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Ebb-and-Flow Bioreactor Regime and Electrical Elicitation: Novel Strategies for Hairy Root Biochemical Production

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ABSTRACT

The ebb-and-flow bioreactor regime and electrical elicitation constitute two novel and promising effective strategies for realizing the large-scale production of secondary metabolites from hairy roots growing in scaled-up bioreactors. Employing the ebb-and-flow regime would provide the forced-convective action that is needed to overcome the mass-transfer resistances offered by the stagnant external boundary layers and/or dead zones which normally exist in the high-density hairy-root bioreactor environment – both in liquid-phase and in gas-phase bioreactors. Meanwhile, employing electrical elicitation could significantly overproduce valuable secondary metabolites produced by hairy roots. Combining the two strategies could certainly help achieve the economic feasibility of the large-scale production from plant hairy roots of specific secondary metabolites.

INTRODUCTION

Plant roots have long been known as prolific manufacturers of an assortment of economically important chemicals. Since Dawson (1942) first showed conclusively that the roots of *Nicotiana tabacum* were the site of synthesis of the secondary metabolite nicotine, numerous root cultures representing a wide range of species have been demonstrated to produce diverse kinds of phytochemicals *in vitro*. The advent of transgenic hairy roots has presented a novel and versatile system for production of plant chemicals (Kamada et al., 1986; Flores et al., 1993; Mahagamasekera and Doran 1998; Medina-Bolivar et al., 2007) and even of transgenic proteins (Medina-Bolivar et al., 2003; Medina-Bolivar and Cramer 2004). Hairy roots have a typical doubling time in culture of 2 to 4 days, grow significantly faster than untransformed roots, are stable in culture, grow significantly faster than most suspension cell cultures, and generally produce the same if not higher levels of metabolites as do normal roots and cell suspension cultures (Hamill et al., 1987; Flores et al., 1987; Kwok and Doran 1995). This paper describes the ebb-and-flow bioreactor regime as a novel and promising effective strategy for overcoming the scale-up challenges that stand in the way of realizing the large-scale production of desired phytochemicals from immobilized hairy root cultures growing in bioreactors. This work also describes electrical elicitation as a novel and promising effective strategy for significantly increasing the production of secondary metabolites by hairy roots in large-scale bioreactors.

EBB-AND-FLOW BIOREACTOR REGIME

Hairy Root Bioreactor Challenges

The mass production of chemicals derived from hairy roots requires the development of scaleable hairy root bioreactors. However, the tangled and fibrous-clump morphology which the hairy roots assume under high-density conditions in bioreactors which is conducive to creating external boundary layers over root surfaces and around the root clumps and stagnant zones within the root clumps presents significant mixing and mass-transfer challenges for liquid-based delivery of nutrients and oxygen to the roots. Oxygen's low solubility in aqueous medium and its poor mass transfer through the medium into the root cells simply make O_2 the most critical limiting factor in hairy root bioreactor design (Yu and Doran 1994; Ramakrishnan and Curtis 1995; Kanokwaree and Doran 1997; Tescione et al., 1997).

In general, a hairy root bioreactor has to fulfill four minimum requirements upon scale up (Cuello 1994): (1) its characteristic configuration (e.g., flow configuration, geometric configuration, etc.) must remain operative, and not degrade, upon scale up; (2) it should make the nutrients and oxygen available, or accessible, to the roots throughout the reaction volume; (3) it should supply both nutrients and oxygen to the roots in sufficient concentrations throughout the reaction volume; and (4) it should lend itself to the scale up process by possessing identifiable and quantifiable characteristic process parameters which can be duplicated at larger scales with relative ease and economy.

Various configurations for small-scale hairy root bioreactors have been employed since the mid 1980's. Rhodes et al., (1986) were the first to demonstrate the successful growth of a hairy root culture in a bioreactor for pharmaceutical production, and were also first to employ a submerged convective-flow configuration in a root bioreactor, growing *Nicotiana rustica* hairy root culture in an 880 mL continuous convective flow bioreactor (CFR). Seminal studies on other types of hairy root bioreactor configuration were conducted by: (1) Taya et al., (1989) for bubble column, immobilized bubble column, immobilized trickle-bed column and immobilized fill-drain (ebb-and-flow) column bioreactors; (2) Kondo et al.,

(1989) for turbine-blade, rotating-drum and immobilized rotating-drum bioreactors; (3) Hilton and Rhodes (1990) for modified batch and continuous stirred-tank bioreactors; and, (4) DiIorio et al., (1992) for a nutrient mist bioreactor. Cuello et al., (1991) were the first to demonstrate the successful growth of a hairy root culture in an ebb-and-flow bioreactor. Subsequent studies on hairy root bioreactors were reviewed by Singh and Curtis (1994) and Choi et al., (2006), among others.

Of the foregoing hairy root bioreactor configurations, only a few can be expected in theory to sustain the successful growth of high-density hairy root cultures upon scale up to significantly larger volumes. For a simple bubble-column design, the dense packing of root clumps would promote bubble coalescing and channeling, leading to poor liquid mixing, localized liquid stagnation and, ultimately, to severe oxygen and nutrient maldistributions and limitations. A fine-mist configuration would be expected to break down into a trickle-bed configuration along the length of the bioreactor, since fine mists would not remain as such after they enter into the dense root bed (Flores and Curtis 1991). Although the trickle-bed configuration appears to be scaleable, it has the potential problem of being susceptible to liquid channeling and liquid stagnation. A stirred-tank bioreactor, with the roots immobilized and securely removed from the impeller zone, would likely have problems of liquid stagnation along the reactor periphery. Meanwhile, a horizontal rotating drum bioreactor appears to have scale-up potential. With the drum partially filled with liquid medium, its immobilized roots would be exposed to alternating gas and liquid phases. This design, however, is complicated by the gradient in linear velocities experienced by the roots along the radius of the reactor.

The continuous CFR and the ebb-and-flow bioreactor (EFBR), both exhibiting saturated tubular convective flow part of the time in the case of the EFBR possess the advantage of liquid and gas mixing through the root matrix, which can be maintained upon scale up. Being continuous systems (with inflow and outflow), both are also ideal configurations for the following scenarios: (1) when the roots require separate biomass accumulation stage and secondary-metabolite production stage, requiring a change in nutrient medium during the course of the growth period; (2) when the concentration of dissolved oxygen in the liquid medium needs to be brought back periodically to a certain level, requiring re-aeration of the liquid medium in a separate well-mixed reservoir; and (3) when the extracellular secondary metabolites excreted by the roots into the liquid medium need to be continually extracted from the liquid medium through *in situ* product removal (ISPR) to prevent feedback inhibition. Both the CFR and EFBR, however, would be expected to have critical limits in their heights to prevent the dissolved-oxygen concentrations in the bulk liquid from becoming growth-limiting to the roots.

The EFBR has the following three principal advantages over the CFR: (1) expected better mixing and mass-transfer capacities owing to the recurrent back-and-forth liquid flows and intermittent liquid and gas phases; (2) intermittent exposures to gas phase, allowing for more effective manipulation of the gas composition (e.g., possible addition or removal of certain gases such as oxygen, carbon dioxide or ethylene) for further optimization of root growth or phytochemical production; and (3) the ability to alleviate the significant energy penalty imposed by the high pressure drop created by the dense root bed by simply flooding the roots with the nutrient medium during the Fill Time (i.e., during the flow phase of the ebb-and-flow regime) and letting the nutrient medium drain out of the root bed by gravity or as assisted by a pump during the Drain Time (i.e., during the ebb phase of the ebb-and-flow regime). In contrast, a CFR requires that its liquid nutrient medium be pushed in bulk through the root bed continuously, incurring the full energy penalty of the root bed’s high pressure drop during the entire bioreactor run.

The Ebb-and-Flow Bioreactor

The ebb-and-flow bioreactor derives its name from the process behavior of its liquid medium which is characterized by its repetitive ebbing and flowing or periodic filling and draining (Figure 1). The reactor may be considered as the common-sense compromise between the two extreme bioreactor configurations of the predominantly liquid-phase and the predominantly gas-phase.

The ebb-and-flow bioreactor has four characteristic operational phases which recur sequentially and intermittently as the liquid medium moves back and forth between the bioreactor vessel and its reservoir (Figure 2). These include the liquid dwell time (LDT), the drain time (DT), the gas dwell time (GDT) and the fill time (FT). The LDT is the phase where the whole reaction volume of the EFBR is completely submerged in liquid, and where the bulk of the liquid medium is neither flowing upward nor downward. The GDT is that operational phase where the EFBR reaction volume is predominantly in the gas phase, and where mass flow of the bulk liquid medium is not occurring. The operational phases where the bulk flow of the liquid medium takes place are the FT, when the bulk flow direction is upward, and the DT, when the bulk flow direction is downward.

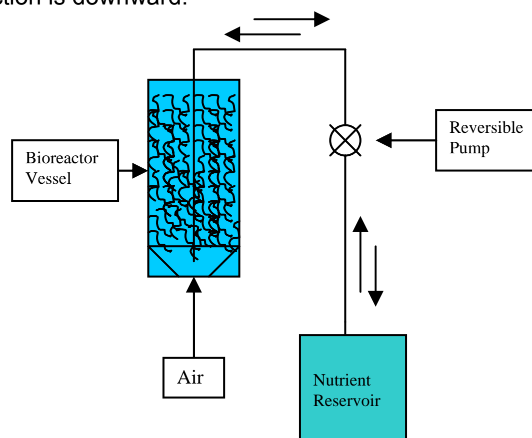

Figure 1. Schematic of an ebb-and-flow bioreactor, showing the bioreactor vessel containing the hairy roots and a programmable reversible pump that moves the liquid nutrient solution back-and-forth between the bioreactor vessel and the reservoir. Compressed air is sparged through the bottom of the bioreactor vessel.

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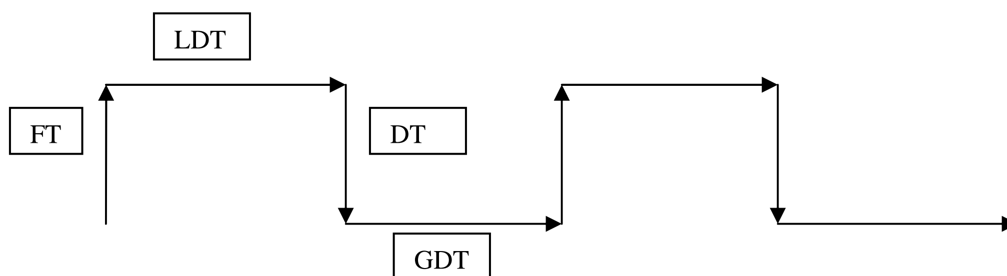

Figure 2. The four characteristic operational phases of the ebb-and-flow bioreactor which recur sequentially and intermittently as the liquid medium moves back and forth between the bioreactor vessel and its reservoir. FT = fill time, LDT = liquid dwell time, DT = drain time and GDT = gas dwell time. Both x and y axes are time scales. The direction of the arrows pertains to process flow.

Ebb-and-Flow Bioreactor Vs. Bubble Column

Cuello et al. (2003a, 2003b) compared the growth performance of a 2.5-L EFBR (Figure 3) against that of a bubble column, also called a submerged sparged bioreactor (SSBR). The EFBR was operated with the following values of operational parameters: LDT = 2 min; GDT = 2 min; FT = 1 min; and DT = 1 min. It was operated in two stages: first, under a bubble column regime for the first seven days of growth; and, second, under the fill-and-drain process for the succeeding time period. The normal EFBR treatment was sparged at an air flow rate of 2.72 mL/s, equivalent to 0.115 vvm, during the entire growth duration. At the start of the reactor run, the EFBR vessel contained 2000 mL of liquid medium while the reservoir contained 500 mL. Note that the working volume inside the bioreactor vessel was only 2000 mL.

Three types of controls were used in discriminating the effects of the ebb-and-flow process. These controls included: (1) the Erlenmeyer shake flasks; (2) a recirculating SSBR; and (3) a nonrecirculating SSBR. The shake flasks used were of two sizes: the 125 mL size and the 1000 mL size containing 50 mL and 250 mL of liquid medium, respectively. The rationale for using a recirculating SSBR control was to keep the total volume of the liquid medium for this control the same as that for the EFBR treatment which was 2500 mL. Similar to the EFBR, the recirculating SSBR contained 2000 mL of liquid medium in the bioreactor vessel and 500 mL in its reservoir at the beginning of a reactor run. Operated as a bubble column with the same sparging rate as the EFBR for the first seven days, the recirculating SSBR was then operated starting on the eighth day so that its liquid medium was recirculated between its cylinder and reservoir at a rate averaging 0.5 mL s^{-1}, a flow rate that was significantly lower than that of the EFBR. During the FT and DT operational phases of the EFBR, the flow reached its minimum value at an average of 14.4 mL s^{-1} on day 18. The third control, a nonrecirculating SSBR which contained only 2000 mL of liquid medium in its cylinder (it had no reservoir), was somewhat of a control for the recirculating SSBR to demonstrate that any effects of the low convective flow produced by the recirculation of liquid medium were negligible. The 125 mL flask control was referred to as 50 mL flask control, the 1000 mL flask as 250 mL flask control, the recirculating SSBR as SSBR, and the nonrecirculating SSBR as $SSBR_{nonrec}$.

The results by Cuello et al., (2003b) clearly demonstrated the EFBR's successful scale up of the 50 mL flask to a 50X scale. The 2.5 L EFBR produced an average dry weight of 21.64 g in 18 d (Figure 3) which was statistically indistinguishable from the combined average dry weights after 18 d of fifty 50 mL shake flasks of 23 g, at α = 0.05, and of ten 250 mL shake flasks of 20.3 g, also at α = 0.05. Note that these comparisons were based on an equal total volume of the liquid medium, 2500 mL. By contrast, the combined average dry weights for the 250-mL flasks significantly fell short (α = 0.05) of those for the 50 mL flasks, showing the failure of the Erlenmeyer flask to be amenable to the scale-up process.

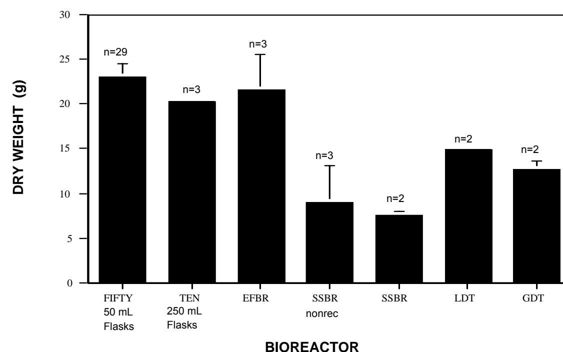

Figure 3. Growth comparison of *Hyoscyamus muticus* hairy root cultures after 18 days in bioreactors with 2500 mL of B5 medium; n = number of observations. EFBR = ebb-and-flow bioreactor; SSBR = submerged sparged bioreactor; LDT = EFBR with LDT of 5 min; GDT = EFBR with GDT of 8 min.

Comparing the EFBR against the SSBR, the EFBR's average dry weight significantly exceeded by 138% (α = 0.05) that for the SSBR of 9.08 g. It was noteworthy that there was no significant difference (α = 0.05) between the average dry weight of 7.56 g for the $SSBR_{nonrec}$ and that for the SSBR, indicating that the SSBR indeed had negligible convective flow and that it was the back-and-

forth convective flow of the EFBR that made it superior to the SSBR. It also showed that air bubbling, which was present in both EFBR and SSBR, was by itself insufficient to maintain root growth at larger scale.

The bioreactors' average specific growth rates (Table 1) reflected the same trends exhibited by their dry weights. No significant statistical differences existed between the average specific growth rates of the EFBR (0.211 d^{-1}) and the two shake-flask controls (0.218 d^{-1} for 50 mL flask and 0.217 d^{-1} for 250 mL flask, both at α = 0.05), but the average specific growth rate of the EFBR significantly exceeded by 41% (α = 0.04) that of the SSBR (0.150 d^{-1}). Also, the average specific growth rate of the SSBR was not significantly different (α = 0.05) from that of the $SSBR_{nonrec}$ (0.142 d^{-1}).

Table 1. Comparison of average specific growth rates (μ) of *Hyoscyamus muticus* hairy root cultures grown in different bioreactor treatments for 18 days; n = number of observations. EFBR = ebb-and-flow bioreactor; SSBR = submerged sparged bioreactor; LDT = EFBR with LDT of 5 min; GDT = EFBR with GDT of 8 min.

Treatment	(day^{-1})	n
50 mL flask	0.219 ± 0.004	29
250 mL flask	0.217 ± 0.0000	3
EFBR	0.211 ± 0.016	3
SSBR	0.150 ± 0.026	3
$SSBR_{nonrec}$	0.142 ± 0.001	2
LDT	0.188 ± 0.001	2
GDT	0.181 ± 0.004	2

Cuello et al., (2003b) found that the EFBR and the SSBR growth profiles over time did not exhibit a marked divergence in their daily dry weights until after day 14. The subsequent rapid increase in the dry weight of the EFBR distinctly contrasted with the smaller growth increments exhibited by the SSBR. This result was supported by visual observations. No appreciable differences were observed between the roots growing in the EFBR and those in the SSBR controls until after the first 10 d of their growth period. It was then when the roots in the EFBR as well as in the SSBR controls started developing into discrete root clumps, which continued to grow and expand and, by day 14, eventually interlocked with neighboring root masses to form a tangled, porous matrix of fibrous hairy roots. At this time, significant coalescing and channeling of the sparged air bubbles during the liquid-phase duration were observed, suggesting the formation of localized stagnant regions within the root clumps as well as the formation of significant external boundary layers. It was also at this time when the difference between the EFBR and the SSBR controls in terms of both growth rate and accumulated biomass sharpened significantly.

Comparing the SSBR and the EFBR hold-up profiles upon drainage (Figure 4), Cuello et al., (2003b) found that the residual hold-up for the SSBR progressively declined after successive draining of its liquid medium. By contrast, the residual hold-up for the EFBR after successive draining remained essentially constant. While the EFBR consistently held up approximately the same volume of medium after each time that the bulk liquid was drained, the SSBR exhibited time-dependency in its residual hold up, with diminishing hold-up levels upon drainage repetition. After repeated filling and draining, the residual hold-up of the SSBR continually decreased and approached that of the EFBR. These results indicated that, in comparison with the EFBR, the SSBR possessed significantly more external boundary layers around the roots and/or stagnant zones that provided significant resistance to mass transfer of oxygen and nutrients. Meanwhile, the bi-directionality of the forced convective flow in the EFBR and the repetitive filling and draining of its bulk medium successfully ensured the periodic attenuation of both the boundary layers and any stagnant zones within the EFBR. The sugar profiles for the EFBR and the SSBR showed that, on day 18, the total sugar level in the EFBR was 8.9 g L^{-1}, a level that was considerably lower than that in the SSBR of 14.0 g L^{-1} for the same day. This result confirmed that the SSBR control, relative to the EFBR, was hampered by sugar mass-transfer limitation. And given the much lower solubility of oxygen in the liquid medium, and the finding that the oxygen consumption rate in the EFBR and SSBR remained statistically the same at an average value of 0.05 mg min^{-1} g^{-1} DW, it could safely be inferred that the SSBR was also hampered by oxygen mass-transfer limitation. The foregoing results on the poor growth performance of the SSBR and the accompanying observations about its hydrodynamic behavior are consistent with those reported by others (Shia and Doran 2000; Kanokwaree and Doran 2000).

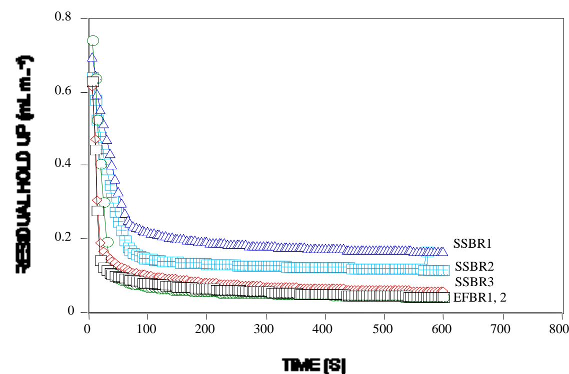

Figure 4. Time profiles of residual hold-up for normal ebb-and-flow bioreactor (EFBR) treatment and submerged sparged bioreactor (SSBR) growing *Hyoscyamus muticus* hairy roots at tissue concentrations of 267 and 283 g L^{-1} FW, respectively. Profiles obtained on successive drainage are designated by 1, 2, etc., in chronological order.

EFBR with Prolonged Liquid Phase and EFBR with Prolonged Gas Phase

Cuello et al., (2003b) also ran an EFBR treatment with a prolonged liquid phase, called LDT treatment, whose LDT = 5 min, GDT = 2 min, FT = 1 min and DT = 1 min. Likewise, an EFBR treatment with a prolonged gas phase, called GDT treatment, whose GDT = 8 min, LDT = 2 min, FT = 1 min and DT = 1 min, was also performed.

Their results (Figure 3) showed that the LDT treatment's average dry weight of 14.99 g and the GDT treatment's average dry weight of 12.74 g fell between those of the EFBR and the SSBR control, that is, below that of the EFBR and above that of the SSBR control. Although the average dry weights of the LDT and GDT treatments

remained statistically indistinguishable from that of the normal EFBR treatment, both at α = 0.05, the average dry weights of the LDT and the GDT treatments were nonetheless 31% and 41% lower than that of the normal EFBR treatment, respectively. Indeed the average dry weights of the LDT and the GDT treatments became significantly lower than that of the normal EFBR treatment at α = 0.12 and α = 0.06, respectively. Equally noteworthy, the average dry weights of both the LDT and the GDT treatments were significantly lower ($\alpha \leq 0.05$) than those of the 50 mL and the 250 mL shake-flask controls. At the opposite lower end, though the average dry weights of the LDT and GDT treatments remained statistically indistinguishable from that of the SSBR, both at α = 0.05, the average dry weights of the LDT and the GDT treatments nonetheless exceeded that of the SSBR by 65% and 40%, respectively. These same trends were exhibited by the resulting growth rates (Table 1).

The sugar profiles showed that, on day 18, the total sugar levels in the LDT and GDT treatments hovered over 8.9 g L^{-1}, a level that was the same as that for the normal EFBR and again considerably lower than that in the SSBR control of 14.0 g L^{-1} for the same day. These results showed that the sugars in the GDT and LDT treatments were consumed to the same extent as that in the normal EFBR treatment, indicating that, just as the normal EFBR treatment did not suffer from sugar mass-transfer limitation, neither the LDT treatment nor the GDT treatment also suffered from sugar mass-transfer limitation. Otherwise, the final sugar concentrations of the LDT and GDT treatments would have been closer to the SSBR's higher residual sugar concentration of 14.0 g L^{-1}.

Despite the LDT and the GDT treatments not experiencing significant sugar mass-transfer limitations, however, the dry-weight and growth-rate results (Figure 1 and Table 1) indicated that the growth performances of both the LDT and GDT treatments were on the verge of becoming significantly poorer than that of the normal EFBR treatment. And, indeed, the average dry weights of both the LDT and the GDT treatments were significantly poorer than those of the 50 mL and the 250 mL shake-flask controls (Figure 3). This was further supported by the resulting maintenance coefficients for the bioreactors. Recall that the maintenance coefficient of a root culture is the portion of the total-sugar substrate it requires to maintain its accumulated biomass. The maintenance coefficients of the LDT and the GDT treatments of 1.09 and 1.49 g sugar g^{-1} DW, respectively, were significantly greater than that of the normal EFBR treatment of 0.30 g sugar g^{-1} DW and statistically indistinguishable from that of the SSBR of 0.70 g sugar g^{-1} DW, indicating that the roots in the LDT and GDT treatments were expending via respiration a great portion of their sugar substrates in maintaining their accumulated biomass. The results demonstrate that the roots in the LDT and GDT treatments were physiologically stressed, though the cause of stress was clearly not sugar mass-transfer limitations. Further, measurements of oxygen consumption rates showed that the LDT treatment and the GDT treatment on day 18 also had an average value of 0.05 mg min^{-1} g^{-1} DW, which was statistically the same as that for the EFBR and the SSBR.

Thus, the roots' relative ability to consume oxygen could not have accounted for the poor growth performance of the LDT and the GDT treatments. All the bioreactors, however, were observed to exhibit extensive bubble entrapment, coalescing and channeling during their respective liquid phases, pointing to the presence of external boundary layers as well as localized stagnant regions within the bioreactors during the liquid dwell time. Thus, without the benefit of more frequent or sufficient forced convective flow generated by the ebb-and-flow action, bioreactors with an extended or permanent liquid phase, like the LDT treatment and the SSBR control, suffered from oxygen mass-transfer limitations. The GDT treatment, also lacking a sufficient forced convective flow to overcome the mass-transfer resistances created by its stagnant root-entrained medium during its prolonged gas phase, must have also experienced oxygen mass-transfer limitation during the prolonged GDT. It was noteworthy that the normal EFBR, the LDT treatment and the GDT treatment all had the same root-entrained medium of 0.5 mL g^{-1} DW. Thus, even though the frequencies of forced convective flow of 22.2% and 16.7% in the LDT and GDT treatments, respectively, were sufficient to obviate overall sugar mass-transfer limitations, such frequencies proved not enough to prevent oxygen mass-transfer limitations in these treatments. Thus, during the prolonged liquid phase of the EFBR, oxygen mass-transfer limitation began to prevail, conforming to normal expectation. During the prolonged gas phase of the EFBR, however, oxygen mass-transfer limitation also began to prevail, if counter-intuitively.

Implications for Scale Up

The study by Cuello et al., (2003b) reinforced that the forced convective delivery of oxygen is the most critical variable for the successful scale up of the EFBR and, indeed, of any hairy root bioreactor. This finding agrees squarely with those from previous studies on the critical necessity of liquid convective flow on the effective delivery of dissolved oxygen to roots grown in liquid medium (Prince et al., 1991; Yu and Doran 1994; Singh and Curtis 1994; Shiao and Doran 2000; Ramkrishnan and Curtis 2004). The study by Cuello et al., (2003b) also underscored, however, the oxygen-delivery limitations that exist in gas-phase bioreactors (also known as liquid-dispersed bioreactors, including the nutrient mist, spray and trickle-bed bioreactors) on account of the existence of stagnant external boundary layers in these bioreactors in addition to the potential occurrence of liquid channeling and water-logging (Shiao and Doran 2000; Williams and Doran 2000; Ramkrishnan and Curtis 2004). Quite simply, there is no convincing proof that oxygen mass transfer limitation does not exist in these gas-phase bioreactors. Weathers et al., (1999), for instance, concluded from their study of 1.5 L nutrient-mist and bubble-column bioreactors, based on mRNA levels of the fermentation enzyme alcohol dehydrogenase (ADH), that the bubble column was oxygen-limited since it exhibited expression of ADH, while the mist bioreactor was not

oxygen-limited since it did not exhibit expression of ADH. (The bubble column yielded 1.7 times more dry biomass than the mist bioreactor.) Unfortunately, however, the measurement of the activity of fermentation enzymes such as ADH as an indicator of hypoxia in hairy root bioreactors has proved unreliable, since cases have been reported where oxygen-limited treatments and the non-oxygen-limited control showed exactly the same levels of ADH activity, or the oxygen-limited treatments failed to show elevated levels of ADH activity (Shiao et al., 2002). Further, the treatments that showed elevated levels of ADH activity ended up yielding greater biomass growth, and the treatments that showed little or no ADH activity ended up yielding lower biomass growth (Weathers et al., 1999; Kim et al., 2002; Shiao et al., 2002). Thus, a successful detection of ADH activity is neither a necessary nor a sufficient indicator of the presence of oxygen limitation. Conversely, failure to detect ADH activity is neither a necessary nor a sufficient indicator of the absence of oxygen limitation.

What is clear, however, is that there is a need both in liquid-phase and in gas-phase bioreactors for a forced-convective action to be employed in proper frequency to overcome the mass-transfer resistances offered by the stagnant external boundary layers and/or dead zones which normally exist in the high-density hairy-root bioreactor environment. One way to generate such forced-convective action is by employing the ebb-and-flow regime, making the ebb-and-flow regime an effective strategy for overcoming the scale-up challenges of hairy-root production in bioreactors.

It is noteworthy that in compiling the results of 22 different studies that presented 26 different hairy-root bioreactor treatments (representing various combinations of different bioreactor sizes, growth periods, and bioreactor types – including stirred tank, bubble column, isolated impeller, convective flow, nutrient mist, trickle/spray), Ramakrishnan and Curtis (2004) found that the bioreactor that yielded the highest average specific growth rate -- for a bioreactor that was at least 2 L in size, had a growth period of at least 18 days, and had a dry biomass output of at least 10 g L^{-1} was the ebb-and-flow bioreactor by Cuello (1994) (Figure 5), which gave an average specific growth rate of 0.22 d^{-1}. It was telling that the one that came the closest to the EFBR was a trickle-bed bioreactor by Ramakrishnan and Curtis (2004), which gave an average specific growth rate of 0.21 d^{-1}, but one which was operated under an enriched-oxygen condition.

Figure 5. A 2.5-L ebb-and-flow bioreactor (EFBR) during gas dwell time and growing 18-day-old hairy roots.

It is worth pointing out that the performance of the normal EFBR in the study by Cuello (1994) and Cuello et al., (2003b) had yet to be optimized. With its FT and DT set constant at 1 min each throughout the entire growth period while the hairy roots were growing, the pump flow rate had to be continually reduced over time so that it would deliver just enough liquid medium into the bioreactor vessel to reach a constant level during each FT of 1 min. With more root biomass accumulating and occupying more space inside the vessel over time, less and less liquid had to be pumped into the vessel during FT. This meant that the flow velocity of the nutrient medium through the root biomass in the normal EFBR treatment kept being reduced just as the mass-transfer resistance offered by the roots was increasing. Clearly, there was room for further improvement of the performance of the normal EFBR treatment.

Incidentally, the results by Cuello et al., (2003b) made clear why the only previous attempt made by Taya et al., (1989) to employ an ebb-and-flow bioreactor to grow immobilized hairy roots (*Armoracia rusticana*) simply failed. Since their 300 mL EFBR used a liquid-phase duration of 15 minutes (i.e., much greater than the 4 min [FT + LDT + DT] used in the foregoing study) plus a GDT of 8 h it could be safely inferred that their system suffered from significant oxygen mass-transfer limitations.

The EFBR can demonstrably meet all of the four minimum requirements of a hairy root bioreactor for scale up. To wit: (1) its characteristic configuration (e.g., flow configuration, geometric configuration, etc.) remains operative, and does not degrade, upon scale up; (2) it can make the nutrients and oxygen available, or accessible, to the roots throughout the reaction volume by proper adjustment of its LDT, GDT, FT and DT; (3) it can supply both nutrients and oxygen to the roots in sufficient concentrations throughout the reaction volume by proper adjustment of its LDT, GDT, FT and DT; and (4) it lends itself to the scale up process by possessing identifiable and quantifiable characteristic process parameters which can be duplicated at larger scales with relative ease and economy. Examples of potential scale-up process parameters for the EFBR include dissolved oxygen concentration at a specified location in the bioreactor, volumetric flow rate, volumetric flow rate per unit volume, Reynold's number, power, and power per unit volume, among others.

Finally, in scaling up the EFBR, there exists a maximum allowable height for the EFBR column since the dissolved oxygen in the liquid medium will be continuously consumed by the roots as the liquid medium passes through the root bed along the length of the EFBR column. It should be noted that the same is true in the case of the continuous convective flow bioreactor (CFR). Cuello (1994) recommended that, in industrial applications, the most efficient and economical way of operating the EFBR would be for two equal-sized EFBR's to be operated in tandem so that when one is drained, its medium is transferred to fill the other. This mode of operation would obviate the need for a costly sterile media reservoir which naturally would have the same

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volume as that of the EFBR. Further, it was recommended that LDT = GDT = 0 in order to maximize the allowable durations for the FT and the DT. Indeed, their calculations showed, adopting a value for the maximum tolerable flow velocity for the roots of 16 cm s^{-1}, that the allowable maximum height for their specific EFBR design was 23 m (or 75 ft).This corresponded to FT = DT = 3 min, specifying that that dissolved oxygen was at saturation level in the liquid when introduced into the EFBR at the beginning of FT and allowed to drop to no less than 40% of saturation when the liquid exited the EFBR at the end of the DT.

ELECTRICAL ELICITATION

Elicitation is the process whereby a molecule or a stress agent induces a plant organ, tissue or cell to synthesize and accumulate phytoalexins. Phytoalexins are antimicrobial chemical compounds or secondary metabolites which in nature are synthesized and accumulated by plants after exposure to microorganisms (Darvill and Albershein 1984). Biotic elicitors are molecules which are either pathogen-derived (so-called exogenous elicitors, that is, with respect to the plant) or plant-derived (so-called endogenous elicitors, also with respect to the plant). Meanwhile, abiotic elicitors are physical or chemical agents that induce physiological stress to plants, leading to the synthesis and accumulation of phytoalexins. Examples of abiotic elicitors include UV radiation, ethylene, antibiotics, fingicides, salts of heavy metals, heat and cold, etc.

The first biotic elicitor to be studied extensively, the peptide monilicolin A, was obtained from the mycelia of the fruit-tree fungal pathogen *Monilinia fructicola* (Darvill and Albershein 1984). Oligosaccharides, polysaccaharides, glycoproteins and organic acids derived from the mycelia and/or cell walls of fungal microorganisms are some of the classes of exogenous elicitors that have been reported, while galacturonosyl residues have been reported to constitute a class of endogenous elicitors (Darvill and Albershein 1984).

Examples of biotic elicitors that have been applied to hairy root cultures to elicit the production of secondary metabolites include those derived from *Aspergillus niger* for *Tagetes patula* hairy roots (Buitelaar et al., 1993), those derived from *Rhizoctonia solani* for *H. muticus* hairy roots (Singh et al., 1994), and those derived from *Botrytis spec.*, *Pythium aphanidermatum*, *Rhodotorula rubra*, *Alternaria zinnae*, *Colletotrichum gloeosporoides*, *Helminthosporium gramineum*, *Sclerotinia sclerotiorum* and *Verticillium dahliae* for *Papaver somniferum* hairy roots (Eilert et al., 1985). Meanwhile, examples of abiotic elecitors that have been applied to hairy root cultures to elicit the production of secondary metabolites include osmotic shock on *H. muticus* hairy roots (Halperin and Flores 1997), and salicylate and methyl jasmonate on *Tanacetum parthenium* hairy roots (Stajokowska et al., 2002).

The mode of action of abiotic elicitors on plants has been postulated in various ways, including through the release of an endogenous elicitor compound from the plant cell (Moesta and Grisebach 1981), through inhibition of the degradative turnover of secondary metabolites (Yoshikawa 1978), and through the involvement of a second messenger molecule that transmits signals from the plasma membrane to trigger the transcription and translation of enzymes (DiCosmo and Misawa 1985).

Electricity as an Elicitor

Elicitation has long been proposed as a potential strategy for increasing the production of secondary metabolites by plant cell or root cultures *in vitro* while they are being grown in bioreactors (Dornenburg and Knorr 1995). There are significant challenges, however, in employing elicitors, including: (1) the labor required to prepare biotic elicitors from their biological sources; (2) loss of capacity of biotic elicitors over time to elicit the biosynthesis of desired secondary metabolites; (3) potential adverse or toxic effects of biotic or abiotic elicitors on the cell or root culture; and (4) difficulty of separating or removing biotic or abiotic elicitors from the culture once they have been added. While with respect to the last point, UV light (an abiotic elicitor) provides the convenience of lending itself to simply being turned on or off as needed, the poor penetration of light through thick clumps of hairy roots in a large-scale bioreactor constitutes a significant challenge to subjecting all the roots in the bioreactor uniformly to the elicitation process, especially when the UV light is applied externally to the bioreactor. Inserting UV lamps into the bioreactor interior, however, would waste bioreactor space or volume, would add complexity to bioreactor maintenance, cleaning, and sterile operation, and could significantly interfere with the bioreactor's hydrodynamic or aerodynamic characteristics.

The use of electricity, however, as an abiotic elicitor obviates the foregoing challenges. Similar to UV light, electricity provides the convenience of lending itself to simply being turned on or off as needed. Further, electricity would be conducted much more efficiently than light through the thick clumps of hairy roots in a large-scale bioreactor when the hairy roots are immersed in their normal nutrient solution containing electricity-conducting ions, allowing for all the roots in the bioreactor to be subjected to the elicitation process in a convenient manner. Also, implementing electrical elicitation would only require two thin metal electrodes to be inserted through the roots inside the bioreactor along the height of the root bed. Alternatively, the electrodes or even metal plates could be positioned against opposite inside walls of the bioreactor if interference with the flow characteristics of the bioreactor would be a concern.

The application of electricity in plant systems has been traditionally focused on the effects of the presence of electric fields or pulses on the following: (1) enhancement of the proliferation rate of plant biomass (Rathore and

Goldsworthy 1985); (2) increase in the rate and efficiency of plant regeneration (Rathore and Goldsworthy 1985); (3) improvement of the rate and success of seed germination (Kazanova 1972); (4) stimulation of protoplast division Rech et al., 1987); and (5) enhancement of DNA synthesis in cultured plant protoplasts (Rech et al., 1988). Takeda et al., (1988) correlated the membrane potential of cultured carrot cells with their synthesis of anthocyanin. They varied the membrane potential of the carrot cells, however, by manipulating the concentration of K^+ ions and by adding various levels of 2,4-D into the culture medium, and not by subjecting the cells to an electric field.

Working with alternating current (AC) and direct current (DC) and how they affect the production of secondary metabolites of *H. muticus* hairy roots, Cuello and Yue (1998) and Johnson and Cuello (1998) appear to be the first to investigate the electrical elicitation of secondary metabolites from hairy root cultures. Both studies employed a 6.3 cm x 6.3 cm x 5.1 cm plexi-glass electrical elicitation box or chamber equipped with two stainless-steel metal plates, serving as electrodes, positioned against opposite inside walls of the box (Figure 6). The hairy roots were placed inside the box and were immersed in their normal nutrient solution before carrying out the electro-elicitation process.

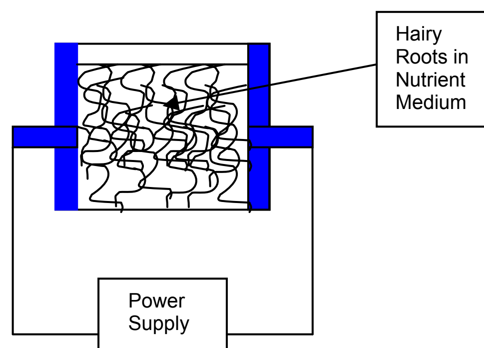

Figure 6. Schematic of an electrical elicitation box equipped with stainless-steel metal plates, serving as electrodes, placed against opposite inside walls of the box.

A decidedly more comprehensive study on electrical elicitation was recently conducted by Kaimoyo et al., (2008), using hairy roots of pea (*Pisum sativum*, L, *var.* Little Marvel) as the primary model system. Kaimoyo et al., (2008) used the same elicitation box that Cuello and Yue (1998) and Johnson and Cuello (1998) had used earlier. Trying different levels of root biomass and electric current, their results showed that 2 g of hairy roots exposed to 100 mA current for 1 h or 10 g of hairy roots exposed to 30 or 50 mA current for 1 h produced very similar levels of the secondary metabolite pisatin, which averaged 32.1 $\pm$ 3.9 μg g^{-1} FW. This amount of pisatin was comparable to that obtained using 0.08 mM aqueous $CuCl_2$ as an abiotic elicitor of *de novo* pisatin biosynthesis (37 $\pm$ 3.3 g g^{-1} FW). They also found that, though the average length of the electro-elicited hairy roots six days after treatment was slightly less than that for the control, the rate of growth from six days on was the same as that of the non-elicited hairy roots. And when these hairy roots were re-elicited with 0.08 mM $CuCl_2$ or a 30 mA current, they produced similar amounts of secondary metabolites as produced by hairy roots that had not been elicited previously. Their gross morphology also did not differ from that of non-elicited controls.

More interestingly, Kaimoyo et al., (2008) tested electrical elicitation on other plant species known to synthesize phytoalexins and other secondary metabolites upon elicitation by various biotic and abiotic elicitors. Cell suspension cultures of Arabidopsis (*Arabidopsis thaliana* L.) and barrel medic (*Medicago truncatula L.*), roots of chickpea (*Cicer arietinum* L.) and fenugreek (*Trigonella foenum-graecum* L.), intact seedlings of sorghum (*Sorghum bicolor* L.), Japanese pagoda tree (*Sophora japonica* L.) and red clover (*Trifolium pratense* L.) were electro-elicited at 30 mA current for 1 h. Secondary metabolites were extracted 24 h later and compared by HPLC or HPLC coupled to mass–spectrometry (HPLC-MS) with those extracted from non-elicited tissues. Except for Arabidopsis and sorghum, there were substantial increases in most of the compounds whose biosynthesis had been shown previously to be stimulated by elicitors. The resulting ratios of compound produced with elicitation to compound produced by the non-elicited control were: 2.8 for medicarpin as produced by fenugreek roots; 4.3 for formononetin as produced by chickpea roots; 44.0 for maackiain, 5.0 for medicarpin and 15.6 for formononetin as produced by red clover seedlings; 34.5 for afrormorsin, 168.1 for medicarpin, 7.0 for irisolidone, 7.6 for formononetin, 3.6 for naringenin, 3.8 for hispidol, 3.5 for tricin and 5.2 for isoflav-3-ene as produced by barrel medic cells in suspension.

The authors noted that, in addition to the expected compounds, the HPLC profiles of the extracts from many of the plants showed the presence of other previously undetected secondary metabolites. For instance, extracts of chickpea roots showed a large increase in formononetin, maackiain and medicarpin as well as over 20 other metabolites. The production of secondary metabolites by cell suspension cultures of barrel medic showed an even greater increase in the number of compounds as 55 compounds showed at least a 2-fold increase in the electro-elicited tissue compared to the non-electro-elicited controls. In the cell suspension cultures of Arabidopsis the expected accumulation of camalexin was not observed. Silver nitrate, previously reported to induce camalexin biosynthesis in leaf tissue, was used as a positive control, but no camalexin was obtained from silver nitrate-treated Arabidopsis cell suspensions. However, two peaks with residence time or RT = 9 min and 13 min, respectively were observed in the HPLC chromatogram of extracts from electro-elicited Arabidopsis cell suspension cultures that were absent in non-elicited cell suspensions. In sorghum seedlings, several different metabolites were detected in the extracts of both elicited and non-elicited tissue, but the differences in the amounts of these compounds were not significant.

The study by Kaimoyo et al., (2008) clearly indicated that electric current constitutes a general abiotic elicitor of

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plant secondary metabolites. As with other abiotic elicitors, the mechanism of action of electricity as elicitor on plants could potentially involve the release of an endogenous elicitor compound from the plant cell (Moesta and Grisebach 1981), the inhibition of the degradative turnover of secondary metabolites (Yoshikawa 1978), and/or the involvement of a second messenger molecule that transmits signals from the plasma membrane to trigger the transcription and translation of enzymes (DiCosmo and Misawa 1985).

Electrical Elicitation of Hyoscyamus muticus Hairy Roots

In our own study, 14-day-old *H. muticus* hairy roots were subjected for 1 h to 12 mA, 67 mA and 158 mA of direct current (DC) and to 11 mA, 28 mA, and 288 mA of 60-Hz alternating current (AC). Weighing an average of 16.3 g per treatment, the roots were treated for alkaloid extraction by the method of Kamada et al., (1986), and alkaloid extracts dissolved in methanol were assayed using HPLC (Shimadzu) equipped with SIL-10A autoinjector and SPD-M10A photodiode array detector. Samples were injected at a volume of 20 mL and were separated isocratically in a Nova-Pak C18 (Waters) steel column (3.9 x 150 mm) using a mobile phase of 12.5% (v v^{-1}) acetonitrile and 87.5% (v/v) aqueous phosphoric acid (0.3% v/v), adjusted to pH 2.2 with triethylamine, at a flow rate of 0.8 mL min^{-1}.

While the average pH of the roots' B5 liquid medium did not significantly change after elicitation, the medium's average electrical conductivity increased significantly for all treatments after elicitation. For the DC treatments, the average electrical conductivity rose from 1.8 to 10.0 mS cm^{-1} for 12 mA, 0.9 to 8.8 mS cm^{-1} for 67 mA, and 0.8 to 19.3 for 158 mA. For the AC treatments, the average electrical conductivity rose from 1.5 to 7.8 mS cm^{-1} for 11 mA, 1.6 to 6.6 mS cm^{-1} for 28 mA, and 0.9 to 27.8 for 288 mA.

The resulting HPLC chromatograms at 215 nm of the roots' alkaloid extracts showed pronounced differences between those for the elicited treatments and those for the unelicited control (Figure 7). Compound I (RT = 8.6 min) was significantly elicited or overproduced when the roots were electro-elicited using either AC or DC elicitation (Table 2). Indeed, the ratios of the level of Compound I in an elicited treatment to that in the control ranged from 11.0 to 13.5 for the AC treatments, while those for the DC treatments ranged from 3.8 to 14.4. By contrast, however, the levels of Compound II (RT = 9.8 min) remained statistically indistinguishable between the elicited AC or DC treatments and the unelicited control (Table 2). And, interestingly, the synthesis of Compound III (RT = 16.0) was significantly inhibited by both AC and DC electrical elicitation.

Thus, the results of this study showed that in the case of *H. muticus* hairy roots, electrical elicitation significantly overproduced compound I, significantly inhibited compound III, and had no appreciable effect on the level of compound II. It is likely that the employment of electrical elicitation to a specific plant species will have these three general effects on the plant's specific sets of secondary metabolites.

EBB-AND-FLOW BIOREACTOR REGIME AND ELECTRICAL ELICITATION

Based on the foregoing sections, the ebb-and-flow regime and electrical elicitation constitute two novel and promising effective strategies for realizing the large-scale production of secondary metabolites from hairy roots growing in scaled-up bioreactors. Employing the ebb-and-flow regime would provide the forced-convective action that is needed to overcome the mass-transfer resistances offered by the stagnant external boundary layers and/or dead zones which normally exist in the high-density hairy-root bioreactor environment – both in liquid-phase and in gas-phase bioreactors. Meanwhile, employing electrical elicitation could significantly overproduce valuable secondary metabolites produced by the hairy roots. Combining the two strategies could certainly help achieve the economic feasibility of the large-scale production from plant hairy roots of specific secondary metabolites.

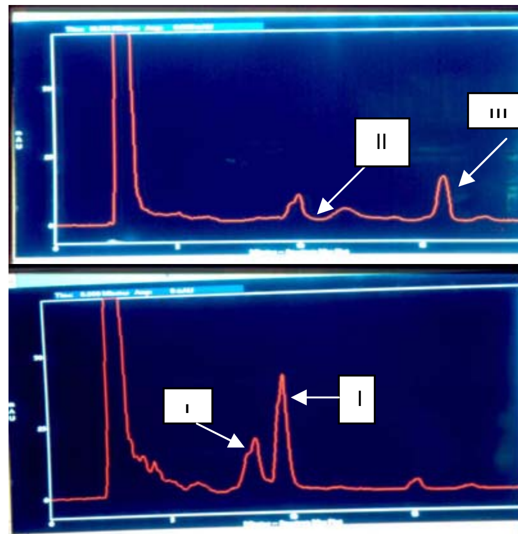

Figure 7. Replicate HPLC chromatograms at 215 nm of the alkaloid extracts from *Hyoscyamus muticus* hairy roots for the control (upper) and for an electro-elicited treatment [12 mA DC] (low). The x-axis is retention time (RT) and the y-axis is absorbance units. Compound I has RT = 8.6 min, Compound II has RT = 9.8 min, and Compound III has RT = 16.0 min.

Table 2. Average levels of compound I (RT = 8.6 min) and compound II (RT = 9.8 min) based on the HPLC chromatograms at 215 nm of the alkaloid extracts from *Hyoscyamus muticus* hairy roots. The letters “a”, “b” and “c” denote statistical differences between the control and the AC and DC electro-elicitation treatments.

	Compound I		**Compound II**	
	Average peak Area (1000x)	**Elicited/Control**	**Average peak Area (1000x)**	**Elicited/Control**
Unelicited control	31.3a	1.0	292.0a	1.0
Elicited AC treatments				
11 mA	423.4b	13.5	298.4a	≈ 1.0
28 mA	366.8b	11.7	345.1a	≈ 1.0
288 mA	346.5b	11.0	325.5a	≈ 1.0
Elicited DC treatments				
12 mA	420.8b	13.4	514.7a	≈ 1.0
67 mA	453.5b	14.4	342.8a	≈ 1.0
158 mA	120.0c	3.8	359.1a	≈ 1.0

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