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THE EFFECT OF IMMOBILIZATION ON ACTIVITY AND STABILITY OF A PROTEASE PREPARATION OBTAINED BY AN INDIGENOUS STRAIN, *BACILLUS LICHENIFORMIS* B 40

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The aim of this work was to immobilize a protease preparation obtained by the fermentation of an indigenous strain, *Bacillus licheniformis* B 40, and to study the influence of several parameters on the activity and stability of the immobilized protease preparation. The tested immobilization methods were physical adsorption on the surface of some inorganic porous supports, silica gel entrapment, and a combined method of entrapment of the enzymes in silica gels deposited on an inorganic support. The immobilization of *B. licheniformis* B 40 protease in silica gels by entrapment or entrapment-deposition proved to be a valuable tool for its thermal and pH stabilization.

INTRODUCTION

Immobilization of enzymes plays an import role within applied biotechnology,[1] in aqueous or organic media. One of the main issues concerning enzyme immobilization is maintaining or even enhancing the structural stability of the catalytic biomacromolecules in view of long-term applications. A large number of techniques and supports are now available for the immobilization of enzymes or cells on a variety of natural and synthetic supports. The choice of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its final application.[2]

Because of commercial applications, proteases have been the focus of many immobilization studies: entrapment, covalent binding, cross-linking and adsorption. A combination of one or more of these techniques may lead to very good results. Commercial success has been achieved when support materials have been chosen for their flow properties, low cost, no toxicity, maximum biocatalysts loading while retaining desirable flow characteristics, operational durability, ease of availability, and ease of immobilization.

More recently, a promising approach to protease immobilization makes use of inorganic and hydrophobic matrixes, such as silica-gel obtained by sol-gel technique.[3-6] Xerogels are synthesized in the presence of enzyme. The resulted nano or mesocomposite material can be dried and grounded, yielding a powder that is stable in both aqueous and organic solvents. The enzymes immobilized by the sol-gel method are used in biotransformations due to their specific properties. These gels show high porosity, high active surface, low density and low thermal conductivity.[7,8] The silica matrix protects biomaterials against external aggression (pH, temperature, solvents).[5,9] This immobilization method possesses the advantages of beneficial qualities, such as transparency, which permits direct spectroscopic assay, and considerable mechanical stability in both

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aqueous and organic solvents, which results in the maintenance of enzymatic activity for several months.[9,10]

Physical bonding is perhaps the simplest of all the immobilization techniques and one which does not alter the activity of the bound enzyme. Physical bonding has a major disadvantage, the easy leaching of the enzyme. Entrapment in polymeric matrixes has also an inherent disadvantage, the diffusional limitations. This steric problem is important especially when the enzyme, a biomacromolecule, has as substrate also a macromolecule. The combination of both immobilization methods, by deposition of the silica gel containing the enzyme in a thin film on an inorganic support, may enhance the advantages and minimize the disadvantages of each technique.

The biological applications of sol-gel chemistry appear to be very promising.[11,12] The aim of this study was to find some simple and efficient methods to immobilize proteases on inorganic supports, with yields as high as possible, in order to obtain insoluble enzymatic preparations with high enzymatic activity, relatively cheap and stable, with potential applications in biotechnology and agriculture (as additives in animal feeding).

In the last several years the feed enzymes have become very well accepted feed additives.[13] The presence of proteases in feed composition is useful and valuable if we consider that protein is an important and very expensive ingredient in animal food diets.[14] The technology of combined fodder involves heat treatment that could cause inactivation of enzymes. A very important issue is maintaining the biocatalytic activity and stability of feed enzymes until and after these will be consumed by animals.

RESULTS

Bacteria and fungi, especially *Bacillus* (*Bacillus subtilis, Bacillus thermoproteolyticus, Bacillus licheniformis, Bacillus amyloliquefaciens)* and *Aspergillus (Aspergillus saitoi, Aspergillus oryzae, Aspergillus niger)* strains, are the most used microorganisms for proteases production for feed industries. These microorganisms produce by fermentation proteases that differ by optimal pH (in acid, neutral or alkaline range) and specificity. *Bacillus* strains produce both alkaline proteases (serine-proteinases, EC 3.4.21) and neutral proteases (metalo-proteinases, EC 3.4.24).[15]

To produce an immobilized enzymatic preparation as stable, cheap and easily obtained as possible, a series of microbial strains were tested, some of them being genetically modified. The strains of *Bacillus subtilis*, *Bacillus globigi*, *Bacillus licheniformis* and *Aspergillus oryzae* are preserved in the collection of industrial microorganisms of the Industrial Microbiology Laboratory of USAMVB Timisoara. [16,17] (Table 1). The enzymatic preparations with protease activity were produced by discontinuous fermentation in submerse medium, in stirred flasks.

The *Bacillus licheniformis* B 40 protease was used in the subsequent immobilizations by physical bonding (on glass, under magnetic stirring and on alumina, in a chromatographic column), entrapment and entrapment-deposition on two different ceramics (Ceramics type I and Ceramics type II), directly from the culture medium, or after the precipitation of the enzyme with ammonium sulphate. A comparison of protease activities of the immobilized enzymes is shown in Table 2.

Table 1

Screening for protease producing *Bacillus* and *Aspergillus* strains

Microbial strains	pH	Fermentation time (hours)	Protease activity[1] (U/100 mL)
Bacillus subtilis USAMVB	8-8.5	48	21.97
Bacillus subtilis 4amy-	8.5	40	43.92
Bacillus globigi R	5.5	48	26.59
Bacillus globigi S	5.5	48	-
Bacillus subtilis Tr 1	7.5	48	48.54
Bacillus subtilis Tr 2	6.5	48	-
Bacillus subtilis Tr 3	6.5	48	19.66
Bacillus subtilis B 52	5.0	40	35.25
Bacillus subtilis B 36	8.5	40	26.57
Bacillus globigi amy+	6.0	40	38.14
***B. licheniformis* B 40**	**6.0**	**48**	**99.50**
Aspergillus oryzae 19 A	5-6.5	48	23.70
Aspergillus oryzae 14 B	5-6.5	48	29.48

[1]All the assays were done in triplicate and the percentage error in each set of readings was within 5%.

Table 2

Immobilization of the proteases produced by a *Bacillus licheniformis* B 40 local strain

Immobilization methods			Protease activity[4] µmol·min^{-1}·g^{-1}	Immobilization yield[5] %
Physical bonding[1]	**PB_1**	Zeolite	0.14	9.3
		Alumina	0.09	6.2
	PB_2	Alumina (24 h fermentation time)	0.12	8.5
		Alumina (48 h fermentation time)	0.13	13.9
	PB_3	Ceramics type I	0.99	74.9
		Ceramics type II	2.29	63.2
Entrapment[2]	**E_1**		0.27	3.5
	E_2	1	0.73	3.3
		2	0.56	0.7
Entrapment-deposition[3]	**ED_1**	1	0.31	14.0
		2	0.32	4.9
	ED_2		0.51	4.2

[1]PB – physical bonding; [2]E – entrapment; [3]ED – entrapment-deposition; [4]All the assays were done in triplicate and the percentage error in each set of readings was within 5%; [5]Immobilization yield (%) = ($U_{tot\ (im)}/U_{tot(i)}$) x100, where $U_{tot\ (im)}$ = protease activity of immobilized enzyme (U/mg) x total weight of immobilized product (mg) and $U_{tot\ (i)}$ = protease activity of native enzyme (U/mL) x total volume of native (initial) enzymatic solution used for immobilization (mL).

For an immobilized preparation with enzymatic activity to be efficient as feed additive it is essential to have some significant properties: no toxicity, low production costs, high activities and stabilities under physiological conditions where they have to act (low pH and 37°C) and in time (stability under ambient storage conditions). Not in the last, it must be solid, in order to be well mixed and homogenized with the solid feed ingredients.

Microbial strains of *Bacillus licheniformis* are used in feed industry. The ceramic supports obtained by PROCEMA SA are ecological and environmental – friendly products, the silica gels are non toxic and create an aqueous microenvironment around enzyme almost similar with that observed in biological media. The discussed immobilization methods are accessible and easy to handle in order to obtain solid preparations with protease activity. Once they have been obtained it is important to establish their activity and stability in physiological conditions.

Immobilization often stabilizes the enzymes structure, thereby allowing their applications even under harsh environmental conditions. To study the influence of temperature on the free and immobilized enzyme behavior, the protease activity was assayed for native and immobilized enzymatic preparation (adsorbed, entrapped and entrapped/deposed) at temperatures from 20 to 75ºC, using the Anson method, at pH 7.0 (Fig. 1).

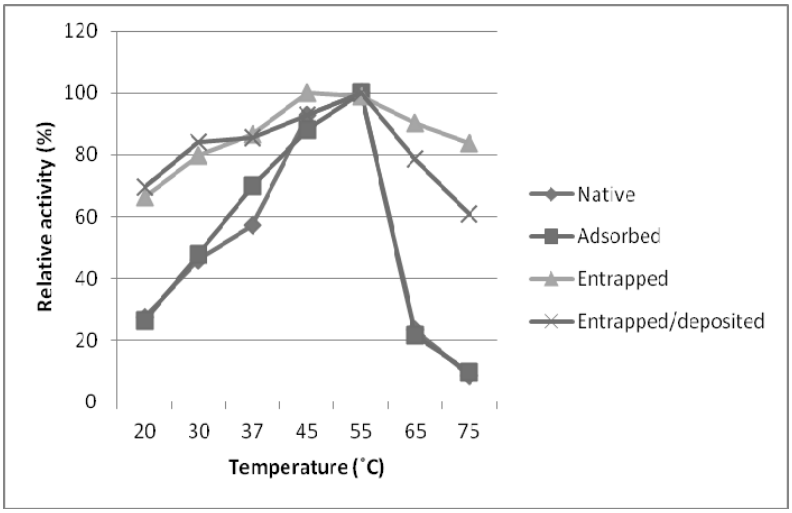


Fig. 1 – Temperature profile of protease activity of free and immobilized enzymatic preparation (all the assays were done in duplicate and the percentage error in each set of readings was within 7%).

In order to study the effect of pH on the enzyme behavior, the protease activity was assayed by Anson method in buffered solutions of pH range 2.2 – 12, at 37ºC (Fig. 2).

The thermal stability of free and immobilized enzymes by physical bonding on ceramics (PB_3) was monitored maintaining the enzymatic preparations for one hour in buffered solutions of pH 7, at different temperatures (37ºC, 50ºC and 60ºC). Protease activity was measured using the Anson method, at 37ºC and pH 7.0 (Fig. 3).

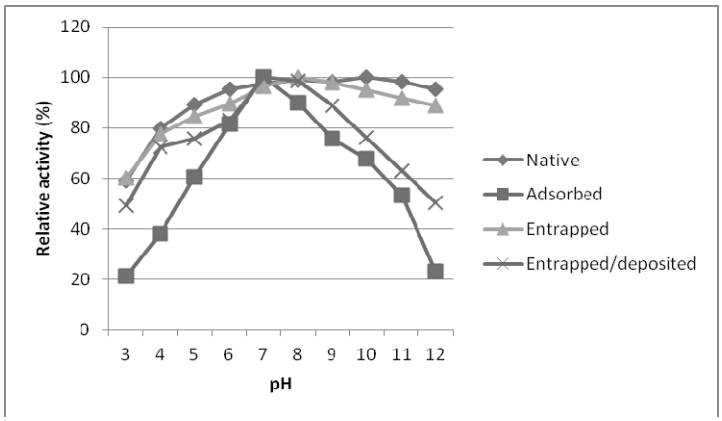
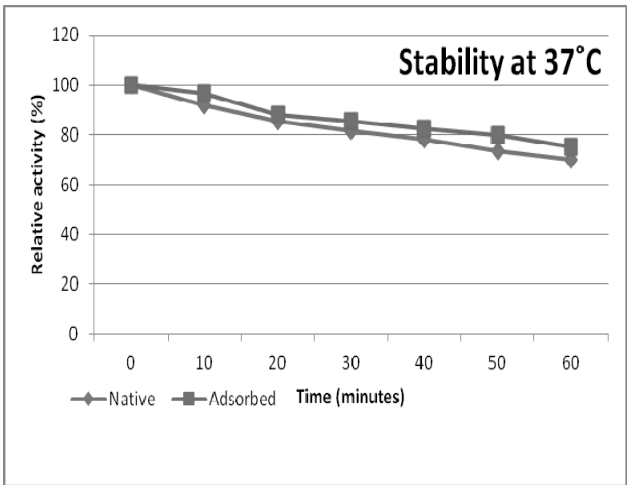
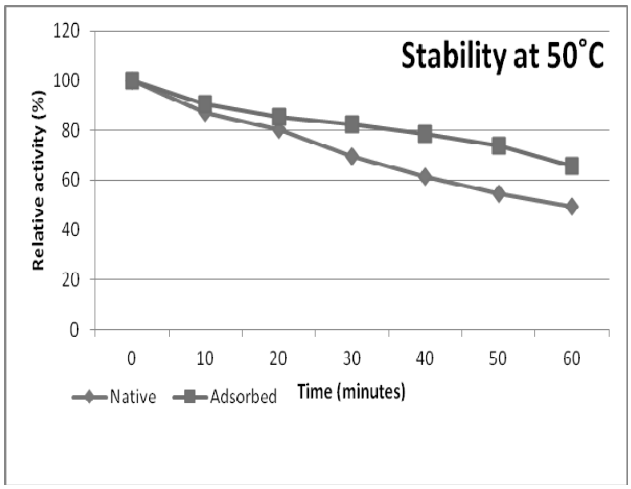


Fig. 2 – pH dependence of protease activity of free and immobilized enzymatic preparation (all the assays were done in duplicate and the percentage error in each set of readings was within 7%).





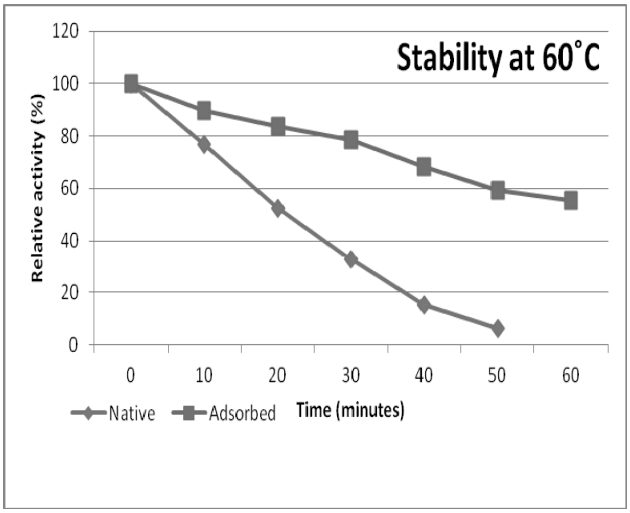


Fig. 3 – Temperature stability of enzymatic preparation, free and immobilized by physical bonding on ceramics (all the assays were done in triplicate and the percentage error in each set of readings was within 5%).

The stability of the adsorbed enzymatic preparation on ceramics type II (PB_3) and entrapped/deposed on ceramics (ED_2) was studied at 37ºC and pH 3, for an hour, to check if the immobilized enzymes could function at a pH and a temperature compatible with the physiological conditions of the proximal region of the digestive tube of the mammals, for application in animal feeding (Fig. 4).

The enzymatic preparation adsorbed on native ceramics (PB_3) was kept at 4ºC and the stability was monitored in time. Enzymatic activities were tested periodically (Table 3).

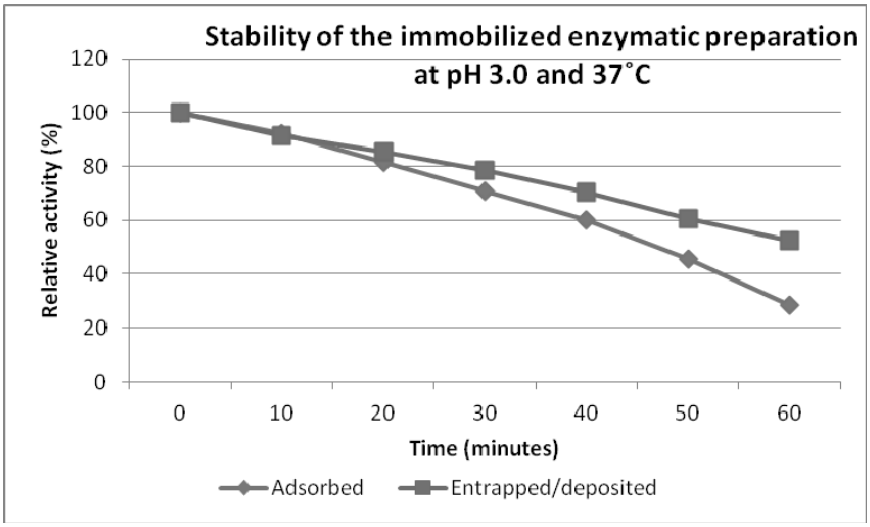


Fig. 4 – Stability of the immobilized enzymatic preparation at pH 3.0 and 37ºC (all the assays were done in triplicate and the percentage error in each set of readings was within 5%).

Table 3

Preservation stability of physically adsorbed enzyme

Period of storage time (months)	0	1	2
Protease activity[1], U/g	0.99	0.59	0.11
Relative activity, %	100.00	59.5	11.4

[1]All the assays were done in triplicate and the percentage error in each set of readings was within 5%.

DISCUSSION

Several cultures of *Bacillus licheniformis* B 40 were prepared and several variants of physical bonding were used. The best protease activity was obtained for the *Bacillus licheniformis* B 40 strain. Considering that the aim of the research was to obtain immobilized proteases, as cheap as possible, the enzyme was not purified initially, but it was immobilized directly from the culture medium. The best results were obtained by adsorption on indigenous ceramics, using the method PB_3 (enzymatic product adsorbed on ceramics type I – protease activity 0.99 $\mu mol \cdot min^{-1} \cdot g^{-1}$, immobilization yield 75%; enzymatic product adsorbed on ceramics type II – protease activity 2.29 $\mu mol \cdot min^{-1} \cdot g^{-1}$, immobilization yield 63.2%).

An entrapped enzymatic product was obtained having an activity of 0.27 $\mu mol \cdot min^{-1} \cdot g^{-1}$, lower than that obtained by adsorption (immobilization yield 3.5%). Using the combined method, an immobilized enzyme was obtained, with activity 20% higher compared to the entrapped one. Comparing the protease activities of the immobilized products it was noticed that the protease activities obtained by entrapment and entrapment-deposition were 8.6 and 7.2 times lower than that obtained by adsorption.

The methods of entrapment and entrapment-deposition of proteases directly from the diluted fermentation medium led to enzymatic preparations with lower protease activities compared to those obtained by adsorption on ceramic support. To avoid this, centrifugates from *Bacillus licheniformis* B 40 submerse cultures were fractionally precipitated with ammonium sulphate up to 60% saturation. A preparation with protease activity of 143.33 U/g and protein content of 0.11 mg_{BSA}/mg of solid product

was obtained. This enzyme was immobilized by entrapment, yielding products with protease activities (0.73 μmol·min^{-1}·g^{-1}and 0.56 μmol·min^{-1}·g^{-1}) 2.5 times higher compared to the case when the raw fermentation medium was used. The enzymatic product obtained by entrapment-deposition had a protease activity of 0.51 μmol·min^{-1}·g^{-1}, slightly lower than that obtained by entrapment, but a slightly greater immobilization yield.

The pH- and temperature-activity profile of the free and immobilized enzymatic preparation and the stability of the immobilized products (at temperature, pH and storage) were studied. The optimum temperature of the free enzymatic preparation was 50-55ºC. The enzyme immobilized by adsorption on ceramic support had temperature dependence almost similar to that of the native enzyme. The preparations immobilized by entrapment and entrapment-deposition showed activities relatively greater than the native enzyme, the protease activity dependence on temperature having a plateau form. All immobilized enzymatic preparations at 37ºC presents better relative activities (PB_1 69.82%, E_1 86.62%, ED_1 85.71%) than the native enzyme (57.08%). At the extremes of the studied temperature range, 20ºC and 75ºC, the relative activities of the entrapped enzyme were 2.5 and 9 times higher than those of the free and adsorbed ones; for the entrapped-deposited enzyme these were also 2.7 and 6.6 times higher.

The free enzymatic preparation had a plateau variation of the enzyme activity on a broad pH domain, from 6 to 12. The entrapped enzymatic preparation, even at the extreme pH values, had a good protease activity, the residual activity being 60% and 88% from the maximum (at pH 8.0-9.2) respectively. In the case of the enzyme immobilized by entrapment-deposition, the pH dependence of the activity was quite similar with that of the entrapped enzyme for pH values lower than 8.0, whereas in the basic pH domain, the decrease was deeper (at pH 12.0, 50.5% of the maximum activity was found). The enzyme immobilized by adsorption had maximum activity at pH 7.0, and for values remote from the optimum, the relative protease activity was smaller than that of the entrapped and entrapped-deposited enzymes.

The comparative study of the behavior of the native and immobilized enzymes proved that the immobilization had a positive effect on the enzyme thermal stability. This effect was obvious especially at 60ºC, when the native enzyme lost its activity after 50 minutes, whereas the adsorbed enzyme showed activity (52%) even after an hour of incubation at this temperature. The half-life time ($t_{1/2}$) of the enzymatic preparation adsorbed on ceramics was higher than that of the native enzyme. At 37ºC, 55ºC and 60ºC, $t_{1/2}$ of the immobilized enzyme was 1.33, 1.33 and 3.0 times higher than $t_{1/2}$ of the native one respectively.

The stability of the immobilized enzymatic preparation was studied at 37ºC and pH 3, for an hour, to find out if it was able to function at a pH and a temperature close to the physiological conditions. After 40 minutes of contact with the pH 3 medium, the residual activity of the adsorbed enzyme was 60% and of enzyme immobilized by entrapment-deposition 70% of the initial activity. These results prove that the inorganic silica matrix has a protective effect on enzyme at physiological values of pH and temperature.

The stability of the enzymatic preparation adsorbed on native ceramics was monitored in time. The residual protease activity was 59% after the first month of storage at 4ºC and dropped to 11% after two months.

EXPERIMENTAL

Materials

Casein Hammerstein and tetraethoxysilane (TEOS) were obtained from Fluka, Folin-Ciocalteu's phenol reagent, alumina (pore diameter 4-10 nm) and trichloroacetic acid from Merck. Zeolite (natural material, pore diameter 80-90 nm) was obtained from a local supplier. The ceramics, supplied by Procema S.A. Timişoara, were obtained from natural row materials (perlytic rock) for agricultural applications. They are environmentally friendly and chemically stable products. Ceramics type I is a granular product with cellular structure without impurities, white–grey color, density – 150-250 kg/m^3, water absorption – min. 200%, granulometry – min. 1.0 mm. Ceramics type II appears like porous pellets, brick color, apparent porosity min 75%, granulometry 4-6 mm. All the others chemicals were commercially available reagent grade products and were used without further purification.

The submerged culture was performed in Erlenmeyer flasks with 50 mL culture broth containing 2% corn meal, 2% soy meal, 0.05% $MgSO_4$, 0.2% $(NH_4)_2HPO_4$, 0.1% vegetal oil and water. The fermentation broth was sterilized 15 min. at 120˚C, than incubated under aeration and stirring (240 r.p.m.), 48 hours, at 37 ± 1 ºC. 5% (V/V) inocullum was used. The purity, pH and protease activity were monitored continuously.

Immobilization protocols

Immobilization of enzymatic preparation from *Bacillus licheniformis* B 40 by **physical bonding (PB)**:

PB_1. 10 mL enzymatic preparation (protease activity 0.3 μmol·min^{-1}·mL^{-1}) and 2 g inorganic support (zeolite or alumina) in 2 mL 0.05 M phosphate buffer, pH 7.0 were mixed 3 h, at room temperature, left overnight, filtered and

washed with 3 mL water for 5 minutes under stirring, filtered and washed with 3 mL cold acetone on the filter paper and dried in desicator at room temperature (25ºC) till constant weight.

PB_2. 45 mL enzymatic preparation obtained after 24 h culture (protease activity 0.3 $\mu mol \cdot min^{-1} \cdot mL^{-1}$) or 30 mL enzymatic preparation obtained after 48 h culture (protease activity 0.31 $\mu mol \cdot min^{-1} \cdot mL^{-1}$) are passed with a low flow rate (1 mL/minute) through a chromatographic column (2 cm diameter, 25 cm height) filled with 10 g alumina (thermal activation, 30 minutes at 200˚C). The support was buffered with 0.05 M phosphate buffer, pH 7.0. The support with the immobilised enzyme was washed in column with 10 mL water.

PB_3. 60 g indigenous Ceramic type I and 77 mL enzymatic preparation (protease activity 0.995 $\mu mol \cdot min^{-1} \cdot mL^{-1}$) in 48 mL 0.05 M phosphate buffer, pH 7.0 were mixed 1 h, at room temperature, then 200 mL cold acetone were added under stirring. The product was filtered and washed with 36 mL cold acetone and dried for 24 h at room temperature (25ºC).

4.5 g indigenous Ceramic type II, 3.6 mL 0.05 M phosphate buffer, pH 7.0 and 12 mL enzymatic preparation (protease activity 1.33 $\mu mol \cdot min^{-1} \cdot mL^{-1}$) were mixed 1 h, 30 mL cold acetone were added under stirring, filtered and washed with 15 mL cold acetone and dried for 24 h under ambient conditions (25ºC).

Immobilization by **entrapment (E)** and **entrapment/ deposition (ED)**:

1. Immobilization of proteases directly from the culture medium. A general immobilization by entrapment procedure uses a buffered solution of an enzyme and alcoxysilane in an aqueous-alcoholic medium as gel precursor. The sol was prepared by stirring 10 mL tetraetoxysilane (TEOS), 10 mL EtOH, 8 mL water and 0.2 mL 1N HCl.

E_1. To 25 mL sol, 25 mL EtOH, 25 mL enzymatic preparation (protease activity 1.33 $\mu mol \cdot min^{-1} \cdot mL^{-1}$) and 25 drops of 25% NH_3 1:1 were added. Gelation time was 30 seconds. The sol-gel was left for gelation and ageing one day, at room temperature, dried and washed under magnetic stirring for 5 minutes with 50 mL water, filtered, washed on the filter paper with 25 mL cold acetone and dried in desicator at room temperature (25ºC).

ED_1. 1.5 g indigenous Ceramics type II were mixed with 3 mL sol, 3 mL EtOH, 8 drops of 25% NH_3 1:1 and 3 mL enzymatic preparation (protease activity 1.33 $\mu mol \cdot min^{-1} \cdot ml^{-1}$). The obtained gel, after 1 day of ageing was dried, washed with 6 mL water under stirring (5 minutes), filtered, washed with 3 mL cold acetone, and dried at room temperature till constant weight.

2. Immobilization of proteases concentrated from the culture medium. Centrifugates from *Bacillus licheniformis* B 40 submerse cultures were fractionally precipitated with ammonium sulphate up to 60% saturation. A preparation with protease activity was obtained (143.33 U/g) and immobilized by entrapment and entrapment/deposition.

E_2. To 7 mL of sol, 7 mL EtOH and 7 mL enzymatic solution (20 mg/mL and 50 mg/mL) in 0.05 M phosphate buffer, pH 7.0, 7 drops of 25% NH_3 1:1 were added. Gelation time was 30 seconds. The sol-gel was left for gelation and ageing 1 day, at room temperature, dried and washed under stirring for 5 minutes with 14 mL water, filtered, washed on filter paper with 7 mL cold acetone and dried in desicator at room temperature (25ºC).

ED_2. 2.5 g indigenous Ceramics type II were mixed with 5 mL sol, 5 mL EtOH, 8 drops of 25% NH_3 1:1 and 5 mL enzymatic solution (50 mg/mL) in 0.05 M phosphate buffer, pH 7.0. The obtained gel, after 1 day of ageing was dried, washed with 6 mL water under stirring (5 minutes), filtered, washed with 5 mL cold acetone, and dried at room temperature till constant weight.

To study the influence of some medium parameters (pH, temperature) on the free and immobilized enzymatic preparation produced by the native *Bacillus licheniformis* B 40 strain, the protease activity was determined in buffered solutions of pH range 2.2-12, at temperatures from 20 to 75ºC. For thermal stability studies, both the native and immobilized enzyme were kept an hour at three different temperatures (37ºC, 50ºC and 60ºC). The stability of the immobilized enzymatic preparation was studied at 37ºC and pH 3, for an hour. The storage stability at 4ºC of the enzymatic preparation adsorbed on ceramics was monitored for 2 months.

The protease activity was measured by UV-VIS spectrometry, according to the Anson method[18, 19] modified for casein, as follows: the enzyme sample (0.2 mL native enzyme solution, 50 mg enzyme immobilized by physical bonding or 100 mg enzyme immobilized by entrapment and entrapment/deposition) was incubated with 2 mL 2% (w/v) casein Hammerstein solution in 0.8 mL 0.05 M phosphate buffer (pH 8.0) at 37°C. After 10 minutes, the reaction was stopped with 4 mL of 5% (w/v) trichloroacetic acid (TCA). The precipitated proteins were removed by filtration, after centrifugation at 6000 rpm for 5 min. The TCA-soluble fraction (1 mL) was incubated (30 minutes at room temperature) with 1 mL 0.2 N HCl, 4 mL 0.5 M NaOH and 1.2 mL Folin-Ciocalteu's phenol reagent (diluted 1:2 right before use) and the absorbance was measured at 578 nm. One unit of activity is defined as the amount of enzyme that hydrolyzes casein Hammerstein liberating 1 $\mu mol_{Tyr} \cdot mL^{-1} \cdot min^{-1}$. The protein content in the supernatants was estimated using the Folin-Ciocalteu's phenol reagent and bovine serum albumin (BSA) as standard (Lowry method).[20]

CONCLUSIONS

Eleven bacterial (*Bacillus*) strains and two fungal (*Aspergillus*) strains were tested for their protease producing capacity. The protease produced by *Bacillus licheniformis* B 40 (activity of 1 $U \cdot mL^{-1}$) was used in further immobilization experiments. The immobilization on ceramics by physical bonding gave the best activities and immobilization yields. The entrapment of *B. licheniformis* B 40 protease in silica gel using the sol-gel technique seams to be a promising, simple and inexpensive method, leading to products with good activity and stability. The silica matrix protects the enzyme against thermal and pH inactivation better than the physical bonding on ceramics. Our experiments prove that proteases immobilization in silica gels by entrapment or entrapment-deposition is a valuable tool for proteins stabilization in applications such as food additives in animal nutrition.

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