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PVA-biocatalyst with entrapped viable *Bacillus subtilis* cells

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Abstract

Bacillus subtilis cells were entrapped in polyvinyl alcohol (PVA)-cryogel beads without decay in their viability and capability of secretion of proteolytic enzymes (metalloproteinase and subtilisin). Conditions for preparation of the PVA-biocatalyst with suitable stability and viability of *B. subtilis* cells were optimized. Diffusion of various compounds into the cryogel (sliced beads) has been monitored *on-line* using image analysis system. Optimal working conditions and kinetic constants for hydrolysis of proteins catalyzed by the PVA-biocatalyst containing whole *B. subtilis* cells were estimated. The PVA-biocatalyst was applied in the hydrolysis of casein. The productivity of the biocatalyst (expressed as an amount of liberated aromatic amino acids) reached a maximal level of 12 mg g^{-1} h^{-1}. Composition of mixture of peptides was dependent on pH, concentrations of Na^+ and glucose, and in the reaction milieu. Protein hydrolysates of desired composition can be obtained using *B. subtilis* viable cells immobilized in PVA-gel. Incubation of the immobilized cells in a nutrient medium with casein successfully regenerated proteolytic activity of the biocatalyst.

Keywords: Immobilized cells; *Bacillus subtilis*; Proteolytic activity; PVA-cryogel

1. Introduction

Immobilized growing cells are thought to belong to the most promising biocatalysts for application in bioprocesses. Recently, development of immobilized biocatalysts containing microbial cells entrapped in a matrix of polyvinyl alcohol (PVA)-cryogel — highly porous hydrophilic gel — is gaining an increasing interest [1–4]. This synthetic polymer is cheap, easily accessible, widely used as biomedical and textile industry materials and frozen food coatings, non-toxic and water-soluble. PVA-based cryogels are obtained by freezing the concentrated solution of the polymer, keeping them frozen for a definite time and, subsequently, thawing them [5]. The purpose of the present work is the creation of a PVA-biocatalyst with viable *Bacillus subtilis* cells, which is able to produce extracellular proteinases (metalloproteinase and subtilisin), and its application in protein hydrolysis.

2. Materials and methods

2.1. Chemicals

PVA (MW 72 kDa, DP 1600, DH 97.5–99.5, Fluka), plant oil, haemoglobin, azoalbumin, azocasein, isoelectric casein (Difco), crystal violet.

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2.2. Microorganisms

B. subtilis IBTC-3 strain, serine proteinase and metalloproteinase producer were obtained from the microbial culture collection at the Institute of Technical Biochemistry of the Technical University of Lodz. Bacterial spores were collected after cultivation of bacteria in a liquid medium containing starch, lactose, casein, corn steep liquor and salts, for 72 h at 30°C. Resulting biomass (containing 90% of spores) was shocked (for 15 min at 80°C) before immobilization.

2.3. Methods

Immobilization of cells was done by "freezing–thawing" (f–t) method [6,7]. Spherical particles of the biocatalyst were obtained by carrying out the PVA-cryogel formation in a hydrophobic phase. The biomass mixed with aqueous solutions of PVA was dropped into plant oil and the mixture was frozen (at −25°C for at least 10 h) and thawed (at a rate of approx. 0.5°C/min) several times. After the polymer had solidified, the beads were collected and washed with 0.14 M NaCl. *Viability* of the immobilized bacteria was determined as colony forming units (CFU) after dissolving the gels (70°C, 2 min). CFU was estimated by plating the samples on Frazier medium (Difco). *Diffusive* properties of cryogels were characterized using image processing and analysis system (IMAL). *Proteolytic activity* of biocatalyst was estimated by modified Anson method: beads of biocatalyst (0.05 g) were added to 1.2 ml of 2% haemoglobin (pH 7.3 or 10.0) and incubated for 1 h at 30°C. Products of proteolysis were determined with Folin reagent. *The specific activity of the biocatalyst was expressed as an amount of μmoles of tyrosine released from the substrate for 1 min by 1 g of beads of biocatalyst.*

2.4. Hydrolysis of proteins

Entrapment of *B. subtilis* cells was performed using a system of three identical reactors (type "plug flow", volume 30 cm^3) filled with this biocatalyst (1–2 g of beads/100 ml of substrate). Casein solutions were recycled through the reactors at a velocity of 20 ml min^{-1}, at 40°C. The substrate was supplied with NaCl and/or glucose, at pH from 6.0 to 10.0. For induction of proteolytic properties (*activation* after the cells immobilization and/or *regeneration* after each hydrolysis cycle), the whole-cell PVA-biocatalyst was incubated in a liquid nutrient medium (18–24 h, 30°C) and washed with 70% ethanol and 0.8% NaCl solution. Only activated or regenerated biocatalyst was applied in hydrolysis.

Determinations during protein hydrolysis were as follows: *aromatic amino acids* concentration (Folin reagent), *free* NH_2-group concentrations in hydrolysates (before and after precipitation of the protein with triacetic acid; Rosen method [8]), amount of free *B. subtilis* cells in hydrolysates (as CFU). Samples of substrates and hydrolysates were collected (at −10°C) and subsequently analyzed by ultrafiltration and HPLC-RP techniques.

3. Results and discussion

3.1. Properties of PVA biocatalyst with entrapped B. subtilis cells

B. subtilis cells were entrapped in PVA-cryogel beads as described in Section 2. Conditions of production of stable, unsoluble at 50°C biocatalyst beads were established before [9]. It was observed that an increase in polymer and cell concentrations, a number of f–t cycles, and a decrease in temperature of freezing, accelerated formation of insoluble PVA beads of biocatalyst. Cryogels displaying high stability can be obtained from 12–15% PVA solution after 4–5 cycles of freezing (at −25°C) and thawing (0.5°C min^{-1}). In this study, an effect of polymer concentration on viability of immobilized bacteria and diffusive properties of the biocatalyst was investigated.

100% viability of bacteria was found after five cycles of f–t of 10–12% PVA-gels, which also contain 0.02% of alginate (Fig. 1). These good results are caused by protective effect of the polymer solution present in macropores, occupied by entrapped bacterial cells. This conclusion is supported

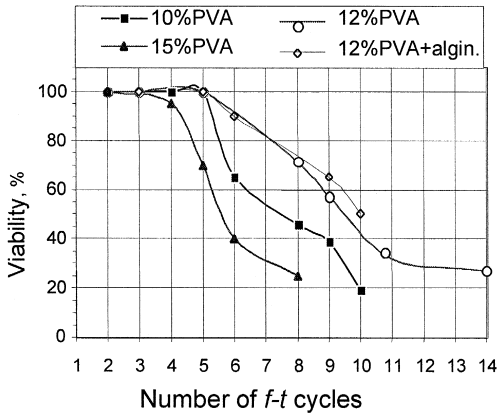

Fig. 1. Viability of *B. subtilis* cells entrapped in cryogels prepared from different solutions of PVA in water, during f–t processes.

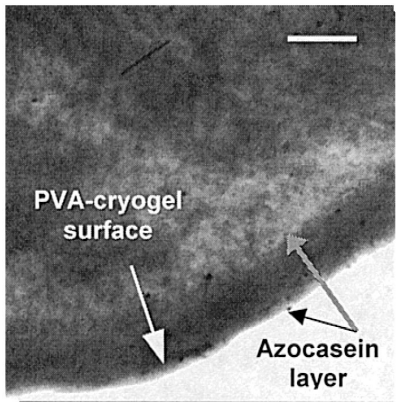

Fig. 3. Example of picture presenting azocasein diffusion into a PVA-cryogel bead for 30 min (bar = 200 μm).

by a decrease in immobilized cell viability during further hardening of the gel matrix (as a result of an increase of f–t cycles number), which results in the decrease of the concentration of the polymer dissolved in the matrix macropores. [10]. Thus, mechanically stable beads of biocatalyst containing 100% of living, entrapped *B. subtilis* cells can be obtained after 4–5 f–t cycles from 12% solution of PVA with these cells.

Diffusion of various compounds, applied as their solutions, into the cryogel (sliced beads) has been monitored *on-line* using IMAL connected to a microscope. Distances between fronts of penetrating molecules and gel border were measured and some images, saved at certain time intervals, were subjected to densitometric analysis. It was revealed that large molecules could penetrate the PVA-biocatalyst. Diffusion distances of haemoglobin (68 kDa), azoalbumin (45 kDa), azocasein (21 kDa) and crystal violet (MW = 372) after 30 min were 64, 91, 161 and 725 μm, respectively. Fig. 2 demonstrates "profiles of penetration" of some proteins into the PVA-biocatalyst (containing 2.5×10^8 of spores/g of beads) detected after densitometric analysis of microscopic images, presented for azocasein in Fig. 3. Under the same conditions of experiment, azocasein does not penetrate alginate, but its molecules accumulate around the surface of alginate beads (Fig. 4).

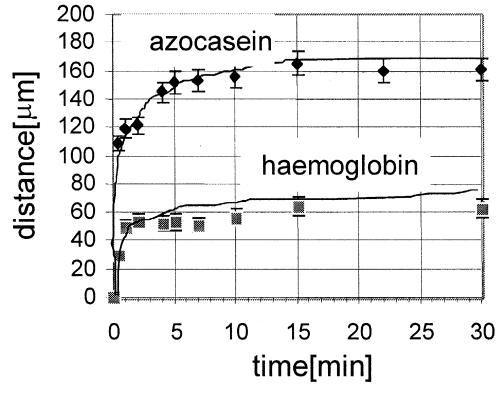

Fig. 2. Plots of penetration of azocasein and haemoglobin into the PVA-cryogel (obtained during *on-line* analysis using IMAL, see text).

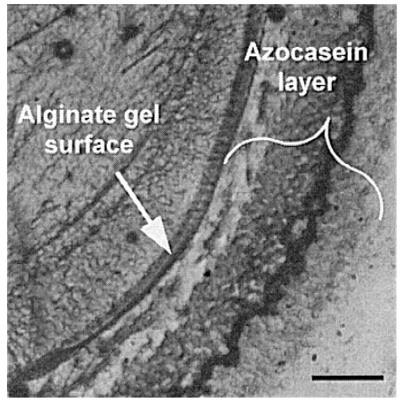

Fig. 4. Microscopic picture of an alginate bead in azocasein solution (2%) showing accumulation of azocasein molecules near the surface of the gel (bar = 200 μm).

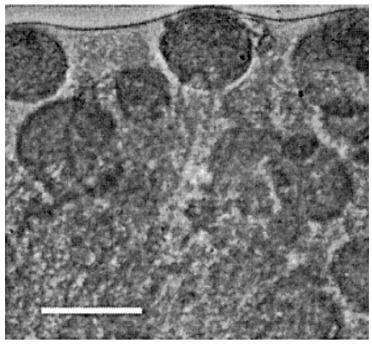

Fig. 5. Microscopic picture of PVA-biocatalyst after 3 years of storage and activation in a nutrient broth medium (microcolonies are distinct) (bar = 50 μm).

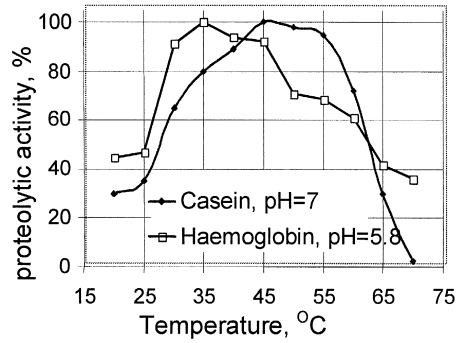

Fig. 6. Effect of temperature on proteolytic activity of the PVA-biocatalyst.

The studies indicate that the entrapment of biocatalysts in PVA-cryogel provides their contact with macromolecular substrates, including proteins that are digested with proteinases produced by viable *B. subtilis* cells.

3.2. Proteolytic activity of immobilized *B. subtilis* cells

Investigation on properties of *B. subtilis* bacteria after their entrapment into the PVA-cryogel confirmed their ability to produce and secrete extracellular proteinases (unpublished data). For this reason, directly after immobilization, the cells should be activated using 18–24-h incubation of beads of biocatalyst in a nutrient medium supplied with 1% of casein. Such activation results in cell reproduction, thus, producing colonies inside the gel (Fig. 5), and the beads of biocatalyst display proteolytic activity (assayed according to Section 2.3). Activation of the biocatalyst is possible even after 3 years of incubation at 4°C (the wet form) or at room temperature (dried form). But in the first case, the biocatalyst shows only the initial metalloproteinase activity (at pH 7.3) and loses even up to 85% of serine proteinase activity (at pH 10.2) (Table 1). Crude preparations of soluble proteinases from *B. subtilis* IBTC-3, entrapped under the same conditions, are completely unstable and lose all activity during both storage and protein hydrolysis. Entrapped viable *B. subtilis* cells are supposed to be "sustainable" source of proteinases.

Table 1
Proteolytic activity[a] of *B. subtilis* cells entrapped in the PVA-cryogel

Sample	Time of storage (months)	Subtilisin	Metalloproteinase
1	0	71	144
2	2	21	170
3	8	14	153
4	16	12	244
5	36	10	159
6	26	50	170

Sample of PVA-biocatalyst.1–5: wet beads, stored at 4°C.6: dried beads, stored at room temperature.

[a]Proteolytic activity of the biocatalyst [μmol min^{-1} g^{-1}], defined in Section 2.3.

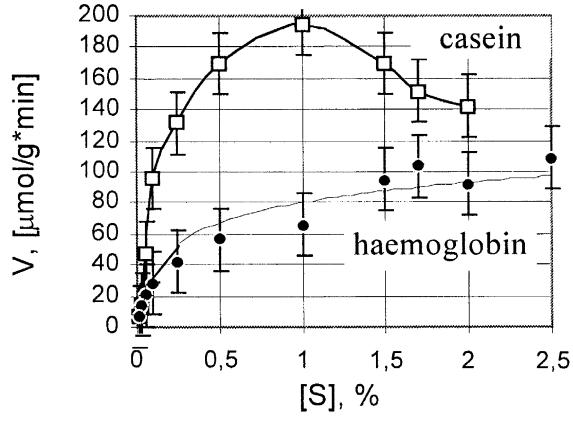

Fig. 7. Effect of substrate concentrations on an initial rate of hydrolysis.

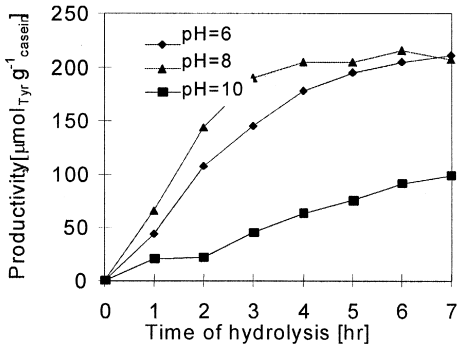

Fig. 8. Effect of pH on casein hydrolysis.

Incubation of activated beads of biocatalyst in solutions of proteins causes their proteolysis. Digestion of haemoglobin (denatured with urea) is most extensive at pH 5.8 and 35°C, and of 1% casein solution at pH 7.0 and 45°C (higher concentration of this protein diminishes the rate of proteolysis) (Figs. 6 and 7). Relative Michaelis constants (K_M) for the activated biocatalyst are 7.9×10^{-5} and 4.1×10^{-5} M against casein and haemoglobin, respectively, in comparison to the K_M value of soluble subtilisin against haemoglobin which is 1.8×10^{-5} M. Properties of the immobilized biopreparation enable its application as a source of proteinases in the hydrolysis of casein.

3.3. Protein hydrolysis using immobilized B. subtilis cells

B. subtilis cells entrapped in PVA-cryogel (6×10^8 cells g^{-1} of biocatalyst) and activated as described above were applied in casein hydrolysis in a process where the substrate is recycled, as described in Section 2.3. The productivity of the biocatalyst (expressed as an amount of released aromatic amino acids) reached maximal level of 12 mg g^{-1} h^{-1}.

Table 2
Composition of casein hydrolysates obtained using the PVA-biocatalyst with whole viable *B. subtilis* cells

Sample of:	HPLC-RP analysis[a]					Ultrafiltration[b]		
	Time of retention [min] (acetonitrile concentration [%]					Percentage of fractions[c] containing peptides of molecular weight, (kDa):		
	0–9 (0)	10–19 (0–37)	20–29 (38–60)	30–39 (61–84)	40–50 (85–100)	> 10	from 0.5 to 10	< 0.5
	Percentage of fraction							
Isoelectric casein	8.3	49.9	27.8	6.7	7.4	99.7	0.3	0
Casein hydrolysate, pH_0 6.0, with 0.54-mM Ca^{+2}[d]	2.9	82.7	7.7	3.8	3.0	0	39.3	60.7
Casein hydrolysate, pH_0 8.0, with 0.54-mM Ca^{+2}	3.5	82.7	6.3	5.1	2.4	9.6	34.3	56.2
Casein hydrolysate, pH_0 10.0, with 0.54-mM Ca^{+2}	46.4	18.0	32.3	3.3	0	43.8	21.5	34.7
Casein hydrolysate, pH 8.0, addition of 100-mM NaCl and 0.5% glucose	6.4	83.3	5.6	1.3	3.4	0.0	32.4	67.6
Casein hydrolysate, pH 8.0, addition of 200-mM NaCl and 0.5% glucose	6.4	85.5	4.2	1.0	2.9	41.8	18.8	39.4
Casein hydrolysate, pH 8.0, without any additions	7.4	83.3	3.9	1.5	3.9	17.3	38.9	43.8

[a] HPLC-RP analysis was performed using a column packed with hydrophobic carrier (type C_8); volume of samples 20 or 100 μl; pH range 6–8; elution: 0.1% TFA in water: acetonitrile (from 0% to 100%); elution rate: 1 ml min^{-1}; monitoring: spectrophotometric, $\lambda = 220$ nm.

[b] Peptides in selected samples were fractionated using membranes: 10 000 Da (PM10) and 500 Da (YC05) and ultrafiltration apparatus (Sartorius).

[c] Expressed as percentage of aromatic amino acids in the fractions.

[d] Ca^{2+} cations were added to stabilize subtilisin.

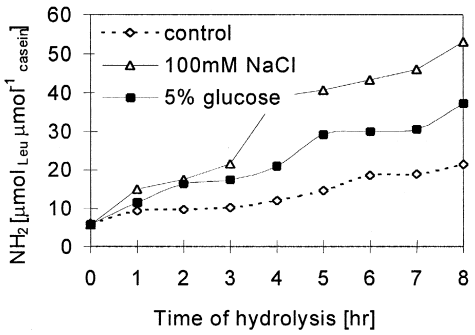

Fig. 9. Effect of NaCl and glucose on productivity of casein hydrolysis.

Dynamics of hydrolysis and composition of hydrolysates depend on the pH of the reaction milieu (Fig. 8, Table 2). The maximal rate of protein hydrolysis was observed at pH 8.0. Enrichment of substrate solution with 100-mM NaCl or glucose, enhanced the hydrolysis rate (Fig. 9). Maximally, 25% of peptide bonds in casein molecules were hydrolyzed after 5 h of this process.

An addition of Na^+ to the reaction medium probably enhanced secretion of subtilisin from immobilized *B. subtilis* cells (mono- and divalent cations can stimulate the serine proteinase secretion as was reported in [11]). More effective proteolysis could also result from changes in casein molecule conformation, giving a form more susceptible to proteolysis. Composition of casein hydrolysates obtained using entrapped *B. subtilis* cells in different conditions was also determined. Hydrolysates obtained with an addition of Na^+ ions and glucose (at pH 8.0), or without any additives at pH 6.0, contained mainly short-chain peptides (see Table 2). More peptides with MW above 500 Da were obtained in the presence of 200-mM NaCl or at pH 10.0. However, the products were qualitatively different, as confirmed by chromatograms of casein hydrolysates, obtained using HPLC-RP (Table 2). It means that the protein hydrolysates of desired peptide composition could be obtained using *B. subtilis* viable cells immobilized in PVA-gel.

It was revealed that immobilized *B. subtilis* cells could be applied in casein hydrolysis several times, providing periodic regeneration of their proteolytic activity by means of incubation in nutrient medium, as described in Section 3.2. Additionally, after proteolysis, the beads of biocatalyst were washed with 70% ethanol (3–5 min, sterilization and probably an enhancement of their permeability [12]) then the biocatalyst was washed with physiologic saline solution. Such processing of immobilized cells successfully regenerates their biocatalytic activity.

4. Conclusions

B. subtilis cells can be entrapped in PVA-cryogel beads without decay in their viability and capability of secretion of proteolytic enzymes (metalloproteinase and subtilisin). Incubation of the immobilized cells in a liquid nutrient medium supplied with casein successfully regenerates their biocatalytic activity and enables their multiple application in protein hydrolysis. The protein hydrolysates of desired peptide composition could be obtained using *B. subtilis* viable cells immobilized in PVA-gel.

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