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Kefiran Production by *Lactobacillus kefiranofaciens* under the Culture Conditions Established by Mimicking the Existence and Activities of Yeast in Kefir Grains

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Kefiran production by *Lactobacillus kefiranofaciens* alone under the culture conditions established by mimicking the presence and activities of yeast cells in kefir grains was investigated. When the pH of the culture broth was controlled at pH 5.5 by adding a 4 N-NaOH solution during cultivation, cell growth and kefiran production were stimulated as compared with those in the cultivation without pH control. The addition of 5 g/l of yeast extract to the medium was essential for kefiran production. By sparging a mixed gas of N_2 and CO_2 at a volume ratio of 9:1 into the culture broth at 0.3 vvm throughout the cultivation, a slight increase in the amount (670 mg/l) of kefiran produced was observed as compared with that (650 mg/l) in the cultivation without aeration. When 10 g/l or 20 g/l of ethanol was added to the medium containing 50 g/l of lactose, the kefiran concentrations were about 930 mg/l or 840 mg/l at 8 days, respectively. The maximum concentration of kefiran obtained was about 1040 mg/l at 10 days, when 10 g/l of ethanol was added to the medium containing 75 g/l of lactose. These results showed that under the culture conditions established by mimicking the actions of yeast cells on *L. kefiranofaciens* in kefir grains the amount of kefiran produced was enhanced even when only the lactic acid bacterium was used.

Keywords: kefiran, polysaccharide, *Lactobacillus kefiranofaciens*, lactic acid bacteria, co-culture with yeast

Lactic acid bacteria which are food-grade organisms possessing the GRAS (generally recognized as safe) status, are widely used in the food industry not only for lactic acid production but also for the formation of minor food components suitable for structure, flavor, and preservation. Several lactic acid bacteria are known to produce extracellular polysaccharides which contribute to the rheology and texture of fermented milk (Crescenzi, 1995). Polysaccharides produced by these bacteria also provide a source of stabilizing, viscosifying, emulsifying, gelling, and water binding reagents for use as natural food additives (Mukai *et al.*, 1991), which may be an alternative to texturizing agents of plant and animal origins.

Kefiran, a water-soluble polysaccharide, is produced in kefir grains which consist of a complex population of lactic acid bacteria and yeasts firmly embedded (Tamime & Robinson, 1988). The presence of yeasts results in the production of ethanol and also of carbon dioxide (CO_2) which gives an effervescent character to these products. Kefiran, which contains approximately equal amounts of glucose and galactose, is produced by *Lactobacillus kefiranofaciens* (Toba *et al.*, 1986). The chemical structure and properties of kefiran extracted from kefir grains were investigated by Kooiman (1968), Mukai *et al.* (1990), and Micheli *et al.* (1999). The antitumor activity of kefiran was reported by Shiomi *et al.* (1982). To utilize kefiran in many applied fields such as food, cosmetic and pharmaceutical industries, it may be necessary to produce a large quantity of the polysaccharide by *L. kefiranofaciens* because extraction of kefiran from kefir grains cultured in milk is complicated and the yield is fairly low.

A homofermentative bacterium, *L. kefiranofaciens* which was isolated from kefir grains by Toba *et al.* (1986) produced kefiran only in medium containing expensive wine, and kefiran was obtained at a low concentration (80 mg/*l*) in the culture. Yokoi *et al.* (1990) isolated a new kefiran-producing homofermentative bacterium, *Lactobacillus* sp. KPB-167B from kefir grains with a newly developed milk whey medium without wine, and examined the optimum culture conditions for production of kefiran by the lactic acid bacterium using MRS medium containing lactose (Yokoi & Watanabe, 1992). Recently, Mitsue *et al.* (1998) obtained a high kefiran-producing strain (*L. kefiranofaciens* KF-75) suitable for industrial production of kefiran by repeated UV irradiation. They found that using the strain KF-75 higher kefiran productivity was successfully obtained by optimizing the medium and culture conditions in a 50-*l* jar fermentor (Mitsue *et al.*, 1999). In particular, they reported that the highest kefiran productivity could be achieved in a co-culture of the strain KF-75 and *Torulaspora delbrueckii*, one of the yeast strains existing in kefir grains (Mitsue *et al.*, 1999).

We reported on not only the high concentration cultivation of several lactic acid bacterial cells using the fed-batch fermentation system involving a bioreactor with a microfiltration module (Taniguchi *et al.*, 1987, 1988), but also the production of the useful substances, superoxide dismutase and an antibiotic polypeptide (nisin), by lactic acid bacteria (Taniguchi *et al.*, 1989, 1994). We have given much attention to efficient production of kefiran by a co-culture of *L. kefiranofaciens* with yeast strains in kefir grains. Based on the results reported by Mitsue *et al.* (1999), we attempted kefiran production using a combination of *L. kefiranofaciens* and some yeast strains. Unfortunately, we were unable to obtain satisfactory results under the co-culture conditions. Accordingly, we focused on the influence of the co-existence of

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yeast on kefiran production by *L. kefiranofaciens* and identified this influence into factors resulting from the presence and activities of yeast strains. Thus, in the present paper, we investigated the effects of addition of yeast extract and ethanol, and the aeration of CO_2 on kefiran production by *L. kefiranofaciens*.

Materials and Methods

Microorganisms The kefiran-producing lactic acid bacterial strains used in this study were *Lactobacillus kefiranofaciens* JCM 6985 and JCM 7446, and *Lactobacillus kefir* JCM 5818 and KL-3. For co-cultures with lactic acid bacteria, *Saccharomyces cerevisiae* IFO 0216, *Candida kefyr* IFO 10287 and *Torulaspora delbrueckii* IFO 1626 were used as yeast strains isolated from kefir grain.

Media and culture conditions Lactic acid bacteria were cultivated usually at 30°C in a modified MRS medium containing 5–10% lactose, 1% Polypepton (Nippon Seiyaku Co., Tokyo), 1% meat extract (Kyokuto Seiyaku Co., Tokyo), 0.5% yeast extract (Oriental Yeast Co., Osaka), 0.5% sodium acetate, 0.2% ammonium citrate, 0.2% K_2HPO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.05% $MnSO_4 \cdot 5H_2O$. The initial pH of the medium was adjusted to 5.5 unless otherwise noted. The medium was sterilized by autoclaving at 121°C for 15 min. Lactic acid bacterial and yeast cells were precultured statically at 30°C in test tubes containing 10 ml of the medium. The precultured cells were separated by centrifugation at 17,000×*g* for 10 min and then inoculated into a fresh medium at an initial turbidity of about 0.5 at 660 nm.

Lactic acid bacteria were cultivated batchwise in 500 ml screw-capped medium bottles or a bioreactor (TBR-2, Sakura Seiki Co., Tokyo) with a working volume of 700 ml. Medium bottles were used for selecting a high kefiran-producing strain, and for examining the effects of basal culture conditions (carbon source, initial pH, temperature, etc.) on cell growth and kefiran production. When the bioreactor was used, the pH of the medium was maintained at 5.5 by adding a 4 N-NaOH solution with a peristaltic pump connected to a pH controller (FC-10, Tokyo Rikakikai Co., Tokyo). Since the presence of yeast in kefir grains results in the production of CO_2 and ethanol, a mixed gas of N_2 and CO_2 at a volume ratio of 9:1 or 5:5 was sparged at 0.3 vvm throughout the fermentation or/and 1 or 2% ethanol was added to the medium described above to evaluate the effects on kefiran production. The agitation rate was usually adjusted to 100 rpm.

Preparation and determination of kefiran Extracellular kefiran was recovered from the culture supernatant and determined. After centrifuging the culture broth at 17,000×*g* for 10 min, kefiran in the supernatant obtained was precipitated by addition of an equal volume of cold ethanol. The resulting precipitate was collected by centrifugation at 17,000×*g* for 10 min and dissolved with distilled water in 10% volume of a corresponding supernatant. The same procedure was repeated three times to purify the polysaccharides. The amount of kefiran was measured as total sugar according to the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Other analytical methods The cell concentration was determined by measuring the turbidity at 660 nm. The number of bacterial and yeast cells was counted under a microscope using a hemocytometer. The supernatant obtained by centrifugation as described above was analyzed for determination of lactose, glucose, galactose, sucrose, and lactic acid concentrations. Lactose, glucose, galactose, and sucrose were determined by HPLC with a Shim-Pack CLC-101C column and a refractive index detector (RID-6A; both instruments from Shimadzu Seisakusho Co., Kyoto). Distilled water was used as the mobile phase at a flow rate of 1.0 ml/min at 80°C. Lactic acid concentration was measured using an HPLC system for analysis of organic acids; the system was equipped with a Shim-Pack SCR 102H column and a conductivity detector (CCD-6A; both from Shimadzu Seisakusho Co.). Three mM of Bis-Tris solution containing 3 mM *p*-toluenesulfuric acid and 100 μM EDTA was used as the mobile phase at a flow rate of 0.8 ml/min at 40°C.

Results and Discussion

Kefiran production using medium bottles To compare the amount of kefiran produced, four lactic acid bacterial strains isolated from kefir grains were cultivated using medium bottles containing the modified MRS medium with 50 g/*l* of lactose at 30°C for 7 days. The initial pH of the medium was adjusted to 5.5. *L. kefir* JCM 5818 and KL-3 grew rapidly on the first day. Although the cell growth of both strains almost stopped after 1 day, lactose was gradually consumed and lactic acid continued to accumulate in the culture broth with gradual decrease in pH. The growth rates of the two *L. kefiranofaciens* strains were slower than those with *L. kefir* and the amounts of lactic acid produced were lower than those by *L. kefir*. However, the final kefiran concentrations were 200–250 mg/*l* and 400–500 mg/*l* for *L. kefiranofaciens* JCM 7446 and JCM 6985, respectively. These values were higher than the concentrations (100–120 mg/*l*) of kefiran produced by the two strains of *L. kefir*. *L. kefiranofaciens* JCM 6985 was selected as one of the most suitable strains for kefiran production and cultivated using glucose, galactose, lactose, sucrose or a mixed sugar of glucose and galactose as a carbon source under the culture conditions described above. Using 50 g/*l* of lactose as a carbon source, the highest kefiran yield (about 500 mg/*l*) was obtained at a cultivation time of 9 days. Moreover, the optimum conditions for initial pH and temperature were examined in the modified MRS medium containing 50 g/*l* of lactose. The initial pH was adjusted to 4.5, 5.5, and 6.5 at 30°C. The highest quantity of kefiran was obtained at the initial pH of 5.5. When cultivations were carried out at 25°C, 30°C, and 37°C under an initial pH of 5.5, the highest yield of kefiran was achieved at 30°C. Therefore, the subsequent cultivations were done using lactose as a carbon source under the conditions of initial pH of 5.5 and temperature of 30°C.

Effect of addition of yeast cells Based on the results reported by Mitsue *et al.* (1999), the effect of addition of yeast cells on kefiran production by *L. kefiranofaciens* JCM 6985 was investigated. In the co-culture, *L. kefiranofaciens* and *S. cerevisiae, C. kefyr*, or *T. delbrueckii* at a concentration of 5×10^5 cells/ml and 1×10^5 cells/ml, respectively, were inoculated simultaneously. Unfortunately, little or no positive effect of the addition of yeast cells on kefiran production by *L. kefiranofaciens* was observed. Similar results were obtained even in the co-cultures where the yeast cells at different concentrations (1×10^3~1×10^5 cells/ml) were added to the culture containing 5×10^5 cells/ml of *L. kefiranofaciens* and/or were added at the midpoint of cultivation.

Effect of addition of yeast extract We considered to separate the positive effects of the presence and activities of yeast

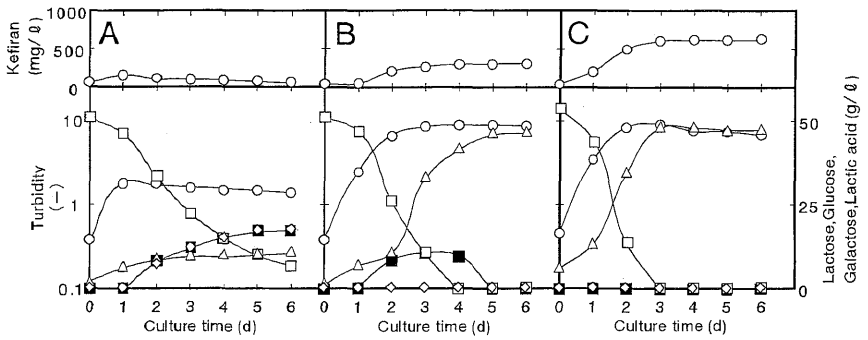

Fig 1. Effects of concentration of yeast extract initially added on kefiran production by *L. kefiranofaciens*. Yeast extract; A: 0 g/*l*, B: 2.5 g/*l*, C: 5 g/*l*. ○: Turbidity, □: Lactose, ■: Galactose, ◇: Glucose, △: Lactic acid.

cells on kefiran production by *L. kefiranofaciens* JCM 6985 into factors of the supplementation of nutrients to the lactic acid bacteria and the provision of conditions suitable for cell growth and kefiran production by formation of CO_2 and/or ethanol. As mentioned above, when the medium bottles were used, the pH of the culture broth dropped and consequently cell growth was inhibited by the accumulation of lactic acid. The concentration of kefiran produced in the cultivation without pH control using a bioreactor was around 460 mg/*l* using the modified MRS medium containing 50 g/*l* of lactose and 5 g/*l* of yeast extract. On the other hand, in other experiments using a bioreactor, cell growth and kefiran production were stimulated by controlling pH at 5.5 during cultivation as will be described below.

Figure 1 shows the effect of addition of yeast extract on kefiran production by *L. kefiranofaciens* JCM 6985. The pH of the culture broth was maintained at 5.5 throughout cultivation by adding 4 N NaOH as described above. In the culture without yeast extract, poor growth was observed and hardly any kefiran was produced. The higher the concentrations of yeast extract added, the higher was the yield of kefiran. The addition of yeast extract at a concentration of 5 g/*l* allowed a drastic increase in kefiran production. The final kefiran concentration was 650 mg/*l* in the culture with gentle agitation (100 rpm), but without aeration. Yokoi *et al.* (1991) also pointed out that the essential nutrient for *Lactobacillus* sp. KPB-167B was yeast extract and that the lack of tryptone or meat extract achieved about 85% of the cell mass obtained in MRS medium. The enhanced cell growth and kefiran production appear to be mainly due to abundant vitamin groups in the yeast extract. It is necessary to examine the medium ingredients affecting the kefiran production; this point is especially important for the industrial production of kefiran.

Effect of aeration of CO_2 Although *L. kefiranofaciens* JCM 6985 grew and produced kefiran in the cultivation without aeration as shown in Fig. 1C, no cell growth and no kefiran production by the bacterium were observed in the cultivation with aeration of N_2 alone at 0.3 vvm (data not shown). Then, a mixed gas of N_2 and CO_2 at a volume ratio of 9:1 or 5:5 was sparged into the culture broth at 0.3 vvm throughout the cultivation. Figure 2 shows the effect of the composition of gas for aeration on kefiran production by *L. kefiranofaciens* JCM 6985. When a mixed gas of 9:1 (N_2:CO_2) was sparged (Fig. 2A), a slight increase in the amount (670 mg/*l*) of kefiran produced was observed as compared with that (650 mg/*l*) in the cultivation without aeration shown in Fig. 1C. The supply of a slight amount of CO_2 was found to be important for cell growth and kefiran production. Moreover, judging from the fact that galactose was detected once at the midpoint (2–4 days) of the cultivation, the aeration of gas containing CO_2 caused a change in uptake and metabolism of lactose. However, the reason for the accumulation of galactose as an intermediate in the culture broth is still unclear.

Effect of addition of ethanol Prior to elucidation of the effect of ethanol addition on kefiran production, the effect of lactose concentration on the production by *L. kefiranofaciens* JCM 6985 was investigated in control cultivation without ethanol. Figure 3 shows the results of cultivations using a modified MRS

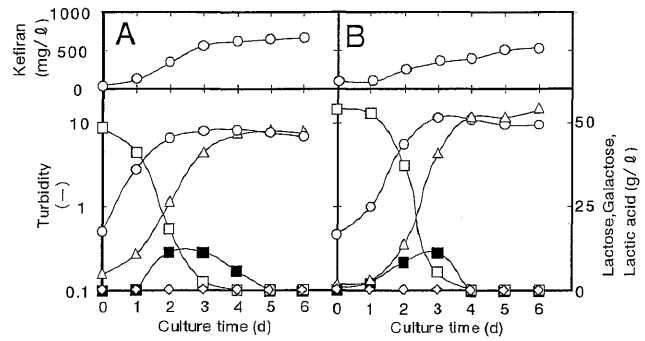

Fig 2. Effects of composition of a mixed gas for aeration on kefiran production by *L. kefiranofaciens*. A mixed gas of N_2 and CO_2 at a volume ratio of 9:1 (A) or 5:5 (B) was sparged at 0.3 vvm. Symbols are the same as shown in Fig. 1.

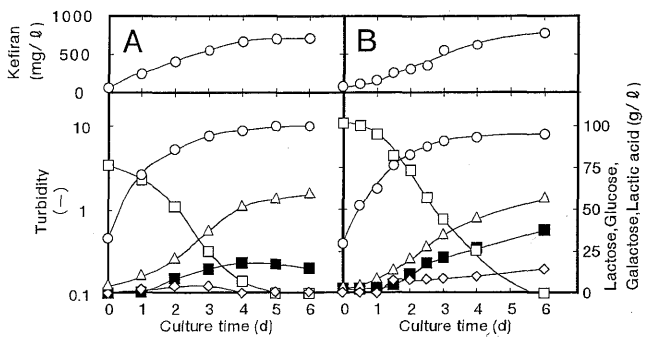

Fig 3. Production of kefiran by *L. kefiranofaciens* using the medium containing 75 g/*l* (A) and 100 g/*l* (B) of lactose as a carbon source. Symbols are the same as shown in Fig. 1.

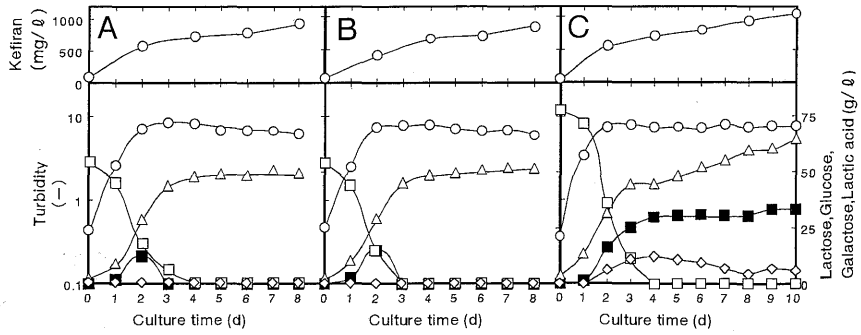

Fig 4. Effects of ethanol addition on kefiran production by *L. kefiranofaciens*. When the medium containing 50 g/*l* of lactose was used as a carbon source, 10 g/*l* (A) or 20 g/*l* (B) of ethanol was added. When the medium containing 75 g/*l* of lactose was used as a carbon source, 10 g/*l* (C) of ethanol was added. Symbols are the same as shown in Fig. 1.

medium containing 75 and 100 g/*l* of lactose as a carbon source. A mixed gas of N_2 and CO_2 at a volume ratio of 9:1 was sparged into the culture broth at 0.3 vvm throughout the cultivations. As shown in Fig.3A, 75 g/*l* of lactose was consumed completely by 5 days, but galactose accumulated gradually and remained in the culture broth at 6 days. Glucose accumulated once for 2–3 days and then was consumed completely by 4 days. The concentration of lactic acid gradually increased and reached a maximum value of 60.3 g/*l* at 6 days. When 100 g/*l* of lactose was used as a carbon source, it was consumed completely by 6 days, but glucose as well as galactose remained in the culture broth after 1 day as shown in Fig. 3B. In this cultivation, the final lactic acid concentration was 57.4 g/*l*. The amounts of kefiran produced from 75 and 100 g/*l* of lactose were about 710 mg/*l* (Fig. 3A) and 770 mg/*l* (Fig. 3B), respectively, which were higher than that (670 mg/*l*) with the cultivation using 50g/*l* of lactose as a carbon source (see Fig. 2A). The yields of kefiran per gram of lactose added could not be enhanced by increasing the initial concentration of lactose. The results show that the accumulation of lactic acid at such a high level causes the inhibition of uptake and metabolism of lactose, just as with the aeration of gas containing CO_2 described above. In addition, it is obvious that the accumulation of lactic acid leads to a decrease in growth rate as reported previously (Taniguchi *et al.*, 1987, 1994; Burgos-Rubio *et al.*, 2000). The removal of lactic acid from culture broth by separation methods such as membrane filtration, electrodialysis, and liquid-liquid extraction seems to allow not only achievement of high cell concentration but also enhancement of kefiran production. Research on the development of a culture system which removes lactic acid for kefiran production is currently under way.

The kefir made by the traditional procedure contains 0.5–1.5% of ethanol and a small amount of acetic acid as well as 0.6–1.0% of lactic acid. By adding initially 10 or 20 g/*l* of ethanol to the medium containing 50 g/*l* or 75 g/*l* of lactose, the effect of the addition on kefiran production by *L. kefiranofaciens* JCM 6985 was examined. Figure 4 shows the results of cultivations with the pH controlled at 5.5 and simultaneously with sparging of a mixed gas of N_2 and CO_2 (9:1). When 10 or 20 g/*l* of ethanol was initially added to the medium containing 50 g/*l* of lactose, the time when galactose could be detected in the culture broth decreased as compared to that without ethanol, although negligible changes in the profiles of lactose consumption and lactic acid production were observed. As shown in Figs. 4A and 4B, kefiran continued to be produced gradually in the culture broth even after all sugars were consumed. The concentration of kefiran obtained when 10 g/*l* or 20 g/*l* of ethanol was added to the medium was about 930 mg/*l* or 840 mg/*l* at 8 days, respectively. These values were significantly higher than that (670 mg/*l*) of the cultivation without ethanol described above. When 10 g/*l* of ethanol was added to the medium containing 75 g/*l* of lactose, the maximum concentration of kefiran obtained was about 1040 mg/*l* at 10 days as shown in Fig. 4C. However, in this cultivation, the added lactose was not utilized efficiently to produce kefiran because both 5.9 g/*l* of glucose and 33 g/*l* of galactose remained in the culture broth. The reduced sugar consumption seems to be due to the accumulation of more than 50 g/*l* of lactic acid, in the same manner as the results of cultivation using 100g/*l* of lactose as a carbon source (see Fig. 3B).

Comparison of amounts of kefiran produced Figure 5 shows a comparison of the amounts of extracellular kefiran obtained under the different culture conditions. By sparging a mixed gas of N_2 and CO_2 at a volume ratio of 9:1 into the culture broth at 0.3 vvm throughout the cultivation, a slight increase in the amount (670 mg/*l*) of kefiran produced was observed as compared with that (650 mg/*l*) in the cultivation without aeration. However, further increase in the CO_2 fraction to one-half of total gas volume caused a decrease in the amount of kefiran produced. When 10 g/*l* or 20 g/*l* of ethanol was added to the medium containing 50 g/*l* of lactose, the kefiran concentrations were about 930 mg/*l* or 840 mg/*l* at 8 days, respectively. The maximum kefi-

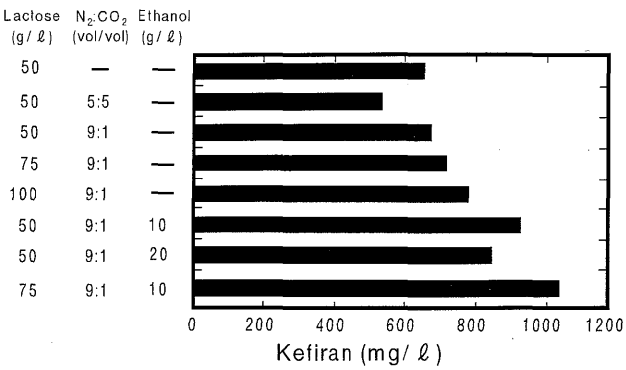

Fig 5. Comparison of the amounts of kefiran produced under different culture conditions.

ran concentration was about 1040 mg/*l* at 10 days, when 10 g/*l* of ethanol was added to the medium containing 75 g/*l* of lactose. Although the time required to reach the maximum concentration of kefiran was longer, the addition of ethanol to the medium made it possible to increase the amount of kefiran produced as shown in Fig. 4.

Based on the results described above, we found that factors resulting from the presence of yeast strains in kefir grains, that is, addition of yeast extract and ethanol, aeration of gas containing CO_2, and their combinations allowed promotion of kefiran production by *L. kefiranofaciens*. In general, to stimulate production of useful substances by cooperative actions between two microorganisms in a co-culture, it seems very difficult to optimize the culture conditions of pH, temperature, composition of aeration gas, ratio of inoculum size, time and amount of inoculation of each microorganism, and composition of nutrients in the medium used. The results obtained in this study may show one good example of production of useful compounds using a single microorganism under culture conditions established by mimicking the actions of yeast cells on *L. kefiranofaciens* in kefir grain as a typically natural co-culture system.

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