PRODUCTION OF ALGINATE FROM AZOTOBACTER VINELANDII AND ITS USE IN WATER AND WASTEWATER TREATMENT

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ABSTRACT

PRODUCTION OF ALGINATE FROM AZOTOBACTER VINELANDII AND ITS USE IN WATER AND WASTEWATER TREATMENT

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Alginates are copolymers of β-D-mannuronic (M) and α-L-guluronic acids (G). In this study, *Azotobacter vinelandii* ATCC® 9046 was used to produce alginate in a fermentor. The effect of parameters such as dissolved oxygen tension (DOT), agitation speed, initial concentrations of sucrose and calcium on the properties of alginate were examined. Changes of DOT in the range of 1 and 10 % affected alginate production. The optimum DOT giving high alginate yield (4.51 g/L) and maximum viscosity was observed as 5 % yielding moderate GG-blocks of 55 %. Both high and low agitation levels reduced alginate production, but these conditions increased GG-block alginates as 76 and 87 % at 200 and 700 rpm, respectively. Moderate sucrose and calcium concentrations, 20 g/L and 50 mg/L, respectively were found better since further increase in their concentrations did not lead to a considerable improvement in alginate production and quality. Sodium alginates produced in this work were investigated for maximum heavy metal uptake with a special focus on copper ion and the highest copper uptake was around 1.9 mM Cu^{2+}/g alginate. Findings showed that the block distribution of alginate was not as important as expected for copper removal

Alginate together with calcium ions was used for the removal of turbidity. The amount of GG-block was found to be important in turbidity removal. Alginate having 55 % GG block and 8.9 cP viscosity resulted in a final turbidity lower than 1 NTU at 2 mg/L of alginate with 60 mg/L of calcium ion.

Keywords: Alginate, *Azotobacter vinelandii*, Heavy Metals, Monomer Distribution, Turbidity

ÖZ

AZOTOBACTER VİNELANDİİ DEN ALGİNAT ÜRETİMİ VE SU VE ATIKSU ARITIMINDA KULLANIMI

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Alginatlar β-D-manuronik (M) ve α-L-guluronik asitin (G) kopolimeridir. Bu çalışmada *Azotobacter vinelandii* ATCC® 9046 fermentörde alginat üretmek için kullanılmıştır. Çözünmüş oksijen konsantrasyonu, karıştırma hızı, sukroz ve kalsiyum konsantrasyonlarının alginatın özellikleri üzerine etkisi izlenmiştir. Çözünmüş oksijen konsantrasyonunun % 1 ve 10 aralığındaki değişimi alginat üretimini etkilemiştir. Yüksek alginat miktarı (4.51 g/L) ve maksimum viskozitenin, % 55' lik ortalama bir GG-blok miktarı ile gözlemlendiği optimum çözünmüş oksijen değeri % 5' tir. Hem yüksek hem düşük karıştırma hızları toplam alginat üretimini düşürmüş, ancak GG-blok yüzdesini 200 rpm' de % 76, 700 rpm' de ise % 87 olacak şekilde artırmışlardır. Orta seviyelerdeki sukroz ve kalsiyum konsantrasyonları (20 g/L and 50 mg/L) alginat üretimi ve kalitesinde iyileşmeye yol açmış, bu parametrelerin daha yüksek değerleri kayda değer bir iyileşme sağlamamıştır. Bu çalışmada üretilen sodyum alginat özellikle bakır iyonuna ağırlık verilerek maksimum ağır metal alımı için araştırılmış ve en yüksek bakır alımı 1.9 mM Cu^{2+}/g alginat olarak bulunmuştur. Sonuçlar alginatın blok dağılımının bakır giderimi için beklendiği kadar önemli olmadığını göstermiştir. Alginat kalsiyum iyonu ile birlikte bulanıklık giderimi için de kullanılmıştır. GG-blok miktarının bulanıklık gideriminde önemli olduğu

bulunmuştur. % 55 GG-blok ve 8.9 cP viskozitesiye sahip 2 mg/L alginat, 60 mg/L kalsiyum iyonu ile bulanıklık değerini 1 NTU' nun altına düşürmüştür.

Anahtar Kelimeler: Alginat, *Azotobacter vinelandii*, Ağır Metaller, Monomer Dağılımı, Bulanıklık

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER I

INTRODUCTION

1.1. GENERAL

The term ‘alginate’ refers to a group of naturally occurring polysaccharides that are linear copolymers of β-D-mannuronic acid and its epimer, α-L-guluronic acid. The residues are arranged in a block-wise fashion along the polymer chain forming homo- and hetero-polymeric regions. While the former contain mannuronic acid (MM-blocks) or guluronic acid (GG-blocks) units, the latter are made up of both monomers in an approximately alternating sequence, being composed of MG dimers and of GGM- and MMG-residues (Sabra et al., 2001).

Alginates are of a great commercial interest because they present a wide range of applications. For instance, in the food industry, they are widely used as additives capable of viscosifying, stabilizing, emulsifying, and gelling aqueous solutions. Also, pharmaceutical industries commonly use highly purified forms of alginates and alginic acid, the former as stabilizers and the latter as a binder and disintegrating agent in tablets. Furthermore, alginates are currently used in a wide range of other industrial applications, such as in textile industry (as thickeners in printing), paper and board treatment, welding road production, water treatment, latex creaming, and ceramics manufacturing (Clementi, 1997).

Alginates are currently extracted from marine brown algae such as Laminaria and Macrocystis. They are located in the intercellular matrix of the algae as a gel containing different cations and represent the major structural component, up to 40 % of the cell dry matter. The habitats of the different species differ with regard to exposure to periodic drying due to the tides and waves, and this is reflected in different requirements for stiffness, elasticity and water-binding capacity between species. These properties are related to the relative content of each block-type in the polymer, and with this why different species need to produce alginates of various properties can be explained (Ertesvåg and Valla, 1998).

Alginates can also be obtained from bacterial sources, such as *Azotobacter vinelandii* and various species of Pseudomonas. Since only a few species of brown algae are suitable for alginate production with regard to abundance, location and uniform quality, there is an increasing industrial interest in an alternative bacterial alginate. Nevertheless, only Azotobacter alginates which are produced as the extracellular polysaccharides show the typical block structure of the polysaccharides derived from marine algae with the three types of sequences: MM-, GG-, and MG-blocks (Clementi, 1997). The main difference between algal and bacterial alginate is that some of the bacterial M-residues are O-acetylated at the 2 and/or 3 position (Ertesvåg and Valla, 1998). The price of algal alginates is generally low, however, there is the need for using raw materials free from seasonal and/or geographical fluctuations, which is the main disadvantage of naturally produced algal alginates. This need can only be satisfied by using selected strains grown under closely controlled operating conditions to produce a product that suits the specific applications in biotechnology and biomedicine. The characteristics of bacterial alginates depend on the strain and/or on the fermentation conditions. Bacterial alginates may become a promising substitute of algal alginates in selected applications if properly produced under well-controlled conditions (Clementi et al., 1999).

From a commercial point of view, the most important characteristics of alginates are the high viscosity of solutions and their capacity as gelling agents. Whereas the gel forming property of alginate depends on the relative content of the two monomers, the viscosity of alginates, which determines their capability as thickening agent, mainly depends on the molecular weight of the polymer and, to a lesser extent, on the polymer composition (Asami et al., 2004). The gelling properties of alginates are due to their capacity to bind a number of divalent or multivalent cations, the most important being the calcium ions. Following the addition of calcium ions, alginate undergoes conformational changes giving rise to well-known 'egg-box' structure of alginate gelation. This is due to chain dimerization and eventually further aggregation of the dimers. In addition, steric arrangements of the GG-blocks were proposed to explain the ion binding tendency of alginate. This is due to the GG-block conformation that leads to the formation of large voids in the alginate chain. These in turn act as preferential binding sites for calcium ions. Therefore, alginates with a low M/G ratio form strong gels, while alginates with a high M/G ratio form weaker and softer but more elastic gels (Clementi, 1997).

Owing to its gel formation ability with calcium ions, alginate from algae was tested as a coagulant for water treatment in previous studies (Çoruh, 2005, Yüksel, 2007). Calcium alginate has been found to be effective in treating waters having different turbidity levels to provide drinking water quality product. Furthermore, algal alginates are capable of removing toxic heavy metals especially from dilute wastewaters. It is known that some functional groups on the surface of the polymer have a role in toxic metal sorption by ionic interactions and complex formation reactions with the metals (Fourest and Volesky, 1996). Even though there are a number of different heavy metal removal studies conducted using algal alginates, there is no work reporting the use of bacterial alginate for a similar purpose. In view of these, apart from other industrial applications, alginate is believed to have applications in water and wastewater treatment industry. However, the characteristics of alginates from algal sources depend very much on the

environmental conditions. Therefore, it is thought that it can be better to produce alginate from bacterial sources having certain set properties by using laboratory fermenters under constant environmental conditions. In addition it would be easy to modify the operating conditions and hence the properties of alginate as a product to test this material for water and wastewater treatment applications.

1.2. HYPOTHESIS

It is hypothesized that properties of alginate (monomer distribution, chain length, etc.) produced by *Azotobacter vinelandii* ATCC® 9046 would be different for changing environmental conditions and medium composition in a laboratory fermentor. Using this approach, it is possible to produce alginate with required quality for a specific application. Similarly, various characteristics created by different fermentation conditions will determine the potential use of bacterial alginate in water and wastewater treatment applications.

1.3. AIM AND SCOPE

The broader goal of this work is to produce alginate, an extracellular polysaccharide from *Azotobacter vinelandii,* by changing the conditions of fermentation with the purpose of using the produced alginate having various characteristics for water and wastewater treatment applications.

The specific aims of this research can be summarized as follows:

- To find out the culture conditions that would maximize the alginate production from *Azotobacter vinelandii*,

- To find out the growth conditions that would maximize the molecular weight and GG-block content of alginate produced,

- To develop reproducible and reliable methods to extract, purify, and analyze the produced alginate,

- To investigate the possibility of using this polymer for wastewater treatment applications, specifically in heavy metal removal,

- To evaluate the possibility of using alginate for water treatment applications, particularly in turbidity removal.

To achieve these purposes, a laboratory fermentor capable of maintaining constant environmental conditions was used in alginate production. Since both the environmental conditions and medium composition influence the properties of alginate, effect of some parameters potentially important for quality and quantity of the polymer were examined. These factors were selected as dissolved oxygen tension, agitation speed, and initial concentrations of carbon source and calcium ion. Then, bacterial alginate samples from fermentors operated at various conditions were extracted, purified and analyzed. Next, alginate produced was tested in water and wastewater treatment applications. For wastewater treatment, the capacity of alginate to remove some heavy metals was examined. Then the applicability of calcium alginate as a potential coagulant for water treatment was evaluated.

CHAPTER II

THEORETICAL BACKGROUND

2.1. ALGINATE

Alginate was first discovered by the British chemist E.C.C. Stanford in 1881 in marine brown algae as the most commonly found polysaccharide containing up to 40% of the cell dry matter. Microbial alginate was described more than 80 years later by Linker and Jones (1964) who recovered alginate from a mucoid strain of *Pseudomonas aeruginosa* and then they partly identified the polymer which was isolated from the sputum of a cystic fibrosis patient. In 1966, Gorin and Spencer also illustrated the production of acetylated alginate by a soil bacterium, *Azotobacter vinelandii*. The primary interest to microbial alginate has been medically motivated. Not only the quick development of pharmaceutical usage of alginate but also the recognition of its unique immunological properties in recent years have led the biochemists to become concerned with determining the optimal conditions for the production this useful material. Since alginates are widely used in food and pharmaceutical industry as additives and because of pathogenic properties of *Pseudomonas* spp, *Azotobacter vinelandii* seems to be a better candidate for alginate production.

2.1.1. STRUCTURE OF ALGINATE

Alginate is a family of unbranched binary co-polymers composed of two monomeric units, (1–4) linked β-D-mannuronic acid (M) and its epimer α-L-guluronic acid (G), of widely varying composition and sequential structure (Figure 2.1) (Sabra et al., 2001). The arrangement of such monomeric units can be comprised of homopolymeric and heteropolymeric regions. While homopolymeric regions contain mannuronic or guluronic acid units, namely MM and GG-blocks, heteropolymeric regions are composed of both monomers interspersed with regions of approximately alternating heteropolymeric structure, MG-blocks. Alginates isolated from different natural sources differ in the length and distribution of the varying block types (Clementi, 1997).

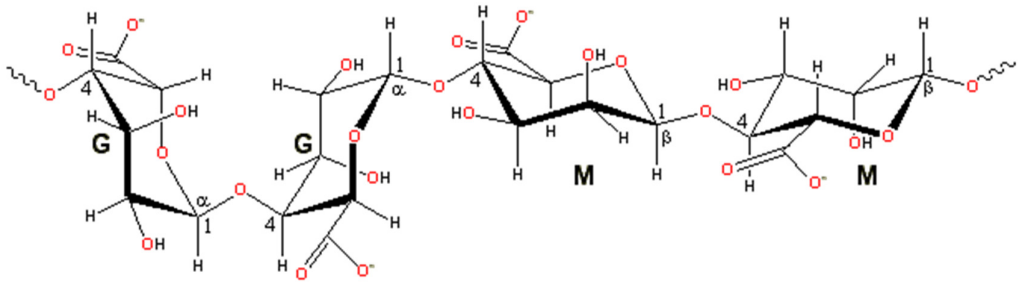

Figure 2.1. Schematical representation of alginate monomers

Since the configuration of mannuronic and guluronic acids are different from each other and so the linkage between the monomers, all these sequences have a different geometry. MM-blocks look like a comparatively straight polymer because they are formed from equatorial groups at C-1 and C-4 positions. Thus, MM-block conformation is meant to a twofold extended ribbon in the alginate polymeric chain. In the case of GG-blocks, however, they are composed of axial groups at both C-1 and C-4 positions, therefore, the resulted chain is a twofold buckled ribbon. This shape is particularly

significant for gelling properties of alginate in the presence of polyvalent cations. These monomers, mannuronic and guluronic acids, constitute alginate polymer by joining at the positions of C-1 and C-4 as in Figure 2.1. In other words, an ether-oxygen bridge joins the carbon at the 1-position in one molecule to the 4-position of another molecule (Mchugh, 1987).

2.1.2. OCCURENCE OF ALGINATES IN NATURE

Alginates are naturally occurring polysaccharides that mainly exist in the cell wall of the brown algae. They are found in the form of the mixed calcium-sodium-potassium salt of alginic acid (Clementi et al., 1995). Although all the numerous species of brown algae contain alginate, only the species *Laminaria hyperborea*, *Macrocystis pyrifera*, *Laminaria digitata*, *Ascophyllum nodosum*, and, to a lesser extent, *Laminaria japonica*, *Eclonia maxima*, *Lessonia nigrescens*, *Durvillea antarctica*, and *Sargassum spp*. are processed for the commercial production of the polymer (Clementi, 1997). Selection of which seaweeds to process is performed depending on the properties of alginate from particular species and availability of the algae for harvesting, because the properties of the polymer changes greatly from one species to another (Mchugh, 1987).

In general, it is believed that the alginate acts as a structural component in marine brown algae due to the fact that alginate is the most abundant polysaccharide found in the plant, which may be included up to 40% of total dry weight. The intercellular gel matrix of the algae supplies both mechanical strength and flexibility to the organism. The algae vary in requirements for stiffness, elasticity and water-binding capacity between species as their habitats differ according to exposure of the species to periodic drying due to the tide and waves. Since these properties are related to the relative content of each block-type in the polymer, this probably explains why different species need to produce different alginates or even alginates vary between different tissues from the same plant (Ertesvåg and Valla, 1998). Thus, there is an obvious relation between structure and function of alginates. For

instance, in *Laminaria hyperborea*, which is an algae grows in very exposed coastal areas, the stipe and holdfast contain a very high content of GG-blocks providing high mechanical rigidity to the plant. On the other hand, the leaves of the same algae having an alginate with low guluronic acid content which might be explained that the leaves float in steaming water need a more flexible texture (Draget et al., 2005).

Alginate production is not limited with marine brown algae because some species of bacteria such as *Pseudomonas*, *Azotobacter vinelandii* and *Azotobacter chroococcum* are able to synthesize alginate. However, only the alginate obtained from *Azotobacter vinelandii* shows the typical block structure with the polymer produced by the seaweeds. On the other hand, the alginates produced by *Pseudomonas spp.* differ in that they do not have GG-blocks structure in the polymer chain which means their alginates are not suitable to use them as gellifying agent. As a result, *Azotobacter vinelandii* replicates the algal alginates best among a number of similar candidates. In addition, *Azotobacter vinelandii* has no pathogenic nature like *Pseudomonas spp*. Furthermore, if the bacterium is cultivated in a medium including Ca^{2+} ions, the alginate synthesized has long homopolymeric sequences of L-guluronic acid residues (Rehm and Valla, 1997).

Pseudomonas aeruginosa is a pathogenic bacterium which causes cystic fibrosis in humans. It produces alginate that provides a protecting coating for the organism against unfavorable conditions. Furthermore, alginate improves adhesion property of the bacterium to solid surfaces. Therefore, *Pseudomonas aeruginosa* form a biofilm giving an advantage to the bacterium to survive under adverse environmental conditions. *Pseudomonas aeruginosa* is also capable of synthesizing a special enzyme, an alginate lyase, which can break the bonds between monomers in the alginate chain leading oligosaccharides. Then, the bacterium is no longer connected to the surface of the lung and able to spread itself to obtain new sites for the colonies (Boyd and Chakrabarty, 1994; 1995; Rehm, 1998).

On the other hand, in *Azotobacter vinelandii*, alginate is a crucial constituent in the cyst production. The alginate coating provides a barrier for the cyst from desiccation and unfavorable environmental conditions. By making the cyst, *Azotobacter vinelandii* can survive in dry soil for several years. The cyst coating around the bacterium swells and germinates when the conditions become better for growth of the bacteria (Nunez et al., 1999). However, the association of alginate production with cyst formation and its structural significance do not explain the abundant production of this polymer during vegetative growth. Under conditions favorable to grow, alginates are synthesized by *Azotobacter vinelandii* as extracellular polysaccharides which have different roles according to the environmental conditions.

Azotobacter vinelandii is a stable producer of acetylated alginate under different circumstances unlike the *Pseudomonads*. Alginate does not serve as an overflow metabolite; rather it plays a role as a protective barrier against heavy metal toxicity or as an ion exchange system with high selectivity particular to calcium ions. Alginate also supplies *Azotobacter vinelandii* to a hydrophilic, negatively charged coating which provides protection against attack and adverse environmental conditions (Sabra et al., 2001). A study conducted by Hammad (1998) showed that alginate-encapsulation prevented *Azotobacter chroococcum* better from the depressive effect of phages compared to the cells which did not have alginate capsule. In another work, *Azotobacter vinelandii* grown in a continuous culture under nitrogen fixation conditions and varying dissolved oxygen concentrations (Sabra et al., 2000) had an alginate capsule around the cells even in the presence of a high shear rate in the medium. Furthermore, it was found that the capsule layer was denser and more compact especially at elevated dissolved oxygen values. Therefore, it was claimed that alginate layer might act as a diffusion barrier between medium and the bacteria in order to protect nitrogenase enzymes used for nitrogen fixation from being damaged by oxygen due to their vulnerability to molecular oxygen. The conclusion to be drawn by these facts is that alginate has no

single function for the vegetative cell itself, but rather provides the cell with a multitude of protective properties under various environmental conditions.

Although the block structures of algal and bacterial alginates are somehow similar, it is evident that there are some differences between them, which are important particularly for the end use of these exopolysaccharides. The most relevant difference between alginate from algae and bacteria is that the bacterial polymers carry *O*-acetyl groups on the D-mannuronic acid residues. Acetylation of these microbial alginates is mainly observed on O-2 than on O-3 positions, nevertheless each mannuronic acid has two hydroxyl groups suitable for substitution. In highly acetylated polymers produced by *Pseudomonas* and *Azotobacter spp*., two or three diacetylated units are observed. A varied degree of acetylation of bacterial alginates is reported in the different species and in different strain of the same species as well because the degree of acetylation is observed to be affected by the cultivation conditions (Clementi, 1997).

Existence of acetylation groups on microbial alginates influences their polyelectrolyte behavior which makes them differ from the algal polymer. They increase ionization degree of the alginates and limit binding ability and selectivity coefficient for cations, such as Ca^{2+} (Clementi, 1997). Therefore, degree of acetylation of the alginates primarily has an effect of gelling capacity of the polymer which is reduced as the degree of acetylation increases. On the other side, the presence of *O*-acetyl groups on bacterial alginates might be advantageous on some properties of the polymer, for example, it has been found that the viscosity of algal alginates increases after random acetylation. Also, the acetylated gels have enhanced swelling ability (Skjak- Bræk et al., 1989).

Furthermore, microbial alginates might have higher molecular weight than the algal product depending on the environmental conditions in cultivation, i.e. especially if they are collected early during fermentation. This property is important for subsequent usage of the polymer. The molecular weights of

algal alginates have been found to range from 48,000 to 186,000 while the values between 64,000 and 1,900,000 Da have been observed for alginates produced by *Azotobacter vinelandii* (Pena et al., 2000).

2.1.3. PROPERTIES OF ALGINATE

The relative amount of each block type, MM-, MG- and GG-blocks, changes between various alginates and the block types show distinct structural differences, all of which influnce the properties of alginates. For instance, while MG-blocks form the most flexible chains and they are more soluble at lower pH than the other two block types, MM-blocks have strong immunostimulating property and GG-blocks are able to form stiff chains (Ertesvåg and Valla, 1998). In view of the commercial exploitation of alginate, the viscosity and gel-forming capacities are the most important characteristics of this polysaccharide. These in turn are affected largely by the block structure and chain length of the polymer (Sabra et al., 2001).

Intrinsic viscosity of alginates determines the capability of inducing viscosity of the polymer into a solution. It can be defined as the ratio of a solution's specific viscosity to the concentration of the solute, extrapolated to zero concentration. This property is primarily determined by molecular weight of the polymer. An empirical equation is developed by Mark–Houwink as follows (Huang et al., 2000):

$$IV = k\,(MW)^{a} \qquad (2.1)$$

Where; IV is the intrinsic viscosity (dL/g), MW the molecular weight and k and a are the Mark–Houwink constants. Mostly, commercially available high viscosity algal alginates have an average molecular weight of around 150,000, which corresponds to a degree of polymerization (the number of uronic acid units per average chain) of about 750. In addition, medium viscosity and low viscosity alginates have a mean molecular weight of about 120,000 and 80,000, respectively (Clementi, 1997). Generally, alginates are

polydisperse (Polydispersity index is the ratio of mean molecular weight to mean number weight) with respect to molecular weight, similar to the other polysaccharides. Because of this polydispersity, the molecular weight of an alginate is evaluated by an average value rather than the whole distribution of molecular weight in alginate chain (Draget et al., 2005).

pH, ionic strength and presence of gelling ions are the factors which influence solubility of alginates in water. The electrostatic charges on the uronic acid residues are affected by the pH of the solvent, and potentiometric titration reveals that the dissociation constants for mannuronic and guluronic acid monomers are 3.38 and 3.65, respectively. An abrupt decrease in pH below the pKa values causes precipitation of alginic acid molecules. On the other hand, the ionic strength of the solute also plays an important role since the solubility of the alginate is limited in solvents with high ionic strength. Also, any change of ionic strength in an alginate solution has a profound effect, especially on polymer chain extension and solution viscosity. Obviously the content of gelling ions in the solvent limits the solubility as well. Especially, the hardness of the water is most likely to be the main problem. While the polymer forms soluble salts with monovalent ions (with the exception of Ag^{+}), alginate forms gels or precipitates when it is introduced to polyvalent metal ions. The affinity of alginate towards divalent ions for the alkaline earth metals is found to be increasing in the order of $Mg^{2+}<<Ca^{2+}<Sr^{2+}<Ba^{2+}$ (Draget et al., 2005).

The most suitable ion for the constitution of an alginate gel is calcium ion because of the fact that the length of its radius fits better into the spaces between alginate monomers particularly those between guluronic acid residues than the other divalent ions. Following the addition of calcium ions, alginate undergoes conformational changes, giving rise to the well known ‘egg-box’ model of alginate gellation (Clementi, 1997). During gellation, two or more chains of alginate bind cooperatively (Figure 2.2). Calcium ions might pack and be coordinated in interstices within buckled chain of guluronic acid monomers having a two-dimensional analogue of a

corrugated egg-box. Mchugh (1987) explains these issues that "the analogy is that the strength and selectivity of cooperative binding is determined by the comfort with which 'eggs' of the particular size may pack in the 'box', and with which the layers of the box pack with each other around the eggs. The model can be extended to be three-dimensional. While calcium helps to hold the molecules together, their polymeric nature and their aggregation bind the calcium more firmly; this has been termed cooperative binding. The structure of the guluronic acid chains gives distances between carboxyl and hydroxyl groups which allow a high degree of coordination of the calcium". Several different chains may become interconnected and this promotes gel network formation. The higher the degree of linkage, the greater the resulting viscosity is.

On the other side, alginic acid gels can be formed by a slow and controlled release of protons. As the pH is decreased, number of carboxyl ions protonated on the alginate chains is increased which results in a reduction of the electrical repulsion between alginate chains. Then, hydrogen bonding between the chains can be easier and more effective since alginate chains become closer with the decrease of repulsion between them. Firstly, this leads to a higher viscosity in the solvent and finally, at around pH 3.5-4.0, an alginic acid gel forms (Mchugh, 1987). The homopolymeric blocks play an important role to form the junctions between the chains in these gels. However, the stability of the gel is mostly determined by the relative content of guluronic acid blocks (Ertesvåg and Valla, 1998). Alginic acid gels differ from calcium-alginate gels in that they are weaker and are reversible at increasing pH.

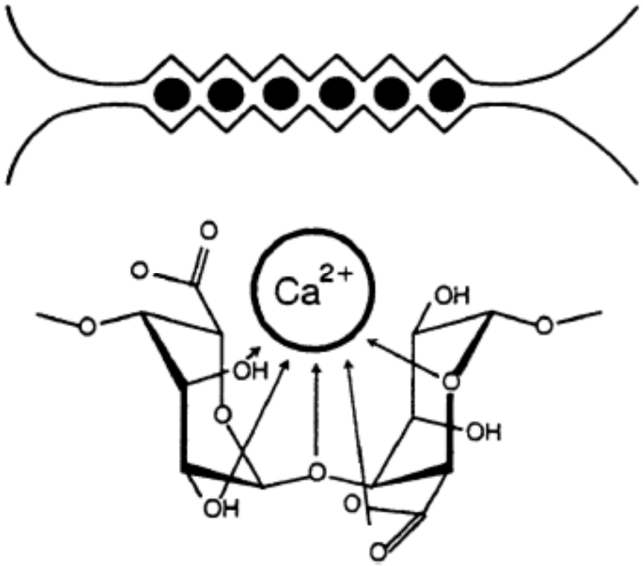

Figure 2.2. Gel formation via GG-blocks: Egg box model (Sabra et al., 2001)

The ratio of mannuronic acid to guluronic acid changes between various alginates and different proportions of MM-, GG- and MG-blocks that can be found. This ratio, and block distribution in alginate chains have a crucial effect on gel formation and gel strength. Also, the strength of alginate gels is influenced by the number of cross-links formed between alginate chains and the type of cross-linking ions (Sabra et al., 2001). Alginates having high GG-blocks constitute rigid gels and they form fairly suddenly as calcium ion concentration is steadily increased. On the other hand, for the alginate with mainly MM-blocks, it is quite the opposite since in that case the gel forms gradually and it is softer and more elastic. This can be explained by differences in the molecular structure of the gels. Also, the source and concentration of the alginate, its degree of polymerization and the calcium concentration might affect the gel strength.

2.1.4. COMMERCIAL APPLICATIONS OF ALGINATE

Alginates that have distinct qualitative features have a clear preference that they might potentially be sold at higher prices than the bulk polymer. Also, such new polysacchraride products may at least theoretically open new kinds of markets. Alginates have different industrial applications as viscosifiers, stabilizers and gel-forming, film-forming or water-binding agents (Rehm and Valla, 1997). Mostly, alginate is used in the form of the readily water-soluble sodium salt, although other salts, like calcium, potassium, and ammonium alginate and the ester derivative propylene glycol alginate (PGA) are commercially available as well (Clementi, 1997). Nowadays, alginates which are used for industrial applications are widely extracted from marine brown algae and the global market for this polymer is around 30,000 ton/year. For majority of the commercial applications, the selling prices for alginate are in the range of 5-20 $ per kilogram. It looks like a challenging work to set up a competitive bacterial production process in this price values because of low prices of such alginates. However, it is possible that the bacterial alginate might become a commercial product owing to both environmental concerns related with algae harvesting and processing and the probability of producing high quality alginate by using microbial processes. For instance, pharmaceutical applications may require high quality alginates of extreme purity that are sold for up to 40,000 $ per kilogram in the market (Rehm and Valla, 1997).

Food industry is the primary user for most of the alginate being harvested from brown seaweeds which may be as high as 50 % of total consumption (Sabra et al., 2001). Alginate is used as a thickener for sauces and syrups and also toppings for ice cream. Furthermore, alginate prevents water-in-oil emulsions such as mayonnaise and salad dressings from separation into their original oil and water phases. In addition, PGA is used in order to stabilize milk proteins under acidic conditions, as found in some yoghurts. Also, alginate enhances the texture, body and sheen of yoghurt. In addition, alginate has an application in fruit drinks with fruit pulp addition in that

sodium alginate or PGA help the pulp stay in suspension, so prevent the drink from unwanted sedimentation under acidic conditions. Similarly, alginate/phosphate mixture keeps the cocoa in chocolate milk in suspension. Small amounts of alginate are able to thicken and stabilize whipped cream. Besides that, alginates have some usages which are not either relevant to their viscosifying character or gelling ability. For example, when alginates are added into ice cream, they have a role as stabilizers resulting in a more smooth product by reducing the formation of ice crystals during freezing. A stable and longer lasting beer foam can also be obtained by just addition of a very low concentration of PGA. For the removal of fine particles and clarification of wine, different chemicals are used, however, it is observed that the addition of sodium alginate in more difficult cases may be much more effective (Mchugh, 2005).

Fruit substitutes mainly depend on another property of alginate, capability of producing gels with cations. Alginates in the presence of calcium are used for the formation of edible dessert jellies. These are usually promoted as fast jellies or desserts since they are simply made by just mixing the powders with water or milk and having no requirement for heating. In addition, alginate gels are utilized for the production of re-structured or re-formed food products. For instance, meat pieces are taken and bound together and then a shape resebling to usual cuts of meat, such as nuggets, roasts, meat loaves, even steaks are given to prepare re-structured meats with the help of a powder sodium alginate. Minced fish might be used for the production of restructured fish fillets by using alginate with the addition of calcium. Frozen fish products can be preserved with the help of calcium alginate films and coatings. Likewise, the oils in oily fish can be protected from the air and rancidity if the fish is frozen in a calcium alginate gel otherwise the oil can become rancid through oxidation even when quick frozen and stored at low temperatures. The last example is that, calcium alginate films help to preserve beef cuts from bacterial contamination before freezing and make the meat juices released during thaw re-absorbed into the meat (Mchugh, 2005).

Alginates are also used as thickeners for the dye containing pastes in textile industry. They attract attention as thickeners with the advent of reactive dyes which react chemically with cellulose in the fabric. In general, usual thickeners like starch combines with the reactive dyes resulting in a decrease in color yields and sometimes yields by-products that are difficult to wash out. On the other hand, alginates have an advantage because of the ability to wash out of the finished textile easily and not reacting with the dyes. Thus, they seem to be the best thickeners for reactive dyes (Mchugh, 2005).

Alginates have a wide range of applications in the pharmaceutical industry. They are used as wound dressings and dental impression materials. In addition, controlled release of the drugs can be achieved by this polymer (Ertesvåg and Valla, 1998) with the help of calcium alginate beads in which the active ingredient is placed. Therefore, a slowed release of the drug might be possible when the bead is exposed in an appropriate environment (Mchugh, 2005). Another application of alginates is related with disintegration ability of the polymer. Although alginic acid is insoluble in water, it swells which makes alginate useful as a disintegrating agent in tablets. Furthermore, sodium alginate is applied as viscosifying agent in some liquid medicines and used to suspend solids. While PGA is able to enhance the stability of emulsions, capsules having sodium alginate together with calcium carbonate have ability for the protection of inflamed areas near the entrance to the stomach. Because the environment is highly acidic in the stomach, this results in insoluble alginic acid and carbon dioxide. Then, the alginic acid rises to the top of the stomach contents and forms a protective layer. Moreover, alginates are constituents of most of diet foods such as biscuits. They turn to alginic acid in the the stomach and fills it by swelling so that the dieter no longer feels hungry (Mchugh, 1987).

One other application of microbial alginates includes their use either as immunostimulants or as gel-forming agents for immobilization of cells. Immobilized cells have applications both in various biotechnological production processes and in medical transplantation technologies. An

important example to that is the reversal of type I diabetes by immobilizing insulin-producing cells in alginate capsules (Rehm and Valla, 1997) by implanting the capsules into the body of whole animals and even humans. This polysaccharide has an ability to activate immune cells to secrete cytokines. It is interesting that the immune system responds depending on the monomer distribution of the alginates. While MM-blocks seem to have the highest response, GG-blocks appear to be non-stimulating. Actually, due to some negative effects of GG-blocks like antibody generation, they cannot be accepted in therapeutic preparations (Sabra et al., 2001). The other examples for the use of alginate as an immobilizer are production of ethanol from starch, beer brewing with immobilized yeast, production of citric acid, continuous yoghurt production, fermentation to produce butanol and isopropanol, continuous acetone-butanol production, pilot-plant production of prednisolone from hydrocortisone, glycerol production from the marine alga, *Dunaliella tertiolecta* (Mchugh, 1987).

Alginate has also several miscellaneous applications such as the coating of tree roots prior to planting and as an inert pesticide adjuvant in the coating of fresh citrus fruits. Furthermore, they are used in paper and board treatment, in welding road production, water-treatment processes, can sealing, latex creaming and ceramics manufacturing (Clementi, 1997).

2.2. ALGINATE PRODUCTION

2.2.1. AZOTOBACTER VINELANDII

Azotobacter vinelandii is a widely distributed free-living soil bacterium which is currently classified as a genus of the family *Pseudomonadaceae*. In addition, it is a gram- negative and strictly aerobic bacterium. The cells have rod shape, being in sizes in the range of 2 - 5 µm (Pena et al., 2002). *Azotobacter vinelandii* gains attention from the scientists because of its

many interesting features, including the ability to grow on a wide variety of carbohydrates, alcohols and organic acids. Moreover, it can form cysts which protect the bacterium under dessication and other physical and chemical agents. The bacteria are also able to fix atmospheric nitrogen gas (N_2) by reducing to ammonium (NH_4^+) with the help of one of three distinct but related nitrogenase enzymes. These enzymes require metals such as molybdenum, vanadium, or iron to activate and they need a huge amount of cellular energy for biosynthesis and activity (Holt et al., 1994). *Azotobacter vinelandii* plays a role for recycling of the hydrogen produced as a byproduct of nitrogen fixation thereby increasing its efficiency. It is interesting to say that the bacteria can fix atmospheric nitrogen gas under fully aerobic conditions unlike other diazotrophic bacteria which must fix nitrogen either anaerobically or microaerobically since nitrogenase enzymes are extremely liable to atmospheric oxygen supply (20% of air saturation). On the other hand, *Azotobacter vinelandii* has developed sophisticated physiological mechanisms to protect their nitrogenase enzymes from oxygen damage.

The other remarkable properties of *Azotobacter vinelandii* are that the bacteria produce two polymers of industrial interest, namely alginates and poly-β-hydroxybutyric acid, both of which have applications particularly in food and biodegradable plastic industries, respectively.

2.2.2. ALGINATE SYNTHESIS MECHANISM IN *AZOTOBACTER VINELANDII*

Azotobacter vinelandii is known to synthesize two different polymers, namely poly-β-hydroxybutyrate (PHB) and alginate. PHB, a biodegradable thermoplastic, is produced by the bacteria intracellularly during unbalanced growth and it is stored by a large variety of bacteria for carbon and energy sources. Species of *Alcaligenes* are widely utilized for industrial production of PHB which has an application as a substitute for bulk plastics (Sabra et al., 2001). On the other hand, alginate is either produced extracellularly by

actively growing *Azotobacter vinelandii* or it participates in formation of the cyst coat in the encystment process.

PHB is required together with alginate in order to achieve a successful cyst. The cyst has a central body which is encased in an inner coat, the intine, and an outer coat, the exine. The body consists of lipid accumulations of PHB while the inner and the outer coats are made of alginate as homo- and heteropolymeric sequences namely mannuronic and guluronic acids blocks. Both the intine and the exine have nearly the same amounts of heteropolymeric blocks. However, the intine is mainly richer in polymannuronic acid and the exine is richer in polyguluronic acid (Sadoff, 1975; Page and Sadoff, 1975).

Alginate produced by actively growing cells possibly plays different roles depending on the environmental conditions. It supplies a protective barrier to the bacteria against unfavorable situations such as heavy metal toxicity, high oxygen concentrations, and high shear rates. The pathway for alginate synthesis in bacteria may be analyzed in four different stages: (i) synthesis of precursor substrate, (ii) polymerization and cytoplasmic membrane transfer, (iii) periplasmic transfer and modification, and (iv) export through the outer membrane and extracellular epimerization (Remminghorst and Rehm, 2006).

2.2.2.1. Alginate Precursor - GDP-Mannuronic acid- Synthesis

All steps comprising degradation of central sugar metabolites into the alginate precursor GDP-mannuronic acid have been examined widely, so they are well understood in the past. GDP-mannuronic acid is known to be synthesized in the cytosol of the bacterial cell. Beale and Foster (1996) who investigated carbohydrate fluxes into alginate biosynthesis in *Azotobacter vinelandii* by NMR studies, showed that alginate production follows Entner-Doudoroff pathway, which is a low energy-yielding pathway common to many gram-negative bacteria (Figure 2.3). The studies showed that there

are three enzymes which are directly responsible for biosynthesis of GDP-mannuronic acid. Fructose 6-phosphate is converted to mannose 6-phosphate by a bifunctional enzyme which is called as phosphomannose isomerase (PMI) / guanosine-diphosphomannose pyrophosphorylase (GMP) and designated by AlgA (PMI–GMP). After the formation of the first intermediate, mannose 6-phosphate, conversion of mannose 6-phosphate into mannose 1-phosphate is catalyzed by phosphomannomutase (PMM, encoded by the AlgC gene) in the second step. Then, the mannose 1-phosphate is converted into GDP-mannose by the GMP reaction of enzyme AlgA with accompanying hydrolysis of GTP. Actually, AlgA favours the opposite reaction; however, the whole pathway proceeds successfully in the direction of alginate biosynthesis because the GDP-mannose is effectively removed in the following step. The step involves production of an immediate precursor for polymerization includes the almost irreversible oxidation of GDP-mannose to GDP-mannuronic acid with help of the enzyme guanosine-diphosphomannose dehydrogenase (GMD, encoded by the AlgD gene) (Rehm and Valla, 1997). AlgD-mediated conversion of GDP-mannose into GDP-mannuronic acid is found as the limiting step and key kinetic control point of the alginate biosynthesis depending on high concentration of GDP-mannose in the cell and its utilization in various pathways (Remminghorst and Rehm, 2006).

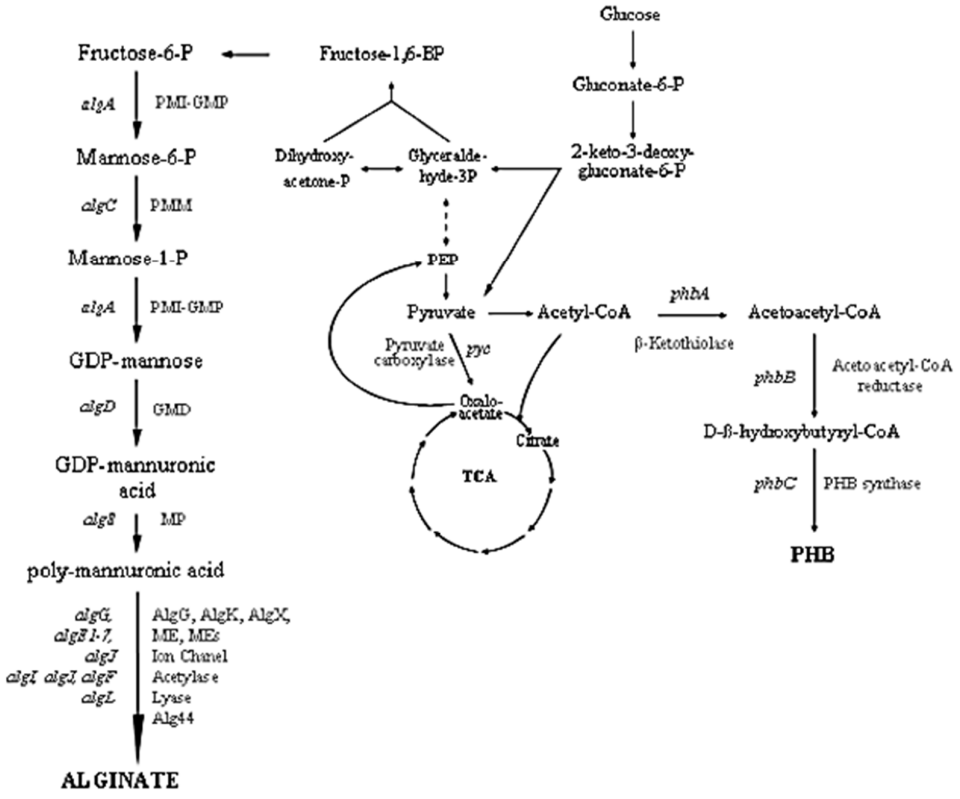

Figure 2.3. Pathways for alginate and PHB biosynthesis in Azotobacter vinelandii (Galindo et al., 2007)

2.2.2.2. Cytoplasmic Membrane Transfer of GDP-Mannuronic acid and Polymerization of the Precursor

The second stage of alginate synthesis involves the transfer of the cytosolic precursor GDP-mannuronic acid across the cytoplasmic membrane and the polymerization of the monomers to polymannuronate. It is because an efficient in vitro system for alginate synthesis has not been constructed yet, the polymerization step is not understood well. It is assumed that polymerization enzymes are located in the cytoplasmic membrane in the form of complexes (Figure 2.4). It is found that the Alg8 and Alg44 proteins catalyze polymerization reaction of GDP-mannuronic acid (Galindo et al., 2007). Although the role of the genes coding these enzymes has not been

known clearly, they form a part of the alg gene cluster. Nevertheless the location and function of protein Alg44 has not been elucidated for a long time. Due to its hydrophobic character, Alg44 was thought as a membrane protein. However, recent works showed that Alg44 functions as part of the periplasmic scaffold and/or bridging Alg8 in the cytoplasmic membrane with AlgE located in the outer membrane. In addition, there is another protein, AlgX that has a potential role in polymerization of GDP-mannuronic acid, although it has not been confirmed by direct experimental evidence (Valla et al., 2001). In the export through the outer membrane, a multi-protein complex might be formed with the help of cytoplasmic and outer membrane proteins as well as periplasmic proteins (Remminghorst and Rehm, 2006).

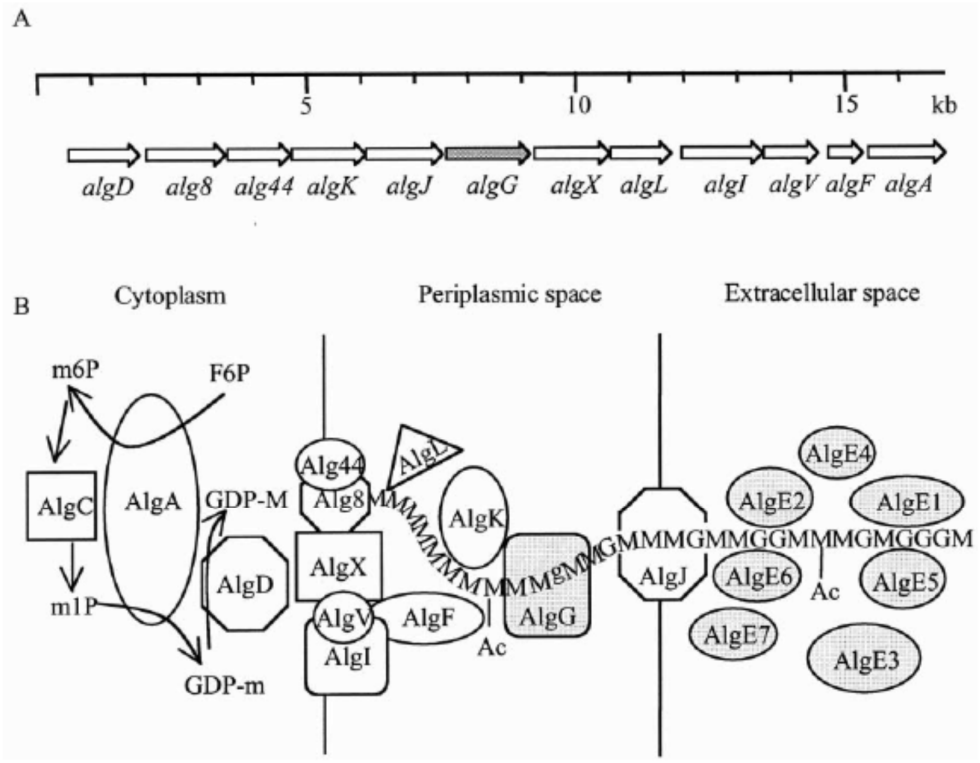

Figure 2.4. A. The alginate biosynthetic gene cluster. B. Schematic model of alginate biosynthesis in Azotobacter vinelandii (Valla et al., 2001)

2.2.2.3. Export of Alginates through the Periplasm and Modification of the Polymer

Alginates are modified with some enzymes which are the ones characterized well in the synthesis process. Elucidating the reaction mechanisms of these alginate modifying enzymes might be helpful in order to produce tailor-made alginates.

The location where the modification of synthesized alginate takes place is the periplasm, so the polysaccharide is first produced as polymannuronate which later being modified at polymer level. Besides that, the genome of *Azotobacter vinelandii* encodes a family of seven extracellular Ca^{2+}-dependend epimerases (designated AlgE1-AlgE7) (Ertesvåg et al., 1999; Valla et al., 2001; Bjerkan et al., 2004). However, the genes encoding these extracellular epimerases are not localized within the alginate biosynthesis gene cluster. On the other hand, all other genes that code alginate modifying enzymes are found within the bacterial biosynthesis operon, comprasing the genes related with the acetylation-complex, namely AlgI, AlgJ, AlgF, the mannuronan C5-epimerase, designated as AlgG, and the alginate lyase, called as AlgL (Figure 2.4) (Remminghorst and Rehm, 2006).

The polymannuronate residues are acetylated by the proteins of AlgI, AlgV, and AlgF that are located in the periplasmic space although acetylation itself is not needed for alginate biosynthesis (Galindo et al., 2007). Transacetylation happens at hydroxyl-groups at C-2 and/or C-3 positions of the mannuronic acid residue. Acetylation makes the mannuronate residues less available to further modification which means it inhibits epimerization of mannuronic acid into guluronic acid as well as degradation of the alginate molecule by lyases (Franklin and Ohman, 1993; Franklin et al., 1994). As a result, the bacteria manage epimerization and degradation processes indirectly by periplasmic acetylation. On the other side, acetylation might be useful to bacteria under dehydrating conditions since the higher the degree

of acetylation in alginates the better the water binding capacity of the polymer.

Alginates are degraded by alginate lyases, which are also called as alginases or alginate depolymerases, by β-elimination reaction. All lyases give a reaction in a similar way to the epimerases although each different lyase is determined by its substrate specificity and cleavage pattern generally. For example, some alginases favor guluronate residues as substrate while some prefer to use only glycosidic bonds between mannuronate residues. It is assumed that the bacteria use alginate lyase as an editing enzyme which controls the length and molecular weight of the polysaccharide. Also, the organism synthesizing alginate cannot metabolize it as a carbon source (Remminghorst and Rehm, 2006).

Alginates also undergo modification by the epimerases which convert unacetylated β-D-mannuronic acid residues to their C-5-epimers α-L-guluronic acid. Although *Azotobacter vinelandii* encodes only one periplasmic mannuronan C5-epimerase (AlgG), the bacteria also have seven extracellular enzymes. Each of these seven enzymes provides various characteristic monomer distributions of G-residues in the mannuronic acid used as a substrate in vitro. As a consequence, this might be an explanation why there is an extensive variability in the alginate synthesized by *Azotobacter vinelandii* (Ertesvåg et al., 1999). This variation in the structure of the alginate can be biologically significant for the formation of the resting-stage, designated as cyst.

Contrary to the extracellular epimerases, AlgG does not require Ca^{2+} ion for activity. Also, this mannuronan C5-epimerase can only produce alternating sequences - MG-blocks - in the polysaccharide chain similar to the case in *Pseudomonas sp.* It was found that the epimerase activity of AlgG was not essential for alginate synthesis, because uronic acids were produced even when the full protein was deleted from the gene cluster (Gimmestad et al.,

2003; Jain et al., 2003). This may be explained by the fact that AlgG may also function in scaffold formation.

There are other proteins which are responsible for the formation of a scaffold. This formation is useful for the nascent alginate chain since it protects and aligns the alginate during traversing through the periplasm. algK and algX are assumed to be the proteins involving in scaffold formation as deletion of these proteins resulted in the secretion of free uronic acids probably due to extensive alginate lyase degradation (Jain and Ohman, 1998; Robles-Price et al., 2004). Although the role of alginate lyase in the scaffold formation is argued, it is still not clear whether the alginate lyase is needed for alginate production or not (Remminghorst and Rehm, 2006).

2.2.2.4. Transfer of Alginate through the Outer Membrane and Extracellular Epimerization by AlgE1-7

AlgE seems to be involved in the export of alginate through the outer membrane according to the biochemical analyses performed in *Pseudomonas sp*. An anion selective pore is constituted by AlgE and as the lipid bilayer studies showed that this pore may be partly blocked by GDP-mannuronic acid. Since AlgE has high similarity in structure with the protein AlgJ in *Azotobacter vinelandii*, it might be suggested that AlgJ has the same functions as its *Pseudomonas* counterpart (Remminghorst and Rehm, 2006). As a result, transfer of the alginate through the outer membrane is achieved with the help of the pore-forming protein AlgJ in *Azotobacter vinelandii* (Galindo et al., 2007).

Alginate is further modified by a series of mannuronan C-5- epiremerases, designated as AlgE1-7, either on the surface of the cell or after transferred into the extracellular environment in *Azotobacter vinelandii*. A family of seven secreted and Ca^{2+}-dependent C-5- epimerases involve in creating different epimerization patterns, comprising GG-blocks of various lengths (Galindo et al., 2007). These enzymes are composed of repetition of two different

protein modules called as A and R modules. The A-modules have 385 amino acid residues which are permanently localized N-terminally in each enzyme. However, there is an exception for two cases in that AlgE1 and AlgE3 epimerases include an additional A-module located internally in these proteins (Figure 2.5). On the other side, the R-modules are shorter than the A-module, about 155 amino acid residues. Also, the R-modules can be repeated up to seven times such as found in AlgE3. Every R-module includes four to seven imperfect repetition of a nine amino-acid residue motif that has some resemblance to known Ca^{2+}-binding motifs in other proteins. In addition to Ca^{2+}-binding, R-modules have an ability to enhance reaction rates, and to stimulate translocation of the enzyme to its target on the cell surface or in the extracellular environment. The A-module can also bind Ca^{2+} ions that are needed for enzymatic activity and surprisingly, this module alone is enough to epimerize the alginate. Thus, it seems that the A-modules are the main determinant for the patterns of epimerization process (Remminghorst and Rehm, 2006).

Among the mannuronan C-5 epimerases, AlgE1 is one of the bifunctional epimerases which implies that it includes two catalytic sites for epimerization, AlgE1-1 and AlgE1-2. Every site produces a different G distribution pattern that while the former introduces the formation of contiguous stretches of GG-blocks in the polysacchraride chain, the latter catalyzes single G residues epimerizing into MG-blocks. As a consequence, the entire AlgE1 forms a mixture of all block structures (Ertesvåg and Valla, 1999). Similarly, AlgE3 has two catalytic sites for the activity like AlgE1, introducing both block types at the same time, MG- and GG-blocks. AlgE1 illustrates similar patterns compared its epimerization properties with AlgE3, but, less amount of AlgE3 is sufficient relative to AlgE1 in order to achieve high degrees of epimerization in alginates.

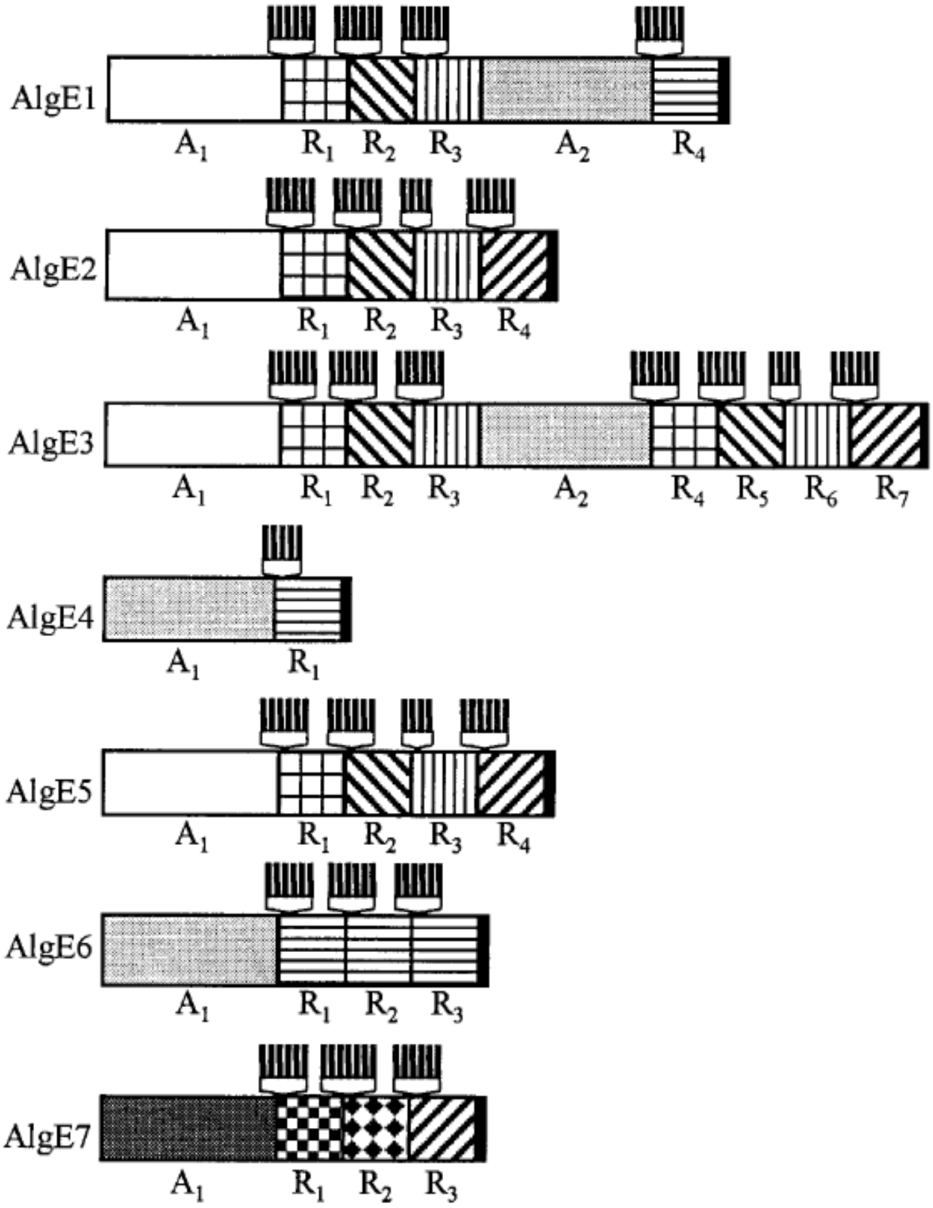

Figure 2.5. The modular structures of the secreted mannuronan C-5-epimerases (Ertesvåg et al., 1999)

The average GG-block length and comparative amount of MG-blocks might vary in some epimerases at relative degrees of epimerization. For instance, AlgE1 introduces higher amount of alternating structures and longer guluronic acid blocks than alginate epimerized by AlgE5 to about the same degree of epimerization. On the other hand, it is not easy to differentiate the

features of some of the enzymes from one another. For example, in the study of Ertesvåg et al (1999), the epimerization characteristics of four enzymes, AlgE2, AlgE5, AlgE1-1 and AlgE3-1, all having A modules belong to the same group were examined at various degrees of epimerization to see if the sequence similarities affect their products. AlgE2 and AlgE5 are also similar in the R modules. It was claimed that there was a structure and function relationship between the enzymes according to results which showed a fairly similar epimerization patterns in these enzymes.

AlgE6 produces longer guluronic acid blocks compared to those epimerized by AlgE2 and AlgE5. It was reported that GG-blocks content of the alginate could be increased to 78% or even higher (Valla et al., 2001), so, this alginate presumably containing very long GG- blocks can be used for the formation of very strong gels (Ertesvåg et al., 1999). Another epimerase, AlgE4 is different from rest of these extracellular epimerases in that it mainly introduces alternating sequences to the alginate chain. However, one work (Høidal et al., 1999) claimed that AlgE4 was able to add GG-blocks at elevated Ca^{2+} concentrations with excessive amount of the enzyme after prolonged incubation times. In addition, another concern about enzymatic activity of AlgE2 and AlgE4 observed was the degree of polymerization of the alginate. If the degree of polymerization of the substrate was lower than 7, AlgE2 and AlgE4 showed no activity for these oligomers. This can be explained that these enzymes require a minimum number of residues, which is 7, to support epimerase activity (Hartmann et al., 2002).

Among all, AlgE7 differs from the others since it includes both strong epimerase and lyase activity (Valla et al., 2001). On the one hand, *Azotobacter vinelandii* might be needed for the lyase activity of AlgE7 in case of germination of the cysts (Ertesvåg et al., 1999). On the other hand, AlgE7 epimerizes the alginate into both alternating sequences and guluronic acid blocks (Svanem et al., 1999). Therefore, the bacterium possibly needs all of the epimerases in order to produce the alginates required at different life stages and under changing environmental conditions.

2.2.3. EFFECT OF MEDIUM COMPOSITION ON ALGINATE PRODUCTION

2.2.3.1. Carbon Source

Azotobacter vinelandii is a free-living, aerobic soil bacterium with a wide range of metabolic diversity. This property makes the organism able to utilize various substrates such as recalcitrant ones (Moreno et al., 1990) if easily degradable compounds are not available in soil.

Alginate is primarily produced from simple, oxidizable sugars although glucose and sucrose are widely used among the others. In an early study by Couperwhite and McCallum (1974), alginate was produced by using Burk's salt medium supplemented with different sugars at 1 % concentration, namely glucose, sucrose, fructose, lactose, maltose, mannitol, sorbitol and raffinose. They investigated the effect of EDTA addition on alginate composition by observing the variation in uronic acid ratios (mannuronic acid/guluronic acid) of alginate. It was found that all microbial alginates synthesized by *Azotobacter vinelandii* cultivated on any one of eight carbon sources as the only C source resulted in low uronic acid ratios having best value as 0.33 by using sucrose, sorbitol, or raffinose. On the other hand, the highest mannuronic acid/guluronic acid ratio was observed in the cases of fructose and lactose as 0.66. When the medium was supplemented with EDTA, uronic acid ratios were observed much higher. For example, alginate samples obtained from the medium containing sucrose and mannitol had mannuronic acid/guluronic acid ratio of 4. This might be explained by the fact that EDTA is a chelating agent which captured the cations particularly calcium found in the cultivation medium. This might alter the enzymatic activity, which was responsible for the epimerization of mannuronic acid residues into guluronic acid blocks, since the epimerases are known for their requirements of calcium ions for their activity.

Another study investigated alginate production under various environmental conditions by two different strains, *Azotobacter vinelandii* NCIB 9068 and *Azotobacter vinelandii* LI. Bacteria were grown in Burk's or NRP (Nitrogen rich phosphorus limited) medium containing either sucrose or glucose at 2 and 4 % of concentration (Savalgi and Savalgi, 1992). The alginate yields were about the same for both strains when the medium was supplemented with 4 % of sucrose. Alginate synthesis was enhanced by using NRP medium in almost all conditions tested in the study. The highest alginate concentrations, 4 g/L and 6.2 g/L, were achieved by NRP medium including sucrose at 2 % by *Azotobacter vinelandii* NCIB 9068 and *Azotobacter vinelandii* LI, respectively. Furthermore, NRP medium containing glucose at concentration of 2 and 4 % was examined for the production alginate at 240 rpm. Maximum amount of alginate could be obtained by *Azotobacter vinelandii* LI as 2.6 g/L for both glucose concentrations. Glucose showed lower alginate production compared to sucrose. Anderson et al. (1987) tried to elucidate pathways of alginate production from glucose and fructose. It was claimed that alginate synthesized from glucose contains only three of the six carbon atoms present in the substrate. Therefore, the conversion efficieny of glucose into alginate was limited to 50 %. This might be the reason for reduced efficiency in alginate production when glucose was substituted with sucrose because sucrose was first degraded into glucose and fructose in alginate biosynthesis pathway and fructose could be a better substrate than glucose for alginate production at least in *Azotobacter vinelandii* (Anderson et al., 1987).

Recently, a study was performed to elucidate the effect of specific growth rate on alginate production by *Azotobacter vinelandii* SML2, a mutant strain unable to produce alginate lyases, in a fed-batch culture (Priego-Jimenez et al., 2005). Carbon limitation slowed down the growth rate of the bacteria. On the other hand, the higher alginate yields were obtained reaching a maximal $Y_{p/x}$ (Yield g alginate / g bacteria) of 2.6 and $Y_{p/s}$ (Yield g alginate / g sucrose) of 1.74 in the cultures conducted at μ of 0.03 hr^{-1}. Also, the average molecular weight of the alginate showed high dependence on

specific growth rate of the bacteria. For the lowest growth rate, μ, (0.03±0.009 hr^{-1}) investigated, a mean molecular weight of 1306±150 kDa was obtained. This value was around 5 times higher than that was achieved in the fed-batch culture grown at a μ= 0.09±0.012 hr^{-1} (275±35 kDa) and 14 times better compared to the value of average molecular weight of 95±8 kDa obtained from the batch culture (μ= 0.21 hr^{-1}). Thus, carbon limitation greatly affected molecular weight and distribution pattern of the alginate produced by *Azotobacter vinelandii* SML2. It was claimed in the study that explanation of the effect of specific growth rate on alginate molecular weight was not possible. However, the genes responsible for the polymerization alginate, alg8, alg44, algX, or algK might be affected due to carbon limitation since the strain used in the study was unable to produce alginases. So low molecular weight alginate may only be evaluated with low activity of the polymerases.

Since *Azotobacter vinelandii* is able to metabolize different substrates as C source, Moreno et al. (1999) investigated the production of exopolysaccharides (EPS) in the presence of some phenolic compounds as carbon and energy source such as 4-hydroxyphenylacetic, 4-hydroxybenzoic or protocatechuic acids. Results obtained throughout this study showed that 4-hydroxyphenylacetic acid resulted in the highest biomass levels, and EPS synthesis, on the other hand, protocatechuic acid only yielded a very small amount of biomass and EPS by *Azotobacter vinelandii* CECT 204. In the case of 4-hydroxyphenylacetic acid, EPS production was higher than with glucose used as the carbon source. Moreover, the uronic acid content of the EPS obtained from 4-hydroxyphenylacetic acid was found to be about twice compared to those obtained from glucose. The other studies involving EPS production from 4-hydroxybenzoic acid were conducted on *Azotobacter vinelandii* ATCC 12387. The characteristics of EPS were investigated in terms of rheological properties and mannuronic/guluronic acid ratio. Then, they were compared with the EPS synthesized by using glucose (Vargas-Garcia et al., 2003; Vargas-Garcia et al., 2002). The results showed that both EPS had lower mannuronic/guluronic acid ratio, which was 0.3 for the

EPS produced by using 4-hydroxybenzoic acid. In addition, amount of uronic acids was higher for the EPS synthesized from 4-hydroxybenzoic acid compared to the case of glucose. Furhermore, higher levels of viscosity were observed, which was between four to seven times higher in EPS from 4-hydroxybenzoic acid compared to that of a commercial alginate.

From all the studies examined related to the carbon source effect of *Azotobacter vinelandii* that there are only few studies (Couperwhite and McCallum, 1974; Vargas-Garcia et al., 2003; Vargas-Garcia et al., 2002) analyzing mannuronic and guluronic acid components of alginate. Also, the researchers did not perform their experiments under well-controlled conditions. Therefore, there seems to be lacking information about the carbon source effect on the monomer distribution from alginate under controlled fermentation conditions.

2.2.3.2. Nitrogen and Phosporus Concentration

Since nitrogen and phosphorus are essential nutrients for the survival of all organisms, production of alginate are investigated under the fixation of atmospheric nitrogen or addition nitrogenous compounds to cultivation medium as well as under phosphorus deficiency by using shake flask and laboratory fermentors.

In an early study (Larsen and Haug, 1971), alginate production was performed by using glucose based medium with/or without ammonium salts by *Azotobacter vinelandii* Strain E in a shake flask. Different ammonium salts, namely NH_4OAc, NH_4Cl, $(NH_4)_3PO_4$, were added and in some experiments, medium pH was set every 24 hour in the range of 6 - 7. Only addition of NH_4OAc, yielded better alginate production although the others reduced alginate synthesis even with the adjustment of the medium pH. By using the medium containing 0.03 M of NH_4OAc, concentration of alginate obtained by adjusting pH 6.5 were increased from 0.65 to 1.98 g/L relative to no addition after 100 hr. This value was the one giving the highest alginate

yield. While the previous study stating positive effect of addition of nitrogenous compounds to culture medium to enhance alginate production, Jarman et al. (1978) claimed the opposite that they investigated limitation of all ingredients of medium used for alginate production by *Azotobacter vinelandii* NCIB 9068. They came into a final conclusion that the highest alginate production could be achieved under nitrogen limitation conditions.

Another work was done to optimize the alginate production by *Azotobacter vinelandii* DSM 576 in terms of C:N ratio and phosphate addition to cultivation media (Clementi et al., 1995). They realized that C:N ratio did not play an important role to maximize alginate production in the range of 60-110 g C/g N. On the other hand, at the lowest phosphate concentration, 0.035 g/L, highest alginate concentration of 6.39 g/L compared to the value of 4.78 which was obtained at 3.5 g/L of phosphate concentration was obtained.

A further study was performed by using two different medium, Burk's nitrogen free and nitrogen-phosphorus rich medium (PNR), by *Azotobacter vinelandii* strain AX (Brivonese and Sutherland, 1989). The highest alginate concentrations were obtained as 2 and 7.5 g/L by using Burk's nitrogen free and PNR medium, respectively, at 280 rpm. In other words, alginate production was increased by changing Burk's medium with PNR. When Burk's medium was supplemented with ammonium nitrate, alginate yield was not enhanced. On the other hand, addition of the nitrogen source to PNR medium with varied peptone sources improved alginate yield up to 30 % reaching maximum level as 6.7 g/L after 5 days of incubation period. They suggest that these results might be elucidated by energy conservation which would be consumed for nitrogen fixation if the medium would be devoid of a nitrogen source. They further claimed that phosphorus could play a role as a buffer, so reduction of the medium pH due to alginic acid secretion would be controlled in a suitable range for the benefit of the bacteria. The reason for these controversial results between the studies might be attributed to the fact that all experiments were conducted in shake flasks which are known to have a heterogeneous oxygen profile during culture time; being higher at the

beginning while reducing during cultivation owing to increased bacterial mass as well as viscosity of medium with alginate production (Sabra et al., 1999).

The other studies to determine the effect of nitrogen and phosphorus on alginate synthesis were performed in controlled bioreactors. In the work of Parente et al. (2000), PNR medium was used in order to produce alginate by *Azotobacter vinelandii* DSM 576 at constant pH and temperature, 7 and 35 ℃, respectively, however, dissolved oxygen left to vary freely since maximum amount of alginate was harvested previously without DO control (Parente et al., 1998). NH_4SO_4 was added as a nitrogen source in the range of 0.45 to 1.05 g/L and it was investigated together with rotation speed (300-700 rpm). Batch experiments conducted at 0.6 and 0.9 g/L of NH_4SO_4 at 400 rpm showed that 0.6 g/L of NH_4SO_4 yielded better production of alginate and microbial mass, about 4 g/L for each compared to 1.2 and 3.8 g/L in the case of 0.9 g/L of NH_4SO_4, respectively. Variation in NH_4SO_4 concentration also affected the molecular weight of the polymer being highest at 0.6 g/L of NH_4SO_4 around 17×10^4 between 20 to 30 hours of fermentation. Furthermore, molecular weight was observed to be mainly dependent on mixing rate and to a lesser on the amount initial NH_4SO_4 concentration. It was thought that both alginate concentration and its composition particularly molecular weight were important at the same time. As a result, the values maximizing alginate production (> 2 g/L) and its molecular weight (>250,000) could be achieved at 0.75- 0.9 g/L of NH_4SO_4 at 500-600 rpm. Although this study was done in a fermentor, it is better to keep in mind that dissolved oxygen concentration was not constant during cultivation; therefore, the results still may not be so reproducible.

There was only one work dealing with alginate production under nitrogen fixation and in a phosphate limited medium by *Azotobacter vinelandii* 93541-b at 3 % of DOT (Sabra et al., 1999). Both biomass and PHB production were improved by increasing phosphate level from 100 mg/L to 400 mg/L. Contrary to these results, highest alginate production was observed under

phosphate limitation, at concentration of 100 mg/L. For example, when the phosphate level was increased from 100 mg/L to 400 mg/L, the amount of alginate synthesized decreased from 4.9 g/L to 3.5 g/L, respectively. These findings were not compatible with those obtained from a shake flask experiment that obtained the maximum alginate was produced at highest phosphate concentration of 800 mg/L. Furthermore, mannuronic acid content and molecular mass distribution were observed during fermentation at different phosphate levels. As expected, mannuronic acid content decreased to around 60 - 70 % till the end of the cultivation since alginates are synthesized as mannuronic acid in the first place and then they are epimerized into guluronic acid. On the other hand, molecular weight of alginate was almost constant until the beginning of a phosphate-limited phase and after that it was reduced drastically under phosphate limitation. The highest molecular mass of alginate was observed as about 0.7×10^6 in case of phosphate limitation.

2.2.3.3. Calcium Ion Concentration

Alginate is first synthesized as mannuronic acid and then guluronic acid residues are introduced at the polymer level by the enzyme mannuronan C-5-epimerases like AlgG, a periplasmic epimerase which has been found in both *Azotobacter vinelandii* and *Pseudomonas aeruginosa*. Also, *Azotobacter vinelandii* genome encodes a family of seven mannuronan C-5-epimerases, designated as AlgE1–AlgE7. In contrast to the AlgG epimerase, these epimerases are extracellular and require Ca^{2+} for their activity. For this reason, some works were directed to analyze the effect of the concentration of calcium ions on alginate composition both in the growth medium and in the post epimerization experiments. However, none of the studies related with alginate production was performed under controlled conditions. There were only two studies (Annison and Couperwhite, 1984; Annison and Couperwhite, 1986) that monomer distribution of alginate was examined at varying calcium concentrations using shake flask experiments.

An early study was performed to establish whether a correlation exists between the ratio of uronic acids in the alginate chain and the concentration of calcium ions in the production medium. Calcium concentration was changed within the range of 0.03 mM - 3.4 mM (Larsen and Haug, 1971). Increase in calcium concentration of the medium up to a certain level, especially from 0.03 to 0.34 mM, resulted in improvement for alginate synthesis and the concentration of the polymer was doubled. Furthermore, the concentration of calcium ions affected the ratio of the uronic acids (mannuronic acid/guluronic acid ratio) in the polymer. In general, low amount of calcium yielded alginates composed of mainly mannuronic acid while intermediate concentrations of calcium ions favored production of alginate rich in guluronic acid. For instance, mannuronic acid/guluronic acid ratio was observed as 4.6 when the concentration of calcium ions was lower than 0.1 mM, however, the ratio was reduced to 0.35 in the case of 0.34 mM of calcium ion which indicated that most of mannuronate was epimerized into guluronate.

Another study also showed the effect of calcium ion on the ratio of the uronic acids of alginates produced by *Azotobacter vinelandii* NCIB 8789 in a shake flask (Couperwhite and McCallum, 1974). The study involved the investigation of EDTA addition into cultivation medium containing different carbohydrates. It is known that EDTA is a chelating agent which is able make bonds with the cations particularly calcium ions that might influence the activity of mannuronan C-5-epimerases. As expected, a significant increase in mannuronic acid/guluronic acid ratio of alginate was observed in the case of EDTA supplemented medium because of impaired activity of the epimerases. The most remarkable example was obtained when sucrose was used as a carbon source at 1 % of concentration. In this case, the uronic acids ratio sharply increased from 0.33 to 4 by the addition of EDTA. Similar results were observed by Obika et al. (1993) that investigation of the influence of calcium ion concentration on the uronic acid ratio of alginate was carried out by *Azotobacter vinelandii* IAM 1078. The amount of calcium was varied in the range of 0.068 and 0.68 mM. The strain was able to produce

alginate at such a high concentration as 3 g/L. Moreover, an alginate having mannuronic acid/guluronic acid ratio in a wide scale, from 0.3 to 8 at 0.68 and 0.068 mM of Ca^{2+}, respectively, could be achieved in only one step production process.

Two further studies were conducted to investigate the effect of calcium ion concentration on monomer distribution of alginate by *Azotobacter vinelandii* Strain E either in a shake flask (Annison and Couperwhite, 1984) or in a vessel operated continuously (Annison and Couperwhite, 1986). The results in batch study revealed that alginate contained mainly GG-blocks together with some MG-blocks leading to total guluronic acid fraction of 0.7 during early cultivation, between 20 and 28 hours. After that mannuronic acid fraction of alginate started to increase while GG-blocks production rate was reduced and MG-blocks were still produced slowly up to 46 hours of the experiment. MG-blocks were the only ones continued to increase to the end of fermentation while mole fractions of other block types decreased although very small amount of alginate was produced between 46 and 100 hours. The reason for the predominant production of guluronic acid containing alginate might be explained by an important reduction in epimerase activity after the period of rapid alginate production. The reason was most probably the drop in free calcium ion concentration observed simultaneously because the alginate produced in early fermentation had high GG-blocks that especially was responsible for binding of calcium ion. As a consequence, the epimerases depend on calcium ion for their activity seems to be self-limiting. Alginate with high guluronic acid content formed some calcium mediated associations at the begining of the study that do not actually form gels or real precipitates, however, these formations make alginate less available for epimerases activity. Then, alginate contanining high mannuronic acid content began to rise after initial rapid alginate production. In the following work (Annison and Couperwhite, 1986), alginate was synthesized by *Azotobacter vinelandii* Strain E in a continuously operated vessel. At lower dilution rates (0.16 and 0.18 hr^{-1}), biomass production was not affected much by variation of calcium ion concentration in the range of 0.34 to 2.72 mM. On

the other hand, it increased from 0.83 to 1.1 g/L at a higher dilution rate of 0.32 hr^{-1}. On the other hand, remarkable changes in alginate production were observed at different dilution rates and calcium levels. Maximum alginate production could be achieved as 1.38 g/L when calcium ion concentration was increased to 2.72 mM at a dilution rate of 0.16 hr^{-1} although the value was maintained around 0.75 g/L between 0.068 and 0.68 mM of calcium concentration. Uronic acid composition of alginate was variable relative to change in calcium ion concentration being most of the alginate with GG-blocks up to 50 % at 0.34 mM of calcium ion, which was produced mainly as MG-blocks at higher calcium levels. Therefore, concentration of calcium ion in cultivation medium had a great influence on both alginate quality and quantity. However, they claimed that the free calcium concentration may not be the only factor since low amounts of guluronic acid were also recovered from the cultures with high levels of free calcium ion. It seems that the epimerase activity yielding high GG-block content of alginate was enhanced by elevation of calcium ion concentration from 0.068 mM to 0.34 mM, but, additional increases in Ca levels (2.72 mM) inhibited the activity, so alginates with primarily MG-block were harvested. As a result, it was suggested that two conditions must exist at the same time for efficient epimerization of alginate. First, calcium ion concentration should be close to 0.34 mM and second, the alginate concentration must be low, less than 0.5 g/L.

Further studies involved post-epimerization of alginate samples by mannuronan-C5-epimerases, designated as Alg E1-E7, and investigation of optimum conditions for efficient epimerization. Since the activities of the epimerases were greatly affected by calcium ion concentration, the effect of calcium ion on epimerization was usually investigated in more detail. In an earlier study (Haug and Larsen, 1971), an enzyme preparation was recovered from the liquid culture medium of *Azotobacter vinelandii* strain E by using ammonium sulphate precipitation after removal of the bacterial cells. The results indicated that activity of enzyme was greatly influenced by varition of calcium ion levels especially if the concentration was higher than

0.5 mM with 1.8 mequiv/L of alginate in solution. Also, no significant activity was observed in the absence of calcium ions. In general, the activity was improved by increasing calcium up to 3.4 mM, but, initial composition of the alginate was another concern that affected the epimerization activity of the enzyme preparation. For instance, a polymer with 92 % of mannuronic acid could be reduced to 28 % with 0.07 mM of calcium ion whereas no change was observed with alginate having 27.5 % of guluronic acid at the beginning even at 3.4 mM of calcium concentration.

Recent studies were mainly interested in the determination of optimal conditions for each AlgE epimerases when the genes coding AlgE1-E7 epimerases were cloned and expressed in *E. coli*. The requirement of calcium ion for the activities of AlgE epimerases are somehow difficult to determine due to calcium binding ability of the enzymes and the alginate at the same time. Some divalents ions, Mg^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+} and Zn^{2+}, were examined in the presence or absence of Ca^{2+} in order to determine the impact on the efficiency of AlgE2 (Ramstad et al., 2001). It was found that only Sr^{2+} supported the activity of AlgE2 in the absence of Ca^{2+}. This result was in agreement with those obtained for AlgE1 (Ertesvåg et al., 1998) and AlgE4 (Høidal et al., 1999). The reason may be explained by the fact that both ions have almost the same ionic radius, so Sr^{2+} can replace Ca^{2+}. The activity of AlgE2 was reduced when Ca^{2+} was used together with all other divalent cations at a high concentration of Ca^{2+} (3.3 mM). On the other hand, Mg^{2+} was the only one that did not cause too much decrease in the activity of the enzyme because it was not a suitable ion for gelling of the polymer. Best results were achieved at the highest concentration, 3.3 mM, of Ca^{2+} (Ramstad et al., 2001).

One other study was conducted to find out the optimal conditions for the activity of another mannuronan-C5-epimerases, AlgE4 (Høidal et al., 1999). The results illustrated that the enzyme showed the maximum epimerization activity at about 1–3 mM Ca^{2+} if the amount of alginate was 0.5 mM or higher. Optimum calcium concentration was a little decreased when the

concentration of alginate was lower. At suboptimal concentrations of Ca^{2+} (0.4 mM), use of Sr^{2+}, Mg^{2+}, or Mn^{2+} with Ca^{2+} enhanced the activity of AlgE4, whereas Ba^{2+} and Zn^{2+} had negative influence on the activity. However, all the ions limited the epimerization of the enzyme at optimal Ca^{2+} levels.

The optimal concentration of Ca^{2+} for AlgE1 activity was found to be somewhat lower compared to AlgE2 and AlgE4 as 0.8 mM (Ertesvåg et al., 1998). On the other hand, the enzyme yielded no activity at 0.05 mM $CaCl_2$, while 0.35 mM of calcium ions was needed to achieve 50 % of the optimal activity. Furthermore, the epimerization activity was somehow decreased at higher Ca^{2+} concentrations than the optimum level. AlgE1 is a bifunctional enzyme having two catalytic domains in the structure namely, AlgE1-1 and AlgE1-2. AlgE1-1 introduces GG-blocks in alginate chain while AlgE1-2 produces alternating sequences. Requirement for each domain was observed to be different that AlgE1-1 needed much higher calcium (optimum around 3 mM) than that of whole AlgE1, while AlgE1-2 required slightly more Ca^{2+} than AlgE1 for optimal activity. Sr^{2+} or Mg^{2+} illustrated positive effect on the activity of AlgE1 at Ca^{2+} concentrations lower than optimum, while Mn^{2+} and Zn^{2+} showed the opposite particularly in case of Zn^{2+} even at very low concentrations (0.1 mM).

The amount of calcium ions also determined the affinity of the enzyme, AlgE2, for the epimerization of different alginates (Ramstad et al., 1999). It was found that AlgE2 showed high affinity for sequences including single G-residues, whereas it had low affinity for the other sequences in the alginate chain particularly when calcium concentration was low. On the other hand, monomer distribution of the polymer became less significant at higher levels of calcium, i. e. Ca^{2+}>1.5 mM. Therefore, the enzyme is able to bind mannuronic acid sequences and the residues having a few consecutive guluronic acid residues. At these higher calcium concentrations, the reaction rate was also sustained stable for several hours and the highest epimerization levels could be achieved after about two days (3.33 mM: 59%

G; 5.25 mM: 60% G; 6.25 mM: 58% G). They concluded that it seemed there was a critical minimum concentration of calcium being in the range of 1.5 and 1.7 mM required to maintain the epimerization process over a longer period of time.

2.2.3.4. Others Parameters

There are some other factors affecting alginate quantity and quality. Some are physical parameters like temperature while some are chemical in character such as pH. For all living things, there is an optimum pH and temperature value or range around which they are able to grow and reproduce better. *Azotobacter vinelandii* is a bacterium that prefers to live at neutral pH values and moderate temperatures (mesophilic values). Because it is difficult to establish a pH control in case of shake flask experiments, generally pH of the cultivation medium was adjusted before autoclaving. Some buffers like MOPS, K_2HPO_4 – KH_2PO_4, were used to compensate pH reduction occurring in connection with alginic acid secretion by *Azotobacter vinelandii* as Chen et al. (1985) argued. Alginate was produced by a mutant C-14 of *Azotobacter vinelandii* NCIMB 9068 in Burk's medium under N_2 fixation by adjusting pH initially 6.5 and in the range of 110 and 200 rpm. Highest alginate concentration and viscosity were observed at 170 rpm resulted with lowest pH value, 5, at the end of 96 hour cultivation. Pena et al. (1997) showed the similar results during production of alginate in an unbaffled flask that pH value was decreased from 7.2 to 5.3. However, no drastic effect of pH reduction on alginate yield was reported by these two works. On the other hand, Clementi et al. (1995) observed inhibition in alginate production by *Azotobacter vinelandii* DSM 576 in a shake flask at 300 rpm when pH was dropped below 5.5. They emphasize the importance of maintaining constant pH values especially around pH 7 during fermentation. An experiment supporting their idea was conducted by supplementing medium with MOPS (effective buffer in range of 7.5-6.5). The results showed that alginate production was increased by 2.5 times compared to the amount of alginate produced in the absence of MOPS.

Only two studies were performed to investigate the effect of temperature on alginate production. In one work, temperature values of 25, 28, 31, 34, and 37 °C were set and for both alginate production and cell growth, 34 °C was found to be optimum value by a mutant C-14 of *Azotobacter vinelandii* NCIMB 9068 (Chen et al., 1985). Also, viscosity of the medium was observed highest at that temperature. In the other study, experiments were done at five different temperature values between 23 and 42 °C (Clementi et al., 1985). Maximum alginate production was achieved at 35 °C compatible to the former work. However, most common temperature value used in order to produce alginate by *Azotobacter vinelandii* was around 30 °C (Sabra et al., 1999; Pena et al., 2000; Savalgi and Savalgi, 1992; Moreno et al., 1999; Vargas-Garcia et al., 2002; Annison and Couperwhite, 1986).

Some studies were involved in determining the effect of temperature and pH on the activity of alginate modifying enzymes which are secreted to extracellular environment and designated as AlgE1-E7. pH is one of the physical parameters that might affect the catalytic activity of the enzymes. Among these enzymes, AlgE4 is predominantly characterized to be responsible for an alternating sequence distribution of the M and G residues (MG blocks). pH optimal for that enzyme was reported between 6.5 and 7.0 (at 1–3 mM Ca^{2+}) and also activity of AlgE4 was found to be very sensitive to alkaline pH values, for instance, at pH values above 8 virtually all activity was lost (Høidal et al., 1999). Similar to the case of AlgE4, AlgE2 which mainly catalyzes the formation of GG-blocks in the alginate chain had a pH optimum between 6.5 and 7 in the presence of 3.3 mM Ca^{2+} for M-rich alginate as substrate (Ramstad et al., 2001).

Temperature is another parameter which has influence on the activity of the epimerases. While the optimum temperature for AlgE4 was observed at near 37 °C (Høidal et al., 1999), it was observed higher in case of AlgE2, at about 55°C in histidine buffer. However, after 3 hours of incubation, the optimum for AlgE2 slightly shifted to a lower and broader temperature range (45 – 55°C), probably due to thermal denaturation of the enzyme. In Tris buffer

(pH 7 and 3 hours of incubation), the optimum temperature was between 40 and 50°C (Ramstad et al., 2001).

2.2.4. ROLE OF ENVIRONMENTAL CONDITIONS ON ALGINATE PRODUCTION

2.2.4.1. Dissolved Oxygen Tension

Dissolved oxygen tension (DOT) can be defined as the partial pressure of oxygen in a gas mixture. The maximum dissolution of oxygen in water is proportional to the partial pressure of oxygen in air. Therefore, at air saturation of a water sample, oxygen concentration in water is about 9 mg/L at 20 °C which corresponds to 100 % DOT.

Azotobacter vinelandii, an obligate aerobic Gram-negative soil bacterium, has an ability to grow by fixing atmospheric nitrogen under aerobic conditions even though nitrogen fixation process is generally limited to situations of reduced aeration or under oxygen deficiency since nitrogenase which is responsible for the reduction of atmospheric dinitrogen to ammonia is highly vulnerable to oxygen (Haddock and Jones, 1977). *Azotobacter vinelandii* has two main mechanisms for the protection of the nitrogenase from damages of atmospheric oxygen so the bacterium is able to survive by nitrogen fixation under fully aerobic growth conditions. First one depends on the removal of oxygen by increasing respiration rate of the organism in order to reduce oxygen level around the cells. Second one involves reversible inactivation of the nitrogenase by complexation (Moshiri et al., 1995; Linkerhägner and Oelze, 1997; Liu et al., 1995; Gallon, 1992).

Respiration is known to be one of the main ways to protect the nitrogen fixation ezymes being damaged by oxygen via reducing the ambient oxygen concentration when the ability of oxygen consumption by the bacterium exceeds the rate of oxygen input into the cells. The hypothesis of respiratory

protection assumes that respiration reduces the oxygen level at the surface of the cells, thus, inside of the organism would be under anaerobic conditions even the concentration of the oxygen is high around the bacteria (Kuhla and Oelze, 1988). This hypothesis depends on the facts that azotobacter species are known with their highest respiratory activities among aerobic bacteria. Furthermore, the bacteria represent high respiratory activities when the organisms are cultivated at higher oxygen levels. When the respiratory activity of the bacterium is increased, electron transfer is also uncoupled resulting in an improvement in oxygen consumption without increasing ATP production. Partial uncoupling of respiration activity possibly happens since the respiratory system of *Azotobacter vinelandii* is branched with either cytochrome o or cytochrome d being terminal oxidases and cytochrome d branch seems to be uncoupled (Linkerhägner and Oelze, 1995; Liu et al., 1995).

Azotobacter vinelandii needs to form different constituents that function in the respiratory chain before increasing the respiratory activity which makes the process slow. Therefore, the protection mechanism via respiration cannot be efficient as soon as the sudden oxygen level increases happen in the medium particularly to higher than 30 % of air saturation. In such a case, the nitrogenase is exposed to a conformational change resulting in switching off the enzyme activity. However, this kind protection of the nitrogenase is temporary contrary to adverse effects of oxygen tension (Post et al., 1983). An iron-sulfur protein (FeSII protein; to the MoFe- and Fe-protein components of nitrogenase) covalently binds during high oxygen concentration period which yields an inactive state of nitrogenase although this state also prevent the enzyme from irreversible inactivation due to high oxygen tension (Moshiri et al., 1995). Complex formation of the enzyme depends upon the oxidation of the electron donor to nitrogenase, and on the reversible oxidation of nitrogenase. Subsequent to a switch-off, nitrogenase is able to restore its activity if oxygen tension reduces to its initial concentration or after the bacteria adjust themselves into new high oxygen level. The latter is followed by an improved formation of components of the

respiratory chain, elevating the cellular respiratory activity (Kuhla and Oelze, 1988).

Many studies claimed that decreasing oxygen level around the organisms seems to be not enough to elucidate the activity of the nitrogen fixing enzymes under fully aerobic conditions even though the hypothesis of respiratory protection has been approved for both azotobacter species and the other diazotrophs in general. Respiratory activity of *Azotobacter vinelandii* cultivating at high levels of oxygen, for instance, does not change greatly with further elevation of dissolved oxygen concentration in the medium. Despite the appreciable differentiation in cellular oxygen consumption, cellular contents of *Azotobacter vinelandii* and activity of nitrogen fixing enzymes are quite similar as well. As a result, *Azotobacter vinelandii* cells are able to use oxygen without any damage in the function of their nitrogenases. The bacteria might have a further protection mechanism for the nitrogenases that involves the reduction of the cellular surface area per cell volume together with an increase of the cellular content of superoxide dismutase due to high oxygen levels in the culture environment. It was shown that in an unbaffled flask (low aeration condition) average diameter of the cells reached up to 35 µm at 72 hours although the cell size was observed only is 2.2 µm by using baffled flasks for the cultivation (high aeration condition) (Pena et al., 2002). Besides, it is suggested that the nitrogen fixing enyzmes can sustain their activity under aerobic conditions on condition that the low redox potential which is required for its function is supplied with the help of sufficiently high flux of electrons through the enzyme complex. This is compatible with the hypothesis of autoprotection of nitrogenase which claims that oxygen is reduced by the nitrogenase when enters the organism. In other words, autoprotection of nitrogenase may be successful only if nitrogenase is found at the reduced state so not switched off (Linkerhägner and Oelze, 1997; Post et al., 1983). Both high levels of MgATP and reducing equivalents are needed to supply the reduced state of nitrogenase enzymes. Especially, MgATP was obviously significant in order to maintain the activity of nitrogenases under aerobic conditions because

reversible inactivation of nitrogenase with the increase of oxygen concentration was accompanied by a reversible decrease in the cellular ATP pool. In addition, adjustment from lower dissolved oxygen levels to higher oxygen concentrations was determined by the regeneration of the ATP pool from stress. As an example, a mutant strain having a partial distruption in respiratory electron transfer, so in energy recovery is not able to survive under nitrogen fixing conditions at elevated dissolved oxygen levels. All these consequnces recommend that there is a quantitative relation between the activity of nitrogenase and the energy status of the cells. Therefore, bacterial oxygen consumption seems to be more significant for nitrogenase activity than the ATP supply when *Azotobacter vinelandii* was grown under aerobic conditions (Linkerhägner and Oelze, 1997).

Sabra et al. (2000) claimed that alginate production may also have a function in handling high oxygen tension and regulating the activity of nitrogenase in *Azotobacter vinelandii*. Actually, the biological function of alginate production in bacteria has not been completely understood yet. It is known that alginate is one of the main constituent of cyst coating that is used as a protective layer especially from dessication in *Azotobacter vinelandii* (Sadoff, 1975). However, vegetatively growing cells of *Azotobacter vinelandii* are also observed to produce alginate under stiuations not favoring encystment (Clementi, 1997). The oxygen concentration should be maintained at low levels in order to protect the nitrogenases in nitrogen-fixing bacteria (Post et al., 1983). Production of alginate and biomass during cultivation of *Azotobacter vinelandii* creates an environment with high viscosity which yields a reduction in oxygen transport rate from the gas phase to the aqueous phase and from the bulk liquid to the cell surface. Moreover, an efficient oxygen barrier on the surface of the bacteria may be even more significant to decrease oxygen transfer into the cell. As a consequence, alginate production has a significant role in overcoming oxygen stress of *Azotobacter vinelandii* especially under phosphate limitation together with the respiratory protection and enzyme inactivation mechanisms. The overall mechanisms for the protection of nitrogenase against oxygen tension in

Azotobacter vinelandii are illustrated in Figure 2.6. The relative significance of various protection mechanisms can change depending on the environmental conditions.

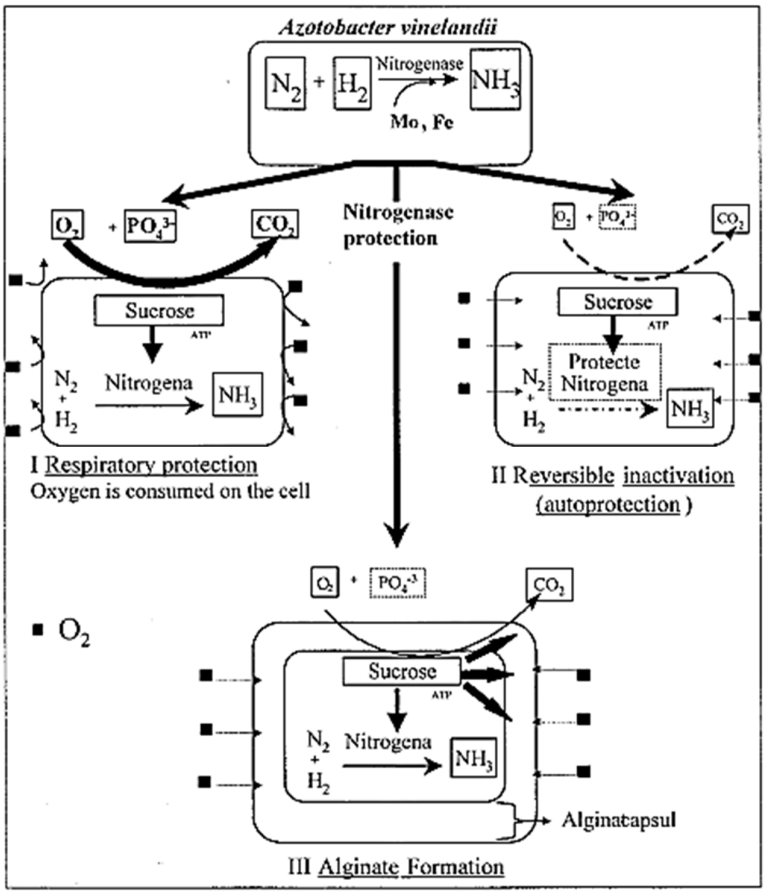

Figure 2.6. Protection mechanisms for nitrogenase against O_2 in *A. vinelandii* (Sabra et al., 2000)

The effect of dissolved oxygen tension on alginate production by *Azotobacter vinelandii* was generally investigated under microaerophilic conditions because of the sensitivity of nitrogenases and key enzymes of alginate biosynthesis to oxygen stress. One study involved alginate production by *Azotobacter vinelandii* DSMZ 93-541b under phosphate-limited continuous culture at different dissolved oxygen tension values

varying in the range of 1-10% air saturation (Sabra et al., 1999). Optimum conditions for the formation of alginate was observed at dissolved oxygen values between 2 and 5 % of air saturation independent of the dilution rate studied, D= 0.08, 0.15, 0.22, and 0.2 h^{-1}. Similar conclusions were found by Parente et al, 1998, that alginate production was performed in a nitrogen and phosphate-rich medium by *Azotobacter vinelandii* DSM576 in a batch culture. Formation of alginate was examined to be greatly affected by changing dissolved oxygen level in the medium. Alginate production rate was getting slower if dissolved oxygen concentration was low (1 %), having a lowest specific alginate production rate around 0.042-0.048 g/g.h, possibly owing to oxygen limitation. On the other side, formation of alginate was enhanced at dissolved oxygen levels between 2 and 5 % of air saturation with specific alginate production rate up to 0.138 g/g.h although *Azotobacter vinelandii* DSM576 yielded no alginate production at 10 % DOT. It was suggested that particularly poly-β-hydroxybutyrate was accumulated when the oxygen level was limited whilst moderate level of aeration is needed in order to achieve high amounts of alginate production. Higher dissolved oxygen levels, however, resulted in inefficient carbon source consumption of *Azotobacter vinelandii* since the carbohydrates were utilized by high cellular respiration to remove the excess oxygen around the surface of the microorganism. Likewise, Pena et al. (2000) showed that the higher the dissolved oxygen tension in the range of 1-5 %, the higher the concentration of alginate by *Azotobacter vinelandii* ATTC 9046 were achieved at any given time obtaining a maximum alginate concentration of about 4.6 g/L at 5 % after 72 hours of fermentation corresponding to the specific alginate production rate of 0.028 g alginate/g biomass.h. Moreover, only 1.3 g/L of the polysaccharide were synthesized at 0.5 % dissolved oxygen tension yielding 0.015 alginate/g biomass.h. The specific sucrose uptake rate was also enhanced by increasing dissolved oxygen level and 0.25 g of alginate was formed per 1 g of sucrose consumed at 5 % dissolved oxygen tension. As a result, effective conversion of carbohydrates into alginate may be possible only if the dissolved oxygen tension is strictly sustained in the range of 1 and 5% of air saturation.

Depending on the aeration conditions, alginate was produced by either during exponential growth phase of the bacteria or partly non-growing bacteria could be involved in the formation of the polymer (Pena et al., 2000). Alginate formation was particularly observed to happen during microbial growth if dissolved oxygen concentration was sustained constant at moderate levels such as in laboratory fermentors. For instance, alginate production was observed to be completely growth-associated at 0.5, 1 and 3 % dissolved oxygen tension values whilst at 5 % DOT, a fraction of the whole alginate yield (20 % of the total) was achieved in the stationary phase. On the other hand, about 50 % of alginate biosynthesis was observed by non-growing bacteria in a shake flask due to the fact that dissolved oxygen concentration (among other factors) cannot be set constant in shake flasks so probably the oxygen profile is the reason for the differences in alginate production patterns. This can be explained that at the beginning of the culture, dissolved oxygen level is higher in shake flasks since the amount of bacteria and the alginate are lower. Therefore, most of the carbohydrates are channeled to remove the excess oxygen from the cell surface. Then, as the bacteria and alginate concentrations are increased, the oxygen level is reduced and become more suitable for alginate production. However, alginate production has been already delayed compared to those in the fermentors where it is possible to adjust a moderate level of oxygen from the beginning of cultivation.

The quality of alginate is also associated with dissolved oxygen level maintained in the cultivation medium. In one study, the increase in dissolved oxygen level from 1 % to 5 % of air saturation was accompanied by an improvement in the specific rate of oxygen consumption as expected due to respiratory protection mechanism of the bacteria (Sabra et al., 2000). However, oxygen consumption was observed almost constant for further increases in dissolved oxygen tension above the values of 5 % which cannot be elucidated by the mechanism mentioned. Therefore, from all these findings it can be said that alginate production seems to participate for the protection of nitrogenase. The specific formation rate of alginate was

examined to understand the role of alginate in nitrogenase protection and interestingly it was found to be reduced by increasing oxygen level to above 5 %. Thus, quality of alginate was further investigated with regard to molecular weight characteristic and guluronic acid content of the polymer to see whether they change with increasing oxygen levels. Results showed that both the molecular weight of the polymer (0.85×10^6) and guluronic acid fraction (45 %) were uniformly increased with the elevation of dissolved oxygen up to 10 % of air saturation. Increase in molecular weight of the alginate was associated with higher viscosities in the medium which reduced oxygen transport. A conclusion can be drawn from the arguments made up to here is that the quality of the polymer, not the quantity seems to be the main factor for the protection of nitrogenase in *Azotobacter vinelandii*. Moreover, morphological changes in alginate capsule were investigated at a low and high oxygen level, 2.5 % and 20 % of air saturation, respectively. Alginate layers were found greatly different at these two oxygen levels. At the low oxygen concentration, alginate layer was very loose with filamentous structures radiating from the surface of the bacteria; whereas a very dense layer of alginate capsule covering the whole cell surface was observed at the higher oxygen tension value. These differences were accompanied by the variation in guluronic acid content of the polymer. G/M ratios of alginate were evaluated as 45 and 88 % at 2.5 and 20 % oxygen tension, respectively. These values are the only information about G/M ratios of alginate produced at different DOT values. No research examined the effect of oxygen tension on monomer distribution of the polymer in detail. Likewise, in the study of Pena et al. (2000) mean molecular weight of the polymer produced was improved by increasing dissolved oxygen tension at low agitation speed (300 rpm) up to 680,000 g/g mol. On the other hand, molecular mass of those obtained at 700 rpm increased to a plateau between dissolved oxygen levels of 1 and 3 %, being maximum as 352,000 g/g mol and then decreased at higher oxygen concentrations. These results were in agreement with the viscosity data since it is mainly determined by the molecular weight of the polymer. At 300 rpm and 5% DOT, the viscosity of alginate was higher relative to those observed at 1 and 3% for the same alginate concentration.

For instance, the viscosity of alginate at 4 g/L measured at 12 s^{-1} shear rate was found to be 420 cP at 5 % dissolved oxygen tension, whilst it was only 20 cP at 3 % oxygen tension. Therefore, it was suggested that polymerization of alginate was greatly dependent on the dissolved oxygen levels (Trujillo-Roldán et al., 2004).

The degree of fluctuation in oxygen profile during cultivation was observed to be important especially for the quality of alginate as much as controlling dissolved oxygen concentration at certain levels favoring alginate synthesis. One study was performed by Trujillo-Roldán et al (2001) that 3 % DOT was selected as the oscillation axis. Amplitude and fluctuation period were changed between 1.0 - 2.2 % of DOT and between 1200 - 4000 s, respectively. It was found that although the kinetics of alginate production was affected to a lesser extent by oscillation of DOT relative to the constant oxygen level, average molecular weight of the polymer produced was influenced greatly. To investigate further, some experiments were performed at various average wave amplitudes, but, at the same wave period (1200 s). These fluctuations resulted in different values of maximum average molecular weight as 240 and 64 KDa and different molecular weight distributions at 1 and 2.2 % of wave amplitudes, respectively. This shows the strong effect of a fluctuating DOT on the molecular properties of alginate. On the other hand, the bacteria synthesized alginates having almost similar molecular weights when the wave period was increased from 1200 to 2400 s at the same wave amplitude (2.2 %). Thus, the wave amplitude had much higher significance on the molecular weight of alginate compared to the wave period. The study concluded that a poor DOT control in alginate formation due to high viscosity of the culture broth and/or inefficient mixing could yield to the loss of polymer quality in terms of its molecular weight (Trujillo-Roldán et al., 2001).

2.2.4.2. Mixing Rate

Fluid mixing is required in fermentation processes in order to supply a homogenous environment to microorganisms so that they are able to take nutrients and enough oxygen particularly in the case of productions performed under aerobic conditions. There are not much studies performed to elucidate the effect of stirring on alginate production. One study was conducted to find the optimum conditions for alginate formation by *Azotobacter vinelandii* DSM® 576 in a shake flask (Clementi et al., 1995) with regard to various operating variables including mixing speed in the range of 250 and 450 rpm. The results showed that maximum alginate production being around 1.7 g/L were achieved when the bacteria was grown at rotation speeds of 250-300 rpm with 20 g/L of glucose at 35 °C. On the other hand, alginate production was continuously reduced with futher increase in shaking rate above 300 rpm yielding the lowest alginate production about 0.3 g/L at 450 rpm. This may be attributable to the oxygen profile common in shake flasks. At higher agitation speeds, the oxygenation of the medium would be very well leading to increased respiration activity to protect nitrogenase enzyme from irreversible oxidation. Thus, this causes wasting of carbon source rather than channeling it to produce alginate. However, the aeration levels obtained at moderate shaking speeds like 250-300 rpm seem to favour alginate production by creating microaerophilic conditions around cells of *Azotobacter vinelandii* DSM 576.

Other studies examining the influence of stirring speed on alginate quantity and quality were performed in fermentors. Parente et al. (2000) investigated the effect of initial inorganic ammonium sulfate concentration and agitation speed on alginate production by *Azotobacter vinelandii* DSM 576 at 35 °C without dissolved oxygen control. Alginate formation was observed quite similar at 300 and 700 rpm with 0.75 g/L of ammonium sulfate although the molecular weight of the polymer was greatly different. For example, highest molecular weight was obtained at around 30 hours of fermentation as 13×10^4 and at 300 rpm while molecular weight was continually increased till

the end of fermentation showing a maximum of 32×10^4 at 700 rpm. Therefore, the authors suggested that polymerization of alginate seemed to be clearly influenced by mixing speed. Thus, experiments were extended by investigating molecular characteristics of alginate at 400, 500, 600 rpm as well. At highest nitrogen concentration with lowest agitation speed, 300 rpm, the mean molecular weight was found to be very low, ca. 3000. The molecular weight of alginate showed a maximum after 30 hours around 12×10^4-16×10^4 at 400 rpm, but, it was reduced to below 10000 at the end of fermentation. Furthermore, at 500 rpm, highest molecular weight was obtained with 0.75 g/L of ammonium sulfate at about 50 hours as 25×10^4 and then stayed constant. On the other hand, at 600 and 700 rpm, the maximum molecular weight of alginate was achieved ca 31×10^4 to 35×10^4 and alginate degradation was not observed contrary to the other values studied. The authors claim that the polymerization seems to be more related to shear stress. Finally, because alginate formation and molecular characteristics were equally significant for ultimate usage of the polymer, an optimization of alginate concentration (> 2g/L) and quality (MW >250 000) was achieved with 500-600 rpm and 0.75-0.9 g/L of ammonium sulfate concentration.

Another study was conducted by *Azotobacter vinelandii* DSMZ 93-541b in a phosphate-limited medium at various mixing speed in a range of 300 to 1,000 rpm at 5% of dissolved oxygen tension and a fixed dilution rate of 0.15 h^{-1} (Sabra et al., 2000). Both alginate and the cell concentration were continually increased up to 600 rpm and then a decrease was observed for further increases in rotation speed. Maximum alginate production and yield were achieved as 0.9 g/L and 0.8 g/g, respectively, while only about 0.3 g/L of alginate could be formed at extreme mixing rates. Variation of agitation speed also affected the quality of alginate. Guluronic acid fraction of the polymer reached about 35-40 %, a typical value observed for 5 % DOT by elevating mixing rate to 800 rpm. This was the only study concerned with M/G ratio of alginate under different agitation speeds although the exact monomer distribution of polymer was not measured. It is a known fact that

alginates with high guluronic acid contents form denser gels than those with a high mannuronic acid fraction. Therefore, the results might be attributable to requirement of a stiff coating of the bacteria to protect from increasing shear forces. Besides, surface area and capsule formation of the bacteria were investigated under different agitation speeds. It was found that increase in shear rate yielded smaller average cell diameter and surface area, the area was observed as a minimum of 8.9 μm^2 at 800 rpm relative to the minimum of 33.8 μm^2 examined at 300 rpm. The reduction in surface area of the cells might be explained by mechanism involved in the protection of nitrogenase against increasing oxygen levels. Capsule formation was observed even at very high mixing rates like 800-1000 rpm around the cells. On the other hand, the specific capsule area (capsule area / cell area) increased with the elevation of mixing rate up to 600 rpm. As a consequence, the effective oxygen tension value on the cell surface was higher at elevated mixing rates owing to decrease of oxygen transport resistance in the bulk liquid and the formation of a thinner liquid film and/or alginate capsule on the surface of the bacteria.

An additional batch experiment was conducted by *Azotobacter vinelandii* ATTC® 9046 at two different mixing speeds, 300 and 700 rpm, at constant DOT of 3 % (Peña et al., 2000). Results showed that alginate production rate was enhanced by elevating agitation speed to 700 rpm even though dissolved oxygen concentration was maintained constant throughout the fermentation possibly due to prevention of diffusional limitations occurring at low rotation speeds. Maximum specific alginate production rate was obtained as 0.07 g alginate/g biomass/h at 700 rpm. These results might be explained by the fact that the bacteria cultivated at 300 rpm are mostly larger, being in agreement with the results obtained by Sabra et al (2000) than those grown at 700 rpm. These bacteria formed aggregates which led to a limitation in transfer of nutrients and gases between the cells and the medium. Thus, the alginate synthesis rate decreased possibly as oxygen might be limited within the aggregates. On the other hand, all cells were observed small, 1.0 to1.2 µm, at 700 rpm with no aggregation, so this case yielded higher alginate

production rate. Furthermore, variation in agitation speeds affected both viscosity and the molecular weight of the alginate. Higher viscosity values were observed at low mixing speeds compared to 700 rpm which were compatible with molecular weight of the polymer. For example, at 300 rpm, molecular weight was enhanced with increasing dissolved oxygen tension reaching maximum at 5 % DOT as 680,000 g/g.mol compared to the highest molecular weight obtained at 700 rpm as 352,000 g/g.mol at 3 % DOT. In addition, a drop in molecular weight was observed at 700 rpm, both with increasing dissolved oxygen level above 3 % and increasing culture age. The former can be attributed to the aggregation observed at low mixing speeds that oxygen level might be considerably lower in aggregates even if it was actually 1, 3, and 5 % in the medium. The latter probably due to alginases produced by the bacteria particularly at stationary growth phase which resulted in degradation of the polymer chain yielding low molecular weight product.

2.3. ENVIRONMENTAL APPLICATIONS OF ALGINATE

2.3.1. HEAVY METAL REMOVAL BY ALGINATE

Environmental pollution is becoming worse with the increase in industrial activities. Particularly aquatic systems are affected by the accumulation of pollutants like heavy metals, synthetic pollutants, etc. (Papageorgiou et al., 2008). Nowadays, one of the most important concerns is heavy metal pollution since various heavy metals are discharged into the environment from different industrial sources. The main industrial activities that cause heavy metal pollution are listed in Table 2.1 (Volesky, 2001). As a result, heavy metal pollution causes crucial environmental problems that threat human health and the ecosystems. There are three types of heavy metals getting attention: 1) toxic heavy metals like Hg, Cr, Pb, Zn, Cu, Ni, Cd,

As,Co, Sn, etc. 2) precious metals such as Pd, Pt, Ag, Au, Ru etc. and 3) radio nuclides like U, Th, Ra, Am, etc. (Wang and Chen, 2009).

Table 2.1. Major industries discharging heavy metals (Volesky, 2001)

Industry	**Metals**
Mining operations	Cu, Zn,Pb, Mn, U, Cr, As, Se, V
Elektroplating operations	Cr, Ni, Cd, Zn
Metal Processing	Cu, Zn, Mn
Coal-fired power generation	Cu, Cd, Mn, Zn
Nuclear industry	U, Th, Ra, Sr, Eu, Am
Special operations	Hg, Au, and precious metals

In today's world, environmental regulations are becoming more stringent for industrial acitivities producing metal containing wastewaters owing to increasing information about the influence of toxic metals in the ecosystems. These heavy metals can not be eliminated by natural processes easily because of high influx to the environment. Treatment of toxic metals is much more complicated if they enter into the ecosystem and it is hard when they accumulate in the ecological pyramid. This affects all living beings and primarily humans (Papageorgiou et al., 2008). The health problems on humans occur both on the acute and chronic levels (Volesky, 2001). Therefore, it is better to remove these heavy metals at their sources before they reach into the complex ecosystem.

The conventional methods used for the removal of toxic heavy metals from industrial effluents are presented in Table 2.2. These methods include chemical precipitation, filtration, chemical oxidation/reduction, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon, reverse osmosis and evaporation. Chemical precipitation and

electrochemical treatment methods are not efficient particularly if the concentration of metal of interest is between 1 and 100 mg/L. In addition, these techniques leave large amount of sludge behind which is difficult to treat. On the other hand, activated carbon adsorption, membrane processes and ion exchange are very expensive when high amounts of effluent with low concentration of heavy metal is treated. Thus, these techniques are not economical to use at larger scales (Wang and Chen, 2009). Furthermore, conventional technologies are getting inefficient as the discharge standards tighten.

Table 2.2. Conventional metal treatment technologies (Volesky, 2001)

Method	**Disadvantages**	**Advantages**
Chemical precipitation and filtration	for higher concentrations, difficult separation, not effective	simple cheap
Chemical oxidation or reduction	chemicals required (not universal) biological system climate sensitive	mineralization
Electrochemical treatment	for high concentrations expensive	metal recovery
Reverse osmosis	high pressures required membrane scaling expensive	pure effluent (for recycle)
Ion exchange	sensitive to particles expensive resins	effective pure effluent metal recovery possible
Adsorption	not for metals	conventional sorbents (carbon)
Evaporation	energy intensive expensive resulting sludges	pure effluent (for recycle)

In general, municipal sewage treatment plants are not able to remove toxic heavy metals. Therefore, these metals and the resulted toxicity remain in sludges and by-product streams. For this reason, heavy metals require in-

situ treatments designed as a pretreatment step. This step should be cheap since it is mostly used for the treatment of large quantities of wastewater. Nowadays, biotechnological applications are gaining interest in order to control and reduce heavy metal pollution and become hot topic due to its potential application. One example to these newer applications is biosorption that uses several natural materials can be an alternative process. These materials originate from biological sources such as algae, fungi, bacteria, etc. Seaweeds especially brown algae have been known to be effective for heavy metal removal by sorption and they have been proposed for industrial usage (Papageorgiou et al., 2008). Nevertheless, the knowledge about the composition and tissue structure of various seaweeds are not enough. In addition, properties of harvested biomass are affected by growth conditions, locations and seasonal variations. These uncertainties in characteristics of most biosorbents limit their chance for use as competitive products comperative to well-known synthetic ion exchangers even though the costs of biosorbents are greatly lower.

In brown algae matrix, biosorption is particularly determined by the properties of the cell wall where complexation reactions and electrostatic attraction may take place. This matrix is mainly composed of alginic acid or alginate (the salt of alginic acid) together with a little amount of sulfated polysaccharide (fucoidan). The alginic acid fraction (10 and 40 % of the dry cell weight) is influenced by growth depth of algae and seasonal changes. Similarly the capability of alginate to remove heavy metals is also greatly affected by the growth conditions of marine brown algae.

2.3.1.1. Factors Affecting Heavy Metal Removal by Alginate

Biopolymers that can be produced in a considerably low cost may have high selectivity for some metals. For these reasons, biosorption is getting attention as an alternative method to conventional techniques for heavy metal removal (Jang et al., 1991). Alginate biopolymer is one of the good candidates for toxic metals treatment from dilute wastewaters. Some

functional groups in the structure of alginate are known to be responsible for the removal of heavy metals. Among them, carboxylic groups are generally the most abundant acidic functional group. Hydroxyl groups are also found in all polysaccharides, however, amount of them is relatively lower. Furthermore, they only have negative charges at pH > 10, so, hydroxyl groups are not efficient sites for metal binding at low pH values. Therefore, the heavy metal reduction capacity mostly depends on the abundance of the carbohydrate functional groups in alginate chain (Davis et al., 2003). In addition to this, alginate conformation is a significant factor influencing the heavy metal uptake capacity of the polymer.

The main mechanisms responsible for toxic metal sorption involve ionic interactions and complex formation between functional groups found in alginate chain and the metals (Fourest and Volesky, 1996). There is a conformational difference between alginate monomers; the regions rich in GG-blocks particularly contribute to gelation with divalent cations. On the other hand, mannuronic acid rich parts of the alginate chain play a significant role in the cation exchange capacity of this biopolymer (Papageorgiou et al., 2008).

Ion exchange takes place due to the electrostatic interactions between metal cations and the functional groups in structure of alginate. Because binding of toxic heavy metals onto available binding sites of the biopolymer leads to the reduced availability of these sites for the other metal cations found in the solution, the mechanism responsible is greatly competitive. As a result, functional groups, such as the carboxylic groups, preferably take metal species showing higher affinity compared to the metal species having lower affinity. On the other hand, a more complicated competitive effect between heavy metals and protons is found in binary systems (Papageorgiou et al., 2008).

Another interaction between alginate functional groups and metal cations involves metal complexation (Pandey et al., 2002). The selective metal

binding ability of alginate is especially determined by the fraction GG-blocks. These regions have a greater selectivity for divalent metal ions and supply a multidentate environment for complexation reaction. The greater selectivity of GG-blocks for divalent metals can be attributed to their zigzag structure that can accommodate the calcium ion and other divalents more readily. These polyguluronic acid sections give an alignment of two chain parts resulting an array of coordination sites with cavities which fit for calcium ions. Therefore, alginate constitutes an ordered solution network with inter chain dimerization of GG-block fractions by calcium and other divalent ions having similar size. This is known as egg-box model. These dimerization sections are terminated by mannuronic acid residues. On the other hand, mannuronic acid containing regions are weaker because they are mostly monodentate (Davis et al., 2003).

Alginates have variable affinity to various heavy metals, which changes according to the relative ratio of mannuronic to guluronic acids. The earlier study claimed that with increasing GG-block content of alginate, the affinity of the polymer increased for divalent cations in the following order of Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ca^{2+}, etc (Haug, 1961). Similar results obtained from another study that affinity of two different alginates between sodium and divalent metals were examined (Haug and Smidsrød, 1965). They found that the selectivity of alginates increased by increasing GG-block fraction of the polymer. This may be explained by selective binding of heavier ions because larger ions may fit better to a binding site with two distant functional groups. A further work was conducted to determine divalent metals uptake capacity of an alginic acid which is originated from *Laminaria digitata*, a specie of brown algae. The amount of protons given to solution decreased in the following order $Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+}$ which supports the findings of Haug (1961). This can be attributed to relative capacity of the metal to compete with protons for organic binding sites.

The other parameters affecting metal uptake can be listed as the concentration range, the solution pH, the reaction kinetics, and the composition of the actual effluent handled. For example, ion exchange ability of alginates is affected by pH of the solution (Fourest and Volesky, 1996) because the charges of functional groups are getting more negative with increasing pH. Therefore, divalent metal uptake capacities of the biopolymer increase. pKa value for mannuronic acid is determined as 3.38 while it is 3.65 for guluronic acid (Fourest and Volesky, 1996, Park et al., 2007). The highest or near maximum metal uptake capacities are achieved at pH values close to the apparent dissociation constant of carboxylic acids for most of the metals such as Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Pb^{2+}. Furthermore, pH of the solution may influence the solubility of the metal ions since higher pH values can cause precipitation of metal hydroxyl salts which are not soluble.

Ionic strength also plays an important role in metal ion uptake studies. Since alginate is a greatly charged polymer, ionized functional groups of the polymer like carboxyl groups create negatively charged areas. They react with anions in the proximity of the polymer leading to their concentration in the vicinity of the surface be lower than that of the bulk solution. Then, ionic strength of the solution can negatively influence the binding of the metals by a screening effect of non-adsorbed ions which compress the layer between alginate molecules. In addition, if adsorbable cations of the salts are used to set the ionic strength, this can result in a competition for the available sites for sorption of the metal ions under study. Thus, the uptake capacity of the metal on alginate decreases (Papageorgiou et al., 2008).

2.3.1.2. Studies Concerning Heavy Metal Removal by Alginate

Biopolymers are selective, efficient, and cheap materials. Therefore, they are highly competitive with the other alternatives for heavy metal removal such as ion exchange resins and activated carbon. Alginate in different forms like sodium alginate, calcium alginate or bead with/without combination of various materials were investigated for heavy metal treatment.

Calcium alginate gels were investigated for the uptake capacities of lead, copper, cadmium, cobalt, nickel and zinc at pH 4 (Jodra and Mijangos, 2001). The results showed that among them, lead is the one having the maximum uptake ability for this algal alginate because the amount of calcium released from gel phase into the solution was higher compared to the other metals studied. Lead was followed by copper and cadmium and then cobalt, zinc and nickel. As a result, the selectivity of alginate for these metals can be ordered as Pb^{2+} > Cu^{2+} > Cd^{2+} > Ni^{2+}, Co^{2+}, Zn^{2+}. This result was in agreement with the results of another study that (Jeon et al., 2002) metal binding ability of the PVA–alginic acid bead was changed in the following order: Pb^{2+} >> Cu^{2+}> Cd^{2+}> Ni^{2+}, Co^{2+}> Ca^{2+}> Mg^{2+}. The selective binding behaviour of alginate depends on relative distribution of alginate monomers and the size of the hydrated ionic radius of cations.

Two different metals investigated due to their hazardous character in the environment and their comparably different behaviour in the chelation, were cadmium and lead (Fourest and Volesky, 1996). Alginic acid and *Sargassum fluitans* (a brown algae) biomass were used to determine the ion exchange capacity at maximum saturation levels of these metals and two different pH values were examined. At pH 2.5, the highest metal uptake of native *Sargassum fluitans* biomass for Cd^{2+} and Pb^{2+} was measured as 0.335 and 0.8 g / g, respectively. On the other hand, sorption capacity was increased by increasing pH to 4.5 that the maximum uptake of Cd^{2+} and Pb^{2+} was 0.91 and 1.06 g / g, respectively. Alginate is the main part of algal biomass structure leading to most of the metal binding. Complex formation between metal and alginate is affected by changing pH. Because two carboxyl groups are required to bind one metal cation, the proximity between ligands is important. Also, the high degree of ionization of functional groups and flexibility of alginate favor the formation of metal complexes. At lower pH values, some of the binding sites are protonated that reduce the ratio between cation uptaken and available carboxyl groups. On the other hand, infrared spectra of alginate in either protonated or cadmium loaded were examined. It was shown that there was an absorbance peak at 1738 cm^{-1}

which reflects the stretching band of free carbonyl double bond from the carboxyl functional group. An obvious shift from 1738 to 1630 cm^{-1} in the carbonyl stretching bond was observed after the experiment conducted at high cadmium concentration. This shift can be attributed to typical complexation reaction between cadmium and the carbonyl group. As a result, cadmium uptake by alginate involves bridging or bidentate complex formation with carboxyl groups in the polymer structure and this is compatible with egg-box model.

Another study concerning maximum cadmium uptake capacity of various alginate extracts was performed by using four different extraction methods for two brown algae species: *Sargassum fluitans* and *Sargassum oligocystum* (Davis et al., 2004). Both algal species have M/G ratio varied in the range of 0.49 to 0.62. The highest cadmium binding values recorded as 1.68 and 1.81 mmol Cd / g alginate extracted from *Sargassum fluitans* by neutral extraction and high-temperature extraction, respectively. Alginate extracted from the other species, *Sargassum oligocystum*, showed close cadmium uptake capacity. Similar to the previous study (Fourest and Volesky, 1996), the authors claimed that the selectivity of alginate for divalent cations is highly affected by structural units of alginate, which can be attributed to egg-box formation of alginate with divalent cations.

A further study examined the significance of macromolecular characteristics of alginate on metal uptake properties in single and mixed metal pair systems (Davis et al., 2003). Sodium alginate with different GG-block content, M/G ratio is ranged from 0.25 to 1.7, was used for cadmium binding studies at pH 4.5. It was aimed to test whether the hypothesis about macromolecular conformation might be the reason for different amounts of metal uptake because of the preferential binding by the regions rich in GG-blocks. However, it was found that there was no important difference for cadmium uptakes by changing GG-block content within the range studied. It can be explained that the selectivity of cadmium binding compared to the monovalent proton, sodium, is so large. Thus, all sites available for binding

of metal ions are satisfied by the divalent cation, cadmium. The authors argue that single metal systems may not be suitable for assessing the effect of conformational change in selectivity of alginate.

In the following part of the study (Davis et al., 2003), mixed metal systems, Cd^{2+}-Ca^{2+}, Ca^{2+}-Mg^{2+}, Cd^{2+}-Mg^{2+}, were used to test the influence of molecular conformation of alginate on metal binding. For Cd^{2+}-Ca^{2+} system, the selectivity coeffcient of cadmium over calcium was found to be similar for alginate extracts and it did not depend on the amount of GG-block in alginate chain over a broad range of X_{Ca} (mole fraction of Ca^{2+}). On the other hand, preferential binding of calcium over magnesium was much more favored since the selectivity coefficient for Ca^{2+}-Mg^{2+} system changed in the range of 2.1 to 18. The alginate extracted from *Sargassum fluitans* having GG-block content of 0.81 obviously showed an improved selectivity for calcium over magnesium relative to the alginate extracted from *Macrocystis pyrifera* having GG-block fraction of 0.23. The selectivity coefficient for Ca^{2+}-Mg^{2+} system for both alginate extracts were higher at comparably low mole fraction of calcium, $X_{Ca} < 0.5$. This value was also decreased by increasing X_{Ca}. Similar to Ca^{2+}-Mg^{2+} system, binding behaviour of alginate in Cd^{2+}-Mg^{2+} system was different by changing GG-block content. The selectivity coefficient was found to increase with increasing GG-block fraction of alginate, but, maximum selectivity was observed at high mole fraction of cadmium, $X_{Cd} > 0.5$. The higher selectivity of alginate for calcium over magnesium at low mole fractions of calcium shows that a small fraction of GG-blocks are responsible for binding calcium. However, for Cd^{2+}-Mg^{2+} system, it is just the opposite and this implies that cadmium binds differently than calcium into alginate structure because the maximum selectivity coefficient is observed at high cadmium mole fractions. Therefore, higher amount of GG-blocks seems to be occupied in the case of cadmium binding.

For the Cd^{2+}-Ca^{2+} system, again no important selectivity of alginate having a broad range of GG-block content was found. It can be said that calcium limits toxic heavy metal, i.e. cadmium, uptake by alginates because of lack of

selectivity in Cd^{2+}-Ca^{2+} system. This behaviour can be explained by the size of the ionic radius of Ca^{2+} and Cd^{2+} which are almost the same. Therefore, they display similar affinity to GG-block fractions in the alginate chain. When two chain sections of guluronic acid come close, the cavity formed supplies a multidentate environment having carboxylate groups, the ring oxygens, and the hydroxyl groups of guluronic acid, all contribute the uptake of the cations. The reason for low selectivity of magnesium for guluronic acid sections is probably due to size of magnesium that has smaller ionic radius. This interferes with the formation of a strong bond coordination environment like in the case of calcium (Davis et al., 2003).

Some works utilized the ability of bead formation of algal alginates with divalent cations in order to remove toxic heavy metals. For instance, copper recovery was studied by in situ formation of alginate beads from sodium alginate by dispersing the solution in a loop fluidized bed reactor (Jang et al., 1990). The highest sorption capacity of copper was found as 0.1 g Cu^{2+}/ g alginate. Most of the total copper sorption (initial concentration of Cu^{2+} > 60 ppm), 90 %, takes place within 1 hour. Furthermore, the final equilibrium binding capacity of copper in alginate beads was reached in the first 2 hours. The regions rich in guluronic acid are effective in copper uptake whereas the regions rich in mannuronic acid are possibly not able to bind copper covalently. However, mannuronic acid blocks having negative charges serve as an ionic environment of the gel phase and therefore, this indirectly improved the partition of sodium and free copper between the gel fluid and the reactor fluid. In a following work (Jang et al., 1991), partially coagulated calcium alginate spheres were used for treatment of a solution having 10-40 ppm of copper concentration. The amount of copper sorbed on alginate was varied in the range of 0.015 to 0.09 g / g if partially coagulated calcium alginate spheres were mixed with synthetic copper solution at low concentration. In this case, an important fraction of available functional groups in the gel phase stayed unbound because of the competition between calcium and low concentration of copper. In addition, the apparent copper uptake affinity increased when the ionic strength of reactor fluid was

decreased especially at low copper concentration. Also, a green algae, *Microcystis*, was examined by combination with sodium alginate to remove copper at high concentration, 100-250 ppm. *Microcystis* and alginate mixture have an ability to bind copper as maximum of 0.36 g copper per g alginate.

In a further study, alginic acid was immobilized by using a novel PVA–boric acid method for the reduction of hydration of alginate beads and application of alginic acid efficiently to wastewater treatment (Jeon et al., 2002). The highest binding capacity of lead on alginic acid beads at pH 4 measured as 390 mg lead / g alginic acid beads that was almost twice of the capacity achieved at pH 2. Performance of alginic acid beads were compared to an inexpensive commercial resin, Amberlite IR-120+, having strongly acidic character. If there was only lead in the solution, the lead uptake capacity of the resin was higher relative to alginic acid beads. However, when lead was found together with calcium and magnesium, Amberlite IR-120+ resulted lower lead sorption capacity independent of initial concentration. Results can be explained by the interference of alkaline metals by binding onto available sorption groups. On the other hand, the influence of calcium and magnesium ions on lead uptake of alginic acid beads was found to be negligible. As a result, the use of alginic acid beads over the resin would be adventageous since Amberlite IR-120+ is not selective for alkaline metals.

Calcium alginate beads were formed together with humic acid for uptake of heavy metals from two industrial wastes having Ni^{2+}, Mn^{2+}, Fe^{2+}, Cd^{2+}, Cu^{2+}, Cr^{2+} and Zn^{2+} (Pandey et al., 2002). Alginate beads are able to remove considerable amount of metals from the leachates. Humic acid integrated alginate beads showed increased heavy metal binding capacity for all metals. This can be attributed to the amount of sites available for heavy metal uptake found in calcium alginate beads is fixed although humic acid containing alginate beads had additional functional groups. These additional sites are the main reason for more sorption of heavy metals in the case of humic acid beads. Although in calcium alginate system the interaction between calcium ions and GG-block sections are a strong cooperative

binding and all functional groups are participate in interaction with calcium, it is claimed that the interaction is different in transition metals like Cu^{2+}, Co^{2+}, Mn^{2+}. In this case, sol-gel transition occurs by complex formation between carboxyl groups in both mannuronic and guluronic acid and coordinated metal ions.

Activated carbon containing alginate beads, AC-AB, were formed to remove eight different heavy metals (Hg^{2+}, Pb^{2+}, Cd^{2+}, Mn^{2+}, Fe^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}) and four mineral ions (Park et al., 2007). AC-AB completely removed heavy metals from solution and meanwhile it leaved important minerals into solution like K^{+}, Na^{+}, Mg^{2+} and Ca^{2+}. Furthermore, AC-AB had an ability to treat heavy metals and toxic organic compounds at the same time due to the presence of AC in alginate beads. When only activated carbon was applied, above 90 % of *p*-toluic acid was removed whereas a small amount of heavy metals could be treated. For example, particularly activated carbon can not remove Cd^{2+}, Zn^{2+}, and Mn^{2+} from the solution. On the other hand, five heavy metals were bind efficiently by alginate beads with only little amount of *p*-toluic acid reduction. The combined system of activated carbon and alginate beads resulted a high removal capacity for both *p*-toluic acid and heavy metal ions.

Calcium alginate extracted from *Laminaria digitata* was examined for heavy metal removal from dilute metal solution (Papageorgiou et al., 2008). Calcium alginate beads display high binding capacity for the heavy metals relative to other low cost adsorbents due to high guluronic acid content. Also, binary metal uptake studies conducted by a solution contaning copper and cadmium showed that calcium alginate beads favor copper sorption over cadmium ion in binary system. This means that a competitive mechanism is responsible in binary sorption process. Therefore, sorption capacities of calcium alginate beads showed great difference for the metals investigated in the following order $Pb^{2+}>Cu^{2+}>Cd^{2+}$.

Application potential of absorbents is influenced by reusability of these materials for successive metal uptake without high loss of their binding capacities for toxic heavy metals. For this purpose, dilute acids like HCl (Park et al., 2007; Papageorgiou et al., 2008), HNO_3 (Park et al., 2007) etc., and EDTA (Jeon et al., 2002; Park et al, 2007) were used for desorption of the metals from the sorbent materials. In order to determine the reusability of sorbent material, an adsorption–desorption cycle of metal ions was performed sequentially. For instance, it was found that the adsorption capacities did not subtantially varied (a maximum 3% change) during the repeated adsorption–desorption operations (Papageorgiou et al., 2008). Another question is the recovery of the heavy metal from concentrated solutions. In general, it is achieved by an independent metal recovery process. For instance, electrowinning procedures are the most feasible alternative for the recovery of metals from desorption solution (Volesky, 2001).

All things considered up to here, studies related with heavy metal removal dealth with alginates from marine algae and brown algal biomass in different forms. One disadvantage of algal alginate is the properties of algal alginates are very much afftected by environmental conditions depending on where the algae is located. Different ion concentrations, salinity conditions, different depths, etc. may bring some fluctuations to the quality of marine algal alginate. On the other hand, it is also possible to produce alginate from bacterial sources at certain characteristics under controlled laboratory fermentors. It seems that no such study exists that uses the alginates from bacterial sources in heavy metal uptake. In this study, alginate was produced by *Azotobacter vinelandii* ATCC® 9046 having various rheological properties and monomer distributions. These samples were investigated for the first time to determine maximum heavy metal uptake capacities of alginates from this bacterial specie.

2.3.2. USE OF ALGINATE IN TURBIDITY REMOVAL

There are lots of solid materials in water and wastewaters which come from land erosion, mineral dissolution, decay of organics, and some effluent discharges of domestic and industrial origin. The materials may contain mineral compounds, silt, bacteria, viruses, some macromolecules, etc. (Bratby, 2006). Therefore, these compounds are required to be removed from potable water supplies since they act as an adsorption surface for microorganisms which are protected from contact with chemicals used in disinfection (Bolto and Gregory, 2007). Most of the solid impurities are in the form of colloidal compounds and their sizes are smaller than 1μm (Hughes, 1990). For this reason, settling velocities of these materials are much lower and thus, use of sedimentation for the treatment is not a feasible option. General approach for the removal of these colloidal solids is agglomeration of these particles by coagulation /flocculation processes which are followed by sedimentation, filtration or flotation (Tebbutt, 1998).

Metal salts are generally used as coagulants and they combine with alkalinity in water in order to form insoluble metal hydroxide flocs which incorporate the colloidal particles. Then a fine precipitate is flocculated and turn into settleable solids. Over a long time, aluminium sulphate has been used widely as a coagulant for turbidity removal, even though it has some health concerns due to aluminium residuals after its application in drinking water production. There are lots of alternatives other than aluminium sulphate such as sodium aluminate, aluminum chloride, polyaluminum chloride, aluminum chlorohydrate, polyaluminum silicate chloride, and forms of polyaluminum chloride with organic polymers. Iron based metal coagulants are also applied like polyferric sulfate, ferrous sulphate, and ferric salts with organic polymers (Bratby, 2006). When iron salts are compared with aluminum salts, they are less expensive. However, if the precipitation reaction is not completed, residual iron left behind can cause some problems especially because of its potential stain-producing properties in washing machines (Tebbutt, 1998).

Usually, polymeric substances that can be natural or synthetic origin are used as coagulant aids together with metal salts. These polymers are mainly applied to destabilize or enhance flocculation process. They are water soluble macromolecular materials (Bratby, 2006). Synthetic polymers are more popular since their properties can be controlled during synthesis like molecular weight of the polymer and the amount and type of charged groups on the surface. Most of synthetic polymers contain polyacrylamide and its copolymers with polyacrylic acid. During synthesis of these polymers, the monomers may be found as impurities in some polyelectrolyte products such as acrylamide residues. It is known that acrylamide is a toxic compound with chronic activity, which is a neurotoxin. Therefore, bioaccumulation of these monomers in drinking water would be hazardous to human health (Hughes, 1990). On the other hand, a lot of natural materials have coagulating abilities and recently some of them like chitosan and starch received quite an interest. These natural substances are originated from seeds, part of trees and plants that include polyelectrolyte (Tebbutt, 1998). Natural polymers are adventageous because of their toxicity free nature and biodegradability in the environment. Also, some microbial natural polyelectrolytes can be produced economically in large scale cultures and at high rates. Moreover, the extracellular flocculants are readily recovered from culture broth (Salehizadeh and Shojaosadati, 2001).

According to current knowledge, chitosan seems to find a wider application in turbidity removal. It was applied to treat wastewaters like palm oil mill effluent (Ahmad et al., 2006), an ionic dye (Reactive Black 5) (Guibal and Roussy, 2007) and flocculation of river silt (Divakaran and Pillai, 2002). There are only two studies concerning the use of alginate as in the form of alginate-g-polyacrylamide for the removal of turbidity from iron ore suspensions (Tripathy et al., 2001) and alginate-g-N-vinyl-2-pyrrolidone for the removal of turbidity from coal suspension (Sand et al., 2010).

2.3.2.1. Double Layer Theory

Agglomeration of small suspended particles is needed for the particles to come together. Figure 2.7 shows schematically the approach of two similarly charged spherical particles. Attracting forces like the London and van der Waals forces are opposed by the interaction of like charges distributed over each particle. Particles come closer only if charges on the particles are decreased (Hughes, 1990).

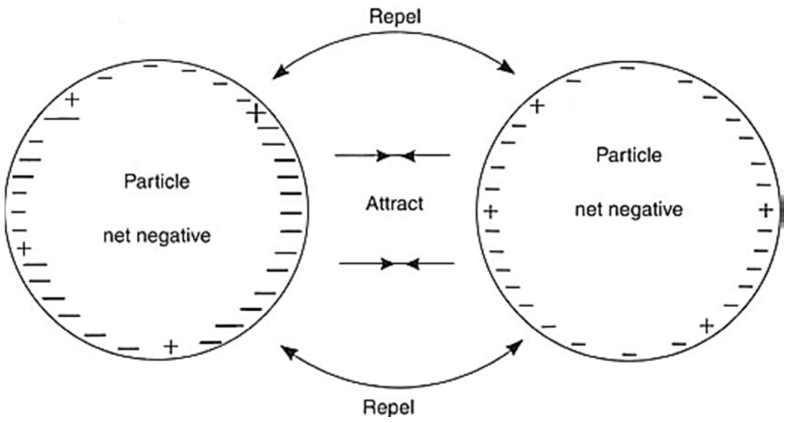

Figure 2.7. The approach for two particles having the same charge (Hughes, 1990)

Most of natural and man-made particles have a residual charge on the surface. Generally this charge is a net negative charge like those found in minerals and clays. Three mechanisms are responsible for surface charges. Firstly, the lattice can be defective in crystalline materials and therefore, a net excess negative or positive charge can be found at the surface. The net charge is balanced by an equivalent charge at the surface and the crystal delivers the compensating ions to produce a double layer when it is in contact with water. This is usually observed in ion-exchange materials like zeolites and clays (Hughes, 1990). Second, surface charges are caused by chemical reactions happening at the surface. Most of the solid surfaces have

functional groups that are easily ionizable such as –COOH and -OH (Bratby, 2006). Third, the reason for surface charges can be the adsorption of some ions from the solution. Generally, adsorption is due to hydrogen-bonding especially in the case of large organics (Hughes, 1990).

The surface charge affects the distribution of closer ions in the liquid. While oppositely charged ions are attracted towards the surface, like charged ions are repelled away from the surface. Together with mutual ionic attraction or repulsion and the mixing tendency of thermal motion cause the formation of an electrical double layer as it is shown in Figure 2.8. This layer is composed of the charged surface and a neutralizing excess of counter ions over co-ions placed in a diffuse manner in the nearby liquid (Bratby, 2006). Usually, there are two distinct regions found in the electrical double layer. First one is an inner region that possibly have adsorbed ions and water molecules. The other one is a diffuse layer. In this layer, ions are distributed depending on the effect of electrical forces and random thermal motion (Bratby, 2006). A shear plane separates these inner region and diffuse layer. The potential between the solid surface and the shear plane is called the Nernst potential whilst the potential found between the bulk phase and the shear plane is named as the zeta potential (Hughes, 1990).

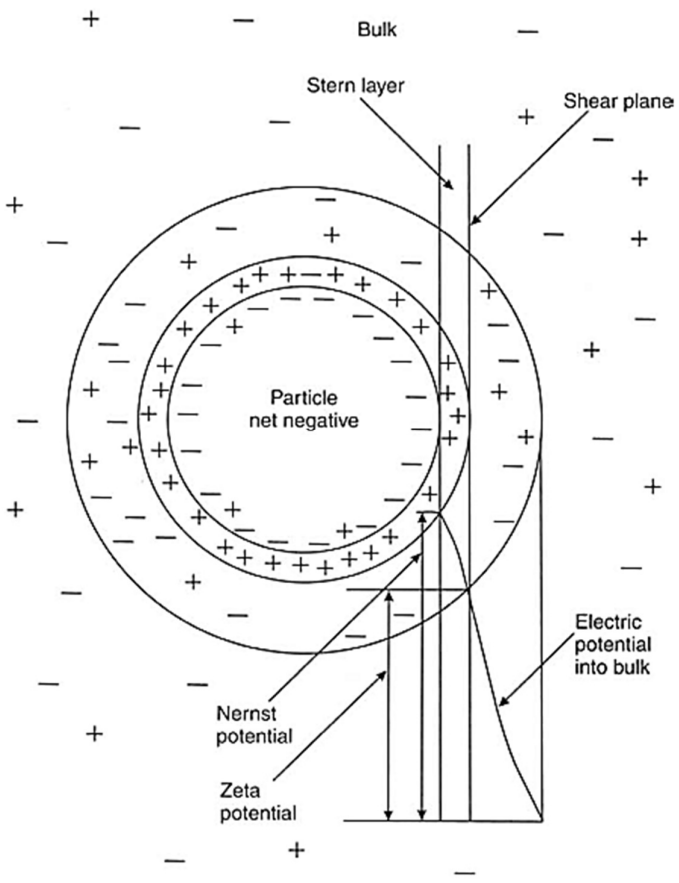

Figure 2.8. The colloidal model (Hughes, 1990)

2.3.2.2. Coagulation

Coagulation methods are mostly applied to destabilize and aggregate colloidal materials in both water and wastewater treatment processes. In water treatment, coagulant materials like polyelectrolytes or metal salts act as added counterions. They are attracted towards the dispersed particles and this eventually leads to the compression of the diffuse layer. This results in a reduction of the energy barrier to supply effective particle collision. When destabilized particles come together, they stick successfully to each other. Rapid mixing is particularly important at this stage because it provides uniform dispersion of the coagulant and opportunity for contact of particles to

each other. Further gentle and prolonged slow mixing leads to formation of larger flocs. These flocs then can combine with suspended material. At the end, the particles having sufficiently high size and weight begin to settle (Maximova and Dahl, 2006).

Various mechanisms are responsible in the coagulation of solid substances such as adsorption, charge neutralization, ionic layer compression, inter-particle bridging and sweep coagulation (Ahmad et al., 2006). If the concentration of suspended material is low, coagulation can be generally achieved by enmeshment in insoluble hydrolysis products produced in the reaction occured between the water and the coagulant. This is called sweep coagulation and it is controlled by the characteristics of the hydrolysis products of the coagulant. Thus, the nature of the original suspended matter is not that important in this mechanism (Tebbutt, 1998). In the case of high suspended solid concentrations, the colloidal theory may be a basis to explain the observed reactions. This means that colloidal particles are destabilized by the adsorption of strongly charged ions. If the added coagulant dose is too high, a charge reversal may occur due to continued adsorption. Hence, suspended particles are restabilized. Under this circumstance, the nature of the suspended matter has an effect on the coagulation process. Moreover, colloidal materials may be either lyophilic or lyophobic. In the case of lyophilic matter, the suspended matter has a high affinity for water and other dispersion media. Lyophilic subtances are macromolecules, for instance, proteins and humic acids. Lyophobic solids like clays and hydrated oxides show a lower affinity for the liquid medium. They are especially sensitive to the addition of electrolytes because lyophobic solids are easily flocculated after such additions. The opposite is true for lyophilic suspended matters and they require very high concentrations of the electrolytes to precipitate (Hughes, 1990).

2.3.2.3. Flocculation

Flocculation is a process in that small particles or small groups of particles form large aggregates. This can be accomplished by perikinetic or orthokinetic flocculation. In the case of perikinetic flocculation, floc formation mainly depends on Brownian motion alone; while inducing a velocity gradient to the solution with the help of mixing is important in orthokinetic flocculation (Hughes, 1990). Furthermore, the perikinetic flocculation is effective for particles less than 1µm diameter and the orthokinetic flocculation dominates for particles larger than 1 µm (Ives, 1990).

Aggregation process requires a low charge on particles and collision of particles. After the pretreatment with an electrolyte, repulsion between particles is reduced and the rate of aggragation is determined by the Brownian motion. However, Brownian motion is usually not sufficient to form large aggregates in a reasonable time. Therefore, mixing of the solution containing suspended matter may enhance the flocculation rate by orthokinetic flocculation (Hughes, 1990).

Stirring of the dispersion by mechanical or hydraulic means leads to the velocity gradients and flocculation process is controlled by the intensity of the gradients. The number of collisions between suspended particles is directly related to the velocity gradient (Tebbutt, 1998). Therefore, flocculation efficiency might be improved by increasing the collision radius of the suspended particles. That is flocculation process is selfenhancing with the formation of larger flocs. However, creation of high shear stress due to increased velocity gradient may cause floc break up, so there should be a limit for the velocity gradient. Furthermore, presence of large particles like observed in solids-contact flocculation or floc-blanket clarifiers, will increase the rate of flocculation. On the other hand, flocculation is selfdiminishing when the number of the suspended particles are decreased because of aggregation (Ives, 1990).

In coagulation/flocculation studies, laboratory experiments must be performed by the jar-test procedure because it is not possible to calculate the dose of coagulant needed. This procedure includes a series of beakers, generally 4 to 6 in number, mixing simultaneously by a paddle in each beaker that supplies the same conditions in all the beakers. The solution is agitated at high speed initially during a short time, i.e. one minute, and then a slow mixing is continued for 10 to 20 minutes. The effect of various conditions such as type and dose of coagulants, pH conditions can be investigated for successful aggregation of suspended particles. As a result, an optimum dose is determined for floc formation having a considerably higher size leading to a quick settlement thereafter (Ives, 1990).

2.3.2.4. Natural Polymers

Coagulation and flocculation processes are widely used for the treatment of turbidity caused by suspended and colloidal matter in order to produce drinking water from most of the raw water sources. Nowadays, there is an increased interest for the use of natural coagulants mostly due to their environmentally benign properties. They can be used as alternative to metal salts, and synthetic polymeric coagulants since natural polyelectrolytes are safe for human health and biodegradable. These compounds are effective for a wider range of doses for the flocculation of different dispersions. Moreover, flocs formed by polymers are generally stronger compared to the ones obtained by metal salts (Özacar and Sengil, 2003).

Polymeric subtances are constituted by a series of repeating chemical units that are colavently bonded. Polyelectrolytes are the polymers having some functional groups in their structure that some of them are ionizable. Ionization of the functional groups makes the polymers charged either positively or negatively. This is determined by properties of the functional groups and called as cationic or anionic polyelectrolytes. If a polymer contains both negative and positive charges, it is known as ampholytic while

the ones having no ionizable functional groups are referred as nonionic polymers (Bratby, 2006).

The most important properties of natural polymers are molecular weights and charge densities. Molecular weights can be in the range from a few thousand up to tens of millions. They may be classified as low, medium and high molecular weight as in the following order $<10^5$,10^5–10^6 and $>10^6$, respectively (Bolto and Gregory, 2007). The most commonly used polymers are anionic polyelectrolytes having carboxyl groups. Especially, anionic polymers with high molecular weight containing low charge density received attention as flocculants for water treatment. Moreover, a small number of non-ionic polyelectrolytes are applied for water treatment (Hughes, 1990).

Flocculation process is also affected by adsorption properties of the polymer on the surface and conformation of the adsorbed polymer. Depending on ionic characteristics of the polymer and its adsorption on the particle surface, flocculation is determined by different mechanisms such as charge neutralization, bridging and charge patch mechanism. Among them, bridging and charge neutralization are more common for flocculation (Besra et al., 2002). For the case of charge neutralization, ability of a polymer to flocculate suspended and colloidal matter depends on charge density on the polymer surface. Polyelectrolytes having high charge density would be more efficient for charge neutralization due to the fact that these polymers can deliver more charge to the particle surface. Also, high charged polymers are not good candidates for bridging interactions because they usually adsorb in a rather flat configuration (Bolto and Gregory, 2007).

When highly charged polyelectrolytes adsorb on weakly charged negative surfaces, neutralization of each surface charged sites by a polymer segment is unlikely, which causes the formation of electrostatic patch mechanism. This might be due to the fact that the average distance between surface sites is greater than that between charged segments in the polyelectrolyte. Therefore, some positively charged surfaces are present as patches

between the negative charged regions even though the surface has an overall charge of neutrality. Patchwise adsorption results closure of particles owing to an electrostatic attraction between positive patches and negative areas, which provide attachment of particles and then flocculation. Charged density of polyelectrolytes is an important concern for electrostatic patch flocculation since bridging flocculation dominates at low charge densities (Bolto and Gregory, 2007).

The collision event generally leads to adsorption on larger particles. For this reason, if mostly very fine particles are present in dispersion, excessively high amount of polyelectrolytes is needed for flocculation. Particularly high molecular weight polyelectrolytes are not efficient for the suspension of very fine particles since the amount of larger particles are less. On the other hand, low molecular weight polymers are found to be successful in these circumstances. However, they still are needed at very high dosages for stable dispersions. Polymeric flocculant materials are usually efficient for previously coagulated dispersions such as charge controlled systems. In these cases, the material possibly acts as a binding agent supplying floc growth to large sizes (Hogg, 1999).

Interparticle bridging is observed if a high molecular weight polymer adsorbs on some particles or share ions in order to form ionic bridges. Particles produce a mesh like matrix having destabilized colloids and polymer branches in interparticle bridging. Flocs entrap smaller particles during settlement, which is known as co-precipitation or sweep floc (Maximova and Dahl, 2006). Both location and the abundance of acidic charges on the surface of the polymers are critical in bridging. For instance, the exposure of carboxylate groups found on side chains greatly enhances flocculation process. On the other hand, accessability of charges found on the back bone of the polymer lowers the possibility of interaction that results in a reduction at flocculation capability for these polymers (Labille et al., 2005). Furthermore, for effective bridging in flocculation, high or total coverage of the particle surface by the polymer is not needed. Ideally, neutralization of a

part of surface charges causing repulsion is enough because full coverage of particle surface by the polymer having flat configuration may cause steric repulsion resulting instable suspension (Besra et al., 2002).

2.3.2.5. Studies Concerning Turbidity Removal Potential of Alginate

There are almost no studies related to the use alginates in turbidity removal. Two different works dealth with turbidity treatment by algal alginates in the form of grafted with synthetic polymers such as alginate-g-polyacrylamide (Tripathy et al., 2001), and alginate-g-N-vinyl-2-pyrrolidone could be found in literature (Sand et al., 2010). However, both focused on solids removal from wastewaters. In the study of Tripathy et al. (2001), alginate-g-polyacrylamide was investigated for the removal of turbidity from iron ore suspensions and the polymer was compared with three different polyacrylamide commercial flocculants. It was found that alginate-g-polyacrylamide resulted in better removal performance compared to the other polyacrylamide flocculants particularly for the treatment of iron ore suspensions having higher density. Turbidity reduction efficiency of alginate-g-polyacrylamide was examined in the range of 0.025 to 5 ppm and turbidity value of 0.5 % iron ore suspension could be decreased to around 2 NTU at 5 ppm concentration. In the other study (Sand et al., 2010), turbidities of coking and non-coking coal dispersions (1%) were tried to be reduced by a grafted copolymer, alginate-g-N-vinyl-2-pyrrolidone. The grafted polymer yielded lower residual turbidity values than alginate alone. They claimed that the reason could be higher bridging opportunities in the case of alginate-g-N-vinyl-2-pyrrolidone due to dangling of the polymer chain.

Some previous work was conducted in Environmental Engineering Department of METU with the use of an algal alginate as a coagulant for turbidity removal of a clay suspension (Çoruh, 2005). Potential use of the alginate was investigated in the presence of calcium ion at various turbidity levels, namely high, medium and low turbidity. Alginate concentration was changed between 0.004 – 40 mg/L while calcium ion dose was varied in the

range of 30 – 200 mg/L. It was found that alginate was especially effective for the treatment of high and medium turbid clay dispersions, 150 and 80 NTU, respectively. In the case of high turbidity, 150 NTU, addition of calcium ion at higher than 80 mg/L of concentrations decreased the turbidity to around 1 NTU at different alginate doses, ranging from 0.8 mg/L to 4 mg/L. Similar results were obtained for water samples with medium turbidity. Better residual turbidity values (about 1 NTU) after jar test experiment were achieved if calcium ion concentration was particularly higher than 80 mg/L while alginate concentration was changing between 0.4 and 0.8 mg/L. However, alginate was not as successful in turbidity reduction at 10 NTU suspension compared to the other levels. In these experiments, calcium ion concentration was changed between 30 and 80 mg/L and the lowest final turbidity value was obtained as 3.9 NTU after 30 min of settling time at 80 mg/L of Ca^{2+} and 0.04 mg/L of alginate concentration. The residual turbidity could only be reduced down to 1.3 NTU by prolonged settling time of 120 min.

A follow-up study was conducted in order to improve coagulation potential of alginate together with calcium ion for low turbidity waters (Yüksel, 2007). The lowest turbidity value was obtained at 120 mg/L of Ca^{2+} (60-120 mg/L of Ca^{2+}) by 0.1 mg/L of a high molecular weight alginate (0.004-2 mg/L of alginate) as 2.44 NTU. Since this turbidity level cannot be accepted for drinking purposes, turbidity removal efficiency was tried to be enhanced by increasing rapid mixing period during coagulation from 2 min to 10 min. This was divided into two parts such as 5 min or 8 min after calcium addition and 5 min or 2 min after alginate dosing, respectively. The best values were achieved as 2.11 and 0.55 NTU at 0.4 and 0.1 mg/L of alginate applying 120 mg/L of Ca^{2+} by 5 min + 5 min and 8 min + 2 min mixing, respectively. Also, the effect of initial pH of clay suspension was examined at pH values of 4, 7 and 9. It was obtained that turbidity removal efficiency was increased by increasing pH. If calcium ion concentration was higher than 80 mg/L, the residual turbidity could be decreased to 1 NTU at pH 9 for 0.2 mg/L of alginate dose. This was attributed to expanded structure of alginate molecule

at basic pH values which contributes to more bridging opportunities, so better flocculation.

The previous work conducted to remove turbidity using alginate was done by a commercially available algal alginate. This alginate was not well-defined by the manufacturer, therefore the exact characteristics (MM-, GG-block etc.) were not known. Despite all the unknown properties, alginate used showed the potential of calcium alginate to act as a coagulant in drinking water treatment. Therefore, these earlier findings constituted the motivation of this work towards the production of well-defined alginate and to be able to manipulate the characteristics of the product so that better coagulant properties can be obtained. For this purpose, bacterial alginate with various characteristics is produced and it is tested in the removal of turbidity with the aim of producing potable water.

CHAPTER III

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. SELECTION OF AZOTOBACTER STRAIN TO BE USED IN THE STUDY

It is seen that both wild type and some mutant strains of *Azotobacter vinelandii* have been used in the literature for alginate production. The widely used strains are ATCC 9046, DSM 576, DSM 93-541b, NCIB 8789, and NCIB 9068. By using the literature information, the suitable *Azotobacter vinelandii* strain was selected for our study.

One of the objectives of this study is to maximize the amount of alginate produced. In fact, *Azotobacter vinelandii* strains aforementioned have somewhat close alginate productions (0.65-1.6 g alginate / g bacteria) under various culture conditions. However, the monomer distribution, particularly the GG-block content of the alginate, and the mean molecular weight (MW) of the polymer are also among the interest of this study. MW is a parameter that has been studied more extensively by the previous research, but there are not many studies involving the analysis of block contents of alginates with respect to growth conditions. Table 3.1 summarizes data reported in the literature on different *Azotobacter* strains and the properties of alginate

Table 3.1. Summary of literature data on alginate production

Bacteria	Growth Environment	Alginate (g/L)	GG-block (%)	Molecular Weight (kDa)	Acetylation Degree (%)	Reference
ATCC 9046	Shake flask	4.5	Nr	1.98×10^{6}g/mol	nr	Peña et al. (1997)
ATCC 9046	Fermentor	4(1g/g)	nr	350	nr	Trujillo-Roldán et al. (2001)
ATCC 9046	Fermentor	8	Nr	1900	nr	Reyes et al. (2003)
DSM 576	Fermentor	5.2(1.4g/g)	0.09	230	18.5±7.9	Clementi et al. (1999)
DSM 93-541b	Fermentor	0.9 (0.85g/g)	45 as guluronic acid	850	nr	Sabra et al. (2000)
DSM 93-541b	Fermentor	4.9(0.75g/g)	Nr	850	nr	Parente et al. (2000)
IAM 1078 (ATCC 9046)	Bubble column	3.5 (1.2g/g)	85	nr	nr	Asami et al. (2004)
Strain E	Shake flask	1.4(0.65g/g)	50	nr	7	Annison and Couperwhite (1984;1986)
NCIMB	Membrane reactor	1 (1.6g/g)	Nr	324	nr	Saude and Junter (2002)

nr: not reported

produced by them. It is worth to mention that all the values could be affected by cultivation conditions besides, the characteristics of the strains. As can be seen from Table 3.1, maximum molecular weights obtained during fermentation of *Azotobacter vinelandii* ATCC 9046, DSM 576, DSM 93-541b were 1900 kDa, 230 kDa, and 850 kDa, respectively. It is seen that the lowest one was obtained by *Azotobacter vinelandii* DSM 576. Nevertheless, almost no investigations were performed for the GG-block contents of the alginate produced by these strains. For *Azotobacter vinelandii* DSM 576, acetylation degree was found to be quite high around 18 ± 7.9 %. Degree of acetylation is a significant issue on the epimerization of MM-blocks into GG-blocks and the higher the acetylation of the polymer, the less epimerization pattern was reported (Franklin et al., 1994). Also, another study showed that GG-block content was almost zero with this strain. Thus, this strain was thought to be a bad candidate for our study. It was found in another study the maximum guluronic acid content was 45 % (at 10% DOT) for the strain of DSM 93-541b, however, it was not reported whether the blocks were in the form of MG- or GG-blocks. On the other hand, *Azotobacter vinelandii* strain E could produce alginate at maximum 50 % of GG-blocks. Unfortunately, the amount of polymer was low at about 1 g/L and no information could be found on molecular weight of this alginate. An equivalent strain to *Azotobacter vinelandii* ATCC® 9046, IAM 1078, used in a work illustrated a promising result indicating that this strain could produce an alginate having up to 85 % of GG-blocks. Furthermore, the alginate produced had a lower acetylation degree of 1- 3.6%. This property is critical for producing high amount of GG-blocks in the alginate chain. Therefore, *Azotobacter vinelandii* ATCC® 9046 (LGC Promochem, U.K.) was selected as the microorganism in this study for alginate production since the literature information indicated that it could produce alginate with high molecular weight, containing high quantities of GG-blocks which were very much in line with the goals of the study.

3.1.2. CULTIVATION MEDIUM

Three different medium formulations were used in this study. First, ATCC medium 12 was used for reviving of the culture because the medium was highly recommended by the depositor of *Azotobacter vinelandii* ATCC® 9046 for this purpose. Second, Burk's medium was applied for growing of preculture before experiments. Burk's medium has no nitrogen source, thus, it supplies selectivity for *Azotobacter vinelandii* ATCC® 9046 due to its ability to fix nitrogen from air. Finally, for the production of alginate, it was decided to use another medium which was widely used in the literature due to its rich content, namely modified Burk's medium.

3.1.2.1. ATCC Medium 12

ATCC medium 12 (ATCC, 2006) content is listed below in Tables 3.2 and 3.3. Soil extract was prepared by adding referred amounts of African violet soil and Na_2CO_3 into 200 mL distilled water. This mixture was boiled for 1 hour and then filtered through a coarse filter to remove soil particles. A 100 mL of soil extract was added to the medium before sterilization. The solution prepared by the chemicals listed below except $FeSO_4$ and mannitol solution. Media was autoclaved together with soil extract for 30 min at 121 °C. pH was adjusted to 7.6 by adding 1 N NaOH or HCl. For mannitol solution, 20 g mannitol was dissolved in 100 mL of distilled water and filtered for sterilization (0.22 µm, Millipore Millex GS). In the same manner, $FeSO_4$ was added after filter sterilization to avoid precipitation during autoclaving.

Table 3.2. Content of ATCC medium 12

Component	Amount
K_2HPO_4, g/800 mL	1.0
$MgSO_4$. $7H_2O$, g/800 mL	0.2
NaCl, g/800 mL	0.2
$FeSO_4$, mg/L	5.0
Soil extract (see below), mL/L	100
Distilled water, mL/L	800
Mannitol soln, mL/L	100

Table 3.3. Soil extract for ATCC medium 12

Component	Amount
African violet soil, g	77.0
Na_2CO_3, g	0.2
Distilled water, mL	200

3.1.2.2. Burk's Medium and Modified Burk's Medium

Burk's medium was used for the growth of preculture. Table 3.4 illustrates the content of the medium. To prepare this medium, first of all, required amount of K_2HPO_4, KH_2PO_4, $MgSO_4.7H_20$, and NaCl were dissolved in distilled water and then autoclaved for 30 min at 121 °C. Glucose solution was autoclaved individually and added thereafter. $CaSO_4$, $FeSO_4.7H_2O$, and $Na_2MoO_4.2H_2O$ were prepared separately and added after filter sterilization (0.22 µm, Millipore Millex GS) to the medium.

Table 3.4. Content of Burk's medium

Component	Amount
Glucose, g/L	20
K_2HPO_4, g/L	0.66
KH_2PO_4, g/L	0.16
$CaSO_4$, g/L	0.05
$MgSO_4$. $7H_20$, g/L	0.2
NaCl, g/L	0.2
$FeSO_4.7H_2O$, g/L	0.027
$Na_2MoO_4.2H_2O$, g/L	0.0029

A modified Burk's medium (Table 3.5) was used for the production of alginate (Peña et al., 1997). Except for $CaSO_4$, $FeSO_4.7H_2O$, and $Na_2MoO_4.2H_2O$, all of the other components were dissolved in distilled water and then pH was adjusted to 7.2 by adding 1 N NaOH or HCl. After that, the medium was sterilized for 30 min autoclaving at 121 ℃. $CaSO_4$, $FeSO_4.7H_2O$ and $Na_2MoO_4.2H_2O$ were added after filter sterilization through 0.22 µm filter (Millipore Millex GS) and the volume was adjusted to 1 L.

Table 3.5. Content of modified Burk's medium

Component	Amount
Sucrose, g	20
Yeast extract, g	3
K_2HPO_4, g	0.66
KH_2PO_4, g	0.16
MOPS, g	1.42
$CaSO_4$, g	0.05
$MgSO_4$. $7H_20$, g	0.2
NaCl, g	0.2
$FeSO_4.7H_2O$, g	0.027
$Na_2MoO_4.2H_2O$, g	0.0029

MOPS : 3-(N-Morpholino) propanesulfonic acid

3.1.3. EXPERIMENTAL SETUP

In the literature, shake flasks, fermentors, bubble columns and membrane reactors found applications in producing alginate from *Azotobacter vinelandii* (Annison and Couperwhite, 1986; Asami et al., 2004; Clementi et al., 1999; Parente et al., 2000; Peña et al., 2000; Priego-Jimenez et al., 2005; Reyes et al., 2003; Sabra et al., 2000; Saude and Junter, 2002) although the fermentors and shake flasks had wider applications among the others. The works revealed that greater amount of alginate might be obtained by using controlled culture environments such as the one provided by the fermentors. Also, the reproducibility of the data obtained with shake flasks in terms of macromolecular characteristic of the alginate was low (Clementi, 1997). Therefore, it was decided to use a fermentor in this study to provide more controlled conditions during cultivation of *Azotobacter vinelandii*, which was expected to help in producing an alginate at desired quality and quantity.

BIOSTAT® Aplus (Sartorious BBI Systems, Germany) which was a single walled autoclavable fermentor was used throughout the study. Working volume of the reactor was 2 L. A number of parameters can be controlled in this reactor. For example, temperature can be controlled by an electrical heater blanket and a cooling finger and is monitored by a temperature sensor (Pt 100). Also, the vessel is stirrer driven having 6-bladed disk impeller for effective mixing. In addition, pH and DOT are measured by a pH-sensor (Hamilton) and pO_2-sensor (Hamilton, a polarographic electrode), respectively, during the process. All these functions are managed by using micro-DCU software. Figure 3.1 represents the fermentor system.

Figure 3.1. Presentation of BIOSTAT® Aplus

3.1.4. ALGINATE SAMPLES FROM FMC BIOPOLYMER

Five different algal alginate samples were obtained from FMC Biopolymer for control purposes during monomer distribution analysis of produced alginate samples by *Azotobacter vinelandii* ATCC® 9046. The properties of these samples are given in Table 3.6. The monomer distribution of these algal alginates was also measured by ^{1}H-NMR spectroscopy and the results (Appendix A) were compatible with the information given by FMC Biopolymer.

Table 3.6. Properties of FMC Biopolymer alginate samples

Name of Sample	Block Distribution		Viscosity (20 °C, 20 rpm, 1%) mPa.sec
	% M-block	% G-block	
LF240D	65-70	30-35	112
LF120M	55-65	35-45	118
HF120RBS	45-55	45-55	744
LF200DL	35-45	55-65	294
LF200S	25-35	65-75	300

3.2. METHODS

3.2.1. REVIVING AND STORAGE OF THE BACTERIA

Azotobacter vinelandii ATCC® 9046 was purchased from LGC Promochem in a double-vial (Figure 3.2) preparation as a freeze-dried culture which was stored below 5 °C before opening the vial.

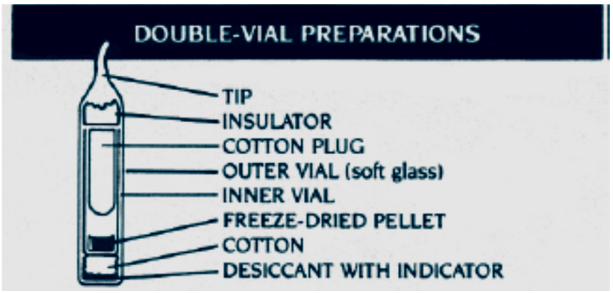

Figure 3.2. Vial preparation for *Azotobacter vinelandii* ATCC® 9046 (ATCC, 2006)

First of all the vial was opened according to the instructions came with the vial and the procedure was followed as explained here. The tip of the outer vial was heated in a flame and a few drops of water were squirt on the hot tip in order to crack glass. Then it was broken with a file and insulation was removed. After that inner vial was removed with forceps and cotton plug was gently raised. To revive the culture, aseptically 1 mL of liquid medium (ATCC medium 12) was added to the freeze-dried material and mixed well. The mixture was transfered to four test tubes of the recommended broth medium (5 mL). The last few drops of this suspension were also transferred to agar slants. Since the culture was freeze-dried, it was activated twice. Following an incubation period of 65 hours at 26 °C, the tubes became turbid due to bacterial growth and this culture was inocculated (1 mL) into other tubes which had 5 mL of fresh ATCC medium 12 and modified Burk's medium incubating for 48 hours at 27 °C. A 1 mL culture broth was inoculated into another tube with 5 mL fresh medium once again for 48 hours at 28 °C same as above.

Various methods are available for storage of bacterial strains. These methods may be classified into two groups, shorth-term and long-term maintenance methods. For long term storage, bacteria at late logaritmic growth phase were frozen in eppendorf tubes having 20 % glycerol. A 1.5 mL of eppendorf tube was filled with 0.5 mL of 40 % glycerol and autoclaved for sterilization. Then, 0.5 mL of liquid medium containing bacteria was inoculated into eppendorf tubes and they were incubated in ice cube trays at around -10 to -20 °C. Also, bacteria which were streaked to petri plates were taken into microbanks for deep freezing at -80 °C. For short-term storage, it was decided to transfer bacteria between liquid medium by taking the stock culture from eppendorf tubes. For that purpose, the bacteria are first activated in shake flasks by inoculating the medium from the stock culture at the ratio of 1 %. These flasks were incubated at 30 °C for the bacteria to reach its logaritmic growth phase. This culture can be stored for short-term such as one month in the refrigerator.

3.2.2. PRECULTURE CONDITIONS AND GROWTH CURVES

Since the culture was frozen, it needed to be activated before the experiments. For this purpose, a preculture was run by using Burk's medium at 30 °C and 200 rpm for 48 hours. Burk's medium does not contain nitrogen source that is helpful to ensure growing only *Azotobacter vinelandii.* Therefore, contamination during inoculation was somewhat avoided. After this period, this culture was used to seed either a flask or the laboratory fermentor.

In order to determine growth characteristics of *Azotobacter vinelandii* ATCC® 9046, growth curves were constructed by using both ATCC medium 12 and modified Burk's medium. Two times activated freeze-dried culture in the related medium was inoculated at the ratio of 0.1% and incubated at 30 °C in six flasks while rotating at 100 rpm. Three of the flasks used ATCC medium 12, and the others contained Burk's medium. Samples were taken every two to four hours and optical density (OD) was read at 600 nm. When OD value was above 0.6, the culture was diluted by the liquid medium to get the OD_{600} < 0.6. Growth curves were conducted for two times.

3.2.3. PRODUCTION OF ALGINATE IN A SHAKE FLASK

The culture taken from eppendorf tubes were activated by runing a seed culture in a flask at 30 °C while rotating at 200 rpm in Burk's medium for 48 hours. Then, this preculture was used to inoculate a shake flask having modified Burk's medium at a ratio of 3 % (v/v). Alginate production was performed during 72 hours at 30 °C and 200 rpm for three times with duplicate sampling. Samples were taken for bacteria and alginate concentration at predetermined time intervals. Culture broth viscosity and monomer distribution of alginate were also analyzed.

3.2.4. PRODUCTION OF ALGINATE IN A FERMENTOR

Similar to the case of shake flask experiments, a preculture was grown at 30 °C and 200 rpm for 48 hours in Burk's medium. After that, fermentor was filled with 2 L modified Burk's medium and autoclaved for 40 minutes at 121 °C. Sterilized medium was inoculated at a ratio of 3 % (v/v). Before inoculation, parameters such as temperature, pH, mixing rate, and DOT were set at the predetermined values. Temperature was set at 30 °C and was measured by a temperature sensor (Pt 100). Heating of the vessel is done by an electrical heater blanket while cooling of the medium was accomplished by a cooling finger. pH was adjusted to 7.2 by the addition of 1 M NaOH or HCl and monitored by a pH-sensor (Hamilton). For efficient agitation of the medium, the reactor was equipped with a stirrer with 6-bladed disk impeller and mixing rate was controlled by a driving shaft. DOT value in the medium was followed by a pO_2-sensor (Hamilton) with a polarographic electrode. Air or O_2/N_2 mixture was fed into fermentor depending on the oxygen requirement of bacteria.

The parameters affecting alginate production were selected after a literature survey as DO tension, mixing intensity, substrate concentration, and calcium ion concentration. If it was not stated specifically, the experiments were done at 5% DOT and 30 °C while mixing at 400 rpm. Moreover, pH was set to 7.2, sucrose and calcium ion concentration were 20 g/L and 50 mg/L, respectively. All of these parameters were maintained constant through 72 hours of experiment. Samples (50 mL) were taken at 6, 12, 24, 32, 48, 56 and 72 hours to determine bacterial dry mass, sucrose concentration and alginate quantity. Also, alginate quality was followed by broth viscosity measurements and block distribution analysis.

First, the reproducibility of the results from fermentor was checked. For this, three experiments were done at 5 % DOT, pH 7.2, 400 rpm, 30 °C, 20 g/L sucrose, 50 mg/L calcium concetration. Once the reproducibility was verified, the following experiments were conducted once with doublicate sampling.

Thus, to determine the effect of various parameters, a series of experiments were started. As mentioned above, the tested parameters were DOT, stirring speed, sucrose concentration and calcium concentration. The temperature and pH were kept at 30 °C and 7.2, respectively during all these studies. The first parameter to study was chosen as the DOT value in the reactors. It was varied as 1, 3, 5 and 10 % ± 1 of air saturation while the sucrose concentration, calcium concentration and stirring speeds were set constant at 20 g/L, 50 mg/L, and 400 rpm, respectively all throughout this part. Once this part is completed by taking enough samples and finishing the necessary analysis, the studies on the next parameter to be investigated was started. The second parameter to work on was chosen as rotation speed. Three different speeds of 200, 400 and 700 rpm values were studied in the fermentor while in each case the DOT, sucrose concentration and calcium concentration were set as constant at 5 %, 20 g/L and 50 mg/L, respectively. Again, after the sample collection and analysis period, the third parameter, the sucrose concentration was studied. Sucrose concentration varied as 10, 20 and 40 g/L in the fermentor while the other parameters were kept constant at the values mentioned above. Finally, the last parameter, calcium concentration was studied at values of 25, 50 and 100 mg/L while DOT, rotational speed and sucrose concentration were kept constant at 5 %, 400 rpm and 20 g/L. During the 72 hours fermentation period, necessary samples were collected and analyzed for parameters of biomass concentration, sucrose concentration, alginate concentration, viscosity of broth and monomer distribution of alginate using the analytical measurements described in part 3.3.

3.2.5. POST - EPIMERIZATION OF ALGINATE SAMPLES

One of the main objectives of this study is the production of alginate by *Azotobacter vinelandii* ATCC® 9046 and the effect of various conditions on the properties of the polymer. However, the ultimate aim is to investigate bacterial alginate in water and wastewater treatment applications such as

heavy metal uptake and turbidity removal. It was proposed that the fraction of GG-blocks in alginate chain had possibly an important role in these applications due to their ability to form egg-box in the presence of cations. Therefore, to elucidate the effect of monomer distribution on metal uptake and turbidity removal, alginates having different amounts of GG-blocks are required. The selected alginate samples obtained from fermentors were directly put into tests of metal removal or turbidity removal without further epimerization since they had different GG-block contents already. However, the alginate samples produced in shake flasks had lower GG-block contents. Therefore, alginate produced by *Azotobacter vinelandii* ATCC® 9046 in shake flasks were subjected to post-epimerization using AlgE1 at two different levels. The products were identified as highly- and moderately-epimerized alginate. All the post-epimerization works were conducted at Norwegian University of Science and Technology in Norway.

3.2.5.1. Production of Epimerase

A plasmid- pHH1- capable of producing AlgE1 was grown overnight at 37 °C and 225 rpm for preculture in 3 x Luria Broth (LB) (Table 3.7.) containing 200 mg/L ampicillin. LB is the standard nutrient media for propagation of *Escherichia coli* for purposes of strain maintenance, cloning, plasmid propagation, and protein expression. On the other hand, ampicillin is often used as a selective agent in molecular biology to select for and to confirm the uptake of genes (e.g., of plasmids) by bacteria (e.g., *E. coli*). Only the bacteria that successfully take up the desired genes become ampicillin resistant, and contain the other desired gene as well. The preculture was used to inoculate a 2 L baffled flask containing 400 mL of 3xLB with ampicillin. The bacteria was grown for 4 hours at 37 °C and after that 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and allowed for further 5 hours growth. The IPTG is used to induce the expression of cloned genes which are under control of the lac operon that is responsible for the transport and metabolism of lactose in *E. coli*. At the end, the cells

were harvested by centrifugation at 7000 rpm for 10 min. Supernatant was discarded and the cells were suspended using 40 mL (4×10 mL) Buffer A (cold + 4 ℃) (Table 3.8) which contains 50 mM MOPS at pH 6.9 and 5 mM $CaCl_2$. This suspension was sonicated for 10 min as 10 mL batches in order to disrupt the cell membranes and proteins were brought into the solution. Finally, the suspension was centrifuged for 30 min at 10000 rpm. Supernatant was filtered and kept refrigerated.

Table 3.7. Content of 3 x Luria Broth

Components	**Concentration (g/L)**
Tryptone	30
Yeast extract	15
NaCl	5

3.2.5.2. Purification of AlgE1

Purification of the enzyme was performed by using ion-exchange chromatography with a column (HiTrap Q HP, 5 mL) operated by a FPLC (Fast Protein Liquid Chromatography) system. HiTrap Q HP is an anion exchange column made of polypropylene. Two buffers were used namely Buffer A as a start buffer (for binding of charged proteins) and Buffer B as an elution buffer (Table 3.8). Both buffers were filtered through a 0.22 µm filter immediately before use. Also, the sample was adjusted to the composition of the start buffer (see in production part; pellets were resuspended in Buffer A). After this adjustment, again the sample should be filtered through a 0.22 µm filter before it was applied to the column.

Table 3.8. Composition of Buffer A and Buffer B

Component	Buffer A	Buffer B
MOPS, mM	50	50
$CaCl_2$, mM	5	5
NaCl, mM	-	1
pH	6.9	6.9

For purification, first, the column was washed out with 5 column volumes of Buffer A at 5 mL/min. Then, it was washed out with 5 column volumes of elution buffer, Buffer B. Finally, the column was equilibrated with 5 - 10 column volumes of Buffer A. The sample was applied at 5 mL/min and it should be washed with at least 5 column volumes of Buffer A or until no materials appear in the effluent. Elution was performed with 5 - 10 column volumes of Buffer B. Sample fractions of 10 mL were automatically collected through the elution. After that, the column was regenerated by washing with 5 column volumes of Buffer B and followed by 5 -10 column volumes of Buffer A in order to prepare the column for a new sample.

3.2.5.3. Measurement of the Enzyme Activity

Three different fractions (A5, A8, and A9) obtained from ion-exchange chromatography were measured to determine which fraction contains AlgE1. This was achieved by conducting a radioactive assay. For this purpose, first 495 µL of Buffer A and 5 µL of probable enzyme-containing fractions were mixed in an eppendorf tube. Also a blank having just Buffer A was prepared. These tubes and an alginate solution (2 mg/mL) already contain 3H, tritium which is radioactive, were prewarmed in a water bath at 37 °C for half an hour. Then, alginate was added into this mixture and all were allowed for incubation for 1 hour at 37 °C. All samples were applied in duplicates. At the

end of incubation, 15 µL of 5 M NaCl and 800 µL of isopropanol were added, mixed and frozen more than 1 hour at – 20 ℃. After waiting for the precipitation of alginate, alginate was separated by centrifugation at 15300 rpm for 30 min. One mL of samples was taken from the eppendorf tubes by caring not to touch to the alginate pellets and put into the counting vials. Then, 5 mL ready safe liquid for scintillation counting was added and all were mixed. Finally, the outside of the vials was washed with 70% of ethanol and measurements were performed by liquid Scintillation counting.

3.2.5.4. Production and Epimerization of Alginate

The culture, *Azotobacter vinelandii* ATCC ®9046, taken from cryo tubes (stock culture at - 80 ℃) was activated by running a seed culture in a flask at 30 ℃ while rotating at 225 rpm in modified Burk's medium for 48 hours. Then, this preculture was used to inoculate a shake flask having modified Burk's medium at a ratio of 1 % (v/v). Alginate production was performed during 72 hours at 30 ˚C and 225 rpm. The alginate extracted from culture broth was used in epimerization experiments. Since the monomer distribution of alginate was determined by ^{1}H-NMR spectroscopy, the experiment was started with 15 mg of alginate. Firstly, 0.25 % of alginate solution was prepared and the conditions were adjusted according to previously determined optimal conditions for AlgE1 to obtain high GG-block alginate (Holtan et al., 2006). That is MOPS (50mM), $CaCl_2$ (0.8 mM), NaCl (20 mM) were added into alginate solution and pH was set to 6.9. Finally, the enzyme was added according to the results obtained from measurement of the enzyme activity. All these constituents were mixed well and the mixture was incubated in a water bath at 37 ℃ during 48 hours. After the incubation period, the solution was dialyzed once against 10 mM of EDTA (pH 8) at 4 ℃ for 24 hours in a dialysis bag (Spectra/Pore® Membrane MWCO: 6–8000) and then against distilled water for 48 hours.

For the epimerization of high amounts of alginate sample, 3 g of alginate was synthesized by shake flasks using the same procedure as above. One gram of alginate was dissolved in 1 L distilled water overnight at 4 ℃ and MOPS (50mM), $CaCl_2$ (0.8 mM), NaCl (20 mM) were added into alginate solution at pH 6.9. The same procedure described above was applied for the epimerization of alginates. However, to epimerize alginate at moderate level, the incubation time was reduced to 8 hours.

3.2.6. DETERMINATION OF HEAVY METAL UPTAKE CAPACITIES OF ALGINATE SAMPLES

3.2.6.1. Relative Uptake of an Alginate Sample for Different Metals

At first place, maximum uptake capacity of an alginate sample for different metals was examined. For this purpose, lead, cadmium, and copper due to their toxic nature and calcium because of being the most suitable ion for egg box formation were selected. The alginate sample used in this part was obtained from a single shake flask experiment conducted during 72 hours at 225 rpm and 30 ˚C (24 % of GG-blocks, 12 % of MG-blocks and 64 % of MM-blocks). It was extracted and purified as explained in section 3.3 before the metal uptake study. The procedure followed (Davis et al., 2004) was first developed by Fourest and Volesky (1996). Accordingly, 2 L of 4.5 mM metal solutions were separately prepared by dissolving required amount of $Pb(NO_3)_2$, $CdCl_2.H_20$, $CuSO_4.5H_20$, and $CaSO_4.2H_20$ in deionized (DI) water and then pH of the solutions were adjusted to 4 by adding HCl or NaOH (1 N, 0.1 N). Changes in pH values were monitored and small variations observed during the experiment were adjusted to 4 instantaneously. Alginate was recovered at 72 hours in the form of sodium alginate. Since sodium alginate was soluble in water, it was confined in dialysis bags (Spectra/Pore® Membrane MWCO: 6–8000). A 50 mg of alginate was placed into dialysis bag and then 2 mL of DI was added to obtain 2.5 % of alginate for each bag. For each metal, 3 sample bags and 2 empty bags (for control) were placed

into metal solution and gently stirred for equilibration for 4 days at room temperature. After that the metal solution was replaced with a fresh one and alginate samples were allowed to equilibrate with the metal for an additional 6 days. All these bags were then transferred into separate flasks for rinsing with DI water for 24 hours. Thereafter, 100 mL of 0.1 N HNO_3 was added into each alginate sample for 24 hours and the system was mixed at 150 rpm to recover the metal sorbed by the biopolymer. This procedure was repeated twice to be sure about desorption of all metals into acid solution. The concentration of metal released into acid solution was measured by atomic adsorption spectroscopy (AAS) (Perkin Elmer AAnalyst 400). By using these data, maximum metal uptake capacity of alginate sample for each metal was calculated.

3.2.6.2. Development of Experimental Procedure for Copper Removal by Alginate

According to the results obtained from the experiments, the alginate sample showed the highest uptake capacity for copper ion. Therefore, copper was selected for further experiments. First of all, it was decided to develop a new procedure for copper uptake studies examining the factors like equilibrium time, alginate and metal concentration. To determine equilibrium time for copper uptake, 3 L of 4.5 mM copper solution was prepared by dissolving $CuSO_4.5H_2O$ in DI water at pH 4. The same alginate sample that was used during multi-metal studies was used at 1 % concentration which was confined in a dialysis bag. The metal solution was replenished at the end of 4 days. 10 sample bags and 10 control bags were placed into metal solution. After certain time intervals, (1, 3, 5, 7 and 10 days) 4 bags (2 sample and 2 control bags) were removed from the solution for washing in DI for 24 hours. After that each bag was transferred into 0.1 N HNO_3 solutions separately and shaken for 24 hours at 150 rpm for desorption of copper. This desorption step was repeated once again. Finally, the amount of copper released into acid solutions was measured by AAS.

Time required to reach equilibrium concentration of copper ion on an alginate sample was decided as 3 days. This time was applied for the following experiments conducted to determine the effect of copper concentration by changing metal solution with a fresh batch every day. To find appropriate alginate and metal concentration, 3 different alginate concentrations such as 0.5, 1 and 2.5 %, and 2 different metal concentrations namely 100 and 250 mg/L of Cu^{2+} were examined by using the same procedure applied above with 3 days of metal uptake duration. For further experiments performed to determine maximum copper uptake of bacterial alginate obtained from different experimental trials, the final procedure was developed as follows: 3 L of 100 mg/L copper solution was prepared by dissolving $CuSO_4.5H_2O$ in DI water at pH 4. pH was controlled and necessary adjustments were done by the addition of NaOH or HCl (1 N, 0.1 N) during the course of experiment. Alginate samples were used at 1 % (20 mg in 2 mL DI) concentration confined in dialysis bags. Three sample bags and two control bags were placed into metal solution by mixing gently for 3 days. Metal solution was changed with a fresh one each day. After that the samples were removed from the solution for rinsing in DI water during 24 hours. Each bag was transferred into 100 mL of HNO_3 (0.1 N) solution for 24 hours at 150 rpm. This desorption procedure was repeated once again and the amount of copper released into acid solutions was measured by AAS.

By using the procedure described above, maximum copper uptake capacities of alginate samples were determined. First set of samples were bacterial alginates obtained from post-epimerization experiments such as non-, moderately- and highly-epimerized alginate samples. Afterwards, bacterial alginates produced in laboratory fermentor at 72 hours by changing conditions like DOT, mixing rates, initial sucrose and calcium ion concentrations were investigated for maximum copper uptake capacities. Finally, three algal alginate samples, which are LF 240D, HF120RBS, and LF 200S, obtained from FMC Biopolymer company were examined in heavy metal uptake experiments.

3.2.7. DETERMINATION OF TURBIDITY CAPACITIES OF ALGINATE SAMPLES

The turbidity level to be studied was selected as 10 NTU since 10 NTU is a value that reflects the turbidity of the typical raw water sources prior to treatment. Besides it is typically more difficult to remove low turbidity levels compared to the higher turbidity levels (Çoruh, 2005). Turbid water was prepared by using kaolinite (Fluka, CAT# 03584). The solution was mixed for 24 hours at room temperature and its turbidity was adjusted to 10 NTU. All experiments were performed with VELP Scientifica JLT6 Jar Test apparatus having six mixers each with two flat blades. The beakers were filled with 500 mL of turbid water samples. Calcium ions and alginate samples were added from stock solutions. A stock solution of 15,000 mg/L for calcium ion and that of 1000 mg/L for alginate solution were prepared. First predetermined amount of Ca^{2+} (15-120 mg/L) was added into the turbid water sample and mixed for 5 min at 120 rpm. Then alginate (0.015 – 20 mg/L) was added and mixed for further 5 min at 120 rpm. After this 10 min rapid mixing period, the aggregates were led to grow for 12 min slow mixing at 40 rpm. Finally, they were allowed to settle for 30 min and final turbidity value was measured by Hach 2100N turbidimeter. The applied ranges for calcium and alginate concentration were determined by referring to the results of a previous study (Çoruh, 2005). On the other hand, the procedure applied for the investigation of turbidity removal was developed by Yüksel (2007).

In these experiments, four different bacterial alginate samples extracted from fermentor experiments and post-epimerized alginates were investigated to elucidate the effect of block structure in alginate on the removal of turbidity. Also, some algal alginate samples were used in turbidity removal experiments to compare their performances with bacterial alginate samples.

3.3. ANALYTICAL MEASUREMENTS

3.3.1. ALGINATE AND BACTERIAL MASS DETERMINATION

Regular samples taken from either the shake flask or the fermentor at predetermined time intervals were analyzed for bacterial mass and alginate concentration as explained below. In this procedure, first the extraction and precipitation of alginate is conducted, then from the remaining sample bacterial mass was determined following the separation of alginate. A 25 mL of culture broth was mixed with 2.5 mL of Na_4EDTA (0.1 M) for 10 minutes. NaCl was added at a ratio of 1.5 % (w/v) into this solution. If the sample was too viscous, it was diluted by 5 times. Then, it was centrifuged at 4000 rpm for 45 min. The centrate was filtered through 0.45 µm membrane filter and concentrated NaOH was added (0.1 M). Solution was shaken at room temperature for 1 hour for deacetylation. After that alginate was precipitated as alginic acid with the addition of HCl by decreasing pH to around 2 and incubated at 4 °C for 30 min. Alginic acid was recovered by centrifugation at 4000 rpm for 10 min. Then, the precipitate was washed with 0.1 N cold HCl solutions and centrifuged again. After that it was dissolved by neutralization in a small amount of distilled water and 0.2 % of NaCl was added before ethanol precipitation (1:1). Precipitated sodium alginate was purified by washing three times with 70 % of ethanol, then pure ethanol and ether. Finally, it was dried under the fume hood for 24 hours and weighed. The bacterial pellet from the first centrifugation was suspended in distilled water and filtered through 0.45 µm membrane filter. Then, it was dried at 105 °C for 1 hour and cooled in a desiccator and weighed for bacterial mass measurement.

3.3.2. SUCROSE DETERMINATION

Dinitrosalicylic acid (DNS) reagent the composition of which is presented at Table 3.9 was used for the determination of sucrose concentration in culture broth. The centrate from the first centrifugation mentioned above was used for sucrose analysis after filtration through 0.45 µm membrane filter. In the test, first, sucrose was degraded by using 1 drop of concentrated HCl solution for each mL of the sucrose solution. Then, the solution was allowed for hydrolysis at 90 ºC for 5 minutes. After that, 3 drops of the 5 N NaOH solution were added to neutralize the acid, because the DNS method must be applied in an alkaline condition to develop the red brown color which represents the presence of reducing sugars (Wang, 2007). In a test tube, 3 mL of sample, 3 mL of DNS reagent were added and boiled for 15 min. Before cooling, 1 mL of potassium sodium tartrate solution (40 %) was added to stabilize the color and absorbance measurements were done at 575 nm (Miller, 1959). A calibration curve (Appendix B) was constructed to correlate the absorbance to the sucrose concentration by making a series of dilutions from 1000 mg/L of stock solution as 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5 g/L.

Table 3.9. Content of DNS reagent used for sucrose determination

Component	**Amount**
Dinitrosalicylic acid, g/100 mL	1
Phenol, g/100 mL	0.2
Sodium hydroxide, g/100 mL	1
Sodium sulfite, g/100 mL	0.05

3.3.3. VISCOSITY MEASUREMENT

Culture broth viscosity was measured to obtain indirect information about the molecular weight of alginate produced because it appeared to be difficult to measure molecular weight of all alginate samples due to requirement of somewhat high polymer mass (300 mg) for the analysis. Furthermore, molecular weights of three alginate samples from 1, 3 and 5 % DOT experiments and two algal alginates, LF240D and HF120RBS, were tried to be determined by using dynamic light scattering (Central Lab, METU). However, due to high quantity of sample requirement and somewhat high error % reported with the results (Table 3.10). It was decided not to continue the analysis of molecular weight. Rather, it was decided to continue with culture broth viscosity measurements to have an idea about molecular weight of the alginates. Viscosity of culture broth was measured by using a rotational viscometer Brookfield LVDVII+ with ultra-low viscosity adapter. Analyses were conducted at 60 rpm corresponding to 73.4 s^{-1} shear rate and 25 ºC. The broth samples were analyzed for two times.

Table 3.10. Molecular weight results from dynamic light scattering measurements of some selected samples

Sample	MW (c), g/mol	error %	MW(q2), g/mol	error %	V, cP	A, g/L
1% DOT	2.27E+06	12.1	1.82E+06	11.2	2.16	2.94
3% DOT	1.46E+06	3.98	5.60E+06	5.09	4.64	3.36
5% DOT	1.29E+06	6.43	2.41E+06	10.1	8.87	4.51
LF240D	1.50E+06	5.91	2.18E+06	7.65	3.81	0.5
HF120RBS	3.35E+06	6.88	1.19E+06	25.7	7.22	0.5

3.3.4. MONOMER DISTRIBUTION ANALYSIS

3.3.4.1. Determination of Monomer Distribution by Acid Extraction and Precipitation

The conventional wet procedure of monomer distribution analysis is presented schematically in Figure 3.3 and goes as follows (Haug et al., 1974). Solid alginate samples (0.1 g) were added into 50 mL of 0.3 M HCl and heated at 100 ºC for 2 hours. After cooling, the mixture was centrifuged for 30 min at 1000 rpm and the amount of carbohydrates in the centrate was measured by the phenol-sulfuric acid method (Dubois et al., 1956) as MG-block. The pellet from centrifugation was resuspended into 50 mL of distilled water and neutralized to pH 7 by adding NaOH. Then the suspension was mixed with a small amount of NaCl (final concentration, 0.1 M) and a small amount of 25 mM HCl to get the pH down to 2.85. The resultant precipitate was separated by centrifugation. In a similar manner, the carbohydrates in the centrate were determined by the phenol-sulfuric acid method as MM-block. Again, the pellet was dissolved into distilled water and the polysaccharides in the solution were determined by the phenol-sulfuric acid method as the GG-block.

In order to check the validity of the procedure (Figure 3.3) for monomer distribution analysis, alginate samples having well-defined properties were obtained from FMC Biopolymer and used to determine their monomer distribution. FMC Biopolymer provided, only the M- and G-block contents of the samples. Using the information provided, M/G ratio ranges were calculated. The calculation of M/G ratio ranges was done by dividing the % M-block value to % G block value so that lowest and highest M/G ratio is obtained and a range is determined. These ranges for each alginate sample used are given in Table 3.11.

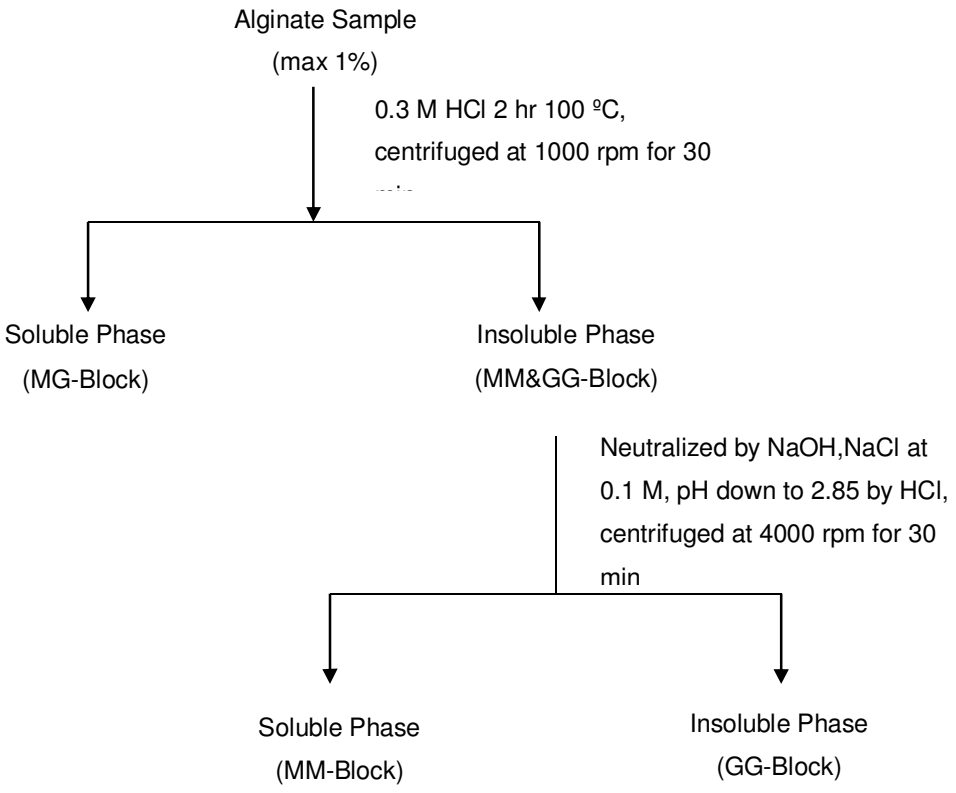

Figure 3.3. Schematical representation of monomer distribution analysis

Table 3.11. M/G ratio of biopolymer samples given by FMC Biopolymer

Alginate	**% M**	**% G**	**Calculated M/G ratio range**
LF240D	65-70	30-35	1.86-2.33
LF120M	55-65	35-45	1.22-1.86
HF120	45-55	45-55	0.82-1.22
LF200DL	35-45	55-65	0.54-0.82
LF200S	25-35	65-75	0.33-0.54

By following the wet analytical procedure above, MG-, MM- and GG-blocks of algal alginate samples were determined. Then, for our analytical results, M/G ratio of each sample was calculated using Eq 3.1 (Grasdelen et al., 1979).

$$M/G = \frac{(\%MM + (\%MG/2))}{(\%GG + (\%MG/2))} \quad (3.1)$$

Equation 3.1 assumes that half of the MG-block is monomer M and half of it is monomer G, and calculates the M/G ratio. Monomer distribution analysis of FMC alginate samples were performed 5 times and averages were calculated and reported.

3.3.4.2. Determination of Monomer Distribution by ^{1}H-NMR Spectroscopy

Alginate samples were also subjected to ^{1}H-NMR analysis in order to determine monomer distribution of the samples. Prior to ^{1}H-NMR analysis, the samples were degraded by partial acid hydrolysis to obtain well resolved signals. For this purpose, 0.1 g of solid alginate samples were added into 100 mL of distilled water and pH of the solution was adjusted to 5.6 by HCl (1 M, 0.1 M) and heated at 100 ºC for 1 hour. Then, pH was decreased further to 3.8 by HCl (1 M, 0.1 M) and the alginate sample was heated at 100 ºC for 30 min. After cooling, the mixture was neutralized to pH 7-8 by adding NaOH (1 M, 0.1 M) and freeze-dried. 10 mg of a sample was dissolved in 0.5 mL of D_2O and 10 µL of 0.3 M EDTA was added into the NMR tube before analysis to prevent traces of divalent cations to interact with alginate (ASTM, 2003). All NMR experiments were carried out on a Bruker spectrometer operating at 400 MHz. ASTM F 2259-03 method was used as a guide and the ^{1}H-NMR signals of alginate monomers were detected accordingly. A typical spectrum of alginate is presented in Figure 3.4 and peak determinations are summarized in Table 3.12.

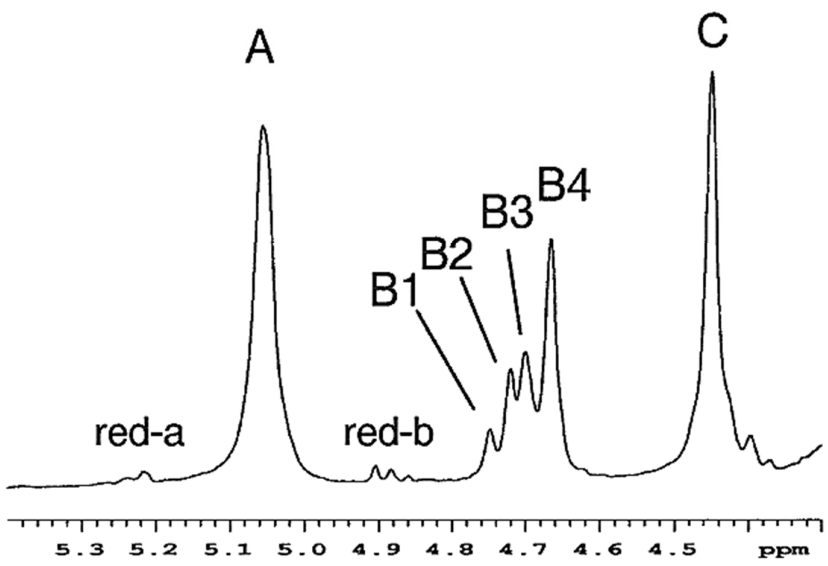

Figure 3.4. The region of the ^{1}H-NMR spectrum of alginate used for quantitative analysis (ASTM, 2003)

The ppm values in Figure 3.4 show chemical shifts that are determined relative to a reference compound used in the analysis. They may alternatively be presented in hertz, but the corresponding hertz values depend on machine magnetic field. For example, the hertz value for a compound would be different between the machines working at 100 MHz and 300 MHz. Also, these chemical shifts are always very small compared with the total field strength such as 100 or 300 million Hz. Therefore, for simple use, the factor 10^6 is introduced and chemical shifts are given in parts per million (ppm) (Balci, 2005).

Table 3.12. Peak determination for 1H-NMR analysis of alginate samples

Signal	Peak
red-a	alpha reducing ends
Signal A	G (proton 1)
red-b	beta reducing ends
Signal B1	GGM (proton 5)
Signal B2	MGM (proton 5)
Signal B3	MG (proton 1)
Signal B4	MM (proton 1)
Signal C	GG (proton 5)

Relative areas of proton signals are estimated by numeric integration of the relevant ^{1}H-NMR signals and the data are calculated from a set of equations (ASTM, 2003). The relations are summarized as follows:

$$G = 0.5(A + C + 0.5(B1 + B2 + B3)) \quad (3.2)$$

$$M = B4 + 0.5(B1 + B2 + B3)) \quad (3.3)$$

$$GG = 0.5(A + C - 0.5(B1 + B2 + B3)) \quad (3.4)$$

$$MG = GM = 0.5(B1 + B2 + B3) \quad (3.5)$$

$$MM = B4 \quad (3.6)$$

$$F_G = G/(M+G) \quad (3.7)$$

$$F_M = M/(M+G) \quad (3.8)$$

$$F_{GG} = GG/(M+G) \quad (3.9)$$

$$F_{MM} = MM/(M+G) \quad (3.10)$$

$$F_{GM} = F_{MG} = MG/(M+G) \quad (3.11)$$

Where, M: relative area of mannuronic acid, G: relative area of guluronic acid, MM: relative area of mannuronic acid blocks, MG; relative area of

alternating blocks, GG: relative area of guluronic acid blocks, F_G: fraction of guluronic acid, F_M: fraction of mannuronic acid, F_{MM}: fraction of mannuronic acid blocks, F_{GG}: fraction of guluronic acid blocks, F_{MG}: fraction of alternating blocks

In the cases that the peaks B1-B4 were not separated well, another approach was applied for the calculation of monomer distribution of alginate samples (Grasdelen et al., 1979). Similar to ASTM method, monomer distribution is determined by using relative areas under the peaks of A (G proton 1), B (M proton 1 and GM proton 5), and C (GG proton 5) (Figure 3.5).

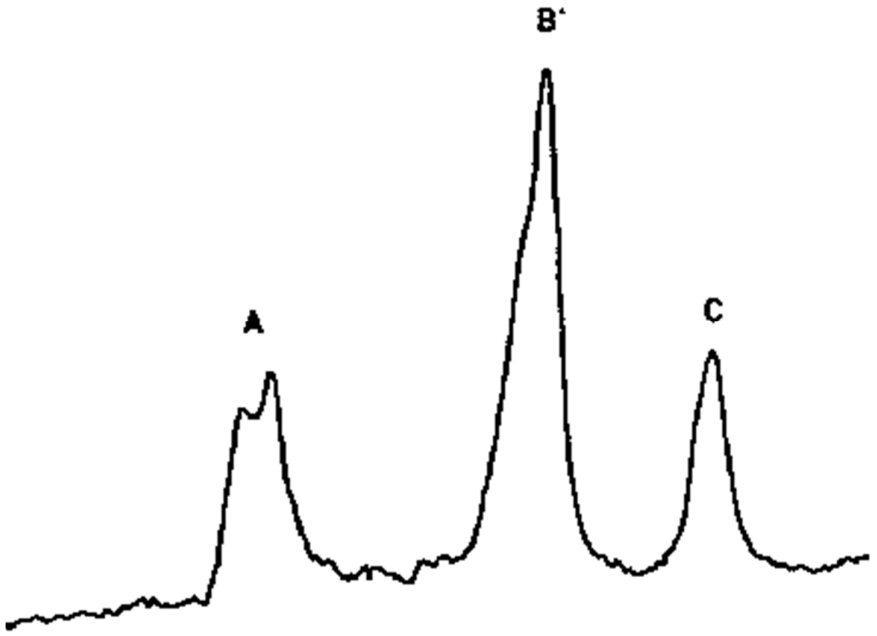

Figure 3.5. The region of the ^{1}H-NMR spectrum of alginate used for quantitative analysis (Grasdelen et al., 1979)

The equations related to the calculation are as follows:

$$F_G = A/(B+C) \quad (3.12)$$

$$F_{GG} = C/(B+C) \quad (3.13)$$

$$F_M + F_G = 1 \quad (3.14)$$

$$F_{GG} + F_{GM} = F_G \quad (3.15)$$

$$F_{MM} + F_{MG} = F_M \quad (3.16)$$

It is worth to remember that both methods assume that $F_{\underline{G}M} = F_{\underline{M}G}$ for long chains, i.e. partially degraded alginate samples as obtained after procedure of ASTM degradation.

Chapter IV

RESULTS AND DISCUSSION

4.1. PRELIMINARY EXPERIMENTS

4.1.1. GROWTH CURVES

Growth curves were constructed for *Azotobacter vinelandii* ATCC® 9046 cultivated in ATCC medium 12 and modified Burk's medium at 100 rpm, and 30 °C. The results are shown in Figure 4.1 and 4.2. As it is illustrated in Figure 4.1, about 12 hours lag phase was observed during the growth of bacteria in ATCC medium 12. This can be attributed to the fact that the cells used for inoculation were taken from two times activated freeze-dried culture which may not be recovered from the effect of freezing completely and/or the number of cells inoculated might be still less that the bacteria need more time to increase its number or mass. The bacteria reached its stationary phase at around 60 hours and OD_{600} of the medium remained constant for a long time which is expected when there is only the cell death rather than cell lysis during cultivation. No lag phase was observed in the case of bacterial growth in modified Burk's medium (Figure 4.2) because the inoculation was done from a culture which was probably in it's exponentially growth phase. After 30 hours, the bacteria attained the stationary phase. However, OD_{600} values decrease after some time that the reason seemed to be the cell lysis.

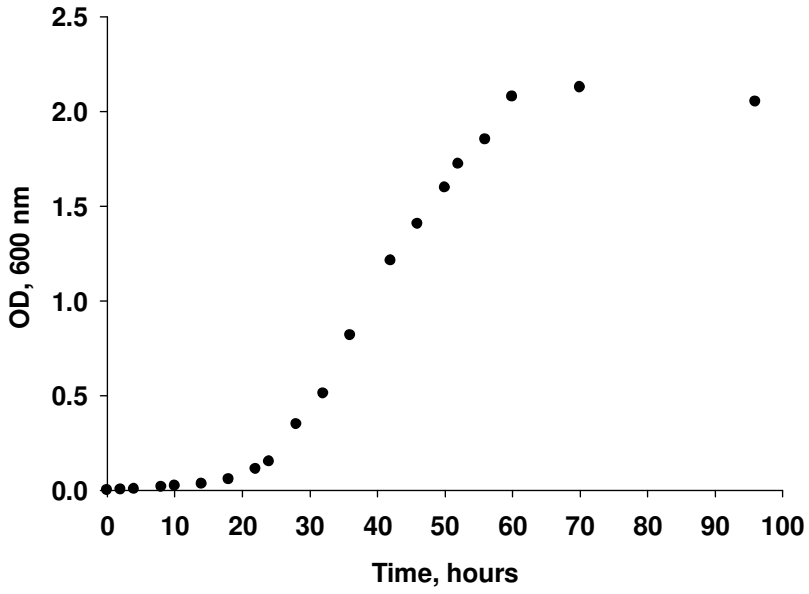

Figure 4.1. Growth curve for *Azotobacter vinelandii* ATCC® 9046 in ATCC medium 12

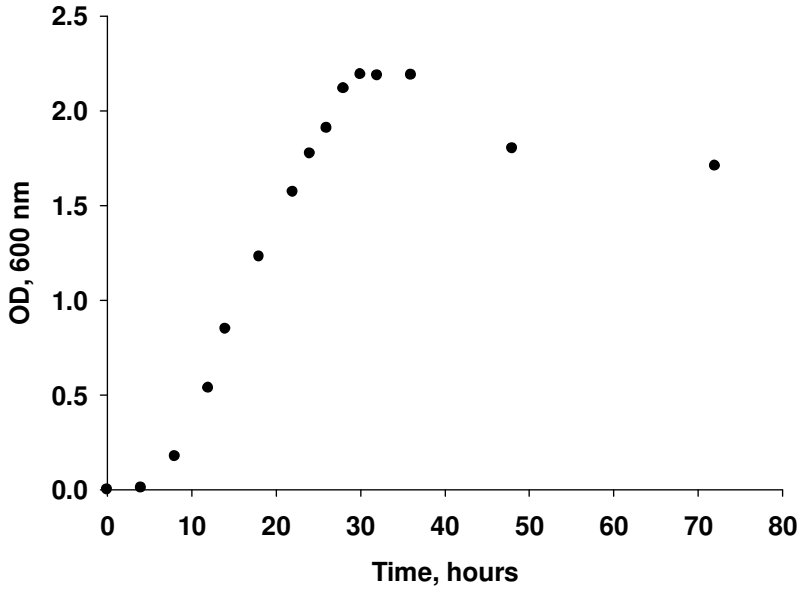

Figure 4.2. Growth curve for *Azotobacter vinelandii* ATCC® 9046 in modified Burk's medium

Table 4.1 represents the growth kinetic parameters of *Azotobacter vinelandii* ATCC® 9046. Specific growth rate (μ) of the bacteria was 0.22 hr^{-1} when modified Burk's medium was used for cultivation, which is 2 times higher than the case of ATCC medium 12. That is because modified Burk's medium is a richer medium and also sucrose utilization rate seems to be higher than mannitol as a carbon source. Specific growth rate was varied in a range between 0.07 and 0.23 hr^{-1} in the literature depending on culture conditions (Peña et al., 1997; Peña et al., 2000; Trujillo-Roldán et al., 2001; Asami et al., 2004; Trujillo-Roldán et al., 2004). With our data, doubling time of *Azotobacter vinelandii* ATCC® 9046 was 3.12 hours for modified Burk's medium while it was 5.97 hours for ATCC medium 12. The first value is similar to the values reported in another study in which doubling times are calculated as 3.22 and 4.18 for *Azotobacter vinelandii* ATCC® 9046 in Burk's medium (Asami et al., 2004). With these findings, modified Burk's medium was decided to be used for further studies.

Table 4.1. Growth parameters of *Azotobacter vinelandii* ATCC® 9046

Medium	**Specific growth rate (μ), (hr^{-1})**	**Doubling time, (hr)**
ATCC medium 12	0.11 ± 0.046	5.97
Modified Burk's medium	0.22 ± 0.032	3.12

4.1.2. ALGINATE PRODUCTION IN A SHAKE FLASK

Shake flask experiments were performed to compare alginate production by *Azotobacter vinelandii* ATCC® 9046 with the production in a fermentor using modified Burk's medium at 200 rpm and 30 °C during 72 hours. These experiments were performed three times and results from one representative batch are shown in Figure 4.3.

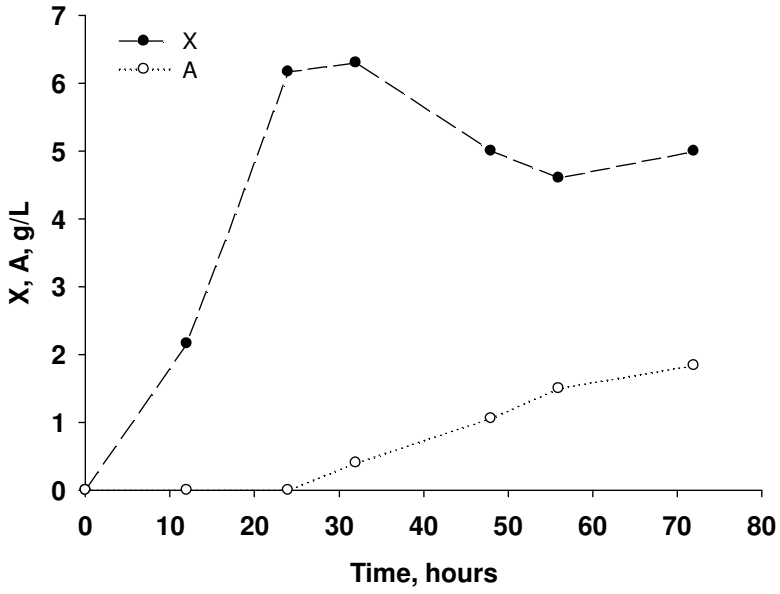

Figure 4.3. Alginate production by *Azotobacter vinelandii* ATCC® 9046 in shake flask

As it is shown in Figure 4.3, the highest bacterial mass was achieved as 6.3 g/L at 32 hours with a specific growth rate of 0.12 hr^{-1}. On the other hand, biosynthesis of alginate was activated towards the end of the exponential growth of bacteria and thereafter continued to increase gradually up to 1.84 g/L at the end of the experiment. These results indicated that alginate was totally produced by non-growing bacteria. *Azotobacter* species have high maintenance requirements that would be used in removing oxygen at the cell surface by oxidizing the available carbon source. This creates a microenvironment of low oxygen concentration suitable for nitrogenase activities in the interior of the cell, in a bulk environment of higher oxygen concentration. In other words, respiration and oxidation of the available carbon are important mechanisms in protecting the enzyme nitrogenase against oxygen damage by lowering the ambient oxygen concentration whenever the capacity for oxygen consumption by the cells exceeds the rate of oxygen input into the culture, and whenever there is enough sugar to be metabolized (Sabra et al., 1999). It seems most of the sucrose was used for

the removal of excess oxygen around the cells at the beginning rather than for alginate production. Therefore, this resulted in considerably low alginate yield at the end of the experiment. The highest alginate yield from biomass was observed as 0.4 ± 0.12 g alginate / g biomass at 72 hours. This yield was quite lower, which changed between 0.62 and 1.33 in other studies and especially it was high in controlled environmental conditions like the one maintained in laboratory fermentors (Peña et al., 2000; Trujillo-Roldán et al., 2003; Seáñez et al., 2001; Trujillo-Roldán et al., 2004).

Properties of this alginate were also determined in terms of monomer distribution in the chain by ^{1}H-NMR spectroscopy and culture broth viscosity. The results are summarized in Table 4.2 and showed that alginate only contained homopolymeric blocks, namely MM- and GG-blocks. Also, it was rich in mannuronic acid even after 72 hours since GG-block content was just increased to around 30 %. On the other hand, culture broth viscosity was considerably high although alginate concentration was 1.84 g/L at maximum. At 72 hours, viscosity value of culture broth was 104.6 cP and it was probably the indication of high molecular weight of produced alginate. These results might be attributed to possible oxygen profile during alginate production in shake flask. At the beginning, oxygen level seems to be high since alginate production was greatly delayed. As the experiment proceded, cell synthesis and increased viscosity may create an extremely limited oxygen concentration nearby the cells at 200 rpm of agitation speed. Moreover, it is a known fact that some key enzymes of alginate biosynthesis are affected by oxygen tension. As a result, both alginate depolymerases (alginate lyases) and epimerases appeared to be influenced negatively by oxygen limitation that leads high molecular weight alginate which is rich in mannuronic acid. The conditions also caused the viscosity to be measured very high.

Table 4.2. Properties of alginate synthesized in shake flask

Time (hours)	Block Distribution (%)			Broth Viscosity (cP)
	MM	MG	GG	
24	0	0	0	1.44
32	78	0	22	6.20
48	67	0	33	19.09
56	66	0	34	68.30
72	70	0	30	104.60

Although considerably high viscosity values could be achieved in shake flasks, the amount of alginate and GG-block fraction were low. Also, it was not possible to control key parameters such as dissolved oxygen levels and pH for alginate production in shake flasks. Therefore, there would be no opportunities for the examination of the effect of important operational parameters on alginate quality and quantity. For this reason, alginate production was continued to be performed in a controlled environment i.e. in a laboratory fermentor.

4.1.3. ALGINATE PRODUCTION IN FERMENTOR

Alginate production was conducted at 5 % DOT in triplicate to observe the reproducibility of the experiments performed in the fermentor. Together with DOT value, the other parameters were maintained constant throughout 72 hours of cultivation time; such that pH, temperature and agitation speed were set to 7.2, 30 ℃ and 400 rpm, respectively. Synthesis of alginate and microbial biomass were observed with respect to time and the results obtained from that experiment are shown in Figure 4.4. Both bacterial mass and alginate concentration were very similar between triplicates, showing differences of less than 15 % of the average except for a few points.

Therefore, the results of fermentation experiments were considered reproducible.

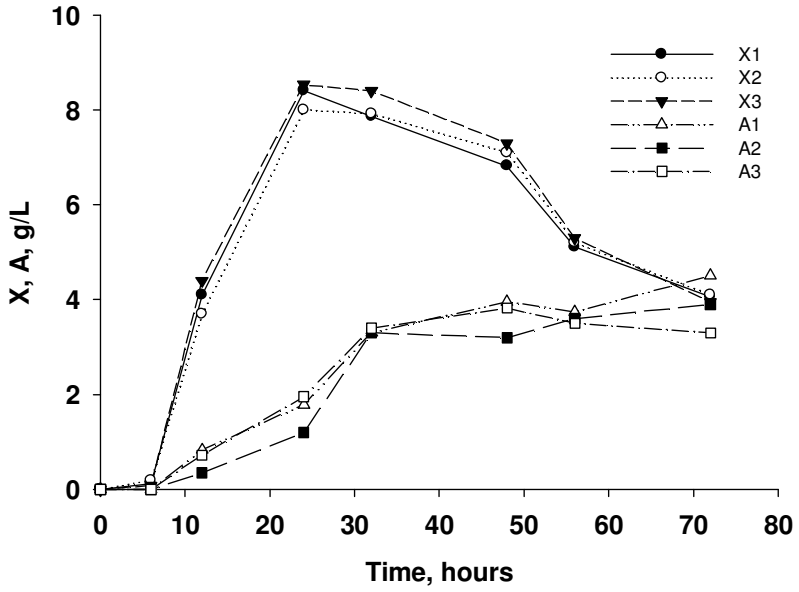

Figure 4.4. Reproducibility of alginate production by *Azotobacter vinelandii* ATCC® 9046 in laboratory fermentor

Microbial growth seemed to be more efficient in the case of fermentor experiments, because the highest average final biomass achieved after 24 hours was 8.31 g/L in the fermentor. The average specific growth rate (μ) was measured as 0.12 hr^{-1} which corresponds to 5.94 hours of doubling time (Table 4.3). This value was almost the same as the value observed in the shake flask. On the other hand, the trend of alginate production in fermentor was different than in shake flask. Moreover, alginate synthesis was activated after 12 hours; although it was delayed till 32 hours in the flask. Furthermore, amount of maximum alginate produced during fermentation was higher in fermentor, which was 4.51 g/L at maximum. The maximum alginate production was 1.84 g/L in the case of shake flask experiments. The reason of this difference might probably be the differences in oxygen profiles that could be maintained constant througout the experiment in laboratory

fermentors. This allows efficient production of alginate in fermentors since bacteria are not required to struggle with high DO level as in the case observed in shake flask when the amount of bacteria and alginate are low at the beginning of the experiments. In other words, bacteria are able to use the carbon source for alginate synthesis in fermentors due to suitable oxygen concentration in the bulk medium other than waste it for the removal of excess dissolved oxygen as in shake flasks.

Table 4.3. Growth parameters of *Azotobacter vinelandii* ATCC® 9046 in fermentor

Specific growth rate (μ), (hr^{-1})	Doubling time, (hr)
0.12 ± 0.002	5.94

4.1.4. DETERMINATION OF THE METHOD FOR MONOMER DISTRIBUTION ANALYSIS

To be able to come up with reliable and simple method, two methods were tested for the determination of monomer distribution of alginate. In the following parts, these tests and the results are being described.

4.1.4.1. Use of Acid Precipitation for Monomer Distribution Analysis

The alginate samples obtained from FMC Biopolymer were subjected to monomer distribution analysis by acid precipitation. The results are summarized in Table 4.4 such that M/G ratios of the samples found by the experiments were within the ranges calculated as described in section 3.3.4.1. A correlation graph (Figure 4.5) for the mid-point of the calculated range in Table 3.6 and the analytically measured M/G value was prepared. Measured values seem to correlate well with the calculated values. As in

Figure 4.5, this graph shows a slope nearly equal to 1 and incercept close to zero. The regression coefficient is 0.9995 which is quite high as well. These numbers indicate that this method measures values close to the real values. As a result, it was decided that the method of monomer distribution led to a good agreement for alginate samples that had defined quality and the method could be used for future analysis. But this approach rested on several approximations, assumptions and calculations. Also, it was a lengthy and wet chemical analysis method. Therefore, a verification of the results with the ^{1}H-NMR analysis, which was an alternative technique, was decided to be conducted as the study proceeded further.

Table 4.4. M/G ratio of biopolymer samples found by wet acid precipitation technique

Alginate	**%MG**	**%MM**	**%GG**	**M/G**
LF240D	42 ± 3	45 ± 4	13 ± 0.1	1.94
LF120M	50 ± 5	34 ± 2	16 ± 6	1.44
HF120	44 ± 2	27 ± 3	29 ± 2	0.96
LF200DL	40 ± 6	21 ± 7	39 ± 4	0.69
LF200S	30 ± 7	17 ± 3	53 ± 0.2	0.47

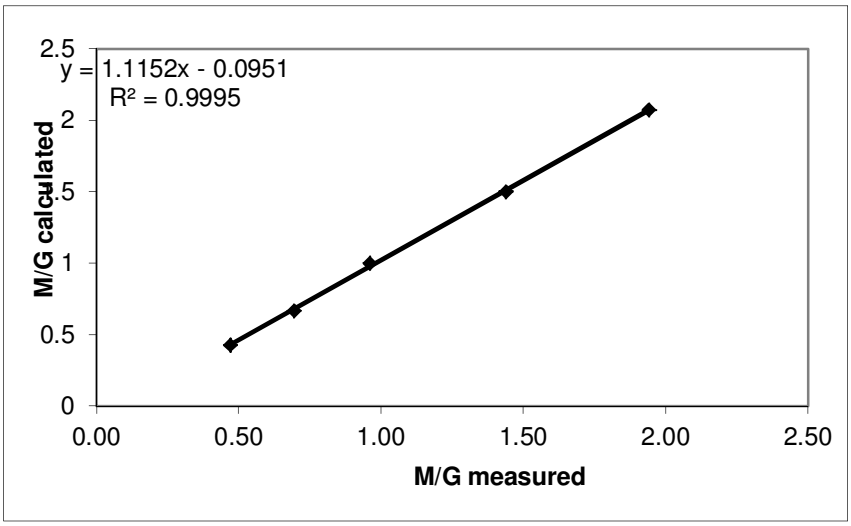

Figure 4.5. Comparison of M/G ratios of FMC biopolymers

4.1.4.2. Use of ^{1}H-NMR for Monomer Distribution Analysis

^{1}H-NMR analysis was applied to one of the alginate samples provided by FMC in order to check acid precipitation method used for monomer distribution analysis. Since D_2O was widely used in the literature for ^{1}H-NMR analysis of alginates, it was decided to use it as the solvent (Larsen et al., 2003; Schürks et al., 2002; Hartmann et al., 2002). However, it was found that the peak of D_2O overlapped with an important peak which was H-1 of mannuronic acid in sample analysis. Thus, studies were continued in order to separate the peaks by seeking a different solvent. Methanol, acetone, and chloroform were tested as potential solvents, but alginate samples were not dissolved in any of these solvents. For that reason, works were continued by using D_2O as a solvent in ^{1}H-NMR analysis.

MM and GG-blocks were isolated by using acid precipitation as mentioned in 3.3.4.1. These blocks together with an FMC alginate sample coded as LF240D were subjected to ^{1}H-NMR analysis. The reason for the selection of LF240D among FMC alginate samples was that MM-block content of

LF240D was the highest and there was the overlaping problem between D_2O and H-1 of mannuronic acid.

First, ^{1}H-NMR analysis of MM- and GG-blocks isolated from LF240D were done at 30 ℃ as shown in Figure 4.6 and Figure 4.7. As it is seen from both of the graphs that we have the solvent peak at around 4.7 ppm at which some parts of the block signal B1-B4 were located according to ASTM F 2259-03.

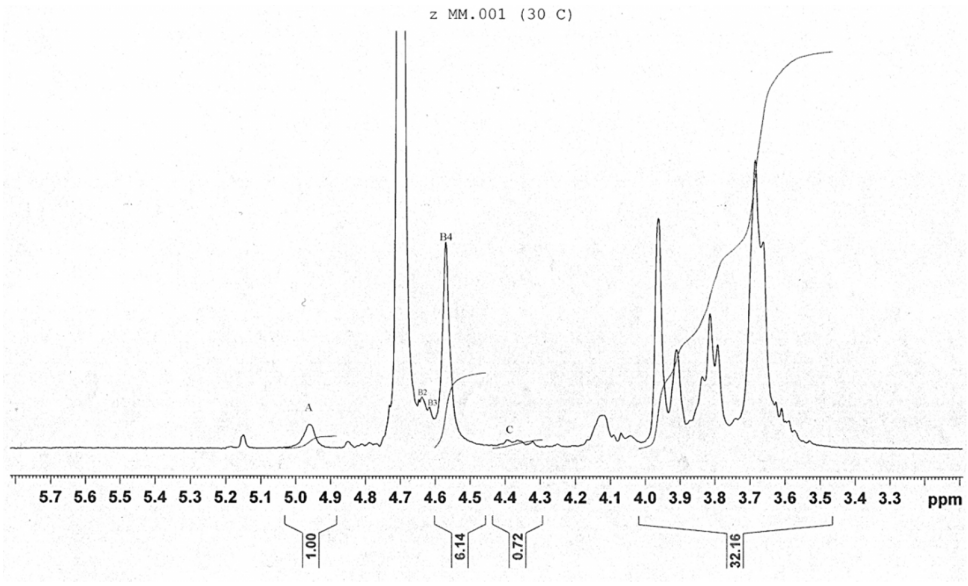

Figure 4.6. ^{1}H-NMR analysis of MM-blocks isolated from LF240D at 30 ℃

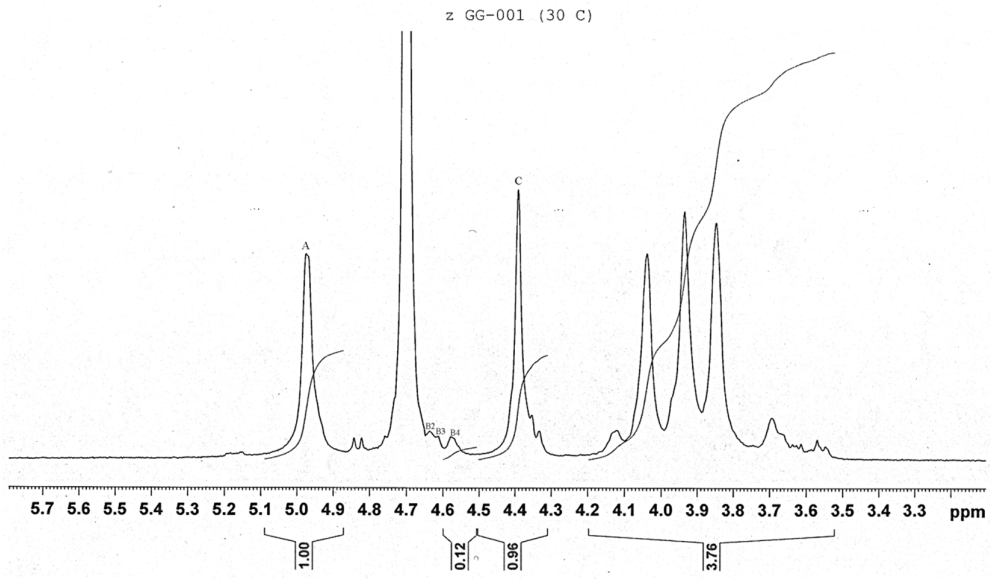

Figure 4.7. ^{1}H-NMR analysis of GG-blocks isolated from LF240D at 30 ℃

When these two figures were compared, it might be said that signals B2-B4 were located near the solvent peak at around 4.6 ppm since these peaks were so much sharp in case of MM-block graph as expected. However, these peaks were not well separated and signal B1 was still again under the solvent peak. ^{1}H-NMR result of LF240D (Figure 4.8) showed almost the same pattern with MM-block. Thus, it was decided to get ^{1}H-NMR analysis at 70 ℃ in order to remove the solvent peak and see how well the peaks were separated.

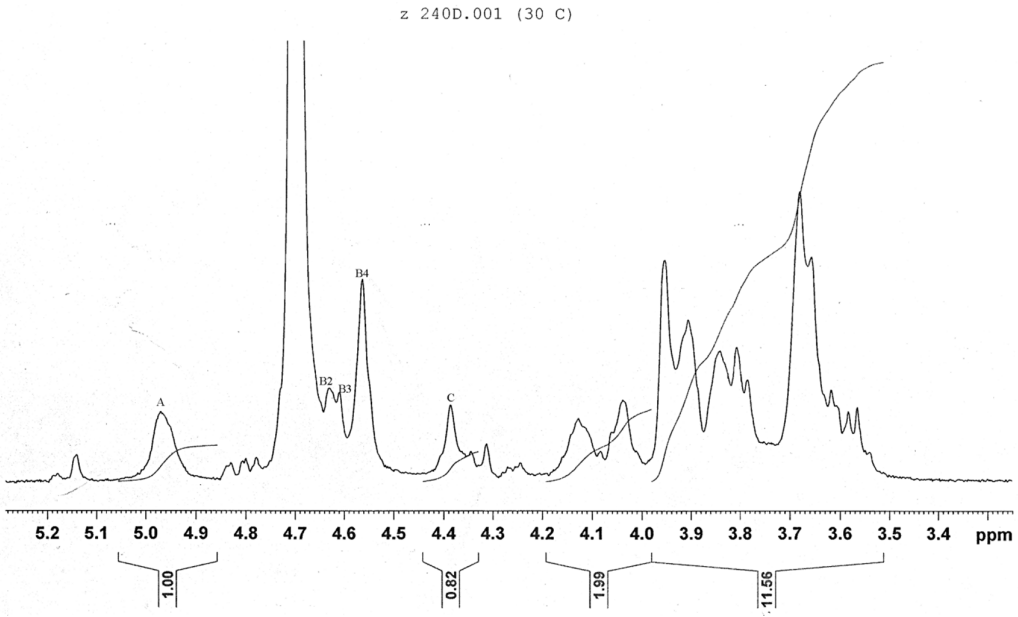

Figure 4.8. ^{1}H-NMR analysis of LF240D at 30 ℃

Figure 4.9 and 4.10 illustrates ^{1}H-NMR analysis of MM- and GG-blocks performed at 70 ℃. The solvent peak is shown at the same location, at 4.7 ppm, and the relevant peaks seemed to shift by about 0.5 ppm. Nevertheless, another problem was come out that signal C was not separated well and it was somewhat located under the solvent peak.

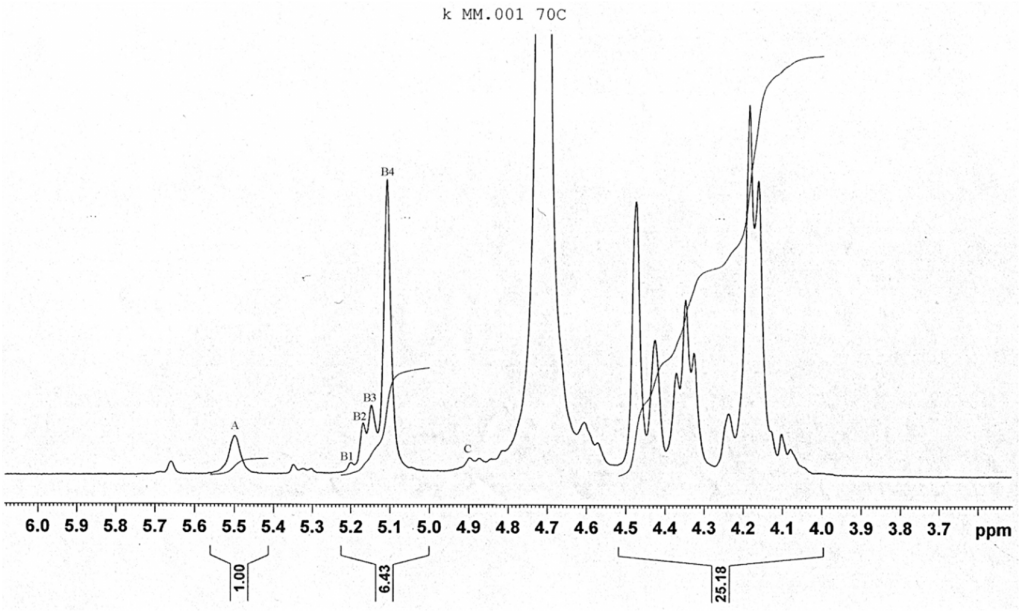

Figure 4.9. [1]H-NMR analysis of MM-blocks isolated from LF240D at 70 °C

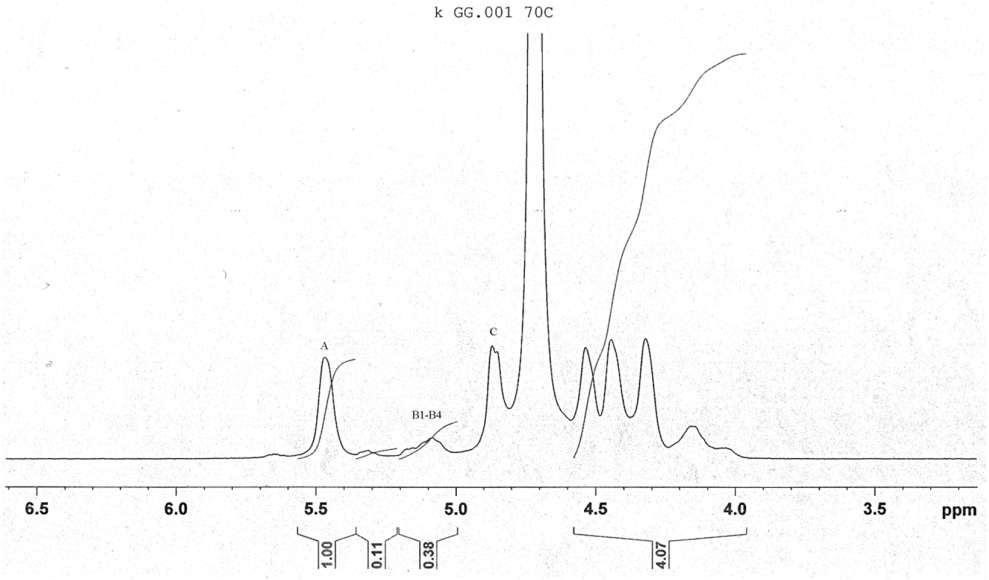

Figure 4.10. 1H-NMR analysis of GG blocks isolated from LF240D at 70 °C

Similary, the same trend was observed in case of [1]H-NMR result of LF240D (Figure 4.11). As a consequence, it was decided to make two [1]H-NMR

analysis for each sample one at 30 °C and the other at 70 °C and compare the relative areas of the peaks observed. Before proceeding, one must be sure that the results of ^{1}H-NMR analysis at 30 °C and 70 °C were compatible. In other words, the areas under the peaks should be the same at 30 °C and 70 °C because the only difference is the analysis temperature which practically affects the location of the peaks not the amount of matter. This hypothesis was proved correct as it is seen from Figure 4.8 and 4.11 that the calculated area of a peak block located from 4 to 4.5 ppm at 70 °C were almost the same as that located from 3.5 to 4 ppm at 30 °C, which are 11.74 and 11.56, respectively. Thus, while the areas of signal A and signal C were obtained from ^{1}H-NMR results performed at 30 °C, the areas of signal B1-B4 were taken from the spectra which was obtained at 70 °C. Then, the whole monomer distribution of alginate samples could be calculated by using these data.

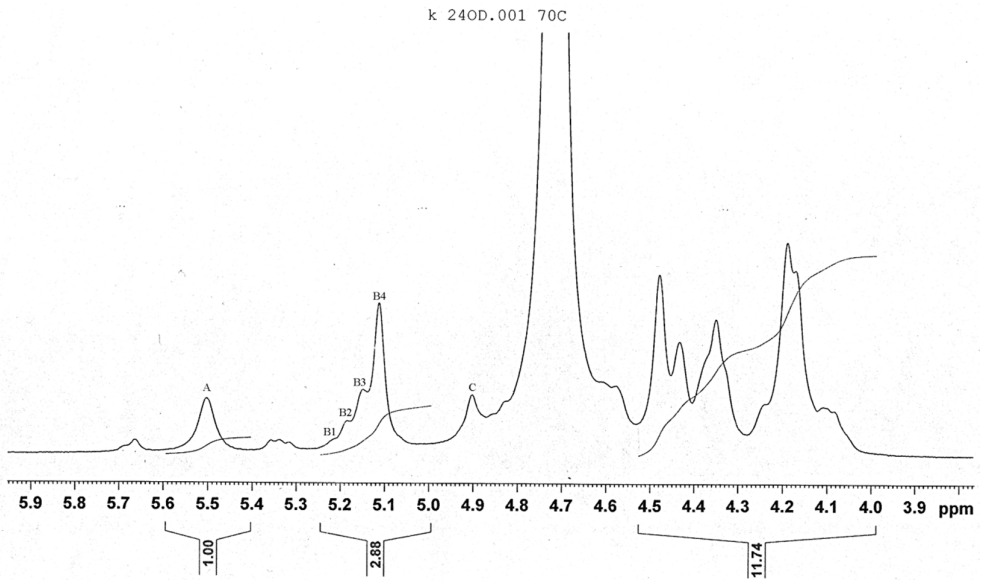

Figure 4.11. ^{1}H-NMR analysis of LF240D at 70 °C

By using the idea above, the monomer distribution of LF240D were calculated by using equations 3.2 - 3.11 and the results are summarized and

compared with the findings of acid precipitation method of the same alginate sample in Table 4.5.

Table 4.5. Comparison of the results of two different methods used for monomer distribution analysis of LF240D

Block Type	Amount, %	
	^{1}H-NMR	Acid Precipitation
MG	22	42 ± 3
MM	56	45 ± 4
GG	22	13 ± 0.1
M/G	2.03	1.94

The results in Table 4.5 showed that the results obtained by ^{1}H-NMR and acid precipitation are not the same. It is thought that there are several sources of error. One of these is the solubilization of the homopolymeric blocks during the partial hydrolysis, which affects the MM-blocks more than the GG-blocks (Haug et al., 1974). This is clearly observed in Table 4.5, where both the MM and GG-blocks of LF240D obtained by acid precipitation are much lower than those obtained from ^{1}H-NMR spectroscopy. Similar erroneous results were encountered for comparison of ^{1}H-NMR and acid precipitation findings of alginates of *C. trinode* and *S. Latifolium* (Larsen et al., 2003). As a result, it was decided to use ^{1}H-NMR spectroscopy for monomer distribution analyses of alginate samples.

4.1.5. POST-EPIMERIZATION OF ALGINATE SAMPLES

Alginate samples produced during the fermentor operation studies normally have a variety of block distributions. These alginates aimed to be tested for their capability of metal removal or turbidity removal. The alginate samples

produced from shake flasks though have limited variety of block distribution. To be able to change the block distribution externally to favor the GG-block structure, epimerization studies were conducted on alginate samples obtained from shake flask experiments by using AlgE1. For this purpose, two different levels of epimerization were tried to be achieved, namely moderately- and highly-epimerized alginate samples. The polymer as harvested from shake flasks was called as non-epimerized alginate. All these experiments were performed at Department of Biotechnology in Norwegian University of Science and Technology, Norway.

4.1.5.1. Production and Purification of AlgE1

First step was the production the epimerase, AlgE1, which was a bimodal enzyme having two units so that it could introduce both MG- and GG-blocks to the structure of the polymer (Ertesvåg and Valla, 1999). A previously developed plasmid, pHH1, was used for the production AlgE1. Then, the epimerase was purified from the culture broth by using ion-exchange chromatography with a column (HiTrap Q HP, 5 mL, an anion exchange column) operated by a FPLC (Fast Protein Liquid Chromatography) system. The peaks obtained from the culture broth containing also AlgE1 after ion-exchange chromatography are shown in Figure 4.12.

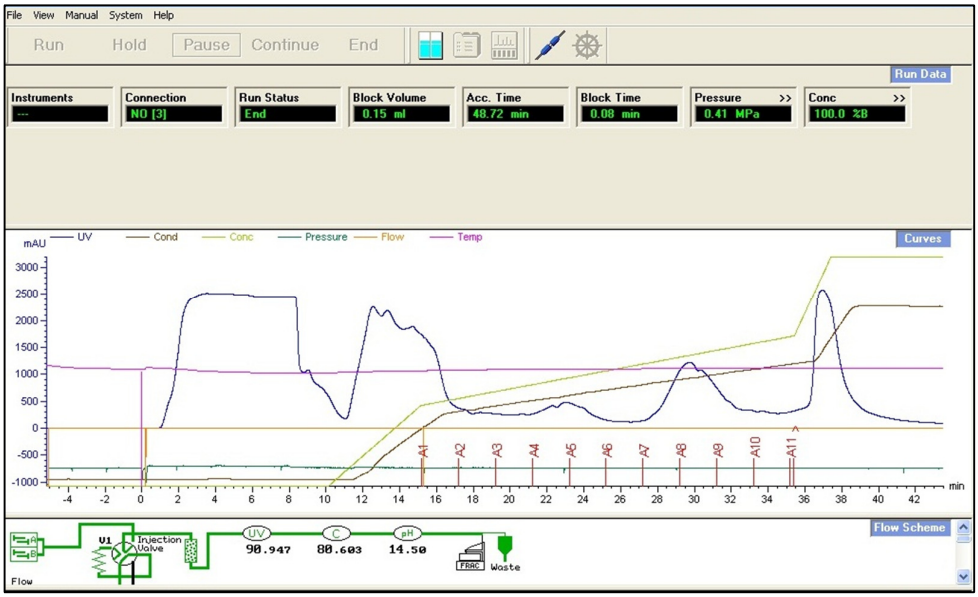

Figure 4.12. Ion-exchange chromatography results for the purification of AlgE1

In ion exchange chromatography, charged molecules bind to oppositely charged molecules found in insoluble matrix by ether linkages. Detection of proteins depends on absorbance measurements at 280 nm. Retention time for each solute is determined by charge strength. According to that, first weakly charged proteins elute and these are followed by strongly charged ones (Satinder, 1989). As a result, first two peak blocks are related with cationic substances that elute first due to their low affinity to the solid matrix of the column. After a certain time, 10 mL fractions through elution process were collected automatically. According to the figure, fraction A5, A8 and A9 are the ones showing high absorption of UV light investigated for the presence of AlgE1.

4.1.5.2. Measurement of Activity of AlgE1

For the measurement of activities for enzyme-containing fractions obtained from ion exchange chromatography, liquid Scintillation counting (LSC) was

applied. In this method, radioactivity of the samples is determined by beta particles emisson such as ^{3}H, ^{14}C, ^{32}P. LSC is particularly efficient for weak beta emitters like tritium, ^{3}H. Beta particles can be described as release of any electrons from the unstable nucleus. The energy discharged from beta particles turn into visible light that is detected by a liquid scintillation counter. For the production of light, the radioactive samples are mixed in a cocktail solution (ready safe liquid) which includes a solvent and a chemical compound known as fluor. Fluors form a flash of light when they interact with a beta particle (Massart et al., 1993). The results of counting are summarized in Table 4.6. As it is seen from the table that only the fraction A5 has an epimerase activity, thus, this fraction can be used for the epimerization of alginate samples. These results were obtained for 5 µL of AlgE1 and the DPM value for A5 was considerably high. For this reason, enzyme activity assay was repeated once again with 1 µL of AlgE1. The average DPM value was found as 2102.25 and it was found to be enough for effective epimerization.

Table 4.6. Results for LSC of possible AlgE1-containing fractions

Matter subjected to LSC	Disintegration's per minute (DPM)
Blank	50.45
A5	2574.60
A8	68.20
A9	58.05

4.1.5.3. Production and Epimerization of Alginate

Alginate was produced by *Azotobacter vinelandii* ATCC ®9046 in a shake flask during 72 hours at 225 rpm and 30 °C. The amount of alginate was found as 1.6 g/L and the ^{1}H-NMR spectrum of the sample is presented in

Figure 4.13. Accordingly, the alginate sample consists of 23 % of GG-blocks, 24 % of MG-blocks and 52 % of MM-blocks. Hence, this alginate is rich in mannuronic acid. This alginate is called as non-epimerized alginate sample and used as an alginate with low GG-block content in further experiments.

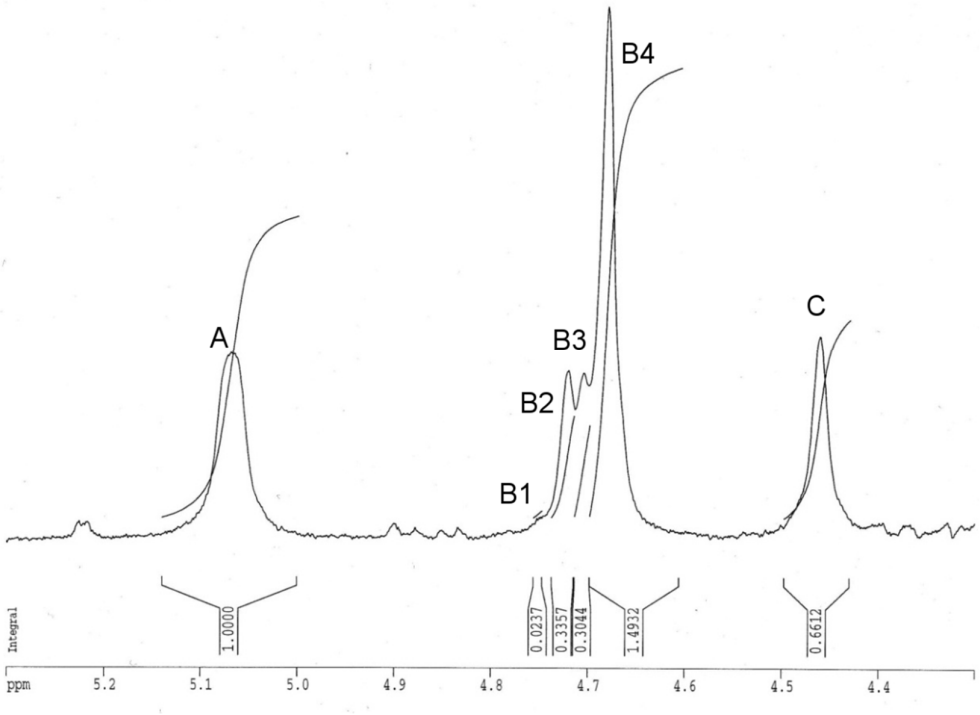

Figure 4.13. ^{1}H-NMR spectrum for non-epimerized alginate sample at 90 ℃

This alginate sample was epimerized by using AlgE1 to obtain highly-epimerized alginate samples. Figure 4.14 illustrates the ^{1}H-NMR spectrum of alginate sample after epimerization. Since the equipment in Norway was able to increase the temperature to 90 ℃, ^{1}H-NMR analysis was performed at 90 ℃. As expected, a high degree of epimerization could be achieved. GG-block fraction was found as 81 % while only 4 % of MM- and 15 % of MG-blocks were observed. These works were done as a reference for the epimerization of high amount of alginate since only 15 mg of alginate was subjected to epimerization.

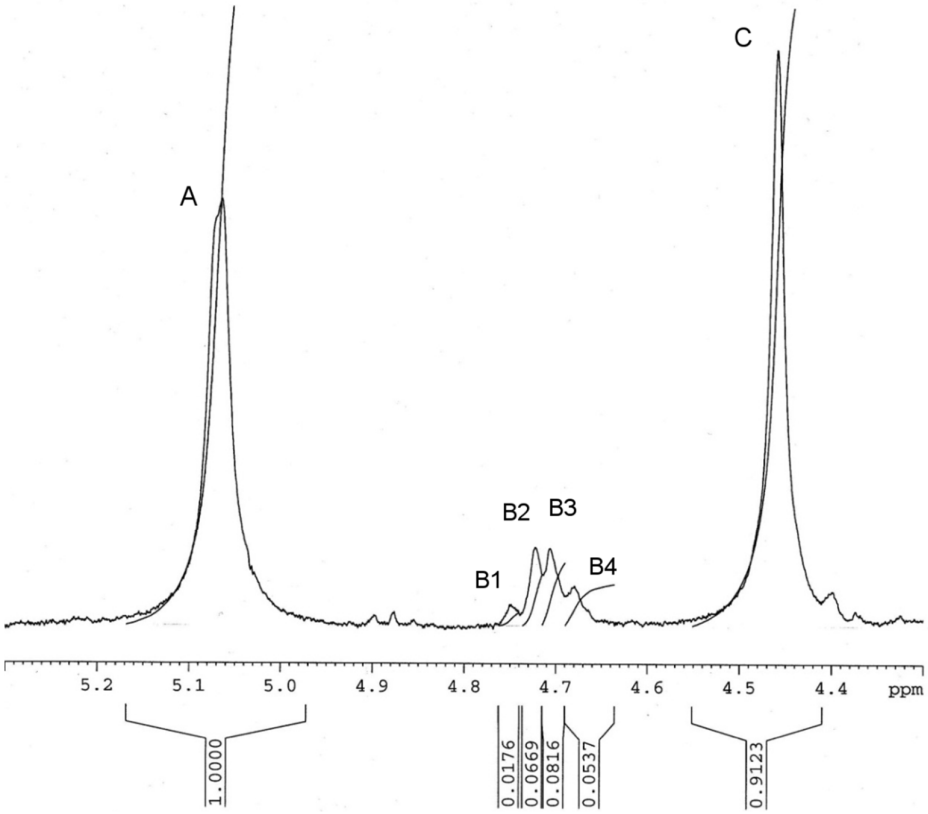

Figure 4.14. ^{1}H-NMR spectrum for epimerized alginate sample at 90 ℃

To be used in the determination of the effect of monomer distribution on heavy metal uptake capacity and turbidity removal efficieny of alginates, about 3 g of alginate was produced by using shake flasks. One third of it was separated to be used as non-epimerized alginate sample. The rest of it was divided into two and they were epimerized to obtain high and moderate degree of epimerization of alginate samples. The non-epimerized alginate sample had similar monomer distribution as previous shake flask runs (24 % of GG-blocks, 12 % of MG-blocks and 64 % of MM-blocks). The ^{1}H-NMR results for highly- and moderately-epimerized sample are shown in Figure 4.15 and Figure 4.16 (See Appendix D for ^{1}H-NMR result for non-epimerized alginate sample). According to these figures, monomer distributions of moderately- and highly-epimerized alginate samples are calculated as in Table 4.7. As a consequence, moderately-epimerized alginate sample has

40 % of GG-block whilst highly-epimerized sample contains 73 % of GG-block. On the other hand, non-epimerized alginate sample has only 24 % of GG-block that all these three samples can be used to elucidate the effect of block type and content (or formation of egg box in the presence of cations) on heavy metal uptake by alginates and flocculation potential of the polymer.

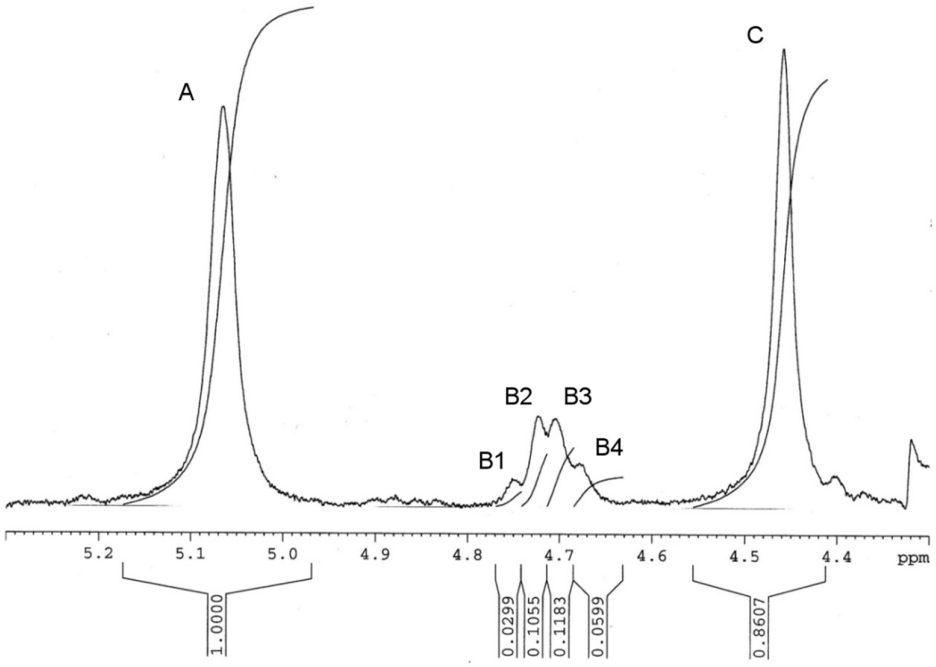

Figure 4.15. ^{1}H-NMR spectrum for highly- epimerized alginate sample at 90 ℃

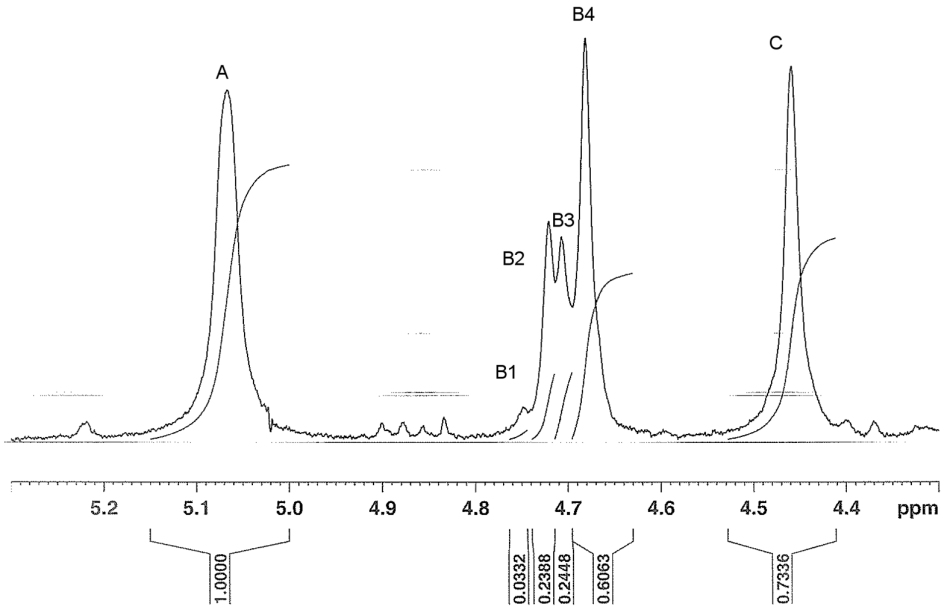

Figure 4.16. ^{1}H-NMR spectrum for moderately- epimerized alginate sample at 90 ℃

Table 4.7. Monomer distribuiton of epimerized alginate samples

Monomers (%)	**Moderately-epimerized Alginate sample**	**Highly-epimerized Alginate sample**
MM-Block	32	3
MG-Block	29	24
GG-Block	40	73

4.2. PRODUCTION OF ALGINATE BY *AZOTOBACTER VINELANDII* ATCC® 9046

4.2.1. DETERMINATION OF EFFECT OF DISSOLVED OXYGEN TENSION

Azotobacter vinelandii cells produce alginate depending on their needs under various environmental conditions. DOT is one of the key parameters affecting alginate formation particularly under nitrogen fixing conditions since nitrogenases that are the enzymes responsible for nitrogen fixation are highly sensitive to high DO levels (Haddock and Jones, 1977). Thus, it is important to control DOT during alginate production. In this part of the study, alginate production was evaluated under controlled DOT conditions in the range of 1 % to 10 % by using a fermentor. The operating conditions for this reactor was at 400 rpm mixing, 20 g/L initial sucrose concentration and 50 mg/L of calcium concentration.

4.2.1.1. Result Obtained at 1 % DOT

Alginate production by *Azotobacter vinelandii* ATCC® 9046 was investigated in a laboratory fermentor at 1 % DOT. Figure 4.17 illustrates changes in cell, alginate and sucrose concentration during 72 hours of fermentation. It seems that there was a small lag period for cell growth since bacterial concentration is very low at 6 hours. The cells grow exponentially during the first 24 hours corresponding to a maximum specific growth rate of 0.099 hr^{-1}. After that, growth slows down and then a slight cell lysis is observed. Maximum bacterial mass was obtained at 32 hours as 7.84 g/L. On the other hand, alginate production was almost continuous throughout the fermentation process. Most of the alginate was synthesized by growing bacteria, thus, alginate production was growth associated, i. e., about 70 % of total alginate was recovered at 32 hours. In addition, highest alginate concentration was

3.18 g/L at 56 hours corresponding to a yield of 0.57 g alginate/g bacteria. Concentration of sucrose, the main carbon source, was concomitantly reduced from 20 g/L to 1.8 g/L leading to 91 % consumption of applied sucrose.

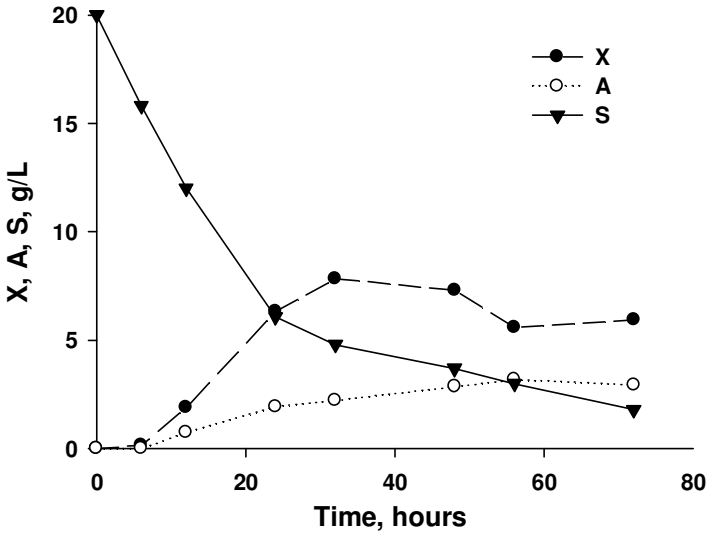

Figure 4.17. Variation in bacterial mass (X), alginate (A) and sucrose (S) concentration at 1 % DOT

Viscosity is one of the important characteristics of alginate as a commercial product because it shows the polymerization degree of alginate and affects thickening properties of the polymer. Generally, it is known that the higher the viscosity is the higher the molecular weight is (Sabra et al., 2001; Rehm and Valla, 1997). Thus, culture broth viscosity was monitored to have an idea about alginate quality in terms of its molecular weight. As it is seen from Figure 4.18, culture broth viscosity increased up to 24 hours to a maximum value of 13.16 cP and then, a sharp decrease was observed at 32 hours. This reduction in viscosity value was continued to the end of fermentation at 1 % DOT. The drop in culture broth viscosity after some time is usually attributed to the activity of alginate lyases that reduce the molecular weight

of the polymer by breaking the bonds between alginate monomers (Rehm and Valla, 1997).

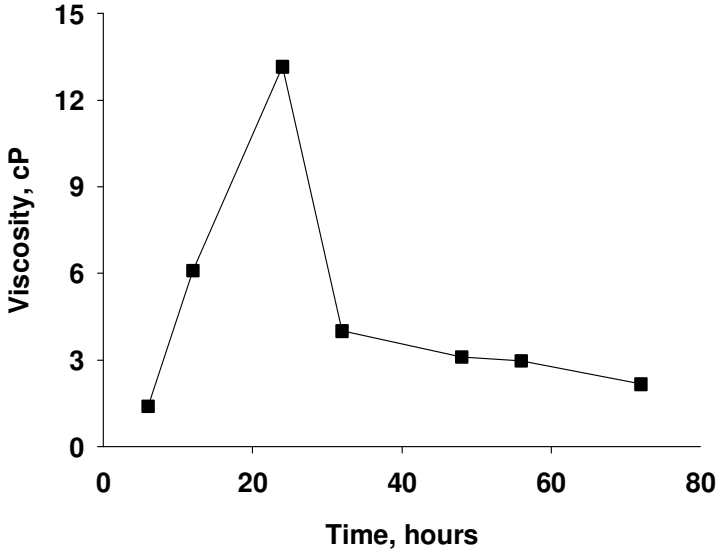

Figure 4.18. Viscosity change of culture broth at 1 % DOT

For the purposes of this work, it is very important to know the monomer distribution of alginate owing to the fact that properties of the polymer are influenced by the block types. Figure 4.19 shows monomer distribution of alginate obtained at 1 % DOT during 72 hours measured by ^{1}H-NMR technique. It seems that alginate is first synthesized as mannuronic acid in the earlier phases of growth and then it is epimerized into guluronic acid. Accordingly, alginate harvested during early fermentation had higher fraction of MM-block which was steadily reduced from 44 % at 24 hours to 14 % at 72 hours. Furthermore, epimerization was faster up to 32 hours and then rate of epimerization slowed down to 72 hours of fermentation. Meanwhile, guluronic acid content was increased by the action of epimerases secreted by *Azotobacter vinelandii* ATCC® 9046. However, it was mainly introduced as GG-block rather than MG-block since only 2-3 % of MG-block was obtained while GG-block content was improved up to 84 % at the end of

fermentation process. *Azotobacter vinelandii* produces seven different extracellular enzymes namely AlgE1-E7. Among them, only AlgE4 epimerases MG-block while the rest introduce both MG- and GG-blocks at various lengths. One of the important factors that affect the activity of these epimerases is calcium ion concentration in the medium (Ertesvåg and Valla, 1998). As a consequence, environmental conditions used in this study somehow favor the epimerases for particular production of GG-block.

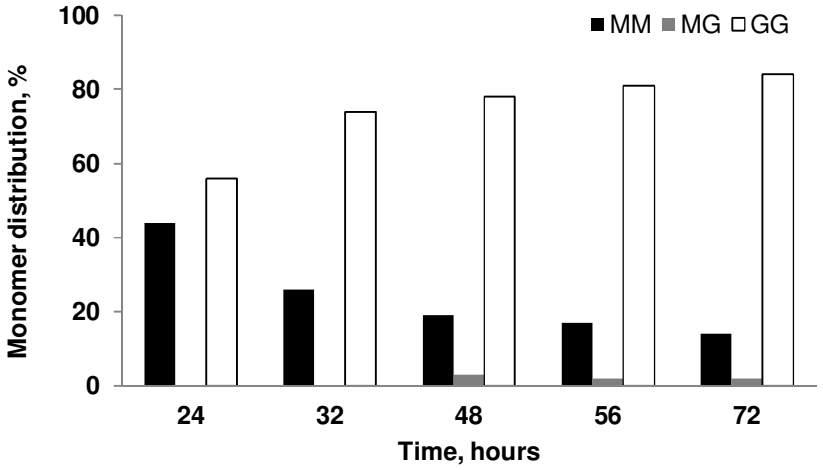

Figure 4.19. Monomer distribution of alginate at 1 % DOT

4.2.1.2. Result Obtained at 3 % DOT

Production of alginate was also examined at 3 % DOT by *Azotobacter vinelandii* ATCC® 9046. Similar to the results for cell culture obtained at 1 % DOT, bacteria showed a lag phase and bacterial mass increased during first 32 hours (Figure 4.20). Furthermore, highest cell concentration was found as 8.21 g/L with maximum specific growth rate of 0.078 hr^{-1}. The latter was smaller than that was observed at 1 % DOT possibly due to oxygen limitation. As it is seen from the figure, cell lysis was more obvious at 3 %

DOT from 32 hours to the end of fermentation leading to a decrease in bacteria concentration down to 4.18 g/L at 72 hours. Synthesis of alginate by *Azotobacter vinelandii* ATCC® 9046 at 3 % DOT was consistent throughout fermentation leading to a maximum alginate concentration as 3.36 g/L. Most of this was produced during 32 hours corresponding to 73 % of the total polymer similar to the case observed at 1 % DOT. In addition, 0.8 g of alginate was produced per 1 g of bacterial mass. Almost all of the sucrose was used up by *Azotobacter vinelandii* ATCC® 9046 for alginate production. The concentration was reduced from 20 g/L to 1.7 g/L at 72 hours of fermentation period.

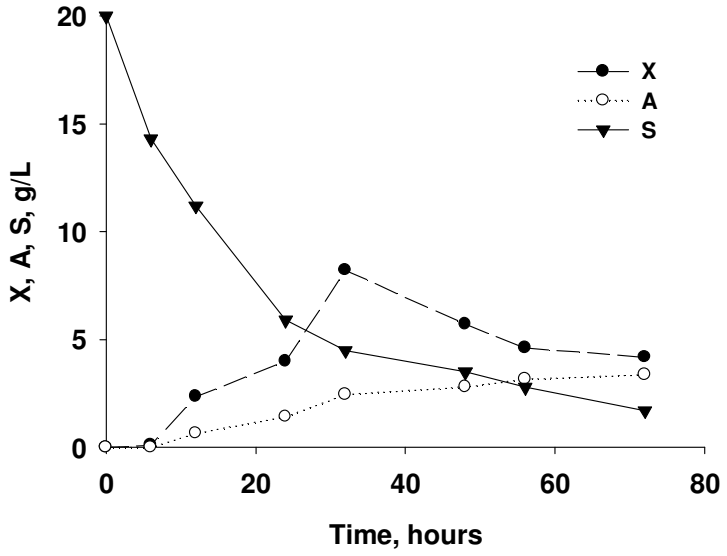

Figure 4.20. Change in bacterial mass (X), alginate (A) and sucrose (S) concentration at 3 % DOT

Figure 4.21 presents the change in culture broth viscosity by alginate production at 3 % DOT by *Azotobacter vinelandii* ATCC® 9046. Viscosity of culture broth increased during 24 hours period at 3 % DOT up to 9.86 cP. Then it gradually decreased to below 5 cP during the course of fermentation process. However, this drop was not as drastic as observed in the case of 1

% DOT. This reduction observed at both 1 % and 3 % DOT was most probably due to the activity of alginate lyases. The activity of alginases, alginate lyases, was shown (Pena et al., 2000) by using a supernatant isolated from the culture grown at 3 % DOT in one study. This study found that the supernatant obtained at 6 hours had a specific alginase activity of 0.013 U/mg protein whilst the activity value was increased to 0.02 U/mg protein at 30 hours.

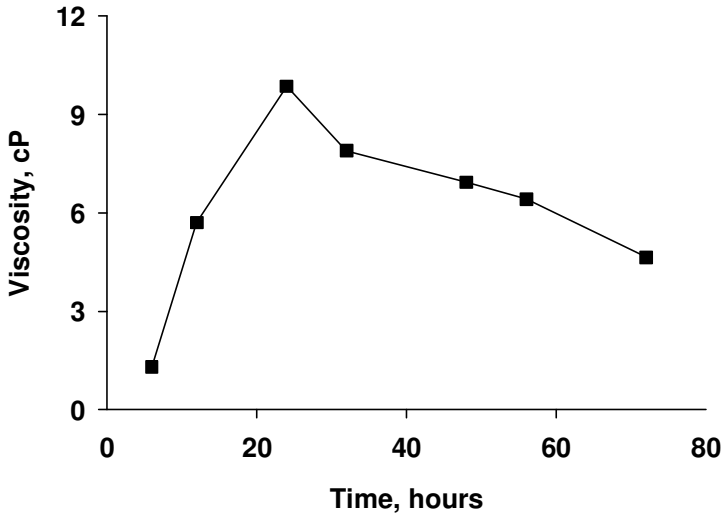

Figure 4.21. Viscosity variation of culture broth at 3 % DOT

The block distribution of the polymer which indicates the relative amounts of MM-, MG- and GG-block constituting alginate was then measured. Alginate block distribution at 3 % DOT showed a similar epimerization pattern evaluated at 1 % DOT (Figure 4.22). Likewise, alginate was rich in MM-block at 24 hours although it then continually decreased down to 29 %. On the other hand, GG-block content of the polymer was improved up to 71 % at the end of fermentation process. Again, very little amount of MG-block synthesis was observed that could be explained by the work of enzymes which are

responsible for epimerization of MM-blocks into MG- and/or GG-blocks. It seems environmental conditions favor particularly GG-block formation.

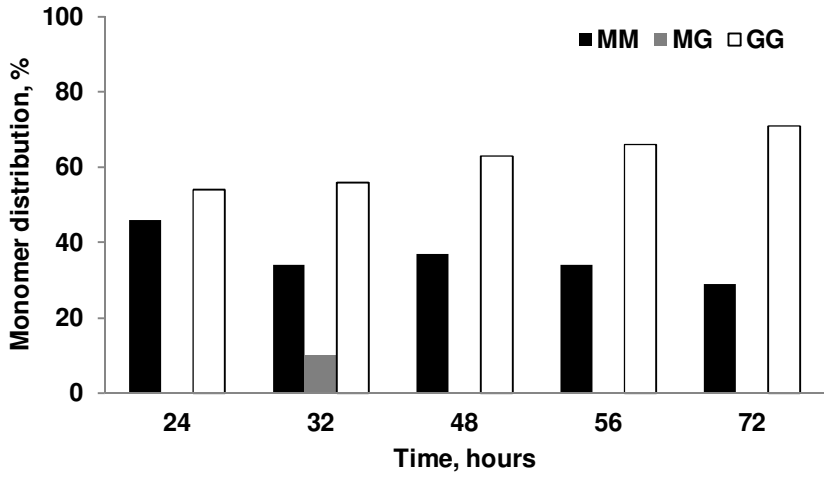

Figure 4.22. Block distribution of alginate produced at 3 % DOT

4.2.1.3. Result Obtained at 5 % DOT

Alginate production was next evaluated at 5 % DOT by *Azotobacter vinelandii* ATCC® 9046. Results showed that the highest alginate yield was achieved under this DOT condition compared to the other DOT values. Changes in bacterial mass, alginate concentration and sucrose concentration are shown in Figure 4.23. Cell growth again showed a small lag period. However, growth was faster than at 1 % and 3 % DOT. Growth was exponential during the first 24 hour period and maximum specific growth rate was observed as 0.117 hr^{-1}. Highest cell mass, 8.7 g/L, was achieved at 24 hours and after this time the cells underwent lysis throughout the rest of the fermentation process. Most of the alginate production was performed by actively growing bacteria although its production continued up to 72 hours. The maximum alginate concentration was found as 4.51 g/L at the end of

fermentation leading to a yield of 1 g alginate/g bacteria. Similar results were obtained by Pena et al (2000) that highest alginate yield was found at 5 % DOT and 300 rpm as 4.6 g/L. Furthermore, Sabra et al (1999) revealed that particularly at 5% DOT, the respiratory quotient (RQ: CO_2 production rate/O_2 uptake rate) value was very close to the optimum value which was calculated theoretically for batch culture under effective production of alginate. The RQ value was found to increase to over 1.0 both at DOT values higher than 5 % and lower than 2 %. In terms of sucrose consumption in this study, it can be seen that sucrose concentration decreased from 20 g/L to 1.65 g/L corresponding to about 92 % of sucrose utilization at 72 hours.

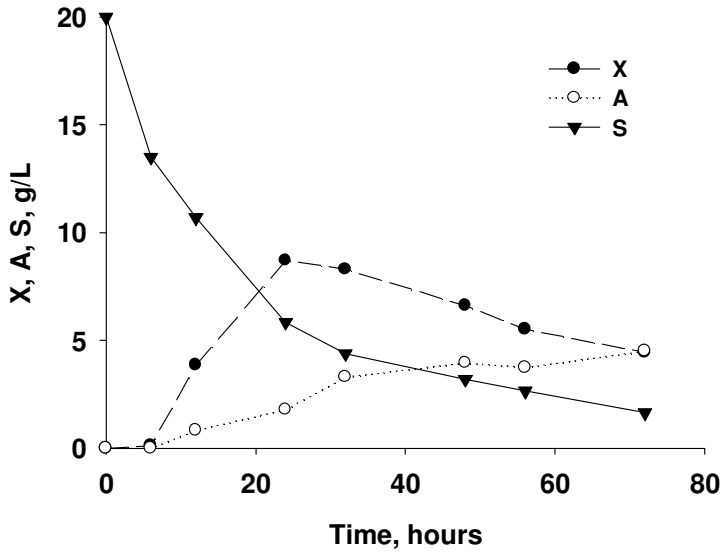

Figure 4.23. Change in bacterial mass (X), alginate (A) and sucrose (S) concentration at 5 % DOT

Figure 4.24 illustrates the changes in culture broth viscosity at 5 % DOT during the course of alginate production. As it is seen from the figure, different from 1 and 3 % DOT, the highest culture broth viscosity value was found at 56 hours as 24.61 cP. Similarly, it started to drop after showing a

maximum. This value was 1.9 and 2.5 times higher than those obtained at 1 % and 3 % DOT. Thus, this result could indicate that best polymerization of alginate was achieved at 5 % DOT compared to the DOT values examined before. This might be due to fact that DOT level affects the activity of alginate lyases. The lowest alginate lyase activity was observed at 5 % DOT in the range of 1 % and 5 % DOT (Trujillo-Roldán et al., 2004). On the other hand, alginate produced by *Azotobacter vinelandii* ATCC® 9046 mainly consisted of homopolymeric regions i.e. it had high amounts of MM- and/or GG-blocks together with lower MG-block content (Figure 4.25). As it is seen from the figure that the biopolymer harvested at 5 % DOT had moderate level of MM-blocks. The maximum value for GG-blocks was found at 56 hours of fermentation process as only 61 %. This value was comparably lower than other DOT values evaluated so far. This result could be related with the needs of bacteria under this specific condition. Although the amount of alginate synthesized was higher at 5 % DOT, the bacteria may not need a dense gel as in the case of high GG-alginate at this DO value. In addition, it is seen here that there is no relation between the broth viscosity and the amount of GG-blocks. Viscosity is more related to the biomass and alginate quantity in the system as well as the molecular weight of the polymer. One other thing to note is that the culture broth viscosity is a gross parameter being influenced by a number of different variables and may or may not reflect the viscosity of alginate produced.

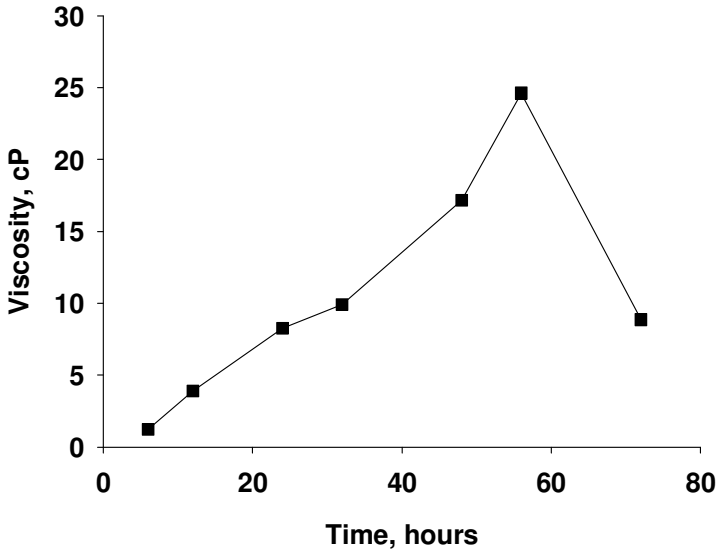

Figure 4.24. Viscosity variation of culture broth at 5 % DOT

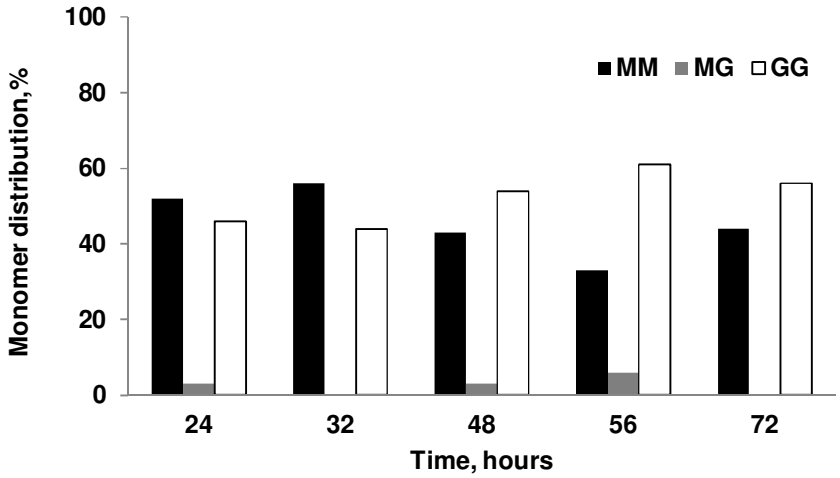

Figure 4.25. Block distribution of alginate produced at 5 % DOT

4.2.1.4. Result Obtained at 10 % DOT

The highest oxygen level tested for alginate production by *Azotobacter vinelandii* ATCC® 9046 was 10 % DOT. For this condition, the variation in concentration of bacteria, alginate and sucrose is shown in Figure 4.26. Bacterial growth was exponential during 24 hours and reached its maximum concentration of 9.29 g/L. Specific growth rate was observed almost the same value as found at 5 % DOT as 0.12 hr^{-1}. After 24 hour period, cell concentration decreased till the end of the fermentation. Unlike the other DOT values investigated, 10 % DOT was obviously not suitable for alginate production as it is seen from the figure. The highest alginate synthesis was observed at 32 hours of fermentation and only 0.78 g/L of alginate was formed at 10 % DOT. After 32 hours, a little reduction in alginate concentration was measured through the end of the process. Most of the sucrose was consumed (from 20 g/L to 1.2 g/L) similar to the other DOT values but conversion of sucrose into alginate was not efficient probably due to wastage of the carbon source in order to reduce high oxygen levels around the cells by respiration.

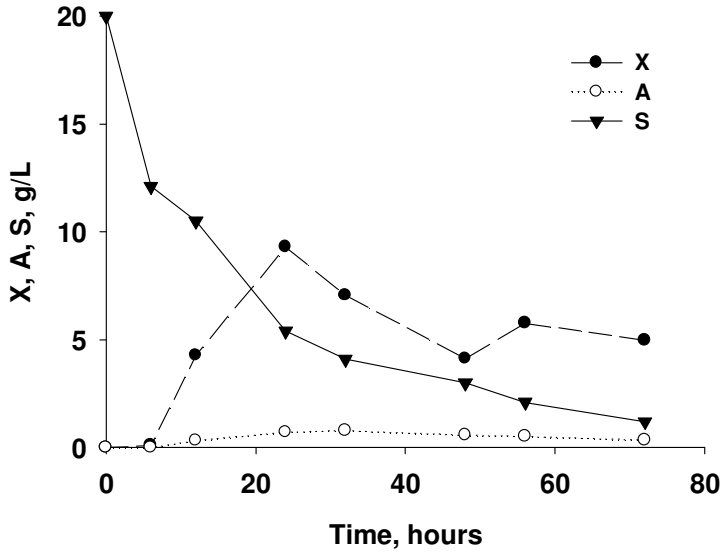

Figure 4.26. Change in bacterial mass (X), alginate (A) and sucrose (S) concentration at 10 % DOT

Viscosity variation during the course of alginate production by *Azotobacter vinelandii* ATCC® 9046 at 10 % DOT is demonstrated in Figure 4.27. The maximum value of viscosity was only 3 cP at 24 hours and then a sharp decrease was observed at 32 hours. This trend was gradual till the end of the fermentation period. Comparably, the lowest culture broth viscosity values were obtained at 10 % DOT. Although bacterial growth was even better, this could be explained by very little amount of alginate formation. Sabra et al (1999) claimed that molecular weight of alginate increased by elevating oxygen concentration from 1 to 10 % of air saturation at dilution rate of 0.08 hr^{-1}. It might be possible to see a parallel trend if viscosity or molecular weight of the alginate was directly measured rather than using the culture broth. Thus, culture broth viscosity increased by increasing the DOT from 1 % to 5 % and then reduced with further increased to 10 % DOT. Figure 4.28 shows monomer distribution of alginate synthesized at 10 % DOT by *Azotobacter vinelandii* ATCC® 9046. Interestingly, relative amount of mannuronic acid was lower compared to DOT range from 1 to 5 %. Even at 32 hours, the GG-block fraction of the polymer increased to around 80 % that was achieved after 72 hours of fermentation at 1 % DOT. All of the mannuronic acid synthesized was epimerized into guluronic acid after 48 hours leading to alginates having 100 % of GG-blocks.

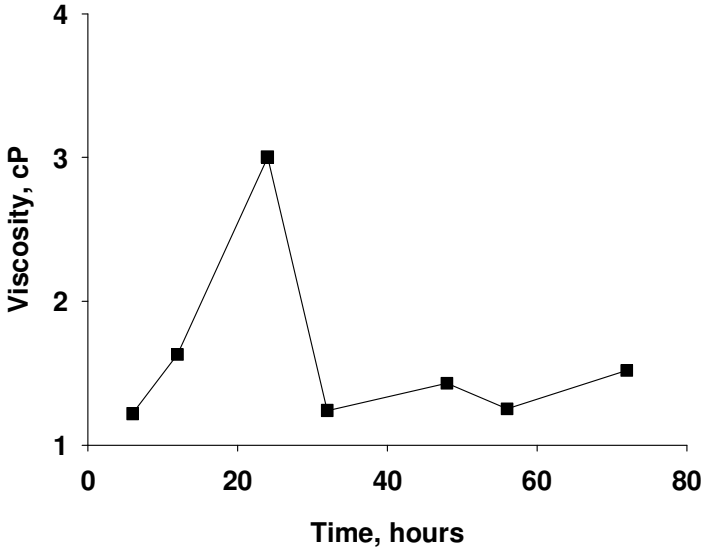

Figure 4.27 Viscosity variation of culture broth at 10 % DOT

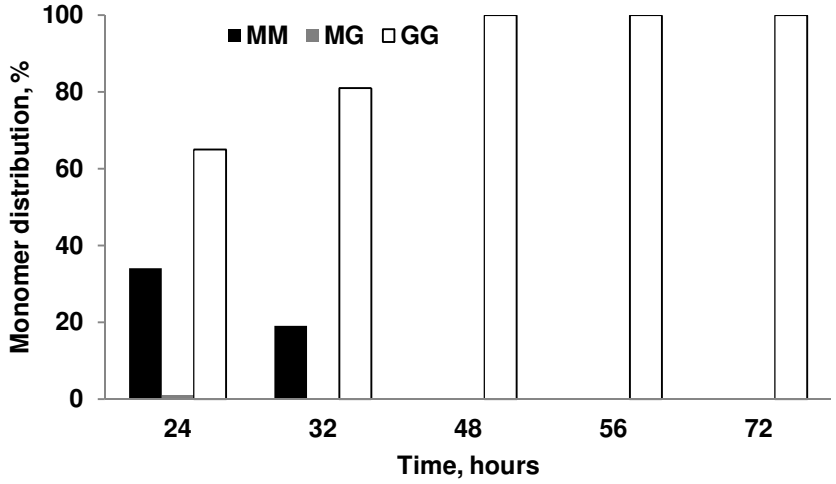

Figure 4.28. Block distribution of alginate produced at 10 % DOT

4.2.1.5. Comparison of Alginates Synthesized at Different Oxygen Values

Alginate production by *Azotobacter vinelandii* ATCC® 9046 was studied in a laboratory fermentor under controlled environmental conditions by varying DOT from 1 to 10 % of air saturation for 72 hour period. Some of the results obtained at different DOT values are summarized in Table 4.8. In general, growth rate was observed to be higher with the increase of DOT from 1 to 10 %. Maximum growth rate was achieved in the case of 10 % of DOT and calculated as 0.120 hr^{-1}. This is in agreement with the literature that Pena et al (2000), observed highest growth rate as 0.20 hr^{-1} at 5 % DOT while it was only 0.07 hr^{-1} at 0.5 % DOT at 300 rpm. Thus, they observed that the higher the DOT (in the range of 0.5 to 5 % DOT) the higher the growth rate. Bacterial growth was found to be exponential within 24 hours at 5 and 10 % DOT with highest cell concentration of 8.7 and 9.29 g/L, respectively. On the other hand, exponential growth was continued up to 32 hours in the case of 1 and 3 % DOT possibly because of limited dissolved oxygen level in the culture medium. After that a pronounced cell lysis was observed for all DOT values and it continued till the end of the fermentation process.

Alginate formation was observed partially growth associated. That is majority of alginate production was performed by actively growing bacteria which corresponded to about 70 % of total production except for the case obtained at 10 % DOT. This phenomenon is generally attributed to the oxygen profile in a production medium. It was claimed that alginate production was partially or completely growth associated in DO controlled laboratory fermentors while most of the alginate formation was observed during stationary growth phase in shake flasks having high aeration conditions which delay alginate production particularly in early cultivation times (Pena et al., 2000). In this work, 1 and 3 % DOT resulted in similar alginate production patterns highest values happening at the end of fermentation process as 3.18 and 3.36 g/L, respectively. On the other hand, it appeared that 10 % DOT level was not suitable for alginate synthesis since only 0.78 g/L of alginate production was

observed at 32 hours which was about 4 times lower than those obtained at 1 % and 3 % DOT. The possible reason might be the wastage of carbon source because it may be mostly channeled to the respiration to reduce high oxygen concentration around the cells. Post et al (1983) showed that in the range of 1 and 30 % DOT, *Azotobacter vinelandii* Strain OP increased respiration rate to protect their nitrogenase from oxygen damage and respiration was claimed to be the prevailing mechanism for the protection. Therefore, alginate concentration at 10 % DOT was observed lower than the other DOT values. Highest alginate yield, 1 g alginate/ g bacteria, was found in the case of 5 % DOT leading 4.51 g/L of alginate at 72 hours. This yield is within the ranges reported in the literature that was around 0.7-1.3 g alginate/ g bacteria (Sabra et al., 1999; Parente et al., 1998; Pena et al., 2000; Trujillo-Roldán et al., 2004). Likewise, Sabra et al. (2000) found 2.5-5 % of DOT values were optimum range for alginate production by *Azotobacter vinelandii* DSM® 93-541b. Furthermore, Pena et al. (2000) showed that alginate synthesis was improved from 1.3 g/L to 4.6 g/L by elevating DOT from 0.5 % to 5 % and the highest value was obtained after 72 hours of fermentation at 5 % DOT and 300 rpm. In addition to these studies, Parente et al. (1998) observed that formation of alginate was enhanced at dissolved oxygen levels between 2 and 5 % of air saturation with specific alginate production rate up to 0.138 g/g.h, whereas *Azotobacter vinelandii* DSM576 yielded no alginate production at 10 % of dissolved oxygen. However, it should be noted that in the study of Parente et al. (1998), DOT levels were controlled by varying mixing speed, so, it was not possible to separate the actual effect of DOT and agitation speed during fermentation. A conclusion can be drawn from the results discussed up to here that oxygen seems to control the alginate production.

Table 4.8. Comparison of some results for DOT experiments

Parameters	**DOT (%)**			
	1	**3**	**5**	**10**
Max biomass conc achieved (g/L)	7.84	8.21	8.70	9.29
Max alginate conc achieved (g/L)	3.18	3.36	4.51	0.78
Time to achieve max alginate conc (hr)	56	72	72	32
Max alginate yield (g alginate/g bacteria)	0.570	0.803	1.00	0.111
Max broth viscosity (cP)	13.16	9.86	24.61	3.00
Time to achieve max broth viscosity (hr)	24	24	56	24
Max GG-block content (%)	84	71	61	100
Time to achieve max GG-block content (hr)	72	72	56	48

In this part of the study, sucrose is used as a carbon source to produce alginate by *Azotobacter vinelandii* ATCC® 9046 at a concentration of 20 g/L. Almost all sucrose was consumed during the course of fermentation process leading to over 90 % of substrate removal at all DOT values. On the other hand, the highest yield based on sucrose consumption was found at 5 % DOT as 0.246 g alginate/ g sucrose. Utilization of sucrose was observed to be faster during early fermentation and shown little differences by changing DOT that is obvious especially at 6 hours. Its concentration was observed to decrease with the increase of DO from 1 % to 10 % DOT at this time. This behavior could be explained by the fact that both alginate and bacterial mass was almost zero at 6 hours and the carbon source should have been removed to supply high respiration rate of the bacterium especially at higher DOT values. On the other hand, sucrose consumption was about identical after 24 hours of fermentation process. Because alginate and cell concentration were comparably high after 24 hours, this increases culture broth viscosity. Eventually that reduces oxygen transfer rate, and so the need for sucrose due to high respiratory activities. This means that after some time although dissolved oxygen was high in the bulk liquid, the concentration around the cells would be much lower because of the existence of highly viscous biopolymer in the medium.

Molecular weight of alginate directly affects the viscosity and other rheological properties of alginate solutions and, therefore, this would determine its utility in specific applications of the polymer in the food and pharmaceutical fields (Galindo et al., 2007). In literature the culture broth viscosity is followed as an indicator of molecular weight of the produced alginate in many studies (Trujillo-Roldán et al., 2003b; Pena et al., 1997; Pena et al., 2000; Reyes et al., 2003). Except for 5 % of DOT, culture broth viscosity has maxima for other DOT values at 24 hours and then the value was reduced continuously towards the end of the fermentation. Like alginate formation, 1 % and 3 % DOT showed quite the same trend for viscosity. Furthermore, highest values were observed as 13.16 and 9.86 cP at 1 and 3 % DOT, respectively. On the other hand, culture broth viscosity was very low

in the case of 10 % DOT probably due to lower alginate production. In addition, best polymerization of alginate was possibly achieved at 5 % DOT indicated by highest culture broth viscosity. The value was increased up to 56 hours of fermentation reaching to 24.61 cP, after which it dropped. Decreases in viscosity levels were obvious for all DOT levels. It is usually explained by release of alginate lyases, also known as alginases or alginate depolymerases that catalyze the β-elimination reaction leading to degradation of alginate (Remminghorst and Rehm, 2006). High viscosity values affect material and gas transfer rate from bulk solution into bacterial cell. Since viscosity of culture broth was comparably higher at 5 % DOT than at 1 % and 3 %, it may be inferred that the stress caused by oxygen on bacteria might force the bacteria to have a polymer with high molecular weight. Therefore, the viscosity of the culture would limit gas transfer that supplies more suitable environment for the bacteria at 5 % DOT. Similar results were obtained by Sabra et al. (2000), that molecular weight of the alginate produced by *Azotobacter vinelandii* DSM® 93-541b was observed to increase from 0.45×10^6 to 0.85×10^6 with the elevation of DOT in the range of 1 % to 10 %. This is similar up to 5 % of DOT in our study. Pena et al. (2000) studied the effect of DOT on alginate production by *Azotobacter vinelandii* ATCC® 9046 in the range of 0.5 % and 5 % DOT showed that both molecular weight of the polymer and culture broth viscosity were increased by increasing DO level at 300 rpm. In this study, the polymer taken from 60 hours of fermentation was analyzed and alginates had mean molecular weight of 680,000 g/g.mol and 266,000 g/g.mol at 5 % and 1 % of air saturation, respectively. Furthermore, it was claimed that oscillation in DOT values could lead to loss of alginate molecular weight from 350 kDa to 240 kDa only with 1 % of deviation from the set DO value (Trujillo-Roldán et al., 2001). As a consequence, it is needed to control oxygen level precisely to produce constant quality product since polymerization/degradation of alginate is strongly influenced by oxygen level.

It is critical to know the monomer block distribution of alginate since the relative amount of the monomers affects greatly its features, so the final use

of the polymer. For instance, MG-blocks form the most flexible chains and they are more soluble at lower pH than the other two block types. Alginates having high GG-blocks constitute rigid gels fairly suddenly as calcium ion concentration is steadily increased. On the other hand, for the alginate with mainly MM-blocks, it is quite the opposite because in that case the gel forms gradually and it is softer and more elastic (Ertesvåg and Valla, 1998). Another important example is that this polysaccharide has an ability to activate immune cells to secrete cytokines. It is interesting that the immune system responds depending on the monomer distribution of the alginates. While MM-blocks seem to have the highest response, GG-blocks appear to be non-stimulating. Actually, due to some negative effects of GG-blocks like antibody generation, they cannot be accepted in therapeutic preparations (Sabra et al., 2001). Similarly, due to the gellation property of polymer with calcium and possibly by other metals too, the monomer distribution is thought to be very critical for environmental applications as well. Therefore, the effect of various DOT values on monomer distribution of alginate was examined. Alginate produced by *Azotobacter vinelandii* ATCC® 9046 was found to be almost homopolymeric in nature that is either contain MM- or GG-blocks with very little amount of MG-blocks. Together with a periplasmic mannuronan C5-epimerase (AlgG), *Azotobacter* additionally modifies alginate by extracellular epimerases (AlgE1-7). Interestingly, each of these seven enzymes introduces a different characteristic sequence distribution of G-residues in the polymannuronate substrate in vitro (Remminghorst and Rehm, 2006). For instance, AlgE2 and AlgE5 seem to predominantly epimerize M residues located next to a preexisting G residue, generating GG-blocks, whilst AlgE4 does not do this, and therefore forms alternating sequences, MG-blocks in the reaction product (Ertesvåg et al., 2009). Thus, it can be commented considering our data that cultivation conditions used in this study favor the enzymes to form mainly GG-blocks. It was found that MM-blocks content decreased from 44 % to 14 % between 24 and 72 hours while GG-blocks content increased up to 84 % at 1 % DOT. Epimerization of MM-blocks into GG-blocks was even faster in the case of 10 % DOT than 1 % DOT, such that all alginate produced at 48 hours had all GG-blocks. On

the other hand, at 3 % DOT, maximum GG-blocks content was obtained as 71 % which was lower than at 1 % DOT at the end of fermentor operation. Furthermore, at 5 % DOT, the value being the most suitable condition for alginate production, alginate was mainly composed of MM-blocks showing a maximum of 61 % GG-blocks at 56 hours. According to current knowledge, there is no study conducted to examine the monomer distribution of alginate produced at controlled DOT, thus, no comparison can be made. To sum up, mannuronic acid was continually epimerized into guluronic acid blocks and it was found that highest GG-block values were achieved at two extreme values of DOT; 1 and 10%. It might be inferred that bacteria need to synthesize alginate having high GG-block content in order to protect themselves from the adverse environmental conditions, for example, stress caused by high oxygen concentration or limited oxygen levels. Although alginate production was not efficient at these DOT values, the polymer concentrations were lower than at 5 % DOT, obviously not the quantity of the polymer but the quality seems to be the main determinant for effective protection of the bacteria by alginate synthesis. It is known that GG-blocks in the polymer chain constitute gel networks with calcium ions found in the culture medium and these gels are dense and strong compared to mannuronic acid gels which are generally softer (Ertesvåg and Valla, 1998). As a result, it may be argued that since guluronic acid gels are highly protective against adverse environmental conditions than mannuronic acid gels, *Azotobacter vinelandii* synthesized high GG-blocks alginates at extreme conditions of this study as well. On the overall with all these evaluations, 5 % DOT was found to be the optimum from among all DOT values studied considering both the maximization of alginate yield and culture broth viscosity although GG-block content of the alginate was lower than some of the other DOT values.

4.2.2. INVESTIGATION OF THE ROLE OF DIFFERENT MIXING RATES

Agitation of culture medium during fermentation processes is crucial to provide a homogenous environment for cultivation and to reduce the mass transfer limitation caused by ineffective mixing. Thus, microorganisms can take necessary nutrients for their growth and product formation. Agitation speed was found to influence alginate synthesis particularly the quality of the polymer produced. According to our knowledge, there are only two studies dealing with the effect of agitation on alginate quality under controlled DOT conditions and none of them monitored block distribution of the biopolymer. Thus, in this part of the study, alginate production was carried out by *Azotobacter vinelandii* ATCC® 9046 at 5 % of air saturation to find out the influence of variation in stirring speeds of 200, 400, and 700 rpm in a laboratory fermentor. The other operating conditions for this part were 20 g/L of initial sucrose concentration and 50 mg/L of calcium concentration. While the results for 700 rpm and 200 rpm are given here, the reader is referred to section 4.2.1.3 for 400 rpm results.

4.2.2.1. Results Obtained at 700 rpm

Alginate production was investigated at a relatively high shear rate, 700 rpm, by *Azotobacter vinelandii* ATCC® 9046 under controlled environmental conditions. Variation in concentration of bacteria, alginate and sucrose during 72 hours of fermentation process is shown in Figure 4.29. A small lag phase was observed during 6 hours period and bacterial growth was exponential up to 24 hours, at which time, 9.87 g/L of bacteria concentration was found as maximum. In addition, maximum specific growth rate was $0.121 hr^{-1}$. After this time cell lysis possibly started which was particularly fast till the end of 48 hours. On the other hand, effective alginate production started at 24 hours since the polymer concentration was only 0.35 g/L at this time. Alginate mass was increased until 56 hours period up to 1.46 g/L and

almost all of alginate synthesis was performed by non-growing bacteria. It seemed that high shear rate, 700 rpm, appeared to be not very efficient for alginate production with a very low yield of 0.244 g alginate/ g bacteria. This might be because of the fact that bacteria form smaller cells at high agitation levels whilst aggregates were found at low mixing speeds which cause transfer limitations. For instance, *Azotobacter vinelandii* DSM® 93-541b cells formed flocs with cell surface area of 33.8 μm^2 at 300 rpm compared to that of 8.9 μm^2 at 800 rpm with no aggregate formation (Sabra et al., 2000). This means that at lower agitation speed values, although oxygen concentration in the bulk medium was 5 % of air saturation, it would be lower inside the cells. When the mixing speed was increased, the bacteria was easily exposed to higher DOT and needed to struggle with this high DO levels. Thus, at 700 rpm, *Azotobacter vinelandii* ATCC® 9046 required to remove high oxygen from the surface causing wastage of the carbon source during increased respiration instead of alginate biosynthesis especially at the beginning of fermentation process. Sucrose was the carbon source in our study and about all of it was consumed at 72 hours which was reduced to 1.04 g/L effectively.

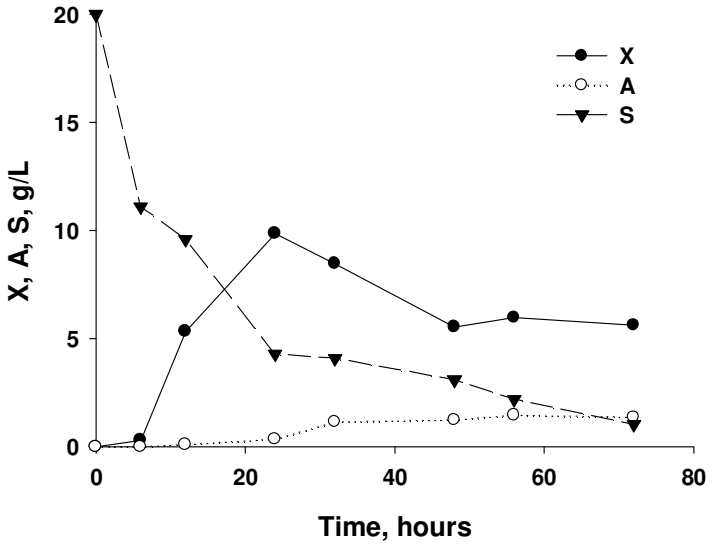

Figure 4.29. Change in concentration of bacteria (X), alginate (A), and sucrose (S) at 700 rpm

Culture broth viscosity was followed to supply information about the polymerization degree of alginate as viscosity is one of the main parameters in deciding commercial use of the biopolymer. Figure 4.30 represents the trend for viscosity of culture broth during the course of fermentation at 700 rpm and 5 % DOT. Very low culture broth viscosities were observed except for the value recorded at 24 hours. Even at this time, the value was measured only as 4.26 cP. Afterwards, the viscosity declined sharply probably due to activity of alginate lyases which are responsible for breaking of bonds between alginate monomers. Low viscosity values may be attributable to DOT level that the bacterium actually exposed during high shear rate. As it was claimed in the previous experiments conducted to elucidate the effect of DOT on alginate quality, alginate polymerization/degradation was greatly affected by oxygenation degree. Although bulk medium DO level was set to 5 % of air saturation which might not impact by the cells at low agitation speed; on the contrary it will be highly sensed by the microorganism at 700 rpm. Thus, polymerization was

observed to be weak at higher oxygenation conditions with elevated shear rate.

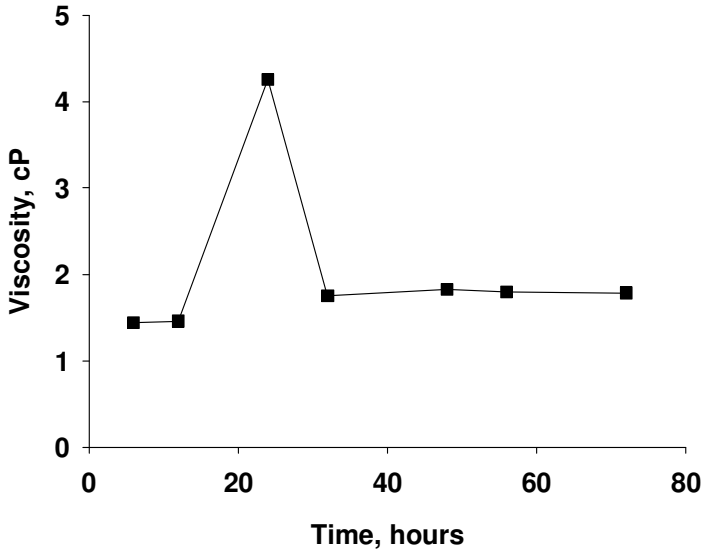

Figure 4.30. Culture broth viscosity variation during the course of fermentation at 700 rpm

Variation in block distribution of alginate produced by *Azotobacter vinelandii* ATCC® 9046 at 700 rpm is illustrated in Figure 4.31. As it is seen from the figure, the polymer synthesized which was even harvested at early fermentation times effectively epimerized into guluronic acid with considerably low MM-block. The amount of GG-block was 78 % at 24 hours and it was steadily improved and reached to 87 % at the end of fermentation batch. Meanwhile, MM-block content increased a little from 18 to 21 % and then dropped to 13 % at 72 hours. On the other hand, amount of alternating sequences, MG-block, was observed almost zero possibly depending on the high activity of epimerases particularly introducing GG-block rather than MG-block into alginate chain. It appeared that *Azotobacter vinelandii* ATCC® 9046 required a stiff coating to cope with the stress caused by high shear

rate applied during the experiment. Sabra et al. (2000) suggested that together with quantity of alginate, quality of the synthesized polymer was even more important to protect the bacteria against high oxygen level due to high mixing rates such as 700 rpm.

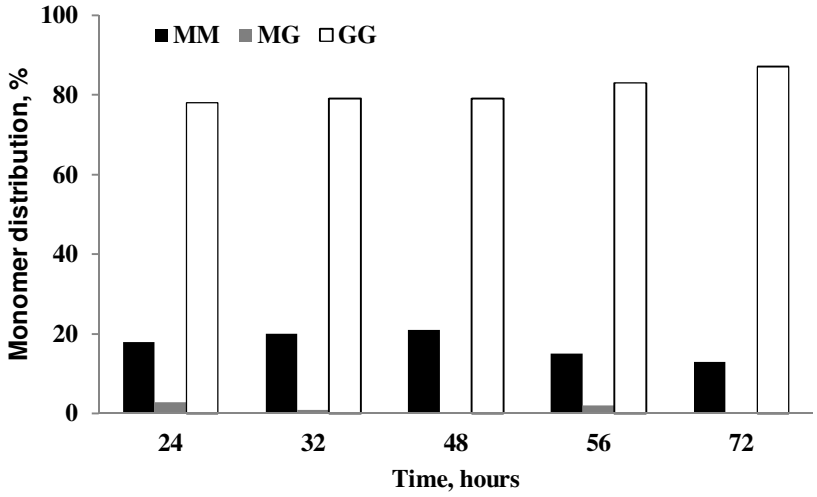

Figure 4.31. Monomer distribution of the polymer obtained at 700 rpm

4.2.2.2. Results Obtained at 200 rpm

Alginate synthesis was also evaluated by *Azotobacter vinelandii* ATCC® 9046 at 200 rpm in a fermentor at 5 % DOT. Figure 4.32 depicts variation in concentration of bacteria, alginate and sucrose during 72 hours of fermentation. The data indicated that low speed of mixing might have caused some limitations such as transfer of nutrients and gases through bacterial membrane due to formation of cell aggregates since bacterial growth rate was observed to be smaller even after 24 hours period. Highest cell mass was achieved at 32 hours as 10 g/L and a quite sharp decrease followed this period till the end of the fermentation. Furthermore, specific growth rate was found as 0.085 hr^{-1} which was quite lower compared to 0.121 hr^{-1} calculated

at 700 rpm. Alginate production started with growth and continued after the growth had stopped reaching a maximum of 2.51 g/L at 56 hours. This value was 1.7 times higher compared to the amount of alginate obtained at 700 rpm. Also, the yield based on biomass was calculated as 0.339 g alginate/ g bacteria. About 67 % of the total production of alginate was observed to be growth associated so alginate formation was continuous throughout the fermentation process. Utilization of sucrose was faster during the first 24 hours. Almost all of it was consumed with only 2 g/L of sucrose being left at 72 hours corresponding to 90 % of utilization.

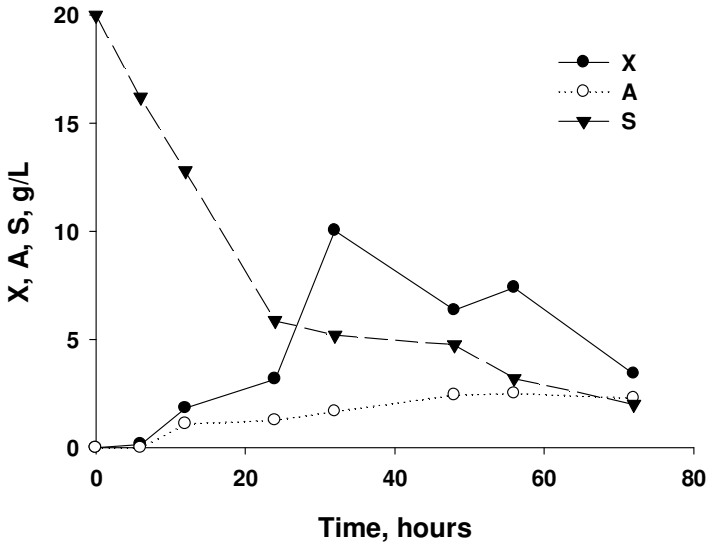

Figure 4.32. Change in concentration of bacteria (X), alginate (A), and sucrose (S) at 200 rpm

Culture broth viscosity was also examined during the course of fermentation (Figure 4.33). Viscosity of culture broth was much higher at 200 rpm compared to the case observed at 700 rpm that very low viscosity values were obtained in such a case. Broth viscosity was continuously enhanced till the end of the fermentation. The increase was faster particularly between 24 and 48 hours which was compatible with trend in alginate synthesis shown in

Figure 4.33. As a result, the highest viscosity value was measured as 12.03 cP at 72 hours of fermentation process. Interestingly, no decrease in viscosity was observed unlike the other experiments conducted up to this point. This might be explained by transfer limitations occurring between culture medium and the cells due to low agitation speed. Although polymerization of alginate in *Azotobacter vinelandii* is known as an intracellular process, mainly extracellular enzymes, alginate lyases, are found to be responsible for the degradation of the polymer. Their transfer into bulk medium may be prevented at 200 rpm and get even more limited with increasing viscosity. In other words, the reduction in culture broth viscosities may not have occurred at low shear rate owing to the limited transfer of alginate lyases into extracellular environment.

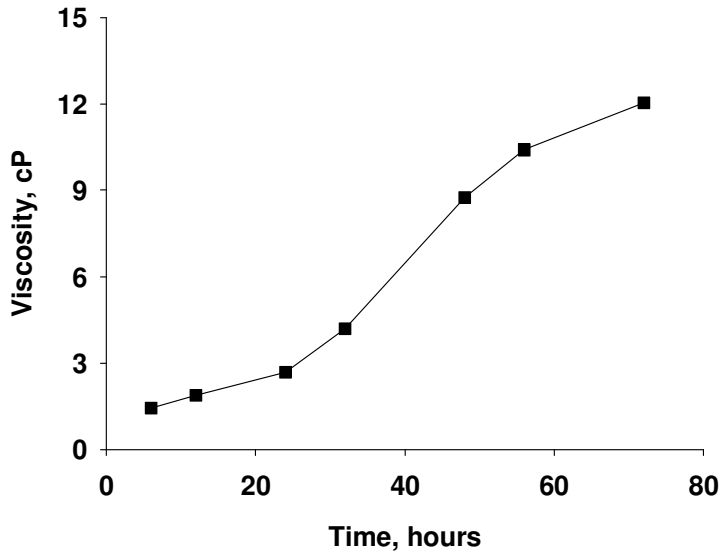

Figure 4.33. Culture broth viscosity variation during the course of fermentation at 200 rpm

Monomer distribution of alginate which was produced at 200 rpm by *Azotobacter vinelandii* ATCC® 9046 was investigated to find the relative

amount of MM-, MG-, and GG-blocks constituting alginate. Figure 4.34 shows changes in block distribution of the polymer during 72 hours of fermentation batch. None of the alginate harvested at any time contains MG-blocks. It appeared that at low agitation speed the bacteria were not required to have an alginate with high GG-block content as high as at 700 rpm because highest epimerization level obtained at 200 rpm was 76 % of GG-block. This value was reached to 78 % even after 24 hours at 700 rpm. It might be explained that *Azotobacter vinelandii* ATCC® 9046 needed a stronger coating against high shear rate compared to low agitation speed. On the other hand, amount of MM-block in alginate was reduced from 35 % to 24 % to the end of fermentation process.

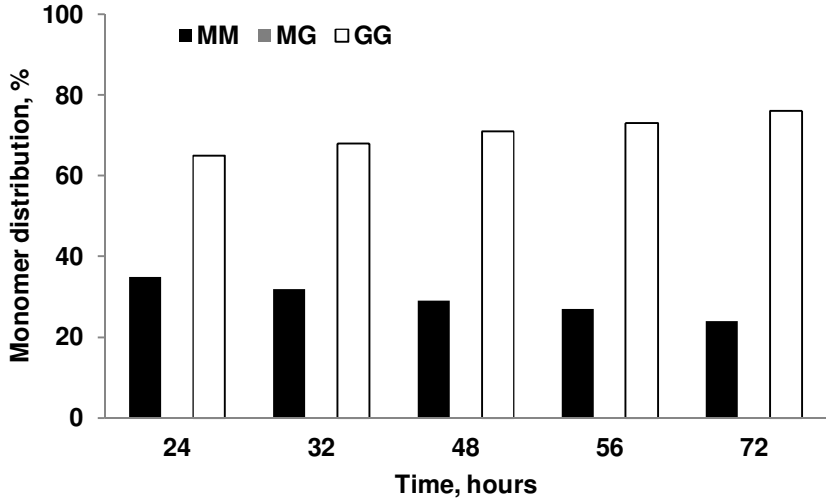

Figure 4.34. Monomer distribution of the polymer obtained at 200 rpm

4.2.2.3. Comparison of Alginates Produced at Various Stirring Speeds

Alginate production by *Azotobacter vinelandii* ATCC® 9046 was examined at three different agitation speeds, 200, 400, and 700 rpm, to elucidate the effect of variation of mixing regime on alginate quality and quantity in a laboratory fermentor at 5 % of air saturation (Table 4.9). Growth rate of the

bacteria was improved by increasing agitation speed from 200 to 700 rpm. It was obvious that bacteria was grown quite slower at 200 rpm compared to the other values tested since cell concentration was only 3.16 g/L after 24 hours at 200 rpm while this value was measured as 9.87 g/L at 700 rpm at the same time. Furthermore, maximum specific growth rate was 0.085 hr^{-1} at 200 rpm whilst it was enhanced up to 0.121 hr^{-1} at 700 rpm. Similarly, Pena et al. (2000) found that the highest specific growth rate of *Azotobacter vinelandii* ATCC® 9046 was 0.23 hr^{-1} at 700 rpm, on the other hand, at 300 rpm it was recorded as 0.14 hr^{-1} at constant DOT of 3 %. Therefore, one can say that low agitation speed might influence transfer rates of some crucial nutrients and gasses to the bacteria because of limited mixing regime occurring in the bulk medium. However, for the growth of bacteria, it appeared that only 200 rpm value was not sufficient for effective mixing due to the fact that growth pattern and maximum cell concentration were close to each other at both 400 rpm and 700 rpm. Highest bacteria concentration was 8.7 and 9.87 g/L at 400 rpm and 700 rpm after 24 hours, respectively, while it was recorded as 10 g/L of after 32 hours at 200 rpm. When the exponential growth phase was ended, cell mass began to decrease with lysis and it was continuous to the end of fermentation batch.

Alginate concentration was followed to evaluate alginate quantity by changing stirring speed from 200 and 700 rpm maintaining constant DOT at 5 % of air saturation by *Azotobacter vinelandii* ATCC® 9046. Alginate production obtained at different mixing speeds was greatly varied. Moderate level of stirring, 400 rpm, resulted in optimum alginate production. On the other hand, although bacterial growth was somewhat limited at 200 rpm, it was found to be better for alginate synthesis compared to 700 rpm which was evidently not suitable for the production of alginate at 5 % DOT. Maximum alginate concentration was achieved as 4.51 g/L at 400 rpm at the end of fermentation, which was only 1.46 g/L at 700 rpm and 2.51 g/L at 200 rpm at 56 hours. Accordingly, the highest yield based on cell mass was observed at 400 rpm as 1 g alginate/ g bacteria which were reported in other studies in the range of 0.3-1.28 g alginate/ g bacteria depending on

experimental conditions (Pena et al., 2000; Parente et al., 2000). Most of the total polymer production was associated with growing bacteria while non-growing bacteria contributed just 30 % of total production during 72 hours. As a conclusion, alginate synthesis was stimulated with increasing agitation speed from 200 to 400 rpm and further elevation of shear rate reduced the amount of the polymer produced at 5 % of air saturation.

This probably is attributable to differences at diffusion rates of necessary nutrients and gases at various mixing speeds in the medium. Bacteria form aggregates at low agitation speed that limit passage of materials and gases between medium and the cells (Sabra et al., 2000) that eventually reduced alginate production yield compared to moderate level of mixing, 400 rpm. At high shear rates, the cells are smaller with no flocs which reduce the problems associated with diffusional limitation. However, the bacteria are required to struggle with higher oxygen concentration compared to lower stirring speeds. This might decrease the amount of sucrose utilized for alginate production since it is wasted during high respiratory activity.

Cultivation medium was supplemented with sucrose as a carbon source to produce alginate by *Azotobacter vinelandii* ATCC® 9046 and variation in concentration of sucrose was observed under different agitation speed at 5 % DOT. About all of the applied sucrose, initially 20 g/L, was utilized at the end of fermentation for all of the stirring speeds. At 72 hours, only 2 g/L of sucrose was left behind at 200 rpm while it was measured as 1 g/L at 700 rpm. The yield based on sucrose utilization was the best at 400 rpm as 0.246 g alginate/g sucrose. Sucrose consumption rate was higher during early fermentation particularly up to 24 hours. Moreover, it was found that the higher the agitation speed the higher the sucrose utilization rate. This behavior might be explained by wastage of the carbon source especially at high mixing rates because at low agitation the cells are exposed to lower oxygen levels due to diffusional limitations. This resulted in decrease in requirements for respiratory protection so the sucrose consumption.

Table 4.9. Comparison of some results for mixing speed experiments

Parameters	**RPM**		
	200	**400**	**700**
Max biomass conc achieved (g/L)	10.00	8.70	9.87
Max alginate conc achieved (g/L)	2.51	4.51	1.46
Time to achieve max alginate conc (hr)	56	72	56
Max alginate yield (g alginate/g bacteria)	0.339	1.00	0.244
Max broth viscosity (cP)	12.03	24.61	4.26
Time to achieve max broth viscosity (hr)	72	56	24
Max GG-block content (%)	76	61	87
Time to achieve max GG-block content (hr)	72	56	72

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Viscosity of culture broth was also monitored during alginate production by *Azotobacter vinelandii* ATCC® 9046 at 5 % DOT with changing mixing speeds in the range of 200 and 700 rpm. Viscosity data gives information about the polymerization degree of alginate and affect the final use of the polymer. Best polymerization was achieved at moderate level mixing, 400 rpm that culture broth viscosity was increased up to 24.61 cP until 56 hours than then it dropped. A reduction in viscosity was also observed after 24

hours at 700 rpm which occurs as a result of activity of extracellular alginate lyases. In addition, high agitation speed led to a little polymerization of alginate corresponding to the lowest culture broth viscosity values compared to the others. The maximum value for viscosity was only 4.26 cP at 700 rpm and it was around 5.8 times lower than 400 rpm. Likewise, in the study of Pena et al. (2000), alginate was produced by *Azotobacter vinelandii* ATCC® 9046 at 5 % DOT and under two different agitation speeds, 300 rpm and 700 rpm. They suggested that the viscosity of the culture broth was higher in the cultures grown at low agitation speed conditions than those grown at high agitation speeds. Their results were also compatible with molecular weight data that 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. On the contrary, molecular weight of alginate was improved with increasing mixing speed from 300 rpm to 700 rpm in the presence of inorganic nitrogen source in batch fermentation without DOT control (Parente et al., 2000). However, not only the change in mixing rates was responsible from these results but also uncontrolled DOT might affect polymerization degree. On the other hand, culture broth viscosity was continually improved throughout the fermentation process under low agitation. The highest culture viscosity was 12.03 cP at 200 rpm, which was quite lower compared to polymerization level obtained at 400 rpm.

One of the significant properties affecting commercial usage of alginate is gelling capacity that is mainly determined by guluronic acid content of the polymer. Thus, block distribution of alginate was assessed and compared during 72 hours of fermentation by *Azotobacter vinelandii* ATCC® 9046 at different agitation speeds and 5 % DOT. In fact ours is the first study in the literature measuring the monomer distribution of alginate at various mixing speeds while all the other culture conditions were controlled. By comparing the results 400 rpm was found to be the best condition for alginate formation compared to the others. Furthermore, at moderate level of mixing, alginate had almost equal amount of MM- and GG-block with very low MG-block content. On the other hand, a paramount epimerization of alginate was

observed at 700 rpm leading to the highest amount of GG-blocks as 87 % at 72 hours of fermentation. Moreover, at 200 rpm, MM-blocks were decreased from 35 % to 24 % while GG-blocks were increased from 65 % to 76 % which was only 10 % lower than that obtained at 700 rpm. It seems that when the environmental conditions become worse like high shear stress due to elevated mixing speeds and/or mass transfer limitations as a result of low agitation, the bacteria need a more rigid coating. It is known that alginate with high guluronic acid content form 'egg box' together with calcium ion in the bulk medium producing dense gels to protect themselves from adverse conditions.

4.2.3. OPTIMIZATION OF CARBON SOURCE – SUCROSE - CONCENTRATION

Azotobacter vinelandii is a bacterium which is able to utilize a wide range of carbon sources for the production of alginate even the ones that are difficult to degrade (Moreno et al., 1990). Dose of carbon sources applied to produce alginate also influenced the amount of alginate and its quality. In this study, a simple carbohydrate, sucrose, was selected as the carbon source for the production of alginate by *Azotobacter vinelandii* ATCC® 9046. The effect of variation in the concentration of the carbon source was examined in the range of 10 to 40 g/L. During experiments, DOT was maintained constant at 5 % of air saturation with mixing at 400 rpm and 50 mg/L of calcium concentration. Only the results for 40 g/L and 10 g/L of sucrose concentration are given here, the reader is referred to section 4.2.1.3 for 20 g/L results.

4.2.3.1. Results Obtained at 40 g/L of Sucrose

Alginate production was investigated by *Azotobacter vinelandii* ATCC® 9046 at 5 % of air saturation and 400 rpm in a laboratory fermentor. In the first set as already discussed in the previous parts, 20 g/L of sucrose was used.

Next, sucrose concentration was applied as 40 g/L i.e. the strength of the medium was doubled in terms of carbon concentration and the results are shown in Figure 4.35. It appeared that a small lag period was observed for bacterial growth during 6 hours similar to the previous observations. After that cell concentration increased up to 32 hours reaching 7.08 g/L then interestingly, some fluctuations were shown by the data and maximum bacterial mass was eventually obtained at 56 hours as 9.5 g/L. This might be explained by the fact that the cell concentration data at 56 hours was either an experimental error or it was claimed that PHB accumulation could be the reason for increase in cell mass through the end of fermentation (Segura et al., 2003). On the other hand, alginate was synthesized continuously until 72 hours with an early lag phase like bacterial mass. Highest alginate concentration was achieved as 4.79 g/L and alginate production was somehow faster between 24 and 48 hours. Sucrose concentration was doubled in this trial compared to the first set. It was reduced to 9 g/L at the end of the fermentation which corresponded to 78 % of utilization of sucrose. In other words, 22 % of sucrose was left in the medium at the end of 72 hours.

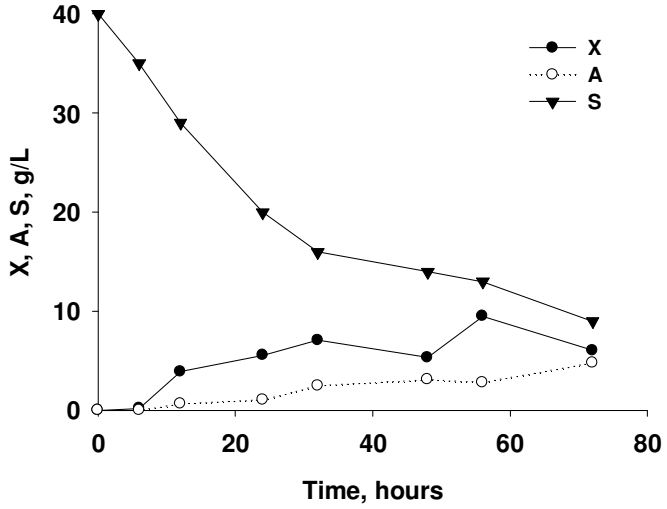

Figure 4.35. Variation in concentration of bacteria (X), alginate (A), and sucrose (S) at 40 g/L of sucrose

Culture broth viscosity was examined during alginate production at 40 g/L of sucrose (Figure 4.36). As is shown in the figure, a steep increase in broth viscosity was shown until 32 hours of fermentation batch. The maximum value was measured as 4.51 cP which indicated quite low degree of polymerization. After that, viscosity declined sharply and this was continuous until 72 hours. Broth viscosity was reduced down to 1.85 cP at this time due to the possible action of alginate lyases which caused breakage of the bonds between alginate monomers. It is thought that the length of alginate chain decreased and it resulted in lower viscosity values.

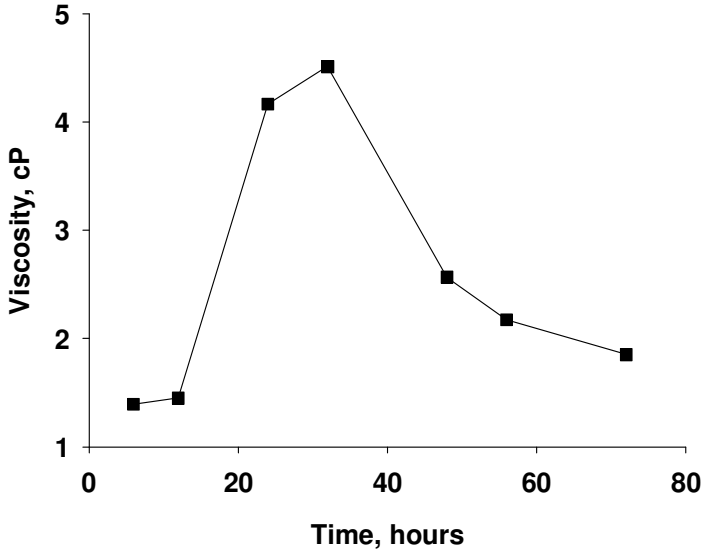

Figure 4.36. Culture broth viscosity changes during fermentation using double sucrose concentration

Quality of alginate was also followed to determine block distribution of the polymer during 72 hours of fermentation process. The results for the effect of using double sucrose dose on monomer distribution of alginate produced by *Azotobacter vinelandii* ATCC® 9046 are depicted in Figure 4.37. At the beginning of fermentation, alginate was composed of MM- and GG-blocks and it was rich in guluronic acid being 63 % at 24 hours. Then, rate of

epimerization seemed to decrease leading to an increase in MM-block content of alginate from 37 to 55 % with a little amount of MG-blocks (5 %). Alginate harvested at 48 hours had 23 % of MG-block which was the highest value obtained up to this part of the work. At the end of the fermentation batch, a polymer with moderate level of GG-block could be obtained by using 40 g/L of sucrose concentration. Fraction of GG-block was increased to 54 % at 72 hours, whilst MG-block content was reduced to 9 %.

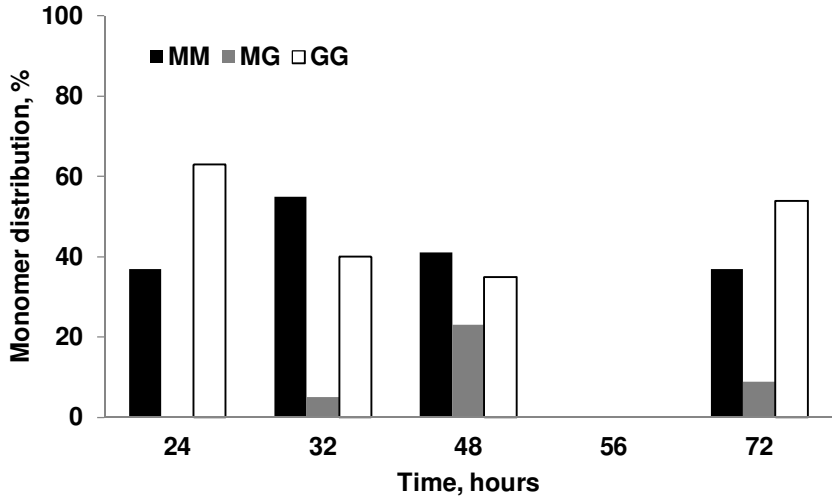

Figure 4.37. Monomer distribution of the polymer observed at 40 g/L of sucrose

4.2.3.2. Results Obtained at 10 g/L of Sucrose

In the last part of carbon dose effect, alginate was produced at low sucrose concentration, 10 g/L, by *Azotobacter vinelandii* ATCC® 9046 at constant DOT of 5 % and 400 rpm. Changes in bacteria, alginate and sucrose concentration are illustrated in Figure 4.38. Growth pattern of the bacteria was again somewhat unexpected that it showed some oscillations during the course of fermentation. The highest cell concentration was obtained at 56 hours as 8.02 g/L. After that a sharp decline in bacterial mass was obtained

due to cell lysis. Since the amount of sucrose applied was somewhat limited, this condition was found to be not efficient for alginate synthesis. Alginate production was observed to be continuous during 72 hours with the maximum alginate formation of 1.63 g/L. On the other hand, as expected, almost all of sucrose was consumed during the course of fermentation. Sucrose concentration was measured at 72 hours as 0.8 g/L that means 92 % of sucrose was utilized for bacterial growth and alginate production.

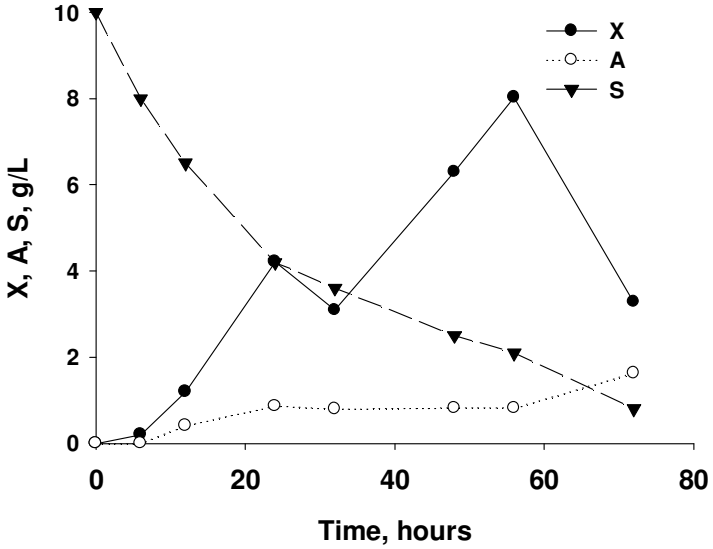

Figure 4.38. Bacteria (X), alginate (A), and sucrose (S) concentration variation at 10 g/L of sucrose

Viscosity of culture broth was monitored to obtain information about polymerization level of alginate produced by *Azotobacter vinelandii* ATCC® 9046 at low sucrose dosage. Figure 4.39 shows the results obtained during 72 hours of the experiment. Polymerization of alginate was improved during the first 24 hours. The highest viscosity value was measured as 2.42 cP. Actually, such a viscosity level indicates a very low degree of polymerization, so, this alginate may not be possibly valuable commercially to be used as a viscosifier. After 24 hours of fermentation batch, a decrease was observed in

broth viscosity. It was continually reduced to the end of fermentation possibly due to the activity of alginate degrading enzymes.

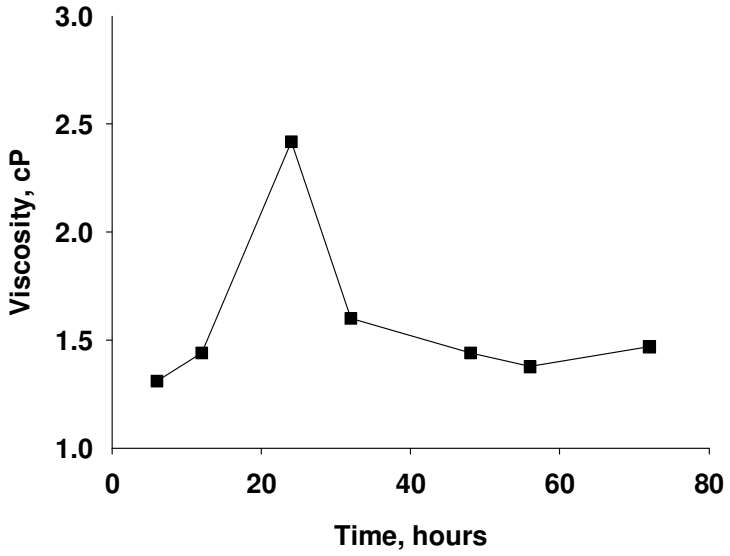

Figure 4.39. Changes in viscosity of culture broth using double sucrose concentration

Effect of using limited carbon source on block distribution of alginate produced by *Azotobacter vinelandii* ATCC® 9046 was also evaluated for alginate quality. Figure 4.40 represents the results obtained from the fermentation batch during 72 hours. The biopolymer synthesized was observed to be rich in guluronic acid which was 80 % even after 24 hours contrary to the results obtained at double sucrose concentration. This result indicates a very efficient epimerization of alginate. This behavior can be attributable to the conditions in which bacteria need to share out their limited carbon source between maintenance and product formation. Thus, bacteria may require an alginate having high GG-block under this stressful circumstance that GG-block is able to form strong coating around the cells with calcium ions in the medium. Furthermore, the highest GG-block content

was achieved after 32 hours as 89 %. On the other hand, the entire polymer produced was epimerized into GG-block other than MG-block. Therefore, fraction of this block was zero throughout the fermentation course. Accordingly, mannuronic acid content was reduced from 20 % to 16 % at the end of the process.

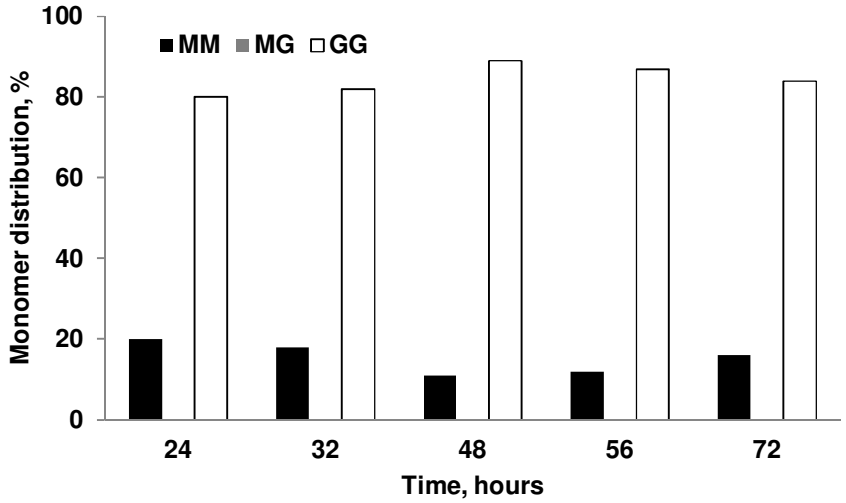

Figure 4.40. Block distribution of the polymer during the course of fermentation at 10 g/L of sucrose

4.2.3.3. Evaluation of the Optimal Carbon Dose

Sucrose was used as a carbon source in order to produce alginate by *Azotobacter vinelandii* ATCC® 9046 under constant DOT, 5 % of air saturation, and at 400 rpm stirring. To evaluate the optimum dose for effective alginate production, three different concentration of sucrose was applied namely 10, 20, and 40 g/L. The results obtained are summarized in Table 4.10 listing the maximum values of parameters obtained in the study. Both at low and high doses of sucrose, some fluctuations in cell concentration were observed. Regardless of these oscillations in bacterial

mass, cell growth appeared to be continuous until 56 hours at 10 and 40 g/L of sucrose. On the contrary, bacteria grew exponentially up to 24 hours in the case of moderate sucrose level. Maximum bacteria concentration achieved at 10 g/L of sucrose was 8.02 g/L, while it was 9.50 g/L at 40 g/L which was only about 20 % higher than the former although the substrate concentration was increased by 4 times. Furthermore, in all cases, a cell lysis was observed and it was more obvious at 20 g/L of sucrose.

Alginate production by *Azotobacter vinelandii* ATCC® 9046 at various sucrose concentrations was examined during 72 hours of fermentation. At all doses of sucrose, the highest polymer concentration was achieved at the end of the fermentation process. Results showed that 10 g/L of sucrose was not enough or the bacteria were not able to direct the limited carbon source efficiently into product formation. Therefore, it resulted in only 1.63 g/L of alginate synthesis at 72 hours. This value was comparably lower than the other two doses. The optimum sucrose in terms of alginate production was found to be 20 g/L, since alginate formation was improved up to 4.51 g/L, most of which was obtained during growth of the bacteria. The last dose evaluated for the effect of the carbon source on alginate production was 40 g/L. However, alginate synthesis was not enhanced as we expected it to be from doubling of the carbon dose. In fact it was even slightly lower until 72 hours compared to the other doses. At the end of fermentation, the highest biopolymer concentration reached 4.79 g/L. Furthermore, the yield was also better at 20 g/L of sucrose that the value was found as 1 g alginate/ g bacteria compared to 0.793 g alginate/ g bacteria at 40 g/L of sucrose. As a result, one can say that 20 g/L of sucrose was sufficient for alginate production. Similar results were obtained during alginate production by *Azotobacter vinelandii* NCIB 9068 and *Azotobacter vinelandii* LI (Savalgi and Savalgi, 1992). Burk's medium was supplemented with 2 % and 4 % of sucrose.

Table 4.10. Comparison of some results for sucrose dose experiments

Parameters	**Sucrose (g/L)**		
	10	**20**	**40**
Max biomass conc achieved (g/L)	8.02	8.70	9.50
Max alginate conc achieved (g/L)	1.63	4.51	4.79
Time to achieve max alginate conc (hr)	72	72	72
Max alginate yield (g alginate/g bacteria)	0.496	1.00	0.793
Max broth viscosity (cP)	2.42	24.61	4.51
Time to achieve max broth viscosity (hr)	24	56	32
Max GG-block content (%)	89	61	63
Time to achieve max GG-block content (hr)	48	56	24

The same alginate yield was observed as 3.5 g/L at both 2 % and 4 % of the carbon source at 240 rpm in a shake flask. Furthermore, only a slight increase was observed from 4.2 g/L to 5.5 g/L by elevating sucrose concentration from 2 % to 4 % by *Azotobacter vinelandii* LI.

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Rate of sucrose utilization was faster for the first 24 hours due to exponential growth of bacteria. In addition, most of alginate was produced during

bacterial growth. Thus, about half of sucrose was consumed until 24 hours. After that sucrose concentration was still decreasing slowly although it was continuous throughout the fermentation. In the cases of 10 and 20 g/L, final sucrose concentration was 0.8 and 1.65, respectively, at 72 hours. That is, almost all of the sucrose was used during the experiment. However, at the highest dose, 40 g/L of sucrose, 9 g/L of sucrose remained in the medium. It appeared that it needed some further time for depletion and 72 hours of experimental time was not enough to see its further decrease. Alginate yield based on sucrose utilization was better at 20 g/L of sucrose as 0.246 g alginate/ g sucrose, while it was only 0.154 g alginate/ g sucrose at 40 g/L.

Viscosity of culture broth was monitored during alginate production by *Azotobacter vinelandii* ATCC® 9046 and compared here for various sucrose concentrations including 10, 20, and 40 g/L. At low and high levels of the carbon source, viscosities of culture broth were higher during early fermentation while the highest value was observed at 56 hours by the application of 20 g/L of sucrose. Since viscosity of the broth gives an idea about the polymerization degree of alginate synthesized during fermentation, it appears that a much better polymerization of alginate is achieved at 20 g/L of sucrose. The maximum viscosity value was observed as 24.61 cP at this concentration. On the other hand, culture broth viscosities only reached to 2.42 cP and 4.51 cP at 10 g/L and 40 g/L of sucrose, respectively. A reduction in viscosities was shown after certain times of fermentation because of the fact that there are some enzymes called alginate lyases which are capable of breaking the bonds between alginate monomers. Thus, the lower the molecular weight of the polymer is, the lower the viscosities in culture broth are.

Monomer distribution of alginate was found to be affected significantly by changing sucrose concentration. For example, carbon limitation resulted in an alginate having high guluronic acid content being around 80 %. GG-block content was improved up to 89 % at 48 hours in the case of 10 g/L of sucrose concentration with no alternating sequences, MG-blocks,

production. This might be due to the active enzymes responsible for the epimerization. It seemed that limited sucrose concentration favor the enzymes mainly producing GG-block and the bacteria could require a more stiff coating to resist that unfavorable condition. On the other hand, at the other sucrose concentrations tested, alginate was mainly synthesized as equal amount of mannuronic and guluronic acids. Most of which were composed of homopolymeric regions with very little amount of MG-blocks particularly at 20 g/L of sucrose. In the case of double carbon concentration, MG-block content was enhanced up to 23 % after 48 hours of fermentation. No comparison with the other studies can be made here since the effect of carbon dose on monomer distribution of alginate has not been investigated before.

4.2.4. VARIATION OF CALCIUM ION CONCENTRATION IN THE MEDIUM

Mannuronic acid is the first product of alginate biosynthesis and it is then epimerized into guluronic acid by a series of enzymes which are known as mannuronan C-5-epimerases. One of them, AlgG, is located in periplasmic space while the others, AlgE1-7, are secreted into extracellular environment during cultivation. Contrary to AlgG, these enzymes depend on calcium ion for their activity. According to our knowledge, there was no study dealing with the effect of calcium ion concentration on monomer distribution of alginate under controlled environmental conditions. Thus, in our work, this issue was investigated by changing calcium ion concentration in the applied medium, ranging from 25 to 100 mg/L. Alginate was synthesized under controlled DOT of 5 % air saturation and at 400 rpm and moderate sucrose concentration (20 g/L). In the parts below the results from 100 mg/L and 25 mg/L of calcium are discussed. The reader is referred to section 4.2.1.3 for the results of 50 mg/L of calcium ion concentration.

4.2.4.1. Results Obtained at 100 mg/L of Calcium Ion Concentration

Production of alginate by *Azotobacter vinelandii* ATCC® 9046 was conducted at 100 mg/L of Ca^{2+} concentration in a fermentor under controlled environmental conditions. This concentration was twice higher compared to the modified Burk's medium. By this way the effect of increase of calcium ion concentration in production medium was investigated. Figure 4.41 illustrates the variations in cell and alginate production and substrate utilization during the course of fermentation. From this figure, bacterial growth seemed to continue until 32 hours exponentially corresponding to highest bacteria concentration of 8.45 g/L and maximum specific growth rate was calculated as 0.103 hr^{-1}. After this period, some fluctuations in cell mass were obtained with a little decreasing trend in concentration of bacteria through the rest of the fermentation process. Thus, cell concentration was reduced to 4.18 g/L due to cell lysis. On the other hand, high calcium level did not favor alginate synthesis as it is seen in the figure. Highest alginate concentration was measured at 56 hours of fermentation as 1.77 g/L which was a bit decreased at 72 hours. According to these results, maximum alginate yield based on biomass was calculated as 0.24 g alginate/ g bacteria. Sucrose concentration was reduced from 20 g/L to 2.5 g/L at 72 hours of fermentation. Most of sucrose was consumed until 32 hours in agreement with alginate production and bacterial growth.

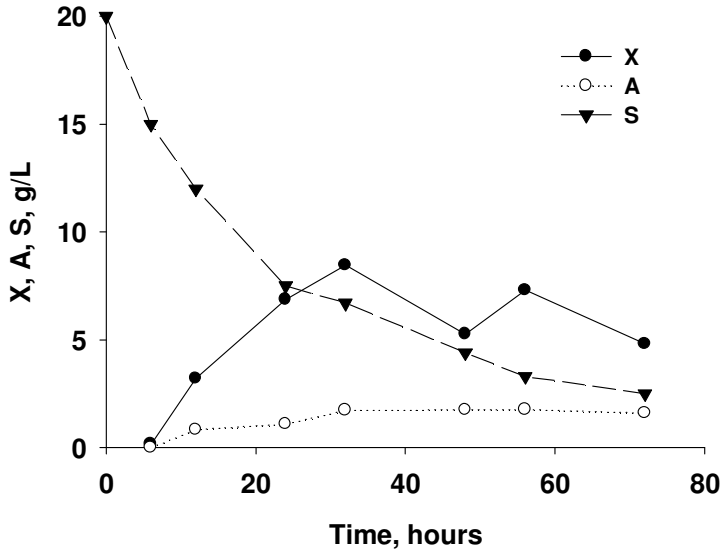

Figure 4.41. Variation in concentration of bacteria (X), alginate (A), and sucrose (S) at 100 mg/L of calcium ion concentration

Figure 4.42 shows changes in culture medium viscosity at 100 mg/L of Ca^{2+} concentration during 72 hours. As it is seen from the figure, polymerization of alginate caused an increase in culture broth viscosity values up to 24 hours. At this time, viscosity showed a maximum with the highest value of 6 cP. However, after this, a sharp declining period in viscosity was obtained especially until 48 hours. Culture medium viscosity was continued to drop until the end of the fermentation period. The reason for this might be explained by secretion of alginases by *Azotobacter vinelandii* ATCC® 9046 that lead degradation of polymer chain. Thus, viscosity of broth was reduced down to 1.75 at 72 hours of fermentation period.

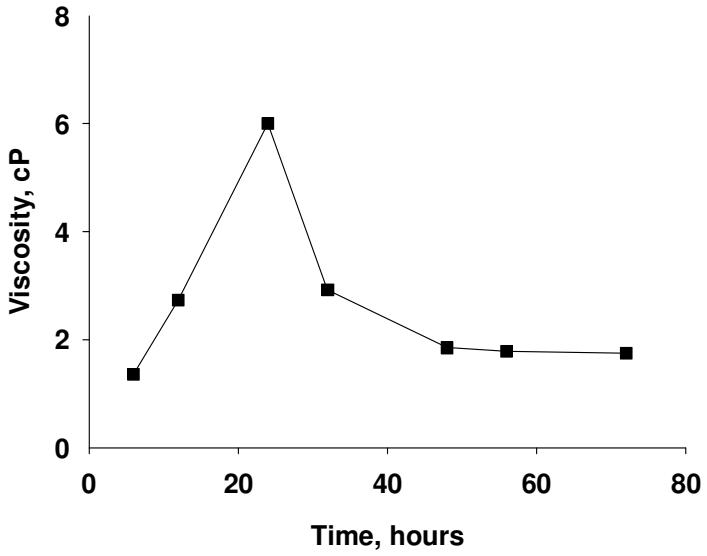

Figure 4.42. Culture broth viscosity changes during fermentation using 100 mg/L of Ca^{2+} concentration

Block distribution of alginate synthesized by *Azotobacter vinelandii* ATCC® 9046 in the presence of 100 mg/L of Ca^{2+} ion concentration is presented in Figure 4.43. All of alginates were composed of homopolymeric regions namely MM- and GG-blocks since none of the polymer was epimerized into alternating sequences, MG-blocks. In addition, the fraction of both monomer types illustrated small variations during 72 hours of fermentation. For example, content of MM-blocks was changed between 32 % and 41 % while amount of GG-blocks was varied in range of 59 % and 68 %. As a consequence, all of mannuronic acid was epimerized into GG-blocks, but one can say that epimerization of alginate was not efficient as expected from doubling of calcium concentration compared to the original production medium since it is a known fact that all of the epimerases depend on calcium ion for their activity. However, 100 mg/L of Ca^{2+} might be a high dose compared to the optimum value required for the active enzyme responsible for epimerization in our study. Therefore, high calcium dose may reduce the activity of the enzyme.

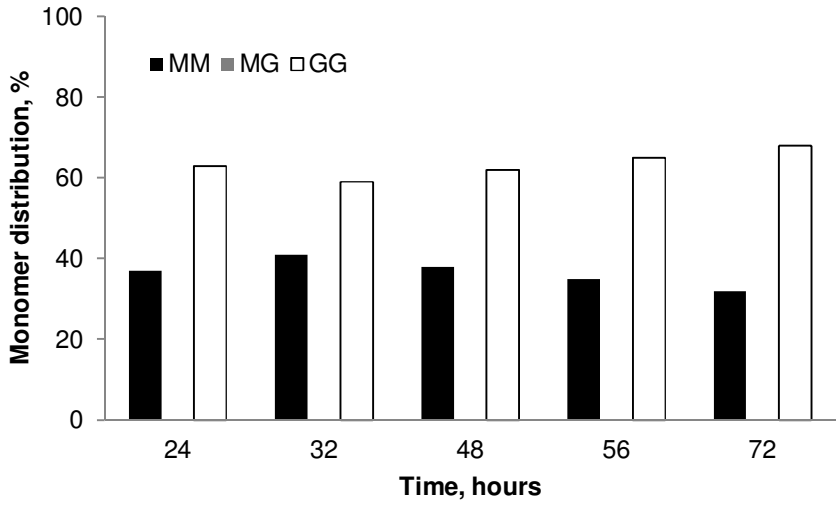

Figure 4.43. Monomer distribution of the polymer observed at 100 mg/L of Ca^{2+}

4.2.4.2. Results Obtained at 25 mg/L of Calcium Ion Concentration

Alginate biosynthesis was performed by *Azotobacter vinelandii* ATCC® 9046 with lower calcium concentration, 25 mg/L of Ca^{2+} at 5 % of air saturation and 400 rpm. The results related with changes in bacteria and alginate concentrations together with sucrose consumption during 72 hours are depicted in Figure 4.44. A small lag period was observed during the first 6 hours and then with exponential growth of the bacteria, cell mass reached its maximum value, 8.54 g/L, at 24 hours of fermentation. This value was almost the same obtained at 100 mg/L of Ca^{2+} after 32 hours. Moreover, a similar maximum specific growth rate was achieved at low calcium dose as 0.116 hr^{-1}. A slight cell lysis caused the reduction in bacteria concentration to 4.65 g/L at the end of the process. Alginate production was also delayed until 12 hours and all of the polymer was synthesized by growing bacteria. In other words, alginate formation was totally growth associated. Accordingly,

alginate concentration was enhanced up to 24 hours corresponding to the highest value of 2.65 g/L. After that amount of alginate was fluctuated a little throughout the fermentation and the concentration showed a small decrease at 72 hours. Furthermore, alginate yield based on biomass was calculated as 0.31 g alginate /g bacteria likewise the one obtained at 100 mg/L of Ca^{2+}. Utilization pattern and the amount of sucrose consumed at 25 mg/L of Ca^{2+} were also similar at both concentrations of calcium ion. Only 2 g/L of it remained in the medium at 72 hours which means 90 % of sucrose was used during the experiment.

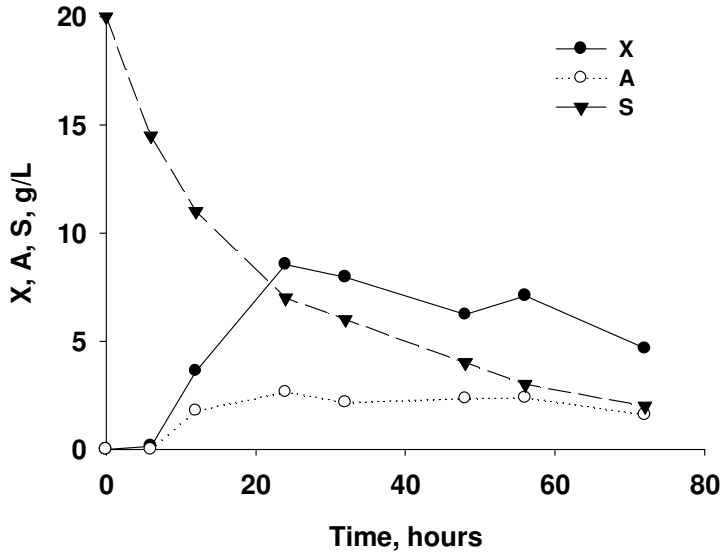

Figure 4.44. Bacteria (X), alginate (A), and sucrose (S) concentration variation at 25 mg/L of Ca^{2+}

Viscosity of cultivation medium was measured during the course of fermentation which was carried out at 25 mg/L of Ca^{2+} concentration (Figure 4.45). As it was a general trend observed in the experiments, viscosity had a maximum value around exponential growth of the bacteria. Then, with the

secretion of alginate lyases into extracellular medium, it was reduced since these enzymes are able to break the bonds between alginate monomers. Accordingly, viscosity of culture broth was improved until 32 hours reaching to the highest level of 29.44 cP. This value was also the maximum viscosity recorded ever. Thus, polymerization of alginate synthesized at this dosage, 25 mg/L of Ca^{2+}, was thought to be better compared to that achieved at high calcium level. After that, viscosity value decreased sharply until 48 hours and then the decrease continued slightly till the end of the fermentation. However, even at 72 hours, medium viscosity was measured as 14 cP that was a quite high value.

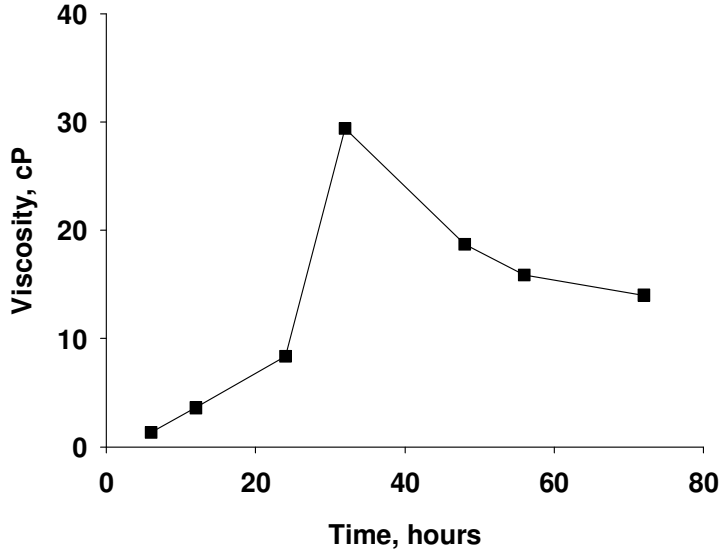

Figure 4.45. Changes in viscosity of culture broth at 25 mg/L of Ca^{2+}

Block distribution of alginate produced by *Azotobacter vinelandii* ATCC® 9046 at low calcium dose was followed to have an idea about quality of the polymer. Figure 4.46 shows the changes in the amount of MM-, MG- and GG-blocks of the biopolymer during 72 hours. It was the first time that an alginate rich in MM-blocks were obtained in this study. Up to now, only a small amount of MG-blocks was observed. Due to the fact that seven different enzymes (Alg E1-E7) are known to be responsible for the

epimerization of alginate in *Azotobacter vinelandii* and these are highly dependent on calcium ion for their activity. Therefore, the use of low calcium dose, 25 mg/L of Ca^{2+}, might be the reason for the epimerization towards MG-block rather than GG-block. Maximum amount of GG-blocks was only 21 % with 6 % of MG-blocks at 24 hours of fermentation. While the amount of MM-blocks decreased from 73 % to 60 % throughout the fermentation process, epimerization was particularly directed into MG-blocks that were improved up to 28 % at 72 hours. Furthermore, GG-blocks of the polymer were reduced down to 12 % at the end. Ramstad et al. (1999) claimed that the enzymes prefer an existing guluronic acid next to MM-blocks to epimerize if the concentration of calcium ions is low in the production medium. On the other hand, they are able to epimerize all monomers without having any preference in the case of high calcium levels.

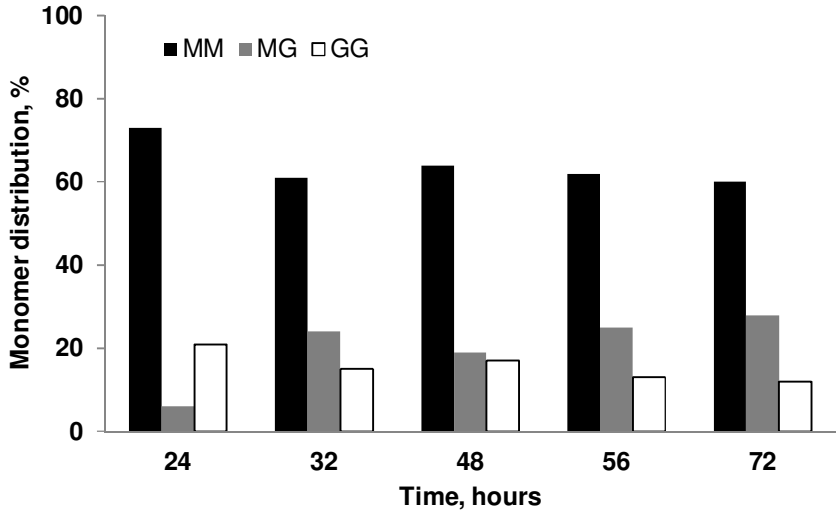

Figure 4.46. Block distribution of the polymer during the course of fermentation at 25 mg/L of Ca^{2+}

4.2.4.3. Comparison of Alginates Produced at Different Calcium Ion Concentration

Alginate production was investigated by *Azotobacter vinelandii* ATCC® 9046 at various calcium ion concentrations (25, 50, and 100 mg/L) in a laboratory fermentor. Other parameters like DOT, agitation speed, temperature and pH were maintained constant at 5 %, 400 rpm, 30 °C, and 7.2, respectively, during the course of experimental batch. Results are summarized in Table 4.11. For all calcium levels, a lag phase was observed during first 6 hours for bacterial growth. After that cell mass started to increase exponentially up to 24 hours in the case of 25 and 50 mg/L of Ca^{2+} while this period was delayed until 32 hours at 100 mg/L of Ca^{2+}. Maximum values obtained for bacterial mass at these calcium doses were similar, but, the highest cell concentration was achieved at 50 mg/L of Ca^{2+} as 8.7 g/L. Likewise, specific growth rates were almost the same at both 25 and 50 mg/L of calcium ion concentration, 0.116 hr^{-1} and 0.117 hr^{-1}, respectively. A cell lysis period started after exponential growth phase with little fluctuations in bacteria concentrations and continued to the end of fermentation period.

Alginate biosynthesis by *Azotobacter vinelandii* ATCC® 9046 was also followed during 72 hours. Alginate production was observed to be continuous throughout the fermentation process at 50 mg/L of Ca^{2+}. However, at the other doses, it was produced during growth and then exhibit small variation with a little decrease at the end. It appeared that moderate level of calcium ions favors the key enzymes of alginate synthesis since 4.51 g/L of alginate was formed at 50 mg/L of Ca^{2+} concentration. In parallel, the highest yield was also found at 50 mg/L of Ca^{2+} as 1 g alginate/ g bacteria. On the other hand, maximum alginate concentration was obtained as 2.65 g/L at 25 mg/L of Ca^{2+}. In this case, alginate synthesis was totally growth associated. Similarly, all of alginates were synthesized by growing bacteria at 100 mg/L of calcium ion concentration. However, high calcium dose somehow inhibited alginate production because the amount of the polymer was 2.5 times higher at 50 mg/L of Ca^{2+}.

Table 4.11. Comparison of some results for calcium dose experiments

Parameters	**Calcium ion (mg/L)**		
	25	**50**	**100**
Max biomass conc achieved (g/L)	8.54	8.70	8.45
Max alginate conc achieved (g/L)	2.65	4.51	1.77
Time to achieve max alginate conc (hr)	24	72	56
Max alginate yield (g alginate/g bacteeria)	0.310	1.00	0.242
Max broth viscosity (cP)	29.44	24.61	6.00
Time to achieve max broth viscosity (hr)	32	56	24
Max GG-block content (%)	21	61	68
Time to achieve max GG-block content (hr)	24	56	72

Sucrose utilization was evaluated during alginate production at various calcium levels. Change in calcium concentration did not lead to a substantial difference in sucrose consumption. There was only slight variation in the case of 50 mg/L of Ca^{2+}. Most of applied sucrose was used for alginate and cell synthesis. Only around 10 % of sucrose was not consumed at 72 hours. As in general, rate of utilization was observed faster during early experimental time. This was somewhat slowed down after 32 hours. Moreover, as the highest value, 0.246 g of alginate could be synthesized per gram of sucrose consumed at 50 mg/L of Ca^{2+}.

In general, viscosity values were almost the same at all calcium doses until 24 hours and they showed maxima at certain times. High viscosity values were observed at 25 and 50 mg/L of Ca^{2+}. For instance, culture broth viscosity was enhanced up to 29.44 cP at 32 hours in the case of 25 mg/L Ca^{2+}. On the contrary, polymerization seemed to be somehow inhibited at high calcium ion dose with the lowest viscosity value of 6 cP after 24 hours. Therefore, it can be said that the enzymes responsible for the polymerization of alginate was affected by variation in calcium ion concentration. Furthermore, it appeared that it was better to keep calcium ion concentration not higher than 50 mg/L for optimum polymerization.

During alginate production, monomer distribution of the polymer was another parameter measured to have knowledge about how the blocks were distributed within the alginate chain. It is known that alginate is first synthesized as mannuronic acid and then epimerized into guluronic acid. Thus, one can said that for all calcium doses tested here, epimerization was not efficient particularly at the lowest dose applied. The reason for that is the enzymes epimerizing mannuronic acid residues depend on calcium ion for the activity. These results were in agreement with the literature (Obika et al., 1993; Couperwhite and McCallum, 1974; Annison and Couperwhite, 1984). As an example, M/G ratio was found to reduce from 8 to 0.3 by increasing calcium ion concentration in the range of 0.068 mM to 0.68 mM (Obika et al., 1993). In our experiments, MM-block content at 25 mg/L of Ca^{2+} was 73 % at

24 hours and it was mainly converted to MG-blocks with limited conversion to GG-blocks. The amount of MG-blocks were improved up to 28 % at the end of fermentation process which was the highest level achieved during the experiments. On the other hand, GG-block content was reduced from 21 % to 12 % at 72 hours at 25 mg/L of Ca^{2+}. When calcium ion concentration was increased to 50 mg/L, GG-block content was enhanced greatly up to 61 % at 56 hours of fermentation. However, the amount of alternating sequences, MG-blocks, was observed to be very low. With further doubling of calcium ion in the applied medium, improvement in epimerized alginate was not effective as expected. As a result, GG-block content at 100 mg/L of Ca^{2+} was only 7 % higher compared to the one obtained at 50 mg/L of Ca^{2+}.

4.2.5. OVERALL SUMMARY

Alginate was produced under controlled conditions by *Azotobacter vinelandii* ATCC® 9046 in this study. Table 4.12 summarizes the conditions maximizing alginate quality and quantity. As it is seen from the table that the highest alginate concentration and yields based on bacterial production and sucrose consumption were achieved at 5 % DOT and 400 rpm with moderate sucrose and calcium concentrations. On the other hand, culture broth viscosity was maximum at the lowest calcium dose applied, 25 mg/L, at 5 % DOT with 400 rpm mixing and 20 g/L of sucrose concentration. Similarly, the highest MM- and MG- block fractions of the polymer were obtained at the same conditions. This might reflect that high viscosities are accompanied by alginates rich in mannuronic acids. Finally, epimerization of mannuronic acids into guluronic acids found to be the best at 10 % DOT and 400 rpm mixing with moderate sucrose and calcium concentrations.

Table 4.12. Conditions maximizing alginate production

Parameters	DOT (%)	RPM	Sucrose Conc (g/L)	Calcium Conc (mg/L)
Alginate production	5	400	20	50
Alginate yield (biomass based)	5	400	20	50
Alginate yield (sucrose based)	5	400	20	50
Broth viscosity	5	400	20	25
MM-block	5	400	20	25
MG-block	5	400	20	25
GG-block	10	400	20	50

4.3. INVESTIGATION OF ENVIRONMENTAL APPLICATION POTENTIAL OF ALGINATE

4.3.1. USE OF ALGINATE IN HEAVY METAL REMOVAL

Industrial activities supply essential products to our lives but eventually cause environmental pollution due to the discharges of the effluents. Heavy metal pollution is one of the major problems. It is critical to remove these metals before being discharged into the environment. In this study, heavy metal uptake capacity of sodium alginate was investigated by giving special

attention to use of alginate samples having different levels of guluronic acid content to elucidate whether it affected heavy metal uptake.

4.3.1.1. Maximum Uptake Capacity of Alginate for Different Metals

An alginate sample recovered from a shake flask experiment at 72 hours was investigated for the sorption of different metals such as Cu^{2+}, Cd^{2+}, Pb^{2+}, and Ca^{2+}. This alginate consists of 24 % of GG-blocks, 12 % of MG-blocks and 64 % of MM-blocks. First three metals are known for their toxicity even at low concentrations. On the other hand, calcium ion gives egg-box formation with alginate particularly having high GG-block content. This experiment was performed at relatively high metal concentration of 4.5 mM at pH 4 (Davis et al., 2004). pH is one of the important parameters affecting metal uptake by alginate because the uptake is accomplished by mainly COO^- groups in the structure of the monomers. These functional sites are occupied by H^+ ions at low pH values and they can not function as binding sites for the metals. Results (Table 4.13) from uptake experiments of these metals showed that copper was the most suitable ion for removal by alginate since the highest metal uptake capacity, 2.4 mM Cu^{2+}/g alginate, was achieved at the end of the experiment. Lead sorption followed copper, while calcium and cadmium uptake capacities of the alginate sample were comparably lower than copper uptake. Haug (1961) reported first time that metal-ion binding to alginic acid extracted from *Laminaria digitata* measured by the amount of protons released into the solution decreased in the order $Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+}$. He explained these results in terms of the relative ability of the binding metal to compete with protons for organic binding sites. Similar results were obtained by others (Papageorgiou et al., 2008; Park et al., 2007; Jodra and Mijangos, 2001). They examined calcium alginate beads and alginate gels for their uptake of different metals. The affinity was found as in the order of $Pb^{2+} > Cu^{2+} > Cd^{2+}$. Therefore, our results are not very much matching with the previous reports in literature for copper and lead uptake preference. The reason might be some variation in copper and lead uptake results (Table

4.13) owing to the limitation on microporous diffusion of the metals from dialysis membrane caused by gel formation between alginate and the metals as also reported by Fourest and Volesky (1996). In fact, the results for copper and lead were close to each other with the other two metals showing significantly lower uptakes.

Table 4.13. Relative metal uptake capacity of an alginate sample

Metal	Metal uptake (mM/g)
Cu^{2+}	2.40 ± 0.10
Pb^{2+}	2.18 ± 0.09
Cd^{2+}	1.57 ± 0.05
Ca^{2+}	1.40 ± 0.02

4.3.1.2. Determination of Maximum Copper Uptake Capacity of Alginate

From preliminary results, it seemed that among the toxic metals investigated above, copper was the most suitable metal for removal by sorption on alginate. Thus, it was selected for further investigations for this part of the study. A procedure was developed to determine maximum copper uptake by alginate samples produced at various conditions. First, the time required for the equilibrium of copper uptake was examined in the range of 1 to 10 days (Table 4.14). Copper ion concentration left in original metal solution and sorbed by alginate was measured by AAS. The metal concentration in original solution did not change much, 4.5 ± 0.21 mM, during 10 days because it was replaced once by a fresh batch after 4 days. The measurements obtained from acid rinse solutions showed that copper uptake of the alginate sample (24 % of GG-blocks, 12 % of MG-blocks and 64 % of MM-blocks) was increased up to 3 days and then a small reduction

was observed towards the end of the experiment. As a result, it was decided that the majority of the copper uptake was achieved in the first day and copper uptake was finished by the end of the third day.

Table 4.14. Changes of copper uptake by an alginate sample with time

Time (days)	**Cu^{2+} uptake (mM/g)**
1	1.95 ± 0.110
3	2.23 ± 0.003
5	2.17 ± 0.005
7	2.14 ± 0.007
10	2.10 ± 0.110

Together with the time needed for maximum copper uptake, effect of alginate and copper concentrations were also examined to finalize the procedure. The purpose here is to come up with a concentration for copper and alginate that participates in the maximum metal uptake so that they can be used for the further studies of metal uptake for different alginate samples from the fermentor. Alginate concentration was varied between 0.5 and 2.5 % whilst two different copper concentrations, 100 and 250 mg/L, were applied for investigation of whether there was an influence of concentration on the highest copper uptake by alginate. Neither alginate nor the metal concentration had any significant impact on the highest copper uptake on alginate as can be seen in Table 4.15. As a consequence, it was decided to continue with 1 % of alginate and 100 mg/L of initial copper concentration for further experiments.

Table 4.15. Investigation of alginate and copper ion concentration copper uptake on an alginate sample

Alginate (%)	Metal uptake (mM/g)	
	Cu^{2+}=100 mg/L	Cu^{2+}=250 mg/L
0.5	2.15 ± 0.06	2.12 ± 0.16
1	1.97 ± 0.03	2.02 ± 0.04
2.5	1.87 ± 0.02	1.99 ± 0.05

4.3.1.3. Maximum Copper Uptake Capacities of Different Alginate Samples

Maximum copper uptake capacities of bacterial alginates synthesized at different environmental conditions either in a shake flask or in a laboratory fermentor were investigated. First, an alginate sample produced in a shake flask was subjected to post-epimerization by AlgE1, one of the epimerases secreted by *Azotobacter vinelandii*, which converts mannuronic acid into guluronic acid. Here, the purpose was to see if there was any effect of GG-block content on copper uptake of alginate. Non-epimerized alginate is the one harvested from the shake flask at 72 hours. This alginate had 24 % of GG-blocks (12 % of MG-blocks and 64 % of MM-blocks) and then it was epimerized at two levels to see the effect of GG-block fraction for copper uptake. Moderately-epimerized sample had 40 % of GG-blocks (29 % of MG-blocks and 32 % of MM-blocks) while highly-epimerized sample contained 73 % of GG-blocks (24 % of MG-blocks and 3 % of MM-blocks). Although GG-block content of each sample was very different, maximum copper uptake values were almost the same (Table 4.16). It seems both mannuronic and guluronic acid contribute complexation reaction between alginate and copper ions.

Table 4.16. Maximum copper uptake by post-epimerized alginate samples

Alginate sample	Cu^{2+} uptake (mM/g)	GG-block (%)
Non-epimerized	1.97 ± 0.03	24
Moderately-epimerized	1.99 ± 0.01	40
Highly-epimerized	1.98 ± 0.01	73

Second group of alginate samples were produced by using a fermentor under controlled environmental conditions. During bacterial alginate production, the effect of DOT, mixing rate, initial sucrose and calcium ion concentrations on alginate quality and quantity was investigated. Copper uptake capacities of alginate samples obtained at the end of these experiments, at 72 hours, were studied. Both the fraction of GG-blocks and culture broth viscosities varied greatly depending on the condition operated. For instance, GG-block content changed from 12 to 87 %. On the other hand, considerably different culture broth viscosities, 1.47 to 14 cP, that may give an idea about chain length of the polymer were also observed (Table 4.17-4.20). From all these results, the average copper uptake of alginate sample was found to be around 1.90 mM Cu^{2+}/g alginate. One can say from the results that neither the amount of GG-blocks nor chain length of alginate samples had a remarkable impact on copper uptake of alginates. Similar results were obtained for the cadmium uptake experiments on sodium alginate from an algal source. They did not achieve any significant difference on cadmium binding of alginate samples with varying guluronic acid content (M/G ratio from 0.25 to 1.70). They claimed that the selectivity coefficients of the divalent cation compared to the monovalent proton, sodium, are so large that almost all sites seem to be satisfied by the divalent cation. Therefore, it was suggested that single metal systems may not be suitable to determine differential selectivity of the alginates (Davis et al., 2003).

Table 4.17. Maximum copper uptake by alginate samples recovered at different DOT (400 rpm, 20 g/L of sucrose conc, 50 mg/L of Ca^{2+})

Alginate sample	GG-block (%)	Viscosity (cP)	Alginate conc. Produced (g/L)	Cu^{2+} uptake (mM/g)
1 % DOT	84	1.9	2.94	1.92 ± 0.03
3 % DOT	71	4.0	3.35	1.95 ± 0.01
5 % DOT	56	8.9	4.51	1.90 ± 0.02

Table 4.18. Maximum copper uptake by alginate samples recovered at various mixing rates (5 % DOT, 20 g/L of sucrose conc, 50 mg/L of Ca^{2+})

Alginate sample	GG-block (%)	Viscosity (cP)	Alginate conc produced (g/L)	Cu^{2+} uptake (mM/g)
200 rpm	76	12	2.28	1.85 ± 0.02
400 rpm	56	8.9	4.51	1.90 ± 0.02
700 rpm	87	1.8	1.36	1.84 ± 0.002

Table 4.19. Maximum copper uptake by alginate samples harvested from different initial sucrose concentration (5 % DOT, 400 rpm, 50 mg/L of Ca^{2+})

Alginate sample	GG-block (%)	Viscosity (cP)	Alginate conc produced (g/L)	Cu^{2+} uptake (mM/g)
10 g/L of sucrose	84	1.47	1.63	1.89 ± 0.06
20 g/L of sucrose	56	8.90	4.51	1.90 ± 0.02
40 g/L of sucrose	54	1.85	4.79	1.92 ± 0.03

Table 4.20. Maximum copper uptake by alginate samples harvested at various initial calcium ion concentration (5 % DOT, 400 rpm, 20 g/L of sucrose conc)

Alginate sample	GG-block (%)	Viscosity (cP)	Alginate conc produced (g/L)	Cu^{2+} uptake (mM/g)
25 mg/L of Ca^{2+}	12	14	1.59	1.91 ± 0.01
50 mg/L of Ca^{2+}	56	8.9	4.51	1.90 ± 0.02
100 mg/L of Ca^{2+}	68	1.75	1.6	1.93 ± 0.03

4.3.1.4. Comparison of Maximum Uptake Capacity of Bacterial Alginates with Algal Alginates and the Other Sorbent Materials

Algal alginate samples purchased from FMC Biopolymer were investigated for copper uptake in order to compare the efficiencies of algal alginates with bacterial alginates produced in this study. As it is seen from Table 4.21, three different algal samples namely, LF240D, HF120RBS, and LF200S were subjected to copper binding. According to ^{1}H-NMR analysis results, GG-block content of alginate samples changes in the range of 19 to 65 %. On the other hand, viscosity of 0.5 g/L of alginate samples is varied from 3.81 to 7.22 cP. LF240D and HF120RBS showed a slightly higher affinity to copper ion under the conditions studied compared to LF200S. However, there was no remarkable influence of GG-block content and/or viscosity of the polymers. The maximum value was recorded for HF120RBS as 2.03 mM Cu^{2+}/g alginate. As a result, bacterial alginate samples synthesized in this study have almost the same copper uptake capacities as the algal alginate samples.

Table 4.21. Maximum copper uptake by FMC Biopolymer algal alginate samples

Alginate sample	GG-block (%)	Viscosity (at 0.5 g/L of alginate) (cP)	Cu^{2+} uptake (mM/g)
LF240D	19	3.81	2.01 ± 0.03
HF120RBS	37	7.22	2.03 ± 0.05
LF200S	54	5.69	1.90 ± 0.06

There are varieties of sorbent materials used for copper removal but the focus here is on marine algal biomass. Especially, brown algae (Sheng et al., 2008; Beolchinia et al., 2006; Vijayaraghavan and Prabu, 2006) is widely used although red (Vilar et al., 2008a; Vilar et al., 2008b) and green algae (Özer et al., 2009) too get attention for the biosorption of copper. Copper uptake capacities of these algal sources were observed in the range of 13-115 mg Cu^{2+}/ g alginate. The other biosorbent materials like white fungus biomass (Yahaya et al., 2009; Mukhopadhyay et al., 2007), bacterial biomass (Ozdemir et al., 2004), waste sludge (Pamukoglu and Kargi, 2006), and agricultural waste sugar beet pulp (Aksu and İşoğlu, 2005) were also investigated for the reduction of copper pollution. But these studies are not very much related to our work so they are not discussed here. The average copper removal to bacterial alginate samples produced in the fermentor was achieved as 1.90 mM Cu^{2+}/g alginate which corresponded to 120 mg Cu^{2+}/g alginate. This value was even higher than that was obtained by other algal biosorbents. This finding is especially important when the facts that properties of bacterial alginates can be controlled during production and they are free from seasonal variations and locations as in the case of algal sources are remembered.

4.3.2. USE OF ALGINATE IN TURBIDITY REMOVAL

Turbidity in water and wastewater is caused by colloidal and suspended solid materials having very low settling velocities. Therefore, their removal are generally accomplished by coagulation and flocculation processes following a sedimentation or a filtration step. Metal salts especially aluminum and iron salts are widely used due to their ability to form metal hydroxides. Although they are inexpensive and easy to handle, these chemicals leave high amounts of sludge and are associated with possible health problems such as aluminium residuals being suspected of causing Alzheimer's disease. Polymers, natural or synthetic, can also be used to improve flocculation. Among them, the use of synthetic polymers is widespread since their properties can be controlled during manufacturing. Most of the synthetic polymers are composed of polyacrylamide and its copolymers with polyacrylic acid. However, acrylamide is known to have a high chronic toxicity, being a neurotoxin (Hughes, 1990). As a result, natural polymers are getting attention as a flocculant such as chitosan, starch, and sodium alginate. These polymers are not toxic to the ecosystem and biodegradable. In addition, microbial polymers can be produced at high rates and they are easily recovered from the fermentation broth (Salehizadeh and Shojaosadati, 2001).

The studies dealing with the potential use of alginates in turbidity removal are very rare. Only some algal alginates grafted with some synthetic polymers were investigated recently (Tripathy et al., 2001; Sand et al., 2010). For this reason, bacterial alginates produced at certain characteristics along with calcium ion were tested as a potential coagulant for turbidity removal of a kaolinite suspension in this work. The role of GG-block content and the possibility of formation of egg-box structure on flocculation are discussed.

4.3.2.1. Turbidity Removal by Post-epimerized Alginate Samples

Kaolinite suspensions having initial turbidity of 10 NTU were treated with alginate samples together with calcium ion in order to reduce the turbidity value down to drinking water level. In Turkey, drinking water quality standards are determined by TS266. According to this standard, maximum allowable turbidity is set to 1 NTU. Furthermore, EPA and WHO standards are set as the same value.

Alginates having high GG-block content are known to form egg-box structure in the presence of calcium ion. It can be proposed that this formation may enhance floc formation and thus, increase the efficiency of turbidity removal process. First, the post-epimerized alginate samples were used to investigate if there is an effect of GG-block fraction on turbidity removal. Properties of post-epimerized alginate samples shown in Table 4.7 indicate that there are three different levels, namely highly-, moderately- and non-epimerized samples. GG-block contents of these polymers are in the order of 73, 40 and 24 %, respectively.

At the beginning of the experiments, the amount of turbidity removed by only addition of calcium ion was examined. This can be considered as a control experiment to show the amount of turbidity removable only by double layer compression by Ca^{2+} ions. Since alginate is an anionic polyelectrolyte, it requires addition of a cation which provides charge neutralization between negatively charged kaolinite particles. Figure 4.47 illustrates the results corresponding to turbidity removal by the addition of calcium ion in the range of 15 – 120 mg/L. As it is seen from the figure that turbidity removal was almost the same in the working range of calcium ion and it was measured as only about 30 %. A somewhat different trend was obtained in a previous study (Çoruh, 2005) that turbidity reduction was increased by increasing calcium dose from 30 mg/L to 80 mg/L. Higher turbidity removal efficiencies, up to 50 % at 80 mg/L, could be achieved by only calcium addition. It might be explained that kaolinite suspension used in our study appeared to be

more stable with higher repulsion forces between particles. After that, only alginate (highly-epimerized alginate sample) was added to determine the contribution of the polymer alone in turbidity reduction. Turbidity removal was decreased by increasing alginate dose from 1 to 10 mg/L probably due to negatively charged functional groups on the surface of the alginate. These groups increase repulsion between the particles although a little opportunity for adsorption of the polymer is also possible and contributes agglomeration (Bolto and Gregory, 2007). Therefore, maximum turbidity removal was obtained at 1 mg/L of alginate as 29 % and it was reduced down to around 20 % for increased doses. One final check done here was to test the effect of stirring and settling only on the turbidity reduction when there were neither of the chemicals added. The results obtained from this part showed that about 10 % of turbidity removal could be achieved without adding any chemicals just by applying the same mixing procedure with 30 min settling. This mechanism was so small that it may be logical to assume that there was no contribution of stirring and settling by gravity on turbidity removal of the stable kaolinite suspension.

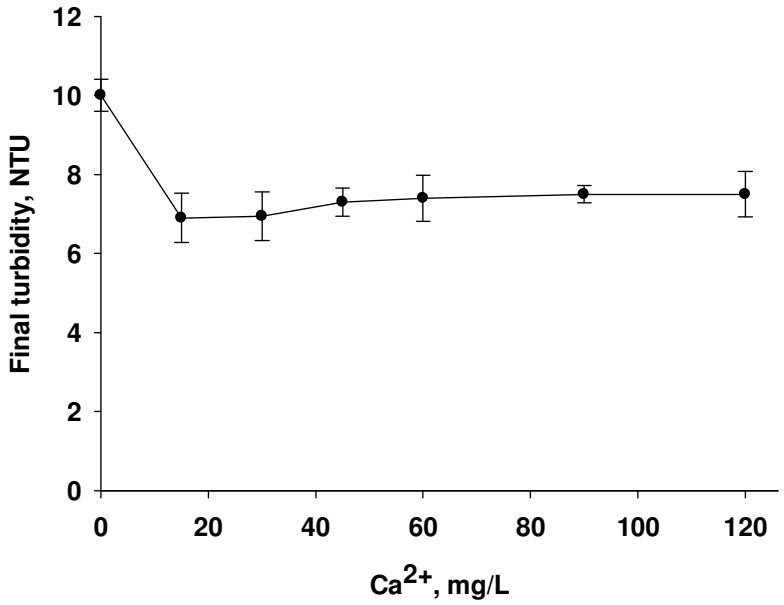

Figure 4.47. Turbidity removal by only calcium ion addition

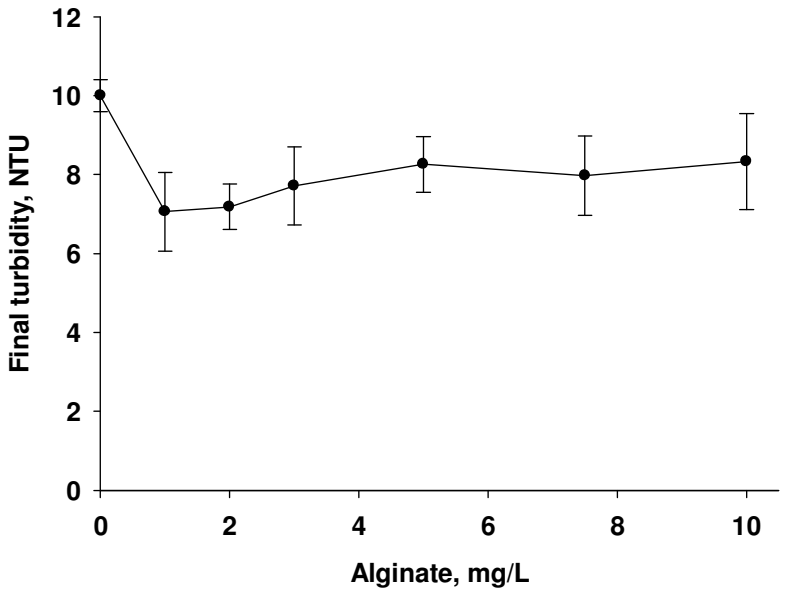

Figure 4.48. Turbidity removal by only alginate addition

Turbidity removal potential of highly-epimerized alginate was investigated in the range of 0.1 – 20 mg/L concentration with the addition of 60 mg/L of calcium ion (Figure 4.49). Results showed that there was a significant removal of turbidity with the calcium alginate system. Turbidity removal efficiency was enhanced by increasing alginate concentration especially if the alginate dose was higher than 3 mg/L. The highest reduction was achieved as 90.8 % by highly-epimerized alginate at 10 mg/L of concentration. This value corresponded to 0.917 NTU. It seems that turbidity removal efficiency for 7.5 mg/L and higher concentrations of highly-epimerized alginate was satisfactory. For further experiments, it was decided to optimize calcium ion concentration with 5 mg/L of alginate since alginate is a valuable source. For this alginate concentration, calcium ion dose was changed between 15 and 120 mg/L and the findings are presented in Figure 4.50. It was found that turbidity removal was enhanced by the increase in calcium concentration first, then it was deteriorated, i.e. the final turbidity increased back up to about 6 NTU at the highest dose of calcium at 120 mg/L. The maximum turbidity removal was observed at 30 mg/L of calcium ion concentration and the final turbidity value was recorded as 1 NTU.

Therefore, the required alginate dose for the same turbidity removal efficieny could be reduced by half with the optimization of calcium concentration.

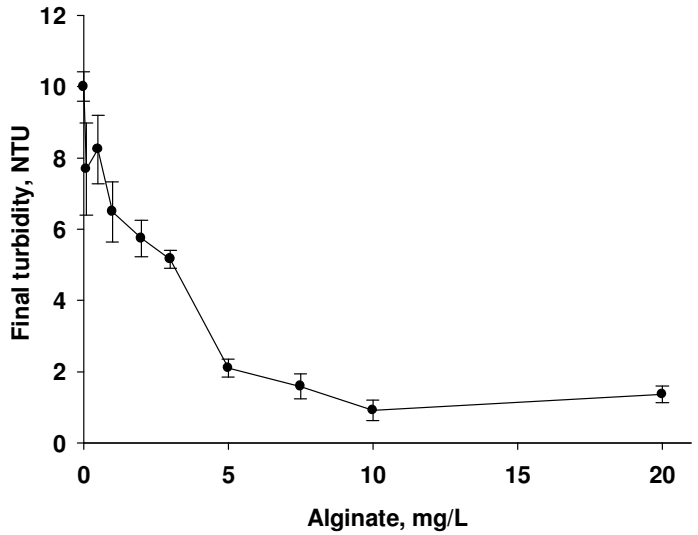

Figure 4.49. Turbidity removal by highly-epimerized alginate at 60 mg/L of Ca^{2+}

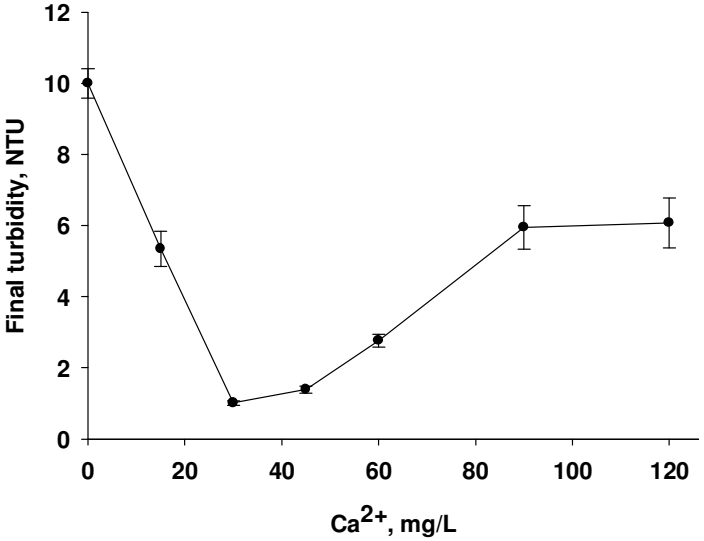

Figure 4.50. Optimization of Ca^{2+} dose for maximum turbidity removal by highly-epimerized alginate of 5 mg/L

The second sample was moderately-epimerized alginate sample. For this sample, alginate concentration was varied in the range of 1 and 10 mg/L for turbidity removal at 60 mg/L of calcium ion concentration. The results are depicted in Figure 4.51. Similar to highly-epimerized sample, turbidity removal was improved by increasing the concentration of moderately-epimerized alginate sample. Also, the maximum reduction was found at 10 mg/L of the alginate sample as around 75 % which corresponded to 2.54 NTU. Thus, although the highest removal was obtained at the same alginate dose for both alginate samples, highly-epimerized alginate with 90.8 % turbidity removal was more efficient than moderately-epimerized one. It might be due to higher GG-block content in high-epimerized sample that favors egg box formation more efficiently. The alginate concentration of 10 mg/L was selected for further calcium dose optimization and Figure 4.52 shows the results from turbidity treatment at different calcium ion concentration. Different from highly-epimerized alginate sample, turbidity reduction was enhanced with the elevation of calcium dose and the best result obtained at 120 mg/L of calcium ion concentration was 92 % (0.804 NTU). As a result, turbidity removal was improved by 17 % compared to 60 mg/L of calcium dose. However, twice higher dose of moderately-epimerized alginate is needed to achieve the same turbidity removal efficiency compared to highly-epimerized alginate.

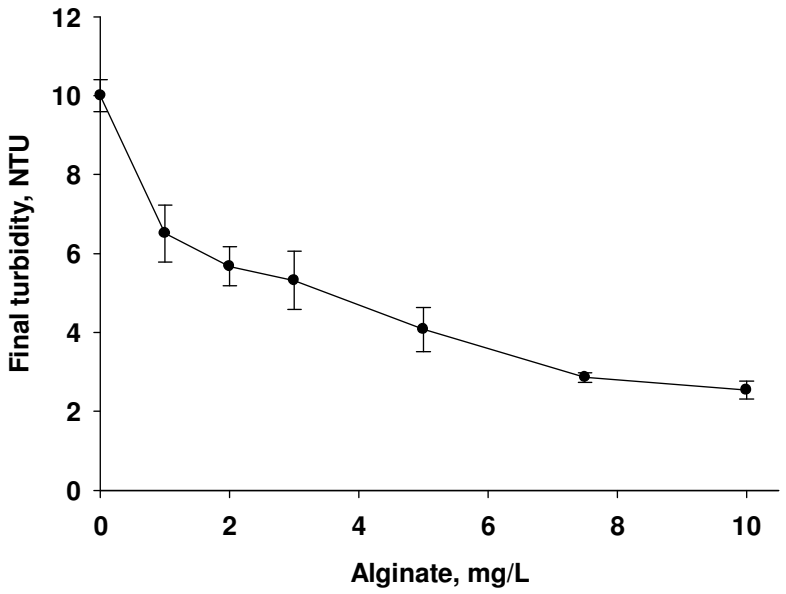

Figure 4.51. Turbidity removal by moderately-epimerized alginate at 60 mg/L of Ca^{2+}

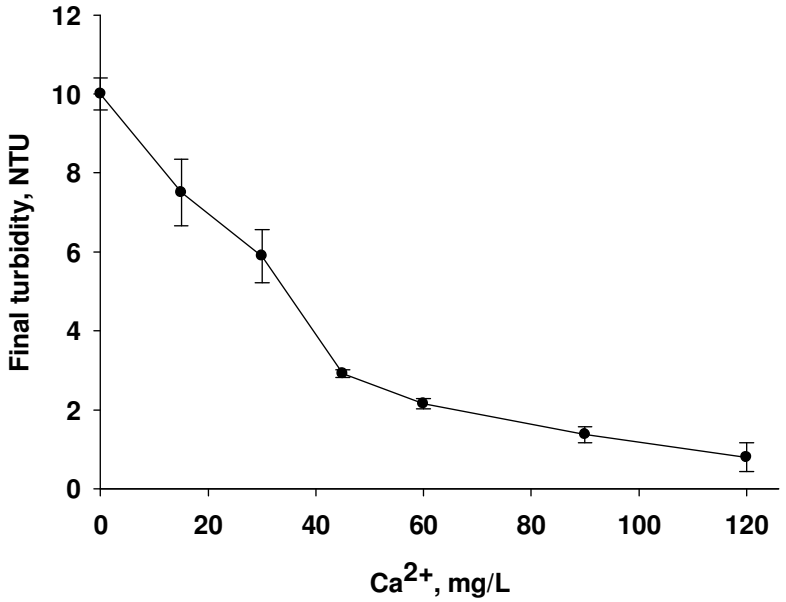

Figure 4.52. Optimization of Ca^{2+} dose for maximum turbidity removal by moderately-epimerized alginate of 10 mg/L

The third sample was non-epimerized alginate having 24 % of GG-block content. Turbidity removal efficiency was examined in the same range, the alginate concentration of 1- 10 mg/L, at 60 mg/L of calcium ion concentration (Figure 4.53). As it is seen from the figure that non-epimerized alginate samples were not at all effective as the others. Only 40 % of turbidity reduction could be achieved at 7.5 mg/L of the alginate sample. This value was considerably lower since the final turbidity value was 5.98 NTU. Then, the removal efficiency was tried to be improved by changing calcium ion concentration in the range of 15 -120 mg/L. During these trials, turbidity removal was only increased to 51.8 % at 30 mg/L of calcium ion concentration. It can be argued that even the amount of GG-block fraction in moderately-epimerized alginate sample is sufficient provided that increased doses of alginate is supplied for enhanced level of turbidity removal. However, 24 % of GG-block fraction seems to be too low for the formation of sufficient egg-box structure with calcium ion. Actually, almost no floc formation was observed during the experiment by non-epimerized alginate.

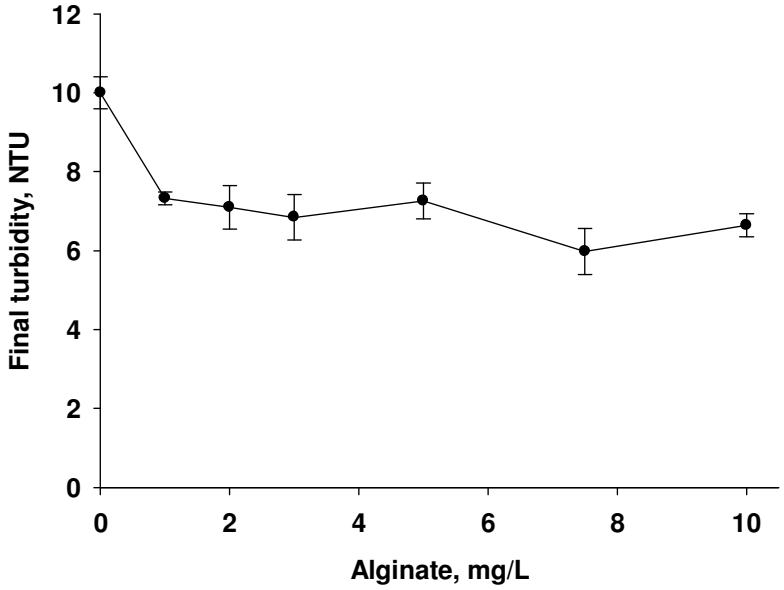

Figure 4.53. Turbidity removal by non-epimerized alginate at 60 mg/L of Ca^{2+}

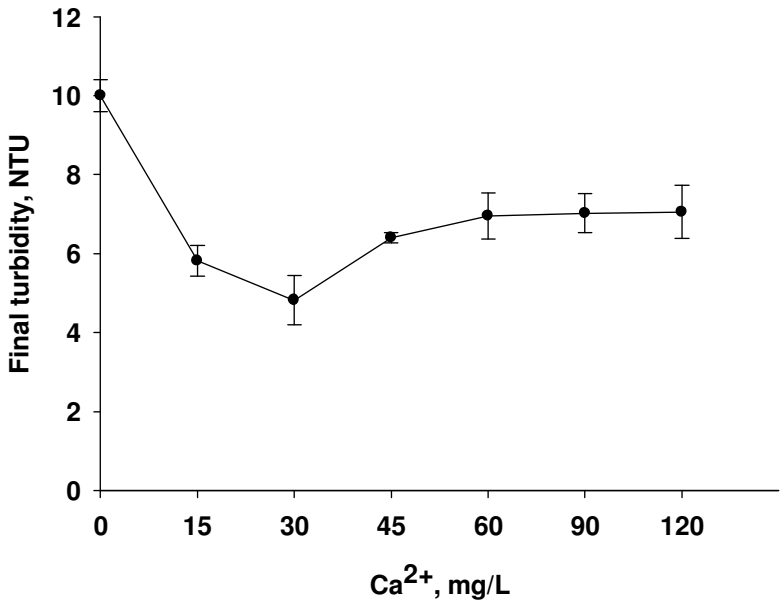

Figure 4.54. Optimization of Ca^{2+} dose for maximum turbidity removal by non-epimerized alginate of 7.5 mg/L

4.3.2.2. Turbidity Removal by Selected Bacterial Alginate Samples

As presented in previous parts, to determine the effect of operational conditions on alginate quantity and quality, alginate production was performed at various DOT values, mixing rates, and initial sucrose and calcium ion concentrations. Alginates extracted from these experiments at the end of 72 hours operational period had different characteristics and four samples were selected for turbidity removal studies. The properties of the samples are summarized in Table 4.22. Alginate sample #1 and #2 were used to decide the role of culture broth viscosity since these two samples had almost the same GG-block content but the viscosity value was much higher in the case of alginate sample #1. The last two samples were selected due to considerably different GG-block contents. Alginate sample #3 contained only 12 % of GG-block in the chain whilst alginate sample #4 constituted 76 % of GG-block. On the other hand, culture broth viscosities for

the last two samples were about the same which allowed to determine the effect of GG-block content on turbidity removal.

Table 4.22. Characteristics of alginate samples used in turbidity removal experiments

Alginate Sample Number	DOT (%)	Rpm	Sucrose (g/L)	Calcium (mg/L)	GG-Block (%)	Broth Viscosity (cP)
1	5	400	20	50	56	8.90
2	5	400	40	50	54	1.85
3	5	400	20	25	12	14
4	5	200	20	50	76	12

A kaolinite suspension having 10 NTU of initial turbidity was treated by alginate sample #1 and the results are shown in Figure 4.55. This alginate sample was found to be greatly effective for turbidity removal since even 2 mg/L of the alginate concentration was sufficient to bring the turbidity down to 1 NTU. This value could be obtained after calcium ion optimization at 5 mg/L of highly-epimerized alginate sample although GG-block content of that alginate was higher than alginate sample #1. To improve turbidity removal efficieny which was 74.6 % at 1 mg/L of alginate sample #1, calcium dose was tried to be optimized in the range of 15 to 120 mg/L. Results of this trials are shown in Figure 4.56 and it was observed that still the best result was obtained at 60 mg/L of calcium ion concentration.

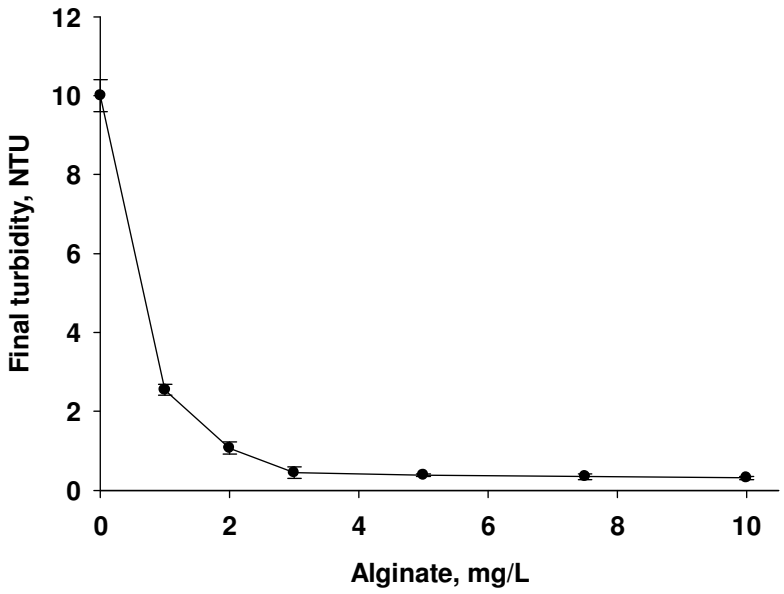

Figure 4.55. Turbidity removal by alginate sample #1 at 60 mg/L of Ca^{2+}

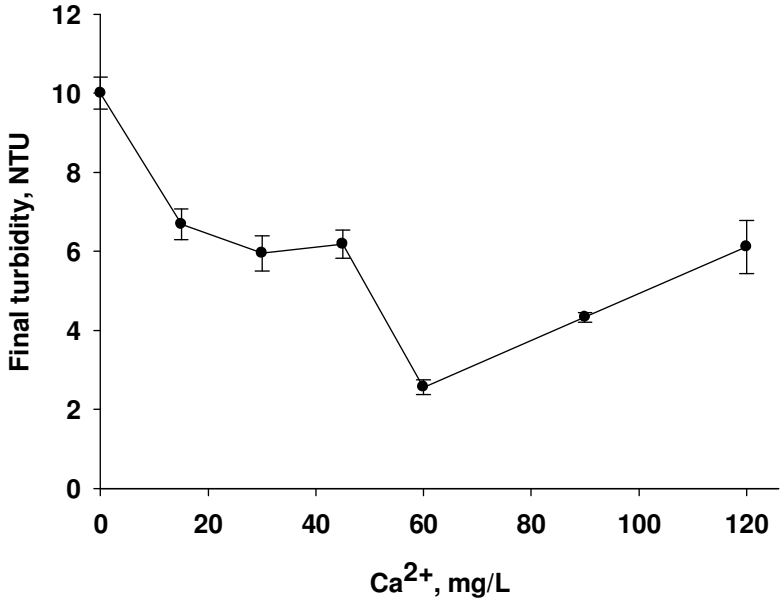

Figure 4.56. Optimization of Ca^{2+} dose for maximum turbidity removal by alginate sample #1 at 1 mg/L

Similar to alginate sample #1, high turbidity removal was obtained in the case of alginate sample #2. Only a decrease in turbidity reduction was observed when the alginate concentration was lower than 3 mg/L compared to alginate sample #1. For instance, at 2 mg/L of the alginate dose, turbidity value was 1.63 NTU for alginate sample #2 while it was recorded as 1NTU for alginate sample #1. This might be explained by the differences of the viscosities of the polymer samples (sample #1 had higher viscosity than sample #2). Therefore, 2 mg/L of alginate sample #2 was subjected to calcium optimization tests and the results showed that 90 mg/L of calcium ion dose gave the highest turbidity reduction as 97 % (Figure 4.57). It seems that the effect of the viscosity value on flocculation process was minor.

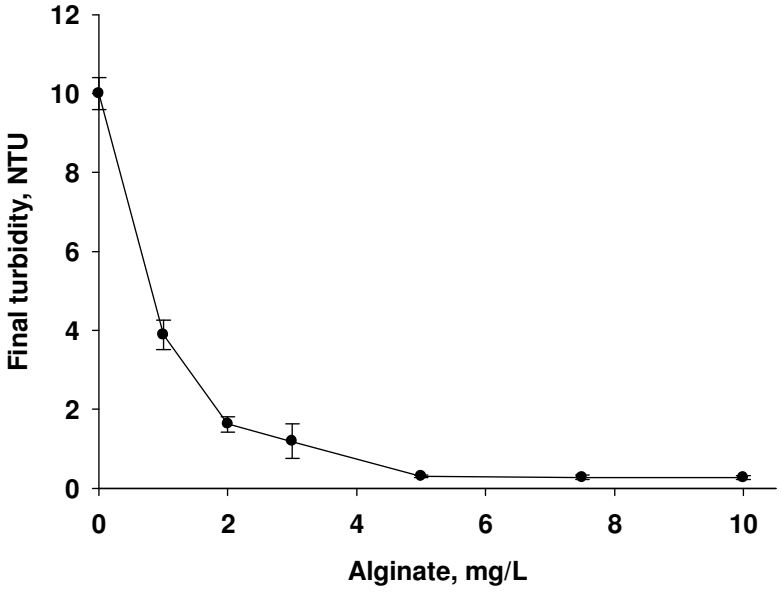

Figure 4.57. Turbidity removal by alginate sample #2 at 60 mg/L of Ca^{2+}

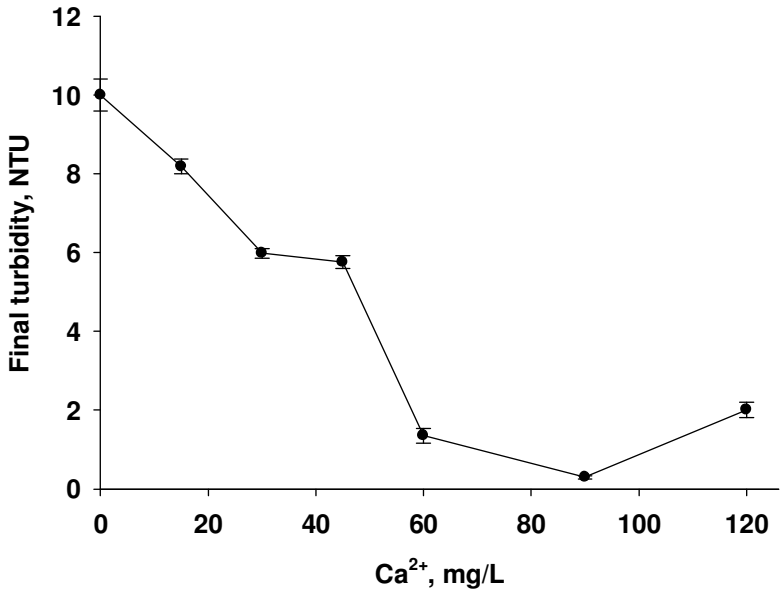

Figure 4.58. Optimization of Ca^{2+} dose for maximum turbidity removal by alginate sample #2 at 2 mg/L

Besides post-epimerized alginate samples, alginate sample #3 and #4 were also examined for turbidity removal to determine the influence of GG-block content of alginate on floc formation. As it is seen from Figure 4.59, alginate sample #3 appeared not to be a good candidate for turbidity removal at least for low turbidity values since the turbidity reduction effciencies were found to be around 20 %. In other words, final turbidity value of kaolinite suspension was about 8 NTU which was too high for drinking purposes. In the next iteration by changing the calcium dose, only a little improvement was obtained for turbidity removal at 2 mg/L of alginate sample #3 (Figure 4.60).

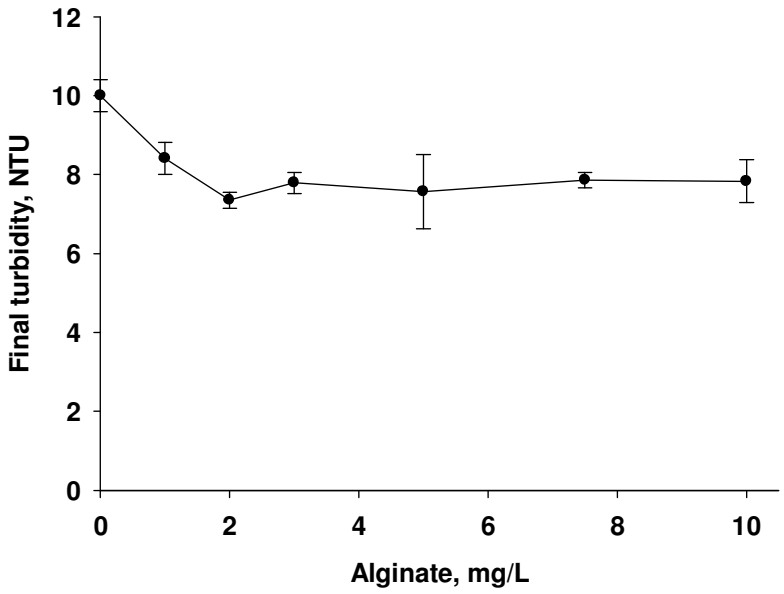

Figure 4.59. Turbidity removal by alginate sample #3 at 60 mg/L of Ca^{2+}

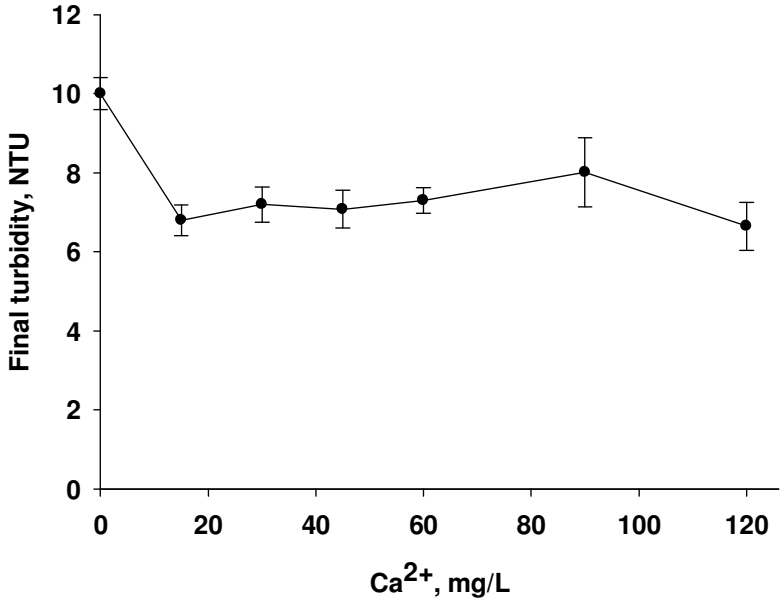

Figure 4.60. Optimization of Ca^{2+} dose for maximum turbidity removal by alginate sample #3 at 2 mg/L

On the other hand, better results could be achieved for the case of alginate sample #4 compared to #3 (Figure 4.61). However, higher doses were required to reach the same turbidity removal efficiencies compared to alginate sample #1 and #2. For example, 88 % of turbidity reduction (1.2 NTU) was observed at 20 mg/L of alginate sample #4, which was obtained at 5 and 10 mg/L of alginate #1 and #2, respectively. Also, turbidity reduction could not be enhanced by calcium ion optimization for alginate sample #4 because the maximum turbidity removal was found at 60 mg/L of calcium ion concentration (Figure 4.62).

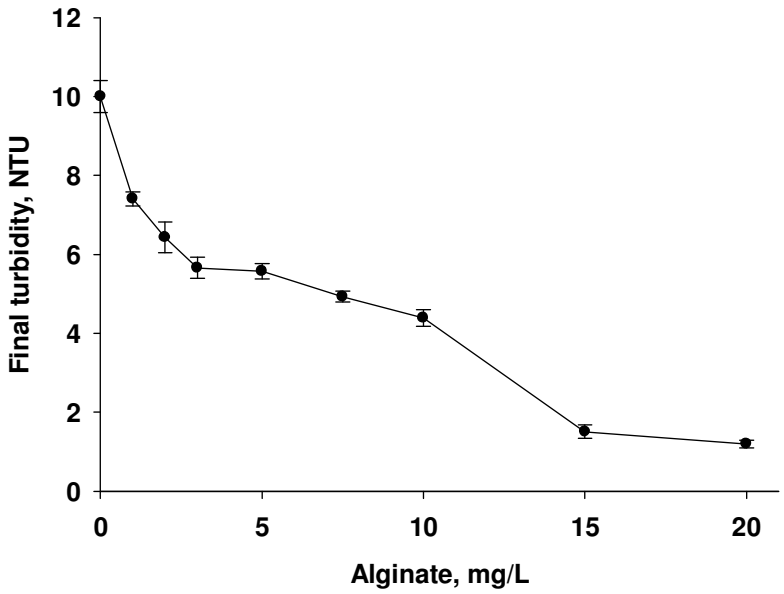

Figure 4.61. Turbidity removal by alginate sample #4 at 60 mg/L of Ca^{2+}

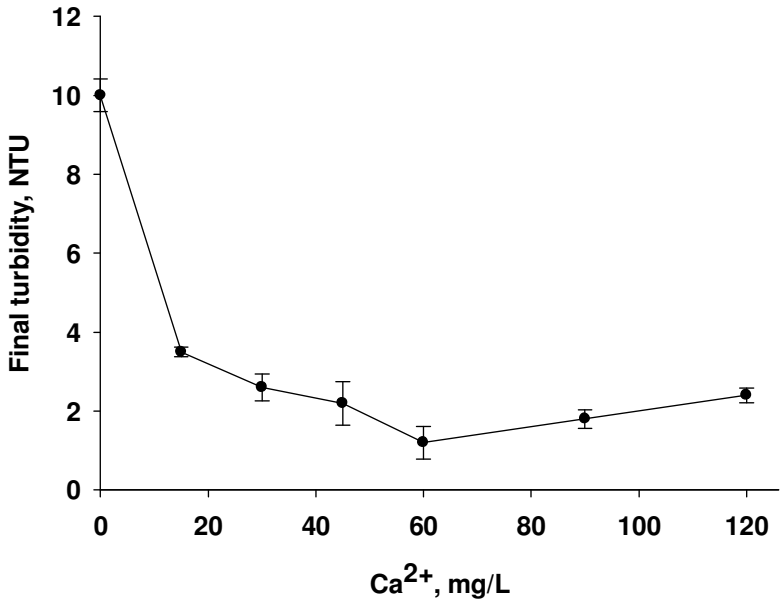

Figure 4.62. Optimization of Ca^{2+} dose for maximum turbidity removal by alginate sample #4 at 15 mg/L

When the results obtained from alginate sample # 3 and #4 are compared, it seems that the results can be explained by the egg box theory. Alginate sample #4 which is the one having high GG-block content resulted in higher turbidity reduction. However, although this sample had higher amount of GG-block and culture broth viscosity, turbidity treatment potential of both alginate sample #1 and #2 were found to be greater. It is expected that the main mechanism for flocculation of alginate sample #4 may be the polymer bridging because of its possible high molecular weight. However, for effective bridging, both charge density of the polymer and coverage ratio of suspended material by the polymer are important. For example, if the charge density of the polymer is high and the charge of the polymer is the same as suspended material such as the case observed in alginate and kaolinite system, adsorption of the polymer on kaolinite surface may somewhat be hindered due to repulsion between alginate and kaolinite particles. Also, high or full coverage of suspended particle surface by alginate is not needed in flocculation. Otherwise, there would be insufficient adsorption sites available.

Complete coverage of the surface with alginate adsorbed with flat configuration would lead to steric repulsion resulting in stable suspension (Besra et al., 2002). These results are consistent with SEM images. Figures 4.63 and 4.65 show SEM images of original kaolinite particles and that of after treatment by alginate sample #1 and sample #4 together with calcium ion, respectively. Most of the kaolinite particles were buried in alginate sample #4 although a little amount of coverage was seen for alginate sample #1. For these reasons, one can conclude that for the effective flocculation of kaolinite by alginate, moderate molecular weight of the polymer is more favorable due to a possible high charge density on alginate surface. Addition of calcium reduces repulsion between negatively charged kaolinite particles and makes a bridge between alginate molecule and kaolinite particles. The amount of GG-block content of the alginate appeared to be important for effective bridging between the particles and at least a moderate level of GG-block was required for efficient turbidity removal. This eventually allows for agglomeration of the flocs and then they settle easily by gravity.

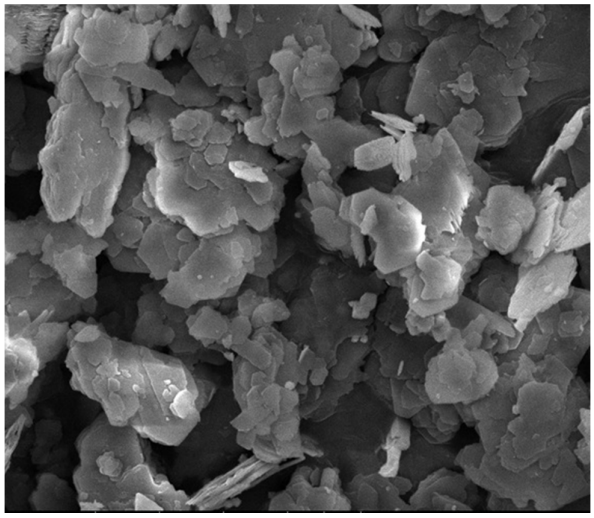

Figure 4.63. SEM images of original kaolinite particles (10,000x)

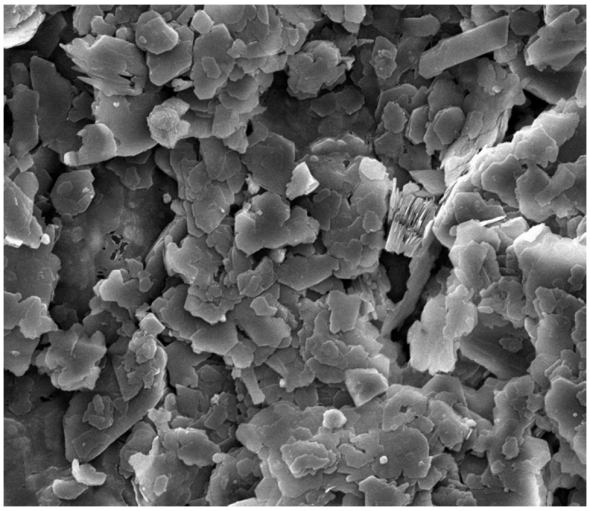

Figure 4.64. SEM images of kaolinite suspension after treatment with alginate sample #1 (10,000x)

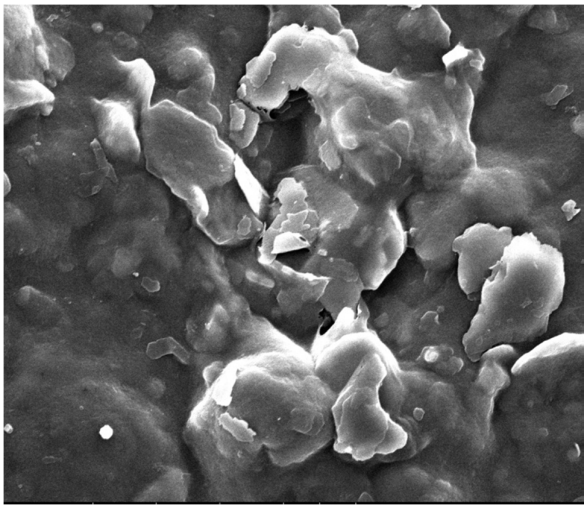

Figure 4.65. SEM images of kaolinite suspension after treatment with alginate sample #4 (10,000x)

4.3.2.3. Turbidity Removal by Algal Alginate Samples

Alginates from algal sources were applied for turbidity removal to compare their effectiveness with bacterial alginate samples. For this purpose, three

different alginate samples were selected having relatively different guluronic acid contents, namely LF240D, HF120RBS, and LF200S. The lowest turbidity value was obtained as 6.52 NTU for LF240D at 2 mg/L of concentration of alginate (Figure 4.66). In other words, this algal alginate sample was not a good candidate for turbidity reduction at low levels. This can be attributed to low GG-block content of alginate, which is only 19 %. A better but not sufficient turbidity reduction of a clay suspension (initially 10 NTU) was achieved down to 3.9 NTU by an algal alginate having 28 % of GG-block after 30 min of settling in the presence of 80 mg/L of Ca^{2+} (Çoruh, 2005). After that LF240D was subjected to calcium optimization at 2 mg/L of alginate concentration, but, there was no improvement in turbidity removal. The highest removal value was obtained at 30 mg/L of calcium ion concentration as 35 %.

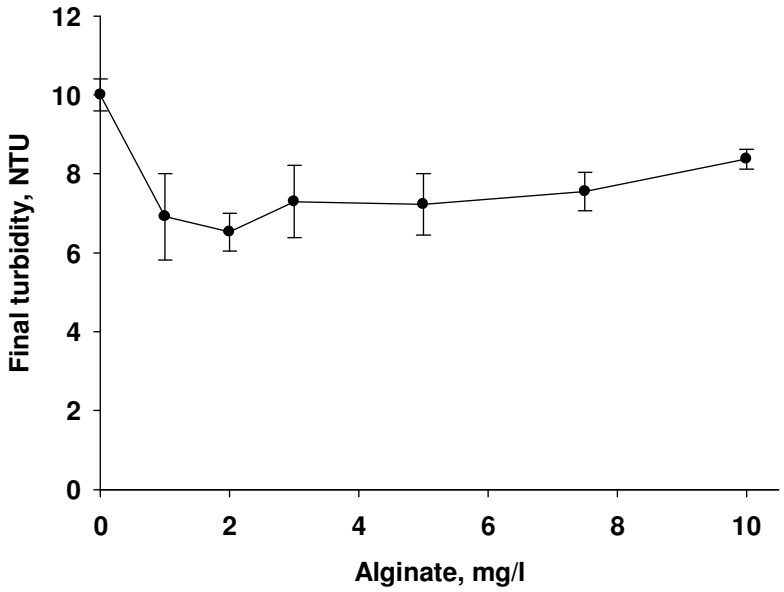

Figure 4.66. Turbidity removal by algal alginate sample, LF240D, at 60 mg/L of Ca^{2+}

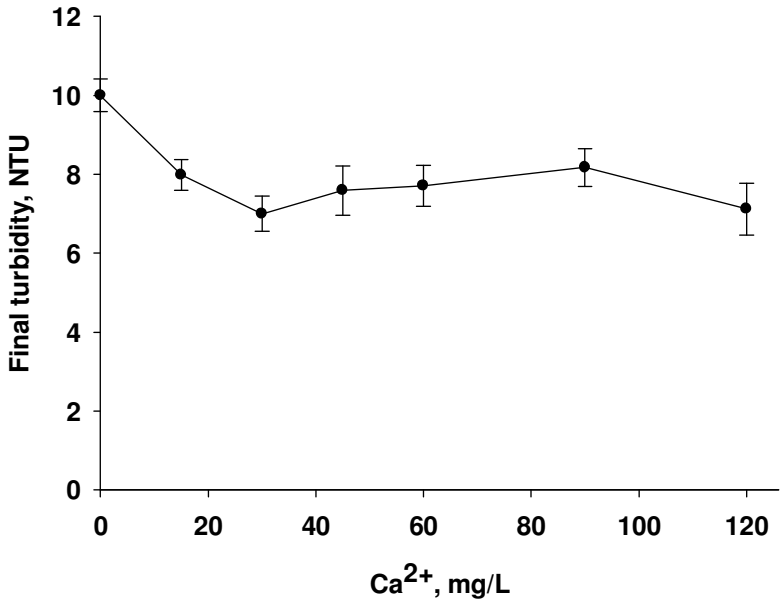

Figure 4.67. Optimization of Ca^{2+} dose for maximum turbidity removal by algal alginate sample, LF240D, at 2 mg/L

On the other hand, both HF120RBS and LF200S resulted in similar turbidity removal efficiencies at 7.5 mg/L of alginate dose as 74.7 and 78.7 %, respectively (Figure 4.68 and 4.70). These alginates had 37 % and 54 % of GG-blocks, respectively. In addition, calcium optimization of these samples was conducted at 7.5 mg/L of alginate concentration in the range of 15 and 120 mg/L of calcium. In the case of HF120RBS, no significant enhancement in turbidity reduction was obtained and the lowest final turbidity was measured as 2.41 NTU at 90 mg/L of calcium ion concentration (Figure 4.69). Moreover, in the case of LF200S, it was possible to bring the turbidity down to water of drinking quality because at 60 mg/L of calcium ion concentration, residual turbidity was measured as 0.26 NTU. One can conclude that only LF200S can be used for the treatment of low turbidity suspensions. All these results were again consistent with the role of egg box formation in flocculation since among the algal alginates tested, LF200S had the highest amount of GG-blocks. However, when these results are compared with bacterial alginate samples, both alginate sample #1 and #2

gave the similar turbidity removal capacities at 2 mg/L of alginate dose. Lower doses were enough to obtain the desired final turbidity values. In other words, bacterial alginate samples produced under controlled environmental conditions seemed to be more efficient for low turbidity treatment.

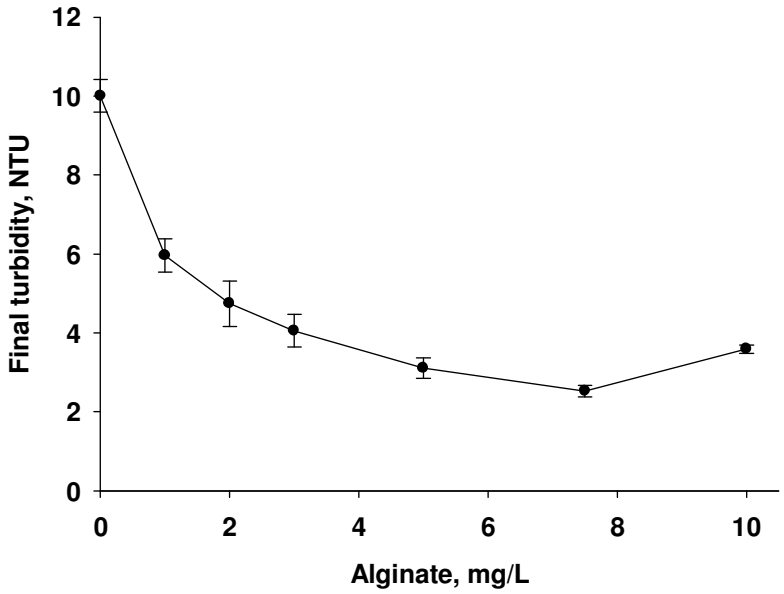

Figure 4.68. Turbidity removal by algal alginate sample, HF120RBS, at 60 mg/L of Ca^{2+}

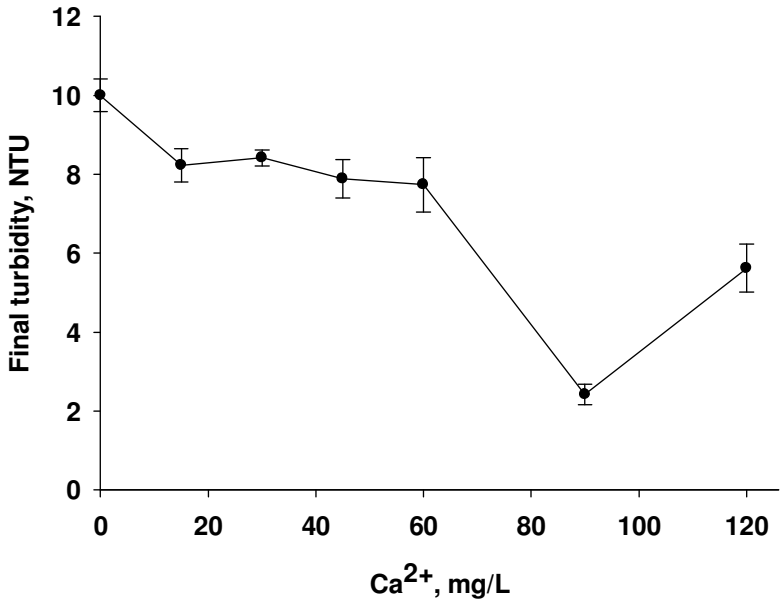

Figure 4.69. Optimization of Ca^{2+} dose for maximum turbidity removal by algal alginate sample, HF120RBS, at 7.5 mg/L

Compared to the algal alginates tested above, turbidity removal efficiencies by the previously tested algal alginate were increased at very low alginate concentrations (Çoruh, 2005). For all calcium doses investigated, the lowest final turbidity value was found at 0.04 mg/L of alginate dose. Final turbidity value could be reduced to 3.9 NTU (80 mg/L of Ca^{2+}) which corresponded to about 60 % of turbidity removal. However, it should be noted that contribution of alginate on total turbidity removal was very low since 50 % of turbidity reduction was achieved by calcium addition only at 80 mg/L. On the other hand, turbidity value was decreased from 10 to 2.44 NTU by 0.1 mg/L of a higher molecular weight algal alginate at 120 mg/L of Ca^{2+} while 60 % of turbidity reduction was obtained by calcium addition only at 120 mg/L (Yüksel, 2007) similar to the previous study.

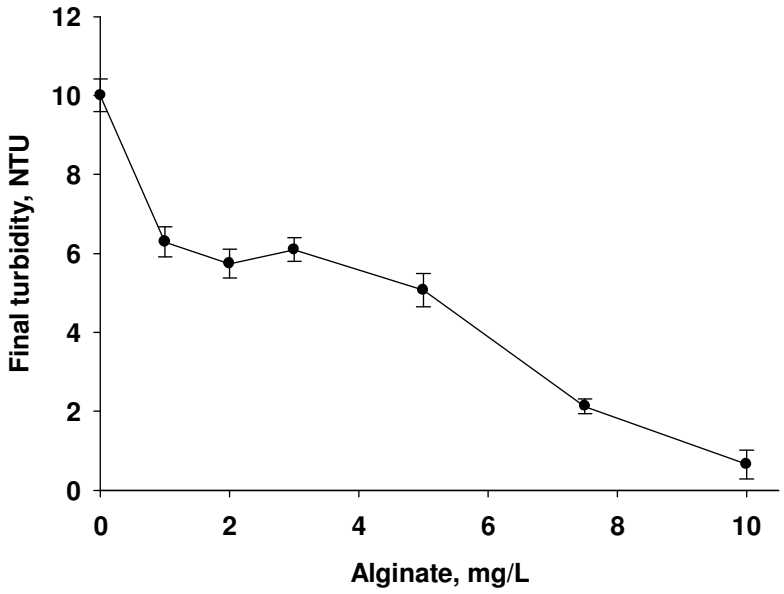

Figure 4.70. Turbidity removal by algal alginate sample, LF200S, at 60 mg/L of Ca^{2+}

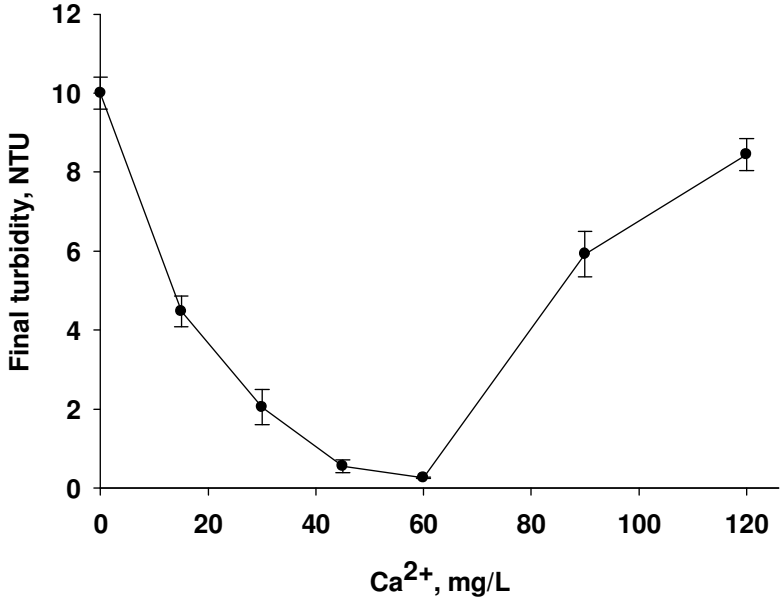

Figure 4.71. Optimization of Ca^{2+} dose for maximum turbidity removal by algal alginate sample, LF200S, at 7.5 mg/L

All things considered, the direct comparison of the results obtained from our work seems to be not possible with the previous studies (Çoruh, 2005; Yüksel, 2007) because the properties of material used for the preparation of synthetic dispersion are somehow different from each other. It was obvious from the finding of only calcium addition that only around 30 % of turbidity removal could be achieved from kaolinite suspension whereas up to 60 % of that was reduced at 120 mg/L of Ca^{2+} in the previous study (Yüksel, 2007). Although the desired turbidity removal appeared to be achieved at much lower alginate doses compared to our findings, both in the case of algal samples and bacterial alginates, the amount Ca^{2+} required to obtain these levels were higher except for some points. As a conclusion, coagulation potential of bacterial alginates was very obvious, that most of the turbidity was removed by bridging between suspended particles and alginate. The removals were enhanced so significantly by the increase of GG-block presence due to egg-box formation of alginate having high GG-blocks in the presence of calcium ion.

CHAPTER V

CONCLUSION

Alginate was produced by *Azotobacter vinelandii* ATCC ®9046 in a laboratory fermentor under controlled environmental conditions. The effect of dissolved oxygen tension, carbon dosage, mixing intensity and calcium ion concentration on alginate quantity and quality in terms of monomer distribution and culture broth viscosity were investigated. Then, bacterial alginates were investigated for potential use in water and wastewater treatment applications. The conclusions can be summarized as follows:

- Alginate production was not efficient in shake flasks possibly due to high oxygen levels at the beginning of the experiments which led to the wastage of carbon source otherwise that would be used for alginate production. For this reason, alginate synthesis was delayed till the end of growth and only 1.84 g/L of alginate could be produced. Furthermore, a considerably high culture broth viscosity value was observed at 72 hours as 104.6 cP in spite of inefficient epimerization (maximum 34 % of GG-blocks at 56 hours). With all these results and since there is also no chance to control the important parameters in shake flasks, it was decided to use a fermentor for alginate production.

- For the analysis of monomer distribution of alginates, two methods, acid precipitation and ^{1}H-NMR spectroscopy were investigated. Alginate monomer blocks, MM- and GG-, were obtained by acid precipitation, then; they were examined together with an algal alginate sample, LF240D, by ^{1}H-NMR analysis. Acid precipitation resulted in lower estimation for homopolymeric blocks due to higher solubility of these blocks under acidic conditions. Therefore, ^{1}H-NMR spectroscopy was found a more suitable method for the determination of monomer distribution of alginates.

- It was possible to produce alginates having different quality by changing the operation conditions of the fermentor and the composition of the culture medium.

- Both the amount of alginate produced and alginate quality were affected by the changes of DOT in the range of 1 and 10 %. The optimum DOT level giving high alginate yield (4.51 g/L) and maximum viscosity level was observed as 5 %. However, GG-block fraction of alginate was found to be moderate being around 55 %. On the other hand, GG-block content of alginate was increased at extreme conditions such as oxygen limitation (1 % DOT) and high aeration (10 % DOT) although alginate synthesis was reduced under these circumstances.

- Mixing intensity was one of the important parameters for alginate quality and quantity that extreme levels detoriated alginate production. In other words, neither high nor low level of stirring did not favor alginate production. At low level of mixing, the bacteria struggle with mass limitations while high shear rates considerably reduce alginate synthesis. These conditions resulted in high GG-block alginates at 76 % and 87 % in the cases of 200 rpm and 700 rpm, respectively. Although epimerization (GG-block formation) of alginates was not as efficient at 400 rpm, the highest alginate concentration and broth viscosities were achieved at moderate agitation speeds.

- Sucrose dose used for the synthesis of alginate by *Azotobacter vinelandii* ATCC ®9046 also affected both concentration and properties of the polymer. A moderate carbon dose, 20 g/L, was found to be the most effective concentration since further increase of sucrose concentration to 40 g/L did not lead to a considerable improvement on neither the yield of alginate produced nor its quality. Epimerization of alginate was moderate at these sucrose concentrations, but, a higher culture broth viscosity was observed at 20 g/L of sucrose concentration. On the other hand, carbon limitation, 10 g/L of sucrose, appeared to force the bacteria to protect themselves from this adverse condition by producing alginate having high GG-block fraction, above 85 %.

- Calcium ion concentration is important because of their critical role in the activity of epimerases. High initial calcium concentration in the production medium did not lead to any significant increase in guluronic acid fraction compared to moderate level; although low calcium doses clearly inhibited activity of epimerases that under this condition only 20 % of GG-block could be produced by the bacteria. For these reasons, it was better to use a moderate calcium dose, 50 mg/L, for the production of alginate which resulted in higher amount of polymer and culture broth viscosity.

- Bacterial alginate samples could be used for water and wastewater treatment applications. Alginates obtained from post-epimerization and fermentor experiments were successfully applied for toxic heavy metal uptake and turbidity removal.

- Sodium alginates were investigated for maximum heavy metal uptake with a special focus on copper ion. Alginate was found effective for copper ion uptake. The maximum copper uptake capacity was around 1.9 mM Cu^{2+} / g alginate. The effect of GG-block content of alginates on copper uptake was also examined. However, no important differences were observed over a wide range of guluronic acid

contents (from 12 % to 87 % of GG-block). Therefore, it seems that a non-selective, more general mechanism valid for all different block types such as a general complexation mechanism dominate the copper uptake by alginate. Furthermore, copper uptake capacities of bacterial alginates were close to algal alginate samples obtained from FMC Biopolymer.

- The last part of the study was conducted to test the effectiveness of alginate in removing low turbidities (10 NTU) from water samples. In these experiments, it was found that the amount of GG-block was important since alginate form egg box with calcium ions. This eventually enhanced the floc formation by increasing bridging opportunities and improved turbidity removal efficiency. At least a moderate level of guluronic acid was required to obtain a water having drinking quality after treatment. Especially, the alginate having 55 % of GG block and 8.9 cP culture broth viscosity was found to be very effective resulting in a turbidity level lower than 1 NTU at 2 mg/L of concentration in the presence of 60 mg/L calcium ion.

CHAPTER VI

6. FUTURE WORK

Alginate was produced by using *Azotobacter vinelandii* ATCC® 9046 which is a wild strain. After a certain stage of growth, the bacteria secrete alginate lyases that break the bonds between alginate monomers, so reduce molecular weight of the polymer. Genetic manipulations on alginate lyases, i.e. erasing of AlgL, could improve alginate molecular weight. Therefore, it would be possible to produce high amount of alginates with high molecular weight.

In this study, alginate production was conducted in a fermentor operated in batch mode. For larger scale of alginate production, first, fermentor needs to operate in continuous mode. Also, the parameters affecting alginate synthesis should be investigated by operating continuously. After these examinations, a pilot plant work would be constructed thereafter.

Sucrose was used as carbon source for alginate production. Since *Azotobacter vinelandii* can utilize a variety carbon sources, alternative carbon sources should be investigated for alginate production in order to reduce the cost for alginate production. For example, possibility of using waste materials to produce a valuable product, alginate, should be evaluated.

For heavy metal removal experiments, sodium alginate confined in dialysis bags was used in this study. Besides, formation of alginate beads would be studied and then calcium alginate beads can be used for heavy metal uptake. Furthermore, performance of alginates for the removal of low turbidity was examined. Alginates can also be applied for other level turbidity removal and natural water sources.

REFERENCES

Ahmad, A.L., Sumathi, S., Hameed, B.H., 'Coagulation of Residue Oil and Suspended Solid in Palm Oil Mill Effluent by Chitosan, Alum and PAC', Chemical Engineering Journal, 2006, 118, 99–105.

Aksu, Z., Işoğlu, I. A., 'Removal of Copper (II) Ions from Aqueous Solution by Biosorption onto Agricultural Waste Sugar Beet Pulp', Process Biochemistry, 2005, 40, 3031–3044.

Anderson, A. J., Hacking, A. J., Dawes, E. A., 'Alternative Pathways for Biosynthesis of Alginate from Fructose and Glucose in Pseudomonas mercodina and Azotobacter vinelandii', Journal of General Microbiology, 1987, 133, 1045–1052.

Annison, G., Couperwhite, I., 'Consequences of the Association of Calcium Ions with Alginate during Batch Culture of *Azotobacter vinelandii*', Applied and Environmental Microbiology, 1984, 19, 321–325.

Annison, G., Couperwhite, I., 'Influence of Calcium on Alginate Production and Composition in Continuous Cultures of *Azotobacter vinelandii*', Applied and Environmental Microbiology, 1986, 25, 55–61.

Asami, K., Aritomi, T., Tan, Y-S., Ohtaguchi, K., 'Biosynthesis of Polysaccharide Alginate by *Azotobacter vinelandii* in a Bubble Column', Journal of Chemical Engineering of Japan, 2004, 37, 8, 1050–1055.

ASTM F 2259-03, 'Standard Test Method for Determining the Chemical Composition and Sequence in Alginate by Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy', 2003.

ATCC, http://www.atcc.org (retrived on 20.06.2006)

Balci, M., 'Basic ^{1}H- and ^{13}C-NMR Spectroscopy', 2005.

Bratby, J., Coagulation and Flocculation in Water and Wastewater Treatment, IWA Publishing, 2nd Edition, 2006.

Beale, Jr. J. M., Foster, J. L., 'Carbohydrate Fluxes into Alginate Biosynthesis in *Azotobacter Vinelandii NCIB 8789*: NMR Investigations of the Triose Pools', Biochemistry, 1996, 35, 4492–4501.

Beolchinia, F., Pagnanellib, F., Torob, L.,Vegliò, F., Ionic Strength Effect on Copper Biosorption by *Sphaerotilus natans*: Equilibrium Study and Dynamic Modelling in Membrane Reactor', Water Research, 2006, 40, 144 –152.

Besra, L., Sengupta, D.K., Royb, S.K., Ay, P., 'Polymer Adsorption: Its Correlation with Flocculation and Dewatering of Kaolin Suspension in the Presence and Absence of Surfactants', International Journal Mineral Processing, 2002, 66, 183–202.

Bjerkan, T. M., Lillehov, B. E., Strand, W. I., Skjåk-Bræk, G., Valla, S., Ertesvåg, H, 'Construction and Analyses of Hybrid *Azotobacter vinelandii* Mannuronan C-5 Epimerases with New Epimerization Pattern Characteristics', Biochemistry Journal, 2004, 381, 813–821.

Bolto, B., Gregory, J., 'Organic Polyelectrolytes in Water Treatment', Water Research, 2007, 41, 2301–2324.

Boyd, A., Chakrabarty, A. M., 'Role of Alginate Lyase in Cell Detachment of *Pseudomonas aeruginosa*', Applied Environmental Microbiology, 1994, 60, 2355–2359.

Boyd, A., Chakrabarty, A. M., '*Pseudomonas aeruginosa* Biofilms: Role of the Alginate Exopolysaccharide', Journal of Industrial Microbiology, 1995, 15, 162-168.

Brivonese, A. C., Sutherland, I. W., 'Polymer Production by a Mucoid Strain of *Azotobacter vinelandii* in Batch Culture', Applied Microbiology and Biotechnology, 1989, 30, 97–102.

Chen, W. P., Chen, J. Y., Chang, S. C., Su, C. L., 'Bacterial Alginate Produced by a Mutant of *Azotobacter vinelandii*', Applied Environmental Microbiology, 1985, 49, 543–546.

Clementi, F., Fantozzi, P., Mancini, F., Moresi, M., 'Optimal Conditions for Alginate Production by *Azotobacter vinelandii*', Enzyme and Microbial Technology, 1995, 17, 983–988.

Clementi, F., 'Alginate Production by *Azotobacter vinelandii*', Critical Reviews in Biotechnology, 1997, 17, 4, 327–361.

Clementi, F., Crudele, M. A., Parente, E., Mancini, M., Moresi, M., 'Production and Characterization of Alginate from *Azotobacter vinelandii*', Journal of the Science of Food and Agriculture, 1999, 79, 602–610.

Couperwhite, I., McCallum, M. F., 'The Influence of EDTA on the Composition of Alginate Synthesized by *Azotobacter vinelandii*', Archieve Microbiology, 1974, 97, 73–80.

Çoruh, H. A., 'Use of Unconventional Coagulants during Water Treatment', MS. Thesis, METU, 2005.

Davis, T. A., Llanes, F., Volesky, B., Mucci, A., 'Metal Selectivity of *Sargassum spp.* and Their Alginates in Relation to Their α-L-Guluronic Acid Content and Conformation', Environmental Science and Technology, 2003, 37, 261–267.

Davis, T. A., Ramirez, M., Mucci, A., Larsen, B., 'Extraction, Isolation and Cadmium Binding of Alginate from *Sargassum spp.*', Journal of Applied Phycology, 2004, 16, 275–284.

Divakaran, R., Pillai, V. N. S., 'Flocculation of River Silt using Chitosan', Water Research, 2002, 36, 2414–2418.

Draget, K. I., Smidsrød, O., Skjåk-Bræk, G., 'Polysaccharides and Polyamides in the Food Industry. Properties, Production, and Patents', 2005.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F., 'Colorimetric Method for Determination of Sugars and Related Substances', Analytical Chemistry, 1956, 28, 3, 350–356.

Ertesvåg, H., Valla, S., 'Biosynthesis and Applications of Alginates', Polymer Degradation and Stability, 1998, 59, 85–91.

Ertesvåg, H., Høidal, H. K., Skjåk-Bræk, G., Valla, S., 'The Azotobacter vinelandii Mannuronan C-5-Epimerase AlgE1 Consists of Two Separate Catalytic Domains', The Journal of Biological Chemistry, 1998, 273, 47, 30927–30932.

Ertesvåg, H, Høidal, H. K., Schjerven, H., Svanem, B. I., Valla, S, 'Mannuronan C-5-epimerases and Their Application for in Vitro and in Vivo Design of New Alginates Useful in Biotechnology', Metabolic Engineering, 1999, 1(3), 262–269.

Ertesvåg, H., Valla, S., 'The Modules of the *Azotobacter vinelandii* Mannuronan-C-5-Epimerase AlgE1 are Sufficient for Both Epimerization and Binding of Ca^{2+}', Journal of Bacteriology, 1999, 3033–3038.

Ertesvåg, H., Valla, S., Skjåk-Bræk, G., 'Enzymatic Alginate Modification', Alginates: Biology and Applications, 2009, 95–115.

Fourest, E., Volesky, B., 'Contribution of Sulfonate Groups and Alginate to Heavy Metal Biosorption by the Dry Biomass of *Sargassum fluitans*', Environmental Science and Technology,1996, 30, 277–282.

Franklin, M. J., Ohman, D. E., 'Identification of algF in the Alginate Biosynthetic Gene Cluster of *Pseudomonas aeruginosa* Which is Required for Alginate Acetylation', Journal of Bacteriology, 1993, 175, 16, 5057–5065.

Franklin, M. J., Chitnis, C. E., Gacesa, P., Sonesson, A., White, D. C., Ohman, D. E., '*Pseudomonas aeruginosa* AlgG is a Polymer Level Alginate C5-mannuronan Epimerase', Journal of Bacteriology, 1994, 176, 7,1821–1830.

Galindo, E., Peña, C., Núñez, C., Segura, D., Espín, 'Molecular and Bioengineering Strategies to Improve Alginate and Polydydroxyalkanoate Production by *Azotobacter vinelandii*', Microbial Cell Factories, 2007, 6, 7.

Gallon, J. R., 'Tansley Review No. 44, Reconciling the Incompatible: N_2 Fixation and O_2', New Phytologist, 1992, 122, 571–609.

Gimmestad, M., Sletta, H., Ertesvåg, H., Bakkevig, K., Jain, S., Suh, S.-J., Skjåk-Bræk, G., Ellingsen, T. E., Ohman, D. E., Valla, S., 'The *Pseudomonas fluorescens* AlgG Protein, but Not Its Mannuronan C-5-epimerase Activity, is Needed for Alginate Polymer Formation', Journal of Bacteriology, 2003, 185, 3515–3523.

Gorin, J. P. A., Spencer, T. J. F., 'Exocellular Alginic Acid from Azotobacter vinelandii', Canadian Journal of Chemistry, 1966, 44, 993–998.

Grasdelen, H., Larsen, B., Smidsrød, O., 'A P.M.R. Study of the Composition and Sequence of Uronate Residues in Alginates', Carbohydrate Research, 1979, 68, 23–31.

Guibal, E., Roussy, J., 'Coagulation and Flocculation of Dye-containing Solutions using a Biopolymer (Chitosan)', Reactive and Functional Polymers, 2007, 67, 33–42.

Haddock, B. A., Jones, C. W., 'Bacterial Respiration', Bacteriological Reviews, 1977, 47–99.

Hammad, A. M. M., 'Evaluation of Alginate Encapsulated Azotobacter chroococcum as a Phage-resistant and an Effective Inoculum', Journal of Basic Microbiology, 1998, 1, 9–16.

Hartmann, M., Holm, O. B., Johansen, G. A. B., Skjåk-Bræk, G., Stokke, B. T., 'Mode of Action of Recombinant Azotobacter vinelandii Mannuronan C-5 Epimerases AlgE2 and AlgE4', Biopolymers, 2002, 63, 77–88.

Haug A., 'The Affinity of Some Divalent Metals to Different Types of Alginates', Acta Chemica Scandinavia, 1961,15, 1794–1795.

Haug A, Smidsrød O., 'The Effect of Divalent Metals on the Properties of Alginate Solutions. II. Comparison of Different Metal Ions', Acta Chemica Scandinavia, 1965, 19, 2, 341–51.

Haug, A., Larsen, B., 'Biosynthesis of Alginate. Part II. Polymannuronic Acid C-5-Epimerase from Azotobacter vinelandii', Carbohydrate Research, 1971, 17, 297–308.

Haug, A., Larsen, B., Smirsrød, O., 'Uronic Acid Sequence in Alginate from Different Sources', Carbohydrate Research, 1974, 32, 217–225.

Hogg, R., 'The Role of Polymer Adsorption Kinetics in Focculation', Colloids and Surfaces A: Physicochemical and Engineering Aspects, 1999, 146, 253–263.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., Williams, S. T., 'Bergey's Manual of Determinative Bacteriology', 1994, 9th Edition, Baltimore.

Holtan, S., Bruheim, P., Skjåk-Bræk, G., 'Mode of Action and Subsite Studies of the Guluronan Block-forming Mannuronan C-5 Epimerases AlgE1 and AlgE6', Biochemical Journal, 2006, 395, 319–329.

Høidal, H. K., Ertesvåg, H., Skjåk-Bræk, G., Stokke, B. T., Valla, S., 'The Recombinant *Azotobacter vinelandii* Mannuronan C-5-Epimerase AlgE4 Epimerizes Alginate by a Nonrandom Attack Mechanism', The Journal of Biological Chemistry, 1999, 274, 18, 12316–12322.

Huang, C., Chen, S., and Pan J. S. "Optimal Condition for Modification of Chitosan: a Biopolymer for Coagulation of Colloidal Particles" Water Research, 2000, 34(3), 1057–1062.

Hughes, M. A., Coagulation and Flocculation, Chapter 4, Part I, 1990, 104–129.

Ives, K. J., 'Coagulation and Flocculation, Part II, Orthokinetic Flocculation, 1990, 130–165.

Jain, S., Ohman, D. E., 'Deletion of algK in Mucoid *Pseudomonas aeruginosa* Blocks Alginate Polymer Formation and Results in Uronic Acid Secretion' Journal of Bacteriology, 1998, 180, 3, 634–641.

Jain, S., Franklin, M. J., Ertesvåg, H., Valla, S., Ohman, D. E., 'The Dual Roles of AlgG in C-5-Epimerization and Secretion of Alginate Polymers in *Pseudomonas aeruginosa*', Molecular Microbiology, 2003, 47, 4, 1123–1133.

Jang, L. K., Harpt, N., Grasmick, D., Vuong, L. N., Geesey, G., 'A Two-Phase Model for Determining the Stability Constants for Interactions between Copper and Alginic Acid', The Journal of Physical Chemistry, 1990, 94, 1, 482–488.

Jang, L. K., Lopez, S. L., Eastman, S. L., Pryfogle, P., 'Recovery of Copper and Cobalt by Biopolymer Gels', Biotechnology and Bioengineering, 1991, 37, 266-273.

Jarman, T. R., Deavin, L., Slocombe, S., Righelato, R. C., 'Investigaton of the Effect of Environmental Conditions on the Rate of Exopolysaccharides Synthesis in *Azotobacter vinelandii*', Journal of General Microbiology, 1978, 107, 59–64.

Jeon, C., Park, J. Y., Yoo, Y. J., 'Novel Immobilization of Alginic Acid for Heavy Metal Removal', Biochemical Engineering Journal, 2002, 11, 159–166.

Jodra, Y., Mijangos, F., 'Ion Exchange Selectivities of Calcium Alginate Gels for Heavy Metals', Water Science and Technology, 2001, 43, 2, 237–244.

Kuhla, J., Oelze, J., 'Dependence of Nitrogenase Switch-Off upon Oxygen Stress on the Nitrogenase Activity in *Azotobacter Vinelandii*', Journal of Bacteriology, 1988, 5325–5329.

Labille, J., Thomas, F., Milas, M., Vanhaverbeke, C., 'Flocculation of Colloidal Clay by Bacterial Polysaccharides: Effect of Macromolecule Charge and Structure', Journal of Colloid and Interface Science, 2005, 284, 149–156.

Larsen, B., Haug, A., 'Biosynthesis of Alginate. Part I. Composition and Structure of Alginate Produced by *Azotobacter vinelandii*', Carbohydrate Research, 1971, 17, 287–296.

Larsen, B., Salem, D. M. S. A., Sallam, M. A. E., Mishrikey, M. M., Beltagy, A. I., 'Characterization of the Alginates from Algae Harvested at the Egyptian Red Sea Coast', Carbohydrate Research, 2003, 338, 2325–2336.

Linker, A., Jones, R. S., 'A Polysaccharide Resembling Alginic Acid from a *Pseudomonas* Microorganism', Nature, 1964, 204, 187–188.

Linkerhägner, K., Oelze, J., 'Cellular ATP Levels and Nitrogenase Switchoff upon Oxygen Stress in Chemostat Cultures of *Azotobacter vinelandii*', Journal of Bacteriology, 1995, 5289–5293.

Linkerhägner, K., Oelze, J., 'Nitrogenase Activity and Regeneration of the Cellular ATP Pool in *Azotobacter Vinelandii* Adapted to Different Oxygen Concentrations', Journal of Bacteriology, 1997, 1362–1367.

Liu, J. K., Lee, F. T., Lin, C. S., Yao, X. T., Davenport, J. W., Wong, T. Y., 'Alternative Function of the Electron Transport System in *Azotobacter vinelandii*: Removal of Excess Reductant by the Cytochrome d Pathway', Applied and Environmental Microbiology, 1995, 3998–4003.

Massart, D. L., Dijkstra, A., Kaufman, L., 'Evaluation and Optimization of Laboratory Methods and Analytical Procedures', Elsevier Scientific Publishing Company, Netherlands, 1993, 337–339.

Maximova, N., Dahl, O., 'Environmental Implications of Aggregation Phenomena: Current understanding', Current Opinion in Colloid and Interface Science, 2006, 11, 246–266.

Mchugh, D. J., 'A Guide to the Seaweed Industry', Fao Fisheries Technical Paper 441, 2005.

Mchugh, D. J., 'Production and Utilization of Products from Commercial Seaweeds', Fao Fisheries Technical Paper 288, 1987.

Miller, G. L., 'Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar', Analytical Chemistry, 1959, 31, 3, 426–428.

Moreno, J., Vargas-Garcia, M.C., Lopez, M.J., Sanchez-Serrano, G., 'Growth and Exopolysaccharide Production by *Azotobacter vinelandii* in Media Containing Phenolic Acids', Journal of Applied Microbiology, 1999, 86, 439–445.

Moshiri, F., Crouse, B. R., Johnson, M. K., Maier, R. J., 'The Nitrogenase-Protective FeSII Protein of *Azotobacter vinelandii*: Overexpression, Characterization, and Crystallization', Biochemistry, 1995, 34, 12973-12982.

Mukhopadhyay, M., Noronha, S.B., Suraishkumar, G.K., 'Kinetic Modeling for the Biosorption of Copper by Pretreated *Aspergillus niger* Biomass', Bioresource Technology, 2007, 98, 1781–1787.

Nunez, C., Moreno, S., Soberon-Chavez, G., Espin, G., 'The *Azotobacter vinelandii* Response Regulator AlgR is Essential for Cyst Formation', Journal of Bacteriology, 1999, 181, 141–148.

Obika, H., Sakakibara, J., Kobayashi, Y., 'Direct Control of the Constituents Ratio in Alginate Produced by *Azotobacter vinelandii*', Bioscience Biotechnology Biochemistry, 1993, 57, 2, 332–333.

Ozdemir, G., Ceyhan, N., Ozturk, T., Akirmak, F., Cosar, T., 'Biosorption of Chromium(VI), Cadmium(II) and Copper(II) by *Pantoea sp. TEM18*', Chemical Engineering Journal, 2004,102, 249–253.

Özacar, M., Sengil, I. A., 'Evaluation of Tannin Biopolymer as a Coagulant Aid for Coagulation of Colloidal Particles', Colloids and Surfaces A : Physicochemical Engineering Aspects, 2003, 229, 85–96.

Özer, A., Gürbüz, G., Çalimli, A., Körbahti, B. K., 'Biosorption of Copper(II) Ions on *Enteromorpha prolifera*: Application of Response Surface Methodology (RSM)', Chemical Engineering Journal, 2009, 146, 377–387.

Page, W. J., Sadoff, H. L., 'Calcium and Uronic Acids in the Encystment of *Azotobacter vinelandii*', Journal of Bacteriology, 1975, 122, 19, 145–151.

Pamukoglu, M. Y., Kargi, F., 'Removal of Copper (II) Ions from Aqueous Medium by Biosorption onto Powdered Waste Sludge', Process Biochemistry, 2006, 41, 1047–1054.

Pandey, A. K., Pandey, S. D., Misra, V., 'Removal of Toxic Metals from Leachates from Hazardous Solid Wastes and Reduction of Toxicity to Microtox by the Use of Calcium Alginate Beads Containing Humic Acid', Ecotoxicology and Environmental Safety, 2002, 52, 92–96.

Papageorgiou,S. K., Kouvelos, E. P., Katsaros, F. K., 'Calcium Alginate Beads from *Laminaria digitata* for the Removal of Cu^{+2} and Cd^{+2} from Dilute Aqueous Metal Solutions', Desalination, 2008, 224, 293–306.

Parente, E., Crudele, M. A., Aquino, M., Clementi, F., 'Alginate Production by Azotobacter vinelandii DSM576 in Batch Fermentation', Journal of Industrial Microbiology and Biotechnology, 1998, 20, 171–176.

Parente, E., Crudele, M.A., Ricciardi, A., Mancini, M., Clementi, F. 'Effect of Ammonium Sulphate Concentration and Agitation Speed on the Kinetics of Alginate Production by *Azotobacter vinelandii* DSM576 in Batch Fermentation', Journal of Industrial Microbiology and Biotechnology, 2000, 25, 242–248.

Park, H. G., Kim, T. W., Chae, M. Y., Yoo, I., 'Activated Carbon-containing Alginate Adsorbent for the Simultaneous Removal of Heavy Metals and Toxic Organics', Process Biochemistry, 2007, 42, 1371–1377.

Peña, C., Campos, N., Galindo, E., 'Changes in Alginate Molecular Mass Distributions, Broth Viscosity and Morphology of Azotobacter vinelandii Cultured in Shake Flasks', Applied Microbiology Biotechnology, 1997, 48, 510–515.

Peña, C., Trujillo-Roldán, M. A., Galindo, E., 'Influence of Dissolved Oxygen Tension and Agitation Speed on Alginate Production and Its Molecular Weight in Cultures of *Azotobacter vinelandii*', Enzyme and Microbial Technology, 2000, 27, 390–398.

Peña,, C., Reyes, C., Larralde-Corona, P., Corkidi, G., Galindo, E., 'Characterization of *Azotobacter vinelandii* Aggregation in Submerged Culture by Digital Image Analysis', FEMS Microbiology Letters, 2002, 207, 173–177.

Post, E., Kleiner, D., Oelze, J., 'Whole Cell Respiration and Nitrogenase Activities in *Azotobacter vinelandii* Growing in Oxygen Controlled Continuous Culture', Archieve Microbiology, 1983, 134, 68–72.

Priego-Jimenez, R., Pena, C., Ramirez, O. T., Galindo, G., 'Specific Growth Rate Determines the Molecular Mass of the Alginate Produced by *Azotobacter vinelandii*', Biochemical Engineering Journal, 2005, 25, 187–193.

Ramstad, M. V., Ellingsen, T. E., Josefsen, K. D., Høidal, H. K., Valla, S., Skjåk-Bræk, G., Levine, D. W., 'Properties and Action Pattern of the Recombinant Mannuronan C-5-Epimerase AlgE2', Enzyme and Microbial Technology, 1999, 24, 636–646.

Ramstad, M. V., Markussena, S., Ellingsen, T. E., Skjåk-Bræk, G., Levine, D. W., 'Influence of Environmental Conditions on the Activity of the Recombinant Mannuronan C-5-Epimerase AlgE2', Enzyme and Microbial Technology, 2001, 28, 57–69.

Rehm, B. H. A., Valla, S., 'Bacterial Alginates: Biosynthesis and Applications' Applied Microbiology and Biotechnology, 1997, 48, 281–288.

Rehm, B. H. A., 'Alginate Lyase from *Pseudomonas aeruginosa* CF1/M1 Prefers the Hexameric Oligomannuronate as Substrate', FEMS Microbiology, 1998, 165, 175–180.

Remminghorst, U., Rehm, B. H. A., 'Bacterial Alginates: from Biosynthesis to Applications', Biotechnology Letter, 2006, 28, 1701–1712.

Reyes, C., Peña, C., Galindo, E., 'Reproducing Shake Flasks Performance in Stirred Fermentors: Production of Alginates by *Azotobacter vinelandii*', Journal of Biotechnology, 2003, 105, 189–198.

Robles-Price, A., Wong, T. Y., Sletta, H., Valla, S., Schiller, N. L. 'AlgX is a Periplasmic Protein Required for Alginate Biosynthesis in *Pseudomonas aeruginosa*', Journal of Bacteriology, 2004,186, 21, 7369–7377.

Sabra, W., Zeng, A.P., Sabry, S., Omar, S., Deckwer, W.D., 'Effect of Phosphate and Oxygen Concentrations on Alginate Production and Stoichiometry of Metabolism of *Azotobacter vinelandii* under Microaerobic Conditions', Applied Microbiology Biotechnology, 1999, 52, 773–780.

Sabra, W., Zeng, A.-P., Lünsdorf, H., Deckwer, W.-D., 'Effect of Oxygen on Formation and Structure of *Azotobacter vinelandii* Alginate and Its Role in Protecting Nitrogenase', Applied and Environmental Microbiology, 2000, 66, 9, 4037–4044.

Sabra, W., Zeng, A.-P., Deckwer, W.-D., 'Bacterial Alginate: Physiology, Product Quality and Process Aspects', Applied Microbiology Biotechnology, 2001, 56, 315–325.

Sadoff, H. L., 'Encystment and Germination in *Azotobacter vinelandii*', Bacteriological Reviews, 1975, 39 (4), 516–539.

Salehizadeh, H., Shojaosadati, S.A., 'Extracellular Biopolymeric Flocculants Recent Trends and Biotechnological Importance', Biotechnology Advances, 2001, 19, 371–385.

Sand, A., Yadav, M, Mishra, D. K., Behari, K., 'Modification of Alginate by Grafting of N-vinyl-2-pyrrolidone and studies of Physicochemical Properties in terms of Swelling Capacity, Metal ion uptake and Flocculation', Carbohydrate Polymers, 2010, 80, 4, 1147–1154.

Satinder, A., 'Selectivity and detectability optimizations in HPLC', 1989, 229–261.

Saude, N., Junter, G.-A., 'Production and Molecular Weight Characteristics of Alginate from Free and Immobilized-cell Cultures of *Azotobacter vinelandii*', Process Biochemistry, 2002, 37, 895–900.

Savalgi, V., Savalgi, V., 'Alginate Production by *Azotobacter vinelandii* in Batch Culture', Journal of General Applied Microbiology, 1992, 38, 641–645.

Schürks, N., Wingender, J., Flemming, H.-C., Mayer, C., 'Monomer Composition and Sequence of Alginates from *Pseudomonas aeruginosa*', Biological Macromolecules, 2002, 30, 105–111.

Seáñez, G., Peña, C., Galindo, E., 'High CO_2 Affects Alginate Production and Prevents Polymer Degradation in Cultures of *Azotobacter vinelandii*', Enzyme and Microbial Technology, 2001, 29, 535–540.

Segura, D., Guzmán, J., Espín, G., '*Azotobacter vinelandii* mutants that overproduce poly-β-hydroxybutyrate or Alginate', Applied Microbiology Biotechnology, 2003, 63, 159–163.

Sheng, P. X., Wee, K. H., Ting, Y. P., Chen, J. P. 'Biosorption of Copper by Immobilized Marine Algal Biomass', Chemical Engineering Journal, 2008, 136, 156–163.

Skjak-Bræk, G., Paoletti, S., Gianferrara, T., 'Selective Acetylation of Mannuronic Acid Residues in Calcium Alginate Gels', Carbohydrate Research, 1989, 185, 119–129.

Svanem, B. I. G., Skjåk-Bræk, G., Ertesvåg, H., Valla, S., 'Cloning and Expression of Three New Azotobacter Vinelandii Genes Closely Related to a Previously Described Gene Family Encoding Mannuronan C-5-Epimerases', Journal of Bacteriology, 1999, 181, 1, 68–77.

Tebbutt, T. H. Y., Principles of Water Quality Control, Coagulation, Chapter 12, 1998, 151–161.

Tripathy, T., Bhagat, R. P., Singh, R. P., 'The Flocculation Performance of Grafted Sodium Alginate and Other Polymeric Flocculants in Relation to Iron Ore Slime Suspension', European Polymer Journal, 2001, 37, 125–130.

Trujillo-Roldán, M. A., Peña, C., Ramírez, O. T., Galindo, E., 'Effect of Oscillating Dissolved Oxygen Tension on the Production of Alginate by *Azotobacter vinelandii*', Biotechnology Progress, 2001, 17, 1042–1048.

Trujillo-Roldán, M. A., Peña, C., Galindo, E., 'Components in the inoculum determine the kinetics of *Azotobacter vinelandii* cultures and the molecular weight of its alginate', Biotechnology Letters, 2003, 25, 1251–1254.

Trujillo-Roldán, M. A., Moreno, S., Segura, D., Galindo, E., Espín, G., 'Alginate Production by an *Azotobacter vinelandii* Mutant Unable to Produce Alginate Lyase', Applied Microbiology Biotechnology, 2003b, 60, 733–737.

Trujillo-Roldán, M. A., Moreno, S., Espín, G., Galindo, E., 'The Roles of Oxygen and Alginate-lyase in Determining the Molecular Weight of Alginate Produced by *Azotobacter vinelandii*', Applied Microbiology Biotechnology, 2004, 63, 742–747.

Valla, S., Li, J., Ertesvåg, H., Barbeyron, T., Lindahl, U., 'Hexuronyl C5-epimerases in Alginate and Glycosaminoglycan Biosynthesis', Biochimie, 2001, 83, 819–830.

Vargas-Garcia, M.C., Lopez, M.J., Elorrieta, M.A., Suarez, F., Moreno, J., 'Physiology of Exopolysaccharide Production by *Azotobacter vinelandii* from 4-hydroxybenzoic Acid', Journal of Industrial Microbiology and Biotechnology, 2002, 29, 129–133.

Vargas-Garcia, M.C., Lopez, M.J., Elorrieta, M.A., Suarez, F., Moreno, J., 'Properties of Polysaccharide Produced by *Azotobacter vinelandii* Cultured on 4-hydroxybenzoic acid', Journal of Applied Microbiology, 2003, 94, 388–395.

Vilar, V. J. P., Botelho, C. M. S., Loureiro, J. M., Boaventura, R. A. R., 'Biosorption of Copper by Marine Algae Gelidium and Algal Composite Material in a Packed Bed Column', Bioresource Technology, 2008a, 99, 5830–5838.

Vilar, V. J. P., Botelho, C. M. S., Boaventura, R. A. R., 'Lead and Copper Biosorption by Marine Red Algae Gelidium and Algal Composite Material in a CSTR ("Carberry" Type)', Chemical Engineering Journal, 2008b, 138, 249–257.

Vijayaraghavan, K., Prabu, D., 'Potential of *Sargassum wightii* Biomass for Copper(II) Removal from Aqueous Solutions: Application of Different Mathematical Models to Batch and Continuous Biosorption Data', Journal of Hazardous Materials, 2006, B137, 558–564.

Volesky, B., 'Detoxification of Metal-bearing Effluents: Biosorption for the Next Century', Hydrometallurgy, 2001, 59, 203–216.

Wang, J., Chen, C., 'Biosorbents for Heavy Metals Removal and Their Future', Biotechnology Advances, 2009, 27, 195–226.

Wang, N. S., 'Sucrose Assay by the Dinitrosalicylic Colorimetric Method', University of Maryland, 2007.

Yahaya, Y. A., Don, M. M., Bhatia, S. 'Biosorption of Copper (II) onto Immobilized Cells of *Pycnoporus sanguineus* from Aqueous Solution: Equilibrium and Kinetic Studies', Journal of Hazardous Materials, 2009, 161, 189–195.

Yüksel, A. M., 'Use of Calcium-Alginate as a Coagulant for Low Turbidity Waters', M.S. Thesis, METU, 2007.

APPENDIX A

^{1}H-NMR RESULTS FOR FMC ALGINATES

Table A.1. ^{1}H-NMR results for FMC alginates

Alginate	MM-block (%)	MG-block (%)	GG-block (%)
LF240D	54	27	19
LF120M	49	22	29
HF120RBS	50	13	37
LF200DL	23	22	29
LF200S	24	22	54

APPENDIX B

CALIBRATION CURVE FOR SUCROSE DETERMINATON

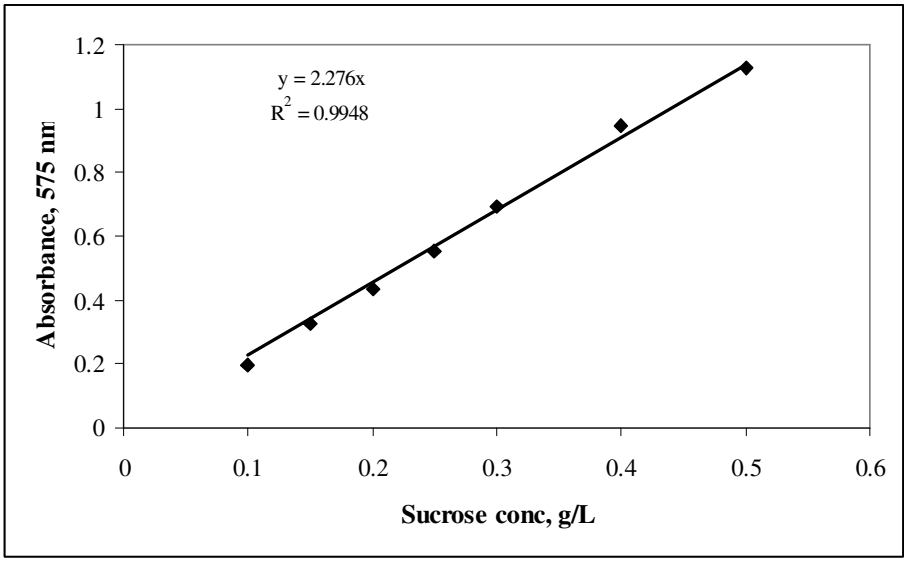

Figure B.1. A sample calibration curve for sucrose determination

APPENDIX C

^{1}H-NMR SPECTRUMS FOR ALGINATES AT DIFFERENT DOT

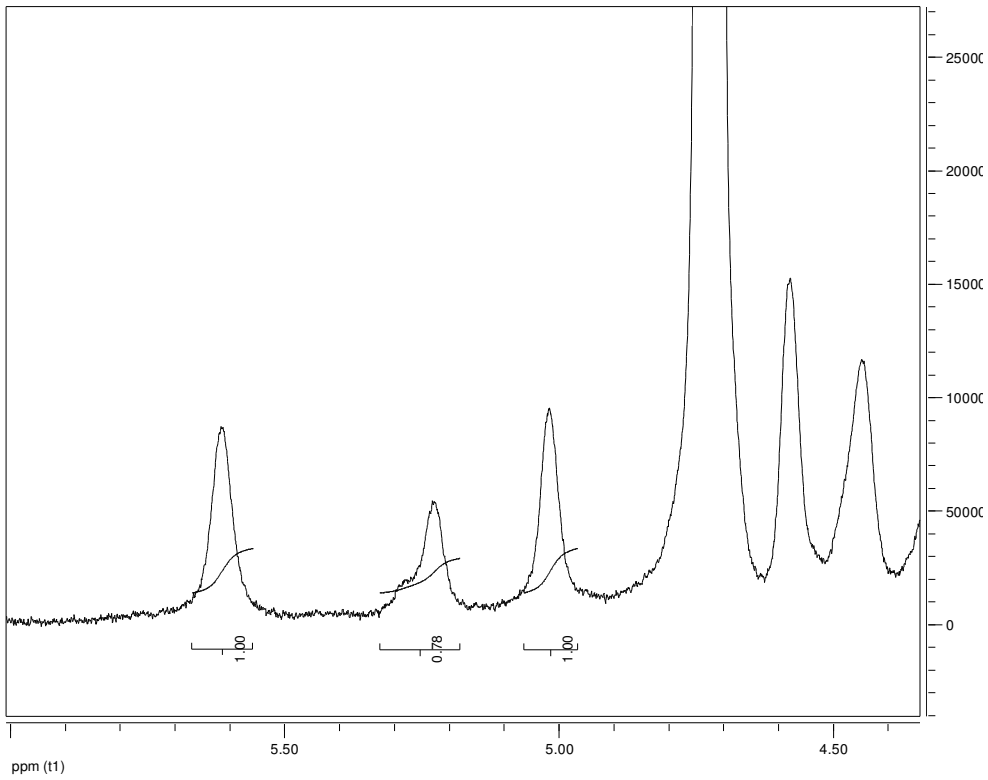

Figure C.1. ^{1}H-NMR spectrum for alginate harvested at 1 % DOT, 24 hours and 70 ℃

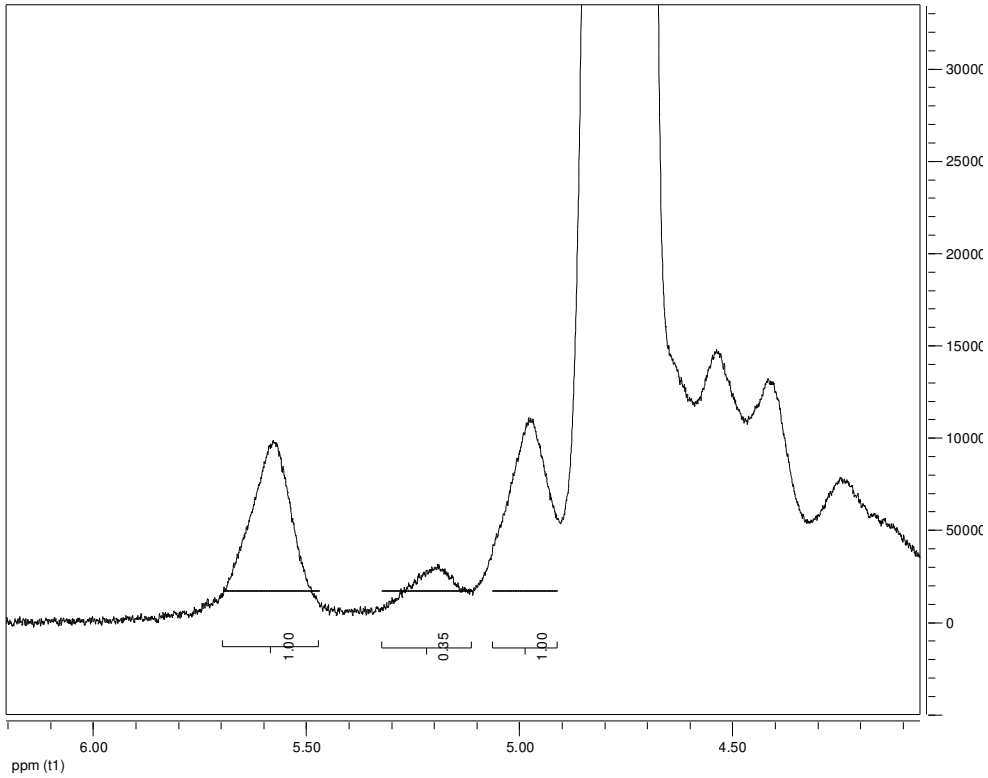

Figure C.2. ^{1}H-NMR spectrum for alginate harvested at 1 % DOT, 32 hours and 70 ℃

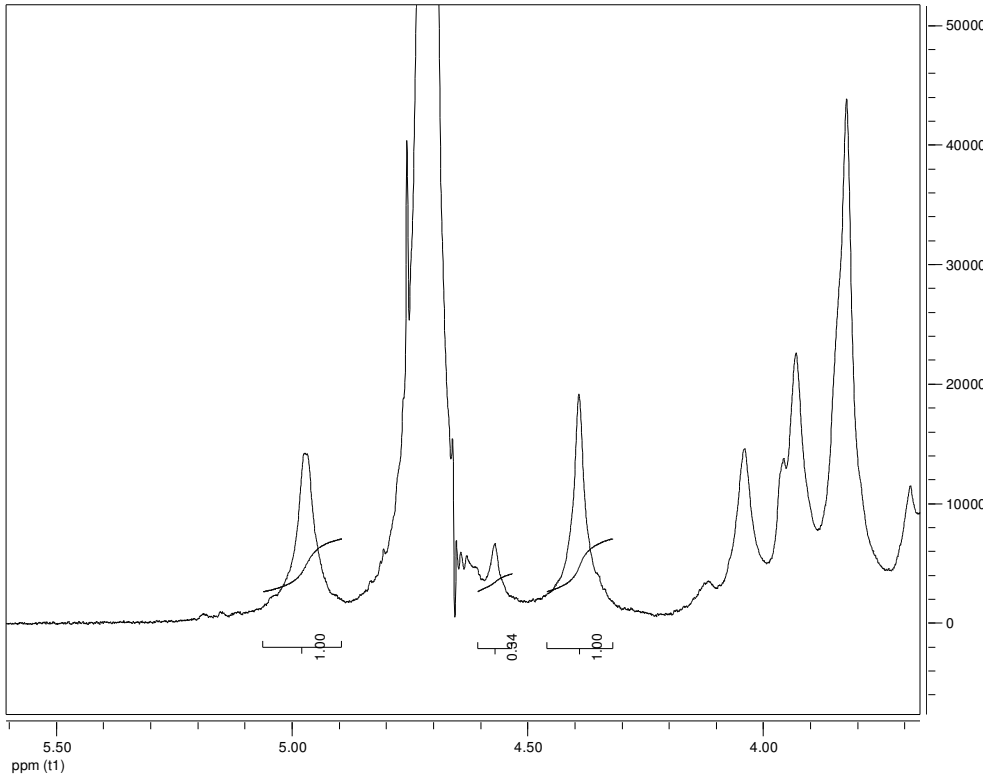

Figure C.3. ^{1}H-NMR spectrum for alginate harvested at 1 % DOT, 24 hours and 30 ℃

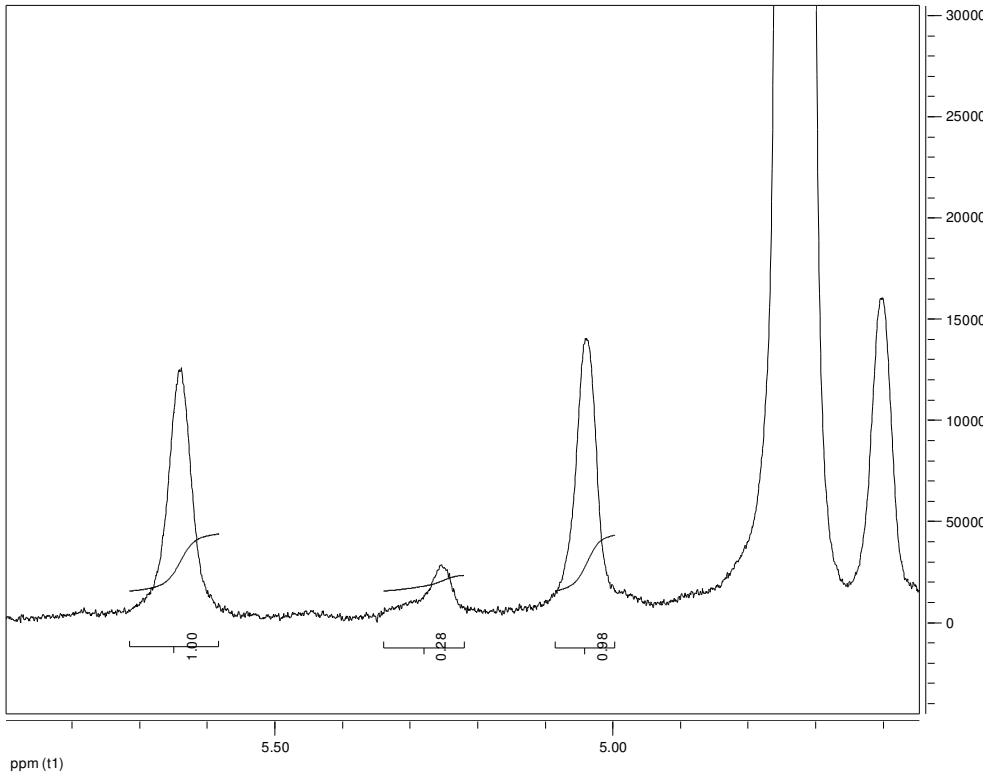

Figure C.4. ^{1}H-NMR spectrum for alginate harvested at 1 % DOT, 48 hours and 70 ℃

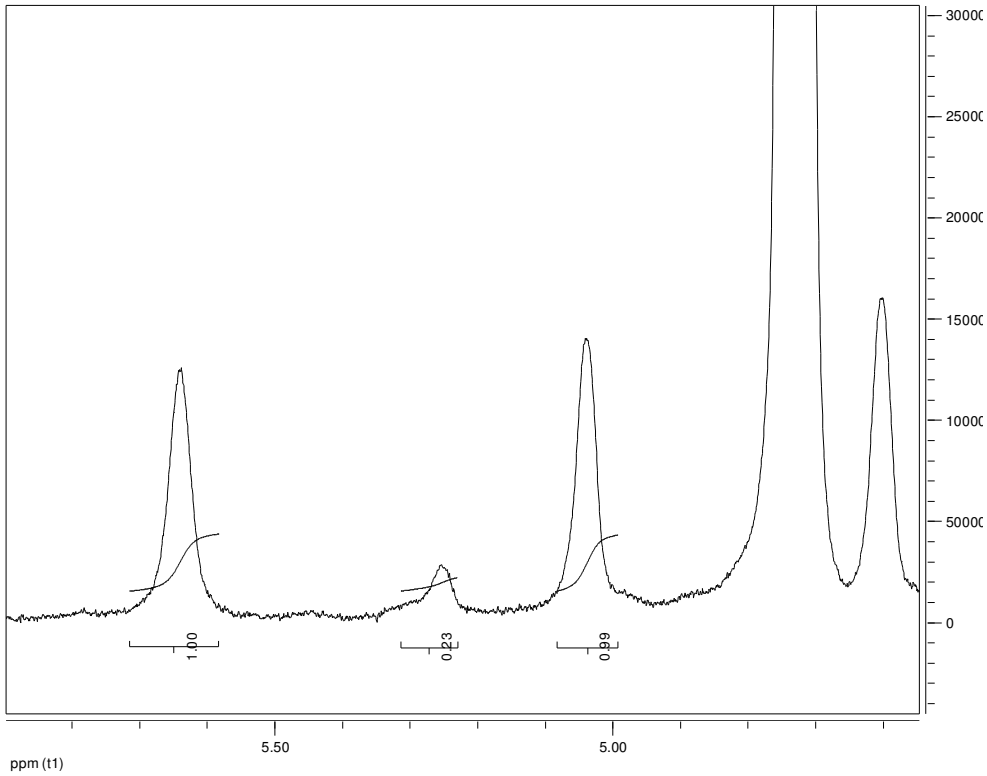

Figure C.5. ^{1}H-NMR spectrum for alginate harvested at 1 % DOT, 56 hours and 70 ℃

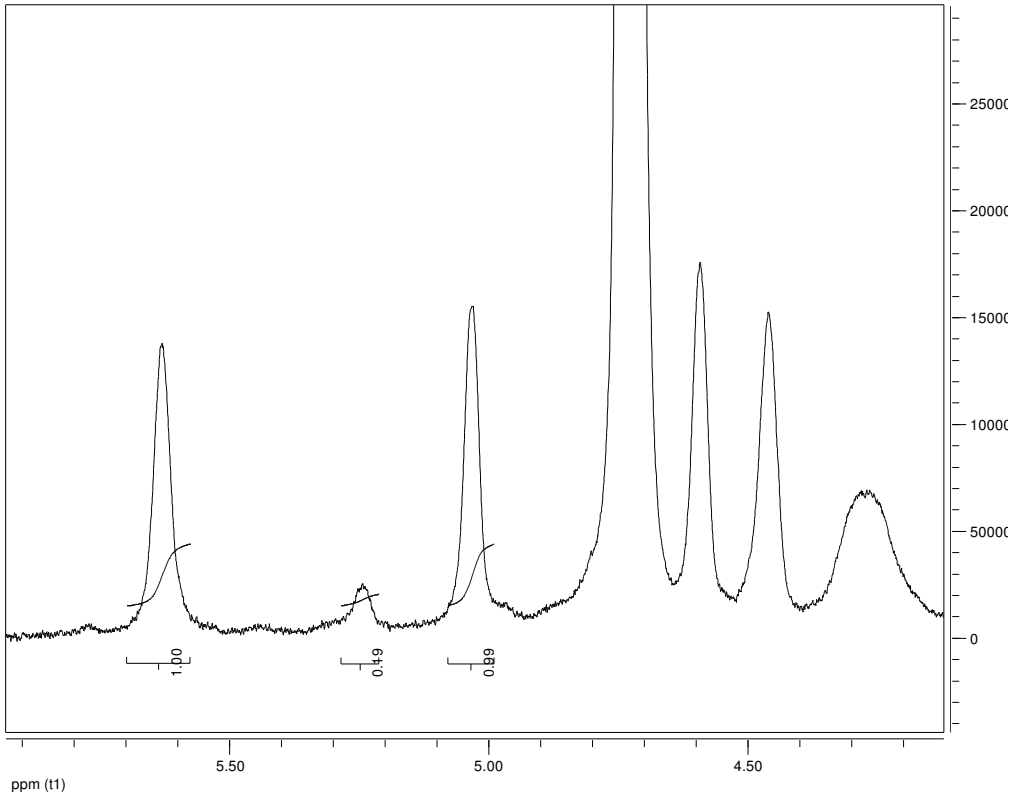

Figure C.6. ^{1}H-NMR spectrum for alginate harvested at 1 % DOT, 72 hours and 70 ℃

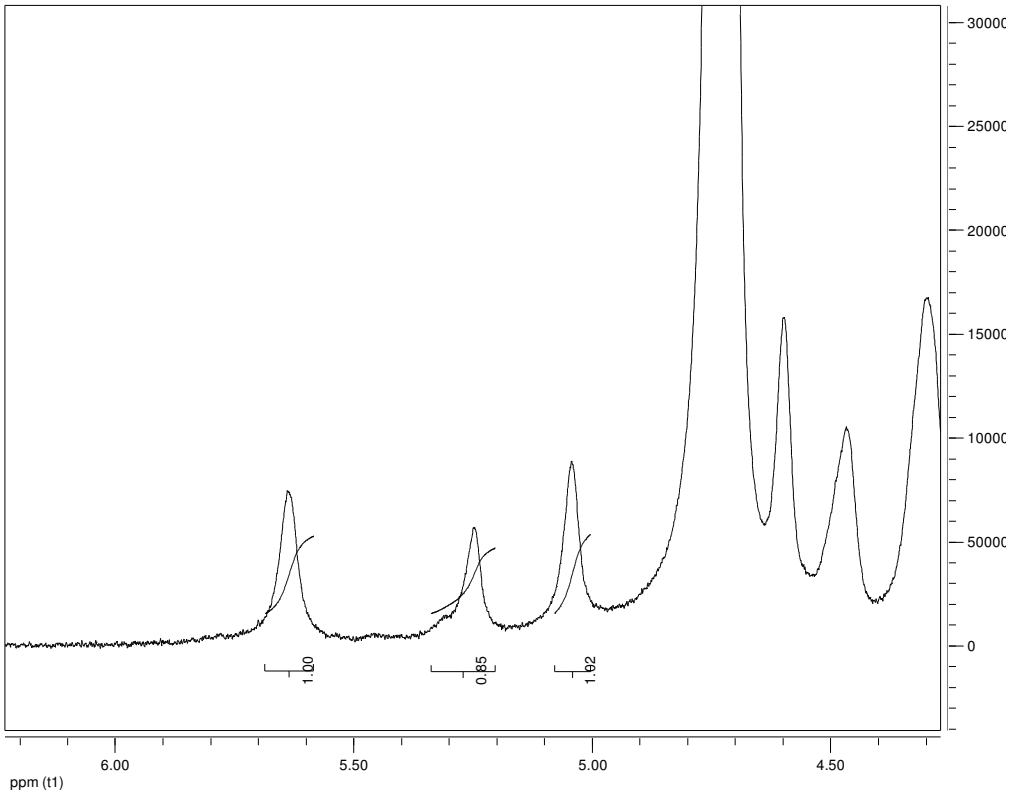

Figure C.7. ^{1}H-NMR spectrum for alginate harvested at 3 % DOT, 24 hours and 70 ℃

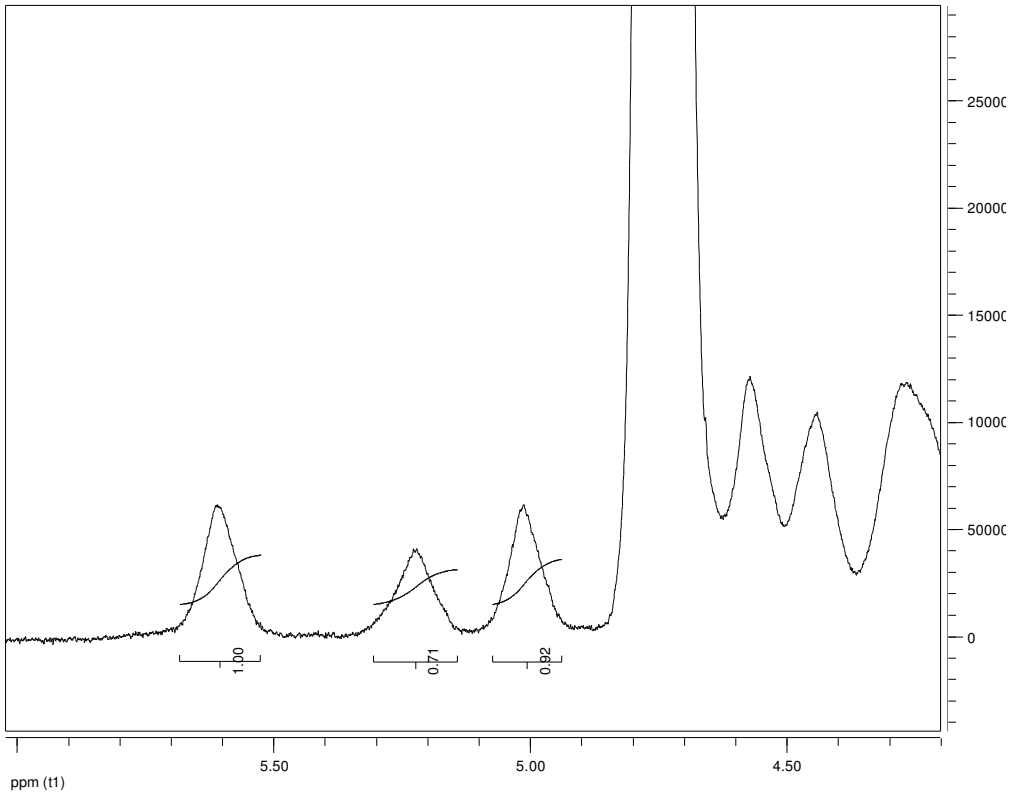

Figure C.8. ^{1}H-NMR spectrum for alginate harvested at 3 % DOT, 32 hours and 70 ℃

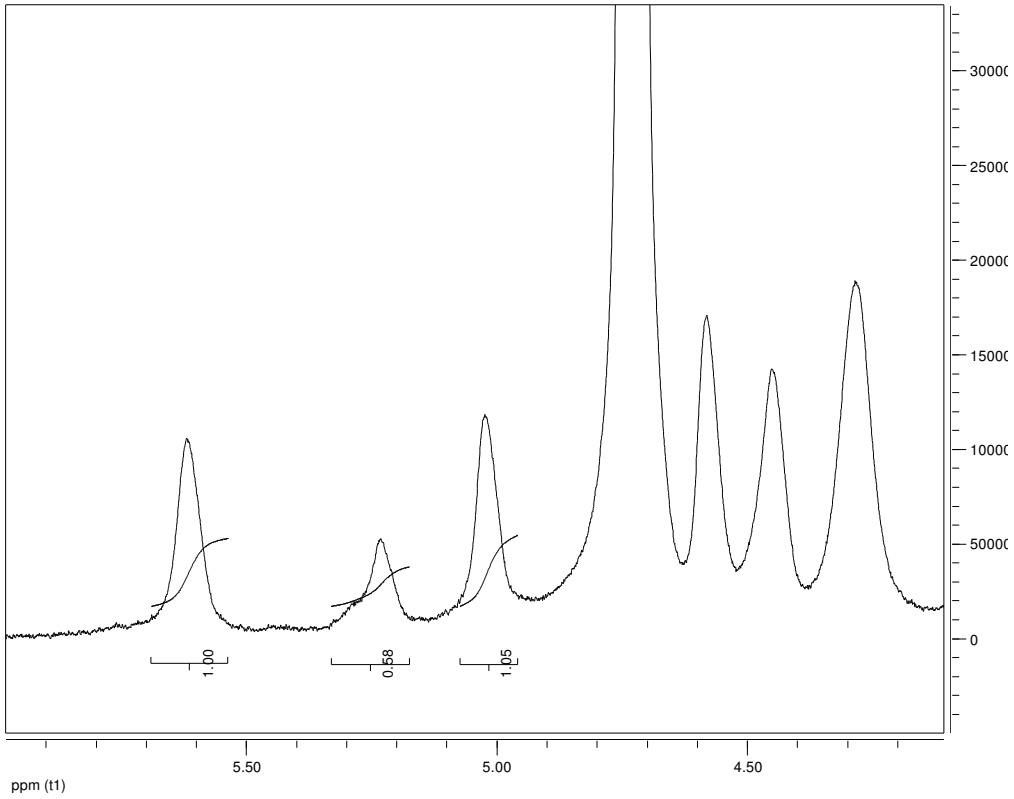

Figure C.9. ^{1}H-NMR spectrum for alginate harvested at 3 % DOT, 48 hours and 70 ℃

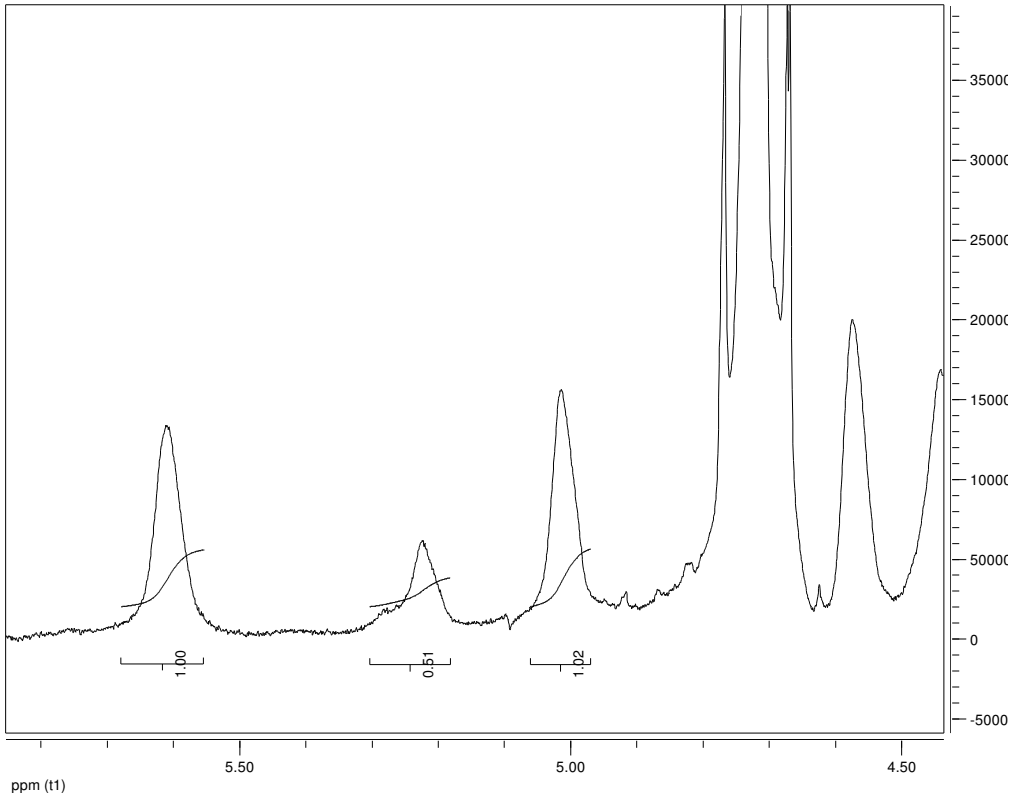

Figure C.10. ^{1}H-NMR spectrum for alginate harvested at 3 % DOT, 56 hours and 70 ℃

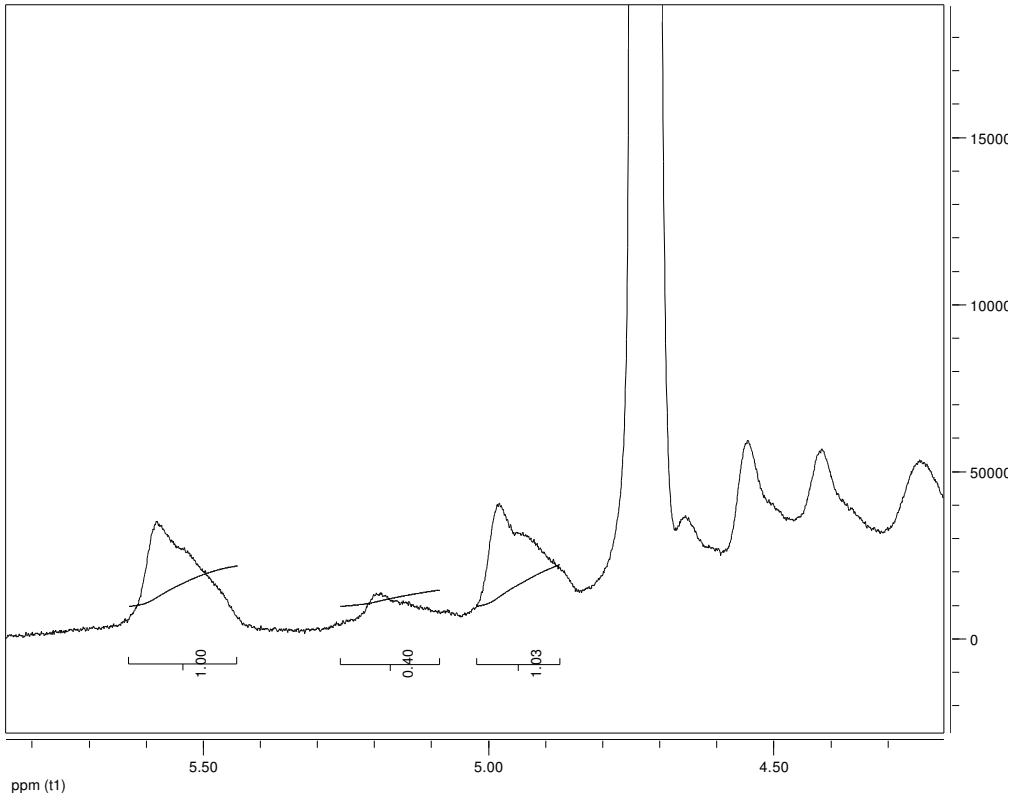

Figure C.11. ^{1}H-NMR spectrum for alginate harvested at 3 % DOT, 72 hours and 70 ℃

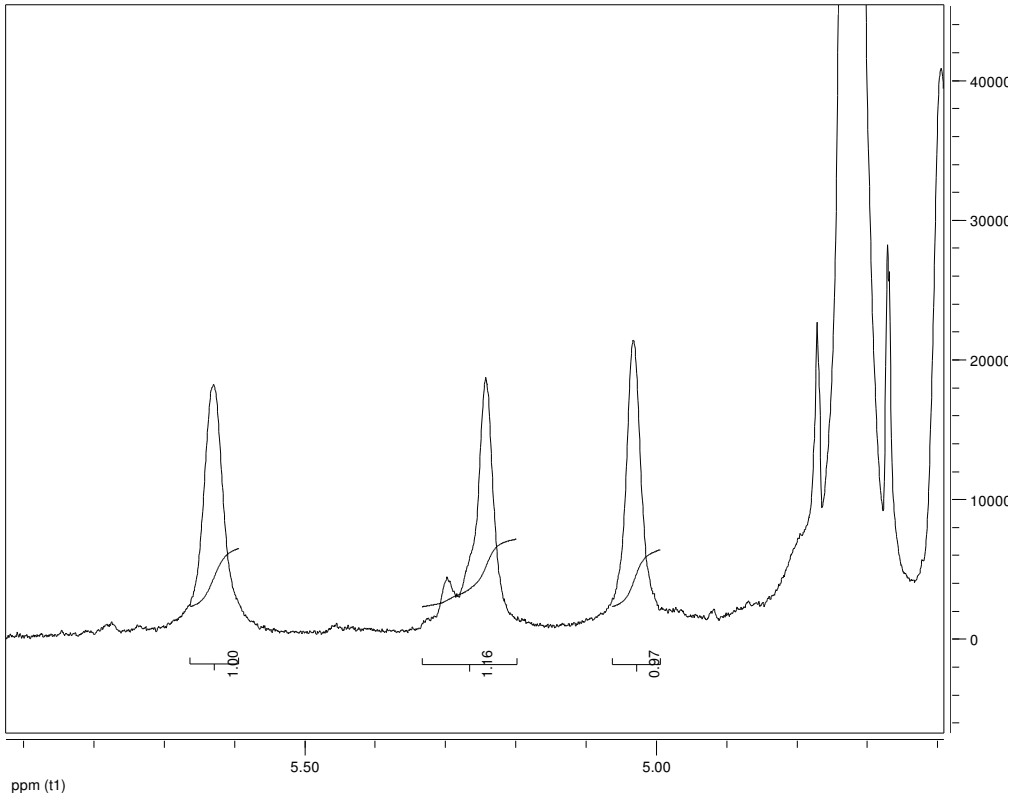

Figure C.12. ^{1}H-NMR spectrum for alginate harvested at 5 % DOT, 24 hours and 70 ℃

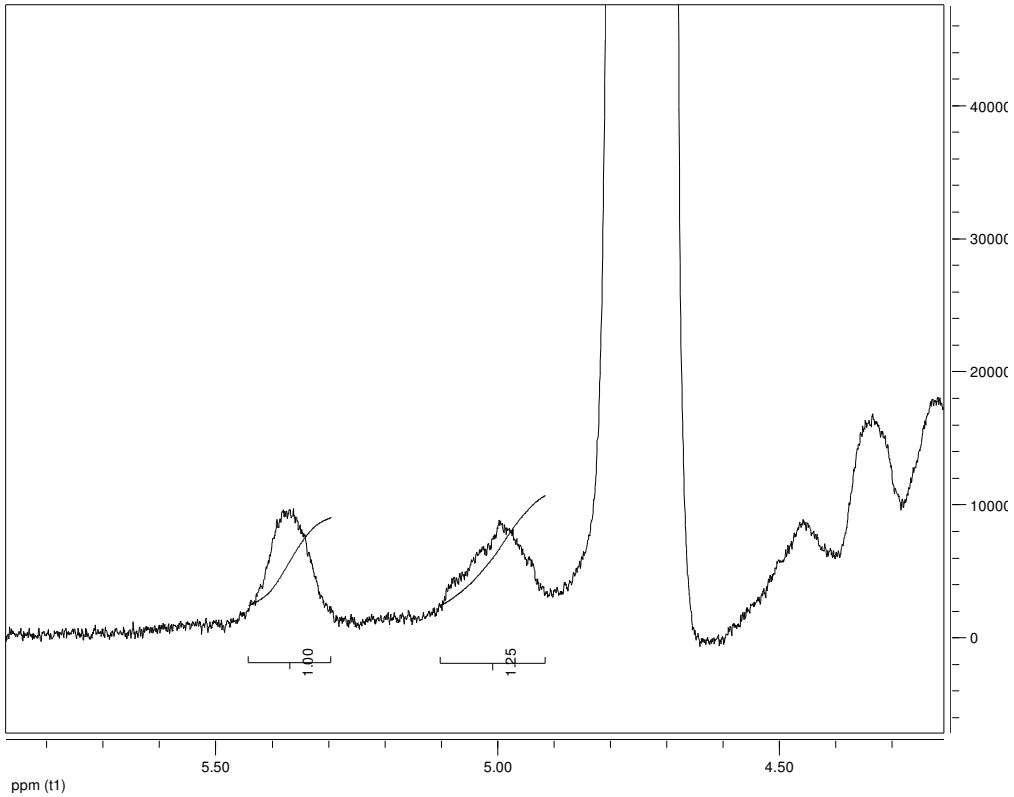

Figure C.13. ^{1}H-NMR spectrum for alginate harvested at 5 % DOT, 32 hours and 70 ℃

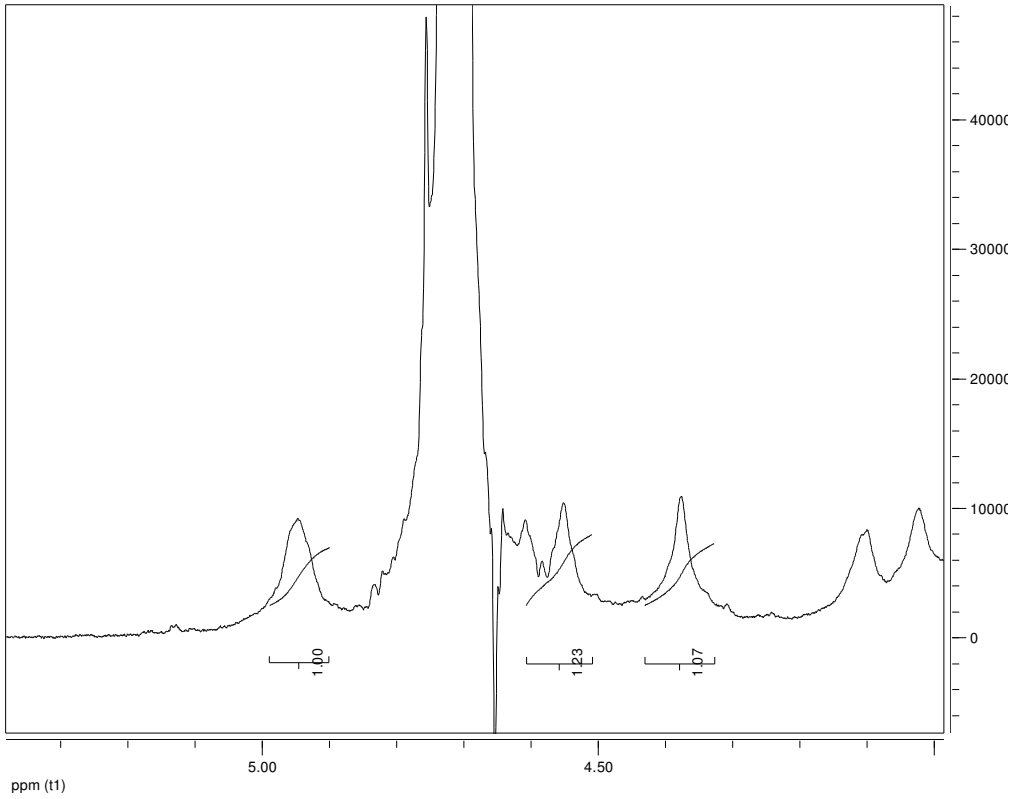

Figure C.14. ^{1}H-NMR spectrum for alginate harvested at 5 % DOT, 32 hours and 30 ℃

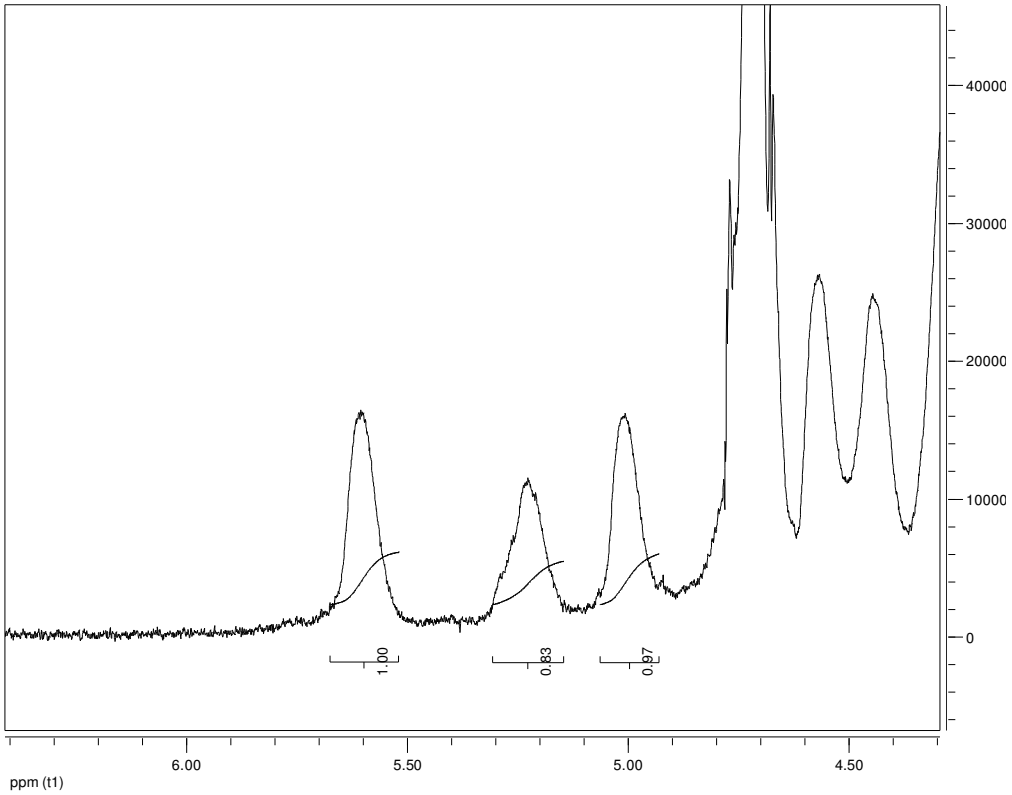

Figure C.15. ^{1}H-NMR spectrum for alginate harvested at 5 % DOT, 48 hours and 70 ℃

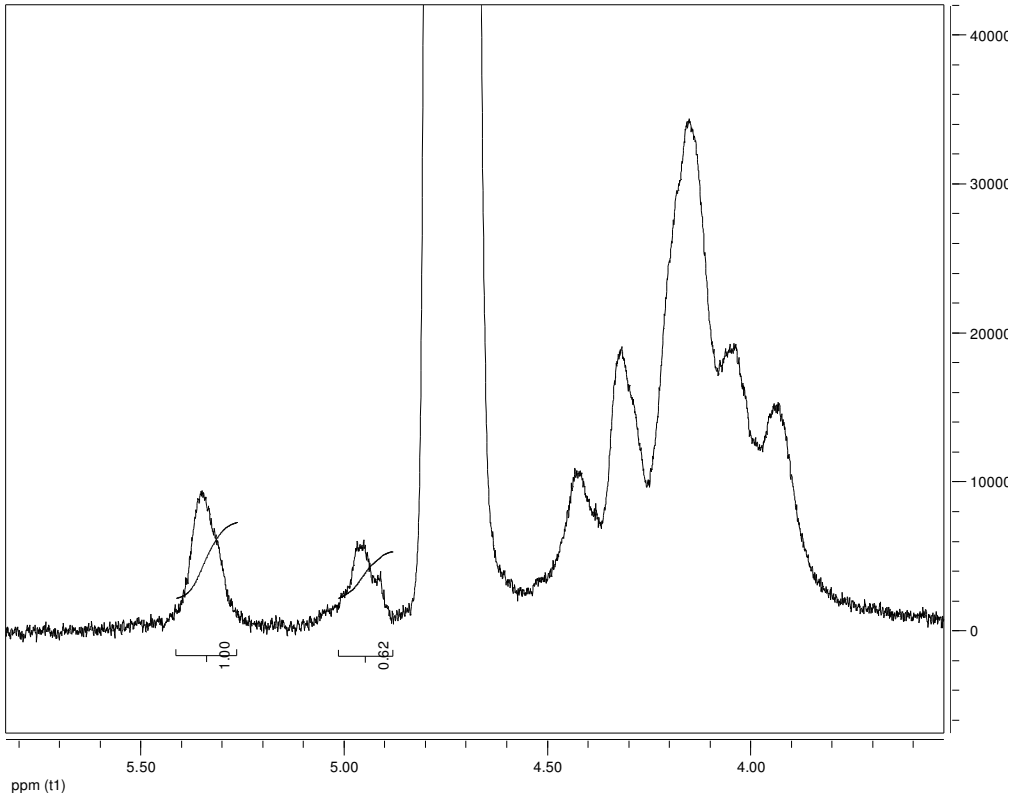

Figure C.16. ^{1}H-NMR spectrum for alginate harvested at 5 % DOT, 56 hours and 70 ℃

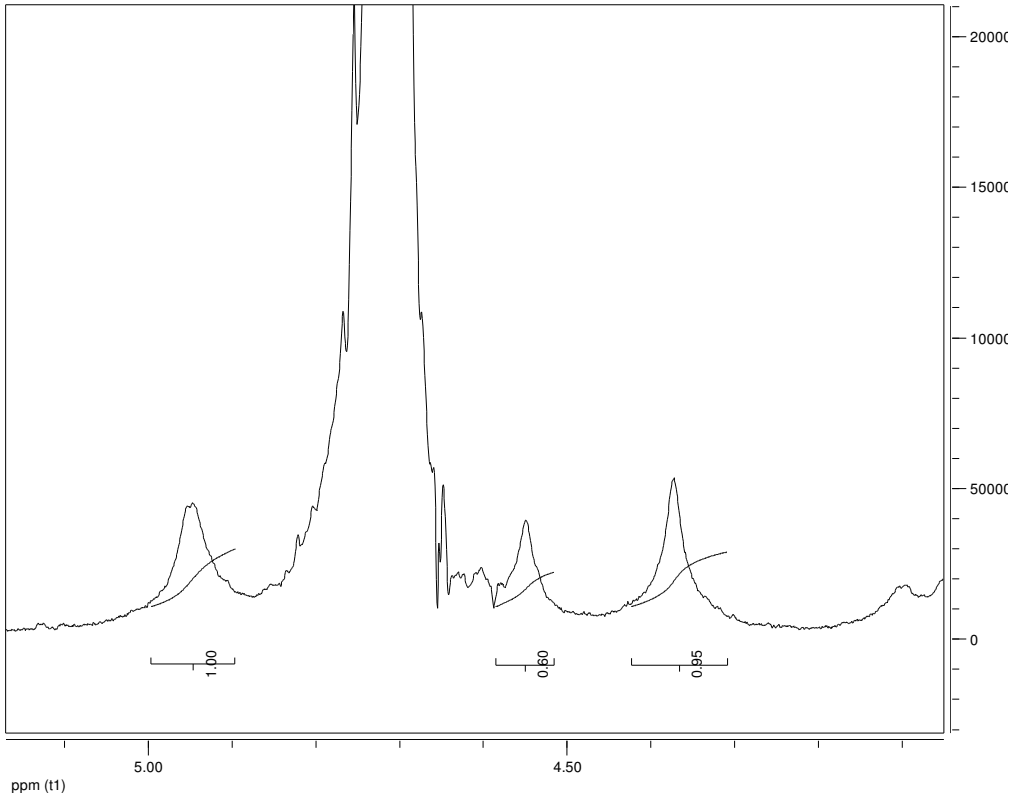

Figure C.17. ^{1}H-NMR spectrum for alginate harvested at 5 % DOT, 56 hours and 30 ℃

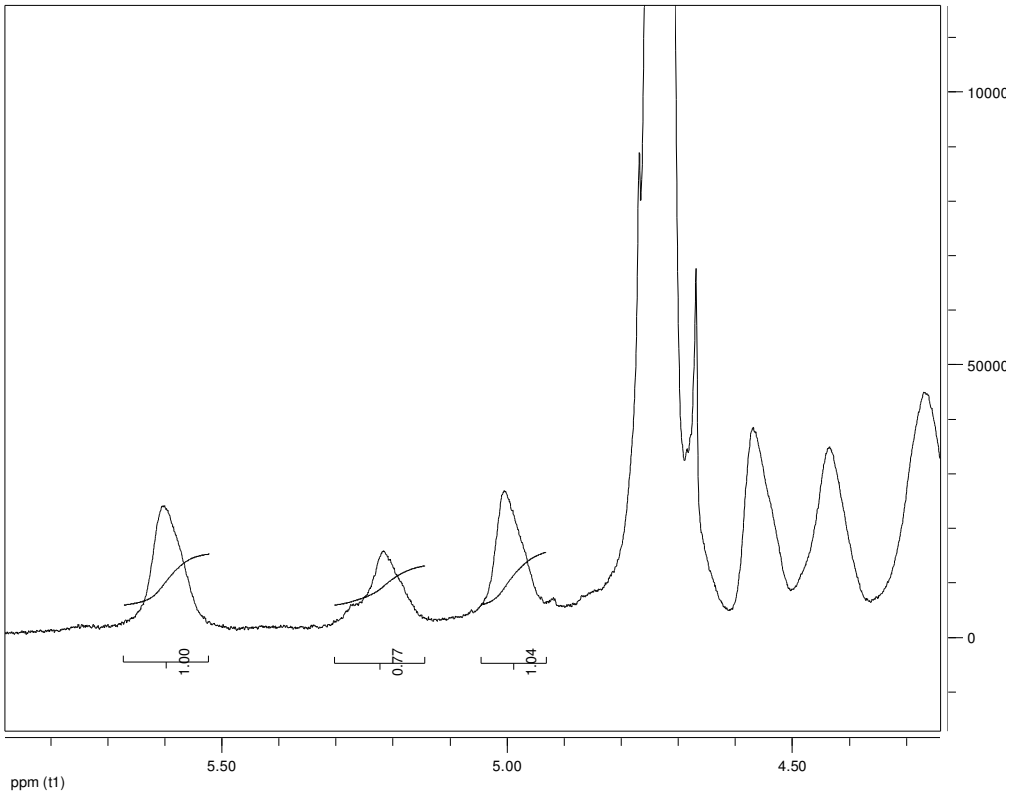

Figure C.18. ^{1}H-NMR spectrum for alginate harvested at 5 % DOT, 72 hours and 70 ℃

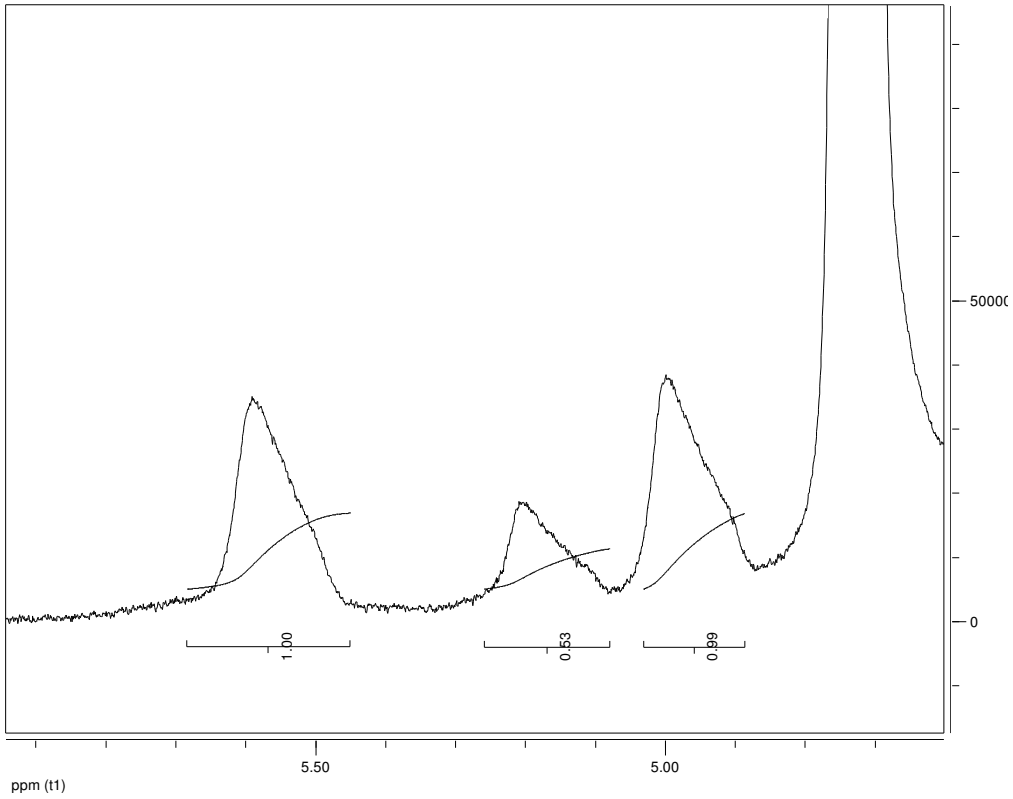

Figure C.19. ^{1}H-NMR spectrum for alginate harvested at 10 % DOT, 24 hours and 70 ℃

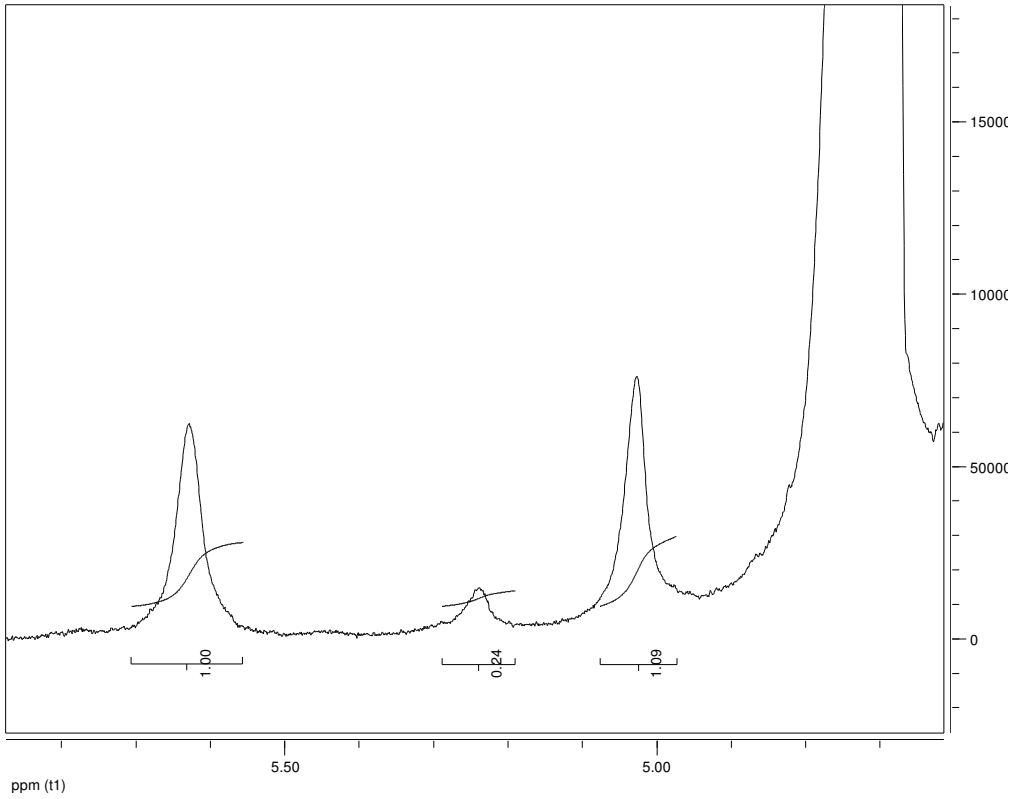

Figure C.20. ^{1}H-NMR spectrum for alginate harvested at 10 % DOT, 32 hours and 70 ℃

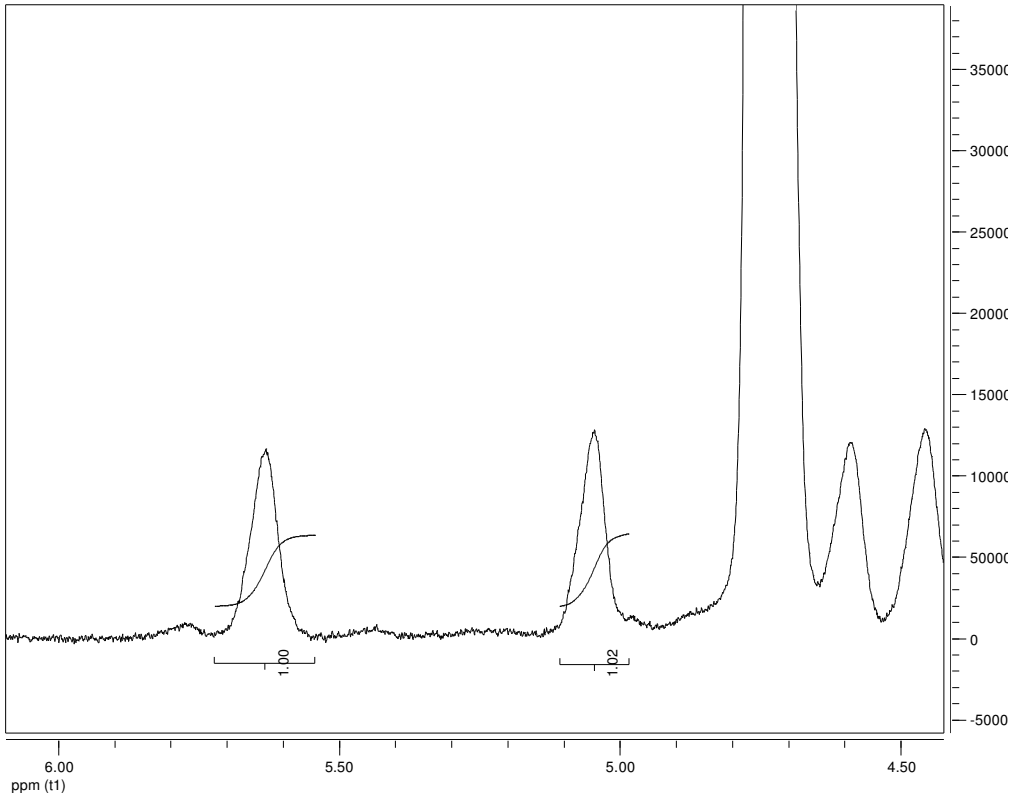

Figure C.21. ^{1}H-NMR spectrum for alginate harvested at 10 % DOT, 48 hours and 70 ℃

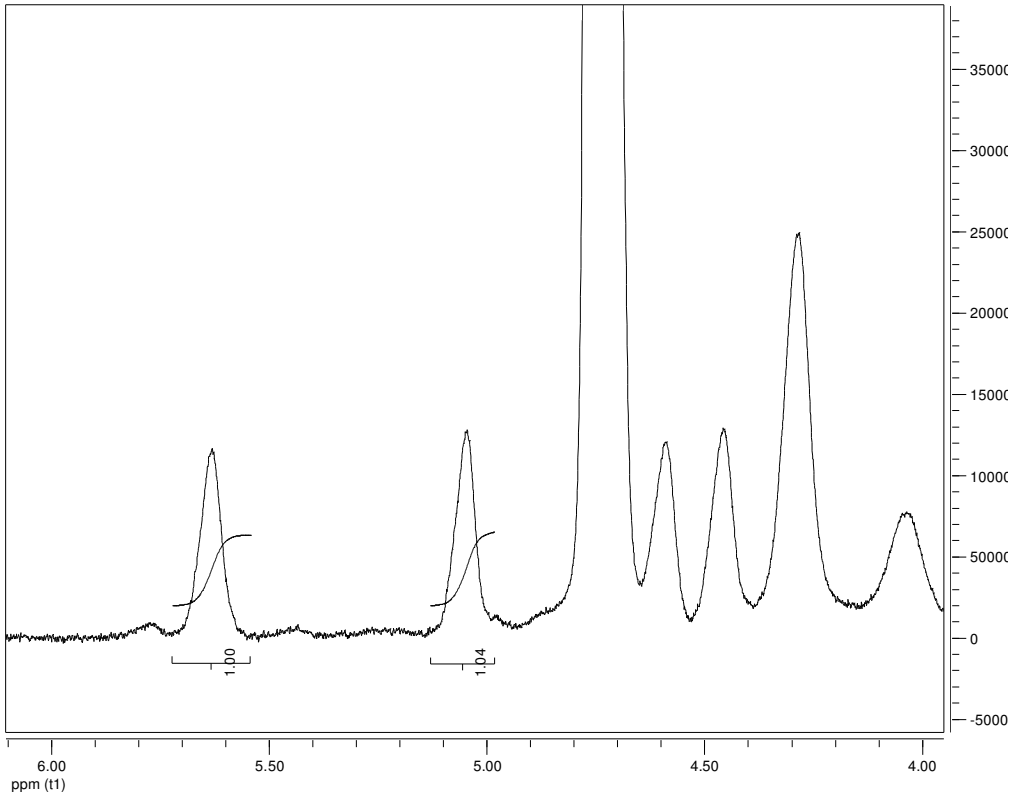

Figure C.22. ^{1}H-NMR spectrum for alginate harvested at 10 % DOT, 56 hours and 70 ℃

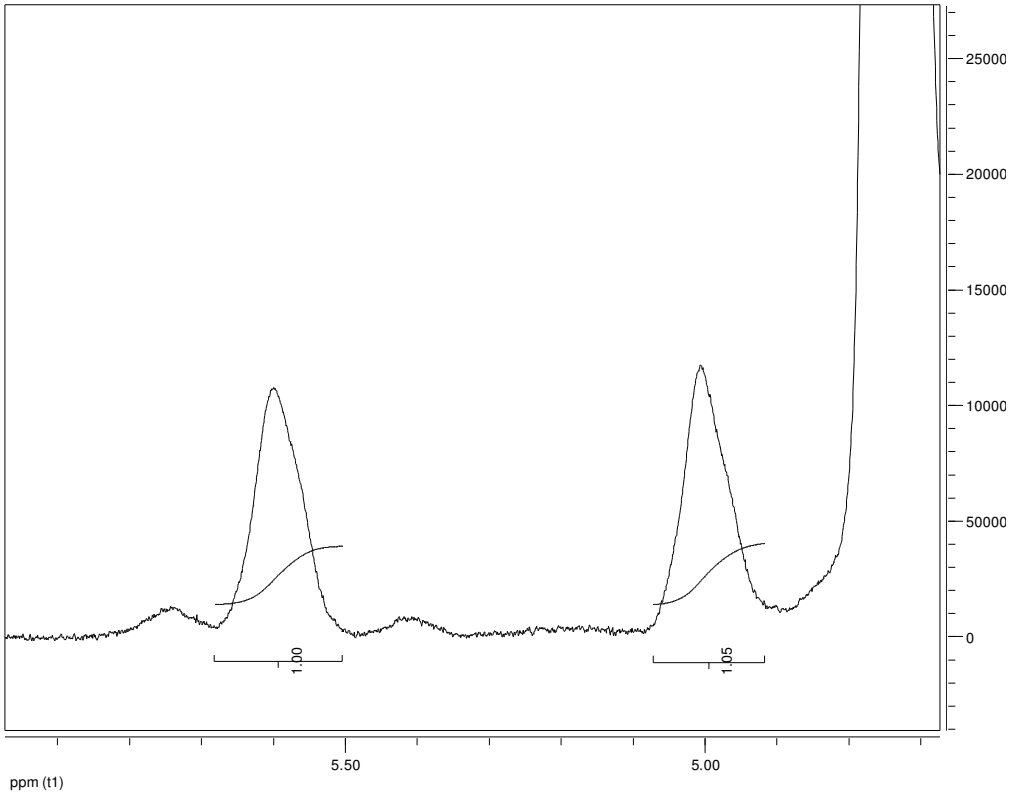

Figure C.23 ^{1}H-NMR spectrum for alginate harvested at 10 % DOT, 72 hours and 70 ℃

^{1}H-NMR SPECTRUMS FOR ALGINATES AT DIFFERENT MIXING SPEED

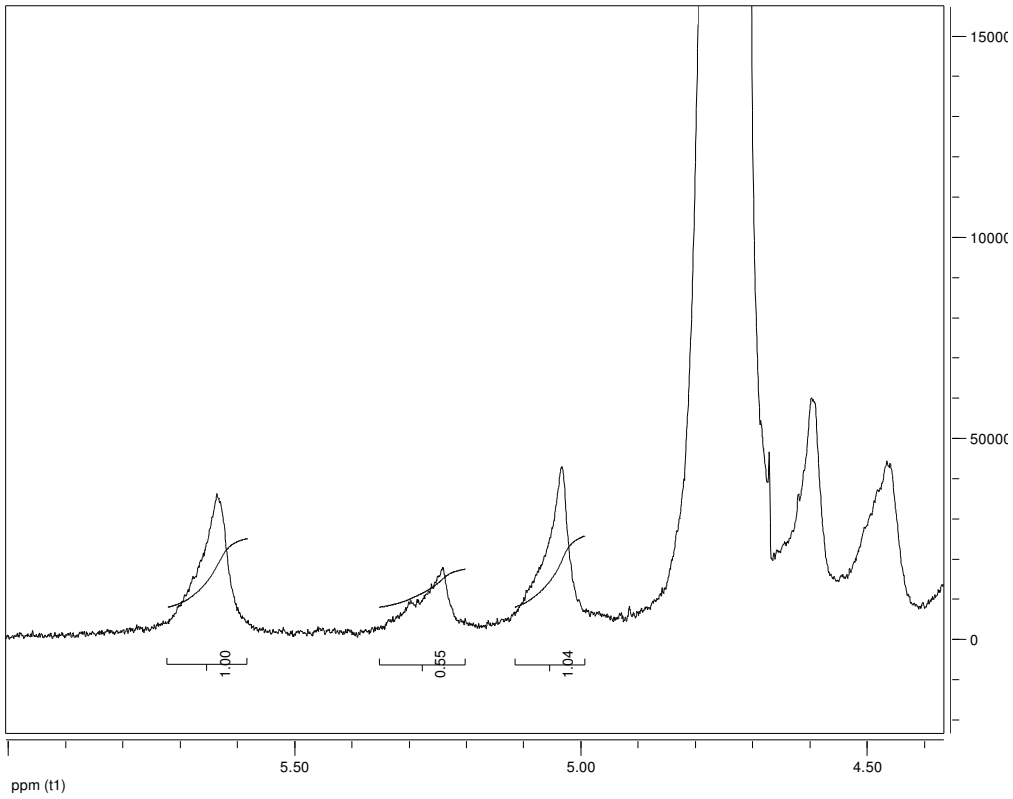

Figure C.24 ^{1}H-NMR spectrum for alginate harvested at 200 rpm, 24 hours and 70 ℃

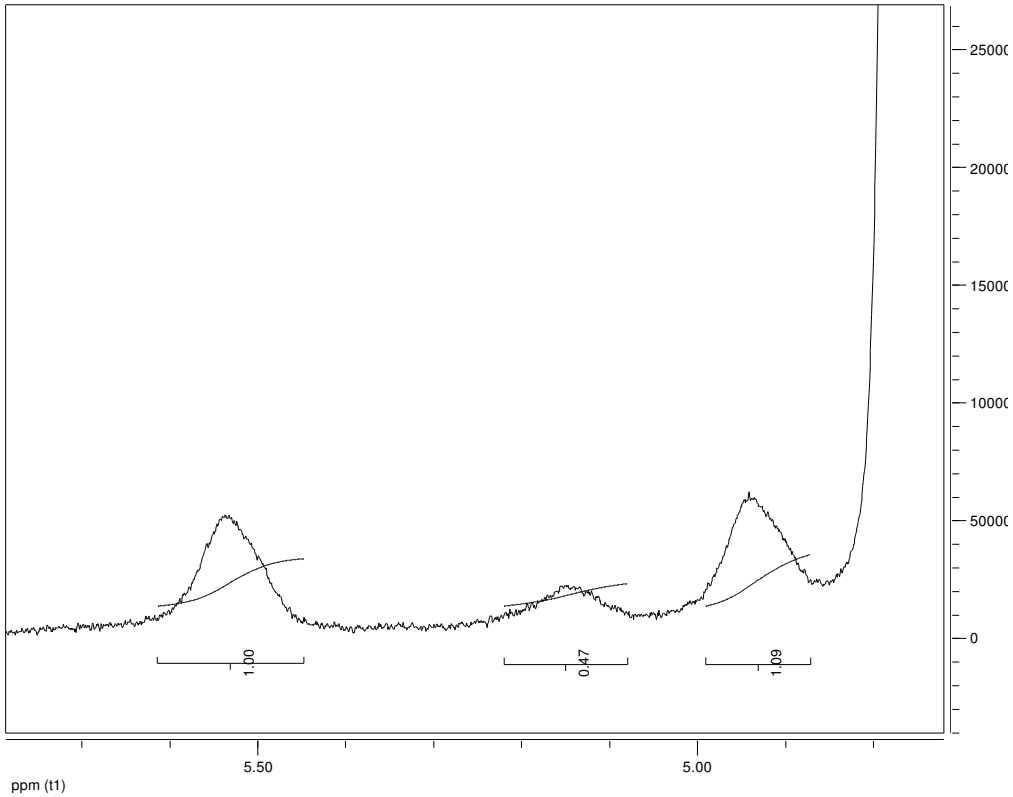

Figure C.25. ^{1}H-NMR spectrum for alginate harvested at 200 rpm, 32 hours and 70 ℃

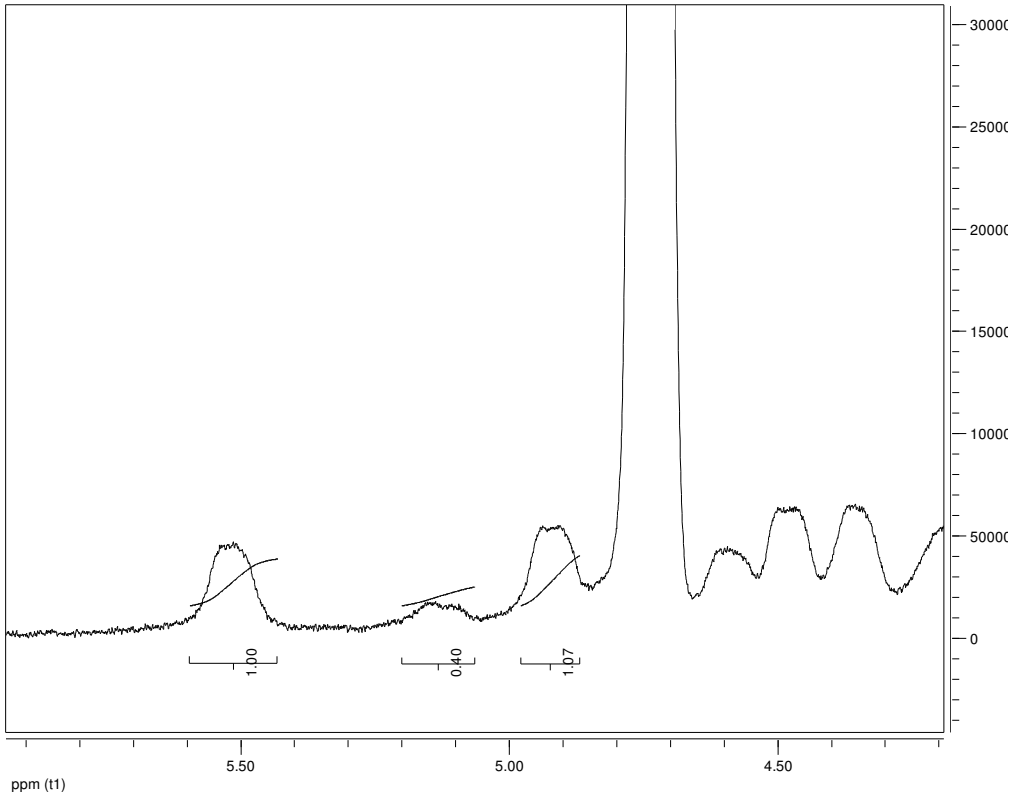

Figure C.26. ^{1}H-NMR spectrum for alginate harvested at 200 rpm, 48 hours and 70 ℃

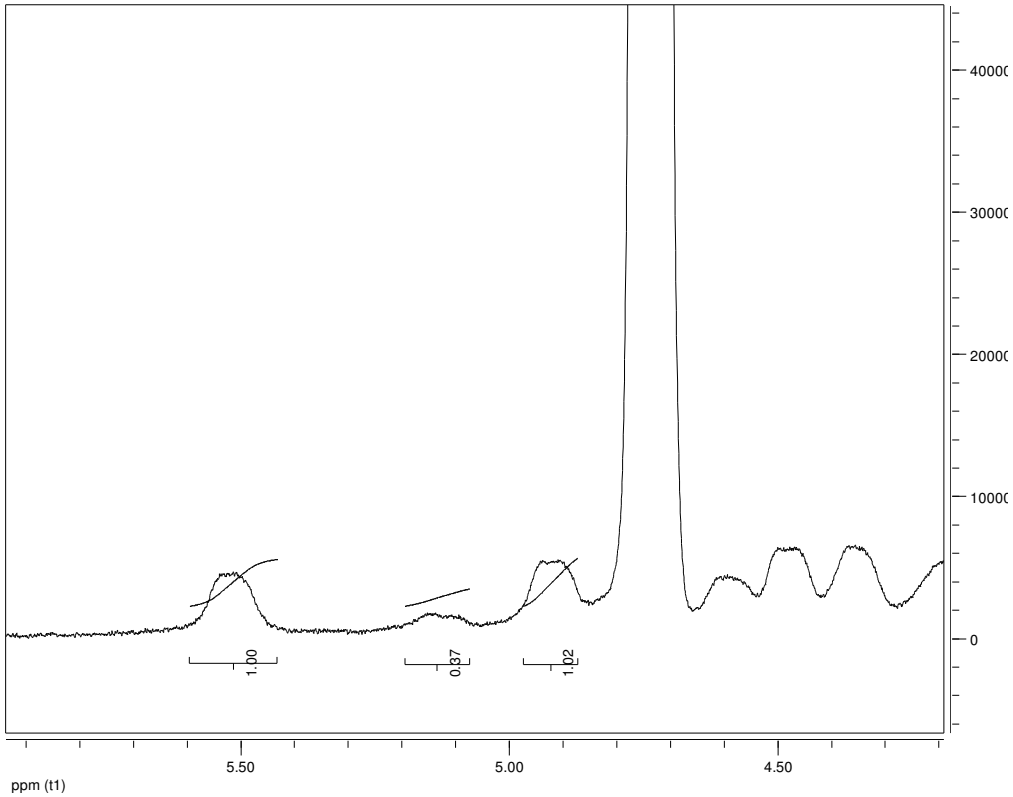

Figure C.27. ^{1}H-NMR spectrum for alginate harvested at 200 rpm, 56 hours and 70 ℃

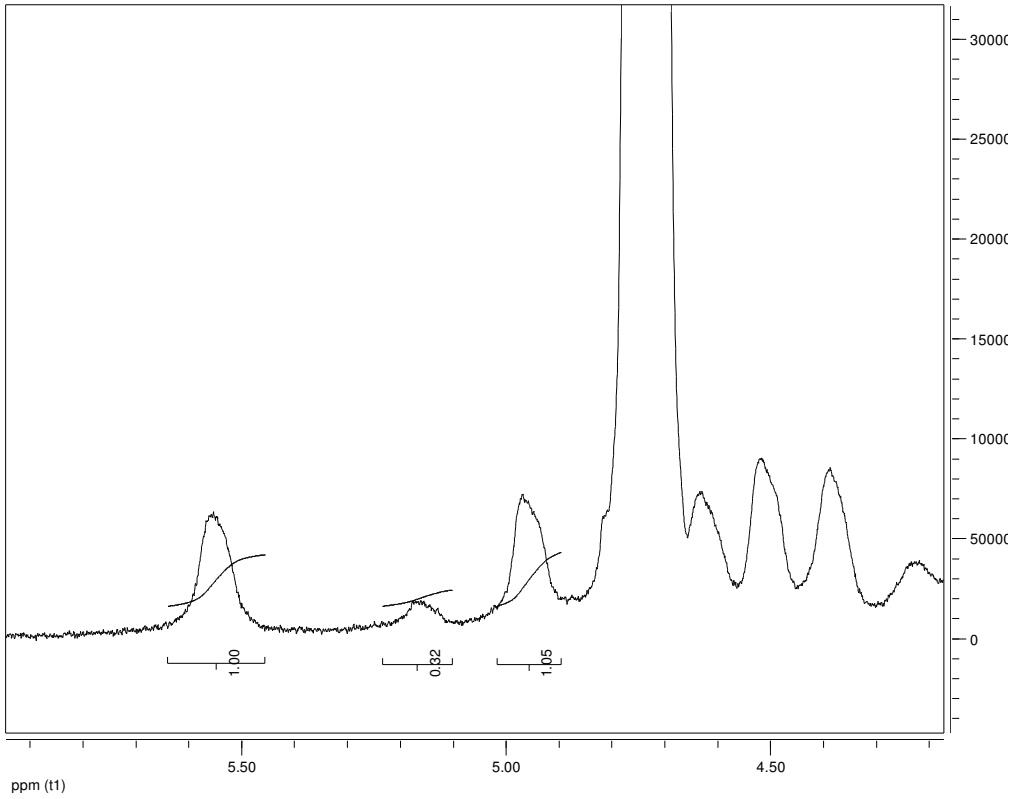

Figure C.28. ^{1}H-NMR spectrum for alginate harvested at 200 rpm, 72 hours and 70 ℃

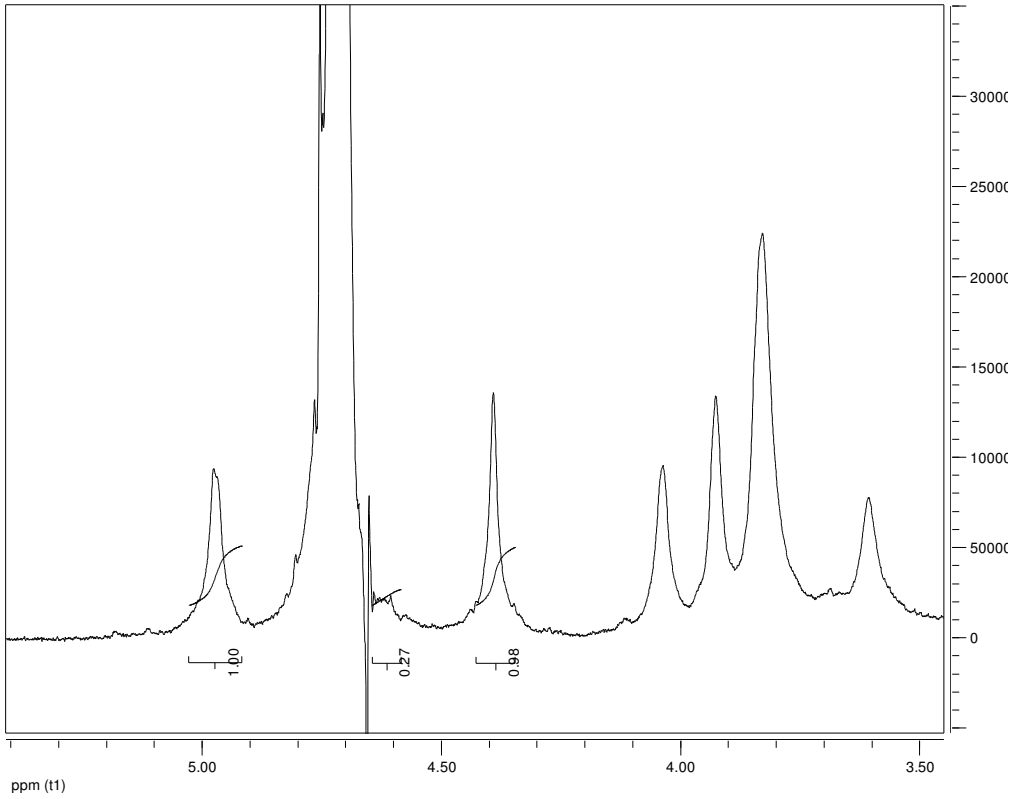

Figure C.29. ^{1}H-NMR spectrum for alginate harvested at 700 rpm, 24 hours and 30 ℃

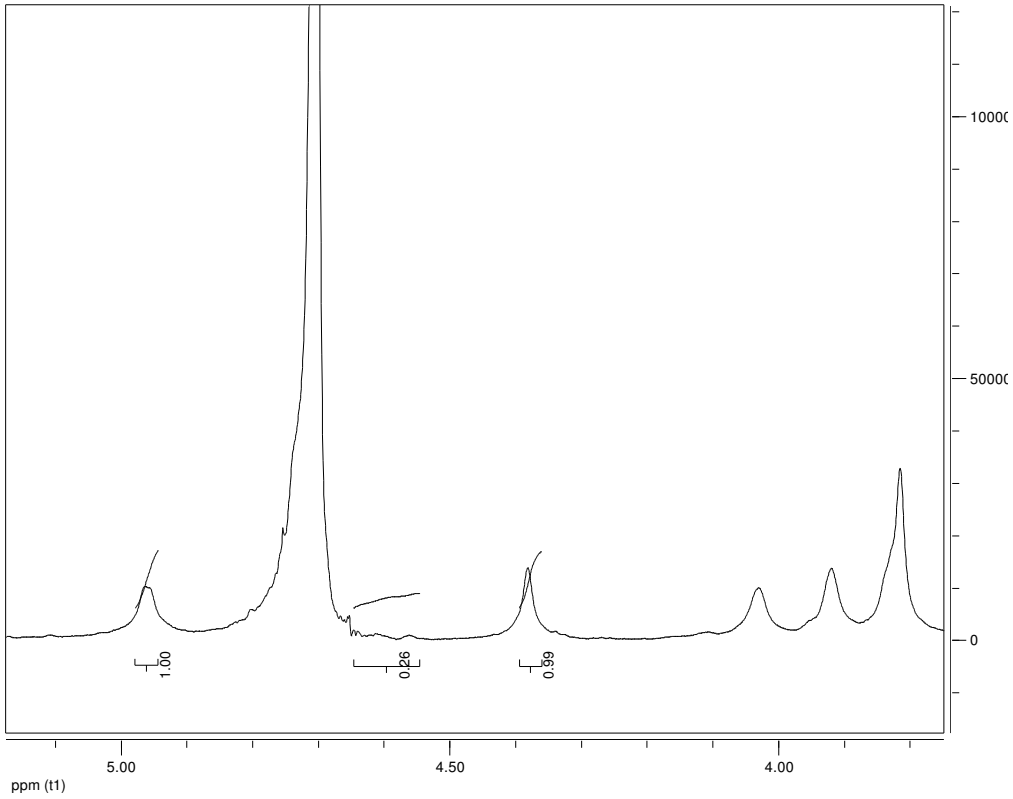

Figure C.30. ^{1}H-NMR spectrum for alginate harvested at 700 rpm, 32 hours and 30 ℃

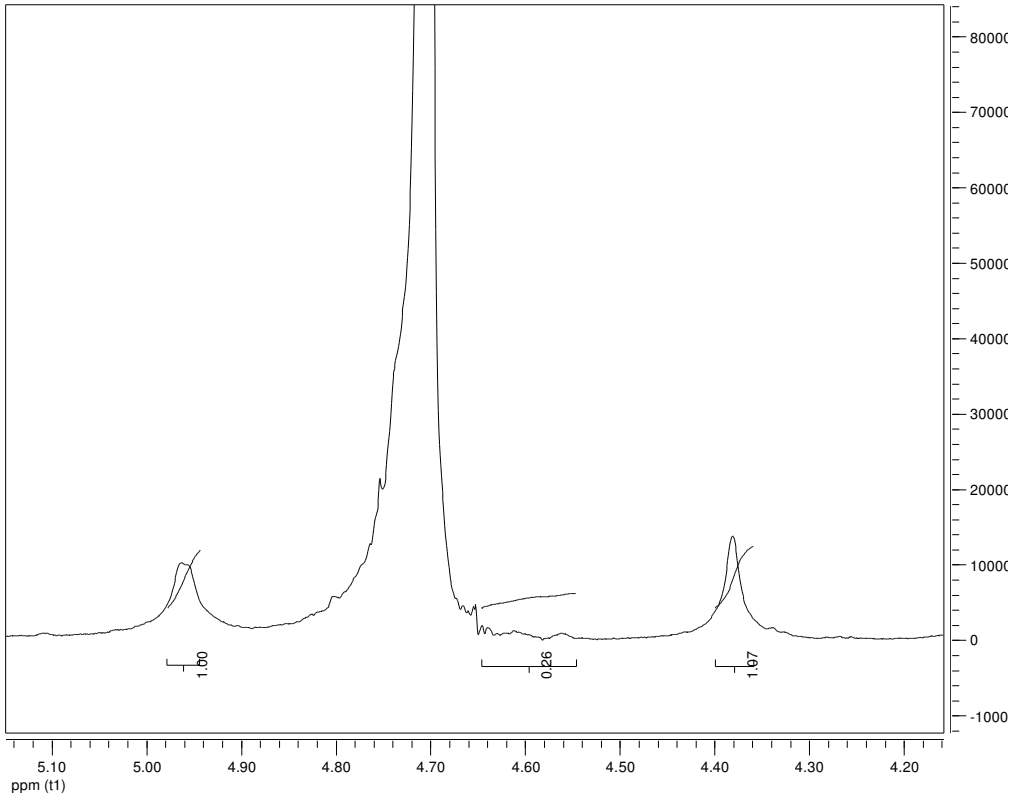

Figure C.31. ^{1}H-NMR spectrum for alginate harvested at 700 rpm, 48 hours and 30 ℃

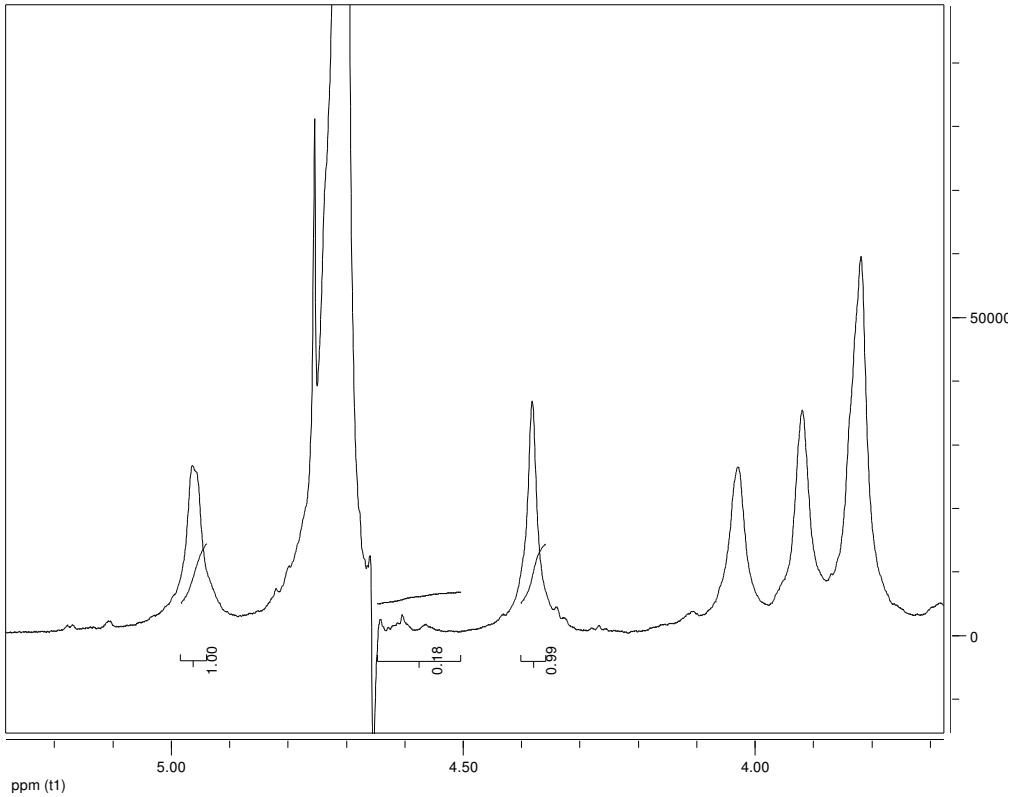

Figure C.32. ^{1}H-NMR spectrum for alginate harvested at 700 rpm, 56 hours and 30 ℃

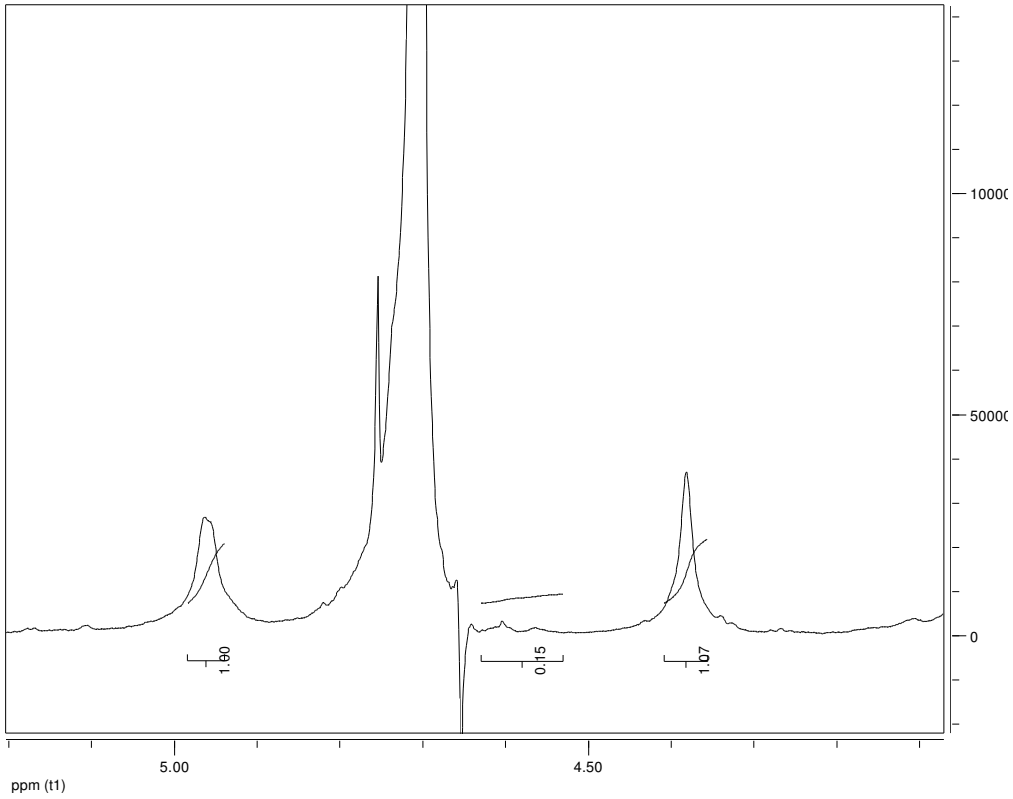

Figure C.33 ^{1}H-NMR spectrum for alginate harvested at 700 rpm, 72 hours and 30 ℃

^{1}H-NMR SPECTRUMS FOR ALGINATES AT DIFFERENT SUCROSE CONCENTRATION

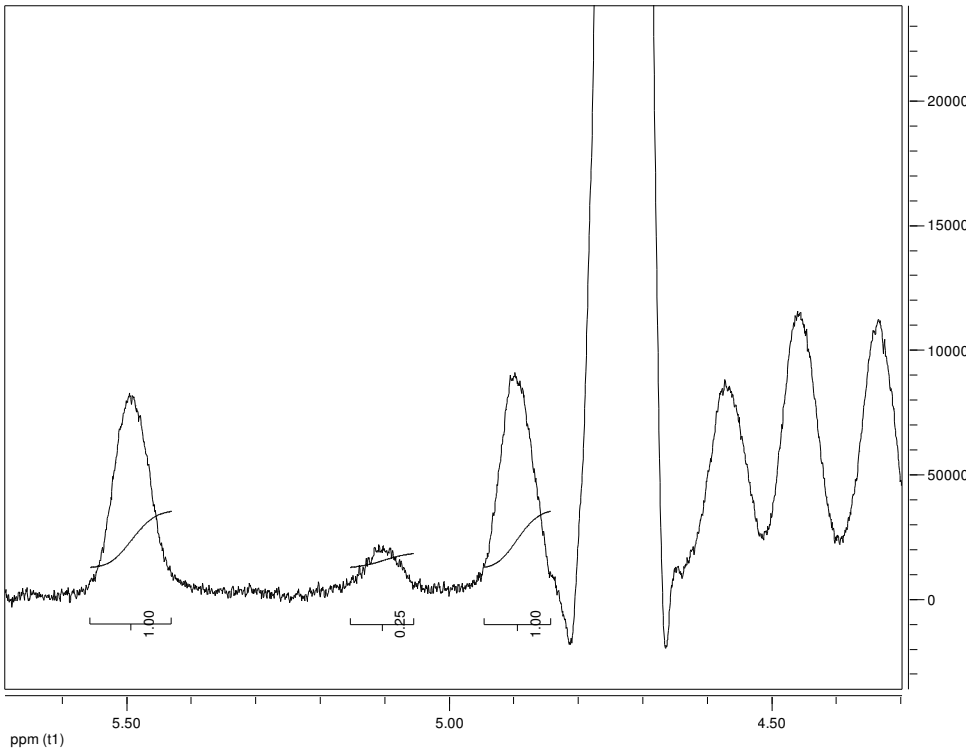

Figure C.34. ^{1}H-NMR spectrum for alginate harvested at 10 g/L of sucrose, 24 hours and 70 ℃

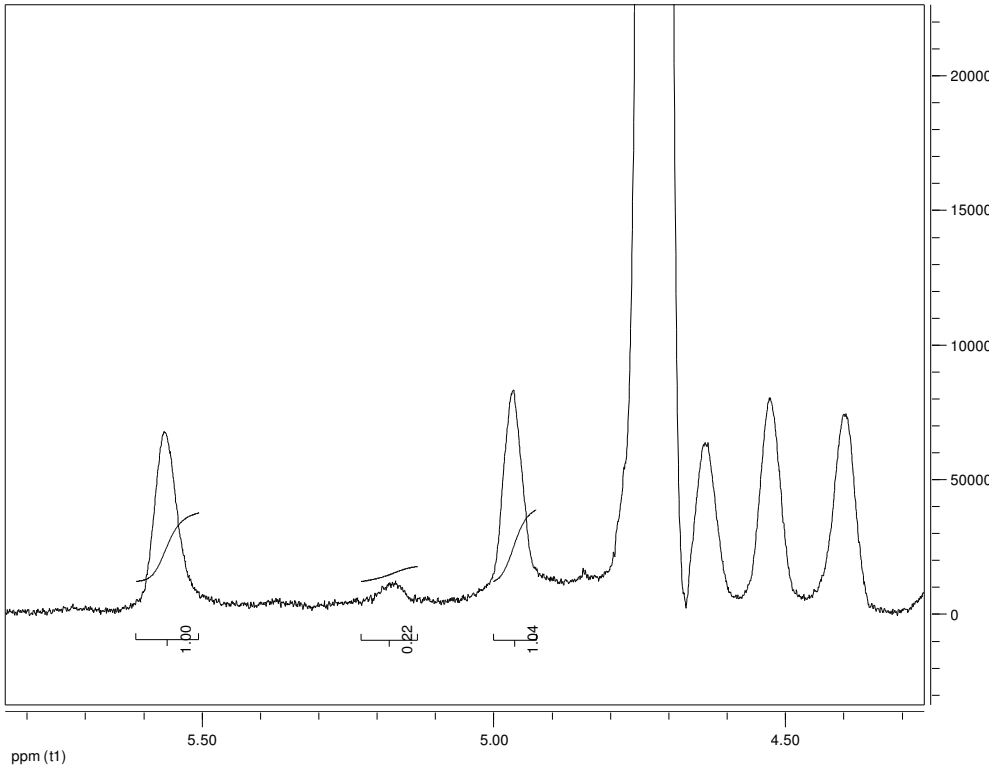

Figure C.35 ^{1}H-NMR spectrum for alginate harvested at 10 g/L of sucrose, 32 hours and 70 ℃

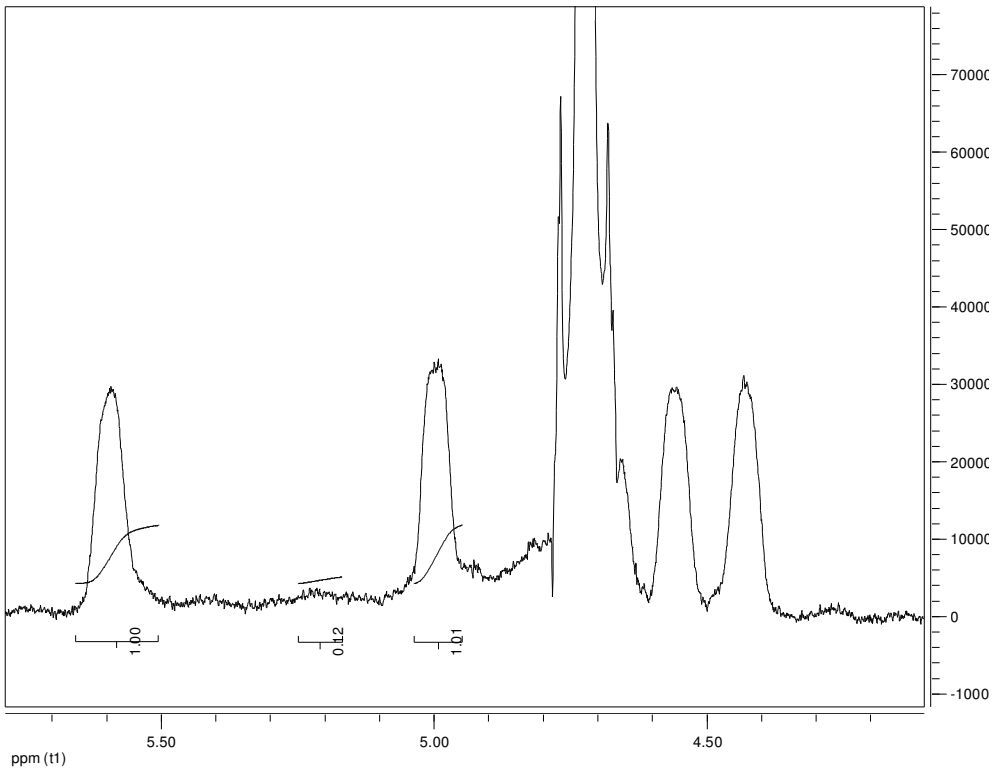

Figure C.36. ^{1}H-NMR spectrum for alginate harvested at 10 g/L of sucrose, 48 hours and 70 ℃

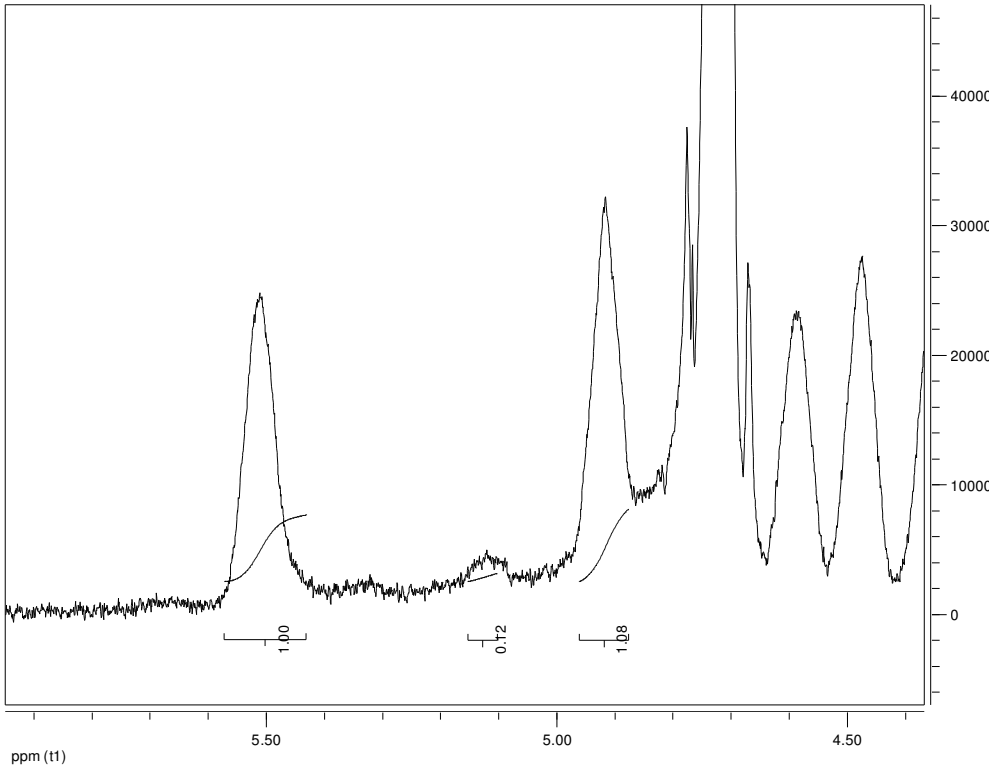

Figure C.37 ^{1}H-NMR spectrum for alginate harvested at 10 g/L of sucrose, 56 hours and 70 ℃

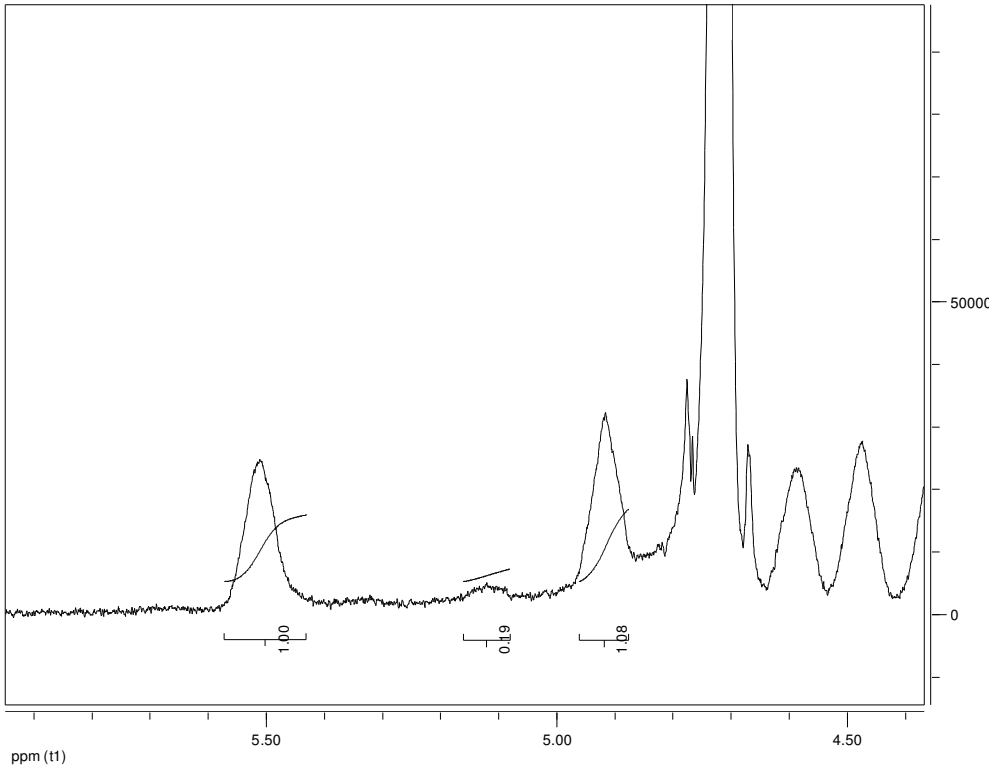

Figure C.38. ^{1}H-NMR spectrum for alginate harvested at 10 g/L of sucrose, 72 hours and 70 ℃

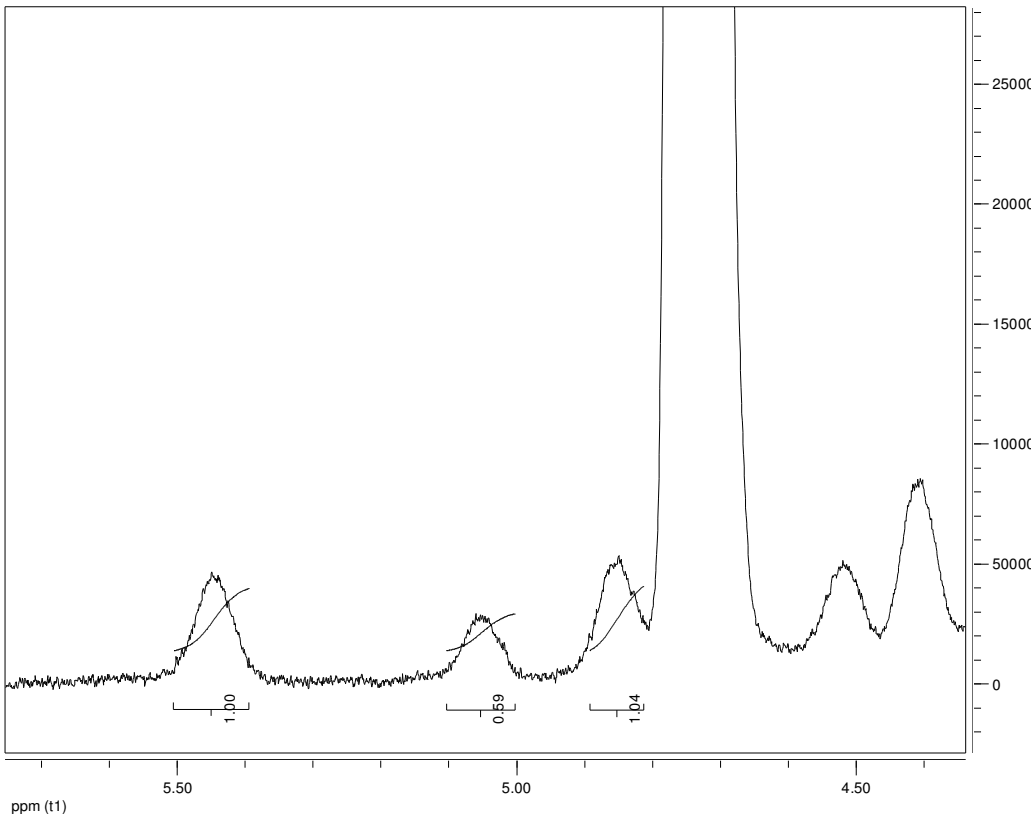

Figure C.39. ^{1}H-NMR spectrum for alginate harvested at 40 g/L of sucrose, 24 hours and 70 °C

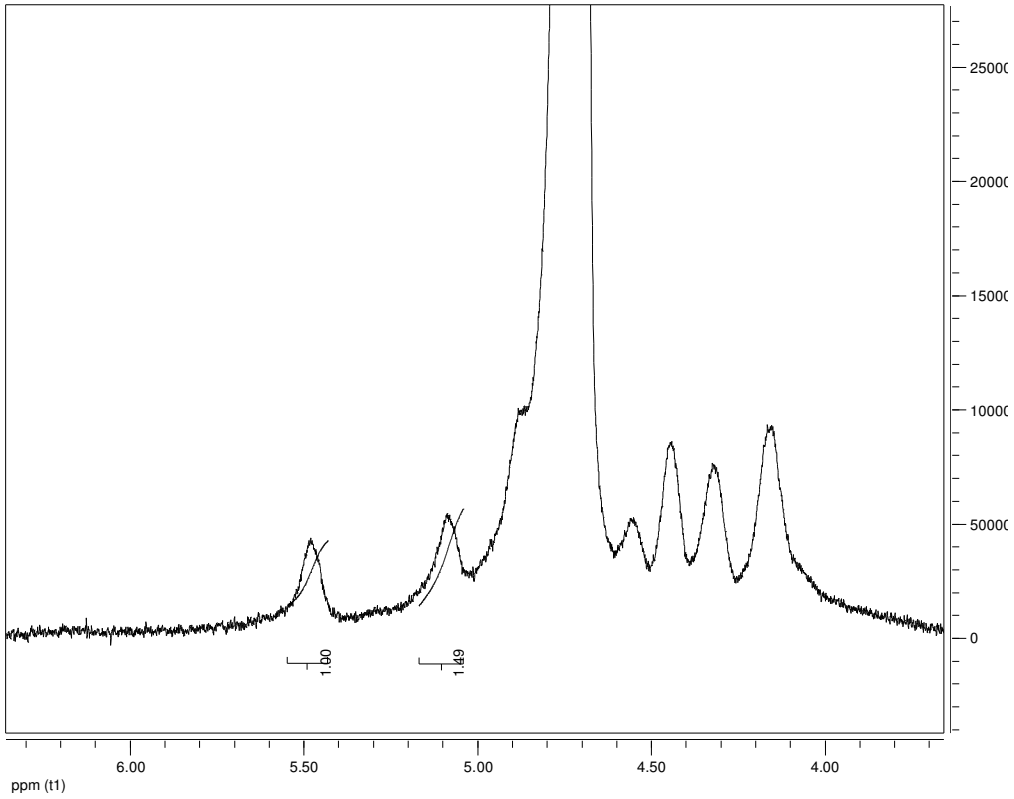

Figure C.40. ^{1}H-NMR spectrum for alginate harvested at 40 g/L of sucrose, 32 hours and 70 ℃

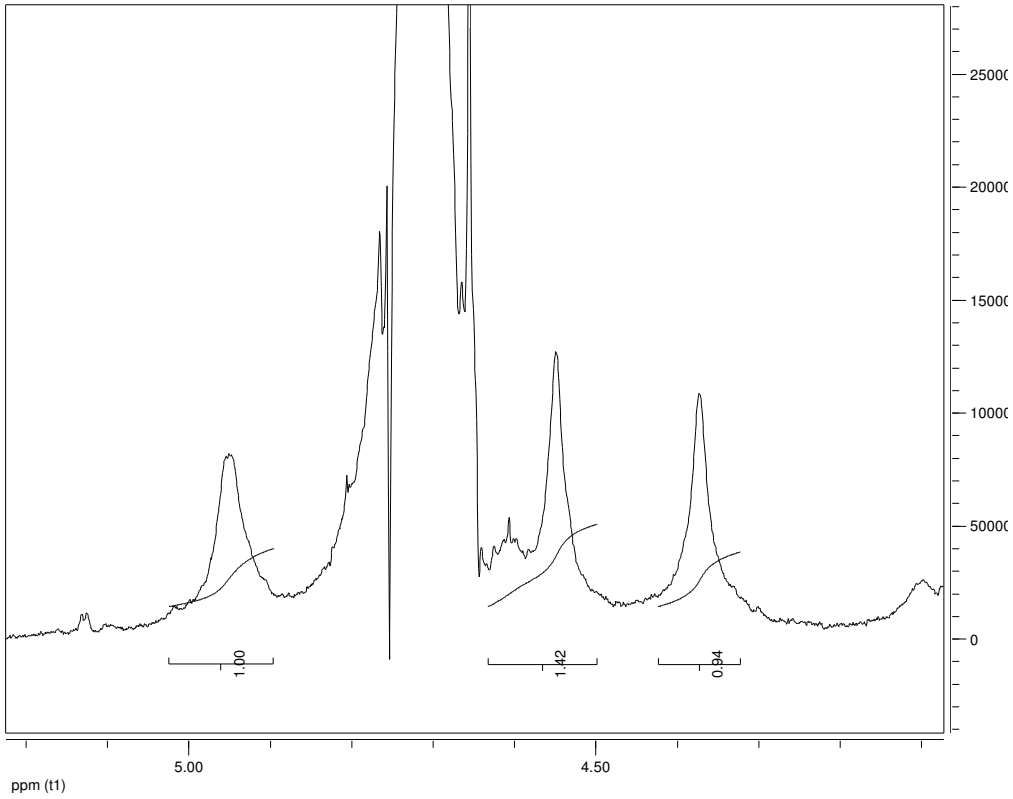

Figure C.41. ^{1}H-NMR spectrum for alginate harvested at 40 g/L of sucrose, 32 hours and 30 ℃

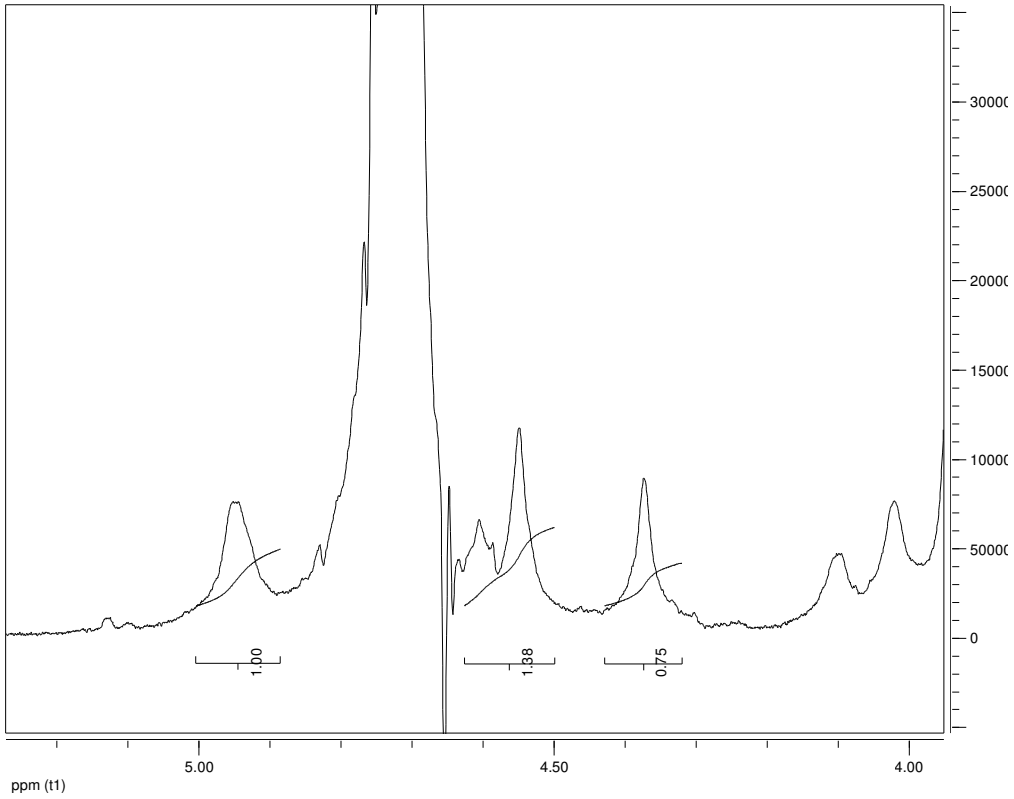

Figure C.42. ^{1}H-NMR spectrum for alginate harvested at 40 g/L of sucrose, 48 hours and 30 ℃

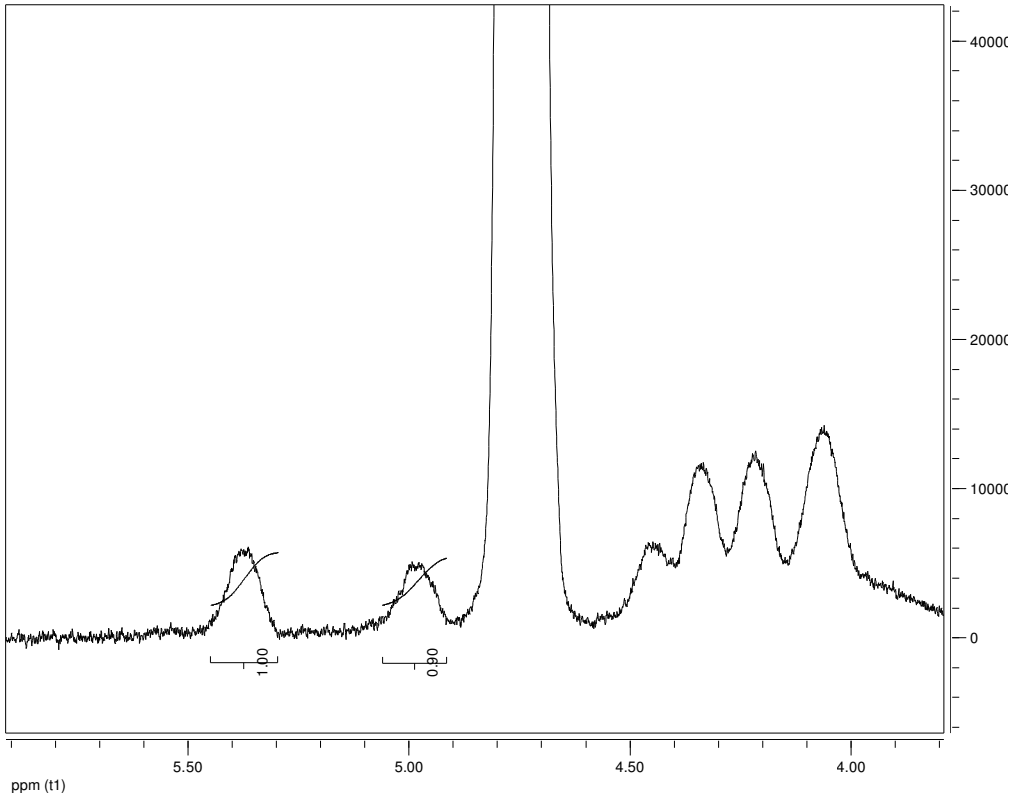

Figure C.43. ^{1}H-NMR spectrum for alginate harvested at 40 g/L of sucrose, 72 hours and 70 ℃

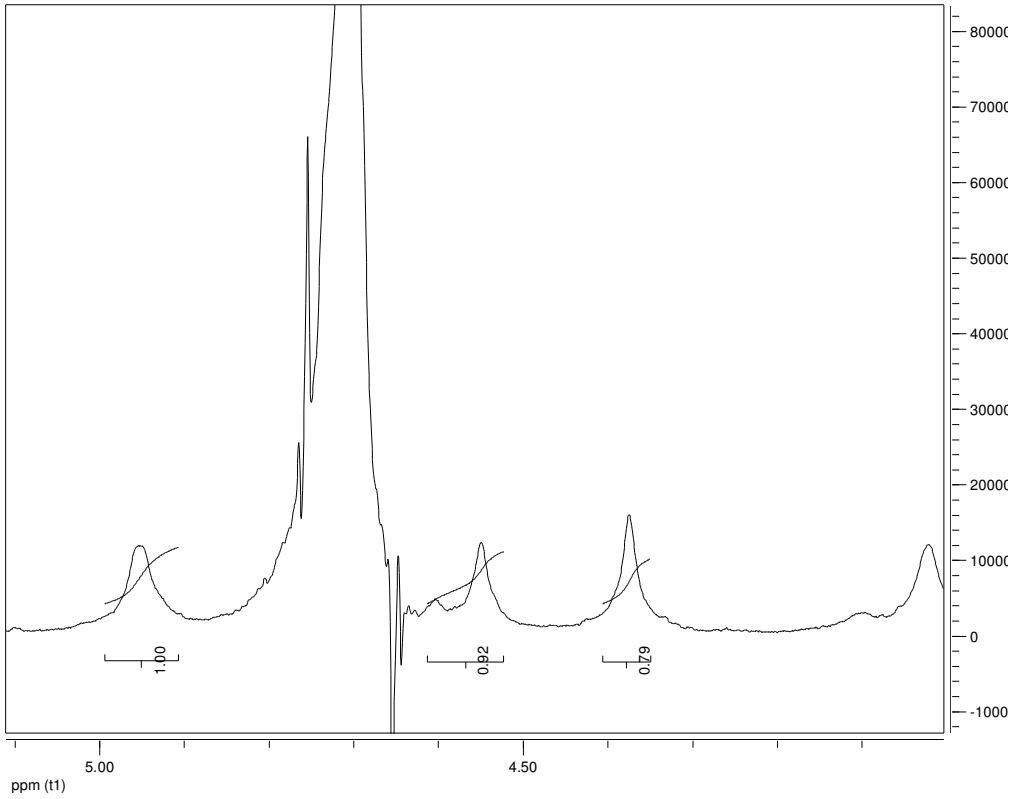

Figure C.44. ^{1}H-NMR spectrum for alginate harvested at 40 g/L of sucrose, 72 hours and 30 ℃

^{1}H-NMR SPECTRUMS FOR ALGINATES AT DIFFERENT CALCIUM CONCENTRATION

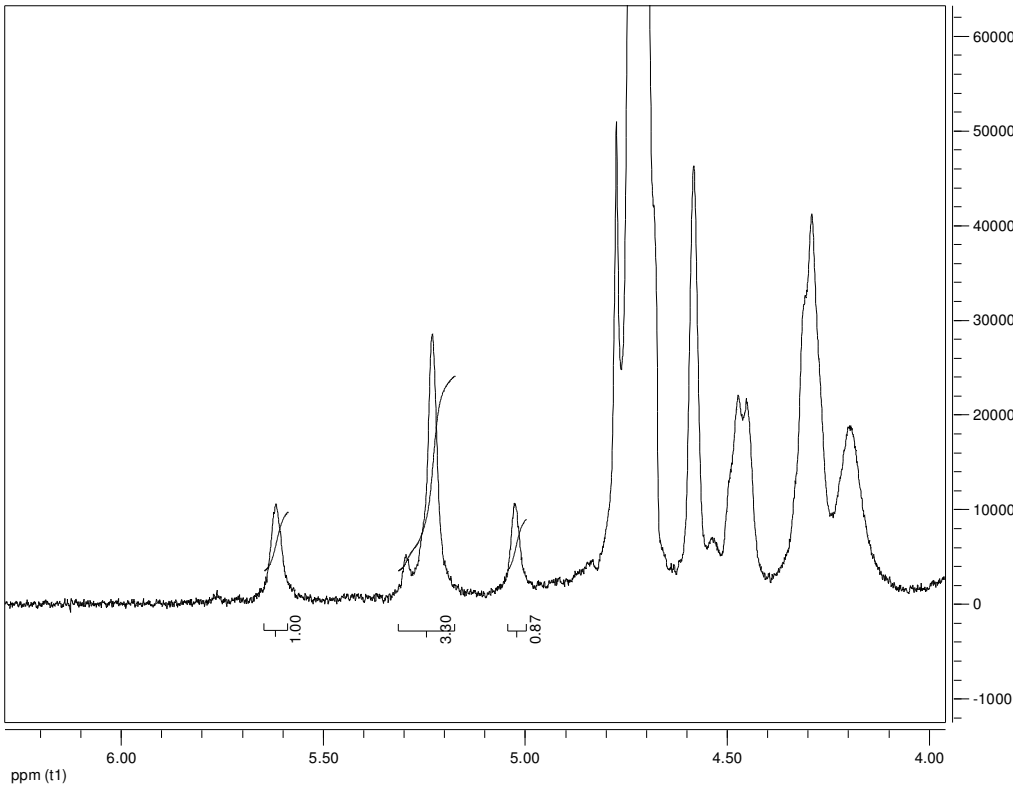

Figure C.45. ^{1}H-NMR spectrum for alginate harvested at 25 mg/L of Ca^{2+}, 24 hours and 70 ℃

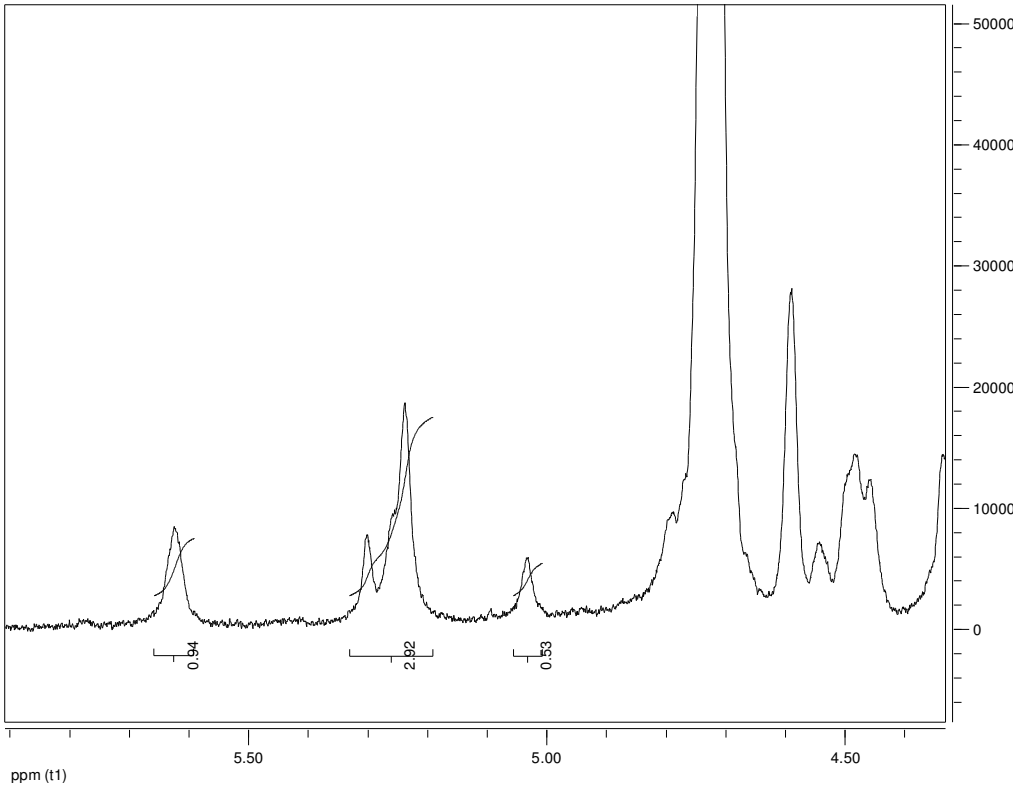

Figure C.46. ^{1}H-NMR spectrum for alginate harvested at 25 mg/L of Ca^{2+}, 32 hours and 70 ℃

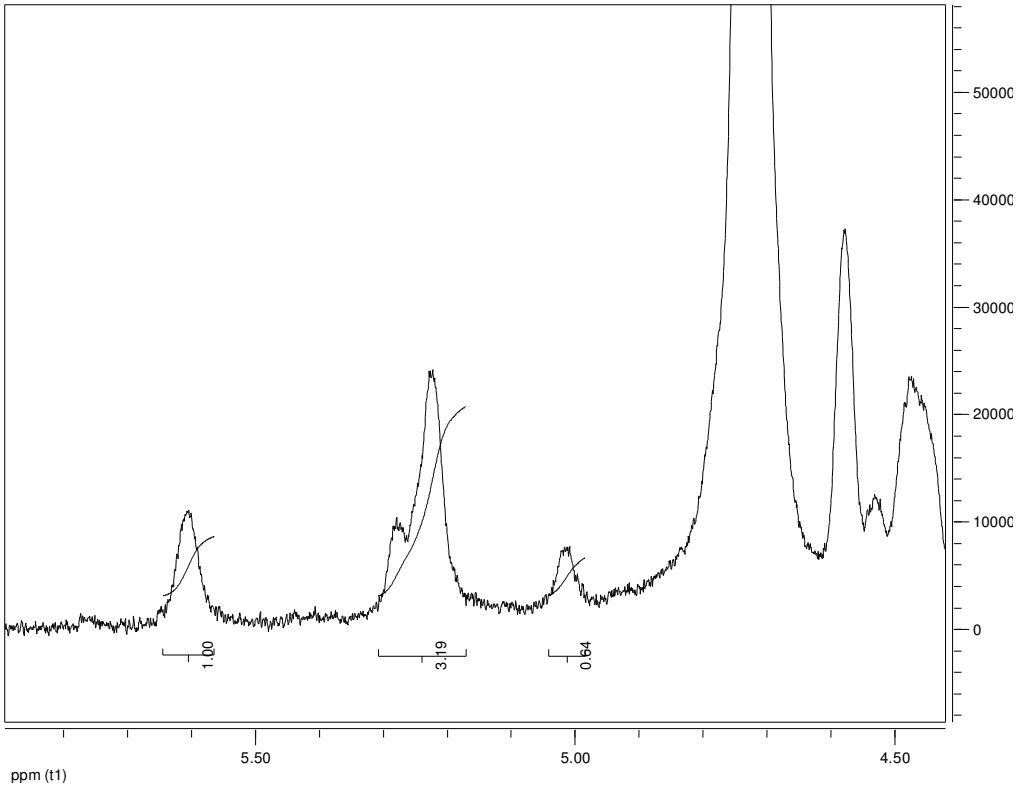

Figure C.47. ^{1}H-NMR spectrum for alginate harvested at 25 mg/L of Ca^{2+}, 48 hours and 70 ℃

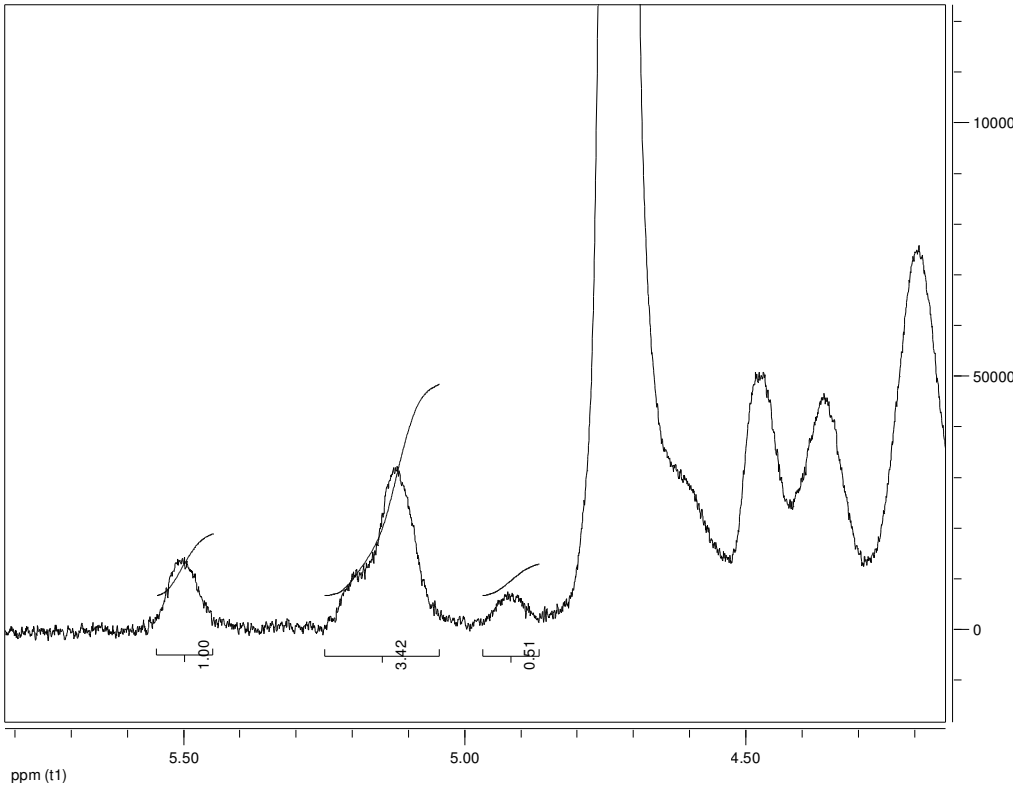

Figure C.48. ^{1}H-NMR spectrum for alginate harvested at 25 mg/L of Ca^{2+}, 56 hours and 70 ℃

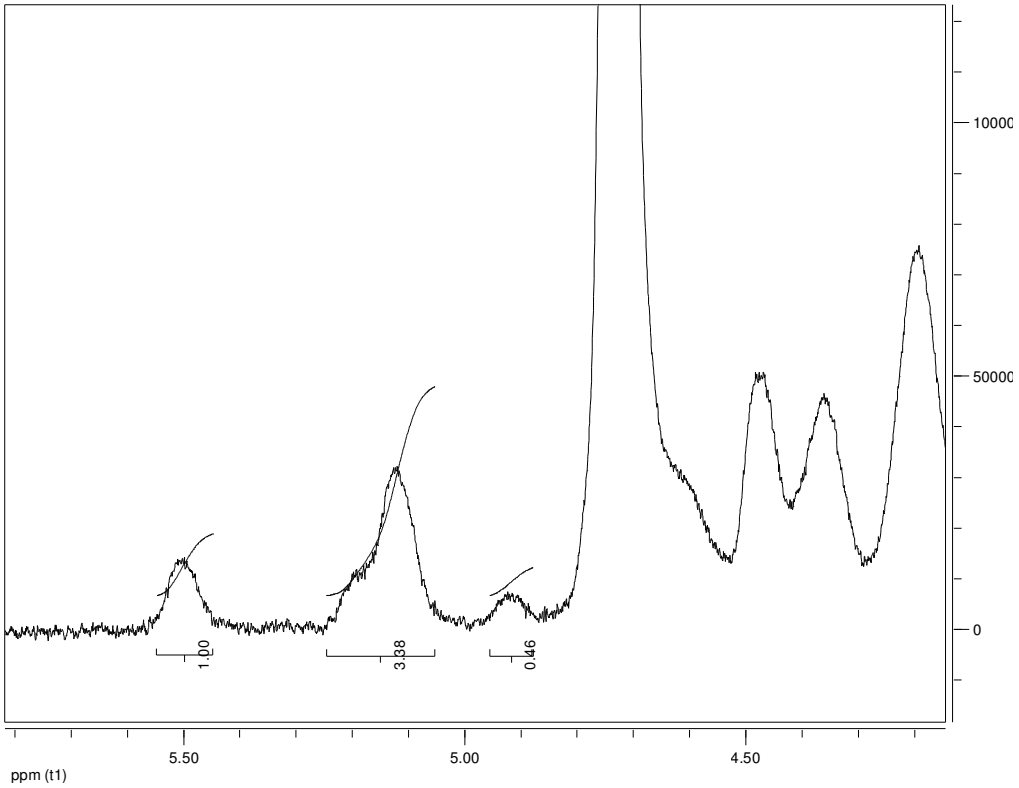

Figure C.49. ^{1}H-NMR spectrum for alginate harvested at 25 mg/L of Ca^{2+}, 72 hours and 70 ℃

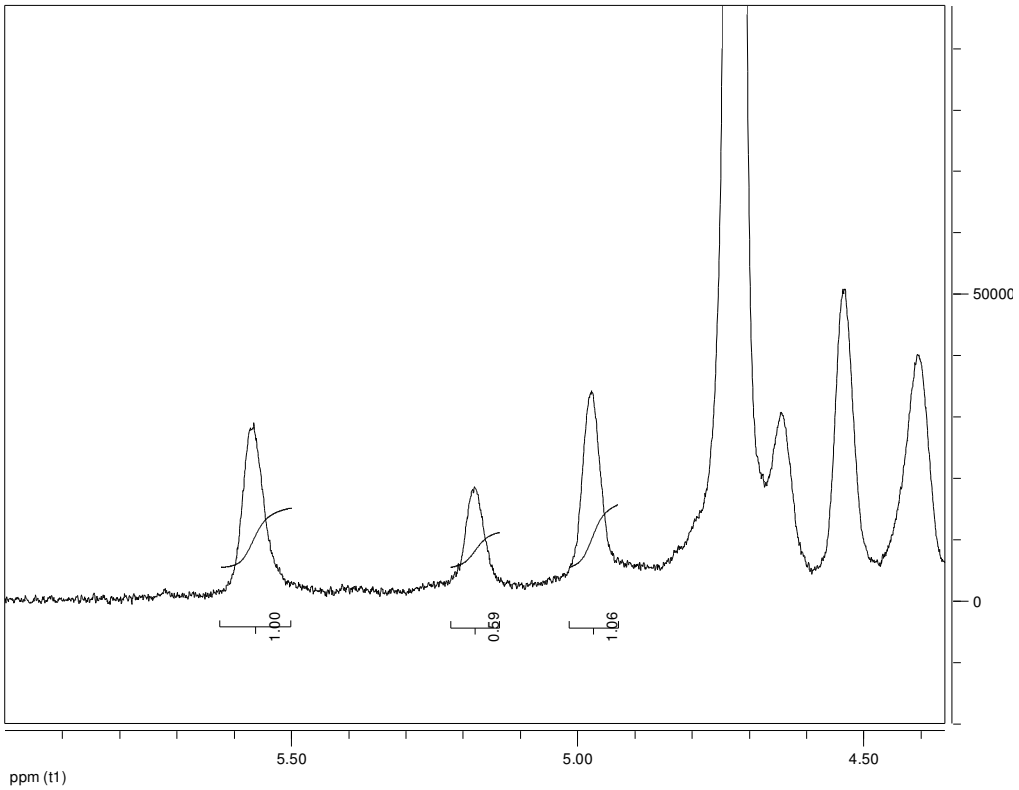

Figure C.50. ^{1}H-NMR spectrum for alginate harvested at 100 mg/L of Ca^{2+}, 24 hours and 70 ℃

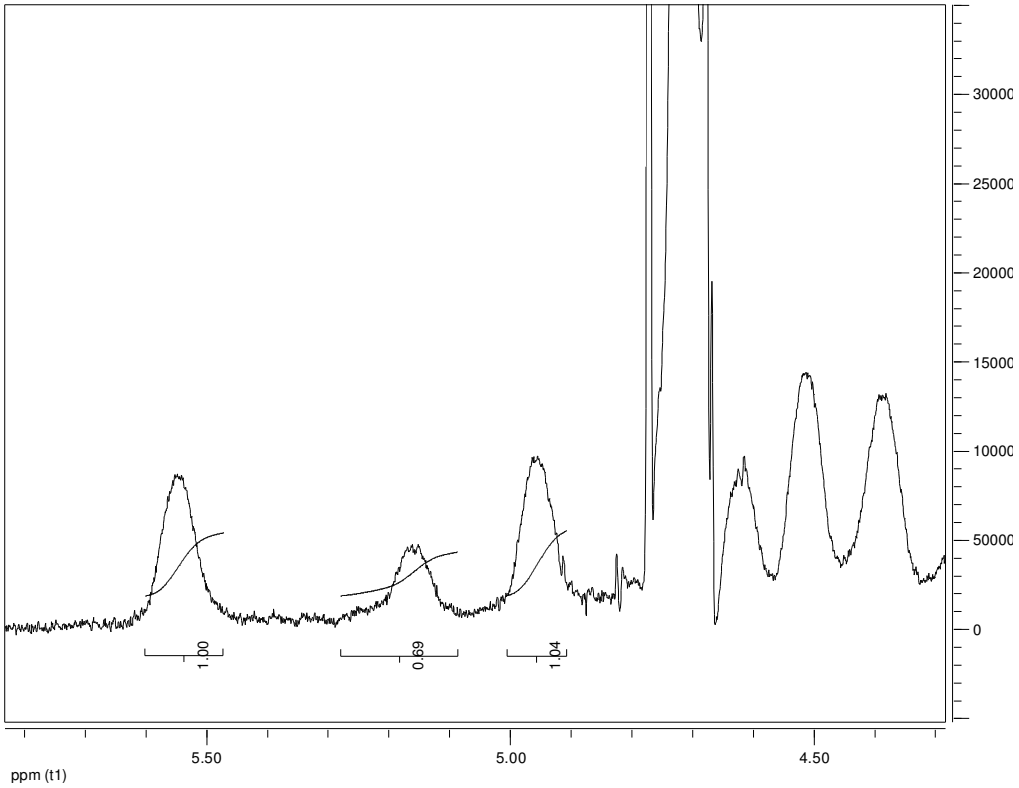

Figure C.51. ^{1}H-NMR spectrum for alginate harvested at 100 mg/L of Ca^{2+}, 32 hours and 70 ℃

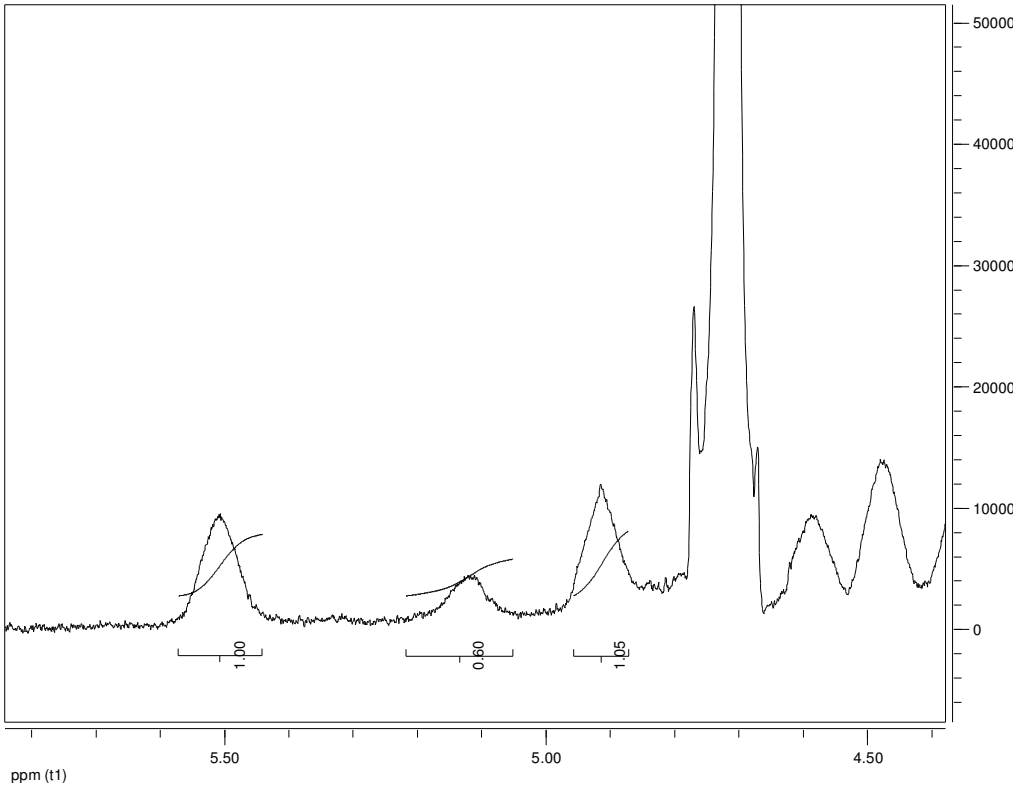

Figure C.52. ^{1}H-NMR spectrum for alginate harvested at 100 mg/L of Ca^{2+}, 48 hours and 70 ℃

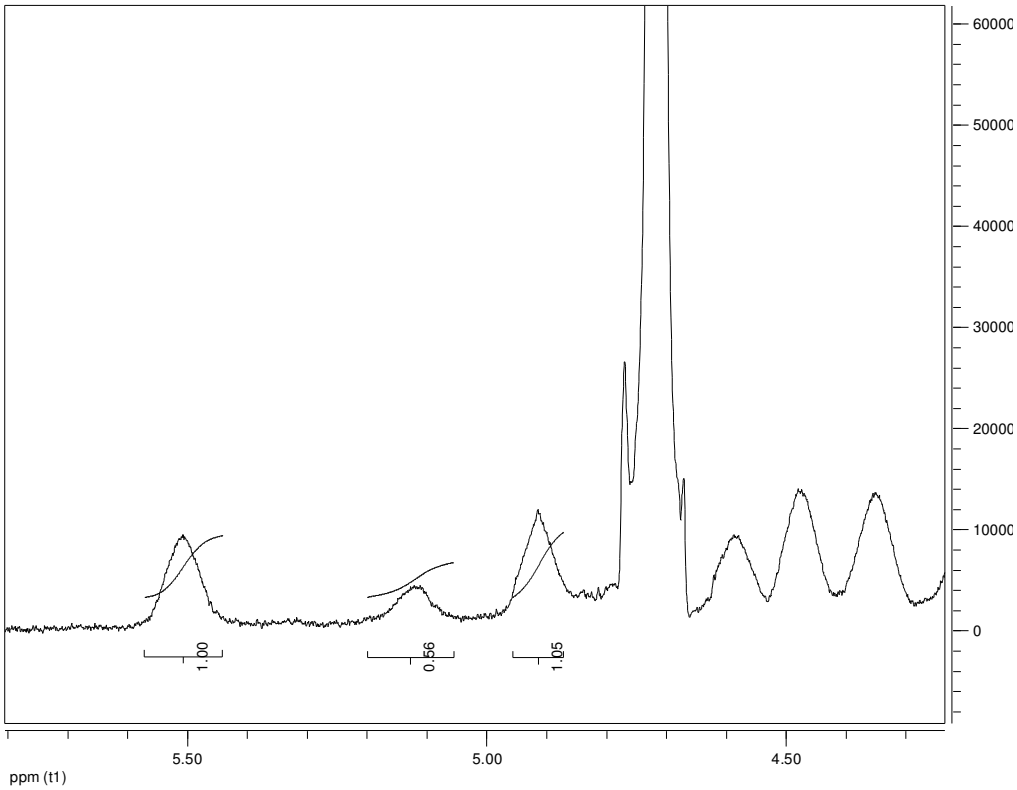

Figure C.53. ^{1}H-NMR spectrum for alginate harvested at 100 mg/L of Ca^{2+}, 56 hours and 70 ℃

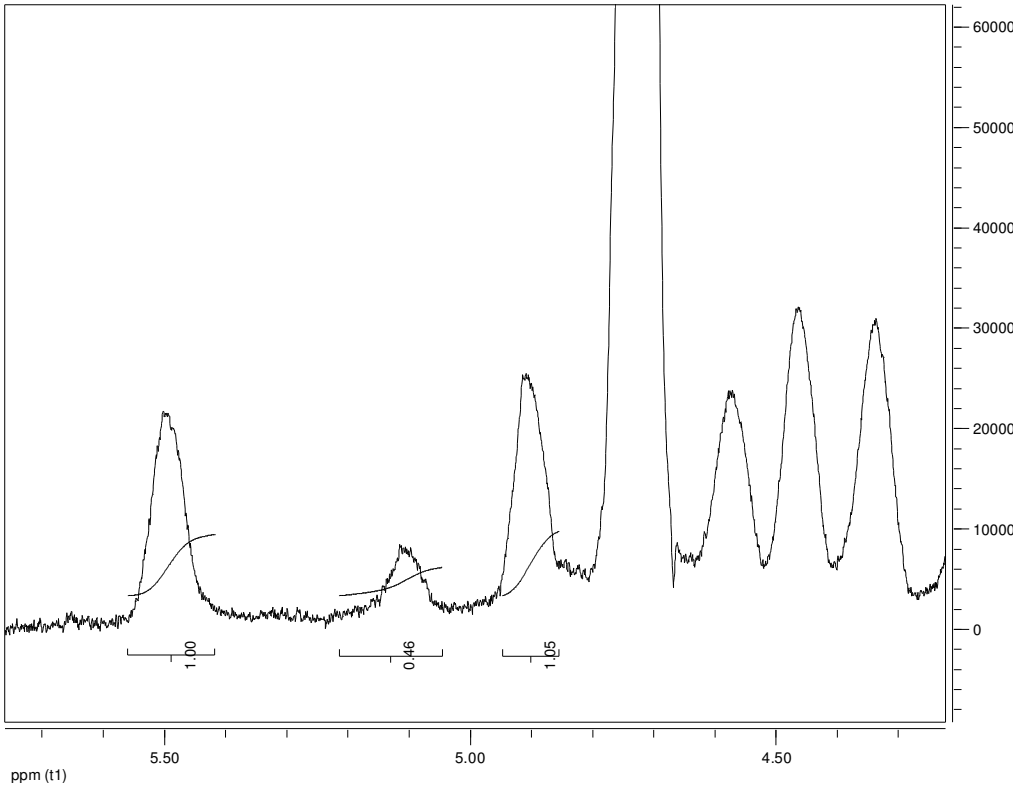

Figure C.54. ^{1}H-NMR spectrum for alginate harvested at 100 mg/L of Ca^{2+}, 72 hours and 70 ℃

APPENDIX D

^{1}H-NMR RESULT FOR NON-EPIMERIZED ALGINATE SAMPLE

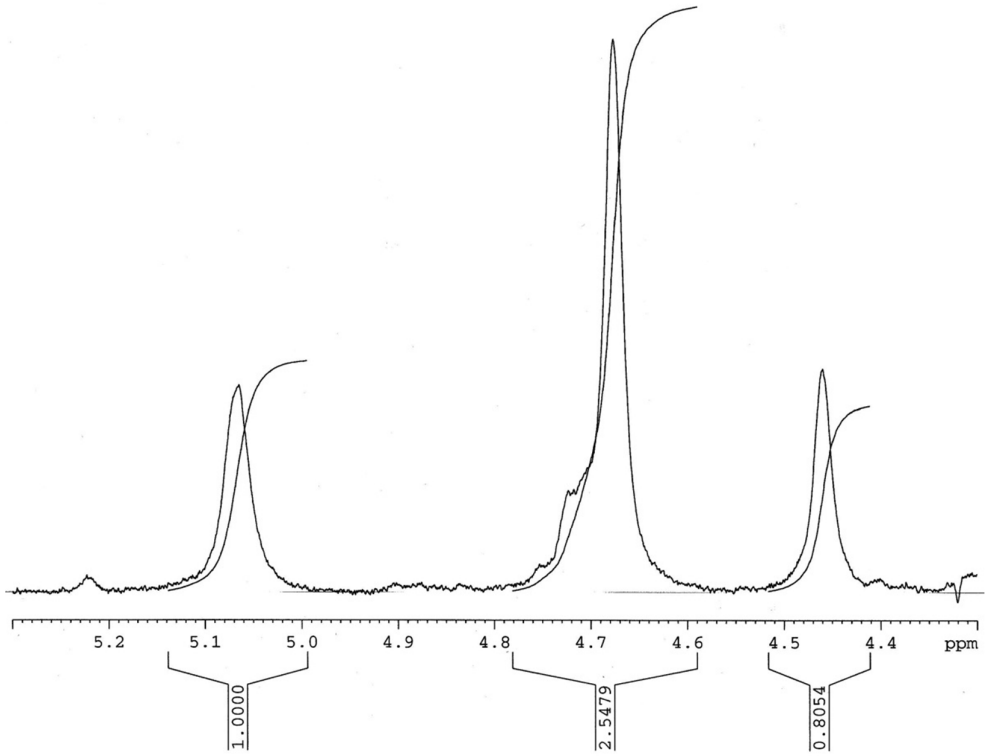

Figure D.1. ^{1}H-NMR result for non-epimerized alginate sample

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PUBLICATIONS

1. I. Akmehmet Balcioglu, C. Sarac, C. Kivilcimdan, E. Tarlan (2006). Application of Ozonation and Biotreatment for Forest Industry Wastewater, *Ozone: Science and Engineering*, 28, 431-436.

2. Balcıoğlu Akmehmet I., Tarlan E., Kıvılcımdan Ç., Türker Saçan M. (2007). Merits of Ozonation and catalytic ozonation pretreatment in the algal treatment of pulp and paper mill effluents, *Journal of Environmental Management*, 85:4, 918-926.

3. Balcıoğlu Akmehmet I., Moral Kıvılcımdan Ç. (2008) 'Homogeneous and Heterogeneous Catalytic Ozonation of Pulp Bleaching Effluent', *Journal of Advanced Oxidation Technologies*, 11:3, 543-550(8).