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Effect of ammonium sulphate concentration and agitation speed on the kinetics of alginate production by *Azotobacter vinelandii* DSM576 in batch fermentation†

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Growth and alginate production by *Azotobacter vinelandii* DSM576 as a function of initial ammonium sulphate concentration (0.45–1.05 g l^{-1}) and agitation speed (300–700 rpm) were studied in batch fermentations at controlled pH. The time course of growth, alginate production and substrate consumption and the effect of nitrogen concentration and agitation speed on kinetic parameters and on maximum alginate molecular weight (MW) was modelled using empirical equations. The kinetics of growth, alginate production and polymerization were deeply affected by agitation speed and, to a lesser extent, by inorganic nitrogen concentration. Average and maximum specific growth rate and maximum alginate MW all increased with agitation speed, and were higher at intermediate ammonium sulphate concentration. Maximum alginate MW (>250,000) was obtained at high agitation speed (600–700 rpm) and alginate depolymerization was limited or did not occur at all when the agitation speed was higher than 500 rpm, while at 400 rpm depolymerization significantly reduced the MW. However, alginate yield was negatively affected by increasing agitation speed. A good compromise between alginate yield (>2 g l^{-1}) and quality (MW>250,000) was obtained with agitation speed of 500–600 rpm and 0.75–0.90 g l^{-1} of ammonium sulphate. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 242–248.

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

Introduction

Alginates are a class of linear heteropolysaccharides composed of residues of β-D-mannuronic acid and α-L-guluronic acid, which are produced by brown algae [17,25] but also by members of the genera *Azotobacter* [7,9] and *Pseudomonas* [6,23]. Because of their ability to form viscous solutions, to stabilize emulsions and to form stable gels in the presence of Ca^{2+} ions, algal alginates are used in a variety of food, pharmaceutical and biotechnological applications [17,18,24]. The rheological properties of algal and bacterial alginates depend on their composition and molecular weight (MW) [25]. The composition of alginates produced by *Azotobacter vinelandii* is similar to those produced by brown seaweeds [3,5], even if the mannurosyl residues of the former are acetylated to a varying degree. This, and the possibility of tailoring the MW and composition of bacterial alginates by affecting the expression of critical genes (alginate lyase, alginate epimerases, acetylase) or by altering medium composition and culture conditions may make bacterial alginates a promising substitute of algal alginates in selected biotechnological and biomedical applications [3,21].

The effect of medium composition and fermentation conditions on alginate production by *A. vinelandii* was studied by several authors [1,2,5,10,11,19,20,22,26]. Alginate production is either growth associated [12,19] or partially associated to growth [1,2,5,19]. The relationship between growth and alginate production is strongly dependent on fermentation conditions. Aeration has a dramatic effect on the amount and quality of the polymer produced and on the kinetics of alginate production: both low and high agitation rates are detrimental to alginate production [1,19]. In continuous cultures at controlled pH, alginate production is maximum when the dissolved oxygen concentration (DO) is between 1% and 5% [11] or between 2% and 5% [22] compared to 10%. Higher alginate productivities were obtained in batch fermentations at DO>2% compared to fermentation without DO control, but the latter showed a higher kinematic viscosity [26]. However, in another study [20] alginate produced at 5% DO had a higher average MW than that produced at 0.5% DO. We reported that although alginate production with *A. vinelandii* DSM576 in a phosphate- and nitrogen-rich medium was faster at 2% or 5% DO, more alginate was produced when no DO control was used; moreover, alginate production appeared to be growth associated at higher DO and partially growth associated at low DO (1%) or without DO control [19]. Unfortunately, alginate MW reached a peak (170,000) before maximum alginate concentration was obtained, and then rapidly decreased to <10,000. Both in shaken flasks [5] and laboratory fermenters [19], alginate degradation started when the inorganic nitrogen in the medium was exhausted. Because alginate MW is critical for the quality of the polymer in food and other applications, the objective of this work was to

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investigate the effects of agitation rate and initial inorganic nitrogen content on alginate production and quality.

Materials and methods

Bacterial strain

A. vinelandii DSM576 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Stock cultures were grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) for 24 h at 35°C and then maintained frozen (−80°C) in TSB containing 25% (vol/vol) glycerol.

Culture media

The seed medium [4,16] had the following composition: 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); 11.56 g (50 mmol l^{-1}) 3(*N*-morpholino)-propane-sulphonic acid monosodium salt (MOPS-Na) buffer (pH 7.2), distilled water 1 l. In the production medium MOPS-Na was omitted because pH was controlled by external addition of NaOH, and the $(NH_4)_2SO_4$ concentration varied between 0.45 and 1.05 g l^{-1} (Table 1). All media were sterilised at 121°C for 15 min.

Growth conditions and fermentation apparatus

One milliliter of the frozen stock culture was used to inoculate a 250-ml baffled Erlenmeyer flask containing 50 ml of seed medium. The culture was incubated on a rotary shaker (Unimax 2010, Heidolph, Germany) at 120 rpm and 35°C for 48 h and used to inoculate (3% 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 revolutions min^{-1}, 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 (usually 60 ml). A 3-l autoclavable stirred tank reactor (Applikon Dependable Instruments, Schiedam, Netherlands) equipped with two six-bladed Rushton turbines (d=45 mm) and an ADI1020 controller was 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 cultures at 35°C, pH 7.0 (by addition of sterile 6 mol l^{-1} NaOH), with constant aeration (80 l h^{-1}, adjusted using a needle valve on a flow meter) and with an absolute pressure of 1.2 bar on the tank top (P_L, adjusted by using a needle valve on the air line downstream of the fermenter). Foam was controlled by automatic addition of a sterile 20% emulsion of Antifoam A (Fluka Chemie, Buchs, Germany). DO was measured by a polarographic electrode (Mettler Toledo AG, Greifensee, Switzerland). Fourteen fermentations were carried out at different agitator speeds (300–700 rpm) and $(NH_4)_2SO_4$ concentrations (AS, 0.45–1.05 g l^{-1}) according to the central composite design described in Table 1. Two replicate fermentations were carried out for treatments 1–4 and 9. The software Biowatch 2.26 (Applikon) was used for in-line 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. The pellet containing bacteria and capsular material was suspended in 10 mmol l^{-1} ethylenediamine tetraacetic acid tetrasodium salt for 2 min to solubilise the cell-associated alginate, and finally centrifuged as described above. The alginate-free biomass was washed with distilled water, centrifuged, dried at 105°C till constant weight to measure biomass concentration (X). The first supernatant was frozen at −20°C until needed for chemical analyses or used immediately to recover the exopolysaccharide by precipitation with three volumes of cold (−20°C) ethanol. The precipitate was recovered by centrifugation (7850×*g*, 10 min, 4°C), resuspended in one volume distilled water and reprecipitated. After a final wash with ethanol, the alginate was redissolved in water and freeze-dried. Average MW was estimated from intrinsic viscosity using a Mark–Houwink regression as described previously [5]. Alginate (P), ammoniacal nitrogen (N) and residual glucose (S) concentrations were measured by spectrophotometric methods as previously described [19].

Statistics

Linear and nonlinear regressions were carried out by using the software Systat 7 for Windows (SPSS Inc., Chicago, IL, USA). Parameters of the kinetic models were estimated directly from the raw data using Madonna 6.0 for MacOS [15].

Reagents

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

Table 1 Experimental values for parameters obtained in the central composite design for the evaluation of the effect of inorganic nitrogen concentration (AS, as g $(NH_4)_2SO_4$ l^{-1}) and agitation speed (rpm) on growth and alginate production by *A. vinelandii* DSM576 in batch fermentation at controlled pHX_{max}, maximum biomass concentration; r_{Xav}, average growth rate; P_{max}, maximum alginate concentration; r_{Pav}, average alginate production rate; MW_{max}, maximum alginate MW; I_{max}, maximum value index of alginate production and quality=P×MW/8. For treatments 1–4 and 9 the average±SD for two replicate fermentations is shown

Treatment	AS (x_1) (g l^{-1})	rpm (x_2) (min^{-1})	X_{max} (g l^{-1})	r_{Xav} (g l^{-1} h^{-1})	P_{max} (g l^{-1})	r_{Pav} (g l^{-1} h^{-1})	MW_{max} ($\times 10^4$)	I_{max} (g l^{-1})
1	0.60	400	4.2±0.4	0.09±0.01	4.2±0.2	0.06±0.01	17±2	3.0±0.3
2	0.90	400	3.9±0.2	0.05±0.01	2.9±0.2	0.07±0.01	14±3	2.0±0.5
3	0.60	600	4.5±0.5	0.11±0.01	2.0±0.4	0.08±0.01	31±5	7.8±0.6
4	0.90	600	4.3±0.1	0.09±0.01	2.4±0.3	0.04±0.01	33±4	8.7±0.5
5	0.45	500	4.8	0.10	3.4	0.07	16	6.9
6	1.05	500	3.8	0.14	1.7	0.07	3	0.4
7	0.75	300	4.6	0.05	1.3	0.02	2	0.2
8	0.75	700	3.7	0.12	1.7	0.05	35	7.2
9	0.75	500	4.0±0.3	0.12±0.01	2.2±0.4	0.06	25±2	5.6±0.6

Results

Fourteen batch fermentations at controlled pH (7.0) and temperature (35°C) were carried out to evaluate the effect of initial ammonium sulphate concentration and agitation speed on alginate production by *A. vinelandii* DSM576. The experimental values for X_{max} (maximum biomass concentration), r_{Xav} (average growth rate, calculated by dividing X_{max} by the elapsed fermentation time), P_{max} (maximum alginate concentration), r_{Pav} (average alginate production rate, calculated by dividing P_{max} by the elapsed fermentation time), MW_{max} (maximum alginate MW) of all fermentations are summarized in Table 1.

The kinetics of growth, alginate production and polymerization were affected by both initial ammonium sulphate concentration and agitation speed. The time course of fermentations at 0.6 g l^{-1} and 400 rpm, 0.9 g l^{-1} and 400 rpm, 0.75 g l^{-1} and 300 rpm, 0.75 and 700 rpm is shown as an example in Figure 1A–D, respectively. Even if constant aeration was provided, DO fell to <0.5% within 26–30 h in all fermentations but rose again to 20–50% when some glucose (1–2 g l^{-1}) was still available (data not shown). Growth started immediately, without any lag, and continued even after ammonium nitrogen decreased to less than 10 mg l^{-1}. Alginate production started after 8–12 h and, if

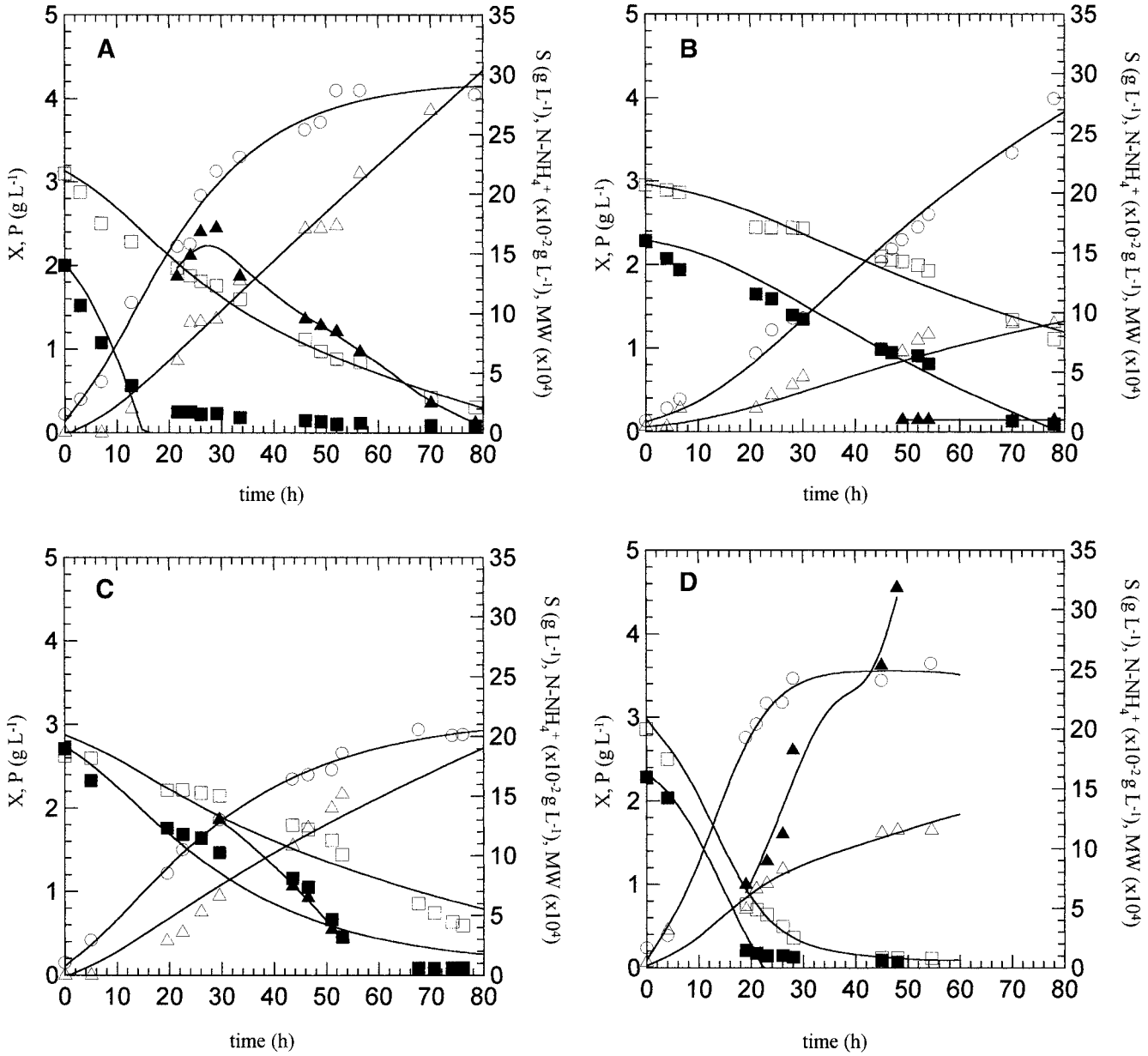

Figure 1 Kinetics of growth (○, biomass concentration; *X*, g l^{-1}), alginate production (△, alginate concentration; *P*, g l^{-1}), sugar (□, glucose concentration; *S*, g l^{-1}), ammonium nitrogen consumption (■, N-NH_4^+ concentration; *N*, $\times 10^{-2}$ g l^{-1}) and alginate polymerization/depolymerization (▲, average MW, $\times 10^4$) by *A. vinelandii* DSM576 in batch fermentation at controlled pH (7.0) and temperature (35°C). (A) 400 rpm, 0.6 g l^{-1} $(NH_4)_2SO_4$; (B) 400 rpm, 0.9 g l^{-1} $(NH_4)_2SO_4$; (C) 300 rpm, 0.75 g l^{-1} $(NH_4)_2SO_4$; (D) 700 rpm, 0.75 g l^{-1} $(NH_4)_2SO_4$. Continuous lines were drawn using the parameters of the model described in the text (X, P, S, N) or distance-weighted least square smoothing (MW). Only part of the data of fermentation (C) are shown to allow the representation of all fermentations on the same scale.

glucose was still available, usually continued after growth had stopped or significantly slowed.

The following models were used to calculate the kinetic parameters for growth, alginate production and substrate (S or N) consumption:

$$r_X = \mu_{max}\{1 - \exp[-K(X_{max} - X)/X]\}X \quad (1)$$

$$r_P = Y_{P/X} r_X + m_P X \quad (2)$$

$$r_X = -r_X(1/Y_{X/S}) - r_P(1/Y_{P/S}) \quad (3)$$

$$r_N = r_X Y_{N/X} \quad (4)$$

where r_X is the growth rate (g l^{-1} h^{-1}), μ_{max} the maximum specific growth rate (h^{-1}), K is a shape parameter of the model (adimensional; as K increases there is a sharper transition from maximum to minimum values of μ), X_{max} maximum biomass concentration (g l^{-1}), X biomass concentration (g l^{-1}), r_P alginate production rate (g l^{-1} h^{-1}), $Y_{P/X}$ alginate yield per unit biomass (growth associated production term, adimensional), m_P the specific production rate at 0 growth rate (non-growth associated production, h^{-1}), r_S glucose consumption rate (g l^{-1} h^{-1}), $Y_{X/S}$ and $Y_{P/S}$ growth and alginate yield per unit glucose consumed, r_N ammoniacal nitrogen consumption rate (g l^{-1} h^{-1}) and $Y_{N/X}$ the ammonium nitrogen consumed per unit biomass produced. Model parameters where estimated directly from experimental data using the curve-fitting option of Madonna 6.0 (a software that uses numerical integration to solve differential equations) and are shown in Table 2. The fit between experimental and calculated data was good ($R^2 > 0.96$) for most fermentations for growth, alginate production and glucose consumption, but rather poor for inorganic nitrogen consumption (Figure 1A to D). Therefore estimates of $Y_{N/X}$ are not shown in Table 2. The models described by Equations 1–4 are only meant to provide an empirical representation of a complex phenomenon such as growth and alginate production DSM576 during batch fermentation. In fact, they do not take into account growth limitation due to reduced oxygen availability and induction of alginate production. Equation 1 was originally used by Frame and Hu [8] to model growth of attachment-dependent cells; however, it provides a better fit than the symmetric logistic equation used in previous papers [5,18] because the growth kinetics of *A. vinelandii* DSM576 becomes asymmetric because of oxygen limitation. Equation 2 is the well known Luedeking–Piret model [14]. Empirical polynomial equations ($y = a_0 + a_1x_1 + a_2x_2 + a_{11}x_1^2 + a_{12}x_1x_2 + a_2x_2^2$ where y is the response variable and x_1 and x_2 are the coded values for AS and rpm; $x_1 = (AS - 0.75)/0.15$; $x_2 = (rpm - 500)/100$) were used to evaluate the relationship between kinetic parameters and initial ammonium sulphate concentration and agitation speed. Statistically significant relationships were obtained only for μ_{max} and K:

$$\mu_{max} = 0.206 + 0.024x_2 - 0.015x_1^2 - 0.020x_2^2 \qquad R^2 = 0.60$$

$$K = 0.311 + 0.197x_1 - 0.200x_2 + 0.288x_1^2 \qquad R^2 = 0.62$$

Due to low R^2 both have limited predictive value. Maximum values of μ_{max} are obtained at 0.75 g l^{-1} and 600 rpm. Both these predictions are in agreement with the experimental data. K increases with agitation speed; at any given agitation speed minimum values of K should be obtained at intermediate (0.6–0.9 g l^{-1}) inorganic nitrogen concentration.

With some exceptions, alginate production was partially associated with growth and started during the exponential phase and continued after growth had stopped (i.e., both $Y_{P/X}$, the yield coefficient for growth associated alginate production, and m_P, the specific alginate production rate at 0 growth rate, were significantly different from 0). However, for the treatment with the lowest ammonium sulphate concentration (treatment 5, Table 2), most of alginate production occurred when growth had stopped, while for the highest ammonium sulphate concentration and the lowest agitation speed (treatments 6 and 7, respectively, Table 2) alginate production was strictly growth associated.

No significant relationship was found between biomass or alginate yield and initial ammonium sulphate concentration and agitation speed. Biomass yield varied only slightly for most treatments (0.29–0.32); however, low values for $Y_{X/S}$ were obtained at high agitation speed, which also resulted in low alginate yield.

The kinetics of alginate polymerization was greatly affected by agitation speed and, to a lesser extent, by initial ammonium sulphate concentration (Figure 2). At the lowest agitation speed (300 rpm, Table 1, Figure 1C) and at the highest nitrogen concentration, alginate MW was very low (ca. 3000). At 400 rpm

Table 2 Estimated values for the parameters of used to model growth, substrate consumption and alginate production by Equations 1–3 *A. vinelandii* DSM576 in batch fermentations at controlled pH with different initial ammonium sulphate concentration (AS) and agitation speed (rpm) X_{max}, maximum biomass concentration; μ_{max}, maximum specific growth rate; K, shape parameter of the growth model; $Y_{P/X}$, alginate yield per unit biomass; m_P, specific alginate production rate at 0 growth rate; $Y_{X/S}$ and $Y_{P/S}$, growth and alginate yield per unit glucose consumed; r_N, ammoniacal nitrogen consumption rate. See text for details. For treatments 1–4 and 9 the average±SD for two replicate fermentations is shown

Treatment	AS (x_1) (g l^{-1})	rpm (x_2) (min^{-1})	X_{max} (g l^{-1})	μ_{max} (h^{-1})	K	$Y_{P/X}$	m_P (h^{-1})	$Y_{X/S}$	$Y_{P/S}$
1	0.60	400	4.1±0.3	0.17±0.01	0.40±0.06	0.33±0.05	0.012±0.001	0.30±0.03	0.24±0.02
2	0.90	400	4.0±0.1	0.15±0.03	0.16±0.05	0.10±0.04	0.017±0.001	0.32±0.01	0.23±0.02
3	0.60	600	4.5±0.4	0.25±0.03	0.63±0.03	0.08±0.01	0.014±0.002	0.29±0.01	0.16±0.03
4	0.90	600	4.3±0.1	0.19±0.04	0.56±0.05	0.15±0.03	0.007±0.001	0.29±0.03	0.17±0.01
5	0.45	500	4.9	0.13	0.72	0.001	0.019	0.29	0.18
6	1.05	500	3.9	0.14	2.56	0.39	0	0.30	0.13
7	0.75	300	4.5	0.06	0.31	0.35	0	0.33	0.15
8	0.75	700	3.8	0.26	1.2	0.27	0.006	0.2	0.09
9	0.75	500	4.1±0.2	0.17±0.05	0.30±0.08	0.50±0.06	0.002±0.001	0.31±0.03	0.18±0.03

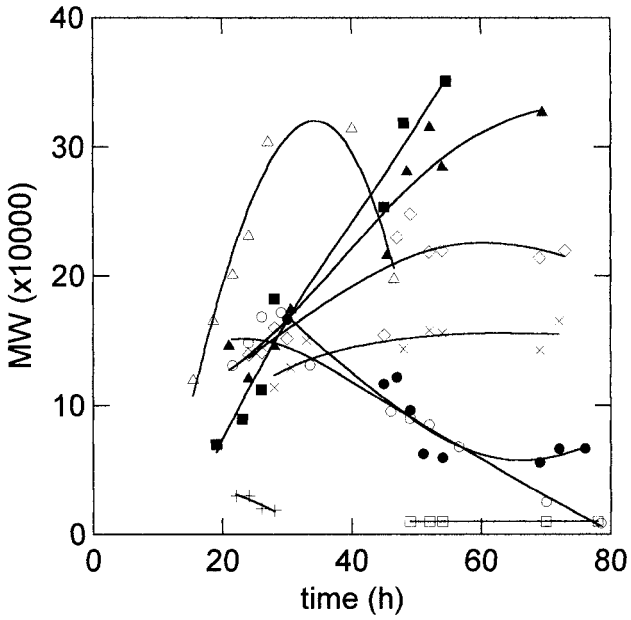

Figure 2 Kinetics of alginate polymerization/depolymerization by *A. vinelandii* DSM576 in batch fermentation at controlled pH (7.0) and temperature (35°C) at different initial ammonium sulphate concentrations (AS, g l^{-1}) and agitation speeds (rpm). (○) AS = 0.6, 400 rpm; (●) AS 0.9, rpm = 400; (△) AS = 0.6, rpm = 600; (▲) AS = 0.9, rpm = 600; (×) AS = 0.45, rpm = 500; (+) AS = 1.05, rpm = 500; (□) AS = 0.75, rpm = 300; (◇) AS = 0.75, rpm = 500; (■) AS = 0.75, rpm = 700. Continuous lines were drawn using distance-weighted least square smoothing.

(Figure 1a) the MW reached a maximum (120,000–160,000) and then decreased to <10,000. At 500 rpm with 0.75 or 0.45 g l^{-1} ammonium sulphate, maximum alginate MW (250,000 and 160,000, respectively) was obtained after ca. 50 h and then remained stable. The highest alginate MW was obtained at 600 and 700 rpm (310,000 to 350,000); no alginate degradation was observed at 700 rpm (Figure 1D; in this case, however, the maximum alginate MW was obtained at the end of fermentation) nor at 600 rpm with 0.9 g l^{-1} ammonium sulphate, while some degradation (with a final MW of 180,000) was observed at 600 rpm and 0.6 g l^{-1} ammonium sulphate (Figure 2). Both product concentration and MW are important in alginate production, because alginate of low MW is of little value. To take this into account, the value of I, an index, which keeps into account both alginate production and quality was calculated ($I = P \times MW/8$; 8×10^4 is the average MW of alginate from *L. hyperborea*, marketed by BDH, taken as an example of commercial alginate). As a consequence of the kinetics of alginate production and polymerization/degradation, the high values of I were obtained at 500–700 rpm and <1.05 g l^{-1}. In fact, even if more alginate was produced at 400 rpm, the low final MW would significantly reduce the value of the product.

Empirical second-order polynomial equations were also used to model the relationship between X_{max}, P_{max} (maximum experimental biomass and alginate concentration), r_{Xav} (average growth rate), r_{Pav} (average alginate production rate, calculated by dividing the final alginate concentration by the time between the start and end of alginate production), MW_{max} (maximum observed MW) and I_{max} (maximum value for I) with AS and rpm. Statistically significant relationships were obtained only for average growth rate and maximum alginate MW:

$$r_{Xav} = 0.064 + 0.017x_2 + 0.0132x_1^2 + 0.006x_2^2 \qquad R^2 = 0.82$$

$$MW_{max} = 28.8 + 8.5x_2 - 4.3x_1^2 - 2.3x_2^2 \qquad R^2 = 0.89$$

Only the coefficients that were significantly different from 0 ($p < 0.05$) are shown. The model for r_{Xav} predicts that growth rate is affected mainly by agitation speed, increasing as agitation increases; at any given speed maximum growth rate would be obtained for low (<0.6 g l^{-1}) or high values (>0.9 g l^{-1}), which is only in partial agreement with experimental data. The model for MW_{max} predicts that maximum MW increases with agitation speed; at any given agitation speed maximum MW would be obtained at intermediate (0.75 g l^{-1}) concentration.

No significant relationship was obtained for maximum biomass and alginate concentration and average alginate productivity. High final biomass concentration with treatments 5 (AS=0.45, rpm=500) and 7 (AS=0.75, rpm=300) may reflect unbalanced growth with synthesis of poly-β-hydroxyibutyrate due to low nitrogen or oxygen availability. At 500 rpm the final alginate concentration was inversely related to the initial AS concentration. The highest final alginate concentrations were obtained with treatments 1 (AS=0.6 g l^{-1}, rpm=400) and 5 (AS=0.45 g l^{-1}, rpm=500); in both cases, alginate production started sooner than with other treatments and lasted longer. The average alginate production rate was between 0.06 and 0.08 g l^{-1} h^{-1} for most treatments. At low agitation speed (300 rpm) both growth and alginate production rate were low.

Discussion

In this study the effect of initial inorganic ammonium sulphate concentration (AS) and agitation speed (rpm) on growth and alginate production by *A. vinelandii* DSM576 in a complex medium was evaluated in batch fermentations at controlled pH using a composite factorial design and empirical modelling. DO was left to vary freely because in a previous study [19] more alginate was produced without DO control compared to fermentations with DO control (1–10%). Moreover, in batch fermentations, DO control by varying agitation speed was effective only for part of the fermentation (i.e., as long as the minimum agitation speed needed for effective mixing did not result in an oxygen transfer rate that exceeds the oxygen uptake rate of the culture), and the DO level was above the set point. A set of semiempirical unstructured models (Equations 1–4) was used to model the kinetics of growth, alginate production and substrate consumption. These models allowed a satisfactory reconstruction of experimental data (with the exception of inorganic nitrogen consumption). Unfortunately, modelling of the relationship between response variables (Tables 1 and 2) and input variables (AS and rpm) using empirical second-order polynomials provided statistically significant models only for maximum MW and parameters related to growth rate. As expected, both average growth rate and maximum specific growth rate (estimated using a semiempirical model), increased as agitation speed (and hence oxygen transfer rate) increased but a reduction in biomass yield was evident at the highest agitation speed, perhaps as a result of a respiratory protection mechanism,

which is well documented in *A. vinelandii* [11,22]. A slight increase of biomass yield was also observed at low (300 rpm) agitation speed, perhaps due to increased synthesis of poly-β-hydroxybutyrate that occurs at very low DOs [11,22].

To our knowledge, there are no studies on the effect of initial inorganic nitrogen concentration on growth and alginate production by *A. vinelandii* during batch or continuous fermentations at controlled pH in laboratory fermenters. In fact, most studies have been conducted under dinitrogen fixation conditions, with no addition of fixed nitrogen [2,11,22] or at a fixed nitrogen concentration in rich media [19,26]. In this study, 0.45–1.05 g l^{-1} ammonium sulphate was added to a rich medium containing 6 g l^{-1} yeast extract. It is known that yeast extract is stimulatory for growth and xanthan production by *Xanthomonas campestris* [12] and a similar behavior has been observed for alginate production by *A. vinelandii* [26]. We found that, except at the lowest agitation speed, ammonium nitrogen was consumed rapidly but, even when it was reduced to very low levels, growth usually continued. Although uptake of organic nitrogen (as measured by reduction of protein content of the supernatant) did take place (data not shown), low levels of ammonium sulphate (0.45 g l^{-1}) resulted in reduced maximum specific growth rate but increased biomass concentration, which in turn may reflect unbalanced growth due to nitrogen limitation. However, at 400 to 600 rpm, AS>0.75 g l^{-1} resulted in reduced maximum biomass concentration and maximum specific growth rate. The effect of the interaction between AS and rpm on biomass yield or growth rate was not statistically significant, but this may only reflect the complex non linear dependency of growth on both substrate composition and oxygen availability.

Although alginate production was partially associated to growth for most treatments, in agreement with previous findings [1,2,5,19], non growth-associated or strictly growth-associated alginate production were also observed (Table 2). In most fermentations alginate production started before inorganic nitrogen was exhausted. At 500 rpm, increasing levels of AS resulted in decreasing alginate production and in different kinetics of production. In fact, at the lowest level of AS, alginate production occurred mainly when growth had stopped, while at 0.75 and 1.05 g AS l^{-1} alginate production was mostly growth associated (Table 2). More than 2.9 g l^{-1} alginate were produced only when agitation speed was 400–500 rpm and AS was between 0.6 and 0.9 g l^{-1}: both higher and lower values of agitation speed and initial ammonium sulphate concentration resulted in reduced alginate production. However, alginate with a high MW would have a higher commercial value [21]: to take this into account we developed an index (I, Table 1) that weights alginate concentration using the MW of a commercial alginate. High values of I (>5) resulted either from a high average MW (>250,000) or from a combination of a medium MW and a high alginate concentration (Table 1). Due to depolymerization, at 400 rpm the value of I was always low. In fact, agitation speed had a dramatic effect on the kinetics of alginate polymerization and depolymerization: even if more alginate was produced at 400 rpm, due to an early depolymerization its final MW was usually low (<40,000); at 600–700 rpm maximum average MW was higher (up to 370,000) and depolymerization was limited. A good compromise between alginate yield (>2 g l^{-1}) and quality (MW>250,000) was obtained with 500–600 rpm and 0.75–0.90 g l^{-1}. The alginate yield (<3.9 g l^{-1}) obtained in this study is still far from being of any commercial interest: increase in alginate yield may be possible by replacing glucose with sucrose, which results in a higher theoretical alginate yield [22], by increasing the initial substrate concentration, or by adopting fed-batch strategies, but further study is needed in this area.

In previous studies [5,19] we found that the onset of depolymerization was related to reduction of the inorganic nitrogen content of the medium below 10–15 mg N-NH_4^+ l^{-1} that corresponded to low values for the specific growth rate (<0.03 h^{-1}). It is also well known that, with some exceptions [26], higher DO values result in higher alginate MW [20,22]. Depolymerization of alginate following nutrient (phosphate) limitation has also been demonstrated [22]. In our study, however, alginate depolymerization did not correlate at all with the residual inorganic nitrogen content of the medium nor with the specific growth rate or DO, but was dependent mainly on agitation speed. Moreover, a phosphate-rich medium (initial phosphate content, 1.34 g l^{-1}) was used and even at the end of fermentation phosphate concentration was never limiting (>0.8 g l^{-1}, data not shown). During dinitrogen fixation, alginate production is considered to be one of the defense mechanisms of the cell against damage caused to nitrogenase by high oxygen tension, and it has been suggested that the size of the capsule may be a self-regulating mechanism to control intracellular oxygen concentration [22]. However, in this study alginate production was carried out under conditions that prevented dinitrogen fixation (because of the levels of inorganic and organic nitrogen): DO was always very low (<0.5%) during most of the fermentation, even at the highest agitation speed, and polymerization seemed to be more related to the oxygen transfer rate or shear stress (which increase with agitation speed) than to DO.

The data gathered in this study are far from sufficient to formulate a satisfactory model to explain the effect of inorganic nitrogen concentration and agitation speed on the kinetics of growth, alginate production and alginate polymerization/depolymerization. The event that triggers depolymerization is also unclear: the onset of depolymerization may be related to the concomitant occurrence of two or more limitations (low levels of oxygen, nitrogen, phosphate) or to the energetic state of the cells. Further experiments with fed-batch and continuous fermentations at different inorganic nitrogen concentrations and controlled DO levels, with measurements of biomass composition, are needed to clarify the relationship between nitrogen concentration, oxygen availability and growth and alginate production.

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