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Unique Properties of Four Lactobacilli in Amino Acid Production and Symbiotic Mixed Culture for Lactic Acid Biosynthesis

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Abstract. With four Lactobacilli—*L. delbrueckii* subsp. *lactis* (ATCC 12315), *L. casei* (NRRL-B1445), *L. delbrueckii* (NRRL-B445), and *L. heveticus* (NRRL-B1937)—the characteristics of cell growth and production of lactate and amino acids were investigated. Especially, the time-course variation in concentration of amino acids (classified into alanine, serine, aspartate, glutamate, aromatic amino acid, and histidine families) was estimated in detail, and the results were systematically compared. It was elucidated that *L. delbrueckii* (NRRL-B445) and *L. helveticus* (NRRL-B1937) had quite different characteristics in growth, lactic acid synthesis, and amino acid production. *L. helveticus* (NRRL-B1937) was superior in the production of amino acids as well as in cell growth, but showed very poor ability in lactic acid production. However, *L. delbrueckii* (NRRL-B445) showed higher yield of lactic acid despite repressed cell growth, but suffered from severe amino acid deficiency in culture. By modulating the initial concentration of each strain in the mixed culture containing both *L. delbrueckii* (NRRL-B445) and *L. helveticus* (NRRL-B1937), the lactic acid production (i.e., the amount of lactic acid produced and lactic acid yield to glucose consumed) was significantly improved, presumably via symbiotic interaction between the two strains.

Lactic acid fermentation has recently received much attention owing to numerous needs for lactic acid in the industry of manufacturing degradable polylactic acid plastics and coatings [8]. Up to date, about 50% of lactic acid has been produced by microbial fermentation, the remainder having been manufactured by chemical synthesis [25]. The lactic acid bacteria are facultative anaerobes that are nutritionally fastidious [13, 19]. Moreover, the lactic acid bacteria generally have limited biosynthetic ability, requiring additional amino acids such as isoleucine, leucine, valine, histidine, methionine, and vitamins for growth [4, 28]. The amino acid requirements and the ability to translocate or intracellularly cleave the oligopeptides may vary among Lactobacillus strains [4, 13, 19, 24]. Furthermore, some lactic acid bacteria do not synthesize essential amino acids and require an exogenous nitrogen source and utilize peptides and proteins from the growth medium by means of more or less complete proteolytic enzyme systems.

Mixed or co-culture systems have been recognized to be effective for certain fermentations. Mixed cultures of lactic acid bacteria are currently used in the dairy industry for manufacturing cheeses and fermented milks. The existence of symbiotic relationships between two bacteria has been clearly demonstrated [2, 7, 15, 18]. Those co-culture systems were attained by cultivating two different strains in a bioreactor with various initial concentration ratios between the two strains having different growth and acidification characteristics [3].

In the present study, we investigated the characteristics of four Lactobacillus strains in cell growth and production of lactate and amino acids. Especially, emphasis was placed on the time-course analysis of amino acid concentration in the culture broth of each Lactobacillus. On the basis of unique properties in amino acid production, two different strains were selected, and various mixed cultures were constituted by varying the

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initial concentration of each strain. The efficacy of the symbiotic bioconsortium culture was clearly demonstrated in improving lactic acid production as well as overcoming nutritional limitations in the culture.

Materials and Methods

Microorganisms. Four Lactobacillus strains were used in this study: *L. delbrueckii* subsp. *lactis* (ATCC 12315), *L. casei* (NRRL-B1445), *L. delbrueckii* (NRRL-B445), and *L. helveticus* (NRRL-B1937). For convenience's sake, the above four bacterial strains were called strains 12315, B1445, B445, and B1937, respectively, in this paper. The strains were stored at −80°C in de Man-Rogosa-Sharpe (MRS) medium [5] supplemented with glycerol (20%).

Media and culture conditions. The bacteria were grown under anaerobic conditions in the MRS broth containing glucose for 48 h at 42°C. The medium composition per liter was as follows: (a) peptone 10 g, beef extract 10 g, yeast extract 5 g, diammonium citrate 3 g, sodium acetate 5 g, Tween 1 g, K_2HPO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $MnSO_4 \cdot 4H_2O$ 0.2 g; and (b) glucose 15 or 20 g. Components (a) and (b) were autoclaved separately and aseptically mixed together before starting the cultivation. Pure or mixed culture experiments were conducted by using a 500-ml Erlenmeyer flask containing 100 ml of the medium above (150 rpm, pH 6.6, 42°C). Inoculum volume of each bacterial strain was 1 ml, and in the case of mixed cultures, various inoculum volumes were used for each strain: 0.1, 0.2, 0.3, 0.4, 0.5, or 1 ml where applicable. Each flask experiment was repeated three times for the accuracy of data analysis.

Analysis of amino acid concentration. Time-course culture samples were filtered onto a membrane (0.45 μm, GS; Millipore), and the filtrate was collected for amino acid analysis. The protein in the filtrate was removed by precipitation with trichloroacetic acid (TCA) (0.5 M final concentration) and then centrifuged at 5,000 *g* for 15 min. The supernatant was filtered on millipore membranes (0.45 μm) and subjected to a derivatization reaction for HPLC analysis with an ACCQ-Tag derivatization kit (Waters Corp., Milford, MA). The analysis results for standard amino acids and MRS medium used were presented in Fig.1A and 1B, respectively. The peak of each amino acid from the culture broth was integrated separately, and the concentration was determined by a standard calibration curve prepared with standard amino acids, the concentration of which ranged from 50 to 100 pM.

Other analytical methods. Bacterial growth was monitored by spectrophotometric measurement at 560 nm. The concentration of glucose was measured enzymatically with a glucose assay kit (Sigma, USA). Lactic acid was analyzed by HPLC equipped with an RI detector (Waters, USA). The column used was an Aminex HPX-87H (Bio-Rad Co., USA) operated at 50°C, and the flow rate was 0.6 ml min^{-1}.

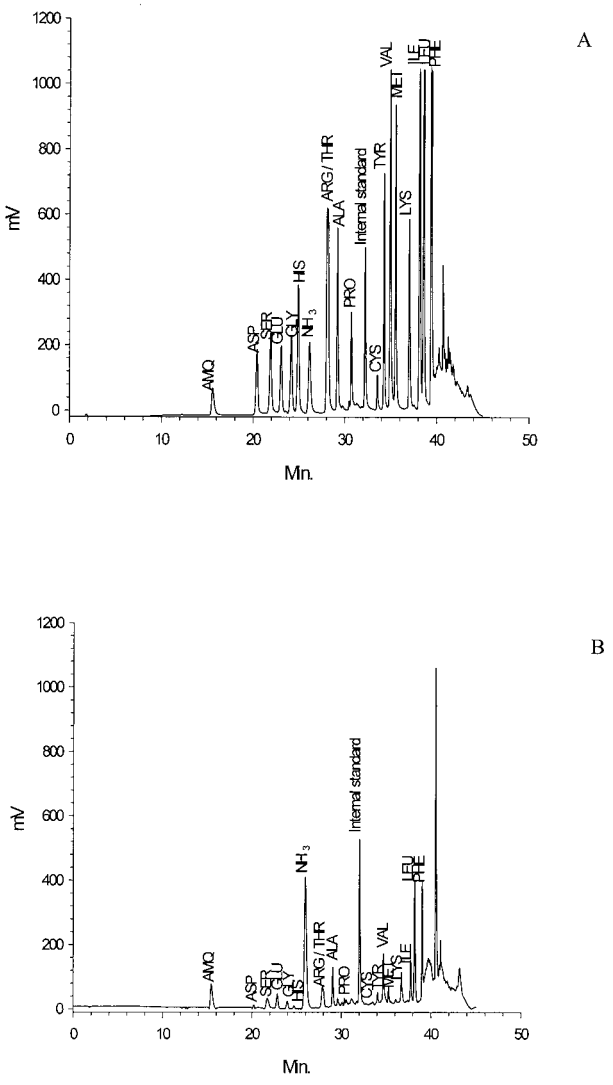

Fig. 1. High-pressure liquid chromatograms of the standard amino acids (A) and amino acids in MRS broth (B). (In Fig. 1A, the concentrations of standard cysteine and the other amino acids are 50 and 100 pmol μl^{-1}, respectively, and in Fig. 1B, the amino acids in MRS medium were analyzed after ×200 dilution of the medium.)

Results

Growth, lactic acid synthesis, and amino acid utilization/production of individual Lactobacillus strain. Every strain showed similar exponential growth for the first 8 h, and afterwards cell growth was significantly differentiated (Fig. 2A). After the first 8-h cultivation, the strains 12315 and B445 entered a typical stationary phase despite the high concentration of residual glucose (≈ 10 g L^{-1}), whereas the B1937 and B1445 strains showed further cell growth with a decreased growth rate. Among the Lactobacilli used, strain B1937 reached the highest culture turbidity (OD_{560}) (≈28) in the course of cultivation and also showed the highest growth yield per glucose consumed (Fig. 2B).

Lactic acid concentration and yield to glucose in the culture of each strain were estimated and compared (Fig. 2C). Contrary to the high growth yield, strain B1937 produced the lowest amount of lactic acid in the culture. The lactic acid yields to glucose of strains 12315, B1445, and B445 were all comparable, 71.4%, 68.8%, and 70.5%, respectively, while the lactic acid yield of strain B1937 was far lower, 41.7%.

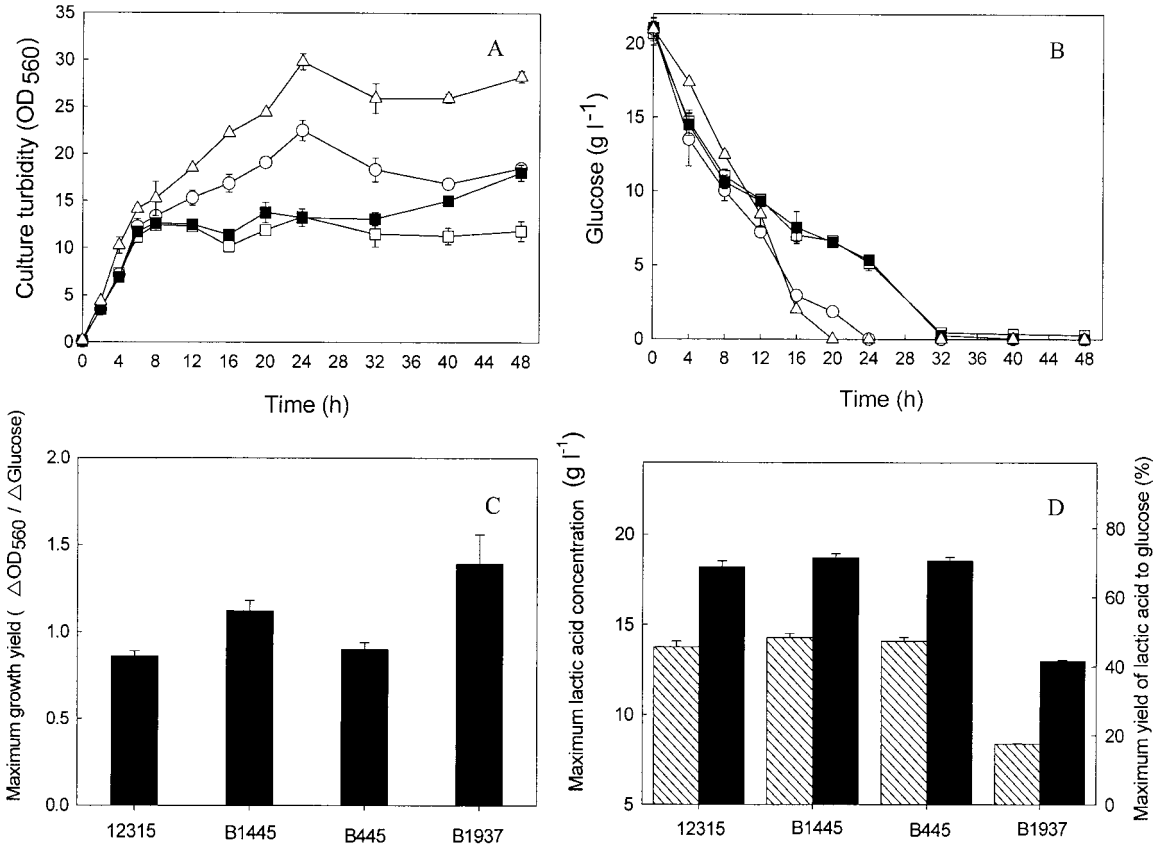

Fig. 2. Time-course variation in culture turbidity (OD_{560}) (A), glucose concentration (B), maximum growth yield to glucose (C), and lactic acid concentration and yield to glucose (D) in the cultures of Lactobacillus strains used. (In Fig. 2A, symbols △, ○, ■, and □ represent strains, B1937, B1445, B445, and 12315, respectively. In Fig. 2D, the slash and solid rectangles represent maximum lactic acid concentration and maximum yield of lactic acid to glucose, respectively.)

From the results in Tables 1A and 1B, the time-course variation of amino acid concentration was on the same trend in the cultures of strains 12315 and B1445. Namely, compared with the first 16 h, the period from the 20^{th} to the 48^{th} h seemed to be such a phase that the production rate of amino acids seemed to be much higher than the utilization rate of amino acids except for cysteine and tyrosine (Tables 1A and 1B). In the culture of strain B445, all amino acids except for alanine were intensively consumed all the way during the cultivation (Table 1C), whereas strain B1937 overproduced most of the amino acids (except for five amino acids, serine, arginine, threonine, cysteine, and tyrosine) from the early stage of growth (Table 1D). It seems to be interesting that in the growth of strain B1937, the quite redundant amount of many amino acids exceeding metabolic need was produced during the whole culture period. The marked difference between strains B445 and B1937 in the time-course production and utilization of amino acids is much better illustrated in Fig. 3. It is worthy of note that strain B1937 looks like an efficient amino acid producer that could well compensate for the amino acid deficiency of strain B445, leading probably to poor growth of the strain.

Improved lactic acid production by symbiotic mixed culture. On the basis of the earlier findings in the individual Lactobacillus culture (Tables 1A–1D, Figs. 2A–2C), strains B445 and B1937 showed quite opposite characteristics in cell growth, lactic acid synthesis, and extracellular amino acid production. Strain B1937 was superior in the production of amino acids as well as in cell growth, but showed very poor ability in lactic acid production. Strain B445 showed a higher yield of lactic acid to glucose, but a low biomass yield and poor ability in amino acid production. The diminished cell growth of the efficient lactic acid producer, strain B445, was due presumably to the lack of amino acid supplement in the culture broth. Hence it is suggested that co-culturing strain B445 with strain B1937 as an amino acid supplier could enhance the growth yield of strain B445 and hence the overall lactic acid production. For the mixed culture

Table 1. Time-course variation of each amino acid concentration in the culture of Lactobacilli. (Considering the initial concentration of amino acids in fresh MRS medium as 100%, symbols in Table 1 represent the relative percentage of amino acid concentration in the culture broth with the following definition: No sign, 90–110%; +, 110–130%; ++, 130–150%; +++, 150–200%; ++++, >200%; −, 90–80%; −−, 80–70%; −−−, 70–50%; −−−−, <50%.)

	Hours after inoculation								
amino acids	4	8	12	16	20	24	32	40	48
1A. *L. delbrueckii* subsp. *lactis* (ATCC 12315)									
Alanine family									
alanine	+	−			+	++		+	
valine	+	−−−			+			+++	
leucine	+	−−−			+	++++			
Serine family									
serine		+	+		+			+++	
cystine	−−	−−−−	−−	−−	−−−		−−−	−−−	−−−
Aspartate family									
aspartic acid	+		+	+	+	+++	+	++++	+
methionine	++	−−−	+	+	+			−	
threonine	−−−	−	−−−	−−−	−−−		−	+++	−−−
isoleucine	+	−−		+	+	+		+++	
lysine		−−−				−−	−		
Glutamate family									
glutamic acid		−−−		+		+++		+++	
arginine	−−−	−	−−−	−−−	−−−		−	+++	−−−
proline	−−−−	−−−−	−−−−	−−−−	−−−−	++++	++++	−−−−	−−−−
Aromatic amino acid family									
phenylalanine	+	−−−				−−	−	+	
tyrosine	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−		−−−−
Histidine family									
histidine	−−	−	+	+	−	+	+	++++	−
1B. *L. casei* (NRRL-B1445)									
Alanine family									
alanine	−−	−	−	−	+	+	+	−−	++++
valine	−−		−	−−−−	+	+	+	−	−−−
leucine	−−−	−	−	−−	+	+	+	−	−−
Serine family									
serine	−−	−	−	−−	+			−	
cystine	−−−	−	−−−	−−−−		−−	++	−−−−	−−−−
Aspartate family									
aspartic acid		−	−	−	+++	+	++		−−
methionine	−−−			−	−	+	++	−−	−−
threonine		−−−−	−−−−	−−−		−	−	−−−−	−−−−
isoleucine		−	−	−	++	+	+	−	−−
lysine	−−−	−−	−−	−−−				−−−	−−−
Glutamate family									
glutamic acid	−−−	−	−	−	+	+	+		−−
arginine		−−−−	−−−−	−−−		−	−	−−−−	−−−−
proline	++++	++++	−−−	++++	++++	++++	++++	−−−−	++++
Aromatic amino acid family									
phenylalanine	−−	−	−−	−−−	+			−−	++++
tyrosine	−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
Histidine family									
histidine	+++	−−−	−	−	++	+	+	−−−	++++
1C. *L. delbrueckii* (NRRL-B445)									
Alanine family									
alanine	+++	++++	++++	++++	++++	++++	++++	++++	++++
valine	−−−−				−	−		−	+
leucine	−−−−				−	−			

Table 1. (*Continued*)

amino acids	Hours after inoculation								
	4	8	12	16	20	24	32	40	48
Serine family									
serine	−−−−			+	−	−	+	−	
cystine	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
Aspartate family									
aspartic acid	−−−−			+	−	−			+
methionine	−−−−				−	−			+
threonine	−−−	−−−	−−−	−−−	−−−−	−−−−	−−−	−−−−	−−−−
isoleucine	−−−−			+	−	−			
lysine	−−−−	−			−−	−−	−	−−	
Glutamate family									
glutamic acid	−−−−			+	−	−		−	
arginine	−−−	−−−	−−−	−−−	−−−−	−−−−	−−−	−−−−	−−−−
proline	−−−−	−−−	−−−−	+++	−−−−	−−	−−	−−−−	−
Aromatic amino acid family									
phenylalanine	−−−−				−−	−−	−	−	
tyrosine	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
glycine	−−−−			+	−		+		+
Histidine family									
histidine	−−−−	−		−	−−	−−		−−−	−
1D. *L. helveticus* (NRRL-B1937)									
Alanine family									
alanine	+	+	+		+		−		
valine		++	++		++	+	−−−	−−−−	−−−
leucine	+	+	+	+	++	+	−	+	+
Serine family									
serine	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
cystine		−−−−	−−−	−−−−	−−−	−−−	−−−−	−−−−	−−−−
Aspartate family									
aspartic acid	+	+	+	+	+	+		++	+
methionine		++	++		++	+	−−−	−−−−	−−−
threonine	−−−	−−−	−−−	−−−−	−−−	−−−−	−−−−	−−−−	−−−−
isoleucine	+	+	−−−−	+	++	+		+	+
lysine		+			+		−	−−−−	
Glutamate family									
glutamic acid		++	++	++	++	++		++	+
arginine	−−−	−−−	−−−	−−−−	−−−	−−−−	−−−−	−−−−	−−−−
proline		+	++	−−	+++	+		−	+
Aromatic amino acid family									
phenylalanine		+	+		+		−−		
tyrosine	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
Histidine family									
histidine	−	+++	+++	+	+++	+++	+	++	+

experiments, per 1 ml inoculum of strain B445, the inoculum volume of strain B1937 was varied as: 0.1, 0.2, 0.3, 0.4, and 0.5 ml. As presented in Fig. 4, the growth of five different mixed cultures was apparently enhanced in terms of culture turbidity (OD_{560}) and growth yield to glucose, compared with strain B445. Figure 4C shows that the amount of lactic acid produced and the lactic acid yield to glucose was significantly improved by co-culturing strain B1937 with strain B445. Especially, in a bioconsortium cultured with a particular inoculum composition (i.e., 0.4 ml of B1937 and 1 ml of B445), the lactic acid yield to glucose was at maximum level, i.e., it increased by about 20% and 45% compared with strains B445 and B1937, respectively (Fig. 4C).

Discussion

Cellular amino acids are produced either by 1) metabolic biosynthesis with intracellular precursor or 2) protease-dependent catabolic production with protein and/or pep-

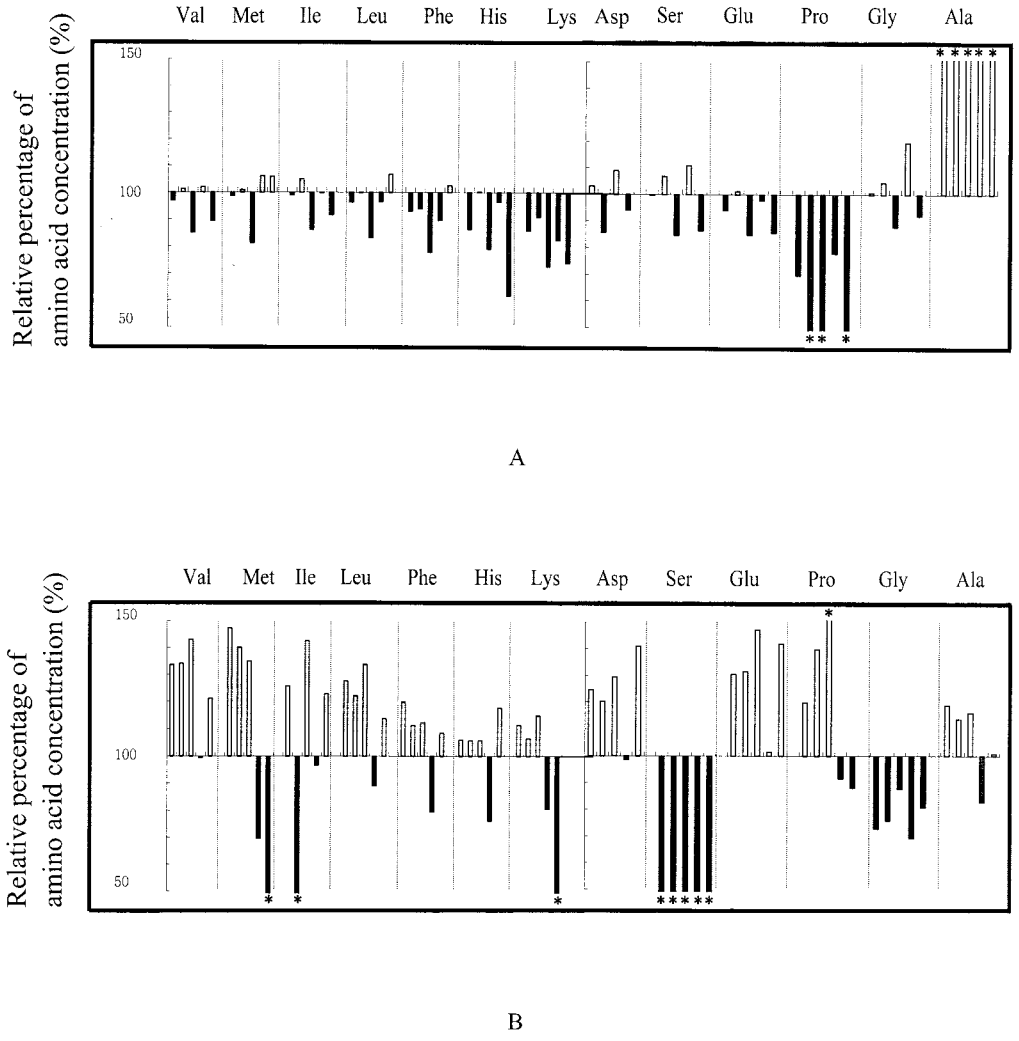

Fig. 3. Comparison between strains B445 (A) and B1937 (B) in the time-course (8, 12, 20, 32, and 40 h) production and utilization of each amino acid (i.e., the relative percentage of amino acid concentration in the culture broth with defining the initial concentration of amino acids in fresh MRS medium as 100%). (Symbol *represents that the relative amount of amino acid is far above 150% or below 50% at the corresponding time point.

tide substrates [1, 10–12, 16, 17, 20–22]. First, bacteria synthesize all of the 20 amino acids necessary for protein biosynthesis, using inorganic ammonium salts as the nitrogen source. In general, biosynthesis of the 20 amino acids is carried out through six independent routes, with different initial precursors. There are six families consisting of different amino acids, as follows: 1) alanine family (alanine, valine, and leucine); 2) serine family (serine and cysteine); 3) aspartate family (aspartic acid, asparagine, methionine, threonine, isoleucine, and lysine); 4) glutamate family (glutamine, arginine, and proline); 5) aromatic amino acid family (tryptophan, phenylalanine, and tyrosine); and 6) histidine. If all amino-acid members belonging to a family above are produced (or utilized) simultaneously in the course of cultivation, it is reasonable to assume that the synthesis of the amino acids is regulated by the family-specific biosynthetic route: Under such assumption, the strains 12315 and B1445 seem to regulate the synthesis of some amino acids belonging to the following families via the intrinsic and family-dependent biosynthetic pathway: the alanine and glutamate families in strain 12315 and the alanine and aspartate families in strain B1445 (Tables 1A and 1B). The concentration of the amino acids belonging to the above families decreased simultaneously in the early culture stage (0–16 h) and increased together in the late culture stage (20–48 h).

Contrary to strains 12315 and B1445, strain B1937 showed unique characteristics in amino acid utilization and production: 1) many amino acids were abundantly produced even from the early culture stage and 2) the concentration of amino acids belonging to each family

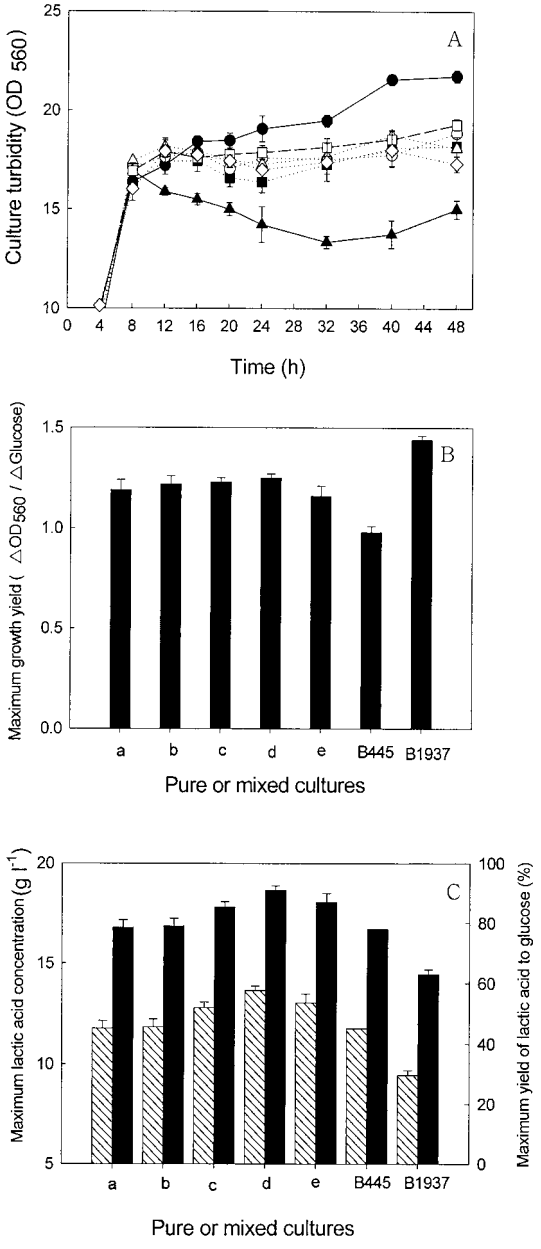

Fig. 4. Time-course variation in culture turbidity (OD_{560}) (A), maximum growth yield to glucose (B), and lactic acid concentration and yield to glucose (C) in the various mixed [(a)-(e)] and pure (B445, B1937) cultures. For mixed culture, inoculum volume of the strain B1937 was varied per 1 ml inoculum of strain B445 as follows: 0.1 ml, (a); 0.2 ml, (b); 0.3 ml, (c); 0.4 ml, (d); and 0.5 ml, (e). In Fig. 4A, symbols ● and ▲ represent strains B1937 and B445, respectively, and symbols ■, ○, △, □, and ◇ represent mixed cultures (a), (b), (c), (d) and (e), respectively. In Fig. 4D, the slash and solid rectangles represent maximum lactic acid concentration and maximum yield of lactic acid to glucose, respectively.

changed quite differently from each other in the course of cultivation except for the serine family. Bacteria can produce amino acid sources via proteolytic degradation of protein/peptide substrates. Proteins or large peptides in media should be degraded into small peptides by exoproteases, and after transport into the cells, the translocated small peptides are then cleaved to amino acids by intracellular peptidases [6]. It has been reported that *L. helveticus* is the most proteolytic species towards a wide range of substrates and has a very efficient proteolytic system involving general aminopeptidase [9, 12, 14]. Therefore, the abundance of amino acids in the culture of the strain B1937 (*L. helveticus* NRRL-B1937) seems to be due to the efficient proteolytic system of the strain. Also, the abundant amino acids in the B1937 culture nearly corresponded to the amino acids that were significantly deficient in the culture of strain B445 (Fig. 3). Therefore, it is expected that if the lactic acid producer, strain B445, is enabled to utilize the efficient proteolytic system of strain B1937, for example by co-culturing both strains, the growth of strain B445 would be derepressed, and the efficiency of lactic acid biosynthesis improved via the symbiotic effect of the mixed culture. To achieve such symbiotic effect, strain B1937 should grow up to a certain level to effectively provide strain B445 with the necessary amino acids, but should not overgrow strain B445 during the co-culture period. Hence, the growth of strain B1937 should be well controlled in the mixed culture, and the initial concentration of each strain can be considered as an important parameter to control the individual culture growth in the mixed culture. As presented in this study, the optimal symbiotic culture maximizing lactic acid production (i.e., the amount of lactic acid produced and the lactic acid yield to glucose) was achieved when a particular inoculum volume of strain B1937 was used (Fig. 4). It seems that the symbiotic interaction between the two strains could overcome the growth repression of the strain B445 and then improve further the biosynthetic efficiency of lactic acid.

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