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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.

1. Introduction

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

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

Nutrients for fermentation consist primarily of nutrient salts, 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.,

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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–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 (NH_4Cl) and potassium dihydrogen phosphate (KH_2PO_4) 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