Pilot-Scale Lactic Acid Production *via* Batch Culturing of *Lactobacillus* sp. RKY2 Using Corn Steep Liquor As a Nitrogen Source

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Summary

In this study, the determination of the efficiency of a pilot-scale fermentation process using corn steep liquor as a nitrogen source was attempted in order to produce lactic acid *via* batch culturing of *Lactobacillus* sp. RKY2. Using pure glucose, fermentation efficiency characteristics, such as final lactic acid, cell growth, yield, and productivity were not substantially influenced by the scale-up of the laboratory-scale fermentation from 2.5- to 30- and 300-litre scale fermentations. In all experiments, the content of lactic acid produced increased in a linear fashion with increases in the initial glucose concentration. In the experiments using wood hydrolyzate, both lactic acid productivity and cell growth were decreased as a result of the scaling-up of the fermentation. This might be attributed to the toxic chemicals contained in the wood hydrolyzates. However, in all experiments, lactic acid yields remained higher than 90 % with regard to the amount of glucose consumed. Therefore, lactic acid was successfully produced by the pilot-scale bioreactor scheme adopted in this study.

Key words: lactic acid, *Lactobacillus*, corn steep liquor, fermentation, wood hydrolyzate, pilot-scale

Introduction

Lactic acid ($CH_3CHOHCOOH$) is an invaluable chemical, initially discovered by the Swedish chemist Scheele in 1780, who identified it in sour milk (*1,2*). Although lactic acid can be manufactured either *via* chemical synthesis or by a biological approach, a great deal of interest has recently become focused on the biological approach, because the chemical synthesis of lactic acid is associated with several serious problems, including environmental issues and the depletion of petrochemical resources (*3,4*). Lactic acid has been classified by the US FDA as GRAS (Generally Recognized As Safe) for use as a food additive, and it has been utilized in a broad range of applications in the food, cosmetic, medical, and pharmaceutical industries (*4–6*). Lactic acid plays a vital role in the chemical industry, where it is used as a precursor for the syntheses of ethyl lactate, propylene oxide, propylene glycol, acrylic acid, 2,3-pentanedione, and dilactide (*7,8*). The worldwide demand for lactic acid is increasing substantially at present, as it can also be employed as a monomer for the synthesis of biodegradable poly-(lactic acid) (PLA), a sustainable bioplastic material (*4,5*). Cargill Dow LLC, the primary US manufacturer of PLA,

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has reported that the global PLA market might expand to 500 000 tonnes per year by 2010. Therefore, considerable increases in the worldwide demand for lactic acid are definitely expected in the coming years (*9*).

The biological production of lactic acid *via* microbial fermentation has been studied extensively by a lot of research groups (*10*). The fermentative production of lactic acid is a fairly interesting subject, owing primarily to the possibility that renewable resources, such as starchy and cellulosic materials, might be employed in the process (*11*). However, the majority of studies regarding lactic acid fermentation have been conducted only on the laboratory-scale, although a few pilot-scale works have also been conducted. Cira *et al.* (*12*) previously reported the pilot-scale lactic acid fermentation of shrimp wastes for chitin recovery, Fukushima *et al.* (*13*) described the production of lactic acid from rice starch in a 5-litre bioreactor, and Miura *et al.* (*14*) conducted a study concerning the production of lactic acid by filamentous fungi in a 100-litre airlift bioreactor. Pilot-scale tests are clearly required before the established laboratory-scale processes can be scaled up to plant-scale (*i.e.* industrial-scale) processes. It is also necessary to find cheaper substrates in the development of a pilot-scale lactic acid production scheme, as the manufacturing cost of lactic acid tends to depend principally on the cost of the raw materials used in the fermentation process. As corn steep liquor (CSL), a waste by-product of the corn-steeping process, contains some of the nutrients essential for microbial growth (*15*), it has been proposed as a potentially effective substrate for the microbial production of lactic acid.

In this study, we produced lactic acid in pilot plant bioreactors (30- and 300-litre scale), and attempted to compare the laboratory-scale fermentation process with the pilot-scale fermentation process. For these objectives, we employed glucose and CSL as the raw materials for the production of lactic acid. We also attempted to produce lactic acid from wood hydrolyzate and CSL in the pilot plant bioreactor, in an attempt to enhance the cost-effectiveness of the process.

Materials and Methods

Strain and culture media

Lactobacillus sp. RKY2 KCTC 10353BP, a homofermentative lactic acid-producing organism, was used in the present study (*16–18*). The strain RKY2 produces DL-lactic acid from glucose with an optical purity of 70 % L(+)-lactic acid (*19*). The organism was maintained in a 5-mL vial containing 50 % glycerol at –70 °C until use.

The medium used for the preparation of the inoculum comprised (in g/L): glucose 30, yeast extract 10, $(NH_4)_2HPO_4$ 2, and $MnSO_4$ 0.1. The primary fermentation media contained (in g/L): CSL 30, yeast extract 1.5, $(NH_4)_2HPO_4$ 2, $MnSO_4$ 0.1, and various concentrations of different carbon sources. The carbon source used for fermentations is glucose or wood hydrolyzate.

Inoculum preparation

Lactobacillus sp. RKY2 cells from stock cultures were transferred to 15 mL of growth medium in a 20-mL vial,

which was then incubated for 12 h at 36 °C in a shaking incubator at 200 rpm. After three or four consecutive transfers to 15 mL of fresh media, 2 mL of the final culture was transferred to 80 mL of growth medium in a 100-mL vial, which was then incubated at 36 °C and 200 rpm for 12 h prior to inoculation into 2.5- and 30-litre scale bioreactors, at a 4 % level. In addition, the 300-litre scale bioreactor was inoculated with a 12-hour culture of 30-litre bioreactor.

Wood hydrolyzates

Oak wood chips (2 × 4 mm) were generously supplied by the Korea Institute of Energy Research (KIER, Daejeon, Korea), and constituted of *w*=49.3 % cellulose, 25.9 % hemicellulose, and 21.7 % Klasson lignin. The wood chips were hydrolyzed enzymatically, in accordance with a previously described method (*20*). The resulting wood hydrolyzate was centrifuged at 15 540 × *g*, and the supernatant, which contained approximately 88 g/L glucose, was diluted with deionized water in order to achieve the desired glucose concentration.

Lactic acid fermentation

The batch fermentations for lactic acid production were conducted in 2.5-, 30-, and 300-litre bioreactors, with working volumes of 1, 20, and 200 L, respectively. In all experiments, the pH of the culture was maintained at 6.0 *via* automatic additions of 10 M NaOH, the temperature was maintained at 36 °C, and the agitation speed was maintained at 200 rpm. The samples were removed aseptically at regular intervals for further analyses.

Analytical methods

Lactic acid was quantified by HPLC analysis coupled with a UV variable wavelength detector set to 210 nm. An Aminex HPX-87H ion-exclusion column was eluted with 5 mM sulfuric acid solution at 0.6 mL/min, and the column temperature was maintained at 35 °C. Glucose was measured enzymatically *via* the glucose oxidase-peroxidase technique, using a Glucose-E kit (YD Diagnostics, Seoul, Korea). Cell growth was determined turbidimetrically by a spectrophotometer at 660 nm, and the values thus obtained were then converted to dry cell mass *via* calculation with the appropriate standard curve.

Results and Discussion

Lactic acid fermentation in laboratory-scale bioreactor (2.5 L)

In order to assess the effects of initial glucose concentrations on lactic acid fermentation, the media were supplemented with 50–125 g/L of glucose. As shown in Fig. 1a, the rates of lactic acid production were almost similar in all cases in this experiment, except when using an initial glucose concentration of 125 g/L. The amount of lactic acid produced increased directly with increases in the quantity of glucose added to the media. Although the maximum lactic acid concentration was 120.8 g/L at an initial glucose concentration of 125 g/L, the lactic acid productivity in this case was no more than 1.3 g/(L·h). This may be attributed to inhibitions of
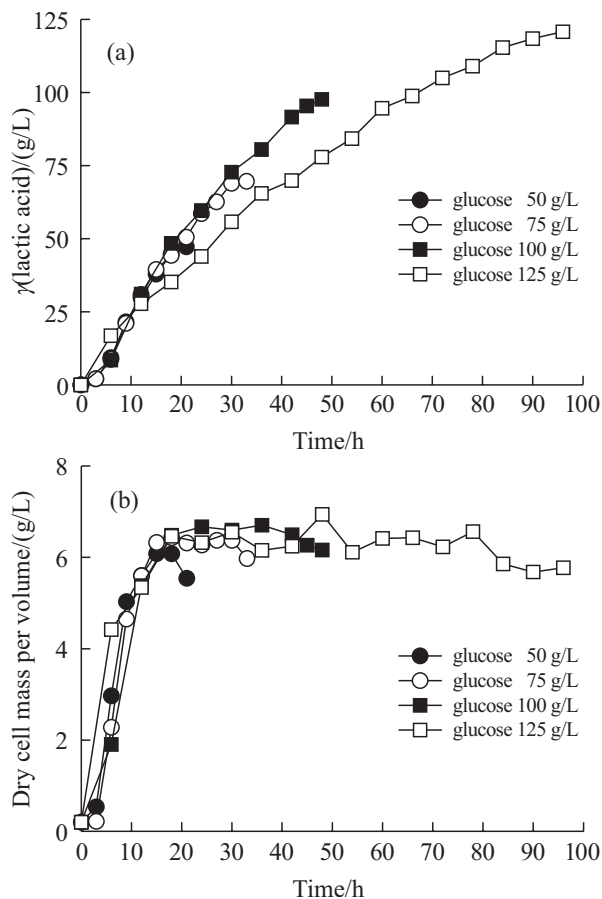
Fig. 1. Lactic acid production (a) and cell growth (b) during the batch culture of *Lactobacillus* sp. RKY2 in a 2.5-L scale bioreactor. Fermentation medium (in g/L): glucose 50–125, corn steep liquor 30, yeast extract 1.5, (NH₄)₂HPO₄ 2, and MnSO₄ 0.1

both the substrate and the product, a well-characterized phenomenon in conventional batch fermentations. In lactic acid fermentations that use high concentrations of glucose, the fermentations are inhibited by high substrate in the first half of the fermentation. As lactic acid is produced, the fermentations are inhibited by the product in the other half of fermentation. Similar observations during lactic acid fermentation processes were reported by Linko and Javanainen (*21*), as well as Hujanen *et al.* (*22*), who investigated lactic acid fermentation by *Lactobacillus casei* in barley starch, and by Ohara *et al.* (*23*), who observed product inhibition in a batch culture of *Streptococcus faecalis*. However, when the added glucose concentration was between 50–125 g/L, the lactic acid yields on the basis of consumed glucose were affected only slightly by the initial glucose concentrations, ranging between 93 and 96 %. The cell growth profiles during fermentation exhibited similar patterns in all experimental cases (Fig. 1b), and the maximum dry cell mass (6.9 g/L) was obtained at an initial glucose concentration of 125 g/L.

Lactic acid fermentation in pilot-scale bioreactor I (30 L)

In order to produce lactic acid in the pilot-scale bioreactor, we first conducted lactic acid fermentations in a

30-L scale bioreactor, containing 20 L of medium supplemented with 50–125 g/L of glucose. The results of this trial are presented in Fig. 2. The amount of lactic acid produced increased in a linear fashion with increases in the initial glucose concentrations, and its maximum values (118.2 g/L of lactic acid) were detected in the medium that had been supplemented with 125 g/L of glucose. The lactic acid production rates were almost similar, except when using an initial glucose concentration of 125 g/L, which resulted in a considerable reduction in lactic acid productivity, to 1.3 g/(L·h). This result is consistent with the data obtained from lactic acid fermentations in the 2.5-litre scale bioreactor. The lactic acid yields based on the amount of glucose consumed were in excess of 94 %, and these were also quite similar to the yields obtained in the laboratory-scale bioreactor. The maximum dry cell mass (9.3 g/L) was achieved using medium supplemented with 75 g/L of initial glucose, and this value corresponded to a 1.3-fold higher maximum dry cell mass than was obtained from the laboratory-scale lactic acid fermentation experiment. These results show that fermentation efficiency characteristics, including lactic acid production, yield, and productivity, should not be affected greatly by scaling-up to 30 L.
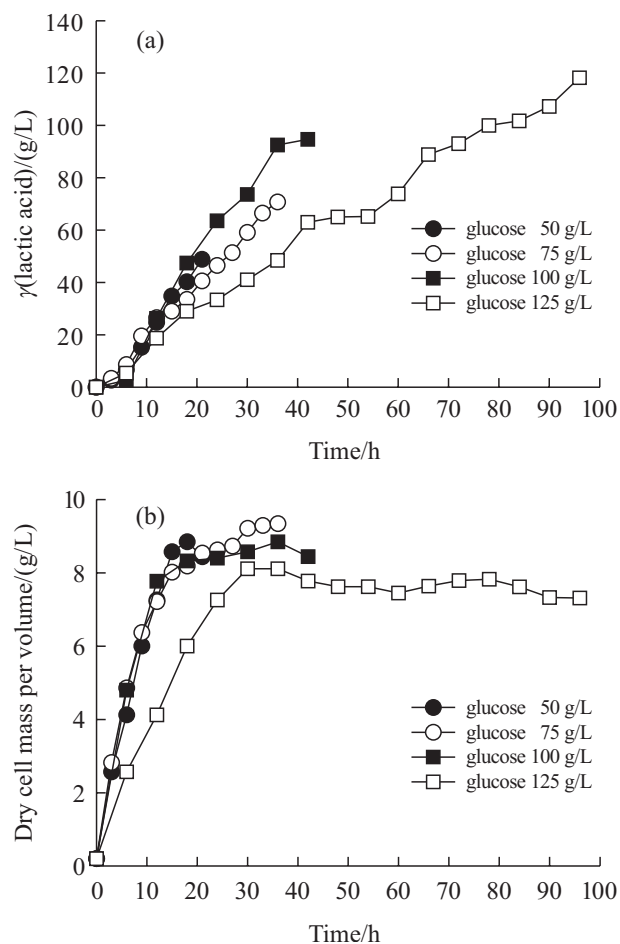

Fig. 2. Lactic acid production (a) and cell growth (b) during the batch culture of *Lactobacillus* sp. RKY2 in a 30-L scale bioreactor. Fermentation medium (in g/L): glucose 50–125, corn steep liquor 30, yeast extract 1.5, (NH₄)₂HPO₄ 2, and MnSO₄ 0.1

Lactic acid fermentation in pilot-scale bioreactor II (300 L)

When a further scale-up of lactic acid fermentation to 300-litre scale was done, a significant increase in fermentation time and a reduction in the amount of cell growth were observed (data not shown). This may be attributed to the toxic effects of oxygen harboured in the headspace of the bioreactor, as the headspace volume of the 300-L bioreactor was 10 times that of the 30-litre bioreactor. However, scale-up of anaerobic fermentations is much easier to perform than of aerobic fermentations, provided that the dissolved oxygen and headspace oxygen in the bioreactor can be decreased to a low value. Therefore, in further trials, we replaced the oxygen in the headspace with nitrogen gas, by sparging it into the bioreactor prior to inoculation.

As can be seen in Fig. 3, during fermentations in the 300-litre bioreactor under anaerobic conditions, the final lactic acid concentrations increased with increases in the added glucose concentrations, eventually reaching a maximum value of 115.1 g/L. However, the maximum lactic acid production rate, 3.0 g/(L·h), was observed in the medium supplemented with 50 g/L of glucose. After this, the lactic acid production rate decreased gradually, probably due to the aforementioned inhibitions. As in the previous experiments, the lactic acid yields were in
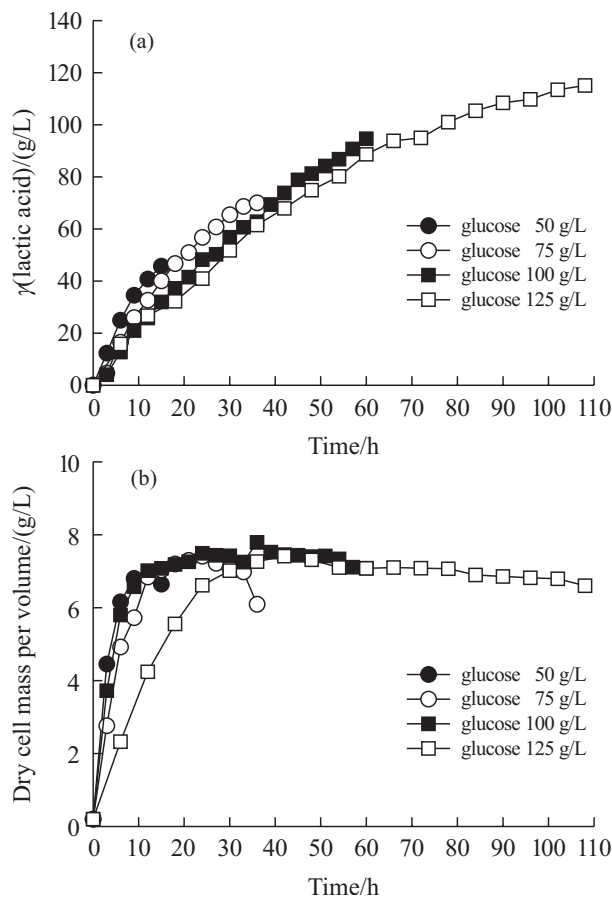
excess of 90 % in all test cases in this study. The profiles of cell growth during fermentation evidenced similar patterns in all cases except when the initial glucose concentration was 125 g/L. The maximum dry cell mass was 7.8 g/L in the medium supplemented with 125 g/L of glucose.

Lactic acid production from wood hydrolyzate in laboratory-scale and pilot-scale bioreactors

Lactic acid fermentations using wood hydrolyzate as a carbon source were conducted in 2.5- and 30-litre bioreactors, and the glucose concentrations in the wood hydrolyzate were 50 and 75 g/L. As shown in Fig. 4, when wood hydrolyzates containing 50 g/L of glucose were used, lactic acid production profiles for fermentation in the 2.5- and 30-litre bioreactors were similar. However, when fermentation was conducted in a 30-litre scale bioreactor supplemented with wood hydrolyzates containing 75 g/L of glucose, the lactic acid production rates decreased gradually after 18 h of fermentation. Lactic acid concentrations and yields were almost similar in all cases. When the media were supplemented with wood hydrolyzates containing 50 g/L of glucose, 48.0 and 46.8 g/L of lactic acid were produced in the 2.5- and 30-litre bio-
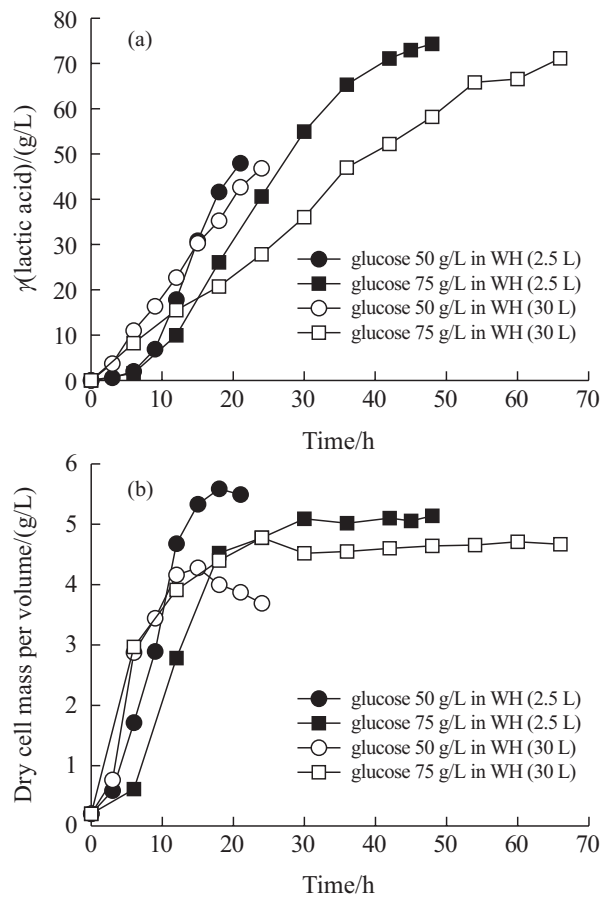

Fig. 3. Lactic acid production (a) and cell growth (b) during the batch culture of *Lactobacillus* sp. RKY2 in a 300-L scale bioreactor. Fermentation medium (in g/L): glucose 50–125, corn steep liquor 30, yeast extract 1.5, (NH₄)₂HPO₄ 2, and MnSO₄ 0.1

Fig. 4. Lactic acid production (a) and cell growth (b) during the batch culture of *Lactobacillus* sp. RKY2 using wood hydrolyzate (WH) as a carbon source in 2.5-L and 30-L scale bioreactors. Fermentation medium (in g/L): glucose in wood hydrolyzate 50–75, corn steep liquor 30, yeast extract 1.5, (NH₄)₂HPO₄ 2, and MnSO₄ 0.1

reactors, respectively, and in both cases the lactic acid yields were in excess of 94 %. Also, when the media were supplemented with wood hydrolyzates containing 75 g/L of glucose, 74.3 and 71.1 g/L of lactic acid were produced in the 2.5- and 30-litre bioreactors, respectively, and in both cases the lactic acid yield was 96 %. The cell growth detected during fermentation in the 30-litre bioreactor was lower than that detected in the 2.5-litre bioreactor.

In fermentations employing wood hydrolyzates containing 50 and 75 g/L of glucose, the maximum dry cell mass obtained in the 30-litre bioreactor was almost half of that obtained in the 2.5-litre bioreactor. The observed reductions in lactic acid productivity and cell growth in the 30-litre bioreactor might be attributed to the inhibition induced by toxic chemicals contained in the wood hydrolyzates. Prior to enzymatic hydrolysis, the wood is steam-exploded in order to disrupt its crystalline structure. During this steam-explosion process, several toxic chemicals are released, such as furfural, 5-hydroxymethyl furfural, and phenolic compounds (*24*). In the present study, the degree to which such inhibition occurred in the 30-litre bioreactor appeared to be larger than that in the 2.5-litre bioreactor, probably due to the fact that the medium had been directly steam-sterilized for a longer time, 20 min (15 min in 2.5-litre bioreactor). A longer sterilization time at high temperature might result in generation of more inhibiting compounds, which might be a possible cause of the reduction of fermentation efficiency. Most of the inhibitory compounds in wood hydrolyzate are commonly generated during the steam-explosion process, but the sterilization time may influence slightly the generation of inhibitory compounds. Lee *et al.* (*25*) reported in a previous study that fermentation efficiency could be enhanced *via* the low-temperature (60 °C) sterilization of the wood hydrolyzates for 120 min. Therefore, with regard to the preparation of the wood hydrolyzates, other methods by which these toxic

chemicals can be neutralized during sterilization should be developed prior to using the hydrolyzates in large--scale applications.

The kinetic parameters of the fermentations are summarized in Table 1. In all experimental cases, lactic acid yields according to the amount of glucose consumed were in excess of 90 %. When pure glucose was employed as a carbon source, fermentation efficiencies were not found to have been affected significantly by a scale--up of the process from 2.5- to 300-litre scale. However, when using wood hydrolyzates, lactic acid productivity and maximum cell growth were both decreased as a result of the scale-up. The wood material must be subjected to pretreatments in order to liberate the fermentable sugars. During the pretreatments of wood, toxic chemicals are released simultaneously with the generation of sugars. However, wood hydrolyzates still constitute a cheap raw material for use in lactic acid production (*10,19*). This is because pure glucose (0.5 US$/kg) is far more expensive than wood (0.025 US$/kg) (*26,27*). Therefore, in order to detoxify or neutralize these toxic chemicals, we will conduct further investigations into the effects of the toxic chemicals, contained in wood hydrolyzates, and assess their effects in the context of lactic acid fermentation.

Conclusions

We successfully conducted a series of pilot-scale fermentations for the production of lactic acid *via* the batch culturing of *Lactobacillus* sp. RKY2 using corn steep liquor as a nitrogen source. During the fermentation process employing pure glucose as a carbon source, fermentation parameters were not substantially altered by the scaling-up of the laboratory-scale fermentation to 30- and 300-litre scale fermentations. However, when using wood hydrolyzates, both lactic acid productivity and cell growth decreased as the result of the scaling-up of

Table 1. Kinetic parameters of lactic acid fermentations by batch culture of *Lactobacillus* sp. RKY2 on laboratory-scale and pilot-scale bioreactors

V(bioreactor)	γ(initial glucose)	γ(lactic acid)	Maximum dry cell mass per volume	η(lactic acid)	Productivity
L	g/L	g/L	g/L	g/g	g/(L·h)
2.5	50	47.2±0.2 (48.0±0.1)	6.1± 0.2 (6.0 ± 0.3)	0.94±0.00 (0.96±0.00)	2.2 (2.3)
	75	69.6±0.5 (74.3±0.6)	6.4±0.1 (5.1±0.2)	0.93± 0.01 (0.96±0.01)	2.1 (1.5)
	100	97.7±0.7	6.7±0.3	0.97±0.01	2.0
	125	120.8±1.1	6.9±0.1	0.96±0.01	1.3
30	50	48.8±0.2 (46.8±0.2)	8.8±0.1 (4.3±0.3)	0.97±0.00 (0.94±0.00)	2.3 (2.0)
	75	70.7±0.1 (71.1±0.4)	9.3±0.4 (4.7±0.1)	0.94±0.00 (0.96±0.01)	2.0 (1.1)
	100	94.7±0.6	8.8±0.1	0.94±0.01	2.3
	125	118.2±0.2	8.4±0.3	0.95±0.00	1.2
300	50	45.7±0.5	6.9±0.4	0.90±0.01	3.0
	75	70.0±0.2	7.4±0.7	0.93±0.00	1.9
	100	94.7±0.1	7.8±0.1	0.96±0.00	1.6
	125	115.1±0.8	7.4±0.3	0.92±0.01	1.1

Data presented in parentheses are from the medium supplemented with wood hydrolyzate as a carbon source, and ± represents standard deviation among the replicates
η(lactic acid)=lactic acid produced/glucose consumed (g/g)

the process, probably due to toxic chemicals in the hydrolyzates. In order to further enhance the production of lactic acid from wood hydrolyzates in a pilot-scale bioreactor, it may be necessary to develop methods by which these toxic chemicals can be neutralized, such as low temperature sterilization.

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