ACS Sustainable Chemistry & Engineering

Research Article

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Antibacterial Peptide Secreted by *Pediococcus acidilactici* Enables Efficient Cellulosic Open L-Lactic Acid Fermentation

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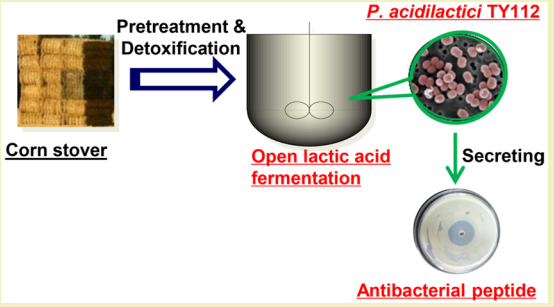
Supporting Information

ABSTRACT: Maintaining aseptic conditions is one of the major cost factors in industrial fermentation. Here, we have demonstrated an open fermentation method for producing cellulosic L-lactic acid by *Pediococcus acidilactici*, which is also capable of secreting an antibacterial peptide to control potential microbial contamination. The peptide secreted by *P. acidilactici* TY112 was isolated and its molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *P. acidilactici* TY112 was applied to an open L-lactic acid fermentation without sterilizing reactors, medium and supporting facilities. No contamination was observed and high L-lactic acid fermentation performances were obtained during open fermentation in synthetic medium and corn stover hydrolysates, both in separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) modes. The open SSF at 30% (w/w) solids loading reached 97.3 g/L of L-lactic acid with a productivity of 1.5 g/(L·h) and yield of 69.4%, which were comparable to the conventional SSF with sterilization. The antibacterial peptide secreted by *P. acidilactici* enabled a successful and more cost-effective open L-lactic acid fermentation method, without energy-consuming sterilization protocols. Potentially, the open fermentation concept presented herein can be applied to other fermentative processes in the future, by expressing appropriate antibacterial peptides in the desired microbial strains.



KEYWORDS: *Microbial contamination, Open fermentation, Pediococcus acidilactici TY112, High solids loading, Simultaneous saccharification and fermentation (SSF), Corn stover*

INTRODUCTION

Preventing microbial contamination is one of the key concerns for most industrial fermentation processes.[1] The most commonly used procedures to prevent contamination include sterilization under high temperatures or strong acidic/alkaline soaking of reactors and pipelines. High-temperature sterilization methods are energy intensive and can potentially produce inhibitory substances to fermentation.[2] Addition of antibiotics is another commonly applied method, but it carries potential health and environmental risks associated with antibiotics resistance.[3,4] Open fermentation is usually carried out under extreme conditions (e.g., thermophilic, alkalophilic, or halophilic), which are suitable for the desired fermenting microorganisms but not appropriate for common microbial contaminants.[5] Previous studies show that open fermentation methods can save up to 40% steam consumption relative to conventional fermentation methods using high temperature sterilization.[6] Thus, open fermentation methods could be economically attractive to replace conventional fermentation processes performed under stringent aseptic conditions.

Lactic acid is a promising biobased chemical that is typically used for production of biodegradable polylactide acid (PLA), which is currently a viable alternative to petroleum-derived plastics.[7] Today, lactic acid is commercially produced via microbial fermentation of starch-derived glucose or sucrose. Lignocellulose feedstocks are abundant, renewable and non-edible resources that could be used as alternative to starch-based sugars for large-scale production of lactic acid.[8] In addition, the cellulosic lactic acid production process can be more cost-effective by eliminating energy-consuming sterilization protocols.[2,9−11]

Researchers have started evaluating open fermentation methods at high temperatures. The thermotolerant *Bacillus* strains, such as *Bacillus coagulans*, *Bacillus* sp. 2−6, and *Bacillus licheniformis* were used to produce lactic acid by an open fermentation method at higher than 50 °C.[2,9−12] High

Received: July 4, 2017
Revised: August 13, 2017
Published: August 24, 2017

DOI: 10.1021/acssuschemeng.7b02212
ACS Sustainable Chem. Eng. 2017, 5, 9254−9262

fermentation temperatures prevent proliferation of contaminant bacteria, while allowing thermotolerant lactic acid bacteria (LAB) cells to grow well and produce lactic acid successfully. However, the neutral pH used for growing thermotolerant *Bacillus* strains does not match the ideal pH for fungal cellulase activity, i.e., pH around 4.8. For this reason, most studies on open fermentation focused on separate hydrolysis and fermentation (SHF). If the optimal operation temperature and pH for enzymes and microorganisms is relatively similar, simultaneous saccharification and fermentation (SSF mode) can be more advantageous, as it alleviates product inhibition to a greater extent during enzymatic hydrolysis.[13] High temperatures also intensify the toxicity of inhibitors present in the pretreated biomass, which reduces microbial growth during fermentation and leads to poor fermentation performance.[2,9,14] Additionally, the reported cellulosic lactic acid titer produced in open fermentation (~80 g/L) is still below the industrial lactic acid fermentation requirement (>100 g/L).[15] Therefore, newly improved open fermentation methods need to be developed to produce economically viable lactic acid titers.

One possible way of performing open lactic acid fermentation is to use a fermenting organism capable of secreting antibacterial agents to prevent microbial contamination. For example, bacteriocins are antibacterial peptides that are secreted by specific LAB strains such as *P. acidilactici*.[16] Our previous studies showed that the engineered strain *P. acidilactici* TY112 exhibited a high L-lactic acid fermentation performance in SSF of lignocellulose materials.[17,18] The combination of the two unique properties may lead to a more attractive open fermentation technology that can operate at moderate fermentation temperatures without the use of expensive and energy-consuming sterilization protocols. This study focuses on developing an open fermentation technology, taking advantage of the intrinsic antibacterial activity of *P. acidilactici* TY112 and its excellent fermentation potential for industrial production of L-lactic acid. The peptide secreted by *P. acidilactici* TY112 was isolated and its antibacterial activity was confirmed. The antibacterial activity of *P. acidilactici* TY112 was applied to an open lactic acid fermentation process using both the simplified MRS medium and corn stover hydrolysate.

MATERIALS AND METHODS

Raw Materials and Enzymes. Corn stover was obtained from Dancheng County, Henan Province, China, in the fall of 2013. Corn stover was water-washed to remove the impurities, air-dried, and milled using a beater pulverizer to pass through a 10 mm diameter screen. The milled corn stover was sealed in plastic bags and stored at room temperature before usage.

The cellulase enzyme Youtell no. 6 was purchased from Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The filter paper activity of Youtell no. 6 was 135 FPU/g (cellulase protein concentration was 90 mg/g cellulase cocktail) determined using the NREL protocol LAP-006,[19] and the cellobiase activity was 344 CBU/g using the method described by Ghose.[20]

Strains and Cultivation Conditions. L-Lactic acid producing strain *P. acidilactici* TY112 was genetically engineered from a wild strain *P. acidilactici* DQ2 by knockout of D-lactic acid dehydrogenase encoding gene *ldhD* and stored in China General Microorganism Collection Center (CGMCC), Beijing, China, with the registration number of 8664.[18] *Staphylococcus aureus* ATCC19095 (purchased from ATCC), *Bacillus subtilis* WB800 (kindly provided by Y.H. Percival Zhang at Virginia Tech), and *Bacillus coagulans* SIIM B179 (purchased from Shanghai Industrial Microbiology Institute, Shanghai, China) were used as indicator strains. *Amorphotheca resinae* ZN1 (CGMCC 7452) was used as a biodetoxification strain to degrade inhibitors from the dry dilute sulfuric acid pretreated corn stover via solid state fermentation.[21]

A simplified MRS medium containing 22 g/L of glucose, 10 g/L of tryptone, 5 g/L of yeast extract, 0.25 g/L of $MnSO_4 \cdot H_2O$, 2.0 g/L of triammonium citrate, 0.58 g/L of $MgSO_4 \cdot 7H_2O$, 2.0 g/L of K_2HPO_4, 5 g/L of CH_3COONa was used for culturing *P. acidilactici* TY112 and lactic acid fermentation. LB medium containing 20 g/L of glucose, 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl was used for culturing *S. aureus* and *B. subtilis*. A medium containing 20 g/L of glucose, 10 g/L of yeast extract, 10 g/L of peptone, 0.25 g/L of $MgSO_4 \cdot 7H_2O$ was used for culturing *B. coagulans*. All the above media were sterilized at 115 °C for 20 min before usage, except for the simplified MRS medium used in open fermentation trials. The simplified MRS and LB media described above were supplemented with 20 g/L agar and used for analyzing the antibacterial activity of *P. acidilactici* and testing cell viabilities by plate count method, respectively.[22]

Pretreatment and Biodetoxification. Corn stover was pretreated using dry dilute sulfuric acid pretreatment (DDAP).[23,24] Briefly, corn stover solids and a dilute sulfuric acid solution at 5.0% (w/w) were cocurrently fed into a reactor at a solid/liquid ratio of 2:1 (w/w), with helically stirring mixing at 50 rpm and pretreated at 175 °C for 5 min. The solids content of the pretreated corn stover was around 50% (w/w), and no wastewater was generated during DDAP.

The pretreated corn stover was biodetoxified using the fungal strain *A. resinae* ZN1 as in our previous studies.[21] Briefly, the pretreated corn stover was neutralized with 20% (w/w) $Ca(OH)_2$ to a pH value of 5.5 and then inoculated with *A. resinae* ZN1 spores and cultured for 7 days at 28 °C. After biodetoxification, the pretreated corn stover solids were autoclaved at 115 °C for 20 min before enzymatic hydrolysis and lactic acid fermentation, except when open SSF trials were performed.

Antibacterial Activity Assays of *P. acidilactici* Spent Media. The antibacterial activity of *P. acidilactici* spent media was assessed by measuring the growth of indicator bacterial strains. First, *P. acidilactici* TY112 was cultured in simplified MRS medium at 42 °C, with agitation at 150 rpm for 16 h, then neutralized to a pH of 6.5 and centrifuged at 16 125 g for 10 min. The supernatant was filtered through a 0.22 μm filter aseptically to prepare the cell-free *P. acidilactici* spent media (mentioned as *P. acidilactici* spent media hereinafter). Second, the fresh culture media for the three indicator strains were supplemented with *P. acidilactici* spent media (20%, v/v), trypsin-treated *P. acidilactici* spent media (20%, v/v), or trypsin itself, respectively. Then the indicator strains (*S. aureus, B. coagulans,* and *B. subtilis*) were inoculated separately and cultured at 42 °C, with agitation at 150 rpm for 12 h. The growth (OD_{600}) of these indicator strains was tested using a spectrophotometer. The trypsin-treated *P. acidilactici* spent media was prepared by adding trypsin to *P. acidilactici* spent media to a final concentration of 1 mg/mL and then hydrolyzed at 37 °C, pH 7.5 for 1 h.

Peptide Purification, Molecular Weight Determination, and Antibacterial Activity Confirmation. Partial purification of the peptide secreted by *P. acidilactici* was done following the adsorption−desorption method, which was based on the fact that the adsorption of pediocins to the producing strains was pH dependent.[25] Briefly, after culturing *P. acidilactici* TY112 in simplified MRS medium for 16 h at 42 °C, the pH of the culture was adjusted to 6.5 with 1.0 M NaOH (most peptides were adsorbed to the cells at this pH value) and then heated at 70 °C for 25 min to kill the viable cells. The peptide and dead cells were collected after centrifugation at 15 000 g for 15 min and then washed twice with sodium phosphate buffer (pH 6.5). The dead cells attached with peptide were resuspended in 50 mL of NaCl solution (100 mM). The pH was adjusted to 2.0 with 5.0% phosphoric acid and the suspension was stirred at 4 °C for 12 h to desorb the peptide from dead cells to the solution. The resultant solution was centrifuged at 12 000*g*, 4 °C for 20 min to discard the cells. The supernatant obtained was dried down to a solid powder in the freeze drier for 24 h. The solid powder was dissolved in 1 mL of ultrapure water for protein content analysis, SDS-PAGE, and antibacterial activity tests.

DOI: 10.1021/acssuschemeng.7b02212
ACS Sustainable Chem. Eng. 2017, 5, 9254−9262

The protein content in the purified powder was analyzed using Bradford method. SDS-PAGE was performed to determine the molecular weight of the purified peptides.

The antibacterial activity of the purified peptide was confirmed. Briefly, 100 μL of separately cultured indicator strains (12 h) were spread on their respective nutrient agar plates, as described in the Strains and Cultivation Conditions section. Then a small well was made aseptically by micropunch in the center of the plates, and 50 μL of diluted peptide solution was added to the well. The plates were incubated at 37 °C for 15 h.

Open Fermentation of Lactic Acid. Open fermentation of L-lactic acid was performed in the simplified MRS medium and corn stover hydrolysate, both in SHF and SSF modes. For open fermentation in the simplified MRS medium, L-lactic acid fermentation was conducted in a 3 L fermenter at both 42 and 37 °C, 150 rpm and pH of 6.5. Four experiments were designed, including (1) conventional fermentation with sterilizing reactors and medium as a control; (2) open fermentation without sterilizing reactors and medium; (3) induced contamination experiments with *B. subtilis* or *S. aureus* inoculated (5%, v/v) into the *P. acidilactici* TY112 culture medium from the beginning of lactic acid fermentation; (4) simultaneous addition of indicator strains and trypsin (1 mg/mL) into the *P. acidilactici* TY112 culture medium, from the beginning of lactic acid fermentation, to test the hypothesis that the antibacterial activity observed in this work is derived from the presence of secreted peptides by *P. acidilactici* TY112. In experiments 3 and 4, sterilized reactors and medium were used for excluding other contamination possibilities.

For open SHF fermentation in corn stover hydrolysate, corn stover hydrolysate was prepared by enzymatic hydrolysis of the pretreated and biodetoxified corn stover at 15% solids loading (w/w) at 50 °C for 48 h, and followed by centrifugation at 8000*g* for 10 min to obtain the hydrolysate liquid. The corn stover hydrolysate contained 69.1 g/L of glucose, 12.0 g/L of xylose, 0.8 g/L of acetic acid, and 0.1 g/L of 5-hydroxymethylfurfural (HMF). The experimental design for open fermentation in corn stover hydrolysate was similar to the simplified MRS medium, except that experiment 4 was not conducted. It is worth noting that during the enzymatic hydrolysis step, the reactors, water, corn stover, and other facilities used were autoclaved to produce the uncontaminated corn stover hydrolysate before fermentation.

For SSF experiments, the corn stover after DDAP and biodetoxification was fed into a 5 L helical stirring bioreactor to a final solids loading of 30% (w/w) and the prehydrolysis was performed at 50 °C, agitated at 150 rpm for 6 h.[26] Then, the SSF was started by inoculating *P. acidilactici* TY112 (10%, v/v) at 42 °C and lasted for 66 h. The cellulase dosage was 15 FPU/g DM. The pH was maintained at 4.8 and 5.5 during prehydrolysis and SSF stage by automatic regulation with 5 M NaOH, respectively. Samples were taken at regular intervals, centrifuged at 11 167*g* for 5 min and the supernatant was analyzed by HPLC. The cell viability during SSF was determined using the plate count method.[22] For open SSF, the reactors, nutrients and corn stover were not sterilized and the samples taken were not controlled in an aseptic condition, while the stringent sterilization was conducted in the conventional SSF as the control. The nutrients supplemented to the lactic acid fermentation system using corn stover were same to the simplified MRS medium except for the glucose.

Analysis and Lactic Acid Yield Calculation. The dry dilute acid pretreated corn stover contained 39.9% of glucan, 3.0% of xylan, 13.5 mg/g DM of glucose, 97.8 mg/g DM of xylose, 13.3 mg/g DM of acetic acid, 5.3 mg/g DM of furfural, 2.1 mg/g DM of 4-HBA, and 0.9 mg/g DM of syringaldehyde determined by the two-step sulfuric acid hydrolysis method described in NREL protocols.[27]

The samples collected at different intervals were determined for glucose, xylose, lactic acid, acetic acid, furfural, and HMF by HPLC. The HPLC was equipped with LC-20AD pump, RI detector RID-10A (Shimadzu, Kyoto, Japan), and a Bio-Rad Aminex HPX-87H column operated at 65 °C with 0.6 mL/min of 5 mM H_2SO_4 as the mobile phase.

Lactic acid yield was defined as the ratio of the obtained L-lactic acid to the theoretical L-lactic acid, which was calculated based on the chemical stoichiometry from cellulose added to the SHF and SSF unit operations.[17]

RESULTS AND DISCUSSION

Identification of Antibacterial Activity in *P. acidilactici* TY112 Spent Media. *P. acidilactici* TY112 spent media was obtained by culturing *P. acidilactici* in simplified MRS medium for 16 h, followed by neutralization, centrifugation and aseptic filtration to remove the cells. The antibacterial activity was determined using *B. subtilis* WB800, *S. aureus* ATCC19095, and *B. coagulans* SIIMB179 as indicator strains under similar cell growth conditions (Figure 1). *S. aureus*, *B. subtilis*, and *B. coagulans* cell growth was significantly inhibited in the experiments where *P. acidilactici* spent media (20%, v/v) was added to their culture media. The lactic acid present in *P. acidilactici* spent media did not cause a pH decrease when added to the indicator strains' culture media, because it was neutralized before addition. An initial hypothesis was to attribute this growth inhibition to antibacterial pediocin-like peptides secreted by *P. acidilactici*, which is a well-known strain for pediocins production.[16] To confirm this assumption, *P. acidilactici* spent media was treated with trypsin at 37 °C for 1 h, prior to antibacterial activity testing. Figure 1 shows that the trypsin-treated *P. acidilactici* spent media practically lost its ability to inhibit the indicator strains' growth and that trypsin itself has no significant effect on their cell growth. These results suggest that peptides, or pediocins-like substances, present in *P. acidilactici* spent media might be responsible for the observed antibacterial activity.

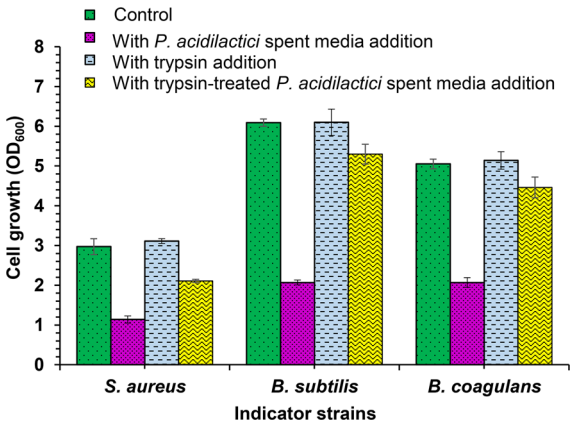


Figure 1. Antibacterial activity of the *P. acidilactici* TY112 spent media.

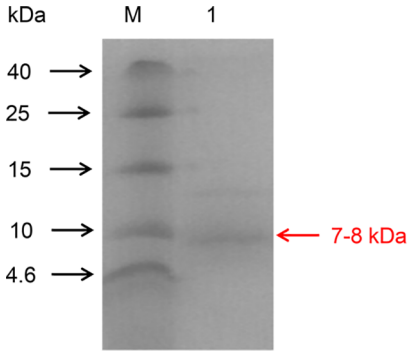


Figure 2. SDS-PAGE of the purified peptide from the culture broth of *P. acidilactici* TY112. Lane M: protein molecular weight markers (kDa). Lane 1: purified peptide band.

DOI: 10.1021/acssuschemeng.7b02212
ACS Sustainable Chem. Eng. 2017, 5, 9254−9262

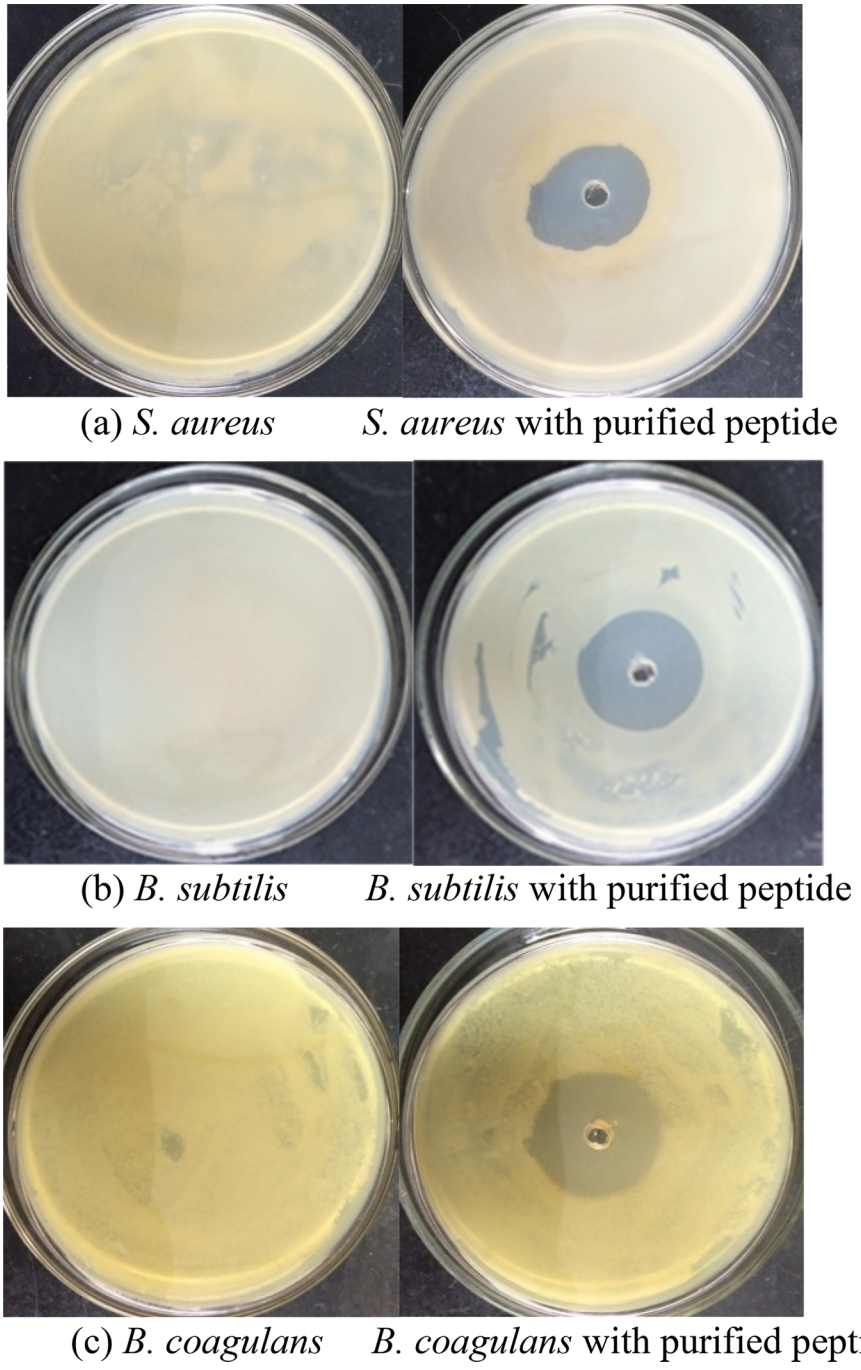


Figure 3. Confirmation the antibacterial activity of the purified peptide by the growth of *S. aureus* (a), *B. subtilis* (b), and *B. coagulans* (c) on nutrient petri-dish agar plates with or without purified peptide.

To further confirm the production of antibacterial proteins or peptides by *P. acidilactici* during lactic acid fermentation, peptides were isolated from the *P. acidilactici* culture broth.[25] A strong band with molecular weight of 7−8 kDa was observed in the SDS-PAGE (Figure 2), which was within the molecular weight range of reported pediocins secreted from *P. acidilactici*. Various reports identified pediocins with distinct molecular weights, such as 4.6,[16] 5.25,[28] 10.3,[29] and 16.5 kDa.[30] The antibacterial activity of the purified peptide secreted by *P. acidilactici* was further confirmed (Figure 3) by pouring the culture broth of the indicator strains onto their respective nutrient agar plates and then digging a small well in the center of each plate, where 50 μL of the purified peptide solution was added prior to a 15 h incubation at 37 °C. A transparent zone was observed in the center of each nutrient agar plate near the area where the peptide was added. In contrast, the indicator cells grew well and spread evenly on the plates without peptide addition (Figure 3).

The combination of these results confirmed the existence of an antibacterial peptide secreted from *P. acidilactici* with molecular weight of 7−8 kDa, which inhibited the growth of typical invading microorganisms such as *S. aureus*, *B. subtilis*, and *B. coagulans*. However, proteomic analysis is still required to confirm if this peptide belongs to the pediocins family.

Open Lactic Acid Fermentation of *P. acidilactici* TY112 in the Simplified MRS Medium. Open lactic acid

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ACS Sustainable Chem. Eng. 2017, 5, 9254−9262

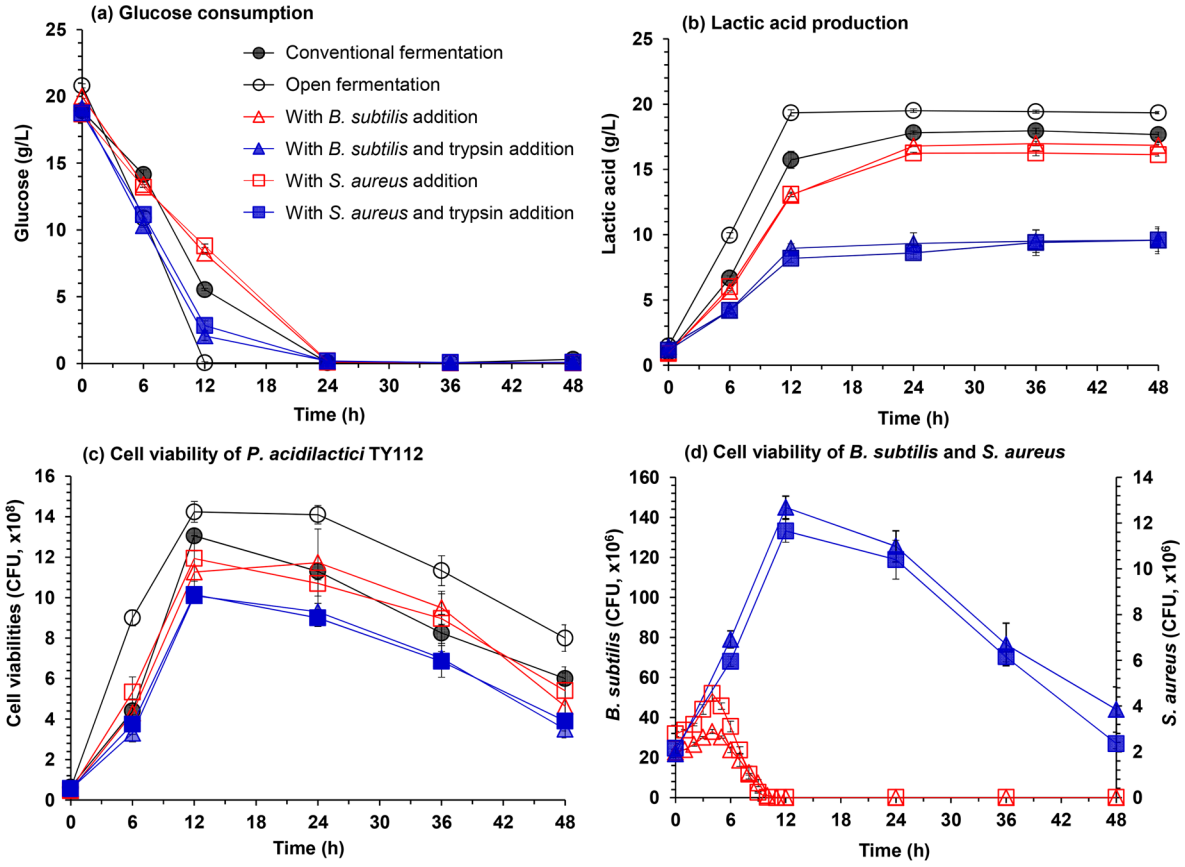

Figure 4. Open lactic acid fermentation in simplified MRS medium at 42 °C: (a) glucose consumption; (b) lactic acid production; (c) cell viability of *P. acidilactici* TY112; (d) cell viability of indicator strains in the cases of with *B. subtilis* addition, with *B. subtilis* and trypsin addition, with *S. aureus* addition, and with *S. aureus* and trypsin addition, respectively.

Table 1. Lactic Acid Fermentation Performance in Simplified MRS Medium at 42 °C

fermentation conditions[a]	lactic acid (g/L)	lactic acid yield (%)
conventional fermentation	18.0 ± 0.2	90.4 ± 1.1
open fermentation	19.5 ± 0.1	87.0 ± 0.5
with *B. subtilis* addition	17.0 ± 0.2	80.4 ± 1.0
with *B. subtilis* and trypsin addition	9.5 ± 0.9	42.6 ± 4.7
with *S. aureus* addition	16.3 ± 0.1	82.8 ± 0.5
with *S. aureus* and trypsin addition	9.6 ± 1.0	45.2 ± 5.4

[a]The detailed fermentation conditions were listed in Figure 4.

fermentation was performed by *P. acidilactici* TY112 in simplified MRS medium at 42 °C (Figure 4 and Table 1). As shown in Figure 4a and b, the rates of glucose consumption and lactic acid generation were greater in open fermentation relative to the conventional fermentation, where sterilization was applied. In addition, *P. acidilactici* TY112 also had a slightly higher cell viability for the open fermentation relative to conventional fermentation (Figure 4c). The final lactic acid concentration was a little higher in open fermentation (19.5 ± 0.1 g/L) than in conventional fermentation with sterilization (18.0 ± 0.2 g/L), while the lactic acid yield was almost the same (87.0 ± 0.5% for open fermentation and 90.4 ± 1.1% for conventional fermentation, respectively). The slightly lower cell viability and fermentation performances in conventional fermentation might be caused by the generation of growth inhibitors during high temperature autoclaving.[4] When the indicator strains *B. subtilis* and *S. aureus* were inoculated separately at the very beginning of lactic acid fermentation, they could grow until 3 h of fermentation, but their viability was rapidly reduced until they died out at about 10 h fermentation (Figure 4c and d). The inoculation of indicator strains resulted in a slight decrease in both the final lactic acid (17.0 ± 0.2 and 16.3 ± 0.1 g/L, respectively) and yield (80.4 ± 1.0% and 82.8 ± 0.5%, respectively) relative to the conventional fermentation with sterilization. When peptide-degrading trypsin was added simultaneously with both indicator strains, they showed good viability throughout the entire fermentation process (Figure 4c and d) and led to a significant decrease (almost by 50%) in final lactic acid (9.5 ± 0.9 and 9.6 ± 1.0 g/L, respectively) and yield (42.6 ± 4.7% and 45.2 ± 5.4%, respectively) relative to that without trypsin addition. These results suggest that the antibacterial peptide secreted by *P. acidilactici* TY112 inhibited the indicator strains' growth, and the antibacterial activity was lost after the peptide was degraded by trypsin. The degradation of such antibacterial peptide further promoted growth of the indicator strains and production of their metabolites other than lactic acid.[31−33]

The antibacterial activity of the secreted peptide was determined using a fermentation temperature of 42 °C, which was a relatively high temperature for fermentation. Thus, open fermentation performance in the presence of indicator strains was also performed at a reduced temperature of 37 °C (Figure S1) to determine if the indicator strains would have a better chance of surviving at lower temperatures. The results showed that comparable fermentation performances were obtained at both 37 and 42 °C, confirming that the

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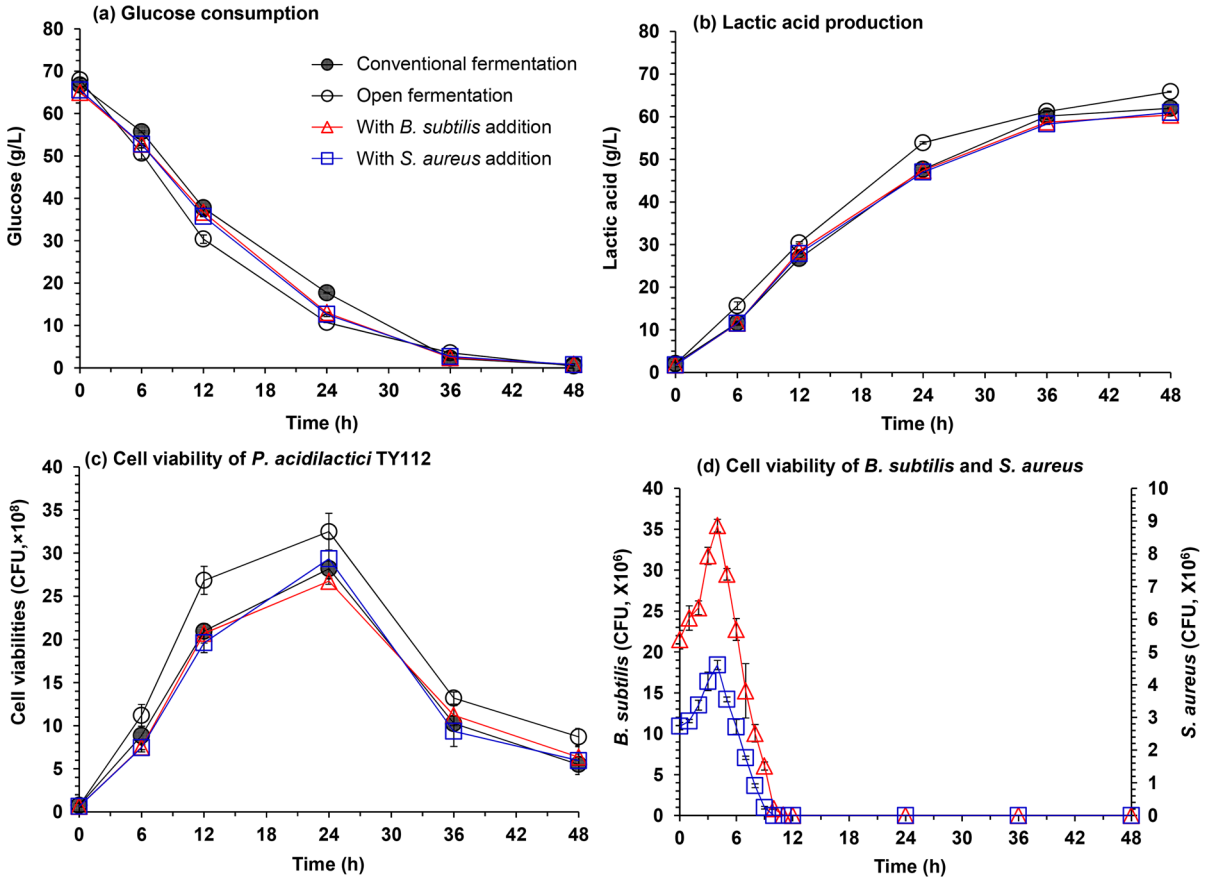

Figure 5. Open lactic acid fermentation using corn stover hydrolysate in SHF mode: (a) glucose consumption; (b) lactic acid production; (c) cell viability of *P. acidilactici* TY112; (d) cell viability of *S. aureus* and *B. subtilis* in cases of with *B. subtilis* addition and with *S. aureus* addition, respectively.

Table 2. Lactic Acid Fermentation Performance in SHF and SSF

fermentation conditions[a]	lactic acid (g/L)	lactic acid yield (%)[b]
	SHF	
conventional fermentation	62.0 ± 0.1	90.6 ± 0.2
open fermentation	65.8 ± 0.1	94.4 ± 0.1
with *B. subtilis* addition	60.4 ± 0.2	91.3 ± 0.3
with *S. aureus* addition	61.0 ± 0	90.5 ± 0
conventional SSF	97.1 ± 0.5	69.3 ± 0.2
open SSF	97.3 ± 0.1	69.4 ± 0.1

[a]The detailed conditions for SHF and SSF are listed in Figures 5 and 6, respectively. [b]The lactic acid yield in SHF was calculated based on glucose in the hydrolysate in the beginning. While the lactic acid yield in SSF was calculated based on glucose equivalents present in pretreated and biodetoxified corn stover.

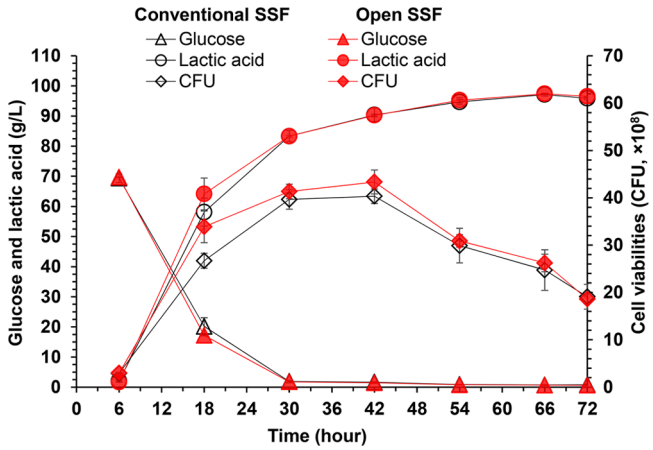

Figure 6. Comparison of open and conventional SSF for lactic acid production at 30% (w/w) solids loading of corn stover.

inability of the indicator strains to grow was due to the presence of the antibacterial peptide and not by the relatively high fermentation temperature of 42 °C.

Open L-Lactic Acid Fermentation of Corn Stover Hydrolysates in SHF and SSF Modes. Open lactic acid fermentation was applied to lignocellulosic hydrolysate in SHF mode using the DDAP pretreated and biodetoxified corn stover at 15% solids loading (w/w). Enzymatic hydrolysis was conducted under stringent aseptic conditions to produce a contaminant-free corn stover hydrolysate before fermentation. A glucan conversion of 88% was obtained from the pretreated and biodetoxified corn stover. Open fermentation experiments for lignocellulosic hydrolysates were designed similarly to those using simplified MRS medium (Figure 5 and Table 2). Figure 5a and b shows that the open fermentation reached a slightly better lactic acid production performance (65.8 ± 0.1 g/L of final lactic acid and 94.4 ± 0.1% of lactic acid yield based on the glucose in the hydrolysate in the beginning) relative to the conventional fermentation, where the hydrolysate was sterilized (62.0 ± 0.1 g/L of final lactic acid and 90.6 ± 0.2% of lactic acid yield). When the indicator strains *B. subtilis* and *S. aureus* were induced separately in the beginning of fermentation, they

DOI: 10.1021/acssuschemeng.7b02212
ACS Sustainable Chem. Eng. 2017, 5, 9254−9262

Table 3. Comparison of Lactic Acid Production in an Open Fermentation Mode Based on Different Contamination Preventing Methods

feedstock	contamination preventing options	pH and temperature	titer (g/L)	ref
Using Starch-Derived Glucose As Substrates				
glucose	high temperature	5.6 and 50 °C	118	2
glucose	high temperature	6.5 and 50 °C	107	14
glucose	high temperature	7.0 and 43 °C	82.4	7
glucose	alkaline	8.5 and 37 °C	129	34
tapioca starch glucose	high temperature	7.0 and 50 °C	99.6	12
white rice bran	high temperature	5.6−5.8 and 50 °C	117	6
Using Lignocellulose Hydrolysate in SHF Mode				
steam explosion pretreated corn stover	high temperature	6.3 and 50 °C	75.0[a]	9
dilute sulfuric acid pretreated wheat straw	high temperature	6.0 and 52 °C	56.5	11
Using Lignocellulose Hydrolysate in SSF Mode				
acid-catalyzed hydrolyzed EFB[b]	high temperature	5.5 and 50 °C	80.6	15
DDAP and biodetoxified corn stover	antibacterial	5.5 and 42 °C	97.3	this study

[a]The lactic acid titer was obtained at a fed-batch fermentation mode. [b]EFB here refers to the empty fruit bunch.

could grow in the first 4 h along with *P. acidilactici* TY112 (Figure 5c and d). After that, the viability of the indicator strains started to decline and died out within 10 h, mainly due to the antibacterial peptide secreted by *P. acidilactici* into the fermentation broth. It is worth to note that the lactic acid fermentation performance (60.4 ± 0.2 and 61.0 ± 0 g/L of final lactic acid, and 91.3 ± 0.3% and 90.5 ± 0% of lactic acid yield for *B. subtilis* and *S. aureus* addition cases, respectively) did not suffer any significant losses after being challenged with induced microbial contamination due to the shorter contamination period.

Although the open fermentation worked well in corn stover hydrolysate (15% solids loading) in SHF mode, product inhibition during enzymatic hydrolysis made it impossible to obtain hydrolysates with high sugar concentrations and, consequently, an industrially relevant lactic acid titer. SSF has a great advantage of alleviating sugar inhibition to a great extent by converting glucose to the final product at it is being produced by enzymatic hydrolysis of cellulose. In addition, optimum fermentation conditions (42 °C and pH 5.5) for *P. acidilactici* are relatively similar to those used for enzymatic hydrolysis with fungal cellulases (50 °C and pH 4.8). Based on this, the open SSF using *P. acidilactici* TY112 could produce industrially relevant lactic acid titers at high solids loading. In the open SSF operation at 30% solids loading of corn stover (w/w), feedstock, bioreactor, nutrients, and water used were not sterilized and samples were taken without aseptic control. Figure 6 and Table 2 show that lactic acid titer steadily increased and reached up to 97.3 g/L after 72 h (productivity of 1.5 g/(L·h) and yield of 69.4% based on glucose equivalents present in pretreated and biodetoxified corn stover), and little differences were observed in the final lactic acid titer and yield relative to the conventional fermentation with sterilization. During open fermentation, the cell viability of *P. acidilactici* TY112 was also similar to that of the control. Random samples were also taken during SSF process for microscopy tests, but no contamination was observed.

Evaluation of Open L-Lactic Acid Fermentation Method Based on Antibacterial Peptide Secretion. Open lactic acid fermentation has been widely studied in the past few years and can be divided into three different categories, based on the used substrates (shown in Table 3). The first category uses starch-derived glucose as substrates. Most of these studies applied thermotolerant LAB at high fermentation temperatures to prevent microbial contamination.[2,6,7,12,14] One exception was the work conducted by Jiang et al., who used the alkalophilic *Exiguobacterium* sp. 8−11 at 37 °C and a higher pH of 8.5.[34] For starch-based substrates, it was possible to obtain a lactic acid titer greater than 100 g/L because high sugar concentrations can be easily achieved after amylase digestion.

The second category uses lignocellulosic hydrolysate in SHF mode. Ouyang et al. obtained 75.0 g/L lactic acid from corn stover hydrolysate using the thermophilic strain *Bacillus* sp. NL01, operated at a fed-batch open fermentation at 50 °C and pH 6.3.[9] Zhang et al. produced 54.6 g/L lactic acid from wheat straw hydrolysate using thermophilic strain *B. coagulans* IPE22 operated at 52 °C and pH 6.0 under nonsterilized conditions.[11] It was apparent that the lactic acid titer was lower than that in the first category, largely because product inhibition during enzymatic hydrolysis was much more evident for cellulase-catalyzed hydrolysis relative to glucoamylase-catalyzed reactions. As a result, the sugar concentrations obtained in lignocellulosic hydrolysates were lower than in starch-based hydrolysates, leading to lower lactic acid concentrations after fermentation.

The third category uses open SSF of lignocellulosic biomass for lactic acid production, which can alleviate product inhibition in a great extent during enzymatic hydrolysis. Ye et al. obtained 80.6 g/L lactic acid using acid-catalyzed empty fruit bunch (EFB) in a high-temperature-based open SSF.[15] However, the low solids loading and high concentration of inhibitors present in the hydrolysate led to a lower lactic acid fermentation performance than that obtained in this study. In addition, the dry biorefinery technology, including DDAP, solid-state biodetoxification, and high solid loading SSF, has superior advantages in terms of lower water and energy consumption compared to those applied in previous studies.[17]

Overall, unlike lignocellulosic biomass, starch-based substrates are competing with human food and animal feed, and are less sustainable for industrial lactic acid fermentation. However, lignocellulosic hydrolysates containing high glucose concentrations are difficult to prepare because of severe enzyme inhibition during high solids loading enzymatic hydrolysis. In this work, the antibacterial-based open SSF was conducted using biodetoxified biomass, lower pH (5.5) and moderate temperatures (42 °C), which helped with inhibitor tolerance of the LAB strain. This approach led to higher SSF performances, as fermentation pH and temperature were relatively close to optimal for fungal cellulases, and the pretreated substrate contained lower concentration of inhibitors for enzymes and LAB. Thus, this approach appears to be an interesting alternative to the current open fermentation methods based on high temperature of operation.

DOI: 10.1021/acssuschemeng.7b02212
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One important aspect of this technology is that these antibacterial peptides are not antibiotics and do not lead to healthcare-related issues, such as proliferation of antibiotic resistant microorganisms. In fact, a wide use of bacteriocin-producing LAB in food fermentations has inadvertently or empirically been made for centuries, and it seems clear that bacteriocin residues are present in our daily food supply.[35] In an industrial setup, peptides present in the fermentation broth after open lactic acid fermentation would lose their activity in subsequent downstream processing that occurs at higher temperatures or could be degraded completely by adding proteases before wastewater treatment.[17]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.7b02212.

Figure S1. Open lactic acid fermentation in simplified MRS medium at 37 °C: (a) glucose consumption; (b) lactic acid production; (c) cell viability of *P. acidilactici* TY112; (d) cell viability of indicator strains in cases of with *B. subtilis* addition, with *B. subtilis* and trypsin addition, with *S. aureus* addition, and with *S. aureus* and trypsin addition, respectively (PDF)

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■ ACKNOWLEDGMENTS

This research was supported by the National Key Research and Development Program of China (2017YFB0309302), the Natural Science Foundation of China (21306048), the Fundamental Research Funds for the Central Universities of China (WF1514325), the Open Funding Project of the Key Laboratory of Motor Vehicle Biofuel Technology (KFKT2014011), and the Chinese Scholarship Council (CSC NO. 201506745002). L.D.C.S. also acknowledges the DOE Great Lakes Bioenergy Research Center (http://www.greatlakesbioenergy.org) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through Cooperative Agreement DE-FC02-07ER64494 between the Board of Regents of the University of Wisconsin System and the U.S. Department of Energy.

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