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Catalase immobilized in capsules in microorganisms removal from drinking water, milk, and beverages

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ABSTRACT

A method for removing microbiological contaminants (bacteria, fungi) present in water, milk, or beverages can be an addition of hydrogen peroxide. The method is known in the literature as "cold pasteurization" and has been used so far in the purification of milk. A possibility of H_2O_2 application in the purification of water and other beverages was considered. Hydrogen peroxide present in liquids must be removed before their application in food and drinks industry. An efficient method of its decomposition is the enzymatic reaction involving catalase. Catalase was immobilized into alginate capsules. Characteristics of the immobilized catalase such as the temperature-activity curve, pH-activity curve, and operational and storage stability were evaluated. Various applications of the preparation were considered. Suitability of the preparation was also investigated using a plug-flow reactor.

Keywords: Clean water; Micro-organism removal; Enzymatic process; Catalase encapsulation; Capsules; Process rate

1. Introduction

The World Organization for Food and Agriculture and the United Nations Committee on Agriculture permit the addition of hydrogen peroxide at a concentration of 0.5–2.5 g l^{-1}, provided that hydrogen peroxide remaining in the preparation is decomposed in a further step [1].

Factors that affect the amount of added hydrogen peroxide include the number and type of micro-organisms, their resistance to hydrogen peroxide, and temperature of the environment. The method is not efficient at high micro-organism concentration [2], but could be considered as an alternative to the treatment of water and other liquids [3–5].

The technique does not have a major impact on the taste of products and nutrients it involves such as amino acids, proteins, vitamins, sugars, and fatty ingredients [1].

Our studies demonstrated that addition of 2.5 g l^{-1} H_2O_2 eliminated all bacterial and fungal contamination in raw milk. *Lactobacillus* strains, occurring naturally in milk at significantly higher concentrations,

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have not been completely destroyed and after removal of H_2O_2, increased at a specific growth rate for itself.

Despite the general information that pure hydrogen peroxide is unstable as a substance used to eliminate bacteria concentrations, it decomposes very slowly. In our study conducted at a concentration of 2.5 g l^{-1}, at pH 5.2 and 7.0, less than 2% of H_2O_2 was decomposed in an hour. A method for an effective, fast decomposition of hydrogen peroxide is based on the use of an enzyme, catalase (H_2O_2 oxidoreductase), which decomposes it into water and molecular oxygen.

Immobilization of catalase into alginate capsules can offer several advantages for industrial (food) applications, including e.g. repeated use, easy separation of purified stream from the biocatalyst, improvement of enzyme stability, and continuous operation in a packed-bed reactor. Alginate is an inexpensive, inert, non-toxic, hydrophilic support, and thus attractive for enzyme immobilization applied in food.

In the present study, an effective method for immobilization of catalase inside the alginate gel capsules is developed. This type of immobilization is called enzyme inclusion. Characteristics of immobilized catalase such as the temperature-activity curve, pH-activity curve, and operational and storage stability were evaluated.

It is expected that:

- the enzymatic preparation presents high catalytic activity in natural medium (water, milk, and juice); high stability under operational and storage conditions; and
- the enzymatic preparation provides easy recovery, reuse, and continuous operation in a packed-bed reactor; presents mechanical resistance.

2. Materials and methods

2.1. Materials

Catalase (hydrogen peroxide oxidoreductase, E.C. 1.11.1.6.) from bovine liver, 2,000–5,000 U mg^{-1}; sodium alginate, HEPES from Sigma Chemical Co.; hydrogen peroxide and other chemicals were obtained from POCh.

2.2. Immobilization of catalase into alginate capsules

For the formation of alginate capsules with catalase, two solutions were prepared. Solution 1—alginic acid sodium salt (20 g l^{-1}) was prepared in a 0.05-M HEPES buffer (pH 7.0 or 6.4) or in a 0.05-M acetate buffer (pH 5.5). Solution 2—catalase solution (2 or 4 mg l^{-1}) was prepared in a 0.05-M HEPES buffer (pH 7.0 or 6.4) or in a 0.05-M acetate buffer (pH 5.5).

This freshly prepared catalase solution (50 ml) was suspended in 50 ml of alginate solution. This suspension was injected into the crosslinking solution. To prepare the crosslinking solution, 30 g of calcium chloride was added to 300 ml of distilled water. The enzyme (0.3 or 0.6 mg) was also added to this solution (to prevent the transport by diffusion of the enzyme molecules in the process of creating a gel structure). The alginate capsules were hardened for one hour at 4˚C and washed twice with the buffer. The capsules were stored at 4˚C in the buffer.

The enzyme concentration was determined by Lowry method [6]. Before and after formation of the capsules, the mass of enzyme in the crosslinking solution was constant. This suggests that the enzyme concentration within the capsules was like in the solution.

2.3. Characterization of immobilized catalase

The catalase activity was determined spectrophotometrically at 230 nm using a standard curve $A(230) = 1.97\ c_S$ [g l^{-1}]. The enzymatic activity was determined in reaction mixture containing substrate H_2O_2 at concentration 0.6 g l^{-1}. The ratio of capsule volume (V_{alg}) to the volume of substrate solution (V_{res}) was 1:20. The enzyme concentration inside capsules was 1 or 2 mg l^{-1}. The reaction was carried out for 1 h.

The effect of pH was investigated in 0.05 M acetate buffer (pH 5.5) or 0.05 M HEPES buffer (pH 6.4 or 7.0) at 24˚C. The effect of temperature was studied in 0.05 M HEPES buffer (pH 6.4) at temperatures ranging 11–40˚C.

The stability of the catalase was studied by measuring the residual activity after incubation of immobilized catalase in 0.05 M acetate buffer (pH 5.5) or 0.05 M HEPES buffer (pH 6.4 or 7.0) at various temperatures (4˚C—storage conditions; 11˚C—the lowest temperature of the process; and 24˚C—the optimal temperature of the process) for different incubation times (every day the first five days, then every 2–3 d for two months).

2.4. Kinetics parameters estimation

To be sure that the influence of the substrate diffusion on process rate is eliminated, kinetic study was carried out in flow system (Fig. 2). The flat alginate structure, $V_{alg} = 0.8$ ml, crosslinked with calcium ions (under the same conditions as described in Section 2.1) containing enzyme at concentration 1.0 or 2.0 mg l^{-1}

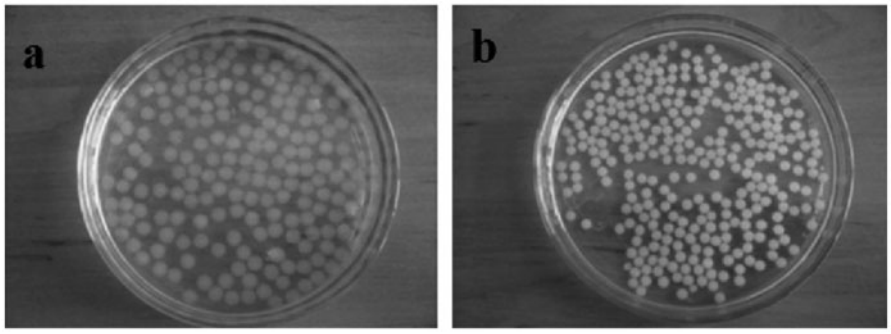

Fig. 1. Alginate capsules kept at T = 24˚C (a) and 4˚C (b).

was prepared. The substrate solution in a volume of 20 ml was circulated through the alginate structure. The activity assay was applied for different H_2O_2 concentrations (up to 3 g l^{-1}). Samples were taken every 20 s for the first five minutes, then every two minutes for 30 min. The concentration of H_2O_2 was determined spectrophotometrically.

2.5. Stability of the catalytic activity in natural media

The catalytic activity was determined in natural media i.e. in fresh cow milk (pH 6.4), tap water (pH 7.0), and Tymbark vegetable juice (pH 5.5). H_2O_2, at concentration 0.6 g l^{-1}, and the immobilized catalase were added to the media. The ratio of capsule volume (V_{alg}) to the volume of solution (V_{res}) was 1:20. The enzyme concentration inside capsules was 2 mg l^{-1}. The process was carried out in BioFlo Bioreactor (New Brunchwick, USA) wherein the oxygen concentration was continuously measured. The reaction was carried out for 2 h.

In order to compare, the reaction in buffers at the same conditions in BioFlo Bioreactor was carried out. Activity in natural media has been compiled with the activity in buffer of the same pH.

2.6. Influence of residence time on the substrate conversion degree

Experiments were carried out at process conditions (11˚C, pH 6.4) in packed-bed reactor. Alginate capsules with the biocatalyst at the concentration of 2 mg l^{-1} inside had a diameter of 2 mm. The total column volume (V_R) was 28.8 ml. The column porosity was checked experimentally using blue dye. The substrate (H_2O_2) concentration in feed stream was 2.5 g l^{-1}.

The residence time in different experiments was in the range 0.1–1 h. Experiments were carried out until steady state, what was equivalent 6–7 volumes exchange.

3. Experimental results

3.1. Characterization of immobilized catalase

The preparation was obtained under conditions chosen experimentally using 1% solution of sodium alginate, 1% (w/v) calcium chloride in a crosslinked bath, at 4˚C. Linking time was 60 min. These conditions eliminate leakage of the enzyme during formation, storage, and application.

The obtained capsules were transparent, round, and almost of the same size (outer diameter at 24˚C in the range of 3.9–4.1 mm). During storage at 4˚C, the capsules had a tendency to shrink to a diameter of 3.2 mm (Fig. 1). After transfer to the aqueous solution at 24˚C they re-took the original size. This behavior resulted from the physicochemical properties of hydrogels.

The preparation was characterized in terms of enzymatic activity and stability depending on temperature and pH (Figs. 3 and 4). Optimum temperature was found at 24–35˚C, but activity was also exhibited at lower temperatures (above 10–11˚C). This is important in view of using the preparations in beverages stored in refrigerated conditions.

The immobilized enzyme showed an optimum pH of 7.5, but the preparation had a broader pH range at relatively high activity. At lower pH values (6.4–7.0) corresponding to the pH of water, milk preparation presents more than 60% of the maximum. The preparation could not be used in fruit juices, beer, and wine (where pH < 5.5). It could be considered applicable in the preparation where the enzyme is immobilized by a covalent bond. Then, the profile of enzyme activity in relation to pH often changes [7]. Thus, the achieved effect can be satisfactory.

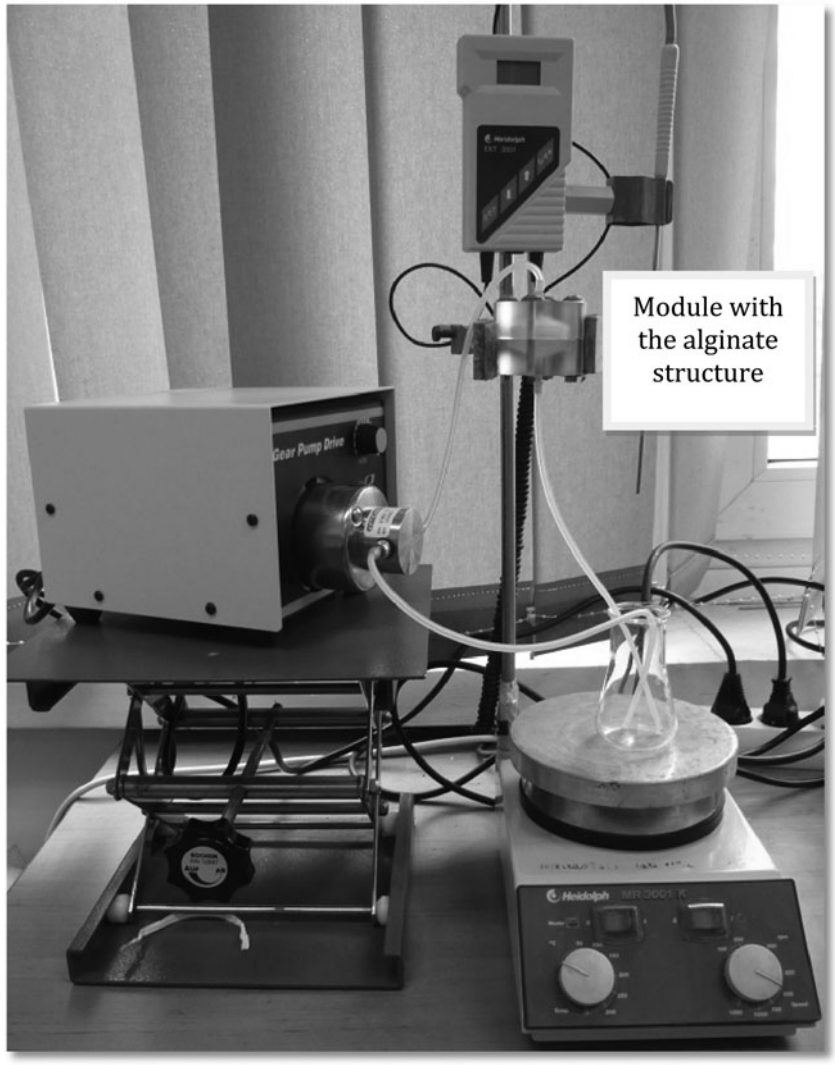

Fig. 2. Set of apparatus for determining kinetics of an encapsulated enzyme.

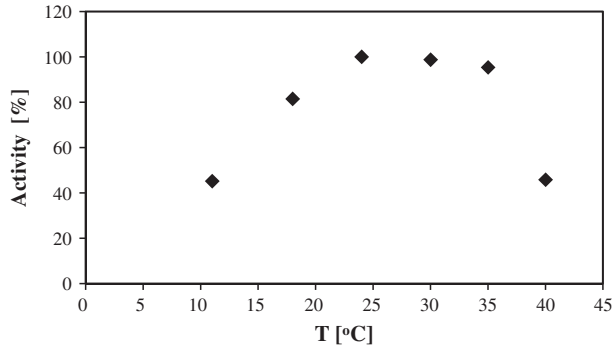

Fig. 3. The temperature profile of catalase immobilized in alginate capsules (pH 6.4). 100% activity corresponds to the activity at 24˚C.

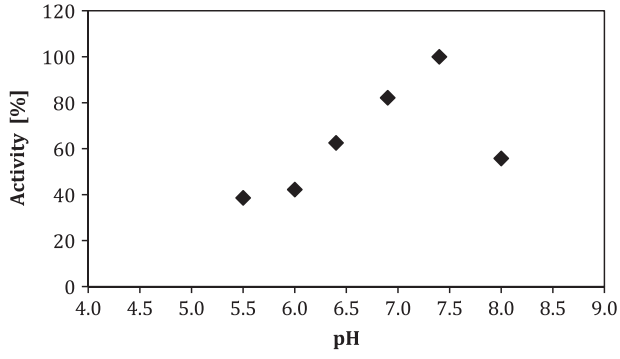

Fig. 4. The pH profile of catalase immobilized in alginate capsules (24˚C). 100% activity corresponds to the activity at pH 7.4.

Thermal stabilities of the immobilized catalase were investigated at 4, 11, and 24 °C and pH 5.5, 6.4, and 7.0. The half-lives were calculated by assuming first-order kinetics of inactivation [8], cf. Table 1.

Regardless of the pH, stability of the enzyme decreases significantly with increasing temperature. Therefore, the preparation should be stored under refrigeration (4–5 °C). For long-term use of the enzyme, it is recommended to carry out the process at lower temperatures (10–15 °C), although this is not the optimum temperature (Fig. 3).

The range of the enzyme concentration is limited by mechanical stability of the immobilized preparation. Oxygen formed during the reaction, which accumulates in the preparation, results in cracking. Thus, molecular oxygen formation rate cannot be greater than its diffusion into the external environment. It was experimentally determined that the maximum concentration of the enzyme was 2 mg l^{-1}. Concentration range of the substrate corresponds to the concentrations used during the cold pasteurization of drinks (up to 3 g l^{-1}).

3.2. Kinetics parameters estimation

For the purpose of determining the reaction kinetics, alginate planar structures were formed. Through these structures, substrate solution was circulated at a pressure of 0.2 MPa—Fig. 2. The structure and the physical and chemical properties of the preparation was the same as in the capsules. Convective flow through the structure eliminated the possible influence of the substrate diffusion on the process rate.

Substrate solution at V_{res} was circulated through the alginate structure containing enzyme (V_{alg}). Total volume of the system in which the decreasing substrate concentration was monitored was $V_{res} + V_{alg}$. To describe the reaction rate in the tested system, the equation described in the literature [7] was applied. In the range of concentrations, when the kinetics is of the first order with respect to the substrate and enzyme, the reaction rate can be expressed by Eq. (1).

$$r_{imm} = -\frac{dc_S}{dt} \cdot \frac{V_{res} + V_{alg}}{V_{alg}} = k_{1,imm} \cdot c_{E,imm} \cdot c_S = k' \cdot c_S \quad (1)$$

Table 2 shows the values of kinetic constants.

3.3. Stability of the catalytic activity in natural media

Usefulness of the preparation and correctness of the determined kinetic parameters was checked under natural conditions. For this purpose, alginate capsules were introduced into vegetable juice (pH 5.5), milk (pH 6.4), and tap water (pH 7.0). In order to determine the activity and stability of the preparation, the process temperature was maintained at 11 °C. The progress of the process was monitored by an oxygen electrode, as the used spectrophotometric method failed with colorful solutions. The following observations were made:

Vegetable juice pH 5.5

(1) The capsules are stable in the environment. Due to dyes, which penetrate into the interior, the stained capsules were red.
(2) Kinetic equations describing the activity and stability of the preparation set out in the acetic buffer (pH 5.5) were slightly different. The enzyme activity decreased by 19.8% ($k_{1,imm}$ = 595.6 l g^{-1} h^{-1}) and the inactivation constant increased and amounted to 0.026 d^{-1}. Hence the half-life was 26.6 d.

Table 1
Thermal stability of the immobilized catalase—the first-order inactivation constant and half-lives (50 mM HEPES buffer).

		Inactivation constant [d^{-1}]	Half-life [d]
pH 5.5 (e.g. some vegetable juices)	4 °C	0.0151	45.9
	11 °C	0.0228	30.4
	24 °C	0.381	1.82
pH 6.4 (e.g. milk)	4 °C	0.0133	52.1
	11 °C	0.0195	35.5
	24 °C	0.339	2.04
pH 7.0 (e.g. water)	4 °C	0.0122	56.8
	11 °C	0.0189	36.7
	24 °C	0.318	2.18

Table 2
Experimental values of kinetic constants (50 mM HEPES buffer, 11 °C).

pH	Kinetic constant $k_{1,imm}$ [l g^{-1} h^{-1}]	Range of application
5.5	742.6	Substrate concentration until 3 g l^{-1}
6.4	1,064.5	Enzyme concentration until 2 mg l^{-1}
7.0	1,568.1	

Milk pH 6.4

(1) The capsules are stable in the environment. Calcium present in milk is likely to positively stabilize the preparation. The capsules do not alter their physical features after storage in milk for several days.
(2) Based on the mass balance of proteins present in the milk before and after incubation of the capsules, no difference was reported. Hence, it is concluded that the milk proteins do not adsorb onto the surface of the capsules in a significant (noticeable) degree.
(3) Kinetic equations describing activity and stability of the preparation set out in the HEPES buffer (pH 6.4) with good accuracy describe the process taking place in milk. The observed differences (+/−4.8%) are within the analytical error.

Tap water pH 7.0

(1) The capsules are stable in the environment.
(2) There was a risk that the enzyme would not exhibit activity at the low ionic strength. Nevertheless, there were no significant differences in the activity of the catalyst (+/−6.1%). However, stability was impaired. The inactivation constant was $0.0212\,d^{-1}$, hence the half-life was calculated to be 32.7 d.

3.4. Influence of residence time on the substrate conversion degree

A model of the packed-bed reactor (with alginate capsules) was elaborated (see Fig. 5). Porosity of the column (the volume of voids in relation to the entire column volume) was determined experimentally. Experiment with the time of flow of the blue dye from the column at a known rate allowed us to estimate the value of the reactor volume. The balance of the differential reactor:

$$Q \cdot c_S = Q \cdot (c_S + dc_S) + r \cdot dV_R \cdot (1 - \varepsilon) \tag{2}$$

Using Eq. (1)

$$-Q \cdot dc_S = dV_R \cdot (1 - \varepsilon) \cdot k' \cdot c_S \tag{3}$$

Introducing residence time τ

$$\frac{dV_R}{Q} \cdot \varepsilon = d\tau = -\frac{\varepsilon \cdot dc_S}{(1 - \varepsilon) \cdot k' \cdot c_S} \tag{4}$$

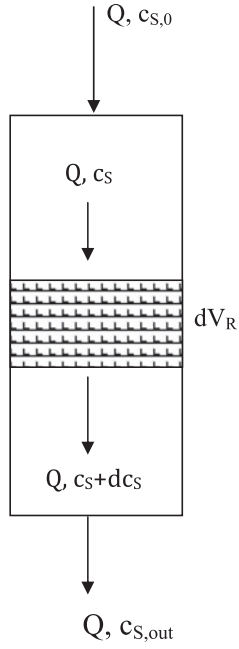

Fig. 5. Scheme of a tubular bioreactor with packed bed.

Integrating

$$\tau = -\frac{\varepsilon}{k' \cdot (1 - \varepsilon)} \int_{c_{S,0}}^{c_{S,out}} \frac{dc_S}{c_S} = \frac{\varepsilon}{k' \cdot (1 - \varepsilon)} \cdot LN \frac{c_{S,0}}{c_{S,out}} \tag{5}$$

A continuous bioreactor with packed bed was designed. Due to the flow resistance, it was found that the diameter of the capsules should not be less than 2 mm. Inside the larger capsules, there is a higher risk of substrate concentration gradient. Then, the enzyme immobilized in the central part of the carrier may be unused. This fact decreases the economy of the process. We remember also that due to diffusion of the formed oxygen, the reaction rate, i.e. concentration of the enzyme, is limited and should not exceed $2\,mg\,l^{-1}$. Inactivation of the enzyme affects the reduction of constant k'.

The process assumptions:

- concentration of the substrate in the fed stream: $2.5\,g\,l^{-1}$,
- concentration of the substrate in the outgoing stream: $<0.025\,g\,l^{-1}$ (conversion > 99%),
- concentration of the enzyme inside the capsules: $2\,mg\,l^{-1}$,
- capsule diameter: 2 mm,
- pH 6.4 (50 mM HEPES buffer) at 11 ℃,
- operation time of the system: 492 h (corresponding to a decrease in enzyme activity by 60%), and
- residence time: 0.1–1 h.

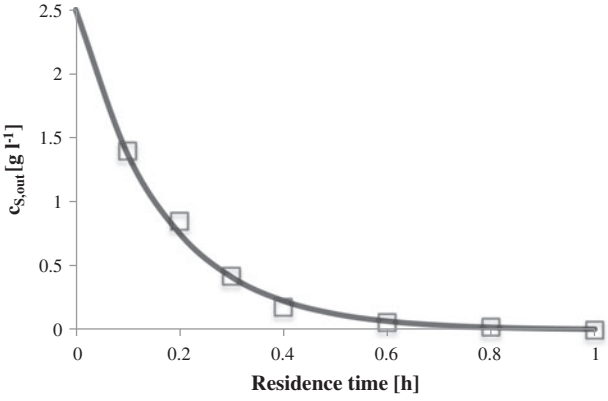

Fig. 6. The outlet concentration of substrate vs. residence time (experimental—points, model-line).

Fig. 6 shows experimental results obtained at different residence times in the initial period of the bed operation. The experimental results were compared with theoretical ones. Good compliance (mean relative error 9.5%) indicates correctly assumed kinetic regime of the process and correctly determined kinetic constant.

At the beginning of the process, the requisite degree of conversion (>99%) is obtained at a residence time of 0.4 h. In further steps, due to a decrease in the biocatalyst activity, the time should be longer. At the time of 492 h (at the biocatalyst activity at 40% of the initial activity), this time must amount to 1 h. Therefore, the bed must work with a variable dispensing.

4. Conclusions

The paper presents the possibility of application of the preparation of included catalase. The carrier used is non-toxic, will not be damaged, or dissolved in the application. This may have direct contact with drinking water and food.

For capsules having a diameter greater than 2 mm, the process can be carried out on a packed-bed reactor. By controlling the enzyme concentration within the formulation (in the range of 2 mg l^{-1}), the size of the capsules and the residence time can achieve the required concentration of hydrogen peroxide in the output stream. In view of the enzymatic activity of the preparation at a pH above 5.5, it is applicable to remove hydrogen peroxide in water, milk, and some vegetable juices. The included enzyme is active at temperatures above 10°C; thus it can be applied in products stored in cooling conditions or in waters of different origins and also during winter.

Acknowledgments

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Nomenclature

$c_{E,imm}$ — enzyme concentration inside alginate structure, g l^{-1}
c_S — substrate concentration, g l^{-1}
$c_{S,out}$ — substrate concentration in the outgoing stream, g l^{-1}
$c_{S,0}$ — substrate concentration in feed the fed stream, g l^{-1}
$k_{1,imm}$ — kinetic constant, l g^{-1} h^{-1}
k' — modified kinetic constant, h^{-1}
Q — stream, l h^{-1}
r_{imm} — reaction rate inside alginate structure, g l^{-1} h^{-1}
t — time, h
V_{alg} — volume of alginate structure, l
V_R — volume of packed-bed reactor, l
V_{res} — volume of substrate reservoir, l
ε — porosity
τ — residence time, h

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