[Chem. Pharm. Bull. 30(3) 782—788 (1982)]

Oxidation of Glucose on Immobilized Glucose Oxidase[1)]

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(Received July 15, 1981)

Oxidation of glucose on immobilized enzymes was carried out to study the effect of intraparticle mass transfer of oxygen on the global rate of the reaction. The catalyst employed, which consisted of both glucose oxidase and catalase supported on activated carbon, was prepared by the carbodiimide method. The experiments were performed in 0.1 M acetate buffer solution (pH=5.5) and at 25°C.

The initial rates of reaction were determined from the absorption rates of oxygen gas, under conditions of no external mass transfer resistances. Nonlinear least-squares analysis gave values of the maximum velocity (V), the Michaelis constant (K_m), the effective diffusivity of oxygen (D_e) and the tortuosity factor of the particles (τ) of $V=8.30\times10^{-6}$ [mol/(g-cat·s)], $K_m=9.46\times10^{-5}$ [mol/cm^3], $D_e=7.70\times10^{-6}$ [cm^2/s] and $\tau=2.0$ [−], respectively. The effectiveness factor calculated from these values indicated that the intraparticle mass transfer of oxygen in the liquid significantly influences the global rate of reaction.

Keywords——oxidation; glucose; immobilized enzyme; glucose oxidase; catalase; activated carbon; kinetics; intraparticle mass transfer

The attachment of enzymes to insoluble supports greatly simplifies their use in many enzymatic reactions. In enzyme catalysis where the reaction is run in a homogeneous batch reactor, it is necessary to separate enzymes from the reaction mixture at the end of the reaction, for example, by ultrafiltration, affinity chromatography, *etc.* In order to avoid these tedious processes for recovery of the enzymes, increasing attention has been given in recent years to the preparation and utilization of immobilized enzymes. When the enzyme is immobilized inside a porous matrix of particles, one of the factors which influences the global rate of enzymatic reaction may be the mass transfer of limiting reactant to the enzyme. In particular, the intraparticle mass transfer will be important.

In this study, the oxidation of glucose by immobilized glucose oxidase on activated carbon was chosen to study the intraparticle mass transfer. The intrinsic kinetics and mechanism of the reaction have been studied,[2-6)] and it is well known that catalase is also necessary to decompose hydrogen peroxide formed by reaction (1) (reaction (3)).

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$\text{glucono-}\delta\text{-lactone} \xrightarrow{H_2O} \text{gluconic acid} \quad (2)$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2}O_2 \quad (3)$$

The experiments were performed in 0.1 M acetate buffer solution (pH=5.5), at atmospheric pressure and 25°C. In a stirred tank reactor, the absorption rate of oxygen gas was measured, then the global rate of reaction was calculated. First, to compare the kinetic parameters (V and K_m) for the glucose oxidase with those given in the literature, data were taken with soluble enzymes under negligible gas-to-liquid mass transfer conditions. Next, the immobilized catalyst was used. The rate data with negligible external mass transfer effects were analyzed by a nonlinear least-squares method to separate the kinetic parameters and the effective diffusivity of oxygen in the liquid (D_e).

Experimental

Chemicals——Glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) were both purchased from Sigma Chemical Company and used as supplied. For catalyst preparation, reagent grade carbodiimide was purchased from Merck Japan Ltd., and granular activated carbon was purchased from Nakarai Chemical Company. All other reagents were purchased from Wako Pure Chemical Industries, Ltd.

Preparation of Immobilized Enzymes——Granular activated carbon (6 to 16 mesh) was crushed and sieved. Two average particle sizes were chosen for the support: $d_P = 0.0069$ cm (200 to 250 mesh) and $d_P = 0.025$ cm (48 to 65 mesh). To achieve covalent enzyme linkage to the support, the carbodiimide method[7] was employed. For one gram of carbon particles (dry weight basis), the procedure was as follows. The particles were immersed in 50 ml of 6 N hydrochloric acid solution and left overnight at room temperature. The suspension was heated for 3 h in a water bath to remove contaminating metals as the chlorides. The particles were washed repeatedly with distilled water until the wash water became neutral, then rinsed three times with 50 ml of 0.1 M acetate buffer (pH=5.5). Next, 150 mg of water soluble carbodiimide (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate) was added to *ca.* 5 ml of buffer containing the particles with gentle shaking. The concentration of carbodiimide in the bulk liquid was followed spectrophotometrically at 260 μm. After 30 minutes, the absorbance of the reaction mixture was about 15% of the initial value. When carbodiimide reacts with acetic acid in the buffer solution, the product has nearly the same absorptivity as carbodiimide. Therefore, this result means that carbodiimide reacted with carboxylic acid residues on the carbon particles or was adsorbed by the particles, and did not react to a major extent with acetic acid. Then, the carbon particles were washed three times with 50 ml of buffer. The concentration of carbodiimide which was washed out from the carbon particles was about 2.5% of the consumed amount, indicating that carbodiimide had reacted nearly quantitatively with the carboxylic acid residues. Subsequently, 70 mg of glucose oxidase and 20 mg of catalase were added to *ca.* 5 ml of buffer containing the above carbon particles. The concentrations of both enzymes in the bulk liquid were measured spectrophotometrically at 280 μm. Coupling was carried out at room temperature for 24 h. Only the specific absorption of carbodiimide at 260 μm was detected after that time. This means that enzymes had displaced the bound carbodiimide to form covalent linkages to the carbon particles. Finally, the immobilized enzyme catalyst was rinsed three times with 50 ml of buffer and stored at 5°C. The physical properties of the catalyst particles are shown in Table I.

Table I. Physical Properties of the Catalyst and the Reaction Mixture

Catalyst	
Glucose oxidase content, wt%	7.0
Catalase content, wt%	2.0
Surface area (N_2, BET method), m^2/g	950 [a,b]
Pore volume, cm^3/g	0.991 [a,c]
Solid phase density, g/cm^3	2.18 [a,d]
Particle density, g/cm^3	0.690 [a,c]
Bulk density, g/cm^3	0.382 [a,c]
Porosity of particle, [−]	0.684 [a,d]
Void fraction of bed, [−]	0.446 [a,d]
Reaction mixture	
Density, g/cm^3	1.006 [c]
Viscosity, g/(cm·s)	9.422×10^{-3} [c]
Solubility of oxygen, mol/cm^3	1.12×10^{-6} [c]

a) For activated carbon. *b*) From Nakarai Chemical Company. *c*) Measured. *d*) Calculated.

Apparatus and Operating Procedure——The glass stirred tank reactor was 7.0 cm in diameter with four baffles (0.78 cm in width). The agitater was a six-bladed turbine impeller (2.50 cm in diameter) located at 2.3 cm above the bottom of the reactor. The liquid height was 5.2 cm. In a constant temperature bath, 200 ml of 0.1 M glucose solution, the pH of which had been adjusted to 5.5 with acetate buffer, was saturated with oxygen by continuous bubbling. The temperature was kept constant at 25°C. Immobilized enzyme or soluble enzyme was added to the solution, and the volume of oxygen gas consumed was measured at regular intervals. In the presence of catalase, the overall reaction can be written as follows:

$$\text{glucose} + \frac{1}{2}O_2 \longrightarrow \text{gluconic acid} + H_2O \qquad (4)$$

The concentration of glucose was plotted as a function of reaction time on semilogarithmic graph paper. Under our experimental conditions, the plots were found to be linear during the first ten min, so that the initial rate of reaction was evaluated from the slope of this line and the initial concentration of glucose.

Results and Discussion

First, experiments were performed by adding the soluble enzymes to the 0.3 M glucose solution (pH=5.5). Fig. 1 shows the cumulative amount of oxygen gas absorbed with respect to reaction time. When glucose oxidase and catalase were both added together to the reaction mixture, the reaction went almost completely, as shown by hollow circles, to the stoichiometric end point. On the other hand, in the absence of catalase, the reaction stopped after about 60 min, as shown by solid circles, and was not completed. In the literature,[6,8] this deactivation of glucose oxidase is reportedly caused by hydrogen peroxide, which is produced in reaction (1).

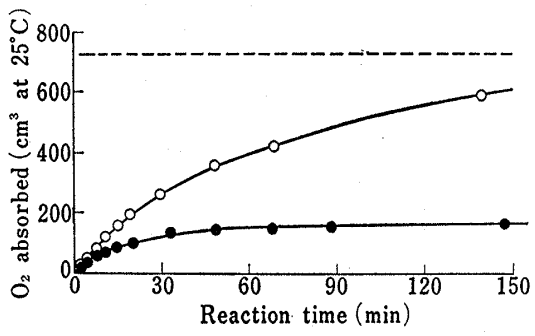

Fig. 1. Absorption Rate of Oxygen Gas in a Stirred Tank Reactor

—○—, glucose oxidase and catalase; —●—, only glucose oxidase; ----, stoichiometric value.

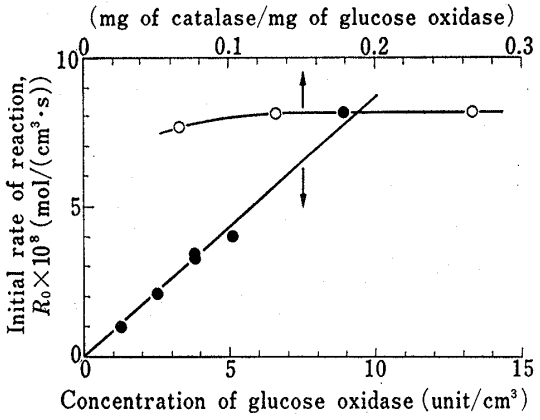

Fig. 2. Effect of the Ratio of Catalase to Glucose Oxidase on the Initial Rate of Reaction (—○—), and the Linear Relationship between the Concentration of Glucose Oxidase and the Initial Rate of Reaction (—●—)

In this reaction system, one mol of oxygen is consumed in reaction (1), and one-half mol of oxygen is produced in reaction (3), due to the decomposition of hydrogen peroxide by catalase. Catalase is also deactivated by hydrogen peroxide.[6,8] Hence, the initial rate of reaction was measured by changing the amount of catalase from 4.7 to 18.7 mg in 200 ml of 0.1 M glucose solution, keeping the amount of glucose oxidase at 70 mg. The effect of catalase concentration on the initial rate of reaction is shown in Fig. 2 by hollow circles. When the concentration of catalase was greater than 10% of that of glucose oxidase, the initial rate of reaction (R_0) was constant. This means that the decomposition rate of hydrogen peroxide was too fast to affect the global rate of reaction in the initial stage,[9] and also the deactivation rates of both enzymes were negligibly small. Then, the absorption rate of oxygen can be considered as the initial rate of reaction according to equation (4). The initial rate of reaction may be expressed by the Michaelis Menten equation as follows:

$$R_0 = \frac{V \times (C_A)_L}{K_m + (C_A)_L} \tag{5}$$

where V is the maximum velocity, K_m is the Michaelis constant and $(C_A)_L$ is the concentration of substrate (glucose).

As interactions between enzyme molecules retard the rate of reaction at high concentrations of enzyme,[10] we checked the relationship between the initial rate of reaction and the

concentration of glucose oxidase. These experiments were performed with 10 to 70 mg of glucose oxidase and 20% of that amount of catalase, at a stirring speed of 1500 rpm. The results are shown in Fig. 2 by solid circles. The linearity of the plots indicates that the interactions between enzyme molecules were negligible, and that the gas-to-liquid mass transfer resistance was also negligible.

Under conditions of negligible mass transfer resistance, the maximum velocity and the Michaelis constant can be determined from the Lineweaver–Burk plot obtained by changing the concentration of glucose. From equation (5),

$$\frac{1}{R_0} = \frac{K_m}{V} \times \frac{1}{(C_A)_L} + \frac{1}{V} \tag{6}$$

In Fig. 3, $(1/R_0)$ is plotted against $(1/(C_A)_L)$. By least-squares analysis, V and K_m were calculated to be 5.6×10^{-8} [mol/(cm^3·s)] and 7.2×10^{-5} [mol/cm^3], respectively. This value of the Michaelis constant is of the same order as those in the literature.[3,4,6]

Next, experiments were performed by adding insoluble (immobilized) enzyme to the reaction mixture. In order to determine the minimum quantity of carbodiimide required to achieve covalent enzyme linkage to the activated carbon, experiments were done with various concentrations of carbodiimide. The catalyst particles of d_P=0.0069 cm (200 to 250 mesh) were used and the stirring speed was kept constant at 1500 rpm. The carbodiimide method to form the covalent enzyme linkage was proposed by Cho and Bailey.[7] The initial rates are plotted against the ratio of carbodiimide to activated carbon weight in Fig. 4. At ratios of above 10%, the initial rates were almost constant. Hence, 15% carbodiimide was used to prepare the immobilized enzymes in all of the following experiments.

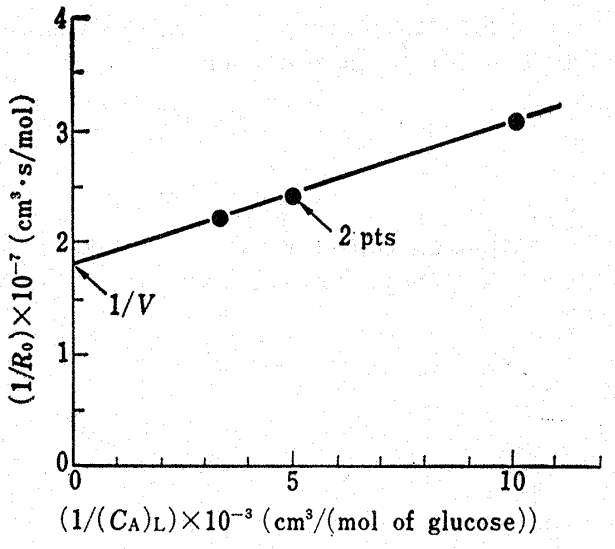

Fig. 3. Reciprocal Glucose Concentration *vs.* Reciprocal Initial Rate of Reaction

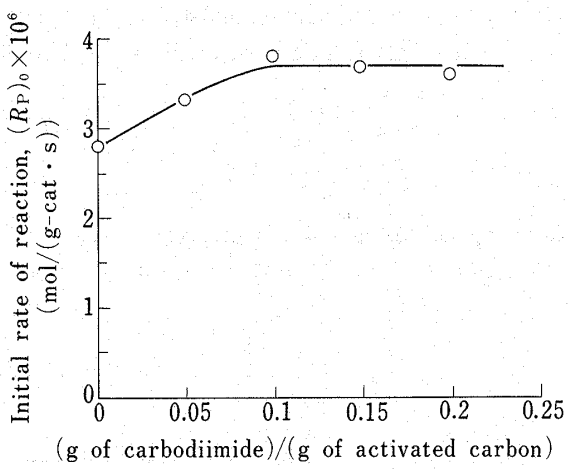

Fig. 4. The Concentration of Carbodiimide required to achieve Covalent Enzyme Linkage to the Activated Carbon

In order to consider the effect of external mass transfer resistances (gas to liquid and liquid to solid), the stirring speed was changed from 900 to 2200 rpm. The catalyst particles of d_P=0.0069 cm were used. The results are shown in Fig. 5 by hollow circles. Above 1300 rpm the initial rates were almost constant, indicating that the external mass transfer resistances were negligible. Under the same experimental conditions, the catalyst of d_P=0.025 cm (48 to 65 mesh) was used at 1500 rpm. The initial rate (solid circle in Fig. 5) was much smaller than that for catalyst of d_P=0.0069 cm. Therefore, it was clear that the intraparticle mass transfer resistance was significant. Hence, the resistance was considered in the following experiments.

In several earlier studies, different dependencies of the apparent rate parameters V and K_m on the concentration of oxygen in the liquid have been reported. Nakamura *et al.*,[4] Gibson *et al.*[3] and Cho *et al.*[6] reported that both V and K_m varied with the concentration of oxygen in such a way that K_m/V was constant. Hsieh *et al.*,[5] on the other hand, found that V changed with the concentration of oxygen, while K_m was independent of the concentration of oxygen. In this study, we assumed that both V and K_m would be proportional to the concentration of oxygen. Under this assumption, the kinetic equation can be expressed as

$$R_0 = \frac{kC_i \cdot (C_A)_L}{KC_i + (C_A)_L} \tag{7}$$

where C_i is the concentration of oxygen in the liquid, and k and K are the proportional coefficients of V and K_m, respectively. Under our experimental conditions, the concentrations of glucose and oxygen were 1.00×10^{-4} [mol/cm^3] and less than 1.12×10^{-6} [mol/cm^3], respectively. Stoichiometrically, one-half mol of oxygen is consumed per mol of glucose. The concentration of glucose was more than 90 times larger than that of oxygen, so that the former can be considered constant in every part of the reaction system, without serious error. When the external mass transfer resistances are negligible, therefore, an internal mass balance equation could be considered only for oxygen. The saturated concentration of oxygen in the reaction mixture, determined by gas chromatography, was about 91% of that in the distilled water. Other physical properties of the reaction mixture are shown in Table I. The catalyst particles may be regarded as spheres. Then, the internal mass balance equation with respect to oxygen can be expressed as

$$\frac{d^2C_i}{dr^2} + \frac{2}{r} \cdot \frac{dC_i}{dr} - \frac{\rho_P}{D_e} \cdot \frac{kC_i \cdot (C_A)_L}{(KC_i + (C_A)_L)} = 0 \tag{8}$$

where ρ_P is the particle density, D_e is the effective diffusivity of oxygen in the liquid, and r is the radial distance in the particle. The boundary conditions can be written as

$$r = 0; \quad \frac{dC_i}{dr} = 0 \tag{9}$$

$$r = r_S; \quad C_i = C_L^* \tag{10}$$

where r_S is the radius of particles, and C_L^* is the saturated concentration of oxygen. As mentioned above, there was no external mass transfer effect under our experimental conditions. Hence, $C_i = C_L^*$ at $r = r_S$.

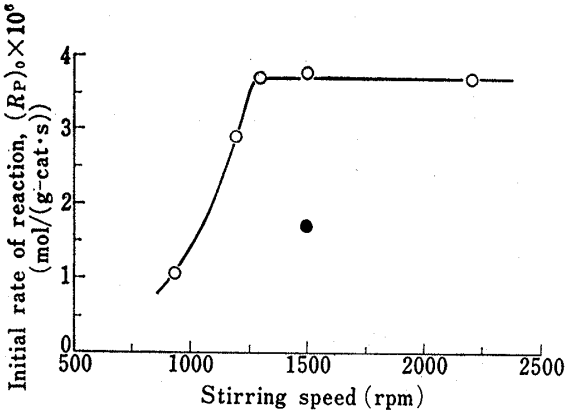

Fig. 5. The Effect of External Mass Transfer Resistances on the Initial Rate of Reaction (—○—), $d_P = 0.0069$ cm, and the Significance of Intraparticle Mass Transfer Resistance (●), $d_P = 0.025$ cm

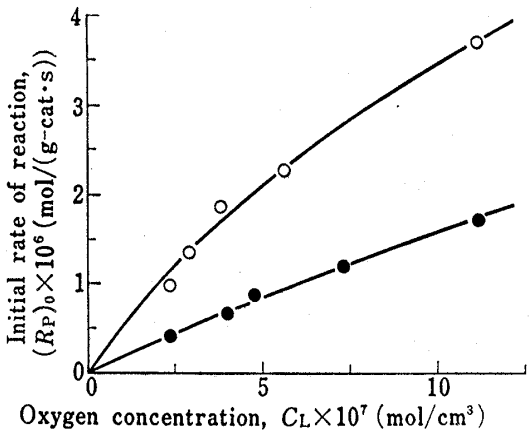

Fig. 6. The Effect of Oxygen Concentration on the Initial Rate of Reaction for Immobilized Enzymes

—○—, $d_P = 0.0069$ cm; —●—, $d_P = 0.025$ cm.

The values of V, K_m and D_e were estimated by nonlinear least-squares analysis, using equations (8) to (10). The data presented in Fig. 6 were used for the analysis. As equation (8) does not hold at $r=0$, it was assumed that $dC_i/dr=0$ at $r=10^{-10}\cdot r_S$. Assigning arbitrary values to V, K_m and D_e, equation (8) was integrated numerically with $\Delta r=0.01\cdot r_S$. Even when $r/r_S=10^{-4}$ and $\Delta r=0.02\cdot r_S$, the errors inherent in calculation were found to be negligible (less than 0.1%). The global rate of reaction can be written as

$$R_0=\frac{3\cdot D_e}{r_S\rho_P}\left(\frac{dC_i}{dr}\right)_{r=r_S} \tag{11}$$

Then, the numerical integration was repeated until $\sum\{(R_0)_{obsd}-(R_0)_{calcd}\}^2$ was minimized. The 95% confidence limits for V, K_m and D_e were all $\pm 20\%$ of the corresponding converged values. Thus, the assumption made above, that V and K_m were both proportional to the concentration of oxygen in liquid, can be considered reasonable. For comparison, these converged values are summarized in Table II together with those obtained in the soluble system. The value of maximum velocity in the immobilized system, which was converted into the dimensions of [mol/(unit·s)], was about 1/3 of that in the soluble system. This decrease of maximum velocity may be mainly caused by enzyme deactivation during the immobilization reaction and by the steric hindrance of fixed enzymes. On the other hand, the Michaelis constants were nearly the same.

Table II. The Values of Apparent Kinetic Parameters

	Insoluble[a]	Soluble[b]
V, mol/(unit·s)	0.46×10^{-8} [c]	1.45×10^{-8}
K_m, mol/cm^3	9.46×10^{-5}	7.21×10^{-5}
V, mol/(g-cat·s)	8.30×10^{-6}	
D_e, mol/cm^3	7.80×10^{-6}	
$\tau=D\cdot\varepsilon_P/D_e$, [−]	2.0	

a) Estimated by nonlinear least-squares analysis.
b) Estimated from Lineweaver-Burk plots.
c) Converted from $V=8.30\times10^{-6}$ mol/(g-cat·s).

The effect of intraparticle mass transfer is to reduce the rate of reaction below what it would be if there were no internal concentration gradient. The effectiveness factor (η) of the catalyst particles is defined as follows;

$$\eta=\frac{\text{actual rate for the catalyst particle}}{\text{rate evaluated at outer surface conditions}} \tag{12}$$

When $\eta\to1$, intraparticle mass transfer has no effect on the rate of reaction; that is, the chemical step controls the rate. In contrast, when $\eta\to0$, only the surface near the outer periphery of the particle is effective; that is, the concentration drops from C_L^* to nearly zero in a narrow region near r_S, so that the central portion of the particle is not utilized. Such a situation is caused by large particle size, low diffusivity, or a rapid intrinsic rate of reaction.

For this reaction system, the global rate of reaction can be expressed with η as follows;

$$R_0=\eta\cdot\frac{kC_L^*\cdot(C_A)_L}{KC_L^*+(C_A)_L} \tag{13}$$

Combining equations (11) and (13) gives

$$\eta=\frac{3[KC_L^*+(C_A)_L]\cdot D_e}{[kC_L^*\cdot(C_A)_L]\cdot r_S\rho_P}\left(\frac{dC_i}{dr}\right)_{r=r_S} \tag{14}$$

For each run, η was calculated by means of equation (14). When pure oxygen was used as a gas phase ($C_L^*=1.12\times10^{-6}$ [mol/cm^3]), the calculated values of η were 0.870 and 0.389 for

d_P=0.0069 cm and 0.025 cm, respectively. When air was used ($C_L^*=2.34\times10^{-7}$), η=0.751 and 0.294 for d_P=0.0069 and 0.025, respectively. These results indicate that the intraparticle mass transfer of oxygen is more significant for the large particle than for the small one, though even for the small particle size, only about 80% of the immobilized enzyme took part in the reaction effectively. Therefore, it is desirable for the effective use of enzymes to reduce the enzyme content in the particle and/or to decrease the particle size.

The tortuosity factor of porous particles (τ), which takes into account the geometrical structure of the pores, is defined as

$$\tau = \frac{D\cdot\varepsilon_P}{D_e} \tag{15}$$

where D is the molecular diffusivity and ε_P is the porosity of particles. The value of D for oxygen in the liquid was assumed to be identical with that in water.[11] With the adopted values of D_e and ε_P, the value of τ was calculated as 2.0. Tortuosity factors of the order of 2 to 5 have been reported for some kinds of porous particles,[12] so that the obtained value of D_e seems to be reasonable.

Our results can be summarized as follows. The rate of oxidation of glucose on immobilized glucose oxidase and catalase on activated carbon can be well explained by equation (7), which may be used in reactor design for this reaction by employing estimated values of V, K_m and D_e. The intraparticle mass transfer of oxygen is a very significant factor in this reaction system, so that, for the effective use of enzymes, the above considerations are important.

In a subsequent paper,[13] an analysis of this reaction carried out in a trickle-bed reactor will be presented, using the results obtained in the present work.

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