HUNGARIAN JOURNAL
OF INDUSTRIAL CHEMISTRY
VESZPRÉM
Vol. 30. pp. 87 - 93 (2002)

KINETIC STUDY AND MATHEMATICAL MODELLING OF GROWTH OF ARTHROBACTER OXYDANS CELLS COVALENTLY IMMOBILIZED ON CELLULOSE GRANULES

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Received: February 12, 2001

The kinetics of covalently immobilized cells' growth in comparison with that of free cells was studied experimentally and numerically. Cells of Arthrobacter Oxydans were covalently immobilized to regenerated and partially deacetylated acetylcellulose granules consecutively activated with sodium periodate, urea and formaldehyde.
The comparison of the data obtained showed that free and immobilized cells have the typical three phases of growth. The initial phase of free cells lasted about 10 hours and for immobilized ones - not more then 5 hours. The second exponential phase showed much more significant difference for both types of cells. The curve for immobilized cells is steeper than that of free ones and attains twice faster the third phase. The glucose utilization by immobilized cells was completed about the 15th hour, for the free cells - about 25th hour. The reproducibility of the data was quite satisfactory (mean error 7.42 %).
A mathematical model was used to describe and analyse the effect of the different mass transfer and reaction kinetic parameters. It shows that replication of immobilized cells is a complex process including mother cells and the new born ones.
The application of the results of this study could be concerned with the use of the immobilized cells as a permanent source of free ones. They are viable and possess a long lasting capacity for urea transformation.

Keywords: Arthrobacter oxydans, covalent immobilized cells of Arthrobacter oxydans, mathematical modelling of immobilized cells' growth

Introduction

Immobilization of microbial cells for the production of useful products has been widely studied in recent years [1]. The use of immobilized cells instead of immobilized enzymes has many advantages like avoiding the stage of isolation and purification of the enzyme, addition of co-factors and increasing the stability of enzyme system. That is the reason of the intensive development of immobilization of intact cells to insoluble carriers over the last years. Microbial cells can be immobilized to insoluble matrices by adsorption [2], microcapsulation [3] or covalent binding [4]. The later technique has not been widely used because of toxic nature of the reagents used for binding, causing the cells' death even during the immobilization. On the other hand the covalent binding offers advantages concerning the absence of diffusion limitations and allows the direct contact between the bound cells and the substrate solution which results in an intense (fast) controlled reaction.

A new method for immobilization of proteins to cellulose derivatives was reported recently. It involves subsequent treatment of the matrix with sodium periodate, urea and formaldehyde [5] and was also applied to covalent binding of *A.simplex* cells [6].

The aim of the present investigation was to study the growth kinetics of covalently immobilized cells to insoluble matrices via hydroxymethyl groups and to compare it with free ones and to propose a mathematical model properly describing the process. For these studies we choose the covalently immobilized cells of *Arthrobacter oxydans*.

Materials and methods

Chemicals

Urea, formaldehyde, potassium hydroxide and sodium periodate were supplied by Merck (Germany); potassium dihydrogen phosphate, sodium hydrogen phosphate and glucose were obtained from Reanal (Hungary). All other chemicals were of reagent grade or better. Granules of triacetylcellulose were obtained by treatment of waste film tapes as described elsewhere [8].

Microorganisms and culture conditions

Arthrobacter oxydans strain 1388 from the National collection for industrial and cell cultures (Bulgaria) was cultured on solid agar medium for 48 hours at 28°C. After this incubation colonies were picked off and suspended in meat peptone broth containing 1 g/l sorbitol and incubated for 48 hours at 28°C. After centrifugation (20 min, 3500 g), the cell mass was suspended in nutrient medium according to Schneider and Kaltwasser [7]. The composition of the nutrient media in respect to 1l quantity was 9 g $Na_2HPO_4.12H_2O$, 2g $KH_2PO_4.7H_2O$, 0.7 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 5g of glucose, 0.3 g of urea and 0.002 g $NiCl_2$. The mineral trace elements solution for 100 ml was as follows: 0.3g $FeCl_3.6H_2O$, 0.1g $Na_2MoO_4.2H_2O$, 0.4g HBO_3, 1.34g $CaCl_2.2H_2O$. One ml of this solution was added to a litre of nutrient medium. The pH of the sterilized nutrient medium was 7.0. The cultivation of free cells was done in a water bath shaker at 28°C for 48 hours. The cell mass was separated by centrifugation, washed with 0.1M phosphate buffer (pH 7) and stored at 4°C.

Immobilization procedure

Treatment of the cellulose carrier

Acetylcellulose granules with diameters in the range 1 - 3 mm were subjected to partial hydrolysis of acetyl groups according to Chen and Tsao [8]. The quantity of 0.28 g dry beads was treated with 0.1M KOH and 5% (v/v) ethanol at hydromodule 1:15 for 24 hours. The urea derivative was then prepared by oxydation of the cellulose granules with 0.25M sodium periodate at 22± 2°C at pH 5 for 2 hours in darkness. After complete removal of sodium periodate by filtration and washing the granules were treated with 15% urea solution for 14 hours in presence of 0.9% (v/v) sulphuric acid at 60°C according to Krysteva et al. [5]. The beads were washed with water in a Buchner funnel until a neutral reaction of the rinsing water. The resulting urea derivative of the regenerated cellulose acetate beads contained 3.3 % nitrogen. This urea derivative cellulose granules were activated in 100 ml of 0.1 N phosphate buffer pH 7.5, containing 12.5% (v/v) formaldehyde and stirred for four hours at 45°C in a closed vessel. Then the granules were thoroughly washed until the complete absence of formaldehyde in the rinsing water.

Immobilization of Arthrobacter oxydans

The activation procedure of the carrier was followed by an immediate addition of *Arthrobacter oxydans* cell suspension with a concentration of 50 mg/ml. The binding was carried out at pH 8 and careful stirring at a temperature 20°C for 20 hours. The cell loaded granules were washed with water and 0.1M phosphate buffer (pH 7) until complete absence of free cells in the rinsing water, followed spectrophotometrically at 660 nm wave length.

After the immobilization procedure the obtained biocatalysts granules were resuspended in the culture medium as already described and incubated for 45 hours to initiate their replication.

Analytical procedures

Measurement of cell growth

Biomass of free cells and those, produced by immobilized cells was measured spectrophotometrically at 600 nm wave length (Perkin- Elmer spectrophotometer, Lambda 2, Germany). Cell growth was also determined by the dry cell weight according to Mallette [10]. The samples were dried until a constant weight at 105°C. The analysis were checked up by determination of protein content, using modified Lowry's method [13].

Enzyme assays

Urease activity of free and immobilized cells was determined according to Melnyk and Olean [12]. The enzymatic reaction was carried out in batch vessel with magnetic stirrer. After 15 min the reaction was interrupted by cooling the mixture in 2°C bath. An aliquot (1 ml) was tested for ammonia presence using Nessler reagent.

Glucose determination

The amount of the glucose consumed was determined colorimetrically at 540 nm by the dinitrosalicylic acid method according to Miller [13].

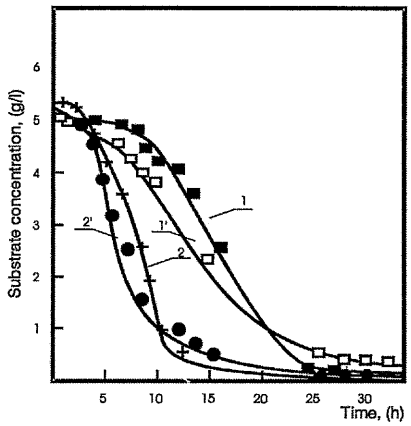

Fig.1a Kinetic of substrate utilization at initial concentrations for the free cells: 40.0 mg/l (1); 118 mg/g (1') and for the immobilized cells: 38.4 mg/l (2); 126mg/l (2')

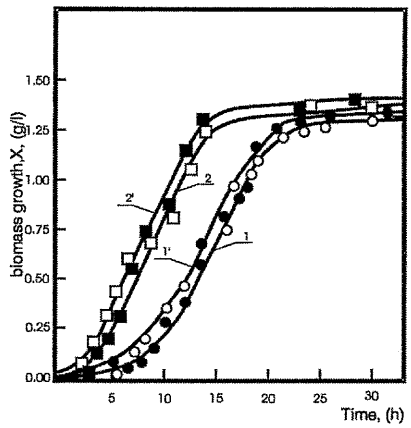

Fig.2a Reproducibility of substrate utilization for free cells: first experiment (1); second experiment (1') and for immobilized cells: first experiment (2); second experiment (2')

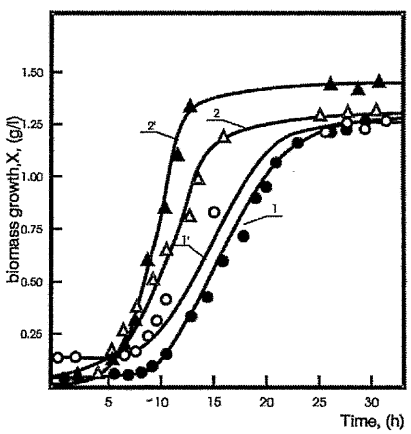

Fig.1b Kinetic of cells' growth at initial concentrations for the free cells: 40.0 mg/l (1); 118 mg/g (1') and for the immobilized cells: 38.4 mg/l (2); 126mg/l (2')

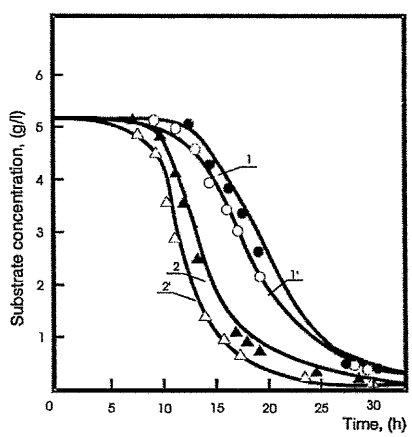

Fig.2b Reproducibility of cells' growth for free cells: first experiment (1); second experiment (1') and for immobilized cells: first experiment (2); second experiment (2')

Kinetic experiments

The experiments on the kinetics of growth of free and immobilized cells of *Arthrobacter oxydans* were performed by batch process in shake flask cultures at pH=7, temperature of 28°C under continuous aeration. During the experiments the biomass and substrate concentrations were measured every 2 hours. The decrease of glucose concentration was also followed. The cells of *Arthrobacter oxydans* were seeded in 50 cm^3 cultural medium. This inoculate was used for kinetic studies (15 cm^3 of the inoculate seeded in 150 cm^3 of nutrient medium). The initial concentration of dry cells was varied from 40 mg/l up to 118 mg/l. The initial concentration of the immobilized cells was varied in the same range as for the free ones (from 38.4 mg/l to 126 mg/l).

Results and discussion

Batch experiments

A set of experiments for monitoring of growth of free and immobilized cells of *Arthrobacter oxydans* was done by analysing the biomass growth (*Fig.1a*) and the substrate consumption (*Fig.1b*).

For both cases the three typical growth phases were observed but their duration for the free and the immobilized cells was different. As it could be seen in *Fig.1*, the initial phase for the free cells was about two times longer that that of the immobilized ones. This difference becomes more significant for the phase of exponential growth. The curve of immobilized cells is much steeper than that of the free ones which results in twice faster reaching of the third phase. These observations are confirmed by monitoring of substrate consumption for the two types of cells: the glucose utilization of the immobilized cells was completed at

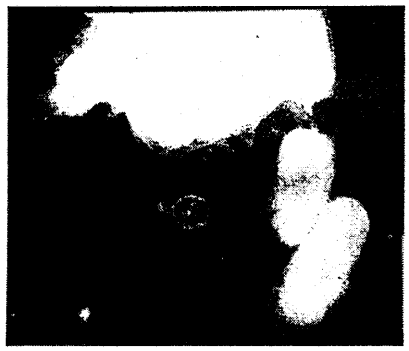

Fig.3 Electronmicroscopic photographs of immobilized cells x 25000

about 15th hour and for the free ones - about the 25th hour.

To check the reproducibility of the results obtained, parallel experiments were done. The results of two of them are given in *Fig.2a* and *b*. The obtained reproducibility of the data is quite satisfactory (mean error 7.42%).

The microscopic picture of immobilized cells of *Arthrobacter oxydans* is presented in *Fig.3*. It was found that the cells of *A. Oxydans* are superficially well situated on the matrix which made us suggest the absence of diffusion limitations. In the same time their covalent binding is probably limited to permit them a free reproduction. The results for the growth of young cells produced by the immobilized ones confirm this suggestion. The urease activities of free, immobilized and new born cells, produced by immobilized cells were respectively: 0.325 mol/(min.g), 0.156 mol/(min.g) and 0.327 mol/(min.g), based on dry cells weight. These values remained almost constant after tenfold transformations after preinoculation of the immobilized cells in a nutrient medium. These results are better than those for *Escherihia coli* OA5 cells with urease activity entrapped in alginate-poly-L-lysine-alginate microcapsules. In this case bacteria leakage has been found to occur when encapsulated bacteria were used for a four-cycle transformation [13]. In other investigations with immobilized fungi in nylon webs for enzymes production the successively repeated batch cultivations were 6 [9] or only 2 [14].

Mathematical modelling

First the free cells runs were analysed using the simple Monod kinetic model:

$$\mu = \mu_m \frac{S}{K_s + S},$$

in order to obtain the parameters μ_m and K_S. As a first approximation the experimental curves were fitted with a linear kinetics (K_S=0) and the obtained value of μ_m was confirmed by the one obtained from the linear part of the plot $\ln(X/X_0)$ vs. $(t-t_{lag})$ of the experimental data. Best results were found for μ_m= 0.2.h^{-1} and t_{lag}= 3h, obtained from the analysis of the experimental data of *Fig.2a* and *b*. Further fit of the experimental data with different values of K_S confirmed the linear kinetics for the free cells growth (K_S=0.01). The plateau of the curve is mainly affected by the yield coefficient Y, so from this part of the curve a value of Y=0.24-0.27 was obtained. The results of the simulation are given on *Fig.5a*, where a satisfactory correspondence between experimental and calculated data is observed.

A mathematical model, proposed to describe the kinetic experiments with immobilized cells, should account for the reaction kinetics and mass transfer limitations in the reactor. First, the statement of the model is based on the following approximations:

1. The reactor is considered as a reactor with perfect mixing;
2. Different kinetic parameters for the immobilized and the free cells is supposed, assuming a simple Monod-type rate equation.
3. The enlargement of the beads size during the fermentation is negligible (i.e. particles of constant radius R are supposed)

The following set of 4 balance equations per unit volume of the reactor will result:

– for the immobilized cells:

$$\frac{dX_R}{dt} = \mu_{mR} \frac{S_R}{K_{sR} + S_R} X_R - k_{fR}'(X_R - X), \quad (1)$$

where index R corresponds to immobilization on the particles surface.

– for the substrate on the surface of the granules:

$$\frac{dS_R}{dt} = -\frac{1}{Y_R} \mu_m \frac{S_R}{K_{sR} + S_R} X_R + k_f'(S - S_R) \quad (2)$$

The mass transfer coefficient across the liquid film around the particles is denoted by k_f' for the substrate and k_{fR}' for the cells, resp.

– for the free cells in the reactor:

$$\frac{dX}{dt} = \mu_m \frac{S}{K_s + S} X + k_{fR}'(X_R - X) \quad (3)$$

– for the substrate in the reactor:

$$\frac{dS}{dt} = -\frac{1}{Y} \mu_m \frac{S}{K_s + S} X - k_f'(S - S_R) \quad (4)$$

The following initial conditions can be added:

for $t = 0$: $S = S_0$; $S_R = 0$; $X = 0$; $X_R = X_{R0}$;

i.e. the influence of the free cells initially in the broth is negligible.

This set of equations can be further simplified, as the experimental data give some evidence about the absence of mass transfer limitation for the new-born cells. As mentioned in the previous paragraph,

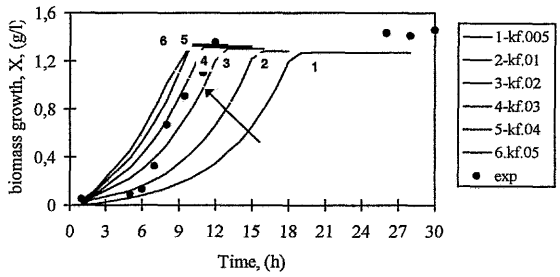

Fig.4a1 Kinetic curves X(t) for different values of the mass transfer coefficient - X_R=0.126g/l

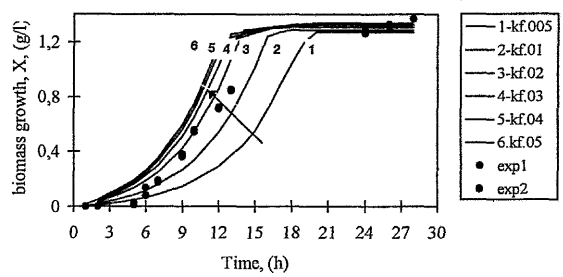

Fig.4a2 Kinetic curves X(t) for different values of the mass transfer coefficient - X_R=0.038g/l

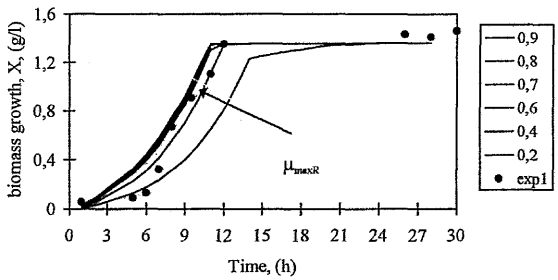

Fig.4b1 Kinetic curves X(t) for different values of the specific growth rate - X_R=0.126g/l

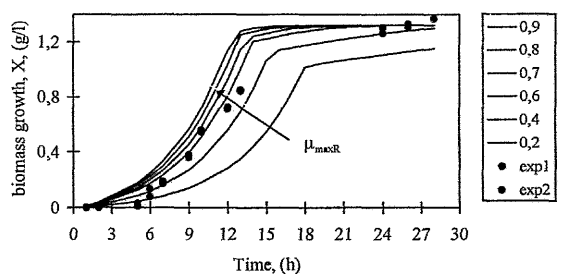

Fig.4b2 Kinetic curves X(t) for different values of the specific growth rate - X_R=0.038g/l

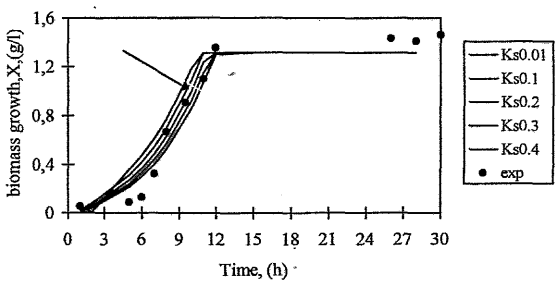

Fig.4c1 Kinetic curves X(t) for different values of the Monod constant - X_R=0.126g/l

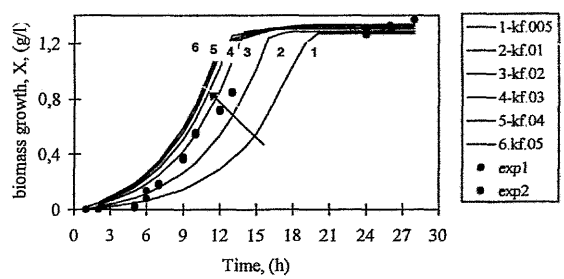

Fig.4c2 Kinetic curves X(t) for different values of the Monod constant 1-X_R=0.126g/l; 2 - X_R=0.038g/l

microscopic pictures of the immobilized cells (as the one shown in *Fig.3*) prove the constancy of the immobilized cells concentration, i.e.:

$$\frac{dX_R}{dt} = 0 \text{ and } X_R = const = X_{R0}. \qquad (a)$$

In such a situation a small or negligible mass transfer coefficient for the substrate is to be expected. This was verified by numerical experiments.

Taking into account *Eq.(a)*, *Eqs.(1)* and *(3)* are reduced to one simple equation for the cells' growth of the immobilized and the new born free cells:

$$\frac{dX}{dt} = \mu_m \frac{S}{K_s + S} X + \mu_{mR} \frac{S_R}{K_{sR} + S_R} X_{R0} \qquad (1')$$

Eq.(1') accounts for two parallel uniformly distributed volume sources of mass - the reaction terms for the free and immobilized cells. For the free (the new born) cells we used the values of μ_m, K_S and Y obtained before from the batch experiments with free cells. . The set of *Eqs.(1')*, *(3)* and *(4)* was used in the simulations to illustrate the sensibility of the kinetic curves to variations of the model parameters, as shown in *Figs. 4a1-4c2.*

In this case a slightly greater value of Y_R=0.3 was used - a fact well coinciding with the experimental data, where a 5-10 % greater yield was found for the immobilized cells in comparison with the free ones. *Figs.* from *4a1* to *4c2* are calculated for two different initial concentrations X_R=0.038 and 0.126g/l, the initial

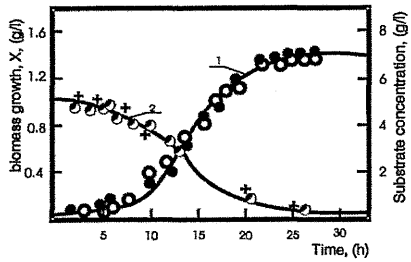

Fig.5a Comparison between experimental and numerical results for free cells: biomass growth (1) - experimental (○) and numerical (+) curves; substrate utilization 2 - experimental (○) and numerical (●) curves

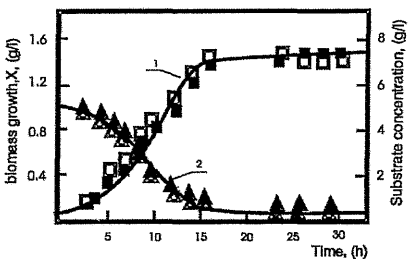

Fig.5b Comparison between experimental and numerical results for free cells: biomass growth (1) - experimental (□) and numerical (■) curves; substrate utilization 2 - experimental (Δ) and numerical (▲) curves

substrate concentration being S_0=5-5.4 g/l. On the same figures the experimental points are shown.

The basic set of values for the parameters, used in the simulations was μ_{mR}=0.6h^{-1}, K_{sR}=0.2-0.3 g/l. They were chosen as physically reasonable and allowing for a satisfactory description of the data with two different initial concentrations. As can be seen from *Fig.4a1* to *Fig.4c2*, the increase of X_R leads to stronger effect of the mass transfer coefficient k_f, while the importance of μ_{mR} and K_{sR} decreases. With fixed k_f and greater immobilized cells source X_{R0} the effect of the mass transfer between free and immobilized cells is greater. High mass transfer flux value makes the kinetic curve less sensible to variations of μ_{mR} and K_{sR}. From the numerical data a value of k_f=0.02-0.03 h^{-1} can be supposed. Calculated on the base of the specific area of the grain it gives a value of k_f=1-1.5.10^{-3} cm/s, corresponding to small mass transfer resistance across the fluid film around the particles. The numerical results were also examined towards the separate importance of each one of the two reaction terms in *Eq.(1')*. *Fig. 6* shows the calculated specific growth rate for the mother and the new born cells, according to *Eq.(1')* and the specified values of the kinetic parameters μ_m, μ_{mR}, K_S, K_{SR}, Y and Y_R. It can be seen that until the 5th hour the two terms are comparable and equal, because of the small amount of reproduced new cells. After the 5th hour the curve increases sharply and the rate of the process is mainly controlled by the new born cells. After the 12th hour it drops sharply because of the substrate exhausting. In the case of substrate addition it

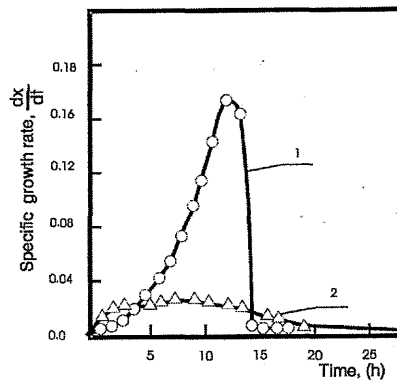

Fig.6 Specific growth rates for newborn (1) and immobilized (mother) cells (2)

should be increasing with the time. These results are in agreement with the experimental evidence. They allow for the description of the status of immobilized (mother cell), as well as the replication status of the new born cell in the nutrient medium and do explain the steeper kinetic curves for immobilized cells (*Fig.2a* and *b*) in comparison with those obtained from experiments with only free ones (*Fig.1a* and *b*). A satisfactory description of the experimental results in both cases was observed, as shown in *Fig.5a* and *b*. The correspondence is poorer in the initial part of the curves (as expected for the lag-phase) and better for the part of the exponential cell growth.

Conclusions

From the results obtained with covalently immobilized *Arthrobacter oxydans* cells the following mechanism of cell growth could be proposed:

The kinetics of growth for immobilized cells is different and their replication is faster than those of the free ones. The complex process of parallel reproduction of mother cells and new born ones can be satisfactorily described by a Monod kinetics. Mass transfer resistance in the liquid film around the particles is small and can be practically neglected.

The immobilized cells are viable and active, possess a long lasting capacity for urea transformation and can be used as a permanent source of free ones. It seems that the special feature of the cell wall of the Gram-positive microorganisms like *Arthrobacter oxydans* allows their covalent binding by this method.

SYMBOLS

k_f'	mass transfer coefficient, m.s^{-1}
K_S	Monod constant, g.l^{-1}
S	substrate concentration, g.l^{-1}
t	time, h
X	dry cells concentration per l fermentation medium, g.l^{-1}

Y yield coefficient, dry cell mass per dry mass of total medium, -

Greek letters

μ specific growth rate, h^{-1}

Indices

m maximum
R at granules surface

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