Using Novel *Lactobacillus plantarum* to Produce Lactic Acid from Lignocellulosic Biomass in an Integrated Simultaneous Saccharification and Fermentation Process

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The isolated bacterium *Lactobacillus plantarum* is strongly tolerant of lignocellulose-derived inhibitory compounds and possesses the ability to produce lactic acid in the presence of 8.0 g/L furfural, 6.0 g/L hydroxymethylfurfural, 4.0 g/L vanillin, and 4.0 g/L syringaldehyde. This bacterium was used in an integrated simultaneous saccharification and fermentation (SSF) process for the production of lactic acid using whole rice straw slurry (with a high solids content of 17%) that had been pretreated with dilute sulfuric acid. This method achieved a lactic acid concentration of 65.6 g/L, which corresponded to a cellulose-to-lactic acid conversion yield of 69%. These results demonstrated that isolated bacterium and the proposed integrated SSF process are able to produce high concentrations of lactic acid. Furthermore, this proposed process does not require detoxification and had a conversion efficiency that is comparable to that obtained using the conventional SSF process, which requires the addition of fresh water. Therefore, it was concluded that the proposed process is promising for commercial lactic acid production and could make the production of lactic acid from lignocellulosic biomass more practical in the future.

Keywords: Lactobacillus plantarum; Tolerant; Lactic acid; Simultaneous saccharification and fermentation (SSF)

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INTRODUCTION

Lactic acid is widely used as an inhibitor, additive, and chemical intermediate in the food, cosmetic, pharmaceutical, and chemical industries. Poly-lactic acid is an environmentally-friendly alternative to petrochemically-derived non-biodegradable plastic (Wang *et al.* 2015). Lactic acid is generally derived through fermentation or chemical technology (Wang *et al.* 2015; Kawaguchi *et al.* 2016). Approximately 90% of the global lactic acid production involves microbial fermentation, with the remainder obtained from petroleum sources (Wang *et al.* 2015). The microorganisms most widely used for lactic acid production are filamentous fungi and lactic acid bacteria (LAB), such as *Lactobacillus* and *Lactococcus*. Lactic acid bacteria can be further grouped into homo-fermentative and hetero-fermentative strains (Martinez *et al.* 2013). Homo-fermentative LABs transform sugars into pure lactic acid, whereas hetero-fermentative LABs produce byproducts, such as ethanol, acetic acid, and carbon dioxide, in addition to lactic acid (Martinez *et al.* 2013).

The economic returns from lactic acid production are determined by the cost of the raw materials. The feedstocks most commonly used in the commercial fermentation of lactic acid are sugar, starch, and whey materials (Wang *et al.* 2015; Balakrishnan *et al.* 2018; Venus *et al.* 2018). The high carbohydrate content of lignocellulose makes it a low-cost alternative compared with these traditional feedstocks.

Lignocellulose obtained from agriculture, forestry, and industrial manufacturing is comprised of cellulose, hemicellulose, and lignin. Rice straw is a good source of lignocellulose and is the most abundant agricultural waste product worldwide (Kumar and Sharma 2017), with an estimated annual production of 700 million ton to 800 million ton. Rice straw has considerable potential as a feedstock for lactic acid production, particularly in Asia (Hsu *et al.* 2010; Chen *et al.* 2011).

The chemical structure of lignocellulose makes it difficult for microorganisms to utilize it directly. Thus, pretreatment methods are required to break down the native structure of the biomass and thereby liberate fermentable sugar (cellulose) for sequential enzymatic hydrolysis prior to fermentation (Kapoor *et al.* 2017). A number of technologies that employ dilute acid, alkane, hot water, steam explosion, and biological processes have been used for pretreatment; however, dilute acid and steam explosion is considered to be the most effective method to render rice straw into a suitable fermentation feedstock (Hsu *et al.* 2010; Chen *et al.* 2011).

Pretreating rice straw with dilute acid generally results in the production of a solid residue that contains cellulose and lignin as well as a liquid fraction that contains soluble sugars derived from hemicellulose and cellulose. Figure 1a shows that solid/liquid separation units enable the simultaneous saccharification and fermentation process (SSF) to produce lactic acid from lignocellulosic biomass (Peng *et al.* 2013; Zhang and Vadlani 2013; Unrean 2018).

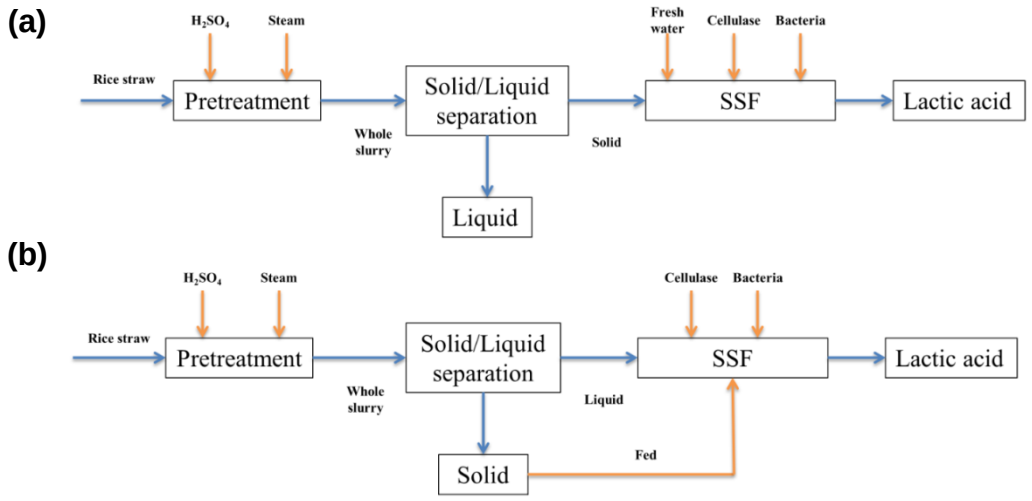

Fig. 1. Proposed lactic acid production processes: (a) conventional SSF with the addition of fresh water; and (b) integrated SSF with whole slurry

Under SSF, fresh water must be added following the separation of solids from liquid to reduce the concentration of fermentative inhibitors, such as furfural, hydroxymethylfurfural (HMF), vanillin, and syringaldehyde (when a dilute acid pretreatment is used), and increase the conversion yield in the hydrolysis and fermentation units. Conversely, whole slurry processes eliminate the need for solid/liquid separation,

thereby reducing equipment installation costs. Nonetheless, pH conditioning and enzymatic hydrolysis can be difficult in whole slurry systems that contain a high lignocellulosic solid content, and few studies have addressed whole slurry processes in the context of lactic acid fermentation (Zhao *et al.* 2013). In this research, an integrated SSF process was developed using a whole slurry of acid-pretreated rice straw (Fig. 1b). Following pretreatment, the solid residue and liquid fraction were separated, and the solid residue was fed into the liquid fraction with cellulase and LAB.

The saccharification of lignocellulose during pretreatment and enzymatic hydrolysis can result in the production of weak acids, furan derivatives, and phenolic compounds, which can have an inhibitory effect on the growth of lactic-acid-producing species (Huang *et al.* 2011; Ling *et al.* 2014). Acetic acid, HMF, and furfural are commonly released during lignocellulosic biomass pretreatment. Although the inhibitory effects of acetic acid can be mediated by maintaining the pH of hydrolysates at 6.0 or higher (Huang *et al.* 2011), phenolic compounds, such as syringaldehyde, vanillin, and aromatic aldehydes (produced during the decomposition of lignin), can interfere with microbial fermentation (Jönsson and Martín 2016). Therefore, in this study, a natural lactic-acid-producing microorganism was developed that is tolerant to fermentation inhibitors and able to ferment sugar from pretreated lignocellulose.

For this, a lactic acid producing bacteria strain, *Lactobacillus plantarum*, which has a strong tolerance to lignocellulose-derived inhibitors, was isolated. This strain was then used in an integrated SSF process for the production of lactic acid using a whole slurry that included non-detoxified rice straw hydrolysate and acid-treated solid residue.

EXPERIMENTAL

Materials

Rice straw was collected from a farm in Tainan, Taiwan. The raw materials were air-dried, milled to approximately 10 mm, and stored at room temperature until further use. The composition of the rice straw was as follows: 34.5% glucan, 21.3% xylan, and 13.3% lignin (Chen *et al.* 2011).

Isolation of L. plantarum

Lactic acid-producing bacteria were first isolated from hydrolysate fermentation broth, and De Man, Rogosa, and Sharpe (MRS) medium was prepared for the enrichment and isolation of the samples. For this, bacteria isolates were obtained using serial dilutions. Bacterial colonies were cultured on growth medium agar plates (57.455 g/L MRS medium and 20 g/L agar) at 37 °C for 24 h before being transferred to MRS agar plates and incubated at 37 °C for another 3 d. Colonies with a lactic acid production capability were identified by first sequencing the 16s rDNA *via* polymerase chain reaction (PCR) and then by performing BLAST analysis on the sequenced rDNA using the GenBank database maintained by the National Center for Biotechnology Information.

The isolated bacterium strain was cultured on growth medium agar plates at 37 °C for 2 d and then transferred to a 250-mL Erlenmeyer flask containing 100 mL of MRS medium. The Erlenmeyer flask was placed on an orbital shaker and the inoculated culture was grown at 37 °C under agitation at 100 rpm for 24 h prior to fermentation.

Culture medium used in the inhibition tolerance experiments

Experiments were conducted in which fermentation was performed in the presence of inhibitory compounds. Specifically, these fermentation experiments were conducted in MRS medium supplemented with various concentrations of HMF, furfural, vanillin, and syringaldehyde. For this, a 0.5-g/L bacteria inoculation and 20 g/L $CaCO_3$ were added to the 250-mL Erlenmeyer flask, and then incubated at 37 °C for 24 h under agitation (100 rpm) by a rotary shaker. The lactic acid production was analyzed three times *via* the general linear model procedure, which was performed using the Statistical Analysis System software (SAS Version 9.2, SAS Institute Inc., Cary, NC, USA). To measure OD, 1 mL of growth medium was removed from each test immediately after inoculation and per 2 hours until 24 hours. OD of the cultural medium was determined using a Double Beam Spectrophotometer U-2900 (HITACHI, Chiyoda, Tokyo, Japan). Each OD value was calculated by subtracting the ODs determined at the beginning of the different tests. OD values < |0.01| were defined as 0.

Preparation of the Hydrolysates and Solid Residues

The rice straw was pretreated with dilute acid in a pilot-scale plant (1 ton/d) at the Institute of Nuclear Energy Research in Taiwan. Pretreatment began with an initial solids content of 40% and 1% H_2SO_4 at 180 °C for 1 min. When the pretreatment process was finished, the solids content decreased to 20% and the mixture was a slurry. The resulting hydrolysates were separated from the acid-treated slurry and adjusted to a pH of 6 using NaOH before *L. plantarum* was added for fermentation analysis.

SSF Assay

Fermentation experiments were implemented using both the conventional SSF process and proposed integrated SSF with whole slurry in 5-L fermenters under agitation (150 rpm) at 37 °C with a 0.5-g/L bacteria inoculation. In all of the SSF experiments, the commercial enzyme Cellic CTec3 (Novozymes, Bagsværd, Denmark) was added at a loading volume of 15 filter paper unit of activity/g cellulose. The pH of the liquid phase was maintained at 5.0 using 10 N NaOH. The solids content increased from 5% to 17% over a period of 36 h, during which time liquid samples were collected every 12 h to determine the glucose and lactic acid concentrations.

Analytical Methods

All of the liquid samples obtained during the SSF experiments were filtered using a 0.45-μm filter and then diluted using deionized water. The glucose and lactic acid concentrations were measured using high performance liquid chromatography (HPLC) in an Agilent 1200 series HPLC (Santa Clara, USA) equipped with a Coregrl-87H3 column (Transgenomic Technologies, San Jose, CA, USA) at 65 °C. For this, 4 mM H_2SO_4 was used as the eluent solution and the flow rate was 1.0 mL/min.

RESULTS AND DISCUSSION

Identification and Characterization of the Isolated Bacterium Strain

Molecular analysis of the lactic acid-producing bacterium isolated from acid-treated rice straw hydrolysates was performed using 16s rDNA sequencing and PCR assay with a recA gene-derived primer. The results revealed that the genetic sequence of the

isolated bacterium shared a 99% similarity with that of existing *L. plantarum* strains in GenBank. However, this lactic acid bacterium does not have the ability to use xylose as a source of carbon. The characteristics of the lactic acid-producing bacterium strain isolated in this study were similar to those of other *L. plantarum* strains (Okano *et al.* 2010; Abdel-Rahman *et al.* 2011; Abdel-Rahman *et al.* 2013). *Lactobacillus plantarum* is generally a facultative hetero-fermentative bacterium that is considered to be a safe probiotic that performs homolactic-like fermentation under specific conditions (Siezen and van Hylckama Vlieg 2011). *Lactobacillus plantarum* is able to use a wide range of fermentable carbohydrate sources and is highly tolerant of acidic environments, alkaline environments, and osmotic stress (Parente *et al.* 2010).

Effects of the Inhibitors on Lactic Acid Fermentation

Lignocellulose is a good source of glucose and xylose; however, lignocellulose must be hydrolyzed to fermentable hexose and pentose before this feedstock can be used by microorganisms (Schubert 2006; Weber *et al.* 2010). Before lignocellulose can be hydrolyzed, it must undergo pretreatment with dilute acid to break down its structure and thereby liberate fermentable sugars. Unfortunately, this process produces fermentation inhibitors and toxic compounds that can interfere with enzymatic hydrolysis (Kim *et al.* 2011), which results in a low fermentation yield (Jönsson *et al.* 2013; Jönsson and Martín 2016). Major inhibitors include furan derivatives (furfural and HMF) derived from fermentable sugars, organic acids (acetic, formic, and levulinic acid) derived from hemicellulose, and phenols (sulfonated lignin, syringaldehyde, and vanillin) derived from lignin polymers. Among these inhibitory compounds, phenols with low molecular weights have inhibitory effects that are particularly pronounced. Phenols are also generally more toxic to microorganisms than furfural and HMF (Palmqvist and Hahn-Hägerdal 2000; Klinke *et al.* 2002; Alvira *et al.* 2010). The concentration of inhibitors depends on the pretreatment conditions and lignocellulose source (Klinke *et al.* 2002).

Figures 2 and 3 illustrate the degree of tolerance exhibited by *L. plantarum* to typical lignocellulose-derived inhibitors, including two types of furans and two phenolic compounds. These results indicated that *L. plantarum* is more sensitive to phenolic compounds than furan. Figures 2a and 2b show that *L. plantarum* tolerated 8.0 g/L furfural and 6.0 g/L HMF, respectively, with no obvious reduction in the lactic acid yield or productivity. In contrast, vanillin and syringaldehyde presented no inhibitory effects on the lactic acid production at concentrations of up to 4.0 g/L (Figs. 2c and 2d). In this study, the inhibitory tolerance of *L. plantarum* was tested with various concentrations of furfural, HMF, vanillin, and syringaldehyde. *Lactobacillus plantarum* had a better tolerance for inhibitors than *L. plantarum* JCL1279, *Pediococcus acidilatici* DQ2, and other species (Zhao *et al.* 2013; Boguta *et al.* 2014). The tolerance of *L. plantarum* to vanillin was close to that of *P. acidilatici* DQ2 and *Bacillus* sp. strain P38 (Peng *et al.* 2013; Zhao *et al.* 2013). *Lactobacillus plantarum* was shown to consume inhibitors with no obvious lag in of the production of lactic acid. For example, the presence of furfural and HMF did not interfere with lactic acid production during hydrolysate fermentation. These results differed considerably from those obtained for hydrolysate fermentation by *Bacillus coagulans* JI12, which only began to convert sugar into lactic acid during hydrolysis after the complete depletion of furfural (Ye *et al.* 2013).

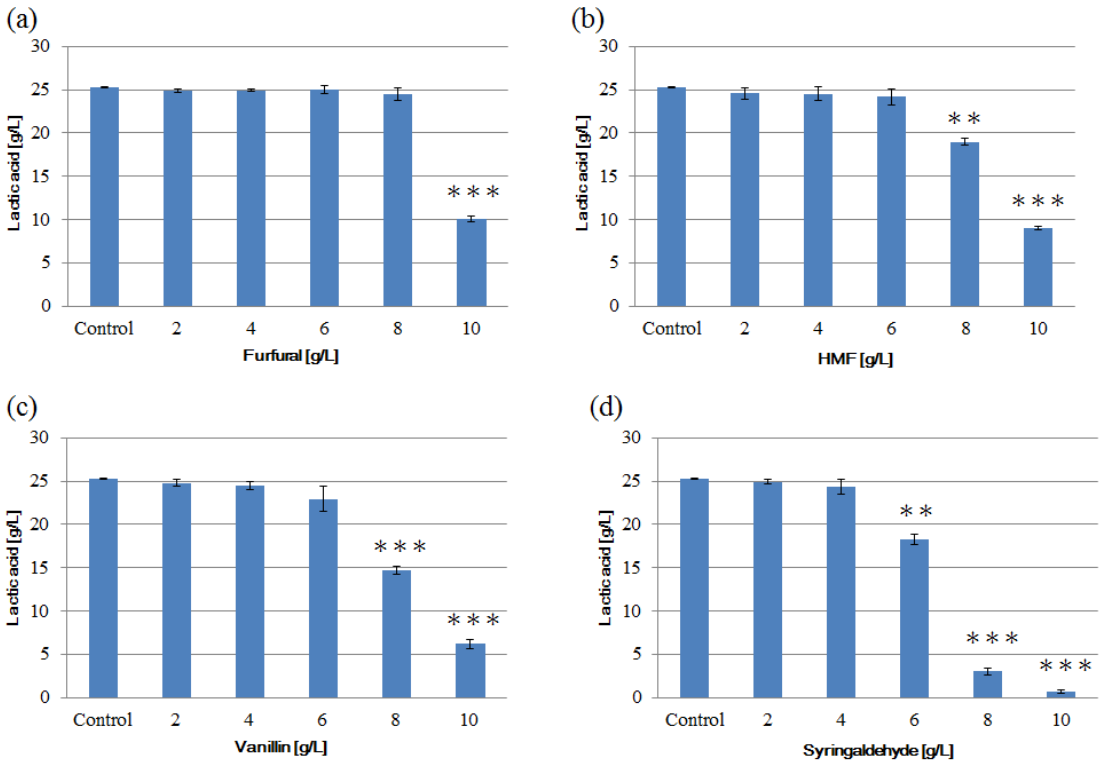

Fig. 2. Inhibitory effects of (a) Furfural, (b) HMF, (c) Vanillin, and (d) Syringaldehyde on *L. plantarum* during Lactic acid production; data is the mean ± standard error (n = 3); ** P < 0.01 and *** P < 0.001 compared with the control group

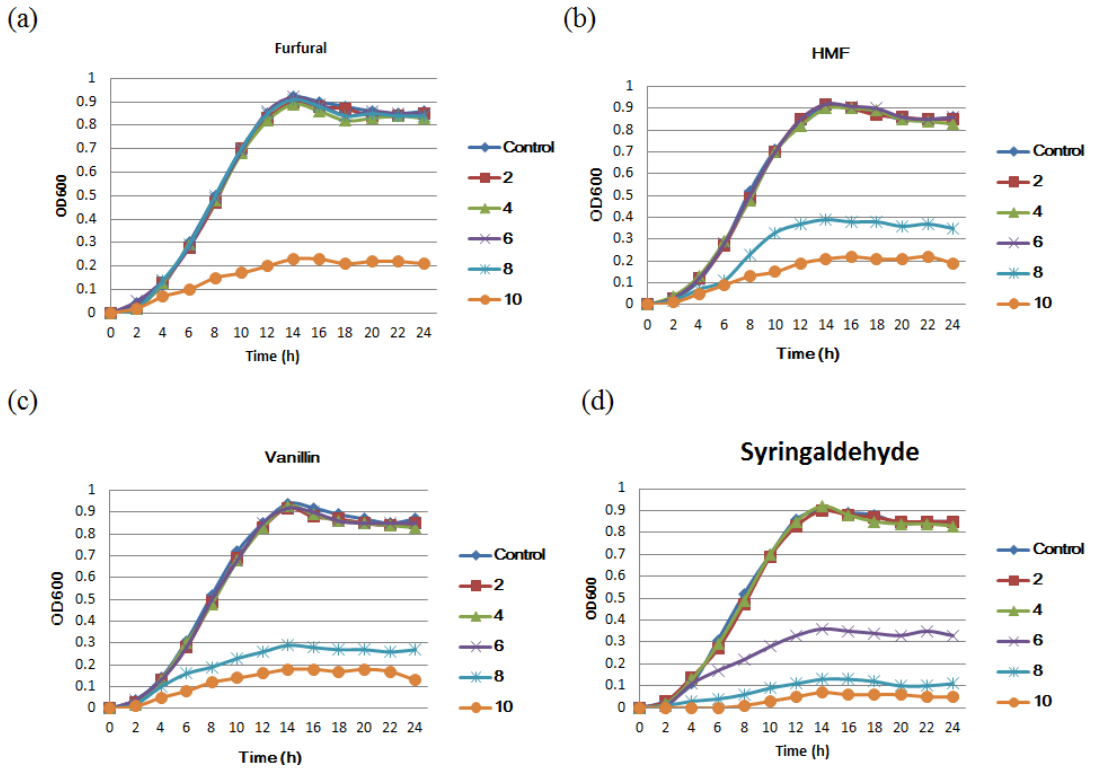

Fig. 3. Optical Density (OD 600) values measured during growth of *L. plantarum* in different tolerant tests of (a) Furfural, (b) HMF, (c) Vanillin, and (d) Syringaldehyde

The detrimental effects of inhibitors can be overcome *via* detoxification using physical, chemical, and biological processes (Moreno *et al.* 2015; Kim 2018); however, these processes can greatly increase production costs (Mussatto and Roberto 2004; Slade *et al.* 2009). *Lactobacillus plantarum* eliminated the need for detoxification, which makes it more economical for lactic acid production.

Evaluation of the Hydrolysate Fermentation

The production of lactic acid by *L. plantarum* was further investigated in lignocellulosic hydrolysates that were obtained from acid-pretreated rice straw that did not undergo detoxification. Figure 4 shows the consumption of glucose and formation of lactic acid in the samples that initially contained the following amounts of inhibitors: 3.8 g/L furfural, 0.19 g/L HMF, 46.3 g/L xylose, and 35 g/L glucose. The strain isolated in this study achieved a lactic acid yield of 89% and maximum productivity of 0.45 g/L/h from glucose. This strain also demonstrated bio-detoxification effects by completely depleting furfural and HMF after 60 h and 48 h, respectively.

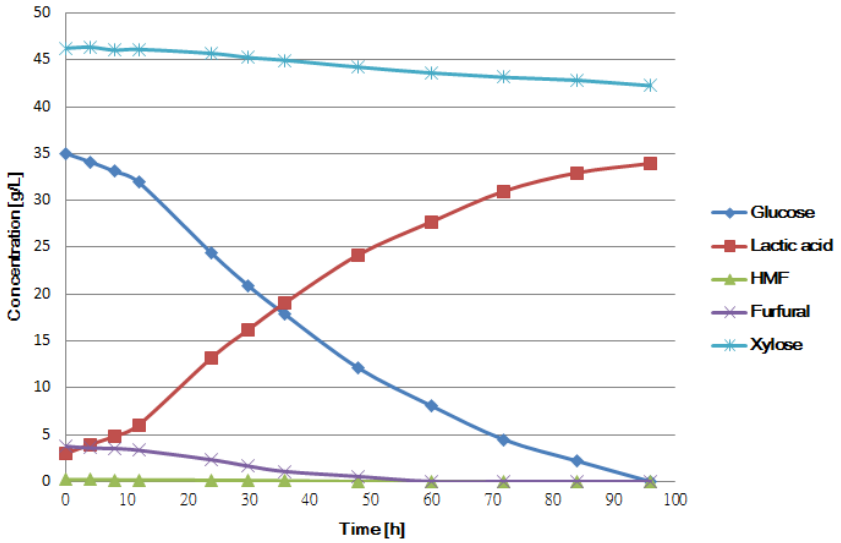

Fig. 4. Graph illustrating the production profile of lactic acid and concentrations of various inhibitors; note that the decreasing concentrations of the inhibitors over time confirmed the detoxification abilities of *L. plantarum*

It should be noted that pretreatment with dilute acid breaks down hemicellulosic structures and releases mono-xylose from the lignocellulosic biomass. In the current study, the xylose in the hydrolysate (36 g/L) provided an additional source of carbon. This xylose was not used for lactic acid production in this study; however, it could be recovered for the production of value-added chemicals, such as xylitol, furfural (Huang *et al.* 2011; Karinen *et al.* 2011), and animal feed (Larsen *et al.* 2012), to improve the economics of lactic acid production from lignocellulosic feedstock. Moreover, if genetic tools are used to further enhance the utilization of xylose, *L. plantarum* could become one of the most economically competitive strains of *Lactobacillus* used in the production of lactic acid.

Chandel *et al.* (2013) found that the concentrations of HMF, furfural, and phenolic compounds in hydrolysates were approximately 1 g/L to 3 g/L after dilute acid pretreatment. However, there was a risk that the concentration of these inhibitors could

suddenly increase in large-scale cellulosic ethanol plants because of non-uniform hydrolysis reactions and diverse feedstocks. The tolerance that *L. plantarum* displayed against typical pretreatment-derived inhibitors should make this LAB practical in applications that produce lactic acid from lignocellulosic biomass. In industrial applications, *L. plantarum* should be able to adapt to a variety of hydrolysate fermentation conditions.

Production of Lactic Acid from the Acid-pretreated Rice Straw Using SSF Processes

Lactobacillus thermophilus, *L. bulgaria*, *Bacillus* sp., and *L. delbrueckii* have been used to produce lactic acid from wood, stalk, corn stover, and sugarcane bagasse using conventional SSF processes (Mussatto and Roberto 2004; Okano *et al.* 2010; Parente *et al.* 2010; Karinen *et al.* 2011; Sasaki *et al.* 2012; Zhao *et al.* 2013). At an initial solids content of approximately 7% to 13%, these species achieved final lactic acid concentrations of approximately 23 g/L to 67 g/L and lactic acid productivity rates of approximately 0.43 g/L/h to 0.93 g/L/h, which were similar to the lactic acid concentration and productivity rate obtained using *L. plantarum* in this study. The proposed scheme also eliminated the need for fresh water and detoxification (Peng *et al.* 2013; Zhao *et al.* 2013). Previous studies have reported that an SSF process that uses *P. acidilactici* and corn stover hydrolysate yields high lactic acid titers and has a good productivity; however, achieving these results required a huge energy input to facilitate biodetoxification (using fungus) over a 5-d period (Chandel *et al.* 2013). The extra energy input could be supplied by steam or electric power generation obtained through the combustion of lignin residue.

In this study, two different SSF processes were used to test the lactic acid productive ability of *L. plantarum*. Comparing with the integrated SSF process, the conventional SSF process was performed, in which fresh water was added to pretreated solid residue to reduce the concentrations of inhibitors after the separation of solid and liquid parts from whole slurry. The major substrate that participated in the metabolic pathway of *L. plantarum* was glucose. The amount of glucose was at the same level in the whole slurry of these two processes. The major different part of these two processes was the speed of enzymatic hydrolysis during SSF process. Because the integrated SSF process used fed-batch method, the speed of enzymatic hydrolysis was slower than the conventional SSF process. That is to say, the fermentable glucose was released at slower speed during the integrated SSF process. But the final concentrations of lactic acid of these two processes were comparison with each other (Figs. 4 and 5), the fermentable glucose amounts of these two processes were almost convert to lactic acid in the metabolic pathway of lactic acid fermentation in *L. plantarum*. Figure 5 shows that this process did not result in any obvious accumulation of glucose *via* cellulose hydrolysis. The rate of sugar utilization was exceeded, which resulted in the production of lactic acid at a rate of approximately 1.0 g/L/h to 1.9 g/L/h over 36 h. The final lactic acid concentration of 61 g/L was achieved with a productivity rate of 0.63 g/L/h, which indicated that 66.7% of the cellulose had been converted to lactic acid in 96 h. The glucose yield that was produced *via* enzymatic hydrolysis by *L. plantarum* was approximately 75%, which indicated an 89% conversion efficiency. On the other hand, the average amount of dry matter used in the tow SSF process was 520 g, and the average operation volume was 3.03 L. According to this, in the conventional SSF process, the average production of lactic acid was 0.35 g/g dry matter.

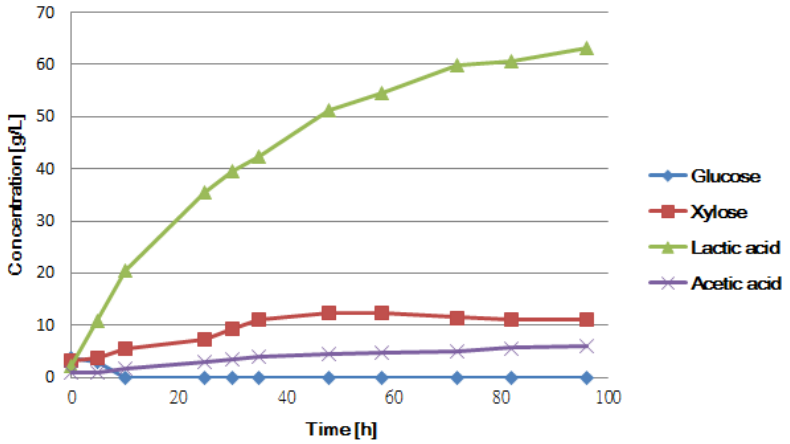

Fig. 5. Production of Lactic acid from the acid-pretreated rice straw using the SSF process with *L. plantarum* and the addition of fresh water

An integrated SSF process using *L. plantarum* and whole rice straw slurry was also performed. The initial solids content increased from 5% to 17% within 36 h. Figure 6 shows the time-course of the product concentration in the integrated SSF process that used whole slurry. The initial furfural and HMF concentrations were 2.2 g/L and 0.15 g/L, respectively. The increase in the solid residue resulted in the accumulation of 25 g/L glucose, which was rapidly consumed and thereby rendered glucose undetectable after 60 h. The isolated *L. plantarum* strain continued consuming furfural, HMF, and solid residue feed. However, the accumulation of lactic acid was slower than in the conventional SSF process because of the dilution effect of wet solid residues at the initial time. The productivity of lactic acid increased by six times after all of the solid residue had been fed into the system. It was found that, in producing lactic acid, the effectiveness of the proposed process was comparable to that of the conventional SSF process. More specifically, under the proposed process, the productivity of lactic acid was estimated at approximately 1 g/L/h to 1.4 g/L/h from approximately 36 h to 60 h, which was comparable to that obtained using the conventional SSF process for 24 h.

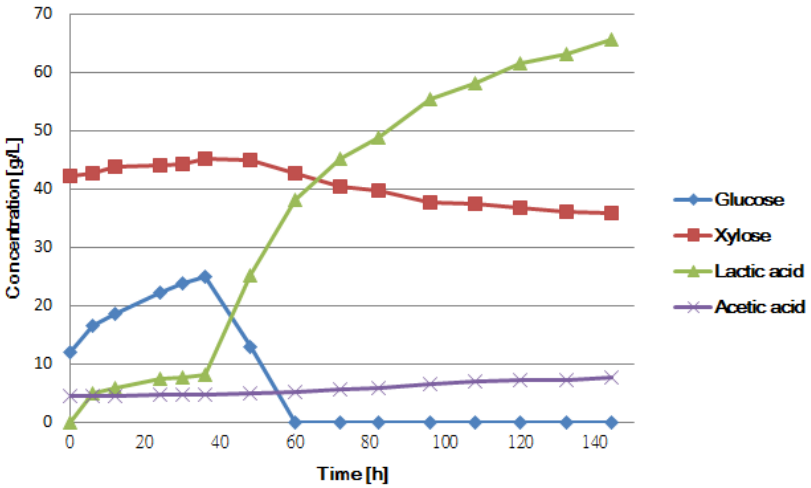

Fig. 6. Production of Lactic acid from the acid-pretreated rice straw using the SSF process with *L. plantarum* and whole slurry

The maximum lactic acid concentration of 65.6 g/L was achieved in 144 h, which resulted in a 69% cellulose-to-lactic acid yield. The average amount of dry matter and the operation volume of the integrated SSF process were at the same level with the conventional SSF process. According to this, the average production of lactic acid was 0.38 g/g dry matter in the integrated SSF process.

CONCLUSIONS

1. In this study, a novel integrated SSF process that uses whole slurry and the bacteria strain *L. plantarum* for the production of lactic acid was proposed. This process does not require the addition of fresh water for enzymatic hydrolysis.
2. The novel bacterium strain eliminated the need for detoxification processes that remove fermentation inhibitors.
3. The proposed process achieved a final lactic acid concentration of 65.6 g/L with a cellulose-to-lactic acid yield of 69%, which makes it a promising cost-effective candidate for the production of lactic acid from lignocellulosic biomass.

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