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Protoplast Formation and Regeneration in *Lactobacillus helveticus*

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*Lactobacillus helveticus*의 Protoplast 형성과 재생에 관한 연구

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Abstract — The optimal conditions for the production and regeneration of *L. helveticus* protoplasts were examined. The protoplast formation of *L. helveticus* was most efficient obtained when the cells grown to mid and late logarithmic phase in MRS medium were used. The maximum number of protoplasts was obtained when lysozyme and mutanolysin were used to lysis the cell wall in 20mM HEPES buffer (pH 7.0) containing 1 M sucrose. Regeneration was accomplished with a complex medium containing 10% sucrose, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 5% gelatin and 0.5% bovine serum albumin. The regeneration frequency of the protoplasts was 10~20% after 5 days of incubation at 30℃.

Members of the genus *Lactobacillus* play an important role in the fermentation of foods for human and animal consumption. They are also among the dominant microorganisms found in the stomach and small intestines of humans and animals. Lactobacilli and other lactic acid bacteria are distributed widely in nature and have been isolated from various sources including human, lower animals, plants, dairy products, wines, and ensilages.

The use of protoplasts for genetic manipulations centres on two major areas: one concerning the use of protoplasts in transformation studies, and the other concerning induction of genetic recombination or new genetic variability by protoplast fusion. Protoplast fusion is a general technique to induce genetic recombination in a variety of procaryotic and eucaryotic microorganisms. The technique is particularly useful for industrial microorganisms which have not been subjected to extensive genetic analysis, because it does not require transducing phages, plasmid sex factors, or a procedure for competency development. It does, however, require establishment of the procedures to form stable protoplasts, to fuse protoplasts, and to regenerate viable cells from fused protoplasts.

Few attempts have been made in the case of *Lactobacillus* spp., because the cell wall of this bacterium is generally resistant to the lytic action of lysozyme (1). Mutanolysin has also been used to produce protoplasts of *L. casei* (2). Techniques for the production and regeneration of protoplasts in *Bacillus* spp. (3) and *Streptococcus* spp. (4) have been established. However, for *Lactobacillus* spp., a few reports on protoplast formation and regeneration have appeared (5).

It seems extremely important to establish genetic techniques applicable to commercially important organism. For this purpose, the optimal conditions for protoplast formation and the subsequent rege-

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neration of the intact cells of *L. helveticus* were examined.

Materials and Methods

Bacterial strains and Media

L. helveticus IAM 12090 was maintained in lyophilized vials and routinely cultured in MRS medium (6). Cells were subcultured through biweekly passage in MRS broth for protoplast experiments. Regeneration was carried out in MRS medium supplemented with 10% sucrose, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 5% gelatin, and 0.5% heat inactivated (56℃ for 30 min.) filter-sterilized bovine serum albumin. Protoplast forming buffer was prepared by addition of 1.0 M sucrose to the 20 mM HEPES buffer (pH 7.0).

Formation of Protoplasts

Cells were statically cultivated at 37℃ to mid and late logarithmic phase (approximately 1×10^8 CFU/m*l*) in MRS broth. The cells were harvested by centrifugation at 4,000×g for 10 min, washed twice in 20 mM HEPES buffer and then suspended in the protoplast forming buffer. Mutanolysin and lysozyme dissolved in the protoplast forming buffer were filter-sterilized and added to the final concentrations of 5 μg/m*l* and 50 μg/m*l*, respectively. The resulting mixture was incubated at 42℃ for 30 min with occational agitation, and protoplasts formed were observed by light microscopy. Lysis of cells during incubation was monitored at intervals by measuring residual turbidity at 650 nm.

Regeneration of Protoplasts

A suspension of protoplasts obtained by the above procedure was diluted with the protoplast forming buffer and then plated on a regeneration medium. The number of colonies was determined after 3 to 5 days of incubation at 30℃. Osmotically, resistant cells were determined by dilution of the protoplast suspension with sterile water and plating onto MRS agar. Colonies were counted after 2 days of incubation at 37℃. Regeneration frequency was expressed as a ratio of regenerants per CFU(colonies before protoplasting).

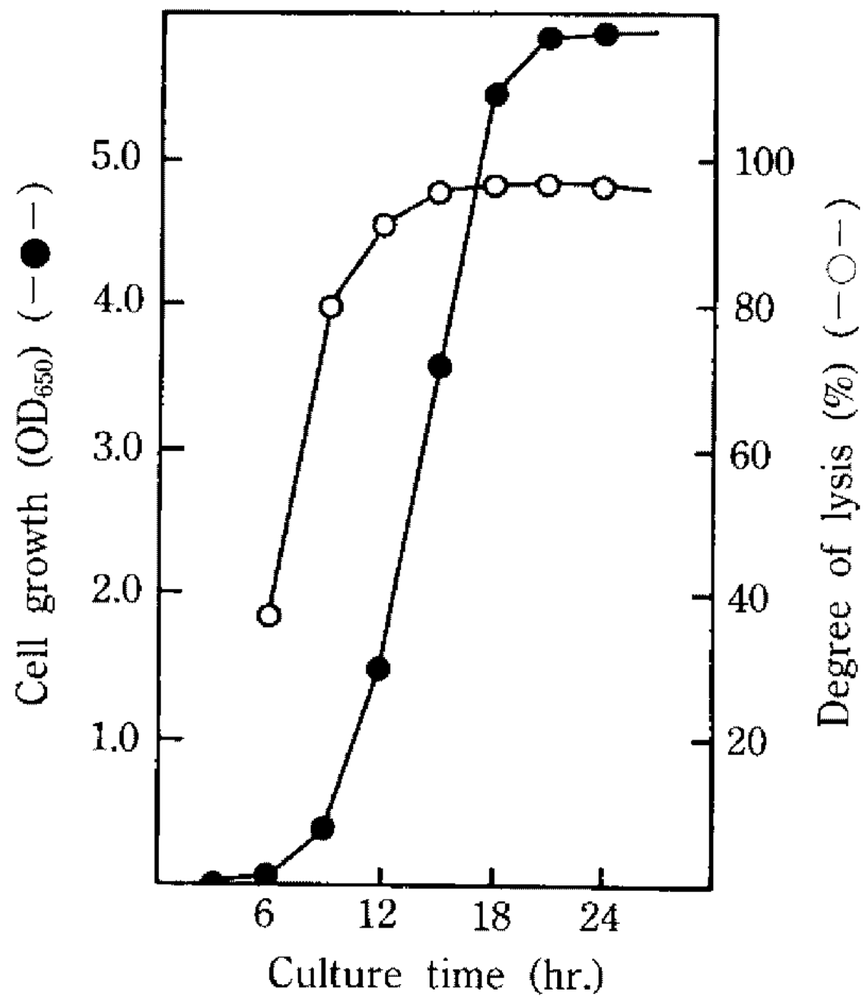

Fig. 1. Effect of growth phase on protoplast formation of *L. helveticus* IAM 12090.
The enzyme concentration added was 50 μg/m*l* lysozyme and 5 μg/m*l* mutanolysin.

Results and Discussion

Production of protoplasts

To determine the optimal physiological state of lysis, a series of cultures was inoculated at 3 hrs intervals, harvested, and subjected to the lytic enzyme. The data presented in Fig. 1 show that mid or late-phase cells were the most easily lysed by the lytic enzyme. This result corresponds with those of Neujahr *et al.* (7) who reported that the protoplast yield was the highest when the cultures of *L. fermenti* at exponential growth phase were used. Whereas, Chassy and Giuffrida (8) reported that the stationary phase cells of *Lactobacillus casei* were more susceptible than those from log-phase cultures.

Most strains of gram-positive bacteria are generally more resistant than gram-negative genera to the action of lysozyme (8). The outer layers of the cell walls of most lactobacilli contain regular arrays of proteins, which may explain why these cells were less susceptible to the lytic action of lysozyme. Kondo and Mckay (9) reported protoplast formation by use of mutanolysin. To obtain complete lysis, we treated with a mixture of lysozyme and mutano-

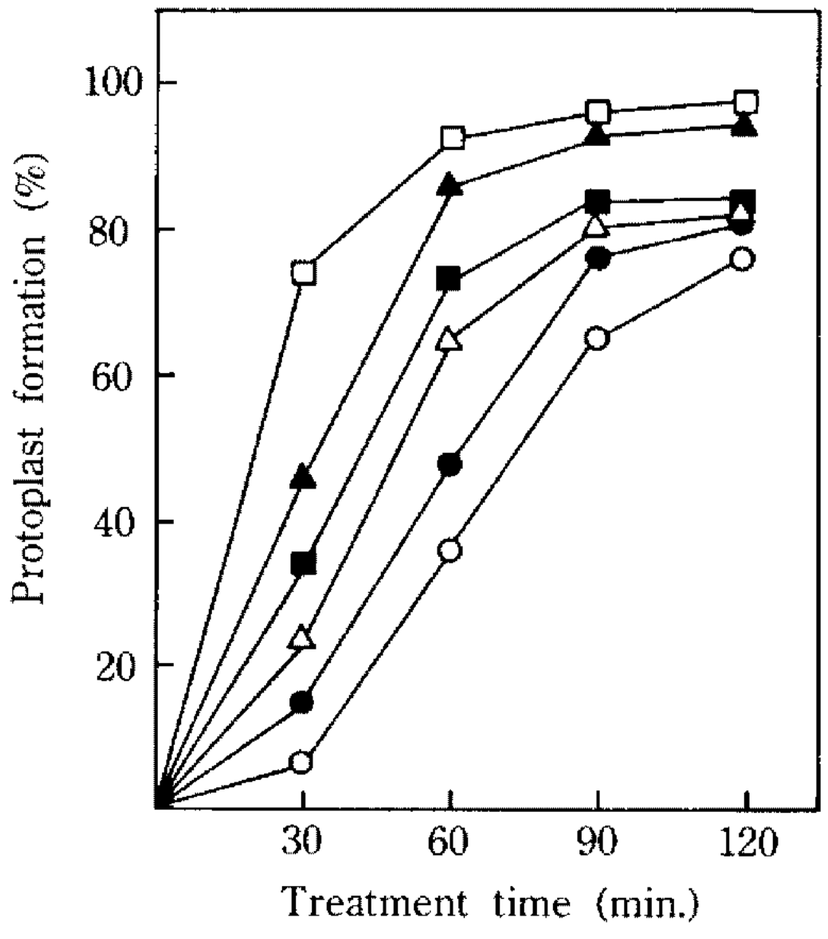

Fig. 2. Effect of sucrose concentrations on protoplast formation of *L. helveticus* IAM 12090.
Reaction was performed with 50 μg/m*l* lysozyme and 5 μg/m*l* mutanolysin dissolved in the 20 mM HEPES buffer containing various concentration's of sucrose.
○; none, ●; sucrose 0.25 M, △; sucrose 0.5 M, ▲; sucrose 0.75 M, □; sucrose 1.0 M, ■; sucrose 1.5 M

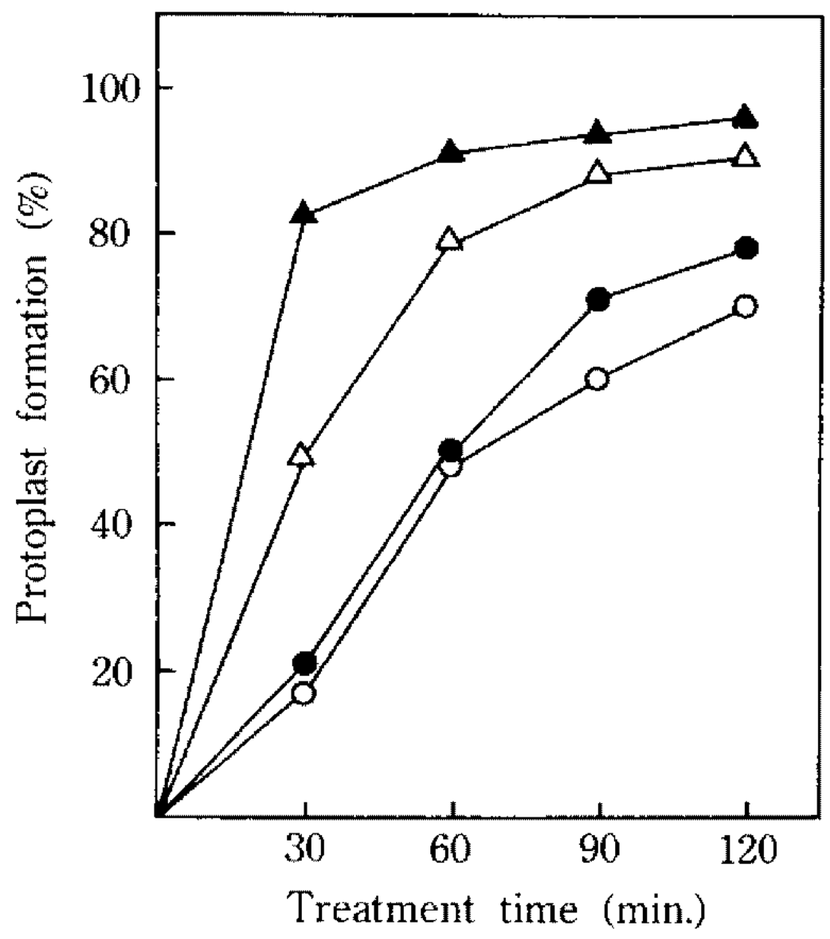

Fig. 3. Effect of reaction temperatures on protoplast formation of *L. helveticus* IAM 12090.
Reaction was performed with 50 μg/m*l* lysozyme and 5 μg/m*l* mutanolysin dissolved in the protoplast forming buffer.
○; 25℃, ●; 30℃, △; 37℃, ▲; 42℃

lysin.

As a osmotic stabilizer for protoplast formation each of sucrose, $MgCl_2$, and $CaCl_2$ added to 20 mM HEPES buffer (pH 7) were examined. As shown in Fig. 2, optimum concentration of sucrose was observed to be 1 M, and the protoplast yield dropped when the sucrose concentration was higher than 1 M. Sucrose was suggested to act not only as an osmoregulator but also as a stimulator for cell wall hydrolysis. But the effects of $MgCl_2$ and $CaCl_2$ were not so significant for preparation of protoplast(data not shown).

Fig. 3 shows how temperature affects the final degree of lysis. It exhibits the hihgest degree of lysis at 42℃. It was higher than the optimum growth temperature of *L. helveticus*.

The optimum pH for the cell lysis was determined using protoplast forming buffer and it was observed that the maximum yield of protoplast was obtained at pH 7.0 (Fig. 4). Baek *et al.* (10) reported that the optimum pH of mutanolysin and lysozyme for the release of protoplast in *L. casei* was pH 7.0.

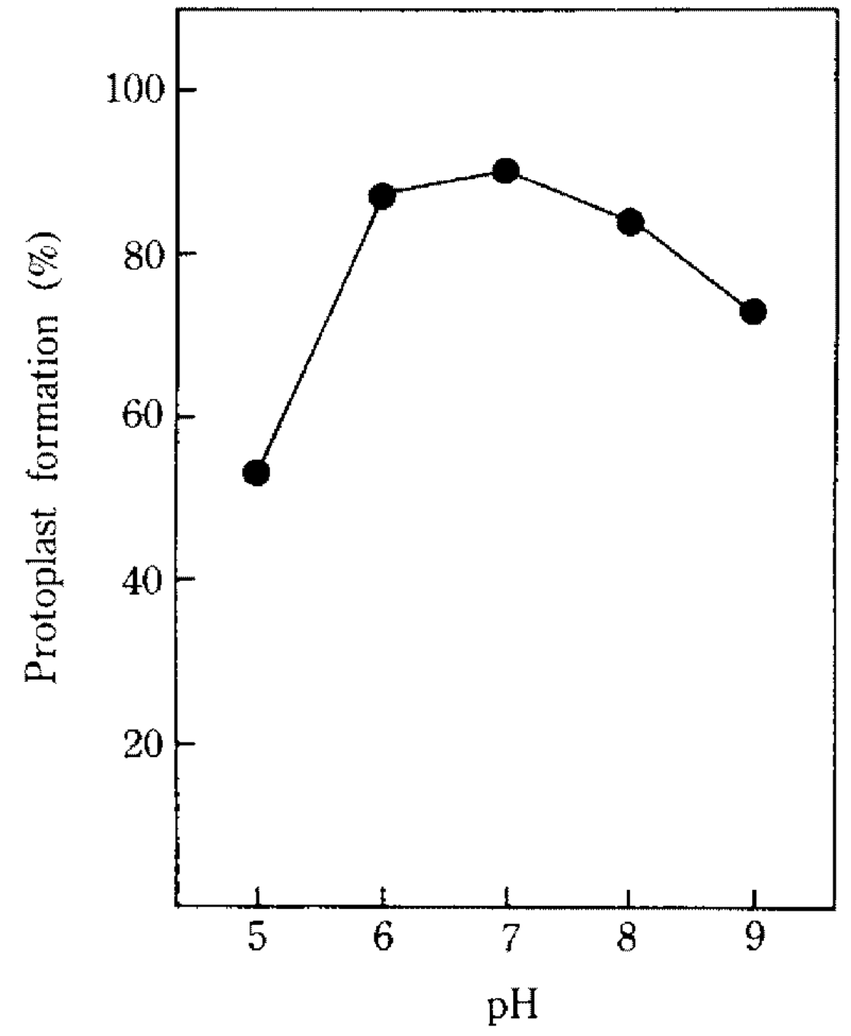

Fig. 4. Effect of pH on protoplast formation of *L. helveticus* IAM 12090.
The enzyme concentration added was 50 μg/m*l* lysozyme and 5 μg/m*l* mutanolysin.

Regeneration of protoplast

One of the general and fundamental properties of all living systems is the ability to repair the injury made to their structures and functions. Protoplast regeneration is no more than an expression of this ability. Baltz (11) has reported on the condi-

tions for effective regeneration of streptomycete protoplasts: a) to everlay soft agar together with protoplasts over the basal agar layer of medium, b) to dry the surface of the underlayer of the agar plates before plating protoplasts, and c) to incubate the protoplasts at lower temperatures for regeneration.

Fig. 5 shows the effect on regeneration frequency of various concentrations of mutanolysin and lysozyme used for formation of protoplasts of *L. helveticus*. To obtain the maximum lysis and regeneration frequency, the enzyme concentration should be 50 μg of lysozyme and 5 μg of mutanolysin per m*l*.

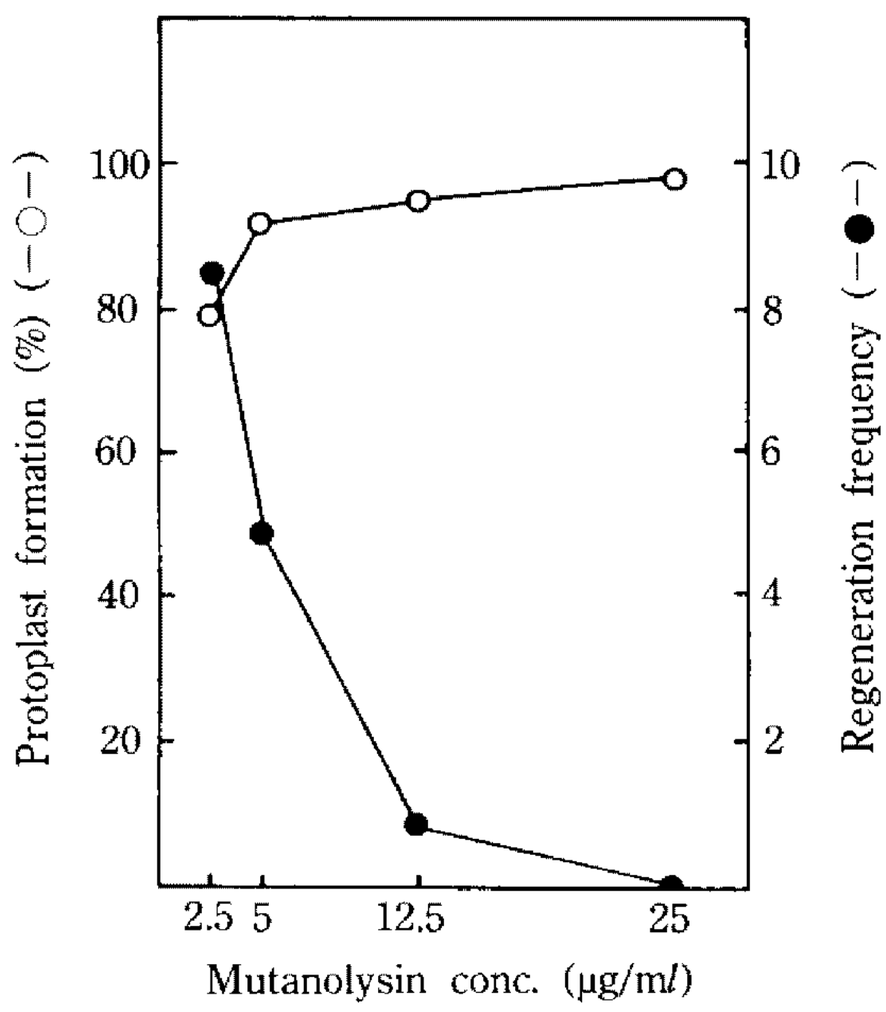

Fig. 5. Effect of mutanolysin concentration on the formation and regeneration of protoplasts of *L. helveticus* IAM 12090.
Cells were treated with mutanolysin and lysozyme (50 μg/m*l*).

Regeneration of protoplasts was dependent upon the length of exposure time of the cells to the lytic enzymes. The higher regeneration frequency was obtained from the protoplasts formed by shorter time of the enzyme treatment (Table 1). However, a short enzyme treatment time for a high regeneration frequency, was not always beneficial, because partial hydrolysis of cell walls could prevent the protoplasts from fusing. On the other hand, a primer of cell walls was necessary for the regeneration of protoplasts. Therefore, the optimal enzyme treatment time should be determined. Similar result was reported by Cha *et al.* (12), who showed that the regeneration frequency decreased with increasing the exposure time.

To find suitable conditions for the regeneration of *L. helveticus* protoplasts, we examined various osmotic stabilizers. Sucrose, $MgCl_2$, and $CaCl_2$ facilitated regeneration (Table 2). The optimal concentrations of sucrose, $MgCl_2$, and $CaCl_2$ for regeneration were found to be 10%, 10 mM, and 20 mM, respectively. However, Kaneko and Sakaguchi (13) used the regeneration medium consisting of 0.5 M sodium succinate for the regeneration of *Brevibacterium flavum* protoplasts.

Recently, Akamatsu and Sekiguchi (14) reported that the regeneration frequency of protoplasts of *Bacillus subtilis* was remarkably enhanced by the addition of an artificial plasma expander and casamino acid. Thus, we examined the effects of two

Table 1. Effect of the enzyme treatment time on formation and regeneration of the protoplasts of *L. helveticus* IAM 12090

Treatment time (min)	Number of regenerated cells (CFU/m*l*)[1]	Number of ORC[2] (CFU/m*l*)	Frequency of protoplasts (%)	Frequency of regeneration (%)
15	4.1×10^8	2.5×10^8	58.3	45.7
30	8.0×10^7	2.3×10^7	96.2	9.9
45	2.9×10^7	7.8×10^6	98.7	3.6
60	5.0×10^6	2.6×10^6	99.6	0.4

Number of initial cells was 6×10^8/m*l*.
The enzyme concentration was lysozyme 50 μg/m*l* and mutanolysin 5 μg/m*l*.
[1]CFU: colony forming unit
[2]ORC: osmotic resistant cell

Table 2. Effect of osmotic stabilizers on regeneration of the protoplasts of *L. helveticus* IAM 12090

Stabilizer	No. of regenerated cells (CFU/m*l*)[1]	Regeneration frequency (%)
Sucrose		
1%	3.5×10^7	4.2
5%	3.8×10^7	4.8
10%	3.9×10^7	5.1
20%	2.8×10^7	2.6
$MgCl_2$		
5 mM	4.3×10^7	5.9
10 mM	4.7×10^7	6.8
20 mM	4.6×10^7	6.6
30 mM	3.9×10^7	5.1
$CaCl_2$		
5 mM	3.4×10^7	4.0
10 mM	3.4×10^7	4.0
20 mM	3.9×10^7	5.1
30 mM	3.1×10^7	3.3

No. of initial cells was 4.7×10^8/m*l*.
No. of osmotic resistant cells was 1.6×10^7m*l*.
[1]CFU: colony forming unit

Table 3. Effect of plasma expanders on regeneration of the protoplasts of *L. helveticus* IAM 12090

Plasma expanders	No. of regenerated cells (CFU/m*l*)[1]	Regeneration frequency (%)
Gelatin		
0%	4.0×10^7	4.5
1.0%	8.6×10^7	10.5
2.5%	1.0×10^8	12.3
5.0%	1.1×10^8	13.6
Gelatin 5.0% +BSA 0.5%	1.3×10^8	16.3

No. of initial cells was 7.7×10^8/m*l*.
No. of osmotic resistant cells was 5.8×10^6/m*l*.
BSA (bovine serum albumin) was inactivated at 56℃ for 30 min., and added to the basal regeneration medium supplemented with 5% gelatin and 10% sucrose.
[1]CFU: colony forming unit

chemicals having similar protperties of plasma expanders, gelatin and BSA, on the regeneration of *L. helveticus* protoplasts using the regeneration medium (MRS medium supplemented with 10% sucrose, 10 mM $MgCl_2$, 20 mM $CaCl_2$) as a basal medium. The result of this series of experiments is shown in Table 3. The presence of gelatin improved the regeneration yield by threefold. The addition of 0.5% BSA improved the regeneration yield by fourfold. Perhaps the gelatin matrix could induce the formation of cell wall only along certain small areas of the membrane.

Table 4. Effect of incubation temperature on regeneration of the protoplasts of *L. helveticus* IAM 12090

Incubation temperature (℃)	No. of regenerated cells (CFU/m*l*)[1]	Regeneration frequency (%)
25	3.0×10^6	–
30	3.1×10^8	12.8
37	2.6×10^8	10.6
42	2.3×10^8	9.3

No. of initial cells was 2.3×10^9/m*l*.
No. of osmotic resistant cells was 1.8×10^7/m*l*.
[1]CFU: colony forming unit

Table 5. Effect of culturing methods on regeneration of the protoplasts of *L. helveticus* IAM 12090

	No. of cells (CFU/m*l*)[1]	Frequency of regeneration (%)
Initial cell	1.6×10^8	
ORC[2]	3.8×10^6	
Surface plate method	3.0×10^7	16.8
Soft agar double layer method	3.6×10^7	20.6
Pour plate method	3.9×10^7	22.5

[1]CFU: colony forming unit
[2]ORC: osmotic resistant cell

As shown in Table 4, the regeneration of *L. helveticus* protoplasts was also affected by the incubation temperature. The regeneration efficiency was found to be highest at 30℃. However, Kanatani *et al.* (15) incubated the protoplasts of *L. plantarum* at 37℃ for efficient regeneration.

To improve the regeneration frequency, the pour plate method used successfully in protoplast regeneration of yeast (16) was applied to *Lactobacillus helveticus*. The modification of the plating procedure by Shirahama *et al.* could improve even higher the regeneration efficiency (17). Table 5 indicates that the frequency was increased 1.3 fold by changing the procedure from spreading to pour plating. In *Streptomyces rimosus*, hypertonic soft agar overlay

onto a solid agar layer enhanced the regeneration of protoplasts (18). Under the above conditions the regeneration frequency was about 20%.

It may also be possible to obtain interspecies genetic recombinants in *Lactobacillus* using these techniques.

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요 약

*L. helveticus*의 protoplast 형성과 재생에 대한 최적 조건에 대하여 검토하였다. MRS 배지에서 *L. helveticus*를 배양했을 때 대수증식기 중반에서 후반이 protoplast 형성에 가장 좋은 시기였다. Sucrose 1 M을 함유한 20 mM HEPES 완충액(pH 7.0)에서 lysozyme과 mutanolysin을 혼합 병용했을 때 가장 좋은 protoplast 형성을 나타내었다. Protoplast의 재생은 10% sucrose, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 5% gelatin과 0.5% bovine serum albumin을 함유한 복합배지에서 가장 양호하였다. Protoplast의 재생율은 30 ℃에서 5일간 배양했을 때 10~20%였다.

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