*fermentation*

MDPI

*Article*

# Plant Proteins as an Alternative Nitrogen Source for Chiral Purity L-Lactic Acid Fermentation from Lignocellulose Feedstock

**Bin Zhang [1], Lei Wu [1], Xiucai Liu [2] and Jie Bao [1,*]**

[1] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
[2] Cathay Biotech Inc., 1690 Cailun Road, Zhangjiang Hi-Tech Park, Shanghai 201203, China
* Correspondence: jbao@ecust.edu.cn

**Abstract:** High optical purity lactic acid is in high demand as the precursor for synthesizing polylactic acid (PLA). The costs of expensive carbohydrates and nitrogen source materials accounts for a large portion of the production costs in lactic homo-fermentation. The use of lignocellulosic biomass for lactic acid production reduces the cost of the carbohydrate feedstock, but the cost of nitrogen sources is a big challenge when considering the high prices of general nitrogen sources. Low-cost nitrogen materials are vulnerable to being contaminated by exogenous mixed L-lactic acid and D-lactic acid; thus, their feasibility as nitrogen sources for the production of optically pure lactic acid products is hindered. The available reports focus on cost reduction using agro-industrial byproducts as nutrient sources, with these presenting fewer concerns on the effect of the optical purity of lactic acid-product monomers for polymerization. In this study, commonly used low-cost nutrient sources were characterized and screened for high optical purity L-lactic acid fermentation. Corn steep liquor (CSL), a widely used and cheap nutrient for lactic acid fermentation, was found not to be suitable because of its high content of mixed D-/L-lactic acids (up to 20%, *w*/*w*). On the other hand, cottonseed meal was found to be completely free of mixed L-/D-lactic acids. Therefore, the cottonseed meal was hydrolyzed with dilute sulfuric acid and used as a nitrogen source for L-lactic acid fermentation using lignocellulose feedstock as a substitution for yeast extract and peptone. The results showed that the final L-lactic acid titer reached 96.5 ± 0.2 g/L from 25% (*w*/*w*)-solids loaded pretreated and biodetoxified wheat straw with a yield of 0.31 g/g feedstock and an optical purity of 99.7%. The techno-economic evaluation indicated that the cost of the cottonseed meal was only USD 0.193/kg of lactic acid product, and the minimum lactic acid selling price (MLSP) was USD 0.813/kg of lactic acid product, which was only 25.1% compared to the use of yeast extract and peptone as the nutrients. Cellulosic L-lactic acid production using cottonseed meal as a complex nutrient source showed competitive performance when compared to starch feedstock from food crops.

**Keywords:** lactic acid; optical purity; lignocellulose; cheap nutrients; cottonseed meal

check for updates

**Citation:** Zhang, B.; Wu, L.; Liu, X.; Bao, J. Plant Proteins as an Alternative Nitrogen Source for Chiral Purity L-Lactic Acid Fermentation from Lignocellulose Feedstock. *Fermentation* **2022**, *8*, 546. https://doi.org/10.3390/fermentation8100546

Academic Editors: Yaohua Zhong and Yinbo Qu

Received: 5 September 2022
Accepted: 14 October 2022
Published: 16 October 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

## 1. Introduction

Optically pure lactic acid is a crucial monomer chemical for the production of polylactic acid (PLA) products [1–3]. The costs of carbon and nitrogen sources for lactic acid production comprise approximately 40–70% of the overall production costs [4,5], which are major variable factors [6]. The use of lignocellulosic biomass for lactic acid production reduces the cost of carbohydrate feedstock, but the cost of general nitrogen sources is a big challenge considering its high price [2,7,8].

The cell growth and metabolism of lactic acid bacterium requires complex nutrients, including vitamins, amino acids, purine, and pyrimidine because of a lack in the incomplete biosynthesis which provides these nutrients [9,10]. The widely used yeast extract (YE) and peptone contribute 38–87% to the total product cost [5,6,11,12] and are not economically

favorable for industrial lactic acid production. There have been a number of efforts to use agro-industrial byproducts to achieve the partial or total replacement of YE and peptone, such as corn steep liquor (CSL) [13,14], malt rootles extract and soybean meal extract [15], wheat bran hydrolysate [1], cottonseed hydrolysate [16], and peanut meal hydrolysate [17]. However, these alternative nutrients are vulnerable to being contaminated by exogenous mixed L-lactic acid and D-lactic acid, thus reducing the optical purity of the lactic acid products. While these nitrogen sources are well used for general lactic acid production, the impact of the minor mixed lactic acids on the high chiral purity has not been fully investigated. When most lactic acid is currently used as a PLA monomer, this drawback becomes a critical factor in cost reduction and optical purity.

In this study, common low-cost alternative complex nutrient sources were characterized and screened for high optical purity L-lactic acid fermentation. Cottonseed meal was found to be completely free of the mixed L-/D-lactic acids; thus, it was selected as the most important candidate as an alternative nitrogen source for L-lactic acid fermentation. The cottonseed meal was hydrolyzed by protease or acid, and then utilized by an engineered *Pediococcus acidilactici* strain to fully substitute yeast and peptone for high optical purity L-lactic acid production from wheat straw. The techno-economic evaluations further demonstrated the potential of using cottonseed meal as a complex nutrient source instead of YE for high optical purity L-lactic acid production.

## 2. Materials and Methods

### *2.1. Raw Feedstock*

The raw wheat straw was harvested in Nanyang City, Henan province, China, June 2022. The main composition of raw wheat straw includes 34.31 $\pm$ 0.14% cellulose, 21.30 $\pm$ 1.78% xylan, 22.12 $\pm$ 0.07% lignin, and 10.63 $\pm$ 0.28% ash on dry base, which were determined by two-step hydrolysis method from NREL protocols [18,19].

### *2.2. Reagents and Enzymes*

The cellulase enzyme Cellic CTec 2.0 was purchased from Novozymes (Beijing, China) with a filter paper activity and protein content of 256 FPU/mL and 81 mg protein/mL. The α-amylase HTAA was purchased from Genencor (Beijing, China), with an activity of 103,900 U/mL.

Yeast extract (LP0021B) and peptone in reagent grade were purchased from Oxoid Co., UK. Yeast extract and peptone in industrial grade were purchased from Angel Yeast Co., Ltd. (Yichang, China).

The two kinds of dried corn steep liquor powder (DCSLP) were purchased from Angel Yeast Co. Ltd. (Yichang, China) (DCSLP #1) and the Xiwang Group Co. Ltd. (Shandong, China) (DCSLP #2), respectively. The cottonseed meal (dephenolized cottonseed) was purchased from Qingdao Create Medium Co., Ltd. (Qingdao, China). The soybean meal was purchased from China Oil and Foodstuffs Corporation (Shanghai, China).

The three kinds of neutral protease were purchased from Novozymes (Beijing, China) (neutral protease #1), SUNSON Industry Group CO., Ltd. (Beijing, China) (neutral protease #2), Vland Biotech INC. (Qingdao, China) (neutral protease #3), respectively, with an enzymatic activity of 0.8 AU/g, $5 \times 10^4$ U/g and $10 \times 10^4$ U/g, according to the manufacturer's instruction. The two kinds of alkali protease were purchased from SUNSON Industry Group CO., Ltd. (Beijing, China) (alkali protease #1) and Vland Biotech INC. (Qingdao, Shandong, China) (alkali protease #2) with the same enzymatic activity ($20 \times 10^4$ U/g). The papain and trypsin were purchased from Sunson Industry Co., Beijing, China with an enzymatic activity of $10 \times 10^4$ U/g and $4 \times 10^4$ U/g.

Glucose, sulfuric acid, and other chemicals in reagent grade were purchased from Sinopharm Chemical Reagent Co., Shanghai, China.

### 2.3. Microorganisms and Medium

The fungus *Paecilomyces variotii* FN89 (CGMCC 17665) was applied to the biodetoxification of the acid-pretreated wheat straw [20] because the weak organic acids and phenolic aldehyde compounds generated from the harsh pretreatment processing severely inhibit the consequent fermenting microbes, which need to be removed. *P. variotii* FN89 can be preserved on potato dextrose agar (PDA) plate at 4 °C for 7 days.

The engineered bacterium *Pediococcus acidilactici* ZY271 (CGMCC 13611) was applied to cellulosic L-lactic acid fermentation [21]. *P. acidilactici* ZY271 can be used for L-lactic homo-fermentation. The whole genome sequencing of *P. acidilactici* ZY271 was submitted to GenBank with the accession number of CP082111.1. A simplified Man-Rogosa-Sharp (MRS) medium containing 20 g/L of glucose, 10 g/L of peptone, 10 g/L of yeast extract, 5 g/L of sodium acetate, 2 g/L of diammonium hydrogen citrate, 2 g/L of $K_2HPO_4$, 0.58 g/L of $MgSO_4{\cdot}7H_2O$, and 0.25 g/L of $MnSO_4{\cdot}H_2O$ was used for *P. acidilactici* ZY271 seed culture. The general medium components for cellulosic L-lactic acid fermentation contained 10 g/L of peptone, 15 g/L of yeast extract, 2 g/L of diammonium hydrogen citrate, and 0.25 g/L of $MnSO_4{\cdot}H_2O$.

### 2.4. Pretreatment and Biodetoxification

The raw wheat straw was pretreated by diluted sulfuric acid according to the previously described protocols [22]. The sulfuric acid dosage was 4.0% (*w*/*w*) on a dry base of wheat straw. An amount of 1200 g of dry wheat straw and 600 g of acid solution were fed-into a 20-L reactor. The pretreatment was maintained at 175 °C for 5 min. The pretreated wheat straw in solid particle form absorbed all of the acid solution and the condensed water and was discharged from the bottom port of reactor. The main compositions of pretreated wheat straw were also determined by a two-step hydrolysis method, which contained 304.8 ± 2.9 mg cellulose, 27.5 ± 0.3 mg glucose, 6.8 ± 0.4 mg xylan, 115.6 ± 2.2 mg xylose, 17.7 ± 4.6 mg acetic acid, 3.1 ± 1.5 mg HMF, and 1.2 ± 1.1 mg furfural per gram of dry matter.

The pH of the pretreated wheat straw was adjusted to ~5.0 by adding $CaCO_3$ powder. The sulfuric acid catalyzed was neutralized to calcium sulfate precipitation. The pretreated wheat straw doesn't need to be washed. The neutralized pretreated wheat straw was then aerobically biodetoxified by *P. variotii* FN89. *P. variotii* FN89 was firstly cultured on a PDA plate at 37 °C for 4 days. The spores were then collected and spread on pretreated and neutralized wheat straw. The wheat straw was statically maintained at 37 °C for 3 days as the seed. The biodetoxification was carried out in a 15 L bioreactor at 37 °C with the aeration of 1 vvm for 48 h. No HMF (5-hydroxymethyfurfural) and furfural were detected by HPLC in the biodetoxified wheat straw.

### 2.5. Preparation of Cottonseed Meal Hydrolysate

The hydrolysis of the cottonseed meal was catalyzed by protease or acid. For protease catalyzed hydrolysis, 20 g (dry matter) of cottonseed meal was mixed with 80 g of deionized water in 250 mL flask. The adding protease dosage was 3% (*w*/*w*) per gram dry matter. The pH value of the mixture was adjusted to 10.0 by adding 5 mol/L NaOH solution when the alkaline proteinase was used as catalyst, while the pH value (~5.0) of the mixture did not need to be adjusted when other proteinase was used as a catalyst. The enzymatic hydrolysis was carried at 50 °C, 150 rpm, for 24 h. The hydrolysate was then inactivated at 90 °C for 10 min to eliminate the negative effect of protease on cellulase and the growth of the subsequent fermentation microorganism. The pH value of enzymatic hydrolyzed cottonseed meal hydrolysate was adjusted to ~5.0 by adding 5 mol/L NaOH solution or 5 mol/L sulfuric acid. The hydrolysate was used as the fermentation medium component to replace YE and peptone. The hydrolysate was added at a 10% (*w*/*w*) mass ratio, equivalent to 20 g/L of cottonseed meal in fermentation medium.

For acid-catalyzed hydrolysis, 20 g (dry matter) of cottonseed meal was mixed with 80 g of acid solution (5–7%, *w*/*w*) in a 250 mL flask. The tested acids included oxalic acid,

hydrochloric acid, and sulfuric acid. The acid dosage was 15–35% (*w*/*w*) of dry matter. The acid hydrolysis was conducted at 90 °C, 150 rpm, for 24 h. The pH of hydrolysate catalyzed by acid was then neutralized to ~5.0 by adding $CaCO_3$ powder. The neutralized hydrolysate was added at a 10% (*w*/*w*) mass ratio to replace YE and peptone, equivalent to 20 g/L of cottonseed meal in a fermentation medium.

### *2.6. Cellulosic L-Lactic Acid SSCF*

One vial of *P. acidilactici* ZY271 was inoculated into 20 mL of MRS medium in a 100 mL flask and cultured at 42 °C, 150 rpm, for 12 h. Then the broth was transferred into 100 mL of MRS medium in a 500 mL flask and cultured at 42 °C, 150 rpm, for 8 h, as the seed. The glucoamylase was added into MRS medium at a 1% (*w*/*w*) mass ratio to break the polysaccharide links among the cell aggregations, thus preventing cell flocculation [23]. The $OD_{600}$ values of seed culture are between 3.5–5.0.

For the simultaneous saccharification and co-fermentation (SSCF) in the 250 mL flask or 5 L bioreactor, the biodetoxified wheat straw was pre-hydrolyzed into a slurry within 6 h at 25% (*w*/*w*)-solids loading at 50 °C and 150 rpm. The cellulase dosage was 4 mg protein/g dry matter. After the pre-hydrolysis, the neutralized plant meal hydrolysate was added at a 10% (*w*/*w*) mass ratio, equivalent to 20 g/L of plant meal. Then the seed was inoculated at the ratio of 20% (*w*/*w*). The anaerobic fermentation was conducted at 42 °C, 150 rpm, for 72 h without aeration. The fermentation pH in the 250 mL flask was neutralized by adding 40 g/L $CaCO_3$ powder. The fermentation pH in the 5 L bioreactor (BaoXing Bioengineering Equipment Co., Ltd., Shanghai, China) was maintained at 5.5 by automatic regulation with 25% (*w*/*w*) of $Ca(OH)_2$ slurry.

### *2.7. Analysis*

The glucose, xylose, lactic acid, acetic acid, HMF and furfural was determined by a Shimadzu HPLC system equipped with a Bio-rad Aminex HPX-87H column and a RID-10A detector. An amount of 20 microliters of the sample was subjected and analyzed at 60 °C using 5 mM $H_2SO_4$ as eluent, with a flow rate of 0.6 mL/min [23]. The chirality of the lactic acid was measured by a D-/L-lactic acid kit (Megazyme International Ireland, Bray Wicklow, Ireland). The glutamic acid was measured by SBA-90D (Biology Institute, Shandong Academy of Sciences, Jinan, Shandong, China). The crude protein content was determined by semi-automatic Kjeldahl apparatus (PeiOu Analysis Instrument, Shanghai, China). The cell growth in the SSCF process was determined by counting the colony-forming units (CFU).

## 3. Results and Discussions

### *3.1. Screening Alternative Nitrogen Sources for High Chiral Purity Lactic Acid Fermentation*

Yeast extract (YE) is the preferred nitrogen source of lactic acid bacteria for providing vitamins, amino acids, and minerals [24,25]. When reagent-grade yeast extract and peptone were used as a nitrogen source, the L-lactic acid titer and yield reached 105.0 ± 0.5 g/L and 0.33 ± 0.01 g/g for biodetoxified wheat straw (dry matter), with an optical purity of 99.5% from the pretreated and biodetoxified wheat straw (Table 1). When the industrial grade YE and peptone were used, the cost was reduced, while the L-lactic acid production and yield was the same or only slightly reduced (Tables 1 and 2). However, the cost of the industrial YE and peptone was still too high (the local selling prices are still over USD 10/kg), considering that lactic acid is a commodity chemical with a price in a range of USD 1.3–2.3/kg [26].

Corn steep liquor (CSL) is a byproduct of wet corn milling processing, which contains natural amino acids, vitamins, minerals, organic acids, and other elemental nutrients [27]. CSL has been widely used as an inexpensive source of essential microbial nutrients for lactic acid production [13,14]. When YE and peptone were replaced by 20 g/L of dried corn steep liquor powder (DCSLP), which was purchased from two suppliers from the commercial market, the final lactic acid titer and yield reached 87.2 ± 1.4 g/L, 85.4 ± 1.0 g/L, 0.26 g/g,

and 0.25 g/g dry matter (Table 1), respectively, with a price of only USD 0.32–1.43/kg (Table 2). However, one serious problem of using corn steep liquor was that the optical purity of the L-lactic acid product sharply reduced to only around 95%, which doesn't meet the requirements of optical purity for PLA production [3]. The further characterizations showed that approximately 20% (*w*/*w*) of the mixed D-/L-lactic acids from the total nitrogen (dry matter), equivalent to 11~13% (*w*/*w*) of D-lactic acid, were found in the dried corn stoop liquor and agreed with the results of previous studies [28]. Therefore, CSL is not suitable as a complex nutrient source for high chiral purity lactic fermentation.

**Table 1.** Nitrogen source use on cellulosic L-lactic acid fermentation performance.

| Nitrogen Sources [a] | Dosage (g/L) | Lactic Acid Titer (g/L) | L-Lactic Acid Purity (%) | L-Lactic Acid Yield (g/g DM) [b] |
|---|---|---|---|---|
| YE + peptone, reagent grade | 15 for YE; 10 for peptone | 105.0 ± 0.5 | 99.5 ± 0.1 | 0.33 ± 0.01 |
| YE + peptone, industrial grade | 15 for YE; 10 for peptone | 102.0 ± 0.8 | 99.6 ± 0.1 | 0.32 ± 0.02 |
| DCSLP #1 | 20 | 87.2 ± 1.4 | 94.9 ± 0.1 | 0.26 ± 0.01 |
| DCSLP #2 | 20 | 85.4 ± 1.0 | 95.3 ± 0.1 | 0.25 ± 0.01 |

Conditions: 5 L bioreactor, 25% (*w*/*w*)-solids loading of biodetoxified wheat straw, 4 mg protein/g dry matter (DM). A 10% inoculation, SSCF at 42 °C, and 150 rpm for 72 h. [a] YE, yeast extract; DCSLP, dried corn steep liquor powder. Other fermentation nutrients included 2 g/L of diammonium hydrogen citrate and 0.25 g/L of manganous sulfate monohydrate. [b] The yield was calculated based on dry pretreated and biodetoxified wheat straw (g/g DM).

**Table 2.** Characterizations of the common complex nutrients used for lactic acid fermentation.

| Nitrogen [a] | Price [b] (USD/kg) | Protein (mg/g DM) | Lactic Acid (mg/g DM) | L-Lactic Acid (mg/g DM) | D-Lactic Acid (mg/g DM) |
|---|---|---|---|---|---|
| YE, reagent grade | 34.92 | 658.6 ± 1.6 | 3.3 ± 0.2 | 1.8 ± 0.1 | 1.5 ± 0.1 |
| YE, industrial grade | 17.46 | 769.3 ± 9.5 | 2.1 ± 0.1 | 0.9 ± 0.1 | 1.2 ± 0.2 |
| Peptone, reagent grade | 80.00 | 760.2 ± 14.3 | 2.0 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.1 |
| Peptone, industrial grade | 11.11 | 728.4 ± 12.5 | ND [c] | ND [c] | ND [c] |
| DCSLP #1 | 1.43 | 451.2 ± 5.3 | 215.8 ± 3.5 | 85.6 ± 1.4 | 130.2 ± 2.1 |
| DCSLP #2 | 0.32 | 467.5 ± 6.1 | 196.3 ± 2.7 | 85.1 ± 0.4 | 111.2 ± 2.3 |

[a] YE, yeast extract; DCSLP, dried corn steep liquor powder. [b] The price was supplied by the local distributors. The exchange rate from US dollars (USD) to the Chinese Yuan (CNY) was set to 6.3. [c] ND, not detected by kit and HPLC.

One crucial prerequisite for selecting cheap protein-rich alternative nutrient sources is their lactic acid content, where the lactic acid product is used as the monomer chemical for PLA polymerization. The D-/L-lactic acid isomers are difficult to separate; thus, alternative nitrogen should be absolutely lactic acid-free. The common agriculture residues, soybean and cottonseed meal, were found to be completely free of lactic acid (Table 3). For protein content, the cottonseed meal was 19.5% higher than the soybean meal (Table 3) and less likely to be used as animal feed for its minor phenolics content [29], leaving versatile room to use it as an alternative nitrogen source for high purity lactic acid fermentation.

**Table 3.** Characterization of cottonseed and soybean meal.

| Nitrogen | Price (USD/kg) | Protein (mg/g DM) | Lactic Acid (mg/g DM) | L-Lactic Acid (mg/g DM) | D-Lactic Acid (mg/g DM) |
|---|---|---|---|---|---|
| Soybean meal | 0.32 | 456.3 ± 9.5 | ND * | ND * | ND * |
| Cottonseed meal | 1.59 | 545.3 ± 11.3 | ND * | ND * | ND * |

* ND, not detected by kit and HPLC. Note: The cellulose and hemicellulose content in the cottonseed and soybean meal were also determined by a two-step hydrolysis method, with 5.0 ± 0.3% and 5.9 ± 1.1% for cottonseed meal, and 10.1 ± 0.3% and 10.9 ± 0.6% for soybean meal on dry matter, respectively.

### *3.2. Hydrolysis of Cottonseed Meal and Consequent Cellulosic L-Lactic Acid Fermentation*

Cellulosic L-lactic acid fermentation performance using cottonseed meal hydrolysate as the complex nutrient source was investigated in the flasks (Figure 1). As lactic acid bacteria, such as *Lactobacillus casei*, *Lactobacillus rhamnosus*, etc., have a limited capacity to synthesize several amino acids [9,12], complex plant protein should be hydrolyzed into its amino acids form before being utilized. Protease enzymatic hydrolysis and acid hydrolysis are the two common methods to decompose protein-rich agriculture residues [30]. Glutamic acid was the most abundant amino acid in the cottonseed meal (up to 10% (*w*/*w*)) [31]; thus, glutamic acid was used as an indicator for the degree of hydrolysis of the cottonseed meal.

Enzymatic hydrolysis by protease was conducted under relatively mild conditions. A total of seven different proteases were used to hydrolyze the cottonseed meal at their optimum pH and temperature. The hydrolysate prepared from 20 g/L of the cottonseed meal was used as the complex nutrient source to completely replace YE and peptone for L-lactic acid fermentation. Higher lactic acid titers of 49.5 ± 1.2 g/L and 52.5 ± 0.4 g/L were obtained using the cottonseed meal hydrolysates by two different alkaline proteases, which were 28.8% and 24.4% lower than the control using YE and peptone, respectively (Figure 1a).

Chemical hydrolysis is low cost and is flexible enough to cleavage the peptide bonds but tends to over-degrade the nutrients [17]. The cottonseed meal was hydrolyzed in a 3, 5, and 7% (*w*/*w*) acid solution of oxalic acid, hydrochloric acid, and sulfuric acid, respectively, and the lactic acid titers reached over 50.0 g/L (Figure 1b).

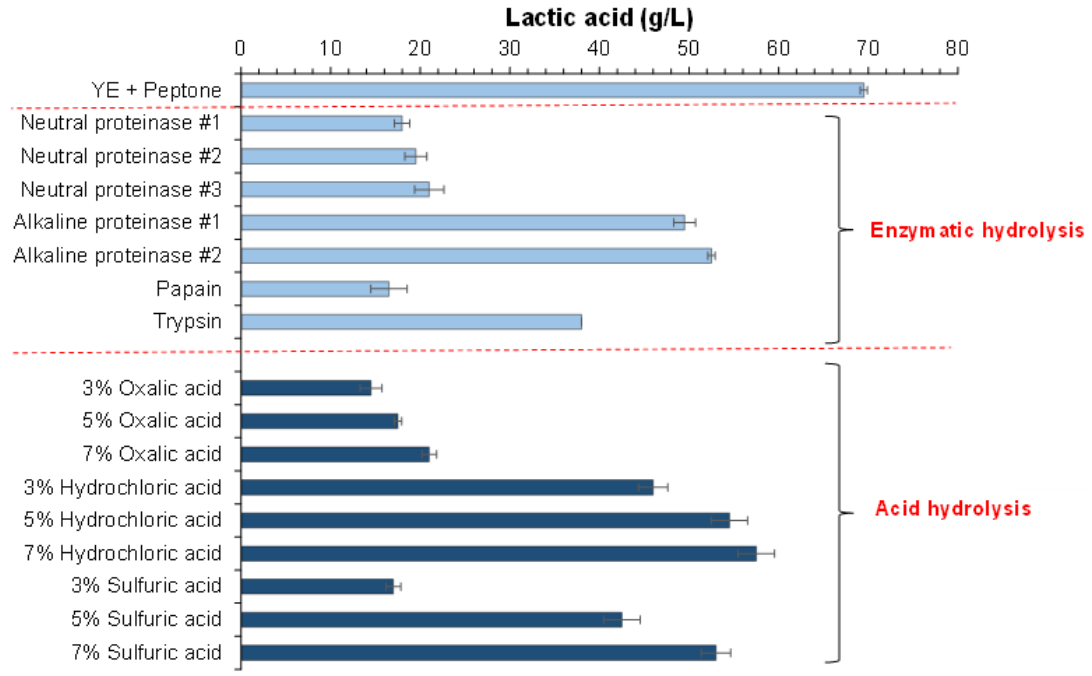
The viable cell number (indicated by CFU) of the lactic acid-producing strain, *P. acidilactici* ZY271, increased with an increasing amino acid content (indicated by glutamic acid content) in both the enzymatic hydrolysate and acid hydrolysate of the cottonseed meal. Under optimal hydrolysis conditions, the free glutamic acid contents in the hydrolysate and the CFU numbers in the fermentation (by acid hydrolysis) of the cottonseed meal were higher than those achieved by enzymatic hydrolysis. This result indicates that the hydrolysis degree of cottonseed meal by enzymatic hydrolysis was lower compared to that by acid hydrolysis. The reason for this might be that the released nutrients from the cottonseed meal by enzymatic hydrolysis were insufficient, resulting in poor bacterial growth and lactic acid production performance. The acid hydrolysate of the cottonseed meal under the moderate acid dosage (5%, *w*/*w*) was used for further evaluation, considering the lessened salt inhibition after acid neutralization [32].

### *3.3. Cellulose L-Lactic Acid Production by SSCF*

As shown in Figure 1a, the cottonseed meal hydrolyzed by 3% (*w*/*w*) alkaline proteinase #2 showed the best fermentation performance among the enzymatic hydrolysis methods. Although fermentation using 7% (*w*/*w*) hydrochloric acid for the hydrolyzed cottonseed meal obtained the highest lactic acid titer, the lactic acid titer using 5% (*w*/*w*) hydrochloric acid was similar. To alleviate the salt inhibition, materials cost, and reactor corrosion, 5% (*w*/*w*) hydrochloric acid was selected as the hydrolysis catalyst for further investigation. Meanwhile, fermentation using 5% (*w*/*w*) sulfuric acid for the hydrolyzed cottonseed meal was also investigated, as compared to the 5% (*w*/*w*) hydrochloric acid hydrolysis. Therefore, the L-lactic acid fermentation performances were evaluated by the simultaneous saccharification and co-fermentation (SSCF) of wheat straw using cottonseed meal hydrolysates catalyzed by 3% (*w*/*w*) alkaline protease #2, 5% (*w*/*w*) sulfuric acid solution and 5% (*w*/*w*) hydrochloric acid solution (Figure 2). The pre-hydrolysis (~6 h) can only hydrolyze partial cellulose and xylan in biodetoxified wheat straw; the glucose and xylose can be continuously released from cellulose and xylan due to the continuous hydrolysis by commercial cellulase during the fermentation. The fermentation performance in a 5 L bioreactor was higher compared to that in the flask due to the accurate pH control and good mixing at high solid loading. When using the cottonseed meal hydrolysate by alkaline protease #2, the lactic acid titer was 67.0 ± 4.3 g/L after 72 h, with residual glucose of 38.7 ± 3.7 g/L. When using the cottonseed meal hydrolysates using sulfuric acid and

hydrochloric acid, the lactic acid titer of the fermentation process was above 93.0 g/L. The optical purity of the L-lactic acid using the cottonseed meal was above 99.5% in all cases.

(**a**) Cellulosic L-lactic acid production using cottonseed meal, hydrolyzed by protease or acid.




(**b**) The CFU in lactic acid broth using cottonseed meal, hydrolyzed by protease or acid.

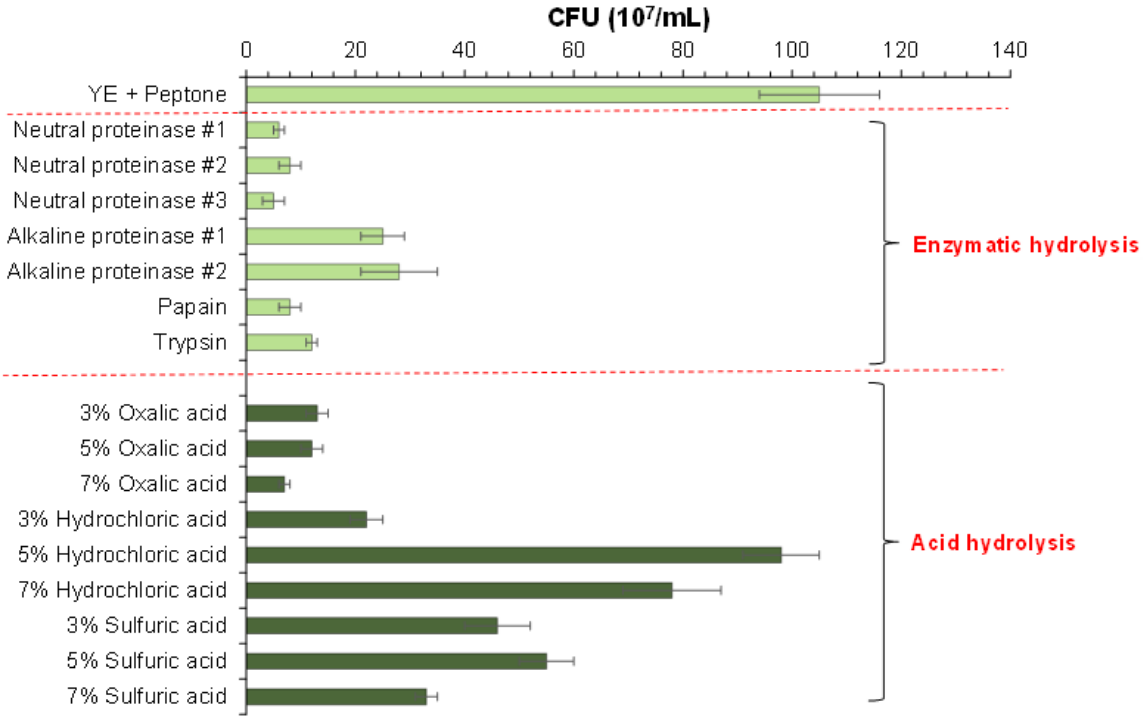



**Figure 1.** *Cont.*

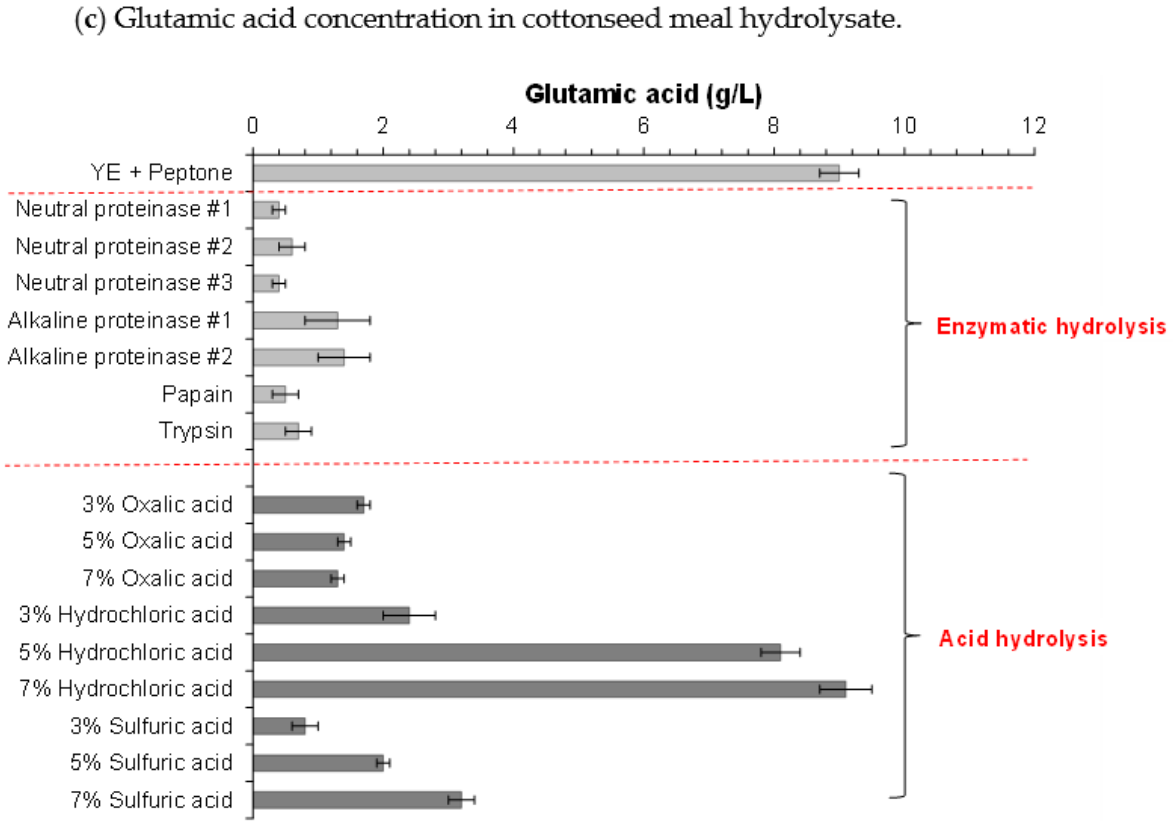



**Figure 1.** Cellulosic L-lactic acid fermentation performance from wheat straw using cottonseed meal hydrolysate as a complex nutrient source in flasks. The cottonseed meal was hydrolyzed by protease or acid. (**a**) Lactic acid titer; (**b**) CFU; (**c**) glutamic acid concentration in hydrolysate. The number of CFUs was measured at 24 h. Conditions: 25% (*w*/*w*)-solids loading: 42 °C, 100 rpm, 72 h. The fermentation pH was controlled by adding 40 g/L of $CaCO_3$ powder. Other fermentation nutrients included 2 g/L of diammonium hydrogen citrate and 0.25 g/L of manganous sulfate monohydrate.

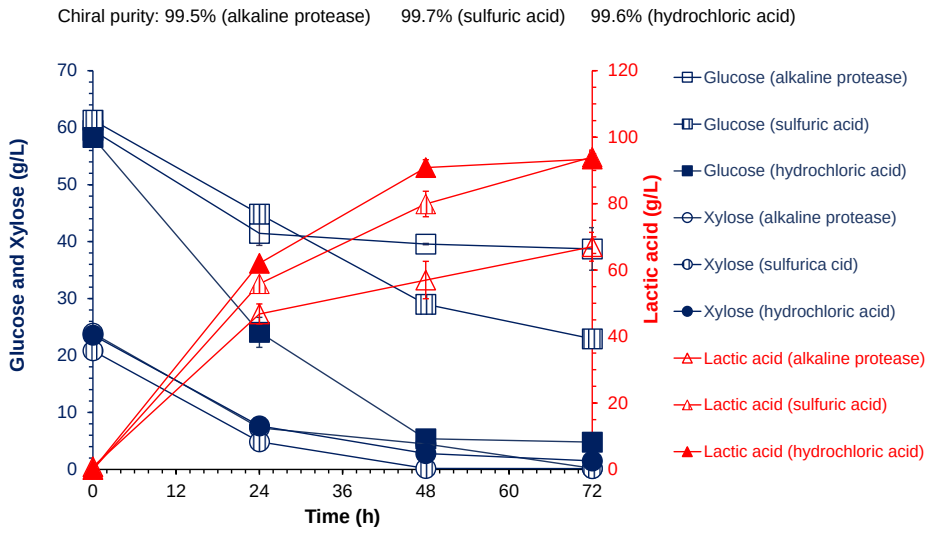



**Figure 2.** L-lactic acid SSCF using cottonseed meal hydrolysate as the complex nutrient source. The cottonseed meal was hydrolyzed by 3% alkaline protease #2, 5% sulfuric acid, or 5% hydrochloric acid. Conditions: 25% (*w*/*w*)-solids loading: 42 °C, 150 rpm, 72 h. The fermentation pH was controlled at 5.5 by automatically adding a 25% (*w*/*w*) $Ca(OH)_2$ solution. Other fermentation nutrients included 2 g/L of diammonium hydrogen citrate and 0.25 g/L of manganous sulfate monohydrate.

It is noteworthy that the consumption rate of glucose in the lactic acid fermentation process using sulfuric acid-hydrolyzed cottonseed meal was lower than that of the fermentation using hydrochloric acid-hydrolyzed cottonseed meal, although the lactic acid titer was similar. In fact, the free glutamic acid contents in those hydrolysates catalyzed by sulfuric acid were significantly lower than that of the hydrolysates catalyzed by hydrochloric acid (Figure 1b). Previous studies showed that the available nitrogen content in the agricultural-residue hydrolysate was related to the free amino acids content [30]. The replacement of YE and peptone by the cottonseed meal hydrolysate catalyzed by sulfuric acid may lead to an insufficient supply of available nitrogen, resulting in inefficient sugar consumption of fermenting strain. Furthermore, the cell biomass in high solids loading wheat straw hydrolysate was difficult to measure due to the interferences of the insoluble solids. It was reported that lactic acid production depends strictly on cell growth [10]. The lower glucose consumption rate and lactic acid titer when using the sulfuric acid-hydrolyzed cottonseed meal as a complex nutrient indicated that lower cell biomass was obtained when compared to using hydrochloric acid-hydrolyzed cottonseed meal.

Although lactic acid fermentation using the cottonseed hydrolysate catalyzed by hydrochloric acid achieved better performance, sulfuric acid was still a preferred option for industrial application. One reason is that chloride acid causes serious corrosion of metal reactors [33]. The other reason is that hydrochloric acid is difficult to remove in the subsequent purification, while sulfuric acid easily forms the precipitate calcium sulfate after neutralization. When sulfuric acid is used as the cottonseed meal hydrolysis catalyst, additional nitrogen sources should be supplemented to enhance the residual sugar conversion (Figure 3). When 10 g/L diammonium hydrogen citrate, 5 g/L diammonium hydrogen citrate combined with 6 g/L ammonium sulfate, or 10 g/L ammonium sulfate was added, the lactic acid titer further increased to 95.1 ± 1.3 g/L, 96.3 ± 0.8 g/L, and 96.5 ± 0.2 g/L, respectively, and the residual glucose was reduced to 2.5 ± 0.9 g/L, 3.6 ± 0.1 g/L, and 3.5 ± 0.2 g/L, respectively. Since the fermenting strain *P. acidilactici* ZY271 was capable of fully utilizing all of the lignocellulose-derived sugars, the residual xylose was consumed below 2 g/L. Generally, the complete removal of residual non-glucose sugars from a lactic acid broth (to a polymer-grade monomer) would cause great difficulties. In this study, the low residual sugar level was satisfied when preparing L-lactic acid monomers for PLA polymerization [34].

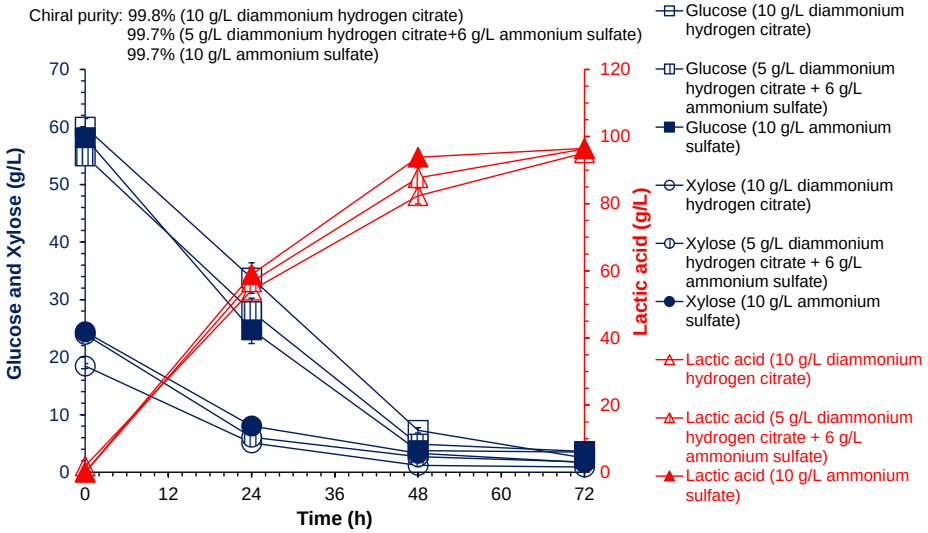



**Figure 3.** L-lactic acid SSCF using sulfuric acid-hydrolyzed cottonseed meal as complex nitrogen, adding different concentrations of diammonium hydrogen citrate and ammonium sulfate. Conditions: 25% (*w*/*w*)-solids loading: 42 °C, 150 rpm, 72 h. The fermentation pH was controlled at 5.5 by automatically adding a 25% (*w*/*w*) $Ca(OH)_2$ solution. Other fermentation nutrients included 20 g/L of cottonseed meal hydrolyzed by 5% (*w*/*w*) sulfuric acid, and 0.25 g/L of manganous sulfate monohydrate.

### *3.4. Preliminary Techno-Economic Evaluations*

Three L-lactic acid production cases were proposed to verify the economic feasibility of using cottonseed meal hydrolysate as an alternative nutrient source. The main production parameters are shown in Table 4. In Case 1, the parental strain *P. acidilactici* TY112 strain, without a xylose utilization pathway, was used for L-lactic acid fermentation [23]. A DCSLP of 20 g/L and diammonium phosphate (2 g/L) were used as a complex nitrogen source. The L-lactic acid optical purity was only 95.3% due to the presence of the D-lactic acid in the DCSLP. The *P. acidilactici* ZY271 strain, with full utilization of all non-glucose sugars, was used in Case 2 with YE and peptone (industrial grade), and in Case 3 with cottonseed meal hydrolysate as the complex nitrogen source.

**Table 4.** Cellulosic L-lactic acid production parameters in different scenarios.

| | **Case 1 [23]** | **Case 2 [This Study]** | **Case 3 [This Study]** |
|---|---|---|---|
| Strain | *P. acidilactici* TY112 | *P. acidilactici* ZY271 | *P. acidilactici* ZY271 |
| Xylose utilization | No | Yes | Yes |
| Raw feedstock | Corn stover | Wheat straw | Wheat straw |
| Pretreatment acid dosage | 5.0%, *w*/*w* (dry matter) | 4.1%, *w*/*w* (dry matter) | 4.1%, *w*/*w* (dry matter) |
| Fermentation solids loading | 30% (*w*/*w*) | 25% (*w*/*w*) | 25% (*w*/*w*) |
| Nitrogen sources | | | |
| Complex nitrogen source [a] | 20 g/L DCSLP | 15 g/L YE + 10 g/L Peptone | 20 g/L cottonseed hydrolysate |
| Available nitrogen source | 2 g/L diammonium phosphate | 2 g/L diammonium hydrogen citrate | 10 g/L ammonium sulfate |
| SSCF period (h) | 72 | 72 | 72 |
| Titer (g/L) | 104.5 | 102.0 | 96.5 |
| Yield (g/g dry raw feedstock) | 0.27 | 0.33 | 0.31 |
| Productivity (g/L/h) | 1.45 | 1.46 | 1.32 |
| Chiral purity (%) | 95.3 | 99.5 | 99.7 |

[a] DCSLP, dried corn steep liquor powder; YE, yeast extract.

The preliminary techno-economic evaluations were based on the operation of a biorefinery plant with an annual processing capacity of 300,000 metric tons of dry lignocellulose feedstock. The process model, total capital investment, and discounted cash flow rate of return analyses were cited from previously established lactic acid production processing based on dry biorefinery technology, with cost updates for the materials and equipment [23]. The price of the material was updated and is shown in Table 5. The minimum lactic acid selling price (MLSP) in Case 1, Case 2, and Case 3 was USD 0.584, 3241, and 0.813/kg lactic acid product, respectively (Table 6). Although the lowest MLSP was obtained by using DCSLP as the complex nutrient source in Case 1, the lactic acid product (95.3% L-purity) cannot be used for polymerization. YE and peptone contribute the highest percentage of MLSP in Case 2, accounting for 81.0%. It has been reported that the raw materials for lactic acid fermentative production usually accounts for over 34% of production costs [35], and the yeast extract only contributed about 38% to the total cost [5]. The contribution of YE and peptone cost to the MLSP was greater in Case 2 than in previous reports, mainly due to the cheap lignocellulose-derived sugar, which only accounted for 8.5% of the MLSP. In Case 3, the cost of the cottonseed meal was only USD 0.193/kg of lactic acid product, accounting for 23.7% of the MLSP. Alves et al. summarized many techno-economic evaluations for lactic acid fermentative production in different processes using different substrates [26]. The results in this study indicated that the cellulosic lactic acid production processing (Case 3, USD 0.813/kg) was comparable and competitive to that from food crops (USD 0.833 or 2.50/kg) [35,36].

**Table 5.** The prices of the materials used in techno-economic evaluations.

| Material | USD Price (2022) |
|---|---|
| Feedstock (wheat straw) | 71.24/ton |
| Sulfuric acid, 98% | 125.06/ton |
| Lime | 99.69/ton |
| Diammonium hydrogen citrate | 3166.11/ton |
| Ammonium sulfate | 87.07/ton |
| Manganese sulfate | 443.26/ton |
| Yeast extract (YE) | 17,413.60/ton |
| Peptone | 11,081.38/ton |
| Cottonseed meal | 1266.44/ton |

**Table 6.** Economic evaluation of lactic acid production in different scenarios.

| | Case 1 | Case 2 | Case 3 |
|---|---|---|---|
| Feedstock handling rate | 300,000 metric tons/year | 300,000 metric tons/year | 300,000 metric tons/year |
| Total capital investment [a] | USD 186 million | USD 193 million | USD 195 million |
| Lactic acid yield | 269 kg/ton corn stover (95.3% L-purity) | 330 kg/ton wheat straw (99.6% L-purity) | 310 kg/ton wheat straw (99.7% L-purity) |
| Plant water usage | 6. 10 kg/kg lactic acid product | 7.29 kg/kg lactic acid product | 8.10 kg/kg lactic acid product |
| Minimum lactic acid selling price (USD/kg lactic acid product) | 0.584 | 3.241 | 0.813 |
| Feedstock | 0.237 | 0.276 | 0.286 |
| Enzyme [b] | 0.130 | 0.101 | 0.104 |
| Complex nitrogen | 0.009 | 2.624 | 0.193 |
| Available nitrogen | 0.016 | 0.045 | 0.007 |
| Sulfuric acid [c] | / | / | 0.005 |
| $CaCO_3$ [d] | / | / | 0.004 |
| None-enzyme conversion | 0.192 | 0.195 | 0.214 |

[a] The increased capital investment in Case 3 was mainly due to the setup of the cottonseed hydrolysis unit compared to Case 2 and more pre-saccharification reactors, SSCF reactors, and helical impellers due to the lower fermentation solids loading (compared to Case 1). [b] The cellulase was produced on-site according to the NREL model [37], and the price was USD 4.34/kg of protein. [c] The sulfuric acid was specifically used for cottonseed hydrolysis. [d] $CaCO_3$ was specifically used for the neutralization of the cottonseed hydrolysate after acid hydrolysis.

## 4. Conclusions

The characterizations of common, low-cost, alternative complex nutrient sources showed that corn steep liquor (CSL), which is a widely used and cheap nutrient for lactic acid fermentation, is not suitable for lactic homo-fermentation due to the fact that it contains about 20% (*w*/*w*) mixed D-/L-lactic acid, which reduces the lactic acid product optical purity. The cottonseed meal was hydrolyzed by a 5% (*w*/*w*) sulfuric acid solution and achieved the efficient substitution of yeast and peptone by supplementing 10 g/L of ammonium sulfate. The final L-lactic acid titer reached 96.5 ± 0.2 g/L from 25% (*w*/*w*)-solids loaded pretreated and biodetoxified wheat straw, with a yield of 0.31 g/g feedstock and an optical purity of 99.7%. The techno-economic evaluations showed that the cost of cottonseed meal was only USD 0.193/kg of the lactic acid product; the minimum lactic acid selling price (MLSP) was USD 0.813/kg of lactic acid product, which was 25.1% of the MESP using YE and peptone as the nutrients. The cellulosic lactic acid production using the cottonseed meal as a complex-nutrient source based on dry biorefinery processing was comparable and competitive to the production from food crops.

**Author Contributions:** Conceptualization, supervision, and resources, J.B.; investigation, writing-original draft and visualization, B.Z.; investigation, formal analysis, L.W.; resources, X.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Natural Science Foundation of China (31961133006, 21978083).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Lu, Z.; He, F.; Shi, Y.; Lu, M.; Yu, L. Fermentative production of L(+)-lactic acid using hydrolyzed acorn starch, persimmon juice and wheat bran hydrolysate as nutrients. *Bioresour. Technol.* **2010**, *101*, 3642–3648. [CrossRef] [PubMed]
2. Abdel-Rahman, M.A.; Xiao, Y.; Tashiro, Y.; Wang, Y.; Zendo, T.; Sakai, K.; Sonomoto, K. Fed-batch fermentation for enhanced lactic acid production from glucose/xylose mixture without carbon catabolite repression. *J. Biosci. Bioeng.* **2015**, *119*, 153–158. [CrossRef] [PubMed]
3. Inkinen, S.; Hakkarainen, M.; Albertsson, A.C.; Sodergard, A. From lactic acid to poly(lactic acid) (PLA): Characterization and analysis of PLA and its precursors. *Biomacromolecules* **2011**, *12*, 523–532. [CrossRef]
4. Ahmad, A.; Banat, F.; Taher, H. A review on the lactic acid fermentation from low-cost renewable materials: Recent developments and challenges. *Environ. Technol. Innov.* **2020**, *20*, 101138. [CrossRef]
5. Tejayadi, S.; Cheryan, M. Lactic acid from cheese whey permeate. Productivity and economics of a continuous membrane bioreactor. *Appl. Microbio. Biotechnol.* **1995**, *43*, 242–248. [CrossRef]
6. Altaf, M.; Venkateshwar, M.; Srijana, M.; Reddy, G. An economic approach for L-(+) lactic acid fermentation by *Lactobacillus amylophilus* GV6 using inexpensive carbon and nitrogen sources. *J. Appl. Microbiol.* **2007**, *103*, 372–380. [CrossRef] [PubMed]
7. Juturu, V.; Wu, J.C. Microbial production of lactic acid: The latest development. *Crit. Rev. Biotechnol.* **2016**, *36*, 967–977. [CrossRef]
8. Zhang, Y.; Yoshida, M.; Vadlani, P.V. Biosynthesis of D-lactic acid from lignocellulosic biomass. *Biotechnol. Lett.* **2018**, *40*, 1167–1179. [CrossRef]
9. Pauli, T.; Fitzpatrick, J.J. Malt combing nuts as a nutrient supplement to whey permeate for producing lactic by fermentation with *Lactobacillus casei*. *Process Biochem.* **2002**, *38*, 1–6. [CrossRef]
10. Wee, Y.J.; Ryu, H.W. Lactic acid production by *Lactobacillus* sp. RKY2 in a cell-recycle continuous fermentation using lignocellulosic hydrolyzates as inexpensive raw materials. *Bioresour. Technol.* **2009**, *100*, 4262–4270. [CrossRef]
11. Sikder, J.; Roy, M.; Dey, P.; Pal, P. Techno-economic analysis of a membrane-integrated bioreactor system for production of lactic acid from sugarcane juice. *Biochem. Eng. J.* **2012**, *63*, 81–87. [CrossRef]
12. Rivas, B.; Moldes, A.B.; Domínguez, J.M.; Parajó, J.C. Development of culture media containing spent yeast cells of *Debaryomyces hansenii* and corn steep liquor for lactic acid production with *Lactobacillus rhamnosus*. *Int. J. Food Microbiol.* **2004**, *97*, 93–98. [CrossRef] [PubMed]
13. Lima, C.; Fontes Coelho, L.; Blanco, K.; Contiero, J. Response surface optimization of D(-)-lactic acid production by *Lactobacillus* SMI 8 using corn steep liquor and yeast autolysate as an alternative nitrogen source. *Afr. J. Biotechnol.* **2009**, *8*, 5842–5846.
14. Yu, L.; Lei, T.; Ren, X.; Pei, X.; Feng, Y. Response surface optimization of L-(+)-lactic acid production using corn steep liquor as an alternative nitrogen source by *Lactobacillus rhamnosus* CGMCC 1466. *Biochem. Eng. J.* **2008**, *39*, 496–502. [CrossRef]
15. Pejin, J.; Radosavljevic, M.; Pribic, M.; Kocic-Tanackov, S.; Mladenovic, D.; Djukic-Vukovic, A.; Mojovic, L. Possibility of L-(+)-lactic acid fermentation using malting, brewing, and oil production by-products. *Waste Manage.* **2018**, *79*, 153–163. [CrossRef]
16. Li, Y.; Wang, L.; Ju, J.; Yu, B.; Ma, Y. Efficient production of polymer-grade D-lactate by *Sporolactobacillus laevolacticus* DSM442 with agricultural waste cottonseed as the sole nitrogen source. *Bioresour. Technol.* **2013**, *142*, 186–191. [CrossRef]
17. Wang, L.; Zhao, B.; Li, F.; Xu, K.; Ma, C.; Tao, F.; Li, Q.; Xu, P. Highly efficient production of D-lactate by *Sporolactobacillus* sp. CASD with simultaneous enzymatic hydrolysis of peanut meal. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 1009–1017. [CrossRef]
18. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C. *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples*; NREL/TP-510–42623; National Renewable Energy Laboratory: Golden, CO, USA, 2008.
19. Sluiter, A.; Hames, B.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Structural Carbohydrates and Lignin in Biomass National Renewable*; NREL/TP-510–42623; National Renewable Energy Laboratory: Golden, CO, USA, 2012.
20. Zhang, B.; Khushik, F.A.; Zhan, B.; Bao, J. Transformation of lignocellulose to starch-like carbohydrates by organic acid-catalyzed pretreatment and biological detoxification. *Biotechnol. Bioeng.* **2021**, *118*, 4105–4118. [CrossRef]
21. Qiu, Z.; Gao, Q.; Bao, J. Engineering *Pediococcus acidilactici* with xylose assimilation pathway for high titer cellulosic l-lactic acid fermentation. *Bioresour. Technol.* **2018**, *249*, 9–15. [CrossRef]
22. Liu, G.; Zhang, Q.; Li, H.; Qureshi, A.S.; Zhang, J.; Bao, X.; Bao, J. Dry biorefining maximizes the potentials of simultaneous saccharification and co-fermentation for cellulosic ethanol production. *Biotechnol. Bioeng.* **2018**, *115*, 60–69. [CrossRef]

23. Liu, G.; Sun, J.; Zhang, J.; Tu, Y.; Bao, J. High titer L-lactic acid production from corn stover with minimum wastewater generation and techno-economic evaluation based on Aspen plus modeling. *Bioresour. Technol.* **2015**, *198*, 803–810. [CrossRef] [PubMed]
24. Li, Z.; Ding, S.; Li, Z.; Tan, T. L-lactic acid production by *Lactobacillus casei* fermentation with corn steep liquor-supplemented acid-hydrolysate of soybean meal. *Biotechnol. J.* **2006**, *1*, 1453–1458. [CrossRef] [PubMed]
25. John, R.P.; Nampoothiri, K.M.; Pandey, A. Fermentative production of lactic acid from biomass: An overview on process developments and future perspectives. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 524–534. [CrossRef] [PubMed]
26. Alves de Oliveira, R.; Komesu, A.; Vaz Rossell, C.E.; Maciel Filho, R. Challenges and opportunities in lactic acid bioprocess design—From economic to production aspects. *Biochem. Eng. J.* **2018**, *133*, 219–239. [CrossRef]
27. Edwinoliver, N.G.; Thirunavukarasu, K.; Purushothaman, S.; Rose, C.; Gowthaman, M.K.; Kamini, N.R. Corn steep liquor as a nutrition adjunct for the production of *Aspergillus niger* lipase and hydrolysis of oils thereof. *J. Agric. Food Chem.* **2009**, *57*, 10658–10663. [CrossRef] [PubMed]
28. Gao, Y.; Yuan, Y.J. Comprehensive quality evaluation of corn steep liquor in 2-keto-L-gluonic acid fermentation. *J. Agric. Food Chem.* **2011**, *59*, 9845–9853. [CrossRef]
29. Selling, G.W.; Hojilla-Evangelista, M.P.; Hay, W.T.; Utt, K.D.; Grose, G.D. Preparation and properties of solution cast films from pilot-scale cottonseed protein isolate. *Ind. Crop. Prod.* **2022**, *178*, 114615. [CrossRef]
30. Brock, S.; Kuenz, A.; Prüße, U. Impact of hydrolysis methods on the utilization of agricultural residues as nutrient source for D-lactic acid production by *Sporolactobacillus inulinus*. *Fermentation* **2019**, *5*, 12. [CrossRef]
31. Tanksley, T.D., Jr.; Knabe, D.A.; Purser, K.; Zebrowska, T.; Corley, J.R. Apparent digestibility of amino acids and nitrogen in three cottonseed meals and one soybean meal. *J. Anim. Sci.* **1981**, *52*, 769–777. [CrossRef]
32. Gao, M.T.; Hirata, M.; Toorisaka, E.; Hano, T. Study on acid-hydrolysis of spent cells for lactic acid fermentation. *Biochem. Eng. J.* **2006**, *28*, 87–91. [CrossRef]
33. Nielsen, H.P.; Frandsen, F.J.; Dam-Johansen, K.; Baxter, L.L. The implications of chlorine-associated corrosion on the operation of biomass-fired boilers. *Prog. Energy Combust. Sci.* **2000**, *26*, 283–298. [CrossRef]
34. He, N.; Jia, J.; Qiu, Z.; Fang, C.; Liden, G.; Liu, X.; Bao, J. Cyclic L-lactide synthesis from lignocellulose biomass by biorefining with complete inhibitor removal and highly simultaneous sugars assimilation. *Biotechnol. Bioeng.* **2022**, *119*, 1903–1915. [CrossRef] [PubMed]
35. Åkerberg, C.; Zacchi, G. An economic evaluation of the fermentative production of lactic acid from wheat flour. *Bioresour. Technol.* **2000**, *75*, 119–126. [CrossRef]
36. González, M.I.; Álvarez, S.; Riera, F.; Álvarez, R. Economic evaluation of an integrated process for lactic acid production from ultrafiltered whey. *J. Food Eng.* **2007**, *80*, 553–561. [CrossRef]
37. Humbird, D.; Davis, R.; Tao, L.; Kinchin, C.; Hsu, D.; Aden, A.; Schoen, P.; Lukas, J.; Olthof, B.; Worley, M.; et al. *Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*; NREL/TP-5100–47764; National Renewable Energy Laboratory: Golden, CO, USA, 2011. [CrossRef]