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Interactions between *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae* in Mixed Culture for Kefiran Production

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Since a positive effect on the growth and kefiran production of *Lactobacillus kefiranofaciens* was observed in a mixed culture with *Saccharomyces cerevisiae*, the elucidation of the interactions between *L. kefiranofaciens* and *S. cerevisiae* may lead to higher productivity. Hence, the microbial interaction of each strain was investigated. Apart from the positive effect of a reduction in the amount of lactic acid by *S. cerevisiae*, a positive effect of *S. cerevisiae* on the growth and kefiran production of *L. kefiranofaciens* in a mixed culture was observed. Various experiments were carried out to study this effect. In this study, the observed increase in capsular kefiran in a mixed culture with inactivated *S. cerevisiae* correlated well to that in an anaerobic mixed culture. Differences in capsular kefiran production were observed for different initial *S. cerevisiae* concentrations under anaerobic conditions. From these fermentation results, it was concluded that the physical contact with *S. cerevisiae* mainly enhanced the capsular kefiran production of *L. kefiranofaciens* in a mixed culture. Therefore, in an anaerobic mixed culture, this direct contact resulted in higher capsular kefiran production than that in pure culture.

[**Key words:** mixed culture, lactic acid bacteria, yeast, interaction, polysaccharides]

Microbial ecosystems with aerobic and anaerobic zones are found in many environments. Examples of these are sediments, soils, biofilms and the rumen (1). Complex microflora in kefir grains, the starter of Caucasian cultured milk, are known to consist of more than 100 types of bacteria and yeasts (2). At the interface of aerobic and anaerobic zones, the concentration of dissolved oxygen is often very low but still sufficient for the growth of aerobes and not sufficiently high for the total inhibition of anaerobes.

Kefiran is an exopolysaccharide embedding the microorganisms inside kefir grains and produced by *Lactobacillus kefiranofaciens*. It contains approximately equal amounts of glucose and galactose (3). Polysaccharide-producing bacteria may either form an amorphous layer of polysaccharides called capsular polysaccharides (CPSs) in the form of a capsule surrounding the cell, or excrete extracellular polysaccharides (EPSs) to the medium. In the case of *L. kefiranofaciens*, the EPSs are called broth kefiran and the CPSs are called capsular kefiran (4). Not only is kefiran used as a thickener, stabilizer, emulsifier, fat substitute or gelling agent, but it also shows antitumor activity (5).

Inside kefir grains, interrelationships between yeasts and bacteria may have a significant influence on the activity of each strain (6) but no significant study has been carried out to verify this hypothesis. It was found that co-inoculation of the lactic acid bacterium *L. kefiranofaciens*, which was isolated from kefir grains, with the lactic acid-assimilating yeast *Saccharomyces cerevisiae*, which was also isolated from kefir grains, not only prevented the accumulation of lactic acid at high concentrations, but also enhanced cell growth and kefiran production. In a previous study (7), it was found that CO_2 and ethanol, known metabolites produced by yeast in kefir grains, did not stimulate kefiran production and the enhancement of kefiran production in mixed culture was not only due to the reduction of lactic acid by *S. cerevisiae* but also to other factors.

Since elucidating the interaction between lactic acid bacteria and yeast may lead to higher productivity, we attempted to analyze the interaction of lactic acid bacteria and yeast by cultivation experiments. In this study, other possible roles of *S. cerevisiae* in mixed culture apart from lactic acid reduction, which may occur, were considered. Since it is well known that yeast possesses catalase activity, one possibility is that yeast might reduce the amount of hydrogen peroxide, which is a growth inhibitor of lactic acid bacteria (8), in mixed culture. Another possibility is that *L. kefiranofaciens* might be sensitive to substances other than CO_2 and ethanol excreted by yeast (9–12). Challinor and Rose (13) also reported that under unfavorable fermentation

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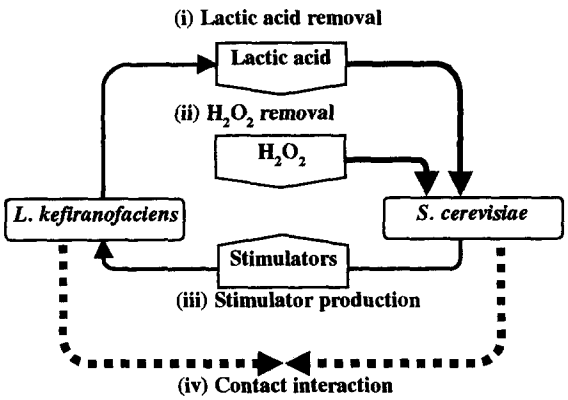

FIG. 1. Interaction between *L. kefiranofaciens* and *S. cerevisiae* for enhancement of kefiran production was investigated in mixed culture.

conditions for cell growth, yeast grown with lactic acid bacteria was able to synthesize and excrete factors required to support bacterial growth. Since high levels of capsular kefiran were detected in mixed culture (7), it was thought that there was some type of physical contact that occurred between the two microorganisms. Following a review of the literature, the possibilities of how *S. cerevisiae* stimulated the growth and kefiran production of *L. kefiranofaciens* are summarized as follows: (i) *S. cerevisiae* reduces concentration of lactic acid, (ii) *S. cerevisiae* removes hydrogen peroxide by catalase activity, (iii) *S. cerevisiae* produces stimulators for *L. kefiranofaciens*, and (iv) *S. cerevisiae* enhances the capsular kefiran production by physical contact.

Figure 1 shows a schematic of these possibilities. It was previously confirmed that the effect of (i) the reduction in the concentration of lactic acid by *S. cerevisiae* in mixed culture enhanced the growth and production of kefiran (7). In this study, the experiments outlined below were designed to investigate the possibilities (ii)–(iv) above: (ii) Hydrogen peroxide (H_2O_2) removal: the effect of H_2O_2 removal due to the catalase activity of *S. cerevisiae* was investigated by treating the medium for *L. kefiranofaciens* cultivation with catalase prior to inoculation with pure culture. (iii) Stimulators production: the spent medium following *S. cerevisiae* cultivation was added to the medium for *L. kefiranofaciens* cultivation to investigate the possibility that *S. cerevisiae* produces stimulators for *L. kefiranofaciens*. In addition, the spent medium following co-cultivation of *L. kefiranofaciens* and *S. cerevisiae* was added to investigate the possibility that *S. cerevisiae* converts products of *L. kefiranofaciens* to stimulators. (iv) Contact interaction: two mixed cultures, one without physical contact and a mixed culture with inactivated *S. cerevisiae*, were constructed to investigate the possibility that *S. cerevisiae* enhances the capsular kefiran production of *L. kefiranofaciens* by contact interaction. In the mixed culture without physical contact, alginate beads were used to entrap *S. cerevisiae* to avoid interaction by any physical contact between the two microorganisms. In the mixed culture with inactivated *S. cerevisiae*, the cells disrupted were with glass beads and by heat treatment. To gain a better understanding of the interaction, we also studied the effect of the initial yeast-to-bacteria ratio on capsular kefiran production under anaerobic conditions.

MATERIALS AND METHODS

Strains, medium, culture and assay *L. kefiranofaciens* JCM6985, which was used to produce kefiran in this study, was obtained from the Japan Collection of Microorganisms (JCM), Wako. The production medium used for kefiran production was a modified MRSL broth medium which consisted of 2% tryptone (Difco Laboratories, Detroit, MI, USA), 1% yeast extract (Difco), 2% meat extract (Extract Ehlrich; Wako Pure Chemical Industries, Osaka), 10% lactose monohydrate (Wako), 0.2% K_2HPO_4 (Wako), 0.4% triammonium citrate (Wako), 0.5% sodium acetate (Wako), 0.1% Tween 80 (Wako), 0.028% $MnSO_4 \cdot 4H_2O$ (Wako), 0.058% $MgSO_4 \cdot 7H_2O$ (Wako) and 0.074% $CaCl_2 \cdot 2H_2O$ (Wako). *S. cerevisiae*, isolated from kefir grains was obtained from the collection at the Institute for Fermentation Osaka (IFO), Osaka. The composition of the YP medium for the seed culture and preculture of the yeast consisted of 1% DL-lactic acid (Wako), 1% Polypepton (Wako), and 1% yeast extract (Difco), pH 5.

Seed cultures of *L. kefiranofaciens* and *S. cerevisiae* were individually prepared and inoculated into 3 *l* of experimental medium in a 7-*l* jar fermentor (MBF-800; Tokyo Rikakikai, Tokyo) using the method previously described (7). The initial concentrations of bacterial and yeast cells were 1×10^7 and 8×10^6 ml^{-1}, respectively.

The procedure for measurement of the viable-cell concentration and the dry-cell concentration of the bacterium was also discussed in the previous report (7). The procedure for measuring the amount of broth kefiran was as previously reported (4). Capsular kefiran was extracted from the cells by boiling in distilled water at 100°C for 30 min. The mixture was centrifuged, the clear supernatant was decanted, and the amount of capsular kefiran in the supernatant was measured using the same method as that for broth kefiran. The glucose concentration was measured with a glucose analyzer (model 2700; YSI Inc., Yellow Springs, OH, USA). Lactose and galactose concentrations were measured using a lactose-galactose F-kit (Roche Diagnostics, Tokyo). DL-Lactic acid concentration was measured by a gas chromatograph (model G-3000; Hitachi, Tokyo), using a capillary column (df = 1.5 μm, i.d. = 0.53 mm, length = 15 m; TC-FFAP type column; GL Science, Tokyo). Detection was performed using a flame ionization detector (FID) and the temperatures of both the injection and detection ports were set at 230°C. The temperature in the column oven was controlled as follows: (i) initially the temperature was kept at 120°C for 1.5 min, (ii) it was then increased at a rate of 25°C/min for 4 min, and (iii) it was then kept at 220°C for 1.5 min. Samples were prepared by mixing with the same volume of butyric acid as an internal standard sample mixture of 1 μl was injected.

Catalase treatment Medium containing catalase (Sigma C-10; 60 mg l^{-1}; Sigma, St. Louis, MO, USA) was used for the pure culture of *L. kefiranofaciens*. The amounts of hydrogen peroxide in pure cultures of *L. kefiranofaciens* with and without catalase treatment under anaerobic conditions were measured. A spectrophotometric method for the determination of hydrogen peroxide was used (14).

Addition of spent medium of *S. cerevisiae* For the preculture of *S. cerevisiae*, lactic acid was used as a carbon source in 100 ml of MRS medium in two 500-ml Erlenmeyer flasks and *S. cerevisiae* was incubated on a reciprocal shaker (120 strokes/min) at 30°C for 2 d after its optical density increased to the same level as it would be in an anaerobic mixed culture. The 200 ml of spent medium fermented by *S. cerevisiae* from which cells were removed by centrifugation and membrane filtration, were added to the fermentor before inoculation of *L. kefiranofaciens*.

Addition of spent medium of *L. kefiranofaciens* and *S. cere-*

visiae One ml of a preculture of *L. kefiranofaciens* and also 1 ml of *S. cerevisiae* was simultaneously inoculated into 100 ml of MRS medium in 500-ml Erlenmeyer flasks. Two flasks were incubated (co-cultured) on a reciprocal shaker (120 strokes/min) at 30°C for 2 d. The 200 ml of mixed culture medium, from which cells were removed by membrane filtration, were added to the fermentor before inoculation of *L. kefiranofaciens*.

Entrapment of *S. cerevisiae* by encapsulation in alginate beads An *S. cerevisiae* suspension was prepared in 100 ml of YP medium (pH 5.0) in two 500-ml Erlenmeyer flasks and incubated on a reciprocal shaker (120 strokes/min) at 30°C for 2 d. Cells were then collected by centrifugation and suspended in 10 ml of saline water (0.85% NaCl). Sodium alginate (2.7 g) (Wako) was dissolved in 90 ml of distilled water and sterilized for 15 min at 121°C. The alginate solution (90 ml) was then left overnight at 4°C to degas, and thoroughly mixed with 10 ml of the *S. cerevisiae* suspension. Calcium alginate beads were aseptically made dropwise by pumping the alginate solution through a Gilson pipette tip into a 3% $CaCl_2$ pH 7 solution. After 30 min the beads were washed three times in sterile distilled water and then stored at 4°C in saline water. The final cell concentration of yeast in the beads was 2.4×10^8 ml^{-1}.

***S. cerevisiae* inactivated by glass bead disruption** An *S. cerevisiae* suspension was prepared in 100 ml of YP medium (pH 5.0) in two 500-ml Erlenmeyer flasks and incubated on a reciprocal shaker (120 strokes/min) at 30°C for 2 d. Yeast cells were collected by centrifugation and suspended in 6 ml of saline water (0.85% NaCl) containing 16.8 g of glass beads (0.5 mm in diameter) and the cell suspension was subsequently vortexed for 10 cycles of 30 s (vortexing) and 30 s cooling on ice. The liquid of the *S. cerevisiae* cells debris was withdrawn from the beads and transferred to the fermentor before inoculation of *L. kefiranofaciens*. Cell viability was checked using the colony-forming cell counting technique to confirm that *S. cerevisiae* were inactivated.

***S. cerevisiae* inactivated by heat treatment** An *S. cerevisiae* suspension was prepared in 100 ml of YP medium (pH 5.0) in two 500-ml Erlenmeyer flasks and incubated on a reciprocal shaker (120 strokes/min) at 30°C for 2 d. Cells were collected by centrifugation, suspended in 10 ml of saline water (0.85% NaCl), and heated at 100°C for 5 min and then added to the fermentor before inoculation of *L. kefiranofaciens*. Cell viability was also checked using the colony-forming cell counting technique to confirm that *S. cerevisiae* were inactivated.

Effect of initial *S. cerevisiae* concentration The initial concentrations of *L. kefiranofaciens* and *S. cerevisiae* were 0.1 g l^{-1} and 0.67 g l^{-1} respectively. To better understand the interaction between *L. kefiranofaciens* and *S. cerevisiae*, two different initial *S. cerevisiae* concentrations 0.32 and 1.11 g l^{-1} were used.

RESULTS

Effect of catalase treatment Figure 2 shows the results of the investigation of the possibility that *S. cerevisiae* might remove hydrogen peroxide by catalase activity. It was found that there was no significant amount of hydrogen peroxide in the medium (Fig. 2A). To verify that a very low concentration of hydrogen peroxide would not affect the productivity of *L. kefiranofaciens*, medium containing catalase was used in the pure culture. No increase in the productivity of the lactic acid bacterium was observed and there was no difference in the level of hydrogen peroxide in the presence or absence of catalase in the medium.

Effect of addition of spent medium of *S. cerevisiae* In mixed culture, the degree of stimulation of kefiran production under aerobic conditions was larger than that under anaerobic conditions. This is due to the large amount of cells and higher lactic acid consumption activity of *S. cerevisiae* under aerobic conditions than under anaerobic conditions (7). To subtract the stimulation effect due to the lactic acid reduction by *S. cerevisiae* from the stimulation effect observed in the mixed culture and identify other stimulatory factors produced by *S. cerevisiae*, an anaerobic mixed culture was used as a control because the lactic acid consumption rate of *S. cerevisiae* was very low under anaerobic conditions.

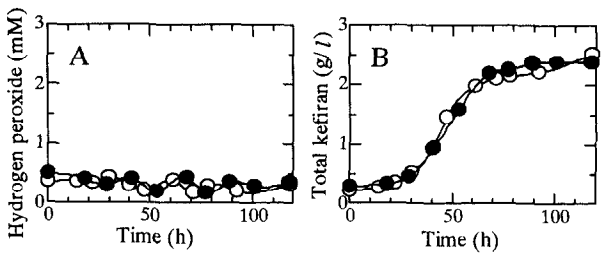

FIG. 2. Hydrogen peroxide concentration (A) and total kefiran production (B) by *L. kefiranofaciens* in pure culture with (closed circles) and without (open circles) catalase treatment.

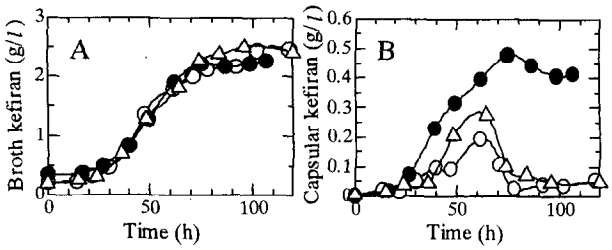

FIG. 3. Broth (A) and capsular (B) kefiran production by *L. kefiranofaciens* in an anaerobic pure culture (open circles) and in an anaerobic mixed culture (closed circles) compared with that following the addition of spent medium of *S. cerevisiae* (triangles).

Figure 3 shows the results of kefiran production by *L. kefiranofaciens* following the addition of spent medium of *S. cerevisiae* compared to that in an anaerobic pure culture and in an anaerobic mixed culture. There was no significant difference in broth kefiran production between the pure and the mixed cultures. The capsular kefiran production in the mixed culture was significantly enhanced compared with that in the pure culture. Figure 3B shows that no significant increase in capsular kefiran was found in the pure culture following the addition of spent medium of *S. cerevisiae*. Therefore, it was concluded that metabolites secreted by *S. cerevisiae* were not the main factor enhancing capsular kefiran production of *L. kefiranofaciens* in mixed culture.

Effect of addition of spent medium of *L. kefiranofaciens* and *S. cerevisiae* Since there was no significant stimulation following the addition of the spent medium of *S. cerevisiae*, the possibility of the conversion to stimulators of some products from lactic acid bacteria by *S. cerevisiae* was investigated. To investigate this possibility, 2 d spent medium of co-culture of *L. kefiranofaciens* and *S. cerevisiae*, in which the stimulation of *L. kefiranofaciens* could be observed (Fig. 3B), was added into a pure culture of *L. kefiranofaciens*. The result is shown in Fig. 4. Although the

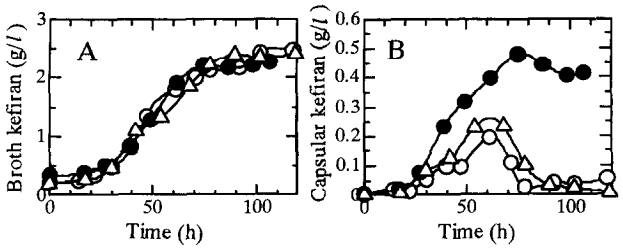

FIG. 4. Broth (A) and capsular (B) kefiran production by *L. kefiranofaciens* in an anaerobic pure culture (open circls) and in an anaerobic mixed culture (closed circles) compared with that in the addition of spent medium of *L. kefiranofaciens* and *S. cerevisiae* (triangles).

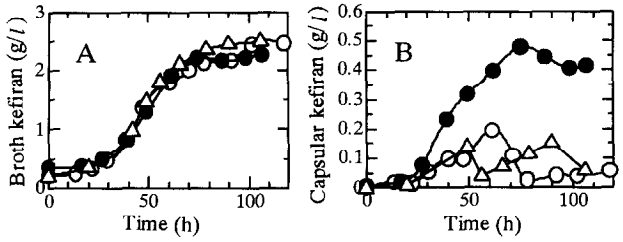

FIG. 5. Broth (A) and capsular (B) kefiran production by *L. kefiranofaciens* in an anaerobic pure culture (open circles) and in an anaerobic mixed culture (closed circles) compared with that in a mixed culture without physical contact (triangles).

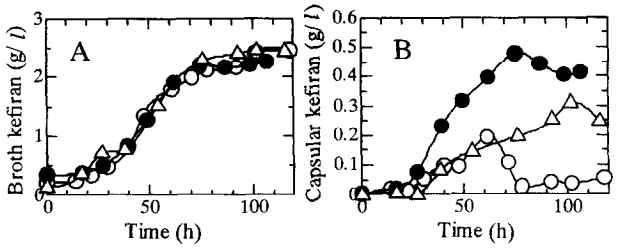

FIG. 6. Broth (A) and capsular (B) kefiran production by *L. kefiranofaciens* in an anaerobic pure culture (open circles) and in an anaerobic mixed culture (closed circles) compared with that in a mixed culture with *S. cerevisiae* inactivated by glass bead disruption (triangles).

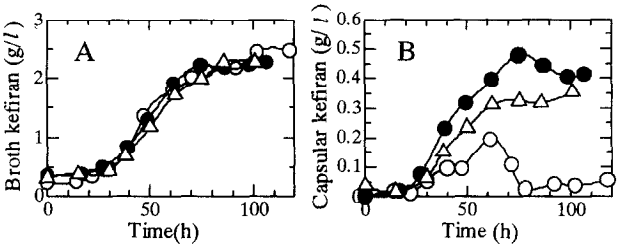

FIG. 7. Broth (A) and capsular (B) kefiran production by *L. kefiranofaciens* in an anaerobic pure culture (open circles) and in an anaerobic mixed culture (closed circles) compared with that in a mixed culture with *S. cerevisiae* inactivated by heat treatment (triangles).

spent medium of co-culture of *L. kefiranofaciens* and *S. cerevisiae* was used, no significant increase in broth or capsular kefiran production by *L. kefiranofaciens* was observed (Fig. 4B).

Finally, the effect of physical contact between *L. kefiranofaciens* and *S. cerevisiae* was investigated. The importance of cell-to-cell interaction in the formation of *Pseudomonas aeruginosa* biofilms has led to an interest in the general role of intercellular signaling in bacterial biofilm formation (15). These data raise the possibility that cell-to-cell interaction is an important requirement for biofilm development in many types of bacteria. Here, the physical contact of both *L. kefiranofaciens* and *S. cerevisiae* in mixed culture might create an ideal environment for effective capsular kefiran production. To study the physical contact in mixed culture, three sets of fermentation experiments, mixed culture without physical contact, mixed culture with *S. cerevisiae* inactivated by disrupting cells with glass beads, and mixed culture with *S. cerevisiae* inactivated by heat treatment, were designed.

Mixed culture without physical contact In a mixed culture without physical contact, alginate beads were used to entrap *S. cerevisiae* in an anaerobic mixed culture to avoid any physical contact between the two microorganisms. In this experiment, there was some exchange of the metabolites of both microorganisms but no contact. Figure 5 shows the time course of broth and capsular kefiran production in a mixed culture without physical contact and no significant increase in broth or capsular kefiran production was observed (Fig. 5B). This result suggested that direct contact between *L. kefiranofaciens* and *S. cerevisiae* is important in enhancing kefiran production.

Addition of *S. cerevisiae* inactivated by glass bead disruption Inactivated *S. cerevisiae* was also used to investigate the physical contact effect. Inactivated *S. cerevisiae* was prepared by disrupting cells with glass beads. In this experiment, no cell metabolites but only cell debris and cell contents were used. Figure 6 shows the results of an anaerobic mixed culture using *S. cerevisiae* inactivated by glass bead disruption. A slight increase in capsular kefiran production in the stationary phase was observed.

Addition of inactivated *S. cerevisiae* by heat treatment *S. cerevisiae* cells inactivated by heat treatment, was added to a pure culture of *L. kefiranofaciens*. The result is shown in Fig. 7. When *S. cerevisiae* inactivated by heat treatment was added into a pure culture of *L. kefiranofaciens*, capsular kefiran production increased (Fig. 7B). This result suggested that the main factor involved in the increased capsular kefiran production in a mixed culture between *L. kefiranofaciens* and *S. cerevisiae* was physical contact between *L. kefiranofaciens* and the cell wall of *S. cerevisiae*.

Effect of initial *S. cerevisiae* concentration The effect of the initial *S. cerevisiae* concentration was investigated. Figure 8 shows the results for anaerobic mixed cultures using three different initial yeast concentrations of 0.32, 0.67, and 1.11 g l^{-1}. The cell concentrations of *S. cerevisiae* (Fig. 8B) were constant throughout the fermentation because *S. cerevisiae* could not grow under anaerobic conditions (7). As shown in Fig. 8C, the *S. cerevisiae* concentration, namely, the amount of likely physical contact, influenced the level of increase in capsular kefiran. However, the effect of physical contact was almost saturated. The reproducibility of experiments was confirmed.

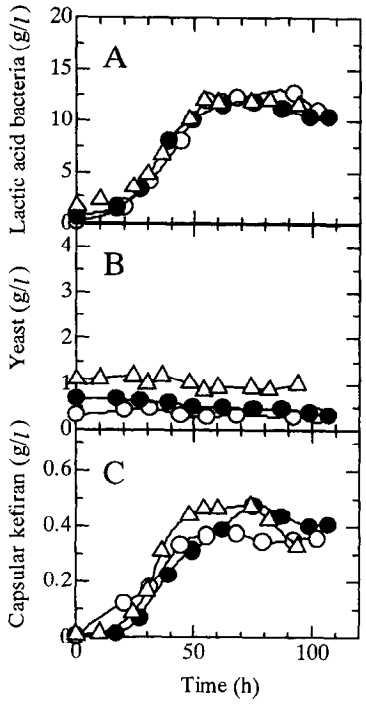

FIG. 8. The results for *L. kefiranofaciens* (A), yeast (B), and capsular kefiran (C) using different initial *S. cerevisiae* concentrations in an anaerobic mixed culture. Open circles, 0.32; closed circles, 0.67; and triangles, 1.11 g l^{-1} dry cell weight.

DISCUSSION

To potentially further enhance kefiran production in a mixed culture of *L. kefiranofaciens* and *S. cerevisiae*, analysis of the interaction between the lactic acid bacterium and yeast using fermentation methods was carried out. Since it is known that yeast can remove hydrogen peroxide, which is a growth inhibitor for lactic acid bacteria (8), the possibility that this underlies the increased kefiran production was investigated. The result shows that no difference in the amount of kefiran produced by *L. kefiranofaciens* was observed in the presence or absence of catalase in the medium. Therefore, the stimulation of the productivity of *L. kefiranofaciens* in mixed culture was not because of the removal of hydrogen peroxide by *S. cerevisiae*.

In a previous report (7), the possibility of lactic acid removal by *S. cerevisiae* was discussed and the enhancement of kefiran production in mixed culture was suggested not only to be due to the reduction of lactic acid by *S. cerevisiae* but also due to other factors. One possible explanation is that lactic acid bacteria in the exponential growth phase are sensitive to substances excreted by the yeast, or yeast grown with lactic acid bacteria was able to synthesize and excrete factors required to support bacterial growth under unfavorable fermentation conditions (9, 10, 12, 13). However, in the present study, stimulation of kefiran production by *L. kefiranofaciens* was not observed either following the addition of the spent medium of *S. cerevisiae* or the spent medium of a co-culture of *L. kefiranofaciens* and *S. cerevisiae*. The amount of ethanol produced by *S. cerevisiae* in our anaerobic and aerobic mixed cultures was less than 1%, and we determined that ethanol at a concentration lower than 1% did not affect *L. kefiranofaciens*. This may be due to the fact that the carbon source for *S. cerevisiae* in the mixed culture was lactic acid, from which *S. cerevisiae* did not produce ethanol. It is well known that yeast metabolism differs for different carbon sources.

Exopolysaccharides are thought to play an important role in the protection against desiccation and adhesion to surfaces, and also in cellular recognition (16). Since an increase in capsular kefiran production by *L. kefiranofaciens* was not found in a noncontact mixed culture (Fig. 5B) but was found in mixed cultures containing inactivated *S. cerevisiae* (Figs. 6B and 7B), it was suggested that *L. kefiranofaciens* may have a mechanism which regulates capsular kefiran production in response to physical contact with yeast. In the mixed culture with *S. cerevisiae* inactivated by glass bead disruption, the increase in capsular kefiran was less than that in a mixed culture with *S. cerevisiae* inactivated by heat treatment. This is assumed to be due to a difference in the number of yeast cells in nonbroken form. This assumption was also supported by the difference for capsular kefiran levels found in the different initial *S. cerevisiae* concentrations (Fig. 8).

From our fermentation results, physical contact between both *L. kefiranofaciens* and *S. cerevisiae* seems to be a highly possible stimulation factor by capsular kefiran production of *L. kefiranofaciens* in mixed culture. It has been reported that capsular polysaccharides may themselves be released into the growth medium as a consequence of the stability of the phosphodiester linkage between the polysaccharide and the phospholipid membrane anchor (17). Since it is considered that the lipids in cell walls are involved in the attachment of capsular polysaccharides to cells, an increase in capsular kefiran production in mixed culture might be because that *S. cerevisiae* affects *L. kefiranofaciens* directly to increase the amount of lipid carrier in the cell wall in the early phase of fermentation. Compared to the capsular kefiran release during the stationary phase found in pure culture, capsular kefiran in mixed culture remained firmly associated with cells until the end of fermentation.

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