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Alginate production and *alg8* gene expression by *Azotobacter vinelandii* in continuous cultures

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Abstract Alginates are polysaccharides that are used as thickening agents, stabilizers, and emulsifiers in various industries. These biopolymers are produced by fermentation with a limited understanding of the processes occurring at the cellular level. The objective of this study was to evaluate the effects of agitation rate and inlet sucrose concentrations (ISC) on alginate production and the expression of the genes encoding for alginate-lyases (*algL*) and the catalytic subunit of the alginate polymerase complex (*alg8*) in chemostat cultures of *Azotobacter vinelandii* ATCC 9046. Increased alginate production (2.4 g l^{-1}) and a higher specific alginate production rate (0.1 g g^{-1} h^{-1}) were obtained at an ISC of 15 g l^{-1}. Carbon recovery of about 100% was obtained at an ISC of 10 g l^{-1}, whereas it was close to 50% at higher ISCs, suggesting that cells growing at lower sucrose feed rates utilize the carbon source more efficiently. In each of the steady states evaluated, an increase in *algL* gene expression was not related to a decrease in alginate molecular weight, whereas an increase in the molecular weight of alginate was linked to higher *alg8* gene expression, demonstrating a relationship between the *alg8* gene and alginate polymerization in *A. vinelandii* for the first time. The results obtained provide a possible explanation for changes observed in the molecular weight of alginate synthesized and this knowledge can be used to build a recombinant strain able to overexpress *alg8* in order to produce alginates with higher molecular weights.

Keywords *Azotobacter vinelandii* · Alginate · Continuous culture · *alg8* · *algL*

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Introduction

Azotobacter vinelandii is an aerobic bacterium that is able to fix nitrogen and produce alginate as an exopolysaccharide comprising a copolymer of (1–4)-linked residues of β-D-mannuronic acid (M) and α-L-guluronic acids (G) in various sequences and ratios. These polysaccharides are used as thickening agents, stabilizers, and emulsifiers in food, medical, and pharmaceutical industries [10]. The production of alginates with specific molecular weights for biomaterials with specific applications can be achieved in a bacterial bioprocess. Studies have demonstrated that the culture conditions of agitation rate and oxygen transfer rate (OTR) affect alginate production in bioprocesses [7, 15, 19]. Peña et al. [19] evaluated the effect of agitation rate and dissolved oxygen tension (DOT) and reported that the highest alginate production (5.0 g l^{-1}) and molecular weight distribution were found at 3% DOT under a high agitation rate (700 rpm). In addition, it has been demonstrated that the OTR affects the molecular weight of alginate polymers in cultures of *A. vinelandii* at a constant specific growth rate [7]. Recently, Lozano et al. [15] showed that the molecular weight of alginate produced under non-oxygen-limited conditions increased by decreasing the OTR of batch cultures of *A. vinelandii*. On the other hand, Díaz-Barrera et al. [8] reported that alginate molecular weight can be influenced by the dilution rate and the carbon source concentration in the inlet medium in chemostat cultures of *A. vinelandii* growing under nitrogen fixation conditions. The changes were attributed to variations in the specific sucrose uptake rate. However, in that study, a low concentration of alginate (about 0.3 g l^{-1}) was obtained using sucrose as the carbon source.

Alginate is synthesized as a polymannuronate from its cytosolic precursor (GDP-M), being degraded by alginate-

lyases (encoded by *algL*) while traversing the periplasm [11]. All of the steps involved in the conversion of central sugar metabolites into GDP-M in *A. vinelandii* have been previously studied and analyzed [2, 21]. Nonetheless, the mechanisms involved in the polymerization and depolymerization steps have not been fully elucidated. Previous studies with *Pseudomonas aeruginosa* (another alginate-producing bacterium) have indicated that the catalytic subunit of the alginate polymerase complex is the *alg8* protein, which is encoded by *alg8* [18]. Remminghorst and Rehm [22] demonstrated that it is possible to increase alginate production in *P. aeruginosa* by at least 10 times by incorporating additional copies of the *alg8* gene, suggesting that *alg8* may be involved in a rate-limiting step of alginate production. Albrecht and Schiller [1] demonstrated that alginate-lyase activity is required for alginate biosynthesis, also in *P. aeruginosa* cells. Trujillo-Roldán et al. [28] reported that alginate-lyase activity is dependent on DOT and that the action of this enzyme is restricted to a post-polymerization step in *A. vinelandii* cultures.

Recently, Díaz-Barrera et al. [9] indicated that alginate production, carbon distribution, and *algL* gene expression were affected by changes in the specific oxygen uptake rate as manipulated by the agitation rate in chemostat cultures of *A. vinelandii* growing diazotrophically (i.e., under nitrogen-fixing conditions). Nonetheless, a systematic study evaluating the effects of agitation rates and/or carbon source concentrations in the inlet medium (at a constant specific growth rate μ) on alginate biosynthesis and genes expression in *A. vinelandii* cultures has not been performed. Taking into account that the agitation rate influences the molecular weight of alginate produced by *A. vinelandii* and that the *alg8* protein and alginate-lyase activity play crucial roles in alginate biosynthesis, this work focused on identifying the effects of the inlet sucrose concentration and the agitation rate on alginate production and gene expression during continuous culture of *A. vinelandii*. From the data obtained, a possible relationship between *alg8* gene expression and alginate molecular weight was established, which, to the best of our knowledge, has never been described until now. The findings obtained in this study provide a possible explanation at the cellular level for changes observed in the quality (in terms of its molecular weight) of the alginate synthesized in continuous culture.

Materials and methods

Bacterial strain and culture medium

Azotobacter vinelandii ATCC 9046 (wild-type strain) was used throughout this study. The culture medium used for continuous cultures contained, per liter of demineralized water, 10, 15, or 20 g sucrose, 0.8 g $(NH_4)_2SO_4$, 0.66 g K_2HPO_4, 0.16 g KH_2PO_4, 0.05 g $CaSO_4$, 0.2 NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.0029 g $Na_2MoO_4{\cdot}2H_2O$, and 0.027 g $FeSO_4$. The solutions of NaCl, $MgSO_4$, Na_2MoO_4, and $FeSO_4$ were autoclaved (121°C for 20 min) separately before being added to the culture medium. Before use, the strain was isolated on agar plates containing the same medium. In preliminary experiments using sucrose and ammonium (i.e., non-nitrogen-fixation conditions), chemostat cultures showed higher alginate production compared with the results of studies by Díaz-Barrera et al. [8] in which *A. vinelandii* was grown under nitrogen fixation conditions.

Inoculum preparation

The inocula were prepared in 500-ml Erlenmeyer flasks, which contained 100 ml of culture medium with 20 g l^{-1} sucrose at an initial pH of 7.0. The cells were grown at 200 rpm and 30°C in an orbital incubator shaker (New Brunswick, USA). After 18 h of cultivation, the cells were transferred (10% v/v) to a bioreactor operated in batch mode.

Bioreactor cultures and sampling

Chemostat experiments were performed in a 3-l Applikon bioreactor (Schiedam, Netherlands) using 2 l of culture medium at 30°C and pH 7.0 as controlled by automatic addition of 2 M NaOH. The continuous mode was operated at a dilution rate of 0.1 h^{-1}, which corresponded to 75% of μ_{max} (data not shown). The culture medium described was fed with sucrose, varying the inlet sucrose concentration (10, 15, or 20 g l^{-1}) depending on the experiment. The working volume was kept constant by withdrawing culture broth through a continuously operating peristaltic pump. The fermentor, which was equipped with two Rushton turbines, was aerated at 2 l min^{-1} (1.0 vvm) and agitated at 300, 500, and 700 rpm. The DOT was measured using a polarographic oxygen probe (Ingold, Mettler-Toledo), and it was not controlled. The continuous culture reached steady state conditions when the optical density at 540 nm (OD_{540}) and the sucrose concentration remained constant (<10% variation) after at least 3–4 residence times under the conditions of the experiment. Samples of cultures (20 or 30 ml) were withdrawn from the bioreactor for analytical measurements. All of the experiments were conducted in triplicate.

Analytical methods

Cell growth was estimated by measuring OD_{540} and establishing a correlation with the dry cell weight. Sucrose concentration was determined by acid hydrolysis with HCl

and subsequently assaying for reducing sugars with the dinitrosalicylic acid (DNS) reagent [16]. Ammonium levels were measured using the phenolhypochlorite method [12]. Alginate concentration was quantified gravimetrically by precipitation with 3 volumes of ice-cold propan-2-ol. The resultant precipitate was filtered (0.22 μm Millipore filters) and dried at 85°C to a constant weight. The molecular weight of alginate was determined by gel permeation chromatography (GPC) using a serial set of Ultrahydrogel columns (UG 500 and Linear Waters) in an HPLC system with a differential refractometer detector (PerkinElmer, USA). 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* as molecular weight standards [6].

Respiratory measurements

Respiratory measurements were based on gas analyses using online measurements of O_2 and CO_2 in the exit gas. A paramagnetic oxygen analyzer (Anarad Inc., USA) and infrared gas analyzer (LI-COR Biosciences, USA) were used for O_2 and CO_2 gas measurements, respectively. The OTR and the carbon dioxide transfer rate (CTR) were determined from gas analysis and calculated by carrying out gas balances [9]. Respiratory quotient (RQ) was determined using the OTR and CTR data (RQ = CTR/OTR) .

Quantitative real-time PCR assay

RNA extraction and cDNA synthesis were performed as previously described [9]. Cells were harvested by centrifugation at 6,870×*g* for 10 min at 4°C and stored at −80°C with RNAlater™ solution (RNA stabilization and protection solution). Total RNA was isolated and purified from the samples using the High Pure RNA Isolation kit (Roche Applied Science), and the concentration of RNA was determined by measuring the ratio of absorbance at 260 and 280 nm. The synthesis of cDNA was carried out using RevertAid™ H First Strand cDNA Synthesis kit (Fermentas Inc.) using random DNA primers according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using LightCycler® FastStart DNA Master SYBR Green I systems (Roche Applied Science). The sequences of the primers used for cDNA synthesis and for qPCR were designed using algorithm Primer 3 as previously indicated [9]. The level of *gyrA* mRNA was used as an internal control [17] to normalize the results obtained for the *algL* and *alg8* mRNA. The relative quantification of gene expression was performed as described previously [9] using the standard curve method with three measurements for each gene for each experimental condition evaluated. A maximum standard deviation (SD) of 10% was obtained. The data are reported as relative expression levels compared to the expression levels of the calibrated value (chemostat culture at 300 rpm with 10 g l^{-1} of sucrose in the inlet medium).

Calculation of the specific uptake/production rates and carbon recovery

The specific alginate production rate (q_p), specific sucrose uptake rate (q_s), specific oxygen uptake rate (q_{O_2}), and specific carbon dioxide rate (q_{CO_2}) in steady state conditions were calculated as follows:

$$q_p = \frac{DP}{X} \quad (1)$$

$$q_s = \frac{D(S_o - S)}{X} \quad (2)$$

$$q_{O_2} = \frac{OTR}{X} \quad (3)$$

$$q_{CO_2} = \frac{CTR}{X} \quad (4)$$

where D is the dilution rate, P is the alginate concentration, X is biomass concentration, S_o is the sucrose concentration in the feed medium, and S is the residual sucrose concentration.

Carbon recovery under steady state conditions was determined from reactor mass balances according to previously described methods [9].

Results and discussion

Substrate concentrations and respiratory activities in steady state conditions at different inlet sucrose concentrations

The DOT levels and ammonium and sucrose concentrations under steady state conditions at different inlet sucrose concentrations (ISCs) are shown in Table 1. During steady state conditions, the level of DOT was nearly zero in all cases, suggesting that the cultures were oxygen-limited. Similar observations have been described previously for continuous cultures of *A. vinelandii* [8, 9]. It is known that adequate oxygen transfer to bioreactors can be evaluated by considering the characteristic time for oxygen uptake (C_L^*/O_2 uptake rate) and the mixing time (t_m) [14]. In order to confirm the existence of oxygen limitation, the characteristic time for oxygen uptake and the circulation time (t_c) (according to the equation described by Delvigne et al. [5]) were estimated from the data obtained in our study. The characteristic time for oxygen uptake was tenfold higher than the calculated t_m ($t_m = 4\ t_c$), indicating that oxygen uptake becomes limiting, and no gradients of dissolved

oxygen are expected, supporting the existence of oxygen limitation in the continuous cultures.

As shown in Table 1, ammonium concentration in the steady state varied between 0.005 and 0.041 g l^{-1}. According to the theory of continuous cultures, the equation $K_s = S(\mu_{max} - D)/D$ was used to calculate the saturation constant (K_s) for ammonium. Under the experimental conditions, a K_s of 0.11 g l^{-1} was obtained, which is least 10 times higher than the ammonium concentrations measured in steady state conditions (Table 1). This evidence suggests that the chemostat cultures were limited by ammonium. It is known that ammonium affects the nitrogenase enzyme complex, which is responsible for reduction of N_2 to ammonia (fixation of nitrogen), inhibiting the activity of the nitrogenase complex or repressing its synthesis [3]. It has been reported that despite the low concentrations of ammonium in the culture medium (only a few millimolar), *Azotobacter* sp. are unable to fix atmospheric nitrogen [4]. Based on this evidence, it is possible to infer that ammonium-assimilating cultures of *A. vinelandii* were conducted under non-nitrogen-fixation conditions.

The sucrose concentration in steady state conditions was higher than 1.2 g l^{-1} at all the ISCs evaluated. Given that K_s values lower than 0.1 g l^{-1} have been observed in sucrose-limited chemostat cultures of *A. vinelandii* [13], the chemostat cultures were not limited by carbon. In light of the data obtained in this study (Table 1), which suggests that ammonium and oxygen may be limiting growth, it is possible that a dual nutrient limitation was observed.

Figure 1 shows the respiratory activity in chemostat cultures operating under steady state conditions at different ISCs. A similar q_{O_2} value (1.0 mmol g^{-1} h^{-1}) was obtained using ISCs of 10 or 15 g l^{-1}, whereas at higher ISCs, the q_{O_2} increased 2.5-fold. Under aerobic conditions, dissimilation and energy regeneration are coupled to the respiratory electron transport chain (oxygen as terminal electron acceptor), which suggests that q_{O_2} may depend on the supply of the carbon substrate or sucrose feed rate. Under our experimental conditions, this phenomenon was only observed at the highest sucrose concentration evaluated. The RQ was influenced by changes in the ISC, decreasing from 1.85 to 0.63 as the ISC increased from 15 to 20 g l^{-1}, which suggests changes in the physiological conditions of *A. vinelandii* as a result of different sucrose feed rates.

Table 1 DOT levels and ammonium and sucrose concentrations in steady state during continuous cultures of *A. vinelandii* conducted at a dilution rate of 0.1 h^{-1} and 300 rpm

	Sucrose in the feed (g l^{-1})		
	10	15	20
DOT (% air saturation)	0–1	0	0–1
Residual sucrose (g l^{-1})	5.6 ± 0.07	1.2 ± 0.05	13.1 ± 0.07
Residual ammonium (g l^{-1})	0.041 ± 0.005	0.011 ± 0.001	0.005 ± 0.001

Values are means of the measurements ± SD. Detection limit for ammonium measurement is 0.002 g l^{-1}

Biomass and alginate production in chemostat cultures conducted at different ISCs

Figure 2 shows the biomass and alginate concentrations under steady state conditions as well as specific rates (q_s and q_p) at different ISCs in chemostat cultures conducted at 300 rpm. A similar biomass concentration was obtained using 10 or 15 g l^{-1}sucrose in the inlet medium, but an increase in the ISC to 20 g l^{-1} caused a decrease in biomass production, varying from 2.4 to 2.0 g l^{-1}. Alginate production was strongly affected by changes in the ISC in the range tested. Higher alginate production (2.4 g l^{-1}) and q_p (0.10 g g^{-1} h^{-1}) were obtained when the ISC was 15 g l^{-1}, representing a critical carbon source concentration in the feed, because both the alginate production and the q_p decreased above this level.

The RQ is a cellular physiological parameter that has been used for analyzing alginate production; certain reports have established that a decrease in alginate production can be related to an increase in the RQ [9, 20, 24]. In our work, an unexpected result was obtained. An increase in alginate production achieved by changing the ISC from 10 to 15 g l^{-1} could be associated with an increase in the RQ (Figs. 1, 2). One possible explanation for this behavior could be that our experiments were conducted under non-nitrogen-fixation conditions (unlike the aforementioned studies), which are known to have effects on cellular metabolism of *A. vinelandii* and, particularly, on alginate production [26].

As shown in Fig. 2, the alginate production in response to changes in the ISCs could be related to variations in the q_s. Thus, the higher alginate concentration (2.4 g l^{-1}) was observed at the higher q_s (0.55 g g^{-1} h^{-1}). In agreement with this evidence, Peña et al. [19] observed that an increase in the q_s was related to higher alginate production, and, in line with these results, Díaz-Barrera et al. [8] recently reported that the q_s calculated under different steady state conditions correlated with alginate molecular weight.

Carbon balance under steady state conditions at different ISCs

In order to evaluate how carbon is distributed during alginate production under the different conditions tested,

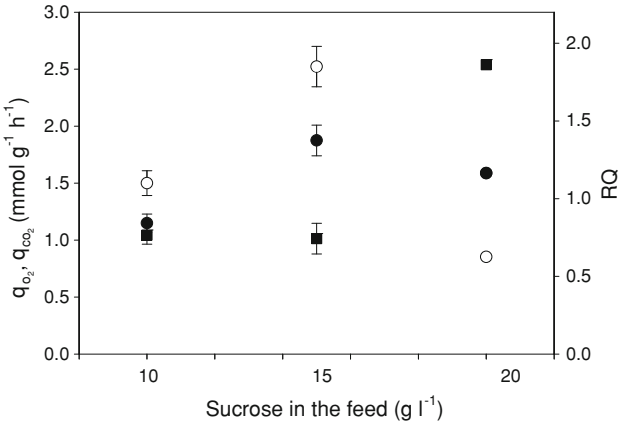

Fig. 1 Respiratory activity of *A. vinelandii* cells in continuous cultures conducted at different ISCs. Chemostat cultures were performed at a dilution rate of 0.1 h^{-1} and 300 rpm. Each point is the mean ± SD. *Filled squares* q_{O_2}, *filled circles* q_{CO_2}, *open circles* RQ

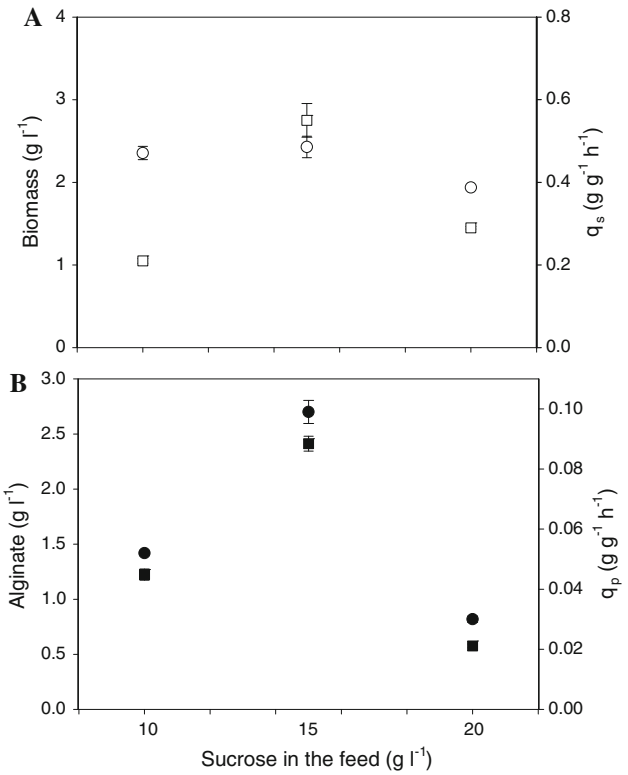

Fig. 2 Biomass, alginate production, and specific sucrose uptake rate under steady state conditions at different ISCs. Chemostat cultures were performed at a dilution rate of 0.1 h^{-1} and 300 rpm. Each point is the mean ± SD. **a** *Open circles* biomass, *open squares* q_s. **b** *Filled squares* alginate, *filled circles* q_p

carbon balance analysis was performed. Table 2 shows sucrose yields, carbon distribution (percentage of carbon atoms from sucrose converted to alginate, biomass, and CO_2), and carbon recovery at each steady state condition. It is clear that a change in the ISC affected the different sucrose yields and the carbon recovery. The sucrose yields on alginate ($Y_{alg/s}$) values obtained in this study were lower than those previously reported by Díaz-Barrera et al. [7] in chemostat cultures, which varied between 0.26 and 0.39 g g^{-1}. It is possible that this difference could be attributed to the nitrogen-fixing conditions used by Díaz-Barrera et al. [7], whereas in our work, the chemostat cultures were carried out under non-nitrogen-fixation conditions. In this regard, Sabra et al. [25] have postulated that under conditions of nitrogen fixation, *A. vinelandii* produces alginate to protect the nitrogenase complex from environmental oxygen.

The percentage of carbon diverted to biomass, alginate, and CO_2 under steady state conditions was highest using an ISC of 10 g l^{-1} (versus 15 or 20 g l^{-1}) of sucrose in the feed. Under this condition (i.e., an ISC of 10 g l^{-1}), the carbon balance was close to 100%, whereas the recovered carbon was only 45 and 52% when 15 or 20 g l^{-1} sucrose was used in the inlet medium. Thus, it is possible that *A. vinelandii* cells growing at lower sucrose feed rates (i.e., lower sucrose in the inlet medium) utilize sucrose more efficiently. Preliminary measurements using HPLC have shown that the unrecovered carbon could be observed owing to accumulation of malate and acetate released in the culture medium. In chemostat cultures conducted at an ISC of 15 g l^{-1}, the percentage of carbon diverted to acetate and malate was nearly 20 and 35%, respectively. A similar carbon distribution (percentage of carbon atoms from sucrose converted to organic acid) in acetate and malate was obtained using 20 g l^{-1} of sucrose in the inlet medium.

An increase in the sucrose feed rate implied changes in the q_s; thus, higher q_s values (0.55 and 0.30 g g^{-1} h^{-1}) were obtained in chemostat cultures conducted at ISCs of 15 and 20 g l^{-1} (Fig. 2). It is possible that carbon flux to the tricarboxylic acid (TCA) cycle was affected by modifications in the sucrose feed rate and, as a consequence, malate and acetate may have been accumulated and released. In light of these observations, further research must be carried out to evaluate variations in the metabolic fluxes of central metabolism in order to understand how carbon distribution into alginate is affected. Based on the experimental evidence obtained, an overview of metabolic pathway in *A. vinelandii* cells using sucrose is presented in Fig. 3.

Effect of the agitation rate on alginate production, molecular weight of alginate, and *algL* and *alg8* gene expression

Previously, Díaz-Barrera et al. [7] reported that alginate production under nitrogen-fixing conditions in chemostat

Table 2 Sucrose yields, carbon distribution, and carbon recovery in chemostat cultures of *A. vinelandii* conducted at a dilution rate of 0.1 h^{-1} and 300 rpm

	Sucrose in the feed (g l^{-1})		
	10	15	20
$Y_{x/s}$ (g g^{-1})	0.49	0.18	0.35
$Y_{alg/s}$ (g g^{-1})	0.25	0.18	0.10
$Y_{CO_2/s}$ (g g^{-1})	0.25	0.15	0.24
Carbon to biomass (%)	59.7	19.7	31.6
Carbon to alginate (%)	24.4	15.6	7.5
Carbon to CO_2 (%)	17.3	9.3	12.6
Recovered carbon (%)	101	45	52

cultures of *A. vinelandii* is affected by the agitation rate. In order to evaluate if the agitation rate influences alginate biosynthesis under non-nitrogen-fixation conditions, chemostat cultures conducted at 500 and 700 rpm were developed using an ISC of 10 g l^{-1}. This sucrose concentration in the inlet medium was selected as the carbon balance approached 100% under this condition (Table 2). Table 3 shows the effects of the agitation rate on OTR values and biomass and alginate production (concentration, $Y_{alg/s}$ and q_p) during steady state conditions. As expected, an increase in the agitation rate from 300 to 700 rpm affected the OTR level, varying from 2.5 to 9.8 mmol l^{-1} h^{-1}. Higher alginate concentration, $Y_{alg/s}$, and q_p values were observed at 500 rpm. In agreement with this evidence and using a bioreactor similar to that used in our work, Díaz-Barrera et al. [9] demonstrated that operating continuous cultures at agitation rates over 500 rpm wastes energy without any benefit to alginate production. At the different agitation rates evaluated, carbon balances were within 98–100% and unlike in chemostat cultures conducted at ISCs of 15 and 20 g l^{-1}, acetate and malate were not detected in the culture medium.

Recently, it has been shown that *algL* and *alg8* gene expression can be affected by the specific oxygen uptake rate (manipulated by changes in the agitation rate) in chemostat cultures of *A. vinelandii* conducted under nitrogen fixation conditions [9]. In order to complement this previous study and evaluate whether gene expression is affected by the agitation rate under non-nitrogen-fixation conditions, the influence of the agitation rate on *algL* and *alg8* gene expression, alginate yield on biomass ($Y_{alg/x}$), and alginate mean molecular weight (MMW) is presented in Fig. 4. An increase in *algL* gene expression was observed by increasing the agitation rate from 300 to 700 rpm, and both the *alg8* gene expression and alginate MMW were higher in chemostat cultures grown at 500 rpm compared with those in cultures grown at 300 and 700 rpm. As shown in Fig. 4, an increase in *algL* gene expression was not related to a decrease in alginate MMW (as expected), and thus, changes in the alginate MMW cannot be attributed to variations in *algL* gene expression under the experimental conditions used in this study. Trujillo-Roldán et al. [28] demonstrated that a decrease in alginate molecular weight in *A. vinelandii* cultures can be due to the action of extracellular alginate-lyase. In disagreement with our results (Fig. 4), Díaz-Barrera et al. [9] reported that a decrease in alginate MMW (about 1.6 times) can be linked

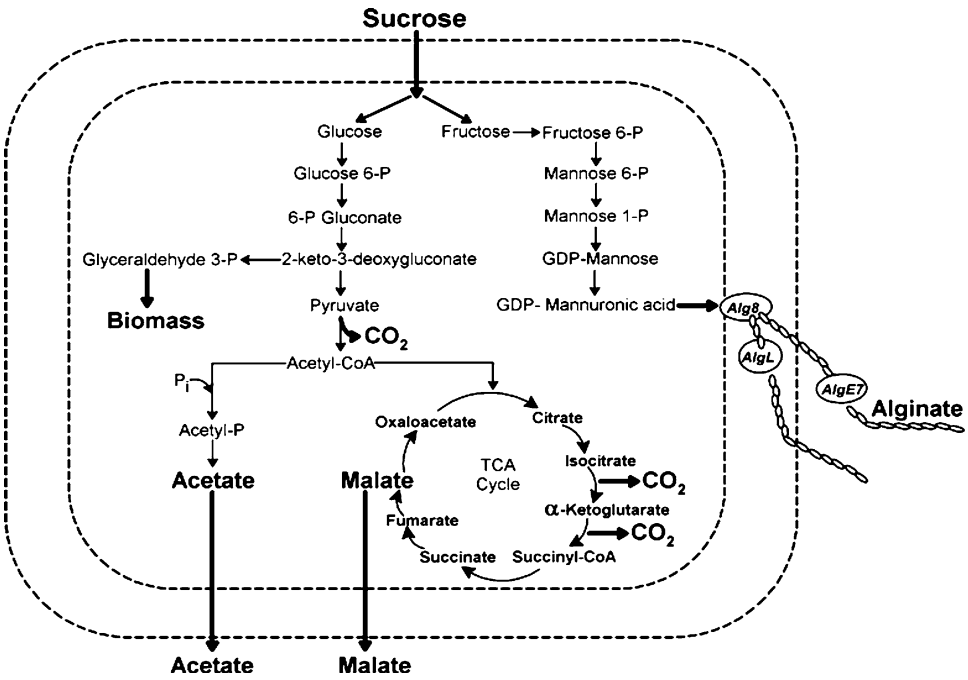

Fig. 3 Schematic metabolic pathway in *A. vinelandii* cells using sucrose as carbon source and ammonium sulfate as nitrogen source (non-nitrogen-fixation conditions). Conditions of cultivation: dilution rate of 0.1 h^{-1} and agitation rate of 300 rpm

Table 3 Characteristic values of OTR and biomass and alginate production at different agitation rates in steady state during continuous cultures of *A. vinelandii* conducted at a dilution rate of 0.1 h^{-1} and 10 g l^{-1} of sucrose concentration in the inlet medium

Agitation rate (rpm)	OTR (mmol $l^{-1}h^{-1}$)	Biomass (g l^{-1})	Alginate production		
			Alginate (g l^{-1})	q_p (g g^{-1} h^{-1})	$Y_{alg/s}$ (g g^{-1})
300	2.5	2.4 ± 0.08	1.2 ± 0.05	0.052 ± 0.001	0.25 ± 0.01
500	4.9	2.0 ± 0.11	1.8 ± 0.08	0.090 ± 0.001	0.31 ± 0.01
700	9.8	3.0 ± 0.14	1.1 ± 0.06	0.037 ± 0.001	0.12 ± 0.01

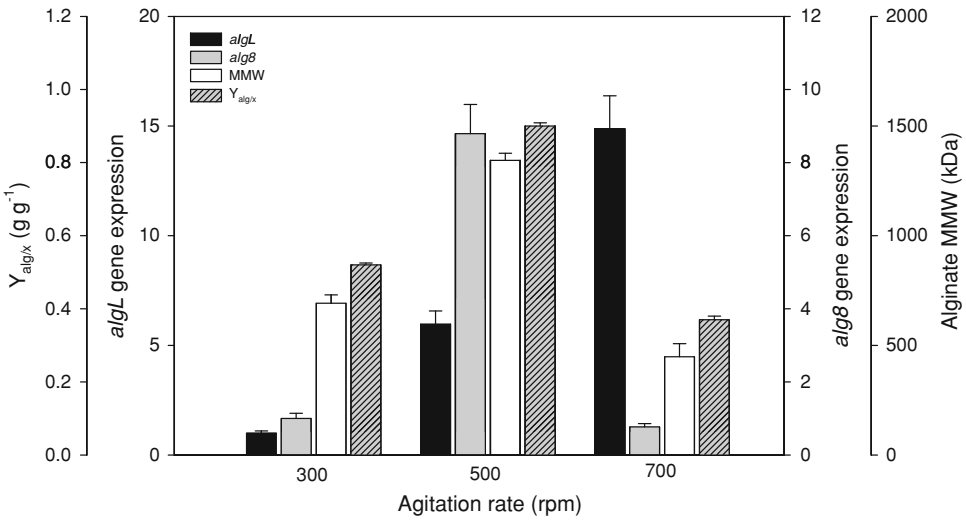

Fig. 4 Effect of the agitation rate on alginate yield, molecular weight of alginate, and gene expression in continuous cultures of *A. vinelandii* performed at a dilution rate of 0.1 h^{-1} and 10 g l^{-1} of sucrose in the inlet medium. Each point is the mean ± SD

to an increase in relative *algL* gene expression (approximately eightfold). The different behavior observed in our work could be explained by considering changes in the alginate-lyase activities due to metabolic differences resulting from the culture conditions used, nitrogen fixation by Díaz-Barrera et al. [9] and non-nitrogen-fixation conditions in this work. In this regard, it is important to note that alginate biosynthesis is constitutive in *A. vinelandii* and that this bacterium produces at least six enzymes that are encoded in the alginate biosynthetic gene cluster with alginate-lyase activities [27]. It is possible that different alginate-lyases could be expressed at different physiological states and, therefore, that analyses of expression patterns would need to be performed. Based on the results obtained in this study, it is possible that some of these alginate-lyase enzymes, which were not evaluated by analysis of *algL* gene expression in our work, may be responsible for altering alginate molecular weight. Thus, *algL* gene expression and alginate-lyase activities in *A. vinelandii* cultures require further investigation.

Interestingly, the results showed that *alg8* gene expression can be associated with changes in the alginate MMW and $Y_{alg/x}$. Thus, the higher alginate MMW (1,343 kDa) and $Y_{alg/x}$ (0.9 g g^{-1}) obtained at 500 rpm are linked to the higher *alg8* gene expression. In agreement with this finding, Remminghorst and Rehm [22] reported that additional copies of *alg8* in *P. aeruginosa* cells result in a higher $Y_{alg/x}$ compared with that of the wild-type strain, suggesting that *alg8* activity is a limiting step in alginate biosynthesis. In order to extend this study's analysis of the relationship between alginate MMW and *alg8* gene expression, all of the data obtained at different ISCs and agitation rates were correlated. Figure 5 shows the relationship between alginate MMW and *alg8* gene expression during steady state conditions under the different conditions evaluated in this study. The results show that a higher alginate MMW can be linked to higher *alg8* gene expression, thereby demonstrating for the first time a relationship between *alg8* gene expression and the molecular weight of alginate synthesized by *A. vinelandii*. The findings obtained suggest that in *A. vinelandii* cells, the gene *alg8* encodes the proposed catalytic subunit of alginate polymerase, as has been demonstrated in *P. aeruginosa* [23]. It is interesting to note that the variations in *alg8* gene expression and alginate molecular weight were obtained under steady state conditions as a result of changes in the agitation rate and the sucrose concentration used in the inlet medium, suggesting that *alg8* gene expression could be modulated by oxygen availability and the carbon source feed rate. The findings obtained in this work provide valuable knowledge about alginate biosynthesis pathways. This information could be used for the construction of recombinant strains able to

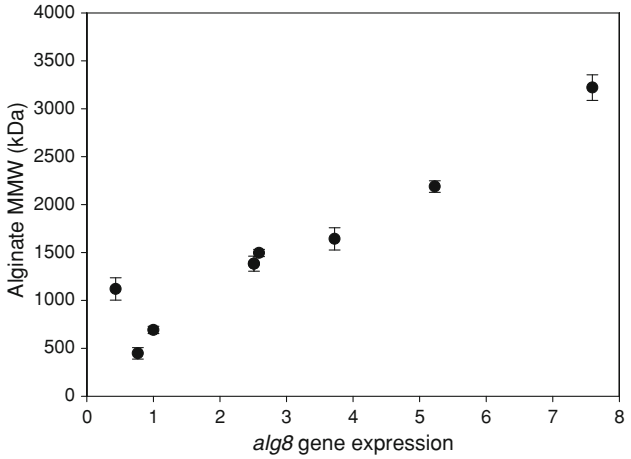

Fig. 5 Relationship between alginate molecular weight and *alg8* gene expression during steady state conditions under the different conditions evaluated. Chemostat cultures performed at a dilution rate of 0.1 h^{-1}, ISCs of 10, 15, and 20 g l^{-1}, and agitation at 300, 500, and 700 rpm. Each point is the mean ± SD

overexpress the *alg8* gene in order to produce alginates with higher molecular weights by fermentation for specific industrial applications.

In this study, alginate production, carbon balance, and expression of the genes *algL* and *alg8* were determined during continuous cultivation of *A. vinelandii*. Alginate production and recovered carbon were affected by the sucrose feed rate. An increase in the molecular weight of alginate can be linked to higher *alg8* gene expression, thereby, for the first time, providing a possible explanation at the cellular level for changes in the molecular weight of the alginate synthesized by *A. vinelandii*.

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