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Purification and Partial Characterization of *Lactobacillus* Species SK007 Lactate Dehydrogenase (LDH) Catalyzing Phenylpyruvic Acid (PPA) Conversion into Phenyllactic Acid (PLA)

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Phenyllactic acid (PLA) is a novel antimicrobial compound synthesized by lactic acid bacteria (LAB), and its production from phenylpyruvic acid (PPA) is an effective approach. In this work, a lactate dehydrogenase (LDH), which catalyzes the reduction of PPA to PLA, has been purified to homogeneity from a cell-free extract of *Lactobacillus* sp. SK007 by precipitation with ammonium sulfate, ion exchange, and gel filtration chromatography. The purified enzyme had a dimeric form with a molecular mass of 78 kDa (size exclusion chromatography) or 39 kDa (SDS-PAGE). The ratio of enzyme activity with PPA to that with pyruvate being almost invariable at every purification step indicated that, in *Lactobacillus* sp. SK007, LDH is responsible for the conversion of PPA into PLA. HPLC profiles of PPA transformation into PLA by growing cells, cell-free extract, and purified LDH of *Lactobacillus* sp. SK007 were also investigated. Results showed that the presence of NADH was found to be necessary for the enzymatic production of PLA from PPA. The purified LDH displayed optimal activity for PPA at pH 6.0 and 40 °C. The K_m values of the enzyme for PPA and pyruvate were 1.69 and 0.32 mM, respectively. Moreover, because other screened LAB strains exhibiting relatively high LDH activity toward PPA produced also considerable amounts of PLA, LDH activity for PPA could be therefore used as a screening marker for PLA-producing LAB.

KEYWORDS: Phenyllactic acid; phenylpyruvic acid; lactic acid bacteria; *Lactobacillus*; lactate dehydrogenase; purification

INTRODUCTION

Foodborne fungi, both yeasts and molds, cause serious spoilage of stored foods. Molds may also produce health-damaging mycotoxins, that is, aflatoxins, trichothecenes, fumonisin, ochratoxin A, and patulin (*1*). Consumer demands for reduced use of chemical preservatives have stimulated research on natural antifungal compounds as biopreservatives. Phenyllactic acid (PLA) is a novel antimicrobial compound (*2*), which has been recently isolated from lactic acid bacteria (LAB) (*3*). PLA had a broad inhibitory activity against a wide range of fungal species isolated from bakery products, flour, and cereals, including some mycotoxigenic species, namely, *Aspergillus ochraceus*, *Penicillium roqueforti*, and *Penicillium citrinum* (*4*). Furthermore, the inhibitory properties of PLA have been demonstrated against both Gram-positive and Gram-negative bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* O157:H7 (*5–7*). Due to its broad inhibitory activity against a variety of foodborne microorganisms, PLA has interesting potential for practical application as an antimicrobial agent in the food industry (*1, 4*).

PLA may be produced by food-related microorganisms, including the fungus *Geotrichum candidum* (*2*), propionibacteria (*8*), and LAB (*1, 3, 7, 9–20*). Lavermicocca and co-workers (*3*) reported the production of PLA by *L. plantarum* 21B used as a starter in sourdough bread, which was the first report showing the production of PLA by LAB (**Table 1**). PLA has also been identified from culture supernatants of several LAB strains, such as *L. plantarum* MiLAB 393 (*9*), *L. coryniformis* Si3, and *L. sakei* (*10*). However, later studies revealed that from the 12 tested species 9 of them included strains that are widely used in the production of fermented foods and able to produce PLA in the range of 0.17–0.57 mM (*11*). Moreover, some commercial probiotic lactobacilli were investigated for their capacity to produce PLA: *L. johnsonii* La1 and *L. acidophilus* IBB 801 produced 0.25 and 0.15 mM PLA, respectively (*15*). In a previous study, 70 of the 112 LAB strains isolated from Chinese traditional pickles could produce relevant amounts of

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Table 1. Review of Some PLA-Producing LAB Strains

strain	source	year	PLA (mM)	ref
L. plantarum 21B[a]	sourdough	2000	0.34	*3*
L. plantarum MiLAB393	grass silage	2002	nr[d]	*9*
L. coryniformis Si3	grass silage	2003	nr	*10*
L. sakei	grass silage	2003	nr	*10*
Enterococcus faecalis	tempeh	2004	nr	*7*
L. plantarum ITM21A	sourdough	2004	0.35	*11*
L. rhamnosus ATCC53103	human origin	2004	0.23	*11*
L. alimentarius ATCC29643	fish products	2004	0.37	*11*
L. fermentum ITM18B	sourdough	2004	0.08	*11*
L. sanfranciscensis IDMC57	sourdough	2004	0.35	*11*
L. acidophilus IDMA2	sourdough	2004	0.20	*11*
L. brevis ATCC14869	human origin	2004	0.46	*11*
Weissella confusa ITM14A	sourdough	2004	0.06	*11*
Enterococcus faecium ATCC882	cheese	2004	0.09	*11*
Leuconostoc mesenteroides subsp. *mesenteroides* ITMY30[b]	olive phylloplane	2004	0.57	*11*
L. citreum ITM22A	sourdough	2004	0.43	*11*
L. plantarum MiLAB 14	grass silage	2005	nr	*1*
L. sanfranciscensis DSM20451^T	ATCC	2006	0.12	*13*
L. plantarum TMW1.468	Technical University of Munich-Weihenstephan	2006	0.50	*13*
L. plantarum VLT01	DIPROVAL collection	2006	0.24	*14*
L. johnsonii La1	LC1, Nestlé	2006	0.25	*15*
L. casei Shirota	Yakult, Yakult Honsha	2006	0.05	*15*
L. rhamnosus GG	Gefilus, Valio	2006	0.10	*15*
L. amylovorus DCE 471	corn steep liquor	2006	0.05	*15*
L. acidophilus IBB 801	dairy product	2006	0.15	*15*
L. plantarum ACA-DC 287	Greek Xynotyri cheese	2006	0.05	*15*
L. plantarum FST 1.7	malted barley	2007	nr	*16*
Lactobacillus sp. SK007[c]	Chinese traditional pickles	2007	0.55	*17*
L. plantarum VLT01	salami	2007	0.28	*18*

[a] *L. plantarum* ITM21B is the first LAB strain reported to produce PLA. [b] The highest level of PLA (0.57 mM) was produced by *L. mesenteroides* subsp. *mesenteroides* ITMY30 grown in MRS broth. [c] PLA production by *Lactobacillus* sp. SK007 used in the present investigation was found to be closer to that of *L. mesenteroides* subsp. *mesenteroides* ITMY30. [d] Not reported.

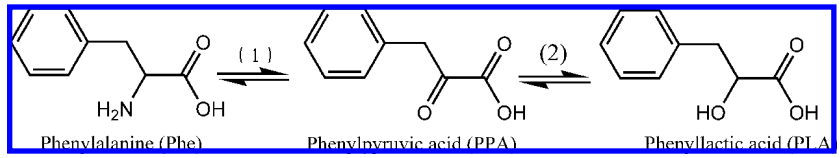

Figure 1. Pathway for PLA production from Phe or PPA by LAB: (1) transamination of Phe to PPA by aminotransferase; (2) reduction of PPA to PLA by dehydrogenase. Transamination (1) is the rate-limited step in PLA production. Modified after refs *4* and *13*.

PLA (*17*). In particular, *Lactobacillus* sp. SK007 produced 0.55 mM PLA, and this amount is similar to that of *L. mesenteroides* subsp. *mesenteroides* ITMY30. Screening of high-PLA-producing LAB strains from different environments has been actively pursued (*18–20*).

Although PLA can be produced by a wide range of *Lactobacillus* species, its production is rather low. PLA was formed by LAB growing in de Man, Rogosa, and Sharpe (MRS) broth in levels up to 0.57 mM (*11, 13*). It has been shown that PLA is a product of phenylalanine (Phe) metabolism (a schematic overview is given in **Figure 1**); in particular, Phe can be transaminated to phenylpyruvic acid (PPA), which is further metabolized into PLA by reduction (*4*). Previously, all studies on PLA production by lactobacilli have employed amino acids as substrates. PLA production by *L. plantarum* ITM21B was improved using increased concentrations of Phe (*11*). Nevertheless, amino acid metabolism by LAB is limited by the availability of amino acceptors in the transamination reaction. Vermeulen and co-workers (*13*) reported that Phe transamination is a limiting factor in PLA production by *L. sanfranciscensis* DSM20451^T and *L. plantarum* TMW1.468. They found also that PLA yields increased from 5 to >30% upon the addition of α-ketoglutarate in *L. plantarum* TMW1.468 culture. In previous research, the rate-limiting step in PLA production by *Lactobacillus* sp. SK007 was also found to be Phe, and this bottleneck could be overcome using PPA instead of Phe as substrate (**Figure 1**), with a 14-fold increase of PLA content (*17*). Traditionally, PPA has been used to produce L-Phe, which is in high demand for the production of the artificial sweetener "aspartame" (*21*). Therefore, compared with Phe, PPA can be obtained easily at a lower price, and PLA production from PPA can be an effective approach. Hence, it is necessary to investigate the enzyme's catalyzing the reduction of PPA to PLA from *Lactobacillus* sp. SK007.

Lactate dehydrogenase (LDH, EC 1.1.1.27) is widely distributed in a variety of sources including LAB, and its biochemical properties have been characterized. It is well-known that LDH catalyzes the reduction of pyruvate to lactate. However, LDH also catalyzes the conversion of PPA into PLA due to its broad substrate specificity. Meister (*22*) first showed the evidence that crystalline beef heart LDH also catalyzes the reduction of PPA to PLA in the presence of NADH. Hummel and co-workers (*23*) described LDH from *L. confusus*, which also converts PPA into PLA for the production of various pharmaceuticals. Although LDH from LAB has been extensively studied for a long time, there have been few reports on the enzyme characterization for the production of PLA. Therefore, the aim of this study was to purify LDH converting PPA into PLA from *Lactobacillus* sp. SK007 and to determine some of

its properties. LDH in some LAB strains and their abilities to produce PLA were also investigated.

MATERIALS AND METHODS

Materials. PPA, PLA, and NADH were purchased from Sigma Chemical Co. (St. Louis, MO). All chromatography columns were obtained from Pharmacia Co. (Amersham Bioscience, Uppsala, Sweden), and electrophoresis reagents were imported from Bio-Rad Laboratories Inc. (Nanjing, China). Other chemicals for enzyme assay and characterization were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Strains and Culture Conditions. *Lactobacillus* sp. SK007 (GenBank accession no. DQ534529), isolated from Chinese traditional pickles, was used in the present investigation. The strain was grown in 1.0 L of MRS broth at 30 °C for 24 h without shaking. The culture was centrifuged at 10000*g* (4 °C) for 20 min, and the cells were collected for further purification. Another nine lactobacilli also isolated from Chinese traditional pickles and maintained in the laboratory were also investigated for their ability to produce PLA.

Enzyme Extraction and Purification. Unless otherwise specified, all purification steps were carried out at 4 °C. Cells harvested were washed twice with sterile 50 mM Tris-HCl buffer (pH 7.1), resuspended in the same buffer, and disrupted by sonication (300 W, pulse on, 1 s; pulse off, 3 s) for 40 min. Cell debris was removed by centrifugation at 10000*g* for 20 min, and the supernatant was used as crude extract.

Purification of LDH catalyzing PPA conversion into PLA was accomplished from cell-free extract by three successive steps. The cell-free extract was subjected to ammonium sulfate precipitation, but the precipitate obtained after saturation up to 60% had no activity for PPA or pyruvate. Hence, the resulting supernatant was saturated up to 100%, and the precipitate was collected by centrifugation at 10000*g* for 20 min, dissolved in a small amount of 50 mM Tris-HCl buffer (pH 7.1), and dialyzed in the same buffer for 24 h with three changes of the buffer during dialysis. The dialyzed enzyme was loaded on a DEAE-Sepharose Fast Flow column (2.6 cm × 30 cm) and equilibrated with 20 mM Tris-HCl buffer (pH 7.1). The absorbed proteins were eluted (1.5 mL/min) with a linear gradient of 0–1.0 M NaCl in the same buffer. Fractions exhibiting activity toward PPA or pyruvate were concentrated and desalted by ultrafiltration. Further purification was carried out using a Sephacryl S-200 HiPrep 16/60 column (1.6 cm × 60 cm, AKTA purifier) previously equilibrated with 20 mM phosphate buffer (pH 6.6) containing 0.15 M NaCl. Proteins were eluted with equilibrating buffer at a flow rate of 0.5 mL/min. The active fraction was pooled and concentrated by ultrafiltration and used as the purified enzyme for further analyses. The purity of enzyme and molecular mass were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue (*24*). Protein concentrations were determined according to the Lowry method (*25*) using bovine serum albumin (BSA) as standard. LDH purity was checked using high-performance liquid chromatography (HPLC) by injecting the enzyme on a Zorbax 300SB RPC18 column (4.6 × 250 mm), via elution with a linear gradient for 20 min using the elution A (100% H_2O, 0.05% TFA) and B (80% acetonitrile, 0.05% TFA) at a flow rate of 1 mL/min.

Enzyme Assay. The activity of LDH toward PPA and pyruvate as substrates was determined by measuring the rate of disappearance of NADH at 340 nm (*22, 23*). The assay mixture contained in 100 mM potassium phosphate buffer (pH 6.5) 0.6 μmol of NADH, 19.6 μmol of PPA or 2.27 μmol of pyruvate, and relevant amounts of enzyme in a total volume of 3.0 mL. The presence of NADH was omitted from the control, and PPA solution was freshly prepared before each use. The enzyme assay was performed at 30 °C, and 1 unit of enzyme activity was defined as the amount of enzyme that catalyzes the degradation of 1 μmol of NADH per minute.

Effects of pH on Enzyme Activity toward PPA. The effect of pH on the activity of purified enzyme toward PPA was measured in 100 mM sodium acetate buffer (pH 3.0–5.0), 100 mM potassium phosphate buffer (pH 6.0–8.0), and 100 mM Tris-HCl buffer (8.0–9.0). The pH stability of LDH was tested by preincubating the enzyme at 30 °C for 60 min in different buffers at the indicated pH values.

Effects of Temperature on Enzyme Activity. For determining the optimum temperature, enzyme activity was measured at different temperatures ranging from 20 to 70 °C. To examine the thermal stability, the enzyme was preincubated at temperatures ranging from 20 to 70 °C for different periods of time before assay at 30 °C as described previously.

Determination of Kinetic Constant of LDH with PPA and Pyruvate. To determine the apparent Michaelis–Menten constant (K_m), enzyme activities using PPA and pyruvate were measured by varying the concentrations of each substrate under optimum conditions of pH and temperature. The K_m value of LDH at each substrate concentration was calculated according to the Lineweaver–Burk method by plotting $1/V$ against 1/[S].

PLA Production from PPA by Growing Cells, Cell-Free Extract, and Purified LDH from *Lactobacillus* Spcies SK007. The production of PLA from PPA by fermentation was carried out according to the methodof Li et al. (*17*). For enzymatic production of PLA, 10 mM PPA and 6 mM NADH were dissolved in 100 mM potassium phosphate buffer (pH 6.5). The reaction was initiated by adding either crude extract or purified enzyme (2 units of activity with PPA). The reaction mixture was maintained at 40 °C for 1 h and then terminated by the addition of 1 M HCl.

Activities of LDH from 10 Isolated LAB Strains and Their Abilities To Produce PLA. The activities of LDH produced by 10 lactobacilli were measured using pyruvate and PPA as substrates in cell-free extract according to the enzyme assay procedure described previously. PLA production by the 10 LAB strains was carried out according to the method of Li et al. (*17*), and PLA content was measured after growth of the strains in MRS broth containing 30.12 mM PPA at 30 °C for 24 h.

HPLC Analysis of PLA and PPA. The product obtained from PPA reduction by growing cells, cell-free extract, and purified LDH was determined using reverse phase HPLC equipped with an Agilent Zorbax SB-C18 column (4.6 mm × 150 mm, 5 μm). Elution was performed with methanol/0.05% TFA (solvent A) and water/0.05% TFA (solvent B) at 1 mL/min and A/B ratios of 10:90, 100:0, 100:0, and 10:90, with run times of 0, 20, 23, and 25 min, respectively. PLA and PPA were analyzed with DAD (Agilent 1100 series) at 210 nm according to the procedure described by Valerio et al. (*11*) with minor modifications.

Statistical Analysis. Analysis of variance (ANOVA) was carried out using SAS software (The SAS System for Windows version 8.1). Experimental data were expressed as the means ± SD of values obtained from triplicate measurements.

RESULTS

Purification of LDH from *Lactobacillus* Species SK007 Catalyzing PPA Conversion into PLA. The cell-free extract of *Lactobacillus* sp. SK007 was subjected to ammonium sulfate precipitation and separated into two fractions: the precipitate obtained after saturation up to 60% and that corresponding to saturation up to 100%. No activity with PPA or pyruvate was detected in the first fraction, whereas >80% of activity was present in the second. After removal of salts by dialysis, the sample was concentrated and then loaded onto a DEAE-Sepharose Fast Flow column. Three different fractions were eluted from the gel matrix, and among these fractions only one showing activity with both PPA and pyruvate could be detected. For further purification, the enzyme was loaded onto a Sephacryl S-200 HiPrep 16/60 column. The elution profile of gel filtration chromatography showed a single sharp peak active toward PPA and pyruvate. Following concentration by ultrafiltration, the fraction reached 57.98 and 516.42 units/mL of activity with PPA and pyruvate, respectively. The purified LDH averaged 19.27% of yield with a 34-fold purification level. The specific activities of purified enzyme with pyruvate and PPA were 203.3 and 22.48

Table 2. Characteristics of LDH from *Lactobacillus* Species SK007 at Different Stages of Purification

step	total activity with PPA[a] (units)	total activity with pyruvate[b] (units)	protein[b] (mg)	specific activity[b] (units/mg)	fold[b]	yield[b] (%)	relative activity[c] (%)
cell-free extract	296.40	2679.95	451.83	5.93	1	100	11.06
ammonium sulfate	237.04	2133.78	125.29	17.03	2.87	79.62	11.11
DEAE-Sepharose Fast Flow	115.96	1073.32	17.98	59.70	10.07	40.05	10.80
AKTA Sephacryl S-200	57.98	516.42	2.54	203.30	34.29	19.27	11.22

[a] Enzyme activity was determined using PPA as substrate. [b] Enzyme activity was determined using pyruvate as substrate. [c] Relative activity is the percentage of activity with PPA to that with pyruvate.

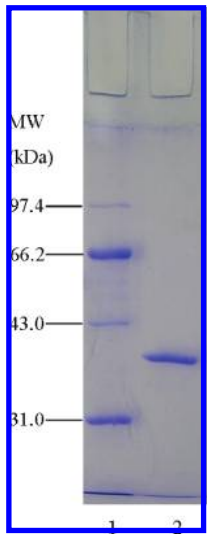

Figure 2. SDS-PAGE of purified LDH by *Lactobacillus* sp. SK007. SDS-PAGE analysis of the enzyme was performed in 12% (w/v) polyacrylamide gel. Lane 1 represents protein makers and lane 2, LDH obtained after purification with AKTA Sephacryl S-200. Rabbit phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa), and hen egg white lysozyme (14.4 kDa) were used as protein markers.

units/mg, respectively. A summary of the LDH purification process is shown in **Table 2**.

Protein purification was successfully achieved to homogeneity as evidenced by a single band corresponding to 39 kDa on SDS-PAGE (**Figure 2**). The apparent molecular mass of native LDH was estimated to 78 kDa by size exclusion chromatography, indicating that LDH produced by *Lactobacillus* sp. SK007 is a dimeric enzyme. The purity of concentrated LDH was determined to be about 98% by HPLC.

Conversion of PPA into PLA by LDH from *Lactobacillus* Species SK007. After purification, only one fraction active toward PPA and pyruvate was isolated from the cell-free extract of *Lactobacillus* sp. SK007. The enzyme activity toward either PPA or pyruvate was determined at each purification step (**Table 2**). The activity of LDH with PPA was always lower than that with pyruvate, but the relative activity (the percentage of enzyme activity with PPA to that with pyruvate) was almost invariable from the crude extract to the purified LDH. Results from **Table 2** show that LDH from *Lactobacillus* sp. SK007 is responsible for the conversion of PPA into PLA by reduction. This finding is in agreement with that of Rijnen (*26*), who reported that LDH from *Lactobacillus lactis* is responsible for PPA reduction to PLA.

HPLC profiles of the transformation of PPA into PLA by growing cells, cell-free extract, and purified LDH from *Lactobacillus* sp. SK007 were also investigated (**Figure 3**). Results showed that growing cells could convert PPA into PLA, as well as crude extract and purified LDH. However, both crude extract and purified LDH required NADH addition as coenzyme to

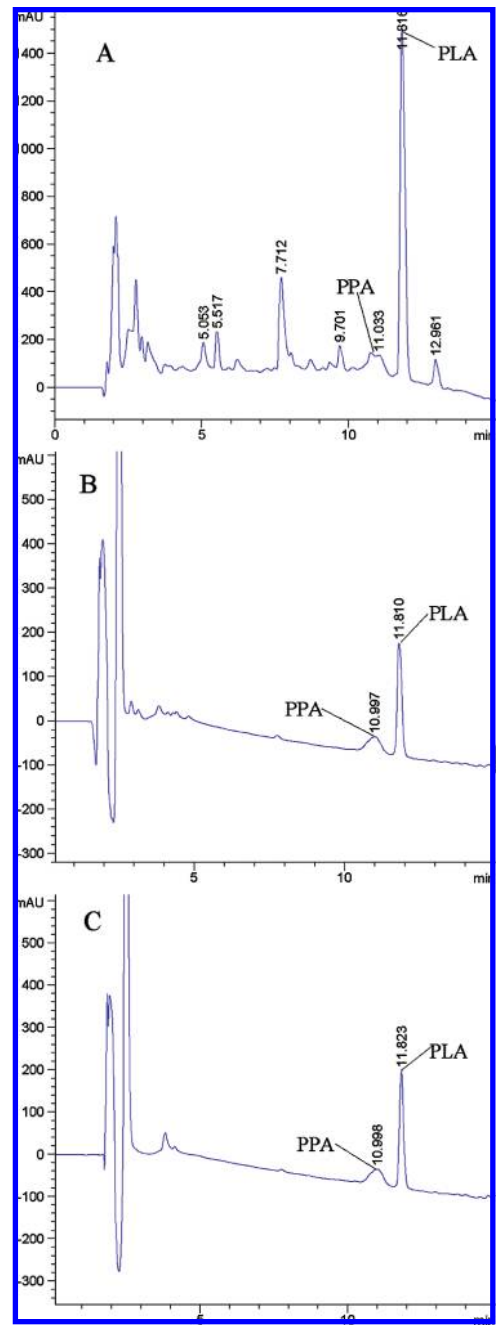

Figure 3. HPLC profiles of PPA conversion into PLA by growing cells, cell-free extract, and purified LDH from *Lactobacillus* sp. SK007: (**A**) conversion of PPA into PLA by growing cells; (**B**) conversion of PPA into PLA by cell-free extract; (**C**) conversion of PPA into PLA by purified LDH. PPA utilization and PLA formation were monitored by HPLC.

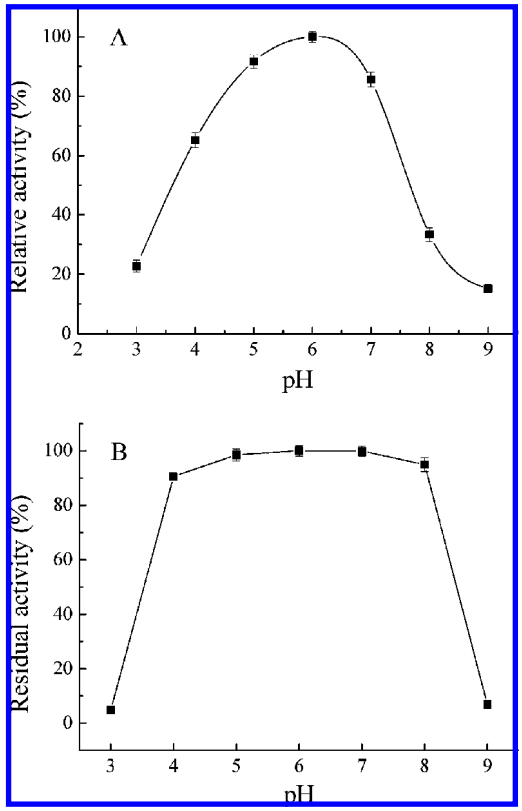

Figure 4. Effects of pH on the activity of LDH from *Lactobacillus* sp. SK007 toward PPA: (**A**) optimal pH of purified LDH; (**B**) pH stability of purified LDH. The enzyme was mixed with various buffers: 100 mM sodium acetate buffer (pH 3.0–5.0), 100 mM potassium phosphate buffer (pH 6.0–8.0), and 100 mM Tris-HCl buffer (8.0–9.0), respectively. The pH stability of LDH was tested by preincubating the enzyme at 30 °C for 60 min in different buffers of respective pH values. Residual activity was expressed as a percentage of the maximum enzyme activity under the assay conditions. Each point represents the mean ($n = 3$) ± standard deviation.

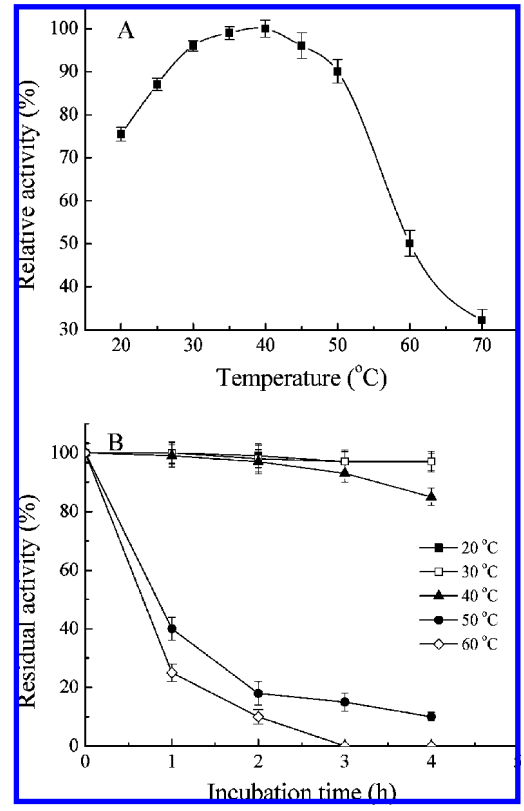

Figure 5. Effects of temperature on the activity of LDH from *Lactobacillus* sp. SK007 toward PPA: (**A**) optimal temperature of purified LDH; (B) thermostability of purified LDH; (■) 20 °C; (□) 30 °C; (▲) 40 °C; (●) 50 °C; (◇) 60 °C. The optimum temperature for the enzyme activity toward PPA was assayed at various temperatures from 20 to 70 °C. Thermostability of the enzyme was determined after incubation of the enzyme in 100 mM potassium phosphate buffer (pH 6.0) at various temperatures for different incubation times. Residual activity was measured, and the relative activity of preincubated sample at 4 °C was regarded as 100%. Each point represents the mean ($n = 3$) ± standard deviation.

initiate the reduction, whereas growing cells did not. Hence, the presence of NADH was found to be necessary for the enzymatic production of PLA from PPA.

Properties of Purified LDH Catalyzing the Conversion of PPA into PLA. *Effects of pH on Enzyme Activity toward PPA.* LDH from *Lactobacillus* sp. SK007 showed an optimum activity at pH 6.0 when PPA was used as substrate (**Figure 4A**) and was found to be virtually inactive below pH 3.0 or above pH 9.0. The effect of pH on LDH stability was studied in the pH range of 3.0–9.0. The enzyme was rather stable from pH 4.0 to 8.0, but a rapid decline in activity was observed below pH 4.0 or above pH 8.0 (**Figure 4B**).

Effects of Temperature on LDH Activity toward PPA. The temperature profile of purified LDH from *Lactobacillus* sp. SK007 was determined from 20 to 70 °C with PPA as substrate. The purified LDH displayed an optimum temperature at 40 °C; the enzyme retained 90% of its relative activity at 50 °C and 50% at 60 °C (**Figure 5A**). The purified LDH lost no activity following exposure at temperatures up to 40 °C for 2 h, but only 20% of initial activity could be retained when the enzyme was exposed at 50 °C for 2 h (**Figure 5B**).

Kinetic Parameters. Using PPA and pyruvate as substrates, the kinetic parameters of purified LDH from *Lactobacillus* sp. SK007 were investigated. The apparent K_m values of enzyme with PPA and pyruvate were 1.69 and 0.32 mM, respectively. K_m values of LDH from other *Lactobacillus* species with either PPA or pyruvate have been compared with that of LDH from *Lactobacillus* sp. SK007 in **Table 3**. Regardless of the strain, LDH affinity for PPA is lower than that for pyruvate. Moreover, the K_m value of LDH toward PPA was found to vary from 0.8 to 20 mM among lactobacilli (*23, 27–29*). For all strains, LDH from *Lactobacillus* sp. SK007 showed a relatively high affinity for PPA.

LDH Activity toward PPA as a Screening Marker for PLA-Producing LAB. In a previous study, 10 lactobacilli, including *Lactobacillus* sp. SK007, isolated from Chinese traditional pickles could produce up to 0.55 mM PLA from Phe in MRS broth (*17*). In this study, activities of LDH from the 10 isolated strains and their abilities to produce PLA were investigated. As shown in **Table 4**, LDH from all tested strains showed activity toward pyruvate and PPA, and the relative activity (the percentage of enzyme activity with PPA to that with pyruvate) varied from 1.08 to 12.51%. With regard to PLA production, strains SK005 and SK008 showed the highest LDH activities toward pyruvate but did not provide also the highest amounts of PLA. In contrast, strains SK006 and SK007 having low LDH activities toward pyruvate displayed the highest yields of PLA. Obviously, strains SK002 and SK004 exhibiting the lowest LDH activities toward pyruvate produced also the lowest quantities of PLA. The correlation between LDH activity with

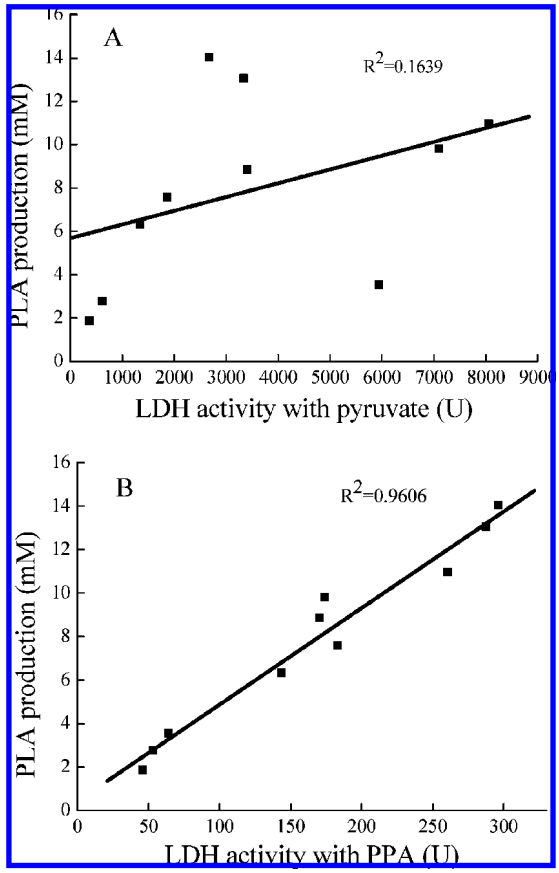

Figure 6. Correlation between LDH activities with PPA or pyruvate and PLA production: (**A**) correlation between LDH activity with pyruvate and PLA production; (**B**) correlation between LDH activity with PPA and PLA production. The activities of LDH produced by 10 isolated lactobacilli were measured using pyruvate or PPA as substrates in cell-free extract. PLA content was measured after the strains had been grown in MRS broth containing 30.12 mM PPA at 30 °C for 24 h.

Table 3. LDH Produced by Some LAB Species: Apparent K_m with PPA or Pyruvate

strain	PPA (mM)	pyruvate (mM)	ref
Lactobacillus sp. SK007	1.69	0.32	this work
L. pentosus	0.8	0.12	*27*
L. pentosus	15	1.8	*28*
L. plantarum	20	1.2	*29*
L. confusus	3.0	0.68	*23*

PPA and PLA production is represented by a linear regression ($R^2 = 0.9606$) in **Figure 6B**. These results demonstrated that lactobacilli possessing high LDH activities toward PPA are able to produce also high amounts of PLA. However, as shown in **Figure 6A**, *Lactobacillus* species with high LDH activities toward pyruvate are not necessarily high PLA producers ($R^2 = 0.1639$). *Lactobacillus* sp. SK007, the LDH activity of which with pyruvate and relative activity were 2679.95 units and 11.06%, respectively, produced 14.04 mM PLA in MRS broth containing 30.12 mM PPA. To our knowledge, this is the highest PLA production by LAB strains reported so far. Therefore, LDH activity may be used as a screening marker for PLA-producing LAB.

DISCUSSION

In this study, LDH from *Lactobacillus* sp. SK007, which catalyzes the reduction of PPA to PLA, was purified and characterized. Results from both SDS-PAGE and HPLC indicated that the enzyme has been purified to homogeneity. The native enzyme was found to have a molecular mass of 78 kDa and to consist of two identical subunits of 39 kDa. In general, monomeric molecular mass for LDH produced by LAB species ranges from 32 to 40 kDa (*30*), and the LDH molecule is supposed to be either a tetramer or a dimer. The molecular mass and the dimeric structure of LDH from *Lactobacillus* sp. SK007 agree with previous findings (*31*).

In *Lactobacillus* sp. SK007, LDH is responsible for the synthesis of PLA from PPA, and this is in agreement with a recent study on *L. lactis* (*26*). Previously, in addition to LDH, Schütte and co-workers (*32*) isolated the so-called L-2-hydroxyisocaproate dehydrogenase (L-HicDH) from *L. confuses*, which catalyzes the stereospecific reduction of straight- and branched-chain aliphatic 2-keto-carboxylic acids including PPA to the corresponding L-2-hydroxycarboxylic acids. Furthermore, the authors found that L-HicDH activity amounted to only 2% of LDH activity when PPA was used as a substrate for both enzymes in *L. confuses* crude extract. L-HicDH activity was measured using the method described by Schütte et al. (*32*). However, in the present study, L-HicDH activity could not be detected in the cell-free extract of *Lactobacillus* sp. SK007 (data not shown). This may be due to the fact that the enzyme is strain-dependent or depends on the strain's nature. Considering the predominance of LDH in LAB strains, it is evident that LDH is mainly responsible for the production of PLA, despite the presence of L-HicDH.

LDH is a NADH-dependent oxidoreductase and requires pyridine nucleotide cofactors for catalysis. In this work, it was shown that NADH is essential for the enzymatic production of PLA from PPA. The high cost of pyridine cofactors, however, necessitates in situ cofactor regeneration for preparative applications. At present, the most profitable methods are the use

Table 4. Activities of LDH Produced by 10 Isolated LAB Strains and Their Abilities To Produce PLA from PPA

LAB strain	species	activity with pyruvate[a] (units)	activity with PPA[a] (units)	relative activity[b] (%)	PLA[a] (mM)
SK007	*Lactobacillus* sp.	2679.95 ± 67.23	296.40 ± 4.80	11.06	14.04 ± 0.42
SK001	*L. plantarum*	3406.80 ± 80.92	170.34 ± 3.37	5.00	8.86 ± 0.33
SK002	*L. plantarum*	620.05 ± 7.29	53.20 ± 0.81	8.58	2.78 ± 0.19
SK003	*L. plantarum*	1344.00 ± 15.12	143.54 ± 1.63	10.68	6.33 ± 0.35
SK004	*L. plantarum*	367.71 ± 14.78	46.00 ± 1.85	12.51	1.87 ± 0.09
SK005	*L. plantarum*	8061.31 ± 127.62	260.38 ± 5.40	3.23	10.97 ± 0.53
SK006	*L. pentosus*	3336.78 ± 94.90	287.63 ± 7.12	8.62	13.06 ± 0.47
SK008	*L. pentosus*	7100.82 ± 128.03	173.97 ± 3.67	2.45	9.82 ± 0.26
SK009	*L. pentosus*	5938.89 ± 147.69	64.14 ± 1.47	1.08	3.55 ± 0.32
SK010	*L. pentosus*	1864.56 ± 76.46	183.10 ± 7.38	9.82	7.59 ± 0.48

[a] Data were expressed as the mean ± SD from triplicate experiments. [b] Relative activity is the percentage of enzyme activity with PPA to that with pyruvate.

of formate dehydrogenase for NADH regeneration (*33*). Recently, PLA production using resting cells was reported to be effective with glucose addition as cosubstrate (*17*). Glucose and glucose dehydrogenase may be applied for NADH regeneration in PLA production by enzyme. Further studies are needed to develop NADH regeneration methods suitable for the enzymatic production of PLA.

PLA is an effective marker of LAB antifungal action, and it is therefore becoming an important selection criterion for LAB (*11, 14*). Previously, very few LAB strains had been shown to produce PLA, but recently, 22 of the 29 tested strains were found to be involved in the production of such metabolites that contribute to food quality preservation along with the sensorial characteristics of fermented products (*11*). PLA production by many LAB strains may be attributed to LDH catalyzing the reduction of pyruvate to lactate, which could convert PPA to PLA, although only a few amounts of PPA are generated from Phe after transamination (**Figure 1**).

Although LDH is widely distributed in LAB, PLA-producing ability was found to vary among the LAB strains tested in this study. In fact, LAB strains producing LDH with high activity toward pyruvate are not necessarily high PLA producers. After transamination, PPA reduction was found to be the second bottleneck in PLA production. LDH has long been recognized in LAB, but its activity toward PPA and pyruvate is different depending on the strain. LDH from *Lactobacillus helveticus* CNRZ 32 had higher activity with pyruvate, but no activity was detected with PPA (*30*). In contrast, LDH from *L. plantarum* had a high activity with PPA (*29*). In this study, a positive correlation between LDH activity toward PPA in 10 isolated LAB strains and their abilities to produce PLA from PPA were demonstrated. In fact, strains possessing high LDH activities toward PPA produced also high amounts of PLA. Therefore, high production of PLA may be achieved by screening a relevant number of LAB strains displaying high LDH activity with PPA.

PLA, being a novel antimicrobial compound produced by LAB, represents a promising natural substance for controlling contaminants in food systems (*1, 4*). On the other hand, the use of functional starter cultures in the food fermentation industry is being explored (*34*). Specifically, LAB strains, being regarded as functional starter cultures, are able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, useful enzymes, or nutraceuticals (*35*). From this point of view, PLA-producing LAB may be applied as functional starter cultures for food preservation due to their broad inhibitory activity against a variety of foodborne microorganisms (*35, 36*). For instance, *L. plantarum* ITM21B, which produced 0.34 mM PLA in MRS medium, when used as a sourdough starter, has been shown to delay the growth of *Aspergillus niger*and *Pennicillium roqueforti* for up to 7 days and to significantly prolong the shelf life of bread (*3*). *Lactobacillus* sp. SK007, which was isolated from Chinese traditional pickles, showed 99% similarity with *L. plantarum* by 16S rDNA sequence (*17*) and produced 14.04 mM PLA in MRS broth containing PPA. These properties provide the possibility of utilizing *Lactobacillus* sp. SK007 as a functional starter culture in the production of some fermented foods. However, further studies are needed to ascertain the contribution of this strain to food quality and preservation.

ABBREVIATIONS USED

PLA, phenyllactic acid; LAB, lactic acid bacteria; PPA, phenylpyruvic acid; Phe, phenylalanine; MRS, de Man, Rogosa, and Sharpe; LDH, lactate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography.

LITERATURE CITED

(1) Schnürer, J.; Magnusson, J. Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci. Technol.* **2005**, *16*, 70–78.

(2) Dieuleveux, V.; Van Der Pyl, D.; Chataud, J.; Gueguen, M. Purification and characterization of anti-*Listeria* compounds produced by *Geotrichum candidum*. *Appl. Environ. Microbiol.* **1998**, *64*, 800–803.

(3) Lavermicocca, P.; Valerio, F.; Evidente, A.; Lazzaroni, S.; Corsetti, A.; Gobbetti, M. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Appl. Environ. Microbiol.* **2000**, *66*, 4084–4090.

(4) Lavermicocca, P.; Valerio, F.; Visconti, A. Antifungal activity of phenyllactic acid against molds isolated from bakery products. *Appl. Environ. Microbiol.* **2003**, *69*, 634–640.

(5) Dieuleveux, V.; Lemarinier, S.; Gueguen, M. Antimicrobial spectrum and target site of D-3-phenyllactic acid. *Int. J. Food Microbiol.* **1998**, *40*, 177–183.

(6) Dieuleveux, V.; Gueguen, M. Antimicrobial effects of D-3-phenyllactic acid on *Listeria monocytogenes* in TSB-YE medium, milk, and cheese. *J. Food Prot.* **1998**, *61*, 1281–1285.

(7) Ohhira, I.; Kuwaki, S.; Morita, H.; Suzuki, T.; Tomita, S.; Hisamatsu, S.; Sonoki, S.; Shinoda, S. Identification of 3-phenyllactic acid as a possible antibacterial substance produced by *Enterococcus faecalis* TH10. *Biocontrol Sci.* **2004**, *9*, 77–81.

(8) Thierry, A.; Maillard, M. B. Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii*. *Lait* **2002**, *82*, 17–32.

(9) Ström, K.; Sjögren, J.; Broberg, A.; Schnürer, J. Lactobacillus plantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.* **2002**, *68*, 4322–4327.

(10) Magnusson, J.; Ström, K.; Roos, S.; Sjögren, J.; Schnürer, J. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiol. Lett.* **2003**, *219*, 129–135.

(11) Valerio, F.; Lavermicocca, P.; Pascale, M.; Visconti, A. Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. *FEMS Microbiol. Lett.* **2004**, *233*, 289–295.

(12) Ström, K.; Schnürer, J.; Melin, P. Co-cultivation of antifungal *Lactobacillus plantarum* MiLAB 393 and *Aspergillus nidulans*, evaluation of effects on fungal growth and protein expression. *FEMS Microbiol. Lett.* **2005**, *246*, 119–124.

(13) Vermeulen, N.; Ganzle, M. G.; Vogel, R. F. Influence of peptide supply and cosubstrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451^T and *Lactobacillus plantarum* TMW1.468. *J. Agric. Food Chem.* **2006**, *54*, 3832–3839.

(14) Armaforte, E.; Carri, S.; Ferri, G.; Caboni, M. F. High-performance liquid chromatography determination of phenyllactic acid in MRS broth. *J. Chromatogr., A* **2006**, *27*, 281–284.

(15) Makras, L.; Triantafyllou, V.; Fayol-Messaoudi, D.; Adriany, T.; Zoumpopoulou, G.; Tsakalidou, E.; Servin, A.; De Vuyst, L. Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Res. Microbiol.* **2006**, *157*, 241–247.

(16) Dal Bello, F.; Clarke, C. I.; Ryan, L. A. M.; Ulmer, H.; Schober, T. J.; Ström, K.; Sjögren, J.; van Sinderen, D.; Arendt, E. K. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. *J. Cereal Sci.* **2007**, *45*, 309–318.

(17) Li, X.; Bo, J.; Pan, B. Biotransformation of phenylpyruvic acid to phenyllactic acid by growing and resting cells of a *Lactobacillus* sp. *Biotechnol. Lett* **2007**, *29*, 593–597.

(18) Coloretti, F.; Carri, S.; Armaforte, E.; Chiavari, C.; Grazia, L.; Zambonelli, C. Antifungal activity of lactobacilli isolated from salami. *FEMS Microbiol. Lett.* **2007**, *271*, 245–250.

(19) Van der Meulen, R.; Scheirlinck, I.; Van Schoor, A.; Huys, G.; Vancanneyt, M.; Vandamme, P.; De Vuyst, L. Population dynamics and metabolite target analysis of lactic acid bacteria during laboratory fermentations of wheat and spelt sourdoughs. *Appl. Environ. Microbiol.* **2007**, *73*, 4741–4750.

(20) Broberg, A.; Jacobsson, K.; Ström, K.; Schnürer, J. Metabolite profiles of lactic acid bacteria in grass silage. *Appl. Environ. Microbiol.* **2007**, *73*, 5547–5552.

(21) Matsunaga, T.; Higashijima, M.; Sulaswatty, A.; Nishimura, S.; Kitamura, T.; Tsuji, M.; Kawaguchi, T. Repeated batch production of L-phenylalanine from phenylpyruvate and NH_4Cl by immobilized cells of *Nocardia opaca* under hydrogen high pressure. *Biotechnol. Bioeng.* **1987**, *31*, 834–840.

(22) Meister, A. Reduction of α,γ-diketo and α-keto acids catalyzed by muscle preparations and by crystalline lactic dehydrogenase. *J. Biol. Chem.* **1950**, *184*, 117–129.

(23) Hummel, W.; Schütte, H.; Kula, M. R. Large scale production of D-lactate dehydrogenase for the stereospecific reduction of pyruvate and phenylpyruvate. *Eur. J. Appl. Microbiol. Biotechnol.* **1983**, *18*, 75–85.

(24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(25) Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(26) Rijnen, L.; Luo, D.; Chambellon, E.; Gitton, C.; Bolotin, A.; Yvon, M. Rôle des hydroxy acide déshydrogenases potentielles de *Lactococcus lactis* dans le catabolisme des acide aminés. In *12ème Réunion du Club des Bactéries Lactiques*; Aurillac, France, 2003; pp 15.

(27) Tokuda, C.; Ishikura, Y.; Shigematsu, M.; Mutoh, H.; Tsuzuki, S.; Nakahira, Y.; Tamura, Y.; Shinoda, T.; Arai, K.; Takahashi, O.; Taguchi, H. Conversion of *Lactobacillus pentosus* D-lactate dehydrogenase to a D-hydroxyisocaproate dehydrogenase through a single amino acid replacement. *J. Bacteriol.* **2003**, *185*, 5023–5026.

(28) Arai, K.; Kamata, T.; Uchikoba, H.; Fushinobu, S.; Matsuzawa, H.; Taguchi, H. Some *Lactobacillus* L-lactate dehydrogenases exhibit comparable catalytic activities for pyruvate and oxaloacetate. *J. Bacteriol.* **2001**, *183*, 397–400.

(29) Taguchi, H.; Ohta, T. D-Lactate dehydrogenase is a member of the D-isomer-specific 2-hydroxyacid dehydrogenase family. Cloning, sequencing, and expression in *Escherichia coli* of the D-lactate dehydrogenase gene of *Lactobacillus plantarum*. *J. Biol. Chem.* **1991**, *266*, 12588–12594.

(30) Bhowmik, T.; Lueck, M.; Steele, J. L. Purification and partial characterization of D-(−)-lactate dehydrogenase from *Lactobacillus helveticus* CNRZ 32. *J. Ind. Microbiol. Biotechnol.* **1993**, *12*, 35–41.

(31) Gasser, F.; Doudoroff, M.; Contopoulos, R. Purification and properties of NAD-dependent lactic dehydrogenases of different species of *Lactobacillus*. *J. Gen. Microbiol.* **1970**, *62*, 241–250.

(32) Schütte, H.; Hummel, W.; Kula, M. R. L-2-Hydroxyisocaproate dehydrogenase—a new enzyme from *Lactobacillus confusus* for the stereospecific reduction of 2-ketocarboxylic acids. *Appl. Microbiol. Biotechnol.* **1984**, *19*, 167–176.

(33) van der Donk, W. A.; Zhao, H. Recent developments in pyridine nucleotide regeneration. *Curr. Opin. Biotechnol.* **2003**, *14*, 421–426.

(34) De Vuyst, L. Technology aspects related to the application of functional starter cultures. *Food Technol. Biotechnol.* **2000**, *38*, 105–112.

(35) Leroy, F.; De Vuyst, L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* **2004**, *15*, 67–78.

(36) Leroy, F.; Verluyten, J.; De Vuyst, L. Functional meat starter cultures for improved sausage fermentation. *Int. J. Food Microbiol.* **2006**, *106*, 270–285.

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