Growth and acid production of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 in the fermentation of algal carcass

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ABSTRACT

Algal carcass is a low-value byproduct of algae after its conversion to biodiesel. Dried algal carcass is rich in protein, carbohydrate, and multiple amino acids, and it is typically well suited for growth and acid production of lactic acid bacteria. In this study, *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 was used to ferment different algal carcass media (ACM), including 2% ACM, 2% ACM with 1.9% glucose (ACM-G), and 2% ACM with 1.9% glucose and 2 g/L amino acid mixture (ACM-GA). Concentrations of organic acids (lactic acid and acetic acid), acetyl-CoA, and ATP were analyzed by HPLC, and activities of lactate dehydrogenase (LDH), acetokinase (ACK), pyruvate kinase (PK), and phosphofructokinase (PFK) were determined by using a chemical approach. The growth of *L. bulgaricus* cells in ACM-GA was close to that in the control medium (de Man, Rogosa, and Sharpe). Lactic acid and acetic acid contents were greatly reduced when *L. bulgaricus* cells were grown in ACM compared with the control medium. Acetyl-CoA content varied with organic acid content and was increased in cells grown in different ACM compared with the control medium. The ATP content of *L. bulgaricus* cells in ACM was reduced compared with that of cells grown in the control medium. Activities of PFK and ACK of *L. bulgaricus* cells grown in ACM were higher and those of PK and LDH were lower compared with the control. Thus, ACM rich in nutrients may serve as an excellent substrate for growth by lactic acid bacteria, and addition of appropriate amounts of glucose and amino acids can improve growth and acid production.

Key words: algal carcass, *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842, organic acid, enzyme activity

INTRODUCTION

In recent years, consumer demand for non-dairy-based probiotic products has increased due to problems of lactose intolerance and cholesterol content associated with the consumption of fermented dairy products (Gupta et al., 2011). In this respect, microalgae as a substrate can offer an alternative means for the production of probiotic and lactic acid because of its nutrient content. Several reports are available on L-lactic acid production by *Lactobacillus paracasei* LA 104 and D-lactic acid production by *Lactobacillus coryniformis* ssp. *torquenes* using green microalgae *Hydrodictyon reticulum* as a substrate (Nguyen et al., 2012a,b) and lactic acid production by *Lactobacillus pentosus* using microalgae *Nannochloropsis salina* as a substrate (Tallukder et al., 2012).

Algal carcass is a low-value byproduct derived from algae after biofuel production. Algal carcass is rich in protein and carbohydrate, both of which make up from 10 to 60% in dried cells (Illman et al., 2000; Gatenby et al., 2003). Algal carcass from *Neochloris oleoabundans* contains 10 to 40% carbohydrate and 20 to 60% protein (Gatenby et al., 2003). Therefore, algal carcass can serve as an excellent substrate for growth and acid production by lactic acid bacteria.

Lactobacillus delbrueckii ssp. *bulgaricus* is widely used in starter cultures, in conjunction with *Streptococcus thermophilus*, for the manufacture of yogurt and particular cheeses. Many papers have reported growth and metabolism conditions of *L. bulgaricus* in different media; for example, *L. bulgaricus* cells grown in medium supplemented with different carbon sources have different resistance to various stress treatments (Chervaux et al., 2000) and those grown in medium supplemented with Tween, edible oils, and amino acids exhibit higher anti-freeze-drying ability (Li et al., 2012). Production of exopolysaccharide by *L. bulgaricus* RR during growth was greater in a developed medium based on de Man, Rogosa, and Sharpe (MRS) broth compared with that in a common medium (Kimmel and Roberts, 1998). In addition, changing the composition of the medium af-

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fects the activity of key enzymes involved in glycolysis in *L. bulgaricus* (Li et al., 2014).

This work is part of an ongoing project to evaluate the potential of algal carcass as a substrate for the development of a probiotic. However, a systematic approach is needed to identify the growth factors needed because of its different composition (such as protein, fat, and carbohydrates) compared with published data from previous studies (Illman et al., 2000; Gatenby et al., 2003). For lactic acid bacteria as starter cultures, growth and acid production should be considered first. For this reason, the present work involved the use of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842 to study the applicability of algal carcass as a medium for growth and acid production.

MATERIALS AND METHODS

Algal Carcass

Dried, ground algal carcass was provided by Qingdao New Technology Development Co. Ltd. (Qingdao, China). Samples were stored at -18°C until analysis and testing.

Culture

Lactobacillus delbrueckii ssp. *bulgaricus* ATCC 11842 was purchased from the American Type Culture Collection (Manassas, VA). It was kept as frozen stock in 40% (vol/vol) glycerol at -80°C . For experimental use, the strain was cultured in MRS broth at 37°C to 10^6 cfu/mL, using 3 successive subcultures (1% vol/vol inoculation each).

Algal Carcass Composition Analysis

To understand the composition of algal carcass and optimize growth medium, some primary indices were analyzed. The protein content of algal carcass was measured by using a Foss Kjeltac 2300 auto-Kjeldahl analyzer (Foss, Hillerød, Denmark) according to the manufacturer's instructions. Total fat was measured by Soxhlet extraction method (AOAC method 963.15-1973).

The carbohydrates were quantified by HPLC using a Waters 2695 separation module with temperature control, a Water 717plus autosampler, Waters 1525 Binary HPLC Pump, and Waters 2414 Refractive Index Detector (Waters, Milford, MA). Equipment and signal were controlled and processed using Waters Empower Pro software. The method used to quantify carbohydrates was based upon a described method (Mirsiaghi and Reardon, 2015) with some modifications. One

gram of algal carcass was mixed with 3 mL of 2 mol/L trifluoroacetic acid and the mixture was added to a vial, which was sealed and placed in an oven at 110°C for 2 h. Then, 0.05 mol/L NaCl was added to neutralize the mixture. Samples were centrifuged at $8,000 \times g$ for 20 min at 4°C . The supernatants were filtered using a $0.2\text{-}\mu\text{m}$ syringe filter directly into an HPLC vial for analysis. The carbohydrates were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, CA) with $9\text{-}\mu\text{m}$ particle size and dimensions of 300×7.8 mm. The mobile phase was 5 mmol/mL sulfuric acid with a flow rate of 0.6 mL/min, the oven temperature was 65°C , and 10 μL of sample was injected. Standards for carbohydrates, including glucose, galactose, fucose, and arabinose, were used to identify and quantify the components in samples.

Fermentation Strategies

To study the applicability of algal carcass as a growth medium for *L. bulgaricus* ATCC 11842, addition of nutritional supplements to the medium was considered because of the limited carbohydrate or AA availability. Glucose (1.9%) and an AA mixture (cystine, glutamate, leucine, isoleucine, and tryptophan; final concentration of 0.4 mg/mL each in mixture) were added to algal carcass solutions. The final ACM used were 2% ACM, ACM with 1.9% glucose (**ACM-G**) and ACM with glucose and 2g/L of the AA mixture (**ACM-GA**). The algal carcass solutions were prepared as follows: algal carcass was ground with a mortar and pestle and prepared as a 2% (wt/vol) solution with sterile distilled water. A defined buffer final concentration of 1.0% (wt/vol) 2-(*N*-morpholino) ethanesulfonic acid (MES) was added to the above solutions to stabilize acidity for growth. The different ACM were then titrated to a pH of 6.5 using 3 mol/L NaOH and sterilized with an autoclave for 30 min at 121°C .

After cooling the sterilized medium, 100 mL of algal carcass solution was added to a 250-mL fermentation vessel and inoculated with 1% (vol/vol) inoculum, and the vessels were placed at 37°C for 24 h in an anaerobic chamber; MRS broth was used as the control. All samples were diluted and plated onto MRS agar, followed by incubation in incubator at 37°C for 48 h for optimized growth, and 10 to 30 cfu in 3 replicates were counted. After 24 h, all solutions were measured for terminal pH with a Delta 320 pH analyzer (Mettler Toledo, Greifensee, Switzerland).

Organic Acid Determination

Each sample of fermented broth was centrifuged at $7,741 \times g$ for 20 min at 4°C . The cell-free supernatant of

fermented broth was used for determination of organic acids (lactic acid and acetic acid) by HPLC (Mirsiaghi and Reardon, 2015) with some modifications.

The analysis was carried out using a Waters 2695 separation module with temperature control, a Waters 717plus autosampler, Waters 1525 Binary HPLC Pump, and Waters 2414 Refractive Index Detector (Waters, Milford, MA). Equipment and signal were controlled and processed using Waters Empower Pro software. An Aminex HPX-87H (Bio-Rad) column was used with 9 μm particle size and dimensions of 300×7.8 mm. Detection of organic acids was carried out at a wavelength of 210 nm. Samples and mobile phases were filtered through a 0.22- μm Millipore filter (Millipore, Bedford, MA) before HPLC injection, and 10 μL of sample was injected. The mobile phase was 5 mmol/mL sulfuric acid with a flow rate of 0.6 mL/min, and the oven temperature was 35°C. Standards for the organic acids were used to identify and quantify the components in samples.

Acetyl-CoA Determination

Quantification of acetyl-CoA was performed using a modified protocol of Vadali et al. (2004). A 150-mL sample was centrifuged at $7,741 \times g$ for 10 min at 4°C. The cell pellet was washed twice with PBS (pH 7.0), 2 mL of 6% perchloric acid including 2 mmol/L dithiothreitol to facilitate cell lysis was added, and then sonicated for 15 min at 300 W (working time: interval time = 1:3) using an ultrasonic cell disruption apparatus (JY92-IIDN, Xinzhi Biotechnology Co. Ltd., Ningbo, China) in an ice bath. The mixture was centrifuged at $16,451 \times g$ for 10 min at 4°C. Then, 3 mol/L potassium carbonate was added to the supernatant while vortexing to neutralize the acid. The solution was centrifuged at $16,451 \times g$ for 10 min at 4°C to pellet the cell debris. The supernatant was collected and filtered through a 0.2- μm filter and stored in the refrigerator pending analysis of acetyl-CoA using HPLC.

Separation of acetyl-CoA was performed with a HPLC system equipped with a Hypersil ODS2 (C18) column (250×4.6 mm, 5 μm , Thermo Fisher, Waltham, MA)

and a Waters 2489 UV-detector. The 2 mobile phase solvents used were buffer A [0.2 M sodium phosphate (pH 5.0)] and buffer B [800 mL of 0.25 M sodium phosphate (pH 5.0) mixed with 200 mL of acetonitrile] at a flow rate of 1 mL/min. The mobile phase profile is shown in Table 1. The HPLC column was operated at 25°C, and 10 μL of sample was injected. Detection was carried out at a wavelength of 254 nm.

ATP Determination

We measured ATP as described by Liu et al. (2006) with some modifications. A 50-mL cell sample was taken from the culture and centrifuged at $4,355 \times g$ for 10 min at 4°C. The pellet was frozen immediately in liquid nitrogen for 3 min. To extract ATP, 10 mL of 0.6 mol/L HClO_4 was added to the cell pellet and mixed thoroughly with a magnetic stirrer for 10 min. The mixture was centrifuged for 10 min at $12,096 \times g$ at 4°C to collect the supernatant. Another 10 mL of 0.6 mol/L HClO_4 was added to the pellets, mixed thoroughly for 10 min, and the supernatant was collected after centrifugation, as before. Two portions of the supernatant were combined in a 25-mL volumetric flask and diluted with 0.6 mol/L HClO_4 to volume. Ten milliliters of the prepared solution was taken and the pH adjusted to 7.0 using 0.8 mol/L potassium hydroxide. After 30 min at 4°C, crystalline KClO_4 was removed from the solution by 0.22- μm filter and then the solution was diluted to 25 mL with PBS (pH 7.0) before HPLC column application.

The concentration of intracellular ATP was measured by HPLC with a Hypersil ODS2 (C18) column (250×4.6 mm, 5 μm , Thermo Fisher) and a Waters 2489 UV-detector. The injection volume for the HPLC analysis was 10 μL . A mixture of 97% 10 mmol/L KH_2PO_4 (pH 7.0) and 3% methanol was used as mobile phase with a flow rate of 1 mL/min. The wavelength of the UV detector was set at 260 nm and the column temperature was controlled at 30°C.

Enzyme Activity Determination

A volume of 5 mL of *L. bulgaricus* cells was collected at $7,741 \times g$ for 10 min at 4°C, the pellets was washed twice with PBS (pH 7.0), and 500 μL of cell lysate suspension (50 mmol/L Tris-HCl, 2 mmol/L EDTA, 100 mmol/L NaCl, 0.5% TritonX-100, pH 8.5 to 9.0, 100 $\mu\text{g}/\text{mL}$ lysozyme, 1 $\mu\text{L}/\text{mL}$ phenylmethylsulfonyl fluoride) was added into the pellets. Cell-free extracts were obtained by sonication for 15 min at 300 W (working time: interval time = 1:3) using an ultrasonic cell disruption apparatus (JY92-IIDN) in an ice bath, followed by centrifugation at $12,000 \times g$ for 10 min at 4°C.

Table 1. The profile of mobile phase in this test

Time (min)	Buffer A (%)	Buffer B (%)
0.00	97	3
5.00	82	18
7.50	72	28
12.50	60	40
18.00	58	42
20.00	97	3

The bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to determine protein concentrations. The absorbance at 562 nm by a model 680 microplate reader (Bio-Rad) was used. According to the standard curve, the protein concentration of the sample was calculated. An LDH kit was used to determine lactate dehydrogenase (LDH), a PFK kit was used to determine phosphofructokinase (PFK), and a PK kit was used to determine pyruvate kinase (PK). These enzyme kits were purchased from Comin Biotechnology Co. Ltd. (Suzhou, China).

The determination of acetate kinase (ACK) was based on the report of Rose (1955). The stock of substrate was made fresh daily using 3.2 mol/L potassium acetate, 1 mol/L Tris-HCl (pH 7.4), and 1 mol/L MgCl_2 in a volume ratio of 25:5:1. A neutral solution of 28% hydroxylamine was made fresh by mixing equal volumes of hydrochloride and KOH. A mixture of 0.3 mL of stock substrate, 0.35 mL of neutral hydroxylamine, 0.1 mL of ATP, and 0.25 mL of cell solution was prepared. The mixture was placed in a 30°C waterbath for 12 min and 1.0 mL of 10% trichloroacetic acid was added. The precipitate was removed by centrifugation at $11,424 \times g$ for 10 min at 4°C, and the color was developed by adding 1.0 mL of FeCl_3 reagent (1.25% FeCl_3 in 1.0 mol/L HCl) at room temperature and read immediately in a ZE6000 colorimeter (Nippon Denshoku, Osaka, Japan). One unit of enzyme was defined as the amount that produced 1 micromole of hydroxamic acid per minute.

Statistical Analysis

All experiments were carried out in triplicate, and each sample was analyzed in duplicate. The results were expressed as means $\pm$ standard deviations. All statistical analyses were carried out using software SPSS 13.0 (SPSS Inc./IBM Corp., Armonk, NY). Differences were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

Composition Analyses of Algal Carcass

In this study, we analyzed the composition of dried algal carcass. The protein and fat contents of algal carcass was $15.32\% \pm 0.22\%$ and $1.66\% \pm 0.51\%$, respectively. The total carbohydrate concentration was 51.9 $\mu\text{g}/\text{mg}$, which included 27.3 $\mu\text{g}/\text{mg}$ glucose, 11.7 $\mu\text{g}/\text{mg}$ galactose, 0.8 $\mu\text{g}/\text{mg}$ fucose, 3.5 $\mu\text{g}/\text{mg}$ mannose, and 8.6 $\mu\text{g}/\text{mg}$ arabinose, respectively. These results are in agreement with published reports (Illman et al., 2000; Gatenby et al., 2003), which stated the range of protein and carbohydrate in dried cells to be 10 to 60%.

Fermentation Analyses

Nutrients are not only involved in the growth of bacteria, but also affect metabolism of lactic acid bacteria and alter metabolites. Therefore, the appropriate nutrient composition and concentration is an important factor for the efficient proliferation of lactic acid bacteria. Algal carcass has significant concentrations of proteins, carbohydrates, and other nutrients that can be used for the production of many co-products, making it a perfect candidate for fermentation substrate of lactic acid bacteria. Several researchers produced lactic acid by *Lactobacillus paracasei* (Nguyen et al., 2012a) and *Lactobacillus coryniformis* ssp. *torquenes* (Nguyen et al., 2012b) and *Lactobacillus pentosus* (Talukder et al., 2012) using undried algae as a substrate. In the current study, 1.9% glucose was directly added to ACM (ACM-G) to reach a total monosaccharide concentration equal to that of MRS broth (2.0%) for comparison. In addition, 2 g/L of an AA mixture was added to ACM with glucose (ACM-GA).

Lactobacillus bulgaricus cells grown in different ACM had different growth curves, and the results are shown in Figure 1. The control medium, MRS broth, provided better nutrient conditions for *L. bulgaricus*. When 2% ACM was used as a fermentation substrate, the growth of *L. bulgaricus* was poor, but addition of nutrients improved growth. The growth of cells in ACM-G and ACM-GA was better than that in 2% ACM. Cell count in ACM-GA reached $8.02 \log_{10} \text{cfu}/\text{mL}$ at 12 h, a count close to that of the control ($P < 0.05$). Thus, glucose and AA can improve the growth of *L. bulgaricus* in ACM. A concentration of 0.25% lactulose has been shown to increase growth of *Bifidobacterium bifidum* when the concentration of available carbohydrates in yogurt is limited (Özer et al., 2005). Supplementation of 0.25% casamino acids in growth medium improved the growth of *Lactobacillus acidophilus* (Azcarate-Peril et al., 2005).

Lactobacillus bulgaricus is a homofermentative strain, the glycolytic pathway is its major metabolic process, and lactic acid is the main product of sugar metabolism. For every 1 mol of monosaccharides entering cells through phosphorylation and enzyme reaction, 2 mol of lactic acid is generated. Other physical activities also occur in the fermentation of lactic acid bacteria; therefore, other metabolites besides lactic acid exist in the homofermentative strain. Lactic acid bacteria under different growth conditions can show different responses and result in a change in metabolic flux (Dawat et al., 1999).

In this study, we examined the effect of algal carcass as the fermentation substrate on production of lactic acid, acetic acid, and acetyl CoA. The results in Table

Figure 1. The growth curve of *Lactobacillus delbrueckii* ssp. *bulgaricus* grown in different algal carcass media (ACM). ACM-G = 2% ACM containing 1.9% glucose; ACM-GA = 2% ACM containing 1.9% glucose and 2 g/L AA mixture; MRS = de Man, Rogosa, and Sharpe. Values shown are mean $\pm$ SD.

2 show that lactic acid content was greatly reduced when *L. bulgaricus* cells were grown in ACM compared with the control medium. Lactic acid content from cells in 2% ACM, ACM-G, and ACM-GA was decreased 96.2%, 57.3%, and 46.1%, respectively. Acetic acid content (Table 2) was also greatly reduced when *L. bulgaricus* cells were grown in ACM compared with the control medium. Acetic acid content from cells in 2% ACM, ACM-G, and ACM-GA was decreased 93.4%, 64.0%, and 45.2%, respectively.

The results in Table 2 for acetyl-CoA content were different from those for organic acids; acetyl-CoA content was increased when cells were grown in ACM compared with the control medium. Acetyl-CoA content from cells in 2% ACM, ACM-G, and ACM-GA was increased 100.6%, 61.4%, and 46.6%, respectively.

The growth and pH of *L. bulgaricus* cells during the 24-h fermentation was not distinctly different in ACM-GA compared with the control medium, but organic acid and acetyl-CoA contents from different

media were significantly different ($P < 0.05$). Addition of glucose and AA mixture to ACM contributed to improving growth and acid production of *L. bulgaricus*. Although total bacterial count was improved, organic acid production was decreased. To determine the causes of this phenomenon, we measured the intermediate metabolite acetyl-CoA in this study. Acetyl-CoA content was remarkably increased compared with the control. The utilization of acetyl-CoA appears to be reoriented toward fatty acid biosynthesis (Fernandez et al., 2008). Therefore, its accumulation may accelerate the formation of phospholipids, which are an important component of the cell membrane, maintaining a functional barrier with normal structure. Organic acids in suboptimal nutrient conditions are used as the carbon source, and of the organic acids, acetic acid is used first and converted to acetyl-CoA, which affects the mobility of the cell membrane and protects the growth of cells (Fu et al., 2006). It is possible that *L. bulgaricus* cells can grow normally when the acid con-

Table 2. The organic acid and acetyl-CoA contents of *Lactobacillus delbrueckii* ssp. *bulgaricus* in different algal carcass media (ACM) at 24 h of fermentation

Medium ¹	pH	Lactic acid (mg/mL)	Acetic acid (mg/mL)	Acetyl-CoA ($\times 10^{-6}$ μ mol/mg)
MRS (control)	4.05 $\pm$ 0.03 ^a	6.14 $\pm$ 0.31 ^a	3.47 $\pm$ 0.51 ^a	8.35 $\pm$ 0.12 ^a
2% (wt/vol) ACM	4.69 $\pm$ 0.02 ^b	0.23 $\pm$ 0.02 ^b	0.23 $\pm$ 0.04 ^b	16.75 $\pm$ 1.04 ^b
ACM-G	4.14 $\pm$ 0.09 ^c	2.62 $\pm$ 0.02 ^c	1.25 $\pm$ 0.04 ^c	13.48 $\pm$ 0.26 ^c
ACM-GA	4.07 $\pm$ 0.01 ^a	3.31 $\pm$ 0.11 ^d	1.90 $\pm$ 0.04 ^d	12.24 $\pm$ 0.15 ^d

^{a-d}Means ($\pm$ SD) within a column are significantly different ($P < 0.05$).

¹MRS = de Man, Rogosa, and Sharpe; ACM-G = 2% ACM containing 1.9% glucose; ACM-GA = 2% ACM containing 1.9% glucose and 2 g/L AA mixture.

Table 3. The activity of primary enzymes¹ (U/mg of protein) and ATP (mmol/g, wet weight of cells) from *Lactobacillus delbrueckii* ssp. *bulgaricus* cells in different growth medium

Medium ²	PFK	PK	LDH	ACK	ATP
MRS (control)	11.78 ± 2.73 ^a	21.78 ± 0.73 ^a	22.95 ± 1.13 ^a	0.54 ± 0.09 ^a	2.07 ± 0.04 ^a
2% ACM	12.31 ± 2.46 ^b	12.17 ± 0.46 ^b	16.83 ± 0.25 ^b	0.70 ± 0.04 ^b	0.35 ± 1.04 ^b
ACM-G	12.48 ± 1.34 ^b	14.86 ± 0.34 ^c	16.31 ± 0.46 ^b	0.73 ± 0.05 ^b	0.78 ± 0.26 ^c
ACM-GA	12.91 ± 1.06 ^b	17.48 ± 0.54 ^d	17.74 ± 0.54 ^c	0.81 ± 0.04 ^d	1.10 ± 0.15 ^d

^{a-d}Means (± SD) within a column are significantly different ($P < 0.05$).

¹PFK = phosphofructokinase; PK = pyruvate kinase; LDH = lactate dehydrogenase; ACK = acetokinase.

²MRS = de Man, Rogosa, and Sharpe; ACM-G = 2% ACM containing 1.9% glucose; ACM-GA = 2% ACM containing 1.9% glucose and 2 g/L AA mixture.

tent is greatly reduced and the content of acetyl-CoA increased (Fu et al., 2006).

Enzyme Activity

Phosphofructokinase is a rate-limiting enzyme of glycolysis in lactic acid bacteria (Viana et al., 2005). The results in Table 3 show that PFK activity of *L. bulgaricus* cells grown in ACM was higher than that in the control medium and the 3 ACM did not differ. The PFK activity of *L. bulgaricus* cells grown in ACM-GA was highest and increased 9.6% ($P < 0.05$). The PFK activity of *L. bulgaricus* cells in 2% ACM and ACM-G increased 4.5% and 5.9%, respectively.

Pyruvate kinase is a key enzyme to regulate carbon metabolism (Ramos et al., 2004). The results in Table 3 showed that PK activity of *L. bulgaricus* cells grown in ACM was lower than that of the control medium. Compared with the control, PK activity of *L. bulgaricus* cells decreased 44.1% in 2% ACM, 31.8% in ACM-G, and 19.7% in ACM-GA.

Lactate dehydrogenase is a key enzyme for lactic acid production during metabolism of lactic acid bacteria. During homofermentation and heterofermentation, LDH catalyzes pyruvate to produce lactic acid. Therefore, monitoring changes in LDH activity is also important. Compared with the control, LDH activity of *L. bulgaricus* cells in ACM was lower and LDH activity of cells in ACM-GA decreased 22.7%.

Acetokinase is involved in pyruvate metabolism (Rose, 1955). From the results in Table 3, ACK activity of *L. bulgaricus* cells in ACM was higher than that of the control. Compared with the control, ACK activity of cells increased 29.6% in 2% ACM, 35.2% in ACM-G, and 50% in ACM-GA.

Adenosine triphosphate is the main source of energy in cells. In this study, ATP content was measured by HPLC. From the results in Table 3, ATP content of *L. bulgaricus* cells in ACM was reduced compared with the control. The ATP content of cells in 2% ACM, ACM-G, and ACM-GA was decreased 83.1%, 62.3%, and 46.9%, respectively.

The adaptation of cells in different environments is very important for cell survival, and the stress response from some bacteria in adverse circumstances is closely related to metabolite flux (Duwat et al., 1999). Activities of PFK and ACK in *L. bulgaricus* cells in the 3 ACM increased slightly ($P < 0.05$), whereas activities of PK and LDH were decreased greatly compared with the control ($P < 0.05$). The high content of ATP restrains PFK and PK activity and slows glycolytic flux in cells (Liu et al., 2006). In this study, decreased ATP content was consistent with decreased lactic acid and acetic acid content, whereas acetyl-CoA content increased. Phosphofructokinase is an allosteric enzyme and its activity is regulated by ATP and several other metabolites. The decomposition rate of carbohydrate under anaerobic conditions depends on the activity of PFK. Enzyme activity in other metabolic paths may be hindered due to the increase of acetyl CoA content, such as LDH activity. Acetokinase regulates the reversible conversion from acetyl-CoA to acetic acid. The findings of decreased acetic acid content and increased enzyme activity imply that an increased quantity of acetic acid is converted to acetyl-CoA through acetyl-P by ACK catalysis.

Microorganisms under different growth environments will have different metabolites (Li et al., 2012). The diversity of the catabolism pathway shows the adaptability of bacteria to the environment in substance metabolism level. Some studies have shown that metabolic pathways of lactobacillus will change significantly in an acidic environment to adapt to acid stress (Burne and Marquis, 2000; Cotter and Hill, 2003). When PK activity was suppressed in our study, other metabolic flow in pyruvate metabolism pathway was enhanced (Fernandez et al., 2008); subsequently, this led to the conversion of fructose-1,6-diphosphate to glyceraldehyde-3-phosphate and then further conversion to phospholipids by fatty acid biosynthesis. The metabolic flow of the conversion to lactic acid was lessened in pyruvate metabolism, whereas the conversion to acetyl-P was enhanced in our study (Fernandez et al., 2008). An increased amount of acetyl-P is expected to be converted to acetyl-CoA

rather than acetic acid. The utilization of acetyl-CoA appears to be reoriented toward fatty acid biosynthesis. In fatty acid synthesis, acetyl-CoA first converts glycerol-3-P and then further converts phospholipids,

the main component of the cell membrane that increase the rigidity and impermeability of the cell membrane. The changes resulting from conversion of acetyl-CoA to phospholipids can strengthen the synthesis of fatty ac-

Figure 2. The pyruvate metabolism of *Lactobacillus delbrueckii* ssp. *bulgaricus* fermenting algal carcass. PFK = phosphofructokinase; PK = pyruvate kinase; LDH = lactate dehydrogenase; ACK = acetokinase. Dashed arrows indicate reinforced metabolic pathway; solid arrows indicate suppressive metabolic pathways; and dotted arrows are speculative based on research of Fernandez et al. (2008).

ids to adapt to stress from the environment (Fernandez et al., 2008).

Most bacteria contain a synthetic gene to synthesize pyruvate decarboxylase; pyruvate decarboxylase can catalyze pyruvic acid to produce acetaldehyde, and then acetaldehyde can be reduced to ethanol by the action of enzymes involved in ethanol production. However, based on the KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>), *L. bulgaricus* does not contain aldehyde dehydrogenase related to ethanol production. According to the above analysis, the pyruvate metabolism profile of *L. bulgaricus* when grown in ACM is shown in Figure 2.

CONCLUSIONS

In this experiment, we studied organic acid metabolism and related enzyme activities when *L. bulgaricus* ATCC 11842 was grown in 2% ACM, ACM-G, and ACM-GA. Algal carcass, a byproduct of biofuel production, contains various nutrients for growth of lactic acid bacteria, and addition of glucose and amino acids can further improve growth and acid production. However, the contents of lactic acid and acetic acid were greatly reduced, and the content of acetyl-CoA, an intermediate metabolite, was increased when cells were grown in ACM. Acetyl-CoA is associated with the formation of the cell membrane and can ensure adaptability of lactic acid bacteria to stress. In addition, according to our results of metabolite flow and related enzyme activities, we propose a pathway for pyruvate metabolism of *L. bulgaricus* fermenting algal carcass.

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