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SHORT COMMUNICATION

Gas supply apparatus using rotational motion of shaking incubator for flask culture of aerobic microorganisms

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Abstract

Shake flask cultivation, a cornerstone in bioprocess research encounters limitations in supplying sufficient oxygen and exchanging gases, restricting its accuracy in assessing microbial growth and metabolic activity. In this communication, we introduce an innovative gas supply apparatus that harnesses the rotational motion of a shaking incubator to facilitate continuous air delivery, effectively overcoming these limitations. We measured the mass transfer coefficient (k_La) and conducted batch cultures of *Corynebacterium glutamicum* H36LsGAD using various working volumes to assess its performance. Results demonstrated that the gas supply apparatus significantly outperforms conventional silicone stoppers regarding oxygen delivery, with k_La values of 2531.7 h^{-1} compared to 20.25 h^{-1} at 230 rpm. Moreover, in batch cultures, the gas supply apparatus enabled substantial improvements in microbial growth, maintaining exponential growth even at larger working volumes. Compared to the existing system, an increase in final cell mass by a factor of 3.4-fold was observed when utilizing 20% of the flask's volume, and a remarkable 9-fold increase was achieved when using 60%. Furthermore, the gas supply apparatus ensured consistent oxygen supply and efficient gas exchange within the flask, overcoming challenges associated with low working volumes. This approach offers a simple yet effective solution to enhance gas transfer in shake flask cultivation, bridging the gap between laboratory-scale experiments and industrial fermenters. Its broad applicability holds promise for advancing research in bioprocess optimization and scale-up endeavors.

KEYWORDS
aerobic cultivation, gas supply apparatus, mass transfer limitation, motion converting, shake flask

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1 | INTRODUCTION

Shake flask cultivation has stood as an indispensable experimental tool in bioprocess research and industrialization [1]. Beyond its role in preparing seed cultures prior to main fermentation, flask cultivation finds wide-ranging applications in small-scale cultures aimed at strain screening, media design, and exploring various fermentation parameters [2–5].

Traditionally, flask cultures rely on shaking flasks for mixing liquid medium and gas exchange [6]. These flasks have stoppers that permit air to enter and exit while protecting against external contaminants. While the stoppers allow some measure of air inflow, they fall short in supplying sufficient oxygen for robust microbial growth. Moreover, the expelling of gas generated is inefficient [7]. Throughout flask cultivation, gas exchange takes place at the interface between bulk gas and liquid medium. Consequently, a need arises to allocate ample headspace for oxygen delivery, thus limiting the available volume. This limitation significantly constrains the practical working volume to 10%–20% of the flask's capacity, reducing productivity per unit flask volume [3].

The ramifications of oxygen limitation manifest problems in aerobic metabolism, such as reduced cellular activity, a shift toward anaerobic metabolism, and diminished yields of desired metabolites [3, 8, 9]. Additionally, assessing the performance of aerobic strains using flasks becomes unfeasible due to the variance in oxygen availability, affecting pre-fermenter scale optimization experiments [10, 11]. The outlined challenges have been acknowledged as inherent limitations of flask culture, spurring a multitude of investigations to enhance oxygen provisioning [12–15].

Following the successful production of penicillin, aerobic bioprocesses predominantly embraced the deep tank fermentation approach [16]. In deep tank fermentation, air is introduced continuously from the lower section of the fermentation tank, with agitation breaking up the bubbles. This method enhances the interaction between the broth and gas, prolonging the residence time of oxygen. However, transposing this concept to the flask culture system necessitates the integration of an additional gas supply pump, potentially involving heightened power consumption and significant modifications either to the flask itself or the entire culture apparatus.

In this communication, we present an innovative system that efficiently delivers external air while retaining the utility of the existing shaking incubator system. We harnessed the rotational movement from the shaking incubator's medium-mixing action, converting it into translational motion to facilitate air pumping. This system introduces fresh external air into the flask continuously, improving oxygen availability.

PRACTICAL APPLICATION

We propose an innovative gas supply apparatus for flask cultivation that harnesses the rotational motion of a shaking incubator to facilitate continuous air delivery, effectively overcoming the challenges of conventional flask culture systems without drastic system changes. The developed gas supply apparatus significantly outperforms typical silicone stoppers regarding gas transfer coefficient as well as microbial growth. Remarkably, this method demonstrates decent performance even at larger working volumes, thus addressing the limitations of low working volumes, such as restricted sampling frequency and many flasks required for seed cultures. Its broad applicability could be envisioned in biological process research endeavors.

To assess mass transfer performance, we compared the k_La of flasks with the newly developed gas supply apparatus against the commonly used silicone stopper in laboratory settings. Subsequently, we conducted batch cultures of the aerobic strain *Corynebacterium glutamicum* H36LsGAD at various working volumes to validate the performance of the gas supply apparatus.

2 | MATERIALS AND METHODS

2.1 | Experimental apparatuses

A shaking incubator (VS-8480SF, Vision Scientific Co., Korea) was partly modified to equip a gas supply system. The 500 mL baffled Erlenmeyer flasks (borosilicate glass, Duran, USA) with GL 45 screw caps were used to evaluate the performance of gas transfer and microbial cell growth. The flask cap was replaced with a silicone stopper in the conventional flask cultivation system.

2.2 | Measurement of mass transfer coefficient k_La

The sodium sulfite (Na_2O_3) oxidation method was employed to determine the mass transfer coefficient k_La [17]. This method quantifies the extent of oxidized Na_2SO_3

in the solution by observing a drop in pH. Measurements were conducted using 500 mL baffled flasks containing 100 mL of the prepared solution at 22°C and 230 rpm.

For flasks connected to the gas supply apparatus, samples were collected every 15 min, while in the case using silicone stoppers, samples were collected every 6 h. The pH of the collected samples was measured using a pH meter (Orion Star A211, Thermo Scientific, USA). To ensure reliable results, all experiments were performed in triplicate.

2.3 | Microorganisms, media, and culture conditions

Corynebacterium glutamicum H36LsGAD was cultivated to evaluate the performance of the gas supply apparatus developed. The seed and cultivation medium used in this study have been previously described in detail [18]. *C. glutamicum* H36LsGAD was cultivated in 500 mL baffled flasks containing 100 or 300 mL of CG50 medium at 30°C and 230 rpm for 12 h. Samples were taken every 3 h and cell growth was determined by measuring optical density at 600 nm (OD_{600}). Lactic acid concentrations in the samples were measured through high-performance liquid chromatography (See Supplementary Methods for details). The inoculum ratio was 1% (v/v) and all cultivations were performed in duplicate. The oxygen partial pressures within the flask's headspace were monitored to check inefficient oxygen influx and carbon dioxide efflux using silicon stoppers (See Supplementary Methods for details).

3 | RESULTS AND DISCUSSION

3.1 | Fabrication of continuous gas supply apparatus for flask culture

We have designed a device that replaces the rotational movement of a shaking incubator with the translational motion for gas pumping (Figure 1) [19]. In the apparatus designed, the shaking part and a fixed section of the shaker are connected to a cylinder with a piston attached, and it acts as a kind of "rack and pinion," allowing the piston's linear movement in an orthogonal direction (See the Video S1 for the actual operation). For this purpose, a disposable 10 mL syringe was used as the piston pump.

A 3-way check valve was connected to the end of the syringe to supply air continuously to the flask while preventing reverse flow.

3.2 | Oxygen transfer performance

Figure 2 depicts the k_La values measured using the sulfite oxidation method [20] under various shaking speeds and liquid volumes. When utilizing the gas supply apparatus with 100 mL liquid volume at 230 rpm, the k_La was 2531.7 h^{-1} compared to 20.25 h^{-1} with the conventional setup, demonstrating the outstanding oxygen transfer capability of the system developed in this study. The relationships between shaking frequency (rpm), filling volume, and k_La for each system are represented by Equations 1 and 2 (dotted lines in Figure 2).

Gas supply apparatus:

$$k_La = 11.0 \cdot 10^{-6} \cdot d^{1.92} \cdot n^{1.795} \cdot d_0^{0.38} \cdot V_L^{-0.87} \quad (1)$$

Conventional setup (silicone stopper):

$$k_La = 3.34 \cdot 10^{-6} \cdot d^{1.92} \cdot n^{1.16} \cdot d_0^{0.38} \cdot V_L^{-0.83} \quad (2)$$

where d [cm] is the maximum inner flask diameter, n [1/min] is the shaking frequency, d_0 [cm] is the shaking diameter, and V_L [mL] is the filling volume.

The constant value of the empirical equation for the gas supply apparatus is $11.0 \bullet 10^{-6}$, which is 3.3 times greater than that for the conventional silicone stopper. While the effect of shaking frequency and filling volume when using the silicone stopper aligns with the findings by Klockner and Buchs [6], the exponent for n in Equation 1 is 1.795, indicating a higher degree of k_La enhancement with increasing rpm. Conversely, although the k_La value with sizeable liquid volume is still much higher, the impact of filling volume exhibited an exponent of −0.87 for the gas supply apparatus, indicating a higher rate of decrease with increasing liquid volume.

On the other hand, when the liquid volume was small (100 mL, 20% filling), k_La decreased sharply at 300 rpm. This sudden drop is probably caused by the gas sparger being exposed to the air without fully submerging in the liquid with low liquid volume and high shaking frequencies.

3.3 | Flask cultivation of *C. glutamicum* H36LsGAD

Batch flask cultures were performed using *C. glutamicum* H36LsGAD to verify whether the excellent gas transfer performance extends to microbial cultivation. In the batch cultures with a 100 mL working volume, the flask system with gas supply apparatus demonstrated superior performance compared to the conventional flask cultivation

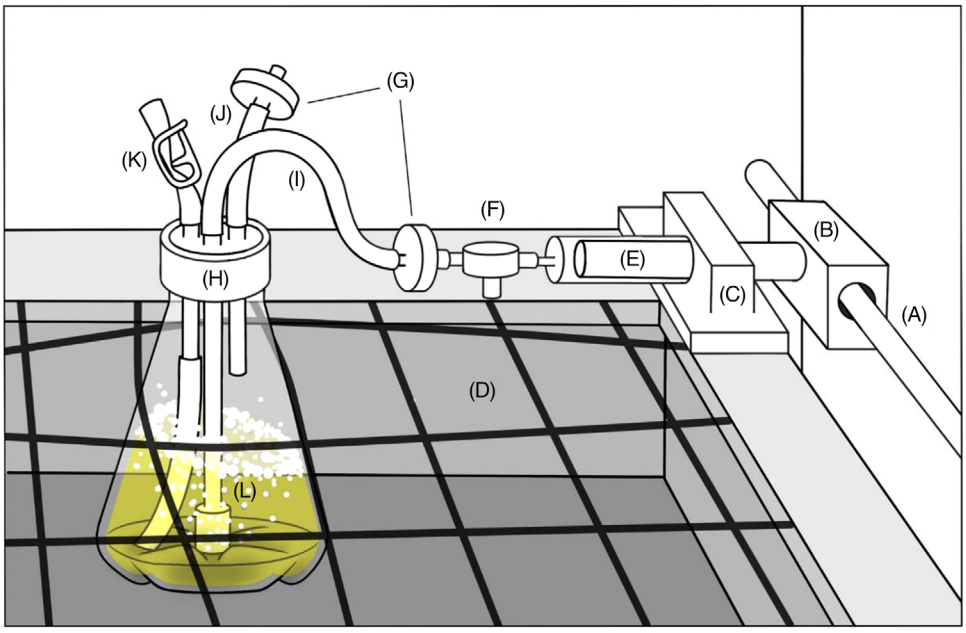

FIGURE 1 Conceptual diagram of flask culture system using gas supply apparatus consisting of (A) supporting body, (B) converting part, (C) fixing part, (D) shaking part, (E) syringe, (F) check valve, (G) syringe filters, (H) 3-port connector cap, (I) gas inlet, (J) gas outlet, (K) sampling line, and (L) sparger.

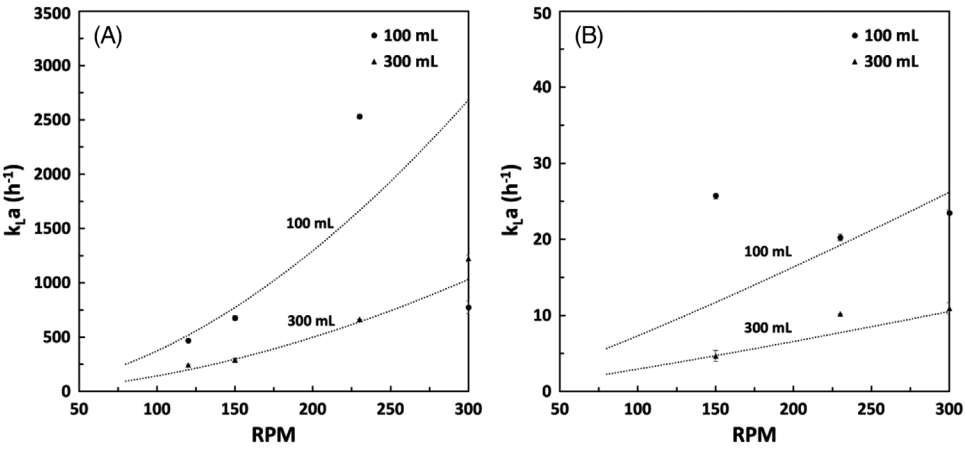

FIGURE 2 k_La values of flasks using gas supply apparatus (A) and silicone stopper (B) under various shaking frequencies and filling volumes. Circle and triangle indicate the k_La values of the flasks with working volumes of 100 and 300 mL, respectively. The dotted lines represent the k_La values estimated by Equations 1 and 2.

(Figure 3A). Both methods exhibited similar cell growth up to 9 h (specific growth rate of ~0.53 h^{-1}); however, the specific growth rate in the flask culture with a silicon stopper drastically decreased to 0.03 h^{-1}, resulting in an OD_{600} of 13.55 after 12 h of culture. Conversely, exponential growth persisted using the gas supply apparatus, achieving a final OD of 45.85, while the specific growth rate decreased slightly to 0.41 h^{-1}.

Subsequently, experiments were conducted with a working volume of 300 mL, significantly higher than the standard liquid ratio (20%) used in flask cultures, to observe whether the gas supply apparatus could address the low volume utilization issue in traditional flask cultures (Figure 3B). Like the 100 mL culture results, both cultivations exhibited a similar initial specific growth rate of approximately 0.43 h^{-1}. However, the specific growth rate in the conventional flask culture rapidly declined after 6 h and eventually reached 0.06 h^{-1}, leading to a final OD_{600} of 3.92. The shaking incubator's rotation speed was increased from 230 to 350 rpm; however, the final

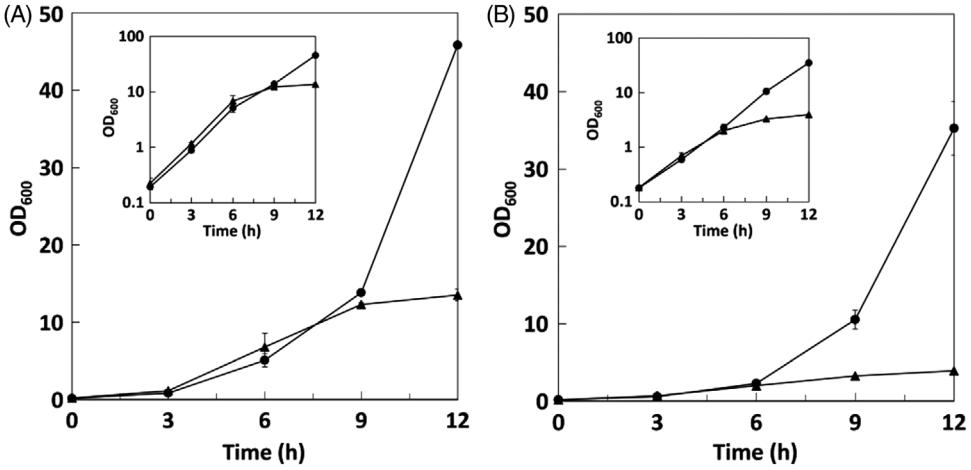

FIGURE 3 Cell growth in the flask culture with a working volume of 100 mL (A) and 300 mL (B) using a gas supply apparatus and silicon stopper. All flask cultures were conducted in a 500 mL baffled flask at 30°C and 230 rpm. Circle and triangle indicate the OD_{600} in the flasks using the gas supply apparatus and silicon stopper, respectively. The inset figures show the semi-log plots of the OD_{600} with time.

OD_{600} still did not exceed 4. In contrast, using the gas supply apparatus, although slower than the 100 mL culture, exponential growth persisted at a specific growth rate of 0.40 h^{-1} in the latter part, resulting in a final OD_{600} of 35.25 at 230 rpm.

The growth rate decreasing in the conventional flask culture is attributed to poor oxygen delivery performance. As dissolved oxygen becomes limited, cell metabolism transitions to an anaerobic state, leading to organic acid accumulation and a subsequent decrease in pH, further inhibiting cell growth. In the 100 mL culture using the gas supply apparatus, the final pH was 6.74, showing a slight difference from the initial pH of 6.46. On the other hand, the conventional flask culture exhibited a final pH of 5.14, which is significantly lower than the initial pH. The lactic acid analysis also reflected the metabolic state change; the final lactic acid concentration was 0.24 g/L with the gas supply apparatus, while 1.49 g/L of lactic acid was accumulated in the conventional flask culture. Conventionally, $CaCO_3$ is commonly used to alleviate these pH-decreasing issues in the flask cultivation of *Corynebacterium* sp. However, it has been pointed out that using the insoluble $CaCO_3$ makes it challenging to monitor cell growth.

Another advantage of the gas supply apparatus is the continuous exchange of headspace gases. In the conventional system, gas exchange solely occurs through the silicon stopper, resulting in inefficient oxygen influx and carbon dioxide efflux. This exacerbates internal oxygen depletion as well as pH drop in the flask. A portion of the headspace gas was replaced with nitrogen, and the oxygen composition was measured to confirm this phenomenon. Recovery from 68% to 80% of atmospheric oxygen partial pressure took more than 8 h, with negligible recovery thereafter (Figure S1). In contrast, atmospheric composition was restored in less than a minute when using the gas supply apparatus. Therefore, utilizing the gas supply apparatus allows for maintaining both high k_La and dissolved oxygen concentrations, ensuring exceptional performance even with large culture volumes. However, evaporation of the culture medium due to vigorous gas injection was observed and 9.0% of the total volume evaporated over 12 h with a 100 mL working volume (See Table S1 for details). Strategies such as reducing the gas supply per pumping should be considered to prevent changes in the culture environment caused by the evaporation of the culture medium.

4 | CONCLUSION

While flask cultures offer the advantage of exploring considerable variables with a simple setup, their poor control over fermentation conditions, including gas transfer, has posed challenges in directly translating obtained results to fermenters. In this communication, we proposed a straightforward approach to achieve gas delivery performance comparable to fermenters by simply converting the rotation motion of a shaking incubator into the pumping energy required for gas supply without drastic system changes. Remarkably, this method demonstrates decent performance even at larger working volumes, thus addressing the limitations of low working volumes, such as restricted sampling frequency and many flasks required for seed cultures. Its broad applicability could be envisioned in biological process research endeavors.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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