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Protoplast Formation and Regeneration of Thermophilic *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*

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고온성 *Clostridium thermocellum*과 *Clostridium thermohydrosulfuricum*의 원형질체 형성 및 재생

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ABSTRACT: **The conditions for protoplasts formation and regeneration of thermophilic anaerobic *C. thermocellum* and *C. thermohydrosulfuricum* were determined under the anaerobic growth conditions. The cells of *C. thermocellum* in initial exponential growth phase were identified to be the most suited for protoplast formation. The optimal conditions for protoplast formation were found to be at 37°C for 2 hours with 0.5 mg/ml of lysozyme in TMG buffer (pH 7.5). On the other hand, *C. thermohydrosulfuricum* grown in the same medium but excluding glycine was optimally protoplasted at the same conditions but with 0.2 mg/ml of lysozyme. The protoplasts of both strains only subjected to lysozyme treatment of the short time were satisfactorily regenerated after 7-10 days incubation at 60°C in regeneration medium containing 0.3-0.4 M sorbitol, 0.5% casamino acid, and high concentration of $CaCl_2$ and $MgCl_2$. The regeneration frequencies of the protoplasts of *C. thermocellum* and *C. thermohydrosulfuricum* were found to be very low level of 4.85×10^{-3} and 4.23×10^{-2}, respectively. The nonregenerated L-form cells were also observed in regeneration medium together with regenerated cells.**

KEY WORDS□ *Clostridium thermocellum, Clostridium thermohydrosulfuricum,* **protoplast formation, protoplast regeneration**

The protoplast formation and regeneration are prerequired essential initial step of research either for a protoplast transformation or a development of a fusion system for strain improvement. However, the study on protoplast formation and regeneration was mainly confined to plant cells, yeasts, and mesophilic aerobic bacterial cells (Fodor *et al.*, 1980).

The specific conditions for the protoplast formation and regeneration of strict anaerobes, including industrially important thermophilic anaerobes, had not been exclusively investigated. The procedures for protoplast formation and regeneration of anaerobic *C. acetobutylicum* (Allcock *et al.*, 1982), *C. pasteurianum* (Minton *et al.*, 1983) and *C. saccharoperbutylacetonicum* (Yoshino *et al.*, 1984) represent the earlier research in this field. Later the plasmid transformation for the anaerobic pathogen *C. perfringens* (Heefner *et al.*, 1984), a chromosomal genetic recombination system and protoplast fusion in obligate anaerobic *C. acetobutylicum* (Jones *et al.*, 1985) were reported. Chen *et al.* (1986, 1987) established the condition for the protoplasts formation and regeneration in dehydrodivanillin-degrading anaerobes of *Fusobacterium varium* and *Enterococcus faecium*. However, there is negligible information on protoplast fusion or genetic transfer system

of strict thermophilic anaerobes. This may be due to the difficulty of manipulation of anaerobic cell which have to be handled in the strictly deoxygenated growth conditions.

A thermophilic anaerobic *Clostridium thermocellum* has a considerable potential for production of a significant amount of cellulase and for direct conversion of cellulosic bimass to ethanol. As a consequence, considerable attention has been given to the utilization of the organism for biofuel production from cellulosic biomass (Cooney *et al.*, 1978; Ha *et al.*, 1987; Kim *et al.*, 1987; Lee *et al.*, 1987; Ng *et al.*, 1981). Coculture of *C. thermocellum* with *C. thermohydrosulfuricum,* which can utilize starch, hexoses and pentoses, enhanced ethanol yield to 3 folds by converting cellulosic biomass (Hyun and Zeikus, 1985; Ng *et al.*, 1981).

A fusant between *C. thermocellum* and *C. thermohydrosulfuricum,* which has a capability of simultaneous conversion of cellulosic biomass and starch to ethanol, would decrease the effort of coculture of above two strains and enhance ethanol yield. So we attempted protoplasts fusion between *C. thermocellum* and *C. thermohydrosulfuricum.* Induction conditions of autolysis and autoplast formation of thermophilic *C. thermohydrosulfuricum* were reported in the previous paper (Kim *et al.*, 1989).

In this work, the procedures for protoplasts formation and regeneration of above two thermophilic anaerobic *Clostridia* were established under strictly anaerobic conditions, to utilize for the development of a genetic exchange and of a fusion system for strain improvement of above strains.

MATERIALS AND METHODS

Strains

The strains used were *Clostridium thermocellum* ATCC 27405 and *Clostridium thermohydrosulfuricum* ATCC 33223.

Media and cultivation

The modified CM3 media (Weimer and Zeikus, 1977) for cell cultivation and protoplast regeneration are shown in Table 1. 1 g/*l* of cysteine-HCl was added as a reducing agent and the initial pH of culture medium was adjusted to 7.5. The medium was deoxygenated with N_2 gas, and sealed with a rubber stopper, and then, cultivated at 60°C. Occationally, 0.2% of glycine was added in CM3 medium to facilitate formation of protoplast. The growth curve of the strains was determined by measuring the optical density at 600 nm.

Table 1. *Composition of CM3 and regeneration media*

Composition (per liter)	CM3 medium	Regeneration medium
Carbon source	10.0 g	10.0 g
Yeast extract	2.0 g	4.0 g
$(NH_4)_2SO_4$	1.3 g	1.3 g
KH_2PO_4	1.5 g	1.5 g
$K_2HPO_4 \cdot 3H_2O$	2.9 g	2.9 g
$MgCl_2 \cdot 6H_2O$	1.0 g	4.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g	0.6 g
$FeSO_4 \cdot 7H_2O$	1.25 mg	1.25 mg
Resazurin	2.0 mg	2.0 mg
Cysteine-HCl	1.0 g	1.0 g
Agar		20.0 g
Casamino acid		5.0 g
Sorbitol		54.7 g

Protoplast formation

The cells harvested by centrifugation at 3,000 × g for 20 min were washed twice with 0.1 M TM buffer (0.1 M Tris-HCl and 20 mM $MgCl_2$, pH 7.5), and then suspended in hypertonic 0.1 M TMG buffer (TM buffer containing 0.5 M glycerol). The final concentration of 0.5 mg/m*l* of filtersterilized lysozyme was added to the cell suspension of *C. thermocellum,* whilst 0.2 mg/m*l* of lysozyme was added to the *C. thermohydrosulfuricum* cell suspension, and then, the suspensions were incubated at 37°C for 2 hours. Protoplast formation was monitored by observing cells using a phase-contrast microscope, and the frequency of protoplast formation was determined by dividing protoplasted cell number with intact cell number.

Protoplast regeneration

For the regeneration of protoplasts, one portion of protoplasts was washed twice with 0.1 M TMG buffer to eliminate lysozyme and diluted in hypertonic solution, thereafter, inoculated in regeneration medium by roll tube method (Gerhart *et al.*, 1981), and then cultivated at 60°C for 7-10 days. Meanwhile, the remained portion of protoplasts was suspended in 5 volumes of distilled water for the disruption of protoplasts by osmotic shock. Thereafter, it was inoculated in regeneration medium and cultivated in similar way. The frequency of protoplasts regeneration was determined as follows;

$$\text{Regeneration frequency} = \frac{B - C}{A}$$

Table 2. *Effect of amino acids on protoplast formation of C. thermocellum*

Amino acids (0.2%)	Degree of protoplast formation (%)
None	68
Threonine	94
Asparagine	80
Valine	96
Glycine	97
Tryptophan	82
Methionine	85
Glutamic acid	93
Histidine	90
Lysine	—

where A; Intact cell number,
B; Colony number regenerated from protoplasts suspended in hypertonic buffer,
C; Colony number regenerated from protoplasts suspended in distilled water.

RESULTS AND DISCUSSION

Protoplasts formation of *C. thermohydrosulfuricum* and *C. thermocellum.*

Effect of amino acid; Gram positive *C. thermohydrosulfuricum* cells, that were cultivated in CM3 medium without amino acid, were well protoplasted with lysozyme treatment. However, Gram negative *C. thermocellum* cells grown in the medium without amino acid were hardly protoplasted with lysozyme treatment. The effect of the various kinds of amino acids on protoplast formation of *C. thermocellum* are shown in Table 2. The cells grown in the medium containing 0.2% of various amino acids were treated with lysozyme in this case. The cell grown in the medium containing valine or glycine was most effectively converted to protoplast. The degree of protoplast formation increased up to 97% in the case of glycine, however, the growth of the strain was slightly delayed in the medium containing glycine, and moreover, the growth was strongly inhibited in lysine containing medium. Protoplasts were well formed under either aerobic or anaerobic conditions, however, the protoplasts that were formed under aerobic condition were not regenerated later.

The similar enhancing effect of glycine of protoplast formation on *C. perfringens* was also observed by Stal and Blaschek (1985), who reported that the cells *C. perfringens* were well protoplasted when 0.4% glycine was added in growth medium. The stimulation mechanism for protoplast formation by glycine was also observed by Hammens *et al.* (1973). According them, glycine added in the medium during cell growth may substitute D-alanine in the cell wall synthesis, which inhibit the transpeptidation between D-alanine and diaminopimelic acid to lead a more loosely cross-linked peptidoglycan and also morphological changes of the cells. Similar interpretation may be applicable in the case of *C. thermocellum.*

Optimal condition of lysozyme treatment; Lysozyme is prevalent enzyme used for production of bacterial protoplasts. To determine the optical lysozyme concentration for protoplast formation, cells were treated at 37 °C for 2 hours with various concentration of lysozyme from 0.05 mg/m*l* to 1.0 mg/m*l*. The degree of protoplast formation was reached to about 95% at 0.2 - 0.5 mg/m*l* of lysozyme in *C. thermocellum* suspension, and to about 96% at 0.1 - 0.2 mg/m*l* of lysozyme in *C. thermohydrosulfuricum* suspension. The use of exceeding amount of lysozyme over 1.0 mg/m*l* hindered the protoplasts formation of both strains.

The optimal time of lysozyme treatment was also determined. The protoplast formation was not further increased beyond 2 hours treatment with 0.5 mg/m*l* of lysozyme in *C. thermocellum*. However, the cells of *C. thermohydrosulfuricum* were more readily protoplasted to about 95% after 1 hour of lysozyme treatment.

Determination of optimal pH; The opitimal pH for protoplast formation was found to be around 7.5 for both strains. Below 50% of cells were protoplasted in the ranges of below pH 6.0 and above pH 8.0.

Effect of osmotic stabilizer; Both cells were highly protoplasted by addition of 0.5 M glycerol as osmotic stabilizer (Table 3). The cells of *C. thermocellum* were also formed the protoplasts well in the case of 0.5 M sorbitol, and *C. thermohydrosulfuricum* cells were also well protoplasted in the case of 2% gelatin. However, the protoplasts produced in the presence of gelatin showed the tendency of aggregation each other. Sucrose and sodium succinate was ineffective for protoplast formation for both microorganisms. The fact that glycerol is optimal osmotic stabilizer for protoplasts formation of above two strains is similar with that of Chen *et al.* (1986), who observed that protoplasts of *F. varium* and *E. faecium* were well formed in phosphate buffer containing 0.2 M glycerol as an osmotic stabilizer. However, Allcock *et al.* (1982) obtained different

Table 3. *Effect of osmotic stabilizers on protoplast formation*

Stabilizers	Conc.	Degree of protoplast formation (%)	
		C. thermocellum	*C. thermohydrosulfuricum*
None		—	50
Sucrose	0.5 M	6	3
NaCl	0.5 M	50	78
KCl	0.5 M	67	80
Sodium succinate	0.5 M	4	7
Lactose	0.5 M	3	10
Glycerol	0.5 M	87	86
Mannitol	0.5 M	76	57
Sorbitol	0.5 M	86	67
Gelatin	2.0%	37	90

Table 4. *Effect of osmotic stabilizers on protoplast regeneration*

Stabilizers	Conc.	*C. thermocellum*	*C. thermohydrosulfuricum*
None		-	±
Sucrose	0.5 M	+	+ +
Glycerol	0.5 M	+	+
Lactose	0.5 M	−	−
KCl	0.5 M	−	+
NaCl	0.5 M	+	−
Sorbitol	0.5 M	+ +	+ +
Mannitol	0.5 M	−	+
Sodium succinate	0.5 M	−	−
PEG	5%	+	+
PVP	10%	+	+
Gelatin	2%	+	+ +

PEG; polyethylene glycol 1000, PVP; polyvinyl pyrrolidone, + +; regeneration and good colony formation, +; regeneration, -; no regeneration.

result that only 80-83% protoplasts were formed from *C. acetobutylicum* after 1 hour at 37 °C in 0.3 M to 0.5 M sucrose and lactose solutions containing 1 mg/m*l* of lysozyme.

Effect of the growth phase; The cells in initial exponential growth phase were most suited for the protoplasts formation for both microorganisms (Fig. 1). Contrastly, the cells in stationary growth phase were inefficient for protoplast formation for two strains.

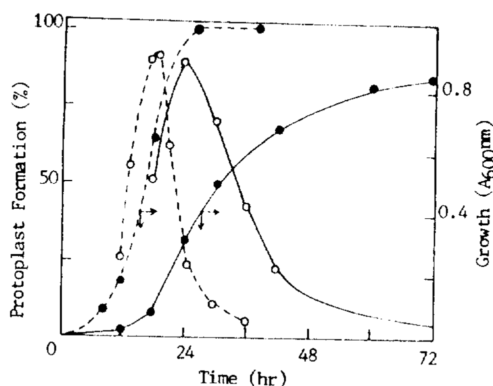

Fig. 1. *Effect of growth phase on protoplast formation.* ●—●; growth, ○—○; protoplast formation of *C. thermocellum*. ●----●; growth, ○----○; protoplast formation of *C. thermohydrosulfuricum*.

Protoplasts regeneration of *C. thermocellum* and *C. thermohydrosulfuricum*.

Effect of various osmotic stabilizers; The protoplasts of *C. thermocellum* were readily regenerated at 60 °C in 7 to 10 days in regeneration medium containing various osmotic stabilizers such as glycerol, NaCl, sucrose, sorbitol, PEG, PVP, and gelatin (Table 4). Both regeneration and colony formation abilities of *C. thermocellum* were increased by adding 0.5 M sorbitol. Meanwhile, sucrose was found to be improper as an osmotic stabilizer due to the formation of foam during regeneration that causes irregular formation of regenerated colony. On the other hand, protoplasts of *C. thermohydrosulfuricum* were successively regenerated in medium containing 0.5 M sorbitol. The ability of colony formation of regenerated cells was also enhanced by sorbitol. The regeneration ability of protoplast was also increased by using glycerol as stabilizer, however, colony forming ability was severely hindered by glycerol.

The optimal concentrations of sorbitol for protoplasts regeneration were found to be 0.3 M and 0.4 M for *C. thermocellum* and *C. thermohydrosulfuricum,* respectively.

Effect of lysozyme treatment time; Overtreatment of lysozyme beyond 1 hour for protoplasts formation tends to decrease the regeneration capability of protoplasts for both strains, which implies that shortening of lysozyme treatment is essential for ef-

Table 5. *Effect of $CaCl_2$ and $MgCl_2$ on protoplast regeneration*

$CaCl_2$ (mM)	$MgCl_2$ (mM)	CFU/m*l* of protoplasts	
		C. thermocellum	*C. thermohydrosulfuricum*
1	5	2.1×10^4	7.0×10^4
2	10	8.2×10^4	1.1×10^5
3	15	1.0×10^5	1.3×10^5
4	20	1.5×10^5	1.0×10^6
8	40	4.2×10^5	6.2×10^5
12	60	4.2×10^4	5.0×10^4

CFU; colony forming units

fective protoplasts regeneration.

Effect of $CaCl_2$; The roles of $CaCl_2$ and $MgCl_2$ in regeneration of the cell wall of *C. thermocellum* and *C. thermohydrosulfuricum* protoplasts were also examined as shown in Table 5. The most promising cell wall regeneration frequencies were obtained when 8 mM $CaCl_2$ and 40 mM $MgCl_2$ were added to the regeneration medium for *C. thermocellum* and 4 mM $CaCl_2$ and 20 mM $MgCl_2$ for *C. thermohydrosulfuricum*. These results are comparable with those of Stal and Blaschek (1985) who identified that the optimal concentration of $CaCl_2$ and $MgCl_2$ for protoplasts regeneration in *C. perfringens is 25 mM. On* the other hand, Allcock *et al.* (1982) reported different results that the addition of $MgCl_2$ and $CaCl_2$ only incr.ased stability of the protoplasts of *C. acetobutylic m,* but decreased their ability of regeneration.

Effect of plasma expanders; Regeneration frequencies of protoplasts of *C. thermocellum* and *C. thermohydrosulfuricum* were not increased by addition of gelatin (1 - 4%) to regeneration medium, instead, it was further decreased when above 4% of gelatin was added to the medium. This result is in good agreement with that of Minton and Morris (1983), who found that the inclusion of even small amount of gelatin (0.5%) in the regeneration medium caused a 60% reduction in the regeneration frequency of *C. pasteurianum* protoplasts. However, our result is contrast with that of Okamoto *et al.* (1983) who reported that the regeneration frequency of *S. lactis* protoplasts was increased about 20 times when 2.5% gelatin was used.

On the other hand, the regeneration frequencies of *C. thermohydrosulfuricum* protoplasts could be increased by the addition of 0.5% of casamino acid, while that of *C. thermocellum* was unaffected. However, other plasma expanders such as bovine serum albumin (BSA) and N-acethyl glucosamine (NAG) was found to be harmful for protoplasts regeneratin for both strains. Above observation is controversial with that of Stal and Blaschek (1985), who watched that the combined addition of BSA and NAG accounted for an additional 100-fold increase in cell wall regeneration frequency of *C. perfringens.* Meanwhile, *Bacillus subtilis* protoplasts could be regenerated on media containing horse serum, bovine serum or gelatin, and these compounds could be replaced by polyvinyl pyrrolidone or dextran (Akamatsu and Sekiguchi, 1981).

Frequencies of protoplast regeneration; Protoplasts regeneration frequencies of *C. thermocellum* and *C. thermohydrosulfuricum* were found to be very low values of below 4.85×10^{-3} and 4.23×10^{-2}, respectively, as shown in Table 6. These low regeneration frequencies are quite different from those of

Table 6. *Regeneration frequencies in C. thermocellum and C. thermohydrosulfuricum*

Strains (exp.)	Starting cells (a)	CFU/m*l* of protoplasts diluted in: Isotonic buffer (b)	Distilled water (c)	Regeneration frequency (b-c)/a
C. thermocellum				
1	5.97×10^8	2.90×10^6	—	4.85×10^{-3}
2	4.66×10^9	5.48×10^6	—	1.18×10^{-3}
3	3.05×10^9	1.01×10^5	9.66×10^2	3.31×10^{-4}
C. thermohydrosulfuricum				
1	6.05×10^7	1.02×10^6	3.00×10^3	1.68×10^{-2}
2	4.10×10^7	7.80×10^5	—	1.90×10^{-2}
3	4.22×10^7	1.8×10^6	—	4.23×10^{-2}

other mesophilic anaerobic microorganisms, that is, 20 - 30% of *F. varium* and *E. faecium* (Chen *et al.*, 1986), 1% of *C. acetobutylicum* (Reyssert *et al.*, 1987), 10% of *C. perfringens* (Stal and Blaschek, 1985).

Morphologies of regenerated cells; Nonregenerated L-form cells were observed together with regenerated cells under phase-contrast microscope after 7 - 10 days of regeneration period. Whereas the colony and cellular morphologies of the regenerated cells were identical to those of the parent culture, nonregenerated protoplast colonies exhibited "fried egg" morphology of L-form colonies. Similar phenomenon was also observed in anaerobic *Clostridium tertium* by Knowlton *et al.* (1984).

적 요

고온 혐기성 *Clostridium thermocellum* 및 *Clostridium thermohydrosulfuricum*의 원형질체 형성 및 재생조건에 대하여 조사하였다. 원형질체 형성은 *C. thermocellum*의 경우, 0.2% glycine을 첨가한 배지에서 초기대수증식기까지 생육한 cell을 0.5 mg/m*l*의 lysozyme을 함유한 100 mM TMG buffer(pH 7.5)에서 37℃, 2시간 처리 했을 때 가장 좋았으며, *C. thermohydrosulfuricum*은 대수증식기의 cell을 동일 조건에서 0.2 mg/m*l*의 lysozyme으로 처리했을 때 양호하였다. 원형질체 재생은 0.3-0.4 M sorbitol, 0.5% casamino acid, 고농도의 $CaCl_2$와 $MgCl_2$를 첨가한 재생배지에서 60℃, 7-10일간 배양했을 때 잘 되었으며, 원형질체 형성시 lysozyme의 처리 시간이 짧을 수록 좋았다. 재생빈도는 *C. thermocellum*의 경우 4.85×10^{-3}, *C. thermohydrosulfuricum*의 경우 4.23×10^{-2} 이하로 낮은 편이었다. 재생배지에 나타난 colony에는 완전히 재생되지 않은 L-form cell도 함께 존재하였다.

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