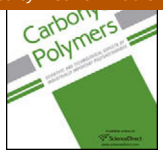
Carbohydrate Polymers 122 (2015) 212–220

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol



High throughput exopolysaccharide screening platform: From strain cultivation to monosaccharide composition and carbohydrate fingerprinting in one day

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ARTICLE INFO

Article history:
Received 5 November 2014
Received in revised form 8 December 2014
Accepted 9 December 2014
Available online 13 January 2015

Keywords:
High throughput screening
Monosaccharide composition
Microbial exopolysaccharide (EPS)
Small scale cultivation

ABSTRACT

Microbial exopolysaccharides (EPS) are multifunctional biogenic polymers, which exist in highly diverse chemical structures. To facilitate a fast determination of the carbohydrate composition of novel isolated strains or modified EPS variants a fast screening and analytical method is required. The platform as realized and described in this article is based on the fast carbohydrate analysis via liquid chromatography coupled with ultra violet and electrospray ionization ion trap detection in 96-well format to detect different sugars, sugar derivatives and substituents such as pyruvate. Monosaccharide analysis from hydrolyzed polysaccharides was validated successfully by 16 commercially available polymers with known structure. The method is sensitive enough to distinguish various types of sphingans which solely differ in small alterations in the monomer composition. Even a quantitative detection of single monomers as present in complex plant polysaccharides like karaya gum, with the lowest recovery, was in accordance with literature. Furthermore, 94 bacterial strains for the validation of the screening platform were completely analyzed and 41 EPS producing strains were efficiently identified. Using the method a carbohydrate-fingerprint of the strains was obtained even allowing a very fast differentiation between strains belonging to the same species. This method can become a valuable tool not only in the fast analysis of strain isolates but also in the targeted screening for polysaccharides containing special rare sugars as well in the screening of strain libraries from genetic engineering for altered structures.

1. Introduction

Carbohydrate polymers play diverse, yet important roles in biological systems. One structurally very diverse class of such molecules is formed by the so called exopolysaccharides (EPS), which are produced by a wide range of bacteria, fungi, algae and archaea (Parolis et al., 1996). The natural function of these molecules typically lies in biofilm formation for the protection of the microorganisms, for the colonization of infected hosts (plants or animals) and also to mediate pathogenicity. Consequently, understanding these molecules and their production has been an important subject in medical science (Costerton, Stewart, & Greenberg, 1999), agriculture (Colegrove, 1983) and increasingly in chemical industry, where EPS as biopolymers can replace petro based polymers and such can contribute to a "greening" of the chemical industry (Baird, Sandford, & Cottrell, 1983) and (Stokes, Macakova, Chojnicka-Paszun, de Kruif, & de Jongh, 2011).

There are numerous different varieties of EPS that are distinguished by their complex structure, which is based on the different types of monosaccharides, the way these are connected and the substitutions that they are carrying. Depending on the different properties, which are conferred by the individual structures, EPS can be applied in various technical or medical fields. For example, they can be used as gelling agent, stabilizer, emulsifier, or for flocculation. Besides having unique properties they also have the advantage of being biobased with the potential to replace petro based polymers such as polyacrylates or polyvinyl alcohol (Tait & Sutherland, 2002) and (Rehm, 2010). In medical applications polysaccharides are also gaining importance e.g. as drug delivery vehicles or due to their antitumoral and antiviral activities (Sosnik, 2014) and (Zhang, Wardwell, & Bader, 2013). For the group of β-glucan polysaccharides, there is currently research taking place whether it has a positive influence on the immune system as well as anti-cancer effects (Chan, Chan, & Sze, 2009).

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http://dx.doi.org/10.1016/j.carbpol.2014.12.021

It is now important to more quickly learn about the EPS offered by nature and to tap their full potential for the various fields as well as for an engineering for the formation of synthetic EPS. In order to do this, a fast and reliable method for the analysis of EPS producing strains including the identity of their polysaccharide is crucial. With this increasing importance of EPS on the one side and the ever growing collections of strains from all different habitats on the other side, such a method should be capable to quickly determine whether a certain microorganism is producing EPS in substantial amounts and to analyze the basic elements of their chemical structure.

Many different techniques have been used to verify microbial EPS production. Mucoid growth is often the method of choice for visual identification of EPS producing strains. However, often they do not show slimy growth leading to false negatives in screening approaches (Ruas-Madiedo & De los Reyes-Gavilan, 2005). Depending on the thickening characteristics of the polysaccharide, observation of viscosity in culture broth can also be an option to screen for EPS production (Garai-Ibabe et al., 2010). Precipitation with different alcohols represents a common detection, isolation and purification method for many EPS (Freitas, Alves, & Reis, 2011), however, not all EPS will easily precipitate. Taken together, these approaches have severe limitations: They are not very reliable; they do not provide quantitative data and actually do not give any information on the identity of the polymer. More thorough analyses require different purification steps for the isolation from the culture broth (e.g. cross flow filtration, protein removal, ion exchange purification) as well as extensive polymer identification (Cerning, 1990). All these steps are, however, tedious and have been performed only in low throughput.

Therefore, we intended to establish and validate a more informative high throughput EPS screening platform, for the detection and rough identification of polymers directly out of small volume cultivations. For this purpose, we combined our recently developed high throughput carbohydrate analysis method (Rühmann, Schmid, & Sieber, 2014) with micro-plate based cultivation and sample preparation to create a powerful and fast tool for screening the highly diverse field of microbial EPS (Fig. 1). The main focus of the screening is to identify novel bacterial producers of EPS in combination with the direct determination of their monomeric composition. Furthermore, the method gives a chance to get more information about already known EPS producers and the different polymers they can produce, additional to the ones already described in literature.

For the validation of this novel high throughput EPS screening platform 16 commercially available polymers with known chemical structure and over 90 putatively EPS producing strains were used and successfully analyzed.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and enzymes were, unless otherwise stated, purchased in analytical grade from Sigma-Aldrich, Carl Roth GmbH (Germany) and Merck KGaA (Germany). The commercial polysaccharides were purchased from: Jungbunzlauer (xanthan gum food grade), Colltec (welan gum Collstab W200), Kelco (gellan gum Kelco®F, diutan gum Kelco-Create®DG, Harke (succinoglycan Rheozan®, konjac gum NI-F4A0), Sigma (hyaluronic acid), Roeper (karaya gum Ceroka®), Cargill (guar gum Viscogum™MP, locust bean gum Viscogum®FA, scleroglucan Actigum®CS 6), Molekular (gum arabic), Lonza (larch gum ResistAid®), IMCD (tara gum KUW, tragacanth KUW) and Roth (xylan).

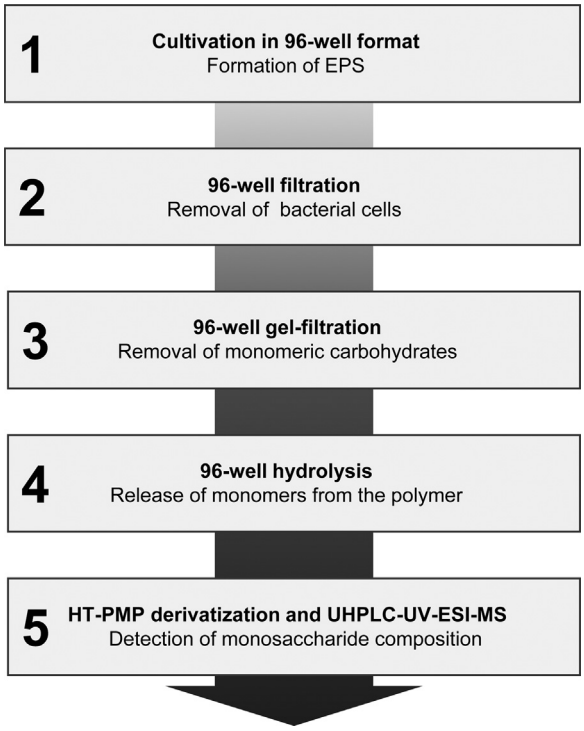

Fig. 1. Overview of the five main screening steps (arrow) of the high throughput exopolysaccharide (EPS) screening platform. After bacteria are grown in 96-well plates, cells are removed by centrifugation (step 1) and a 96-well filtration (step 2) before monomeric sugars from the growth media are removed by a 96-well gel filtration (step 3). Following the hydrolysis of the polymers (step 4), their monomeric composition can be analyzed via HT-PMP method (high throughput 1-phenyl-3-methyl-5-pyrazolone, step 5). (UHPLC–UV–ESI–MS, Ultra high performance liquid chromatography with ultra violet and electrospray ionization ion trap mass spectrometer).

2.2. Culture conditions (step 1)

The strains were stored at −80 °C in a 96-well micro titer plate with screening media containing 20% of glycerol. Precultures were grown in a 96-deep-well plate containing 1 mL screening media with 1.5 g/L casein peptone, 1.33 g/L magnesium sulfate heptahydrate, 10 g/L 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 0.05 g/L calcium chloride dihydrate, 11 g/L glucose monohydrate, 1.67 g/L potassium dihydrogenphosphate, 2 mL/L RPMI 1640 vitamins solution (Sigma) and 1 mL/L trace element solution (2.5 g/L iron-(II)-sulfate heptahydrate, 2.1 g/L sodium tartrate dihydrate, 1.8 g/L manganese-(II)-chloride tetrahydrate, 0.075 g/L cobalt-(II)-chloride hexahydrate, 0.031 g/L copper-(II)-sulfate heptahydrate, 0.258 g/L boric acid, 0.023 g/L sodium molybdate and 0.021 g/L zinc chloride) in each well. Inoculation was performed with a 96-pin replicator. After incubation of the preculture for 48 h at 30 °C in a microplate shaker (1000 rpm) equipped with an incubator hood (TiMix 5 control and TH 15, Edmund Bühler GmbH). The main culture was inoculated with 10 μL of the preculture in 990 μL of fresh screening media and incubated in the same way as described above.

2.3. General workflow of the screening (step 1–4)

Removal of the cells from the main culture occurred via centrifugation at 3710 × *g* for 30 min at 20 °C. An aliquot (200 μL) of the supernatant was transferred to a 96-well filtration plate (1 μm A/B Glass, Pall Corporation) and filtered by centrifugation (3000 × *g* for 10 min at 20 °C). In the next step 30 μL of filtrated supernatant were placed in the center of each well of a gel-filtration plate (96-well SpinColumn G-25, Harvard Apparatus), that had been equilibrated by washing three times with 150 μL ammonium acetate buffer (pH

5.6). The plate was centrifuged at 1000 × *g* for 2 min at 20 °C. 20 μL of the eluate were mixed with 4 M trifluoroacetic acid (TFA) 1:1 (v/v) in a 96-well PCR-plate for hydrolyzation of the polysaccharides, which was performed in a sand bath for 90 min at 121 °C. To avoid evaporation of the samples, the silicone cap mat was secured with a specially designed clamping device. After cooling down to room temperature the pH of all samples was adjusted to approximately 8.0 by use of 3.2% aqueous ammonia solution. An aliquot of 25 μL was transferred into a 96-well-PCR micro titer plate (781350, Brand) for derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP). The neutralization of the samples was controlled by addition of 12.5 μL phenol red solution (1 g/L in 20% ethanol) to the remaining hydrolysate. This step is crucial since the PMP-derivatization process is significantly inhibited when performed below pH 7.

2.4. 96-Well derivatization method for monomer analysis (step 5)

Previously we demonstrated that the high throughput 1-phenyl-3-methyl-5-pyrazolone analysis (HT-PMP) is capable for high throughput carbohydrate determination. Detailed parameters are given in (Rühmann et al., 2014). The calibration standards were diluted with neutralized TFA-matrix to compensate the influence on the derivatization process.

2.5. Precipitation

50 μL of sample were precipitated with 150 μL of isopropanol and mixed at 1000 rpm for 15 min. Successful precipitation was indicated by flakes or fibers in each single well of the plate.

2.6. Glucose assay

50 μL of sample or standard solution were placed into a 96-well plate. Reaction was started by adding 50 μL of assay mixture (40 mM potassium phosphate (pH 6.0), 1.5 mM 2,2-azino-bis-(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS, A1888, Sigma-Aldrich), 0.4 U glucose oxidase (G2133, Sigma-Aldrich) and 0.02 U horseradish peroxidase (P6782, Sigma-Aldrich). After incubation on a micro-plate shaker at 400 rpm for 30 min at 30 °C the resulting extinction was measured at 418 nm. The absorption at 480 nm was subtracted to eliminate background signals in the UV. The calibration curve was compiled in the range from 2.5 to 500 μM ($n = 3$) of glucose.

3. Results and discussion

3.1. Method overview

The core of the method is the identification of polysaccharide formation during microbial growth as well as the quantitative analysis of the monosaccharide composition in 96-well format. Until now, identification of EPS production and compositional analysis typically consisted of several separated steps, including isolation, purification, drying, decomposition and a lengthy chromatographic analysis. These separated approaches take a couple of days or even weeks and are only manageable in low throughput. Analysis of the monomeric composition of the EPS produced directly out of the supernatant without any precipitation has never been described so far. The high performance of the HT-PMP analysis of the carbohydrate monomers now offers the chance to work in high throughput. So, while it is not possible to avoid all separation steps we thought it could be possible to simplify the separation and analysis procedure to the smallest possible extent, such that it would allow downscaling for higher throughput. In our eyes unavoidable steps to realize this sophisticated time and work saving analysis, are for example the complete removal of the bacterial cells as well as of the remaining carbohydrates from the cultivation, as they interfere with the correct determination of the carbohydrates from the polymer. Furthermore, the purified polymer has to be hydrolyzed and derivatized in small scale to enable a reliable high throughput analysis by HPLC. The major challenge in our intention to develop a fast EPS identification method was to combine all these steps within a reliable miniaturized growth and analysis scheme.

An overview of the devised workflow is given in Fig. 1: (1) Bacteria are grown in 96-well plates using a growth medium which was found to induce increased EPS production for many bacterial strains in previous experiments (data not shown). (2) After 48 h of growth, cells are removed by centrifugation and an additional filtration step in a 96-well plate to ensure complete cell removal for the subsequent analyses and process steps. (3) A 96-well gel filtration removes remaining monomeric sugar components from the growth medium to eliminate false signals in the subsequent analysis of the carbohydrate content of the polysaccharides. (4) The polymer containing filtrate is used for hydrolysis of the polymers in 96-well micro scale. (5) The most important step within the method is the analysis of the monomers by HT-PMP, which reveals the monomeric composition of the polymers by double detection via UV-detector and mass spectrometer.

3.2. Validation of the screening procedure with defined polymer solutions

For the validation of manageability and accuracy of the screening procedure 16 known and commercially available polysaccharides (1 g/L) were subjected to the main process steps. They consisted of six bacterial polymers (xanthan gum, welan gum, gellan gum, diutan gum, succinoglycan and hyaluronic acid), nine polysaccharides from plants (karaya gum, konjac gum, guar gum, gum arabic, larch gum, locust bean gum, tara gum, tragacanth and xylan) and scleroglucan, produced from a filamentous fungus. By choosing this diversity of microbial polymers in combination with the highly complex structure of the plant gums the capability of the screening platform for a broad range of polymers was ensured. The aqueous polymer solutions were handled along all relevant steps of the screening platform (Fig. 1, steps 2 to 5).

3.2.1. 96-Well hydrolysis and detection of the monosaccharide composition of known polysaccharides (step 4 and 5)

The carbohydrate analysis was performed via liquid chromatography coupled with ultra violet and electrospray ionization ion trap detection (HPLC–UV–ESI–MS) in 96-well format as recently published (Rühmann et al., 2014). The micro scale hydrolysis in combination with the high throughput determination of carbohydrates (both in 96-well format) makes this method perfectly suitable to be applied in a screening platform. The main benefits of this method are the high resolution separation (12 min) and the simultaneous determination of deoxy-, amino- and anhydro-sugars as well as hexoses, pentoses, dimers, uronic acids and carbohydrate degradation products like furfural and hydroxymethylfurfural (HMF). Furthermore, the potential to quantify via UV- as well as MS-detection in the same range make the method highly reliable. Additionally, unknown sugar compounds and substituents like pyruvate ketal can be identified via MS-detection. In order to validate this method for the EPS screening platform the monomeric composition of 16 polymer solutions of known structure was analyzed. Results are given in Table 1.

Chemically, the galactomannans of plant origin (guar-, tara- and locust bean gum) are composed of galactose and mannose with a linear backbone chain of β 1,4 linked mannose. Solely the number of branches of β 1,4 linked galactose distinguishes these three polysaccharides. Guar gum contains on average one

Table 1
Comparison of the monosaccharide compositions and ratios as described in literature and as detected by the screening platform: Abbreviations: Man, D-mannose; GlcUA, D-glucuronic acid; GalUA, D-galacturonic acid; Rha, L-rhamnose; Gen, D-gentiobiose; Cel, D-cellobiose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; Gal, D-galactose; Ara, L-arabinose; Xyl, D-xylose; Fuc, L-fucose ($n = 3$).

Polymer	Carbohydrate ratio				Qualified dimers
	Monosaccharides	Described	Ref.	Detected	
Diutan gum[a]	GlcUA:Rha:Glc	1:3:2	Sonebi (2006)	0.3:3:2.2	GlcUA–Glc
Gellan gum[a]	GlcUA:Rha:Glc	1:1:2	Sutherland (2005)	0.3:1:1.5	GlcUA–Glc
Guar gum[b]	Man:Gal	2:1	Spiridon and Popa (2005)	1.8:1	
Gum arabic[b]	GlcUA:Rha:Gal:Ara	1:1:3.5:2.5	Alban (2010)	0.4:1:3.3:2.4	GlcUA–Gal
Hyaluronic acid[a]	GlcUA:GlcNAc	1:1	Robyt (1998)	2:1	
Karaya gum[b]	GlcUA:GalUA:Rha:Gal	1:2:2:2	Weiping (2005)	2:2:2:2.8	
Konjac gum[b]	Man:Glc	1.6:1	Takigami (2005)	1.7:1	
Larch gum[b]	Gal:Ara	6.7:1	Goellner et al. (2011)	6.8:1	GlcUA–Gal
Locust bean gum[b]	Man:Gal	3.5:1	Spiridon and Popa (2005)	3.4:1	
Scleroglucan[c]	Glc	1	Rinaudo and Vincendon (1982)	1	Gen
Succinoglycan[a]	Glc:Gal	7:1	Sutherland (2005)	4.5:1	
Tara gum[b]	Man:Gal	2.8:1	Spiridon and Popa (2005)	3.3:1	
Tragacanth[b]	GalUA:Gal:Ara:Xyl:Fuc	2.6:1:2:2:0.6	Weiping (2005), Tischer, Iacomini and Gorin (2002)	0.8:1:2:2.2:0.6	
Welan gum[a]	GlcUA:Rha:Glc:Man	1:1.6:2:0.3	Sutherland (2005)	0.3:1.6:1.2:0.9	GlcUA–Glc
Xanthan gum[a]	Man:GlcUA:Glc	2:1:2	Sutherland (2005)	1.7:0.3:2	GlcUA–Man Cel
Xylan[b]	Xyl	1	Buslov et al. (2009)	1	

Polymer origin:
[a] Bacteria,
[b] Plant and
[c] Filamentous fungus.

galactose residue on every second mannose and locust bean gum on every third to fourth mannose residue. After performing all process steps, the HT-PMP method confirms these molar ratios of Man:Gal of 1.8:1 for guar gum and 3.4:1 for locust bean gum (Table 1).

Xanthan gum has a neutral cellulosic back bone with anionic side chains containing mannose and glucuronic acid in the ratio of 2:2:1 as well as pyruvic- and acetyl-groups as substituents. In the screening the monomer composition of xanthan gum was determined as Glu:Man:GlcUA in the ratio of 2:1.7:0.3 (Table 1). The content of uronic acid and mannose monomers was lower than expected. The more pronounced effect of GlcUA results from the high degradation rate of the monomeric uronic acid. The loss in mannose monomers results from a stable [β-D-GlcUA-(1 → 2)-D-Man] dimer after hydrolysis. Dimers of uronic acids and various hexoses exhibit strong glycosidic bounds, which are not easily cleaved under standard hydrolysis conditions. In the case of xanthan this results in incomplete hydrolysis of GlcUA–Man dimers. Prolonging the time or harshening the conditions of hydrolysis would lead to higher degradation of uronic acids. In their work on the degradation kinetics of uronic acids during hydrolysis Tait, Sutherland, and Clarke-Sturman (1990) recommended that uronic acids can also be quantified in the form of their aldobiouronic acid (dimer of uronic acid and a hexose). This provides a significant advantage of milder hydrolysis conditions. Applying such conditions the degradation rate of uronic acids is noticeably reduced and a more accurate determination can be obtained via the aldobiouronic acid. Accordingly, in the case of xanthan gum the aldobiouronic acid was identified via mass spectrometry (m/z 687). Unfortunately, only limited variants of aldobiouronic acids are commercially available, so calibration and quantification of these compounds is currently impractical.

These findings in combination with the high degradation rate of the uronic acids under standard hydrolysis conditions explain the lower ratio of GlcUA and Man (bound in the dimer) compared to the published values. While the ratios of more stable sugars like mannose and glucose reflect values very close to literature, all ratios for uronic acids are lower than expected for the polymer. This has to be taken into account for the carbohydrate ratios in Table 1.

The phenomenon of dimer formation in incomplete hydrolyses like aldobiouronic acids represents a valuable source of additional information for the identification of the sugar sequence within the polymer. Dimers can be used to identify typical glycosidic linkages in the polymer structure. For example, in the case of xanthan, cellobiose [β-D-Glc-(1 → 4)-D-Glc] was identified in the hydrolyzed sample. Further detected dimers are explained in detail in Section 3.3.3. Additional information on the polymer structure provided by the MS-detection in the case of Xanthan is the identification of hexose pyruvate ketals (m/z 581). In combination with information from the retention time even assignment of the sugar to which the ketal is linked is possible.

In the case of gellan, diutan and welan gum the stoichiometric ratios were also altered and [β-D-GlcUA-(1 → 4)-D-Glc] uronic acid dimer was detected. The differentiation of those three polymers was still possible by the individual ratios of the monomers, even so their backbone structure is identical and differences are only on the side chains. The rhamnose content of diutan gum is three times higher than in gellan gum, which results from the α-1,4 rhamnose dimeric side chain of diutan gum. Welan gum was identified via the rhamnose content and the additional mannose.

Even plant gums (gum arabic, karaya gum, larch gum and tragacanth), which have a very complex structure and show high variability within their stoichiometry, resulting from their origin as well as from the isolation process, were verified according to the literature.

For polymers, which contain monosaccharides with a high degradation rate (e.g. hyaluronic acid) in the hydrolysis process further optimization is possible. In order to analyze such polymers, the hydrolysis conditions (acid concentration, incubation temperature and time) can be adjusted to obtain reliable results.

In sum, the 96-well hydrolysis and the detection of the monosaccharide composition was successfully validated. The monosaccharide compositions of a broad range of diverse polymers, including complex plant polysaccharides, could be detected and discriminated by use of the screening platform.

3.2.2. *Polymer permeability of the 96-well filtration (step 2)*

In order to ensure a low background of the screening approach, only cell free EPS containing supernatants should be analyzed. Cell removal can most easily be achieved by centrifugation. However, when working with polysaccharides high viscosity can be

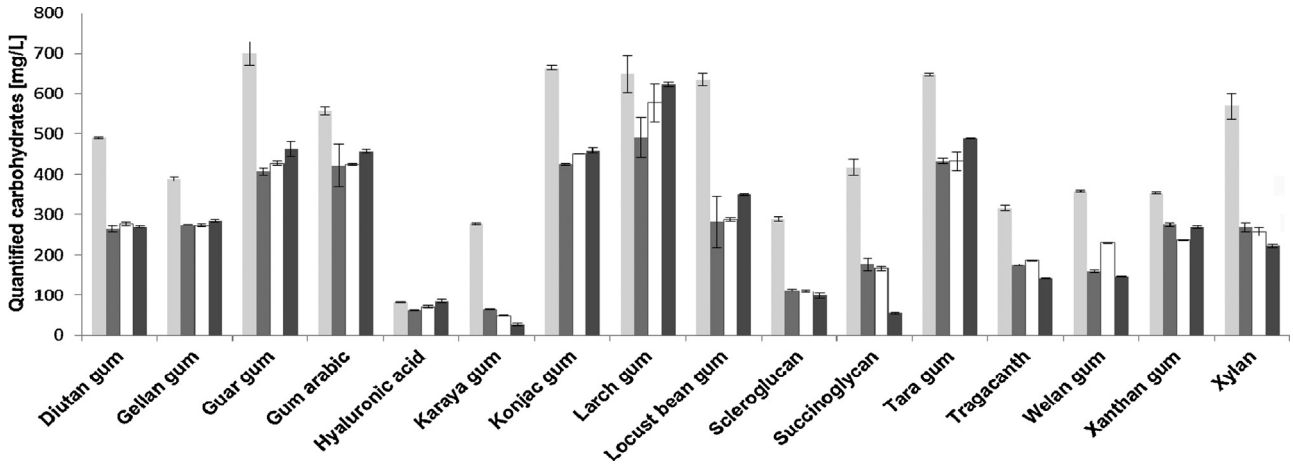

Fig. 2. Recovery of all quantifiable monosaccharides analyzed from hydrolyzed polymers (1 g/L) in the different main process steps: light gray, only hydrolyzed (step 4–5); gray, gel-filtrated with 1 g/L glucose (step 3–5); white, gel-filtrated (step 3–5), dark gray complete screening (step 2–5) ($n = 3$).

obstructive. Therefore, for complete cell removal from viscous samples an additional filtration step was implemented using 1 μm glass filter membrane. Here, a crucial point is the polymer permeability of the filtration membrane. To evaluate the influence of the filtration membrane on polymer concentration, all polymer solutions were handled according to step 2 (cell removal) and subsequently put through steps 3 to 5 (Fig. 1), once subjected to the filtration and once without the filtration step. The results are shown in Fig. 2. It was found that 13 out of the 16 polymers could pass the filter membrane with a yield of 75% or higher. Only for three polymers permeability was lower (succinoglycan 33.4 ± 3.7%, karaya gum 53.4 ± 15.0% and welan gum 63.6 ± 1.3%).

3.2.3. Removal of monomeric carbohydrates via 96-well gel-filtration (step 3)

Non-metabolized glucose from the cultivation media would lead to several problems in the screening platform. Neither the HT-PMP-derivatization nor the HPLC–UV–ESI–MS system can handle high concentrations of glucose (>1 g/L). In addition monomeric glucose would hinder the detection of glucose as component of the polymers. Therefore, a gel-filtration step was implemented to remove the remaining glucose after cultivation. Additionally, the gel-filtration purifies the polymer containing supernatant from salts and further small molecules like monomeric carbohydrate compounds, others than glucose to minimize the analytical background in the monomer analysis. To validate the efficiency of gel-filtration (Fig. 1, step 3), the polymer solution was spiked with 1 g/L of glucose. Glucose concentration was determined before and after gel-filtration via glucose assay. The gel-filtration step reduced glucose concentration of all spiked polymer solutions by more than 93% (Fig. 3). At the same time 45–78% of each polymer passed the gel-filtration matrix, except for karaya gum 20.8 ± 1.7%, scleroglucan 37.8 ± 3.3%, and succinoglycan 39.6 ± 2.8%.

The final recovery of both filtration steps (Fig. 1, step 2 and 3) was >40% in sum, except for karaya gum 11.0 ± 6.6%, succinoglycan 13.2 ± 1.2% and scleroglucan 34.1 ± 5.6%. However, to analyze the monomer composition of the polysaccharides a high recovery in the main process steps is not essential. Accordingly, for all tested polymers, even for karaya gum with the lowest recovery, determination of the monomeric composition was possible.

3.3. Screening of 94 novel isolated bacterial strains

After validation of feasibility and accuracy of the screening platform with known commercial polymers, a 96-well microtiter plate with 94 novel isolated bacterial strains, including some known EPS producing bacteria genera e.g. *Xanthomonas* and *Agrobacterium*, from different environmental habitats with an expected high potential for EPS production, was analyzed via the high throughput EPS screening platform (Supplementary Table 1). All steps including cultivation, filtration, gel-filtration, hydrolysis and final analysis of the monomeric composition of the polymer were applied. Two negative controls (media without inoculation) were used to identify cross contaminations and to verify the removal of glucose via gel-filtration. After hydrolysis and derivatization with PMP the negative controls only displayed signals for small amounts of remaining glucose, excluding interfering signals from media components (Table 2 and Supplementary Table 1).

3.3.1. Cultivation and removal of bacterial cells (step 1 and 2)

The 94 strains were cultivated for EPS formation in a special screening medium. All 21 different genera showed good growth behavior at the selected screening conditions. After cultivation cells were separated via centrifugation. As described above, due to increased viscosity by strong EPS formation for some strains, no cell pellet could be obtained in this step, demonstrating the necessity for the filtration step. However, for five of the 94 screened strains filtration of the culture broth was not possible, indicating a very high viscosity and potentially a complete retention of these polymers on the glass fiber membrane. Therefore, a precipitation of the supernatant with isopropanol was performed before and after the first filtration step (Fig. 1, step 2) in order to identify and verify an adsorption of those polymers on the filter membrane. The five highly viscous cultures showed polymer fibers in the first, but not in the second precipitation. This correlates with the fact that only small amounts of carbohydrates are detected after hydrolysis (<56 mg/L). Therefore, the retention of the polymers on the filter membrane leads to false negative results.

3.3.2. Gel-filtration and glucose analysis (step 3)

The removal of remaining glucose from the fermentation broth was performed via gel-filtration and showed high performance with all different types of supernatants. In order to evaluate the amount of glucose consumed by the bacterial strains from the growth media, a glucose measurement was performed. Depending on the isolates the glucose consumption reached from non to complete metabolization (Supplementary Table 1). Additionally, two glucose assays performed after step 3 and 4 were applied to

Table 2
Exemplary EPS analysis results from the bacterial screening: Sugar detection via HT-PMP method (step 5) Abbreviations: +, positive; –, negative; Glc, D-glucose; Gal, D-galactose; Man, D-mannose; GlcUA, D-glucuronic acid; GlcN, D-glucosamine; Rha, L-rhamnose; Fuc, L-fucose; Rib, D-ribose; UA, uronic acid; Cel, Cellobiose; Gen, Gentiobiose.

MTP well	Carbohydrate content [mg/L]								Unknown sugar	Substituent	Dimers			Genus
	Glc	Gal	Man	GlcUA	GlcN	Rha	Fuc	Rib	Sum	Deoxy-hexose	$Hexose^{4,6\,Pyr}$	UA + hexose	UA + deoxy hexose	Cel	Gen	
A1	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	*Negative control*
H8	497	–	392	42	–	30	2	–	964	–	+	+	–	+	–	*Xanthomonas*
H12	2056	280	13	–	–	–	–	–	2349	–	–	–	–	+	+	*Agrobacterium*
A4	891	698	84	85	12	13	262	9	2053	+	+	–	–	–	–	*Microbacterium*
C1	896	104	223	108	12	57	48	9	1551	–	–	+	–	–	–	*Microbacterium*

determine the correct glucose content within the polymer. Therefore, a delta value between monomeric glucose after gel-filtration (step 3) and the glucose content after hydrolysis (step 4) was calculated. The delta values showed that the glucose content of the polymer reached up to 2.4 g/L (Supplementary Table 1). For the correct determination of such high glucose concentrations a glucose assay with a broader linearity range, than that of the HT-PMP analysis was chosen.

3.3.3. Analysis of monomeric compositions and closer discussion of four exemplary EPS producers (step 5)

The polymers were hydrolyzed via micro scale hydrolysis using TFA and the released monomers were derivatized with PMP. Then, those derivatized sugars were measured via UHPLC–UV–ESI–MS and all results of the monomeric carbohydrate contents, dimers and unknown deoxy hexoses as detected in the 94 screened strains are given in Supplementary Table 1. 41 strains showed a carbohydrate content higher than 300 mg/L. Additionally, eleven strains showed a carbohydrate content in the range of 150–300 mg/L, indicating a putative EPS production.

Four out of the 94 analyzed strains were exemplarily selected for closer discussion (Table 2): The successful analysis of commercial xanthan gum was verified under screening conditions by use of a newly isolated *Xanthomonas* strain (MTP H8). Hydrolysis of the polymer produced by this strain displayed the ratio: Glc:Man:GlcUA of 2:1.5:0.1. Furthermore, cellobiose, an uronic acid-hexose dimer as well as pyruvate ketal were detected, all being in accordance with the results of the commercial xanthan sample.

The second example is a polymer produced by *Agrobacterium* sp. (MTP H12). Next to glucose and galactose, gentiobiose and cellobiose were detected; both are typical dimers of succinoglycan, produced by *Agrobacterium strains*. The ratio of Glc:Gal was determined as 7.3:1 and a pyruvate substitution was detected. These results (with exception of trace amounts of mannose) again represent the typical composition of succinoglycan.

A third polymer to be discussed was produced by a *Microbacterium* (MTP A4) strain and contained Glc:Gal:Man:Fuc in a ratio 10:8:1:3. A glucuronic acid content of 85 mg/L was measured. Concerning the high degradation rate of uronic acids the content in the polymer is assumed to be higher. Most interesting in the polymer analysis was the observation of a peak with an *m*/*z* value of 495 at 5.9 min (Fig. 4), representing an unknown deoxy hexose. Signal response for this hexose was similar to that of mannose so the compound is probably a significant part of the polymer. The retention time of this unknown deoxy hexose differs from the three calibrated deoxy hexoses rhamnose, fucose and 2-deoxy-glucose. Nevertheless, a closer analysis of the MS-spectra can reveal the identity of this monomer (Supplementary Fig. 1). The MS-spectra of the unknown deoxy hexose showed high accordance with the MS-spectra of rhamnose and fucose but not to that of 2-deoxy-glucose. Furthermore, the comparison of the elution profiles of hexoses and deoxy hexoses leads to the assumption that this sugar could be either 6-deoxy-allose or 6-deoxy-talose. Takeuchi, Yokota, and Misaki (1990) described a 6-deoxy talose in the polysaccharide structure of the genus *Microbacterium*. This indicates that the polymer described above probably also contains a 6-deoxy talose. This example demonstrates the advantage of the combined UV- and MS-detection to identify unknown sugars in the polymer structure. Furthermore, pyruvate ketal (*m*/*z* 581) was also detected in this polymer, demonstrating the power of this method to also determine substituents. Another benefit is the linearity over a broad calibration range. In case of this strain, also trace amounts of glucosamine, ribose and rhamnose could be identified, although they were not significant, with less than 1% of the carbohydrate content of the polymer. Finally, this analytical method enables the

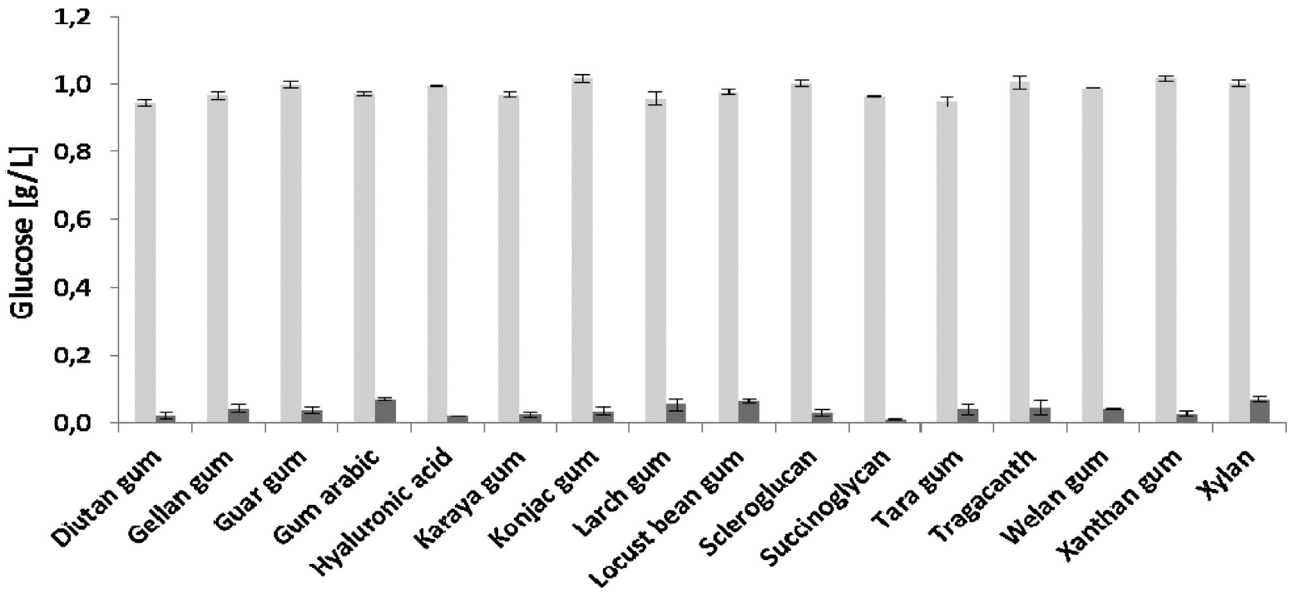

Fig. 3. Glucose concentration of 1 g/L glucose spiked polymer solutions (1 g/L): light gray, before gel-filtration; dark gray, after gel-filtration ($n = 3$).

direct identification of a broad range of various sugars, even for overlaying signals in the UV-detection, as exemplarily given in Fig. 4.

The fourth polymer was also produced from a *Microbacterium* strain (MTP C1). The monomers determined in this polymer were similar to the ones in the isolate (MTP A4) discussed above. However, with 16:2:4:1:1 a different ratio of Glc:Gal:Man:Fuc:Rha was detected, which suggests another polymer. For both polymers glucose was the main sugar, but the ratios of galactose and fucose differed substantially. Furthermore, glucuronic acid and an uronic acid dimer with an m/z value of 687 were determined via mass spectrometer. This indicates that the uronic acid is linked to a hexose which was not detected in the other *Microbacterium* polymer. Additionally, the glucuronic acid and the rhamnose content were higher, than in the polymer of the strain MTP A4. At the same time, no pyruvate ketal was found in the *Microbacterium* polymer (MTP

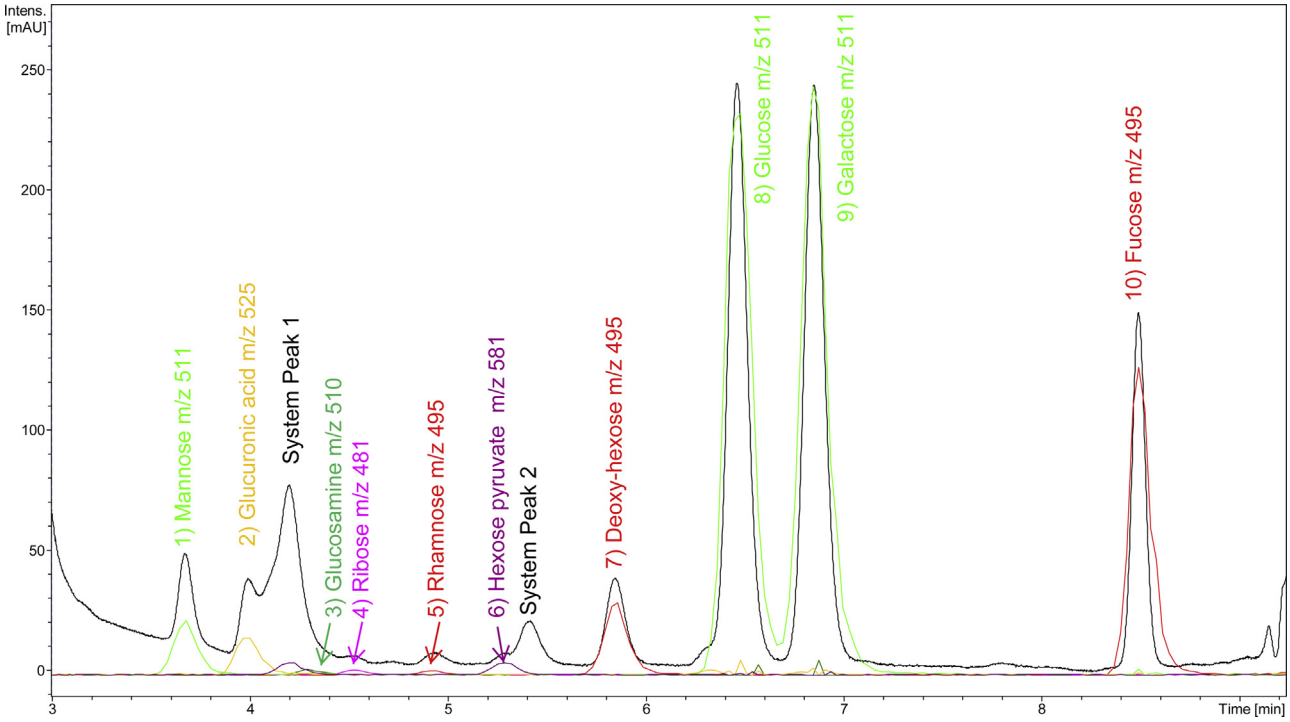

Fig. 4. Overlay of the UV- and MS-chromatogram (3–9 min) of the *Microbacterium* (MTP A4) monosaccharide analysis. UV 245 nm (black); extracted ion chromatograms (EIC): hexoses (m/z 511, green); uronic acid (m/z 525, orange); pyruvate ketal (m/z 581, violet); amino hexoses (m/z 510, dark green); pentoses (m/z 481, pink); deoxy hexoses (m/z 495, red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

C1). These findings represent impressive examples of the benefits of the HT-PMP derivatization with UV- and MS-detection. If these two *Microbacterium* strains were only screened via a standard method for e.g. detection of total carbohydrates, such as phenol sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), they might not have been identified as two *Microbacterium* strains producing completely different EPS.

The direct analysis of the monosaccharide composition gives a first hint for the polymer structure and enables the fast identification of completely unknown EPS producers or of superior producers of already known polymers, provided that only one EPS is produced. Trace amounts of monomers released from cell fragments, capsular EPS and protein glycosylation should be taken into account. However, the concentration of EPS originating sugars is much higher than the trace amounts of monomers released from other sources. This method is not a replacement of a detailed polymer analysis or characterization of polymer properties, but it enables a much more targeted and faster screening. For example, strain productivity and EPS inducible parameters, like salt concentration, pH-value, *C*/*N* ratio, C-source etc. can be screened very quickly by the total carbohydrate content present in the samples. Also, the influence of these parameters on the monomeric composition of the polymer is directly detectable. Additionally, the method offers the possibility to create a very fast polymer fingerprint and therefore, to verify and validate the genera of many different strains.

Summarizing the results, the screening platform is able to identify already known EPS producers and verify their polymer structure e.g. screening of genera from *Xanthomonas* and *Agrobacterium*. Furthermore, different polymers from the same genus can be identified. Furthermore, the platform can also be supplemented with new analytical modules depending on the field of interest. In future, one additional analytical module e.g. could be the parallel detection of different substitutes with a fast HPLC-method to get more structural information from the polymers. When combined with genome analysis of novel EPS producing strains, the preliminary structural information as obtained by this method will lead to enhanced information of gene structure relationship. A fast insight in the putative function of the various genes involved in EPS biosynthesis can be obtained. With current methods this still represents a bottleneck in microbial polysaccharide research. Additionally several knock out variants, or further genetically modified EPS producers can be screened for alteration of the monomer composition of their polymers in high throughput and short time.

4. Conclusion

The high throughput exopolysaccharide screening platform in 96-well format was developed for the identification of EPS producing bacterial strains, isolated from different habitats. Thereby, the main focus is to identify novel bacterial EPS producers in combination with the direct determination of their monomeric composition. The analysis of the monomeric composition was validated by 16 commercial available polymers. The method was successfully proven via the screening of 94 novel isolated strains from 21 different genera. The benefits of the screening platform were highlighted by the discussed of four exemplarily selected strains.

The results obtained in this study proof the suitability of the platform for screening novel EPS producers within only 24 h excluding time of cultivation. The highly intuitive sample preparation in 96-well format makes this high throughput EPS screening platform a powerful tool. Reliable results can be achieved for sugar monomers, their derivatives, di-and trimers and even substituents, such as pyruvate-ketal. The detailed monosaccharide analysis allows a targeted screening for polysaccharides containing special rare sugars like fucose, galacturonic acid or even unknown deoxy hexoses. Furthermore, different sugar combinations in defined ratios can be detected and therefore enable the simple identification of novel EPS or structurally related variants of already known ones. Therefore, this platform will speed up polysaccharide research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.12.021.

References

Alban, S. (2010). Kohlenhydrate, II: Polysaccharide und Polysaccharidderivate. In O. S. R. Hänsel (Ed.), *Pharmakognosie—Phytopharmazie* (pp. 461–590). Heidelberg: Springer Medizin Verlag.

Baird, J. K., Sandford, P. A., & Cottrell, I. W. (1983). Industrial applications of some new microbial polysaccharides. *Biotechnology*, *1*(9), 778–783.

Buslov, D. K., Kaputski, F. N., Sushko, N. I., Torgashev, V. I., Solov'eva, L. V., Tsarenkov, V. M., et al. (2009). Infrared spectroscopic analysis of the structure of xylans. *Journal of Applied Spectroscopy*, *76*(6), 801–805.

Cerning, J. (1990). Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiology Reviews*, *87*(1–2), 113–130.

Chan, G., Chan, W., & Sze, D. (2009). The effects of beta-glucan on human immune and cancer cells. *Journal of Hematology & Oncology*, *2*(1), 25.

Colegrove, G. T. (1983). Agricultural applications of microbial polysaccharides. *Industrial & Engineering Chemistry Product Research and Development*, *22*(3), 456–460.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science*, *284*(5418), 1318–1322.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Freitas, F., Alves, V. D., & Reis, M. A. M. (2011). Advances in bacterial exopolysaccharides: From production to biotechnological applications. *Trends in Biotechnology*, *29*(8), 388–398.

Garai-Ibabe, G., Areizaga, J., Aznar, R., Elizaquivel, P., Prieto, A., Irastorza, A., et al. (2010). Screening and selection of 2-branched (1,3)-β-D-glucan producing lactic acid bacteria and exopolysaccharide characterization. *Journal of Agricultural and Food Chemistry*, *58*(10), 6149–6156.

Goellner, E. M., Utermoehlen, J., Kramer, R., & Classen, B. (2011). Structure of arabinogalactan from *Larix laricina* and its reactivity with antibodies directed against type-II-arabinogalactans. *Carbohydrate Polymers*, *86*(4), 1739–1744.

Parolis, H., Parolis, L. A. S., Boan, I. F., Rodriguez-Valera, F., Widmalm, G., Manca, M. C., et al. (1996). The structure of the exopolysaccharide produced by the halophilic Archaeon *Haloferax mediterranei* strain R4 (ATCC 33500). *Carbohydrate Research*, *295*, 147–156.

Rehm, B. H. A. (2010). Bacterial polymers: Biosynthesis, modifications and applications. *Nature Reviews Microbiology*, *8*(8), 578–592.

Rinaudo, M., & Vincendon, M. (1982). Carbon-13 NMR structural investigation of scleroglucan. *Carbohydrate Polymers*, *2*(2), 135–144.

Robyt, J. F. (1998). Polysaccharides I. Structure and function. In J. F. Robyt (Ed.), *Essentials of carbohydrate chemistry* (pp. 157–228). New York, NY: Springer-Verlag.

Ruas-Madiedo, P., & De los Reyes-Gavilan, C. G. (2005). Invited review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science*, *88*(3), 843–856.

Rühmann, B., Schmid, J., & Sieber, V. (2014). Fast carbohydrate analysis via liquid chromatography coupled with ultra violet and electrospray ionization ion trap detection in 96-well format. *Journal of Chromatography A*, *1350*, 44–50.

Sonebi, M. (2006). Rheological properties of grouts with viscosity modifying agents as diutan gum and welan gum incorporating pulverised fly ash. *Cement and Concrete Research*, *36*(9), 1609–1618.

Sosnik, A. (2014). Alginate particles as platform for drug delivery by the oral route: State-of-the-art. *ISRN Pharmaceutics*, *2014*, 17.

Spiridon, I., & Popa, V. I. (2005). Hemicelluloses: Structure and properties. In S. Dumitriu (Ed.), *Polysaccharides structural diversity and functional versatility* (pp. 475–489). New York, NY: Marcel Dekker.

Stokes, J. R., Macakova, L., Chojnicka-Paszun, A., de Kruif, C. G., & de Jongh, H. H. J. (2011). Lubrication, adsorption, and rheology of aqueous polysaccharide solutions. *Langmuir*, *27*(7), 3474–3484.

Sutherland, I. W. (2005). Microbial exopolysaccharides. In S. Dumitriu (Ed.), *Polysaccharides structural diversity and functional versatility* (pp. 431–457). New York, NY: Marcel Dekker.

Tait, K., & Sutherland, I. W. (2002). Antagonistic interactions amongst bacteriocin-producing enteric bacteria in dual species biofilms. *Journal of Applied Microbiology*, *93*(2), 345–352.

Tait, M. I., Sutherland, I. W., & Clarke-Sturman, A. J. (1990). Acid hydrolysis and high-performance liquid chromatography of xanthan. *Carbohydrate Polymers*, *13*(2), 133–148.

Takeuchi, M., Yokota, A., & Misaki, A. (1990). Comparative structures of the cell-wall polysaccharides of four species of the genus *Microbacterium*. *Journal of General and Applied Microbiology*, *36*(4), 255–271.

Takigami, S. (2005). Konjac mannan. In T. Heinze (Ed.), *Polysaccharides I. Structure, characterization and use* (pp. 425–436). Berlin Heidelberg: Springer-Verlag.

Tischer, C. A., Iacomini, M., & Gorin, P. A. J. (2002). Structure of the arabinogalactan from gum tragacanth (*Astragalus gummifer*). *Carbohydrate Research*, *337*(18), 1647–1655.

Weiping, W. (2005). Tragacanth and karaya. In T. Heinze (Ed.), *Polysaccharides I. Structure, characterization and use* (pp. 247–262). Berlin Heidelberg: Springer-Verlag.

Zhang, N., Wardwell, P., & Bader, R. (2013). Polysaccharide-based micelles for drug delivery. *Pharmaceutics*, *5*(2), 329–352.