Plant Cell Reports (1985) 4:220–223

Plant Cell Reports
© Springer-Verlag 1985

Cultivation of cell cultures of *Berberis wilsonae* in 20-l airlift bioreactors

M. Breuling, A. W. Alfermann, and E. Reinhard

Pharmazeutisches Institut (Pharmazeutische Biologie), Universität Tübingen, Auf der Morgenstelle 8, D-7400 Tübingen, FRG

Received June 3, 1985 / Revised version received July 11, 1985 – Communicated by W. Barz

ABSTRACT

Suspension cultures of Berberis wilsonae produce 4 berberine-type alkaloids: berberine, palmatine, columbamine and jatrorrhizine. In particular the formation of the phenolic alkaloids columbamine and jatrorrhizine and of berberine proves to be dependent on the concentration of dissolved oxygen. With higher aeration rates, berberine and jatrorrhizine yields can be increased considerably. Thus we reached an alkaloid yield of more than 3 g x l^{-1} with 50% dissolved oxygen tension in the medium. As far as we know this is one of the best results in fermenting of alkaloid-producing cell cultures.

ABBREVIATIONS

pO_2, dissolved oxygen concentration in % saturation (using air); HPLC, high-performance liquid chromatography; vvm, volume air x volume $medium^{-1}$ x $minute^{-1}$; rpm, revolutions per minute; IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxy acetic acid.

INTRODUCTION

Protoberberine-type isoquinoline alkaloids are characteristic of the Berberidaceae. Members of this family are used in medicine, especially in the Far-East, because of the anti-bacterial and anti-inflammatory properties of their alkaloids (Kondo 1976). In addition, berberine-type alkaloids are known to be capable of inducing cytostatic activity in macrophages in vitro (Kumazawa et al. 1984).
Berberis species synthesize 4 main berberine-type alkaloids: berberine, columbamine, jatrorrhizine and palmatine (fig. 1, Ikram 1975).

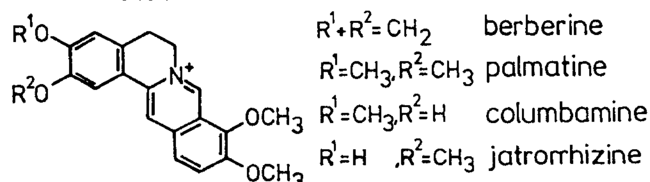

Figure 1. The 4 main alkaloids of Berberis wilsonae.

Offprint requests to: M. Breuling

Berberine is the major alkaloid in the root bark of the plant; in cell cultures, however, jatrorrhizine predominates (Hinz et al. 1981, Rothenberger 1982).
In 1981 Hinz et al. reported a yield of about 1.7 g alkaloids x l^{-1} medium from Berberis stolonifera using 100-ml flasks. By selecting a highly productive cell line of Berberis wilsonae in 1982 Rothenberger was able to increase the alkaloid yield up to 2.26 g x l^{-1} medium. Experiments with 1-l flasks resulted in a decrease in the alkaloid content of about 25% as compared with the results in 100-ml flasks.
Alkaloid formation as a function of dissolved oxygen tension has been discussed for Coptis japonica by Yamada et al. (1981) and by Sato et al. (1982).
Within the scope of our work about alkaloid formation in fermenting cultures of Berberis wilsonae, the present study will report a few aspects of the influence of dissolved oxygen tension in 20-l airlift reactors.

MATERIALS AND METHODS

Culture conditions

Cell cultures of Berberis wilsonae Hemsl. & Wils., line WSG3, were used for these investigations. This high alkaloid producing cell line was selected by Rothenberger (1982) from Berberis wilsonae callus cultures because of its dark orange colour. The cells were subcultivated every 10 days by inoculating 36 g cells (fresh weight) into 300 ml of medium in 1-l Erlenmeyer-flasks. They were kept on rotary shakers (120 rpm) in the dark at 24°C. The basal medium of Murashige and Skoog (1962) was used with the following modifications: 40 g x l^{-1} sucrose, 0.2 mg x l^{-1} IAA, 0.2 mg x l^{-1} 2,4-D, 2.0 mg x l^{-1} kinetin.
Eight 10-day-old flasks were used to inoculate the Wahl (1977) laboratory airlift reactor containing the same medium used during preculture with the sucrose concentration reduced to 20 g x l^{-1}. The working volume was kept constant at 19 l by adding sterile water. If necessary an antifoam material was added (2.5 g polypropylenegly-

col, mol weight 2025, E. Merck, Darmstadt, FRG, x l^{-1} water). Because of the gradual depletion of the carbon source, a 30% glucose solution (w/v) was fed to the reaction mixture from day 6 on. Fermentation was carried out with a constant aeration rate or a constant dissolved oxygen concentration (pO_2). The pO_2 was measured in situ with a polarographic oxygen electrode (Dr. W. Ingold, Urdorf, Switzerland) and regulated using an oxygen control unit from B. Braun (Melsungen, FRG).

Analytical procedures

100 mg of the dried and powdered tissue were extracted under reflux with 10 ml methanol. After cooling the extract was filtered and diluted with methanol 1:20.
The alkaloid content of the solution was analyzed by HPLC (Beckman Gradient Liquid Chromatograph Model 332) using a 250 x 4.6 mm stainless steel column with Nucleosil 5C18 (Macherey & Nagel, Düren, FRG) as the stationary phase. The liquid phase was water adjusted to pH3 with H_3PO_4 and 84% acetonitrile redistilled in water adjusted to pH_3 with H_3PO_4. We used a modification of Rüffer's gradient (personal communication 1984) as shown in fig. 2.
The flow rate was 1.5 ml x $min.^{-1}$, measuring wave length 228 nm, injection volume 20 ul.

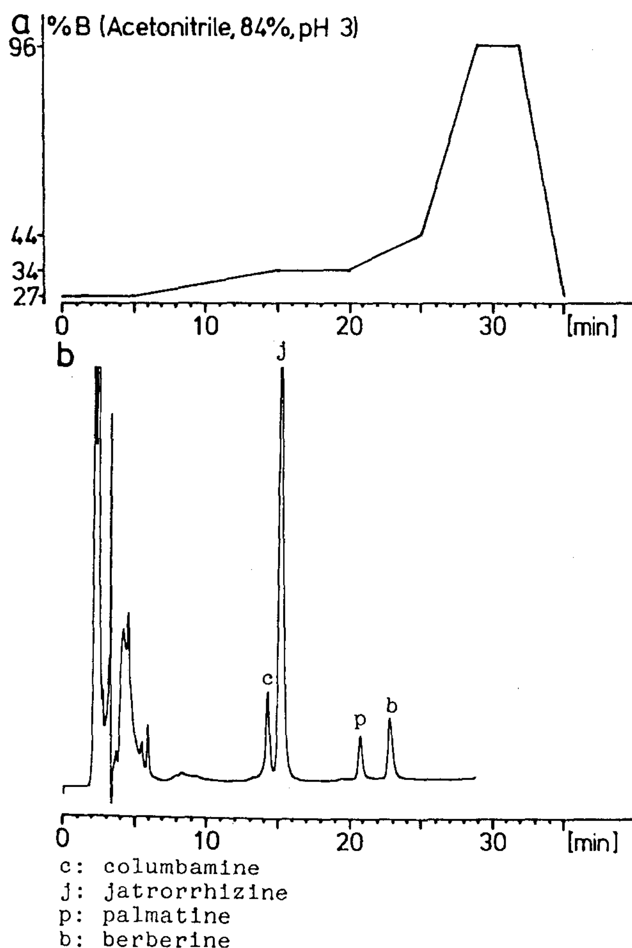

Figure 2.a: Percentage of acetonitrile in the liquid phase. Rinsing cycle between minute 25 and 35. b: HPLC-chromatogram of the 4 protoberberine alkaloids.

This simple method results in good separation and quantification of the four alkaloids.

RESULTS AND DISCUSSION

It has been shown by various authors that airlift reactors are suitable for secondary product formation by plant cell cultures (see i.e. Wagner and Vogelmann 1977, Fowler 1981, Smart and Fowler 1984). Only at high cell densities can mixing problems occur that might make it advantageous to use a stirred-tank reactor (Tanaka 1981, Ulbrich et al. 1985, Spieler 1985).
In preliminary experiments we found that airlift reactors can be used for cultivating Berberis cell cultures for alkaloid production. Here we will demonstrate the importance of controlling the dissolved oxygen tension (pO_2) in the bioreactor in order to achieve optimal product yields.

Influence of pO_2 on cell growth

Table 1 shows that a dissolved oxygen tension between 20 and 50% saturation using air for aeration has no influence on cell growth. The cell yield on day 14 is about 25 g x l^{-1}, the specific growth rate during the logarithmic phase of growth is between 0.13 and 0.15 $days^{-1}$, and the doubling time is between 4.6 and 5.5 days. By feeding more glucose to the reaction mixture during a cultivation time of 20 days, dry weights of more than 40 g x l^{-1} can be achieved (see also figs. 4 and 5).

Under such conditions differences in cell yields can be observed as a result of insufficient mixing with low aeration rates (only up to 0.3 vvm or a pO_2 of 20%). The higher aeration rates necessary to guarantee 40 or 50% pO_2 also result in a good mixing of the culture broth. 0.5 vvm and more causes growth reduction because of shear stress and high ventilation (cf. Smart and Fowler 1984).

aeration rate	(vvm)	0.1-0.3	0.1-0.4	0.1-0.5
dissolved oxygen	(%pO_2)	20	40	50
spec. growth rate	($days^{-1}$)	0.15	0.15	0.13
doubling time	(days)	4.6	4.6	5.5
dry weight at day 14	(g x l^{-1})	25.5	25.1	24.8

Table 1. Growth data at various dissolved oxygen concentrations.

Effect of pO_2 on alkaloid formation

Fig. 3 shows that increasing the oxygen tension and aeration rate enhances the accumulation of berberine, columbamine and jatrorrhizine, whereas palmatine is only affected to a very minor extent. This reflects the close biosynthetic interrelationship of the first three alkaloids; the final steps in palmatine biosynthesis may be somewhat divergent. Whereas fig. 3 only shows the maximal amount of alkaloids, fig. 5 demonstrates the kinetics of accumulation for the four alkaloids during the growth cycle of the cells. Maximal berberine accumulation occurs very early (days 2-4) during the lag phase in the growth cycle, diminishing when columbamine and jatrorrhizine accumulation increase during the logarithmic and early

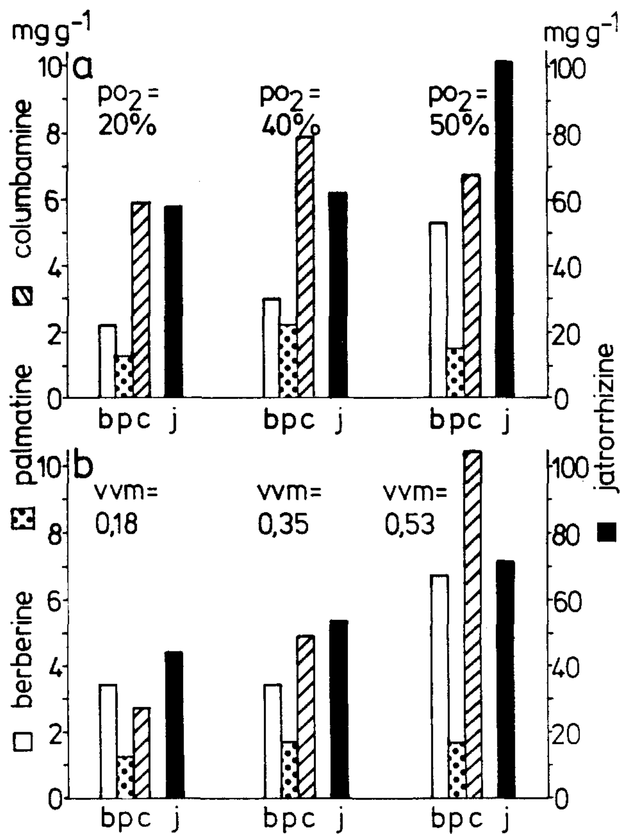

Figure 3. The dependence of alkaloid formation on various aeration rates.
a: increasing pO_2.
b: increasing vvm.

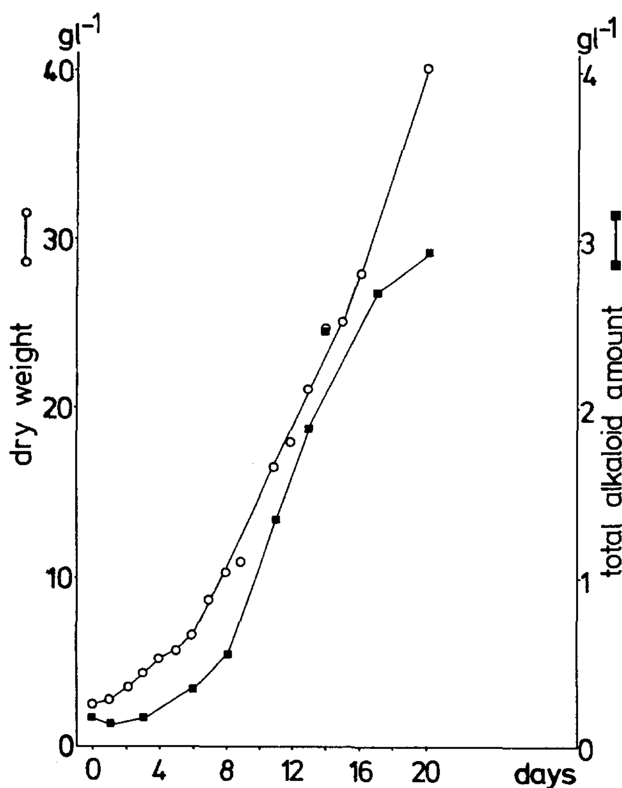

Figure 4. The yields in dry weight and total alkaloids show parallel courses during the whole fermentation cycle.

stationary growth phase. This, too, demonstrates the intermediate function of berberine in columbamine and jatrorrhizine biosynthesis, as was found in Beecher and Kelleher's enzymatic studies (1983).
The maximum jatrorrhizine accumulation (10.1%) in percent of dry matter can be observed on day 15. Alkaloid biosynthesis, however, continues until day 20, as is shown in fig. 4.

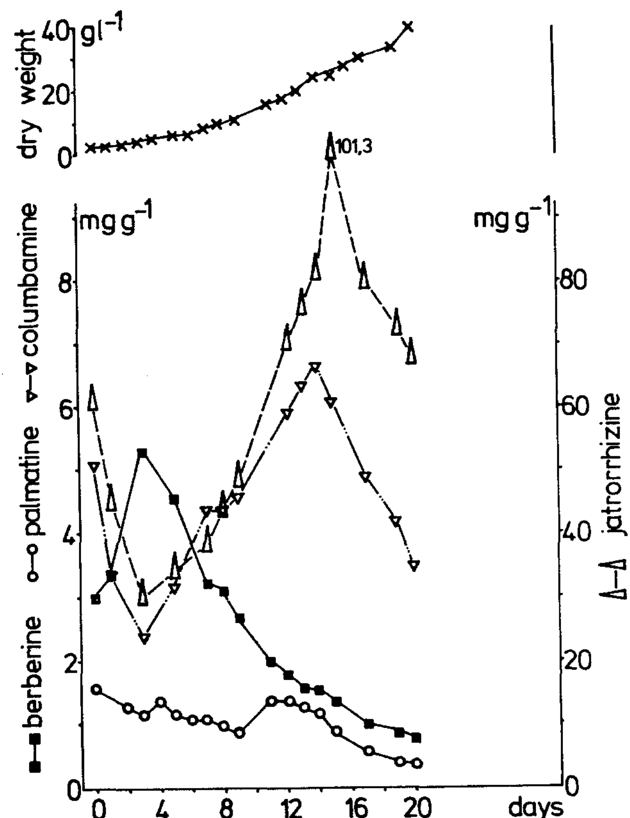

Figure 5. The kinetics of alkaloid accumulation during the growth cycle and the yield in dry weight at pO_2=50% in the culture broth.

Because of the significant increase in cell dry weight there is still an increase in the total alkaloid yield up to more than 3 g x l^{-1}. To our knowledge, these are the highest alkaloid yields from plant cell cultures in bioreactors.
Although the oxygen demand of plant cells is quite low when compared with microorganisms the experiments presented here show very clearly that it may be very important to control the oxygen tension in the bioreactor in order to obtain optimal secondary product yields from plant cell cultures. Similar results were obtained by Spieler et al. (1985). Moreover, it can be seen that by providing the appropriate cultural conditions it is possible to improve the product yields in bioreactors substantially as compared with the results in shake flasks.

ACKNOWLEDGEMENTS

We would like to thank Dr. M. Rüffer (Munich, FRG) for her kind advice and valuable information on alkaloid separation by HPLC.

This work was supported by the Bundesministerium für Forschung und Technologie.

REFERENCES

Beecher CWW, Kelleher WJ (1983) Tetrahedron letters 24: 469-472

Fowler MW (1981) Chem Ind 7: 229-233

Fukui H, Nakagawa K, Tsuda S, Tabata M (1982) in: Fujiwara A (ed) Plant Tissue Culture Tokyo, p 313

Hinz H, Zenk MH (1981) Naturwissenschaften 68: 620-621

Ikram M (1975) Planta Med 28: 353-358

Kondo Y (1976) Heterocycles 4: 197-219

Kumazawa Y, Hagaki A, Fukumoto M, Fujisawa H, Nishimura C, Nomoto K (1984) Int J Immunopharmac 6: 587-592

Murashige T, Skoog F (1962) Physiol Plant 15: 473-497

Rothenberger S (1982) Untersuchungen zur Bildung von Protoberberin-Alkaloiden in Zellkulturen von Berberis wilsonae Hemsl. & Wils. Doctoral Thesis, Universität Tübingen

Sato F, Endo T, Mashimoto T, Yamada Y (1982) in: Fujiwara E (ed) Plant Tissue Culture Tokyo, p 319

Smart NJ, Fowler MW (1984) J exp Botany 35: 531

Spieler H, Alfermann AW, Reinhard E (1985) Appl Microbiol Biotechnol, in press

Tanaka H (1982) Biotechnol Bioengin 24: 425-442

Ulbrich B, Wiesner W, Arens H (1985) in: Neumann KH, Barz W, Reinhard E, Bender L, Hüsemann W, Alfermann AW (eds) Primary and secondary metabolism in Plant Cell Cultures Springer Berlin Heidelberg New York, in press

Wagner F, Vogelmann H (1977) in: Barz W, Reinhard E, Zenk MH (eds) Plant Tissue Culture and its Bio-technological Application Springer Berlin Heidelberg New York, pp 245-252

Wahl J (1977) Fermentation von pflanzlichen Zellkulturen und 12ß-Hydroxylierung von ß-Methyldigitoxin durch Zellkulturen von Digitalis lanata. Doctoral Thesis, Universität Tübingen

Yamada Y, Sato F (1981) Phytochem 20: 545-547