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Production and Regeneration of *Lactobacillus bulgaricus* Protoplasts

JUN, HONG KI*, HYUN JEONG PARK, HYUNG SUK BAIK, AND JAE CHUL SONG[1]

Department of Microbiology, College of Natural Sciences
Pusan National University, Pusan 609-735, Korea
[1]Department of Food and Nutrition, College of Natural Sciences
University of Ulsan, Ulsan 680-749, Korea

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Conditions for the production and regeneration in *Lactobacillus bulgaricus* protoplasts were investigated. Protoplasts of *L. bulgaricus* strains were obtained by treatment with mutanolysin and lysozyme together in a protoplast forming buffer containing 0.02 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.0) and 0.5 M sucrose. High protoplast yield was obtained from cells cultured in the de Man, Rogosa and Sharpe(MRS) medium at the middle to late logarithmic growth phase. Regeneration was efficiently accomplished with a complex medium containing 1% sucrose, 20 mM $MgCl_2$, 5% gelatin, and 0.5% bovine serum albumin. The frequency of regeneration of protoplasts was 10~20% after 5 days of incubation at 30°C.

Characteristics that make the lactobacilli important in foods are their ability to ferment sugars with the production of considerable amounts of lactic acid, which makes it possible to use them in the production of fermented plant and dairy products or the manufacture of industrial lactic acid. Also, the lactobacilli has the heat resistance, or thermoduric properties, of most of the high-temperature lactobacilli, enabling them to survive pasteurization or other heating processes, such as that given the curd in the manufacture of Swiss and similar cheeses.

Protoplast fusion is one of the most promising methods of gene transfer in both genetical studies and improvement of starter strains for practical use in dairy industries. Basic techniques for genetic manipulation involving protoplast fusion and transformation are well established in certain bacteria of industrial importance (7, 8, 16) but are less advanced in lactobacilli.

The cell wall of *Lactobacillus* is generally assumed to be resistant to the lytic action of egg white lysozyme. Lactobacilli are thought to be more resistant to muramidase than are streptococci. Since Tomochika *et al.* (19) produced protoplasts of *L. casei* by treatment with mutanolysin, an endo-N-acetyl muramidase isolated from *Streptomyces globisporus*, Lee-Wickner and Chassy (13) have reported the preparation of *L. casei* protoplasts by using mutanolysin in combination with lysozyme.

First of all, to develop a system for protoplast fusion in lactobacilli, we investigated the optimal conditions for protoplast formation and regeneration of *L. bulgaricus*.

*Corresponding author
Key words: *Lactobacillus bulgaricus*, protoplast formation, regeneration

MATERIALS AND METHODS

Bacterial Strains and Media

L. bulgaricus IFO 13953 was maintained in lyophilized vials and routinely cultured in MRS medium (6). Cells were subcultured one time in MRS broth for protoplast experiments. The medium for cell wall regeneration was MRS medium containing 1% sucrose, 20 mM $MgCl_2$, 5% gelatin and 0.5% bovine serum albumin (BSA). A solution of BSA was heat inactivated at 56℃ for 30 min and sterilized by filtration through a membrane filter. Protoplast forming buffer (PB) consisted of 20 mM HEPES (pH 7.0) and 0.5 M sucrose.

Formation of Protoplasts

Cells were grown at 37℃ to mid and late logarithmic phase in MRS broth [about 1×10^8 CFU (colony forming unit)/ml]. The cells were harvested by centrifugation at 4000×g for 10 min, washed twice in 20 mM

HEPES buffer and then suspended in PB. Mutanolysin and lysozyme were added to final concentrations of 2.5 μg/ml and 50 μg/ml, respectively.

The suspension was incubated at 37℃ for 10 min with occasional agitation. Protoplast formation was judged by noting the appearance of spherical cells under light microsocopy. Also, protoplast formation was observed by measuring the decrease in turbidity at 650 nm.

Regeneration of Protoplasts

The protoplast suspension was diluted with PB and plated with the regeneration agar medium. Colonies were counted after 3~5 days of incubation at 30℃, because the regeneration protoplasts grew very slowly. 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℃. The frequency of regeneration was defined as the ratio of net regenerants per initial cell number.

RESULTS AND DISCUSSION

Production of Protoplasts

The conditions suitable for protoplast formation by lytic enzyme treatment were examined. Baltz (4) reported a critical "transition" stage between exponential and stationary growth for the preparation of protoplasts of *Streptomyces fradiae* and *S. griseofuscus* that would be capable of good regeneration and use in fusion. In preliminary experiments, it was found that the middle or late logarithmic growth phase was the best time for the efficiency of protoplast formation (Fig. 1). Kang *et al.* (10) reported that *Lactobacillus casei* cells from the logarithmic growth phase to the early stationary phase were most susceptible to mutanolysin treatment.

Lysozyme hydrolyzes repetitive N-acetylglucosamine-β-1,4-N-acetyl muramic acid bonds present in the bacterial cell wall. This hydrolysis frequently causes overt cellular lysis of gram-negative bacteria unless an osmotic stabilizer is present to protect the osmo-fragile spheroplasts that result from the action of muramidase. It is generally well recognized that the cell wall of *Lactobacillus* is susceptible to mutanolysin although they are rather resistant to egg white lysozyme (5). In contrast to other lactic acid bacteria, cell walls of *L. bulgaricus* were better lysed by lysozyme. To obtain more complete lysis in the absence of an osmotic stabilizer, we treated together with lysozyme and mutanolysin.

In order to increase the stability of the protoplasts, osmotic stabilizer was added to 20 mM HEPES buffer. As shown in Fig. 2, 0.5 M sucrose increased the stability of protoplasts and the protoplast yield dropped when

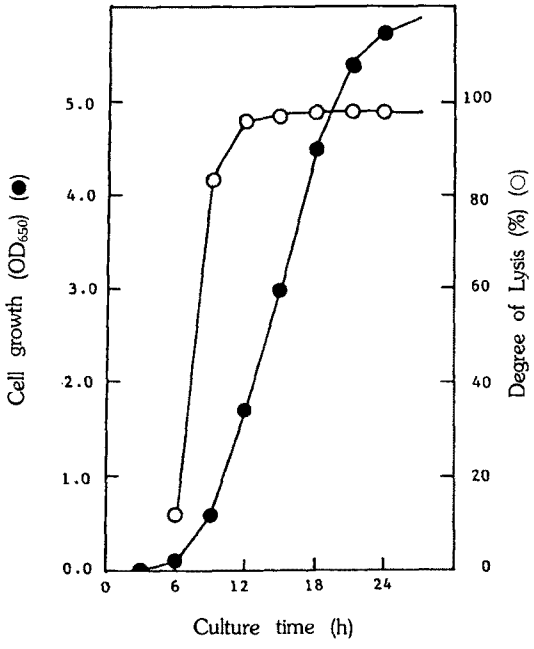

Fig. 1. Effect of growth phase on protoplast formation of *L. bulgaricus* IFO 13953.
The enzyme concentration was 50 μg/ml lysozyme and 2.5 μg/ml mutanolysin.

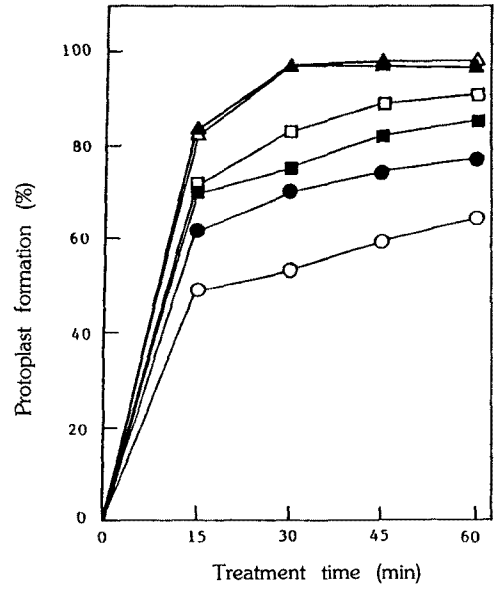

Fig. 2. Effect of the sucrose concentration on protoplast formation of *L. bulgaricus* IFO 13953.
Reaction was performed with 50 μg/ml lysozyme and 2.5 μg/ml mutanolysin dissolved in the 20 mM HEPES buffer containing various sucrose concentrations.
○; none, ●; sucrose 0.25 M, △; sucrose 0.5 M, ▲; sucrose 0.75 M, □; sucrose 1.0 M, ■; sucrose 1.5 M

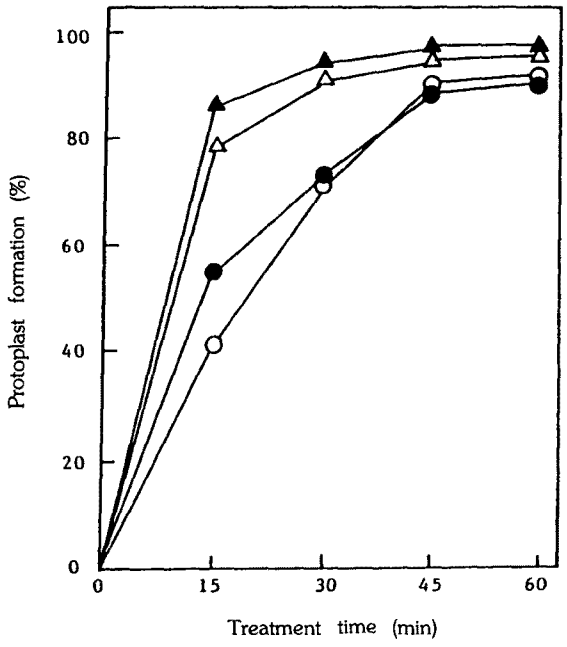

Fig. 3. Effect of reaction temperature on protoplast formation of *L. bulgaricus* IFO 13953.

Reaction was performed with 50 μg/ml lysozyme and 2.5 μg/ml mutanolysin dissolved in the protoplast forming buffer.

○; 25℃, ●; 30℃, △; 37℃, ▲; 42℃

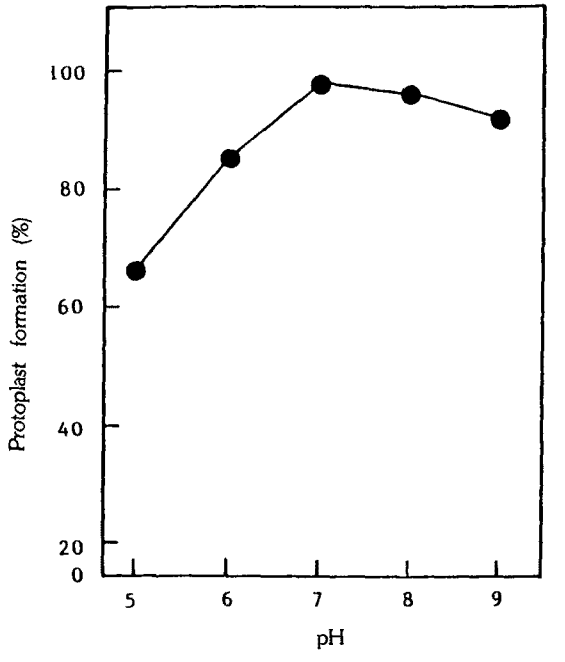

Fig. 4. Effect of pH on protoplast formation of *L. bulgaricus* IFO 13953.

The enzyme concentration was 50 μg/ml lysozyme and 2.5 μg/ml mutanolysin.

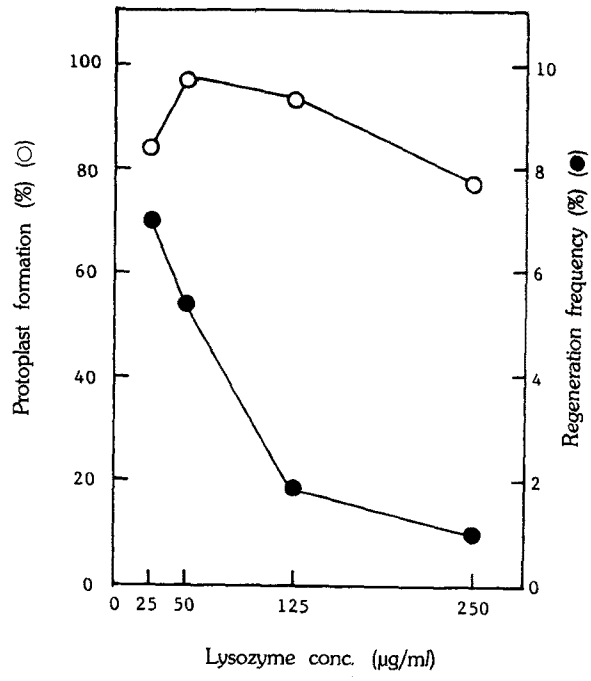

Fig. 5. Effect of lysozyme concentration on protoplast formation and regeneration of *L. bulgaricus* IFO 13953.

Cells were treated with lysozyme and mutanolysin (2.5 μg/ml).

the sucrose concentration was higher than 0.5 M. Sucrose was suggested to act not only as an osmotic stabilizer but also aid the lysozyme action (18). However, Mg^{2+} or Ca^{2+} was not effective for protoplast formation (data not shown).

As shown in Fig. 3, the highest yield of protoplasts was obtained by incubating at the temperature range of 37℃ to 42℃. It was similar to optimum growth temperature of *L. bulgaricus*.

Optimum pH for protoplast formation 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.* (3) reported that the optimum pH of mutanolysin and lysozyme for the release of protoplast in *L. casei* was pH 7.0.

Regeneration of Protoplasts

One of the general and fundamental properties of all living systems is the ability to repair injury made to their structures and functions. Protoplast regeneration is no more than an expression of this ability. Fig. 5 shows the effect on regeneration frequency of various concentrations of lysozyme and mutanolysin. When the concentration of lysozyme was greater than 50 μg/ml, a decrease in frequency of protoplast formation was observed. It was found that 2.5 μg mutanolysin per ml and 50 μg lysozyme per ml were suitable for for-

Table 1. Effect of the enzyme treatment time on protoplast formation and regeneration of protoplast of *L. bulgaricus* IFO 13953

Treatment time (min)	Number of regenerated cells (CFU/ml)[1]	Number of ORC[2] (CFU/ml)	Frequency of protoplasts (%)	Frequency of regeneration (%)
10	1.5×10^7	4.6×10^6	96.7	7.7
20	6.8×10^6	9.4×10^5	99.3	4.2
30	1.4×10^6	4.2×10^5	99.9	0.9
40	9.2×10^4	4.2×10^4	99.97	0.04

Number of initial cells was 1.4×10^8/ml.
The enzyme concentration was lysozyme 50 μg/ml and mutanolysin 2.5 μg/ml.
[1]CFU: colony forming unit
[2]ORC: osmotic resistant cell

Table 2. Effect of osmotic stabilizers on the regeneration of protoplasts of *L. bulgaricus* IFO 13953

Stabilizer	No. of regenerated cells (CFU/ml)[1]	Regeneation frequency (%)
Sucrose		
1%	1.6×10^8	7.2
5%	7.9×10^7	2.0
10%	2.0×10^5	–
20%	3.4×10^4	–
$MgCl_2$		
5 mM	1.86×10^8	8.9
10 mM	2.17×10^8	10.9
20 mM	2.36×10^8	12.1
30 mM	2.40×10^8	12.4
$CaCl_2$		
5 mM	8.3×10^7	2.2
10 mM	9.1×10^7	2.8
20 mM	8.0×10^7	2.1
30 mM	7.9×10^7	2.0

No. of initial cells was 1.6×10^9/ml.
No. of osmotic resistant cells was 4.8×10^7/ml.
[1]CFU: colony forming unit

mation of protoplasts that could be efficiently regenerated on regeneration medium.

The lytic enzyme treatment time affected the regeneration frequency (Table 1). The longer the treatment time was, the lower the regeneration frequency was. The optimum treatment time for the formation and regeneration of protoplasts was determined to be 10 mins. This result suggested that prolonged enzyme treatment caused almost complete hydrolysis of the cell walls without leaving a residual primer which is necessary for regeneration.

Since the successful regeneration of protoplasts depends on the composition of the regeneration medium, regeneration medium was developed. In order to examine the effect of osmotic stabilizers on protoplast regeneration, various osmotic stabilizers were added to the regeneration medium. As shown in Table 2, the regeneration frequency was significantly improved by the addition of 1% sucrose and 20 mM $MgCl_2$ to the medium. The effect of $CaCl_2$ was not significant. These results were slightly different from those in protoplast formation where 0.5 M sucrose was the most effective as osmotic stabilizer.

Table 3. Effect of plasma expanders on the regeneration of protoplast of *L. bulgaricus* IFO 13953

Plasma expanders	No. of regenerated cells (CFU/ml)[1]	Regeneration frequency (%)
Gelatin 0%	1.7×10^8	6.7
1.0%	1.9×10^8	8.5
2.5%	2.1×10^8	10.3
5.0%	2.6×10^8	14.9
Gelatin 5.0% + BSA 0.5%	2.9×10^8	17.6

No. of initial cells was 1.2×10^9/ml.
No. of osmotic resistant cells was 9.6×10^7/ml.
BSA (bovine serum albumin) was inactivated at 56℃ for 30 min, and added to the basal regeneration medium supplemented with 5% gelatin and 1% sucrose. [1]CFU: colony forming unit

Since gelatin, horse serum, and bovine serum albumin (BSA), often used for regeneration media (1, 15), are all plasma expanders, it seemed interesting to examine the relationship between plasma expanders and the stimulative factors for regeneration frequency. For this purpose, we chose two chemicals having the properties of plasma expanders, gelatin and bovine serum albumin, and examined the stimulative effect on regeneration frequency. Gelatin increased survival of protoplasts about 2 times and the addition of 0.5% BSA improved the regeneration frequency greater (Table 3). Gelatin was suggested to serve as a stabilizer of protoplasts by coating the membrane and as a direct source for cell wall synthesis. In *B. subtilis*, Gabor and Hotchkiss (8) reported a similar stimulative effect by the use of a low concentration, 0.5%, of gelatin.

The regeneration of protoplasts was affected by the incubation temperature for the regeneration. The data presented in Table 4 demonstrate that the best regeneration was obtained by incubating at 30℃ below the optimum growth temperature of *L. bulgaricus*.

For cell wall regeneration, four types of culturing methods are often used, i.e. gelatin medium (11), agar

Table 4. Effect of incubation temperature on the regeneration of protoplasts of *L. bulgaricus* IFO 13953

Incubation temperature (℃)	No. of regenerated cells (CFU/ml)[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/ml.
No. of osmotic resistant cells was 1.8×10^7/ml.
[1]CFU: colony forming unit

Table 5. Comparison of culture methods on the regeneration of protoplasts of *L. bulgaricus* IFO 13953

	No. of cells (CFU/ml)[1]	Frequency of regeneration (%)
Initial cell	1.2×10^9	
ORC[2]	9.6×10^7	
Surface plate method	2.9×10^8	17.6
Soft agar double layer method	3.0×10^8	18.5
Pour plate method	3.3×10^8	21.2

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

medium (4), soft agar layer (2, 9, 17), and pour plate methods (12). To improve the regeneration frequency, the overlaying method which was successfully used in protoplast regeneration of yeast (14) was applied to *L. bulgaricus*. As shown in Table 5, we found that the regeneration frequency of protoplasts was significantly affected by the embedding procedure. The perfect embedding of protoplasts into agar is very improtant.

These methods can be widely applicable to *Lactobacillus* strains and provide a basis for protoplast fusion and transformation experiments with these organisms.

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REFERENCES

1. **Akamatsu, T. and J. Sekiguchi.** 1981. Studies on regeneration media for *Bacillus subtilis* protoplasts. *Agric. Biol. Chem.* **45**: 2887-2894.
2. **Akamatsu, T. and J. Sekiguchi.** 1984. An improved method of protoplast regeneration for *Bacillus* species and its application to protoplast fusion and transformation. *Agric. Biol. Chem.* **48**: 651-655.
3. **Baek, Y.J., H.S. Bae, M. Yoo, Y.K. Kim, and H.U. Kim.** 1986. A study on the protoplast formation and regeneration of *Lactobacillus casei* YIT 9018. *Kor. J. Appl. Microbiol. Bioeng.* **14**: 251-257.
4. **Baltz, R.H.** 1978. Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. *J. Gen. Microbiol.* **107**: 93-102.
5. **Chassy, B.M. and A. Giuffrida.** 1980. Method for the lysis of gram-positive, asporogenous bacteria with lysozyme. *Appl. Environ. Microbiol.* **39**: 153-158.
6. **de Man, J.C., M. Rogosa, and M.E. Sharpe.** 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23**: 130-135.
7. **Fodor, K. and L. Alfoldi.** 1976. Fusion of protoplasts of *Bacillus megaterium*. *Proc. Natl. Acad. Sci. USA.* **73**: 2147-2150.
8. **Gabor, M.H. and R.D. Hotchkiss.** 1979. Parameters governing bacterial regeneration and genetic recombination after fusion of *Bacillus subtilis* protoplasts. *J. Bacteriol.* **137**: 1346-1353.
9. **Kaneko, H. and K. Sakaguchi.** 1979. Fusion of protoplasts and genetic recombiantion of *Brevibacterium flavum*. *Agric. Biol. Chem.* **43**: 1007-1013.
10. **Kang, Y., J.H. Kim, and D.D.Y. Ryu.** 1987. Protoplast fusion of *Lactobacillus casei*. *Agric. Biol. Chem.* **51**: 22 21-2227.
11. **King, J.R. and H. Gooder.** 1970. Reversion to the streptococcal state of enterococcal protoplasts, spheroplasts, and L-forms. *J. Bacteriol.* **103**: 692-696.
12. **Koo, Y.J., W.S. Park, D.H. Shin, and T.J. Yu.** 1985. Regeneration of yeast protoplast in *Hansenula anomala* var. *anomala* and *Saccharomyces cerevisiae*. *Kor. J. Appl. Microbiol. Bioeng.* **13**: 145-149.
13. **Lee-Wickner, L.J. and B.M. Chassay.** 1984. Production and regeneration of *Lactobacillus casei* protoplasts. *Appl. Environ. Microbiol.* **48**: 994-1000.
14. **Necas, O.** 1961. Physical conditions as important factors for the regeneration of naked yeast protoplasts. *Nature* **192**: 580-581.
15. **Okamoto, T., Y. Fujita, and R. Irie.** 1983. Protoplast formation and regeneration of *Streptococcus lactis* cells. *Agric. Biol. Chem.* **47**: 259-263.
16. **Schaeffer, P., B. Cami, and R.D. Hotchkiss.** 1976. Fusion of bacterial protoplasts. *Proc. Natl. Acad. Sci. USA.* **73**: 2151-2155.
17. **Shirahama, T., T. Furumai, and M. Okanishi.** 1981. A modified regeneration method for streptomycete protoplasts. *Agric. Biol. Chem.* **45**: 1271-1273.
18. **Shirai, M., C. Ishii, and T. Aida.** 1983. Protoplast formation of *Bacillus colistinus*. *Agric. Biol. Chem.* **47**: 877-879.
19. **Tomochica, K., M. Funabashi, A. Nagata, T. Fujii, and Y. Kanemasa.** 1982. Protoplast formation of *Lactobacillus casei* induced by endo-N-acetylmuramidase. *Jap. J. Microbiol.* **37**: 777-785.