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D-lactic acid biosynthesis from biomass-derived sugars *via Lactobacillus delbrueckii* fermentation

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Abstract Poly-lactic acid (PLA) derived from renewable resources is considered to be a good substitute for petroleum-based plastics. The number of poly L-lactic acid applications is increased by the introduction of a stereocomplex PLA, which consists of both poly-L and D-lactic acid and has a higher melting temperature. To date, several studies have explored the production of L-lactic acid, but information on biosynthesis of D-lactic acid is limited. Pulp and corn stover are abundant, renewable lignocellulosic materials that can be hydrolyzed to sugars and used in biosynthesis of D-lactic acid. In our study, saccharification of pulp and corn stover was done by cellulase CTec2 and sugars generated from hydrolysis were converted to D-lactic acid by a homofermentative strain, *L. delbrueckii,* through a sequential hydrolysis and fermentation process (SHF) and a simultaneous saccharification and fermentation process (SSF). 36.3 g L^{-1} of D-lactic acid with 99.8% optical purity was obtained in the batch fermentation of pulp and attained highest yield and productivity of 0.83 g g^{-1} and 1.01 g L^{-1} h^{-1}, respectively. Luedeking-Piret model described the mixed growth-associated production of D-lactic acid with a maximum specific growth rate 0.2 h^{-1} and product formation rate 0.026 h^{-1} ,obtained for this strain. The efficient synthesis of D-lactic acid having high optical purity and melting point will lead to unique stereo-complex PLA with innovative applications in polymer industry.

Keywords D-lactic acid, fermentation, corn stover, pulp, biosynthesis

List of symbols

μ_{max} Maximum specific growth rate (h^{-1})

C_0 Initial glucose concentration (g L^{-1})

C_p Product concentration (g L^{-1})

Y_{PS} Product yield (g lactic acid g^{-1} glucose)

Y'_{PS} Product overall yield (g lactic acid g^{-1} biomass)

Y_{XS} Yield of cell dry mass from substrate (g cell dry mass g^{-1} glucose)

Y_{PX} Yield of product from cell dry mass (g D-lactic acid g^{-1} cell dry mass)

q_{PS} Product formation rate (h^{-1}) calculated based on the equation $q_{PS=}\frac{1}{S}\times\frac{dP}{dt}$

Q_p Productivity (g L^{-1} h^{-1})

Introduction

Lignocellulosic biomass is gaining importance as a potential source of renewable energy and chemicals as the fossil fuel reserves are eventually getting depleted. Demand continues to increase for production of high-value chemicals and materials from renewable resources to attain domestic self-sufficiency and enhanced national security. Lactic acid is an important and multifunctional organic acid that has wide applications in the food, pharmaceutical, and chemical industries [1, 2]. It exists in two optical isomeric forms, L (+) and D (-) poly-lactic acid (PLA), which are being developed as a substitute for petroleum-derived plastics. The high chemical resistance of poly lactic acid is advantageous in the manufacture of fibers, nonwoven fabrics, and films [3]; however, the application of poly L-lactic acid (PLLA) is limited by its melting point [4]. This problem can be obviated by blending it with poly D-lactic acid (PDLA). The melting point of the resulting stereocomplex polymer is approximately 50 °C higher than that of the respective single polymers [5]. The optical purity of lactic acid accentuates the physical properties of poly D-lactic acid-based polymers [6]. The chemical process of making lactic acids usually yields a mixture of these two enantiomers, which is an undesirable feature; therefore, the biological process of making pure lactic acid is preferred [7].

To date, intense studies have been conducted on the production of L-lactic acid from different biomass through microbial fermentation [8-11], but information on biosynthesis of D-lactic acid from biomass is limited. A few wild-type strains such as *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Sporolactobacillus inulinus* [12], *Lactobacillus coryniformis* subsp. *torquens* [13], and *Lactobacillus delbrueckii* subsp. *lactis* QU41 [14]

have been identified as D-lactic acid producers. Traditional production of lactic acids typically uses starch derived from food crops as the fermentation substrate [12, 15], but this process may affect the global food supply. Lignocellulosic materials are favorably structured to produce lactic acids, which require the breakdown of cellulose to sugars [16]. This step usually can be done by acid hydrolysis and enzymatic hydrolysis. The enzymatic hydrolysis method is preferred, because it can be done under mild reaction conditions avoiding the use of toxic and corrosive chemicals [17]. The hydrolysis and fermentation steps can be done sequentially (SHF) or simultaneously (SSF). The SSF process offers better yields because it avoids product inhibition and results in higher productivity [18, 19].

Production of D-lactic acid from cardboard [20, 21], cellulose [13], peanut meal [22], and rice bran [3] has been studied. Other sources include pulp and corn stover, which have the potential to become cheap and abundant sources for production of ethanol, organic acids, and other chemicals [7, 21]. Pulp is prepared by chemically or mechanically separating cellulose fibers from wood, fiber crops, or waste paper [23]. Corn stover, which includes the leaves, stalks, and cobs of corn plant, is the most abundant agricultural residue in the U.S. [24]; to the best of our knowledge, no research has been reported on D-lactic acid fermentation via pulp and corn stover as substrates.

The purpose of this study was to produce D-lactic acid with high yield and optical purity from pulp and corn stover by *lactobacillus delbrueckii* ATCC 9649. *L. delbrueckii* is a homofermentative lactic acid bacterium that can provide a continuous bioprocess with high volumetric productivity and optically high purity of D-lactic acid under anaerobic

conditions [25]. In addition, kinetic analyses of enzyme hydrolysis and fermentation of glucose to D- lactic acid also have been studied in this work.

Materials and methods

Raw materials and chemical treatment

Regular pulp and mechanically modified pulp were obtained from the MeadWestvaco's Crompton mill. Corn stover was obtained from fields in Manhattan and Tribune, Kansas. Alkali treatment was performed on corn stover before hydrolysis. Corn stover was suspended in 20 g L^{-1} NaOH and heated at 121 °C for 30 min in an autoclave (Tomy SS-325E, Tomy SEKO CO., LTD, Tokyo, Japan), then washed under running distilled water and filtered through muslin cloth until no color was visible in the wash water. The alkali-treated corn stover was dried at 80 °C for 24 h and ground to fine particle size in a laboratory mill (3303, Perten Instruments, Springfield, IL) for further enzymatic hydrolysis.

Enzyme hydrolysis

CTec2 (cellulase) obtained from Novozymes Inc. (Franklinton, NC) was used in this experiment. Enzyme hydrolysis assays were carried out at 45 °C in 250 mL screw capped plastic conical flasks with orbital agitation (150 rpm). The substrate concentration was 2% (w/v). pH was kept at 4.8 using 0.05 mol L^{-1} citric acid-sodium citrate buffer. The cellulase activity of CTec2 was measured by the filter paper assay [26], and the activity was expressed in terms of filter paper units (FPU). CTec2 was added on a dosage of 2, 4,

and 8 FPU g^{-1} of dry biomass, respectively. Product yield is based on the amount of glucose released divided by the amount of biomass consumed.

Microorganism and culture conditions

Lactobacillus delbrueckii ATCC 9649 obtained from the American Type Culture Collection (Manassas, VA) was used in this work. *L. delbrueckii* inoculum was prepared by growing cells in a 100 mL Wheaton serum bottle containing 50 mL of liquid MRS medium (MRS broth, Difco Laboratories, Detroit, MI) and incubated at 37 °C in a temperature-controlled shaker (Innova 4300, New Brunswick scientific, NJ) at 120 rpm for 15 h. CO_2 (3 vvm) was sparged into the bottle to create anaerobic growing conditions.

Sequential hydrolysis and fermentation (SHF)

Shake flask fermentation was modified according to the procedure described by Mukhopadhyay [27]. Fermentation was performed in 100 mL Wheaton serum bottles containing 50 mL of synthetic medium, pulp, modified pulp, or corn stover hydrolyzate, and lasted for 30 h. The synthetic medium consisted of 10 g L^{-1} of glucose, 10 g L^{-1} of peptone, 5 g L^{-1} of yeast extract, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of sodium acetate, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, 0.05 g L^{-1} of $MnSO_4.4H_2O$, and 1 g L^{-1} of Tween 80. Pulp, modified pulp, and corn stover hydrolyzate were supplemented with all the components (except glucose) of the synthetic medium. pH of the media was adjusted to 6.5 by 10 mol L^{-1} NaOH, and 3% (w/v) of calcium carbonate was added to control the pH. Temperature was maintained at 37 °C, and agitation was 120 rpm. Batch and fed-batch fermentation were performed in a 7 L fermenter with a working volume of 5 L (Bioflo 110, New Brunswick Scientific Inc.

Enfield, CT). In the batch fermentation experiment, paper pulp was added in quantity (270 g) that would possibly achieve a glucose concentration of 40 g L^{-1} in the medium. After hydrolysis, the pulp hydrolyzate was supplemented with all the components (except glucose) of the synthetic medium. The synthetic medium was used in fed-batch fermentation as a control. After 36 h, 1 L of fermentation medium was taken out and 1 L of feeding medium, which consisted of 40 g L^{-1} of glucose, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of sodium acetate, 2 g L^{-1} of ammonium citrate, 2 g L^{-1} of K_2HPO_4, 0.1 g L^{-1} of $MgSO_4.7H_2O$, and 0.05 g L^{-1} of $MnSO_4.4H_2O$, was added. During the fermentation, the temperature was maintained at 37 ºC; agitation speed at 100 rpm; and pH at 6.5. CO_2 was sparged at 3 vvm through the vessel to maintain anaerobic conditions.

Simultaneous saccharification and fermentation (SSF)

SSF process was modified according to the procedure described by Mukhopadhyay [27]The optimal temperature and pH for the enzymatic hydrolysis and the bacterial fermentation are different; In SSF, temperature was at 40 °C and pH was at 5.5, which were conducive for both enzymatic hydrolysis and bacterial activity. 2 g of dried pulp and corn stover was suspended in 50 ml 0.05 mol L^{-1} sodium citrate buffer (pH 5.5) with all the components (except glucose) of the synthetic medium. 3% (w/v)calcium carbonate was added to control the pH. CTec2 was added at 8 FPU g^{-1} of biomass, and *L.delbrueckii* was inoculated at 5% (v/v), and agitation rate was 150 rpm.

Analyses

Fermentation samples were centrifuged at 15,000×g for 10 min in an Eppendorf centrifuge (5415R, Eppendorf, Hauppauge, NY). The supernatant was collected in sample vials and stored at -4 °C for product and residue glucose analyses.

Sugars were quantified by a binary HPLC system (Shimadzu Scientific Instruments, Columbia, MD) equipped with a refractive Index detector (RID-10A) and phenomenex RPM monosaccharide column (300×7.8 mm, Phenomenex, Torrance, CA). Deionised water was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. The oven (Prominence CTD-20A) temperature was maintained at 80 ^{o}C.

Lactic acids were quantified by a Chirex Chiral column (150×4.6 mm, Phenomenex, Torrance, CA) with isocratic 1 mmol L^{-1} copper (II) sulfate mobile phase at 1 mL min^{-1}. Peaks were monitored using a UV detector at 254 nm (Shimadzu, PDA).

Results and discussion

Enzymatic hydrolysis

Experiments with different loads of cellulase were performed to determine a suitable enzyme loading for enzymatic hydrolysis of pulp, modified pulp, and alkali-treated corn stover. The maximum reaction rate (v_{max}) was calculated from the Michaelis-Menten equation ($v = \frac{v_{max}[S]}{K_m + [S]}$). v_{max} increased almost linearly with the increase of enzyme concentration in all three biomass cases (Fig. 1). The hydrolysis rate of corn stover and modified pulp was about to reach a plateau when the enzyme loading increased, perhaps due to substrate saturation [28]. Increased enzyme loading from 2 to 8 FPU g^{-1} of

substrate increased glucose yield by 24% after 48 h of pulp saccharification (Fig.2a); however, increasing the enzyme dosage did not significantly change the final glucose yield in the saccharification of mechanically modified pulp (12%) (Fig. 2b) and alkali treated corn stover (11%) (Fig. 2c). The highest glucose yield was observed at 24 h for mechanically modified pulp as well as corn stover. The initial saccharification rate of mechanically modified pulp and corn stover was higher than that of pulp. Mechanically modified pulp had finer fiber size, which made it much easier for the enzymes to break down. Alkali treatment caused the cellulose in corn stover to swell, which led to an increase in the internal surface area and a decrease in the degree of crystallinity of cellulose [29], therefore making cellulose in alkali-treated corn stover much easier for the enzyme to access.

Production of D-lactic acid by SHF

The purpose of this portion of the study was to produce D-lactic acid by *L. delbrueckii* using sugars derived from biomass as a cheap carbon source. We also tested another strain *Sporolactobacillus inulinus* ATCC 15538. Unlike in the results obtained by Fukushima *et al.* [12], *S. inulinus* produced L-lactic acid instead of D-lactic acid in our experiments. This result may be due to the difference in strain or the possible alternation of bacterial character after receiving it.

In shake flask fermentation, the amount of pulp (1 g), mechanically modified pulp (1.3 g), and corn stover (1.2 g) was set up to obtain 10 g L^{-1} glucose after enzymatic hydrolysis. No residual glucose was observed after 30 h fermentation, and the final pH of the medium was between 5 to 5.5. The optical purity of D-lactic acid was 99.9%. These

results were in close agreement with Demirci and Pometto [30]. The highest yield of D-lactic acid was observed in corn stover hydrolyzate (Table 1). Besides glucose, 5.6 g L^{-1} xylose and 1.7 g L^{-1} arabinose were also present in the corn stover hydrolyzate; however, xylose remained unused, and arabinose was below detectable levels at the end of fermentation. *L.delbrueckii* cannot use xylose due to the lack of xylose isomerase and xylulokinase, two key enzymes in xylose assimilation [31].

In fed-batch fermentation, almost all glucose was consumed within the first 36 h (first stage). In the second stage, feeding medium was added, and fermentation was completed within 80 h. The Luedeking-Piret equation ($\frac{1}{X}\frac{dP}{dt} = \alpha \frac{1}{X}\frac{dX}{dt} + \beta$) was used to describe the D-lactic acid production from synthetic medium in the first stage. Growth-associated constant (α) and non-growth associated constant (β) can be calculated from the graph of the specific production rate (q_p) versus the specific growth rate (μ); the correlation coefficient (R^2) was 0.88 (Fig. 3). Compared with other strains listed in Table 2, in our study *L. delbrueckii* had lower μ_{max} and higher α values. Lower μ_{max} suggests lower growth efficiency, and a high α value indicates a higher contribution of the cell growth to D-lactic acid production [32]. The value of α multiplied by μ_{max} was 1.56, which was larger than the β value, indicating that the specific growth rate played an important role in specific D-lactic acid production.

Figures 4 and 5 show the fermentation profile of the synthetic medium and pulp hydrolyzate, respectively. Table 3 summarizes the results of the first stage of fed-batch fermentation and batch fermentation. 37.4 g L^{-1} of D-lactic acid was obtained by the end of first-stage fermentation, and the product yield and productivity obtained were 0.93 g g^{-}

[1] and 1.04 g L^{-1} h^{-1}, respectively. These results were in agreement with other studies in literature [8, 33]. Fed-batch fermentation was completed within 80 h; at the end of fermentation, about 5.5 g L^{-1} glucose was left and up to 57.3 g L^{-1} D-lactic acid with optical purity of 99.8% was accumulated, which led to a productivity of 0.72 g L^{-1} h^{-1}. After pulp hydrolysis, the glucose concentration was 50 g L^{-1} and was used in the batch fermentation. After 30 h, glucose was hardly consumed, and even if we extended the fermentation time to 36 h, 6.2 g L^{-1} residual glucose remained. At the end of fermentation, 36.3 g L^{-1}lactic acid was produced, the yield of D-lactic acid was calculated by the amount of D-lactic acid produced divided by the amount of glucose consumed, which was 0.83 g g^{-1}, and productivity was 1.01 g L^{-1} h^{-1}. In a similar study undertaken in our laboratory, L-Lactic acid was synthesized from cheese whey and a yield (0.98 g g^{-1}) and productivity (1.14 g L^{-1} h^{-1}) was obtained [8]. The product formation rate of batch fermentation of pulp hydrolyzate was quite close to the product formation rate of first-stage fed-batch fermentation using the synthetic medium. The yield of D-lactic acid (0.83 g g^{-1}) from pulp hydrolyzate was lower than the first-stage yield (0.93 g g^{-1}) from synthetic medium. The reason might be due to substrate inhibition; therefore, the SSF process was preferred in subsequent experiments.

Production of D-lactic acid by SSF

After demonstrating the feasibility of producing D-lactic acid from biomass hydrolyzate in the batch process, SSF was carried out using pulp and corn stover in a shake flask. In SSF, samples were collected after 4 h of incubation; the profiles obtained for corn stover and pulp SSF experiments are shown in Figure 6.. In SSF, cellulose hydrolysis and

glucose assimilation were combined into a single fermentation process [34]. During the first 8 h, bacteria were in low activity and glucose accumulated to around 8 g L^{-1} and 14 g L^{-1} in the case of pulp and corn stover, respectively. After the first 8 h cultivation, glucose concentration was kept low, which indicated that bacterial cells were metabolically active during the entire course of the fermentation and also meant that enzymatic hydrolysis of cellulose was the rate limiting step for D-lactic acid production as already observed by other groups [35, 36]. Xylose accumulated and remained nearly constant throughout the process. It was impossible to know the exact amount of glucose consumed in the SSF process; therefore, in order to compare SSF and SHF, results were expressed as an overall yield (the amount of D-lactic acid produced divided by the amount of biomass used)(Table 1). The highest D-lactic acid overall yield was 0.48 and 0.38 g g^{-1} of pulp in SSF and SHF, respectively. For corn stover, the maximum D-lactic acid overall yield was 0.58 and 0.41 g g^{-1} in SSF and SHF, respectively, demonstrating that the SSF process was more efficient than the SHF process. The reason for the higher overall yield in SSF may be that glucose released during the hydrolysis step was rapidly consumed as substrate during the fermentation step, therefore reducing the end-product inhibition of hydrolysis [37].

Conclusions

In this study, we demonstrated efficient D-lactic acid production with high optical purity from pulp, modified pulp, and corn stover by *L. delbrueckii* ATCC 9649. Enzymatic hydrolysis of biomass was achieved effectively by CTec2 enzyme system. D-lactic acid productivity was not only high, but also cost-effective because pulp and modified pulp

need no pretreatment. The SSF process demonstrated the advantages of avoiding substrate inhibition and increasing the productivity and yield of D-lactic acid. The yield obtained in the present study would have been even higher if xylose from corn stover hydrolyzate could be completely used by the microorganism. Future study should be directed toward complete use of the available carbohydrate for efficient D-lactic acid production.

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Table 1 D-lactic acid production through SHF and SSF process in shake flask

		C_0[a]	C_P[b]	Y_{PS}[c]	Y'_{PS}[d]	Q_P[e]
SHF	Synthetic medium	10	7.7±0.05	0.77±0.01	---	0.25±0.01
	Pulp	9.7±0.17	7.5±0.47	0.77±0.66	0.38±0.02	0.25±0.03
	Modified pulp	11.2±0.09*	8.5±0.39	0.76±0.03	0.42±0.02	0.28±0.01
	Corn stover	9.9±0.05	8.3 ±0.04	0.83±0.01	0.41±0.01	0.27±0.01
SSF	Pulp	---	19.2±1.63*	---	0.48±0.04*	0.31±0.04
	Corn stover	---	20.1±0.65*	---	0.58±0.03*	0.32±0.07

Each mean is based on three replications ($p < 0.05$; REGWQ; one-way ANOVA)

[a] Initial glucose of modified pulp hydrolyzate was significantly different

[b] D-lactic acid concentration in SSF process was significantly different in SHF process

[c] Product yield was not significantly different in SHF process; product yield was calculated by the amount of D-lactic acid produced divided by the amount of glucose consumed.

[d] Product overall yield was significantly different between SSF and SHF; product overall yield was calculated by the amount of D-lactic acid produced divided by the amount of biomass used.

[e] Productivity was not significantly different.

Table 2 Kinetic parameters of different lactic acid bacteria

Microorganism	Substrate	μ_{max}	α	β
L. delbrueckii (this study)	Glucose	0.2	7.8	0.18
L. lactis [38]	Lactose	1.1	0.392	3.02
E. faecalis RKY1 [39]	Molasses	1.6	0.26	---
Lactobacillus helveticus [40]	Whey permeate	0.48	2.33	0.77

Table 3 Kinetic parameters of fed-batch and batch fermentation

	C_p	Y_{PS}	Y_{PX}	Y_{XS}	q_{PS}	Q_P
Fed-batch (stage I)	37.4	0.93	10.9	0.086	0.026	1.04
Pulp hydrolyzate batch	36.3	0.83	---	---	0.023	1.01

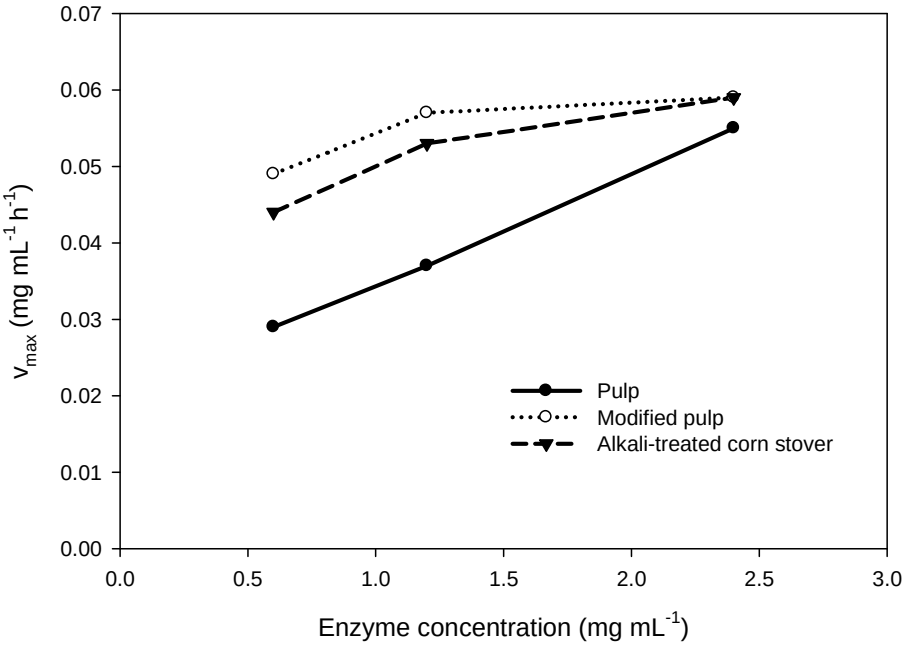
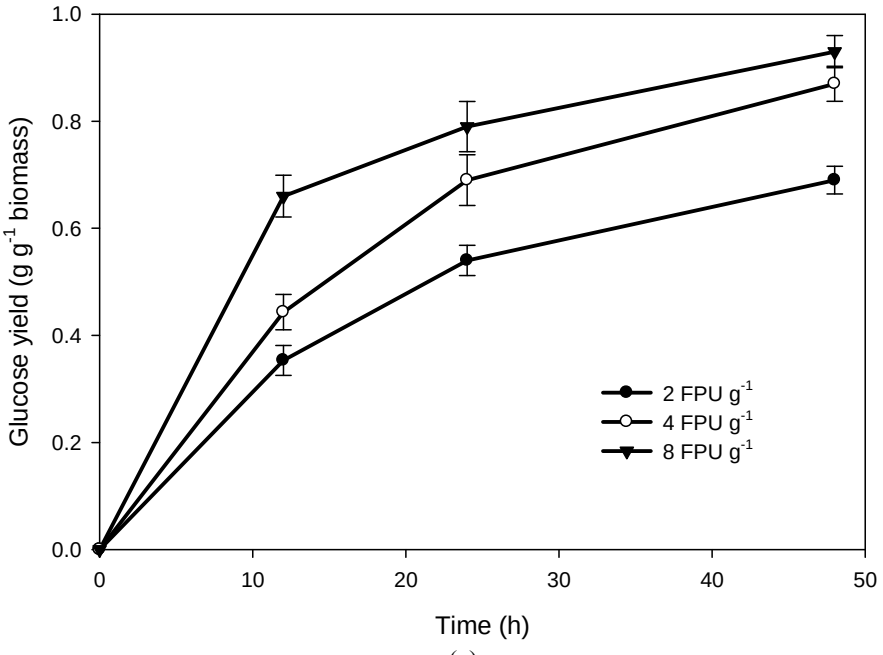
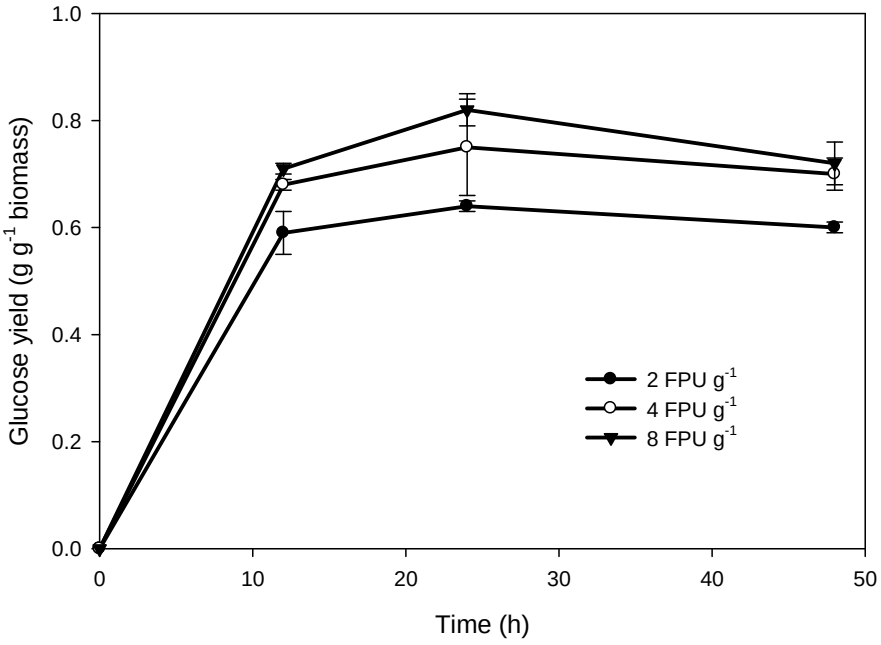


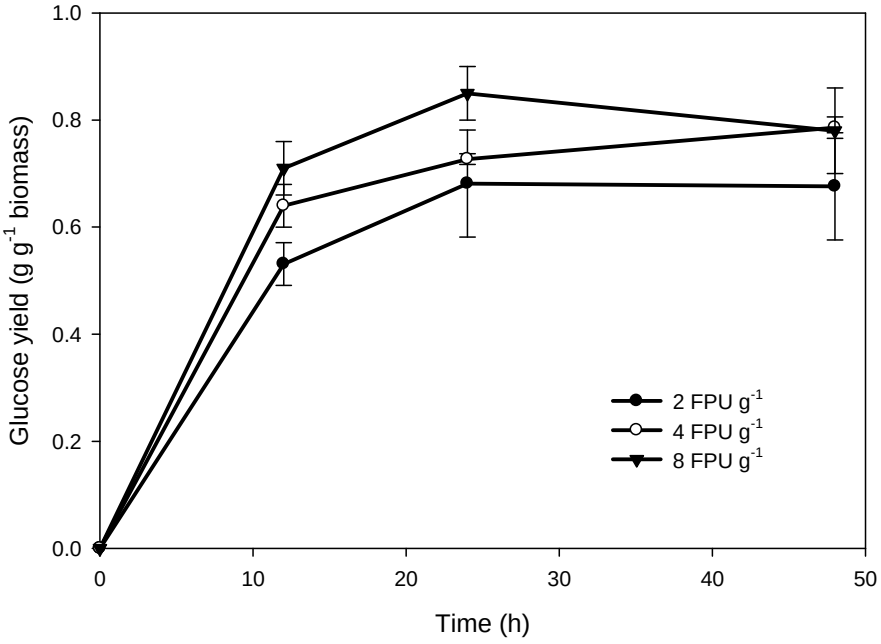
Fig. 1 Plot of v_{max} of different biomass versus enzyme concentration



(a)



(b)



(c)

Fig. 2 Enzymatic hydrolysis of pulp (a), mechanically modified pulp (b), and alkali-treated corn stover (c) at varying cellulase levels

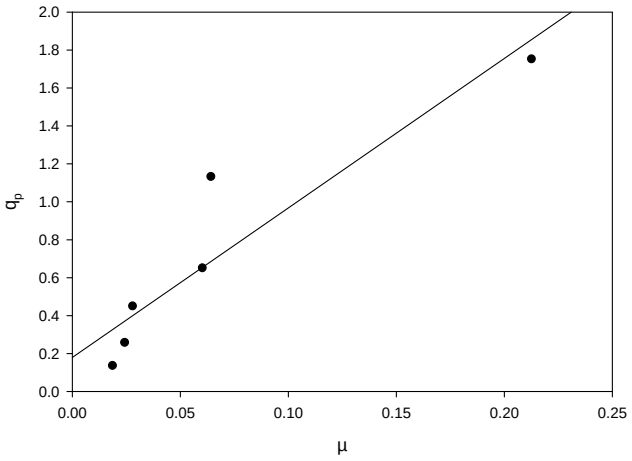


Fig. 3 Specific production rate versus specific growth rate for *L. delbrueckii* growing on the synthetic medium

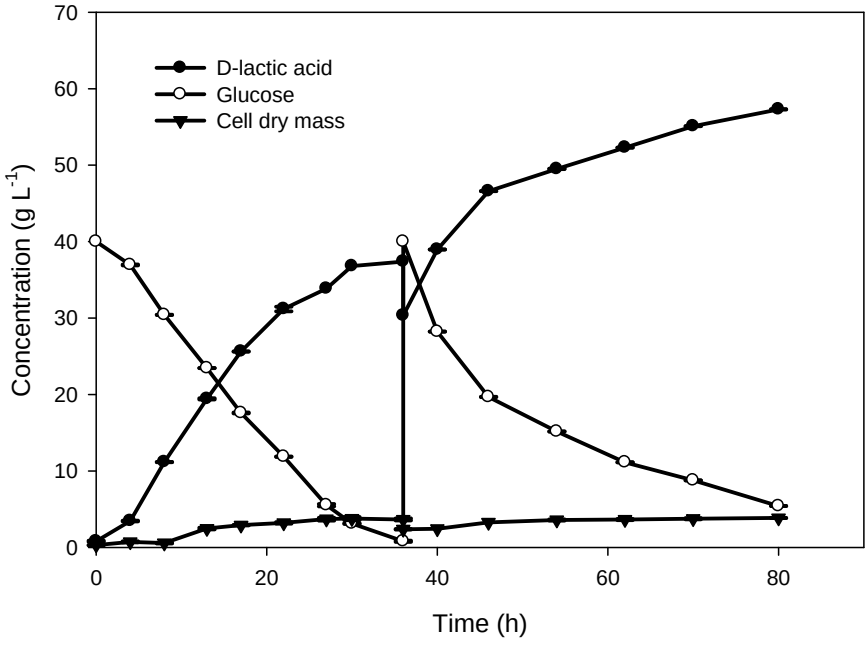


Fig. 4 Fed-batch fermentation profile of D-lactic acid from the synthetic medium

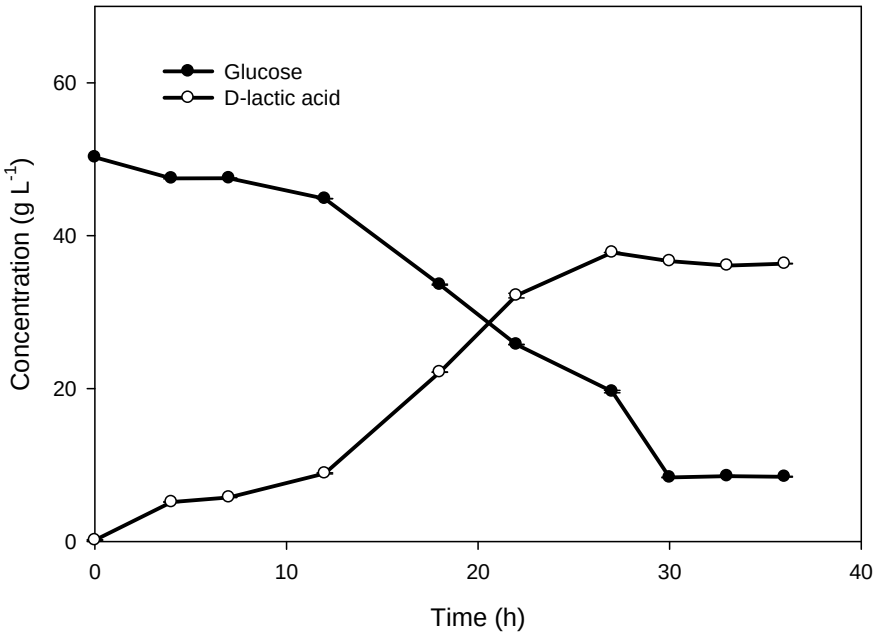
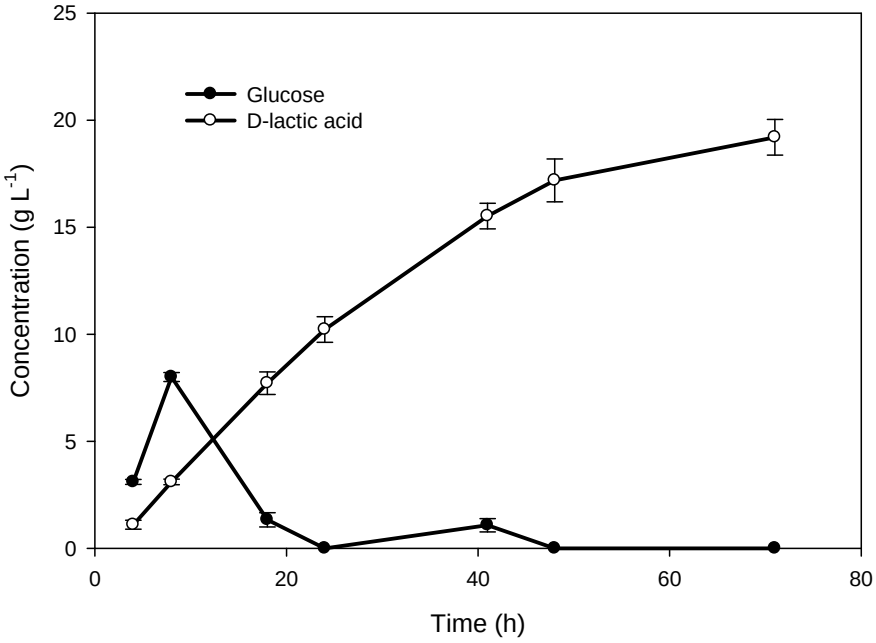
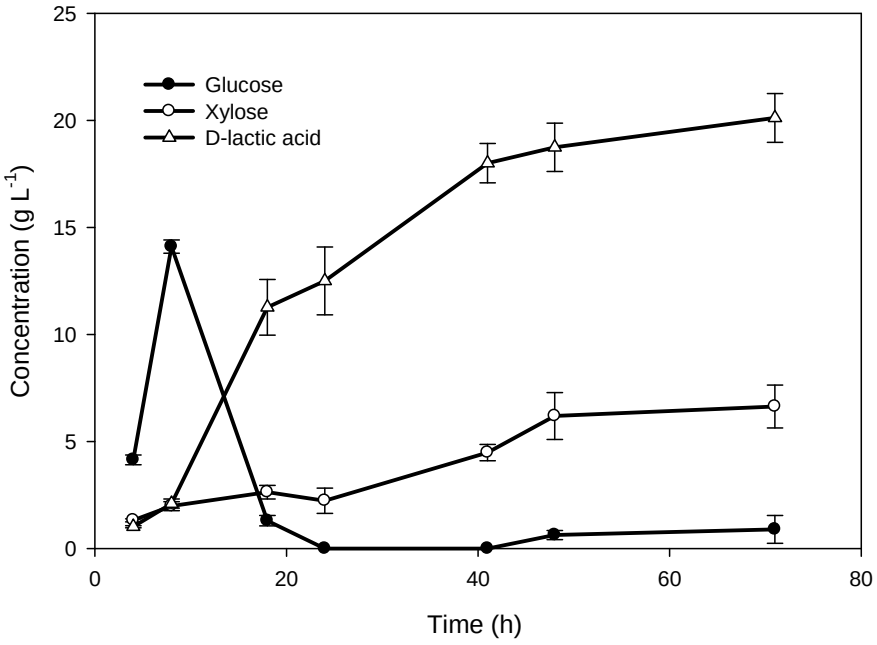


Fig. 5 Batch fermentation profile of D-lactic acid production from pulp hydrolyzate



(a)



(b)

Fig. 6 Time course of SSF process with *L.delbrueckii* using pulp (a) and alkali-treated corn stover (b)