Chapter IV

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

It was assumed that effective killer yeasts could be used to inhibit the growth of lactic acid-utilizing yeasts and to prevent silage from undergoing aerobic spoilage. The objective for this chapter is to describe the selection of killer yeast strains for this purpose, evaluation the effect of killer protein and killer yeast addition in a silage fermentation model system. Furthermore, I speculate that dried whey permeate, the high BOD by-product of cheese making, can be used as a lactose source for the production of killer yeast biomass and as a silage additive to produce good quality silage, at the same time, the pollution load in the environment in dairy regions can be reduced. Thus I studied cell productivity of killer yeast from whey permeate.

1) Selection of killer strain

Five strains of killer yeast were tested against 15 identified yeast strains and 8 wild yeast strains isolated from silage. Their killer spectra were measured at pH 4.5 (Table IV-1). Incidentally, the killing activity was more effective at a low pH than at neutral pH conditions. Most of the silages are in low pH range that the killing activity may expressed in silage. Among the killer strains tested, *Kluyveromyces lactis* IFO 1267 and *Williopsis mrakii* (*Hansenula mrakii*) NCYC 500 showed wide killer spectra. Although *W. mrakii* NCYC 500 had almost no effect on *C. krusei* and YG5 on the 2nd days, it killed these target strains on the 5th days. These results are explained by the fact that the first killing action of *W. mrakii* NCYC 500 resulted in the inhibition of cell wall synthesis in the target strain[79]. However, *K. lactis* IFO 1267 extensively inhibited the growth of the target strains from the 2nd days. *Kluyveromyces* species has been used to make Kefyr and Koumiss from milk in Asia for many centuries[80]. Furthermore, since the *K. lactis* killer strain was able to assimilate and ferment lactose as a sole carbon source, which rarely assimilated by other yeasts, *K. lactis* and lactic acid bacteria may preferably grow in silage than other wild yeasts when lactose was used as a supplemental carbon source. Thus I selected the *K. lactis* killer strain as one of the best, in terms of safety, for use in the field.

TableIV-1. Activity of various killer yeasts against yeasts grown on silage.

Killer strains	Killer type	Culture time (day)	Killing of strains: *Candida krusei* IFO 0011 MAFF0433 MAFF 4085	*S.cerevisiae* IFO 0304 IFO 1953 IFO 2048 ABXR-11A B511-4C	*Pichia anomala* IFO 0121 IFO 0145 IFO 0146 IFO 0963 CBS 251	*P. fermentans* AHU 3822 AHU 3844	*P. membrani-faciens* CBS 107	Yeasts isolated from silage YG-5 Sil-2 YS-1 YG-3 YS-2 It-1 Sil-1 YG-2
Kluyveromyces lactis IFO1267	pGKL 1,2	2	△△△	○△○○○	○○○○○	△△	○	△△○○△○○×
		5	△△△	○△○○○	○××○○	△△	○	○△○○○○○△
Saccharomyces cerevisiae ATCC 38976	K_1	2	×××	△×○○×	××××△	××	×	×△○××××××
		5	△△×	××○○△	×××××	××	△	×△○×××××△
Williopsis mrakii NCYC 500	K_9	2	×××	○×○○○	○○○○○	××	△	×○○○○○○×
		5	◎◎◎	○△○○○	○○○○○	××	△	◎○○○○○△×
Kluyveromyces drosophilarum NCYC 575	K_{10}	2	△△△	×△○△−	○○△○○	△△	△	△△○△○△××
		5	△○△	△△○○−	△△△△△	△△	△	△△○△△○△△

Assay was done at 30 °C, pH 4.5. Symbol: ◎, strong killing; ○, killing; △, weak killing; **X**, no killing.

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

The killer protein secreted by *K. lactis* IFO 1267 has been characterized as a macromolecular glycoprotein[81]. Killing activity was tested by the addition of the crude killer protein into the rapid-silage fermentation model system of liquid and solid culture of the target strain. Crude killer protein, extracted from the culture broth of *K. lactis* IFO 1267 by ultrafiltration, was added to the liquid culture of *S. cerevisiae* IFO 0304 (Fig. 4-1). The higher the protein concentration, the stronger the inhibition of the growth of the target strain. The decrease in the lactic acid content and increase of pH then were inhibited. In the solid culture, the inhibition was comparatively stronger than in the liquid culture because the addition of 2-fold dilution of the crude protein repressed the target strains growth completely (Fig. 4-2) in solid culture, but less suppressive in liquid culture (Fig. 4-1).

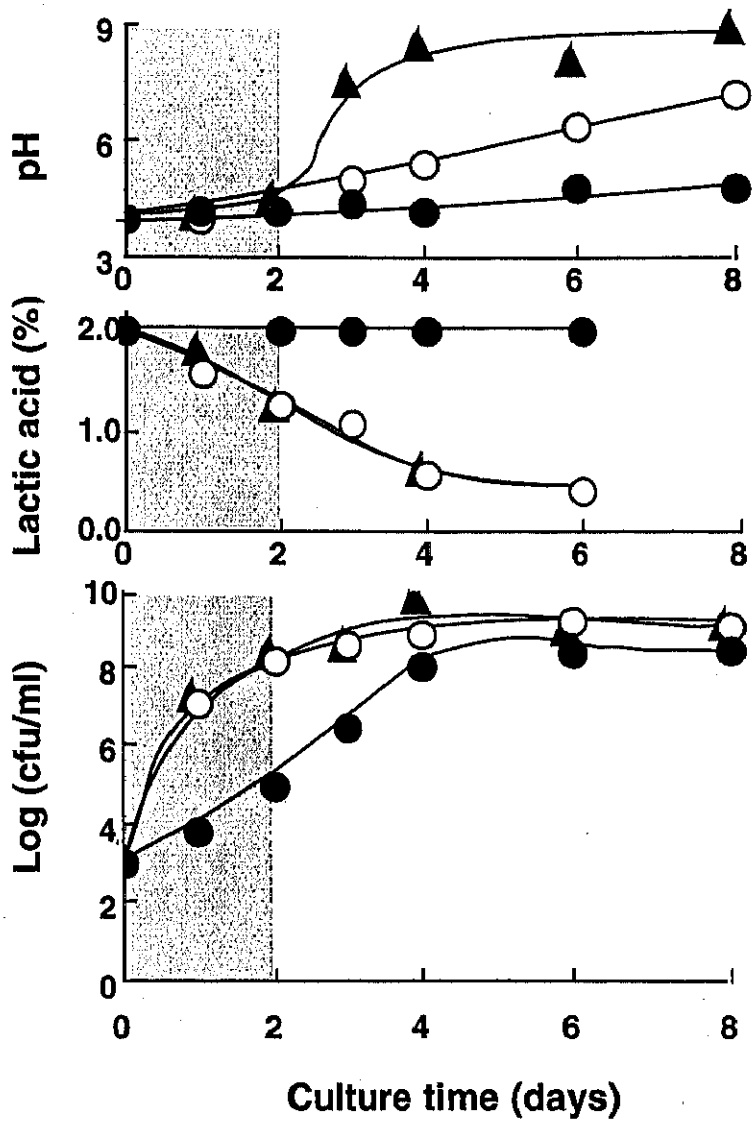

Fig. 4-1. Time course of target strain in liquid culture with crude killer protein.

The shaded area indicates anaerobic conditions and blank area, aerobic conditions. Target strain only (▲), target strain with killer protein at 10-fold dilution of the crude solution (○), and target strain with killer protein at 2-fold dilution of the crude solution (●).

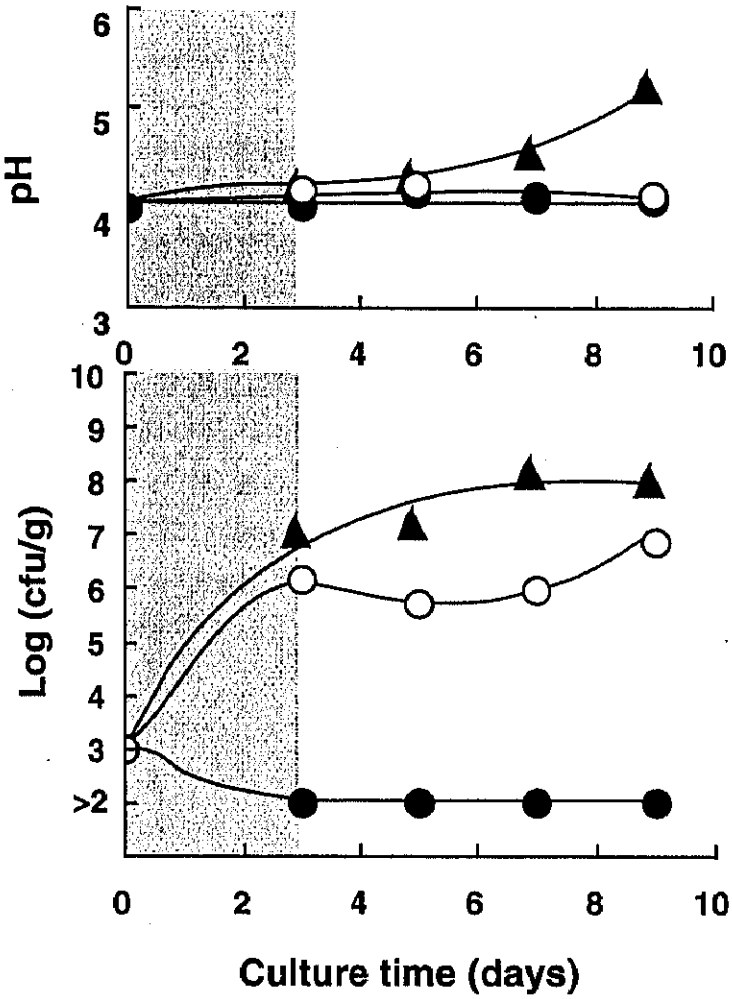

Fig. 4-2. Time course of target strain in solid culture with crude killer protein.

The shaded area indicates anaerobic conditions and blank area, aerobic conditions. Target strain only (▲), target strain with killer protein at 10-fold dilution of the crude solution (○), and target strain with killer protein at 2-fold dilution of the crude solution (●).

2.2) Effect of killer yeast addition on target strain in the liquid model system of silage fermentation

Because the killer protein is too expensive to use for ensiling, the killing activity was tested using the killer strain, *K. lactis* IFO 1267. The killing effect of killer yeast, *K. lactis* IFO1267, against a target strain, *S. cerevisiae* IFO 0304, in the rapid-liquid model system was investigated. Figure 4-3 illustrates the growth curve of *S. cerevisiae* IFO 0304 when glucose was used as the carbon source. The maximum cell number of the target strain in the mixed culture decreased by approximately 10% of that of the pure culture when the same amount (10^3 cells/ml) of killer yeast was inoculated. When 10^5 killer yeast cells/ml were inoculated, the growth of the target strain temporarily was repressed but eventually resumed. This result may account for the *K. lactis* IFO 1267 bacteriostatic action. Moreover, the growth of target strain was repressed completely when 10^8 cells/ml of killer yeast were inoculated. When lactose was used as a carbon source (Fig. 4-4b), the inoculation of 10^5 cells/ml of killer yeast repressed the target strain growth completely, indicating that the killing activity was more effective when lactose, instead of glucose (Fig. 4-3), was used as a carbon source.

However, the ability of *K. lactis* IFO 1267 to assimilate lactic acid was confirmed by growing the strain on a SD plate containing 0.5 % calcium lactate as the sole carbon source. Namely, *K. lactis* IFO 1267 assimilated lactic acid, led to increased pH (Fig. 4-4a) of the culture, and contributed to the aerobic spoilage of silage.

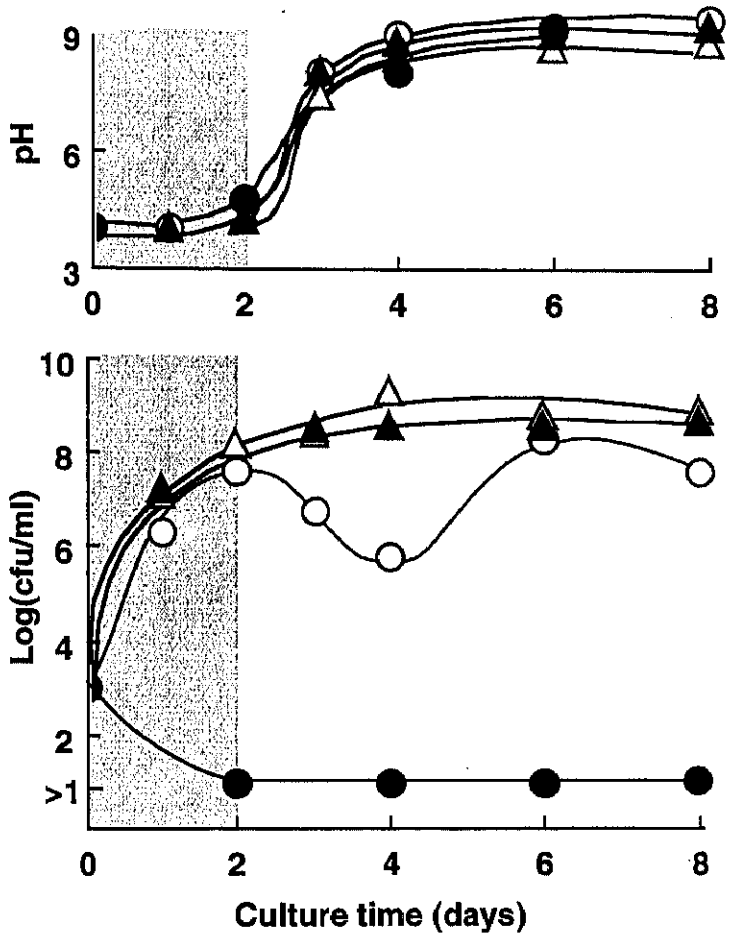

Fig. 4-3. Time course of target strain in mixed liquid culture with killer yeast.

The shaded area indicates anaerobic conditions and blank area, aerobic conditions. Ratio of inoculum size of *K. lactis* against target strain are $0:10^3$ (▲), $10^3:10^3$ (△), $10^5:10^3$ (○), and $10^8:10^3$ (●).

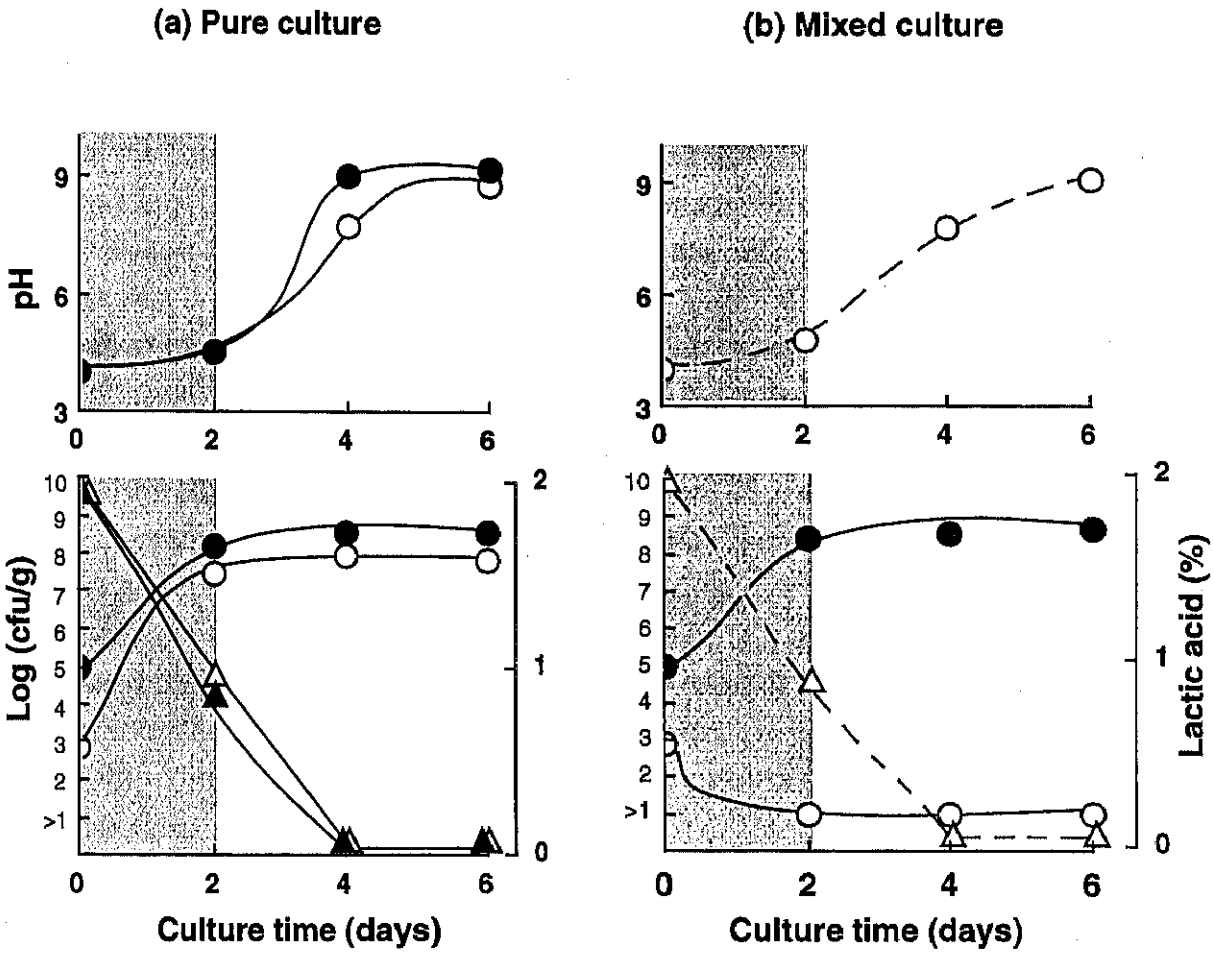

Fig. 4-4. Time course of pure (a) and mixed (b) liquid culture with lactose.

The mixed culture contains the killer yeast *K. lactis* IFO 1267. Ratio of inoculum size of *K. lactis* against target strain is 10^5:10^3. The shaded area indicates anaerobic conditions and blank area, aerobic conditions. The symbols refer to the growth and pH of *K. lactis* (●-●), target strain (○-○), residual lactic acid of *K. lactis* (▲-▲), target strain (△-△), mixed culture pH (○...○) and mixed culture residual lactic acid (△...△).

2.3) Effect of killer yeast addition on target strain in the solid model system of silage fermentation

Figure 4-5 shows the time course of the solid mixed culture. Maximum cell numbers of the target strain decreased by approximately 10% of the amount in the pure culture when 10^3 cells/g of fresh matter of the target strain and 10^8 cells/g of fresh matter of the killer yeast were inoculated. When the inoculation amount of the target strain decreased to 10^1 cells/g of fresh matter, the target strain growth was repressed completely (Fig. 4-5b). Unlike the addition of killer protein, the inhibition in the solid culture was weaker than that in the liquid culture (Fig. 4-1 and 4-2). This phenomenon was ascribed to diffusional mass transfer as well as to the propagation of *K. lactis* IFO 1267 in the liquid culture; the cell number decreased in the solid culture under anaerobic conditions. It is well known that, in many yeasts, multiplication is repressed under strictly anaerobic or high CO_2 pressure conditions. *S. cerevisiae*, is one of the yeast species that is tolerant to such conditions[82]. The number of *K. lactis* IFO 1267 cells decreased to approximately 1% of the inoculated mass (10^8 cells/g of fresh matter), while *S. cerevisiae* IFO 0304 displayed faster cell proliferation when inoculated separately on the silage fermentation medium in an anaerobic jar for 3 days, regardless of whether the carbon source was glucose or lactose. However, both strains propagated under the same conditions except when they were incubated aerobically, suggesting that the gas composition affected the growth of *K. lactis* IFO 1267. Therefore, the solid mixed culture was tested under aerobic conditions. The growth of the target strain was repressed completely when 10^3 cells/g of fresh matter of the target strain and 10^8 cells/g of fresh matter of the killer yeast were inoculated using lactose as a carbon source (Fig. 4-6). The inhibition effect was the same as that in the liquid mixed culture mentioned before.

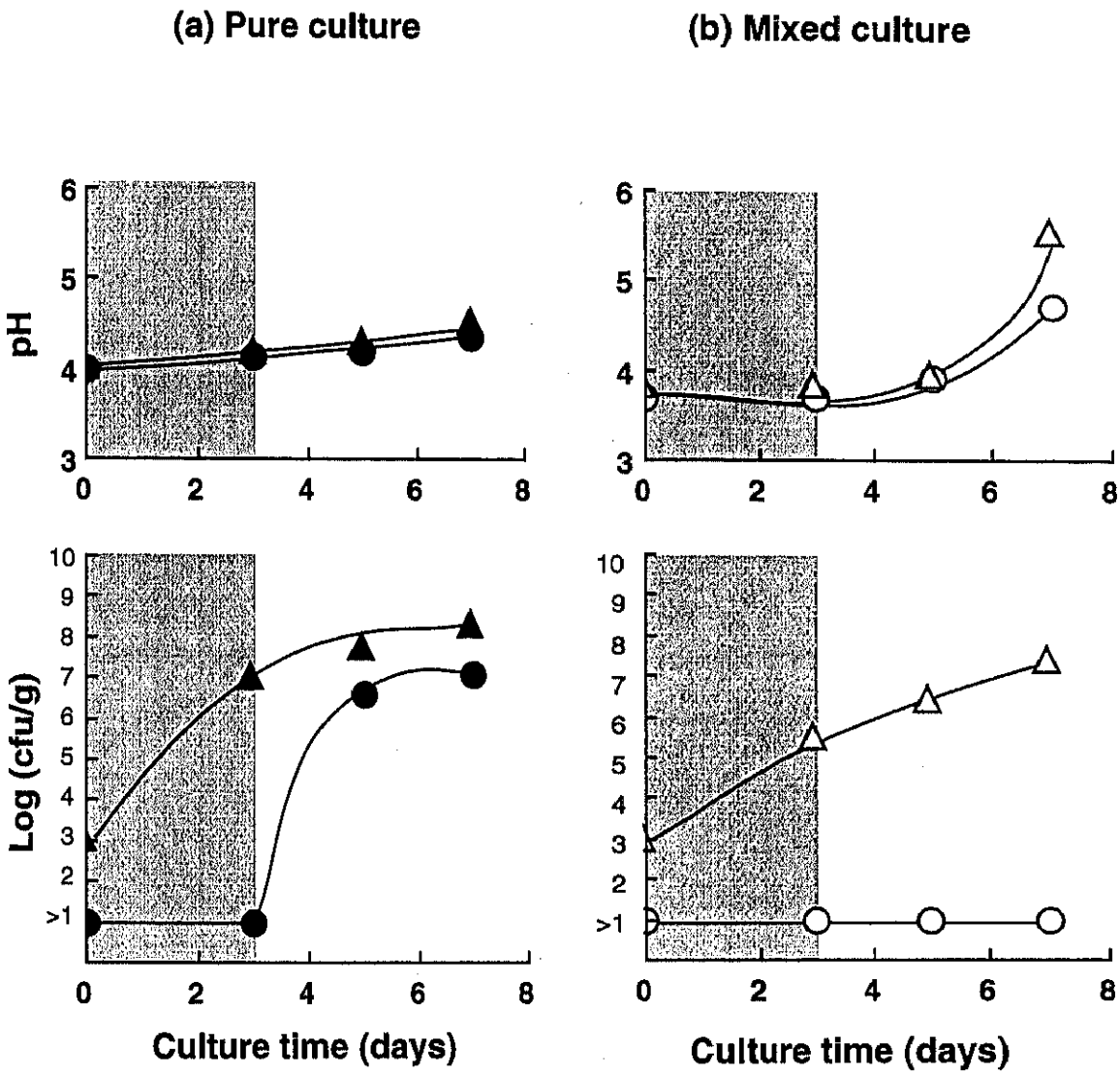

Fig. 4-5. Time course of pure (a) and mixed (b) solid culture with glucose.

The shaded area indicates anaerobic conditions and blank area, aerobic conditions. Ratio of inoculum size of *K. lactis* against target strain are $0:10^{3}$ (▲), $10^{0}:10^{1}$ (●)$10^{8}:10^{3}$ (Δ) and $10^{8}:10^{1}$ (○).

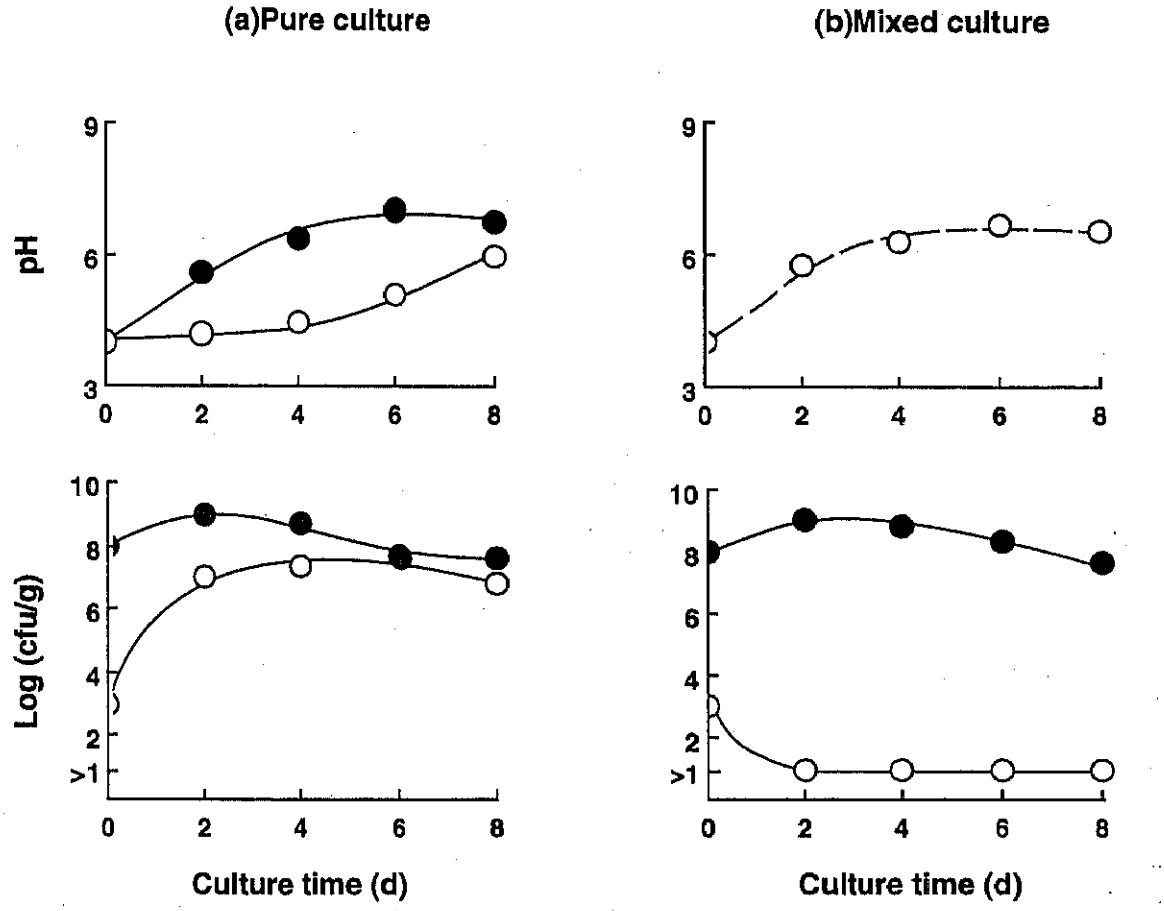

Fig. 4-6. Time course of pure (a) and mixed (b) solid culture with lactose under aerobic conditions.

Ratio of inoculum size of *K. lactis* against target strain is 10^8:10^3. The symbols refer to the growth and pH of *K. lactis* (●-●), target strain (○-○) and mixed culture pH (○...○).

2.4) Effect of killer yeast unable to metabolize lactic acid on target strain

The killer strain, *K. lactis* IFO 1267 assimilated lactic acid as a sole carbon source, led to increased pH of the culture, and contributed to the aerobic spoilage of silage. Lactic acid is one of the non-fermentable carbon sources. Enzymes, with regard to the tri-carboxylic acid cycle, exist in mitochondria. Hence, the yeasts, which lost mitochondria, designated as respiratory deficient mutant, cannot assimilate lactic acid. The killer strain, *S. cerevisiae* F-102 which was produced by protoplast fusion between a *S. cerevisiae* strain and *K. lactis* IFO 1267[83] cannot assimilate lactic acid because it is a respiratory deficient mutant. Though it cannot assimilate lactose either, the possibility of using the killer yeast, deficient in assimilate lactic acid as silage additive was examined.

When 10^3 cells/ml of target strain, *S. cerevisiae* IFO 0304 and 10^8/ml of the killer strain, *S. cerevisiae* F-102 were inoculated in the liquid culture containing glucose as a carbon source, *S. cerevisiae* F-102 failed to supress the growth of target strain. The same result was obtained in the case of the solid culture, presumably due to the competitive exclusion of *S. cerevisiae* F-102 from the mixed culture with *S. cerevisiae* IFO 0304 for the carbon source before it could exhibit the killing ability. The respiratory deficient mutant lost respiratory energy-yielding metabolism.

3) Growth of *K. lactis* on whey permeate

Nine *Kluyveromyces* strains were tested their ability to growth on whey permeate medium (WM, Table IV-2a). Furthermore, I compared the growth of *K. lactis* IFO 1267 against other yeast, most widely used as feed yeast (SCP, single cell protein) in animal diets. Yeasts growth on WM which carbon source was glucose instead of whey permeate was shown on Table IV-2 b. *K. lactis* IFO 1267 showed relatively high growth among the strains tested on WM of which lactose and glucose as a carbon source. All the tested yeast, except for *K. fragilis* IFO 1777 and *K. lactis* IFO 0648, consumed carbon source (2.5% as glucose) within 24 h.

Table IV-2. Biomass production of lactose-assimilating yeast strains from whey permeate (a) and common SCP yeasts grown on glucose (b).

(a) Whey permeate		(b) Glucose	
Strain	Dry cell weight	Strain	Dry cell weight
Kluyveromyces fragilis		*K. fragilis*	
IFO 0288	10.1	IFO 0541	9.2
IFO 0541	9.3		
IFO 1735	7.0	*K. lactis*	
IFO 1777	2.7	IFO 0648	5.2
		IFO 1267	9.2
Kluyveromyces lactis			
IFO 0433	9.0	*Saccharomyces cerevisiae*	
IFO 0648	6.5	IFO 0304	9.1
IFO 1090	4.4		
IFO 1267	8.9	*Candida utilis*	
IFO 1903	7.9	IFO 1086	8.8

4) Discussion

K. lactis IFO 1267 displayed rapid killing activity and wide spectrum killing; it was highly effective at a low pH against the target strains causing aerobic spoilage. On the basis of these results, I suggest that the killing action of *K. lactis* IFO 1267 may prevent silage from undergoing aerobic spoilage. In addition, *K. lactis* IFO 1267 can assimilate and ferment lactose as a sole carbon source, unlike most yeasts. Therefore, if lactose and a killer yeast with a lactose assimilation capacity are added to forage crops before ensiling, active growth of lactic acid bacteria and specific inhibition of wild yeasts growth may occur as the microflora of *moto* and sake mash with killer sake yeast. Accordingly, I selected *K. lactis* IFO 1267 as a killer strain for use in the following study. However, the killing zone of target strains by *K. lactis* IFO 1267 did not increase as the one by *W. mrakii* NCYC 500. This phenomenon accounts for the action of *K. lactis* IFO 1267 on the target strain, which resulted in the inhibition of growth at the G_1 stage[79] was more bacteriostatic than killing.

The crude killer protein, secreted by *K. lactis* IFO1267, displays a inhibition against the propagation of *S. cerevisiae* both in liquid and solid model system of silage fermentation. It was suggested that the killer protein produced by a strain of killer yeast, *K. lactis* IFO 1267, was able to prevent silage from undergoing aerobic spoilage. However, the killer yeast addition at the point of silage making seems to be more practical, because killer protein is too expensive to use for ensiling and the killer protein can be inactivated by proteinase digestion or heat production during ensiling. I investigated the effect of killer yeast addition on target strain in the model system of silage fermentation.

Indeed, the growth of the target strain, *S. cerevisiae* IFO 0304, was repressed by *K. lactis* IFO 1267, both in liquid and solid mixed cultures. The repression was more effective when lactose, instead of glucose, was used as a carbon source because of the repression of the growth of the target strain. *K. lactis* IFO 1267 showed good growth not only on glucose but also on whey permeate. If whey permeate, the waste from cheese manufacture, could be used as a source of lactose, the efficient utilization of unused resources could be promoted: thus, the closed ecosystem of forage and dairy products in the dairy industry could be maintained.

K. lactis IFO 1267, however, may be involved in the aerobic spoilage because of its ability to assimilate lactic acid and it can be involved in aerobic spoilage of silage. The respiratory deficient killer strain of *S. cerevisiae*, that cannot assimilate lactic acid, fails to multiply, and the killing activity did not appear. Further studies should be carried out to induce or to isolate the killer strain that cannot assimilate lactic acid, and prevent silage from undergoing aerobic spoilage.