University of Groningen

Diffusion of Oxygen in Alginate Gels Related to the Kinetics of Methanol Oxidation by Immobilized *Hansenula polymorpha* Cells

Hiemstra, Harry; Dijkhuizen, Lubbert; Harder, Willem

Published in:
European Journal of Applied Microbiology and Biotechnology

DOI:
[10.1007/bf00501507](https://doi.org/10.1007/bf00501507)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1983

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Hiemstra, H., Dijkhuizen, L., & Harder, W. (1983). Diffusion of Oxygen in Alginate Gels Related to the Kinetics of Methanol Oxidation by Immobilized *Hansenula polymorpha* Cells. *European Journal of Applied Microbiology and Biotechnology*, 18(4), 189-196. <https://doi.org/10.1007/bf00501507>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Diffusion of Oxygen in Alginate Gels Related to the Kinetics of Methanol Oxidation by Immobilized *Hansenula polymorpha* Cells

Harry Hiemstra, Lubbert Dijkhuizen, and Wim Harder

Department of Microbiology, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands

Summary. In the yeast *Hansenula polymorpha* an oxygen-requiring enzyme, alcohol oxidase, catalyzes the conversion of methanol into formaldehyde. After growth on methanol cells of the organism were harvested and entrapped in barium-alginate gels. The diffusion of oxygen towards these cells is seriously hindered by the polymer network. This caused significant changes in the kinetics of methanol oxidation by the immobilized cells as compared to that observed with cells free in suspension. The apparent $K_s(\text{O}_2)$ of the immobilized cells was dependent upon the density of the cells in the alginate beads and the bead radius. Using a well-known theoretical model, originally developed to describe the kinetics of oxygen diffusion in mold pellets, the diffusion coefficient for oxygen in barium-alginate gels was estimated. This coefficient was only 25% of that in water. The model also allowed an adequate simulation of the observed kinetics of methanol oxidation by the immobilized cells.

Introduction

Alginate gels have been widely used for the immobilization of animal, plant, and microbial cells, mainly because the method is considered to be mild, inexpensive and easy to perform. However, immobilization based on entrapping cells in a polymer matrix generally suffers from at least one major disadvantage, namely that of a reduced rate of diffusion of reactants towards the entrapped biocatalyst. This has been repeatedly recognized as a rate-limiting step in the conversion of substrate, thus lowering the efficiency of the immobilized biocatalyst (Cheetham et al. 1979; Klivanov and Huber 1981; Nilsson et al. 1980; Ohlson et al. 1979; Tramper et al. 1983). Despite this, little is known about the extent of the diffusion problems in alginate gels.

In this paper an analysis of oxygen diffusion is presented. As a model system for such an analysis we used cells of the methylotrophic yeast *Hansenula polymorpha* entrapped in a barium-alginate matrix. This organism is able to utilize methanol as a carbon source for growth and contains under this condition an oxygen requiring enzyme, alcohol oxidase, for the oxidation of the C_1 substrate (Van Dijken et al. 1976). A comparison of the kinetics of methanol oxidation by cell suspensions and suspensions of alginate beads containing different concentrations of cells was performed which allowed us to estimate the diffusion coefficient of oxygen in the polymer matrix.

Materials and Methods

Microorganism, Media and Culture Conditions. *Hansenula polymorpha* CBS 4732 was grown in batch culture on a medium containing per dm^3 of deionized water: $(\text{NH}_4)_2\text{SO}_4$, 2.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; NaH_2PO_4 , 3.0 g; K_2HPO_4 , 0.7 g; yeast extract, 0.5 g; trace elements solution (Vishniac and Santer 1957),

List of Symbols C , local oxygen concentration inside the alginate bead; $\text{mol} \cdot \text{cm}^{-3}$; $\bar{C}$, external oxygen concentration; $\text{mol} \cdot \text{cm}^{-3}$; $D(\text{O}_2)_{\text{alg}}$, diffusion constant for oxygen in a barium-alginate matrix; $\text{cm}^2 \cdot \text{s}^{-1}$; $K_s(\text{O}_2)$, Michaelis-Menten constant for oxygen at excess (200 mM) methanol; mM; p , ratio of $D(\text{O}_2)_{\text{alg}}$ over $D(\text{O}_2)_{\text{water}}$; Q_{O_2} , rate of oxygen consumption by free cells (at $C = \bar{C}$); $\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ cells; $\bar{Q}_{\text{O}_2}$, rate of oxygen consumption by the alginate beads; $\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ cells; Q'_{O_2} , rate of oxygen consumption by immobilized cells (at $C = \bar{C}$); $\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ cells; Q''_{O_2} , maximum rate of oxygen consumption; $\text{mol} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$ cells; R , radius of an alginate bead; cm; r , radial distance; x , dimensionless radius (r/R); y , dimensionless oxygen concentration ($C/\bar{C}$); α , Thiele modulus given by Eq. 5; β , dimensionless Michaelis-Menten constant for oxygen [$K_s(\text{O}_2)/\bar{C}$]; l_m , cell density of an alginate bead; g cells $\cdot \text{cm}^{-3}$ alginate beads; η , effectiveness factor given by Eq. 7

Offprint requests to: W. Harder

0.2 cm³ and 10 cm³ of a vitamin/methanol solution containing per cm³: biotin, 10 µg; thiamine, 300 µg and 0.5 cm³ pure methanol as the carbon and energy source. Cultivation was at 37° C in a New Brunswick rotary shaker set at 200 rpm. Cells were harvested by centrifugation in the early stationary phase at a cell density of 0.8–1.0 mg dry weight · cm⁻³. Before use, the cells were washed twice with a 100 mM Tris-HCl buffer, pH 7.5 or a 50 mM K-phosphate buffer, pH 7.5.

Cell Dry Weight Determination. The dry weight values were calculated from the total organic carbon content of the cell suspensions, measured with a Model 915A Beckman Carbon Analyzer. The observed values were multiplied by a factor of 2 to give the dry weight since 50% of the cell material consists of carbon.

Immobilization and Storage Conditions. A stock solution of 2.5% (w/v) sodium alginate (BDH, Poole, UK) in water was used throughout. A known amount of cells was suspended in a volume of 500 mM Tris-HCl buffer, pH 7.5. This suspension was used to dilute the alginate stock solution, to give a 2.0% (w/v) alginate solution in 100 mM Tris-HCl, pH 7.5. The resulting suspension was mixed thoroughly in order to obtain a homogeneous distribution of the cells. A peristaltic pump was used to extrude the alginate-cell mixture through a Pasteur pipette into a 2% (w/v) BaCl₂ solution in 100 mM Tris-HCl, pH 7.5. Small beads (0.05–0.1 cm diameter) were produced by pumping the mixture through a hypodermic needle and blowing the nascent drop from the tip of the needle with a longitudinal flow of air. The beads formed immediately in the BaCl₂-solution and were allowed to cure at room temperature for 1–2 h. Further hardening of the beads was completed overnight at 4° C. To remove excess barium-ions the beads were then washed with 100 mM Tris-HCl buffer and stored in the same buffer. Under these conditions 1 cm³ of the 2.0% alginate mixture yielded approximately 500 mg barium-alginate beads (see below). The diameter of the beads was determined by means of a microscope equipped with a scale in the ocular. A variation in the diameter of the beads of approximately 10–20% was observed. The half-life of the methanol oxidizing activity of the immobilized cells (2 mg cells · cm⁻³ alginate beads; radius 0.05 cm) was 17 days for beads stored at 4° C and 10 days for beads stored at 31° C. Generally the beads were used within 24 h following their immobilization.

Measuring the Weight of the Alginate Beads. The weight of the alginate beads was recorded after the removal of adherent water, by placing the beads on three layers of filterpaper for 1 min. The water content of the alginate beads was determined by drying overnight at 75° C.

Solubilization of the Alginate Beads. Calcium-alginate beads loaded with 1 mg cells · cm⁻³ alginate were prepared as described for barium-alginate beads except that a CaCl₂ solution was used. The three dimensional alginate network was disintegrated by shaking the beads for 4 h in a 0.1 M K-phosphate buffer, pH 7.5 at 20° C (Nilsson et al. 1980). The subsequent separation of cells from the remaining alginate debris by centrifugation, however, was unsatisfactory. The pellet, containing both cells and alginate, was therefore diluted in 100 mM Tris-HCl buffer, pH 7.5 and this suspension used for the determination of the remaining methanol oxidizing activity (Nilsson et al. 1980).

Methanol Oxidation by Free and Immobilized Cells. Oxygen consumption by free and immobilized cells was measured with a Clark type oxygen electrode. The electrode vessel contained 4 cm³ 100 mM Tris-HCl buffer, pH 7.5. After equilibrating the buffer with air or air/oxygen mixtures at 37° C, cells (50 µg dry weight) or alginate beads (0.02–0.1 cm³) were added to the electrode vessel. Subsequently the oxygen electrode was inserted into the vessel,

leaving no gas phase in the vessel and preventing gas exchange between the buffer solution and the gas atmosphere. After recording the endogenous rate of oxygen consumption for 3–5 min, methanol (final concentration 200 mM) was added to the vessel by means of a Hamilton syringe. The decrease in the oxygen concentration in the vessel upon methanol addition was followed in time and the initial oxygen consumption rate, corrected for the endogenous oxygen consumption, was expressed as µmol O₂ · min⁻¹ · mg⁻¹ dry weight of cells or as µmol O₂ · min⁻¹ · cm⁻³ alginate beads. During the measurements the contents of the electrode vessel was stirred continuously with a magnetic stirrer, to ensure proper mixing. This did not result in disintegration of the beads, which were quite stable mechanically and stayed fully intact.

Determination of the Apparent $K_s(O_2)$ Values of Free and Immobilized Cells. Oxygen consumption rate measurements were performed as described above. The various oxygen concentrations in the electrode vessel were established by pre-equilibrating the buffer with various amounts of pure oxygen (0.19–0.93 mM O₂ at 37° C) or gassing with nitrogen (0–0.19 mM O₂). The reciprocals of the observed rate and oxygen concentration were plotted according to Lineweaver-Burk and the apparent $K_s(O_2)$ was calculated from the best fit through the experimental points using the method of least squares.

Theoretical Considerations

The kinetic problems in oxygen-requiring processes caused by the low solubility of oxygen in water, are not limited to immobilized systems but have also been encountered in mold pellets (Aiba and Kobayashi 1971; Bharvarju and Blanch 1975) and other microbial aggregates (Ngian and Lin 1976). The model of Aiba and Kobayashi (1971) for oxygen diffusion in mold pellets, with the minor changes suggested by Bharvarju and Blanch (1975), has been adapted for *Hansenula polymorpha* cells entrapped in a barium-alginate gel. The following assumptions were made: (i) the alginate beads are spherical; (ii) oxygen is the only rate-limiting substrate. The enzyme alcohol oxidase requires two substrates namely oxygen and methanol which both can limit the reaction rate. We observed that the apparent K_s -(methanol) at air saturation [$(O_2) = 0.19$ mM] is 1 mM for freely suspended cells and 1–2 mM for cells in alginate beads of 0.05 cm diameter containing 2–40 mg cells · cm⁻³ alginate beads (results not shown). This is in contrast to the results of Couderc and Baratti (1980), who reported an apparent K_s -(methanol) of 21 mM for *Hansenula polymorpha* cells entrapped in a polyacrylamide gel. According to their explanation this high apparent K_s -(methanol) value was due to an extra diffusion barrier caused by cell wall and peroxisomal membrane. This is probably not correct because the K_m -(methanol) of the purified alcohol oxidase (Van Dijken et al. 1976) is close to the apparent K_s -(methanol) of free or entrapped cells. In the present work an external concentration of 200

mM methanol has been used in all oxygen consumption measurements in order to make the rate of oxygen consumption independent of the methanol concentration; (iii) the diffusion process is as described by Fick's Law; (iv) the cells are homogeneously distributed in the alginate beads; (v) there is no partitioning between oxygen in the buffer and oxygen in the alginate matrix; (vi) external diffusion of oxygen plays no role in the process. This was achieved by setting the rate of stirring by the magnetic stirrer in the oxygen electrode vessel sufficiently high (results not shown).

Using the above assumptions, we can describe the process of oxygen diffusion in the alginate matrix and oxygen utilization by the immobilized cells mathematically (for explanation see: Aiba and Kobayashi 1971; Bharvarju and Blanch 1975; list of symbols):

$$\frac{dC}{dt} = D(O_2)_{\text{alg}} \left(\frac{d^2C}{dr^2} + \frac{2}{r} \cdot \frac{dC}{dr} \right) - \ell_m \cdot Q'_{O_2} \quad (1)$$

where at high concentrations of methanol

$$Q'_{O_2} = Q''_{O_2} \cdot \frac{C}{K_s(O_2) + C}$$

$$= Q_{O_2} \left(\frac{K_s(O_2) + \bar{C}}{\bar{C}} \right) \left(\frac{C}{K_s(O_2) + C} \right) \quad (2)$$

$$\text{if } Q''_{O_2} = Q_{O_2} \cdot \frac{K_s(O_2) + \bar{C}}{\bar{C}}$$

In steady state $\left(\frac{dC}{dt} = 0 \right)$ it follows from Eq. (1) and (2):

$$D(O_2)_{\text{alg}} \left(\frac{d^2C}{dr^2} + \frac{2}{r} \cdot \frac{dC}{dr} \right) =$$

$$\ell_m \cdot Q_{O_2} \left(\frac{K_s(O_2) + \bar{C}}{\bar{C}} \right) \left(\frac{C}{K_s(O_2) + C} \right) \quad (3)$$

Introducing the dimensionless terms $y = \frac{C}{\bar{C}}$, $x = \frac{r}{R}$ and $\beta = \frac{K_s(O_2)}{\bar{C}}$, Eq. 3 can be rewritten as follows:

$$\frac{d^2y}{dx^2} + \frac{2}{x} \cdot \frac{dy}{dx} = R^2 \cdot \frac{\ell_m \cdot Q_{O_2}}{D(O_2)_{\text{alg}} \cdot \bar{C}} (\beta + 1) \cdot \frac{y}{\beta + y} \quad (4)$$

Defining

$$\alpha = R \sqrt{\frac{\ell_m \cdot Q_{O_2}}{D(O_2)_{\text{alg}} \cdot \bar{C}}} \quad (5)$$

reduces Eq. (4) to:

$$\frac{d^2y}{dx^2} + \frac{2}{x} \cdot \frac{dy}{dx} = \alpha^2 (\beta + 1) \cdot \frac{y}{\beta + y} \quad (6)$$

with boundary conditions $x = 0, \frac{dy}{dx} = 0$
 $x = 1, y = 1$

The efficiency of an immobilized biocatalyst in terms of the oxidation of a certain substrate can be described with an effectiveness factor η (Goldstein 1976) which is defined as the ratio of the rate of oxygen consumption by immobilized cells to that of the free cells. In mathematical form this is expressed as follows:

$$\eta = \bar{Q}_{O_2} / Q_{O_2} = \frac{3}{\alpha^2} \cdot \frac{dy}{dx} \Big|_{x=1} \quad (7)$$

Computer Calculations

The differential equation (Eq. 6) cannot be solved analytically and therefore must be solved numerically. This was done using a 4th order Runge-Kutta method (Aiba and Kobayashi 1971).

1. Estimation of $D(O_2)_{\text{alg}}$
 α (Eq. 5) is a function of the cell density (l_m) and $D(O_2)_{\text{alg}}$ (the other factors contained in α are considered constant). $D(O_2)_{\text{alg}}$ can be replaced by p , which is the ratio of $D(O_2)_{\text{alg}}$ over $D(O_2)_{\text{water}}$ ($0 < p < 1$). For a given value of p , l_m defines α and for that value of α the differential equation can be solved, which gives η (Eq. 7). This results in a number of η values at various cell densities (l_m) for one particular value of p .

2. Calculation of the kinetics of methanol oxidation in immobilized cells

The value $\bar{C}$ defines α and β (other factors in α , and β , are considered constant). For these values of α and β , specified by $\bar{C}$, the differential equation can be solved. This gives η as a function of $\bar{C}$. When $\eta \times Q_{O_2}$ is plotted versus $\bar{C}$, the kinetics of methanol oxidation by the immobilized cells is obtained. These calculations have been performed for various combinations of cell densities and radii of the alginate beads.

Results and Discussion

Effect of Immobilization on Methanol Respiration

Immobilization in alginate gels is generally regarded as a very mild procedure for the cells. Usually 80–85% of the cells remain viable after immobilization (Nilsson et al. 1980). However, Holcberg and

Table 1. Effect of various buffer concentrations, ions, and immobilization of cells in alginate on the methanol oxidizing activity of *Hansenula polymorpha*

	Oxygen consumption rate ^a
Cells in 500 mM Tris-HCl (pH 7.5) after harvesting and two washings	100% ^b
Cells stored in 500 mM Tris-HCl (pH 7.5) for 16 h at 4° C	100%
Cells stored in 100 mM Tris-HCl (pH 7.5) for 16 h at 4° C	109%
Cells stored in 2% (w/v) CaCl ₂ for 16 h at 4° C	105%
Cells stored in 2% (w/v) BaCl ₂ for 16 h at 4° C	95%
Cells immobilized in calcium-alginate beads, stored for 16 h at 4° C, followed by desintegration of the beads in 0.1 M K-phosphate buffer (pH 7.5) at 20° C for 4 h	91%

^a The oxygen consumption rates were determined at the following substrate concentrations: O₂ = 0.19 mM; methanol = 200 mM

^b This activity was 0.7 μmol O₂ · min⁻¹ · mg⁻¹ dry weight of cells and was set at 100%

Margalith (1981) reported that calcium ions in the alginate matrix had an inhibitory effect on alcoholic fermentation by yeast. For this reason we examined the effect of immobilization in alginate and the presence of various buffers and ions used during the immobilization procedure, on the methanol oxidizing activity of *Hansenula polymorpha* cells.

In our hands disintegration of the barium-alginate network, which is essential for recovering cells for estimating the remaining activity, was unsuccessful. We therefore used calcium-alginate gels to study the effect of the immobilization procedure on the oxidizing capacity of the cells. In addition, the effect of various buffer concentrations and ions was studied in cell suspensions (Table 1). The recovery of methanol oxidizing activity from the alginate gels was 91%. This observation, together with the finding that the efficiency factor approaches unity at low cell densities (< 0.3 mg cells · cm⁻³ alginate beads, see below), indicates that immobilization in alginate does not cause inactivation of methanol oxidizing activity. Moreover, no inhibitory effect of various buffer concentrations or barium and calcium ions (2%, w/v) on methanol respiration by *Hansenula polymorpha* was detectable. This may be due to the fact that alcohol oxidase is compartmentalized in peroxisomes and thus well-protected by the cell wall and by cytoplasmic and peroxisomal membranes.

Effect of Cell Concentration in the Gel on the Rate of Oxygen Uptake

The effect of cell-loading in the alginate beads on methanol oxidation was studied in beads with a radius of approximately 0.05 cm. At the lowest weight ratio of cell-alginate tested the effectiveness factor η approaches unity (Fig. 1), indicating that there is no diffusion limitation under these conditions. However, as the cell density in the beads is increased, the effectiveness factor η decreases strongly. Since there is no external oxygen and methanol diffusion limitation, or internal methanol diffusion limitation, in these experiments (see above) the results indicate that, with increasing cell densities in the beads, the rate of reaction becomes increasingly limited by diffusion of oxygen from the liquid through the gel matrix. The cells present in the outer layers of the beads will consume most of the available oxygen rapidly and as a result an oxygen concentration gradient develops in the bead. In beads highly loaded with cells this, most likely, even results in oxygen depletion in the core of these beads. This of course reduces the effectiveness of methanol oxidation as the cell density increases because only part of the cells present in the beads is actively engaged in the oxidation of the substrate.

Determination of the Diffusion Constant for Oxygen in the Alginate Matrix

Figure 2 shows the relationship between the effectiveness factor η and the cell density in alginate beads, with a radius of approximately 0.05 cm, as a function of the diffusion constant for oxygen in the alginate matrix. These curves have been calculated using the model and the kinetic parameters of free cells. In this figure the experimental data have also been plotted. The results show that a curve calculated for a p value of approximately 0.25 gives the best fit with the experimental observations. For cell densities less than 2 mg cells · cm⁻³ alginate and more than 15 mg cells · cm⁻³ alginate, a deviation from the curve for $p = 0.25$ is seen. An explanation for this cannot be given at the moment but, most likely, p is not completely independent of the cell density.

The observed p value of 0.25 corresponds to an oxygen diffusion coefficient in the barium-alginate gel of 7.10^{-6} · cm² · s⁻¹. Since the water content of the alginate beads is approximately 95%, it is clear that the alginate network surrounding the cells constitutes an important barrier for the diffusion of oxygen. Comparable low values for the diffusion coefficient of oxygen have been determined for various microbial aggregates (see Ngian and Lin 1976).

Fig. 1. Effect of the concentration of *Hansenula polymorpha* cells in barium-alginate beads on the rate of oxygen consumption. The radius of the beads was approximately 0.05 cm. The rate of oxygen consumption was measured with an external concentration of 0.19 mM O_2 and 200 mM methanol. The effectiveness factor η is given by Eq. 7. ●—●—●, oxygen consumption rate; ×—×—×, η

Fig. 2. Effect of the cell density on the effectiveness factor η . ●—●—● experimental points for beads with a radius of approximately 0.05 cm. The rate of oxygen consumption was measured with an external concentration of 0.19 mM O_2 and 200 mM methanol. The curves were calculated using the following parameters: $Q_{O_2} = 5 \cdot 10^{-6} \text{ mol } O_2 \cdot \text{s}^{-1} \cdot \text{g}^{-1} \text{ cells}$, $\bar{C} = 0.19 \cdot 10^{-6} \text{ mol } O_2 \cdot \text{cm}^{-3}$, $K_s(O_2) = 0.26 \text{ mM } O_2$, $\beta = 1.4$, $R = 0.05 \text{ cm}$, and $D(O_2)_{\text{water}} = 2.6 \cdot 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$

Fig. 3. Calculated rate of oxygen consumption by immobilized cells of *Hansenula polymorpha* as a function of the external oxygen concentration. Parameters used: $D(O_2)_{\text{alg}} = 7 \cdot 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$, $Q''_{O_2} = 11.5 \cdot 10^{-6} \text{ mol } O_2 \cdot \text{s}^{-1} \cdot \text{g}^{-1} \text{ cells}$, $Q_{O_2} = 6.3 \cdot 10^{-6} \text{ mol } O_2 \cdot \text{s}^{-1} \cdot \text{g}^{-1} \text{ cells}$, and $K_s(O_2) = 0.26 \text{ mM } O_2$. A: $R = 0.15 \text{ cm}$, $l_m = 1 \text{ mg cells} \cdot \text{cm}^{-3} \text{ beads}$; B: $R = 0.05 \text{ cm}$, $l_m = 2 \text{ mg cells} \cdot \text{cm}^{-3} \text{ beads}$

Fig. 4. Lineweaver-Burk plots of the measured rate of oxygen uptake by immobilized *Hansenula polymorpha* cells at various oxygen concentrations. A: $R = 0.15$ cm, $l_m = 1$ mg cells $\cdot$ cm $^{-3}$ beads; B: $R = 0.05$ cm, $l_m = 2$ mg cells $\cdot$ cm $^{-3}$ beads

A Comparison of Experimental and Calculated Values of Apparent $K_s(O_2)$ for Beads of Various Dimensions

Figure 3 shows the relationship between the external oxygen concentrations and the rate of methanol oxidation by beads of two different diameters containing different concentrations of cells. The curves have been calculated using the above model, the kinetic parameters of free cells and an oxygen diffusion coefficient in alginate of 7.10^{-6} $\cdot$ cm 2 $\cdot$ s $^{-1}$. From these curves the apparent $K_s(O_2)$ values were derived.

It is also possible to determine the apparent $K_s(O_2)$ values under these conditions experimentally. The results of these measurements, plotted according to Lineweaver-Burk, are shown in Fig. 4. For a comparison various calculated and experimental values of the apparent Michaelis-Menten constant for oxygen (under conditions of excess methanol) thus obtained are summarized in Table 2. From the results it is clear that the oxygen concentration at air saturation [$(O_2) = 0.19$ mM] lies far below the apparent $K_s(O_2)$ of the beads, irrespective of their size or the cell concentration contained in them. In addition, the results show that the apparent $K_s(O_2)$ of the beads depends on two factors, namely the cell density (l_m) and radius R of the beads.

When comparing the calculated and experimental apparent $K_s(O_2)$ values, it is evident that the experimental values are 30–40% lower than those that were calculated. This may be caused by several factors. Firstly, it is exceedingly difficult to produce completely spherical beads with a uniform radius. A certain size distribution of the beads is unavoidable.

Table 2. Calculated and experimental apparent $K_s(O_2)$ values of free and immobilized *Hansenula polymorpha* cells in the presence of an external methanol concentration of 200 mM

Radius beads (cm)	Cell density (mg cells $\cdot$ cm $^{-3}$ beads)	Exp. $K_s(O_2)$ (mM)	Calc. $K_s(O_2)$ (mM)
Free cells		0.26	—
Immobilized cells			
0.05	2	0.33	0.5
0.05	40	2.5	3.6
0.15	1	0.67	1.1
0.15	20	6.0	9.0

Table 3. Calculated rates of oxygen consumption by immobilized *Hansenula polymorpha* cells at various cell densities and bead diameters. Data were calculated using the experimental apparent $K_s(O_2)$ values^a and an external oxygen concentration of 0.19 mM^b

Radius beads (cm)	Cell density (mg cells $\cdot$ cm $^{-3}$ beads)	(%)	Rate of oxygen (μ mol $\cdot$ min $^{-1}$ $\cdot$ cm $^{-3}$ beads)	%
0.15	20	100	$2.3 \cdot 10^{-1}$	100
0.15	1	5	$8.4 \cdot 10^{-2}$	36
0.05	40	100	1.7	100
0.05	2	5	$2.7 \cdot 10^{-1}$	36

^a Comparable results were obtained when the calculations were performed using the calculated values of $K_s(O_2)$

^b The other parameters used are given in the Legend to Fig. 3

Secondly, as indicated in Fig. 1 for an oxygen concentration of 0.19 mM, the experimental error in the determination of O_2 consumption rates by the immobilized cells varies between 10–20%. Thirdly, the experimental apparent $K_s(O_2)$ values have been obtained from Lineweaver-Burk plots using the assumption that a linear relationship exists between the reciprocals. However, Engasser and Horvath (1973) showed that for entrapped cells or enzymes this is not always the case, particularly not at high oxygen concentrations so that apparent K_s values can only be accurately obtained from such plots by extrapolation from very low substrate concentrations. Unfortunately, oxygen consumption measurements at low oxygen concentrations may give large errors. For these reasons we neglected possible deviations from a linear relationship in the Lineweaver-Burk plots.

Pseudostability

The preceding results show that an increase in the cell density contained in the alginate beads causes a decrease in the effectiveness factor η because trans-

port of oxygen towards the biocatalyst becomes rate-limiting. In addition, this results in an increase in the apparent $K_s(\text{O}_2)$. In the course of time a decrease in the catalytic activity of these alginate beads may be expected due to inactivation of the methanol-oxidizing system in the entrapped yeast cells. Assuming homogeneous inactivation in the alginate beads this will result in a steady decline in the rate of oxygen consumption by the beads. However, a consequence of this decrease in the amount of biocatalyst is that the apparent $K_s(\text{O}_2)$ decreases (Table 2). This phenomenon was studied in more detail by a calculation of the rates of oxygen consumption by immobilized *H. polymorpha* cells at various cell densities and bead diameters. The data presented in Table 3 show that a 20-fold decrease in cell density (for instance via inactivation) results in only a 3- to 7-fold decrease in the oxygen consumption rate. This shows that the stability of the methanol oxidizing capacity in the beads cannot be estimated accurately from measurements of the decrease in the rate of oxygen uptake of suspensions of beads with time. If such a procedure is adopted, it will lead to an overestimation of the number of active cells, a phenomenon which is called pseudostability. The obvious explanation for these observations is that in the course of time, during the inactivation process the effectiveness factor η increases (compare Fig. 1), either because the rate of reaction becomes less strongly limited by diffusion of oxygen into the alginate beads, or that a larger part of the immobilized cells now becomes actively involved in methanol oxidation, or both. A method for separating pseudostability from real stability for an immobilized enzyme system has been described by Oshima and Harano (1981).

Conclusions

(i) Cells of the yeast *Hansenula polymorpha* immobilized in barium alginate retain the ability to oxidize methanol. The effectiveness factor approaches unity at low cell densities in the beads but decreases strongly at the higher cell densities. (ii) Using a theoretical model for oxygen diffusion the oxygen diffusion coefficient in a barium-alginate gel has been estimated. The diffusion coefficient in the alginate matrix is only 25% of the value in water. (iii) Oxygen diffusion through the alginate matrix to *H. polymorpha* cells influences the kinetics of methanol oxidation of the immobilized cells, but also affects stability measurements (pseudostability). The apparent $K_s(\text{O}_2)$ of the alginate beads containing *H. polymorpha* cells depends on the cell density and the radius, whereas the apparent $K_s(\text{methanol})$, at a methanol concentration of 200 mM, is independent of the cell

density in the beads. (iv) On the basis of the theoretical model, the experimental kinetic parameters of free cells and the oxygen diffusion constant in alginate gels it was also possible to predict the kinetic behaviour of the immobilized cells. (v) Theoretical calculations show that a decrease in the amount of active cells in the alginate gel does not give a corresponding decrease in the rate of oxygen consumption of the immobilized cells. This phenomenon is called pseudostability. (vi) One of the criteria for the practical use of an immobilized system must be a high rate of conversion. Which conditions must be met in case of an oxygen requiring system? Firstly, in order to reduce the effect of oxygen diffusion problems the beads must have a radius as small as possible. Secondly, the beads must be loaded highly with cells. Such systems will have a low efficiency and a high apparent K_s for oxygen but they show the highest oxygen consumption rate. In such systems the effectiveness factor will increase when in the course of time inactivation occurs.

Acknowledgements. The investigations were supported (in part) by the Foundation for Fundamental Biological Research (BION) which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO). The authors are indebted to Dr. A. J. Schilstra and Mr. L. J. Steijvers for the computer analysis. We are grateful to Mr. J. Zagers for his help in preparing the figures and to Dr. J. Tramper and Dr. K. Ch. A. M. Luyber for their suggestions during the preparation of the manuscript. We thank Marijke Smits for her assistance in part of the experimental work.

References

- Aiba S, Kobayashi K (1971) Comments on oxygen transfer within a mold pellet. *Biotechnol Bioeng* 13: 583–588
- Bharvarju SM, Blanch HW (1975) Mass transfer in mycelial pellets. *J Ferment Technol* 53: 413–415
- Cheetham PSJ, Blunt KW, Bucke C (1979) Physical studies on cell immobilization using calcium alginate gels. *Biotechnol Bioeng* 21: 2155–2168
- Coudere R, Baratti J (1980) Immobilized yeast cells with methanol oxidase activity: preparation and enzymatic properties. *Biotechnol Bioeng* 22: 1155–1173
- van Dijken JP, Otto R, Harder W (1976) Growth of *Hansenula polymorpha* in a methanol-limited chemostat. *Arch Microbiol* 111: 137–144
- Engasser JM, Horvath CH (1973) Effect of internal diffusion in heterogeneous enzyme systems: evaluation of the true kinetic parameters and substrate diffusivity. *J Theor Biol* 42: 137–155
- Goldstein LC (1976) Kinetic behavior of immobilized enzyme systems. *Meth Enzymol* 44: 397–443
- Holcberg IB, Margalith P (1981) Alcoholic fermentation by immobilized yeast at high sugar concentrations. *Eur J Appl Microbiol Biotechnol* 13: 133–140
- Klibanov AM, Huber J (1981) Application of immobilized hydrogenase for the detritiation of water. *Biotechnol Bioeng* 23: 1537–1551
- Ngian KF, Lin SH (1976) Diffusion coefficient of oxygen in microbial aggregates. *Biotechnol Bioeng* 18: 1623–1627

- Nilsson I, Ohlson S, Häggström L, Molin N, Mosbach K (1980) Denitrification of water using immobilized *Pseudomonas denitrificans* cells. Eur J Appl Microbiol Biotechnol 10: 261–274
- Ohlson S, Larsson PO, Mosbach K (1979) Steroid transformation by living cells immobilized in calcium alginate. Eur J Appl Microbiol Biotechnol 7: 103–110
- Ooshima H, Harano Y (1981) Effect of intraparticle diffusional resistance on apparent stability of immobilized enzymes. Biotechnol Bioeng 23: 1991–2000
- Tramper J, Luyben KChAM, van den Tweel WJJ (1983) Kinetic aspects of glucose oxidation by *Gluconobacter oxydans* cells immobilized in calcium alginate. Eur J Appl Microbiol Biotechnol 17: 13–18
- Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21: 195–213

Received May 11, 1983