J. Microbiol. Biotechnol. (2010), **20**(1), 101–109
doi: 10.4014/jmb.0905.05032
First published online 5 November 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Enhancement of L-Lactic Acid Production in *Lactobacillus casei* from Jerusalem Artichoke Tubers by Kinetic Optimization and Citrate Metabolism

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Received: May 10, 2009 / Revised: August 3, 2009 / Accepted: August 4, 2009

Efficient L-lactic acid production from Jerusalem artichoke tubers, by *Lactobacillus casei* G-02, using simultaneous saccharification and fermentation (SSF) in a fed-batch culture, is demonstrated. A kinetic analysis of the SSF revealed that the inulinase activity was subjected to product inhibition, whereas the fermentation activity of G-02 was subjected to substrate inhibition. It was also found that the intracellular NADH oxidase (NOX) activity was enhanced by the citrate metabolism, which dramatically increased the carbon flux of the Embden–Meyerhof–Parnas (EMP) pathway, along with the production of ATP. As a result, when the SSF was carried out at 40°C after an initial hydrolysis of 1 h and included a sodium citrate supplement of 10 g/l, an L-lactic acid concentration of 141.5 g/l was obtained after 30 h, with a volumetric productivity of 4.7 g/l/h. The conversion efficiency and product yield were 93.6% of the theoretical lactic acid yield and 52.4 g lactic acid/100 g Jerusalem artichoke flour, respectively. Such a high concentration of lactic acid with a high productivity from Jerusalem artichokes has not been reported previously, making G-02 a potential candidate for the economic production of L-lactic acid from Jerusalem artichokes on a commercial scale.

Keywords: Lactic acid, *Lactobacillus casei*, Jerusalem artichoke, kinetic optimization, citrate metabolism

Lactic acid is widely used in the food, cosmetic, pharmaceutical, textile, and chemical industries, and much work has already been done to improve the production of lactic acid from inexpensive raw substrates with cheap and energy-saving processes using genetically modified organisms and multi-stage fermentation systems [8, 11, 12, 24, 27, 31, 38]. Recently, the increased manufacture of biodegradable polylactide polymers has attracted global interest in the production of L-lactic acid using a fermentative route. Jerusalem artichokes, which are nearly 20% (w/w) carbohydrate that is composed of 70–90% (w/w) inulin, can grow well in poor soil and show a high tolerance to frost and various plant diseases. Although this biomass source has already been proposed as a possible substrate for ethanol production in a simultaneous saccharification and fermentation process (SSF) [15, 26, 28], there have been few studies on an SSF process for lactic acid production from Jerusalem artichokes.

In SSF, since the inulin from Jerusalem artichoke flour is unfermentable by *Lactobacillus casei*, inulinase is supplied exogenously by the addition of the culture supernatant of *Aspergillus niger*. In this study, the inulinase activity was found to be subjected to product inhibition in SSF, whereas the fermentation activity of *Lactobacillus casei* G-02 was subjected to substrate inhibition. To enhance the lactic acid productivity, a previous study increased the inoculation volume to 35% of the medium [13], but this is impractical for industrial-scale production. To maximize the rate of saccharification, the fructose concentration in the culture should be kept as low as possible, but this decreases the lactic acid productivity. Therefore, the SSF kinetics need to be investigated to maximize the productivity of lactic acid from Jerusalem artichokes.

Since *Lactobacillus* sp. lacks an efficient respiration metabolism, the major metabolic energy is gained from glycolysis [10]. However, when large amounts of ATP are required for maintaining the proton motive force (PMF) to resist acid stress, the energy generated *via* glycolysis is not sufficient to allow the maximum rates of the biosynthetic machinery, thereby hampering the lactic acid production by this strain in SSF [6, 9]. Previous studies have found that the growth of *Lactobacillus helveticus* is improved by the citrate metabolism, which enhances the ratio of dissociated to nondissociated forms of acetic acid produced and gains

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extra ATP [36]. Recently, Sánchez *et al.* [33] reported that the citrate metabolism endowed the cells of *Lactococcus lactis* with an extra ability to counteract acid toxicity; that is, citrate/lactate exchange and less expenditure of ATP to achieve pH homeostasis.

However, little data are currently available on the influence of the citrate metabolism on glycolysis in *Lactobacillus casei*, which is the most common and preferred bacteria for L-lactic acid production [3, 7, 13, 16, 18, 21, 30]. Accordingly, to further enhance the production of lactic acid, this study supplemented the media with sodium citrate to maximize the specific growth rate and specific fructose consumption rate of G-02, plus the influence of citrate on the metabolism and physiology of the strain was also investigated. The results revealed that the citrate metabolism enhanced the NADH oxidase (NOX) activity, as well as the ratios of $[NAD^+]/[NADH]$ and [ATP]/[ADP] in the G-02 cells, plus the accumulation of pyruvic acid was decreased and the carbon flux of glycolysis increased. As a consequence, the lactic acid productivity in the SSF was significantly improved. Thus, the results presented here demonstrate a novel way to enhance the production of lactic acid from Jerusalem artichokes using SSF, which will promote the use of Jerusalem artichokes as a promising raw material for L-lactic acid production in the bio-industry.

Materials and Methods

Biological Materials

The Jerusalem artichoke tubers were given by Xiongying Agriculture Co. from GanSu Province, P. R. China, and prepared as described previously [15]. The Jerusalem artichoke flour used in this study contained 56% (w/w) total reducing sugars. The soybean flour was obtained from a local source and milled commercially. All the chemicals were purchased from Sigma Chemical Co. (MI, U.S.A.). *Lactobacillus casei* G-02, a homofermentative lactic acid bacterium that can produce *L*(+)-lactic acid with an optical purity of 95%, was isolated from a Chinese traditional pickled vegetable and identified to the genus level. In addition, this strain can cometabolize citrate with fructose, and has been deposited in the China Center of Type Culture Collection (CCTCC) as CCTCC M 208232. A BLAST comparison of this strain with other related *Lactobacillus* showed that G-02 had a 98.83% similarity with *Lactobacillus casei* KLDS 1.0720. The generation and identification of *Aspergillus niger* SL-09 are described elsewhere [14].

Medium for Inocula

The *Lactobacillus casei* G-02 was grown on a medium containing (per liter) 30 g of glucose, 10 g of peptone, 10 g of yeast extract, 2 g of $(NH_4)_2HPO_4$, 0.2 g of $MnSO_4 \cdot H_2O$, and 20 g of $CaCO_3$, pH 7.0, for 20 h at 30°C with shaking at 140 rpm.

Enzyme Preparation

The medium used for the production of inulinase contained (per liter) 50 g of Jerusalem artichoke flour, 50 g of soybean flour, and 5 g of sucrose ester, pH 5.4. This medium was cooked at 100°C for 1 h, and then filtered through a cheese-cloth to remove any insoluble materials. The shake flasks were incubated at 30°C for 60 h on a rotary shaker (140 rpm), and the submerged culture of *A. niger* SL-09 was filtered through a cheese-cloth to obtain a clear supernatant, which was considered as the crude enzyme preparation.

Simultaneous Saccharification and Fermentation

The saccharification and fermentation of the Jerusalem artichoke flour proceeded simultaneously in a 7-l jar fermentor (KF-7 L; Korea Fermentor Co., Inchon, South Korea) with a working volume of 4 l. Using the optimum fermentation conditions determined in previous studies [13], the current experiments were carried out in a lactic acid production medium containing 50 U/ml of inulinase activity and 50 g/l of $CaCO_3$ with an initial pH of 6.5 and no pH control. Considering the requirements of industrial-scale production, the inoculation volume of G-02 in the tests was decreased to 10% of the medium. To study the kinetic characteristics of inulinase, the SSF experiments were carried out under various temperatures (from 20 to 60°C) with various concentrations of Jerusalem artichoke flour (from 10 to 160 g/l). During the fermentation process, liquid culture samples were periodically withdrawn and used to analyze the L-lactic acid and total sugar. The medium was agitated at the beginning of the fermentation to submerge the solid matter in the liquid, and then incubated without agitation. Each condition was examined in triplicate.

Previous studies have found that the conversion efficiency decreases dramatically when the initial Jerusalem artichoke flour concentration in the medium is over 230 g/l [13]. Therefore, to determine the initial hydrolysis time for lactic acid productivity, the liquid medium containing 200 g/l of Jerusalem artichoke flour was incubated at 50°C for 0.5 to 2 h, and then used for lactic acid production with a 10% inoculation volume. The experiments were all performed in triplicate.

To further enhance the lactic acid productivity from Jerusalem artichoke flour, the above fermentations were carried out at 40°C for 36 h with a supplement of sodium citrate at concentrations from 5 to 25 g/l, and a culture without the supplement was used as the control. All the experiments were performed in triplicate, and the values expressed as the means of duplicate measurements of three independent samples. The data were analyzed using SAS version 8.0 software (U.S.A.).

Influence of Citrate Metabolism on Glycolysis

From the time course of lactic acid production in the SSF, the maximum lactic acid accumulation rate was after 15 to 20 h of fermentation. This stage can be regarded as the pseudo steady state, since the accumulation rate of intracellular metabolites is near zero [34]. To determine the effect of the citrate metabolism on the carbon flux distribution of G-02 at 40°C, samples were aseptically withdrawn from the fermentation vessel at 15 and 18 h. All the samples were immediately cooled on ice to determine the extracellular metabolites.

To determine the concentrations of the intracellular metabolites, the G-02 culture was filtered through a cheese-cloth to obtain a clear supernatant, and then centrifuged at 5,000 $\times g$ for 15 min at 4°C to harvest the cells. The cell pellets were washed twice with ice-cold saline [0.85% NaCl (w/v)] and resuspended in 25 ml of a 200 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA. Next, the cells were disrupted ultrasonically at 4°C for 40 cycles of

5 s (ACX 400 sonicator at 20 kHz; Sonic and Materials, Newton, MA, U.S.A.), and any cell debris was removed by centrifugation (1,0000 ×*g* for 10 min at 4°C), leaving the cell extract (CE). The extracellular and intracellular metabolites (fructose, lactate, acetate, and pyruvate) were analyzed using high performance liquid chromatography (HPLC, Waters Associates model 209, equipped with a differential refractive index R1401 detector), using a Merck Lichrosorb-NH_2 column (4.6×250 mm, 5 μm), at a temperature of 30°C. A mixture of acetonitrile:water (60:40, v/v) was used as the mobile phase at a flow rate of 1.0 ml/min. All the samples were injected twice. To detect the concentration of ATP, ADP, NADH, and NAD, cell samples were removed from the culture, frozen immediately in liquid nitrogen for 60 s, and stored at -20°C for tests.

Analytical Methods

The inulinase activity was assayed as described previously [15]. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of fructose equivalent from inulin per minute. The protein concentrations were determined using the Bradford method [1] with bovine serum albumin as the standard. The residual glucose concentration was measured using the Miller method [25] with fructose as the standard. The total reducing sugar was assayed using the same method after acid hydrolysis (adjusted to pH=1.0 with sulfuric acid and heated for 30 min at 100°C). The pH was measured using a pH meter. The L-lactic acid was determined using an SBA-40C immobilized enzyme biosensor. The biomass concentration was determined by measuring the OD_{600} or DCW per liter, where 5 ml of the culture broth was centrifuged (1,0000 ×*g* for 10 min), washed twice with distilled water, and dried at 105°C until achieving a constant weight. Under these experimental conditions, an OD_{600} of 1.0 was equal to 0.41 g DCW/l.

The intracellular pH was measured using the fluorescence method developed by Breeuwer *et al.* [2] with 5 (and 6-)-carboxyfluorescein succimidyl ester (cFSE) as the fluorescent probe. The harvested cells were then washed and resuspended in a 50 mM potassium HEPES buffer (pH 8.0). Thereafter, the cells were incubated for 10 min at 30°C in the presence of 1.0 μM cFDASE, washed, and resuspended in a 50 mM potassium phosphate buffer (pH 7.0). To eliminate any nonconjugated cFSE, glucose (final concentration, 10 mM) was added, and the cells were incubated for an additional 30 min at 30°C. The cells were then washed twice, resuspended in a 50 mM phosphate buffer (pH 7.0), and placed on ice until used. The cells containing the fluorescent probe were diluted to a concentration of approximately 10^7 cells/ml in a 3-ml glass cuvette and placed in a thermostated cuvette holder of a spectrofluorometer (30°C). The fluorescence intensities were then measured at excitation wavelengths of 490 and 440 nm by rapidly alternating the monochromator between both wavelengths. The emission wavelength was 520 nm, and the excitation and emission slit widths were 8 and 10 nm, respectively. At the end of each assay, the extracellular fluorescence signal (background) was determined by filtration of the cell suspension through a 0.22-mm-pore-size membrane filter and measurement of the filtrate. The ratios of the 490- to 440-nm measurements were corrected for a background signal.

For the ATP detection, the cells were harvested by centrifugation at 2,000 ×*g* for 10 min, and then washed twice with distilled water. To extract the ATP, 10 ml of 0.6 mol/l $HClO_4$ was added to the cell pellets and mixed thoroughly using a magnetic stirrer for 10 min. The mixture was then centrifuged for 10 min at 10,000 ×*g* 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 collected after centrifugation. Two portions of the supernatant were combined in a 25-ml volumetric flask and increased to 25 ml with 0.6 mol/l $HClO_4$. Ten ml of the prepared solution was taken and the pH adjusted to 7.0 with 0.8 mol/l potassium hydroxide. After being kept at 4°C for 30 min, the crystal $KClO_4$ was removed from the solution by filtration (pore size=0.22 m) and then diluted to 25 ml using phosphate buffer (pH 7.0) before applying to the HPLC column. The injection volume for the HPLC analysis was 10 μl.

NADH and NAD are acid and alkali labile, respectively. Therefore, measuring the oxidized and reduced forms requires extraction from separate samples. To extract the NADH, 20-ml cell samples were freeze-dried for 24 h and transferred to a 20-ml solution containing 50 mmol/l KOH, 30% ethanol, and 22 mmol/l borate (pH 10). The extracted samples were left on ice for 30 min before the pH was adjusted to 9.0–9.4 using 3 mol/l HCl under vigorous agitation. After centrifugation at 4°C for 10 min at 10,000 ×*g*, the supernatant was immediately tested for NADH without neutralizing to avoid any destruction of NADH by high local concentrations of acid.

Meanwhile, to extract the NAD, 20 ml of cell samples was freeze-dried for 24 h and transferred to 20 ml of 70% cold ethanol containing a 10 mmol/l potassium phosphate buffer, pH 6.5, and extracted for 30 min at room temperature (25°C). During the extraction procedure, the pH was kept below 7.0 using 0.5 mol/l HCl under vigorous agitation to avoid any NAD degradation. After centrifugation for 10 min (4°C and 10,000 ×*g*), the supernatant was immediately used for metabolite concentration measurements.

The NOX activity in the CE was assayed spectrophotometrically at 25°C in a total volume of 1 ml containing 0.8 ml of a 62.5 mM

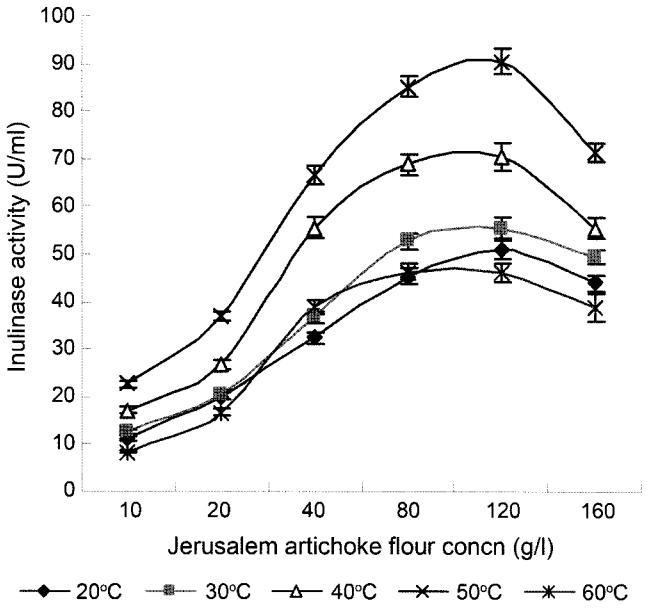

Fig. 1. Inulinase activity in media at various temperatures and concentrations of Jerusalem artichoke flour in SSF.

The inulinase activity was assayed in a 7-l jar fermentor containing 4 l of the liquid culture with a 10% inoculation volume of G-02 and no pH control. The results are expressed as the means of duplicate measurements under the same conditions in three independent experiments. The standard deviations were lower than 5% of the values (for inulinase activities).

potassium phosphate buffer with different pH values (5.3, 5.8, 6.3, 6.8, and 7.3), 0.1 ml of 2.9 mM NADH, and 0.1 ml of 3 mM EDTA. The reaction was initiated by the addition of a suitable amount of the CE of strain G-02 (0.5 to 5 μl) and monitored based on the decrease in *A*340, as described by Lopez de Felipe *et al.* [23]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of NAD^+ per minute when using an ε of 6,220 $M^{-1}cm^{-1}$.

Results

Kinetic Characterization of Inulinase

Several experimental runs were carried out to determine the influence of temperature and the Jerusalem artichoke flour concentration on the inulinase activity. As shown in Fig. 1, the maximum inulinase activity was obtained at 50°C with a substrate concentration of 120 g/l, 2-fold higher than that obtained at 30°C with the same substrate concentration. The inhibitory effects of fructose on the inulinase activity were further tested, and the results are shown in Fig. 2. Increasing the fructose concentration decreased the inulinase activity, owing to product inhibition, and the activity was almost completely eliminated when the fructose concentration exceeded 20 g/l, which falls into the category of slow-binding reversible inhibition kinetics [29]. Therefore, the rates of the enzyme reaction (n) in the SSF process were estimated using Eq. (1),

$$V = \frac{V_{max[S]}}{K_m\left(1 + \frac{[I]}{[K_I]}\right) + [S]} \quad (1)$$

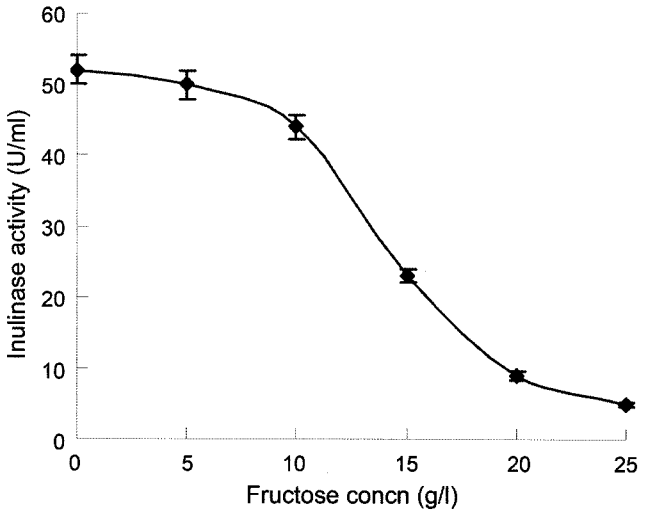

Fig. 2. Inulinase activity in media with various concentrations of fructose.

The inulinase activity was assayed in a 7-l jar fermentor containing 4 l of the liquid culture with an inulinase activity of 50 U/ml. The results are expressed as the means of duplicate measurements under the same conditions in three independent experiments. The standard deviations were lower than 5% of the values (for inulinase activities).

where [I] is the fructose concentration, K_m is the Michaelis constant, [S] is the Jerusalem artichoke flour concentration, and K_I is the inhibition constant.

Equation (1) shows that an increased v_{max} value and decreased K_m value were essential to enhance the rate of the enzyme reaction. The progress-of-substrate-reaction method was then applied to determine the inhibition kinetics in more detail (Fig. 3). The results signified that the V_{max} for inulinase at 40°C (714.2 μmol/min) and 50°C (909.5 μmol/min) was 1.5- and 1.9-fold higher, respectively, than that at 30°C (476.2 μmol/min). The value of K_m at 40°C (59.1 mmol/l) and 50°C (34.9 mmol/l) was also significantly lower than that at 30°C (68.3 mmol/l). Thus, based on these results and because the conversion efficiency decreased dramatically in the SSF when the Jerusalem artichoke flour concentration was over 230 g/l

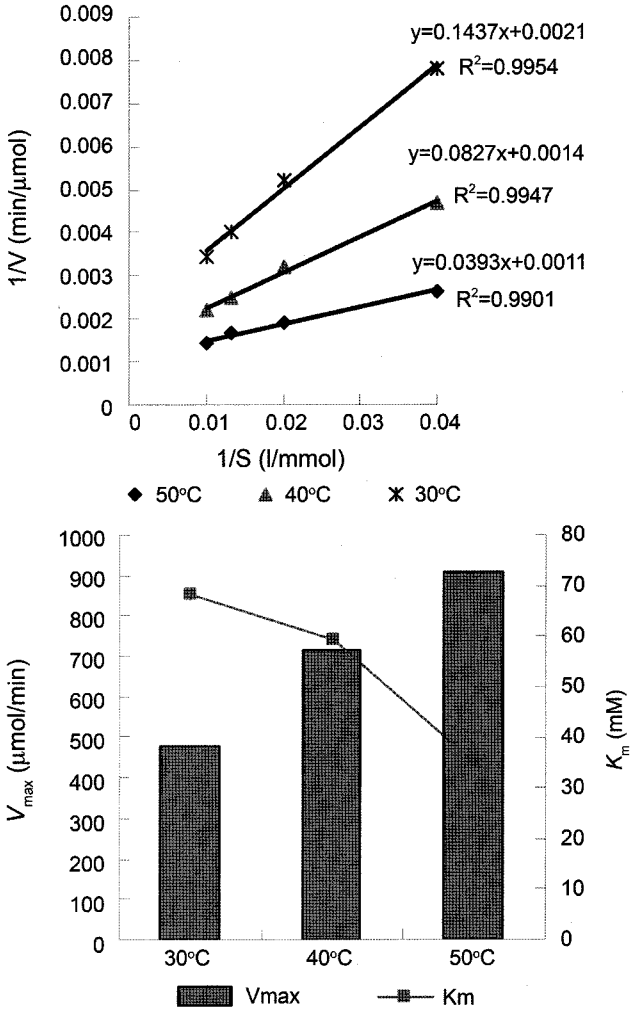

Fig. 3. Kinetic analysis of inulinase activity in SSF at 30°C, 40°C, and 50°C.

The kinetic parameters were calculated using the Michaelis–Menten equation: $\frac{1}{V} = \frac{K_m}{V_m} \times \frac{1}{S} + \frac{1}{V_m}$.

Table 1. Effect of initial hydrolysis time on fructose concentration and lactic acid productivity from Jerusalem artichoke flour by G-02 in sequent SSF[a].

Initial hydrolysis time (h)	0	0.5	1	1.5	2
Fructose conc. (g/l)	10.5±0.21	38.6±0.42	62.4±0.52	82.6±0.61	97.3±0.82
Lactic acid productivity (g/l/h)	2.3±0.11	2.8±0.12	3.3±0.14	3.1±0.14	2.7±0.13

[a]Experiments were performed in a 7-l jar fermentor containing 4 l of a liquid culture with 200 g/l of initial Jerusalem artichoke flour incubated at 50°C for 0.5 to 2 h with a 10% inoculation volume of G-02 and no pH control. The results are expressed as the means of duplicate measurements under the same condition in three independent experiments. The standard deviations were lower than 5% of the values (for fructose concentrations and lactic acid productivity).

[13], it was concluded that the lactic acid productivity was enhanced when hydrolyzing the substrate at 50°C with a concentration of 200 g/l before inoculation, and then conducting the fermentation at a more favorable temperature of 40°C.

Effect of Initial Hydrolysis Time on Lactic Acid Productivity

The experiments were designed to investigate the lactic acid production from Jerusalem artichoke flour at 40°C with various initial hydrolysis times, and the results are summarized in Table 1. Although increasing the initial hydrolysis time increased the fructose concentration, the maximum lactic acid productivity in the SSF was achieved with an initial hydrolysis time of 1 h. Disregarding the osmotic effect resulting from the increased molar concentration in the medium by the initial hydrolysis, the fermentation activity of G-02 was subjected to substrate inhibition [35], and the rate of lactic acid yield (γ_p) in the SSF process was estimated using Eq. (2):

$$r_p = Y_{p/x} \cdot \mu C_x \quad (2)$$

where $Y_{p/x}$ is the apparent yield coefficient, μ is the cellular growth rate, and C_x is the biomass of G-02. When considering the anaerobic conditions, C_x was near constant in this process, and therefore, a high μ and $Y_{p/x}$ were essential for an enhanced γ_p. In the substrate inhibition kinetics, μ was expressed as follows:

$$\mu = \frac{\mu_{max} C_S}{K_S + C_S + \frac{C_S^2}{K_{SI}}}; \quad C_{S,max} = \sqrt{K_S K_{SI}} \quad (3)$$

where K_S is the saturation constant, K_{SI} is the substrate inhibition constant, and C_S is the fructose concentration. Equation (3) shows that the maximum μ was obtained in the medium with a substrate concentration of $C_{S,max}$. It was also found that the maximum lactic acid productivity was obtained in the culture with a fructose concentration of 62 g/l. Thus, to further enhance the lactic acid production, it was necessary to enhance the $Y_{p/x}$ of the process.

Effect of Citrate on Lactic Acid Productivity

The effect of sodium citrate on the lactic acid production of G-02 was investigated at 40°C in SSF, and the results showed that the lactic acid productivity was significantly influenced by the addition of different concentrations of sodium citrate, where the maximum productivity of 4.7 g/l/h was obtained when increasing the sodium citrate concentration to 10 g/l. Surprisingly, the addition of a high concentration of sodium citrate led to a dramatic decrease in the lactic acid production.

The saccharification and fermentation of the Jerusalem artichoke flour proceeded simultaneously within the 7-l fermentor containing 4 l of a medium with enzyme activity 50 U/ml, sodium citrate 10 g/l, $CaCO_3$ 50 g/l, and Jerusalem artichoke flour 200 g/l, with an initial pH of 6.5 and no pH control. After hydrolysis at 50°C for 1 h, the culture was

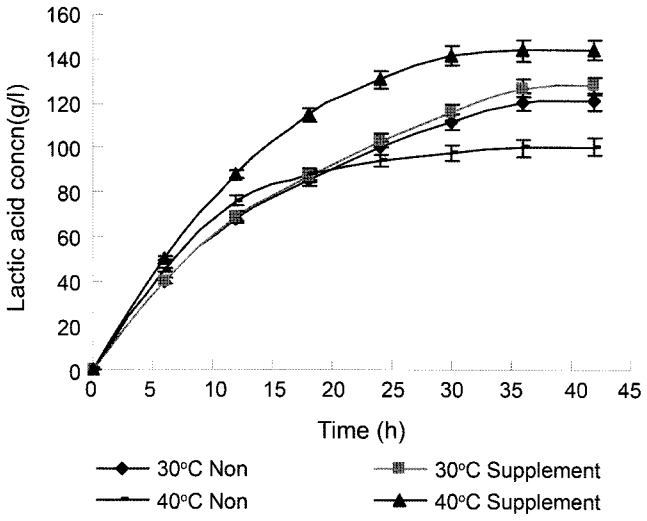

Fig. 4. L-Lactic acid production from Jerusalem artichoke flour in fed-batch culture at 30°C and 40°C with or without sodium citrate supplement.

The sodium citrate was supplemented at a concentration of 10 g/l, and the fermentations were performed in a 7-l jar fermentor containing 4 l of the liquid culture. After hydrolysis at 50°C for 1 h, the culture was continued with an inoculation volume of 10% at 30°C and 40°C, respectively. In the fed-batch fermentation, 180 and 100 g of Jerusalem artichoke flour were added to the medium after 12 h and 24 h of fermentation, respectively. The medium was agitated at the beginning of the fermentation to submerge the solid matter in the liquid, and then incubated without agitation. Cultures without the supplement were used as the control. The results are expressed as the means of duplicate measurements under the same conditions in three independent experiments. The standard deviations were lower than 5% of the values (for lactic acid concentrations).

continued with an inoculation volume of 10% at 30°C and 40°C, respectively. In the fed-batch fermentation, 180 and 100 g of Jerusalem artichoke flour were added to the medium after 12 and 24 h of fermentation, respectively. The medium was agitated at the beginning of the fermentation to submerge the solid matter in the liquid, and then incubated without agitation. As shown in Fig. 4, in the media without the sodium citrate supplement, increasing the fermentation temperature from 30°C to 40°C resulted in a dramatic decrease of lactic acid production after 18 h of fermentation. However, this phenomenon was removed by adding 10 g/l of sodium citrate to the media, which then resulted in a high L-lactic acid concentration of 141.5 g/l after 30 h and a favorable productivity of 4.7 g/l/h. The yield of this process was 52.4 g L-lactic acid/100 g Jerusalem artichoke flour, and the conversion efficiency of the total available sugar to lactic acid was 93.6% of the theoretical lactic acid yield. However, this significant enhancement with the addition of sodium citrate was only found at 40°C, a temperature that is very close to the upper limit for this bacterium. The results also showed that fermentation without the supplement of sodium citrate essentially stopped when the lactic acid concentration reached about 80 g/l at 40°C, but not at 30°C. This could be an additive effect of lactic acid toxicity and temperature.

Table 2. Extracellular parameters of G-02 after 15 and 18 h in medium with or without 10 g/l of citrate.

Parameter	Time (h)	Control	With citrate
Fructose[a]	15	44.32±0.5	25.09±0.42
180.00[b]	18	37.12±0.4	11.01±0.31
Pyruvic acid[a]	15	0.28±0.02	0.15±0.01
88.06[b]	18	0.33±0.02	0.16±0.01
Lactic acid[a]	15	82.20±0.81	101.62±1.10
90.08[b]	18	87.92±0.92	115.11±1.13
Acetic acid[a]	15	0.31±0.03	1.88±0.05
60.05[b]	18	0.45±0.04	2.42±0.08
Biomass[c]	15	5.34±0.16	5.73±0.18
	18	5.41±0.17	6.11±0.21
μ^d	15	<0.01	0.02±0.01
P_s^e	18	16.25	18.84
$pH_{ex}{}^f$	18	4.78±0.11	4.16±0.10
Y_f^g	18	87.83%	91.21%
Y_t^h	18	64.08%	83.89%

[a]Concentrations of metabolites expressed in g/l.
[b]Molecular weight of metabolites.
[c]Biomass of G-02 expressed in g DCW/l.
[d]Specific growth rate of G-02 after 15 h of fermentation expressed in 1/h.
[e]Specific productivity of G-02 after 18 h expressed in g lactic acid/g DCW/h.
[f]pH of liquid culture after 18 h of fermentation.
[g]Lactic acid yield after 18 h expressed in g lactic acid/g fructose consumed.
[h]Lactic acid yield after 18 h expressed in g lactic acid/g total available sugars.
Note: After incubating for 15 and 18 h under anaerobic conditions by SSF, 20 ml of the culture was removed from the fermentation vessel, frozen immediately in liquid nitrogen for 60 s, filtrated through cheese-cloth, and centrifuged at 5,000 ×*g* for 15 min at 4°C to obtain a clear supernatant, as described under Materials and Methods. The extracellular metabolite concentrations are expressed as the amount present in 1 ml of the incubation mix just before quenching. The measurements are averages of three supernatants. The standard deviations were lower than 10% of the values.

Influence of Citrate on Metabolism of G-02 at 40°C

The extracellular metabolite concentrations of G-02 after 15 and 18 h are presented in Table 2, and the metabolite accumulation rates were calculated using the following equation (Table 3):

$$V = \frac{(C_{18h} - C_{15h}) \times 1,000}{MW \times 3} \quad (4)$$

where V (mmol/l/h) is the metabolite accumulation rate, C_{18h} and C_{15h} are the metabolite concentrations measured after 15 and 18 h, respectively, and MW is the molecular weight of the metabolites. As seen, the fructose consumption rate of G-02 after 18 h of fermentation was significantly enhanced by the citrate metabolism, and more than 2-fold higher than that in the control. Meanwhile, the pyruvate accumulation rate of G-02 in the control was nearly 10-fold higher than that in the medium supplemented with citrate. Summarizing the results in Tables 2 and 3, the extracellular acetate concentration and accumulation rate of G-02 after 18 h were both significantly enhanced by the citrate metabolism, and 5.4- and 3.7-fold higher, respectively, than those of the control. This was mainly due to the cleavage of the citrate by lyase. Although the media were supplemented with 50 g/l of $CaCO_3$ to alleviate the pH decrease caused by the lactic acid production, the pH_{ex} value decreased to near 4.1 after 18 h in the medium with citrate, which was about 0.6 pH units lower than that of the control.

Summarizing the results in Tables 2 and 4, the conversion efficiency and lactic acid productivity of G-02 were both significantly enhanced by the citrate metabolism. Thus, to understand the mechanism leading to this increase, the intracellular metabolites of G-02 after 18 h of fermentation were quantitatively analyzed (Table 5). The NOX activity in G-02 was enhanced by the citrate metabolism, and nearly 2-fold higher than that in the control. A comparison of the [NADH]/[NAD^+] ratio between the control and the

Table 3. Extracellular metabolite accumulation/consumption rate of G-02 after 18 h in medium with or without 10 g/l of citrate.

Metabolites[a]	Control	With citrate
Fructose	11.47	26.07
Pyruvic acid	0.21	0.02
Lactic acid	21.17	49.97
Acetic acid	0.81	2.98

[a]Fructose is expressed as consumption rate in mmol/l/h, and the others are expressed as accumulation rate in mmol/l/h.
Values of extracellular metabolite concentrations between control and medium with citrate differed significantly (α=0.05).

Table 4. Extracellular metabolite distribution of G-02 in medium with or without 10 g/l of citrate after 18 h.

Metabolites[a]	Control	With citrate
Fructose	100.00	100.00
Pyruvic acid	0.87	0.04
Lactic acid	92.28	95.78
Acetic acid	2.37	3.83

[a]Calculation when regarding fructose as 100.

medium with citrate showed that the citrate metabolism decreased the ratio of [NADH]/[NAD^+] from 0.48 to 0.21, which was mainly due to the enhanced NOX activity. Further studies found that citrate metabolism resulted in an ATP concentration that was nearly 1.5-fold higher than that in the control, whereas the ratio of [ATP]/[ADP] was enhanced from 0.11 to 0.13. When comparing the pH_{ex} and pH_{in} values in Tables 2 and 5, although the pH_{ex} value for the medium with citrate was 0.6 pH units lower than that for the control, the pH_{in} value was 0.3 pH units higher, implying that more protons were translocated by the G-02 cells to achieve pH homeostasis, which was mainly due to the enhanced ATP concentration caused by the citrate metabolism, thereby improving the specific growth and productivity, as shown in Table 2. Nonetheless, the high citrate concentration results enhanced the *in vivo* acetic acid concentration [36], which may have detrimental effects on the cell physiology [17].

Table 5. Intracellular parameters of G-02 in medium with or without 10 g/l of citrate after 18 h.

Parameters	Control	With citrate
NAD[a]	20.88±0.21	25.61±0.26
NADH[a]	10.21±0.12	5.29±0.18
ADP[a]	11.03±0.14	11.97±0.13
ATP[a]	1.18±0.03	1.62±0.02
Pyruvic acid[b]	3.56±0.04	1.86±0.03
Lactic acid[b]	346.56±8.03	326.71±7.35
Acetic acid[b]	5.26±0.17	12.92±0.19
NOX activity[c]	547.91±11.19	926.56±14.15
pH_{in}[d]	5.15±0.16	5.48±0.14

[a]Concentration expressed as nmol/ml.
[b]Concentration expressed as g/l.
[c]NOX activity expressed as nmol/min/mg cell protein.
[d]Intracellular pH of G-02 after 18 h of fermentation.
Note: After incubation for 18 h under anaerobic conditions by SSF, the culture of G-02 was removed from the fermentation vessel, frozen immediately in liquid nitrogen for 60 s, filtrated through cheese-cloth to obtain a clear supernatant, and then centrifuged at 5,000 ×*g* for 15 min at 4°C to harvest the cells, as described under Materials and Methods. The intracellular data are expressed as the amount present in 1 ml of the incubation mix just before quenching (the cytosolic volume was approximately 2% of the volume of the incubation mixture). The measurements are the averages of three extracts. The standard deviations were lower than 5% of the values.

DISCUSSION

Although SSF has long been proposed as a beneficial process for ethanol and lactic acid production from raw substrates, its low conversion efficiency and easy contamination make it industrially impractical [5]. To enhance the conversion efficiency of lactic acid from Jerusalem artichokes using SSF, this study found that a high fermentation temperature was necessary to enhance the inulinase activity and denaturation of the macromolecular inulin, making the substrate more accessible to the enzyme. This would also be attractive to the lactic acid industry, as this would dramatically decrease the cost of cooling in the summer. However, increasing the culture temperature enhances the lactic acid toxicity on the cell physiology [32, 37], and therefore, the kinetic properties of inulinase were investigated with a concentrated culture supernatant of *A. niger* SL-09 under SSF conditions. The results showed that the V_{max} and K_m values for inulinase at 50°C were 909.5 μmol/(mg·min) and 34.9 mmol/l, respectively. Chen *et al.* [4] previously reported that the V_{max} and K_m values for fungal inulinase at 60°C were 67.11 μmol/(mg·min) and 6.25 mmol/l, respectively. Thus, when disregarding the difference in the assay temperatures, the V_{max} and K_m values for inulinase in the SSF were over 13 and 5-fold higher, respectively, than those in the previous report, indicating that the enzyme activity was enhanced significantly in the SSF, most likely due to the lack of product inhibition. Nonetheless, as the macromolecular inulin in the SSF made the substrate more inaccessible to the enzyme, the substrate concentration required for the maximum enzyme activity was also enhanced.

Fig. 5 shows the metabolic pathway of G-02 in the medium supplemented with citrate, where the carbon source, energy, and reducing force for lactic acid production were mainly provided by the Embden–Meyerhof–Parnas (EMP) pathway from fructose. The citrate, transported *via* a plasmid-encoded carrier (CitP), was cleaved by citrate lyase into acetate and oxaloacetate, the latter of which was subsequently decarboxylated into pyruvate by the action of oxaloacetate decarboxylase. Thus, to enhance the lactic acid production, it is necessary to increase the carbon flux of the EMP pathway, and decrease the acid toxicity on the cells. Previous studies found that a high concentration of ATP is required to maintain the proton motive force (PMF) to resist acid stress [6, 9], but increasing the ATP concentration in the cells decreases the flux of the EMP pathway [20, 22]. Torino *et al.* [36] found that effect of citrate on cell growth was partially dependent on the extra ATP gained by the cells. More recently, Sánchez *et al.* [33] reported that the beneficial effect of citrate on cell growth under acid stress conditions primarily stemmed from less ATP expenditure [33]. In the present study, the citrate metabolism caused a 2-fold higher NOX activity in the G-02 cells than in the control, which significantly improved

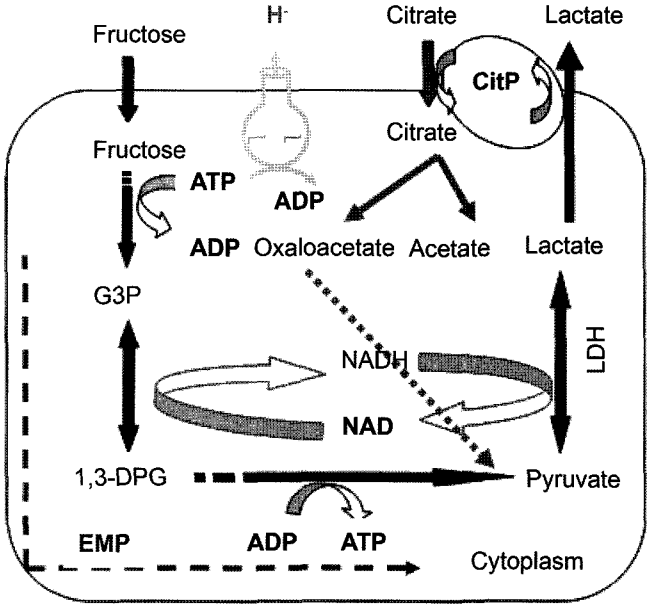

Fig. 5. Metabolic flux distribution maps for G-02 in fermentation with citrate supplement.

the carbon flux of the EMP pathway by decreasing the accumulation of pyruvate. As a result, the specific productivity of lactic acid after 18 h was 1.16-fold higher than that for the control, plus the [ATP]/[ADP] ratio increased from 0.11 to 0.13. Therefore, these results suggest that the enhanced ATP concentration mainly resulted from the increased carbon flux of the EMP pathway in the G-02 cells.

According to Torino *et al.* [36], the enhanced NOX activity in *Lactobacillus helveticus*, when cometabolizing citrate with lactose, promoted the synthesis of succinate and changed the fermentation pattern of this strain from a homolactic to a mixed-acid profile. Nonetheless, this phenomenon was not found in G-02, and no succinate was detected in the control or media with citrate, which was mainly due to the high lactate dehydrogenase activity of *Lactobacillus casei*, the most common and preferred bacteria for L-lactic acid production [3, 7, 13, 16, 18, 21, 30]. This was further corroborated by the low concentration and accumulation rate of pyruvate observed for the fermentation with the sodium citrate supplement.

Interestingly, although the extracellular lactic acid concentration was enhanced by the citrate metabolism from 87.92 to 115.11 g/l after 18 h of fermentation, the intracellular lactate concentration was decreased from 346.56 to 326.71 g/l, indicating that the citrate metabolism effectively improved the lactic acid secretion in the G-02 cells. This was also consistent with a recent report that showed that the citrate metabolism endowed *Lactococcus lactis* cells with an extra ability to counteract acid toxicity by acting as a citrate/lactate exchanger [33]. The present observations of the pH_{ex} and pH_{in} values after 18 h of fermentation also confirmed this conclusion.

It has already been reported that a high concentration of lactic acid in the medium gives *Lactobacillus* sp. a competitive advantage over known pathogens and other undesirable bacteria [19, 32]. Thus, to avoid contamination, a high lactic acid productivity is essential to obtain a desirable concentration within a short time. Therefore, in this study, the initial hydrolysis of Jerusalem artichoke flour was performed at 50°C for 1 h before the inoculation to maximize the lactic acid productivity in the SSF, plus the inoculation volume was decreased to 10% of the medium. Furthermore, to enhance the resistance of the G-02 cells to acid stress at 40°C, the media were supplemented with sodium citrate to enhance the carbon flux of the EMP pathway. As a result, an L-lactic acid concentration of 141.5 g/l was obtained at 40°C after 30 h, with a productivity of 4.7 g/l/h in the medium with 10 g/l of sodium citrate. Therefore, the present results confirmed that a high lactic acid productivity could be obtained from Jerusalem artichokes when simultaneously removing the *in intro* repression (fructose repression) and *in vivo* stress (acid stress) in the SSF, allowing further application in the lactic acid industry.

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