New Guidelines For Selecting Best Extraction Methods of EPS Using Atomic Force Microscopy

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ABSTRACT

A new guideline with atomic force microscopy (AFM) to select the effective extraction method for extracellular polymeric substances (EPS), was proposed and validated by 'classical' biochemical ways. The classical biochemical guidelines showed that efficiencies of four extraction methods were comparable regardless of different microbial species and the extraction using NaOH or Formaldehyde-NaOH was the best. The efficiencies of extraction were further investigated using AFM by analyzing interaction forces between bio-probe, which a single cell of *S. cerevisiae* was attached at the end of the cantilever, and flat surface. Interaction forces fitted with steric model showed the thickness of residual polymer layer had good agreement with biochemical results. The comparison of results from two guidelines explained the basic mechanisms of extraction methods.

Keywords : extracellular polymeric substances, atomic force microscopy, extraction, steric model, cell lysis

INTRODUCTION

Extracellular polymeric substances (EPS) are metabolic products accumulated on the bacterial cell surface [1]. It has been believed that EPS plays key roles during the floc formation, the adhesion and the maturation of biofilm, and the self-protection of microorganisms [1-7]. There are several physico-chemical methods for extraction of EPS, but they have exhibited wide variation as a function of extraction methods and conditions [8-10]. Also, the evaluation guidelines to select the best extraction method have been limited to indirect bio-chemical assay such as the comparisons of carbohydrate, protein, uronic acid, and DNA contents. However, indirect characterization methods cannot exclude the potential contamination of extracted solution by extracting reagents [8]. In this study, we established a new and potential guideline for the direct evaluation for the effectiveness of EPS extraction methods using atomic force microscopy.

MATERIALS AND METHODS

Four extraction methods (Heat, EDTA, NaOH, formaldehyde with NaOH) were compared using 2 g VSS/L of four microbial communities (*S. cerevisiae*, *P. aeruginosa*, activated sludge from biological denitrification reactor (BNR), activated sludge from DeN process (DeN)). Extractions were performed according to Liu and Fang [9] and Shin *et al*.[11]. The total amount of extracted EPS was measured by the sum of protein [12] and polysaccharides contents [13]. The DNA in EPS solution was measured by the spectro-photometric method with DNA of *E. coli* as a standard [14].

A single cell of wild and EPS-extracted *S. cerevisiae* was attached at the end of the cantilever [15], and interaction forces were measured using atomic force microscopy (AFM). The approach curves were analyzed by the steric force model as equation 1 [16].

$$F_{St} = 50 k_B T r L \Gamma^{3/2} e^{-2\pi h / L} \qquad (1)$$

where F_{st} is steric interaction force, k_B is Boltzmann constant, T is temperature (^{o}K), r is the radius of S. *cerevisiae*, L is the equilibrium height of the polymer layer,

Γ is the grafted polymer density (m^{-2}) and h is the distance between the two surfaces.

RESULTS AND DISCUSSIONS

Results showed that each extraction methods gave similar trends regardless of bacterial communities. The extraction by NaOH gave the largest amount of EPS from all bacterial communities, and followed by formaldehyde+NaOH, heat, and EDTA (Figure 1).

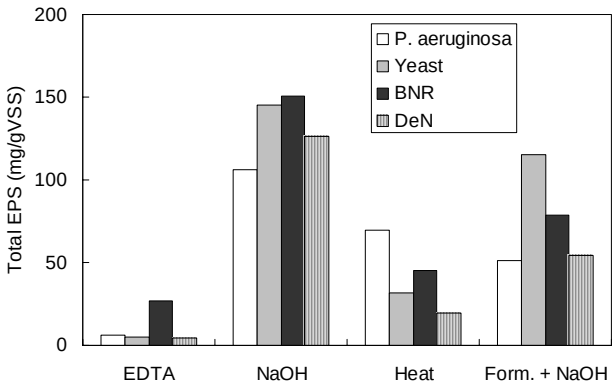

Figure 1: The amount of extracted EPS by various methods from various bacterial communities

The presence of NaOH resulted the dissociation of acidic groups in EPS with the stronger repulsion between the negative-charged extracted EPS, which led to the increase of solubility in water. Formaldehyde with NaOH showed minimal DNA concentration with large amount of EPS in extracted solutions (Figure 2).

Formaldehyde could fix the cell by reacting with amino, hydroxyl, carboxyl and sulfo-hydryl groups of proteins and nucleic acids of cell membrane, and thus prevent the excessive cell lysis by NaOH [8]. The extraction by EDTA showed low amount of EPS and high DNA concentration because of the removal of cations by EDTA from the cell membrane, causing cell lysis and the release of intracellular DNA.

Figure 3 showed the representative attraction curves of AFM. The thickness of EPS layers for all extraction methods was smaller than that of the untreated (control) *S. cerevisiae*, which clearly showed the effectiveness of extraction directly. The calculation of thickness based on the steric model showed that extraction by EDTA and formaldehyde with NaOH were effective to remove the EPS layers (Table 1).

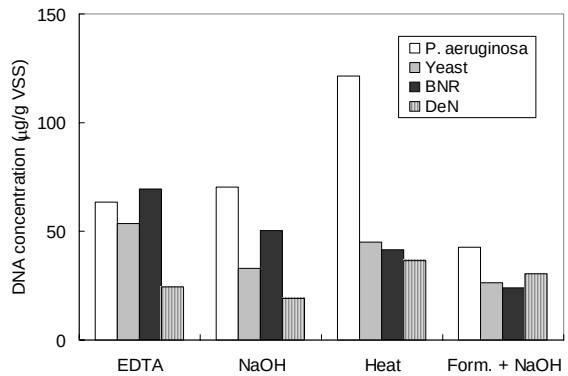

Figure 2: The concentration of DNA in the solutions extracted by various methods

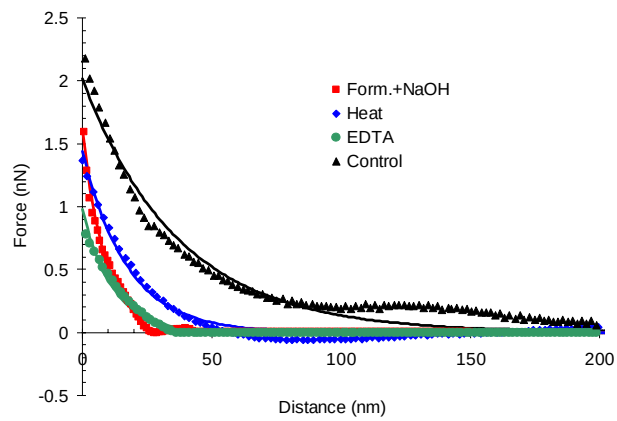

Figure 3: Representative approching curves of *S. cerevisiae* cell-prove treated by various extraction methods

METHODS	CONTROL	EDTA	HEAT	FORM+NaOH
Γ ($X10^{15}$)	0.67±0.14	0.9±0.21	0.9±0.17	1.5±0.23
L (nm)	233±47	73±29	108±24	55±16

Table 1. Summary of attracting curve analysis using steric model.

The bio-probe extracted by formaldehyde with NaOH showed minimal polymer thickness due to the effective extraction of EPS and the crosslink of the protein of cell membranes [17].

Considering above results, formaldehyde with NaOH was the best method to extract EPS. Also, atomic force microscopy could be applied to select, or to investigate the effectiveness of extraction methods in direct way.

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