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Simultaneous Saccharification and Fermentation of Cellulose for Lactic Acid Production

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Lactic acid production from α-cellulose by simultaneous saccharification and fermentation (SSF) was studied. The cellulose was converted in a batch SSF using cellulase enzyme Cytolase CL to produce glucose sugar and *Lactobacillus delbrueckii* to ferment the glucose to lactic acid. The effects of temperature, pH, yeast extract loading, and lactic acid inhibition were studied to determine the optimum conditions for the batch processing. Cellulose was converted efficiently to lactic acid, and enzymatic hydrolysis was the rate controlling step in the SSF. The highest conversion rate was obtained at 46°C and pH 5.0. The observed yield of lactic acid from α-cellulose was 0.90 at 72 hours. The optimum pH of the SSF was coincident with that of enzymatic hydrolysis. The optimum temperature of the SSF was chosen as the highest temperature the microorganism could withstand. The optimum yeast extract loading was found to be 2.5 g/L. Lactic acid was observed to be inhibitory to the microorganisms' activity.

Key words: lactic acid, cellulose, enzymatic hydrolysis, SSF

INTRODUCTION

Lactic acid has a wide range of food-related and industrial applications. Furthermore, new applications, such as biodegradable plastics made from polylactic acid [1, 2], have the potential to greatly expand the market for lactic acid. In view of this growing demand, cellulosic biomass is currently being regarded as important feedstocks for lactic acid production [2, 3]. Cellulosic substances are the most abundant renewable resources [4]. The process schemes for the production of lactic acid from cellulosic biomass can share with those proposed for production of ethanol from biomass. One such scheme is simultaneous saccharification and fermentation (SSF). SSF is a bioprocess capable of directly converting lignocellulosic materials to end product. It has been extensively investigated in connection with ethanol production from cellulosic biomass [5, 6]. Recently it has also been brought up as a means of producing lactic acid [7]. There is one technical aspect in lactic acid fermentation that makes it particularly suitable for SSF operation. Many of the lactic acid producing bacteria are thermo-tolerant. The operating temperature of the SSF can thus be brought to the level close to the optimum of the cellulase enzymes, making the overall process more efficient, especially in the use of enzymes. Furthermore, since no carbon dioxide is formed in the fermentation of lactic acid, substrate carbon is more efficiently utilized in lactic acid than ethanol fermentation.

The SSF is a single-step process in which enzymatic hydrolysis of cellulose into glucose and subsequent glucose fermentation are carried out in a single vessel. In the SSF, the rate of hydrolysis is much slower than the rate at which the microorganism can consume glucose. Consequently, faster saccharification rates result because the glucose product is immediately removed, considerably diminishing its inhibitory effect on the cellulase system. To effectively apply the SSF method to produce lactic acid from cellulosic substrates, the enzymatic hydrolysis and fermentation conditions, such as pH and temperature, must be coincident. And cellulase inhibition by lactic acid must be less than that by glucose and cellobiose. This research was undertaken to assess the feasibility of producing lactic acid by the SSF process. The effects of temperature, pH, lactic acid inhibition, and yeast extract loading were studied to determine the optimum conditions for the batch processing.

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MATERIALS AND METHODS

Materials

A commercial α-cellulose from Sigma(93% glucan and 5% xylan) was used as the cellulosic substrate. The cellulase enzyme, Cytolase CL was obtained from Environmental Biotechnologies, Inc., U.S.A. It has filter paper cellulase of 96 FPU/mL, β-glucosidase activity of 80 p-NPGU/mL, and endo-glucanase activity of 613 CMCU/mL.

Microorganism and Medium

Lactobacillus delbrueckii (IFO 3534, KCCM No. 40001) was used for fermentation. The culture was grown at 37°C for 24 h, and stored at 4°C in agar slants made of MRS broth and 2% agar (Difco). The seed culture was grown at 37°C for 36 h in MRS broth.

Batch Experiment of SSF

Batch experiments of SSF were done in a 1000-mL fermenter with 600 mL working volume. The com-

position of the SSF medium was (per liter): cellulose substrate (10-30 g), yeast extract (30 g), NaOH (1.25 g), K_2HPO_4 (0.2 g), KH_2PO_4 (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.6 g), $MnSO_4 \cdot H_2O$ (0.03 g), and $FeSO_4 \cdot 7H_2O$ (0.03 g). For each run, substrate and yeast extract were dissolved in 550 mL fermentation medium. After the fermenter containing substrate and medium was autoclaved and cooled down to reaction temperature, 10% (40 mL) inoculum and enzyme (45 IFPU/g cellulose) were added to the fermenter. The pH of the SSF broth was controlled using 5 N NH_4OH.

Enzymatic Hydrolysis

The saccharification reaction of cellulose substrate was performed in 100-mL glass bottles containing 50 mL buffer (sodium citrate). It was agitated at 150 rpm on a shaker bath. The enzyme loading was 45 IFPU/g cellulose, and initial cellulose concentration was 1%(w/v).

Analytical Methods

The samples were analyzed for sugars, lactic acid, and acetic acid by HPLC, equipped with an RI detector. Bio-Rad's HPX-87H column was used at 65°C, with 0.005M H_2SO_4 as mobile phase. The flow rate was set at 0.6 mL/min.

RESULTS AND DISCUSSION

Lactic Acid Production by SSF

Typical lactic acid production from α-cellulose by SSF is illustrated in Fig. 1. Accumulation of glucose was seen in the initial phase of the SSF. The fermentation then proceeded under glucose-limited conditions, indicating that the enzymatic hydrolysis is the rate-limiting step in the SSF. Lactic acid concentration increased steadily. The observed overall yield of lactic acid (lactic acid formed) from α-cellulose was calculated to be 0.90 at 72 hours based on cellulose content. During the SSF process, ammonium hydroxide was used to control fermentation pH. The neutralization agent also serves as a nitrogen source. Because the hydrolysis is the rate controlling step in the SSF, the subsequent work in the SSF was focused on the improvement of the hydrolysis process.

Effect of Temperature

The typical temperature for the fermentation by *Lactobacillus delbreuckii* was reported to be in the range of 42-46°C [8]. However, the optimum temperature for enzymatic hydrolysis was observed to be about 50°C or higher. Fig. 2 depicts the effect of temperature on the enzymatic hydrolysis of α-cellulose. The enzymatic hydrolysis was carried out at three temperature levels, 37, 42, and 50°C. Glucose concentrations at 6 hours of reaction time were analyzed. It is clearly seen that the production rates of glucose increased with temperature within the scope of this experiment, indicating that higher temperature is favorable for the enzymatic hydrolysis than the optimum temperature for *Lactobacillus delbreuckii*. Therefore, the optimum temperature for SSF could be in the range close to that for the enzymatic hydrolysis because the enzymatic hydrolysis is the rate-limiting step in the SSF.

However, when a temperature above 47°C was applied in the SSF, it was observed that the microorganism's activity was dramatically reduced and showed low lactic acid formation. Fig. 3 shows time course of the lactic acid and glucose production by SSF performed at temperatures of 46 and 50°C. In the SSF at 50°C, glucose was accumulated throughout the entire SSF period, and very little lactic acid was formed. In the SSF at 46°C, however, glucose was accumulated at the early phase of the SSF. It was converted to lactic acid rapidly after the microorganism was well grown. The SSF proceeded under glucose-limiting conditions thereafter. Although not shown in the Fig. 3, it was observed in this study that the microorganism's activity was dramatically reduced at temperatures above 47°C and showed very little lactic acid formation. The temperature of the SSF was chosen as the highest temperature the microorganism could withstand. In this work, it was chosen to be 46°C, one degree below the upper limit for allowing a safety factor.

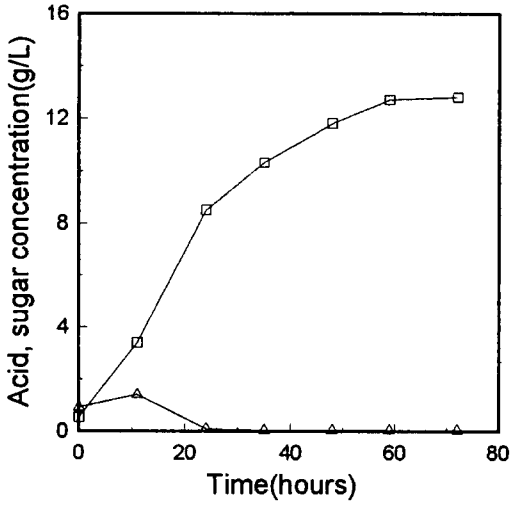

Fig. 1. Time course of glucose and lactic acid production from cellulose by SSF. (SSF conditions: temperature 46°C, pH 5.0, α-cellulose 12.5 g/L enzyme 45 IFPU/g cellulose, symbol: △; glucose, □; lactic acid).

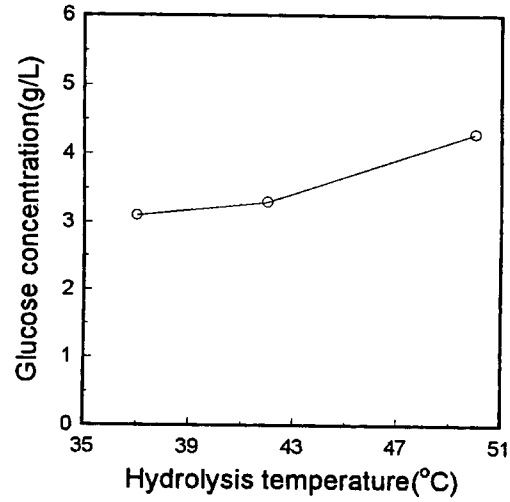

Fig. 2. Effect of temperature on glucose formation in enzymatic hydrolysis of α-cellulose. (hydrolysis conditions: pH 5.0, α-cellulose 1.0 g, enzyme 45 IFPU/g cellulose, 50 mL buffer, hydrolysis time 6 hrs).

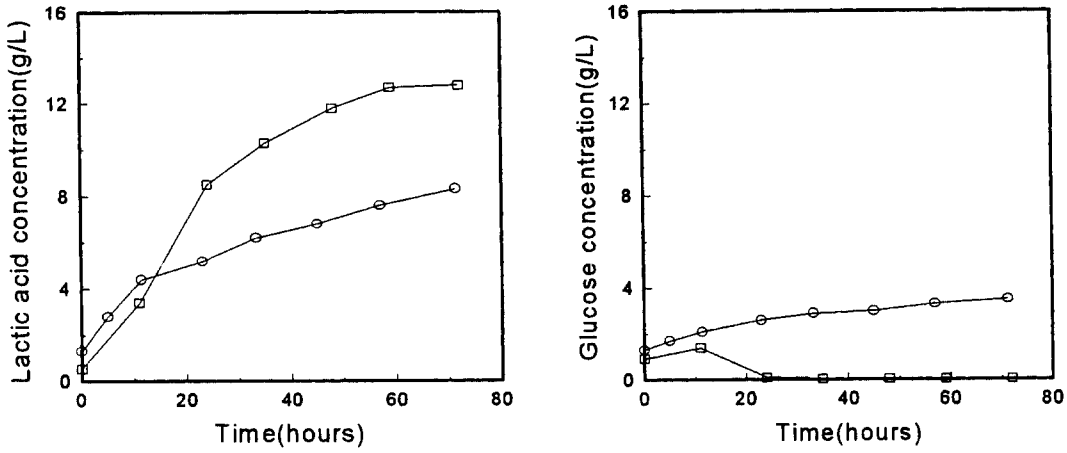

Fig. 3. Effect of temperature on glucose and lactic acid production in SSF of α-cellulose. (SSF conditions: Temperature 46°C, 50°C, pH 5.0, α-cellulose 12.5 g/L, enzyme 45 IFPU/g cellulose, symbol: □ ; 46°C, ○ ; 50°C).

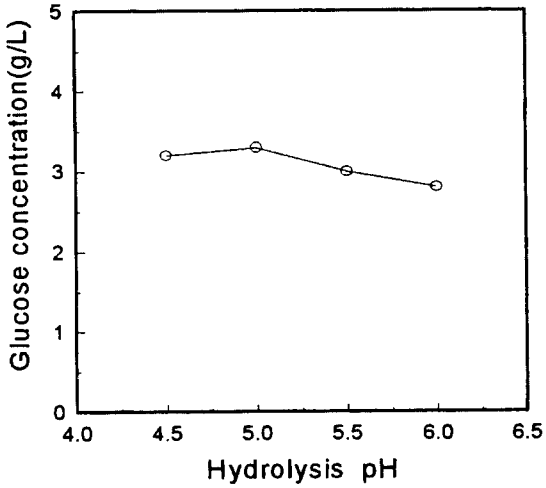

Fig. 4. Effect of pH on glucose formation in enzymatic hydrolysis of α-cellulose. (hydrolysis conditions: temperature 42°C, α-cellulose 1.0 g, enzyme 45 IFPU/g cellulose, 50 mL buffer, hydrolysis time 6 hours).

Effect of pH

The effect of pH on enzymatic hydrolysis was shown in Fig. 4. The optimum pH of the enzyme used in this study was found to be 4.5-5.0. It has been reported that the optimum pH for lactic acid production by *Lactobacillus delbreuckii* was between 5 and 6 [8]. If the two processes are combined (SSF), the optimum pH would be 5.0. Fig. 5 shows the effect of pH on SSF. Three pH levels (5.0, 4.7, and 4.3) were applied in this experiment. In the SSF at pH 4.3, glucose was accumulated throughout the entire SSF period, and lactic acid was formed slowly. This indicates that cell growth was suppressed and the process was limited by the fermentation. At pH 5.0 and 4.7, glucose was observed to be accumulated at the early phase of the SSF.

After the cells were well grown, it was converted to lactic acid rapidly. Glucose was consumed more rapidly at pH 5.0 than at pH 4.7, indicating that a higher pH of 5.0 was favored over 4.7 for cell growth. Therefore, the optimum pH in SSF was chosen to be 5.0.

Effect of Yeast Extract Loading

One of the major costs in lactic acid fermentation is the consumption of yeast extract. Large amount of yeast extract is required as a nutrient in the fermentation for lactic acid. Three yeast extract levels (2.5, 12.5, 30 g/L) were tested for its effect on the SSF. The time courses of the SSF runs are shown in Fig. 6. Glucose was accumulated more in the SSF with lower yeast extract

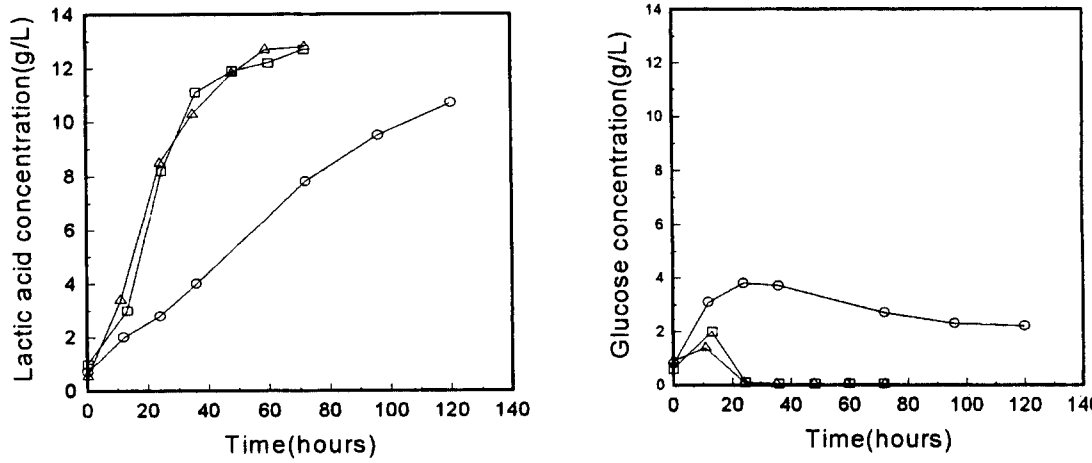

Fig. 5. Effect of pH on glucose and lactic acid production on SSF of α-cellulose. (SSF conditions: pH 4.3, 4.7, 5.0, temperature 46°C, α-cellulose 12.5 g/L, enzyme 45 IFPU/g cellulose, symbol: ○ ; pH 4.3, □ ; pH 4.7, △; pH 5.0).

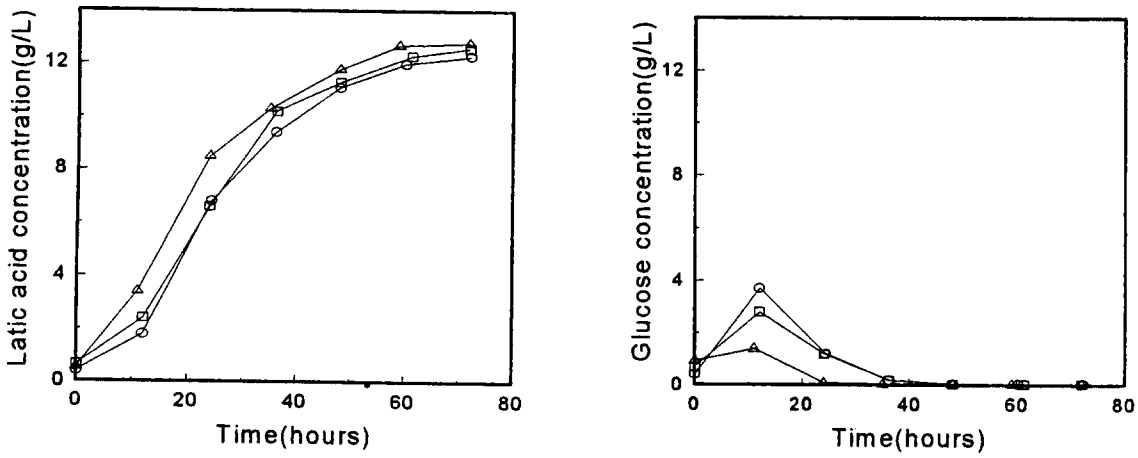

Fig. 6. Effect of yeast extract loading in SSF of α-cellulose. (SSF conditions: pH 5.0, temperature 46°C, α-cellulose 12.5 g/L, enzyme 45 IFPU/g cellulose, yeast extract loading: ○; 2.5 g/L, □; 12.5 g/L, △; 30 g/L).

concentration. However, the final lactic acid formation was very close with each other. Therefore, low concentration of 2.5 g/L was recommended for the SSF.

Lactic Acid Inhibition

Organic acids, depending on whether they are in an ionic form or undissociated form, have different inhibitory effects on the growth of the microorganism. It was reported that the specific growth rate decreased from 0.41 to 0.02 h^{-1} when free lactic acid (undissociated one) concentration increased from 0.4 to 6.0 g/L, and lactate (dissociated one) had little effect on lactic acid fermentation [9].

The effect of lactic acid on the SSF was investigated. The lactic acid concentration was analyzed by HPLC. The analytic result gave the total amount of lactic acid (free lactic acid and lactate). The amount of free lactic acid and lactate were then calculated from pKa of lactic acid (3.86) at a given pH level. Fig. 7 shows an SSF run with initial input of 64 g/L lactic acid. The pH was controlled at 5.0. The amount of free lactic acid accounts for 7% (4.5 g/L) of total lactic acid in the SSF broth. During the SSF, glucose was accumulated, and no lactic acid was produced. This result indicates that lactic acid severely inhibited the growth of microorganism. The enzymatic digestibility of α-cellulose was normally more than 80% after 72 hours hydrolysis. The yield of glucose formation after 72 hours in this SSF was only 40% based on initial α-cellulose input. Therefore, lactic acid was not only an inhibitor of cell activity, but inhibited the enzyme activity as well. A product separation process is strongly recommended for the SSF.

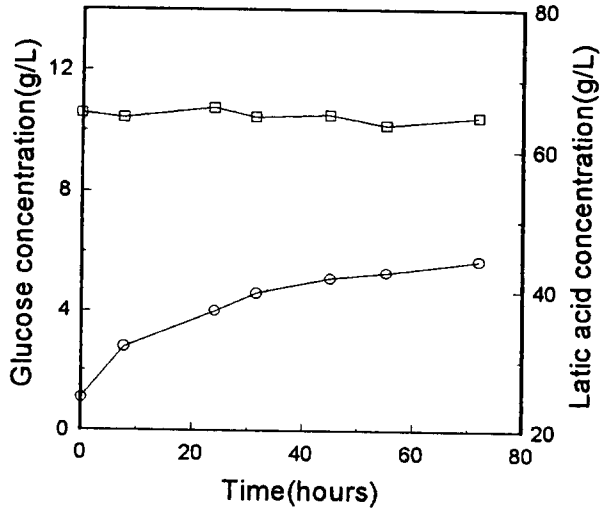

Fig. 7. Lactic acid inhibition on SSF of α-cellulose. (SSF conditions: initial latial latic acid input 64 g/L (free lactic acid 3.8 g/L), temperature 46°C, pH 5.0, α-cellulose 12.5 g/L, enzyme 45 IFPU/g cellulose, symbol: ○; glucose, □; lactic acid).

CONCLUSIONS

A process termed simultaneous saccharification and fermentation (SSF) using α-cellulose for lactic acid production was demonstrated. Cellulose was converted efficiently to lactic acid, and the enzymatic hydrolysis was the rate controlling step in the SSF. The highest conversion rate was obtained at 46°C and pH 5.0. The observed yield of lactic acid from α-cellulose was 0.90 at 72 hours. The optimum pH of the SSF was coincident with that of enzymatic hydrolysis. The optimum temperature of the SSF can be chosen as the highest temperature the microorganism can withstand. The optimum yeast extract loading was found to be 2.5 g/L. Lactic acid was observed to be inhibitory to the microorganisms' activity.

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