Protoplast formation and regeneration in *Lactobacillus delbrueckii*

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Abstract Method for production and regeneration of *Lactobacillus delbrueckii* protoplasts are described. The protoplasts were obtained by treatment with a mixture of lysozyme and mutanolysin in protoplast buffer at pH 6.5 with different osmotic stabilizers. The protoplasts were regenerated on deMan, Rogosa and Sharpe (MRS) with various osmotic stabilizers. Maximum protoplast formation was obtained in protoplast buffer with sucrose as an osmotic stabilizer using a combination of lysozyme (1 mg/ml) and mutanolysin (10 µg/ml). Maximum protoplast regeneration was obtained on MRS medium with sucrose (0.5 M) as an osmotic stabilizer. The regeneration medium was also applicable to other species of lactobacilli as well. This is, to our knowledge, the first report on protoplast formation and efficient regeneration in case of *L. delbrueckii*.

Keywords *Lactobacillus delbrueckii* · Protoplast formation · Protoplast regeneration

Introduction

Protoplast fusion is a useful technique in the improvement of microorganisms. This technique allows the exchange of entire genomes between unrelated genera of microorganisms. It is considered as classical or fundamental method of gene transfer in which multiple genes can be introduced in contrast to the advanced methods in molecular biology which are suitable for introducing only one or few genes. We have been successful in transferring cellulase genes (multiple phenotype) using protoplast fusion approach leading to changed properties of enzymes such as cellulases [1, 2] and amylases [3]. Recent reports on genome shuffling in *Lactobacillus* proved the success of protoplast fusion approach which generated new population of strains with additional improvements in acid [4, 5] and glucose tolerance [6]. This genome shuffling approach was also used to obtain *Lactobacillus delbrueckii* strain capable of converting starch to lactic acid [7].

Protoplast fusion in lactobacilli [8, 9] has been reported. However, this technique has been applicable to certain strains of lactobacilli mainly due to the difficulty in both protoplast isolation and regeneration. Though protoplast regeneration on agar media with 0.3 M raffinose has been successful, frequencies of regeneration often remained low [10]. There are reports on regeneration of protoplasts of *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus rhamnosus* [11] and *Lactobacillus casei* [12]. There are no reports on systematic studies on protoplast isolation and regeneration in case of *L. delbrueckii*.

We have isolated several mutants of *L. delbrueckii* using acclimatization and ultraviolet irradiation that produced increased lactic acid concentrations [13]. One of these mutants, Uc-3 was used to produce lactic acid from

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bagasse derived cellulose since it contained high amounts of cellobiase [14]. However, these mutants lack in acid tolerance ability and hence cannot produce lactic acid at acidic pH (<4.5). We would like to employ genome shuffling approach through recursive protoplast fusion which might help in widening the substrate utilization range as well as in improving the acid tolerance of *L. delbrueckii* mutants.

In this study, we attempted the production and regeneration of protoplasts of *L. delbrueckii* mutant Uc-3 and optimized the conditions for both isolation and regeneration. This method has been tested for isolation and regeneration of protoplasts from other species of *Lactobacillus* genus.

Materials and methods

Chemicals and enzymes

Lysozyme and mutanolysin were purchased from Sigma Chemical Company, USA. MRS medium, yeast extract, sucrose and glycine were from Hi-Media, Mumbai, India. All the other chemicals used were of analytical grade and obtained from local sources.

Bacterial strains and growth media

The strains of lactobacilli used were *L. lactis* NCIM 2368, *L. brevis* NCIM 2090, *L. casei* NCIM 2364, *L. plantarum* NCIM 2083, *L. fermentum* NCIM 2165, *L. delbrueckii* NCIM 2025, *L. delbrueckii* NCIM 2365 mutant Uc-3 were obtained from NCIM Resource Center, National Chemical Laboratory, Pune, India. Cultures were maintained in MRS liquid medium with 1% CaCO₃.

MRS medium with 0.5 M sucrose and 2% agar was used as regeneration medium (RM). Regeneration was also checked on cane sugar medium containing 10% hydrolyzed cane sugar, 1% yeast extract and 4% CaCO₃ supplemented with 0.5 M sucrose and 2% agar. All media were sterilized at 121°C for 20 min. The pH of all media adjusted to 7.0 prior to sterilization. Protoplast buffer (PB) consisted of 0.5 M sucrose, 10 mM Tris-HCl and 20 mM MgCl₂ with pH adjusted to 6.5.

All cultures were grown by transferring 0.5 ml of working stock culture to 10 ml MRS broth and incubated anaerobically for 24–48 h at 37°C or 42°C. These grown cultures (5 ml) were further transferred to 100 ml MRS broth in screw capped 250 ml conical flasks and incubated at appropriate temperatures anaerobically with shaking at 150 rpm for 16–20 h to obtain fresh exponential phase cells. These cells were further processed for protoplast formation and regeneration studies.

Protoplast formation

Cells grown in MRS liquid medium were harvested by centrifugation at 5,000 rpm, washed twice with PB without sucrose and suspended in PB to adjust the OD 660 to 0.6–0.8. A portion of cell suspension (5 ml) was treated with various concentrations of lysozyme and mutanolysin or combination of both and the mixture was incubated at 37°C for 2 h with shaking at 80 rpm. The formation of protoplasts was monitored by decrease in optical density at 660 nm. The protoplasts were also observed as spherical cells by microscopy or by enumeration of osmotically resistant cells before and after protoplast formation.

Table 1 Effect of lysozyme and mutanolysin concentration and time of incubation on production of protoplasts of *Lactobacillus delbrueckii* mutant Uc-3

Enzyme concentrations	Protoplast formation* (%)			
	0 h	1 h	2 h	3 h
Control	0	0	0	0
Lysozyme (10 mg/ml)	0	48 ± 3	63 ± 3.5	62 ± 3.0
Lysozyme (10 mg/ml) + mutanolysin (10 µg/ml)	0	70 ± 4.0	81 ± 5.0	82 ± 4.5
Lysozyme (5 mg/ml) + mutanolysin (10 µg/ml)	0	74 ± 3.5	82 ± 4.0	83 ± 4.0
Lysozyme (1 mg/ml) + mutanolysin (10 µg/ml)	0	73 ± 3.0	82 ± 4.5	82 ± 5.0
Mutanolysin (10 µg/ml)	0	42 ± 2.5	63 ± 4.5	65 ± 4.0

*The decrease in optical density (OD) at 660 nm or enumeration of the osmotically fragile cells (the difference between the number of osmotically resistant colonies before and after protoplast formation treatment) was used as indices of protoplast formation. The values are the average of three independent experiments.

Table 2 Formation of protoplasts in different osmotic stabilizers

Osmotic stabilizers used	Protoplast formation* (%)
Sucrose (0.5 M)	83 ± 5.0
Sorbitol (0.5 M)	78 ± 4.5
Mannitol (0.5 M)	83 ± 3.0
Raffinose (0.3 M)	33 ± 3.5
Raffinose (0.5 M)	35 ± 4.0

*The protoplasts were formed in PB with 0.5 M sucrose containing lysozyme (1 mg/ml) and mutanolysin (10 µg/ml) and the protoplast formation was checked after 2 h. The values are average of three independent experiments.

Protoplast regeneration

The cell pellet, after protoplast formation, was washed with PB to remove traces of lytic enzymes by centrifugation at 3,000 rpm for 10 min and the cell pellet was re-suspended in the same buffer. The cell suspension was suitably diluted in PB and 100 µl of each dilution was plated on RM. The cell suspension was also plated on non-osmotically stabilized medium (RM without osmotic stabilizers, NOS) to check the osmotically resistant cells. Plates were incubated anaerobically at respective temperatures and colonies were counted after 2 days of incubation. The frequency of protoplast regeneration was calculated from following equation:

$$[(\text{CFU/ml of protoplast on RM}) - (\text{CFU/ml of protoplast on NOS RM})]$$

$$[(\text{CFU/ml of initial no. cells}) - (\text{CFU/ml of protoplast on NOS RM})]$$

Results and discussion

Protoplast formation

We attempted to standardize the protocol for protoplast formation for *L. delbrueckii* mutant Uc-3 and the same procedure was adapted to different species of *Lactobacillus* genus. Lysozyme or mutanolysin individually failed to

produce protoplasts but mixture of lysozyme (1 mg/ml) and mutanolysin (10 µg/ml) produced maximum protoplasts (Table 1). Such requirement of mixture of lysozyme and mutanolysin was found to be necessary for formation of protoplasts from various *Lactobacillus* strains [11, 12]. Higher concentrations of lysozyme and mutanolysin did not help in increasing the protoplast formation efficiency. The growth of *L. delbrueckii* Uc-3 in presence of glycine did not enhance the protoplast isolation significantly (data not shown). Among all osmotic stabilizers tested sucrose was almost as effective as mannitol at 0.5 M concentration (Table 2). Fewer protoplasts were observed in raffinose at both 0.3 M and 0.5 M. It is reported that raffinose at 0.3 M concentration favors the protoplast formation in *Lactobacillus* strains [11].

Regeneration of protoplasts

MRS with different osmotic stabilizers such as sucrose, sorbitol and mannitol were evaluated as potential osmotic stabilizers for protoplast regeneration. We found that sucrose at 0.5 M concentration was the best which gives maximum protoplast regeneration in case of *L. delbrueckii* mutant Uc-3 (Table 3). It was also observed that sucrose derived protoplasts regenerated well on sucrose based RM. Complex media supplemented with bovine serum albumin, gelatin, polyvinylpyrrolidone and raffinose were used for regeneration of *Lactobacillus* protoplasts which led to more than 10–99% regeneration of protoplasts [11]. However, there was no report on formation and regeneration of protoplasts from *L. delbrueckii*. We also used hydrolyzed sucrose based medium which was as effective as MRS-based RM (data not shown). MRS-based RM with sucrose as an osmotic stabilizer was found to be effective for *L. lactis*, *L. brevis*, *L. casei*, *L. plantarum*, *L. fermentum* protoplasts (Table 4).

In conclusion, this study demonstrates that there is no established universal method for protoplast regeneration of all genera of lactobacilli. This could be due to the requirement of suitable osmotic stabilizer for regeneration. However, we found that sucrose was the best osmotic stabilizer for regeneration of protoplasts of lactobacilli since we get more than 30% efficiency of protoplast regeneration in case of all

Table 3 Protoplast regeneration of *L. delbrueckii* mutant Uc-3 on regeneration media containing different osmotic stabilizers

Protoplasts derived in	Protoplast regeneration* (%) on MRS regeneration medium with		
	Sucrose (0.5 M)	Sorbitol (0.5 M)	Mannitol (0.5 M)
Sucrose (0.5 M)	25.5 ± 2.5	25.0 ± 3.0	21.0 ± 3.5
Sorbitol (0.5 M)	23.5 ± 3.0	11.0 ± 3.0	19.0 ± 4.0
Mannitol (0.5 M)	20.0 ± 3.0	25.0 ± 4.0	6.0 ± 2.5

*The values are average of three independent experiments.

Table 4 Protoplast formation and regeneration of different *Lactobacillus* strains

Strains used	Protoplast regeneration* (%)
<i>Lactobacillus delbrueckii</i> mutant Uc-3	25.5 ± 3.5
<i>Lactobacillus lactis</i> NCIM 2368	30.6 ± 3.0
<i>Lactobacillus brevis</i> NCIM 2090	38.0 ± 4.5
<i>Lactobacillus casei</i> NCIM 2364	36.5 ± 4.5
<i>Lactobacillus plantarum</i> NCIM 2083	30.5 ± 3.0
<i>Lactobacillus fermentum</i> NCIM 2165	33.0 ± 4.0
<i>Lactobacillus delbrueckii</i> NCIM 2025	25.0 ± 2.5

*The protoplasts were formed in PB with 0.5 M sucrose and regenerated on MRS with 0.5 M sucrose as an osmotic stabilizer. The values are average of two independent experiments.

the species of *Lactobacillus* tested. This work will provide a basis for the genetic improvements of *L. delbrueckii* using protoplast fusion approach.

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