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TRANSFORMATION OF *LEUCONOSTOC OENOS* BY ELECTROPORATION

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SUMMARY

An electroporation-induced transformation system was developed for *Leuconostoc oenos*. Of the three plasmids tested (pGK13, pAMS100 and pIL253), only pGK13 was expressed. Several parameters were studied with possible influence on transformation efficiency. Electroporation conditions of 2000 V/cm, 25μF and 200 ohm proved successful. Transformation efficiencies (number of cells/μg plasmid DNA) of 1 X 10^3 were obtained.

INTRODUCTION

Leuconostoc oenos plays an important role in the secondary fermentation of wines, especially red wines with a high acid content and pH below 3.5 (Davis *et al.*, 1985; Wibowo *et al.*, 1985). Despite the fact that *L. oenos* is used commercially to conduct MLF, surprisingly few basic studies have been done (reviewed by Van Vuuren and Dicks, 1993). No genetic studies have been published, probably due to the lack of a transformation method. Recent studies on the transport systems for malate, lactate, and the malolactic enzyme in *L. oenos* (Cox and Henick-Kling, 1989, 1990) are likely to focus the attention on genetic studies in the future.

In the present study, an electroporation protocol for three starter cultures of *L. oenos* (PSU-1, ML-34 and 19Cl) is described. Several parameters were studied with possible influence on tranformation efficiency.

MATERIALS AND METHODS

Cells of *L. oenos* PSU-1, ML-34 and 19Cl were inoculated (2%, v/v) into 100 ml acidic grape broth (Dicks *et al.*, 1990) and incubated at 30°C to OD_{600} = 0.3, 0.5, 1.0, 1.5 and 2.0, respectively. The cells were harvested by centrifugation (9820 *g*, 20 min, 4°C), washed three times with 100 ml sterile incubation buffer (0.6 M sucrose, 7 mM potassium phosphate, 1mM $MgCl_2$, pH 7.5), and resuspended in the same buffer to an OD_{600} of 0.8. To this cell suspension, lysozyme was added (20 kU/ml) and incubated for 30 min at 30°C. The cells were harvested (3500 *g*, 20 min, 4°C), washed with four volumes ice-cold electroporation buffer (0.6 M sucrose, 1 mM K-phosphate, 1mM $MgCl_2$, pH 4.8), and gently resuspended in the same buffer to an OD_{600} of 0.8 (approx. 1 X 10^5 cells/ml). Fifty μl of this cell suspension was mixed with 1 μg plasmid DNA (pGK13, pAMS100 or pIL253, respectively), transferred to a sterile Gene Pulser cuvette

with an inter-electrode distance of 2 mm (Bio-Rad Laboratories, Richmond, Calif.), and left on ice for 5 min prior to electroporation. The cells were exposed to a single electric pulse (capacitor at 25μF and 200 ohm) at 2000, 4000 and 6500 V/cm, respectively. The cells were then immediately transferred to 0.5 ml acidic grape broth, supplemented with 0.6 M sucrose. Cells were incubated for 2 h at 30°C to allow for the expression of the antibiotic resistance marker. The cell suspension was diluted and 50 μl plated onto acidic grape medium (2% agar) with 0.3 to 5 μg chloramphenicol or erythromycin, respectively. Cells electroporated without any plasmid DNA and plated onto medium with and without antibiotics, and cells not electroporated, served as controls. All plates were incubated at 30°C in air-tight flasks in an atmosphere of 19.8% CO_2, 11.4% H_2 and N_2 (balance). Transformants were visible after one week of incubation. Successful transformation was confirmed by agarose gel electrophoresis of plasmid DNA from randomly selected antibiotic-resistant clones.

RESULTS

The three strains of *L. oenos* included in this study were successfully transformed with pGK13, but not with plasmids pAMS100 and pIL253 (Table 1). No transformants were obtained when cells were electroporated with 4000 and 6500 V/cm. Transformation efficiencies of 1 X 10^3 (i.e. cells per μg plasmid DNA) were obtained with all three strains of *L. oenos* when harvested in the early exponential growth phase (OD_{600} = 0.5). No transformants were obtained when cells were harvested at OD_{600} = 0.3, 1.5 and 2.0, respectively. The number of transformants decreased to 1 X 10^2 cells per μg plasmid DNA when cells from the mid exponential growth phase (OD_{600} = 1.0) were electroporated. The survival rate after electroporation was 20% (Table 1). No spontaneous antibiotic-resistant mutants could be detected on control plates.

Table 1. Transfer efficiencies of *Leuconostoc oenos* PSU-1, ML-34 and 19Cl with three different plasmids

Plasmid	*L. oenos* transformants per μg DNA			Cell count of cells not electroporated[a]	Percentage transformants
	PSU-1	ML-34	19Cl		
pGK13	1 X 10^3	1 X 10^3	1 X 10^3	5 X 10^3	20
pAMS100	ND[b]	ND	ND	5 X 10^3	
pIL253	ND	ND	ND	5 X 10^3	

[a] Colony forming units (cfu) from 50 μl of cells resuspended at OD_{600} = 0.8 and incubated at 30°C for 2 h.

[b] No transformants detected.

DISCUSSION

Leuconostoc oenos is resistant to most mechanical methods used for the disruption of lactic acid bacteria and is not easily lysed when treated with lysozyme. Furthermore, some strains produce exopolysaccharides which forms a thick electron-dense layer on the cell surface (Van Vuuren and Dicks, 1993).

Electroporation of *L. oenos* could only be achieved when cells were treated with lysozyme. Cells of *W. paramesenteroides* and *Lactobacillus curvatus* were made more compatible by adding DL-threonine to the growth medium (David *et al.*, 1989; Gaier *et al.*, 1990). Powell *et al.* (1988) recorded higher transformation efficiencies when cells of *Lactococcus lactis* subsp. *lactis* were treated with lysozyme prior to electroporation.

The age of *L. oenos* cells are important, since only cells in the early exponential growth phase could be transformed. Similar results were obtained for *Lactobacillus sake*, *L. curvatus* (Gaier *et al.*, 1990) and *L. casei* (Watanabe *et al.*, 1994). Higher transformation efficiencies were reported for *Lactobacillus helveticus* subsp. *jugurti* (Hashiba *et al.*, 1990) and *L. lactis* subsp. *lactis* (McIntyre and Harlander, 1989) when cells from the stationary growth phase were electroporated. It is not clear why *L. oenos* cells from the lag growth phase could not be transformed. It might be that cell size, chain length, degree of cell aggregation, and the structure of the cell wall play an important role, as suggested by Knight and Scrutton (1986).

The low transformation efficiency obtained for *L. oenos* (1×10^3 cells/μg plasmid DNA) can be ascribed to the fact that only 20% of the cells survived electroporation (Table 1). This value might even be lower since cell chains are disrupted during electroporation, giving rise to more colony forming units (cfu). Luchansky *et al.* (1988) reported similar low tranformation efficiencies for *Leuconostoc mesenteroides* subsp. *dextranicum* (1×10^4 cells/μg DNA) and *L. lactis* subsp. *lactis* (7.7×10^3 cells/μg DNA). It might also be that *Leuconostoc* spp., including *L. oenos*, contains active endonucleases. This would explain the difficulties we often encounter when isolating plasmid DNA from *L. oenos*.

The inability to transform *L. oenos* at voltages higher than 2000 V/cm could be ascribed to a weakened cell wall due to lysozyme treatment. *Lactobacillus casei* is also optimally transformed at 2000 V/cm (Watanabe *et al.*, 1994).

The electroporation method described allowed the successful transformation of three strains of *L. oenos* and open the field for future genetic research. The technique might have to be slightly modified for other strains of *L. oenos*.

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