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External and Internal Glucose Mass Transfers in Succinic Acid Fermentation with Stirred Bed of Immobilized *Actinobacillus succinogenes* under Substrate and Product Inhibitions

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This paper is dedicated to the study on the external and internal mass transfers of glucose for succinic acid fermentation under substrate and product inhibitions using a bioreactor with stirred bed of immobilized *Actinobacillus succinogenes* cells. By means of the substrate mass balance for a single particle of biocatalysts, considering the kinetic model adapted for both inhibitory effects, specific mathematical models were developed for describing the profiles of the substrate concentration in the outer and inner regions of biocatalysts and for estimating the substrate mass flows in the liquid boundary layer surrounding the particle and inside the particle. The values of the mass flows were significantly influenced by the internal diffusion velocity and rate of the biochemical reaction of substrate consumption. These cumulated influences led to the appearance of a biological inactive region near the particle center, its magnitude varying from 0 to 5.3% of the overall volume of particles.

Keywords: Succinic acid fermentation, stirred bed, immobilized cells, *Actinobacillus succinogenes*, internal diffusion, mass transfer

Succinic acid is a dicarboxylic acid with numerous applications in the chemical industry (reagents, synthetic resins, biodegradable polymers, electroplating, green solvents, inks), agriculture (pesticides, growth regulators and stimulators), and pharmaceutical and food industries (amino acids, antibiotics, vitamins, surfactants, additives) [17, 22, 29, 31].

Succinic acid is industrially produced using liquefied petroleum gas, namely butane, by chemical synthesis *via* maleic anhydride [31]. The cost of this technology varies between 4.1 to 6.3 EUROs depending on the acid's final purity, with the contribution of raw material to this cost being of 1 EURO/kg succinic acid [17, 31]. Besides the difficulty, the downstream processes and the chemical technology raise important problems concerning environmental protection [17].

The "white biotechnology," concept that has been promoted at the International Conference "European Bioperspectives - En Route to a Knowledge-Based Bio-Economy" (Cologne, Germany, May 31 – June 1, 2007), sustains the priority of the use of renewable sources for chemicals production by low-expense and eco-friendly biotechnologies [3]. In these circumstances, the interest in producing succinic acid by fermentative low-cost technologies has increased in the last years. Thus, a large number of microorganisms that are potentially producers of succinic acid have been tested: bacteria (*Veillonella parvula, Selenomonas ruminatum*, *Succiniclasticus ruminis*, *Corynebacterium glutamicum, Enterococcus fecalis*, *Actinobacillus succinogenes*, *Actinobacillus succiniproducens*, *Mannheimia succiniproducens*, *Escherichia coli*) [5, 7, 11–14, 16, 19, 20, 25], yeasts (*Saccharomyces cerevisiae*) [1], and fungi (*Aspergillus niger*, *Aspergillus fumigatus*, *Byssochlamys nivea*, *Lentinus degener*, *Paecilomyces varioti*, *Penicillium viniferum*) [6, 15, 18].

However, either as a result of the low yield of substrate bioconversion into succinic acid, or of the non-Newtonian

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rheology and complex composition of the final broth, only the strain types of *Actinobacillus succinogenes* and *Actinobacillus succiniproducens* have been considered as important producers of this acid. These microorganisms possess the ability to convert various carbon sources under anaerobic conditions into succinic acid and secondary acids (formic, acetic, pyruvic acids). *A. succinogenes* has been cultivated on media containing glucose, saccharose, molasses, glycerol, starch, cellulosic hydrolysates, or milling by-products as the carbon source [4, 7, 14, 16, 27].

Most of the fermentative systems for succinic acid production have been carried out using free *A. succinogenes* cells, the process being affected by the substrate and product inhibition phenomena [14, 16]. There are no information concerning the use of immobilized *A. succinogenes*, excepting its growth as biofilm on inert supports of composite materials [23, 24]. However, the formation of biofilm cannot avoid the inhibitory effects.

As was previously concluded, the use of immobilized microorganisms or enzymes offers the advantages of the increase of the thermal, chemical, and to the shear forces resistance of the biocatalysts. Other advantages include the diminution or avoidance of the inhibition processes, the easier recovery of the biocatalysts from the final broths, and, consequently, the increase of the number of repeated biosynthesis cycles re-using the same particles of biocatalysts [9]. The bioreactors using immobilized biocatalysts can be designed as column, stirred, gas-lift, or membrane bioreactors, being operated in batch, continuous, or semicontinuous systems, with a fixed, mobile/stirred, expanded, or fluidized bed.

Although the bioreactors with a fixed bed of biocatalysts are widely preferred, they have some major disadvantages [9]. The flow inside the bed is laminar, thus leading to low rates of mass and heat transfers and inducing the back-mixing or reverse-flow phenomenon. On the other hand, the solid particles from the effluent can clog the biocatalyst bed, thus leading both to the reducing of the flow rate inside the bed, and to the biocatalyst inactivation. Another important undesirable phenomenon is the formation of the preferential flow channels inside the bed at the beginning of the feed with medium or during the bioreactor working. The formation of these channels induces the deviation from the plug flow and the inefficient conversion of the substrate.

For these reasons, the bioreactors with stirred/mobile beds have become more attractive. Moreover, because of their constructive and operational similitude to the well-known stirred bioreactors, higher rates of heat and mass transfers can be reached. The models describing the hydrodynamics or the heat and mass transfers in stirred bioreactors, as well as their design and optimization could be easily adapted for the stirred bed bioreactors. However, these models are valid only for the continuous phase inside the bioreactor. Owing to the deposition tendency of the solid phase at the bioreactor bottom, to the internal diffusion of the substrate or product into the biocatalyst particle, the mixing, and, consequently, the flow of these suspensions, as well as the mechanism and kinetics of the processes occurring into the solid phase become more complex than those in the homogeneous systems. Therefore, new models have to be established for the biocatalyst phase.

The previous studies indicated that the immobilization of yeast or bacterial cells in alginate and their utilization in systems with a stirred bed of biocatalysts can represent a viable alternative to the fermentation with free cells [9, 10]. By selecting the optimum operating regime of the bioreactor, the activity and physical integrity of the immobilized cells remain unaffected for many fermentation cycles, even if the fermentation is carried out under substrate or product inhibition conditions.

In this context, this work investigates the external and internal mass transfers of glucose under substrate and product inhibition limitations in succinic acid production by immobilized *A. succinogenes*, using a stirred bed bioreactor. Based on the experimental results, a new mathematical model describing the implication of internal diffusion on the distribution of substrate concentration inside the biocatalyst particle has been established.

Materials and Methods

Microorganism, Medium, Fermentation, and Analytical Equipments

The experiments have been carried out in 100 ml (80 ml working volume) small anaerobic bioreactors, each containing 54 ml of medium with the following composition (per liter): glucose 30 g, yeast extract 5 g, $NaH_2PO_4 \cdot H_2O$ 1.16 g, Na_2HPO_4 0.31 g, NaCl 1.0 g, $MgCl_2 \cdot 6H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, vitamin B_{12} 1 μg, biotin 20 μg, folic acid 20 μg, thiamine 50 μg, riboflavin 50 μg, niacin 50 μg, pantothenate 50 μg, *p*-aminobenzoate 50 μg, lipoic acid 50 μg, vitamin B_6 100 μg, $MgCO_3$ 30 g, and silicone antifoam 1 ml [14]. The bioreactors were placed on a rotary shaker at 100 rpm and incubated at 37°C. During the fermentation, the broth was sparged with 0.1 vvm CO_2.

A. succinogenes ATCC 55617 cells immobilized in alginate were used in the experiments. The microorganism was provided by the American Type Culture Collection and was preserved at −70°C. The inoculum was prepared by incubating *A. succinogenes* at 30°C in 100 ml Duran bottles, each containing 50 ml of trypticase soya broth. The bottles were stirred at 100 rpm on a rotary shaker for 48 h.

The immobilization was carried out by bacterial cells inclusion into the alginate matrix, respecting the method given in the literature [28]. The biocatalysts were prepared separately for each bioreactor in aseptic conditions. For this purpose, 6 ml of inoculum was mixed with 20 ml of 5% aqueous solution of sodium alginate. The biocatalysts particles were obtained by dripping this suspension through a capillary into a solution of 0.2% $CaCl_2$ under constant pressure. Capillaries with three different diameters were used and the obtained particles of immobilized *A. succinogenes* had the

following diameters: 3.0, 3.6, and 4.2 mm, respectively. In all cases, the volumetric fraction of the immobilized cells into the medium was 0.23. Any mechanical damage of the biocatalyst due to the shear forces was recorded during the experiments.

The fermentation end was considered as when either the glucose was completely consumed or its concentration remained constant for 12 h.

Glucose concentration was determined every 2 or 3 h by using a glucose analyzer of GL 6 type (Analox Instruments, UK).

Theoretical Aspects and Mathematical Model for Glucose Transfer

Internal diffusion is important, especially for the biocatalysts obtained by immobilizing cells or enzymes inside of an inert matrix. In this case, the substrate has to migrate to the cells or enzymes through nonlinear channels, its diffusion being described by the effective/ apparent diffusion coefficient or diffusivity. The rate of the biochemical reactions occurring inside the biocatalyst particle is inferior to that corresponding to the homogeneous system, due to the lower substrate concentration compared with its value in the liquid bulk.

The values of glucose concentrations at the particle surface and inside the particle can be obtained by means of its mass balance related to a single biocatalyst particle. For this purpose, the following assumptions have been considered:

- the kinetics of succinic acid production can be described by a Jerusalimsky model for substrate and product inhibition, adapted to the immobilized *A. succinogenes* cells [5]:

$$v_P = V \cdot C_C \cdot \left(\frac{K_{iS}}{K_{iS}+C_S}\right) \cdot \left(\frac{K_{iP}}{K_{iP}+Y_{P/S}\cdot C_S}\right) \tag{1}$$

- the biocatalyst particle is spherical;
- the yeast cells are uniformly distributed inside the particle;
- there are no interactions between the substrate or products and support;
- the internal diffusion is described by Fick law and effective diffusivity.

In this case, according to the Bird equation [2], the expression for the mass balance of glucose related to the biocatalyst particle is

$$\frac{dC_{SP}}{dt} = D_{Se}\cdot\left[\frac{1}{r^2}\cdot\frac{d}{dr}\left(r^2\cdot\frac{dC_{SP}}{dr}\right)\right] - V\cdot C_C\cdot\left(\frac{K_{iS}}{K_{iS}+C_{SP}}\right)\cdot\left(\frac{K_{iP}}{K_{iP}+Y_{P/S}\cdot C_{SP}}\right) \tag{2}$$

Considering the steady-state conditions, Eq. (2) becomes

$$\frac{d^2C_{SP}}{dr^2} + \frac{2}{r}\cdot\frac{dC_{SP}}{dr} = \frac{V\cdot C_C}{D_{Se}}\cdot\left(\frac{K_{iS}}{K_{iS}+C_{SP}}\right)\cdot\left(\frac{K_{iP}}{K_{iP}+Y_{P/S}\cdot C_{SP}}\right) \tag{3}$$

and can be solved under the following boundary limits:

(i) r = 0 (at particle center), $\frac{dC_{SP}}{dr} = 0$

(ii) r = R_P (at particle surface), $-D_{Se}\cdot\frac{dC_{SP}}{dr} = k_L\cdot(C_{SL}-C_{Si})$

In these circumstances, the solution of Eq. (3) describes the glucose concentration profile inside the biocatalyst particle:

$$C_{SP} = \frac{Bi\cdot(C_{SL}-C_{Si})\cdot\cosh(3\varphi\cdot R_p)}{R_P^{\,2}}\cdot\left[\frac{3\varphi}{R_p} - R_p\cdot\tanh(3\varphi\cdot R_p)\right]\cdot\frac{\sinh(3\varphi\cdot r)}{r} \tag{4}$$

The substrate concentration at the particle surface is obtained using the following relationship:

$$C_{Si} = \frac{Bi\cdot C_{SL}\cdot\cosh(3\varphi\cdot R_p)\cdot[3\varphi - R_p^{\,2}\cdot\tanh(3\varphi\cdot R_p)]\cdot\sinh(3\varphi) - C_{SL}\cdot R_p^{\,4}}{Bi\cdot\cosh(3\varphi\cdot R_p)\cdot[3\varphi - R_p^{\,2}\cdot\tanh(3\varphi\cdot R_p)]} \tag{5}$$

The Thiele modulus, φ, and the Biot number, Bi, quantify the influence of the internal diffusion. Thus, the Thiele modulus indicates the magnitude of the influence of internal diffusion on the biochemical reaction rate [10, 30]. For the studied fermentation system, it is defined by the modified expression

$$\varphi = \frac{R_p}{3}\cdot\sqrt{\frac{V\cdot C_C}{D_{Se}}\cdot\frac{Y_{P/S}\cdot K_{iS}+1}{K_{iP}\cdot K_{iS}}} \tag{6}$$

The Biot number represents the ratio between the resistance to the diffusion in the boundary layer surrounding the particle and that corresponding to the internal diffusion:

$$Bi = \frac{k_L\cdot R_P}{D_{Se}} \tag{7}$$

By means of the substrate concentration values in the liquid bulk and inside the biocatalyst particle, the external and internal mass flows of glucose can be calculated. Therefore, the substrate flux from the liquid phase to the particle surface is

$$n_L = k_L\cdot(C_{SL}-C_{Si}) \tag{8}$$

where k_L represents the mass transfer coefficient in the boundary layer at the particle surface, being calculated with the expression [21]

$$\left(\frac{k_L\cdot d_P}{D_{SL}}\right)\cdot\left(\frac{\rho_L\cdot D_{SL}}{\eta_L}\right)^{1/3}\cdot\left(\frac{\eta_L}{\phi\cdot v_s\cdot\rho_L\cdot d_P}\right) = 1.90\cdot\left[\frac{(1-\phi)\cdot\eta_L}{v_s\cdot\rho_L\cdot d_P}\right]^{1/2} \tag{9}$$

The internal mass flow can be obtained by combining the Fick law

$$n_P = -D_{Se}\cdot\frac{dC_{SP}}{dr} \tag{10}$$

with Eq. (4), resulting in the following expression adequate for the succinic acid fermentation under substrate and product inhibition:

$$n_p = D_{Se}\cdot\frac{Bi\cdot(C_{SL}-C_{Si})\cdot\cosh(3\varphi\cdot R_p)}{R_P^{\,3}}\cdot[3\varphi - R_P^{\,2}\cdot\tanh(3\varphi\cdot R_p)]\cdot\left[\frac{3\varphi\cdot\cosh\left(\frac{3\varphi\cdot r}{R_P}\right)}{R_P\cdot r} - \frac{\sinh\left(\frac{3\varphi\cdot r}{R_P}\right)}{r^2}\right] \tag{11}$$

The values of the parameters used for calculating the external and internal mass transfers have been previously established and are given in Table 1.

Table 1. Parameters used for calculations.

Parameter	Value	Source
D_{SL}, m^2/s	6.47×10^{-10}	[26]
D_{Se}, m^2/s	4.39×10^{-10}	[8]
K_{iS}, kg/m^3	80	[5]
K_{iP}, kg/m^3	48	[5]
V, kg/kgs	1.75×10^{-4}	[5]
$Y_{P/S}$, kg/kg	1.10	[14]

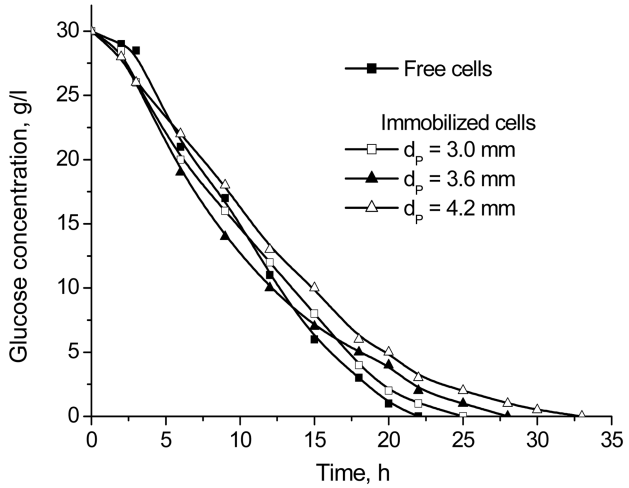

Fig. 1. Variation of glucose concentration during succinic acid fermentation for free and immobilized *A. succinogenes* cells.

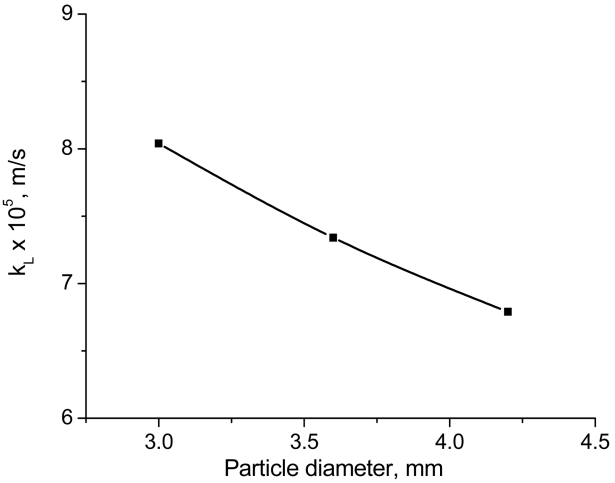

Fig. 3. Influence of biocatalyst particles size on k_L.

RESULTS

From Fig. 1, it can be observed that the duration of succinic acid fermentation with free *A. succinogenes* is about 22 h, the lag period being 3 h. The fermentation duration increased when immobilized cells were used, becoming 25 h for biocatalysts particles of 3.0 mm diameter, 28 h for 3.6 mm diameter, and 33 h for the largest particles of 4.2 mm diameter, respectively.

Equation (5) suggests that the value of the superficial concentration of glucose and its consumption rate depend on the size of the biocatalyst particles. According to Fig. 2, for the considered domain of biocatalysts particles diameter, the minimum value of the C_{Si}/C_{SL} ratio corresponds to the diameter of 3.6 mm.

The dependence between k_L and the biocatalysts size is graphically presented in Fig. 3.

The external mass flow of glucose was calculated by introducing the values of the above-discussed parameters in Eq. (8), considering various concentrations of glucose corresponding to different moments of the fermentation. Therefore, as can be observed from Fig. 4, the mass flow increased with the increase of particles size, reached a maximum value for the biocatalysts particles of 3.6 mm diameter, and decreased for larger particles.

Because the glucose concentration inside the biocatalyst particle depends strongly on its concentration at the particle surface, the variation of the ratio between these two concentrations with the particle radius can describe more accurately the influence of the internal diffusion. From Fig. 5, it can be seen that the ratio C_{SP}/C_{Si} is significantly reduced towards the particle center, this effect being more pronounced for biocatalyst particles with

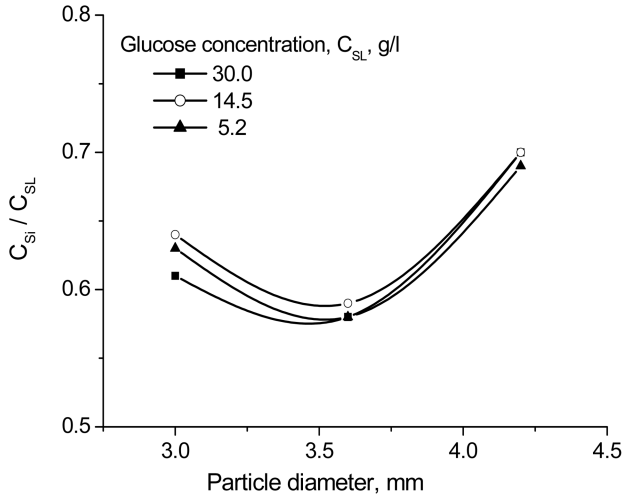

Fig. 2. Influence of biocatalyst particles size on ratio C_{Si}/C_{SL}.

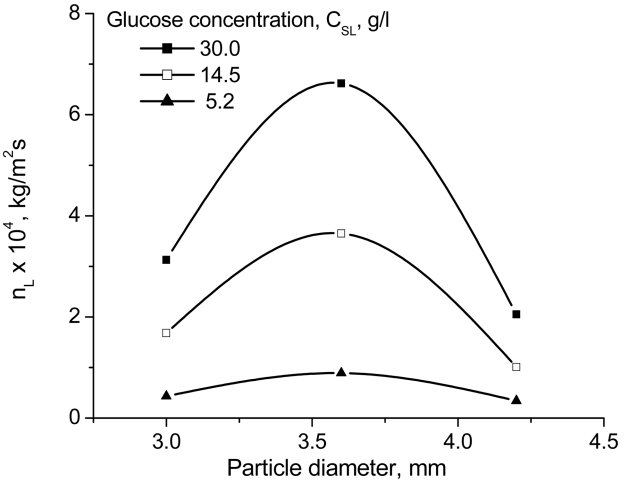

Fig. 4. Influence of biocatalyst particles size and volumetric fraction on external glucose mass flow.

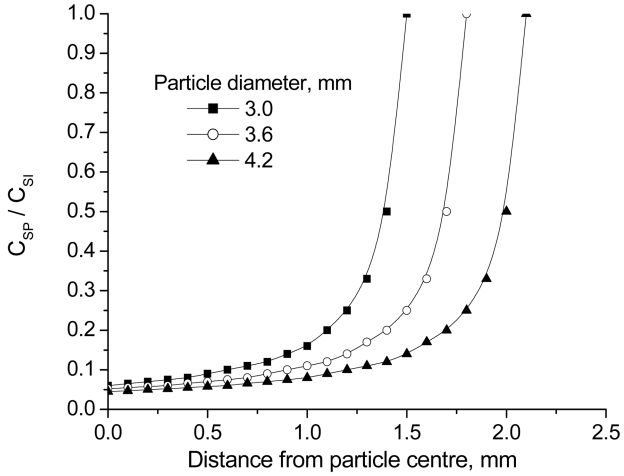

Fig. 5. Variation of C_{SP}/C_{Si} ratio with distance from the particle center.

higher diameters.

By means of Eq. (11) and using the above-calculated data, the values of the internal mass flow of glucose was estimated. The variation of internal mass flow with the biocatalyst radius is plotted in Fig. 6.

DISCUSSION

Owing to the internal diffusion of substrate inside the biocatalyst particle, the substrate concentration is diminished in the vicinity of the biological active center when immobilized cells are used. Generally, this effect leads to the reduction of biochemical process rate (of biomass growth or product formation) as compared with the systems using free cells. However, if the substrate concentration induces the inhibitory phenomenon, the internal diffusion could attenuate this effect and increase the process rate.

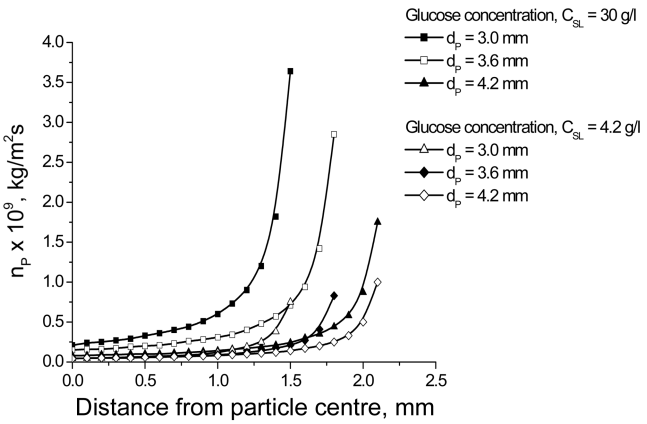

Fig. 6. Variation of internal glucose mass flow with distance from the particle center.

The differences between the durations of succinic acid fermentation for free and immobilized *A. succinogenes* cells, indicated in Fig. 1, are the consequence of the glucose internal diffusion, which reduces the substrate concentration inside the particle and, implicitly, its consumption rate. Apparently, the lag step is not so evident in the case of immobilized *A. succinogenes*, possible due to the diffusion of glucose from the liquid phase to the biocatalysts one. However, in the first hours of fermentation, the rate of glucose consumption becomes higher for the immobilized cells, this effect being more important for the particles with 3.6 mm diameter. Thus, for this size of biocatalysts particles, the highest substrate consumption rate is reached in the first 12 h. This result underlines the positive effect of internal diffusion in the case of succinic acid fermentation under substrate inhibition. Over 12–15 h, when the glucose concentration in the medium becomes lower than that inducing the inhibitory effect, the internal diffusion represents the limiting factor of the fermentation process and, therefore, the substrate consumption rate decreases, an evolution that is more pronounced by increasing the particles size.

The results graphically presented in Fig. 2, respectively the correspondence between the minimum value of the C_{Si}/C_{SL} ratio and the intermediary diameter of biocatalyst particle, correlated with those presented in Fig. 1, suggesting that the intermediary size of the particles allows to reaching the highest rate of glucose consumption (the lowest superficial concentration of glucose), owing to the equilibrium between the antagonistic processes of internal diffusion and the substrate inhibition. The substrate inhibition is more pronounced for the smaller immobilized cells particles, whereas the internal diffusion becomes the main limiting step for the larger ones.

Fig. 3 indicates the negative effect of the particle diameter on the mass transfer coefficient through the liquid boundary layer surrounding the particle, owing to the increase of this layer thickness. As can be observed from Fig. 4, the initial variation of external mass flow of substrate is contrary to that of k_L. This effect is due to the amplification of the substrate concentration gradient inside the liquid boundary layer, an effect that exceeds the diminution of k_L from the smallest particles to the largest ones. According to the above-discussed results, the maximum substrate concentration gradient is recorded for the intermediary particles of immobilized *A. succinogenes*.

The dependence between the C_{SP}/C_{Si} ratio and distance towards the particle center is plotted in Fig. 5 and suggests that the reduction of this ratio becomes more pronounced for biocatalyst particles with higher diameters. Thus, the value of this ratio decreases from 1 to 0.06 for the smallest particles of immobilized *A. succinogenes*, to 0.052 for the

medium particles, and to 0.045 for the largest ones, respectively. This variation is not affected by the modification of substrate concentration in the medium.

In these circumstances, depending on the superficial concentration of glucose, the glucose concentration at the particle center could reach a very low value as compared with that from the liquid bulk, this becoming an important threat for the succinic acid fermentation process. Owing to the increase of the magnitude of diffusional resistance for the larger particles, the central substrate concentration for particles with 3 mm diameter is about 1.2 times higher than that for particles with 3.6 mm diameter, and respectively about 2.5 times higher than that corresponding to the particles having the diameter of 4.2 mm.

The primary analysis of the plotted curves from Fig. 6 indicates that the values of the internal mass flow are about 10^5 times lower than those recorded for the external mass flow. Moreover, Fig. 6 shows the direct correlation between the internal mass flow and substrate concentration, both being significantly reduced near the particle center. For a certain inner position, the highest levels of internal mass flow have been reached for the smallest particles, owing to the lowest resistance to the substrate internal diffusion. Taking into consideration the order of magnitude of effective diffusivity, it can be assumed that for values of internal mass flow lower than 1×10^{-10} kg/m^{2}s, the mass transfer of glucose is insignificant. The region corresponding to the lowest values of substrate internal mass transfer could be considered as a "biologically inactive region." The extent of this region depends on the particle size and glucose concentration in the liquid phase. Therefore, for C_{SL} = 30 g/l, the glucose mass flow becomes negligible for the largest particles in a region with radius of 0.4 mm, for the intermediary particles in a region with radius of 0.25 mm, while for the smallest biocatalysts the negligible value of internal mass flow has not been reached. However, for C_{SL} = 4.2 g/l, the radius of the biologically inactive region becomes of 0.8 mm for the largest particles, of 0.5 mm for the intermediary particles, and respectively of 0.3 mm for the smallest ones. Because the studied bioreactor contains mobile beds of immobilized biocatalysts, the size of the biologically inactive region is significantly diminished compared with that previously reported for similar fermentation using a fixed bed bioreactor [30].

In conclusion, the proposed models can be used for calculating the glucose concentrations at the surface and inside the particles of immobilized *A. succinogenes* cells, as well as its external and internal mass flows. The results indicate that it is possible to reach very low values of mass flow near the particle center, values that could be negligible. The region inside the particle corresponding to the insignificant mass transfer of glucose could be considered a "biologically inactive region," its magnitude varying from 0 to 5.53% of the overall volume of particles.

Notations

Bi - Biot number
C_C - cells concentration, kg/m^3 d.w.
C_S - substrate concentration, kg/m^3
C_{Si} - substrate concentration at the biocatalyst particle surface, kg/m^3
C_{SL} - substrate concentration in the liquid bulk, kg/m^3
C_{SP} - substrate concentration inside the biocatalyst particle, kg/m^3
d_P - biocatalyst particle diameter, m
D_{Se} - effective diffusivity, m^2/s
D_{SL} - liquid-phase diffusivity, m/s
K_{iP} - product inhibition constant, kg/m^3
K_{iS} - substrate inhibition constant, kg/m^3
k_L - liquid-phase mass transfer coefficient, m/s
n_L - external mass flow, kg/m^{2}s
n_P - internal mass flow, kg/m^{2}s
R_P - biocatalyst particle radius, m
v_S - liquid superficial velocity, m/s
V - maximum biochemical reaction rate, kg/m^{3}s
$Y_{P/S}$ - yield of substrate conversion to product, kg/kg

Greek Letters

φ - Thiele modulus
η_L - liquid-phase viscosity, Pa.s
ρ_L - liquid-phase density, kg/m^3

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