The Production of Industrially Important Bacterial Polysaccharides

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Until the 1950s plant tissues were the only practical commercial sources of polysaccharide gums and mucilages, but the development of the exopolysaccharides dextran from *Leuconostoc mesenteroides* in the early 1950s and xanthan gum from *Xanthomonas campestris* in the following two decades demonstrated the potential of fermentation in the production of industrially important gums. For a number of reasons (Whistler, 1973, pp. 513–542) dextran usage has been limited to high-value small-volume applications such as extension of blood plasma and the manufacture of chromatographic support media, and this has restricted the world production to a large extent. However, in the case of xanthan the situation is different, and world production is already several thousand tons. The unique physical properties shown by this gum (Whistler, 1973, pp. 473–497) have produced an increasing demand, and industry throughout the world has been stimulated to investigate the possibilities of exploiting other micro-organisms to produce further similarly valuable polysaccharides. Examples of gums in development at the present time are the glucan pullulan from *Aureobasidium pullulans* (Yuen, 1974) and a heteropolysaccharide containing glucose, galactose, uronic acid and fucose from *Erwinia carotovora* (Kang & Kovacs, 1974).

We became interested in microbial polysaccharides through reports that certain bacteria produce extracellular polysaccharides similar in many respects to the polyuronide alginic acid, which was previously known only from the brown algae. Alginic acid is a commercially important gum with gelling and viscoelastic properties of use in the food, textile, pharmaceutical and paper industries (Percival & McDowell, 1967). The bacteria concerned were *Pseudomonas aeruginosa* (Linker & Jones, 1966; Carlson & Mathews, 1966) and *Azotobacter vinelandii* (Gorin & Spencer, 1966). The former was rejected for study because of its association with pathogenic conditions in man, and *A. vinelandii* was selected for examination. Studies based on small-scale fermentation experiments suggested that the *Azotobacter* polysaccharide would be sufficiently valuable for the process to be feasible if high yields could be obtained.

*Azotobacter vinelandii* (N.C.I.B. 9068) cells were grown initially in shake flasks under the fermentation conditions described by Gorin & Spencer (1966), and a 5% yield of polysaccharide was obtained. Analysis of the monosaccharide components both by hydrolysis and by reduction followed by hydrolysis confirmed that the material contained a high proportion of mannuronic acid and guluronic acid. No other sugars were detected in the purified polymer, but the polysaccharide was partly acetylated. The position of linkage of both monosaccharide units was supported as being 1→4 both by methylation and by periodate oxidation studies, and the acetyl groups were found to be present to a low and variable degree. Block structure analysis by the method of Penman & Sanderson (1972) indicated that the features typical of algal alginate were present in the bacterial polysaccharide. The behaviour of aqueous solutions of the gum toward multivalent metal cations was also very similar to that shown by algal material (Whistler, 1973, pp. 49–81). By using light-scattering techniques (Smidsrød & Haug, 1968) a weight-average molecular weight of $5 \times 10^5$ was obtained, and by osmometry (G. Vane, unpublished work) a number-average value of $5 \times 10^5$ was found. By these and other techniques it was shown that bacterial alginate was similar in many ways to its algal counterpart. However, as indicated by the molecular-weight data and supported by polyacrylamide-gel electrophoresis (Bucke, 1974), the polysaccharide appeared to be polydisperse in comparison with many algal samples tested.

The physical properties of bacterial alginate, namely solution rheology and gel behaviour, were compared with algal samples, and it was found that the bacterial product
was inferior in several respects. The poor quality was attributed largely to the polydisperse nature of the material and its insufficiently high molecular weight.

Two major objectives of further early studies therefore were to improve both product yield and quality, and the initial approach was by the empirical development of medium composition in shake flasks and conventional stirred tank fermenters in batch culture. Of the many parameters investigated, that which produced the most significant increase in the yield of alginic acid was the lowering of the phosphate content to one-twentieth of that in the standard Burk’s medium (Gorin & Spencer, 1966). Owing to the lowered buffering capacity of the medium, pH control then became necessary to maintain the rate of polysaccharide production as, through the production of the acid polysaccharide and CO₂, the culture pH was found to fall until cell growth and polysaccharide production ceased. By these means the yield of pure alginic acid was raised to 25% of the sucrose supplied. A second result of this work was that the molecular-weight distribution of the product was found to have narrowed and that the weight-average value was substantially higher. This change in molecular weight was found to be accompanied by a concomitant increase in product quality, and the physical properties shown by the polymer now fell into the range covered by many commercially useful algal materials.

At this stage it was decided to scale up the laboratory process in order to examine economic and technical problems and to obtain larger samples for evaluation. Pilot production by a batch process was therefore commenced.

In a more thorough economic evaluation of the process based on the results described above and data from the pilot plant it was found that it would be necessary to make further basic progress in certain specific areas of research. The objectives defined were as follows. (a) To select a cheaper substrate than pure sucrose, e.g. glucose, glucose syrup or a cheap form of sucrose such as molasses. (b) To obtain further increases in yield. (c) To increase absolute polysaccharide concentration in the fermenter. (d) To shorten the effective fermentation time. (e) A further objective, though not vital to the basic process economics, was recognized as important in the development of more advanced technology. This was to achieve control over polysaccharide biosynthesis in order to manufacture a range of alginates each with a chemical structure significantly different. As there is evidence that the physical properties of alginates are related to their chemical structures, it would then be possible to ‘tailor’ products for specific customer requirements.

The approaches to these problems were as follows: (i) strain selection and development; (ii) biochemical engineering (fermenter design); (iii) studies in continuous culture; (iv) further medium development; (v) biochemical studies.

It was recognized that it would be necessary to obtain much more detailed knowledge of the biochemistry of alginate production if the approaches described above were to be fully exploited. Because very little was known about the biosynthetic pathways, a fairly basic approach was required.

The biosynthesis of alginate has been studied in the alga *Fucus gardeneri* (Lin & Hassid, 1966a,b) with the conclusion that precursors of the polysaccharide were GDP-mannuronic acid and GDP-guluronic acid. The detection of a mannuronate 5-epimerase in cultures of *A. vinelandii* (Haug & Larsen, 1971) and in extracts of the alga *Laminaria digitata* (Madgwick et al., 1973) has been reported by Norwegian workers. They concluded that the initial polymeric product in alginic acid biosynthesis was polymannuronic acid and that mannuronic acid residues were epimerized to guluronic acid at the polymer level.

The approach was to employ such possible radioactive precursors of alginic acid as were commercially available to trace the reaction sequence from sucrose to the final product and to study the properties of the individual enzymes involved. Sucrose was inverted slowly by an invertase (β-fructofuranosidase) with a pH optimum of 7.0, and the enzymes hexokinase, glucose phosphate isomerase, mannose phosphate isomerase, phosphomannomutase and GDP-mannose pyrophosphorylase were each detected and assayed separately. The later enzymes in the sequence were assayed in combination by
supplying radioactive GDP-mannose to cell-free extracts that retained particulate material. High-voltage electrophoresis of the resulting products demonstrated the formation of GDP-mannuronic acid and a polymeric material that remained at the origin of the electrophoretogram. This polymeric material was degraded by an alginate lyase partially purified from a marine *Pseudomonas* species and was co-precipitated with authentic algal alginic acid by both acid and propan-2-ol. Partial acid hydrolysis by the method of Haug & Larsen (1966) followed by pH fractionation of the homopolymeric material into polymannuronic acid and polyguluronic acid blocks indicated that the polymer consisted of 10% polymannuronic acid blocks, 4% polyguluronic acid blocks and the remaining 86% alternating sequences. These results combined to demonstrate that the radioactive polymeric material produced on supplying GDP-[14C]mannose to extracts of *A. vinelandii* cells was similar to alginic acid (Scheme 1).
Each of the constituent enzymes of the pathway was present with sufficient activity to account for the observed rate of alginate biosynthesis.

The information obtained from this work, together with other biochemical information, e.g. knowledge of the mechanisms of nitrogen fixation and respiration (Yates & Jones, 1974) and of the deposition of poly-β-hydroxybutyrate in A. vinelandii (Dawes & Senior, 1973) has been useful in several respects. For example, in the selection of mutants for further study, the organisms will be examined for their stability to grow and produce polysaccharide on fixed nitrogen, for changes in the biosynthetic steps described above and for lack of poly-β-hydroxybutyrate deposition. Of the enzymes involved in the biosynthesis of alginate, that with the greatest commercial potential is the alginate epimerase, which could be used to improve the properties of low-quality alginates.


THE STRUCTURES OF SOME CARBOHYDRATE-METABOLIZING ENZYMES: a Colloquium organized on behalf of the Carbohydrate Biochemistry Group by R. J. Sturgeon (Edinburgh) and K. Decker (Freiburg)

Active-Site-Directed Inhibitors of Glycosidases

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Inhibitors for the labelling of functional groups at the active site of glycosidases should fulfil the following requirements: (1) structural similarity with the substrate to facilitate interaction with the substrate-binding site of the enzyme; (2) a reactive group (e.g. epoxide, α-haloacetyl, diazoacetyl, azido) able to form a stable covalent bond with an amino acid side chain at the active site. If this is to be a group directly involved in catalysis the reactive group of the inhibitor should be near a position in the enzyme–inhibitor complex that corresponds to the substrate bond cleaved during glycoside hydrolysis. For β-glucosidases from various sources this is the case with 1,2-epoxy-3,4,5,6-tetrahydroxycyclohexane (conduritol B epoxide, I) (Legler, 1966). α-Glucosidase from yeast seems to react with 6-bromo-3,4,5-trihydroxycyclohex-1-ene (II) with similar specificity (Legler & Lotz, 1973). The high glycone-specificity of most glycosidases precludes an