.7 ± 0.3 and 10.8 ± 1.8 h^{-1}), while higher and lower values of k_La did not show significant differences. In addition, the specific consumption rates (q_S) below a k_La of 10 h^{-1} were almost three-fold lesser than those above a k_La of 26 h^{-1} (Table 3). Similarly, the final production of exopolysaccharides and the viscosity of the culture medium were significantly increased under k_La values of 6.7 ± 0.3 h^{-1} (1.05 ± 0.31 g L^{-1} and 3.0 ± 0.5 cps, respectively) and 10.8 ± 1.8 h^{-1} (0.94 ± 0.25 g L^{-1} and 2.7 ± 0.9 cps, respectively) compared with the other conditions evaluated. Low but non-limiting levels of DOT (4-20%) have previously been shown to determine improved growth and production of polyhydroxybutyrate in *R. trifolii* cultures (Thompson and Leps, 1986). In our experiments, we evaluated the DOT in the four intermediate values of k_La. The DOT was measured and recorded online as reported Reynoso-Cereceda *et al.*, (2016). A sharp decrease in DOT (Fig. 2C) until 10 h of culture was seen at a k_La of 6.7 h^{-1} where DOT remained very close to 0%, indicating a limitation of oxygen in these cultures. When cultures were carried out a t a k_La of 10.8 h^{-1}, the DOT decreased to ~30% at 14 h of culture. With higher k_La values, there was no marked decrease in DOT.

3.4 *Scale-up of R. phaseoli cultures from shake flasks to a pneumatic bioreactor*

Due to the geometric differences between shake flasks and BCBs, scale-up empirical correlations can hardly be used. In this sense, a criterion that could be easily measured in both and that encompassed a mass transport phenomenon was used, due to that k_La was the scaling-up criterion.

Table 4. Effect of the volumetric oxygen mass transfer coefficient (k_La) on kinetic and stoichiometric parameters for cultures of *R. phaseoli* growth in 5 L lab pneumatic bioreactors. Specific growth rate (μ), maximum biomass (Xmax), residual C-Source (RC), yield biomass-substrate ($Y_{X/S}$) and specific substrate consumption (q_s). The mean and standard deviation for at least three biological replicates are shown. The data with the same letter are not statistically different (Tukey multiple comparisons).

	Lab 5 l, bubble column bioreactor		
Aireation (vvm)	**0.1**	**0.3**	**1.0**
k_La (h^{-1})	4.3±0.1	9.5±0.3	40.1±1.2
X_{max} (A.U. 600 nm)	1.38±0.41	2.56±0.30 [a]	3.0±0.6 [a]
X_{max} (CFU mL^{-1}) × 10^9	3.0±0.7	4.8±0.8 [a]	6.0±1.1 [a]
μ (h^{-1})	0.147±0.022 [a]	0.183±0.032 [a,b]	0.241±0.031 [b]
RC (g L^{-1})	6.0±0.1 [a]	5.9±0.1 [a]	5.8±0.2 [a]
$Y_{X/S}$ (CFU pg^{-1})	0.75±0.17	1.20±0.20 [a]	1.42±0.27 [a]
q_S (pg CFU^{-1} h^{-1})	0.19±0.02 [a]	0.16±0.02 [a]	0.17±.01 [a]
EPS (g L^{-1})	0.25±0.06	0.41±0.11	1.78±0.13
Viscosity (cps)	1.0±0.5	5.2±0.6	20.6±0.3

Criteria commonly used and easily measurable in pneumatic bioreactors such as holdup gas, radial distribution of liquid velocities, back-mixing of the liquid cannot be measured in shake flasks. To cover at least three values of k_La, *R. phaseoli* cultures were grown in a 5-liter BCB using YEG media at aeration rates of 0.1, 0.3, and 1.0 vvm (0.5, 1.5, and 5.0 slpm), with initial values of k_La between 4.0 and 40 h^{-1} (Table 4). In contrast to shake-flask cultures, the highest k_La value (40.1 ± 1.2 h^{-1}) produced statistically similar results to the mid k_La value (9.5 ± 0.3 h^{-1}), in biomass growth (2.56 ± 0.30 and 3.0 ± 0.6 A.U., respectively), viability (4.8 ± 0.8 and 6.0 ± 1.1 ×10^9UFC mL^{-1}, respectively), and μ (0.183 ± 0.032 and 0.241 ± 0.031 h^{-1}, respectively), while both the higher k_La values produced higher results than the lowest k_La value (4.3 ± 0.3 h^{-1}) (Table 4, Fig. 3A).

A similar C-source consumption was also observed (Fig. 3B), with residual glucose concentrations (~6.0 g L^{-1}), although there was a clear difference in bacterial growth (Fig. 3A, Table 4). Consequently, $Y_{X/S}$ values were statistically similar at k_La values of 9.5 ± 0.3 and 40.1 ± 1.2 h^{-1}, but higher than the yield obtained at 4.3 ± 0.1 h^{-1} (Table 4). Like shake flasks, the q_S did not show significant differences between the k_La values evaluated.

In this study, we proposed to determine the effect of the volumetric mass transfer coefficient (measured before inoculating the cultures), with the assumption that the k_La remains relatively constant during cultivation. In the BCB at the high k_La (40.1 ± 1.2 h^{-1}), an increase in viscosity was observed during the exponential phase of growth (Table 4). This increase in viscosity (up to 20 cps at the end of culture) was a consequence of the production of exopolysaccharides (1.78 ± 0.13 g L^{-1}, Table 4) and could modify the mass transfer rate; this does not happen in shake flasks as the viscosity at the completion of shake-flask cultures was always close to that of water (1.0 to 3.0 cps). A clear demonstration of this phenomenon may be the fact that in cultures incubated at a k_La of 10.8 ± 1.8 h^{-1} in shake flasks, the DOT does not reach zero (Fig. 3C). Meanwhile, the cultures carried out at 9.5 ± 0.3 h^{-1} in BCBs do reach zero for approximately 15 h of culture (Fig. 4C). Furthermore, the DOT of the cultures grown at a k_La of 40.1 ± 1.2 h^{-1} in the BCB show a similar decrease to cultures grown at 10.8 ± 1.8 h^{-1} in shake flasks. In *R. radiobacter* the culture broth became viscous when the DOT was over 40% (Wu *et al*., 2003) in a similar trend as reported here. Apparently, the differences in hydrodynamic conditions between the BCB and shake flasks, like the local energy dissipation rate, shear rates or the turbulent kinetic energy, allow an increase in the production of exopolysaccharides that modify the

viscosity of the broth fermentation.

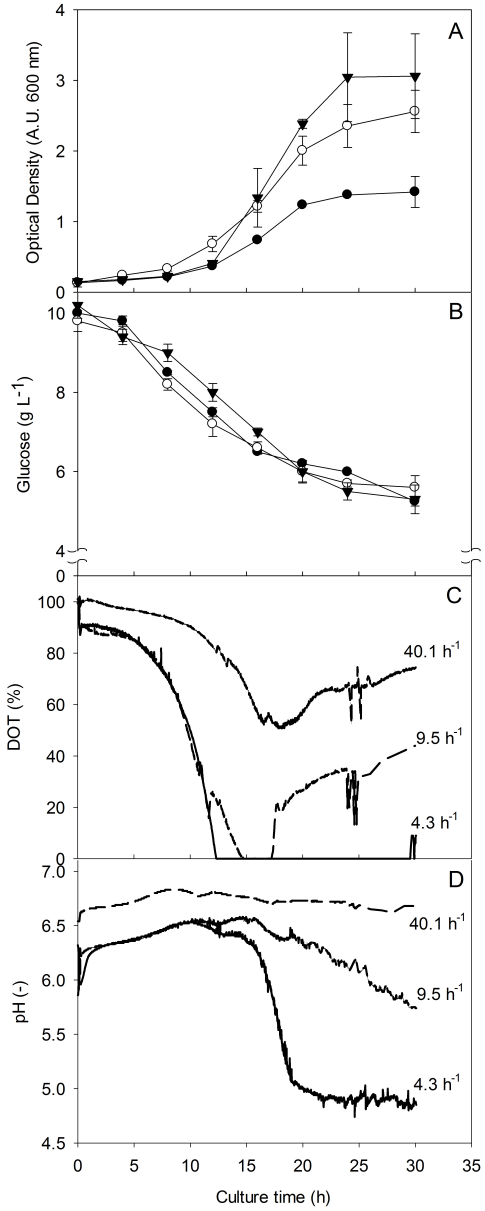

Fig. 3 Effect of the volumetric oxygen mass transfer coefficient (K_La) in bubble column bioreactor on cultures of *R. phaseoli* growth evaluated as optical density (A), glucose consumption (B), dissolved oxygen tension (C) and pH (D). K_La values of 4.3±0.1 (closed circles), 9.5±0.3 (open circles) and 40.1±1.2 (close downside triangle) h^{-1} were evaluated. Data show the mean and standard deviation for at least three biological replicates per condition. In the case of DOT and pH a representative trend of one culture at each K_La is shown.

Similar phenomena of increases in viscosity associated to molecular weight regulation of an exopolysaccharide in a nitrogen-fixing bacterium have been previously reported (Trujillo-Roldán *et al*., 2004; Castellane *et al*., 2015), as also the formation of bacterial clusters associated to EPS at low agitation, phenomenon documented in Peña *et al*. (2000).

A similar trend of the change in DOT can be seen in pH values (measured online in the BCB); where the greater the decrease in DOT, the greater the decrease in pH (Fig. 3D). The acidification of the culture medium under conditions of oxygen limitation has been demonstrated in similar cultures previously (Kannenberg and Brewin, 1989). This is probably due to the presence of acetate and other monocarboxylic acids, and it has been demonstrated that these acidic conditions can inhibit bacterial growth (Perez-Galdonaf and Kahn, 1994).

3.5 R. phaseoli inoculant from bioreactor cultures can nodulate Phaseolus vulgaris

One of the main issues with *Rhizobium* sp. is the possibility that during a bioprocess, bacteria may lose a proportion of their plasmids that are required for subsequent symbiosis (Brom *et al*., 1992; Kaminsky *et al*., 2019). Samples of *R. phaseoli* in chemically defined medium containing 1.5 g L^{-1} K_2HPO_4 and glucose (in shake-flask cultures) and samples from YEG medium (from both shake flasks and BCB) were used as inoculant for *Phaseolus vulgaris* (Table 5). The controls were non-inoculated seeds (negative control) and seeds treated with bacteria inoculated in TY medium (positive control). The height of plants and the wet weights of shoots and roots were not significantly different from values obtained after the treatments (Table 5), with the exception of an increased wet weight of roots from the seeds treated with bacteria inoculated in TY medium (0.74 ± 0.17 g). However, the number of nodules per plant obtained with the inoculant harvested in undefined medium (8.1 ± 6.6 nodules per plant) was significantly higher than the nodules obtained after the inoculation in TY medium (2.0 ± 2.0 nodules per plant). The differences between inoculant from TY and YEG media were not significant (Table 5). Expectedly, we did not find any nodules in the non-inoculated plants.

Table 5. Inoculation of *Phaseolus vulgaris* seeds with *R. phaseoli* growth in defined medium with glucose and 1.5 g L^{-1} K_2HPO_4 harvest from shake flasks (SF, n=28) cultures and undefined media (YEG) harvest from SF (n=10) and bubble column bioreactor (BCB, n=10). Non-inoculated (NI, n=10) and inoculated with *R. phaseoli* grown in undefined medium (TY, n=9) were made as controls. The effect in plant growth as height (H), shoot wet weight (SWW), root wet weight (RWW) and number of nodules (NN), were tasted for the different treatments.

Treatment	H (cm)	SWW (g)	RWW (g)	NN (-)
NI	27.0±4.1 [a]	1.0±0.2 [a]	0.52±0.14 [a]	0 [a]
TY	25.2±3.8 [a]	1.2±0.2 [a]	0.74±0.17 [b]	2.0±2.0 [ab]
Defined media (SF)	27.2±4.7 [a]	1.0±0.3 [a]	0.51±0.21 [a]	8.1±6.6 [c]
Undefined media (YEG-SF)	26.8±3.8 [a]	1.1±0.5 [a]	0.66±0.19 [ab]	6.1±5.0 [bc]
Undefined media (YEG-BCB)	24.6±7.2 [a]	0.9±0.4 [a]	0.55±0.19 [ab]	4.7±3.8 [bc]

[a,b,c] Tukey multiple comparisons, The data with the same letter are not statistically different.

4 Discussion

Although the exact composition of a production media for commercial inoculants is generally patented or the components are not reported, it is known that mannitol and sucrose are the main C-sources used industrially (Supplementary Material, Table S1). Rhizobia are extensively reported as a class of symbiotic diazotrophic bacteria that use C_4 acids (as succinic acid) in preference to C_6 sugars (as glucose), while C_6 sugar utilization is repressed as long as C_4 acids are present (Arwas *et al*., 1985; Yurgel and Kahn, 2004; Iyer *et al*., 2016; Iyer and Rajkumar, 2019). Moreover, the main C-sources consumed by *Rhizobium* bacteroids in the nodule are dicarboxylates (such as malate or fumarate, but mainly succinate) that produce sufficient energy for biological nitrogen fixation (Geddes and Oresnik, 2014; Iyer *et al*., 2016). In several cases, yeast extract is added as a C- and N-source and a source of growth factors in undefined culture media such as YMB or YEM (Stowers, 1985; Ben-Rebah *et al*., 2007; Hossain *et al*., 2019). Inexpensive raw materials derived from agroindustry have also been evaluated as culture media components (Bissonnette *et al*., 1986; Tittabutr *et al*., 2005; Ben-Rebah *et al*., 2007; Lobo *et al*., 2019). However, glucose is normally consumed by *Rhizobium* sp. (Stowers, 1985) and is a suitable substrate to scale-up production due to its relatively low cost. In the current study, we showed that glucose was the best C-source for axenic *R. phaseoli* submerged cultures, in both defined and undefined (YEG) media. Additionally, the use of a chemically defined medium could reduce the risk of contamination during the culture, as also in shelf-life formulation due to the exhausted culture media is normally used as a part of the formulation of inoculants (Mutturi *et al*., 2016).

Rhizobium sp. is exposed to extremely low phosphate concentrations in the nodules but to high concentrations depending on the type of soils to which they are exposed. The performance of *Rhizobium* sp. has shown strain-to-strain differences surely due to from the soil from which they were isolated (Beck and Munns 1984; Cassman *et al*., 1981a, 1981b). To deal with this variation, *Rhizobium* sp. presents an ability to store large quantities of phosphate, an efficient utilization of internal phosphate, and a robust uptake at low external concentrations. We found that when glucose was used and K_2HPO_4 was increased 7.5-fold from the original concentration (0.22 g L^{-1}) used by Sherwood (1970), the maximal biomass (measured as CFU mL^{-1}) increased by almost threefold (Fig. 1A), μ by more than twofold (Table 1), $Y_{X/S}$ also by more than threefold, and the Q_p was also approximately threefold (Table 1). However, when the K_2HPO_4 concentration was increased to 2.0 g L^{-1}, maximal biomass, kinetic, and stoichiometric parameters decreased (Fig. 1A and 1B, Table 1). An inhibition in *Bradyrhizobium japonicum* growth was attributed to elevated biotin concentrations used in a chemically defined medium with mannitol as C-source (Murphy and Elkan, 1963). However, Date (1972) explained this behavior by using relatively high potassium concentrations (1 g L^{-1} K_2HPO_4 and 1 g L^{-1} KH_2PO_4). In our experiments, we observed

that the medium turned turbid when formulated the medium with K_2HPO_4 concentration up to 2.0 g L^{-1}. However, changes in K_2HPO_4 with succinate as the C-source did not alter the growth profile for any of the concentrations of the salt evaluated (Fig. 1D and 1E). Moreover, the kinetic and stoichiometric parameters did not show any significant changes (Table 1). The differences in the maximum number of cells reached when either glucose or succinate was used as C-source (Fig. 1) might be expected because it is known that the activity of enzymes from the Entner-Doudoroff pathway are higher when sugars are used instead of dicarboxylates (Romanov *et al*., 1994).

The concentration of oxygen present in the soil, and therefore the oxygen transfer rates, are fundamental in the free-living rhizobia (Rutten and Poole, 2019; Kosmachevskaya *et al*., 2020) and in establishing symbiosis (Hérouart *et al*., 2002; Rutten and Poole, 2019). The oxygen transfer rate is also an important parameter in liquid rhizobial cultures (Bissonnette *et al*., 1986; Blunt *et al*., 2018; Kosmachevskaya *et al*., 2020), and especially when scaling to bioreactors (Trujillo-Roldán *et al*., 2013). Higher k_La values showed lower values of growth and viability, but more than double the q_s, implying that a high OTR can induce increased bacterial consumption of the C-source to contend with the oxidative stress (Crockford *et al*., 1995). Fortunately, *R. phaseoli* has a small optimal interval of k_La, meaning a reduced range of OTR for optimal growth, which demonstrates the strong relationship between OUR and bacterial metabolism (Blunt *et al*., 2018). The relationship between low values in OTR, bacterial growth and the production of intracellular and extracellular compounds in nitrogen-fixing bacteria has been widely discussed (Trujillo-Roldán *et al*., 2001; Trujillo-Roldán *et al*., 2004; Trujillo-Roldán *et al*., 2013; Blunt *et al*., 2018). It is previously reported that pneumatic bioreactors, are more effective than mechanically agitated bioreactors producing EPS, like pullulans (Özcan *et al*., 2014) and xhantan gum (Suh *et al*., 1992) being the aeration rate one of the main factors involved in the modification of the EPS physicochemical characteristics (Menniti and Morgenroth, 2010). It was shown that EPS production is directly associated with OTR, in both upward and downward flows in pneumatic bioreactors and the fact that high aerations avoid stagnation zones in BCBs (Suh *et al*., 1992). However, to the best of our knowledge, growth of a nodule-forming bacterium in a small interval of mass transfers has not been specifically reported. Moreover, under a review of the literature, there are no articles that present a hydrodynamic or mass transfer comparison of shaken flasks and bubble columns, which may be useful in terms of a better understanding all the aspects related to scale-up between these two models. Although no formulations were evaluated in this project to extend shelf life, samples of all cultures were kept for up to 24 weeks in closed 1.0 mL plastic tubes at room temperature in the respective exhausted culture medium. In glucose cultures, in both defined and undefined culture media, cell viability decreased by two orders of magnitude at the end of the 24 weeks term (Supplementary Material, Fig. S1 and Table S2). A direct relationship has been shown between the amount of intracellular reserve polymers, for example polyhydroxybutyrate, and the shelf life of the cultures (Encarnación *et al*., 1995; Povolo and Casella, 2004; Ratcliff *et al*., 2008; Trujillo-Roldán *et al*., 2013). The main effect of using a bacterial inoculant formulated with both chemical defined and undefined media in greenhouse experiments was the improvement in the number of nodules per plant with respect to the controls. Previous data suggested that the utilization of exogenous dicarboxylates is essential for effective nodule development by *R. leguminosarum* (Arwas *et al*., 1985).

Conclusions

In the present study, *R. phaseoli* was able to grow in C4, C6, and C12 sugars in both chemical defined and undefined media, which validated its capacity to form nodules in legumes and the possibility of being an effective inoculant in leguminous production. In rhizobia, symbiotic genes are normally in plasmids and can be lost in bacterial sub-culturing. Therefore, growing *R. phaseoli* in the culture media proposed in this study, and in the scale-up from shake flasks to a pneumatic bioreactor, indicates that the plasmids required for optimal symbiotic performance were maintained as nodulation was successful.

Nomenclature

A.U.:	absorbance units (-)
BCB:	bubble column bioreactor
C_L:	bulk dissolved oxygen concentration (mmol O_2 L^{-1})
C_L^*:	saturation dissolved oxygen concentration (mmol O_2 L^{-1})

DOT: dissolved oxygen tension (% of air saturation)
gC/gN: Mass carbon/nitrogen ratio in grams
H: plant growth measure as height (cm)
k_La: volumetric oxygen transfer coefficient (h^{-1})
NN: number of nodules per plant (-)
OTR: oxygen transfer rate (mmol O_2 L^{-1} h^{-1})
OUR: oxygen uptake rate (mmol O_2 L^{-1} h^{-1})
Q_p: volumetric biomass productivity (CFU mL^{-1} h^{-1})
q_s: specific substrate consumption rate (pg CFU^{-1} h^{-1})
RC: residual C-source (g L^{-1})
RWW: plant root wet weight (g)
S: substrate as C-source (g L^{-1})
slpm: standard air liters per minute (L min^{-1})
SWW: plan shoot wet weight (g)
t: culture time (h)
T°: temperature (ºC)
vvm: volume of air per volume of culture medium
X: biomass dry weight (CFU mL^{-1}, A.U., g L^{-1})
$Y_{X/S}$: biomass / substrate yield (gbiom $gglucose^{-1}$)

Greek symbols

μ: specific growth rate (h^{-1})

Subscripts

max: maximum value achieved in a culture
f: final value achieved in a culture
i: initial value achieved in a culture

Acknowledgements

RIGC is grateful for the scholarship from the National Council for Science and Technology, Mexico (CONACYT, Consejo Nacional de Ciencia y Tecnología, México, 220150). RIGC is a doctoral student from Programa de Doctorado en Ciencias Bioquímicas, Universidad Nacional Autónoma de México (UNAM). We also thank Betsabé Valencia de la Luz Eng., Ce Akatl Mikiztli Arias-Poblano Chem., César F. Gonzalez-Monterrubio M.Sc., Esperanza Martínez-Romero Ph.D., and Martha López-Guerrero Ph.D. for their scientific and technical assistance. This project was developed under the Institutional Program of the Institute of Biomedical Research, National Autonomous University of Mexico: "The production of biomolecules of biomedical interest in bacteria and fungi (Instituto de Investigaciones Biomédicas-UNAM: La producción de biomoléculas de interés biomédico en bacterias y hongos".

Funding: This work was partially funded by PAPIIT-UNAM, Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica, Universidad Nacional Autónoma de México (PAPIIT-UNAM IT-200719, IN-208415, IN-210419). This work was also partially financed by the "Consejo Nacional de Ciencia y Tecnología" (CONACYT 247473).

References

Ángeles-Argáiz, R.E., Carmona-Reyes, I.A., Quintero-Corrales, C.A., Medina-Macías, F.J., Blancas-Cabrera, A., Valdez-Cruz, N.A., Ulloa, M., Trujillo-Roldán, M.A., and Garibay-Orijel, R. (2020). From field sampling to pneumatic bioreactor mycelia production of the ectomycorrhizal mushroom Laccaria trichodermophora. *Fungal Biology 124*, 205-218. https://doi.org/10.1016/j.funbio.2020.02.003

Aparicio-Fernandez, X., Yousef, G.G., Loarca-Pina, G., de Mejia, E., and Lila, M.A. (2005). Characterization of polyphenolics in the seed coat of black Jamapa bean (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry 53*, 4615-4622. https://doi.org/10.1021/jf047802o

Arwas, R., McKay, I.A., Rowney, F.R.P., Dilworth, M.J., and Glenn, A.R. (1985). Properties of organic acid utilization mutants of Rhizobium leguminosarum strain 300. *Microbiology 131*, 2059-2066. https://doi.org/10.1099/00221287-131-8-2059

Barbour, W.M., Hattermann, D.R., and Stacey, G. (1991). Chemotaxis of Bradyrhizobium japonicum to soybean exudates. *Applied and Environmental Microbiology 57*, 2635-2639.

Bashan, Y., Trejo, A., and de-Bashan, L.E. (2011) Development of two culture media for mass cultivation of Azospirillum spp. and for production of inoculants to enhance plant growth. *Biology and Fertility of Soils 47*, 963-969. https://doi.org/10.1007/s00374-011-0555-3

Beck, D. P., Munns, D. N. (1984) Phosphate nutrition of Rhizobium spp. *Applied and Environmental Microbiology 47*, 278-282.

Ben-Rebah, F., Prévost, D., Yezza, A., and Tyagi, R.D. (2007). Agro-industrial waste materials and wastewater sludge for rhizobial inoculant production: a review. *Bioresource Technology 98*, 3535-46. https://doi.org/10.1016/j.biortech.2006.11.066

Bergersen, F.J. (1961). The growth of Rhizobium in synthetic media. *Australian Journal of Biological Sciences 14*, 349-360. https://doi.org/10.1071/BI9610349

Beringer, J.E. (1974). R factor transfer in *Rhizobium leguminosarum. J Gen Microbiol 84*, 188-198. https://doi.org/10.1099/00221287-84-1-188

Bissonnette, N., Lalande, R., and Bordeleau, L.M. (1986). Large-scale production of *Rhizobium meliloti* on whey. *Applied and Environmental Microbiology 52*, 838-841.

Blunt, W., Sparling, R., Gapes, D.J., Levin, D.B., and Cicek, N. (2018). The role of dissolved oxygen content as a modulator of microbial polyhydroxyalkanoate synthesis. *World Journal of Microbiology and Biotechnology 34*, 106. https://doi.org/10.1007/s11274-018-2488-6

Bosworth, A.H., Williams, M.K., Albrecht, K.A., Kwiatkowski, R., Beynon, J., Hankinson, T.R., Ronson, C.W., Cannon, F., Wacek, T.J., and Triplett, E.W. (1994). Alfalfa yield response to inoculation with recombinant strains of Rhizobium meliloti with an extra copy of dctABD and/or modified nifA expression. *Applied and Environmental Microbiology 60*, 3815-3832

Brahmaprakash, G.P., and Sahu, P.K. (2012). Biofertilizers for Sustainability. *Journal of the Indian Institute of Science 92*, 37-62

Brom, S., De Los Santos, A.G., Stepkowsky, T., Flores, M., Dávila, G., Romero, D., and Palacios, R. (1992). Different plasmids of Rhizobium leguminosarum bv. phaseoli are required for optimal symbiotic performance. *Journal of Bacteriology 174*, 5183-5189. https://doi.org/10.1128/jb.174.16.5183-5189.1992

Büchs, J. (2001). Introduction to advantages and problems of shaken cultures. *Biochemical Engineering Journal 7*, 91-98. https://doi.org/10.1016/S1369-703X(00)00106-6

Buntić, A.V., Stajković-Srbinović, O.S., Knežević, M.M., Kuzmanović, D.Ž., Rasulić, N.V., and Delić, D.I. (2019). Development of liquid rhizobial inoculants and pre-inoculation of alfalfa seeds. *Archives of Biological Sciences 71*, 379-387. https://doi.org/10.2298/ABS181008062B

Cassman, K.G., Munns D.N., Beck D.P. (1981a). Phosphorus nutrition of Rhizobium japonicum: strain differences in phosphate storage and utilization. *Soil Science Society of America Journal 45*, 517- 520. https://doi.org/10.2136/sssaj1981.03615995004500030015x

Cassman, K.G., Munns D.N., Beck D.P. (1981b). Growth of Rhizobium strains at low concentrations of phosphate. *Soil Science Society of America Journal 45*, 520-523. https://doi.org/10.2136/sssaj1981.03615995004500030016x

Castellane, T.C.L., Persona, M.R., Campanharo, J.C., and de Macedo Lemos, E.G. (2015). Production of exopolysaccharide from rhizobia with potential biotechnological and bioremediation applications. International *Journal of Biological Macromolecules 74*, 515-522. https://doi.org/10.1016/j.ijbiomac.2015.01.007

Chang, H., Li, J., Zhu, Y., Chen, L., Shi, Y., Li, S., Qin, H., Huang, S., Li, Q., Lv, X., Tian, S., Hu, Z., Qi, Z., Liu, C., Wu, X., Xin, D., and Chen, Q. (2019). Identification of a gene encoding a sugar transporter as a potential target of a novel LysR-type transcriptional regulator rluD from rhizobium required for symbiotic nodulation in soybean (Glycine max Merr.). *Plant Breeding 138*, 325-335. https://doi.org/10.1111/pbr.12685

Crockford, A.J., Davis, G.A., and Williams, H.D. (1995). Evidence for cell-density-dependent regulation of catalase activity in *Rhizobium leguminosarum* bv. phaseoli. *Microbiology 141*, 843-851. https://doi.org/10.1099/13500872-141-4-843

Date, R.A. (1972). Sources and quantities of yeast extract for growth of Rhizobia. Journal of Applied Bacteriology 35, 379-387. https://doi.org/10.1111/j.1365-2672.1972.tb03713.x

Date, R.A. (2001). Advances in inoculant technology: a brief review. *Australian Journal of Experimental Agriculture 41*, 321-325. https://doi.org/10.1071/EA00006

de Souza, J.E.B., and Ferreira, E.P.D.B. (2017). Improving sustainability of common bean production systems by co-inoculating rhizobia and azospirilla. *Agriculture, Ecosystems & Environment 237*, 250-257. https://doi.org/10.1016/j.agee.2016.12.040

Deaker, R., Hartley, E., Gemell, G., Herridge, D.F., and Karanja, N. (2016). Inoculant production and quality control. In: Howieson JG, Dilworth MJ (eds) Working with rhizobia. *Australian Centre for International Agricultural Research*, Canberra, 167-186

Didonet, A.D., and Magalhaes, A.C. (1997). Growth and nitrite production by *Azospirillum* strains subjected to different levels of dissolved oxygen in the medium. *Soil Biology and Biochemistry 29*, 1743-1746. https://doi.org/10.1016/S0038-0717(97)00066-7

Dudman, W.F. (1964). Growth and extracellular polysaccharide production by Rhizobium meliloti in defined medium. *Journal of Bacteriology 88*, 640-645

Encarnación, S., Dunn, M., Willms, K., and Mora, J. (1995). Fermentative and aerobic metabolism in *Rhizobium etli. Journal of Bacteriology 177*, 3058-3066. https://doi.org/10.1128/jb.177.11.3058-3066.1995

Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *The Journal of General Microbiology 16*, 374-381. https://doi.org/10.1099/00221287-16-2-374

García-Cabrera, R.I., Valdez-Cruz, N.A., Blancas-Cabrera, A., and Trujillo-Roldan, M.A. (2020). Oxygen transfer rate affect polyhydroxybutyrate production and oxidative stress response in submerged cultures of *Rhizobium phaseoli. Biochemical Engineering Journal 162*, 107721.

Geddes, B.A., and Oresnik, I.J. (2014). Physiology, genetics, and biochemistry of carbon metabolism in the alphaproteobacterium *Sinorhizobium meliloti. Canadian Journal of Microbiology 60*, 491-507. https://doi.org/10.1139/cjm-2014-0306

González-Leos, A., Bustos, M., Rodríguez-Castillejos, G., Rodríguez Durán, L., and Del Ángel Del Ángel, J. (2019). Kinetics of lactic acid fermentation from sugarcane bagasse by *Lactobacillus pentosus. Revista Mexicana De Ingeniería Química 19*, 377-386. https://doi.org/10.24275/rmiq/Alim618

Hernández-Forte, I., and Nápoles-García, M.C. (2018). Effect of different carbon sources on the growth of a rhizobia strain. *Cultivos Tropicales 39*, 87-90.

Hérouart, D., Baudouin, E., Frendo, P., Harrison, J., Santos, R., Jamet, A., Van de Sype, G., Touati, D., and Puppo, A. (2002). Reactive oxygen species, nitric oxide and glutathione: a key role in the establishment of the legume-Rhizobium symbiosis?. *Plant Physiology and Biochemistry 40*, 619-624. https://doi.org/10.1016/S0981-9428(02)01415-8

Hossain, A., Gunri, S.K., Barman, M., Sabagh, A.E., and da Silva, J.A.T. (2019). Isolation, characterization and purification of Rhizobium strain to enrich the productivity of groundnut (*Arachis hypogaea* L.). *Open Agriculture 4*, 400-409. https://doi.org/10.1515/opag-2019-0040

Iyer, B., Rajput, M.S., Jog, R., Joshi, E., Bharwad, K., and Rajkumar, S. (2016). Organic acid mediated repression of sugar utilization in rhizobia. *Microbiology Research 192*, 211-220. https://doi.org/10.1016/j.micres.2016.07.006

Iyer, B., and Rajkumar, S. (2019). Succinate irrepressible periplasmic glucose dehydrogenase of *Rhizobium* sp. Td3 and SN1 contributes to its phosphate solubilization ability. Archives of Microbiology 201(5), 649-659. https://doi.org/10.1007/s00203-019-01630-2

Jagjot, K., Gulab, P., Madhurama, G., and Brar, S.K. (2018). Development of liquid inoculants: an innovative agronomic practice for sustainable

agriculture. *Journal of Experimental Biology and Agriculture Sciences JEBAS 6*, 472-481. https://doi.org/10.18006/2018.6(3).472.481

Kaminsky, L.M., Trexler, R.V., Malik, R.J., Hockett, K.L., and Bell, T.H. (2019). The inherent conflicts in developing soil microbial inoculants. *Trends in Biotechnology 37*, 140-151. https://doi.org/10.1016/j.tibtech.2018.11.011

Kannenberg, E.L., and Brewin, N.J. (1989). Expression of a cell surface antigen from *Rhizobium leguminosarum* 3841 is regulated by oxygen and pH. *Journal of Bacteriology 171*, 4543-4548. https://doi.org/10.1128/jb.171.9.4543-4548.1989

Kosmachevskaya, O.V., Osipov, E.V., Van Chi, T., Mai, P.T.T., and Topunov, A.F. (2020) Effect of cultivation conditions on poly (3-hydroxybutyrate) synthesis by nodule bacteria *Rhizobium phaseoli. Applied Biochemistry and Microbiology 56*, 64-71. https://doi.org/10.1134/S000368382001010X

Lobo, C.B., Tomás, M.S.J., Viruel, E., Ferrero, M.A., and Lucca, M.E. (2019). Development of low-cost formulations of plant growth-promoting bacteria to be used as inoculants in beneficial agricultural technologies. *Microbiology Research 219*, 12-25. https://doi.org/10.1016/j.micres.2018.10.012

López-Guerrero, M.G., Ormeño-Orrillo, E., Acosta, J.L., Mendoza-Vargas, A., Rogel, M.A., Ramírez, M.A., Rosenblueth, M., Martínez-Romero, J., and Martínez-Romero, E. (2012). Rhizobial extrachromosomal replicon variability, stability, and expression in natural niches. *Plasmid 68*, 149-158. https://doi.org/10.1016/j.plasmid.2012.07.002

López-López, A., Rogel, M.A., Ormeño-Orrillo, E., Martínez-Romero, J., and Martínez-Romero, E. (2010). *Phaseolus vulgaris* seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum* sp. nov. *Systematic Applied Microbiology 33*, 322-327. https://doi.org/10.1016/j.syapm.2010.07.005

Maier, B., Dietrich, C., and Büchs, J. (2001). Correct application of the sulphite oxidation methodology of measuring the volumetric mass transfer coefficient k_La under non-pressurized and pressurized conditions. *Food and Bioproducts Processing 79*, 107-113. https://doi.org/10.1205/096030801750286267

Maier, U., Losen, M., and Büchs, J. (2004). Advances in understanding and modeling the gas-liquid mass transfer in shake flasks. *Biochemical Engineering Journal 17*, 155-167. https://doi.org/10.1016/S1369-703X(03)00174-8

Mandal, N.C., and Chakrabartty, P.K. (1993). Succinate-mediated catabolite repression of enzymes of glucose metabolism in root-nodule bacteria. *Current Microbiology 26*, 247-251. https://doi.org/10.1007/BF01575912

Menniti, A., and Morgenroth, E. (2010). The influence of aeration intensity on predation and EPS production in membrane bioreactors. *Water Research 44*, 2541-2553. https://doi.org/10.1016/j.watres.2009.12.024

Murphy, S.G., and Elkan, G.H. (1963). Growth inhibition by biotin in a strain of *Rhizobium japonicum. Journal of Bacteriology 86*, 884-885

Mutturi, S., Sahai, V., Sharma, S., and Bisaria, V.S. (2016). Strategies for high-density cultivation of bio-inoculants in submerged culture with special reference to pseudomonads. In: Singh DP, Singh HB, Prabha R (eds) *Microbial Inoculants in Sustainable Agricultural Productivity*, 1st edn. Springer, New Delhi, 181-196

O'Hara, G.W., Hungria, M., Woomer, P., and Howieson, J.G. (2016). Counting rhizobia. In: Howieson JG, Dilworth MJ (eds) Working with rhizobia. *Australian Centre for International Agricultural Research*, Canberra 109-124

Okon, Y., and Itzigsohn, R. (1995). The development of Azospirillum as a commercial inoculant for improving crop yields. *Biotechnology Advances 13*, 415-424. https://doi.org/10.1016/0734-9750(95)02004-M

Ona, O., Van, I.J., Prinsen, E., and Vanderleyden, J. (2005). Growth and indole-3- acetic acid biosynthesis of Azospirillum brasilense Sp245 is environmentally controlled. *FEMS Microbiology Letters 246*, 125-132. https://doi.org/10.1016/j.femsle.2005.03.048

Özcan, E., Sargin, S., and Göksungur, Y. (2014). Comparison of pullulan production performances of air-lift and bubble column bioreactors and optimization of process parameters in air-lift bioreactor. *Biochemical Engineering Journal 92*, 9-15. https://doi.org/10.1016/j.bej.2014.05.017

Perez-Galdonaf, R., and Kahn, M.L. (1994). Effects of organic acids and low pH on *Rhizobium meliloti* 104A14. *Microbiology 140*, 1231-1235. https://doi.org/10.1099/13500872-140-5-1231

Povolo, S., and Casella, S. (2004). Poly-3-hydroxybutyrate has an important role for the survival of *Rhizobium tropici* under starvation. *Ann microbiol 54*, 307-316.

Quijano, G., Franco-Morgado, M., Córdova-Aguilar, M., Galindo, E., and Thalasso, F. (2020). Oxygen transfer in a three-phase bubble column using solid polymers as mass transfer vectors. *Revista Mexicana De Ingeniería Química 19*, 483-494. https://doi.org/10.24275/rmiq/Proc1486

Ratcliff, W.C., Kadam, S.V., and Denisonm, R.F. (2008). Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. *FEMS Microbiology Ecology 65*, 391-399. https://doi.org/10.1111/j.1574-6941.2008.00544.x

Reynoso-Cereceda, G.I., García-Cabrera, R.I., Valdez-Cruz, N.A., and Trujillo-Roldán, M.A. (2016). Shaken flasks by resonant acoustic mixing versus orbital mixing: Mass transfer coefficient k_La characterization and *Escherichia coli* cultures comparison. *Biochemical Engineering Journal 105*, 379-390. https://doi.org/10.1016/j.bej.2015.10.015

Romanov, V.I., Hernández-Lucas, I., and Martínez-Romero, E. (1994). Carbon metabolism enzymes of *Rhizobium tropici* cultures and bacteroids. *Applied and Environmental Microbiology 60*, 2339-2342.

Rutten, P.J., and Poole, P.S. (2019). Oxygen regulatory mechanisms of nitrogen fixation in rhizobia. *Advances in Microbial Physiology 2019*, 325-389. https://doi.org/10.1016/bs.ampbs.2019.08.001.

Sahonero-Canavesi, D.X., Sohlenkamp, C., Sandoval-Calderón, M., Lamsa, A., Pogliano, K., López-Lara, I.M., and Geiger, O. (2015). Fatty acid-releasing activities in *Sinorhizobium meliloti* include unusual diacylglycerol lipase. *Environmental Microbiology 17*, 3391-3406. https://doi.org/10.1111/1462-2920.12814

Schwinghamer, E.A. (1960). Studies on induced variation in the rhizobia. I. Defined media and nodulation test techniques. *Applied Microbiology 8*, 349.

Sherwood, M.T. (1970). Improved synthetic medium for the growth of Rhizobium. *Journal of Applied Bacteriology 33*, 708-713. https://doi.org/10.1111/j.1365-2672.1970.tb02253.x

Sierra, S., Rodelas, B., Martínez-Toledo, M.V., Pozo, C., and González-López, J. (1999). Production of B-group vitamins by two *Rhizobium* strains in chemically defined media. *Journal of Applied Microbiology 86*, 851-858. https://doi.org/10.1046/j.1365-2672.1999.00765.x

Stowers, M.D. (1985). Carbon metabolism in *Rhizobium* species. *Annual Reviews of Microbiology 39*, 89-108.

Suh, I.S., Schumpe, A., and Deckwer, W.D. (1992). Xanthan production in bubble column and air-lift reactors. *Biotechnology and Bioengineering 39*, 85-94. https://doi.org/10.1002/bit.260390113

Thompson, B.G., and Leps, W.T. (1986). Effect of dissolved oxygen on growth and production of exopolysaccharide by *Rhizobium trifolii*. *Journal of Fermentation Technology 64*, 335-338. https://doi.org/10.1016/0385-6380(86)90127-5

Tittabutr, P., Payakapong, W., Teaumroong, N., and Boonkerd, N. (2005). Cassava as a cheap source of carbon for rhizobial inoculant production using an amylase-producing fungus and a glycerol-producing yeast. *World Journal of Microbiology and Biotechnology 21*, 823-829. https://doi.org/10.1007/s11274-004-2240-2

Trujillo-Roldán, M.A., Peña, C., Ramírez, O.T., and Galindo, E. (2001). Effect of oscillating dissolved tension upon the kinetics of growth, alginate production and molecular

weight in cultures of *Azotobacter vinelandii*. *Biotechnology Progress 17*, 1042-1048. https://doi.org/10.1021/bp010106d

Trujillo-Roldán, M.A., Moreno, S., Espín, G., and Galindo, E. (2004). The roles of oxygen and alginate-lyase in determining the molecular weight of alginate produced by *Azotobacter vinelandii*. *Applied Microbiology and Biotechnology 63*, 742-747. https://doi.org/10.1007/s00253-003-1419-z

Trujillo-Roldán, M.A., Valdez-Cruz, N.A., Gonzalez-Monterrubio, C.F., Acevedo-Sánchez, E.V., Martínez-Salinas, C., García-Cabrera, R.I., Gamboa-Suasnavart, R.A., Marín-Palacio, L.D., Villegas, J., and Blancas-Cabrera, A. (2013). Scale-up from shake flasks to pilot-scale production of the plant growth-promoting bacterium *Azospirillum brasilense* for preparing a liquid inoculant formulation. *Applied Microbiology and Biotechnology 97*, 9665-9674. https://doi.org/10.1007/s00253-013-5199-9

Tzintzun-Camacho, O., Sánchez-Segura, L., Minchaca-Acosta, A., Rosales-Colunga, L., Hernández-Orihuela, A., and Martínez-Antonio, A. (2016). Development of bacterial culture medium from avocado seed waste. *Revista Mexicana De Ingeniería Química 15*, 831-842. Retrieved from http://rmiq.org/ojs311/index.php/rmiq/article/view/1046

Valenzuela-Cobos, J., Grijalva-Endara, A., Marcillo-Vallejo, R., and Garcés-Moncayo, M. (2020). Production and characterization of reconstituted strains of Pleurotus spp. cultivated on different agricultural wastes. *Revista Mexicana De Ingeniería Química 19*, 1493-1504. https://doi.org/10.24275/rmiq/Bio1126

Villamarín-Gallegos, D., Oviedo-Pereira, D., Evangelista-Lozano, S., Sepúlveda-Jiménez, G., Molina-Torres, J., and Rodríguez-Monroy, M. (2020). Trichoderma Asperellum, an inoculant for production of steviol glycoside in Stevia Rebaudiana bertoni plants micropropagated in a temporary immersion bioreactor. *Revista Mexicana De Ingeniería Química 19*, 1153-1161. https://doi.org/10.24275/rmiq/Bio947

Vincent, J.M. (1970). A Manual for the Practical Study of the Root-Nodule Bacteria. Blackwell Scientific Publications, Oxford, 164 pp.

Weaver, R.W., Wei, G.R., and Berryhill, D.L. (1990). Stability of plasmids in Rhizobium phaseoli during culture. *Soil Biology and Biochemistry 22*, 465-469. https://doi.org/10.1016/0038-0717(90)90179-4

Wu, Z., Du, G., and Chen, J. (2003). Effects of dissolved oxygen concentration and DO-stat feeding strategy on CoQ10 production with *Rhizobium radiobacter*. *World Journal of Microbiology and Biotechnology 19*, 925-928 https://doi.org/10.1023/B:WIBI.0000007322.19802.57

Yurgel, S.N., and Kahn, M.L. (2004). Dicarboxylate transport by rhizobia. *FEMS Microbiology Reviews 28*, 489-501. https://doi.org/10.1016/j.femsre.2004.04.002

Zahran, H.H. (2017). Plasmids impact on rhizobia-legumes symbiosis in diverse environments. *Symbiosis 73*, 75-91. https://doi.org/10.1007/s13199-017-0476-5

Zapata-Vélez, A.M., and Trujillo-Roldán, M.A. (2010). The lack of a nitrogen source and/or the C/N ratio affects the molecular weight of alginate and its productivity in submerged cultures of *Azotobacter vinelandii*. *Annals of Microbiology 60*, 661-668. https://doi.org/10.1007/s13213-010-0111-7