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Phenolics production by encapsulated *Nicotiana tabacum* cells

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ABSTRACT

Plant cells of tobacco (Nicotiana tabacum L.) were grown for several generations in suspension cultures. Cells were immobilized in continuous bioreactors in calcium alginate (Ca Alg) beads or in poly-L-lysine (PLL) encapsulated calcium alginate-hydrogels. In each case, the cells were fed continuously a modified Linsmaier-Skoog plant cell culture medium. The bioreactor effluent was analyzed for total phenolic compounds. The net specific productivity of phenolics was calculated on a daily basis for several test runs. For comparison, productivity in suspension cultures was monitored. Productivity of suspended cells declined to zero within 9 d; both immobilized and encapsulated cells remained productive for 16 d following inoculation. Specific productivity by encapsulated cells was higher than that by immobilized cells; in both types similar rates of decline in productivity occurred.

INTRODUCTION

Plants provide a wide variety of useful biochemicals, including medicinal compounds, flavors, fragrances, and agricultural chemicals. If plant cells were grown in culture and the microenvironments of the individual cells were controlled in a manner to maximize production, as opposed to plant survival, then it should be possible to far exceed the production rate of cells in a whole plant (Sahai and Knuth, 1985; Staba, 1980; Curtin, 1983; Shuler, 1981).

Plant cells are less amenable to commercial secondary metabolite production than are bacterial or fungal cells. Plant cells grow much more slowly, they produce targeted compounds more slowly, they are more easily disrupted by physical stress, and their behavior (growth and synthesis) is influenced by chemical signals from neighboring cells (Sahai and Knuth, 1985). Low growth rate increases the cost of producing a given quantity of cell material. Conventional fermentations utilize cells for a short time, after which they are harvested from batch cultures or washed out with the product stream from continuous cultures. But if cells can be immobilized and kept productive, the high one-time cost of producing the cells can be reckoned against an extended period of productivity. Immobilized cells also are protected from liquid shear forces. In addition, if plant cells are immobilized in close proximity, cell-cell interactions that foster passage of biochemical signals may consequently increase productivity. Prenosil et al. (1987) and Breteler et al. (1987) correlated aggregation of cultured plant cells with enhanced productivity. Aichison et al. (1977) quote several pre-1977 studies where differentiated cells exhibited increased production of secondary metabolites. Dougall (1979) reasoned that this increase in productivity results not from differentiation but from the conditions that encourage differentiation.

In this study, cells of the plant species Nicotiana tabacum were entrapped in two different matrices. Cells were immobilized in calcium alginate beads, a method used in numerous studies with plant cells; or, cells were encapsulated in alginate hydrogel-polylysine capsules, a technique used for mammalian cells (Jarvis, 1986) but not previously applied to plant cells. The anticipated advantage of the latter method was for cells to grow in contact with one another and develop structures which would achieve higher productivity. In each case, the entrapped cells were placed in bioreactors that were fed continuously a medium designed to maintain viability and maximize productivity. The effluent from the bioreactors was collected and analyzed; the productivity (production per unit time per unit biomass) of phenolics was studied under a variety of conditions.

MATERIALS AND METHODS

Surface sterilized explants of tobacco (Nicotiana tabacum cv. Kentucky 147) leaves were placed on Linsmaier and Skoog (1965) solid nutrient medium containing 3.3 mg/l 2,4-dichlorophenoxyacetic acid and 1.0 mg/l kinetin. Callus material was subcultured for seven generations at 20-25°C with continuous low intensity lighting from two 36 W cool white fluorescent bulbs. Primary suspension cultures were started by aseptically chopping up 2-5 g of callus and placing the pieces in 100 ml of liquid medium in 500 ml baffled Erlenmeyer flasks (Bellco Biotechnology, Vineland, NJ). Each culture flask was capped with a cotton and cheesecloth plug that was covered with aluminum foil, and agitated at 20-25°C under fluorescent room lighting at 120 rpm in a shaker-incubator (New Brunswick Scientific, New Brunswick, NJ). Suspension subcultures were maintained by adding 15 ml of healthy suspension culture to 100 ml of fresh medium in similar flasks

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every 14 d. The medium for all suspension cultures followed Hashimoto et al. (1982).

Suspension cultures of various ages, from 2-18 d since inoculation, were sampled. No single culture was sampled more than three times (each sample being 10 ml), because the change in culture volume affected the behavior of the remaining cells. The samples were centrifuged (500 x g for 5 - 7 min) in graduated centrifuge tubes. The packed volume was calculated as a fraction of the sample volume. The supernatant was analyzed for unspecified phenolics by a spectrophotometric technique (Singleton and Rossi, 1985; Hallsby, 1986), in which Folin and Ciocalteu's Reagent attacks the hydroxyl bond in the phenolic molecule and produces a blue color. Intracellular phenolic concentrations were determined following Hallsby (1986).

Seventeen day old suspension culture (7.5 ml) cells were aseptically immobilized in calcium alginate (Ca Alg) beads by adding to sterile 2% sodium alginate solution (15 ml). The mixture was peristaltically pumped through a manifold ending with 3.5 mm glass tubing, from which drops fell into sterile Hashimoto et al. (1982) medium containing 5.6 g/l calcium chloride. The calcium and alginate ions coprecipitated, forming soft spheres of about 5 mm diameter in which viable cells were entrapped.

Cell-laden CaAlg beads were placed in an aqueous solution of 0.05% poly-L-lysine (PLL, MW of about 50,000) and shaken gently by hand for 15 minutes. A coating of PLL formed on the beads. The PLL solution was decanted and replaced with 2% sodium citrate in water. The vessel was covered and shaken in the shaker-incubator at 120 rpm for 2 hours. This dissolved the calcium alginate complex, leaving PLL encapsulating the hydrogel and cells.

The biocatalyst particles, either beads or capsules, were poured into a plastic chromatography column (FLEX-COLUMN,™ Kontes Scientific Glassware/Instruments, Vineland, NJ). Each column was 2.5 cm in diameter by 13 cm long; one third of the column volume was filled with biocatalyst. The column was closed and sealed with silicone sealant. The aseptic operations, from Ca Alg immobilization to loading of the bioreactor columns, took place in a laminar flow hood. Four bioreactor columns were set up to operate in parallel; typically, one or two of them developed plugs and failed to operate. The feed to the bioreactors was the medium of Hallsby (1986). Although 2,4-dichlorophenoxyacetic acid was included, the medium composition had reduced phosphate which was intended to limit cell growth and maximize productivity. The concentrations of phenolics in the feed and effluent were measured by the same method as was used for suspension culture tests. The medium flow rate was acquired by weight difference (full versus empty) of the sample vial over a known sampling time. This flow was kept below 1.0 ml/h (using a Watson-Marlow Model 501Z peristaltic pump set for intermittent operation), to assure a phenolics concentration enhancement in the reactors that would be large enough to be measured.

The quantity of cell mass in each column was estimated by taking a 15 ml sample of the suspension culture at the same time 7.5 ml of the suspension culture was added to the sodium alginate. From the dry weight of the sample (corrected for bead material that was lost in transferring to the column) the total cell mass was calculated. The uncertainty was high, however, because it was difficult to draw identical portions of a suspension culture.

The specific productivity, σ , of extracellular phenolics was calculated by

$$\sigma = \frac{[p\ (out)-p(in)]q}{M},$$

where p(out) is the outlet phenolics concentration (mg/ml), p(in) is the inlet phenolics concentration (μg/ml), q is the flow rate through the column (ml/h), and M is the total cell mass in the column (dry weight). The units of σ become $\mu g\text{-}hr^{-1}\text{-}g_{dw}^{-1}$.

RESULTS AND DISCUSSION

Free Cell Suspension Cultures

Two response variables were used for *N. tabacum* free cell suspension cultures: the fraction of biomass in the culture (given as fractional packed volume), and the specific productivity of phenolics (given as μg of product per dry weight of biomass per h). The time course study shows the average values for packed volume and extracellular phenolics concentration from 3 to 5 different cultures of the same age (Fig. 1). Samples were taken from cultures 2, 4, 6, 8, 10, 12, 14, 16 and 18 d of age. Cell growth, reflected by changes in packed volume, occurred through 18 days in culture. The most rapid growth occurred from 4 to 14 days after inoculation, the maximum specific growth rate, μ, was 0.33 d^{-1}. In a report by Hashimoto et al. (1982), large-scale continuous cultures of tobacco cells were grown with a doubling time of the order of 24 h (μ = 0.693 d^{-1}).

The net productivity of extracellular phenolics was calculated for each 2-d period (Fig 1). The extracellular phenolics concentration (mg/ml) was multiplied by the non-cell volume of the culture (115 ml times [one minus the fractional packed volume]). The change in this quantity was divided by the average dry weight of biomass and, in turn, by the 48 h time period. Productivity was highest very soon after inoculation, during the lag or very early log phase. Productivity remained positive until 8 days after inoculation.

Starting 8 d after inoculation, when biomass growth remained strong, net productivity of

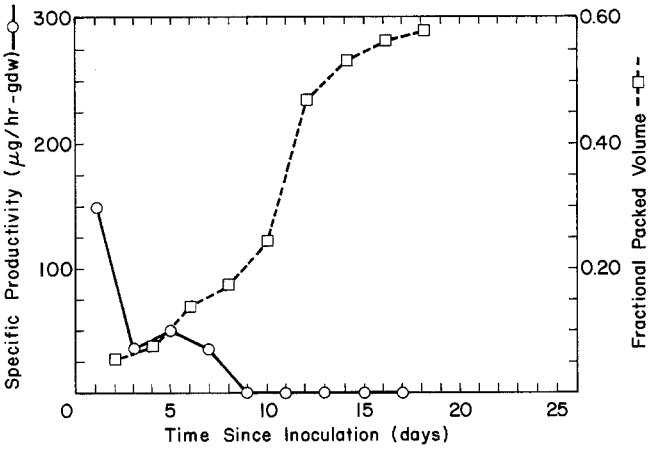

Figure 1. Growth and specific productivity of phenolics of suspended *N. tabacum* cells culture.

extracellular phenolics became negative (shown as zero in Fig. 1). This was probably the result of at least two factors. First, the cells may have absorbed large quantities of phenolics as the concentrations of both cells and phenolics increased. Indeed, an increase in intracellular phenolics concentrations was observed in the small number of analyses performed; intracellular phenolics rose from 160 µg/g dry weight to 300 µg/g between 4 and 8 d after inoculation. However, these levels of intracellular phenolics were too small to account for the observed decrease in extracellular phenolics. Secondly, some phenolics are unstable in solution; e.g., the gallic acid solutions used as analysis standards were not stable at room temperature. By day 8, the breakdown rate of some extracellular phenolics may have exceeded the production rate. Therefore, gross production of phenolics almost certainly declined during the period of highest specific growth rate in the cultures, while the period of highest productivity of extracellular phenolics in suspension culture preceded the period of highest cell growth rate.

Immobilized Cell Cultures

Production kinetics of immobilized N. tabacum cells were also studied in small packed bed bioreactors. Three bioreactors were operated in parallel from the same feed reservoir; two reactors were operated with CaAlg-immobilized cells and one with PLL-encapsulated cells. The results of two test series are presented here for specific productivities as a function of time. These tests were terminated after 26 d operation when feed tank contamination was observed, but data collected through 16 d was considered in comparing productivities of the two types of immobilized cells.

As with the suspension cell cultures, the CaAlg-immobilized cell specific productivities began with a relatively large value and declined to near zero by day 16 in the first test series (Fig. 2). Specific productivities of CaAlg-immobilized cells in the second series (Fig. 3) were stable and relatively high. Both bioreactors exhibited their highest productivities from 3 to 5 d and slowly diminished thereafter. Both had a minimum productivity after 16 d operation.

The specific productivities of PLL-encapsulated cells were comparable or higher than those of CaAlg-immobilized cells (Figs. 2-3). During the first test series, flow was interrupted through the PLL-encapsulated cells; when flow was restarted, the cells exhibited phenolics production. During the second test series, the encapsulated cells maintained high productivity throughout the test; productivity peaked on day 3, and declined until day 16 when it was close to zero.

Productivity in the suspension cultures declined in a similar manner as immobilized and encapsulated cells. Productivity was higher, and the decline in productivity was slower, in the immobilized and encapsulated cells compared to suspension cultures. In the suspension cultures, productivity decline can be attributed to either depletion of a medium component important to biosynthesis, or accumulation of a compound inhibiting phenolics synthesis. In the bioreactors, where fresh medium was pumped through continuously, neither depletion nor buildup should have occurred. Here, the productivity decline may be attributed to lack of oxygen; the bioreactor columns did not have an air feed line separate from the liquid feed. The flow rate of dissolved oxygen to bioreactors was estimated to be one-tenth that consumed by the same quantity of cells in suspension culture in the presence of excess oxygen (Halsby, 1986). Effectiveness factor calculations for the alginate biocatalyst (Haigh, 1988) show that oxygen starvation was not a consequence of mass transfer limitation within the beads.

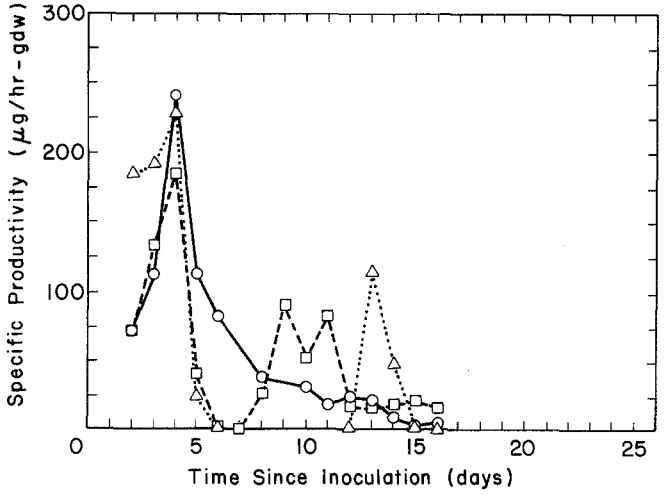

Figure 2. Specific productivities of phenolics in Test Series 1: Immobilized and Encapsulated Cells

-□- CaAlg-immobilized cells reactor 1

—○— CaAlg-immobilized cells reactor 2

··△·· PLL encapsulated cells reactor 3

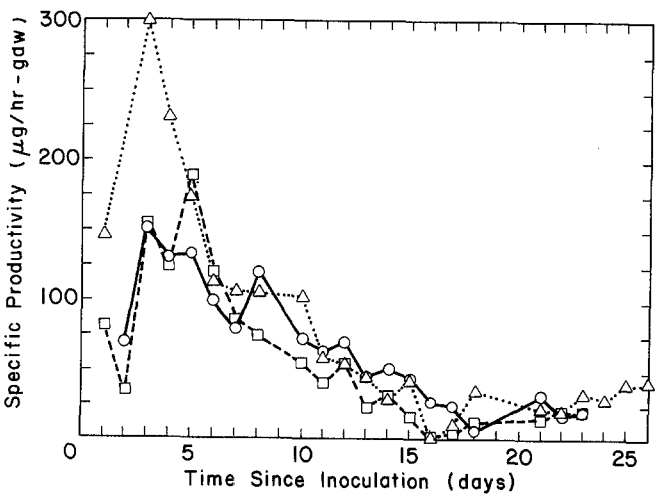

Figure 3. Specific productivities of phenolics in Test Series 2: Immobilized and Encapsulated Cells

-□- CaAlg-immobilized cells reactor 1

—○— CaAlg-immobilized cells reactor 2

··△·· PLL encapsulated cells reactor 3

The specific productivity early in each test (2 to 5 d) was used for comparison (Table I) because uninterrupted flow occurred over that period in all reactors. Also, the large productivity values reduce the relative uncertainty. In both test series, the encapsulated cells showed the higher productivity, but by less than one standard deviation. The integrated phenolics production throughout each test may be meaningful, although flow irregularities occured through the reactors at times during each test. These data more greatly differentiate the phenolics production between the PLL-encapsulated and CaAlg-immobilized cells; the former produced twice as much as the latter. The specific productivities of soluble phenolics by these immobilized systems are 5 to 10 times greater than those reported by Sahai and Shuler (1984) for tobacco cells in a two-stage chemostat.

Enhanced productivity of the PLL-encapsulated system compared to Ca-Alg immobilized cells may result for several reasons. First, the PLL membranes may provide better mass transfer of products from the microenvironment of the entrapped cells, thus reducing product inhibition relative to the alginate beads (Kargi and Rosenberg, (1987)). Secondly, the limited amount of dissolved oxygen supplied in the feed allows greater rates of survival of PLL encapsulated cell because the dry weight of cells in this case was less than in CaAlg-immobilized case. Thirdly, cell contact and aggregation in PLL spheres might improve cellular phenolics production. However, the initially greater specific productivity in the PLL capsules was not sustained because non-growth medium was supplied to the columns.

Table I. Productivity of phenolics from CaAlginate immobilized and poly-L-lysine encapsulated N. tabacum cell bioreactors.

Test	Immobilization Matrix	Cells [gdw]	Specific Productivity[a] [ug/h/gdw]	Integrated Production[b] [ug/gdw]
1	CaAlg	0.044	134 ± 74	22,157
	CaAlg	0.049	107 ± 64	22,464
	PLL	0.008	156 ± 91	36,096
2	CaAlg	0.038	122 ± 38	32,064
	CaAlg	0.052	117 ± 34	27,533
	PLL	0.025	274 ± 69	47,578

[a] Specific productivity (mean ± standard deviation) during uninterrupted flow period from 2 - 5 d after inoculation in the test. (For the PLL in Test 2, productivity for day 3 was calculated by interpolation.)

[b] Total integrated production of phenolics (per unit dry weight) through the first 16 d of each bioreactor test.

REFERENCES

Aitchison P A, Macleod A J, Yeoman M M (1977) In H E Street (ed), Plant Tissue and Cell Culture 2nd edition, Univ of Calif Press, pp. 267-306

Breteler H, Hulst A C, Tramper J (1987) Proc 4th Eur Cong Biotechnol 2:430

Curtin M E (1983) Bio/technology 1:649-657

Dougall D K (1979) In W R Sharp, P O Larsen, E F Paddock, and V Raghaven, eds, Plant Cell Tissue Culture Principles and Applications, Ohio State Univ Press, pp 727-743

Haigh J R (1988) Production of Phenolics by Immobilized and Encapsulated Cells of Nicotiana tabacum M S Thesis, Colorado State Univ, Fort Collins, CO

Hallsby G A (1986) Immobilized Plant Cells for the Production of Specialty Chemicals PhD Dissertation, Cornell Univ, Ithaca, NY

Hashimoto T, Azechi S, Sugita S, Suzuki K (1982) In A Fujiwara, ed, Plant Tissue Culture, Japanese Assoc for Plant Tissue Culture, pp 403-404

Goosen MFA (1988) Chem Eng Education Fal:196-200

Kargi F, Rosenberg M Z (1987) Biotechnol Prog 3:1-8

Linsmaier E M, Skoog F (1965) Physiol Plant 18:100-127

Prenosil J E, Hegglin M, Baumann T W, Frischknecht P M, Kappeler A W Brodelius P, Haldimann D (1987) Enzyme Microb Technol 9:450-458

Sahai O, Knuth M (1985) Biotechnology Progress 1:1-9

Sahai O, Shuler ML (1984) Biotechnol Bioeng 26:27-36

Shuler M L (1981) Annals of NY Acad of Sci 369:65-79

Singleton V L, Rossi J A Jr (1984) Amer J Enol Viticult 16:144-158

Staba E J (ed) (1980) Plant Tissue Culture as a Source of Biochemicals CRC Press Roca Baton, Florida

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