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Increases in alginate production and transcription levels of alginate lyase (*alyA1*) by control of the oxygen transfer rate in *Azotobacter vinelandii* cultures under diazotrophic conditions ☆

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ABSTRACT

Background: Alginates are polysaccharides used in a wide range of industrial applications, with their functional properties depending on their molecular weight. In this study, alginate production and the expression of genes involved in polymerization and depolymerization in batch cultures of *Azotobacter vinelandii* were evaluated under controlled and noncontrolled oxygen transfer rate (OTR) conditions.

Results: Using an oxygen transfer rate (OTR) control system, a constant OTR (20.3 ± 1.3 mmol $L^{-1}h^{-1}$) was maintained during cell growth and stationary phases. In cultures subjected to a controlled OTR, alginate concentrations were higher (5.5 ± 0.2 g L^{-1}) than in cultures under noncontrolled OTR. The molecular weight of alginate decreased from 475 to 325 kDa at the beginning of the growth phase and remained constant until the end of the cultivation period. The expression level of *alyA1*, which encodes an alginate lyase, was more affected by OTR control than those of other genes involved in alginate biosynthesis. The decrease in alginate molecular weight can be explained by a higher relative expression level of *alyA1* under the controlled OTR condition.

Conclusions: This report describes the first time that alginate production and alginate lyase (*alyA1*) expression levels have been evaluated in *A. vinelandii* cultures subjected to a controlled OTR. The results show that automatic control of OTR may be a suitable strategy for improving alginate production while maintaining a constant molecular weight.

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1. Introduction

Alginates are polysaccharides with variable proportions of (1–4)-β-D-mannuronic acid (M) and α-L-guluronic acid (G), and they are used as food additives and encapsulation agents in biotechnology [1]. Alginates can be produced by *Azotobacter vinelandii* [2], and several *Pseudomonas* sp. [3]. *A. vinelandii* is a nitrogen-fixing bacterium with a high respiration rate that can synthetize the polyester poly-3-hydroxybutyrate (PHB) [2]. *A. vinelandii* is able to produce alginates with different compositions and characteristics, mainly in terms of their G/M distribution, molecular weights and degree of acetylation [4]. Alginate biosynthesis in *A. vinelandii* cells is an energy-demanding process characterized by different stages that occur in the cytoplasm and periplasm. The alginate biosynthesis steps of the precursor (GDP-mannuronic acid) have been described [4] while the molecular mechanisms of polymerization and modification are less understood [5]. In the periplasm, starting from the precursor, the nascent polymer is polymerized and modified. The polymerization of the precursor is carried out by the enzymes glycosyltransferase/polymerase (Alg8, encoded by *alg8*) and copolymerase (Alg44, encoded by *alg44*), located in the inner membrane. Modifications are carried out by different enzymes which depolymerize (AlgL, encoded by *algL*; AlyA1–3, encoded by *alyA1*, *alyA2* and *alyA3*), acetylate (AlgV, encoded by *algV*) and epimerize (AlgE1–7) [5]. In particular, AlgE7 (encoded by *algE7*) is a bifunctional enzyme with lyase and epimerase activity [6].

Some studies have evaluated the expression of particular genes involved in alginate biosynthesis [7,8,9,10]. In batch cultures of *A.*

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vinelandii at a constant dissolved oxygen tension (DOT) of 1%, higher expression levels of *alg8* and *alg44* were obtained than in cultures with 5% DOT [9]. On the other hand, in cultures performed in continuous mode, the alginate mean molecular weight (MMW) and *alg8* gene expression levels were found to be higher at a lower oxygen transfer rate (OTR) [7]. However, further study is required, especially to understand the polymerization and depolymerization in alginate synthesis.

In batch cultures of *A. vinelandii* it has been established that the alginate molecular weight increases during the cell growth period and decreases in the stationary phase [9,11,12]. Different strategies for alginate production by *A. vinelandii* have been evaluated [13,14,15], demonstrating that it is possible to produce alginates of different molecular weights by varying the OTR of the cultures. In batch cultures of *A. vinelandii* the OTR reaches a maximum level then remains constant during the cell growth phase, later decreasing when the cells are in stationary phase [14,16]. Therefore, in the stationary phase a decrease in the alginate molecular weight can be related to variations in the OTR or the oxygen uptake rate (OUR) [12]. To overcome the limitation of producing alginate with different molecular weights during cultivation, from a technological perspective, it is possible to produce alginate at a constant OTR during the cultivation of *A. vinelandii*. Recently, we reported the design of a control system for maintaining a constant OTR in batch cultures of *A. vinelandii* [17]. Previously, Beronio and Tsao [18] developed oxygen transfer rate control during oxygen-limited culture to enhance the production of 2,3-butanediol by *K. oxytoca.* The aim of this study was to evaluate alginate production in batch cultures carried out under controlled OTR conditions using an automatic control system based on variation of the oxygen gas input to the bioreactor. Alginate production (concentration and molecular weight) and the expression of genes involved in alginate polymerization (*alg8*, *alg44)* and depolymerization (*algE7*, *alg*L and *alyA1*) were evaluated under conditions of controlled and non-controlled OTR.

2. Materials and methods

2.1. Microorganism and culture medium

Azotobacter vinelandii ATCC 9046 (wild-type strain) was used in this study. The bacterium was cultivated under nitrogen fixation conditions. The strain was cryopreserved at −80°C in a 30% glycerol solution. The culture medium used contained 20 g L^{-1} sucrose, 0.66 g L^{-1} K_2HPO_4, 0.16 g L^{-1} KH_2PO_4, 0.05 g L^{-1} $CaSO_4$, 0.2 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.0029 g L^{-1} $Na_2MoO_4{\cdot}2H_2O$, and 0.027 g L^{-1} $FeSO_4{\cdot}7H_2O$. Sucrose, K_2HPO_4, and KH_2PO_4 were dissolved in a bioreactor and autoclaved at 121°C for 20 min. $CaSO_4$ was sterilized separately (121°C for 20 min) and added to the medium. Solutions of NaCl, $MgSO_47H_2O$, $Na_2MoO_4{\cdot}2H_2O$, and $FeSO_4{\cdot}7H_2O$ were separated and sterilized in an autoclave at 121°C for 20 min. The initial pH was adjusted to 7.0 with 2 M NaOH [10].

2.2. Inoculum preparation

Cells from cryopreserved vials were transferred to Erlenmeyer flasks (500 mL) containing 100 mL of culture medium and cultured at 200 rpm and 30°C in an orbital incubator shaker (shaking radius 20 mm) (Daihan LabTech CO, Korea). Cells in the growth phase (10% v v^{-1}) were collected and transferred to the bioreactor [12].

2.3. Cultivation conditions

Batch culture was performed in a 3 L bioreactor (Applikon, Schiedam, Netherlands) using a working volume of 1.5 L. The bacterium was grown in the culture medium described previously. The bioreactor was equipped with 3 Rushton turbines (4.5 cm diameter). Culture was carried out at 30°C with an air flow of 1.5 L min^{-1} (1.0 vvm) at pH 7.0 ± 0.1, controlled automatically by the addition of 2 M NaOH. Culture was conducted under controlled OTR and noncontrolled conditions. The bioreactor was operated at 300, 500 and 700 rpm under noncontrolled OTR conditions and 500 rpm under controlled OTR conditions. The DOT was measured with a polarographic oxygen probe (Ingold, Mettler-Toledo) and was not controlled. Culture samples (10 mL) were collected from the bioreactor. The batch cultures were carried out in triplicate.

2.4. Description of an automatic system for OTR control

In *A. vinelandii* cultures under some conditions, alginate molecular weight decreases during the stationary phase of culture [11]. In addition, it has been reported that OTR affects alginate production [15], and that OTR decreases during the stationary phase [12,14]. Consequently, we evaluated alginate production in cultures subjected to controlled OTR during their culture period. To control the OTR throughout the culture, an OTR control system for batch cultures of *A. vinelandii* was previously designed [17]. Briefly, a PI control algorithm for the OTR was established based on the relationship between oxygen gas concentrations measured with a gas analyzer. The analyzer is a closed-loop control system modulating oxygen gas output and input. The manipulated variable is the oxygen input to the bioreactor and the controlled variable is the oxygen gas output of the fermenter, indicative of bacterial oxygen consumption. Under non-controlled OTR conditions, oxygen input corresponds to the composition of air (i.e., 20.9%), and this value varies depending on the development of the culture. After 12 h of cultivation the OTR control system was initiated and the oxygen input was varied to control output at 20.3% [17]. This control approach permits the OTR to be controlled indirectly through oxygen output. Thus, the batch cultures for producing alginate were evaluated under controlled OTR and noncontrolled conditions.

2.5. Respiration parameters

The OTR and carbon dioxide transfer rate (CTR) were calculated by measuring oxygen and carbon dioxide in the exit gas using a gas analyzer (Teledyne Instruments, model 7500), according to previously described methods [12]. The OTR and CTR were determined according to Equation 1 and Equation 2:

$$OTR = \frac{CF_G^{in}}{V_R V_M}\left(X_{O_2}^{in} - X_{O_2}^{out}\left(\frac{1 - X_{O_2}^{in} - X_{CO_2}^{in}}{1 - X_{O_2}^{out} - X_{CO_2}^{out}}\right)\right) \quad \text{[Equation 1]}$$

$$CTR = \frac{CF_G^{in}}{V_R V_M}\left(X_{CO_2}^{in}\left(\frac{1 - X_{O_2}^{in} - X_{CO_2}^{in}}{1 - X_{O_2}^{out} - X_{CO_2}^{out}}\right) - X_{CO_2}^{out}\right) \quad \text{[Equation 2]}$$

where C is the unit conversion factor (1000), F_G^{in} is the volumetric inlet air flow under standard conditions (L h^{-1}), V_R is the working volume (L), V_M is the mol volume of the ideal gas under standard conditions (L $mmol^{-1}$), $X_{O_2}^{in}$ is the molar fraction of oxygen in the inlet air (mol mol^{-1}), $X_{O_2}^{out}$ is the molar fraction of oxygen in the outlet gas stream of the bioreactor (mol mol^{-1}), $X_{CO_2}^{in}$ is the molar fraction of carbon dioxide in the inlet air (mol mol^{-1}), and $X_{CO_2}^{out}$ is the molar fraction of carbon dioxide in the outlet gas stream of the bioreactor (mol mol^{-1}).

The respiratory quotient (RQ) was determined according to Equation 3:

$$RQ = \frac{CTR}{OTR} \quad \text{[Equation 3]}$$

In oxygen-limited cultures, the OUR is equal to the OTR, and the specific oxygen uptake rate(q_{O_2}) can then be calculated according to Equation 4:

$$q_{O_2} = \frac{OTR}{X} \quad \text{[Equation 4]}$$

where X is the biomass (g L^{-1}) during cell growth.

2.6. Analytical measurements

The biomass concentration was quantified gravimetrically. The culture broth (10 mL) was mixed with 1 ml of 0.1 N Na_4EDTA and 1 mL of 1.0 N NaCl, followed by centrifugation at 7650 *g* for 10 min. The obtained pellet was dried at 80°C and weighed until a constant weight was reached. The sucrose concentration was determined through acid hydrolysis followed by determination of reducing sugars with dinitrosalicylic acid (DNS) reagent [19]. PHB was extracted from the cells and quantified as crotonic acid using HPLC-UV. The dry biomass (2–3 mg) was hydrolyzed with concentrated H_2SO_4 (1 mL) in a Thermo-Shaker (H5000-H-E, Multi-Therm) for 1 h at 90°C and 700 rpm. Crotonic acid (Sigma-Aldrich) was quantified using an HPLC-UV system with an Aminex HPX-87H column (300 × 7.8 mm, 9 μm, Bio-Rad). Elution was performed with 5 mM H_2SO_4 at 0.6 mL min^{-1} and 55°C [10]. Alginate concentrations were measured gravimetrically by precipitation with 3 volumes of 2-propanol [14,20], and the resultant precipitate was filtered (0.22-μm Millipore filters) and dried at 65°C to a constant weight. Five milligrams of dry alginate was dissolved in 1 ml of 0.1 M $NaNO_3$ followed by agitation for 1 h at 35°C, and then left to stand for at least 12 h at room temperature. The samples were filtered through 0.22 μm nylon membranes before being injected into the HPLC system. The alginate MMW was determined using gel permeation chromatography (GPC) using a serial set of Ultrahydrogel columns (500 Å, 10 μm, 7.8 × 300 mm, Waters) in an HPLC system with a differential refractometer detector (Jasco, Japan). Elution was performed with 0.1 M $NaNO_3$ at 35°C at a flow rate of 0.9 ml min^{-1} using pullulans from *Aureobasidium pullulans* (Shodex) as molecular weight standards between 6.2 and 736 kDa [21].

2.7. Gene expression

RNA was isolated from cells grown using a High Pure RNA Isolation Kit (Roche Life Sciences, Germany) and treated with RNase-free DNase (Roche) according to the manufacturer's recommendations. The RNA was quantified using a BioSpec-nano system. Reverse transcription-real time PCR was performed with the primers used for *alg8*, *alg44*, *algL*, *alyA1*, *algE7* and *gyrA* (Table 1). cDNA was synthesized using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. For RT-qPCR, 100 ng of total RNA was retrotranscribed. Real-time PCR was performed in an AriaMx Real-Time PCR system (Agilent Technologies, USA) using Brilliant II SYBR@ Green QPCR Master Mix (Agilent Technologies, USA). The samples were denatured 5 min at 95°C. Forty cycles of amplification for reverse transcription-real time PCR were used (95°C for 15 s, 59°C for 15 s and 72°C for 15 s). Melting curve analysis confirmed the amplification of a single product for each primer pair. The results were analyzed using the $2^{-\Delta\Delta C}_{T}$ method [22,23]. Relative gene expression values were normalized using *gyrA* as a housekeeping gene [10], and are reported as the fold changes with respect to the transcript levels of the cultures under noncontrolled OTR conditions.

2.8. Fermentation parameters calculations

The specific growth rate (μ), biomass produced/g sucrose ($Y_{x/s}$), alginate produced/g sucrose ($Y_{alg/s}$), alginate produced/g biomass ($Y_{alg/x}$) and the specific sucrose uptake rate (q_s) were calculated as follows:

$$\frac{dX}{dt} = \mu X\left(1 - \left(\frac{X}{X_m}\right)\right) \quad \text{[Equation 5]}$$

$$Y_{\frac{x}{s}} = \frac{X_{max} - X_o}{S_o - S_t} \quad \text{[Equation 6]}$$

$$Y_{\frac{alg}{s}} = \frac{P_{max} - P_o}{S_o - S_t} \quad \text{[Equation 7]}$$

$$Y_{\frac{alg}{x}} = \frac{P_{max} - P_o}{X_o - X_{max}} \quad \text{[Equation 8]}$$

$$q_s = \frac{\Delta S}{\Delta t\, X'} \quad \text{[Equation 9]}$$

where X is the biomass (without PHB) concentration during the culture (g L^{-1}), X_m is the maximum biomass concentration (without PHB) (g L^{-1}), X_{max} is the maximum biomass concentration (g L^{-1}), X_o is the initial biomass concentration (g L^{-1}), P_o is the initial alginate concentration (g L^{-1}), P_{max} is the maximum alginate concentration (g L^{-1}), S_o is the initial sucrose concentration (g L^{-1}), and S_t is the sucrose concentration (g L^{-1}) when the biomass or alginate concentration is maximum. The q_s was calculated considering the slope of sucrose vs time of cultivation curve ($\frac{\Delta S}{\Delta t}$) during the cell growth period, and X′ is the biomass (without PHB) produced (g L^{-1}) in this same period.

2.9. Statistical analysis

The results are expressed as the mean value for three independent cultures. All data were analyzed using one-way ANOVA with Tukey's post hoc test ($P \leq 0.05$) after assessment of the fundamental assumptions of ANOVA regarding the normality of the distribution (Shapiro-Wilk test, *p*-value >0.05). The statistical software Minitab® (version 18.1-© 2017 Minitab, Inc.) was used for all statistical analyses.

Table 1
Primers design for gene expression by qPCR.

Gene	Primers	Gene	Primers
alg8-F	5′-TGTTGAACCAGCTCTGGAAG-3′	*alg8*-R	5′-CCTACCCGCTGATCCTCTAC-3′
alg44-F	5′-CGACAACTTCACCGAAGGG-3′	*alg44*-R	5′-TGACGAAGTAGAGGTCGTAGAG-3′
algE7-F	5′-AGATAGGTGCGGTTGGTTTC-3′	*algE7*-R	5′-CTCCGACCTGATTCTCGATT-3′
algL-F	5′-GCCCAGTAGGAGTGGTTGTT-3′	*algL*-R	5′-CTGAAATTCTCCAGTTCGCA-3′
alyA1-F	5′-CGGTCGGTATTGCACATAGA-3′	*alyA1*-R	5′-CAAGATCCACCGTTTGAGTG-3′
gyrA-F	5′-ACCTGATCACCGAGGAAGAG-3′	*gyrA*-R	5′-AGGTGCTCGACGTAATCCTC-3′

3. Results and discussion

3.1. Alginate production at different agitation rates

Batch cultures of *A. vinelandii* were grown at different agitation rates (300, 500 and 700 rpm) under non-controlled OTR conditions. Cell growth and alginate production were evaluated under the different tested conditions. Table 2 shows μ, the maximum biomass concentration, $Y_{x/s}$, the maximum alginate concentration and the maximum alginate MMW obtained. The maximum biomass concentration (6.73 ± 0.31 g L^{-1}) was found in the cultures grown at 500 rpm, while the lowest biomass concentration (3.40 ± 0.04 g L^{-1}) was obtained at 700 rpm. μ was higher at 500 rpm (0.21 ± 0.0 1 h^{-1}) than under the other conditions evaluated. At 300 and 500 rpm $Y_{x/s}$ was similar (approximately 0.33 g g^{-1}), but at 700 rpm, $Y_{x/s}$ decreased approximately 2.4-fold compared to the cultures grown at 500 rpm. At 700 rpm the highest alginate concentration (4.17 ± 0.18 g L^{-1}) was obtained, which can explain the lowest $Y_{x/s}$ observed at 700 rpm. Previously, Moral and Sanin [24] used the same strain to produce alginate in cultures grown at 200, 400 and 700 rpm but under controlled DOT (5% DOT) conditions. In contrast to our findings, those authors found that in cultures grown at 400 rpm 4.51 g L^{-1} alginate was produced, while at 700 rpm lower alginate production (1.46 g L^{-1}) was achieved. Peña et al. [25] and Lozano et al. [15] reported similar results to those of our study. These authors demonstrated that in cultures grown under controlled DOT the alginate concentration could be increased by increasing the agitation rate. The agitation rate affects the OTR and has been demonstrated to be important for alginate production [12,14,15]. In this sense, it is possible that the differences observed with other authors [15,24,25] concerning alginate production may be related to different OTR levels reached in the cultures.

Cultures grown at 500 rpm yielded the highest alginate MMW during cell growth (594 ± 28 kDa). The properties of alginate depend on the G-M content, degree of acetylation and molecular weight [26]. Alginates are commercially important due to their viscosifying power, which increases at a higher molecular weight [16,27]. Therefore, for a particular application the evaluation of culture conditions in which a higher molecular weight can be produced is of interest. As higher μ and alginate MMW were obtained in the cultures at 500 rpm (Table 2) the cellular growth and alginate produced in cultures grown at 500 rpm under a controlled OTR will be analyzed below.

3.2. Characterization under controlled OTR and non-controlled conditions

To evaluate the biomass, PHB and alginate production under controlled OTR and non-controlled conditions the growth of *A. vinelandii* was conducted in batch cultures at 500 rpm. Fig. 1 shows the OTR, DOT, biomass, sucrose, PHB, and CTR evolution in batch cultures with and without OTR control. The OTR profile obtained under non-controlled OTR conditions was typical of *A. vinelandii* cultures [14,16], in which a constant OTR of 16.8 ± 0.8 mmol L^{-1}-h^{-1} was obtained from 12 to 40 h of cultivation (Fig. 1a). After 40 h of cultivation, the OTR decreased until reaching values near 2.6 mmol $L^{-1}h^{-1}$. Under controlled OTR conditions, the OTR remained constant from 20 h of cultivation onward at 20.3 ± 1.3 mmol $L^{-1}h^{-1}$, kept constant during fermentation for 72 h (Fig. 1a). This constant OTR for 52 h of cultivation demonstrates the possibility of controlling the OTR in cultures. In cultures of *A. vinelandii* and *P. putida* it has been demonstrated that oxygen demand is so high that the DOT level decreases and approaches zero [14,28,29]. The DOT increased after 50 or 55 h of cultivation depending on the conditions evaluated. It is well established that the dissolved oxygen concentration (C_L) in cultures depends on the OTR and oxygen consumption by microorganisms (OUR) ($dC_L/dt = OTR - OUR$) [30]. Because in cultures grown under a controlled OTR, the OTR remained constant (approximately 20 mmol $L^{-1}h^{-1}$), after 52 h of cultivation, it is likely that the increase in the DOT is due to a change in oxygen consumption by *A. vinelandii*. Under the conditions evaluated (controlled and noncontrolled OTR), different profiles of the biomass concentration were observed (Fig. 1b). A higher biomass concentration (6.7 ± 0.3 g L^{-1}) was obtained in cultures with non-controlled OTR. During the cell growth phase the DOT was nearly zero (Fig. 1a), indicating that the cultures were limited by oxygen, as has been reported for *A. vinelandii* [12]. This finding demonstrates the high level of respiration of *A. vinelandii* cells [2,31].

In cultures under OTR control biomass concentration decreased from 5.0 to 3.9 g L^{-1} during the stationary phase, not observed in the cultures without OTR control. In light of this evidence, the control of the OTR affected the evolution of biomass during the stationary phase. From the start of the cultures PHB content increased (Fig. 1c). A similar intracellular PHB evolution was observed by Millán et al. [32] using the same strain in cultures conducted with controlled DOT (1%) under oxygen limitation. Under OTR control a lower accumulation of PHB was obtained, reaching 60 ± 2% (w/w) at 64 h of cultivation (Fig. 1c). PHB accumulation was affected by OTR control, affecting the cell dry weight. Thus, for cultures grown under controlled OTR, the resulting lower cell dry weight (5.0 g L^{-1}, Fig. 1b) may be explained by a lower PHB accumulation (60% of cell dry weight) (Fig. 1c) compared with that obtained under non-controlled OTR.

Under both conditions, sucrose was totally consumed (Fig. 1b), indicating that the carbon source limited cell growth. Under non-controlled OTR a decrease in the OTR during the stationary phase is indicative of the depletion of sucrose. Fig. 1d exhibits the CTR evolution observed under both conditions. A similar profile for the CTR was obtained under both conditions, reaching 14.0 ± 1.0 mmol L^{-1}-h^{-1} in the culture without OTR control and 10.3 ± 0.4 mmol $L^{-1}h^{-1}$ under OTR control. Interestingly, in both conditions, the CTR decreased in the stationary phase (after 40 h of cultivation), and because the OTR in this phase was higher under OTR control (Fig. 1a), the RQ (= CTR/OTR) was lower under OTR control, as will be presented below.

Table 2
Cell growth and alginate production in batch cultures of *A. vinelandii* conducted at different agitation rates.

Agitation rate (rpm)	μ (h^{-1})	Maximum biomass (g L^{-1})	$Y_{x/s}$ (g g^{-1})	Maximum alginate (g L^{-1})	Maximum alginate MMW during the cell growth (kDa)
300	0.15 ± 0.01	6.10 ± 0.10	0.33 ± 0.02	3.09 ± 0.02	326 ± 22 (24 h)
500	0.21 ± 0.01**	6.73 ± 0.31	0.34 ± 0.02	2.87 ± 0.14	594 ± 28** (32 h)
700	0.14 ± 0.01	3.30 ± 0.18**	0.14 ± 0.03**	4.17 ± 0.19**	187 ± 5 (16 h)

The values are means ± standard errors for triplicate experiments.
** $P < 0.05$ was statistically significant between the three conditions assessed using ANOVA and Tukey's HDS test.

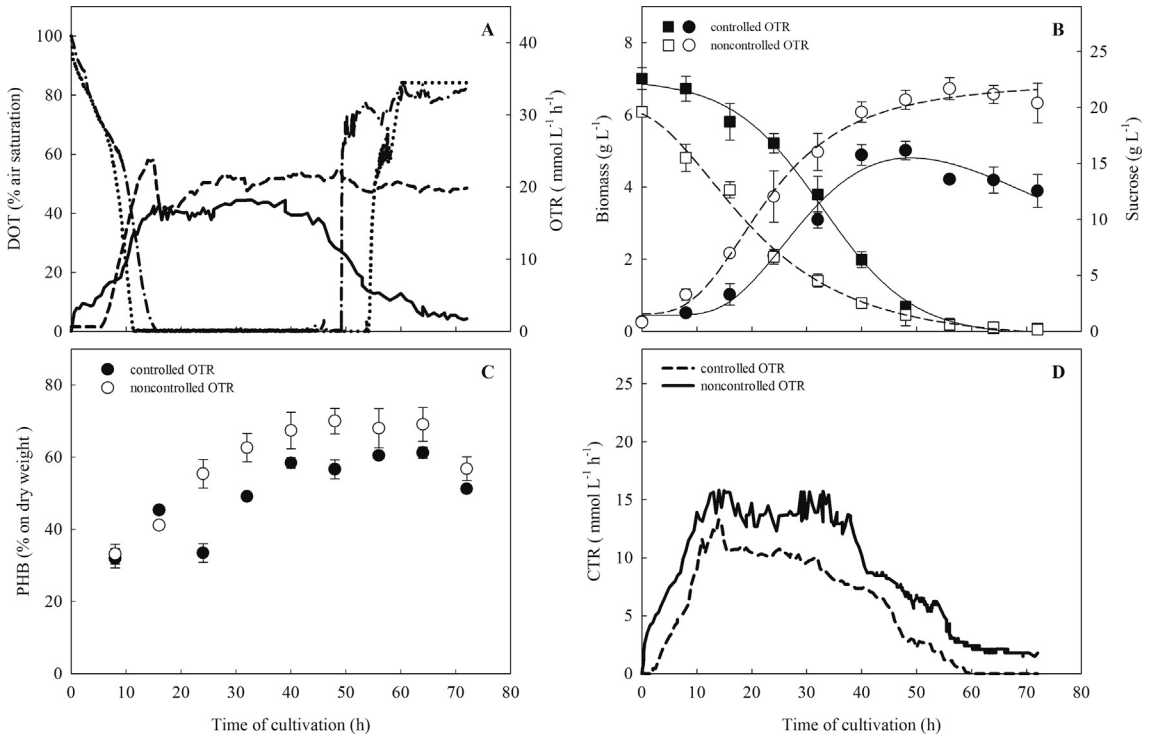

Fig. 1. Time-course profile of the OTR (a), DOT (a), biomass (b), sucrose (b), PHB (c) and CTR (d) in batch cultures of *A. vinelandii* conducted in a 3 L bioreactor at 500 rpm under controlled and non-controlled OTR. In panel A: OTR profile under non-controlled OTR (fx1), OTR profile under controlled OTR (fx2), DOT profile under non-controlled OTR (fx3), DOT profile under controlled OTR (fx4). In panel B: Biomass (circles), sucrose (square). DOT, OTR and CTR data are showed as mean value with differences of <10%. OTR profile was adapted from Farias et al. [17].

3.3. Evolution of the alginate concentration, q_{O_2} and RQ under controlled OTR and non-controlled conditions

Fig. 2 shows the alginate concentration, q_{O_2} and RQ recorded under the conditions evaluated. Under OTR contro, the alginate concentration was higher (5.5 ± 0.2 g L^{-1}) than with a noncontrolled OTR, 2.9 ± 0.1 g L^{-1} (Fig. 2a). To our knowledge, the alginate concentration that we obtained under OTR control is the highest reported in batch cultures of *A. vinelandii* in a bioreactor under nitrogen fixation [5]. During the cell growth phase (between 18 and 40 h, Fig. 1b) a significant difference in the alginate concentration was observed (Fig. 2a). Previous studies have reported that alginate production is affected by the OTR of the culture [14,33]. Díaz-Barrera et al. [14] demonstrated that a change in the OTR (approximately 25%) increased alginate production by only 0.2 g L^{-1}; instead, a variation of 67% in the OTR increased the alginate concentration approximately 3-fold. Similarly, Peña et al. [33] showed that an increase in the OTR from 2.6 to 6.0 mmol $L^{-1}h^{-1}$ (increase of 57%) improved the alginate concentration by 1.0 g L^{-1}. Given this evidence and considering that under the evaluated conditions (controlled and non-controlled OTR), a difference of less than 15% of the OTR was observed during cell growth, it is possible that the higher alginate production obtained under OTR control (1.9-fold) can be explained by other factors. There are some reports in the literature that have indicated that a different q_{O_2} or RQ could affect alginate production in *A. vinelandii* [33,34]. Fig. 2b shows q_{O_2} during the cell growth. The q_{O_2} in the cultures under OTR control varied from 23.7 to 4.7 mmol $g^{-1}h^{-1}$, while under non-controlled conditions it only varied from 8.3 to 3.0 mmol $g^{-1}h^{-1}$. The higher value of q_{O_2} obtained under OTR control could indicate that the carbon flux through the tricarboxylic acid (TCA) cycle was enhanced, as has been previously reported [35]. A more active TCA cycle could favor energy generation, fundamental for the synthesis of alginate precursors [36]. A lower concentration of acetyl-CoA available for PHB biosynthesis could explain the lower PHB accumulation under controlled OTR conditions (Fig. 1c). Based on these observations, under controlled OTR conditions a metabolic change determined by the carbon entering the TCA cycle could explain the higher alginate production observed (Fig. 2a). Jiménez et al. [37] compared two strains of *A. vinelandii* (wild type and AT6, with impaired PHB production) and observed that a higher q_{O_2} in cultures of the AT6 strain (62% compared to the wild-type strain) could explain a higher alginate production. Further studies must be developed to evaluate the carbon flux in *A. vinelandii* under OTR control.

In cultures grown under OTR control a lower RQ was obtained compared with that observed without OTR control. Under OTR control the RQ was constant between 12 and 31 h of cultivation, reaching a value of 0.49 ± 0.05 after which the RQ strongly decreased (Fig. 2c). In contrast, under non-controlled OTR conditions a higher RQ (0.79 ± 0.05) was obtained between 12 and 45 h of cultivation. Notably, under OTR control the highest increase in alginate production (2.4–4.3 g L^{-1}, Fig. 2a) was obtained during the cell growth period, indicating that the lowest RQ increased alginate production under OTR control. Under this condition, the CTR decreased during the stationary phase and, as the OTR was constant, the RQ was lower under OTR control. In agreement with this evidence, a lower RQ value could be associated with the highest conversion of sucrose to alginate, previously described in *A. vinelandii* cultures [33].

Table 3 shows the fermentation parameters obtained under the evaluated conditions. Compared with noncontrolled OTR conditions, OTR control strongly increased q_s and alginate yields. In contrast, under controlled OTR conditions, μ and $Y_{x/s}$ were lower, indicating that without OTR control carbon is used mostly for

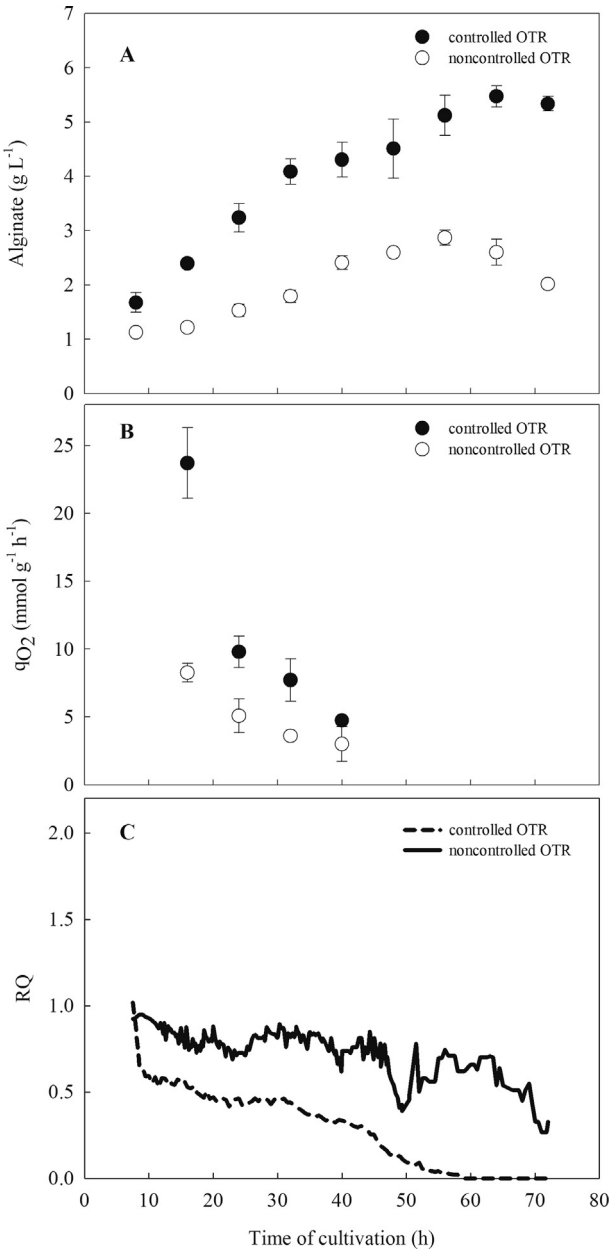

Fig. 2. Time-course profile of alginate, q_{O_2} and RQ in batch cultures of *A. vinelandii* conducted in a 3 L bioreactor at 500 rpm under controlled and non-controlled OTR. Alginate profile was adapted from Farias et al. [17].

cellular growth. Under OTR control, q_{O_2} was higher (Fig. 2b) and the cultures presented a higher q_s (0.37 ± 0.02 g $g^{-1}h^{-1}$), in line with higher alginate production (Fig. 2a). Accordingly, Peña et al. [25] and Lozano et al. [15] demonstrated that an increase in q_s improved alginate synthesis.

3.4. Evolution of alginate molecular weight

The evolution of the alginate MMW during culture was affected by the controlled OTR (Fig. 3). Up to 24 h of cultivation the alginate MMW was similar under the two experimental conditions, reaching 475 ± 16 kDa (controlled OTR) and 513 ± 76 kDa (non-controlled OTR). After 24 h of cultivation under controlled OTR conditions the alginate MMW decreased to 325 ± 19 kDa then remained constant, while the molecular weight in cultures conducted under non-controlled OTR increased to 607 ± 43 kDa at 40 h of cultivation.

It is interesting to note that under OTR control it is possible to keep the alginate MMW constant over 40 h of cultivation (Fig. 3). From a production perspective it is desirable to improve alginate production while maintaining a constant molecular weight during cultivation. Thus, the use of an automatic control system to maintain a constant OTR could be a suitable strategy for producing more alginate with constant quality (in terms of molecular weight) for a longer time of cultivation. According to the application, bacterial alginates with different molecular weights can be produced. For example, alginates with a low MMW (approximately 200 kDa) can be adequate as a prebiotic source, while as a viscosifier and gelling agent, a polymer with a high molecular weight is recommended. Based on the evidence, to produce alginates with a higher molecular weight and maintain a constant value during the stationary phase, *A. vinelandii* can be cultured at a lower OTR level under controlled OTR conditions.

To our knowledge, this is the first time that the concentration of alginate and its molecular weight have been analyzed under OTR control in *A. vinelandii* cultures. Previously, Trujillo-Roldán et al. [11] reported the use of an *A. vinelandii* mutant lacking alginate lyase (SML2) to produce alginate. Those authors found that in batch cultures under DOT control (3%) it was possible to obtain a constant alginate molecular weight during the stationary phase. Nevertheless, the alginate concentration obtained by Trujillo-Roldán et al. [11] (3.3 g L^{-1}) was lower than that obtained here, although yeast extract was used as a nitrogen source in their cultures. Previous reports have indicated that the alginate molecular weight can be increased by decreasing the OTR, principally to OTR below 10 mmol $L^{-1}h^{-1}$, whereas in cultures grown at higher OTR, generally, the alginate molecular weight seems to be independent of the OTR [38]. In light of this evidence the different alginate molecular weights obtained between 30 and 40 h of cultivation (Fig. 3) could be more related to the q_{O_2} than the OTR since the OTR was approximately 17 mmol $L^{-1}h^{-1}$ (non-controlled OTR) and 20 mmol $L^{-1}h^{-1}$ under controlled OTR.

Under non-controlled OTR conditions, the alginate MMW decreased dramatically from 607 to 385 kDa between 40 and 48 h of cultivation. A decrease in the alginate MMW during culture can be explained by the action of alginate lyases [9,11] encoded by six alginate lyase genes in *A. vinelandii* [39]. As in our study, a decrease in alginate molecular weight was observed at different times of cultivation (Fig. 3) depending on OTR conditions (controlled or noncontrolled), and it is possible that changes in lyase genes could explain this behavior. Therefore, the transcription of

Table 3
Parameters of fermentation obtained in batch cultures of *A. vinelandii* conducted under controlled and non-controlled OTR.

Culture conditions	μ (h^{-1})	$Y_{x/s}$ (g g^{-1})	q_s (g $g^{-1}h^{-1}$)	$Y_{alg/s}$ (g g^{-1})	$Y_{alg/x}$ (g g^{-1})
controlled OTR	0.16 ± 0.02	0.23 ± 0.02	0.37 ± 0.02**	0.21 ± 0.02**	0.99 ± 0.08**
Non-controlled OTR	0.21 ± 0.01**	0.34 ± 0.02**	0.27 ± 0.02	0.14 ± 0.01	0.41 ± 0.03

The values are means ± standard errors for triplicate experiments.
** $P > 0.05$ was statistically significant between the conditions assessed using ANOVA and Tukey's HDS test.

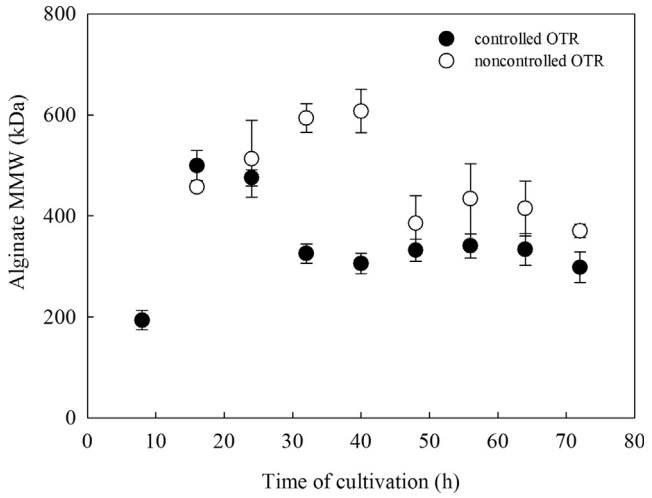

Fig. 3. Comparison of alginate molecular weight obtained in batch cultures of *A. vinelandii* conducted in a 3 L bioreactor at 500 rpm under controlled and non-controlled OTR.

genes involved in alginate polymerization and depolymerization under controlled OTR and noncontrolled conditions was evaluated.

3.5. Gene expression related to alginate biosynthesis

The relative expression of the genes involved in alginate biosynthesis under controlled and noncontrolled OTR conditions was studied. Fig. 4 shows the relative gene expression of the genes related to polymerization in three stages of cultivation: the cell growth phase (24 h), the pre-stationary phase of growth (40 h) and the stationary phase of growth (72 h). Regardless of the stage of cultivation transcription levels of *alg44* were lower under OTR control. After 30 h of cultivation a lower alginate MMW was produced under controlled OTR (Fig. 3). During this period a low transcription level of *alg44* (low polymerase activity could be expected) could be associated with the lower molecular weight observed. Similarly, Flores et al. [9] reported that a high alginate molecular weight obtained at low DOT (1%) was associated with the high expression of *alg44*.

In the cell growth phase (24 h), the transcription levels of *alg8* were higher (1.7-fold) under OTR control but lower in the stationary phase under the same conditions. It was previously found that in chemostat cultures, a decrease in the gene expression of *alg8* was related to an increase in the OTR [7]. Considering that in cultures under OTR control, the OTR was constant, changes in the transcription level of *alg8* could be more closely related to the phase of culture than to the OTR level. One possible approach for addressing this question is to evaluate how the specific growth rate could affect *alg8* gene expression, which might be studied in chemostat culture.

Fig. 5 shows the relative gene expression of *algL*, *alyA1* and *algE7* under controlled and noncontrolled OTR. The expression of these genes was evaluated in the same three stages of cultivation. In the cell growth phase (24 h), similar gene expression of *algL* was observed, whereas the transcription of *algE7* was lower under OTR control (Fig. 5a). AlgE7 is a bifunctional enzyme with lyase and epimerase activity, introducing G-blocks into alginate [6]. In the stationary phase (72 h) higher gene expression of *algE7* (1.6-fold) was obtained under OTR control (Fig. 5c), whereas during the cell growth phase, lower transcription levels of *algE7* were observed. Based on this evidence, a relationship between the changes in the alginate molecular weight and the transcription levels of *algE7* is not clear.

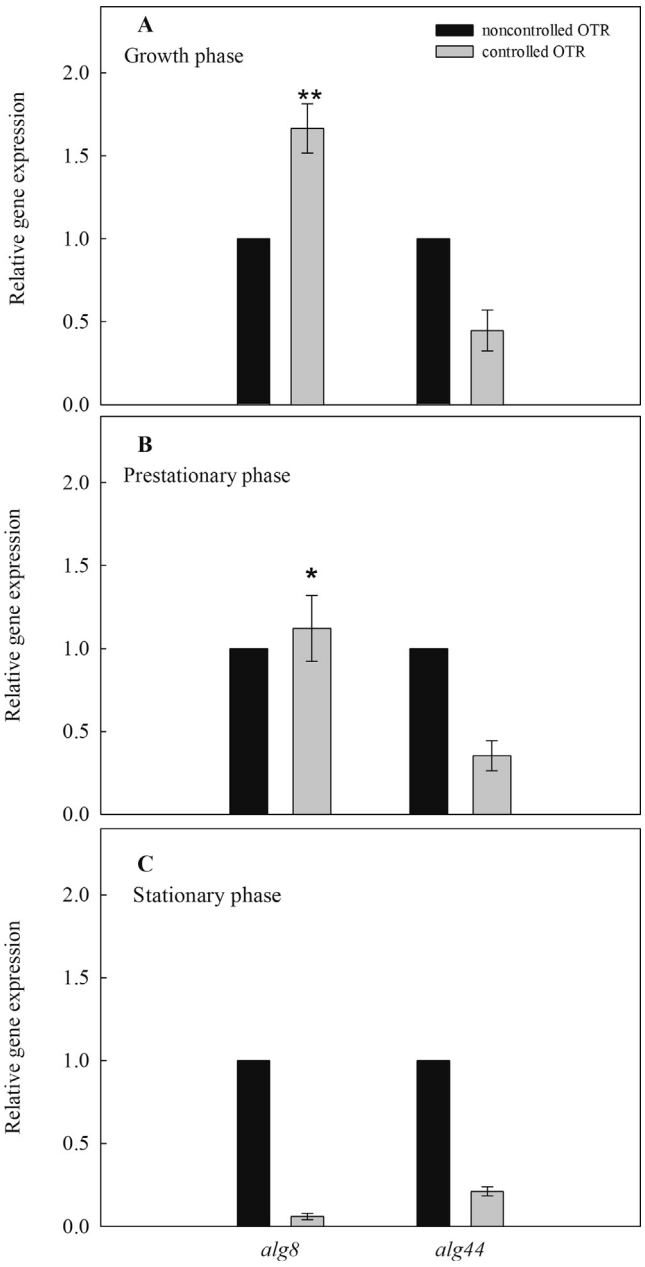

Fig. 4. Gene expression of genes involved in polymerization (*alg8* and *alg44*), during growth phase (24 h cultivation), prestationary phase (40 h cultivation) and stationary phase (72 h cultivation) *A. vinelandii* batch cultures under controlled and non-controlled OTR. The level of the *alg8* and *alg44* transcripts was normalized according to the level of the *gyrA* mRNA as described in **Section Materials and Methods**. The data are presented as fold changes respect of calibrator value (noncontrolled OTR condition). Results are presented as the means ± standard error. $^{*}P > 0.05$ was not statistically significant and $^{**}P < 0.05$ was statistically significant using ANOVA and Tukey's HDS test.

One possibility is that changes in the transcription levels of *algE7* could be related to changes in the guluronic composition in alginate. Further studies must be performed to examine alginate composition (G/M ratio) under the conditions evaluated.

Regardless of the state of cultivation a higher level of *alyA1* transcription (5.8- and 12-fold) was obtained under OTR control. The transcription levels of *alyA1* were more strongly affected by OTR control conditions. In contrast, Flores et al. [9] reported that in the stationary phase in batch cultures grown under a constant DOT, the *alyA1* gene was not expressed. Those authors developed cultures under controlled DOT conditions and used a culture

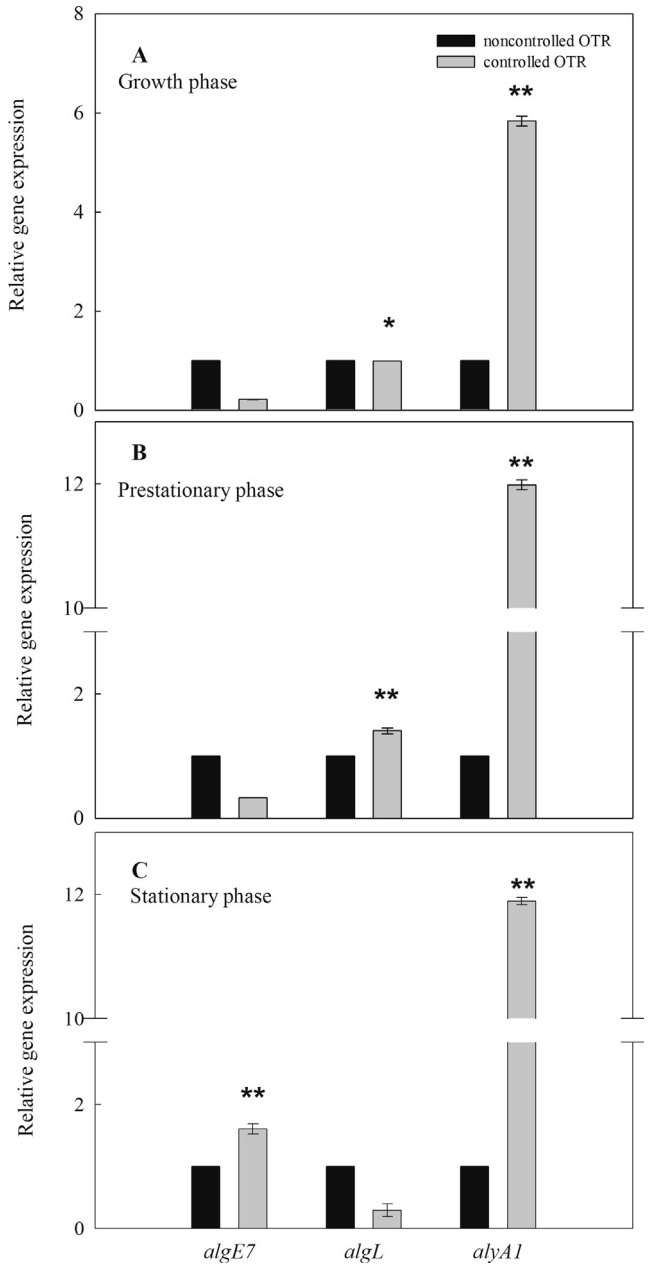

Fig. 5. Gene expression of genes involved in depolymerization and acetylation (*algE7, algL and alyA1*) during growth phase (24 h cultivation), prestationary phase (40 h cultivation) and stationary phase (72 h cultivation) *A. vinelandii* batch cultures under controlled and non-controlled OTR. The level of the *algE7, algL and alyA1* transcripts was normalized according to the level of the *gyrA* mRNA as described in **Section Materials and Methods**. The data are presented as fold changes respect of calibrator value (non-controlled OTR condition). Results are presented as the means ± standard error. $^{*}P > 0.05$ was not statistically significant and $^{**}P < 0.05$ was statistically significant using ANOVA and Tukey's HDS test.

medium that prevented nitrogen fixation (containing ammonium acetate), which could explain the different behaviors observed.

In bioreactors there is no comparative experimental evidence about alginate production (or the expression level of genes) under fixation and nonfixation of nitrogen in cultures grown under similar cultivation conditions. Tec-Campos et al. [40] developed a genome-scale metabolic model for *Azotobacter vinelandii* DJ to evaluate metabolic processes associated with nitrogen fixation and ammonium assimilation. These authors demonstrated that the average flux distributions of carbon and nitrogen decreased under diazotrophic conditions, which can be due to low available nitrogen due to the high energy cost of nitrogen fixation. Likewise, the proposed model also predicted an increase in the cell growth rate (28%) and alginate production rate (27%) when ammonium was used as the nitrogen source. Further studies must be performed to evaluate alginate production and molecular composition under fixation and nonfixation of nitrogen under controlled OTR conditions.

Alginate biosynthesis is a process that has a high energy demand, and it has been suggested that alginate biosynthesis imposes a high rate of oxygen consumption on the cell, correlated to the energy demand for their synthesis [36]. Furthermore, the respiratory system in *A. vinelandii* is very complex, with cytochromes and dehydrogenases activated differently under fixation of nitrogen, thus affecting the energetic efficiency [2] and possibly alginate synthesis. Based on these antecedents and considering that under our conditions of cultivation the cells have a higher energy cost for nitrogen fixation, it is possible that using ammonium as a nitrogen source could result in higher alginate production.

Considering that under controlled OTR conditions during the cell growth phase a decrease in the alginate MMW (from 475 to 325 kDa) was observed (Fig. 3), it is possible that higher transcription of the *alyA1* gene could explain this decrease in the molecular weight. The high gene expression of *alyA1* observed under controlled OTR (Fig. 5) could be determinant to maintain a constant molecular weight between 32 h and the end of cultivation.

As a higher q_{O_2} was obtained under OTR control (Fig. 2b), it is possible that higher transcription of the gene that encodes *alyA1* could be related to higher cellular respiration by *A. vinelandii*. To analyze this possibility, a derivative *alyA1*- could be built by insertion of an antibiotic resistance marker [39]. This strain mutant could be assayed in cultures conducted to high q_{O_2}, evaluating the alginate molecular weight and the G/M ratio of the polymer produced. Alginate biosynthesis has been studied using genetic and regulatory approaches, since most of the genes involved in this metabolic pathway are regulated by the presence of oxygen [41]. In particular, information about the signals that modulate alginate production and polymerization is limited. Recently, Ahumada-Manuel et al. [42] demonstrated that OTR (and hence the q_{O_2}) affects c-di-GMP accumulation, regulating the production and the molecular weight of alginate produced by *A. vinelandii*.

Díaz-Barrera et al. [43] previously suggested that higher oxygen availability affects lyase expression levels in chemostat cultures. In *A. vinelandii* it has been suggested that AlyA1 is an intracellular enzyme [39], but its function is unknown. Some studies on gene expression involved in the biosynthesis of microbial polysaccharides have been reported [44,45]. Zhu et al. [44] demonstrated that increasing the OTR by adding a water-immiscible organic phase (oxygen vector) can stimulate the transcription levels of genes involved in welan gum biosynthesis. The evidence obtained thus far permits the identification of genes more affected by OTR control and has a greater influence on the determination of the characteristics of alginate, particularly its molecular weight. Further studies must be carried out to evaluate other characteristics of alginate produced under OTR control, such as the degree of acetylation or the G/M distribution. These results contribute to an understanding of alginate biosynthesis, with the understanding that regulation of biosynthesis is critical to production of tailor-made alginates.

4. Conclusion

A control system based on oxygen gas composition allowed the maintenance of a constant OTR during the cell growth and stationary phases in batch cultures of *A. vinelandii*. Alginate production was improved under controlled OTR conditions. The transcription levels of *alyA1* were more strongly affected by OTR control than

those of other genes involved in alginate biosynthesis. OTR control during the culture of *A. vinelandii* can be a suitable strategy for enhancing alginate production while maintaining a constant molecular weight during cultivation.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] Soto MJ, Retamales J, Palza H, et al. Encapsulation of specific *Salmonella* Enteritidis phage f3αSE on alginate-spheres as a method for protection and dosification. Electron J Biotechnol 2018;31:57–60. https://doi.org/10.1016/j.ejbt.2017.11.006.

[2] Castillo T, García A, Padilla-Córdova C, et al. Respiration in *Azotobacter vinelandii* and its relationship with the synthesis of biopolymers. Electron J Biotechnol 2020;48:36–45. https://doi.org/10.1016/j.ejbt.2020.08.001.

[3] Vásquez-Ponce F, Higuera-Llantén S, Pavlov MS, et al. Alginate overproduction and biofilm formation by psychrotolerant *Pseudomonas mandelli* depend on temperature in Antarctic marine sediments. Electron J Biotechnol 2017;28:27–34. https://doi.org/10.1016/j.ejbt.2017.05.001.

[4] Hay ID, Rehman ZU, Moradali MF, et al. Microbial alginate production, modification and its applications. Microb Biotechnol 2013;6(6):637–50. https://doi.org/10.1111/1751-7915.12076. PMid:24034361.

[5] Urtuvia V, Maturana N, Acevedo F, et al. Bacterial alginate production: An overview of its biosynthesis and potential industrial production. World J Microbiol Biotechnol 2017;33(11). https://doi.org/10.1007/s11274-017-2363-x. PMid: 28988302.

[6] Svanem BIG, Strand WI, Ertesvåg H, et al. The catalytic activities of the bifunctional *Azotobacter vinelandii* mannuronan C-5-epimerase and alginate lyase AlgE7 probably originate from the same active site in the enzyme. J Biol Chem 2001;276(34):31542–50. https://doi.org/10.1074/jbc.M102562200. PMid: 11390391.

[7] Díaz-Barrera A, Aguirre A, Berrios J, et al. Continuous cultures for alginate production by *Azotobacter vinelandii* growing at different oxygen uptake rates. Process Biochem 2011;46(9):1879–83. https://doi.org/10.1016/j.procbio.2011.06.022.

[8] Díaz-Barrera A, Soto E, Altamirano C. Alginate production and *alg8* gene expression by *Azotobacter vinelandii* in continuous cultures. J Ind Microbiol Biotechnol 2012;39(4):613–21. https://doi.org/10.1007/s10295-011-1055-z. PMid: 22072437.

[9] Flores C, Moreno S, Espín G, et al. Expression of alginases and alginate polymerase genes in response to oxygen, and their relationship with the alginate molecular weight in *Azotobacter vinelandii*. Enzyme Microb Tech 2013;53(2):85–91. https://doi.org/10.1016/j.enzmictec.2013.04.010. PMid: 23769307.

[10] Díaz-Barrera A, Maturana N, Pacheco-Leyva I, et al. Different responses in the expression of alginases, alginate polymerase and acetylation genes during alginate production by *Azotobacter vinelandii* under oxygen-controlled conditions. J Ind Microbiol Biotechnol 2017;44(7):1041–51. https://doi.org/10.1007/s10295-017-1929-9. PMid: 28246966.

[11] Trujillo-Roldán MA, Moreno S, Espín G, et al. Roles of the oxygen and alginate-lyase in determining the molecular weight of alginate produced by *Azotobacter vinelandii*. Appl Microbiol Biotechnol 2004;63(6):742–7. https://doi.org/10.1007/s00253-003-1419-z. PMid: 12928755.

[12] Díaz-Barrera A, Gutierrez J, Martínez F, et al. Production of alginate by *Azotobacter vinelandii* grown at two bioreactor scales under oxygen-limited conditions. Bioprocess Biosyst Eng 2014;37(6):1133–40. https://doi.org/10.1007/s00449-013-1084-2. PMid: 24173209.

[13] Priego-Jimenéz R, Peña C, Ramírez OT, et al. Specific growth rate determines the molecular mass of the alginate produced by *Azotobacter vinelandii*. Biochem Eng J 2005;25(3):187–93. https://doi.org/10.1016/j.bej.2005.05.003.

[14] Díaz-Barrera A, Peña C, Galindo E. The oxygen transfer rate influences the molecular mass of the alginate produced by *Azotobacter vinelandii*. Appl Microbiol Biotechnol 2007;76(4):903–10. https://doi.org/10.1007/s00253-007-1060-3. PMid: 17598106.

[15] Lozano E, Galindo E, Peña C. Oxygen transfer rate during the production of alginate by *Azotobacter vinelandii* under oxygen-limited and non oxygen-limited conditions. Microb Cell Factories 2011;10(1):13. https://doi.org/10.1186/1475-2859-10-13. PMid: 21352581.

[16] García A, Castillo T, Ramos D, et al. Molecular weight and viscosifying power of alginates produced by mutant strains of *Azotobacter vinelandii* under microaerophilic conditions. Biotechnol Rep 2020;26:. https://doi.org/10.1016/j.btre.2020.e00436. PMid: 32140445e00436.

[17] Farias G, Fabregas E, Diaz-Barrera A, et al. Automatic control for the production of alginate by *Azotobacter vinelandii*. IEEE Access 2019;7:168606–12. https://doi.org/10.1109/ACCESS.2019.2954180.

[18] Beronio PB, Tsao GT. Optimization of 2, 3-butanediol production by *Klebsiella oxytoca* through oxygen transfer rate control. Biotechnol Bioeng 1993;42 (11):1263–9. https://doi.org/10.1002/bit.260421102.

[19] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31(3):426–8. https://doi.org/10.1021/ac60147a030.

[20] Saeed S, Mehmood T, Irfan M. Statistical optimization of cultural parameters for the optimized production of alginic acid using apple (*Malus domestica*) peels through solid-state fermentation. Biomass Conv Bioref 2020. https://doi.org/10.1007/s13399-020-01143-9.

[21] Díaz-Barrera A, Silva P, Berrios J, et al. Manipulating the molecular weight of alginate produced by *Azotobacter vinelandii* in continuous cultures. Bioresour Technol 2010;101(23):9405–8. https://doi.org/10.1016/j.biortech.2010.07.038. PMid: 20675122.

[22] Livak KL, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 2001;25(4):402–8. https://doi.org/10.1006/meth.2001.1262. PMid: 11846609.

[23] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. Nat Protoc 2008;3(6):1101–8. https://doi.org/10.1038/nprot.2008.73. PMid: 18546601.

[24] Moral ÇK, Sanin F. An investigation of agitation speed as a factor affecting the quantity and monomer distribution of alginate from *Azotobacter vinelandii* ATCC®9046. J Ind Microbiol Biotechnol 2012;39(3):513–9. https://doi.org/10.1007/s10295-011-1043-3. PMid: 22009058.

[25] Peña C, Trujillo-Roldán M, Galindo E. Influence of dissolved oxygen tension and agitation speed on alginate production and its molecular weight in cultures of *Azotobacter vinelandii*. Enzyme Microb Tech 2000;27(6):390–8. https://doi.org/10.1016/S0141-0229(00)00221-0.

[26] Remminghorst U, Rehm BHA. Bacterial alginates: from biosynthesis to applications. Biotechnol Lett 2006;28(21):1701–12. https://doi.org/10.1007/s10529-006-9156-x. PMid: 16912921.

[27] Gómez-Pazarín K, Flores C, Castillo T, et al. Molecular weight and viscosifying power of alginates produced in *Azotobacter vinelandii* cultures in shake flasks under low power input. J Chem Technol Biotechnol 2016;91(5):1485–92. https://doi.org/10.1002/jctb.4747.

[28] Gomez E, Santos VE, Alcon A, et al. Oxygen uptake and mass transfer rates on growth of *Pseudomonas putida* CECT5279: Influence on biodesulfurization (BDS) capability. Energy Fuels 2006;20(4):1565–71. https://doi.org/10.1021/ef050362y.

[29] Mohanakrishnan AS, Easwaran SN, Ravi DP, Mahadevan S. Understanding the biocalorimetric and respirometric behaviour of co-culture (*R. eutropha, P. putida* and *A. vinelandii*) in poly (3-hydroxybutyrate) batch production. Biochem Eng J 2020;155:107334. https://doi.org/10.1016/j.bej.2019.107334.

[30] Garcia-Ochoa F, Gomez E, Santos VE, et al. Oxygen uptake rate in microbial processes: An overview. Biochem Eng J 2010;49(3):289–307. https://doi.org/10.1016/j.bej.2010.01.011.

[31] Oelze J. Respiratory protection of nitrogenase in *Azotobacter* species: Is a widely held hypothesis unequivocally supported by experimental evidence?. FEMS Microbiol Rev 2000;24(4):321–33. https://doi.org/10.1111/j.1574-6976.2000.tb00545.x. PMid: 10978541.

[32] Millán M, Segura D, Galindo E, et al. Molecular mass of poly-3-hydroxybutyrate (P3HB) produced by *Azotobacter vinelandii* is determined by the ratio of synthesis and degradation under fixed dissolved oxygen tension. Process Biochem 2016;51(8):950–8. https://doi.org/10.1016/j.procbio.2016.04.013.

[33] Peña C, Galindo E, Büchs J. The viscosifyng power, degree of acetylation and molecular mass of the alginate produced by *Azotobacter vinelandii* in shake flasks are determined by the oxygen transfer rate. Process Biochem 2011;46 (1):290–7. https://doi.org/10.1016/j.procbio.2010.08.025.

[34] Sabra W, Zeng A, Sabry S, et al. Effect of phosphate and oxygen concentrations on alginate production and stoichiometry of metabolism of *Azotobacter vinelandii* under microaerobic conditions. Appl Microbiol Biotechnol 1999;52 (6):773–80. https://doi.org/10.1007/s002530051590.

[35] Castillo T, Heinzle E, Peifer S, et al. Oxygen supply strongly influences metabolic fluxes, the production of poly(3-hydroxybutyrate) and alginate, and the degree of acetylation of alginate in *Azotobacter vinelandii*. Process Biochem 2013;48(7):995–1003. https://doi.org/10.1016/j.procbio.2013.04.014.

[36] Mærk M, Jakobsen ØM, Sletta H, et al. Identification of regulatory genes and metabolic processes important for alginate biosynthesis in *Azotobacter vinelandii* by screening of a transposon insertion mutant library. Front Bioeng Biotechnol 2020;7. https://doi.org/10.3389/fbioe.2019.00475. PMid: 32010681.

[37] Jiménez L, Castillo T, Flores C, et al. Analysis of respiratory activity and carbon usage of a mutant of *Azotobacter vinelandii* impaired in poly-β-hydroxybutyrate synthesis. J Ind Microbiol Biotechnol 2016;43(8):1167–74. https://doi.org/10.1007/s10295-016-1774-2. PMid: 27154760.

[38] Flores C, Díaz-Barrera A, Martínez F, et al. Role of oxygen in the polymerization and de-polymerization of alginate produced by *Azotobacter vinelandii*. J Chem Technol Biotechnol 2015;90(3):356–65. https://doi.org/10.1002/jctb.4548.

[39] Gimmestad M, Ertesvag H, Heggeset TMB, et al. Characterization of three new *Azotobacter vinelandii* alginate lyases, one of which is involved in cyst germination. J Bacteriol 2009;191(15):4845–53. https://doi.org/10.1128/JB.00455-09. PMid: 19482920.

[40] Tec-Campos D, Zuñiga C, Passi A, et al. Modeling of nitrogen fixation and polymer production in the heterotrophic diazotroph *Azotobacter vinelandii* DJ. Metab Eng Commun 2020;11. https://doi.org/10.1016/j.mec.2020.e00132. PMid: 32551229.

[41] Setubal JC, dos Santos P, Goldman BS, et al. Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. J Bacteriol 2009;191(14):4534–45. https://doi.org/10.1128/JB.00504-09. PMid: 19429624.
[42] Ahumada-Manuel CL, Martínez-Ortiz IC, Hsueh BY, et al. Increased c-di-GMP levels lead to the production of alginates of high molecular mass in *Azotobacter vinelandii*. J Bacteriol 2020;202:. https://doi.org/10.1128/JB.00134-20. PMid: 32989088e00134.
[43] Díaz-Barrera A, Martínez F, Guevara Pezoa F, et al. Evaluation of gene expression and alginate production in response to oxygen transfer in continuous culture of *Azotobacter vinelandii*. PLoS ONE 2014;9(8):. https://doi.org/10.1371/journal.pone.0105993. PMid: 25162704e105993.
[44] Zhu P, Dong S, Li S, et al. Improvement of welan gum biosynthesis and transcriptional analysis of the genes responding to enhanced oxygen transfer by oxygen vectors in *Sphingomonas* sp. Biochem Eng J 2014;90:264–71. https://doi.org/10.1016/j.bej.2014.06.011.
[45] Schmid J, Sieber V, Rehm B. Bacterial exopolysaccharides: Biosynthesis pathways and engineering strategies. Front Microbiol 2015;6:496. https://doi.org/10.3389/fmicb.2015.00496.