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Alginate production by *Azotobacter vinelandii* DSM576 in batch fermentation

E Parente, MA Crudele, M Aquino and F Clementi

Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Universita della Basilicata, I-85100 Potenza, Italy

The kinetics of growth and alginate production from glucose in a nitrogen and phosphate-rich medium by *Azotobacter vinelandii* DSM576 were studied in a laboratory fermenter at pH 7 and 35°C. Batch fermentations were carried out both without control of dissolved oxygen concentration (DO) and at 1, 2, 5 and 10% DO. Although growth was faster at higher DO, maximum biomass concentration was lower. No alginate was produced at 10% DO. Alginate production was faster at 5 and 2% DO but higher alginate concentrations and yields were obtained without DO control. Alginate production was growth-associated at 5% DO, but significant amounts of alginate were produced after growth had stopped at lower DO values. In fermentations without DO control the molecular weight of the polymer reached a maximum ($11–17.6 \times 10^4$) when specific growth rate was between 0.02 and 0.04 h^{-1} and residual concentration of ammoniacal nitrogen was between 0.01 and 0.02 g L^{-1} and then sharply decreased.

Keywords: alginate; *Azotobacter vinelandii*; kinetics; batch fermentation

Introduction

Alginates are linear copolymers, composed of (1→4)-linked residues of β-d-mannuronic acid and its C-(5)-epimer-α-l guluronic acid, which naturally occur as the major intercellular polysaccharide of marine brown algae (*Phaeophyceae*). From a commercial point of view the most important characteristics of alginates are their ability to form viscous solutions and gels. Their intrinsic viscosity, which determines their ability to induce viscosity in solution, mainly depends on the molecular weight of the polymer, and, secondarily, on its composition, whereas the capability of forming strong brittle gels reacting with Ca^{2+} ions is due to the long sequences of homopolymeric regions of l-guluronic acid residues along the chain [26]. Due to these properties, alginates can be used in a wide range of industrial applications mainly in the food, biomedical, pharmaceutical, agricultural and textile fields [21,25].

At present all commercial alginates are extracted from a few species of brown algae [26]. However, synthesis of alginate-like polymers has been demonstrated in several bacterial species belonging to the genera *Azotobacter* and *Pseudomonas* [1,7,8,11,17,22,24]. Since several useful microbial polysaccharides are today commercially produced through fermentation processes [29], alginate-producing bacteria could be of interest as an alternative industrial source of alginates. *Azotobacter vinelandii* seems to be the best candidate given the absence of pathogenic organisms in the *genus* and its capability of synthesizing polymers with long homopolymeric sequences of l-guluronic acid residues when cultivated in media containing Ca^{2+} ions [2]. Moreover, present knowledge of the genetics and biosynthesis of alginates in *A. vinelandii* may allow the tailoring of composition and block distribution of the polymer through expression of the appropriate epimerase genes in high alginate-producing strains [10].

Alginate production by *A. vinelandii* has been studied in nitrogen and phosphate-limited [12,13], nitrogen-limited [4] or rich [3,30] media, but very little information on the kinetics of fermentation at controlled pH and on the effect of dissolved oxygen (DO) on alginate yields is available [13,23,30]. In previous studies the optimal conditions for alginate production by *A. vinelandii* DSM576 were determined in shaken flask fermentations in phosphate and nitrogen-rich buffered media [5] and the kinetics of this process were investigated [6]. Product formation was partially growth-associated with a marked increase in the polymer biosynthesis after the cessation of growth. However, alginate production occurring during the late phase of growth may be of no practical value because of its low average molecular weight (MW). In shaken flask fermentations we found that the MW of the secreted polymer increased during the exponential cell growth phase until the ammoniacal nitrogen content fell below a critical value of approximately 0.01 g L^{-1}, after which the MW decreased linearly with time [6]. This may be due to the release of periplasmic alginases by cells lysing at the end of growth [15].

The aim of this work was to investigate the kinetics of production and of alginate polymerization/degradation during batch growth of *A. vinelandii* DSM576 in a laboratory fermenter at controlled pH.

Materials and methods

Bacterial strain

Azotobacter vinelandii DSM576 was obtained as a freeze-dried culture from Deutsche Sammlung vono Mikroorganismen (DSM, Braunschweig, Germany). Stock cultures were grown on Tryptose Soy Broth (TSB) (Difco, Detroit, MI, USA) for 24 h at 35°C and then maintained frozen (–80°C) in TSB containing 25% glycerol.

Correspondence: Dr E Parente, Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Universita della Basilicata, Via Anzio, 10, 85100 Potenza, Italy
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Culture media

The seed medium [5,19] had the following composition (per liter of distilled water): 20 g glucose; 0.6 g $(NH_4)_2SO_4$; 2 g Na_2HPO_4; 0.3 g $MgSO_4 \cdot 7H_2O$; 6 g yeast extract (Oxoid, Basingstoke, UK); 11.56 g (50 mM) 3(*N*-morpholino)-propane-sulphonic acid monosodium salt (MOPS-Na) buffer (pH 7.2). MOPS-Na was omitted in the production medium, since pH was controlled by external addition of NaOH. All the media used were sterilised at 121°C for 15 min.

Growth conditions and fermentation apparatus

One millilitre of the frozen stock culture was used to inoculate a 100-ml Erlenmeyer flask containing 27 ml of seed medium. The seed culture was incubated on a rotary shaker at 300 rpm and 35°C for 48 h and used to inoculate (4% vol/vol) two 250-ml baffled Erlenmeyer flasks containing 50 ml of the seed medium. After an incubation of 24 h at 35°C and 120 rpm the seed culture was used to inoculate the production medium in the fermenter to obtain an initial optical density (OD) of 0.4 at 600 nm. A 3-L (working volume) stirred tank reactor (Applikon Dependable Instruments, Schiedam, Netherlands) equipped with two six-bladed Rushton turbines and an ADI 1020 controller were used. The fermenter was filled with 2.14 L of the production medium and sterilized at 121°C for 15 min. All fermentations were carried out in batch at 35°C, pH 7.0 (by addition of sterile 6 M NaOH) and with a pressure on the tank top (P_L) of 1.2 bar. Foam was controlled by automatic addition of a sterile 10% solution of Antifoam A (Fluka Chemie, Buchs, Germany). Dissolved oxygen concentration (DO) was measured by a polarographic electrode (Mettler Toledo AG, Greifensee, Switzerland). Two replicate fermentations were carried out at a fixed agitator speed (n) of 400 rpm and the DO was left to vary freely. Six further fermentations were carried out at controlled (by automatically varying agitator speed) DO (one at 1%, two at 2%, one at 5%, two at 10%): the measured DO values during most of the fermentations were close to the setpoints, with mean values of 0.8, 1.8, 5.3 and 10.0, respectively. The software Biowatch 2.26 (Applikon) was used for data acquisition (pH, temperature, DO, speed, cumulated alkali and antifoam addition).

Analytical methods

Culture broth samples were centrifuged at 7850 × *g* at 10°C for 30 min by using a refrigerated superspeed centrifuge (Centrikon model T124, Kontron Instruments, Milan, Italy) to remove bacteria and capsular material. The pellet containing bacteria and capsular material was suspended in 10 mM ethylenediamine tetraacetic acid tetrasodium salt for 2 min to solubilise the cell-associated alginate, and finally centrifuged as described above. The alginate-free cell precipitate was washed twice with distilled water, centrifuged, dried at 105°C till constant weight to yield the biomass concentration (X). The supernatant was frozen at −20°C until needed for chemical analyses or immediately used to recover exopolysaccharide by adding 3 volumes of 95% v/v ethanol [5]. After centrifugation at 7850 × *g* at 4°C for 20 min, the alginate-based sediment was washed twice with 95% v/v ethanol, recentrifuged and desiccated under vacuum at 40°C in a centrifugal evaporator (Centrivap, Labconco, Kansas City, MI, USA). Spectrophotometric assays were used for the measurement of alginate (P) [14] and ammoniacal nitrogen N [27] and residual glucose (S) [20] concentrations.

Intrinsic viscosity values (μ) were measured as previously described [6] and used to predict the average molecular weight (MW) of all the bacterial alginates under study *via* a Mark-Houwink regression [6].

Statistics

Linear and nonlinear regressions were carried out by using the software Systat 5.2.1 for Macintosh (Systat Inc, Evanston, IL, USA).

Reagents

Unless otherwise indicated all reagents were obtained from BDH (Poole, UK).

Results

The kinetics of growth and alginate production by *A. vinelandii* DSM576 in batch culture were studied in a laboratory fermenter at controlled pH. Two replicate fermentations were carried out at fixed agitation speed (400 rpm). The kinetics of growth, alginate production and glucose consumption were almost identical, and only slight differences were observed in the kinetics of nitrogen consumption and alginate polymerization (data not shown). The time course of one of the replicate fermentations is shown as an example in Figure 1. Dissolved oxygen (DO) concentration fell below 2% within a few hours and increased only when both growth and alginate production were completed (after about 75 h). Alginate production was almost linear from 7 to 70 h, with an average productivity (r_P) of 0.06 g L^{-1} h^{-1}. Although most alginate was produced

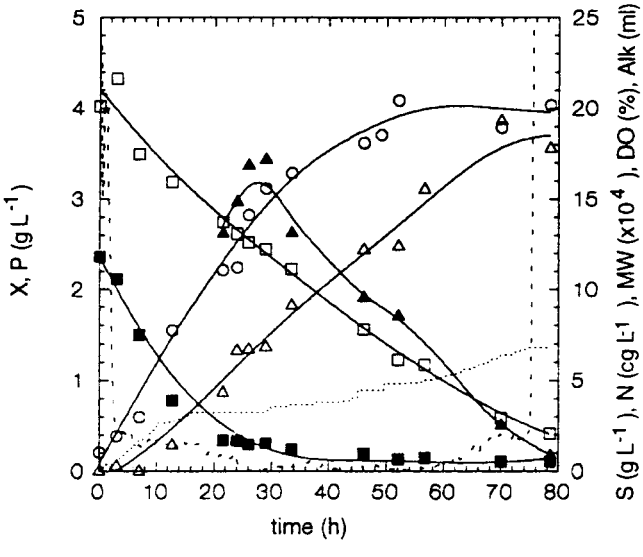

Figure 1 Kinetics of growth (cell dry weight ○, X, g L^{-1}), alginate production (△, P, g L^{-1}), glucose (□, S, g L^{-1}) and ammoniacal nitrogen (■, N, cg L^{-1}) consumption, and molecular weight (▲, MW, x 10^4) of the polymer during batch fermentation by *A. vinelandii* DSM576. Continuous lines were obtained by Distance Weighted Least Square Smoothing. Dissolved oxygen concentration (DO, %, dashed line) and alkali consumption (Alk, ml, dotted line) are also shown.

during growth, production continued after growth had significantly slowed down. The molecular weight (MW) of the polymer reached a peak (17.6×10^4) at 30 h and then decreased. Although the maximum alginate concentration (P_{max}, 3.9 g L^{-1}, with a yield, $Y_{P/S}$, of 0.23 g alginate per g of glucose consumed) was obtained at the end of fermentation (70 h), at this point the average MW had decreased to 2.5×10^4.

To investigate the effect of dissolved oxygen concentration (DO) on growth and alginate production, six further fermentations were carried out at controlled DO (one at 1%, two at 2%, one at 5% and two at 10%). Growth, alginate production and glucose consumption are compared in Figure 2a, b and c, respectively; the kinetics of replicate fermentations (2% and 10%) was very similar and only one of the replicates is shown.

Growth was faster when DO was kept at values ≥2%, but maximum biomass concentration (X_{max}) and biomass yield per unit of glucose consumed ($Y_{X/S}$) decreased significantly (Figure 2a, Table 1). At DO 5 and 10%, a pronounced cell lysis occurred at the end of fermentation (with significant increase in pH and foaming). At DO 1% oxygen limitation may have severely slowed down growth compared to fermentations without DO control: DO was below 1% for 90% of the time and agitation speed was always lower than 400 rpm.

No alginate was produced at DO 10%. At DO 5%, alginate production ceased at the end of growth, when residual glucose concentration was below 2 g L^{-1} (Figure 2a–c). In the other fermentations alginate production continued after growth had significantly slowed down (Figure 2a and b). Higher alginate concentrations and yields were obtained in fermentations with DO control (Table 1). Alginate production was slower when DO was kept at 1% probably because of oxygen limitation. At DO 2% and 5%, alginate production ceased before glucose was exhausted and a slight decrease in alginate concentration was observed thereafter. When compared to fermentations without DO control, alginate productivities were higher, but final alginate concentration and yield were lower (Figure 2b, Table 1).

With the exception of the fermentation carried out at DO 1%, where 5 g glucose L^{-1} were still present after 80 h, more than 90% of the glucose had been consumed by the end of fermentation (Figure 2c) in all fermentations.

A generalized logistic equation was used to smooth the raw data and obtain estimates of X (cell dry weight) and P (alginate concentration) [9]:

$$C_i = K/[1 + \exp(a_0 + a_1 \cdot t + a_2 \cdot t^2 + a_3 \cdot t^3)] \quad (1)$$

where C_i is cell dry weight or alginate concentration, K is a constant which was arbitrarily set to 1.2 times the

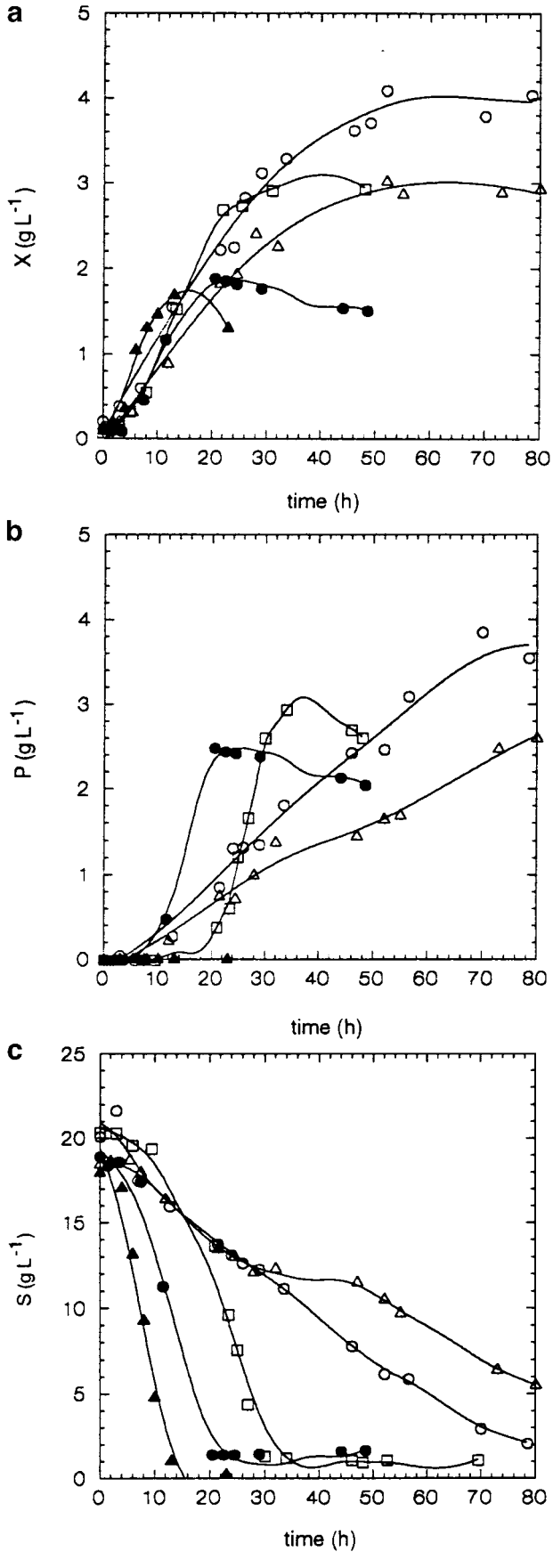

Figure 2 (a) Kinetics of growth (cell dry weight, X, g L^{-1}) of *A. vinelandii* DSM576 during batch fermentation. (b) Kinetics of alginate production (P, g L^{-1}) by *A. vinelandii* DSM576 during batch fermentation. (c) Kinetics of glucose consumption (S, g L^{-1}) by *A. vinelandii* DSM576 during batch fermentation. (○) no DO control; (△) DO = 1%; (□) DO = 2%; (●) DO = 5%; (▲) DO = 10%. Continuous lines were obtained by Distance Weighted Least Square Smoothing.

Table 1 Growth and alginate production by *Azotobacter vinelandii* DSM576 in batch fermentations

DO_{set}[a]	X_{max}[b]	P_{max}[c]	$Y_{X/S}$[d]	$Y_{P/S}$[e]	r_P[f]
None	4.1	3.9	0.22	0.20	0.06
1%	2.9	2.6	0.22	0.20	0.03
2%	2.9	2.9	0.31	0.18	0.22
5%	1.9	2.4	0.10	0.14	0.20
10%	1.7	0	0.09	0	0

[a]DO setpoint.
[b]Maximum biomass concentration, g L^{-1}.
[c]Maximum alginate concentration, g L^{-1}.
[d]Biomass yield per unit glucose consumed, g g^{-1}.
[e]Alginate yield per unit glucose consumed, g g^{-1}.
[f]Average alginate productivity, g L^{-1} h^{-1}.

maximum value of C_i, a_i are parameters of the equation and t is the time in h. The equation provided an excellent fit for the experimental data (adjusted $r^2 > 0.98$). The first derivative of Equation (1) can be calculated as follows:

$$dC_i/dt = -C_i \cdot (1 - C_i/K) \cdot (a_1 + 2 \cdot a_2 \cdot t + 3 \cdot a_3 \cdot t^2) \quad (2)$$

and used to estimate growth rate (r_X) and alginate production rate (r_P). The specific growth rate (μ) and the specific alginate production rate (π) were calculated by dividing the absolute rates by the smoothed values of X.

The relationship between specific alginate production rate (π) and specific growth rate (μ) was non linear (Figure 3). At low DO concentrations (fermentations without DO control and with DO 1%), π increased rapidly from 0 to 0.034 g g^{-1} h^{-1} as μ increased to 0.08 h^{-1} and then increased slowly to 0.04 g g^{-1} h^{-1} as μ increased to 0.20 h^{-1}. At DO 2%, π increased sharply at μ between 0.03 and 0.32 h^{-1}. At DO 5%, π reached a peak at $\mu = 0.3$ h^{-1} and then decreased sharply at higher μ values.

In fermentations without DO control the molecular weight (MW) of the polymer reached a maximum (11–17.6×10^4) when the specific growth rate was between 0.02 and 0.04 h^{-1} (Figure 4), and then decreased. Only a limited number of data points were available for fermentations with DO 1% or 2%. Therefore we are unable to make any inference on the relationship between μ and MW in fermentations at controlled DO.

Since μ was correlated with residual ammoniacal nitrogen concentration (N, data not shown), and the molecular weight (MW) of the polymer was related to μ, residual N could be used, at least in fermentations without DO control, to estimate the harvesting time in order to maximize MW (maximum MW was obtained when N was between 0.01 and 0.02 g L^{-1}). Although N can be measured on-line to predict when the average MW reaches a peak, a relatively expensive instrumentation is needed. Alkali consumption may provide a simple in-line estimate of N. In fact, a good linear correlation was found between consumed ammonia nitrogen and meq alkali L^{-1} added to control pH to 7 (N_0–N = 0.50 + 0.747·meq NaOH, $r^2 = 0.94$) in all fermentations (data not shown).

Discussion

We studied alginate production by *A. vinelandii* DSM576 in a laboratory fermenter at controlled pH and evaluated the effect of dissolved oxygen concentration (DO) on growth and alginate production. Studies on alginate production by *A. vinelandii* at controlled pH and/or DO are rare. Horan *et al* [13] evaluated the effect of DO in continuous fermentations under phosphate limitation with a dilution rate of 0.1 h^{-1}. A 'respiratory protection' response with increase in growth rate, hydrolysis of poly β-hydroxybutyrate and oxidation of the carbon source was observed when DO was increased from 1 to 10%. Although some

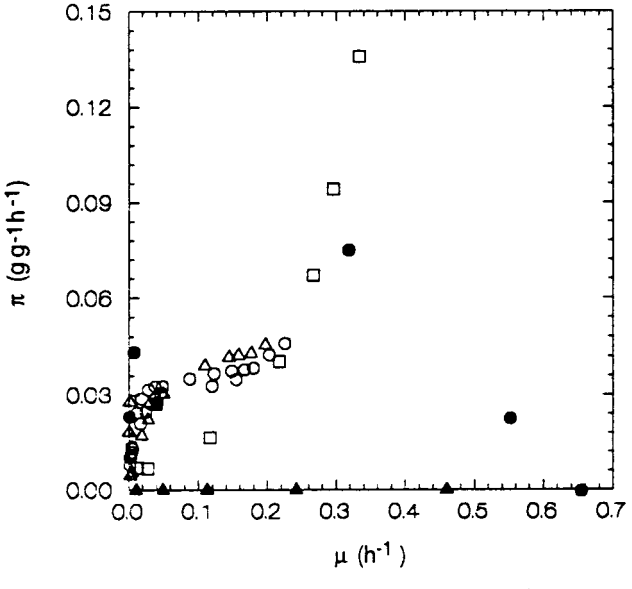

Figure 3 Relationship between specific growth rate (μ, h^{-1}, calculated by generalized logistic smoothing) and specific alginate production rate (π, g g^{-1} h^{-1}) during batch fermentation by *A. vinelandii* DSM576. (○) no DO control; (△) DO = 1%; (□) DO = 2%; (●) DO = 5%; (▲) DO = 10%.

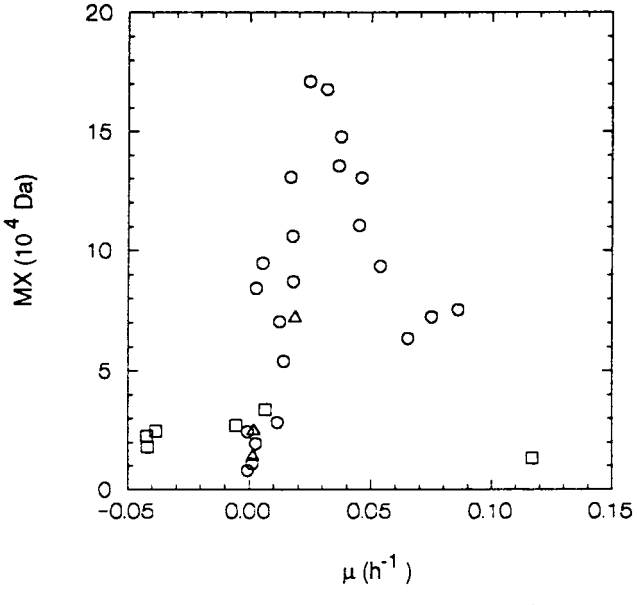

Figure 4 Relationship between specific growth rate (μ, h^{-1}, calculated by generalized logistic smoothing) and molecular weight of the polymer (MW, x 10^4) during batch fermentation by *A. vinelandii* DSM576. (○) no DO control; (△) DO = 1%; (□) DO = 2%.

of the key variables studied by Horan *et al* [13] were not monitored, we found that growth of *A. vinelandii* DSM576 in batch fermentation showed a similar response: in fact, growth rate increased and biomass yield significantly decreased at DO ≥5%. These results are consistent with those obtained by Pena and Galindo [23] who found that specific growth rate was significantly reduced at DO 0.5% (0.06 h^{-1}) compared to DO 5% (0.22 h^{-1}).

We found that alginate production was strongly influenced by dissolved oxygen concentration. Maximum alginate concentration was obtained in fermentations without DO control. Alginate production was significantly faster at DO 2 or 5%, but no alginate was produced at DO = 10%. This finding is in agreement with the fact that poly-β-hydroxybutyrate is accumulated under oxygen limitation, whereas a good level of aeration is required to obtain high alginate production [3]. However, at too high DO an inefficient carbohydrate utilisation occurs due to the high respiration rate of *A. vinelandii* [3]. Horan *et al* [13] found that the rate of alginate production in continuous culture was maximum when DO was between 1 and 5% and then decreased, though alginate was still produced at DO 10%. Lower alginate yields compared to batch fermentations were attributed to high activity of alginate lyases at both low (0%) or high (10%) dissolved oxygen concentrations. Tinoco *et al* [30] obtained higher specific productivities (π) at high DO (0.034 g g^{-1} h^{-1}) compared to fermentations without DO control (0.024 g g^{-1} h^{-1}) although alginate produced in the former condition apparently had a lower viscosity. On the other hand Pena and Galindo [23] found that alginate concentration, productivity and viscosity of the broth were higher at DO 5% compared to DO 0.5%. In our study, the specific alginate production rate (π) was influenced by both DO concentration and specific growth rate (μ). In fermentations without DO control and at DO = 1%, maximum values of π (0.042–0.048 g g^{-1} h^{-1}) were comparable with the values reported at DO = 10–15% by Tinoco *et al* [30]. The specific alginate production rate (π) increased dramatically with increasing DO concentration up to 5% and specific growth rate (up to 0.138 g g^{-1} h^{-1}), but a decrease in π was observed at DO 5% when μ was higher than 0.3 h^{-1}. This may be in agreement with the hypothesis that limited availability of the lipid carrier [28] may cause a reduction of alginate synthesis at high growth rates, when the carrier is used mostly for the synthesis of cell envelope.

Alginate production has been described as either completely growth-associated [16] or as partially growth-associated [3,4,12]. The well known Luedeking and Piret (LP) equation [18] has been used to model polysaccharide production [6,16,30]:

$$r_P/X = \pi = Y_{P/X} \cdot \mu + m_P \qquad (3)$$

where $Y_{P/X}$ is the yield of alginate per unit cell biomass (growth associated production term) and m_P the specific production formation rate at zero cell growth rate (non-growth associated production term). According to the LP model the relationship between π and μ should be linear. In a previous study [6] the kinetics of alginate production by *A. vinelandii* DSM576 in shaken flasks was modeled using the LP model: the contribution of non-growth associated production was found to be 78%. In the present study alginate production by *A. vinelandii* DSM576 appeared to be strongly related to growth at DO 5% while non-growth associated production was observed at lower DO values. The relationship between π and μ was non linear, especially at high DO values although this may be due to the approach used to estimate μ and π. Preliminary attempts to fit the LP model to the data using the procedure previously described [6], showed that the model was completely inadequate for fermentations at DO 2 or 5% but a reasonably good fit ($r^2 = 0.97$) was obtained for fermentations at DO 1% and without DO control. Estimates of $Y_{P/X}$ (which ranged from 0.34 ± 0.06 to 0.41 ± 0.09 g g^{-1}) were in the same order as those obtained in shaken flask fermentations (0.40 ± 0.15 g g^{-1}), but m_P was significantly lower (0.009 ± 0.001 to 0.010 ± 0.002 compared to 0.032 ± 0.005 g g^{-1} h^{-1} in shaken flasks) thus showing that in batch fermentations at controlled pH, growth-associated alginate production is prevalent. Since the same strain and substrate (except for the presence of the buffering agent Na-MOPS in shaken flasks) were used in both cases, the difference in kinetics did not depend on strain differences [3] but on differences in growth conditions (ie pH control and aeration).

The molecular weight (MW) of alginate is critical for the intrinsic viscosity and the thickening properties of the polymer [26]. Studies on the kinetics of polymerization and depolymerization of alginate are rare [6,23]. Pena and Galindo [23] found that high MW fractions were more abundant in alginate produced at DO 5% rather than at DO 0.5%. On the other hand we obtained both in shaken flasks [6] and in laboratory fermenters (this work), alginate with the highest average MW ($>10 \times 10^4$) in fermentations without DO control. However, only a few data points were available for fermentations at controlled DO because of difficulties in collecting alginate for MW measurements, and we are unable to make any inference on the effect of DO on maximum MW of the polymer. We found that rate of alginate polymerization/depolymerization by *A. vinelandii* DSM576 in batch fermentations at controlled pH followed a pattern similar to that observed in shaken flasks [6], although maximum MW was obtained later in shaken flasks (40 h) compared to laboratory fermenters (24–30 h). In both cases the rate of polymerization was apparently related to μ and residual ammoniacal nitrogen (N) concentration, decreasing sharply when N was lower than 0.011 (shaken flask) or 0.01–0.02 (fermenter) g L^{-1}. Although further experiments are needed to confirm the value of this relationship in other media, on-line measurement of N or its indirect estimation by in-line neasurement of alkali addition may provide a useful decisional tool to prevent reduction of average MW. The decrease in molecular weight of alginates produced by *Azotobacter* species [15] and *Pseudomonas fluorescens* and *P. putida* [7] has been attributed to periplasmic [15] or intracellular [7] alginate lyases released in the medium after cell lysis at the end of growth. In shaken flasks polymerization of the alginate continued for some time after growth had significantly slowed down, until the critical N concentration was reached. In the present study significant increase in cell dry weight occurred after the critical values for specific growth

rate (μ) and N were reached and after maximum MW was obtained. The increase in cell dry weight after the exhaustion of N may have been due to the accumulation of poly-β-hydroxybutyrate [3] and significant release of alginate lyase may have occurred before the end of growth. However, neither of these parameters was measured, and further study is in progress to elucidate the kinetics of polymerization and depolymerization of alginate in *A. vinelandii* DSM576.

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