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Effect of Fermentation Conditions on L-Lactic Acid Production from Soybean Straw Hydrolysate

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Four types of straw, namely, soybean, wheat, corn, and rice, were investigated for use in lactic acid production. These straws were mainly composed of cellulose, hemicellulose, and lignin. After pretreatment with ammonia, the cellulose content increased, whereas the hemicellulose and lignin contents decreased. Analytical results also showed that the liquid enzymatic hydrolysates were primarily composed of glucose, xylose, and cellobiose. Preliminary experiments showed that a higher lactic acid concentration could be obtained from the wheat and soybean straw. However, soybean straw was chosen as the substrate for lactic acid production owing to its high protein content. The maximum lactic acid yield (0.8 g/g) and lactic acid productivity (0.61 g/(l/h)) were obtained with an initial reducing sugar concentration of 35 g/l at 30°C when using *Lactobacillus casei* (10% inoculum) for a 42 h fermentation period. Thus, the experimental results demonstrated the feasibility of using a soybean straw enzymatic hydrolysate as a substrate for lactic acid production.

Keywords: Agriculture straw, enzymatic hydrolysate, *Lactobacillus casei*, L-lactic acid

Introduction

Lactic acid can be classified into L-, D-, and DL-lactic acids, according to its configuration and optical rotation. However, only L-lactic acid can be completely metabolized by the human body without any production of toxic substances or side effects due to the L-lactic acid dehydrogenation enzyme. Moreover, excessive absorption of D- or DL-lactic acid can cause a disordered metabolic mechanism or toxicity [3].

Two methods are currently used to produce lactic acid, where one involves chemical synthesis that yields only DL-lactic acid, and the other involves biological fermentation that can yield a particular lactic acid by selecting specific microorganisms, substrates, and conditions. Therefore, studies on L-lactic acid and its application as a health supplement are of great importance worldwide. Sugar and starch are the primary raw materials for traditional lactic acid fermentation [1, 23, 31]. However, plant straw fiber [21, 22], kitchen waste [24], and paper sludge [17, 19, 20] can also be used as substrates. In addition, previous studies have reported on the use of agriculture wastes, such as cotton cellulose [4], corn stover [2, 14], and wheat straw [5, 16], for lactic acid production.

Soybean, wheat, corn, and rice are the major cereal crops in China, with an output of approximately 350 million tons annually. This then produces approximately 480 million tons of straw annually, representing 80% of the total straw production in China. Most of this straw is currently burnt in the fields. However, the discovery that straw can be hydrolyzed into soluble sugar for L-lactic acid production using biological fermentation has both enlarged the resource for lactic acid production and solved the problem of environmental pollution and resource wastage.

When analyzing the hydrolysis conversion of soybean straw, Xu *et al*. [25] found that the cellulose in smashed dry

soybean straw increased by 70.27%, whereas the hemicellulose and lignin decreased by 41.45% and 30.16%, respectively, after pretreatment with a 10% ammonia solution at room temperature for 24 h. Meanwhile, another study on the solid-substrate fermentation and enzymolysis of soybean stalk cellulose pretreated with ammonia found that the resulting cellulase activity reached 798.84 filter paper units (FPU)/g [26]. The maximum enzymatic hydrolysis efficiency of 51.22% was also achieved when using cellulose with a 5% (w/v) substrate concentration at 50°C and pH 4.8 for 36 h [27]. Moreover, the fermentation of a soybean straw hydrolysate with *Lactobacillus casei*, *Lactobacillus sake*, and a co-inoculation of both strains yielded lactic acid conversions of 48.27%, 56.42%, and 71.05%, respectively [28]. However, the effect of the fermentation conditions on L-lactic acid production from the enzymatic hydrolysis of soybean straw has not yet been discussed in detail, and the optimum conditions have not yet been established. Furthermore, the feasibility of L-lactic acid fermentation using different straws has not yet been investigated.

Accordingly, the current study investigated the chemical compositions of soybean straw, wheat straw, corn straw, and rice straw. Enzymatic hydrolysates of these different straws were then used as substrates for L-lactic acid production by *L. casei*. Soybean straw was finally selected as the raw material owing to its high reducing sugar content and high L-lactic acid production. The effects of temperature, the inoculum size, and sugar concentration were also examined, to achieve the optimal fermentation conditions.

Materials and Methods

Materials

The straw was supplied by a local processing factory. The cellulase with a filter enzymatic activity of 15,000 FPU/g was purchased from Wuxi Enzyme Company (China). *L. casei* was obtained from the China Committee for Culture Collection of Microorganisms. The bacteria were maintained on de Man, Rogosa, and Sharpe (MRS) medium.

Experiment Methods

Straw pretreatment. The dry straw was smashed and pretreated with a 10% ammonia solution in a flask at room temperature for 24 h. After filtration, the solids were dried at 80°C and then used as the material for enzymatic hydrolysis.

Enzymatic hydrolysis. The hydrolysis was performed in a 0.05 M citrate buffer (pH 4.8) at 50°C for 36 h, with 5% (w/v) pretreated straw and an enzyme loading of 50 FPU/(g straw). The final reducing sugar content in the enzymatic hydrolysates was measured.

L-Lactic acid fermentation of enzymatic hydrolysates. All the experiments were performed using batch fermentation under strictly anaerobic conditions with 100 ml of the sterilized fermentation medium in stoppered 250 ml Erlenmeyer flasks that contained (in g/l) peptone, 5.0; yeast extract, 5.0; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 0.5; NaCl, 0.1; and $CaCO_3$, 20.0 mixed with 1,000 ml of the enzymatic hydrolysate. The fermentation was performed at 25°C on a shaking bed (140 rpm) for 54 h with a 13.3 g/l initial reducing sugar concentration and the addition of a 10% (v/v) inoculum. The L-lactic acid concentration was measured periodically.

Analytical Methods

The cellulose, hemicellulose, lignin, protein, and moisture content were all determined according to previously reported methods [5]. The reducing sugar and lactic acid were determined using a high-performance liquid chromatograph from the Waters Corporation (USA). The sugar was determined using a high-performance liquid chromatograph with an NH_2 column (4.6 mm × 250 mm) and refractive index detector, where ethyl nitrile (ethyl nitrile:water = 70:30) was used as the mobile phase with a flow rate of 1 ml/min. The lactic acid was determined using a C_{18} column (4.6 mm × 250 mm) and ultraviolet absorption detector, where sulfuric acid (0.01 mol/l) at 0.7 ml/min was used as the mobile phase and the detection was performed at 210 nm. For both columns, the injection volume was 10 μl and the tests were conducted at room temperature.

Results and Discussion

Substrate Selection for L-Lactic Acid Production

Preliminary experiments were performed to compare the soybean, wheat, corn, and rice straw substrates for lactic acid production. Additionally, the chemical composition and lactic acid concentration were determined before and after the ammonia pretreatment. The major chemical compositions of the four kinds of straw are presented in Table 1. The results show that the cellulose content was higher than the hemicellulose and lignin contents in all four substrates.

The X-ray diffraction (XRD) patterns in Fig. 1 also show the crystallinity of the four different types of straw. Although the compositions were very similar, the cellulose level varied slightly, where the soybean straw showed relatively low cellulose and low hemicellulose levels, but high lignin and protein levels.

Whereas the complex structures formed by cellulose, hemicellulose, and lignin in the straw fibers effectively block the degradation of cellulose [6], the ammonia pretreatment showed a positive effect by eliminating some of the lignin, thereby enhancing the cellulose content and porosity of the straw and changing the external surface

Table 1. Major chemical composition of various straws prior to pretreatment.

Name	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Protein (%)
Soybean straw	30.92	22.43	17.64	9.12
Wheat straw	42.04	25.06	16.07	5.60
Corn straw	32.42	23.12	10.19	3.18
Rice straw	33.94	26.02	15.42	4.55

construction. These conditions are then favorable for cellulose degradation and conversion. Moreover, the addition of ammonia also supplemented the nitrogen source and supported the growth of the microorganism.

The mechanism of ammonia pretreatment includes the saponification of the intermolecular ester bonds crosslinking xylan hemicelluloses and other components, such as lignin and other hemicelluloses [30], which in turn enhances the accessibility of hemicelluloses and cellulose to hydrolytic enzymes. The effect of the ammonia pretreatment is shown in Fig. 2, where the pretreatment clearly increased the cellulose by an average of 13.1%, yet the hemicellulose and lignin levels were decreased by 9.95% and 4.47%, respectively. Thus, while the ammonia pretreatment was effective in solubilizing the lignin, the hemicellulose was also partially degraded. Hydroxyl ions are believed to disrupt the hydrogen bonds between cellulose and hemicellulose, and hydrolyze the ester bonds connecting cell-wall polysaccharides, resulting in significant solubilization of hemicellulose [8]. This phenomenon has also been reported by other researchers. When Kim *et al*. [11] used an ammonia recycled percolation process to pretreat corn stover, they reported a reduction of 70–85% of lignin and 40–60% of hemicellulose. When Kim and Lee [12] pretreated corn stover by soaking in aqueous ammonia (SAA) at room temperature, 11.3% of the xylan was dissolved after 10 days. Notwithstanding, for the current study, no measures were taken to decrease the hemicellulose dissolution, as the main hemicellulose-derived sugars (*i.e*., xylose and arabinose) cannot be utilized by the homofermenter *L. casei* used in this study.

The sugar compositions measured for the straw enzymatic hydrolysates are shown in Fig. 3. The average reducing sugar concentration for the enzymatic hydrolysates was approximately 12.34 g/l. In all four straws, glucose, xylose, and cellobiose were the three main components at an average ratio of 3.9:1.7:1. About 39.05% of the cellulose in the pretreated soybean straw was hydrolyzed to glucose and cellobiose, which was slightly higher than that in the other straws (34.05% for wheat straw, 32.85% for corn straw, and 31.36% for rice straw). These sugar recovery yields are relatively low when compared with other reported results. When Kim and Lee [13] pretreated corn stover using SAA at 60°C, the enzymatic digestibility of the glucan content increased from 17% to 85%. Meanwhile, when Kang *et al*. [10] pretreated rapeseed straw using SAA at the optimum temperature of 69°C, they obtained 60.7% of theoretical glucose. One explanation is that the present study performed the ammonia pretreatment at room

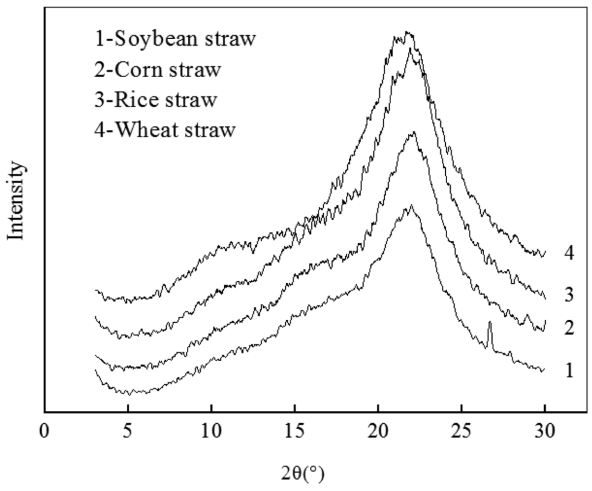

Fig. 1. XRD patterns of various straws prior to ammonia pretreatment.

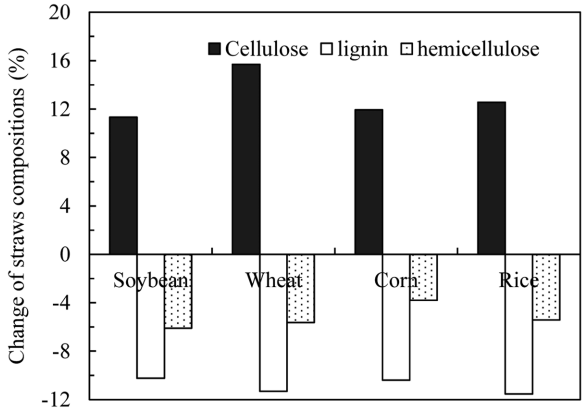

Fig. 2. Changes in major chemical composition of substrates after ammonia pretreatment.

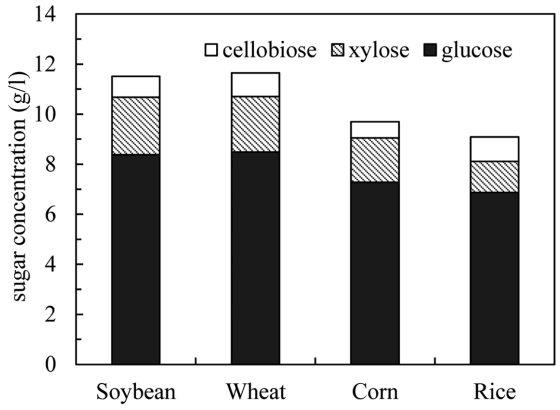

Fig. 3. Sugar composition of enzymatic hydrolysates from various substrates.

temperature (~20°C), which may have significantly affected the total fermentable sugar production, and thereby the sugar recovery yield [29].

Table 2 presents the L-lactic acid fermentation results when using the enzymatic hydrolysates of the four different straws. All four substrates achieved a high L-lactic acid production, confirming the feasibility of L-lactic acid fermentation from straw hydrolysates. As shown in Table 2, the reducing sugar concentrations and lactic acid production from the soybean and wheat straw hydrolysates were higher than those from the corn and rice straw hydrolysates. Although the lactic acid production from the wheat straw was very close to that from the soybean straw (2.9% discrepancy), this study chose to use soybean straw as the substrate for lactic acid production for the following reasons: (i) a large amount of soybean straw is burned in fields every year, which causes huge environmental problems. For example, approximately 1/3 of the soybean production in China comes from Heilongjiang Province, where 10 million tons of soybean straw is burned annually; (ii) in contrast to other straws, soybean straw has a high lignin content and hard texture, making machine treatment and digestion as animal feedstuff difficult; (iii) soybean straw has a higher protein content than other straws, which satisfies the need of C/N for fermentation and also facilitates future utilization of the residue after lactic acid fermentation.

Table 2. L-Lactic acid concentrations from four substrates after fermentation.

Substrate	Reducing sugar (g/l)	Lactic acid (g/l)
Wheat	13.82	5.685
Soybean	13.07	5.521
Rice	10.98	5.394
Corn	11.49	5.148

Effects of Fermentation Conditions on L-Lactic Acid Production

Effect of temperature on L-lactic acid production. A suitable temperature is one of the necessary conditions for the growth of all microorganisms. Growth and metabolic responses occur within a certain temperature range, which affects the microorganism growth and reproduction, along with enzyme secretion and metabolite formation.

In this study, the L-lactic acid fermentation was performed at different temperatures: 25°C, 30°C, 35°C, and 40°C. Fig. 4 shows that the maximum lactic acid yield and sugar utilization were achieved at 30°C, whereas a remarkably lower L-lactic acid yield and sugar utilization efficiency were obtained at temperatures below or above 30°C. This phenomenon was primarily caused by the restriction of *L. casei* at the lower temperatures, whereas higher temperatures can reduce bacteria and cause mass death, resulting in restricted metabolic activity.

Effect of inoculum size on L-lactic acid production. The inoculum size has an incremental effect on the growth speed of a microorganism. Thus, the larger the inoculum size within a certain range, the less time a microorganism needs to enter the log phase. Because of this shortened lag period, the total fermentation time is also shortened, resulting in enhanced lactic acid productivity. In this study, 5%, 10%, 15%, and 20% inoculations of *L. casei* were

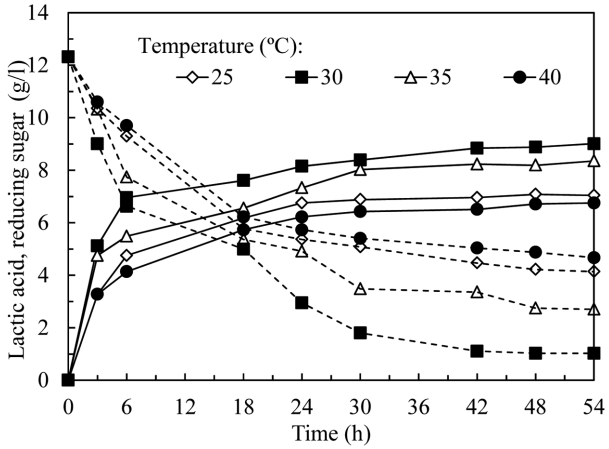

Fig. 4. Effect of temperature on L-lactic acid fermentation using soybean straw liquid enzymatic hydrolysate.
Solid lines represent lactic acid concentrations, while dotted lines represent reducing sugar concentrations.

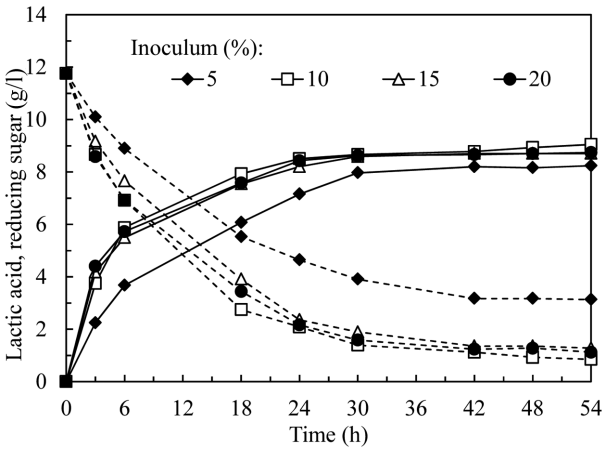

Fig. 5. Effect of inoculation on L-lactic acid fermentation using soybean straw liquid enzymatic hydrolysate.
Solid lines represent lactic acid concentrations, and dotted lines represent reducing sugar concentrations.

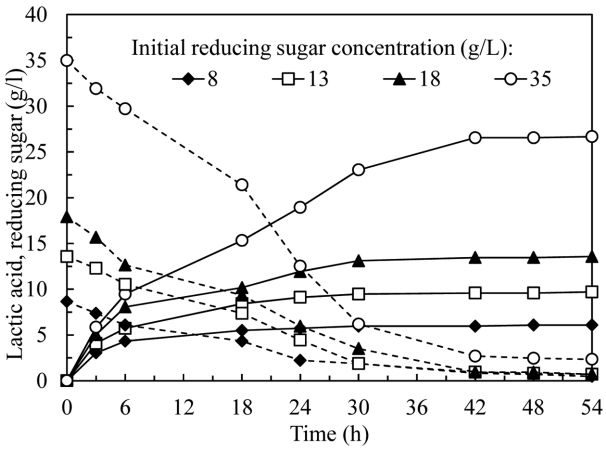

Fig. 6. Effect of initial reducing sugar concentration on L-lactic acid fermentation using soybean straw liquid enzymatic hydrolysate.
Solid lines represent lactic acid concentrations, and dotted lines represent reducing sugar concentrations.

used for the L-lactic acid fermentation. As shown in Fig. 5, an inoculum size of 10% or more resulted in similar lactic acid productivity, whereas the 5% inoculation resulted in a much lower productivity. After fermentation for 54 h, the lactic acid concentration with the 5% inoculum (8.24 g/l) was slightly lower than that with the 10% inoculum (9.05 g/l). As a higher sugar concentration (3.13 g/l) remained in the medium with the 5% inoculum, it was predicted that more lactic acid would be produced if the residual sugar could be utilized by the bacteria. Thus, the decreased sugar utilization and lactic acid production by the bacteria may have been due to the lower pH caused by acid accumulation. Consequently, the results demonstrated that a low inoculum size was not beneficial for the accumulation of the target product. Meanwhile, the lactic acid production only increased minimally when the inoculation was higher than 10%. Therefore, the optimal inoculum size for *L. casei* was determined as 10%.

Effect of initial reducing sugar concentration on L-lactic acid production. The soybean straw enzymatic hydrolysate was concentrated by evaporation or diluted to obtain various sugar concentrations of 8, 13, 18, and 35 g/l. The fermentation was then conducted at 30°C with a 10% inoculum. As shown in Fig. 6, the product/substrate ratio for the four different initial sugar concentrations was 0.70, 0.72, 0.76, and 0.76 g lactic acid/g reducing sugar, respectively. This indicates that the low sugar concentration resulted in a low lactic acid production, plus an initial sugar concentration up to 35 g/l did not restrict the metabolism of the bacteria. Thus, the L-lactic acid yield increased gradually when increasing the substrate concentration.

Fermentation Under Optimum Conditions

The L-lactic acid fermentation from the soybean straw hydrolysate was conducted under the following optimum conditions: initial sugar concentration, 35 g/l; temperature, 30°C; and inoculum size, 10%. As shown in Fig. 7, the yield of lactic acid based on the substrate consumed, defined as the ratio of the lactic acid produced to the reducing sugar consumed, was 0.80 g/g after 42 h of fermentation. The lactic acid volumetric productivity, calculated as the ratio

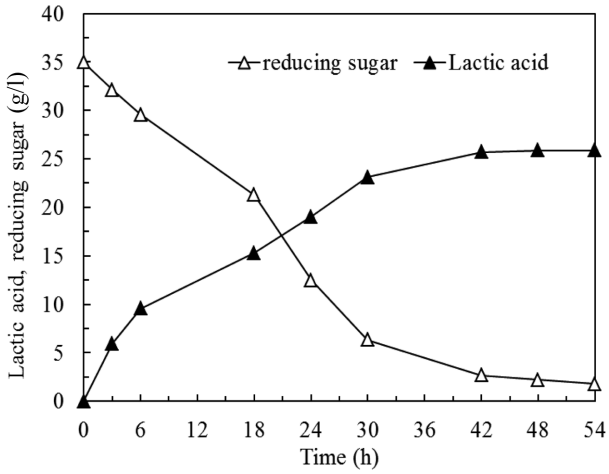

Fig. 7. L-Lactic acid production from soybean straw under optimum conditions.

of the lactic acid concentration to the fermentation time, decreased gradually from 1.99 g/(l·h) during the first 3 h to 0.61 g/(l·h) during 42 h of fermentation. The decreased productivity with the prolonged fermentation period was due to the lower availability of the substrate, inhabitation of the product, and lower pH. Therefore, these results indicate that soybean straw could be utilized for lactic acid production to recycle agricultural waste and save costs.

The L-lactic acid yield and productivity obtained in this study are comparable with those previously reported by Moldes [18], who used *Lactobacillus pentosus* to produce lactic acid from an acidic hydrolysate of vine shoots with a yield and productivity of 0.76 g/g and 0.51 g/(l·h), respectively. However, the current results are relatively low when compared with other studies that used *L. casei* to produce lactic acid. Hujanen and Linko [7] used two homofermentative strains, *L. casei* NRRL B-441 and *L. casei* subsp. *rhamnosus* NRRL B-445, for lactic acid production from glucose and obtained a maximal volumetric productivity of 4.1 g/(l·h) and 3.5 g/(l·h), respectively. Li *et al*. [15] used a corn steep liquor-supplemented acid-hydrolysate of soybean meal to produce lactic acid with *L. casei* LA-04-1, and obtained a yield of 0.9 g/g and productivity of 1.69 g/(l·h). The saccharification and fermentation of cassava bagasse with *L. casei,* conducted by John *et al.* [9], also achieved a maximum yield of 0.96 g/g starch and productivity rate of 1.40 g/(l·h). Therefore, the relatively low yield and productivity in the present study may have been due to the following reasons: (i) as a homofermenter, *L. casei* is incapable of utilizing xylose and arabinose to produce lactic acid, thereby partially wasting the sugar components in the hydrolysate and resulting in a lower yield. Thus, the use of a facultatively heterofermenter or the development of recombinant bacteria capable of co-fermentating pentoses and hexoses would likely increase the lactic acid yield; (ii) in this study, as the pH was not controlled during the fermentation process, the adverse effects of acidification during the fermentation process were not alleviated; (iii) the initial reducing sugar concentration used in the present study was 35 g/l, which is much lower than that reported by Hujanen and Linko [7] (80–160 g/l). As shown in Fig. 7, *L. casei* can probably tolerate higher reducing sugar concentrations, resulting in a higher lactic acid productivity. Thus, further research is needed to enhance the yield and productivity.

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