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ORIGINAL PAPER

Sol-Gel Entrapment of *Bacillus licheniformis* CMIT 1.33 Proteases in Silica-Gels

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Abstract

In the present work, the immobilization technique by entrapment in silica gels of Bacillus licheniformis CMIT 1.33 local strain proteases was studied. Enzymes entrapment in solid mesoporous silica gels obtained by sol-gel method was performed by in situ polymerization of tetraethoxisilane (TEOS) and tetramethoxisilane (TMOS). The native enzymatic preparation was entrapped at neutral pH, in presence of a pore forming agent. Entrapped enzymes in silica gels have been clearly shown to retain their biocatalitic activity and exhibit increased stability at environmental effects. The enzyme-substrate affinity modification was investigated based on the kinetics of native and immobilized enzymes respectively, in the hydrolysis of a model substrate with low molecular mass.

Keywords: protease, *Bacillus*, immobilization, sol-gel, silica gel, inorganic support

Introduction

Active and stable biocatalysts are required by the different fields of biotechnology [1-4] and the immobilized enzymes have an essential role [5-7]. The immobilized biocatalysts are used very efficiently in food and feed technologies, as biocatalysts and biosensors in industrial processes, in bioremediation, in medicine and in organic synthesis in aqueous or organic media [8-10].

Over the last years the efforts of researchers from feed industry have been directed toward studying the influence of exogenous enzymes in feed additives to improve the availability of nutrients from feed composition [11-14]. Unprotected potential exogenous feed enzymes can be inactivated in the feed additives technology and they also can be inefficient in specific conditions of animal digestive tract. It is very important to use stabilized feed enzymatic preparations. The immobilization by entrapment of enzymes within porous matrices in order to improve their activity and stability becomes more and more popular. One useful enzyme immobilization method is the entrapment in silica gels using the sol-gel method [15-20]. The sol–gel process is a well-known method to obtain hybrid organic-inorganic ceramic mesoporous materials under mild conditions [21]. The activity of enzymes immobilized in sol-gel matrixes is due to the interaction between enzymes and the surrounding silica network [22, 23]. Silica mesoporous matrices are compatible with biomolecules and retain their biological activity [24-28].

The proteins are a very important and, at the same time, a very expensive part of the feed. For the young animals amino acids requirement dictates a fast and complete hydrolysis of vegetable proteins. Exogenous proteases as feed supplements are useful to

improve proteins nutritive value from animal feed by all type of protein hydrolysis, even proteins that can not be digested by endogenous enzymes, or by completing the animal's own digestive enzymatic system [29, 30].

The aim of this work was the stabilization of a protease preparation obtained by submerged fermentation of *Bacillus licheniformis* CMIT 1.33 local strain. Two immobilization methods were used: entrapment in silica gels and entrapment-deposition on local ceramics [31, 32]. The silica gels were obtained by using different alkoxide precursors (TEOS, TMOS). The activity and stability of native and entrapped enzymatic preparations were studied comparatively, as well as their kinetic behavior.

Materials and methods

Tetraethoxysilane (TEOS), tetramethoxysilane (TMOS) and casein Hammerstein were obtained from Fluka, Folin-Ciocalteus phenol reagent, trichloroacetic acid, $MgSO_4$, $(NH_4)_2HPO_4$ and vegetal oil from Merck. The ceramic, supplied by Procema S.A. Timişoara, was obtained from natural row materials (perlytic rocks) for agricultural applications. They are environmentally friendly and chemically stable products (Table 1).

Table 1. Properties of ceramic

Properties	Description
Product appearance	Granular product with cellular structure without impurities
Colour	White – grey
Density	150-250 kg/m^3
Water absorption	min. 200%
Granulometry	min. 1.0 mm

All the others chemicals were commercially available reagent grade products and were used without further purification.

Four strains of *Bacillus licheniformis* were cultivated under aerobic conditions (rotary shaker, 240 rpm), at 37ºC, for 48 hours in Erlenmeyer flasks containing 50 mL culture medium (sterilized 15 min. at 120˚C) - 2% corn meal, 2% soy meal, 0.05% $MgSO_4$, 0.2% $(NH_4)_2HPO_4$, and water. 5% (V/V) inoculum was used. The purity of culture, pH and protease activity were monitored continuously.

The protease activity was measured by UV-VIS spectrometry, according to the Anson method modified for casein [33, 34]. One unit of activity is defined as the amount of enzyme that hydrolyzes Hammerstein casein liberating 1 $\mu mol_{Tyr} \cdot mL^{-1} \cdot min^{-1}$. The protein content in the supernatants was assayed using the Folin - Ciocalteus phenol reagent (Lowry method) [35].

Immobilization protocols

1. **Entrapment** of enzymatic preparation from *Bacillus licheniformis* CMIT 1.33

A sol-gel entrapment method was performed using a two-step procedure – the acid hydrolysis of alkoxysilane, followed by entrapment of enzymatic preparation, directly from the culture medium, at neutral pH [21].

The sol was prepared by stirring tetraalkoxysilane (TEOS, TMOS, 40 mL), EtOH (40 mL), water (32 mL) and HCl (1N, 0.8 mL) until it becames a clear solution. In the second step the sol was mixed with EtOH, enzymatic preparation (0.62 $\mu mol \cdot min^{-1} \cdot mL^{-1}$ protease activity, 3.88 $mg_{BSA} \cdot mL^{-1}$ protein content) and 25% NH_3 1:1 in 1:1:1:1 volume ratio. Gelation occurred within a minute. The gel is aged one day, at room temperature, in a closed glass and washed with water (sol – water volume ratio 1:2) under magnetic stirring for 5 minutes, filtered, washed on the filter paper with cold acetone (sol – acetone volume ratio 1:1) and dried over $CaSO_4$ at room temperature.

Entrapment in the presence of glucose was done by replacing a part of EtOH volume with the corresponding 0.2 mg/mL glucose solution.

2.**Entrapment/deposition** of enzymatic preparation from *Bacillus licheniformis* CMIT 1.33

The following components: sol (18 mL), EtOH (18 mL), 48 drops 25% NH_3 1:1 and enzymatic preparation (0.62 μmol·min^{-1}·mL^{-1} protease activity, 3.88 mg_{BSA}·mL^{-1} protein content, 18 mL), were added to the inorganic ceramic support (9 g), under magnetic stirring. The gelation occurred in several seconds up to a few minutes. The obtained gel was aged for one day, washed for 5 minutes with distilled water under active stirring (36 mL), filtered in vacuum, washed with cold acetone (18 mL) and dried at room temperature till constant weight.

Influence of environment parameters on immobilized enzyme activity

Studies on the effect of some environmental conditions (pH, temperature) on the activities of enzymatic preparation upon immobilization were done by measurement of the protease activity in buffered solutions of pH range 2.2 – 12.0 and at temperatures ranging from 20 to 95ºC.

The stability of the native and immobilized enzymes in the temperature range of 37-65ºC was monitored by maintaining the immobilized enzymes for an hour in NaH_2PO_4/Na_2HPO_4 buffer solution (0.05M, pH 7.0) at different temperatures. For pH stability studies, the immobilized enzymes were kept one hour at pH 3 and 37ºC. Enzymes stability was analyzed as well after four months of storage at 4ºC.

Kinetic studies

The kinetic study was carried out on the native enzyme and on the enzyme entrapped in silica gels (TEOS and TMOS) obtained in presence of D-glucose, using p-nitrophenyl acetate (NPA) as substrate.

To determine the kinetic parameters, the substrate hydrolysis was performed for 20 minutes, at 37ºC, by varying the concentrations of $5\cdot10^{-3}$ M NPA solution in CH_3CN (0.083-0.333 μmol/mL) with native (200 μL) and immobilized (200 mg) enzymes in NaH_2PO_4/Na_2HPO_4 buffer solution 0.05 M, pH 7.5. Samples were taken at every 2 minutes; the absorbance was measured at 400 nm using a UV-VIS spectrophotometer (PG Instrument T60U Spectrophotometer, 37°C) comparatively with a control. The product concentration (p-nitrophenol, PNF) was obtained from the calibration curve. The initial reaction rates were calculated from the d[PNF]/dt slope at all initial NPA concentrations for the native and entrapped enzymes.

Results

Microorganisms largely used in dietary enzyme supplements industry to manipulate animal digestion are bacteria (e.g. *Bacillus* sp.) and fungi (e.g. *Aspergillus* sp.). Submerged fermentation has been especially used for the production of industrial proteases by microbial strains because of the ease handling and greater possibility to improve enzyme production by optimization of process parameters (medium composition, environmental factors - pH, temperature etc.) [36].

In our study a series of microbial *Bacillus licheniformis* strains were tested. The enzymatic preparations with protease activity were produced by discontinuous fermentation in submerse medium, in stirred flasks (Table 1, Table 2, Figure 1).

Table 1. Screening for protease producing *Bacillus licheniformis* strains

Strain	pH	Fermentation time h	Protease activity U·mL^{-1}	Protein content mg·mL^{-1}
B licheniformis CMIT 1.33	7	72	0.617	3.876
B.licheniformis CMIT 1.35	6.5	72	0.442	1.983
B.licheniformis CMIT 1.34	5.5	72	0.397	1.727
B.licheniformis CMIT 1.32	5.5	72	0.095	1.824

Table 2. The kinetics of protease and protein production by *Bacillus licheniformis* CMIT 1.33 strain

Fermentation time (h)	pH	Protease activity $U \cdot mL^{-1}$	Protein content $mg \cdot mL^{-1}$	Specific activity $U \cdot mg_{prot}^{-1}$
8	6	0.050	1.543	0.032
16	5-5.5	0.098	0.986	0.095
24	5.5	0.148	1.589	0.093
32	5.5-6	0.362	1.692	0.214
40	5.5-6	0.528	2.094	0.252
48	6	0.568	2.244	0.253
56	6.5	0.707	2.425	0.292
64	6.5-7	0.977	3.774	0.259
72	6.5-7	0.617	3.876	0.159

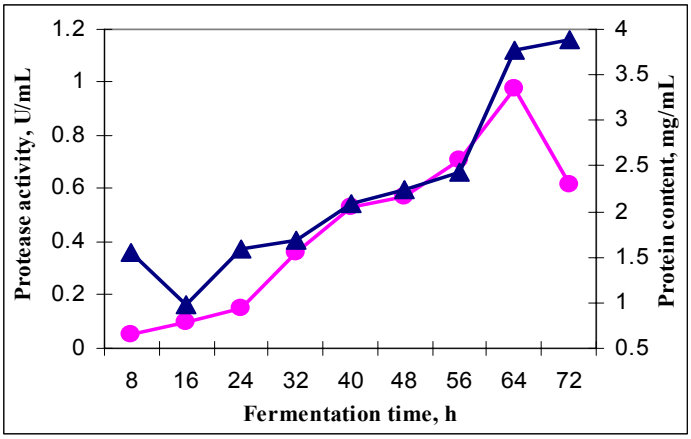

Figure 1. The kinetics of protease and protein production by *Bacillus licheniformis* CMIT 1.33 strain, (●) protease activity, (▲) protein content

The *Bacillus licheniformis* CMIT 1.33 strain (Table 1) was used in the subsequent immobilization studies. The immobilization methods used were entrapment and entrapment-deposition on ceramics, directly from the culture medium (Table 3). The combined method was a way to increase enzymatic activity of immobilized preparations by associating two different immobilization methods, entrapment in gels and physical bonding on the surfaces of porous inorganic supports.

The use of some organic molecules in the gel formation process that may influence the dimensions of the forming pores represents another way to increase the immobilized enzyme activity. Within such meso- or macroporous silica gels, substrate molecules diffusion is much eased, explaining the increase of the enzymatic activity [37, 38]. D-glucose is such a templating agent and was tested for both of the precursors used in entrapment, TEOS and TMOS (Table 3). The general immobilization procedure was used, but parts of the water volumes were replaced with solutions of the templating agent, in the volume ratios admitted by the synthesis recipe of the gel. The glucose concentration in the matrix during gelation was about 30%.

Table 3. Protein content and protease activity of the immobilized enzymatic preparations

Precursor	Immobilization method	Weight g	Protein content $mg_{BSA} \cdot g^{-1}$	Protease activity $\mu mol \cdot min^{-1} \cdot g^{-1}$	Immobilization yield[a] %
TEOS	Entrapment	5.90	8.31	0.31	7.40
	Entrapment (glucose)	14.44	4.77	1.24	72.33
	Entrapment/ deposition	11.15	2.76	0.45	45.27
TMOS	Entrapment	9.80	5.24	0.27	17.08
	Entrapment (glucose)	12.45	5.00	0.71	57.33
	Entrapment/deposition	12.82	3.53	0.14	16.15

[a]Immobilization yield (%) = $100.U_{tot(im)}/U_{tot(i)}$, where $U_{tot(im)}$ = protease activity of immobilized preparation (U/mg).total weight of immobilized preparation (mg), $U_{tot(i)}$ = protease activity of native enzymatic preparation (U/mL).total volume of native enzymatic preparation used for immobilization (mL)

To study the influence of some medium parameters on the free and immobilized enzyme (entrapped, entrapped/deposited) behavior, the protease activity was determined in buffered solutions of pH range 2.2 – 12 (citric acid/Na_2HPO_4 and glycine/NaOH buffer solutions), at temperatures ranging from 20 to 95ºC (Figure 2 and Figure 3).

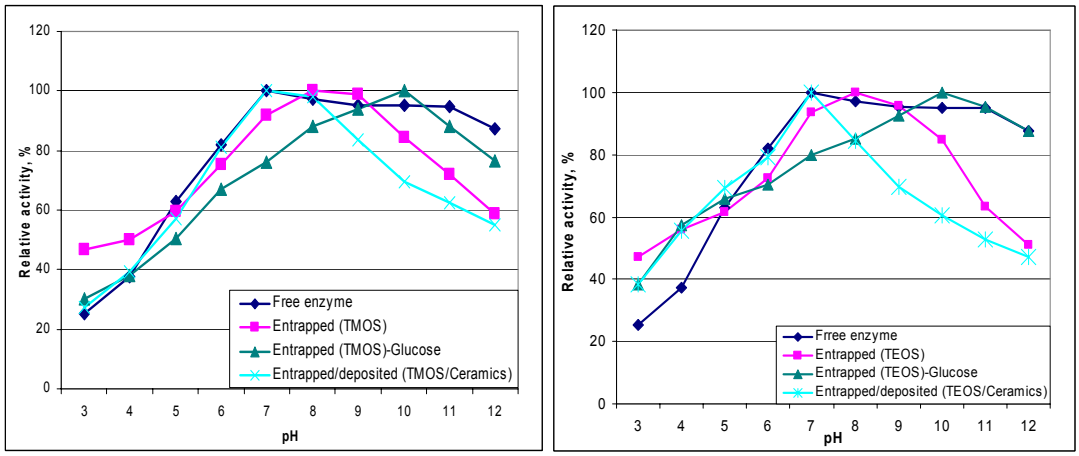

Figure 2. pH dependence of protease activity of free and immobilized enzymatic preparation

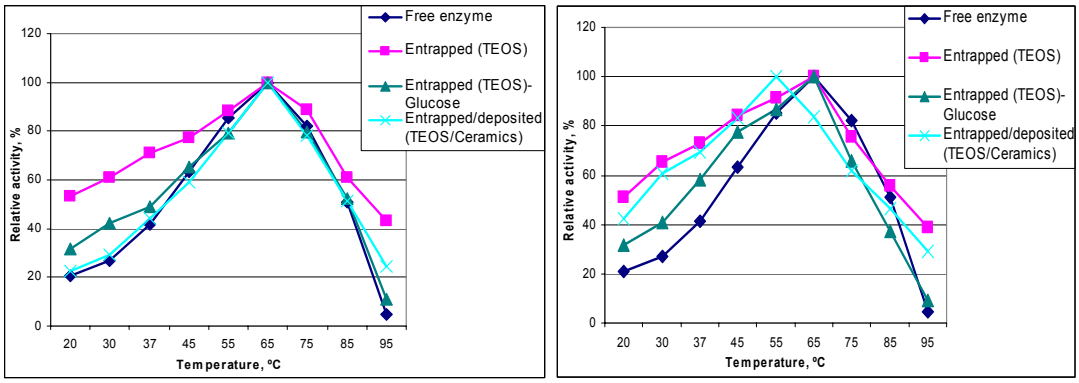

Figure 3. Temperature profile of protease activity of free and immobilized enzymatic preparation

The stability of these enzymes at the temperature range of 37-65ºC was monitored maintaining the immobilized enzymes for a certain period of time at different temperatures. Protease activity was assayed using the Anson method, at 37ºC and pH 7.0 (Figure 4).

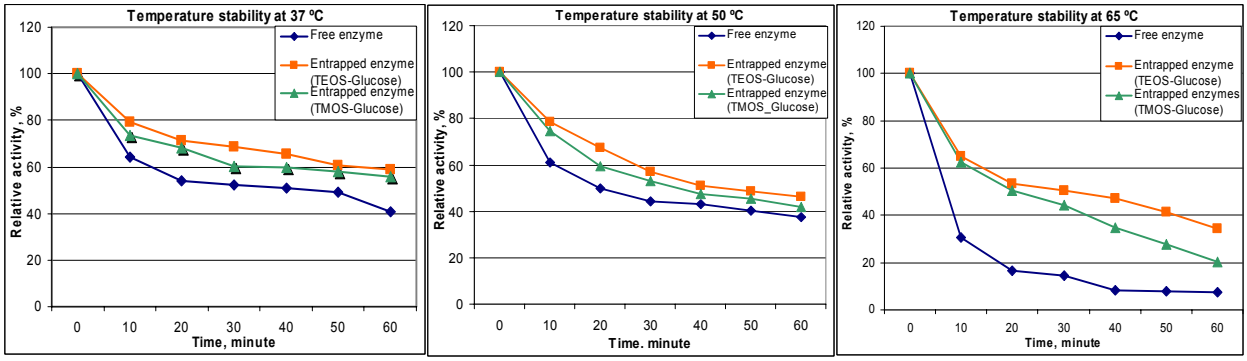

Figure 4. Temperature stability of enzymatic preparations (free (♦) and entrapped enzymes: (■) TEOS-glucose, (▲) TMOS-glucose)

Enzymes stability was analyzed as well after several months of storage at 4ºC. The enzymatic preparations of entrapped and entrapped/deposited were kept at 4ºC and the stability was monitored in time. Enzymatic activities were determined periodically (Table 4).

Table 4. Preservation stability of immobilized enzyme

Immobilization method	Relative activity %			
	initial	one month	two months	four months
Entrapment (TEOS)	100.00	67.10	63.25	51.54
Entrapment (TEOS - glucose)	100.00	63.63	59.44	48.06
Entrapment/deposition (TEOS/ceramics)	100.00	50.22	45.54	32.22
Entrapment (TMOS)	100.00	78.17	69.87	52.74
Entrapment (TMOS - glucose)	100.00	74.82	66.66	49.23
Entrapment/deposition (TMOS/ceramics)	100.00	38.44	10.28	-

To find if the immobilized enzymes could function at a mammalian physiological pH and temperature values and if they are appropriate as feed additives, the stability of entrapped (TEOS and TEOS-glucose) and entrapped/deposed on ceramics (TEOS/ceramics) enzymatic preparations were studied at 37ºC and pH 3, for an hour and in the absence of any preservation agent (Figure 5).

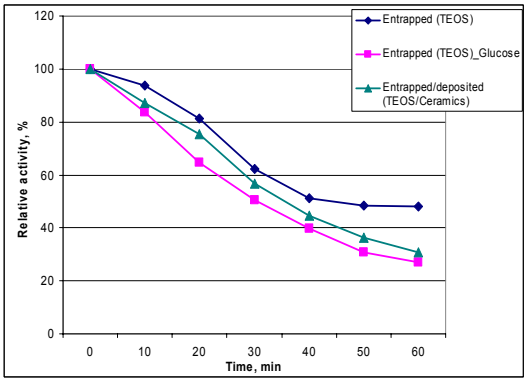

Figure 5. Stability of the immobilized enzymatic preparation at pH 3.0 and 37ºC

The kinetic parameters of the free and the entrapped enzymatic preparation by using the two precursors TEOS and TMOS, in the presence or in the absence of glucose, were obtained using an ester with low molecular weight, p-nitrophenyl acetate, as substrate.

The initial reaction rate dependence on the substrate concentration was monitored to determine the kinetic constants of the free and immobilized enzymes. Samples were taken from time to time, product concentration was obtained from the calibration curve; the initial rates were determined at different substrate concentrations from the d[P]/dt slope and the kinetic parameters V_{max} and K_M were calculated using the Michaelis-Menten equation and the Lineweaver – Burk linearization plot (Table 5 and Figure 6).

Table 5. The kinetics parameters of the free and immobilized enzymatic preparation

Kinetics parameters		K_M µmol.mL^{-1}	$V_{max}\cdot10^3$ µmol.mL^{-1}·min^{-1}	$V_{max}\cdot10^3/K_M$ min^{-1}
Native	Michaelis-Menten equation	0.33	7.99	24.21
	Lineweaver-Burk plot	0.33	8.05	24.40
Entrapped (TEOS)	Michaelis-Menten equation	0.37	2.54	6.86
	Lineweaver-Burk plot	0.35	2.46	7.03
Entrapped (TEOS-glucose)	Michaelis-Menten equation	0.39	3.09	7.92
	Lineweaver-Burk plot	0.59	4.36	7.39
Entrapped (TMOS)	Michaelis-Menten equation	0.27	2.07	7.67
	Lineweaver-Burk plot	0.36	2.56	7.11
Entrapped (TMOS-glucose)	Michaelis-Menten equation	0.15	1.97	13.13
	Lineweaver-Burk plot	0.15	1.92	12.80

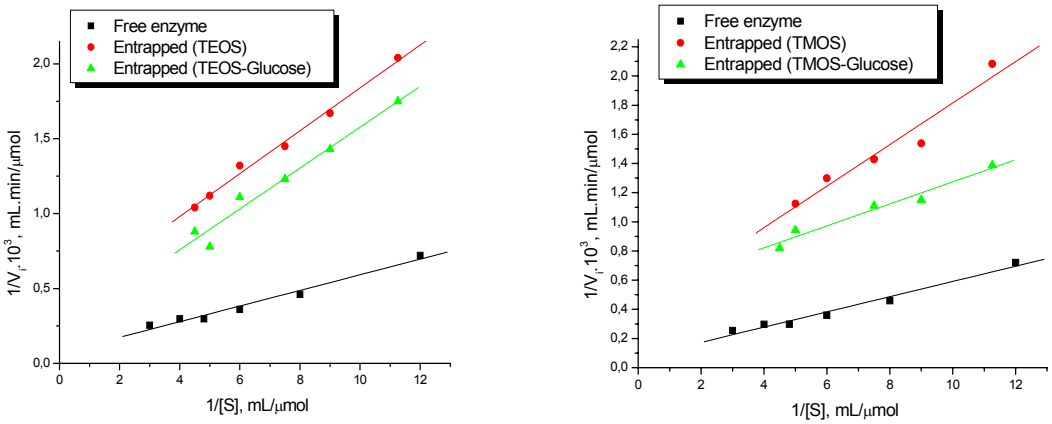

Figure 6. Lineweaver – Burk linearization plots for native and entrapped *B. licheniformis* CMIT 1.33 enzymatic preparations.

Discussion

Four proteases producing microbial strains were tested. The best protease activity was obtained using the *Bacillus licheniformis* CMIT 1.33 strain (Table 1). This enzyme was used directly from the culture medium without any intermediary purification steps, in the subsequent immobilization studies. Production of *Bacillus licheniformis* CMIT 1.33 protease reaches maximum levels (0.977 $U.mL^{-1}$) at 64 h, after that the enzyme production tends to decline (Figure 1).

The results showed that all the enzymatic preparations entrapped in silica gels obtained in the presence of D-glucose had an increased activity compared to the enzymatic activity of the control by 2.6 to 4 times.

Protease activities and immobilization yield of preparations obtained by entrapment/deposition on ceramics, in case of TEOS precursor, were higher than for entrapment, for similar enzyme loadings. In enzyme immobilization using TMOS precursor the increases in enzyme activity and immobilization yield were significant just in the case of entrapment in presence of D-glucose forming pore agent.

The free enzymatic preparation has the optimum pH 7. The enzyme immobilized by entrapment/deposition on ceramics has the same optimum pH as the native enzyme, but in the acidic domain (pH 3-5) the immobilized enzyme activity is increased by more than 10%. In the case of the entrapped enzyme, the activity is slightly reduced in the basic domain, whereas in the acid domain it has an increase in protease activity, for both precursors, TEOS and TMOS, in the presence or in the absence of D-glucose. The increase is more significant in case of TEOS. Entrapment in presence of D-glucose changes the pH-activity dependence profile, the enzymatic activities obtained in basic domain, pH 10-12, were higher (77-100%) than the activities of entrapped (51-85%) and entrapped/deposited enzyme (47-70%).

The optimum temperature of the native and immobilized microbial enzymatic preparation with protease activity is 65ºC. For temperatures lower and higher than the optimum, the relative activity of immobilized enzyme is higher than in the case of the free enzyme. At 37°C all immobilized enzymatic preparation (except entrapped/deposited obtained by TMOS precursor) presented better relative activities than the native one (42%). The best results were obtained for the immobilization in xerogel (TEOS and TMOS precursor) in the absence of D-glucose (73-74%).

The comparative study of the heat stability of native and immobilized enzymes showed that the greatest stability was obtained for the entrapped enzyme in the presence of glucose resulted by using TEOS precursor, than for the enzyme immobilized by using precursor TMOS, and, at last, for the native one. At the 37ºC, native enzyme residual activity

was lower (40.66%) than that of the entrapped enzyme (more than 55%). The enzyme immobilized, after an hour of storage at 65ºC, had a residual activity 4.5 times higher than that of the native one in case of TEOS precursor and 2.75 times higher in case of TMOS.

Enzymatic preparations entrapped in the absence of glucose, for both of precursors TEOS and TMOS, had a very good stability in time, after 4 months from immobilization more than 50% of the initial activity was found. For the entrapped enzyme in the presence of glucose, TEOS precursor, the residual activity was 48% after 4 months of storage at 4ºC, whereas in the case of the enzyme immobilized by entrapment -deposition on silica it was approximately 32% after 4 months of storage.

The enzyme entrapped in matrixes obtained by TEOS provided the best stability at 37ºC and pH 3. After an hour the residual activity of the entrapped enzyme was almost 50% and of enzyme immobilized by entrapment-deposition more than 30% of the initial activity. Immobilization by entrapment in the presence of D-glucose decreases the stability of enzymes under these conditions (27%). The silica gels matrix synthesized in the absence of pore forming agent increased dramatically the stability of the enzyme at physiological values of pH and of the temperature.

The kinetic study revealed that both the native and immobilized enzymes follow a Michaelis – Menten type kinetics. In the case of the immobilized enzymes, the *apparent Michaelis constant*, K_M, is almost similar with that of the native ones, which means that the enzyme affinity for its substrate does not decrease significantly. V_{max}/K_M ratio indicated that immobilization decreased the catalytic efficiency by 4 times.

The entrapped enzyme in presence of pore forming agent, using TMOS precursor, provides a calculated Michaelis constant, K_M, two times lower and a maximum reaction rate, V_{max}, four times lower than that obtained for the native enzyme. This increase in the affinity of enzymes to their substrate that we observed is probably a result of the conformational rearrangement of protein molecule during the immobilization method. This structural modification enhances the substrate molecule access to active catalytic centre of enzymes. V_{max}/K_M ratio exhibited a two times higher catalytic efficiency than other immobilized enzymatic preparations presented previously.

Conclusions

Enzymatic preparations using proteases obtained by submerged fermentation of a local *Bacillus licheniformis* strain were immobilized in/on inorganic supports, using the sol-gel procedure. The influence of immobilization on enzyme activity and stability was studied.

Our results clearly show that immobilization has a remarkable stabilizing effect on enzyme activity, even in the absence of a preserving agent. The exogenous immobilized microbial enzymatic preparations with protease activity can act on available substrate at low pH in stomach and can be used as feed additives. They were stable for a time sufficiently long to act in proximal segment of digestive tract.

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References

1. P.V. IYER, L. ANANTHANARAYAN, *Process Biochem*, 43, 1019-1032 (2008)
2. P. GADLER, K. FABER, *Trends Biotechnol*., 25 (2), 83-88 (2007)
3. J.M. WOODLEY, *Trends Biotechnol*., 26 (6), 321-327 (2008)
4. A. SCHMID, F. HOLLMANN, J.B. PARK, B. BÜHLER, *Curr. Opin. Biotechnol*., 13, 359-366 (2002)

5. L. BETANCOR, H.R. LUCKARIFT, *Trends Biotechnol*., 26 (10), 566-572 (2008)
6. J. KIM, J.W. GRATE, P. WANG, *Trends Biotechnol*., 26 (11), 639-646 (2008)
7. J. E. SMITH, *Biotechnology*, Cambridge University Press, Cambridge (2004)
8. M.E.M. ZAMORA, F.R. MELGAREJO, F.G. CANOVAS, P.A.G. RUIZ, *J. Biotechnol*. 139, 163-168 (2009)
9. S.K. MOCCELINI, A. SPINELLI, I.C. VIEIRA, *Enzyme Microb. Technol*., 43, 381-387 (2008)
10. E. ROSINI, G. MOLLA, M.S. PILONE, L. POLLEGIONI, S. SACCHI, *J. Biotechnol*., 135, 377-384 (2008)
11. J.M. TRICARICO, J.D. JOHNSTON, K.A. DAWSON, *Anim. Feed Sci. Technol,* 145, 136-150 (2008)
12. M. ONDERCI, N. SAHIN, G. CIKIM, A. AYDIN, I. OZERCAN, S. AYDIN, *Poultry Sci.*, 85 (3), 505-510 (2006)
13. M.J. SCHANG, J.O. AZCONA, *Harnessing nature, Proceedings of Alltech's 17th European, Middle East and African Lecture Tour*, 34-43 (2004)
14. O.A. OLUKOSI, A.J. COWIESON, O. ADEOLA, *Poultry Sci*., 86 (1), 77-82 (2007)
15. L. FERREIRA, M. A. RAMOS, J. D. DORDICK, M. H. GIL, *J. Molec. Catal. B: Enzymatic,* 21, 189-199 (2003)
16. T. CORADIN, J. LIVAGE, *C. R. CHIMIE*, 6, 147-152 (2003)
17. M.T. REETZ, A. ZONTA, J. SIMPELKAMP, *Biotechnol. Bioeng*., 49 (5), 527-534 (1996)
18. D. AVNIR, S. BRAUN, O. LEV, M. OTTOLENGHI, *Chem. Mater.,* 6, 1605-1614 (1994)
19. W. JIN, J. D. BREMAN, *Anal. Chim. Acta*, 461, 1-36, (2002)
20. I. GILL, A. BALLESTEROS, *Trends Biotechnol*., 18, 282-296 (2000)
21. C. J. BRINKER, G. W. SCHERER, *Sol Gel Science. The Physics and Chemistry of Sol – Gel Processing*, Academic Press, Boston (1990)
22. J. LIVAGE, T. CORADIN, C. ROUX, *J. Phys.: Condens. Matter*., 13, 673-691 (2001)
23. C.B. PARK, D.S. CLARK, *Biotechnol. Bioeng.,* 78 (2), 229-235 (2002)
24. K. SANGEETHA, V.B. MORRIS, T.E. ABRAHAM, *Appl. Catal. A: General*, 341, 168-173 (2008)
25. B. SAMUNEVA, P. DJAMBASKI, E. KASHCHIEVA, G. CHERNEV, L. KABAIVANOVA, E. EMANUILOVA, I.M.M. SALVADO, M.H.V. FERNANDES, A. WU, *J. Non-Crystall. Solids*, 354, 733-740 (2008)
26. M. PALJEVAC, M. PRIMOŽIČ, M. HABULIN, Z. NOVAK, Z. KNEZ, *J. Supercritic. Fluids*, 43, 74-80 (2007)
27. J. KIM, J.W. GRATE, P. WANG, *Chem. Eng. Sci*., 61, 1017-1026 (2006)
28. P. VIDINHA, V. AUGUSTO, J. NUNES, J.C. LIMA, J.M.S. CABRAL, S. BARREIROS, *J. Biotechnol.,* 135, 181-189 (2008)
29. I. GUTIERREZ, A. ESPINOSA, J. GARCIA, R. CARABANO, C. DE BLAS, *Anim. Research,* 52 (5), 461-471 (2003)
30. S. GHAZI, J.A. ROOKE, H. GALBRAITH, *British Poultry. Sci.,* 44 (3), 410-418 (2003)
31. B. VLAD-OROS, M. DRAGOMIRESCU, G. PREDA, F. PETER, A. CHIRIAC, *Rev. Roum. Chim*., 52 (11), 1083-1087 (2007)
32. M. DRAGOMIRESCU, G. PREDA, B.OROS, F. PETER, *Rev. Chim., Bucureşti*, 57(6), 610-615 (2006)
33. J. F. ANSON, *Gen. Phys. Chem*., *22*, 79 (1939)
34. *Novo Enzymes Catalog*, Novo Industrii, AIS, Bagsvaerd
35. O. H. LOWRY, N. J. ROZBROUGH, L. A. PAN, R. J. RANDALL, *J. Biol. Chem*., 193, 165-151 (1951)
36. ST. JURCOANE, E. SASARMAN, A. ROSU, A. BANU, I. LUPESCU, R.B. TAMBA, F. RADOI, *Tratat de Biotehnologie*, vol. I, Editura Tehnica, Bucuresti (2004)
37. Y. WEI, J. XU, H. DONG, M. LIN, *Mater. Letters*, 44, 6-11 (2000)
38. B. TAN, S.E. RANKIN, *J. Non-Cryst. Solids*, 352, 5453-5462 (2006)