Intelligent invertase micro and nanocapsules make and use

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Abstract

The analytical conditions and underlines the efficiency of alginate encapsulation a gross enzymatic product of β-fructofuranosidase (invertase) activity obtained from the strain Aspergillus niger strain MIUG 1.15 by applying new immobilization techniques based on ion gelation emulsification were studied. Preliminary studies made it possible the identification of limiting parameters that condition the immobilization process efficiency. Thus it has been established that the nature and composition of the immobilization support, calcium ion vector and some process parameters (pH, temperature and enzyme stability) are major factors enzyme immobilization and their catalytic activity.

Keywords: *bio-encapsulation, emulsification-gelation, invertase, Aspergillus niger*

Introduction

Immobilizations of biologically active molecules or microbial cells are processes of advanced biotechnology perspectives since they can provide extraordinary possibilities for profitable and elegant biocatalyst conversions. Thus, using biocatalyst under immobilization state makes bioconversion processes more efficient by improving productivity, providing continuing process control and possibilities to reuse the biocatalyst.

The extensive use of enzymes induces wide applications in new fields which ten years ago were unconceivable. In addition to fields regarded as classical in the food industry, fodder, textile and leather industries where enzyme application is intensive and manifold, new fields have emerged such as detergents, pharmacy, cosmetics, chemicals, environment monitoring.

At present quite a large number of enzyme immobilization procedures are known that, although relatively easy to implement, involve complex processes due to the numerous factors, which simultaneously or independently affect the process and therefore are hard to control.

The most widely used bio-molecule immobilization methods are based on extrusion and emulsion techniques. Extrusion consists in encapsulated transfer, as drops (generated under the action of gravity, electrostatic force or microwaves), gel substances (natural poliglucides, synthesis polymers, etc) thus micro-spheres of uniform distribution of the 2-3 mm sizes [4].

Out of the natural polymers the most widely used for encapsulation are the alginates, carragenans, agar, gelatine etc.

The immobilization by encapsulation involves blending of an aqueous solution containing the encapsulated product and the gel with a vegetable oil in the presence of an emulgator. By stirring a direct or reverse emulsion is produced that, under proper chemical conditions, generates microcapsules of 10-50 μm.

Immobilization of free enzymes or enzymatically active entire cells by inclusion into alginate hydrogel, obtained by changing the sodium ion into calcium ion is a widely spread encapsulation method. There is, however, a disadvantage with this method, namely large capsules of large size distribution are obtained.

Internal gelation of a sodium alginate solution can be achieved by fast release of calcium ions in an insoluble calcium complex to be found as fine dispersion in the aqueous phase and acid medium [2,3].

This approach advances an alternative to this method, namely internal ionotropic gelation simultaneously with an emulsion process, which should ensure enzyme encapsulation in microcapsules of small sizes and smaller diameter distribution [1].

The researches were focused on: establishing a correlation between the sodium alginate structure and the micro-capsule size; studying the influence of the gelation vector on the ionotropic exchange process; establishing the optimum physical-chemical conditions for fungal invertase bio-encapsulation; evaluating the efficiency of the immobility techniques by studying the mass transfer which reflects retaining or release of the enzymes into or out of the microcapsules.

Material and Methods

Reagents:

Immobilization supports: sodium alginate (A_1) taken from the algae *Macroyistis pirifera* (Sigma) and sodium alginate (A_2) taken from the algae *Laminaria hyperborea* (Proton AS-Norw).

Enzyme: extracellular fungal invertase produced by selected stain *Aspergillus niger* MIUG 1.15 (belongs to the collection of the Microbiology laboratory, University of Galati, Romania – coded MIUG) obtained by stationary cultivation on liquid medium with glucose, peptone and Czapek salts [5].

Calcium vectors - calcium carbonate, calcium oxalate, calcium citrate.

Emulsification factors: Sorbitane trioleate (Span 80), Tween.

Soy oil

Buffer solutions: 0,02 M phosphate buffer, pH=6.0-8.0

Invertase encapsulation by emulsification and internal gelation method

The sodium alginate solution of given concentration (1-3%) was blended with a calcium salt suspension (calcium carbonate, oxalate and citrate) in 0.02M phosphate buffer pH=6,0. The mixture thus obtained (300 ml) was subsequently dispersed into vegetal oil (100 ml of the soy oil) in the presence of 10 ml of the surfactant (Span 80) resulting a reverse emulsion A/U. During stirring a drop of 1-5 ml of the glacial acetic acid was added when the sodium-calcium ion exchange takes place along with the alginate gelation. After 10 minutes a calcium chloride solution ($0,1 mol \cdot l^{-1}$) is further added to generate transition of the reverse emulsion into direct emulsion. The phase reversing was generated by the conductive method. Upon completion of the oily phase, the microcapsules have been separated during water phase by filtering and rinsed with a Tween 1% solution. After drying, the microcapsules have been analyzed in terms of grains size and microscopically by horizontal sizing with an optical microscope type Olympus MG-400.

Evaluation of invertase mobility efficiency

Uniform size micro-spheres have been suspended in a glass column of 1 cm diameter and 10 cm height. In order to establish the system thermodynamic balance a mixture of sodium alginate and invertase was used. The enzyme concentration in said mixture was arbitrarily regarded as initial concentration, $C_{(0,t)}$ (expressed in spectrum photometric units). After 30 minutes, the column was wash by buffer phosphate solution 0,02 M (pH=6,0) establishing a yielding speed of 0, 5 $ml \cdot s^{-1}$. Every 5 minutes, the enzyme concentration of the eluates was analyzed by spectro-photometric method by evaluate OD at 289 nm.

Results and Discussions

The grain analysis of the microcapsules obtained by means of the two samples of alginate revealed a rather high percentage of small microcapsules ($4\text{-}5 \cdot 10^{-4}$ m) with the alginate A1 as compared with the alginate A2 where the microcapsules distribution is quite high implying an increased non-homogeneity of the enzyme immobility (Figure1)

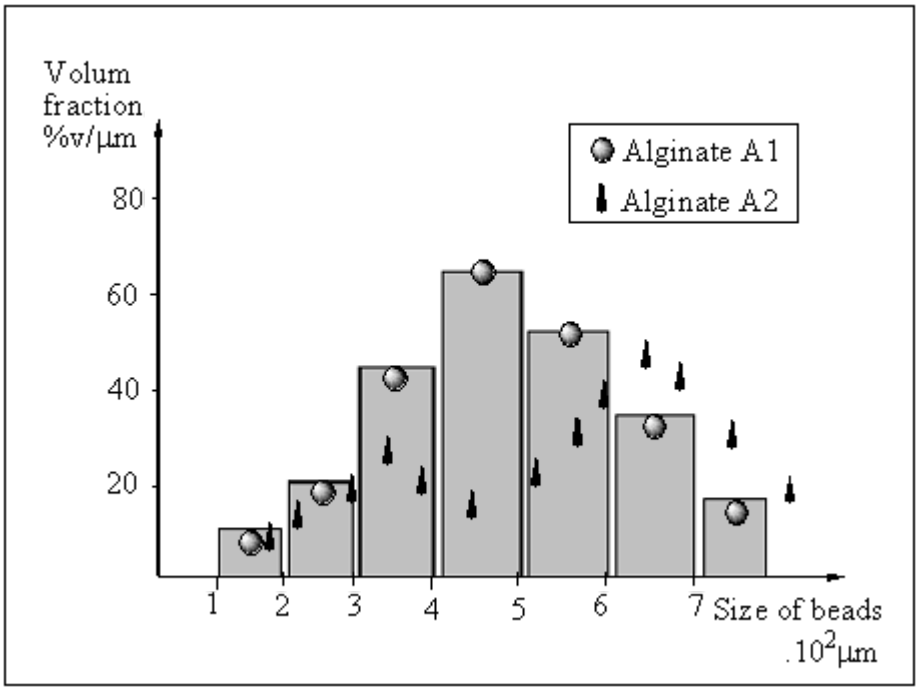

Figure 1. Histogram of micro-spheres distribution depending on the support nature and composition

Considering the foregoing, the alginate A1 has been considered the most efficient for enzyme encapsulation and therefore has been used in all the other experimental measurements. The investigations of the calcium ion generating substances revealed a higher capacity of gelation as compared with the calcium oxalate or citrate. Also, when calcium carbonate is used as ion generator the most homogenous microcapsule system was obtained (Figure 2).

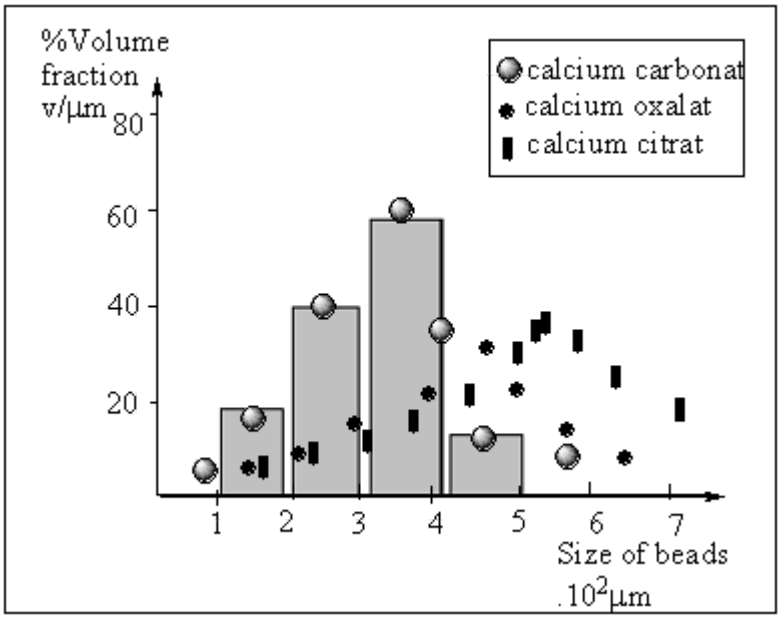

Figure 2. Distribution of microcapsule produced by alginate A1 invertase encapsulation and variation of the calcium ion vectors

The kinetic analysis of the gelation process also revealed the more difficult gelation of the sodium alginate under the action of the calcium organic salts maybe due to the low calcium ion release speed. Thus, in case of calcium citrate, gelation takes place after 80 minutes while in the presence of calcium carbonate gelation takes place quickly within max 15 minutes after salt has been added.

Phase inversion control in the emulsioned system is evaluated by measuring the electrical conductivity at 20 degrees temperature by using a gauge Metler Tolledo MC 226. The results show an increased electrical conductivity proportional to the increase in salt concentration ($CaCl_2$) in aqueous solution. The spectacular jump

of the electrical conductivity values is recorded from a concentration of the $CaCl_2$ equal to 58 ml which is precisely when emulsion A/U is converted into U/A and phase separation respectively resulting in alginate microcapsules (Figure 3).

To analyze the enzyme diffusion inside the microcapsules the model of diffusion across a spherical area as used based on the Fick law (eq. 1) applied to a homogenous system with microparticles of $2r = 2{,}510^{-4}$ m diameter

$$J_{gel} = -D_{gel}\frac{dc}{dr} \qquad (1)$$

where:

J - flux of enzymatic diffusion in the radius direction (r), $mol \cdot l^{-1} \cdot cm \cdot s^{-1}$;

D - enzyme diffusion through the alginate gel, $cm^2 \cdot s^{-1}$;

c - enzyme concentration expressed in arbitrary units.

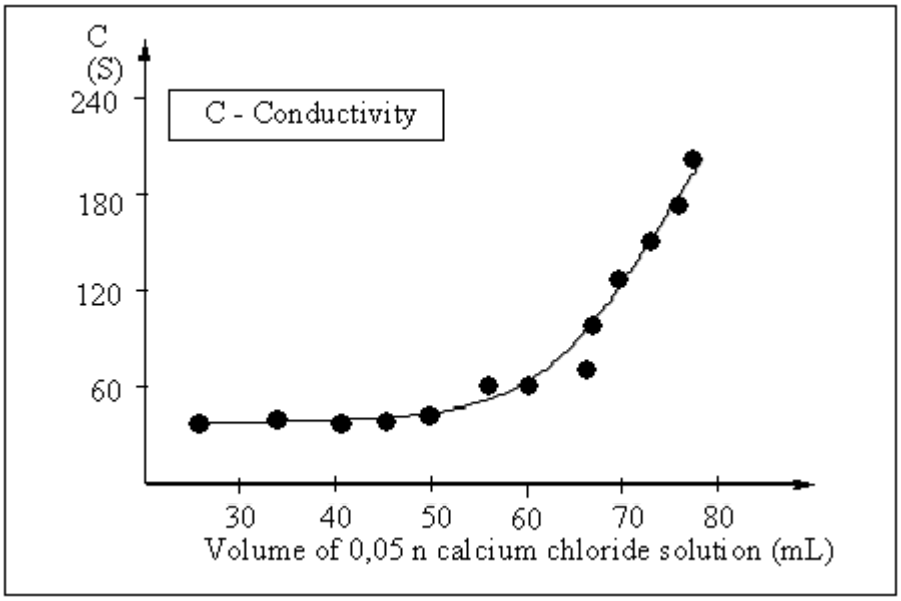

Figure 3.Effect of the $CaCl_2$ solution concentration on the formation of microcapsules

According to the mass conservation law, for a volume unit of a spherical particle we can write (eq.2):

$$\frac{\partial c_{(r,t)}}{\partial t} = D_{gel}K_{gel}\left(\frac{\partial^2 c_{(r,t)}}{\partial r^2} + \frac{2}{r}\frac{\partial c_{(r,t)}}{\partial r}\right) \qquad (2)$$

For the spherical microcapsule the following limit/boundary conditions can be defined:

a) in the center of the sphere, when *r=0* (eq.3):

$$\frac{\partial c_{(0,t)}}{\partial r} = 0 \qquad (3)$$

b) at interface flux when *r = R* for any time (eq. 4):

$$\left(\frac{\partial^2 c(R,t)}{\partial r^2}\right) = K_L\left[c_{(R,t)} - c_{(R,0)}\right] \qquad (4)$$

Under these conditions the equation 5 can be written as:

$$\frac{\Delta c}{\Delta t} = D_{gel}K_L\left[c_{(R,t)ext} - c_{(R,o)int}\right] \qquad (5)$$

where:

$c_{(R,t)}$ = concentration of enzyme diffused at time t

$c_{(R,\ 0)}$ = initial enzyme concentration

K_L = mass transfer coefficient

$$K_L = \left[\frac{R^3 \cdot v_s}{D_{apa}^{\frac{3}{2}}}\right]^{\frac{1}{2}} \cdot \left[\frac{\rho}{\upsilon_s}\right]^{\frac{1}{6}}$$

where:

R = microcapsule radius

v_s = effluent solution kinetic velocity, ml·s^{-1};

ρ = density of the effluent solution, g·cm^{-3};

υ_S = kinetic viscosity, cm^2·s^{-1};

$D_{apã}$ = water diffusion coefficient in the alginate gel, cm^2·s^{-1}

Based on the enzyme concentration released into the effluent and making use of the vales of the variables involved in the calculation of the mass transfer coefficient listed below: R=1,25·10^{-4} m; v_s = 0,4 -0,5 ml·s^{-1}; ρ =1,1243 g·cm^{-3}; υ_S = 6,463·10^{-3} cm^2·s^{-1}; $D_{apã}$ = 0,61·10^{-5} cm^2·s^{-1}.

By means of equation 5 the coefficient of enzyme diffusion through the alginate microcapsule is calculated. Calculations have revealed the variability of this parameter with pH. Thus, $D_{gel(A1)}$ = 0,85 10^{-6} cm^2·s^{-1}, at pH=6,7, and $D_{gel(A1)}$ = 1,75 10^{-6} cm^2·s^{-1}, at pH=7,5.

The analysis of the enzyme diffusion process showed the existence of three main stages (Figure 4). The first stage, which lasts for about 20 minutes, involves a process of equilibrium stabilization inside and outside the microcapsule. In this stage the enzyme loss within the system is very slow.

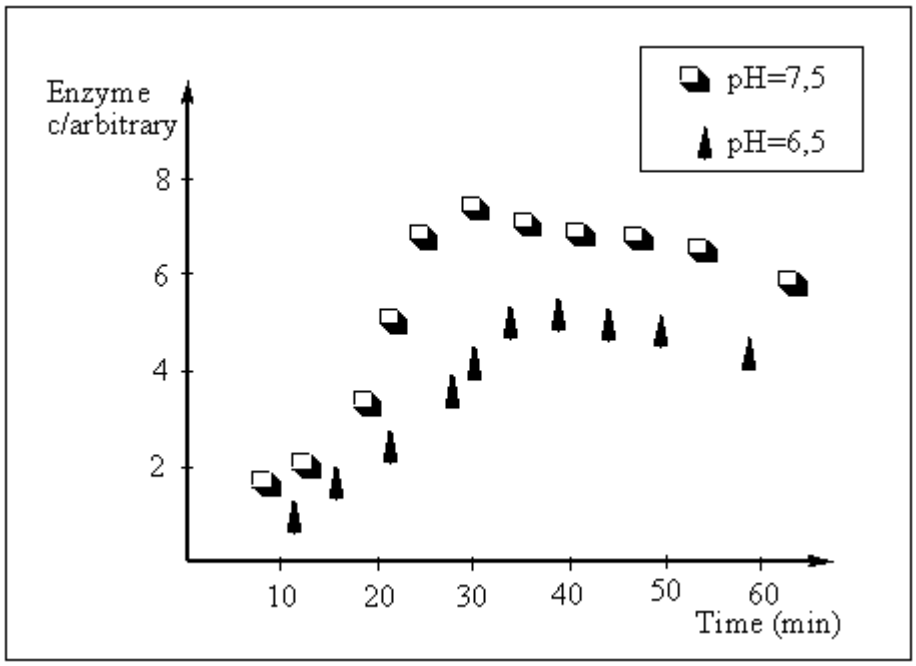

Figure 4. Dynamics of enzyme loss within the system

In a 20-40 minutes' interval the protein diffusion process gets intense, the lost enzyme concentration increasing almost linearly (stage II). It is found that this stage varies in terms of time with the pH, which is 30-40 minutes at a pH= 6.5 and only for 20 minutes, at a pH = 7.5.

During this period when the highest loss of enzyme takes place is followed by a new stage (stage III) when the diffusion process gets down to a minimum between he inside and the outside of the micro-spheres until reaching a thermodynamic equilibrium. After this stage there a no losses of enzyme and the activity of the substance immobilized can be further used in the substrate bioconversion.

Conclusions

The researches carried out represent a preliminary study for the identification of some limiting parameters during the fungal invertase immobilization by encapsulation, applying the ionotrope gelation emulsion method.

This is quite an efficient method for invertase immobility due to the small sizes of the microcapsules and the system homogeneity, elements that can significantly contribute to the improvement of the immobilized enzyme action conditions.

Immobilization efficiency depends on both structure and composition of the polymer chosen as support as well as on the nature of the Ca^{2+} ion vector. Thus the calcium carbonate proved more efficient for the sodium alginate gelation as compared with the organic calcium salts.

The mass transfer through the alginate gel takes place easily which is also confirmed by the average values of the diffusion coefficients.

The stability of the immobilized substance referring to the retaining of the encapsulated enzyme within the system depends on the concentration and type of alginate used as support, pH and temperature.

To establish the conditions under which enzymatic activity is affected along with the behavior and stability of the immobilized enzymatic preparation under special conditions of hydrolysis of the saccharose as substrate, new researches are needed to focus on the invertase activity to be determined by the standard analysis procedure.

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