The production, molecular weight and viscosifying power of alginate produced by *Azotobacter vinelandii* is affected by the carbon source in submerged cultures

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Abstract

Alginate is a linear polymer composed of β-1,4 linked mannuronic acid and its epimer, α-L-guluronic acid, and frequently extracted from marine algae, as from bacteria such as *Azotobacter* and *Pseudomonas*. Here, we show the impact of conventional and unconventional carbon sources on *A. vinelandii* growth, alginate production, its mean molecular weight (MMW) and its viscosifying power. Starting with 20 g/L of sugars, the highest biomass concentration was obtained using deproteinized and hydrolyzed whey (6.67±0.72 g/L), and sugarcane juice (6.68±0.45 g/L). However, the maximum alginate production was achieved using sucrose (5.11±0.37 g/L), as well the highest alginate yield and specific productivity. Otherwise, the higher alginate MMW was obtained using sugarcane juice (1203±120 kDa), and the higher viscosifying power was obtained using deproteinized/ hydrolyzed whey (23.8±2.6 cps L/galg). This information suggests that it is possible to manipulate the productivity and molecular characteristics of alginates, as a function of the carbon source used. All this, together with the knowledge of the effects of environmental conditions will allow for high yields of high added value biopolymers.

Keywords: alginates; *Azotobacter vinelandii*; viscosifying power; unconventional carbon sources.

La producción, el peso molecular y el poder viscosificante del alginato producido por *Azotobacter vinelandii* es afectado por la fuente de carbono en cultivos sumergidos

Resumen

El alginato es un polímero lineal compuesto por ácidos β-1,4 manurónico y su epímero, α-L-gulurónico y con frecuencia se extrae de algas marinas, como también de bacterias como *Azotobacter* y *Pseudomonas*. En este trabajo, se presenta el impacto de diferentes fuentes de carbono convencionales y no convencionales en el crecimiento de *A. vinelandii*, producción de alginato, su peso molecular promedio (PMP) y su capacidad viscosificante. Todos los experimentos se iniciaron con 20 g/L de azúcares totales, donde la más alta concentración de biomasa se obtuvo utilizando suero de leche hidrolizado y desproteinizado (6.67±0.72 g/L), y jugo de caña de azúcar (6.68±0.45 g/L). Sin embargo, la producción máxima de alginato se logró utilizando sacarosa (5.11±0.37 g/L), así como el más alto rendimiento de alginato y productividad específica. Por otra parte, el mayor PMP de alginato se obtuvo con jugo de caña de azúcar (1203±120 kDa). Además, la capacidad viscosificante más alta se obtuvo utilizando suero de leche desproteinizado y hidrolizado (23.8±2.6 cps L/galg). Esta información sugiere que es posible manipular la productividad y las características moleculares de alginatos como función de la fuente de carbono utilizada. En conjunto con el conocimiento de los efectos de las condiciones ambientales se lograrían altos rendimientos de biopolímeros de alto valor agregado.

Palabras clave: alginatos, *Azotobacter vinelandii*, capacidad viscosificante, fuentes de carbono no convencionales.
1. Introduction

Alginites are polysaccharides composed of β-1,4 linked to mannuronic acid and its epimer, α-L-guluronic acid, and is used as a thickener, stabilizer, and gelling agent in the textile, pharmaceutical, and food industries [1,2]. These polymers are extracted from marine algae, and can also be obtained from bacterial sources such as the Azotobacter and Pseudomonas species [2-4]. The industrial importance of alginites is their ability to modify rheological properties of aqueous systems, and this is determined by the composition of the polymer. Alginate composition (or quality) is ruled by many factors, such as: the mean molecular weight (MMW), the polydispersity index (PI), the monomers ratio (mannuronic and guluronic acid residues, M/G ratio), the sequence pattern and the O-acetylation degree in mannuronic acid residues [5-9]. One of the main challenges in the production of microbial alginites is the ability to influence their quality by controlling the environmental conditions (such as dissolved oxygen, pH and temperature) and the culture medium, on bioreactors. This has been widely reported and reviewed [2,10].

Previous work has demonstrated the significant role of the culture medium components on alginate productivity by A. vinelandii, such as carbon source (C-source), calcium and phosphates concentration [6,11-13], and carbon to nitrogen ratio (C/N) [12,14]. In recent years, the effect of different nutrients on the alginate molecular characteristics has been reported, such as the effect of different nitrogen sources and the C/N ratio on MMW [14]. Moreover, other components of the culture medium such as 3-(N-morpholino)-propane-sulfonic acid (MOPS) affect the acetylation of alginate but not the MMW [15]. Also, the alginate produced by A. vinelandii by using 4-hydroxybenzoic acid as C-source is different to that produced using glucose in terms of its M/G ratio and acetyl groups [6].

The growth of A. vinelandii, the alginate production and its molecular characteristics are affected by operational factors in bioreactors, such as agitation speed [16-18], the concentration of dissolved CO2 [19], and dissolved oxygen tension (DOT) [17, 20-22]. In particular, DOT affects the M/G ratio [18], the acetylation degree [9] and the MMW [17,20,21,23]. Other studies succeeded in demonstrating that under the manipulation of the specific growth rate, the productivity and the MMW of alginate can be controlled. This through modification of the influx of carbon sources by setting up the dilution rate (D as the ratio of flux inlet/tank volume), in fed-batch cultures [24] and in continuous cultures [25,26]. However, in these continuous cultures both the manipulation of the growth rate and the concentration of the C-source in the influent medium modify the MMW of the alginate obtained [25].

Previous research reported that the specific consumption rate of sucrose is highly related with changes in alginate productivity [17]. Furthermore, a possible link between the specific carbon source consumption rate and the MMW of alginate has been proposed [25]. It can be assumed that the changes in metabolizing different C-sources may modify the production rate of mannuronic acid residues (through the enzymes AlgA, AlgC and AlgD), and in turn might be affecting the polymerization rate, where the key enzymes Alg8, Alg60 and Alg44 are involved [21,23]. As far as we know, there are no previous papers documenting the direct effects of non-conventional and conventional C-sources on alginate molecular weight, and its polydispersity index by A. vinelandii. Therefore, in the search for higher value-added alginites, we evaluated different carbon sources on the production, the mean molecular weight, the polydispersity index, the viscosifying power of alginate, the growth of A. vinelandii and the consumption of these sources.

2. Materials and Methods

2.1. Microorganism, culture media and growth conditions

The strain of Azotobacter vinelandii ATCC-9046 was used, and maintained by monthly subculture on Burk's agar (18%) slopes, and stored at 4°C [16,17]. A. vinelandii was grown in a modified Burk's medium of the following composition (in g/l): yeast extract (Difco) 3.0, K2HPO4 0.66, KH2PO4 0.16, MOPS 1.42, CaSO4 0.05, NaCl 0.2, MgSO4-7H2O 0.2, Na2MoO4-2H2O 0.0029, FeSO4-7H2O 0.027 [16]. The C-source for each experiment was added at a concentration of 20 g/L. A concentrated NaOH solution was used to adjust the initial pH to 7.2 in all cultures. 500X solution of salts MgSO4-7H2O, FeSO4-7H2O and Na2MoO4-2H2O were prepared and sterilized independently to avoid precipitation. The required volume of sterile salts was added at the moment of inoculation. Previously to sterilization, sugarcane juice was filtered through 0.45 µm filter membrane (MilliporeTM, USA). Acid hydrolysis of the whey was conducted at pH 5.5, 90°C for 20 minutes, protein was recovered by centrifugation at 5000 xg for 10 min. The pH was adjusted to 1.5 with HCl, and brought to 121°C for 30 minutes. Pre-cultures were performed in a rotary shaker (with a rotatory diameter of 2.54 cm) at 200 rpm and 29°C, for 24 h [16] by using three colonies (from Petri dishes) inoculated in 250-mL flasks, containing 50 mL of the medium.

Washed cells from pre-cultures were inoculated to start with an optical density near 0.15 absorbance units in 250 mL shake flask, containing 50 mL of modified Burk's medium, and incubated in a rotary shaker (with a rotatory diameter of 2.54 cm) at 200 rpm and 29°C. Under these conditions, the cells were grown under oxygen limitation [16]. The aim of using washed cells as inoculum was to avoid the exhaust inoculum broth components (as extracellular alginate-lyase activity and alginate). It has been shown that the exhausted broth components from the inoculum play important regulatory roles in alginate biosynthesis, and in determining its molecular weight [27]. Two flasks were removed from the incubator for kinetic analysis as needed [16].

2.2. Analytical determinations

Biomass and alginate were determined gravimetrically as previously described [16]. The total reducing sugars (TRS) were evaluated by the dinitrosalicicyc acid (DNS) method, and an acid hydrolysis was undertaken when necessary. A standard correlation was made for each carbon source. Viscosity was measured on a cone and plate viscometer (Wells-Brookfield,
USA) using a CP-52 cone at a rotational speed of 6 rpm, which corresponds to a shear rate of 12 s$^{-1}$ at room temperature [16]. Mean molecular weight (MMW), polydispersity index (PI) and molecular weight distributions (MWD) of alginates were estimated by gel-permeation chromatography as previously reported [16,21], and pullulans from Aureobasidium pullulans (from 5,800 to 1,600,000 Da) were used as standards. The polydispersity index (PI) was defined as the ratio of MMW (weighting the polymer molecules based on the weight of those having a specific molecular mass) to mean number weight (weighting the molecules based on the number of those with a specific molecular mass) [12,13]. All cultures were carried out at least in triplicate. Figs 1 and 2 show the mean value of at least three independent cultures and the standard deviation among replicas.

3. Results and Discussion

Five different carbon sources were evaluated (fructose, galactose, glucose, lactose, and sucrose) in modified Burk’s medium. Moreover, hydrolyzed and deproteinized liquid whey and sugarcane juice were also evaluated alone, or as a complement of Burk’s medium, always maintaining an initial concentration of 20 g/L of total reducing sugars (TRS). All cultures were carried out for 120 h and the final concentrations of biomass, alginates and TRS are shown in Fig. 1. If considering just the conventional carbon sources, the carbon source that yields the highest biomass (5.51±0.3 g/L) and alginate (5.11±0.37 g/L) production was sucrose, followed by glucose (5.47±0.65 and 3.1±0.70 g/L for biomass and alginate, respectively). Lower biomass growth and alginate production were obtained using fructose and galactose (Fig. 1A and 1B). These results confirm that A. vinelandii can use any of these three monomers to produce alginate as previously reported by Pindar and Bucke [28]. However, no biomass growth (nor alginate production) was observed using lactose as a carbon source in Burk’s medium [28].

Page et al. [29] demonstrated that large production of PHB (19 to 22 g/L) is supported when A. vinelandii is grown with complex, unrefined carbohydrate sources such as cane and beet molasses. In addition, these complex substrates may have other desirable effects on the production of PHB, such as culture growth promotion and reducing the time for PHB formation. Taking into account data reported by Page et al. [29], we evaluated two complex carbon sources (hydrolyzed and deproteinized whey and sugarcane juice) in modified Burk’s medium. Moreover, hydrolyzed and deproteinized liquid whey or sugarcane juice (without Burk’s medium) were used, less than half the C-source was consumed (Fig. 1C). In glucose, sucrose, galactose, hydrolyzed and deproteinized whey and sugarcane juice with Burk’s medium, the consumption of the C-source was nearly complete, and only residual values of less than 2.0 g/L were observed (Fig. 1C).

The highest alginic/biomass yield was found using sucrose as C-source with a value of 0.928±0.048 galginate/gbiomass (Table 1), and also the best alginic/C-source yield was obtained (0.281±0.043 galginate/gsucrose). Alternatively, the best yield biomass/C-source was obtained using hydrolyzed and deproteinized whey (0.454±0.064 gbiomass/gwhey) and sugarcane juice (0.406±0.042 gbiomass/gsugarcane) not fortified with Burk’s medium (Table 1). However, the lowest alginic/C-source yields of all were obtained for hydrolyzed and deproteinized whey (0.153±0.046 galginate/gc-source) and sugarcane juice (0.098±0.018 galginate/gc-source). This might be due to the lower concentrations of a nitrogen source, decreasing alginate production, but increasing biomass growth [14].
Using sugarcane juice-Burk culture medium, the highest viscosity was obtained (85±9 cps), with 3.55±0.42 g/L of alginate, and a MMW of 1203±120 kDa (Fig. 1B, 1D and 1E). Compared to sucrose-Burk medium, the viscosity was 50±9 cps with a final alginate concentration of 5.11±0.37 g/L and a MMW of 990±80 kDa. In glucose, the viscosity was 41±7 cps with 3.08±0.70 g/L of alginate and MMW of 1100±90 kDa (Fig.s 1B, 1D and 1E). The polydispersity index of alginites obtained from all C-sources was less than 2.0. These values demonstrated that alginates are monodisperse, without significant differences (Fig. 1F), at least in the experiments carried out in this work for shake flasks.

Peña et al. [10] propose "viscosifying power" as the way to compare the viscosifying capacity of alginates from different sources or processes, which can be defined as the viscosity generated per unit of polymer concentration. In order to compare the differences in the viscosifying power of alginates obtained from each C-source, viscosity/concentration ratio were calculated from final cultures of A. vinelandii (Table 1). The higher viscosifying power was found in those cultures carried out with sugarcane juice + Burk’s medium (23.8±2.6 cps L/galginate), and was twice the lowest viscosifying power obtained by using sucrose + Burk’s components (9.69±0.95 cps L/galginate). The most interesting result was the viscosifying power of 18.57±2.16 cps L/galginate obtained from cultures carried out with hydrolyzed and deproteinized whey + Burk’s components (Table 1), due to the lower MMW of the alginate obtained (121±27 kDa) (Fig. 1D). These results cannot be explained only by MMW differences, because there are other factors such as the monomers ratio (M/G ratio), the sequence pattern and the O-acetylation degree in mannuronic acid residues that might also affect viscosifying power [6,9,10]. On the other hand, Peña et al. [30] reported that the viscosifying power, the degree of O-acetylation and the MMW of the alginate produced by A. vinelandii in shake flasks are determined by the oxygen transfer rate (OTR).

Those data are in agreement with our data in terms that the OTR might be affected by the C-source used, as previously reported in E. coli cultures [31,32]. There is a well documented strong relationship between alginate production, its molecular characteristics and OTR [9, 10,33,34].

The alginate viscosity production and MMW differences between this work and those reported by Peña et al. [16], where sucrose + Burk’s medium were used, might be due to the Erlenmeyer shake flasks dimensions, volumes and inoculation form. Peña et al. [16] used shake flasks of 500 mL containing 100 mL of medium, inoculating 10% of the pre-culture (around 0.1-0.3 g/L, dry weight), reporting a maximum alginate concentration (obtained at 72 h) of 4.5 g/L, viscosity of 520 cps and a MMW of 1.98x10^6 Da with a polydispersity index (PI) of 1.50, whereas, in this work, we used 250 ml shake flasks with 50 mL of culture medium, inoculated with washed cells. On the other hand, Peña et al. [16] reported that alginate production change as a function of the filling volume in shake flasks, modifying also its MMW. Thus, it is not surprising—comparing our results with those previously reported—that alginate molecular characteristics were different in spite of using the same strain of A. vinelandii.

In order to dissect the role of non-conventional carbon sources in A. vinelandii growth, C-source consumption and alginate molecular characteristics, a complete kinetics of A. vinelandii growth and alginate production was undertaken by using sucrose, deproteinized and hydrolyzed whey and sugarcane juice in Burk’s medium (Fig. 2). A similar final biomass was obtained using whey and sugarcane juice in Burk’s medium (6.7±0.7 g/L and 6.7±0.4 g/L, respectively), with similar specific growth rates (0.047±0.003 and 0.046±0.004 h⁻¹, respectively). But lower biomass was obtained using sucrose (5.5±0.2 g/L) with a higher specific growth rate (0.052±0.002 h⁻¹) (Fig. 2.A). However, lower alginate production, and lower specific alginate production rates were obtained by using hydrolyzed whey (2.4±0.3 g/L and 3.77x10⁻³ ±0.3x10⁻³ galginate/gbiomass, respectively), and sugarcane juice (3.6±0.4 g/L and 5.54x10⁻³±0.6x10⁻³ galginate/gbiomass, respectively) in Burk’s medium, than in sucrose (5.1±0.4 g/L and 25.8x10⁻³±2x10⁻³ galginate/gbiomass, respectively) (Fig. 2.B). In terms of C-source consumption, almost all were consumed, but no significant differences were obtained in specific consumption rates with an average of 3.46x10⁻³±0.27x10⁻³ gC-source/gbiomass (Fig. 2.C). A similar viscosity kinetics was found between those cultures grown using deproteinized and hydrolyzed whey and sucrose in Burk’s medium (Fig. 2.D). These similarities are not correlated to MMW kinetics, where an almost five times lower MMW was obtained in those cultures grown using hydrolyzed and deproteinized whey (Fig. 2.E). To explain this behavior, the viscosifying power was plotted as a function of culture time (Fig. 3). This relation can...
be a quantitative approach to define the quality of alginates [10]. Viscosifying power increases during culture, and decreases at the end of the exponential phase, possibly due to the action of alginate lyases [21,35]. Moreover, there is no relationship between biomass growth kinetics, Alginate MMW and its production and the viscosifying capacity. This may be due to differences in the biopolymer molecular characteristics, such as the M/G ratio and the acetylation degree [15,30]. Table 1 outlines the final viscosifying power of cultures. The highest viscosifying power was obtained using deproteinized and hydrolyzed whey, and sugarcane juice in Burk’s culture medium. However, it is clear that higher values of viscosifying capacity can be achieved during cultivation (Fig. 3) as a response of the increases in the MMW [2,9,15,30], and the final values presented in Table 1 are the result of the alginate lyase activity [21,23,35].

Here, we could suggest that using alternative C-sources, and consequently more complex but less expensive ones, alginates with different molecular properties than those obtained with conventional C-sources can be produced.

4. Concluding remarks

To our knowledge, this is the first work reporting the effect of non-conventional and conventional C-sources on alginate MMW and viscosifying power produced by A. vinelandii. Although the highest concentration of biomass was obtained with the use of alternative C-sources (deproteinized and hydrolyzed whey and sugarcane juice), the highest concentration of alginates was obtained using sucrose in Burk’s medium. Furthermore, the best alginate yields and specific alginate production rates were obtained using sucrose, compared to unconventional C-sources. Moreover, differences in viscosifying power clearly demonstrated that the alginates produced by A. vinelandii when using deproteinized and hydrolyzed whey are surely different in composition than those produced with sugar cane or sucrose, said differences lying depending on the monomers ratio (M/G), the sequence pattern and the acetylation degree in mannuronic acid residues. Based on the data presented here, it can be assumed that it is possible to produce alginates with different molecular characteristics by modifying only the components of the culture, such as the C-source. This knowledge associated with the understanding of the effects of environmental conditions, would yield high concentrations of high added value alginates.

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