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Optimization and Comparison of Two Electrotransformation Methods for Lactobacilli

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Abstract: To date, *lactobacilli* are widely used in food industries and new probiotic products; hence these are considered as an attractive target for genetic modifications. This study was conducted to improve electroporation of probiotic lactobacilli which is a necessary prerequisite for genetic manipulations. Four strains of probiotic lactobacilli from different sources were grown in Man Rogosa and Sharp (MRS) broth medium containing glycine, as a cell wall weakening agent or a pulse of glycine for evaluation of glycine effect on electroporation efficiency. After evaluation of various parameters such as washing buffers, washing times, electric field strength, pulse duration and plasmid concentration, a practical electroporation protocol was presented to improve electrotransformation of lactobacilli in comparison with two standard protocols. This new protocol exhibited higher transformation efficiency (transformants/μg plasmids) than standard methods ($p<0.05$) with no differences between species (*Lb. casei*, *Lb. crispatus, Lb. salivarus* and *L. rhamnosus*). The pulse of glycine had no effect on the number of the transformants in three methods. In contrast to plasmid concentration, plasmid size had no influence on the transformation efficiency. The modified method enabled to transform plasmid into the resistant lactobacilli against transformation. These bacteria have potential for bioengineering research to improve special performance.

Key words: Probiotic, lactobacilli, eletroporation, plasmid size

INTRODUCTION

Food grade microorganisms such as lactobacilli bacteria are one of the most important groups of microorganisms that are exploited for commercial proposes; hence these are considered as an attractive target for genetic modifications. Recently, DNA technology offers controllable approaches to improve performance of lactic acid bacteria (Liu *et al*., 2007). An essential part of these approaches for strain improvement is introduction of DNA into the bacterial cells (Aukrust *et al*., 1995).

There are two general methods for transferring of DNA into the lactic acid bacteria; protoplast fusion and electrotransformaion as a physical method. Lin and Savage (1986) firstly reported transfection of a rifampicin resistance gene into *Lactobacillus acidophilus* by protoplast fusion technique. The reported efficacy of this method was very low in protoplast fusion, from 0.2×10^{-8} to 1×10^{-3} transformants per μg of DNA (Iwata *et al*., 1986; Lin and Savage, 1986; McCarthy *et al*., 1988). Electrotransformaion is considered as a newer technique with a higher efficacy which has been successfully employed for introducing DNA into the *Lactobac illus* species (Natori *et al*., 1990; Walker *et al*., 1996), although the transformation efficiency is extremely variable, ranging from 10^2-10^7 transformants per μg of DNA.

Because of heterogeneity of the genus lactobacilli, electroporation protocols should be optimized for each species, even within the strains of one species (Aukrust *et al*., 1995). Consistent with this notion, Sieo *et al*. (2006) electrotransformed DNA plasmid into *L. crispatus* I12 with 10^4 transformants per μg DNA, whereas others obtained less than 50 transformants for different strains of *L. crispatus* using the same procedure (Beasley *et al*., 2004). All these evidences suggest that some elements such as growth phase, medium composition and electrical condition need to be optimized for each strain of lactobacillus.

Gastrointestinal tract of chicken is a potential survival niche for variety species of lactobacilli including *L. crispatus, L. salivarus* and *L. reuteri*. There are many

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studies investigating benefits and special properties of these bacteria, such as their probiotic activities or enzyme production (Ehrmann *et al*., 2002; Taheri *et al*., 2009). Beside this, it is declared that lactobacilli may show improvement in their performance by genetic manipulations (Beasley *et al*., 2004). However, there are some challenges to overcome in introducing of DNA into the lactobacilli, originated from GI tract of chickens because of their recalcitrant in electrotransformation (Beasley *et al*., 2004; Mason *et al*., 2005).

At the present study, a new practical electroporation protocol was presented for electrotransformation of lactobacilli which are isolated from different sources and compared with two standard protocols (Aukrust *et al*., 1995).

MATERIALS AND METHODS

Bacterial strains and plasmids: Lactobacillus strains were isolated from chicken GI and human breast milk (Table 1). Lactobacilli were anarobically cultured on Man Rogosa and Sharp (MRS) medium at 37°C. Ten micrograms per micro litter and 5 μg mL^{-1} of erythromycin were added to the culture medium for the selection of *lactobacillus rhamnosus* and other lactobacilli electrotransformants, respectively. Plasmids pNZ3004 (NIZO, 4.9 k bp) and pBU003 (Bu-ali Research Institute, 6.2 kbp) originated from pWV01 were used for transformation. The plasmids were amplified in *E. coli* MC1061 and were grown at 37°C with agitation in Luria-Bertani medium supplemented with 400 μg mL^{-1} of erythromycin. Plasmid extraction from *E. coli* was carried out by the PrimePrep Plasmid DNA Isolation Kitaccording to the protocol (Genet Bio). The purity and concentration of the plasmid DNA were determined by the agarose gel electrophoresis and spectrophotometric analysis.

Glycine tolerance test: Tolerance to glycine as a cell wall weakening agent was evaluated in the present study strains. Glycine concentrations from 0.4 to 10% were made in MRS broth and each lactobacillus was cultured in all concentrations at 37°C overnight.

Preparation of electrocompetent cells: The standard protocol was used for electrotransformation of lactobacilli (Aukrust *et al*., 1995). Briefly, the strains were refreshed in MRS broth containing glycine (standard 1) or without glycine (standard 2) for overnight at 37°C. Overnight cultures were diluted in 12 mL competent medium

Table 1: The lactobacillus strains used in this study

Strain	Reference	Origin
L. salivarus	Heravi *et al.* (2011)	Chickens' crop
L. crispatus	Heravi *et al.* (2011)	Chickens' ceca
L. rhamnosus	Nasiraii *et al.* (2011)	Human breast milk
L. casei	DSM, Delft, The Netherlands	

Table 2: The summary of procedures and related buffers

Procedure	Refresh medium	Competent medium	Washing buffer	Electroporation buffer
Standard I	MRS	MRS+2% glycine	952 mM Sucrose+3.5 mM MgCl	952 mM Sucrose+3.5 mM $MgCl_2$
Standard II	MRS+2% glycine	MRS+2% glycine	952 mM Sucrose+3.5 mM MgCl	952 mM Sucrose+3.5 mM $MgCl_2$
Modified	MRS	0.5 Sucrose+ MRS+2% glycine	DDW[a], EDTA, DDW, 0.3 M sucrose	0.3 M Sucrose

a Double distillate water

the exponential phase (OD = 0.5- 0.6). The bacterial cells were harvested by centrifugation (6000 x g, 10 min, 4°C) and washed by specific buffers depending on the procedure (Table 2). Finally, the cells were resuspended in 100 μL of electroporation buffer.

In the modified procedure, an overnight culture of lactobacillus was used to inoculate 12 mL MRS broth containing 2% glycine and 0.5 M sucrose and incubated at 37°C anaerobically. The initial absorbance after inoculation was 0.02 and finally reached to 0.2 absorbance unit (early exponential phase) after 2 or 3 h at 37°C. The centrifuged cells (6000 x g, 10 min, 4°C) were washed twice with 10 mL cold distilled water and followed by a 5 min incubation in EDTA. After washing with distilled water and 0.3 M sucrose, the cells were resuspended in 100 μL of 0.3 M sucrose as electroporation buffer.

Electroporation protocol: Electrotransformation was carried out by mixing of 100 μL of resuspended cells with 0.3 to 1 μg plasmid DNA. The suspension was transfer into a disposable cuvette (Bio-Rad Laboratory, Richmond, CA) with an 0.2 cm electrode gap and subjected to an electric pulse at 8 ms, 1.5 kv, 600 Ω and 10 μF using a MicroPulser (Bio-Rad Laboratory, Richmond, CA). The electrporated cells were immediately diluted in 1 mL recovery media and incubated in at 37°C for 2 h. Recovery media for standards procedure was MRS containing 0.3 M sucrose and 20 mM $MgCl_2$ and for modified procedure was only MRS. The transformed cells were cultured on MRS agar containing erythromycin and incubated for 2 to 3 days. The transformation efficiency was expressed in number of transformants per microgram of plasmid DNA.

factorial method. Treatment means were separated using the Duncan's multiple rang test at 5% level of probability. The final absorbance of bacteria in competent medium was use as covariate for correction of observations.

RESULTS

The modified procedure showed significantly ($p<0.05$) higher efficiency than other procedures (Table 3). Using of glycine as a pulse in modified and standard I methods exhibited higher transformants bacteria but difference was not significant ($p>0.05$). Transformation efficiency was improved after addition of glycine to the competent medium as a pulse.

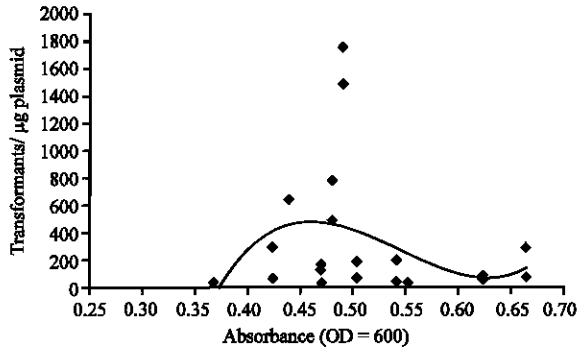

Fig. 1: Effect of cell growth phase on the trend of transformation efficiency.

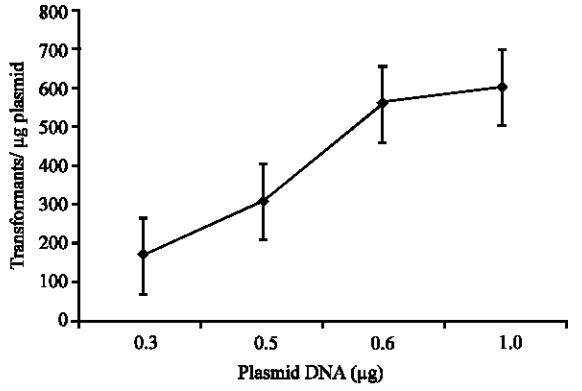

Fig. 2: The relation of plasmid DNA concentration with the rate of transformation.

Table 3: Mean of transformation efficiency in different procedures

	Modified	Standard I	Standard II	p-value
Efficiency*(transformants µg DNA^{-1})	732.08[a]	221.61[b]	140.99[b]	<0.05
SE[†]	163.276	165.713	85.508	

*Means within a column lacking a common superscript differ, [†]Standard Error

Table 4: The effect of plasmid size on transformation efficiency

	pBu003	pNZ3004	p-value
Efficiency (transformants µg DNA^{-1})	417.23	312.56	>0.05
SD[a]	130.046	130.046	

[a]Standard deviation

Comparison of standard I and modified procedures suggests an important role for sucrose in preparing of bacterial cells for electroporation. The presence of sucrose in the competent medium improved electrotransformation efficiency in modified method (Table 3).

In the present report, exponential phase cells were used for electrocompetence. Middle exponential phase exhibited high efficiency in electrotransformation while early exponential phase displayed low transformants probably because of a low cell concentration (Fig. 1).

Count of tranformants was raised after increasing of the plasmid concentration (Fig. 2). Using 0.6 µg versus 0.3 µg of DNA plasmid exhibited 3.4 times higher of lactobacillus transformants and using a concentration of 1 µg DNA showed no difference from that of 0.6 µg.

Higher concentrations of plasmid resulted in a plateau effect. Also plasmid size has no effect on the transformation efficiency (Table 4).

The electric field strength [kV $(cm^2)^{-1}$] and pulse duration (ms) were also considered as variable parameters for electroporation with constant resistant (600 Ω) and capacity (10 µF). The optimized field strength and pulse duration were 7.5 kV $(cm^2)^{-1}$ and 8 ms, respectively, for all the lactobacilli used in this study. More electric field strength and less pulse duration resulted in low numbers of transformants. For example, 4 ms electric pulses at a field intensity of 10 kV$(cm^2)^{-1}$ displayed no transformed lactobacilli.

DISCUSSION

Mason *et al.* (2005) showed that a pulse of glycine rather than growth in glycine was effective on a successful electroporation. Addition of glycine to the growth medium inhibits cross-linking in cell wall and this appears to enhance transformability of some lactobacilli that are recalcitrant to transformation (Aukrust *et al.*, 1995). Although, some bacteria showed a high tolerance to glycine like *Lactobacillus crispatus* that tolerates 6% glycine in medium, increase of glycine concentration from 2 to 6% revealed no improvement in transformation efficacy (data not shown). Therefore, 2% glycine was used for all species. This result is consistent with the other studies that showed the presence of glycine was necessary for improvement of transformation efficiency (Serror *et al.*, 2002; Sieo *et al.*, 2006). The positive effect of glycine may be increased further by growing of the

cells in osmotically stabilized media (Bringel and Hubert, 1990). It is also reported that addition of sucrose, as an osmotic stabilizer, to the wash solution increased transformation efficiency (Serror *et al*., 2002). Detection of this property was impossible in this study, since sucrose was used in wash buffer of all procedures.

It was demonstrated that a cell density higher than optimum alters the threshold of pulse intensity or duration (Luchansky *et al*., 1989). A perfect cell density enhances the interaction of cells and plasmid DNA and reduces electrical pulse damage (Sieo *et al*., 2006; Aukrust *et al*., 1995) recommended middle growth phase for preparation of cells, whereas (Serror *et al*., 2002) used the cells in early exponential phase. All the mentioned studies shows that cells in exponential growth phase usually are more susceptible to penetration by DNA.

Concentration of plasmid is the other important parameter that affects electrotransformation efficiency. Results of current study are consistent with the others which indicated a linear relationship between concentration of plasmid DNA and transformation efficiency (Serror *et al*., 2002; Sieo *et al*., 2006). Based on (Beasley *et al*., 2004) study, plasmid size has a significant impact on transformation efficiency. The transformation frequency of most competent protoplasts generally decreases with increasing size of plasmid (Gasson and Fitzgerald, 1994). At the present study, there was no significant difference between two plasmids in transformation of lactobacilli (Table 4). This may be due to a small difference (1.3 kbp) in size of tested plasmids.

Although, there have been several reports about transformation of different lactobacilli species, feasibility of transformation in some lactobacilli is very low, especially species that originated from gastrointestinal tract of chickens. It is necessary to optimize electrotransformation parameters for lactobacilli and to improve an efficient procedure for this species. Our experiments resulted in a modified procedure with a better efficiency than standard methods. This procedure also perfectly exhibited high efficiency in *Lactococcus lactis*. Therefore, it is suggested that the modified method could be replace with standard method for an efficient transformation of lactobacilli.

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