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Covalent Immobilization of β-Galactosidase Enzyme onto Modified Alginate Gel Beads

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Abstract

Alginate / H0 (acrylamide-co-acrylic acid) gel beads was generated and modified by sequential soaking with polyethyleneimine (PEI) and glutaraldehyde (GA) as a functional carrier suitable for enzymes immobilization. The modification was carried out by using response surface methodology. Maximum immobilization yield (78.2%) of β-galactosidase was obtained by soaking the gel beads with 3.5% of (PEI) for 5hrs. followed by soaking the treated gel beads with 4 % of (GA) for 6hrs. Analysis of variance (ANOVA) showed a high coefficient of determination value (R^2) of 0.90, ensuring a satisfactory adjustment of the quadratic model with the experimental data. Thermo gravimetric analysis (TGA) of gel beads during different improvement steps showed a remarkable increase in their thermal stability from 190, 200 and 210 °C for Alginate-H0, Alginate-H0 / PEI and Alginate-H0 / PEI / GA respectively. The reusability test proved the durability of the modified Alginate / H0 (acrylamide-co-acrylic acid) gel beads for 7 cycles with retention of 100% of the immobilized enzyme activity losing only 10% of its activity after 17 cycles. To be more convenient for industrial uses , considerable stability and reusability of bound enzyme maybe advantageous for its industrial application.

1. Introduction

There are of paramount importance for natural polymers, where it found in nature as a result of all organisms growth cycle. In recent years, there is an attraction goes to utilize biodegradable polymers which produced from renewable resources. Production and development as well as research on biodegradable biopolymers have been fasted in recent years. These natural biodegradable polymers called biopolymers. Polysaccharides, as alginate, starch, chitosan and cellulose, represent the most characteristic family of these natural polymers [1].

Alginate, a non-branched binary copolymer, is a biopolymer found in brown algae. It is composed of β-D-mannuronic acid monomer linked to α-L-guluronic acid monomer, through a 1,4-glycoside linkage. Alginate is able to form gels in the presence of counter ions, as divalent cations, such as Ca^{2+} [2]. This gelling property makes it important biopolymer [3].

Copolymers of acrylamide with ionic comonomers are of high interest for a multitude of industrial applications, and have been studied by academics [4]. It presents an interesting and useful model for academic copolymerization studies. On the other hand, both the homopolymers and copolymers are of practical interest with productions in the thousands of tons scale. Both homo and copolymers were also subject of characterization studies [5, 6].

The combination between alginate and acrylamide copolymers has many advantages in immobilization procedure. As the carrier should have great attention by choosing the one that has good mechanical strength, resistant for microorganisms, cheap and has available function groups in its large surface area [7]. There are many methods of immobilization such as adsorption, crosslinking, entrapment, encapsulation and covalent binding method [8].

One-variable-at-a-time considered as the classical optimization method does not allow determination of the interactive effects of the parameters in the investigated process [9, 10]. Due to these drawbacks, response surface methodology (RSM) considered as an excellent alternatives currently used. Box and Wilson (1951), was firstly used RSM as a statistical tool that enables to evaluate the effect of the independent variables,

individually or in combination, and their interactions on response variables in the investigated process allowing improving, developing, and optimizing such process [11]. Moreover, the mathematical model generated by the experimental methodology could describe the process [10].

In the present work Alginate / H0 (acrylamide-co-acrylic acid) gel beads were prepared and modified by using polyethyleneimine (PEI) and glutaraldehyde (GA). Optimization was done by using response surface methodology techniques, 30 runs were done to reach to the optimum concentration and soaking time for (PEI) and (GA) aiming to achieve maximum recovery in activity of the immobilized enzyme. The immobilization efficiency of the generated polymers was tested by its immobilization with β-galactosidase moreover the re-usability of the selected polymer was tested.

2. Material and Methods

2.1. Materials

Sodium alginate low molecular weight (Alg) was obtained from Fluka. polyethyleneimine (PEI) (50%) and glutaraldehyde (GA) (50%), were obtained from Sigma-Aldrich. Acrylic acid (AA), chemically pure grade inhibited with 180-220 ppm methyl ethyl hydroquinone (MEHQ), (M.wt. (72.06 g/mol), m.p.(13.5^{o}C), b.p.(141^{o}C), d.(1.045 g/ml)was obtained from EIF chem. (ATO) company, France. Acrylamide (AAm) M.wt. (71.08 g/mol), C.F. [C3H5NO], m.p. (84.5 ^{o}C), d.(1.13 g/ml) was purchased from Merck- Shuchardt Company, Germany. Benzoyl peroxide (BPO, initiator) M.wt. (242.23 g/mol), C.F. [C14H10O4], melting range (103-105 ^{o}C) was supplied by Merck. Methanol (M.wt. (32.04 g/mol), C.F. [CH4O], b.p. (64.7 ^{o}C), d. (0.79 g/ml) was obtained from El-Naser pharmaceutical chemical company, Egypt. Other chemicals were of Analar or equivalent quality. The Encapsulator, model IE-50 was purchased from Innotech Encapsulator in Switzerland.

2.2. Methods

2.2.1. Preparation of alginate beads

Alginate gel beads were prepared according to Ghada *et al*, (2016) [12]. Sodium alginate (Alg) was dissolved in distilled water to give a final concentration of 2 % (w/v). The alginate solution was dropped through a nozzle of 300 µm using the Inotech Encapsulator (fig. 1) in a hardening solution containing 2.5 % (w/v) $CaCl_2$ solution and was soaked for 3 hrs.

2.2.2. Preparations of different polymers

Alginate was modified by adding another polymer. Alginate / H0 (acrylamide-co-acrylic acid), Alginate / SO3 (acrylamide-co-acrylic acid-co-3 allyloxy-2-hydroxy-1-propanesulfonic acid sodium salt solution), Alginate / NMAM (acrylamid e-co-acrylic acid-co-N, N dimethylacrylamid) and Alginate / HEMA (acrylamide-co-acrylic acid-co-2 hydroxy ethyl methyl methacrylate), were prepared in ratio 1:1 and dissolved in distilled water using an overhead mechanical stirrer. After complete dissolution, the polymer solutions were dropped through a nozzle of 300 µm using the Inotech Encapsulator (fig. 1) in a hardening solution containing 2.5 % (w/v) $CaCl_2$.

Figure 1 Encapsulator for making uniform gel beads

2.2.3. Modification of Alginate / H0 (acrylamide-co-acrylic acid) by central composite design

To examine the cumulative effect of beads formation, response surface methodology was employed. A 2^4 full factorial central composite design (CCD) with 16 trials for factorial design (1), 8 trials for axial point and 6 replicate trials at the central point, leading to a set of 30 experiments was designed. The range and levels of five coded levels ($-\alpha$, -1, 0, 1, $+\alpha$) and the experimental design is shown in Table1. All the variables were taken at a central value represented by '0'. The response value from each experiment of CCD was the average of triplicates. The beads were first soaked in an amine solution (PEI) in different concentration for the desired time

and the excess of amine washed thoroughly by using distilled water. Afterwards, beads were soaked in a GA solution prepared in different concentrations for the desired time, washed twice with distilled water, and directly used to immobilize β-galactosidase [13].
The response values from each experiment of CCD were the average of triplicates. Four variables (PEI percent (%), PEI contact time, GA percent (%) and GA contact time) were studied; immobilization yield percent (%) obtained was taken as the dependent variable or response (*Y*). The second-order polynomial coefficients were calculated and analyzed using the 'SPSS' software (Version16.0,) Second-degree polynomials, Eq. (1), which includes all interaction terms, β were used to calculate the predicted response:

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta \Sigma_{ij} X_i X_j \qquad \text{Eq. (1)}$$

where *Y* represents response variable, β_0 is the interception coefficient, β_i the coefficient of the linear effect, β_{ii} the coefficient of quadratic effect and β_{ij} are cross product coefficients, $X_i X_j$ are independant variables which influence the response variable *Y*. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination for each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated by using STATISTICA (0.6).

2.2.4. Covalent Immobilization of β-Galactosidase onto the Activated beads.
About 1 g of the activated beads was mixed with 5 ml citrate-phosphate buffer (pH 4.5) containing 7 U of β-galactosidase. This mixture was agitated, using a roller stirrer, at room temperature for 16 hrs. Afterwards, the beads were filtered off, washed thoroughly with distilled water, and directly assayed for β-galactosidase activity. Immobilization Yield (I.Y.) has been calculated from the following equation:

I.Y. % = (*C*/*A* − *B*)*100

Where *A* is the activity of free enzyme, *B* is the activity of remaining enzyme, whereas *C* is the activity of immobilized enzyme.

2.2.5. Determination of β-Galactosidase Activity.
β-galactosidase activity was assayed as follow, 1 gram of β-galactosidase loaded beads were soaked in 4 ml of 0.1 M acetate buffer (pH 4.6), followed by the addition of 1 ml of a 10 mM O-Nitrophenyl b-D-Galactopyranoside (ONPG) solution. The reaction was left to proceed for 15 min in a thermo-stated shaking water bath at 37 °C. Beads were then removed, and the absorbance of the supernatant was measured at 405 nm. Regarding the free β-galactosidase, it was assayed by mixing 0.25 ml of the enzyme solution with 3.75 ml of 0.1 M acetate buffer (pH 4.6). Afterwards, 1 ml of a 10 mM ONPG solution was added to the reaction mixture. The reaction was left to proceed for 15 min in a thermos-stated water bath at 37 °C. Then, the absorbance of the solution was measured at 405 nm. One unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1.0 nmol of o-nitrophenol from the ONPG per min under standard assay conditions [14].
2.2.6. Fourier Transform infrared (FT-IR)
Infrared spectra of all formulations were evaluated with Fourier transform infrared spectroscopy (FTIR-850, Tianjin Gangdong Sci & Tech development Co., Ltd, China). FTIR spectra were taken in wavelength region from 400 to 4000 cm^{-1} at ambient temperature.

2.2.7. Scanning Electron Microscope (SEM)
The surface of different gel formulations gel beads, gel beads + PEI, gel beads + PEI + GA and gel beads + PEI + GA + Enzyme was evaluated by using scanning electron microscopy (SEM, SU3500, HITACHI) to show the changes occurred on the surface after each reaction.

2.2.8. Operational stability
The reusability of immobilized β-galactosidase was studied by using the modified alginate gel beads. One gram of the grafted gel beads was added to 4ml of 0.1 M acetate buffer (pH 4.6) and 1 ml of a 10 mM o-nitrophenyl b-D-galactopyranoside (ONPG). The mixture was incubated for 15 min at 37 °C in a shaking water bath, and the substrate solution was assayed as above. The same gel beads were washed twice with acetate buffer and re-incubated with another substrate solution; this procedure was repeated for 17 times, and the initial activity was considered as 100%. Relative activity expressed as a percentage of the 100% activity and calculated according to the following equation:

$$\text{Relative activity (\%)} = \frac{\text{enzyme activity in the nth cycle}}{\text{enzyme activity in the 1st cycle}} \times 100$$

3. Results and discussion

At the beginning of our study different polymers were prepared by combination of alginate with different polymers preparing, Alginate / H0 (acrylamide-co-acrylic acid), Alginate / SO_3 (acrylamide-co-acrylic acid-co-3 allyloxy-2-hydroxy-1-propanesulfonic acid sodium salt solution), Alginate / NMAM (acrylamid e-co-acrylic acid-co-N, N dimethylacrylamid) and Alginate / HEMA (acrylamide-co-acrylic acid-co-2 hydroxy ethyl methyl methacrylate) polymers. The generated polymers were examined for its efficiency for β-galactosidase immobilization. The results represented in Fig. 2. showed that the maximum immobilization yield (70%) was obtained at the polymer formation by the combination of alginate with H0 (acrylamide-co-acrylic acid). This result may be referred to the carboxylic groups found naturally in H0 (acrylamide-co-acrylic acid), leading to increase the reaction probability consequently increase the amount of immobilized enzyme [15].

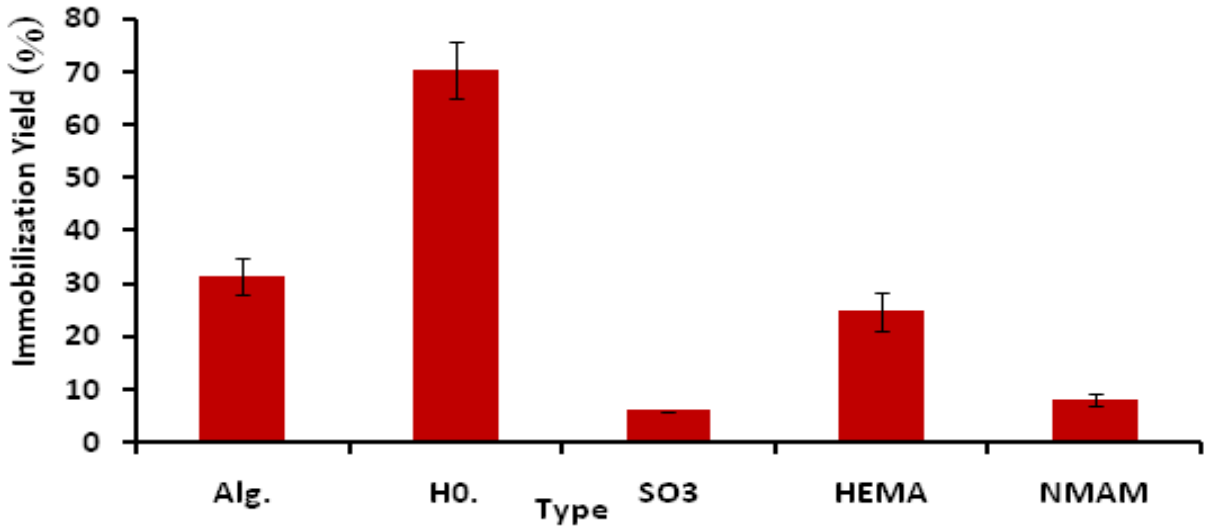

Figure 2 different types of modification for alginate beads

3.1. Optimization of the modification conditions by central composite design (CCD)

For modification of Alginate/ H0 (acrylamide-co-acrylic acid) gel beads , four different variables (PEI percent (%), X_1; PEI contact time, X_2; GA percent (%), X_3 and GA contact time, X_4) were chosen to determine their optimum response region of the immobilization yield (%) of β-galactosidase. Table 1 represents the design matrix of the coded variables together with the experimental results of the immobilization yield (%). All experimental trials were performed in triplicate and the average of the observations was used. The maximum immobilization yield (%) of β-galactosidase was 78.20 in run 29 when PEI percent was 4(%); PEI contact time was 6 hrs.; GA percent was 2.5(%) and GA contact time was 3hrs. The following regression equations obtained after the standard analysis of variance (ANOVA) presented the level of the immobilization yield (%) of β-galactosidase as a function of PEI percent(%),PEI contact time, GA percent (%) and GA contact time. Regression analysis was used to analyze the data and thus a polynomial equation was derived from regression analysis as follows:

Immobilization yield (%)= $-4.107 + 11.267X_1 - 13.664X_2 + 27.209X_3 + 16.340X_4 + 1.709\ X_1^2 - 1.272\ X_2^2 - 1.454\ X_3^2 + 1.404\ X_4^2 - 0.724\ X_1X_2 - 0.141X_1X_3 - 1.122\ X_1X_4 - 0.004X_2X_3 - 2.881X_2X_4 - 4.107X_3X_4$

Table 2 shows a significant *F*-value (4.584) which implied the model to be significant. Model terms having values of Prob > F (0.003) are less than 0.05 which considered significant. The regression equation obtained after ANOVA indicating that the determination of coefficient (R^2) was, calculated as 0.9 for immobilization yield (%) of β-galactosidase (a value of $R^2 > 0.75$ indicated the aptness of the model) that means the statistical model can explain 90% of variability in the response, in reasonable agreement with the adjusted R^2 of 0.811. The goodness of the model can be checked by the determination of coefficient (R^2) and correlation coefficient (R). The R^2 value is always between 0 and 1. The closer the R^2 is to 1, the stronger the model and the better it predicts the response [16, 17]. The value of R^2 being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation.

Three-dimensional response surfaces **(Fig.3 a-f)** were plotted on the basis of the model equation, to investigate the interaction among the variables to determine the optimum value. 2^4 full-factorial central composite design was used by many authors for optimization of immobilization yield (%) of β-galactosidase [18]. Fig (4) showed the close relation between observed results and predicted values obtained by the polonomial equation.

Table 1 represents the design matrix of the coded variables together with the experimental results of the immobilization yield (%)

Trial No.	X_1	X_2	X_3	X_4	Immobilization yield (%)	
					Experimental	predicted
1	-1	-1	-1	-1	46.20227	49.31
2	-1	1	-1	-1	46.19816	44.55
3	0	0	0	+ ∞	52.62508	49.30
4	0	0	0	0	55.09793	62.878
5	0	0	0	- ∞	52.61274	54.19
6	-1	1	1	1	52.39055	55.37
7	0	0	0	0	69.12442	62.878
8	-1	1	-1	1	49.55974	53.32
9	0	0	0	0	49.69964	62.878
10	-1	1	1	-1	65.33904	63.88
11	1	-1	-1	-1	51.27551	48.64
12	0	0	0	0	71.11998	62.878
13	1	1	1	-1	67.75016	70.12
14	1	1	1	1	61.66886	60.55
15	-1	-1	-1	1	60.0683	58.12
16	0	0	- ∞	0	52.93367	51.1
17	1	1	-1	-1	53.06534	54.40
18	1	-1	-1	1	53.03654	56.39
19	+ ∞	0	0	0	56.23354	56.44
20	- ∞	0	0	0	49.75724	47.82
21	-1	-1	1	-1	74.18532	75.38
22	0	- ∞	0	0	75.73239	75.08
23	0	0	0	0	62.15849	62.87
24	1	1	-1	1	62.37245	62.12
25	0	0	0	0	70.38348	62.87
26	-1	-1	1	1	66.68038	66.90
27	0	0	+ ∞	0	70.55217	69.78
28	1	-1	1	-1	73.45704	71.09
29	0	+ ∞	0	0	78.20523	76.42
30	1	-1	1	1	59.1343	61.55
Levels	(%)	(h)	(%)	(h)		
- ∞	1	0.5	1	0.5		
-1	2.5	1.5	1.5	1.5		
0	4	3	2.5	3		
1	5	4.5	3.5	4.5		
+ ∞	6	6	5	6		

x_1 is the codded *value of PEI percent* ; x_2 is the codded value of PEI contact time; x_3 is the codded *value of GA percent* x_4 is the codded *value* contact time

3.2. FT- IR spectroscopic analysis

The FTIR bands of Alginate-H0, Alginate-H0 / PEI, Alginate-H0 / PEI / GA and Alginate-H0 / PEI / GA / Enzyme gels beads were shown in Figure 5. Spectrums of Alginate-H0 (A) show the characteristic two bands one at 3444 cm^{-1} which belonging to NH_2 and OH groups found in Poly(acrylamide-co-acrylic acid) and alginate, the other band at 1412 cm^{-1} which belonging to C=O and COOH groups found in the polymer. After treatment of polymer with polyethyleneimine (PEI) as in (B) we can notice that the band at 3444 cm^{-1} increased due to the increase in NH_2 of PEI. And in (C) after treatment with glutaraldehyde we can also see the appearance of the new characteristic band at 1631 cm^{-1} proving the presence of C=N group resulting from the reaction between PEI and glutaraldehyde. And (D) has the two bands one for NH_2 and increased due to the amine group of the enzyme and the second band of C=N group resulting from the reaction between PEI and glutaraldehyde and also between amine group of enzyme and glutaraldehyde [19, 20].

Table 2 Model coefficients estimated by multiples linear regression (significance of regression coefficients)

Variables	Regression coefficients	Standard error	t- test	P-value	
Intercept	-4.107	1.350	1.664	0.117	
X_1	11.267	-1.896	-2.482	0.025	
X_2	-13.664	2.609	3.388	0.004	
X_3	27.209	2.268	2.968	0.010	
X_4	16.340	-1.439	-2.312	0.035	
X_1^2	1.709	-0.683	-1.272	0.223	
X_2^2	-1.272	-1.287	-2.614	0.020	
X_3^2	-1.454	0.924	1.826	0.088	
X_4^2	1.404	-0.351	-0.627	0.540	
X_{12}	-0.724	-0.093	-0.184	0.857	
X_{13}	-0.141	-0.522	-1.161	0.264	
X_{14}	-1.122	0.003	-0.007	0.995	
X_{23}	-0.004	-1.341	-2.982	0.009	
X_{24}	-2.881	1.350	1.664	0.117	
X_{34}	-4.107	-1.896	-2.482	0.025	
ANOVAs					
	Df	*SS*	*SM*	*F test*	*Significance F (P)*
Regression	14	2156.397	154.028	4.584	0.003
Residual	15	504.068	33.605		
Total	29	2660.465			

$x_1$is the codded *value of PEI percent* ; x_2 is the codded value of PEI contact time; x_3 is the codded *value of GA percent* x_4 is the codded *value* contact time; *df* Degree of freedom; *SS* Sum of squares; *MS* Mean sum of squares; *F* Fishers's function; *Significance F* corresponding level of significance R^2 0.90, Adjusted R^2 0.811

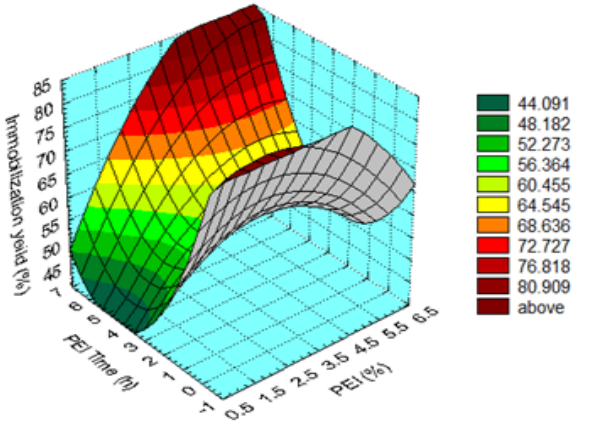

Fig 3a Response surface plot showed the effect of the PEI percent and PEI contact time on immobilization yield percent of β- galactosidase at X_3= 0 and X_4= 0

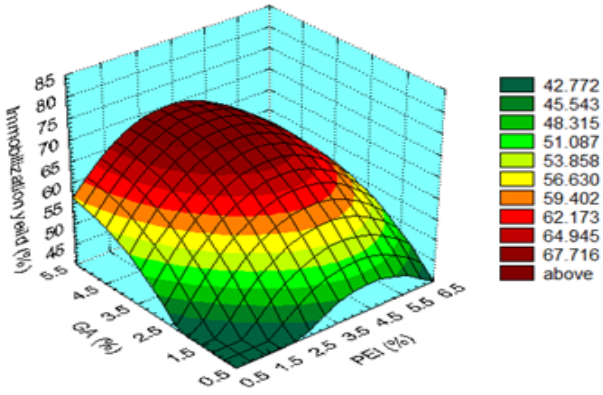

Figure 3b Response surface plot showed the effect of the PEI percent GA percent on immobilization yield percent of β- galactosidase at X_2= 0 and X_4= 0

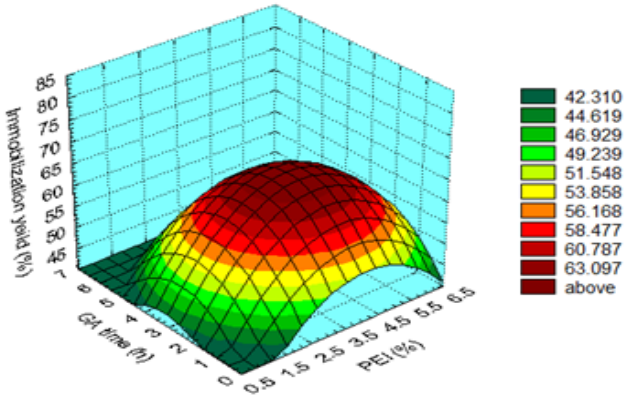

Figure 3c Response surface plot showed the effect of the PEI percent and GA contact time on immobilization yield percent of β- galactosidase at X_2 = 0 and X_3= 0

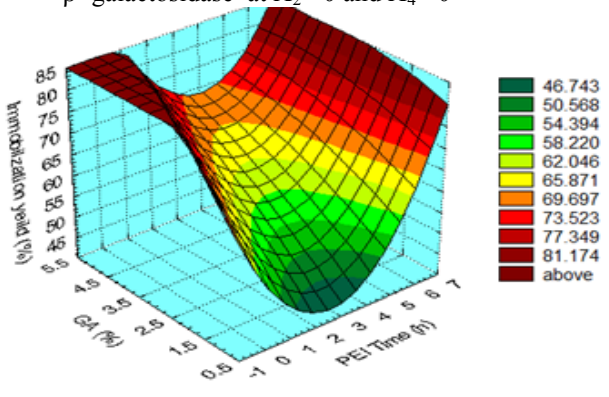

Figure 3d Response surface plot showed the effect of the GA percent and PEI contact time on mmobilization yield percent of β- galactosidase at X_1= 0 and X_4= 0

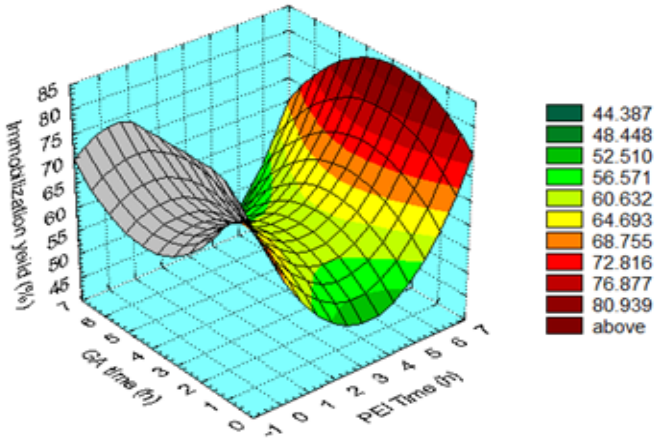

Figure 3e Response surface plot showed the effect of the GA contact time and PEI contact time on immobilization yield percent of β- galactosidase at X_1= 0 and X_3= 0

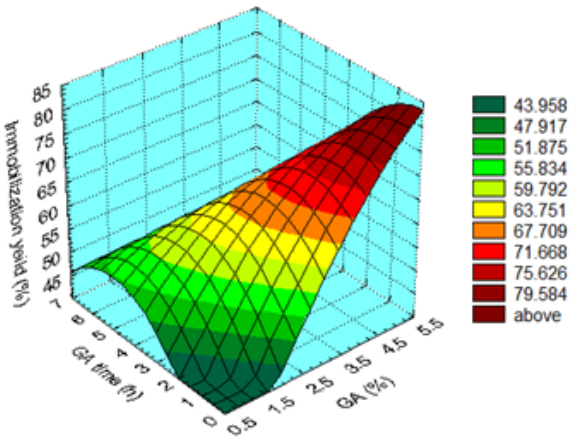

Figure 3f Response surface plot showed the effect of the GA contact time and GA percent contact time on immobilization yield percent of β- galactosidase at X_1= 0 and X_2= 0

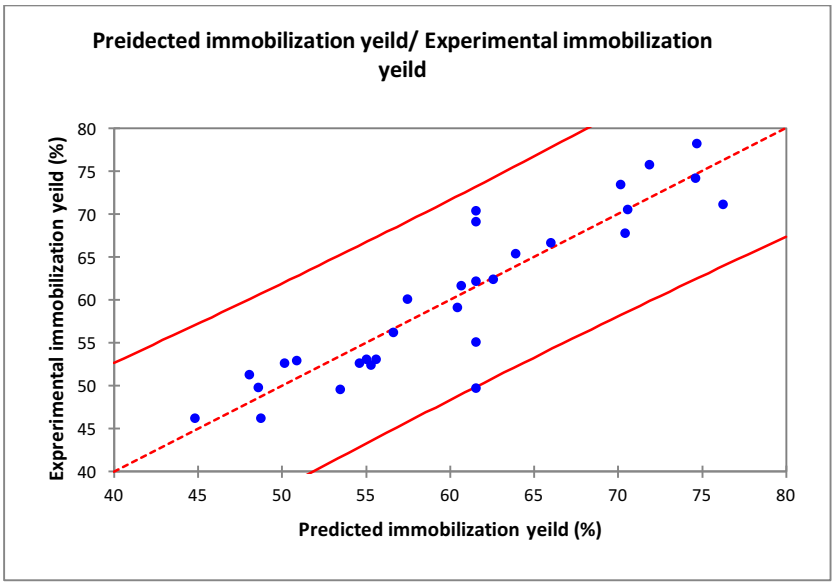

Figure 4 Correlation between the observed and predicted values immobilization yeild (%) determined by the first-order polynomial equation.

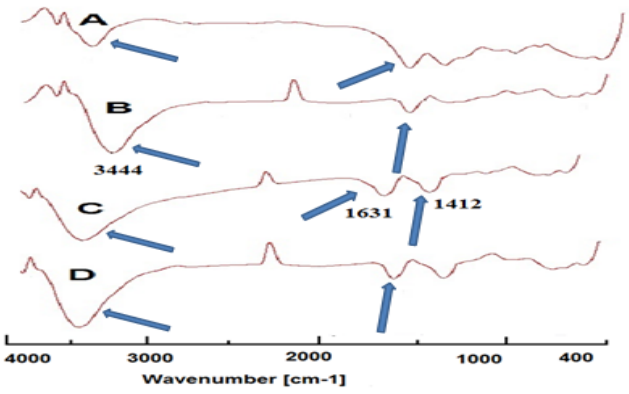

Figure 5 FT-IR of H0. Gel beads (A), aminated beads (B), activated beads (C) and immobilized one (D).

3.3. Thermal Gravimetric Analysis (TGA)

The TGA thermogram of Alginate-H0, Alginate-H0 / PEI, Alginate-H0 / PEI / GA and Alginate-H0 / PEI / GA / Enzyme gels are shown in Figure 6 and data were tabulated in Table (3). The treatment of Alginate-H0 with PEI followed by GA showed a gradual and obvious improvement in their TGA. The TGA of Alginate-H0 was 190 °C compared to Alginate-H0 / PEI which is 200 °C and Alginate-H0 / PEI / GA which is 210 °C and after

immobilization which becomes 300 °C. The gels' thermal improvement could be explained by the formation of polyelectrolyte interaction between the polyanions (-COO-) of Alginate-H0 and the polycations (-NH3+) of the PE. Further hardening of the gel beads using GA showed further increase in the TGA of Carr. / PEI / GA 210 °C. These improvements in their TGA could be attributed to the formation of a stronger crosslinking of the gel beads due to the formation of Schiff's base between the free PE's amino groups and GA [21].

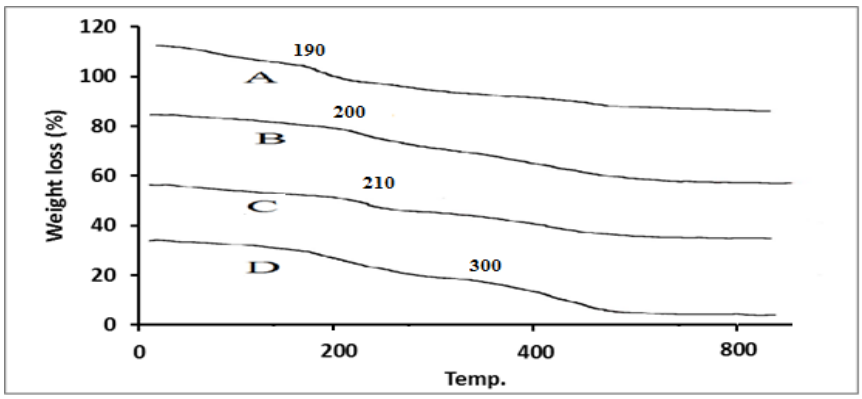

Figure 6 TGA Thermographs of Alginate-H0. Gel beads (A), aminated beads (B), activated beads (C) and immobilized one (D).

Table 3 TGA data of Alginate-H0 Gel beads, aminated beads, activated beads and immobilized one

Type	TGA Temp.
Alginate-H0	190
Alginate-H0 + PEI	200
Alginate-H0 + PEI + GA	210
Alginate-H0 + PEI + GA + Enz.	300

3.4. Scanning Electron Microscope (SEM)

Figures 7, 8 displayed SEM for Alginate-H0, aminated Alginate-H0, activated Alginate-H0 and immobilized one.

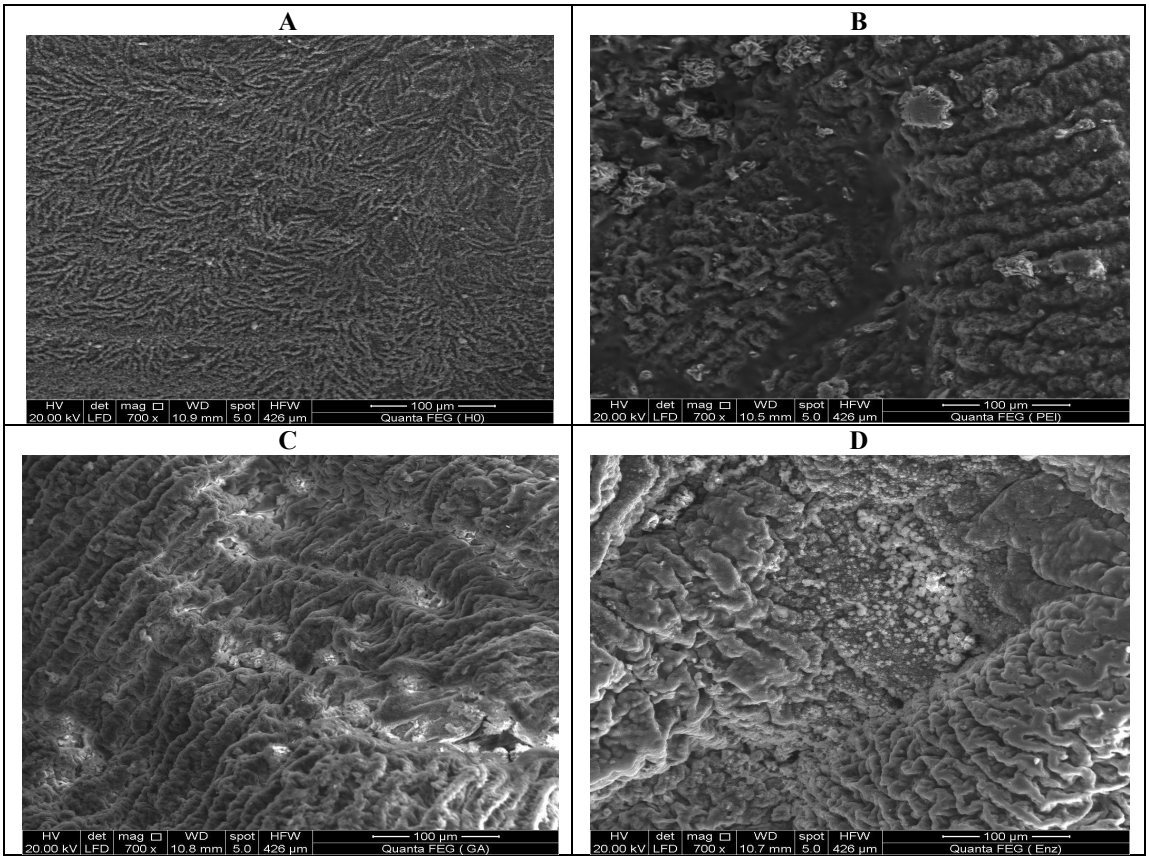

Figure 7 SEM for surface of gel beads. Gel beads (A), aminated beads (B), activated beads (C) and immobilized one (D). At magnification of 700x

From these (A, B, C and D) we noticed the changes which happened in each step. As we can see there is difference in the surface after each step. Even in low magnification or in higher one we can observe the aggregates on the surface which differ from step to another [22].

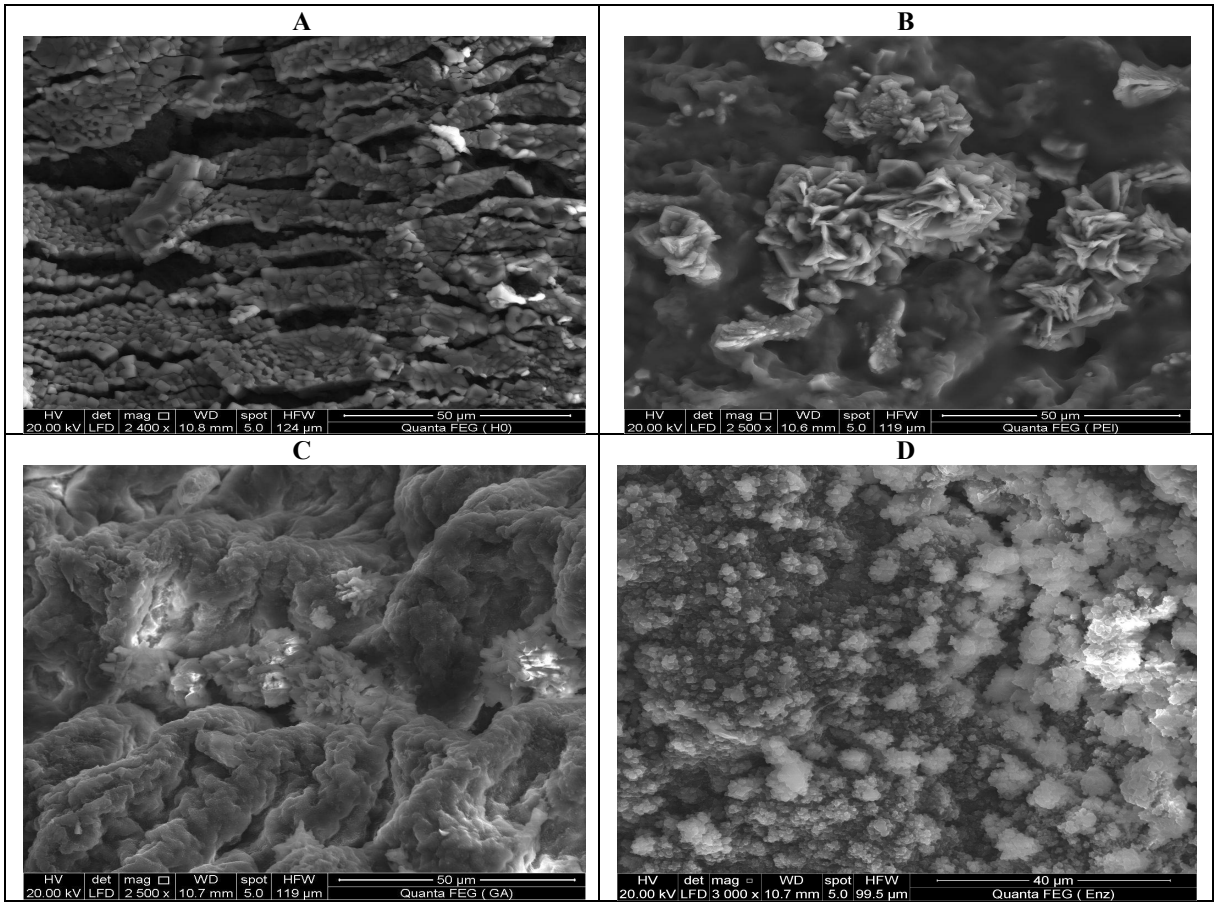

Figure 8 SEM for surface of gel beads. Gel beads (A), aminated beads (B), activated beads (C) and immobilized one (D). At magnification of 2500x

3.5. Operational stability of Alginate / H0 (acrylamide-co-acrylic acid) gel beads immobilized with β-galactosidase

Recycling of Immobilized enzymes are preferred as they can be reused many times lowering the production costs and it considered as a marker indicating the efficiency of the immobilization process. β-galactosidase immobilized on Alginate / H0 (acrylamide-co-acrylic acid) beads could be reused for 17 consecutive cycles retaining 100% of its initial activity **(Fig. 9).** The enzyme activity retains 100 % of its activity for the first7 cycles after that, the enzyme loss only 10% reaching the17cycles. The loss in activity was attributed to inactivation of enzyme due to continuous use. This result is in agreement with Ghada *et al.;* (2016) who use naringinase immobilized onto modified alginate for 20 cycles [12].

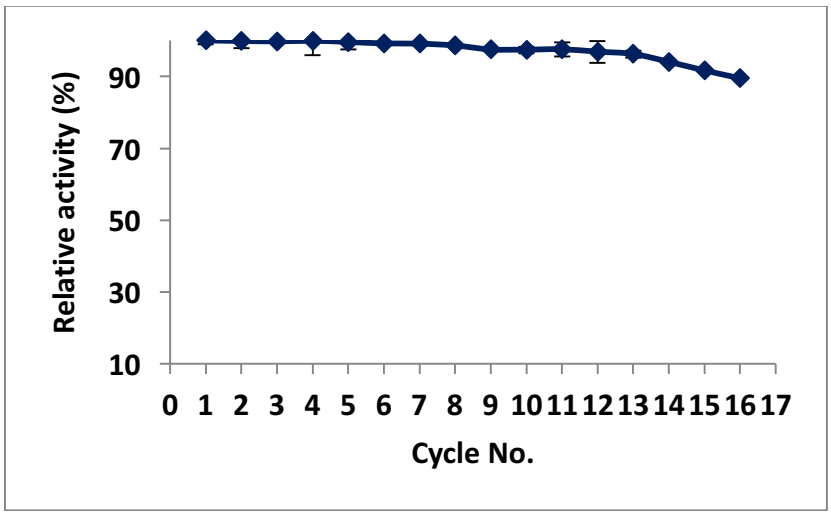

Figure 9 Operational stability of Alginate / H0 gel beads immobilized with β-galactosidase

Conclusion

This study justifies the use combination alginate with H0 (acrylamide-co-acrylic acid forming grafted beads as a suitable matrix for the covalent binding immobilization. Generated beads were modified by using PEI and GA, optimization of concentrations of PEI and GA and their contact time with gel beads were optimized by using 2^4 full factorial central composite design (CCD). β-galactosidase was immobilized on the generated gel beads. The immobilization efficiency was evaluated. Immobilized β-galactosidase beads can be reused for more than 17 consecutive cycles retaining of its activity.

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