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Positive effect of reduced aeration rate on growth and stereospecificity of DL-malic acid consumption by *Azospirillum brasilense*: Improving the shelf life of a liquid inoculant formulation

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ABSTRACT

Azospirillum brasilense has significance as a growth promoter in plants of commercial interest. Two industrial native strains (*Start* and *Calf*), used as a part of an inoculant formulation in Mexico during the last 15 years, were incubated in laboratory-scale pneumatic bioreactors at different aeration rates. In both strains, the positive effect of decreased aeration was observed. At the lowest (0.1 vvm, air volume/liquid volume × minute), the highest biomass were obtained for *Calf* (7.8×10^{10} CFU/ml), and *Start* (2.9×10^{9} CFU/ml). These were higher in one magnitude order compared to cultures carried out at 0.5 vvm, and two compared to those at 1.0 vvm. At lower aeration, both stereoisomeric forms of malic acid were consumed, but at higher aeration, just L-malate was consumed. A reduction in aeration allows an increase of the shelf life and the microorganism saved higher concentrations of polyhydroxybutyrate. The selected fermentation conditions are closely related to those prevalent in large-scale bioreactors and offer the possibility of achieving high biomass titles with high shelf life at a reduced costs, due to the complete use of a carbon source at low aeration of a low cost raw material as DL-malic acid mixture in comparison with the L-malic acid stereoisomer.

1. Introduction

Among the plant growth promoting rhizobacteria (PGPR), *Azospirillum brasilense* is one of the most used and studied for its ability to increase crops yield, attributed mainly to an enhancement in root development by the uptake of water and minerals (Fibach-Paldi et al., 2012). This is because *A. brasilense* has the ability to produce key signals and components of plant growth promotion in association with several plants, including wheat, sorghum, barley, rice and maize (Fibach-Paldi et al., 2012; Okon and Itzigsohn, 1995).

A large number of literature has been published on the mechanisms of action and benefits of the consortium plant-microorganism (Bashan and de-Bashan, 2005; Fibach-Paldi et al., 2012). Furthermore, the effect of submerged culture nutrients on *A. brasilense* growth has been studied with the aim of increasing bacterial productivity (Albrecht and Okon, 1980; Bashan et al., 2011; Fallik and Okon, 1996; Joe et al., 2010; Kefalogianni and Aggelis, 2002). Malic acid is one of the most common nutrients for *A. brasilense*. An important field of study with significant industrial implications can be the understanding of how operating conditions can modify biomass yields on cultures, based on the consumption of malic acid as a carbon source. This is because there is a misunderstanding in the literature about the consumption of both stereoisomers D- and L-malic acid. Even more, the highest biomass yields achieved using the mixture of these stereoisomers has been reported in comparison with other carbon sources, without specifying whether both were consumed by the microorganism (Kamnev et al., 2012; Konnova et al., 2003; Rodelas et al., 1994; Westby et al., 1983). However, Fallik and Okon (1996) reported that *A. brasilense* is not able to use the D-stereoisomer. Certainly, as a consequence of this, L-malic acid is commonly used as a carbon source (Cappuyns et al., 2007; Ona et al., 2005), supported by the fact that roots selectively secrete the L-stereoisomer, which is used by plants as a signal to recruit beneficial rhizobacteria (Rudrappa et al., 2008; Westby et al., 1983). Nevertheless, D-malic consumption was previously reported in wild-type strain Sp7 and also in mutant defective to produce polyhydroxybutyrate (PHB) (Kadouri et al., 2003).

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In addition, the effect of oxygen in the metabolism and growth of *A. brasilense* has been studied. For example, low levels of oxygen can be determinant factor in regulating bacterial growth rate and production of nitrites (Didonet and Magalhaes, 1997), and in the production of indole-3-acetic acid, a well-known auxin (Ona et al., 2005). Furthermore, high oxygen levels are highly related to floc formation by *A. brasilense* (Joe et al., 2010), and to the inhibition of nitrogenase activity (Fadel-Picheth et al., 1999; Hartmann and Burris, 1987). On the other hand, in order to obtain large amounts of inoculants based on *A. brasilense*, some culture strategies have been proposed, such as fed-batch cultures (Fallik and Okon, 1996) or scale-up strategies using the volumetric oxygen transfer coefficient (K_La) (Trujillo-Roldán et al., 2013). The use of low K_La values to successful scale-up this bioprocess supports the idea of the sensitivity to oxygen by *A. brasilense* in submerged cultures (Didonet and Magalhaes, 1997; Ona et al., 2005). Here, we evaluate the effect of three different aeration conditions on *A. brasilense* kinetics of growth of two native Mexican strains, named *Start* and *Calf*, used as a part of an inoculant formulation in México during the last 15 years, with an application between 50×10^3 and 200×10^3 hectares per year. Moreover, we evaluated the effect of the aeration on D- and L-malate consumption as carbon source, PHB production, following culture variables such as pH and dissolved oxygen tension, and one year shelf life of a liquid inoculant formulation based on *A. brasilense* from bubble column submerged cultures.

2. Materials and methods

2.1. Microorganisms and culture conditions

Two native Mexican strains of *A. brasilense*, named *Start* and *Calf*, from the company Biofabrica Siglo XXI S.A. de C.V. culture collection were used (Trujillo-Roldán et al., 2013). An additional cell bank is in the Bioprocess Unit at the Biomedical Research Institute at UNAM. These strains were firstly isolated by Professor Jesus Caballero-Mellado Ph.D. (CCG-UNAM, Mexico). The difference between both strains is the ability to adapt to different types of soils within Mexico (Trujillo-Roldán et al., 2013). Pre-cultures were performed in 500-ml Erlenmeyer flasks (Pyrex, México) at 30 °C, 150 rpm, containing 100 ml of modified NFb, which contains (g/l): 5.0 DL-malic acid, 0.5 K_2HPO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 0.1 NaCl, 0.02 $CaCl_2$, 0.012 $FeSO_4$, 0.0025 Na_2MoO_4, 0.01 $MnSO_4$, 4.8 KOH, 0.3 NH_4Cl, 0.01 H_3BO_4 and 0.3 of yeast extract.

2.2. Analytical determinations

Bacterial growth was determined by three methods: optical density at 600 nm (Beckman DU730 spectrophotometer, IN USA), dry weight, and colony forming units (CFU/ml). CFU/ml were determined by 1:10 serial dilutions with NaCl 0.85%, cultured in solid NFb medium with Congo red at 30 ± 2 °C for 48 h. Solid NFb-Congo red medium contains (g/l): 5.0 DL-malic acid; 0.5 K_2HPO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 0.1 NaCl, 0.015 $FeCl_3$, 0.3 of yeast extract, and 16.0 of bacteriological agar. Final CFU/ml was determined by multiplying the number of colonies in the Petri dish by the dilution factor. CFU/ml 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 Biofabrica Siglo XXI S.A. de C.V., and trials were made at 4 and 12 months by triplicate.

Carbon source consumption was performed using an enzymatic kit for D-malate (Catalog 11215558035, R-Biopharm, Roche, Darmstadt, Germany) and L-malate (Catalog 10139068035, R-Biopharm, Roche, Darmstadt, Germany). In summary, D-malate is oxidized to oxaloacetate by nicotinamide adenine dinucleotide (NAD) in the presence of D-malate dehydrogenase. Oxaloacetate is converted by the same enzyme to pyruvate and CO_2. Wherein the amount of NADH formed was stoichiometrically equal to the amount of D-malate. On the other hand, L-malate is oxidized to oxaloacetate by NAD in the presence of L-malate dehydrogenase. In order to remove all oxaloacetate, a second reaction takes place catalyzed by the glutamate-oxaloacetate transaminase, converting all oxaloacetate to L-aspartate in the presence of L-glutamate. Finally, the amount of NADH formed was stoichiometrically equal to L-malate. In both cases, NADH was measured at 334, 340, and 365 nm (Sheng et al., 2010).

Polyhydroxybutyrate (PHB) was quantified by using the method reported by Law and Slepecky (1961), but with some modifications. Briefly, vacuum dried cells (from 1.0 ml of culture medium) were incubated for 1 h at 30 °C with 1.0 ml of sodium hypochlorite (6%, v/v). After centrifugation (11,000 × *g*, 15 min), the hypochlorite was removed, and the centrifuged material was washed with 1.0 ml of distilled water. After washing, 1.0 ml of cold acetone was added, centrifuged again (11,000 × *g*, 15 min) and finally, suspended in 300 μl of ethanol. For PHB hydrolysis to crotonic acid, ethanol was evaporated by heating the sample at 70 °C. Five milliliters of concentrated H_2SO_4 were added and the covered tubes were keep in an oven for 4 h at 70 °C. Cooled samples were measured at 235 nm using H_2SO_4 as blank. The standard linear relation was PHB (mg/l) = 0.1606 × Abs (235 nm), quantified by using crotonic acid (Sigma-Aldrich, St. Louis, MO, USA). It has been reported that the method used in this work showed a strong correlation when compared with a gas chromatography based quantification method (Hohenschuh et al., 2012).

2.3. Culture conditions in pneumatic bioreactors, data acquisition and control

A. brasilense Start and *Calf* strains were grown in individual 500-ml Erlenmeyer shake flasks (Pyrex, México) containing 100 ml of modified NFb culture media at 30 °C, and 220 rpm (C25I, New Brunswick-Eppendorf Co., USA). Cultures were carried out in homemade bubble columns with a nominal volume of 6 liters, and working volume of 5 liters (diameter height ratio of 5:1). Aeration rates of 0.1, 0.5, and 1.0 volume of air per medium volume (vvm) were evaluated for both *A. brasilense* strains using also the modified NFb culture media. This bubble columns were designed to have enough ports for pH and dissolved oxygen tension (DOT) electrodes, as well as inoculation and venting. Air was filtered through a 0.45 μm filter (Millex, Merck-Millipore, USA) before entering the column and bubbled through a sintered glass diffuser. The only controlled parameter was the temperature (30 °C). DOT, temperature and pH were displayed online and stored in a hard drive for further analysis using a homemade data acquisition control system based on LabView™ (Trujillo-Roldán et al., 2001), and in ADI 1010/1030 Applikon™ data acquisition control system. Off-line optical density 600 nm (Beckman DU730 spectrophotometer, IN USA) was follow every 4 h during cultures.

2.4. Primers design, RNA extraction, cDNA synthesis, quantitative RT-PCR and data analysis

Reference genes (*gyrA* and *glyA*) were selected from genes previously used in quantitative RT-PCR assays for *A. brasilense* (McMillan and Pereg, 2014) (Table 1). NCBI's primers blast was used to design the others primers in Table 1 (Ye et al., 2012). Primer efficiencies were determined by construction of a standard curve using 4-fold serial dilutions of pooled cDNA template (Table 1).

Total RNA was collected at 2 and 20 h of culture near the beginning and ending of the exponential growth phase. The RNA was isolated using the ZR Fungal/Bacterial RNA MiniPrep (Zymo