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A Process Study of Lactic Acid Production from *Phragmites australis* Straw by a Thermophilic *Bacillus coagulans* Strain under Non-Sterilized Conditions

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Abstract: *Phragmites australis* straw (PAS) is an abundant and renewable wetland lignocellulose. *Bacillus coagulans* IPE22 is a robust thermophilic strain with pentose-utilizing capability and excellent resistance to growth inhibitors. This work is focused on the process study of lactic acid (LA) production from *P. australis* lignocellulose which has not been attempted previously. By virtue of thermophilic feature of strain IPE22, two fermentation processes (i.e., separated process and integrated process), were developed and compared under non-sterilized conditions. The integrated process combined dilute-acid pretreatment, hemicellulosic hydrolysates fermentation, and cellulose utilization. Sugars derived from hemicellulosic hydrolysates and cellulose enzymatic hydrolysis were efficiently fermented to LA in a single vessel. Using the integrated process, 41.06 g LA was produced from 100 g dry PAS. The established integrated process results in great savings in terms of time and labor, and the fermentation process under non-sterilized conditions is easy to scale up for economical production of lactic acid from PAS.

Keywords: lactic acid; SSCF; *Bacillus coagulans*; *Phragmites australis*; hemicellulosic hydrolysates

1. Introduction

Lactic acid (LA) is an important chemical commodity with versatile applications in food, chemicals and pharmaceutical industries [1]. Currently, the studies on converting lignocellulosic feedstock into LA were extensively reported [2–4]. *Phragmites australis* straw (PAS) is an abundant and renewable lignocellulosic feedstock with worldwide distribution [5]. In China, there are at least 0.67 million hectares for planting *P. australis*, with a PAS output of more than 3 million ton [6]. Large scale output of PAS offers a huge potential as a renewable resource for LA production.

Lignocellulose, including PAS, consists primarily of cellulose, hemicellulose, and lignin [7]. Commercial use of lignocellulose for LA production faces at least three major technical barriers. Firstly, the formed soluble byproducts during raw materials pretreatment inhibit microbial growth and retard fermentation [8]. Secondly, to improve product yield, the completely utilizing lignocellulose-derived sugars is essential and economical [9]. Thirdly, the cost of cellulase hydrolysis is a critical factor for lignocellulose usage [10]. Simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and co-fermentation (SSCF) is an effective way to eliminate feedback inhibition of cellulase hydrolysis, since the released glucose could be rapidly fermented to product [7]. Recently, a new integrated process was applied to produce lignocellulose-based biochemical [3,11–13]. Figure 1 shows a schematic diagram of the conventional separated process and the new integrated process. The integrated process consists of lignocellulose pretreatment and the following SSCF operation.

Obviously, SSCF is at the heart of the integrated process and the robust thermophilic strain with pentose-utilizing capability and excellent resistance to growth inhibitors is essential.

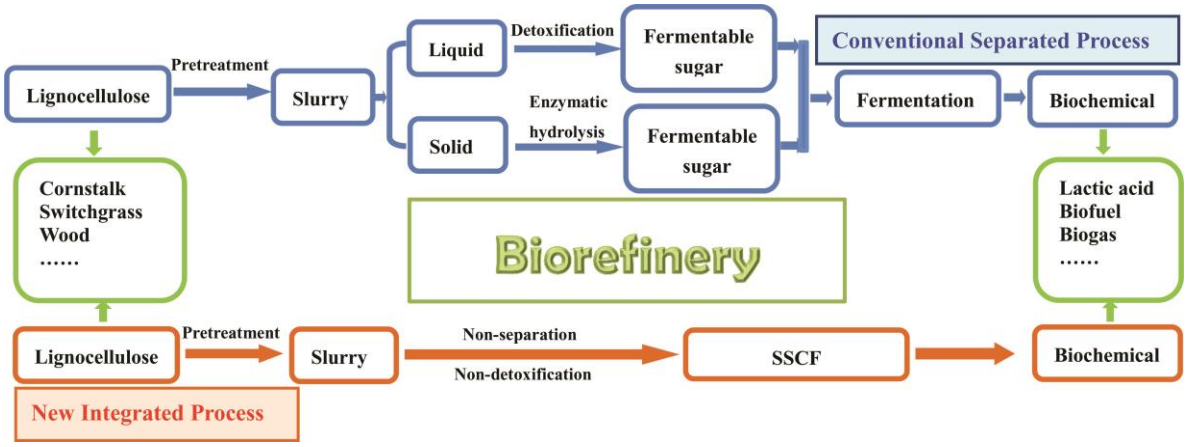

Figure 1. A schematic diagram of the separated process and the integrated process.

As a lignocellulosic feedstock, PAS has been widely studied to produce biofuel [5,6,14]. However, to the best of our knowledge, no report has investigated LA production from PAS. *Bacillus coagulans* IPE22, a thermophilic LA producing strain reported in our previous study, displayed prominent ability to produce LA from glucose, xylose, and arabinose under non-sterilized conditions, and the strain also tolerated inhibitors derived from lignocellulose pretreatment [15]. Based on the strain's excellent features, an integrated process could be established to economically produce LA from PAS including biomass pretreatment, hemicellulosic hydrolysates fermentation and cellulose utilization. In addition, a separated process was also conducted aiming to calculate product yield and titer in comparison with the integrated process.

2. Materials and Methods

2.1. Strain, Culture Medium, and Fermentation

B. coagulans IPE22 was used in the present work. The strain was isolated from soil and the thermophilic strain has robust property to resist contamination. Thus, all fermentations in this work were conducted under non-sterilized conditions, and medium was used directly without sterilization. Medium of mMRS (Modified De Man–Rogosa–Sharpe) was employed for strain culture, the medium contained 10 g/L peptone, 10 g/L beef extract, 5 g/L yeast extract, 2 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $MnSO_4 \cdot H_2O$. The type and concentration of carbon sources varied in different fermentation experiments. For seed culture, 20 g/L glucose was employed. Method of seed culture preparation was the same as our previous report [15]. Fermentations were performed in a 5 L stirred fermenter (Bailun Bio, Shanghai, China). Temperature and pH were controlled automatically at 50 °C and 6.0, respectively. Samples were collected from fermenter at different time to measure concentration of substrate and LA.

2.2. Pretreatment of Raw Materials and Dilute Acid Hydrolysates Fermentation

The PAS was collected from Baiyangdian Lake wetland of China in November, 2017. The naturally dried PAS was cleaned, smashed, and then grinded to 20 mesh, followed by treatment with 2% (w/v) sulfuric acid at a 10% (w/v) loading in an autoclave (MLS-3780, SANYO, Osaka, Japan) at 121 °C for 60 min. Then, the obtained slurry was separated by filtration, and the liquid and solid fractions were collected. The liquid fraction was defined as dilute acid hydrolysates. The solid fraction was washed by deionized water until the filtrate was neutral. Subsequently, the solid residue was dried to constant weight at 60 °C. The obtained dry solid residue was defined as WIS (water insoluble solids). WIS was

used for enzymatic hydrolysis and SSF experiments. Composition of PAS (cellulose, hemicellulose, and lignin) was detected according to the method of reported literature [14].

Sugars in dilute acid hydrolysates were analyzed and fermented to LA by the addition of mMRS medium without carbon source. The seed culture of *B. coagulans* IPE22, ratio of the inocula of seed culture to fermentation broth was 5% (v/v). Deionized water was used to wash WIS until the filtrate was neutral and the washed WIS was dried to constant weight at 80 °C for the subsequent SSF experiments.

2.3. SSF Process

SSF was conducted using commercial cellulase derived from *Trichoderma reesei*. The enzyme was purchased from Sunson Group Ningxia Enzyme Preparation Plant, China. The filter paper units (FPU) activity, cellobiase (CBU) activity, and protein content of the cellulase preparation were as follows: 26.22 FPU/mL, 0.51 CBU/mL, and 8.22 mg/mL, respectively. SSF was conducted in fermenter at 50 °C, 150 r/min and pH 5.0 using deionized water as solvent with cellulase loading of 20 FPU/g cellulose. Medium of mMRS without carbon source was used as nutrient supplement and 5% (v/v) inocula of the strain IPE22 seed culture was employed to generate SSF. The initial cell concentration of the strain during SSF process was 0.25 g dry cell per liter fermentation broth.

2.4. SSCF Process

PAS was pretreated by 2% (w/v) sulfuric acid at 121 °C for 60 min with a solids loading of 10% (w/v) as described in Section 2.2. After pH value of the generated slurry was adjusted to pH 6.0, a two-step SSCF process was triggered by the supplement of mMRS medium. The culture conditions during step 1 were 50 °C and 150 r/min. With the growth of strain IPE22, glucose released from pretreatment was metabolized by biocatalysts to produce LA, leading to a pH decline of fermentation broth. When the pH value dropped to 5.0, the step 2 stage of SSCF started. Enzyme was added to fermentation broth and the fermentation conditions were altered to pH 5.0, 50 °C and 150 r/min. During SSCF, Cellulase loading and seed culture inocula ratio were the same with the SSF experiments.

2.5. Analytical Methods

The concentration of sugars (glucose, xylose and arabinose) and LA in broth was measured by HPLC (Shimadazu Corp., Kyoto, Japan) using the same method reported in our previous study [15]. The HPLC instrument is equipped with UV/Vis-detector (SPD-20A, Shimadzu Corp., Kyoto, Japan) and refractive index (RI) detector (RID-10A, Shimadzu Corp., Kyoto, Japan).

3. Results

3.1. Pretreatment of PAS and Bioconversion of Sugars in Dilute acid Hydrolysates to LA

The composition of the untreated PAS (on dry weight basis) was as follows: cellulose, 42.45%; hemicellulose, 29.34%; lignin, 17.28%. After pretreatment, the generated slurry of PAS was filtrated to achieve dilute acid hydrolysates and WIS. In the WIS, the composition of cellulose, hemicellulose, and lignin changed to 56.49%, 6.58%, and 23.22%, respectively. By means of pretreatment, 18.80 g mixed sugar (glucose 2.49 g, xylose 13.88 g, arabinose 2.43 g) was generated from 100 g PAS. Figure 2 shows the profile of the dilute acid hydrolysates fermentation by the strain IPE22 with mMRS as nutrient supplement. Compared with pentose, utilization of glucose was usually easy for microbe [16]. Thus, glucose was consumed up in 10 h by the strain IPE22, while the metabolism of xylose and arabinose lasted for 19 h. Finally, a titer of 20.78 g/L LA was obtained. In the form of quality description, LA of 17.86 g was obtained by fermenting sugars in dilute acid hydrolysates.

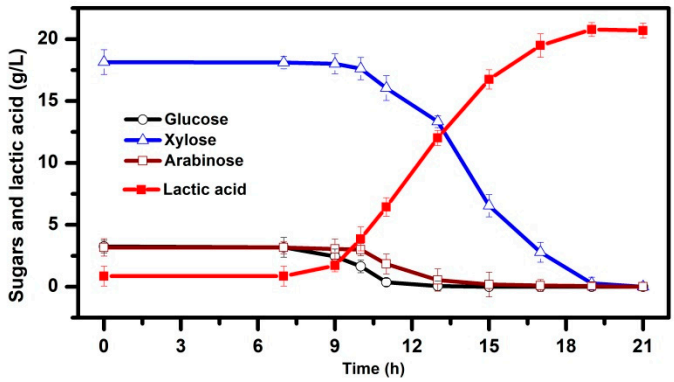

Figure 2. Fermentation of dilute acid hydrolysates of PAS by *B. coagulans* IPE22. Fermentation was conducted using dilute acid hydrolysates of PAS (glucose, 3.25 g/L; xylose, 18.15 g/L; arabinose, 3.18 g/L) as carbon source. Symbols represent: lactic acid, filled squares; glucose, open circles; xylose, open triangles; arabinose, open squares.

3.2. SSF Process

B. coagulans IPE22 had the ability of fermenting glucose and cellobiose to LA, and its fermentation temperature well matched the enzymatic hydrolysis condition of fungal cellulase [15]. After pretreatment of 100 g PAS, 53.42 g WIS was available (containing cellulose 30.18 g). SSF were conducted using the obtained WIS by *B. coagulans* IPE22. SSF was carried out at 5.3% (*w*/*v*) solid loading, which corresponds to 30.18 g of cellulose per 1 L liquid mMRS medium at the enzyme loading of 20 FPU/ g cellulose. Cellulose in PAS was hydrolyzed and LA was produced by the strain IPE22 as shown in Figure 3. At the beginning of SFF, glucose and cellobiose were detected indicating feedback inhibition existed. With the growth of the strain IPE22, the generated glucose and cellobiose from cellulose during SSF was efficiently fermented to LA with a final titer of 17.51 g/L. In the form of quality description, 20.66 g LA were obtained from the above 53.42 g WIS.

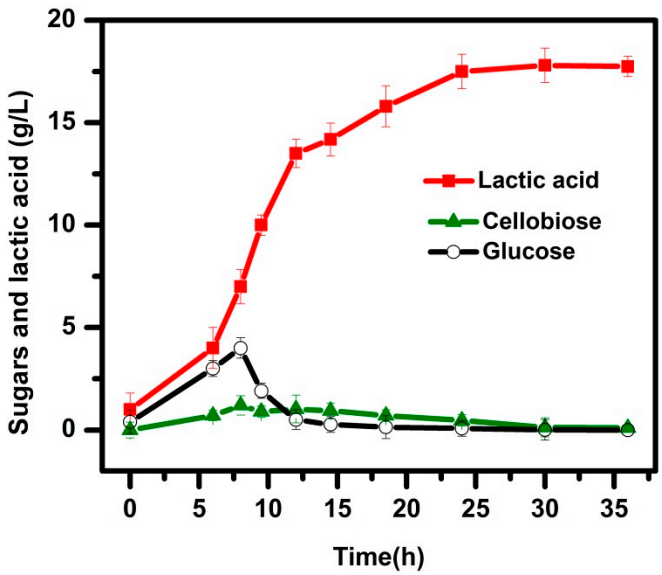

Figure 3. Time courses of SSF by *B. coagulans* IPE22 using mMRS as nutrient supplement. SSF was carried out at 5.3% (*w*/*v*) solid loading, which corresponds to 30.18 g of cellulose per 1 L liquid mMRS medium at the cellulase loading of 20 FPU/g cellulose. Symbols represent: lactic acid, filled squares; glucose, open circles; cellobiose, filled triangles.

3.3. Integrated Process for LA Production via SSCF

After dilute sulfuric acid pretreatment of PAS, 24.66 g/L mixed sugars were detected in the hydrolytes. The mixed sugars consisted of glucose 3.12 g/L, xylose 18.17 g/L and arabinose 3.37 g/L, respectively. Because of the feedback inhibition effect of glucose on cellulose hydrolysis, a two-step SSCF was carried out after biomass pretreatment and pH adjustment. The process of two-step SSCF with mMRS as nutrient supplement was illustrated in Figure 4. During step 1 of SSCF (0–13 h), concentration of glucose was dropped from 3.12 g/L to 0.96 g/L because of the growth of bacteria. Due to carbon catabolite repression, xylose, and arabinose concentration remained constant before glucose consumed up. Step 2 of SSCF (13–55 h) began with cellulase addition when pH of the both decline to 5.0. Then, concentration of glucose and cellobiose increased rapidly on account of cellulase hydrolysis. After 20 h fermentation, biocatalysts entered into exponential phase. Glucose and cellobiose were consumed quickly and LA titer increased markedly. After 27-hour fermentation, concentration of both cellobiose and glucose dropped to a low concentration and the utilization of xylose and arabinose was triggered. As a result, LA concentration of 35.05 g/L was obtained after 55 h experiment. Under the condition tested, LA of 41.06 g was obtained from 100 g dry PAS.

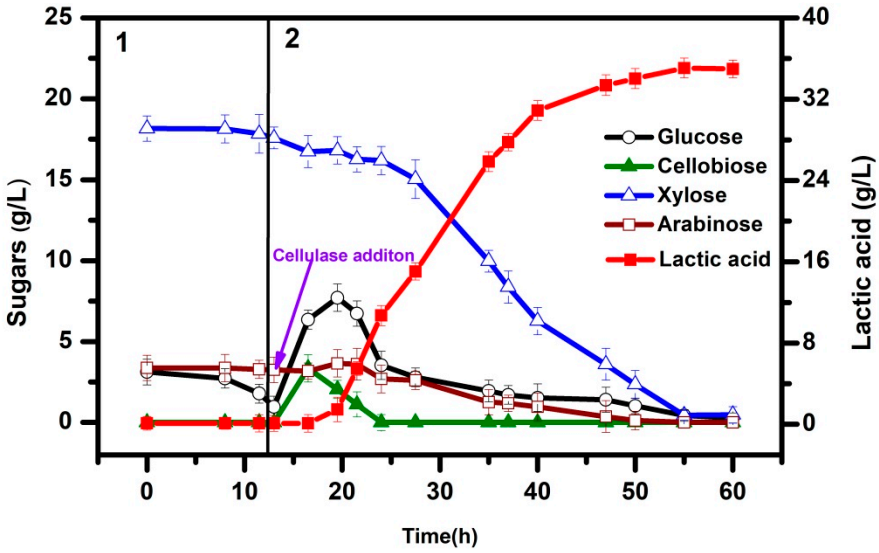

Figure 4. Profile of two-step SSCF with PAS by *B. coagulans* IPE22. During step 1, fermentation was conducted at 50 °C, 150 r/min and pH was not controlled. During step 2, fermentation was conducted at 50 °C, 150 r/min and pH 5.0. Cellulase loading was 20 FPU per gram cellulose during SSCF process. Symbols represent: lactic acid, filled squares; glucose, open circles; xylose, open triangles; arabinose, open squares; cellobiose, filled triangles.

4. Discussion

In the past decade, the studies on converting lignocellulosic feedstock into LA are extensively reported [17,18]. The most commonly used lignocellulosic sources include agricultural residues (cereal straw, sugarcane bagasse, corn stover, etc.) and forestry woody feedstocks (spruce, eucalyptus, birch, etc.). As an abundant and renewable lignocellulose, PAS are attracting worldwide attention to produce bio-based chemicals [19,20]. However, to the best of our knowledge, no report has investigated LA production from PAS. As we all know, there are few strains which could directly ferment lignocellulose to produce lactic acid [21]. Thus, lignocelluloses need to be subjected to a hydrolysis step for converting the cellulose and hemicellulose into potentially fermentable sugars [22]. Lactic acid bacteria (LAB), such as *Lactobacillus rhamnosus* and *Lactococcus lactis*, are usually used in industrial production of LA. However, most of them lack the ability to ferment pentose sugars [7,10]. In addition, LAB strains are not suitable for SSF or SSCF process due to their mesophilic growth feature. Due to the limitation of strain, the process of LA production from lignocellulose was usually divided into

several individual operating units, including feedstock pretreatment, hemicellulosic hydrolysates fermentation, cellulose saccharification, and hexose fermentation [1]. Figure 5 illustrates a schematic diagram of the conventional separated process (containing dilute acid hydrolysates fermentation, cellulose hydrolysis and fermentation or SSF) and the integrated process. The integrated strategy combines several individual operation units and consists of pretreatment and subsequent SSCF. Under integrated process, solid-liquid separation, detoxification, and separated fermentation steps are avoided. The slurry of pretreated lignocellulose is directly fermented in a single vessel. Obviously, SSCF is at the heart of the integrated process and the robust thermophilic strain is also essential to establish the economical way for bio-refinery [13].

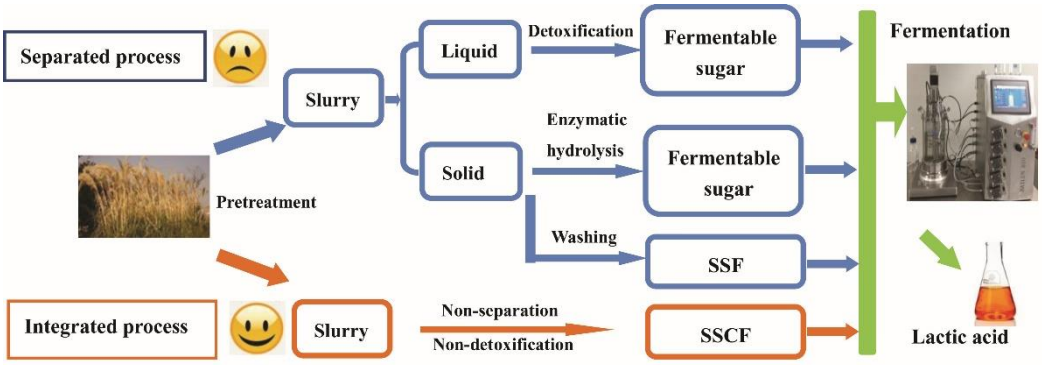

Figure 5. A schematic diagram of the separated process and the integrated process.

Production of LA through integrated process was first proposed using *Lactobacillus brevis* [13]. However, *L. brevis* was mesophilic bacteria, and produced LA by heterofermentation pathway. So, the products of the strain were a mixture of LA, acetic acid and ethanol. An eco-friendly method of biomass pretreatment using ionic liquids followed by subsequent hydrolysis for efficient SSCF process has been explored using *L. brevis* [11]. An integrated system for LA production in combination with lignocellulose pretreatment, separate hydrolysis and fermentation was developed via *L. casei* from sugarcane bagasse [3]. However, the suitable growth temperature for the above strain was 37 °C, which did not match the enzymatic temperature of cellulase. *B. coagulans* IPE22 was an ideal biocatalyst for SSCF process for its hexose and pentose co-fermentation ability and excellent thermophilic growth feature [23]. In this work, the developed integrated strategy simplified the LA production process by combining pretreatment, cellulose hydrolysis, and fermentation, and the fermentation process under non-sterilized conditions is easy to scale up for economical production of lactic acid from PAS. *B. coagulans* IPE22 could ferment both cellobiose and glucose to LA, thus feedback inhibitions during SSCF could be relieved, leading to efficient conversion of cellulose to LA [23].

For a better understanding of the integrated process, Figure 6 shows the mass balance analysis of the integrated process and separated process along with experimental results in this work. For the separated process, filtration operation was employed after PAS pretreatment. Mixed sugars in dilute acid hydrolysates were fermented to 20.78 g/L LA and WIS was converted to 17.51 g/L LA by SSF. In the form of quality description, 17.86 g and 20.66 g LA were obtained from dilute acid hydrolysates and SSF, respectively. In other words, total LA of 38.52 g was produced from 100 g dry PAS by the separated process. In the integrated process, after pretreatment of PAS, the obtained slurry was directly fermented to LA through two-step SSCF. So, in comparison to separated process, a higher LA concentration could be achieved. As a result, LA of 35.05 g/L was attained using mMRS as nutrient supplementation, and the LA yield was 41.06 g per 100 g dry PAS, respectively.

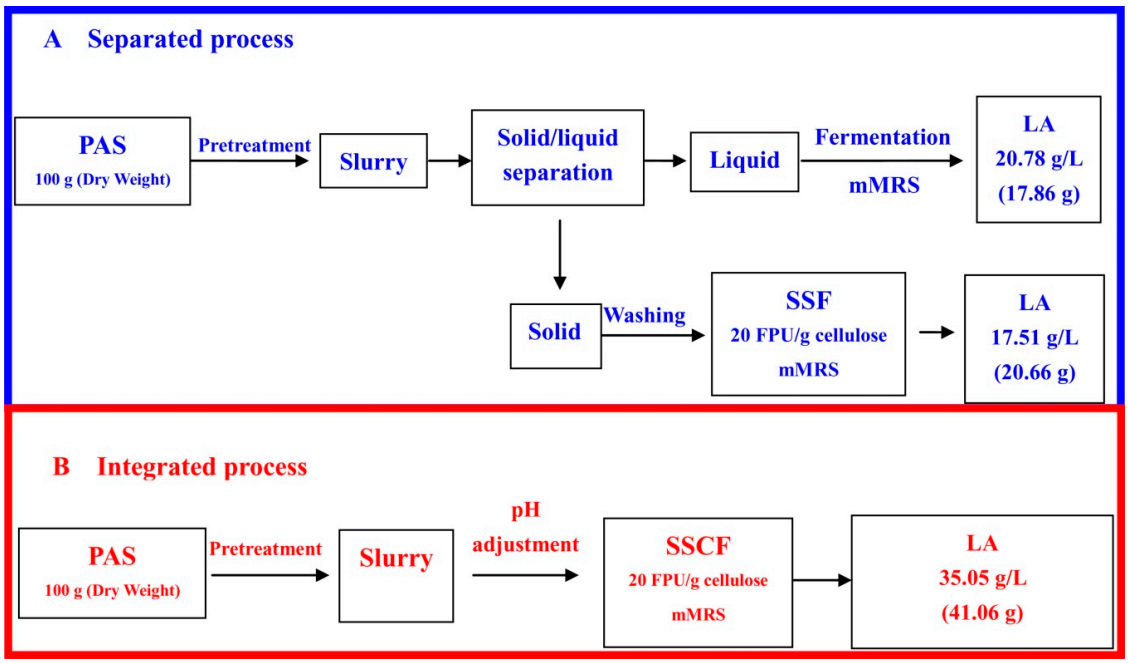

Figure 6. Mass balance analysis of lactic acid (LA) production from PAS by the separated process (**A**) and the integrated process (**B**).

Though, a relatively high LA concentration was obtained by integrated strategy, the titer is also unable to compete with industrial LA fermentation from starch-derived glucose [24]. The low loading of PAS in our work resulted in the low sugars concentration in hydrolysates, thus the concentration of LA was limited to a low value. After pretreatment of PAS, the obtained slurry was a soil-like mixture. The excessive improvement of PAS loading was difficult to achieve by dilute acid pretreatment due to the high viscosity. Moreover, the slurry obtained from excessive high solids loading would be too viscous to mix effectively during the subsequent fermentation. The optimized pretreatment methods, such as ammonia fiber expansion technology, could achieve high solids loading of lignocellulose [25]. Using further optimized methods, such as partial saccharification before fermentation [26] and fed-batch SSF [27], viscous of the slurry could be reduced. The high concentration fermentable hydrolysates could be achieved and the high LA concentration will be obtained [28]. Thus, the integrated process will be more commercially attractive by means of improvement of pretreatment and saccharification. The further studies are ongoing to optimize the established integrated process for economical and efficient LA production from PAS.

5. Conclusions

In conclusion, the present work is focused on study of lactic acid production process using PAS, this has not been attempted previously. An integrated process for LA production from PAS was established using *B. coagulans* IPE22 under non-sterilized conditions. The operation was performed in a single vessel by combining dilute-acid pretreatment, hemicellulosic hydrolysates fermentation, and SSF. Sugars generated from pretreatment and cellulose enzymatic hydrolysis were efficiently converted to LA by IPE22. As a result, 41.06 g LA was achieved from 100 g dry PAS.

Author Contributions: Conceptualization, Y.Z.; methodology, M.L. and T.N.; validation, Z.N.; writing—original draft preparation, Z.N.; writing—review and editing, Y.Z.; funding acquisition, Y.Z. and Z.N.

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

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