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A SIMPLE AND MODIFIED MANOMETRIC METHOD FOR MEASURING OXYGEN UPTAKE RATE OF PLANT CELLS IN FLASK CULTURES

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SUMMARY

A simple and convenient technique was developed based on the principle of Warburg manometric method to measure O_2 uptake rate (OUR) and CO_2 evolution rate (CER) of suspended cells in a shake flask culture. It was successfully applied to suspension cultures of rice (*Oryza sativa*) and *Panax notoginseng* cells, and some important bioprocess parameters, such as OUR, CER, respiratory quotient (RQ), specific OUR (SOUR) and specific CER (SCER), were quantitatively obtained. The measuring system is easy to operate, able to treat many samples simultaneously and is economical.

INTRODUCTION

Several methods for measuring O_2 uptake rate (OUR) of cultured cells are used routinely: Warburg manometric method (Umbreit *et al.*, 1972); O_2 balance method by monitoring O_2 levels in the inlet and exhaust gases in a bioreactor; and dynamic method using O_2 electrode. In the manometric method, a Warburg respirometer is required and it is also difficult to aseptically measure the cultured cells during cultivation. The oxygen balance method is usually used in a large-scale bioreactor, and it requires a gas analyzer (Bond *et al.*, 1988; Zhong *et al.*, 1994) or a mass spectrometer (Nikolova *et al.*, 1991). The dynamic method is also usually used for bioreactor cultivations; for shake flask cultivations, it requires a special shake flask besides an O_2 electrode. It is obvious that an experimental system using these methods is rather complicated and relatively expensive; it

is difficult to monitor OUR of cells aseptically during flask cultivation, or impossible to treat many samples simultaneously.

In plant cell cultures, shake flask culture is an indispensable stage of cultivation. Effects of various culture conditions such as medium components on the physiological and metabolic behaviours of plant cells are usually investigated in a shake flask. Selection of cell lines and preliminary scale-up studies are also conducted using a flask cultivation system. These investigations are very essential and critical to bioprocess control and optimization in a bioreactor on a large-scale. However, to the best of our knowledge, there is yet no research regarding the monitoring of OUR, CO_2 evolution rate (CER) and respiratory quotient (RQ) in shake flask cultures of plant cells. In this article, based on the principle of Warburg manometric method, we first developed a simple and convenient system for measuring the parameters OUR and CER of plant cells in a shake flask, then successfully applied the system to suspension cultures of both rice and notoginseng cells in a flask culture.

MATERIALS AND METHODS

Plant cells, medium and culture conditions Suspension cultures of rice cells (*Oryza sativa*) and notoginseng cells (*Panax notoginseng*) were used in the present study. Rice cells were cultured in N_6 medium (Chu *et al.*, 1975) plus 30 g sucrose/*l*, 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/*l* and 0.2 mg kinetin (KT)/*l* (Wen and Zhong, 1995). Notoginseng cells were cultured in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 30 g sucrose/*l*, 2 mg 2,4-D/*l* and 0.7 mg KT/*l* (Zhong and Zhu, 1995). Both of them were inoculated in a 250 m*l* shake flask containing 50 m*l* medium, and cultivated at 25°C under darkness (Wen and Zhong, 1995; Zhong and Zhu, 1995).

Medium feeding During cultivation of rice cells, both carbon and nitrogen sources were fed into the flask cultures on the 14th day of cultivation. The feed medium for each 250 m*l* flask was 1.5 g sucrose, 141.5 mg KNO_3 and 23.2 mg $(NH_4)_4SO_4$.

Analyses Dry cell weight was measured as follows. The cells of a sample were filtered under vacuum, washed with deionized water for several times, then dried under 60°C for ca. 48 h until the cell weight is constant (Wen and Zhong, 1995). Residual sugar concentration was determined using the method of phenol-concentrated sulfuric acid as described previously (Zhong *et al.*, 1993).

RESULTS AND DISCUSSION

1. A System for Measuring OUR and CER of Cell Cultures in a Shake Flask

An experimental system for measuring OUR and CER of a flask culture is developed as shown in Fig. 1. The whole system with incubation shaker is under temperature-controlled environment. During cultivation, the cells take up O_2 and give out

CO_2 which is rapidly absorbed by the alkaline solution of the little bottle in the flask (Fig. A). For the measurement of OUR and CER, the pinch valve (as shown in Fig. 1) is closed, and the pressure of the closed system decreases due to the consumption of O_2 in the air. Thus, the OUR value can be obtained by monitoring the dynamic decrease of the system pressure. If the cell concentration is known, specific OUR (SOUR) can be further obtained. The calculation formula are as follows.

$$OUR = K \cdot dh/dt \quad (1)$$

$$SOUR = OUR/X \quad (2)$$

Here $$K = \rho \cdot g \cdot v/RT \quad (3)$$

where X is cell concentration; ρ is the density of liquid in manometer; g is acceleration of gravity (a constant); v is the gas volume inside the whole system (determined as described below); R is gas constant; T is temperature; h is the height of liquid column in manometer; t = time.

The value of v is determined by subtracting the volume of fluid in vessel(s) (i.e., 250 m*l* flask for Fig. 1B, and both 250 m*l* flask and 20 m*l* bottle for Fig. 1A) from the total volume within the system (Fig. 1). The total volume within the system is determined as follows: In a temperature-constant room (25°C), the clean dry whole system is weighed, and it is then filled with distilled water which has been boiled to remove dissolved gases and cooled to 25°C. Water is added until the level just reaches the reference point on closed arm of manometer, then the whole system is weighed. From the temperature of the water its density can be ascertained, and the volume of the whole system can be calculated. To improve the accuracy of the method, in our case the v value is determined for different flasks, and a rubber bung used to seal the system is pushed in tightly to give the same internal volume for a specific flask.

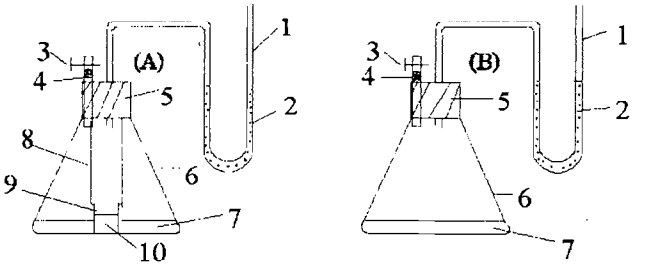

Figure 1. Experimental system for measuring OUR and CER of shake flask cultures. A, system with a 20 m*l* alkaline solution-containing bottle; B, system without the bottle. In the figure: 1, glass tube; 2, water; 3, pinch valve; 4, cotton plug; 5, rubber bung; 6, 250 m*l* flask; 7, medium; 8, stainless steel wire for securing of the bottle 9; 9, 20 m*l* bottle; 10, 10% KOH solution (3 m*l*).

For system B, the pressure change inside the flask is determined by the consumption of O_2 over the production of CO_2, i.e.,

If CER > OUR K dh/dt = CER - OUR (4)

If CER < OUR K dh/dt = OUR - CER (5)

Because OUR value is available from the system A, thus the CER value can be obtained from Eq. 4 or 5.

2. Application

2.1 Monitoring of OUR and CER in batch and fed-batch cultures of rice cells

Figures 2A and 2B show the time profiles of both the cell growth and residual sugar concentration in batch and fed-batch cultures of rice cells in a shake flask. The dynamic changes of OUR and CER during cultivation were measured using the above-described technique (Fig. 2C). The corresponding values of SOUR and SCER were obtained after measuring the cell mass in a flask. Figure 2D indicates that at the lag phase, the values of SOUR, SCER and RQ (respiratory quotient, i.e., CER/OUR) were quite low. These parameters reached maximum at an early stage of linear growth phase, i.e., 2.30 μmol O_2/g DW·min, 2.65 μmol CO_2/g DW·min and 1.15, respectively. Later, the values of SOUR and SCER suddenly decreased. The RQ value was maintained at about 1.0 from the 4th to 14th day of cultivation with little change. After the 14th day, the RQ decreased rapidly.

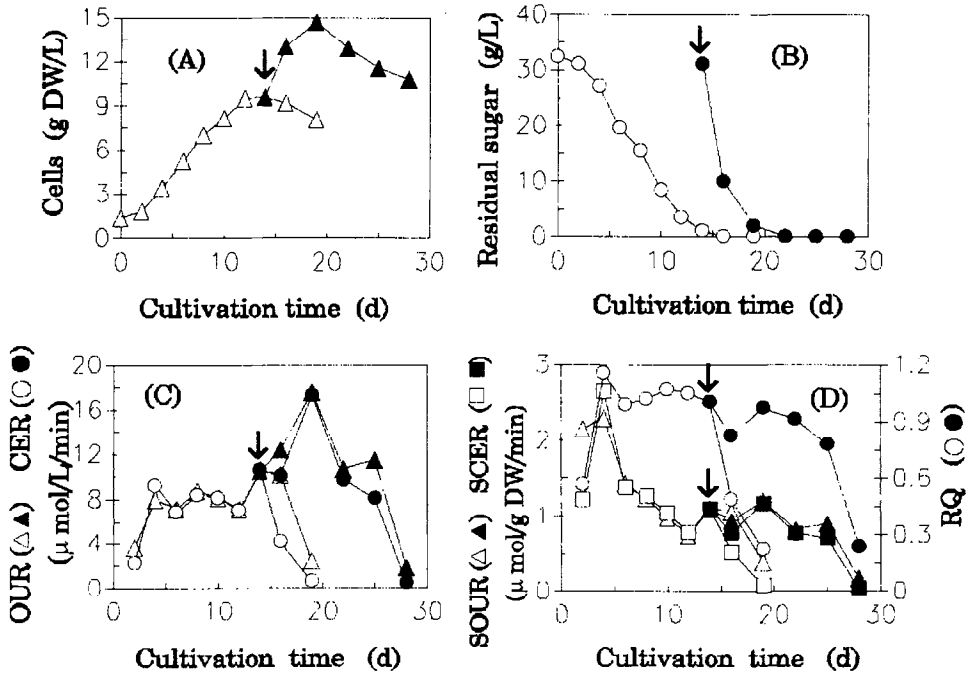

Figure 2. Time profiles of the growth (A), residual sugar concentration (B), OUR and CER (C), as well as SOUR, SCER and RQ (D) in batch and fed-batch cultures of rice cells in a 250 ml shake flask. In the figure, the arrow indicates the point of medium feeding, and the dark symbols represent the data after medium feeding.

In a fed-batch culture, carbon and nitrogen sources were supplemented to the medium on the 14th day of cultivation. The OUR and CER after feeding were also measured using this method. Figure 2 shows that the cell mass, OUR and CER values increased after feeding. The values of SOUR, SCER and RQ of the cells under feeding (fed-batch culture) were much higher than those without feeding (batch culture) (Fig. 2D). This indicates that the effect of nutrient feeding on the cells' physiological and metabolic behaviours can be accurately monitored by the system. A further study of the nutrient feeding strategy is however required.

2.2 Monitoring of OUR and CER in notoginseng cell cultures

Suspension cultures of *Panax notoginseng* were also used in our experiments to further verify the applicability of the developed measuring system. Figure 3 shows the time profiles of the cell growth (A), OUR, CER (B), as well as SOUR, SCER and RQ (C) of the cell cultures in a 250-m*l* shake flask. The maximum values of SOUR and SCER were 2.52 μmol O_2·g DW·min and 1.83 μmol CO_2·g DW·min, respectively (at the early stage of growth phase). The values of these two parameters maintained at a relatively high level until the 13th day of cultivation, then decreased rapidly until the end of cultivation (the 18th day). Their change pattern is different from that of rice cell cultures, but similar to the case of *Perilla frutescens* cell cultures in a bioreactor (Zhong *et al.*, 1994). The value of RQ maintained at about 0.7-0.8 during the whole growth phase, and had a sharp decrease at the end of cultivation. The change pattern of RQ is similar to that of both rice cell cultures as described above and *P. frutescens* cell cultures under a low agitation speed in a bioreactor (Zhong *et al.*, 1994).

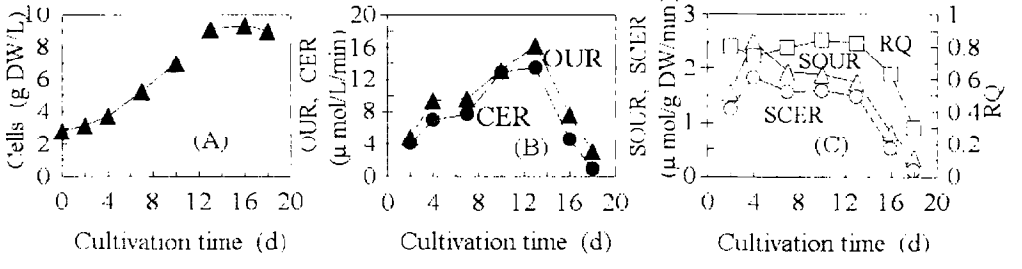

Figure 3. Dynamic changes in the cell growth (A), OUR and CER (B), as well as SOUR, SCER and RQ (C) in suspension cultures of *Panax notoginseng* cells in a flask.

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