Combined use of sugars and nutrients derived from young maize plants for
thermophilic L-lactic acid fermentation

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Abstract

Fermentation of lignocellulosic biomass requires auxiliary materials such as nutrients for fermentation. Because of the low prices of fermentation products such as bioethanol, the costs of nutrients are not negligible. We have developed the concept of using substances natively present in lignocellulosic biomass as nutrients for the downstream fermentation. The leaves and stalks of young dent corn plants were used as biomass and the nutrients were recovered by soaking them in water before alkaline pretreatment, followed by enzymatic saccharification and fermentation. Performing thermophilic L-lactic acid fermentation using these recovered nutrients support their use as commercially feasible alternatives. A level of recovered nutrients of 40% was sufficient to support the fermentation of recovered sugars derived from the same corn biomass. However, the amount of recovered nutrients required for fermentation was almost double that when using yeast extract based on the amounts of nitrogen and/or phosphorus added. The nitrogen and phosphorus balances in the process indicated that adding nutrients was crucial for promoting fermentation based on the amounts of nitrogen and phosphorus. The nutrients proposed were considered to be effective for fermenting biomass pretreated by alkaline, dilute acid, and hydrothermal methods.

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38   Keywords: nutrient supplementation; corn stover; alkaline pretreatment;

39   enzymatic saccharification; *Bacillus coagulans*; catch crop

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42 **1. Introduction**

43 Utilization of sugars derived from non-edible parts of plant biomass
44 has become almost practicable owing to extensive studies on the
45 pretreatment, saccharification, and fermentation of lignocellulosic biomass
46 (Davis et al., 2013). These sugars are usually intended as fossil fuel
47 alternatives for producing products such as ethanol or organic acids, which
48 can be widely used as fuel for vehicles, raw materials for plastics, and
49 commodity chemicals. Therefore, an industry converting the sugars can be
50 considered as a primary bulk chemical manufacturer.

51 The fermentation step in the bulk manufacturing process requires
52 not only sugars as the main raw material but also auxiliary materials;
53 nutrients for fermentation and acids and bases for pH adjustment. Although
54 these auxiliary materials are inexpensive, their costs are considerable
55 relative to the very low prices of fermentation products as commodity
56 chemicals (Davis et al., 2013). In particular, adding nutrients markedly
57 increases the process costs (Lau et al., 2008) so it is necessary to reduce
58 nutrient use and/or identify alternative nutrient sources.

59 Nutrients for fermentation consist primarily of nutrient salts,
60 vitamins, and trace elements (Lau et al., 2012). For auxotrophic fermenting

organisms, further additives will be needed. For example, lactic acid-producing bacteria are known to be fastidious so other substances, such as amino acids, peptides, and nucleic acid derivatives, must be added to the growth medium (Thomsen et al., 2007). To supply all these ingredients, yeast extract has been used for bench- and pilot-scale fermentations, resulting in increased production costs.

Alternatives to yeast extract, based on agro-industry waste, have been studied intensively; corn steep liquor (Kang et al., 2011), corn distiller's dried grains with solubles (Lau et al., 2008), wheat gluten (Hetényi et al., 2010), potato juice (Dishisha et al., 2013), and plant juice (Thomsen, 2005). However, these all pose a supply problem and incur transportation costs, representing a constraint on production costs for using lignocellulosic biomass. One possible option is to obtain nutrients from the primary lignocellulosic biomass itself as Thomsen (2005) has suggested for plant juice.

Lignocellulosic biomass contains substances that function as nutrients during fermentation. Plant juice, including green and brown juices, is a byproduct when grass is converted to fodder pellets. Several studies have investigated the use of plant juices for supplying nutrients for fermentation; L-lysine (Thomsen and Kiel, 2008) and lactic acid (Andersen and Kiel, 2000;

Sreenath et al., 2001; Venus and Richter, 2006). These studies have suggested that lignocellulosic biomass might contain an adequate range of nutrients for the growth of the fermenting organisms.

However, the amounts of nutrients supplied by lignocellulosic biomass itself for use as a raw material for fermentation remain unclear. To address this question, Lau et al. (2008) demonstrated that ethanol could be produced completely from sugars obtained from corn stover with no added nutrients. They applied a unique pretreatment, Ammonia Fiber Expansion (AFEX), which used no water or other solvent and so avoided leaching nutrients from the biomass. Their results suggested that the raw materials contained sufficient nutrients for fermentation. Lau et al. (2012) also found that biomass pretreated by AFEX had enough nutrients for the downstream fermentation.

On the other hand, common pretreatments, such as dilute acid, alkaline, and hydrothermal pretreatments, use water as the solvent and result in the leaching out of nutrients (Lau et al., 2008). In fact, producing ethanol by the fermentation of sugars derived from wheat straw using a hydrothermal pretreatment with no added nutrients did not provide a sufficient yield compared with the process using added nutrients (Jørgensen, 2009). This was also the case for lactic acid production (Sreenath et al., 2001).

Therefore, for a conventional water-based pretreatment, the use of leached nutrients is conceivable for fermentation with only a slight modification of the process.

In the present study, we investigated the use of nutrients naturally present in lignocellulosic biomass as supplements for the production of L-lactic acid by fermentation of the biomass. A simple nutrient recovery step was introduced before the processes of pretreatment, saccharification, and fermentation of the biomass. The nutrient balances were also elucidated in a series of experimental processes.

2. Materials and Methods

2.1 Materials

Dent corn (*Zea mays* L., KD730) was grown for 60 days in the late fall (mid-October to mid-December) at Kochi University (33°33.1' N, 133°40.7' E) and was harvested green. The plant density was set at 7 plants m⁻². The corn was cultivated as a catch crop, whose purpose was to take up the nutrients remaining in the soil to prevent their leaching into underground water (Fujiwara, 2012). The corn biomass above ground was used for the present study. The biomass was dried at 70°C, ground in a mill (Oster No. 6630, Jarden Consumer Solutions, Boca Raton, FL, USA), and kept in a desiccator

until use. The biomass hydrolyzate or analytical grade glucose was used as the sugar source in the following L-lactic acid fermentation.

Bacto™ yeast extract (Difco Laboratories, Franklin Lakes, NJ, USA), analytical grade ammonium chloride (NH₄Cl) and potassium dihydrogen phosphate (KH₂PO₄) were used as nutrients in the fermentation experiments to determine the required amounts of nitrogen and phosphorus. Meiselase (Meiji Seika Pharma Co. Ltd., Tokyo, Japan) was used for the saccharification of the pretreated biomass (Koreishi et al., 2009). Before Meiselase was used, its cellulase activity (filter paper unit; FPU) was determined under the saccharification conditions detailed in section 2.5 using the National Renewable Energy Laboratory protocol (NREL protocol; Adney and Baker, 1996).

2.2 Thermophilic L-lactic acid fermentation

Bacillus coagulans JCM 2258, purchased from Riken Bioresource Center (Tsukuba, Japan), was used to produce L-lactic acid (Akao et al., 2007). The strain was pre-incubated in LB medium at 55°C for 2 days with continuous shaking and then stored at room temperature as the stock culture solution.

Thermophilic L-lactic acid fermentation was performed in a 15-mL

sterile disposable tube, in which 10 mL of culture medium and 0.1 mL of the stock culture solution were mixed and kept at 55°C for 5 days in an incubator with continuous shaking. The culture medium consisted of glucose solution (25 g glucose L⁻¹; 4 mL), yeast extract solution (5 mL), and calcium carbonate solution (5.6 g L⁻¹; 1 mL). The glucose, yeast extract, and calcium carbonate solutions were autoclaved separately (121°C, 15 min) and mixed. The amount of yeast extract added was determined on the basis of the ratios of nitrogen and/or phosphorus to carbon (CN and/or CP ratios, respectively). When corn biomass was used, the glucose and yeast extract solutions were replaced with its hydrolyzate and nutrient solutions as described in sections 2.5 and 2.3, respectively.

2.3 Recovery of nutrients

Two types of nutrient recovery conditions were examined: one for recovering nutrients to investigate their availability during **thermophilic L-lactic acid fermentation** and the other for elucidating the nutrient balances in the experimental process. In the former case, nutrients were prepared using 300 g of dried and ground biomass soaked for 24 h in 3.0 L of distilled water at ambient temperature (Nagare et al., 2012). After extraction, approximately 1.7 L of primary nutrient solution was recovered after which

the residue was washed with 1.2 L of distilled water. The primary nutrient solution and washwater recovered were combined so that a total of 3.0 L of nutrient solution was obtained to use as an alternative yeast extract solution. A glass micro filter (GF/A; Advantec Toyo Kaisha, Ltd. Tokyo, Japan) was used for solid-liquid separation in the present study.

To evaluate the nitrogen and phosphorus balances in the experimental process, small-scale nutrient recovery was performed in triplicate using a 50-mL disposable tube to which 3 g of corn biomass and 30 mL of distilled water were added. After 1 h of continuous mixing using a shaker (200 rpm; NR-2, Taitec Co. Ltd., Koshigaya, Japan), approximately 30 mL of nutrient solution was recovered by the protocol mentioned above. The biomass recovered here was used in the following alkaline pretreatment and enzymatic saccharification.

2.4 Alkaline pretreatment

Alkaline pretreatment was used to promote the saccharification of the biomass. Following the recovery of the nutrient solution from each of the three 50-mL tubes described in section 2.3, 0.4 g of sodium hydroxide and distilled water to a total volume of 45 mL were added to each tube. After 1 day of continuous mixing using a shaker (100 rpm; NR-2) at ambient

temperature, the pretreated biomass was recovered and washed with 0.1 M sodium acetate buffer (pH 4.5) three times. The recovered biomass was dried at 80°C for 2 days after which the weight in each tube was measured to determine the recovery ratio through the nutrient recovery and pretreatment steps. The biomass was then crushed again using a mill (WB-1, Osaka Chemical Co. Ltd., Osaka, Japan) and passed through a 1-mm mesh screen.

2.5 Saccharification

Two conditions for enzymatic saccharification were used: one for determining the sugar recovery efficiency and the other for recovering sugars for **thermophilic L-lactic acid fermentation**. The first saccharification was performed in triplicate according to the NREL protocol (Selig et al., 2008). The conditions used were 45°C, pH 4.5 with 0.1 M sodium acetate buffer, and 3 days of incubation with continuous shaking (150 rpm; NR-2). Meiselase was added at 60 FPU per g of pretreated biomass. Sodium azide was used as an antibiotic.

For the sugar recovery saccharification, 3.0 g of pretreated biomass was saccharized in a 250-mL glass bottle. The conditions used were 45°C and 3 days incubation with continuous shaking using a bottle shaker (NK-3,

Thermal Kagaku Sangyo Co. Ltd., Tokyo, Japan). The biomass and 200 mL of 0.01 M sodium acetate buffer (pH 4.5) were separately autoclaved. Before the biomass and the buffer solution were mixed, 180 FPU of Meiselase was dissolved in the buffer solution and the solution was filtered using a sterile 0.2- μ m syringe filter. After incubation, the reaction was stopped by boiling and the solution containing sugars was separated using the GF/A filter. The solution was then concentrated using a centrifugal concentrator (VC-36R, Taitec Co. Ltd., Koshigaya, Japan) to obtain a glucose concentration of 25 g L⁻¹. The Meiselase solution (180 FPU in 200 mL acetate buffer) with no substrates after the above-mentioned incubation contained a marginal amount of sugars ($n = 3$; glucose, none detected; reducing sugars, 0.6 ± 0.0 g L⁻¹).

2.6 Determination of required CN and CP ratios

The performance-limiting factors for **thermophilic L-lactic acid fermentation** were assumed to be the amounts of nitrogen and phosphorus in the culture medium, hence the CN and CP ratios were defined to describe the amounts of nutrient required for **thermophilic L-lactic acid fermentation**. The carbon in glucose was considered in the calculation of CN and CP ratios as follows:

$$\text{CN or CP ratio} = \frac{\text{glucose weight (g) in the reactor} \times 0.4}{\text{nitrogen or phosphorus weight (g) in the reactor}} \quad (1)$$

The NH_4Cl and KH_2PO_4 were presumed to be able to compensate for any deficiency in nitrogen and phosphorus, respectively, in the culture media used for the investigation of the required CN and CP ratios.

The procedure was as follows: a gradual reduction in the amount of yeast extract added to **thermophilic L-lactic acid fermentation** of glucose was designed to determine the required CN and CP ratios. Once significant decreases in the L-lactic acid yield had been observed in the sequential experimental design, the limiting factor and its required additive level were identified by adding NH_4Cl and/or KH_2PO_4 . After one limiting factor had been determined, the required additive level of the other limiting factor was assessed by a further gradual reduction in the amount of yeast extract added and compensating for the first limiting factor by adding NH_4Cl or KH_2PO_4 . The effects of inoculum (stock culture solution) on the CN and CP ratios in the culture medium were also considered.

2.7 Availability of nutrients

If glucose yield is assumed to be 0.2 g g^{-1} based on the initial biomass weight, 60 g of glucose should be recovered from 300 g of corn biomass. Because 3 L of the solution was recovered from 300 g of the biomass

described in section 2.3, the glucose yield (60 g of glucose) means that a maximum of 50 mL of the solution is available for 1 g of recovered glucose, if they have both originated from the same biomass. The glucose concentration in the culture medium was set at 10.0 g L⁻¹ (0.1 g of glucose in 10-mL tube fermentation; section 2.2), indicating that a maximum 500 mL L⁻¹ of the nutrient solution can be supplied in the medium (5.0 mL of nutrient solution in 10-mL tube fermentation).

The **thermophilic L-lactic acid fermentation** of glucose with recovered nutrient solution was performed to test the substitutability of nutrient solution as a nutrient source. The glucose concentration was set at 10.0 g L⁻¹ in the culture medium and the additional amounts of recovered nutrient solution in the test tube were set at 100, 200, and 300 mL L⁻¹ (1, 2, and 3 mL addition to 10-mL tube fermentation; 20%, 40%, and 60% (v/v) addition to the available supply, i.e., 500 mL L⁻¹ of the nutrient solution). To evaluate the effect of nutrient compound hydrolysis, hydrochloric acid (12 N) was added to the nutrient solution before autoclave treatment. In total, 5 mL of diluted nutrient solution with distilled water and 125 or 250 µL of hydrochloric acid were used for hydrolysis. After autoclave treatment, the solution was neutralized with calcium carbonate before incubation.

2.8 Analytical method

The cellulose, hemicellulose, and lignin contents of the corn biomass were determined using the NREL protocol (Sluiter et al., 2008) with slight simplification (Akao et al., 2012). The total carbon, nitrogen, and phosphorus concentrations of the biomass, yeast extract, and Meiselase on a dry basis were determined using a CN coder (Vario EL III, Elementar Analysensysteme, Frankfurt, Germany) and ICP-AES (Vista-pro, SII, Chiba, Japan) following the dry ashing protocol (Park, 1996). The total nitrogen and phosphorus contents in the nutrient solution, the waste waters from pretreatment, and the concentrated hydrolyzate solution were determined using a total organic carbon and total nitrogen analyzer (TOC-VCSN/TNM-1, Shimadzu Co. Ltd., Kyoto, Japan) and ICP-AES following nitric acid–hydrochloric acid digestion (APHA, AWWA, WEF, 1998).

The reducing sugars content was determined by the DNS method using the NREL protocol (Adney and Baker, 1996). The glucose content was evaluated using a glucose kit (Glucose CII, Wako Pure Chemical Industries Ltd., Osaka, Japan). The D-,L-lactic acid content was determined by high-performance liquid chromatography (mobile phase, 5% 2-propanol and 1 mM CuSO₄ solution; column, OA-5000, Sumika Chemical Analysis Service Ltd., Osaka, Japan; UV detector, 254 nm). The optical purity of L-lactic acid

was calculated as follows:

$$OP_L = \frac{(L-LA) - (D-LA)}{(L-LA) + (D-LA)} \times 100\%, \quad (2)$$

where OP_L is the optical purity of L-lactic acid (%) and D^-,L -LA is the concentrations of D-,L-lactic acid (mg L^{-1}).

The L-lactic acid yield (g g^{-1}) was calculated by dividing the final L-lactic acid amount (g) by the initial glucose amount (g) in the reactor. Each experimental condition was repeated five times. The Student's t test or the Games–Howell's multiple range test were performed using SPSS version 19.0.0 (IBM, Chicago, IL, USA) to assess the significance of the differences in the L-lactic acid yield ($p < 0.05$).

3. Results and Discussion

3.1 Composition of experimental materials

The estimated composition of the corn biomass on a dry weight basis was determined as 24.8% cellulose, 14.9% hemicellulose, and 18.0% ash. The total carbon, nitrogen, and phosphorus concentrations in the corn biomass, yeast extract, and Meiselase on a dry basis are summarized in Table 1.

3.2 Requisite CN and CP ratios

Thermophilic L-lactic acid fermentation with reducing yeast extract

concentrations indicated that the minimum added nutrients were CN = 26.5 and CP = 277, based on the L-lactic acid yield (Figure 1A). Fermentation accompanied by the addition of NH_4Cl (CN = 22.5) and/or KH_2PO_4 (CP = 234) under even lower levels of added yeast extract (CN = 30.3 and CP = 318) showed that phosphorus was the limiting factor, with an adequate CP ratio of 234 (Figure 1B). In view of the L-lactic acid yield under the conditions of CN = 26.5 and CP = 277 (Figure 1A), the required CP ratio was concluded to be ≤ 277 . Increasing the CN ratio with a reduced addition of yeast extract, at a fixed CP ratio of 234 using KH_2PO_4 , increased the required CN ratio to 69.6 (Figure 1C).

The CN ratio of approximately 70 is somewhat higher than those reported for L-lactic acid production (e.g. CN = 37; Smith and Holtzapple, 2010). One reason for this might have been the long fermentation period, meaning that a gradual depression of the L-lactic acid production rate might occur with an increasing CN ratio without lowering the yield during the 5-day fermentation. Uppugundla et al. (2014) have demonstrated that the ethanol fermentation of corn stover pretreated by a dilute acid method led to a reduction in the ethanol production rate when no nutrients were added, although the ethanol yields with or without nutrient supplements were almost the same.

Rittmann and McCarty (2001) reported that the nitrogen content of bacterial cells can be reduced to approximately one half of its usual percentage by weight during nitrogen starvation conditions, thus reducing the potential growth rate. Under normal bacterial cell conditions, they also reported that the required phosphorus amount was one fifth to one seventh of that for nitrogen on a weight basis. In the present study, the required CP ratio was determined to be ≤ 277 , so that the CN ratio would be expected to be less than 40–55 on a weight basis. This almost corresponds to 35 under normal bacterial cell conditions and one half of the CN ratio of 70 obtained under nitrogen starvation conditions.

The required CN ratio was confirmed using a stoichiometric equation (equation 3) for microbial synthesis and growth (Rittmann and McCarty, 2001). To use this equation, the product yield is needed. The average measured product yield in complete fermentations in Figure 1 was 0.84 ($n = 30$; D-,L-lactic acid, $8.4 \pm 0.3 \text{ g L}^{-1}$; optical purity, $97.4 \pm 1.9\%$). Using equation 3, this yield corresponds to a CN ratio of 27. Therefore, the sufficient CN and CP ratios for **thermophilic L-lactic acid fermentation** would be 27–35 and 234, respectively. The CN ratio obtained of 27–35 agreed with reported values (Smith and Holtzapple, 2010).

3.3 Nutrient supply by corn biomass extract

Approximately 3.0 L of nutrient solution was obtained from 300 g of the corn biomass. The concentrations of total carbon, nitrogen, and phosphorus were 4,559 mg L⁻¹, 956 mg L⁻¹, and 139 mg L⁻¹, respectively. Marginal amounts of sugars and D-,L-lactic acid were present in the nutrient solution (glucose, none detected; reducing sugars, 0.3 g L⁻¹; D-,L-lactic acid, 0.2 g L⁻¹). Based on the nitrogen and phosphorus concentrations, adding 100 mL L⁻¹ (1 mL addition in the tube reactor; 20% (v/v) addition to the available supply) of the nutrient solution to the total culture media volume would provide almost enough nitrogen and phosphorus for **thermophilic L-lactic acid fermentation** (CN = 35.4, CP = 265) when the glucose concentration in the medium was 10 g L⁻¹. The experimental design and results are summarized in Figure 2.

Fermentation with a 20% (v/v) addition of nutrient solution resulted in a low yield of L-lactic acid ($n = 5$; L-lactic acid, 1.8 ± 0.1 g L⁻¹; optical purity, $93.8 \pm 0.5\%$), although the required amounts of nitrogen and phosphorus were provided. It may be difficult for *B. coagulans* to use the nitrogen and phosphorus directly in the nutrient solution. Fermentation with the 20%

addition of nutrient solution and the given amounts of hydrochloric acid improved L-lactic acid production but resulted in insufficient yields ($n = 5$; L-lactic acid, $4.7 \pm 0.2 \text{ g L}^{-1}$; optical purity, $97.7 \pm 1.1\%$). The experimental conditions with a 40% (v/v) addition of nutrient solution (CN = 19.2, CP = 139) led to significant improvement in L-lactic acid yields compared with the conditions mentioned above, based on the multiple-range test ($p < 0.05$).

The sufficient fermentation of L-lactic acid using the 40% (v/v) addition of nutrient solution indicates that **thermophilic L-lactic acid fermentation** can be performed using only corn biomass as sources of sugar and nutrient material derived from the biomass itself. During the industrial fermentation of sugars, byproducts of the agro-industry such as corn steep liquor can be used as nutrient sources because they are inexpensive (Davis et al., 2013). However, these byproducts incur some costs, which would not be negligible relative to the prices of final products such as bioethanol. Using organic wastes as nutrients for fermentation is thus attractive and has been considered for commercial industrial use (Thomsen, 2005).

The present study has shown that extracting nutrients from a primary raw material simply by soaking in water could provide an alternative nutrient source in fermentation. Furthermore, the nutrients in the present study could also be supplied at the site of the fermentation plant,

thus reducing the transportation cost of nutrients. However, the required amounts of nitrogen and phosphorus supplied by the nutrient solution for *thermophilic L-lactic acid fermentation* were higher compared with those supplied by the yeast extract.

3.4 Sugar recovery and its fermentation using nutrients from corn biomass

A nutrient recovery step was introduced before the ordinary water-based pretreatment (Figure 3). The saccharification efficiency of the alkaline pretreatment of corn biomass following nutrient recovery was evaluated in 50-mL disposable tubes in triplicate. The resulting weight recovery ratio was 0.51 ± 0.00 ($n = 3$). Using this value, the recovery ratios of glucose and reducing sugars based on the dry weight of the original corn biomass were calculated as 0.23 ± 0.01 and 0.35 ± 0.01 , respectively ($n = 3$). Indeed, the glucose recovery ratio was low compared with reported saccharification efficiencies (Uppugundla et al., 2014) but can be accounted for by the low cellulose content (24.8%) in the biomass tested. The low cellulose content may have been caused by a relatively short cultivation period (60 days) and a low ambient temperature during this period (average daily temperature approximately 15°C; <http://www.data.jma.go.jp/gmd/risk/obsdl/index.php>). The glucose recovery

ratio based on the cellulose content and an anhydro correction (0.90; Sluiter et al., 2008) was determined as 0.84, which can be considered acceptable (Gould, 1984).

Sugars were recovered from the same pretreated biomass using a 200-mL glass bottle for **thermophilic L-lactic acid fermentation**. The ratios of glucose recovery before and after autoclave sterilization were 0.23 and 0.20, respectively, based on the original dry biomass weight; those of reducing sugars were 0.37 and 0.31, respectively. An approximately 15% loss of sugars was observed in the autoclave treatment. **Thermophilic L-lactic acid fermentation** of the autoclaved hydrolyzate and adding a 40% (v/v) recovered nutrients was used to assess the feasibility of the proposed process. Based on the L-lactic acid yield from the initial glucose weight in the media ($n = 5$; L-lactic acid yield, 1.04 ± 0.05 ; optical purity, $97.1 \pm 0.3\%$), the recovered nutrients provided a sufficient yield for **thermophilic L-lactic acid fermentation** (Figure 4). The CN and CP ratios were calculated as 13.0 and 104, respectively. The proposed process could thus be demonstrated as successful, at least when using young corn biomass as the sugar and nutrient source and *B. coagulans* as the fermenting organism. The proposed process could be used on other lignocellulosic materials to test its potential. Confirming the assimilation of the recovered nutrients by other

microorganisms is another challenge for applying the process to other types of fermentation such as ethanol fermentation, given that auxotrophy for growth depends on species.

3.5 Nutrient balances in the experimental process

The **thermophilic L-lactic acid fermentation** of the hydrolyzate with no nutrient supplement in Figure 4 was halved ($n = 5$; L-lactic acid yield, 0.53 ± 0.05 ; optical purity, $86.2 \pm 2.0\%$). A similar observation was reported by Sreenath et al. (2001) and Jørgensen (2009), indicating that the hydrolyzate contained some nutrients for bacterial growth. In this case, the CN and CP ratios were calculated as 38.7 and 444, respectively. Indeed, nutrients originating from the corn biomass may remain after pretreatment and saccharification. Cellulase, added in the saccharification step, could also be a possible nutrient source. However, the effects of these nutrient sources on the product yield are unknown. Accounting for these possible nutrient sources in the calculation of the required amounts of nutrients would promote a cost-effective operation for processing lignocellulosic biomass. Therefore, the nitrogen and phosphorus balances in the proposed process were evaluated (Figure 5).

When using the 40% (v/v) addition of recovered nutrients, the

amounts of added nitrogen and phosphorus accounted for 67 and 77%, respectively, of their total weight in the fermentation. The additional nutrients exerted a large effect on the fermentation conditions, especially regarding the amounts of nitrogen and phosphorus. The addition of cellulase would provide enough nitrogen for the subsequent fermentation. However, autoclave treatment (heat treatment) for sterilization followed by precipitation and filtration (GF/A) reduced the amount of nitrogen by 89%. Cellulase is easily denatured and precipitated by heat treatment. The amounts of phosphorus carried over and added in the saccharification step were marginal so a further addition was required in the fermentation step, although 85% of the phosphorus in the saccharification step remained after autoclave treatment. Phosphorus in native biomass can easily be recovered by aqueous pretreatment so it would be reasonable to use it.

Large amounts of nitrogen and phosphorus present in raw materials, such as corn biomass and enzymes, might not remain available until the subsequent fermentation step if sterilized by heat. Such a treatment would lead to the loss of nutrients, especially nitrogen and phosphorus, as well as sugars mentioned in section 3.4. Avoiding heat treatment, by using a non-sterile fermentation, might increase the final product yield and reduce the amount of nutrients added for the fermentation step.

4. Conclusions

A process for the production of thermophilic L-lactic acid by fermenting sugars and nutrients derived from the same lignocellulosic biomass has been proposed and demonstrated. Nutrients were recovered from young dent corn biomass by soaking in water before alkaline pretreatment. The recovered nutrients could be used as a commercially feasible alternative in thermophilic L-lactic acid fermentation. The amounts of nutrients recovered were sufficient to support the thermophilic L-lactic acid fermentation of recovered sugars derived from the same biomass. The nitrogen and phosphorus balances in the proposed process indicated that nutrient recovery during the initial stage was reasonable and that adding the recovered nutrients was essential for the subsequent thermophilic L-lactic acid fermentation.

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Figure Captions

Fig. 1. L-Lactic acid yield in each batch incubation ($n = 5$). Bars indicate standard deviations. A: yeast extract added for a CN ratio of 22.5 - 34.0. B: yeast extract added for a CN ratio of 30.3. CN and/or CP were adjusted to 22.5 and/or 234. C: yeast extract added for a CN ratio of 64.9 - 78.7, and the CP ratio was adjusted to 234. Different lowercase letters indicate significant differences ($p < 0.05$).

Fig. 2. L-Lactic acid yields in batch incubations using recovered nutrients with and without acidic hydrolysis ($n = 5$). Bars indicate standard deviations. Percentage of nutrient addition was consistent with the available supply amount based on possible recovered glucose and that of hydrochloric acid addition was consistent with the total culture media volume. 20% (v/v) nutrient addition, CN = 35.4, CP = 265; 40% (v/v) nutrient addition, CN = 19.2, CP = 139; 60% (v/v) nutrient addition, CN = 13.1, CP = 94.

Fig. 3. An experimental process equipped with a nutrient recovery step.

Fig. 4. L-Lactic acid yields from corn biomass hydrolyzate with and without

recovered nutrients ($n = 5$). Bars indicate standard deviations and asterisks indicate significant differences ($p < 0.05$). The hydrolyzate and nutrients were derived from the same corn biomass. The amounts of nutrients available were set as the total amounts of nutrient divided by the total amounts of recovered glucose. For the nutrient addition case, a 40% amount was added to the reactor.

Fig. 5. Nitrogen and phosphorus balances in the experimental process based on the use of 1 g of young corn biomass. Cellulase (Meiselase, 60 FPU per g-substrate after alkaline treatment) and recovered nutrients (40% amounts recovered in a nutrient recovery step) were added in this process. The values in boxes were determined by experimental analysis and those in italics by calculation. Glucose amounts are also presented; initial values were calculated using the cellulose content (24.8%) and an anhydro correction (0.90).

617   **Table 1** Average carbon, nitrogen, and phosphorus contents in young corn
618   biomass, yeast extract, and cellulase (weight %).

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	Carbon (<i>n</i> = 3)	Nitrogen (<i>n</i> = 3)	Phosphorus (<i>n</i> = 2)
Young corn biomass ^a	41.78	3.37	0.28
Yeast extract ^a	39.25	11.06	1.22
Cellulase ^a	43.47	6.94	0.07

620   ^a See 2.1. Data taken from the lots used in the present study.

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Fig. 1.

Fig. 2.

Experimental conditions			Significant differences								
Level	Extract	HCl	in L-lactic acid concentration								
	Addition	Addition	Level ¹								
	(% v/v)	(% v/v)	1	2	3	4	5	6	7	8	9
1	20	0		*	*	*	*	*	*	*	*
2	20	1.25	*		-	-	*	*	*	*	*
3	20	2.5	*	-		-	*	*	*	*	*
4	40	0	*	-	-		-	-	-	-	-
5	40	1.25	*	*	*	-		-	-	-	*
6	40	2.5	*	*	*	-	-		-	*	*
7	60	0	*	*	*	-	-	-		-	-
8	60	1.25	*	*	*	-	-	*	-		-
9	60	2.5	*	*	*	-	*	*	-	-	

¹ *, significant difference ($p < 0.05$); -, no significant difference

Fig. 3.

Fig. 4.

Fig. 5.

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