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Original Article

OPTIMIZATION OF ELECTROPORATION MEDIATED TRANSFORMATION OF *LACTOBACILLUS PLANTARUM* FOR INDUSTRIAL EXPLOITATION

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ABSTRACT

Objective: The objective of our study was to evaluate different established electrotransformation protocols for their suitability on *Lactobacillus plantarum*.

Methods: We tested strategies of electrical parameters in addition to already published electroporation methods proven to be successful in other *Lactobacillus* strains.

Results: Our strain differed highly in transformation efficiencies and optimal growth conditions. Among the various methods performed, we observed high-efficiency protocol yielded 8X10^6 transformants, and the optimum electroporation parameter was found to be at 1500V.

Conclusion: Independent of electroporation method *Lactobacillus plantarum* strain remained reluctant to high-efficiency transformation protocol.

Keywords: *Lactobacillus plantarum*, Transformation, Electroporation, Lithium acetate

INTRODUCTION

Lactic acid bacteria belong basically to the order Lactobacillales; trustworthy classification has to be based on both genotypic and phenotypic methods [1]. Lactobacillus plantarum is a facultatively heterofermentative bacterium that produces DL-lactate and contains meso-diaminopimelic acid in its cell wall. It is closely related to *L. paraplantarum, L. pentosus*, and *L. arizonensis* and can be distinguished from these species by 16S rRNA sequencing only [2]. In addition, *L. plantarum* has a repertoire of osmoprotection, which enhances its ability to thrive in different environments [3]. Lactobacilli are used to produce metabolites, enzymes and proteins of therapeutic value including antigens, cytokines, and pro and prebiotics [4]. Metabolic processes like the production of GOS by *L. plantarum* can be enhanced by introducing strong constitutive promoters upstream of genes coding for the relevant enzymes [5].

The capacity to secrete heterologous proteins varies from species to species. Hols *et al.* [6] demonstrated the ability of *L. plantarum* to secrete up to 10 mg/l of a specific antigenic fusion protein. Efficient expression vectors [7] have already been developed for Lactococcus where expression of recombinant proteins is ensured by constitutive promoters or inducible systems. A crucial advantage of lactic acid bacteria and their applications is that most lactic acid bacteria have GRAS status (= generally regarded as safe), which means that they have no recorded toxic or pathogenic activity. Xie *et al.* [8] worked on *E. coli* and proposed that DNA is absorbed on the cell surface and transfers into the cell after formation of membrane pores by induction of an overcritical membrane potential during the electric pulse by electroporation. Similar reports were carried out by in lactic acid bacteria that usually require additional steps to transcend the rigid cell wall [9]. PEG is widely used as electroporation or storage solution and was also shown to increase electroporation of *L. plantarum* [10], and a different strain [11] described the loss of electrocompetence of some strains when kept too long on ice before electroporation. The milestones in establishing methods for electroporation of lactic acid bacteria include growth in glycine and sucrose [12], the use of PEG as electroporation or storage solution [13], and thorough testing of suitable electrical parameters [14] of pulse length and time shown increase electroporation of lactobacillus strains.

MATERIALS AND METHODS

Microorganism, chemicals and media

Lactobacillus plantarum was procured from MTCC, Chandigarh (No. 5422), media were prepared with deionized water and sterilized by autoclaving at 120 °C for 20 min. Sensitive reagents like glycine or antibiotics were sterile-filtered. MRS media (HiMedia) was used for the cultivation of *Lactobacillus plantarum.* MRS was supplemented with 5 µg/ml ampicillin, with 25µg/ml. All enzymes and vectors for transformation purpose were procured from Fermentas.

Optimization parameters of electroporation

Based on Axelsson and Ahrne [14] method, an overnight culture of *L. plantarum* 5422 was grown in MRS at 37 °C and diluted in 40 ml fresh MRS to an OD600 of 0.2. After growth at 37 °C cells were washed in ice-cold ddH2O and once in 30% PEG1500. The cells were suspended in 30 % PEG1500 leading to an OD600 of 10 to 20 and aliquoted into 50µL portions. The suspensions were electroporated without the addition of plasmids to determine survival rates. Different combinations of voltage and resistance were applied (table 1); capacity was set at 25 µF [15].

Table 1: Different combinations of voltage and resistance for electropration

V	1000	1000	1500	1500	2000	2000	2500	2500
Ω	800	400	800	400	800	400	800	400

The electrotransformation protocol was repeated, adding 1 µg of pUC plasmids, respectively, to the aliquoted cells. DNA and cells were mixed and held 10 min on ice prior to electroporation. After application of the electric pulse, cells were diluted in 500 µl MRS and incubated for 2 h at 37 °C, before plating them on MRS agar containing 5 µg/ml ampicillin.

Electroporation method with glycine as additive

Lactobacillus plantarum 5422 is incubated at 30 °C without aeration. The culture was diluted to an OD600 of 0.2 in MRS supplemented with 1% glycine [16]. The cells were harvested at an optical density of approximately OD600 0.6. Centrifugation was performed at 10, 000 rcf for 10 min to pellet the cells. They were washed three times with dH2O, once with 30 % PEG1500 and suspended in fresh 30 % PEG1500 (4 ml). Centrifugation were increased in speed and time when necessary. 40 µl aliquots of resuspended cells were stored at −80 °C or used immediately. OD600 of the aliquoted cells was between 11 and 19. Thawed or freshly prepared aliquots were held 10 min on ice after mixing 1 µg of plasmid [17] with each aliquot. The cell solution was transferred into precooled electroporation cuvettes; during pipetting trapping of air bubbles was avoided. A pulse of 12KV was applied before incubation in 500 µl MRS at 30 °C for 5 min. Undiluted and serial dilutions of recovered cell suspensions were spread and incubated on either MRS ampicillin agar or plain MRS agar. For evaluation of cell survival and transformations efficiency, it is further necessary to spread cell suspensions before the appliance of an electric pulse. Transformant colonies are visible after two days in general.

Lithium-acetate method

Overnight cultures were grown under strain-specific conditions as per MTCC instructions and diluted to an OD600 of 0.2. Cells were harvested after reaching an OD600 of 0.4–0.6. Cells were centrifuged for 15 min and resuspended in 30 ml lithium acetate solution [19] (0.6 M sucrose, 100 mM lithium acetate, 10 mM Tris-HCl, pH 7.5). 120 ml of lithium acetate-DTT (0.6 M sucrose, 100 mM lithium acetate, 10 mM Tris-HCl, pH 7.5, 10 mM DTT) solution were added, and the cell solution was incubated for 30 min at room temperature and 50 rpm. The cells were resuspended in 0.3 M sucrose. Electrical parameters were set as described for the initial protocol.

High-efficiency protocol for Lactococcus *plantarum*

High-efficiency protocol as described earlier by Papagianni *et al.* [20] for the transformation of *Lactobacillus lactis* was used to transform Lactobacillus plantarum, Overnight cultures of L. lactis were grown in M17 supplemented with 0.5 % glucose and 40 mM threonine and diluted 1:12.5 in 25 ml of M17 with glucose. Cells were harvested at OD600 ~ 0.3 and suspended at room temperature for 30 min in 8 ml of 100 mM LiAc, 10 mM DTT, 0.6 M sucrose, and 10 mM Tris-HCl, pH 7.5. Following pretreatment, cells were washed twice with ice-cold ddH2O, once with 50 mM EDTA, ddH2O and twice with 0.3 M sucrose. Cells were immediately electroporated by a single pulse at 2.5 kV, 200 Ω and 25 µF using 1 µg of DNA. The cell suspension was diluted in 5 ml M17 containing glucose and 1% sucrose and incubated for 2 h at 30 °C.

High salt protocol

The high salt protocol as described earlier by Palomino *et al.* [21] for the transformation of *Lactobacillus lactis* was used to transform *Lactobacillus plantarum*, Cells were cultured for 21 h at 37 °C without aeration. 1 ml of preculture was diluted in 49 ml MRS supplemented with 0.4 M–0.7 M NaCl and incubated for 22 h. 50 µl aliquots were electroporated with 2.5 kV and 200Ω (25 µF) for 5 min.

Polymerase chain reaction

Primers were designed using Primer Express (Applied Biosystems), and oligonucleotides were synthesized by Bioserve, Hyderabad (100 pMol/µL). The following primers 5-TGTA-AAACGACGGCCAGT-3 for sense strand and 5-AACAG CTATGACCATG-3 for anti-sense strand were used in standard polymerase chain reactions (PCR) as to verify the presence of the *bla* gene [22] in the recombinant *Lactobacillus* DNA. PCRs were performed to confirm transformation of lactobacilli with pUC plasmids. Master mix was usually made to 50ul with PCR buffer 5ul, dNTPs (2 mM) 5ul, MgCl2 (25 mM) 3ul, DNA polymerase (2.5U/ul(0.4ul, sense and anti-sense primer (10pml/ul) 2ul, template DNA (~5ng) of 1ul. PCR cycling conditions for 35 cycles were set up with denaturation at 98C for 35s, annealing at 49C for 30s and extension at 72C for 45s.

Gel electrophoresis

Agarose gel electrophoresis was performed at 120 V for analytical gels (15–20 min) and with 80 V for preparative gels (30–40 min). Samples were mixed with 6x BX buffer (0.25 % bromphenol blue, 0.25 % xylen cyanol, 30 % glycerol). 6 µl of 2-log DNA ladder (0.1–10 kb; New England Biolabs, UK) were applied to a 1 % agarose gel (1% agarose in 1xTAE buffer) to determine DNA length of fragments. Gels were supplemented with ethidium bromide and run in TAE buffer (40 mM Tris-acetate, 100 mM EDTA).

RESULTS AND DISCUSSION

Optimization of electrical parameters

We determined survival rates after 4 washes and electroporation under various voltage and resistance combinations (table). The resulting disparity in survival depending on the different settings was only moderate. Thus we agreed to use 2000 V/400 Ω/25 µF and 2500 V/400 Ω/25 µF for further experiments as they displayed the lowest survival rates and should, therefore, have the highest impact on cell structure. Washed *L. plantarum* were treated with different combinations of voltages and resistances with a capacity of 25 µF and plated on MRS agar (surviving cells/aliquot containing 8 ~10 CFU) were reported.

Table 2: Cell survival at different voltage and resistance combinations

V	Ω	Surviving cells
1000	800	$4*10^6$
1000	400	$5*10^6$
1500	800	$4*10^6$
1500	400	$8*10^6$
2000	800	$1*10^6$
2000	400	$2*10^6$
2500	800	$7*10^6$
2500	400	$3*10^6$

Electroporation protocols

Although the efficiencies are highly dependent on the plasmids used. For instance, of the four different methods under electroporation carried, high-efficiency protocol as shown the optimum transformation efficiency for *Lactobacillus plantarum*. We repeated a slightly altered electrotransformation protocols for determination of electrical parameters and added 1 µg of pUC plasmids, respectively, to the aliquoted cells. Post-pulse incubation time was set at 2 h, which is assessed to give cells enough time to recover and resume bacterial protein synthesis is known to be rapidly expressed. pUC18 of 5ng was added to 50ul of a suspension of *L. plantarum* cells, and transformation frequency is calculated as transformants/survivors.

Transformation efficiencies of *L. plantarum* strains grown in 1 % glycine; lithium acetate-mediated, high efficiency and high salt method. Cells were aliquoted to an OD600 of 11-19. Survival = CFU/aliquot after electroporation, plated on MRS agar (fig. 1).

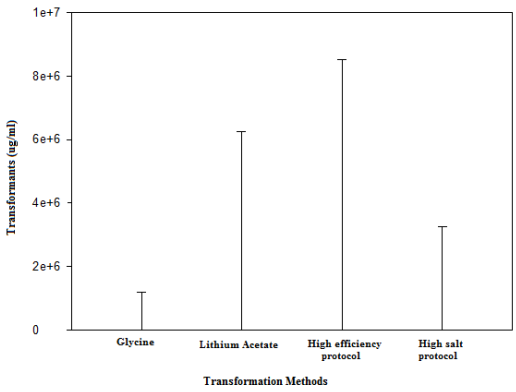

Fig. 1: Transformation efficiencies with different methods of *L. plantarum* strain

Amplification and analysis of recombinants

PCR amplification of *bla* gene fragments was carried out using the primer sense and antisense primers under standard conditions as previously described. The quality of pUC18 plasmid (fermentas) was evaluated by agarose gel electrophoresis, indicating relative purity. Samples of the PCR products of 10ul were analyzed on a 1.5% agarose gel and stained with ethidium bromide. Lane 1 shows size markers of a DNA ladder; lane 2 is the position of migration of the bla gene product (predicted size, 917 bp). Gels were photographed under UV light on a UVI-Tech BioImager (fig. 2).

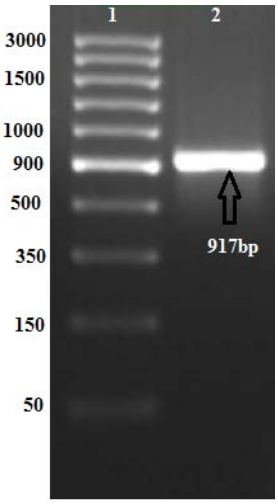

Fig. 2: PCR Amplicon of *bla* gene of pUC18 analyzed on 1.5% agarose gel

CONCLUSION

In summary, the high-level transformation of *bla* gene in *Lactobacillus plantarum* have been achieved easily by high-efficiency method. Even if assigned to the same species, strains show huge variation in transformability and optimal growth conditions. A protocol working for one strain might not be suitable for the other one. Independent of electroporation protocol or plasmid choice *L. plantarum* remained reluctant to high-efficiency electroporation. It might, therefore, be more promising to conclude the effort on *L. plantarum* and to turn towards ideal strain for commercial exploitation. Our work provides the genetic tools which should be useful for the development of recombinant strains amenable to practical applications in the field of biotechnology.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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