The Production and Characterization of Alginate Produced by Pseudomonas Syringae.

Richard David Ashby

Louisiana State University and Agricultural & Mechanical College

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The production and characterization of alginate produced by
*Pseudomonas syringae*

Ashby, Richard David, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1994
THE PRODUCTION AND CHARACTERIZATION
OF ALGINATE PRODUCED
BY PSEUDOMONAS SYRINGAE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Microbiology

by

Richard David Ashby
B.S., Brigham Young University, 1987
August 1994
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Finally, I dedicate this work to my wife, Shelley, my daughter, Kailee, and to both sides of my extended family. Their love, patience and understanding made this work possible.
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ABSTRACT

Alginate has many industrial uses because of its unique colloidal behavior, and its ability to thicken, stabilize, emulsify, suspend, form films, and produce gels. Currently, all commercial alginate comes from brown algae, where it exists naturally as a structural material. Many different types of brown algae produce alginate but it can only be obtained in sufficient quantity and quality from a limited number of species. On the basis of location, at least half of the world's resources of this polymer are potentially at risk due either to political instability or industrial pollution. Bacteria provide a potentially unlimited alternate source for alginate. Pseudomonas syringae pv phaseolicola ATCC 19304, produced an acetylated alginate-like polysaccharide with a weight average molecular weight ($M_W$) of $1.2 \times 10^5$. This bacterial polymer was composed of 82% mannuronic acid and 18% guluronic acid. Compositional analysis of the reduced alginate polymer showed that L-gulose was more sensitive to acid degradation than D-mannose. The percentage of gulose recovered at various hydrolysis times was extrapolated to zero hydrolysis time to account for the loss of gulose. Acetylation affected the solution and gelling properties of the polymer. Acetylated bacterial alginate showed increased viscosity, and water holding capacity, and altered cation precipitability over unacetylated alginates. By controlling the degree of acetylation on the bacterial alginate, the solution and gelling properties of the polymer can be manipulated and the polymer targeted to specific applications.
INTRODUCTION

Alginate is a water-soluble gum used in the food and chemical industry primarily as an emulsifier, stabilizer, or thickening (gelling) agent (117, 144). The commercial sources of alginate are the brown marine seaweeds of the family Phaeophyceae, specifically, those of the genera *Ascophyllum*, *Ecklonia*, *Fusarium*, *Laminaria*, and *Macrocystis*. These alginates form viscous solutions at low concentrations, show polyelectrolytic behavior, and form gels and films (97). Typically, these alginates are block copolymers, linked 1-4, of β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). The ratio of M/G varies from one algal species to the next and dictates the gelling properties of the polymer. Alginates with high M/G ratios are more extended and produce elastic, pliable gels due to the smaller regions of poly-G blocks. Alginates with low M/G ratios produce strong, brittle gels, because of the higher affinity of poly-G for Ca++, and the greater compaction of the molecule.

Alginate is one of the few eukaryotic polysaccharides that have a potential prokaryotic source. Many species of bacteria produce "alginate-like" polysaccharides. This was first discovered in studies on the "slime" produced by *Pseudomonas aeruginosa* (13, 75, 76) in infections of cystic fibrosis patients. *Azotobacter vinlandii* (51, 99) was also studied as a potential source of alginate because of its nonpathogenic nature. At high respiration rates the majority of the carbon used by *A. vinlandii* goes to respiration and the formation of poly β hydroxybutyrate (PHB, 23). This bacterium
produces alginate only during encystment and does not produce sufficient polymer for industrial production.

Other nonpathogenic pseudomonads, including the fluorescent pseudomonads (58, 120), and the phytopathogenic pseudomonads, specifically *Pseudomonas syringae* (37, 38, 91), produce alginates. *P. syringae* produces an alginate conspicuously different from that of the brown seaweeds. As with other bacterial alginates, *P. syringae* alginates are generally acetylated at position C-2 and/or C-3 of the mannuronic acid residues. Unlike seaweed alginate, *P. syringae* polymers do not contain an extensive block structure. The bacterial alginate produces bulky, elastic gels with high water retention because of its lack of extensive poly-G blocks, O-acetylation, and high M/G ratio (47).

The goals of this study were to determine the conditions which influence alginate production from *P. syringae* and to characterize the properties of the polymer in order to determine if the use of this organism is feasible for large scale production of alginate.
I. Bacterial Exopolysaccharides: General Characteristics.

Most species of bacteria produce exopolysaccharides (EPS), i.e., polysaccharides found outside the cell wall, either attached in the form of a capsule or secreted into the extracellular environment as a slime (135). There have been many theories proposed to explain the role of bacterial EPS and all relate to the survival advantages it offers to the organisms. Originally, these polysaccharides were studied because of their link to pathogenicity. This was especially true of the capsules of strains of Streptococcus pneumoniae, Bacillus anthracis, and Klebsiella pneumoniae (2). The virulence of these organisms is due, in part, to the protection provided by the capsular polysaccharide surrounding the cell. Within animal hosts, capsules interfere with both phagocytosis and antibody binding (87).

Some bacteria secrete EPS as an aid to colonization by assisting in surface attachment (16, 50). The ability of Streptococcus mutans and Streptococcus salivarius cells to adhere to the surface of teeth is partially a function of the EPS produced by these oral bacteria (87). Other roles of EPS include preventing desiccation, energy storage, concentration and uptake of charged molecules, particularly metal ions (11, 17, 46), protection against the effects of ultraviolet radiation (17), and mediation of biofilm and microcolony formation (18).

Bacterial EPS is species specific. Sugars are common components in most bacterial EPS. D-glucose, D-mannose, and D-galactose occur frequently, and L-rhamnose and L-fucose are less
common. The presence of negative charges is a feature of some bacterial EPS. Usually these charges are the result of the incorporation of uronic acids into the polymer. The most common negatively charged sugar present in bacterial EPS is D-glucuronic acid which is a component of hyaluronan, xanthan gum, and most teichuronic acids. D-mannuronic acid, D-galacturonic acid and L-guluronic acid are also components of some bacterial polymers. D-mannuronic acid and L-guluronic acid are associated primarily with bacterial alginates. D-galacturonic acid is found in some lipopolysaccharides produced by *Proteus mirabilis*, (77) and *Xanthomonas campestris* (142).

Biosynthesis of EPS normally occurs by either of two mechanisms. EPS may be produced at the cytoplasmic membrane using precursors formed intracellularly, or they may be formed from specific precursors in the extracellular environment. Generally, the first type is characteristic of heteropolysaccharide production, while the second is characteristic of certain homopolysaccharides including levans and dextrans (133). Both pathways require the production of activated nucleotide diphosphate forms of the monosaccharides and the precursors for substituent groups, specifically acetate, pyruvate, and succinate (25, 37). Acetyl CoA was recently confirmed as the source of acetate in xanthan gum biosynthesis by *Xanthomonas campestris* (66). The precursor for pyruvate is phosphoenolpyruvate (71). These substituents introduce negative charges into the polysaccharides. A high number of negative charges, coupled with a high molecular weight, produce polymers that form highly hydrated gels. While
the ability to produce EPS is widespread, under laboratory conditions this ability is often unstable and lost on repeated culture (17).

Bacterial EPS varies enormously in structure and composition, ranging from simple homopolysaccharides to complex, highly substituted and branched heteropolysaccharides (Table 1). These polysaccharides can be divided into five distinct groups: 1) dextrans and levans, or homopolysaccharides produced by bacteria using sucrose as a specific substrate, 2) homopolysaccharides other than dextran and levan, *i.e.*, cellulose, 3) heteropolysaccharides containing more than one type of monosaccharide synthesized from a specific carbon substrate, 4) heteropolysaccharides formed from repeating unit structures, *i.e.*, xanthan gum, and 5) heteropolysaccharides composed of two types of monomer with no repeating unit, *i.e.*, alginate (134).

II. Alginate: Overview

1. Compositional Differences, Seaweed vs. Bacterial Alginate

Alginate, the major structural polysaccharide of the brown algae (Phaeophyceae), was first discovered by E. C. C. Stanford in 1881 (19). It was not until 30 years later that uronic acids were identified as the major components (118). Initially, it was assumed that $\beta$-D-mannuronate was the only monosaccharide present in alginate. Subsequent research established the copolymeric nature of alginate and the presence of variable amounts of a second uronic acid, $\alpha$-L-guluronate (41).
Table 1. The structures of some bacterial exopolysaccharides.

<table>
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<tr>
<th>Species</th>
<th>Polysaccharide</th>
<th>Structure</th>
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<tr>
<td><em>Leuconostoc mesenteroides</em></td>
<td>Dextran</td>
<td>(1(\alpha)6 glucose)(_n)</td>
</tr>
<tr>
<td><em>Acetobacter xylinum</em></td>
<td>Cellulose</td>
<td>(1(\beta)4 glucose)(_n)</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em> var. <em>myxogenes</em></td>
<td>Curdlan</td>
<td>(1(\beta)3 glucose)(_n)</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td>Mutan</td>
<td>(1(\alpha)3 glucose)(_n)</td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td>Levan</td>
<td>(2(\beta)6 fructose)(_n)</td>
</tr>
<tr>
<td><em>Azotobacter vinlandii</em></td>
<td>Alginate</td>
<td>1-4 linked ManA and GulA</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Alginate</td>
<td>1-4 linked ManA and GulA</td>
</tr>
<tr>
<td><em>Streptococci</em> (Haemolytic group A)</td>
<td>Hyaluronan</td>
<td>(-3GlcNAc1(\beta)4GlcA1(\beta))(_n)</td>
</tr>
<tr>
<td><strong>Xanthomonas campestris</strong></td>
<td>Xanthan</td>
<td>(-4Glc1(\beta)4Glc1(\beta)4Glc1(\beta))(_n)</td>
</tr>
</tbody>
</table>

Abbreviations: Gal, galactose; Glc, glucose; GlcA, glucuronic acid; GulA, guluronic acid; Man, mannose; ManA, mannuronic acid; GlcNAc, N-acetylglucosamine; OAc, O-acetyl; Pyr, pyruvate.

\(A^a = (-3\alpha1\text{Man(OAc)2}\beta1\text{GlcA4}\beta1\text{Man4-Pyr})\)
Within the last 30 years, alginate was found to be produced by some prokaryotes. The observation that *Pseudomonas aeruginosa* was able to synthesize an "alginate-like" polysaccharide was first reported by Linker and Jones (75), although mucoid strains of this bacterium were isolated much earlier. They demonstrated that this polymer had components identical to algal alginate. In a subsequent study, they showed that an O-acetylated alginate was the major component of *P. aeruginosa* slime (76). Carlson and Matthews (13) confirmed the composition of bacterial alginates in a study of 13 different mucoid isolates from cystic fibrosis patients. More recently, the related pseudomonads *P. fluorescens*, *P. mendocina*, *P. putida* (53), and many pathovars of *P. syringae* (37) have been identified as alginate producers. The same year that *P. aeruginosa* was reported to produce alginate, Gorin and Spencer (51) showed that the polysaccharide produced by strains of *Azotobacter vinlandii* was the same as the acetylated alginate produced by pseudomonads. Later it was determined that alginate biosynthesis by *A. vinlandii* was closely associated with the formation of vegetative cysts (93).

Alginates are unbranched copolymers of β-D-mannuronic acid and its C-5 epimer α-L-guluronic acid, linked 1-4 (Fig. 1). The actual composition and sequence of the polymer is dependent upon the source (73, 96, 130). Alginates from seaweed and *A. vinlandii* generally contain extensive regions of homopolymeric blocks of mannuronic acid and guluronic acid together with some random or alternating sequences. Alginates derived from strains of *Pseudomonas* contain few block homopolymeric regions. Instead,
Figure 1. Structures of the uronic acids of alginates.
they are composed predominantly of random sequences. In addition, the mannuronic acid residues are highly O-acetylated (21, 125). The structure, as well as the position of acetylation in these alginates, has been extensively studied. The O-acetyl groups are associated exclusively with the C-2 and/or C-3 positions of the D-mannuronate residues. $^1$H nuclear magnetic resonance spectroscopy (NMR) revealed that some of the mannuronate residues were 2,3 di-O-acetylated, although the mono-O-acetylated forms were more common (21, 125, 127). The reason for the O-acetylation in bacterial alginates is not entirely clear. It was suggested that acetylation may be part of a control mechanism that operates during biosynthesis that controls the M/G ratio by preventing epimerization of the carboxyl group on the C-5 mannuronate carbon (128). Alternatively, it may play a role in dictating the solution properties of the polysaccharide (129).

Seaweed and bacterial alginates differ in their M/G ratios. Most alginates isolated from the brown seaweeds show M/G ratios in the range of 0.45 ($Laminaria hyperborea$) to 1.85 ($Ascophyllum nodosum$, 117). The most common industrially available alginate is extracted from the brown alga, $Macrocystis pyrifera$. This polymer contains 60% 4-linked mannuronate and 40% 4-linked guluronate residues giving an M/G ratio of 1.5. The alginates produced by $P. aeruginosa$ contain 80% 4-linked mannuronate and 20% 4-linked guluronate residues, giving an M/G ratio of 4.0 (150).

Haworth projections of the $\beta$-D-mannuronate and the $\alpha$-L-guluronate residues show little difference in the two structures. Epimerization of the carboxyl group at C-5 does, however produce a
marked change in the three dimensional conformation of these monosaccharides. Since the carboxyl group is the most bulky substituent on the ring, the most energetically favorable conformation orients this group to the equatorial position. As a result, β-D-mannuronate exists preferentially in the $4C_1$ chair form, whereas the α-L-guluronate residues adopt the $1C_4$ conformation (Fig. 1). Chains of sugars containing combinations of these two forms produce polymers with different three dimensional structures. The β-D-mannuronate linkage positions, C-1 and C-4, are equatorial to the plane of the sugar ring. These linkages are axial for the α-L-guluronate residues. X-ray fiber diffraction studies of alginates containing high proportions of mannuronate residues indicate a flat ribbon-like conformation in the solid state (5, 109, 110). This conformation is similar to the β 1-4 diequatorially linked polymers such as cellulose. Alginates rich in polyguluronate, which is 1-4 diaxially linked, adopt a buckled 2-fold chain conformation (6, Fig. 2).

2. Properties

The physical composition of alginates dictates the solution and gelling properties of the polymers. Alginate is a structural component in the brown seaweeds where it contributes to both the tensile strength and the flexibility of the algal tissue (64, 143). In *A. vinlandii* it serves a structural function for the metabolically dormant cysts. Evidence exists that it is an important component of both the cyst exine and intine, which are microscopically distinct regions outside the central body of the cyst (115). Alginate
Figure 2. The block structures of alginate. A. The ribbon-like structure of poly \( \beta \)-D-mannuronate. B. The buckled chain conformation of poly \( \alpha \)-L-guluronate. C. Alternating sequences of \( \beta \)-D-mannuronate and \( \alpha \)-L-guluronate.
production by \textit{P. aeruginosa} was related to resistance to antibiotics, phage and bacteriocins and protection against phagocytosis and antibody attachment. This suggests, that for \textit{P. aeruginosa}, alginate functions as a general protective barrier.

Alginates with low M/G ratios produce strong but brittle gels, whereas alginites with high M/G ratios form more elastic gels (62, 110). This difference is due to the arrangement of the block structures within the polymers and is particularly evident when the polysaccharide interacts with cations. Alginate, being a polyanion, is a natural ion-exchanger where the selectivity and strength of binding depend on the cation, the conformational characteristics, and the linear charge density of the polymer. Most multivalent cations bind alginates and cross-link the polysaccharide to form a gel matrix. Some regions of the polysaccharide form stronger chelation complexes than others with divalent cations, especially calcium ions (Ca$^{2+}$). The "egg box model" was proposed to explain this interaction (82, 83, 88, 109, 110, Fig. 3.). The binding of Ca$^{2+}$ is strong because in addition to the ionic binding to the carboxyl groups, various ring and hydroxyl oxygen atoms are able to chelate the cations. Polyguluronate binds Ca$^{2+}$ very strongly. Polymannuronate or mixed sequences do not bind Ca$^{2+}$ with as high an affinity. When various block sequences were isolated from intact alginate, polyguluronate blocks showed an enhanced binding of Ca$^{2+}$ in polymers above 20 residues (61). This indicated a cooperative mechanism where binding sites exist in an ordered array, and binding of one ion facilitates binding of a second. Similar
Figure 3. The "egg box" model for Ca\textsuperscript{++} induced gelation of poly α-L-guluronate.
effects were not seen with polymannuronate blocks or alternating sequences (68).

Bacterial alginates that contain few polyguluronate blocks (a high M/G ratio), and that are O-acetylated, produce relatively bulky, flexible gels with high water retention in the presence of Ca\(^{++}\) (109). The high number of mannuronate residues and O-acetyl groups reduce the cooperative binding of Ca\(^{++}\) and weaken the gel network. This fact, in association with the greater positive osmotic pressure caused by the increased number of dissociated counterions, significantly enhances the water holding capacity of bacterial gel matrices (129). The resulting gel provides cells with hydrophilic capsules that protect them from other microorganisms, chemicals, antibiotics, and desiccation (116). In *P. syringae*, alginate is thought to play an important role in the pathogenicity of the bacterium, allowing adhesion to the host surface (137).

3. Applications

While alginate production is widespread among members of the brown seaweed (Phaeophyceae), only a few species of brown seaweed are used for commercial production. The principal source of the world's supply of alginate is the giant kelp, *Macrocystis pyrifera*. Other seaweeds that are used in alginate manufacture are *Ascophyllum nodosum* and species of *Laminaria* and *Ecklonia*.

Alginate is used in foods and for general industrial applications because of its unique colloidal behavior and its ability to thicken, stabilize, emulsify, suspend, form films, and produce gels. The primary properties on which the widespread use of
alginate is based are: 1) the formation of viscous solutions at relatively low concentrations, 2) the polyelectrolytic behavior in solution, 3) the ability to form gels by chemical reaction, 4) the formation of films on surfaces, and 5) the base exchange properties (97).

Food products in which alginites are used include frozen foods, pastry fillings, bakery products, and syrups. Because of their gelling ability, alginites are also used in instant and cooked puddings, and pie fillings. Alginites are excellent emulsifiers for salad dressings and stabilizers for beverages, whipped toppings, and sauces (Table 2). Alginites are also of value in a number of industrial applications, such as the manufacture of paper, adhesives, textiles, air fresheners, explosives, polishes, antifoams, ceramics, and cleaners (19, 97, Table 3).

Since the industrial production of seaweed alginites began, several commercially important derivatives of alginites have been developed. Commercial derivations include the sodium, potassium, ammonium, calcium, and mixed ammonium-calcium salts of alginic acid, propylene glycol alginate, and alginic acid. The propylene glycol ester is the only commercial, organic derivative of alginate. It has improved acid stability and resistance to precipitation by calcium and other multivalent metal ions (19).

4. Commercial Manufacture

*Macrocystis pyrifera* grows in relatively calm marine waters in large, dense beds. It is a very rapidly growing plant that allows for up to four cuttings per year. At the time of harvesting, a dense
<table>
<thead>
<tr>
<th>Property</th>
<th>Product</th>
<th>Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water holding</td>
<td>Frozen foods</td>
<td>Maintains texture during freeze-thaw cycles.</td>
</tr>
<tr>
<td></td>
<td>Pastry fillings</td>
<td>Produces smooth, soft texture</td>
</tr>
<tr>
<td></td>
<td>Syrups</td>
<td>Suspends solids, controls pouring consistency</td>
</tr>
<tr>
<td></td>
<td>Bakery icings</td>
<td>Counteracts stickiness and cracking</td>
</tr>
<tr>
<td>Gelling</td>
<td>Puddings</td>
<td>Firms body and texture</td>
</tr>
<tr>
<td></td>
<td>Pie and pastry fillings</td>
<td>Acts as a cold water gel base; develops soft gel body with</td>
</tr>
<tr>
<td></td>
<td></td>
<td>broad temperature tolerance; gives improved flavor release.</td>
</tr>
<tr>
<td></td>
<td>Dessert gels</td>
<td>Produces clear, firm, quick-setting gels.</td>
</tr>
<tr>
<td>Emulsifying</td>
<td>Salad dressings</td>
<td>Emulsifies and stabilization</td>
</tr>
<tr>
<td></td>
<td>Sauces</td>
<td>Emulsifies oils and suspends solids</td>
</tr>
<tr>
<td>Stabilizing</td>
<td>Beer</td>
<td>Maintains beer foam</td>
</tr>
<tr>
<td></td>
<td>Juices</td>
<td>Stabilizes pulp in concentrates and finished drinks.</td>
</tr>
<tr>
<td></td>
<td>syrups and toppings</td>
<td>Suspends solids; produces uniform body</td>
</tr>
<tr>
<td></td>
<td>Whipped toppings</td>
<td>Stabilizes fat dispersion, and freeze-thaw breakdown.</td>
</tr>
<tr>
<td></td>
<td>Sauces and gravies</td>
<td>Thickens and stabilizes for a broad range of applications</td>
</tr>
</tbody>
</table>

*a* Table was adapted from Sandford and Baird (117).
Table 3. Some Industrial Applications of Alginate\textsuperscript{a}

<table>
<thead>
<tr>
<th>Property</th>
<th>Product</th>
<th>Performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water holding</td>
<td>Paper coating</td>
<td>Controls rheology of coatings</td>
</tr>
<tr>
<td></td>
<td>Paper sizings</td>
<td>Improves surface properties, and ink acceptance</td>
</tr>
<tr>
<td></td>
<td>Adhesives</td>
<td>Controls penetration to improve adhesion and application</td>
</tr>
<tr>
<td></td>
<td>Textile printing</td>
<td>Produces very fine line prints</td>
</tr>
<tr>
<td>Gelling</td>
<td>Air freshener</td>
<td>Firm, stable gels are produced from cold water systems</td>
</tr>
<tr>
<td></td>
<td>Explosives</td>
<td>Elastic gels produced by reaction with borates</td>
</tr>
<tr>
<td></td>
<td>Toys</td>
<td>Nontoxic materials made for impressions</td>
</tr>
<tr>
<td></td>
<td>Hydromulching</td>
<td>Holds mulch to inclined surfaces; promotes seed germination</td>
</tr>
<tr>
<td>Emulsifying</td>
<td>Polishes</td>
<td>Emulsifies oils and suspends solids</td>
</tr>
<tr>
<td></td>
<td>Antifoams</td>
<td>Emulsifies and stabilizes</td>
</tr>
<tr>
<td></td>
<td>Latexes</td>
<td>Stabilizes latex emulsions; provides viscosity</td>
</tr>
<tr>
<td>Stabilizing</td>
<td>Ceramics</td>
<td>Suspend solids</td>
</tr>
<tr>
<td></td>
<td>Cleaners</td>
<td>Suspends and stabilizes</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Table was adapted from Sandford and Baird (117)
mat of fronds floats on the ocean surface. Harvesting is actually a massive pruning of the kelp beds. Underwater blades mow the kelp approximately three feet below the water surface. The kelp is then conveyed into the hold of a barge by a moving belt.

The process for alginate extraction is based on an ion-exchange process. In seaweed, the alginate is present as a mixed salt of sodium and/or potassium, calcium, and magnesium (19). The exact composition of the polymer varies with the type of seaweed but this does not affect processing. The extraction process begins by grinding the seaweed and washing it with water. A strong alkali is then added to the washed seaweed and the mixture is heated to extract and dissolve the alginate. The crude alginate solution is then clarified and precipitated by the addition of calcium chloride. The calcium alginate is acid treated to produce an alginic acid precipitate. Sodium carbonate is then added to make a sodium alginate paste which is dried, and milled into sodium alginate powder (Fig. 4).

The extraction costs of seaweed alginate are higher than other industrially available gums due to harvesting and purification costs (117, Table 4). Because of the unique properties of alginates, and the high number of potential food and industrial applications, there are no good replacements for this polymer. Bacteria have been studied as potential alternate sources of alginate. A bacterial source would give an unlimited supply of alginate and may reduce costs.
Figure 4. Flow diagram for the extraction of sodium alginate from seaweed (117).
Table 4. Estimates of U. S. Consumption and Price of Polysaccharides.

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>% U. S. Consumption&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Retail Price (per 100 g)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Food</td>
<td>Industrial</td>
</tr>
<tr>
<td>Agar</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Alginate</td>
<td>11.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>11.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Guar gum</td>
<td>19.3</td>
<td>53.8</td>
</tr>
<tr>
<td>Gum arabic</td>
<td>29.5</td>
<td>10.8</td>
</tr>
<tr>
<td>Gum ghatti</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Gum tragacanth</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Karaya gum</td>
<td>1.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Locust bean gum</td>
<td>11.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Pectin</td>
<td>9.6</td>
<td>0</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>2.9</td>
<td>9.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent U. S. consumption is based on 1980 figures. Percentages represent the amount of polysaccharide used for food and industrial applications relative to each other (117).

<sup>b</sup> Retail price is based on 1994 figures from Sigma Chemical Co., St Louis, Missouri.
III. Alginate Biosynthesis

1. Pathway

In 1966, the biosynthetic route of alginate production was established for the marine brown seaweed *Fucus gardneri* (73, 74). Later, a similar pathway was proposed for alginate production by *Azotobacter vinlandii* (99). In 1981, Piggott *et al* (98) proposed a pathway for alginate production in *Pseudomonas aeruginosa* (Fig. 5). This pathway was based on the detection of all the enzymes necessary for biosynthesis of bacterial alginate, except for the epimerase and the O-acetyl transferase.

Fructose 6-phosphate is considered to be the precursor in the alginate biosynthetic pathway. The primary route of glucose catabolism in pseudomonads is through the Entner-Doudoroff pathway, producing glyceraldehyde 3-phosphate and pyruvate. Carlson and Matthews (13) showed that the C-6, but not the C-1 of glucose was incorporated into alginate. This implied that carbon atoms 1, 2, and 3 of glucose were converted to pyruvate by the 2-keto 3-deoxyphosphogluconate aldolase reaction and eventually were lost to the alginate biosynthetic pathway as CO$_2$ and acetyl CoA. Carbon atoms 4, 5, and 6 were channeled into alginate through glyceraldehyde 3-phosphate. Fructose 6-phosphate can be produced either through gluconeogenesis or by condensation of glyceraldehyde 3-phosphate with dihydroxyacetone phosphate. Subsequent studies with a *P. aeruginosa* mutant deficient in fructose 1,6 diphosphate aldolase showed that there was preferential $^{14}$C incorporation from C-6 into alginate compared with C-1 labeled glucose in the wildtype. Incorporation of C-6 and
Figure 5. The proposed biosynthetic pathway of alginate in *Azotobacter vinlandii* and *Pseudomonas aeruginosa*. The first 4 enzymatic steps have been identified in both organisms. The polymerase and epimerase have been identified in *A. vinlandii* only, and the acetyl transferase has not yet been identified in either organism.
C-1 of glucose were similar in the mutant (7, 78). A summary of the overall routes of incorporation of fructose and glucose in alginate can be seen in Figure 6.

Fructose 6-phosphate can be isomerized to mannose 6-phosphate by phosphomannose isomerase. Transfer of the phosphate group by phosphomannomutase produces mannose 1-phosphate from mannose 6-phosphate. Mannose 1-phosphate and GTP are then converted into GDP-mannose, catalyzed by GDP-mannose pyrophosphorylase. The subsequent oxidation through GDP-mannose dehydrogenase results in the formation of GDP-mannuronic acid. Polymerization of GDP-mannuronic acid results in polymannuronate which is secreted from the bacterial cell and becomes the substrate for an extracellular acylase and/or epimerase in the production of the mature polymer (47).

The transport mechanism of the bacterial alginate across the cytoplasmic membrane is believed to be similar to that of some bacterial cell wall polymers, which use isoprenoid lipid carriers (134). The final step in the biosynthesis of bacterial alginate is the selective epimerization of the mannuronate residues to guluronate by an extracellular epimerase. The selectivity is thought to be dictated by the O-acetylation of the mannuronate residues. Acetylation is believed to inhibit epimerization, thereby dictating the final composition of the alginates. Acetyl CoA is the probable source of the acetyl group in bacterial alginates (136), just as it is on the mannosyl residues of xanthan gum produced by Xanthomonas campestris (66). The mechanism of bacterial O-acetylation is not well defined.
Figure 6. A comparison of the overall routes of incorporation of fructose and glucose in alginate produced by *Ps. aeruginosa* (47).
2. Enzymology

Only four of the seven proposed enzymatic steps in the biosynthetic pathway of alginate from fructose 6-phosphate to the mature polymer have been positively identified in Pseudomonas aeruginosa (98). Six of the steps have been identified in Azotobacter vinlandii (86). The first four enzymes in the pathway have been found in both organisms and have been the subject of much investigation. In P. aeruginosa the genes encoding these enzymes are located at approximately 34 minutes on the chromosome linkage map (89, Fig. 7). What is known about these enzymes is summarized below.

**Phosphomannose isomerase**: This enzyme catalyses the reversible conversion of fructose 6-phosphate to mannose 6-phosphate. The enzyme is the product of the algA gene in P. aeruginosa, and has a molecular weight of approximately 56,000 on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 82). Overexpression of the algA gene produces increased activity of the next two enzymes of the pathway, *i.e.*, phosphomannomutase and GDP mannose pyrophosphorylase (48, 114). In the overexpressed state, phosphomannose isomerase was separated from phosphomannomutase but could not be separated from GDP mannose pyrophosphorylase (114). The inability to separate these two activities has led to the proposal that, in P. aeruginosa, the algA gene encodes a single protein having phosphomannose isomerase and GDP mannose pyrophosphorylase activities.
Figure 7. The relative location of the alginate (alg) genes on the chromosome linkage map of Pseudomonas aeruginosa. (89).
Phosphomannomutase: This enzyme converts mannose 6-phosphate to mannose 1-phosphate. The enzyme is the product of the \textit{algC} gene, and has a molecular weight of 38,000. It was detected in the soluble cytoplasmic fraction of both mucoid and nonmucoid strains of \textit{P. aeruginosa}, from clinical and nonclinical sources (92). Phosphomannomutase has an absolute requirement for glucose 1,6-diphosphate for its activity (114, 151). Induction of the \textit{algA} gene increases phosphomannomutase activity by about 10 fold.

GDP mannose pyrophosphorylase: This enzyme catalyses the formation of GDP mannose from mannose 1-phosphate and GTP. This enzyme was detected in \textit{P. aeruginosa} by Piggott et al (98). It was suggested that the activity of this enzyme may represent one of the two enzymatic activities of the \textit{algA} gene product. This enzyme may be an example of a bifunctional protein where the two activities catalyze noncontiguous steps in a biosynthetic pathway.

GDP mannose dehydrogenase: This enzyme catalyses the oxidation of GDP mannose to GDP mannuronic acid (98, 105). The product of the \textit{algD} gene, GDP mannose dehydrogenase has been purified and found to be a hexamer with a molecular weight of 290,000 (9). Its absence in alginate negative mutants suggests that it is essential for alginate biosynthesis. The \textit{algD} gene has been cloned and sequenced. It is transcriptionally activated in mucoid, but not in nonmucoid strains of \textit{P. aeruginosa} (26, 27). The gene contains regions that have considerable sequence homology with two outer membrane protein genes (\textit{ompF} and \textit{ompC}) from \textit{E. coli}
Like ompF and ompC, expression of the algD gene is regulated by external osmolarity (9).

The final reactions in the biosynthesis of bacterial alginate in *P. aeruginosa* are not yet fully understood. From the structural composition of alginate, it has been proposed that the final steps include polymerization of the mannuronate residues, O-acetylation of some of those residues, export of the polymer, and epimerization of the carboxyl group at C-5. The assumption is that the polymerase uses GDP mannuronic acid as its substrate and that the product of this reaction is polymannuronate. The evidence for this is based on the observation that in *Azotobacter vinlandii* and the brown seaweeds there is an epimerase that converts certain mannuronate residues to guluronate at the polymer level. The polymerase in *P. aeruginosa* has proved exceptionally difficult to measure and the enzyme has not been purified. Very low levels of activity of this enzyme have been measured in membrane fractions prepared from cell extracts of mucoid *P. aeruginosa* (47), but the characterization of this polymerase is still at a preliminary stage.

The mechanism by which guluronate residues are incorporated into *Pseudomonas* alginate remains a mystery. The assumption has been that a polymannuronic acid C-5 epimerase acts at the polymer level to convert some mannuronate residues to guluronate residues. The epimerase from *A. vinlandii* has been purified to homogeneity (126), and recently Piggott et al (98) isolated the gene encoding a C-5 epimerase (*algG*) from *P. aeruginosa*. The enzyme required Ca$^{++}$ for activity, much like the enzyme isolated from *A. vinlandii*. *AlgG* mutants were found to be
incapable of incorporating guluronic acid residues into bacterial alginate.

The epimerase of P. aeruginosa appears to be different from the enzyme isolated from A. vinlandii. The M/G ratio of P. aeruginosa alginate is never less than 1.0, and this ratio is generally unaltered by changes in growth conditions. In A. vinlandii the M/G ratio may be less than 1.0 and the resulting block structure may be altered by changes in Ca$^{++}$ concentrations in the growth medium (70).

The alginates of P. aeruginosa are highly O-acetylated with the O-acetyl groups being associated exclusively with the mannuronic acid residues. It is thought that one of the functions of acetylation is to protect the mannuronic residues in the polymer from epimerization to guluronate residues (126). Recently, an alginate modification gene, algF, was sequenced. This gene codes for a 28 kd protein which controls the addition of O-acetyl groups to the mannuronic acid residues. The algF gene was reported to be nonessential for alginate biosynthesis, but is required for acetylation of the alginate polymer (44).

A major difference between the alginates of A. vinlandii, and P. aeruginosa is the arrangement of mannuronate and guluronate residues within each polymer. The polymer from A. vinlandii is arranged into homopolymeric blocks of mannuronate and guluronate, whereas in P. aeruginosa alginate polyguluronate blocks are absent. This difference reflects differences in O-acetylation and epimerization activities between these two bacteria. These
differences may indicate alterations in the latter stages of alginate biosynthesis in *P. aeruginosa* from those proposed in Figure 5.

3. Regulation

In the lungs of cystic fibrosis patients Alg (-) strains of *P. aeruginosa* usually convert to the mucoid Alg (+) form. *In vitro*, the Alg (+) phenotype is unstable and Alg (-) revertants appear over time. Genetic mapping experiments have shown that spontaneous alginate conversion is based on the gene products of the *algB*, *algR*, *algS*, and *algT* genes (42, 45, 79, 90). The primary genetic event that regulates the "on/off" switch in alginate production is handled primarily by the *algS* and *algT* genes located at approximately 68 minutes on the chromosomal linkage map of *P. aeruginosa* (89, Fig. 7).

Spontaneous conversion between the mucoid and nonmucoid state is the result of a genetic alteration at the *algS* gene locus. The *algS* gene is a genetic switch that controls the expression of *algT* (42). Formation of the AlgS protein results in the activation of the *algT* gene. The gene product of *algT* acts as a regulatory protein in the biosynthesis of bacterial alginate in *P. aeruginosa* by promoting the activation of the structural genes involved in the biosynthetic pathway (42, 43).

Along with its role in regulation of the structural genes in alginate production, the *algT* gene product also is involved in the expression of the *algB* gene (149). The *algB* gene is located at approximately 12 minutes on the chromosome map (Fig. 7). Its gene product is not directly involved in the biosynthetic pathway of
alginate but is apparently involved in high level production of alginate in *P. aeruginosa* (49). The *algB* gene product belongs to a class of proteins that control gene transcription in response to environmental stimuli (149).

The last gene involved in the regulation of alginate biosynthesis is the *algR* gene. This regulatory gene controls transcription of the *algD* gene, which encodes for GDP mannose dehydrogenase, an essential enzyme in the biosynthesis of bacterial alginate (98). DNA sequence analysis showed a high degree of homology between the *algR* gene and other environmentally responsive bacterial regulatory genes, including *ompR, phoB, ntrC,* and *spoA* (28). This indicates that the production of bacterial alginate is affected by environmental stimuli or specific chemical compounds present in the environment.

Recently, the mechanism of alginate production control by *algB, algR,* and *algT* has been examined. Compared with Alg (+) strains, deletion mutations in the *algB* and *algT* genes showed highly reduced transcriptional activity in the biosynthetic gene cluster (at 34 minutes, 49). This indicated that the pathway of alginate biosynthesis is under control not only by the *algR* gene product but also by the gene products of *algB* and *algT*. Whether the *algT* and *algB* gene products act directly on any promoters in the biosynthetic gene cluster or on other regulatory genes is not known.
IV. *Pseudomonas syringae* Alginates

Numerous pathovars of *P. syringae* were reported to produce bacterial alginates (37). El Banoby and Rudolph (34) reported that the EPS from several plant pathogenic pseudomonads were capable of inducing water soaked lesions on compatible leaf tissue. This suggested that alginate may be necessary for successful colonization of plant host tissue. In common with other bacterial alginates, those produced by *P. syringae* are highly acetylated. The M/G ratio is variable among strains and among different preparations from the same strain. Fett *et al* (38) reported that the percentage of guluronic acid in *P. syringae* alginates varied from < 1% to 28% when grown *in planta*, and that the *in planta* samples had a higher degree of acetylation than the alginates produced *in vitro*. It was also reported that the *P. syringae* alginates were smaller, on the average, than those from *P. aeruginosa* (37). The number average molecular weights range from $3.8 \times 10^3$ for alginates from *P. syringae pv glycinea* produced *in vitro*, to $47.1 \times 10^3$ for *P. syringae pv papulans* alginate produced *in vitro*, compared to *P. aeruginosa* alginates whose size was reportedly in the range of $10^5$ (37). It appears that alginate biosynthesis is a common property of the majority of pseudomonads in rRNA-DNA homology group 1 (29, 40, 94).

V. polysaccharide Analysis

1. Depolymerization

Characterization of any polysaccharide starts with a compositional analysis. Acid hydrolysis is common to most methods
of determining the physical composition of a polysaccharide. Hydrolysis is usually conducted with dilute mineral acids, the most common being sulfuric acid, hydrochloric acid, or trifluoroacetic acid at 100°C for varying lengths of time. Many factors influence the rate of hydrolysis of any polysaccharide, including ring size, configuration, conformation, and polarity of the component sugars (8).

In studying the composition of alginic acid, most researchers reduce the uronic acids to a neutral polymer to facilitate acid hydrolysis without thermal destruction of the component monosaccharides (13, 37, 38, 91, 150). Acid hydrolysis of neutral polysaccharides has been studied, and the mechanism now accepted was first suggested by Edward (33) and is depicted in Figure 8 for the hydrolysis of methyl β-D glucopyranoside. The process involves protonation of the glycosidic oxygen atom to form the conjugate acid, followed by the formation of a cyclic carbonium-oxonium ion which probably exists in the half chair conformation having C-2, C-1, O, and C-5 in a plane. Reaction with water then gives the protonated reducing sugar and from it the reducing sugar is formed.

Alginic acid, being a polyuronide, contains a carboxyl group at the C-5 position of each component monosaccharide. This carboxyl group confers acid resistance to the glycosyluronic acid linkages (15). Many theories have been advanced as to why. There is evidence that the enhanced stability may be attributed to either steric factors or to inductive effects (145). Ranby and Marchessault (107, 108) formulated an induction-stabilization theory, which proposed that the glycosidic bond is stabilized by the inductive
Figure 8. The mechanism of acid hydrolysis of glycosides. The carbonium ion intermediate (3) is in the half-chair conformation (8).
effect of the polar carboxyl group. According to this theory, the presence of an electronegative group, such as a carbonyl or carboxyl group, can exert inductive influences on the glycosidic oxygen atom. In a polyuronide, the carboxyl group would oppose the protonation of the glycosidic oxygen atoms by making the electron pairs on the glycosidic oxygen atoms less basic, resulting in a more stable bond. A possible formation of six membered rings, where hydrogen stabilizes the negatively charged carboxyl group and the glycosidic oxygen atom has also been postulated (107). Although the exact explanation for the stability of glycosyluronic acid bonds has not been pinpointed, suffice it to say that these bonds are more difficult to hydrolyze than the neutral O-glycosidic bonds.

Many factors influence the rate of hydrolysis of polysaccharides. The ease of hydrolysis at a particular temperature and acid concentration increases in the order glucopyranoside < fructopyranoside < fructofuranoside (63). This indicates that ring size affects hydrolysis. There is a direct relationship between the strain (or free energy) associated with a molecule and the rate of hydrolysis (121). In general, aldoburanosides and aldoheptanosides are hydrolyzed more rapidly than the corresponding aldopyranosides. The five and seven membered rings are strained because of the distortion of the tetrahedral angle of the ring carbon atoms. The pyranoid rings can pucker to eliminate strain (35).

The anomeric configuration in pyranosides also plays a role in the stabilities of these sugars under acid hydrolysis conditions. Feather and Harris (36) used anomeric pairs of methyl aldopyranosides to study the affects of the anomeric configuration
on acid stability. They found that an anomer with an equatorial methoxyl group hydrolyzes more rapidly than an anomer containing an axially oriented group. Two explanations have been proposed. First, equatorial bonds are considered to be more accessible than axial bonds, thus making them more available for proton transfer from a hydronium ion to the glycosidic oxygen atom. Alternatively, the greater reactivity of the equatorial substituent is due to its higher free energy caused by a polar interaction between the equatorial methoxyl group and the ring oxygen atom (33).

2. Acid Sensitivity

Monosaccharides are degraded by acid to a greater or lesser extent depending on the sugar and strength of the acid (119). In addition to degradation, acids may convert sugars into anhydro derivatives. Spontaneous conversion to a 1,6 anhydroaldopyranose occurs in acidic solutions of several aldoses and ketoses having the indol (100, 102, 146), altro (101, 112), and gulo (131, 132) configurations. In dilute acid solutions those sugars of the gluco, manno, and galacto configurations are almost completely hydrolyzed to the free aldoses. Reeves (111) first suggested an explanation for this different behavior in terms of conformational interactions. He showed that β-D-idose, with all hydroxyl groups equatorial, has a higher propensity to form 1,6 anhydrides than does β-D-glucose whose hydroxyl groups are all oriented axially. This system represents a good example of conformational control of
an equilibrium. The position of the equilibrium is dependent on the steric arrangement of the groups not taking part in the reaction.

The solutions of any reducing sugar contain an equilibrium mixture of the $1C_4$ and $4C_1$ forms of the $\alpha$ and $\beta$ anomers. Only the $1C_4$ form of the $\beta$ anomer can directly form the anhydride without change in configuration and conformation. 1,6 Anhydride formation is believed to occur in two steps: 1) conversion of other forms of the sugar into the $1C_4$ form of the $\beta$-D anomer, and 2) formation of the anhydride. Pratt and Richtmyer (104) showed that an axial hydroxyl group at C-3 is the most important axial constituent in determination of 1,6 anhydride formation. An axial hydroxyl group at C-3 can interact with an anhydride bridge and destabilize the anhydride. The four hexoses having axial hydroxyl groups at C-3 (D-glucose, D-mannose, D-galactose, and D-talose) form less anhydride than those hexoses whose C-3 hydroxyl group is oriented equatorially (D-allose, D-altrose, D-gulose, and D-idose). The overall ability for 1,6 anhydride formation then is:

\[
\text{id}o > \text{al}to, \text{gulo} > \text{talo} > \text{ allo} > \text{galacto} > \text{manno} > \text{gluco}
\]

Some debate still exists about the placement of talose compared to allose in the above scheme. Some researchers claim talose is more prone to 1,6 anhydride formation. Others claim allose forms 1,6 anhydrides more readily (103, 141).
VI. Goals of This Study

This study was designed to optimize the conditions for production of bacterial alginate from *Pseudomonas syringae* pv *phaseolicola*, ATCC 19304 to determine the feasibility of large scale production, and to characterize the physical properties of the product, relating those properties to the resulting solution and gelling properties of the polymer.
MATERIALS AND METHODS

I. Organisms, Growth Conditions, and Maintenance

*Pseudomonas syringae* subsp. *phaseolicola* ATCC 19304 was obtained from the American Type Culture Collection, Rockville, MD. It was selected for this research because of its lack of human pathogenicity and its potential for producing a highly acetylated bacterial alginate (39).

Cultures were maintained at 4°C on Dworkin Foster (DF) agar (32) supplemented with 2% (w/v) gluconic acid. This medium contained (in grams per liter of deionized water): KH$_2$PO$_4$, 4.0; Na$_2$HPO$_4$, 6.0; NaCl, 0.4; KNO$_3$, 9.1; (NH$_4$)$_2$SO$_4$, 0.9; MgSO$_4$•7H$_2$O, 0.2; gluconic acid, 20; and agar, 15. The gluconic acid was aseptically added to the salts medium after separate sterilization in an autoclave at 121°C, at 15 lbs./in$^2$ of pressure for 15 minutes. The pH of the agar was between 6.9 and 7.0 prior to sterilization. Plates were inoculated with 0.1 ml of a standardized 48 hour DF broth culture of *P. syringae*. This culture showed an absorbance between 1.9 and 2.0 at 660 nanometers. The inoculum was spread using a flame sterilized, bent glass rod. After growth at 30°C for 48 hours, the plates were stored at 4°C. Cultures were transferred every fourth week.

All broth starter cultures used in this work were prepared by inoculating *P. syringae* ATCC 19304 from agar plates into 100 ml of DF broth in 250 ml Erlenmeyer flasks. Cultures were incubated at 30°C for 48 hours at 180 rpm on a NBS Model G25-KC rotary shaker (New Brunswick Scientific Co. Inc., Edison, NJ). Cultures were
standardized with sterile water to an absorbance at 660 nanometers between 1.9 and 2.0 on a Gilford Response II spectrophotometer (Gilford Instrument Lab., Oberlin, OH) prior to use.

II. Analytical Methods

1. Cell Mass Determination

Dry cell weight for calculation of specific yield of alginate or percent acetylation was measured directly. Broth cultures were centrifuged at 17,000 x g for 15 minutes in a Sorval Superspeed Model RC-5B centrifuge (Du Pont Co., Wilmington, DE) to remove bacterial cells. Cells from agar media were resuspended in 100 ml of deionized water. The suspension was centrifuged at 27,000 x g for 60 minutes to pellet the cells from the highly viscous solution. Once the cells were separated, the pellets were treated equally. The pellets were washed twice in 10 ml of deionized water. The supernatant was discarded and the pellet was resuspended in an equal volume of deionized water. The cell suspension was poured into a pre-dried, tared, aluminum weighing dish and was dried in a drying oven at 100°C to a constant weight.

2. Total Carbohydrate Quantitation

The total carbohydrate present in *P. syringae* EPS was determined by the phenol-sulfuric acid assay (31). The protocol was as follows:
1) To 2 ml of carbohydrate solution containing 10 to 100 µg of carbohydrate, 0.05 ml of 80% (v/v) aqueous phenol was added.

2) The solution was shaken in a Vortex mixer for a count of 5. Then, 5 ml of concentrated sulfuric acid was added and the solution mixed again for another count of 5.

3) The tubes were incubated at room temperature for 30 minutes to allow the color to develop. The absorbance was measured at 485 nanometers on a Gilford Response II spectrophotometer (Gilford Instrument Lab., Oberlin, OH). This value was compared to a standard curve of sodium alginate from *Macrocystis pyrifera* (Sigma Chemical Co., St Louis, MO) for determination of the total carbohydrate present in the solution.

3. Alginate Quantitation

Alginate concentrations were determined by two different methods. These methods included the uronic acid assay described by Blumenkrantz and Asboe-Hansen (10), and the carbazole assay of Knutson and Jeanes (69). Each method has its benefits. The uronic acid assay is rapid, but is only accurate up to 150 µg/ml of uronic acid. The carbazole assay takes much longer to perform, but it is accurate up to 1000 µg/ml.

The uronic acid assay was used predominately with alginates produced in broth cultures. This assay allowed direct testing of the supernatant without color interference from the remaining salts in solution. The protocol was as follows:
1) To 0.2 ml of sample containing from 0.5 to 30 μg uronic acid, 1.2 ml of 12.5 mM tetraborate in concentrated sulfuric acid was added.
2) The tubes were chilled in an ice bath for 10 minutes.
3) The mixture was shaken in a Vortex mixer, and the tubes heated in a water bath at 100°C for 5 minutes.
4) After cooling in a water-ice bath, 20 μl of 0.15% (w/v) meta-hydroxydiphenyl in 0.5% (w/v) sodium hydroxide was added to the above mixture.
5) The tubes were shaken for a count of 5 and the absorbance read at 520 nm as described previously. This value was compared to a standard curve of sodium alginate from *Macrocystis pyrifera* to obtain the concentration of uronic acid.

The carbazole assay was used for high concentrations of purified alginate. Experimental error diminished with this assay because of fewer dilution steps. The protocol for the carbazole assay was as follows:

1) 0.5 ml of a purified alginate solution containing 0 to 500 μg of alginate was equilibrated in a water-ice bath for 10 minutes.
2) Three ml of cold concentrated sulfuric acid was added to this solution. The mixture was re-equilibrated in a water-ice bath.
3) The solution was then mixed with a Vortex mixer for a count of 4 and heated at 55°C for 20 minutes in a water bath.
4) The solution was then re-equilibrated in a water-ice bath for 10 minutes, and 0.2 ml of a 0.2% (w/v) carbazole solution (Eastman Kodak, Rochester NY) in ethanol was added.

5) The sample was mixed with a Vortex mixer for a count of 10 and allowed to incubate at room temperature for 3 hours for color development.

6) The sample absorbance was read at 530 nm as described previously. This value was compared to a standard curve of sodium alginate from *Macrocystis pyrifera* to determine bacterial alginate concentrations.

4. Acetyl Quantitation

The percent acetylation was determined by the method described by McComb and McCready (80). A standard curve for percent acetylation was prepared with glucose pentaacetate (Sigma Chemical Co., St. Louis, MO). Prior to assay, all samples were desalted by dialysis for 48 hours against deionized water at room temperature. The protocol was as follows:

1) One volume of 9.4% (w/v) sodium hydroxide was added to one volume of 3.75% (w/v) hydroxylamine solution.

2) To 2 ml of the above mixture, 0.5 ml of the sample solution was added with agitation.

3) After 5 minutes, 0.5 ml of acid methanol was added with agitation, then 1.3 ml of the ferric perchlorate solution was added.
4) After 5 minutes, the precipitated hydroxamic acid and ferric complex was removed by microcentrifugation for 3 minutes in a Sorval Microspin 24s Microcentrifuge (Du Pont Co., Wilmington, DE).

5) Color intensity was determined by measuring absorbance at 520 nm as described above. This value was compared to the standard curve of glucose pentaacetate to determine the percent acetylation.

The reagents for acetyl quantitation were prepared as follows:

1) Acid Methanol Solution

Chilled reagent grade absolute methanol was added to 35.2 ml of chilled 70% perchloric acid to make a 500 ml solution. This solution was used as the acidic methanol solution.

2) Ferric Perchlorate Solution

Ferric chloride (1.93 g) was dissolved in 5 ml of 70% perchloric acid and evaporated almost to dryness. It was then diluted to 100 ml with water for use as the stock ferric perchlorate. Then 8.3 ml of 70% perchloric acid was added to 60 ml of stock ferric perchlorate solution. This solution was cooled in an ice bath and made to 500 ml with chilled reagent grade absolute methanol.

3) Glucose Pentaacetate Standard Solution

Pure crystalline β-D-glucose pentaacetate (108.9 mg) was dissolved by heating with gentle agitation in about 5 ml of ethyl alcohol, and made to 50 ml with deionized water. 2, 4, 5, and 7 ml of this solution were then taken and made to 50 ml with deionized water. These solutions represent 120, 240, 300, and 420 µg/ml of acetyl.
III. Optimization of Bacterial Alginate Production

1. Media Composition:

A. Carbon Source

*P. syringae* ATCC 19304 was tested for its ability to produce an acetylated bacterial alginate when grown on different carbon sources. The carbon sources tested were fructose, glucose, sucrose, glycerol, and gluconic acid. Each separate solution of test carbon source was autoclaved and then aseptically added to the DF broth. The concentration of each stock solution was 20% (w/v or v/v). Each carbon source was tested at a final concentration of 2% (w/v or v/v). After inoculation of DF broth from starter cultures, (3%, v/v), *P. syringae* was incubated for 48 hours with shaking as described previously. At 48 hours, the culture broth was centrifuged to remove bacterial cells, and the cell mass determined as described previously. The cell free broth was analyzed by the phenol-sulfuric assay (total carbohydrate production), the uronic acid assay (alginate production), and the acetyl assay (degree of acetylation).

B. Nitrogen Source

*P. syringae* was tested for its ability to produce acetylated bacterial alginate on different nitrogen sources. The nitrogen sources tested were ammonia, [(NH$_4$)$_2$SO$_4$], nitrate, (KNO$_3$), nitrite, (KNO$_2$), and urea. Each DF broth was made by incorporating only the test compound as a potential nitrogen source for the organism. Each nitrogen source was placed into the medium at initial concentrations of 2 mM, 5 mM, 9 mM, 12 mM, and 15 mM (w/v). Gluconic acid was used as the carbon source at a final concentration
of 2% (w/v). After inoculation, (3%, v/v), from standardized starter cultures, *P. syringae* was incubated for 48 hours with shaking as described previously. At the appropriate time the culture broth was centrifuged to remove bacterial cells, and the cell mass determined as described previously. The broth was analyzed by the uronic acid assay for alginate production.

2. pH and Temperature

The effect of pH on bacterial cell yield and alginate production was investigated in DF broth. The initial pH's were 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, and 7.2. Cell yield and alginate production were measured, as described previously, after incubation at 30°C for 48 hours at 180 rpm in a NBS Model G25-KC rotary shaker (New Brunswick Scientific Co. Inc., Edison, NJ). In each case, phosphate buffer (0.03 M) was used to maintain the pH of the culture broth. Gluconic acid, at a final concentration of 2% (w/v), was the carbon source.

The effect of temperature on bacterial cell yield and alginate production was investigated in DF broth. The temperatures tested were 25°C, 28°C, 29°C, 30°C, 31°C, and 32°C. Both cell yield and alginate production were measured, as described previously. Each culture was grown under the same conditions as the pH cultures.

3. Agar vs. Broth Culture

Cell yields and alginate production were measured in 5 different media, both on agar and in broth culture. All media were made up in deionized water. The media included DF media, nutrient media (Difco Lab. Detroit, MI), media composed of 3 g/L
beef extract (Difco Lab. Detroit, MI), media containing 5 g/L peptone (Difco Lab. Detroit, MI), and media containing a mixture of 3 g/L beef extract and 5 g/L peptone. All media were supplemented with 2% gluconic acid (w/v). The gluconic acid was sterilized separately by autoclaving as a stock solution of 20% (w/v), and added to the culture media aseptically after sterilization. Agar media were prepared with 15 g/L agar. The pH of each test media was between 6.9 and 7.0 prior to sterilization. All inocula were from a standardized 48 hour starter culture of *P. syringae* grown in DF broth at 30°C with shaking as described previously. Liquid cultures were inoculated from the standardized 48 hour starter culture to a final concentration of 2% (v/v). Solid cultures were inoculated with 0.1 ml of the same standardized starter culture per 100 x 15 mm petri dish containing 25 ml of medium. The inoculum was spread using a flame sterilized, bent glass rod. All test cultures were allowed to grow for 48 hours at 30°C. The liquid cultures were incubated with shaking as described previously. At the appropriate time, the cell yield and alginate production of each culture were measured as described previously. Alginate production was measured by the uronic acid assay. Total alginate production on solid media was measured by scraping the bacterial growth from half of a petri dish (allowing 2 measurements per petri dish) and resuspending that growth in 10 ml of deionized water. The cells were then removed by centrifugation and the cell mass determined, as described previously. Alginate production was assayed by the uronic acid assay. The total alginate production for the solid cultures was reported in µg/cm².
IV. Batch Fermentations

Batch fermentations were conducted in 1 liter volumes in a NBS 2.5 liter Bioflo II batch/continuous culture fermenter (New Brunswick Scientific Inc., Edison, NJ) in DF broth supplemented with 2% (w/v) gluconic acid. Temperature was maintained at 30°C, and the pH was maintained at 7.0 by titration with 3 M sodium hydroxide. Air, filtered through a sterile Whatman Hepavent filter (Whatman Inc., Clifton NJ) was supplied through a sparger at a rate of 1 standard liter per minute (SLPM), and agitation was at 100 rpm. Samples were periodically withdrawn aseptically, throughout the bacterial growth cycle. Growth was measured by absorbance at 660 nanometers as described previously. Alginate was measured in the cell free supernatant by the uronic acid assay.

V. Purification of Bacterial Alginate

The bacterial alginates used for characterization studies were obtained from either DF agar or Nutrient agar plates supplemented with 2% (w/v) gluconic acid. Bacterial growth was scraped off the agar plates using a bent glass rod and resuspended in 150 ml of deionized water. The samples were vortexed until evenly suspended and then the bacterial alginate was separated from the cells by centrifugation as described previously. Three volumes of isopropanol were added to one volume of clarified supernatant to precipitate the polysaccharide. This solution was mixed for 15 minutes and the precipitated alginate was removed by winding around a glass rod. The precipitate was then dried in acetone followed by air drying.
Purity was determined by the carbazole assay using sodium alginate from *Macrocystis pyrifera* as the standard, and by wavelength scans between 200 nm and 300 nm in increments of 0.5 nm on a Gilford Response II spectrophotometer (Gilford Instrument Lab., Oberlin, OH). Absence of detectable peaks at 260 nm and 280 nm indicated an absence of nucleic acid and protein respectively. A large peak at 230 nm indicated the presence of large amounts of carbohydrate. This carbohydrate was determined to be alginate by the carbazole assay. The purity of the bacterial alginate produced from *P. syringae* ATCC 19304 was more than 98% (w/v).

VI. Deacetylation of Bacterial Alginate

The alginates from *P. syringae* ATCC 19304 were deacetylated for comparison with seaweed alginate and acetylated bacterial alginate. The comparisons were to determine the effects of acetylation on the solution and gelling properties of the polymer. Purified, acetylated bacterial alginate was dissolved in deionized water at a concentration of 1 mg/ml. Three volumes of this solution were mixed with one volume of 1 N sodium hydroxide solution. After incubation for 20 minutes at room temperature, with gentle agitation, one volume of 1 N hydrochloric acid was added to neutralize the solution (final pH was about 7.0) and stop the reaction. The deacetylated bacterial alginate was extensively dialyzed against deionized water. The effectiveness of the process was determined from concentrations of acetyl groups in the preparation.
VII. Alginate Size and Quantity Determinations

Molecular weights were determined by gel permeation chromatography (GPC) from alginate solutions [100 µg/ml (w/v)] in deionized water. Alginate sizes and polydispersity indices were determined by measurement of multiangle light scattering intensities using a DAWN-Photometer (Wyatt Technology, Santa Barbara, CA). The DAWN GPC detector measures the scattering intensities of a sample at 15 different angles and transmits the data to a computer for digital conversion and subsequent processing under control of the ASTRA™ (or ASTRA 202) software (Wyatt Technology, Santa Barbara, CA). Acetone and cyclohexane were used for instrument calibration. Concentrations were obtained from a Waters Model 410 Differential Refractometer (Millipore Corp., Milford, MA). Alginate sizes were based on the GPC calculation and the following external standards: T10, T40, and T500 (Pharmacia Co., Uppsala, Sweden) dextrans. Sample injection volumes were 100 µl and the GPC column was an Ultrahydrogel Linear column (Waters, Millipore Corp., Milford, MA). The running buffer was 0.1 M NaNO₃, and the temperature was 45°C.

VIII. Sugar Sensitivity to Acid
1. Thin Layer Chromatography

Standards of D-mannose and L-gulose (Sigma Chemical Co., St. Louis, MO) were prepared at a concentration of 6.66 mg/ml (w/v) in deionized water. One ml of acid (1 N HCl or 1 N H₂SO₄) and 1 ml of standard sugar solution were mixed and the solution heated at 100°C for 0.5, 1, 2, 3, and 4 hours. Each solution was then
neutralized with 1 ml of 1 N NaOH giving a final sugar concentration of 1.67 mg/ml (w/v) and a final pH of between 6.0 and 7.0.

Thin layer chromatography was performed on the hydrolysates on 0.25 mm plates coated with silica gel 150A (Whatman Co., Maidstone, England), or kieselgel 60 F254 (E. Merck Co., Darmstadt, Germany). The hydrolyzed sugar solutions were spotted at a concentration of 50 μg sugar/spot. The running solvent was n-propanol and water in a ratio of 85:15 (v/v). The plates were developed by spraying with 20% (v/v) H2SO4 in methanol and charring at 100°C for 15-20 minutes.

2. Ion Chromatography

Standards of D-mannose and L-gulose (Sigma Chemical Co., St. Louis, MO) were prepared at a concentration of 1 mg/ml (w/v) in deionized water. One ml of sugar solution was mixed with 1 ml of acid (1 N HCl or 1 N H2SO4). Each solution was hydrolyzed at 100°C for 0.5, 1, 2, 3, and 4 hours. After hydrolysis each solution was neutralized with 1 ml of 1 N NaOH giving a final sugar concentration of 0.33 mg/ml (w/v) and a final pH between 6.0 and 7.0.

Quantitative determinations of the acid sensitivities of each sugar were made with ion chromatography. Ion chromatography was performed on each hydrolyzed standard, using a Dionex ion chromatography system (Dionex Corp., Sunnyvale, CA) fitted with a Carbopac PA1 column (4 x 250 mm). A 100 mM solution of NaOH was used as eluent and pumped at 0.5 ml/minute by a gradient pump. A 50 μl sample was injected and the signal was detected by a pulse amperometric detector. Integration was accomplished by a
Dionex 4400 integrator. The relative peak areas were used to quantitate the percent degradation of each sugar under the experimental conditions employed.

IX. Identification of the Acid Hydrolysis Product of L-Gulose

1. Thin Layer Chromatography

Thin layer chromatography was used to help identify the acid hydrolysis product of L-gulose. L-Gulose (Sigma Chemical Co., St. Louis, MO) was acid hydrolyzed and separated on a TLC plate as described previously. At the end of a run, the TLC plate was air dried. The silica gel in the region corresponding to the Rf value of the spot of interest was scraped from the plate and eluted in deionized water for 10 minutes. The solution was microcentrifuged in a Sorval Microspin 24S microcentrifuge (Du Pont Co., Wilmington, DE) for three minutes to pellet the silica gel. The supernatant was freeze dried in a Flexi-Dry freeze dry apparatus (FTS Systems, Stone Ridge, NY).

The freeze dried sample was resuspended in 0.2 ml of deionized water and again spotted on a 0.25 mm TLC plate coated with kieselgel 60 F254 (E. Merck Co., Darmstadt, Germany) as described previously. The sample was run in n-propanol and water in a ratio of 85:15 (v/v) with samples of 1,6 anhydro β-D-mannopyranose, and 1,6 anhydro-β-D-glucopyranose (Sigma Chemical Co., St. Louis, MO) and the migration of the unknown compared to the known standards.
2. Stability of 1,6 Anhydro β-L-Gulopyranose

The stability of 1,6 anhydro β-L-gulopyranose was measured by molecular modeling on SYBYL molecular modeling software version 6.2 (Tripos Assoc. Inc., St. Louis, MO). The molecule was drawn using the above software and then minimized to its lowest energy level at pH 7.0 with a dielectric constant of 78.8 (equivalent of water). Once the molecule was at its lowest energy levels for the above conditions, it was solvated in water by computer and heated to 400°K for a total of 100,000 femtoseconds (10^{-11} seconds). This allowed the solvent to attain equilibrium. A distance dependent dielectric constant was used to avoid the conditions of a vacuum. Energy measurements were made at 250 femtosecond intervals.

X. Alginate Reductions

Alginites were chemically reduced prior to acid hydrolysis of the polymers. The method used for the reduction of the uronic acids in alginites to the corresponding neutral sugars was that of Taylor et al (140). An aqueous solution of alginate containing 100 microequivalents of carboxylic acid in 10 ml of deionized water was adjusted to pH 4.75 with 0.1 M NaOH. One millimole of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to the alginate solution to convert the uronides to esters. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 M HCl. The reaction was allowed to continue until hydrogen ion uptake ceased (45-60 minutes). Then, 25 ml of a 3 M NaBH4 solution was added dropwise over a 1 hour period to reduce the uronides to the more readily hydrolyzable neutral polymers. The pH was maintained at
7.0 by titration with 4 M HCl. 1-Propanol was added dropwise, as necessary to minimize foaming. The reaction mixture was then made slightly acidic to destroy any remaining sodium borohydride, and the solution was dialyzed exhaustively against deionized water. Each reduced alginate sample was then concentrated in a Buchi Model R110 rotary evaporator (Buchi Lab., Flawil, Switzerland) and precipitated by addition of three volumes of isopropanol and dried by washing in acetone.

XI. Alginate Compositions

Composition measurements of the alginites from *Macrocystis pyrifera* (Sigma Chemical Co., St. Louis, MO), and *P. syringae* ATCC 19304 were made by ion chromatography of the acid hydrolyzed, reduced polymers. Each alginate sample was prepared in the same manner as the D-mannose, and L-gulose sugar standards for ion chromatography described previously. The reduced samples were mixed at a concentration of 1 mg/ml and 1 ml of solution mixed with 1 ml of acid (1 N HCl or 1 N H2SO4). The samples were then hydrolyzed at 100°C for 0.5, 1, 2, 3, and 4 hours. After hydrolysis, 50 μl of each sample was injected into the Dionex ion chromatography system described above. The resulting peak areas were then correlated and extrapolated back to time zero to determine the percentage of mannose and gulose present in the reduced polymer. This composition was then directly correlated to the composition of both *Macrocystis pyrifera* and *P. syringae* ATCC 19304 alginites.
XII. Properties of Bacterial Alginates and Effects of Acetylation

1. Viscosity

The viscosities of *Macrocystis pyrifera* alginate and acetylated and deacetylated *P. syringae* ATCC 19304 alginate were measured as a function of temperature, concentration, and degree of acetylation. In each case, viscosities were determined by the method of Allison and Matthews (1) using a simple U-shaped Ostwald capillary viscometer designed for small volumes. The time taken for a sample to fall a fixed distance under gravity (N), divided by the time taken by water to fall that same distance (N₀) was expressed as a measure of comparative viscosity (Visc.\text{com} = N/N₀).

The effect of temperature on alginate solution viscosity was measured at alginate concentrations of 400 µg/ml (w/v) over a temperature range of 30°C to 85°C. The effects of alginate concentration on viscosity was measured at 50°C at concentrations ranging from 50 µg/ml (w/v) to 1000 µg/ml (w/v). The effect of acetylation on viscosity was measured at 50°C at alginate concentrations of 50 µg/ml (w/v), 100 µg/ml (w/v), and 200 µg/ml (w/v) and the values averaged. Deacetylation of bacterial alginate was as described previously with minor variations. A 400 µg/ml sample of highly acetylated bacterial alginate was deacetylated to various degrees by varying the concentration of NaOH used in the reaction and by altering the reaction time. Sodium hydroxide concentrations ranged from 0.25 M-1.0 M while reaction times were between 5 minutes and 20 minutes. Acetylated seaweed alginate was obtained from Jin W. Lee (72) and partially deacetylated as described previously.
2. Water Holding Capacity

Water holding capacities of alginate gels were measured by determining the amount of water lost from the gels upon drying. Alginate gel beads were made from a 0.6% (w/v) solution of seaweed, acetylated or deacetylated bacterial alginate. Using a 5 ml pipet tip, 4 ml of the alginate solution was dripped slowly into 30 ml of CaCl$_2$, FeCl$_3$, or PbCl$_2$ solution. Each metal solution was tested at concentrations of 0.05 M, 0.1 M, 0.25 M, or 0.5 M. The beads were allowed to form in each metal solution for 15 minutes after which they were washed thoroughly in deionized water (5 minutes). After air drying for 5 minutes, 10 beads from each sample were placed into predried, tared aluminum weigh dishes and weighed. The weighing dishes were then placed in a drying oven at 100°C and the sample dried until a constant weight was reached. The dishes were then reweighed and the water holding capacity of each bead calculated as g water/ g dry alginate gel.

3. Surface Tension

The relative surface tensions of beads were measured using bead diameter, the smaller the bead diameter the higher the surface tension on the bead. Gel beads were made as described previously in 0.5 M CaCl$_2$. Upon formation, 1/3 of each bead sample was washed in deionized water for 5 minutes and allowed to dry for 5 minutes prior to diameter measurement with a dial caliper (L. Starrette Co., Athol, MA). The second portion of each sample was incubated at 4°C for 24 hours in deionized water prior to measurement, and the final third of each sample was incubated
at 4°C for 24 hours in the 0.5 M CaCl₂ solution prior to measurement. Water holding capacity was directly related to the relative surface tension on each bead. A lower surface tension on the bead resulted in a larger bead diameter and higher water holding capacity of the gel.

4. Precipitation by Metal Ions

The precipitation of seaweed alginate and acetylated and deacetylated bacterial alginate by metal ions was compared. Purified alginates were dissolved in deionized water at a concentration of 400 μg/ml (w/v). Metal salts were dissolved in deionized water to prepare for the solutions with concentrations of 0 to 25 or 100 mM. The metal ions tested were: Cs⁺, Rb⁺, Mg²⁺, Ca²⁺, Sr²⁺, Mn²⁺, Fe³⁺, Co²⁺, Cu²⁺, Zn²⁺, Pb²⁺, and U⁶⁺. All metal salts were obtained from Sigma Chemical Co., St. Louis, MO, except for uranyl acetate (Eastman Kodak Co., Rochester, NY). Four volumes of seaweed alginate solution or acetylated or deacetylated bacterial alginate solution were mixed with one volume of each metal solution, respectively. The mixtures were incubated for 12 hours at room temperature and centrifuged (17,000 x g for 30 minutes) in a Sorval Superspeed Model RC-5B centrifuge (Du Pont Co., Wilmington, DE). The supernatants were separated and the concentration of alginate remaining in each supernatant was measured by the uronic acid assay. The amounts of alginate separated as a gel were calculated by difference and those values were used to determine the relative precipitation of the alginate solutions by the metal ions.
RESULTS

I. Production of Bacterial Alginate
1. Media Compositions and Conditions

In broth culture, alginate production by *P. syringae* ATCC 19304 followed the growth curve of the organism (Fig. 9). Maximum cell mass (dry weight) and alginate yields were obtained 48 hours post inoculation. The type of carbon source used affected the alginate yield of *P. syringae* ATCC 19304, as well as the degree of acetylation within the polymer (Table 5). *P. syringae* grew well on glucose, sucrose, glycerol, and gluconic acid, but did not grow on fructose. Sucrose grown cells yielded an EPS, only 77% percent of which was uronic acid. Gluconic acid grown cells yielded the most alginate (approximately 200 µg/mg cell dry weight), with the highest degree of acetylation (approximately 100%). Gluconic acid was the carbon source of choice due to the increased alginate yield of gluconic acid grown *P. syringae*.

*P. syringae* ATCC 19304 utilized ammonia as a nitrogen source, but was unable to utilize nitrate, nitrite, or urea. There was an inverse correlation between alginate yield and the initial concentration of ammonia in the media. As initial ammonium concentration increased from 2 mM to 15 mM the cell mass increased 2.4 fold, from 0.66 mg cell dry weight/ml to 1.58 mg cell dry weight/ml. At the same time, alginate yield decreased 2.4 fold, from 1200 µg/ml to 500 µg/ml (Fig. 10). This indicated that at higher initial ammonium concentrations the carbon normally destined for alginate production was used by the cells for cell
Figure 9. The relationship between cell mass (♦), and alginate (uronic acid) accumulation (□) by *P. syringae* ATCC 19304 with time in shake flask culture.
Table 5. Effects of Carbon Source on Alginate Yield from *P. syringae* ATCC 19304

<table>
<thead>
<tr>
<th>Carbon Source&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Cell Yield (mg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Yield Total EPS (µg/ml)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>% Alginate&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Yield Alginate (µg/mg cell)&lt;sup&gt;f&lt;/sup&gt;</th>
<th>% Acetylation&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.25</td>
<td>175</td>
<td>100</td>
<td>140</td>
<td>107 (± 23)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.23</td>
<td>211</td>
<td>77</td>
<td>132</td>
<td>4 (± 1)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.96</td>
<td>134</td>
<td>100</td>
<td>140</td>
<td>84 (± 17)</td>
</tr>
<tr>
<td>Gluconic acid</td>
<td>1.04</td>
<td>213</td>
<td>100</td>
<td>205</td>
<td>97 (± 30)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cultured in DF broth supplemented with 2% carbon source, and grown for 48 hours at 30°C with shaking at 180 rpm.

<sup>b</sup> Fructose was also tested but there was no growth.

<sup>c</sup> Cell yield was measured as mg cell dry weight/ml broth.

<sup>d</sup> Yield of total EPS was measured as µg EPS/ml broth.

<sup>e</sup> The percent of total EPS that was alginate.

<sup>f</sup> Yield of alginate was measured as µg alginate/mg cell dry weight.

<sup>g</sup> The molar ratio (%) of acetyl to uronic acid.
Figure 10. The effect of the initial ammonium concentration on cell mass (♦), and total alginate (uronic acid) accumulation (□), by *P. syringae* ATCC 19304 at 48 hours.
division. As initial ammonium concentrations increased from 2 to 9 mM, the specific yield of alginate decreased from 2100 μg/mg cell dry weight to 500 μg/mg cell dry weight. Initial ammonium concentrations above 9 mM resulted in a consistently lower specific alginate yield (Fig. 11).

Initial pH of the DF broth and temperature both affected the cell mass and alginate yield of *P. syringae* ATCC 19304. Maximum growth of *P. syringae* ATCC 19304 occurred in DF broth with initial pH's between 6.4 and 7.2. Alginate yield was greatest in DF broth with initial pH's between 6.8 and 7.0. Above pH 7.0 the alginate yield declined (Fig. 12). The optimum temperature for alginate production was 30°C. There was little change in cell mass with temperatures between 25°C to 32°C. Alginate production was extremely temperature dependent, with a sharp optimum at 30°C (Fig. 13).

2. Agar vs. Broth Culture

The ability of *P. syringae* ATCC 19304 to grow and produce alginate was compared on agar and in broth culture. Upon successive transfers in broth culture, alginate yield decreased dramatically. Initially, alginate yield reached a maximum of 550 μg alginate/ mg cell dry weight at 27 hours. After one transfer back into broth culture, the yield decreased to only 50 μg alginate/ mg cell dry weight. After a second transfer, there was less than 10 μg alginate/ mg cell dry weight. On agar media alginate production by *P. syringae* ATCC 19304 was consistent upon transfer from one
Figure 11. The effect of the initial ammonium concentration on specific yields of alginate (uronic acid) by P. syringae ATCC 19304 at 48 hours.
Figure 12. The effect of initial pH on cell mass (♦), and alginate (uronic acid) accumulation (□), by *P. syringae* ATCC 19304 at 48 hours.
Figure 13. The effect of temperature on cell mass (♦), and alginate (uronic acid) accumulation (○), by *P. syringae* ATCC 19304 at 48 hours.
culture to another. The organism averaged approximately 1870 µg alginate/mg cell dry weight over 5 successive transfers (Table 6).

Total and specific alginate yields were determined in various media. *P. syringae* was tested in broth and on an agar surface. DF salts, nutrient media, peptone media, and beef extract media were tested for their ability to support bacterial growth and promote alginate production by *P. syringae* ATCC 19304 (Table 6). In both broth and on agar, nutrient media supported the highest total alginate yield in a 48 hour period. The specific alginate yield was 1.5 to 2 fold greater in beef extract media than in any of the other media tested. In all cases, growth on agar media increased the specific yield of bacterial alginate by about 3 fold. In DF broth the specific yield was approximately 500 µg/mg cell dry weight and was constant from 24 hours to 96 hours. This indicated that the total yield of alginate increased at a constant rate over this period. On agar media specific alginate yields reached approximately 1850 µg/mg cell dry weight between 24 and 72 hours and then began to decrease after 72 hours.

Ferric ion affected alginate production. By increasing the initial concentrations of ferric ion from 0 mM to 1 mM, the specific alginate yield by *P. syringae* ATCC 19304 decreased by 87% (Fig. 14). This indicated that iron starvation may have a role as a trigger for alginate production.
Table 6. Alginate Yield by *P. syringae* ATCC 19304 on Different Media.

<table>
<thead>
<tr>
<th>Mediaa,b</th>
<th>Yield in Liquid Media</th>
<th>Yield on Solid Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific (μg/mg cell)c</td>
<td>Total (μg/ml)d</td>
</tr>
<tr>
<td>DF Salts</td>
<td>550</td>
<td>480</td>
</tr>
<tr>
<td>Beef Extract, Peptone</td>
<td>580</td>
<td>620</td>
</tr>
<tr>
<td>Nutrient</td>
<td>590</td>
<td>640</td>
</tr>
<tr>
<td>Peptone</td>
<td>680</td>
<td>540</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>1320</td>
<td>450</td>
</tr>
</tbody>
</table>

*a* Cultures were grown at 30°C for 48 hours. Liquid cultures were shaken at 180 rpm. All cultures were supplemented with 2% (w/v) gluconic acid.

*b* Beef extract was used at a 3 g/L concentration and Peptone was used at a 5 g/L concentration. Beef extract, Peptone, and Nutrient media were from Difco Labs., Detroit, MI.

*c* Specific production was measured as μg alginate/mg cell dry weight.

*d* Total production in liquid culture was measured as μg alginate/ml broth.

*e* Total production on solid culture was measured as μg alginate/cm² of agar surface.
Figure 14. Effect of initial ferric ion (Fe³⁺) concentration on the specific yields of alginate (uronic acid) by *P. syringae* ATCC 19304 at 48 hours.
II. Characterization of Bacterial Alginate

1. Recovery

Alginate produced by *P. syringae* ATCC 19304 was recovered from the surface of DF agar plates supplemented with 2% (w/v) gluconic acid. Several different alcohols were tested for their ability to precipitate bacterial alginate. Recoveries were compared. All of the alcohols tested: methanol, ethanol, n-propanol, and iso-propanol, precipitated 100% of the alginate at a final alcohol concentration of 50% (v/v). In order to determine the effect of acetylation on alcohol precipitation, equal amounts of acetylated and deacetylated alginate were precipitated with various concentrations of iso-propanol. Acetylation did not affect product recovery.

2. Molecular Weight

The weight average molecular weight \( (M_W) \) and number average molecular weight \( (M_n) \) were determined for both acetylated and deacetylated bacterial alginates by gel permeation chromatography (GPC). The \( M_W \) and the \( M_n \) of the seaweed alginate were approximately 65% smaller than the native bacterial alginate. Deacetylation with sodium hydroxide did not alter the molecular weights appreciably. Upon deacetylation, the \( M_W \) of the bacterial alginate decreased by 6%, and the \( M_n \) decreased by 11%. In each case, the polydispersity was approximately 3.00. This indicated that there was a wide range of molecular sizes in each alginate sample (Table 7).
<table>
<thead>
<tr>
<th>Alginate sample</th>
<th>$M_n^a$ (x 10^4)</th>
<th>$M_w^b$ (x 10^4)</th>
<th>$M_w/M_n^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Macrocystis</em></td>
<td>1.4</td>
<td>4.7</td>
<td>3.36</td>
</tr>
<tr>
<td></td>
<td>($\pm$ 1.5 x 10^3)</td>
<td>($\pm$ 2.6 x 10^3)</td>
<td></td>
</tr>
<tr>
<td>Acetylated</td>
<td>4.3</td>
<td>12.7</td>
<td>2.95</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>($\pm$ 8.2 x 10^3)</td>
<td>($\pm$ 7.2 x 10^3)</td>
<td></td>
</tr>
<tr>
<td>Deacetylated</td>
<td>3.8</td>
<td>11.9</td>
<td>3.13</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>($\pm$ 7.7 x 10^3)</td>
<td>($\pm$ 8.9 x 10^3)</td>
<td></td>
</tr>
</tbody>
</table>

*a* Number average molecular weight.

*b* Weight average molecular weight.

*c* Polydispersity
3. Composition

The determination of alginate composition required the reduction of the uronic acids to their corresponding neutral sugars prior to acid hydrolysis of the polymer. This reduction facilitated the acid hydrolysis of the glycosidic bonds by conversion of the acid resistant glycosyluronic acid bonds to the more acid labile glycosyl bonds. The acid sensitivities of D-mannose and L-gulose (Sigma Chemical Co., St. Louis, MO) were studied using thin layer chromatography (TLC) and ion chromatography. TLC indicated that D-mannose and L-gulose have marked differences in their acid sensitivity. Acid hydrolyzed (HCl) D-mannose resulted in only one spot on TLC (Rf=0.40). The intensity of this spot remained constant between 0 and 4 hours hydrolysis time (Fig. 15). L-gulose produced a second spot (Rf=0.56) after more than 1 hour hydrolysis. The intensity of this spot increased with hydrolysis time. At the same time, the intensity of the gulose spot (Rf=0.37) decreased proportionally (Fig. 16). Both D-mannose and L-gulose reacted similarly in HCl and H2SO4 (Table 8).

The relative acid sensitivity of each sugar was measured by Dionex ion chromatography. D-Mannose was acid stable. After 4 hours of HCl or H2SO4 hydrolysis, 98% of the original sugar was recovered. L-gulose was relatively stable for 1 hour, after which it began to rapidly breakdown. After 4 hours hydrolysis, only 22% of the original sugar was recovered (Fig. 17).

The acid breakdown product of L-gulose was not definitively identified. TLC of this compound showed a migration (Rf=0.58) equal to 1,6 anhydro β-D-mannopyranose (Rf=0.58), and very close to 1,6
Figure 15. Thin layer chromatograph of HCl hydrolyzed D-mannose. Lane 1 = unhydrolyzed D-mannose in H₂O, Lane 2 = unhydrolyzed D-mannose in HCl, Lanes 3-7 = hydrolyzed D-mannose in HCl for 0.5, 1, 2, 3, and 4 hours respectively at 100°C.
Figure 16. Thin layer chromatograph of HCl hydrolyzed L-gulose. Lane 1 = unhydrolyzed L-gulose in H₂O, Lane 2 = unhydrolyzed L-gulose in HCl, Lanes 3-7 = hydrolyzed L-gulose in HCl for 0.5, 1, 2, 3, and 4 hours respectively at 100°C.
Table 8. Thin Layer Chromatography (Rf values)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spot A</th>
<th>Spot B</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-mannose in HCl</td>
<td>0.40 (± 0.03)</td>
<td>N/A\textsuperscript{c}</td>
</tr>
<tr>
<td>L-gulose in HCl</td>
<td>0.37 (± 0.03)</td>
<td>0.56 (± 0.03)</td>
</tr>
<tr>
<td>D-mannose in H\textsubscript{2}SO\textsubscript{4}</td>
<td>0.37 (± 0.04)</td>
<td>N/A</td>
</tr>
<tr>
<td>L-gulose in H\textsubscript{2}SO\textsubscript{4}</td>
<td>0.34 (± 0.03)</td>
<td>0.50 (± 0.04)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Each Rf value was calculated by the distance of the spot from the origin divided by the total distance traveled by the running solvent.
\textsuperscript{b} Each sample was hydrolyzed in an equal volume of 1 N acid and hydrolyzed for 4 hours at 100°C.
\textsuperscript{c} N/A = Not Applicable
Figure 17. The stability of monomeric D-mannose and L-gulose in HCl and H2SO4 under hydrolysis conditions at 100°C, as determined by ion chromatography. L-gulose in HCl (□), D-mannose in HCl (♦), L-gulose in H2SO4 (■), D-mannose in H2SO4 (○).
anhydro β-D-glucopyranose (Rf=.60, Fig. 18). This indicated that the molecule may be a 1,6 anhydride. Molecular modeling of 1,6 anhydro β-L-gulopyranose (Fig. 19) showed that this molecule has a low total energy (approximately -1400 kcal/mol) and thus is stable (Table 9). This lent emphasis to the possibility of 1,6 anhydride formation upon acid hydrolysis of L-gulose.

Destruction of the reduced sugars in the alginates on acid hydrolysis paralleled the results seen with the D-mannose and L-gulose monomers. After correction for gulose destruction by extrapolation back to time 0, a composition of 60% mannuronic acid and 40% guluronic acid was obtained for Macrocystis alginate, and 82% mannuronic acid and 18% guluronic acid for P. syringae ATCC 19304 alginate (Fig. 20). This correlated well to the reported composition for alginate produced by P. aeruginosa of 80% mannuronic acid and 20% guluronic acid, and for Macrocystis alginate of 60% mannuronic acid and 40% guluronic acid (150).

III. Functional Properties

1. Viscosity

The viscosity of a solution depends on the molecular weight and the rigidity of the solute, as well as environmental factors, especially temperature. The concentration, percent acetylation, and temperature all contributed to variations in the flow properties of alginates. At an alginate concentration of 0.1% (w/v) the viscosity of acetylated bacterial alginate was 2.7 fold greater than seaweed alginate. Deacetylated bacterial alginate was 2.0 fold more viscous than seaweed alginate. As alginate concentrations increased from
Figure 18. Thin layer chromatograph of the acid hydrolyzed product of L-gulose. Lane 1 = unhydrolyzed L-gulose in H2O, Lane 2 = standard 1,6 anhydro β-D-mannopyranose, Lane 3 = standard 1,6 anhydro β-D-glucopyranose, Lane 4 = acid hydrolysis product of L-gulose.
Figure 19. Computer derived image of 1,6 anhydro β-L-gulopyranose. The image is depicted in cross stereo view.
Table 9. The Energetics and Stability of 1,6 Anhydro β-L-Gulopyranose

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Time (Femtosec) b</th>
<th>Potential Energy c</th>
<th>Kinetic Energy</th>
<th>Total Energy</th>
<th>Temperature (°K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>250</td>
<td>-2307.73</td>
<td>861.11</td>
<td>-1446.62</td>
<td>387.77</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>-2432.09</td>
<td>886.92</td>
<td>-1545.17</td>
<td>399.39</td>
</tr>
<tr>
<td>4</td>
<td>750</td>
<td>-2475.61</td>
<td>855.84</td>
<td>-1619.77</td>
<td>385.40</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>-2512.66</td>
<td>878.87</td>
<td>-1633.79</td>
<td>395.77</td>
</tr>
<tr>
<td>7</td>
<td>1500</td>
<td>-2542.10</td>
<td>870.16</td>
<td>-1671.94</td>
<td>391.85</td>
</tr>
<tr>
<td>14</td>
<td>3250</td>
<td>-2554.33</td>
<td>865.87</td>
<td>-1688.46</td>
<td>389.92</td>
</tr>
<tr>
<td>31</td>
<td>7500</td>
<td>-2512.04</td>
<td>820.92</td>
<td>-1691.11</td>
<td>369.67</td>
</tr>
<tr>
<td>33</td>
<td>8000</td>
<td>-2569.74</td>
<td>875.05</td>
<td>-1694.70</td>
<td>394.05</td>
</tr>
</tbody>
</table>

a Values were obtained using SYBYL molecular modeling software (Tripos Assoc. Inc., St. Louis, MO).
b 1 femtosecond equals 10^{-15} seconds.
c All energy values are measured in kcal/mol.
Figure 20. Gulose recovered, expressed as a percent of the total sugar present in the HCl hydrolyzed reduced alginates, from *Macrocystis pyrifera* (□), and *P. syringae* ATCC 19304 (●), as determined by ion chromatography.
0 μg/ml to 1000 μg/ml (w/v), the comparative viscosity (N/N₀) of each solution increased non linearly. In each case, this increase exhibited non-newtonian flow dynamics (Fig. 21). Seaweed alginate deviated least from newtonian flow. Acetylated bacterial alginate deviated most. The difference in the viscosities of acetylated and deacetylated alginates indicated that acetylation linearly affected the flow dynamics of alginate solutions. An average of the comparative viscosity of seaweed alginate measured at concentrations of 50, 100, and 200 μg/ml (w/v) increased approximately 8% per a 50% increase in acetylation. An average of the comparative viscosity of bacterial alginate measured at the same concentrations, increased 16% per a 50% increase in acetylation (Fig. 22). This difference was probably due to the increased average molecular weight of the bacterial alginate polymer, and its more extended structure due to high amounts of D-mannuronic acid.

Temperature also affected the viscosity of alginate solutions. Each alginate solution responded similarly to the affects of temperature. The viscosity of each alginate solution at a concentration of 400 μg/ml (w/v) was constant from 30°C to approximately 52°C. At 52°C the viscosities of each alginate solution decreased dramatically. Between 52°C and 85°C the viscosities of the seaweed alginate sample decreased 3.4%, on the average, per °C. The viscosities of the acetylated bacterial and deacetylated bacterial samples decreased 9.2% per °C, and 6.5% per °C respectively. At 85°C the comparative viscosities of each alginate solution were approximately equal (Fig. 23).
Figure 21. Comparative viscosity, \( (N/N_0) \), of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (■), and deacetylated *P. syringae* alginate (♦), as a function of alginate concentration, (w/v).
Figure 22. The effects of acetylation on the comparative viscosity, \((N/N_0)\), of *Macrocystis* alginate (□), and *P. syringae* alginate (♦).
Figure 23. The effects of temperature on the comparative viscosity, \((N/N_0)\), of *Macrocystis* alginate (II), acetylated *P. syringae* alginate (I), and deacetylated *P. syringae* alginate (•).
2. Physical Effects

The affects of acetylation on gelation, water holding capacity, surface tension, and gel porosity were determined. Calcium induced gelation was altered by both acetylation, and calcium concentration. The gels produced from acetylated bacterial alginate held approximately 100 g water/g dry alginate gel compared to the deacetylated bacterial alginate gel which held approximately 32 g water/g dry alginate gel (Table 10). As the concentration of CaCl₂ increased from 0.05 M to 0.50 M, the water holding capacity of each Ca-alginate gel decreased linearly. The water holding capacities of bacterial alginate gels made with 0.50 M CaCl₂ were approximately 54% that of gels made with 0.05 M CaCl₂. In contrast, Ca-alginate gels made with seaweed alginate showed a 41% decrease in water holding capacity over the same calcium ion increase.

The water holding capacities of both Fe-alginate (ferric), and Pb-alginate gels were determined for seaweed alginate and acetylated and deacetylated bacterial alginates. The results were compared to those obtained from Ca-alginate gels. In each case, Fe-alginate gels held less water than their Ca-alginate counterparts, but more than Pb-alginate gels. The water holding capacity of Fe-alginate (0.05 M FeCl₃) gels made with seaweed alginate was approximately 82% that of Ca-alginate gels. Pb-alginate (0.05 M PbCl₂) gels held water at 67% that of Ca-alginate gels (Fig. 24). As the concentration of metal (Fe³⁺ or Pb²⁺) increased, the water holding capacity of the resulting gels decreased. By increasing the metal concentration from 0.05 M to 0.50 M in increments of 0.05 M, the water holding capacity in Fe-alginate gels decreased by an
Table 10. Effects of Calcium Concentration on the Water Holding Capacities\(^a\) of Alginate Gels.

<table>
<thead>
<tr>
<th>Alginate sample</th>
<th>Calcium Concentration(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 M</td>
</tr>
<tr>
<td><em>Macrocystis</em></td>
<td>65 (± 3)</td>
</tr>
<tr>
<td><em>Acetylated</em></td>
<td>100 (± 6)</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>32 (± 3)</td>
</tr>
</tbody>
</table>

\(^a\) Water holding capacities are calculated as g water/g dry alginate gel.

\(^b\) Calcium was derived from CaCl\(_2\) (Sigma Chemical Co., St. Louis, MO).
Figure 24. Percent difference in water holding capacity of *Macrocystis* alginate gels made with ferric iron (□), and lead (♦), as compared to calcium alginate gels.
average of 2.2% per 0.05 M increase, and in Pb-alginate gels by an average of 3.7% per 0.05 M increase. The water holding capacities of Fe-alginate gels and Pb-alginate gels made with acetylated and deacetylated bacterial alginate were similar to those of seaweed alginate. The water holding capacity of Fe-alginate (0.05 M FeCl₃) gels made with acetylated and deacetylated bacterial alginate averaged approximately 79% that of the corresponding bacterial Ca-alginate gels. The water holding capacity of Pb-alginate (0.05 M PbCl₂) gels made under the same conditions averaged only 46% that of the corresponding bacterial Ca-alginate gels (Fig. 25, 26). As in seaweed alginate gels, the water holding capacity for both acetylated and deacetylated bacterial alginate gels decreased as the metal concentration increased. The water holding capacities of Fe-alginate gels made with acetylated and deacetylated bacterial alginate decreased by an average of 2.0% per 0.05 M increase in iron concentration. The water holding capacity of Pb-alginate gels decreased by an average of 3.0% per 0.05 M increase in lead concentration (Fig. 25, 26).

The bead size is a function of the surface tension of the gel that makes up the bead, the smaller the bead the higher the surface tension. Acetylated bacterial alginate forms gel beads in 0.5 M CaCl₂ that averaged 4.04 millimeters in diameter (Table 11). These beads are 37% larger than equivalent beads made with seaweed alginate, and 45% larger than beads made with deacetylated bacterial alginate.

Incubation of the gel beads in deionized water or 0.5 M CaCl₂ at 4°C for 24 hours altered the size of the gel beads. Beads
Figure 25. Percent difference in water holding capacity of acetylated *P. syringae* alginate gels made with ferric iron (□), and lead (♦), as compared to calcium alginate gels.
Figure 26. Percent difference in water holding capacity of deacetylated *P. syringae* alginate gels made with ferric iron (□), and lead (♦), as compared to calcium alginate gels.
Table 11. Effects of Acetylation on Bead Surface Tension$^a$

<table>
<thead>
<tr>
<th>Alginate sample</th>
<th>Bead Diameters (mm.)</th>
<th>Time in Water$^b$</th>
<th>Time in CaCl$_2$$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 minutes</td>
<td>24 hours</td>
</tr>
<tr>
<td><em>Macrocystis</em></td>
<td></td>
<td>2.95 (± 0.05)</td>
<td>2.69 (± 0.04)</td>
</tr>
<tr>
<td>Acetylated</td>
<td></td>
<td>4.04 (± 0.06)</td>
<td>2.82 (± 0.04)</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td></td>
<td>2.79 (± 0.05)</td>
<td>1.96 (± 0.04)</td>
</tr>
</tbody>
</table>

$^a$ The relative surface tension was measured by bead diameter (mm.), the smaller the bead the higher the surface tension on the bead.

$^b$ Water values were measured after 15 minutes in 0.5 M CaCl$_2$ and incubation in water at 4°C for the relevant times.

$^c$ CaCl$_2$ values were measured after incubation in 0.5 M CaCl$_2$ at 4°C for 24 hours.
incubated in deionized water made from acetylated bacterial alginate had diameters averaging 2.82 millimeters, a decrease of 42% from the initial size. These beads were still 5% larger than the seaweed alginate gel beads and 44% larger than the deacetylated bacterial alginate gel beads. This indicated that the relative surface tension of acetylated gels was less than deacetylated alginate gels. Extended incubation (24 hours) of Ca-alginate gel beads in 0.5 M CaCl2 produced different results. The Ca-alginate gel beads made from seaweed alginate were the largest with an average diameter of 2.64 millimeters. These beads were 17% larger than the acetylated alginate beads and 57% larger than the beads made from deacetylated bacterial alginate. This indicated that acetylation decreased the surface tension on the Ca-alginate gel beads, and extended exposure to calcium decreased the size of the beads and increased surface tension on the beads.

The relative porosity of the Ca-alginate and Fe-alginate (ferric) gel beads were determined from the rate of water loss with time (Fig. 27). A large absolute slope indicated a more rapid water loss resulting from larger relative pore sizes. Ca-alginate gels, made from acetylated bacterial alginate, showed the most rapid loss of water (absolute slope= 6.8 x 10^{-2}). The rate of water loss in the gels made from deacetylated bacterial alginate (absolute slope= 5.2 x 10^{-2}) was 24% slower than the acetylated bacterial polymer and 13% faster than seaweed alginate (absolute slope= 4.6 x 10^{-2}). The rates of water loss of Fe-alginate gels were also determined and compared as a percentage of Ca-alginate gels. Both the gels made from seaweed alginate and deacetylated bacterial alginate lost
Figure 27. Rate of water loss of calcium alginate gels made from *Macrocystis* alginate, l slope l = 4.6 x 10^{-2} (□), acetylated *P. syringae* alginate, l slope l = 6.8 x 10^{-2} (♦), and deacetylated *P. syringae* alginate, l slope l = 5.2 x 10^{-2} (▌), as a function of incubation time at 50°C.
water approximately 2 fold faster than their Ca-alginate counterparts, indicating a more open structure. The gels produced by the acetylated bacterial alginate showed a rate of water loss approximately equal to that of Ca-alginate gel beads indicating comparable pore sizes between these gels (Fig. 28).

3. Cation Precipitation by Alginates

Precipitation of acetylated and deacetylated bacterial alginates, measured as gelation, by cations was compared to that of seaweed alginate. Twelve metal ions were screened and then classified into 3 groups, depending on their ability to precipitate acetylated and/or deacetylated bacterial alginate. The groups were: 1) those cations having the ability to precipitate half the available bacterial alginate at a concentration of less than 20 mM metal, (U$^6+$, Cu$^{2+}$, Pb$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, and Fe$^{3+}$), 2) those cations having the ability to precipitate half the available bacterial alginate at a concentration of greater than 20 mM metal, (Zn$^{2+}$, Co$^{2+}$, and Mn$^{2+}$), and 3) those cations unable to precipitate half the available bacterial alginate at a concentration up to 100 mM metal, (Mg$^{2+}$, Cs$^{1+}$, and Rb$^{1+}$).

Of all the cations tested, uranium, copper, lead and ferric ions precipitated both acetylated and deacetylated bacterial alginate at low concentrations. They were able to precipitate more than 90% of the alginate at metal concentrations less than 5 mM. Acetylation did not significantly affect the ability of these ions to precipitate bacterial alginate. Uranium precipitated greater than 90% of deacetylated bacterial alginate at a metal concentration of 1 mM and greater than 90% of the acetylated bacterial alginate at a
Figure 28. Comparison of the water loss of ferric iron alginate gels of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (♦), and deacetylated *P. syringae* alginate (■), compared to calcium alginate gels as a function of incubation time at 50°C.
concentration of 5 mM (Fig. 29). Cupric ion reacted much the same way, precipitating 98% of the deacetylated bacterial alginate at 2.5 mM copper, and greater than 90% of acetylated bacterial alginate at a metal concentration of 5 mM (Fig. 30). Lead induced gelation of bacterial alginates was least affected by acetylation. Greater than 90% of both acetylated and deacetylated bacterial alginates precipitated in the presence of 1 mM lead chloride (Fig. 31). Ferric ions precipitated both acetylated and deacetylated bacterial alginate. Acetylation increased the precipitability of the bacterial polymer by ferric ion. Approximately 90% of the acetylated bacterial polymer precipitated with 1 mM ferric chloride. In contrast, 90% of the deacetylated bacterial alginate precipitated with 2 mM ferric chloride (Fig. 32).

A high affinity of calcium ions for polyguluronate residues is the basis for the current gelling theory (the "egg box model") for seaweed alginates. As expected, almost 100% of the seaweed alginate was precipitated by 5 mM calcium chloride (Fig. 33). Calcium precipitation was less efficient for bacterial alginates, probably due to the absence of extensive polyguluronate blocks. The amount of bacterial alginate precipitated by calcium ions increased as the calcium concentration increased. Approximately 95% of the deacetylated bacterial alginate and 65% of the acetylated bacterial alginate precipitated with 60 mM calcium chloride.

As might be expected from its chemical similarity to calcium, strontium was also an effective precipitant of both acetylated and deacetylated bacterial alginate. As with calcium, strontium showed a greater ability to precipitate deacetylated than acetylated
Figure 29. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (●), and deacetylated *P. syringae* alginate (■) by uranium ions. Relative precipitation is expressed as the % alginate precipitated by U⁶⁺ ions.
Figure 30. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (●), and deacetylated *P. syringae* alginate (■) by copper ions. Relative precipitation is expressed as the % alginate precipitated by Cu$^{2+}$ ions.
Figure 31. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (♦), and deacetylated *P. syringae* alginate (‖) by lead ions. Relative precipitation is expressed as the % alginate precipitated by Pb$^{2+}$ ions.
Figure 32. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (♦), and deacetylated *P. syringae* alginate (●) by ferric ions. Relative precipitation is expressed as the % alginate precipitated by Fe$^{3+}$ ions.
Figure 33. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (●), and deacetylated *P. syringae* alginate (●) by calcium ions. Relative precipitation is expressed as the % alginate precipitated by Ca$^{2+}$ ions.
bacterial alginate. Strontium precipitated 100% of the deacetylated bacterial alginate at a final concentration of 20 mM. Approximately 78% of the acetylated bacterial alginate was precipitated with 60 mM strontium chloride (Fig. 34).

The second set of cations were those that precipitated half the available bacterial alginate at a concentration of greater than 20 mM metal. Zinc ions fell into this category. Zinc precipitated deacetylated bacterial alginate better than its acetylated counterpart. The precipitation of both acetylated and deacetylated bacterial alginate did not begin until zinc concentrations were above 10 mM. The concentration of precipitated bacterial alginate increased as the concentration of zinc increased. Approximately 55% of the acetylated polymer precipitated in 60 mM zinc chloride, and 95% of the deacetylated polymer precipitated at the same metal concentration (Fig. 35). It appears that acetylation decreased the precipitability of alginate by zinc ions.

Manganese and cobalt ions showed a limited affinity for deacetylated bacterial alginate and no affinity for the acetylated polymer. Manganese precipitated 100% of the deacetylated bacterial polymer at a concentration of 75 mM (Fig. 36). Cobalt precipitated approximately 87% of the deacetylated bacterial polymer at that concentration (Fig. 37). Acetylation completely inhibited the ability of manganese and cobalt to precipitate bacterial alginate. Neither acetylated nor deacetylated bacterial alginate precipitated with cesium, rubidium, or magnesium ions (Fig. 38, 39).
Figure 34. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (●), and deacetylated *P. syringae* alginate (■) by strontium ions. Relative precipitation is expressed as the % alginate precipitated by Sr$^{2+}$ ions.
Figure 35. Precipitation of *Macrocystis* alginate (Ⅰ), acetylated *P. syringae* alginate (●), and deacetylated *P. syringae* alginate (Ⅱ) by zinc ions. Relative precipitation is expressed as the % alginate precipitated by Zn$^{2+}$ ions.
Figure 36. Precipitation of *Macrocystis* alginate (Ⅱ), acetylated *P. syringae* alginate (●), and deacetylated *P. syringae* alginate (■) by manganese ions. Relative precipitation is expressed as the % alginate precipitated by Mn$^{2+}$ ions.
Figure 37. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (♦), and deacetylated *P. syringae* alginate (■) by cobalt ions. Relative precipitation is expressed as the % alginate precipitated by Co$^{2+}$ ions.
Figure 38. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (♦), and deacetylated *P. syringae* alginate (‖) by cesium ions. Relative precipitation is expressed as the % alginate precipitated by Cs$^{1+}$ ions.
Figure 39. Precipitation of *Macrocystis* alginate (□), acetylated *P. syringae* alginate (♦), and deacetylated *P. syringae* alginate (■) by magnesium ions. Relative precipitation is expressed as the % alginate precipitated by Mg$^{2+}$ ions.
The relative ability of these metal ions to precipitate alginates were compared to the P<sub>1/2</sub> values for seaweed alginate and recorded as the fold difference from those values (Table 12). The P<sub>1/2</sub> value is defined as the concentration of metal ions (mM) required to precipitate 50% of a polymer from a 400 μg/ml (w/v) solution. The relative order of precipitation of acetylated and deacetylated bacterial alginate by these ions, as determined by P<sub>1/2</sub>, were as follows:

Acetylated bacterial alginate: Pb<sup>2+</sup>=Fe<sup>3+</sup>=U<sup>6+</sup> > Cu<sup>2+</sup> > Sr<sup>2+</sup> > Ca<sup>2+</sup> > Zn<sup>2+</sup> > Mg<sup>2+</sup>=Co<sup>2+</sup>=Cs<sup>1+</sup>=Mn<sup>2+</sup>=Rb<sup>1+</sup>

Deacetylated bacterial alginate: Pb<sup>2+</sup>=Fe<sup>3+</sup>=U<sup>6+</sup> > Cu<sup>2+</sup> > Sr<sup>2+</sup> > Ca<sup>2+</sup> > Zn<sup>2+</sup> > Mn<sup>2+</sup> > Co<sup>2+</sup> > Mg<sup>2+</sup>=Cs<sup>1+</sup>=Rb<sup>1+</sup>
Table 12. The Precipitation of *Macrocystis* Alginate and Acetylated and Deacetylated *P. syringae* Alginate by Metal Ions

<table>
<thead>
<tr>
<th>Ions</th>
<th>Periodic Group</th>
<th><em>Macrocystis</em> Alginate (P1/2)</th>
<th>Acetylated <em>P. syringae</em> Alginate</th>
<th>Deacetylated <em>P. syringae</em> Alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cs⁺⁺</td>
<td>I A</td>
<td>No affinity</td>
<td>No affinity</td>
<td>No affinity</td>
</tr>
<tr>
<td>Rb⁺⁺</td>
<td></td>
<td></td>
<td>No affinity</td>
<td>No affinity</td>
</tr>
<tr>
<td>Mg⁺⁺</td>
<td>II A</td>
<td>No affinity</td>
<td>3.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td></td>
<td></td>
<td>No affinity</td>
<td>3.4</td>
</tr>
<tr>
<td>Sr⁺⁺</td>
<td></td>
<td></td>
<td>No affinity</td>
<td>3.7</td>
</tr>
<tr>
<td>Mn⁺⁺</td>
<td>VII A</td>
<td>30.0</td>
<td>No affinity</td>
<td>0</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>VIII A</td>
<td>1.8</td>
<td>-0.7</td>
<td>-0.6</td>
</tr>
<tr>
<td>Co²⁺</td>
<td></td>
<td>9.6</td>
<td>No affinity</td>
<td>3.7</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>I B</td>
<td>0.5</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>II B</td>
<td>7.0</td>
<td>4.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>IV B</td>
<td>0.5</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>U⁶⁺</td>
<td>Actinide metal</td>
<td>0.9</td>
<td>-0.2</td>
<td>-0.4</td>
</tr>
</tbody>
</table>

a  
P1/2 is the concentration of metal ions (mM) required to precipitate 50% (w/v) alginate from 400 μg/ml (w/v) alginate solutions.

b  
Values expressed are the fold difference from P1/2 of *Macrocystis* alginate (positive values = less ability to precipitate, Negative values = greater ability to precipitate, zero values = equal ability to precipitate).

c  
"No affinity" signifies that the ion did not precipitate 50% of the alginate sample up to 100 mM ion concentration.
DISCUSSION

Alginate biosynthesis is a common characteristic of a majority of pseudomonads in rRNA-DNA homology group I (94). This group includes all the fluorescent and a few nonfluorescent pseudomonads. Until recently, only certain strains of *P. aeruginosa* isolated almost exclusively from cystic fibrosis patients, and strains of *A. vinlandii* were known to produce O-acetylated alginate as EPS (67). The fluorescent pseudomonads, *P. fluorescens*, *P. mendocina*, and *P. putida*, were subsequently found to produce alginate, but only under conditions of stress (bacteriocin, bacteriophage, antibiotic, 53, 58). Within the past seven years, many plant pathogenic fluorescent pseudomonads have been recognized as alginate producers under the appropriate conditions. This strongly indicates that the ability to synthesize alginate may have an important evolutionary role.

Little is known about the synthesis of alginate by the phytopathogenic pseudomonads. *Pseudomonas syringae* pv *phaseolicola* ATCC 19304 produces high amounts of O-acetylated alginate *in vitro*. The amount of alginate, as well as the degree of acetylation of the polymer, varied with carbon source. *P. syringae* ATCC 19304 grown on sucrose produced a mixed population of EPS. Although alginate was the predominant EPS produced, *P. syringae* ATCC 19304 also produced levan (C-2 → C-6 fructan backbone) on sucrose (57). Levan production indicates that the bacterium also has the ability to synthesize levansucrase. Sucrose was not utilized as the carbon source in this research since the presence of levan in
the exopolymer extracts made purification of the alginate more difficult.

Gluconic acid was chosen as the carbon source in this work because cells grown on gluconic acid produced approximately 21% more alginate than cells grown on glucose. It is well established that the primary route of glucose catabolism in pseudomonads is through the Entner-Doudoroff pathway, which produces pyruvate and glyceraldehyde 3-phosphate (52). Glucose conversion to 6-phosphogluconate is required prior to entry into the Entner-Doudoroff pathway. This conversion can be accomplished in one of two ways. Glucose can be phosphorylated to glucose 6-phosphate by a hexokinase and subsequently oxidized to 6-phosphogluconate by a glucose 6-phosphate dehydrogenase (148), or glucose can be converted to gluconate at the surface of the cell by an NAD(P) dependent glucose dehydrogenase followed by gluconolactonase. Gluconate is then oxidized to 2-ketogluconate by a membrane bound gluconate dehydrogenase during transport of the sugar into the cell. Once inside the cell the 2-ketogluconate is phosphorylated to 6-phosphogluconate (47). Most pseudomonads, i.e., *P. aeruginosa*, *P. fluorescens*, and *P. putida* oxidize glucose to gluconate using the second mechanism prior to transport of the sugar into the cell (52). The increased production of alginate by gluconate grown cells over glucose grown cells probably results from a decreased energy requirement for the enzymatic oxidation of glucose to gluconate prior to transport into the cell.

In *P. aeruginosa*, the C-6 of gluconate incorporates into alginate (13). Carbon atoms 1, 2, and 3 of gluconate are converted
to pyruvate through the 2-keto 3-deoxyphosphogluconate aldolase reaction and are eventually lost as CO₂ and acetyl CoA. Carbon atoms 4, 5, and 6 are channeled into alginate through glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate can condense with dihydroxyacetone phosphate to produce fructose 1,6-diphosphate and ultimately fructose 6-phosphate, which is the starting material for alginate biosynthesis (7, 78). Besides being a source of fructose 6-phosphate, gluconic acid can also be oxidized to acetyl CoA (7). Acetyl CoA is reportedly the source of the O-acetyl groups on the mannosyl residues of xanthan gum (66), and is the probable source of acetyl in bacterial alginates (136).

P. syringae ATCC 19304 utilized ammonia as a nitrogen source for growth. It was unable to use nitrate, nitrite, or urea. All pseudomonads produce energy by respiration. In some cases, nitrate can be used as an alternate electron acceptor allowing growth to occur anaerobically. Those pseudomonads that can conduct nitrate respiration can reduce nitrate beyond the toxic nitrite stage to molecular nitrogen by "denitrification." P. syringae was unable to utilize nitrate as a nitrogen source and therefore lacks the cellular makeup to conduct nitrate respiration. This may be due to the absence of cytochrome C in the electron transport chain of P. syringae (94). In plants, free ammonium ions are present at very low levels. Ammonium is toxic to the plants, because it inhibits the production of ATP in the mitochondrial and photosynthetic electron transport systems. Most nitrogen present in plants is found associated with organic compounds. In plants, ammonium is converted into organic compounds primarily through
three different reactions: 1) formation of glutamic acid by reaction with \( \alpha \)-ketoglutarate, 2) formation of glutamine by reaction with glutamic acid, and 3) formation of carbamyl phosphate in arginine biosynthesis and pyrimidine biosynthesis. \( P. \) \textit{syringae} is a proteolytic bacterium (94). This bacterium probably provides itself with ammonia through deamination of the component amino acids allowing the survival of the bacterium on the host leaf surface.

Polysaccharide production by microorganisms is enhanced in nitrogen limited, high carbon content media (147). The changing carbon/nitrogen ratio exhibited the same effect on alginate production in \( P. \) \textit{syringae} that has been reported for other pseudomonads. Higher yields were obtained with high carbon/nitrogen ratios (low nitrogen content, high gluconate). It has been proposed that limitation of essential nutrients, in this case nitrogen, inhibits growth and directs the course of total polysaccharide biosynthesis from cell wall material (LPS and peptidoglycan) to extracellular polysaccharide synthesis (133). It was further postulated that during active growth the same pool of isoprenoid lipid carriers involved in polysaccharide synthesis are utilized by cell wall and LPS precursors (133). These conditions direct alginate biosynthesis toward production as a secondary metabolite. In the case of \( P. \) \textit{syringae} ATCC 19304, alginate biosynthesis did not fully fit this hypothesis. Alginate production by \( P. \) \textit{syringae} is necessary for successful colonization of plant host tissue (113). Although alginate production did increase as the carbon/nitrogen ratio increased, alginate was produced by \( P. \) \textit{syringae} coincidental with cellular reproduction.
Bacteria secrete EPS for many different reasons. Bacterial EPS may aid colonization (16, 50), prevent desiccation, store energy, or help concentrate and take up charged molecules, particularly metal ions (11, 17, 46). In cystic fibrosis patients *P. aeruginosa* secretes alginate to help colonize the lungs and to protect itself against phagocytosis. *P. syringae* produces alginate to aid in colonization and parasitization of leaf surfaces (30, 138).

The ability of *P. syringae* to produce alginate decreased rapidly upon successive transfers in liquid media. Alginate production remained stable upon transfer on solid media. The pathogenicity of *P. syringae* is dependent upon its ability to produce alginate (113). Colonization of a leaf surface by *P. syringae* causes halo blight (leaf spots) of the leaf. Alginate must be present to produce leaf spots (34). These leaf spots are probably due to an interruption of the photosynthetic pathway in the leaf. All pseudomonads respire, and thus must have a source of ferric iron for synthesis of cytochromes. On the leaf surface, the sources of ferric iron are limited to ferredoxin and the cytochrome bf complex. The breakdown of ferredoxin or the cytochrome bf complex interrupts photosynthesis by stopping electron transport and aids in leaf spotting. Acetylated *P. syringae* alginates have a high affinity for ferric iron. By increasing the ferric iron concentration in the growth media alginate production decreased. The parasitization of *P. syringae* ATCC 19304 may be due to the ability of the acetylated alginate to scavenge the ferric iron from ferredoxin or the cytochrome bf complex. By scavenging the iron, photosynthesis
would be interrupted and leaf spots would occur due to the inability of the plant to make energy for chlorophyll biosynthesis.

The native alginates of \textit{P. syringae} ATCC 19304 were polydisperse. This indicated that there was a high degree of variability in size of the alginate produced by the biological system. The $M_W$ for the native bacterial polymer was $1.3 \times 10^5$. Upon deacetylation, the $M_W$ dropped approximately 6\% to $1.2 \times 10^5$. This drop was due to the loss of the acetyl groups at C-2 and/or C-3 of the mannuronic acid residues within the polymer. By monitoring the difference in the acid stability of D-mannose and L-gulose from the acid hydrolyzed, reduced alginates, more accurate alginate compositions were obtained for the bacterial polymer. The alginate produced by \textit{P. syringae} ATCC 19304 had a final composition of 82\% mannuronic acid and 18\% guluronic acid. This composition was in contrast with the previously reported composition of greater than 95\% mannuronic acid and less than 5\% guluronic acid where the relative acid sensitivity of each sugar was not considered (38).

Both the solution and gelling properties of bacterial alginate were altered by the degree of acetylation on the polymer. The viscosity of the acetylated polymer was higher than the deacetylated bacterial or seaweed alginates at equivalent concentrations. The native bacterial alginates were acetylated with an average of 1.2 acetyl units per uronic acid residue. This produced a 6\% increase in $M_W$ and a 26\% increase in viscosity at a concentration of 1 mg/ml. Acetylation reportedly increases the viscosity of alginate solutions (129). These increases may be due to the increase in total molecular weight of the
polymer, or altered conformations of the polymer in aqueous solution. Alginates high in mannuronic acid exhibit a flat ribbon-like conformation. High amounts of guluronate produce a more puckered structure. It is possible that the introduction of acetyl groups shifts the conformational energy without increasing the accessible surface of the molecules, eventually producing a flexible polymer with an increased number of conformations. Because the acetylated polymer showed a 4 fold increase in viscosity over molecular weight, it is probable that both factors play a role.

Acetylation altered the affinity of the polymer for many cations, including calcium. Except for ferric and to some extent copper ions, the ability of multivalent cations, particularly calcium, to induce gelation of the alginate was reduced in the acetylated polymer. In parallel, there was a marked decrease in the strength of the gels. The effects of acetylation on the gelling properties of bacterial alginates were seen in the water holding capacities, the surface tensions, and the relative porosities of the Ca-alginate gels.

Acetylation profoundly affected the rigidity of the Ca-alginate gels and their ability to hold water. Generally, the volume of an ionic gel is dependent upon a positive osmotic pressure. The osmotic pressure of the gel is due mainly to the positive entropy of the mixing of counterions with water, which is counterbalanced at equilibrium by a negative pressure due to the elasticity of the network (139). For Ca-alginate gels, which are enthalpic rather than entropic (3), the elasticity depends on the number and strength of the crosslinks. Since the introduction of acetyl groups impairs the cooperative binding of calcium ions, the number of
dissociated counterions per polymer chain increases with increasing degree of acetylation. The high number of dissociated counterions enhances the positive osmotic pressure. At the same time, it weakens the forces holding the network together. Reduction in the cooperative binding of calcium ions reduces both the strength and the number of crosslinks in the network. Fewer crosslinks lessen the surface tension on the gel and as a result allows higher water holding capacities within the gel network. This was observed with the \textit{P. syringae} alginate. Acetylated bacterial alginate gels had a much lower surface tension, and as a result a water holding capacity that was approximately 68 percent higher than its deacetylated counterpart. Fe-alginate gels and Pb-alginate gels showed the same general properties, however neither the ferric nor lead alginate gels held as much water as the Ca-alginate gels. These differences are attributable to the relative affinities of each cation to the alginate. Ferric and lead ions had a higher affinity than calcium ions for both acetylated and deacetylated alginates.

Because of its higher viscosity and lower affinity for many polyvalent cations, acetylated bacterial alginate may be a possible substitute for seaweed alginate in many applications. More viscous solutions can be made with lower concentrations of bacterial alginate than with seaweed alginate, reducing the polymer requirements in a given application. By varying the degree of acetylation, it should be possible to produce alginates with designer properties.

As a polyelectrolyte, alginate has the potential to concentrate toxic, heavy and/or valuable metals from the environment. The
removal of metals by microorganisms reportedly depends on physicochemical adsorption to cellular components such as polysaccharides and proteins (84). Because of the high negative charge on the cell walls of bacteria, fungi, and algae, microorganisms have been used to concentrate metal ions including uranium, copper, manganese, cadmium, molybdenum, gold, and mercury (20, 56, 65). The polyanionic nature of alginates also allows the binding and concentration of heavy metals through metal induced gelation of the polymers. The formation of alginate gels in the presence of polyvalent cations is altered by acetylation of the alginate, as well as the native conformation of the polymer. The physical binding of calcium ions to guluronate residues in seaweed alginate is due to charge charge interactions between the positive charges from calcium and the negative charges from the carboxyl groups. The size of calcium ions is such that they fit into the space formed by the polyguluronate stretches of seaweed alginates (83). Randomly organized bacterial alginate also gels in the presence of many polyvalent cations. This casts some doubt on the principal tenant of the "egg-box" theory; that only polyguluronate residues in alginate play a key role in gelation. The ability of bacterial alginates, without polyguluronate blocks, to gel in the presence of calcium indicates a reevaluation of this theory is necessary.

Multivalent cations interact differently with alginates (59, 60). Each cation examined showed variations in its ability to precipitate seaweed and bacterial alginate. With the exception of ferric and uranium ions, polyvalent cations more readily precipitated seaweed alginate over its bacterial counterpart.
most instances, deacetylation of the bacterial polymer enhanced its cation precipitability. This ability to adjust the affinities of alginates for specific polyvalent cations increases the feasibility of using this polymer to remove toxic metals, i.e., lead or uranium from drinking water.

Acid hydrolysis is used to depolymerize polymers for compositional analysis. Ideally, hydrolysis goes to completion without loss of the components. Practically, however, some sugar loss occurs and must be accounted for to accurately determine polysaccharide compositions. Compositional analysis of alginates requires the reduction of the uronic acid residues to their corresponding neutral sugars prior to acid hydrolysis. This reduction facilitates the acid hydrolysis of the glycosidic bonds by converting the acid resistant glycosyluronic acid bonds to the more acid labile glycosyl bonds. Reduction of alginate polymers liberates D-mannose and its C-5 epimer L-gulose after acid hydrolysis. Both D-mannose and L-gulose react differently under acid conditions. D-Mannose is relatively acid stable. L-gulose is markedly acid sensitive. In general, aldose containing polysaccharides are completely hydrolyzed with minimal sugar loss by 1 M H2SO4 at 100°C for 5 hours (119).

At equilibrium D-mannose residues favor the $^4C_1$ chair conformation while the L-gulose residues adopt the $^1C_4$ chair conformation because the bulky group at C-5 orients to an equatorial position. These sugars are not locked into these conformations. In solution these monomers exist in a dynamic equilibrium. This equilibrium allows alternation between both $^4C_1$
and 1C4 conformations, as well as the boat, half-chair, and open conformations. The open chain conformation allows anomeration around C-1 producing both the α and β anomer. This equilibrium allows numerous conformational possibilities for each monomer including the possible formation of 1,6 anhydrohexopyranoses in acid solutions.

Sugars of the gulo., ido., and altro. configurations undergo spontaneous conversion to 1,6 anhydrides in acids. Sugars of the gluco., manno., and galacto. configurations produce very little 1,6 anhydride at equilibrium and are almost completely hydrolyzed to the free aldoses under acid conditions (4, 81). This is because of the orientation of the hydroxyl groups at C-2, C-3, and C-4. Axially oriented hydroxyl groups, particularly at C-3, destabilize anhydride formation. Equatorially oriented hydroxyl groups favor the formation of 1,6 anhydrides. The equilibrium is dependent on the steric arrangement of hydroxyl groups which do not take part in the reaction. Once a 1,6 anhydride bond is formed, the conformational equilibrium is shifted so the sugar no longer exists in a dynamic state. The formation of a second ring within the molecule locks it into that particular conformation.

D-Mannose, whose free energy favors the 4C1 chair conformation, does not readily form the 1,6 anhydride bond when it is in the 1C4 chair conformation and the hydroxyl group attached to the anomeric carbon is in the β position. Although this conformation brings C-6 and the anomeric hydroxyl group into axial positions around the ring and into proximity, this conformation also brings the hydroxyl groups at C-2 and C-3 into axial positions which
destabilize and inhibit anhydride formation. The axial hydroxyl group at C-3 interacts with the potential anhydride bond blocking its formation. In the β conformation D-mannose, D-glucose, and D-galactose all have axial hydroxyl groups at C-3 and therefore do not readily form 1,6 anhydrides.

The free energy of L-gulose favors the 1C4 chair conformation. At dynamic equilibrium it can also exist in the 4C1 conformation, which places the bulky group at C-5 in an axial orientation. If the hydroxyl group at the anomeric carbon is in the β position the C-6 and the anomeric hydroxyl group come into close enough proximity to allow 1,6 anhydride formation. An exception is that the anhydride bond forms below the plane of the ring with L-gulose rather than above it. This orientation places the hydroxyl groups at C-3, and C-4 in equatorial positions around the ring and the hydroxyl group at C-2 axial. In this position the free energy favors 1,6 anhydride formation due to the absence of a destabilizing axial hydroxyl group at C-3.

Upon acid hydrolysis, L-gulose locked into a new conformation. This was seen by the increase in intensity of a second spot on TLC over time. Although the acid hydrolysis product of L-gulose was not definitively identified, the molecule was tentatively identified as a 1,6 anhydride because it had the same migration as 1,6 anhydro β-D-mannopyranose, and 1,6 anhydro β-D-glucopyranose on TLC. Computerized molecular modeling of 1,6 anhydro β-L-gulopyranose using SYBYL software indicated that the molecule has a low total energy, favorable for its production and stability.
Existing methods for determination of the composition of alginates recommend hydrolysis in 1 M H$_2$SO$_4$ for 90 minutes at 100°C (38). Since L-gulose is more susceptible to conformational modification under these conditions than is D-mannose, lack of accounting for gulose destruction has resulted in inaccurate reports of composition.

Analysis of acid hydrolyzed reduced alginates paralleled those obtained with the D-mannose and L-gulose monomers. When correcting for gulose destruction, by extrapolation back to time 0, the results of the seaweed alginate analysis correlated exactly with the published results of 60% mannuronic acid and 40% guluronic acid as determined by the reductive cleavage method of Zeller and Gray (150). The alginates produced by the phytopathogenic pseudomonads apparently have a higher concentration of L-guluronic acid than had been previously reported. The reported composition of the *P. syringae* ATCC 19304 alginate is greater than 95% D-mannuronic acid and less than 5% L-guluronic acid (38). After correcting for the relative acid sensitivities of each corresponding neutral sugar, the actual composition of the bacterial alginate was 82% D-mannuronic acid and 18% L-guluronic acid. This ratio correlates well to the reported composition of *P. aeruginosa* alginate of 80% D-mannuronic acid and 20% L-guluronic acid (150).

*P. syringae* ATCC 19304 produced large amounts of acetylated alginate when grown on gluconic acid. While production of this polymer decreased dramatically on subculture in liquid media, production was stable on solid media. Maximum production was
dependent on the pH, temperature, and a high carbon/nitrogen ratio within the growth media. The $M_W$ of the bacterial polymer was approximately 1.7 times larger than the seaweed counterpart with a composition of 82% mannuronic acid and 18% guluronic acid, and an average of 1.2 acetyl groups per monomer. These physical characteristics allowed for much higher solution viscosities at equal concentrations, and cation induced gels with increased water holding capacity. By controlling the degree of acetylation it was possible to control the solution and gelling properties of the polymer to some extent. The polyanionic nature of the polymer allows for the binding of many toxic and heavy metals. Ultimately, it should be possible to create inexpensive designer alginates whose properties are controlled by the degree of acetylation on the polymer.
REFERENCES


78. Lynn, A. R., and J. R. Sokatch. 1984. Incorporation of isotope from specifically labelled glucose into alginate of 


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Major Field: Microbiology

Title of Dissertation: The Production and Characterization of Alginate Produced by Pseudomonas syringae

Approved:

Major Professor and Chairman
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