Open Fermentative Production of L-Lactic Acid from Distillers' Grains by *Lactobacillus casei* CICC 6056

Jin Zheng,[a] Yingying Liu,[b] Xiaohong Sun,[c,*] Qunhui Wang,[a,*] Hui Zou,[a] Juan Wang,[a] and Ming Gao [d]

Distillers' grains (DG) are potential fermentable sugar substrates for the production of value-added products. This study focused on the development of an open fermentation process for the production of L-lactic acid from DG using *Lactobacillus casei* CICC 6056. The open fermentation process was feasible, resulting in an L-lactic acid yield similar to that obtained with sterilized fermentation. However, a decrease in pH below 5.0 after 24 h resulted in a poor L-lactic acid concentration of 9.18 g/L and an inferior reducing sugar conversion rate of 53.6%. Therefore, an optimal pH adjustment method was sought. A pH adjustment to 6.5 every 24 h effectively improved the L-lactic acid concentration to 21.3 g/L with a 97.8% reducing sugar conversion rate. Furthermore, the application of a simultaneous saccharification and fermentation process at 50 °C in open condition increased the L-lactic acid concentration to 25.0 g/L. This yield was superior to L-lactic acid fermentation from DG using a separate hydrolysis and fermentation method (21.3 g/L) and from a commercial sugar in batch culture (23.6 g/L). These results demonstrated that *L. casei* CICC 6056 has great industrial potential as a superior candidate strain for the production of L-lactic acid from DG due to its broad applicability in a wide temperature range (from 37 to 50 °C).

Keywords: Open fermentation; L-Lactic production; Distillers' grains; Lactobacillus casei

Contact information: a: Department of Environmental Engineering, School of Civil and Environmental Engineering, University of Science and Technology Beijing, No.30 Xueyuan Road, Haidian District, Beijing 100083, China; b: China National Institute of Standardization, No.4 Zhi Chun Road, Haidian District, Beijing 100088, China; c: Beijing Academy of Agricultural and Forestry Sciences, No.11 Shuguang Garden Road, Haidian District, Beijing 100097, China; d: Laboratory of Microbial Technology, Division of Systems Bioengineering, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan;
** Corresponding authors: sunxiaohong19675@aliyun.com; wangqh59@sina.com*

INTRODUCTION

Lactic acid is a valuable industrial chemical with various industrial applications. Lactic acid can be utilized as an acidulant, flavoring agent, and preservative in the food, pharmaceutical, leather, and textile industries. It can also be used for the production of base chemicals for polymerization to generate biodegradable polylactic acid (PLA) (John *et al*. 2007). PLA is considered one of the most promising biodegradable and biocompatible polymers. PLA has an increasingly important role in reducing the net emission of carbon dioxide and petroleum demand (Sun *et al*. 2012). Therefore, the demand for L-lactic acid, which can be produced using fermentative processes, has substantially increased due to its use as a monomer in the preparation of PLA (Wee *et al*. 2006). However, there are two major challenges in the production of L-lactic acid *via*

microbial fermentation, namely, the high cost of feedstock such as traditional food crops and the high operational cost associated with sterilization, downstream separation, and purification.

Many renewable raw materials from agricultural industries, such as residual starch, lignocellulose, and industrial and municipal waste products have been used as substrates for L-lactic acid production to reduce the cost of raw material (Wang *et al*. 2014). Distillers' grains (DG), a byproduct of the dry grind ethanol process, have high polymeric sugar contents and are relatively easy to hydrolyze (Kim *et al*. 2008). DG is mainly composed of seed hull, germ, proteins, and oil, and is generally marketed as animal feed due to its high nutritional value. In addition, DG has various biotechnological applications to produce value-added products, such as antioxidants, monosaccharides, oligosaccharides, bioethanol, biogas, and lactic acid (Xiros and Christakopoulos 2012; Mussatto 2014; Chatzifragkou *et al*. 2015).

During lactic acid fermentation, an open or non-sterilized fermentation is favorable because it reduces equipment needs, energy consumption, and labor costs. In addition, without autoclaving, the degradation of sugar substrates and other nutritional elements for lactic acid fermentation, and the generation of unfavorable furfural compounds that can inhibit bacterial growth can be avoided (Ma *et al*. 2014). However, widespread indigenous microorganisms have a similar optimal fermentation temperature, which ranges from 30 to 40 °C. Therefore, contamination is often unavoidable without sterilization of the medium (Qin *et al*. 2009). The application of thermophilic L-lactic acid-producing bacteria can be effective to reduce contamination. Thermophilic *Bacillus* species are novel lactic acid producers that have a higher range of fermentation temperature of 50 to 60 °C, simple nutrition requirements, and the ability to produce L-lactic acid with high optical purity (Sakai and Ezaki 2006a; Wang *et al*. 2013). Lactobacilli are typically used in L-lactic acid fermentation, producing a high yield of lactic acid (Bai *et al*. 2003; Ding and Tan 2006). However, lactobacilli have an optimal temperature range of 35 to 45 °C. To date, no report has indicated the use of lactobacilli to produce L-lactic acid using an open fermentation system.

In previous studies, an open fermentation method was utilized to produce lactic acid from kitchen waste by controlling fermentation conditions including temperature, oxygen, and pH (Wang *et al*. 2002; 2003). The present study is the first to demonstrate an open L-lactic acid fermentation of DG by *Lactobacillus casei* at an elevated temperature. The effects of critical factors such as pH and temperature during open fermentation were also investigated. Therefore, this study provided important bases for adopting *L. casei* in open L-lactic acid fermentation from DG.

EXPERIMENTAL

Materials

Microorganism and culture media

L. casei CICC 6056 was purchased from the China Center of Industrial Culture Collection (Beijing, China). *L. casei* was maintained on Man-Rogosa-Sharpe (MRS) agar slants (10 g/L peptone, 8 g/L beef extract, 4 g/L yeast extract, 20 g/L glucose, 2 g/L triammonium citrate, 10 g/L $CaCO_3$). The initial pH was adjusted to 6.5 and the slants were incubated at 37 °C. Fully grown slant cultures were stored at 4 °C and sub-cultured every 2 months. MRS liquid medium was sterilized at 121 °C for 20 min and used for

seed preparation. The seed culture was prepared by inoculating a group of cells from a fully-grown slant culture into 50 mL of seed medium in a 100 mL Erlenmeyer flask using an inoculation loop. The flask was incubated at 37 °C for 12 h with an agitation rate of 150 rpm. The inoculum size for subsequent open or sterilized fermentation was 10% (v/v).

Distillers' grains (DG)

The DG used in this study was kindly provided by the Niulanshan Winery (Beijing Shunxin Agriculture Co., Ltd; Beijing, China) and contained 12.5% cellulose, 12.3% hemicellulose, and 25.38% lignin. The DG was dried at 80 °C for 24 h in an air circulation oven, ground to a uniform length of less than 1 mm using an electric grinder, and subsequently packaged and stored in plastic bags at room temperature until use. Ground DG was mixed with tap water at 25% (w/v) for L-lactic acid fermentations.

Methods

Open fermentation of DG

In order to assess the feasibility of an open fermentation of DG, the production of L-lactic acid under open conditions was compared to that performed under sterilized conditions. A medium containing 25 g of DG and 100 mL of tap water without nutrient supplementation was used as a fermentation substrate in a 250 mL Erlenmeyer flask. Before the fermentation, a cellulase hydrolysis was performed at 50 °C for 72 h. Cellulase with an enzymatic activity of 400 U/g was obtained from Beijing Donghua Biotechnology Co., Ltd. (Beijing, China) and used for cellulase loading at 10 U/g of DG. The pH for cellulase hydrolysis was 4.8. Subsequently, the resulting hydrolysate with solid fraction was used as the substrate for open fermentation of DG. For the sterilized fermentation, the same medium was autoclaved at 121 °C for 20 min. The initial pH was adjusted to 6.5. In both conditions, fermentation was performed anaerobically at 37 °C for 144 h at an agitation rate of 150 rpm.

To evaluate the effect of pH adjustments on open L-lactic fermentation, the pH of the medium was adjusted using 10% ammonium hydroxide in three pH swing modes: 1) the initial pH was adjusted to 6.5 (pH 6.5); 2) the initial pH was adjusted to 6.5 and subsequently swung to 6.5 every 12 h (pH 6.5-12 h); and 3) the initial pH was adjusted to 6.5 and subsequently swung to 6.5 every 24 h (pH 6.5-24 h). The fermentation medium was prepared as described above, but without sterilization. Samples were taken every 12 h for analysis.

Simultaneous saccharification and fermentation (SSF) was performed using different temperatures for L-lactic acid production from DG. For the SSF, cellulase was used at the same loading condition as in the previous experiment. Cellulase hydrolysis and L-lactic acid fermentation were performed simultaneously at different temperatures [37 °C (SSF 37), 43 °C (SSF 43), and 50 °C (SSF 50)] for 168 h using the pH 6.5-24 h adjustment mode.

The separate hydrolysis and fermentation (SHF) was compared to the SSF. For the SHF, cellulase hydrolysis was performed for 72 h. Subsequently, L-lactic acid fermentation was initiated using 10% inoculum for 96 h. For the control experiment, commercial glucose was used at 24 g/L and MRS medium was utilized for the L-lactic acid production. This concentration of commercial glucose was equivalent to that of the reducing sugars in DG hydrolysate. Samples were collected every 24 h to determine the concentration of L-lactic acid and reducing sugars.

In order to evaluate the growth of *L. casei* CICC 6056 at a higher temperature, 10% *L. casei* CICC 6056 was grown in MRS medium with 25 g/L glucose at 50 °C. Fermentation at the established optimal temperature of 37 °C was conducted as a control. The cells were cultured at an agitation speed of 150 rpm without aeration for 52 h, using the pH 6.5-24 h adjustment mode. OD_{600} and L-lactic acid concentration were measured every 4 h.

Analytical methods

Reducing sugars were measured using the 3,5-dinitrosalicylic acid method (Miller 1959). L-Lactic acid concentration was measured using an SBA-40C biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, Jinan, China) based on the specific enzymatic reaction. The cellulose, hemicellulose, and Klason lignin contents were determined based on differences between the neutral (NDF) and acid detergent fiber (ADF) contents, the ADF and acid detergent lignin (ADL) contents, and the ADL and ash contents, respectively (Van 1963).

Statistical analysis

The experiments were done in triplicate. All values are expressed as the mean ± standard deviation. Values from different treatment groups were compared using the analysis of variance. Differences at $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Comparison of Open and Sterilized Fermentation Methods

The effects of medium sterilization were assessed (Fig. 1). After cellulase hydrolysis for 72 h, the initial reducing sugar concentration was 26.3 and 23.5 g/L for conditions with and without sterilization, respectively (Fig. 1a). The slightly higher reducing sugar concentration in the sterilized fermentation may have been caused by lignocellulose hydrolysis in DG during autoclaving. A mild thermal pretreatment temperature (100 to 140 °C) and a lower pH (1 to 4) in acidic pretreatment can lead to a high polysaccharide solubilization (Pedersen *et al*. 2011). In this study, an autoclaving temperature of 121 °C may have caused an increase in reducing sugar concentration. Following 96 h of fermentation, a maximum L-lactic acid concentration of 11.6 and 9.18 g/L was obtained, leading to a yield of 0.758 and 0.729 g/g in the sterilized and open fermentations, respectively (Fig. 1b). Despite a higher initial reducing sugar concentration in the sterilized fermentation, a comparable L-lactic acid yield was obtained from both fermentation methods. These results demonstrated the feasibility of an open fermentative production of L-lactic acid from DG.

Regardless of whether open or sterilized fermentation was used, the sugar conversion rate at 96 h was only approximately 50%, indicating that half of the initial reducing sugars in the hydrolysate were not converted to L-lactic acid. This might be due to the sharp decrease in pH during the first 24 h. As shown in Fig. 1c, the pH sharply decreased from 6.5 to 4.5 in the sterilized fermentation, and to 4.9 in the open fermentation at 24 h, and pH in both methods decreased gradually to approximately 4.1 at 144 h. Although *L. casei* was reported to be acid-tolerant and is relatively insensitive to product inhibition by lactic acid (Bruno-Barcena *et al*. 1999), the optimal pH for *L. casei* to produce a significantly higher level of acid was above 5.0 (Yoo *et al*. 1996; Roberto *et*

al. 2007). In this experiment, the pH of the broth was below 5.0 after 24 h of fermentation, which may adversely affect the growth of *L. casei*. Because pH of the environment can alter the charge distribution on cell membrane and its permeability, the low pH may affect enzyme activities *in vivo*, leading to poor cell growth (Ouyang *et al*. 2013). Therefore, a subsequent experiment was aimed to enhance sugar consumption and L-lactic acid concentration by optimizing the pH adjustment method.

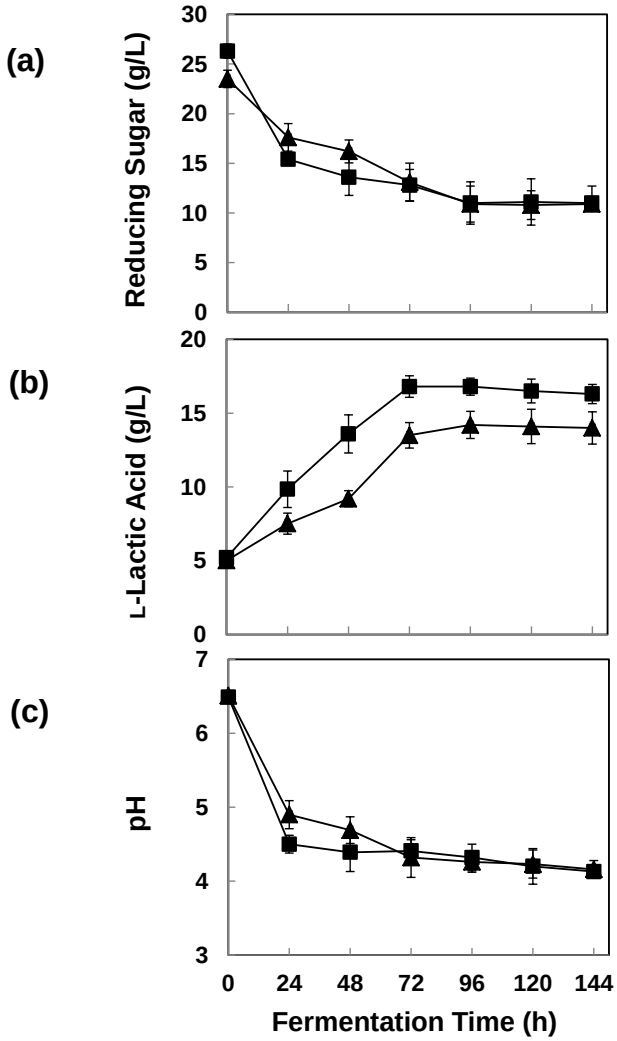

Fig. 1. The fermentation of L-lactic acid using open or sterilized conditions at 37 °C. The ratio of distillers' grains to water was 1:4 (w/v), and the initial pH was adjusted to 6.5. (a) Reducing sugar concentration, (b) L-lactic acid concentration, and (c) pH are shown. (■) in a sterilized condition, (▲) in an open condition

Effect of pH Adjustments on L-Lactic Acid Production in Open Fermentation

The pH of the medium is a critical factor that may influence microbial growth and L-lactic acid production. In this study, three pH adjustment modes were compared to determine the optimal pH conditions. As shown in Fig. 2, reducing sugars were consumed to a final concentration of less than 1 g/L after adjusting the pH to 6.5 during fermentation. This suggested a final sugar conversion rate of >96%. Regardless of the frequency of pH adjustments during fermentation (every 12 or 24 h), approximately 21

g/L of L-lactic acid was produced. This concentration was >2-fold higher than that observed in the process without pH adjustments during fermentation. The pH adjustments during fermentation also improved the L-lactic acid yield from 0.729 g/g to 0.924 and 0.941 g/g using the pH 6.5-24 h and pH 6.5-12 h modes, respectively. Others have reported that the optimal pH for cell growth and lactic acid production was between 5 and 7 (Yoo *et al*. 1996; Roberto *et al.* 2007). Therefore, periodic pH increase to 6.5 may improve lactic acid fermentation. In addition, Taniguchi *et al.* (2005) and Tanaka *et al.* (2006) have reported that a pH environment of 4.5 to 5.0 could suppress the growth of indigenous bacteria during SSF in lactic acid fermentation of unsterilized defatted rice bran. An effective lactic acid production was achieved by periodic pH reductions (using pH swings in the acidic range) to inhibit the proliferation of non-lactic acid bacteria (Sakai *et al*. 2004). In the present study, the pH was swung from 4.0 to 6.5 by periodic pH adjustments (every 12 or 24 h; Fig. 3).

The results showed that when compared to the condition without pH adjustments, periodic pH adjustments to 6.5 not only inhibited the proliferation of non-lactic acid bacteria, but also provided suitable environment for the growth of *L. casei* to produce L-lactic acid. Sakai *et al*. (2006b) also reported that intermittent pH adjustments (adjusted to 7.0 every 12 h) were effective to attain lactic acid accumulation by lactic acid fermentation from model kitchen refuse. These findings suggested that periodic pH adjustments to 6.5 are necessary during L-lactic acid fermentation.

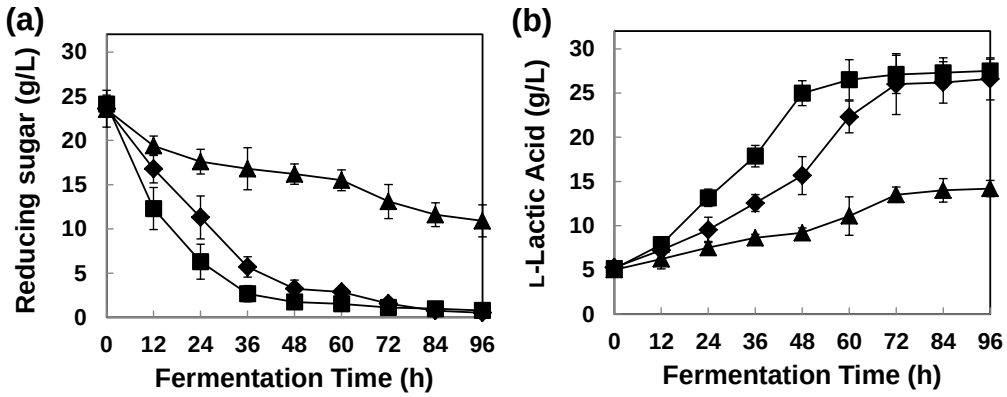

Fig. 2. L-Lactic acid production in open fermentation using different pH conditions. The ratio of distillers' grains to water was 1:4 (w/v). The concentrations of (a) reducing sugar and (b) L-lactic acid are shown. (▲) pH 6.5, (■) pH 6.5–12 h, and (◆) pH 6.5–24 h.

Moreover, the maximum L-lactic acid production rate using the pH 6.5-12 h mode (0.593 g/L/h, obtained between 36 and 48 h) was slightly higher than that obtained using the pH 6.5-24 h mode (0.553 g/L/h, obtained between 48 and 60 h). However, the final L-lactic acid production were similar (22.0 and 21.3 g/L for pH 6.5-12 h and pH 6.5-24 h, respectively).

According to a previous study, ammonium hydroxide is an appropriate pH regulation reagent (among five regulation reagents evaluated, namely ammonium hydroxide, sodium hydroxide, dimethylamine, trimethylamine, and calcium carbonate) based on a technological perspective, such as dilution (Hetenyi *et al*. 2011); however, excess ammonia may be toxic for *L. casei*. Therefore, considering that the aim of the study was to seek a time-saving and cost-effective pH adjustment method, pH adjustment

to 6.5 every 24 h was an optimal method to enhance L-lactic acid production from DG in open fermentations.

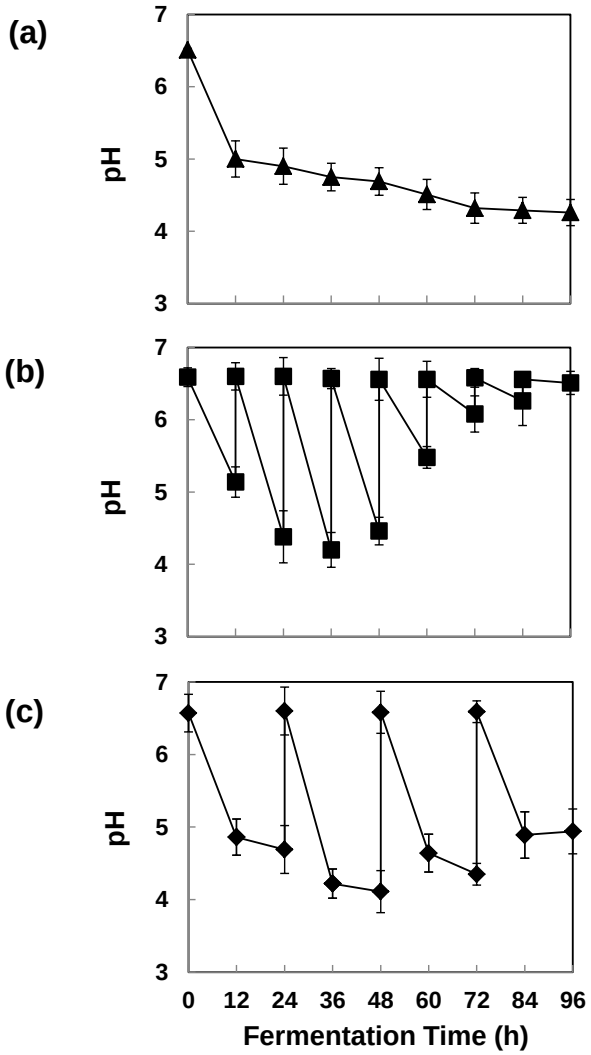

Fig. 3. The change of pH in open fermentation under different pH conditions. The ratio of distillers' grains to water was 1:4 (w/v). (a) pH 6.5, (b) pH 6.5-12 h, and (c) pH 6.5-24 h.

Comparison of SSF and SHF in Open L-Lactic Acid Fermentation from DG

The SSF in experiments described above entailed a 72-h enzymatic hydrolysis of DG and a 96-h L-lactic fermentation step. Although SSF provided optimal conditions for both enzymatic hydrolysis and fermentation in lactic acid production, the time-consuming nature of the process is a major disadvantage. Thus, an experiment was subsequently performed to compare SSF and SHF.

Before the experiment was performed, the optimal conditions for the two biocatalysis processes in SSF, the enzymatic hydrolysis and the microbial fermentation, were considered (Wang *et al*. 2015). The optimal temperature, which can differ significantly for the two biocatalyses, is one of the most important factors in SSF. The optimal temperature for enzyme hydrolysis is approximately 50 °C (Zhang *et al*. 2013; Zhou *et al*. 2014); however, 37 °C is more favorable for the production of lactic acid by

L. casei (Hujanen and Linko 1996). Therefore, 50 °C was chosen for a preliminary examination of the thermal-tolerant property of *L. casei*. Compared to an L-lactic acid fermentation at 37 °C in MRS medium, a long lag phase of cell growth (28 h) was observed at 50 °C (Fig. 4a). This observation was in agreement with a study by Qin *et al*. (2012) that showed a repression of *L. casei* G-03 cell growth when the culture temperature was increased from 38 to 50 °C. Although the maximum cell density at 50 °C was 33.5% lower than that observed at 37 °C, the maximum L-lactic acid concentration was only 9.64% lower than that at 37 °C (Fig. 4b). These results demonstrated that *L. casei* CICC 6056 can tolerate a higher temperature of up to 50 °C. Therefore, the effects of different temperatures (37, 43, and 50 °C) on L-lactic acid production were assessed in a subsequent experiment. In addition to temperature, pH can also affect both the cellulase hydrolysis and *L. casei* growth. The optimal pH for cellulase hydrolysis is 4.8 (Shang *et al*. 2014; Ko *et al*. 2015), which is within the range of pH change (pH 4.0 to 6.5) in the optimized pH condition (pH 6.5-24 h) for lactic acid production described above. Thus, the effect of pH on cellulase activity can be considered negligible within this specific pH range during lactic acid production.

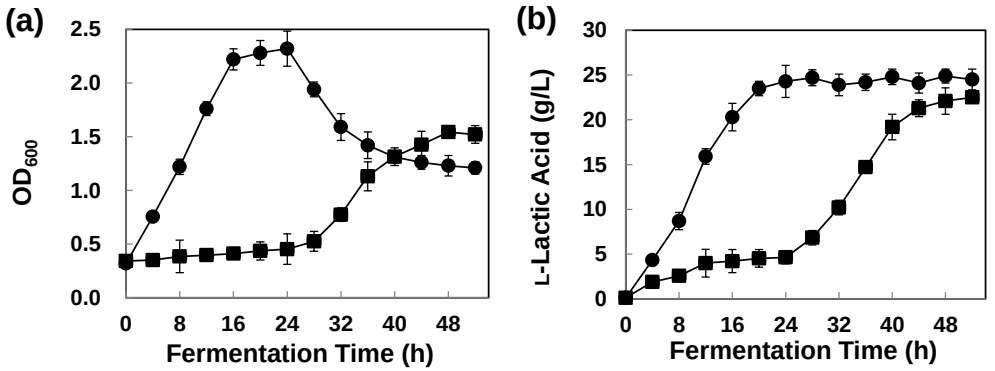

Fig. 4. The thermal-tolerant property of *L. casei* CICC 6056 at 50 °C. (a) OD_{600} and (b) L-lactic acid concentration in an MRS medium with 20 g/L glucose. The pH 6.5-24 h adjustment mode was used. (●) 37 °C (control), (■) 50 °C.

First, the effects of temperature on lactic acid production by open fermentation using SSF were evaluated (Fig. 5). At all temperatures evaluated, the concentration of reducing sugars increased during the first 24 or 48 h, but it sharply decreased with lactic acid accumulation. A higher reducing sugar concentration of 26.1 g/L at 24 h was observed at 50 °C, demonstrating that 50 °C was conducive for the generation of reducing sugars during SSF. Accordingly, a higher L-lactic acid production of 25.0 g/L was obtained at 50 °C when compared to those obtained at other temperatures. Previous studies demonstrated highly efficient L-lactic acid production by *L. casei* at 37 °C (Hujanen and Linko 1996; Buyukkileci and Harsa 2004). Chronopoulos *et al.* (2002) found that the optimal temperature for the growth and lactic acid production of *L. casei* was 43 °C. Qin *et al.* (2012) demonstrated that *L. casei* G-03 produced less L-lactic acid at 50 °C than at a lower temperature range of 35 to 45 °C. However, in the current study, 50 °C was found to be more permissive for cellulase hydrolysis and lactic acid production. These differences in optimal temperature for lactic acid production by *L. casei* may have been due to different characteristics of the *L. casei* strain used and the fermentation conditions. In this study, lactic acid fermentation was performed using open conditions and a combined enzymatic hydrolysis and fermentation step. Here, 50 °C was

determined to be the optimal temperature for both enzymatic activity and cell growth (SSF 50; Table 1). The elevated temperature might have also suppressed the growth of indigenous bacteria, which resulted in improved L-lactic acid production by *L. casei* under the open conditions. Therefore, a future study will focus on the analysis of microbial population during open lactic acid fermentation.

Furthermore, SHF and SSF 50 (SSF at 50 °C) was also compared (Fig. 5). As a control, fermentation from a commercial glucose, used at the same reducing sugar concentration as that from DG after cellulase hydrolysis (24 g/L), was conducted. After hydrolysis for 72 h, 24.4 g/L of reducing sugars were generated in the SHF. This concentration was lower than that observed in SSF at 24 h (26.1 g/L), because sugar accumulation in the hydrolysis step during SHF can inhibit the activity of cellulase, leading to a lower reducing sugar concentration. In SSF, the hydrolysis and fermentation steps are carried out simultaneously in a single vessel. Thus, glucose released by the action of cellulases is converted directly to lactic acid by fermenting microorganisms. Additionally, continuous removal of sugars from the medium minimizes the inhibition of enzyme activity by the feedstock. This resulted in enhanced saccharification and lactic acid yield in SSF relative to those in SHF (Singhania *et al*. 2014). L-lactic acid production in SSF was 17% higher than that in SHF. In addition, the maximum rate of L-lactic acid production in SSF (0.462 g/L/h, obtained at 48–72 h) was higher than that in SHF (0.430 g/L/h, obtained at 120–144 h). This finding suggested that SSF was able to accelerate the fermentation, thus shorten the fermentation time. Furthermore, L-lactic acid production in SSF 50 (25.0 g/L) was similar to that in glucose fermentation (23.6 g/L), indicating that DG was a good feedstock candidate for lactic acid production.

During grain-based ethanol production, a liquid fraction (thin stillage) and solid fraction (wet distillers' grains) are produced as byproducts of ethanol distillation and dehydration (Chatzifragkou *et al*. 2015). The utilization of liquid distillery stillage as a substrate for lactic acid production has been extensively studied. However, the concentration of reducing sugars in the sterile liquid stillage was only 12 g/L; thus, glucose supplementation is needed to improve the initial reducing sugar concentration for lactic acid production (Djukic-Vukovic *et al.* 2012; 2013). In this study, approximately 25 g/L of reducing sugar was obtained from the enzymatic hydrolysis of DG without optimizing the substrate loading. Therefore, one benefit of the method used in this study was the ability to adjust the amount of initial reducing sugars by enzymatic hydrolysis of different DG quantities for lactic acid production, without supplementation with commercial sugars. In addition, most lactic acid fermentation methods from distillery waste have been performed in sterilized condition and with nutrient supplementation (Pejin *et al*. 2015; Zhang *et al*. 2011). The present study evaluated open fermentation without nutrient supplementation. Following the optimization of fermentation process, L-lactic acid production was improved by 2.7-fold (Table 1).

Open fermentation processes using thermophilic *Bacillus* species at a relatively high temperature of approximately 50 °C have been commonly used for lactic acid production. In order to obtain active *Bacillus* species, it is critical that strain refresh, seed culture, and fermentation are conducted at 50 °C (Ma *et al*. 2014; Wang *et al*. 2015). In the present study, *L. casei* CICC 6056 was used because it can be cultivated at 37 °C and tolerate a high temperature of 50 °C in lactic acid fermentation. For the application of *L. casei* in industrial manufacturing of lactic acid, it is important that lactic acid production by *L. casei* CICC 6056 can be performed in a wide range of temperature.

Due to differences in fermentation mode and conditions, it was challenging to compare the results of this study with those of other studies. Nevertheless, L-lactic acid production was improved from 9.18 to 25 g/L in this study. Further optimization of fermentation parameters such as the initial substrate concentration may further enhance the efficiency of L-lactic acid production. Because *L. casei* CICC 6056 can tolerate a higher temperature of 50 °C, a significant amount of L-lactic acid can be produced from DG using SSF at 50 °C.

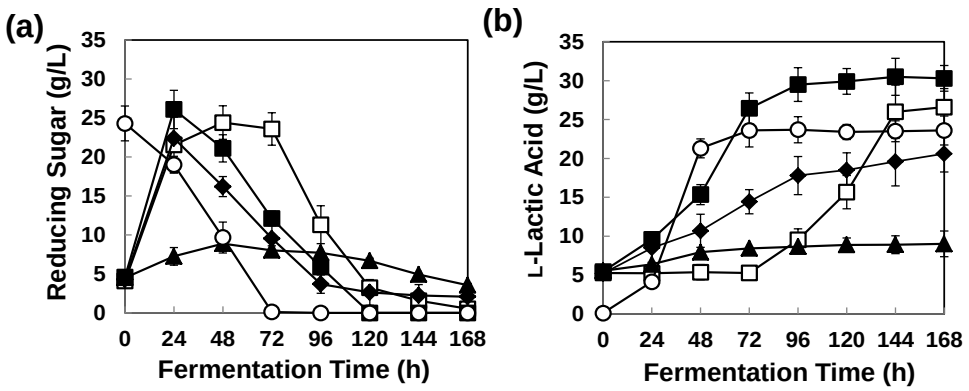

Fig. 5. The effect of temperature on L-lactic acid production in an open fermentation of distillers' grains (DG). The levels of (a) reducing sugar and (b) L-lactic acid are shown. The ratio of DG to water was 1:4 (w/v). During the lactic acid fermentation, the initial pH was adjusted to 6.5, and the pH was subsequently swung to 6.5 every 24 h. (■) SSF at 50 °C, (◆) SSF at 43 °C, (▲) SSF at 37 °C, (□) SHF, and (○) control (24 g/L glucose).

Table 1. Maximum L-Lactic Acid Concentrations

Experiments	Sterilized or Open Fermentation	Fermentation Mode	pH Adjustment	Temperature (°C)	Maximum L-Lactic Acid Concentration (g/L)
Sterilized condition	Sterilized	SHF	pH 6.5	H: 50; F: 37	11.6 ± 0.6
Open condition (pH 6.5)	Open	SHF	pH 6.5	H: 50; F: 37	9.18 ± 0.87
pH 6.5-12 h	Open	SHF	pH 6.5-12 h	H: 50; F: 37	22.0 ± 1.5
pH 6.5-24 h	Open	SHF	pH 6.5-24 h	H: 50; F: 37	21.3 ± 1.6
Glucose (24 g/L)	Open	—	pH 6.5-24 h	50	23.6 ± 2.1
SSF 37	Open	SSF	pH 6.5-24 h	37	3.46 ± 0.53
SSF 43	Open	SSF	pH 6.5-24 h	43	15.4± 1.1
SSF 50	Open	SSF	pH 6.5-24 h	50	25.0 ± 1.4

SHF: separate hydrolysis and fermentation
SSF: simultaneous saccharification and fermentation
H: hydrolysis
F: fermentation
pH 6.5: the initial pH was adjusted to 6.5
pH 6.5-12 h: the initial pH was adjusted to 6.5, and the pH was subsequently swung to 6.5 every 12 h
pH 6.5-24 h: the initial pH was adjusted to 6.5, and the pH was subsequently swung to 6.5 every 24 h
—: neither SHF nor SSF, batch fermentation only

CONCLUSIONS

The production of L-lactic acid from DG in open fermentation was feasible and resulted in a comparable L-lactic acid yield to those obtained using sterilized fermentation (0.729 and 0.758 g/g for the open and sterilized conditions, respectively). An adjustment of the initial pH of the medium to 6.5 and subsequently adjusting it to 6.5 every 24 h resulted in a pH change during fermentation from 4.0 to 6.5. This led to a 2.3-fold increase in L-lactic acid concentration compared to those obtained using a condition without pH adjustment during fermentation. The L-lactic acid yield was improved from 0.729 to 0.924 g/g when the pH was periodically adjusted every 24 h. Lastly, *L. casei* CICC 6056 can tolerate a high temperature of 50 °C, and L-lactic acid production was improved 1.7-fold by optimizing the pH and temperature in an open fermentation of DG using SSF.

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REFERENCES CITED

Bai, D. M., Wei, Q., Yan, Z. H., Zhao, X. M., Li, X. G., and Xu, S. M. (2003). "Fed-batch fermentation of *Lactobacillus lactis* for hyper-production of L-lactic acid," *Biotechnol. Lett.* 25(21), 1833-1835. DOI: 10.1023/A:1026276925649

Bruno-Barcena, J. M., Ragout, A. L., Cordoba, P. R., and Sineriz, F. (1999). "Continuous production of L(+)-lactic acid by *Lactobacillus casei* in two-stage systems," *Appl. Microbiol. Biotechnol.* 51(3), 316-324.

Buyukkileci, A. O., and Harsa, S. (2004). "Batch production of L(+) lactic acid from whey by *Lactobacillus casei* (NRRL B-441)," *J. Chem. Technol. Biotechnol.* 79(9), 1036-1040. DOI: 10.1002/jctb.1094

Chatzifragkou, A., Kosik, O., Prabhakumari, P. C., Lovegrove, A., Frazier, R. A., Shewry, P. R., and Charalampopoulos, D. (2015). "Biorefinery strategies for upgrading distillers' dried grains with solubles (DDGS)," *Process Biochem.* 50(12), 2194-2207. DOI: 10.1016/j.procbio.2015.09.005

Chronopoulos, G., Bekatorou, A., Bezirtzoglou, E., Kaliafas, A., Koutinas, A. A., Marchant, R., and Banat, I. M. (2002). "Lactic acid fermentation by *Lactobacillus casei* in free cell form and immobilized on gluten pellets," *Biotechnol. Lett.* 24(15), 1233-1236. DOI: 10.1023/A:1016274121642

Ding, S. F., and Tan, T. W. (2006). "L-Lactic acid production by *Lactobacillus casei* fermentation using different fed-batch feeding strategies," *Process Biochem.* 41(6), 1451-1454. DOI: 10.1016/j.procbio.2006.01.014

Djukic-Vukovic, A. P., Mojovic, L.V., Jokic, B. M., Nikolic, S. B., and Pejin, J. D. (2013). "Lactic acid production on liquid distillery stillage by *Lactobacillus rhamnosus* immobilized onto zeolite," *Bioresour. Technol.* 135, 454-458. DOI: 10.1016/j.biortech.2012.10.066

Djukic-Vukovic, A. P., Mojovic, L.V., Vukasinovic-Sekulic, M. S., Rakin, M. B., Nikolic, S. B., Pejin, J. D., and Bulatovic, M. L. (2012). "Effect of different fermentation parameters on L-lactic acid production from liquid distillery stillage," *Food Chem.* 134(2), 1038-1043. DOI: 10.1016/j.foodchem.2012.03.011

Hetenyi, K., Nemeth, A., and Sevella, B. (2011). "Role of pH-regulation in lactic acid fermentation: Second steps in a process improvement," *Chem. Eng. Process*. 50(3), 293-299. DOI: 10.1016/j.cep.2011.01.008

Hujanen, M., and Linko, Y. Y. (1996). "Effect of temperature and various nitrogen sources on L(+) lactic acid production by *Lactobacillus casei*," *Appl. Microbiol. Biotechnol.* 45(3), 307-313.

John, R. P., Nampoothiri, K. M., and Pandey, A. (2007). "Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives," *Appl. Microbiol. Biotechnol.* 74(3), 524-534. DOI: 10.1007/s00253-006-0779-6

Kim, Y., Mosier, N., and Ladisch, M. R. (2008). "Process simulation of modified dry grind ethanol plant with recycle of pretreated and enzymatically hydrolyzed distillers' grains," *Bioresour. Technol*. 99(12), 5177-5192. DOI: 10.1016/j.biortech.2007.09.035

Ko, J. K., Ximenes, E., Kim, Y., and Ladisch, M. R. (2015). "Adsorption of enzyme onto lignins of liquid hot water pretreated hardwoods," *Biotech. Bioeng.* 112(3), 447-456. DOI: 10.1002/bit.25359

Ma, K. D., Maeda, T., You, H. Y., and Shirai, Y. (2014). "Open fermentative production of L-lactic acid with high optical purity by thermophilic *Bacillus coagulans* using excess sludge as nutrient," *Bioresource Technol.* 151, 28-35. DOI: 10.1016/j.biortech.2013.10.022

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chem.* 31(3), 426-428. DOI: 10.1021/ac60147a030

Mussatto, S. I. (2014). "Brewer's spent grain: A valuable feedstock for industrial applications," *J. Sci. Food Agric*. 94(7), 1264-1275. DOI: 10.1002/jsfa.6486

Ouyang, J., Ma, R., Zheng, Z. J., Cai, C., Zhang, M., and Jiang, T. (2013). "Open fermentative production of L-lactic acid by *Bacillus* sp. Strain NL01 using lignocellulosic hydrolyzates as low-cost raw material," *Bioresource Technol.* 135, 475-480. DOI: 10.1016/j.biortech.2012.09.096

Pedersen, M., Johansen, K. S., and Meyer, A. S. (2011). "Low temperature lignocellulose pretreatment: effects and interactions of pretreatment pH are critical for maximizing enzymatic monosaccharide yields from wheat straw," *Biotechnol. Biofuels* 4, 11. DOI: 10.1186/1754-6834-4-11

Pejin, J., Radosavljevic, M., Mojovic, L., Kocic-Tanackov, S., and Djukic-Vukovic, A. (2015). "The influence of calcium-carbonate and yeast extract addition on lactic acid fermentation of brewer's spent grain hydrolysate," *Food Res. Int.* 73, 31-37. DOI: 10.1016/j.foodres.2014.12.023

Qin, J. Y., Zhao, B., Wang, X. W., Wang, L. M., Yu, B., Ma, Y. H., Ma, C. Q., Tang, H. Z., Sun, J. B., and Xu, P. (2009). "Non-sterilized fermentative production of polymer-grade L-lactic acid by a newly isolated thermophilic strain *Bacillus* sp 2-6," *PLOS ONE* 4(2), e4359. DOI: 10.1371/journal.pone.0004359

Qin, H., Gong, S. S., Ge, X. Y., and Zhang, W. G. (2012). "The effect of temperature on L-lactic acid production and metabolite distribution of *Lactobacillus casei*," *Prep. Biochem. Biotechnol.* 42(6), 564-573. DOI: 10.1080/10826068.2012.665114

Roberto, I., Mussatto, S., Mancilha, I., and Fernandes, M. (2007). "The effects of pH and

nutrient supplementation of brewer's spent grain cellulosic hydrolysate for lactic acid production by *Lactobacillus delbrueckii*," *J. Biotechnol*. 131(2), S181-S182. DOI: 10.1016/j.jbiotec.2007.07.920

Sakai, K., and Ezaki, Y. (2006a). "Open L-lactic acid fermentation of food refuse using thermophilic *Bacillus coagulans* and fluorescence in situ hybridization analysis of microflora," *J. Biosci. Bioeng.* 101(6), 463-475. DOI: 10.1263/jbb.101.457

Sakai, K., Fujii, N., and Chukeatirote, E. (2006b). "Racemization of L-lactic acid in pH-swing open fermentation of kitchen refuse by selective proliferation of *Lactobacillus plantarum*," *J. Biosci. Bioeng.* 102(3), 227-232. DOI: 10.1263/jbb.102.227

Sakai, K., Mori, M., Fujii, A., Iwami, Y., Chukeatirote, E., and Shirai, Y. (2004). "Fluorescent in situ hybridization analysis of open lactic acid fermentation of kitchen refuse using rRNA targeted oligonucleotide probes," *J. Biosci. Bioeng.* 98(1), 48-56. DOI: 10.1263/jbb.98.48

Shang, Y. P., Su, R. X., Huang, R. L., Yang, Y., Qi, W., Li, Q. J., and He, Z. M. (2014). "Recycling cellulases by pH-triggered adsorption-desorption during the enzymatic hydrolysis of lignocellulosic biomass," *Appl. Microbiol. Biotechnol.* 98(12), 5765-5774. DOI: 10.1007/s00253-014-5761-0

Singhania, R. R., Saini, J. K., Saini, R., Adsul, M., Mathur, A., Gupta, R., and Tuli, D. K. (2014). "Bioethanol production from wheat straw via enzymatic route employing *Penicillium janthinellum* cellulases," *Bioresour. Technol.* 169, 490-495. DOI: 10.1016/j.biortech.2014.07.011

Sun, J., Zhu, J., and Li, W. (2012). "L-(+) lactic acid production by *Rhizopus oryzae* using pretreated dairy manure as carbon and nitrogen source," *Biomass Bioenerg.* 47, 442-450. DOI: 10.1016/j.biombioe.2012.09.011

Tanaka, T., Hoshina, M., Tanabe, S., Sakai, K., Ohtsubo, S., and Taniguchi, M. (2006). "Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation," *Bioresource Technol.* 97(2), 211-217. DOI: 10.1016/j.biortech.2005.02.025

Taniguchi, M., Hoshina, M., Tanabe, S., Higuchi, Y., Sakai, K., Ohtsubo, S., Hoshino, K., and Tanaka, T. (2005). "Production of L-lactic acid by simultaneous saccharification and fermentation using unsterilized defatted rice bran as a carbon source and nutrient components," *Food Sci. Technol. Res.* 11(4), 400-406. DOI: 10.3136/fstr.11.400

Van, S. (1963). "Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin," *J. Assoc. Off. Agric. Chem.* 46, 829-835.

Wang, L. M., Xue, Z. W., Zhao, B., Yu, B., Xu, P., and Ma, Y. H. (2013). "Jerusalem artichoke powder: A useful material in producing high-optical-purity L-lactate using an efficient sugar-utilizing thermophilic *Bacillus coagulans* strain," *Bioresource Technol.* 130, 174-180. DOI: 10.1016/j.biortech.2012.11.144

Wang, Q. H., Narita, J. Y., Ren, N. Q., Fukushima, T., Ohsumi, Y., Kusano, K., Shirai, Y., and Ogawa, H. I. (2003). "Effect of pH adjustment on preservation of kitchen waste used for producing lactic acid," *Water Air Soil Poll.* 144(1), 405-418. DOI: 10.1023/A:1022919716627

Wang, Q. H., Narita, J., Xie, W. M., Ohsumi, Y., Kusano, K., Shirai, Y., and Ogawa, H. I. (2002). "Effects of anaerobic/aerobic incubation and storage temperature on preservation and deodorization of kitchen garbage," *Bioresource Technol.* 84(3), 213-220. DOI: 10.1016/S0960-8524(02)00062-7

Wang, Y., Cai, D., He, M. L., Wang, Z., Qin, P. Y., and Tan, T. W. (2015). "Open fermentative production of L-lactic acid using white rice bran by simultaneous

saccharification and fermentation," *Bioresource Technol.* 198, 664-672. DOI: 10.1016/j.biortech.2015.09.010

Wang, Y., Yang, Z. X., Qin, P. Y., Tan, T. W. (2014). "Fermentative L-(+)-lactic acid production from defatted rice bran," *RSC Adv.* 4(17), 8907-8913. DOI: 10.1039/c3ra46140h

Wee, Y. J., Kim, J. N., and Ryu, H. W. (2006). "Biotechnological production of lactic acid and its recent applications," *Food Technol. Biotechnol.* 44, 163-172.

Xiros, C., and Christakopoulos, P. (2012). "Biotechnological potential of brewers spent grain and its recent applications," *Waste and Biomass Valorization* 3(2), 213-232. DOI: 10.1007/s12649-012-9108-8

Yoo, I. K., Chang, H. N., Lee, E. G., Chang, Y. K., and Moon, S. H. (1996). "Effect of pH on the production of lactic acid and secondary products in batch cultures of *Lactobacillus casei*," *J. Microbiol. Biotechnol*. 6(6), 482-486.

Zhang, L. A., Shi, K. Q., and Shi, G. Y. (2011). "Production of L-lactic acid with very high gravity distillery wastewater from ethanol fermentation by a newly isolated *Enterococcus hawaiiensis*," *J. Chem. Technol. Biotechnol.* 86(2), 213-216.

Zhang, W., Lin, Y., Zhang, Q., Wang, X., Wu, D., and Kong, H. (2013). "Optimisation of simultaneous saccharification and fermentation of wheat straw for ethanol production," *Fuel* 112, 331-337. DOI: 10.1016/j.fuel.2013.05.064

Zhou, J., Ouyang, J., Zhang, M., and Yu, H. (2014). "Simultaneous saccharification and fermentation of bagasse sulfite pulp to lactic acid by *Bacillus coagulans* CC17," *BioResources* 9(2), 2609-2620. DOI: 10.15376/biores.9.2.2609-2620

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