Chapter VI

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

In this chapter, I propose a new process involving the addition of a genetically modified killer yeast to improve the aerobic stability of silage. By gene disruption of *KlPCK1*, the gene coding for phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme of gluconeogenesis, a killer strain defective in growth on lactic acid, *Kluyveromyces lactis* PCK27 was constructed from a wild killer strain *K. lactis* IFO 1267. Comparison was made between the action of wild killer yeast strain and that of the gene disruptant strain as well as with a killer defective strain, *K. lactis* m8, in silage. The results of the model fermentation system indicate that PCK27 inhibited the growth of target yeast, *Pichia anomala*, inoculated as aerobic spoilage yeast, and prevented the rise in pH. This suppressive effect of PCK27 was not only due to growth competition between killer strain and target strain but also to the killer protein produced. This latter possibility was suggested by the weaker inhibition of the target yeast growth by *K. lactis* m8. In the laboratory scale experiment of maize silage, the addition of killer yeast aerobically prevented both the growth of lactic acid-assimilating yeast, and pH increase during the first 5 days after opening the silo bag. This result might have been caused by the change in the yeast flora. From these results, I concluded the strain PCK27 can be used as an additive to prolong the aerobic stability of maize silage. Furthermore, I studied the biosafty of using the genetically modified killer yeast as a silage additive.

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

I studied the differences in growth between the wild killer strain [*K. lactis* IFO 1267(*KlPCK1*)] and the PEPCK gene-disruptant killer strain [*K. lactis* PCK27(*klpck1*)] in the model system (Fig. 6-1). After ensiling, the growth of both strains was observed in the first week but declined following further incubation. Under aerobic condition, the growth curve was similar in both strains until the 5th day after which that of strain PCK27 decreased while that of strain IFO1267 significantly increased between 5th and 8th day after opening the silo bag. Under this condition, lactic acid content did not change and pH increased slightly in the PCK27 inoculated sample, while a significant decline in lactic acid content and an increase in pH were observed in IFO1267 inoculated sample between 5th and 8th day. The total sugar content remaining in each sample was 4.2%, 0.2%, and less than 0.02%, at the beginning of ensiling, at opening the silo bag, and at the 5th day after opening the silo bag, respectively.

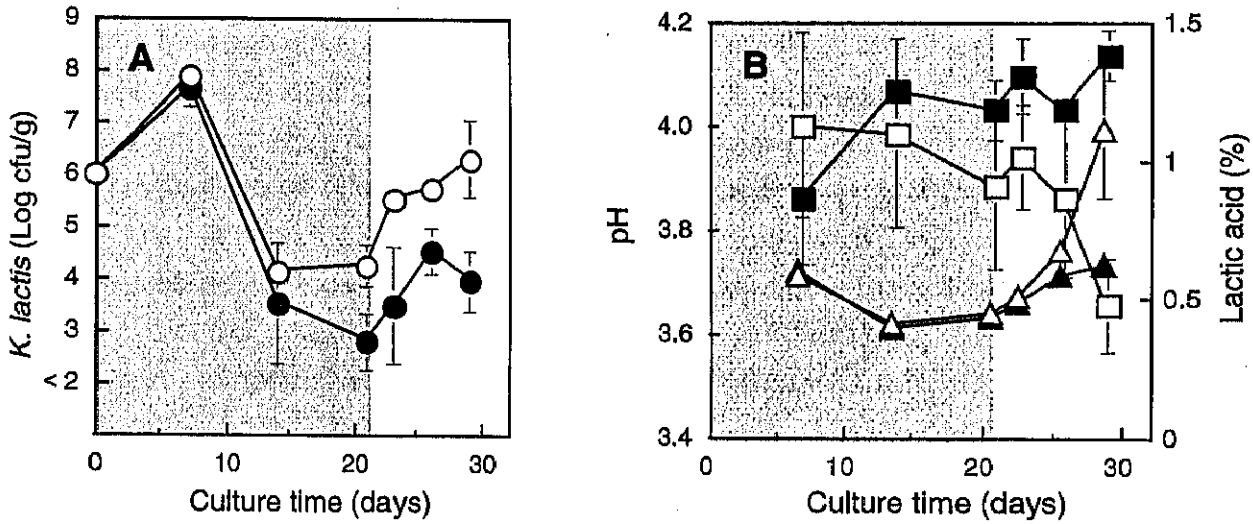

Fig. 6-1. Growth curve of *K. lactis* wild strain and transformant (A) and time course of pH and lactic acid content (B) in maize silage model fermentation system.

The shaded area indicates anaerobic conditions, and the blank area, aerobic conditions. The closed and open symbols refer to the growth (●,○), (▲, △), lactic acid content (■, ❑) of *K. lactis* PCK27 and *K. lactis* IFO1267 inoculated silage, respectively.

1.2) The killer effect on different target yeast strains in the model system

I investigated the killer effect of *K. lactis* PCK27 against each of the target yeast strains, *P. anomala* AHU 3936, 3937, and 3938, in the model system (Fig. 6-2). *P. anomala*, which has a wide fermentative ability for sugars and high respiration capacity for organic acids[90], plays an important role in aerobic spoilage of silage[8]. Under both anaerobic and aerobic condition, killer yeast addition significantly suppressed the growth of target strains (Fig. 6-2A). After opening the silo bag, significant increase in pH and decrease in residual sugar were observed in the samples without killer yeast addition, while in those with killer yeast addition both the pH increase and consumption of sugar were suppressed (Fig. 6-2B, D). Under this condition, lactic acid content had a tendency (P=0.06) to be different between the samples with and without killer yeast (Fig. 6-2C). After opening the silo bag, the lactic acid content in the killer yeast co-inoculated samples did not change until the 5th day which then decreased between the 5th and 8th day. On the other hand, in the sample without killer yeast addition, lactic acid decreased promptly after opening the silo bag. The growth curve of killer yeast was almost the same in all samples (data not shown).

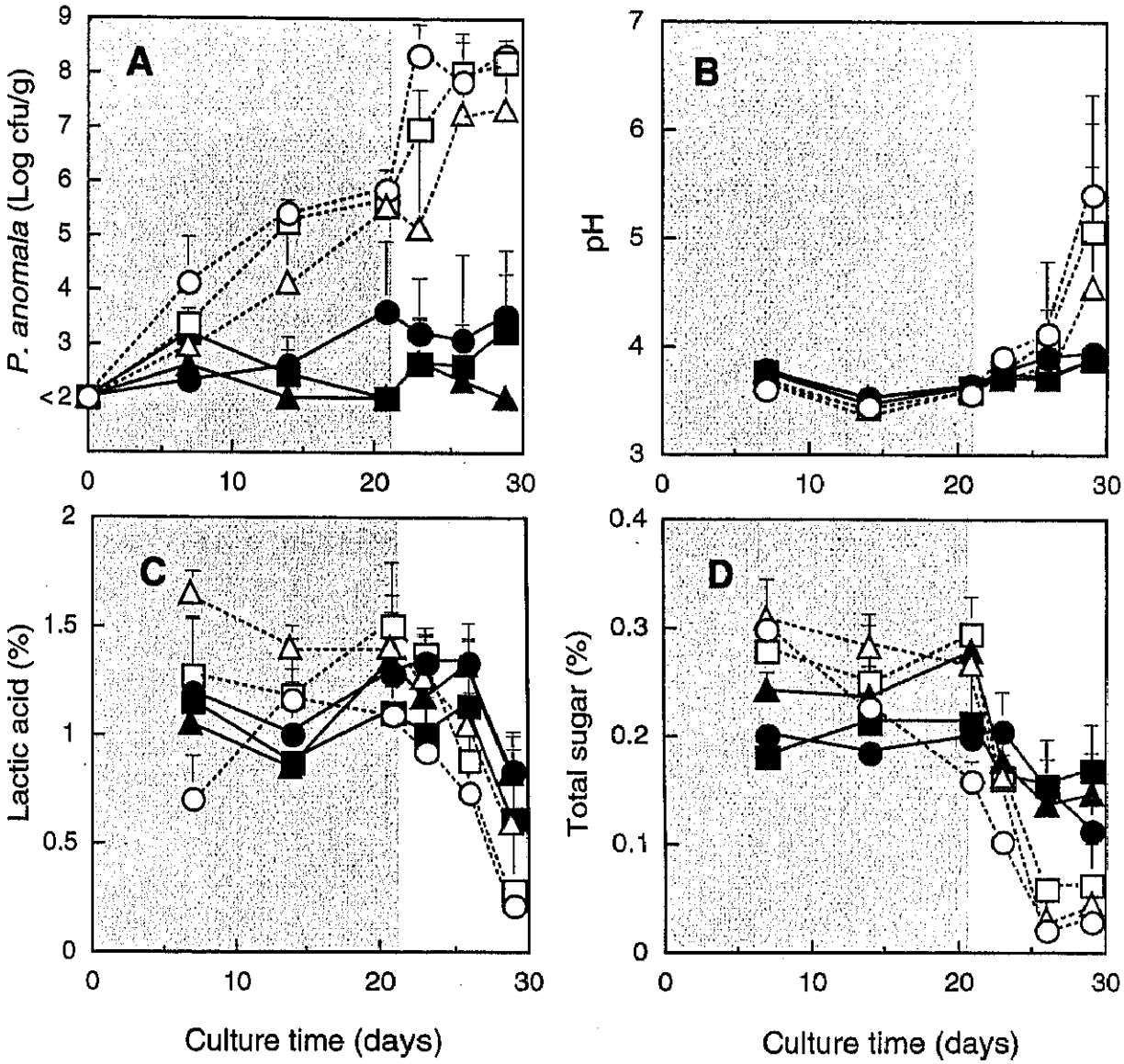

Fig. 6-2. Growth curve of *P. anomala* strains (A) time course of pH (B), lactic acid content (C) and residual sugar (D) in maize silage fermentation model system with and without killer yeast.

The shaded area indicates anaerobic conditions, and the blank area, aerobic conditions. Symbols: Target strain *P. anomala* AHU 3936 (○, ●); AHU 3937(△, ▲) and AHU3938 (❑, ■). Open symbols refer to the single yeast culture and the closed symbols, to mixed culture of killer yeast, PCK27 and target strain.

1.3) Growth of *P. anomala* in the model system with co-inoculation of *K. lactis* killer strain or killer defective strain.

To evaluate the production of the killer protein and its killer effect in silage, I compared the differences in growth of target strains between samples with addition of *K. lactis* killer strain and those with *K. lactis* m8, a strain defective in the killer gene. The presence of two linear plasmids, pGKL1 and pGKL2, confers the killer phenotype in *K. lactis*[30]. UV irradiation frequently cures these killer plasmids[88]. I constructed killer defective *K. lactis* strain m8 by UV irradiation and confirmed that the strain showed the same growth pattern on sugar and lactic acid as the wild strain, although this strain was defective in pGKL plasmids (Fig. 6-4) and defective in killer activity (Fig. 6-3). *P. anomala* AHU 3936 was inoculated in the model system together with either killer strain IFO1267or PCK27, or with killer-defective strain, m8 (Fig. 6-5). The growth of *P. anomala* AHU3936 was found to be markedly suppressed by addition of *K. lactis* strains under both anaerobic and aerobic conditions. After opening the silo bag, the growth of target strain was more significantly suppressed by the addition of *K. lactis* killer strains than by the addition of strain m8. No differences in inhibition of target strain were observed between the two killer strains. The growth curves of all *K. lactis* strains were similar.

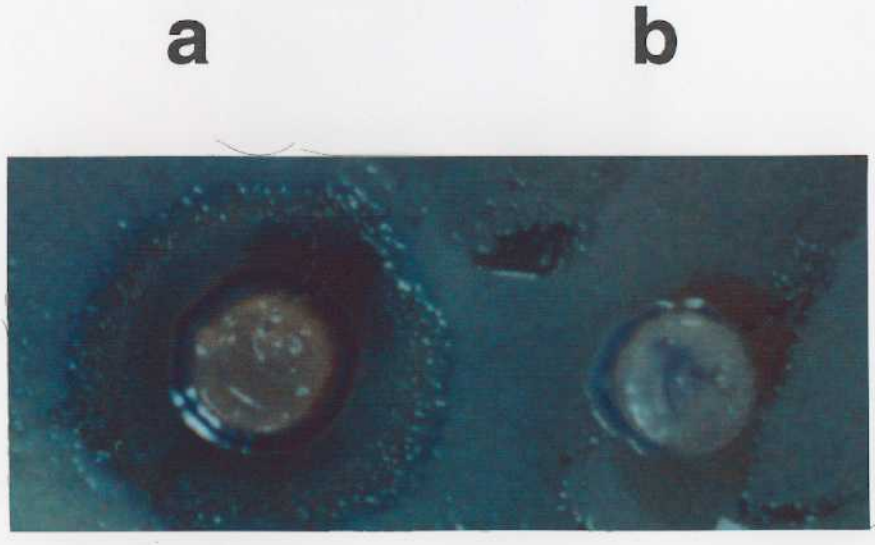

Fig. 6-3. Agar plate assay for killer activity.

K. lactis IFO1267 (a) and mutant strain m8 (b).
Target strain: *Saccharomyces cerevisiae* B511-4C

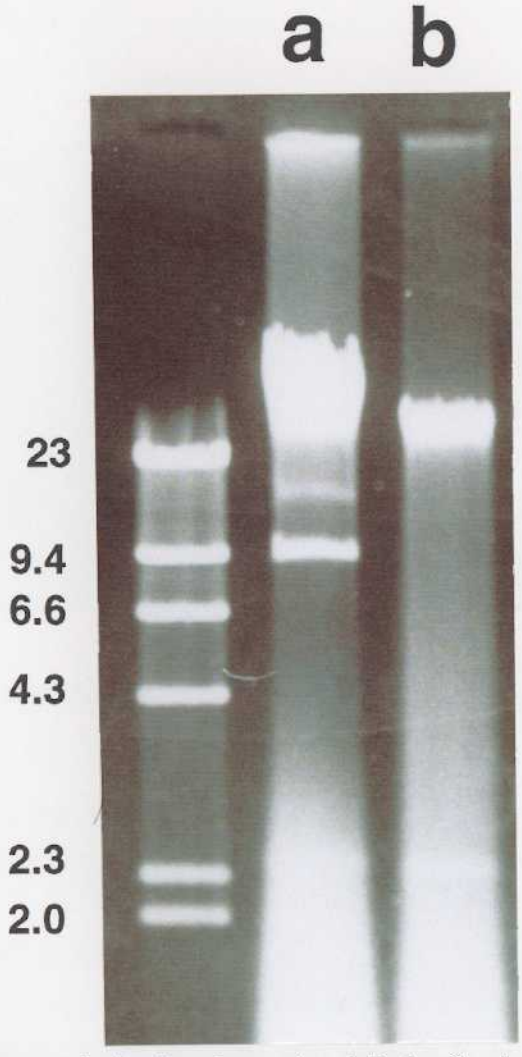

Fig. 6-4. Agarose gel electrophoresis of *K. lactis* plasmids.

DNA extracts from *K. lactis* IFO1267 (Lane a) and mutant strain m8 (Lane b).

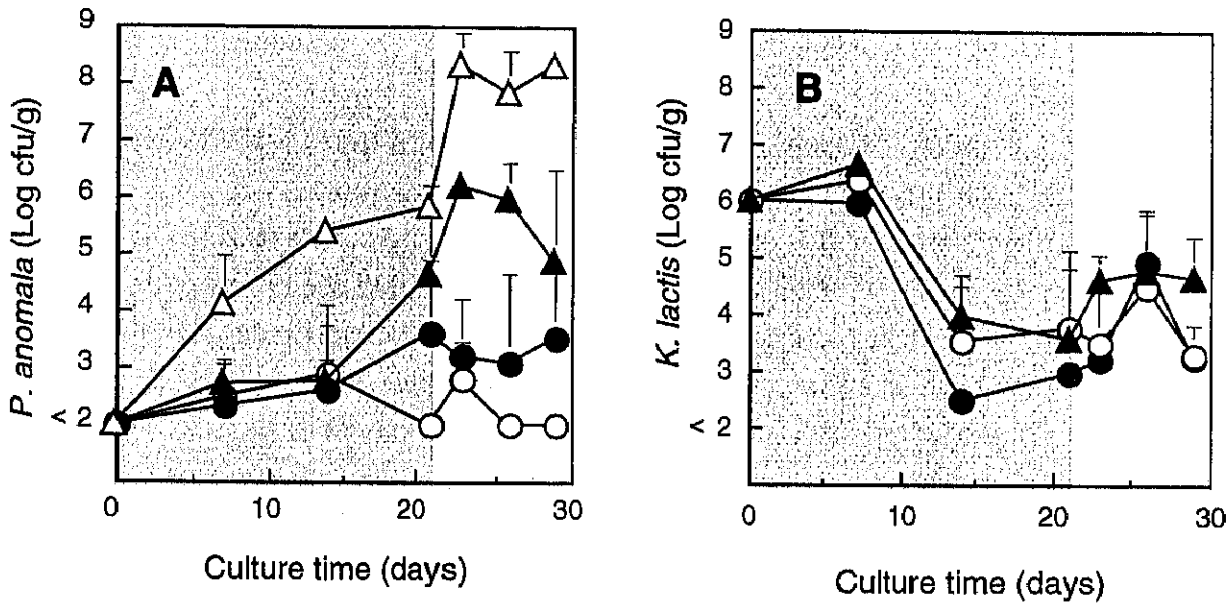

Fig. 6-5. Growth curve of *P. anomala* AHU 3936 (A), and *K. lactis* strains (B) in mixed culture with *P. anomala* in maize silage fermentation model system.

The shaded area indicates anaerobic conditions, and the blank area, aerobic conditions. Symbols: Mixed culture with *K. lactis* m8 (▲); with *K. lactis* IFO 1267 (○); with *K. lactis* PCK27 (●) and without *K. lactis* (△).

2) Study in the laboratory scale maize silage

Finally, I evaluated the effect of both killer strains in silage made from freshly harvested maize and added with 1% lactose. Silage with and without 1% lactose addition were used as control 1 and control 2, respectively.

2.1) The growth of *K. lactis* on maize silage

Both *K. lactis* strains showed similar significant increase in number after three days of anaerobic incubation (Fig. 6-6). However, their population decreased with further incubation and failed to show any colonies on selective medium even after 14 days of anaerobic incubation.

2.2) Effect of killer yeast addition on wild yeast present in maize silage

Figure 6-7. shows the growth curve of lactic acid-assimilating yeast (A) and total yeast (B) both excluding *K. lactis*, and time course of pH (C) and lactic acid content (D). At opening the silo bag, the population of total yeasts and lactic acid-assimilating yeasts were higher in lactose and killer yeast added samples than both control samples (Fig. 6-7B). However, after opening the silo bag, the increase of lactic acid-assimilating yeasts was significantly higher in the control samples. While the growth constant of lactic acid-assimilating yeasts were about 1/2 in killer yeast co-inoculated samples compared to that of control samples between the 2nd and 5th day (Fig. 6-7A). The density of lactic acid-assimilating yeasts was 75% and 10% of total yeasts in all silage samples using fresh maize used and after 1 week of anaerobic incubation, respectively. During the last 2 weeks of anaerobic condition, the density of lactic acid-assimilating yeasts remained at less than 50% in all control samples. On the other hand, in the lactose and killer yeast added samples, it increased to more than 50% at the end of anaerobic condition, but changed promptly upon opening the silo bag. Furthermore, in these samples, the density decreased to less than 50% between the 2nd and 5th day of incubation. More than 90% of total yeast assimilated lactic acid after 2 days of aerobic incubation in control samples. The pH of silage rose significantly from 3.7 to more than 6.0 in the control samples, while in those samples inoculated with the killer yeast the pH increased only slightly (3.7 to 3.9) until the 5th day and finally to 4.5 on the 8th day (Fig. 6-7C). Lactic acid was not detected at the 5th and 8th day in non-enriched sample and other samples, respectively. There were no differences seen among the decrement curves (Fig. 6-7D). At the start of silage fermentation, the total sugar including inoculated lactose was 10.7%. At the end of anaerobic incubation, the residual sugar concentration in lactose-enriched and non-enriched samples were 0.3% and 0.03%, respectively. This residual sugar promptly consumed within 2 days after opening the silo bag (data not shown). There were no differences in populations of lactic acid bacteria and aerobic bacteria among samples.

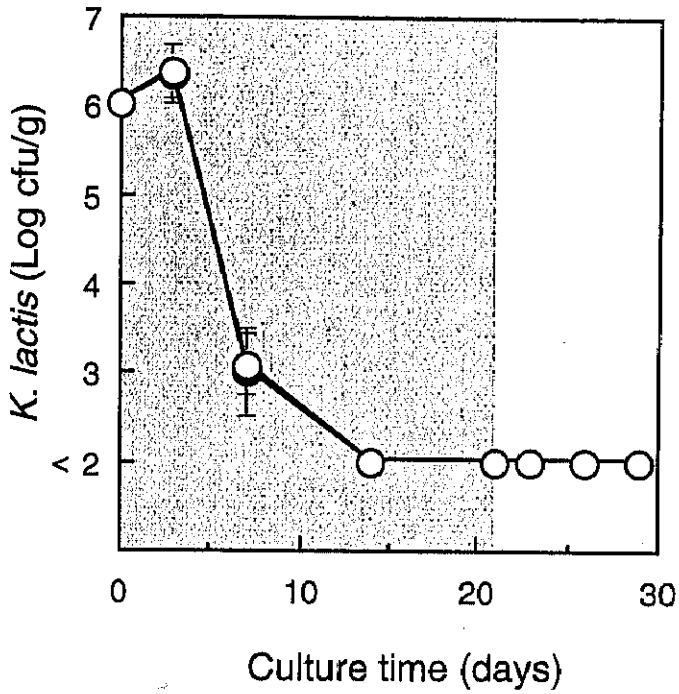

Fig. 6-6. Growth curve of *K. lactis* wild strain and transformant in laboratory scale silage.

The shaded area indicates anaerobic conditions, and the blank area, aerobic conditions. The symbols refer to the growth of *K. lactis* PCK27 (●) and *K. lactis* IFO1267 (○).

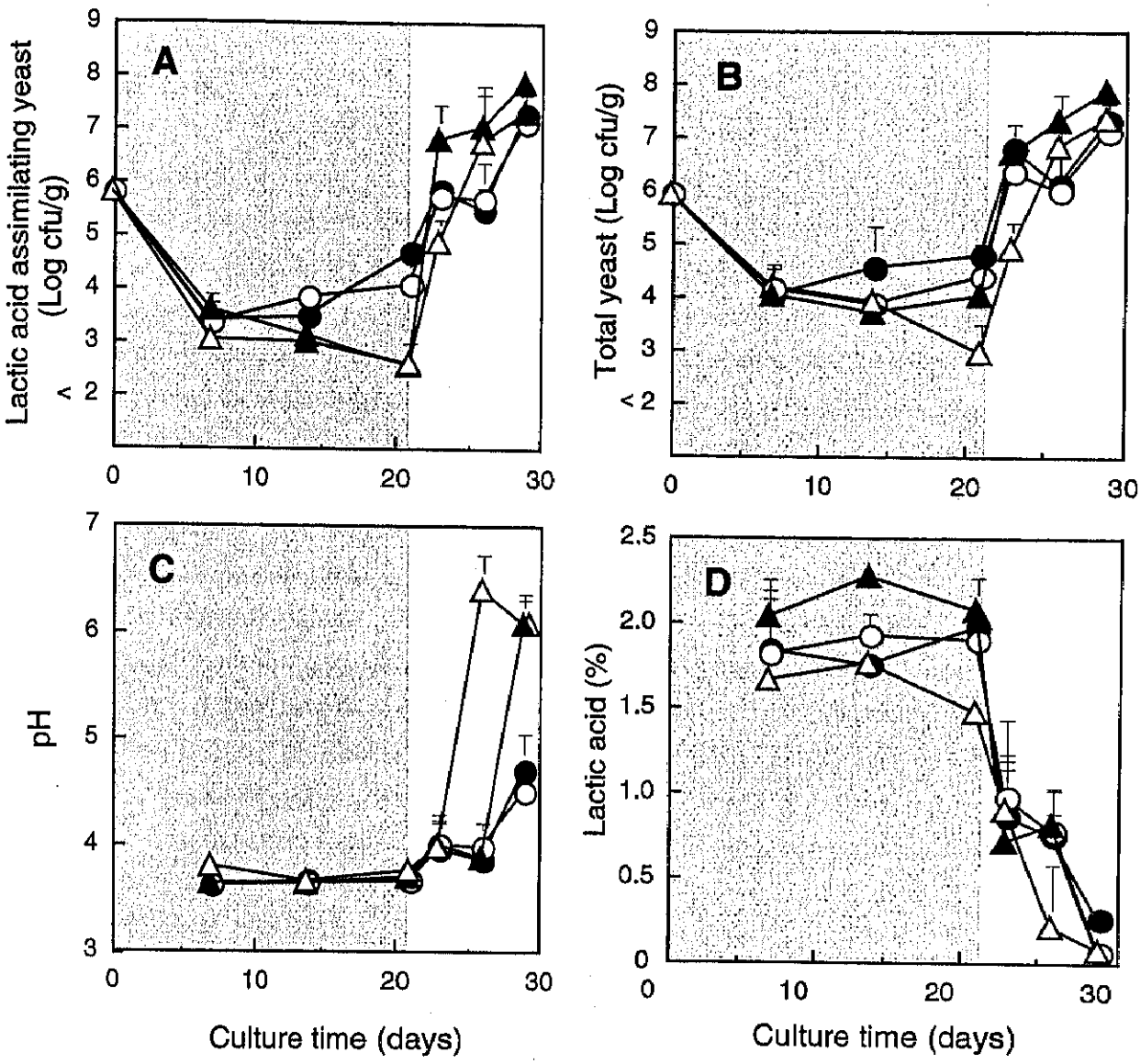

Fig. 6-7. Growth curve of lactic acid-assimilating yeasts (A) and total yeasts (B) both excluding *K. lactis*, and time course of pH (C) and lactic acid content (D) in laboratory scale silage added with killer yeast.

The shaded area indicates anaerobic conditions, and the blank area, aerobic conditions. Symbols: Addition of *K. lactis* PCK27 (●), *K. lactis* IFO1267 (○) in 1% lactose-enriched silage; addition of 1% lactose only (▲) and without any additive (△).

2.3) Effect of killer yeast addition on bacteria present in maize silage

The populations of lactic acid bacteria and aerobic bacteria were not affected by the addition of the killer yeast. The silage was unstable if it was prepared without the addition of either lactose or killer yeast. The pH increased rapidly in non-treated silage after five days of aerobic exposure, and the increment of aerobic bacteria accompanied a decrease of lactic acid.

4) Discussion

In this study, I evaluated the mode of action of genetically modified killer *K. lactis* strain on aerobic spoilage yeast in silage. For my first step, I used a modified model system[58] to analyze the growth of yeasts and bacteria and the change in chemical composition of silage. This system can evaluate the interaction of intended microorganisms without being influenced by variation of chemical composition and the microflora of crops used for silage. Later, I investigated the effect of killer yeast addition in a laboratory scale silage.

In previous chapter, I described that *K. lactis* IFO1267 inhibited the propagation of target yeast, *S. cerevisiae* in a model system. The *KlPCK1* gene coding for phosphoenolpyruvate carboxykinase, a key enzyme of gluconeogenesis, was disrupted in *K. lactis* PCK27. Accordingly, the strain PCK27 can not grow on silage limited in carbohydrates even if it contains non-carbohydrate substrates, i. e. lactic acid, acetic acids, ethanol, and 2, 3-butanediol. As expected, strain PCK27 stopped growing in silage after sugar was completely consumed (Fig. 6-1). Consequently, slight pH increase was observed. On the other hand, the wild strain IFO 1267 grew in sugar-limited silage by aerobically assimilating lactic acid.

K. lactis is unable to grow under low redox condition[51] and can be classified as weak fermentative. As shown in Fig. 6-1A, Fig. 6-5B and Fig.6-6, my results reveal that *K. lactis* exhibited the same culture response as type II yeasts (Fig. 1-1), according to the clasification of silage yeast by fermentation ability. The decrements in growth at later stage of fermentation were conspicuous in laboratory scale silage. It may be due to growth competition, inhibition of sugar fermentation and possibly by killer effect of killer yeast, which present in silage. The low redox condition caused by respiration of microorganisms and plants may have inhibited fermentation of sugars by *K. lactis*. Yeasts require energy from fermentation of sugars to withstand the toxic short-chain organic acids, such as lactic and acetic acids[12], and ethanol. These given conditions may have led to death of *K. lactis* at the later stage of fermentation in laboratory scale silage.

The genetically modified killer yeast clearly inhibited the growth of target *P. anomala* strains and caused a pH increase in the model system(Fig. 6-2). Towards end of aerobic condition, lactic acid concentration decreased but surprisingly, no rapid pH increase

was observed. These results suggest the possibility of oxidation of lactic acid to acetic acid by inoculated *L. plantarum* under glucose limited, aerobic conditions. This pathway is known in *L. plantarum*[12]. Also, the possibility of lactic acid decomposition by target strains can not be ruled out because this decrement was limited in *K. lactis* PCK 27 inoculated sample only (Fig. 6-1).

Comparison of growth inhibition of *P. anomala* shows that killer defective strain was less effective than the wild killer strain (Fig. 6-5). The growth of killer defective strain in silage was similar to that of wild killer strain. From these results it can be inferred that the inhibition of growth of target strains was due not only to growth competition but also to the killer protein produced by the killer yeasts.

On the basis of the results of the model system, I tried to evaluate the effect of killer yeast inoculation in laboratory scale silage (Fig.6-6, 6-7). I observed that the inoculation of killer yeast changed the yeast flora in silage; this change in yeast flora could have affected the results obtained. My results show that under anaerobic condition the ratio of lactic acid-assimilating yeasts to total yeasts, except for *K. lactis*, was higher in lactose and killer yeast added samples than in control samples. However, at opening the silo, I found that this ratio was completely reversed. According to the classification of yeast in silage, I assume that the main yeast population in the lactose and killer yeast added samples at the last stage of anaerobic incubation belongs to type III (Fig. 1-1). Because of the continuous decrease in number of lactic acid-assimilating yeasts present in control samples under anaerobic condition, I thought that those yeasts belong to type II. The main population of yeasts in lactose and killer yeast added samples could not assimilate lactic acid during 2, 5 days of aerobic incubation. They, however, can possibly utilize residual sugar and xylose produced as the result of acid hydrolysis or action of natural hemicellulases[12] and are therefore considered not involved aerobic spoilage[8]. There are several possible reasons for the low growth constants of lactic acid-assimilating yeast in lactose and killer yeast added samples. These include the low lactic acid-assimilating ability of the strain, growth competition due to the presence of large population of yeasts, which can not assimilate lactic acid, and the existing killer effect of killer yeasts. I can not conclude yet whether the change in yeast flora was due to effect of killer protein or to growth competition. However, the suppression of type II yeast is coincides with the killer spectrum of *K. lactis* IFO1267. Because I selected the killer yeast by wide killer spectrum against type II yeasts in Chapter IV. Hara et al. reported a change in yeast flora by addition of killer *S. cerevisiae* in wine making[35]. Further ecological studies will conduct the effect of killer protein. Lactic acid-assimilating yeasts in control samples easily grew under aerobic condition. Although I did not see any differences in lactic acid consumption among samples, significant differences in pH increase were observed. This could be explained by the presence of various organic acids including lactic and acetic acids in silage. Over the 5

days after opening the silo bag, the higher population of lactic acid-assimilating yeasts in the control samples can be expected to consume lactic acid completely in the early stage, and may consume other residual acids until the 8th day after opening the silo bag.

In the model system, I was able to confirm that the PEPCK defective *K. lactis* killer strain did not grow in the silage by utilizing lactic acid as a carbon source. The killer yeast prevented the growth of target strain, *P. anomala*, not only by growth competition but probably also by the killer protein it produced. Consequently, the degradation of sugar and lactic acid and pH increase were suppressed. Furthermore, in the laboratory scale silage, the addition of killer yeast might have led to a change in the yeast flora during silage fermentation, and as a result, delayed the aerobic increase of yeasts that have a high respiration capacity for lactic acid. While in the model system, I observed a significant effect on silage quality by addition of genetically modified killer strain 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 yeasts decreased to a number below the limit of detection before opening the silo bag. I therefore concluded that the addition of PEPCK defective killer *K. lactis* strain delayed the spoilage of silage under aerobic conditions.