Development of a Bottle-Free Multipurpose Incubator for Generating Various Bacterial Culture Conditions

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The purpose of this study was to develop a multipurpose incubator, without the gas cylinders (bottles) which are required for H_2 and CO_2 supplementation. In our bottle-free multipurpose incubator, the H_2 and CO_2 were generated by chemical reactions induced within the chamber. The reaction between sodium borohydride and acetic acid at a molar ratio of 1:1 was used to generate H_2 according to the following formula: $4NaBH_4 + 2CH_3COOH + 7H_2O \rightarrow 2CH_3COONa + Na_2B_4O_7 + 16H_2$, whereas the other reaction, citric acid and sodium bicarbonate at a 1:1 molar ratio, was used to generate CO_2, according to the following formula: $C_6H_8O_7 + 3NaHCO_3 \rightarrow Na_3(C_6H_5O_7) + 3H_2O + 3CO_2$. Five species of obligate anaerobic bacteria, one strain of capnophilic bacterium, and one strain of microaerophilic bacterium were successfully cultured in the presence of their respective suitable conditions, all of which were successfully generated by our bottle-free multipurpose incubator. We conclude that, due to its greater safety, versatility, and significantly lower operating costs, this bottle-free multipurpose incubator can be used for the production of fastidious bacterial cultures, and constitutes a favorable step above existing anaerobic incubators.

Key words: bottle-free multipurpose incubator, anaerobic incubator, anaerobic, capnophilic, microaerophilic

Anaerobic incubators (anaerobic glove boxes) have been used principally for the culture of obligate anaerobic microorganisms. The main advantage of a readily available anaerobic incubator is that it operates automatically, and can maintain absolute anaerobic conditions. However, current incubators are saddled with several serious disadvantages: 1) They require several different gas cylinders (bottles) for the supplementation of 5% CO_2, 15% H_2 and 80% N_2 necessary to establish anaerobic condition, and to maintain continuous positive pressure within the chamber. The maintenance of these gas bottles is costly. 2) They are inconvenient, as the bottles cause safety concerns, and are not easily moved. 3) They are only used for culturing obligate anaerobic microorganisms, since the concentrations of carbon dioxide and oxygen cannot be regulated.

In this study, in order to overcome the above shortcomings of existing anaerobic incubators, we developed a bottle-free multipurpose incubator, the design of which was predicated on two principles: first, H_2 and CO_2 would be generated by chemical reactions induced within the chamber instead of being provided by external bottles; second, the control of H_2 and CO_2 generation should enable the regulation of O_2 and CO_2 concentrations in the chamber,

thus allowing the generation of various culture conditions for fastidious bacterial cultures, in addition to anaerobic culture. This is the reasoning behind the designation, 'bottle-free multipurpose incubator'. In addition, we used a long-lasting and inexpensive honeycomb-sectioned palladium cylinder as a catalyst, for the conversion of O_2 and H_2 into H_2O. Finally, we successfully cultured several fastidious bacteria in a variety of culture conditions, all of which were generated by the bottle-free multipurpose incubator. Here, we report the principles of the new incubator, and assess its versatility.

Materials and Methods

Main structure and accessory items

Two differently sized bottle-free multipurpose incubators were manufactured, in cooperation with the Hansol Unit Co. in Gwangju. The small-scale incubator (total volume; 18.8 L) was initially used for preliminary study. On the basis of the principles, and the data obtained from the small-scale incubator, we developed a large-scale incubator (total volume; 402 L) for the culturing of tested bacteria. In this paper, we are referring to the small-scale incubator, unless otherwise noted. Each bottle-free multipurpose incubator consists of six main parts, including the main body, the two chemical reaction baths for the gen-

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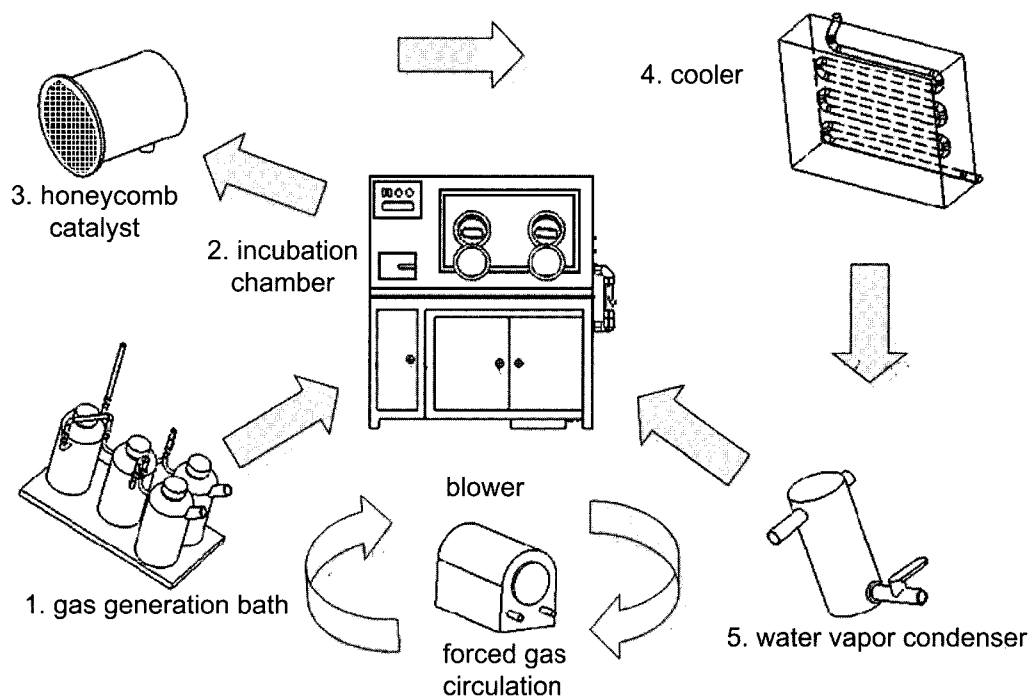
Fig. 1. The diagram of gas conditions generated in the multipurpose incubator. Hydrogen (or carbon dioxide) generated from the gas generation bath (1) is injected into the incubation chamber (2). The blower then circulates the gas throughout the incubation chamber via the honeycomb catalyst (3), the cooler (4), the water vapor condenser (5), and the incubation chamber, sequentially, until the desired gas condition is generated. Arrows indicate the gas flow for the gas conditions.

eration of H_2 and CO_2, the two gas wash bottles for the passing through of H_2 and CO_2, a honeycomb-sectioned palladium cylinder (DC Chemical Co. Korea), a charcoal filter (Shinkwang, Chem. Co., Korea), and a latex air-bag. The main body of the incubator consists of an instrument board, an interchange area, and a culture chamber. Generated H_2 or CO_2 was passed through each gas wash bottle, in order to increase the quality of the gas. We utilized a palladium cylinder with a honeycomb section (40 slits/inch^2), the surface of which was coated with 5.25 g of palladium. An impregnated activated charcoal filter was also incorporated, and used to remove volatile and noxious by-products, including sulfur acids (H_2S, SO_2), caproic acid, and other organic acids, such as propionic, butyric, isovaleric, and valeric acids. The air bag was constructed by injection molding, and was used to counterbalance both the positive pressure caused by the inward flow of H_2 and CO_2, and the negative pressure caused by the elimination of O_2. A diagram of this multi-purpose incubator is shown in Fig. 1.

Measurement of hydrogen gas volume and the reaction time and analysis of hydrogen gas purity

We also developed a chemical reaction bath for the generation of H_2. Hydrogen gas was produced via the addition of 3.78 g of sodium borohydride ($NaBH_4$ Finish Chemicals, USA) with 25 ml of 2, 4, 6, and 16 M acetic acid solution (CH_3COOH, Merck, USA) at molar ratios of

$1:0.5$, $1:1$, $1:1.5$, and $1:4$ in the reaction bath. The hydrogen gas generated at each molar ratio was then passed through the gas wash bottle, and collected in a polyvinyl chloride bag, in order to verify the volume and reaction times required for H_2 generation, which were measured with a gas meter (DC-2, Shinagawa, Seiki, Japan) and a timer, respectively. In order to analyze the purity of the H_2, we collected H_2 at a $1:1$ molar ratio, as the generation of H_2 gas at a $1:1$ molar ratio evidenced the most rapid reaction times, and was a sufficient volume of H_2 for use in the small-scale incubator. In brief, H_2 was passed through the gas wash bottle at 10°C and at 25°C, collected in a polyethylene terephthalate bag (PET bags, OMI, Japan), then analyzed with a Precision Gas Mass Spectrometer (271. FINNIGAN, Germany) at the Korean Testing and Research Institute for the Chemical Industry (Korea).

Chemical analysis of precipitates and extracts during hydrogen gas generation

Precipitates in the reaction bottle, and clouded water in the gas wash bottle, were detected during the chemical reaction for the generation of hydrogen gas at a $1:1$ molar ratio. Therefore, the precipitates were dried at 70°C to extract the powder, and the clouded water was also dried at 90°C, in order to obtain extracts of the dissolved materials, which were analyzed with an SEM/EDX (scanning electron microscopy/energy dispersive X-ray analyzer,

Noran Instruments, USA, Model No.220A-1SPS), XRD (X-ray diffraction, D/MAX, Rigaku, Japan), and ICP (Inductively coupled plasma, Labtam 8440, Australia), at the Korean Testing and Research Institute for the Chemical Industry.

Carbon dioxide production

Carbon dioxide was generated according to the following reaction formula: $C_6H_8O_7 + 3NaHCO_3 \rightarrow Na_3 (C_6H_5O_7) + 3H_2O + 3CO_2$ (Ferguson *et al.*, 1976). In brief, 100 mM citric acid ($C_6H_8O_7$, DC Chemical Co., Ltd. Korea) was allowed to react with 50, 100, 200, and 300 mM of sodium bicarbonate ($NaHCO_3$, DC Chemical, Korea) at molar ratios of 1:0.5, 1:1, 1:2, and 1:3. In each case, reaction time and CO_2 concentration were measured with a timer and a portable CO_2 analyzer (Anagas CD 98, Geotechnical Instruments, UK). The carbon dioxide, like the hydrogen gas, was then passed through another gas wash bottle.

Measurement of reagent quantities and the time required to create an absolute anaerobic, 5% microaerophilic or a 5~10% CO_2 conditions in the large-scale bottle-free multi-purpose incubator

The hydrogen-generating reaction between 4 M $NaBH_4$ and 4 M CH_3COOH (1:1 molar ratio) was used to create an absolute anaerobic condition, and a 5% microaerophilic condition, within the large-scale bottle-free multipurpose incubator (402 L). The atmospheric conditions were measured with a portable oxygen meter (COSMOS Mini Sensor, New Cosmos Electric Co. Japan). A 5% CO_2 concentration was achieved via the reaction of 0.65 M $NaHCO_3$ with 0.65 M $C_6H_8O_7$, at a 1:1 molar ratio, and a 10% CO_2 concentration was obtained by allowing 1.3 M $NaHCO_3$ to react with 1.3 M $C_6H_8O_7$, at a 1:1 molar ratio. The CO_2 concentration was measured with a portable CO_2 analyzer. The time required to reach the anaerobic state, the 5% microaerophilic state, and the 5% and 10% CO_2 concentrations in the large-scale incubator were monitored and recorded.

Sources of bacteria tested, and the optimal media for culture in the large-scale bottle-free multipurpose incubator

Five species of obligate anaerobic bacteria were cultured in their own optimal solid media, as follows: *Bacteroides fragilis* (ATCC 25285) was cultured in Brucella agar (BBL, USA), which was supplemented with hemin (5 μg/ml), vitamin K1 (10 μg/ml), and 5% sheep blood (BAKH). *Peptostreptococcus asaccharolyticus* (KCTC 3321) was cultured in Reinforced Clostridial Medium (RCM, Media No.8, KCTC). *Fusobacterium nucleatum* subsp. *polymorphum* (KCTC 2488) was cultured in Peptococcus Medium (PM, Media No.184, KCTC). *Mobiluncus mulieris* (ATCC 35239) was cultured in Brucella agar (BBL, USA), which was supplemented with vitamin K1 (10 μg/ml), cysteine

$HCl \cdot H_2O$ (0.5 mg/ml), and 5% sheep blood (BASB). *Propionibacterium acnes* (KCTC 3314) was cultured in Phenylethyl alcohol agar (BBL, USA), which was supplemented with vitamin K1 (10 μg/ml) and 5% sheep blood (PAKS). These obligate anaerobic microbes were also inoculated on Wilkins-Chalgren agar, which was supplemented with 5% sheep blood (WCSB). This agar variant is primarily used as a basal medium for the evaluation of antibiotic susceptibility of anaerobic bacteria (Kato *et al.*, 1993). As a representative microaerophilic bacterial strain, *Campylobacter fetus* subsp. *jejuni* Smibert (ATCC 29428) was cultured in Brucella broth (BBL, USA), supplemented with 0.16% agar (BA). As a representative capnophilic bacterial strain, *Mycoplasma hominis* (Freundt) Edward (ATCC 14017) was cultured in Mycoplasma Medium (MM, BBL, USA). These anaerobes, a microaerophilic and a capnophilic bacterium, were incubated for 48 h. These cultures were then repeatedly tested three times.

Results

Measurement of hydrogen gas volume and the rate of hydrogen gas generation

Sodium borohydride was allowed to react with acetic acid solutions at 0.5:1, 1:1, 1:1.5 and 1:4 molar ratios, for the generation of H_2. The reaction rate at each molar ratio is listed in Table 1. At molar ratio of 1:1, hydrogen gas was generated at the most rapid rate (90 s), and 8.56 L of hydrogen gas were achieved, which was sufficient to achieve an anaerobic state in the incubator. A great deal of noxious fumes were generated at the 1:4 molar ratio. Therefore, the 1:1 molar ratio was selected for the generation of hydrogen gas in the large-scale incubator, as well as for the gas analysis.

Analysis of hydrogen gas purity

The hydrogen gas produced by the reaction of 3.78 g of $NaBH_4$ with 25 ml of 4 M CH_3COOH was collected in a gas wash bottle at either 10°C or at 25°C. This gas was then subjected to analysis at the Korean Testing and Research Institute for the Chemical Industry. The purities

Table 1. The amount of H_2 volume generated at each different molar ratio of acetic acid and sodium borohydride and its reaction time

Molar Ratios H_2 (CH_3COOH : $NaBH_4$)	Gas volume (L)	Reaction time (second)
1:0.5	8.22	>300
1:1.0	8.56	90
1:1.5	8.55	180
1:4.0	*	100

*Generation of noxious fumes

of the hydrogen samples obtained at 25°C and at 10°C were 98.94% and 99.14%, respectively.

Chemical analysis of the precipitates and extracts after the hydrogen gas generation reaction

The main elements of the precipitates and extracts were boron and sodium. The precipitates consisted of sodium acetate and sodium borate (Table 2). The deduced chemical reaction formula, therefore, was based on the chemical analyses of the gases, precipitates, and extracts, as follows: $4NaBH_4 + 2CH_3COOH + H_2O \rightarrow 2CH_3COONa + Na_2B_4O_7 + 16H_2$.

Optimal molar ratio of the reagents required for the production of CO_2

Reaction time at the 1:1 molar ratio of 100 mM $C_6H_8O_7$ and 100 mM $NaHCO_3$ was found to be relatively rapid,

Table 2. The analysis of precipitates in the reaction bath and extracts from gas wash solution

Samples	Test Methods*	Components
	XRD	Sod. acetate & Sod. borate
Precipitates	SEM/EDX	Na
	ICP	B, Na
	XRD	Sod. acetate
Extracts	SEM/EDX	Na, Ca
	ICP	B, Na

*The abbreviations for test methods are described in Materials and Methods section.

Table 3. The CO_2 concentration generated at each different molar ratio of citric acid and sodium bicarbonate and its reaction time

Molar ratios $(C_6H_8O_7 : NaHCO_3)$	Final CO_2 concentration (%)	Reaction time (min)
1:0.5	2.4	3
1:1.0	5.1	5
1:2.0	10.6	45
1:3.0	15.0	90

and appropriate for the generation of practical CO_2 concentrations, like 5% and 10%. The greater the stoichiometric amount of $C_6H_8O_7$ versus $NaHCO_3$ was, the more CO_2 was generated, as is shown in Table 3. Considering the rapid reaction time (5 min), the reaction at the 1:1 molar ratio was selected for the production of CO_2 gas in the large-scale incubator, and for the culturing of the tested bacteria.

Reaction time required for creating various conditions in the large-scale bottle-free multipurpose incubator

A reaction time of 45 min was required to achieve an anaerobic state in the large-scale incubator, via the addition of 80 g of $NaBH_4$ to 530 ml of 4 M CH_3COOH solution at a 1:1 molar ratio, as derived from the results observed in the small-scale incubator. With 60 g of $NaBH_4$ and 400 ml of 4 M CH_3COOH solution at the same molar ratio, it took 35 min to reach a 5% microaerophilic environment in the large-scale incubator. At a 1:1 molar ratio of 0.65 M $NaHCO_3$ with 0.65 M $C_6H_8O_7$, it took 5 min to reach a 5% CO_2 concentration condition in the large-scale incubator: at the same molar ratio, a twofold increase in the amounts of $NaHCO_3$ and $C_6H_8O_7$ enabled the large-scale incubator to reach a 10% CO_2 concentration condition within the same time (Table 4).

Bacterial cultures under various culture environments generated in the bottle-free multipurpose incubator

Five strains of obligate anaerobic bacteria, one microaerophilic bacterial strain, and one capnophilic bacterial strain were cultured in the large-scale bottle-free multipurpose incubator, when we allowed them to grow under their preferred culture conditions. The five strains of obligate anaerobic bacteria grew well on their own appropriate media under absolute anaerobic conditions, even though *Mobiluncus mulieris* ATCC 35239 exhibited smaller colonies on the basal medium, WCSB, with regard to the antibiotic susceptibility testing of anaerobic bacteria than those on BASB, its typical medium. *Campylobacter fetus* and *Mycoplasma hominis* also exhibited normal growth characteristics on their optimal media under 5% microaerophilic and capnophilic conditions, which were generated as described in the Materials and Methods section. The results from these tests are shown in Table 5.

Table 4. Molar ratio and the time required for creation of each environmental condition in the large-scale bottle-free multipurpose incubator

Culture conditions	Molar ratios	Reaction time (min)
5% CO_2	0.65 M : 0.65 M $(C_6H_8O_7 : NaHCO_3)$	5
10% CO_2	1.30 M : 1.30 M $(C_6H_8O_7 : NaHCO_3)$	5
Absolute anaerobic	1 : 1 molar ratio (addition of 80 g of $NaBH_4$ to 530 ml of 4 M CH_3COOH)	45
5% microaerophilic	1 : 1 molar ratio (addition of 60g of $NaBH_4$ to 530 ml of 4 M CH_3COOH)	35

Table 5. Results of bacterial cultures under absolute aerobic, microaerophilic, and capnophilic conditions

Culture conditions & bacterial strains	Media[#]	Growth results after 48 h incubation
Absolute anaerobic		
Bacteroides fragilis ATCC 25285	BAKH	Growth
	WCSB	Growth
Peptostreptococcus asaccharolyticus	RCM	Growth
	WCSB	Growth
KCTC* 3321		
Fusobacterium nucleatum	PM	Growth
	WCSB	Growth
KCTC 2488		
Mobiluncus mulieris ATCC 35239	BASB	Growth
	WCSB	Less growth
Propionibacterium acnes KCTC 3314	PAKS	Growth
	WCSB	Growth
5% Microaerophilic		
Campylobacter fetus ATCC 29428	BA	Growth
Capnophilic		
Mycoplasma hominis ATCC 35239	BASB	Growth

*KCTC: Korean Collection for Type Cultures
[#]The abbreviations for media tested are described in Materials and Methods section.

Discussion

We were interested in developing a new incubator with greater safety, versatility, and significantly lower operating costs than existing anaerobic incubators. In this paper, we reported the principles and performance of our newly developed bottle-free multipurpose incubator. The main principle underlying the construction of our bottle-free multipurpose incubator is the generation of H_2 and CO_2 via chemical reactions between $NaBH_4$ and CH_3COOH, as well as via chemical reactions between $NaHCO_3$ and $C_6H_8O_7$. Since these chemical reactions proved to be inducible in the bottle-free incubator itself, instead of being the result of external supplementation by external gas cylinders (bottles), the bottles were obviously not a necessity, and could be done away with in our design. As the H_2 reacted with oxygen in the bottle-free multipurpose incubator, it was transformed into water by the palladium catalyst, and the oxygen could thus be eliminated.

Ferguson *et al.* (1976) first reported on anaerobic microbes cultured in anaerobic jars which were supplemented by H_2 and CO_2, and these gases were generated by the reaction of $NaBH_4$ with water, according to the formula: $NaBH_4 + 2H_2O \rightarrow NaBO_2 + 4H_2$, and by the reaction of $NaHCO_3$ with $C_6H_8O_7$, according to the formula: $3NaHCO_3 +$ $C_6H_8O_7 \rightarrow Na_3(C_6H_5O_7) + 3H_2O + 3CO_2$. Our previous study (Yang, 2002), however, found that the chemical reaction between $NaBH_4$ and H_2O was too slow to achieve desirable anaerobic conditions. Gribble *et al.* (1985) used acetic acid instead of water, according to the following: $NaBH_4 + 3CH_3COOH \rightarrow NaBH(CH_3COO)_3 + 3H_2$. Therefore, in this study, we utilized diluted acetic acid instead of water, in order to achieve a rapid reaction time for the generation of H_2, and to generate a greater quantity of H_2. The reaction used for the generation of H_2 was rendered much more rapid by the use of 4 M acetic acid, especially at a 1:1 molar ratio between $NaBH_4$ and CH_3COOH, as shown in Table 1. Moreover, by regulating these two reagents at the same molar ratios, we were able to conveniently control oxygen levels, and create a microaerophilic environment, as well as the anaerobic state in the large-scale bottle-free multipurpose incubator. Subsequent to the analysis of the precipitates formed by reacting $NaBH_4$ with 4 M CH_3COOH at a 1:1 molar ratio, our deduced reaction formula was as follows: $4NaBH_4 + 2CH_3COOH + 7H_2O \rightarrow 2CH_3COONa + Na_2B_4O_7 + 16H_2$. As shown in Tables 3 and 4, CO_2 production was completed within 5 min at a 1:1 molar ratio between $NaHCO_3$ and $C_6H_8O_7$. The greater the amount of these reagents at that molar ratio, the more CO_2 can be produced. Taken together, this bottle-free mul-

tipurpose incubator proved able to generate the variety of CO_2 and O_2 concentrations which are required for the culturing of bacteria.

In the bottle-free multipurpose incubator, practically all of the tested bacteria cultured well on their optimal media, under each of their preferred culture conditions, all of which were generated in the bottle-free multipurpose incubator, except for that of *Mobiluncus mulieris*. This was one of the anaerobes tested, and it exhibited the formation of smaller colonies on WCSB than when grown on its proper medium, BASB.

Here, we have obtained and reported good, solid evidence that our bottle-free multipurpose incubator can be used for the production of fastidious bacterial cultures with greater safety and versatility, and a significantly lower cost, than the incubators currently in use. Our novel incubator constitutes a favorable replacement for existing anaerobic incubators.

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