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A comparative study of mist and bubble column reactors in the in vitro production of artemisinin

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Abstract *Artemisia annua* hairy roots grown in nutrient mist reactors produced nearly three times as much artemisinin as roots grown in bubble column reactors, 2.64 µg/g DW and 0.98 µg/g DW, respectively.

Keywords *Artemisia annua* · Artemisinin · Hairy roots · Bubble column reactor · Nutrient mist reactor

Abbreviations *DW:* Dry Weight · *FW:* Fresh weight · *HPLC:* High-performance liquid chromatography

Introduction

Hairy roots can be a good alternate source of phytochemicals because transformed roots grow rapidly and produce secondary metabolites at levels comparable to those of the parent plants (Bhadra et al. 1993; Rodriguez-Mendiola et al. 1991). In some cases the levels of secondary metabolites in hairy roots exceed those of whole plants (Jung and Tepfer 1987). We have hairy root clones of *Artemisia annua* L. that have been routinely subcultured in shake flasks and have been producing the antimalarial sesquiterpene artemisinin for more than 5 years (Weathers et al. 1994, 1996). Making commercially useful quantities of any secondary metabolite in hairy root cultures, however, is challenging and requires large-scale culture in bioreactors.

The reactors used for hairy root culture can be classified as either liquid-phase or gas-phase. Conventional fermentors and bubble column reactors are liquid-phase reactors because the roots are submerged in the growth medium. In gas-phase reactors, the roots are intermittently exposed to ambient air, or another gas mixture, and the nutrient liquid; trickle-bed and nutrient mist reactors belong in this category. McKelvey et al. (1993) were the first to study the fundamental differences between liquid- and gas-phase reactors in delivering nutrients, including oxygen, to roots. They evaluated the effect of the reactor environment on hairy root growth and demonstrated that different culture environments affected biomass accumulation. In liquid-phase reactors, Nuutila et al. (1994) showed that different shear levels affected both growth and the alkaloid content in *Catharanthus roseus* hairy roots. The strength of salts in the culture medium and the culturing temperature affected hyoscyamine production in *Datura stramonium* in stirred tank reactors (Hilton and Rhodes 1990). In addition, in the same study, operating the reactor in continuous rather than batch mode resulted in a threefold increase in the rate of production of hyoscyamine (Hilton and Rhodes 1990). Most studies reporting the effects of culture conditions on secondary metabolite production have been conducted using liquid cultures, and very few studies have directly compared liquid- and gas-phase culture systems.

Over the past 5 years we have carried out an extensive comparative study of hairy root growth productivity in two very different reactors: a nutrient mist (gas-phase) reactor and a bubble column (liquid-phase) reactor. The results of that study will be published in an engineering journal. Our goal has never been to optimize growth or artemisinin production; rather, we focused on understanding how hairy roots respond to the two disparate reactor environments. We chose *Artemisia annua* hairy roots as a model because we have extensive experience with the YUT16 clone and because the species produces, among other terpenoids, an important antimalarial therapeutic. During our studies we measured artemisinin levels and were surprised to find a dramatic difference in production that consistently correlated with reactor type. Here we report those results.

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Materials and methods

Hairy root culture

The hairy root clone YUT16 originated from a Yugoslavian strain of *Artemisia annua* L. (Weathers et al. 1994). The clone was grown in 125-ml flasks using full-strength Gamborg's B5 medium (Gamborg et al. 1968) with the exception that $CaCl_2 \cdot 2H_2O$ was at half the normal concentration (Wobbe et al. 1998). Sucrose was added to the medium (3%, w/v), and the pH of the medium was adjusted to 5.7 before autoclaving. Details of subculturing and maintenance procedures can be found in Smith et al. (1997). The temperature of all culture flasks and reactors was maintained at 23°±2°C. Roots grown in shake flasks for 2 weeks were used as inoculum for the reactor experiments.

Reactor experiments

The nutrient mist reactor (mist reactor) consisted of a root culture chamber, a medium reservoir, and a mist chamber; the geometry of the culture chamber was identical to that of the bubble column reactor (Fig. 1). The working volume of the reactors was 1.5 l, and both reactors initially ran as bubble columns at an air flow rate of 250 ml/min (0.167 vvm) to distribute and anchor the roots onto the stainless steel mesh support structure. For the mist reactor, the medium in the culture chamber was drained into the reservoir after 6–21 days of bubble column mode, and the mist mode began. During mist mode, mist feeding usually lasted for 5 min followed by a mist-off period for 15 min. Air at 5 l/min was introduced into the mist chamber as the carrier gas to transport the mist droplets into the culture chamber. Air only flowed during the mist-on period of the feeding cycle. In two mist reactor runs, different carrier gas phase compositions were tested: (1) 1% O_2, 99% N_2, and (2) air enriched to 0.5% CO_2. A more detailed description of reactor configurations and operation can be found in Weathers et al. (1999).

The inoculum level in the reactors varied from 1.9 g FW/l to 7.1 g FW/l. In most cases, one mist and one bubble column reactor ran side by side, forming a set of concurrent experiments. The main purpose of the concurrent experiments was to minimize any effect from other culture conditions such as light, temperature, or the subculture generation of the hairy root inoculum. In each set of concurrent experiments, both reactors had similar inoculum levels and, therefore, can be directly compared with each other. After 18–38 days the roots were harvested, and fresh and dry weights were measured, and the ratio of dry weight to fresh weight was determined for each reactor.

Shake flask experiments

An inoculum level of 6 g/l was used for all shake flask experiments. Shake flasks contained the same medium composition as mentioned previously. Three shake flasks were harvested every 3 days for a total of 4 weeks, and fresh weight, dry weight, and artemisinin content of the roots were then measured.

Artemisinin content analysis

To extract artemisinin, we sonicated 1 g FW of roots for 30 min in 3 ml of HPLC-grade toluene (Fisher Scientific, Pittsburgh, Pa.), followed by 10 min of centrifugation at 4,390 *g* (HN-S Centrifuge, International Equipment, Needham Heights, Mass.). The supernatant was saved, and the pellets were extracted again in toluene. Both supernatants were pooled, aliquoted into four test tubes, dried under nitrogen flow, and stored at –20°C until HPLC analysis.

Artemisinin was assayed using the Q260 HPLC method of Smith et al. (1997) as modified below. The dried extract was solubilized with 100 µl methanol (HPLC grade, EM Science, Gibbstown, N.J.), and 400 µl of NaOH solution (0.2% w/v) was added. The mixture was hydrolyzed for 45 min at 50°C. The reaction was stopped by adding 400 µl of acetic acid (0.2 *M*) and placing the test tube in iced water. To make the final volume of 1 ml, we added 100 µl of methanol to the test tube. The sample was analyzed by HPLC using a Microsorb C18 column (5 µm, 150×4.6 mm, 100 *Å*; Varian Analytical Instruments, Walnut Creek, Calif.) and a tunable absorbance detector (model 446, Waters, Milford, Mass.) at 260 nm. The mobile phase was 45% methanol and 55% 0.01 *M* sodium phosphate buffer. The pH of the mobile phase was adjusted to 7.0. The injection volume was 100 µl and artemisinin eluted at about 12 min using a flow rate of 1.0 ml/min. Artemisinin was identified using an authentic standard. All chemicals, unless otherwise indicated, were from Sigma Chemical, St. Louis, Mo. The artemisinin content (µg/g DW) was calculated from the measured fresh weight of the roots using the dry weight to fresh weight ratio of the roots determined for each reactor.

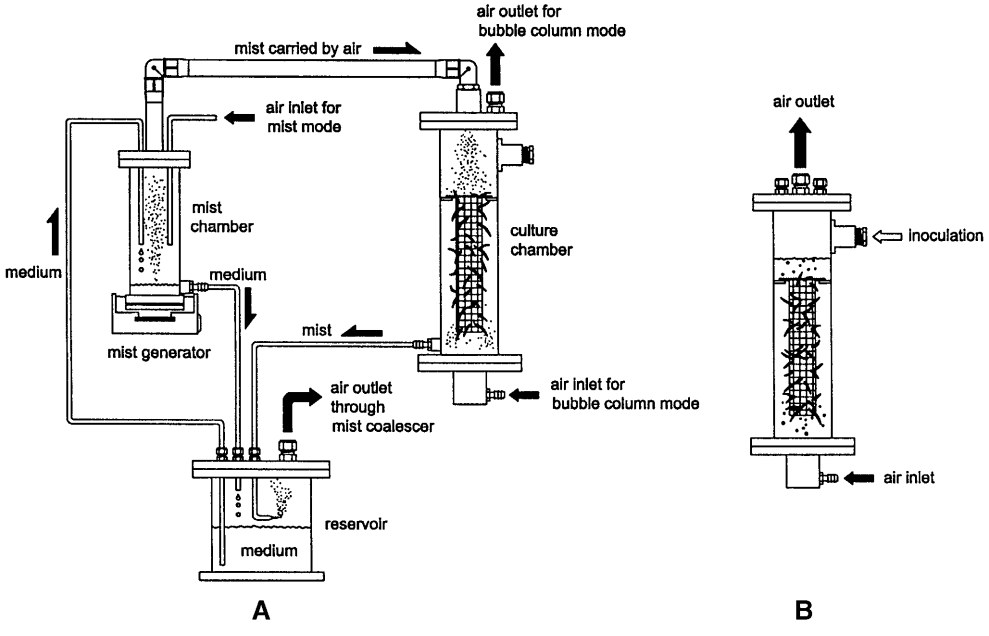

Fig. 1A, B Schematic of the nutrient mist and the bubble column reactors used in these experiments. **A** The nutrient mist reactor, **B** bubble column reactor

Statistical analysis

At least six root samples were harvested from different locations of each reactor, extracted, and assayed for artemisinin. The results were then averaged. The significance of the averaged differences in artemisinin level between reactors was evaluated using the Student's *t*-test.

Results and discussion

The artemisinin content of roots harvested from the reactors is shown in Table 1. Data from a total of ten concurrent experiments are presented, as well as data from three mist (M1–M3) and three bubble column (BC1–BC3) reactors that did not have successful counterparts, mainly due to contamination of the paired reactor. Each line in Table 1 represents one set of concurrent experiments.

In all concurrent experiments, roots grown in the mist reactor had a higher artemisinin content than those grown in the bubble column reactor. In some cases, the difference was more than a factor of ten (compare reactors within set no. 4, and within set no. 8). Furthermore, the highest artemisinin content found in a mist reactor (reactor no. M3) was higher than the highest artemisinin content detected in any bubble column reactor (reactor no. BC1). Pooling the results of the concurrent reactors (reactor sets nos. 1–10) shows that, on average, roots grown in the mist reactors produced three times as much artemisinin per gram dry weight as roots grown in the bubble column reactors (Table 1). The control shake flasks harvested from 3–28 days with same medium composition did not show any detectable artemisinin (Table 2).

Although the average level of artemisinin production in these reactors was on the low end of the levels typically obtained from roots grown in shake flasks (Table 2; Weathers et al. 1994, 1996), the results from reactor M3 (Table 1) indicate higher levels can potentially be achieved. *A. annua* hairy roots habituated to and then grown in half-strength-calcium B5 medium produce negligible amounts of artemisinin (Table 2). In contrast, the same clone grown in B5 medium can produce higher artemisinin levels, although these have declined since the clone was first initiated in 1993 (Table 2). During the course of these comparative studies (more than 70 reactor runs, each ranging from 7–38 days), we did not alter the contents of the culture medium to either optimize growth or artemisinin production. Indeed, a series of fractional factorial studies have been completed (Weathers et al. 1997) or are in progress to optimize both biomass and artemisinin productivity in these roots. The best conditions for maximizing both biomass and artemisinin production, as determined by those studies, should subsequently be tested in reactors.

Table 1 Artemisinin content of roots grown in mist and bubble column reactors (*N.D.* not detected)

	Culture period (days)	Nutrient mist reactor		Bubble column reactor		Notes
		Biomass (g DW/l)	Artemisinin[a] (µg/g DW)	Biomass (g DW/l)	Artemisinin[a] (µg/g DW)	
Concurrent experiment (paired reactor) set						
1	21	4.24	2.67A	6.27	1.03AB	General operation[b]
2	21	3.33	2.87A	6.65	1.42AB	General operation
3	28	3.44	3.49A	5.98	2.14B	General operation
4	28	7.39	2.07A	8.83	0.21B	General operation
5	28	3.57	4.27A	10.23	1.08B	General operation
6	28	5.56	0.85A	6.21	0.51A	5 min on/ 5 min off
7	28	5.44	3.32A	9.23	1.36AB	5 min on/ 5 min off
8	21	0.62	1.82A	3.61	0.07B	1% O_2 and 99% N_2
9	28	9.54	2.86A	8.95	0.34B	Fed-batch[c]
10	38	14.42	2.14A	15.27	1.62A	Fed-batch[d]
Average			2.64±0.97A		0.98±0.68B	
Unpaired						
M1	18	4.47	0.91			9 days bubble column
M2	28	3.81	0.89			Air with 0.5% CO_2
M3	28	2.52	16.33			12 days bubble column
BC1	28			9.14	11.12	
BC2	21			5.18	N.D.	
BC3	37			15.87	0.78	Fed-batch[d]

[a] The results of the statistical analysis should be read together but only for each concurrent experiment set as (letter for the mist reactor, letter for bubble column reactor). A, A: Not significantly different at 95% confidence level; A, B: significantly different at 95% confidence level; A, AB: not significantly different at 95% confidence level, but significantly different at 90% confidence level

[b] General operation conditions for mist reactors are: 6 days of bubble column mode; mist cycle, 5 min on/15 min off; carrier gas, air. Any changes to these conditions are noted in the table

[c] For both reactors, the culture medium was removed and fresh medium was supplied at day 14. The bubble column mode for the mist reactor lasted for 14 days

[d] The culture medium was removed and fresh medium was supplied at days 21 and 31. The bubble column mode for any mist reactor lasted for 21 days

Table 2 Artemisinin content of *A. annua* clone YUT16 hairy roots grown in shake flasks

Culture medium used with age of culture at assay (days)	Artemisinin content (μg/g DW)[a]	Reference and notes
Gamborg's B5, 3% sucrose		
14	250	Weathers et al. 1994
14	375	Weathers et al. 1996
17	40	Souret and Weathers, unpublished
Gamborg's B5, 3% sucrose with half calcium		
14	0.8	Wobbe et al. 1998
15	Negligible	This report

[a] *A. annua* hairy roots are 8% dry weight when grown in liquid media

Artemisinin is a sesquiterpene lactone endoperoxide found mainly in the aerial parts of *A. annua* L. plants. In 1999, Wallaart et al. reported a photochemical conversion of dihydroartemisinic acid to artemisinin in vivo. Dihydroartemisinic acid is first converted to dihydroartemisinic acid hydroperoxide by singlet oxygen ($^1O_2$) via photooxidation, followed by an air oxidation involving triplet oxygen ($^3O_2$) to yield artemisinin (Wallaart et al. 1999). Artemisinin contains the therapeutically active endoperoxide bridge (Meshnick et al. 1996). Both dihydroartemisinic acid and dihydroartemisinic acid hydroperoxide are present in the plant (Wallaart et al. 1999). Since synthesis of artemisinin requires a considerable involvement of oxygen, our initial hypothesis was that the higher content of artemisinin in the mist reactor is due to a greater availability of oxygen. We have already shown that, in contrast to roots grown in shake flasks and in bubble column reactors, oxygen is not limited in the mist reactor (Weathers et al. 1999).

Although oxygen availability would seem to be crucial in increasing the artemisinin levels in roots grown in mist reactors, the results from reactor set no. 8 suggest that this may not be the only factor. In reactor set no. 8, roots were deliberately oxygen-limited in the mist reactor by using 1% O_2 and 99% N_2 as the carrier gas. Although the roots grew very poorly (Table 1), they still produced more artemisinin per unit biomass than their bubble column counterparts. During operation, the medium in the mist reactor can become concentrated as a result of water evaporation. Thus, the roots in the mist reactor are exposed to high concentrations of nutrients longer than roots in the bubble column reactor. In shake flasks the concentration of artemisinin in the roots is greatest before the roots begin exponential growth (6 days after inoculation; Weathers et al. 1996). As the culture grows, the medium becomes depleted of mineral salts and sugars, and the artemisinin level per gram of roots decreases fivefold after 22 days in culture, although the total artemisinin in the culture increases threefold (Weathers et al. 1996). The higher levels of artemisinin found in roots grown in the mist reactor may thus be a response to the increased osmotic strength of the medium.

In summary, we have demonstrated that the type of reactor environment can significantly affect the concentration of secondary metabolites in hairy root cultures. Our work and those of others clearly show that the production of artemisinin in reactors is feasible, although not yet economical (Liu et al. 1998), especially when compared to field production (Ferreira et al. 1997). These results, along with additional studies that employ optimum growth and production media, may lead to a commercially useful process strategy that uses hairy roots for the production of artemisinin and other secondary metabolites.

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