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Full Length Research Paper

Application of a stir-tank bioreactor for perfusion culture and continuous harvest of *Glycyrrhiza inflata* suspension cells

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We developed a perfusion stir-tank bioreactor that allowed continuous cultivation and harvest of plant cells and challenged for *Glycyrrhiza inflata* cell suspensions. Continuous separation of cell cultures from the medium occurred using a gravitational sedimentation column. We then conducted cell retention studies of this bioreactor by detecting the cell loss in the discarded medium. We determined that complete cell retention had been achieved when the perfusion rate (perfusion rate is the ratio of medium feeding volume per day to bioreactor working volume) was lower than 100% d^{-1} and the cell suspension recirculation speed was more than 0.5 ml s^{-1}. Growth kinetic measurements showed that the maximum cell concentration reached 25 ± 0.5 g l^{-1} DW (dry weight). The maximum growth rate occurred on Day 18. Under the given perfusion medium feed rate, the bioreactor operated smoothly, maintaining a relatively stable concentration of 20 g l^{-1} through continuous cell harvest. The bioreactor we used could be an efficient cell culture system and demonstrates industrial potential.

Key words: Continuous harvest, glycyrrhiza inflate, medicinal herbs, perfusion bioreactor, suspension cells.

INTRODUCTION

In vitro culture of higher plants is an alternative source for raw materials and secondary metabolites. Secondary metabolites released in the culture medium can also be reused by the cells or degraded in the medium (Dobbeleer et al., 2006). In addition, the substances released from cells may inhibit cell growth or hinder the further production of target products due to feedback inhibition (Rao et al., 2002).

Glycyrrhiza inflate is a common Chinese medicinal herb used to harmonize the effects of the other ingredients in most of Chinese herbal prescriptions (Yang et al., 2009). The main effective constituent is flavonoids and saponins (Nomura et al., 2002; Yang et al., 2008), but we fail to detect the saponins in *in vitro* cells. Tissue and cell browning has been a major impediment of *G. inflata* culture *in vitro*. The traditional methods applied to overcoming the harmful effects of browning have no conclusive ameliorative effect on the browning of plant *in vitro* tissue and cells. Application of two liquid-phase bioreactor to remove metabolites from medium cannot be extended, because most plant cells were sensitive to solvent toxic effect (Bassetti and Tramper 1994).

Perfusion culture in bioreactors has high potential for propagation of plant cells and can offset the possibility of browning and feedback inhibition. To maximize this propagation potential, we continuously replaced the medium in our bioreactor to prevent cell metabolites and other substances from harming growing cells, while promptly supplemented the cultures with fresh medium. Continuous cell/medium separation is a difficult problem in perfusion culture and the approaches developed for mammalian cell retention cannot be applied to plant cells. Retaining cells by continuous centrifugation is not applicable because plant cells are very sensitive to shear stress (Johnson et al., 1996). Also, *in situ* filtration by membrane or steel mesh can lead to filter clogging when cell density is high (Kawahara et al., 1994). Gravitational sedimentation is considered the most effective way to separate cells from medium in the perfusion culture of

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Abbreviations: BA, 6- Benzylaminopurine; **NAA,** naphthaleneacetic acid; **MS,** Murashige and Skoog medium.

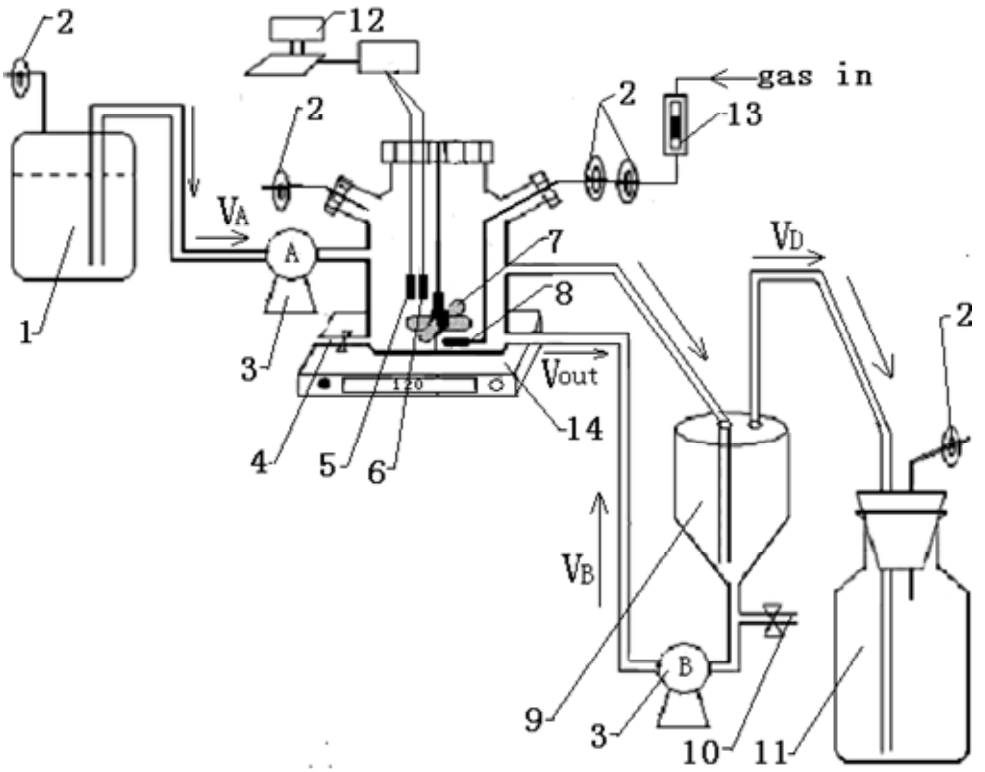

Figure 1. Configuration of perfusion stir-tank bioreactor system. **1)** Fresh medium; **2)** Air filter; **3)** Pump; **4)** Sample pipe; **5)** pH probe; **6)** DO probe; **7)** Impeller; **8)** Air sparger; **9)** Settlement device; **10)** Cell harvest pipe; **11)** Discarded medium; **12)** Computer; **13)** Air flow meter; **14)** Magnetic churn-dasher.

plant cells. Wei and Arias (2003) obtained complete cell retention under the conditions of perfusion rate 40% d^{-1} and packed cell volume (PCV) of 60% by using a perfusion bioreactor based on cell sedimentation. Wei et al. (1996) also reached maximum cell retention efficiency of 100 percent at the perfusion rate of 100% d^{-1} and 20% PCV using an air-lift bioreactor, which incorporated a cell sedimentation zone delimited by a rectangular baffle in the lower downcomer.

However, such bioreactors with built-in cell-settling devices tend to have numerous cells accumulating at the bottom of the reaction tank, causing difficulties with liquid mixing and mass transferring. Dobbeleer et al. (2006) developed a perfusion stir-tank bioreactor with four sedimentation columns fixed vertically on the lid of the reaction tank, but failed to find a suitable position for the gas sparger they used. Combining a high perfusion rate with high cell concentration for perfusion bioreactors with built-in cell settling devices is thus highly challenging.

This paper focuses on the application of a perfusion stir-tank bioreactor coupled with a cell/medium separation device positioned in the external loop of the system, with the goal of preventing clogging of the settling device and attendant failure to propagate suspension cells. We cultivated *G. inflate* in this bioreactor and achieved continuous *in vitro* cell harvest. We plan further studies to apply this system to other plant species.

MATERIALS AND METHODS

Cell culture and maintenance

Cell culture was derived from callus induced from *G. inflata* hypocotyls on solid medium of MS (Murashige and Skoog, 1962) supplemented with 2 mg L^{-1} naphthalene acetic acid (NAA). Cell suspensions were established from friable callus in liquid MS media supplemented with 30 g L^{-1} sucrose, 0.4 mg L^{-1} 6- benzylaminopurine (6-BA) and 0.4 mg L^{-1} NAA shaking at 120 rpm. The suspensions were subcultured every 7 days in a 500-ml Erlenmeyer flask containing 200 ml fresh medium on a rotary shaker at 120 rpm. The culture was kept at 25°C under darkness.

Bioreactor configuration and perfusion culture

The configuration of the perfusion stir-tank bioreactor system is depicted in Figure 1. This bioreactor has a working volume of 1.0 liter (total volume 1.5 liters). An important component of our system is an external, 0.3-liter gravitational sedimentation column, with which we achieved continuous cell/medium separation. Two liquid pumps controlled the perfusion medium feed rate and liquid circulation speed. A 4–bladed impeller was used for agitation and a microporous glass flake embedded in the pipe acted as the gas sparger. A microprocessor monitored dissolved oxygen (DO) and pH values.

Bioreactor cultures were established containing 1 liter MS medium supplemented with 30 g l^{-1} sucrose, 0.4 mg l^{-1} 6-BA and 0.4 mg l^{-1} NAA. Cell suspensions from shake flasks were inoculated into the bioreactor at an initial concentration of 5 g l^{-1} dry weight. The perfusion medium was continuously added to bioreactor at a feed rate of 30% (v/v) each day and the agitation and aeration rates were set at 150 rpm and 0.3 vvm, respectively. A batch mode was also designed at the same conditions as the perfusion run, except there was no medium exchange.

Determination of cell weight and cell retention efficiency

The samples from the bioreactor were put through a filter paper under vacuum and washed three times with distilled water to remove residual sugar. The filtered cells were transferred to pre-weighted dishes and weighed to obtain the fresh weight (FW), and finally dried at 60°C for two days to obtain cell dry weight (DW). The specific growth rate was calculated as the formula (Jeong et al., 2007):

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\ln(x/x_0)}{t}$$

where X is biomass weight, t refers to the cultivation period during which the maximum dry cell mass was obtained, x_0 is inoculation size and μ is specific growth rate (day^{-1}). The cell retention efficiency was determined by measuring cell loss in the discarded medium.

Determination of volumetric oxygen mass transfer coefficient (K_La)

The K_La was measured with water by a dynamic gassing-in and gassing-out method (Robert et al., 1981) at different aeration rates and stirring speeds. Degassing was performed using N_2 gas fed at the same flow rate as air. The value of K_La is defined as:

$$\ln\frac{c^*}{c^* - c} = k_L a \cdot t$$

where c is oxygen concentration in the liquid (water) at time t (mg l^{-1}) and c^* is oxygen solubility in the liquid (mg l^{-1}).

Determination of cell viability and flavonoid content

Cell viability was determined by color reaction with 0.02% Evans blue and calculated as a percent of colorless cells (Demidova et al., 2006). To obtain the flavonoid content, samples of dry cells were ground to fine powder in a mortar and filtrated with a 100-mesh sieve. Ethanol of 80% (v/v) was added to the filtrated powder and extracted with ultrasonic for 90 min. When evaporation of the solvent under reduced pressure, the ethanol extract liquids was re-extracted with equivalent volume ethyl acetate for tree times, followed by redissolution with ethanol of 95%. After centrifugation, the supernatant was adjusted to a constant volume of 50 ml with methanol. The sample of 0.5 ml was added by 0.5 ml hydroxide sodium (10%) and shaken up for 5 min for color reaction, then diluted to 10 ml. The absorbance of samples was measured at 410 nm by spectrophotometer. The standard sample was 0.1 mg ml^{-1} rutin solution and the regression equation of standard curve was Y = 0.1182x + 0.0012, r = 0.996 (n = 5).

Table 1. Effect of cell suspensions recirculation speed (v_B)[b] on cell survival rate[a].

v_B (ml s^{-1})	Cell survival rate (%)
0.0	81.7
0.05	76.0
0.2	69.3
0.5	68.0
1.0	49.3
2.0	33.3

[a]These experiments were carried out at a controlled medium feed rate and agitation rate of 50 %(v/v) per day and 150 rpm, respectively. Cell viability was determined 10 days after inoculation and about 2000 cells were calculated in each of three replicates.
[b]A liquid pump (diameter of the match piper is 1.0 cm) controlled the cell suspensions recirculation speed (vB).

RESULTS AND DISCUSSION

Cell retention efficiency of the bioreactor

Cell cultures were continuously separated from the medium in the settling device and then pumped to the reaction tank. The perfusion medium feed rate (v_A) and cell culture recirculation speed (v_B) were controlled by liquid Pumps A and B (pipe diameter 1.0 cm). Thus the equality of all components, represented by the equation $v_A=v_D=v_{out}-v_B$, was established once the system began to operate smoothly (Figure 1). The cell retention efficiency of this bioreactor was determined by detecting the cell loss in the discarded medium. Figure 2 depicts the cell retention efficiency at different perfusion rates and cell suspension recirculation rate. These experiments were carried out at controlled agitation (150 rpm) and aeration (0.3 vvm) rates. The results suggest that both the v_B and perfusion rates influence cell retention efficiency. Cell loss was detected in the exhaust medium with a perfusion rate of more than 100% d^{-1} and v_B less than 0.5 ml s-1. V_B was positively correlated with cell retention rate.

Complete cell retention was achieved with v_B 1.0 ml s^{-1} and a perfusion rate lower than 100% d^{-1}. However, plant cells are very sensitive to shear stress during recirculation of culture medium. Table 1 shows that more than half of the cells died once the v_B exceeded 1 ml s^{-1}. Hence, we determined that the appropriate conditions are v_B 0.5 ml s^{-1} with a perfusion rate of 50% d^{-1} (Figure 2).

Volumetric oxygen mass transfer coefficient (k_La)

For this bioreactor system, two parameters can be used

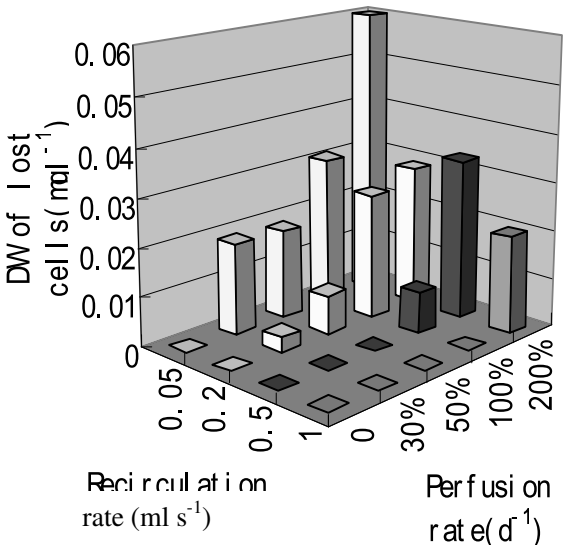

Figure 2. Effect of perfusion rate and cell suspension recirculation speed on cell retention efficiency in bioreactor. The cell retention efficiency is defined as: $(C_M - C_L)/C_M$, where C_M is cell density in bioreactor, and C_L is cell density in discarded medium.

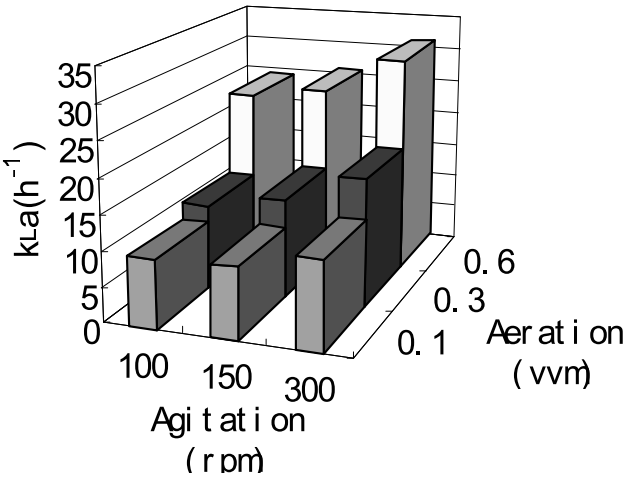

Figure 3. K_La of bioreactor at different agitation and aeration velocities.

to modulate k_La. Figure 3 shows that stirring rates and aeration rates are positively associated with k_La, but *in vitro* cells are subject to shear forces from excessive mixing and aeration. Generally, the appropriate k_La for plant cell growth in bioreactors is 10 - 20 h^{-1} (Zhong, 2001). Therefore, the mass transfer ability of this bioreactor system meets the requirement of plant cell growth when the agitation and aeration rates are over 150 rpm and 0.3 vvm, respectively.

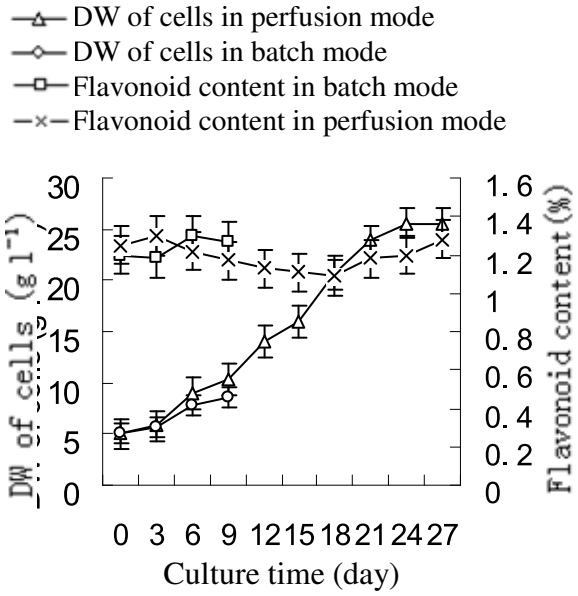

Figure 4. Comparison of growth kinetics and flavonoid content of *Glycyrrhiza inflata* cells between perfusion mode and batch mode. These experiments were repeated for three times.

Cell growth and harvest

While culturing cells in the bioreactor, the number of cells entering the settling column increased as cell density rose. When the packed cell volume (PCV) reached 60%, the cells in the settling column formed a stable sedimentation front evolved in a homeostasis. However, as cell concentration was allowed to rise, the cultures became very thick in the bioreactor, which worked against mass transfer and cell growth.

In perfusion cell cultures, manipulation of the cell bleed rate has been shown to be effective in achieving high specific growth rates and reducing dead cell accumulation (Hiller et al., 1993; Wei et al., 1996). In this study, the bioreactor was operated with a bleed stream to transfer cells out of the bioreactor. The cells were continuously harvested at a harvest pipe (refer to Figure 1). Figure 5 shows that the maximum specific growth rate occurred on Day 18 and the maximum cell concentration was 20 g l^{-1} at that point (Figure 4). To keep a high cell growth rate under the given perfusion medium feed rate (that is, 30% per day), cells concentration was maintained at about 20 g l^{-1} through continuous cell harvest, with cell density on-line monitored. For continuous perfusion culture of plant cells, the maximum growth rate mainly depends on the perfusion medium feed rate. Thus, we conclude that the growth rate of *G. inflata* could be further improved by increasing the perfusion rate. However, the cell culture recirculation speed must be increased to retain cells in the settling device if the bioreactor is operated with a high perfusion rate, which will further increase the damaging shear force on plant cells.

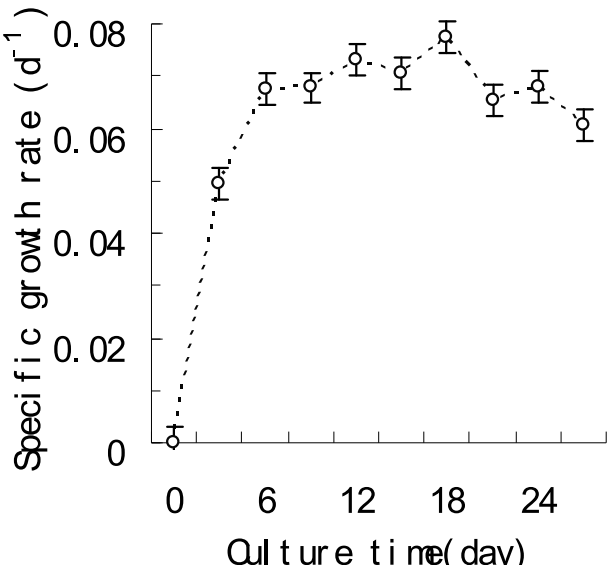

Figure 5. Changes in Specific growth rates during the perfusion culture.

Cell and tissue browning have also been major impediments to *in vitro* culture of *G. inflata*. In this study, we effectively mitigated cell browning through perfusion culture; with suspension cells dying of browning in about 9 days when cultivated in batch mode (refer to Figure 4). In addition, no significant difference in flavonoid content was observed between perfusion and batch run. Growth kinetic measurements (Figure 4) showed that the maximum cell concentration reached 25 ± 0.5 g l^{-1} DW much higher than that of 16.4 g l^{-1} DW reported in literature (Yang et al., 2008). This bioreactor culture system is stable, convenient and inexpensive, with potential Industrial applications. We plan further studies on scale-up and volume increase of this bioreactor system.

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