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NATURAL PRODUCTS

An investigation of agitation speed as a factor affecting the quantity and monomer distribution of alginate from *Azotobacter vinelandii* ATCC® 9046

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Abstract Alginate is a copolymer of β-D-mannuronic and α-L-guluronic acids. Distribution of these monomers in the alginate structure is one of the important characteristics that affect the commercial value of the polymer. In the present work, the effect of agitation speed in the range of 200–700 rpm on alginate production by *Azotobacter vinelandii* ATCC® 9046 was investigated at a dissolved oxygen tension of 5% of air saturation. Experiments were conducted in a fermentor operated in batch mode for 72 h while the production of biomass and alginate, the consumption of substrate and the change in culture broth viscosity and monomer distribution of the polymer were monitored. Results showed that the growth rate of the bacteria increased from 0.165 to 0.239 h^{-1} by the increase of mixing speed from 200 to 400 rpm. On the other hand, alginate production was found to be the most efficient at 400 rpm with the highest value of 4.51 g/l achieved at the end of fermentation. The viscosity of culture broth showed similar trends to alginate production. Viscosity was recorded as 24.61 cP at 400 rpm while it was only 4.26 cP at 700 rpm. The MM- and GG-block contents were almost equal in most of the culture times at 400 rpm. On the other hand, GG-blocks dominated at both low and high mixing speeds. Knowing that GG-blocks make rigid and protective gels with divalent cations, due to the higher GG-block content, the gel formation potential is higher at 200 rpm as well at 700 rpm, which might originate from the unfavorable environmental conditions that the bacteria were exposed to.

Keywords Alginate · Agitation speed · *Azotobacter vinelandii* · Monomer distribution · Viscosity

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Introduction

Alginate is composed of two monomers, β-D-mannuronic acid (M) and α-L-guluronic acid (G). It is a natural polysaccharide that is mainly found in the cell wall structure of marine brown algae [6]. However, alginate production is not limited to brown algae since some species such as Pseudomonas and *Azotobacter vinelandii* also produce alginate. Nevertheless, only the alginate synthesized by *A. vinelandii* has a similar block structure to the polymer produced by brown algae containing all block types, namely MM- , MG-, and GG-blocks [4]. Since the block types show distinct structural differences, they strongly influence the properties of alginate; for example the MG-blocks form the most flexible chains, whereas the GG-blocks form the stiffest chains. Gel-forming capacities and viscosity of the polymer are known to be critical parameters for industrial applications. While the viscosity particularly depends on the molecular weight, the gelling ability is determined by the GG-block content of alginate [16].

Alginate is one of the important constituents for *Azotobacter* species during the formation of cysts under adverse environmental conditions. In addition, the bacteria are known to produce alginate even during vegetative growth as a protective coating depending on the environmental conditions, such as in high-shear stresses [14].

On the other hand, efficient stirring of bulk liquid is essential to provide a homogeneous environment. At low agitation speeds, surface area of *A. vinelandii* cells was observed to be higher and the cells tended to form aggregates. On the contrary, smaller cells without any floc formation were obtained at high shear rates [11]. Properties of bacterial aggregates, which are formed by bacteria and alginate, directly affect material and gas transfer in the cultivation medium together with the increase in viscosity. It was claimed that even though a constant oxygen concentration was maintained in the bulk liquid, the actual oxygen level around the cells was determined by agitation speed. Therefore, typically the bacteria are exposed to higher oxygen concentrations under high mixing rates, compared to the oxygen levels encountered at low agitation speeds [12, 17]. Although *A. vinelandii* is known to be an obligate aerobic bacterium, the enzymes responsible for nitrogen fixation are highly sensitive to high oxygen concentrations [5]. The bacteria are required to remove excess oxygen around the cells by increasing the respiration rate, which might lead to the wastage of carbon source at high shear rates instead of the production of alginate.

Agitation speed was found to influence alginate formation, particularly the quality of the polymer produced. Specific alginate production rate by *A. vinelandii* ATCC® 9046 was observed to increase with increasing mixing speed from 300 to 700 rpm at a dissolved oxygen tension (DOT) of 3% of air saturation [12]. Similarly, both bacterial mass and alginate production by *A. vinelandii* DSM® 93-541b were found to increase by increasing stirring speeds up to 600 rpm at 5% DOT [17]. Furthermore, guluronic acid content was observed to increase by the increase of stirring speed up to 800 rpm. Beyond 800 rpm, guluronic acid quantity showed a decrease. On the other hand, Peña et al. [12] found that alginate having high molecular weight was obtained at low agitation speed (300 rpm) at all DOT values studied in the range of 1 and 5%. Therefore, together with the amount of alginate synthesized, the quality of alginate in terms of molecular weight and guluronic acid content was found to be greatly affected by the agitation speed.

According to the current literature, there are only two studies examining the effect of agitation speed on alginate quality under controlled DOT conditions. However, none of these studies monitored the block distribution of the biopolymer despite the aforementioned industrial importance of monomeric blocks. In this study, alginate production was carried out by *A. vinelandii* ATCC® 9046 to find out the influence of stirring speeds of 200, 400, and 700 rpm on the monomer distribution of alginate in a laboratory fermentor at a DOT of 5%.

Materials and methods

Microorganism and cultivation medium

Azotobacter vinelandii ATCC® 9046 was the bacterium used for alginate production in this study. The bacteria was preserved at −20°C in 20% glycerol solution during the course of the work. Before any experiment, the bacterium was activated by conducting a preculture in Burk's medium. A shake flask was used for that purpose at 200 rpm and 30°C. This medium was composed of glucose (20 g/l), K_2HPO_4 (0.66 g/l), KH_2PO_4 (0.16 g/l), $CaSO_4$ (0.05 g/l), NaCl (0.2 g/l), $MgSO_4{\cdot}7H_2O$ (0.2 g/l), $Na_2MoO_4{\cdot}2H_2O$ (0.0029 g/l) and $FeSO_4{\cdot}7H_2O$ (0.027 g/l). A modified form of Burk's medium was used for alginate production in fermentor. It contains sucrose (20 g/l), yeast extract (Merck) (3 g/l), K_2HPO_4 (0.66 g/l), KH_2PO_4 (0.16 g/l), MOPS (1.42 g/l), $CaSO_4$ (0.05 g/l), NaCl (0.2 g/l), $MgSO_4{\cdot}7H_2O$ (0.2 g/l), $Na_2MoO_4{\cdot}2H_2O$ (0.0029 g/l) and $FeSO_4{\cdot}7H_2O$ (0.027 g/l). For Burk's medium, the salts, K_2HPO_4, KH_2PO_4, NaCl, and $MgSO_4{\cdot}7H_2O$, were sterilized together by autoclaving at 121°C for 20 min. Then, glucose and $CaSO_4$ were sterilized separately and added into these salts. Finally, $FeSO_4{\cdot}7H_2O$ and $Na_2MoO_4{\cdot}2H_2O$ were added separately after filter sterilization through a 0.22-μm filter (Millipore Millex GS). For modified Burk's medium, all of the components, except for $CaSO_4$, $FeSO_4{\cdot}7H_2O$, and $Na_2MoO_4{\cdot}2H_2O$, were dissolved in distilled water and sterilized at 121°C for 40 min. $CaSO_4$ was then sterilized separately in an autoclave and added to the medium. Finally, $FeSO_4{\cdot}7H_2O$ and $Na_2MoO_4{\cdot}2H_2O$ were filter-sterilized and added as mentioned above. pH was set to 7.2 by the addition of NaOH or HCl (0.1 or 1 M).

Alginate production in the fermentor

A laboratory fermentor, BIOSTAT® A plus (Sartorious BBI Systems, Germany), with 2 l of working volume was used for alginate production. The vessel was equipped with six-bladed disc impellers with a stirrer diameter of 53 mm. During experiments, the effect of three different mixing speeds was tested: 200, 400, and 700 rpm. DOT was maintained constant at 5% ± 1 with respect to air saturation with the help of an oxygen probe (Hamilton, Switzerland) built in the fermenter and a controller system. The output of the pO_2-controller triggers the set point input of the servo controller. This system supplies air via a pulsed control valve or oxygen into N_2 input depending on the oxygen requirement of the bacteria. For optimum adaptation of the control system, the PID (proportional-integral-derivative) parameters for servo controller were adjusted accordingly. pH was adjusted to 7.2, by the addition of 1 N NaOH or HCl and was controlled by a pH probe (Hamilton,

Switzerland). Preculture, 1.6 g/l, was fed into the fermentor at a ratio of 3% (60 ml/2,000 ml). All experiments were performed during the batch operation of fermentor for 72 h and at 30°C. At the beginning of the study, the fermentor was operated under the same operational conditions three times for the case of 400 rpm. The results were found to be very reproducible, with the percent standard deviation varying between 5 and 12%. For this reason, the study was continued with one batch reactor for the experiments conducted at 200 and 700 rpm. Analytical measurements were conducted by taking 50 ml samples for predetermined time intervals, which were analyzed in duplicate.

Analytical measurements

Determination of bacterial mass and alginate concentration

A 2.5 ml of Na_4EDTA (0.1 M), and 1.5% (w/v) NaCl were added into 25 ml of culture broth and mixed for 10 min. For viscous samples, dilution was applied up to five times and then all are centrifuged at 4,000 rpm for 45 min. After that, the supernatant was filtered (0.45-μm membrane filter) and deacetylated after addition of NaOH (0.1 M) by shaking at room temperature for 1 h. Alginate was recovered as alginic acid by reducing the pH to about 2. The precipitate was incubated for 30 min at 4°C and separated by centrifugation at 4,000 rpm for 10 min. A further washing was applied by using 0.1 N cold HCl solution. After another centrifugation, alginic acid pellets were dissolved by neutralization and then precipitated by ethanol (1:1) with the addition of NaCl (0.2% w/v). Recovered alginate was washed three times with 70% of ethanol, then once with pure ethanol and then with ether. Finally, alginate was dried under fume hood and weighed. For bacterial mass determination, the precipitate from the first centrifugation was resuspended in distilled water and filtered (0.45 μm membrane filter). After that, it was dried at 105°C for 1 h, and then cooled and weighed.

Determination of sucrose concentration

Sucrose, in supernatants taken from the first centrifugation mentioned above, was degraded with the addition of concentrated HCl by heating at 90°C for 5 min. Then, the solution was neutralized by NaOH (5 N). After that, DNS method was applied for the measurements of residual sucrose concentration [8].

Determination of culture broth viscosity

Measurement of culture broth viscosity was performed by a rotational viscometer (Brookfield LVDVII+) with an ultra-low viscosity adapter. All analyses were done at the shear rate of 73.4 s^{-1} and 25°C.

Determination of monomer distribution of alginate

^{1}H-NMR spectroscopy was utilized for the determination of monomer distribution of alginates produced during experiments. To obtain good signals, alginate samples were required to be degraded by using partial acid hydrolysis before any measurements. Alginate (0.1 g) was added to 100 ml of distilled water. pH was set to 5.6 (using 1 M, or 0.1 M HCl) and heated at 100°C for 1 h. A further degradation was performed by decreasing pH to 3.8 and heating at 100°C for 30 min. The solution was neutralized to around pH 7 (using 1 M, or 0.1 M NaOH) and freeze-dried. For analysis, 10 mg of alginate was added into 0.5 ml of D_2O. Also, 10 μl of 0.3 M EDTA was used to prevent interaction of divalent cations with alginate [2]. ASTM F 2259-03 method was used as a guide and the ^{1}H-NMR signals of alginate monomers were detected according to Moral [9].

Results and discussion

Alginate production by *A. vinelandii* ATCC® 9046 was examined at three different agitation speeds, 200, 400, and 700 rpm, to elucidate the effect of mixing speed on alginate quality and quantity in a laboratory fermentor. All experiments were conducted at a DOT of 5% of air saturation, which was the value previously determined as the most suitable level of aeration for alginate production in the range of 1–10% of air saturation [9]. Figure 1 illustrates changes in bacteria concentration observed during 72 h of fermentation process at various stirring speeds. The bacterial growth started out with a lag period that lasted for about 6 h, independent of the mixing rate. Right after this period, bacterial cell concentration rose sharply for the case of 700 rpm; which was closely followed by 400 rpm. Mixing rate at 200 rpm caused the slowest rise in the bacterial concentration with respect to time (Fig. 1). The bacterial cell concentration was only 3.16 g/l at 200 rpm, while this value was measured as 9.87 g/l at 700 rpm at the end of 24 h. When the exponential growth phase ended, cell mass began to decrease, possibly due to lysis, which continued until the end of fermentation batch. Parallel to these, the growth rate of bacteria was measured higher with the increasing agitation speed from 200 to 400 rpm. The maximum specific growth rate was 0.165 h^{-1} at 200 rpm, which then increased to 0.239 h^{-1} at 400 rpm. At 700 rpm, a slight decrease was observed and growth rate was measured as 0.192 h^{-1}. Similarly, Peña et al. [12] found that the highest specific growth rate of *A. vinelandii* ATCC®

9046 as 0.23 h^{-1} at 700 rpm; on the other hand, at 300 rpm the growth rate was recorded as 0.14 h^{-1} (at constant DOT of 3%). For the growth of bacteria, it appeared that only a 200-rpm value may not be preferred as an effective mixing, since the growth pattern was a little delayed compared to the ones at both 400 and 700 rpm.

Alginate concentration was also measured to evaluate how alginate quantity changes by changing the stirring speed from 200 and 700 rpm under a constant DOT of 5%. Alginate production was varied greatly depending on mixing speeds as it is shown in Fig. 2. Most of the total polymer production (about 65%) was associated with growing bacteria at 200 rpm while non-growing bacteria contributed to 60 and 75% of the total alginate production at 400 and 700 rpm, respectively, during 72 h. Although some of the cells were undergoing decay after a certain time, the rest of the microorganisms produced alginate to protect themselves from unfavorable conditions. A stirring speed of 200 rpm was found to be better for maximizing alginate synthesis compared to 700 rpm which, was evidently not suitable for the production of alginate. The stirring level of 400 rpm, which is considered a moderate level stirring, resulted in maximum alginate production. Alginate concentration was achieved as 4.51 g/l at 400 rpm at the end of fermentation, whereas it was only 1.46 g/l at 700 rpm and 2.51 g/l at 200 rpm at 56 h. Peña et al. [12] showed that specific alginate production rate was 0.07 g alginate/g biomass/h at 700 rpm, which decreased to 0.026 g alginate/g biomass/h at 300 rpm (DOT at 3%). In our study, the highest yield based on cell mass was observed at 400 rpm as 1 g alginate/g biomass. Other researchers reported this value to be in the range of 0.3–1.6 g alginate/g biomass at various experimental conditions [1, 10, 12, 15, 18, 19]. From our results, it was assessed that alginate synthesis was stimulated with increasing agitation speeds from 200 to 400 rpm, but further increase in mixing, therefore the increase of shear, reduced the amount of the polymer produced. This is possibly attributable to differences in diffusion rates of nutrients and gases at various mixing speeds in the medium. As the previously published works also indicate, at low agitation speeds, bacteria form aggregates that limit passage of materials and gases between the growth medium and the cells [12]. This eventually reduces alginate production yield compared to a moderate level of mixing at 400 rpm. In line with the previous studies, at high shear rates, the cells are possibly smaller, without many flocs present, which reduces the problems associated with diffusional limitation [11, 12]. In addition, one possible reason might be the variation in the maximum oxygen transfer rate (OTR_{max}). A very recent study [7] showed that alginate synthesis depends strongly on OTR_{max} even when the DOT was maintained constant at 5% at two different agitation speeds of 300 and 700 rpm. This study reported that the highest alginate concentration, 3.1 g/l, and the maximum specific alginate production rate, $0.031 g_{alg}/g_{bio}$ h, were obtained at OTR_{max} of 100 mmol/l h, which was the highest value observed at 700 rpm.

Cultivation medium was supplemented with sucrose as the carbon source to produce alginate by *A. vinelandii* ATCC® 9046. During the fermentation process, the decrease of sucrose concentration was monitored under different agitation speeds (Fig. 3). Sucrose consumption rate was higher during early fermentation, particularly up to 24 h due to cellular growth and partly for alginate production. On the other hand, after the exponential growth phase, sucrose appeared to be utilized by the bacteria mainly for the production of alginate. Almost all of the 20 g/l of sucrose, which was initially present in the system, was utilized at the end of the fermentation for all of the

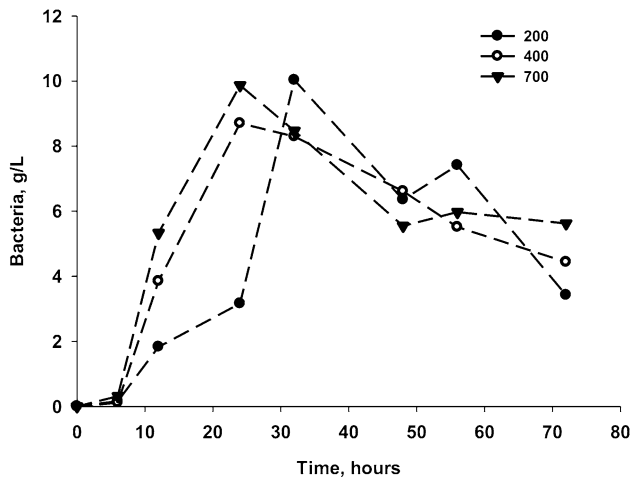

Fig. 1 Changes in bacterial mass during alginate production at different agitation speeds

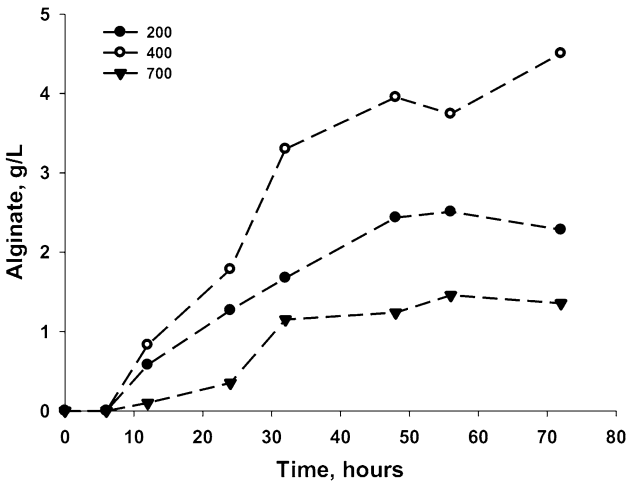

Fig. 2 Variations in alginate concentration during fermentation at different agitation speeds

stirring speeds. At 72 h, only 2 g/l of sucrose was left at 200 rpm, whereas only 1 g/l was left at 700 rpm. It was found that the higher the agitation speed was, the higher the sucrose utilization rate was. On the other hand, the alginate yield based on sucrose utilization was the best at 400 rpm as 0.246 g alginate/g sucrose. This behavior might be explained by the wastage of the carbon source especially at high mixing rates.

Viscosity is one of the important characteristics of the polymer for industrial applications. It indirectly gives an idea about the polymerization degree of alginate produced. Additionally, it is known that the higher the viscosity the higher the molecular weight [13, 16]. For this reason, viscosity of culture broth was also monitored during alginate production by *A. vinelandii* at various mixing rates. The results are depicted in Fig. 4. The figure shows that the stirring speed has a great importance on the culture broth viscosity; such that low stirring speeds, especially 400 and then 200 rpm value to a lesser extent, cause much higher culture broth viscosities compared to 700 rpm. It is well known that culture broth viscosity is affected by microorganism concentration, alginate concentration, and degrees of acetylation and polymerization of alginate. The dominant increase in alginate concentration as shown in Fig. 2 at 400 rpm (followed by at 200 rpm) could be due to one of these reasons. Our preliminary results obtained in a shake flask under controlled mixing conditions (data not included) indicated that culture broth viscosity may still be high even when the overall alginate concentration was measured low. This result indicated that it is not enough to explain the viscosity data by only alginate concentration. For this reason, we believe that the increase observed in viscosity value could originate from a difference in the degree of polymerization (i.e., a change in molecular mass) of alginate. With this finding in mind, it can indirectly be inferred

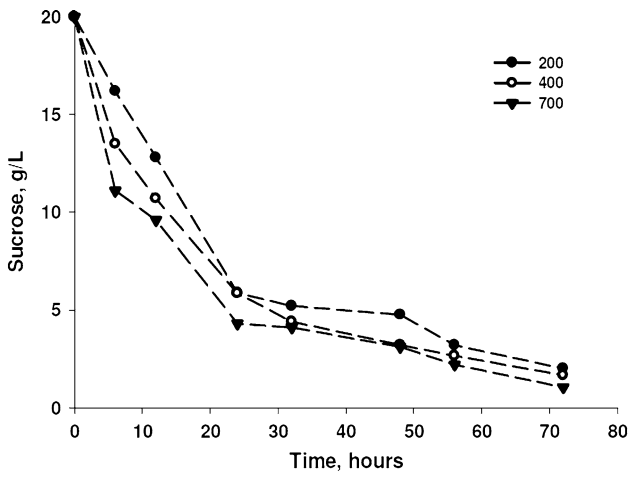

Fig. 3 Changes in sucrose concentration during alginate production at different agitation speeds

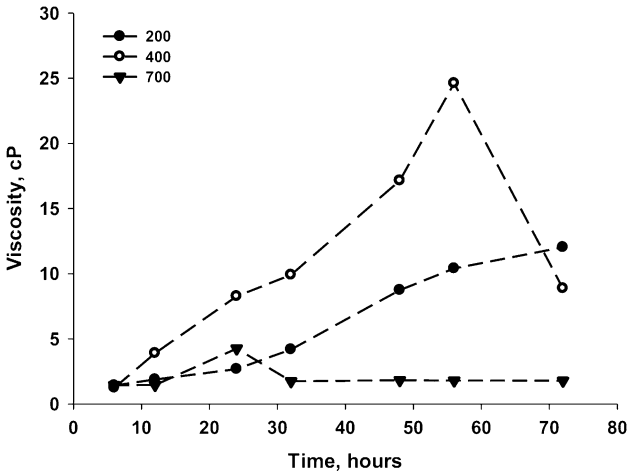

Fig. 4 Variations in culture broth viscosity during fermentation at different agitation speeds

that possibly a higher degree of polymerization was achieved at a moderate level of mixing at 400 rpm. Culture broth viscosity increased up to 24.61 cP until 56 h, which then decreased until the end of 72 h, possibly as a result of activity of extracellular alginate lyases that break the bonds between monomers in the alginate chain leading to oligosaccharides. A smaller reduction in viscosity was also observed after 24 h at 700 rpm. It is assumed that the bacteria use alginate lyase as an editing enzyme that controls the length and molecular weight of the polysaccharide [3, 14]. On the other hand, broth viscosity was continually increased throughout the fermentation process under low agitation conditions. The highest culture broth viscosity was 12.03 cP at 200 rpm, which was still quite low compared to the values achieved at 400 rpm. High agitation speed possibly led to little polymerization of alginate that corresponded to the lowest culture broth viscosity values, only 4.26 cP, compared to the other agitation speeds. Likewise, in the study of Peña et al. [12], alginate was produced by *A. vinelandii* ATCC® 9046 at 5% DOT and under two different agitation speeds, 300 and 700 rpm. They suggested that the viscosity of the culture broth was higher in the culture that was grown at low agitation speed than those grown at high agitation speeds. They also obtained well-matching molecular weight data. For instance, at 5% DOT, mean molecular weight of the polymer at 300 rpm was observed as 680,000 g/g mol compared to 160,000 g/g mol at 700 rpm. A contradicting study showed that molecular weight of alginate increased with increasing mixing speed from 300 to 700 rpm in the presence of inorganic nitrogen source without DOT control [10]. However, it is critical to keep in mind that since the DOT was not controlled, a combined effect of mixing rate and DOT might be responsible for the results obtained by Parente et al. [10] since the uncontrolled DOT might affect

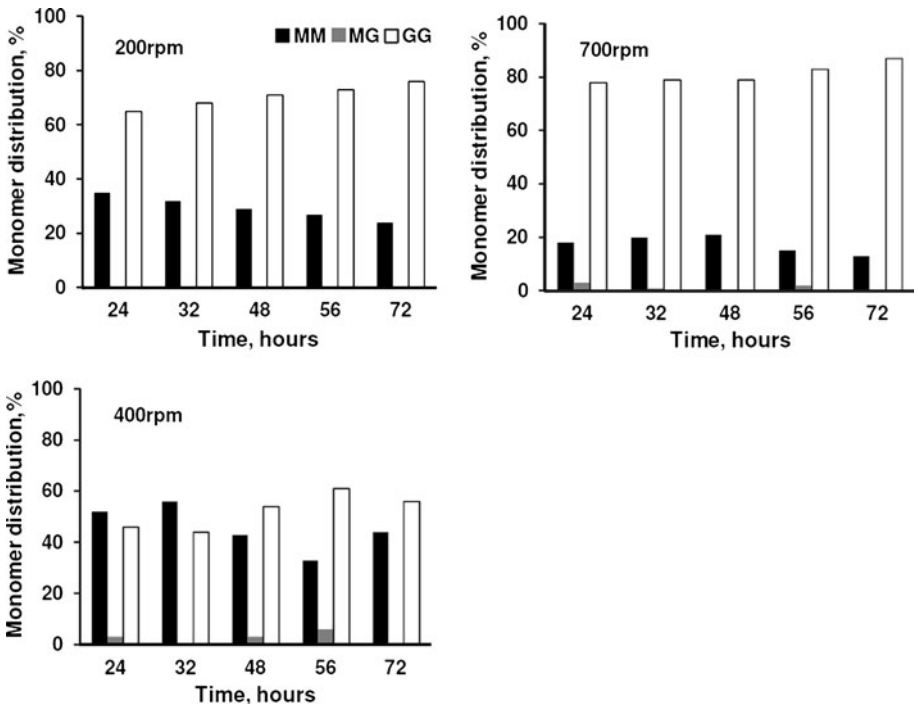

Fig. 5 Variations in monomer distribution during fermentation at different agitation speeds

the molecular weight of alginates. Recently, it was found that the molecular weight of the alginate was greatly influenced by OTR_{max}, rather than mixing rate [7]. In this study, two different mixing rates, 500 and 700 rpm, corresponding to similar OTR_{max} (90 and 100 mmol/l h, respectively) were investigated. Although the agitation speeds were varied, mean molecular weights of alginate was the same around an average value of 160 ± 40 kDa.

Another critical parameter monitored in this work is the block distribution of alginate due to the fact that the relative amount of the monomers affects the polymer's features as well as its final use. Generally, the presence of G residues in alginates introduces the potential for gel formation in the presence of divalent cations such as Ca^{2+}, but harder and denser gel formation can only take place if the G residues are found as consecutive stretches. Alginates rich in MM-blocks cannot form cationic gels, but can form acidic gels [3]. The lengths and numbers of GG-blocks also affect several important physical properties of the gels, such as stiffness, swelling, and porosity. Block distribution of alginate produced by *A. vinelandii* ATCC® 9046 at different agitation speeds was assessed and compared during 72 h of fermentation (Fig. 5). In fact, this study constituted the first study in the literature measuring the monomer distribution of alginate at various mixing speeds while all the other culture conditions were controlled. With the aforementioned results, 400 rpm has been found as the best condition for the highest amount of alginate synthesis compared to the others (Fig. 2). Further results showed that at this value, alginate had almost equal amounts of MM- and GG-blocks with a very low amount of MG-blocks. Therefore, one can say that under this condition, alginate has a moderate level of gel-formation capacity. On the other hand, a paramount epimerization of MM- and MG-blocks into GG-blocks of alginate was observed at 700 rpm. At 700 rpm and 72 h of fermentation, the highest amount of GG-blocks was obtained as 87%, whereas MM-blocks were only about 15%. This finding possibly indicates that bacteria strive to protect themselves from the effects of high shear induced by high mixing speeds by producing a high quantity of GG-block-containing alginate. Similarly, in a previous work, the amount of guluronic acid in alginate produced by *A. vinelandii* DSM® 93-541b (at 5% of air saturation and a fixed dilution rate of 0.15 h^{-1}) increased with increasing mixing rate up to 800 rpm [17]. Results showed (Fig. 5) that in the case of 200 rpm, the amount of mannuronic acid blocks was 35% at 24 h, which was then directly epimerized into only GG-blocks, without any MG-block formation. Therefore, MM-block content of alginate decreased to 24%, whereas GG-block content increased to 76% at the end of the fermentation. It is obvious that this condition, too, similar to the one at 700 rpm, yielded alginate that is ready to form an 'egg box' structure with calcium ions due to the high presence of guluronic acid to produce denser gels. It can be concluded that due to the higher GG-block content, the

gel formation potential is higher at 200 rpm as well at 700 rpm, which might originate from the unfavorable environmental conditions that the bacteria were exposed to.

Conclusions

Alginate production by *A. vinelandii* ATCC® 9046 was investigated at a constant DOT of 5% to evaluate the effect of agitation speed on alginate quality and quantity. Alginate synthesis was found to be effective at a moderate level of mixing speed, 400 rpm, which can possibly overcome the diffusional limitations that were observed at 200 rpm. Besides, the conditions at 400 rpm did not result in wastage of sucrose caused by high respiration activity at high shear rates. Viscosity of the culture broth was also affected by changing the stirring speed: the highest culture broth viscosity possibly corresponding to the best polymerization of alginate was achieved at 400 rpm. Almost half of the mannuronic acid was epimerized into guluronic acid in the case of moderate level of mixing. On the other hand, maximum GG-block content was obtained as 87% at 700 rpm, possibly as a response to the high shear rate.

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