Passive Diffusion in Organic Acid Fermentations: Diffusion as a Primary Uptake Mechanism in Citric Acid Fermentation

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ABSTRACT

Simple and facilitated diffusion models for glucose through the cell membrane were prepared and compared with fermentation data from published studies on the citric acid fermentation process. It was found that the physical and uncontrolled process of diffusion could explain the specific rate of glucose uptake observed during the production phase in several different fermentation.

KEYWORDS: diffusion, citric acid fermentation

1. INTRODUCTION

The accumulation of organic acids, such as citric acid, in commercial processes is strongly influenced by the nutrient composition of the production medium. This is typically a "light syrup" containing 10 % to 22 % (w/v) sugar and in the case of citric acid is deficient in manganese ions. The glucose uptake rate has been identified by Torres [1, 2] as an important factor in the rate of citric acid production, using mathematical modeling to show that the glycolytic reactions of *Aspergillus niger* are limited by the supply of the initial substrate and removal of the final product.

Transport proteins for glucose have been identified and characterized in citric acid producer *Aspergillus niger*. There are now two different carriers known, a high-affinity carrier that is expressed at all times and a low-affinity, expressed only in the presence of high glucose concentrations [3, 4]. The characteristic parameters of these carriers at pH 2 have the following values [4]:

	High affinity carrier	**Low affinity carrier**
V_{max}	0.052 μmol s^{-1} g^{-1}	0.75 μmol s^{-1} g^{-1}
K_m	3.7 x 10^3 μM	260 μM
$K_{i\ (citric\ acid)}$	2.3 x 10^5 μM	9 x 10^5 μM

In industrial media the concentration of glucose is high enough to saturate the mediated transport mechanism during the whole fermentation process. However, it is of interest to note that the specific uptake rate of glucose appears to be related to the external concentration of glucose during almost all of the period of citric acid production and it is not restricted in the expected fashion [5]. This is a situation that would appear to run contrary to mediated methods of transport and control.

To our knowledge, no direct study comparing simple diffusion with other fermentation parameters has been published. In the present study, models for simple diffusion and protein-

mediated mechanisms were created to determine whether simple diffusion through the membrane could explain the observed relationship between glucose uptake rate and concentration at high glucose concentrations.

2. MATERIALS AND METHODS

Work was carried out by the Strathclyde group as described earlier in Papagianni [5] and Papagianni *et al.* [6]. Values for the diffusive flux constant were calculated from data given by Kubicek and Rohr [7], Krzystek *et al.* [8], Charley [9] and Papagianni *et al.* [6].

Modeling aspects

To analyze previously published works, trend lines were fitted to data by optimizing the least-third-quartile-squares fit of a straight line through the origin, represented by

$$q_s = m \cdot S \tag{1}$$

Only data points from the period of significant citric acid production were used. For mediated transport the model was based on the application of kinetic parameters published by Torres [1, 2] to the conditions found in the fermentation with the Michaelis-Menten equation:

$$V = \frac{V_{max} \cdot S}{K_m + S} \tag{2}$$

where, the quoted units for V were then converted to specific uptake rate q_s. Concerning the citric acid inhibition of the glucose transporters, the mechanism was assumed to be non-competitive because of the lack of similarity between the molecular structures of citrate and glucose and also because of the data shown in Mishak *et al.* [3]. K_i values for the high and low-affinity carriers were calculated from the data given by Torres *et al.* [1, 2, 4] using the equations:

$$K_i = [I] \cdot \left(\frac{V_{max}}{Vi_{max}} - 1 \right) \tag{3}$$

and

$$Vi_{max} = \frac{V_{max}}{1 + ([I]/K_i)} \tag{4}$$

Some assumptions were made to construct a model for simple diffusion. Biomass was assumed to be a single cylinder with a hyphal diameter having a volume equivalent to that of all branched mycelium. The internal glucose concentration was assumed to be zero. The third assumption was that not all of the biomass is active and the value of 18% biomass activity (X_A) was used to reflect the fact that only the growing tips of the mycelium are active.

The flux of a solute (J) can be determined using experimentally determined permeability coefficients (P_j) from

$$J_j = P_j \cdot (c_j - c_o) \tag{5}$$

The glucose permeability coefficient for *A. niger* was estimated to be 1.0×10^{-7} cm s^{-1} [10]. The specific uptake rate q_s can be calculated from the flux, using the equation

$$q_s = A_x \cdot X_A \cdot J \tag{6}$$

The specific surface area, A_x, was calculated from:

$$A_x = \frac{2}{r_{hyp} \cdot \rho_x \cdot (1 - X_{H2O})} \tag{7}$$

This was derived from geometric equations for the surface area and volume of a single, long thin cylinder, to which the hyphae of the mycelium can be approximated for these purposes.

The effect of diffusion through the stationary aqueous layer adjacent to the membrane was calculated using eqs. (5) and (7) and the values of cellular attributes are summarized below:

Cellular Attribute	Value
Stagnant layer thickness	100 μm
Hyphal diameter	2.5 μm
Biomass density	1.1 g cm^{-3}
Water content of wet biomass	70%
Specific surface area	4.8×10^4 cm^2 g DW^{-1}

The calculated effect of diffusion through the stationary 100 μm layer surrounding the cell was a reduction in the effective concentration at the membrane boundary of 1 %, which is negligible.

3. RESULTS AND DISCUSSION

The relationship between the glucose concentration and the specific glucose uptake rate was investigated using three sets of previously published batch fermentation data produced using a range of different experimental methods. To eliminate any effect of the falling glucose concentration during the batch processes, the models was also compared with data collected from a series of fed-batch runs in which the concentration of glucose was maintained constant, but in which other fermentation parameters were not controlled (glucostat).

Figures 1, 2 and 3 contain data from shake flask cultures, air-lift and stirred-tank bio-reactors, respectively. The calculated values of m (eqn. 1) for each of these fermentation systems are similar and the fact that they were obtained using different fermentation systems implies that a simple relation between q_s and S exists, which is independent of variations in fermenter hydrodynamics arising from different reactor configuration. In Figure 4 (glucostat data), the value obtained for the gradient of the trend line correlates very closely to the values of m obtained from the batch culture models. In glucostat, this value is independent of the effects of time and, consequently, of citric acid concentration. The similarity of the value of the constant m for the various systems suggests that the effects of time and citric acid concentration on q_s are small, and the only significant parameter appears to be the concentration of glucose.

In Figure 5, there appears to be no effect of the concentration of citric acid on the specific glucose uptake rate in glucostat runs. However, both mediated carriers were inhibited by citrate under producing conditions, as shown in Figure 6. Since these effects cannot be observed in the glucostat experiments, glucose must therefore be taken up by some mechanism that is not inhibited by the accumulation of citric acid.

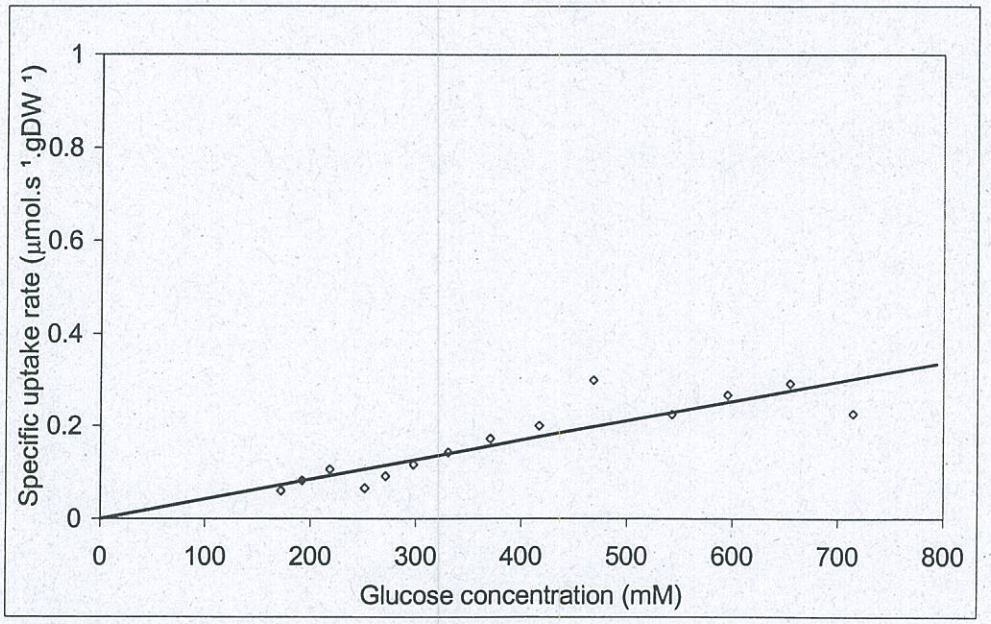

Figure 1 Comparison of q_s and S from data published by Kubicek and Rohr [7]

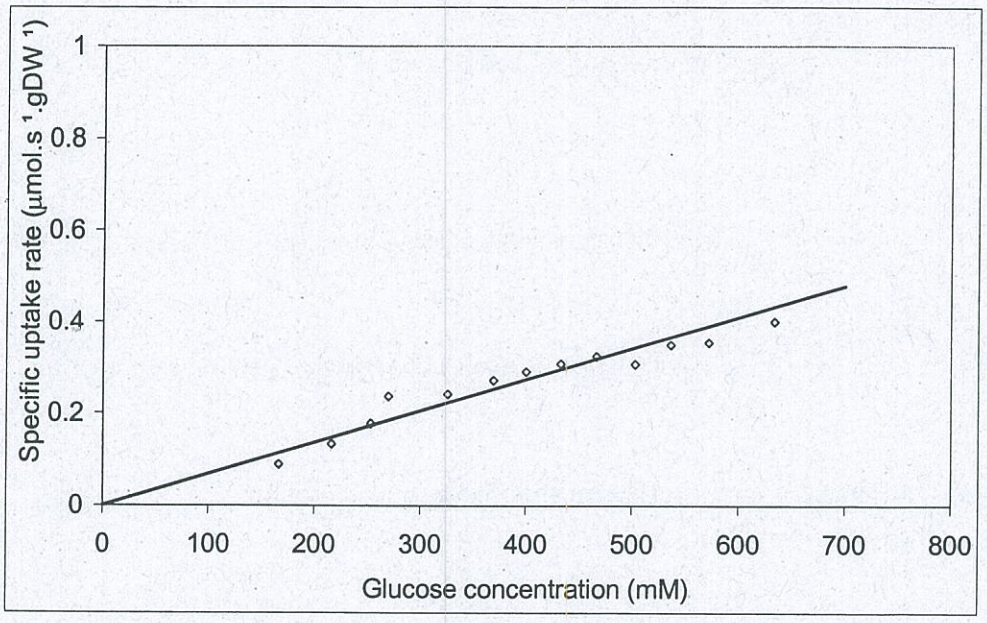

Figure 2 Comparison of q_s and S from data published by Charley [9]

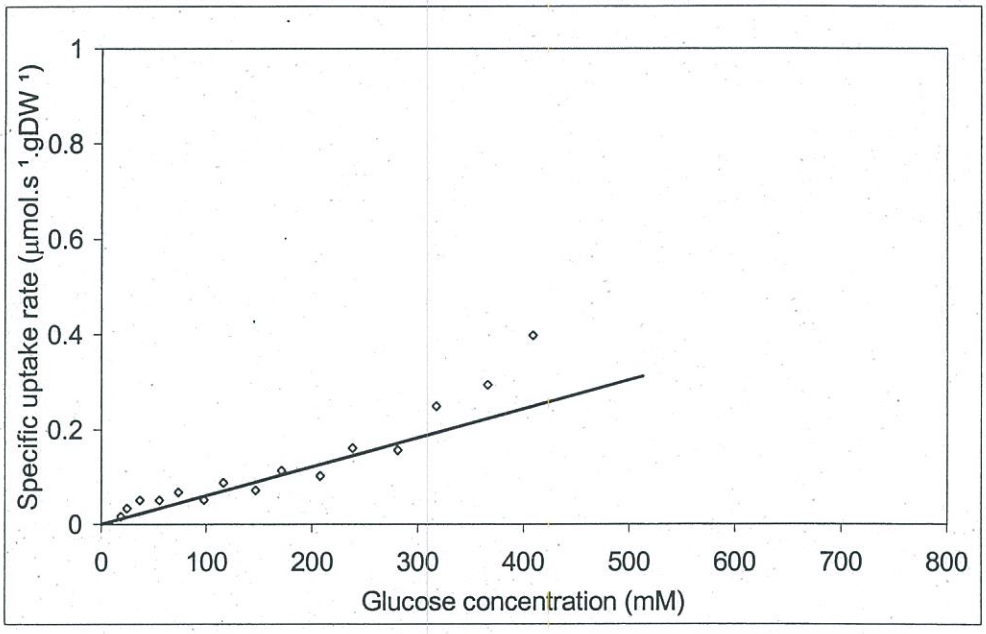

Figure 3 Comparison of q_s and S from data published by Krzystek *et al.* [8]

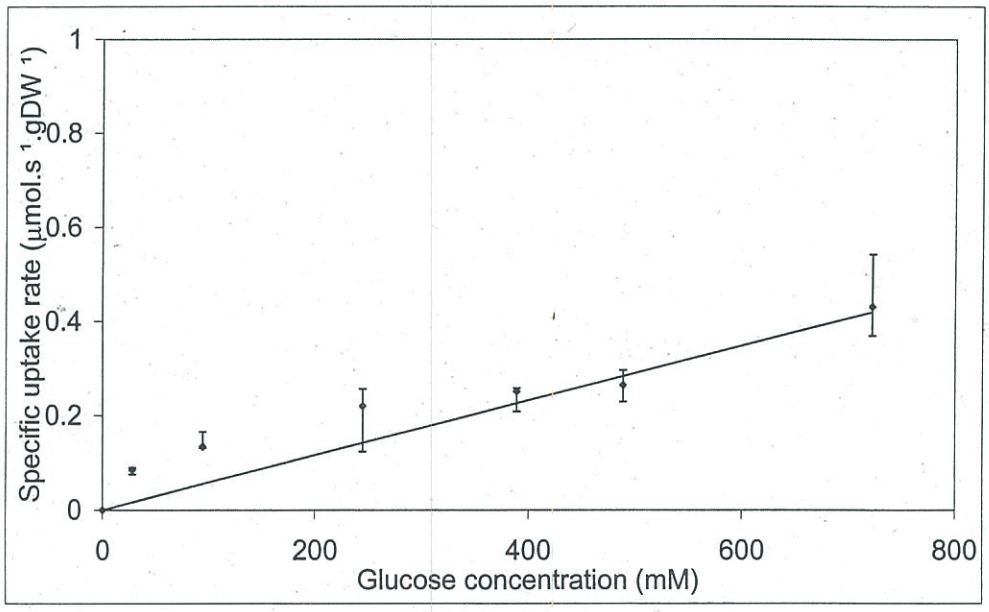

Figure 4 Comparison of q_s and glucose concentration in glucostat experiments [5]

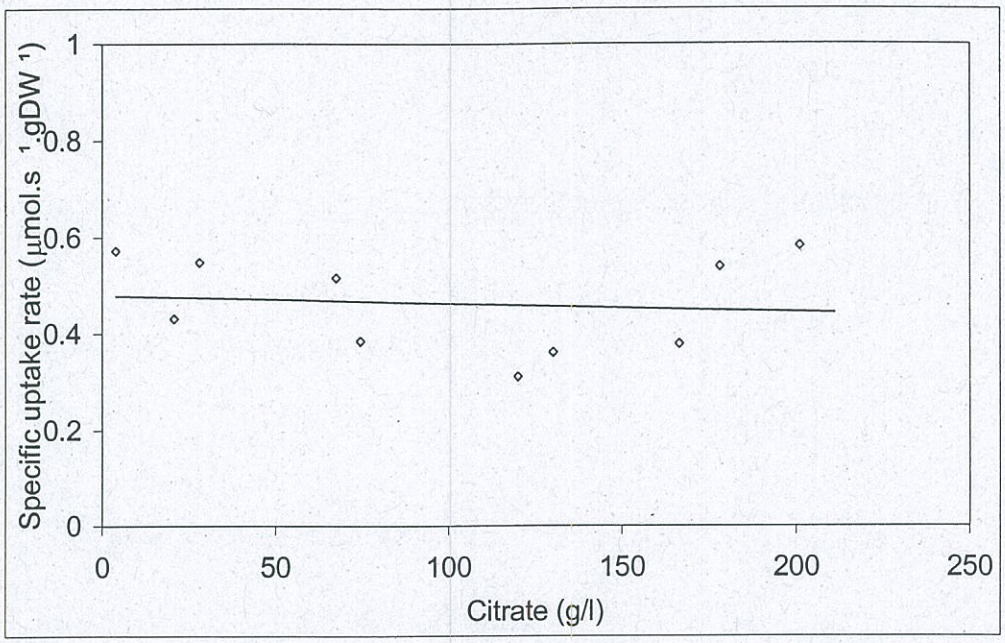

Figure 5 Effect of citric acid on specific uptake rate

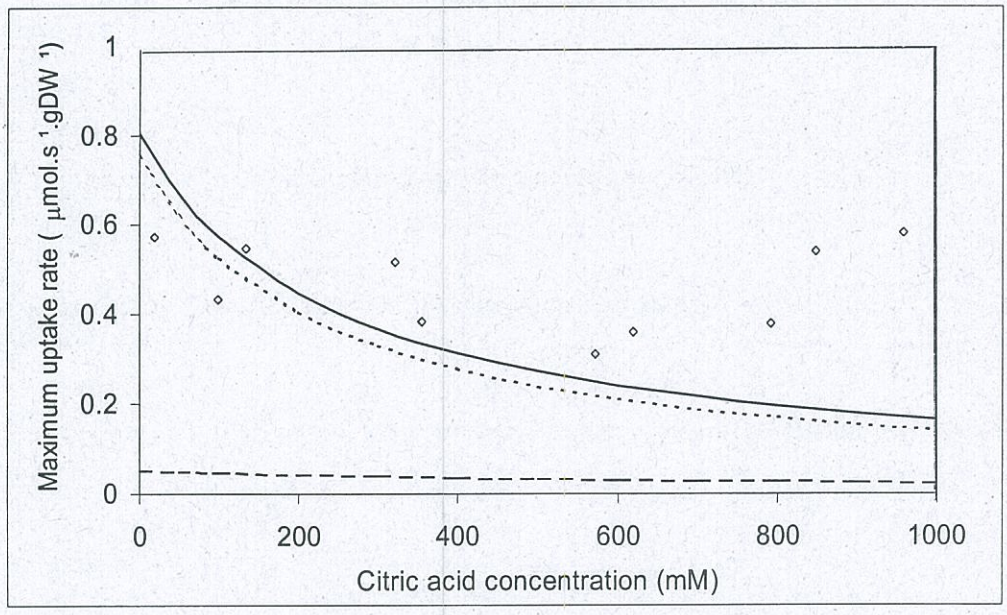

Figure 6 Effect of citric acid concentration on maximum specific uptake rate

Figure 7 presents the results from the mediated transport and diffusion models, showing the effect of glucose concentration on the specific glucose uptake rate (at pH 2). The total uptake, mediated by both high and low-affinity carriers is shown by the dashed line, while the uptake by simple diffusion is shown by the solid line. The median observed values for q_s from the glucostat runs are also shown for comparison. Figure 7 clearly shows that the model for non-mediated uptake for glucose is a better match for the experimental data than the protein carrier model. There is some uptake that is above the predicted level for simple diffusion at low glucose concentrations (< 150 mM), and this would appear to be protein-mediated.

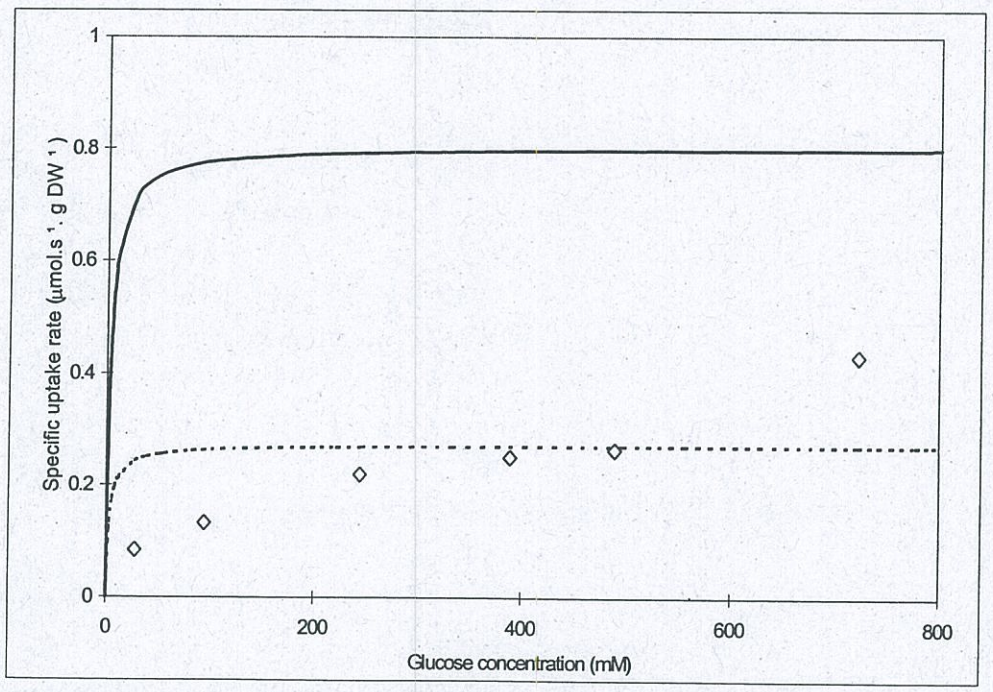

Figure 7 Effect of glucose concentration on the specific glucose uptake rate (total uptake mediated by high and low affinity carriers in solid line and maximum inhibition in dashed line). Also shown superimposed for comparison, the median observed values from the glucostat experiments.

In Figure 8 the uptake mediated by the high affinity carrier, is shown by the long dashed lines, while for the low-affinity carrier by the short dashed lines. The solid line represents the total q_s, while glucostat data is shown again for comparison. In this figure no inhibition by citric acid can be observed, so glucose must be taken up by a mechanism not inhibited by citric acid. The effect of glucose concentration on the specific glucose uptake rate is shown in Figure 9, where the median observed values for q_s from the glucostat runs are also shown again. The mediated model does not explain the positive linear relationship between observed uptake rate and glucose concentration. It can be seen in this figure that diffusional rates match the observed data.

However, one difficulty that remains is an explanation for the obvious sigmoidal shape of the experimental data. The observed uptake rate falls below the model prediction when the glucose concentration exceeds 400 mM. This appears to be in agreement with the observation by Kubicek [11] that the internal concentration of trehalose-6-phosphate only reaches inhibitory levels when the external glucose concentration is increased to about 400 mM. With hexokinase inhibited, glucokinase becomes the only means for the phosphorylation of glucose, with a resultant loss in phosphorylating activity. An intracellular pool of glucose will then accumulate with two direct effects upon the specific uptake rate. The first of these is a reduction in the concentration gradient across the cell membrane, which reduces the flux though the membrane. This is overcome by

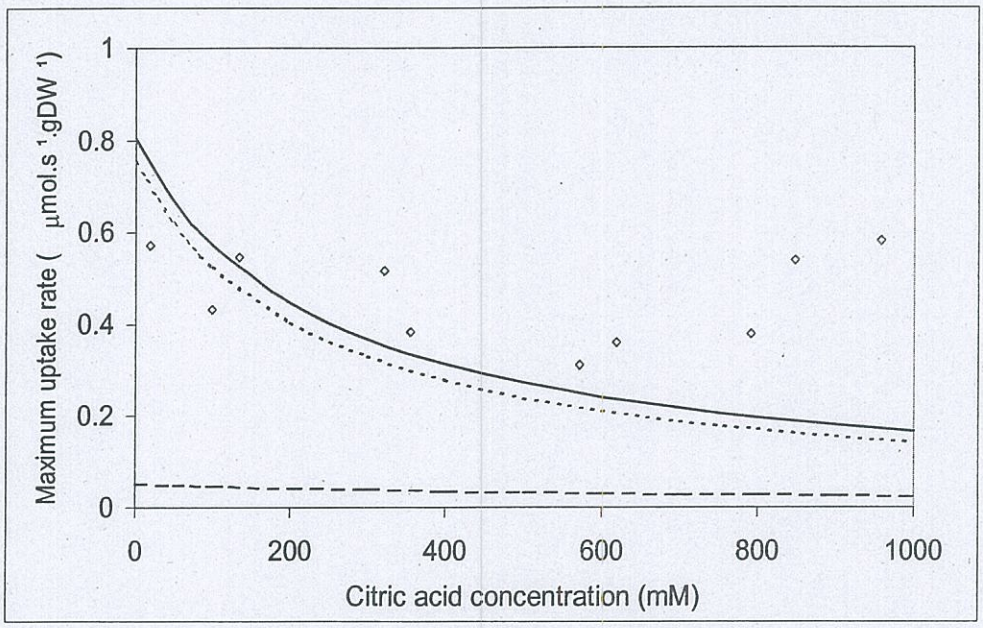

Figure 8 Effect of citric acid concentration on the maximum specific uptake rate of glucose: high affinity carrier shown by long dashed line, low affinity carrier by short dashed lines, total qs by the solid line and glucosta data

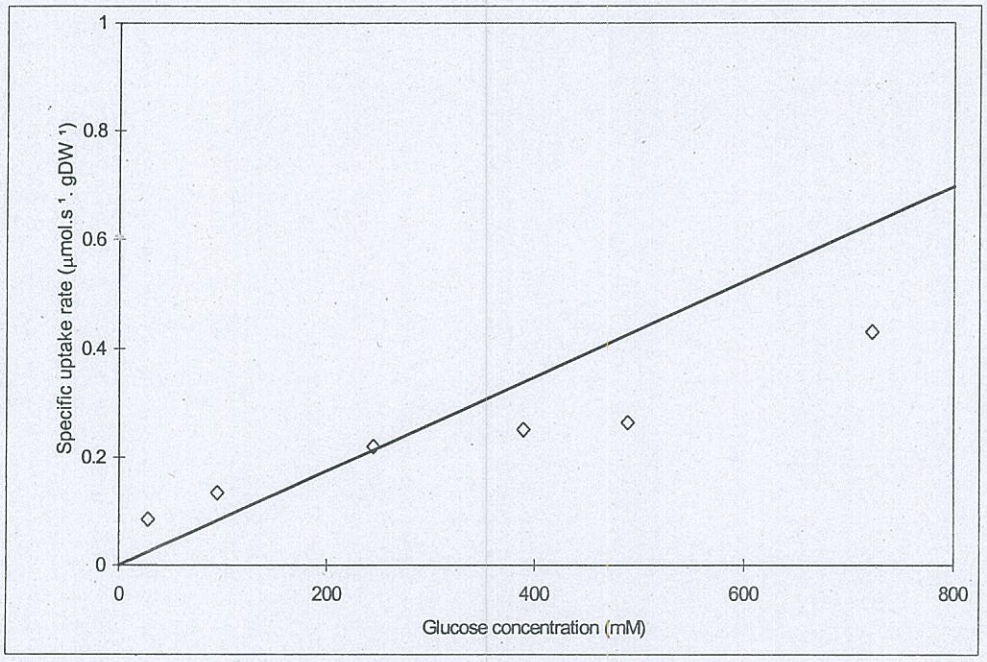

Figure 9 Experimental results and diffusion model

further increasing the external glucose concentration. The second effect is the increase in flux through the glucokinase metabolic step, due to the increased concentration of its substrate.

4. CONCLUSIONS

The mediated model does not reflect the relationship between the observed uptake rate and glucose concentration, nor for the lack of sensitivity to citrate. This is due in part of the low value of K_m in relation to the actual substrate concentration, which means that the carriers are saturated until the end of the process. The membrane barriers must be strongly inhibited under the standard production conditions.

The simple diffusion model fits all the observed data and it explains the relationship between the specific uptake rate and the concentration of glucose, which should not exist under carrier-saturated conditions. Simple diffusion is an inevitable physical process, which cannot be regulated by the organism. This may account for the overproduction of organic acids under the specific process conditions. The simple nature of this mechanism also explains the similarity of the uptake relationships from different sources, despite the use of different strains and culture conditions.

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NOMENCLATURE

A_x	specific surface area (cm^2 g DW^{-1})
c_j, c_o	concentration; internal, external (μmol)
D_j	diffusivity of solute j (cm^2 s^{-1})
$[I]$	concentration of inhibitor (μmol)
J_j	flux of solute j (μmol s^{-1} cm^{-2})
K_i	inhibition constant (μmol)
K_m	Michaelis constant (μmol)
m	diffusive flux constant (cm^3 s^{-1} g DW^{-1})
P_j	permeability j (cm s^{-1})
q_s	specific substrate uptake rate (μmol s^{-1} g DW^{-1})
r_{hyp}	radius of the hyphal filament (cm)
S	concentration of substrate (μmol)
V_i, V_{max}, V_{imax}	rate; maximum rate (of an enzyme-catalyzed reaction); Maximum rate under inhibition (μmol s^{-1} g DW^{-1})
X_A	active fraction of biomass
X_{H2O}	water content of wet biomass
ρ_x	density of wet biomass (g cm^{-3})