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Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium *Pediococcus acidilactici* DQ2

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ABSTRACT

A lactic acid bacterium with high tolerance of temperature and lignocellulose derived inhibitor was isolated and characterized as *Pediococcus acidilactici* DQ2. The strain used in the simultaneous saccharification and fermentation (SSF) for high titer lactic acid production at the high solids loading of corn stover. Corn stover was pretreated using the dry sulphuric acid pretreatment, followed by a biological detoxification to remove the inhibitors produced in the pretreatment. The bioreactor with a novel helical impeller was used to the SSF operation of the pretreated and biodetoxified corn stover. The results show that a typical SSF operation at 48 °C, pH 5.5, and near 30% (w/w) solids loading in both 5 and 50 L bioreactors was demonstrated. The lactic acid titer, yield, and productivity reached 101.9 g/L, 77.2%, and 1.06 g/L/h, respectively. The result provided a practical process option for cellulosic lactic acid production using virgin agriculture lignocellulose residues.

1. Introduction

Lactic acid is an important commodity chemical and also a monomer compound of the booming biodegradable polylactic acid (PLA) (Nampoothiri et al., 2010). The great expectation for replacing petroleum based polymer materials such as polyethylene (PE), polypropylene (PP), and polystyrene (PS) by PLA requires the lactic acid monomer supply produced from non-food feedstocks such as the abundant lignocellulose biomass.

Many studies on lactic acid production from lignocellulose biomass had been conducted using the simultaneous saccharification and fermentation (SSF) process as reviewed by John et al. (2009), besides the recent works on using other non-food biomass such as algae as feedstock (Nakano et al., 2012; Nguyen et al., 2012). The process parameters using agricultural residues and wood residues as lignocellulose feedstocks were compared: Woiciechowski et al. (1999) obtained 19.98 g/L of lactic acid from 86 g/L of the steam-exploded *Pinus taeda* chips in 120 h SSF using *Rhizopus oryzae* NRRL 395; Moldes et al. (2000) obtained 62 g/L of lactic acid from 66 g/L of the delignified and NaOH treated *Eucalyptus globulus* wood chips in 124 h SSF using *Lactobacillus delbrueckii* NRRL B-445, at the high cellulase dosage of 28 FPU and 364 IU β-glucosidase per gram of substrate; Sreenath et al. (2001) obtained 46.4 g/L of lactic acid from 100 g/L of the hot water pretreated Alfalfa fiber substrate in 96 h SSF using *Lactobacillus plantarum* NRRL 14431; Miura et al. (2004) obtained 24 g/L of lactic acid from 100 g/L of corncob in 96 h SSF at 10 FPU cellulase per gram of substrate using a *Rhizopus* strain; Maas et al. (2008) obtained 40.7 g/L of lactic acid from 230 g/L of the lime-treated wheat straw in 56 h SSF using *Bacillus coagulans* DSM 2314; Cui et al. (2011) obtained 20.95 g/L of lactic acid from 30 g/L of the NaOH-treated corn stover in 48 h SSF using the mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*.

There are at least two major technical barriers in the present lactic acid production processing technologies using lignocellulose feedstock: (1) the high energy consumption of the steam usage in the pretreatment, and the waste water generation released in the pretreatment and in the washing step for inhibitor removal (detoxification); (2) the low lactic acid titer and productivity in its fermentation process. In this study, efforts were made to give the practical resolutions to the technical barriers on the cellulosic lactic acid production.

The resolution for the first technical barrier is the application of a new lignocellulose processing technology developed in our previous studies for cellulosic ethanol production were applied the cellulosic lactic acid production. First, a dry dilute sulfuric acid pretreatment on lignocellulose residues were used and the significant reduction of steam energy and zero waste water generation were realized to give a completely dry pretreated materials, while the pretreatment efficiency was well maintained (Zhang et al., 2011). Then the pretreated materials were quickly detoxified using a "kerosene fungus" *Amorphotheca resinae* ZN1 for degrading the inhibitors generated in the pretreatment (Zhang et al., 2010b) to give a qualified feedstock for the SSF for production of high titer lactic acid using a unique helical ribbon stirring bioreactor (Zhang et al., 2010a).

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The present work concerned the second technical barrier. The high final titer is crucially important for reduction of the overall separation and concentrating costs of lactic acid. The lactic acid in the present processing technologies was only 50–60 g/L, far below the requirement for industrial organic acid production (generally above 100 g/L). Although Wee et al. (2004) obtained 93 g/L of lactic acid from the vacuum evaporation concentrated oak wood hydrolysate using an *Enterococcus faecalis* strain at high cellulose dosage of 30 FPU per gram of substrate, but apparently, the method using vacuum evaporation requires huge energy input and apparently was not possible for industrial practice. Elevating the lactic acid titer requires a robust lactic acid bacterium (LAB) and the high substrate content in the SSF.

In this study, a new lactic acid bacterium (LAB), *Pediococcus acidilactici* DQ2, with high temperature tolerance, high lignocellulose derived inhibitor resistance, and high lactic acid production performance was isolated and used for the simultaneous saccharification and fermentation (SSF) for high titer lactic acid production at the high solids loading of corn stover. The results show that a typical SSF operation at 48 °C, pH 5.5, and near 30% (w/w) solids loading of corn stover (300 gram of dry corn stover solids in 1 kg fermentation slurry) in both 5 and 50 L bioreactors was demonstrated. An excellent performance was obtained with the lactic acid titer, yield, and productivity of 101.9 g/L, 77.2%, and 1.06 g/L/h, respectively. The result provided a practical process option for cellulosic lactic acid production process using virgin agriculture lignocellulose residues.

2. Methods

2.1. Raw materials and enzymes

Corn stover (CS) was grown in Shandong, China and harvested in fall, 2009. After collection, the materials were milled coarsely using a beater pulverizer and screened through a mesh with the circle diameter of 10 mm. The milled raw materials were washed to remove the field dirt, stones and metals, then dried at 105 °C until the weight was constant and stored in sealed plastic bags for use.

The cellulase enzyme used was Accellerase 1000 from Genencor International (Rochester, NY, USA). The filter paper activity and the cellobiase activity of Accellerase 1000 were determined to be 55.1 FPU/ml and 158.5 IU/ml, respectively, equivalent to 0.27 mL/g DM. L-lactic acid standard sample used was sodium L-lactate from Sigma–Aldrich (St. Louis, MO, USA) and the purity of the sodium L-lactate sample was over 98%. Inhibitors chemicals including acetic acid, formic acid, levulinic acid, vanillin were from Sinopharm Chemical Reagent Co. (Shanghai, China), and furfural, 5-hydroxymethylfurfural (HMF) from J&K Scientific Co. (Beijing, China).

2.2. Medium and strains

The potato–dextrose–agar (PDA) plates contained 200 g of potato extract juice, 20 g of glucose, 20 g of agar in 1 L of deionized water. The yeast extract–peptone–dextrose (YPD) medium solution contained 20 g of glucose, 20 g of peptone, 10 g of yeast extract in 1 L of deionized water. The De Man-Rogosa-Sharpe (MRS) medium contained 10 g of enzymatic digest of animal tissue, 10 g of beef extract, 5 g of yeast extract, 20 g of dextrose, 5 g of sodium acetate trihydrate, 1 g of polysorbate 80, 2 g of dipotassium phosphate, 2 g of triammonium citrate, 0.1 g of magnesium sulfate heptahydrate, 0.05 g of manganese sulfate tetrahydrate in 1 L deionized water (De Man et al., 1960). The MRS medium was simplified by deleting the expensive and unnecessary ingredients for the isolated strain culture. The simplified MRS composition contained 20 g of glucose, 5 g of yeast extract, 2 g of triammonium citrate, 5 g of sodium acetate trihydrate, 0.6 g of magnesium sulfate heptahydrate, 2 g of dipotassium phosphate, 0.25 g of magnesium sulfate heptahydrate in 1 L deionized water. The medium used in Petri dishes was added with 15 g of agar. Medium and water used above was autoclaved at 115 °C for 20 min.

Amorphotheca resinae ZN1 was obtained in our laboratory and used in the biodetoxification of the inhibitor compounds in the pretreated CS materials (Zhang et al., 2010b). Stock cultures and activation cultures were carried out in PDA medium at 4 and 25 °C, respectively. The strain is under the processes of the registration in CGMCC (China General Microorganism Collection, Beijing, China) and Chinese Patent application before open to the academic community. The whole genome sequencing was also in process by BGI (Shenzhen, China).

2.3. Isolation and characterization of P. acidilactici DQ2

The original LAB strain was isolated from the corn stover slurry used for ethanol fermentation. Ten grams of the biodetoxified CS samples were diluted with 90 ml of sterilized water and incubated for 2 h at 37 °C and 150 rpm to obtain the 1×10^{-1} suspension. The suspension was further diluted into 10^{-2}, 10^{-3} and 10^{-4} suspensions. The 10^{-4} dilution was streaked onto the YPD and the simplified MRS gels for enrichment culture. The culture was incubated for 3 days at 25, 37 and 42 °C, respectively, then the colonies were re-streaked onto the YPD and the simplified MRS gels based on the morphology of the colonies. The single colony was isolated, re-streaked and transferred for five times to obtain the purified single colony. The single colony was inoculated onto the YPD medium and the simplified MRS medium for lactic acid fermentation, respectively. The cell mass cultured in the simplified MRS medium was collected for extraction of the genomic DNA using the EZNA Bacterial DNA Kit (Omega Biotek, Norcross, GA, USA). The 16S rDNA genes were amplified using PCR by the universal primers TH1f (5′-AGAGTTTGATCMTGGCTCAG-3′) and TH1r (5′-ACGGCTA CCTTGTTACGACTT-3′). The purified PCR products were sequenced in Shanghai Biotech Service (Shanghai, China). Then the 16S rDNA gene sequences were blasted in the NCBI genebank and the phylogenetic tree was constructed based on the neighbor-joining method using Bioedit 7.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and Mega 4 (http://www.megasoftware.net/).

The stock cultures were maintained at −80 °C freezer in the simplified MRS broth containing 30% (v/v) glycerol solution. One stock vial was inoculated into the simplified MRS broth and cultured at 42 °C, 150 rpm for 12 h for activation and seeds culture. The culture was carried out in a 250 mL flask containing 50 mL simplified MRS medium with 10% (v/v) inoculation ratio at 42 °C, 150 rpm for 80 h. The pH was controlled by addition 60 g of $CaCO_3$ per 100 g of glucose. All experiments were done in triplicates.

2.4. Inhibitor tolerance, thermo-tolerance, and pentose utilization of P. acidilactici DQ2

Inhibitor tolerance experiments were conducted in a 250 mL flask containing 50 mL of the simplified MRS broth with different inhibitor concentrations of furfural, HMF, acetic acid, formic acid, levulinic acid, vanillin, and cultured at the 10% inoculation ratio, 42 °C, 150 rpm for 24 h. The xylose utilization for lactic acid production was also carried out in the same way by replacing glucose with xylose at 10% of inoculation ratio, 42 °C, 150 rpm for 48 h. Calcium carbonate was added at a proportional of 60% (w/w) of the carbon resources in the broth for roughly controlling the pH. All experiments were done in triplicate.

The thermo-tolerance experiment of *P. acidilactici* DQ2 was performed in the 5 L bioreactor equipped with a helical impeller

as described in Zhang et al. (2010a). The pretreated and biodetoxified CS was autoclaved at 115 °C for 30 min, and 121 °C for 60 min. Then the autoclaved CS was fed into the bioreactor at 15% (w/w) for the SSF operation. First, the prehydrolysis was carried out at 50 °C, pH 5.0 for 8 h; then the temperature was reduced to 42 °C and the pH was increased to 5.5 to start the SSF operation.

2.5. Pretreatment and biodetoxification of CS

The dry dilute acid pretreatment followed the procedure in Zhang et al. (2011). The dried CS was presoaked at the presoaking ratio of the solid (the dry materials) to the liquid (the sulfuric acid solution) of 2:1 (w/w) at 2.5 g sulfuric acid per 100 g of the dried CS and was pretreated at the conditions of 190 °C, 1.2 MPa, for 3 min. The pretreated CS contained the dry solid matter (DM) approximately 50% (w/w) and no free waste water was generated in the pretreatment. The CS hydrolysate at 15% (w/w) solids content contained 33.95 g/L of glucose, 18.18 g/L of xylose, 7.21 g/L of cellobiose, 4.70 g/L of acetic acid, 1.20 g/L of 5-HMF, 1.10 g/L of furfural, and 0.32 g/L of levulinic acid.

The biological removal of inhibitor compounds in the pretreated CS followed the biodetoxification procedure in Zhang et al. (2010b). The spores of the biodetoxification fungus of *A. resinae* ZN1 were inoculated onto the solids pretreated CS containing 50% solids (w/w) for the removal of the inhibitors generated in the pretreatment. The biodetoxification process lasted for 5 days at 25 °C, pH 6.0, with 60% (w/w) moisture until 90% of furfural and HMF were removed. Then the biodetoxified CS were autoclaved at 115 °C for 30 min and stored at 4 °C for use. The biodetoxified CS hydrolysate at 15% (w/w) solids content contained 31.25 g/L of glucose, 8.01 g/L of xylose, 0 g/L of cellobiose, 1.50 g/L of acetic acid, 0.05 g/L of 5-HMF, furfural and levulinic acid were not detected. The CS was autoclaved for 20 min at 115 °C and ready for SSF operation.

2.6. SSF of the pretreated and biodetoxified CS

The SSF operation of the dilute sulfuric acid pretreated and biodetoxified CS was performed in the 5 L bioreactor equipped with a helical impeller (Zhang et al., 2010a). The detailed information of the bioreactor used, including the figuration, the operation procedure description, the impeller drawing, and the materials of the bioreactor, were described in Zhang et al. (2010a). The SSF was operated at two stages, the prehydrolysis stage started at the beginning and then the real SSF stage was followed until the end of the operation. In the prehydrolysis stage, the CS was fed into the bioreactor within 8 h for prehydrolysis at the Accellerase 1000 cellulase of 15.0 FPU/g DM, at 50 °C and pH 5.0. The SSF started afterwards by inoculating the *P. acidilactici* DQ2 seeds culture into the bioreactor and operated for 48–96 h. The extra nutrients contained 1.0 g/L of ammonium sulfate; 2.0 g/L of dipotassium phosphate, 1.0 g/L of magnesium sulfate heptahydrate, and 1.0 g/L of yeast extract in the SSF experiments. The pH was maintained during the hydrolysis and SSF stages by addition of 5 M NaOH solution and 4 M H_2SO_4 solution. The samples were withdrawn at regular intervals and centrifuged at 10,000 rpm for 5 min to obtain the supernatant for analysis on HPLC.

The scale-up experiment was performed in a 50 L bioreactor equipped with a helical ribbon impeller. The configuration of the impeller was similar to the one used in 5 L bioreactor but with proportionally scaled-up.

2.7. Analysis of CS composition

The composition of CS was analyzed using ANKOM 220 Cellulose Analyzer (ANKOM Technology, Macedon, NY, USA). The original CS contained 34.2% cellulose, 31.5% hemicellulose and 8.1% lignin (w/w, dry weight base). The moisture content of CS was measured at 105 °C until the weight was constant.

2.8. Analysis of sugars, lactic acid and inhibitors on HPLC

Glucose, lactic acid, acetic acid and levulinic acid were analyzed using high performance liquid chromatography (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-Rad Aminex HPX-87H column at the column temperature 65 °C. The mobile phase was 5 mM H_2SO_4 at the rate of 0.6 mL/min. Furfural, HMF and vanillin were analyzed using the reversed-phase HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Japan) with a YMC-Pack ODS-A column at ambient temperature. The mobile phase was 50% methyl cyanide solution at the rate of 1.0 mL/min. All samples were centrifuged to remove the cell mass and other water insoluble substances, then filtered through a 0.22 μm filter before analysis.

The D/L chiral purity of lactic acid was analyzed using the Megazyme D/L-lactic acid kit K-DLATE purchased from Megazyme International Co. (Wicklow, Ireland).

2.9. Calculations

The lactic acid yield of the SSF based on the pretreated CS was calculated according the following equation:

$$\text{Lactic acid yield} = \frac{[Lac]_f \times V_f - [Lac]_0 \times V_0}{f \times W_{CS} \times 1.111 \times 1.0} \times 100\%$$

where $[Lac]_f$ and $[Lac]_0$ are the lactic acid concentrations at the end and the beginning of the fermentation (g/L), V_f and V_0 are the volume of liquid at the end and the beginning of the fermentation (L), W_{CS} is the weight of the dry CS used in the SSF (g), f is the cellulose fraction of the dry CS (g/g), 1.111 is the conversion factor for cellulose to equivalent glucose, 1.0 is the conversion factor for glucose to lactic acid on the mass basis of stoichiometric biochemistry. The unit g/L refers to the volume of the liquid fraction calculated based on the water mass balance of the SSF operation.

3. Results and discussion

3.1. Identification and characterization of the isolated LAB strain P. acidilactici DQ2

The 16S rDNA fragment of the isolated LAB bacterium was amplified using the universal primers TH1f and TH1r, and the sequence was shown in Table 1. The sequence was blasted in the NCBI genebank and the phylogenetic tree was constructed as shown in Fig. 1. The result shows that the 16S rDNA sequence of the isolated bacterium had a 99.9% similarity to that of *P. acidilactici*, thus the isolated LAB strain was designated as *P. acidilactici* DQ2 based on the analysis. *P. acidilactici* DQ2 is a lactic acid bacterium (LAB) strain, but rarely used for production of lactic acid (Okano et al., 2010). Instead, the strain was usually used for production of bateriocin, an antibiotic for animal feed use. Currently, the *P. acidilactici* DQ2 strain is in the process of the registration into the China General Microorganism Collection Center (CGMCC, Beijing, China) and it will be open to the academic community after the registration.

Fig. 2 shows the utilization of both glucose and xylose of *P. acidilactici* DQ2 for lactic acid fermentation. Fig. 2(a) shows that the lactic acid increased with the increasing glucose concentration in the simplified MRS medium. The least lactic acid yield reached 0.9 g/g glucose, and the least lactic acid tolerance of *P. acidilactici* DQ2 was up to 68 g/L without obvious substrate inhibition. Fig. 2(b) shows that no lactic acid formation and xylose consumption

Table 1
16S rDNA sequence of the isolated LAB strain *P. acidilactici* DQ2.

GGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTACGGCTACCTTGTTACGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAAAAGGTTA
CCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACChGGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGAT
CCTCTAGAGATTACGGCTACCTTGTTACGACTTCACCCTAATCATCTGTCCCACCT GGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTACGGCTACCTT
GTTACGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAAAAG
GTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACChGGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGG
ATCCTCTAGAGATTACGGCTACCTTGTTACGACTTCACCCTAATCATCTGTCCCACCT GGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTACGGCTACC
TTGTTACGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAAAAG
GTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACChGGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGA
TCCTCTAGAGATTACGGCTACCTTGTTACGACTTCACCCTAATCATCTGTCCCACCT GGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTACGGCTACCTTG
TTACGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAAAAG
GTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACChGGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGA
TCCTCTAGAGATTACGGCTACCTTGTTACGACTTCACCCTAATCATCTGTCCCACCT GGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTACGGCTACCTTG
TTACGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAAAAGGTTA
CCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACChGGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCT
CTAGAGATTACGGCTACCTTGTTACGACTTCACCCTAATCATCTGTCCCACCT GGGCGGCGGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTACGGCTACCTTGTTA
CGACTTCACCCTAATCATCTGTCCCACCTTAGACGGCTAGCTCCTAAAAGGTTACCCCACCGGCTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGG
AACGTATTC

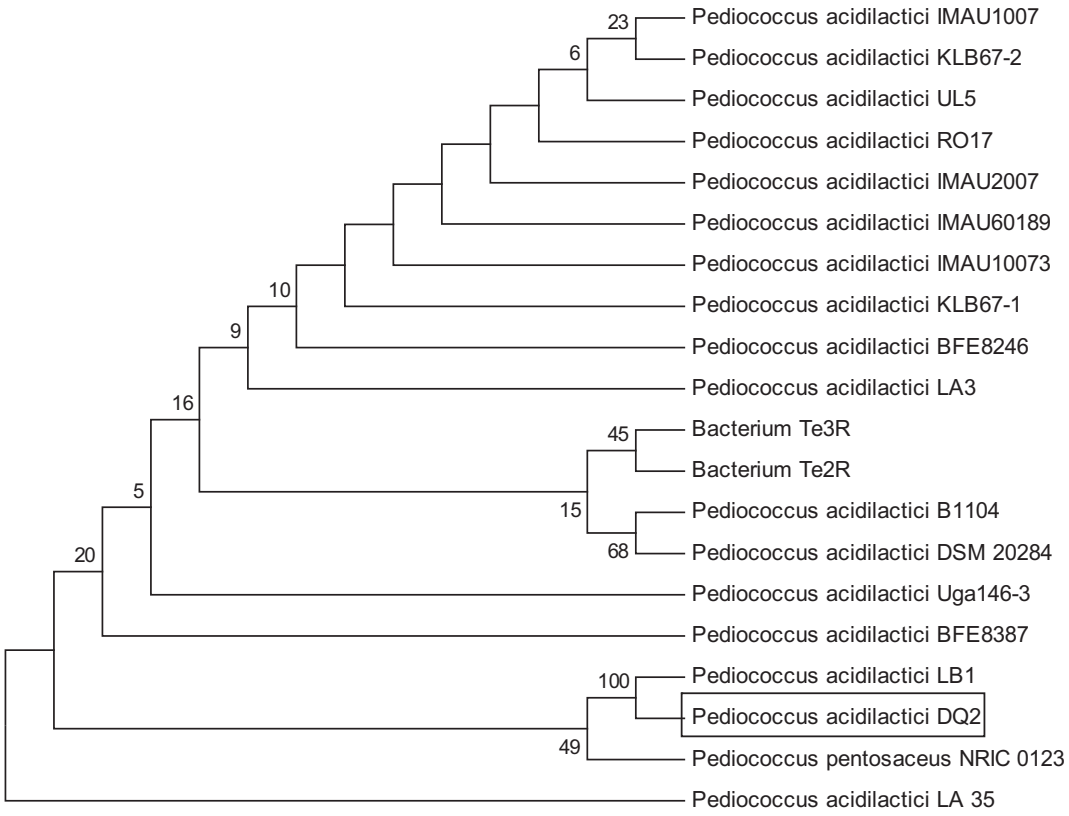


Fig. 1. Phylogenetic tree of *P. acidilactici* DQ2 based on the 16S rDNA sequence analysis.

were observed in the *P. acidilactici* DQ2 fermentation at different xylose concentrations in the simplified MRS medium (pH 5.5). However, it was also found that xylose was consumed at few specific conditions (higher pH at 6.5) after glucose was completely consumed, but no lactic acid formation was observed (data not shown), indicating the xylose utilization pathway may exist in *P. acidilactici* DQ2 but require a specific initiation action. Currently the xylose utilization in *P. acidilactici* DQ2 is under investigation by the ways of metabolic regulation as well as the genetic pathway establishment from xylose to xylulose by xylose isomerase, if the metabolic regulation fails to initiate the possible xylose utilization pathway.

The D/L chiral purity of the lactic acid produced by *P. acidilactici* DQ2 was analyzed by the Megazyme D/L-lactic acid assay Kit. The result shows that the purity of the L-lactic acid in the lactic acid produced was 63.4% and the D-lactic acid was 36.6%, indicating the lactic acid products of *P. acidilactici* DQ2 fermentation were hetero-lactic acids with approximately 2/3 of L-lactic acid and 1/3 of D-lactic acid. The chiral disunity of lactic acid made the product not suitable as the PLA monomer. Currently the knock-out of the D-lactic acid dehydrogenase gene (D-*ldh*) is under the way to yield an engineered L-lactic acid producing strain, and the knock-out of the L-lactic acid dehydrogenase gene (L-*ldh*) to a D-lactic acid producing strain is also under investigation.

The unique properties of the isolated *P. acidilactici* DQ2 were demonstrated by its excellent thermo- and inhibitor-tolerance. Fig. 3 shows the unusual tolerance of *P. acidilactici* DQ2 under the typical autoclave temperatures: after 30 min at 115 °C or 1 h

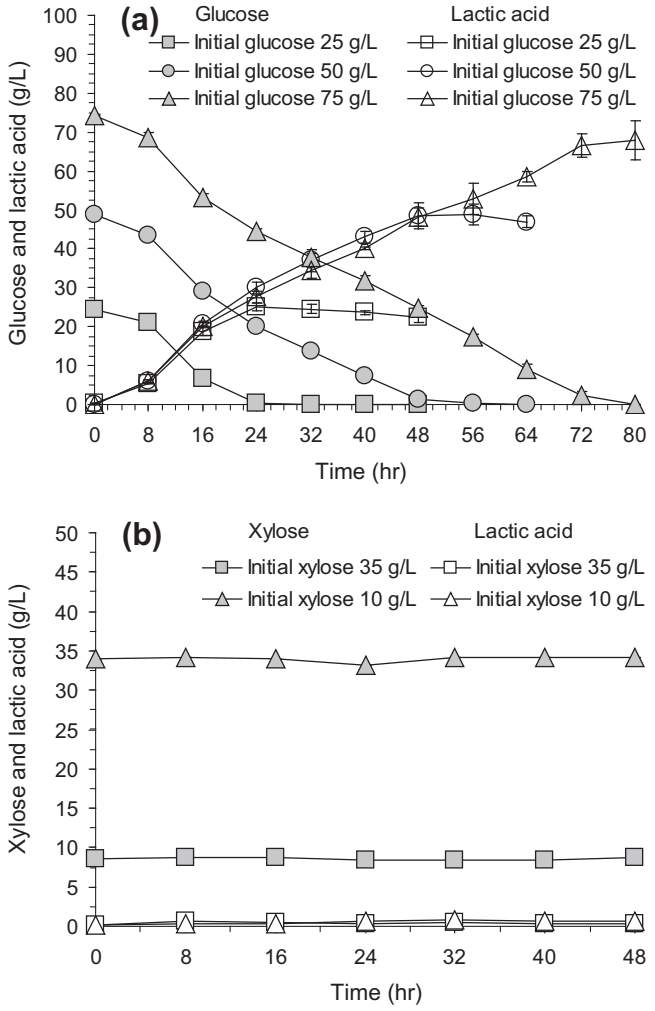


Fig. 2. Lactic acid fermentation at different initial glucose and xylose concentrations in the simplified MRS broth. (a) Different initial glucose concentrations; (b) different initial xylose concentrations. Conditions: 250 mL flask containing 50 mL of the simplified MRS broth, 10% inoculation ratio, 42 °C, 150 rpm for 80 h. The pH was controlled by addition 60 g $CaCO_3$ per 100 g glucose.

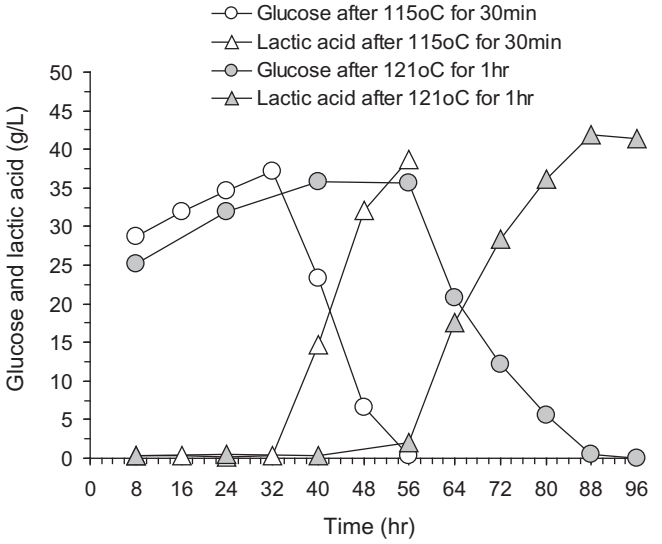


Fig. 3. Lactic acid fermentation of *P. acidilactici* DQ2 on the pretreated and biodetoxified CS after the extremely high temperature (autoclave) treatment. Autoclaved at 115 °C for 30 min, or 121 °C for 60 min. Conditions: CS at 15.0% (w/w), Accellerase 1000 at 15.0 FPU/g DM; 50 °C, pH 5.0 for 8 h prehydrolysis and 42 °C, pH 5.5, 150 rpm for fermentation.

at 121 °C of the autoclave operation, the strain still survived and produced considerable lactic acid in the simultaneous saccharification and fermentation (SSF) at 42 °C after a relatively long lag phase. As a control experiment, no lactic acid formation was observed in the SSF at the same temperature (42 °C) when the *P. acidilactici* DQ2 cells on the CS were thoroughly washed to remove the microbes and then autoclaved, indicating the lactic acid formation was not due to the microbial contamination of LAB strains during the SSF operation. This unique thermo-tolerant property of *P. acidilactici* DQ2 may benefit for the SSF at a high temperature, which matches the optimal cellulase activity (50 °C or higher) and reducing microbial contamination risks.

The tolerance of *P. acidilactici* DQ2 to the inhibitor compounds derived from lignocellulose degradation in the pretreatment is shown in Fig. 4. The selected inhibitors included two furan derivatives (furfural and HMF), three weak organic acids (acetic acid, formic acid, and levulinic acid), and one phenolic compound (vanillin) (Almeida et al., 2007; Zhang et al., 2010b). Fig. 5(a) and (b) show that *P. acidilactici* DQ2 had an extraordinary tolerance to the two furan derivatives, furfural and HMF; the cell growth and the lactic acid productivity did not show any obvious decrease at 3.0 g/L of furfural and 3.0 g/L of HMF, which were almost the lethal levels to ethanol fermenting strains (Palmqvist and Hahn-Hagerdal, 2000; Klinke et al., 2004; Almeida et al., 2007). Fig. 4(c) shows that growth of *P. acidilactici* DQ2 was not affected until the acetic acid reached 3.6 g/L, and the lactic acid productivity was affected up to a high level of 7.2 g/L. Fig. 4(d) shows that both the cell growth and the lactic acid productivity of *P. acidilactici* DQ2 was relatively sensitive to formic acid but still maintained a normal performance to 0.5 g/L of formic acid, which was already a high value in the pretreated lignocellulose. Fig. 4(e) shows that levulinic acid had almost no obvious effect on both the cell growth and the fermenting performance of *P. acidilactici* DQ2 up to 3.2 g/L, similar to other ethanol fermenting strains. Fig. 4(f) shows that *P. acidilactici* DQ2 was relatively sensitive to vanillin in both the cell growth and lactic acid fermentation performance, but when vanillin was below 0.2 g/L, both the cell growth and the lactic acid yield maintained satisfactory. Vanillin or other lignin derivatives were minor in most of the pretreatment processing and the level of 0.2 g/L vanillin was hardly detected.

3.2. SSF at the high temperature and high solids loading of CS feedstock

The SSF using *P. acidilactici* DQ2 was performed at the high temperature and high solids loading of the pretreated and biodetoxified CS for achieving the high lactic acid titer, yield, and productivity required by industrial practice. The helical ribbon impeller stirring bioreactor was used for the SSF operation which was able to process the high solids loading (Zhang et al., 2010a). The results are shown in Fig. 5.

Fig. 5(a) shows the SSF at different solids loadings (15%, 20%, 25% and 30%, w/w). The SSF was well performed at the solids loadings from 15% to 25% (w/w) until it reached 30% (w/w), in which the residual glucose appeared and the SSF was not complete. The result indicates that the SSF of *P. acidilactici* DQ2 could be operated at high solids loading of the pretreated and biodetoxified CS and possessed the potential to achieve the high lactic acid titer to 64.8 g/L and the satisfactory product yield of 60.6%. It was also noticed that increasing the lactic acid titer did not worsen the other fermentation performances. Fig. 5(a) shows that with increase of the solids loading from 15%, 20%, to 25% (w/w), the final lactic acid titer increased from 41.6, 59.2, to 64.8 g/L, the productivity was

almost constant (0.87, 0.82, to 0.90 g/L h), and the yield only slightly decreased from 66.4%, 64.1%, to 60.6%, respectively.

Fig. 5(b) shows the SSF in a high temperature range from 42 to 50 °C. The SSF was operated at the solids loading of 25% (w/w) and the maximum yield of lactic acid was found at 48 °C with the lactic acid titer and yield of 73.5 g/L and 63.7%, respectively. The SSF at 50 °C was not complete with the significantly decreased lactic acid yield and the existence of the residual glucose, indicating the temperature of 50 °C might exceed the maximum tolerance of *P. acidilactici* DQ2. The SSF reached an excellent temperature matching between the enzymatic hydrolysis of CS (50 °C) and the lactic acid fermentation by *P. acidilactici* DQ2 (48 °C).

Fig. 5(c) shows the SSF at different pH values from 5.0 to 6.5. It was found that *P. acidilactici* DQ2 preferred the elevated pH in the SSF. The generally applied pH at 5.0 for cellulase and ethanol fermenting strains such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* showed a very poor performance for *P .acidilactici* DQ2. However, a minor elevation of pH to 5.5 could significantly change the situation. In the pH range tested, the lactic acid fermentation at pH 5.5, 6.0, and 6.5 showed the similar performance without significant difference. Considering the lower pH facilitates the cellulose decomposition and reduces contamination risks, the optimum pH for the SSF was preferred to the lower value at 5.5. The optimal temperature and pH was a tradeoff between the cell growth and the saccharification when the pretreated CS was used as the feedstock. While in the synthetic MRS medium, the higher pH was benefit for the cell growth and lactic acid fermentation in the experimental range of pH 5.0–6.5, and almost no effect from temperature in the range tested (42–48 °C).

When lignocellulose was used as feedstock for ethanol fermentation, generally the saccharification step is regarded as the rate-limiting step in the SSF and cut down the process performance to the low productivity and yield (Gauss et al., 1976; Kim et al., 2008.). The similar phenomenon was found for the SSF of lignocellulose for lactic acid production. The acceleration of saccharification rate should play an important role in the SSF of lignocellulose into either ethanol or lactic acid.

3.3. Scale-up of lactic acid production at the high solids loading of the pretreated CS

Fig. 5(a) demonstrated that at the solids loading of 30% (w/w), the SSF was not performed well with considerable residual glucose and decreased lactic acid yield. It was estimated that the cell viability may perform a crucial role for the SSF and the increase of cell density was conducted by increasing the inoculation ratio

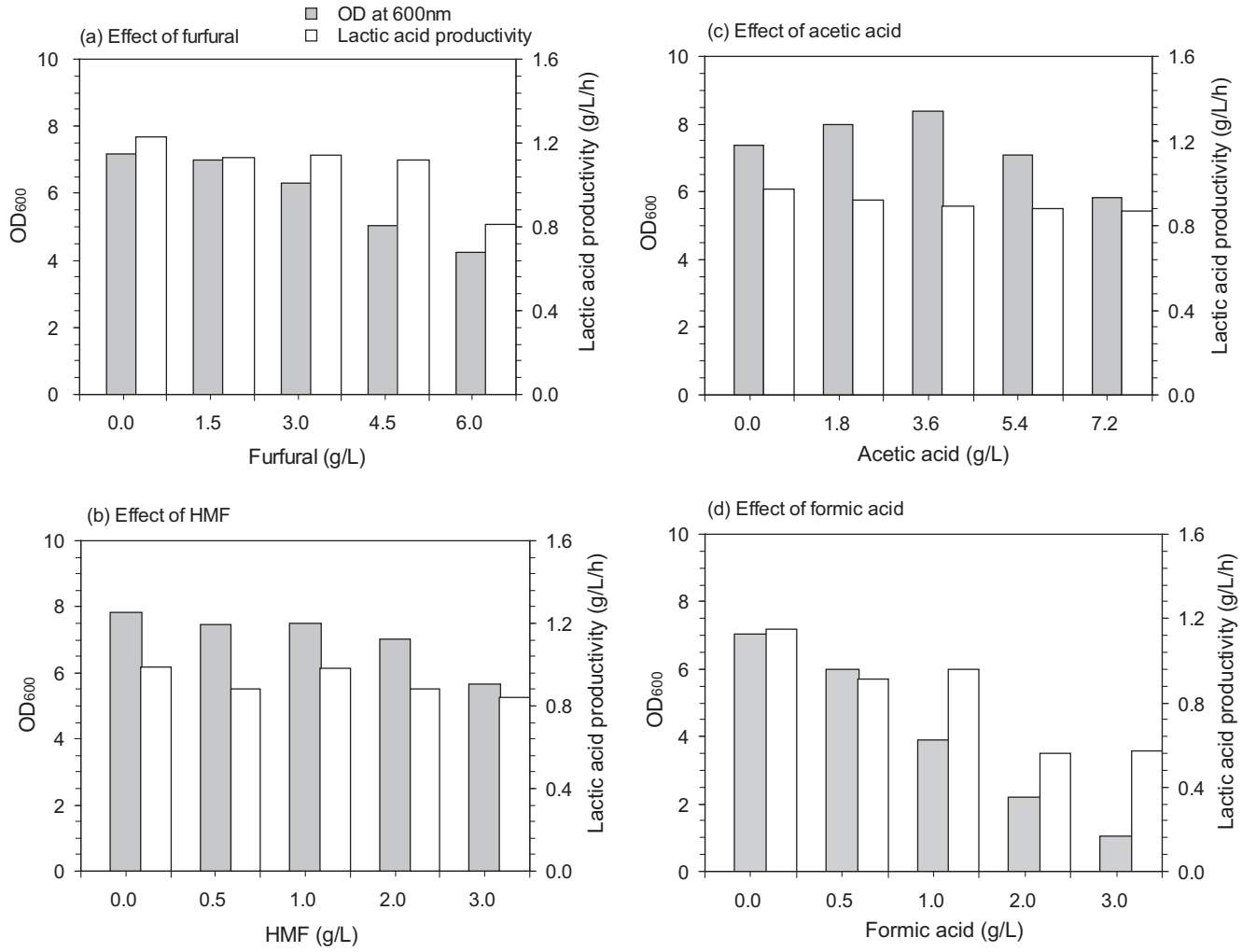


Fig. 4. Inhibitor tolerance of *P. acidilactici* DQ2 to the lignocellulose degradation compounds. (a) Furfural tolerance; (b) HMF tolerance; (c) acetic acid tolerance; (d) formic acid tolerance; (e) levulinic acid tolerance; (f) vanillin tolerance. Conditions: 250 mL flask containing 50 mL of the simplified MRS broth, 10% (v/v) inoculation ratio, 42 °C, 150 rpm for 24 h. The pH was controlled by addition 60 g $CaCO_3$ per 100 g glucose.

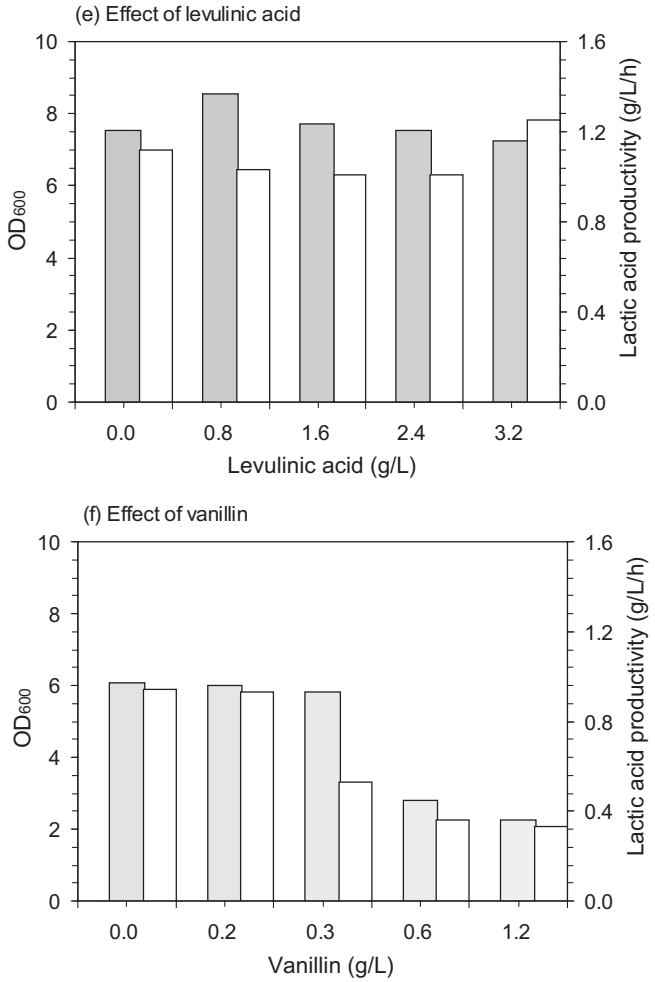
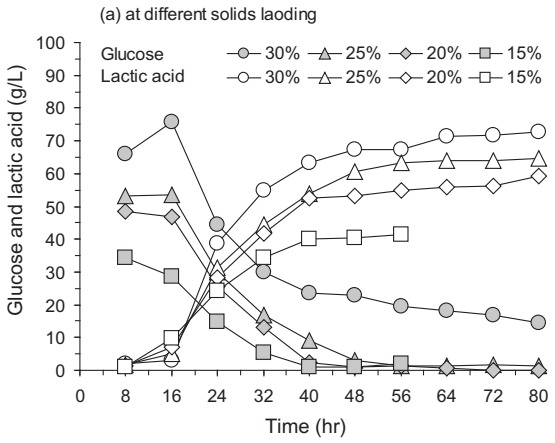
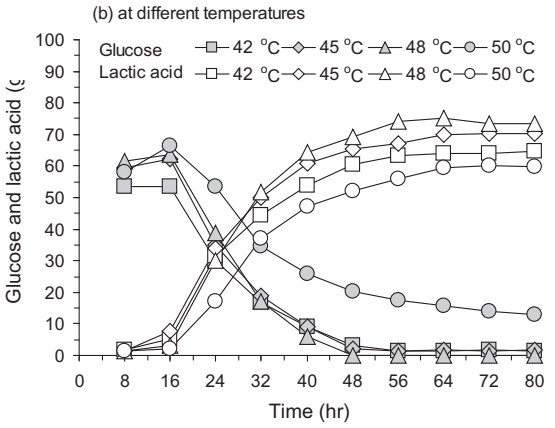


Fig. 4. (*continued*)



Solid loading (w/w, %)	30	25	20	15
Lactic acid titer (g/L)	72.7±0.7	64.8±0.5	59.2±2.0	41. 6±0.9
Lactic acid yield (%)	50. 7±0.5	60. 6±0.4	64. 1±2.1	66.4±1.4
Lactic acid productivity (g/L/h)	1.01±0.01	0.90±0.01	0.82±0.03	0.87±0.01

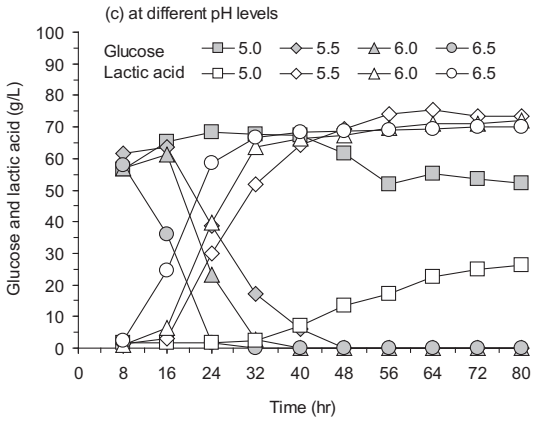


Fermentation temperature (°C)	42	45	48	50
Lactic acid titer (g/L)	64.8±0.5	70.4±0.03	73.5±0.03	59.6±0.3
Lactic acid yield (%)	60.6±0.4	61.2±0.03	63.7±0.03	51.5±0.3
Lactic acid productivity (g/L/h)	0.90±0.006	0.98±0.001	1.17±0.001	0.83±0.004

Fig. 5. SSF of the pretreated and biodetoxified CS at different fermentation parameters. Conditions: 50 °C, pH 5.0 for 8 h prehydrolysis (before inoculation), Accellerase 1000 at 15 FPU/g DM, 5% of inoculation ratio, 150 rpm stirring rate. (a) At different solids loading, 42 °C, pH 5.5; (b) at different temperatures, pH 5.5, solids loading 25.0% (w/w); (c) at different pH levels, 48 °C, solids loading 25.0% (w/w).

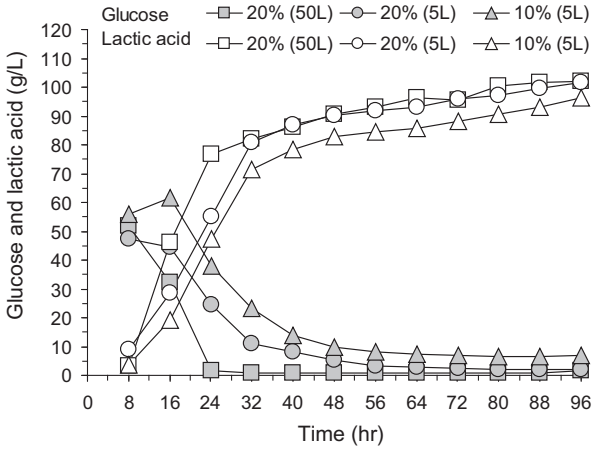
of *P. acidilactici* DQ2 seeds to 20% (v/v) as shown in Fig. 6. The titer, yield and productivity of lactic acid of the SSF increased significantly to 101.6 g/L (the theoretical lactic acid concentration was 141.5 g/L), 71.8%, and 1.06 g/L/h within 96 h, respectively. The similar fermentation operation was applied to the greater bioreactor scale of 50 L with slight lowered solids loading of 27% (w/w), ten folds of the bioreactor used, very close results were obtained with the titer, yield and productivity of lactic acid 101.9 g/L, 77.2%, and 1.06 g/L/h within 96 h, respectively. Up to our knowledge, this lactic acid titer was the maximum value of the reported cellulosic lactic acid studies and the lactic acid titer of 100 g/L showed the potential of industrial applications. Although Wee et al. (2004) obtained 93 g/L of lactic acid from the vacuum evaporation concentrated oak wood hydrolysate using an *Enterococcus faecalis* strain at high cellulase dosage of 30 FPU per gram of substrate, but apparently, the method using vacuum evaporation requires huge energy input and not possible to be accepted by industrial practice. Although the result was obtained at the high inoculation ratio (20%, v/v), the same result might be obtained by using high cell density culture of *P. acidilactici* DQ2 as the fermentation seeds at a reasonable inoculation ratio (10%, v/v). However, the cells began to flocculate when a high cell density was reached caused by the formation of polysaccharides for current *P. acidilactici* DQ2 strain. Currently the high cell density culture is under investigation by lessening the flocculation by amylase addition to the cell culture and the very positive result was obtained.

A very preliminary evaluation was calculated on the value added from corn stover to ethanol and lactic acid: 150 kg ethanol could be produced from 1 ton of CS based on the practical yield of 75% from cellulose to ethanol, and the ethanol equals to $150 assuming $1,000 per ton of fuel ethanol. On the other hand, 300 kg lactic acid could be produced based on the same yield of 75% from cellulose to lactic acid as shown in this study, and the lactic acid equals to $360 assuming $1,200 per ton for chemical grade lactic acid, or equals to $750 assuming $2,500 per ton for polymer monomer grade lactic acid. In both lactic acid cases (chemical or monomer), the value added for production of lactic acid was 2–5 folds greater than that for ethanol production, although the process flowsheet and cost are very similar except the product purification



Fermentation pH values	5.0	5.5	6.0	6.5
Lactic acid titer (g/L)	26.3±1.0	73.5±0.03	72.2±0.7	70.2±0.08
Lactic acid yield (%)	18. 8±0.8	63. 7±0.03	62.9±0.6	60.1±0.07
Lactic acid productivity(g/L/h)	0.36±0.010	1.17±0.001	1.01±0.01	0.97±0.001

Fig. 5. (*continued*)



Bioreactor scale	5L	5L	50L
Solids loading of CS (w/w)	30%	30%	27%
Inoculation ratio (%)	10	20	20
Lactic acid titer (g/L)	96.1±2.2	101.6±1.3	101.9±2.1
Lactic acid yield (%)	67.9±1.5	71.8±0.9	77.2±1.6
Lactic acid productivity (g/L/h)	1.00±0.03	1.06±0.02	1.06±0.02

Fig. 6. SSF of the pretreated and biodetoxified CS at different bioreactor scales and inoculation ratios. Conditions: 50 °C, pH 5.0 for 8 h prehydrolysis (before inoculation) and then SSF (after inoculation), bioreactor with 5 and 50 L in volume, inoculation ratio at 10% or 20%, solids loading 30.0% or 27.0% (w/w), Accellerase 1000 at 15 FPU/g DM, 48 °C, pH 5.5, 150 rpm stirring rate.

step. The results may indicate that, for lignocellulose biorefinery industry, the chemical may go first before biofuels as the target products to make the industry independent of the government subsidy.

4. Conclusion

A new lactic acid bacterium *P. acidilactici* DQ2 was isolated and used for the SSF of corn stover at the high solids loading for lactic acid production. The corn stover was pretreated using the dry dilute sulphuric acid pretreatment and biologically detoxified using *A. resinae* ZN1, and finally used for the SSF operation. A typical SSF operation at 48 °C and pH 5.5, and near 30% (w/w) solids loading in both 5 and 50 L bioreactors was demonstrated with the lactic acid titer, yield, and productivity of 101.9 g/L, 77.2%, and 1.06 g/L/h, respectively.

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