Model identification and diffusion coefficients determination of glucose and malic acid in calcium alginate membranes

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Abstract

Cell-free and cell-occupied calcium alginate membranes were placed in a plate diffusion cell. The measurements of the diffusion coefficients for glucose and malic acid were performed for each isolated solute, for cocurrent diffusion and with simultaneous CO₂ evolution.

The results indicate that neither CO₂ release nor solute concentration or cocurrent diffusion affect the diffusion coefficients of either solute. The presence of cells is the main factor responsible for the decrease in solute transport across alginate membranes.

The mass transfer mechanism in cell-occupied membranes is better characterized by the random pore model.

1. Introduction

The immobilization of whole microbial, plant and animal cells in support materials is a technique that offers many advantages [1–3]. By immobilizing cells, high density cell cultures can be obtained, leading to faster reaction rates, while at the same time cell product stream concentration is reduced, simplifying downstream processing. Furthermore, the activity of immobilized cells is kept at a higher value, for a larger period of time, than in cell-free systems.

The support material most frequently employed is calcium alginate [4, 5]. Alginate is a naturally occurring polymer of β -D-mannuronic acid and α -L-guluronic acid units linked by 1,4-glycosidic bonds. In the presence of polyvalent cations, cross-linking between macromolecular chains is observed and a gel is formed. Calcium ions are the more frequently used for the latter purpose.

Being of low cost and easy to use, the success of alginate gel as an immobilization support is also due to the gentle environment that it provides for the entrapped material. However, several disadvantages are known, namely its low stability (affected by substances such as citrate and phosphate) and its high porosity. This characteristic limits the application of calcium alginate gels to high molecular weight compounds and whole cells or organelles [5, 6].

Nevertheless, the immobilization of cells poses some practical problems [7–9]. Since substrate and

reaction products have to be transported to and from the immobilized cells, mass transfer processes occurring in gels can become rate limiting, decreasing the cells' overall productivity. Substrate and product accumulation may occur, altering the metabolism of entrapped cells. Also, cell growth at the gel surface, decreasing product yields and causing cell leakage and break-up of the support, may be important.

In order to optimize the operating conditions of immobilized cell reactors using reaction–diffusion models, the characterization of mass transfer mechanisms inside gels is needed. Diffusion coefficients of substrates and products in various supports have been determined by several workers [6, 7, 9–13].

In this work, a diaphragm diffusion cell is used to determine the influence of glucose and malic acid concentration, diffusing either individually or cocurrently, biomass concentration and CO₂ evolution on the diffusion coefficient of glucose and malic acid in a calcium alginate gel.

2. Materials and methods

2.1. Materials

Sodium alginate was obtained from Riedel–de Haen, glucose from Merck and L-malic acid from Sigma Chemical Co.

Baker's yeast, obtained from a local bakery, was the organism used in all immobilized cell experiments.

2.2. Analysis

Glucose concentration was measured by the glucose-oxidase enzymatic method (Sigma Chemical Co.).

Malic acid concentration was determined by an enzymatic assay using malate dehydrogenase [14].

Yeast cell viability was assayed by methylene blue staining [15].

2.3. Preparation of calcium alginate membranes

In all experiments, sodium alginate concentration was 3.0% (w/v). Membranes of defined thickness and diameter were cast in a Perspex ring held between two porous glass plates and hardened for 3 h in a bath of 2% (w/v) calcium chloride.

To obtain membranes containing inactivated baker's yeast cells the following procedure was adopted: commercial baker's yeast was weighed and suspended in distilled water and a heat shock (50 °C for 30 min) was applied to the suspension. Microscopic observation and methylene blue staining allowed us to verify that cell death was complete with no lysis. The yeast suspension was then centrifuged, washed and resuspended in the sodium alginate solution of the desired concentration. Membranes were hardened as previously described.

For the experiments done with active cells, baker's yeast cells were previously activated by suspending them in a 1% (w/v) glucose solution, for 30 min at 30 °C. The yeast suspension was centrifuged, washed and resuspended in the alginate solution. Two groups of experiments with activated yeasts were done: in the first, hardened calcium alginate membranes were immediately placed in the diffusion apparatus. In the second one the membranes were previously immersed in a 1% (w/v) glucose solution, for 3 h, to ensure that no glucose gradients were observed, inside the membrane, at the beginning of the experiments. With this procedure, it was possible to assess the influence of CO₂ evolution in malic acid diffusion.

2.4. Diffusion cell

The diffusion cell (Fig. 1) is a modification of the apparatus described by Hannoun and Stephanopoulos [7]. It is made of Perspex and consists of two chambers divided by the gel plate. The volume of each chamber is 120 ml. Half-cells, corresponding to the diffusion chambers, are held together with screws. The calcium alginate membrane is supported

Fig. 1. Diffusion cell (all dimensions are in millimetres).

by a poly(vinyl chloride) squared mesh (mesh number 3.5) and sealed with O-rings. Agitation in the upper chamber was obtained using a stirrer (150 rev min⁻¹) and in the lower chamber a magnetically driven bar (150 rev min⁻¹).

Samples were collected either by a hole drilled in the upper chamber or through a septum in the lower chamber. This chamber was connected to a capillary column acting as a reservoir of solution to replace the liquid removed during sampling. Experiments with malic acid and glucose solutions placed in either of the two chambers were carried out.

The diffusion cell was placed at a temperature-controlled water bath to ensure that all measurements were made at 30 °C.

3. Theory

Diffusion coefficients were calculated using the lag-time analysis [7].

The diffusion mass transfer process that occurs through an alginate membrane suspended between two well-mixed chambers of concentrations C_1 and C_2 , assuming that the chambers are well mixed and the component concentrations are the same at the surface of the membranes and the bulk fluid, is represented by

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \quad (1)$$

where C is the concentration, D the diffusion coefficient, t the time and x the distance with the following boundary conditions:

$$C = C_1 \quad x = 0 \quad (2)$$

$$C = C_2 \quad x = l \quad (3)$$

where l is the membrane thickness.

If the components diffuse from the chamber where $C = C_1$ to the chamber where $C = C_2$ and assuming that, for sufficiently large chambers, changes in concentration are negligible and that C_2 is approximately zero, we may apply to eqn. (1) the initial conditions

$$C = 0 \quad 0 < x < l \quad \text{at } t = 0 \quad (4)$$

and, after integration, obtain [16]

$$C = C_1 \frac{l-x}{l} + \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{-C_1}{n} \sin\left(\frac{n\pi x}{l}\right) \exp\left(\frac{-Dn^2\pi^2 t}{l^2}\right) \quad (5)$$

The solute flux F is

$$F|_{x=l} = -D \left(\frac{\partial C}{\partial x} \right)_{x=l} \quad (6)$$

On integration of eqn. (6) with respect to t from $t = t_0$ to $t = t_s$, and multiplication by the membrane area A , the total amount Q of solute transferred through the membrane is, for significantly large times,

$$Q_{ts} = \frac{ADC_1}{l} \left(t_s - \frac{l^2}{6D} \right) \quad (7)$$

The intercept of the linear part of the curve obtained by plotting Q vs. time is the so-called "lag time".

Diffusion coefficients are then calculated from the lag time and the membrane thickness.

4. Results and discussion

4.1. Diffusion coefficients of glucose and malic acid in cell-free alginate (3.0% w/v) membranes

Figure 2 represents an experimental plot obtained for the diffusion of glucose through the alginate membranes.

Two different glucose concentrations were tested (10 and 100 g l⁻¹) to assess the influence of solute concentration on the diffusion coefficient. The values obtained for the diffusion coefficient were identical

Fig. 2. Transferred glucose through the membrane vs. time for a diffusion experiment ($C_1 = 10$ g l⁻¹).

for the 10 g l⁻¹ ($D = 6.59 \times 10^{-6} \pm 0.20 \times 10^{-6}$ cm² s⁻¹) and 100 g l⁻¹ ($D = 6.72 \times 10^{-6} \pm 0.09 \times 10^{-6}$ cm² s⁻¹) experiments and of the same order of magnitude as the diffusion coefficient of glucose in water ($D = 6.80 \times 10^{-6}$ cm² s⁻¹). These results agree with those reported by several workers [7, 12] for glucose diffusion through Ca-alginate membranes.

For malic acid diffusion the only concentration tested was 10 g l⁻¹, since this is the amount of malic acid that is normally found in practical applications. In this case, the diffusion coefficient ($D = 4.36 \times 10^{-6} \pm 0.09 \times 10^{-6}$ cm² s⁻¹) was significantly lower (54%) than in aqueous solution ($D = 8.02 \times 10^{-6}$ cm² s⁻¹). Such a reduction may be attributed to the higher charge density of malic acid, compared with glucose with a consequent stronger interaction with the alginate membrane. Such a hypothesis supports the results obtained by Furui and Yamashita [10] for malic acid and other charged solutes diffusing through polyacrylamide and k-carrageenan gels, although disagreeing with the data of Chresand *et al.* [17]. These results also indicate that the hydrodynamic theory which is regarded as satisfactory by Hannoun and Stephanopoulos [7] for solutes diffusing in gels does not apply in most cases because of the existence of interactive mechanisms between gel and solute. It must be remembered that malic acid is a smaller molecule than glucose and, as predicted by the hydrodynamic theory, its diffusion coefficient in water is 1.2 times the diffusion coefficient of glucose. However, such a relationship is not valid for the diffusion in alginate gels, and in this case the diffusion coefficient of malic acid is 0.7 times the diffusion coefficient of glucose.

4.2. Cocurrent diffusion of glucose and malic acid

Glucose and malic acid are two compounds that may be simultaneously metabolized during fermentation. Thus it is important to analyse the influence that each has on the other's diffusion coefficient.

Using the same range of concentrations that were tested before, cocurrent flow experiments were done. The diffusion coefficients obtained ($D = 6.61 \times 10^{-6} \pm 0.21 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for glucose and $D = 4.45 \times 10^{-6} \pm 0.28 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for malic acid) clearly demonstrate that there was no interaction between glucose and malic acid when they diffuse cocurrently through Ca-alginate membranes. Such a result was not unexpected since the physical parameters that control diffusion coefficients are not significantly affected by the solute concentrations tested. Again it must be noted that the hydrodynamic theory does not agree with the results obtained.

4.3. Influence of the concentration of immobilized cells on the diffusion coefficients of glucose and malic acid

The results obtained showing the influence of immobilized cell concentration on the diffusion coefficients of glucose and malic acid are represented in Fig. 3. For these experiments the initial glucose and malic acid concentrations were, in both cases, 10 g l^{-1} . It is evident, from the plotted values, that for both glucose and malic acid the diffusion coefficient decreases linearly with increasing biomass concentration. The slopes of these relations are of the same order of magnitude (9 ± 3 for glucose diffusion and 7 ± 2 for malic acid diffusion, with a confidence interval of 95%) for the two solutes, confirming that the cell concentration is the main factor responsible for the reduction in the diffusion

Fig. 3. Variation in the diffusion coefficients of glucose (●) and malic acid (○) with concentration of immobilized yeast cells.

coefficients in alginate gels as suggested in the review by Westrin and Axelsson [3]. Anyway, recent data [13] indicate that the presence of dead biomass does not influence the diffusion coefficient of glucose in alginate gels. The contradiction between this set of results demonstrates the need for further work on mass transfer phenomena in gels.

In order to check some of the models that describe diffusion through gel membranes, the diffusion coefficients obtained were converted into effective diffusion coefficients D_e , according to [3]

$$D_e = \epsilon D \quad (8)$$

where

$$\epsilon = 1 - (\alpha\phi_c + \phi_p) \quad (9)$$

α represents the volume fraction of individual cells that is not accessible to solute (it was assumed that $\alpha = 1$, meaning that the outer cell membrane totally excludes the solute), ϕ_p is the polymer volume fraction in the gel and ϕ_c is the cell volume fraction.

The model that gives a better description of the influence of cell concentration in the effective diffusion coefficient for the experimental conditions tested is the "random-pore" model (Fig. 4), described by the equation

$$\frac{D_e}{D_{e0}} = (1 - \phi_c)^2 \quad (10)$$

where D_{e0} is the effective diffusion coefficient in the absence of cells.

The approximately constant percentage deviation that is observed for this model is most probably related to a slightly higher biomass concentration in the lower side of the gel, which may cause a more pronounced effect of cell concentration.

Fig. 4. Influence of cell volume fraction on the effective diffusion coefficients of (●) glucose and (○) malic acid calculated according to experimental data using eqns. (10)–(12).

All the other tested models given by the following equations presented significant deviations:

$$\frac{D_e}{D_{e0}} = \frac{1 - \phi_c}{1 + \frac{1}{2}\phi_c} \quad (11)$$

$$\frac{D_e}{D_{e0}} = 1 - \phi_c \quad (12)$$

4.4. Influence of CO₂ evolution rate

In fermentation, when glucose and malic acid are being consumed, ethanol and CO₂ are produced. Stoichiometrically, for each mole of consumed glucose, 2 mol of ethanol and 2 mol of CO₂ will be formed and, for each mole of consumed malic acid, 2 mol of CO₂ and 1 mol of ethanol will be produced. It might be expected that CO₂ molecules released through the membrane play an important role in the rate of diffusion of soluble solutes to and from the interior of membranes, as suggested by Ruggeri *et al.* [13]. These are the only reported results dealing with this subject. Anyway they are not based on a realistic situation, as low pH values induce structural changes in the gel.

In order to try to clear up this aspect, it was decided to measure the transport of malic acid through Ca-alginate membranes when CO₂ was being actively produced and released. Malic acid is a substrate that is metabolized at very low reaction rates in the presence of glucose [18] by yeasts belonging to the genera *Saccharomyces* (such as baker's yeast). This characteristic was responsible for a significant reduction in the biochemical reaction factor and consequently allowed to obtain a realistic assessment of the influence of CO₂ evolution rate on malic acid diffusion.

An experiment was performed by placing in the upper chamber of the diffusion cell an aqueous solution of glucose and malic acid, diffusing through a membrane containing immobilized activated yeasts.

Two kinds of experiment were done. In the first case, immobilized viable cells (the viability was confirmed by methylene blue staining) did not experience any contact either with glucose or with malic acid, prior to the diffusion experiment. In the second case, alginate membranes were previously immersed in a 10 g l⁻¹ solution of glucose to ensure that, when malic acid was added to the upper chamber of the diffusion cell, CO₂ was being actively released. The concentration of immobilized viable cells in the alginate membrane was 5% (v/v).

In both cases, the malic acid diffusion coefficient was of the same order of magnitude as that obtained

with inactivated cells, within an experimental error of $\pm 10\%$ which is an unexpected result.

In the first set of experiments it might be argued that the experiment did not last sufficiently long to take CO₂ evolution into account. However, such an argument could not be used for the second set of experiments since CO₂ was effectively being released when malic acid was added to the upper chamber of the diffusion cell.

A possible explanation may be that the turbulence caused by CO₂ release eliminates any film mass transfer resistance between the bulk fluid and the membrane and that convective mass transfer may become significant inside the membrane.

Unfortunately, it was not possible to obtain accurate control of the CO₂ evolution rate in this group of experiments, although visual observation allowed us to ensure that several CO₂ rates were assayed. In spite of these different experimental situations the malic acid diffusion coefficient was always of the same magnitude.

Experiments were tried with a higher concentration of immobilized cells (20% v/v) but the CO₂ released caused alginate membrane disruption.

These results suggest that CO₂ acts by increasing convective mass transfer inside the membranes, thereby having the opposite effect to the reduction in available area for solute flux. The main consequence of CO₂ release will be the mechanical stress that it causes inside the membranes, leading eventually to alginate release into the medium and, in extreme cases, to total membrane disruption.

5. Conclusions

From these experiments several conclusions concerning solute diffusion through Ca-alginate membranes can be made.

(1) Solute concentration or cocurrent diffusion of two solutes does not affect the respective diffusion coefficients.

(2) The presence of cells decreases the transport of solutes through Ca-alginate membranes.

(3) CO₂ release does not change the diffusion coefficient of soluble solutes. This may be explained by the internal pore turbulence caused by CO₂ release.

(4) The hydrodynamic theory is not applicable to these results.

(5) The effective diffusion coefficient can be correlated with cell concentration by the random pore model.

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