Process Biochemistry 46 (2011) 1879–1883

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

# Continuous cultures for alginate production by *Azotobacter vinelandii* growing at different oxygen uptake rates

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ARTICLE INFO

*Article history:*
Received 1 April 2011
Received in revised form 22 June 2011
Accepted 23 June 2011

*Keywords:*
Alginate
Oxygen transfer rate
Continuous culture
Carbon balance
*alg8*
*algL*

ABSTRACT

Continuous cultures for alginate production from *Azotobacter vinelandii* were conducted at different specific oxygen uptake rates ($q_{o2}$) under oxygen-limited conditions. As a result of varying the agitation rate, the $q_{o2}$ increased from 2.2 to 6.0 mmol $g^{-1}$ $h^{-1}$. An increase in $q_{o2}$ up to 4.8 mmol $g^{-1}$ $h^{-1}$ resulted in an increase of alginate concentration, as well as in the specific alginate production rate, which can be attributed to that carbon flux was less diverted towards biomass production. A lower alginate molecular weight (869 kDa as compared to 1350 kDa) was obtained by increasing the $q_{o2}$ from 2.2 to 4.8 mmol $g^{-1}$ $h^{-1}$ and a higher gene expression of *algL* (about 8-fold) was observed, which could explain the decrease in the alginate molecular weight. The results showed for the first time that alginate production and carbon distribution are affected by the $q_{o2}$. The manipulation of this parameter could be used as a control strategy for alginate production.



## 1. Introduction

Alginates are exopolysaccharides produced by algae and bacteria such as *Azotobacter vinelandii*, an obligate aerobe that can grow under nitrogen-fixing conditions and that also produces poly-β-hydroxybutyrate (PHB) [1]. Alginates are co-polymers of (1-4)-linked residues of β-D-mannuronic acid (M) and α-L-guluronic acids (G) that are widely used as thickening agents, stabilizer, emulsifier, and drug carriers in the food, pharmaceutical and chemical industries [2]. At present, to our knowledge, the extraction from marine algae is the only economic way to produce alginate. However, alginates for some specific applications in the biomedical field cannot be achieved with algal alginates, whereas bioprocesses to produce bacterial alginates can be implemented in order to manipulate the characteristic of the polymer. It is known that differences in the M/G ratio and the alginate molecular weight determine the chemical and physical properties of the polymer, and hence its application potential [3]. Previous studies have showed the importance of the dilution rate ($D$), the dissolved oxygen tension (DOT) and the oxygen supply conditions on alginate production by *A. vinelandii* [4–6]. Up to now, few studies have focused on the influence of the oxygen transfer rate (OTR) on alginate production in terms of quantity and quality, such as the molecular weight and the viscosifying power [5,7]. Regarding alginate biosynthesis, it has been proposed that the catalytic subunit of the alginate polymerase complex is the Alg8 protein (encoded by *alg8*), which is located in the cytoplasmatic membrane and has been reported as essential for alginate polymerization [2,8]. Previous evidence has demonstrated that additional *alg8* gene copies in *Pseudomonas aeruginosa* cells (other alginate-producing bacterium) impact on alginate yield, suggesting that Alg8 is a key bottleneck for alginate biosynthesis [10]. On the other hand, different studies have established that alginate-lyase (encoded by *algL*) is responsible of the degradation of the polymer produced by *A. vinelandii* [9]. Taking into account that the OTR influences the alginate molecular weight [5] and that *A. vinelandii* has a very high respiration rate [11] under oxygen-limited conditions, the oxygen uptake rate (OUR) could be related to the alginate production and the expression of some genes involved in its biosynthesis, as was suggested by Díaz-Barrera et al. [12]. However, to our knowledge there is not experimental evidence to support this possibility. In order to expand the understanding on the production of alginate in a bacterial bioprocess, this work focused on the influence of the specific oxygen uptake rate ($q_{o2}$) on carbon distribution (percentage of carbon atoms from glucose converted to alginate, biomass and $CO_2$) and alginate production during continuous culture in a stirred tank bioreactor. A possible relationship between alginate molecular weight, and expression of genes *algL* and *alg8* was also evaluated. To our knowledge, this is the first report where carbon distribution in alginate fermentation and genes expression of *algL* and *alg8* in cultures of *A. vinelandii* is reported.

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doi:10.1016/j.procbio.2011.06.022

## 2. Materials and methods

### 2.1. Microorganism and culture medium

The wild-type *A. vinelandii* ATCC-9046 was used. The bacterium was grown in a medium of the following composition (per litre): 10 g glucose, 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 $Na_2MoO_4$ $2H_2O$, 0.027 g $FeSO_4$. Under our experimental conditions, cultures conducted under nitrogen fixation have showed a highest alginate production as compared to non-fixation conditions (data not shown).

### 2.2. Inoculum preparation

The cultures were carried out in 500 ml Erlenmeyer flasks with 100 ml of culture medium. The initial pH was adjusted to 7.0, using 2 M NaOH. The microorganism was incubated at 30 °C at 200 rpm in an orbital shaker (New Brunswick, USA). After 18 h the cells were transferred (10%, v/v) to a bioreactor operated in batch mode.

### 2.3. Bioreactor operation

Chemostat cultures were conducted in a 3-l bioreactor system (Applikon, Schiedam, Netherlands) with 2 l working volume. The cultures were carried out at 30 °C and pH 7.0 controlled by automatic addition of 2 M NaOH. DOT was not controlled but was monitored with a polarographic oxygen probe (Ingold, Mettler-Toledo). The bioreactor was agitated at 300, 400, 500, 600 and 700 rpm, aerated at 2 l $min^{-1}$ (1.0 vvm), and operated in continuous culture mode with a $D$ of 0.08 $h^{-1}$. This $D$ was selected considering a 75% of $\mu_{max}$, which was determined in batch cultures (data not shown). The OTR was manipulated by changing agitation rates. The continuous culture reached steady-state when the optical density at 540 nm ($OD_{540}$) and the glucose concentration remained constant (<10% variation) after at least 3 residence times. Samples of cultures (20 or 30 ml) were withdrawn from the bioreactor for analytical measurements. All analyses were carried out in triplicate. The results shown are the mean value of two independent chemostat cultures and the standard deviations among replicates are given.

### 2.4. Biomass, glucose, PHB quantification, and viscosity

Biomass was estimated gravimetrically. The culture broth (10 ml) was mixed with a mixture (2 ml) of $Na_4EDTA$ (0.1 M) and NaCl (1.0 M) and then centrifuged at 7650 × $g$ for 10 min. The pellet was washed with 10 ml of distilled water and filtered through previously weighed Millipore filters (0.45 μm pore size). The filters were then dried at 85 °C to constant weight. Glucose was assayed for reducing power with a DNS reagent [13]. PHB was extracted from the cells using a mixture of chloroform/hypochlorite solution (30%, v/v) as described by Peña et al. [14]. PHB was hydrolyzed to crotonic acid using concentrated $H_2SO_4$. The crotonic acid was assayed using a HPLC-UV system with an Aminex HPX-87H ion-exclusion organic acid column. Elution was performed with 0.014 N $H_2SO_4$ at 0.7 ml $min^{-1}$ and 50 °C [15]. Culture broth viscosity was measured using a rheometer (Brookfield, LVDV II) at room temperature (20 °C), according to the instrument manufacturer.

### 2.5. Quantification of alginate concentration and molecular weight

Alginate concentration was quantified gravimetrically. Three volumes ice-cold of propan-2-ol was added to supernatant, and the resultant precipitate was filtered (0.22 μm Millipore filters), dried at 85 °C to constant weight. The mean molecular weight (MMW) of the 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) under the operating conditions previously described [5].

### 2.6. Gas analysis and respiratory measurements

Gas analysis was performed by online measurements of $O_2$ and $CO_2$ in the exit gas and compared with measurements taken at the inlet gas flow rate. 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 outlet gas stream was dried by a cooler and the volume flow (0.3 l $min^{-1}$) through the gas analyzer was kept constant with a thermal mass flow controller. The OTR and the carbon dioxide transfer rate (CTR) were determined from gas analysis and calculated by carrying out gas balances [16]. Respiratory quotient (RQ) was determined by its definition (RQ = CTR/OTR) using the OTR and CTR data.

### 2.7. Elemental analysis

Elemental biomass and alginate composition was determined using an analyzer for elemental CHN determination (CE Instruments, UK). Two milligrams sample were oxidized at 1000 °C and the resulting gases were determined using a thermal conductivity probe for carbon. Analyses were made on cells and alginate obtained at each steady-state. Carbon distribution at steady-state conditions was determined from reactor mass balances according to the previously described [16].

**Table 1**
Primers used in qPCR analysis.

| Gene | Forward primers (5′→3′) | Reverse primers (5′→3′) |
|---|---|---|
| *gyrA* | CGTGATGCTGATCAAGTTGG | TCCTCGTCGTCGAATAGCTC |
| *algL* | ATGCGCTACATGGAGAAAGG | TTGTACTCGGTGGTGAGCAG |
| *alg8* | GATCATCGGCTCCATCAAAT | GTCCGGAAAGAGACAACAGC |

### 2.8. RNA extraction and cDNA synthesis

*A. vinelandii* cells were harvested by centrifugation at 6870 × $g$ for 10 min at 4 °C and stored at −80 °C with RNAlater™ solution (RNA stabilization and protection solution) according to the manufacturer's protocol. Total RNA was isolated and purified from the samples using High Pure RNA Isolation kit (Roche Applied Science) and its concentration measured by 260/280 nm ratio absorbance. 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.

### 2.9. Quantitative real-time PCR assay

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 qPCR were designed using algorithm Primer 3 (Table 1). The level of the *gyrA* mRNAs was used as internal control [17] to normalize the results obtained for the *algL* and *alg8* mRNAs. The relative quantification of gene expression was performed using the standard curve method [18] with three measurements for each gene of each condition (300 and 500 rpm), giving a maximum standard deviation of 10%. The data are reported as relative expression levels compared to the expression levels of calibrator value (chemostat culture at 300 rpm).

### 2.10. Calculation of the specific alginate production rate and $q_{o2}$

Specific alginate production rate ($q_p$) and $q_{o2}$ were calculated in steady-state at each agitation rate, considering $D$, alginate concentration ($P$, mean value), OTR value (experimentally determined) and biomass concentration ($X$, mean value) in the bioreactor using the following equations:

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

$$q_{o2} = \frac{OTR}{X} \quad (2)$$

## 3. Results and discussion

### 3.1. Alginate, biomass and PHB production at different agitation rates

The effect of the agitation rate on biomass, alginate, PHB and glucose concentration in steady-state is shown in Fig. 1. A change in the agitation rate caused only a small variation on biomass concentration, varying between 1.5 and 1.9 g $l^{-1}$ (Fig. 1a). The glucose concentration in steady-state was higher than 3.5 g $l^{-1}$ at all the agitation rates tested. Considering that saturation constant ($K_s$) values lower than 0.1 g $l^{-1}$ in glucose-limited chemostat cultures has been reported [19], our chemostat cultures were not limited by carbon. On the other hand, at all the steady-states evaluated the level of DOT was nearly zero (data not shown), suggesting that the cultures of *A. vinelandii* were oxygen-limited. In order to confirm this condition, maximum OUR ($OUR_{max}$) values were calculated at all the steady-state, according the previously described [5]. $OUR_{max}$ values between 20 and 30 times higher as compared with the measured OTR were obtained, which support the existence of oxygen limitation in our continuous cultures.

As shown in Fig. 1b, an increase in agitation rate from 300 to 500 rpm improved alginate concentration from 0.3 g $l^{-1}$ to 1.1 g $l^{-1}$ (3.7-fold), and PHB accumulation decreased from 31 to 18% (w/w). A similar behavior has been previously described for batch cultures of *A. vinelandii*, obtaining between 0.5 and 1.5 g $l^{-1}$ of alginate and from 63 to 17% (w/w) of the dry cell weight of PHB by increasing the agitation rate (from 260 to 560 rpm) [5]. In this regard, it is known the difficult is to separate the synthesis of alginate and PHB in a wild-type organism [20] such as the one used in our work.