Journal of Industrial Microbiology, 12 (1993) 109–113
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Published by The Macmillan Press Ltd

# Analysis of microbial exopolysaccharides from industrial water systems

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(Received 26 February 1993; revision received 1 May 1993; accepted 4 May 1993)

*Key words:* Industrial slimes; Exopolysaccharides; Biofilms

## SUMMARY

The monosaccharide composition of exopolysaccharides extracted from mixed microbial slimes from cooling towers and process waters of paper machines was determined. Gas chromatographic evidence demonstrated that the microbial slimes found in these industrial systems were heteropolysaccharides, each composed of more than one monosaccharide. Monosaccharides identified in deposit samples were glucose, mannose, galactose, rhamnose, fucose, and glucuronic acid. The methods reported allow efficient extraction and analysis of microbial exopolysaccharides, and the results have implications for slime control strategies in industrial systems.

## INTRODUCTION

Microorganisms and the slimes they produce are responsible for the formation of deposits in the papermaking process and industrial cooling water systems. Bacterial slimes are composed of exopolysaccharides (EPS), which exist as capsules or slime layers outside of the cell walls [11]. When these slimes form on surfaces in paper or cooling systems, they trap organic and inorganic components and debris present in the process waters.

As the microorganisms grow within paper system deposits, portions of the deposit may detach from the surface and cause paper breaks and spots in produced paper, which reduces the paper quality and increases machine downtime. Microbial growth and slime formation in cooling systems results in reduced heat exchange caused by biofouling and plugging of heat exchanger tubes, excessive fouling of the cooling water, tower decks and fill, and is a potential cause of under-deposit corrosion. Control of microbial slime formation in cooling and paper systems is an economic necessity.

Approaches to microbial slime or biofilm control have included the use of biocides [2], biodispersants [13], or enzymes [3]. These approaches attempt to control slime by either killing the organisms that produce the slime, by disrupting the attachment process so that slime formation is prevented, or by hydrolyzing the EPS produced by the microorganisms after attachment, respectively. To be used successfully, the latter two approaches require understanding of the attachment process and the synthesis of EPS subsequent to attachment. For example, use of an enzyme to control slime will require knowledge of the composition of the slime, so that an appropriate enzyme–substrate combination is chosen.

Different views of the composition of industrial slime deposits exist, but no data directly supporting those views have been published. Hatcher [3] suggested that slimes are composed of levan, a homopolysaccharide composed of repeating units of fructose. This is inconsistent with literature related to the biosynthesis of levans, which shows that levans can only be produced by bacteria growing on sucrose [9]. During levan biosynthesis, the fructose unit of sucrose is incorporated into levan, while the glucose unit is used for energy and growth by the microorganism. It is unlikely that bacteria in paper or cooling systems will encounter sucrose in significant amounts, and hence levans should not be a significant component of industrial slimes. A recent Canadian patent suggested that EPS of industrial water systems may be composed of alginate [4], a polysaccharide composed of mannuronic and guluronic acids [7]. A U.S. patent claimed that microbial slime in industrial systems can be controlled using α-amylase, cellulase, and protease [14], which presupposes that the slimes result from polymers of α- and β-linked glucose residues and protein. Finally, a recent European patent application [5] also suggested that algal slimes in cooling systems contain alginates.

These differing views can be contrasted with those more commonly accepted in the technical literature, that slime-producing bacteria form heteropolysaccharides consisting of monosaccharides such as glucose, mannose, galactose, and glucuronic acid. Studies have shown that the composition of EPS is usually independent of the substrate or carbon source used for growth and polysaccharide production [11]. It was therefore felt that slimes found in industrial cooling and

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papermaking systems were likely to be composed of mixed heteropolysaccharides. Composition analysis of mixed microbial slimes would clarify the issue.

In this paper we report findings from the direct determination of the monosaccharide compositions of EPS extracted from actual slime deposits. Sixteen samples of slime were obtained from operating cooling towers and paper mills across the United States. Using published procedures, the deposits were extracted, hydrolyzed, and the monosaccharide composition of each EPS determined by gas chromatographic techniques.

## MATERIALS AND METHODS

### *Extraction techniques*

Deposit samples were obtained from operating cooling or paper mill systems. These were shipped by overnight courier, and were received within 24–48 h of sampling. The samples were lyophilized on arrival. Once dried, the samples were mixed in a Waring blender in order to obtain a uniform dry powder for extraction.

### *Polysaccharide extraction from deposit samples*

Between 250 and 500 mg of lyophilized slime sample was suspended in 100 ml of 10 mM ethylenediaminetetraacetate (EDTA) [8], mixed for 1 min in a Waring blender, and centrifuged in a Sorvall RC-5 refrigerated centrifuge at 12000 × *g* at 4 °C. The supernatant was decanted and saved, while the pellet was resuspended in 100 ml of 10 mM EDTA. The suspended pellet was extracted again by mixing for 3 h on a magnetic stirring plate. The mixture was centrifuged and separated as described above. The resulting pellet was stored at 4 °C until needed. Each supernatant was combined with 150 ml of isopropanol and refrigerated overnight to precipitate the polysaccharide. The precipitated material was harvested by centrifugation at 12000 × *g* for 10 min at 4 °C, and the pellet was suspended in deionized water. The polysaccharide was desalted by dialysis in 10000 molecular weight cut-off dialysis tubing against deionized water for 48 h, and the water was replaced after 24 h. The solution obtained contained the extracted polysaccharide.

### *Hydrolysis of polysaccharides and gas chromatographic analysis of monosaccharides*

Both the pellets and the dialysates processed above were lyophilized overnight, and resuspended in 24 ml of deionized water in 25-ml serum vials. These were hydrolyzed by adding 1 ml of concentrated HCl, capping the vials and digesting in a 100 °C oven for 24 h. The hydrolysates were neutralized with 1 M NaOH and lyophilized overnight. The lyophilized hydrolysates were then transferred to 5-ml reaction vials and prepared for gas chromatographic analysis. The monosaccharides were converted to oxime derivatives with 2 ml of STOX (25 mg $ml^{-1}$ hydroxylamine HCl and 6 mg $ml^{-1}$ phenyl-β-D-glucopyranoside as internal standard in pyridine, Pierce Chemical Co.) and heated to 70 °C for 45 min. Trimethylsilane (TMS) ethers were then prepared by adding 2 ml of *N*-trimethylsilylimidazole, TMSI (Pierce Chemical Co.) and heating to 70 °C for 15 min. All samples were allowed to cool at ambient temperature for 30 min. Derivatives of all monosaccharide standards were prepared in a similar fashion. Monosaccharides used as standards included ribose, rhamnose, fucose, fructose, galactose, mannose, xylose, arabinose, glucose, glucuronic acid, and *N*-acetylglucosamine.

Gas chromatography of the derivatized monosaccharides was performed on a Hewlett Packard 5880A Level 4 Gas Chromatograph equipped with a flame ionization detector (FID). Separation of the TMS ethers was achieved on a J&W Scientific DB-1701 fused silica capillary column 30 m × 0.25 mm. Analysis conditions included an initial column temperature of 150 °C for 10 min followed by a 5 °C $min^{-1}$ increase in temperature to a final column temperature of 280 °C which was held for 10 min. A sample size of 0.1 μl was placed in a split injector with a 60:1 split ratio; septum purge was 2 ml $min^{-1}$ with helium. The injector and detector temperatures were 280 °C and 300 °C, respectively. The carrier gas was helium at a rate of 1.5 ml $min^{-1}$. Hydrogen and air to the FID detector were at rates of 30 and 400 ml $min^{-1}$, respectively. The identities of the monosaccharides from the slime samples were established by comparison of their retention times with the retention times of the monosaccharide standards, with respect to the retention time of the internal standard. The retention times of the standards are shown in Table 1 and a typical chromatogram is shown in Fig. 1.

## RESULTS AND DISCUSSION

Slime deposits from industrial systems may be composed of many different components and are highly variable. Any attempt to determine the monosaccharide composition of the microbial EPS of paper mill or cooling water deposits requires that any non-microbial polysaccharide present be eliminated during sample preparation, or that its likely contribution to the monosaccharide composition be established. It was expected that slime samples from cooling

TABLE 1

Retention times for major peaks of trimethylsilyl derivatives of monosaccharide standards

| Monosaccharide | Retention time (min) |
|---|---|
| Arabinose | 12.60 |
| Xylose | 12.68 |
| Ribose | 13.23 |
| Rhamnose | 14.08 |
| Fucose | 14.17 |
| Fructose | 16.60 |
| Galactose | 17.48 |
| Mannose | 17.55 |
| Glucose | 17.73 |
| Glucuronic acid | 18.81 |
| *N*-acetylglucosamine | 23.10 |

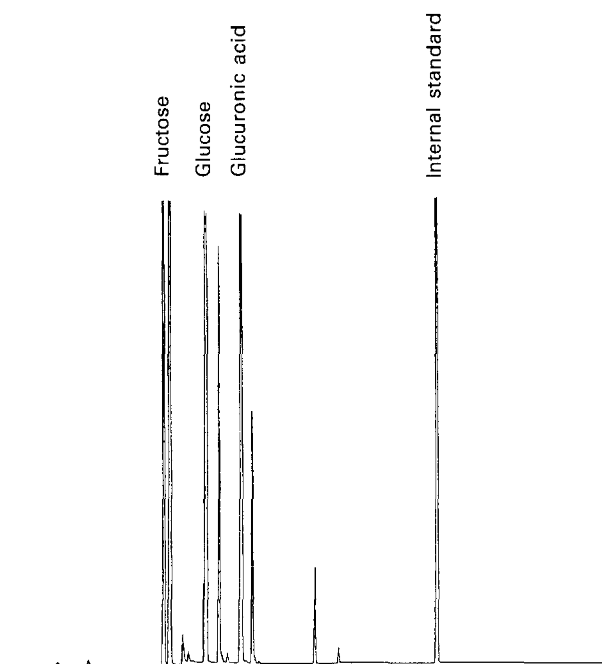


Fig. 1. GC profile of TMS-ether derivatives of monosaccharide standards by gas chromatography.

systems would have a relatively simple composition, with few, if any, non-microbial polysaccharides present, other than those leached from wooden structures within the towers. Monosaccharides found in genetic materials and cell wall components will be present, but the contribution to the monosaccharide profile by these materials should be very small, compared to that of EPS. Paper mill deposits, however, could contain significant amounts of cellulose, hemicellulose, and starch, since these are present in high concentrations in papermaking systems. The contributions cellulose and starch make to the monosaccharide compositions of paper mill deposits will be glucose, while xylose, and to a lesser extent, galactose, arabinose, and mannose could be obtained by hydrolysis of the hemicellulose fraction of wood [10]. Small amounts of ribose, deoxyribose, and other sugars might result from the hydrolysis of microbial cells.

Microbial EPS were expected to be composed of hexoses, such as glucose, galactose, and mannose, uronic acids, and deoxyhexoses, such as rhamnose and fucose, since these have been reported in heteropolysaccharides obtained from slime-forming and capsule-producing microorganisms [11]. Standards of all potential monosaccharides were obtained and derivatized, and their gas chromatographic characteristics were determined. Figure 1 shows an example of the elution profile of fructose, glucose, glucuronic acid, and the internal standard. It is evident that these compounds could be easily distinguished. The retention times of the other monosaccharides were determined in a similar fashion, and all expected compounds were well separated, making identification of each sugar possible (Table 1).

Note that the methods used resulted in a major and minor peak for each monosaccharide. When the EPS hydrolysates were analyzed, some of the minor peaks tended to merge, and this made quantitation of those sugars impossible. However, since the field samples contained EPS that was contributed by an unknown spectrum of organisms, quantitation would not have given useful results. As a consequence, no attempts to quantify these sugars were made.

The gas chromatographic (GC) profile of a paper mill sample which had not been extracted before hydrolysis is shown in Fig. 2. The largest peaks evident are those of glucose and xylose. Also present is a variety of hexose and deoxyhexose sugars and uronic acids, probably contributed by the microbial EPS. There is no evidence of ribose, deoxyribose, or fructose. It seems likely that the majority of the glucose came from cellulose or starch, and the xylose came from hemicellulose.

To determine whether bacterial EPS could be separated from the non-slime fraction of the sample, we extracted a paper mill deposit by methods developed by Platt and Geesey [8], using EDTA. Figure 3 is a chromatogram of the material remaining from a typical deposit after the EPS has been extracted with EDTA. Figure 4 shows the composition of the EDTA extract of the same sample. Comparison of the profiles shows that the magnitude of the glucose peak of the extract is diminished, and no xylose is present. The remaining peaks are characteristic of heteropolymers and, thus, attributed to the slime EPS. This supports the belief that the majority of the glucose shown in Fig. 2 came from the hydrolysis of cellulose or starch present in the paper mill deposit, and that the xylose came from the hydrolysis of the hemicellulose fraction. EDTA extraction seemed to separate microbial EPS from non-microbial polysaccharides. However, as is evident from Fig. 3, extraction of the EPS fraction was not complete, since a large

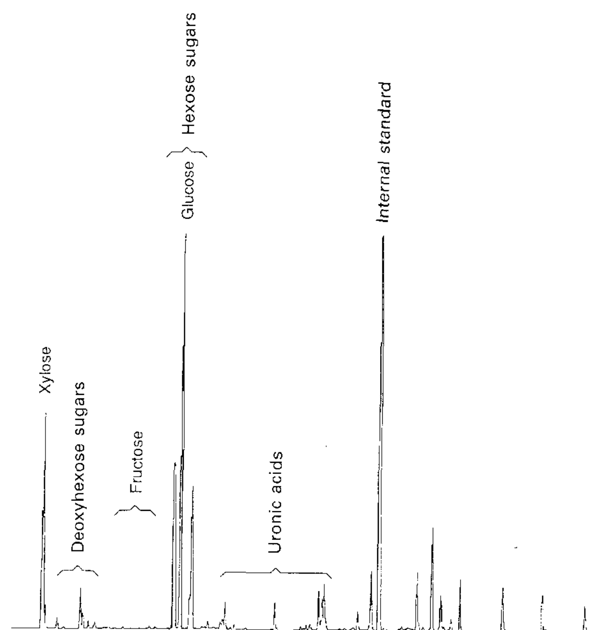


Fig. 2. GC profile of TMS-ether derivatives of an acid hydrolyzed paper mill deposit sample.

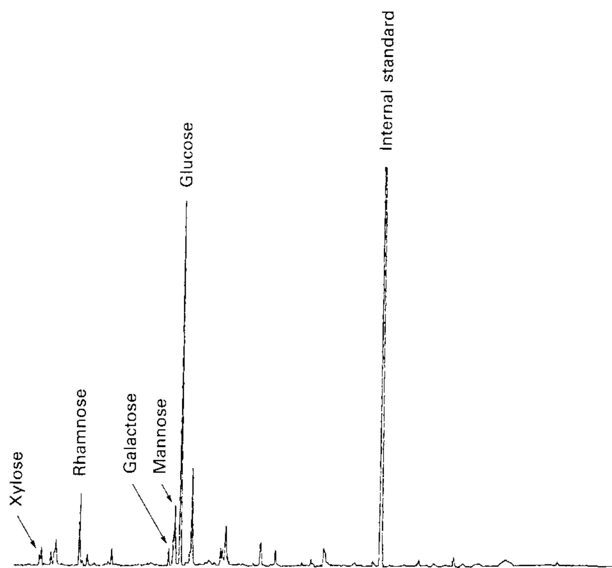


Fig. 3. GC profile of TMS-ether derivatives of a paper mill deposit after extraction with EDTA. Deposit was acid hydrolyzed prior to derivatization.

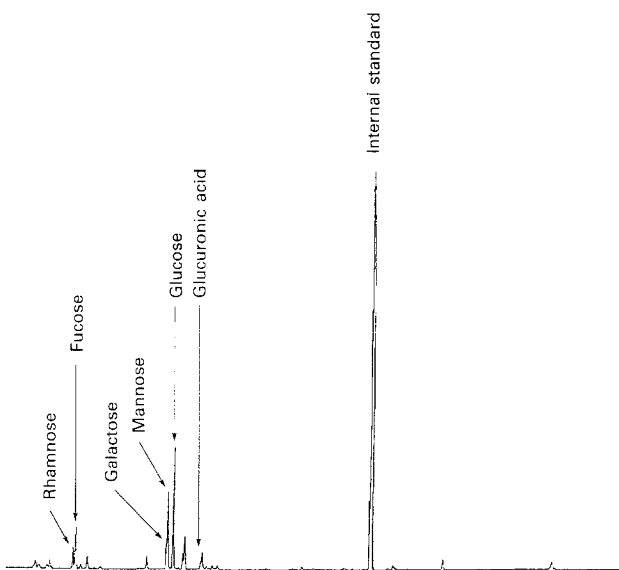


Fig. 4. GC profile of TMS-ether derivatives of the EDTA-extracted EPS of deposit shown in Fig. 3. EPS was acid hydrolyzed prior to derivatization.

fraction of rhamnose remained. In spite of this, recovery was sufficient to determine the content of the EPS fraction and the pattern was repeated in other analyses. This work confirmed that the extraction procedure produced substances that resembled EPS.

Using these techniques, slime deposits from 16 sites located in different areas of the United States were extracted and analyzed (Table 2). These samples were from operating cooling towers and paper systems. Monosaccharides identified were those reported in the literature to be found in heteropolysaccharides isolated from clinically important microbes. These included glucose, mannose, galactose, rhamnose, fucose and glucuronic acid.

All sample extracts analyzed had compositions similar to those reported by Sutherland [11], typical of EPS. Although no ribose, deoxyribose, or cell wall constituents were identified in the extracts, these could occasionally be found in the non-extractable portions of the deposits, but in very small amounts. The constituents identified were those expected from a polymicrobic slime. Thus, it appears that the extraction procedure and analytical methods employed were capable of providing information on the composition of the EPS.

No support was found for the contention that levans are important components of paper mill slimes or typical of industrial process waters, since no fructose could be detected in any of the deposit samples analyzed. The monosaccharides detected in all the deposits were similar, and any of them might be considered as a 'signature' compound for a bacterial EPS. Since rhamnose and fucose are found less frequently in non-microbial polysaccharides, the presence of these sugars could be considered diagnostic for bacterial EPS in industrial slimes.

The findings reported here suggest that enzymatic control of slime in industrial systems will be difficult to achieve. The presence of cellulose and starch in paper mill systems offers an obstacle to the use of cellulase and α-amylase for slime control, since these enzymes will attack essential process water components. Effective enzymes for slime control will need to accommodate the large number of possible linkages between the many monosaccharides of various EPS. Enzymes reported in the literature to have activity against EPS [12,1,6] are usually very specific to certain linkages and cannot hydrolyze slimes of other EPS-producing organisms or other strains of the same organism. The task of enzymatic slime control in industrial waters would therefore require an array of enzymes capable of hydrolyzing the many potential linkages that could be present in different slimes or require an enzyme with extremely broad specificity. Biocides and biodispersants, because of their broad specificity, remain preferred methods for industrial slime control.

Concerns for the environment continue to be the driving force for the introduction of safer products and environmentally acceptable control strategies should be the goal of any biological control technology. Future needs will encompass use of all these technologies.

## ACKNOWLEDGEMENTS

We would like to thank Bruce R. Keppler and Cato R. McDaniel of the Betz Analytical Services Group for developing the gas chromatographic techniques used in this study.

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TABLE 2

Summary of carbohydrate compositions of biological field samples

| Source | Composition | | | | | |
|---|---|---|---|---|---|---|
| | Rhamnose | Fucose | Galactose | Mannose | Glucose | Glucuronic acid |
| Cooling tower, Georgia | | √ | | √ | √ | √ |
| Cooling tower, Louisiana | | √ | √ | | √ | √ |
| Cooling tower, Louisiana | √ | √ | √ | √ | √ | √ |
| Cooling tower, Louisiana | √ | | √ | √ | √ | |
| Refinery cooling tower, Louisiana | √ | √ | √ | √ | √ | |
| Refinery cooling tower, Texas | √ | √ | √ | √ | √ | |
| Paper mill AES, Pennsylvania | √ | √ | √ | √ | √ | |
| Paper mill Saveall, Pennsylvania | √ | | √ | √ | √ | |
| Paper mill, California | √ | √ | √ | √ | √ | |
| Paper mill, Georgia | √ | √ | √ | √ | √ | √ |
| Paper mill, Georgia | √ | √ | √ | √ | √ | √ |
| Paper mill, Georgia | √ | √ | √ | √ | √ | |
| Paper mill, Pennsylvania | √ | √ | √ | √ | √ | √ |
| Paper mill, Pennsylvania | √ | √ | √ | √ | √ | √ |
| Paper mill, Washington | √ | √ | √ | √ | √ | |
| Sulfur bacteria, well, Florida | √ | √ | √ | √ | √ | √ |

√ signifies that this monosaccharide was present in this specific sample.


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