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Changes in Cell Size and Buoyant Density of *Pseudomonas diminuta* in Response to Osmotic Shocks

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Abstract *Pseudomonas diminuta* (ATCC 19146) has been typically used in the bacterial challenge test for validation of the sterilizing filtration process. Cell size is critical for determining the retention characteristics of membrane filters with pore-size of 0.2 μm. The changes of cell sizes after osmotic shocks at 150, 260, 500, and 700 mosM were measured by a particle size analyzer and the changes of their buoyant densities were analyzed with a Percoll gradient. The results indicated that there were no significant differences when cells were cultured in 260 mosM medium and osmotically shocked at 500 and 700 mosM. However, the osmotically shocked cells at 150 mosM showed a 38% increase of the cell size compared to the cells at 260 mosM. From these study, we concluded that the worst case condition for validation of a sterilizing filter would be 500 mosM, not because of changes in the cell size, but due to decrease in cell viability under those conditions.

Key words: *Pseudomonas diminuta*, filter validation, osmolarity, buoyant density

The safety requirements of drugs directly affect human life, and the requirements become more strict each year. Therefore, more accurate validation is required on sterile drug products produced by aseptic processing. Regulatory guidelines for the aseptic manufacture of pharmaceuticals recommend the validation of sterilizing filters by a bacterial challenge test under "worst case" conditions [5, 9]. The use of *P. diminuta* (ATCC 19146) provides a realistically suitable case to challenge the sterilizing filters.

The validation of sterilizing filtration processes requires the use of bacterial suspensions in the drug product. The physical and chemical attributes, such as pH, vicosity, osmolarity, ionic strength, and surface-active agents, could interfere with the effectiveness of membrane filters which retain particles by adsorption [7, 8].

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Among these factors, changes of osmolarity have an effect on the size and viability of microorganisms used in the bacterial challenge test. Baldwin *et al.* [2, 3] reported that *E. coli* cultured in 150 mosM NaCl medium and osmotically shocked at 300 or 500 mosM showed a 15 or 21% decrease of the cell size, respectively. It was assumed on the basis of the experimental data that high osmolarity would constitute a worst-case condition.

Contrary to our expectation, in our study with *P. diminuta*, we could not show the shrinkage of the cell size after osmotic shocks. Buoyant densities of cells separated by size showed also similar results. *P. diminuta* (ATCC 19146), a standard test organism for validation of 0.2 μm-rated membrane filters, was used in this study. Cells were prepared to challenge according to the standard procedures [1, 6]. Lyophilized cells were incubated in 5 ml of tryptic soy broth (TSB) (Difco, MD, U.S.A.) at 30±2°C for 24 h. Seventy-five microliters of the culture were transferred to tryptic soy agar (TSA) slants. The TSA slants were incubated at 30±2°C for 48 h and then stored at 4°C. At this point, the purity of the cultures was verified by Gram staining and the cultures were checked for uniform colonial morphology. A culture tube containing 10 ml of TSB was aseptically inoculated with a loop full of cells from the slant and incubated at 30±2°C for 24 h. Seed culture (2 ml) was inoculated to 50 ml of saline lactose broth (SLB) and incubated at 30±2°C for 24 h. The osmolarity of SLB media, which contained 97% of NaCl (0.76 g) and 3% of lactose broth (0.13 g/10 ml, Difco, MD, U.S.A.), was about 260 mosM. Cultured cells in SLB were centrifuged at 10,000 rpm for 10 min at 4°C, and then the cells were exposed to different osmolarities by adjusting with NaCl (150, 500, and 700 mosM). The osmotic shock reaction was performed for 1 h on ice.

After the reaction, cell sizes were measured using a particle size analyzer (Coulter, U.S.A.). This method was rapid and easy to size the cells compared to the scanning electron microscopy. As shown in Fig. 1(a), when cells were cultured in 260 mosM medium and then osmotically

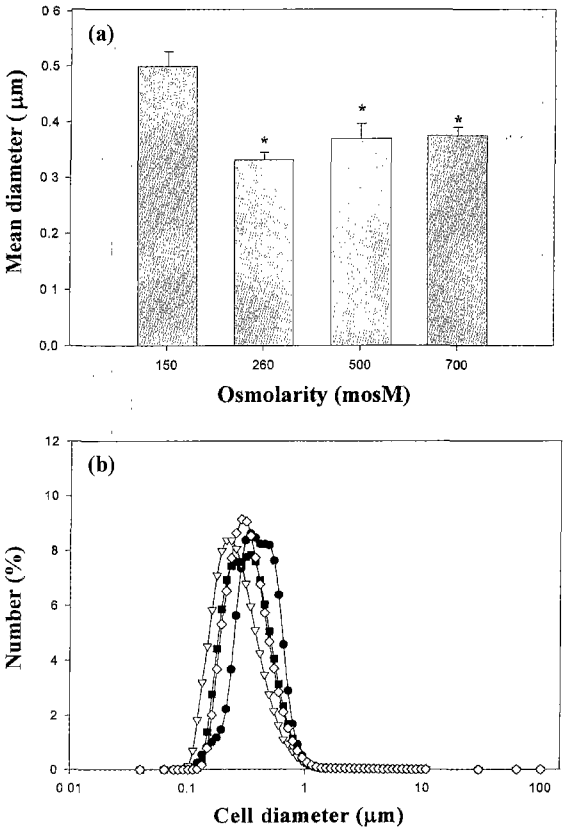

Fig. 1. Changes in cell size of *P. diminuta* in response to osmotic shocks measured by a particle size analyzer.
(a) Comparision of cell size after exposure to various osmolarities. Results are expressed as the mean±SD. *P<0.001 (n=7). (b) Size distribution of cell diameter. Symbols indicate -●-, 150 mosM; -▽-, 260 mosM; -■-, 500 mosM; and -◇-, 700 mosM.

shocked at 500 and 700 mosM, there was no change in the cell size. When cells were grown in the same medium and then osmotically shocked to 150 mosM, cells showed an increase in their size, 38% larger than that in 260 mosM. Also, when the distribution of cell size at 150 mosM was compared to the other osmotically, the mean cell diameter was noticeably larger in size (Fig. 1b). When microorganism grown in TSB was transferred to the SLB media, which contained limited sources of carbon and nitrogen, we also observed the shrinkage of the cells by the particle size analyzer. It was expected on the basis of the above results that low assimilable carbon levels would offer a worst-case condition by a possibility to reduce the cell size. In a viability test for the microorganism, those cells which were shocked at 500 or 700 mosM showed decrease of 15.6% or 26.6% in 260 mosM, respectively.

Percoll (Amersham Pharmacia Biotech, Sweden) was used to make density gradients. To make density gradients, concentrated NaCl (1.5 M) was added to the Percoll solutions to adjust isoosmolarity (260 mosM) or nonisoosmolarity (150, 500, 700 mosM) to the growth medium. Osmolarity

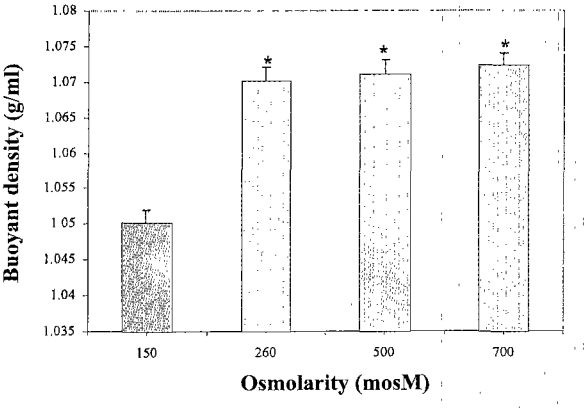

Fig. 2. Buoyant density of *P. diminuta* exposed to an osmotic shock.
Buoyant density of the cell measured using a Percoll gradient. Results are expressed as the mean±SD. *P<0.001 (n=3).

was confirmed with an osmometer (Gonotec, Germany). In order to optimize discontinuous gradient centrifugation, various combinations of gradients were tested and evaluated. Among the tested, the cells separated in gradients formed by 1.06, 1.08, and 1.10 g/ml of Percoll. The Percoll gradient separation was modified as described previously [4].

To determine the buoyant density of the cells, 200 μl of cells after the osmotic shock reaction were layered onto a preformed Percoll gradient. Next, density marker beads (Amersham Pharmacia Biotech, Swedden) were carefully layered on top of the gradient. The tube was then centrifuged at 10,000 rpm for 10 min at 4°C. After the centrifugation, the distance from the bottom of the Percoll gradient tube to the cell band and the marker band was measured with the calipers. By using linear interpolation, the buoyant density was determined. As cells shocked at the lower osmolarity, the buoyant density decreased from about 1.07 g/ml at 260 mosM to about 1.05 g/ml (Fig. 2). Under the higher osmolarity, however, there was no change in the buoyant density.

In conclusion, the present study suggested that the worst-case condition for a bacterial challenge test could be 500 mosM. Under this condition, microorganism viability would decrease rather than showing any change in the cell size. Therefore, organisms to be challenged will not be smaller than the size of the membrane pores during the course of the filtration process. Based on the present study, the bacterial challenge test system could be developed into a laboratory-scale equipment and directly carried out with *P. diminuta* under different osmotic conditions.

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