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The Use of a Gas Chromatography/Milli-whistle Technique for the On-line Monitoring of Ethanol Production Using Microtube Array Membrane Immobilized Yeast Cells

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Hollow, poly(L-lactic acid) microtube array membranes (MTAM) were used in preparing membranes that contained immobilized yeast cells. To evaluate the performance of the developed system for continuous and fed-batch fermentation, a gas chromatography/milli-whistle device was used to on-line monitor the production of ethanol. The milli-whistle was connected to the outlet of a GC capillary, and when the fermentation gases and the GC carrier gas passed through it, a sound with a fundamental frequency was produced. The online data obtained for frequency-change *vs.* retention time can be recorded after a fast Fourier transform. In typical bioethanol fermentation, the yeast cells cannot be recycled, whereas the artificial yeast-MTAMs can be. The hollow-MTAM containing immobilized yeast cells significantly enhanced to bioethanol productivity, and represent a novel, promising technology for bioethanol fermentation. Our data indicate that the gas chromatography/milli-whistle device, which is economical and stable, is a very useful detector for long-term monitoring.

Keywords Gas chromatography, whistle, ethanol, yeast immobilization, yeast-MTAM

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Introduction

Immobilized yeast can effectively reduce the negative effects of inhibitors and the processing cost of preparing an inoculum for use in the continuous or fed-batch fermentation of microorganisms can be reduced to a considerable extent.[1-6] This is possibly because that in the case of the fermentation by microtube array membrane (MTAM)-immobilized yeasts, the yeasts are contained in the MTAM with a local higher cell density, whereas the inhibitors are evenly dispersed in solution. This leads to a higher ratio of cells to inhibitors inside MTAM (as compared to free cells), making the cells in a better position to metabolize the inhibitors into something less toxic to cells, such as to change furans into furfuryl alcohols. Thus, as compared to the free cells, MTAM-immobilized cells could be more tolerant to the inhibitors. Although the current techniques available for the immobilization of yeast cells have some drawbacks, we were able to use a highly porous hollow MTAM to immobilize *Saccharomyces cerevisiae* to form yeast-MTAMs for bioethanol fermentation.[7-11] The porous hollow yeast-MTAMs were developed by using a coaxial electrospinning technique. They were functionalized by creating nanopores on the surface using a pore-forming agent, followed by a leaching-out process, as described previously.[12-16] In order to evaluate the efficiency of the porous hollow yeast-MTAMs for bioethanol fermentation, we used glucose as the model biofuel according to the literature.[17] The gases generated from the bioethanol conversion process was monitored by a GC (gas chromatography)/whistle system.[18-20] In fact, we previously reported on the development of a novel universal detector for gas detection in which a milli-whistle was used as a detector. The milli-whistle was connected to the outlet of the GC capillary and when the GC-eluants and carrier gas pass through the whistle, a sound is produced. After a fast Fourier transform, the sound wave that is generated from the milli-whistle is picked up by a microphone. Thus, by this technique, the online quantitative determination of ethanol produced from glucose by using porous hollow yeast-MTAMs was achieved. Poly-L-lactic acid (PLLA) was used to prepare nano-porous MTAMs, since it is an environment friendly-material;[9] further *Saccharomyces cerevisiae* was selected as the model yeast. The conditions for preparing the yeast-MTAMs and the potential for repeated fed-batch bioethanol fermentation were evaluated and optimized. Details of the experimental conditions are reported and the real-time relationship between the frequency-shifts and ethanol concentration are also discussed.[21]

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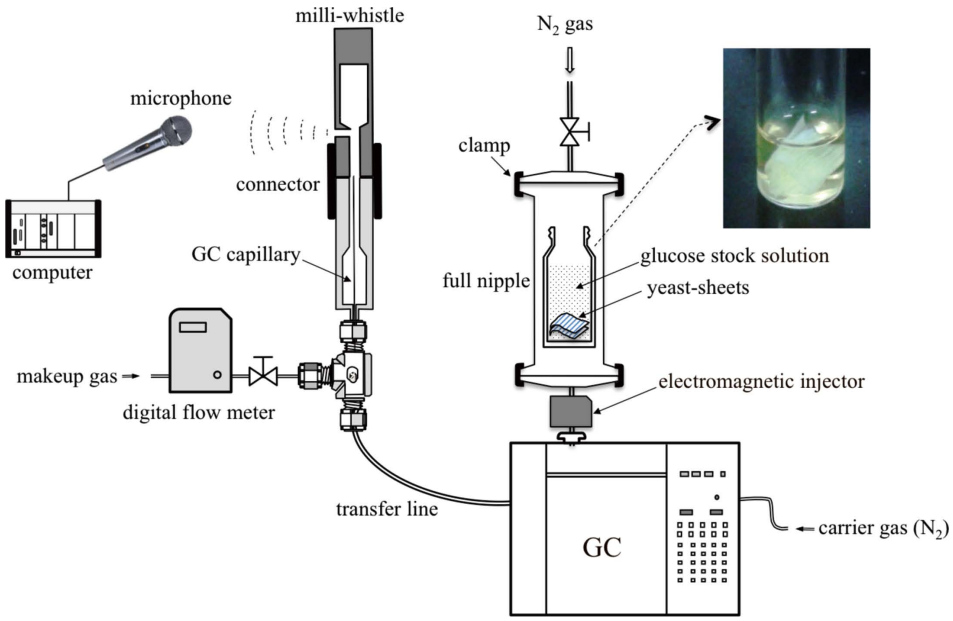

Fig. 1 Schematic diagram of the GC/whistle-accelerometer setup. The inset photo shows the actual yeast-MTAMs in a glass vial.

Experimental

Materials

The *Saccharomyces cerevisiae* (BCRC 21686) was purchased from Bioresources Collection and Research Center (BCRC) in Hsinchu, Taiwan. A commercial dry yeast powder (*Saccharomyces cerevisiae*; Fermiport, France) was purchased from a local supermarket. Peptone and the yeast extract were obtained from Fromedium (UK). D-Glucose (99%) was purchased from Showa Co. (Japan). DISPERBYK (wetting and dispersing additive) was obtained from BYK Additives & Instruments (Germany). PLLA (poly-L-lactic acid; M_w, 10 kDa) was obtained from BioTechOne, Taiwan. Polyethylene glycol/polyethylene oxide (PEG; M_w, 35 kDa/PEO; M_w, 900 kDa) was purchased from Sigma-Aldrich. *N*,*N*-Dimethyl formamide (DMF) and dichloromethane (DCM) were purchased from Merck (USA) and Seedchem (USA), respectively.

Apparatus

A gas chromatograph (GC 5890; Hewlett-Packard, Avondale, PA) equipped with a DB-VRX column (30 m × 0.45 mm × 2.55 μm) was used in this study. A commercial electrospinning machine (Nanon-01A; MECC Co., Ltd., Japan) was used for preparing the artificial yeast-MTAMs, *i.e.* immobilized yeasts. A tabletop scanning electron microscope (TM3030; Hitachi, Japan) and a viscometer (SV-10; A&D Co., Ltd., Japan) were also used.

Solutions

The *Saccharomyces cerevisiae* was cultured in a yeast peptone dextrose media (containing 1% yeast extract, 2% bactopeptone and 5% dextrose, respectively; pH no control) at 26°C to reach a cell density of 10^9 cfu/mL for immobilization;[9,21,22] cfu, colony-forming unit. To prepare the electrospinning dope, PLLA was dissolved in mixed DCM/DMF (8/2; v/v) solvents at room temperature to obtain a 10% solution. PEG was added to the PLLA solution to obtain a PEG/PLLA (30/70; v/v) solution.[9]

Shell solution. DCM (32 mL) and DMF (8 mL) were mixed in a glass vial (50 mL). Following this, 4.2 g of PLLA and 1.8 g PEG were added to the vial, and the mixture was dissolved using an electromagnetic stirring bar (300 rpm) overnight. DISPERBYK (0.4 mL) was then added to the vial and the mixing continued for 2 additional hours. The mixing velocity of this solution was 300 – 600 cp.

Core solution. DI water (40 mL) was added to a 50-mL glass vial. Following this, 2.0 g PEG and 2.0 g PEO were added to the vial, respectively, and the resulting mixture was dissolved by stirring (300 rpm) overnight. The *Saccharomyces cerevisiae* cells (4.89 g; cell density of 10^9/mL) were added to the mixed solution and the resulting suspension was stirred for 2 more hours. The stirring velocity of this solution was 1300 cp. Preparation of PLLA hollow-MTAMs with immobilized *Saccharomyces cerevisiae*.

A commercial electrospinning machine was used to prepare the hollow-MTAMs, using an in-house fabricated co-axial spinneret. The shell and core solutions were delivered, by passing them through the co-axial spinneret. Syringe pumps were set at rates of 5 mL/h and 5 mL/h for the shell and core solutions, respectively, where a 5-kV voltage was applied. The distance between the moving spinneret (speed, 1 mm/s) and rotating drum collector (200 rpm) was 5 cm. All electrospinning procedures were performed in a chamber at a relative humidity of 50 ± 5% and a temperature of 25 ± 1°C.

Results and Discussion

Figure 1 shows a schematic diagram of the GC/milli-whistle setup, which was similar to that used in our previous study.[18-20] The whistle, made of brass, was 1 mm in diameter and 5 mm in length. The sound, generated from the whistle, was detected and recorded by a computer microphone and built-in sound card, respectively; data points were acquired at 0.15 s intervals. An standard microphone was used to pick up the frequency changes, in which nitrogen gas was used as the carrier gas and

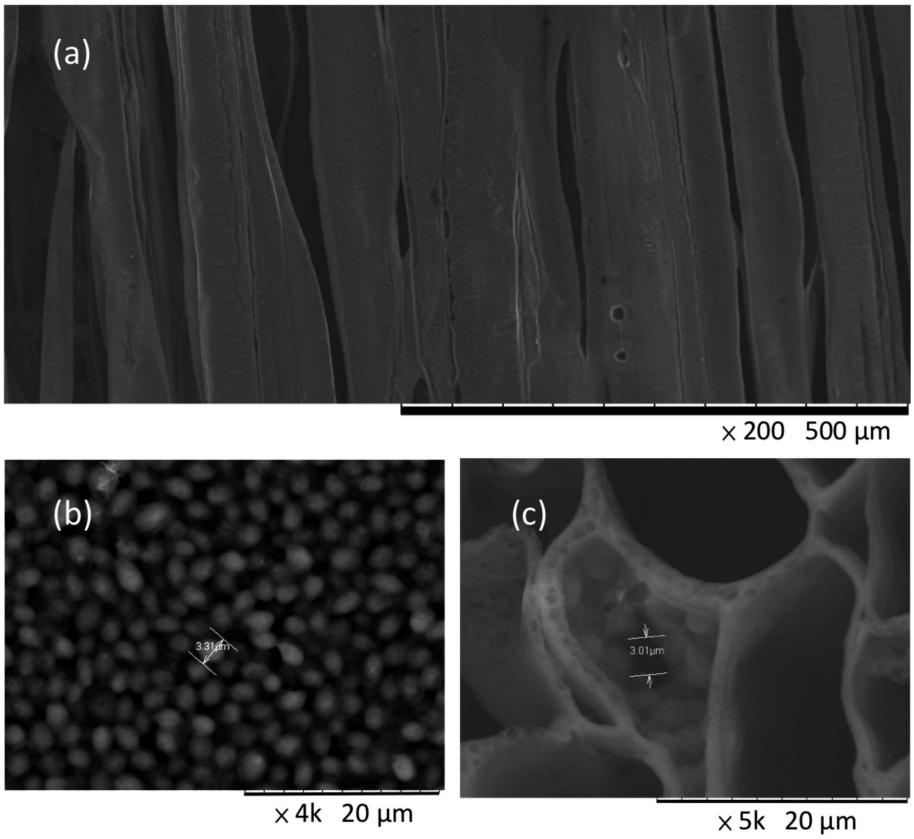

Fig. 2 SEM section images of (a) PLLA yeast-MTAMs and light microscopy images of (b) free *Saccharomyces cerevisiae* and (c) PLLA yeast-MTAMs immobilized *Saccharomyces cerevisiae* prepared by an *in situ* method.

makeup gas. The LabVIEW program (National Instruments, USA) was used for real-time frequency monitoring. A NW full nipple (flange, KF 16; material, 304 S.S.) was used as the reservoir to hold the solution vial. Both sides of the NW full nipple were connected with NW clamps. A tape heater was used to maintain the temperature at 30°C during the entire period of the experiments.[21-25] The gases, produced from glucose, were injected into the GC liner (i.d., 2 mm) by a programmed injector, which was modified from a commercial electromagnetic valve (24 V). The flow meter and a K-type thermal couple were used to continuously monitor the flow rate and temperature, respectively. Herein, the background pressure for injection used was nitrogen gas (2 kg/cm^2); the flow rate of the carrier gas and makeup gas were usually maintained at 5 – 6 and 38 – 40 mL/min, respectively. The injection interval was 5 min and the injection period was 100 ms. Under these conditions, the averaged sample injection volume was estimated to ~167 µL. Without pH control, the glucose stock solution consisted of 1% yeast extract, 2% bactopeptone and 15% dextrose; the size of the PLLA hollow yeast-MTAMs was 1 × 3 cm. Figure 2 shows an SEM section image of (a) PLLA yeast-MTAMs and light microscopy images of (b) free *Saccharomyces cerevisiae* and (c) PLLA yeast-MTAMs immobilized *Saccharomyces cerevisiae* prepared by the *in situ* method. The average diameter of a single layer with a hollow tube diameter of approximately 20 – 50 µm provided sufficient space for the *Saccharomyces cerevisiae* to thrive. Nanopores with a diameter of approximately 30 nm were located on the surface of each hollow tube, allowing simple sugars, alcohol, and other small molecules to diffuse in and out. Frames (b) and (c), show free *Saccharomyces cerevisiaes* and when they were associated with inside the PLLA yeast-MTAMs, respectively. In the beginning, to a 1-mL aliquot of a yeast solution, the amount of *Saccharomyces cerevisiae* was about 10^6 cfu/mL and this value increased to 10^8 cfu/mL after 36 h. During the electrospinning process, the total amount tended to decrease to 10^4 cfu/mL, but then grew back to 10^8 cfu/mL after 55 h. Using *Saccharomyces cerevisiae* (amount, 10^8 cfu/mL), 150 mg of glucose was converted into 48 mg of ethanol. Figure 3 shows the raw data for the real-time vibrational frequency of the whistle when the gas samples (produced from glucose by the yeast) were injected into the GC capillary (every 5 min). The X-axis and Y-axis show the retention time (min) of the GC chromatogram and the values for the sound frequency (Hz), respectively. When the carrier/make-up gases continuously pass through the whistle, the fundamental frequency was determined to be ~6671.3 Hz. When the additional component, CO_2 and gas formed during the production of ethanol in this case, passes through the whistle, a sharp frequency change occurs. The molecular weights of CO_2 and ethanol are larger than that of nitrogen, so that the frequency change is lower than the fundamental frequency. The "*" mark shows the system peak. This is because, when the gas sample is suddenly injected into the GC inlet, the background pressure dramatically decreased and then increased, resulting in frequency changes (down and up, respectively). The inset in Fig. 3 shows the expanded region for a retention time of 10 min. As can be seen, in addition to the injection system peak (marked as "*"), a huge peak corresponding to CO_2 gas and a minor peak corresponding to ethanol vapor with frequency changes of 4.669 and 0.075 Hz

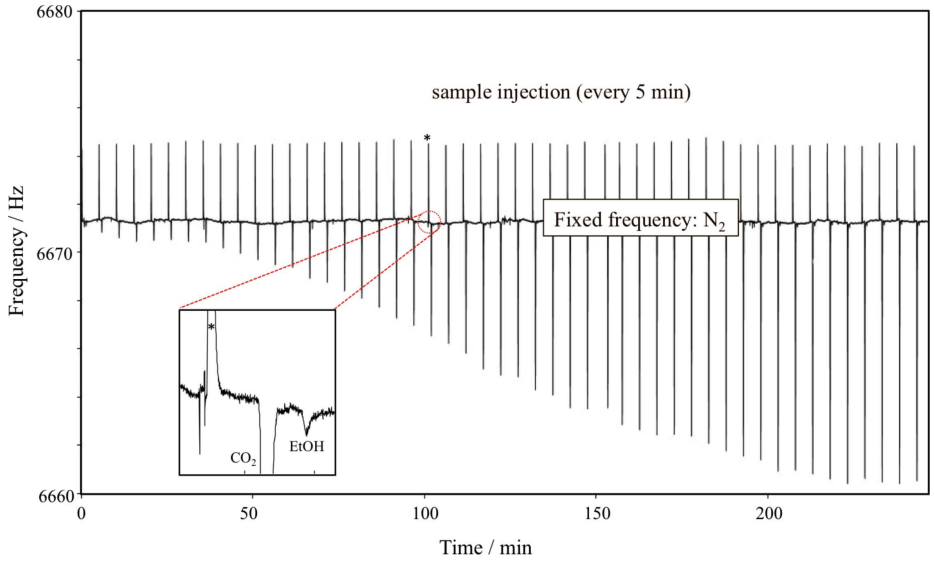

Fig. 3 Typical GC chromatogram of the real-time frequency changes when the gas samples were injected into the GC capillary (every 5 min). The X-axis and Y-axis show the retention time (min) of the GC chromatogram and the values for the sound frequency (Hz), respectively. The inset shows the expanded region for a retention time of 100 min.

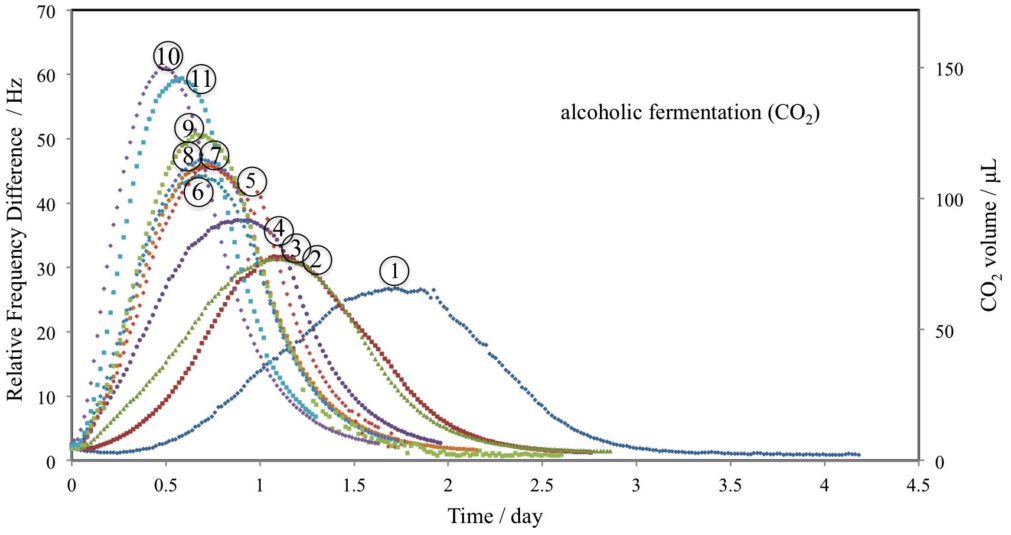

Fig. 4 Relationship between frequency changes and monitoring days, during 11 cycles. The X-axis shows the time of monitoring periods (day). The Y-axis (left) shows the values for the sound frequency changes (Hz) and Y-axis (right) shows the detected volume of CO_2, respectively.

can be seen, respectively. Based on the calibration curves, the concentration of CO_2 was 2.7 µL/injection (each injection volume, 167 µL); the concentration of ethanol was 4.2% (v/v). Figure 4 shows the raw data for the real-time vibrational frequency of the whistle when the gas samples were injected into the GC capillary (every 5 min), for 11 cycles. The X-axis shows the time of the monitoring periods (day). The Y-axis (left) shows the values for the changes in sound frequency (Hz) and the Y-axis (right) shows the volume of CO_2 detected. It was found that, during the first cycle (100 h), the amount of CO_2 production was low, compared to the other cycles. This is because the total amount of immobilized *Saccharomyces cerevisiae* was too low to generate high concentrations of CO_2. When the glucose stock solution (1% yeast extract, 2% bactopeptone and 15% dextrose) was reloaded in a second cycle, however, the level of CO_2 was similar to the first cycle, but the time needed for completion of the fermentation was shorter. This indicates that higher amounts of *Saccharomyces cerevisiae* had been grown, thus generating higher amounts of CO_2 gas. This indicates that the amount of ethanol produced was also increased, since the amount of ethanol produced is directly proportional to that for CO_2. Between the third and 11th cycle, more CO_2 was generated, indicating that the concentration of ethanol were correspondingly increased. As shown in Fig. 5, the X-axis shows the time of the monitoring periods (days). The Y-axis (left) shows the values for the sound

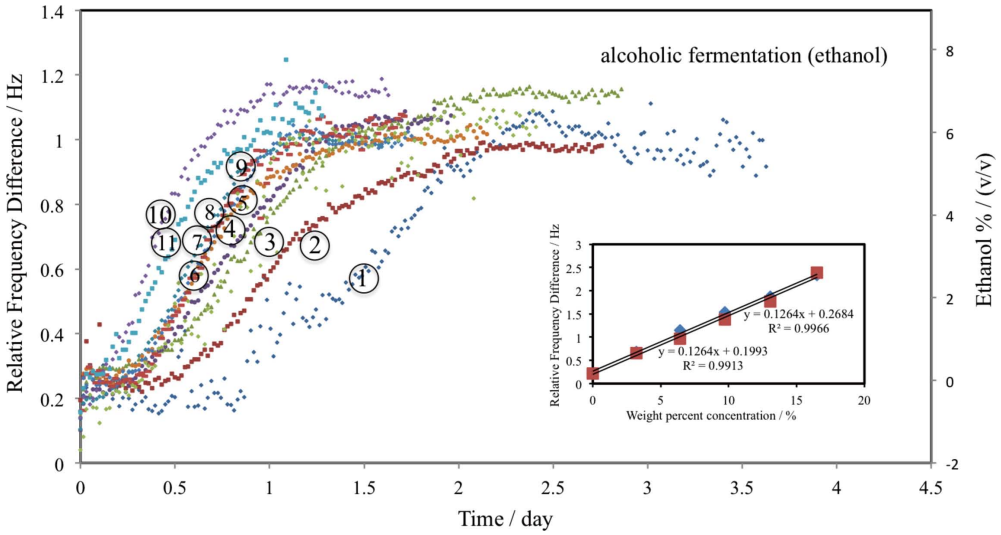

Fig. 5 Relationship between frequency changes and monitoring days, during 11 cycles. The X-axis shows the time of monitoring periods (day). The Y-axis (left) shows the values for the sound frequency changes (Hz) and Y-axis (right) shows the detected volume of ethanol formed, respectively. Inset, the calibration curve used for the quantification of ethanol.

frequency changes (Hz) and the Y-axis (right) shows the percentage of ethanol in the stock solution (based on the calibration curve), respectively. During the first cycle, the amount of ethanol produced was low, compared to the other cycles. However, in subsequent cycles the production increased significantly. Regarding the use of *Saccharomyces cerevisiae,* under conditions of 30°C, maximum production is thought to be reached in about 1 day, with an ethanol concentration of 8% (theoretical value). Our data confirmed this, although various yeasts present different fermentation behavior. Furthermore, the yeast-MTAMs can be recycled at least 11 times, representing a completely new technique in the fermentation industry. The major merit of this technique is that immobilized yeast cells can effectively reduce the negative effects of inhibitors and preparing an inoculum for the continuous or fed-batch fermentation of microorganisms is relatively low cost.

Conclusions

In this study, using a compact and economical gas chromatography/ milli-whistle device, we successfully demonstrated the on-line detection of gases (CO_2 and ethanol vapor) released from fermentation when glucose was used as the biofuel. Novel porous MTAMs were used to immobilize yeast cells, leading to an accelerated bioethanol fermentation. The findings also show that the use of yeast-MTAMs is highly efficient and can be recycled many times. We believe the new type of yeast-MTAMs, combined with an on-line monitoring technique would be useful in the fermentation industry. The method described herein is simple, is consistent with sustainable science and has the potential for use in practical analyses.

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