Chapter III

Development of methods for this study

To undertake this study, I needed to develop improved, quick, and low-cost analytical method to measure the sugar and lactic acid content of silage and high efficiency transformation technique to construct suitable killer *Kluyveromyces lactis* strain. This chapter describes the development of both techniques.

1) Analysis of chemicals in silage

Although enzymatic assay and HPLC are available to measure lactic acid and residual sugar in silage, I needed analytical method that can measure a number of samples at a time. I selected a colorimetric measurement using a 96-well microplate for analysis of D(L)-lactic acid, glucose, fructose, sucrose and lactose. The procedure involves the NAD(P)H reduction of water-soluble tetrazolium salt, WST-1 (Fig. 2-3).

Figure 3-1 shows the standard curves of D(L)-lactic acid (A) and glucose, fructose, sucrose, and lactose (B). The standard curves exhibit good linearity. The relative standard deviations that were determined by four separate runs for each point did not exceed five percent. I determined the sensitivity of WST-1 to D (L)-lactic acid as 0.53 $\Delta A/\Delta$ [D-lactic acid] (OD/mg) and 0.35 $\Delta A/\Delta$ [L-lactic acid] (OD/mg). WST-1 exhibited linear responses to NAD (Fig. 3-2A). There was no report of oxidation of NADPH by WST-1. However, Fig. 3-2-B shows the WST-1 that is also available as an indicator for NADPH. I measured fructose and sucrose, and a half amount of lactose, as glucose. I determined the sensitivity of WST-1 toward glucose (fructose and sucrose) and lactose, as 0.33 $\Delta A/\Delta$ [glucose] (OD/mg) and 0.14 $\Delta A/\Delta$ [lactose] (OD/mg).

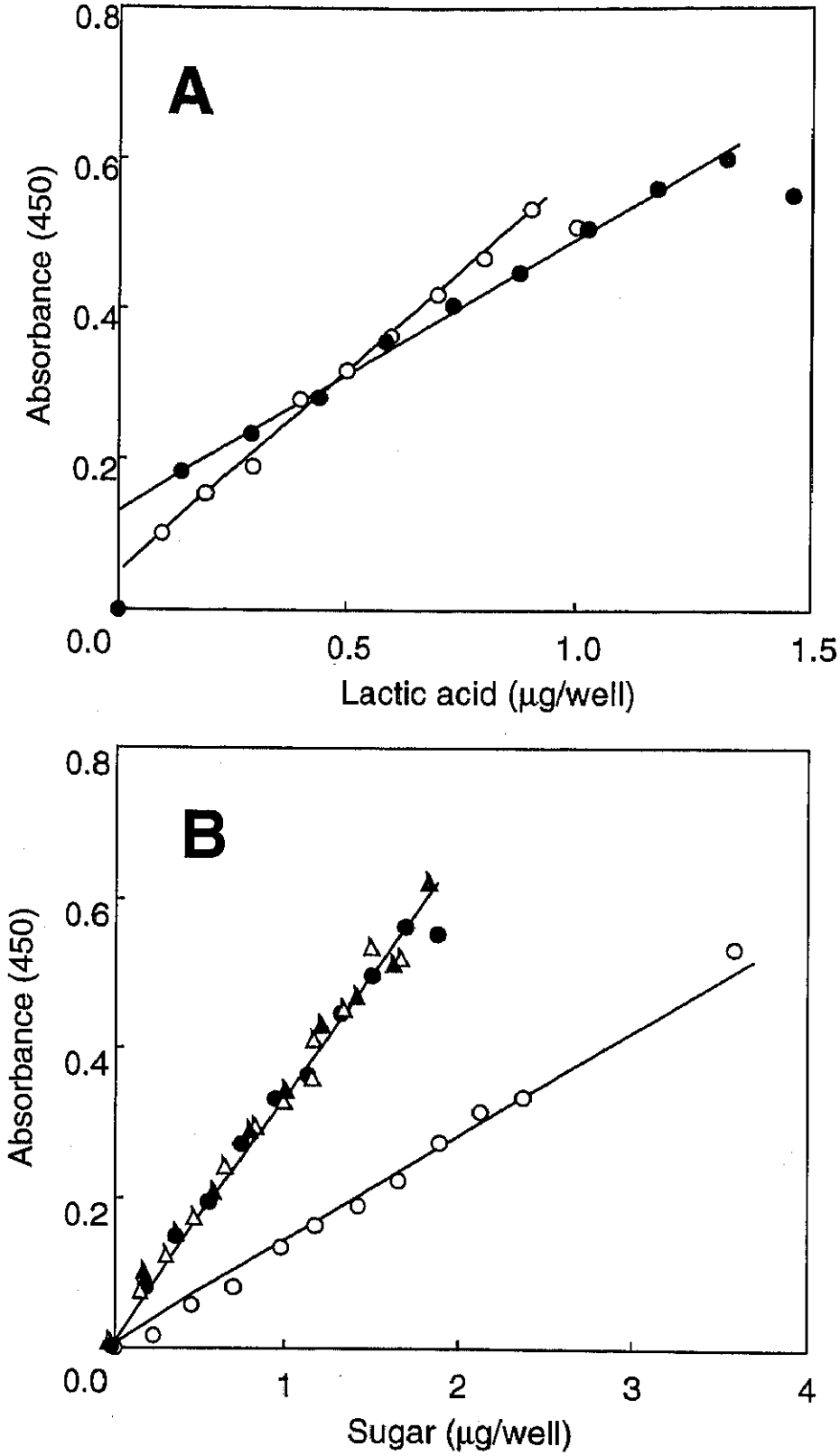

Fig. 3-1. Standard curves of D, L- lactic acid (A), and sugar (B) assays.

Symbols: L-lactic acid (●), D-lactic acid (○), glucose (●), sucrose (△), fructose (▲) and lactose (○).

2) Transformation of *K. lactis*

The use of electroporation procedures has been very popular as a mean of introduce exogenous DNA into cells. Several transforming techniques in general use for transforming yeast suffer from significant limitations, namely, lithium acetate transformation[63] and spheroplast transformation[73]. Lithium acetate transformation, although relatively fast and simple, provides only a low efficiency of DNA transfer in *K. lactis*[51]. Spheroplast transformation, while more efficient, is complicated and time consuming.

2.1) Construction of electroporation apparatus

A simple device for electroporation by alternating current was constructed (Fig. 3-2). The mains (200 V) were used as power supply and pulse duration was controlled by combined capacity of condensers. The narrow electrode distance (0.5 mm) come off the high field strength (4 KV/Cm) by the safety equipment of relatively low voltages. The protoplast fusion chamber (FTC-01, Shimadzu) was used as the electroporation chamber.

2.2) Effect of buffer concentration and pulse length on electroporation

The pulse shape and length were measured by an oscilloscope. The waveform exhibits an exponential-decay as shown in Fig. 3-3. Transformation efficiency is known to depend on electrolysis strength and pulse length. I selected and fixed the field strength to 4 KV/Cm as suggested by data of several reports about yeast electroporation[74-78]. Pulse length (τ) is the product of capacitance of condenser (C) and resistance (R=Rc + Rb) of the circuit of equipment (Rc) and buffer (Rb).

τ=CR

The pulse length varied among the three buffer solutions used for electroporation (Fig. 3-4). When the capacitance of condenser was set from 0 to 470 μF, pulse length of electroporation buffer (0.001 N KCl) containing 5% of TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA) increased correspondingly from 0 to 21 msec, and it increased from 0 to 91 m sec when 5% of TE buffer was suspended in water. I selected 0.001N KCl because the range of pulse length difference was narrower than water by addition of DNA solution.

Dependency of survival on pulse capacitance was studied. Figure 3-5 shows the capacitance increase simultaneously decrease the number of surviving cell. Furthermore, the survival rate decrease in the buffer of high conductivity in the same capacitance of condenser (Table III-1). It is because of the shortening the pulse length.

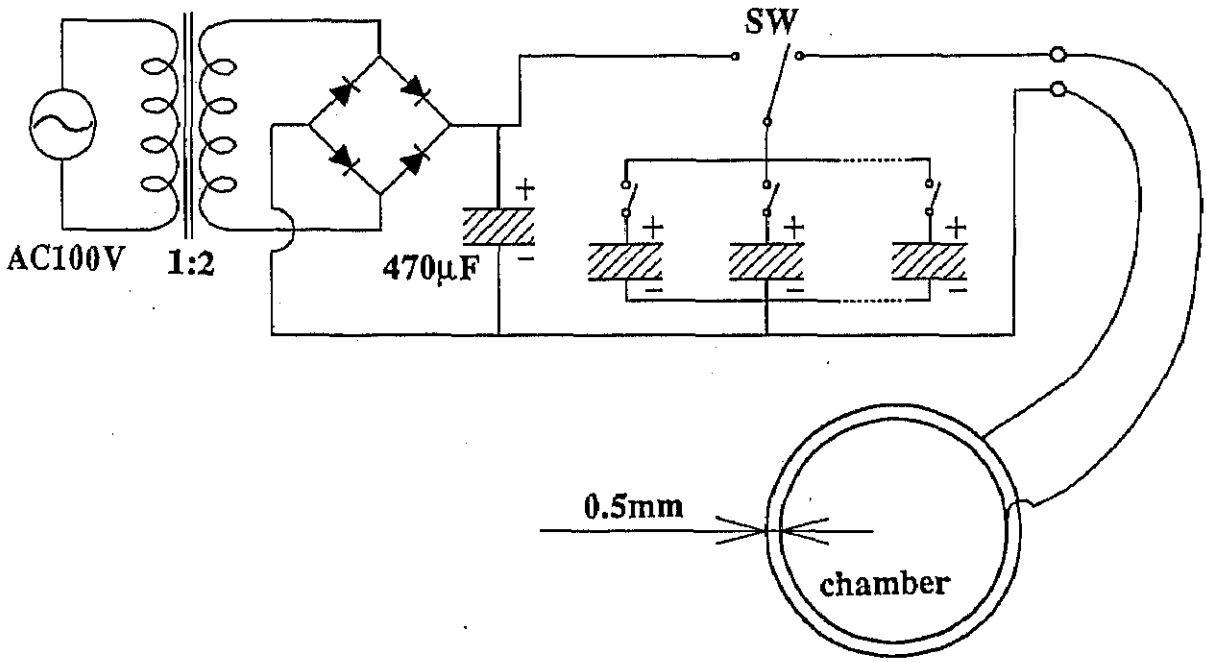

Fig. 3-2. Circuit diagram of the electroporator.

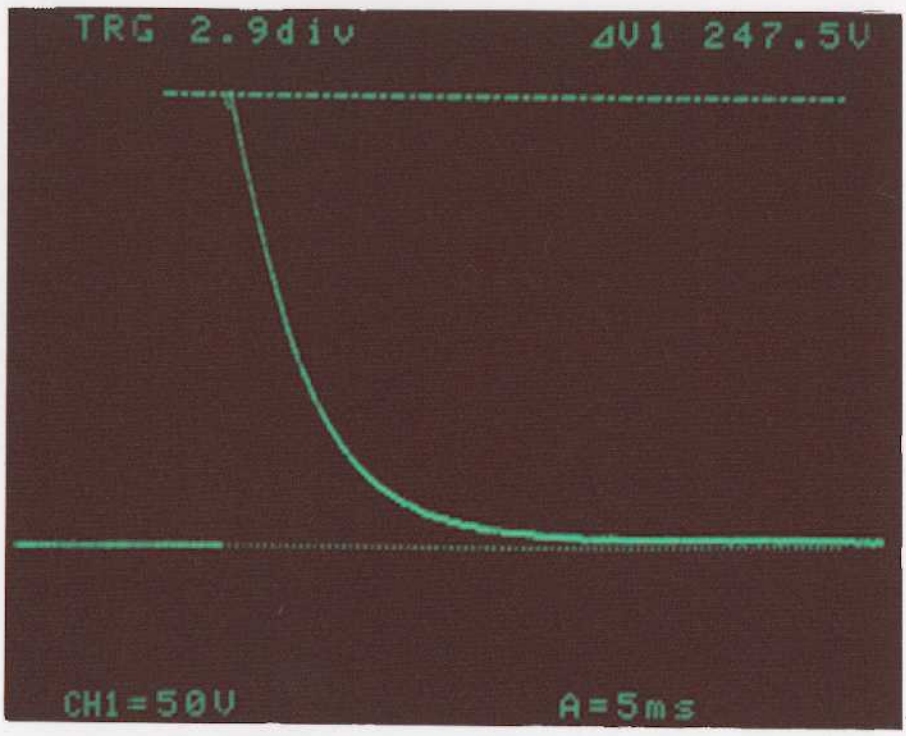

Fig. 3-3. The waveform of the electroporator.

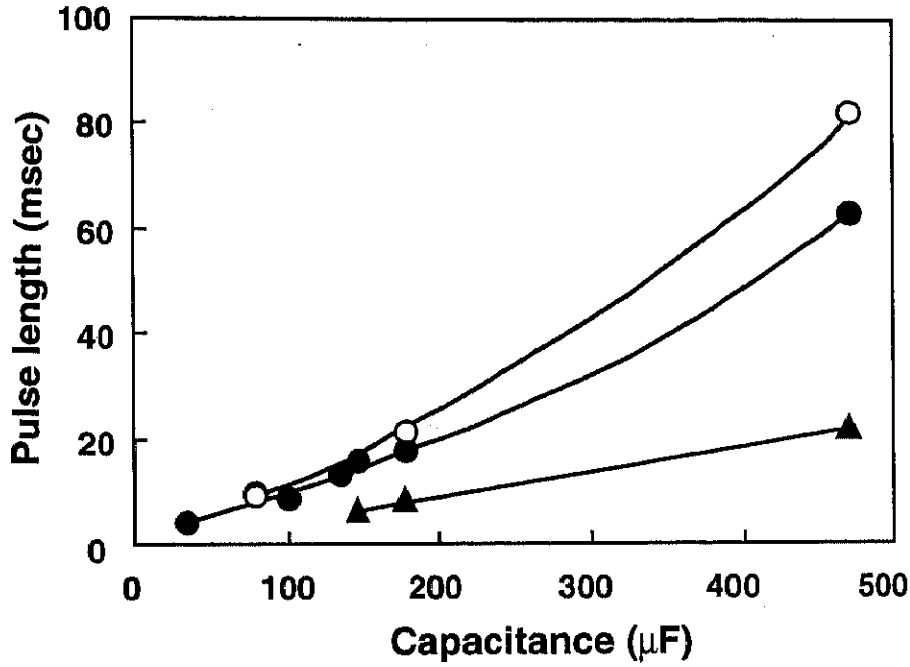

Fig. 3-4. Effect of buffer concentration on pulse length.

○: distilled water with DNA solution, ●: 0.001N KCl, ▲: 0.001N KCl with DNA solution.

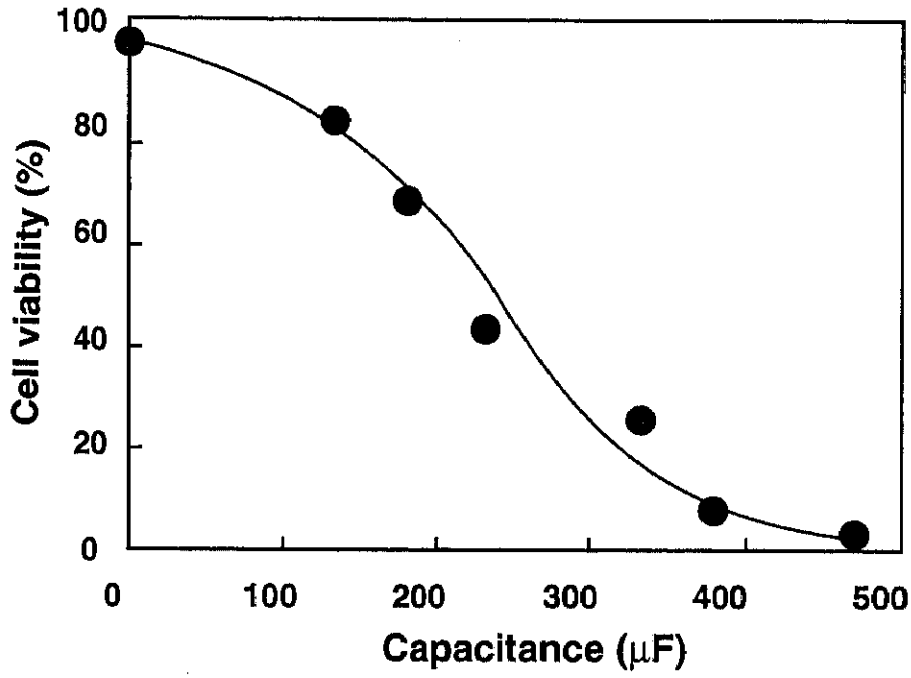

Fig. 3-5. Effect of capacitance on cell viability.

Table III-1. Effect of buffer concentration on cell viability.

KCl concentration (N)	Cell viability (%)
0.1	100
0.001	65
0.0001	60

1/10 volume of DNA solution (TE buffer) was added.
Electroporation was performed at 180 μF.

2.3) Effect of capacitance on transformation efficiency

The derived vector pE1 was transformed in *K. lactis* MW98-8C. The transformants selected on SD plate deficient in uracil (Fig 3-6) shows the efficient transformation under various capacitance setting. The highest transformation efficiency was obtained at 180 μF. At that time, the transformation efficiency was 4 x 10^2/μg DNA. The survival rate at 180 μF is about 67% as shown in Table III-1. These data agree with the report of the best transformation efficiency which was observed when 50 to 70% of the cells are still viable.

3) Discussion

I developed a technique for the convenient assay of lactic acid and sugar content of silage. The chromogenic regent, WST-1, is quantitatively reduced by NAD(P)H. The amount of D, L-lactic acid, glucose, sucrose, fructose, and lactose were reproductively measured very quickly and inexpensively. I concluded that these analytical methods are practical enough for measuring the concentration of lactic acid and sugars in silage. This technique can be applied to the measurement of other components that can be measured by enzymes with the production of NAD(P)H, e.g., ethanol, malic acid, starch, and so forth.

The yeast *K. lactis* was efficiently transformed by electroporation. None of the transformants were isolated by lithium acetate procedure in my trial. Electroporation is preferred when high-frequency transformation is required. The condition of pulse length and the field strength of hand-made electroporation apparatus can be changed by selection of condenser capacity and buffer of various conductances. Voltage of electroporation apparatus is fixed mains voltage as 200V;however, the smaller field strength can be selected by selecting wide electrode distance (e. g. 1 mm come off the field strength 2 KV/Cm).

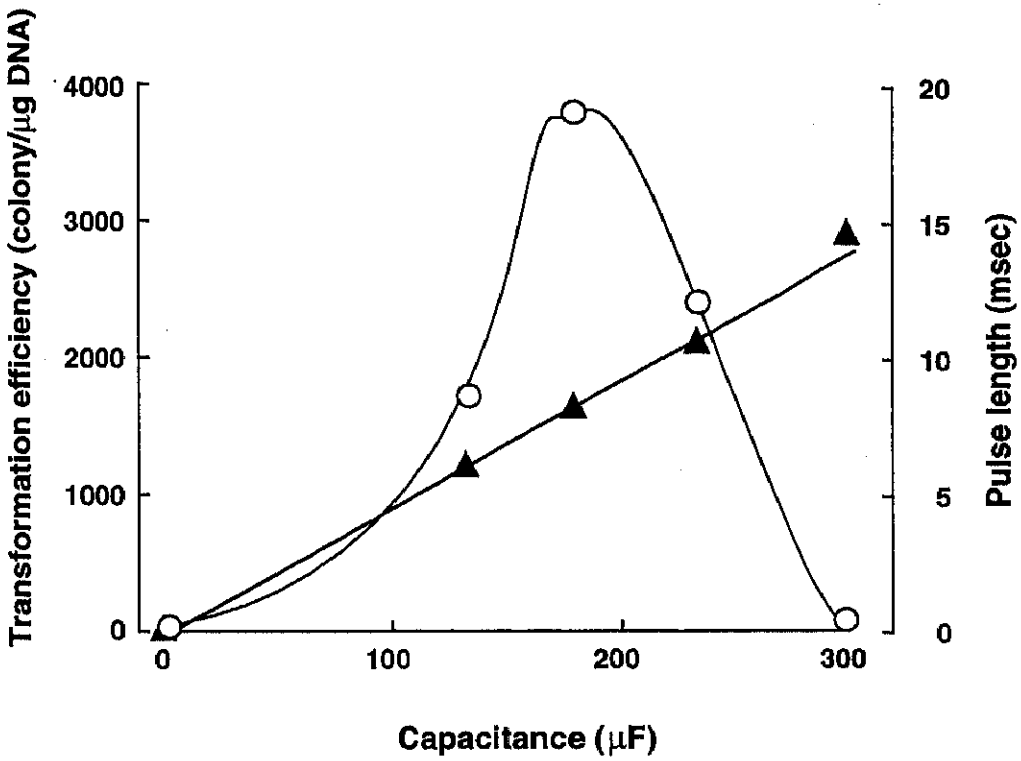

Fig. 3-6. Transformation of pE1 plasmid on various capacitances.

○: transformation rate, ▲: pulse length.