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Oxygen uptake rate of immobilized growing hybridoma cells

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Summary. The specific oxygen uptake rate of hybridoma cells immobilized in calcium alginate gel particles was measured, and the observed data was compared with those of non-immobilized cells. The uptake rate of the immobilized cells coincided with that of the non-immobilized hybridoma cells just after immobilization, but increased with cell growth. On the other hand, the cellular glucose consumption rate decreased slightly during the experiments. The increased oxygen uptake rate by immobilized cells was closely related to the formation of cell colonies in the gel particles.

Introduction

In industry, cultivation of animal cells at high concentrations is greatly advantageous, and perfusion cultures are widely used. In this method it is necessary to remove a part of medium and to supply fresh medium simultaneously, while maintaining the cells in a fermentor, in order to dilute the waste metabolites accumulated. Such cell maintenance can be more easily achieved by immobilizing the cells in non-noxious gel particles. Several studies (Posillico 1986; Nilsson et al. 1983; van Brunt 1986; Shirai et al. 1987) have been reported on cultivation of immobilized animal cells at high concentrations of over 1×10^7 cells/g gel.

In immobilized animal cell cultures, oxygen dissolved in the medium, the saturated concentration of which is very low (7 ppm), is transferred to gel particles only by intraparticle diffusion. Thus, it is necessary to evaluate oxygen uptake rates of immobilized animal cells for the rational design of fermentors using such cells. Gosmann and Rehm (1986) previously reported the oxygen uptake rate of various kinds of microbial cells immobilized in calcium alginate gel particles. No studies, however, have been published on the oxygen uptake rate of immobilized animal cells. In this study, the oxygen uptake rate of hybridoma cells immobilized in calcium alginate gel particles was measured using a dissolved oxygen (D.O.) electrode, and the experimental data was compared with those obtained with non-immobilized hybridoma cells.

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Materials and methods

Materials. Cells of the mouse-human hybridoma, 4H11, and the mouse-mouse hybridoma, 4C10B6, were used. The former produced a human IgA monoclonal antibody, whereas the latter produced a mouse IgG monoclonal antibody. Shirai et al. (1987) obtained the monoclonal antibody from 4H11 cells which were immobilized in calcium alginate and were suspended in an expanded bed fermentor. A serum-free RDF medium, a mixture of RPMI 1640, DME and F12 media, was used. Its detailed composition was reported in the previous paper (Shirai et al. 1987). Bovine serum albumin was not added to the medium for cultivation of the 4C10B6 cells.

Cultivation of immobilized cells. The 4H11 and 4C10B6 cells were entrapped in calcium alginate gels for immobilized culture by the method described previously (Shirai et al. 1987). Gel particles 3 mm in diameter for the 4H11 cells, and 2 mm in diameter for the 4C10B6 cells were obtained. Gel particles with entrapped cells (4 g) were suspended in 15 cm^3 of medium in a Petri dish 9 cm in diameter, and were incubated at 37° C in a 95% air/5% CO_2 atmosphere. The medium was replenished once a day for both the cells.

The 4H11 cells immobilized in calcium alginate gel particles of 2 mm in diameter were incubated in a fermentor (Fig. 1) under adjusted conditions. Glucose and oxygen concentrations in the medium were monitored by the glucostat method (Toren 1967) and with a D.O. electrode (Oriental Denki, Niizashi Japan, 10AN), respectively. The concentrations were controlled at 5 mM glucose and 4 ppm oxygen in the me-

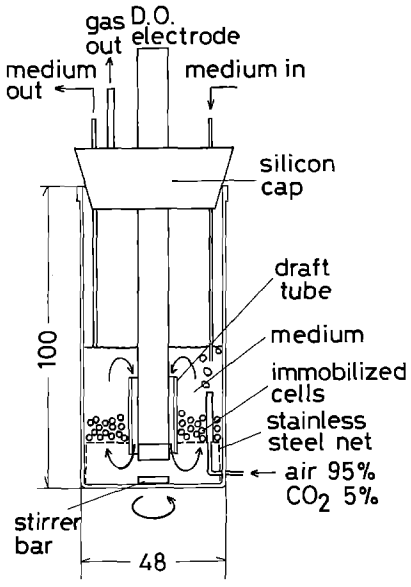

Fig. 1. A fermentor for cultivation of the immobilized cells. The medium is mixed with a magnetic stirrer and circulated around a draft tube without gel destruction

dium by adjusting the feeding rates of the fresh medium and the gas. It was confirmed that the cells could proliferate in a logarithmic growth phase at these concentrations.

Measurement of oxygen uptake rate. The specific oxygen uptake rates of non-immobilized 4H11 and 4C10B6 cells were measured using a D.O. electrode in a glass vessel (Fig. 2a). A glass bar was used as an agitator instead of a Teflon bar because oxygen is adsorbed by the latter. The vessel always contained 5.5 cm^3 of cell suspension. Cells were harvested in various known stages of the growth phase. Cell concentrations were adjusted by adding fresh medium to within $1\text{-}2 \times 10^6$ cells/cm after removal of the old medium by centrifugation. Each cell suspension was then loaded into the vessel, which was immediately put in a water bath at 37° C. Changes in oxygen concentration in the vessel were then measured, to monitor the oxygen uptake rates of the non-immobilized cells at different cultivation stages.

Figure 2b shows the vessel used for measurement of the oxygen uptake rate of immobilized hybridoma cells. The vessel had a draft tube, around which the medium was circulated, and the stirrer bar was screened by a stainless steel net in order to prevent gel destruction. The vessel contained 12.3 cm^3 of medium and immobilized cells. The immobilized gel particles were weighed after their surfaces had been wiped with adsorbent paper. Oxygen uptake by the immobilized cells was measured by the same method as that described for non-immobilized cells. Cell numbers were counted twice before and after measurement of the oxygen uptake rate, as described previously (Shirai et al. 1987), and viability was determined by trypan blue dye exclusion.

Determination of glucose consumption and lactate production rates. The 4H11 cells immobilized in calcium alginate gel particles 3 mm in diameter were cultivated in a Petri dish, 6 cm in diameter, in order to determine the mean glucose consumption and lactate production rates per cell. Gel particles (2.3 g) were added to the Petri dish, and then 7 cm^3 of medium was added, and replenished twice a day. Since the cell concentration in the gel particles was measured each time the medium was replenished during the logarithmic growth phase, the change of cell concentrations between successive measurement points was small. Therefore, the mean glucose consumption and lactate production rates per cell was calculated by dividing these changes per unit gel by the averaged cell concentration in the gel between the two points.

The concentration of glucose in the medium was measured by the glucostat method (Toren 1967). The concentration of lactate accumulated was determined from changes in the concentration of reduced nicotinamide adenine dinucleotide (NADH) in an enzymatic reaction.

Results and discussion

Oxygen uptake rate of non-immobilized hybridoma cells

The oxygen uptake rate of non-immobilized 4H11 cells was measured. The concentration of oxygen decreased linearly with time. The specific oxygen

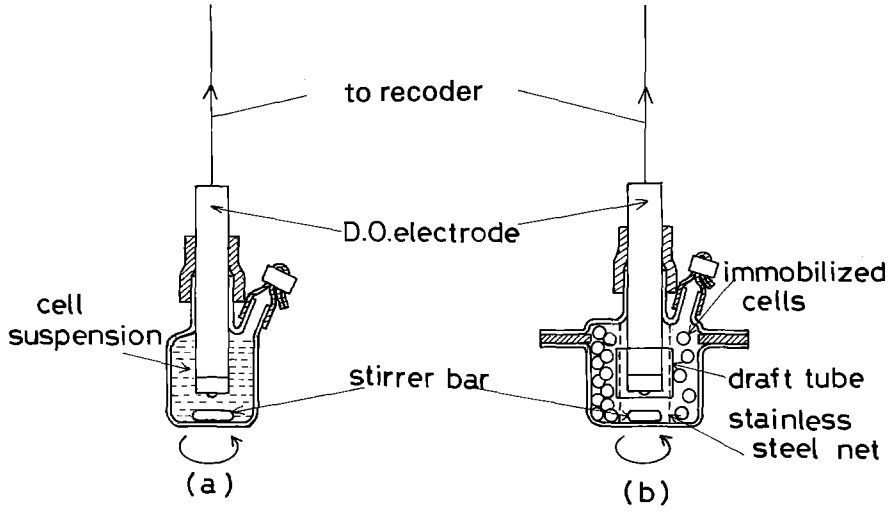

Fig. 2a, b. Vessels for measurement of the oxygen uptake rate. **(a)** Non-immobilized cells. **(b)** Immobilized hybridoma cells

uptake rate per cell was determined from the slope of the curve.

Figure 3a shows an increase in the number of 4H11 cells while Fig. 3b demonstrates oxygen uptake rate and the concentration changes in glucose and lactate. The 4H11 cells' specific oxygen uptake rate was almost constant regardless of the cell growth phase. The average value of the oxygen uptake rate measured was 3.44×10^{-13} mol/h per cell. In addition, the specific oxygen uptake rate of non-immobilized 4C10B6 cells was found in similar experiments to be 2.31×10^{-13} mol/h per cell.

Oxygen uptake rate of immobilized hybridoma cells

The oxygen uptake rates of both hybridoma cells 4H11 and 4C10B6, immobilized in calcium alginate gel, were measured. The ratios of specific oxygen uptake rates of the immobilized hybridoma cells to those of the non-immobilized cells are plotted on Fig. 4 against the cultivation time. Shortly after immobilization, the specific oxygen uptake rates of the cells were the same as those of the non-immobilized cells, but they increased with cultivation time. The growth curve of immobilized cells is also shown, but only for 4H11. Figure 4 demonstrates that the oxygen uptake rate increased with increasing cell concentration in the gel particles, but increased further even after the cell concentration was gradually decreasing.

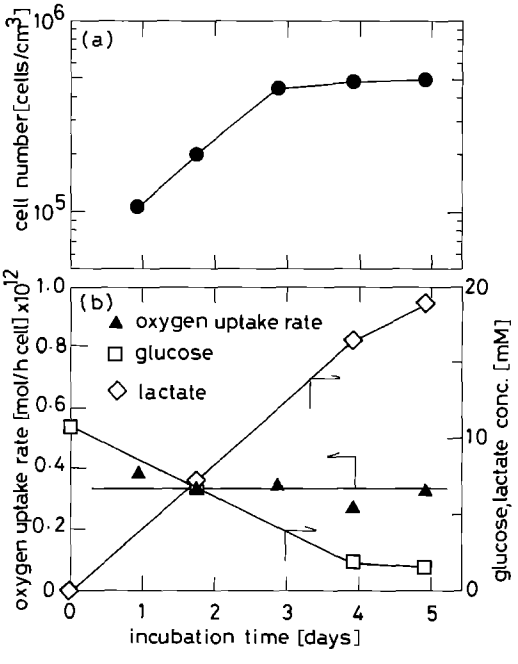

Fig. 3a, b. Oxygen uptake rate, glucose consumption and lactate accumulation during the growth of non-immobilized 4H11 cells. **(a)** Growth curve for 4H11 cells. **(b)** Oxygen uptake rates of 4H11 cells, and changes in concentration of glucose and lactate

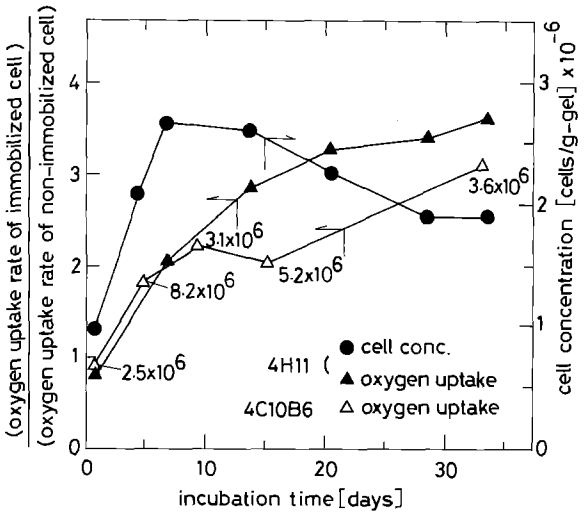

Fig. 4. Oxygen uptake rate of immobilized 4H11 and 4C10B6 cells. *Numbers* attached to *open triangles* represent cell concentrations at the time of immobilized 4C10B6 oxygen uptake measurement

With 4C10B6 cells the experiments were carried out using immobilized gel particles seeded with different cell concentrations. The numbers attached to each data point in Fig. 4 refer to the cell concentrations when their oxygen uptake rates were measured (cells/g gel). The oxygen uptake rate of immobilized 4C10B6 cells also increased with cultivation time. The data in Fig. 4 also demonstrates that the increase of the specific oxygen uptake rate of immobilized 4C10B6 cells was independent of their apparent concentration in the gel particles because no relationship was found between specific oxygen uptake rate and cell concentration.

Since the measured values of oxygen uptake rate of the immobilized hybridoma cells included the effect of intraparticle diffusion of oxygen, the intrinsic oxygen uptake rate might have been higher than the values shown in Fig. 4. The specific oxygen uptake rate of each immobilized cell type increased at least 3–3.6-fold above initial levels.

Gosmann and Rehm (1986), however, found that the oxygen uptake rate of microbial cells grown in Ca-alginate gel particles decreased with cell concentration, and other researchers (Sato and Toda 1983; Kobayashi et al. 1973) have found that the specific oxygen uptake by immo-

bilized growing microorganisms or mycelial pellets did not increase with either cultivation time or pellet density. On the other hand, Frame and Hu (1985) observed an increase in oxygen uptake by animal cells cultured on microcarriers when glucose in the medium was depleted. Controls of metabolism in animal cells may be quite different from those of microorganisms.

Relationship between increase in the oxygen uptake rate of immobilized hybridoma cells and the formation of cell colonies in gel particles

The 4H11 cells immobilized in Ca-alginate gel particles were incubated in the fermentor shown in Fig. 1. Concentrations of the medium components including oxygen were adjusted to be constant, and the medium was well mixed. The cells were seeded at a concentration sufficient for all the cells in the gel to survive, even at the harvest for the measurement of the oxygen uptake rate. Therefore, the cell concentration in the gel particle was not very high at the time of the measurement. It was confirmed that the cell viability in the gel particles did not decrease during cultivation. Figure 5 shows the relationship between the cell concentration in the gel and the oxygen uptake rate. In this figure, Λ and Ψ denote the ratio of the cell concentration in the gel at the time of measurement of oxygen uptake rate to that at the initial stage, and the ratio of the specific oxygen uptake rate measured to that of the non-immobilized 4H11 cells, respectively. A linear correlation was found between them. It was found from this figure that the cell numbers composing a colony in a gel particle affected the specific oxygen uptake rate.

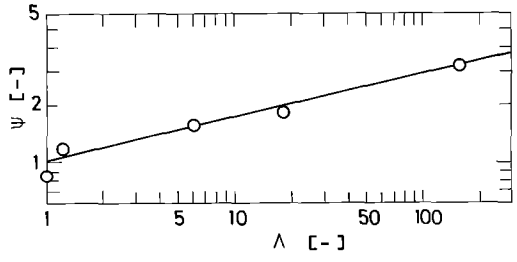

Fig. 5. Effect of cell numbers composing a colony in a gel particle on the specific oxygen uptake rate.
$\Lambda = X/X_0$, $\Psi = r_{O_2}/r_{O_2N}$
X, cell concentration in a gel particle at the time of the measurement; X_0, cell concentration at the beginning of the experiment; r_{O_2}, specific oxygen uptake rate of an immobilized cell; r_{O_2N}, specific oxygen uptake rate of a non-immobilized cell

Table 1. Oxygen uptake rate of immobilized 4H11 cells and cell concentration in the gel particles

Cell concentration (cells/g gel)	Oxygen uptake rate (mol/h·cell)
8.8×10^5	2.67×10^{-13}
1.0×10^6	2.91×10^{-13}
1.7×10^6	4.09×10^{-13}
5.8×10^6	4.52×10^{-13}
1.1×10^7	3.60×10^{-13}
2.3×10^7	2.14×10^{-13}
	Av. 3.32×10^{-13}
Non-immobilized	3.44×10^{-13}

As shown in Fig. 4, however, the oxygen uptake rate increased independently of the apparent cell concentration in the gel particles. The cells near the surface of the gel particles were able to grow remarkably well because they were in contact with oxygen-rich medium. However, dead cells accumulated from the centre outwards due to lack of oxygen caused by the growth of those cells nearer the surface. The cell concentration near the surface, therefore, increased locally, although the cell concentration in the gel particles as a whole decreased gradually (Fig. 4). Therefore, the increase in oxygen uptake rate was correlated with a locally high cell concentration in the gel where cells were alive.

The oxygen uptake rate of 4H11 cells at various concentrations in the gel particles was measured one day after immobilization in order to clarify whether the increased oxygen consumption rate was related to the cell concentration. As shown in Table 1, the specific oxygen uptake rate of immobilized 4H11 cells at a concentration of 2.9×10^7 cells/g gel was almost the same as that of non-immobilized cells, suggesting that this the enhancement of oxygen uptake rate is not linked to the cell concentration.

The hybridoma cells immobilized in gel particles were partially concentrated into colonies which seemed to be compressed by the gel matrix. The colonies expanded as the cells grew. After measurement, immobilized 4C10B6 cells cultured for 16 and 33 days in a Petri dish were removed from the gel particles by dissolving them, and cultivated under suspension conditions. Oxygen uptake rates of these cells were measured and Table 2 shows the results. In each case, the oxygen uptake rate decreased sharply.

The results shown in Fig. 5 and Table 2 suggest that the increased oxygen uptake rate is correlated with the number of cells in a colony and

Table 2. Change in oxygen uptake rate between the 4C10B6 cells immobilized and non-immobilized after dissolution of the immobilized gel particles

Cultivation time (days)	Oxygen uptake rate (mol/h·cell)		
	Immobilized cells	Just after dissolution	2 days after dissolution
16	3.89×10^{-13}	2.08×10^{-13}	2.47×10^{-13}
33	7.20×10^{-13}	3.53×10^{-13}	—

an increase in the number of cells adhering together, or at least in very close contact with one another.

Glucose consumption and lactate accumulation by immobilized hybridoma cells

The oxidation of glucose under anaerobic and aerobic conditions are represented by Eqs. (1) and (2), respectively.

$$C_6H_{12}O_6 + 2P_i + 2ADP \longrightarrow 2CH_3CH(OH)COOH + 2ATP + 2H_2O \quad \text{(glycolytic pathway)} \tag{1}$$

$$C_6H_{12}O_6 + 6O_2 + 36P_i + 36ADP \longrightarrow 6CO_2 + 36ATP + 42H_2O \quad \text{(through TCA cycle)} \tag{2}$$

The selectivity of lactate can be calculated stoichiometrically from the above equations of multiple reactions when the glucose consumption and oxygen uptake rates are known.

The 4H11 cells immobilized in Ca-alginate gel particles were cultivated in a Petri dish to determine the glucose consumption and lactate production rates. Figure 6 shows changes in the selectivity of lactate and the glucose consumption rate by cells during the cultivation period. The glucose consumption rate in the logarithmic growth phase were slightly higher than that in the rest of cultivation period, and the values were regarded as constant in each period. The selectivity might be equal to unity when glucose is oxidized completely under anaerobic conditions.

In Fig. 6 the selectivity of lactate tends to decrease. It was found stoichiometrically that the decrease in the selectivity indicates enhancement of glucose oxidation under aerobic conditions. In order to obtain the calculated points of lactate selectivity in Fig. 6, the values of oxygen uptake rate in Fig. 4 were utilized, while the averaged values of glucose consumption rates in the logarithmic

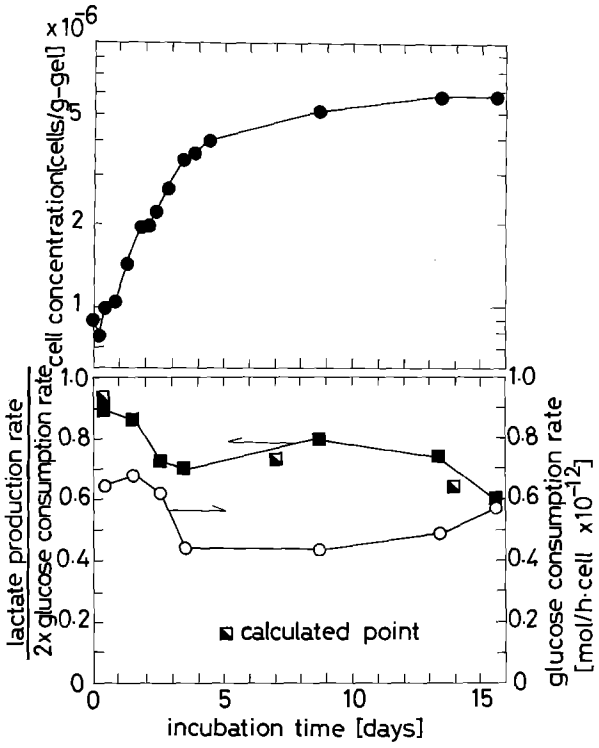

Fig. 6. Changes of selectivity of lactate and glucose consumption rate by the immobilized 4H11 cells. The *upper* graph indicates a change of the cell concentration in the gel particles during the same period

growth phase and remaining cultivation period shown in Fig. 6 were used. Both the selectivities which were obtained experimentally and from calculations agree well, indicating that the increase in oxygen uptake rate of the immobilized cells during cultivation is also supported stoichiometrically.

In our experiments, the specific oxygen uptake rate increased, whereas the specific glucose consumption rate did not change so much. These experimental facts and the stoichiometric relationship represented by Eqs. (1) and (2) indicate that the ATP-producing reaction represented by Eq. (2) was enhanced.

Although the ATP-generation rate of the 4H11 cells immobilized in Ca-alginate gel particles must have been enhanced, an increase in the cell proliferation rate was not observed. Much energy would be needed for cell maintenance when the cells were cultivated under a compressed condition such as in immobilized gel particles.

It may be concluded that the specific oxygen uptake rate of immobilized hybridoma cells increased with cultivation time, a phenomenon that could be correlated with the formation of cell col-

onies in the gel particles. On the other hand, the specific glucose consumption rate of the cells slightly decreased during cultivation.

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