Chapter VII

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

In this chapter, I discussed the biosafety of using the genetically modified killer *Kluyveromyces lactis* as a silage additive to prevent aerobic spoilage. I discussed it under the following concepts: familiarity, risk/safety analysis and risk management which were recommended by Organization for Economic Co-Operation and Development (OECD).

The Organization for Economic Co-Operation and Development (OECD) recommends the consideration of using genetically modified microorganisms for bio-safety, operation of stepwise development, and evaluation[91]. I believe that this present study is in conformation with the recommendation of OECD. My approach enabled me to gather data and information needed for the safe utilization of genetically modified microorganisms for food storage and for the protection of the environment.

1) Concept of familiarity

1.1) Familiarity with microorganisms and environment

I selected the *K. lactis* killer strain as one of the best, in terms of safety, for use in the field. *K. lactis*, a non-pathogenic yeast, is similar to *Saccharomyces cerevisiae*[50, 51, 92] and is quite known for its use as food and feeds. *K. lactis* is one of the fewer yeast species that assimilate both lactose and α-glucosides[93]. The yeasts can be found in dairy products such as milk, yogurt and cheese. *K. lactis* has been used to produce Kefyr and Koumiss for many centuries, and has been affirmed as Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration (FDA) as a direct ingredient of food[94]. *Kluyveromyces* species are particularly good lactose fermenters and are therefore useful in alcohol and yeast biomass production from whey. The results of testing inactivated *K.lactis* powder as a protein supplement revealed its high nutritional qualities and a broad range of therapeutic effects was determined with no problems. Furthermore, the use of *K. lactis* has been permitted in a number of countries world wide as a host strain for producing the heterologous protein of the milk-clotting enzyme, chymosin. Like *S. cerevisiae* or *Schizosaccharomyces pombe, K. lactis* is rapidly becoming a model organism. An European-based international group of geneticists interested in *K. lactis* has agreed to adopt a standard nomenclature of mutations and chromosomes for the species. They have chosen strain CBS 2359 (*K. lactis* IFO 1267) as standard for genetic analysis. Thus, further genetic information including sequencing the genome will become clear near future.

1.2) Familiarity with trait

Disability to growth on lactic acid and antibiotic G418 resistance were attributed to the disruption of PEPCK gene due to insertion of marker gene. The introduced marker gene is the most commonly used marker gene, bacterial aminoglycoside phosphotransferase II (*APT2*), also known as neomycin phosphotransferase II (*NPTII*) gene. The gene, isolated from *E. coli* transposon Tn903 as kanamycin resistance gene, functioned in yeast as G418 resistance gene. It has been used in most commercialized transgenic plant varieties and also in those being planned for commercialization. Many reports on biosafety of genetically modified plants using *NPTII* gene included studies on characterization of plant with kanamycin resistant gene, RNA products from plants with *NPT II*, as well as the level of NPTII protein production[95, 96]. Quick digestion of NPTII protein by animals and human, absence of homology to known toxic proteins and allergenic proteins, absence of compromise of NPTII protein in food on the therapeutic use of kanamycin, led to the conclusion that NPTII protein is not itself harmful to animal or humans[96]. Based on these findings, *NPTII* became the first marker gene to be included in the list of approved markers for transformed plants and food. From the above considerations I concluded that the genetically modified *K. lactis* by *APT2* (*NPTII*) gene is one of the most suitable strains for this study.

1.3) Familiarity with genetically modified killer *K. lactis* strain by this study

Data needed to asses the safety concerns of genetically modified *K. lactis* killer yeast were generated. During the process of developing transformed *K. lactis*, it underwent a series of selection indices including 1) positive response on G418 resistant assay, 2) negative response on growth on lactic acid, 3) low level of PEPCK, 4) growth on sugars including lactose, 5) killer activity 6) growth and killer effect on silage fermentation model system, 7) growth and killer effect on laboratory scale silage. Such selection indices are analogues to typical selection process for development of silage additive by conventional microorganism breeding.

K. lactis PCK27 is a prototype construct used to demonstrate that levels of PEPCK could be reduced by gene disruption and such reduction altered only the characteristics of growth on non-fermentable carbon source as predicted. Data of this study support the safety of transformed gene and other characteristics were unchanged. This strain contains only a gene required for the integration and selection process. Integration and structure of inserted DNA were analyzed and was indicated as stably integrated into *KlPCK1* location of *K. lactis* genome. Once inserted, the *APT2* gene behaved like any other *K. lactis* gene (i. e., were stable and segregated in a Mendelian fashion).

2) Risk/safety analysis

2.1) Exposure considerations

2.1.1) Survival, persistence and dispersal

As shown in Chapter VI (Fig. 6-1A, Fig. 6-5B and Fig.6-6), *K. lactis* can not survive the low redox condition in the presence of short-chain organic acids. Furthermore, *K. lactis* PCK27 stopped growing on the silage without sugar. Its survival and persistence is desirable for strong killer effect, but undesirable from the point of view of environmental safety.

2.1.2) Gene transfer

Conjugation (mating) is the direct transfer of gene between microorganisms in the environment. *K. lactis* PCK27 possesses a reduced mating efficiency (Chapter V). Therefore, the *APT2* gene rarely transferred by sexual reproduction. The presence of two linear plasmids, pGKL1 and pGKL2, confers the killer phenotype in *K. lactis* IFO 1267[30]. The killer plasmids is well studied by several workers. Very similar, if not identical, plasmids were also present in several other *K. lactis* isolates. Both pGKL1 and pGKL2 have inverted terminal repeates whose 5' ends[97] have a covalently attached protein[98]. Replication is probably initiated by these terminal proteins. pGKL plasmids have been transferred to petit-negative (respiratory deficient, *rho*$^-$) *S. cerevisiae* by cell fusion, but not to *rho*$^+$ strain[83, 99]. pGKL plasmids have been stable transferred to *K. marxianus* strains by illegitimate mating[100]. However, the frequency of killer gene transfer is quite low because of the difficulty to transfer both plasmids including mature protein, and of low mating efficiency. I have previously tried to transform pGKL plasmids into *K. lactis* killer defective strain. But, the transformed pGKL plasmids were very unstable. Thus I conclude that the transfer frequency of integrated *APT2* and *K. lactis* killer plasmid are relatively low.

2.2) Scale-dependent considerations

To better understand the killer effect on silage the experiment was scale up from silage fermentation model system (1.25 g) to laboratory scale silage (50 g). For the practical use, further study with larger size of silo, effect on ruminal microorganisms and study of risk management will be needed to satisify the information for biosafety.

2.3) Potential adverse effects

2.3.1) Target effects

In the model system, I observed a significant effect of addition of genetically modified killer strain on silage quality compared to wild killer strain. I was not able to make a complete comparison between these two strains in the laboratory scale silage

because the density of killer yeast decreased to a number below the limit of detection before opening the silo bag (Fig. 6-6). However, the killer yeast addition was found to cause a change in the yeast flora of silage (Fig. 6-7). To avoid the possible aerobic spoilage by reduced *K. lactis* killer strain, I concluded that the genetically modified killer strain is suitable for silage additive.

2.3.2) Non-target effects

Effect of addition of genetically modified killer strain on lactic acid bacteria and aerobic bacteria, considered in this study as non-target microorganisms, was also studied. Initial tests indicate no difference between samples with and without killer yeast. However, I have not started the study on effect of killer protein against ruminal microorganisms. Killer *S. cerevisiae* are used in brewing field. Killer yeasts are widespread in natural habitats[101]. Animals as well as humans may take in such killer yeast with food. However, the effect on ruminal microorganisms may be essential if it is feed as silage additive. The gene, product and mode of action of killer protein are variable. *K. lactis* killer protein blocks sensitive *S. cerevisiae* in the G_1 phase of the cell division cycle[31], leading to unbudded cells with unreplicated DNA[102]. The protein is a large heterotrimeric ($\alpha\beta\gamma$) N-glycosylated protein[103], but the smallest (γ) subunit alone is responsible for the cell cycle arrest. However, the α and β subunits are required for native killer protein to act from the cell exterior . The α polypeptides shows sequence similarity to plant and bacterial chitinases, and exhibits a chitinase activity[104]. Several genes required to resist the effect of *K. lactis* killer protein were reported[105, 106 , 107, 108 , 109 , 110]. Among them is the gene essential for cell wall synthesis. But the protein which directly interact with γ polypeptide subunit of killer protein and the mechanism of G_1 arrest by γ subunit are not known yet.