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Sparge gas composition affects biomass and ajmalicine production from immobilized cell cultures of *Catharanthus roseus*

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Abstract

Despite their low solubility in aqueous medium, dissolved gases play important roles in the cultivation and successful scale-up of plant cell cultures. In this paper, the effects of O_2 and CO_2 on growth and secondary metabolism were investigated using the production of ajmalicine from *Catharanthus roseus* cultures. The effects of gas composition were investigated using shear-protected alginate-immobilized cells (diameter < 2 mm) cultured in bubble columns sparged with premixed gases, including nine combinations of O_2 and CO_2. A wider range of concentrations (10–95% O_2, 0.03–10% CO_2 by mole) was studied to explore potential benefits or drawbacks.

Sparge gas composition significantly altered growth and ajmalicine production. Low and high O_2 concentrations (10, 90, 95% O_2) were either inhibitory or toxic to growth and ajmalicine production. The effects of CO_2 depended on O_2 concentration. At lower O_2 concentrations (21% O_2), increasing the CO_2 concentration decreased both growth and specific ajmalicine production. At higher O_2 concentrations (78.4% O_2), increasing the CO_2 concentration decreased growth while specific ajmalicine production was not affected. In these studies, extracellular ajmalicine concentration was maximized with a gas mixture of 50% O_2 + 0.03% CO_2.

Keywords: *Catharanthus roseus* cell cultures; Gas composition; Oxygen; Carbon dioxide; Secondary metabolism; Ajmalicine; Alginate immobilization; Amberlite® XAD-7 resin; Bubble column reactors

1. Introduction

Dissolved gases such as O_2, CO_2, and ethylene have generally not been optimized for growth and secondary metabolism in plant cell cultures. However, literature indicates that dissolved gases do impact growth and secondary metabolism and ultimately impact the success of scale-up [1–16]. Although gases have low solubility in aqueous medium, their role or effects are not negligible with respect to growth and secondary metabolism.

For instance, molecular oxygen plays a key role in the cell's bioenergetics as the final electron acceptor in respiration. In addition, molecular oxygen may also be involved in cytochrome P450-mediated oxygenation reactions in the biosynthesis of secondary metabolites such as terpenoid indole alkaloids [17] and taxanes [18]. Since different enzymes are involved, growth and secondary metabolism may be optimized at different O_2 concentrations [10,19–21].

Carbon dioxide and ethylene are other gases produced in plant cell cultures that can act as essential nutrients or hormones, altering growth-related and secondary metabolic activities. For instance, CO_2 stripping is associated with high aeration rates in bioreactors and has been implicated in lowering growth rates, lengthening lag periods, and causing browning [5–7,12,13,22]. Although the mechanism is not fully understood, maintaining a minimum CO_2 concentration in the cultures (i.e. 20 mbar in *Catharanthus roseus* cultures) is essential for promoting growth and culture viability [23–25].

Abbreviations: 2,4-D, 2,4-dichlorophenoxy-acetic acid; FW, fresh weight; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; k_La, mass transfer coefficient; LB, Luria-Bertani media; MS, Murashige-Skoog; *n*-HS, *n*-heptanesulfonic acid; SD, standard deviation; TLC, thin layer chromatography; W/v, weight per volume ratio; V/v, volume to volume ratio; Vvm, volume of gas per volume of culture per min

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The endogenous plant hormone ethylene is involved in many physiological responses in the whole plant [26]. In plant cell cultures, ethylene either stimulated [27–30], inhibited [31], or had no effect [32] on the production of various secondary metabolites. Carbon dioxide and ethylene can also interact and modulate each other's effects [3,26,29,33].

This paper investigates the effects of a wide range of O_2 and CO_2 concentrations on growth and secondary metabolism in *C. roseus* cultures, using the production of ajmalicine as a model system. Potential benefits or toxicities associated with operating outside the range achieved with air or air supplemented with CO_2 (typical sparge gas compositions) could then be explored. This gas effect study was designed to (1) investigate a wider range of O_2 and CO_2 concentrations in the gas phase (i.e. 10–95% O_2 and 0.03–10% CO_2) than that previously reported in the literature, (2) vary O_2 and CO_2 independently, and (3) alter gas composition while minimizing effects due to shear.

The effects of gas composition were investigated using shear-protected alginate-immobilized cells cultured in bubble columns sparged with premixed gases. A Box-Wilson statistical design [34] was used to prescribe the minimum number and the specific combinations of O_2 and CO_2 in the study. Ethylene was not included in this study since previous experiments indicated that ethylene inhibited ajmalicine production above 1 ppm [31]. The effect of oxygen transport on dissolved oxygen profile within immobilized cells (spherical beads of less than 2 mm in diameter) was also analyzed. This present study illustrates the impact of a wide range of dissolved gas compositions on growth and secondary metabolism and suggests that gas composition, like media composition, should be optimized for growth and secondary metabolism.

2. Materials and methods

2.1. Maintenance of C. roseus cell suspension cultures

The *C. roseus* cell suspension cultures were maintained as previously described in Lee and Shuler [35]. Every 9 days, 3.0 g FW of suction-filtered cells was transferred to 100 ml of fresh growth medium in a 500 ml Erlenmeyer flask. The growth medium [36] consisted of MS minimal organics [37] (GIBCO Life Technologies Inc., Grand Island, NY), 30 g/l sucrose, and 5 μM 2,4-D. The growth medium was autoclaved at 121 °C for 15 min. The cultures were incubated in the dark at 25 °C on a gyratory shaker at 120 rpm.

2.2. Priming C. roseus cell suspension cultures for alkaloid production

After 9 days in growth medium, the cultures were suction-filtered and 5.0 g FW of cells was transferred to 50 ml of MS production medium in 250 ml Erlenmeyer flasks for 10 days, as previously described in Lee and Shuler [35]. MS production medium consisted of MS minimal organics, 80 g/l sucrose, and 10 μM IAA [36]. The production medium was autoclaved at 121 °C for 15 min. The cultures were incubated in the dark at 25 °C on a gyratory shaker at 120 rpm.

2.3. Cell immobilization procedure

After being primed in production medium for 10 days, cells were immobilized in alginate under sterile conditions, as previously described in Lee and Shuler [35]. Primed cell suspensions were sieved for cell aggregates of less than 500 μm. The large cell aggregates were removed to prevent clogging the pipette tip used in the extrusion of alginate beads. The sieved fraction was then suction-filtered and mixed with 2% (w/v) sodium alginate solution (medium viscosity; Sigma Chemical Co., St. Louis, MO) to achieve an initial cell concentration of 25% by cell weight. The cell-alginate mixture was extruded through a 1000 μl pipette tip with concentric forced air flow. The cell-alginate droplet size was controlled by altering the air flow rate. Alginate-immobilized cells in the form of spherical beads were dropped and hardened in MS production medium plus 50 mM $CaCl_2 \cdot H_2O$ for 30–40 min. After hardening, the final cell concentration in the beads was approximately 33% by cell weight. The hardened beads (less than 2 mm in diameter) were rinsed in production medium to remove the excess calcium and suction-filtered; the cell-alginate beads, containing 5 g FW cells, were cultured in 100 ml of MS production medium supplemented with 5 mM $CaCl_2 \cdot H_2O$ in a 500 ml Erlenmeyer flask. The MS production medium promoted fresh weight accumulation in immobilized cells. The growth and ajmalicine production profiles of immobilized cells in shake flasks were shown in Lee and Shuler [35].

2.4. Bubble column reactors

The effects of gas composition on growth and ajmalicine production were investigated using immobilized cells cultured in bubble column reactors (Fig. 1). The bubble column reactors were fritted cylindrical filter funnels (Kontes, Vineland, NJ), which were modified to include glass ports for dissolved oxygen probes (New Brunswick Scientific, Edison, NJ). The bubble column dimensions were 45 mm × 310 mm (diameter versus height). Humidified gas was forced through the fritted glass disk to disperse the gas as bubbles and to provide mixing. The mass transfer coefficient ($k_L a$) of each bubble column was determined using the unsteady state method, i.e. after displacing dissolved O_2 with N_2, air was sparged into the bubble column and the dissolved O_2 concentration was monitored with time. The mass transfer coefficient of the bubble columns was greater than 30 h at 1.5 vvm of air (i.e. 150 ml/min). The variation in growth and ajmalicine production between three replicate bubble columns was determined and is presented in Fig. 2 and in Section 3.1.