Chapter II

Materials and Methods

1) Materials

1.1) Microorganisms

1.1.1) Bacteria

Escherichia coli JM109 strain (*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* Δ(*lac-proAB*)/F'[*traD36, proAB+, lacI*^{q}*. lacZ*Δ*M15*], Takara Shuzo, Japan) was used for DNA manipulation. *Lactobacillus plantarum* MAFF 516001 (Ministry of Agriculture, Forestry and Fisheries, Japan) isolated from silage was used as lactic acid bacterium in the silage fermentation model system.

1.1.2) Yeasts

Yeast strains used in this study are shown in Table II-1.

1.2) Plasmids

Plasmids used in this study are shown in Table II-2 and Fig. 2-1.

1.3) Chemical products

All chemicals were obtained from commercial sources and were of reagent grade. ECL direct nucleic acid labeling and detection systems (ECL system: Amersham International) and DIG-High Prime DNA labeling and detection kit I (Boehringer Mannheim) were used for genomic/chromosome hybridization and colony hybridization[57], respectively. Protein concentration was determined by Protein assay kit (BioRad) with bovine serum albumin (238031, Boehringer Mannheim) as a standard. Oligonucleotides were prepared by Espec Oligo Service.

Table II-1. Yeast strains used in this study.

Strain	Genotype or description	Reference or source
Kluyveromyces lactis		
IFO 1267	a, killer (pGKL1, pGKL2)	IFO*1
IFO 0433	growth comparison study*2	do.
IFO 0648	do.	do.
IFO 1090	do.	do.
IFO 1903	do.	do.
MW98-8C	α, *ura*A, *arg* A1, *lys* A1, cir 0, killer (pGKL1, pGKL2)	H.Fukuhara*3
MW98-8C/MD2/1	isogenic to MW98-8C, cir+	do.
PCK27	a, killer (pGKL1, pGKL2) *klpck*1	this study
PCK50	do.	do.
m8	killer defective strain	do.
Kluyveromyces fragilis		
IFO 0288	growth comparison study	IFO
IFO 0541	do.	do.
IFO 1735	do.	do.
IFO 1777	do.	do.
Saccharomyces cerevisiae		
ATCC 38976	killer (K_1)	ATCC*4
F-102	rho-, killer (pGKL1, pGKL2)	N.Gunge*5
IFO 0304	killer tester strain	IFO
IFO 1953	killer tester strain	do.
IFO 2018	killer tester strain	do
B511-4C	killer tester strain	N.Gunge
CJM150	(*pck1, leu2, ura3*)	C.Gancedo*6
YNN27	*trp 1, ura 3, gal 1*	YGSC*7
Williopsis mrakii		
NCYC 500	killer (K_9)	NCYC*8
Kluyveromyces drosophilarum		
NCYC 575	killer (K_{10})	do.
Candida utilis		
IFO 1086	growth comparison study	IFO

*1 Institute for Fermentation, Osaka, Japan

*2 Used for comparison study of growth on whey

*3 Institut Curie-Biologie, Orsay, France

*4 American Type Culture Collection, Rockville, Maryland, USA.

*5 Kumamoto University of Engineering, Kumamoto, Japan

*6 Instituto de Enzimologia del C.S.I.C., Universidad Autonoma, Madrid, Spain

*7 Yeast Genetic Stock Center, U. S. A.

*8 National Collection of Yeast Cultures, Norwich, U K.

Table II-1(continued).

Strain	Genotype or description	Reference or source
Candida krusei		
IFO 0011	killer tester strain*9	IFO
MAFF 4033	do.	MAFF*10
MAFF 4085	do.	do
Pichia anomala		
IFO 0121	do.	IFO
IFO 0145	do.	do.
IFO 0146	do.	do.
IFO 0963	do.	do.
CBS 251	do.	CBS*11
AHU 3936	killer tester strain isolated from silage*12	AHU*13
AHU 3937	do.	do.
AHU 3938	do.	do.
Pichia fermentans		
AHU 3822	killer tester strain	do.
AHU 3844	do.	do.
Pichia membranifaciens		
CBS 107	do.	CBS
Yeast, isolated from silage		
YG-2	killer tester strain isolated from silage	O. Tanaka*14
YG-3	do.	do.
YG-5	do.	do.
YS-1	do.	do.
YS-2	do.	do.
Sil-1	do.	do.
Sil-2	do.	do.
It-1	do.	do.

*9 Known to be involved in aerobic spoilage[10, 53].

*10 Ministry of Agriculture, Forestry and Fisheries, Japan

*11 Centraalbureau voor Schimmelcultures, The Netherlands

*12 Isolated by T. Sasaki, Seishu Junior College, Sapporo, Japan

*13 Hokkaido University, Sapporo, Japan

*14 National Grassland Research Institute, Japan

Table II-2. Plasmids used in this study.

Plasmid	Description	Reference or source
E. coli vector		
Bluescript II (KS^+)	*Amp*	Stratagene
S. cerevisiae vector		
YCp50	*URA3, CEN4, ARS1 Amp, Tet,*	ATCC*1
pYCDEΔG11	*ADH1*p-*APT2, TRP1* 2μDNA, *Amp*	T. Ogata[55]
pYCDEΔG12	do.	This study
pPCK	*KlPCK1, Amp*	do.
pPCKl	*KlPCK1*::*ADH1*p-*APT2 Amp*	do.
pPDKD	do.	do.
K. lactis vector		
pE1	pKD1(*K. lactis* 2μ) *URA3, Amp, Tet*	H. Fukuhara*3 (Fig. 2-1)
pEPGK41	S11b, *URA3, Amp*	do.

*1 American Type Clture Collection, USA.

*2 Applied Technology Research Laboratory, Asahi Breweries

*3 Institut Curie-Bioligie, Orsay, France

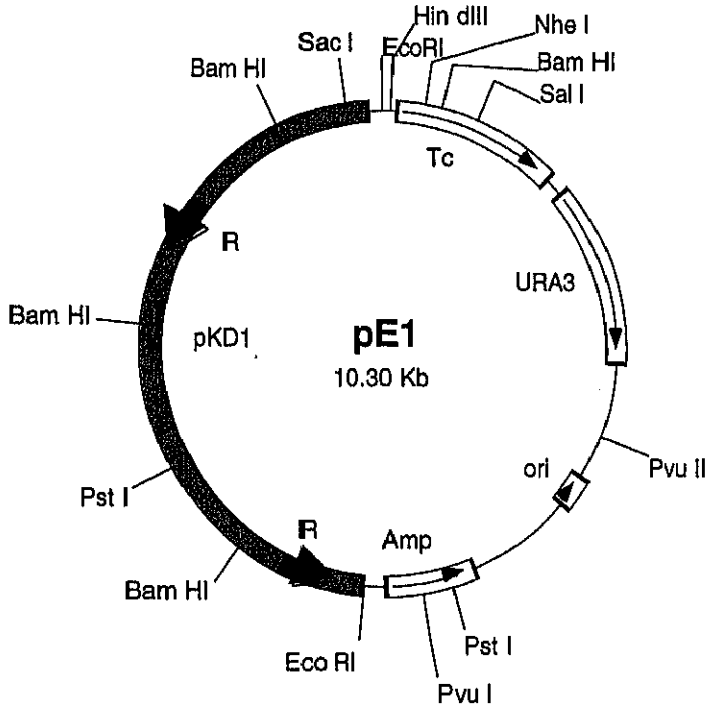

Fig. 2-1. Structure of pE1 plasmid used in this study.

This plasmid was supplied by H. Fukuhara (Curie-Biologie). pE1 contains the total sequence of pKD1, the 2-μ family plasmid derived from *K. drosophilarum* UCD 51-130[56], into the *Eco* RI site of the pBR322-derived plasmid YIp5. pKD1 autonomously replicate in *K. lactis*. Transformation marker is *URA3* for *K. lactis uraA* hosts.

1.4) Biological products

All enzymes were obtained from commercial sources. Whey permeate was prepared from cheese whey powder supplied by Yukijirushi as follows. Cheese whey powder was dissolved in warm water at a concentration of 10% as lactose and then heated at 121°C for 5 min. After cooling, it was centrifuged at 4 °C at 7000rpm for 20 min to remove insoluble proteins. The supernatant was filtered through Watman No.2 filter paper and the filtrate filtered using Labomodule ACL 1010 (AsahiKasei) membranes with a molecular weight cut off of 1,3000 daltons to remove soluble proteins. The clear filtrate was used as whey permeate. All crops for silage were obtained from National Grassland Research Institute. Powdered alfalfa hay cube and maize (*Zea mays*, total sugar of 105 g kg^{-1} DM) were used for the model system of silage fermentation and fresh maize (total sugar of 278 g kg^{-1} DM, 65% water content) were used for the laboratory scale-silage preparation.

2) Methods

2.1) Culture condition

2.1.1) Culture media and growth conditions

2.1.1.1) Bacteria

E. coli was cultured in LB (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.5 and 50 μg/ml of ampicilin if needed) at 37 °C and the seed culture of *L. plantarum* was cultivated in GPY medium (0.2% peptone, 0.1% yeast extract, 0.4% glucose, 0.1% sodium acetate, 0.005% $MnCl_2$ in tap water, pH adjusted to 5.3 by the addition of acetic acid) on a shaker at 37 °C for 48 hours.

2.1.1.2) Yeast

The yeast was grown in YPD/YPG (1% yeast extract, 2% peptone, 2% glucose/glycerol) and SD [0.67% Yeast nitrogen base without amino acids (Difco Laboratories), 2% glucose or 0.5% calcium-lactate supplement with appropriate amino acids] on a shaker at 30 °C for 48 hours. YED medium consisted of 1% yeast extract (Difco Laboratories) and 1% glucose was used for electroporation. For study of *K. lactis* growth on whey, CM [4% lactose, 1% peptone (Difco Laboratories), 0.05% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract and tap water] and WM [whey permeate solutions (ca. 2.5% lactose) with 0.36% $(NH_4)_2SO_4$, 0.05% each of KH_2PO_4, $MgSO_4 7H_2O$, 0.1% yeast extract and distilled water with 0.4% aliquot of $CaCO_3$, sterilized separately] were used for seed culture and main culture, respectively.

2.1.2) Preparation of silage fermentation model system

2.1.2.1) Rapid system

Yeast strains were cultured separately in 30 ml of YPD medium in a 300-ml Erlenmeyer flask at 30 °C for 48 hr on a rotary shaker (220 rpm). Each seed culture was inoculated into 10 ml of liquid medium containing 0.45% yeast extract, 0.75% peptone, 2% glucose, or lactose and 2% L-lactic acid, pH 4.0 in a 100-ml Bunsen-bulb type Erlenmeyer flask and shaken at 30 °C for 2 days anaerobically. Then the Bunsen-bulb was replaced with a cotton plug, and the cultivation was continued for 1, 2, 4, and 6 days, aerobically. Solid culture was maintained on alfalfa hay cube powder (AHC), sterilized with ethylene oxide gas (37 °C, 3hr), containing 70% water in the same manner as for the silage fermentation model described by Tanaka and Ohmomo[15]. Each seed culture was added with 3.3 ml of distilled water, which contained 0.1 g each of glucose or lactose and L-lactic acid sterilized by autoclaving, in a 50-ml screw-capped bottle that contained 1.7 g of sterilized AHC. The solution contained a final concentration of 2% each of carbon source and 70% water, pH 4.0. The loosely capped bottles were incubated in an anaerobic jar (BBL) at 30 °C for 3 days, and then the cultivation was continued for 2, 4 and 6 days, aerobically.

2.1.2.2) Conventional system

I selected maize as a forage crop for this study because aerobic spoilage by lactic acid-assimilating yeast is a serious problem in maize silage[12, 13]. 1% of lactose was added in silage making as a carbon source for lactic acid bacteria and killer yeast. K-nylon layered polyethylene bag (Hiryu, Asahi Kasei) was used as a silo bag for both the model system of silage fermentation (model system) and laboratory scale silage. To create anaerobic condition, air was ejected from the bag and then the bag was sealed. Aerobic condition was established by opening the silo bag.

A previously reported model system of silage[58] was used with some modifications. Appropriate amounts of lactose and water were added into the sterilized maize powder and then was inoculated with lactic acid bacteria and yeasts. Seed culture of the yeast strains was prepared in YPD medium and *L. plantarum*, in GPY medium on a rotary shaker for 48 hours at 30 °C and at 37 °C, respectively. Powdered dried maize was sterilized using ethylene oxide gas at 37 °C for 3 hours. I introduced *L. plantarum* [10^6 cfu g^{-1} (wt/wet wt.)] into sterilized crops with 1% lactose and 70% water content (1.25g final weight). Depending on the objective of the experiment, the killer strain (10^6 cfu g^{-1}) and the target strain (10^2 cfu g^{-1}) were inoculated separately or in combination into the sterilized crops. The silo bag was incubated at 28 °C for three weeks under anaerobic condition and then for 2, 5, and 8 days under aerobic condition.

2.1.3) Laboratory scale silage preparation

I prepared a laboratory scale silage following that previously reported[58, 59]. In this system the killer yeast strain (10^6 cfu g^{-1}) and 1% of lactose were introduced into 50 g portions of fresh maize (20 mm lengths) and packed into the bags. Treatment of bags and incubation conditions were the same as the model system.

2.1.4) Count of viable cells

Number of yeast cells in seed culture was calculated based on average microscopic field.

The microbial counts of silage were determined as colony forming units (cfu) using selective agar media. After incubation, I added four volumes (vol./wet wt.) of sterilized water to the silo bag, sealed the bag and suspended them by shaking at 4 °C for one hour. I diluted the resulting cell suspension appropriately, and spread it onto selective agar plates. Selective agar plates contain 0.67% dehydrated Yeast nitrogen base (Difco Laboratories) with 2% glucose (MC medium) for counting total yeast, 0.5% calcium lactate (MA medium) for counting lactic acid-assimilating yeast, or 2% lactose (ML medium) for counting lactose-assimilating yeast as a sole carbon source, respectively. *K. lactis* was detected as distinct pink colonies on MC or ML medium. Its color is due to the production of the red pigment pulcherrimin[51] (Fig. 2-2). The colonies of *S. cerevisiae* F-102 (auxotroph, respiratory deficient) were counted on the SD agar plate to which 0.1% of peptone had been added. I counted the number of *P. anomala* colonies on a minimum-nitrogen medium (MN medium) agar plate containing 1.5% Yeast carbon base (Difco Laboratories) and 1% KNO_3 in distilled water. After sterilization, pH of all the yeast media was adjusted to 3.5 by HCl, with methyl orange as a pH indicator. The colonies were counted after incubation at 28 °C for 2 days. Lactic acid bacteria were counted on GPY agar plate containing 1% $CaCO_3$ after anaerobic incubation at 37 °C for 2 days in an AneroPack anaerobic jar (Mitsubishi Gas Chemical). Aerobic bacteria were counted on nutrient agar plate (0.5% beef extract, 1% peptone and 0.5% NaCl; Eiken Chemical) after incubation at 28 °C for 2 days.

For study of *K. lactis* growth on whey permeate, cell growth of preinocula and subsequent culture were measured and expressed as turbidity of the culture read at 580 nm (OD_{580}) and dry cells weight, respectively. Dry cell weight was measured after the remaining $CaCO_3$ had been dissolved by 0.1 N HCl, washed with distilled water and then dried in an oven at 105°C for 24 h.

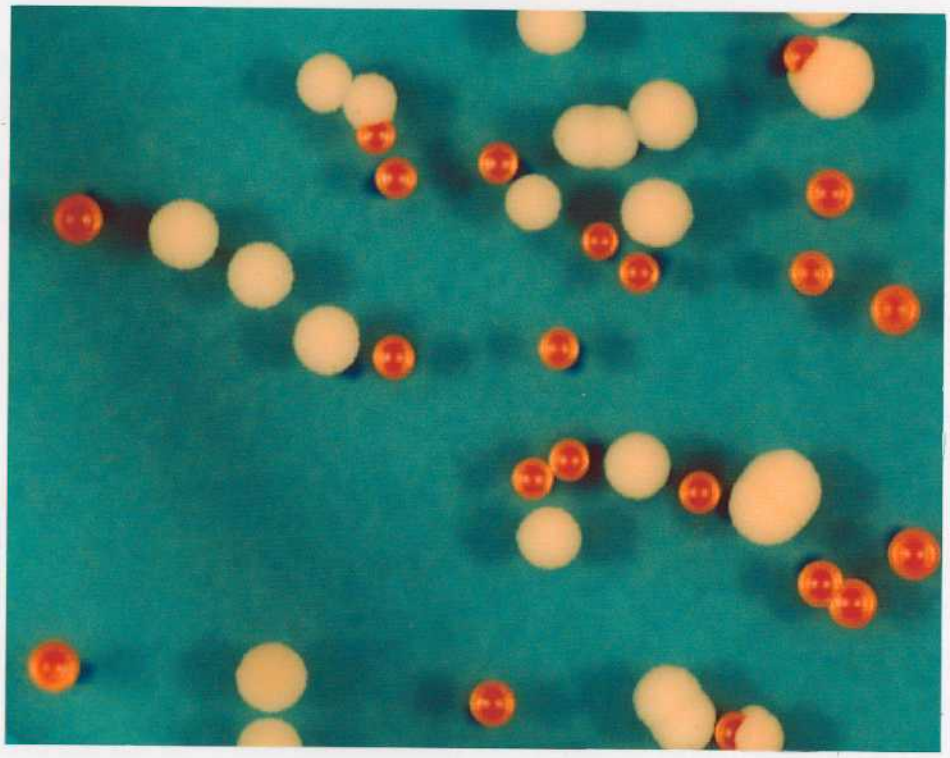

Fig. 2-2. Characteristic of colonies.

The colonies of *K. lactis* IF0 1267 were red, and those of *S. cerevisiae* IFO 0304 were white on SD plate.

2.2) Isolation of mutants

The mutants were produced by random mutagenesis which was done by exposure to UV light. Cells grown on SD for 24 h were washed and suspended in 0.2 M potassium phosphate buffer (pH 8.0). The cell suspension was irradiated with UV light to induce a 99% decrease in viability. The mutagen-treated cell suspension was spread on SD plates supplemented with 2% lactose.

2.3) DNA manipulation

2.3.1) General DNA techniques

Standard DNA manipulations were performed as described by Sambrook et al.[60] using the *E. coli* JM109 strain (Takara Shuzo). Genomic Southern hybridization[61] with the ECL direct nucleic acid labeling and detection systems (ECL system: Amersham International) and colony hybridization[57] with DIG-High Prime DNA Labeling and detection kit I (Boehringer Mannheim) were performed for cloning the *KlPCK1* gene with *PCK1*, the PEPCK gene of *S. cerevisiae* , as the probe. *PCK1* was amplified by polymerase chain reaction[60] (PCR) using genomic DNA of *S. cerevisiae* YNN27 as template and two primers selected to hybridize with the 5' and 3' ends of the Pck1 ORF[45]. Sequencing was performed on the DNA of deletion mutants prepared using a Kilo-Sequence Deletion Kit (Takara Shuzo). The nt sequences of the deletion mutants were analyzed using the technique of Sanger et al.[62] and the Taq/ Dye Primer and Terminator PRISM kits (Perkin Elmer) by DNA sequencing system 373A (Perkin Elmer). Plasmid carrying the *KlPCK1* was transformed into yeast by lithium acetate according to Ito et al.[63].

2.3.2) Isolation of genomic DNA from yeasts

Genomic DNA was prepared as described by Kaiser, C. et al.[64]. Restriction endonucleases were purchased from Takara Shuzo. Southern hybridization analysis was conducted by using ECL Direct Nucleic Acid Labelling and Detection Systems (Amersham International) following the protocols described in the supplier's instruction.

2.3.3) Isolation of killer plasmids from *K. lactis*

The isolation of pGKL plasmids was performed according to the osmotic lysis procedure as described by Stam et al.[65] as follows. *K. lactis* cells were grown in 200 ml YPD to a density of 2 x 10^8 cells per ml. Cells were treated with 0.2% 2-mercaptoethanol and 60 mM ethylenediamine-tetraacetic acid, disodium salt (EDTA) at 30 °C for 30 min. Cells were washed with 1.2 M sorbitol and converted to protoplasts in 10 mM Tris-HCl (pH8.0) containing 1.2 M sorbitol, 20 mM EDTA and 0.2 mg / ml zymolyase 100T (Seikagaku Kogyo). Protoplasts were washed once in 1.2 M sorbitol.

The protoplasts were incubated in 0.2% SDS containing proteinase K (0.5 μg/ml) at 37 °C for 45 min. DNA was extracted with phenol:chloroform:isoamylalcohol treatment and concentrated by ethanol precipitation.

2.3.4) Separation of yeast chromosome by pulsed field gel electrophoresis

The chromosomes of yeasts were separated using a contour-clamped homogeneous electric field (CHEF) as described by Wésolowski-Louvel et al.[51]. Yeast is grown overnight in liquid YPD medium to early stationary phase. The collected cells were treated with 50 mM EDTA containing 50 μg/ml zymolyase 100T at a density of 2 x 10^9 cells/ml. An equal volume of 1% low melting temperature agarose (SeaPlaque, FMC, dissolved in 125 mM EDTA and held at 50°C) was added. The mixture was poured in 100 μl molds and allowed to harden at 4°C. The agarose blocks were placed in 5 μl of a solution containing 500 mM EDTA, pH8-9, 7.5% 2-mercaptoethanol, 50 mM Tris, pH8. These blocks were incubated at 37°C for at least 4 h to overnight. The solution was changed to 500 mM EDTA, pH 8-9, 1% sarkosyl, and proteinase K (1 mg/ml). The incubation was continued for 6-24h at 50°C, after which the blocks were stored at 4°C in 500 mM EDTA/1% Sarkosyl. The CHEF electrophoresis was performed on a 15 cm gel using a 1.5% of standard low melting temperature agarose (Biorad). Electrophoresis was run at 130 volts for 73 hours. The temperature of the buffer was maintained at 9-10°C. For the first 16 hours the pulse time was set to 100 sec, and then varied from 180 sec to 360 sec with a linear rap during the following 51 hours, with the last 6 hours set to 360 sec. The gel was treated with prewarmed 0.25 M HCl at 30°C for 5 min, and was neutralized with 0.5 M Tris, pH7 at room temperature for 30 min. The DNA was transferred from gel to filter and then Southern hybridization was performed using conducted by ECL Direct Nucleic Acid Labelling and Detection Systems.

2.4) Transformation of yeast

2.4.1) Transformation by LiCl

Yeast cells were grown at 30 °C to a cell density of 10^7-2x10^7 cells/ml. The cells were pelleted at 5,000 rpm for 5 min and washed with 1/5 vol. 0.1 M LiCl in TE. After centrifugation the cells were resuspended in 1/100 vol. 0.1 M LiCl in TE and were shaken for 1 h at 30 °C. 10 μl of LiCl-treated cells were incubated with 1-5 μg of transforming DNA and 5 μl of sonicated salmon sperm DNA (10 mg/ml) at 30 °C for 30 min. After adding 0.8 ml of 40% PEG-4,000, the suspension was further incubated at 30 °C for 30 min. Dimethylsulfoxide (DMSO) was added to a final concentration of 10% and the tube was incubated 5 min at 42 °C. Cells were harvested by centrifugation at 4,000 rpm for 3 min, washed with sterilized water and centrifuged again. Cells were plated on selective media.

2.4.2) Transformation by electroporation

2.4.2.1) Basic procedure

The yeast were grown on SD at 30 °C for 17 hr. The harvested cells were treated with 0.001 N KCl containing 1 M dithiothreitol (DTT) at 30°C for 10 min and were washed with 0.001 N KCl. 190 µl of cell suspension containing 2 x 10^7 cells and 10 µl of DNA solution containing 0.1 µg pE plasmids in TE was incubated on ice for 20 min, transferred to the electroporation chamber and pulsed. The treated cells were incubated in 1 ml YPD at 30 °C for 1 hr. The cells were spread on selection medium.

2.4.2.2) Enhanced transformation

Plasmid DNA was transferred into *K. lactis* strains following the basic procedure as described by Sánchez, M. et al.[66] with some modification. A culture of *K. lactis* strain grown at 30 °C overnight was used as seed culture. The cells were then grown in YPD for 3.5 h until the early exponential phase, centrifuged, washed with ice cold water and resuspended into YED containing 25 mM DTT. The cell suspension was incubated for 30 min at 30 °C with shaking, centrifuged, and resuspended in electroporation buffer (EB, 1 mM Tris-HCl [pH7.5], 270 mM sucrose, 1 mM lithium acetate) at a density of 5 x 10^8 cells per ml. A 200 ml aliquot of the cell suspension was mixed with 1 ml DNA solution (1 mg in TE buffer) and placed on ice for 15 min. Electroporation was performed in a 0.5 mm sterile cuvette FTC-01 (Shimadzu) using the hand made electroporator. One attenuation pulse at a setting of 200 V (4 KV/cm) with a capacitance of 100 to 200 µF was applied. Pulse duration was about 16 to 24 ms under this condition. The cell suspension was transferred to a sterile Eppendorf tube containing 1 ml of cold YED broth, and incubated for 15 min on ice and for 6 hr at 30 °C before plating.

2.5) Enzymatic assay

2.5.1) Preparation of cell free extracts

S. cerevisiae were grown on SD medium containing glucose for 2 days at 30 °C on a rotary shaker. The harvested cells were washed, transferred to the SD medium containing ethanol as the sole carbon source and shaken for 24 hr. *K. lactis* strain was grown on YPD medium for 24 hr, the washed cells were transferred to the YPG or YPD medium and shaken for 13 hr. *In vivo* inactivation of the enzymes was achieved by addition of 10 ml YPD medium into 10 ml of culture broth and incubation at 30 °C for 80 min with shaking. Cell-free extract were prepared as described by Perea and Gancedo[42]. One hundred mg (wet weight) of cells were washed with cold water, shaken in a vortex with 1 g glass beads (0.5 mm diameter) in 0.5 ml of 20 mM imidazole

buffer (pH7) for five periods of 1 min with interval of 1 min. After centrifugation at 27,000 x g for 10 min at 4 °C, the enzyme activities of supernatants were assayed.

2.5.2) Analysis of enzyme activity

All enzyme assays were carried out at 30 °C. PEPCK activity was assayed according to Perea, J and Gancedo, C.[42] with some modifications. The reaction mixture consisted of 150 μmol imidazole, 150 μmol $NaHCO_3$, 6 μmol $MnCl_2$, 6 μmol glucose, 1 μmol reduced glutathione, 1 μmol ADP, 0.5 μmol NADH, 0.1 μmol phosphoenolpyruvate, 80 n kat malate dehydrogenase, 40 nkat hexokinase in a total volume of 3 ml and 50 μl cell free extract. The pH of the assay was 7.0. To ascertain the ATP dependency of the PEPCK, the enzyme activity of crude protein was assayed as above with and without ADP. Lactic acid dehydrogenase was assayed by the supplier's instruction (Oriental Yeast) with some modifications. The reaction mixture consisted of 85 μmol potassium phosphate buffer, pH 7.0, 0.5 μmol NADH, 1.6 μmol Na-pyruvate in a total volume of 1030 μl and 50 μl cell free extract. Malate dehydrogenase was assayed as described by Wolfe, R. G. et al.[67] with some modifications. The reaction mixture consisted of 90 μmol glycine-NaOH buffer, pH 10, 2.5 μmol NAD, 1 μmol sodium L-malate in a total volume of 1050 μl and 50 μl cell free extract. Fructose-1,6-bisphosphatase of the dialyzed crude enzyme solution was assayed as described by Foy, J. J.[68]. The reaction mixture consisted of 0.1 M KCl, 10mM $MgCl_2$, 0.1mM fructose-1,6-bisphosphate, 1mM EDTA, 50 mM imidazole buffer (adjusted to pH 7 with HCl), 0.25 mM NADP, 0.5 unit of phosphoglucose isomerase, 0.5 unit of glucose-6-phosphate dehydrogenase in a total volume of 1 ml. Crude protein of *K. lactis* was prepared from cell free extract by dialysis in 100 volumes of cold 0.05 M potassium phosphate buffer, 1 mM EDTA and 2 mM 2-mercaptoethanol, pH 7.0 for 18 hr with three changes of buffer. To measure the enzyme activities, 200 ml of crude protein solution was added to the assay mixture. The substrate, fructose-1,6-bisphosphatase, was omitted from the control. The amount of protein was determined with Protein Assay kit with bovine serum albumin as a standard.

2.6) Assay of killer spectrum

2.6.1) Killing activity

The killing ability of killer yeasts was tested as described by Somers and Bevan[69] using *S. cerevisiae* B511-4C as a target strain. The target strains initially were cultured in 30 ml of the YPD medium put in a 300-ml Erlenmeyer flask at 30 °C for 24 hr on a rotary shaker (220 rpm). The cell suspension containing approximately 5×10^6 cells was spread on buffered methylene blue medium. The killer strains, cultured on YPD plates at 25 °C for 24 hr, were taken out with a cork borer (diameter 5 mm) and placed on cells of the target strain, which were spread on the assay medium. After 2 or 5 days

of incubation at 25 °C, a killing zone was observed around the agar plugs of killer yeast. The sensitivity was estimated by the width of the killing zone.

2.6.2) Preparation of the crude killer protein

About 750 ml of the culture broth in which *K. lactis* IFO 1267 had been cultivated for 19 hr was centrifuged. The supernatant was concentrated and washed with 2.0 L of 1:10 McIlvaine buffer (pH 6.0) at 4 °C using labomodule ACL-1010 (Asahi Kasei) membranes with a molecular weight cutoff of 13,000 dalton. The concentrate (190 ml) was filter-sterilized by passing through a 0.20 μm membrane filter (Advantec). The filtrate thus obtained was stored at -20 °C in the presence of 30% glycerol.

2.7) Assay of yeast mating reaction

To analyze the mating efficiency of *K. lactis* PCK27, we tried to cross with it laboratory *K. lactis* strains. Wild strain *K. lactis* IFO 1267 has been reported as mating **a** type[30]. I crossed *K. lactis* MW 98-8C (α *ura A, argA1, lys A1*) and strain PM6-7A (**a**, *ura A, ade2*) with *K. lactis* PCK27 on YPD plate after over night incubation on YPD plate. The cell mixture was transferred to SD plate containing 0.5% calcium lactate as a carbon source. Zygote were selected on the selective agar plate.

2.8) Chemical analytical methods

2.8.1) High performance liquid chromatography

The concentration of glucose, lactose, and lactic acid were determined by HPLC using a LC5A (Shimadzu) with a column, Shim-pack SCR 101H (7.9 mm x 30 cm, 40 °C). Distilled water adjusted to pH 2.2 with $HClO_4$ was used as the mobile phase at a flow rate of 1.2 ml/min. A reflectometer and spectrophotometric detector (210 nm) were used to detect sugar and lactic acid, respectively.

2.8.2) Enzymatic assay

Lactic acid and sugar content of each sample were measured by a colorimetric assay on a 96-well plate as follows. NAD(P)H was quantitatively produced from D (L) lactic acid or sugar using enzymatic determination F-kit [Roch diagnostic], and a highly reduced water soluble tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium, monosodium salt, (Dojindo Laboratories)] in the presence of 1-methoxy PMS (1-methoxy-5-methylphenazium methylsulfate, Dojindo Laboratories) to produce formazan dye[70] (Fig. 2-3).

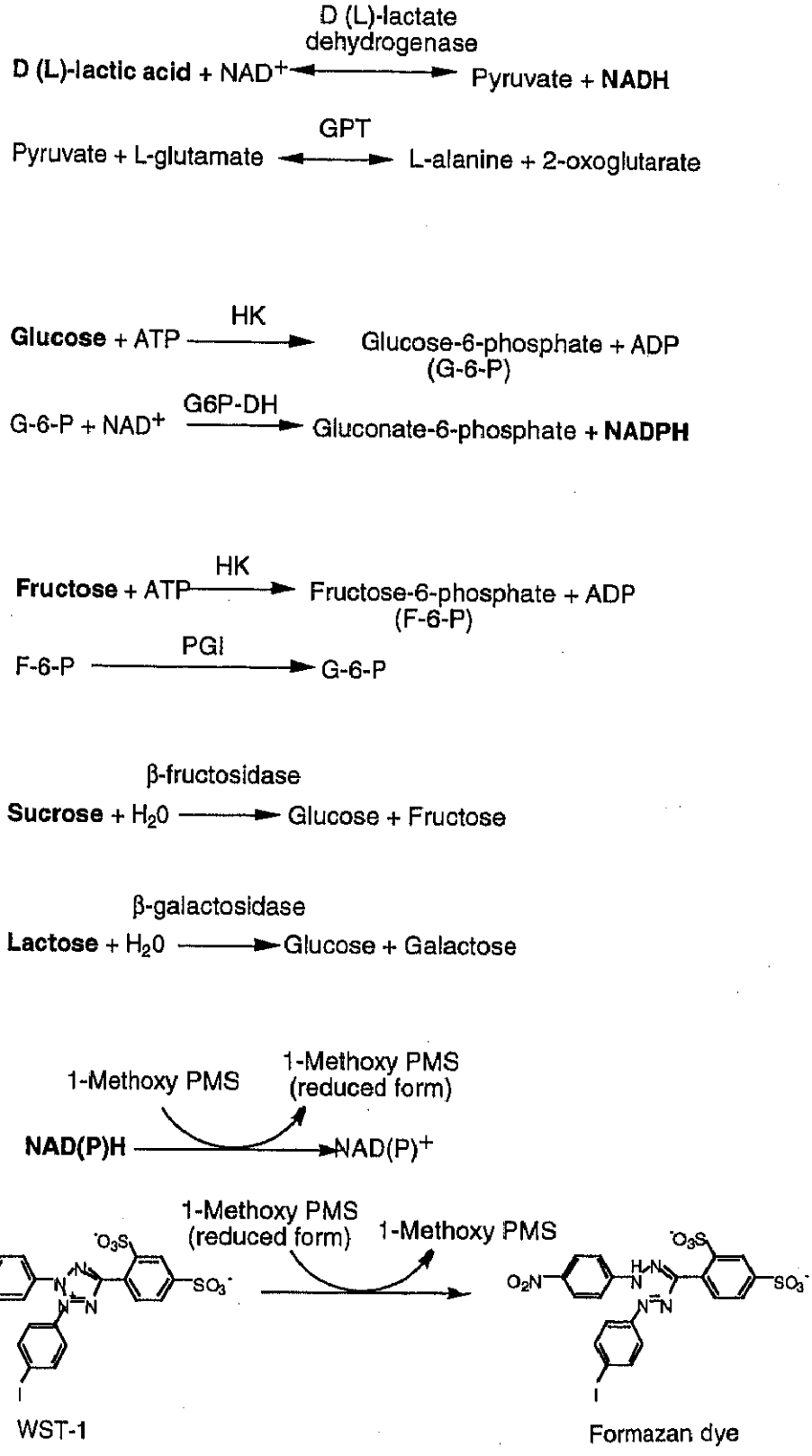

Fig. 2-3. Principle for assay of lactic acid and residual sugar.

GPT: glutamate-pyruvate transaminase, HK: hexokinase, PGI : phosphoglucose isomerase.

15 µl of each sample solution was mixed with 10 µl of each of 1mM WST-1 and 50 µM 1-methoxy PMS. 1/30 volume of each buffers, β-NAD or NADP, enzymes and other materials recommended in manufacture's instruction were mixed and treated, respectively, on 96 well microplate at 33 °C for 15 min. Final volume of each well was 90 µl. Sucrose and lactose were previously dehydrated on well as manufacture's instruction at 33 °C for 30 min. 15 µl of 0.1 N HCl was added immediately after enzymatic reaction. The absorbance of the produced formazan dye was measured at 450 nm. The concentration of D (L)-lactic acid, glucose, fructose, sucrose and lactose in silage were measured separately, and calculated as % total lactic acid and % total sugar of wet matter.

2.8.3) Chemical assay

In the study of yeast growth on whey permeate, the amount of reducing sugars was determined by the modified Somogyi's method[71].

2.9) Computer analysis

2.9.1) DNA and amino acids analysis

Sequences were analyzed using the MP search program (Intelli Genetics).

2.9.2) Statistical analysis

In chapter VI, data are presented as the means and standard deviations of results from triplicate assays for each experiment. All data were subjected to ANOVA using General Linear Model procedures of the Statistical Analysis System[72]. Growth, pH, lactic acid and residual sugar were analyzed by the Duncan's Multiple Range test. A value of $P < 0.05$ was considered to be significant.