Induction of Autolysis and Autoplast Formation of Anaerobic *Clostridium thermohydrosulfuricum*

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혐기성 *Clostridium thermohydrosulfuricum* 의 Autolysis 및 Autoplast 형성유도

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ABSTRACT: Induction conditions for autolysis and autoplast formation of thermophilic *Clostridium thermohydrosulfuricum* were studied. The cells in the initial exponential growth phase were well autolysed in Tris-HCl buffer or inorganic buffers containing univalents, such as K^+ and Na^+, and chemicals such as cysteine-HCl, sorbitol and glycerol. Meanwhile, autolysis induction was slightly inhibited by divalents, such as Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+}, and strongly by divalents, such as Fe^{2+}, Cu^{2+} and citric acid. The autolysis was stimulated when the cells were grown in the medium containing ampicillin that inhibites cell wall synthesis, meanwhile, it was slightly inhibited by nucleic acids and protein synthesis inhibitors. The optimal pH and temperature for the induction of autolysis were 7.5 and 60°C, respectively. On the other hand, the cells were autoplasted without lysozyme treatment during autolysis due to the stabilization of protoplasmic membrane in the presence of divalents such as Mg^{2+}, Ca^{2+}, Mn^{2+} and Ni^{2+}. Autoplast formation was mostly induced at 37°C in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM $MgCl_2$ and 0.3 M glycerol, and in the late exponential growth phase growing cell.

KEY WORDS □ ***Clostridium thermohydrosulfuricum*, autolysis, autoplast formation, induction conditions.**

The roles of bacterial cell wall are to preserve the cell form and to protect the cell from physical shock. But the bacterial cell walls can be decomposed by the action of lytic enzymes, such as, lysozymes, digestive enzyme of a snail, and autolysin. Especially autolysin plays an important role in cell elongation, septum formation, cell separation and competence for transformation (Pooley, 1976; Rogers, 1979; Horne and Tomasz, 1985). The bacterial autolysin exists in an inactive form under normal conditions, however, it is activated under abnormal environmental conditions, and lyses its own cell wall and causes cellular autolysis.

This autolysis phenomenon is known to be induced by several factors such as pH, temperature, growth condition, and osmotic environment (Carson and Daneo-Moore, 1979). The conditions for induction or inhibition of autolysis and its mechanism have been studied for *Streptococcus* sp. (Sayare *et al.*, 1972; Horne *et al.*, 1975; Carvalho *et al.*, 1987),

Pseudomonas sp. (Parente *et al.*, 1984), *E. coli* (Kusser *et al.*, 1987; Leduc, 1980), *Bacillus* sp. (Fan, 1970), *Clostridium tertium* (Knowlton *et al.*, 1984), *C. saccharoperbutylacetonicum* (Ogata *et al.*, 1973, 1974, 1980a, b) and *C. acetobutylicum* (Allcock *et al.*, 1981).

The unstability of cells caused by cellular autolysis prevents the stable protoplast formation for transformation and occationally causes the conversion of normal cell to L-form cell in isotonic solution. This autolysis phenomenon may be applied in artificial excretion of inner cellular materials such as DNA, RNA and protein and also in autoplast formation for gene transformation. Autoplast formation was observed in *C. saccharoperbutylacetonicum* (Ogata and Hongo, 1975), *S. faecalis* (Joseph and Shockman, 1974) and *Listeria monocytogenes* (Tyrrell *et al.*, 1973). *L. monocytogenes* was hardly protoplasted with the conventional treatment of lysozyme, however, it could be efficiently autoplasted during growth in the medium containing sucrose and $MgCl_2$ as stabilizers. Heefner *et al.* (1984) used autoplasts for the purpose of transformation, that is, 38.8 kb of pJU124 plasmid having Tc resistant genetic marker can be transferred to L-phase cell or autoplast in *C. perfringernes* with the treatment of PEG. *C. saccharoperbutylacetonicum* was also well autolysed in the exponential growth phase with the treatment of 0.3-0.4 M sucrose, meanwhile, the formed autoplast was scarecely regenerated (Yoshino *et al.*, 1984).

A thermophilic *Clostridium thermohydrosulfuricum* is known to have the capability of the production of saccharolytic enzymes and ethanol from pentose, hexose and starch etc., however, it cannot use cellulose as carbon source (Hyun and Zeikus, 1985a, b, c; Ng *et al.*, 1981). Meanwhile, *Clostridium thermocellum* can produce cellulolytic enzymes and ethanol from cellulosic biomass (Ha *et al.*, 1987; Lee *et al.*, 1987; Kim *et al.*, 1987; Ng *et al.*, 1977). So we attempted protoplasts fusion between *C. thermocellum* and *C. thermohydrosulfuricum* to obtain fusant which may be able to convert both cellulose and starch to high yield of ethanol.

However, the physiological properties and genetic characteristics on *Clostridium thermohydrosulfuricum* are not well investigated. The purpose of this paper is to investigate the various conditions for the induction and inhibition of autolysis and autoplast formation of above strain. This research will contribute not only for investigation of physiological characteristics of above strains but also for investigation of protoplast formation and regeneration for gene transformation.

MATERIALS AND METHODS

Strain

The strain used in this study was *Clostridium thermohydrosulfuricum* ATCC 33223 deposited in KCTC.

Media and cultivation

C. thermohydrosulfuricum was grown anaerobically at 60°C in modified CM3 medium (Weimer and Zeikus, 1977) containing 1% glucose as a carbon source and 0.1% cysteine-HCl as a reducing agent. In some cases, chemicals such as antibiotics and osmotic stabilizers were added in medium. The medium was deoxygenated with N_2 gas and sealed with a rubber stopper. The initial pH of the medium was adjusted to 7.5. The growth of the strain was determined as optical density at 600 nm.

Autolysis induction

The culture broth of *C. thermohydrosulfuricum* in exponential stage was centrifuged at 8,000×g for 20 min. The precipitated cells were washed twice with sterilized distilled water and suspended in 50 mM Tris-HCl buffer (pH 7.5) and then autolysis was induced under various conditions. The degree of autolysis was determined as follows.

$$\text{Degree of autolysis (\%)} = \frac{A-B}{A} \times 100$$

A; The initial optical density at 600 nm

B; The final optical density at 600 nm after the induction of autolysis.

Antibiotics treatment

To determine the effects of antibiotics on autolysis induction, antibiotics were added to the medium when optical density of culture broth reached to 0.2. Thereafter, the cells were further grown to 0.6 as optical density, then the cells were recovered and condensed by centrifugation (8,000×g, 20 min) and then autolysis was induced

by the same way as described previously. The antibiotics used were erythromycin (0.01 μg/ml) as the inhibitor of protein synthesis, nalidixic acid (0.1 μg/ml) as the inhibitor of DNA synthesis, rifampicin (0.01 μg/ml) as the inhibitor of RNA synthesis, and ampicillin (0.1 μg/ml) as the inhibitor of cell wall synthesis.

Induction of autoplast formation

The cells in initial exponential growth phase were collected and induced without lysozyme treatment at 37°C in 50 mM TM buffer (50 mM Tris-HCl and 20 mM $MgCl_2$, pH 7.5). The autoplast formed under above condition was observed with the phase-contrast microscope with 1,500 folds magnification. Autoplast was observed as protoplast-like globular shape and the degree of autoplast formation (%) was determined dividing formed autoplast number with total cell number.

RESULTS AND DISCUSSION

Autolysis induction of *C. thermohydrosulfuricum*

The cells of *C. thermohydrosulfuricum* in exponential growth phase were autolysed by incubation at 60°C in various buffers such as 50 mM Tris-HCl buffer (pH 7.5), 50 mM sodium phosphate buffer (pH 7.5) and 50 mM potassium phosphate buffer (pH 7.5). However, the cells were nearly autolysed in distilled water. The intensity of autolysis was most strong in potassium phosphate buffer (Fig. 1).

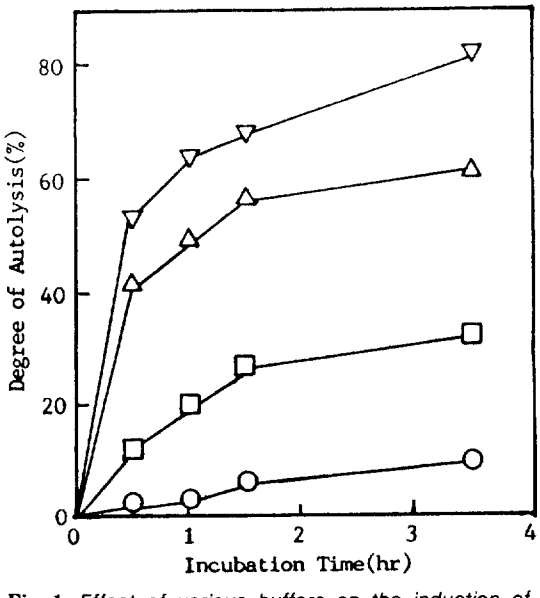

Fig. 1. *Effect of various buffers on the induction of atuolysis.*
○—○; Distilled water, □—□; 50 mM Tris-HCl buffer, △—△; 50 mM Na-phosphate buffer, ▽—▽; 50 mM K-phosphate buffer.

The effect of various cations and chemicals on autolysis induction is appeared in Table 1. It shows that the autolysis induction is severely effected by several cations and chemicals.

Table 1. *Effect of metal ions, chemicals, and stabilizers on the induction of autolysis of C. thermohydrosulfuricum*

Added materials	Concentration	Relative Lysis Rate(%)
None		100
Metal ions		
KCl	0.3M	168
NaCl	0.3M	131
CsCl	0.3M	167
$MgCl_2$	0.02M	65
$CaCl_2$	0.02M	65
$MnCl_2$	0.02M	56
$NiSO_4$	0.02M	26
$FeSO_4$	0.02M	10
$CuSO_4$	0.02M	10
Chemicals		
Citric acid	0.01M	0
EDTA	0.01M	83
o-Phenanthrolin	0.001M	131
Sodium azide	0.01M	131
β-Mercaptoethanol	0.01M	152
Mono iodoacetate	0.01M	152
Sodium cyanide	0.01M	157
Cysteine-HCl	0.01M	160
p-CMB	0.001M	160
Gelatin	2%	126
PVP	5%	126
PEG	5%	137
Sucrose	0.3M	151
Sorbitol	0.3M	174
Glycerol	0.3M	174

Autolysis was induced at 60°C for 2 hrs in 50 mM Tris-HCl buffer (pH 7.5) containing each compound.

Autolysis of the strain was enhanced by the addition of the univalent cations such as K^+, Na^+ and Cs^+. The higher autolysis induction in sodium or potassium phosphate buffers (pH 7.5) over Tris-HCl buffer may be caused by the presence of univalent ions such as Na^+ and K^+ in each buffer. However, autolysis induction was decreased in the presence of bivalent cations such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+}, especially strongly influenced by Cu^{2+} and Fe^{2+}. Autolysis was also completely inhibited by 0.01 M citric acid and slightly by 0.01 M EDTA. Meanwhile, autolysis was activated by most chemicals such as o-phenanthroline, sodium azide, β-mercaptoethanol, monoiodoacetate, sodium cyanide, cysteine-HCl, *p*-CMB, sorbitol, and glycerol.

The reason of autolysis activation by various chemicals is not known exactly, however, according to Ogata *et al.* (1975), lysis inhibition by bivalent cations may be due to the two different actions; one is the inhibition of autolysin activity, and the other is the stabilization of developed protoplast-like cells. Cu^{2+} and Fe^{2+} may be due to the former action, meanwhile, Mg^{2+}, Ca^{2+} and Mn^{2+} may be due to the latter action because these bivalent cations are well known to stabilize bacterial protoplast.

Fig. 2 shows that 0.3 M KCl is most suitable concentration for the induction of autolysis in *C. thermohydrosulfuricum*. Autolysis of *C. thermohydrosulfuricum* by univalent cations is similar to those of other strains. Ogata *et al.* (1973) reported that growing cells of *C. saccharoperbutylacetonicum* were lysed by univalent cations, such as K^+, Na^+, Rb^+, Cs^+, Li^+ and NH_4^+, however, bivalent cations such as Ca^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+} and Ni^{2+} at above 0.05 M concentration inhibited the lysis. On the other hand, Carvalho *et al.* (1984) reported that fresh *S. faecium* cells was not autolysed either in phosphate or in Tris-HCl buffer (pH 8.0); however, when they are frozen-thawed (−70°C) in Tris-HCl buffer before incubation in the same buffer, some autolysis is observed. Ogata *et al.* (1980) also suggest that the lysis of *C. saccharoperbutylacetonicum* by sucrose treatment is catalysed by autolysin which acts on peptidoglycan and then causes local weakening of the rigid cell wall and so that facilitate the lysis of newly synthesized cell wall material. According

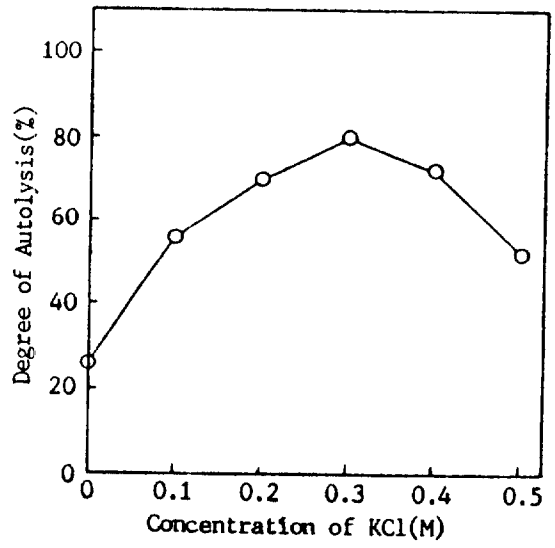

Fig. 2. *Induction of autolysis at various concentration of KCl.*
Autolysis was induced at 60 °C for 1 hr in 50 mM Tris-HCl buffer (pH 7.5) containing each concentration of KCl.

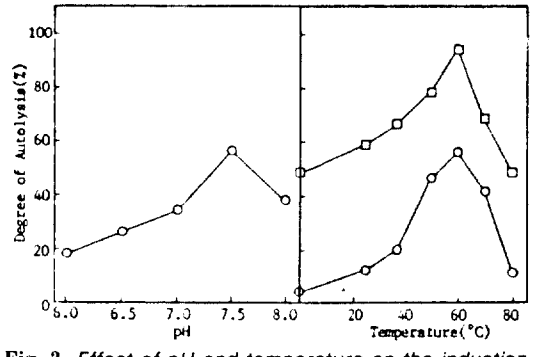

Fig. 3. *Effect of pH and temperature on the induction of autolysis.*
Autolysis was induced at 60 °C for 2 hrs in 50 mM Tris-HCl buffer (○—○) and 50 mM Tris-HCl buffer containing 0.3 M KCl (□–□).

to Parente and Silva (1984), autolysis of exponentially growing cell of *Pseudomonas fluorescens* could be induced by a rapid treatment with distilled water before treatment with 0.5 M sodium acetate, pH 6.5, and incubation for 2 hr at 30°C.

Effect of pH and temperature on autolysis induction

As shown in Fig. 3, autolysis of the cells was mostly induced in pH 7.5 and 60°C which were the same conditions used for the initial cultivation. The autolysis was also increased at 60°C by the

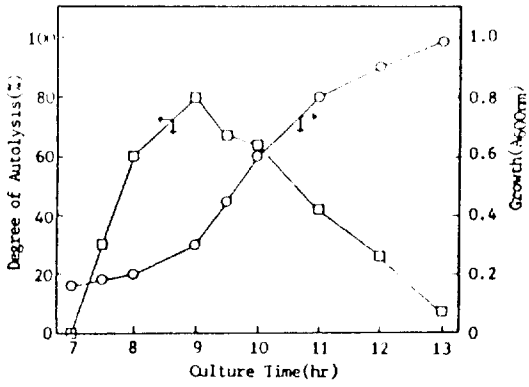

Fig. 4. *Effect of growth phase on the induction of autolysis.*
○—○; Growth curve, □–□; Autolysis.
Autolysis was induced at 60 °C for 1 hr in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M KCl.

addition of 0.3 M KCl to Tris-HCl buffer.

Effect of growth phase on autolysis induction

Fig. 4 shows the induction patterns of autolysis of the cells at each growth phase. Autolysis was highly induced in the early exponential phase in which division of the cells was extensively progressed, however, the cells of late exponential and stationary phases were not autolysed at above conditions.

Above result is similar with that of Ogata *et al.* (1975) who reported that the most rapid lysis of *C. saccharoperbutylacetonicum* occurred in the early exponential phase cultures, but no lysis was observed on the late exponential and stationary growth phase growing cells.

Effect of antibiotics on autolysis induction

C. thermohydrosulfuricum was treated with various kinds of antibiotics as shown in Fig. 5. Protein synthesis inhibitor, erythromycin, showed a more pronounced inhibitory effect on the induction of autolysis than nucleic acid synthesis inhibitors, such as, nalidixic acid and rifampicin. On the other hand, cell wall synthesis inhibitor (ampicillin) has a stimulatory effect on the induction of autolysis. The inhibitory effect caused by the inhibitor of RNA synthesis may presumably be more closely correlated with concomitant secondary inhibition of protein synthesis. Similar observations were obtained on the autolysis of *Streptococcus faecalis* (Sayare *et al.*, 1972) and *Clostridium saccharoperbutylacetonicum* (Ogata *et al.*, 1975).

Treatment with antibiotics as inhibitors on the

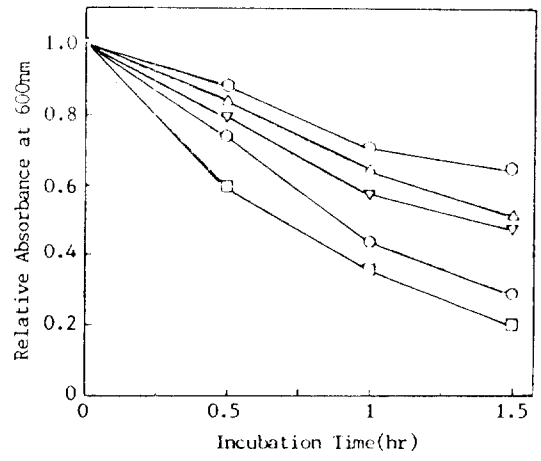

Fig. 5. *Effect of antibiotics on the induction of autolysis.*
Autolysis was induced at 60 °C in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M KCl.
○—○; None, ○—○; Erythromycin, △–△; Rifampicin, ▽–▽; Nalidixic acid, □–□; Ampicillin.

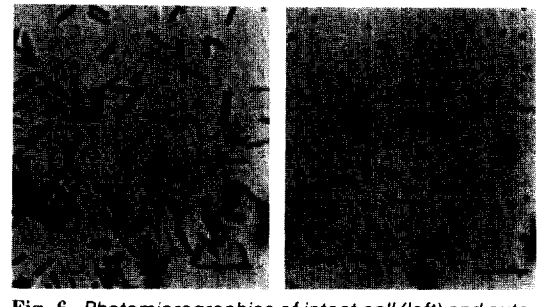

Fig. 6. *Photomicrographies of intact cell (left) and autoplast (right) of Clostridium thermohydrosulfuricum.*
Bar represents 10 μm.

biosynthesis of DNA, RNA and protein resulted in rapid decrease in the rate of autolysis of other strains (Shockman, 1965; Higgins *et al.*, 1970; Pooley and Shockman, 1970; Stewart and Marmur, 1970; Ogata *et al.*, 1980).

Autoplast formation of *C. thermohydrosulfuricum*

The normal cells of *C. thermohydrosulfuricum* were converted to protoplast forms (autoplasts) in the presence of divalent cations such as Mg^{2+}, Ca^{2+}, Ni^{2+} and Mn^{2+} in Tris-HCl buffer (pH 7.5) without the addition of lysozyme (Fig. 6). As shown in Table 2, degree of autoplast formation was reached up to about 98% by Mg^{2+}, which is

Table 2. *Effect of bivalent ion on the induction of autoplast formation*

Kinds of bivalent ion	Concentration	Degree of autolysis (%)	Degree of autoplast formation(%)
None		74	0
$MgCl_2$	0.02M	49	98
$CaCl_2$	0.02M	52	96
$MnCl_2$	0.02M	48	88
$NiSO_4$	0.02M	12	35
$FeSO_4$	0.02M	0	0
$CuSO_4$	0.02M	0	0

Autoplast formation was induced at 37 °C for 50 hrs in 50 mM Tris-HCl buffer (pH 7.5) containing each bivalent metal ion.

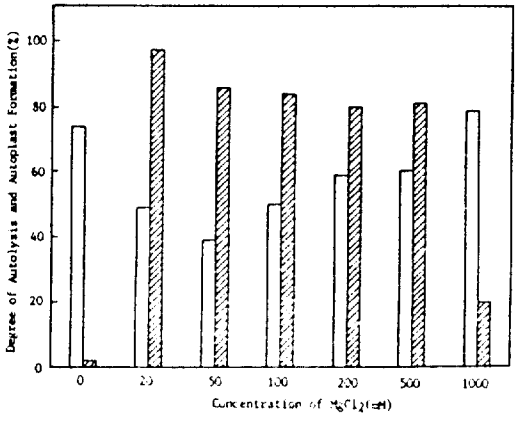

Fig. 7. *Effect of $MgCl_2$ concentration on the induction of autoplast formation.*
Autoplast formation was induced at 37 °C for 50 hrs in 50 mM Tris-HCl buffer (pH 7.5) containing each concentration of $MgCl_2$.
□; Autolysis, ▨; Autoplast formation.

thought to be the most proper autoplast stabilizer among other divalent cations. On the other hand, the cells were not converted to autoplast by the addition of Fe^{2+} or Cu^{2+} to the same buffer. The reason is thought that Fe^{2+} and Cu^{2+} inhibit the action of a autolysin in cells to prevent cells from autolysis.

Fig. 7 shows that the cells were well autoplasted in the concentration of 20 to 500 mM Mg^{2+}. 20 mM Mg^{2+} was thought to be the most proper concentration for autoplast formation, however, above 1 M of Mg^{2+} showed stimulatory effect on autolysis instead of autoplast formation. On the

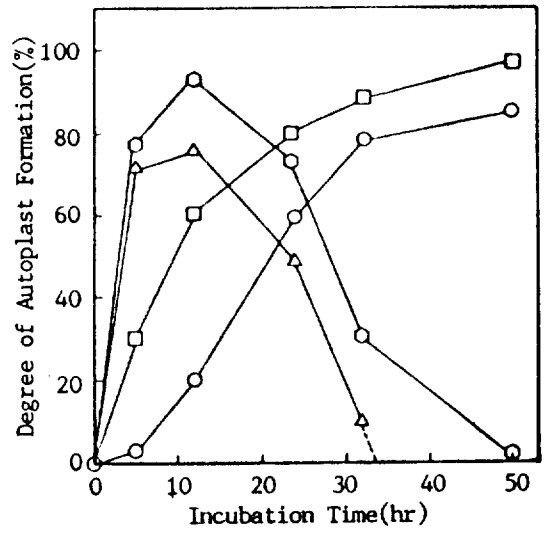

Fig. 8. *Effect of temperature on the induction of autoplast formation.*
Autoplast formation was induced in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM $MgCl_2$. ○—○; 25 °C, □–□; 37 °C, ○—○; 50 °C, △–△; 60 °C.

other hand, the organism was not autoplasted by univelent cations such as K^+ and Na^+ which promoted the autolysis. These results indicate that the divalent cation such as Mg^{2+} and Ca^{2+} may be closely related to the stability of protoplast membrane for autoplast formation after the lysis of cell wall.

The autoplast formation phenomenon caused by the action of autolysin without the treatment of lysozyme could be utilized for protoplast formation for transformation. Though *Lactobacillus casei* could not be protoplasted with the treatment of only lysozyme, 99% of the cells were protoplasted with the simulataneous treatment of autolysin and lysozyme (Lee-Wickner and Chassy, 1984). Also Heefnerr *et al.* (1984) could transfer the pJU 124 plasmid to the autoplast of *C. perfringens.*

Effect of temperature and pH on autoplast formation

The degree of autoplast formation of *C. thermohydrosulfuricium* was variable at each temperature as shown in Fig. 8. The degree of autoplast formation was increased up to 98% at 37°C for 50 hours, and autoplasts formed were stable for a long period of time at this temperature. Though 93% of the cells were autoplasted after the

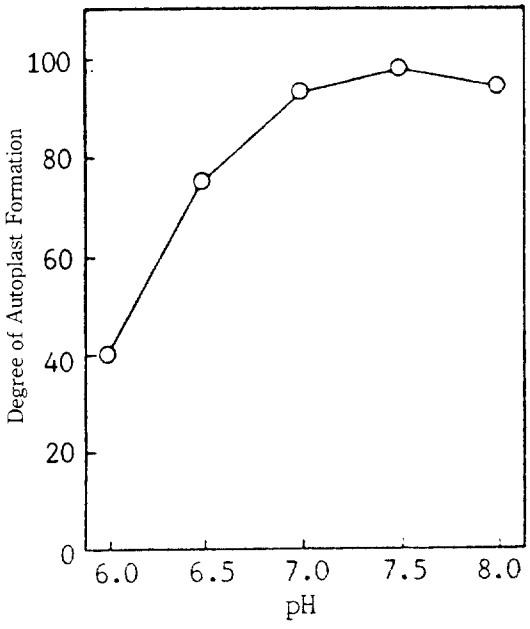

Fig. 9. *Effect of pH on the induction of autoplast formation.*
Autoplast was induced at 37 °C for 50 hrs in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM $MgCl_2$.

treatment for 12 hours at 50°C, however, most of the formed autoplasts were lysed with the passage of time.

On the other hand, the cells were rapidly autolysed after autoplast formation at 60°C, and they were nearly autoplasted at below 20°C.

The degree of autoplast formation was similar between pH 7.0 and 8.0, as shown in Fig. 9. The cells were optimally autoplasted at pH 7.5 as same with the optimal pH for autolysis.

Effect of TM buffer concentration on autoplast formation

Fig. 10 shows the effect of the TM buffer concentration on autoplast formation. The degree of autoplast formation was increased to 98% by increasing buffer concentration from 0.01 M to 0.05 M, however, it was slightly decreased in buffer concentration above 0.05 M. On the other hand, the strain was not autolysed and autoplasted in distilled water.

Joseph and Shockman (1974) reported that *S. faecalis* autolysed rapidly in ammonium acetate buffer and the rate of cellular autolysis in this buffer remains constant over a rather broad range of

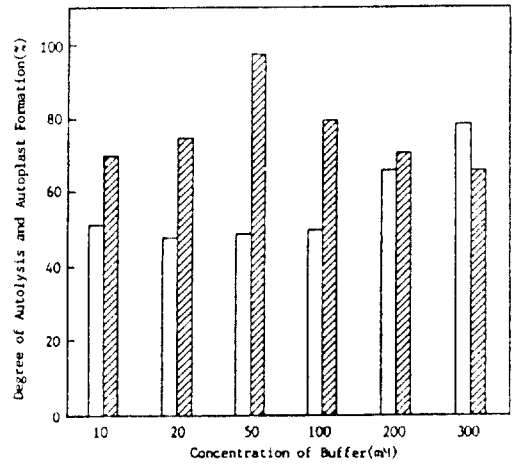

Fig. 10. *Effect of Tris-HCl buffer concentration on the induction of autoplast formation.*
Autoplast formation was induced at 37 °C for 50 hrs in each buffer (pH 7.5) containing 20 mM $MgCl_2$.
□; Autolysis, ▨; Autoplast formation.

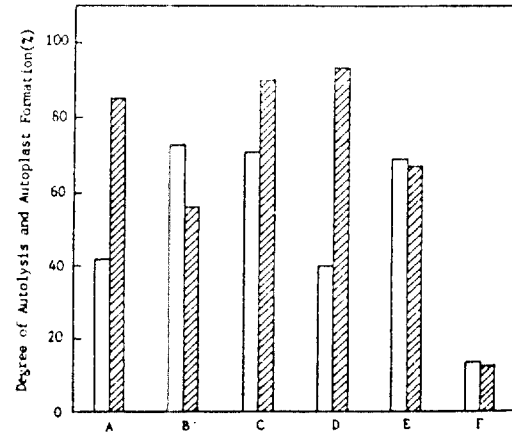

Fig. 11. *Effect of stabilizers on the induction of autoplast formation.*
Autoplast formation was induced at 37 °C for 36 hrs in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM $MgCl_2$ and each stbilizer: A; none, B; KCl (0.3 M), C; NaCl (0.3 M), D; glycerol (0.3 M), E; sorbitol (0.3 M), F; sucrose (0.3 M).
□; Autolysis, ▨; Autoplast formation.

ammonium acetate concentrations from 0.02 M to 0.3 M.

Effect of osmotic stabilizers on autoplast formation

As shown in Fig. 11, the degree of autoplast for-

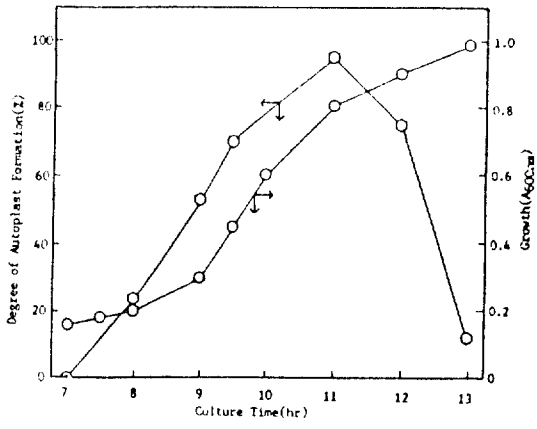

Fig. 12. *Effect of growth phase on the induction of autoplast formation.*
○—○; Growth curve, ○—○; Autoplast formation.
Autoplast formation was induced at 37°C for 50 hrs in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM $MgCl_2$.

mation was slightly increased by the addition of 0.3 M NaCl or 0.3 M glycerol compared with control. However, it was decreased by 0.3 M KCl and 0.3 M sucrose.

C. saccharoperbutylacetonicum was effectively stabilized by 0.4 M sucrose solution containing 5 mM Mg^{2+} (Ogata *et al.*, 1980). On the other hand, cellular lysis of *S. faecalis* in 0.04 M ammonium acetate was prevented by 0.38 or 0.5 M sucrose, and the presence of 0.1 mM Mg^{2+} resulted in a further increase in prolonged osmotic stability in both 0.3 M and 0.5 M sucrose (Joseph and Shockman, 1974). Use of high concentration of magnesium can increase osmotic stability even in the case of low sucrose concentration.

Effect of growth phase on autoplast formation

Fig. 12 shows that the autoplast formation is highly induced in the late exponential phase in which the cell wall may be more stable than in initial exponential phase, as dissimilar with the cellular autolysis highly induced in the exponential phase. However, the autoplast formation was nearly induced in stationary phase where the lysis of cell wall was also not recognizably induced.

적 요

고온성 *Clostridium thermohydrosulfuricum* 의 autolysis 및 autoplast 형성에 대하여 조사하였다. 초기대수증식기 상태의 세포가 K^+나 Na^+ 등의 1가이온을 함유한 buffer나 Tris-HCl buffer 속에서 autolysis 현상을 나타내었다. 이 현상은 1가이온이나 cysteine-HCl, sorbitol, glycerol 등의 화학물질이 존재할 때 강하게 촉진되었으나 Ca^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+} 등의 2가이온에 의해 다소 저해되었으며, 특히 Fe^{2+}, Cu^{2+} 등의 2가이온 및 citric acid에 의해서는 강하게 저해되었다. 또한 세포벽합성 저해제인 ampicillin을 함유한 배지에서 생육한 세포는 autolysis가 촉진되었으나 핵산 및 단백질합성 저해제의 경우 autolysis가 저해되었다. Autolysis가 유도되는 최적 pH는 7.5, 최적 온도는 60℃였다. 한편, autolysis가 일어나는 도중 Mg^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+} 등의 2가이온에 의해 원형질막이 안정화될 때 lysozyme의 첨가없이 autoplast가 형성되었다. 이 autoplast는 대수증식기 말기 세포에서 형성이 잘되었으며, autoplast 형성의 최적 pH는 7.5, 최적 온도는 37℃, 최적 $MgCl_2$ 농도는 20 mM이었으며, 화학물질로는 0.3 M glycerol이 효과적이었다.

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