Adsorbent Culture of Tobacco Cell Suspensions with Different Adsorbents

R. Maisch, B. Knoop, and R. Beiderbeck

Botanisches Institut der Universität Heidelberg, Im Neuenheimer Feld 360, D-6900 Heidelberg, Bundesrepublik Deutschland

Z. Naturforsch. **41c**, 1040–1044 (1986); received July 7/September 18, 1986

Nicotiana tabacum, Cell Culture, Adsorbents, Secondary Substances

The release of secondary substances from plant cell suspensions can be enhanced by the addition of adsorbents to the culture medium. Tobacco cells react to a variety of different adsorbents (Florisil, raw silk, Polyclar AT, and the adsorber resins XAD-4 and XAD-7) with important qualitative and quantitative changes of their secondary substance pattern, in some cases without changes of growth.

Many plant species are synthezising secondary substances used by man *e.g.* as drugs, perfume bases or insectizides. Rapidly growing tissue cultures of the same plants however usually do not produce them or at best in traces. A commercially profitable yield is the rare exception and described up to now for shikonin only [1, 2].

One of the proposed ways out of this bottleneck is the adsorbent culture [3]. This technique is based on the addition of solid adsorbents to growing cell cultures. The adsorbent phase – replacing plant storage compartments – accumulates even traces of secondary substances from the medium thus avoiding any type of feed back inhibition on subsequent output.

The effectiveness of this technique could be demonstrated for coniferyl aldehyde from *Matricaria chamomilla* using activated charcoal as an adsorbent [3, 4]. The use of activated charcoal however is subject to some disadvantages and to make the technique generally applicable it seemed to be useful to investigate other kinds of adsorbents.

Material and Methods

Cell culture

A suspension culture of *Nicotiana tabacum* var. Maryland was established in 1981 [4]. All culture passages were made with a volume of 24 ml suspension in baby food jars (210 ml) shaken with 100 rpm at 25 °C in 4600 lux and 14 h daylength.

Reprint requests to Dr. B. Knoop.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/1100–1040 $ 01.30/0

Fungus culture

Erlenmeyer flasks (200 ml) containing 20 ml Czapek-Dox medium were inoculated with 0.1 g mycelium of *Penicillium purpurogenum* Stoll. obtained from Centraal-bureau voor schimmelcultures, 3740 AG Baarn, Netherlands.

Viability

The viability of cells was determined by staining with fluoresceine diacetate [5] and/or Evan's Blue [6].

Adsorbents

The adsorbents had to be added to the cell suspensions in a form which allowed a quick and complete separation from the cells. Those adsorbents available as granules were applicated directly, others representing fine powders were first imbedded into an alginate matrix according to published methods [7]. For this purpose 0.56 g alginate were suspended in 1 ml ethanol, then diluted with 50 ml hot water (80–90 °C) and stirred to a clear solution. After addition of the powdered adsorbent (in the case of Polyclar AT 3 g/10 ml alginate solution) this suspension was dripped from a pipette into a bath of $CaCl_2{*}2H_2O$ (60 mM in H_2O) thus forming droplets of about 3–4 mm diameter. After 5 h the alginate beads were thoroughly washed with water to remove the unbound $CaCl_2$. All adsorbents were autoclaved together with the medium before the cells were added.

If not mentioned otherwise the usual amounts were 0 (control), 0.2, 0.5, 1.5, and 3.0 g of pure adsorbent per culture (24 ml).

Harvest of cells and adsorbents

After 5 days of co-culture (standard conditions) the adsorbents were separated from the cells and repeatedly washed with water. In the experiments using imbedded adsorbents the alginate matrix was solubilized with 0.2 M Tri-sodium-citrate. After 1 h the adsorbent was removed by filtration and thoroughly washed with water. All adsorbents were carefully dried before the extraction procedure.

The cell mass was determined as fresh weight. For controls adsorbent-free cultures were filtered and the cell-free spent medium was incubated with the adequate amount of adsorbent for about 8 h on a shaker. During this time the secondary substances released by control cells into their medium were bound to the adsorbent.

Extraction of substances from the adsorbents

The extraction methods depended on the adsorbent used and had been worked out in preliminary experiments investigating the desorption of metabolites of a standardized betula leaf extract. The solvents were evaporated, residues taken into 300 µl methanol and separated by thin layer chromatography (TLC).

TLC

The extracts were separated using silicagel plates with fluorescent dye (Merck, Darmstadt FRG, No. 5553). The solvent systems were:

a. Ethyl acetate + formic acid + acetic acid + water (100 + 11 + 11 + 26, v/v);

b. chloroform + methanol + ethyl acetate (40 + 10 + 10, v/v).

The resulting substance patterns were evaluated under UV-light (254 and 365 nm) with and without Natural Compound Reagent A (NCR; Roth, Karlsruhe FRG, No. 9920). Quantitative determination of single compounds was made by spectrophotometry or fluorometry after isolation of the single TLC-bands.

Identification

Chlorogenic acid was identified by fluorescence with and without staining, co-chromatography of a pure reference and NMR.

Results

Culture growth

Starting with about 1.0–1.5 g cell fresh weight in 24 ml medium the suspension culture of tobacco cells yielded up to 10 g within 10 days. The logarithmic growth phase was between the 2nd and 6th day (Fig. 1).

All experiments were started with 7 days old cultures. Adsorbents were added in the course of a cell transfer and harvested 4 or 5 days later. Since at this time more than 95% of the cells were viable the pattern of secondary substances released into the medium is not markedly influenced by substances leaking from dead cells.

Culture with different adsorbents

Zeolith Taylor (Serva, Heidelberg FRG, No. 36610)

The addition of Zeolith Taylor (a synthetic Na-Al-silicate) to a cell suspension led to a marked growth inhibition depending on the added amount of the adsorbent and was accompanied by a pH-shift in the medium to higher values (Table I). Nevertheless the

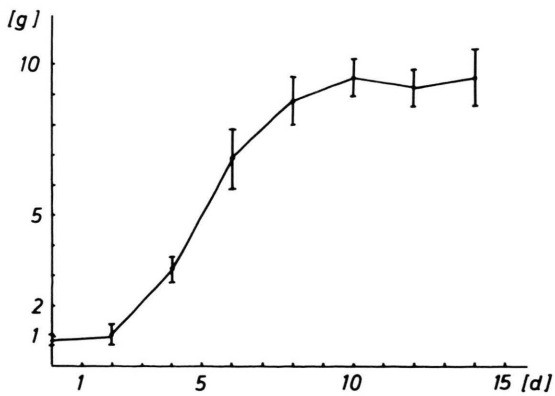

Fig. 1. Growth curve of a tobacco suspension culture (from 4 experiments).

Table I. Growth parameters of tobacco cells together with Zeolith as an adsorbent. a and b represent results from two independent experiments.

Zeolith [g]	Fresh weight [g]			pH at harvest
	a	b	average [%]	
0	4.90	5.20	100	5.70
0.2	4.25	4.10	83	5.75
0.5	3.30	3.30	65	6.10
1.5	2.10	2.40	45	6.35
3.0	2.10	2.15	42	6.55

cell viability was more than 90% even with 3 g adsorbent per culture flask.

At the end of the co-culture the adsorbent showed a light brown colour indicating the presence of bound cell products but all elution methods tested failed to remove them from Zeolith. Only some few weakly fluoresceing bands could be found on the chromatogram after extraction with ethanol/diethylamine (4 + 1) as well as with a saturated KCl-solution.

Furthermore the growth inhibition could not be overcome by preincubating the Zeolith with large quantities of fresh medium to diminish the adsorption of medium components during cell culture.

Florisil (Serva, Heidelberg FRG, No. 21528)

Florisil, a Mg-tri-silicate, inhibited cell growth completely already with 0.2 g per culture. After 5 days 90% of the cells were dead and the pH of the suspension had risen to 7.8. Higher amounts of this adsorbent still amplified these effects. This means that Florisil is unsuitable to be used in the adsorbent technique.

Silk (unstained, not spun, Gütermann, Gutach-Breisgau FRG)

Raw silk fibres were thoroughly washed with different organic solvents and finally water, then knotted to strings and added to the fresh media. During autoclaving this scleroprotein released some brownish colour into the medium and subsequently cell growth was inhibited completely with 0.5 g silk. Nevertheless silk adsorbed several fluoresceing cell products which could be removed either by acetone or by ethanol/diethylamine (4 + 1). However, the growth inhibition together with some difficulties to separate the fibres completely from cells let this kind of silk appear not very suitable as a second phase although unsoluble proteins might principally be interesting adsorbents.

Polyclar AT (Serva, Heidelberg FRG, No. 33162)

This water-insoluble polyvinyl pyrrolidone is available as a fine powder and since its specific density is similar to that of tobacco cells, it is difficult to separate it from the cultured cells. Therefore Polyclar AT had to be immobilized by imbedding it to alginate beads. The pH of the culture remained unchanged but higher amounts slightly reduced the fresh weight yield of cell material, *e.g.* to 60% with 3 g of adsorbent. The viability of the cells however was more than 90% although the final cell size was markedly increased compared to the adsorbent-free control. The latter is usually found in old cultures and under nutrient deficiencies.

Extracts of Polyclar AT with ethanol/diethylamine (4 + 1) followed by TLC yielded several fluoresceing compounds not detectable either in cell-free medium, in cell extracts or in Polyclar AT alone. Therefore these substances must have been released by the cultivated cells as a result of the treatment.

XAD-7 (Serva, Heidelberg FRG, No. 42851)

XAD-7 is an adsorbent consisting of acrylic acid esters. Small amounts of this adsorbent (up to 0.5 g per 24 ml culture) did not affect the growth rate of a tobacco suspension culture, and more than 95% of the cells were viable. Increased amounts of XAD-7 occasionally led to lower cell yields (Table II) and increased cell lengths. However this depended on the cell line used and may vary in a wide range.

Table II. Cell fresh weight and pH of a suspension culture with different amounts of added XAD-7. Measurements after 4 days of culture. The initial cell mass was 0.8 g. Standard deviations in brackets.

XAD-7 [g]	Fresh weight [g]	pH at harvest
0	4.38 (0.20)	5.60
0.2	4.55 (0.07)	5.78
0.5	4.45 (0.07)	5.45
1.5	2.65 (0.35)	5.08
3.0	2.60 (0.57)	4.80

Using acetone a number of fluoresceing substances could be eluted not appearing in cell free medium or the pure adsorbent and in part not even detectable in the extract of 1 g living cells. Compared to the content of normal culture medium the total amount of many secondary substances was drastically enhanced by the addition of the adsorbent XAD-7 (Fig. 2).

XAD-4 (Serva, Heidelberg FRG, No. 42831)

XAD-4 is an adsorbent based on a polystyrene matrix which is even more compatible with tobacco cells than XAD-7. XAD-4 adsorbed more fluoresceing substances from a tobacco culture than XAD-7 and its acetone extract gave more and clearer fluoresceing bands (Fig. 3).

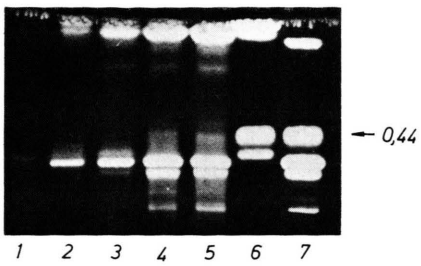

Fig. 2. TLC of substances released from a tobacco suspension culture into XAD-7. Traces from left to right: 1 = control; 2–5 = increasing amounts of XAD-7 (see Table II); 6 = mixture of Rutin (R_f 0.40), chlorogenic acid (0.45), umbelliferone (0.95), scopoletine (0.99); 7 = acetone extract of complete cells. Solvent system 1 and detection under UV 366 nm after staining with NCR.

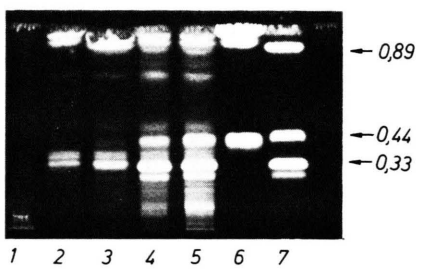

Fig. 3. TLC of substances released from a tobacco suspension culture into XAD-4. Traces from left to right: 1 = control; 2–5 = increasing amounts of XAD-4 (see Table III); 6 = mixture of chlorogenic acid (0.44), umbelliferone (0.95), scopoletine (0.99); 7 = acetone extract of complete cells. Solvent system 1 and detection under UV 366 nm after staining with NCR.

Three of these substances (Rf 0.33; 0.44; 0.89; solvent system 1) were of special interest because their production was correlated to the amount of added adsorbent. One of them (R_f 0.44) could clearly be identified as chlorogenic acid (Table III).

The content of chlorogenic acid in the medium of the adsorbent-free controls was close to the limit of detection whereas in XAD-4 loaded cultures the enhancement of chlorogenic acid accumulation could roughly be estimated as at least 20-fold (Table III). Fluorometry of this compound gave similar results [8].

XAD-4 is suitable for adsorbent culture also from two other reasons: After extraction and washing with acetone it can be reused without any restriction. Additionally XAD-4 can be imbedded into alginate beads to fascilitate the separation of adsorbent and cells without any disadvantage to the method.

Table III. Fresh weight and production of chlorogenic acid per culture (24 ml) depending on the added amount of adsorbent. Control values are close to the limit of detection. Standard deviations in brackets.

XAD-4 [g]	Fresh weight [g]	Absorbence 328 nm	Concentration [µg/ml]
0	4.29 (0.79)	0.012	0.5
0.2	4.20 (0.43)	0.072	1.3
0.5	4.28 (0.46)	0.277	5.5
1.5	4.85 (0.88)	0.432	8.0
3.0	4.63 (0.18)	0.477	9.5

XAD-4 was also used with a liquid culture of *Penicillium purpurogenum* Stoll. This ascomycete releases a number of red coloured products to the Czapek-Dox-medium (Merck) [9].

After 7 days of co-culture of *Penicillium* with XAD-4 the adsorbent was removed and the medium of the adsorbent-free control postextracted with XAD-4 as already described. All samples of the adsorbents yielded acetone extracts coloured yellowish. The colour intensity increased with increasing amounts of XAD-4.

The analysis of these extracts by means of TLC demonstrated again a pronounced higher production of secondary substances released from the cells in the presence of an adsorbent (Fig. 4).

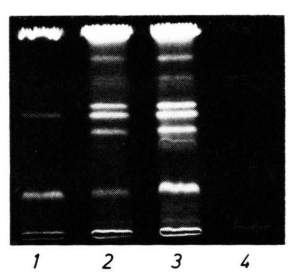

Fig. 4. TLC of substances released from *Penicillium purpurogenum* into XAD-4. Traces from left to right: 1–3 = 0.5, 1.5, 3.0 g XAD-4; 4 = control. Solvent system 2; detection under UV 366 nm.

Conclusion

Of all adsorbents tested XAD-4 and to a lesser degree XAD-7 and Polyclar AT were suited for an application in tobacco suspension cultures and in a

mycelium culture of *Penicillium purpurogenum*. The patterns of secondary substances extractable from these adsorbents demonstrated the possibility to influence the secondary substance production in a complex way: The yield of some substances is enhanced, that of others is reduced or unchanged. In the case of XAD-4 and certain cell lines secondary substance production changes are promoted without accompanying growth inhibition. The addition of XAD-7 or Polyclar AT leads to different secondary substance changes combined with a slight growth reduction. Anyway a maximum growth rate of plant cell cultures is not necessarily the ultimate goal if secondary substance production is intended because it is known from many experiments that maximum substance production excludes maximum growth rate and *vice versa*.

Since different adsorbents collect and bind different patterns of secondary substances and since each adsorbent can be eluted fractionally by subsequent elution steps (results not published) this method offers high flexibility.

Acknowledgements

The authors are indepted to Deutsche Forschungsgemeinschaft for financial support.

[1] B. E. Ellis, IAPTC Newsletter **38,** 13 (1982).
[2] Y. Yamada and Y. Fujita, in: Handbook of Plant Cell Culture (D. A. Evans, W. R. Sharp, P. V. Ammirato, and Y. Yamada, eds.), **Vol. 1,** p. 717, New York 1983.
[3] B. Knoop and R. Beiderbeck, Z. Naturforsch. **38c,** 484 (1983).
[4] R. Beiderbeck and B. Knoop, Z. Naturforsch. **39c,** 45 (1984).
[5] J. M. Widholm, Stain Tech. **47,** 189 (1972).
[6] D. F. Gaff and O. Okong'O-Ogola, J. Exp. Bot. **22,** 756 (1971).
[7] E. N. A. Mbanaso, Plant Sci. Lett. **25,** 61 (1982).
[8] R. Maisch, Diplomarbeit, Universität Heidelberg (1985).
[9] J. I. Pitt, The Genus Penicillium and its Telomorphic States *Eupenicillium talaromyces*. Acad. Press London, New York, Toronto, Sydney, San Francisco 1979.
[10] R. Beiderbeck and B. Knoop, in: Biotechnology in Agriculture and Forestry (Y. P. S. Bajaj, ed.), Springer, Berlin, Heidelberg, New York (in press).