Plant Cell Reports (1989) 8:255–258

Plant Cell Reports

Effect of oxygen supply on berberine production in cell suspension cultures and immobilized cells of *Thalictrum minus*

Y. Kobayashi, H. Fukui, and M. Tabata

Faculty of Pharamceutical Sciences, Kyoto University, Kyoto 606, Japan

Received February 6, 1989/Revised version received May 22, 1989 – Communicated by F. Constabel

Abstract

The ample supply of O_2 proved to be of great importance for berberine production in cell suspension culture of Thalictrum minus, as the specific O_2 consumption rate of berberine-producing cells was twice as high as that of non-producing cells. Furthermore, berberine yield increased with increases in the volumetric O_2 transfer coefficient (K_La). Estimation of the optimum conditions of oxygen supply in suspension cultures and immobilized cells according to a known theoretical model assuming O_2 uptake by cells to be a zero-order reaction was in good agreement with the experimental data. The O_2 supply to immobilized cells could be improved by reducing the cell density and radius of the bead.

Introduction

The application of immobilized plant cells for the production of useful compounds has attracted increasing attention in recent years (Brodelius 1985). To improve aeration conditions for berberine production by immobilized T. minus cells, we devised a liquid-gas two phase bioreactor (Kobayashi et al. 1988). However, active proliferation of cells entrapped in a Ca-alginate gel bead was observed only in the peripheral layer of the bead, as reported for immobilized microbial(Wada et al. 1980, Chibata et al. 1983), animal(Hashimoto et al. 1988) and plant cells (Nakajima et al. 1985). Such a phenomenon could be due to diffusional limitation in a gel bead (Dalili et al. 1987). Cells deep in the gel are susceptible to a lack of O_2 because of the low saturation concentration of O_2 in the medium and the high respiration rate of cells, as shown in mycelial pellets of Aspergillus niger (Yano et al. 1963). In the present study, the effect of O_2 on berberine production in T. minus cells was examined to determine the optimum conditions for cell immobilization.

Material and methods

Cell suspension culture

A cell line of T. minus L. var. hypoleucum Miq. (Nakagawa et al. 1984) has been maintained as a suspension culture in "growth medium" i.e. Linsmaier-Skoog (LS) medium containing 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) by subculturing every two weeks.

For induction of berberine production, 17-day-old cells (1 g fresh wt. = 75 mg dry wt.) of the stock culture were transferred to 30 ml of "production medium" i.e. LS medium containing 100 μM 1-naphthaleneacetic acid (NAA) and 10 μM benzyladenine (BA), in a 100 ml Erlenmeyer flask with three replicates. The cultures were agitated on a reciprocal shaker at a speed of 100 strokes/min at 25°C in the dark.

Culture of immobilized cells

T. minus cells (17-day-old) were entrapped in Ca-alginate beads according to the method of Kierstan and Bucke (1977) with minor modifications. Cells (10 g fresh wt.) suspended in 2 % alginate (25 ml) were dripped from a wide-mouthed pipette (3 mm in diameter) into a 50 mM $CaCl_2$ solution; the Ca-alginate beads (ca 5 mm in diameter) formed were left in the solution for 3 hr at 25°C in the dark, then washed with 30 ml of LS basal medium.

Beads containing a total of 1 g fresh cells were inoculated into 30 ml of the production medium in a 100 ml Erlenmeyer flask and incubated under the same conditions as mentioned above.

Quantitative analysis of berberine

The quantitative analysis of berberine was carried out by HPLC as described elsewhere (Nakagawa et al., 1984) using SEP-PAK C_{18} instead of Amberlite XAD-2 for the separation column.

Estimation of uptake rate of O_2 and macronutrients

The uptake rate of O_2 by cultured cells was estimated by measuring the decrease of O_2 concentration in the air-saturated nutrient medium using DO electrode at 25°C. Quantitative analysis of sucrose, PO_4^{3-}, NO_3^-, and NH_4^+ in the medium were carried out by the methods of Dubois (1956), Takahashi (1955), Japanese Industrial Standard(1966), and Iwaeda and Ohsawa (1974), respectively.

Determination of volumetric O_2 transfer coefficient

The O_2 transfer coefficient (K_La) was determined as follows according to the method used by Fujita (1985). After reducing the dissolved O_2 concentration (DO) in an aqueous solution of 1 mM $CuSO_4$ to 0-1 ppm by adding 10% Na_2SO_3 solution, air was supplied to the solution at a fixed rate on a reciprocal shaker at 25°C. The value of K_La was calculated from the increasing DO by the formula:

$$\ln(C_s - C) = -K_La\,t + \ln(C_s - C_o)$$

where C_s is the saturated DO (ppm) in water at 25°C (8.1 ppm), C is the DO (ppm) in the solution at t (min), and C_o is the DO (ppm) at t = 0.

Offprint requests to: Y. Kobayashi

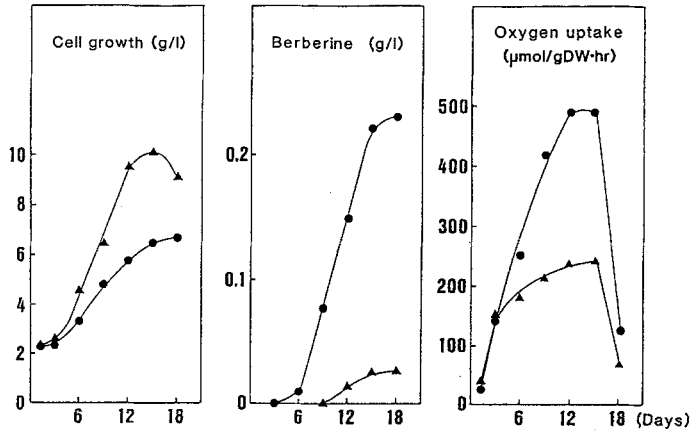

Fig. 1. Cell growth, berberine production, and O_2 uptake rate of T. minus cell suspension cultures in growth medium (—▲—) and those in production medium (—●—).

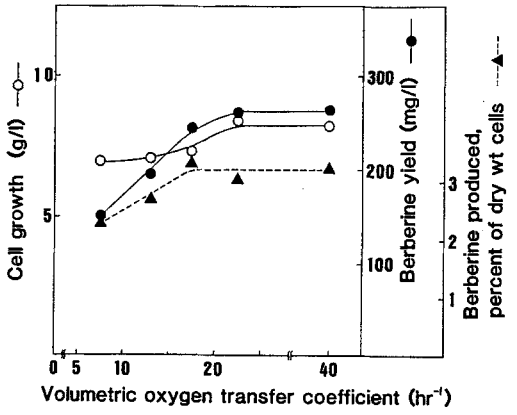

Fig. 3. Effects of volumetric O_2 transfer coefficient (K_La) on cell growth and berberine production of T. minus cell suspension cultures in production medium (culture period : 15 days).

Results and Discussion

Effect of O_2 supply on berberine production

T. minus cell suspension cultures in growth medium and those in production medium showed marked differences in cell growth, berberine production, and O_2 uptake (Fig. 1). In growth medium, cells grew faster, but produced little berberine. By contrast, cells in production medium produced a large amount of berberine, which was mostly secreted into the medium, in spite of their inferior growth. Interestingly, the specific O_2 uptake rate in production medium increased linearly with time during the period of berberine production which lasted from day 3 to 12, while that in growth medium showed only a slight increase after day 3. These results suggest an important role of O_2 in berberine production. The relationship between the O_2 supply and berberine production in production medium was studied by varying the value of the volumetric O_2 transfer coefficient (K_La) which is dependent on the volume of medium in a flask (Fujita, 1985), as shown in Fig. 2. The inoculum size was adjusted according to the medium volume to keep the starting cell density constant. Fig. 3 shows the effect of K_La value on cell growth and berberine production. Under standard conditions (30 ml medium in 100 ml Erlenmeyer flask), the K_La value is 17.5 hr^{-1}. Although increasing K_La above 20 hr^{-1} affected neither cell growth nor berberine production, decreasing K_La caused a significant reduction of the latter without markedly affecting the former.

The specific O_2 uptake rate is considered a zero-order reaction, as the O_2 uptake rate was not affected by the DO in medium and DO was reduced linearly (Fig. 4). Thus, the value of C_m can be calculated from the following equation (Wise, 1951):

$$K_La(C_s - C_m) = Q\,d \qquad (1)$$

where C_s is the saturated DO in water at 25°C (253 μmol/l), C_m is the DO (μmol/l) in medium at an equilibrium state between O_2 supply and O_2 consumption, Q is the specific O_2 uptake rate (μmol/gDW•hr), and d is the cell density in medium (gDW/l).

Fig.5 shows the relation between K_La and C_m after 15 days of culture calculated from equation (1) and data (K_La and d) in Fig.3 by setting Q at 500 and 250 μmol/gDW•hr, which were the maximum O_2 uptake rate for berberine production and cell growth, respectively (Fig.1). When K_La is smaller than 15, the calculated C_m value at Q = 500 becomes lower than 0, which means that the O_2 supply limits berberine production. However, the calculated C_m values at Q = 250 are higher than 0 at any K_La values between 7.5 to 40, indicating that the O_2 supply is sufficient for cell growth. These estimations are in good agreement with the data presented in Fig. 3 and support the conclusion that a lack of O_2 supply would affect berberine production more seriously than cell growth. Thus, from the equation (1), moderate conditions for O_2 supply could be obtained.

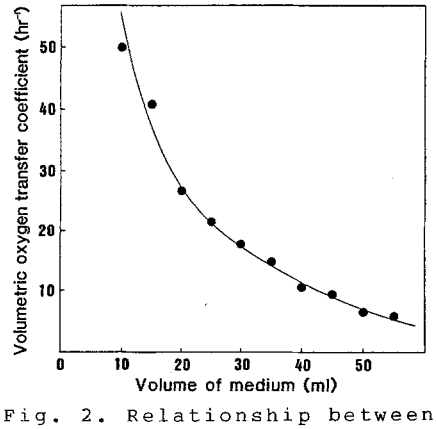

Fig. 2. Relationship between volumetric O_2 transfer coefficient (K_La) and medium volume in 100 ml Erlenmeyer flask.

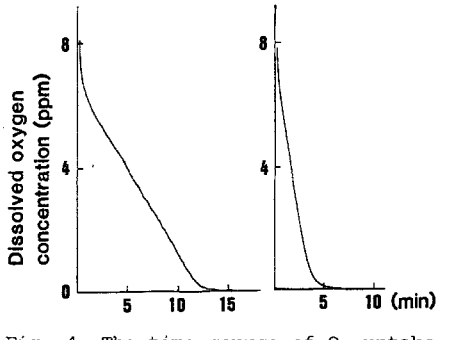

Fig. 4. The time-course of O_2 uptake from air-saturated production medium by T. minus cell cultures 6 days (left) and 15 days (right) after inoculation (medium volume: 30 ml).

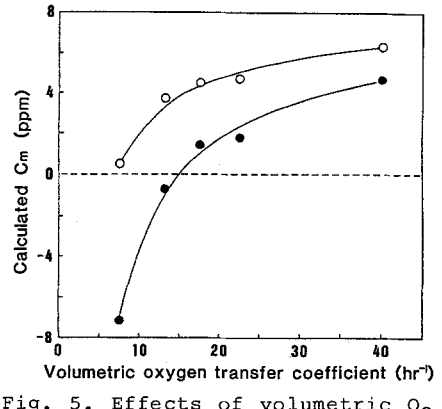

Fig. 5. Effects of volumetric O_2 transfer coefficient (K_La) on DO concentration in production medium when the specific O_2 uptake rate was 500 μmol/gDW•hr (—●—), and 250 μmol/gDW•hr (—○—) after 15 days of culture.

Limiting factor of mass transfer within gel beads

As we have reported earlier (Kobayashi et al. 1988), the berberine productivity of immobilized cells of T. minus could be improved by periodic exposure of the alginate gel beads to air in a special bioreactor. Nevertheless, the O_2 supply to the entrapped cells inside the gel seemed to be insufficient not only for berberine production but also for cell growth, since cell proliferation was observed only near the surface of gel beads, whereas the cells at the center of the beads were almost dead. Such serious limitation of O_2 transfer has also been reported for immobilized animal cells (Hashimoto et al. 1988) and mycelial pellets (Yano et al. 1963). This is probably due to a low saturation concentration of O_2 in water (8.1 ppm at 25°C) in addition to a high O_2 uptake of the cells, which would consume all the dissolved O_2 in a few minutes if the O_2 supply were stopped (Fig. 4).

To determine the limiting factor in the beads, the "effectiveness factors" for the uptake of O_2 and some macronutrients (sucrose, phosphate, nitrate, and ammonium) were examined. The effectiveness factor, which indicates the degree of substrate supply, is expressed as the ratio of the rate of reaction in the presence of diffusional barriers to the rate of that if all the catalyst were exposed to the same concentration of substrates in medium. In this experiment, we estimated the effectiveness factor by dividing the uptake rate of the total immobilized cells by that of freely suspended cells, when the cells of both systems were at the end of the lag phase (3 days after inoculation). At this stage, cells were not yet dividing, being distributed uniformly within the beads. The effectiveness factors obtained from this experiment are shown in Table 1. The data clearly show that the O_2 supply whose effectiveness factor was as small as 0.39 is the only limiting factor among the substrates examined.

Table 1. Effectiveness factors for the uptake of oxygen and some macronutrients by immobilized Thalictrum cell cultures after 3 days of culture

Substrate	Initial concentration (mM)	Amount of uptake: Free cells (mM/3d)	Amount of uptake: Immobilized cells* (mM/3d)	Effectiveness factors
Sucrose	86.7	9.28	9.19	0.99
Phosphate	1.25	0.30	0.30	1.00
Nitrate	39.4	1.46	2.17	1.49
Ammonium	20.6	0.78	1.01	1.29
Oxygen	253*2	507*3	199*3	0.39

* As the controls for substrate uptake by immobilized cells, Ca-alginate beads without cells were incubated under the same conditions.
*2 μmol/l
*3 μmol/l•hr

Theoretical estimation of the optimum immobilization conditions

It is expected that the O_2 supply to the cells within a gel bead would be improved by manipulating the particle size and the cell density. The optimal values for these factors may be estimated by theoretical analysis of O_2 diffusion in a particle using equation (2) (Yano et al. 1961), which expresses a steady-state one-dimensional mass balance, on the following assumptions: 1) the cell density within a spherical particle is uniform, 2) the rate of molecular diffusion is constant throughout the particle, 3) the specific O_2 uptake rate is expressed by a zero-order reaction.

$$D\left(\frac{d^2C}{dr^2} + \frac{2dC}{dr}\right) - \rho Q = 0 \qquad (2)$$

r: the arbitrary radius (cm) of a gel particle,
D: the diffusion coefficient of O_2 (cm²/sec) in the gel particle, which is estimated as 90 % of the O_2 diffusion coefficient in water (1.8×10^{-5} cm/sec) according to Kurosawa et al. (1988),
C: the DO (μmol/cm³) at r,
Q: the specific O_2 uptake rate of cells (μmol/gDW•sec),
ρ: the cell density (gDW/cm³) within the gel particle.

Whether or not the O_2 supply is sufficient may be estimated by simple equations (Yano at al. 1963) derived from equation (2):

$$\frac{6C_mD}{R^2\rho Q} \geq 1 \quad : \quad O_2 \text{ supply is sufficient}$$
$$\frac{6C_mD}{R^2\rho Q} < 1 \quad : \quad O_2 \text{ supply is insufficient} \qquad (3)$$

R: the radius (cm) of the gel particle,
C_m: the DO (μmol/cm³) at R = r, which is considered to be equal to the DO concentration in medium.

In order to improve the O_2 supply within a gel particle, R and ρ should be smaller. In the experiment, smaller beads (R = 1 mm) were prepared by dripping a 1.5 % alginate solution containing T. minus cells from a syringe into a $CaCl_2$ solution instead of the wide-mouthed pipette and 2 % alginate solution used for the preparation of ordinary beads (R = 2.5 mm). Supposing that the cell mass would double to 5 gDW/l and that the specific O_2 uptake (Q) must be set for 250 to 500 μmol/gDW•hr, it would be theoretically necessary for enough O_2 supply to adjust the initial cell density (ρ_o) in the beads (R = 1 mm) to 3.8 - 12.5 mgDW/cm³, which is calculated from equation (3), using the following values:

C_m = 0.18 to 0.11 μmol/cm³ calculated from equation (1) using C_s = 253 μmol/cm³, K_La = 17.5 hr^{-1}, and d = 5 gDW/l;
D = $0.9 \times 1.8 \times 10^{-5}$ cm²/sec;
R = 0.1 cm;
ρ = $2\rho_o$, where ρ_o is the initial cell density (mgDW/cm³);
Q = 0.7×10^{-4} to 1.4×10^{-4} μmol/mgDW•sec.

Fig. 6 shows the effect of the initial cell density (2.9 to 22.4 mgDW/cm³) on the growth and berberine production of cells in 100 ml-flasks containing 30 ml of production medium. Berberine

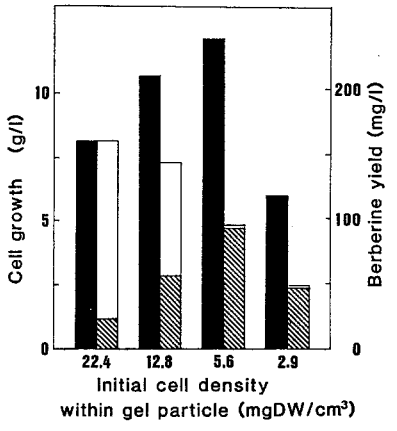

Fig. 6. Effects of initial cell density within Ca-alginate beads on cell growth and berberine production after 18 days of culture in production medium. ■: berberine yield, ▨: cells in beads, and □: leaked cells.

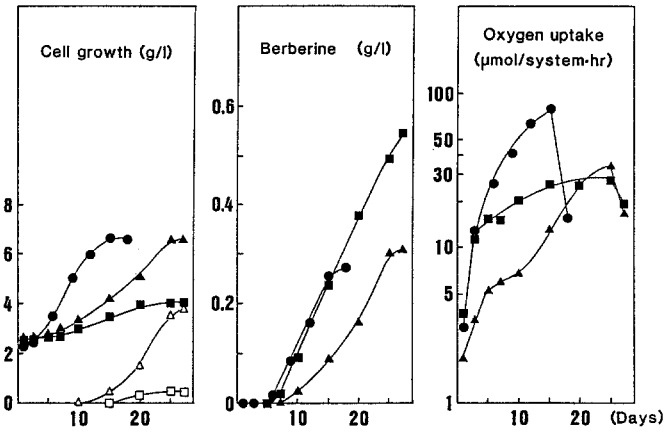

Fig. 7. Time-course of cell growth, berberine production, and O_2 uptake in different culture systems; (A) suspension culture(—●—), (B) immobilized cells before (—▲—) and (C) after (—■—) the improvement of immobilization conditions. —Δ—, —□— = leaked cells in system (B) and (C), respectively.

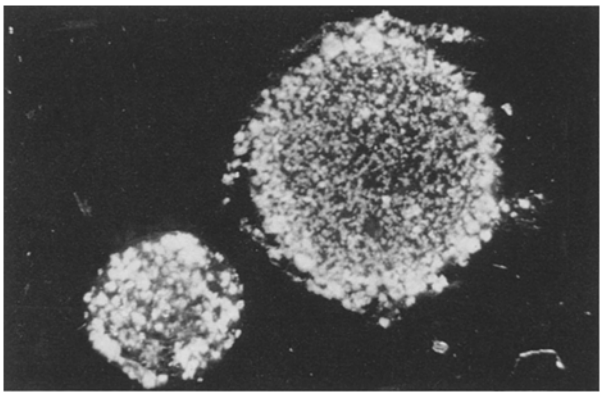

Fig. 8. Cross sections of Ca-alginate gel beads showing the distribution and proliferation of T. minus cells in large (R = 2.5 mm, ρ_o = 22.4 mgDW/cm³) and small (R = 1 mm, ρ_o = 5.9 mgDW/cm³) beads.

yield increased by 1.5 times and specific productivity increased by 2.5 times as the initial cell density (ρ_o) was decreased from 22.4 to 5.6 mgDW/cm³. These experimental results agree well with the theoretical expectation. Furthermore, the leakage of cells from the beads could be prevented nearly completely by lowering the initial cell density, presumably owing to the slow and uniform proliferation of cells within the beads. When initial cell density was decreased to 2.9 mgDW/cm³, cell growth was suppressed considerably in spite of a sufficient O_2 supply. This would be partly due to limited cell-to-cell contact within the beads at low cell density.

These results indicate that the moderate combination of beads radius (R) and initial cell density within beads (ρ_o) for berberine production by immobilized T. minus cells could be determined theoretically by the estimation of oxygen demand by cells for berberine production.

Performance of immobilized cells under improved conditions

Comparisons were made between the following culture systems in flasks with regard to cell growth, berberine production, and O_2 uptake: (A)free cells (inoculum size: 2.5 mgDW/cm³); (B)immobilized cells (R = 2.5 mm, ρ_o = 22.4 mgDW/cm³); and (C)immobilized cells (R = 1 mm, ρ_o = 5.9 mgDW/cm³). The results are shown in Fig. 7. As might have been expected, the O_2 uptake rate of system C was much higher than that of B during the culture period from day 1 to 15 and the cells were uniformly distributed in the beads (Fig. 8), indicating a distinct improvement in O_2 supply. A sudden increase of the O_2 uptake by B after 10 days of culture was apparently due to the rapid growth and respiration of cells that had leaked out of the beads.

As for the berberine production, system C not only showed a pattern similar to that of A, but maintained a high production rate (25 mg/l•day) twice as long as did A. Since cell proliferation is greatly limited in the beads, the specific production rate of immobilized cells in system C was twice as high as that of free cells in system A.

Thus, immobilized cell system of T. minus capable of secreting berberine can be more productive than the cell suspension system, if the cells are immobilized in beads under appropriate conditions especially with respect to the O_2 supply.

Acknowledgment

We wish to thank Dr.Yoshihito Shirai, Department of Industrial Chemistry, Kyoto University, for helpful discussions.

References

Brodelius P (1985) Trends in Biotechnology 3: 280-285
Chibata I, Tosa T, Fujimura M (1983) Ann. Rep. Ferment. Process 6: 1-22
Dalili M, Chau PC (1987) Appl. Microbiol. Biotechnol. 26: 500-506
Dubois M (1956) Anal. Chem. 28: 350
Fujita Y (1985) 'Production of shikonin derivatives by cell suspension culture of Lithospermum erythrorhizon' Doctoral Thesis, Kyoto University
Hashimoto K, Shirai Y, Kawahara H, Yamachi H (1988) Advances in Soc. Chem. Eng., Series No.17: 133-137 (In Japanese)
Iwaeda K, Ohsawa K (1974) 'Colorimetrical analysis of ammonium' In: Applied Colorimetrical Analysis in Inorganic Chemistry, Vol. 3 (Hirano Y et al. ed) Kyoritsu Shuppan, Tokyo, pp 419-420 (In Japanese)
Japanese Industrial Standard K-1306 (1966)
Kierstan M, Bucke C (1977) Biotech. Bioeng. 19: 387-397
Kobayashi Y, Fukui H, Tabata M (1987) Plant Cell Rep. 6: 185-186
Kobayashi Y, Fukui H, Tabata M (1988) Plant Cell Rep. 7: 249- 253
Kurosawa J, Matumura M, Tanaka H (1988) Abstract of 53th Ann Meeting of Soc. Chem. Eng., Sendai, Japan pp 29 (In Japanese)
Nakagawa K, Konagai A, Fukui H, Tabata M (1984) Plant Cell Rep. 3: 254-257
Nakajima H, Sonomoto K, Usui N, Sato F, Yamada Y, Tanaka A, Fukui S (1985) J. Biotech. 2: 107-117
Takahashi Y (1955) Seikagaku (Biochemistry) 26: 690 (In Japanese)
Wada MJ, Kata J, Chibata (1980) Eur. J. Appl. Microbiol. Biotech. 10: 275-287
Wize WS (1951) J. gen. Microbiol. 5: 167-177
Yano T, Kodama T, and Yamada K (1961) Agr. Biol. Chem. 25(7): 580584
Yano T, Yamada K (1963) In: 'Biochemical Engineering', Symposia Res. Inst. Appl. Microbiol. University of Tokyo, No. 5, University of Tokyo Press, pp 16-36 (In Japanese)