Studies on the Prevention of Aerobic Spoilage of Silage by Killer Yeast, *Kluyveromyces lactis*
# CONTENTS

## Chapter I
**Introduction**

1) **Feed resources for animal production – The framework**
   1.1) Livestock-environmental interactions
   1.2) Self-sufficiency of food in Japan

2) **Feed and by-product production in the dairy industry**
   2.1) Silage production
   2.2) Aerobic spoilage of silage
   2.3) Yeasts found during ensiling
   2.4) Recent studies on silage microorganisms and additives
   2.5) Whey production
   2.6) Utilization of whey in the dairy industry

3) **Yeast physiology and biotechnology**
   3.1) Killer yeast - Biotic factors influencing yeast growth
   3.2) Utilization of killer phenomena
   3.3) Carbon metabolism of yeast – Gluconeogenesis
   3.4) Molecular biology of yeast, *K. lactis*

## Chapter II
**Materials and Methods**

1) **Materials**
   1.1) Microorganisms
   1.1.1) Bacteria
   1.1.2) Yeasts
   1.2) Plasmids
   1.3) Chemical products
   1.4) Biological products

2) **Methods**
   2.1) Culture condition
   2.1.1) Culture media and growth conditions
   2.1.1.1) Bacteria
2.1.1.2) Yeast
2.1.2) Preparation of silage fermentation model system
2.1.2.1) Rapid system
2.1.2.2) Conventional system
2.1.3) Laboratory scale silage preparation
2.1.4) Count of viable cells
2.2) Isolation of mutants
2.3) DNA manipulation
2.3.1) General DNA techniques
2.3.2) Isolation of genomic DNA from yeasts
2.3.3) Isolation of killer plasmids from *K. lactis*
2.3.4) Separation of yeast chromosome
   by pulsed field gel electrophoresis
2.4) Transformation of yeast
2.4.1) Transformation by LiCl
2.4.2) Transformation by electroporation
2.4.2.1) Basic procedure
2.4.2.2) Enhanced transformation
2.5) Enzymatic assay
2.5.1) Preparation of cell free extracts
2.5.2) Analysis of enzyme activity
2.6) Assay of killer spectrum
2.6.1) Killing activity
2.6.2) Preparation of the crude killer protein
2.7) Assay of yeast mating reaction
2.8) Chemical analytical methods
2.8.1) High performance liquid chromatography
2.8.2) Enzymatic assay
2.8.3) Chemical assay
2.9) Computer analysis
2.9.1) DNA and amino acids analysis
2.9.2) Statistical analysis
Chapter III
Development of methods for this study

1) Analysis of chemicals in silage  
2) Transformation of *K. lactis*  
2.1) Construction of electroporation apparatus  
2.2) Effect of buffer concentration and pulse length on electroporation  
2.3) Effect of capacitance on transformation efficiency  
3) Discussion

Chapter IV
Selection of killer yeasts (*Kluyveromyces lactis*) to prevent aerobic spoilage of silage

1) Selection of killer strain  
2) Effect of killer protein and killer yeast on target strain in the model system of silage fermentation  
2.1) Effect of killer protein addition on target strain in the model system of silage fermentation  
2.2) Effect of killer yeast addition on target strain in the liquid model system of silage fermentation  
2.3) Effect of killer yeast addition on target strain in the solid model system of silage fermentation  
2.4) Effect of killer yeast unable to metabolize lactic acid on target strain  
3) Growth of *K. lactis* on whey permeate  
4) Discussion

Chapter V
Isolation of *Kluyveromyces lactis* killer strain defective in growth on lactic acid

1) Isolation and nucleotide sequence of the gene encoding phosphoenolpyruvate carboxykinase from *K. lactis*  
1.1) Cloning of *K. lactis* PEPCK  
1.2) Complementation of *S. cerevisiae pck1*  
1.3) Mapping of the *KIPCK1* gene
1.4) Sequence analysis of KIPCK1
1.5) Comparison of deduced PEPCK amino acid sequences of K. lactis, S. cerevisiae and other organisms

2) Construction of K. lactis killer strains
defective in growth on lactic acid by gene disruption
2.1) Selection of marker gene
2.2) Gene disruption of KIPCK1
2.3) Isolation of the KIPCK1 disruptant
2.4) Southern hybridization of chromosomes
2.5) Enzyme activity
2.6) Growth curve of disruptants
2.7) Killing activity of disruptants
2.8) Mating assay of K. lactis PCK27

3) Discussion

Chapter VI
Prevention of aerobic spoilage of maize silage
by a genetically modified killer yeast,
defective in ability to grow on lactic acid

1) Study in the model system of silage fermentation
1.1) Comparison of the growth of K. lactis wild strain and its transformant in the model system
1.2) The killer effect on different target yeast strains in the model system
1.3) Growth of P. anomala in the model system with co-inoculation of K. lactis killer strain or killer defective strain.

2) Study in the laboratory scale maize silage
2.1) The growth of K. lactis on maize silage
2.2) Effect of killer yeast addition on wild yeast present in maize silage
2.3) Effect of killer yeast addition on bacteria present in maize silage

4) Discussion
Chapter VII

Safety aspect of using a genetically modified strain as a silage additive

1) Concept of familiarity
   1.1) Familiarity with microorganisms and environment 81
   1.2) Familiarity with trait 82
   1.3) Familiarity with genetically modified killer *K. lactis* strain by this study 82

2) Risk/safety analysis
   2.1) Exposure considerations 83
   2.1.1) Survival, persistence and dispersal 83
   2.1.2) Gene transfer 83
   2.2) Scale-dependent considerations 83
   2.3) Potential adverse effects 83
   2.3.1) Target effects 83
   2.3.2) Non-target effects 84

Chapter VIII

General Discussion

Acknowledgments 87
References 88
Paper lists 98
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AHC</td>
<td>alfalfa hay cube</td>
</tr>
<tr>
<td>BOD</td>
<td>biological oxygen demand</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CBS</td>
<td>Centraalbureau voor Schimmelcultures, The Netherlands</td>
</tr>
<tr>
<td>CHEF</td>
<td>contour-clamped homogeneous electric field</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DDBJ</td>
<td>DNA Data Bank of Japan</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double strand RNA</td>
</tr>
<tr>
<td>DM</td>
<td>dry matter</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory, Germany</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>G418</td>
<td>geneticine</td>
</tr>
<tr>
<td>GDP</td>
<td>gross domestic product</td>
</tr>
<tr>
<td>GenBank</td>
<td>GenBank in National Center for Biotechnology Information (NCBI), USA</td>
</tr>
<tr>
<td>GRAS</td>
<td>Generally Recognized As Safe</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IFO</td>
<td>Institute for Fermentation, Osaka, Japan</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-Operation and Development</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAFF</td>
<td>Ministry of Agriculture, Forestry and Fisheries, Japan</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCYC</td>
<td>National Collection of Yeast Cultures, UK.</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>NRRL</td>
<td>Agriculture Research Service Culture Collection, USA.</td>
</tr>
<tr>
<td>SCP</td>
<td>single cell protein</td>
</tr>
<tr>
<td>YGSC</td>
<td>Yeast Genetic Stock Center, USA.</td>
</tr>
</tbody>
</table>