Journal of Bioscience and Bioengineering
VOL. 127 No. 1, 121–127, 2019

www.elsevier.com/locate/jbiosc

Development of novel on-line capillary gas chromatography-based analysis method for volatile organic compounds produced by aerobic fermentation

Hyeok-Won Lee,[1,2,‡] Jung-Ho Park,[3,‡] Hee-Suk Lee,[1,4] Chun-Suk Kim,[1] Jin-Gyeom Lee,[1] Won-kyo Kim,[1] Kyoung-Hwa Ryu,[1] Jung-Oh Ahn,[1,4] Eun-Gyo Lee,[1,4] Seon-Won Kim,[5] Jong-Min Jeon,[2] Yung-Hun Yang,[2] Eui-Sung Choi,[1,4] and Hong-Weon Lee[1,4,*]

Biotechnology Process Engineering Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 30 Yeongudanji-ro, Cheongwon-gu, Cheongju, Chungcheongbuk-do 28116, Republic of Korea,[1] Department of Microbial Engineering, College of Engineering, Konkuk University, 120 Neungdong-ro, Gwanngjin-gu, Seoul 05029, Republic of Korea,[2] Bio-Evaluation Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), Yeongudanji-ro, Cheongwon-gu, Cheongju, Chungcheongbuk-do 28116, Republic of Korea,[3] Department of Bioprocess Engineering, University of Science and Technology (UST) of Korea, 217 Gajeong-ro, Yuseong-gu, Daejeon 34113, Republic of Korea,[4] and Division of Applied Life Science (BK21 Plus), PMBBRC, Institute of Agricultural and Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea[5]

Received 16 May 2018; accepted 8 July 2018
Available online 30 July 2018

Many volatile compounds, such as isoprene, a precursor used in the synthesis of natural rubber, have been produced through fermentation using genetically engineered microorganisms. Despite this biotechnological success, measuring the concentrations of volatile compounds during fermentation is difficult because of their high volatility. In current systems, off-line analytical methods usually lead to product loss, whereas on-line methods raise the production cost due to the requirement of complex devices. Here, we developed a novel on-line gas chromatography (GC)-based system for analyzing the concentration of isoprene with the aim to minimize the cost and requirement for devices as compared to current strategies. In this system, a programmable logic controller is used to combine conventional GC with a syringe pump module (SPM) directly connected to the exhaust pipe of the fermentor, and isoprene-containing samples are continuously pumped from the SPM into the GC using an air cylinder recycle stream. We showed that this novel system enables isoprene analysis during fermentation with convenient equipment and without the requirement of an expensive desorption tube. Furthermore, this system may be extended to the detection of other volatile organic compounds in fermentation or chemical processes.

[**Key words:** Isoprene production; On-line monitoring; Isoprene; Programmable logic controller; Syringe pump module]

Isoprene is a highly volatile organic compound (VOC) that can be utilized as a fossil fuel alternative and as a platform chemical in the manufacture of products such as rubber, elastomers, and isoprenoid-based medicines (1–3). Isoprene is chemically produced from the isobutene-containing C4 fraction and formaldehyde, compounds that are obtained from fossil fuel (4–6). Given recent environmental concerns about the use of fossil fuels, biological production of isoprene fermentation from renewable sources is considered (7). Successful biological production of isoprene by fermentation has been reported, and the mevalonate (MVA) and 1-deoxy-D-xylulose-5-phosphate (DXP) biosynthetic pathways have been manipulated to enhance isoprene production (8–10).

Despite the successful production of isoprene using synthetic biology by engineered microorganisms, quantitative analysis of isoprene is problematic because of its high volatility and reactivity. Gas chromatography (GC) has been widely used for the characterization of VOCs, such as isoprene and volatile fatty acids (VFAs), through either off-line (direct injection) or on-line (with auto-sampler) analysis. Several strategies have been described, such as direct injection, solvent extraction, solid phase micro-extraction (SPME) of the aqueous or gas phase, and SPME sampling of the static head-space followed by GC (11). In particular, the determination of the concentration of isoprene in the head-space of a liquid culture using a GC-mass spectrometry (GC–MS) system coupled with an auto-sampler (CTC PAL system) and a SPME syringe has been described (12). Kayser's group established a GC method using a Tenax TA trap to detect isoprene after liquid culture in an air-tight flask (13). However, all of these methods, either off-line or on-line, have weaknesses, such as being time-consuming, requiring extensive sample preparation and expensive devices, and being limited to anaerobic culture conditions. Therefore, an automated control system for rapid monitoring is necessary to deal with the fast dynamic changes in isoprene levels under aerobic conditions.

In this study, we developed a novel online GC-based system for isoprene analysis using a syringe pump module. Notably, a direct connection from a fermentor to a GC system for the purpose of isoprene analysis has not been previously reported. For on-line

* Corresponding author at: Biotechnology Process Engineering Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 30 Yeongudanji-ro, Cheongwon-gu, Cheongju, Chungcheongbuk-do 28116, Republic of Korea. Tel.: +82 43 240 6612; fax: +82 43 240 6609.
E-mail address: hwlee@kribb.re.kr (H.-W. Lee).
‡ The first two authors contributed equally to this work.

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.07.007

monitoring of isoprene production during fermentation, we developed an algorithm to control the SPM and the air cylinder for isoprene analysis, and this was applied to the system using a programmable logic controller (PLC) steered via a touch screen. We showed that the novel on-line monitoring system can be easily installed and successfully used to perform isoprene analysis using a series of injections from an isoprene flow. Further, the on-line system worked properly in a fed-batch culture mode, with accurate analysis of isoprene levels.

MATERIALS AND METHODS

Bacterial strains and plasmids *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, USA) was used for genetic manipulation and isoprene production in this study. A pS-NA plasmid, derived from pSTV28 containing p15A origin of replication and chloramphenicol resistance gene and encoding the following six biosynthetic enzymes: hydroxymethylglutaryl-CoA synthase (*mvaS*) and hydroxymethylglutaryl-CoA reductase (*mvaE*) from *Enterococcus faecalis*, mevalonate kinase (*mvaK1*), phosphomevalonate kinase (*mvaK2*) and mevalonate diphosphate decarboxylase (*mvaD*) from *Streptococcus pneumoniae*, and isopentenyl pyrophosphate isomerase (*idi*) from *E. coli* (10), was used. All six enzymes were encoded on the plasmid as an operon. The plasmid pT-PtispS, derived from pTrc99A containing pBR322 origin of replication and ampicillin resistance gene and encoding isoprene synthase from *Populus trichocarpa* was also used in this study (3,14).

Culture conditions The pS-NA and pT-PtispS plasmids were simultaneously transformed into *E. coli* DH5α cells, which were plated onto LB agar plates (5 g/L yeast extract, 10 g/L tryptone-peptone, 5 g/L NaCl, and 20 g/L agar supplemented with 50 μg/mL each of ampicillin and chloramphenicol). A single colony grown on the agar plate was transferred to a 200-mL Erlenmeyer flask containing 20 mL of broth medium (5 g/L yeast extract, 10 g/L tryptone-peptone, 5 g/L NaCl, and 2 g/L glycerol) and then transferred to a 5-L Erlenmeyer flask containing 1 L of flask broth. The flask medium was supplemented with 50 μg/mL of each of ampicillin and chloramphenicol for preculture. After 6 h of cultivation at 37°C on a rotary shaker (200 rpm), the culture was used as seed culture. The seed culture (1 L) was inoculated into a 30-L fermentor (KFC LA-150; Kobiotech Co., Ltd., Incheon, Korea) containing 9 L of initial medium (10 g/L glycerol, 20 g/L yeast extract, 10 g/L casein peptone, 5 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 3 g/L Na_2HPO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 0.4 mL/L antifoam, 50 μg/mL ampicillin, 50 μg/mL chloramphenicol) and 1 mL of a trace element solution in 1 N HCl (13.2 g/L $CaCl_2 \cdot 2H_2O$, 8.4 g/L $FeSO_4 \cdot 7H_2O$, 2.4 g/L $MnSO_4 \cdot 4H_2O$, 2.4 g/L $ZnSO_4 \cdot 7H_2O$, 0.48 g/L $CuSO_4 \cdot 5H_2O$, 0.48 g/L $CoCl_2 \cdot 6H_2O$, 0.24 g/L $Na_2MoO_4 \cdot 2H_2O$, and 0.06 g/L $K_2B_4O_7 \cdot XH_2O$) for fed batch culture. The phosphate-containing compounds (KH_2PO_4 and Na_2HPO4) were sterilized separately from the main medium and were added separately into the 30-L fermentor after sterilization of the culture medium at 121°C for 30 min. When the initial glycerol was consumed, a feeding medium was continuously fed into the fermentor, and the feeding rate was regulated by a peristaltic pump. The dissolved oxygen (DO) level was maintained above 20% by adjusting agitation and aeration using mixed pure oxygen and air. The main culture was carried out in a 30-L fermentor with a 10-L working volume at 37°C and adjusted to pH 7.0 with 10 N NaOH. The continuous feed medium, which was designed to achieve high isoprene production and high bacterial cell mass, was composed of 800 g/L glycerol and 80 g/L yeast extract.

Apparatus and instrumentation for on-line monitoring The on-line analysis system was established with a direct connection from the fermentor to a GC system (Fig. 1B). To maintain the DO level, the concentration of pure oxygen in the culture broth was continually maintained at 0.2 vvm. Pure oxygen was mixed with air during the fermentation procedure. The exhaust gas pipeline was directly connected to the SPM via an exhaust bottle. An auto-sampler (Locas, Daejeon, Korea) was established to measure both the optical density (OD) and the glycerol concentration in the culture broth. Several apparatuses were installed for on-line monitoring of isoprene using GC: syringe needle (Hamilton, Boston, MA, USA), 1-L air-Tedlar bag (Toptrading Eng, Seoul, Korea), cooling water bath (Jisico, Korea), syringe pump module (SPM) XLP 6000 (Cavro, USA), air cylinder TPC1B10-75 (TPC, Seoul, Korea), PLC (LS, Seoul, Korea), touch screen CM-XT04CA-D (Samin, Siheung, Korea), auto switch D-C73K (TPC), and solenoid valve CKS210-06 (Samin) (Fig. 1).

Monitoring and control systems for on-line monitoring The PLC communicated with the several hardware components over a field bus and was controlled locally by using a touch panel. The PLC has a software view of the master panel (Fig. 2A) with sub-screens for the initial settings (Fig. 2B) and detail settings of the SPM (Fig. 2C). The master panel displays the various available options and allows input of configurable parameters, as shown in Fig. 2A. Full operation of the overall analysis procedure is controlled by the signals for START or STOP. There are two modes (Auto or Man) for controlling the operation of the air cylinder. In the sub-screen for these options, the Initial SPM set both the 1st and 2nd aspiration and elimination times (Fig. 2B). Fig. 2C shows the sub-screen that represents the Detail screen with which the following parameters were controlled: the order of aspiration, elimination rate, cycle time, cycle number, 1st and 2nd delay times, and aspiration and elimination volumes. The PLC software was programmed using Microsoft Visual Basic. The digital outputs from the control program were converted to analog signals by the PLC interface card.

Analytical methods Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Uvicon 941 Plus; Kontron Instruments Co., Zurich, Switzerland). The glycerol concentration was analyzed using an HPLC system (RI detector ERC-7515A, ERC Instrument Co., Kawaguchi, Japan) equipped with an Aminex 87H ion exclusion column (Bio-Rad, Hercules, CA, USA). The column temperature was kept at 85°C, and the mobile phase was deionized water, which was applied at a flow rate of 0.5 mL/min. The isoprene concentration was measured with a gas chromatograph (Varian X-3300, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector. To increase the detection level of isoprene, the 1-mL sample was injected into an Agilent J&W GC column (30 m × 0.53 mm ID). The temperature program used was 3 min at 50°C followed by an increase to 150°C for 10 min; the column was maintained at this temperature for 12 min before lowering to 50°C again.

RESULTS AND DISCUSSION

Comparison of isoprene analytical methods To date, most studies have used one of three systems for isoprene analysis that are either anaerobic or aerobic culture-based (Fig. 1A). The anaerobic culture-based analysis of isoprene is performed using an airtight flask. An autosampler (CTC PAL system) with SPME syringe was used to absorb the headspace sample in an air-tight flask (Fig. 1A, system 1). With this analysis system, it is difficult to identify the capacity for isoprene production because of the anaerobic condition and the fact that oxygen loss in the flask can become problematic owing to the large number of sampling events required. For example, in a 500-mL flask, 15 mL of the air sample was sampled every 15 min during a period of culture (13,15). In contrast, aerobic culture-based analysis is performed in a fermentor system equipped with aeration capability (Fig. 1A, system 2). However, with this system, because of the high degree of aeration, it is difficult to obtain an accurate sample amount when sampling using the manual SPME holder; furthermore, loss during sampling or during the concentrating process can occur (16,17). Both analysis methods rely on a complex process of sample concentration using a Tenax TA trap after off-gas sampling, sample thermal desorption at a high temperature (180°C), and GC analysis under a flow of nitrogen at 2.5 mL/min. Thus, both these systems require expensive and complex machinery and both are limited by problems of sample loss and the fact that they are time-consuming. Meanwhile, Kim et al. reported isoprene production in an air-tight flask and analysis by applying a dodecane overlay—because of its low toxicity to the microorganism—for hydrophobic-isoprene recovery, although this process is laborious and time-consuming (Fig. 1A, system 3) (14). Considering the problems of isoprene analysis with any of the current systems, we designed an on-line analysis system using a GC instrument that could be used during aerobic isoprene fermentation (Fig. 1B).

To determine the accuracy of the method for isoprene analysis during fermentation, we compared the above methods using a Tenax TA trap, dodecane method, and novel on-line monitoring method (Fig. 3). Isoprene gas samples were prepared using a 1-L air-Tedlar bag with a serial dilution consisting of known concentrations (10, 50, 100 mg/L) of isoprene. When using the Tenax TA trap, the isoprene samples with different concentrations were pumped through a Tenax TA trap, desorbed at 180°C, and analyzed by GC. When using dodecane, the isoprene samples (100 μL) were added in a mixed sample of culture broth (1 mL) and dodecane (1 mL). After vortexing, the dodecane containing isoprene was analyzed using GC. When using our novel method, 1-mL samples of

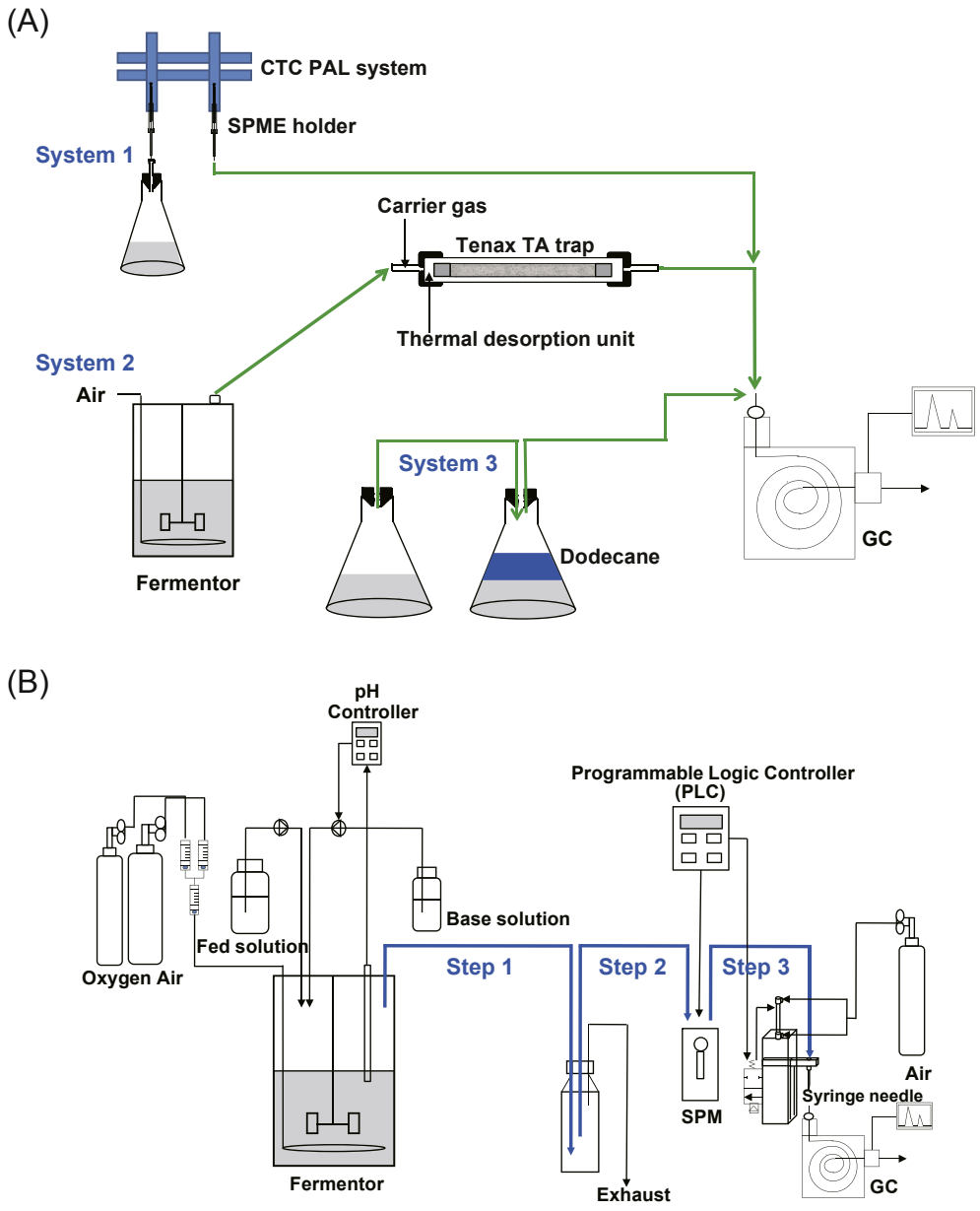

FIG. 1. Schematic diagram of the apparatus and instrumentation for on-line monitoring of isoprene analysis: (A) the three commonly used systems for isoprene analysis using anaerobic or aerobic culture-based culture conditions: system 1, analysis with CTC PAL system and SPME holder; system 2, analysis with Tenax TA trap; system 3, analysis of dodecane. The green arrows show the flow of the steps of isoprene analysis. (B) The on-line isoprene analysis system adopting a direct connection from the fermentor to a GC system. The blue arrows represent the flow of isoprene-containing air from the fermentor (step 1→step 2→step 3).

the Tedlar bag were injected into the GC instrument using the on-line monitoring program. When 10 mg/L isoprene was analyzed by each method, the isoprene concentrations were 9.9 ± 0.4 mg/L for the Tenax TA trap, 9.87 ± 0.62 mg/L for dodecane, and 9.94 ± 0.58 mg/L for the novel method (Fig. 3A). When 50 mg/L isoprene was injected; the measured isoprene concentrations were 45.8 ± 2.3 mg/L for the Tenax TA trap, 43.6 ± 1.8 mg/L for dodecane, and 49.25 ± 1.0 mg/L for the novel method (Fig. 3B). When using 100 mg/L isoprene, measured isoprene concentrations were 89.6 ± 3.3 mg/L for the Tenax TA trap, 86.3 ± 2.8 mg/L for dodecane, and 97.8 ± 1.58 mg/L for the novel method (Fig. 3C). These results show that the three analysis methods produced similar results at low isoprene concentration (10 mg/L). However, as the concentration of isoprene increased, the measured concentrations were substantially lower than the injected concentrations for the Tenax TA trap and dodecane methods, indicating these two methods induce sample loss at high isoprene concentration, while the novel method did not result in significant sample loss. Thus, the on-line monitoring method is more accurate than the other methods at high isoprene concentrations. Therefore, the on-line monitoring system allows the analysis of a wider range of isoprene levels, and can be applied to analysis during fermentation.

On-line monitoring was investigated in this study in order to overcome the complexity of the anaerobic system and avoid the product loss that occurs with the aerobic system. In partial support of this, there have been several reports describing the use of headspace GC (HSGC) for off-line analysis of volatile products. However, only a few studies, such as that by Boe et al. (18), have reported the use of on-line HSGC in the application of biogas production. Notably, our system consisted of an SPM, which pumped

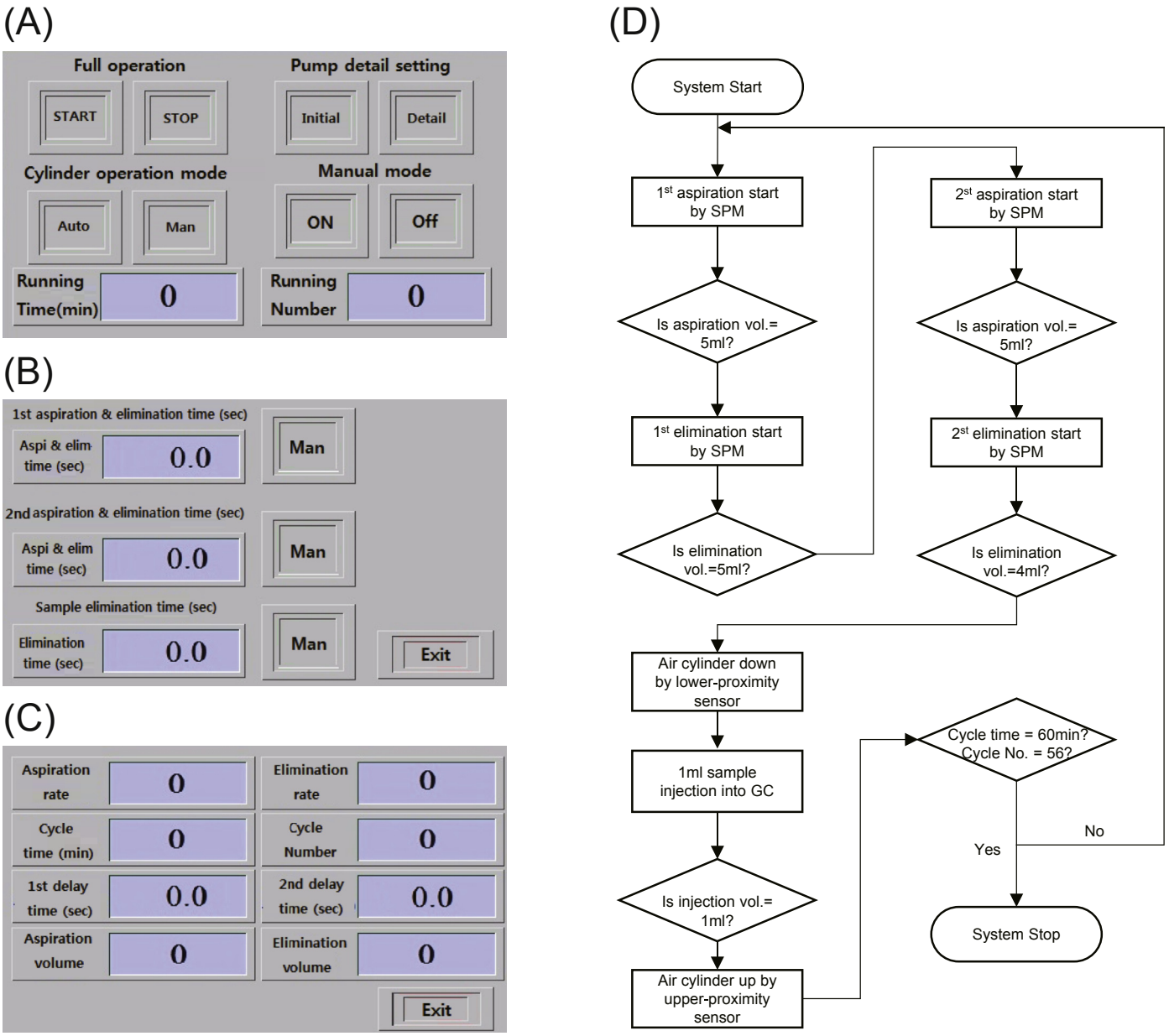

FIG. 2. Programmable logic controller (PLC) panels for on-line analysis of isoprene (A–C): (A) the master PLC panel, (B) the sub-screens for the initial settings of the SPM, (C) the sub-screens for the detail settings of the SPM. Flowchart for on-line monitoring of isoprene production using capillary GC (D).

the gas sample from the fermentor headspace within a specified time interval for gas analysis. In contrast to traditional methods, this approach allowed efficient analysis of the isoprene produced from the aerobic culture in real-time, thereby simplifying the overall VOC analytical process.

Operating principle of the on-line monitoring system The on-line monitoring and control system for the analysis of isoprene production during fermentation was accomplished by connecting the SPM to a syringe needle (Fig. 2D). The whole system can be started at once using the touch screen on the PLC. The air stream containing isoprene from the fermentor flows continuously from the SPM (by means of a three-way valve) to a syringe needle. The three-way SPM valve repeats an operation that consists of 1st aspiration, 2nd aspiration, and elimination of the air sample. The remaining 1 mL of air sample in the SPM is then simultaneously loaded and analyzed by GC. When controlling the rate and operation time of SPM, the operation time of the air cylinder should also be considered because the whole operation rate of on-line GC could be changed. The biggest difference between this system and the GC (or LC) auto-sampler is the use of an air cylinder driven by a solenoid valve. Under control of the PLC, the air cylinder operates at the appropriate time to inject the remaining air sample in the SPM into the GC. Subsequently, the volatile isoprene in the air stream migrates into the GC because the air acts as a mobile phase.

When the SPM receives the motion instruction, this is turned on and the entire air sample from the fermentor is transferred to the air cylinder through a syringe needle after taking a 5-mL air sample. The SPM then expels 4 mL of the air sample to the outside. The air cylinder then receives a signal to downturn through a digital input via the upper-proximity sensor (P01-signal) from the PLC (P20-signal). When the air cylinder downturn-operation signal is processed, the remaining 1 mL of air sample in the SPM is simultaneously loaded

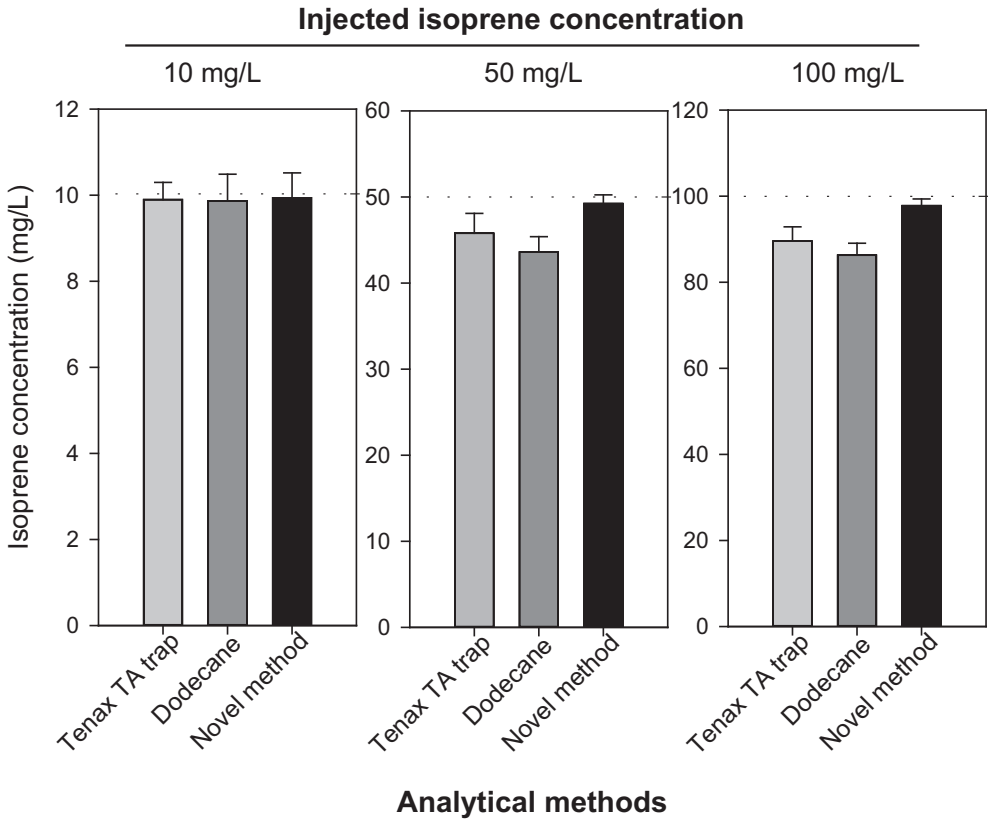

FIG. 3. Comparison of isoprene analysis methods using Tenax TA trap, dodecane, and novel on-line monitoring and various concentration of isoprene. Isoprene production given as mg/L was presented as the mean of three replicated experiments.

into the GC. Finally, when the isoprene analysis is initiated, the air cylinder receives an upturn operation signal through a digital-input via the lower-proximity sensor (P00-signal) from the PLC (P20-signal). As mentioned above, controlling the rate and the time of SPM is an important point for accurate analysis of isoprene levels in this system. After introduction of the air sample into the GC, the air cylinder returns to its home position to complete the process, thus allowing the isoprene analysis process to be repeated over a number of time intervals.

Standard curve and detection limit of isoprene To generate a standard curve and evaluate the detection limit for isoprene analysis, isoprene standard gas samples were prepared using an air-Tedlar bag with a serial dilution consisting of known concentrations of isoprene. To this end, after injection of the known isoprene volume into a 1-L air-Tedlar bag, pure air was added. After passing through the SPM, these isoprene standards were analyzed using the on-line monitoring system to generate the isoprene standard curve (Fig. 4A). The isoprene standard curve

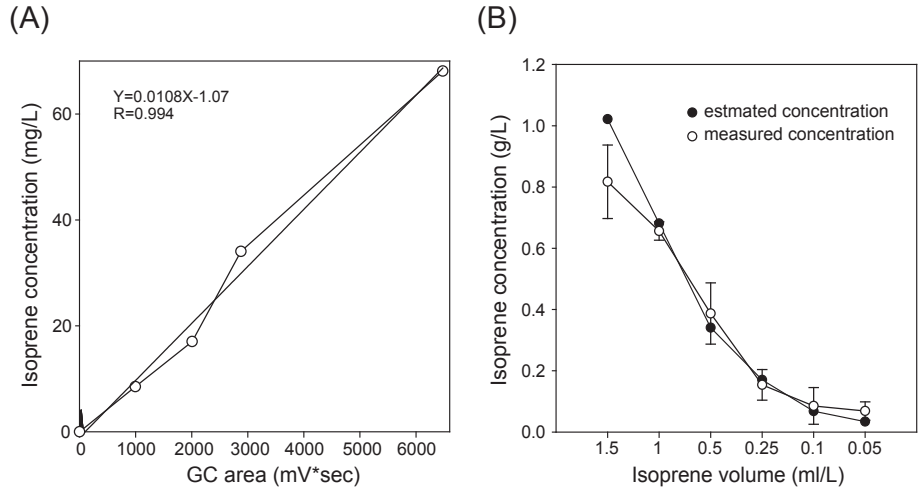

FIG. 4. Isoprene standard curve and detection limit: (A) peak for isoprene standard, (B) isoprene detection limit at various concentrations: estimated (closed circles) and measured (open circles) isoprene concentrations. Results given as g/L of each isoprene concentration are presented as the mean of three replicated experiments.

shows the isoprene concentration relative to the area detected by the GC. The quadratic equation describing the relationship between isoprene concentration and GC area was as follows: $Y = 0.0099X + 0.7401$ ($R = 0.9978$), where Y is the isoprene concentration (mg/L) and X is the GC area (mV s). The margin of error for the quadratic equations was maintained in the range of −3% to +3% and the correlation coefficients were significant at the 99% confidence level (Fig. 4A). As a result, this isoprene standard curve was of sufficient reproducibility and accuracy for it to be applied to monitoring the isoprene fermentation process. To evaluate the reproducibility of isoprene detection, different sample volumes were tested (Fig. 4B). From the results, if the injection volume exceeded 1 mL, a difference between the injected and the measured concentrations was found (Fig. 4B). Therefore, a sample volume of 1 mL was determined to be optimal for the GC analysis of isoprene and was used in further experiments.

An important point when considering the flow of an isoprene stream from the fermentor and its introduction into the GC column is to ensure that the optimized injection volume containing the minimum isoprene concentration can be detected.

Application of the on-line monitoring system in 30-L fed-batch cultures for isoprene production A 30-L fed-batch cultivation study was carried out to explore the ability of the on-line monitoring system to detect isoprene during fermentation. The *E. coli* strain DH5α containing the plasmids pT-ispS, encoding isoprene synthase, and pS-NA, encoding all the genes of the MVA pathway, was used as an isoprene producer. Glycerol and NaOH (10 N) were used as the main carbon source and the pH control reagent, respectively. The time profile of isoprene production by *E. coli* DH5α in complex culture medium was determined (Fig. 5). To increase the isoprene detection using on-line GC, 0.2 vvm of aeration and the DO level (20%) were maintained with pure oxygen. The mixture of air and oxygen increased the detection range of isoprene. The exponential growth phase began immediately after inoculation of the seed culture into the 30-L main fermentor. As initial glycerol (10 g/L) was depleted after 7.5 h, a feeding medium, containing glycerol (800 g/L) and yeast extract (80 g/L) as a carbon and nitrogen source, was continuously fed into the culture. The feeding rate was gradually increased using a step-wise gradient depending on the cell growth. The cells were grown for a total of 56 h and the cell concentration achieved an OD of 201 by the end of the culture. By using the novel on-line monitoring system, we showed that, as a result of the co-expression of isoprene synthase and all genes of the MVA pathway, the engineered *E. coli* DH5α accumulated 12.7 g/L of isoprene. Further, the total consumption of glycerol was 524 g glycerol/L and the isoprene yield on glycerol was calculated to be 33 mmol isoprene/mol glycerol.

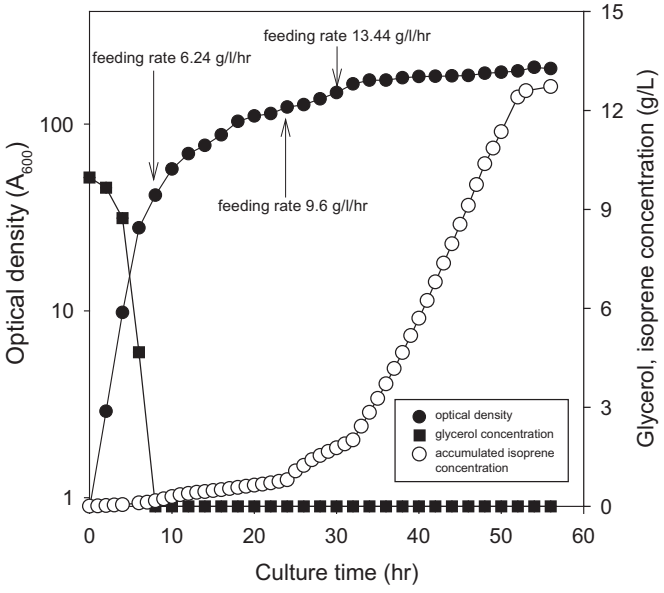

FIG. 5. Time profiles for 30-L fed-batch cultivation using on-line analysis for isoprene production: closed circles, optical density; closed rectangulars, glycerol concentration; open circles, accumulated isoprene concentration.

In this study, a novel on-line monitoring system using GC was employed for the analysis of production of isoprene during aerobic fermentation. This system avoids complex analytical processes and unstable concentrations that previously arose from the generally used anaerobic or aerobic culture conditions. This system has the advantages that it is easily installed and is fully automated. In a fed-batch fermentation test by using this system, an engineered *E. coli* strain DH5α accumulated 12.7 g isoprene/L. Based on these data, this on-line monitoring system might be easily adapted for the detection of VOCs and volatile gas production in microbial fermentation and chemical processes.

ACKNOWLEDGMENTS

This research was supported by grants from KRIBB (Korea Research Institute of Bioscience & Biotechnology) Research Initiative Program funded by the government of Korea (KGM-4231811) and from Korea Research Institute of Bioscience & Biotechnology (KRIBB) through the Research Initiative Program and from the Animal Disease Management Technology Program (No. 315039-3) of the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET), Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

References

1. **Alianell, G. A., Derwitsch, F., Wells, D., and Taylor, T.:** Isoprene compositions and methods of use, US2010/0099932 A1 (2010).
2. **Lindberg, P., Park, S., and Melis, A.:** Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism, Metab. Eng., **12**, 70–79 (2010).
3. **Wendisch, V. F., Bott, M., and Eikmanns, B. J.:** Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids, Curr. Opin. Microbiol., **9**, 268–274 (2006).
4. **Reis, T.:** Isoprene production-2. Synthesis based on isobutylene, Chem. Process Eng., **53**, 68–71 (1972).
5. **Ushio, S.:** Extract isoprene with DMF, Chem. Eng. Prog., **79**, 82–83 (1972).
6. **DiGiacomo, A., Maerker, J., and Schall, J.:** Isoprene by dehydrogenation, Chem. Eng. Prog., **57**, 3540 (1961).
7. **Clomburg, J. M. and Gonzalez, R.:** Biofuel production in Escherichia coli: the role of metabolic engineering and synthetic biology, Appl. Microbiol. Biotechnol., **86**, 419–434 (2010).
8. **Seemann, M., Bui, B. T., Wolff, M., Tritsch, D., Campos, N., Boronat, A., Marquet, A., and Rohmer, M.:** Isoprenoid biosynthesis through the methylerythritol phosphate pathway: the (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (GcpE) is a [4Fe-4S] protein, Angew. Chem. Int. Ed. Engl., **41**, 4337–4339 (2002).
9. **Anthony, J. R., Anthony, L. C., Nowroozi, F., Kwon, G., Newman, J. D., and Keasling, J. D.:** Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene, Metab. Eng., **11**, 13–19 (2009).
10. **Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., and Keasling, J. D.:** Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids, Nat. Biotechnol., **21**, 796–802 (2003).
11. **Kuzma, J., Nemecek-Marshall, M., Pollock, W. H., and Fall, R.:** Bacteria produce the volatile hydrocarbon isoprene, Curr. Microbiol., **30**, 97–103 (1995).
12. **Strobel, G. A., Dirkse, E., Sears, J., and Markworth, C.:** Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus, Microbiology, **147**, 2943–2950 (2001).

13. **Julsing, M. K., Rijpkema, M., Woerdenbag, H. J., Quax, W. J., and Kayser, O.:** Functional analysis of genes involved in the biosynthesis of isoprene in *Bacillus subtilis*, Appl. Microbiol. Biotechnol., **75**, 1377–1384 (2007).
14. **Kim, S. W. and Keasling, J. D.:** Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production, Biotechnol. Bioeng., **72**, 408–415 (2001).
15. **Xue, J. and Ahring, B. K.:** Enhancing isoprene production by genetic modification of the 1-deoxy-D-xylulose-5-phosphate pathway in *Bacillus subtilis*, Appl. Environ. Microbiol., **77**, 2399–2405 (2011).
16. **Yang, J., Nie, Q., Liu, H., Xian, M., and Liu, H.:** A novel MVA-mediated pathway for isoprene production in engineered *E. coli*, BMC Biotechnol., **16**, 5 (2016).
17. **Yang, C., Gao, X., Jiang, Y., Sun, B., Gao, F., and Yang, S.:** Synergy between methylerythritol phosphate pathway and mevalonate pathway for isoprene production in *Escherichia coli*, Metab. Eng., **37**, 79–91 (2016).
18. **Boe, K., Batstone, D. J., and Angelidaki, I.:** An innovative online VFA monitoring system for the anerobic process, based on headspace gas chromatography, Biotechnol. Bioeng., **96**, 712–721 (2007).