

# Production of zoogloea gum by *Zoogloea ramigera* with glucose analogs

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Zoogloans with altered sugar composition were synthesized by *Zoogloea ramigera* by varying the glucose concentration and initial medium pH. The relative mol % of the sugar components, glucose and galactose, in the exopolymer made with 2% (w/v) glucose as the carbon source was 66 and 34%, respectively. By varying the glucose concentration and initial medium pH, the mol % ratios of glucose to galactose in zooglan ranged from 70 : 30 to 58 : 42. Also, glucose analogs, 3-*O*-methyl-D-glucose, 2-amino-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-glucose, were used as a co-substrate with glucose to produce modified zoogloans. The mol % ratios of glucose to galactose in exopolymers produced by co-feeding glucose analogs ranged from 70 : 30 to 9 : 91.

## Introduction

Zoogloea gum (zooglan) is the bacterial exopolysaccharide produced by *Zoogloea ramigera* and consists of glucose, galactose, and pyruvic acid (Friedman and Dugan, 1964; Ikeda *et al.*, 1982; Franzen and Norberg, 1984). In one study, the relative mol % of these components in the zooglan produced by *Z. ramigera* 115 was 71, 19, and 10, respectively (Ikeda *et al.*, 1982). Another investigation described the relative mol % of glucose and galactose in the zooglan by *Z. ramigera* ATCC 25935 as 67 and 33, respectively (Franzen and Norberg, 1984). Zooglan is a long chain polysaccharide consisting of mainly  $\beta$ -1, 4-linked glucose residues and  $\beta$ -1, 4- and  $\beta$ -1, 3-linked galactose residues with branches of glucose residues at the C-3 or C-6 positions of the galactose residues (Ikeda *et al.*, 1982).

The bacterium plays an important role in flocculation during waste water treatment (Joyce and Dugan, 1970). Extracellular polymers made by *Z. ramigera* can accumulate certain metal ions such as Cu, Fe, Ni, Co, and Zn (Friedman and Dugan, 1968; Norberg and Persson, 1984; Kuhn and Pfister, 1990). The weak acidity and structural features of zooglan may explain this behavior (Ikeda *et al.*, 1982); its combined functional properties of viscoelasticity, surface-activity, acid stability, and salt compatibility enable it to function as an effective stabilizer and emulsifier when used in oil-in-water emulsion systems (Stauffer *et al.*, 1980).

The effects of physiological conditions on zooglan yields and composition have not been explored. In the present

work, variability in the composition of zooglan and the possibility that repeat units other than glucose and galactose might be incorporated were explored. Specifically, the effects of physiological parameters such as the glucose concentration and the initial medium pH on the molar ratio of glucose to galactose in products were studied.

## Materials and methods

### Bacterial strain and growth medium

*Z. ramigera* ATCC 25935 was grown on the mineral salts medium (MSM) with the following composition (g/l): K<sub>2</sub>HPO<sub>4</sub>, 2.0; KH<sub>2</sub>PO<sub>4</sub>, 1.0; NH<sub>4</sub>Cl, 1.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; yeast extract, 0.01; and glucose, 20 (Norberg and Enfors, 1982). The pH of medium was adjusted to 6.5–6.7 before sterilization. The carbon source was autoclaved separately for 20 min at 121°C and added under aseptic conditions.

### Production of zooglan

Starter cultures were prepared by transferring cells from agar slants to 50 ml MSM with 2% (w/v) glucose in 250 ml Erlenmeyer flasks. These cultures were incubated for 3 days at 30°C and 180 rpm. This starter culture was used as a 3% (v/v) inoculum for 150 ml of MSM with carbon source (2 to 3%, w/v) in 500 ml Erlenmeyer flasks. Cultures were incubated for 5 days under the same conditions used in preparing the starter cultures. Since the glucose analogs, 3-*O*-methyl-D-glucose (3-*O*-methylglucose), 2-amino-2-deoxy-D-glucose (glucosamine), and 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine), did not support cell growth,

co-substrate mixtures of 1% glucose and 2% of glucose analog were used for the 5 day cultivations. Samples were periodically withdrawn from the cultures to determine cell growth at 650 nm and exopolymer production.

Cell broths were centrifuged at  $15,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  to remove cells. To determine the biomass, cells were washed with distilled water and dried at  $100\text{--}105^{\circ}\text{C}$  to constant weight. Purification of zooglan was carried out exactly as was described elsewhere (Ikeda *et al.*, 1982). The precipitated material was repeatedly washed with acetone and ether, dissolved in deionized water, and dialyzed against deionized water using dialysis tubing with a molecular weight cut off of  $12,000\text{--}14,000$  Da. After dialysis for 2–3 days with 4–5 changes of deionized water, the solution was lyophilized and the exopolymer yield was determined by weighing.

### Instrumental measurements

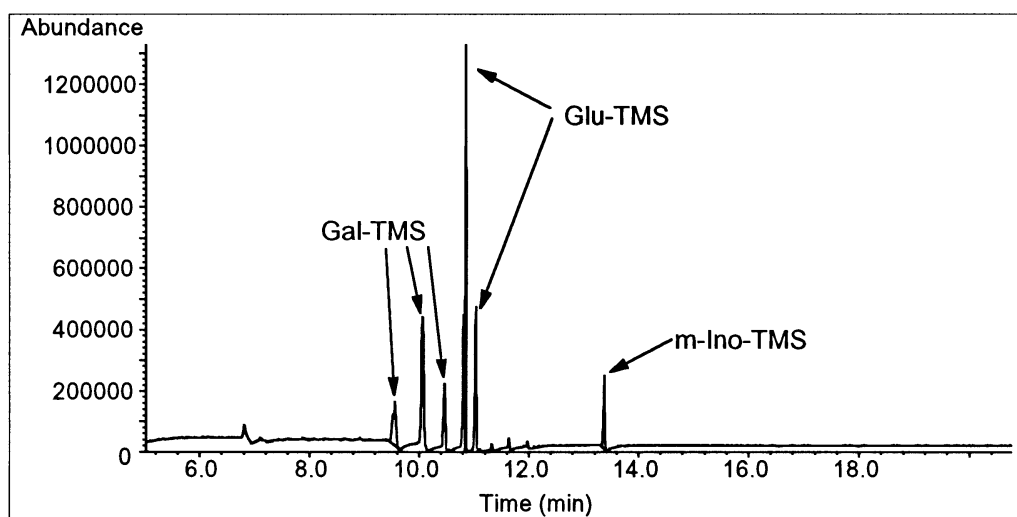
Preparation of samples for GC/MS analyses was carried out as described elsewhere (Chaplin, 1982; Lee *et al.*, 1997). GC/MS analyses were performed on a Hewlett Packard gas chromatograph, model 5890 Series II, also equipped with an HP model 7673 injector and coupled to a Mass Selective Detector (HP 5971 Series). The capillary column was a cross-linked 5% phenyl methyl silicone fused silica (HP Ultra MS 5,  $30\text{ m} \times 0.25\text{ mm}$ , film thickness  $0.33\text{ mm}$ ). Dry oxygen-free helium ( $0.8\text{ ml/min}$  flow rate) was used as the carrier gas and the column temperature program was  $140^{\circ}\text{C}$  for 2 min, then increased at  $8^{\circ}\text{C}$  per min up to  $260^{\circ}\text{C}$ . Sample volumes of 1 ml were injected and the injector was purged for 0.6 min after injection. To quantitate the repeat unit

composition of products, response factors were generated from the relative values of GC/MS peak area using an equimolar mixture of pure sugars and *m*-inositol as the internal standard.

### Results

When *Z. ramigera* was cultured on glucose, 3-*O*-methylglucose, glucosamine, and *N*-acetylglucosamine as carbon sources (2%, w/v), only glucose supported growth. The yield ( $\mu\text{g/ml}$ ) and specific yield ( $\mu\text{g/mg}$ , yield per dry cell weight) of zooglan from the 5 day culture grown on glucose were 135 and 118, respectively. The major components in the exopolymer were glucose and galactose, identified by the peak retention times and the area ratio of their anomers in the GC/MS chromatogram (Fig. 1). Based on relative peak areas and response factors of corresponding standard sugars, the relative mol % of glucose and galactose in this exopolymer was calculated to be 66 and 34%, respectively.

Cell growth and exopolymer yield increased steadily as the medium glucose concentration was increased from 0.5 to 4% and 0.5 to 2%, respectively (Table 1). As the glucose concentration was increased to 3%, the specific yield of exopolymer increased to  $132\text{ }\mu\text{g/mg}$  dry cells. A further increase in the glucose concentration from 3 to 4% had no substantial effect on the specific yield. Increasing glucose concentration above 4% resulted in a decrease in both exopolymer yield and specific yield. The relative mol % of galactose in the exopolymers showed a modest increase from 33 to 42% as glucose concentration was increased from 0.5 to 5.0%.



**Figure 1** GC/MS spectrum of trimethylsilylated (TMS) repeat units of zooglan made by using 2% glucose as the sole carbon source. The abbreviations used are as follows: glucose is Glu, galactose is Gal and *meso*-inositol is *m*-Ino.

**Table 1** Effect of glucose concentration on cell growth, exopolymer production, and composition after 5 day culture of *Z. ramigera*

Glucose (% w/v)	pH <sup>1)</sup> (mg/ml)	DCW <sup>2)</sup> (µg/ml)	Yield Yield <sup>3)</sup>	Specific Yield	Glucose : Galactose (Mol ratio in polymer)
0.5	4.7	0.8	71	86	67 : 33
1.0	4.6	0.9	86	96	66 : 34
2.0	4.5	1.0	112	108	66 : 34
3.0	4.5	1.2	112	132	62 : 38
4.0	4.3	1.6	112	129	62 : 38
5.0	4.1	1.7	91	55	58 : 42

1) The final pH after 5 day culture.

2) Dry cell weight.

3) µg exopolymer/mg dry cells.

For the cultures grown on 2% glucose, cell growth and exopolymer yield as well as the sugar repeat units in the product varied with the initial medium pH (Table 2). As the initial medium pH was increased from 5.6 to 7.6, the specific yield of exopolymer decreased, while cell growth increased. The minimum specific yield of exopolymer and maximum dry cell weight were 25 mg/mg of cells and 4.0 mg/ml, respectively, when the initial medium pH was 7.6. The relative mol % of galactose in the exopolymers increased from 30 to 42% as the initial pH was increased from 5.6 to 7.6. The highest yield and specific yield of the exopolymer was found for cultures having an initial medium pH of about 5.6 and a final medium pH of about 4.0. For cultures which had initial medium pH values of 6.8 and higher, the final medium pH did not decrease below 6.1.

Cultivations with glucose analogs as a co-substrate showed an increase in the time required to reach maximal growth and decrease in the yield and specific yield of exopolymer relative to cultivations carried out

**Table 2** Effect of initial medium pH on the cell growth, exopolymer production and composition after 5 day culture of *Z. ramigera*

Initial pH	pH	DCW (mg/ml)	Yield (µg/ml)	Specific Yield	Glucose : Galactose (Mol ratio in polymer)
5.6	3.9	0.8	160	200	70 : 30
5.9	4.1	1.1	152	138	70 : 30
6.4	4.9	1.2	150	125	68 : 32
6.8	6.1	2.3	152	66	69 : 31
7.2	6.5	2.7	103	38	62 : 38
7.6	7.1	4.0	100	25	58 : 42
8.0	7.4	2.6	101	39	67 : 33

using 3% glucose as the carbon source (Table 3). Of the glucose-related co-substrates, 3-O-methylglucose was least detrimental to cell growth and exopolymer production. Glucose analogs as co-substrates resulted in altered zooglan composition. In general, these co-substrates increased the relative mol % of galactose in zooglan from 35% to more than 50%. Interestingly, the culture on 1% glucose and 2% 3-O-methylglucose resulted in an exopolymer which contained 91 mol % of galactose.

## Discussion

The relative mol % of glucose to galactose in the zooglan by *Z. ramigera* with glucose as the carbon source was reported as 67 : 33 (Franzen and Norberg, 1984) and 79 : 21 (Ikeda *et al.*, 1982), respectively. In this study, it was shown that the sugar repeat unit composition in the exopolymer produced by *Z. ramigera* ATCC 25935 varied in response to changes in the media glucose concentration and the initial pH (Tables 1 and 2). In this way, the relative mol % of galactose in products was varied from 30 to 42%. Based on these results, the low galactose content of zooglan in the previous report (Ikeda *et al.*, 1982) may be due to differences in culture conditions. The observation that microbial growth conditions can lead to modification of exopolysaccharide structure is consistent with other reports (Slodki, 1987). For example, the ratio of mannuronate to guluronate and the degree of O-acetylation of bacterial alginate produced by *Pseudomonas aeruginosa*, was changed according to the carbon source in nutrient media (Marty *et al.* 1992). The composition as well as the viscosity of xanthan gum produced by *Xanthomonas campestris* was also changed with concentration of mineral salts (Sutherland, 1990).

The glucose analogs, when used as sole carbon sources, did not support the cell growth of *Z. ramigera*. However,

**Table 3** Effect of glucose analogs as co-substrates with glucose on cell growth, zooglan formation and composition after 5 day culture of *Z. ramigera*

Carbon <sup>1)</sup>	pH	DCW (mg/ml)	Yield (µg/ml)	Specific Yield	Glucose : Galactose (Mol ratio in polymer)
Glucose <sup>2)</sup>	4.8	1.2	144	120	65 : 35
3-O-Methylglucose	5.6	1.6	83	52	9 : 91
Glucosamine	4.5	1.6	46	29	33 : 67
N-Acetylglucosamine	6.2	1.4	38	27	33 : 67

1) Glucose analogs (2%, w/v) as co-substrates were mixed with 1% glucose.

2) Carbon source was 3% glucose.

the mixtures of glucose and these sugars as co-substrates can support cell growth. There was no evidence for the direct incorporation of glucose analogs into zooglan unlike the case of 3-O-methylglucose and N-acetylglucosamine that were directly incorporated into the exopolymers produced by *Agrobacterium* sp. (Lee *et al.*, 1997). Cell growth, exopolymer production, and the zooglan sugar repeat unit composition varied significantly depending on the glucose analogs used as the co-substrates with glucose. Contents of galactose as high as 91 mol % resulted from this strategy (Table 3). Non-metabolizable glucose analogs inhibit the bacterial growth on a wide variety of carbon sources (Kornberg and Lambourne, 1994). These compounds probably compete with glucose at the level of transport and/or metabolism (Cloherty *et al.*, 1996; Versa *et al.*, 1996). Instability in exopolysaccharide production by *P. aeruginosa* (Darzins and Chakrabarty, 1984), *X. campestris* (Sutherland, 1990), and *Z. ramigera* (Easson, Jr. *et al.*, 1987) has been reported. Apparently, the introduction of these glucose analogs as a co-substrate into *Z. ramigera* cultivations results in dramatic changes in sugar metabolism, due to their competition with glucose (Cloherty *et al.*, 1996; Versa *et al.*, 1996) and alteration in gene expression involved in biosynthetic pathway of zooglan (Easson, Jr. *et al.*, 1987) so that pathways leading to the formation of galactose monomer are greatly enhanced at the expense of the formation of polymerizable glucose. Thus, it is concluded that glucose analogs can function as powerful agents for modulating sugar metabolism and, ultimately, exopolysaccharide structure.

Metal adsorption by *Z. ramigera* is attributed mainly to zooglan which has a slight negative charge and multiple hydroxyl groups. The affinity for metal ions can be altered by changing the chemical composition and/or structure of the biopolymer (Ikeda *et al.*, 1982; Kuhn and Pfister, 1990). The modified zooglans synthesized in this study likely have altered affinities for metal ions due to the changed chemical structure. Further work

will focus on elucidating the relationship between biopolymer composition, metal binding affinities, and other functional properties of these zooglan products.

## References

- Chaplin, M. (1982). *Anal. Biochem.* 123, 336–341.
- Cloherty, E.K., Diamond, D.L., Heard, K.S., and Carruthers, A. (1996). *Biochem.* 35, 1323–13239.
- Darzins, A., and Chakrabarty, A.M. (1984). *J. Bacteriol.* 159, 9–18.
- Easson, Jr., D.D., Sinskey, A.J., and Peoples, O.P. (1987). *J. Bacteriol.* 169, 4518–4524.
- Franzen, L., and Norberg, A.B. (1984). *Carbohydr. Res.* 128, 111–117.
- Friedman, B.A., and Dugan, P.R. (1964). *J. Bacteriol.* 55, 205–207.
- Friedman, B.A., and Dugan, P.R. (1968). *Develop. Ind. Microbiol.* 9, 381–388.
- Fukui, T., Ito, M., and Tomita, K. (1982). *Eur. J. Biochem.* 127, 423–428.
- Ikeda, F., Shuto, H., Saito, T., Fuui, T., and Toita, K. (1982). *Eur. J. Biochem.* 123, 437–445.
- Joyce, G.H., and Dugan, P.R. (1970). *Develop. Ind. Microbiol.* 11, 377–386.
- Kornberg, H., and Lambourne, L.T.M. (1994). *Proc. Natl. Acad. Sci. USA*, 91, 11080–11083.
- Kuhn, S.P., and Pfister, R.M. (1990). *J. Ind. Microbiol.* 6, 123–128.
- Lee, J.W., Yeomans, W.G., Allen, A.L., Kaplan, D.L., Deng, F., and Gross, R. A. (1997). *Can. J. Microbiol.* 43, 149–156.
- Marty, N., Dournes, J., Chabanon, G., and Montrozier, H. (1992). *FEMS Microbiol. Lett.* 98, 35–44.
- Norberg, A.B., and Enfors, S. (1982). *Appl. Environ. Microbiol.* 44, 1231–1237.
- Norberg, A.B., and Persson, H. (1984). *Biotechnol. Bioeng.* 26, 239–246.
- Peoples, O.P., and Sinskey, A.J. (1989). *J. Biol. Chem.* 264, 15293–15297.
- Slodki, M.E. (1987). New bacterial polysaccharides. In: *Industrial Polysaccharides*, S. Stivala, V. Crescenzi, and I.C.M. Dea, eds. pp. 3–13, New York: Gordon and Breach Science Pub.
- Stauffer, K.R., Leeder, J.G., and Wang, S.S. (1980). *J. Food Sci.* 45, 946–952.
- Sutherland, I.W. (1990). Physiology and industrial production. In: *Biotechnology of Microbial Exopolysaccharides*, pp. 70–88, Cambridge, England: Cambridge Univ. Press.
- Vera, J.C., Reyes, A.M., Carcamo, J.G., Velasquez, F.V., Rivas C.I., Zhang, R.H., Strobel, P., Tribarren, R., Scher, H.I., Slebe, J.C., and Golde, D.W. (1996). *J. Biol. Chem.* 271, 8719–8724.

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