Chapter VIII

General Discussion

Aerobic spoilage of silage by microorganisms is an important problem facing ruminant animal production. Little attention, however, has been given to the control of undesirable microflora in silage. Aerobic spoilage is primarily attributable to lactic acid utilization by wild yeasts present in forage crops used in silage making. In view of the considerable economic and environmental significance of silage production, this study was conducted to examine the prevention of aerobic spoilage using killer yeast additives to silage. Here I describe the selection of killer yeast strains, construction of the killer yeast strain and evaluation of the feasibility of addition of killer yeast to prevent aerobic spoilage.

To initiate this study, I developed a new analytical method to measure lactic acid and sugar content of silage. By this practical method, I could measure a number of samples together at one time. Periodic analysis of the silage quality was made possible by this procedure. Furthermore, I developed an efficient transformation strategy by electroporation. To avoid the possible disruption of useful characteristics that may occur during mutation, *Kluyveromyces lactis* was genetically modified by homologous recombination between the original gene and disrupted counterparts. Both techniques enabled me to undertake further studies.

The killer yeast *K. lactis* IFO 1267 was characterized by its rapid killing activity and wide spectrum against yeasts which cause aerobic spoilage of silage. The killer strain and its crude killer protein suppressed the growth of wild yeasts in the rapid model system for silage fermentation. *K. lactis* has been known to assimilate lactose, an ability that is unusual in yeasts. The addition of lactose to silage effectively propagated the killer yeast and prevented the growth of target strains. However, because of its ability to assimilate lactic acid, the possibility that the killer strain may also be involved in aerobic spoilage of silage can not be ruled out.

I therefore decided to disable the killer strain from utilizing lactic acid by gene disruption. I focused my target on a specific enzyme of gluconeogenesis based on the fact that gluconeogenesis is essential for yeast to grow on gluconeogenic carbon sources such as ethanol, lactic acid or amino acids. I disrupted the gene (*KlPCK1*) coding for phosphoenolpyruvate carboxykinase (PEPCK). Following confirmation of this gene by hybridization with the *Saccharomyces cerevisiae* PEPCK gene (*PCK1*) used as a probe, *KlPCK1* was cloned from the *K. lactis* genome. *KlPCK1* complements the *pck1* phenotype of *S. cerevisiae* as indicated by its positive growth in lactic acid as well as the presence of PEPCK activity. Prior to cloning, *S. cerevisiae* (*pck1*) showed negative results for both parameters.

I disrupted *KlPCK1* by site-directed recombination *in vitro*. To construct a killer strain defective in its ability to grow on lactic acid, native *KlPCK1* was exchanged for the disrupted *KlPCK1* by homologous recombination. The gene disruptant was not able to grow on lactic acid and showed no PEPCK activity, and so gene disruption was confirmed as stable. The growth rate on sugar and killer activity were the same as the wild strain.

I evaluated the effect of killer yeast addition in silage by the silage fermentation model system and laboratory scale silage. The genetically modified killer yeast stopped growing at the sugar limiting stage and did not increase the pH of silage. The addition of recombinant killer yeast with appropriate amounts of lactose effectively prevented the propagation of target yeasts. Lactic acid consumption and pH increment were delayed by killer yeast addition. Addition of the killer defective *K. lactis* strain did not suppress the growth of target strains as the killer *K. lactis* did. This preventive effect is not only due to growth competition among the microbial species present but also by the killer effect of *K. lactis*. Thus I conclude that the genetically modified killer yeast, defective in its ability to grow on lactic acid is not involved in the aerobic spoilage of silage.

Finally, I tried to evaluate the effect of killer yeast addition on laboratory scale. Growth of *K. lactis*, both the wild and transformant strain, decreased immediately after increasing in the early stages after ensiling. However, inhibition of the growth of lactic acid-assimilating yeast by killer yeast addition was observed resulting from a change in yeast flora. pH increase was also delayed. These results indicate that *K. lactis* killer yeast, defective in growth on lactic acid, can prevent aerobic spoilage.

Prevention of aerobic spoilage by killer yeast addition is considered as a suitable technique from an economic and environmental management perspective. The fermentation control by microorganisms would be a safe and inexpensive technique. Whey use for stable silage production is an efficient method of recycling the waste effluent generated in a dairy producing region. My results showed that biomass of *K. lactis* killer strain can be produced from whey permeate added with small amount of ammonium sulfate, vitamins and salts. Thus, whey permeate can be used for production of killer yeast biomass as well as be used as silage additive as carbon source. The use of genetically modified microorganism in the field requires steps that would ensure biosafety. Stepwise development and evaluation of killer yeast utilization as a silage additive were therefore considered important components of this study. My findings have shown that *K. lactis* killer strain is one of the best strains to use in the field. This is the first study to demonstrate the feasibility for prevention of aerobic spoilage of silage by genetically modified killer yeast.