Production of D-lactic acid from sugarcane bagasse using steam-explosion

Chizuru Sasaki, Ryosuke Okumura, Ai Asakawa, Chikako Asada and Yoshitoshi Nakamura

Department of Life System, Institute of Technology and Science, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan

E-mail: csasaki@bio.tokushima-u.ac.jp

Abstract. This study investigated the production of D-lactic acid from unutilized sugarcane bagasse using steam explosion pretreatment. The optimal steam pressure for a steaming time of 5 min was determined. By enzymatic saccharification using Meicellase, the highest recovery of glucose from raw bagasse, 73.7%, was obtained at a steam pressure of 20 atm. For residue washed with water after steam explosion, the glucose recovery increased up to 94.9% at a steam pressure of 20 atm. These results showed that washing with water is effective in removing enzymatic reaction inhibitors. After steam pretreatment (steam pressure of 20 atm), D-lactic acid was produced by Lactobacillus delbrueckii NBRC 3534 from the enzymatic hydrolyzate of steam-exploded bagasse and washed residue. The conversion rate of D-lactic acid obtained from the initial glucose concentration was 66.6% for the hydrolyzate derived from steam-exploded bagasse and 90.0% for that derived from the washed residue after steam explosion. These results also demonstrated that the hydrolyzate of steam-exploded bagasse (without washing with water) contains fermentation inhibitors and washing with water can remove them.

Introduction

Lactic acid is widely used in food, pharmaceutical, and chemical industries. In particular, lactic acid has been used as a raw material for manufacturing poly(lactic acid), one of the most promising biodegradable polymers. For this reason, many researchers have studied
L-lactic acid production strategies using unutilized cellulosic plant material and starchy biomass such as paper sludge, water wastes, agro wastes, and beer production byproducts [1-4]. Recently, it was discovered that D-type and L-type lactic acids form stable stereocomplexes that have thermal properties different from those of the original poly(lactic acid) made from only L-type or D-type lactic acid [5-7]. However, few studies have focused on strategies for producing D-lactic acid from unutilized plant biomass [8, 9].

The main components of unutilized cellulosic biomass are cellulose, hemicellulose, and lignin. Because cellulose is surrounded by a hard network of lignin, it is necessary to decompose it using an environmentally friendly pretreatment method prior to its conversion into glucose using an enzymatic method.

Steam explosion is a hydrothermal pretreatment method for plant biomass that uses high-pressure and high-temperature steam without the addition of any chemicals and involves chemical effects such as auto hydrolysis, defibration, and delignification. Compared to the generally used chemical treatments, it has several potential advantages: it eliminates the use of toxic substances, such as strong acids or alkalis, chemical tolerance equipment, waste solution processing, and preliminary feedstock size reduction.

Sugarcane bagasse, a byproduct of the sugar industry, is an abundant source of lignocellulose. Although it is used as fuel for boilers, a large quantity of this material is accumulated in sugar processing plants, which leads to environmental problems. Advanced utilization of this material as a carbon resource holds promise for the bioproduction of useful chemicals like lactic acid.

In the present study, optimal steam explosion pretreatment conditions for sugarcane bagasse followed by enzymatic saccharification were investigated. In addition, efficient microbial conversion of hydrolyzates derived from sugarcane bagasse to D-lactic acid by *Lactobacillus delbrueckii* was investigated.

**Materials and Methods**

**Sugarcane bagasse**

The raw sugarcane bagasse (bagasse) from Kyuyo Sugar Co. Ltd. (Okinawa, Japan) used in these experiments was cut into 1–3 cm-sized chips. The chemical composition (based on dry material) of bagasse was 45.1% α-cellulose, 22.3% Klason lignin, 27.9% hemicellulose, and 4.7% other.

**Steam explosion**

Pretreatment of the bagasse was conducted in a steam explosion apparatus (Japan Chemical Engineering and Machinery Co. Ltd., Osaka, Japan). The reactor was charged with 100 g (dry
matter) of feedstock per batch. Saturated steam from the boiler was then allowed to enter the reactor to heat the bagasse at a controlled pressure of 15, 20, 25, 30 or 35 atm. Each pressure was maintained for 5 min and then the reactor was suddenly depressurized. The exploded bagasse was recovered in a cyclone and cooled to room temperature.

**Components analysis of bagasse and steam-exploded bagasse**

Components analysis of untreated bagasse and steam-exploded bagasse was conducted by Wayman’s method [10] using distilled water and methanol as extractive solvents. The method is illustrated in Fig. 1. The amount of components, i.e., water-soluble material (WSM), methanol-soluble material, acid-soluble lignin, holocellulose (cellulose and hemicellulose), and Klason lignin, was determined as follows.

![Diagram](image)

**Fig. 1** Component analysis method of untreated bagasse and steam exploded bagasse

Five grams of dry untreated or steam-exploded bagasse was added to 100 mL of distilled water and extracted for 24 h at room temperature. The solid (Residue 1) and liquid materials were separated by filtration, the filtrate was recovered from the liquid, then concentrated, dried, and weighed (water-soluble material). Residue 1 was extracted at 80°C for 24 h in a Soxhlet extractor using 150 mL methanol to dissolve the methanol-soluble material. After concentration and drying of the extract, the methanol-soluble material was weighed. Residue 2 from the methanol extraction consisted of holocellulose and Klason lignin, a high-molecular weight lignin. This residue (1 g) was added to 15 mL of 72% (w/v) sulfuric acid and kept at...
room temperature for 4 h. The residue was placed in a 100 mL conical flask, washed with 560 mL distilled water, and then autoclaved for 1 h. After the insoluble material was washed with distilled water, it was heat-dried at 105°C to a constant weight and weighed (Klason lignin). The weight of holocellulose was calculated by subtracting the weight of Klason lignin from 1 g of Residue 2.

**Enzymatic hydrolysis**

Steam-exploded bagasse was enzymatically hydrolyzed using Meicellase (produced by *Trichoderma viride*) provided by Meiji Seika Co., Ltd. Enzymatic hydrolysis was performed using a 0.05 M sodium phosphoric acid buffer (pH 4.5) at 50°C on a rotary shaker at 140 rpm for 48 h. The substrate concentration and enzyme loading were 100 g/L and 50 FPU/g substrate, respectively. The supernatant was centrifuged to remove solid waste and analyzed for glucose. All enzymatic hydrolysis experiments were performed in duplicate.

**Microorganism and inoculum cultivation**

*Lactobacillus delbrueckii* NBRC 3534, a homofermentative D-lactic acid producer, a high optical purity D-lactic acid producing strain (content of D-type lactic acid is 98.5%) [8], was used to produce D-lactic acid from the hydrolyzate of steam-exploded bagasse. The microorganism was subcultured every four weeks. The strain was precultured in 100 mL of medium in a 300 mL flask at 37°C for 24 h using a static incubator. The preculture media comprised 0.1 g/L (NH₄)₂SO₄, 10 g/L glucose, 0.1 g/L KH₂PO₄, 0.1 g/L MgSO₄·7H₂O, and 1.0 g/L yeast extract. Later, the cells were harvested by centrifugation (2000×g, 15 min), rinsed thoroughly with sterile distilled water, centrifuged again, and then resuspended in sterile distilled water.

**Optimization of initial glucose concentration of the medium**

To determine optimal initial glucose concentration for D-lactic acid fermentation using *Lactobacillus delbrueckii* NBRC 3534, four glucose concentrations of 21.0, 32.3, 44.5, and 53.6 g/L were examined. The other nutrients added were 5.0 g/L yeast extract, 10.0 g/L peptone, 5.0 g/L sodium acetate, 2.0 g/L ammonium citrate, 2.0 g/L K₂HPO₄, 0.1 g/L MgSO₄·7H₂O, 0.05 g/L MnSO₄·H₂O, 1.0 g/L Tween 80[12]. The strain was cultured in 100 ml of medium in a 300 ml flask at 37°C for 72 h using a static incubator.

**D-lactic acid fermentation using bagasse hydrolyzate**

The nutrient medium for D-lactic acid fermentation contained hydrolyzate derived from steam-exploded bagasse (substrate concentration of 10% w/v), nutrient medium, 0.05 M
acetate buffer (pH 5.0), and 10% (v/v) preculture solution. The nutrient medium and buffer were autoclaved at 121°C for 20 min. The hydrolyzate was added after sterilizing with a 0.22 µm pore size filter. Cultivation was carried out in a 300 mL flask with 100 mL of the medium using a static incubator at 37°C. Aliquots of the samples were collected and assayed for determining ethanol and residual glucose concentrations.

**Determination of residual glucose and produced D-lactic acid**

Residual glucose and produced D-lactic acid concentrations in the fermentation medium were determined by the mutarotase GOD method (Glucose C-Ⅱ test, Wako Pure Chemicals Co., Ltd., Japan) and an enzymatic UV test with D-lactate dehydrogenase (D-LDH) (enzymatic fluid D-lactic acid, R-Biofarm, GmbH, Germany), respectively.

**Results and discussion**

**Composition analysis of steam-exploded bagasse**

To break down the lignin network that surrounds cellulose and increase the accessibility of the enzyme to cellulose, steam explosion was used to treat the bagasse. Table 2 shows the chemical composition of untreated bagasse and steam-exploded bagasse (steam pressures of 15, 20, 25, 30, and 35 atm; steaming time 5 min) separated by the extraction method illustrated in Fig. 1. The highest amount of WSM (17.8%), which contains sugars derived from holocellulose, was obtained at a steam pressure of 15 atm. In general, hemicellulose in the holocellulose hydrolyzes under less severe conditions than cellulose [13, 14]; therefore, mainly degradation of hemicellulose occurred. Above 20 atm, the amount of WSM decreased owing to the degradation of pentose, i.e., xylose or arabinose sugars derived from hemicellulose, which changed to insoluble materials such as furfural [15]. With increased production of sugars and further degraded materials, the amount of holocellulose decreased (steam pressures of 15 and 35 atm resulted in 48.6% and 34.4%, respectively). The amount of methanol-soluble material increased with steam pressure. Steam pressures of 15 and 30 atm resulted in 7.9% and 23.7%, and it contained low-molecular lignin [16-18]. Furthermore, the maximum reduction of Klason lignin (reduction rate 19.8%) was observed at a steam pressure of 20 atm. At higher pressure, a gradual increase was observed owing to the combination of lignin with low-molecular lignin and water-soluble materials [16-18].
Table 1  Chemical composition (%) of untreated and steam exploded bagasse at different steam pressures (steaming time 5 min)

| Components             | Untreated bagasse | Steam pressure (atm) [Steaming temperature] |
|------------------------|-------------------|---------------------------------------------|
|                        |                   | 15 [200°C] | 20 [213°C] | 25 [224°C] | 30 [234°C] | 35 [243°C] |
| Water soluble material | 1.9               | 17.8      | 13.7      | 11.2      | 9.7       | 10.0       |
| Methanol soluble material | 1.5         | 7.9       | 22.0      | 22.8      | 23.7      | 22.6       |
| Acid soluble lignin    | 1.8               | 0.9       | 0.4       | 0.4       | 0.4       | 0.4        |
| Holocellulose          | 72.6              | 53.3      | 46.2      | 44.6      | 41.7      | 39.0       |
| Klason-lignin          | 22.2              | 20.1      | 17.8      | 21.0      | 24.5      | 28.0       |
| Total                  | 100               | 100       | 100       | 100       | 100       | 100        |

Enzymatic saccharification of steam-exploded bagasse

The effect of steam explosion conditions on the enzymatic saccharification of bagasse was studied using a substrate concentration of 50 g/L and results are presented in Fig. 2(A). Steam pressures of 15, 20, 25, 30, and 35 atm for a steaming time of 5 min were investigated. The maximum amount of glucose produced was observed at a steam pressure of 25 atm and steaming time of 5 min: 364 mg/g dry steam-exploded bagasse, which corresponds to a 73.7% glucose yield from untreated bagasse. For steam pressures of 20, 30, and 35 atm, the glucose yield decreased more than that for steam pressure of 15 atm. Fig. 2(B) shows the enzymatic saccharification of washed steam-exploded bagasse, i.e., WISM, after steam explosion as a substrate (substrate concentration is 50 g/L). Under all conditions, the amount of glucose produced increased. Similar higher glucose concentration was achieved at steam pressures of 20 and 25 atm. The value was 469 mg in 1 g of steam-exploded dry bagasse, corresponding to a 94.9% glucose yield from untreated bagasse. These results indicated that if inhibitors for saccharification, such as low-molecular lignin and water-soluble decomposed saccharide, i.e. furfural and 5-hydroxymethylfurfural (5-HMF), the amount of these materials were 16.7, 3.5 mg in 1 g of steam-exploded dry bagasse at steam pressure 25 atm (data not shown), were washed with water, the enzyme could access cellulose in the treated bagasse. Dunlop et al. [15], Palmqvist et al.[19] reported that during high temperature or acid pretreatment of wood, water-soluble inhibitors such as sugar- and lignin-derived materials are formed. From the viewpoint of low energy input, this study indicated that the optimal condition for steam explosion of bagasse was steam pressure of 20 atm for a steaming time of 5 min.
Fig. 2 Enzymatic saccharification of steam exploded bagasse (A) and water insoluble residue after steam explosion (B). Symbols; (○) steam pressure 15 atm, (□) 20 atm, (■) 25 atm, (△) 30 atm and (▲) 35 atm, at steaming time 5 min.

D-lactic acid production from bagasse

The optimal initial glucose concentration for D-lactic acid by *Lactobacillus delbrueckii* NBRC 3534 using was investigated before production of D-lactic acid from bagasse. Maximum D-lactic acid yield (from initial amount of glucose) and the residual ratio of glucose for a fermentation period of 72 h are summarized in Table 2. The maximum yield was observed at an initial glucose concentration of 21.0 g/L, i.e., 78.6%. In the case of an initial glucose concentration greater than 21.0 g/L, the residual ratio of initial glucose increased. For D-lactic acid production from bagasse in this study, an initial glucose concentration was adjusted at 20.0 g/L.

Table 2 D-lactic acid production and amount of residual glucose by *Lactobacillus delbrueckii* NBRC 3534 at different initial glucose concentrations after 72 h cultivation.

| Initial glucose Conc. (g/l) | D-lactic acid Conc. (g/l) | Yield (%) | Residual glucose Conc. (g/l) | Residual ratio of initial glucose (%) |
|-----------------------------|---------------------------|-----------|-----------------------------|-------------------------------------|
| 21.0                        | 16.5                      | 78.6      | 0.8                         | 3.8                                 |
| 32.3                        | 17.7                      | 54.8      | 10.8                        | 33.4                                |
| 44.5                        | 18.7                      | 42.0      | 23.0                        | 51.6                                |
| 53.6                        | 17.7                      | 33.0      | 33.9                        | 63.2                                |
Fig. 3 shows the time courses of glucose consumption and D-lactic acid production by *Lactobacillus delbrueckii* NBRC 3534 using available glucose, steam-exploded bagasse (steam pressure of 20 atm, steaming time 5 min), and water-insoluble residue after steam explosion (20 atm, 5 min) as substrates. When water-insoluble residue after steam explosion was used as the substrate, D-lactic acid production behavior similar to the results obtained using commercially available glucose was observed. In this case, the maximum amounts of D-lactic acid produced were 16.5 g/L (available glucose) and 17.1 g/L (water-insoluble residue), at a 72 h cultivation time, which corresponds to 81.7% and 90.0% of the conversion rate of the initial glucose concentration, respectively. On the other hand, when steam-exploded bagasse was used as the substrate, i.e., without washing with water after steam explosion treatment, the amount of D-lactic acid produced decreased (conversion rate was 66.5%) and no dramatic glucose consumption was observed. This phenomenon also showed the presence of fermentation inhibition materials in the steam-exploded bagasse, similar to the case of enzymatic saccharification. This study found that a simple method with low environmental loading, such as washing only with water, could remove the inhibitors for D-lactic acid producers and cellulase from steam-exploded bagasse. It was determined that 48.9 g of D-lactic acid can be produced from 100 g of water-insoluble residue after steam explosion (steam pressure of 20 atm and steaming time 5 min) using *Lactobacillus delbrueckii* NBRC 3534. Moreover, from 100 g of bagasse, 42.2 g of D-lactic acid can be produced by this method.

![Residual glucose concentration and D-lactic acid production profiles by Lactobacillus delbrueckii NBRC3534. Symbols; (●) Glucose with available glucose, (○) D-lactic acid derived from available glucose, (■) Glucose with steam exploded bagasse, (□) D-lactic acid](image-url)
derived from steam exploded bagasse, (▲) Glucose with water insoluble residue after steam explosion and (△) D-lactic acid derived from water insoluble residue after steam explosion.

Conclusions
This study showed that high amounts of glucose could be produced from enzymatic hydrolyzate of steam-exploded bagasse. In particular, washing the steam-exploded sample with water effectively removes the inhibitors that interrupt the reaction between cellulose and the enzyme (cellulase). Furthermore, it was determined that higher amounts of D-lactic acid could be produced from a carbon source derived from steam-exploded bagasse washed with water after steam explosion than from that prepared without washing using Lactobacillus delbrueckii NBRC 3534. Washing with water is a simple and environmentally friendly method for removing inhibitors from the pretreated residue. This study will contribute to improving large-scale industrial methods for the production of D-lactic acid from unutilized plant materials.

Acknowledgements
A part of this study was funded by Shiseido Female Researcher Science Grant and Grant-in-Aid for Young Scientist (B)(No. 21750159) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References
[1] Marques S, Santos J.A.L, Girio F.M, Roseiro J.C. 2008 Eng. J. 41 210
[2] Nakasaki K, Adachi T 2003 Biotechnol. Bioeng. 82 263
[3] John R.P, Nampoothiri M, Pandey A 2006 Process Biochem. 41 759
[4] Shindo S, Tachibana T 2004 J. I. Brewing 110 347
[5] Ikeda Y, Jamshidi K, Tsuji H, Hyon S.H. 1987 Macromolecules 20 904
[6] Tsuji H, Hyon S.H, Ikeda Y 1991 Macromolecules 24 2719
[7] Slivniak R, Domb A.J 2002 Biomacromolecules 3 754
[8] Fukushima K, Sogo K, Miura S, Kimura Y 2004 Macromol. Biosci. 4 1021
[9] Lu Z, Lu M, He F, Yu L 2009 Bioresource Technol. 100 2026
[10] Chua M.G.S, Wayman M 1979 Can. J. Chem. 57 141
[11] Mikami, H. Ishida Y 1983 Bunseki Kagaku (in Japanese) 32 E207
[12] Mercier P, Yerushalmi L, Rouleau D, Dochain D, 1992 J. Chem. Technol. Biot. 55 111
[13] Palmqvist E. Hahn-Hagerdal B 2000 Bioresource Technol 74 25
[14] Ando H, Sakaki T, Kokusho T, Shibata M, Uemura Y, Hatate Y 2000 Ind. Eng. Chem. Res. 39 3688
[15] Dunlop A.P 1948 *Ind. Eng. Chem.* **40** 204
[16] Nakamura Y, Sawada T, Inoue E 2001 *J. Chem. Technol. Biot.* **76** 879
[17] Kobayashi F, Take H, Asada C, Nakamura Y 2004 *J. Biosci. Bioeng.* **97** 426
[18] Asada C, Nakamura Y, Kobayashi F 2005 *Biotechnol. Bioprocess E* **10** 346
[19] Palmqvist E, Hahn-Hagerdal B, Galbe M, Zacchi G. 1996 *Enzyme Microb. Tech.* **19** 470