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Full Paper

The effect of sodium acetate on the growth yield, the production of L- and D-lactic acid, and the activity of some enzymes of the glycolytic pathway of *Lactobacillus sakei* NRIC 1071^T and *Lactobacillus plantarum* NRIC 1067^T

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The effect of sodium acetate was studied on the change of the growth yield, the production of L- and D-lactic acid, and the activity of lactate dehydrogenases (LDHs; L-lactate dehydrogenase [EC 1.1.1.27, L-LDH] plus D-lactate dehydrogenase [EC 1.1.1.28, D-LDH]), fructose-1, 6-bisphosphate aldolase [EC 4.1.2.13, FBP-aldolase], and phosphofructokinase [EC 2.7.1.11, PFK] of *Lactobacillus sakei* NRIC 1071^T and *Lactobacillus plantarum* NRIC 1067^T. The growth yield of *L. sakei* NRIC 1071^T was increased 1.6 times in the presence of sodium acetate compared with its absence. The activity of LDHs in *L. sakei* NRIC 1071^T and *L. plantarum* NRIC 1067^T was retained longer under the addition of sodium acetate in the reaction mixture. As a result, these strains produced much more lactic acid in the presence of sodium acetate compared with its absence. Furthermore, the activity of L-LDH in *L. sakei* NRIC 1071^T cultivated in the presence of sodium acetate increased three times or more compared with the activity of the cells cultivated in its absence. Consequently, the type of stereoisomers of lactic acid produced by *L. sakei* shifted from the DL-type to the L-type because the ratio of L-lactic acid to D-lactic acid produced became larger with the addition of sodium acetate to culture media. This phenomenon was not observed in *L. plantarum* NRIC 1067^T. Further, the participation of lactate racemase is discussed from the viewpoint of the production of D-lactic acid by *L. sakei.*

Key Words——effect of sodium acetate; D-lactate dehydrogenase; L-lactate dehydrogenase; lactate racemase; *Lactobacillus plantarum*; *Lactobacillus sakei*; type of stereoisomers of lactic acid

Introduction

In a previous paper, Iino et al. (2001) reported the good growth and the high production of lactic acid by a large number of lactic acid bacteria when they were cultivated in the presence of sodium acetate. Of 60 strains studied, only *Lactobacillus sakei* strains shifted the type of stereoisomers of lactic acid from the DL-type to the L-type due to the high production of L-lactic acid and the low production of D-lactic acid.

L. sakei was reported to possess L-lactate dehydrogenase [EC 1.1.1.27, L-LDH] for the production of L-lactic acid (Katagiri and Kitahara, 1938; Malleret et al.,

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1998) and lactate racemase [EC 5.1.2.1] for the production of D-lactic acid from L-lactic acid (Hiyama et al., 1968; Kandler and Weiss, 1986; Katagiri and Kitahara, 1937; Kitahara et al., 1957; Ôbayashi and Kitahara, 1959; Stetter and Kandler, 1973). Furthermore, *L. sakei* was described as producing L-lactic acid exclusively when the production of lactate racemase was repressed by sodium acetate (Kitahara et al., 1957; Stetter and Kandler, 1973; Toyoda et al., 1979). However, lactate racemase would not explain the mechanism of enhancing the production of L-lactic acid because this enzyme participates in the production of D-lactic acid from L-lactic acid and is not concerned in the high production of L-lactic acid. The participation of D-lactate dehydrogenase [EC 1.1.1.28, D-LDH] has not been clarified yet in the case of *L. sakei.* Iino et al. (2001) suggested that sodium acetate played roles in the activation of LDHs, the production of LDHs, and/or the enhancement of the glycolytic pathway that will result in the high production of pyruvic acid by lactic acid bacteria. It is of interest to note that the ratio of L-form to D-form of biological substances such as L-lactic acid and D-lactic acid is largely shifted back and forth by environmental conditions.

This paper deals with the effect of sodium acetate on the growth yield, the production of L- and D-lactic acid by resting cells, and the enzyme activity of L-LDH, D-LDH, fructose-1, 6-bisphosphate aldolase [EC 4.1.2.13, FBP-aldolase], and phosphofructokinase [EC 2.7.1.11, PFK] by the cell-free extract of *L. sakei* NRIC 1071^T and *Lactobacillus plantarum* NRIC 1067^T. Further, the participation of lactate racemase is discussed from the viewpoint of the production of D-lactic acid by *L. sakei.*

Materials and Methods

Bacterial strains. *L. sakei* NRIC 1071^T and *L. plantarum* NRIC 1067^T were used in this study.

Cultivation. Strains were cultivated in 5 ml of GYP broth as reported in a previous paper (Iino et al., 2001). *L. sakei* NRIC 1071^T was stationarily cultivated at 25°C for two days, and *L. plantarum* NRIC 1067^T at 30°C for two days. These strains were stationarily precultured in GYP broth for two days. Cells were collected by centrifugation at 3,500 rpm for 15 min at room temperature, and washed twice with sterile saline. The washed cells were resuspended in sterile saline, and 50 µl of the suspension was inoculated into GYP broth and other liquid media with a pipette.

Determination of biomass. Cells were collected by centrifugation, washed twice with distilled water, and put in preweighed tubes. The cells were then dried to a constant weight by lyophilization and reweighed.

Monitoring of bacterial growth. The monitoring of bacterial growth was performed by the method described in the previous paper (Iino et al., 2001).

Determination of a total amount of lactic acid and an amount of acetic acid during the growth. Cells were cultivated in liquid media, collected by centrifugation at every two hours, and the resulted supernatant was used for analysis. A total amount of lactic acid and an amount of acetic acid were determined by the method described in the previous paper (Iino et al., 2001).

Preparation of resting cells. Cells at a late logarithmic phase cultivated in 5 ml of liquid media were collected by centrifugation, and washed twice with 50 mM Tris-HCl buffer (pH 7.5). The cells were used for the resting cells in this study.

Determination of a total amount of lactic acid and the type of stereoisomers produced from glucose by resting cells. Resting cells were suspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) for the production of lactic acid from glucose. A reaction mixture containing 100 µl of resting cells and 800 µl of 50 mM Tris-HCl buffer (pH 7.5) was preincubated at the below-mentioned temperatures. A reaction was started by adding 100 µl of 500 mM glucose in the reaction mixture. The resting cells of *L. sakei* NRIC 1071^T were incubated at 25°C for 24 h, and those of *L. plantarum* NRIC 1067^T at 30°C for 24 h. The reaction mixture was boiled for 10 min, and centrifuged at 15,000 rpm for 15 min at 4°C. After pellets were discarded, 250 µl of the resulting supernatant was used for the determination of total lactic acid, and 750 µl was used for the determination of the type of stereoisomers of lactic acid by the method previously described (Iino et al., 2001). The reaction was performed with or without 50 mM sodium acetate in the reaction mixture.

Production of DL-lactic acid from L- or D-lactic acid by resting cells. The resting cells were suspended in 900 µl of 50 mM imidazole-HCl buffer (pH 6.0), and used for the production of DL-lactic acid from L- or D-lactic acid. After preincubation at the below-mentioned temperatures, a reaction was started by adding 100 µl of 500 mM lithium L-lactate or 500 mM lithium D-lactate in the reaction mixture. The total amount of lactic acid and the type of stereoisomers were determined by the

method mentioned above. The resting cells of *L. sakei* NRIC 1071^T were incubated at 25°C for 24 h, and those of *L. plantarum* NRIC 1067^T at 30°C for 24 h. The reaction was performed with or without 50 mM sodium acetate in the reaction mixture.

Preparation of the cell-free extract. Cells cultivated in 5 ml of liquid media were collected by centrifugation (a total 100 ml of culture broth was prepared from 20 tubes in order to make uniform cultural conditions), washed with 50 mM Tris-HCl buffer (pH 7.5) twice, and suspended in 50 mM Tris-HCl buffer (pH 7.5). The cells were then suspended in 1 ml of a cell lysis solution containing 1 mg of lysozyme (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) and 0.1 mg of Labiase (Seikagaku Kogyo Co., Ltd.) for 60 min, and then disrupted by sonication on ice (output control 3×duty cycle 30%). The lysed cells were centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant was used for the cell-free extract.

Production of DL-lactic acid from L- or D-lactic acid by the cell-free extract. The production of DL-lactic acid from L- or D-lactic acid was determined by the method described by Hiyama et al. (1968). The enzyme activity of the production of DL-lactic acid from L- or D-lactic acid by the cell-free extract was determined by the ratio of L-lactic acid to D-lactic acid produced. DL-Lactic acid was produced in a 40 mM imidazole-HCl buffer (pH 6.0) containing an appropriate amount of the cell-free extract. A reaction was started by adding 1 mM lithium L-lactate or 1 mM lithium D-lactate (a final concentration) to the reaction mixture. After the incubation for 60 min at the below-mentioned temperatures, the reaction mixture was boiled for 10 min, and centrifuged at 15,000 rpm for 30 min at 4°C. The reaction mixture of *L. sakei* NRIC 1071^T was incubated at 25°C for 60 min, and that of *L. plantarum* NRIC 1067^T at 30°C for 60 min. After pellets were discarded, 10 µl of the supernatant was submitted to HPLC for the determination of L- and D-lactic acid produced as reported in the previous paper (Iino et al., 2001).

Assay of enzymes. The total activity of L-LDH plus D-LDH of the cell-free extract was expressed as LDHs, and assayed by the NADH oxidation in a 40 mM imidazole-HCl buffer (pH 6.0) containing 0.3 mM NADH, 3 mM fructose-1,6-bisphosphate (FBP), and 10 mM $MnSO_4$. A reaction was started by adding 2 mM sodium pyruvate (a final concentration) at the below-mentioned temperatures, and the decrease in absorbance was monitored at 340 nm. The reaction mixture of *L. sakei* NRIC 1071^T was incubated at 25°C, and that of *L. plantarum* NRIC 1067^T at 30°C. The activity of L-LDH and D-LDH of the cell-free extract was determined on the basis of the activity of LDHs and the ratio of L-lactic acid to D-lactic acid produced because L-form and D-form were not determined separately by the above-mentioned method. L-Lactic acid and D-lactic acid were produced in a 40 mM imidazole-HCl buffer (pH 6.0) containing 3 mM NADH, 3 mM FBP, 10 mM $MnSO_4$, and 2 mM sodium pyruvate. A reaction was started by adding an appropriate amount of cell-free extract to the reaction mixture. After the incubation for 60 min at the above-mentioned temperature, the reaction mixture was boiled for 10 min, and centrifuged at 15,000 rpm for 30 min at 4°C. After pellets were discarded, 10 µl of the supernatant was submitted to HPLC for the determination of L- and D-lactic acid produced by cell-free extract as reported in the previous paper (Iino et al., 2001). In this study, the activity of enzymes producing D-lactic acid was shown as the activity of D-LDH, which may include the activity of D-LDH and lactate racemase.

The activity of PFK was assayed in a 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 mM NADH, 2.0 mM ATP, 2.5 mM $MgCl_2$, 0.54 U/ml of aldolase (rabbit muscle; Roche Diagnostics GmbH, Mannheim, Germany), and 0.8 U : 2.3 U/ml of a glycerol-3-phosphate dehydrogenase (GDH)/triosephosphate isomerase (TPI) mixture (rabbit muscle; Roche Diagnostics GmbH). The assay was coupled with the NADH oxidation by the use of aldolase, GDH, and TPI. A reaction was started by adding 2 mM fructose-6-phosphate (a final concentration) at the above-mentioned temperatures, and the decrease of absorbance was monitored at 340 nm.

The activity of FBP-aldolase was assayed by the NADH oxidation in a 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 mM NADH, 5 mM cysteine-HCl (monohydrate), 5 mM sodium hydroxide, and 0.8 U : 2.3 U/ml of a GDH/TPI mixture (rabbit muscle, Roche Diagnostics GmbH). A reaction was started by adding 3 mM FBP (a final concentration) at the above-mentioned temperatures, and the decrease of absorbance was monitored at 340 nm.

All enzyme activities were assayed with or without 5 mM sodium acetate in the reaction mixture, replacing the atmosphere in a sealed cell with nitrogen gas, and incubating the reaction mixture anaerobically. One unit of enzyme activity was defined as the amount of enzyme (mg protein) that catalyzed the oxidation of 1 µM

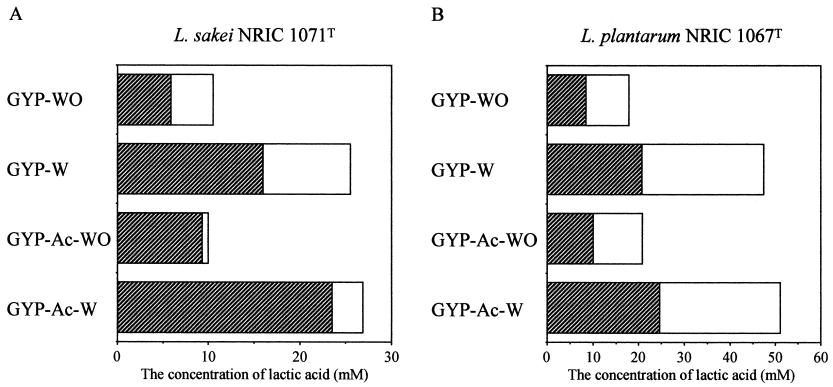

Fig. 1. The effect of sodium acetate on the production of stereoisomers of lactic acid from glucose by the resting cells of *L. sakei* NRIC 1071^T and *L. plantarum* NRIC 1067^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture; hatched bars, L-lactic acid (mM); open bars, D-lactic acid (mM).

NADH min^{-1}. Protein was determined by the dye-binding method (Bradford, 1976) using bovine serum albumin as a standard.

Results

The effect of sodium acetate on the growth yield

The growth yield of *L. sakei* NRIC 1071^T was 13.6 g of dry weight per mol of glucose when the strain was cultivated in the absence of sodium acetate; 21.3 g, in the presence of 50 mM sodium acetate; and 13.3 g, in the presence of 50 mM potassium phosphate buffer. The growth yield of *L. plantarum* NRIC 1067^T was 16.0 g of dry weight per mol glucose when the strain was cultivated in the absence of sodium acetate; 19.9 g, in the presence of 50 mM sodium acetate; and 17.7 g, in the presence of 50 mM potassium phosphate buffer.

The shift of pH in culture media and the assimilation of acetic acid added in culture media by L. sakei NRIC 1071^T and L. plantarum NRIC 1067^T

L. sakei NRIC 1071^T produced lactic acid during the growth in the absence of sodium acetate, and the pH of the culture medium gradually decreased from 6.8 to 3.8. This strain produced about two times the amount of lactic acid in the presence of 50 mM sodium acetate, and about 1.7 times the amount of lactic acid in the presence of 50 mM potassium phosphate buffer compared with the amount of lactic acid in the absence of sodium acetate. During the growth of this strain, the pH of the medium decreased from 6.8 to 4.1 in the presence of 50 mM sodium acetate, and from 6.8 to 4.2 in the presence of 50 mM potassium phosphate buffer.

L. plantarum NRIC 1067^T produced lactic acid during the growth in any of culture media. As a result, the pH of the medium gradually decreased from 6.8 to 3.4 in the absence of sodium acetate, from 6.8 to 4.0 in the presence of 50 mM sodium acetate, and from 6.8 to 3.8 in 50 mM potassium phosphate buffer.

Moreover, *L. sakei* NRIC 1071^T and *L. plantarum* NRIC 1067^T did not assimilate acetic acid during the growth in the presence of 50 mM sodium acetate.

The effect of sodium acetate on the production of the total amount of lactic acid and of the amount of stereoisomers of lactic acid from glucose by the resting cells of L. sakei NRIC 1071^T and L. plantarum NRIC 1067^T

Experiments about the effect of sodium acetate on the production of the total amount of lactic acid and of the amount of stereoisomers from glucose were carried out by the use of resting cells cultivated in the absence of sodium acetate and with 50 mM sodium acetate, and the presence of 50 mM sodium acetate in the reaction mixture.

Regardless of the presence of sodium acetate in the media, the resting cells of *L. sakei* NRIC 1071^T produced a total amount of lactic acid to the same extent from glucose (Fig. 1A; GYP-WO and GYP-Ac-WO). The above-mentioned resting cells produced about 2.5 times the amount of lactic acid under the addition of 50 mM sodium acetate to the reaction mixture (Fig. 1A; GYP-W and GYP-Ac-W), as compared with the amount of lactic acid under the absence of 50 mM sodium acetate (Fig. 1A; GYP-WO and GYP-Ac-WO).

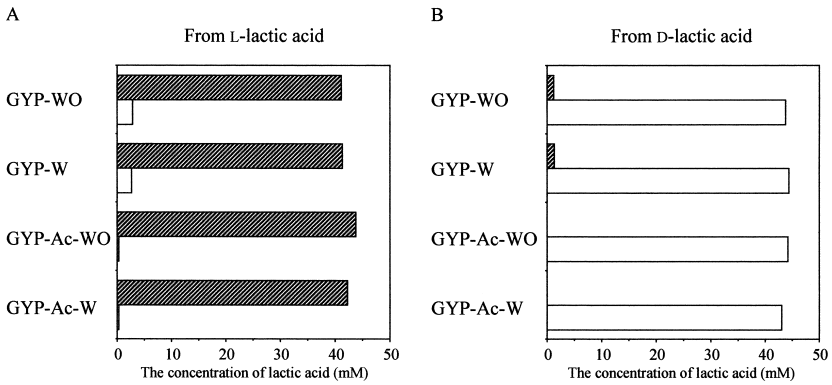

Fig. 2. The effect of sodium acetate on the production of DL-lactic acid from L- or D-lactic acid by the resting cells of *L. sakei* NRIC 1071^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture; hatched bars, L-lactic acid (mM); open bars, D-lactic acid (mM).

The resting cells of *L. sakei* NRIC 1071^T cultivated in the absence of sodium acetate produced L- and D-lactic acid to almost the same extent from glucose (L-lactic acid : D-lactic acid≒3 : 2) (Fig. 1A; GYP-WO and GYP-W), regardless the addition of 50 mM sodium acetate to the reaction mixture. In contrast, the resting cells cultivated in the presence of 50 mM sodium acetate produced about 1.5 times the amount of L-lactic acid (Fig. 1A; GYP-Ac-WO and GYP-Ac-W), regardless of the addition of 50 mM sodium acetate to the reaction mixture, as compared with cells cultivated in the absence of sodium acetate (Fig. 1A; GYP-WO and GYP-W), and produced barely any D-lactic acid.

Regardless of the presence of sodium acetate in the media, the resting cells of *L. plantarum* NRIC 1067^T produced lactic acid to the same extent from glucose (Fig. 1B; GYP-WO and GYP-Ac-WO). Moreover, the above-mentioned cells produced two times or more the amount of lactic acid under the addition of 50 mM sodium acetate in the reaction mixture (Fig. 1B; GYP-W and GYP-Ac-W), as compared with the cells under no addition of sodium acetate to the reaction mixture. However, the ratio of L-lactic acid to D-lactic acid was almost the same (ca. 1 : 1), irrespective of the culture media used and the addition of sodium acetate in the reaction mixture.

The effect of sodium acetate on the production of DL-lactic acid from L- or D-lactic acid by the resting cells of L. sakei NRIC 1071^T and L. plantarum 1067^T

The resting cells of *L. sakei* NRIC 1071^T cultivated in the absence of sodium acetate produced scarcely any DL-lactic acid from L-lactic acid (Fig. 2A; GYP-WO and GYP-W), or from D-lactic acid (Fig. 2B; GYP-WO and GYP-W), regardless of the addition of sodium acetate to the reaction mixture. The resting cells cultivated in the presence of 50 mM sodium acetate did not produce DL-lactic acid from L-lactic acid (Fig. 2A; GYP-Ac-WO and GYP-Ac-W), or from D-lactic acid (Fig. 2B; GYP-Ac-WO and GYP-Ac-W), regardless of the addition of 50 mM sodium acetate in the reaction mixture.

The resting cells of *L. plantarum* NRIC 1067^T produced DL-lactic acid from L-lactic acid or D-lactic acid (about 50%) (Fig. 3A and B; GYP-WO, GYP-W, GYP-Ac-WO, and GYP-Ac-W), regardless of the addition of 50 mM sodium acetate to the reaction mixtures.

The effect of sodium acetate on the activity of LDHs in the cell-free extract of the cells from L. sakei NRIC 1071^T and L. plantarum NRIC 1067^T

The cell-free extract from *L. sakei* NRIC 1071^T cultivated in the absence of sodium acetate showed an increase of absorption at 340 nm under the addition of 5 mM sodium acetate to the reaction mixture, as compared with the absorption in the absence of sodium acetate in the reaction mixture. The increase was retained longer, and amounted to up to 1.3 times (Fig. 4A). This finding was recognized for the cells cultivated in the presence of 50 mM sodium acetate and for those in the presence of 50 mM potassium phosphate buffer.

The cell-free extract from *L. plantarum* NRIC 1067^T cultivated in the absence of sodium acetate showed an increase of absorption at 340 nm under the addition of 5 mM sodium acetate to the reaction mixture, as compared with the absorption in the absence of sodium ac-

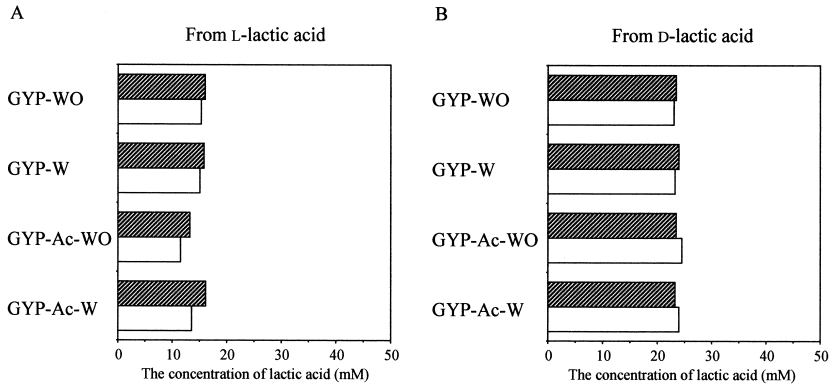

Fig. 3. The effect of sodium acetate on the production of DL-lactic acid from L- or D-lactic acid by the resting cells of *L. plantarum* NRIC 1067^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture; hatched bars, L-lactic acid (mM); open bars, D-lactic acid (mM).

etate in the reaction mixture (Fig. 4B). The increase was retained longer, and amounted to up to 2.5 times. This finding was also recognized for the cells cultivated in the presence of 50 mM sodium acetate, and the cells cultivated in the presence of 50 mM potassium phosphate buffer.

In contrast, the activity of L-LDH from rabbit muscle (Roche Diagnostics GmbH) and the activity of D-LDH from *Lactobacillus leichmannii* (Roche Diagnostics GmbH) were not affected by the addition of 5 mM sodium acetate to the reaction mixture (Fig. 4C).

The effect of sodium acetate on the activity of L-LDH and D-LDH in the cell-free extract of the cells from L. sakei NRIC 1071^T and L. plantarum NRIC 1067^T

The activity of L-LDH of the cell-free extract from the cells of *L. sakei* NRIC 1071^T cultivated in the presence of 50 mM sodium acetate increased two to two and half times (0.8 to 0.9 U/mg) (Fig. 5A; GYP-WO, GYP-W, GYP-Ac-WO, and GYP-Ac-W) compared with the activity from the cells cultivated in the absence of sodium acetate. In contrast, the activity of L-LDH was almost the same in the cell-free extract from *L. sakei* NRIC 1071^T cultivated in the presence of 50 mM potassium phosphate buffer (Fig. 5A; GYP-P-WO and GYP-P-W), regardless of the addition of 5 mM sodium acetate to the reaction mixture. The activity of D-LDH from *L. sakei* NRIC 1071^T did not show a significant difference among the cell-free extracts from the cells cultivated in the absence of sodium acetate, from those cultivated in the presence of 50 mM sodium acetate, or from those cultivated in the presence of 50 mM potassium phosphate buffer (Fig. 5B), regardless of the addition of 5 mM sodium acetate to the reaction mixture.

The activity of L-LDH of *L. plantarum* NRIC 1067^T was almost the same for the cell-free extract from the cells cultivated in the absence of sodium acetate, as for those cultivated in the presence of 50 mM sodium acetate, and those cultivated in the presence of 50 mM potassium phosphate buffer (Fig. 6A), regardless of the addition of 5 mM sodium acetate to the reaction mixture. The activity of D-LDH in the cell-free extract from *L. plantarum* NRIC 1067^T was almost the same, irrespective of the culture media and the addition of sodium acetate to the reaction mixture (Fig. 6B).

The effect of sodium acetate on the production of DL-lactic acid from L- or D-lactic acid by the cell-free extract of the cells from L. sakei NRIC 1071^T and L. plantarum NRIC 1067^T

The cell-free extract of the cells from *L. sakei* NRIC 1071^T cultivated in the absence of sodium acetate did not produce DL-lactic acid from L-lactic acid or from D-lactic acid, regardless of the addition of sodium acetate to the reaction mixture (Fig. 7A and B; GYP-WO and GYP-W). The production of DL-lactic acid from L-lactic acid or from D-lactic acid was not shown by the cell-free extract from the cells cultivated in the presence of 50 mM sodium acetate, or by that in the presence of 50 mM potassium phosphate buffer (Fig. 7A and B; GYP-Ac-WO, GYP-Ac-W, GYP-P-WO, and GYP-P-W).

The cell-free extract of the cells from *L. plantarum* NRIC 1067^T did not produce DL-lactic acid from L-lactic acid or from D-lactic acid (Fig. 8A and B), regardless of the cultivation in the presence of 50 mM sodium ac-

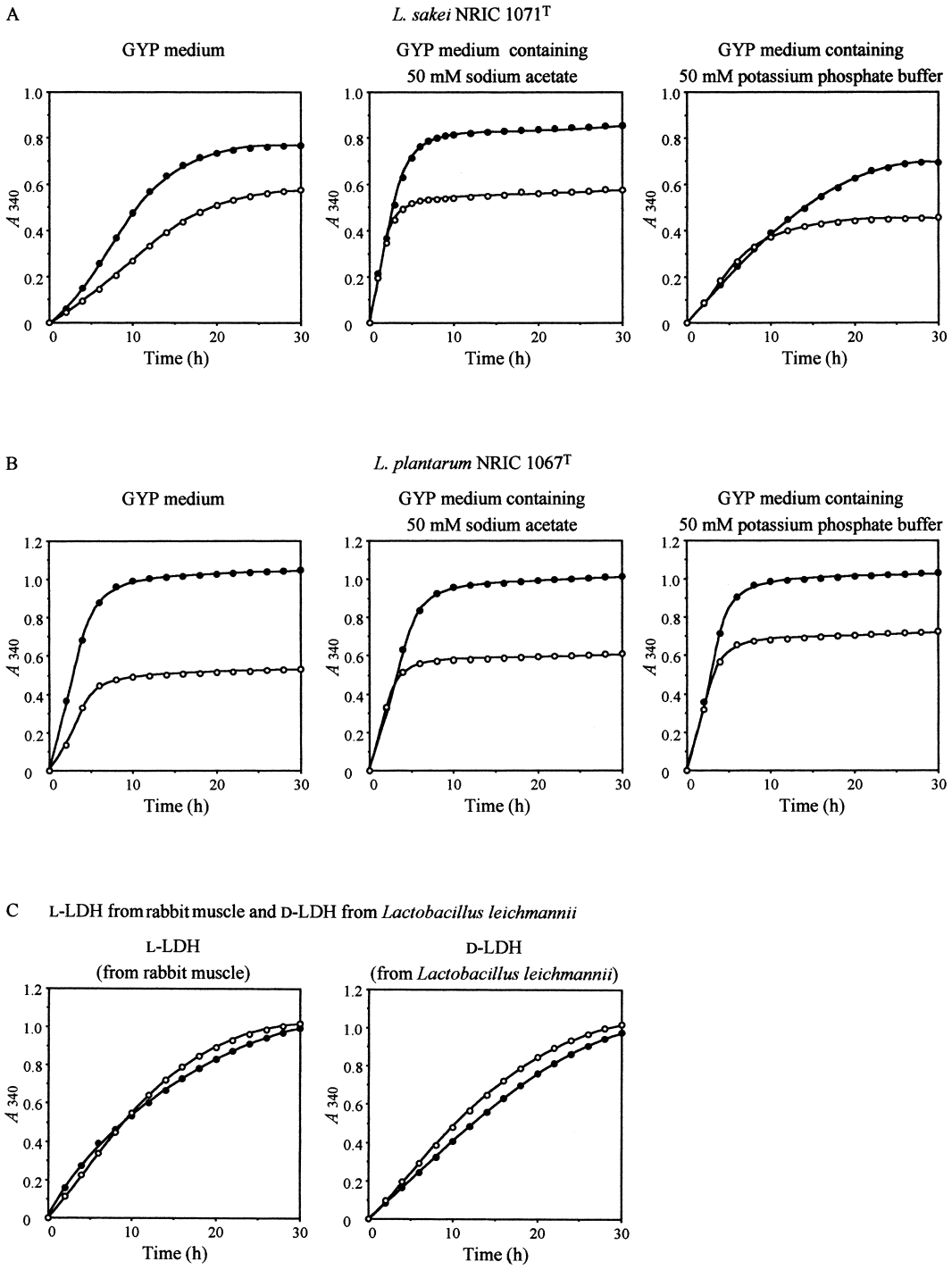

Fig. 4. The effect of sodium acetate on the activity of LDHs in the cell-free extracts of the cells from *Lactobacillus sakei* NRIC 1071^T and *L. plantarum* NRIC 1067^T.

Symbols: ○, the activity of LDHs without 5 mM sodium acetate; ●, the activity of LDHs with 5 mM sodium acetate.

etate and in the presence of 50 mM potassium phosphate buffer, and the addition of 5 mM sodium acetate to the reaction mixture.

The effect of sodium acetate on the activity of PFK and FBP-aldolase in the cell-free extract of the cells from L. sakei NRIC 1071^T and L. plantarum NRIC 1067^T

The activity of PFK in the cell-free extract from *L. sakei* NRIC 1071^T cultivated in the absence of sodium acetate changed slightly with the addition of 5 mM

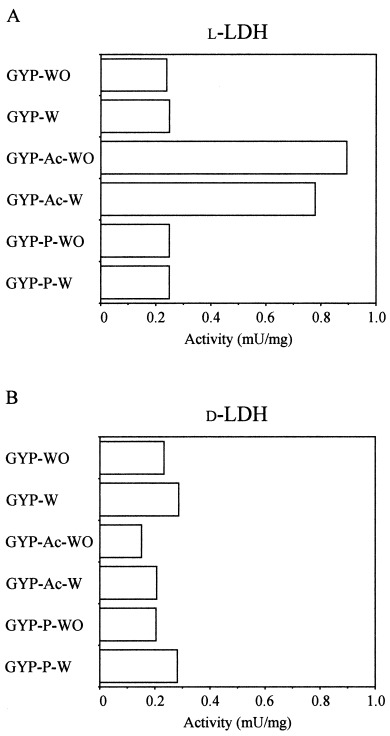

Fig. 5. The effect of sodium acetate on the activity of L-LDH and D-LDH in the cell-free extracts of the cells from *L. sakei* NRIC 1071^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; GYP-P, GYP medium containing 50 mM potassium phosphate buffer; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture.

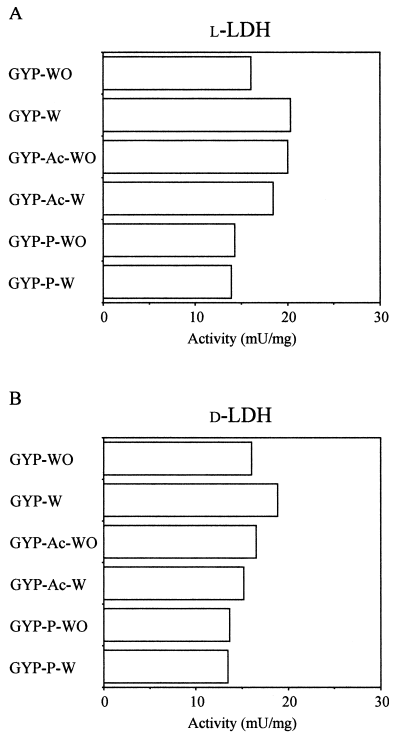

Fig. 6. The effect of sodium acetate on the activity of L-LDH and D-LDH in the cell-free extracts of the cells from *L. plantarum* NRIC 1067^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; GYP-P, GYP medium containing 50 mM potassium phosphate buffer; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture.

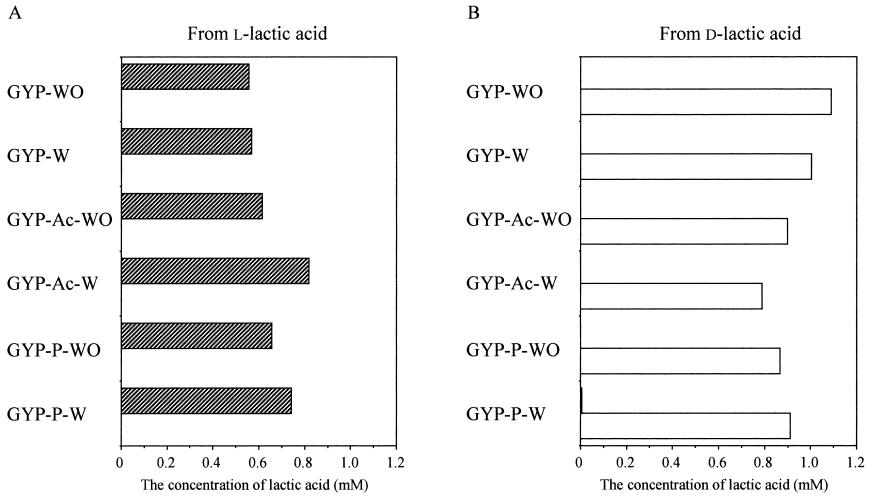

Fig. 7. The effect of sodium acetate on the production of DL-lactic acid from L- or D-lactic acid by the cell-free extracts of the cells from *L. sakei* NRIC 1071^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; GYP-P, GYP medium containing 50 mM potassium phosphate buffer; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture; hatched bars, L-lactic acid (mM); open bars, D-lactic acid (mM).

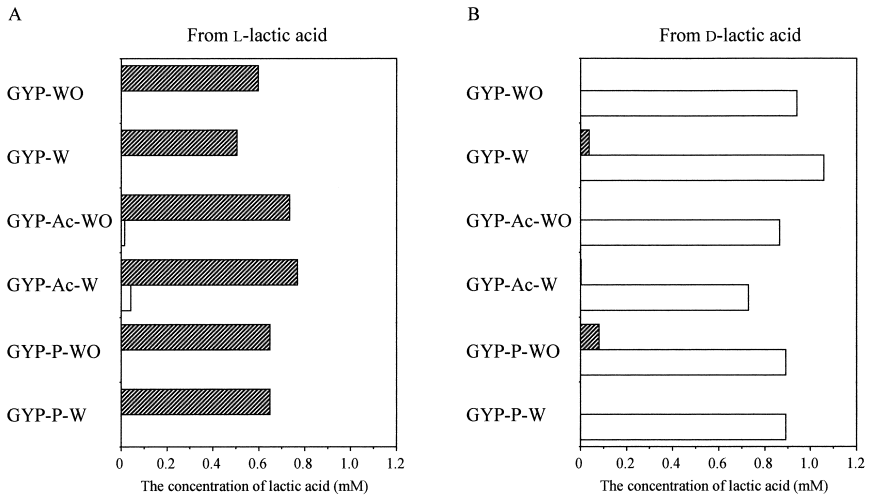

Fig. 8. The effect of sodium acetate on the production of DL-lactic acid from L- or D-lactic acid by the cell-free extracts of the cells from *L. plantarum* NRIC 1067^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; GYP-P, GYP medium containing 50 mM potassium phosphate buffer; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture; hatched bars, L-lactic acid (mM); open bars, D-lactic acid (mM).

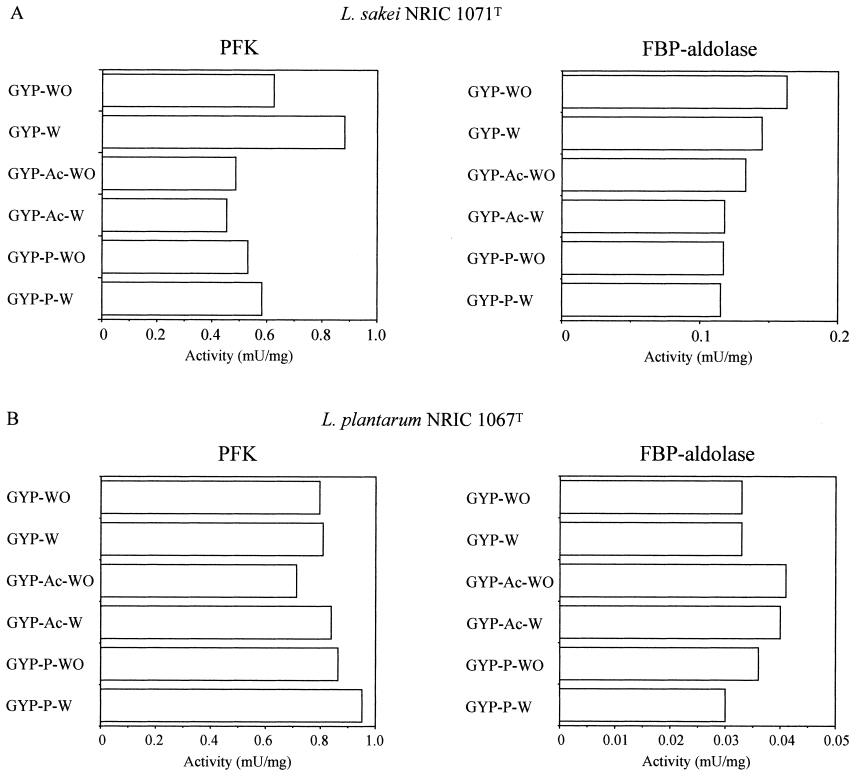

Fig. 9. The effect of sodium acetate on the activity of PFK and FBP-aldolase in the cell-free extracts of the cells from *L. sakei* NRIC 1071^T and *L. plantarum* NRIC 1067^T.

Symbols: GYP, GYP medium; GYP-Ac, GYP medium containing 50 mM sodium acetate; GYP-P, GYP medium containing 50 mM potassium phosphate buffer; WO, without sodium acetate; W, with 50 mM sodium acetate in the reaction mixture.

sodium acetate to the reaction mixture (Fig. 9A). The activity of FBP-aldolase in this cell-free extract did not change with the addition of 5 mM sodium acetate to the reaction mixture (Fig. 9A). Furthermore, the activity of PFK and FBP-aldolase of the cells of *L. sakei* NRIC 1071^T remained mostly unchanged (Fig. 9A), regardless of cultivation in the presence of 50 mM sodium acetate or 50 mM potassium phosphate buffer.

The activity of PFK and FBP-aldolase in the cell-free extract from *L. plantarum* NRIC 1067^T cultivated in the absence of sodium acetate did not change, regardless of the addition of 5 mM sodium acetate to the reaction mixture (Fig. 9B). Furthermore, this finding was recognized for the cells cultivated in the presence of 50 mM sodium acetate and in 50 mM potassium phosphate buffer. Moreover, the activity of PFK from rabbit muscle (Roche Diagnostics GmbH) was not affected by the addition of 5 mM sodium acetate to the reaction mixture (data not shown).

Discussion

The growth and energy yield of lactic acid bacteria are expressed by Y_{ATP} in the case of homofermentatives (Bauchop and Elsden, 1960; Oxenburgh and Snoswell, 1965). Assuming Y_{ATP} to be approximately 10.5, the data obtained in this study indicated that the production of ATP from 1 mol of glucose metabolized was estimated at 1.3 mol in the absence of sodium acetate, 2.0 mol in the presence of 50 mM sodium acetate, and 1.3 mol in the presence of 50 mM potassium phosphate buffer. When *L. sakei* NRIC 1071^T was cultivated in the presence of 50 mM sodium acetate, Y_{ATP} increased 1.6 times compared with that in the absence of sodium acetate. Consequently, *L. sakei* will acquire more ATP in the presence of 50 mM sodium acetate than in the absence of sodium acetate. The growth yield of *L. plantarum* NRIC 1067^T indicated the production of 1.5 mol ATP from 1 mol of glucose metabolized in the absence of sodium acetate, 1.9 mol in the presence of 50 mM sodium acetate, and 1.7 mol in the presence of 50 mM potassium phosphate buffer, respectively. The above-mentioned facts would indicate the favorable influence of sodium acetate on the energy yield of lactic acid bacteria. Furthermore, the pH of the culture medium gradually decreased during the cultivation in the absence of sodium acetate, in the presence of 50 mM sodium acetate, and in the presence of 50 mM potassium phosphate buffer. Thus, sodium acetate is considered to play roles other than a role in buffering.

The resting cells of *L. sakei* NRIC 1071^T, regardless of the cultivation in the absence or the presence of 50 mM sodium acetate, produced almost the same amount of lactic acid from glucose. In addition, the cells cultivated in the above conditions produced two and half times or more the amount of lactic acid from glucose under the addition of 50 mM sodium acetate to the reaction mixture, as compared with the production of lactic acid without the addition of sodium acetate to the reaction mixture. This fact was also found in the case of *L. plantarum* NRIC 1067^T. Consequently, the yet-functioning glycolytic pathway in the cells would be activated by sodium acetate, and this activation suggested the high production of lactic acid from glucose under the addition of sodium acetate in the reaction mixture.

The increase of absorption at 340 nm in the reaction mixture was found in the cell-free extract from *L. sakei* NRIC 1071^T cultivated in the absence of sodium acetate under the addition of sodium acetate to the reaction mixture, as compared with the activity without the addition of sodium acetate. The increase amounted to up to 1.3 times. The increase of absorption was also found for the cells cultivated in the presence of 50 mM sodium acetate and in 50 mM potassium phosphate buffer. The remarkable increase of the absorption in the cell-free extract would show the activation of LDHs by sodium acetate, indicating the high production of lactic acid. Such a phenomenon was also found in the case of *L. plantarum* NRIC 1067^T. Therefore, it would be concluded that sodium acetate further activates LDHs and stabilizes the activity in the cell-free extract of *L. sakei* NRIC 1071^T and *L. plantarum* NRIC 1067^T. It is worthy of mention that sodium acetate did not increase the activity of L-LDH from rabbit muscle or D-LDH from *Lactobacillus leichmannii.*

The activity of L-LDH in the cell-free extract of *L. sakei* NRIC 1071^T cultivated in the presence of 50 mM sodium acetate increased three times or more compared with the activity of the cells cultivated in the absence of sodium acetate, and in 50 mM potassium phosphate buffer. The production of L-LDH in *L. sakei* NRIC 1071^T might be activated by sodium acetate because the activity of this enzyme was almost the same under the addition of 5 mM sodium acetate to the reaction mixture. The activity of D-LDH from *L. sakei* NRIC 1071^T did not show a significant difference one in any

of culture media or with the addition of 5 mM sodium acetate to the reaction mixture. In contrast, L-LDH and D-LDH in the cells of *L. plantarum* NRIC 1067^T were not influenced by sodium acetate. Moreover, the activity of PFK and FBP-aldolase was not affected by sodium acetate. Consequently, the activation and stabilization of L-LDH by sodium acetate would favor the production of L-lactic acid by *L. sakei* NRIC 1071^T.

L. sakei was reported to produce D-lactic acid from L-lactic acid by lactate racemase (Hiyama et al., 1968; Katagiri and Kitahara, 1937; Stetter and Kandler, 1973). However, the participation of lactate racemase has not been clarified yet for the production of D-lactic acid by *L. sakei.* Previously, the DL-formers such as *L. sakei* and *L. plantarum* had been suggested to produce L-LDH or D-LDH and lactate racemase (Katagiri and Kitahara, 1937, 1938; Kitahara and Ôbayashi, 1955; Kitahara et al., 1952). In contrast, *L. plantarum* was reported to have both L-LDH and D-LDH (Dennis and Kaplan, 1960), and the participation of the enzymes in *L. plantarum* has clearly been shown for the production of DL-lactic acid (Hiyama et al., 1965; Mizushima et al., 1964). In addition, the resting cells of *L. sakei* NRIC 1071^T did not produce DL-lactic acid from L-lactic acid or D-lactic acid. Contrary to the study of Hiyama et al. (1968), the cell-free extract of *L. sakei* NRIC 1071^T did not produce DL-lactic acid from L- or D-lactic acid in this study. Consequently, the participation of lactate racemase has not been clarified yet for the production of DL-lactic acid in this strain. Further, the cell-free extract from *L. plantarum* NRIC 1067^T did not produce DL-lactic acid from L- or D-lactic acid. In contrast, the resting cells of *L. plantarum* NRIC 1067^T produced DL-lactic acid from L- or D-lactic acid to almost the same extent, but the mechanism has not been clarified. As reported previously (Iino et al., 2001), a large number of DL-formers of lactic acid bacteria produced L-form and D-form of lactic acid at almost the same ratio (ca. 5 : 5). The production of stereoisomers of lactic acid might be regulated in the cells of the DL-former such as *L. plantarum* NRIC 1067^T.

The resting cells of *L. sakei* NRIC 1071^T cultivated in the absence of sodium acetate produced DL-lactic acid, regardless of the addition of 50 mM sodium acetate to the reaction mixture. In contrast, the cells of *L. sakei* NRIC 1071^T cultivated in the presence of 50 mM sodium acetate produced L-lactic acid, regardless of the addition of 50 mM sodium acetate to the reaction mixture. As mentioned above, this shift of the type of stereoisomers by *L. sakei* would be ascribed to the activation of the activity of L-LDH and the enhancement of the production of L-LDH due to the increase of biomass. Consequently, the change of the ratio of L-lactic acid to D-lactic acid was due to the high production of L-lactic acid and the low production of D-lactic acid. The shift of the stereoisomers of lactic acid appears to be a characteristic of *L. sakei.* In fact, a large number of *L. sakei* strains produced a large amount of L-lactic acid but not D-lactic acid when the strains were cultivated in the presence of sodium acetate (Iino et al., 2001).

The good growth of lactic acid bacteria and the enhancement of the production of L-lactic acid with sodium acetate will be useful for the application of lactic acid bacteria such as probiotics (Ray, 1996).

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