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Morphological Variation of *Enterobacter* sp. BL-2 in Acetate-mediated pH Environment for Excretive Production of Cationic Microbial Polyglucosamine Biopolymer

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***Enterobacter* sp. BL-2 excretively produced a unique cationic polyglucosamine biopolymer PGB-1 comprised of more than 95% D-glucosamine in an acetate-mediated culture condition. The excretion of the biopolymer PGB-1 was closely associated with the cellular morphology of *Enterobacter* sp. BL-2, a feature highly dependable on the pH of the medium. The initially formed uneven and irregular surface cells were aggregated into the cell-biopolymer network structure connected by the adhesion modules of the cell-bound biopolymer. The excretive production of the biopolymer PGB-1 coincided with the disruption of the cell-biopolymer network, most actively at the medium pH of 8.0.**

Keywords: Acetate-mediated pH control, cationic polyglucosamine biopolymer, cellular morphology, *Enterobacter* sp. BL-2, excretive production

Most microbial polysaccharides are produced in an anionic or neutral form; however, a unique cationic-type bioflocculant from *Citrobacter* sp. TKF04 for wastewater treatment has been reported [2, 4]. Moreover, two novel cationic polyglucosamine biopolymers, PGB-1 and 2, composed of more than 95% glucosamine monomer units, showing similar FT-IR and NMR spectra to chitosan from crab shells, have been produced from *Enterobacter* sp. BL-2 [7] and *Citrobacter* sp. BL-4 [5], respectively.

The above unique cationic polyglucosamine biopolymers were excretively produced by a pH-stat fed-batch cultivation using acetic acid as the feeding stock, indicating that the pH control is the most critical factor for excretive overproduction in an acetate-mediated culture condition. Gram-negative enteric bacteria are known to form polysaccharide molecules at the cellular surface to protect them from an organic acid environment [1]. However, the cellular physiology regulating the excretion of adhesive biopolymers in order to protect them from an acetate-mediated pH-environment has not been elucidated yet; in particular, the excretion of the positively charged cationic polyglucosamine biopolymer PGB-1 from the negatively charged bacterial surface due to the phosphoryl and carboxylate substituents in the outer cell envelope [6, 8] remains unclear.

In this work, *Enterobacter* sp. BL-2 was cultivated in different acetate-mediated pH environments, and the effect of the pH levels on the excretion of the cationic polyglucosamine biopolymer PGB-1 was elucidated, along with the highly pH-dependable morphological variation of *Enterobacter* sp. BL-2 cells.

Enterobacter sp. BL-2 reported in our previous work [7] was cultivated in a basal medium (pH 7.0) composed of 0.1% $(NH_4)_2SO_4$, 0.01% yeast extract, 2% $MgSO_4 \cdot 7H_2O$, 2% $CaCl_2$, 0.5% NaCl, and 1.5% sodium acetate at 30°C for 48 h. For the pH-stat fed-batch cultivation, the cell was cultivated in a 5-l jar fermenter batch-wisely until the medium pH changed to 7.0, 8.0, and 9.0, respectively, and then a 2 M acetic acid solution was fed intermittently to maintain the constant pH levels of 7.0, 8.0, and 9.0 for 72 h.

The excreted polyglucosamine biopolymer PGB-1 from *Enterobacter* sp. BL-2 was measured as follows. It was precipitated in three volumes of absolute ethanol, and then deproteinized by 2 M NaOH before lyophilization. The lyophilized PGB-1 was hydrolyzed in 6 M HCl, and then the amount of glucosamine was measured using the modified Elson-Morgan method [3]. The cell-bound PGB-1 was extracted in a 0.1 M Tris-HCl buffer (pH 8.0) at 25°C for 12 h, and then the amount of glucosamine was measured similarly.

The morphological features of the cell and cell-polymer network were observed using a field emission scanning electron microscope (S-4200, Hitachi). The solubility at the different pHs was measured in a 0.1 M sodium citrate-

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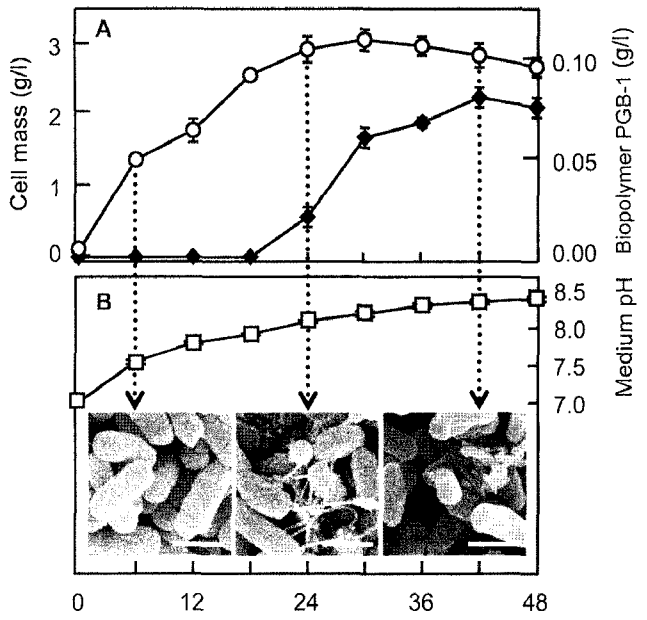

Fig. 1. Profiles of batch cultivation of *Enterobacter* sp. BL-2 in basal medium containing sodium acetate.
Cell ( ), polyglucosamine biopolymer PGB-1 (◆), medium pH ( ), and cellular morphology. The strain was cultivated in a basal medium composed of 0.1% $(NH_4)_2SO_4$, 0.01% yeast extract, 2% $MgSO_4 \cdot 7H_2O$, 2% $CaCl_2$, 0.5% NaCl, and 1.5% sodium acetate as the carbon source in a shake flask at 30°C, 150 rpm, for 48 h. Scale bar = 1 μm; magnification: ×20,000.

citric acid buffer for pH 5.0 and 6.0, a 0.1 M Tris-HCl buffer for pH 7.0 and 8.0, and a 0.1 M sodium bicarbonate-NaOH buffer for pH 9.0 and 10.0, respectively. The dissolved PGB-1 in each buffer was precipitated in three volumes of absolute ethanol, and then weighed after drying.

Fig. 1 illustrates the profiles of the cell growth, polyglucosamine biopolymer PGB-1 concentrations, medium pH, and cellular morphology of *Enterobacter* sp. BL-2 during the batch cultivation in a basal medium supplemented with sodium acetate as the sole carbon source. As shown in Fig. 1A, the cell growth occurred until 24 h, while the excretive production of the biopolymer PGB-1 was initiated from 18 h at the stage where the cell growth was nearly completed, indicating a typical mixed growth-associated product formulation pattern.

As shown in Fig. 1B, the medium pH increased gradually from the initial pH 7.0 to the final pH 8.5. The cellular morphology of *Enterobacter* sp. BL-2 also changed noticeably from the initial smooth surface to uneven and irregular surface cells at an early 6 h cultivation period, indicating the morphological adaptation of cells to an acetate-mediated culture condition. The cell-polymer network was connected by the adhesion modules of the cell-bound biopolymer thereafter, and then the cell-polymer network structure was disintegrated to release the cell-bound biopolymer into the culture broth at the late active excretion phase, indicating a close relationship with the morphological

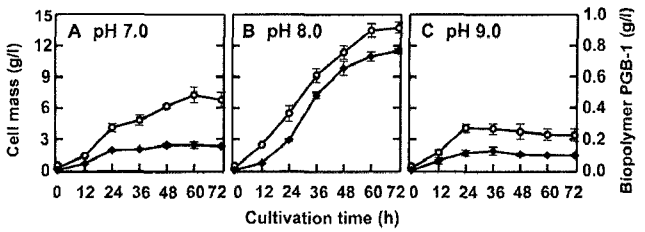

Fig. 2. pH-stat fed-batch cultivation of *Enterobacter* sp. BL-2 at different pH levels.
Cell ( ) and polyglucosamine biopolymer PGB-1 (◆). The strain was cultivated in the same basal medium in a 5-l jar fermenter at 500 rpm and 0.3 vvm batch-wisely, and then 2 M acetic acid was fed intermittently to maintain a constant pH level of 7.0, 8.0, and 9.0, respectively.

variation of *Enterobacter* sp. BL-2 cells possibly induced by the pH levels. When the *Enterobacter* sp. BL-2 was cultured in a medium containing glucose as a carbon source or LB medium, neither a significant morphological variation nor the cell-polymer network structure was observed (data not shown). The morphological variations were uniquely observed only in an acetate-mediated culture condition, especially related to pH levels of the acetate medium.

To compare the effect of an acetate-mediated pH level on the PGB-1 production, the pH-stat fed-batch cultivations constantly maintaining the medium pH at 7.0, 8.0, and 9.0 were carried out as shown in Fig. 2. The highest cell growth and production of biopolymer PGB-1 were observed at pH 8.0. At pH 7.0, the cell grew relatively well; however, the active excretion of the biopolymer PGB-1 did not occur. The cell growth was inhibited at an alkaline pH of 9.0, and consequently, there was only a limited production of the biopolymer PGB-1.

Fig. 3 compares the morphological features of *Enterobacter* sp. BL-2 cells and the distribution of the cell-bound and

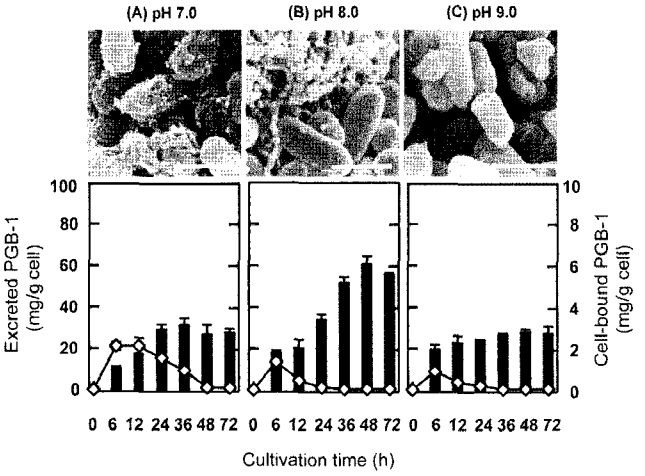

Fig. 3. Morphological feature of *Enterobacter* sp. BL-2 cells observed after 24 h and distribution of cell-bound ( ) and excreted (■) PGB-1 during pH-stat fed-batch cultivation.
Scale bar=1 μm; magnification: ×20,000.

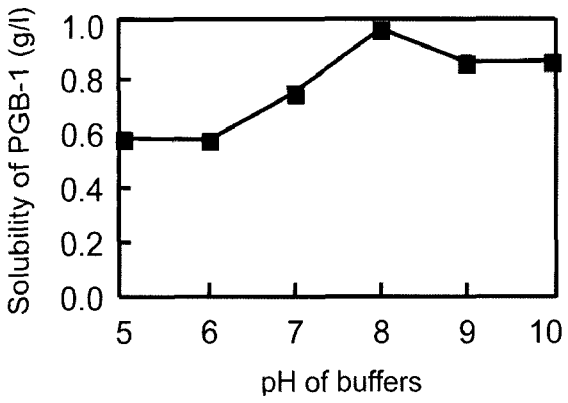

Fig. 4. Effect of pH levels on the solubility of cationic biopolymer PGB-1.

excreted forms of the biopolymer PGB-1 during the pH-stat fed-batch cultivations at different pH levels. The biopolymer PGB-1 was mostly produced as an excreted form, whereas only a small portion was the bound form, except for the initial stage of the pH-stat fed-batch cultivations, especially at pH 7.0.

The morphological variations of *Enterobacter* sp. BL-2 cells at the different pH levels were also compared in Fig. 3. Fiber-like polymer meshes released from the cells were observed at an optimal pH of 8.0 (Fig. 3B). The aggregated cell-polymer network connected by the adhesion modules of the cell-bound biopolymer was formulated at the neutral pH of 7.0 (Fig. 3A), and even the disrupted and inactive form cells at an alkaline pH of 9.0 (Fig. 3C).

The solubility of the biopolymer PGB-1 at the different pH buffers was illustrated, as shown in Fig. 4, and the highest solubility was obtained at pH 8.0. The high solubility can facilitate the release of the biopolymer PGB-1 from the cell-polymer network the most readily. However, the genomic regulations in *Enterobacter* sp. BL-2 controlling the excretion of this unique cationic polyglucosamine biopolymer PGB-1 from the negatively charged cells under the acetate-mediated culture condition needs to be further elucidated.

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