J Ind Microbiol Biotechnol (2013) 40:715–723
DOI 10.1007/s10295-013-1274-6

The acetylation degree of alginates in *Azotobacter vinelandii* ATCC9046 is determined by dissolved oxygen and specific growth rate: studies in glucose-limited chemostat cultivations

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Received: 27 November 2012 / Accepted: 12 April 2013 / Published online: 3 May 2013

Abstract Alginates are polysaccharides that may be used as viscosifiers and gel or film-forming agents with a great diversity of applications. The alginates produced by bacteria such as *Azotobacter vinelandii* are acetylated. The presence of acetyl groups in this type of alginate increases its solubility, viscosity, and swelling capability. The aim of this study was to evaluate, in glucose-limited chemostat cultivations of *A. vinelandii* ATCC9046, the influence of dissolved oxygen tension (DO) and specific growth rate (μ) on the degree of acetylation of alginates produced by this bacterium. In glucose-limited chemostat cultivations, the degree of alginate acetylation was evaluated under two conditions of DO (1 and 9 %) and for a range of specific growth rates (0.02–0.15 h^{-1}). In addition, the alginate yields and PHB production were evaluated. High DO in the culture resulted in a high degree of alginate acetylation, reaching a maximum acetylation degree of 6.88 % at 9 % DO. In contrast, the increment of μ had a negative effect on the production and acetylation of the polymer. It was found that at high DO (9 %) and low μ, there was a reduction of the respiration rate, and the PHB accumulation was negligible, suggesting that the flux of acetyl-CoA (the acetyl donor) was diverted to alginate acetylation.

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Keywords *Azotobacter vinelandii* · Alginate acetylation · Specific growth rate · Dissolved oxygen tension · Poly(3-hydroxybutyrate)

Introduction

Alginates are a family of linear copolymers conformed by (1-4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) [9]. These polymers are covalently linked in different sequences, which may be found as homopolymeric blocks (MMMMM, GGGGG) or heteropolymeric blocks (MGMGMG) [2, 23]. Alginates are obtained from brown algae and bacteria of the genera *Azotobacter* and *Pseudomonas* [10]. Alginates isolated from bacteria differ from those obtained from algae because these polymers are acetylated on the C-2 and/or C-3 residues of the mannuronic acid units [30]. Alginates have been used in several industries as viscosifiers and gel- or film-forming agents. In recent years, these polymers have been used as matrices for cell immobilization and controlled delivery systems for drugs [2, 23].

The chemical structures of alginates influence their properties and potential applications. Specifically, the presence of *O*-acetyl groups modifies the alginates conformation [32]. The acetylation of alginates increases their solubility in aqueous solutions and reduces the interaction with divalent cations [31]. Small changes in the degree of acetylation positively affect the viscosities of alginate solutions independent of the effects of mean molecular weight (MMW) [21, 32]. In addition, the gels of acetylated alginates show high swelling capabilities resulting in softer and more-hydrated gels [31, 35].

It has been observed that alginate acetylation plays important biological functions in *A. vinelandii* and

Pseudomonas. In the case of *A. vinelandii*, this bacterium produces cysts resistant to desiccation, and alginate is an important component of these structures [34]. In a mutant strain of *A. vinelandii* (AJ34) that produces unacetylated alginates, Vázquez et al. [34] observed important reductions in encystment efficiency and resistance to desiccation when compared with the wild type. In the case of *Pseudomonas aeruginosa,* an opportunistic pathogen, it has been observed that the organism requires acetylated alginate for the formation and stability of biofilms during its infection processes; in addition, the acetylation of alginates enhances the resistance of this bacterium to complement-mediated and opsonic antibody-mediated phagocytosis [3, 17, 33]. It must be emphasized that cysts and biofilms occur in environments under nutrient limitations [36] and, therefore, low specific growth rates [15]. In addition, it has been proposed that the acetyl groups confer protection to the polymer from the activities of alginate lyases and epimerases [19, 32].

A. vinelandii also produces poly(3-hydroxybutyrate) (PHB), which is an intracellular polymer belonging to the family of polyhydroxyalkanoates (PHAs). This product is accumulated under unbalanced growth conditions, mainly oxygen limitation, as a carbon and energy reserve material. Recently, PHB has acquired importance because it is a biodegradable and biocompatible thermoplastic that can be used in a wide variety of products as a substitute for plastics derived from oil [10].

The effects of dissolved oxygen tension (DO) and specific growth rate (μ) on the production of alginate and its chemical composition, mainly its MMW, have been widely studied in batch and chemostat cultivations of *A. vinelandii* [16, 20, 24–26].

Nevertheless, it is important to note that the fermentation parameters that affect the degree of acetylation of alginates produced by *A. vinelandii* have been little studied. It has been reported that changes in the medium composition, such as the addition of pH buffers such as MOPS [21], as well as changes in calcium and phosphate concentrations [1] impact the degree of alginate acetylation.

Other studies have described the effect of μ on the acetylation degree of alginates [1]. Those authors observed that the acetylation degree of alginates increased from 0.05 mol_{Acetyl} mol_{Alg}^{-1}, obtained at $\mu = 0.16\ h^{-1}$, to 0.35 mol_{Acetyl} mol_{Alg}^{-1}, when μ was 0.32 h^{-1}, using calcium and phosphate concentrations of 1.5 and 2 mM, respectively.

Recently, Peña et al. [22] showed that in cultures developed in shake flasks at low shaking frequency, in which the oxygen transfer rate (OTR) was low, the acetylation degree of the polymer was higher (5.8 ± 0.3 %) compared with the values obtained (3.5 ± 0.3 %) at a higher shaking rate and therefore higher OTR. However, in those experiments, it was not possible to discriminate between the influence of oxygen in the bulk liquid and that of the specific growth rate on the degree of acetylation of the alginates.

The aim of this study was to evaluate how dissolved oxygen (DO) and specific growth rate (μ) affect the degree of acetylation of alginates produced by *A. vinelandii* ATCC9046 in chemostat cultures.

Materials and methods

Microorganism

Experiments were carried out using wild-type *A. vinelandii* ATCC 9046 (American Type Culture Collection). This strain was maintained using monthly subcultures on Burk′s agar slopes and stored at 4 °C.

Culture medium

The medium used was the nitrogen-free modified Burk's medium [20] with the following composition (in g l^{-1}): K_2HPO_4 0.66, KH_2PO_4 0.16, MOPS 1.42, $CaSO_4{\cdot}2H_2O$ 0.05, NaCl 0.2, $MgSO_4$ 0.2, $NaMoO_4{\cdot}2H_2O$ 0.0029, and $FeSO_4{\cdot}7H_2O$ 0.027. The glucose concentration used for batch stage cultivation was 6 g l^{-1}, and for chemostat stage cultivation, it was 4 g l^{-1}. The initial pH was adjusted to 7.2 with 1 N NaOH before autoclaving.

Chemostat cultures

Chemostat experiments were performed in a 3-l bioreactor (Applikon Schiedam, Netherlands) using a 2-l working volume. The pH was kept constant at 7.2 ± 0.1, and it was controlled by the addition of 1 N NaOH. Temperature was maintained at 29 °C. The bioreactor was equipped with two Rushton turbines ($D_T/T = 1/3$, where D_T was the turbine diameter, and T the bioreactor diameter), and it was agitated at 300 rpm. The aeration rate was 0.5 vvm. The working volume was kept constant by feeding fresh medium and withdrawing culture broth through a continuously operating peristaltic pump (Easy load Masterflex, USA). Three DO conditions were evaluated (1, 5 and 9 %). Based on the chemostat theory, we considered $\mu = D$ (where μ was the specific growth rate and D the dilution rate). The range of D evaluated for the cultivations conducted at 1 and 5 % DO were from 0.02 to 0.08 h^{-1}. The cultivations conducted at the DO of 9 % were evaluated for values of D of 0.04, 0.08, and 0.15 h^{-1}. The upper limits of D used for the experiments (0.08 and 0.15 h^{-1}, respectively), were 80 % of μ_{max} for each DO. The values of μ_{max} were previously determined in batch cultivations of *A. vinelandii* at

the different DOs using the same culture conditions (pH = 7.2, 29 °C, 300 rpm and 0.5 vvm). DO was measured using a polarographic oxygen probe (Applikon Schiedam, Netherlands), and it was controlled at the operational temperature (29 °C) by gas blending, automatically varying the proportions of nitrogen and oxygen in the inflowing gas through two 5850F mass flow controllers (Brook Instruments B.V., Netherlands) and using a system based on a PID control that has been previously described [16]. The steady-state condition was achieved after three residence times, when biomass concentration remained constant (≤10 % variation). For each condition, three independent cultures were run, and three independent samples were taken during three residence-time after the steady state was reached. For all the conditions, there was no detected residual glucose (the limiting substrate).

Analytical methods

Cell growth, alginate, and glucose concentration assessments

Microbial growth was evaluated using optical density (O.D.) at a wavelength of 560 nm. Measurements of absorbance were performed in duplicate for each time point. In addition, dry-weight measurements were performed using 10-ml samples of culture broth. Each sample was centrifuged at 11,000 rpm for 15 min, the pellet was recovered and filtered, dried and weighed using cellulose acetate filters (0.2-μm pore size, Sartorius Biolab Products) as detailed by Peña et al. [20]. Alginate quantification was performed by dry weight as described previously by Peña et al. [20]. From each sample used for biomass dry weight determination, the supernatant was recovered, and alginate was precipitated by adding three volumes of propan-2-ol. The precipitated alginate was filtered and dried at 80 °C for 24 h, to a constant weight, as was described previously [20]. Glucose quantification was performed by HPLC using an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, Hercules, CA, USA). The eluent used was H_2SO_4 (7 mM) at a flow rate of 0.8 ml min^{-1}. Glucose detection was achieved using a refractive index (RI) detector (Waters 2414 detector).

Quantification of alginate acetylation

The degree of acetylation was quantified using HPLC [4]. Each lyophilized alginate sample (5–10 mg) was weighed and placed in an Eppendorf tube, and it was dissolved with 500 μl Milli Q water. Subsequently, 500 μl of 1 N NaOH were added, and the sample was mixed by vortexing. This mixture was incubated at 80 °C for 2 h. Later, the sample was cooled and acidified by the addition of 625 μl of 1.5 M H_3PO_4. The sample was centrifuged at 11,000 rpm for 15 min, and the aqueous phase was recovered for acetate quantification. Acetyl groups were quantified by HPLC using an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, Hercules, CA, USA) at 50 °C and using H_2SO_4 (7 mM) as the eluent with a flow rate of 0.65 ml min^{-1}. The acetic acid concentration was determined by UV absorption at 210 nm using a photodiode array detector (Waters 2996), under these chromatographic conditions, acetate eluted at 13.17 ± 0.05 min (Fig. 1a). Figure 1b shows a chromatographic profile for the acetate quantification of an acetylated alginate sample after hydrolysis with 1 M NaOH, in which the acetate peak is separated from those of other, unknown, hydrolysis products of alginate (Fig. 1c).

Poly(3-hydrohybutyrate) (PHB) quantification

PHB content was quantified by HPLC, after its conversion into crotonic acid. Firstly, the biomass was dried under vacuum at 60 °C. For each sample, 3 mg of biomass was weighed in a 1.5-ml Eppendorf tube; 1 ml of H_2SO_4 was added, and the sample was heated at 90 °C for 1 h. Subsequently, the sample was cooled at room temperature and diluted with Milli-Q water to concentrations within the range of the calibration curve. The PHB quantification was performed using an HPLC system with an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad, Hercules, CA, USA) at 50 °C and using H_2SO_4 (7 mM) as the eluent with a flow rate of 0.65 ml min^{-1}. Crotonic acid was quantified using UV absorption at 220 nm. The standard was a commercial PHB (Sigma-Aldrich) that was treated identically as the samples.

Oxygen transfer rate and specific oxygen uptake rate determinations

Equations used for determinations of specific oxygen uptake rates were as follows:

$$C_g = \frac{F_{O_2}}{F_T}[O_2] \quad (1)$$

$$OTR = k_l a(C_g - C_L) \quad (2)$$

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

where C_g (mmol l^{-1}) was the dissolved oxygen concentration at equilibrium, calculated based on the proportion of the flux of oxygen at the inlet (F_{O_2}; l h^{-1}) of the system and the total flux of gas at the inlet (F_T; l h^{-1}), OTR (mmol l^{-1} h^{-1}) was the OTR, $k_l a$ (h^{-1}) was the volumetric oxygen transfer coefficient, C_L (mmol l^{-1}) was the dissolved oxygen concentration, q_{O_2} (mmol g^{-1} h^{-1}) the

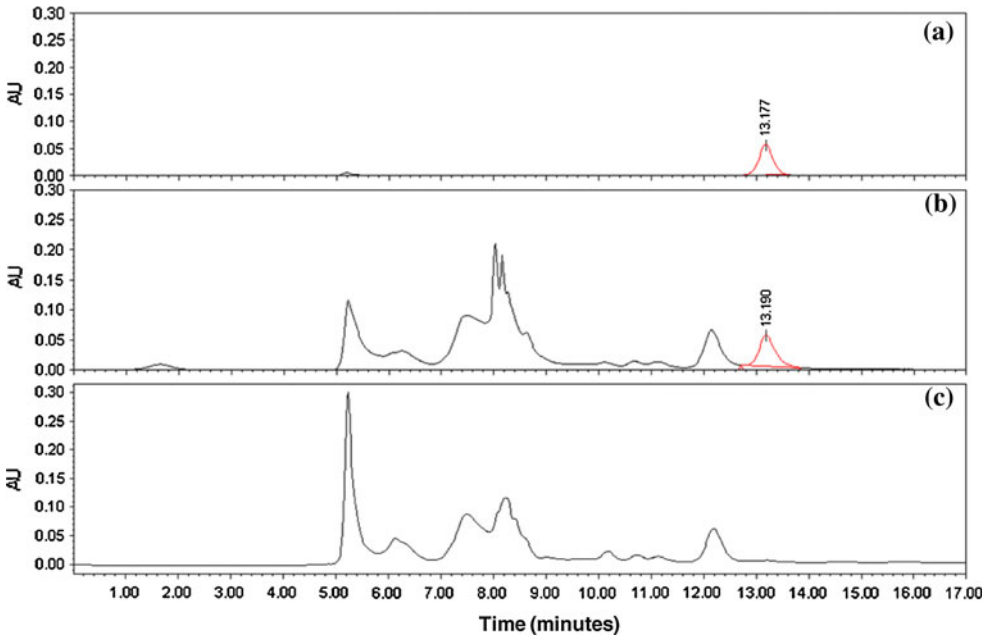

Fig. 1 Acetate standard 0.05 mM (**a**). Chromatographic profile of acetylated alginate hydrolyzed with 1 M NaOH at 80 °C (**b**). Chromatographic profile of algal alginate hydrolyzed with 1 M NaOH at 80 °C, negative control (**c**)

specific oxygen uptake rate and X (g l^{-1}) was the biomass concentration. For chemostat cultures at constant DO and for conditions of steady state, the OTR is equal to the oxygen uptake rate (OUR).

Results

Dissolved oxygen profiles, biomass, and PHB production

To evaluate the effect of DO on the acetylation of alginates, three DO conditions were tested: 1 %, which is known as a condition that promotes production of alginate and PHB by *A. vinelandii* [20], and two conditions, 5 and 9 % DO, under which there are no oxygen limitations and alginate production is present [25]. Figure 2a shows dissolved oxygen profiles for the DOs evaluated in this study. The oxygen control during cultivations at the different dilution rates (D) was achieved with coefficients of variation of $\leq$15 %.

Figure 2b shows the steady-state biomass concentrations in the chemostats and Table 1 summarizes the biomass and product yields on glucose. The highest biomass concentration was of 0.61 ± 0.02 g l^{-1} and this was obtained at 1 % DO, regardless of the μ employed (Fig. 2b) and this was coincident with the high PHB content observed under these set of experiments (Fig. 2c). The PHB content in the cultures grown at 1 % DO reached up to 33 % of the total biomass at $D = 0.02$ h^{-1}; while the lowest PHB content for the three DO evaluated was obtained at 9 % (3.1 ± 1.5 % w/w).

Oxygen uptake rate (OUR) and specific oxygen consumption rates (q_{O_2})

Figure 3a, b shows the OUR and the q_{O_2} for the different conditions evaluated. For the three DO conditions, these parameters showed increases when the specific growth rate was higher. Significant lower values of OUR and q_{O_2}, (OUR = 2.9 mmol l^{-1} h^{-1}; q_{O_2}, = 4 mmol g^{-1} h^{-1}) were obtained at 1 % DO and $D = 0.02$ h^{-1} as compared with the culture developed at 5 % at the same dilution rate (Fig. 3b). In contrast, the highest values for the two parameters were obtained at 9 % DO and $D = 0.15$ h^{-1} (OUR = 18.36 mmol l^{-1} h^{-1}; q_{O_2} = 39.7 mmol g^{-1} h^{-1}).

Alginate production and the acetylation degree of alginates

Because there were no significant differences in alginate production parameters, including alginate acetylation, between the cultivations at 1 and 5 % DO (data not shown), the following discussion will be based on the results at 1 and 9 % DO. For the cultivations conducted at 1 % DO, the alginate concentrations and alginate yields based on glucose ($Y_{Alg/G}$) and biomass ($Y_{Alg/X}$) did not show significant differences among the dilution rates evaluated (Fig. 4 a, b; Table 1). In contrast, in the cultures conducted at 9 % DO, the highest values of alginate concentration (1.19 ± 0.22 g_{Alg} l^{-1}), $Y_{Alg/G}$ (0.30 ± 0.06 g_{Alg} g_G^{-1}) and $Y_{Alg/X}$ (3.4 ± 0.82 g_{Alg} g^{-1}) were achieved for the cultivations developed at $\mu = 0.04$ h^{-1}. These values dropped to 0.74 ± 0.04 g l^{-1} ($Y_{Alg/G}$ = 0.19 ± 0.01 g_{Alg} g_G^{-1};

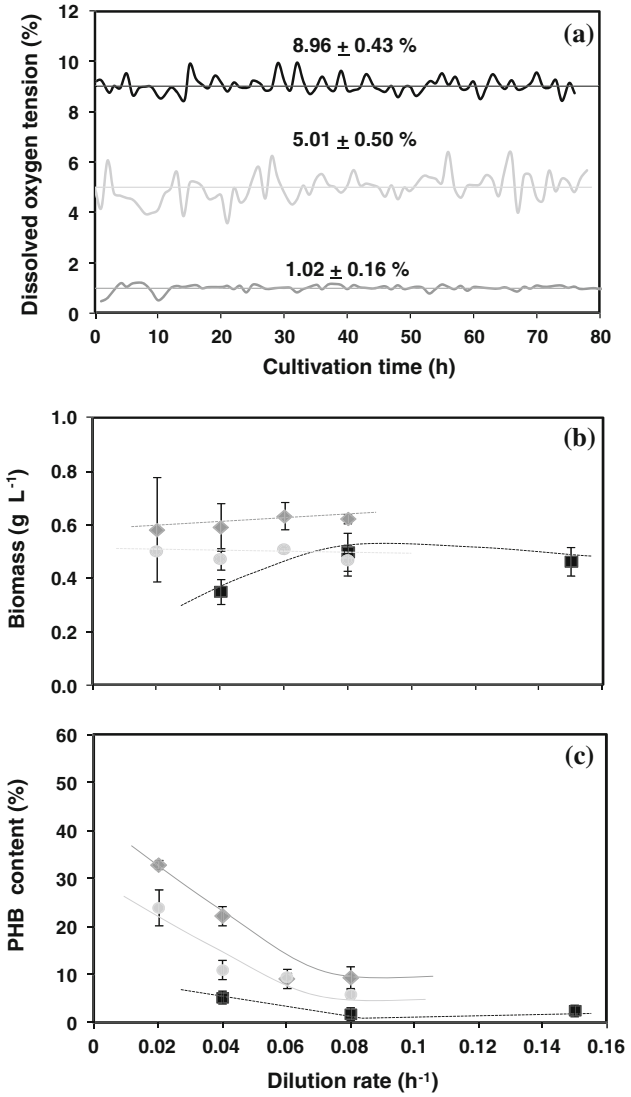

Fig. 2 Dissolved oxygen profiles of the chemostat cultivations of *A. vinelandii* at 1, 5, and 9 % DO (**a**). Steady-state biomass concentration (**b**) and PHB concentration (**c**) for cultivations at 1 % (*filled diamond*), 5 % (*filled circle*), and 9 % (*filled square*) of DO in chemostat cultivations of *A. vinelandii* at different dilution rates

$Y_{Alg/X} = 1.61 \pm 0.12$ g_{Alg} g^{-1}) when the μ was increased to 0.15 h^{-1}.

Figure 4c shows the degree of acetylation of alginates as a function of μ and DO. This was higher in the cultivations developed at 9 % DO, as compared with the degree of acetylation obtained at 1 % DO for the same dilution rates. For both conditions of DO (1 and 9 %), the highest value of acetylation degree of alginates was achieved for $D = 0.04$ h^{-1}; however, they showed different trends. A bell-shaped trend was observed for the condition at 1 % DO, with a maximal value at $D = 0.04$ h^{-1}; whereas a decreasing profile was evident for the case of 9 %, decreasing acetylation degree from 6.94 ± 0.81 % to 2.25 ± 0.07 % at $D = 0.15$ h^{-1} (Fig. 4c).

Table 1 Biomass, PHB, and alginate yields

DOT (%)	D (h^{-1})	$Y_{X/G}$ (g g_G^{-1})	$Y_{PHB/G}$ (g_{Alg} g_G^{-1})	$Y_{Alg/G}$ (g_{Alg} g_G^{-1})	Global yields
1	0.02	0.15 ± 0.05	0.048	0.26 ± 0.05	0.41
	0.04	0.15 ± 0.02	0.033	0.22 ± 0.03	0.37
	0.06	0.16 ± 0.01	0.015	0.21 ± 0.04	0.35
	0.08	0.16 ± 0.00	0.015	0.20 ± 0.01	0.34
5	0.02	0.13	0.030	0.24	0.37
	0.04	0.12 ± 0.01	0.013	0.19 ± 0.04	0.31
	0.06	0.13 ± 0.01	0.008	0.16 ± 0.03	0.29
	0.08	0.12 ± 0.01	0.007	0.17 ± 0.06	0.29
9	0.04	0.09 ± 0.01	0.004	0.3 ± 0.06	0.39
	0.08	0.12 ± 0.02	0.002	0.22 ± 0.05	0.34
	0.15	0.12 ± 0.01	0.003	0.19 ± 0.01	0.31

Discussion

Effect of the dissolved oxygen

The results of the present study show (Fig. 4c and 5a) that DO (under constant agitation rate and D) plays an important role in the acetylation of alginate by *A. vinelandii*. The acetylation degree of alginates was higher in the cultivations conducted at 9 % DO (within a range of μ between 0.04 and 0.08 h^{-1}) than in those developed at the low DO (1 %). Although the molecular mechanisms involved in the regulation of alginate acetylation as a function of DO are not completely understood, the following explanation may be given.

It has been proposed that acetyl-Coenzyme A (acetyl-CoA) is the acetyl donor for alginate acetylation [5], and it is also the precursor for PHB biosynthesis [29]. Under low oxygen concentration and therefore low oxygen-consumption rate conditions, some enzymes of the tricarboxylic acid cycle (TCA), such as citrate synthase and isocitrate dehydrogenase (ICDH), could be down-regulated by the accumulation of $NADPH^+$ and $NADH^+$ reducing the flux of acetyl-CoA through the TCA cycle [13, 28] and increasing the availability of acetyl-CoA for processes such as PHB synthesis [13, 28] and alginate acetylation [22]. In our study, the highest alginate acetylation was obtained at 9 % DO and low μ (0.04 h^{-1}); under those conditions, the oxygen uptake rate was very low (6.9 ± 1.1 mmol l^{-1} h^{-1}), a similar value to that obtained from the cultivations at 1 % (Fig. 3a). It is possible that under those conditions, a reduction of the flux of acetyl-CoA through the TCA cycle could be occurring, which would increase the availability of acetyl-CoA for PHB biosynthesis and acetylation degree.

On the other hand, the % C-mol distribution showed remarkable differences between the cultivations at 1 and 9 % DO (Table 2). In the cultivations conducted at 1 %

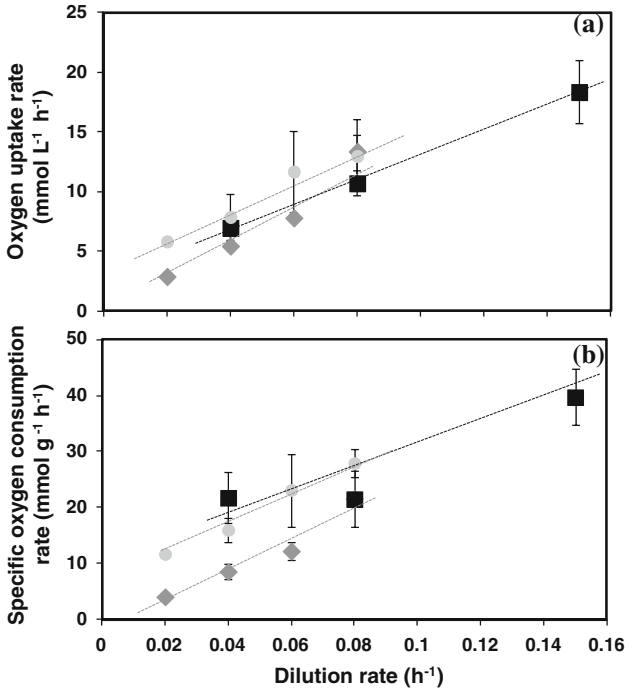

Fig. 3 Oxygen uptake rate (**a**) and specific oxygen consumption rate (**b**) for cultivations at 1 % (*filled diamond*), 5 % (*filled circle*), and 9 % (*filled square*) of DO in chemostat cultivations of *A. vinelandii* at different dilution rates

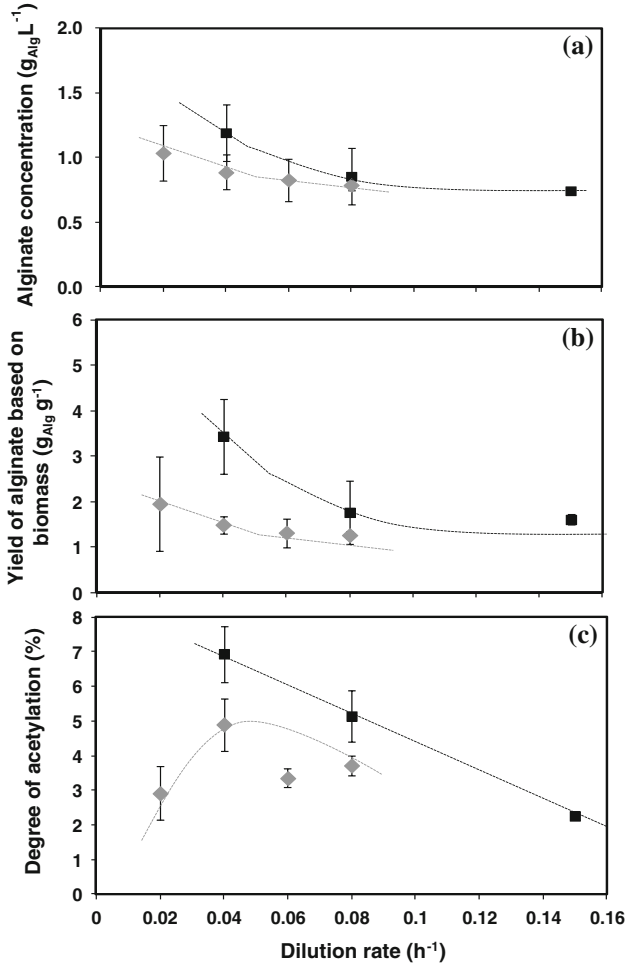

Fig. 4 Steady-state alginate concentration (**a**), alginate yield based on total biomass (**b**), and degree of alginate acetylation (**c**) for cultivations at 1 % (*filled diamond*) and 9 % (*filled square*) of DO

DO, the % C-mol that was channeled into PHB biosynthesis (mainly provided by acetyl-CoA) was higher (3.8 %) compared with that used for alginate acetylation (1.5 %). In contrast, in the cultures developed at 9 % DO, the % C-mol used for PHB biosynthesis was negligible (0.3–0.5 %), suggesting that acetyl-CoA could be diverted to alginate acetylation, because the % of C-mol used for this purpose was of 2.8 % (Table 2). The fact that content of PHB was higher in the cultivations conducted at 1 % DO could have been the result of the increased levels of $NADPH^+/NADP$ [28] under low DO. However, it would be of interest to evaluate alginate acetylation in mutant strains of *A. vinelandii* unable to produce PHB to confirm this behavior.

In the cultivations conducted at 9 % DO, not only were the highest levels of alginate acetylation achieved but also increases in alginate yields were obtained relative to the other conditions evaluated, and this behavior was reflected in the % C-mol used for alginate, mainly at $D = 0.04\ h^{-1}$ (Table 2). It must be emphasized that the values of $Y_{Alg/X}$ reached under such growth conditions were much higher than those reported previously for batch [20] and some chemostat cultures of *A. vinelandii* [6–8, 26]. Our results suggested that under high DO (9 %), the whole process of alginate biosynthesis was favored, including the alginate acetylation. In this line, previously Leitáo and Sá-Correia [15] in *P. aeruginosa* found that expression of the genes *algA*, *algC*, and *algD* and the activities of their encoded enzymes (the bifunctional enzyme phosphomannose isomerase/guanosine diphosphomannose pyrophosphorylase, the phosphomannomutase, and the GDP-mannose dehydrogenase, respectively) were up-regulated by increases in oxygen concentration within a range of 0–10 % DO.

Effect of the specific growth rate

The acetylation degree was also affected by the dilution rate (μ) (Fig. 5b). The highest values were achieved at a very low D ($0.04\ h^{-1}$). This is highly relevant because, even though the behavior of *A. vinelandii* in chemostat cultivations has been widely studied, in general, the values of D evaluated by other authors were higher than $0.05\ h^{-1}$ [1, 6, 8, 12, 14, 26]. Previously, Annison and Couperwhite [1] evaluated alginate acetylation in chemostat cultures of *A. vinelandii* at different values of D. In contrast to the

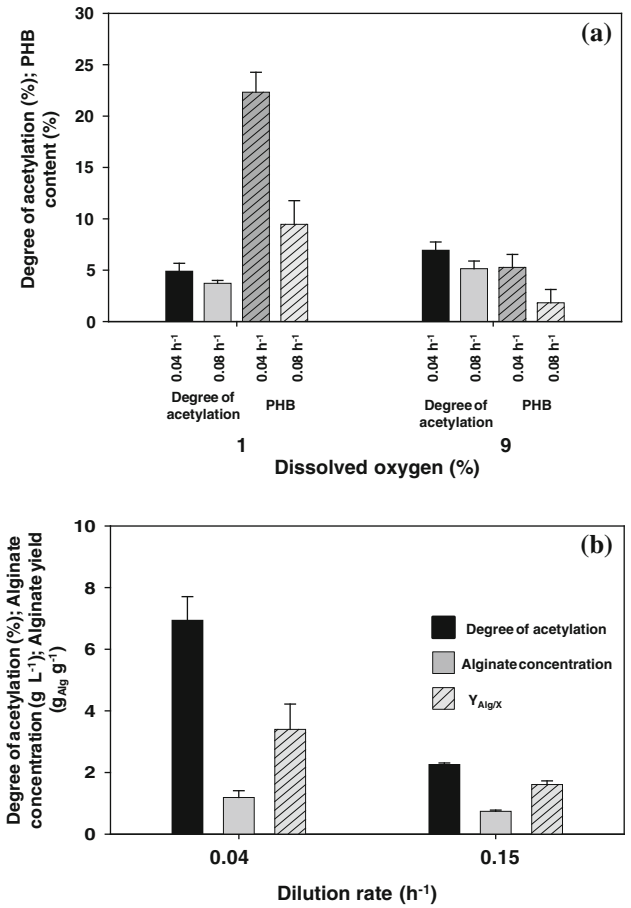

Fig. 5 Degree of alginate acetylation and PHB content at 1 and 9 % DO for $D = 0.04$ and 0.08 h^{-1} (**a**); degree of alginate acetylation, alginate concentration and alginate yield based on biomass at 0.04 and 0.15 h^{-1} for cultivations at 9 % DO (**b**)

results found in the present study, those authors observed that the acetylation degree of alginates increased a high dilution rate (0.32 h^{-1}). However, the conditions and experimental range reported by Annison and Couperwhite [1] were considerably different to those used in the present study. Firstly, a strict control of DO at 1 and 9 % DO was achieved in our study; while in the mentioned study, the DO oscillated between 20 and 30 %. Another important difference was the range of D evaluated; Annison and Couperwhite [1] tested values of D of 0.16, 0.18, and 0.32 h^{-1}, in contrast to the present study in which the range of D evaluated was lower (0.02–0.15 h^{-1}).

It should be emphasized that the highest values for alginate acetylation were achieved under low dilution rates, mainly at 9 % DO, correlating with the increase in the alginate yields observed under such conditions (Fig. 5b). Previously, other authors have reported that the highest alginate yields ($Y_{Alg/X}$ and $Y_{Alg/G}$) were obtained at low values of μ in cultivations of the *A. vinelandii* mutant strain

Table 2 Carbon usage

DOT (%)	D (h^{-1})	% C-mol$_{Alg}$	% C-mol$_{Acetyl}$	% C-mol$_{Biomass}$	% C-mol$_{PHB}$	% C_{used}
1	0.04	19.5	1.5	14.2	3.8	38.9
	0.08	17.6	1.0	17.2	1.7	37.5
9	0.04	25.7	2.8	9.3	0.5	38.2
	0.08	18.8	1.5	14.9	0.3	35.5

For the calculation of each % C-mol, the following considerations were done: 24.6 g_{CDW} = 1C-mol for % C-mol$_{biomass}$; 26.0 g $_{Hydroxybutiric\ acid}$ = 1C-mol for % C-mol$_{PHB}$; 32.3 g $_{Mannuronic}$ = 1C-mol for % C-mol$_{Alg}$; 21.5 g $_{Acetyl}$ = 1C-mol for % C-mol$_{Acetyl}$.

SML2 using exponential, fed-batch cultivations [24], and in chemostat cultivations of *Pseudomonas mendocina* [27]. In *A. vinelandii*, alginate production is involved in cyst formation under desiccation conditions in which nutrient limitations are occurring and therefore very low specific growth rates might be obtained. The results obtained in the present study support the hypothesis that low specific growth rates may have an important role in alginate biosynthesis. Although the regulation of alginate biosynthesis is a complex process that is not completely understood, it is well known that alginate biosynthesis could be positively regulated at the gene expression level by sigma factors such AlgU and RpoS [18]. The latter is a central regulator present during the stationary growth phase (in which growth rates are close to zero) in bacteria, and it also participates in transcriptional regulation of the PHB biosynthetic genes (*phbBAC*) [11], in agreement with the increased PHB accumulation observed at the lowest dilution rates at 1 % DO.

Conclusions

It has been demonstrated in glucose-limited chemostat cultivations of *A. vinelandii*, that DO and μ determine the degree of acetylation of alginates produced by this bacterium. Changes on DO and μ affect the carbon fluxes used for respiration and PHB production and indirectly the degree of acetylation of alginates, suggesting that alginate acetylation is highly dependent of the acetyl-CoA availability. In addition, alginate acetylation was also affected by the alginate biosynthetic process, showing that the increased alginate acetylation correlated with increased alginate yields.

Acknowledgments Financial support of DGAPA-UNAM (grant IN110310-3). PhD grant of Tania Castillo from CONACYT. The authors acknowledge the helpful discussions with Dr. Cinthia Núñez and Dr. Álvaro Lara.

Conflict of interest The authors declare that they have no competing interests.

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