

# Microbial production of water-soluble non curdlan type exopolymer-B with controlled composition by *agrobacterium* sp

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The cell growth, production of exopolymers, and the molar ratio of glucose to mannose in the water-soluble non curdlan type exopolymer-B (WSNCE-B), which is one of three exopolymers purified from the culture of *Agrobacterium* sp., varied with carbon source, culture medium, and initial medium pH. The molar percentage of rhamnose, a minor component in WSNCE-B, varied up to 13%, dependent on physiological conditions. No rhamnose was found in the WSNCE-B purified from the culture with initial medium pH  $\geq$  6.8. The relative amount of mannose in WSNCE-B increased regularly with the amount of yeast extract added to the mineral salts medium. The relative amounts of glucose, mannose, and rhamnose in the WSNCE-B can be controlled by varying culture conditions.

## Introduction

*Alcaligenes faecalis* var. *myxogenes* 10C3K produces an extracellular unbranched homo- $\beta$ -(1–3)-glucan called curdlan which is water-insoluble at neutral pH (Harada *et al.*, 1966a; 1968). The complex medium for the production of curdlan-type polysaccharides was developed with yeast extract as the best nitrogen source (Harada *et al.*, 1966a, b). Yeast extract is a complex mixture of amino acids, peptides, and proteins as well as a good source of vitamin B and some mineral salts (Sharmila *et al.*, 1989; Shen *et al.*, 1993). A defined medium, containing mineral salts and phosphate buffer to maintain a suitable pH during culture, was also developed (Harada *et al.*, 1966c). The defined medium was used in continuous culture (Philips *et al.*, 1983) and in a two stage continuous process for improved productivity (Lawford *et al.*, 1982).

An unidentified water-soluble exopolymer named exopolymer B was isolated from a nitrogen-limited chemostat culture (dilution rate  $< 0.2 \text{ h}^{-1}$ ) of *Agrobacterium* sp. ATCC 31749 (Phillips *et al.*, 1983). Three different types of exopolymers, including curdlan, were purified from cultures of *Agrobacterium* sp. The water-soluble non curdlan type exopolymer-A (WSNCE-A) consists of glucose and galactose with rhamnose as a minor component. The major components of the WSNCE-B are glucose and mannose and the minor one

is rhamnose (Lee *et al.*, 1997). In this study, the production of exopolymers by *Agrobacterium* sp. ATCC 31749 was studied and the physiological factors that affect the production and composition of WSNCE-B were investigated.

## Materials and methods

### Microorganism

*Agrobacterium* sp. (formerly named *Alcaligenes faecalis* var. *myxogenes*) ATCC 31749 was grown and maintained on slants containing 4% glucose, 0.5% yeast extract, 1.0% calcium carbonate, and 1.5% agar.

### Media for production

Complex medium (CM) and mineral salts medium (MSM) were used for the production of exopolymers. The CM contained 0.5% yeast extract, 1.0% calcium carbonate, and 4.0% carbon source (Harada *et al.*, 1966b). The MSM contained the following components (g/l):  $\text{KH}_2\text{PO}_4$ , 1.74;  $\text{K}_2\text{HPO}_4$ , 0.49;  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ , 3.7;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.25;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.024;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.015;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.01; citrate, 0.21;  $\text{NH}_4\text{Cl}$ , 2.1; and 2% (w/v) carbon source (Lawford *et al.*, 1982). Carbon sources included glucose and glucose analogs such as 3-O-methyl-D-glucose (3-O-methylglucose), 2-amino-2-deoxy-D-glucose (glucosamine), 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine), and 2-deoxy-D-glucose (2-deoxyglucose) that were autoclaved

separately for 20 min at 120°C and added to media under aseptic conditions.

### Production of exopolymers

Production of exopolymers was carried out as described elsewhere (Lee *et al.*, 1997). The culture after 5 days under aerobic conditions was centrifuged at  $12,000 \times g$  for 30 min. The pellet was added to an equivalent volume of 0.5 N sodium hydroxide at 3°C, the mixture was stirred for 10 min, and then left to stand for 3 hr at the same temperature. The resulting solution was centrifuged at  $12,000 \times g$  for 40 min, and curdlan in the clear supernatant was precipitated by neutralization with 10% acetic acid and repeatedly washed with water, acetone, and ether.

The supernatant (SN) after centrifugation of the neutralized solution was treated with two volumes of ethanol. The precipitate was dissolved in deionized water (DW) and dialyzed against DW with a MW cut off of 12,000–14,000. After dialysis for 2–3 days with 4–5 changes of DW, the solution was lyophilized. This fraction is termed Exopolymer A and represents the water-soluble non curdlan type exopolymer-A (WSNCE-A). The SN remaining after centrifugation of the cell culture was purified by the same procedure as WSNCE-A. This fraction is termed Exopolymer B and represents the water-soluble non curdlan type exopolymer-B (WSNCE-B) (Phillips *et al.*, 1983).

### Analytical methods

Dry cell weight (DCW) was determined by direct weighing of the cell fraction after drying to constant weight at 100–105°C. The yield of the curdlan was determined by the same procedure. The concentration of WSNCEs was determined colorimetrically by the phenol-sulfuric acid method (Dubois *et al.*, 1956). A standard curve for WSNCEs was prepared from glucose. Gas chromatographic analysis after methanolysis of the polysaccharides and subsequent trimethylsilylation

(TMS) was used to determine the composition of carbohydrates in the exopolymers (Chaplin, 1982). Preparation of samples for GC analyses was carried out as was described previously (Lee *et al.*, 1997).

### Results

The total production of WSNCE-B in CM was higher than in MSM (Table 1 and 2). Each WSNCE-B purified from all the cultures had glucose and mannose as the major components. Most samples contained rhamnose as a minor component. The highest molar percentage of rhamnose in WSNCE-B was 13% when N-acetylglucosamine was used as the carbon source in MSM. The relative amount of rhamnose in WSNCE-Bs purified from each culture did not vary significantly. The molar ratios of glucose to mannose in WSNCE-B purified from MSM ranged from 1.0: 0.7 to 1.0: 1.1 and those purified from CM ranged from 1.0: 4.6 to 1.0: 5.9, depending on the carbon source.

Cell growth increased as the initial medium pH was increased from 5.6 to 7.6. After a 5 day culture, the final pH of all the cultures was close to 4.0 (Table 3). The optimal initial pH for the production of curdlan and WSNCE-A was 6.4 and 7.2, respectively. Unlike curdlan and WSNCE-A, the optimal initial pH for the production of WSNCE-B was 7.6 the as same as that for cell growth. Depending on the initial pH of MSM, the molar ratio of glucose to mannose in WSNCE-B ranged from 1.0: 0.9 to 1.0: 1.3. At low initial medium pH, the molar ratio of glucose to rhamnose was 1.0: 0.2 and molar percentage of rhamnose was up to 10%. When the initial medium pH was 6.8 or greater, rhamnose was not detected in WSNCE-B.

The highest cell growth and total yield of exopolymers were obtained when 0.5% yeast extract was added to MSM (Table 4). When yeast extract was not added to MSM, the highest specific yield of curdlan and lowest yields of WSNCE-A and B resulted. The molar ratio of

**Table 1** Effect of carbon source on the cell growth, production of exopolymers, and composition of WSNCE-B after 5 day culture of *Agrobacterium* sp. in CM<sup>1</sup>

Carbon	pH <sup>2</sup>	Exopolymers ( $\mu$ g/ml)			Molar ratio in WSNCE-B		
		Curdlan	WSNCE-A	WSNCE-B	Glucose	Mannose	Rhamnose
Glucose	6.6	75	103	182	1.0:	4.7	0.1
3-O-Methylglucose	8.4	48	35	185	1.0	5.3	0.1
N-Acetylglucosamine	8.1	84	106	205	1.0	4.6	0.1
2-Deoxyglucose	6.9	63	57	216	1.0	5.9	0.1

<sup>1</sup>Results obtained after 5 day culture in the complex medium (CM) with 4% carbon source.

<sup>2</sup>Final pH after 5 day culture at 30°C (The initial pH of the media was adjusted to 6.5.)

**Table 2** Effect of carbon source on the cell growth, production of exopolymers, and composition of WSNCE-B after 5 day culture of *Agrobacterium* sp. in MSM<sup>1</sup>

Carbon	pH	DCW <sup>2</sup> (mg/ml)	Exopolymers ( $\mu$ g/ml)			Molar ratio in WSNCE-B		
			Curdlan	WSNCE-A	WSNCE-B	Glucose	Mannose	Rhamose
Glucose	4.0	1.10	70	111	13	1.0	0.9	0.1
3-O-Methylglucose	6.1	0.87	14	15	16	1.0	1.0	0.1
N-Acetylglucosamine	7.4	4.69	158	455	78	1.0	1.1	0.3
2-Deoxyglucose	5.9	0.86	28	17	16	1.0	0.7	0.2

<sup>1</sup>Results obtained after 5 day culture in the mineral salt medium (MSM) with 2% carbon source.<sup>2</sup>Dry cell weight.**Table 3** Effect of initial medium pH on the cell growth, production of exopolymers, and composition of WSNCE-B after 5 day culture of *Agrobacterium* sp. in MSM

Initial pH	pH	DCW (mg/ml)	Exopolymers ( $\mu$ g/ml)			Molar ratio in WSNCE-B		
			Curdlan	WSNCE-A	WSNCE-B	Glucose	Mannose	Rhamose
5.6	4.0	0.66	21	69	28	1.0	0.9	0.2
6.0	4.0	1.09	41	58	22	1.0	1.0	0.1
6.4	4.1	1.43	69	65	13	1.0	1.0	0.1
6.8	4.1	1.79	50	94	14	1.0	1.3	0.0
7.2	4.1	2.23	41	159	21	1.0	1.2	0.0
7.6	4.1	2.63	20	143	35	1.0	1.1	0.0
8.0	4.2	2.62	17	121	28	1.0	1.0	0.0

**Table 4** Effect of yeast extract on the cell growth, production of exopolymers, and composition of WSNCE-B after 5 day culture of *Agrobacterium* sp. in MSM

Yeast extract (%, w/v)	pH <sup>1</sup>	DCW (mg/ml)	Exopolymers ( $\mu$ g/ml)			Molar ratio in WSNCE-B		
			Curdlan	WSNCE-A	WSNCE-B	Glucose	Mannose	Rhamose
0.00	4.1	1.96	60	107	21	1.0	1.2	0.0
0.25	4.0	4.12	108	251	133	1.0	2.8	0.0
0.50	5.2	5.16	140	473	329	1.0	3.5	0.0
1.00	6.9	5.07	102	365	159	1.0	4.8	0.0

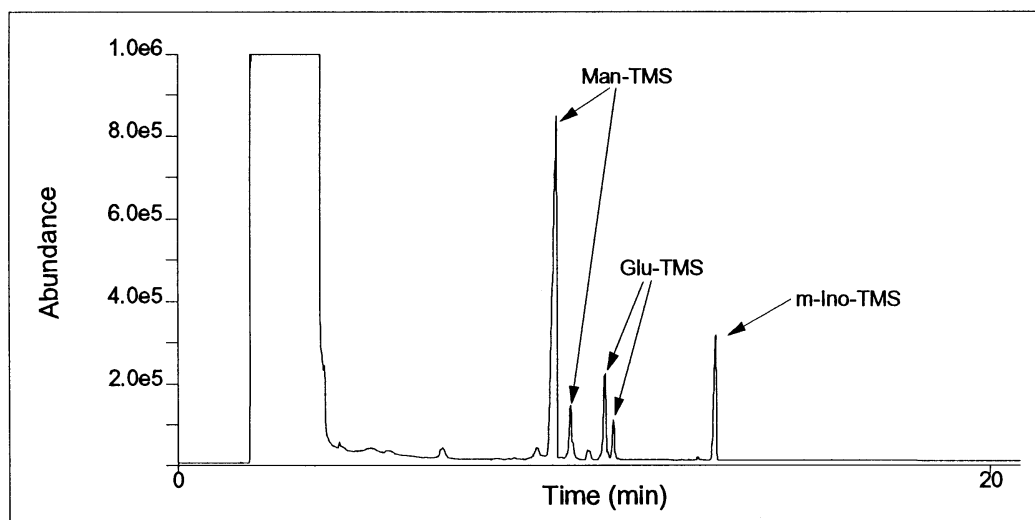
<sup>1</sup>Final pH after 5 day culture at 30°C (The initial pH of the media was adjusted to 6.8).

glucose to mannose in WSNCE-B purified from the culture grown in MSM without yeast extract was 1.0: 1.2, but this ratio increased regularly as the amount of yeast extract added to MSM was increased. A gas chromatogram of TMS-sugars derived from WSNCE-B purified from cultures grown in MSM with 1.0% yeast extract showed that the major components were glucose and mannose identified by the peak retention times and the relative peak area for the corresponding  $\alpha$  and  $\beta$  anomers (Fig. 1). Rhamnose was not detected for in any cultures where the initial pH of media was adjusted to 6.8 (Table 4). The detection limit of rhamnose was 0.3  $\mu$ g per 0.5 mg exopolymer samples analyzed by GC. The molar ratio of glucose to mannose in WSNCE-B with 1.0% yeast extract was 1.0: 4.8, a ratio very similar

to that of WSNCE-B purified from the culture grown in CM with glucose as the sole carbon source.

## Discussion

*Alcaligenes faecalis* var. *myxogenes* 10C3 simultaneously produces four different types of exopolysaccharides; curdlan, succinoglycan, octasaccharide repeating-unit of succinoglycan, and cyclic (1 $\rightarrow$ 2)- $\beta$ -D-glucan (Hisamatsu *et al.*, 1982). As was shown in our previous report (Lee *et al.*, 1997), three different types of exopolymers; curdlan, WSNCE-A, and WSNCE-B, were purified from cultures of *Agrobacterium* sp. ATCC 31749. The relative amount of each exopolymer and the monomeric composition of WSNCE-B varied with the carbon source and medium used (Tables 1 and 2). The ingredient(s) of CM



**Figure 1** Gas chromatogram of trimethylsilylated (TMS) sugar components of WSNCB from cultures grown in MSM with addition of yeast extract (1%, w/v) and glucose (2%, w/v), as carbon sources. The abbreviations used are as follows: glucose is Glu, Mannose is Man, and *m*-inositol is *m*-Ino.

may affect the cell growth and the production of exopolymers as well as the molar ratio of glucose to mannose in WSNCB. Microbial culture conditions, including carbon source, can affect the production of exopolysaccharides and lead to modification of the exopolysaccharide structure (Slodki, 1987). For example, the molar ratio of components and degree of O-acetylation in bacterial alginate produced by *Pseudomonas aeruginosa* changed according to carbon source (Marty *et al.*, 1992). The composition of xanthan gum and its viscosity was changed based on the concentration of mineral salts in media (Sutherland, 1990)

Rhamnose was not detected in WSNCB when the initial medium pH was 3–6.8. This included WSNCBs purified from cultures grown in MSM with addition of yeast extract for which initial medium pHs were adjusted to 6.8 (Fig. 1). The use of lower initial medium pH, rather than the addition of yeast extract to MSM, may lead to the metabolism and/or polymerization of rhamnose to form the WSNCB containing more rhamnose.

The relative amount of mannose in WSNCB increased regularly with increasing yeast extract added to MSM. This strategy gave a molar ratio of glucose to mannose up to 1.0: 4.8 (Table 4). Because yeast extract can be used as an alternative carbon source (Fava *et al.*, 1995) and contains growth promoting substances (Sharmila *et al.*, 1989), it normally has a strong positive effect on

bacterial growth rates (Armenante *et al.*, 1995; Shen *et al.*, 1993), enzyme activity (Kadowaki *et al.*, 1988), and productivity of metabolites (Aeschlimann and Stockar, 1990). Since vitamins as well as trace elements present in the yeast extract are involved in the activity of some enzymes responsible for substrate uptake and/or metabolism, some pathways may be regulated by the concentration of yeast extract in the medium (Fava *et al.*, 1995). Thus, it is reasonable that the addition of yeast extract to MSM found herein was useful as a method to increase exopolymer production and regulate the content of mannose in WSNCB (Table 4).

As a result of this study, an exopolymer which consists of only glucose and mannose can be produced by adjustment of the initial medium pH. The molar ratio of glucose to mannose in this exopolymer was controllable over the range from 1.0: 1.2 to 1.0 to 4.8, based on the amount of yeast extract added to the medium. Further work will focus on elucidating the relationship between composition and functional properties of these WSNCBs.

## References

- Aeschlimann, A. and von Stockar, U. 1990. *Appl. Microbiol. Biotechnol.* 32:398–402.
- Armenante, P.M, Fava, F. and Kafkewitz, D. 1995. *Biotechnol. Bioeng.* 47:227–233.
- Chaplin, M. 1982. *Anal. Biochem.* 123:336–341.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). *Anal. Chem.* 28, 350–56.

- Fava, F., Armenante, P.M., Kafkewitz, D., and Marchetti, L. 1995. *Appl. Microbiol. Biotechnol.* 43:171-177.
- Harada, T., Masada, M., Fujimori, K., and Maeda, I. 1966a. *Agr. Biol. Chem.* 30:196-198.
- Harada, T., Masada, M., Hidaka, H., and Takada, M. 1966b. *J. Ferment. Technol.* 44:20-24.
- Harada, T., Fujimori, K., Hirose, S., and Masada, M. 1966c. *Agri. Biol. Chem.* 30:764-769.
- Harada, T., Misaki, A., and Saito, H. 1968. *Arch. Biochem.* 124:292-298.
- Hisamatsu, M., Amemura, A., Matsuo, T., Matsuda, H., and Harada, T. 1982. *J. Gen. Microbiol.* 128:1873-1879.
- Kadowaki, S., Takegawa, K., Yamamoto, K., Kumagai, H., and Tochikura, T. 1988. *Agri. Biol. Chem.* 52:2105-2106.
- Lawford, H. G., Philips, K. R., and Lawford, G. R. 1982. *Biotechnol. Lett.* 4:689-694.
- 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.
- Phillips, K. R., Pik, J., Lawford, H. G., Lavers, B., Kligerman, A., and Lawford, G. R. 1983. *Can. J. Microbiol.* 29:1331-1338.
- Sharmila, M., Ramanand, K., and Sethunathan, N. 1989. *Can. J. Microbiol.* 35:1105-1110.
- Shen, C. F., Kosaric N., and Blaszczyk, R. 1993. *Appl. Microbiol. Biotechnol.* 39:132-137.
- Slodki, M. E. 1987. New bacterial polysaccharides. In *Industrial Polysaccharides*. Edited by Stivala S. S. *et al.* Gordon and Breach Science Pub., New York. pp. 3-13
- Sutherland, I. W. (Editor). 1990. Physiology and industrial production In *Biotechnology of Microbial Exopolysaccharides*. Cambridge Univ. Press, Cambridge. pp. 70-88.

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