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High CO_2 affects alginate production and prevents polymer degradation in cultures of *Azotobacter vinelandii*

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Abstract

The effect of carbon dioxide on the production and quality of alginate by *Azotobacter vinelandii* was evaluated in batch cultures conducted in a 1 L bioreactor under constant dissolved oxygen tension of 3%, using different levels of CO_2 (0–25% v/v) in the income gas stream. The effect of CO_2 on the process was assessed in terms of biomass growth, product formation, and substrate consumption. The impact of CO_2 addition on the polymer molecular weight was also quantified. Biomass growth and alginate yield was first inhibited (4–8% CO_2) and then stimulated (13% CO_2). For 25% CO_2, bacterial growth and alginate production were totally inhibited. For low added CO_2 ($<$4%) the mean molecular weight at the end of the culture dropped dramatically. This drop was not observed when 8% or 13% CO_2 was added. The results suggest that high CO_2 concentrations inhibit the synthesis or activity of polymer-degrading enzymes (alginate- lyases).
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Keywords: Carbon dioxide; Alginate; Molecular weight; *Azotobacter vinelandii*

1. Introduction

CO_2 is produced in aerobic and anaerobic fermentations through decarboxylation reactions in a number of metabolic steps, and hence has an influence on the performance of microbial cultures. High CO_2 partial pressures occur in processes in which there is a high total pressure, in regions where the hydrostatic pressure is elevated (i.e. in the bottom of large fermenters) and in poorly ventilated systems, such as shake flasks [1] or inefficiently aerated bioreactors [2]. A dramatic example of the effect of CO_2 in large-scale fermenters has been reported by Geraats [3] for the industrial production of a lipase using *Pseudomonas alcaligenes.* This author found that high CO_2 was responsible for a 70% decrease in enzyme titer when scaling-up the process.

The effect of increased CO_2 partial pressures has been investigated in many biological systems. Ho et al. [4] and Onken & Liefke [5] reviewed different aspects of the effects of CO_2 on microbial processes. More specific reviews have focused on the effect on yeasts [6], bacteria [7] and filamentous fungi [8]. Recent papers, not covered in the previous reviews, have been published [9–14] studying the effect of CO_2 in various biological systems. In the last few years, information has been published about the effect of CO_2 for bacterial cellulose production [15,16]. However, there are no reports for other microbial polysaccharides.

Microbial polysaccharides are gaining acceptance in a variety of applications [17] as viscosifiers, suspending agents and, in some cases, as gelling agents. Among them, microbial alginate [18] is of particular interest because has the potential to substitute alginates obtained from marine algae (the current commercial method to produce them). Microbial alginates are produced by bacteria of the genus *Pseudomonas* and *Azotobacter* [19,20], the latter being the most promising because its non-pathogenic nature.

Azotobacter vinelandii is a Gram-negative bacterium exhibiting a high respiration rate [21] and, therefore also has a high CO_2 generation rate. The alginate produced by this bacterium is highly dependent on the specific culture conditions. Under low dissolved oxygen tension (DOT), the organism accumulates the intracellular storage polymer poly-beta-hydroxybutyrate (PHB); whereas at high DOT, *A. vinelandii* uses the carbon source mainly for biomass production. Efficient conversion of sucrose to alginate is

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achieved only if the oxygen is accurately controlled between 1 and 5% of oxygen saturation [22–24]. In shake flasks, it produces an alginate of high molecular weight with a relatively high broth viscosity [25]. However, when the process is scaled-up to a laboratory fermenter, the molecular weight (and hence the broth viscosity) are up to two orders of magnitude lower than that obtained using shake flasks [23]. Nonetheless, alginate productivity is higher in fermenters.

The high CO_2 generation rate of *A. vinelandii* and the inefficiency of the shake flasks system to remove this gas, may increase the concentration of dissolved CO_2 during the fermentation process. As this system is very different to sparged bioreactors, CO_2 could be playing a key role in promoting the production of high molecular weight alginate in shake flasks. Therefore, the aim of the present study was to evaluate the effect of carbon dioxide on the production and quality of the alginate produced by *A. vinelandii* in a bioreactor under conditions of constant dissolved oxygen tension.

2. Materials and methods

2.1. Microorganism

Azotobacter vinelandii ATCC 9046 was used. The bacterial strain was preserved and sub-cultured as described previously [23,25].

2.2. Culture medium and fermentation system

A. vinelandii was grown in a modified Burk's medium with the following composition (in g/liter): sucrose 20; yeast extract (Difco) 3; 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; $FeSO_4$ 0.027 [25]. The cultures were carried out in a 1.5 L vessel with a working volume of 1.0 L. The bioreactor was equipped with three Rushton impellers (impeller/tank diameter = 0.5) agitated at 700 rpm. The pH was controlled at 7.2 ± 0.1 through automatic addition of NaOH (2N). The foam produced was controlled by the addition of silicon (10% w/v, Dow Corning), when necessary. The dissolved oxygen tension (DOT) was measured using a dissolved oxygen probe (Ingold) and the signal recorded by a Macintosh II SI computer via a Mac Adios II A/D & D/A (GW Instruments) interface. The DOT was controlled online by gas blending as described elsewhere [23]. DOT was controlled within a ± 0.5% band with respect to the set point. The cultures were conducted at a temperature of 29°C with a constant total gas flow rate of 800 ccpm. The exhaust gas was analyzed by a CO_2 infrared detector (Columbus Instruments Corporation, model 1410) and the dissolved CO_2 concentration was hence calculated using the expression reported by Royce and Thornhill [26].

A series of fermentations were performed with operating conditions held constant at 3% of DOT, under the same conditions described previously. These conditions were chosen because we have obtained the maximal alginate concentrations and cell growth in previous experiments performed in our laboratory [23]. These parameters were maintained constant so as to distinguish the effect of CO_2 from the other variables. A blend of gases (O_2, N_2 and CO_2) was used to keep the DOT constant, and maintain the CO_2 concentration in the inlet gas stream at 0, 4, 8, 13 and 25%. Biomass, alginate and sucrose concentrations were measured at regular intervals throughout culture. From these data, bacterial specific growth rate (SGR), alginate production rate (APR) and sucrose uptake rate (SUR), were calculated.

2.3. Analytical determinations

Sucrose, biomass, alginate, broth viscosity and molecular weight distributions were measured as reported previously [23,25]. Experiments were conducted in duplicates and the average reported.

2.4. Identification of alginase activity

A 10 ml-sample of culture broth was mixed with 1 ml Na_4EDTA (0.1 M) and 2 ml NaCl (1.0 M) and then centrifuged at 12,000 rpm during 20 min. To remove slime polysaccharides, 3 ml of calcium chloride (10%) were added to 10 ml of supernatant. After storage at 4°C for 3 h, the resultant precipitate was removed by centrifugation (12,000 rpm, 20 min). The supernatant was dialyzed against 0.05 M Tris-HCL buffer (pH 7.2). The extract (1 ml) was incubated with 4 ml (5 mg/mL in 0.05 M TRIS solution) of commercial alginate from *Macrosystis pyrifera* (Sigma) for 4 h at room temperature with agitation. Alginase activity was identified by following the changes on the molecular weight by gel-permeation chromatography [23]. A control in which an inactivated extract (by boiling it during 30 min) was incubated with alginate, was also included.

3. Results and discussion

3.1. CO_2 evolution in cultures

Fig. 1 shows the CO_2 profiles of the cultures for the different concentrations of CO_2 added to the inlet gas. The calculated dissolved CO_2 concentration increased during the culture for all the conditions tested, the exception being 25% CO_2 for which no bacterial growth was observed. The control experiment (i.e. no CO_2 added) reached a maximum dissolved CO_2 concentration of 4% towards the end of the culture. The culture in which 13% CO_2 was added reached a maximum dissolved CO_2 concentration of about 20%. Calculating the total CO_2 produced (by measuring the area under the curves of Fig. 1) showed that the experiment with

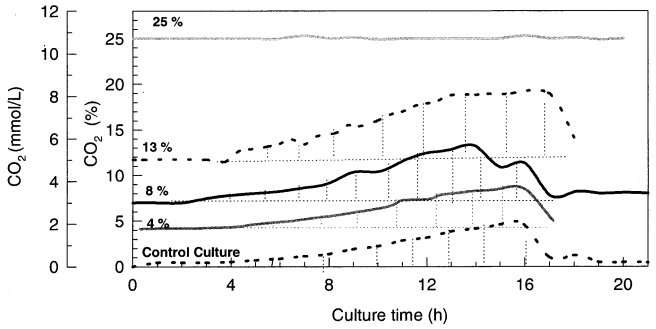

Fig. 1. Evolution of calculated dissolved CO_2 concentrations as a function of CO_2 content in the inlet gas stream and culture time.

4% added CO_2 produced an almost identical quantity of CO_2 to the control experiment. However, for 8% and 13% added CO_2, the total amount of CO_2 generated during fermentation increased 19 and 63%, respectively, as compared to the control experiment (no CO_2 added). These results are consistent with the sucrose uptake rate (see Fig. 3 c) and with the overall balance of carbon estimated for total CO_2, biomass and alginate produced (data not shown). On the other hand, it is known [22] that a high sucrose uptake rate is associated with a high production of CO_2 in an energy –wasting and carbon-wasting mechanism.

3.2. Influence of CO_2 on bacterial growth and alginate production

Fig. 2 shows, as an example, the CO_2 evolution, biomass growth, alginate production and sucrose consumption in the culture with 8% CO_2 addition, as compared to no CO_2 addition (control). Both bacterial growth and alginate production were affected by the CO_2 addition, whereas sucrose consumption was only slightly affected. Specific growth rate (0.22 h^{-1}) and alginate production rate (0.12 g alg/g bio h) were higher in the culture without CO_2 addition than the values found in the culture in which 8% CO_2 was added (Fig. 3). In both conditions, alginate production was growth- associated and it reached 4.6 g/liter at the end of the process for the control culture; whereas in the culture enriched with 8%, CO_2 alginate concentration was 2.2 g/liter. Sucrose consumption was 87.5% at the end of the culture for both conditions evaluated. In the control culture, the level of CO_2 increased as a result of microbial growth and it reached a maximum concentration of 4.8% after 16 h. CO_2 production dropped as the culture reached the stationary phase.

The effect of carbon dioxide content in the inlet gas stream on specific growth rate, alginate production rate and sucrose uptake rate is summarized in Fig. 3. There was an inhibitory effect of CO_2 on the specific growth rate and alginate production rate for 4% and 8% CO_2 addition; whereas at 13% CO_2, both parameters were stimulated. In the case of sucrose uptake rate, a slight difference was found in the range from 0 to 8% CO_2, increasing at 13% CO_2. *A.*

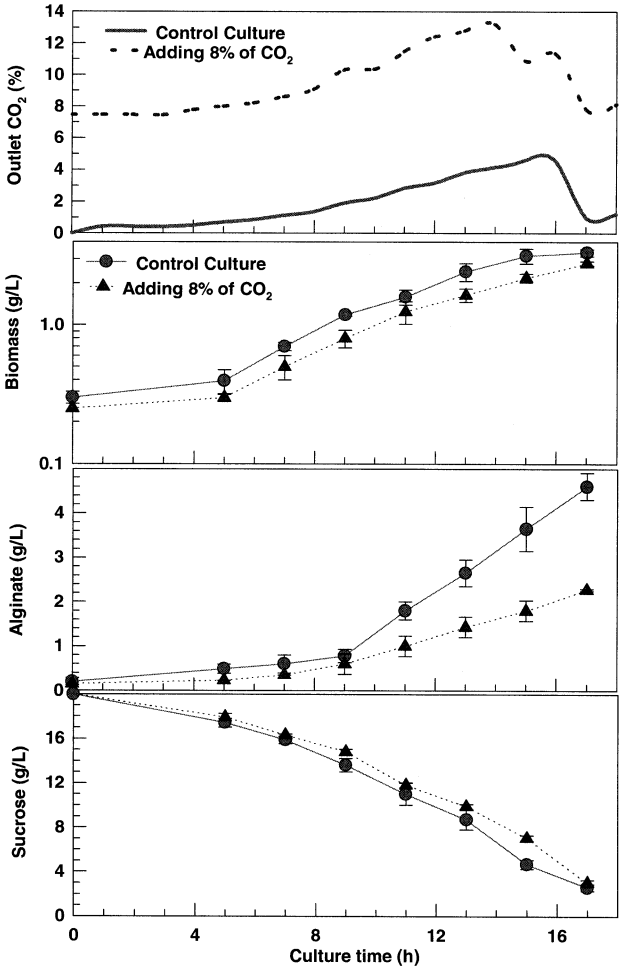

Fig. 2. Evolution of calculated dissolved CO_2, biomass, alginate and sucrose as a function of culture time for an inlet gas stream containing 8% CO_2.

vinelandii growth and alginate production were totally inhibited when a CO_2 concentration of 25% was used. Table 1 shows the alginate yield on a biomass basis (Y p/x) and on a substrate basis (Y p/s). The lowest yields were obtained when 8% CO_2 was added. Using 13% CO_2 addition, a statistically significant increase in both yields (Y p/x and Y p/s) was observed as compared to the results for 8% CO_2 addition. Although it is not possible to fully explain this stimulatory effect, a similar behavior has been reported by Mc Intyre and Mc Neil [14] for *Aspergillus niger* when a similar (13%) fraction of CO_2 was added to the inlet gas stream. These authors found that both the biomass concentration and the yield factor for biomass (Y x/s) increased in the range of 7.5% to 12% CO_2, decreasing both parameters at 15% CO_2.

The values of CO_2 used in the inlet gas stream (8%, 13%, and 25%) were similar or higher than those that could be necessary to simulate the CO_2 generated in cultures performed in shake flasks for *Saccharomyces cerevisiae* cultures, which have values of up to 25% CO_2 [1]. Also, the CO_2 concentrations used were higher than the dissolved

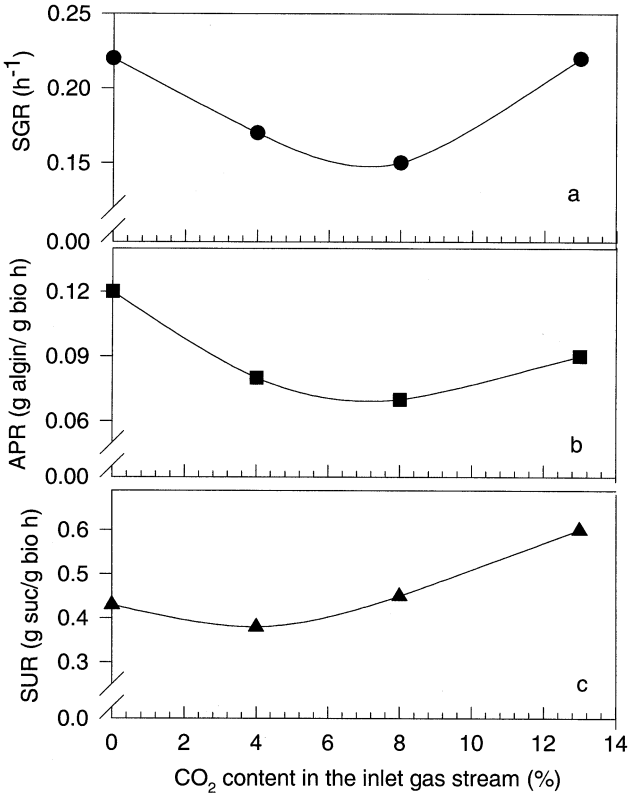

Fig. 3. Influence of CO_2 content in the inlet gas stream on the specific growth rate (SGR) (a), alginate production rate (APR) (b) and sucrose uptake rate (SUR) (c), during the culture of *A. vinelandii*.

CO_2 values that are found in the bottom of industrial bioreactors (7–12%), where increased levels are the result of the microbial metabolism and the hydrostatic pressure at the base of the vessel [5].

If compared with other microorganisms, including bacteria [7], yeasts [6] and fungi [8], *Azotobacter vinelandii* turned out to be highly resistant to high CO_2 concentrations. However, when *A. vinelandii* was cultured using less than 13% CO_2 in the inlet gas, the bacteria consumed most of the carbon source with a sucrose consumption rate not varying greatly for all the conditions tested (Fig. 3 c). This indicates that CO_2 did not affect the carbon source intake system. On the other hand, when the bacteria were cultured using 13%

Table 1
Yield factors and broths viscosity of cultures of *A. vinelandii* conducted at various CO_2 inlet gas contents

CO_2 content %	Y p/x (g algin/g biom)	Y p/s (g algin/g suc)	Viscosity (cps)
0	1.36	0.26	7
4	1.11	0.22	7
8	0.92	0.2	7
13	1.37	0.19	7
25	*	*	1

* No growth nor alginate production was observed.

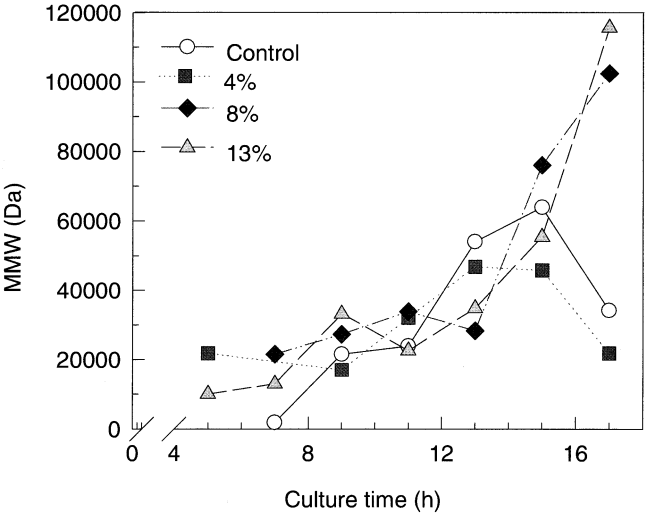

Fig. 4. Evolution of the alginate mean molecular weight during culture as a function of the CO_2 content in the inlet gas stream.

CO_2, the uptake rate of sucrose was higher than the control culture (Fig. 3 c). This behavior confirms the stimulatory effect of CO_2 on the *A. vinelandii* metabolism. It has been reported [27,28] that the CO_2 can stimulate the growth of microorganisms and therefore the substrates consumption. Stimulation of growth may occur because some anabolic reactions involve CO_2 fixation, such as the formation of oxaloacetate from pyruvate, which could be stimulated in the presence of external CO_2. On the other hand, Jones & Greenfield [6] showed that a range of enzymes that participate in the anabolic metabolism in yeasts, are inhibited by high CO_2 (or HCO_3^+) concentrations, including phospoenolpyruvate carboxykinase and pyruvate carboxylase. These enzymes are involved in the alginate production pathway, via the formation of oxaloacetate and subsequent gluconeogenesis under conditions with high energetic charge [20]. However, for a conclusive explanation of the stimulatory-inhibitory effects of CO_2, further work will be necessary to identify the affected enzymes and the metabolic consequences.

3.3. Influence of dissolved CO_2 on alginate quality

The alginate obtained under the different conditions evaluated was characterized in terms of their mean molecular weight (MMW). Fig. 4 shows the evolution of the mean molecular weight along the culture, for all the conditions evaluated. Until 13 h of culture time, all conditions exhibited a relatively similar behavior. However, after this time, two distinct profiles are evident. One of them is representative of cultures conducted under low levels of CO_2 (0 and 4%), where the maximum mean molecular weight was reached between 13–15 h of culture. However, at the end of fermentation (17 h), a fall of more than 50% in the MMW was observed. This drop in MMW has been described by

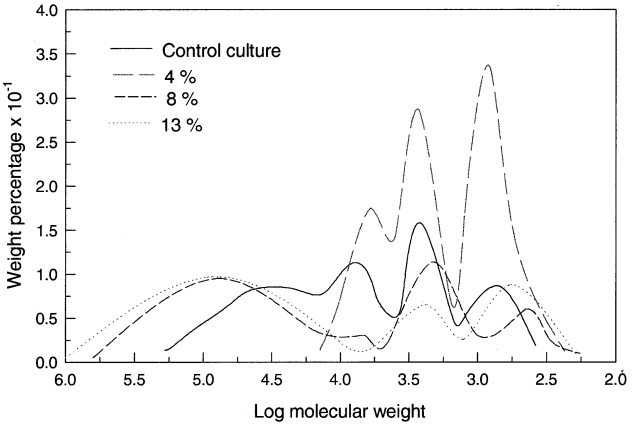

Fig. 5. Alginate molecular weight distributions as a function of the CO_2 content in the inlet gas stream.

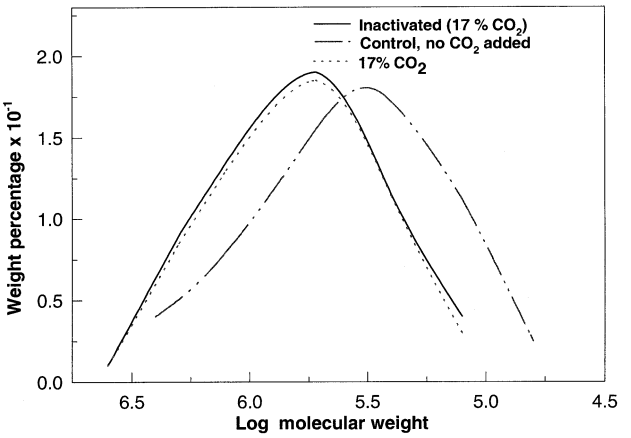

Fig. 6. Molecular weight distributions of a commercial alginate incubated with a broth extract obtained from a culture conducted using 17% CO_2 in the inlet gas stream, as compared to that obtained when no CO_2 was added. Also shown is the molecular weight distribution of alginate incubated with an inactivated (heat-treated) extract.

Peña et al. [23] for cultures grown under the same conditions of agitation (700 rpm) and differing dissolved oxygen tensions (1, 3, 5 and 7%). This decrease in MMW might be mediated by the action of alginate-degrading enzymes, which may be released in response to a given dissolved oxygen level during the cultivation and/or as a function of the age of the culture [23].

The cultures conducted at 8% and 13% addition of CO_2 showed a considerable increase in the MMW at the end of the fermentation process (after 15 h of culture). MMW of 102,000 and 105,000 Da were obtained for 8 and 13% addition of CO_2, respectively. These results are the first reported where no drop in alginate MMW was observed at the end of the culture, under constant dissolved oxygen and agitation conditions. Although the mechanism through which the CO_2 exerts its effect remains unclear, the fact that biomass growth and sucrose consumption were practically unaffected suggests the CO_2 principally affects the final steps in alginate biosynthesis. In general, the effects of CO_2 are found at the level of the cellular membrane as well as due to changes in internal pH of the microorganism [5]. This causes induction/repression of enzymes, mainly by the production of HCO_3^- which regulates cell metabolism [8]. However, as CO_2 is involved in all carboxylation/decarboxylation reactions, this gas could have an impact on the cell metabolism at various metabolic points. It may be possible that high levels of CO_2 (8% and 13%) inhibit either the synthesis or the activity of an alginate degrading enzyme (i.e. lyase) produced by *A. vinelandii*.

Fig. 5 shows the MMW distribution at the end of the culture, for all the conditions tested. For the control, and for the culture with 4% of CO_2 addition, it is possible to see three well-defined families of MMW. These fractions correspond to MMW peaks lower than 10,000 Da. On the other hand, the cultures where 8 and 13% of CO_2 were added, exhibited a very similar behavior among them, having a family which correspond to 100,000 Da and another two low-molecular-weight fraction (lower than 2,000 Da).

The broth viscosities obtained under the different conditions evaluated in the present study were about 7 cps (Table 1). Compared to values obtained in shake flasks [25], the bioreactor's broth viscosities were lower by two orders of magnitude. Some of the CO_2 values in the inlet gas stream (8 and 13%) are higher than the CO_2 levels found for other biological system in the headspace of shake flasks [1]. Therefore, the data suggest that the high viscosity observed in shake flasks is not solely caused by the relatively high partial pressures of carbon dioxide present in shake flasks.

3.4. Polymer-degrading activity under high CO_2 concentration

De-polymerization of alginate was tested using a culture conducted at 17% CO_2 concentration in the inlet gas stream. In this culture, final biomass and polymer concentration were very similar to those obtained when 8 and 13% of CO_2 were added to the culture (data not shown). In this experiment, only one sample was taken at the end of the fermentation (24 h). For this condition, the MMW was 90,000 Da, a similar value to that found in the cultures where 8% and 13% CO_2 was added. As shown in Fig. 6, the molecular weight distribution of the commercial alginate was unaffected when placed in contact with the broth extract of the culture conducted using 17% CO_2, showing a MMW similar to that determined for the control system (alginate with inactivated extract). In contrast, when the commercial alginate was incubated with extract from the culture without CO_2 addition, a drop in alginate MMW from 375,000 to 307,000 was observed. These results confirm the fact that high CO_2 concentrations inhibit either the synthesis or the activity of polymer-degrading enzymes (lyases).

4. Conclusions

Both bacterial growth and alginate production were affected by the CO_2 addition. Total growth inhibition was observed when using 25% CO_2 in the inlet gas stream. In terms of growth rate and alginate production, an inhibitory (0–8%)- stimulatory effect (8–13%) was observed. CO_2-enriched cultures exhibited low viscosity (7 cp) broths. This fact makes very unlikely that the high viscosity obtained in shake flasks cultures can be mediated exclusively by high CO_2 levels. CO_2 addition (at 8% or higher) prevents the normally observed drop in alginate mean molecular weight, suggesting that high CO_2 concentrations inhibit either the synthesis or the activity of polymer-degrading enzymes (lyases).

Acknowledgments

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