Distribution of extracellular polysaccharides in anaerobic granular sludges

T. Zhang and H.H. P. Fang*

Department of Civil Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong, China
(E-mail: *hrechef@hkucc.hku.hk*)

Abstract Four fluorescent lectin probes specific for individual saccharides were applied to reveal the distribution of extracellular polysaccharides in methanogenic and hydrogen-producing granular sludges. Results indicated that most of extracellular polysaccharides were distributed in the outer layer of both granules. The main constituents of extracellular polysaccharide in the methanogenic granule were glucose/mannose and N-acetyl-galactosamine, whereas those in the hydrogen-producing granules were only glucose/mannose. There were little fucose and galactose in both granules.
Keywords Anaerobic; distribution; EPS; granule; polysaccharide; staining

Introduction

Extracellular polymeric substances (EPS) facilitate the aggregation of microbes, and thus play an important role in the development of biofilm, granular sludge and activated sludge flocs. Polysaccharides are the major component of EPS (Sutherland, 1997; Fang and Jia, 1996; Liu and Fang, 2002). The polysaccharide contents in EPS of activated sludge (Frølund *et al.*, 1996), anaerobic granule (Fang and Jia, 1996) and biofilm (Jahn and Nielsen, 1998) are traditionally characterized from those fractions that can be extracted by physical and/or chemical means (Fang and Jia, 1996; Liu and Fang, 2002). However, polysaccharides may also be stained *in situ* for microscopic characterization. The staining agents generally used include calcofluorwhite M2R (Del Gallo *et al.*, 1989), congo red (Allison and Sutherland, 1984) and alcian blue (Wetzel *et al.*, 1997). Lectins were developed recently for the staining of polysaccharide with better specificity than the traditional agents (Michael and Smith, 1995; Lawrence et al., 1998; Neu, 2000).

Lectins are a group of proteins, each of which has a specific binding characteristic for certain saccharides. Lectins labeled with fluorescent dyes can thus be used to map the distribution of specific saccharides in the microbial community. Although the distribution of polysaccharides in biofilm has been studied using lectin probes (Lawrence *et al.*, 1998; Neu, 2000), little information is available so far on the distribution of polysaccharides in activated sludge and granular sludge. In this study, four fluorescent lectin probes specific for respective saccharides were applied to examine *in situ* the polysaccharides distribution in two types of anaerobic granular sludge.

Materials and Methods

Granular sludges

Two anaerobic granular sludges from previous studies were used. The methanogenic granule was sampled from a reactor treating brewery wastewater (Fang *et al.*, 1995; Liu *et al.*, 2002), whereas the hydrogen-producing granule was a reactor treating sucrose-rich wastewater (Fang *et al.*, 2002).

Water and Environmental Management Series **ISBN 1 84339 503 7 pp 153-158** © IWA Publishing 2004

Probes

Table 1 lists the probes used in the study, including two DNA-targeting probes SYTO9 and propodium iodide-PI (Molecular Probes, Eugene, OR), and four saccharides-targeting lectin probes labelled with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Sigma). The DNA-targeting SYTO9 and PI probes were used to stain bacteria cells, while the lectin probes were used to stain saccharides in the EPS. When excited by a laser at proper wavelengths, the SYTO9 and the FITC-labeled lectin probes emit green light, whereas the PI and the TRITC-labeled lectin probes emit red light. In order to reveal simultaneously the distributions of both EPS and bacterial cells for a given sample, SYTO9 was used jointly with the TRITC-labeled lectin probes, and PI with the FITC-labeled lectin probes. All the probe working solutions were prepared using a pH 7.2 phosphate-buffered saline solution (PBS; 0.13M NaCl, plus 10 mM Na_2HPO_4) and stored in -20°C before use.

Table 1 Cell and lectin probes and their key characteristics

Probe description	Probe abbreviation	Concentration (μM)	Specific target	Ex/Em* (nm)
Cell probes				
SYTO9	SYTO9	5	nucleic acid	488/525
propodium iodide	PI	30	nucleic acid	536/617
Lectin probes				
from *Canavalia ensiformis* labeled by TRITC	ConA -TRITC	1	glucose and mannose	540/566
from *Ulex europaeus* labeled by TRITC	UEA I -TRITC	1	L-fucose	540/566
from *Erythrina cristagalli* labeled by FITC	EC -FITC	1	N-acetyl-galactosamine and galactose	490/525
from *Arachis hypogaea* labeled by FITC	AH -FITC	1	Galactose	490/525

* Ex/Em: maximum excitation and emission wavelengths.

Confocal laser scanning microscopy

A confocal laser scanning microscope (CLSM), (LSM 5 Pascal, Zeiss, Jena, Germany) was used to examine the granules after staining. It was equipped with two lasers (488 and 543 nm), a beam splitter NFT545, two filter sets (BP515-530 and LP560) and three lenses (10X/NA0.5, 40X/NA0.8 and 63X/NA1.4). To avoid interference between the emitted fluorescent lights, the two-track mode was applied in all image acquisition. For the first track, the sample was excited by the laser of 488 nm and the emitting light was collected by the filter set BP515-530; for the second track, the laser of 543 nm and the filter set LP560.

Cell and EPS staining

The hydrogen-producing granules were stained and were observed by CLSM at various depth levels. Due to poor light penetration, the methanogenic granules could not be observed. Instead, only the cross-sections of sliced methanogenic granules were stained and observed. The granules were first gently washed with PBS, embedded with the OCT compound (Miles, Elkhart, IN), and then sectioned

into 20 µm slices with a rotary cryo-microtome (CM 1510-Cryostat, Leica, Germany). The cross-sections of granules were collected on the wells of the glass slide coated by 0.01% poly-L-lysine solution. The slide was then immersed in PBS for 10 min to remove the OCT compound and air-dried.

The methanogenic granule sections and hydrogen-producing granules were placed into wells on the glass slides. Each sample in the well was covered with 20 µl of a staining solution, containing a cell probe and a lectin probe listed in Table 1. The slides containing methanogenic granule sections were then incubated in a dark moist chamber at room temperature for 20 min, whereas those containing hydrogen-producing granules were incubated for 60 min. After incubation, the sample wells were carefully rinsed with filtered PBS three times to remove the residual staining solutions.

Results and Discussion

Under proper conditions, microbes could aggregate forming granules in anaerobic (Lettinga *et al.*, 1980; Fang *et al.*, 1995) as well as aerobic (Beun *et al.*, 1999) reactors. Although it has been extensively studied for over two decades, the granulation mechanism is still not fully understood (Fang, 2000). It is generally believed that EPS play an important role in the formation of methanogenic (Jia *et al.*, 1996) as well as hydrogen-producing granules (Fang *et al.*, 2002). However, little information is available on the distribution of EPS in these granules. In this study, lectin probes with fluorescent labels were used for the *in situ* analysis of polysaccharides and, thus, EPS in two types of anaerobic granules.

EPS in methanogenic granule

Figure 1 illustrates the images of a methanogenic granule stained by (a) the glucose/mannose-specific ConA-TRITC and (b) the DNA-specific SYTO9 probes. It clearly shows that the glucose/mannose-containing EPS were mostly distributed at the outer layer of the granule, whereas the distribution of bacteria was rather uniform without forming layers.

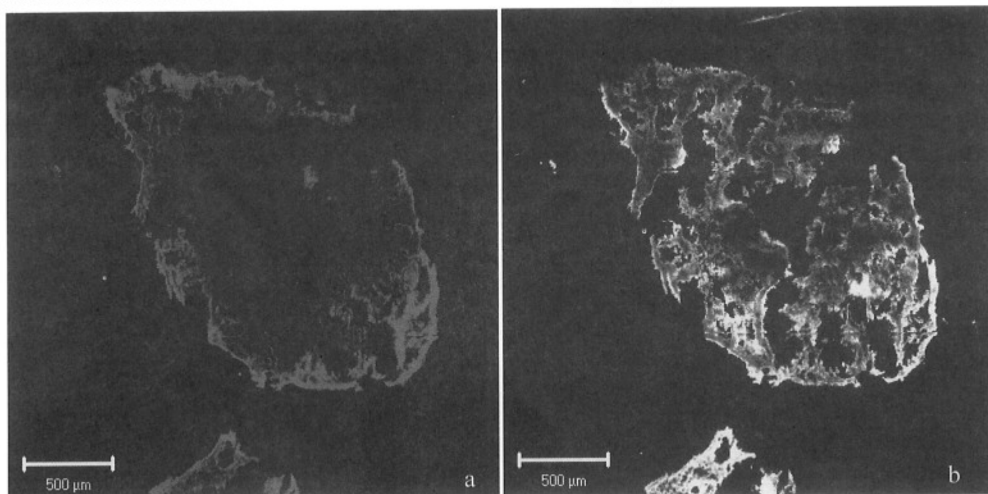

Figure 1 Images of a methanogenic granule stained using (a) ConA-TRITC, and (b) SYTO9. (bar = 500 µm)

Figure 2 illustrates the images of another methanogenic granule stained by (a) the N-acetyl-galactosamine/galactose-specific EC-FITC and (b) the DNA-specific PI probes. It shows that the N-acetyl-galactosamine/galactose-containing EPS were also mostly distributed at the outer

layer of the granule, whereas the bacterial distribution was uniform without forming layers.

Images of methanogenic granules stained by L-fucose-specific UEA I and galactose-specific AH probes had a very weak fluorescent signal, indicating that there was little L-fucose and galactose in the EPS of methanogenic granules. Thus, the image in Figure 2(a) was most likely due to the presence of N-acetyl-galactosamine, not galactose, in the EPS.

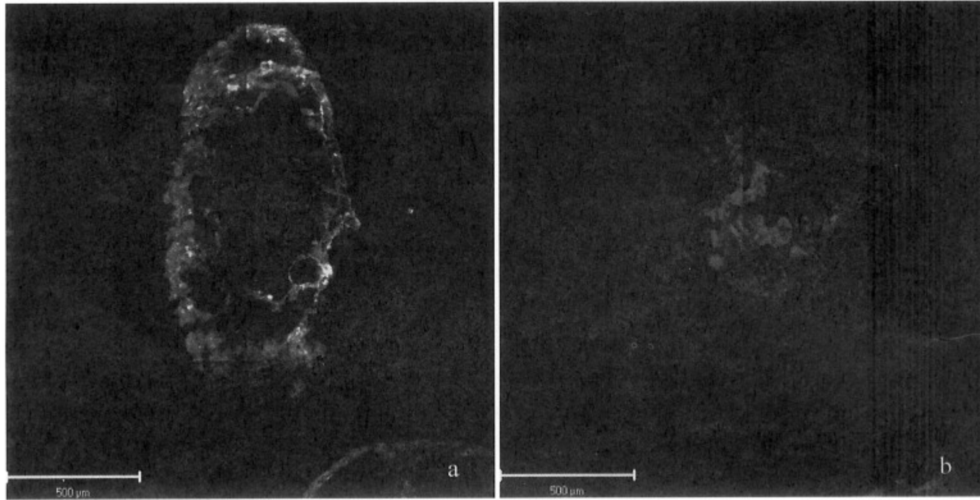

Figure 2 Images of a methanogenic granule stained using (a) EC-FITC, and (b) PI. (bar = 500 μm)

Results show that glucose, mannose and N-acetyl-galactosamine were the three major constituents of the extracellular polysaccharide of methanogenic granules. These constituents were previously reported in bacterial EPS by other researcher (Bryers and Drummond, 1998).

EPS in hydrogen-producing granule

Figure 3 illustrates the images of a hydrogen-producing granule stained by (a) the glucose/mannose-specific ConA-TRITC and (b) the DNA-specific SYTO9 probes. It also shows that the glucose/mannose-containing EPS were mainly distributed at the outer layer of the granule, whereas the bacteria cells were uniformly distributed.

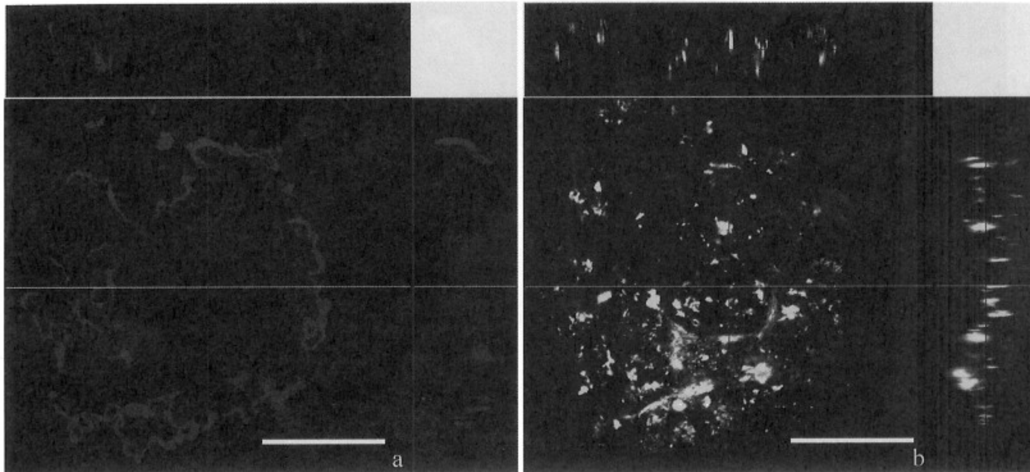

Figure 3 Cross-section images of a hydrogen-producing granule stained using (a) ConA-TRITC, and (b) SYTO9. (bar = 200 μm)

Images of hydrogen-producing granules staining by EC-FITC, UEA I-TRITC and AH-FITC probes produced very weak fluorescent signals, suggesting that there were little L-fucose and N-acetyl-galactosamine and galactose in the hydrogen-producing granules.

Conclusion

Results in this study show that extracellular polysaccharides of both methanogenic and hydrogen-producing granules were mostly distributed at the outer layer of the granules, whereas the bacterial distributions were rather uniform without a layered structure. EPS of the methanogenic granule were mostly composed of glucose/mannose and N-acetyl-galactosamine, whereas those of hydrogen-producing granule were mostly composed of glucose/mannose. There were little L-fucose and galactose in both granules.

Acknowledgement

The authors wish to thank the financial support of the Hong Kong Research Grants Council (HKU7007/02E), and the University Development Fund of the University of Hong Kong.

References

Allison D.G. and Sutherland I.W. (1984). A staining technique for attached bacteria and its correlation to extracellular carbohydrate production. *J. Microbiol. Meth.*, **2**, 93–99.

Beun J.J., Hendriks A., van Loosdrecht M.C.M., Morgenroth E., Wilderer P. A. and Heijnen J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Wat. Res.*, **33**, 2283-2290.

Del Gallo M., Negi, M. and Neyra, C.A. (1989). Calcofluor- and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. *J. Bacteriol.*, **171**, 3504–3510.

Fang H.H.P., Chui H.K. and Li Y.Y. (1995). Micro-structural analysis of UASB granules treating brewery wastewater. *Wat. Sci. Tech.*, **31**(9), 129-135.

Fang H.H.P. and Jia X.S. (1996). Extraction of extracellular polymer from anaerobic sludge. *Biotechnol. Technol.*, **10**, 803-808.

Fang, H.H.P. (2000). Microstructure of UASB granules and its resulting effects. *Wat. Sci. Tech.*, **42**(12), 201-208.

Fang H.H.P., Liu, H. and Zhang, T. (2002). Characterization of a hydrogen-producing granular sludge. *Biotechnol. Bioeng.*, **78**(1), 44-52.

Frølund B., Palmgren R., Keiding K. and Nielsen P.H. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Wat. Res.*, **30**, 1749-1758.

Jahn A. and Nielsen P.H. (1998). Cell biomass and exopolymer composition in sewer biofilms. *Wat. Sci. Tech.* **37**(1),17-24.

Jia X.S., Furumai H. and Fang H.H.P. (1996). Yields of biomass and extracellular polymers in four anaerobic sludges. *Environ. Tech.*, **17**, 283-291.

Lawrence J.R., Neu T.R. and Swerhone G.D.W. (1998). Application of multiple parameter imaging for the quantification of algae, bacterial and exopolymer components of microbial biofilms. *J. Microbiol. Meth.*, **32**, 253-261.

Lettinga G., van Velsen A.F.M., Hobma S.W., De Zeeuw W. and Klapwijk A. (1980). Use of upflow sludge blanket reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnol. Bioengr.*, **22**, 699-734.

Liu W.T., Chan O.C. and Fang H.H.P. (2002). Microbial community dynamics during start-up of acidogenic anaerobic reactors. *Wat. Res.*, **36**(13), 3203-3210.

Liu H. and Fang H.H.P. (2002). Extractions of extracellular polymeric substances (EPS) in sludges, *J. Biotechnol.*, **95**(3), 249-256.

Michael T. and Smith C.M. (1995). Lectins probe molecular films in biofouling: characterization of early films on non-living and living surfaces. *Mar. Ecol. Prog. Ser.* **119**, 229–236.

Neu T.R. (2000). *In situ* cell and glycoconjugate distribution in river snow studied by confocal laser scanning microscopy. *Aquat. Microbiol. Ecol.* **21**, 85–95.

Sutherland I.W. (1997). Microbial exopolysaccharides - structural subtleties and their consequences. *Pure Appl. Chem.* **69**, 1911-1917.

Wetzel R.G., Ward A.K. and Stock M. (1997). Effects of natural dissolved organic matter on mucilaginous matrices of biofilm communities. *Arch. Hydrobiol.* **139**, 289-299.

Zhang T. and Fang H.H.P. (2001). Quantification of extracellular polymeric substances in biofilm by confocal laser scanning microscopy. *Biotechnol. Lett.* **23**(5), 405-409.