Effective D-lactic acid production from corncobs by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*

Jun-ichi Horiuchi, * *Syuka Naito, Yoichi Kumada, Kenji Okano, Akihiko Kondo and Tsutomu Tanaka*

Prof. J. Horiuchi, * S. Naito and Prof. Y. Kumada

Department of Functional Chemistry, Kyoto Institute of Technology, Hashigami-cho, Matsugasaki, Sakyo-ku, Kyoto, 606-8585, Japan

E-mail: horiuchi@kit.ac.jp

Prof. K. Okano
International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan

Prof. T. Tanaka
Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

Prof. A. Kondo
Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

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A metabolically engineered *Lactobacillus plantarum* mutant, which could produce D-lactic acid from both glucose and xylose, was applied for the production of optically pure D-lactic acid from corncobs by simultaneous saccharification and fermentation (SSF). Using a corncob hydrolysate obtained by a combination of dilute acid treatment using 1.5% H_2SO_4 followed by enzymatic saccharification, the *L. plantarum mutant* completely assimilated both glucose and xylose in the corncob hydrolysate within 20 hours, resulting in the successful production of D-lactic acid with high optical purity in a batch culture. To improve the performance of D-lactic acid production from corncobs, SSF experiments from 100 to 250 g/L of acid-hydrolyzed corncobs using 1.5% H_2SO_4 were performed, and 49.7 to 101 g/L of D-lactic acid with 96.8-98.6% of optical purity was successfully produced. The D-lactic acid yield from corncobs ($Y_{L/C}$) was approx. 0.61 when 100-150 g/L of acid-hydrolyzed corncobs was used; however, the $Y_{L/C}$ decreased to 0.49 as the concentration of acid-hydrolyzed corncobs because of insufficient acid hydrolysis of the corncobs. Therefore, by increasing the H_2SO_4 concentration to 3.5%, D-lactic acid production from corncobs significantly increased to 134 g/L with $Y_{L/C}$ of 0.63 and 2.88 g/(L·h) of productivity from 250 g/L of acid-hydrolyzed corncobs.

1. Introduction.

Biotechnological production of various chemicals from renewable resources such as lignocellulosic biomass containing hemicellulose and cellulose has drawn much attention from industries because it has the potential to realize the cheaper production of chemicals with low environmental impact.[1] Among the various chemicals produced from renewable resources, lactic acid has been recognized as a valuable monomer chemical not only in the food and pharmaceutical industries but also in chemical industries that produce biodegradable plastics such as poly-lactic acid (PLA)[2]. PLA has been mainly produced from L-lactic acid to date. [3] However, it was recently reported that a stereocomplex PLA consisting of poly L-lactic acid (PLLA) and poly D-lactic acid (PDLA) had greater thermostability than PLA;[4] this has led to an increase in the demand for D-lactic acid. [5]

In response to these circumstances, D-lactic acid production from renewable resources has been extensively studied. [6] Several lactic acid bacteria, such as *Lactobacillus delbrueckii* produce pure D-lactic acid; [7, 8] however, most of them produce D-lactic acid from glucose and cannot effectively assimilate xylose, which is the main pentose obtained from the hemicellulosic content of various lignocellulosic biomasses. A novel homo-lactic acid bacterium that produces L-lactic acid from xylose was isolated recently; [23] however, there are few reports on lactic acid bacteria producing optically pure D-lactic acid from xylose. To effectively utilize lignocellulosic biomass for chemicals production, and to minimize the discharge of residual wastes during D-lactic acid production process, it is extremely important to use both hemicellulose and cellulose because they constitute more than 20 - 30% of the lignocellulosic biomass, respectively. Therefore, we metabolically engineered *Lactobacillus plantarum* [9, 10, 12], to produce optically pure D-lactic acid from both glucose and xylose, and applied it to D-lactic acid production from starch, rice bran and brown rice. [11, 13]

However, the D-lactic acid production from lignocellulosic biomass composed of hemicellulose, cellulose and lignin has not been sufficiently investigated. [14]

Among the various lignocellulosic feedstocks applicable for biological production of chemicals, corncobs are regarded as a promising agricultural resource for D-lactic acid production because huge amounts of such cobs, which are disposed in corn processing industries, are available worldwide and are rich in hemicellulose and cellulose. However, corncobs have not been effectively utilized thus far, although they are partially used in animal feed and mushroom cultivation media, and mostly returned to farmlands as fertilizer. [15] We have previously reported microbial xylitol production using *Candida magnoliae* from xylose obtained by dilute sulfuric acid treatment of corncobs, wherein the hydrolysis conditions of the cobs and the removal of inhibitors generated during acid hydrolysis were investigated. [16, 17]

For D-lactic acid production from corncobs using metabolically engineered *L. plantarum*, the hemicellulose and cellulose contained in the cobs need to be hydrolyzed to xylose and glucose with high yield, respectively. Since the hydrolysis and saccharification of hemicellulose and cellulose are more difficult than those of biomass containing starch or mono-sugars, it is important to develop an efficient hydrolysis process for D-lactic acid production from lignocellulosic biomass. [18, 19] To obtain xylose from corncobs effectively, we previously applied dilute acid treatment using sulfuric acid. Sulfuric acid hydrolysis decomposed hemicellulose into xylose with a high yield. [16, 17] It is well known that enzymatic saccharification using cellulase is effective for the hydrolysis of cellulose to produce glucose. [18, 19] On the other hand, it has also been pointed out that the enzymatic saccharification of cellulose is extremely time-consuming; this diminishes the process performance, including the productivity for D-lactic acid production. To overcome this

shortcoming, simultaneous saccharification and fermentation (SSF), which combines enzymatic hydrolysis with fermentation in one bioreactor, has been developed and widely applied in various fermentation processes. [20] Since SSF allows the simultaneous fermentation along with the hydrolysis of corncobs, the application of SSF is considered useful for the effective production of D-lactic acid from corncobs.

In this study, to examine the possibility of producing optically pure D-lactic acid effectively from corncobs using metabolically engineered *L. plantarum,* we performed a batch experiment using a corncob hydrolysate medium containing glucose and xylose, and SSF experiments using acid hydrolyzed corncobs which mainly contains xylose and cellulose,. The performance of the SSF using the *L. plantarum* mutant was then evaluated in terms of D-lactic acid concentration, optical purity, productivity and yield.

2. Experimental Section

Microorganism and culture media: A metabolically engineered *L. plantarum mutant* (NCIMB 8826 *Δldhl1::PxylAB-xpk1::tkt-Δxpk2::PxylAB*) was used in this study. [12] Figure 1 illustrates the main metabolic routes for D-lactic acid biosynthesis in this strain. Briefly, to achieve D-lactic acid production from xylose, the *ldhL1* gene for L-lacitic acid production was replaced with the *xylAB* operon consisting of xylose isomerase and xylulokinase genes. Deletion of the *ldhL1* gene induced production of optically pure D-lactic acid, whereas introduction of the *xylAB* operon enabled xylose assimilation. In addition, by replacing the phosphoketolase gene 1 (*xpk1*) with the transketolase gene *(tkt*) and the phosphoketolase gene 2 (*xpk2*) with the additional *xylAB* operon, acetic acid production was almost terminated by redirecting the PK pahtway to the PP pathway and xylose assimilation was further enhanced. It has been reported that this strain can produce optically pure D-lactic acid from glucose and xylose.[12] Supplemental figures (Figure S1) shows the difference in batch

cutivations between the original strain and the *L. plantarum mutant* used in this study with respect to D-lactic acid production from xylose and glucose. De Man-Rogosa-Sharpe (MRS) medium, [24] which had the following composition (per liter of demineralized water), was used; glucose 20 g/L, pepton 10.0g, meat extract 10.0g, yeast extract 5.0g, $(NH_4)_2(CO_3) \cdot H_2O$ 4.0g, K_2HPO_4 2.0g, CH_3COONa 5.0g, diammonium hydrogen citrate 2.0g, $MgSO_4 \cdot 7H_2O$ 0.2g, and $MnSO_4 \cdot nH_2O$ 0.05g. These components excluding glucose and CH_3COONa were added to the corncob hydrolysate media for batch and SSF experiments. The strain was stored at -80 °C as frozen glycerol stocks containing MRS medium and 25% glycerol. One milliliter of the glycerol stock was melted moderately and transferred to a 500-mL flask containing 100mL MRS medium. The culture was incubated at 37 °C for 24h on a reciprocating shaker (70 stokes/min), and the flask culture broth was used to seed the main culture.

Feedstock and hydrolysis: Dried corncobs, with a particle size smaller than 5mm, purchased from China, were used as feedstock for D-lactic acid production in this study. The average composition of the corncobs used in this study was approx. 32% cellulose, 35% hemicellulose, 20% lignin, 4% ash, and others. The water content of the corncobs was analyzed using a moisture analyzer (ML-50, A&D Co. Ltd., Japan). Various amounts of corncobs were mixed with 1.0 L of dilute sulfuric acid in a 5 L flask and steam-autoclaved at 121°C for 60-80 min. Therefore, the concentration of corncobs (g/L) are expressed as weight (g) of corncobs over volume (L) of dilute sulfuric acid. The concentration of sulfuric acid was varied from 1.0% to 3.5% as the concentration of corncobs was increased. After the acid hydrolysis, pH of the acid hydrolyzed corncobs was adjusted to 5.0 using 10M NaOH. The acid-hydrolyzed corncobs were then supplemented with the components of MRS medium excluding glucose and CH_3COONa and used for SSF experiments.

Enzyme: Meicelase (Meiji corp., Japan); activity: 518 FPU/g-enzyme, which contains cellulases obtained from *Trichoderma viride,* was used for saccharification of corncobs in this study. Meicelase powder (0.05 g) was dosed to 1.0 g-corncobs (corresponding to 25.9 FPU/g-corncobs) for saccharification. Meicelase powder contains sugars as stabilizers for enzymes, accordingly, approx. 0.4 g of lactic acid can be produced from 1 g of Meicelase powder. Time course data of D-lactic acid concentration in this study included lactic acids generated from Meicelase. D-lactic acid generated from Meicelase was excluded in the calculation of total D-lactic acid production.

Analytical methods: The concentration of D/L-lactic acid was analyzed by an enzymatic method (F-kit for D/L-lactic acid, J.K. International Inc., Japan). Xylose, glucose, and acetic acid concentrations were determined by HPLC using a Shodex SUGAR SH1821 column (8.0 mm I.D.×300 mm; Showa Denko K.K., Tokyo). The absorbance at 280nm (A_{280}) was employed as an index of the inhibitors including furfural and 5-HMF in the corncob hydrolysates because A_{280} value has been widely used as an index of phenolic compounds such as furfural which is known as the main inhibitory byproduct in chemically hydrolyzed biomass solutions. Cell concentration was determined by measuring the optical density at 660 nm (OD_{660}). All samples were analyzed in duplicate.

Calculation of optical purity, productivity and yield from corncobs of D-lactic acid:

The optical purity (enantiomeric excess, ee) of D-lactic acid was determined according to the following equation;

$$ee = \frac{DLA - LLA}{DLA + LLA} \times 100(\%)$$

where, *DLA* and *LLA* denote the concentrations of D-lactic acid and L-lactic acid, respectively.

The productivity of D-lactic acid (PD) was calculated using the following equation;

$$PD = \frac{DLA}{Fermentation\,time}\left(\frac{g}{L \cdot h}\right)$$

Fermentation time (h) is the culture time from the beginning of fermentation to the culture time when the increase in D-lactic acid concentration terminated.

The yield of D-lactic acid over corncobs ($Y_{L/C}$) was calculated using the following equation;

$$Y_{\frac{L}{C}} = \frac{Final\,DLA \times Final\,volume\,of\,liquid\,fraction}{Dry\,weight\,of\,corn\,cobs}$$ ¿

Batch experiments using corncob hydrolysate medium containing glucose and xylose:

To prepare a corncob hydrolysate medium containing xylose and glucose for batch experiments, a combination of dilute sulfuric acid hydrolysis and enzymatic saccharification of corncobs was employed. Flow chart for preparation of corncob hydrolysate was shown in Figure S2 as supplemental information. One hundred grams of corncobs (water content: 6.3%) were mixed with 1.0 L of 1.5 % diluted sulfuric acid in a 5 L flask and were hydrolyzed at 121°C for 60 min. using an autoclave. After acid hydrolysis, the pH of the acid-hydrolyzed corncobs was adjusted to 5.0 by the addition of 10M NaOH and enzymatic saccharification was carried out for 60 hours at 50 °C on a shaker (100 rpm) after adding 5 g of Meicelace to the 1.0 L of the acid hydrolyzed corncobs. After saccharification, the hydrolysate was filtered using cotton gauze. To remove the inhibitors generated during the acid hydrolysis of the corncobs, the filtered hydrolysate was treated with 30g of activated carbon (Shirasagi M, Osaka Gas Chemicals Co., Ltd., Japan), which is commercially used for the de-colorization of foods and sugars. After adsorption treatment with activated carbon, the A_{280} of the hydrolysate decreased to 2.0. Finally, the components of the MRS medium were

184 added to the hydrolysate, which was then used for batch experiments. A computer-controlled
185 2 L jar fermenter with a 1 L working volume (BMZ-02NP3, Biott Corp., Japan) was used
186 for batch experiments. One hundred milliliters of the pre-culture were added to 1.0 L of
187 hydrolysate medium in the fermenter, and the agitation rate and temperature were
188 automatically controlled at 300 rpm and 37 °C, respectively. The culture pH was maintained
189 at 6.0, using 10 M NaOH solution.

190 *Simultaneous saccharification and fermentation using acid hydrolyzed corncobs*:

191 Acid-hydrolyzed corncobs prepared under various H_2SO_4 and corncobs concentrations were
192 employed for SSF experiments with the same jar-fermenter. The SSF experiments were
193 initiated by simultaneously adding 25.9 FPU (0.05g) /g-corncobs of Meicelase and 100 mL
194 of the pre-culture to the fermenter, and the temperature and agitation rate were automatically
195 controlled at 37 °C and 200 or 400 rpm, respectively. The culture pH were automatically
196 controlled at 6.0, using 10M NaOH. Since it has been reported that the formation of calcium
197 lactate effectively suppresses product inhibition by decreasing the concentration of lactic
198 acid, [25] $CaCO_3$ was used as a neutralizing agent for the pH control of SSF instead of NaOH
199 when 250 g/L of acid-hydrolyzed corncobs was used. Based on the stoichiometric
200 calculations, 120 g of $CaCO_3$ was added to the acid-hydrolyzed corncobs prepared from 250
201 g/L (w/v) corncobs. Since the increase in H_2SO_4 and corncobs concentrations resulted in an
202 increase in A_{280} which seriously inhibited the growth of lactic acid bacteria, 15g of activated
203 carbon was added to the acid hydrolyzed corncobs to remove inhibitors such as furfural and
204 hydroxymethylfurfural. Upon the addition of 15g of activated carbon to 1.0 L of acid
205 hydrolyzed corncobs, the A_{280} of the liquid fraction of the acid-hydrolyzed corncobs
206 decreased to less than 80.

207

3. Results

3.1. D-Lactic acid production using corncob hydrolysate medium containing xylose and glucose

To evaluate the possibility of using metabolically engineered *L. plantarum* for D-lactic acid production from corncobs, batch experiments using a corncob hydrolysate medium containing glucose and xylose were performed. The corncob hydrolysate medium contained 25.2 g/L of glucose and 22.4 g/L of xylose was obtained from 100 g/L (w/v) of corncobs using a combination of dilute sulfuric acid hydrolysis with enzymatic saccharification. A_{280} of the hydrolysate after acid hydrolysis was approx. 100. The glucose and xylose yields from corncobs was approx. 0.25g-glucose/g-corncobs and 0.22g-xylose/g-corncobs, respectively. The results are shown in Figure 2. Glucose was firstly consumed and then xylose was smoothly assimilated. Xylose assimilation was delayed to the consumption of glucose; however, clear catabolite repression was not observed as previously reported. [13] Both glucose and xylose in the corncob hydrolysate medium were completely utilized within 20 hours, resulting in the successful production of 43.5 g/L of lactic acid from 47.6 g/L of total sugars, which corresponds to 0.91 of D-lactic acid yield from sugars in the corncob hydrolysate medium. Accordingly, the D-lactic acid yield from corncob ($Y_{L/C}$) was 0.51 g-D-lactic acid/g-dried corncob. Since the concentration of L-lactic acid was 0.89 g/L, the optical purity of D-lactic acid was 96.1 % which is sufficiently high for PDLA production. Thus, metabolically engineered *L. plantarum* could effectively produce optically pure D-lactic acid from a corncob hydrolysate medium containing glucose and xylose.

3.2. Simultaneous saccharification and fermentation for D-lactic acid production using acid hydrolyzed corncobs

231 SSF experiments using acid-hydrolyzed corncobs were performed to develop an effective
232 process for D-lactic acid production using the metabolically engineered *L. plantarum*. Table
233 1 summarizes the results of analysis of the liquid fraction in the acid-hydrolyzed corncobs
234 employed for the SSF experiments. The concentrations of corncobs and H_2SO_4 were varied
235 from 100 to 250 g/L and from 1.5% to 3.5%, respectively. The concentration of glucose
236 ranged from 3.4 to 5.1 g/L and was relatively low considering the cellulose content of the
237 corncobs (approx. 35%). On the other hand, the concentration of xylose increased from 22.0
238 g/L to 45.6 g/L when the concentrations of corncobs and H_2SO_4 were increased. These results
239 show that the hemicellulosic content of the corncobs was mainly hydrolyzed to xylose by the
240 dilute acid treatment, whereas the cellulosic content remained unhydrolyzed. The weight of
241 residual corncobs after acid hydrolysis was decreased to 55-60% of that of the corncobs used
242 for hydrolysis. The acetic acid concentration was approx. 3.0 g/L when the concentration of
243 corncobs was 250 g/L.

244 Firstly, an SSF experiment using an acid-hydrolyzed corncob medium containing 3.1 g/L
245 glucose and 18.7 g/L xylose, which was obtained using 1.5% of H_2SO_4 from 100 g/L
246 corncobs, was conducted. The SSF experiment was initiated by inoculating 100 mL of pre-
247 culture with 5.2 g of Meicelase. Figure 3 shows the time courses of xylose, glucose, and D-
248 lactic acid concentrations in SSF experiment. The SSF using acid-hydrolyzed corncobs
249 successfully progressed and completed within 40 h, resulting in 45.2 g/L D-lactic acid
250 production. The concentration of glucose temporarily increased; however, it promptly
251 decreased as fermentation progressed. This indicated that the rate-limiting step of the SSF
252 was the hydrolysis of cellulose contained in the corncobs, discounting the initial lag phase of
253 *L. plantarum*. The utilization of xylose by *L. plantarum* mutant was also steady; however,
254 compared with the results in Figure 2, it should be noted that the assimilation of xylose was
255 seriously delayed. Since the final volume of culture increased to 1.35 L in this experiment

256 due to the addition of 100 mL inoculum and NaOH solution for neutralization and pH
257 control, the total D-lactic acid production was 59.0 g from 100 g-corncobs; accordingly, $Y_{L/C}$
258 became 0.59 g-D-lactic acid/g-dried corncobs in this experiment.

259 To increase the concentration of D-lactic acid produced in the SSF, the concentration of
260 corncobs was increased from 100 to 150, 200 and 250 g/L under the hydrolysis condition
261 using 1.5% H_2SO_4. Table 2 summarizes the results of SSF experiments. The final
262 concentrations of D-lactic acid successfully increased from 49.7 g/L using 100 g/L of acid-
263 hydrolyzed corncobs to 70.7, 88.4 and 101 g/L for 150, 200 and 250g/L of acid-hydrolyzed
264 corncobs, respectively. The $Y_{L/C}$ was approx. 0.61 g-D-lactic acid/g-dried corncob at 150g/L
265 of corncobs; this was sufficiently high. Figure 4 shows the time courses of the D-lactic acid
266 concentration during SSF for experimental run using 100, 150 and 250g/L of acid-
267 hydrolyzed corncobs. D-Lactic acid production increased with the concentration of acid-
268 hydrolyzed corncobs. Thus, the concentrated D-lactic acid production from corncobs was
269 achieved by SSF using metabolically engineered *L. plantarum*. Conversely, the $Y_{L/C}$
270 decreased to 0.49 g-D-lactic acid/g-dried corncobs when 250g/L of acid hydrolyzed corncobs
271 was applied. This could have been caused by insufficient acid hydrolysis of corncobs. This
272 insufficient acid hydrolysis probably affected the performance of the SSF because the
273 saccharification of cellulose by Meicelace was the rate-limiting step in the SSF of the acid-
274 hydrolyzed corncobs.

275 Therefore, to improve the hydrolysis of 250 g/L of corncobs, the concentration of H_2SO_4 for
276 acid-hydrolysis was increased to 2.5% and 3.5%, as indicated in Table 1. Figure 5 (A) shows
277 the time courses of xylose, glucose, and D-lactic acid concentrations in SSF experiment using
278 250 g/L of corncobs hydrolyzed with 2.5% H_2SO_4. The SSF using the acid-hydrolyzed
279 corncobs successfully progressed, resulting in an increase in the production of D-lactic acid

to 121.1 g/L. $Y_{L/C}$ was improved to 0.56 g-D-lactic acid/g-dried corncobs. Thus, the increase in H_2SO_4 concentration together with the use of activated carbon was effective in improving concentrated D-lactic acid production from corncobs by SSF using metabolically engineered *L. plantarum*. Based on this result, the H_2SO_4 concentration for acid hydrolysis was increased to 3.5% to further improve D-lactic acid production. Figure 5 (B) shows the time course of the SSF experiment using 250 g/L of corncobs hydrolyzed with 3.5% H_2SO_4. The increase in the H_2SO_4 concentration for hydrolysis, greatly enhanced the saccharification of cellulose by Meicelace, resulting in the highest D-lactic acid production of 134 g/L. As summarized in Table 2, $Y_{L/C}$ and productivity were also significantly improved to 0.63 and 2.89 g/(L·h) , respectively. The optical purity of the produced D-lactic acid was sufficiently high in all the experimental runs.

4. Discussion

In this study, optically pure D-lactic acid was produced from corncobs using a batch culture and SSF with metabolically engineered *L. plantarum*. The batch culture using a corncob hydrolysate medium containing glucose and xylose showed that the metabolically engineered *L. plantarum* could successfully metabolize both xylose and glucose and produce optically pure D-lactic acid. Compared with the results of a batch culture using synthetic MRS medium, shown in Figure S1(B), the assimilation rates of xylose and glucose were similar, although a corncob hydrolysate medium containing glucose and xylose was used. As described in the Introduction, it is important to use both hemicellulose and cellulose to effectively utilize lignocellulosic biomass such as corncobs; this strain was extremely useful for this purpose. In all the SSF experiments, the glucose concentrations remained low except at the initial stage, indicating that the saccharification of cellulose by Meicelase was the rate limiting step for D-lactic acid production from corncobs. On the other hand, compared with

304 the results in Figure 2, the assimilation rate of xylose was delayed in all the SSF experiments,
305 leading to a decrease in D-lactic acid productivity. Presently, it is not clear why the xylose
306 assimilation was delayed in the SSF experiments; however, there may be some explanations,
307 including the effects of inhibitors generated during acid hydrolysis, product inhibition under
308 high lactic acid concentration or the glucose effect caused by continuous saccharification of
309 cellulose in SSF. Further investigation of these aspects will help improve the process
310 performance of D-lactic acid production from lignocellulosic biomass.

311 To utilize both the hemicellulose and cellulose contained in corncobs, we employed the
312 dilute acid hydrolysis of corncobs using 1.5 - 3.5% sulfuric acid, based on our previous
313 studies. [16, 17] Using dilute acid hydrolysis, approximately 75-80 % of hemicellulose in
314 corncobs was recovered as xylose. The advantages of dilute acid hydrolysis are the selective
315 extraction of xylose from hemicellulose with a high yield and simple operation at low cost.
316 On the other hand, the glucose generation from corncobs by acid treatment was relatively
317 low, and was less than 10% of the cellulose contained in the corncobs being recovered;
318 however, the residual cellulose in the corncobs was successfully saccharified to glucose by
319 Meicelase. The saccharification of cellulose in 250 g/L of corncobs improved upon
320 increasing the concentration of sulfuric acid from 1.5% to 3.5%. This was probably caused
321 by the change in the crystalline structure of cellulose to an amorphous structure under the high
322 sulfuric acid concentration, which increased the surface area of cellulose accessible to
323 cellulase.

324 An SSF experiment using an acid-hydrolyzed corncob medium containing 3.1 g/L of
325 glucose and 18.7 g/L of xylose, which was obtained from 100 g/L of corncobs, was
326 successfully performed (Fig.3), resulting in 45.2 g/L of D-lactic acid production with $Y_{L/C}$ of
327 0.6 g-D-lactic acid/g-dried corncob. Upon increasing the corn cob concentration in the series

of SSF experiments, the concentration of D-lactic acid finally increased to 134 g/L when 250 g/L of corncobs hydrolyzed with 3.5% H_2SO_4 was used. There are many studies on the production of concentrated D-lactic acid from glucose or starch feedstocks; however, with respect to D-lactic acid production from lignocellulosic biomass containing hemicellulose and cellulose such as corncobs, the 134g/L of D-lactic acid concentration achieved in this study was extremely high. This result also shows that the host strain *L. plantarum* NCIMB 8826, can tolerate high lactic acid concentrations because the growth and lactic acid production of lactic acid bacteria was not seriously inhibited by more than 100g/L of lactic acid.

The maximum L-lactic acid concentration was 2.8 g/L (Table 2); accordingly, the optical purity of D-lactic acid was more than 95% (Table 2), which is sufficiently high for PDLA production.

Table 3 compares the results of this study with those of previous studies aimed at D-lactic acid production from lignocellulosic biomass by SSF. The D-lactic acid concentrations in previous studies [7, 21, 22] were limited to the range of 25 - 36.6 g/L since the strains used could utilize only glucose in lignocellulosic biomass. On the other hand, by using metabolically engineered *L. plantarum,* the concentration of D-lactic acid has been greatly increased to 102.3 g/L[13] and 137 g/L in this study.

A high D-lactic acid yields from corncobs ($Y_{L/C}$) 0.56-0.63 were obtained in this study, indicating that both xylose and glucose obtained from the cobs were successfully converted to D-lactic acid by the *L. plantarum* mutant during SSF. Notably, this corresponded that approx. 90% of the hemicellulose and cellulose contained in the corncobs were converted to D-lactic acid, because the average contents of hemicellulose and cellulose in the corncobs

were approx. 35% and 32%, respectively. This result shows that the use of acid hydrolysis as a pretreatment of corncobs promotes the effective utilization of hemicellulose and cellulose in the cobs and is advantageous for D-lactic acid production. If only the cellulose content in the cobs was available for D-lactic acid production, the maximum $Y_{L/C}$ will decrease to approx. 0.35. Thus, the addition of xylose assimilation pathway to *L. plantarum* significantly improved the $Y_{L/C}$, which would contribute to the reduction of wastes and waste waters discharged from production plant as well as the cost-effective production of D-lactic acid.

The productivity of D-lactic acid increased with the concentration of corncobs and a maximum productivity of 2.89 g/(L · h) was achieved when 250 g/L of corncobs hydrolyzed with 3.5% sulfuric acid was used. (Table2) Since the productivity increased significantly when 3.5% sulfuric acid was applied, the use of this higher sulfuric acid concentration enhanced the conversion of the crystalline structure of cellulose to amorphous cellulose. However, it should be noted that a high sulfuric concentration acid concentration for hydrolysis results in the excessive degradation of xylose, leading to a decrease in xylose concentration together with the generation of growth inhibitors such as furfural and 5-hydroxymethylfurfural (5-HMF).

In conclusion, we successfully applied metabolically engineered *L. plantarum* to optically pure D-lactic acid production from corncobs by SSF. An SSF experiment using 250 g/L of acid-hydrolyzed corncobs produced 134 g/L of D-lactic acid with a productivity of 2.88 g/L/h. By utilizing both xylose and glucose effectively, the D-lactic acid yield over corncobs increased to 0.56-0.63 which corresponds to a conversion of 90% of the hemicellulose and cellulose in the corncobs for D-lactic acid production. These results surpass those of previous studies. We believe this is a good application of metabolic engineering for the production of D-lactic acid from renewable resources, which will contribute to further progress in this field

to realize the cost-effective production of biodegradable plastics for the sustainable development of our society.

Conflict of Interest

All the authors declare that they have no conflict of interest.

Author Contributions

JH contributed to the study design and drafted the manuscript. SN performed the experiments. KO, KA and TT contributed the development of genetically engineered lactic acid bacteria. All the authors analyzed the data. All the authors contributed to revising the manuscript, and read, and approved the submitted version.

Keywords

D-lactic acid, simultaneous saccharification and fermentation, corncobs, metabolic engineering, *Lactobacillus plantarum*

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Table 1. Analysis of liquid fraction of acid-hydrolyzed corn cobs used for SFF experiments.

No.	Corn cobs conc. g/L (w/v)	H_2SO_4 conc. %	Glucose conc. g/L	Xylose conc. g/L
1	100	1.5	5.1±2.8	22.0±4.7
2	150	1.5	3.4±0.7	30.0±2.3
3	200	1.5	N/A	38.8
4	250	1.5	N/A	45.6
5	250	2.5	4.5	41.4
6	250	3.5	3.9±0.8	43.2±2.6

Results for No.1, 2 and 6 were shown as mean values ±SD, n=2.

20

Table 2. Results and performance of SSF for D-lactic acid production using corn cobs hydrolyzed under various cocetrations of H_2SO_4.

No.	Corn cobs conc.	H_2SO_4 conc.	D-lactic acid conc.	L-lactic acid conc.	D-lactic acid yield	Productivity	Optical purity
	g/L (w/v)	%	g/L	g/L	g-DLA/g-corncobs	g-DLA / (L· h)	%
1	100	1.5	49.7±2.9	077±0.61	0.56±0.07	1.62±0.15	96.8±2.47
2	150	1.5	70.7±4.5	0.96±0.35	061±0.0	1.53±0.03	97.8±0.2
3	200	1.5	88.4	0.75	0.53	1.76	98.0
4	250	1.5	101	0.74	0.49	1.71	98.6
5	250	2.5	121	2.8	0.56	2.11	95.4
6	250	3.5	134±13.4	2.8±0.01	0.63±0.07	2.89±0.01	95.6±0.42

Results for No.1, 2 and 6 were shown as mean values ±SD, n=2.

427

21

Table 3 Comparison of D-lactic acid production from lignocellulosic biomass by SSF

Biomass	Microorganism	Fermentation method	D-lactic acid	D-lactic acid yield	Productivity	Optical purity	Reference
			g/L	g-DLA/g-biomass	g-DLA / (L· h)	%	
pulp	*L. delbrueckii*	SSF	36.3	0.83	1.01	99.8	7
cellulose	*L. coryniformis*	SSF	approx. 25	0.89	0.5	N/A	21
dry microalga	*L. coryniformis*	SSF	36.6	0.458	2.38	95.8-99.6	22
hardwood pulp	*L. plantarum*	SSF	102.3	0.879 (g/g-sugar)	2.61	99.2	14
corn cobs	*L. plantarum*	SSF	134±13.4	0.63±0.07	2.89±0.01	95.6±0.42	This study

22

Figure captions

Figure 1. Metabolic pathways for D-lactic acid production and genetic modification in metabolically engineered *L. plantarum.*

Figure 2. Time courses of batch cultivation for D-lactic acid production using metabolically engineered *L. plantarum* with corn cob hydrolysate medium containing xylose and glucose. Symbols; ●, Glucose conc. (g/L); ▲, D-lactic acid conc. (g/L); ■, Xylose conc . (g/L); ○, L-lactic acid conc. (g/L)

Figure 3. Time courses of SSF for D-lactic acid production using metabolically engineered *L. plantarum* employing acid hydrolyzed corncobs.
Corncobs conc. was 100 g/L

Figure 4. Time course of D-lactic acid concentration during SSF of various concentrations of acid-hydrolyzed corncobs using metabolically engineered *L. plantarum*. Symbols: Corncobs conc., ▲,100 g/L; ■, 150 g/L; ●, 250 g/L.

Figure 5. Time courses of SSF for D-lactic acid production from 250 g/L of acid-hydrolyzed corncobs using metabolically engineered *L. plantarum*. (A) SSF experiment using corncobs hydrolyzed in 2.5% H_2SO_4 solution. (B) SSF experiment using corncobs hydrolyzed in 3.5% H_2SO_4 solution. Symbols: ●, Glucose conc. (g/L); ▲, D-Lactic acid conc. (g/L); ■, Xylose conc. (g/L).

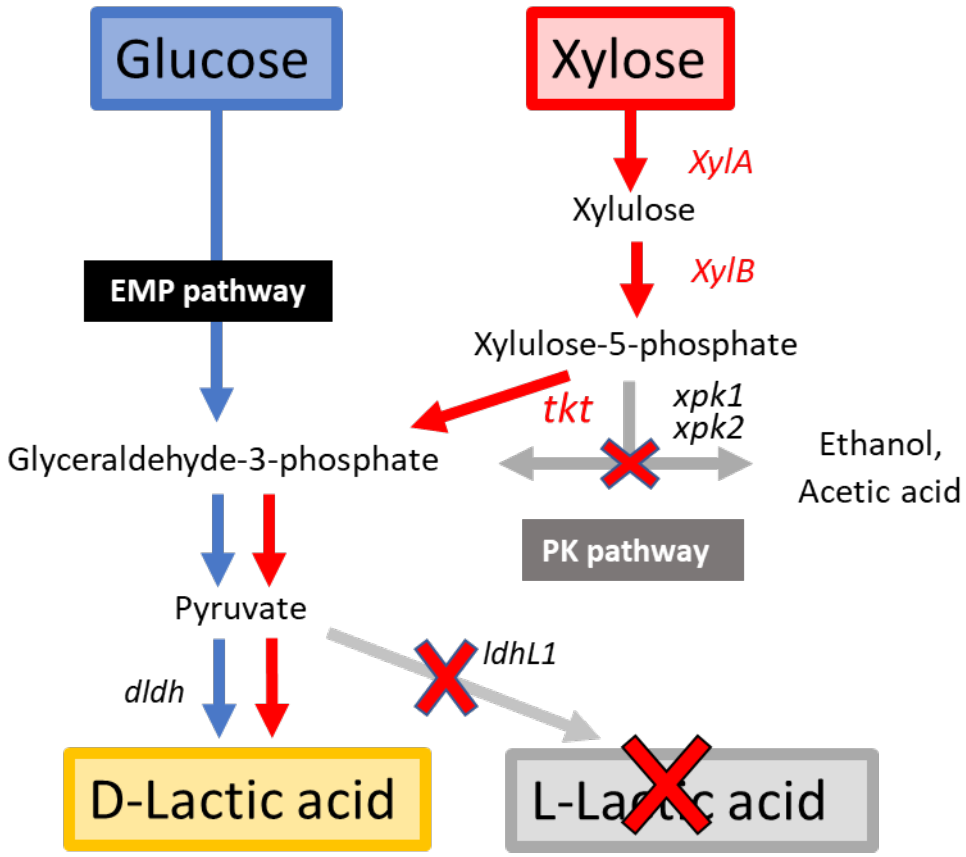

Figure 1. Metabolic pathways for D-lactic acid production and genetic modification in metabolically engineered *L. plantarum.*

24

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459

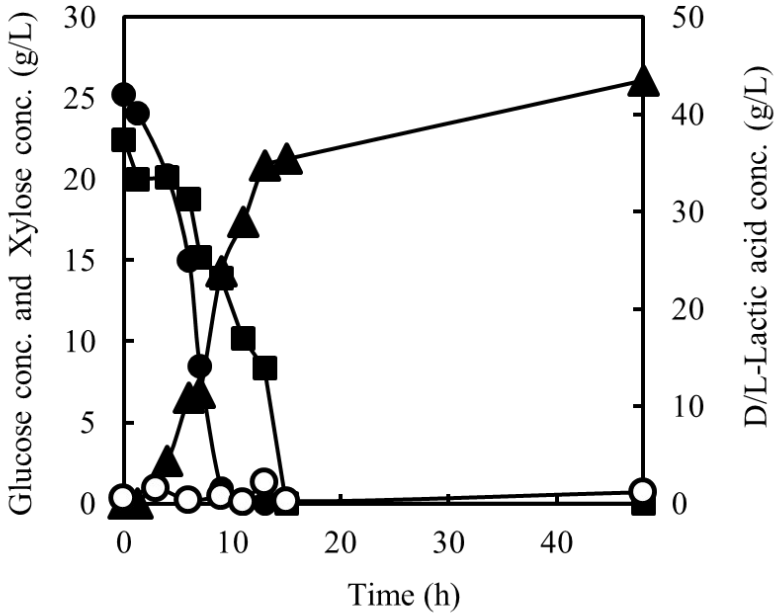

Figure 2. Time course of batch cultivation for D-lactic acid production using metabolically engineered *L. plantarum* with corncob hydrolysate medium containing xylose and glucose. Symbols: ●, Glucose conc. (g/L); ▲, D-Lactic acid conc. (g/L); ■, Xylose conc. (g/L); ○, L-Lactic acid conc. (g/L).

25

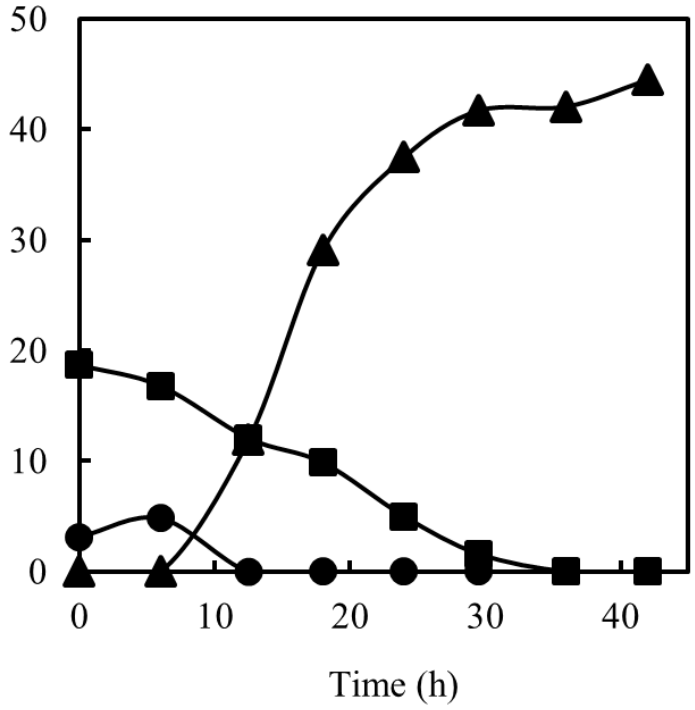

Figure 3. Time courses of SSF for D-lactic acid production using metabolically engineered *L. plantarum* employing acid hydrolyzed corn cobs. Corn cobs conc. was 100 g/L. Symbols; ●, Glucose conc. (g/L); ▲, D-lactic acid conc. (g/L); ■, Xylose conc . (g/L);

26

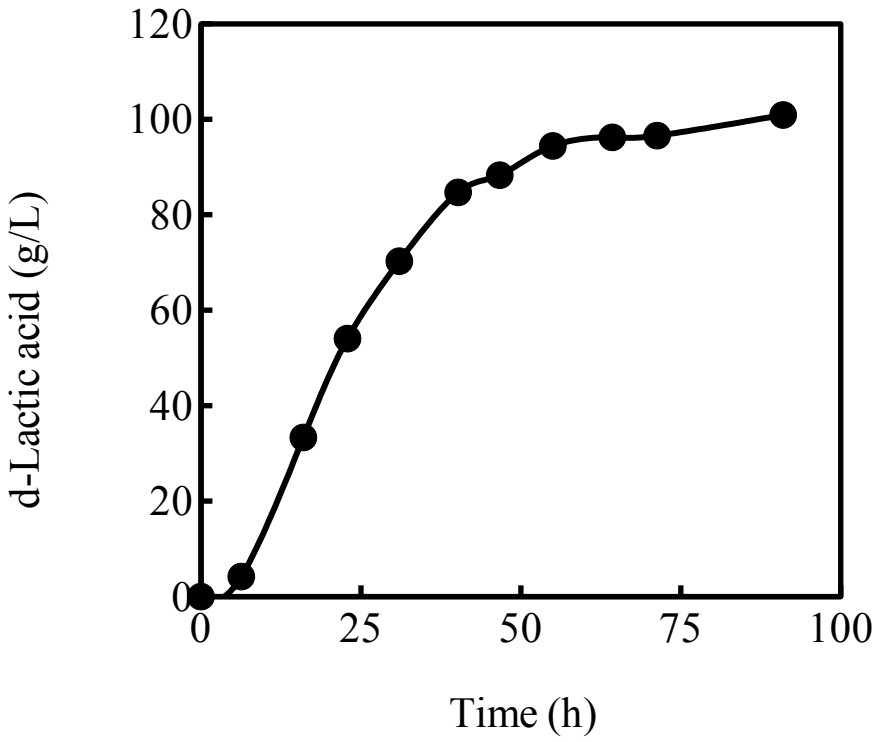

Figure 4. Time courses of D-lactic acid concentration during SSF using metabolically engineered *L. plantarum* employing acid hydrolyzed corn cobs with various concentrations. Symbols;Corn cobs conc., ▲,100 g/L, ■, 150 g/L, ●, 250 g/L;

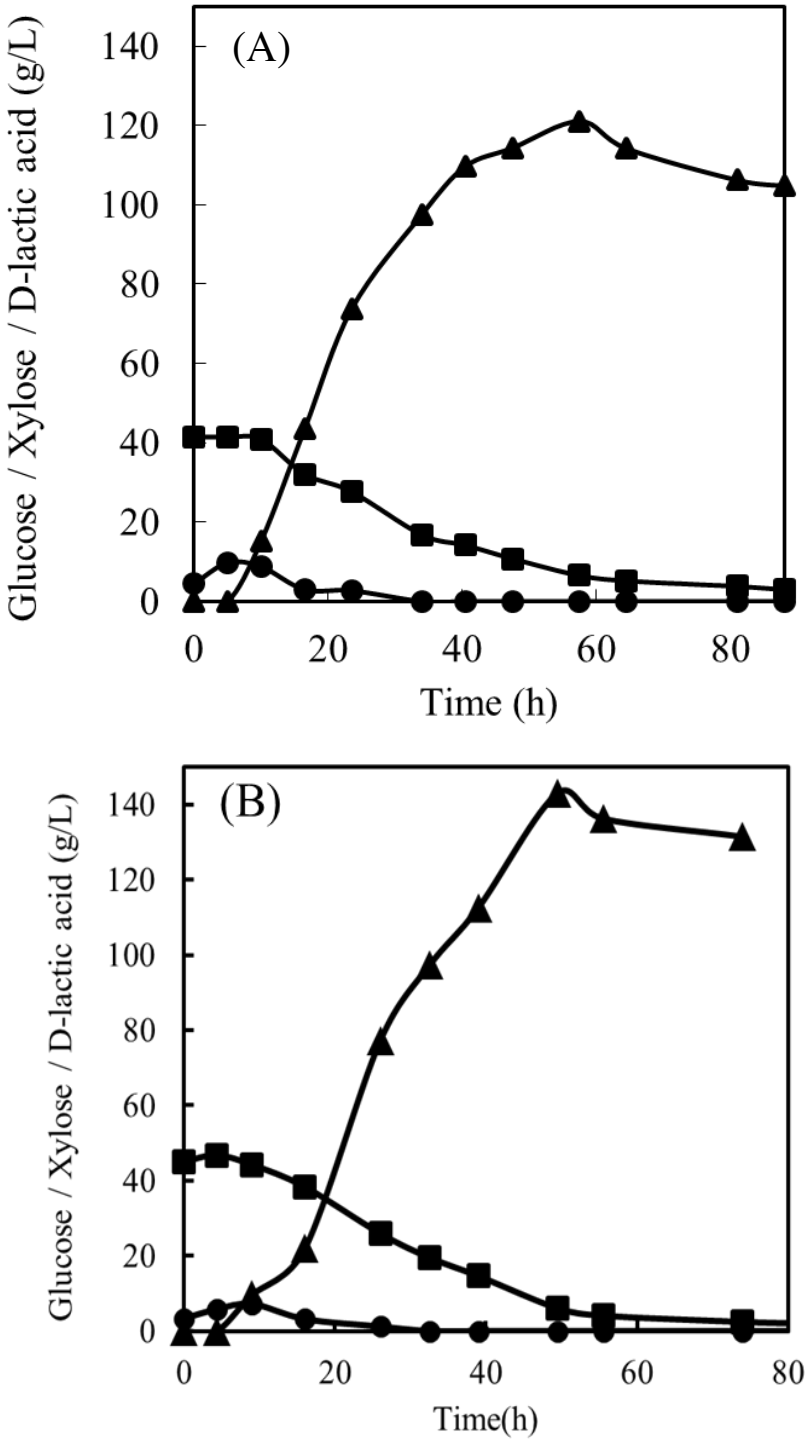

Figure 5. Time courses of SSF for D-lactic acid production using metabolically engineered *L. plantarum* employing 250g/L of acid hydrolyzed corn cobs. (A) SSF experiment using corn cobs hydrolyzed in 2.5 % of H_2SO_4 solution. (B) SSF experiment using corn cobs hydrolyzed in 3.5 % of H_2SO_4 solution. Symbols; ●, Glucose conc. (g/L); ▲, D-lactic acid conc. (g/L); ■, Xylose conc . (g/L);

28