Enzymatic modification of bacterial exopolysaccharides

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ENZYMATIC MODIFICATION OF BACTERIAL EXOPOLYSACCHARIDES

Xanthan lyase as a tool for structural and functional modification of xanthan

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Proefschrift ter verkrijging van de graad van doctor op gezag van de rector magnificus van Wageningen Universiteit, prof. dr. ir. L. Speelman, in het openbaar te verdedigen op woensdag 2 mei 2001 des namiddags om vier uur in de Aula.

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STELLINGEN

1. De in dit proefschrift geconstateerde resistentie van het EPS van *Lactococcus lactis* ssp. *cremoris* B40 tegen biologische afbraak, wordt ondersteund door de observaties van Van Casteren et al. (1998, 1999).
(Van Casteren, W.H.M., C. Dijkema, H.A. Schols, G. Beldman, and A.G.J. Voragen. 1998. Carbohydr. Polym. 37: 123-130; Van Casteren, W.H.M., M.A. Kabel, C. Dijkema, H.A. Schols, G. Beldman, and A.G.J. Voragen. 1999. Carbohydr. Res. 317: 131-144)

2. De veelgebruikte term "decoration" voor andere groepen dan suikers in polysachariden veronderstelt ten onrechte dat deze groepen er slechts voor de sier zitten en niet bijdragen aan de functionele eigenschappen.

3. Het geringe aantal genen in het menselijk genoom ten opzichte van het aantal menselijke eiwitten, rechtvaardigt, ook voor prokaryote organismen, een kritische herbeschouwing van de manier waarop aan een DNA-sequentie een eiwit-coderende functie wordt toegekend.
(International Human Genome Sequencing Consortium. 2001. Nature 409: 860-921)

4. De aanwezigheid, in een bacteriepopulatie, van individuen met een op enigerlei wijze verhoogde mutatiesnelheid, biedt een verklaring voor de werkzaamheid van de "acclimation"-ophopingstechniek.
(Wery, J., B. Hidayat, J. Kieboom, and J.A.M. de Bont. 2001. J. Biol. Chem. 276: 5700-5706; Boe, L., M. Danielsen, S. Knudsen, J.B. Petersen, J. Maymann, and P.R. Jensen. 2000. Mutation Res. 448: 47-55; Asano, Y., K. Fujishiro, Y. Tani, and H. Yamada. 1982. Agric. Biol. Chem. 46: 1165-1174)

5. Met het spellen van het woord "nutraceuticals" als "neutraceuticals" geven Kleerebezem et al. (1999) aan weinig vertrouwen te hebben in de werkzaamheid van deze verbindingen.
(Kleerebezem, M., R. van Kranenburg, R. Tuinier, I.C. Boels, P. Zoon, E. Looijesteijn, J. Hugenholtz, and W.M. de Vos. 1999. Antonie van Leeuwenhoek 76: 357-365)

6. Het bewezen gunstige effect van nicotine op patiënten die lijden aan psychiatrische aandoeningen zoals ADHD en schizofrenie had al vermoed kunnen worden uit de oude volkswijsheid: "een tevreden roker is geen onruststoker".
(Levin, E.D., and A.H. Rezvani. 2000. Eur. J. Pharmacol. 393: 141-146)

7. Het gevoerde reorganisatiebeleid aan de Wageningen Universiteit valt niet te rijmen met de huidige profilering als "university for life sciences" .

8. Zitten is een werkwoord.
(Gerrit Th. Rietveld)

Stellingen behorende bij het proefschrift "Enzymatic modification of bacterial exopolysaccharides – xanthan lyase as a tool for structural and functional modification of xanthan".

Harald Ruijssenaars
2 mei 2001

CONTENTS

1. GENERAL INTRODUCTION

EXTRACELLULAR POLYSACCHARIDES

General Bacteria produce polysaccharides in various qualities, such as cell wall components, storage components and extracellular polysaccharides (EPSs). Like other polysaccharides, EPSs are linear or branched polymeric molecules consisting of monosaccharide units coupled by glycosidic bonds. EPSs can contain one type of monosaccharide (homoglycans) or different types of monosaccharides (heteroglycans). The monosaccharides in EPSs are mostly common hexoses, but also N-acetylhexosamines and uronic acids can be found. The sugar residues may be decorated with organic or inorganic groups such as pyruvic ketals, acetyl groups, sulphate or phosphate.

The production of EPS is a trait found in many bacterial species. A division is made between EPS that is relatively tightly associated with the cell (capsular polysaccharide or CPS) and EPS that is more loosely associated or excreted (exopolysaccharide or 'slime') [123]. In contrast to plant polysaccharides, bacterial (hetero-type) EPSs have a very regular structure, which reflects their mode of synthesis. EPSs are built up from oligosaccharide units that are synthesized intracellularly from sugar nucleotides. The complete, lipid-linked, oligosaccharide units are translocated across the cell membrane and polymerized, resulting in a sequence of identical repeating oligosaccharide units [48] (**Fig. 1.1**).

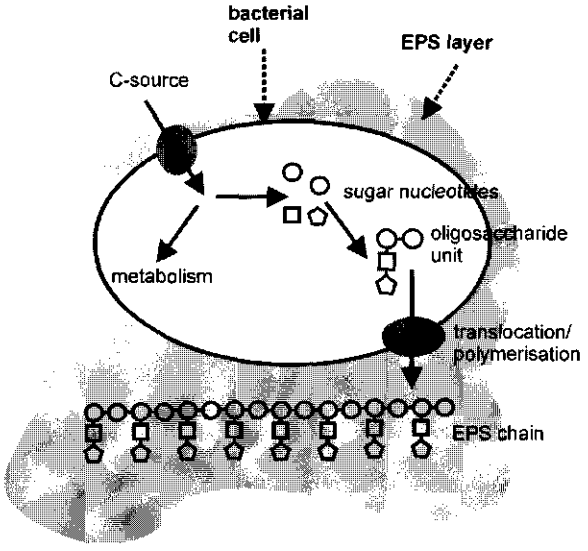


Fig. 1.1. *Schematic representation of hetero-type EPS biosynthesis.*

A completely different class of EPSs is represented by the homoglycans dextran (composed of α-1,6-linked glucosyl units with varying degrees of branching at position 3) and levan (composed of β-2,6-linked fructosyl units). These EPSs are produced extracellularly by specific glycosyltransferases.

Biological functions of EPS Several biological functions have been ascribed to EPS, the principal function being protection against hostile environmental conditions such as desiccation, harmful compounds (toxic metal ions, antibiotics), and predation by protozoa [66, 63, 123]. Furthermore, EPS may play a role in pathogenecity and protection against bacteriophage infection by shielding the parts of the cells that may act as recognition sites for the host's immune system or phages [81, 32]. For phages that specifically infect EPS-producing bacteria, however, EPS itself may act as a recognition site [61, 3, 78]. Finally, EPS is important in adhesion to surfaces and the formation and maintenance of biofilms [106]. The role of EPS as a storage component is probably limited as EPS-producing microorganisms generally cannot degrade the EPS they produce.

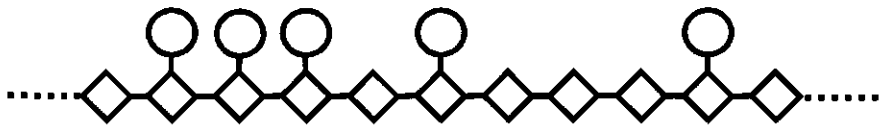
Application of EPS A number of bacterial EPSs (e.g., xanthan, bacterial alginate, pullulan, gellan, acetan) are applied on an industrial scale as a thickener, gelling agent or stabilizer of emulsions and dispersions. They can be found in products as diverse as oil-drilling muds, explosives, pesticides, paints, polishes, fire-fighting liquids, and cosmetics [5]. Only xanthan, gellan, and curdlan (U.S. and Japan) are permitted for food use. The latter two may be used to replace gelatine, which is an increasingly important issue in view of the concerns raised by BSE.

Besides being added to food to act as a stabilizer or thickener, EPS can be produced *in situ* like the EPS produced by lactic acid bacteria in fermented dairy products such as yoghurt. EPS is at least partly responsible for the peculiar rheology and texture of these products [112]. Like other polysaccharides, lactic acid bacterial EPSs may have valuable functional characteristics. An additional advantage of lactic acid bacteria is their food-grade status, allowing the use of these organisms or their products for food applications. However, lactic acid bacterial (hetero-type) EPS is generally produced in such a low amount (0.05-1.0 g/l [28]) that the application is largely restricted to systems in which the EPS is produced *in situ*.

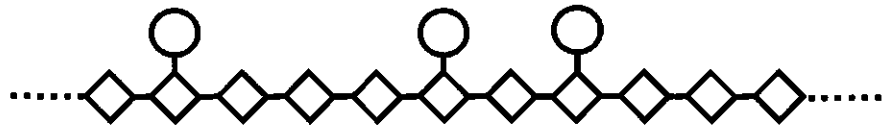
EPS can also be used as a source of unusual monosaccharides like L-rhamnose or L-fucose, which may be abundant in certain EPSs but are otherwise difficult to obtain [116]. These valuable monosaccharides can be released from the EPS by enzymatic or chemical degradation. Similarly, EPS-derived oligosaccharides can be obtained, which may be useful as prebiotic factors in food [23].

STRUCTURE-FUNCTION RELATIONSHIPS OF POLYSACCHARIDES

General The physical properties of a polysaccharide, such as gelling capacity or viscosifying ability, are the resultant of its primary structure and molecular weight. The primary structure comprises the glycoside composition, the linkage type between the glycoside residues, the presence of side chains and the decoration of the glycosyl residues. The impact of the primary structure on the functional properties can be illustrated by comparing the plant galactomannans locust bean gum (LBG) and guar gum. LBG and guar gum are β-1,4 linked mannans carrying α-galactosyl side chains at C6 of the mannosyl residues. In LBG, 20 to 25% of the mannosyl residues are substituted with galactose, whereas in guar gum the degree of substitution is 50% (**Fig. 1.2**). Although the only difference is in the degree of substitution, the functional properties of these polysaccharides are quite different: guar gum is a stronger viscosifier than LBG, whereas LBG has the capacity to form thermoreversible gels with xanthan.



guar gum



locust bean gum

Fig. 1.2. Schematic representation of guar gum and locust bean gum (LBG).

The primary structure of a polysaccharide determines the physical characteristics to a large extent. Therefore, a profound understanding of the relationship between polysaccharide structure and physical properties may be used to predict how a specific structural modification influences the functionality. This knowledge could be used to design *tailor-made* polysaccharides with specific, desired properties.

In order to gain insight into structure-function relationships of polysaccharides, systematic research on sets of closely related polysaccharides is required. The structures of the related polysaccharides should be characterized in detail and the physical properties should be established. The main chain length should remain constant to ensure that the observed effects can only be attributed to the altered primary structure. Sets

of related polysaccharides can be obtained either as such from natural sources or by specific post-synthetic modification of a parent-polysaccharide. Because of their regular structure, bacterial (hetero-type) EPSs would be ideal starting materials both for studying structure-function relationships and for manufacturing well-defined tailor-made polysaccharides.

Obtaining related EPS structures from natural sources One of the approaches to obtain sets of related EPSs from natural sources is by simply selecting EPS-producing strains and characterising the EPS produced. This approach is highly dependent on chance and extremely time-consuming. After laborious strain-selection procedures, an extensive chemical characterization of the EPS is required to determine if a new EPS is related to a known structure and therefore useful, or not.

Another possibility to obtain different, but related, EPSs can sometimes be achieved by manipulating the culture conditions during EPS-production, such as fermentation time, nutrient exhaustion, growth rate and oxygen levels. This approach to EPS modification, however, is often restricted to changes in decoration and can also result in an undesired change of the main chain length [108, 104, 99, 102, 62].

Rather than randomly screening bacteria for EPS-production or manipulating culture conditions, EPS-producing bacteria may be genetically modified to obtain sets of related EPSs [117, 64, 22]. New opportunities are created by the rapidly increasing number of EPS gene clusters being elucidated, enabling directed mutagenesis of EPS-biosynthetic genes [54]. Genetic modification of EPS-producing bacteria may, however, go hand in hand with unintentional adverse side effects. The three stages of EPS biosynthesis, i.e., sugar nucleotide synthesis, repeating unit synthesis and translocation-polymerization of the repeating units, are fully optimized with respect to each other. Therefore, it is most likely that an intervention by genetic modification at one of the levels will cause detrimental effects at the other levels. This may result in phenomena such as an altered main chain length, a completely different sugar composition (i.e., an unrelated EPS is produced), a decreased EPS-yield or even a complete loss of EPS-producing capacity [117, 46, 13].

Post-synthetic modification of EPS A different approach to obtain sets of related polysaccharide structures is by post-synthetic modification of a parent EPS, either chemically or enzymatically. Chemical modification usually employs an acid or alkaline treatment to break glycosidic bonds or to remove decorating groups. An example is the removal of different side chain residues from the EPS of *Lactococcus lactis* ssp. *cremoris* NIZO B40, using rather harsh treatments with 2 M NaOH, 28 M HF and 0.3 M H_2SO_4 [114, 115]. Although effective, chemical modification is usually not very specific and the main chain is easily affected.

Alternatively, exo-acting polysaccharide-degrading enzymes can be used for modification of polysaccharides. These enzymes generally cleave mono- or disaccharides from the non-reducing ends of the backbone or the side chains. An example is the modification of the plant galactomannan guar gum with α-galactosidase. This enzyme was used to remove α-galactosyl side groups until a polysaccharide was obtained that was similar to LBG, both in structure and functional properties [14]. Polysaccharide-degrading enzymes are generally very specific and therefore the effect of a step-by-step removal of side chain residues can be studied, leaving the backbone unaffected.

Polysaccharide-degrading microorganisms can be used as sources of polysaccharide-modifying enzymes. Such microorganisms generally produce a complex mixture of extracellular as well as intracellular polysaccharide-degrading enzymes. For use as a defined tool in polysaccharide modification, the modifying enzyme should be free from contaminating polysaccharide-degrading enzyme activities. Recombinant production of single polysaccharide modifying enzymes may be an alternative to the extensive and laborious purification procedures otherwise required.

XANTHAN: A MODEL EXOPOLYSACCHARIDE

General Xanthan, an EPS produced by various *Xanthomonas campestris* pathovars, is the major commercially produced EPS (10,000-20,000 tonnes/yr.). Xanthan is produced at a high yield: 60-70% of the substrate is converted into polymer in continuous culture [51]. In batch culture, depending on the production strain and on the fermentation conditions, xanthan can accumulate to 10-25 g/l in 48-72 h [38, 111, 79]. Xanthan has very useful functional characteristics, such as reversible shear thinning behaviour, a high viscosity at low polymer concentrations, and stability over a wide range of temperatures, pHs and ionic strengths [73]. The bulk of xanthan is applied in drilling muds for oil recovery, keeping sand and small rocks in suspension and lubricating the drill head. Xanthan received approval for food use from the U.S. Food and Drug Administration in 1969 and from the European Union in 1974 (registered as E415). No acceptable daily intake (ADI) was specified. Xanthan is used in many foods, e.g., juices, drinks, ice cream, salad dressings and desserts.

Xanthan, with its relatively complex but regular structure (**Fig. 1.3**), is an ideal model polysaccharide for systematic study of structure-function relationships. Xanthan can be viewed as a substituted cellulose: the pentasaccharide repeating units form a β-1,4 glucan backbone with a trisaccharide side chain at every second glucosyl residue. The side chain, consisting of α-mannose, β-glucuronic acid and β-mannose, may carry an O-

acetyl group at C6 of the α-mannosyl residue and a 4,6-carboxyethylidene group (pyruvate ketal) at the β-mannosyl residue. Instead of the pyruvate ketal, a second O-acetyl group may be present at C6 of the terminal side chain mannosyl residue [43, 96]. The degree of acetylation and pyruvation may vary, depending on the growth conditions and the production strain. In commercial xanthan samples, usually all internal mannosyl residues are acetylated and about 30% of the terminal mannosyl residues are pyruvated.

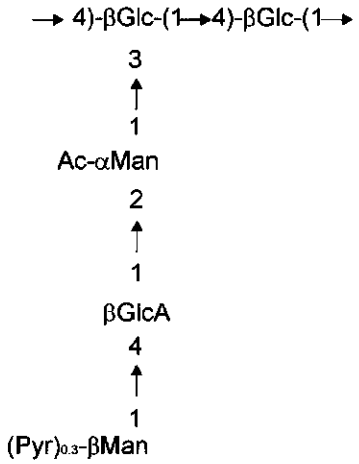


Fig. 1.3. Structure of the xanthan repeating unit. The degree of acetylation and pyruvation may vary, depending on production strain and fermentation conditions.

In solution, xanthan can be present in two conformations, depending on temperature, pH and ionic strength: as a stiff, ordered helical structure or as an unordered 'random coil' (**Fig. 1.4**). In the ordered conformation, the xanthan side chains are folded in and associated with the backbone. It has not been fully established whether the ordered conformation is a single or a double helix structure, but most evidence points towards a double helix [124]. In the unordered conformation, the side chains project away from the backbone, into the solution.

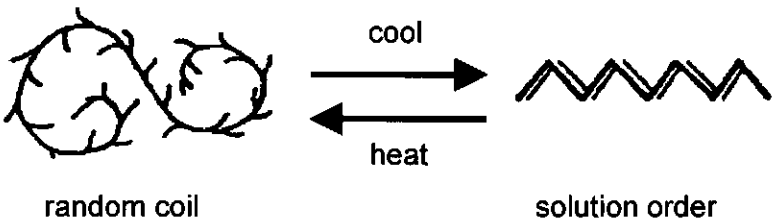


Fig. 1.4. Helix-coil transition of xanthan in solution (from Dea et al. [27]).

Xanthan interacts with galactomannans such as guar gum and LBG. With LBG, a thermoreversible gel is formed. With guar gum, the interaction results merely in a viscosity increase. The exact nature of the gel forming interactions between xanthan and LBG are still subject of controversy. Several authors suggest that the interaction involves ordered xanthan [27, 124], whereas others state that unordered segments of xanthan interact with LBG [129, 20]. Tako [109] proposed a specific interaction of the xanthan side chain with the mannan backbone by hydrogen bonding. Whichever model is true, it appears that gel formation requires a certain amount of disorder for the two polysaccharides to enter into an interaction, followed by a restoration of a certain amount of order to consolidate the gel structure (cf. Cheetham and Mashimba [19]).

Structure-function relationships of xanthan Several structural variants of xanthan have been obtained by some of the methods described earlier in this chapter. Xanthans differing in acyl-decoration (acetate and pyruvate) were obtained from different *Xanthomonas* strains [100, 104]. Also by altering fermentation conditions and by mild alkaline or acid treatment, the degree of xanthan decoration can be modified [108, 99, 10]. The acyl-decoration has a marked effect on the physical properties of xanthan. Several studies indicate that the presence of acetyl groups has an adverse effect on the viscosity and the ability to interact with other polysaccharides. This may be explained by a stabilising effect of the acetyl groups on the ordered conformation of xanthan, reducing the possibilities for intermolecular interactions [43, 92, 93, 94, 83]. The pyruvate groups have a reverse, but less pronounced, effect.

The effect of different side chain lengths on the physical properties of xanthan has been studied as well. Xanthans with truncated side chains, consisting of tetrameric or trimeric repeating units, were obtained from mutants of *X. campestris* that lacked one or more glycosyltransferases (**Fig. 1.5**) [6, 107, 117, 43, 46]. The terminal side chain mannosyl residue can also be removed chemically, by a treatment with dilute acid [21]. Furthermore, an *Acetobacter xylinum* strain was obtained that produced acetan, a xanthan-related EPS (**Fig. 1.5**). Like xanthan, acetan has a β-1,4 glucan backbone carrying a side chain at every second glucosyl. Unlike xanthan, however, acetan has a pentasaccharide side chain of which only the first two residues are identical to xanthan [50]. A mutant of this strain, *A. xylinum* CR1/4, produces a truncated side-chain acetan that is identical to mutant polytetramer xanthan except for the O-acetyl groups on the backbone (**Fig. 1.5**) [64, 22].

The side chain length appears to have a dramatic effect on the physical properties of xanthan. Compared to native polypentamer xanthan, the mutant polytetramer is a much weaker viscosifier whereas the polytrimer

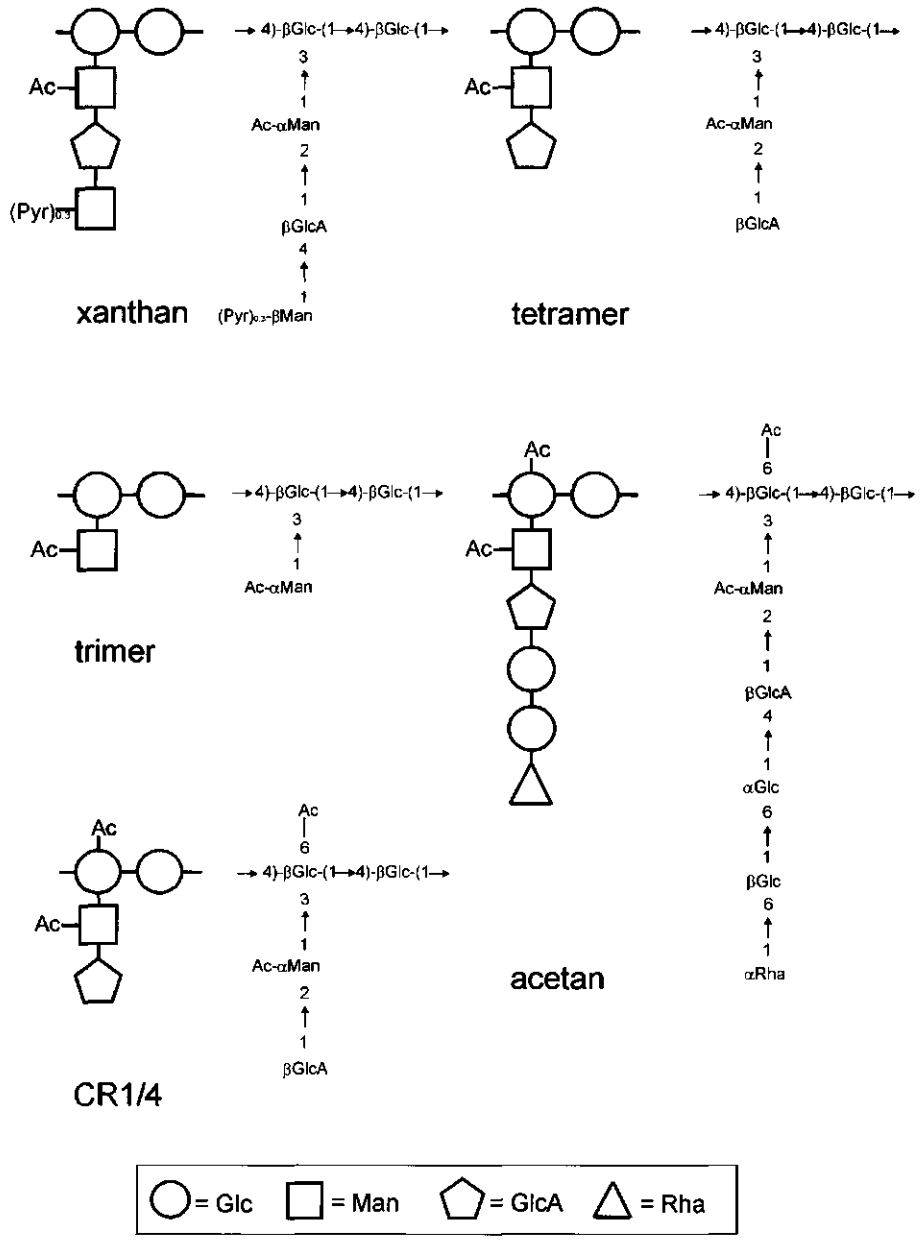


Fig. 1.5. Repeating units of xanthan and xanthan-related EPS structures.

is a superior viscosifier on a weight basis [107, 43, 6]. Acetyl-free polytetramer, on the other hand, produces a higher viscosity than native xanthan [43]. Levy et al. [60] suggest that the major difference between the low viscosity acetylated polytetramer and the polypentamer is the higher side chain flexibility of the polypentamer. They also state that the non-acetylated polytetramer shows more open helical backbone than the low viscosity acetylated polytetramer, thus giving rise to more intermolecular interactions and a higher viscosity. The (acetylated) polytetramer has been reported to adopt an ordered structure that is similar to native xanthan [69, 21]. Although similar to native xanthan in this respect, polytetramer xanthan forms an exceptionally weak gel with LBG [92], suggesting a specific role of the side chain in gel formation. The model of Tako [109] discussed above, however, cannot explain this observation, since the proposed interaction does not include the terminal residue of the xanthan side chain.

The primary structure of xanthan has been elucidated and the functional properties have been studied extensively. However, much is still poorly understood, e.g., the exact nature of the interaction between xanthan and LBG, and the precise organization of the ordered helical conformation. Furthermore, in none of the studies on the variant xanthans the main chain length or the molecular weight were defined. Therefore, the results in these studies should be interpreted with some reservation; an effect of variations in the main chain length cannot be excluded. Studies on modified xanthans, that are defined also with respect to the backbone length, may provide clarity in these matters. Because of the high specificity required, enzymatic modification of native polypentamer xanthan is the method of choice to obtain such defined variant xanthan structures.

XANTHAN-MODIFYING ENZYMES

General Xanthan-modifying enzymes can be obtained from xanthan-degrading micro-organisms. Xanthan-degrading pure cultures [101, 103, 15, 76] as well as mixed cultures [17, 47, 103] have been described. Based on the reaction products obtained with various enzyme mixtures, it has been postulated that the enzymes required to break each glycosidic bond in the xanthan molecule are available in nature [16]. It is improbable, however, that every such enzyme can attack intact xanthan. Rather, a sequential degradation is to be expected, as observed with *Bacillus* sp. GL1 [76]. In this case, the terminal side chain mannosyl residue is first removed by an extracellular xanthan lyase (see below). Then, the backbone of the resulting polytetramer is attacked by an extracellular endoxanthanase, releasing tetramer units. These are subsequently transported into the cell and further degraded intracellularly to monosaccharides by various glycosidases.

Enzymatic modification of xanthan **Figure 1.6** shows two hypothetical routes for a stepwise enzymatic modification of polypentamer xanthan to polytetramer and polytrimer xanthan. One route starts with a β-mannosidase, resulting in a polytetramer that is identical to that obtained from the *Xanthomonas* mutants described earlier. For the second modification step in this route, a β-glucuronidase could be employed. This step is equivalent to the modification of mutant polytetramer xanthan with commercial β-glucuronidase from *Escherichia coli* [107], which yielded a (partial) polytrimer xanthan with improved viscosifying properties.

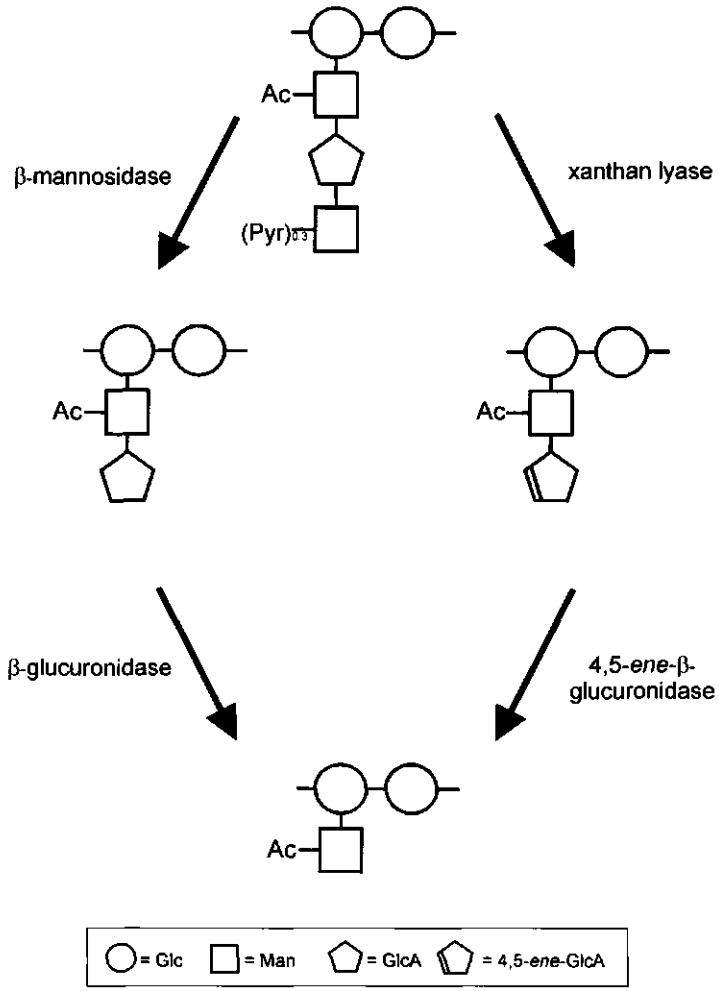


Fig. 1.6. Two hypothetical routes for enzymatic modification of xanthan. More enzymes than indicated may be required if the activity of the enzymes is affected by the acyl decorations.

The other route employs xanthan lyase as a first-step enzyme. Xanthan lyase removes the terminal mannosyl residue via β-elimination yielding mannose and a polytetrasaccharide (**Fig. 1.7**). This polytetrasaccharide is different from the mutant-polytetramers in that the terminal side chain residue is a 4,5-*ene*-β-glucuronyl instead of a β-glucuronyl. This double bond may be exploited for further chemical modification. For the second enzymatic modification step, a rather unusual enzyme is required, i.e., 4,5-*ene*-β-glucuronidase. Such an enzyme, that released 4,5-*ene*-β-glucuronyl residues from xanthan lyase-treated xanthan as well as gellan lyase-treated gellan, was recently purified and characterized by Hashimoto et al. [40].

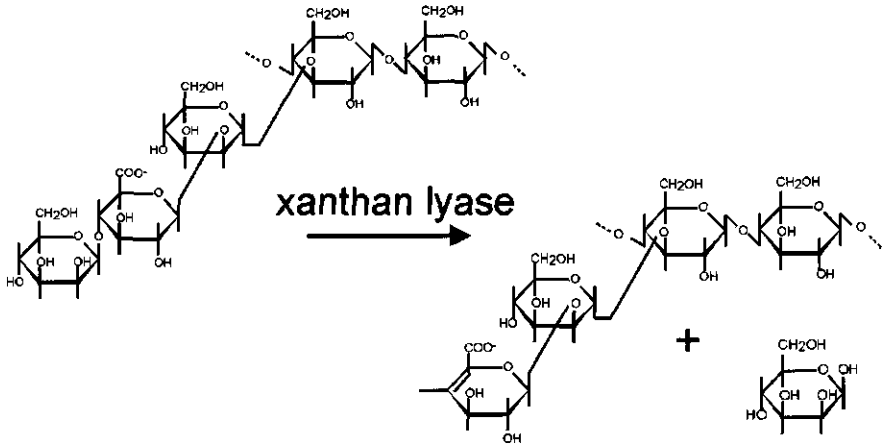


Fig. 1.7. Action of xanthan lyase on polypentamer xanthan. Acyl decorations are omitted for clarity.

It should be noted that the enzyme responsible for the removal of the terminal side chain mannose may be specific for either pyruvated or non-pyruvated xanthans. In this case, two enzymes will be necessary for a complete removal of the terminal side chain mannosyl residues. The acetyl group may cause similar problems in the second modification step.

Xanthan lyase As described above, xanthan lyase is one of the enzymes that can be employed for xanthan modification. Like other polysaccharide lyases, xanthan lyase cleaves glycosidic bonds using a β-eliminative insteadof a hydrolytic mechanism, introducing a double bond between C4 and C5 of the uronosyl residue. The formation of this double bond can be monitored by measuring A_{235} or by determining the increase of thiobarbituric acid-reactive material [105]. Lyases appear to be favoured above hydrolases for cleavage of 1,4-uronosyl linkages. Possibly, β-elimination requires less energy than hydrolysis to break a glycosidic bond

next to a uronosyl residue [105]. Also for the degradation of xanthan, β-mannosidases appear to be uncommon: only in one report such an enzyme is mentioned [101]. Among polysaccharide lyases, xanthan lyase is a unique enzyme in that it is exo-acting, removing a residue from the side chain. All other polysaccharide lyases known to date are endo-acting enzymes, cleaving the backbone.

Few reports exist on xanthan lyases. The first indication for the existence of such an enzyme was found by Lesley [59], who reported the presence of unsaturated oligosaccharides in enzymatic degradation products of xanthan. Xanthan lyase was first described as such by Sutherland [103], who partially purified xanthan lyases from a *Bacillus*, a *Corynebacterium* and a mixed culture. The action of these relatively small enzymes (30-33 kDa) was independent on the degree of pyruvation and acetylation of xanthan. The first purified xanthan lyase was described by Ahlgren [1]. This enzyme, obtained from a salt-tolerant mixed culture, had a molecular mass of 33 kDa and was specific for pyruvated mannosyl residues. An enzyme with a similar specificity, but much larger (75 kDa), was purified from *Bacillus* sp. GL1 [41].

Xanthan lyases often appear to act in conjunction with endoglucanases. The xanthan lyases described by Sutherland [103] were most active on partially depolymerized xanthan. Ahlgren [1] also suggested that xanthan lyase acted synergistically with endoglucanase: the terminal side chain mannosyl residues would be more accessible to xanthan lyase after hydrolysis of the backbone by endoglucanase. Also *Bacillus* sp. GL1 degrades xanthan with the use of an extracellular endoglucanase, however, this enzyme can only attack *after* xanthan lyase has removed the terminal side chain mannosyl residue.

No information exists about xanthan lyases on a molecular level. Hashimoto et al. [42] claimed that they had cloned the gene encoding xanthan lyase from *Bacillus* sp. GL1, however, to date no report on this work has been published.

OUTLINE OF THE THESIS

The aim of the research described in this thesis was to produce, purify and characterize specific EPS-degrading enzymes. The enzymes were to be applied for two purposes: 1) as a tool in the elucidation of structure-function relationships of EPSs and 2) for the production of tailor-made EPSs by specific, partial degradation of a parent EPS.

In Chapter 2, the biodegradability of various EPSs was assessed in order to evaluate the probability of obtaining microorganisms capable of degrading a specific EPS, and thus, potential EPS-modifying enzymes. The higher the resistance of an EPS to biological degradation, the lower this probability. As an outcome of this study, the initial target EPS, i.e., the EPS of

Lactococcus lactis ssp. *cremoris* B40, was abandoned. Instead, xanthan was chosen as a subject of further study, being readily biodegradable and a good model polysaccharide for studying structure-function relationships.

Chapter 3 reflects the studies performed on testing and developing plate screening methods for the detection of polysaccharide-degrading microorganisms. Such methods are a prerequisite for efficient screening of polysaccharide-degrading bacteria or (expression) gene libraries.

Eventually, a xanthan-degrading bacterium, *Paenibacillus alginolyticus* XL-1, was isolated from a xanthan-degrading mixed culture. This strain produces a xanthan lyase, which was purified and characterized (Chapter 4). Chapter 5 describes the isolation and cloning of the gene encoding this enzyme and its heterologous expression in *E. coli*. Chapter 6 discusses some molecular aspects of xanthan lyase as well as the opportunities for further research on the xanthan-modifying enzymes of *P. alginolyticus* XL-1.

2. BIODEGRADABILITY OF FOOD-ASSOCIATED EXTRACELLULAR POLYSACCHARIDES

Harald J. Ruijssenaars, Francesca Stingele, and Sybe Hartmans

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Abstract

Exopolysaccharides (EPSs) produced by lactic acid bacteria, which are common in fermented foods, are claimed to have various beneficial physiological effects on humans. Although the biodegradability of EPSs is important in relation to the bioactive properties, knowledge on this topic is limited. Therefore, the biodegradability of eight EPSs, six of which were produced by lactic acid bacteria, was compared with microorganisms from human faeces or soil. EPS-degradation was determined from the decrease in polysaccharide-sugar concentration and by high performance size exclusion chromatography (HPSEC). Xanthan, clavan, and the EPSs produced by *Streptococcus thermophilus* SFi 39 and SFi 12 were readily degraded, in contrast to the EPSs produced by *Lactococcus lactis* ssp. *cremoris* B40, *Lactobacillus sakei* 0-1, *S. thermophilus* SFi20, and *Lactobacillus helveticus* Lh59. Clearly, the susceptibility of exopolysaccharides to biological breakdown can differ greatly, implying that the physiological effects of these compounds may also vary a lot.

INTRODUCTION

Polysaccharides are important compounds in many foods. They are present as endogenous constituents (e.g., cell walls) or added as ingredients (thickeners, gelling agents, stabilizers). Furthermore, polysaccharides can be produced *in situ* like the exopolysaccharides (EPS) produced by lactic acid bacteria in fermented dairy products such as yoghurt.

Depending on their digestive fate, the effect of food-associated EPSs on the consumer's health can be very different. Polysaccharides that are degraded in the colon can be fermented by the colonic microflora resulting in formation of short chain fatty acids (SCFAs). SCFAs provide energy to the epithelial cells and have been claimed to play a role in the protection against colorectal cancer [25, 39]. If polysaccharides are not degraded, they can offer protection against colorectal cancer by increasing the stool bulk or by specific adsorption of carcinogens [39]. If these specific effects are involved, polysaccharides occurring in foods in small amounts can be disproportionately important in protection against colorectal cancer.

It follows from the above that the biodegradability of an EPS is an important characteristic in relation to its physiological effects. However, very

little is known about the biodegradability of these compounds. Given that one of the proposed functions of EPS in nature is protection against dessication and predation [123], EPSs can be expected to be rather resistant to biodegradation since easily degradable EPSs will not offer much protection. In view of the lack of knowledge concerning the biodegradability of food-related exopolysaccharides, we have compared the biodegradation of eight structurally characterized EPSs by microorganisms present in soil and human faeces. Six of the EPSs tested were produced by lactic acid bacteria that were isolated from various foods or food-related sources. *Lactococcus lactis* ssp. *cremoris* strain B40 was isolated from Finnish ropy milk or 'viili' [119]. *Lactobacillus sakei* 0-1 was isolated from a naturally fermented Belgian sausage [82]. *Streptococcus thermophilus* SFi12, SFi39, SFi20 and *Lactobacillus helveticus* Lh59 were isolated from several yoghurt starter cultures. Xanthan, produced by the plant pathogen *Xanthomonas campestris*, was also tested as it is the most widely applied food-grade EPS. Also, a non food-grade EPS, clavan, produced by the plant pathogen *Clavibacter michiganensis* LMG5604 [35], was tested.

MATERIALS AND METHODS

Exopolysaccharides Exopolysaccharides from *Streptococcus thermophilus* SFi12 (NCC (Nestlé Culture Collection) 2002), SFi39 (NCC 2130), SFi20 (NCC 2033), and *Lactobacillus helveticus* Lh59 (NCC 975) were obtained as described previously [58, 98, 97]. The EPS of *Lactococcus lactis* ssp. *cremoris* strain B40 was kindly provided by Remco Tuinier (Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands) [113]. The EPS of *Lactobacillus sakei* 0-1, previously named *Lactobacillus sake* 0-1 [82] was a kind gift from Aat Ledeboer (Unilever Research Laboratory, Vlaardingen, The Netherlands). Clavan was kindly provided by Petra Vanhooren (University of Gent, Belgium). Xanthan was obtained from Sigma. The structures of the EPSs are presented in **Fig. 2.1**.

Preparation of inoculum material Soil samples containing organic debris from plants and trees, taken a few cm below the soil surface, were pooled and thouroughly mixed 1:1 (wt:vol) with buffered mineral salts medium (pH 6.9) (medium D) (containing in mg l^{-1}: EDTA: 10.0; $ZnSO_4 \cdot 7H_2O$: 2.0; $CaCl_2 \cdot 2H_2O$: 1.0; $FeSO_4 \cdot 7H_2O$: 5.0; $Na_2MoO_4 \cdot 2H_2O$: 0.2; $CuSO_4 \cdot 5H_2O$: 0.2; $CoCl_2 \cdot 6H_2O$: 0.4; $MnCl_2 \cdot 4H_2O$: 1.0; $(NH_4)_2SO_4$: 2,000; $MgCl_2 \cdot 6H_2O$: 100; K_2HPO_4: 1,550; $NaH_2PO_4 \cdot H_2O$: 850). The resulting slurry was filtered through a paper filter using a Buechner funnel and a vacuum pump, and the filtrate was used as inoculum. Fresh faeces were collected from one healthy volunteer in a bottle that was preflushed with nitrogen. The sample was diluted 1:1 (wt:vol) with CO_2-saturated 2 × concentrated medium D. The

resulting slurry was continuously bubbled with CO_2 to maintain anaerobiosis. The faecal slurry was filtered through two layers of cheese cloth, and the filtrate was used as inoculum.

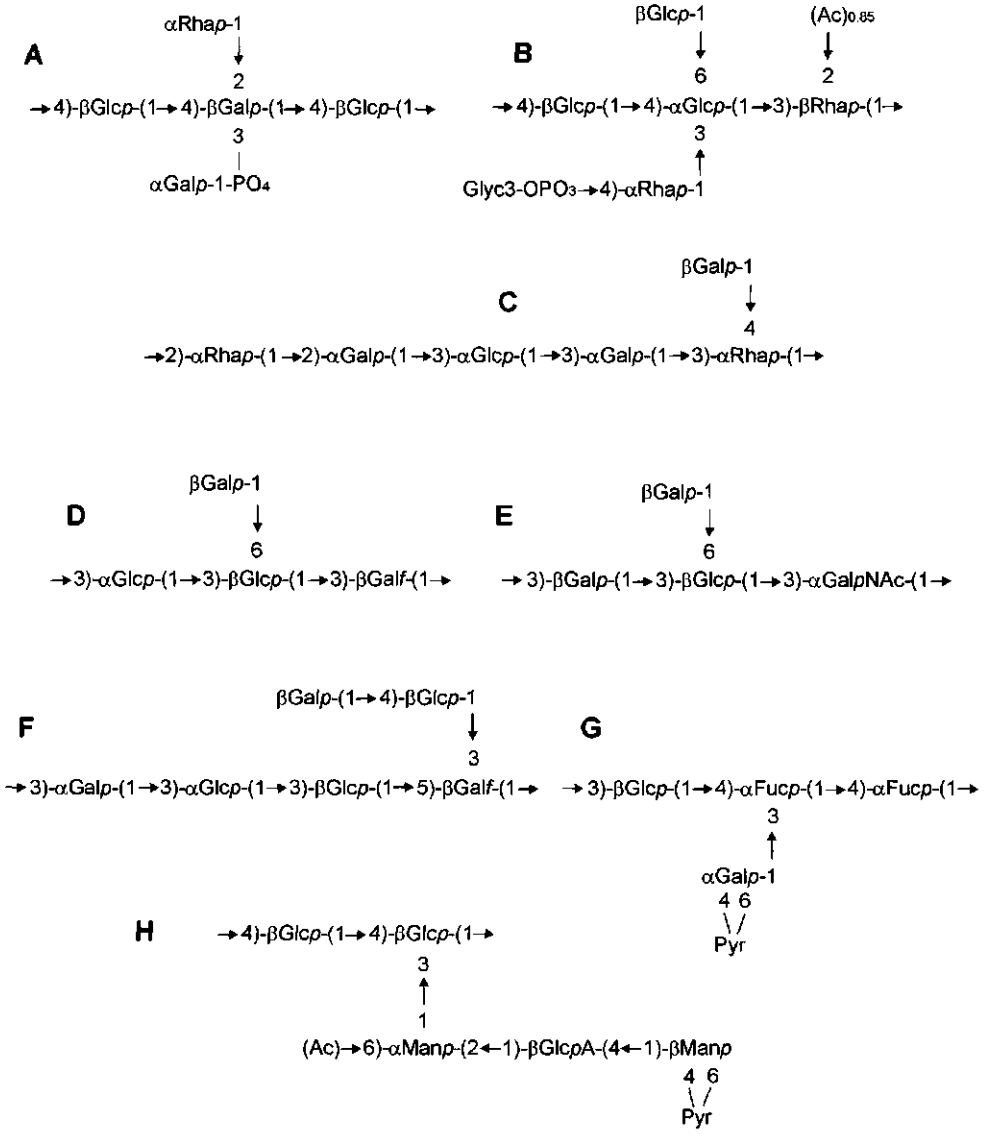

Fig. 2.1. Structures of the exopolysaccharides tested in this study. A: EPS produced by *Lactococcus lactis* ssp. *cremoris* B40 [114]; B: EPS produced by *Lactobacillus sakei* 0-1 [82]; C: EPS produced by *Streptococcus thermophilus* SFi12 [58]; D: EPS produced by *Streptococcus thermophilus* SFi39 [58]; E: EPS produced by *Streptococcus thermophilus* SFi20 [58]; F: EPS produced by *Lactobacillus helveticus* Lh59 [97]; G: clavan [35]; H: xanthan.

Media and incubations For the incubations with soil-filtrate as inoculum, the EPSs were dissolved in medium D at a concentration of 2.65 g l^{-1}. Serum bottles (130 ml) were filled with 5 ml of the EPS-media and autoclaved. Each bottle was inoculated with 100 µl of inoculum and incubated with shaking at 30°C. For the incubations with faecal slurry-filtrate as inoculum, bottles (120 ml) were filled with 2.5 ml of 2 × concentrated EPS-media and sealed. Air was removed from the bottles by applying vacuum to the bottles, with subsequent flushing with N_2. This procedure was performed twice prior to autoclaving. The bottles were inoculated with 2.5 ml of faecal slurry, resulting in a final EPS-concentration of 2.65 g l^{-1}, and incubated statically at 37°C. All incubations were carried out in duplicate. Samples (500 µl) were drawn after 0, 1, 2, and 5 days of incubation. The samples were centrifuged and the supernatants were stored at -20°C until analysis.

Determination of EPS degradation The decrease of the EPS-sugar concentration in the cultures (i.e., the consumption of polysaccharide sugars) was determined as a measure for the biodegradation of EPS. The EPS-sugar concentration was calculated for each sample according to EPS = TS – RS (EPS = EPS-sugar concentration, TS = total soluble carbohydrate concentration, RS = reducing sugar concentration). The total soluble carbohydrate concentration (TS) was measured by the phenol-sulfuric acid method [29]. TS comprises both polysaccharide sugars and non polysaccharide sugars (i.e., low-molecular-weight (LMW) sugars). The reducing sugar concentration (RS), determined according to the dinitrosalicylic acid (DNS) method [70], was used as a measure for non polysaccharide sugars and subtracted from the total sugar concentration to obtain the EPS-sugar concentration. Glucose was used as a standard in both sugar assays, and all concentrations were expressed as mmol l^{-1} glucose equivalents. The sugar analyses were performed in duplicate on samples from duplicate cultures. The EPS-sugar consumption was corrected for adsorption of EPS to inoculum material (control incubations on EPS-media with autoclaved inocula) and for consumption of polysaccharides from the inoculum (control incubations on medium without polysaccharide).

HPSEC analyses were carried out on twofold diluted and centrifuged culture supernatant samples with an SP 8700 HPLC-system equipped with 3 Bio-Gel TSK columns in series: 60 XL, 40 XL, and 30 XL (each 300 x 7.5 mm, Bio-Rad), together with a TSK XL guard column (40 x 6 mm). The system was eluted with 0.4 mol l^{-1} Na acetate (pH 3.0) at a flow rate of 0.8 ml min^{-1} at 30°C. Peaks were detected with a refractive index (RI) detector.

RESULTS

Inoculum treatment Exopolysaccharides from various sources were dissolved in buffered mineral salts medium and inoculated with soil or human faecal inocula. The inocula were filtered to minimize the amount of suspended particles to which EPS may adsorb or that could interfere with HPSEC analysis. As a consequence, the number of microorganisms in the inocula were reduced: filter paper retained about 90%, and two layers of cheese cloth retained about 30% of the CFU's.

EPS-degradation by soil microorganisms Culture supernatants were analysed for total soluble carbohydrate and reducing sugar content after 0, 1, 2 and 5 days. **Table 2.1** shows the EPS-sugar concentrations for day 0 and day 5, as well as the EPS-consumption, for the cultures with soil as the inoculum. The data in the table are the means of four EPS concentrations, calculated from duplicate sugar analyses on samples from duplicate cultures. EPS was considered not to be degraded if the standard deviation (SD) exceeded the amount of EPS-sugars consumed.

Table 2.1. EPS-sugar concentrations and consumption in the incubations with soil as inoculum at t = 0 and t = 5 days[a].

EPS-source	EPS-sugar (mmol l^{-1} glc-equivalents)		EPS-sugar consumed (mmol l^{-1} glc-equivalents)	Degradation
	t = 0	t = 5 d		
L. lactis ssp. *cremoris* B40	9.0 ± 0.7	8.0 ± 0.5	1.0 ± 1.2	-
L. sakei 0-1	7.5 ± 0.7	6.2 ± 0.8	1.3 ± 1.5	-
S. thermophilus SFi12	9.4 ± 3.5	5.2 ± 0.5	4.2 ± 4.0	±
S. thermophilus SFi39	14.8 ± 0.7	10.7 ± 0.7	4.1 ± 1.4	+
S. thermopilus SFi20	6.8 ± 1.8	7.4 ± 2.1	-0.6 ± 3.9	-
L. helveticus Lh59	6.0 ± 0.8	6.4 ± 0.9	-0.4 ± 1.7	-
C. michichanensis LMG5604	9.0 ± 0.4	6.7 ± 1.1	2.3 ± 1.5	+
X. campestris	14.0 ± 3.8	0.1 ± 0.1	13.9 ± 3.9	+

[a]Sugar analyses were performed in duplicate on samples from duplicate incubations.

It is clear from **Table 2.1** that the majority of the EPSs were not degraded by the soil inoculum. The amount of EPS-sugars consumed of the EPS produced by *S. thermophilus* SFi 12 was just above the standard deviation; the EPS produced by *S. thermophilus* SFi 39, clavan, and xanthan were significantly degraded. The soil inoculum did not contain detectable amounts of carbohydrate (results not shown). Therefore, no correction was required for polysaccharide consumption from the inoculum. The control

cultures with autoclaved soil suspension did not show a decrease in EPS concentration with either inoculum (results not shown), indicating that EPS did not adsorb to the soil material which was removed during centrifugation of the samples. Hence, no correction for this phenomenon was required. Degradation of EPS in the soil culture samples was also assessed by HPSEC. For the EPSs that were not degraded, like the EPS produced by *Lactococcus lactis* ssp. *cremoris* B40, no change in the HPSEC elution pattern was observed, confirming the results of the sugar measurements. If an EPS was significantly degraded, the EPS peak disappeared, as indicated in **Fig. 2.2 A** for the EPS of *S. thermophilus* SFi39. It is clear that most of the EPS (eluting between 31 and 37 min) present at day 0 had disappeared after 5 days of incubation. Also some LMW compounds (eluting after 42 min) had disappeared. With clavan, only very little EPS consumption was found based on the sugar measurements. The HPSEC chromatogram (**Fig. 2.2 B**) shows an EPS peak at day 0 eluting between 29 and 36 min, whereas at day 5 a peak could be observed eluting between 34 and 42 min. Thus, a decrease of the molecular weight rather than disappearance of the EPS was observed here.

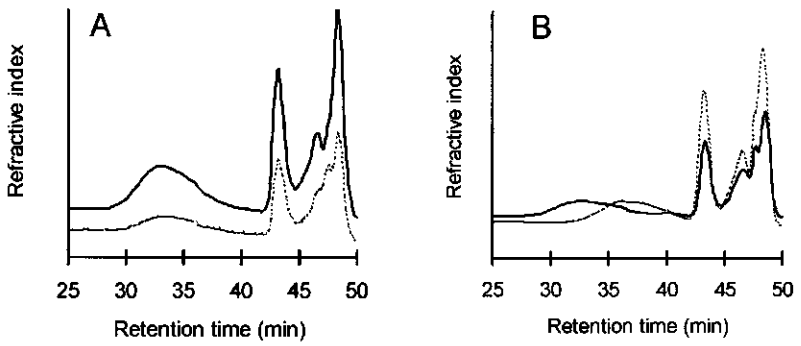

Fig. 2.2. A. HPSEC chromatogram of the incubation containing SFi39-EPS, inoculated with soil suspension at t = 0 (solid line) and t = 5 days (dashed line). **B.** HPSEC chromatogram of the incubation containing clavan, inoculated with soil suspension at t = 0 (solid line) and t = 5 days (dashed line).

EPS-degradation by faecal microorganisms The culture supernatants of the faecal slurry cultures were analysed similarly to the soil culture supernatants. The results in **Table 2.2** demonstrate that, also in the faecal slurry cultures, most EPSs were not degraded. As with the soil cultures, the EPS produced by *S. thermophilus* SFi 39 and xanthan were readily degraded. Also, the EPS produced by *S. thermophilus* SFi 12 was clearly degraded by

the faecal slurry inoculum, whereas clavan was not. No correction was required for polysaccharide consumption from the inoculum nor for adsorption of EPS to the inoculum material (results not shown).

Table 2.2. EPS-sugar concentrations and consumption in the incubations with faecal slurry as inoculum at t = 0 and t = 5 days[a].

EPS-source	EPS-sugar (mmol l^{-1} glc-equivalents)		EPS-sugar consumed (mmol l^{-1} glc-equivalents)	Degradation
	t = 0	t = 5 d		
L. lactis ssp. *cremoris* B40	9.1 ± 2.9	8.2 ± 1.5	0.9 ± 4.4	-
L. sakei 0-1	3.4 ± 1.5	4.9 ± 0.4	-1.5 ± 1.9	-
S. thermophilus SFi12	10.2 ± 1.3	0.0 ± 0.5	10.2 ± 1.8	+
S. thermophilus SFi39	16.5 ± 1.5	4.2 ± 0.2	12.3 ± 1.7	+
S. thermophilus SFi20	9.6 ± 1.5	9.8 ± 1.2	-0.2 ± 2.7	-
L. helveticus Lh59	8.2 ± 2.0	7.1 ± 1.7	1.1 ± 3.7	-
C. michiganensis LMG 5604	10.0 ± 2.5	8.7 ± 1.2	1.3 ± 3.7	-
X. campestris	11.5 ± 3.1	3.5 ± 0.2	8.0 ± 3.3	+

[a]Sugar analyses were performed in duplicate on samples from duplicate incubations.

The initial rate of degradation of the EPSs that were degraded both by the faecal slurry and the soil inocula, was faster in the faecal slurry cultures, as demonstrated in **Fig. 2.3.** No clear relation, however, could be observed between the inoculum material and the extent to which EPS was degraded. Xanthan was almost completely mineralized in the soil culture, whereas the degradation in the faecal slurry culture ceased at about 35%. In contrast, the EPSs produced by *S. thermophilus* SFi 39 and SFi12 were degraded to a greater extent in the faecal slurry culture. Degradation of EPS in the faecal slurry cultures was not determined by HPSEC. This technique proved to be unsuitable for the faecal slurry culture samples, as low signals and drifting baselines were obtained.

DISCUSSION

In this study we have compared the biodegradability of EPSs from a number of bacteria, the majority of which were food-grade. The EPSs were dissolved in mineral salts medium and inoculated with soil or faecal inocula. Human faeces were used to assess the *in vitro*-biodegradability by colonic microflora, whereas soil was chosen as an environmental inoculum material.

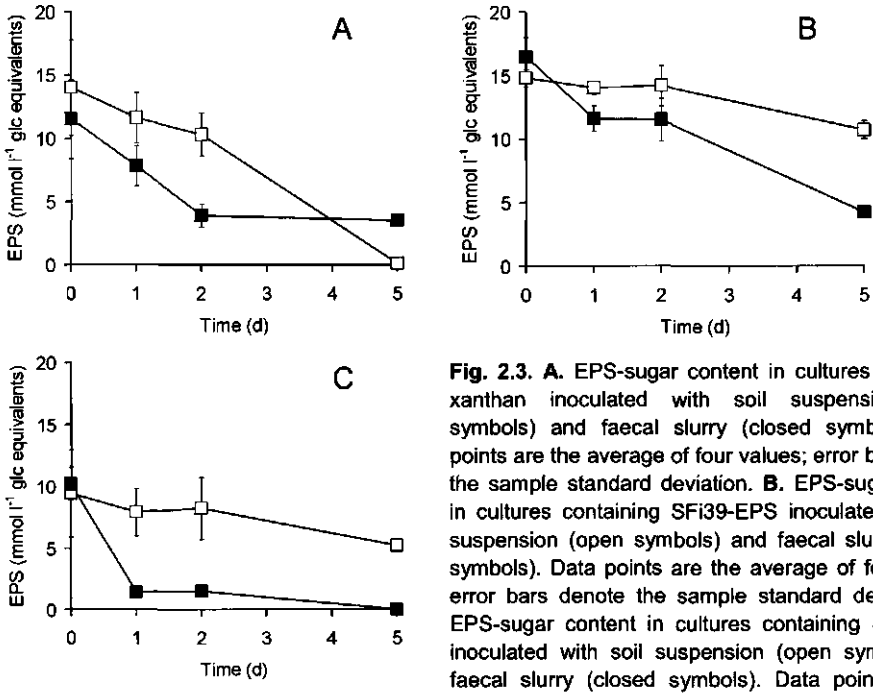

Fig. 2.3. A. EPS-sugar content in cultures containing xanthan inoculated with soil suspension (open symbols) and faecal slurry (closed symbols). Data points are the average of four values; error bars denote the sample standard deviation. **B.** EPS-sugar content in cultures containing SFi39-EPS inoculated with soil suspension (open symbols) and faecal slurry (closed symbols). Data points are the average of four values; error bars denote the sample standard deviation. **C.** EPS-sugar content in cultures containing SFi12-EPS inoculated with soil suspension (open symbols) and faecal slurry (closed symbols). Data points are the average of four values; error bars denote the sample standard deviation.

Most EPS preparations used were obtained from EPS-producing bacterial cultures by alcohol or acetone precipitation without additional purification. Commonly used methods to determine the biodegradability of organic compounds, such as the measurement of the biological oxygen demand (BOD) or the formation of gases like CO_2 or CH_4, were considered unsuitable for monitoring EPS degradation, because metabolization of impurities like protein would be wrongly interpreted as degradation of EPS. Therefore, the EPS-sugar consumption was determined, calculated from the total carbohydrate and the reducing sugar concentrations, to quantify the EPS-degradation. It was assumed that the LMW sugars, represented by the reducing sugar concentrations, were monosaccharides. Since the absence of oligosaccharides was not verified, the EPS-sugar concentrations may have been slightly overestimated. Adsorption of EPS to inoculum material and consumption of polysaccharides from the inoculum material could be neglected.

Under the conditions used in this study, most EPSs tested were resistant to biodegradation, in accordance with the proposed protective function of EPS in the natural environment. Only xanthan, clavan and the EPSs produced by *S. thermophilus* SFi 12 and SFi 39 were consumed significantly. HPSEC analysis confirmed the degradation results in the soil

cultures. The chromatograms showed that EPS either disappeared or stayed intact. In the case of clavan, HPSEC showed that the EPS was not completely metabolized; rather its molecular weight decreased, in accordance with the limited consumption of EPS-sugar as established from the sugar analyses. Furthermore, the amount of LMW compounds (eluting after 42 min) had increased after 5 days, suggesting that clavan was partially degraded to oligosaccharides that were consumed only to a limited degree.

In the soil cultures, the initial number of microorganisms was much smaller than in the faecal slurry cultures owing to the smaller inoculum size. This probably explains the higher initial biodegradation rates found in the faecal slurry cultures. No relationship could be observed, however, between the net biodegradation results and the inoculum material, and thus, indirectly, by the initial number of microorganisms present. The four EPSs that were degraded at all were degraded in the soil cultures, whereas only three of these EPSs were degraded in the faecal slurry cultures. Therefore, it can be assumed that filtration of the inocula did not seriously affect the net biodegradation results despite the loss of a number of microorganisms.

The structures of the EPSs tested in this study differ in many aspects. Therefore, it is not possible to relate the susceptibility to biological degradation directly to the primary structure. Several observations may, however, be made. The repeating units of the two lactic acid bacterial EPSs that were readily degraded had relatively simple structures, with only a single β-galactosyl residue as a side chain. In contrast, the repeating units of the EPSs of *L. lactis* ssp. *cremoris* B40 and *L. sakei* 0-1, which were both not degraded, contain a backbone residue that is substituted by two residues, one uncharged and one charged. This double substitution may greatly impair the accessibility of the EPS to degrading enzymes. The EPS of *L. lactis* ssp. *cremoris* B40 was indeed resistant to degradation by many enzyme preparations: only after chemical removal of the galactosylphosphate residue could one of the tested enzyme preparations degrade this EPS [114]. When the rhamnosyl residues were also partially removed, degradation could proceed even further. The other EPSs that appeared to be rather recalcitrant (SFi20 and Lh59-EPS) do not share any structural similarities.

Although the degradation conditions used may have been somewhat restrictive (one type of soil, faeces from one human volunteer, loss of microorganisms from the inocula by filtration, and a relatively short incubation period), it is clear that EPSs can differ greatly in their susceptibility to biodegradation. As the biodegradability of EPSs is expected to be directly related to the potentially beneficial health effects, further studies are required. Especially, studies on the colonic microflora of humans who regularly consume EPS-containing foods, as well as the digestive fate of EPS, could provide more insight into this matter.

Acknowledgements

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3. PLATE SCREENING METHODS FOR THE DETECTION OF POLYSACCHARASE-PRODUCING MICROORGANISMS

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Abstract

Polysaccharide-degrading enzymes (polysaccharases) are widely applied in industry. One of the sources of these enzymes are polysaccharide-degrading microorganisms. To obtain such microorganisms from enrichment cultures, strain collections or gene libraries, efficient plate screening methods are required that discriminate between intact and degraded polysaccharide. This can be achieved by making use of specific physicochemical properties of the polysaccharide, like complex formation with dyes and gelling capacity, or by application of dye-labelled polysaccharides. In this review, a survey is presented of plate methods based on these principles. Both theoretical and practical aspects of the methods are discussed.

INTRODUCTION

Polysaccharides are linear or branched polymeric molecules consisting of monosaccharide units coupled by glycosidic bonds. Polysaccharides can contain one type of monosaccharide (homoglycans) or different types of monosaccharides (heteroglycans). Furthermore, non-sugar substituents like pyruvic ketals, acetyl groups or sulphate groups can be present. Polysaccharides, both of vegetable and microbial origin, are widely applied in industry as thickener, gelling agent and stabilizer of emulsions and dispersions.

Besides polysaccharides, also polysaccharide-degrading enzymes (polysaccharases) are widely applied in industry (pulp-and-paper, textile, food) and in products like detergents. Polysaccharases cleave glycosidic bonds using either a hydrolytic mechanism (glycosidases) or a β-eliminative mechanism (lyases). In addition, these enzymes act either in an endo- or an exo-fashion. Endopolysaccharases cleave glycosidic bonds inside the main chain of the polysaccharide. These enzymes are applied in processes that require depolymerization of polysaccharides, e.g., in fruit juice manufacture, pectinases and cellulases are used to degrade fruit cell walls. Purified endopolysaccharases are also used as analytical tools to elucidate polysaccharide structures: the complex polysaccharides are specifically cleaved to oligosaccharides that are relatively easy to analyse [120].

Exopolysaccharases cleave glycosidic bonds from the exterior of the polysaccharide, generally releasing mono- or disaccharide units from the non-reducing ends of the backbone or the side chains. Exopolysaccharases can be applied to obtain 'tailor-made' polysaccharides, i.e., the structure of the polysaccharide is specifically modified to introduce altered functional properties. An example of this application is the use of α-galactosidase to decrease the degree of substitution of the galactomannan guar gum, resulting in a polysaccharide that resembles the functionally superior (and more expensive) locust bean gum [14]. Purified exopolysaccharases can also be applied as analytical tools for sequencing and structural characterization of oligosaccharides [49].

Like their substrates, polysaccharases are widespread in nature. They can be found in every type of organism including mammals, plants, algae, moulds, bacteria and phages. For the production of polysaccharases, microorganisms are usually most convenient. Many microorganisms excrete polysaccharases to degrade polysaccharides to mono-, di- or oligosaccharides that are transported into the cell for further metabolization. Polysaccharase-producing microorganisms can be obtained from enrichment cultures on the target polysaccharide or from strain collections, libraries obtained by directed evolution, and (expression) gene banks. For the screening of these large numbers of microorganisms, efficient plate screening methods are a prerequisite.

In principle, polysaccharide degradation can be determined by monitoring microbial growth on plate media with the polysaccharide as a sole source of carbon and energy. In this case, however, the polysaccharide must be very pure. Growth on organic impurities like protein, which are common in polysaccharide preparations, may be interpreted as growth on the polysaccharide. Furthermore, polysaccharase-producers will not be detected if the polysaccharide is degraded but not used as a growth substrate. Therefore, more reliable plate screening methods are required that allow unambiguous discrimination between intact and degraded polysaccharide. Preferably, these methods should not only demonstrate polysaccharide degradation, but also provide an indication of the quantity of polysaccharase that is produced. Such methods may be based on specific physico-chemical properties of the polysaccharide of interest; these properties should make the presence of the polysaccharide detectable in the plate medium and they should change or disappear when the polysaccharide is degraded. Alternatively, dye-labelled polysaccharides can be incorporated into plate media.

A number of assays for the degradation of polysaccharides have been described in literature. In this review, a survey is presented of plate methods for the detection of polysaccharase-producing microorganisms. Some methods were originally developed as plate assays, whereas others were set

up as liquid enzyme assays and have been adapted for use in plate media. The principles of the methods are described as well as their assets and limitations. Furthermore, some practical aspects are discussed.

PLATE SCREENING METHODS BASED ON COMPLEX FORMATION BETWEEN POLYSACCHARIDES AND DYES

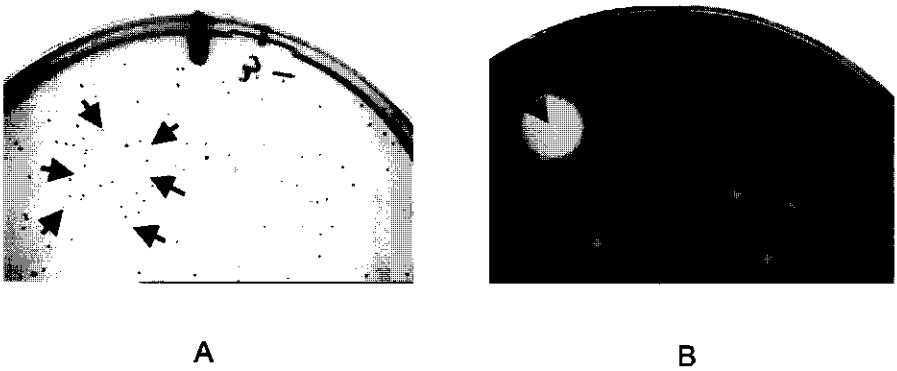
Some polysaccharides interact non-covalently with dyes. This interaction can be used to make the polysaccharide visible in a plate medium. The plates are flooded with a dye solution and incubated to allow the interaction to take place. After washing off unbound dye, stained areas will be visible that contain the intact polysaccharide whereas unstained spots will appear where the polysaccharide has been degraded.

The dye Congo red (CR) interacts with (1→4)-β-D-glucans, (1→3)-β-D-glucans, (1→3)(1→4)-β-D-glucans and (1→4)-β-D-xylans [125]. This interaction has been used in plate screening methods, e.g. to enumerate cellulolytic bacteria in the bovine rumen [110]. CR has also been used to detect cellulolytic enzyme activity in electrophoretic gels (zymogram technique) [11] and in enzyme fractions by means of a gel-diffusion assay [126]. In this case, enzyme fractions were incubated in holes punched in an agar plate containing carboxymethyl-cellulose (CMC). After subsequent staining with CR, haloes could be observed around the holes containing the active fractions.

For the CR assay, microorganisms are plated on a suitable medium with 5-10 g/l of substrate (e.g. CMC or oat gum). After incubation, the plates are flooded with an aqueous CR solution (1 g/l) and left to stand for 10-15 min. Subsequently, the CR solution is poured off and replaced by 1 *M* NaCl for 10-15 min to maximize the binding of the dye to the polysaccharide. Finally, the plates are washed with several changes of demineralized water to remove unbound dye. Degradation zones are visible as non- or lightly coloured haloes around the polysaccharide-degrading colonies. This is demonstrated in **Fig. 3.1**, showing a CMC-plate with colonies from an enrichment culture on CMC, before and after CR-staining. Only a small portion of the colonies appeared to be surrounded by haloes, which differed in size. The diameter of the halo provides a preliminary quantification of the enzyme activity produced. After the CR staining procedure, the plates may be flooded with 1 M acetic acid or HCl. The colour will change from red to blue, sometimes increasing the contrast between degradation zones and background. Congo red can also be added as a medium component [44], but the results are not always as satisfactory.

In our laboratory, CR staining has also been used to demonstrate the degradation of xanthan side chains by *Paenibacillus alginolyticus* XL-1 in a plate medium. Xanthan can be viewed as a cellulose molecule with a

trisaccharide side chain on every second glucosyl residue. Instead of a colourless zone as for CMC degradation, a red zone was observed around the colonies. Apparently, the backbone retained its polymeric character whereas the side chains were removed to such an extent that the cellulosic backbone could interact with the dye [85].



A B

Fig. 3.1. Detail of a plate containing mineral salts medium with carboxymethylcellulose (CMC) (5 g/l), solidified with agar (20 g/l). **A**: unstained plate showing colonies from an enrichment culture on CMC. **B**: plate after staining with Congo red (CR); the colonies were washed off prior to staining. Arrows indicate the CMC-degrading colonies and the corresponding haloes after CR staining.

Also other complexing dyes have been used to demonstrate polysaccharide-degradation in plate screening methods. Ruthenium red was used with alginate and pectin [33, 34]. The fluorescent complex that is formed between calcofluor white and β-glucans has been employed to detect β-glucanase in a gel-diffusion assay [65]. Another well-known interaction, although not strictly a dye-polysaccharide interaction, is the formation of a blue coloured complex between amylose and iodine. Flooding of starch plates with Lugol's iodine solution results in dark blue plates with uncoloured zones where the starch has been degraded.

The flooding procedure may cause several problems. Colonies may be dislodged and cross-contamination may occur, impairing proper isolation of microorganisms. Furthermore, colonies may not be dislodged until after the dye-incubation step. In this case, the dye had no access to the polysaccharide and unstained spots will appear that may -mistakenly- be interpreted as a degradation zones. In addition, the staining reagent may be toxic to the microorganisms on the plate. These problems can be overcome by using replica plates that are washed prior to staining to remove the colonies and allow the dye full access. Colonies corresponding with haloes on the stained plates can subsequently be isolated from the master plates.

Alternatively, an overlay technique can be used, like the triple-layer technique discribed by Wood et al. [126]. Here, an agar layer containing the cells is poured on top of a basal medium layer, followed by a third layer containing the substrate. After incubation, the plates can be stained as described above without the risk of cross-contamination or dislodging of colonies. Colonies can easily be isolated by jabbing a sterile toothpick through the top layer.

PLATE SCREENING METHODS USING GEL-FORMING (MIXTURES OF) POLYSACCHARIDES

Some polysaccharides have the capacity to form gels: the polysaccharide-chains can associate, forming a three-dimensional network in which the solvent (water) is immobilized. Sometimes metal ions are required for gelation, e.g., K^+ for κ-carrageenan and trivalent metal ions like Cr^{3+}, Fe^{3+} or Al^{3+} for xanthan [77]. Furthermore, combining two polysaccharides may result in a mixture with gelling properties that are different from those of the polysaccharides individually. Xanthan and locust bean gum (LBG) alone do not gel, whereas the two polysaccharides combined form a thermoreversible gel. Pectin gels under acidic conditions at a low water activity, whereas alginate requires Ca^{2+} for gelation and precipitates at a low pH. A mixture of pectin and alginate, however, forms gels under acidic conditions at a high water activity, in the absence of Ca^{2+} [124].

Gel-forming polysaccharides or mixtures of polysaccharides can be used in plate media as the solidifying agent instead of agar. Polysaccharide-degrading colonies can be detected as they will sink into the solid medium. The speed at which this phenomenon occurs, provides an indication for the amount of polysaccharase activity. Kennedy and Sutherland [53] isolated gellan lyase-producing bacteria using a synthetic plate medium, solidified with gellan. **Figure 3.2** shows a similar plate with colonies obtained from an enrichment culture on gellan. The tiny colony indicated by the arrow at day 2 proved to be a gellan-degrader, which gradually destroyed the plate. Plates solidified by a mixture of xanthan and LBG (5 g/l each) were used in our laboratory to obtain glucose-derepressed mutants of the xanthan-degrading bacterium *P. alginolyticus* XL-1. Wild-type *P. alginolyticus* XL-1 does not affect xanthan-LBG plates containing 10 g/l glucose as the production of xanthan-degrading enzymes is strongly repressed by glucose [85]. After 1-methyl-3-nitro-1-nitrosoguanidine mutagenesis, however, several colonies could be isolated that liquefied xanthan-locust bean gum plates in the presence of glucose (unpublished results).

If a polysaccharide or polysaccharide-mixture is to be used as a gelling agent in plates, attention should be paid to several aspects. The gel should be firm enough to withstand rubbing with a spatula during the plating procedure. The xanthan-LBG gel mentioned previously is much weaker than

an agar gel and plating should be done with caution. If salts are required for gelling, these should not be toxic to microorganisms or inhibitory to polysaccharases, as can be the case for trivalent metal ions. When a mixture of polysaccharides is used, it should be well established which polysaccharide is degraded, if not both. Furthermore, as illustrated by **Fig. 3.2**, the colonies should be isolated at an early stage as the destruction of the gel may impair the isolation of single colonies.

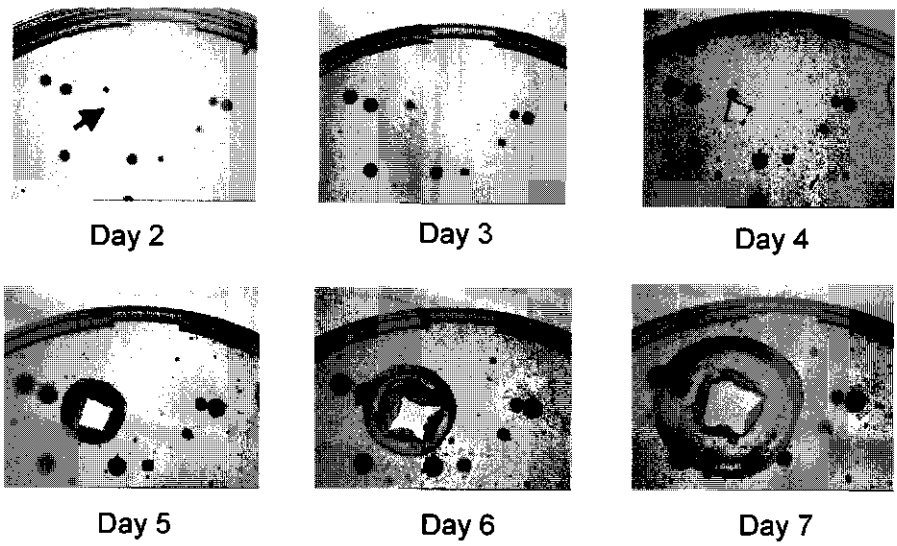


Fig. 3.2. Detail of a plate containing mineral salts medium solidified by 15 g/l gellan (Phytagel, Sigma) after 2, 3, 4, 5, 6 and 7 days of incubation at 30°C. The arrow indicates the gellan-degrading colony at day 2.

PLATE SCREENING METHODS BASED ON THE SOLUBILITY CHARACTERISTICS OF POLYSACCHARIDES

Soluble polysaccharides can usually be precipitated from a solution by adding water-miscible solvents like ethanol or acetone, which lower the water activity and hence decrease the solubility of the polysaccharide. Another precipitation mechanism is the formation of insoluble complexes of polyanionic polysaccharides with detergent salts like cetylammonium bromide (CTAB) or cetylpyridinium chloride, but also with dyes like Congo red and calcofluor white [125]. As polysaccharides in plate media are also in solution, they can be precipitated by flooding the plate with a precipitant. Precipitation of undegraded polysaccharide will give the plate an opaque, milky white appearance. Degradation zones will be visible as clear haloes, the diameter of which is a measure for the amount of activity that was produced.

Ethanol was used as a precipitant in plate methods to determine the degradation of pullulan and alginate [72, 90]. Aqueous detergent salt-solutions were used as a precipitant in plate methods to show the degradation of pectin, CMC and alginate [37, 36, 24, 33]. The plates, containing 0.5-7.5 g/l of polysaccharide and solidified with agar, were flooded with the precipitant until haloes appeared, which took 30 min to several hours. **Figure 3.3** shows a pectin plate with colonies from an enrichment culture on pectin. The fungal colony indicated by the arrow was capable of degrading pectin as shown by the halo, which emerged upon flooding the plate with a CTAB solution.

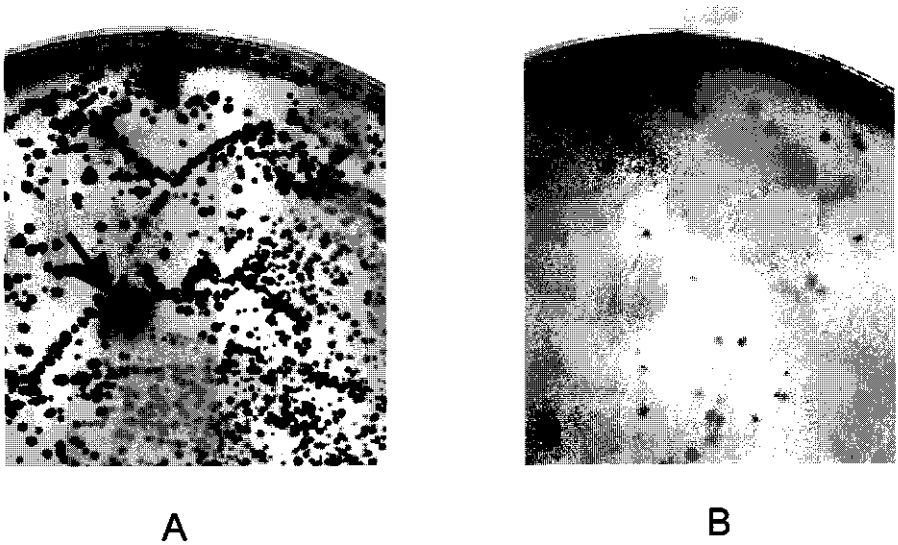


Fig. 3.3. Detail of a plate containing mineral salts medium with apple pectin (5 g/l), solidified with agar (20 g/l). **A**: untreated plate showing colonies from an enrichment culture on pectin; the fungal colony that degrades pectin is indicated by the arrow. **B**: plate after flooding with 1% (w/v) CTAB and subsequent incubation at room temperature for 3h. The colonies were washed off prior to flooding; traces of colonies are still visible, however, as the colonies partially grew in the agar. A halo is visible on the spot where the fungal colony was present.

This method is not so much restricted to specific polysaccharides and is therefore more generally applicable. However, not all polysaccharides precipitate equally efficiently in plate media, especially not with ethanol. Furthermore, also agar, which is commonly used as a solidifying agent, will eventually precipitate, possibly masking degradation zones. As also this method requires the plates to be flooded, precautions must be taken to avoid toxicity problems, cross-contamination and dislodging of colonies as described for the methods using dye-polysaccharide interactions.

Also polysaccharides which have a limited solubility in water can be used in plate screening media. Furthermore, some polysaccharides which are otherwise soluble in water, precipitate as a result of autoclaving. To prepare the screening plates, the polysaccharide is suspended in the medium, mixed with agar and autoclaved. After solidification, the plates have a turbid appearance because of the insoluble polysaccharide. If the polysaccharide is degraded, clear zones will appear. Using this principle, microorganisms degrading the exopolysaccharides of *Klebsiella pneumoniae* and *Clavibacter michiganensis* were isolated [118]. Cellulose-degrading bacteria were isolated from plates containing 0.1% (w/v) ball-milled filter paper [55].

PLATE SCREENING METHODS USING DYE-LABELLED POLYSACCHARIDES

Instead of having to rely on their specific physicochemical properties, polysaccharides can be chemically modified to make them detectable in plate media. This can be achieved by covalently attaching a label molecule to the polysaccharide that is either directly visible or that can be made visible, like fluorescent labels. The labelled polysaccharide can be incorporated into a plate medium and upon degradation of the polysaccharide, labelled oligosaccharides are formed. In contrast to the intact polysaccharide, the oligosaccharides can easily diffuse through the plate. This causes a local dilution of label around the polysaccharase-producing colonies, which becomes visible as a halo in which the label is (nearly) absent. The diameter of the halo gives an indication of the amount of polysaccharase produced.

Usually, azo-dyes are used for labelling polysaccharides. Remazol Brilliant Blue (RBB), Direct Green I, Ostazin Brilliant Red H-3B and Mikacion Brilliant Red 5BS have been coupled to different polysaccharides like amylose, locust bean gum, pullulan, CM-cellulose, CM-amylose, CM-barley-glucan, beechwood-xylan, hydroxyethylcellulose and inulin [80, 18, 52, 8, 11, 68]. Dye-labelled polysaccharides have been applied in liquid assays [80, 68], zymogram techniques [7] and in plate screening methods [18, 84, 127]. Kanno and Tomimura [52] developed an elegant plate assay for simultaneous detection of bacteria producing starch- and/or pullulan-degrading enzymes, incorporating both red-dyed pullulan and blue-dyed amylose in one plate medium. A blue halo around a colony indicated pullulanase activity, a red halo amylase activity and a colourless halo both activities.

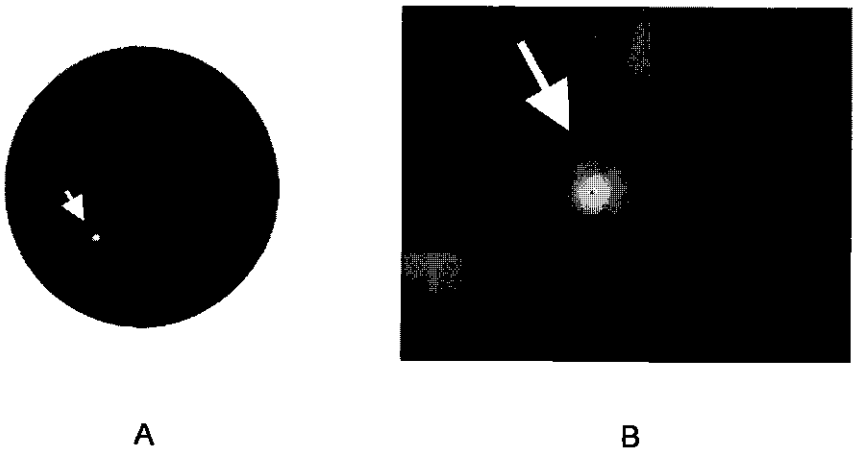
All soluble polysaccharides can be labelled with an azo-dye. Moreover, insoluble polysaccharides can be dyed after chemical modification to a soluble polysaccharide, e.g., by attaching CM or carboxyethyl groups [68]. As azo-dyes also bind to proteins, extensive purification of the polysaccharide may be required prior to labelling to ensure that only the polysaccharide is dyed. The polysaccharide solution should furthermore not be too viscous. A

low viscosity polysaccharide-preparation may be obtained by partially degrading the polysaccharide with endopolysaccharases [68], provided that these enzymes are available. Alternatively, the polysaccharide may receive a mild acid treatment, although it should be remembered that the side chains, if present, may also be affected.

A number of common polysaccharides are commercially available as azo-dyed substrates (Megazyme, Warriewood, Australia). For other polysaccharides, the following RBB-labelling procedure, adapted from McCleary [68] and Rinderknecht et al. [80], can be used. The purified polysaccharide (usually 5-10 g/l) is dissolved in demineralized water and heated to 60°C. The maximum concentration of polysaccharide must be established for each individual polysaccharide: all polysaccharide material should dissolve and the solution should not be very viscous. When the polysaccharide is dissolved, RBB (0.2 g/g of polysaccharide) and anhydrous Na_2SO_4 (0.2 g/g of polysaccharide) are added. After everything is dissolved, 0.2 g of Na_3PO_4 per g of polysaccharide is added and the solution is stirred for 2 h at 60°C. After cooling to room temperature, the dyed polysaccharide is precipitated with 3 volumes of ice cold ethanol and redissolved in demineralized water. The precipitation procedure is repeated until the supernatant is colourless. After dialysis to remove final traces of unbound dye, the dyed polysaccharide may be freeze-dried or used directly. The degree of substitution with RBB can be determined by measuring the OD_{590} after acid hydrolysis of the labelled polysaccharide (3 h at 100°C in 0.75 M H_2SO_4), using free RBB as a standard [67]. However, the OD_{590} of a dilute RBB-polysaccharide solution also provides a good estimate. Using the procedure described above, the ratio RBB : glycosyl units typically ranges from 1 : 20 to 1 : 50. The concentration of dye-labelled polysaccharides in plate media (usually 0.1-0.5% (w/v)) should be optimized for each batch as the substitution degree may vary. The colour intensity of the background should provide sufficient contrast with possible degradation zones. **Figure 3.4** shows an RBB-guar gum plate with colonies from an enrichment culture on guar gum; colourless haloes are surrounding the galactomannan-degrading colonies.

Using dye-labelled polysaccharides, degradation can be observed directly on the plate, without any manipulations like flooding with a reagent. The dye-label, however, may interfere with polysaccharase action by steric hindrance. Furthermore, the label itself may be affected by enzymes: azo-dyes can be decolourized by enzymatic oxidation [121]. In this case, haloes will appear around colonies although the polysaccharide is not degraded. We isolated bacteria that produced haloes on plates containing RBB-labelled exopolysaccharide (EPS) of *L. lactis* ssp. *cremoris* B40 [114]. None of the isolated strains, however, were able to degrade this EPS [88]. Apparently, dye-attacking microorganisms were obtained from the assay plates rather

than polysaccharide-degrading microorganisms, which may be inherent to the resistance of B40-EPS to biological degradation [87]. If the dye-labelled polysaccharide is resistant to biological degradation, dye-affecting microorganisms may be found more easily than polysaccharide-degrading microorganisms because the latter are probably less ubiquitous.



A B

Fig. 3.4. Plate containing mineral salts medium with Remazol Brilliant Blue-labelled guar gum (4 g/l, Megazyme), solidified with agar (20 g/l). **A**: whole plate, the largest halo is indicated by an arrow, **B**: detail of plate with the indicated halo (arrow); the guar gum-degrading colony is visible in the centre.

CONCLUDING REMARKS

The plate screening methods described in this review provide an array of relatively straightforward and simply applicable tools for specific detection of polysaccharase-producing microorganisms. Moreover, the methods provide a preliminary quantification of the activity that is produced: larger haloes or faster liquefaction of a gel indicate a higher polysaccharase activity. **Figures 3.1, 3.2, 3.3** and **3.4** clearly demonstrate that growth is an unreliable indicator for polysaccharide-degradation. In most cases, many colonies were present on the polysaccharide-plates. However, relatively few colonies –and those generally small- actually degraded the polysaccharide as detected by the various methods, underlining the importance of specific methods for the detection of polysaccharase-production. The majority of the colonies on the plates have probably been growing on impurities that were present in either the polysaccharide or the agar preparations.

Although the plate screening methods described in this review are based on different principles, they have in common that the presence of high-molecular-weight polysaccharide is visible or can be made visible. Thus, degradation of the polysaccharide will be detected only if the polysaccharide

is depolymerized. For this reason, the methods are biased towards microorganisms that produce endopolysaccharases. Microorganisms that degrade polysaccharides exclusively by exopolysaccharases may escape detection: the polymeric nature of the polysaccharide is not necessarily affected, for only the side chains may be attacked. On the other hand, some specific physicochemical properties may be lost as a result of exopolysaccharase activity, e.g., the gel-forming capacity of xanthan with LBG is lost when the terminal side chain residues are partially removed [86].
It may be clear that not every detection method is equally suitable for each polysaccharide. Which detection method or methods can or should be used is largely dependent on the polysaccharide being studied. Each polysaccharide has its own unique chemical and physical characteristics, which may sometimes lend themselves to exploitation in a plate screening method. Thus, as much information as possible should be obtained about the polysaccharide in order to make a well-considered choice from among the available methods, adapt an existing method or develop a new method. In practice, however, the information to hand may be limited as not all polysaccharides are equally well studied. Selecting a plate screening method will therefore often be a matter of trial and error, for which, it is hoped, this review may offer some some starting points.

4. A PYRUVATED MANNOSE-SPECIFIC XANTHAN LYASE INVOLVED IN XANTHAN DEGRADATION BY *Paenibacillus alginolyticus* XL-1

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Abstract

The xanthan-degrading bacterium *Paenibacillus alginolyticus* XL-1, isolated from soil, degrades approximately 28% of the xanthan molecule and appears to leave the backbone intact. Several xanthan-degrading enzymes were excreted during growth on xanthan, including xanthan lyase. Xanthan lyase production was induced by xanthan and inhibited by glucose and low-molecular-weight enzymatic degradation products from xanthan. A xanthan lyase with a molecular mass of 85 kDa and a pI of 7.9 was purified and characterized. The enzyme is specific for pyruvated mannosyl side chain residues and optimally active at pH 6.0 and 55°C.

INTRODUCTION

Xanthan, the extracellular polysaccharide (EPS) produced by *Xanthomonas campestris* has many industrial applications as a thickener of aqueous solutions and as a stabilizer of emulsions, foams, and particulate suspensions. Xanthan is used in many foods, e.g., juices, drinks, ice cream, salad dressings and dry mix formulations such as desserts. The bulk of xanthan, however, is used for enhancing oil recovery and in the manufacturing of explosives, paints, polishes, fire-fighting liquids, and cosmetics [73].

Xanthan has a pentasaccharide repeating unit: the β-1,4-glucan backbone is substituted on alternate glucosyl residues with a trisaccharide side chain consisting of α-mannose, β-glucuronic acid, and β-mannose (**Fig. 4.1 A**). Also "variant xanthans" with truncated side chains have been described [6, 107]. These variants, consisting of tetrasaccharide or trisaccharide repeating units (**Fig. 4.1 B** and **4.1 C**), are produced by *X. campestris* mutants. Truncation of the side chain affects the viscometric properties of xanthan. Compared to the polypentamer, the acetylated polytetramer is a weaker viscosifier [60], whereas the polytrimer is reported to be a superior viscosifier on a weight basis [43]. However, these variant xanthans, especially the polytrimer, are produced at low yields [117]. An attractive alternative method to produce xanthans with truncated side chains could be enzymatic modification of polypentamer xanthan.

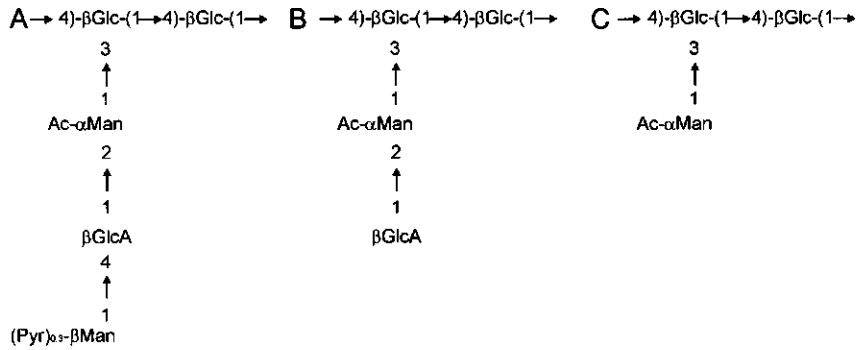

Fig. 4.1. Native (**A**) and mutant xanthan structures (**B** = polytetramer, **C** = polytrimer). The extent of acetylation and pyruvation varies with bacterial source and culture conditions.

Xanthan-modifying enzymes can be obtained from xanthan-degrading microorganisms. Xanthan-degrading pure cultures [101, 103], as well as mixed cultures [17], have been described. In some cases pure cultures were isolated from mixed cultures but, compared to the mixed cultures, growth rates and production of xanthan-degrading enzymes were considerably lower [15, 47, 103].

Xanthan lyase is one of the enzymes that can be used for xanthan modification. This enzyme removes the terminal mannosyl residue via β-elimination, yielding a free mannose and a tetrasaccharide repeating unit as in **Fig. 4.1 B**; however, with a 4,5-*ene*-glucuronyl residue on the side chain. Xanthan lyase activity can easily be monitored by measuring the increase of A_{235} caused by the conjugation of the formed C=C bond with the carboxylate group in the uronic acid residue. Alternatively, the double bond introduced by xanthan lyase can be oxidized with periodate. This yields an oxidation product that reacts with thiobarbituric acid (TBA) to a chromophore at 590 nm [105]. Xanthan lyases were first obtained by Sutherland [103] from a *Bacillus* sp., a *Corynebacterium* sp., and a mixed culture. The action of these enzymes was independent of the degree of pyruvation and acetylation of xanthan. Pyruvated mannose-specific xanthan lyases have been purified from a salt-tolerant mixed culture by Ahlgren [1] and, very recently, from a *Bacillus* sp. by Hashimoto et al. [41].

In this study, the isolation of *Paenibacillus alginolyticus* XL-1 from an enrichment culture on xanthan is described. This strain was tested for xanthan-degrading enzyme activities. Several xanthan-degrading enzymes were excreted, including a pyruvated mannose-specific xanthan lyase that was purified and characterized.

MATERIALS AND METHODS

Enrichment of xanthan-degrading bacteria Xanthan-degrading bacteria were enriched on mineral salts medium, pH 6.9, with 3 g l^{-1} xanthan (Sigma G-1253, practical grade). Mineral salts medium contained (in mg l^{-1}): EDTA: 10.0; $ZnSO_4 \cdot 7H_2O$: 2.0; $CaCl_2 \cdot 2H_2O$: 1.0; $FeSO_4 \cdot 7H_2O$: 5.0; $Na_2MoO_4 \cdot 2H_2O$: 0.2; $CuSO_4 \cdot 5H_2O$: 0.2; $CoCl_2 \cdot 6H_2O$: 0.4; $MnCl_2 \cdot 4H_2O$: 1.0; $(NH_4)_2SO_4$: 2,000; $MgCl_2 \cdot 6H_2O$: 100; K_2HPO_4: 1,550; $NaH_2PO_4 \cdot H_2O$: 850. In a 500-ml Erlenmeyer flask, 100 ml of xanthan medium was inoculated with 1 ml of a 1 : 1 (wt/vol) mixture of soil and 0.9% (wt/vol) NaCl. The cultures were incubated with shaking at 30°C, and 1 ml was transferred daily to 100 ml of fresh medium. After repeated transfers, pure cultures were isolated and maintained on solid mineral salts medium containing 5 g l^{-1} of mannose or xanthan and yeast extract (0.05 g l^{-1}, added after autoclaving from a filter-sterilized 5 g l^{-1} stock solution).

Strain and culture conditions *Paenibacillus alginolyticus* XL-1, isolated from a mixed culture growing on xanthan, was maintained on solid xanthan medium supplemented with filter-sterilized yeast extract. Liquid cultures were incubated at 30°C with shaking, on mineral salts medium containing 5 g of carbon source and 0.05 g of filter-sterilized yeast extract l^{-1}. For enzyme production, 1 l of xanthan medium in a 5-l Erlenmeyer flask was inoculated with 5 ml of a xanthan-grown overnight culture. After 20 h of incubation, the culture was centrifuged (15,000 × *g*, 15 min, 4°C), and the supernatant was used for enzyme purification.

Polysaccharides Practical-grade xanthan was obtained from Sigma (G-1253). Native and chemically modified xanthans (EPS of *X. campestris* X646 and *Xanthomonas phaseoli* X556, Kelzan (Kelco), and Flocon 4800C (Pfizer)) were kind gifts of Ian Sutherland (Division of Biological Sciences, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, United Kingdom). The capsular polysaccharide of *Klebsiella* serotype K5 [30] was kindly provided by Harm Snippe (Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation, Academic Hospital Utrecht, Utrecht, The Netherlands).

Purification of xanthan Practical-grade xanthan was dissolved to 20 g l^{-1} in demineralized water and 17% (vol/vol) of an ice cold trichloroacetic acid solution (80% (wt/vol)) was added to precipitate proteins. The mixture was stirred for 20 min at 4°C and centrifuged (25,000 × *g*, 15 min, 4°C). After neutralization of the supernatant with 5 M NaOH, xanthan was precipitated by adding 3 volumes of ice cold absolute ethanol. The precipitate was collected by filtration and dissolved in demineralized water. After extensive dialysis at 4°C against demineralized water, the purified xanthan solution was stored at -20°C or lyophilized.

Chemical modification of xanthan Pyruvic acetals were removed from xanthan using the procedure of Bradshaw et al. [10]. Purified xanthan (5 g l^{-1}) was heated at 100°C for 90 min in 5 mM trifluoroacetic acid (TFA). After dialysis against demineralized water, the preparation was stored at -20°C. Acetyl groups were removed according to Shatwell et al. [91]. Purified xanthan (2.5 g l^{-1}) in 0.1 M NH_4OH was incubated at 60°C for 1 h. After dialysis, the solution was stored at -20°C.

Preparation of enzymatic low molecular weight (LMW) degradation products of xanthan To obtain LMW degradation products released from xanthan by the enzyme system of *P. alginolyticus* XL-1, filter-sterilized supernatant (15 ml) of a xanthan-grown overnight culture was incubated at 30°C with 250 ml of an autoclaved xanthan solution (10 g l^{-1} in 15 mM phosphate buffer [pH 6.9]). Reducing sugars were measured every 3 to 4 days to monitor xanthan degradation. Between days 13 and 17, the reducing sugar formation ceased, and the incubation mixture was centrifuged to remove insoluble xanthan residues. The supernatant was dialysed against 300 ml of demineralized water, and the dialysate containing the LMW fraction was autoclaved for use as a medium component.

To obtain the LMW product released from xanthan by purified xanthan lyase, 2 ml of purified xanthan solution (7 g l^{-1}) was incubated with 50 mU of xanthan lyase for 4 h at 30°C. The reaction mixture was dialysed against 50 ml of demineralized water. The dialysate containing the LMW fraction was lyophilized and stored at -20°C.

Xanthan lyase purification *P. alginolyticus* XL-1 was cultured as described above. The supernatants of 2 cultures (1 l each) were pooled and the extracellular enzymes were concentrated by ammonium sulphate precipitation (60% saturation). After centrifugation, the pellet was resuspended in 24 ml of 10 mM Tris (pH 8.0) and centrifuged to remove insolubles. To the supernatant, solid $(NH_4)_2SO_4$ was added to a final concentration of 1 M. Subsequent purification steps were carried out on an FPLC system (Pharmacia) operated at room temperature. The enzyme solution was applied to a hydrophobic interaction chromatography (HIC) column (Pharmacia HiTrap Phenyl Sepharose HP, 1 ml) in 6 ml batches. Proteins were eluted with a linear gradient of 1 to 0 M $(NH_4)_2SO_4$ in 10 mM Tris (pH 8.0) at a flow rate of 0.5 ml min^{-1} in a total volume of 10 ml. The xanthan lyase-containing fractions of 4 subsequent runs were pooled and dialysed overnight at 4°C against 10 mM Tris (pH 8.0). Subsequently, the HIC pool was applied to an anion exchange chromatography (AEC) column (Source 15Q HR 5/5). Proteins were eluted with a linear gradient of 0 to 0.15 M NaCl in 10 mM Tris (pH 8.0) at a flow rate of 1 ml min^{-1} in a total volume of 25 ml. Xanthan lyase-containing fractions were pooled and stored at -20°C.

Protein electrophoresis Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of

Laemmli [57] by using a Hoeffer Mighty Small system (Pharmacia). Gels were stained with Coomassie brilliant blue. Isoelectric focusing (IEF) was carried out on a Phast Gel system (Pharmacia).

Enzyme assays To assess total xanthan-degrading enzyme ("xanthanase") activity, equal volumes of culture supernatant and purified xanthan solution (5 g l^{-1} in 15 mM phosphate buffer [pH 6.9]) were mixed and incubated at 30°C. Samples were drawn at set intervals and assayed for reducing sugars. β-1,4-Glucanase activity was measured like xanthanase by using carboxymethylcellulose (CM-cellulose) instead of xanthan as the substrate. Similar incubations were carried out to determine enzyme activity releasing uronic acid-containing fragments from xanthan. In these samples, high-molecular-weight xanthan residues were precipitated with 3 volumes of ice-cold absolute ethanol. After centrifugation, the supernatant containing the LMW degradation products was concentrated by lyophilization and assayed for uronic acids. To determine glycosidase activities, equal volumes of culture supernatant and a 10 mM solution of *p*-nitrophenyl-D-glycoside (10 mM) were incubated. Released *p*-nitrophenol was measured at 410 nm after the reaction was stopped with 3 volumes of ice-cold 0.2 M Na_2CO_3. Xanthan lyase activity was determined spectrophotometrically in a Perkin-Elmer λ-1 spectrophotometer. Xanthan lyase (100 µl of crude enzyme or 0.5 to 1 µg of purified enzyme) was added to 500 µl of purified xanthan solution (0.05 g l^{-1} in 15 mM phosphate buffer [pH 6.9]), mixed quickly and the A_{235} was recorded continuously. One unit of xanthan lyase activity is defined as the amount of enzyme that forms 1 µmol of 4,5-*ene*-glucuronyl residues per minute (ε_{235} = 8.0 cm^2 μmol^{-1}). To determine the optimal pH for xanthan lyase, activity was measured as described above but xanthan was dissolved in McIlvaine buffer of different pH values. Tenfold concentrated McIlvaine buffer was prepared by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4 to obtain the desired pH. To assess the thermal stability of xanthan lyase, the purified enzyme was diluted to 40 µg ml^{-1} in McIlvaine buffer at pH 5, 6, and 7. The enzyme solutions were incubated for 15 min at different temperatures and immediately stored on ice. Subsequently, activity was measured according to the standard procedure.

Determination of ε_{235} of the xanthan lyase reaction product Purified xanthan lyase (150 mU) was incubated with 1.2 ml of purified xanthan (6 g l^{-1}). Both A_{235} and the reducing sugar concentration relative to a mannose-standard were measured at set intervals. The A_{235} was measured after inactivating the enzyme with 0.5 volume of 5 M NaOH and appropriate dilution with water. A_{235} was plotted against the concentration of reducing sugars, and from the slope an ε_{235} of 8.0 cm^2 μmol^{-1} was determined.

Calculation of xanthan degradation To determine the extent of xanthan degradation by *P. alginolyticus* XL-1, the bacterium was cultured in closed 500-ml serum bottles containing 40 ml of xanthan medium. Total sugar, reducing sugar, CO_2 evolution, and biomass formation were monitored during

incubation. The following assumptions were made to be able to calculate xanthan degradation on a C-mol basis: the number of xanthan repeating units equals the total sugar content, corrected for the reducing (i.e., nonpolysaccharide) sugars, divided by 5; the average molecular formula for a xanthan repeating unit is $C_{32.7}H_{48.9}O_{26.8}$ (molar mass: 869.3 g; 96% acetylation and 26% pyruvation, see **Table 4.3**); the carbon content of biomass is 50% (wt/wt); and the reducing sugars released are hexoses.

Analytical procedures The growth of *P. alginolyticus* XL-1 on xanthan was monitored by measuring CO_2 evolution in closed 500-ml serum bottles containing 40 ml of medium with a HP 6890 gas chromatograph (Hewlett-Packard) equipped with a Poraplot Q column (25 m). Growth on carbon sources other than xanthan was determined by measuring the optical density at 660 nm. Dry biomass was measured gravimetrically after the cells were collected by centrifugation, washed twice with demineralized water, and dried overnight at 110°C.

Protein was measured by using the bicinchoninic acid protein assay kit (Pierce) according to the supplier's instructions. Reducing sugars were determined with the dinitrosalicylic acid method of Miller [70] by using glucose or mannose as the standard. Total sugar was determined with the phenol-sulfuric acid method of Dubois et al. [29] by using glucose as the standard. Uronic acids were determined by the method of Blumenkrantz and Asboe-Hansen [9] with glucuronic acid as the standard. For qualitative determination of 4,5-*ene*-glucuronyl residues formed by xanthan lyase, the TBA method of Weissbach and Hurwitz [122] was used. Glucose was determined enzymatically with the Boehringer Mannheim D-glucose test kit according to the supplier's instructions.

To determine the pyruvate content of xanthan, xanthan (1 g l^{-1}) was hydrolysed in 1 M HCl for 1.5 h at 100°C. Subsequently, free pyruvate was determined enzymatically by using the Sigma Diagnostics pyruvate kit according to the supplier's instructions. The acetyl content of xanthan was determined by the method of Hestrin [45] with acetylcholine as the standard.

Thin-layer chromatography (TLC) of the LMW xanthan lyase product was carried out on silica gel plates (Merck Kieselgel 60 F_{254}). The eluent was 2-propanol-acetone-1 M lactic acid (2: 2: 1). For the detection of sugars, the plates were sprayed with phenol-sulfuric acid reagent (3 g of phenol and 5 ml of concentrated sulfuric acid in 95 ml of ethanol) and heated at 110°C for 5 to 10 min.

RESULTS

Isolation of a xanthan lyase-producing *Paenibacillus alginolyticus* Microorganisms were enriched from soil samples in liquid medium with xanthan as the sole source of carbon and energy. After repeated transfers, a xanthan-degrading mixed culture was obtained that grew to a high optical density within 24 h. The presence of xanthan lyase activity in the supernatant of the mixed culture was demonstrated by incubating the supernatant with xanthan and measuring an increase of the A_{235} as well as the formation of TBA-reactive material. No β-1,4-glucanase activity was observed.

After 30 daily transfers, dilutions of the mixed culture were plated on solid mineral salts medium with mannose and filter-sterilized yeast extract. Initial attempts to isolate xanthan lyase-producing bacteria failed when yeast extract was autoclaved with the medium, suggesting a requirement for a heat-labile component from yeast extract. After 2 days of incubation at 30°C, 12 visibly different colonies were selected and obtained as pure cultures. The pure cultures were grown in mineral salts medium with xanthan and filter-sterilized yeast extract. After overnight incubation at 30°C, cultures were assayed for xanthan lyase production. One of the isolated strains produced xanthan lyase activity, and this strain was designated strain XL-1.

Identification of strain XL-1 was carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (data not shown). Physiological tests pointed to *P. alginolyticus* / *Paenibacillus chondroitinus* and fatty acid analysis showed the pattern for the *Bacillus circulans* / *Paenibacillus* group. Partial 16S ribosomal DNA sequencing showed 97.8% similarity to *P. alginolyticus*. Although strain XL-1 did not degrade alginate, the name *P. alginolyticus* will be maintained as the description of the species [95, 75] does not exclude strains that are incapable of alginate degradation.

P. alginolyticus XL-1 was able to use xanthan, glucose, glucuronic acid, mannose, mannitol, and glycerol as the sole carbon and energy source; no growth was observed on CM-cellulose. Strain XL-1 did not grow without filter-sterilized yeast extract or with autoclaved yeast extract. Filter-sterilized yeast extract could be replaced by a mixture of vitamins (containing *p*-amino benzoic acid, folic acid, lipoic acid, riboflavin, biotin, Ca-pantothenic acid, nicotinic acid, pyridoxamin, pyridoxin, thiamin, and cobalamin). To determine which vitamins were essential, *P. alginolyticus* XL-1 was cultured in media in each of which a single vitamin was omitted. No growth occurred when thiamin or biotin was absent, the latter probably being the heat-labile compound that is destroyed when yeast extract is autoclaved.

Xanthan utilization by *P. alginolyticus* XL-1 When plates with strain XL-1 on solid medium with xanthan were stained with Congo red, red haloes were observed around the colonies, indicating the presence of β-1,4-glucan.

This suggested that long stretches of the xanthan backbone remained intact whereas the side chains were removed, exposing the backbone and allowing interaction with Congo red. To determine the extent of xanthan degradation, *P. alginolyticus* XL-1 was cultured on xanthan in liquid medium, and total amounts of sugar, reducing sugar, CO_2 evolution, and biomass formation were monitored during incubation. CO_2 evolution stopped after 24 h, but the incubation was carried on for another 72 h to allow growth-independent xanthan degradation to proceed to completion.

At the start of the incubation, 1.37 mmol of total xanthan-sugar (8.96 mmol of C) was present. After 96 h, 1.00 mmol of CO_2 (1.00 mmol of C), 19.6 mg of dry biomass (0.82 mmol of C), and 0.11 mmol of reducing sugars (0.66 mmol of C) were formed. The amount of total soluble sugar at 96 h was 0.57 mmol, of which 0.11 mmol was reducing sugar. Therefore, only 0.46 mmol of soluble polysaccharide sugar (3.0 mmol of C if intact pentasaccharide repeating units are assumed) was still present after incubation. Thus, 28% of the xanthan-C initially present was converted to CO_2, biomass, and reducing sugar but only 33% was recovered as remaining polysaccharide-sugar. The unrecovered 39% was probably modified xanthan with a decreased solubility, present in the slime-like layer that was observed on top of the cell pellet after centrifugation. This layer was lost upon washing the cells for biomass determination.

The extent of xanthan degradation by the extracellular enzyme system of *P. alginolyticus* XL-1 was also determined. A sterile xanthan solution was incubated with 2.5% (vol/vol) filter-sterilized crude supernatant of a xanthan-grown culture of *P. alginolyticus* XL-1. Reducing sugar formation was monitored weekly, and by week 4 no further increase in reducing sugar concentration was observed. The final amount of reducing sugars formed was 26% of the total sugar initially present. This value is in accordance with the 28% breakdown calculated from CO_2, biomass and reducing sugar formation in the *P. alginolyticus* XL-1 culture and suggests that xanthan is degraded to monosaccharides that are subsequently metabolized.

Production of xanthan-degrading enzymes by *P. alginolyticus* XL-1

P. alginolyticus XL-1 produces various xanthan-degrading enzyme activities. **Table 4.1** summarizes the activities observed in the supernatant of a 20-h culture of *P. alginolyticus* XL-1 grown on xanthan. Xanthan lyase activity could be detected with unmodified, depyruvated, and deacetylated xanthan as substrates. Also LMW compounds reacting as uronic acids were released from xanthan upon incubation with culture supernatant. These compounds could be either uronic acids or uronic acid-containing oligosaccharides. β-1,4-Glucanase, β-mannosidase, α-mannosidase, β-glucuronidase, and β-glucosidase activities could not be detected.

With carbon sources other than xanthan, very low titers of xanthan lyase were produced, indicating that xanthan lyase was induced during

growth on xanthan. To demonstrate the inducing effect of xanthan, xanthan (0.2 g l^{-1}) was added to two cultures of *P. alginolyticus* XL-1, growing exponentially on mannose. **Figure 4.2 A** shows that xanthan lyase production started 2 h after the addition of xanthan. At 4 h after the addition of xanthan, glucose (0.25 g l^{-1}) was added to one of the induced cultures, causing an immediate stop in xanthan lyase production. When glucose was exhausted, xanthan lyase production started again. In the induced culture without additional glucose, xanthan lyase production stopped 6 h after the addition of xanthan, although growth continued. In the control culture without xanthan, a small amount of xanthan lyase activity was produced towards the end of the exponential growth phase.

Table 4.1. Activities of xanthan-degrading enzymes present in a 20-h culture of *P. alginolyticus* XL-1 grown on xanthan.

Enzyme	Substrate	Activity (mU ml^{-1}) [a]
total xanthanase	xanthan (Sigma)	39.0
xanthan lyase	xanthan (Sigma)	15.6
	xanthan DP[b] (Sigma)	1.7
	xanthan DA[b] (Sigma)	15.6
uronic acid-releasing activity	xanthan (Sigma)	2.9

[a] 1 Unit (U) is defined as the amount of enzyme releasing 1 µmol of the assayed compound per min.
[b] DP = chemically depyruvated; DA = chemically deacetylated

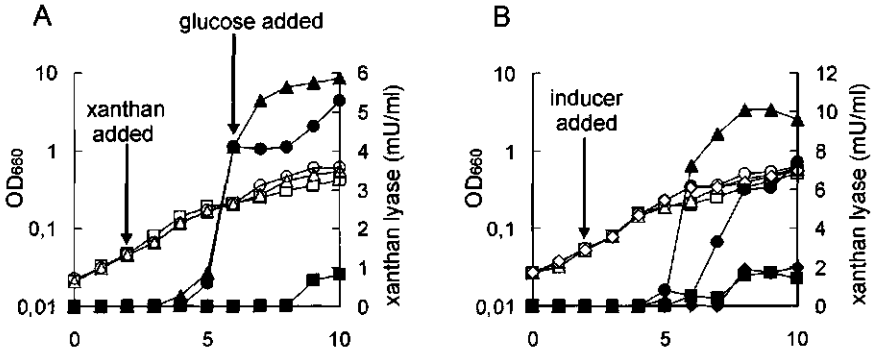

Fig. 4.2. A. Growth (open symbols) and xanthan lyase activity (closed symbols) in mannose-grown cultures of *P. alginolyticus* XL-1: ■, control; ▲, xanthan (0.2 g l^{-1}) added after 2h; ●, xanthan (0.2 g l^{-1}) added after 2h and glucose (0.25 g l^{-1}) added after 6 h. **B.** Growth (open symbols) and xanthan lyase activity (closed symbols) in mannose-grown cultures of *P. alginolyticus* XL-1: ■, control; ▲, xanthan (0.2 g l^{-1}) added after 2h; ●, xanthan (0.2 g l^{-1}) and LMW xanthan degradation products (0.54 mM) added after 2 h; ◆, LMW xanthan degradation products (0.54 mM) added after 2h.

It is unlikely that xanthan is the true xanthan lyase inducer since xanthan molecules are too large to enter a bacterial cell. Probably, xanthan lyase production is triggered by an LMW enzymatic degradation product of xanthan. This was tested by monitoring xanthan lyase activity after the addition of LMW xanthan degradation products (0.54 mM reducing sugars), xanthan (0.2 g l^{-1}) or both xanthan and LMW xanthan degradation products to log-phase cultures of *P. alginolyticus* XL-1 growing on mannose. **Figure 4.2 B** shows that rather than having an inducing effect, the enzymatic xanthan-hydrolysate appeared to inhibit xanthan lyase production. In the culture with only LMW xanthan degradation products, xanthan lyase was not produced above the level in the control culture without additional potential inducers.

Xanthan lyase purification Xanthan lyase was purified 26-fold from 2 l of culture supernatant. The enzyme eluted from the Phenyl Sepharose column at approximately 0.7 M $(NH_4)_2SO_4$ in three fractions. The enzyme eluted from the Source 15Q column at approximately 0.05 M NaCl in a single peak at A_{280} in two subsequent fractions. The purification results are summarized in **Table 4.2**. SDS-PAGE and subsequent staining of the gel with Coomassie brilliant blue showed a single band (**Fig. 4.3**). The molecular mass was estimated to be 85 kDa compared to the relative mobilities of the protein standards in the SDS-PAGE gel. IEF showed a single band at pH 7.9.

Table 4.2. Purification of xanthan lyase from 2 l of culture fluid of *P. alginolyticus* XL-1 grown on xanthan.

Purification step	Total protein (mg)	Total units*	Specific activity (U mg^{-1} protein)	Purification factor
Cell-free culture broth	517	50.0	0.10	1
$(NH_4)_2SO_4$ precipitation (60% saturation)	75	32.8	0.44	4.4
Phenyl Sepharose HP chromatography + overnight dialysis	8.5	19.5	2.29	22.9
Source 15Q chromatography	4.2	11.0	2.62	26.2

* 1 unit is defined as the amount of enzyme that produces 1 µmole of 4,5-*ene*-glucuronic acid residues per min. Activity was calculated from the increase of A_{235}; ε_{235} was 8.0 cm^2 μmol^{-1}.

Properties of purified xanthan lyase The purified xanthan lyase was active over a broad pH range with an optimum at pH 6.0. The optimum temperature for xanthan lyase was 55°C. **Figure 4.4** shows, however, that the enzyme

was not stable at temperatures higher than 45°C. The enzyme was a little more stable at acidic pH values, but at 55°C enzyme activity was lost at all pH values tested.

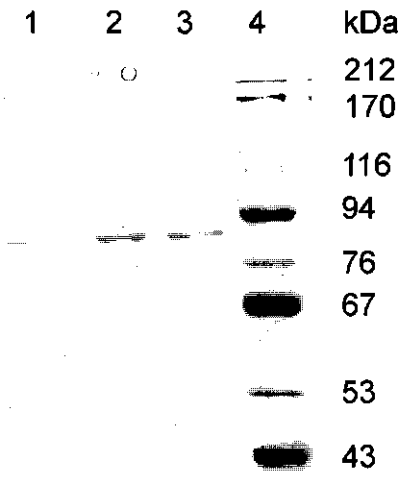

Fig. 4.3. SDS-PAGE gel of different purification stages of xanthan lyase; lane 1: ammonium sulfate precipitate, lane 2: dialyzed HIC-pool, lane 3: AEC-pool, lane 4: protein standards.

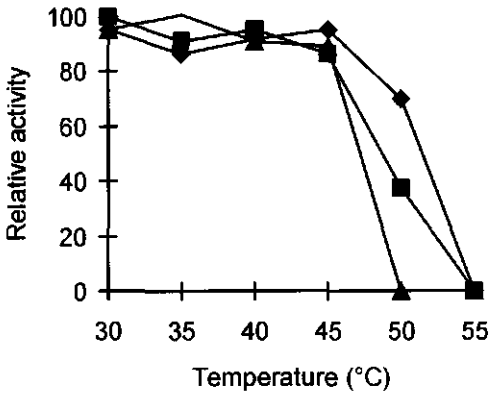

Fig. 4.4. Residual xanthan lyase activity after 15 min incubation at indicated temperature at pH 5 (◆), 6 (■) and 7 (▲). 100 = 105 mU ml^{-1}.

Various salts affected xanthan lyase activity. $CuCl_2$ caused a 70% activity drop at a concentration of 0.1 mM, whereas $HgCl_2$ completely inhibited the enzyme at 0.1 mM. EDTA did not show any effect at a concentration of 10 mM. NaCl had no effect at 10 mM but higher concentrations of NaCl inhibited the enzyme: at 85 mM NaCl, 60% of the initial activity was found, and at 450 mM all activity was lost. $CoCl_2$, $MgCl_2$,

and $CaCl_2$ at a concentration of 1 mM and $MnCl_2$ at a concentration of 0.1 mM had no effect on xanthan lyase activity.

To characterize the LMW reaction product of xanthan lyase, the purified enzyme was incubated with xanthan, and the LMW fraction was isolated. After it was freeze-dried, the material was dissolved in demineralized water and divided into two aliquots and one aliquot was hydrolysed with TFA. The untreated aliquot contained 5.7 mM reducing sugars (mannose equivalents) and 0.1 mM pyruvate. The TFA-hydrolysed aliquot contained 5.9 mM mannose equivalents and 4.6 mM pyruvate. TLC analysis showed an unidentified spot and a slight mannose spot for the untreated aliquot, whereas the TFA-treated aliquot showed only a mannose spot. These results suggested that the enzyme preferentially removes pyruvated mannose from xanthan. To confirm that the purified xanthan lyase was specific for pyruvated mannosyl residues, the enzyme was incubated with various xanthans that were chemically depyruvated, deacetylated, or both depyruvated and deacetylated. The *Klebsiella* K5 capsular polysaccharide was tested as well. The K5 polysaccharide has the Pyr4,6Manβ(1→4)GlcAβ bond of the xanthan side chain in its backbone [30], but it was not a substrate for xanthan lyase. No xanthan lyase activity could be detected on chemically depyruvated xanthans (**Table 4.3**). The enzyme was active on chemically deacetylated Sigma xanthan, as well as on xanthan that is naturally low in acetyl content (Flocon 4800C and X556-EPS). However, the enzyme was not active on deacetylated X646-EPS.

DISCUSSION

The xanthan-degrading bacterium *P. alginolyticus* XL-1 was isolated from a mixed culture growing on xanthan. This organism requires both thiamin and biotin for growth. Since the organism proliferated in the mixed culture without the addition of biotin and thiamin, it is likely that these compounds were provided for by other organisms. This phenomenon may explain other researchers' findings that the growth rate and the production of xanthan-degrading enzymes is greatly reduced when pure xanthan-degrading cultures are separated from the mixed cultures they originated from [15, 17, 47, 103]. Possibly, these mixed cultures also produced some growth factor required by the xanthan degraders. Alternatively, two or more strains may have acted concertedly in xanthan degradation.

Xanthan is not degraded to completion by *P. alginolyticus* XL-1. The formation of Congo red-stainable material around colonies on agar plates with xanthan and the semisoluble, slime-like material observed in liquid cultures indicate that the enzymes excreted by strain XL-1 only remove residues from the xanthan side chains, whereas long stretches of the β-1,4-glucan backbone remain intact. As only 28% of xanthan-C was recovered as CO_2,

biomass, or reducing sugars, the side chains are apparently removed only partially.

Table 4.3. Activity of purified *P. alginolyticus* XL-1 xanthan lyase on various substrates. All polysaccharides were dissolved to 0.05 g l^{-1} except X556-EPS (dissolved to 0.025 g l^{-1} due to high background absorption at 235 nm). 100 = 0.7 mU ml^{-1}.

Substrate	Acetylation (%)[a]	Pyruvation (%)[a]	Relative activity
Xanthan (Sigma)	96	26	100
Xanthan (Sigma), DA[c]	21	21	100
Xanthan (Sigma), DP[c]	83	12	0
Flocon 4800C[b]	40	61	80
Flocon 4800C, DP[b]	54	12	0
X646[b]	90	54	50
X646, DP[b]	88	7	0
X646, DA[b]	26	44	0
X646, DAP[b]	20	7	0
X556[b]	32	74	67
X556, DP[b]	22	12	0
Kelzan	54	23	80
K5	58	23	0

[a] Expressed as a percentage of the number of repeating units. The number of repeating units was determined from total sugar measurements divided by 5 for xanthans and 3 for K5-cps.
[b] Data adapted from Shatwell *et al.* [91].
[c] DP = chemically depyruvated, DA = chemically deacetylated.

The outer side chain mannosyl residue can be removed either by a xanthan lyase or a β-mannosidase. Only xanthan lyase activity was detected in the culture supernatant, but it cannot be excluded that a β-mannosidase is produced that is not active on *p*-nitrophenyl-β-D-mannoside. Extracellular xanthan lyase production by strain XL-1 is induced by xanthan and repressed by glucose. Also, LMW enzymatic degradation products of xanthan inhibited rather than induced xanthan lyase production. Possibly, the true xanthan lyase inducer is an intermediate enzymatic degradation product of xanthan (e.g., an oligosaccharide) that is further converted by other degrading enzymes to a repressor (e.g., a monosaccharide). This would explain the inhibiting effect of LMW xanthan degradation products: degradation has proceeded to such an extent that all inducer has been converted to repressor. In cultures with xanthan as inducer, xanthan lyase production stopped after a relatively short period of time, while exponential growth continued. This may also be explained by the conversion of an inducing xanthan fragment to a repressor over time, resulting in a stop in xanthan lyase production.

The purified xanthan lyase of strain XL-1 is different from the xanthan lyases described by Ahlgren [1] and Sutherland [103] in a number of

respects. The *Paenibacillus* enzyme (molecular mass, 85 kDa) is much larger than these xanthan lyases, which had a molecular mass in the range of 30 to 33 kDa. The optimal pH is in between the values reported for these enzymes: pH 7.25 for the *Bacillus* enzyme [103] and pH 5 for the xanthan lyase from the mixed culture [1]. The pI of 7.9 is much higher than the pI of the xanthan lyase purified by Ahlgren [1] (pI 3.7). Furthermore, the xanthan lyase from *P. alginolyticus* XL-1 is not as salt tolerant as the enzyme described by Ahlgren [1], which is not surprising as strain XL-1 was not selected for its salt tolerance. The xanthan lyase of *P. alginolyticus* XL-1 is more similar to the recently described xanthan lyase of *Bacillus* sp. strain GL1 [41]. The molecular mass of this enzyme is in the same order of magnitude (75 kDa), and the optimal pH (5.5) is near that of the *Paenibacillus* enzyme (pH 6.0). There are, however, also differences: the *Paenibacillus* enzyme is more stable at higher temperatures. Furthermore, the *Paenibacillus* xanthan lyase is not affected by 1 mM $CoCl_2$, $MgCl_2$, $CaCl_2$, or 10 mM EDTA, whereas the *Bacillus* sp. strain GL1 xanthan lyase was stimulated by $CoCl_2$ and inhibited by the other compounds. On the other hand, $CuCl_2$ and $HgCl_2$ had a much stronger inhibiting effect on the *Paenibacillus* enzyme.

Like the other xanthan lyases described in literature, the *Paenibacillus* enzyme was active on intact, nondepolymerized xanthan. The enzymes described by Ahlgren [1] and Hashimoto et al. [41], however, probably act in conjunction with depolymerases. Also, the lyases described by Sutherland [103] were associated with endoglucanases and showed a higher activity on xanthan-derived oligosaccharides than on intact xanthan. The *Paenibacillus* xanthan lyase was not found to be associated with endoglucanases either in the pure culture or in the mixed culture from which strain XL-1 originated. Therefore, the true substrate for this xanthan lyase is probably intact xanthan.

Like the enzyme described by Ahlgren [1] and Hashimoto et al. [41], the purified xanthan lyase is specific for pyruvated mannosyl residues. The LMW fraction released by xanthan lyase from xanthan contained a little more mannose than pyruvate and on TLC plates a slight mannose spot in the untreated sample was visible. Possibly, xanthan lyase releases a small amount of unpyruvated mannose from xanthan during prolonged incubation. However, the purified enzyme showed no activity at all on chemically depyruvated xanthans. Therefore it is clear that the enzyme prefers pyruvated xanthan to nonpyruvated xanthan. Unexpectedly, xanthan lyase was not active on pyruvated, deacetylated X646-EPS. It is not likely that the acetyl group is required for activity, since the enzyme was active on xanthans that are originally low in acetyl substituents as well as on chemically deacetylated Sigma xanthan. Possibly, the deacetylated X646-EPS has adopted a structure which renders the pyruvated side chains inaccessible to xanthan lyase. The *Klebsiella* K5 polysaccharide was not a substrate suggesting that the purified xanthan lyase is a true "exolyase".

In chemically depyruvated xanthans, ca. 12% of the repeating units are still pyruvated. Apparently, the pyruvate groups were removed incompletely by the acid hydrolysis treatment, which was mild to prevent the cleavage of glycosidic bonds. However, the purified xanthan lyase was not active on these substrates despite the presence of pyruvate groups. Possibly, removal of pyruvate groups was incomplete because parts of the polysaccharide molecules are inaccessible to H^+ molecules, e.g., due to aggregate formation. If so, it would also be unlikely that enzymes can act on these parts of the polysaccharide molecules.

The 4,5-*ene*-glucuronyl residue in the xanthan side chain resulting from xanthan lyase activity is identical to the 4,5-*ene*-galacturonyl residue that is formed by pectate lyase in pectate oligomers. The ε_{235} for the 4,5-*ene*-glucuronyl residue determined in this study (8.0 cm^2 μmol^{-1}) is, however, higher than the values reported for products of pectate lyase, i.e., 4.6 [56] and 5.2 [71] cm^2 μmol^{-1}. This difference is probably due to the different molecular environments that surround the 4,5-*ene*-glucuronyl residue in modified xanthan and in pectate-derived oligomers, respectively.

With the xanthan lyase described here, the pyruvated mannosyl residues of xanthan side chains are preferentially removed, resulting in a modified xanthan consisting of tetrameric and pentameric repeating units. Obviously, the extent of modification is dependent on the extent of pyruvation of xanthan. Most xanthans are pyruvated to ca. 30%; therefore, if complete conversion to a polytetramer is desired, a second xanthan lyase or a β-mannosidase is required that either is specific for nonpyruvated mannosyl residues or is nonspecific. Strain XL-1 probably produces a second xanthan lyase as the crude culture broth exhibited xanthan lyase activity with chemically depyruvated xanthan as a substrate (see **Table 4.1**). To further modify the tetrameric repeating units formed by xanthan lyase to trimers, an enzyme removing the unsaturated uronic acid residue is required. Such an enzyme, a "4,5-*ene*-β-D-glucuronidase" has to our knowledge not yet been described in literature. Since an enzyme activity releasing uronic acid(-containing fragment)s from xanthan was detected in the culture supernatant (see **Table 4.1**), it may be possible that strain XL-1 produces such an enzyme. Considering the various xanthan-degrading enzyme activities produced by *P. alginolyticus* XL-1, we think this strain is a valuable source of enzymes for structural modifications of xanthan.

Acknowledgements

We thank Ian Sutherland for his generous gift of xanthan samples with different pyruvate and acetate content and Harm Snippe for kindly providing the *Klebsiella* K5-cps. We thank André Pots for his assistance with IEF and Joost Wijnen for identification of the vitamins required for growth of *P. alginolyticus* XL-1.

5. A NOVEL GENE ENCODING XANTHAN LYASE OF *Paenibacillus alginolyticus* strain XL-1

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Abstract

Xanthan-modifying enzymes are powerful tools in studying structure-function relationships of this polysaccharide. One of these modifying enzymes is xanthan lyase, which removes the terminal side chain residue of xanthan. In this paper, the cloning and sequencing of the first xanthan lyase-encoding gene is described, i.c., the *xalA* gene, encoding pyruvated mannose-specific xanthan lyase of *Paenibacillus alginolyticus* XL-1. The *xalA* gene encoded a 100,823-Da protein, including a 36-amino-acid signal sequence. The 96,887-Da mature enzyme could be expressed functionally in *Escherichia coli*. Like the native enzyme, the recombinant enzyme showed no activity on depyruvated xanthan. Compared to production by *P. alginolyticus*, a 30-fold increase in volumetric productivity of soluble xanthan lyase was achieved by heterologous production in *E. coli*. The recombinant xanthan lyase was used to produce modified xanthan, which showed a dramatic loss of the capacity to form gels with locust bean gum.

INTRODUCTION

Xanthan is an extracellular polysaccharide produced by *Xanthomonas campestris* that is widely applied as a thickener of aqueous solutions and as a stabilizer of emulsions, foams, and particulate suspensions [73]. Xanthan consists of pentasaccharide repeating units: the β-1,4-glucan backbone is substituted on alternate glucosyl residues with a trisaccharide side chain consisting of α-mannose, β-glucuronic acid, and β-mannose (see **Fig. 5.1**).

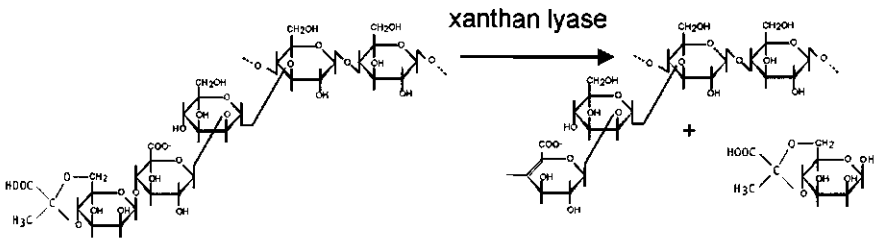

Fig. 5.1. Action of pyruvated mannose-specific xanthan lyase on the pentasaccharide repeating unit of xanthan. Acetyl groups are omitted for clarity.

The inner mannosyl residues are usually acetylated, whereas about 30% of the terminal mannosyl residues are pyruvated.

Also, xanthans carrying truncated side chains, produced by mutants of *X. campestris*, have been described [6, 107]. Studies of these variant xanthans showed that truncation of the side chain affects the rheological properties of xanthan. The absence of the terminal side chain mannosyl residue results in a weaker viscosifier than xanthan [60], whereas the absence of both the terminal mannosyl and the glucuronyl residue results in a viscosifier superior to xanthan [43]. These truncated side chain xanthans are interesting polysaccharides, both from a scientific and a practical point of view. They are, however, produced at low yields, especially the polytrimer [117]. Furthermore, the chain length of the backbone may differ from native xanthan, hampering correct comparison of xanthans with different side chains. Enzymatic modification of the side chains would be a preferable method to obtain tailor-made xanthans, as the effect of a step-by-step removal of side chain residues can be studied, leaving the backbone unaffected.

Mixed or pure bacterial cultures that grow on xanthan generally produce a mix of xanthan-degrading enzymes [17, 41, 47, 85, 105]. One of these degradative enzymes that is potentially useful for xanthan modification is xanthan lyase. Xanthan lyase removes the terminal mannosyl residue via β-elimination yielding a free mannose and a tetrasaccharide repeating unit (**Fig. 5.1**). Both nonspecific [103] and pyruvated mannose-specific xanthan lyases [1, 41, 85] have been described. The resulting polytetrasaccharide differs from the polytetrasaccharide produced by *X. campestris* mutants: xanthan lyase-modified xanthan bears a 4,5-*ene*-glucuronic acid instead of a glucuronic acid as a terminal side chain residue. So far, no amino acid sequences of xanthan lyases have been published and hence no information exists on homology or relatedness with other polysaccharide-degrading enzymes.

Previously, a xanthan-degrading bacterium, *Paenibacillus alginolyticus* XL-1, was isolated, and the pyruvated mannose-specific xanthan lyase produced by this organism was purified and characterized [85]. In this report, we present the first nucleotide sequence of a gene encoding xanthan lyase. The gene encoding pyruvated mannose-specific xanthan lyase from *P. alginolyticus* XL-1 was cloned, sequenced, and functionally expressed in *Escherichia coli*.

MATERIALS AND METHODS

Strains, plasmids and DNA manipulations For DNA isolation, *P. alginolyticus* XL-1 was cultured at 30°C on mineral salts medium [85]

supplemented with 5 g/l mannose and 0.05 g/l filter sterilized yeast extract. E. coli strains XL1-Blue MRF' (Stratagene, La Jolla, Calif.) and BL21(DE3) (Novagen, Madison, Wis.) were used for cloning and expression studies. E. coli strains and transformants were cultured at 37°C on Luria-Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl), supplemented with the proper antibiotic when required.

The ZAP Express vector (Stratagene) was used for construction of a genomic library of *P. alginolyticus* XL-1. The vector pUC19 [128] was used for construction of sublibraries of genomic *P. alginolyticus* DNA. The vector pGEM-T Easy (Promega, Madison, Wis.) was used for cloning of PCR products. The vector pET28a (Novagen) was used for heterologous expression of the xanthan lyase-encoding gene.

Plasmid DNA was isolated from *E. coli* using Qiagen miniprep spin columns (Westburg BV, Leusden, The Netherlands). Agarose-trapped DNA was extracted using the Qiaex II gel extraction kit (Westburg BV). Plasmid DNA was introduced into *E. coli* by electroporation using a Bio-Rad gene pulser. Genomic DNA of *P. alginolyticus* XL-1 was isolated using the cetyltrimethylammoniumbromide procedure [4]. Other standard molecular biology techniques were carried out according to Sambrook et al. [89].

Construction of a genomic library of *P. alginolyticus* XL-1 in *E. coli* Genomic DNA of *P. alginolyticus* XL-1 was partially digested with *Bsp*143I (an isoschizomer of *Sau*3A) (MBI Fermentas, Vilnius, Lithuania), and fragments of 4 to 14 kb were isolated after agarose gel electrophoresis. The purified fragments were ligated to *Bam*HI-digested and calf intestine alkaline phosphatase-treated ZAP Express vector (Stratagene, La Jolla, CA) according to the suppliers' instructions. After transfection of the packaged ligation mix, a primary phage library containing 14,500 PFU was obtained. After amplification and mass excision of the phage library, the resulting pBK-CMV-phagemid library was transfected to *E. coli* XL-1 Blue MRF' and plated on LB agar containing ampicillin (100 µg/ml) as well as X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) for blue-white screening. It appeared that 75% of the colonies contained an insert with an average size of 4.5 kb. If a genome size of 4.2 Mb is assumed for *P. alginolyticus* XL-1 (as for *Bacillus subtilis*), the primary library represented 12 times the genome.

PCR experiments PCRs were carried out in an automated thermal cycler (Perkin-Elmer). Standard reaction conditions used were 10 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and ending with 10 min at 72°C. Primer R1 (5'-CTG CGC TTC CGC GGT AAG CG-3') was designed on the reverse strand of the *xalA* gene, near the start codon. This primer was used, in combination with primer M13 (5'-CCC AGT CAC GAC GTT GTA AAA CG-3') or M13 reverse (5'-AGC GGA TAA CAA TTT CAC ACA GG-3'), to obtain the sequence upstream of the

xalA gene, including the start of the gene (see Results for details). Primer C5 (5'-GAA TTC *ATG* **TCG** GAT GAG TAT GAT ACG CTG C-3') was designed on the 5' end of the *xalA* gene, starting at the codon for the N-terminal Ser-residue (bold face) of the mature protein. An *Eco*RI site (underlined) was introduced for cloning purposes; a start codon (in italics) was included for expression in vectors lacking a transcription start. Primer C7 (5'-AAG CTT CCC TCC CCA AAG CTG C-3') was designed on the reverse complement strand of the *xalA* gene, upstream of the putative terminator sequence, including a *Hin*dIII site (underlined) for cloning purposes. Degenerate oligonucleotide primers are listed in **Table 5.1**.

Table 5.1. Amino acid sequences of the N terminus and internal peptides of purified pyruvated mannose-specific xanthan lyase from *P. alginolyticus* XL-1 and nucleotide sequences of the derived degenerate primers.

Peptide	Sequence[a]	Degenerate oligonucleotide primer[b]
N terminus	SDEYDTLRLKWRDM (residues 1-14)	D2: 5'-GAY GAR TAY GAY ACV TTR C-3'
Peptide 15	SXISSENSIGT (residues 324-334)	D5: 5'-TCS GAR AAY TCS ATY GGH AC-3'
		D7[c]: 5'-GTD CCR ATS GAR TTY TC-3'
Peptide 17	TSAQVSSYASNPNISVL (residues 626-642)	D12: 5'-CTR ATR TTN GGR TTR CTN GC-3'
Peptide 25	TPGGTTNYLXVDLR (residues 49-62)	-
Peptide 28	ENTLNVVGVNFX (residues 653-664)	-

[a] X can be either cysteine or tryptophan. Cysteine was not determined with the method used. Tryptophan was masked by diphenylthiourea, which is formed from phenylisothiocyanate in the Edman degradation. Underlined amino acids were used to design degenerate primers. Residue numbers refer to the position in the deduced amino acid sequence of the mature enzyme encoded by the *xalA* gene (1 = the N-terminal residue).

[b] D = (A + T + G); H = (A + T + C); N = (A + C + T +G); R = (A + G); S = (C + G); V = (A + C + G); Y = (C + T).

[c] primer D7 is complementary to D5

Determination of internal amino acid sequences of xanthan lyase

P. alginolyticus XL-1 xanthan lyase was produced and purified as described previously [85], followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The Coomassie-stained xanthan lyase band was cut from the gel and transferred to an Eppendorf tube for in-gel digestion. Briefly, washing was carried out in 0.2 M ammoniumbicarbonate containing 50% acetonitrile. The protein was reduced with dithiothreitol and alkylated with iodoacetamide followed by in-gel digestion with 0.5 to 3 µg of trypsin (Promega, modified) in 0.2 M ammoniumbicarbonate overnight at 37°C. The

tryptic peptides were extracted using acetonitrile in 0.1% trifluoroacetic acid, first at 60% and then at 40%. The peptide extract was separated and fragments were isolated on polyvinylidene difluoride membrane for sequence analysis using a microblotter system (Perkin-Elmer). Edman degradation of polyvinylidene difluoride-bound peptides was carried out with a Procise cLC sequencer (Perkin-Elmer).

Expression of xanthan lyase in *E. coli* BL21(DE3) Plasmid pEXL1 (pET28a carrying the *xalA* gene as a 2.7-kb *Eco*RI/*Hin*dIII fragment) was transformed to a λDE3 lysogen of *E. coli* BL21 for expression testing. Strain BL21(DE3) harbors the T7 RNA polymerase gene required for transcription of the target gene in pET28a. Both the T7 RNA polymerase and the target gene are under control of the *lac* repressor (*lacI*), thus allowing tight control of the expression of the target gene. For expression of the *xalA* gene, BL21(DE3) carrying pEXL1 was cultured on 50 ml of LB medium containing 50 µg of kanamycin/ml. At an optical density at 600 nm of 0.5, 1 mM IPTG was added for induction, and the culture was incubated for another 3 h. The cells were harvested and the cell pellet was resuspended in 5 ml of 15 mM potassium phosphate (pH7). After sonication, the cell extract was assayed for xanthan lyase activity.

Xanthan lyase assay Xanthan lyase activity was determined as described previously [85], by incubating xanthan lyase with xanthan and measuring the increase of A_{235}, the increase of reducing sugars [70], or the increase of thiobarbituric acid-reactive material [122].

Protein electrophoresis SDS-PAGE was carried out according to Laemmli [57] using a Hoeffer Mighty Small system (Pharmacia). For estimation of molecular mass, prestained precision standards (10 kDa to 250 kDa; Bio-Rad) were used. Gels were stained with Coomassie brilliant blue.

Rheological measurements Rheological measurements on xanthan-locust bean gum (LBG) gels were performed in a Bohlin CVO Rheometer using a C14 cup-and-bob geometry. Gels were formed by mixing 5 g of xanthan (native or modified)/l and 5 g of LBG/l solutions (in 15 mM potassium phosphate buffer [pH7]). Modified xanthan was prepared by incubating 10 ml of a 5 g of xanthan/l solution in 15 mM potassium phosphate (pH 7) with 500 µl of cell extract of an induced BL21(DE3) culture harboring pEXL1 for 2 h at 30°C. After transfer to the rheometer, the gel was heated from 25 to 80°C and subsequently cooled from 80 to 25°C. After the gel was allowed to set at 25°C for 15 min, the gel was submitted to a stress sweep regime from 0.08 to 3 Pa at a frequency of 1 Hz to determine the rigidity (elastic modulus, G') and the viscosic aspect (viscous modulus, G'') of the molecular bonding in the gel.

Nucleotide sequence accession number The DNA sequence of *xalA* has been deposited in the EMBL/GenBank/DDBJ database under the accession number AF242413.

RESULTS

Amino acid sequences of xanthan lyase peptides and synthesis of a specific *P. alginolyticus* xanthan lyase probe by PCR Xanthan lyase was purified and both N-terminal and internal amino acid sequences were determined (**Table 5.1**). Degenerate primers, designed from these amino acid sequences, were used in PCR reactions with genomic *P. alginolyticus* XL-1 DNA as a template to obtain xanthan lyase specific DNA fragments.

The combination of primers D2 and D7 yielded a 1-kb PCR product (product A), whereas primers D5 and D12 yielded a 0.9-kb PCR product (product B). As D5 and D7 were designed from the same peptide, but complementary to each other, the amplified sequences must be overlapping parts of the xanthan lyase-encoding gene. PCR products A and B were cloned in pGEM-T Easy to obtain pGA and pGB, respectively. The nucleotide sequences of the inserts of pGA and pGB were determined. The deduced amino acid sequences contained the peptide sequences, including the amino acid residues that were omitted in the primer design (see **Table 5.1**). Furthermore, the sequence of peptide 25 was found in the deduced amino acid sequence of PCR product A. These results strongly suggested that the cloned fragments contained parts of the gene encoding pyruvated mannose-specific xanthan lyase. The insert of plasmid pGA was excised with *Eco*RI, and the resulting 1-kb fragment was isolated. The insert fragment was used as a template in a PCR reaction with primer D2 and D7 using digoxigenin-labeled nucleotides to obtain a specific digoxigenin-labeled xanthan lyase probe.

Isolation of the *P. alginolyticus* xanthan lyase-encoding gene Initially, it was attempted to isolate the xanthan lyase-encoding gene from the *P. alginolyticus* XL-1 genomic library, both by expression screening and by colony hybridization with the specific xanthan lyase probe. For expression screening, the library was plated on LB medium solidified with a mixture of xanthan and LBG. As purified xanthan lyase liquefies xanthan-LBG gels (unpublished observations), these plates should be liquefied by colonies expressing xanthan lyase. Although liquefying colonies were obtained, none of these expressed xanthan lyase, based on activity measurements. Also, screening by colony hybridization proved to be unsuccessful: a total of 22,000 colonies were screened but no positive clones could be detected. As the complete xanthan lyase-encoding gene could not be obtained from a representative genomic library, it was decided to construct sublibraries to try to obtain the gene, if not in its entirety, then in parts.

Genomic DNA of *P. alginolyticus* XL-1 was digested with several restriction enzymes, and the resulting fragments were separated on an agarose gel. After Southern blotting and hybridization with the probe, a 4.3-kb *Bam*HI fragment, a 2.9-kb *Eco*RI fragment, and two *Hin*dIII fragments (0.6 kb

and 3.6 kb) were visible. The latter observation was in accordance with the nucleotide sequence of PCR fragment A, which contained a *Hin*dIII site at 556-bp from the 5'-end. Genomic *Bam*HI fragments (fraction of 4.2 to 4.4 kb), *Eco*RI fragments (fraction of 2.7 to 3.0 kb) and *Hin*dIII fragments (fractions of 0.5 to 0.7 kb and 3.5 to 3.7 kb) were isolated and ligated into the corresponding sites of pUC19. After transformation, colony hybridization was carried out with the xanthan lyase probe to determine which clones carried the DNA fragments of interest. No hybridizing transformants containing a *Bam*HI or an *Eco*RI fragment could be detected. Both *Hin*dIII fragments, however, could be obtained from the corresponding sublibraries. Plasmid DNA was isolated, and the complete nucleotide sequences of the *Hin*dIII fragments were determined. Based on the nucleotide sequences and the Southern blot analysis, a restriction map was constructed (**Fig. 5.2**).

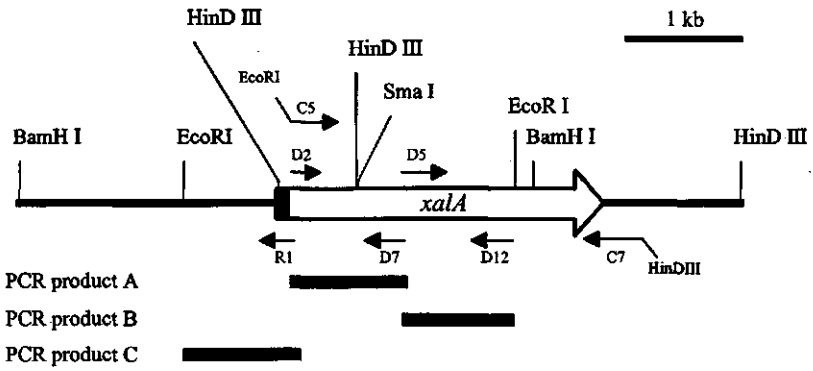

Fig. 5.2. Schematic representation of the genomic DNA fragment carrying the *P. alginolyticus* XL-1 *xalA* gene. The *xalA* gene is indicated by an open arrow, the part of the gene encoding the signal peptide is in black. Primer R1 was used to obtain the sequence upstream of the 5' *Hin*dIII site in the *xalA* gene. Primers D2 and D7 yielded PCR product A, which was used as a template to obtain a specific xanthan lyase probe. Primers D5 and D12 yielded PCR product B. PCR product C was obtained from a reaction on the ligation mixture of the *Eco*RI sublibrary, using primers R1 and M13 reverse. Primers C5 and C7 were used to synthesize the part of the gene encoding mature xanthan lyase for heterologous expression.

The sequence obtained from the two *Hin*dIII fragments encoded mature xanthan lyase as well as a large part of the signal sequence. The start of the gene, however, was missing. From **Fig. 5.2** it can be observed that the hybridizing *Eco*RI and *Bam*HI fragments should contain the missing part of the gene. These fragments could not, however, be isolated from the sublibraries. Apparently the introduction of plasmids containing these inserts, which carry a truncated xanthan lyase gene with the complete signal sequence, as well as probably a promoter region, is lethal to *E. coli*.

Therefore, it was attempted to amplify a part of the *Eco*RI fragment by PCR. A reaction was carried out on the ligation mixture of the *Eco*RI sublibrary, using specific primers for the xanthan lyase-encoding gene (R1) and the regions of pUC19 flanking the insertion site (M13 or M13 reverse). The primer combination R1 and M13 reverse yielded the expected 1-kb product (product C). The complete nucleotide sequence of this PCR product was determined. The sequence overlapped the sequence of the xanthan lyase-encoding gene by 200 bp and contained the missing part of the gene as well as about 800 bp upstream of the start codon.

Nucleotide sequence of the xanthan lyase-encoding gene From the two genomic *Hin*dIII fragments and PCR product C, the complete xanthan lyase-encoding gene and its flanking regions could be reproduced (see "Nucleotide sequence accession number" above). The sequence revealed a putative promoter sequence, a putative ribosome-binding site, and a 2,811-bp open reading frame starting with an ATG start codon and ending with TAA. A putative terminator sequence (inverted repeat) was observed 60 bp downstream of the stop codon. The open reading frame, designated *xalA*, encoded a 100,823-Da protein, of which the first 36 amino acids represent a *Bacillus*-like signal sequence [74]. The signal peptide displays three positively charged residues (Arg) in the N-terminal region, a 20 amino acid hydrophobic core terminated by two helix-breaking Pro residues, and a signal peptidase recognition site (Ala-X-Ala). Behind this putative cleavage site, the N terminus of the mature enzyme should start, which is indeed identical to the determined N-terminal amino acid sequence of purified *P. alginolyticus* XL-1 xanthan lyase. Furthermore, all determined internal peptide sequences (see **Table 5.1** for residue numbers) could be observed in the deduced amino acid sequence, confirming that the proper gene was obtained.

The mature xanthan lyase consists of 900 amino acids with a calculated molecular mass of 96,887 Da. A comparison of the deduced amino acid sequence of *xalA* with the SwissProt database revealed homology with other polysaccharide lyases, i.e., hyaluronidases and chondroitinases. The observed homologies were, however, very low. The highest scores were 36% identity in a 690-amino-acid overlap for *Streptomyces griseus* hyaluronidase (accession number BAA78618) and 33% identity in a 770-amino-acid overlap for a putative lyase secreted by *Streptomyces coelicolor* (accession number CAA19982). *P. alginolyticus* XL-1 xanthan lyase did not show any activity on hyaluronic acid or chondroitin.

Functional expression of mature xanthan lyase in *E. coli* The *xalA* gene, without the signal sequence, was cloned into the expression vector pET28a in order to prove the functionality of the gene. The oligonucleotides C5 and C7 were designed to synthesize the gene by PCR while introducing the appropriate restriction sites (i.e., *Eco*RI and *Hin*dIII, respectively) for cloning the gene into pET28a. A reaction with these primers on genomic *P.*

alginolyticus XL-1 DNA yielded the expected 2.7-kb fragment, which was cloned in pGEM-T Easy to obtain pGXL1. Unfortunately, the restriction sites to be used for cloning were also present in the *xalA* gene itself (see **Fig. 5.2**). Thus, to avoid the necessity of partial restriction with *Eco*RI and *Hin*dIII, pGXL1 was digested with *Eco*RI/*Sma*I and with *Sma*I/*Hin*dIII. The *Eco*RI/*Sma*I fragment containing the 5' part of the *xalA* gene and the *Sma*I/*Hin*dIII fragment containing the 3' part were isolated and ligated into *Eco*RI/*Hin*dIII-digested pET28a to obtain plasmid pEXL1. After transformation and amplification of pEXL1 in *E. coli* XL1-Blue MRF', the plasmid was transformed to *E. coli* BL21(DE3) for expression analysis. The BL21(DE3) transformants were plated on LB medium, supplemented with 1mM IPTG and 50 µg of kanamycin/ml and solidified by xanthan and LBG. The gel was liquefied, confirming that xanthan lyase was expressed.

Induced BL21(DE3) cells carrying pEXL1 were harvested and the cell pellet was sonicated. Upon incubation of the soluble fraction of the sonicated cells with xanthan, xanthan lyase activity could be demonstrated by measuring the increase of A_{235}, the increase of reducing sugars, and the increase of thiobarbituric acid-reactive material. The soluble xanthan lyase activity in the cell extract, as quantified by measuring A_{235}, amounted to 8.3 U/ml. A total soluble xanthan lyase activity of 41.5 U was obtained from one 50 ml culture. Also, the insoluble fraction of the cell extract, resuspended in 15 mM potassium phosphate buffer, contained a trace of xanthan lyase activity (about 2% of total activity). This activity was doubled after overnight incubation at 4°C, suggesting that the recombinant enzyme was partially captured in inclusion bodies that resolubilized and became active upon incubation in buffer.

SDS-PAGE analysis (**Fig. 5.3**) showed a 100-kDa band, corresponding to recombinant xanthan lyase, appearing after induction in the cell extract of *E. coli* carrying pEXL1 (lane 2). Also, the insoluble fraction of the cell extract (lane 3) contained this band in a huge amount, confirming that at least 95% of the xanthan lyase was deposited as inclusion bodies. The molecular mass of the recombinant xanthan lyase as observed in lane 2 is slightly higher than the molecular mass of native xanthan lyase (lane 4). This can be explained by the pET28a-encoded N-terminal His tag that is attached to the recombinant xanthan lyase. The estimated molecular mass of mature xanthan lyase is in accordance with the molecular mass predicted from the deduced amino acid sequence (97 kDa). Previously, however, a molecular mass of 85 kDa was reported [85]. This difference can be attributed to the 94 kDa marker (phosphorylase b, lane 5) that was initially used as a reference in estimating the molecular mass of xanthan lyase. As can be observed from **Fig. 5.3**, phosphorylase b migrates more slowly than the 100 kDa marker of the Bio-Rad precision protein standard (lane 6). Thus, the molecular mass of

xanthan lyase was previously estimated to be smaller than 94 kDa, whereas its actual size is 97 kDa.

Like the purified *P. alginolyticus* enzyme, the recombinant xanthan lyase showed no activity on depyruvated xanthan. As shown in **Fig. 5.4 A**, the pH profiles of native and recombinant xanthan lyase are identical. The temperature profiles, however, are different (**Fig. 5.4 B**). Recombinant xanthan lyase still shows activity at 65°C whereas native xanthan lyase rapidly loses activity above 55°C. Considering that purified native xanthan lyase is not stable above 45°C [85], the results suggest that the recombinant xanthan lyase is slightly more stable.

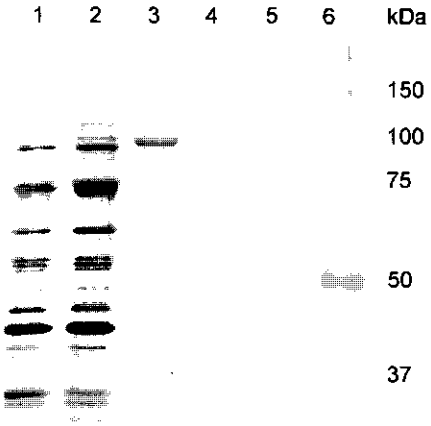

Fig. 5.3. SDS-PAGE gel of fractions of uninduced and induced *E. coli* BL21(DE3) cells carrying pEXL1. Lane 1: uninduced cells, total cell protein; lane 2: induced cells, cell extract (soluble fraction); lane 3: induced cells, cell extract (insoluble fraction, the relative amount loaded is 1/10 of lane 1 and 2); lane 4: native purified xanthan lyase; lane 5: marker proteins (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa); lane 6: Bio-Rad precision protein standards.

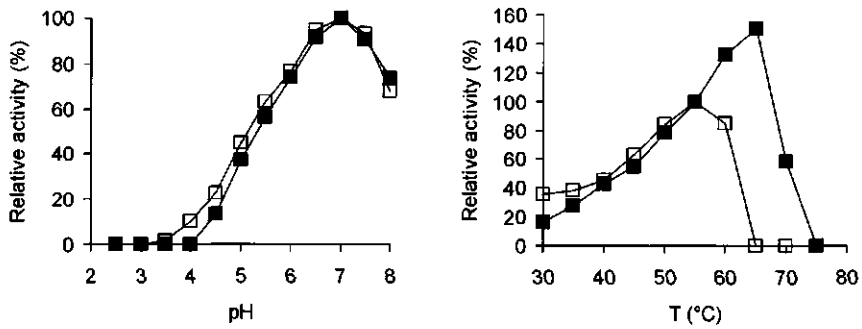

Fig. 5.4. A. Relative activity of native xanthan lyase (purified, open symbols) and recombinant xanthan lyase (cell extract, closed symbols) at different pH values, on xanthan in McIlvaine buffer. **B.** Relative activity of native xanthan lyase (purified, open symbols) and recombinant xanthan lyase (cell extract, closed symbols) at different temperatures, on xanthan in 15 mM potassium phosphate buffer (pH7). The activity of both enzymes was set at 100% at the conditions under which the native xanthan lyase was most active.

Modification of xanthan with recombinant xanthan lyase To illustrate an effect of recombinant xanthan lyase on the physical properties of xanthan, xanthan-LBG gels were prepared with unmodified xanthan and xanthan lyase-modified xanthan. After preparing the gels, the elastic modulus (G') and the viscous modulus (G") of the gels were determined. The G' value, which is a measure for the "rigidity" of the gel, was 35 for the the gel prepared with unmodified xanthan. The gel prepared with xanthan lyase-modified xanthan, however, had a G' value of only 1.4. Based on this value, the mixture of modified xanthan and LBG could hardly be classified as a gel. Consequently, tan δ (= G"/ G'), which is a measure for the "fluidity" of the gel, is much higher for the modified xanthan-LBG gel (1.0 as opposed to 0.12 for the unmodified xanthan-LBG gel), confirming the visual observation that this gel has a far more liquid character than the other gel.

DISCUSSION

This report describes the first nucleotide sequence of a xanthan lyase-encoding gene. The gene, encoding a pyruvated mannose-specific xanthan lyase of *P. alginolyticus* XL-1, was designated *xalA*, as probably a second xanthan lyase with a different substrate specificity is also produced by this strain [85]. Comparison of the deduced amino acid sequence of xanthan lyase to the Swiss-Prot database showed only slight homologies to other polysaccharide lyases, indicating that this enzyme constitutes a new family of polysaccharide lyases.

The mature xanthan lyase, devoid of its signal sequence, was functionally expressed in *E. coli*. The N terminus of mature xanthan lyase was identical to the deduced N terminus located behind the putative signal peptidase cleavage site, confirming the start of the mature enzyme. Furthermore, the internal peptide sequences obtained from native purified pyruvated mannose-specific xanthan lyase were all present in the deduced amino acid sequence of *xalA*. The recombinant enzyme was, like the *P. alginolyticus* XL-1 enzyme, not active on depyruvated xanthan. Furthermore, native and recombinant xanthan lyases showed identical pH profiles. The temperature profiles, however, indicated that the recombinant xanthan lyase is a bit more stable than the native xanthan lyase. The increased stability can probably be attributed to a stabilizing effect of proteins from the cell extract that were present in the case of recombinant xanthan lyase. Alternatively, the His tag may stabilize the enzyme, or the conformation of the enzyme may be slightly different in the heterologous host. The deduced amino acid sequence predicted a 97-kDa protein, which was in accordance with the observed molecular mass of native as well as recombinant xanthan lyase on SDS-PAGE (**Fig. 5.3**).

The undisrupted xanthan lyase-encoding gene could not be isolated from a representative genomic library. In addition, fragments containing the 5' terminus of the gene could not be obtained from various sublibraries. These results suggest that the 5' end of the xanthan lyase-encoding gene in combination with the DNA upstream of the start codon (i.e., the promoter sequence and the ribosome-binding site) cannot be rescued in *E. coli*. Possibly, the signal sequence linked to the (truncated) xanthan lyase protein is expressed, which may be lethal to *E. coli*, e.g., by blocking the protein export system or accumulation in the cell membrane. Nevertheless, the remainder of the *xalA* gene could be obtained by PCR, using a ligation mixture for the construction of one of the sublibraries as a template.

Although most of the recombinant xanthan lyase was captured in inclusion bodies, the production of soluble xanthan lyase by recombinant *E. coli* was greatly enhanced compared to that by *P. alginolyticus* XL-1. A 50 ml culture of *E. coli* BL21(DE3) harboring pEXL1 yielded 40 U of soluble xanthan lyase, whereas 2 l of *P. alginolyticus* XL-1 culture yielded only 50 U [85]. Thus, recombinant production of xanthan lyase provides the possiblity of producing relatively large amounts of enzyme. Furthermore, no extensive purification is required, as no interfering xanthan-affecting enzyme activities are produced by *E. coli*. This makes the recombinant xanthan lyase an excellent tool for studying structure-function relationships of xanthan. This was illustrated by preparing xanthan lyase-modified xanthan and determining its ability to form gels with LBG. Whereas native xanthan formed a firm gel with LBG, modified xanthan hardly showed any interaction. This radical change in functional properties was caused by the action of a single enzyme that only removes the pyruvated terminal side chain residues. The availability of xanthan lyase opens the possibility for detailed further studies of the physical properties of xanthan lyase-modified xanthan. Furthermore, xanthan lyase treatment can be followed by further modifications. These could be enzymatic to obtain a (partial) polytrimer using enzymes like unsaturated glucuronyl hydrolase (4,5-*ene*-β-glucuronidase) [40] or chemical modification at the double bond in the 4,5-*ene*-β-glucuronyl residue.

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6. DISCUSSION

GENERAL

The aim of the research in this thesis was to obtain specific EPS-degrading enzymes. These enzymes were to be used as a tool for structural and functional modification of EPSs. Xanthan, the most important food-grade EPS, was chosen as the target EPS.

P. alginolyticus XL-1, a bacterium related to *Bacillus circulans* and *Paenibacillus chondroitinus*, was used as the source of xanthan-modifying enzymes. Strain XL-1 was the single xanthan lyase producer among twelve strains isolated from a xanthan-degrading mixed culture obtained from soil. *P. alginolyticus* XL-1 partially degrades xanthan, using a mixture of enzymes. Xanthan degradation appeared to be restricted to the side chains. No endoglucanase activity was observed in the culture broth and a high-molecular-weight (HMW) xanthan remnant persisted in the medium. This HMW product had a limited solubility and showed interaction with Congo red, indicative of a β-1,4 glucan, i.e., a (probably partially) bare xanthan backbone.

XalA, the pyruvated mannose-specific xanthan lyase of *P. alginolyticus* XL-1, was the principal enzyme of interest. In order to be able to use XalA as a tool for structural modification of xanthan, the enzyme had to be produced in rather large amounts. In order to increase the productivity of XalA, the gene encoding this enzyme, *xalA*, was isolated, cloned and expressed heterologously in *E. coli*. Heterologous production of XalA by *E. coli* yielded a 30-fold increased volumetric productivity, compared to *P. alginolyticus* XL-1. Thus, sufficient enzyme could be obtained for the modification of xanthan.

OTHER XANTHAN-DEGRADING ENZYMES OF *P. alginolyticus* XL-1

Besides the pyruvated mannose-specific xanthan lyase, several other enzyme activities, potentially useful for xanthan modification, were excreted during growth on xanthan. Although the titers of these activities in the culture broth were very low, the enzymes could be partially purified. An overview of the enzymes and their sites of action is presented in **Fig. 6.1**.

Xanthan lyase B In the culture supernatant, xanthan lyase activity could be measured with native as well as depyruvated xanthan as a substrate. The purified xanthan lyase (xanthan lyase A), however, showed no activity on depyruvated xanthan (Chapter 4). Therefore, it is suggested that a second xanthan lyase is excreted that is either non-specific or specific for non-pyruvated xanthan. Unfortunately, this enzyme, designated xanthan lyase B, could not be precipitated from the culture broth with ammonium sulphate at less than 60% saturation. This is a critical value: above this point, also the HMW xanthan remains will precipitate from the culture broth. This results in a

very viscous slurry from which no enzymes can be purified. Since other concentration techniques like ultrafiltration yielded similar problems, the enzyme was not purified further and the exact substrate specificity was not established.

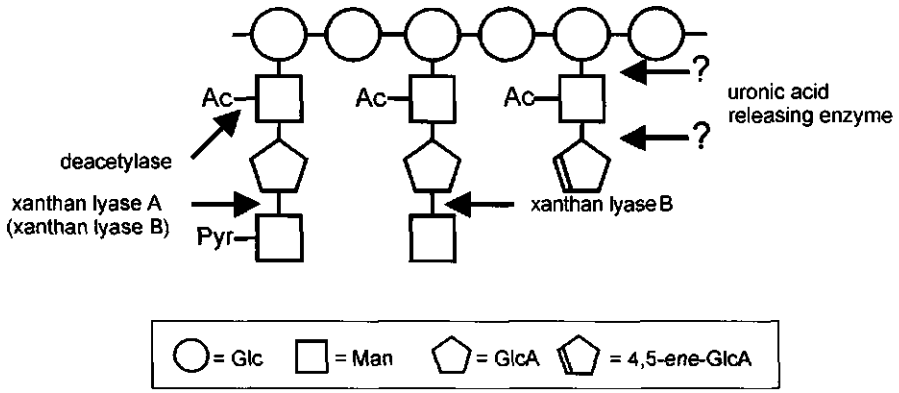

Fig. 6.1. Xanthan-degrading enzymes of *P. alginolyticus* XL-1 and their sites of action.

Xanthan deacetylase *P. alginolyticus* XL-1 excreted an acetylase that was able to release acetyl groups from xanthan. The 'xanthan deacetylase' was recovered at about 90% purity and had a molecular mass of approximately 60 kDa. This enzyme could be useful for the removal of acetyl groups from xanthan as a mild alternative to the alkaline treatment of Bradshaw et al. [10].

Uronic acid releasing enzyme A 'uronic acid releasing' enzyme was purified from the crude culture broth to about 80% homogeneity. This enzyme, approximately 100 kDa in molecular mass, was only active on xanthan that had been treated with xanthan lyase, or on native xanthan in the presence of xanthan lyase. Considering that the xanthan backbone remained intact, it must be assumed that either 4,5-*ene*-β-glucuronic acid or 4,5-*ene*-β-glucuronyl-α-mannosyl disaccharide was released. In the first case, the enzyme would be a 4,5-*ene*-β-glucuronidase. Such an enzyme, similar to the 42-kDa 'unsaturated glucuronyl hydrolase' of *Bacillus* sp. GL1 [40], could be used in conjunction with xanthan lyase to obtain polytrimer xanthan (see Chapter 1). Whichever product is released by the 'uronic acid releasing' enzyme, the enzyme is different from the *Bacillus* sp. GL1 'unsaturated glucuronyl hydrolase' . The *Paenibacillus* enzyme is produced extracellularly and has a much higher molecular mass.

Xanthan-LBG-gel liquefying enzyme activities While screening the *P. alginolyticus* XL-1 gene library for xanthan lyase activity, indications were found for the existence of two more enzymes that are active on xanthan. The library was plated on xanthan-LBG solidified plates as described in Chapter 5.

Three clones were isolated that liquefied the assay plates; two of these contained identical plasmids. The two different plasmids were designated pXLY4 and pXLY5 (GenBank accession no.'s AF242414 and AF242415, respectively). Unfortunately, the xanthan-LBG-gel-liquefying enzyme activity encoded by these plasmids could not be identified. No pyruvate or reducing sugars were released from xanthan (or LBG) and no xanthan lyase activity could be measured in the cell extracts of the gel-liquefying *E. coli* transformants. Database searches with the (translated) insert sequences of the plasmids did not reveal any homologies with known proteins or genes.

MOLECULAR ASPECTS OF XalA

The xanthan lyases decribed in literature can be divided into two groups based on their molecular masses: 'large xanthan lyases' and 'small xanthan lyases'. These groups may represent members of different polysaccharide lyase families. The 'large' group contains the 97-kDa XalA enzyme and the 75-kDa xanthan lyase of *Bacillus* sp. GL1 [41]. The latter enzyme has been suggested to be excreted as a 95-kDa precursor from which a 20-kDa C-terminal peptide is removed posttranslationally [42]. No indication for such a posttranslational modification was found for XalA. The 'small' group contains the xanthan lyases obtained from a *Corynebacterium* sp., a *Bacillus* sp. and a mixed culture, with molecular masses ranging from 30 to 33 kDa [1, 103]. It appears that the natural substrate for the two large xanthan lyases is intact xanthan [41; Chapter 4]. The small xanthan lyases, on the other hand, have been suggested to act preferentially on partially depolymerized xanthan [103, 1]. The specificity for pyruvated or non-pyruvated xanthan and the molecular mass of the xanthan lyase do not appear to be connected.

XalA was placed in polysaccharide lyase family 8 (see http://afmb.cnrs-mrs.fr/~pedro/CAZY/lya_8.html), based on the results of gapped BLAST searches [2]. This family so far contained only chondroitinases and hyaluronidases. The domain structure of XalA is presented in **Fig. 6.2**. The protein contains an N-terminal signal sequence for excretion, a polysaccharide lyase family 8 domain (PFAM entry: Lyase_8) and a C-terminal domain, designated CXAL, that is preceded by a proline-rich motif. No preliminary function could be assigned to the CXAL domain based on the amino acid sequence. Database searches revealed no significant homology to any known domain or protein.

XalA is different from the other polysaccharide lyases in family 8 with respect to the substrate that is degraded, the presence of a large C-terminal domain, and its mode of action. Xanthan lyases are exo-acting enzymes, attacking from the outer end of the polysaccharide side chain. In contrast, all other polysaccharide lyases known to date are endo-acting, attacking the polysaccharide backbone. In glycosyl hydrolases, the mode of action is

determined by the active site topology. Endo-glycosidases have a 'cleft'-like active site that can accomodate a polysaccharide chain, allowing the enzyme to hydrolyse glycosidic bonds in the backbone. Exo-glycosidases, on the other hand, have a 'pocket'-like active site that can only accomodate terminal sugar residues from the backbone or the side chains [26].

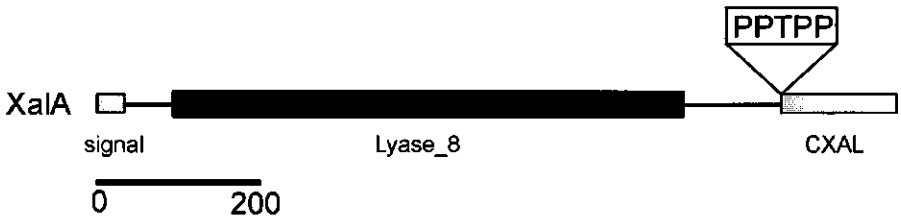

Fig. 6.2. Domain structure of XalA.

Similar active site topologies may apply to polysaccharide lyases. At present, only one 3D structure of a family 8 polysaccharide lyase is publicly available from the PDB database, i.e., chondroitin AC lyase from *Cytophaga heparina* (PDB entry: 1CB8; two more are currently on hold: 1C82 and 1EGU). Indeed, this enzyme shows a cleft, similar to endoglucanase CelD, which harbours the hypothetical active site residues [31]. Possibly, the C-terminal domain of xanthan lyase, which is absent in other family 8 polysaccharide lyases, affects the catalytic site topology, enforcing an 'exo' mode of action. The proline-rich region preceding the CXAL domain, indicates a strong 'bend' in the protein chain. This may allow the CXAL domain to incline towards and partially block the active site, which should be situated in the Lyase_8 domain.

XANTHAN MODIFICATION BY RECOMBINANT XalA

The recombinant xanthan lyase was used to modify xanthan. Since XalA is specific for pyruvated mannosyl residues, and the degree of pyruvation of an average commercial xanthan sample is about 30%, no more than 30% of the side chains could have been modified. As a result of the modification with xanthan lyase, the ability of xanthan to form a gel with LBG was lost (Chapter 5).

The observed change in the functional properties of XalA-modified xanthan may have been caused by the simultaneous removal of pyruvate groups with the mannosyl residues. It has been noted by several authors that the removal of pyruvate groups stabilizes the ordered conformation and weakens the interaction with LBG [92, 109], or even prevents gelation [19]. Thus, the absence of pyruvate groups in XalA-modified xanthan may result in

a conformation that is too rigid for any interaction with LBG, supporting the view that unordered xanthan segments interact with LBG [129, 20].

Alternatively, a specific interaction may occur between the xanthan side chain and LBG, which is lost upon the removal of the terminal side chain residue. The model proposed by Tako [109], however (see Chapter 1), seems to be contradicted by our results. In this model, the terminal xanthan side chain residue is not involved in the intermolecular bonding.

A third possibility is that the interaction with LBG is lost as a result of the very partial nature of the modification by XalA. It is not known how such a partial modification affects the properties of xanthan. The irregular structure of the XalA-modified xanthan may prevent the polysaccharide from adopting an ordered conformation. As discussed in Chapter 1, a certain amount of order appears to be required for xanthan to form a gel with LBG, as much as a certain amount of disorder. The ordered conformation may be required either for a direct interaction with LBG, as suggested by several authors [27, 124], or perhaps to provide sufficient rigidity to the gel.

xalB, A SECOND XANTHAN LYASE ENCODING GENE?

While screening the genomic *P. alginolyticus* XL-1 library for the *xalA* gene, a clone was isolated that hybridized with a probe specific for *xalA*. The nucleotide sequence of the genomic DNA insert of the isolated plasmid was very similar to *xalA*, however, not identical. This observation suggested that this clone contained (a part of) a gene encoding a different xanthan lyase, possibly the enzyme that was active on depyruvated xanthan, i.e., xanthan lyase B. Like *xalA*, this gene could not be isolated in its entirety from the genomic library. Inverse PCR yielded the remainder of the structural gene, designated *xalB*, as well as 470 bp upstream of the gene, including the ribosome binding site and the promoter region (GenBank accession no. AF318176). No terminator sequence was observed downstream of *xalB*, in contrast to *xalA*.

Molecular aspects of the XalB protein The *xalB* gene encoded a 1058-amino acid, 110.5-kDa protein that was very similar to XalA (55% identity, 69% similarity in a 913 amino acid overlap). Like XalA, XalB can be placed in polysaccharide lyase family 8. As can be observed from **Fig. 6.3**, the domain structure of XalB is similar to XalA, including a Pro-rich motif preceding the C-terminal domain. However, instead of a single CXAL domain, XalB contains two such domains in tandem. The individual CXAL domains of XalB are almost identical, and both are very similar to the CXAL domain of XalA.

Heterologous expression of XalB In order to assess the functionality of the *xalB* gene, the part supposedly encoding mature XalB was cloned into the expression vector pET28a, as described previously for *xalA* (Chapter 5). A protein of the expected size was expressed in *E. coli*, but no xanthan lyase

activity could be measured. This may be due to problems associated with the heterologous expression, e.g., protein misfolding in *E. coli* or an adverse effect of the pET28a-derived N-terminal His-tag. However, no such problems occurred with the heterologous expression of XalA.

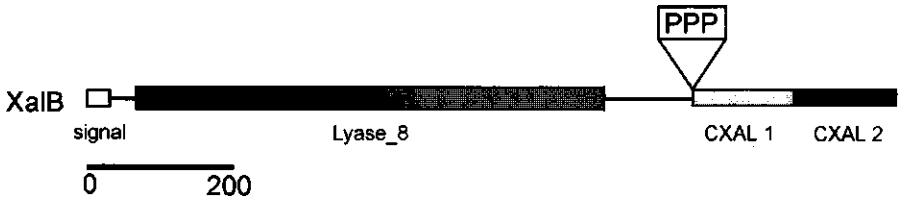

Fig. 6.3. Domain structure of XalB.

Alternatively, a postranslational modification, which is unlikely to occur in *E. coli*, may be required in order to obtain an active enzyme, e.g., the removal of the second CXAL domain. As described above, such a posttranslational modification has been suggested for the xanthan lyase of *Bacillus* sp. GL1 [42]. However, in this particular case, the removal of the 20-kDa C-terminal peptide was not required to obtain an active enzyme.

A third possibility is that XalB is not a xanthan lyase. If the C-terminal domain does affect the active site topology as suggested above, the double CXAL domain in XalB may indicate a substrate specificity different from XalA. Finally, the duplication of the C-terminal domain in XalB could indicate that a partial gene duplication has occurred, which may have resulted in a disfunctional gene.

Since *xalA* is the only xanthan lyase-encoding gene reported to date, the suggestions made with respect to the function of *xalB*, and also the role of the C-terminal CXAL domains in XalA and XalB, are quite speculative. Until the functionality of the *xalB* gene has been confirmed, it will be unclear whether XalB is a (functional) xanthan lyase or not.

INDUSTRIAL RELEVANCE AND FUTURE PROSPECTS

From an industrial point of view, xanthan is the most important EPS, finding widespread applications both in food and in non-food industry. Xanthan-modifying enzymes, such as those produced by *P. alginolyticus* XL-1, may be very helpful in controlling and understanding the functionality of xanthan. Many questions with respect to the relation between the structure and the physical properties of xanthan still remain unanswered. Xanthan-modifying enzymes may help to gain more insight into this matter, enabling us to study the effect of specific structural modifications. Using the knowledge generated in this fashion, xanthan variant structures with a specific functionality could be

designed, depending on the desired application. Such designs could be executed by applying, again, specific xanthan-modifying enzymes.

In this thesis, one such enzyme, XalA, was developed and used as a tool for the structural modification of xanthan, which resulted in a marked change of the functionality. To explain the observed change unequivocally, more research regarding the exact structure and physical behaviour of the modified xanthan will be required. It is clear, however, that the partial modification of xanthan by a single enzyme may have a dramatic effect on its functional properties.

Besides XalA, other enzymes that are possibly useful for xanthan modification are produced by *P. alginolyticus* XL-1. These enzymes, however, are excreted in prohibitively low amounts. Therefore, instead of a production organism for xanthan-modifying enzymes, *P. alginolyticus* XL-1 may be more useful as a source of genes encoding xanthan-modifying enzymes for heterologous production. The XalB protein, derived from the *xalB* gene of *P. alginolyticus* XL-1, is probably a family 8 polysaccharide lyase and considering the similarity to XalA, it is probably a xanthan lyase. However, more research will be required to asses the functionality of the *xalB* gene, in order to clarify whether *xalB* encodes a xanthan lyase or a different enzyme, or that it is a non-functional evolutionary relict.

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SUMMARY

Bacterial extracellular polysaccharides (EPSs) can be applied, e.g., in foods, as a thickener or stabilizer. The functional properties that make a polysaccharide suitable for such applications are largely determined by the primary structure, i.e., the sugar composition, the linkage types between the sugar units, and the presence of side chains and non-sugar substituents. The aim of this research was to obtain EPS-modifying enzymes that could be used as tools both for studying structure-function relationships of (food-grade) EPSs and for the production of tailor-made EPSs with a specific, desired functionality. EPS-degrading microorganisms could serve as a source of such enzymes.

To get an idea of the probability of finding EPS-degrading microorganisms, a comparative biodegradability study was carried out on eight EPSs, six of which were produced by lactic acid bacteria (Chapter 2). Human faeces or soil were used as inocula. Xanthan, clavan, and the EPSs of *Streptococcus thermophilus* strains SFi39 and SFi12 were readily degraded. The four other EPSs, produced by *Lactococcus lactis* ssp. *cremoris* B40, *Lactobacillus sakei* 0-1, *S. thermophilus* SFi20, and *Lactobacillus helveticus* Lh59, were not. Xanthan, the most relevant food-grade EPS, was chosen as the target for further studies.

For efficient screening of polysaccharide-degrading microorganisms, plate methods are required that discriminate between intact and degraded polysaccharide. Such methods can make use of specific physicochemical properties of the polysaccharide, such as complex formation with dyes and gelling capacity. Alternatively, dye-labelled polysaccharides can be applied. Chapter 3 presents a survey of plate methods based on the above principles.

A mixed xanthan-degrading culture was obtained from soil by enrichment on xanthan. From this culture, *Paenibacillus alginolyticus* XL-1 was isolated. This strain degraded 28% of the xanthan molecule and appeared to leave the backbone intact. Several xanthan-degrading enzymes were excreted during growth on xanthan, including a xanthan lyase. Xanthan lyase removes the terminal mannosyl residue of the trisaccharide xanthan side chain by a β-eliminative mechanism, resulting in a double bond in the side chain glucuronyl residue. Xanthan lyase is the only polysaccharide lyase that is exo-acting, releasing residues from the outside of a polysaccharide molecule. All other polysaccharide lyases described to date are endo-acting, attacking the polysaccharide backbone. In *P. alginolyticus* XL-1, xanthan lyase production is induced by xanthan and inhibited by glucose and low-molecular-weight enzymatic degradation products from xanthan. A 97-kDa xanthan lyase was purified and characterized. The enzyme is specific for pyruvated mannosyl side chain residues and optimally active at pH 6.0 and 55°C (Chapter 4).

The gene encoding the pyruvated mannose-specific xanthan lyase of *P. alginolyticus* XL-1, designated *xalA*, was isolated. The *xalA* gene encodes a 936-amino acid protein, including a 36-amino acid signal sequence. The XalA protein belongs to polysaccharide lyase family 8, which until now only contained chondroitinases and hyaluronate lyases. The part of the *xalA* gene encoding the 900-amino acid, 96,887-Da mature enzyme was expressed functionally in *Escherichia coli*. Like the native enzyme, the recombinant enzyme is specific for pyruvated xanthan. Heterologous production of XalA in *E. coli* increased the volumetric productivity by a factor 30, compared to production by *P. alginolyticus*. The recombinant xanthan lyase was used as a tool to modify xanthan, which resulted in a dramatic loss of the capacity to form gels with locust bean gum.

Besides xanthan lyase, *P. alginolyticus* XL-1 produces other enzymes that could be useful for xanthan modification, such as a xanthan deacetylase and an enzyme releasing uronic acid, or uronic acid-containing oligosaccharides, from xanthan lyase-modified xanthan. Since these enzymes were produced at very low titers, *P. alginolyticus* XL-1 is not a suitable production organism for xanthan-modifying enzymes. Strain XL-1 may be very useful, however, as a source of genes for heterologous production of xanthan-modifying enzymes.

SAMENVATTING

Bacteriën produceren extracellulaire polysachariden (EPSen), die vanwege hun specifieke fysische eigenschappen toegepast kunnen worden als verdikkingsmiddel of stabilisator, o.a. in levensmiddelen. Deze fysische eigenschappen worden grotendeels bepaald door de primaire structuur van het EPS, m.a.w. door de suikersamenstelling, de wijze waarop de suikers onderling verbonden zijn en de aanwezigheid van eventuele niet-suiker groepen. Het hier beschreven onderzoek had tot doel specifieke, EPS-afbrekende enzymen te verkrijgen. Deze zouden als hulpmiddel ingezet kunnen worden bij het bestuderen van structuur-functie relaties van ("food-grade") EPSen en bij de productie van EPSen met specifieke, gewenste eigenschappen. Als bron van dergelijke enzymen zouden EPS-afbrekende microorganismen kunnen dienen.

Om een beeld te krijgen van de kans om zulke EPS-afbrekende microorganismen te vinden, werd een vergelijkende studie uitgevoerd naar de biologische afbreekbaarheid van een achttal EPSen (Hoofdstuk 2). Zes van de bestudeerde EPSen waren afkomstig van melkzuurbacteriën. Grond of faeces werd gebruikt als entmaterialen. Xanthaan, clavaan, en de EPSen van *Streptococcus thermophilus* stammen SFi39 en SFi12 werden snel afgebroken, in tegenstelling tot de vier andere EPSen, afkomstig van *Lactococcus lactis* ssp. *cremoris* B40, *Lactobacillus sakei* 0-1, *S. thermophilus* SFi20 en *Lactobacillus helveticus* Lh59. Xanthaan, dat voor de industrie het meest belangrijke EPS is, werd gekozen als het onderwerp voor verdere studie.

Voor een efficiënte beoordeling van de EPS-afbrekende capaciteit van microorganismen, zijn plaatmethoden vereist waarbij onderscheid wordt gemaakt tussen intact en afgebroken EPS. Bij zulke methoden kan gebruik gemaakt worden van specifieke fysisch-chemische eigenschappen van het EPS, zoals het vermogen een complex te vormen met kleurstoffen of het vermogen een gel te vormen. Tevens kan als substraat een EPS gebruikt worden, waaraan covalent een kleurstof gekoppeld is. Hoofdstuk 3 geeft een overzicht van plaatmethoden gebaseerd op deze principes.

Uit een grondmonster werd een xanthaan-afbrekende mengculture verkregen door ophoping op xanthaan. Uit deze culture werd de bacterie *Paenibacillus alginolyticus* XL-1 geïsoleerd. Deze bacterie brak 28% van het aangeboden xanthaan af, waarbij de hoofdketen ongemoeid bleef. Verscheidene enzymen, betrokken bij de afbraak van xanthaan, werden uitgescheiden in het medium tijdens groei op xanthaan, waaronder een xanthaanlyase. Xanthaanlyase splitst de terminale mannoseresiduen af van de zijketen van xanthaan, via een β-eliminatief mechanisme. Hierbij ontstaat een dubbele binding in het glucuronylresidue van de zijketen. Xanthaanlyase is het enige polysacharidelyase dat middels een exomechanisme werkt.

Hierbij worden suikerresiduen gesplitst vanaf de niet-reducerende uiteinden van het polysacharidemolecuul. Alle andere, tot nu toe bekende polysacharidelyases zijn endoenzymen, die bindingen in de hoofdketen van polysachariden verbreken. In *P. alginolyticus* XL-1 wordt de productie van xanthaanlyase geïnduceerd door xanthaan en geremd door glucose en laagmoleculaire enzymatische afbraakproducten van xanthaan. Een xanthaanlyase met een molecuulmassa van 97 kDa werd gezuiverd en gekarakteriseerd. Dit enzym is specifiek voor gepyruvileerde mannoseresiduen en optimaal actief bij pH 6,0 en 55°C (Hoofdstuk 4).

Het gen dat codeert voor het hierboven genoemde enzym, *xalA*, werd geïsoleerd (Hoofdstuk 5). Het *xalA*-gen codeert voor een 936 aminozuren tellend eiwit, inclusief een signaalsequentie van 36 aminozuren. Het XalA-eiwit behoort tot de polysacharidelyase-familie 8, die overigens chondroitinases en hyaluronaatlyases bevat. Het deel van het *xalA* gen dat codeert voor het 900 aminozuren tellende, rijpe enzym, werd functioneel tot expressie gebracht in *Escherichia coli*. Evenals het oorspronkelijke enzym, is het recombinante enzym specifiek voor gepyruvyleerd xanthaan. Middels heterologe productie van XalA in *E. coli* werd een 30-voudige toename in volumetrische productiviteit bereikt, in vergelijking met *P. alginolyticus*. Na modificatie met recombinant xanthaanlyase, verloor xanthaan het vermogen een gel te vormen met locust bean gom.

Naast xanthaanlyase produceert *P. alginolyticus* XL-1 andere enzymen die mogelijk nuttig zijn voor de modificatie van xanthaan, zoals een xanthaandeacetylase en een enzym dat uronzuren of uronzuurbevattende oligosachariden vrijmaakt uit (met xanthaanlyase behandeld) xanthaan. Deze enzymen worden echter in zeer lage hoeveelheden geproduceerd. Daarom is *P. alginolyticus* XL-1 mogelijk meer van nut als een bron van genen voor de heterologe productie van enzymen voor de modificatie van xanthaan, dan als productieorganisme voor deze enzymen.

NAWOORD

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Harald

CURRICULUM VITAE

Harald Johan Ruijssenaars werd geboren op 24 juni 1970 te Driebergen-Rijsenburg. In 1989 behaalde hij het diploma gymnasium-β aan de RSG Schoonoord in Zeist. Aansluitend begon hij aan de studie Levensmiddelentechnologie, oriëntatie Biotechnologie, aan de toenmalige Landbouwuniversiteit Wageningen. In november 1994 werd deze studie afgesloten met afstudeervakken Industriële Microbiologie (constructie van een genbank van *Phaffia rhodozyma*) en Proceskunde (geïntegreerde nitrificatie en denitrificatie in een arlift loop reactor), alsmede een bedrijfsstage bij Gist-brocades te Delft. Dezelfde maand begon hij aan het promotieonderzoek beschreven in dit proefschrift bij de sectie Industriële Microbiologie, alwaar hij tot februari 2001 werkzaam bleef. Vanaf 1 februari 2001 is hij in dienst als post-doc onderzoeker bij het Hercules European Research Center in Barneveld.

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