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Xanthan

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Introduction

During the second half of the 20th century, many new and useful polysaccharides of scientific and commercial interest have been discovered which can be obtained from microbial fermentations. Microorganisms such as bacteria and fungi produce three distinct types of carbohydrate polymers: (1) extracellular polysaccharides, which can be found either as a capsule that envelops the microbial cell or as an amorphous mass secreted into the surrounding medium; (2) structural polysaccharides, which can be part of the cell wall; or (3) intracellular storage polysaccharides. The scientific and industrial success of polysaccharides of microbial origin is due to several factors. First, they can be produced under controlled conditions with selected species; second, they usually present a high structural regularity; and third, different microorganisms can synthesize a wide range of very specific ionic and neutral polysaccharides with widely varying compositions and properties. Such variety is not found among plant polysaccharides and, perhaps more importantly, it cannot be imitated by means of synthetic chemistry.

The usefulness of water-soluble carbohydrate polymers in industry relies on their wide range of functional properties. The most important characteristic is their ability to modify the properties of aqueous environments, that is their capacity to thicken, emulsify, stabilize, flocculate, swell and suspend or to form gels, films and membranes. Another very important aspect is that polysaccharides obtained from natural, renewable sources are both biocompatible and biodegradable.

Xanthan, a microbial biopolymer produced by the Xanthomonas bacterium, has provoked great scientific and industrial interest since its discovery in the late 1950s. In 1999 alone, more than 300 references of articles or patents dealing with xanthan are listed in Chemical Abstracts. Since 1990, more than 2000 patents have been listed in Derwent World Patents Index. This interest is due to the extraordinary properties of xanthan as well as to the successful establishment of an industrial process for its production.

Historical Outline

Xanthan gum was discovered in the 1950s by scientists of the Northern Regional Research Laboratory of the U.S. Department of Agriculture in the course of a screening which aimed at identifying microorganisms that produced water-soluble gums of commercial interest. The first industrial production of xanthan was carried out in 1960, and the product first became available commercially in 1964. Toxicology and safety studies showed that xanthan caused no acute toxicity, had no growth-inhibiting activity, and did not alter organ weights, hematological values or tumors when fed to rats or dogs, neither in short-term, nor in long-term feeding studies. The approval for food use was given by the U.S. Food and Drug administration in 1969, and the FAO/OMS specification followed in 1974. Authorization in France was given in March 1978, and approval in Europe was obtained in 1982, under the E number E415. The official definition of the EU food regulations for E415 is: “Xanthan gum is a high molecular-weight polysaccharide gum produced by a pure culture fermentation of a carbohydrate with natural strains of Xanthomonas campestris, purified by recovery with ethanol or propane-2-ol, dried and milled. It contains d-glucose and d-mannose as the dominant hexose units, along with d-glucuronic acid.
and pyruvic acid, and is prepared as the sodium, potassium or calcium salt. Its solutions are neutral. The molecular weight must be approximately 1 MDa and the color must be cream. Xanthan gum is approved as food additive with an acceptable daily intake (ADI) “not specified”; that is, no limit for ADI is defined and the gum may be used quantum satis, which means with just the quantity useful for the application. Today, xanthan is produced commercially by several companies, such as Monsanto/Kelco, Rhodia, Jungbunzlauer, Archer Daniels Midland, and SKW Biosystems. For the past few years xanthan gum has also been produced in China. Annual volumes worldwide are estimated to be about 35,000 tons in 2001.

3 Structure

3.1 Chemical Structure

Xanthan is a heteropolysaccharide with a very high molecular weight, consisting of repeating units (Figure 1). The sugars present in xanthan are d-glucose, d-mannose, and d-glucuronic acid. The glucose are linked to form a β-1,4-d-glucan cellulosic backbone, and alternate glucose have a short branch consisting of a glucuronic acid sandwiched between two mannose units. The side chain consists therefore of β-d-mannose-(1,4)-β-d-glucuronic acid-(1,2)-α-d-mannose. The terminal mannose moiety may carry pyruvate residues linked to the 4- and 6-positions. The internal mannose unit is acetylated at C-6. Acetyl and pyruvate substituents are linked in variable amounts to the side chains, depending upon which X. campestris strain the xanthan is isolated from. The pyruvic acid content also varies with the fermentation conditions. On average, about half of the terminal mannoses carry a pyruvate, with the number and positioning of the pyruvate and acetate residues conferring a certain irregularity to the otherwise very regular structure. Usually, the degree of substitution for pyruvate varies between 30 and 40%, whereas for acetate the degree of substitution is as high as 60–70%. Some of the repeating units may be devoid of the trisaccharide side chain.

3.2 Superstructure/Secondary Structure

The secondary structure of xanthan depends on the conditions under which the molecule is characterized. The molecule may be in an ordered or in a disordered conformation. The ordered confirmation can be either native or renatured; in the native form the conformation is present at temperatures below the melting point of the molecule, a temperature which depends on the ionic strength of the medium in which xanthan is dissolved. The secondary structure of xanthan and the methods to analyze it have been recently reviewed in detail by Stokke et al. (1998). X-ray scattering results indicate that
native xanthan in the ordered conformation exists as a right-hand helix with five-fold symmetry with a pitch of 4.7 nm and a diameter of 1.9 nm (Moorhouse, 1977). Two models, a single-strand helix and a double-strand or multi-strand helix, have been proposed, though most authors currently support the idea of a double helix. The helix is stabilized by noncovalent bonds, such as hydrogen bonds, electrostatic interaction, and steric effects; its structure can be described as rigid rod. In aqueous solution, the molecule may undergo a conformational transition which can be driven by changes in temperature and ionic strength, and which depends on the degree of ionization of the carboxyl groups and acetyl contents. The temperature-induced transition from an ordered to a disordered conformation is generally attributed to a complete or partial separation of the double-strand form. Renaturation may occur under favorable conditions, which means temperatures below the transition temperature and high salt concentrations. The transition from the denatured to the renatured state is reversible, whereas that from the native to the denatured state is irreversible. The model of a double-strand structure has been supported by several studies. Capron et al. (1997) demonstrated that upon heating to temperatures above the order–disorder transition temperature, denaturation of the native ordered conformation occurred, together with a reduction in molecular weight. The molecular weight is roughly halved, which supports the model of a double-strand conformation for the native form. The molecular weight was found to be invariant after renaturation on cooling. The renaturation probably occurs as an intramolecular process, which means that the restoration of the ordered form of xanthan seems to take place within a single molecule. Most likely, the conformation for the renatured form of xanthan is that of an anti-parallel, double-stranded structure consisting of one chain folded as a hairpin loop (Liu et al., 1987). The viscosity of renatured xanthan is higher than the viscosity of native xanthan however, thereby supporting the hypothesis that single-stranded xanthan molecules associate during renaturation to form supramolecular structures.

4 Occurrence

Xanthan is produced by *X. campestris*, a plant-associated bacterium that is generally pathogenic for plants belonging to the family Brassicaceae. *Xanthomonas* causes a variety of disease symptoms such as necrosis, gummosis and vascular parenchymatous diseases on leaves, stems or fruits; an example is “black rot” of crucifers such as cabbage, cauliflower or broccoli. *Xanthomonas* does not form spores, but it is very resistant to desiccation during relatively long periods. Survival at room temperature for 25 years has been reported by Leach et al. (1957). The resistance is usually due to the protective effect of the xanthan gum produced and exuded by the bacteria. Xanthan also protects the bacteria from the effects of light, and generally causes wilting of the leaves by blocking water movements (Leach et al., 1957). Exopolysaccharides, like xanthan, are also known to provide protection against bacteriophages by building a physical barrier against the phage attack (Mac-Neely, 1973). The polysaccharide is not a reserve energy source because in general the bacterium is not able to catabolize its own extracellular polysaccharide.
5
Physiological Function

The physiological function of the exopolysaccharide xanthan has received little attention compared with investigations into the molecule's production, properties, and applications. The bacteria (*Xanthomonas* sp.) that produce xanthan gum as a secondary metabolite are usually phytopathogenic, or may live in asymptomatic association with plant tissues or epiphytes. *Xanthomonas* infections have been observed in over 120 monocotyledonous and over 150 dicotyledonous plant species.

Xanthan is the predominant component of the bacterial slime. The physiological function of xanthan has been deduced as being analogous to the functions of other exopolysaccharides (Yang and Tseng, 1988; De Crecy et al., 1990; Daniels and Leach, 1993; Chan and Goodwin, 1999).

Enclosure of bacterial cells in the exopolysaccharide (EPS) results in prolonged survival, and increased resistance to both temperature and ultraviolet (UV) light (Leach et al., 1957). In rice, the wilting induced by EPS seems to play a role in pathogenesis (Kuo et al., 1970). EPS may increase cell membrane leakage, which in turn leads to wilting (Vidhyasekaran et al., 1989). In general, bacterial EPS induce water-soaking of the intercellular space which is important for bacterial colonization. It is also possible that xanthan forms a gel-like slime in the intercellular space as a result of synergy with other plant polysaccharides. This gel may then promote bacterial colonization of plant tissue by retarding the desiccation of the bacterial colony, by protecting the bacteria from bacteriostatic compounds, and by preventing close morphological contact of the colony with the cell wall, thus preventing the triggering of plant defense reactions. The amount of EPS produced by *Xanthomonas* is correlated to the organism's virulence; strains with attenuated virulence usually produce less EPS (Ramirez et al., 1988) and the distribution of the polysaccharide in infected leaves coincides with that of bacteria. This was seen in a study in rice, where EPS and bacteria were distributed in both the xylem and transverse veins (Watabe et al., 1993).

6
Analysis and Detection

In order to characterize xanthan, different parameters must be taken into consideration, such as chemical structure, acetaetate and pyruvate contents, molecular weight, secondary structure, and rheological behavior.

6.1
Chemical Characterization

By using chemical analysis, the sugar composition of the molecule as well as the nature and the degree of substituent content can be ascertained.

6.1.1
Sugar Composition

The sugar composition of xanthan is difficult to obtain as the cellulosic backbone is highly resistant to hydrolysis. Moreover, in the side chains the presence of uronic acid prevents complete hydrolysis of the aldobioionic (β-D-GlcAp-(1 → 2)-α-Manp) acid without degradation of the glucuronic acid. Well-documented reports of this situation have been made (Tait and Sutherland, 1989; Tait et al., 1990) in which the most suitable conditions to determine the neutral sugars, the aldobiouronic (β-D-GlcAp-(1 → 2)-α-Manp) acid and the substituents are described. A single condition with one form of hydrolysis is insufficient to characterize all the constit-
struments of xanthan quantitatively. Due to these problems, the official description of xanthan, e.g., by the JECFA (Joint Expert Committee for Food Additives), does not refer to its chemical composition, but only to its ability to gelify in the presence of locust bean gum (LBG). In the official description, there is no reference to acetyl groups, only to pyruvic acid.

6.1.2 Pyruvic Acid Determination

After hydrolysis (Cheetham and Punruckvong, 1985; Tait et al., 1990), the pyruvic acid content of xanthan can be determined in several ways. The oldest method described is a colorimetric procedure using 2,4-dinitrophenylhydrazine (DNPH) (Slonecker and Orentas, 1962), and this is still the reference method for the JECFA. Duckworth and Yaphe (1970), have developed an enzymatic method using lactate dehydrogenase (LDH), the reaction being as follows:

\[
\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+ \quad (1)
\]

The amount of NAD released is measured at 340 nm. More recent determinations use high-pressure liquid chromatography (HPLC) (Cheetham and Punruckvong, 1985; Tait et al., 1990) or nuclear magnetic resonance (NMR) methods, both of which permit the simultaneous detection of both pyruvate and acetate.

6.1.3 Acetate Determination

In 1949, Hestrin published a method for the determination of acetate which uses hydroxamic acid, but today (see Section 6.1.2) NMR and HPLC methods are more often used (Cheetham and Punruckvong, 1985; Tait et al., 1990).

6.2 Physical Characterization

Besides the chemical composition of xanthan, its physical characteristics such as molecular weight, secondary structure and rheological properties are the most important determinants of the behavior of this molecule in its final application.

6.2.1 Molecular Weight

Values reported in the literature for the molecular weight ($M_w$) of xanthan are usually between 4 and $12 \times 10^6$ g mol$^{-1}$. Accurate determination of the $M_w$ of xanthan is difficult for several reasons, including: (1) the very high molecular weight; (2) the stiffness of the molecule; and (3) the presence of aggregates. Nonetheless, several techniques have been used to determine the molecular weight of xanthan, including GPC-MALLS (gel-permeation chromatography with multi-angle laser light scattering), AFFF-MALLS (asymmetrical flow field fractionation combined with multangle laser light scattering) and electron microscopy.

**GPC-MALLS**

GPC-MALLS is a technique that permits the estimation of absolute molecular weight and gyration radius of polysaccharides, without the need for column calibration methods or standards. Often used in the field of polymer analysis, this technique is constituted by a GPC system which allows molecules to be separated as a function of their molecular size, and also by MALLS, which allows information to be obtained on the molecular weight of the fraction eluted from the column. With xanthan, the GPC technique presents several difficulties: first, the high molecular weight of the xanthan combined with its rod-like structure causes the xanthan...
molecules to have a very high hydrodynamic volume. The columns which are used today are unable to separate molecules with such high hydrodynamic volumes, and so xanthan appears as a monodisperse molecule. Second, for the same reason – and also due to the tight stationary phase of the column – the xanthan is submitted to a very high shear rate when eluted across the column, and this can degrade the molecule. In addition, the MALLS detection for xanthan analysis is problematic as, due to its high molecular weight, extrapolation to zero angle is not easy.

There are two classical methods to determine the molecular weight: the Zimm method; and the Debye method. In the Zimm method, we express \( K_c/R_0 = f(\sin 0/2) \), whereby \( K \) is an optical parameter and \( R_0 \) is the Rayleigh ratio. Since \( 1/M \) is obtained by extrapolation to zero angle, this method is not suitable for xanthan. The values obtained for \( 1/M \) are in the order of \( 10^{-7} \), which leads to a significant variation of the value calculated for the molecular weight. In the Debye method, we express \( \Delta R_0/K_c = f(\sin 0/2) \); hence, by extrapolation to zero angle, \( M \) can be determined directly. This method is preferable for xanthan, but even in this case the very great angular dependence prevents the linear extrapolation and a polynomial of 3rd or 4th order is needed in order to obtain reproducible results. Some authors (Capron et al., 1997) prefer to use LALLS (low-angle laser light scattering), which provides a measure at a very low angle (5°) and avoids this problem, but this type of apparatus does not provide any information on the gyration radius. Another problem is the presence of aggregates in the xanthan solution, and these are probably responsible for the very high molecular weight values reported in the literature. Such aggregates, even when present in very low quantities only, give very important signals in light scattering.

**AFF-MALLS**

In order to avoid the problems which occur with GPC, recent investigations have used AFFF (Janca, 1988). AFFF uses a narrow channel in which a solvent flows, and a field is applied perpendicularly to this channel. Usually, the perpendicular field is created by a perpendicular flow. The sample is injected into the inlet of the channel and eluted by the solvent. At the same time, the field applied across the channel presses the sample against the wall (accumulation wall) of the channel. Due to the gradient of velocity across the channel coming from the laminar flow, the particles – depending on their distances from the wall – have different velocities. In AFFF, the separation is governed by the diffusion coefficient, and so this technique allows the separation of molecules on the basis of their hydrodynamic radius up to a size of several micrometers. An advantage of this method is that no packing material is needed, and this also avoids the problem of shear. The problem is that the different parameters – the two flows, the injected volume, and the solvent – must be carefully adjusted in order to obtain good results.

**Electron Microscopy**

This technique allows direct measurements of the xanthan molecule to be made after vacuum drying in the presence of glycerol and covering the molecule with a platinum film (Stokke et al., 1998). The contour length \( L \) of individual xanthan chains can be visualized by electron microscopy, and the average value of \( L \) reflects the molecular weight. Electron microscopy can also be used to detect the formation of microgels.

### 6.2.2 Secondary Structure

The physico-chemical properties of xanthan in aqueous solutions can be studied by
means of various experimental techniques, such as light scattering measurements, hydrodynamic measurements, thermodynamic properties such as ion activity, dependence of the transition temperature on the ionic strength and calorimetric measurements. A relatively new method for studying the superstructure of xanthan is atomic force microscopy (AFM) (Capron et al., 1998a; Morris et al., 1999). AFM allows visualization of the surface of a sample, with a resolution close to the atomic scale. The mechanical properties of both native and denatured xanthan can be measured on the molecular scale (Li et al., 1999; Morris et al., 1999). AFM measures the interaction between the sample and the tip of the measuring device. A force exists between the atom of the tip of the microscope and those of the sample, separated by only a few Angstroms. By moving the tip, it is possible to follow the variation of this force on the surface of the sample and so obtain an image of the sample and estimate its shape. No modification of the sample is needed for the measurement, and this technique can be applied to both conducting and nonconducting samples. AFM avoids the drying step of the sample, and can provide images of individual xanthan molecules as well as of aggregated molecules. Molecules and molecular interactions can be imaged in the liquid environment.

Capron et al. (1997) have studied the size and conformation changes associated with the temperature-induced denaturation and renaturation of native xanthan under different salt conditions. The different methods used were LALLS, size exclusion chromatography coupled with multi-angle light scattering (SEC-MALLS), low shear intrinsic viscosity measurement, and circular dichroism. The conformational transition can be monitored using NMR, optical rotation or calorimetric measurements. The specific optical rotation changes suddenly at the melting temperature of the molecule, from about −120 to almost zero. Circular dichroism studies near 200 nm show a decrease in overall ellipticity when passing through the transition region.

6.2.3 Rheology

Depending on the medium conditions, that is polymer concentration, salts or addition of other hydrocolloids, xanthan systems can be a Newtonian or pseudo-plastic solution or a gel. The rheological behavior can be determined using viscometers by applying shear rate and measuring shear stress and viscosity or using controlled shear stress or deformation rheometers to perform dynamic viscoelastic or flow measurements. Xanthan solutions can be characterized by classical rheological parameters, such as intrinsic viscosity [η]. The intrinsic viscosity corresponds to the hydrodynamic volume of the polymer chain in a given solvent. Classically, it can be obtained by measuring the viscosity at different low concentrations in the Newtonian domain, where the overlap between hydrodynamic volume of individual polymer chains is negligible and applying the equations of Huggins (Eq. 2) and Kraemer (Eq. 3) (Launay et al., 1986):

\[
\eta_s/C = [\eta] + \lambda_1[\eta]^2C
\]

\[
\ln(\eta_s/C) = [\eta] + \lambda_2[\eta]^2C \text{ with}
\]

\[
\eta_s = \eta/\eta_b
\]

\[
\eta_s = (\eta - \eta_b)/\eta_b
\]

where \( \eta_s \) is the solution viscosity, \( \eta_b \) the solvent viscosity, \( \eta \) the relative viscosity, \( \eta_s \) the specific viscosity, \([\eta]\) the intrinsic viscosity and \( \lambda_{1,2} \) are constants which are functions of the hydrodynamic interaction between molecules. In dynamic measurement, the sample is submitted to a defor-
mation $\gamma(t)$ or a stress $\sigma(t)$ which are sinusoidal function of time. When the system is viscoelastic linear, the stress $\sigma(t)$ is a sinusoidal function of time with the same frequency $\omega$ as $\gamma(t)$ and a phase angle gap $\delta$.

$$\gamma(t) = \gamma_0 (\cos \omega t + i \cdot \sin \omega t)$$  \hspace{1cm} (6)

$$\sigma(t) = \sigma_0 (\cos (\omega t + \delta) + i \cdot \sin (\omega t + \delta))$$  \hspace{1cm} (7)

If $\delta = 0$, stress and deformation are proportional at every moment, which means that the behavior is elastic linear. If $\delta = \pi/2$, stress and deformation speed are proportional at every moment, the behavior is Newtonian. If $0 < \delta < \pi/2$ the behavior is viscoelastic. The complex modulus $G^*$ can be defined as:

$$G^* = \sigma^* / \gamma^* = G' + i \cdot G''$$  \hspace{1cm} (8)

$$G' = (\alpha / \gamma_0) \cdot \cos \delta$$  \hspace{1cm} (9)

$$G'' = (\alpha / \gamma_0) \cdot \sin \delta$$  \hspace{1cm} (10)

$$\tan \delta = G'' / G'$$  \hspace{1cm} (11)

$G'$ is the storage modulus which corresponds to the elastic component of the system, and $G''$ is the loss modulus which corresponds to the viscous component of the system. We can also define the complex viscosity:

$$\eta^* = \sigma^* / \omega = \eta' + i \cdot \eta''$$  \hspace{1cm} (12)

$$\eta' = G' / \omega$$  \hspace{1cm} (13)

$$\eta'' = G'' / \omega$$  \hspace{1cm} (14)

The gel-like structure of xanthan solutions can be characterized by deformation measurements. Small deformation measurements characterize the intact network, whilst large deformation measurements destroy the network and therefore give lower values. It is possible to measure the static yield point of a xanthan solution by applying an increasing deformation at constant shear rate and measuring the shear stress. Initially, the stress generated in resistance to the deformation increases linearly with the deformation, as in an elastic solid. Ultimately, the resistance reaches a maximum corresponding to the breaking point of the network (i.e., the yield point) and then drops again, settling down at a constant value which defines the steady shear viscosity. Xanthan gum solutions can also be characterized using dynamic light scattering. This technique allows characterization of the boundary between dilute and semi-dilute solutions. The degree of dilution is important because in a truly diluted state, the xanthan coils occupy a defined hydrodynamic volume, whereas above a critical concentration the molecules interact. Dynamic light scattering experiments can demonstrate the onset of molecular interaction and the onset of anisotropic aggregation (Rodd et al., 2000).

### 7 Biosynthesis

The path of xanthan biosynthesis has been described and reviewed by several authors (Leigh and Coplin, 1992; Becker et al., 1998). Xanthan synthesis starts with the assembly of the pentasaccharide repeating units, and these are then polymerized to produce the macromolecule. The oligosaccharide repeating units of xanthan are formed by the sequential addition of monosaccharides from energy-rich sugar nucleotides, involving acetyl-CoA and phosphoenolpyruvate. A polyisoprenol phosphate from the inner membrane functions as an acceptor (Ielpi et al., 1993). The first step of the pentasaccharide assembly is the transfer of glycosyl-1-phosphate from UDP-glucose to polyisoprenol phosphate. This transfer is followed by sequential transfer of the other sugar residues, D-mannose and D-glucuronic acid.
from GDP-mannose and UDP-glucuronic acid, respectively, which gives the complete lipid-linked repeating pentasaccharide unit. Acetyl and pyruvyl residues are added at this lipid-linked pentasaccharide stage; these are donated by acetyl-CoA and phosphoenolpyruvate, respectively. Depending on the strain and on the fermentation conditions, O-acetyl groups are attached in varying quantities to the internal mannose residue, and pyruvate is added to the terminal mannose. The xanthan chains grow at the reducing end (Figure 2). The final steps of the biosynthesis, which means the secretion from the cytoplasmic membrane, the passage across the periplasm and outer membrane and the excretion into the extracellular environment has not yet been entirely elucidated. The process requires energy, and it is probably accomplished via a specific transport system, which ensures the release of the polymer from the lipid carrier and the transport across the outer membrane (Daniels and Leach, 1993).

Many genes which are involved in xanthan biosynthesis have been identified, isolated and characterized (Figure 3). In *Xanthomonas campestris* pv. *campestris*, the biosynthesis is directed by a cluster of 12 genes, *gumB* to *gumM* (Vanderslice et al., 1989; Vojnov et al., 1998). Seven gene products are required for the transfer of the monosaccharides and for the acylation at the lipid intermediate level to form the complete acylated repeating unit. This gene cluster is not linked to the genes which are required for the synthesis of the sugar nucleotide precursors. The 12 genes of the cluster are