Chapter I

Introduction

1) Feed resources for animal production – The frame work

1.1) Livestock-environmental interactions

Enhanced animal production systems to satisfy demand for high value animal protein are needed. The driving force behind the demand for livestock products is a combination of population growth (1.5 percent per year), rising incomes (capita GDP has grown over 3 percent per year) and urbanization (over 80% of the world's population growth occurs in cities of developing countries) world wide, particularly in the developing countries[1]. However, the rapidly increasing demand for livestock products contacts with the traditional resource base for livestock production that cannot expand at the same pace. Finding the balance between increasing food production and the preservation of the world's natural resources is a major challenge. Policies, technologies and strategies need to be designed to correct negative environmental effects of livestock production which are not reflected in product and input prices. Their purpose is not simply to reduce the environmental damage by reducing pollution, but also to enhance and save natural resources, through the use of livestock, and to turn waste into useful products.

1.2) Self-sufficiency of food in Japan

Japanese self-sufficiency of food has been dropping in recent decades. The degree (caloric bases) was 73% in 1965 and 41% in 1997[2]. Due to the concern for food security threatened by unstable factors in the world food trade and the environmental importance of Japanese agriculture, The Basic Law on Food, Agriculture and Rural Areas was enacted by Japanese government in 1999[3], to secure a stable food supply to Japanease people with domestic agricultural production as its basis, together with an appropriate combination of food importation and food stockpiles. The decrese in food self-sufficiency in Japan is mainly due to an increase in consumption of foods which can not be produced in sufficient quantities such as fat, meat, milk and eggs. Such products are in demand at three times than 1965 level. Furthermore, reduction of rice intake, of which can support 100% in Japan, spur on the decrese in food self-sufficiency. In Japan, while 68% of the stock farm products domestically produced, 80% of feed is imported[4]. Consequently, the increment of animal feed production is essential to increase the degree of self-sufficiency.

2) Feed and by-product production in the dairy industry
2.1) Silage production

Silage is a fermented feed for ruminant animals. Silage fermentation is performed on crops having a high moisture content with lactic acid bacteria under anaerobic conditions. Anaerobiosis and acidic conditions inhibit the growth and survival of contaminating microorganisms, resulting in stable silage. Ensilage is now a major method for forage conservation in many countries. Japan has the smallest area in the world for producing crops to feed animals. Furthermore, Japan has a rainy season. We therefore, have to produce the highest amount of nutritional harvest and stock them without being influenced by rainfall. Consequently, the production of silage is increasing. In 1996, about 30 million tons of silage was produced in Japan and that accounts for 68% of dairy feed[4]. Nowadays, the method, so-called silage feeding method for the whole year, is very common in Japan. Silage is prepared at the time of the highest production amount by a definite machinery system with cooperating nearby dairy farmers. Animals are fed silage the whole year. This work-saving-system enables Japan to produce animal feed at a low cost[5].

2.2) Aerobic spoilage of silage

After ensiling, quick acidification by lactic acid bacteria under anaerobic conditions is important to produce good-quality silage in competition with other microorganisms. When the silo is opened for feeding or sealed inadequately, silage is exposed to oxygen. This exposure leads to aerobic spoilage whereby aerobic microorganisms degrade lactic acid and residual water soluble carbohydrates to CO_2, and break down protein and amino acids to amines, amides, and ammonia in silage[6]. The importance of yeasts in aerobic spoilage has been confirmed by several workers[7-11]. Lactic acid-utilizing yeasts have a major role in initiating spoilage processes in silage made from a variety of forage crops. Most yeasts found in silage grow on acidic silages with pH a between 3.5 and 6.5. These yeasts utilize a variety of substrates including organic acids, such as lactic acid, acetic acid, among others that are commonly present in silage. Such actions consequently result in an increase in the pH of silage. Yeasts appear to grow on silage first, followed by bacteria, particularly those with proteolytic properties[12]. Aerobic spoilage results in considerable heat generation, pH increases, and the decrease in digestibility. This decrease in digestibility is greatest for protein with a decrease in silage palatability[11]. It has also been reported that aerobic spoilage results in dry matter (DM) losses during ensilage ranging from 4 to 30% of the original herbage which varies with type of silo and herbage[10]. Feeding spoiled material to animals should be avoided because it can lead to clinical diseases of the animals[12].

Even though the addition of propionic acid at high rate is effective against aerobic spoilage, its use has been limited because of its corrosive nature, relatively high cost,

involvement in the depression of animal intake and the various sensitivities of yeast[12, 13]. The fermentation control of silage by microorganisms would be one safe and inexpensive alternative. Inoculation of lactic acid bacteria has been recommended to improve the aerobic stability of silage, but is not effective at high oxygen concentration[12].

2.3) Yeasts found during ensiling

Several investigations have classified the main yeasts occurring in silage into three physiological types[12] (Fig. 1-1, Table I-1). The majority of the yeasts found on fresh crops are non-fermentative species (Type I) of the genera *Cryptococcus*, *Rhodotorula*, *Sporobolomyces and Torulopsis*, ranging in numbers from 10^1 to 10^7 /g. Many of these yeasts are pigmented, the carotenoid pigments giving them protection from ultraviolet radiation. After harvesting the crops, yeast numbers often increase during wilting, partly due to favorable growth conditions. Jonsson and Phalow[8] found that there was a rapid increase in total yeasts (10^3 to 10^6) during the first 12 hours of the 36 hour wilting period, including lactic acid assimilating and fermentative species (>10^4 /g) which at the time of harvest had been below the level of detection (<100 /g). After anaerobiosis has been achieved in the silo, the aerobic species are succeeded by fermentative yeasts. The main yeasts occurring in silage have been classified into two physiological groups:

1) The top-growing or pellicle yeasts, which have a weaker fermentation ability but a high respiration ability for lactic acid, i. e. genera *Pichia* and *Candida* (Type II).
2) Yeasts with high fermentative ability for sugars but variable ability to assimilate lactic acid, i. e. genera *Saccharomyces* (Type III).

The predominant species during ensiling depend on the level of anaerobiosis achieved. If the silage is exposed to air during fermentation, lactic acid-assimilating yeasts (Type II) predominated while, if anaerobic conditions are achieved and maintained, type II yeasts increase initially, but further anaerobic ensiling results in a reduction of type II yeasts and predominance of type III yeasts. If air is present in the silage during fermentation, type II yeasts predominate and for this reason they are considered to play an important role in aerobic spoilage of silage[8, 12, 14].

2.4) Recent studies on silage micoorganisms and additives

Silage is a solid mixed culture. The chemical composition and the microflora of raw plant materials, incubation temperature and anaerobic condition of silos are important factors affecting silage fermentation. These factors make analysis of silage difficult. Tanaka et al. have described a repeatable model system for silage fermentation[15,16]. They inoculated intentional microorganisms into sterilized milled plant materials on a small scale and incubated it anaerobically in a incubator. This system made it possible to analyze the silage microflora with ease.
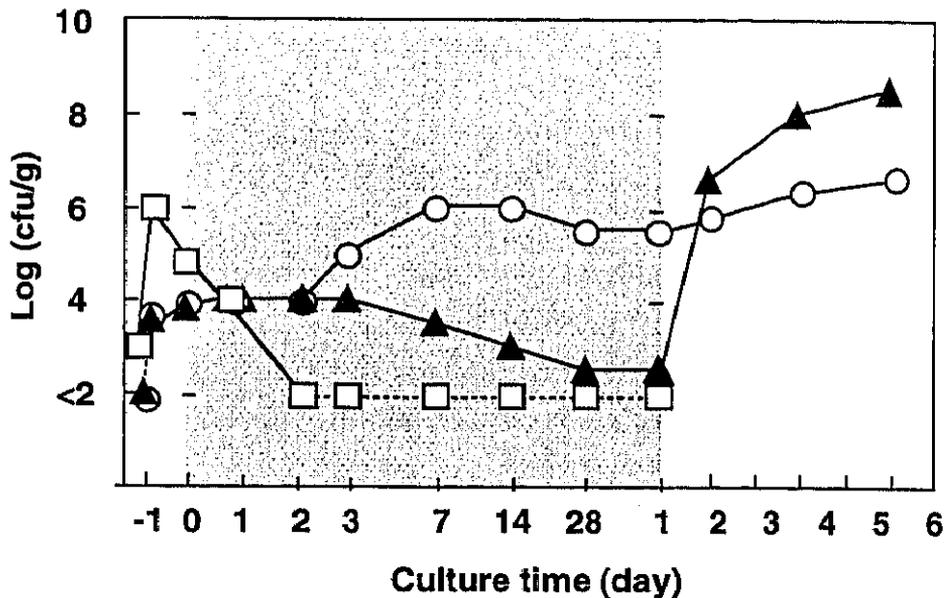
10

8

6

4

<2

Log (cfu/g)

-1 0 1 2 3 7 14 28 1 2 3 4 5 6

Culture time (day)

Figure 1-1. Growth of yeasts during wilting, silage fermentation and after opening the silo[8, 12, 14].

The shaded area indicates anaerobic conditions, and the blank area, aerobic conditions. Symbols: type I yeast (□), type II yeast (▲), and type III yeast (○).

Table I-1. Yeasts found in silage[14].

	Fermentation ability	Lactic acid assimilation	
Grass			
Rhodotorula glutinis	-	v	
R. rubra	-	v	
Cryptococcus laurentii	-	v	type I
Candid albidus	-	v	
Sporobolomyces roseus	-	v	
S. salmonicor	-	-	
Silage			
Saccharomyces cerevisiae	+	v	
S. exiguus	+	-	type III
Torulaspora delbrueckii	+	v	
Pichia anomala (Hansenula anomala)	+	+	
P. fermentans	+	+	
P. membranifaciens	w/-	v	type II
Issatchenkia orientalis (Candida krusei)	+	+	
Debaryomyces hansenii	w/-	v	
C. pulcherrima	+	+	
C. tropicalis	+	v	

Codes for responses to tests: positive (+), negative (-), weak positive response (w), and variable response (v).

4

2.5) Whey production

The consumption of cheese in Japan and total production of domestic cheeses in 1996 was 222,593 tons and 34,186 tons, respectively. The consumption and domestic production cheese is increased by 200 and 160 %, respectively from 1985 to 1996. Global cheese production is as 12 million tons in 1996[17]. Whey accounts for about 85 to 90% of the volume of the milk used for transformation into ripened cheese. That is more than 100 million tons of whey was produced world wide. The BOD of whey ranges from 30,000 to 60,000 mg/L, depending on the cheese manufacturing process, and about 90% of the whey BOD is due to the lactose component[18]. Thus, disposal of cheese whey is a major problem for the dairy industry. It is the largest cause of water pollution from dairy food plants. Ultrafiltration is widely employed to recover whey proteins and this generates whey permeates which contain approximately 6% total solids. 70% or more of the solids of whey permeates are lactose[19]. Whey permeate is still being produced world-wide in such a large amount that it creates disposal problems in countries with a dairy industry.

2.6) Utilization of whey in the dairy industry

There is a growing interest in using whey permeate as a substrate for fermentation to produce ethanol[20], single cell protein[21, 22] and enzymes such as β-galactosidase, polygalacturonase or carboxypeptidase[23]. Several species of the yeast genus *Kluyveromyces* have a good lactose assimilating/fermentation ability, and are therefore useful in the whey bio-conversion process[24, 25]. Single cell protein of yeast including *Kluyveromyces* species have been used for ruminant animal feed to improve the rumen flora activity and to improve the performance of milk protein and fat synthesis[26, 27]. In ruminants, the live yeasts may be used as a protein source and act as to scavenge O_2 and prevent oxidative stress to rumen bacteria, or they may provide malic and other dicarboxylic acids to stimulate rumen bacterial growth[25].

Furthermore, dried whey has been used successfully in a number of silage studies[12]. Carbohydrate-rich materials are added to silage crops in order to increase the supply of available energy for the growth of the lactic acid bacteria, and they are of particular importance in crops deficient in soluble carbohydrate content. Materials which have been used for this purpose include sugars, molasses, whey, beet pulp, citrus pulp and potatoes. Whey added to a range of different crops increased the DM and lactic acid contents of silages. Addition of lactic acid bacteria with whey increased the lactic acid content of the silage, in general. However, the high cost of drying whey limits its use. Progress in membrane technology has contributed to the development of the sugar concentration process, thus saving on energy.

3) Yeast physiology and biotechnology

3.1) Killer yeast - Biotic factors influencing yeast growth

Almost 30 years ago, some strains of *Saccharomyces cerevisiae* were found to secrete a protein (killer toxin) lethal to other strains (sensitive) of the same species[28]. These killer strains were immune to their own toxins. A classification of killer yeasts exists which is based on cross-reactivity between different species. In the yeast killer phenomenon, killer proteins are encoded by different genetic determinants of different bioaction pathways[29]. For example, *Kluyveromyces lactis* killer protein, first found in *K. lactis* IFO 1267 (NRRL Y-1140, CBS 2359)[30], encoded by two linear DNA plasmids, which blocks completion of G_1 phase of the cell division cycle of sensitive cells[31]. While *Williopsis mrakii* killer protein is chromosomally inherited and which acts by firstly binding to cell wall receptors on sensitive cell walls followed by the creation of channels in the cell membrane to affect membrane permeability[32]. All killer proteins of *S. cerevisiae* are encoded on linear dsRNA plasmids; among them some affect membrane permeability, and one block G_1 phase of the cell division cycle[25, 33].

3.2) Utilization of killer phenomena

Possible uses for killer phenomena include the protection of industrial fermentations against contaminating yeasts. In brewing, sake mash and grape musts are usually not pasteurized before fermentation, and thus many wild yeasts from natural materials are brought to fermenting mash / musts. There is a high probability of producing inferior quality products, one potential approach of handling this problem is the construction of useful sake killer yeast[34] and wine killer yeast[35-38]. They have been reported to produce sake and wine of good quality. These strains can exclude sensitive yeast contaminants from fermenting sake mash and grape musts. The seed culture of sake, so-called *moto*, is made from water, malted rice (*koji*) and cooked rice under low temperature. During the initial stage of making *moto*, nitrite, produced from nitrate, and lactic acid, produced by lactic acid bacteria kill wild yeasts. Lactic acid bacteria are killed by the acidity of lactic acid. After one month, only sake yeast is propagated in *moto* [39]. The initial microflora transition of *moto* making is similar to silage fermentation. And the final stage of *moto* making, the microflora transition is similar to aerobic spoilage of silage. Several fermented foods and fodder are produced by fermentation of lactic acid bacteria together with yeast as sake, miso, soy sauce, pickles, Kefyr, bread dow and silage[40].

3.3) Carbon metabolism of yeast – Gluconeogenesis

Yeasts are grown on non-carbohydrate substrates as the sole carbon energy sources such as lactic acid, ethanol, glycerol, succinic acid and acetic acid. However, to grow on such carbon sources the synthesis of sugars required for macromolecular biosynthesis is necessary. Several yeast species have the ability to synthesize complex cellular polysaccharides from simple short-chain carbon compounds. Central to this capability is the conversion of pyruvate to glucose, which is referred to as gluconeogenesis, the reversal of glycolysis. Many yeasts, including S. cerevisiae, can alternate between a glycolytic mode of metabolism in the presence of glucose and a gluconeogenic mode in its absence. Two enzymes are characteristic in gluconeogenesis, fructose-1,6-bisphosphatase (E. C. 3.1.3.11) and phosphoenolpyruvate carboxykinase (E. C. 4.1.1.49/32) (PEPCK). PEPCK catalyzes the decarboxylation of oxaloacetate in the presence of ATP/GTP and a divalent metal ion to give CO_2, ADP/ GDP and phosphoenolpyruvate. PEPCK catalyze phosphoenolpyruvate formation as the first committed step in gluconeogenesis[25, 41].

A mutant of S. cerevisiae lacking PEPCK was isolated[42]. The PCK1 gene coding PEPCK was cloned by functional complementation of the mutant[43] and sequenced[44, 45]. The expression of the PCK1 gene in S. cerevisiae is strictly regulated and dependent on the carbon source provided[46]. The yeast, K. lactis, based on DNA sequences is closely related to S. cerevisiae. However, these two yeasts differ considerably in their physiologies. A study of their galactose catabolism[47] and fructose-1,6-bisphosphatase[48] concluded that S. cerevisiae demonstrates strong glucose repression, whereas K. lactis displays only partial glucose repression in most strains.

3.4) Molecular biology of yeast, K. lactis

K. lactis, a budding yeast is closely related to S. cerevisiae with respect to the predicted protein sequences of structural genes (the mean amino acid identity of 83.5%; standard deviation, 19.1%[49]) and random sequence of 1.3 % of the K. lactis genome, (mean amino acid identity, 63.6 %; standard deviation, 49.7%[50]). Thus K. lactis has limited sequence divergence from S. cerevisiae. The entire sequence of the genome of the yeast S. cerevisiae was made public in April, 1996. Such sequences from databases are now powerful tools for predicting the functions of K. lactis genes. Many biochemical studies of K. lactis had been reported because of the industrial and biochemical interest in its carbon metabolism differences with S. cerevisiae. For S. cerevisiae, several laboratories have developed genetically labeled strains and vectors as well as methods for molecular studies of this species[51]. For example, homologous recombination of the original gene for β-galactosidase gene of K. lactis and modified counterparts have been reported[52]. This report describes the development of a K. lactis strain as a host organism for the stable production of the milk-clotting enzyme, chymosin, using an integrating vector system.

Prompted by these studies, I initiated an investigation on the use of killer yeast as an inoculant to prolong the aerobic stability of silage. This doctoral thesis describes, materials and methods in Chapter II, the development of procedures to undertake this study in Chapter III, selection of killer yeast strains to prevent growth of aerobic spoilaging-yeasts in Chapter IV, construction of appropriate killer yeast for silage additives in Chapter V, and the study of prevention of aerobic spoilage by constructed killer yeast in a model system of silage fermentation and laboratory scale silage in Chapter VI. The biosafety of addition of genetically modified killer yeast in silage is discussed in Chapter VII.