STABILIZATION OF MICROBIAL ENZYMATIC PREPARATIONS USED IN FEED INDUSTRY

STABILIZAREA PREPARATELOR ENZIMATICE MICROBIENE UTILIZATE ÎN INDUSTRIA FURAJERĂ

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The enzymatic compound with protease activity obtained by Bacillus licheniformis CMIT 1.33 cells fermentation was entrapped in sol-gel matrixes, using TEOS as precursor. The sol-gel technique is a soft method for silica glass synthesis at room temperature. In silica gels, the enzymes maintain their catalytic activity for a longer period of time and present high stability at environmental factors. Bacillus licheniformis CMIT 1.33 enzyme was also immobilized by entrapment in silica gels deposited on a ceramic support. The optimal temperature and pH of the native and immobilized enzyme did not vary significantly. At temperature and pH values lower than the optimum, the relative activities have been higher for the immobilized compound. The immobilization of the enzymatic compound increased the stability. The enzymatic compounds with protease activity obtained by silica gels entrapment and entrapment/deposition on ceramic support can be used as forage additive.

Key Words: feed enzymes, microbal proteases, *Bacillus licheniformis***,** immobilization, sol-gel, TEOS.

Introduction

Enzymes used in feed industry are isolated especially from microorganisms. Enzymes from microbial sources are most suitable for industrial purposes for the following reasons: the production cost is low, the conditions for production are not restricted by location reasons, the time required for production is short, mass production is possible, and microbes may be manipulated by genetic engineering and the strains developed in this manner will be able to produce abnormal amounts of enzymes inherent in this organisms, as well as to synthesize foreign proteins derived from animal cells. Until now efficient enzymatic compounds are available only in some specific activities. That's why it is very important for the future to obtain and use some enzymatic systems with specific characteristics, with competitive costs and a large applicability in animal feeding.

The forage enzymes frequently used today in animal feeding will be certainly replaced when new enzymes will be available, with improved characteristics, including a better stability. The characteristics demanded for the new enzymes are: higher specific activities, high resistance to inactivation by thermal treatment, at low pH or protease activity, low production costs and high stability in time [1, 2].

Materials and Methods

Casein Hammerstein and tetraethoxysilane (TEOS) were obtained from Fluka, Folin-Ciocalteus phenol reagent and L-tyrosine were purchased from Merck, ceramics and all the other chemicals were obtained from local suppliers or were commercially available reagent grade products. The *Bacillus licheniformis* CMIT 1.33 strain was obtained from the Industrial Microbiology Laboratory of USAMVB Timisoara. The immobilization methods [3] used in this study were:

1. Entrapment – to the sol (tetraethoxysilane, ethyl alcohol and water at pH 3.5– 4.0) (40 mL), ethanol (40 mL) and buffered enzymatic solution (40 mL) were added, after adjusting pH to 8.0 (25% NH_3). Gelation time was 1–2 min. The sol–gel was left for gelation and ageing, at room temperature, washed with water (80 mL) and acetone (40 mL), than crushed and dried in desicator under ambient conditions till constant weight.

2. Entrapment-deposition on ceramic - to the inorganic support (9 g), sol (18 mL), ethanol (18 mL), ammonia and enzymatic solution (18 mL) were added under magnetic stirring. The gelation occurred in several seconds up to minutes. The gel was aged for one day. Afterwards it was washed with distilled water (36 mL) under active stirring, washed with acetone (18 mL) and dried at room temperature.

Stability test – the immobilized enzyme (500 mg enzyme immobilized by entrapment or entrapment/deposition on ceramic) in 0.05 M phosphate buffer, pH 3.0 (5 mL) was incubated at 37ºC for one hour. Samples were withdrawn at every 10 minutes and protease activity was assayed.

The protease activity was measured by UV-VIS spectrometry, according to the Anson method, using casein as substrate [3]. 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 was assayed according to the Lowry method [6].

Results and Discussions

To produce an immobilized enzymatic preparation as stable, cheap and easily obtainable as possible, a series of microbial *Bacillus* strains were tested. The strains were isolated in the Laboratory of Industrial Microbiology from BUASVM Timişoara and in the Laboratory of Microorganism Genetics from UASVM Bucharest. The enzymatic preparations with protease activity were produced by discontinuous fermentation in submerse medium, in stirred flasks. The best protease activity was obtained for the *Bacillus licheniformis* CMIT 1.33 strain (Table 1). This protease was used in the subsequent immobilization studies.

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

Strain	pH	Fermentation time , (h)	Protease activity, (U/100 mL)	Protein content (mg/100 mL)
Bacillus licheniformis CMIT 1.33	7	72	61.78	387.64
Bacillus licheniformis CMIT 1.35	6.5	72	44.23	198.32
Bacillus licheniformis CMIT 1.34	5.5	72	39.67	172.74
Bacillus licheniformis CMIT 1.32	5.5	72	9.53	182.39

The enzymatic preparation (activity 0.618U/mL, protein content 3.88mg/mL) obtained by fermentation of a *Bacillus licheniformis* CMIT1.33 strain was immobilized by entrapment in silica gel obtained from TEOS by sol-gel method and entrapment–deposition on a ceramic support, directly from the culture medium.

The highest protease activity was found for the immobilized preparation obtained by entrapment – deposition on ceramic support (0.45 $\mu mol \cdot min^{-1} \cdot g^{-1}$, 45.27% immobilization yield). Comparing the protease activities of the immobilized products, it was noticed that the protease activity obtained by entrapment was 1.5 times lower than that obtained by entrapment - deposition (0.31 $\mu mol \cdot min^{-1} \cdot g^{-1}$, 7.4% immobilization yield)(Table 2).

Table 2. Protein content and protease activity of the immobilized enzyme

Immobilization method	**Weight (g)**	**Protein content ($mg_{BSA} \cdot g^{-1}$)**	**Protease activity ($\mu mol \cdot min^{-1} \cdot g^{-1}$)**	**Immobilization yield, (%)**
Entrapment	5.90	8.31	0.31	7.40
Entrapment/Deposition	11.15	2.76	0.45	45.27

In the most cases, the enzymatic compounds stability increases after immobilization. A change in the pH and/or temperature profile of enzyme activity can be also observed. The influence of some environmental parameters (pH, temperature) on the free and immobilized enzymatic preparation and the stability of the immobilized products (at temperature and pH) were studied (Figure 1, Figure 2 and Table 3). The free enzymatic preparation had a low variation of the enzyme activity on a broad range of pH, from 7 to 12. At pH 3, the entrapped and entrapped/deposited enzymatic preparations had a better protease activity then the free enzyme, the relative activity being 47% and 39% from the maximum (at pH 8.0-7.0), respectively (Figure 1). The optimum temperature of the free enzymatic preparation was 65ºC (Figure 2). The preparations immobilized by entrapment and entrapment-deposition showed greater relative activities than the native enzyme, especially at temperatures less then the optimum. At 37ºC the activity of immobilized enzyme was 73% and 69% from the maximum (65ºC for entrapped and 45ºC for entrapped/deposited, respectively).

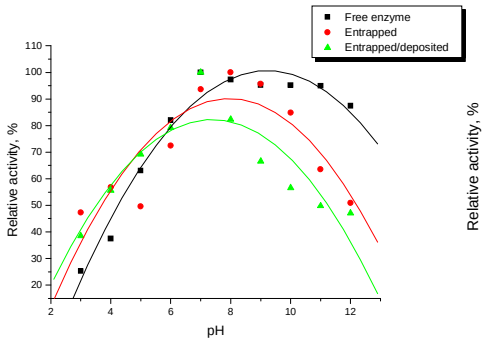

Figure 1. pH dependence of free and immobilized enzyme activity

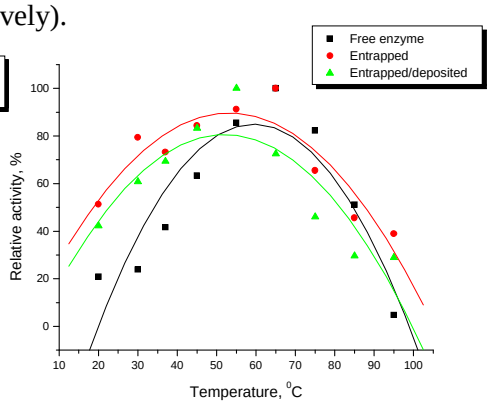

Figure 2. Temperature dependence of the protease activity in free and immobilized enzyme

For using as efficient forage additives, the enzymes have to be stable in the physiological conditions from the proximal segment of the intestine tract in the mammal's organism. The immobilized enzymatic preparations were tested "in vitro". The stability for one hour at 37ºC and pH 3 was studied (Table 3).

After one hour, the relative activity of the entrapped enzyme was 48%. The enzyme immobilized by entrapment-deposition after one hour of contact with the pH 3 medium exhibited 31% of the initial activity.

The stability of the immobilized enzymatic preparations was monitored in time. The relative protease activity was 67% for entrapped enzyme and 50% for entrapped/deposited enzyme after the first month of storage at 4ºC.

Table 3. Stability of the immobilized enzymatic preparation at pH 3.0 and 37ºC

Time minute	Entrapped enzyme		Entrapped/deposited enzyme	
	Protease activity ($\mu mol \cdot min^{-1} \cdot g^{-1}$)	Relative activity, (%)	Protease activity ($\mu mol \cdot min^{-1} \cdot g^{-1}$)	Relative activity, (%)
0	0.29	100.00	0.40	100.00
10	0.27	93.75	0.33	84.17
20	0.23	81.32	0.31	77.36
30	0.16	56.40	0.20	49.72
40	0.15	51.32	0.18	44.68
50	0.14	48.42	0.14	36.24
60	0.14	47.90	0.12	30.95

Conclusions

The culture broth with protease activity obtained by *Bacillus licheniformis* CMIT 1.33 cells fermentation was immobilized by entrapment in silica gel and entrapment /deposition on ceramic support. The immobilization has led to enzymatic preparations with improved stability.

At physiological temperature, 37˚C, the protease activity of the immobilized compound is 1.75 times higher than that of the free one. The immobilization reduces the negative effect of the acidic pH on protease activity. After keeping for one hour at 37˚C and pH 3, the relative activity of silica gel – enzyme preparation is close to 50%.

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