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Comparison Between Experimental and Theoretical Values of Effectiveness Factor in Cephalosporin C Production Process with Immobilized Cells

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ABSTRACT

Cells of *Cephalosporium acremonium* ATCC 48272 immobilized in calcium alginate beads were utilized for cephalosporin C production and the results were compared with those obtained with free cells. The experiments were performed with synthetic medium containing glucose and sucrose as carbon and energy sources. Experimental effectiveness factor values were obtained at various cell and dissolved-oxygen concentrations, considering Monod kinetics for the respiration rate, and were compared with the values calculated with zero-order kinetics in spherical bioparticle. The results showed that the assumption of oxygen limitation by diffusion in the bioparticle was correct, and that cephalosporin C production with immobilized cells is perfectly viable, although a slightly lower rate than that obtained in the free cell process was observed.

Index Entries: Cephalosporin C production; immobilized cells; respiration rate; effectiveness factor

INTRODUCTION

Cephalosporin C is a β-lactam antibiotic with some biological activity; its importance lies in the fact that it is the raw material for obtaining several semisynthetic antibiotics. Industrially, high-yield strains of the strictly aerobic filamentous fungus *Cephalosporium acremonium* are utilized through submerged cultures in aerated stirred-tank bioreactors, resulting in highly viscous non-Newtonian fermentation broths. The use of immobilized filamentous fungi in fluidized-bed tower bioreactors is a promising alternative

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for minimizing gas–liquid oxygen mass transfer problems, since viscosity reduction is brought about in fermentation broth *(1,2)*. Despite the fact that these unconventional bioprocesses present many advantages when compared to processes utilizing free cells, a rigorous examination of the intraparticle mass transfer limitations, mainly concerning oxygen transport, should be carried out in order to get a better appraisal of the advantages of these processes.

The effect of diffusion limitation of several essential nutrients in gel matrices has been investigated by many authors *(3–6)*. However, most of these studies were conducted using gel either with entrapped dead cells or without cells, and in several studies transfer phenomena involving these biocatalysts have been evaluated only through numerical techniques and modeling, without experimental observations.

In this work, cephalosporin C production processes with both free and immobilized cells were compared through the investigation of the limitation created by oxygen diffusion into gel beads on the process rate. Kinetic parameters for the respiration rate, considering Monod kinetics model, were previously estimated by using experimental data of free cell assays. The effective oxygen diffusivity during the immobilized cell process was estimated considering a dead core model for bioparticle *(7,8)*. These data allowed calculation of experimental effectiveness factor values *(8–10)*, which were compared with a theoretical zero order effectiveness factor vs Thiele modulus curve. Furthermore, the influence of sugar type and concentration on the respiration rate was investigated for the process carried out with free cells.

MATERIALS AND METHODS

Microorganism

C. acremonium ATCC 48272 (C-10).

Culture Media

Inocula cultures were prepared in a synthetic medium containing (in g/L): glucose (30.0), ammonium acetate (8.8), DL-methionine (5.0), oleic acid (1.5), K_2HPO_4 (5.8), KH_2PO_4 (2.3), $CaCO_3$ (2.0), $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.16), Na_2SO_4 (0.81), $MgSO_4 \cdot 7H_2O$ (0.384), $CaCl_2 \cdot 2H_2O$ (0.08), $MnSO_4 \cdot H_2O$ (0.032), $ZnSO_4 \cdot 7H_2O$ (0.032), $CuSO_4 \cdot 5\ H_2O$ (0.002), pH 7.0 ± 0.1.

The main fermentation medium was based on Shen et al. *(11)*, and adapted to the nutritional requirements of the current strain. Its composition was (in g/L): glucose (27.0), sucrose (36.0), ammonium acetate (8.8), DL-methionine (5.0), oleic acid (1.5), K_2HPO_4 (2.97), KH_2PO_4 (1.08), $CaCO_3$ (2.0), with the same inorganic salts as the inoculum medium composition, pH 7.0 ± 0.1. For the immobilization cell process, the same medium was

used, but, to maintain the integrity of the gel beads, K_2HPO_4 was suppressed, KH_2PO_4 concentration was changed to 1.5 g/L, and $CaCl_2 \cdot 2H_2O$ (1.0 g/L) was supplied to the medium.

Analytical Methods

Cell Concentration

The cell mass was represented as volatile suspended solids (VSS), in g/L. For the measurement of biomass in the gel beads, they were previously dissolved in a 0.5 *M* K_2HPO_4 solution (~20 mL/50 gel beads), according to the procedure used by Khang et al. *(12)*.

Sugar Concentration

To determine glucose concentration, GOD-PAP enzymatic method was used. For sucrose measurement, samples were previously hydrolyzed in an acid medium and GOD-PAP method was carried out.

Antibiotic Concentration

Cephalosporin C titers were determined by an agar diffusion bioassay using *Alcaligenes faecalis* ATCC 8750 *(13)*.

Experimental Procedure

Cephalosporin C Production Processes

The fermentation runs were performed in shaken flasks at 250 rpm, 26°C. The inoculum preparation procedure was the same for both processes (free and immobilized cells). Initially, resuspended lyophilized cells of *C. acremonium* were cultivated in slants containing complete medium *(11)* for 7 d at 26°C. The main fermentation inoculum was obtained after two consecutive precultures carried out in shaken flasks, for 48 h (germination) and 24 h, respectively. A percentage of 10% vol inoculum/vol culture medium was maintained between the several stages. Main fermentation periods with free and immobilized cells were of 144 and 168 h, respectively. Samples were taken every 24 h to measure pH, cell concentration, sugars, cephalosporin C, and respiration rate.

Gel bead preparation was similar to that proposed by Khang et al. *(12)*, but for the present work some modifications were made. Initially, a suspension, composed of 17% volume of the final preculture broth per total desired volume mixture of gel, alumina, and cells, was centrifuged for 2 min at 1106*g*, 6°C. Part of the supernatant was then discarded, so that the resulting concentrated cell suspension volume was approximately half the initial one. This suspension was added to a mixture of sodium alginate (20.0 g/L) and alumina of less than 325 mesh (10.0 g/L), to complete the desired volume. Afterwards, the mixture of gel, alumina, and cells was dripped through a needle into a stirred solution $CaCl_2 \cdot 2H_2O$

0.1 *M* (~600 mL solution for each 150 mL of the mixture). After preparation, the gel beads were cured in this solution for approx 1.5 h at room temperature, and then washed twice with 300 mL deionized water. The initial average diameter of the gel beads obtained was 2.2 ± 0.1 mm. For the immobilization process, the main fermentation broth was inoculated at a ratio of 15% vol gel beads/volume culture medium, which corresponded approximately to the amount of inocula from the fermentation with free cells. The diameter of the gel beads was determined with a magnifying glass throughout fermentation. An average diameter of 2.7 ± 0.15 mm was obtained during the whole process.

Measurements of Respiration Rate

For measurement of respiration rate, Yellow Springs Instrument model 5300 was used, attached to a recorder and to a dissolved oxygen analyzer. The equipment is comprised of controlled temperature (26°C) water bath, with appropriate ports in which measuring flasks (3 mL working volume), supplied with magnetic stirrers, can be inserted. To these flasks a dissolved oxygen electrode was attached *(9)*.

Investigation of sugar influence on respiration rate was carried out with free cells. For the measurements, 10–20 mL fermentation broth was centrifuged (1106*g*, 6°C), the supernatant was discarded, and the compacted cells were diluted to 1:10 in fresh production medium and added to the measuring flasks. Seven glucose concentration values, between 0.2 and 19.0 g/L, and eight sucrose concentration values, between 0.5 and 31.0 g/L were utilized during free cell measurements. Blank measurements were carried out, with no sugar medium. The biomass varied from 0.9 to 1.45 g cells/*L*. Measurements were replicated 4–6×.

For experimental effectiveness factor determination, free and immobilized cell respiration measurements were performed in a fresh production medium containing 10.0 g/L glucose. For immobilized cell measurements, a given amount of gel beads (30–35 bioparticles) were introduced into the flasks containing fresh production medium. The free and immobilized cell concentrations, C_x and $C_{x\,biop}$, were determined following respiration rate measurements. Their values were between 0.9 and 1.8 g cells/L for the free cells, and 1.0 and 6.8 g cells/L for the immobilized cells. Replicate measurements were made, as in sugar influence on respiration study.

Estimation of Oxygen Intraparticular Diffusion Coefficient

The oxygen diffusivity, De_{O_2}, in Ca-alginate gel beads containing viable cells was determined by utilizing apparatus similar to that used by Kurosawa et al. *(14)*. By means of this system, fresh medium saturated with oxygen passed continuously at a controled flow rate through a measuring flask of 220 mL containing a given number of gel beads. This flask was equipped with a magnetic stirrer and a water bath at 26°C. The mea-

surements were made with gel beads taken after 92 h of the main fermentation *(8)*. By cutting the particle with a blade, a biolayer of cells inside the gel bead of about 200 μm was observed through a microscope with scaled eye-pieces, evidencing the existence of a dead core in the bioparticle. The measured values of the bioparticle radius, R_p, and the critical radius, R_{cr}, were 1.35 ± 0.05 and 1.15 ± 0.05 mm, respectively.

The reaction–diffusion model was then developed, considering the existence of a dead core in the bioparticle. This model was obtained through differential mass balances for oxygen in quasisteady state over the measuring flask and inside the gel bead along r, from R_p to R_{cr}. The oxygen diffusivity coefficient was estimated by nonlinear regression method, according to Marquardt's numerical method *(15)*, with 95% confidence interval *(8)*.

Determination of Effectiveness Factor

The effectiveness factor is defined as follows *(7,16)*:

$$\eta = \text{rate of reaction with pore diffusion resistance/ rate of reaction with surface conditions} \quad (1)$$

Applied to the respiration rate at a cell concentration C_x, the effectiveness factor definition (Eq. 1) results in Eq. 2:

$$\eta = r_{obs}/R_{O_2} \cdot C_x \quad (2)$$

where r_{obs} and $R_{O_2} \cdot C_x$ refer to respiration rate with and without oxygen diffusion limitation, and C_x is the free-cell concentration expressed as biomass contained in the gel bead by culture medium volume.

In order to develop a mathematical model of the immobilized cell respiration rate, r_{obs}, the following assumptions were made:

1. The respiration rate follows Monod kinetics.
2. The gel beads are approximately spherical (mean radius R_p).
3. The biomass is homogeneously distributed in the bioparticle.
4. The medium is perfectly stirred in relation to the liquid phase.
5. Resistance to oxygen transfer through the external film around gel bead is negligible.
6. Radial oxygen flow and oxygen diffusion rate follow Fick's law.
7. The effective intraparticular diffusivity of oxygen remains constant during the process.

In quasisteady state, the global oxygen consumption rate of the encapsulated cells equals the diffusive flow of oxygen through the surface and the variation in oxygen concentration with time at the surface of the gel bead, expressed as:

$$r_{obs} = A_p/V_p \cdot De_{O_2} \cdot (dC_{O_2}/dr|_{r=R_p}) = dC_{O_2}/dt|_{r=R_p} \quad (3)$$

where V_p and A_p are the particle volume and external surface area, respectively.

Values of r_{obs} were determined in several oxygen and cell concentrations, by means of the derivative of the curves obtained during respiration rate measurements of the immobilized cells. The experimental effectiveness factors were then calculated using Eq. 2.

By applying zero order reaction equation to the Monod kinetics, the Thiele modulus, ϕ_0, can be calculated as follows:

$$\phi_0 = \frac{R_p}{3}\sqrt{R_{max} \cdot C_x^{biop}/2 \cdot De_{O_2} \cdot C_{O_2}|_{r=R_p}} \quad (4)$$

where R_{max} is the maximum specific respiration rate and C_x^{biop} is the gel bead cell density in g cell/L gel.

RESULTS AND DISCUSSION

Comparison Between Cephalosporin C Fermentation by Free and Immobilized Cells

The experimental results obtained in processes by either free or immobilized cells, using synthetic medium containing glucose and sucrose as the main carbon and energy sources, are shown in Figs. 1 and 2, respectively. As can be observed, both processes present two consecutive and distinct phases, as in most secondary metabolite production processes. The first phase is characterized by high cellular growth rate with the assimilation of the rapidly metabolizable sugar (glucose), resulting, however, in low production rate. In the second phase, the consumption of the more slowly assimilated carbohydrate (sucrose) results in higher production rates and insignificant biomass increase. This diauxic behavior occurs in such a way that, in the initial fermentation period, the synthesis of enzymes responsible for antibiotic production is repressed by glucose. When this sugar concentration value becomes sufficiently low, the β-lactam synthetases production is released and the microorganism is able to synthesize cephalosporin C at high rates *(17)*.

The process with immobilized cells (Fig. 2) showed slower global rate and productivity: about 75%, compared with the process with free cells (Fig. 1). However, the productivity in relation to sucrose consumption obtained in fermentation with bioparticles was around 1.4 times higher than that obtained with free cells, showing that the new process is a promising alternative to be carried out in continuous or semicontinuous tower type bioreactors for long periods of time.

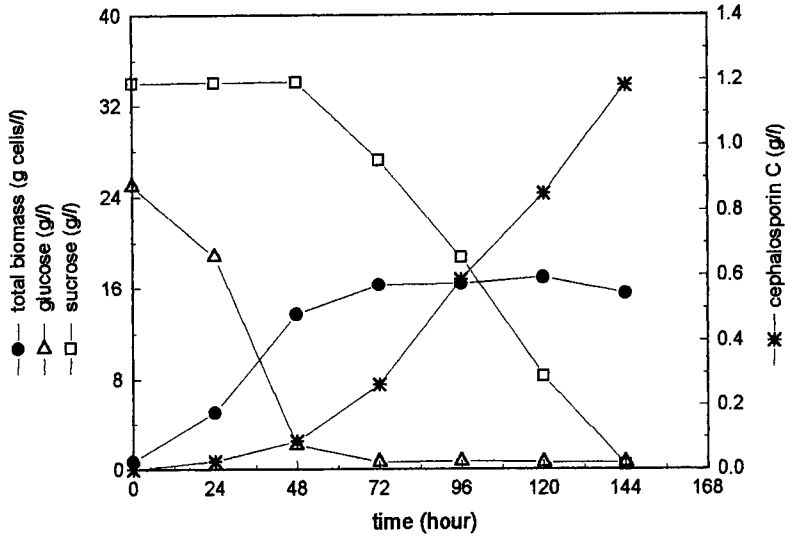

Fig. 1. Time-course of cephalosporin C batch process with synthetic medium in shaken flasks by free cells of *C. acremonium* ATCC 48272.

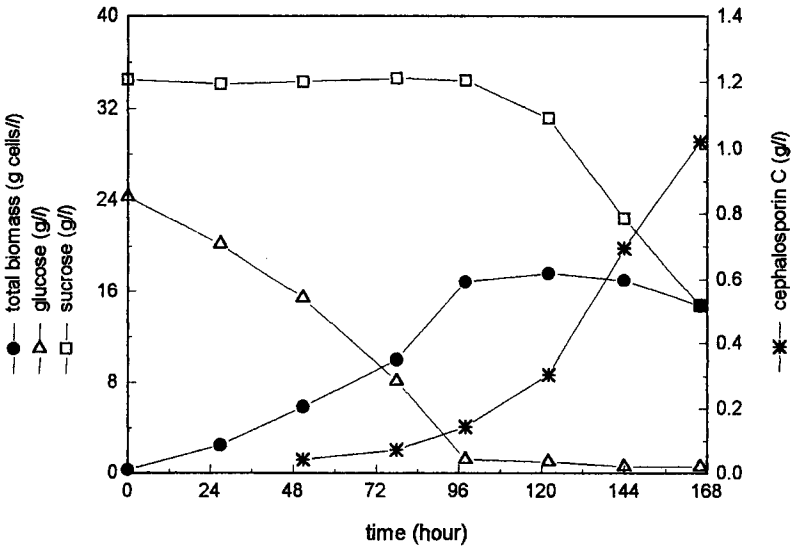

Fig. 2. Time-course of cephalosporin C batch process with synthetic medium in shaken flasks by immobilized cells of *C. acremonium* ATCC 48272 in gel beads of Ca-alginate containing alumina.

Free and Immobilized Cell Respiration Rate Measurements

Because the diauxic phenomenon strongly affects the production characteristics of this process, it was initially supposed that the specific respiration rate would be influenced by the oxygen concentration, and also by the type and concentration of sugar according to Monod equation.

The following equation represents the differential mass balance of oxygen inside the measuring flask during measurements with free cells:

$$-dC_{O_2}/dt = R_{O_2} \cdot C_x = R_{max} \cdot [C_{s_i}/(k_{s_i} + C_{s_i})] \cdot [C_{O_2}/(k_{O_2} + C_{O_2})] \cdot C_x \qquad (5)$$

where C_{O_2} is the dissolved oxygen concentration, R_{O_2} is the specific respiration rate, C_x is free cell concentration, C_{s_i} is the specific sugar concentration (glucose or sucrose), and k_{O_2} and k_{s_i} are the Monod saturation constants for oxygen and type of sugar, respectively.

Figure 3 shows the specific respiration rate data calculated from analysis of the 72 h cultivation time measurements with free cells. The oxygen consumption behavior was observed to follow a zero order kinetics in relation to glucose as well as sucrose, so that the respiration rate was not affected by the type and concentration of sugar. Furthermore, the same behavior described above was observed in blank measurements, proving that oxygen is the sole respiration rate limiting reagent.

Therefore, based on the above results, respiration rate expression can be simplified to:

$$-dC_{O_2}/dt = R_{O_2} \cdot C_x = R_{max} \cdot [C_{O_2}/(k_{O_2} + C_{O_2})] \cdot C_x \qquad (6)$$

Typical graphics of oxygen concentration vs time obtained with free and immobilized cells are shown in Fig. 4. In the free-cell systems, oxygen consumption is observed to be directly associated with the respiration rate, which seems to follow zero-order type reaction kinetics; the oxygen profile observed during measurements with immobilized cells shows that diffusion rate remarkably affects oxygen consumption rate.

Free cell measurements allowed estimation of the kinetic parameters, R_{max} and k_{O_2}, as 3.254 ± 0.608 ($molO_2/g$ cell. s) × 10^7 and 2.771 ± 0.730 ($molO_2/L$) × 10^6, respectively, through classical nonlinear least-square regression method, according to Marquardt's procedure *(15)*, with 95% confidence intervals *(9)*. Immobilized cell respiration rates were determined through the derivatives calculated along the oxygen profiles.

Determination of Experimental Effectiveness Factor

The experimental effectiveness factor values were calculated using the estimated kinetic parameters, and effective oxygen diffusivity, De_{O_2},

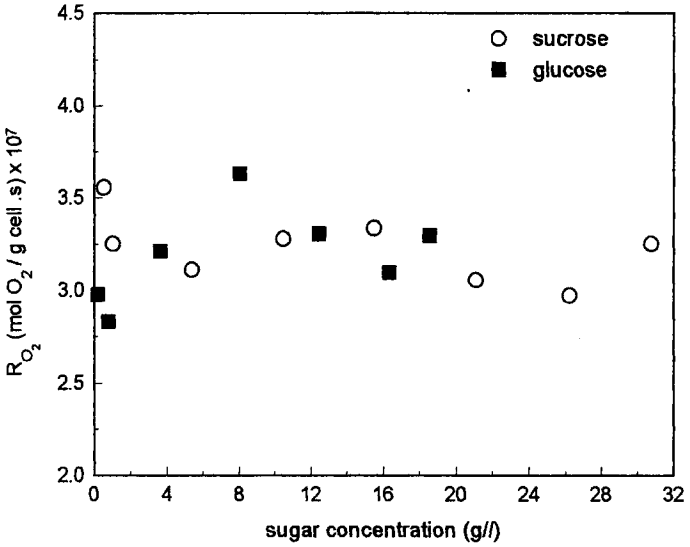

Fig. 3. Typical data of specific respiration rate of *C. acremonium* ATCC 48272 vs sugar concentration obtained during free cells process, after 72 h fermentation time.

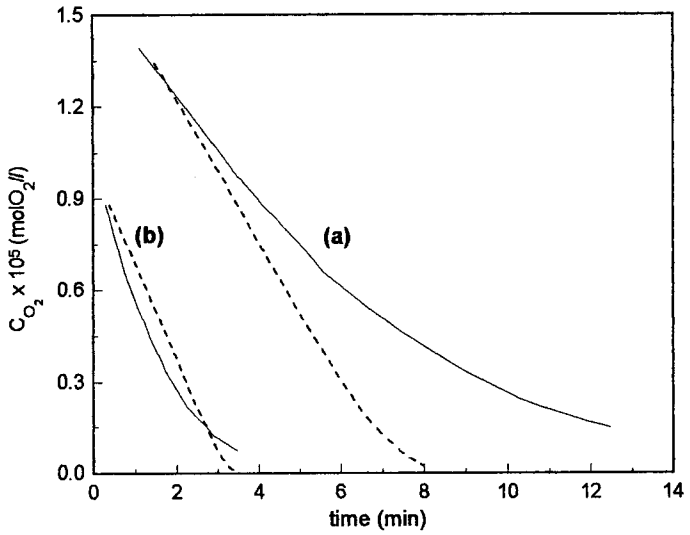

Fig. 4. Typical curves of respiration measurements with free cells (---) and immobilized cells (—) obtained after (a) 24 h cultivation time (C_x = 1.04 g cell/L; $C_{x\ biop}$ = 1.0 g cell/L) and (b) 96 h cultivation time (C_x = 1.89 g cell/L; $C_{x\ biop}$ = 6.3 g cell/L).

estimated as 1.913 ± 0.227 (m^2/s) × 10^9 *(8)*. The effectiveness factor data are presented in Table 1.

The relationship of the dissolved oxygen concentration variation values vs cell mass concentration is shown in Fig. 5. From the slopes of the

Table 1
Experimental Effectiveness Factor Values Obtained Through Respiration Rate Measurements with Free and Immobilized Cells

C_{O_2} (molO_2/L) $\times 10^5$	8.8	7.7	6.6	5.5	4.4	3.3	2.2
C_x^{biop} (g cell/L gel)	η_{exp}						
9.4	0.685	0.648	0.594	0.514	0.441	0.363	0.325
21.3	0.509	0.471	0.432	0.377	0.317	0.258	0.228
33.0	0.565	0.535	0.474	0.405	0.317	0.283	0.253
58.4	0.533	0.509	0.445	0.370	0.313	0.258	0.223
66.4	0.501	0.471	0.410	0.347	0.296	0.247	0.218

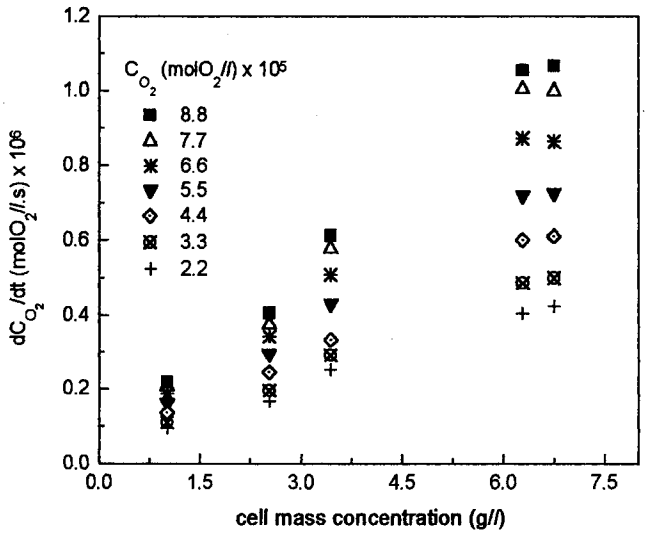

Fig. 5. Experimental respiration rate data obtained at various dissolved oxygen concentration and cell mass concentration values.

straight lines corresponding to each oxygen concentration, the plot of Fig. 6 was obtained, showing a pseudo first order type relationship. This clearly suggests that the process with the bioparticles is limited by intraparticular oxygen mass transfer. This has already been shown that, for free cells, oxygen consumption rate behaves as a zero-order type reaction most of the time.

Plot of experimental effectiveness factor values vs calculated Thiele modulus for zero-order reaction (ϕ_0), compared with theoretical effectiveness factor η vs ϕ curve, is presented in Fig. 7.

In this figure, the experimental data obtained with low cell density bioparticles (Table 1), withdrawn at the beginning of the process, agree satisfactorily with the theoretical zero-order curve. However, the effective-

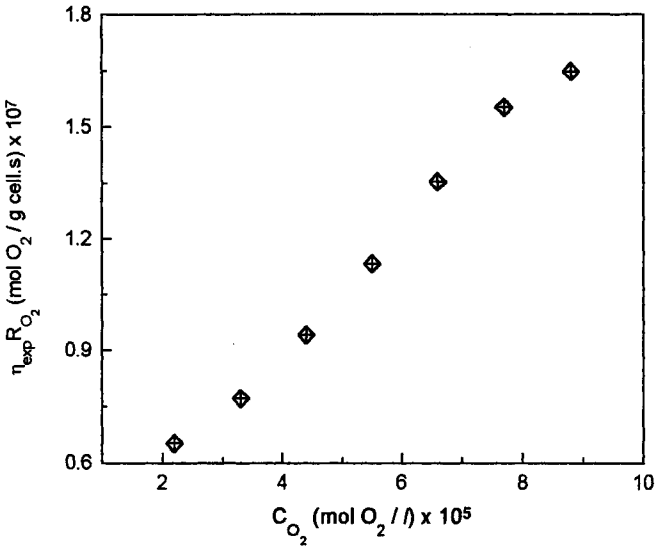

Fig. 6. Plot of immobilized cell specific respiration rate vs dissolved oxygen concentration.

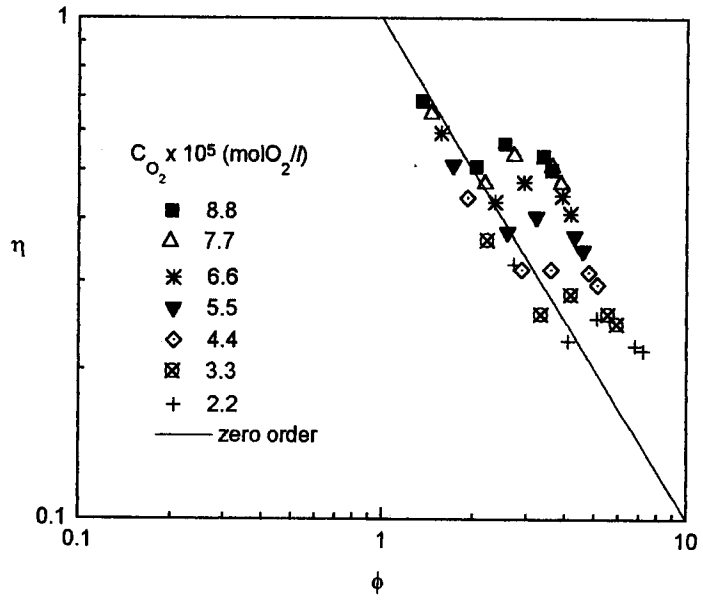

Fig. 7. Plot of the experimental effectiveness factor data vs calculated ϕ_o, superimposed on theoretical η vs ϕ_o curve.

ness factor experimental values obtained with higher cell density particles, withdrawn after approx 48 h process time, begin to deviate from the theoretical values. The cause of this behavior probably lies in the fact that, although the intraparticular cell density remains at low values, the oxygen

diffusion rate is sufficiently high to support maximum respiration rate, and, when the gel bead density increases in the course of the process, the diffusional limitations predominate, leading to more difficult oxygen penetration into the particle, and resulting in the reaction rate becoming oxygen-concentration dependent. Furthermore, the observed biolayer presence near the internal bioparticle surface, where total biomass was concentrated, results in a higher gel bead cell mass density, and a modified Thiele modulus based on dead core model must be utilized. At present, a study considering the effectiveness factor calculations for such a biolayer inside the particle is being carried out to compare with the experimental effectiveness factor values obtained in this work.

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