Journal of Polymer and Biopolymer Physics Chemistry, 2016, Vol. 4, No. 1, 28-39
Available online at http://pubs.sciepub.com/jpbpc/4/1/4

DOI:10.12691/jpbpc-4-1-4

A Peptidase Enzyme from *Bacillus cereus* with Antimicrobial Properties: Optimizing the Immobilization in Chitosan Beads Using Box-Behnken Design

Catalina Kotlar[1,2], Sara Inés Roura[1,2], Alejandra Graciela Ponce[1,2,*]

[1]Grupo de Investigación en Ingeniería en Alimentos, Departamento de Ingeniería Química y en Alimentos, Facultad de Ingeniería, Universidad Nacional de Mar del Plata, Juan B. Justo 4302, 7600 Mar del Plata, Argentina
[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
*Corresponding author: agponce@mdp.edu.ar

Abstract Enzymes are exploited as catalysts in many industrial, biomedical, and analytical processes. There has been considerable interest in the development of carrier systems for enzyme immobilization because immobilized enzymes have enhanced stability compared to soluble enzymes, and can easily be separated from the reaction. In the current study, microbial peptidases liberated by *B. cereus* were immobilized in cross-linked chitosan beads and characterized using azocasein as a substrate. The Box-Behnken design was applied to determine the optimal conditions to maximize proteolytic activity. An empirical second-order model was determined by multiple regression analysis of the experimental data to describe the relationship between tested variables and the response. The determination coefficients (R^2) were above 90%. Under optimal conditions (2.2 mm bead diameter, 1.06 enzyme/ bead ratio, 5.82% v/v glutaraldehyde and 18°C) the proteolytic activity was 0.938 U/ml. The retained immobilized enzyme can be reused up to five times. The storage stability of immobilized peptidases at 4°C was up to 10 days, while at 32°C the enzyme lost its activity within three days. Finally, novel antimicrobial properties of the immobilized peptidases were found. These results could have important benefit for the food industry.

Keywords: *Bacillus cereus, Box-Behnken experimental design, immobilization enzymes, proteolytic enzymes, immobilization process optimization, Response surface methodology*

Cite This Article: Catalina Kotlar, Sara Inés Roura, and Alejandra Graciela Ponce, "A Peptidase Enzyme from *Bacillus cereus* with Antimicrobial Properties: Optimizing the Immobilization in Chitosan Beads Using Box-Behnken Design." *Journal of Polymer and Biopolymer Physics Chemistry*, vol. 4, no. 1 (2016): 28-39. doi: 10.12691/jpbpc-4-1-4.

1. Introduction

Peptidases have enormous field of research and usage in food, pharmaceutical and detergent industries due to their pivot proteolytic activity [1,2]. The continuous peptidase requirement constitutes about the 60% of the total commercial enzymes involve in the industries [3].

In order to improve the economy and exploitation of enzymatic, continuous operations could be used for a long period of time. Another possibility is to recover the enzyme and to stop the reaction without drastic treatments which could alter its activity allowing a possible reuse. Immobilized enzymes offer the following benefits: stability, repetitive uses, possibility to stop the reaction easily and obtain product and get a free enzyme product. Enzyme immobilization by means of covalent coupling to a reactive insoluble support is the choice method when a long operational performance is required.

Chitosan is obtained by thermo-chemical deacetylation of crustacean chitin [4]. Chitosan is an abundant and cheap raw material due to the long extent of sea coasts and the high activity of the seafood industries [5]. Chitosan beads draw much attention for the enzyme immobilization because the immobilized enzyme in this support can improve mechanical properties, thermal stability and bactericidal activity [6]. The poor acid stability and mechanical strength are the main disadvantages of using chitosan beads [7]. Several modification methods, such as chemical cross-linking of the chitosan beads surface [6] using agents as glutaraldehyde, have been performed to improve acid stability, mechanical strength, pore size, hydrophilicity and biocompatibility [8].

There are many parameters involved in the immobilization process that can affect the matrix and the native activity of biomolecules [9]. Moreover the efficiency of this process must be improved mainly due to its economic disadvantage. Therefore, immobilization parameters should be studied and optimized to preserve the native activity of biomolecules and to achieve high immobilization efficiency.

Different strategies can be used to optimize the enzyme immobilization. The classical method involves changing one variable at a time keeping the others constant. This

method, however, requires a large experiment number to illustrate individual factor effects and does not consider the effect of various parameters interactions [10]. Use of statistical methods helps to select significant parameters from a large number of factors and the interactions between variables can be understood easily. Response Surface Methodology (RSM) is a statistical and mathematical method that involves both main interactions and interaction effects to improve optimal process settings. RSM has been widely used to evaluate the interactions among various process parameters and their optimization to apply in various biotechnology processes [11,12].

In the present research an evaluation of the variables that affect the activity and stability of the immobilized peptidase from a newly isolated strain of *B. cereus* [13] was done. The enzyme was immobilized in cross-linked chitosan beads, and was optimized by using RSM. Different factorial designs are available in RSM techniques [14,15]. Here three level-four-factors Box-Behnken designs were used. The predicted results were then validated with the experimental data. Also the antimicrobial capacity of the immobilized enzyme was determined loading for potential effects peptidases against human pathogens [16]. Finally the stability of the newly peptidase was determined through the repeated use of the immobilized enzyme and its storability at two temperatures.

2. Materials and Methods

2.1. Bacterial Strain

A previous isolated strain *Bacillus cereus* from fermented cabbage with demonstrated high proteolytic activity [13] was used in this study.

The strain maintained on soft Brain and Heart agar (3.5% w/v of agar- agar) at -18°C was activated in two steps: First a *B. cereus* loop was subcultured in 8 mL Brain and Heart Infusion (BHI) and incubated at 32°C for 24h. Subsequent to which 2 mL of culture was centrifuged at 1,000 rpm for 3 min at 4°C. Precipitate was then added to 25 mL fresh BHI and statically incubated at the above described conditions.

2.2. Peptidase Production

Five mL of an overnight culture of *B. cereus* strain was used to inoculate 100 mL aliquots of Minimal Broth [consisted of (% w/v): 0.1 bacteriological glucose (Britania, Lot.095, Buenos Aires, Argentina) and 0.25 yeast extract (Acumedia, Lot. 66-22, Maryland, United States)] [13] buffered at pH 8 with Tris-HCl 0.2 M. The inoculated flask was incubated at 37°C for 24 h [17]. The bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The obtained supernatant, named as crude extract (CE), was filtrated using a 0.22 µm filter (Merk Millipore, Millipak 40) and the supernatant was used as the starting point for immobilization procedure, designed as enzyme solution (E).

2.3. Preparation of Chitosan Beads

Three batches with different bead diameter (2, 5 and 8 mm) were performed. Chitosan solution was prepared by dissolving 7g of chitosan into 100 mL of 5% (v/v) acetic acid solution with stirring for 1h. The mixture was extruded at room temperature drop by drop with a syringe needle into 200 mL of alkali coagulating solution (H_2O:MeOH: NaOH 4:5:1 w/w/w) and maintained with periodic stirring for 2 h in order to allow formed chitosan beads to harden. The wet chitosan gel beads were extensively rinsed with destilled water to remove any NaOH, filtered and stored in distilled water at 4°C.

2.4. Chitosan Beads Cross-linking

For immobilization coupling with glutaraldehyde, the chitosan beads were immersed in a 5, 7.5 or 10 % (w/v) glutaraldehyde solution, gently shaken at room temperature for 1 h and then the beads were washed with distilled water to remove any glutaraldehyde excess. The mixture was subsequently agitated at room temperature for 4 h and then stayed at 4°C overnight. Once more the cross-linked beads were washed several times with distilled water to remove the glutaraldehyde excess. The absorbance of the solution was continuously monitored with UV at 280 nm till the absorbance was less than 0.01 indicating unbounded glutaraldehyde was washed away.

2.5. Peptidase Immobilization

The glutaraldehyde cross-linked chitosan beads (B) were immersed in the enzyme solution (E) with a given E/B ratio. The mixture was gently stirred for 10 min and then placed in refrigerator at 4°C for 24 h. The supernatant was removed, and the beads were washed three times with deionized water. The immobilized peptidase was recovered from the solution and stored at 4°C for next use. The beads were washed again three times with phosphate buffer. The resulting immobilized enzyme (IE) was stored in buffer pH 7.04 at 4°C. During the immobilization course, the leftover glutaraldehyde solution was analyzed for protein and enzyme activity to ascertain leaching, if any.

2.6. Characterization of Chitosan Beads

2.6.1. Solubility

Chitosan and cross-linked chitosan beads were tested with regard to their solubility in each of acetic acid 5% (v/v), distilled water and sodium hydroxide solution 0.10 M by adding 0.10 g of chitosan and cross-linked chitosan beads in each of the above solutions for a period of 24 h with stirring.

2.6.2. Diameter and Porosity

The diameter (D) and porosity (ε) of chitosan beads were determined by the amount of water within the pores of the chitosan beads [8] using the following equations:

$$D = \left[6 * \frac{W_D / \rho_{cs} + \left(W_W - W_D\right) / \rho_W}{\pi} \right]^{\frac{1}{3}} \quad (1)$$

$$\varepsilon = \frac{\left(W_W - W_D\right) / \rho_W}{W_W / \rho_{CS} + \left(W_W - W_D\right) / \rho_W} * 100\% \quad (2)$$

Where *Ww* (g) is the weight of the wet chitosan beads before drying; W_D (g) is the weight of the chitosan beads after drying; ρ_w is the water density, 1.0 g cm^{-3}; and ρ_{cs} is the chitosan density, 0.47 g cm^{-3}. Chitosan density represents the dry weight of chitosan materials in the volume of wet chitosan beads containing water.

2.6.3. Proteolytic Activity

Proteolytic activity of the immobilized enzyme (IE) was assessed by using azocasein as substrate. Briefly, 6 beads representing an equivalent volume to 120 µl aliquot of crude enzyme with a bead diameter of 2.333 ±0.006 mm were incubated with 480 µl of 10 g/L azocasein in buffer 0.2 M Tris- HCl pH 7.04 for 30 min at 32 °C. The reaction was stopped by the addition of 480 µL of trichloroacetic acid (TCA) to a final concentration of 100g/L and incubated for 30 min at 4°C before being centrifuged at 10,000 rpm for 10 min. 800 µl of the supernatants from the centrifuged reactions were added to 200 µL of 1.8 N sodium hydroxide and the absorbances at 420 nm were measured in Spectrum SP-2000 UV (Zhejiang, China) spectrophotometer.

For the control, the reaction was stopped with TCA immediately after the supernatant addition. One enzyme activity unit (U) was expressed as the amount of enzyme that cause an absorbance change of 0.01 at 420 nm under the assayed conditions (30 min at 32°C). Proteolytic activity values were expressed as U/mL. The specific activity was expressed in unit of enzyme activity/g of protein (U/g).

The immobilization percentage was defined as the total activity in immobilized beads/total activity of the soluble enzyme loaded x 100.

2.8. Soluble Protein Content

Total soluble protein content was determined according to the method of Lowry *et al*. [15], using the Folin Ciocalteu's phenol reagent (Sigma-Aldrich, St. Louis, Missouri, United States) and bovine serum albumin as standard [18].

2.9. Antimicrobial Assay

The antimicrobial activities of crude enzyme, beads, cross-linked beads and IE were evaluated using a shake flask method. The indicators used were: *Listeria monocytogenes* (Gram-positive indicator) provided from CERELA (Tucumán, Argentina) and *Escherichia coli* O157:H7, ATCC 43895 (American, Type Culture Collection), provided by CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, La Plata, Argentina) (Gram-negative indicator). The microorganisms were individually first overnight cultured in Brain and Heart Infusion (BHI) at 37°C before use. The bacterial cultures were subsequently diluted with BHI to a concentration of approximately 10^6 CFU/mL. Meanwhile, crude enzyme, beads, cross-linked beads and IE were dispersed separately in BHI at the same concentrations in flasks (120 µL or the equivalent volume of beads). BHI without a tested sample was used as a blank. After 24 h of incubation, the whole dispersions of each tested sample in the bacteria cultures were analyzed by a plate count technique in Plant Count Agar to antimicrobial activity of each tested material.

2.10. Experimental design and optimization by RSM

To find out the optimum level of the most effective variables and to study their relationships, RSM using Box-Behnken [18] design was applied. Four critical selected variables were: bead diameter (mm), beads/enzyme ratio (v/v); glutaraldehyde concentration (% v/v) and cross-linking reaction temperature (°C). Every variable at three coded levels: low (−1), middle (0) and high (+1); and non-coded valued was shown in Table 1.

In developing the regression equation the factors were coded according to the equation:

$$X_i = \frac{(x_i - x_o)}{\Delta x_i} \quad (3)$$

Where X_i is the coded value of the i^{th} independent variable, x_i is the natural value of the i^{th} independent variable, x_{0i} is the natural value of the i^{th} independent variable at the center point and Δx_i is the steep change value.

According to this design, the total number of experimental runs was $2^k + 2k + x_0$, where k is the number of variables and x_o is the number of the experiment repetitions at the center points. Thus for this design, a total of 29 experiments (k= 4; x_0= 5) were performed according to the Box-Behnken design given in Table 2.

Once the proteolytic activity was measured for each trial, data was subjected to a second order multiple regression analysis to explain the behavior of the system using the least square regression methodology. The general polynomial equation is in the following form:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2 \quad (4)$$

Where *Y* is the predicted response (proteolytic activity), β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient for the interaction effect, and X_i is a dimensionless coded value of x_i (independent variable).

Table 1. Coded and non-coded values of the experimental variables

Variables	Symbols	-1	0	1
Bead diameter (mm)	BD	2	5	8
Beads/ enzyme ratio (v/v)	BE	0.5	1	1.5
Glutaraldehyde concentration (% v/v)	GC	5	7.5	10
Temperature (ºC)	T	0	10	20

Data were analyzed using the software package SAS (version 8.0, Inst. Inc., Cary, N.C., U.S.A. 1999). This software was used for regression analysis of the data obtained and to estimate the coefficient of regression equation. ANOVA (analysis of variance) which is statistical testing of the model in the form of linear term, squared term and interaction term was also utilized to test the significance of each term in the equation and goodness of fit of the regression model obtained [19]. This response surface model was also used to predict the result by isoresponse contour plots and three dimensional surface plots.

Statistical testing of the model was done by the Fisher's statistical test. The goodness of model could be checked by the determination coefficient (R^2), correlation coefficient (R) or *F*-test. All data are the mean of triplicates obtained from three independent runs.

3. Results and Discussion

3.1. Characterization of Chitosan Beads

Table 3 shows the diameter (D) and the porosity (ε) of the non-cross-linked and cross-linked chitosan beads. A slight reduction in the diameter of chitosan beads was found after cross-linking with glutaraldehyde. This is related to an increase in the hydrophobicity of the chitosan beads by the addition of an alkali group in the cross-linking reaction [16]. An increase the surface area due to diameter of chitosan beads reduction, increases the accessibility to enzymes, facilitating the hydrolysis, in this sense the cross-linking with glutaraldehyde, did the hydrolysis more efficiently.

3.2. Interpretation of Regression Analysis

Table 2 presents the Box-Behnken (BB) experimental design matrix together with the results of the observed and predicted results on the bead diameter (BD), bead/ enzyme ratio (BE), glutaraldehyde concentration (GC) and temperature (T) effects on proteolytic activity for the immobilized peptidase.

A second-order full polynomial equation was fitted by applying multiple regression analysis to the experimental data. The empirical relationship between proteolytic activity (Y) and the four variables in coded units is given below (Equation 5):

$$\begin{aligned} Y = {} & 0.609 - 0.252 * BD + 0.031 * BE + 0.036 * GC \\ & + 0.104 * T - 0.051 * BD^2 - 0,002 * BE^2 \\ & + 0.016 * GC^2 - 0.116 * T^2 + 0.015 * BD * BE \\ & - 0.020 * BD * GC - 0.035 * BD * T \\ & - 0.046 * BE * GC - 0.035 * BD * T \\ & - 0.046 * BE * GC + 0.095 * BE * T - 0.133 * GC * T \end{aligned} \quad (5)$$

Where Y is the immobilized enzyme relative activity; BD is the beads diameter (mm), BE is the chitosan beads/ enzyme ratio; GC is the glutaraldehyde concentration; and T is the temperature.

Table 2. Response surface Box- Behnken design and experiments

Run	BD	BE	GC	T	Measured proteolytic activity (U/mL)	Predicted proteolytic activity (U/mL)
1	2	0,5	7,5	10	0,820	0,791
2	8	0,5	7,5	10	0,285	0,257
3	2	1,5	7,5	10	0,864	0,822
4	8	1,5	7,5	10	0,390	0,349
5	2	1	5	10	0,658	0,769
6	8	1	5	10	0,249	0,306
7	2	1	10	10	0,917	0,881
8	8	1	10	10	0,427	0,337
9	2	1	7,5	5	0,576	0,555
10	8	1	7,5	5	0,090	0,122
11	2	1	7,5	15	0,816	0,832
12	8	1	7,5	15	0,189	0,258
13	5	0,5	5	10	0,608	0,510
14	5	1,5	5	10	0,761	0,664
15	5	0,5	10	10	0,528	0,673
16	5	1,5	10	10	0,497	0,643
17	5	0,5	7,5	5	0,456	0,451
18	5	1,5	7,5	5	0,316	0,323
19	5	0,5	7,5	15	0,455	0,469
20	5	1,5	7,5	15	0,693	0,720
21	5	1	5	5	0,195	0,237
22	5	1	10	5	0,628	0,574
23	5	1	5	15	0,725	0,710
24	5	1	10	15	0,627	0,516
25	5	1	7,5	10	0,609	0,609
26	5	1	7,5	10	0,639	0,624
27	5	1	7,5	10	0,820	0,791
28	5	1	7,5	10	0,285	0,257
29	5	1	7,5	10	0,864	0,822

Table 3. Porosity and diameter of cross-kinked and non cross-linked chitosan beads

Chitosan beads	Non-cross-linked	Cross-linked
Wet weight (W_W, mg)	6.590 ±0.020	6.395 ±0.021
Dry weight (W_D, mg)	0.237±0.006	0.225 ±0.007
Porosity (ε, %)	92.656 ±0.191	92.542 ±0.475
Diameter (D, mm)	2.357 ±0.002	2.333 ±0.006
Density (g/ml)	1.055 ±0.031	1.036 ± 0.005

Results are average of triplicate experiments ± standard deviation.

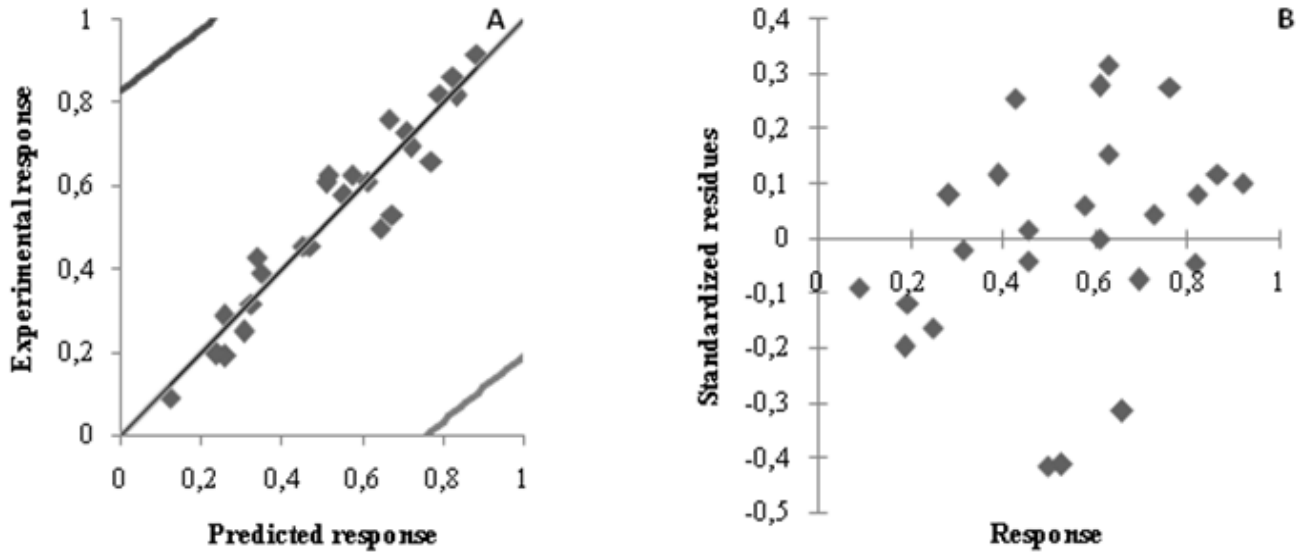

Figure 1. (A) Observed proteolytic activity versus predicted proteolytic activity from the empirical model (Y= x + 2E-15; R^2 = 0.9935); (B) Plot of internally studentized residual versus predicted response

The model adequacy (Figure 1) was assessed as the correlation between predicted and observed data (Figure 1 A) and residual analysis (Figure 1 B). Results corroborated the model capacity to describe the proteolytic activity by response surface. From correlation between predicted response and experimental values (Figure 1 A) is evident that the regression model can well represent the experimental data. The normality of the data can be checked by plotting the normal probability plot of the residuals. Figure 1B plots the residuals versus the fitted values (predicted response). The residuals are scattered randomly about zero i.e. the errors have a constant variance. No evidence exists that the regression terms are correlated with one another.

On the basis of the ANOVA outputs evaluation (Table 4), the statistical significance of a quadratic model for the immobilized peptidase proteolytic activity response was confirmed and it can be concluded that the model can be used for further analysis of process variables.

The model F-value (6.920) indicated that the model was significant. As values of "Prob>F" were less than 0.05, the model terms were significant, also a "Lack of Fit F-value" of 76.160 implied that Lack of Fit was not significant relative to the pure error. Non-significant lack of fit is good. The coefficient of determination (R^2) calculated was 0.906, indicating that the model explain 90.60% of the variability. A relatively lower value of the coefficient of variation (CV= 3.26%) indicated a better precision and reliability of the experiments [14].

Residual were plotted against Y (response) as horizontal band (Figure 1.B) indicated no abnormality (no unusual behavior), confirming adequacy of regression model. Thus this model could be used to navigate the design space [20].

Obtained results (Figure 2 and Table 5) demonstrate the statistical significance of linear quadratic coefficients BD (p <0.0001) and T (p= 0.008), and interaction coefficient GC*T (p = 0.034). Other quadratic coefficients not confirmed the statistical significance.

The sign of the coefficients confirmed that the proteolytic activity increase with a decrease of the bead diameter.

Table 4. Analysis of variance (ANOVA) for the model of immobilized peptidase relative activity

Sources	DF	Sum of squares	F value	P value Prob> F
Model	14	1.127	6.920	0,002
Residual	14	0.116	54.400	
Lack of fit	10	0.116	76.160	
Pure error	2	0.000	0.000	
Total	28	1.244		

R2= 0.906, adjusted R2= 0.775, CV= 3.26; Adeq Precision= 52.915.

Table 5. Analysis of variance (ANOVA) for all the terms of the model of immobilized peptidase relative activity

Source	Coefficient estimate	Standard error	t- value	P value Prob>F
Intersect	0,609	0,108	5,646	0,000
BD	-0,252	0,031	-8,084	< 0,0001
BE	0,031	0,031	0,987	0,347
GC	0,036	0,031	1,145	0,279
T	0,104	0,031	3,329	0,008
BD^2	-0,051	0,064	-0,802	0,441
BE^2	-0,002	0,064	-0,038	0,970
GC^2	0,016	0,064	0,248	0,809
T^2	-0,116	0,064	-1,804	0,101
BD*BE	0,015	0,054	0,283	0,783
BD*GC	-0,020	0,054	-0,375	0,715
BD*T	-0,035	0,054	-0,654	0,528
BE*GC	-0,046	0,054	-0,853	0,414
BE*T	0,095	0,054	1,752	0,110
GC*T	-0,133	0,054	-2,461	0,034

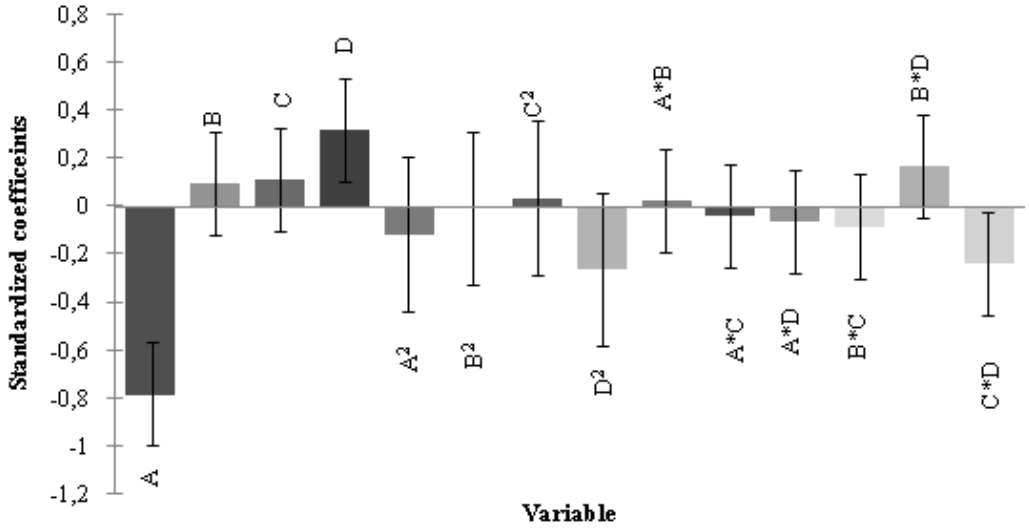

Figure 2. Pareto chart showing the effects of observed factors and their combined impact on the response (n=3)

An increase in the cross-linking ratio did not reduce the available sites of the immobilized enzyme but increased the steric hindrance for diffusion through the chitosan beads [21]. Over the chosen concentration range a positive effect on the response was observed (Figure 2). This could indicate that glutaraldehyde binding causes a slight conformational change in the enzyme, which results in increased catalytic sites exposure, which was occluded in the enzyme native state. Several authors [22,23,24] have described some effects on enzyme stabilization when immobilization of each enzyme molecule occurs through several residues, by multipoint attachment. The higher the concentration of anchor groups, the greater is the amount of bound protein and/ or the higher is the probability of multipoint attachment of protein to a carrier. Owing to such a relationship, three kinds of responses in the immobilized enzymatic activity could be seen. First, carrier's surface overloading may cause steric hindrance of substrate and enzyme active sites, thus decreasing the enzymatic activity. Secondly, a decrease in activity as a result of charges in the enzyme structure by multipoint covalent modification could be seen. Finally, the greater the number of linkages between protein and a carrier's surface, the more stable is the preparation obtained [24]. On the other hand, a high concentration of aldehyde groups on the support surface may lead to several multipoint connections between enzyme and the matrix, promoting distortions of its three-dimensional structure and of its active site. Therefore a control of the experimental conditions is critical to achieve a significant multipoint covalent attachment.

The enzyme activity responds to its microenvironment and the interactions to the solid matrix [25]. The protein matrix interaction is of critical importance because its affect not only the protein conformation, but also the activity and stability [26].

Non significant coefficients were eliminated and the reduced model can be expressed as follow (Equation 6):

$$Y = 0.609 - 0.252 * BD + 0.104 * T - 0.133 * GC * T \quad (6)$$

Where Y is the immobilized enzyme relative activity; BD is the beads diameter (mm); GC is the glutaraldehyde concentration; and T is the temperature.

The predicted values for proteolytic activity were calculated by using the simplified mathematical model given by Equation 6. These values were tabulate in Table 2.

As calculated with Equation 6, the optimum point is localized at BD= -0.93; BE= 0.12; GC= 0.328 and T= 0.8, corresponding to the actual values of 2.2 mm; 1.06 enzyme volume: bead volume; 5.82% v/v glutaraldehyde; and 18°C. Predicted proteolytic activity in the optimum, obtained by applying the regression analysis on the model was 0.938 U/mL.

The Pareto chart (Figure 2) illustrates the standardized effects of the independent variables and their interactions on the dependent variable (immobilized peptidase activity). In the Pareto chart, the effect of each factor as well as the level of its effects on responses is expressed by the length of its bar in the graph. A clear significant negative effect of linear factor BD (bead diameter) was found, indicating the increase of immobilized peptidase proteolytic activity with the bead diameter reduction. Since adsorption is a prerequisite step in the hydrolytic process, it seems intuitive that specific surface area would have an effect on hydrolysis rates since a higher surface area-to-weight ratio should mean more available adsorption sites per mass of substrate [27]. On the other side, linear factor T (temperature) has a positive effect on the response.

As can be seen from Figure 2, it is evident that interaction effect GC*T have a significant negative quadratic effect on the response. However, the effect bead/ enzyme ratio and glutaraldehyde concentration have no significant effects on immobilization process. The interpretation of the interactions was simplified by using three-dimensional plots for the regression model.

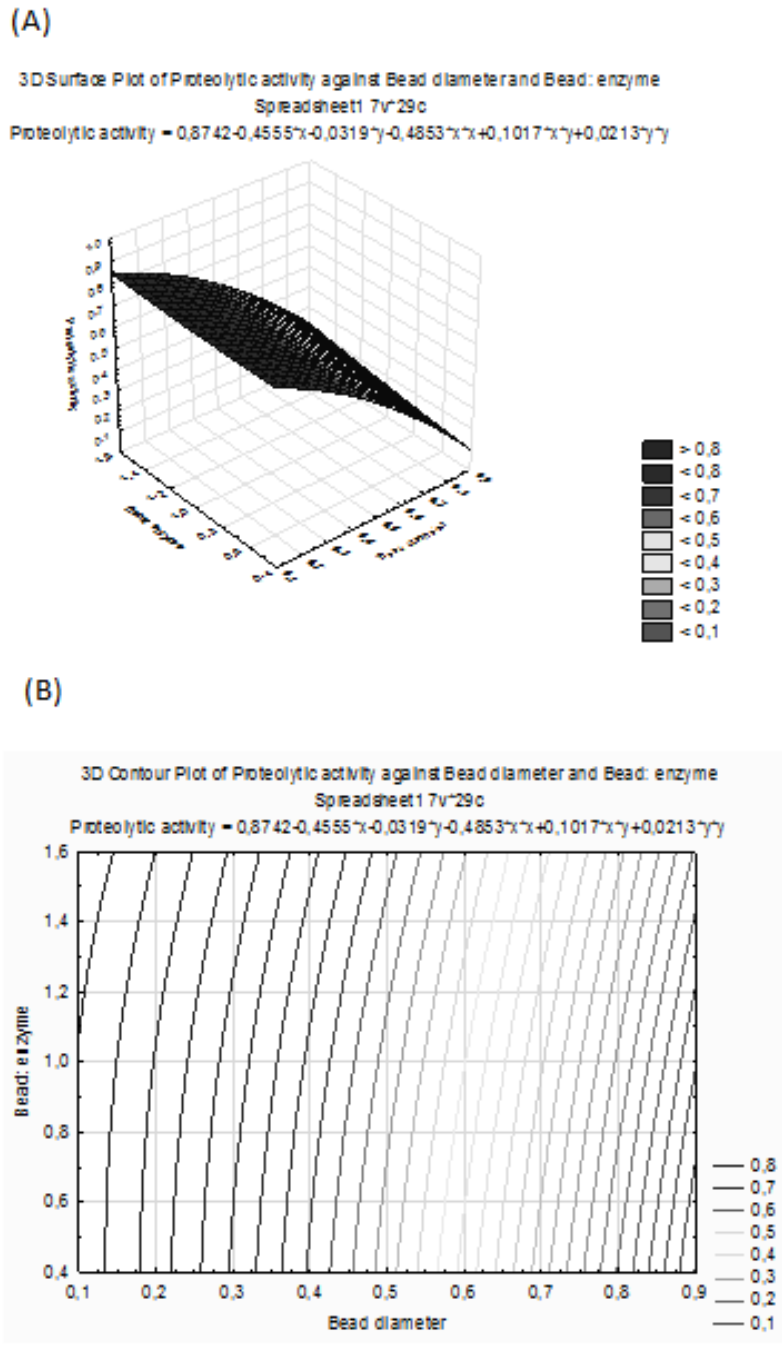

Figure 3. Response surface plot (A) and its contour plot (B) of proteolytic activity by peptidase immobilized in cross-linked chitosan beads showing interaction between bead diameter and beads/ enzyme ratio. Other variables were held at zero level

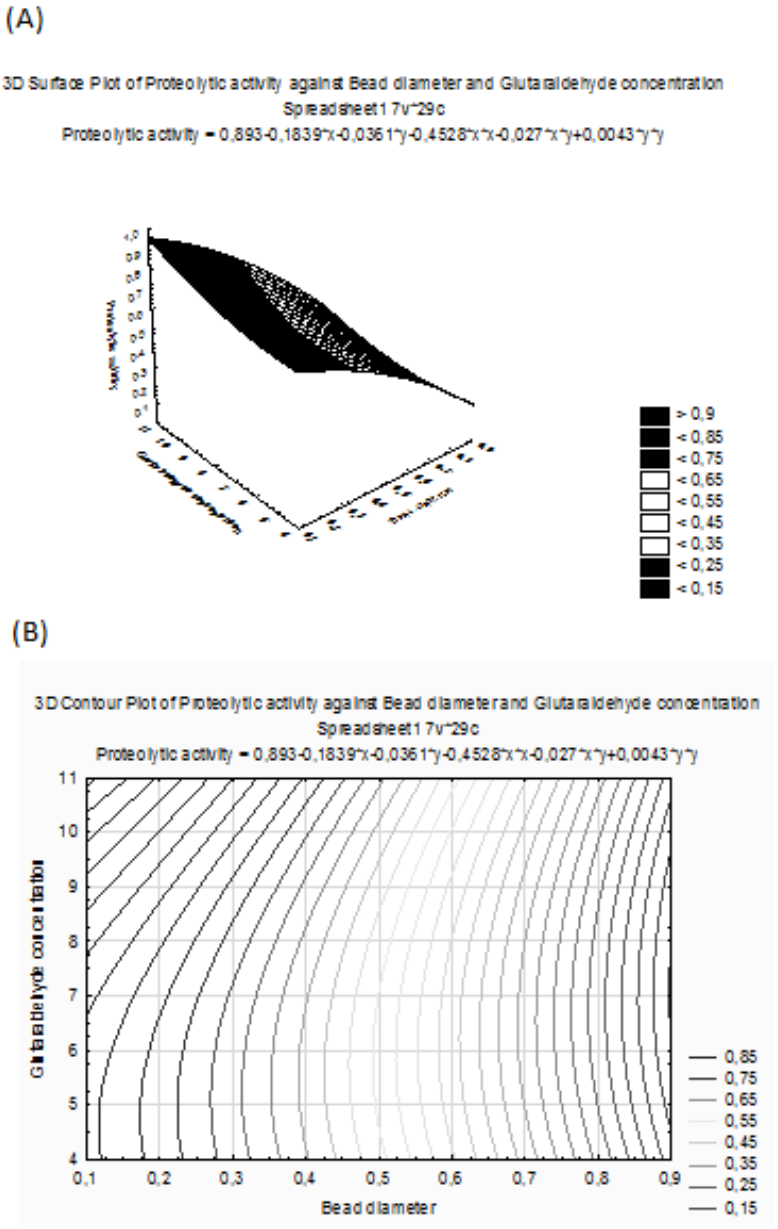

Figure 4. Response surface plot and its contour plot of proteolytic activity by peptidase immobilized in cross-linked chitosan beads showing interaction between bead diameter and glutaraldehyde concentration. Other variables were held at zero level

3.3. Interpretation of Response 3D Surface Plots and Contour Plots

There were 12 pairs of response surface and corresponding contour plots. Figure 3, Figure 4, Figure 5 and Figure 6 depict four typical pairs.

In surface plot of Figure 3 (A), the proteolytic activity (U/mL) was represented by varying simultaneously bead diameter (BD) and bed volume: enzyme volume (BE). From this plot, it is clear than a reduction in the bead diameter and bead/ enzyme ratio increased the response. The bead diameter drastically affected the response. The isoresponse contour plot between bead diameter and bead volume: enzyme volume is given in Figure 3 (B). The lines of contour plots predicting the value of proteolytic activity of the immobilized enzyme for different bead diameter at different bead volume: enzyme volume. These values are more or less similar to the experimental values.

The effect of different bead diameter (BD) at different glutaraldehyde concentration (GC) on the proteolytic activity of the immobilized enzyme is present in Figure 4 (A and B). It was observed that a reduction in the bead diameter drastically increased the proteolytic activity; however an increase in the glutaraldehyde concentration enhanced slightly the response.

Figure 5 (A and B) presented the interaction between the bead diameter (BD) and the temperature (T) on the proteolytic activity of immobilized peptidase. The contour plot is convex, containing the maximum response inside the design boundary. Moreover, these plots exhibit a general uphill-trend with the response level moving higher with the increase of the three variables. This means that higher temperature and lower bead diameter (within the experimental ranges) were favorable for proteolytic activity of immobilized peptidase.

The effect of the interaction amongst the glutaraldehyde concentration (GC) and the temperature (T) on the proteolytic activity on immobilized peptidase was presented in Figure 6 (A and B). The predicted response surface of the stationary point was shaped like a saddle

without a unique optimum. The saddle-shaped response surface indicates that there is significant interaction between some factors and implies that the behavior of the response with respect to temperature depends on the values of glutaraldehyde concentration. The saddle-shaped response surface plots depicting dual conditions for the enhanced production indicated the presence of proteolytic activity with optima at different conditions.

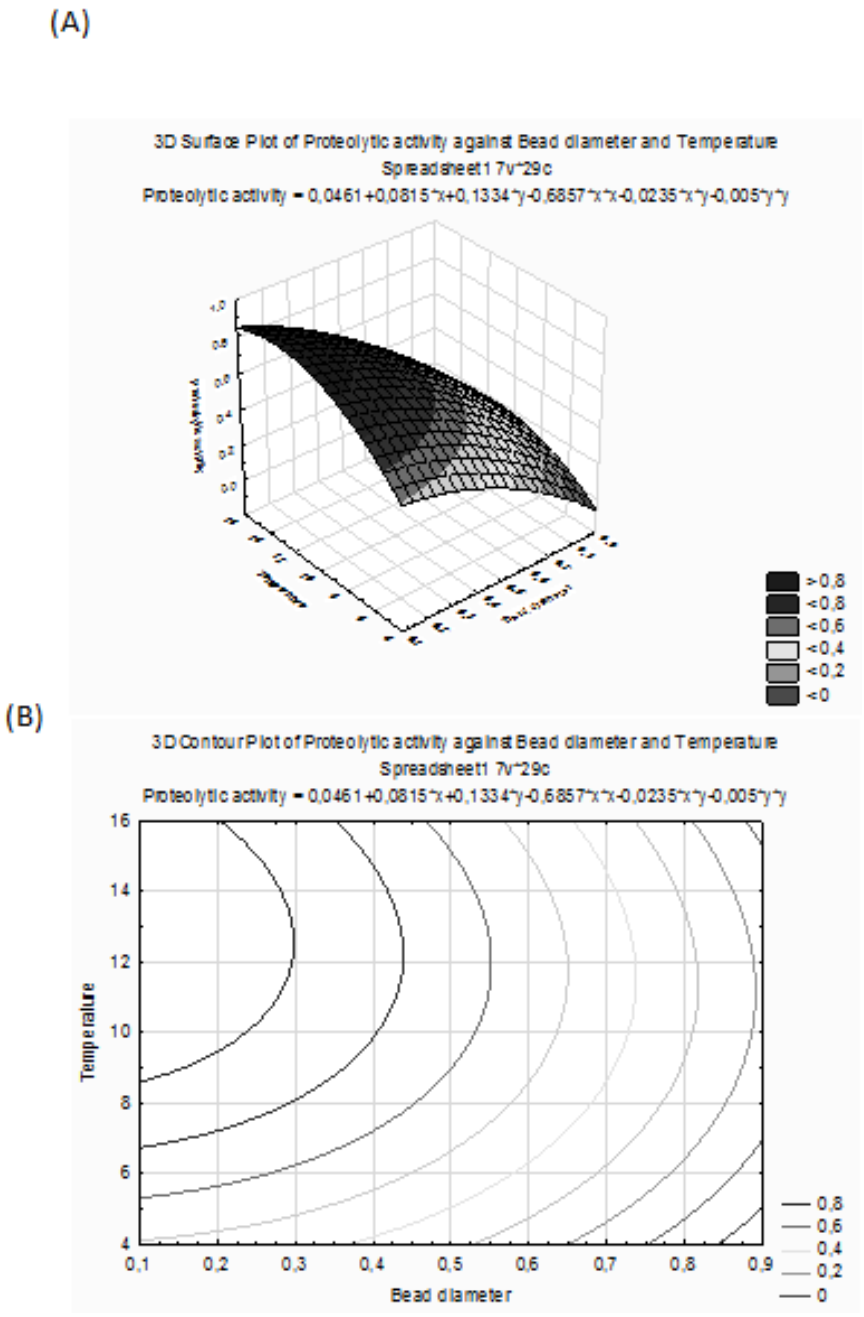

Figure 5. Response surface plot and its contour plot of proteolytic activity by peptidase immobilized in cross-linked chitosan beads showing interaction between bead diameter and temperature. Other variables were held at zero level

3.4. Validation

In order to test the reliability of the model in predicting maximum proteolytic activity, three additional experiments were performed at optimal conditions.

The three replicate experiments yielded an average maximum proteolytic activity of 0.928 U/mL ± 0.025 U/mL. The maximum proteolytic activity for the immobilized enzyme obtained from the experiments (0.928 U/mL) was very close to those estimated using RSM at the optimal conditions (0.938 U/mL). The good agreement between the experimental and estimated responses verified the existence of maximum points and the accuracy of the mathematical models.

3.5. Antimicrobial Activity

The antibacterial activity was evaluated against both Gram-positive, i.e., *Listeria monocytogenes*, and Gram-negative, i.e., *Escherichia coli*, bacteria, using a shake flask method on Brain and Heart Infusion (BHI). The microbial reduction of chitosan beads, glutaraldehyde-chitosan beads and peptidase immobilized on glutaraldehyde-chitosan beads prepared under the

optimal conditions respect to the control were present in Table 6. In the sequence presented above, these beads demonstrated decreased antagonist effect against the indicators microorganisms. Reductions around one and two order log were observed against the assayed pathogens. No significantly statistic differences were observed in chitosan and glutaraldehyde-chitosan beads antimicrobial activity against *Listeria monocytogenes*. However, chitosan beads were most effective against *E. coli* O157:H7 than glutaraldehyde-chitosan beads.

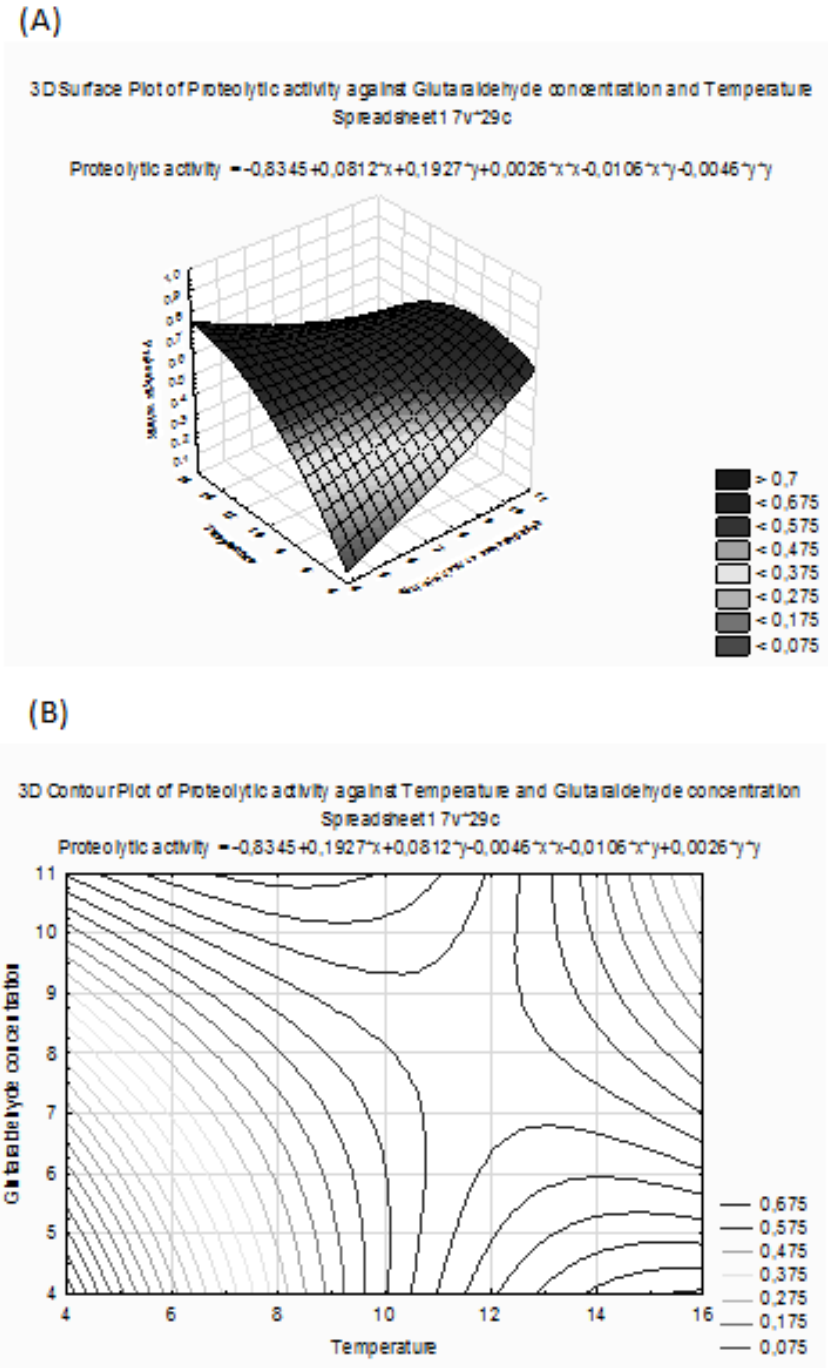

Figure 6. Response surface plot and its contour plot of proteolytic activity by peptidase immobilized in cross-linked chitosan beads showing interaction between glutaraldehyde concentration and temperature. Other variables were held at zero level

Table 6. Reduction in viable count from *Listeria monocytogenes* and *Escherichia coli* O157:H7 (n= 3) related to control

Microorganism	*Reduction in log UFC/ml*		
	Chitosan beads	*Glutaraldehyde-chitosan beads*	*Immobilized peptidase on cross-linked chitosan beads*
Escherichia coli	1.76[a]	1.20[b]	1.10[c]
Listeria monocytogenes	1.87[a]	1.75[a]	1.19[b]

(*) Values with different letters in each row are significantly different ($P < 0.05$).

Not only chitosan presents antimicrobial activity, but also a previous result indicated that the partially purified peptidase of *Bacillus cereus* inhibited the growth of *Escherichia coli O157:H7* [16], however no synergist effect was found after the covalent binding between chitosan and the enzyme. It was found that the enzyme did not present antimicrobial capacity against *Listeria monocytogenes* [16], but the interaction of this enzyme with the chitosan matrix produced an antimicrobial activity. From the industrial point view, the antimicrobial abilities of the immobilized enzyme could result in a greater stability from the microbiological point during its storage.

The antimicrobial activity of the beads against *L. monocytogenes* could be explained by the natural properties of the employed matrix. Chitosan antimicrobial activity against bacteria could be due to the polycationic nature of the molecule, which allows interaction and formation of polyelectrolyte complexes with polymers produced at the bacteria cell surface [28].

The novel antimicrobial property of the immobilized peptidase liberated by *B. cereus* could have important benefit for the food industry.

3.6. Reusability of Immobilized Peptidase

The activity of the immobilized enzyme was assayed for five cycles with azocasein as substrate, in order to find out the reuse of the immobilized enzyme. The enzyme showed 75% activity during the second recycle; and 58.5% and 31.6% activity in their third and four uses while complete loss in the immobilized enzyme activity was observed during the fifth cycle (Figure 7). The tendency line drawn for the proteolytic activity during the reusability cycles was represented by the following regression equation (Equation 7):

$$Y = -0.2435x + 1.2607. \quad (7)$$

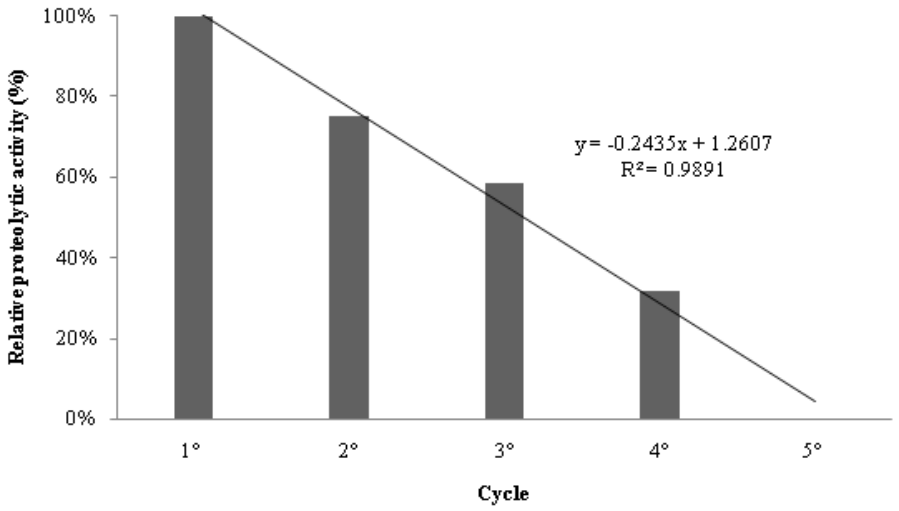

Figure 7. Repeated use of immobilized peptidase showing the number of times the immobilized enzyme can be used (Y= -0.2435x + 1.2607; R^2= 0.9891)

This decrease in activity was due to the leakage of peptidase from the beads, occurred during the beads washing at the end of each cycle. In another study was reported that peptidase entrapped in Ca-alginate beads was reused for 3 cycles with ≈35% loss in activity [29].

3.7. Storability of Immobilized Enzyme

The immobilized enzyme was stored at two different temperatures (4°C and 32°C) and activity was measured for 14 days to determine the storage stability of immobilized enzymes. Beads stored at 4°C showed 2.7% activity loss after 2nd days (48 hours) and 39% activity loss after 10th days (240 hours). Major loss in enzyme activity of immobilized enzyme was observed at 32ºC, showing 68% loss in activity on the 2nd day (24 hours) and no activity was found on the 3rd day. These results suggested that the enzyme is more stable at 4°C as compared with 32°C.

4. Conclusions

In this work statistical techniques were applied as tools to determine the necessary conditions to obtain optimum yields in the enzymatic activity of the exoproteases of the strains of a Bacillus cereus strain immobilized on glutaraldehyde crosslinked chitosan beads.

From the results obtained from the optimization of the immobilization process of enzymes, it can be concluded that the correct selection of the parameters for immobilization of the proteases, and their subsequent optimization, is necessary to achieve an efficient enzymatic activity. This methodology maximizes amount of information that can be obtained while limiting the numbers of individual experiment. This useful statistical tool can help to reduce cost during enzymatic process, resulting in a cost reduction of the desired final product.

Thinking in the real application of the described model and its scaling at an industrial level, the results obtained could be useful in manipulating the properties of immobilized enzymes in crosslinked chitosan beads to achieve maximum efficiency in their hydrolytic activity.

The novel antimicrobial properties of the proteases of the *B. cereus spp*. immobilized against both Gram positive and Gram negative pathogens could result in benefits for the application of the systems in various bioprocesses, where the control of the microbial load of the substrates or environmental contamination is a critical factor to be consider.

It should be noted that immobilization leads to stabilizing effects of enzymes on solid supports. This stabilization may on the one hand prevent aggregation and even proteolysis caused by other proteases present in the extract, but in turn may also cause changes in conformations at the level of the proteins which are detrimental to their catalytic activity.

However, the immobilization of the protease in a matrix results in a potential reduction of the unfolding of its structure, induced by denaturing agents such as temperature and pH extremes or organic solvents. This protection, added to the operating conditions that were established (storage under refrigeration and reusability) are desirable parameters considering a potential industrial application.

Acknowledgments

This work was partially supported by grants from CONICET, UNMdP and Secyt, Agencia. Buenos Aires, Argentina.

References

[1] Wellington, S.A., Edilson, H.F., James, A.S., Gonçalves, L.R. (2005). *Appl. Biochem.* Biotech. 41, 201-207.

[2] Kalisz, H.M., Ed. (1988). Advances in Biochemical Engineering/ Biotechnology, Springer-Verlag, Berlin Heidelberg, pp: 1-65.

[3] Potumarthi, R., Subhaker, C., Pavani, A. (2008). *Bioresour Technol.* 99, 1776-1786.

[4] Dutta, P.K., Dutta. J., Tripathi. V.S. (2004). *J. Sci. Ind. Res.* 63, 20-31.

[5] Ward, O.P. (1983). *Proteinases*. In Microbia1 Enzymes and Biotechnology, Applied Science Publishers, London, 251-317.

[6] de Azeredo, H.M.C. (2009). Food Research International 42, 1240-1253.

[7] Sotitopoulou, G., Chaimotakis, N. A. (2005). *Biomat.* 26, 6771-6779.

[8] Chatterjee, S., Lee, D.S., Lee, M.W., Woo, S.H. (2009). *J. Hazard Mater.* 166, 508-513.

[9] Gupta, R., Chaudhury, N.K. (2007). *Biosens. Bioelectron* 22, 2387-2399

[10] Myres, R.H., Montgomery, D.C. (2008). Response surface methodology: process and product optimization using designed experiments, Hoboken, pp. 290-296.

[11] Kotlar, C.E., Ponce, A.G., Sansevero, R., Roura, S.I. (2009). *Internet J. Microbiol.* 8 (1), 1937-8289.

[12] Mundra, P., Desai, K., Lele, S.S. (2007). *Bioresour Technol.* 98, 2892-2896.

[13] Pérez Borla, O., Davidovich, L., Roura, S.I. (2010). *LWT - Food Sci. and Technol.* 3(2), 298-301.

[14] Khuri, A., Cornell, J.A., Eds. (1987). Response Surface Design and Analysis of Experiment, AQSA Quality Press, New York, pp. 177-186.

[15] Masson, R.L., Gunst, R.F., Hess, J.L., Eds. (1989). Statistical Design and Analysis of Experiments, New York, pp.518-526.

[16] Kotlar, C.E., Ponce, A.G., Roura, S.I. (2013). *LWT - Food Sci. and Technol.* 50, 378-385.

[17] Kotlar, C.E., Agüero, M.V., Roura, S.I. (2010). *Ind. Biotechnol.* 6(6), 364-374.

[18] Box, G.E.P., Hunter, J.S. (1957). *Ann. Stat.* 28, 195-241.

[19] Huiping, L., Guoqun, Z., Shanting, N., Yiguo, L. (2007). *Comp. Mater. Sci.* 38, 561-570.

[20] Nahit, A. (2005). *Enzyme Micro. Tech.* 37, 441-447.

[21] Chiou, M.S., Li, H.Y. (2002). *J. Hazard Mater.* B93, 233-248.

[22] Blanco, R.M., Guisán, J.M. (1988). *Enzyme Microb. Tech.* 10, 227-232.

[23] Blanco, R.M., Calvete, J.J., Guisán, J.M. (1989). *Enzyme Microb. Tech.* 11, 353-359.

[24] Bryjac, J. (2003). *Biochem. Eng. J.* 16, 347-355.

[25] Vidinha, P., Augusto, V., Almeida, M. (2006). *J. Biotechnol.* 121, 23-33.

[26] Wang, Q., Zhang, J.,Wang, A. (2009). *Carbohyd. Polym.* 78, 731-737.

[27] Mansfield, S.H., Mooney, C., John, N., Saddler, J.N. (1999). *Biotechnol. Prog.* 15, 804-816.

[28] Durango, A.M., Soares, N.F.F., Andrade, N.J. (2006). *Food Cont.* 17, 336-341.

[29] Anwar, A., Qader, S.A., Raiz, A., Iqbal, S., Azhar, A. (2009). *J. Appl. Behav. Sci.* 7, 1281-1286.