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/g_{alg}). 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- glurónico 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 e hidrolizado (23.8±2.6 cpsL/g_{alg}). 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

Alginates 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 alginates 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 alginates 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 these produce by 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 CO_2 [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 alginates, 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* ATTC-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, K_2HPO_4 0.66, KH_2PO_4 0.16, MOPS 1.42, $CaSO_4$ 0.05, NaCl 0.2, $MgSO_4$-$7H_2O$ 0.2, Na_2MoO_4-$2H_2O$ 0.0029, $FeSO_4$-$7H_2O$ 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 $MgSO_4$-$7H_2O$, $FeSO_4$-$7H2O$ and Na_2MoO_4-$2H_2O$ 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 (Millipore™, 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 29ºC, 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 29ºC. 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 dinitrosalicylic acid (DNS) method, and an acidic 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, alginate 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 was 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 the production of alginates by *A. vinelandii* and determined the effect of this process on their molecular characteristics, *i.e.* mean molecular weight and polidispersity index.

In cultures using hydrolyzed and deproteinized whey, a biomass concentration of 3.55±0.79 g/L and 1.20±0.13 g/L of alginate were obtained. While, using sugarcane juice 1.87±0.59 g/L of biomass and 0.45±0.20 g/L of alginate were obtained. It should be mentioned that those unconventional C-source cultures were carried out by just adjusting the initial pH to 7.2, TRS to 20 g/L and adding any buffer or salt. However, by complementing these unconventional C-sources with Burk´s medium (without sucrose), 6.67±0.72 g/L and 6.68±0.45 g/L of biomass were obtained, and 2.42±0.28 g/L and 3.55±0.42 g/L of alginate were achieved

by using hydrolyzed and deproteinized whey or sugarcane juice, respectively.

When reviewing the consumption of C-sources, measured as total reducing sugars, as shown in Fig. 1C, no C-source uptake was observed using lactose, which is congruent with no obtaining biomass growth, nor alginate production. Furthermore, when hydrolyzed and deproteinized 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 alginate/biomass yield was found using sucrose as C-source with a value of 0.928±0.048 $g_{alginate}/g_{biomass}$ (Table 1), and also the best alginate/C-source yield was obtained (0.281±0.043 $g_{alginate}/g_{sucrose}$). Alternatively, the best yield biomass/C-source was obtained using hydrolyzed and deproteinized whey (0.454±0.064 $g_{biomass}/g_{whey}$) and sugarcane juice (0.406±0.042 $g_{biom}/g_{sugarcane}$) not fortified with Burk´s medium (Table 1). However, the lowest alginate/C-source yields of all were obtained for hydrolyzed and deproteinized whey (0.153±0.046 $g_{alginate}/g_{C-source}$) and sugarcane juice (0.098±0.018 $g_{alginate}/g_{C-source}$). This might be due to the lower concentrations of a nitrogen source, decreasing alginate production, but increasing biomass growth [14].
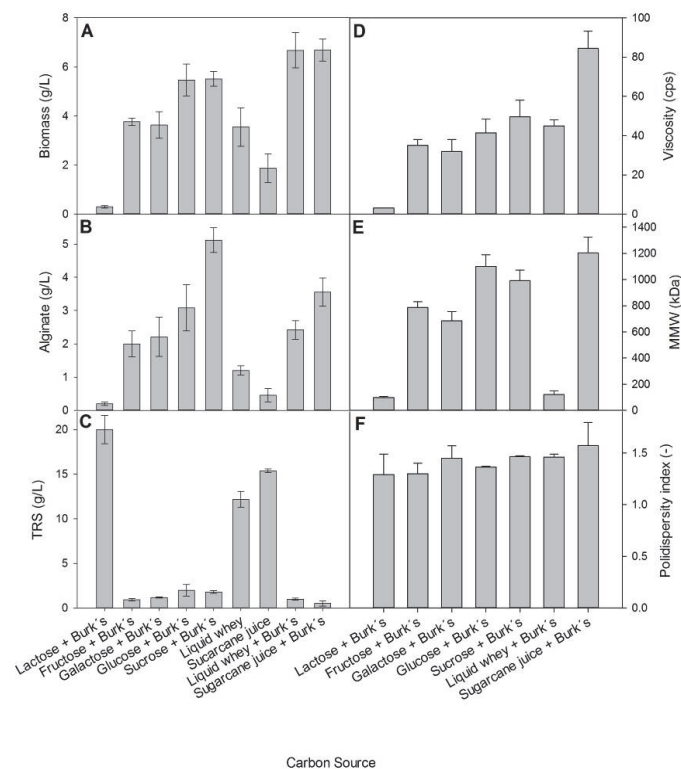

Figure 1. Effect of different carbon sources on the final concentrations of *A. vinelandii* growth (A), alginate production (B), and total reducing sugars (C), culture broth viscosity (D), alginate mean molecular weight (E), and polidispersity index (F) in cultures carried out in 250 mL shake flask, containing 50 mL of modified Burk's medium and incubated at 200 rpm and 29ºC.
Source: The Authors

Table 1.
Impact of the carbon source on the kinetics and stoichiometric parameters of alginate production in cultures of A.vinelandii, by using 20 g/L of different carbon sources with and without Burk´s medium components. Source: Authors' own

C-source	$Y_{alg/biom}$ (g_{alg}/g_{biom})	$Y_{alg/C-souce}$ ($g_{alg}/g_{C-source}$)	$Y_{biom}/C-souce$ ($g_{biom}/g_{C-source}$)	Viscosifying power (cps/g_{alg}/L)
Lactose + Burk´s medium	0.667±0.003	0.100±0.010	0.150±0.005	15.5±2.5
Fructose + Burk´s medium	0.531±0.038	0.105±0.029	0.197±0.006	17.5±1.8
Galactose + Burk´s medium	0.608±0.045	0.117±0.090	0.193±0.030	14.5±2.2
Glucose + Burk´s medium	0.564±0.307	0.171±0.068	0.303±0.082	13.4±1.6
Sucrose + Burk´s medium	0.928±0.048	0.281±0.043	0.303±0.036	9.7±0.9
Liquid whey	0.338±0.110	0.153±0.046	0.454±0.064	ND
Sugarcane juice	0.242±0.138	0.098±0.018	0.406±0.042	ND
Liquid whey + Burk´s medium	0.362±0.005	0.127±0.017	0.351±0.048	18.6±2.2
Sugarcane juice + Burk´s medium	0.532±0.098	0.182±0.021	0.343±0.022	23.8±2.6

ND: not determined
Source: The Authors

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 alginates 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/$g_{alginate}$), and was twice the lowest viscosifying power obtained by using sucrose + Burk´s components (9.69±0.95 cps L/$g_{alginate}$). The most interesting result was the viscosifying power of 18.57±2.16 cps L/$g_{alginate}$ 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 1.98×10^6 Da with a polidispersity 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 $3.77 \times 10^{-3} \pm 0.3 \times 10^{-3}$ $g_{alginate}/g_{biomass}$h, respectively), and sugarcane juice (3.6±0.4 g/L and $5.54 \times 10^{-3} \pm 0.6 \times 10^{-3}$ $g_{alginate}/g_{biomass}$h, respectively) in Burk´s medium, than in sucrose (5.1±0.4 g/L and $25.8 \times 10^{-3} \pm 2 \times 10^{-3}$ $g_{alginate}/g_{biomass}$h, 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 $3.46 \times 10^{-3} \pm 0.27 \times 10^{-3}$ $g_{C-source}/g_{biomass}$.h (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),
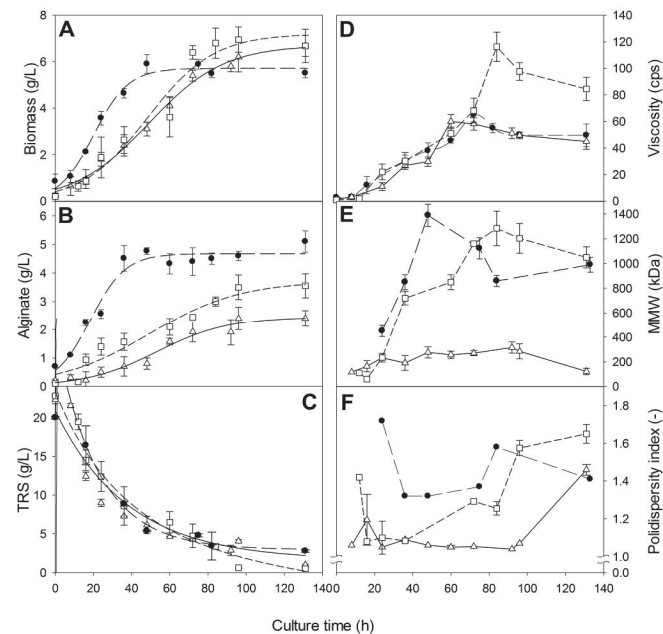

Figure 2. Kinetics of biomass growth (A), alginate production (B), total reducing sugars (C), culture broth viscosity (D), alginate mean molecular weight (E), and polidispersity index (F) of cultures carried out in 250 mL shake flask, containing 50 mL of modified Burk's medium and incubated at 200 rpm and 29ºC, by using sucrose (closed dots), deproteinized and hydrolyzed whey (open triangles), and sugarcane juice (open squares) as a carbon source in Burk´s medium.
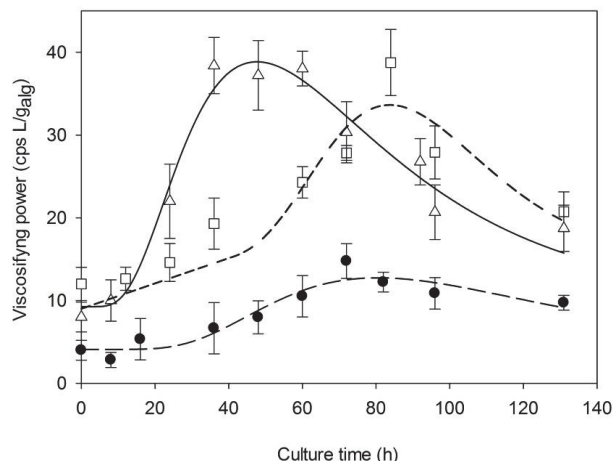Source: The Authors

Figure 3. Kinetics of the relation viscosity/alginate concentration (viscosifying power) in culture media carried out using sucrose (closed dots), deproteinized and hydrolyzed whey (open triangles), and sugarcane juice (open squares) as a carbon source in Burk´s medium.
Source: The Authors

the highest concentration of alginate 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.

Acknowledgements

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