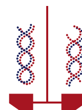

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Production of bioethanol from Napier grass via simultaneous saccharification and co-fermentation in a modified bioreactor

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The aim of this study was to use a modified bioreactor system for simultaneous saccharification of cellulose and bioethanol production. We tested *Aspergillus niger* and *Trichoderma reesei* for cellulose saccharification and *Zymomonas mobilis* for bioethanol production simultaneously in this modified bioreactor. The results showed that various carboxymethylcellulose (CMC) concentrations (10, 15, or 20 g/L) as a substrate for *A. niger* and *T. reesei* yielded bioethanol production of 0.51, 0.78, and 0.89 g/L and a CMC conversion rate of 10.2%, 10.7%, and 8.89%, respectively. These data suggested that at 10 g/L CMC as a substrate, the CMC conversion rate was higher than that at the other concentrations. When CMC concentration exceeded 15 g/L, bioethanol production was prolonged to 40 h. These results were attributed to the viscosity of CMC. This study also tested Napier grass (an agricultural byproduct) for bioethanol production. The results revealed bioethanol production and the theoretical Napier grass conversion rate were 0.38 g/L and 12.6%, respectively, for 13-h cultivation when the feeding concentration of Napier grass was 10 g/L. When Napier grass concentration was increased to 15 g/L, bioethanol production and the Napier grass conversion rate reached 0.51 g/L and 23%, respectively, after 14-h cultivation. Eventually, the experimental results indicated using agricultural waste for bioethanol production has been become a potential strategy.

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[**Key words:** Saccharification; Carboxymethylcellulose; Bioethanol; Modified bioreactor; Napier grass]

With the developing industrialization, energy consumption increases year after year (1). Because the price of fossil fuels increases gradually, reductions in the CO_2 release have become a main trend (2,3). The raw materials for bioethanol production are widely available, but to avoid food deficits or a steep rise in food prices, researchers made lignobioethanol a mainstream product (4). Many types of fruit in Taiwan can yield agricultural waste after food processing. This agricultural waste is rich in celluloses. Conventionally, the solution to the waste problem depends on composting on farms or burying of waste in soil resulting in a loss of cellulose. Nevertheless, learning how to utilize this agricultural waste and to transform it into bioenergy can balance waste processing and new energy development.

Several merits of lignocellulose as a raw material for bioenergy production can be listed: (i) low cost, (ii) multiple sources, (iii) the absence of a conflict with food production, and 4) fuel from lignin. Agricultural waste in Taiwan is mainly Napier grass and rice stalks. These types of waste consist of cellulose (40%–50%), hemicelluloses (25%–35%), and lignin (15%–25%) (5–7). The composition of lignocellulosic biomass depends on its type and components. Because Napier grass is 50% cellulose, how to utilize this grass effectively is a major problem for the development of industrial biotechnological applications.

The steps for transforming agricultural waste into bioethanol include pretreatment of raw materials, saccharification, and fermentation by microorganisms (8,9). The pretreatment of agricultural waste is the most effective step for enhancement of the saccharification efficiency and for lowering the costs. The purposes of pretreatment are to reduce the proportion of lignocellulose and crystallization level of lignocellulose, to degrade lignocellulose, and to increase specific area of lignocellulose (10). The pretreatment of lignocellulose mainly involves chemical processing (including an acid or alkali [e.g., ammonia]). The polymerization degree of hemicelluloses and cellulose can be reduced to increase the saccharification efficiency when lignocellulose is processed by the acid method. The purposes of pretreatment with alkali are to increase the porosity and specific surface area and thus to decrease the crystallization degree of cellulose and to degrade cell cellulose (11,12). Finally, these degraded celluloses are transformed into reducing sugars (glucose) by saccharification enzymes from a fungus such as *Trichoderma reesei* and *Aspergillus niger*. These reducing sugars are then utilized by *Zymomonas mobilis* to produce bioethanol (13–19). A modified bioreactor that utilizes

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simultaneous saccharification and co-fermentation (SSCF) was used for the effective conversion of Napier grass into bioethanol. Previous studies have reported the conversion of cellulose into monosaccharides using microorganisms (11,13). Furthermore, SSCF also presents several advantages such as higher product yield, shorter processing time, and lower reactor volume.

The aim of this study was to enhance bioethanol production by means of various pretreatments of Napier grass for mixed cultivation of *T. reesei* and *A. niger* in an immobilization system and then for production of bioethanol by *Z. mobilis* to achieve SSCF (20–22).

MATERIALS AND METHODS

Microorganisms and media *T. reesei* BCRC 31863 and *A. niger* BCRC 31130 were obtained from the Bioresource Collection and Research Centre (BCRC) of Taiwan. The stock culture was maintained aseptically on potato-dextrose-agar (PDA) petri plates (BD, NJ, USA). The PDA plates were incubated at 30°C for 7 days until good sporulation and then were stored at 4°C. *Z. mobilis* BCRC 10809 was obtained from the BCRC of Taiwan. The stock culture medium in a plate consisted of yeast extract (5.0 g/L, BD), glucose 20 g/L (Sigma-Aldrich, MD, USA), and agar 20 (g/L, Sigma) at pH 6.8, with cultivation at 30°C for 2 days. Bushnell-Haas selection E medium (BHSD) was used for cultivation of *T. reesei* spores, *A. niger* spores, and *Z. mobilis*, and consisted of carboxymethylcellulose (CMC, 10 g/L, Sigma), $MgSO_4 \cdot 7H_2O$ (0.4 g/L, Sigma), KH_2PO_4 (1 g/L, Sigma), $(NH_4)_2HPO_4$ (1 g/L, Sigma), $CaCl_2$ (0.02 g/L, Sigma), and $FeCl_3$ (0.04 g/L, Sigma).

Dilute-alkali pretreatment of Napier grass Air-dried Napier grass samples were cut into 2-cm pieces. Fifty grams of Napier grass was mixed with 750 mL of 1% NaOH (Sigma) in a 1-L bottle and autoclaved at 121°C for 15 min, then washed three times with 500 mL of distilled water, and dried at 100°C for 48 h. Finally, the pretreated samples were ground, sieved, and stored separately (23).

Dilute-acid pretreatment of Napier grass The air-dried Napier grass samples were cut into 2-cm pieces. Fifty grams of Napier grass was mixed with 750 mL of 2% H_2SO_4 (pH 2.0) (Sigma) in a 1-L bottle and autoclaved at 121°C for 20 min, washed three times with 250 mL of distilled water, and dried at 100°C for 48 h. Finally, the pretreated samples were ground, sieved, and stored separately (24).

Cell immobilization Concentrated *Z. mobilis* (3.25×10^6 cells/mL) were mixed with sodium alginate (to obtain a 4.0% [w/v] sodium alginate solution). The alginate beads were prepared by syringing the well-mixed 3% sodium alginate solution by means of a 16-gauge needle into a 3% $CaCl_2$ (Sigma) solution. Finally, the alginate beads with bacteria were harvested by washing with phosphate buffer.

Bioethanol production in the modified bioreactor Fig. 1 shows a schematic diagram of a modified bioreactor. The operation process of this modified bioreactor was according to our previous study (25). Briefly, this procedure involved cocultivation of the three species (*T. reesei*, *A. niger*, and *Z. mobilis*). First, *T. reesei* and *A. niger* were cultivated on a polyurthane (PU) (Sigma) carrier with a fresh BHSD medium to significantly increase microbial proliferation (the precultivation step), and *Z. mobilis* was entrapped into alginate (Sigma) beads in the meantime. Second, the BHSD medium was removed, and the microbe-laden alginate beads were placed into the lower anaerobic section of the bioreactor after the precultivation step. The fresh medium for bioethanol production was fed into the bioreactor to produce bioethanol.

Analysis of reducing sugars and bioethanol For comparative purposes, total reducing sugar present in the hydrolysate was also estimated by the dinitrosalicylic acid (DNS) method (17). Briefly, 3 mL of DNS was added to an equal volume of the reducing sugar sample in a lightly capped test tube. The mixture was then heated at 90°C for 5–15 min to develop the characteristic reddish-brown color. One milliliter of a 40% potassium sodium tartrate solution was added to the mixture to stabilize the color. After cooling to room temperature in a cold water bath, absorbance was recorded using a spectrophotometer at a wavelength of 575 nm. The OD 575 values for the reducing sugar sample and blanks were subtracted from those of the analyzed sample. Samples for HPLC analysis were centrifuged and passed through 0.2-μm filters. Concentration of bioethanol was determined using an Aminex HPX-87H column (Bio-Rad, USA) at 65°C with an isocratic mobile phase elution of 0.05 mM H_2SO_4 at 0.6 mL/min. Standards were prepared and used to quantify unknown samples. All samples were analyzed in triplicate (n = 3) to obtain a mean value.

RESULTS AND DISCUSSION

The CMC is artificial cellulose that is often used as a substrate for bioethanol production by microorganisms. Thus, in this study, we also utilized CMC as a substrate for bioethanol production to evaluate the cellulose digestion ability and bioethanol production potential of *T. reesei*, *A. niger*, and *Z. mobilis* when the three stains were cocultivated.

Effects of CMC concentrations on bioethanol production The inocula (*A. niger* 5.5×10^9 spores and *T. reesei* 6.5×10^9 spores) were added into the HSD medium for 72-h precultivation and then, we changed the medium to BHSD for bioethanol production. As shown in Fig. 2, the bioethanol production, the lignocellulose conversion rate, and biomass reached 0.56 g/L, 11.1%, and 1.93 g, respectively, at the above-mentioned inoculum composition. Cellobiose started to accumulate at the time point 6 h of cultivation, and reached 0.082 g/L at 9 h of cultivation and then decreased to zero by the end of the experiment. This result suggested that cellobiose was rapidly converted to glucose by a saccharification enzyme to serve as a nutrient for *Z. mobilis* during production of bioethanol.

On the basis of the results mentioned above, various high CMC concentrations (10, 15, and 20 g/L) were tested to verify the bioethanol production. The results in Fig. 3A show that bioethanol production amounted to 0.78 g/L at 45 h of cultivation, and glucose was consumed almost completely after 18 h of cultivation. Fig. 3B shows similar results, namely, that bioethanol production was 1.2 g/L after 45 h of cultivation, and glucose was consumed almost completely by 21 h of cultivation. These data could be attributed to viscosity of the high CMC concentration resulting in slow substrate circulation by the pump. Therefore, increasing the CMC concentration may significantly improve bioethanol production. On the other hand, the fermentation duration can also be extended when CMC concentration exceeds 10 g/L.

An experiment with repeated fed-batch cultivation According to the above results, to overcome the disadvantages of a high CMC concentration, the repeated fed-batch cultivation strategy was evaluated by replacing the CMC substrate

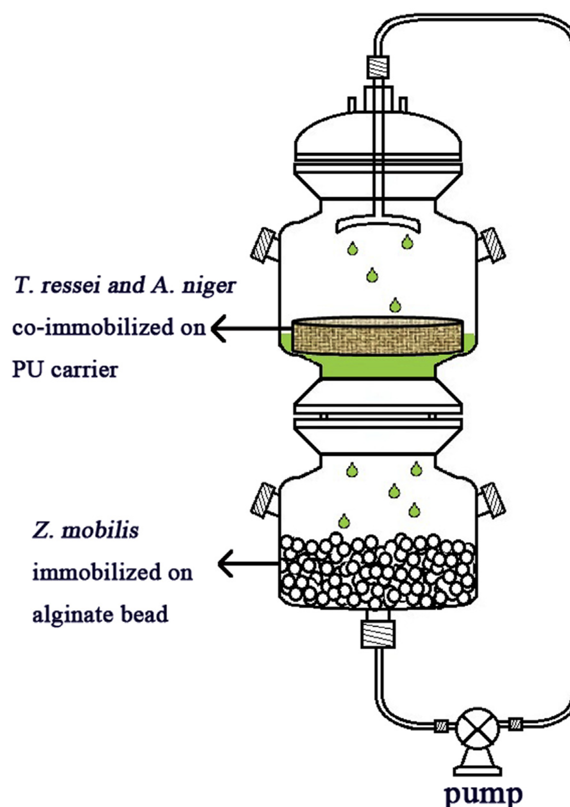

FIG. 1. The scheme of the modified bioreactor used in this study (25).

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