8
Microbial Polysaccharide Products

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Introduction

Exopolysaccharide-synthesising micro-organisms are of widespread occurrence. After much experimental work and examination of physical properties, a small number of microbial polysaccharides are now accepted products of biotechnology. Several others are in various stages of development. The uses and value of such polymers vary widely. Some are employed because of their unique physical properties. Others are superior to other natural or synthetic polymers. In this category are xanthan and gellan (gelrite) which, although they have found various food applications, also find diverse uses in non-food systems. Gellan has proved especially valuable in plant cell biotechnology. Xanthan is still the 'benchmark' product. It received food approval in the US many years ago; it is a relatively inexpensive product because of the very high conversion (*c.* 70%) of substrate to polymer and relative ease of processing and recovery. It is an accepted product in various industrial applications, including oil exploration and development. Because of lower yields or production and processing problems, other microbial polysaccharides are generally more expensive, some markedly so. At the top of the scale is bacterial hyaluronic acid from *Streptococcus equi* and related bacteria. Many of the major applications of microbial polysaccharides utilise their gel-forming ability or their high viscosity in aqueous solution, others use more specialised properties.

Many other exopolysaccharides (EPS) have been the subject of studies into microbial pathogenicity and in some bacteria there is a direct correlation between the presence of EPS as capsules surrounding the cell and the pathogenic state. The majority of plant-pathogenic bacteria produce copious quantities of EPS both as loose slime and capsules. Other microbial EPS play important roles in adhesion to exposed surfaces where they may be found as major constituents of 'biofilms' (Sutherland, 1997). Among these is a very unusual positively charged polysaccharide composed of D-glucosamine (*Figure 1*) (Mack *et al.*, 1996). This polymer has been demonstrated to be closely associated with the intercellular adhesion of strains of *Staphylococcus epidermidis* and with attachment to polystyrene and various types of plastic surfaces.

Abbreviations: CD, circular dichroism; EPS, exopolysaccharide; LBG, locust bean gum

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EPS I

→ [6-β-D-Glc*p*NAc-(1→ 6)-β-D-Glc*p*NAc-(1→6)-β-D-Glc*p*-N-(1→6)-β-D-Glc*p*NAc-(1→ 6)-β-D-Glc*p*NAc-(1 →

Ratio: Glc*p*NAc : Glc*p*N c. 5:1

EPS II

→ [-6-β-D-Glc*p*NAc-(1→ 6)-β-D-Glc*p*NAc-(1→6)-β- D-Glc*p* N-(1→6)-β-D-Glc*p*NAc-(1→ 6)-β-D-Glc*p*NAc-(1→

Ratio: Glc*p*NAc : Glc*p*N c. 16:1 Also contains: phosphate and succinyl half esters

Figure 1. The positively charged polysaccharides from *Staphylococcus epidermidis* strains. Results of Mack *et al.* (1996).

As a result of these diverse interests, there have now been structural studies on a very large number of microbial exopolysaccharides. We now have a better idea of the relationships which exist between structure and function in microbial exopolysaccharides (Sutherland, 1994), but it is still difficult to predict from a knowledge of polysaccharide structure which microbial polymers are likely to prove worth developing. Many reports in the literature which have suggested potential industrial usage have proved over optimistic. The series of mutants yielding xanthan with abbreviated side-chains provides an excellent example of the unpredictability of physical properties (*Figure 2*). Such mutant bacterial strains fail to yield as much polymer as the wild type and may also be less stable. The mass of the polymer produced may also be changed. At the same time, the availability of xanthan with a second acetyl group (Stankowski *et al.*, 1993) (and little if any pyruvylation) enabled us to compare such a product with the traditional mono-acetylated polysaccharide (e.g. Shatwell *et al.*, 1990). Acetan has the same cellulosic main-chain as xanthan and shows some structural similarities in its side-chain although this is a pentasaccharide as opposed to the pyruvylated trisaccharide of xanthan (Couso *et al.*, 1987). Solutions of acetan have also been shown to be highly viscous. The physical properties of acetan are indeed very similar to those of xanthan (Berth *et al.*, 1996; Ojinnaka *et al.*, 1996). Acetan, like xanthan, can be represented as forming stiff, double-stranded chains. The persistence length is *c.* 100 nm (Harding *et al.*, 1996). A similar series of altered polysaccharides with truncated side-chains, is now also being developed from acetan-synthesising *Acetobacter xylinum* (Colquhoun *et al.*, 1995).

Polysaccharide properties and applications

Many polysaccharides are used as gelling agents. This is true of starch and also of alginate, agar and carrageenan obtained from various marine algae. All these are traditional products, which have been used over long time periods. Gelation may be an inherent property of the polysaccharide or may require the presence of either monovalent or multivalent cations. Further, non-gelling polysaccharides such as xanthan may form gels in admixture with plant galacto- or gluco-mannans (synergistic gelling). The process of gel formation by microbial polysaccharides has thus come under intense scrutiny. Ross-Murphy and Shatwell (1993) divided such polymer gels and networks into three categories: systems in which junctions were formed by covalent

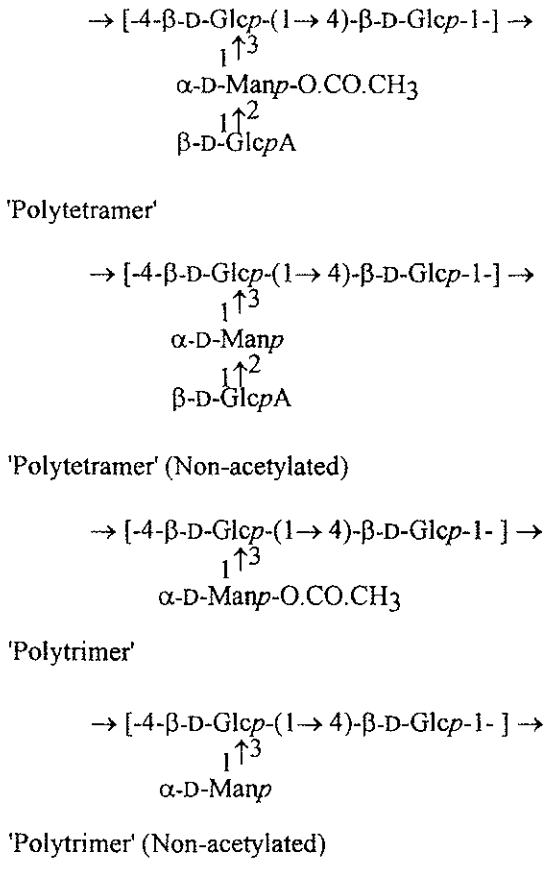


Figure 2. Mutant forms of xanthan with truncated side-chains.

cross-linking; physical junctions which could be disrupted; and entanglement networks. The structure and properties of microbial polysaccharides including curdlan and gellan have been known for many years. Other gel-forming polymers such as mutan and a neutral polysaccharide from *Rhizobium* (Gidley *et al.*, 1987) have also been well studied. Various novel bacterial gelling polysaccharides have been reported more recently. Several of these resemble gellan in requiring deacylation prior to discovery of their ability to form gels in association with divalent actions. One of these interesting new polymers was discovered accidentally through a study of mutants of *Rhizobium meliloti*. This polysaccharide proved to be a cryptic product, synthesis of which was normally suppressed. It was a homopolysaccharide, a polymer of 1,4-β-D-glucuronic acid carrying *O*-acetyl groups on the C2 and C3 positions with molecular weight ranging from 6×10^4 to 5×10^5 (*Figure 3*) (Dantas *et al.*, 1994). As might be expected, some of its properties were very similar to those found in some algal alginates. Thus, it formed gels in the presence of monovalent, divalent or trivalent cations (Heyraud *et al.*, 1994) but these gels were thermoreversible unlike calcium or strontium alginate gels (Dantas *et al.*, 1994). A second gel-forming polymer, the galacturonic acid-containing polysaccharide 'beijeran' is produced by *Azotobacter*

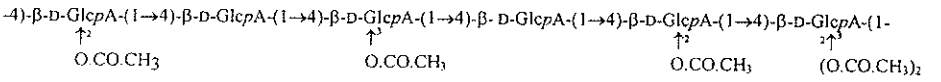


Figure 3. The structure of poly-D-glucuronic acid from *Rhizobium meliloti* M5N1. Results of Courtois *et al.* (1994).

beijerinckia strain YNM1 (Ogawa *et al.*, 1996). The repeat unit of this EPS is a linear trisaccharide carrying *O*-acetyl substituents on the C6 position of the glucose residue (*Figure 4*). Physical studies suggested that the polymer formed an extended two-fold helix and that the deacetylated macromolecule exhibited higher crystallinity (Ogawa *et al.*, 1997). Studies on each of these polymers are still at an early stage. The unique properties of exopolysaccharides and the influence of relatively minor substituents are also exemplified by the *Enterobacter* XM6 polymer (Nisbet *et al.*, 1984). In aqueous solutions XM6 yielded highly viscous solutions, as did the polymer from *Klebsiella aerogenes* type 54. At higher concentrations, XM6 formed gels in the presence of both monovalent and divalent cations and showed a very sharp transition between the ordered and disordered form at 39°C. However, the acetylated polymers with exactly the same carbohydrate structure from *K. aerogenes* type 54 failed to form gels, as also did the polymer from *Escherichia coli* K27 in which the side-chains are D-galactosyl residues replacing D-glucose (Sutherland *et al.*, 1970). This was observed both for type 54 polymers which carried *O*-acetyl groups on each tetrasaccharide repeat unit and for those which were acetylated on alternate repeat units. On deacetylation with mild alkali, all the polymers were capable of gel formation. X-ray fibre diffraction studies indicated that XM6 was highly crystalline due to strong polymer-polymer interactions, whereas the acetylated polymers were poorly crystalline with weak intermolecular interactions (Atkins *et al.*, 1987). Morris and Miles (1986) suggested that acetylation did not appear to alter the helical conformation of the polysaccharides but controlled gelation through the effects it exerted on intermolecular association and crystallisation of segments of the polymer chains.

Curdlan is a linear 1,3-β-D-glucan synthesised by several Gram negative bacteria including *Agrobacterium* spp. and is used for various food applications in Japan. Although curdlan was insoluble in water, it could be dissolved in sodium hydroxide solutions. It appeared to form triple helical structures under appropriate conditions (Deslandes *et al.*, 1980). Curdlan was also unusual in that it formed two distinct types of gel (Harada *et al.*, 1994). The first type (low-set gel) was formed when alkaline solutions of the polysaccharide were neutralised or when aqueous solutions were heated to 60°C and cooled. A second type of gel (high set gel) formed when solutions were heated above 80°C leading to a hydrophobic interaction and to stronger gels than those obtained at lower temperatures. The higher temperature followed by cooling converted the polysaccharide to the triple helical form. The polysaccharide differed from other microbial polysaccharides in forming a gel on heat treatment, with gel strength increasing with both temperature and time of heating. The high temperature gels were also much more resistant to hydrolysis by 1,3-β-D-glucanases and by acid treatment and failed to melt on further heating. This alteration in properties was ascribed to the formation of a pseudocrystalline form with greatly reduced hydration of the curdlan following high temperature treatment (Kanzawa *et al.*, 1989). Chemical introduction of *O*-acetyl groups altered the conformation and properties of curdlan and

Azotobacter beijerinkia YNM1 Exopolysaccharide (Beijeran)

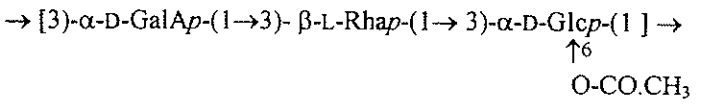


Figure 4. The structure of beijeran.

yielded a single helical structure in which there was one molecule of water per glucose residue (Okuyama *et al.*, 1996). Although it has been less studied than curdlan, the linear 1,3-α-D-glucan mutan produced by *Streptococcus mutans* also exhibits crystalline structure (Ogawa *et al.*, 1981)

Gellan has proved to be one of the most interesting recent microbial polysaccharide introductions. It is produced commercially from a strain of *Sphingomonas elodea* and has a linear tetrasaccharide repeat unit (Jansson *et al.*, 1986). The gelling properties of this polysaccharide are only fully revealed after chemical deacylation to remove the *O*-acetyl and *O*-glyceryl substituents, both of which are on the 3-linked D-glucose residue. The native polymer only formed soft, elastic gels but progressive removal of the acyl groups increased the brittleness of the gels produced in the presence of divalent cations such as Mg^{2+}. The extent of acetylation was thought to control the local crystallisation of parts of the polysaccharide chains (Morris and Miles, 1986). Chandrasekaran and Rhada (1995) indicated that monovalent ions such as K^+ promoted antiparallel alignment of the double helices of gellan. Divalent ions linked the gellan molecules causing gelation. The actual gel strength varied depending on the counterion added with the monovalent ions increasing from $Li^+<Na^+<K^+<Cs^+$, while Mg^{2+}, Ca^{2+} and Sr^{2+} gels were broadly similar in strength but $<Zn^{2+}$ $<Cu^{2+}$ $<Pb^{2+}$. Even stronger gels were obtainable when the polysaccharide was in the proton form (Larwood *et al.*, 1996). The L-glyceryl substituents on native gellan caused significant shielding of carboxylate groups and weakened the linkage between chains. The resultant gels were therefore weak and rubbery as opposed to the hard, brittle gels of the deacylated polysaccharide. Doner and Douds (1995) demonstrated that if gellan were first freed of all multivalent cations, it could form gels in a manner similar to algal alginates on the addition of Ca^{2+} despite the very different chemical structures of the two polysaccharides. Beads of gellan were produced when solutions of the monovalent salt form were dropped into solutions of divalent cations. This extended the applications of gellan for biotechnology in the area of cell and plant culture and enzyme immobilisation. Moritika *et al.* (1992) observed that the gelling and melting temperatures of gellan increased with both increased gellan concentration and salt concentration. This was attributed to the increased number of junction zones and decreased rotational freedom of the parallel helices. Gellan is only one of a series of polysaccharides from *Sphingomonas* spp. All possessed closely related chemical structures but none of the others formed gels although most yielded highly viscous solutions with considerable thermostability.

Modified polysaccharides

As has already been mentioned, many polysaccharides require chemical modification before they exhibit useful properties. This usually entails alkali treatment to remove acyl groups. Thanks to genetical studies or to the availability of spontaneous mutants or strains which naturally produce variants on the normal EPS structure, a number of polymers are readily available in modified form. The availability of 'families' of closely related structures has also provided considerable information on the effect of specific substituents or alterations. These EPSs and products from mutant bacteria have the advantage over material chemically modified by mild acid hydrolysis, that they are likely to be more uniform in composition and molecular weight. Xanthan has been one of the most productive polymers in this respect. Betlach *et al.* (1987) prepared a series of mutant xanthans with truncated side-chains (*Table 1*) in which either the terminal sugar (β-linked D-mannose and its attached pyruvate ketal) or the terminal disaccharide were absent. Further variants lacked *O*-acetyl groups on the internal mannose residue. Another mutant product lacking the terminal mannose residue was isolated and studied by Tait *et al.* (1989). It could be further modified by treatment with β-D-glucuronidase which removed some, though not all, of the uronic acid residues. The product lacking terminal mannose residues yielded lower solution viscosity than the wild type xanthan but removal of some uronosyl residues gave a product with higher viscosity than wild type. Modelling experiments performed by Levy *et al.* (1996) have provided some possible explanations for the observed behaviour of the truncated xanthans. These included greater flexibility of the side-chains in native xanthan and an increased quantity of open helical backbone in polymer which lacked the terminal mannose but was acetylated on the internal mannose. It was suggested that this might account for the higher viscosity of this polymer than was found for either native xanthan or the deacetylated mutant product. This hypothesis is however questionable, as no account was taken of the duplex structure which is considered to be a major feature of the ordered state of 'wild type'xanthan.

Xanthan with modified side-chains in which the terminal β-D-mannosyl residues were removed by mild acid treatment, proved capable of maintaining the ordered, double-stranded state (Christensen *et al.*, 1993a,b). Even removal of over 70% of the remaining aldobiouronic acid side-chains failed to affect the order-disorder transition. Thus, transition was not dependent on the terminal mannose of the side-chains. Removal of side-chain sugars did however affect both transition enthalpy and the intensity of the major peak at 204 nm in the CD spectrum of ordered xanthan. It also yielded sharper transition within a smaller temperature range than that seen with native xanthan. This may well result from production of polymer molecules more uniform in structure and in mass. Callet *et al.* (1987) demonstrated that in xanthans of the same molecular weight, neither acetyl nor pyruvate substituents influenced dilute solution viscosity. They did note that acetyl groups had a stabilising effect on the conformational transition of xanthans while pyruvate groups had the opposite effect. This was confirmed for a series of xanthans differing naturally in acylation by Shatwell *et al.* (1990). It was also clear that irrespective of the cations present, the salt concentration had a very marked effect on the transition temperature for all the xanthan variants studied.

When dilute solutions of native or deacetylated xanthan were mixed with either

Table 1. Comparison of 'gel strengths' (as indicated by the G' and tan δ values) for a range of xanthan/gluco- or galacto-mannan mixed systems (0.5% xanthan – 1% gluco- or galacto-mannan)

Xanthan	% substituent		Xanthan/LBG		Xanthan/KM	
	Acetate	Pyruvate	G'/Pa	Tan d	G'/Pa	Tan d
X646	4.5	4.4	430	0.095	300	0.106
X1128	7.7	1.7	93	0.25	–*	–*
X1128 DAC	0	1.3	330	0.049	114	0.15
X556	1.6	6.0	510	0.048	230	0.075
X556 Depyr	1.1	1.0	520	0.049	93	0.11
XBD9a	2.3		62	0.41		

- No data is shown as these systems failed to gel at ambient temperature
- DAC = deacetylated; Depyr = depyruvylated
- XBD9 is xanthan defective in terminal mannose (Tait *et al.*, 1989).

locust bean gum (LBG) or Konjac mannan, the viscosity was greatly enhanced (Goycoolea *et al.*, 1995) as a result of the interaction between these polymers. The actual viscosity depended on the proportions of the different polysaccharides present. Another marked difference from the individual solutions was the observation of significant thixotrophy. Most modifications have been achieved through loss or removal of acyl substituents or of side-chain monosaccharides. Scleroglucan or very similar polymers are produced by several fungal species. These are 1,3-β-D-glucans to which 1,6-β-D-glucose residues are attached on approximately every third main-chain glucose; they yield highly viscous aqueous solutions. It does not form gels in the presence or absence of ions such as Cr^{3+}. Stokke *et al.* (1995) modified scleroglucan through the introduction of varying amounts of carboxyl groups. The products then gelled in the presence of Cr^{3+}. The transition from viscoelastic solution to gel depended on the ion concentration and on the degree of carboxylation.

SYNERGISTIC GELLING

Xanthan and acetan show considerable structural similarities (*Figure 5*). Both also yield highly viscous aqueous solutions and undergo a thermally reversible order-disorder transition in solution, but differences are seen in synergistic gelling. The formation of synergistic gels when mixed aqueous solutions of xanthan and plant galactomannans are heated and cooled has received both study and application. Ross-Murphy *et al.* (1996) used a series of different xanthan preparations to demonstrate that the acyl groups of xanthan played a significant role in the interactions with guar gum, LBG and konjac mannan. Removal of the acetyl groups from xanthan enhanced gelation. Most xanthans formed a relatively strong gel network with LBG. An exception was a preparation in which there was a high acetate and low pyruvate content. The tan δ and *G'* values were rather higher and lower respectively than for the other mixtures. Removal of the acetate from this xanthan with mild alkali increased the *G'* value by almost 250% and the tan δ fell. Removal of pyruvate had little effect, indicating that it probably played little if any role in gelation. When mixed with konjac mannan, the xanthans showed similar behaviour to the interaction with LBG but higher polysaccharide concentrations were needed. However, the acetylated, non-pyruvylated xanthan failed to gel whereas the deacetylated material formed a strong

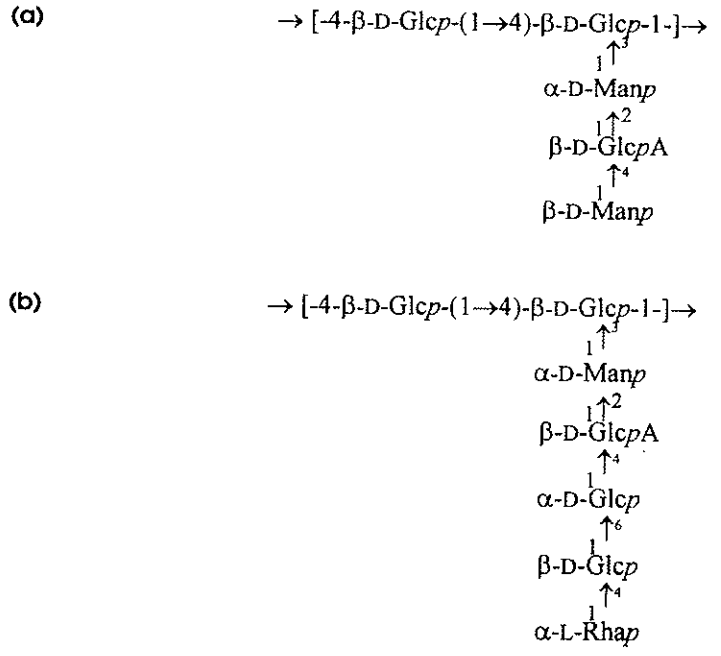


Figure 5. Comparison of the structures of xanthan and acetan. (a) The structure of the exopolysaccharide from strains of *Xanthomonas campestris* (Xanthan). Typically the polymer carries an *O*-acetyl group on each repeating unit and 0.3 pyruvate ketals on the terminal mannose residue. (b) The structure of the exopolysaccharide from strains of *Acetobacter xylinum* (Acetan). Typically the polymer carries 2 *O*-acetyl groups on each repeating unit, one of which is possibly on a main-chain glucose residue.

gel. This again demonstrated the inhibitory effect of the *O*-acetyl groups (*Table 1*). Initially, mixtures of xanthan and LBG showed areas enriched in xanthan but, after heating above the transition temperature, these disappeared, probably due to more uniform distribution of the two component polysaccharides and disappearance of the liquid crystal mesophases (Schorsch *et al.*, 1995). Variations in the ratio of mannose to galactose in LBG also affect the properties of the mixed gels. A difference in gelation temperature of almost 13°C was observed by Lundin and Hermansson (1995) when comparing mixtures of xanthan and LBG with high and low mannose:galactose ratios.

It has now been found that although native acetan does not form gels with LBG or konjac mannan, deacetylation of the bacterial polysaccharide promotes synergistic interactions with both (Ojinnaka *et al.*, 1998). Acetan resembles xanthan in that it adopts a similar conformation in the solid state and shows the same thermally reversible transition from ordered (helical form) to disordered coil in solution. The failure of the native, acetylated acetan to form mixed gels was attributed to the solubility promoted by the presence of the *O*-acetyl groups and the resultant inhibition of intermolecular association. The role played by acyl groups and the frequent need for their removal to reveal useful properties suggest that genetic manipulation of several bacterial strains to delete the polysaccharide acetylase genes might prove a useful approach. As the relevant gene or genes have already been identified in a number of

systems, such as succinoglycan production by *Rhizobium* sp. (Glucksman *et al.*, 1993) and xanthan synthesis in *X. campestris* (Betlach *et al.*, 1987), this should be feasible. Indeed, Franklin and Ohman (1996) have identified the two acetylase genes responsible for acetylation on the C2 and C3 positions of *Pseudomonas* alginate and have produced a mutant yielding non-acetylated alginate in good yield.

Biomimetics and other properties

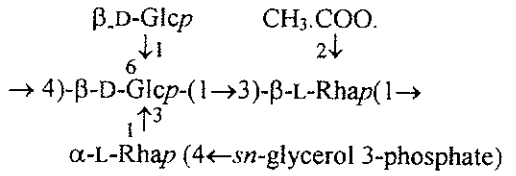
Although high solution viscosity or the ability to form gels have resulted in commercialisation of several polysaccharides, other properties may also be very useful. These may depend on either physical or biological properties. Two most valuable exopolysaccharide products have proved to be bacterial cellulose and hyaluronic acid. The former has found several specialist applications, including use in audio-membranes. It can also be manufactured into wound dressings which show excellent retention of fluid and stimulation of healing in extensive burns or similar wounds (Joris and Vandamme, 1993). Bacterial hyaluronic acid owes its acceptance to the very high capacity for water regain and to its compatibility with the human immune system. It can be found as a replacement for hyaluronic acid in human fluids or as an effective moisturising agent in high quality cosmetics. Another example of biological properties leading to novel polysaccharide applications can be found in the range of fungal 1,3-β-D-glucans which include scleroglucan. These have proved to be potent immunomodulators, a property that is still poorly understood (Misaki *et al.*, 1993).

Possible new products

Despite examination of many bacterial isolates from different parts of the world (Dasinger *et al.*, 1994) and novel environments including deep-sea thermal vents (Guezennec *et al.*, 1994; Rougeaux *et al.*, 1996), few new polymers with interesting properties have been discovered. Among the products from five deep-sea isolates, one yielded high viscosity in aqueous solution and appeared to have some properties in common to xanthan. It also possessed high affinity for certain heavy metal ions including cadmium, lead and zinc (Loaëc *et al.*, 1997). One of the few to have been fully characterised and found to be worth further investigation is the exopolysaccharide from *Alteromonas* strain 1644 (Bozzi *et al.*, 1996a,b). This polysaccharide formed a gel in the presence of divalent cations which proved to be clear and very elastic. Exopolysaccharide from a Venezuelan soil isolate (Dasinger *et al.*, 1994) also yielded highly viscous aqueous solutions insensitive to high concentrations of NaCl or $CaCl_2$. Its composition resembled that of some Rhizobium polymers in containing D-mannose, D-glucose, D-galactose and D-glucuronic acid in the molar ratio 1:4:1:2. It also contained 10–15% acetate.

Another possible area for development lies in bacterial alginates. Earlier attempts to develop these commercially were unsuccessful, primarily because of the low molecular mass of the products. This was caused by release of alginate lyases present in the polysaccharide-synthesising bacteria. While those from *Azotobacter* spp. are in effect acylated variants of the algal material, use of the extracellular poly-D-mannuronosyl-4-epimerase enzyme (Skjåk-Bræk and Larsen, 1985) may permit tailoring of the composition and hence the physical properties of alginates of bacterial or algal origin.

The structure of the exopolysaccharide from *Lactobacillus sake* 0-1



The structure of the exopolysaccharide from *Lactobacillus acidophilus* LMG9433

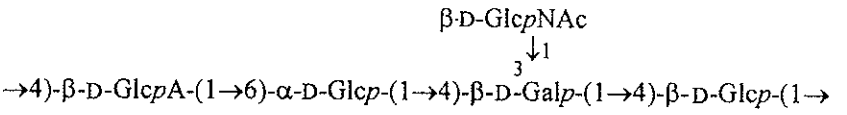


Figure 6. Structures of some *Lactobacillus* exopolysaccharides. Robijn *et al.* (1995a,b; 1996).

Future products

What emphasis will there be in the search for new exopolysaccharide products. The search for microbial polysaccharides specifically for food use has switched to studies on bacterial species such as *Lactobacillus* spp. which are already accepted food micro-organisms. This might preclude the necessity for much of the extensive testing required to obtain approval for food use. Several of these have now been characterised (*Figure 6*) (Robijn *et al.*, 1995a,b; 1996). Production of this group of polymers is beset with difficulties – yields are low and complex media and growth conditions may be required. Apart from these, it is always posible that, given the current volume of research in this area, some new polymers will be found which possess really useful and possibly unique properties. Chemically modified polymers may also yield novel properties and applications. Perhaps more useful biological properties will also be discovered.

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