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A study on the protoplast formation and regeneration of *Lactobacillus casei* YIT 9018

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Lactobacillus casei YIT 9018의 원형질체 생성과 재생에 관한 연구

백영진 · 배형석 · 유 민 · 김영기 · 김현욱*

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Optimum conditions for the protoplast formation and regeneration of *Lactobacillus casei* have been searched for. *L. casei* cells were converted to protoplast by treating with 10μg/ml of mutanolysin in 20mM potassium phosphate buffer (pH6.8) containing 6mM $CaCl_2$, 6mM $MgCl_2$ and 1M sucrose. Maximum number of protoplasts was obtained when cells were taken from Tomochika's medium containing 0.5% glycine at the middle to late logarithmic growth phase. Regeneration was efficiently accomplished on the regeneration medium containing 6mM $CaCl_2$, 6mM $MgCl_2$, 0.8M sucrose and 10% of horse serum. The efficiency of the cell wall regeneration from protoplasts was 2-5% after 3-4 days of incubation at 30°C.

Bacteria belonging to the genus *Lactobacillus* have very intimate relationship with man, as the important microorganisms used in dairy fermentations, and capabilities. They assist man to digest his food better, to make capabilities. The assist man to digest his food better, to make his food more delicious and more preservable, and to keep his gastro intestinal tract and other mucous intestine membrane healthy and intact. Futhermore other functions of lactobacilli for the fermented foods and the human health are known to be farmore important and diverse[9, 12]. Accordingly, new needs are raised for more informations on the genes and the genetic manipulations of this important genus.

With the progress of molecular genetic, useful genetic transfer systems of lactic acid bacteria, such as transduction[10], conjugation[4, 11] and transformation[7] have been demonstrated. Protoplast fusion has another potential for improving strains of lactic acid bacteria.

Chassy and Giuffrida[3] have reported that lactobacilli can be lysed by lysozyme in the presence of polyethylene glycol (PEG). Mutanolysin has also been reported to produce protoplasts of *L. casei*[13, 14]. Lee-Wickener and Chassy[8] obtained the protoplast of *L. casei* cells after treated with mutanolysin and lysozyme, which required fairly long time.

We attempted to develop the protoplast fusion system as one of the practical gene-mixing tool for the strain development of *L. casei*. First of all, this study has been carried out to elucidate the optimum conditions for the production and regeneration of protoplasts of *L. casei*.

Key Words: *Lactobacillus casei* – protoplast formation, regeneration.

Materials and Methods

Bacterial strains and media

L. casei YIT 9018 used throughout this study has been maintained in ILS stab stock culture and transferred to reconstituted skimmilk 10% (wt/vol) before use. Culture were grown in TCM media[14] for cell crops. Cells were subcultured two or three times in TCM broth. The cells were cultured overnight at 37°C in 40ml TCM broth containing 0.5% glycine. At the mid or late logarithmic growth phase (about 0.8-0.9 absorbance at 650nm), the cells were harvested by centrifugation, washed in 20mM potassium phosphate buffer, and suspended in 10-12ml of protoplast formation buffer (20mM potassium phosphate (pH6.8), 6mM $CaCl_2$, 6mM $MgCl_2$, and 1 M sucrose as a stabilizer. Regeneration medium (RM) was TCM with 0.2% glucose, 0.8M sucrose and agar (Hard medium 1.3%, soft medium 0.6%). Three ml of sterilized soft medium containing the final concentrations of 6mM $MgCl_2$, 6mM $CaCl_2$ and 0.3ml of horse serum (GIBCO LAB) were stirred gently before pouring onto the hard regeneration medium plate.

Protoplast formation

Mutanolysin (Dainippon Pharmaceutical Co. LTD., Japan) dissolved in 20mM potassium phosphate buffer was added to the cell suspension at the final concentration of 10μg per ml of *L. casei* strain. The cell-mutanolysin mixture was incubated at 37°C for 10 to 30min with gentle agitation in a shaking water bath. Protoplasts were searched for under the phase contrast microscope at 1600x. The protoplast formation was observed by measuring turbidity decrease at 650nm.

Regeneration of protoplasts

The protoplast suspension was diluted with protoplast formation buffer, plated on the RM, and incubated at 30°C for 3-5days for the cell regeneration. Colonies of normal cells were enumerated by plating saline-diluted cell suspension on TCM agar medium. The regeneration frequency was the ratio of net regeneration per initial cell number.

Regeneration frequency was calculated as follows;

$$\text{Regeneration frequency (\%)} = \frac{\text{CFU on RM-CFU on ORC}}{\text{Initial cell number-CFU on ORC}} \times 100$$

where, CFU on RM: colony forming units appeared on regeneration medium

CFU on ORC: osmotically resistant cell

Initial cell number: cell number before protoplast formation

Electron microscopic examination of protoplasts

Samples (ca, 10^{10}/ml cells) for the scanning electron microscopy (SEM) were prefixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH7.4) for 2hrs at 4°C, washed three times with 0.1M phosphate buffer. After centrifugation, the pellets were postfixed with 1% OsO_4 in 0.1M phosphate buffer, and dehydrated with ethanol. The samples were treated with isoamyl-acetate for 30 min, dried with CO_2 gas. After gold coating, the specimens were observed and photographed by Hitach electron microscope (Model 2-450, Japan).

The specimen of transmission electron microscopy (TEM) were prepared by the procedure described previously[8]. The ultra thin sections were mounted on copper grids, stained with uranyl acetate followed by Reynolds lead citrate. The ultra thin sections were observed and photographed by Hitachi transmission electron microscope (Model H-50, Japan).

Results and Discussion

Protoplast formation of *L. casei* cells

Lactobacillus casei is one of homofermenting and Gram-positive, rod-shaped lactic bacteria. It is generally well

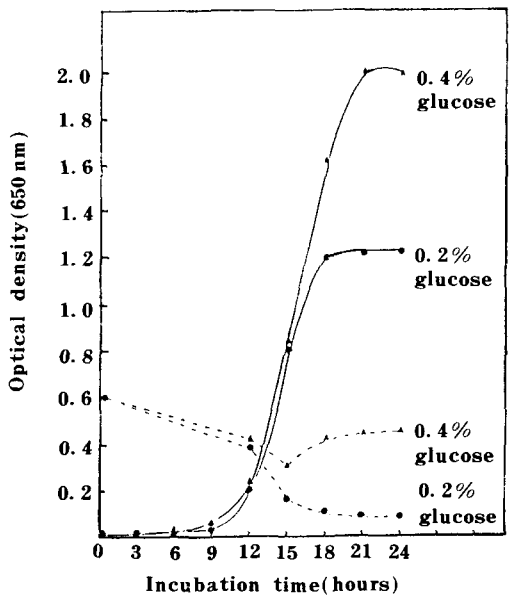

Fig. 1. Effect of growth phase on the formation of protoplasts of *L. casei* YIT 9018 by mutanolysin.

—— : growth, ····· : cell lysis

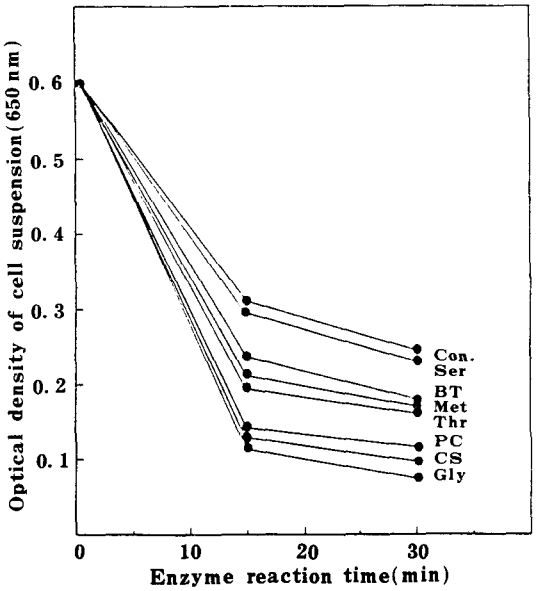

Fig. 2. Effect of several chemicals in TCM broth on the protoplast formation of *L. casei* YIT 9018 cells by mutanolysin.

Con : Control
Ser : Serine (1.5%)
BT : Bacitracin (0.048 I.U./ml)
Met : Methionine (0.1%)
Thr : Threonine (2.0%)
PC : Penicillin (0.15 I.U./ml)
CS : Colistin (142.4 μg/ml)
Gly : Glycine (1.5%)

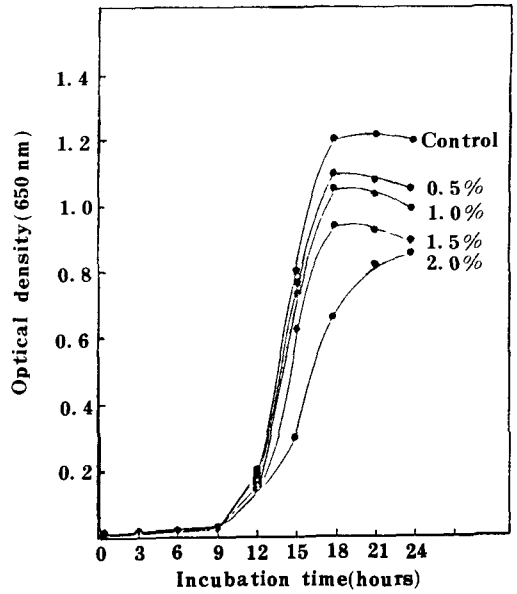

Fig. 3. Effect of glycine concentration in TCM broth on the growth of *L. casei* YIT 9018.

recognized that most lactic acid bacteria are susceptible to mutanolysin (N-acetyl-muramidase) although they are rather resistant to egg white lysozyme[3].

A number of factors appear to influence the lysis of cell walls by mutanolysin, and especially the composition of the growth medium are likely to modify the cell wall properties to cell wall-lysing enzymes. When *L. casei* YIT 9018 was grown in TCM broth containing 0.2% or 0.4% glucose, the cells at the different growth phase illustrated the different sensitivity to mutanolysin, as shown in Fig. 1.

L. casei YIT 9018 cells performed the best growth when TCM broth was supplemented with 0.4% glucose. When these cells were subjected to mutanolysin, the cells grown in TCM broth containing 0.2% glucose were lysed consistantly much better than the cells grown in TCM broth containing 0.4% glucose. The middle or late logarithmic growth phase were found to be the best time for the efficiency of protoplast formation. The earlier informations on several chemicals like DL-threonine and glycine[2,3] and penicillin[6], which are known to affect the lytic properties of the cells in the growth media, have been examined and the results are shown in Fig. 2 and Fig. 3.

As shown in Fig. 2, several amino acids like serine, methionine, threonine, and glycine, and several antibiotics like penicillin, bacitracin, and colistin, actually improved the protoplast formation of *L. casei* cells by mutanolysin. Among the amino acids tested, glycine appears to be the promising candidate for inducing *L. casei* cells more susceptible to mutanolysin. When TCM broth was supplemented with

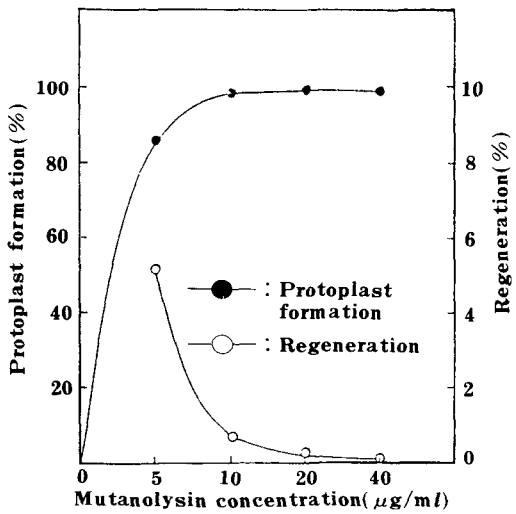

Fig. 4. Effect of mutanolysin concentrations on protoplasts formation and regeneration of *L. casei* YIT 9018 cells.

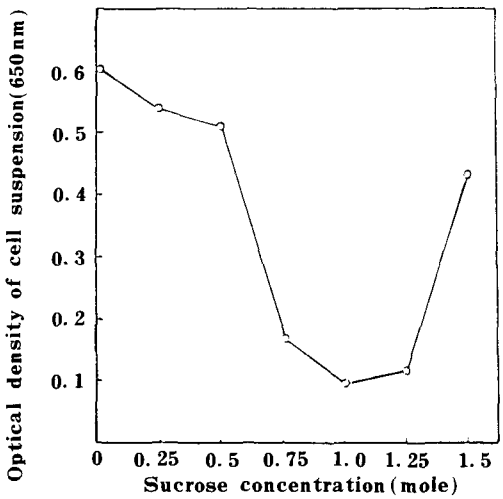

Fig. 5. Effect of sucrose concentration of cell suspension on the formation of protoplasts of *L. casei* YIT 9018 cells by mutanolysin(10 μg/ml).

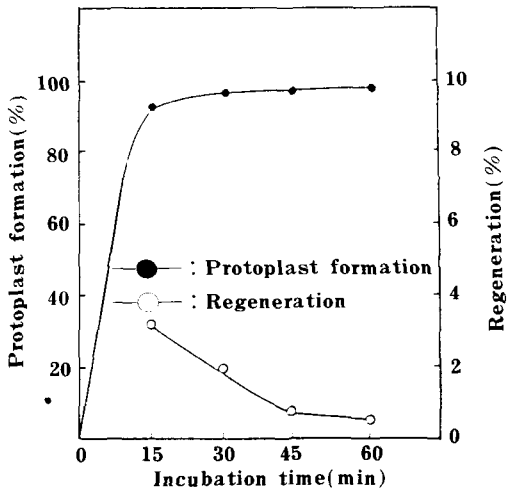

Fig. 7. The formation of protoplasts and regeneration of *L. casei* YIT 9018 cells under the optimum conditions.

0.5% glycine, *L. casei* cells evidenced the good protoplast formation by mutanolysin treatment as well as the near-normal growth as shown in Fig. 3.

Like other lactic acid bacteria, cell walls of *L. casei* YIT 9018 were better lysed by mutanolysin while they are fairly resistant to egg white lysozyme. Although mutanolysin and lysozyme could be used in mixtures, mutanolysin alone was found to be sufficient to induce the efficient protoplast formation of *L. casei* YIT 9018 cells. As shown in Fig. 4, the optimum concentration of mutanolysin appears to be 10μg/ml which produced the maximum number of protoplasts of *L. casei* YIT 9018 cells. When 5μg/ml of mutanolysin was used, the better cell regeneration of the protoplasts was obtained constantly, but protoplast formation was below 90% of the cells.

In order to increase the stability of the induced protoplasts, the protoplast formation buffer has been studied for supplementation with several concentration of stabilizer. As illustrated in Fig. 5 and Fig. 6, the best protoplast crops of *L.*

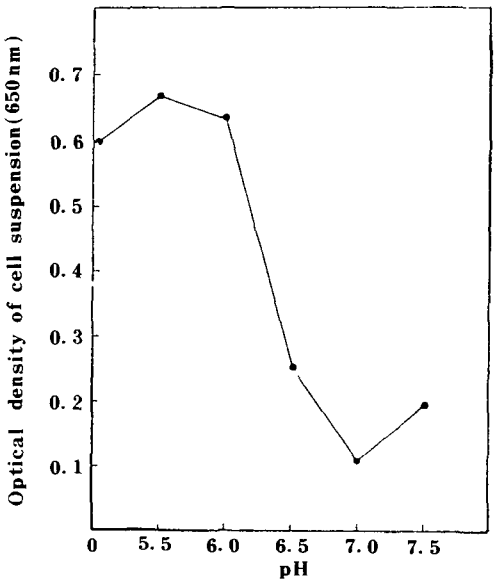

Fig. 6. Effect of pH cell suspension on the formation of protoplast of *L. casei* YIT 9018 cells by mutanolysin.

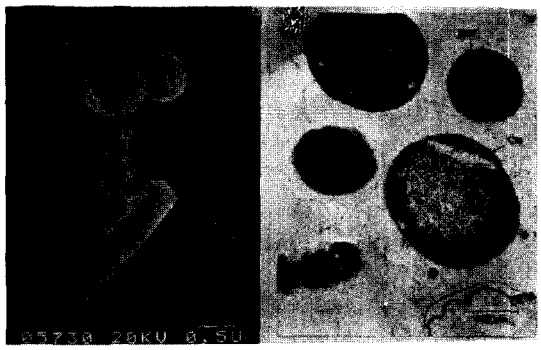

Fig. 8. Electron micrographs of protoplasts of *L. casei* YIT 9018.

A) Scanning electron micrographs (Protoplasts : spherical shape, Intact cells: rod shape)
B) Transmission electron micrographs (CM : cell membrane of protoplast, CW : cell wall of intact cell)

Table 1. Effects of Ca^{2+} and Mg^{2+} on the protoplasts formation and their regenerat on of *L. casei* YIT 9018.

Reaction buffer	OSC* (10^6 CFU/m*l*)	ORC* (10^6 CFU/m*l*)	Protoplasts formation (%)	Regeneration (%)
Sucrose (1 M)	1.5	1.3	99.9	0.02
Sucrose+6 mM Mg^{2+}	11	7.9	99.3	0.28
Sucrose+6 mM Ca^{2+}	19	15	98.6	0.37
Sucrose+6 mM Mg^{2+} +6 mM Ca^{2+}	20	12	98.9	0.74
Sucrose+12 mM Mg^{2+} +12 mM Ca^{2+}	270	190	82.7	8.79
Sucrose+24 mM Mg^{2+} +24 mM Ca^{2+}	560	490	55.5	48

The number of total viable cells before enzyme treatment was 1.1×10^9 per m*l*.
*OSC : Osmotically sensitive cells (protoplasts+ORC)
*ORC : Osmotically resistant cells

casei YIT 9018 cells were obtained when the cells were treated by mutanolysin at 37°C in the protoplast formation buffer of pH 7.0 containing 1 mole sucrose.

When *L. casei* YIT 9018 cells grown in TCM broth containing 0.5% glycine, we could induce more than 90% of cells to form the protoplasts and almost 4% of protoplasts could be regenerated into cells within 10 minutes as illustrated in Fig. 7. The shape of *L. casei* YIT 9018 cells and their protoplasts could be observed in electron micrographs of Fig. 8, in which the cell membrane and chromosome region could be easily distinguishable.

Cell regeneration of protoplasts

Cell regeneration of protoplast was konwn to be affected by serveral factors like culture media, growth phase, temperature, dilution buffer, and osmotic stability. The effects of salts on protoplast formation and regeneration of *L. casei* YIT 9018 cells have been tested in the medium containing different concentrations of Ca^{2+} and Mg^{2+}. As shown in Table 1, the regeneration of *L. casei* YIT 9018 protoplasts was significantly improved by adding 6mM $CaCl_2$ and 6mM $MgCl_2$ to the medium. Cell regeneration reached 8% of the protoplasts when 12mM Ca^{2+} and 12mM Mg^{2+} were supplemented while higher concentration of these salts decreased the protoplast formation. Divalent salts such as $CaCl_2$ and $MgCl_2$ increased the stability of *L. casei* protoplasts and the regeneration frequency. The results of Lee-Wickner and Chassy[8] were compatible with ours, but the higher concentration of these salts decreased the protoplast formation. Considering the results obtained, it was apparent that the optimum concentrations of these salts appear to be around 6mM $CaCl_2$ and 6mM $MgCl_2$, which helped the regeneration of *L. casei* protoplasts while giving the best crops of pro-

Table 2. Effects of agar concentration of soft agar overlay on the regeneration efficiency *L. casei* YIT 9018 protoplasts.

Agar concentration (%)	OSC* (10^7 CFU/m*l*)	ORC* (10^7 CFU/m*l*)	Regeneration (%)	Cells regenerated (10^7 CFU/m*l*)
0	7.2	5.3	1.3	1.9
0.2	6.9	5.3	1.1	1.6
0.4	7.7	5.3	1.7	2.4
0.6	8.2	5.3	2.0	2.9
0.8	7.9	5.3	1.8	2.6
1.0	7.9	5.3	1.8	2.6

The number of total viable cells before enzyme treatment was 1.5×10^9 per m*l*.
*OSC : Osmotically sensitive cells (protoplast+ORC)
*ORC : Osmotically resistant cells

Table 3. Effects of incubation temperature on the regeneration efficiency of *L. casei* YIT 9018 protoplasts.

Incubation temperature (℃)	OSC* (10^7 CFU/ml)	ORC* (10^7 CFU/ml)	Regeneration (%)	Cells regenerated (10^7 CFU/ml)
25	7.5	5.3	1.5	2.2
30	8.5	5.3	2.3	3.2
37	7.5	5.3	1.5	2.2
42	0	5.3	0	0

The number of total viable cells before enzyme treatment was 1.5×10^9 per ml.
* OSC : Osmotically sensitive cells (protoplast + ORC)
* ORC : Osmotically resistant cells

Table 4. Effects of horse serum in soft agar overlay on the regeneration of *L. casei* YIT 9018 protoplasts.

Media containing $MgCl_2$ (mM)	$CaCl_2$ (mM)	HS* (%)	OSC* (10^7 CFU/ml)	ORC* (10^7 CFU/ml)	Regeneration (%)	Cells regenerated (10^7 CFU/ml)
0	0	0	8.0	5.3	1.9	2.7
6	0	0	9.0	5.3	2.6	3.7
0	6	0	9.2	5.3	2.7	3.9
6	6	0	9.3	5.3	2.8	4.0
6	6	10	1.2	5.3	4.7	6.7

The number of total viable cells before enzyme treatment was 1.5×10^9 per ml.
* HS : Horse serum
* OSC : Osmotically sensitive cells (protoplasts + ORC)
* ORC : Osmotically resistant cells

toplasts.

As a method of enhancing the cell regeneration of protoplasts, Kaneko and Sakaguchi[6] introduced the soft agar overlay on the hard regeneration media. Although the differences are not very great, the agar concentration in the overlay soft agar affected the cell regeneration efficiency and the best regeneration was obtained when the soft agar contained 0.6% agar as evident in Table 2.

As shown in Table 3, the regeneration of *L. casei* YIT 9018 protoplasts was affected by the incubation temperature for the regeneration. Although the protoplasts of *L. casei* YIT 9018 were regenerated into cells at the temperature range of 25°C to 37°C, the best regeneration was obtained when the medium was incubated at 30°C below the optimum growth temperature of *L. casei* YIT 9018. Furthermore, Table 4 demonstrated that 10% horse serum in soft agar overlay almost doubled the cell regeneration of *L. casei* YIT 9018 protoplast from around 2% to 4.8%, as reported earlier by several authors.[1, 5]

요 약

Lactobacillus casei 균주의 protoplast 형성과 정상세포로의 재생에 관한 최적조건이 연구되었다. *Lactobacilus casei* 균주들은 sucrose 1 mole이 함유된 20 mM potassium phosphate 완충액 (pH 6.8)에서 mutanolysin을 10 μg/ml 농도로 처리하였을 때 용이하게 protoplast가 형성되었다. Protoplast 의 최대형성은 균주가 0.5%의 glycine이 함유된 TCM 배지에서 생장이 대수기 중기에서 말기에 도달된 세포를 수확하여 사용했을 때에 이루어졌다. 세포 재생은 6 mM $CaCl_2$, 6 mM $MgCl_2$, 0.8 M sucrose 그리고 horse serum 10%가 함유한 재생배지에서 효율적으로 이루어졌다. Protoplast의 세포벽 재생 빈도는 30℃에서 3-4 일간 배양후 2-5% 범위로 나타났다.

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