Spheroplast Formatation and Regeneration of *Zymomonas mobilis*

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*Zymomonas mobilis*의 원형질체 형성과 재생

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Abstract: The aims of the present studies were to develop conditions for the spheroplast formation of *Zymomonas mobilis* and regeneration of the spheroplasts to normal cells in synthetic media. *Z. mobilis* cells harvested from exponential growth phase were treated with lysozyme, mutanolysin, and glycine in various conditions. It was found that spheroplasts were formed only with the treatment of glycine but not with the enzymes treatments. It was therefore considered that the tetrapeptide strand of peptide strand of peptidoglycan might play more important roles than the glycan strand in maintaining the vital mechanical function of *Z. mobilis*. It was found also that removal of outher membrane was the major problem in protoplast formation of *Z. mobilis*. As results, It was observed that over 85% of cells were readly converted to spheroplasts with sole glycine treatment for 4 hr and 7-10% of the spheroplasts were regenerated to normal cells in synthetic media.
Key words: *Zymomonas mobilis*, spheroplast, lysozyme, mutanolysin, glycine.

The bacterium *Zymomonas mobilis* is the gram-negative bacteria fermenting ethanol by the Entner-Doudoroff Pathway (Swing *et al.*, 1977). It has economic potential for industrial ethanol production (Rogers *et al.*, 1979 ; Lee *et al.*, 1979) but it has one disadvantage for the industrial application, which can utilize only glucose, fructose, and sucrose. In this sense to extend the substrate range is considered to be next research objectives (Goodman *et al.*, 1982 ; Skonicki *et al.*, 1980 ; Cary *et al.*, 1983 ; Walia *et al.*, 1984 ; Browne *et al.*, 1984, Lee *et al.*, 1984).

Since the protoplast fusion techniques were established in plant cells (Kao *et al.*, 1974), it has been proved also to be a potential technique for strain improvement in fungi and bacteria (Ferenczy *et al.*, 1975 ; Fodor *et al.*, 1976 ; Shaeffer *et al.*, 1976 ; Hopwood *et al.*, 1977). Bacterial fusion techniques have been used more successfully in gram-positive bacteria than gram-negative bacteria and very few reports were published on the protoplast formation using the gram-negative bacteria. The reason was considered that effective methods for the removal of the outer membrane of the gram-negative bacteria have not been well developed, consequently, spheroplasts having part of outer membrane attached to cytoplasmic membrane were formed instead of proto-

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plast.

The current study was objected to develop optimal conditions for the spheroplast formation of *Z. mobilis* and attempted to fuse the spheroplasts in synthetic media.

MATERIALS AND METHODS

Strains and media

Zymomonas mobilis ZM4 (CP4 de Lima *et al.*, 1970, now designated ATCC 31821, Lee *et al.*, 1980) was used in this studies. Maintenance of the strain and culture procedures have described in earlier publications (Rogers *et al.*, 1979 ; Lee *et al.*, 1979).

Spheroplast formation and regeneration

Spheroplast formation and regeneration in rich media was followed the previous reports (Lee *et al.*, 1984). Synthetic medium used for the regeneration of spheroplasts was formulated as reported by Goodman *et al.* (1982). The efficiency of the spheroplast formation was compared with the methods reported by Osborn *et al.*, (1972), Weiss (1976), Matsushita *et al.* (1981), Laddaga *et al.* (1982), Coetzee *et al.* (1979).

RESULTS AND DISCUSSION

Spheroplast formation

Various methods, which have been used in the formation of protoplast or spheroplast of the gram-negative bacteria, were applied to *Z. mobilis*. As shown in Table 1, it was not possible to obtain any spherical form of cells with the methods except the method reported by Coetzee where glycine was used. It was interest to note that the cell wall lytic enzyme, lysozyme, was not very effective in the formation of protoplast.

It was considered that the lysozyme is too large (M.W. 15,000) to penetrate the outer membrane of *Z. mobilis*. It was shown that only small (M.W. 650) and water soluble molecules were allowed to penetrate the outer membrane of the gram-negative bacteria (Nikaido *et al.*, 1979). Since glycine treatment which was consided to inhibit the formation of the tetrapeptide strand was essential in the formation of spheroplasts, it was considered also that the tetrapeptide strand of the peptidoglycan layer might play more important roles than glycan strand in maintaining the vital mechanical function of *Z. mobilis*.

Table 1. *Comparisons of various methods for the formation of Spheroplast of Zymomonas mobilis.*

Schemes	Methods suggested by	Spheroplast formation
Tris-acetate buffer(pH7.5) lysozyme-EDTA	Osborn *et al.* (1972)	Not formed
Tris buffer (pH 8.0) lysozyme-EDTA	Weiss, R. L. (1976)	〃
Tris buffer(pH8.0) with 0.5M NaCl, lysozyme-EDTA	Matsushita *et al.* (1981)	〃
Tris buffer (pH8.0, 0.5M NaCl-0.5M sucrose lysozyme	Laddaga *et al.* (1982)	〃
Tris buffer (pH 8.0) lysozyme-EDTA-glycine	Coetzee *et al.* (1979)	99%

In order to clarify the considerations, it was attempted to alter the permeability of the outer membrane by treatment of cells in various conditions. It was believed that the ineffectiveness of most macrolide antibiotics against gram-negtive cells is mainly due to their inability to penetrate the bacterial outer membrane (Omura *et al.*, 1977). In this sense, a macrolide antibiotic, tylosin, was used as an indicator of changes in the permeability by the determination of susceptibility. The susceptibility of *Z. mobilis* to tylosin was evaluated by comparing viability of cells before and after tylosin treatment. As shown in Figure 1, normal cells were not affected by 100mg, l^{-1} of tylosine, thus, 100mg, 1^{-1} of tylosin was applied to the cells in order to know the alteration of outer membrane permeability.

Effects of pH on cell viability

Cell numbers counted from the media containing tylosin and from without tylosin were compared after washing the normal cells

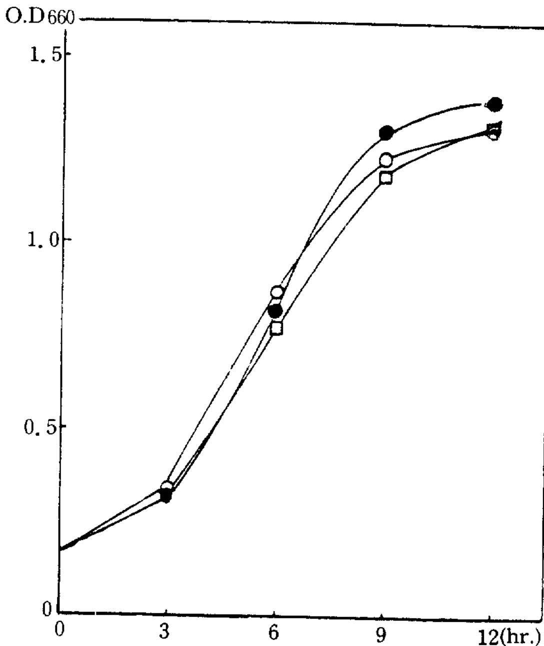

Fig. 1. *Effect of tylosin concentration on the growth of Z. mobilis.*
○—○ contiol, ●—● 50mg ml^{-1}
□—□ 100mg ml^{-1}.

with various buffers in different pH. Table 2 shows that cell numbers were varied according to the treating conditions. However very constant cell numbers were obtained from the culture treated with the buffer of citrate-phosphate pH5, phosphate pH6 and 7. It indicated that the changes of permeability was not significant. However it was apparent that much less cell numbers were counted from the cultures treated with the buffers of citrate pH4 and tris pH8 and 9. Further decrease in cell numbers were observed also with the treatment of tylosin compared to those of without tylosin treatment. The results suggested that the effects of tylosin was resulted from the alteration of outer membrane permeability.

Table 2. *Effects of pH and buffer on viable cell numbers*

pH and buffer (0.1M)	Cells, ml^{-1}	
	without tylosin	with tylosin
Citrate-phosphate pH 4	3.2×10^{8}	3.8×10^{8}
Citrate-phosphate pH 5	1.5×10^{9}	1.5×10^{9}
Phosphate pH 6	1.5×10^{9}	1.5×10^{9}
Phosphate pH 7	1.5×10^{9}	1.5×10^{9}
Tris pH 8	8.2×10^{8}	4.9×10^{8}
Tris pH 9	2.3×10^{8}	1.6×10^{8}

Table 3. *Effects of Ca^{++}, Mg^{++} and EDTA treatment on cell viability*

Pretreatment	Cells, ml^{-1}	
	without tylosin	with tylosin
Mg^{++} (0.125 M)	5.9×10^{7}	3.2×10^{7}
Ca^{++} (0.125 M)	17.0×10^{7}	4.6×10^{7}
EDTA (0.125 M)	7.9×10^{7}	2.8×10^{7}

It has been reported that tris-buffer has cations and can alter the permeability of the outer membrane of gram-negative bacteria (Irvin *et al.*, 1981; Hosmin *et al.*, 1983). It was believed also that the lower pH of tris-buffer exerted more cations, which could be more easily interacted with the outer membrane, therefore, increased in the permeability for the large molecules could be expected.

The optimum pH for the growth of *Z. mobilis* was about pH5 (Lee *et al.*, 1979), hence, it was decided that the optimum pH for the protoplasting was 5-6 and tris-maleate was considered to be suitable for the increase of the outer membrane permeability.

Effect of Ca^{++}, Mg^{++}, and EDTA on outer membrane permeability

Cells of *Z. mobilis* harvested from exponential growth phase were treated with high concentration (0.125 M) of Ca^{++} and Mg^{++} for 30 min and then plated on rich media and also on the rich media containing tylosin (100mg, l^{-1}). As can be seen in Table 3, it was evident that viable cells on the media containing tylosin were lower than those of obtaining from the media without tylosin. It was considered that the loss of viability at the divalent treatment might be resulted from the alteration of the outer membrane, which permitting the tylosin penetration. Schindler *et*

Table 4. *Comparisons of viable cells regenerated from the spheroplasts prepared with glycine and EDTA-lysozyme.*

Spheroplasting condition	Cells, ml^{-1}	
	without tylosin	with tylosin
Glycine	3.4×10^5	2.9×10^5
EDTA-Lysozyme Glycine	7.9×10^4	2.8×10^4

al. (1979) reported that the outer membrane of gram-negative bacteria had two binding sites of Mg^{++} and Ca^{++} and that the divalent ions were very important to make membrane structure and also in permeability. It was considered also that the ratio of the divalent ions could be changed by treatment with high concentration of each ion(Lugtenberg *et al.*, 1983). It revealed that Ca^{++} treatment was more effective than Mg^{++} in the alteration of permeability. The results suggested that the replacement of Mg^{++} with Ca^{++} resulted in the alteration of the outer membrane to increase the permeability of tylosin in *Z. mobilis*.

The permeability changes of the outer membrane were made also by treatment with EDTA as given in Table 3. The results indicated that interference of ionic balance on outer membrane could alter the permeability. It was confirmed that spheroplasts formed with sole glycine treatment were not susceptible to

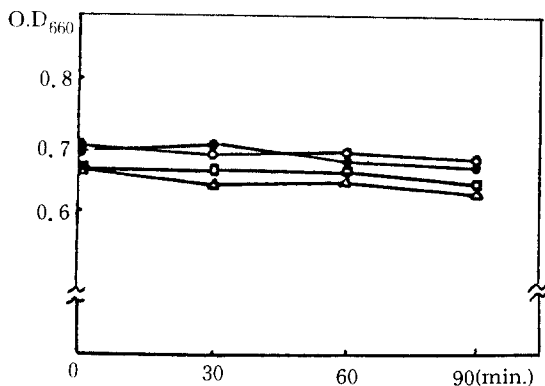

Fig.2. *Effect of mutanolysin on the lysis of cell wall*

○—○ 0mg ml^{-1}, ●—● 25mg ml^{-1}
□—□ 50mg ml^{-1}, △—△ 100mg ml^{-1}

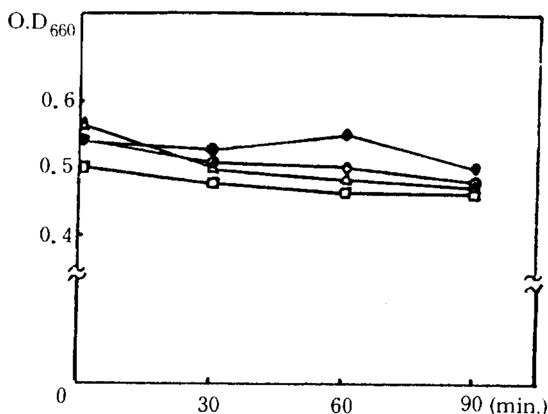

Fig.3. *Effect of lysozyme on the lysis of cell wall*

○—○ 0mg ml^{-1}, ●—● 100mg ml^{-1}
□—□ 500mg ml^{-1}, △—△ 1,000mg ml^{-1}

tylosin, while the spheroplasts formed with combined treatment of glycine and EDTA were more susceptible as shown in Table 4.

Effects of lysozyme and mutanolysin on spheroplast formation

The previous reports showed that lysozyme treatment at the given conditions was not effective in the formation of spheroplast. At the present studies, it was attempted to evaluate the effects of the pretreatment on the formation of spheroplast with the cell wall lytic enzymes, lysozyme and mutanolysin. It was assumed that the removal of the cell wall of gram-negative was also an important factor in protoplast formation, although its thickness is not comparable to the gram-positive bacteria. Osmotically fragile cells were considered to be protoplast or spheroplast, consequently, the optical density of cells reacted with the enzymes without any osmotic stabilizers were measured at 660 nm. As given to Figure 2, 3 and 4, it was clear that the enzymes were still not effective to form the spheroplasts.

It is our utmost interests why these enzymes were not useful in the shperoplast formation. Since the enzymes can hydrolyze glycan strand (β-1, 4-glycosidic linkage), it was thought that cell wall structure of *Z. mobilis* might be different from that of gram-posive bacteria. In other words, the cell wall may contain different residues resulted in that it was no more sub-

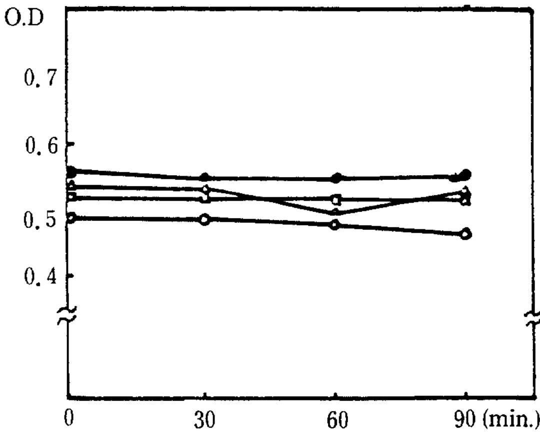

Fig.4. *Effect of the combined treatment of mutanolysin and lysozyme on the lysis of cell wall.*

○—○ mutanolysin 50mg ml^{-1}, lysozyme 100mgml^{-1}, ●—● mutanolysin 50mg ml^{-1}, lysozyme 250mg ml^{-1}, □—□ mutanolysin 50mgml^{-1}, lysozyme 500mg ml^{-1}, ◇—◇ mutanolysin. 50mg ml^{-1}, lysozyme 1,000 mg ml^{-1}.

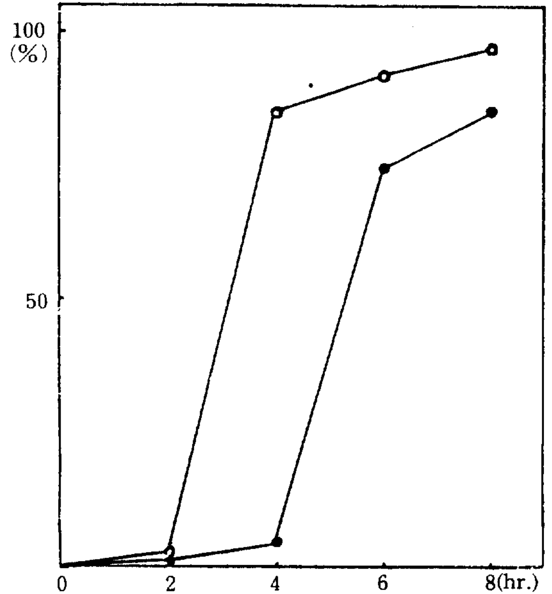

Fig. 5. *Effect of spheroplasting conditions on the spheroplast formation.*

○—○ Lysozyme-EDTA-Glycine
●—● Glycine only.

strate to the enzymes. It has been reported that the cell wall carring O-acetyl, free amino group, N-nonsubstituted glucosamine residues was not easily hydrolyzed with the treatment of lysozyme (Brunfitt *et al.*, 1958 ; Krause *et al.*, 1961 ; Perkins *et al.*, 1965 ; Stromingar *et al.*, 1967 ; Amano *et al.*, 1983 ; Araki *et al.*, 1972). The profiles of the fatty acids of the *Z. mobilis* were compared with different strains and showed that *Z. mobilis* has very unusual lipo-polysaccharides (Barrow *et al.* 1983).

Spheroplast formation efficiencies were compared with the sole glycine treatment and with the EDTA-Ca-lysozyme-glycine treatment and the data were shown in Figure 5. It was evident that the sequencial treatment was much more efficient in spheroplasting. Over 80-90% cells were very readly converted to spherical form and it was found that less than 1% of cells were osmotolerant when the spherical cells were shocked with diluent without any osmotic stabilizers. These results revealed that the combind treatment of glycine -lysozyme-glycine was synergistic in the spheroplast formation. And it was considered again that the tetrapeptide strand of peptidoglycan might more important roles than the glycan strand in maintaining the vital mechanical function of *Z. mobilis*.

Regeneration of spheroplast to normal cell

In order to increase the regeneration frequency, it was necessary to optimize the formulation of regeneration media and also spheroplasting conditions. As shown in Table 5, the period of glycine treatment was considered to be very important for the higher regeneration frequency. It was also found that the plasma expander (viz. bovine serum albumin, polyvinylpyrolidone) was not effective to

Table 5. *Effect of spheroplasting time on the regeneration of the spheroplasts.*

Time (hr.)	Spheroplast formation(%)	Regeneration frequency
4	85	7.3
6	92	3.02
8	98	0.75

increase the regeneration frequency (data were not shown here). 0.7M sorbitol was used as stabilizer in to avoid the cell lysis during the spheroplasting. It was found that the synthetic medium formulated by Goodman *et al.* (1982) was suitable to regenerate the spheroplasts. As a result, 7-10% of regeneration frequency was obtained in the synthetic media.

적 요

Zymomonas mobilis의 spheroplast 형성과 재생조건의 개량과 최소배지에서의 재생에 대해서 연구하였다. Tris-maleate buffer (pH 5~6), Ca^{++} ions 그리고 EDTA등은 outer membrane의 투과성 변화를 야기시켰다. ***Zymomonas***의 세포벽은 lysozyme, mutanolysin 같은 세포벽 분해효소에 의해 분해가 되지 않는 것처럼 보였고, spheroplast 형성에 glycine 처리는 필수적이었다. 그러나 lysozyme 처리에 의해서 spheroplast 형성이 촉진되는 것을 확인하였다. 따라서 lysozyme과 glycine을 함께 처리함으로서 spheroplasting 배지에서의 처리시간을 4시간으로 단축할 수 있었고 재생율이 7-10%로 증가되었다.

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