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# Application of Plackett–Burman Screening Design to the Modeling of Grafted Alginate–Carrageenan Beads for the Immobilization of Penicillin G Acylase

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**ABSTRACT:** Grafted alginate–carrageenan beads were used to immobilize the industrial enzyme penicillin G acylase (PGA). Sixteen factors were screened with the Plackett–Burman design (PBD) to test their significance on the gel beads formation and enzyme immobilization process. The results of PBD showed a wide variation of 30-fold in the amount of immobilized penicillin G acylase (iPGA) from 11.9 to 354.16 U/g of beads; this reflected the importance of the optimizing process. Among the 16 tested factors, only 3 were proven to be significant. These factors were the enzyme buffer pH (*N*), enzyme soaking time (*Q*) with the gel beads, and enzyme concentration (*P*). The Pareto chart revealed that both *Q* and *P* exerted significant positive effects on the amount of iPGA, whereas *N* had a negative effect. We recommend further study to optimize only these three significant, distinctive enzyme factors. The PGA covalent attachment to the gel beads were proven by Fourier transform infrared spectroscopy, elemental analysis, and NaCl and reusability tests. The best gel bead formula succeeded in the immobilization of 354.16 U/g of beads and proved to be reusable 14 times, retaining 84% of the initial enzyme activity. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40295.

**KEYWORDS:** catalysts; composites; gels

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## INTRODUCTION

Penicillin G acylase (PGA; EC 3.5.1.11) is a hydrolytic enzyme that acts on the side chain of penicillin G to produce 6-amino penicillanic acid (6-APA). 6-APA is a $\beta$-lactam antibiotic intermediate that is among the building blocks of the semisynthetic $\beta$-lactam antibiotics industry; these antibiotics include ampicillin, amoxicillin, cloxacillin, cephalexin, and cefatoxime. The $\beta$-lactam antibiotics industry has annual sales of about $15 billion; this makes up 65% of the total antibiotics market. Moreover, the annual consumption of PGA is estimated to be in the range of 10–30 million tons.[1] Consequently, the immobilization of such an important industrial enzyme will be of great use. Immobilization prevents the contamination of the final product with the enzyme and allows the easy separation of the immobilized enzymes. Moreover, immobilization usually increases the stability of the enzyme.

However, efficient commercial carriers suitable for the immobilization of enzymes are fairly expensive.[2] Available carriers, including such as Eupergit C or agarose, are sold for € 6000 and € 3250/kg, respectively.[3] Hence, there is a persistent need to prepare new enzyme carriers that are cheap. Carrageenans (Car's) and alginates (Alg's) are two hydrogels that are available at reasonable costs. Moreover, they are permitted for use in the pharmaceutical and food industries, and they have diverse features.[4] Thus, they are ideal candidates for enzyme immobilization.

Alg's are naturally derived polysaccharides that are composed of (1,4)-linked $\beta$-D-mannuronic acid (M units) and $\alpha$-L-guluronic acid (G units) monomers (Figure 1).[5] Alg gel is formed by ionic network formation in the presence of cations, such as calcium ions or other multivalent counter ions. This method qualifies as safe, mild, fast, and cheap. However, chelating agents, such as phosphates and citrates, which are present in lots of buffers, can disrupt the gel structure by binding calcium ions.[6] However, the $\kappa$-Car gel lattice is not affected by the presence of phosphates or citrates. Hence, the incorporation of $\kappa$-Car with Alg is

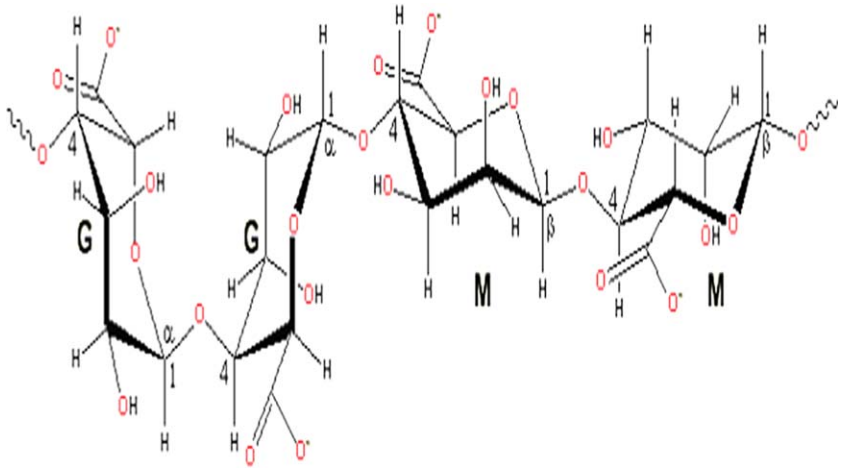


**Figure 1.** Schematic diagram illustrating the structures of the Alg, M units, and G units. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expected to improve the gel's stability and prolong its lifetime. That is why, in our study, we incorporated κ-Car with Alg.

κ-Car is a naturally abundant biopolymer isolated from red seaweed polysaccharides (carrageenose 4′-sulfate).[7] This polyanion contains one sulfate group (—$SO_4^-$) per molecule (Figure 2).[8] One can form a Car gel easily by dissolving its powder in hot water and letting it sit out at room temperature. The gel formation process involves a conformational transition of the Car molecule according to a mechanism offered by Wang and Qiang,[9] that is, coil to helix to gel. This advantage enables the Car gel to be shaped in any form.[10]

There are many techniques for immobilizing enzymes; these include adsorption, covalent immobilization, encapsulation, entrapment, and crosslinking.[11] Nevertheless, the covalent technique has the advantage of keeping the enzyme well bound to the carrier, and this prevents enzyme leakage.[12] This enables the reuse of the immobilized enzyme 10s of times and reduces its cost and the cost of its products. This is why covalent immobilization is widely preferred on the industrial scale. However, both Car and Alg lack active functional groups for immobilizing enzymes covalently. This problem was solved by the incorporation of a new functional group (aldehyde) into either Alg or Car.[13,14] In this study, Alg–Car gel beads were formulated and activated for the covalent immobilization of PGA.

It is worth mentioning that to our knowledge, previous reports have not dealt with Alg–Car polyelectrolyte as a matrix for the covalent immobilization of enzymes. Alg–Car uniform gel beads were prepared with the encapsulator and hardened with $CaCl_2$. Afterward, the gel beads were activated via a two-step method (Figure 3) as follows:

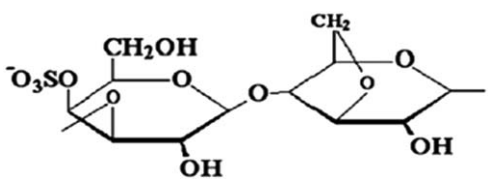


**Figure 2.** Chemical structures of κ-Car.

- First, we added the synthetic polyamine compound polyethylenimine (PEI) to the Alg–Car gel beads. The PEI contributed both protonated and free amino groups to the activation process. The PEI's protonated amino groups formed an ionic complex (network) with anions of the Alg–Car gel, where Alg contributed its carboxylate groups and Car offered sulfate groups. This network helped to strengthen the gel and increase its thermal stability.[15,16]
- On the other hand, the PEI's free amino groups were used during the second activation step. This step involved the

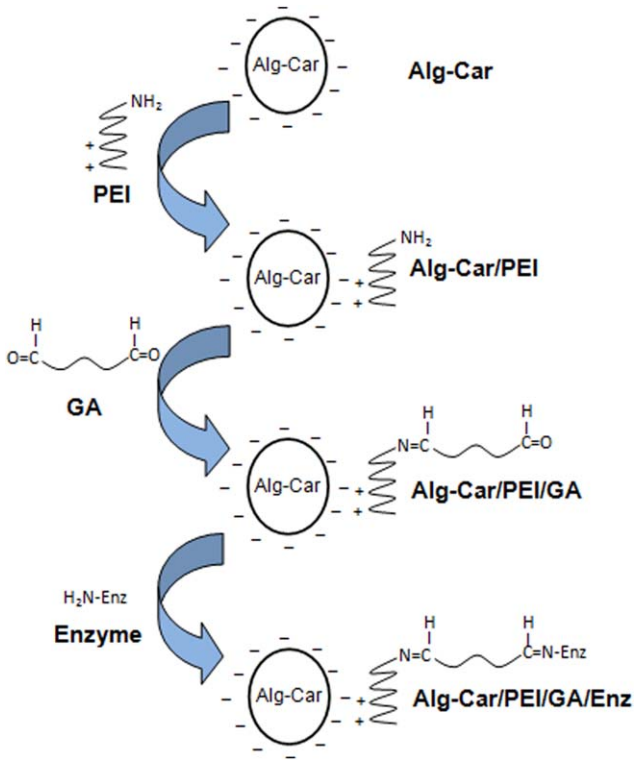


**Figure 3.** Schematic representation of Alg–Car gel activation and enzyme (Enz) immobilization. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

addition of the dialdehyde spacer arm, glutaraldehyde (GA). The aldehyde groups present at one end of the GA molecule reacted with the free amine moieties of PEI to form a Schiff's base. Meanwhile, the unreacted GA side created free aldehyde groups for the covalent immobilization of PGA. The existence of this free aldehyde group was elucidated by Fourier transform infrared (FTIR) spectroscopy.

To optimize the formation, the activation of the Alg–Car gel beads, and the immobilization of PGA, numerous process variables needed to be optimized. Optimizing these numerous factors with the one-factor-at-a-time approach would be extremely tedious and time-consuming. Consequently, we used statistically planned experiments instead. Here, we used the Plackett–Burman design (PBD). PBD is a screening statistical design that identifies significant factors from a large number of suspected contributor factors for the desired response.[17] Moreover, PBD considerably reduces the number of experiments required. In fact, a $k$ number of factors can be evaluated and screened for significant factors with only $k+1$ experiments.[18]

In this study, we used PBD to screen 16 possibly influential factors for their effect on the Alg–Car gel bead formation, activation, and PGA immobilization onto these beads. Out of these 16 factors, only 3 were found to be significant [enzyme buffer pH ($N$), enzyme soaking time ($Q$) with the gel beads, and enzyme concentration ($P$)], and thus, they had to be further optimized to reach the maximum amount of immobilized penicillin G acylase (iPGA).

## EXPERIMENTAL

### Materials

Lyophilized PGA was acquired from CPC (Italy). $\kappa$-Car was purchased from Fluka. Sodium alginate was obtained from Winlab. GA solution (25% v/v in water), PEI, and 98% 4-dimethylaminobenzaldehyde were purchased from Sigma-Aldrich. All of the other fine chemicals were Analar or equivalent quality. An Innotech Encapsulator model IE-50R was acquired from Innotech Co. in Switzerland for the preparation of uniform gel beads on the semipilot scale.

### Methods

**Formulation and Activation of the Gel Beads.** Alg and Car were dissolved in distilled water at 60°C. Afterward, uniform gel beads were formed through injection of the gel solution with the encapsulator instrument through a 300- or 700-$\mu$m nozzle under a pressure of 1 bar. The formed beads were received in a $CaCl_2$ solution and left in it to harden. The beads were then removed from the $CaCl_2$ solution and were directly activated for the covalent immobilization of PGA. First, the beads were soaked in a PEI solution for a specified period of time. The excess PEI was then removed by the thorough washing of the beads with distilled water. Afterward, the beads were soaked in a GA solution, washed with distilled water, and directly used to immobilize PGA. It is worth mentioning that the color of the Alg–Car beads changed from white to orange brown during the activation process. This orange brown color was due to the formation of the Schiff's base (—N=CH—) between the PEI's amino groups and the GA's aldehyde groups. It is noteworthy that the presence of the aldehyde group required for PGA immobilization was proven through the FTIR technique.

**Morphological Analysis.** For comparative purposes, three different sets of beads (Alg, Car, and Alg–Car) were prepared and activated as mentioned in in the previous section. Afterward, the activated gel beads were either directly lyophilized or lyophilized after they were soaked in 0.05$M$ citrate–phosphate buffer for 18 h. The surface morphology of the lyophilized beads was examined with scanning electron microscopy (SEM; S-590, Hitachi) to investigate the porosity of the gel beads. The size of the lyophilized beads was also examined with optical and polarized microscope.

**Immobilization of PGA.** About 1 g of the activated Alg–Car gel beads was soaked in 8 mL of PGA solution. This mixture was shacked, using a roller stirrer, at room temperature for 0.5 or 18 h. Afterward, the gel beads were filtered off, washed thoroughly with distilled water, and directly assayed for PGA activity.

**FTIR Spectroscopy.** The infrared spectra of all of the formulations were recorded with FTIR spectroscopy (FTIR-4100, Jasco). FTIR spectra were taken in the wavelength region from 4000 to 400 $cm^{-1}$ at ambient temperature. Formulas 1 (Alg–Car), 2 (Alg–Car/PEI), 3 (Alg–Car/PEI/GA), and 4 (Alg–Car/PEI/GA/PGA) were examined for the presence of the new functionalities with FTIR spectroscopy.

**Elemental Analysis.** The elemental composition of the three samples (Alg–Car, Alg–Car/PEI, and Alg–Car/PEI/GA) was determined by an elemental analyzer (Vario El, Elementar, Germany).

**Determination of the PGA Activity.** The PGA activity was assayed with the $p$-dimethyl amino benzaldehyde method.[19] The method comprised two stocks: A and B. Stock A was formed by the mixture of 0.125 g of $p$-dimethyl amino benzaldehyde with 14.2 mL of methanol, 20 mL of 20% v/v acetic acid, and 2.5 mL of 1$N$ NaOH; then, the volume was completed to 100 mL with distilled water. Stock B consisted of a 50 m$M$ penicillin G (substrate) solution in 0.1$M$ phosphate buffer at pH 7. Equal amounts of both stocks were mixed to produce the assay solution. We assayed iPGA by soaking 0.25 g of the enzyme-loaded beads in 4.5 mL of the assay solution. The reaction was left to proceed for 5 min. The beads were then removed, and the absorbance of the supernatant was measured at 415 nm. On the other hand, we assayed the free PGA by mixing 0.5 mL of the enzyme solution with 4.5 mL of the assay solution. The reaction was left to proceed for 5 min. Then, the absorbance of the solution was measured at 415 nm. One enzyme unit (U) was defined as the amount of enzyme that catalyzed the formation of 1 $\mu$mol of 6-APA/min under the assay conditions.

**Assessment of the Covalent Bond Formed between PGA and the Activated Alg–Car Beads.** The PGA-loaded beads were incubated with 1$M$ NaCl at 25°C for 1 h. After that, the enzyme activity was analyzed in the supernatant to check if any unbound enzyme had leaked.[20]

**Operational Stability (Reusability).** The reusability of PGA covalently immobilized onto the activated Alg–Car beads was

**Table I.** Factors Tested by the PBD

| Factor's name | Code | Group | Low level (−) | High level (+) | Unit |
|---|---|---|---|---|---|
| Nozzle diameter | A | I | 300 | 700 | μm |
| Car % | B | | 1 | 2 | % w/v |
| Alg % | C | | 1 | 2 | % w/v |
| $CaCl_2$ concentration | D | | 1 | 5 | % w/v |
| $CaCl_2$ soaking time | E | | 1 | 6 | h |
| PEI concentration | F | II | 1 | 4 | % v/v |
| PEI pH | G | | 6.62 | 8.42 | pH |
| PEI soaking time | H | | 10 | 120 | min |
| GA concentration | J | | 0.5 | 5 | % v/v |
| GA pH | K | | 4 | 6.5 | pH |
| GA temperature | L | | 2.5 | 29 | °C |
| GA soaking time | M | | 0.5 | 3 | h |
| *N* | N | III | 4.7 | 7 | |
| Enzyme buffer molarity | O | | 0.01 | 0.05 | *M* |
| Enzyme concentration | P | | 523 | 2124 | U/g of beads |
| Enzyme soaking time | Q | | 0.5 | 18 | h |
| Dummy 1 | R | — | − | + | |
| Dummy 2 | S | — | − | + | |
| Dummy 3 | T | —h; | − | + | |

tested. The gel beads were assayed as mentioned earlier. Afterward, the same gel beads were washed thoroughly with distilled water and reassayed. This procedure was repeated 14 times, and the initial activity was considered to be 100%. The relative activity was expressed as a percentage of the starting operational activity.

**PBD.** The effects of 16 factors on the amount of iPGA were evaluated with PBD. These factors were grouped into three groups (Table I). Group I included factors affecting the Alg–Car gel bead formation. Group II factors were concerned with the bead activation, whereas group III factors dealt with the PGA immobilization process. Each of the tested factors was studied at two levels, namely, a high level (denoted by +) and a low level (denoted by −), as listed in Table I. We tested these 16 factors and 3 dummy (unassigned) variables conducting 20 runs ($k+1$, where $k$ is the number of factors; Table II). All of the experiments were conducted in triplicate, and the average value of the iPGA/g of beads was used for statistical analysis. The results of the experimental designs were analyzed and interpreted with Design Expert statistical software (Stat-Ease 8.0.7.1, trial version).

## RESULTS AND DISCUSSION

### FTIR Spectroscopy

The FTIR bands of the Alg–Car, Alg–Car/PEI, Alg–Car/PEI/GA, and Alg–Car/PEI/GA/PGA are shown in Figure 4. With regard to the Alg–Car formulation, Alg showed its characteristic functional group (—$COO^-$) as a band at 1600 $cm^{-1}$ and a band at 1430 $cm^{-1}$. Moreover, the sulfate group (—$OSO_3^-$) of the Car appeared as a band at 1050 $cm^{-1}$. With regard to the Alg–Car/PEI formulation, the PEI was expected to reveal two characteristic bands for the amine groups. These two bands were a stretching vibration at 3400–3300 $cm^{-1}$ and a bending vibration at 1650–1590 $cm^{-1}$. Both bands were overlapped with the broadband of the —OH and the —COOH groups at 3300–3600 and 1590–1630 $cm^{-1}$, respectively. It is worth mentioning that the sulfate band of the Car was perturbed from 1050 to 1030 $cm^{-1}$ after the formation of the gel complex with PEI. This could have been due to the strong polyelectrolyte interaction between the positively charged —$NH_3^+$ of the PEI and the negatively charged —$OSO_3^-$ of Car.

As for the Alg–Car/PEI/GA formulation, we postulated that the aldehyde groups present at one end of the GA molecules reacted with the free amine moieties of the PEI to form a Schiff's base. This Schiff's base (—C=N—) appeared as a shoulder at 1700 $cm^{-1}$. Moreover, we also assumed that the unreacted GA side created free aldehyde groups for the covalent immobilization of PGA. These aldehyde groups appeared as a slight indentation at 1320 $cm^{-1}$. This slight indentation disappeared in the Alg–Car/PEI/GA/PGA formulation, and this indicated that the free GA aldehyde groups were used to covalently immobilize PGA through the formation of the Schiff's base with the PGA amino groups. This Schiff's base appeared as a shoulder at 1700 $cm^{-1}$. The Schiff's base formed between the aldehyde groups of GA and the amino groups of PGA was also noticed by Bahman et al.[21] and Ren et al.[22] at 1648 and 1639 $cm^{-1}$, respectively.

### Elemental Analysis

The elemental analysis data for the Alg–Car gel beads was 19.47% (C), 5.4% (H), 0% (N), 1.32% (S), and 73.81% (O). Meanwhile, the elemental composition of the Alg–Car/PEI beads was 30.64% (C), 7.2% (H), 5.636% (N), 1.45% (S), and

**Table II.** PBD Matrix for the Screening of Variables Influencing the Amount of iPGA

| Run | A | B | C | D | E | F | G | H | J | K | L | M | N | O | P | Q | R | S | T | iPGA (U/g of beads) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | + | − | + | + | − | − | + | + | + | + | − | + | − | + | − | − | − | − | 76.91 |
| 2 | − | − | − | + | + | − | + | + | − | − | + | + | + | + | − | + | − | + | − | 150.91 |
| 3 | − | + | − | − | − | − | + | + | − | + | + | − | − | + | + | + | + | − | + | 215.26 |
| 4 | − | + | − | + | − | − | − | − | + | + | − | + | + | − | − | + | + | + | + | 11.90 |
| 5 | + | + | + | − | + | − | + | − | − | − | − | + | + | − | + | + | − | − | + | 206.17 |
| 6 | − | − | + | + | − | + | + | − | − | + | + | + | + | − | + | − | + | − | − | 96.69 |
| 7 | + | + | − | + | − | + | − | − | − | − | + | + | − | + | + | − | − | + | + | 108.87 |
| 8 | + | − | − | + | + | + | + | − | + | − | + | − | − | − | − | + | + | − | + | 290.08 |
| 9 | + | − | + | − | − | − | − | + | + | − | + | + | − | − | + | + | + | + | − | 354.16 |
| 10 | − | + | + | − | − | + | + | + | + | − | + | − | + | − | − | − | − | + | + | 74.88 |
| 11 | − | − | + | + | + | + | − | + | − | + | − | − | − | − | + | + | − | + | + | 220.11 |
| 12 | + | − | + | − | + | − | − | − | − | + | + | − | + | + | − | − | + | + | + | 64.90 |
| 13 | − | + | + | − | + | + | − | − | + | + | + | + | − | + | − | + | − | − | − | 230.47 |
| 14 | − | + | + | + | + | − | + | − | + | − | − | − | − | + | + | − | + | + | − | 298.29 |
| 15 | − | − | − | − | + | + | − | + | + | − | − | + | + | + | + | − | + | − | + | 163.47 |
| 16 | + | + | − | − | + | + | + | + | − | + | − | + | − | − | − | − | + | + | − | 106.12 |
| 17 | + | − | + | + | − | − | + | + | + | + | − | + | − | + | − | − | − | − | + | 117.02 |
| 18 | + | + | + | + | − | + | − | + | − | − | − | − | + | + | − | + | + | − | − | 76.20 |
| 19 | + | − | − | − | − | + | + | − | + | + | − | − | + | + | + | + | − | + | − | 302.15 |
| 20 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | 191.74 |

55.074% (O). The absence of nitrogen in the Alg–Car gel beads and its presence in the PEI-treated beads proved the incorporation of PEI into the Alg–Car gel. Elnashar et al.[13] also proved the incorporation of PEI into Car gel with the elemental analysis, where nitrogen was absent in case of the Car gel, but it was present at a concentration of 15.059% in the PEI-treated Car gel.

The elemental analysis data of the Alg–Car/PEI/GA beads were 40.35% (C), 7.1% (H), 4.322% (N), 1.2% (S), and 47.028% (O). The rise in the carbon percentage from 30.64% in the case of the Alg–Car/PEI beads to 40.35% in the case of the Alg–Car/PEI/GA beads reflected the incorporation of GA ($C_5H_8O_2$) in the gel structure.

### Assessment of the Covalent Bond Formed between PGA and the Activated Alg–Car Beads

PGA was covalently immobilized onto the activated Alg–Car gel beads through the formation of a Schiff's base between its amino groups and the free aldehyde groups of the GA molecules (Figure 3). The covalent immobilization of PGA was proven by the lack of enzyme release from the gel beads that were incubated in 1*M* NaCl. It was noteworthy that PGA has been shown by many authors to be covalently immobilized onto GA-activated Car,[13] Alg,[14] glutamate-pillared-layered double hydroxides,[22] composite PEI/$SiO_2$,[23] and grafted nylon membranes.[24]

### Reusability

The main advantage of covalent immobilization is the formation of strong covalent bonds between the enzyme and the carrier. Accordingly, the enzyme's leakage is minimized, and its reusability is enhanced. The reusability of PGA covalently immobilized onto the activated Alg–Car beads was evaluated (Figure 5). The iPGA retained 84% of its initial activity after 14 cycles. This result was somewhat consistent with those reported in the literature, whereas PGA covalently immobilized onto the

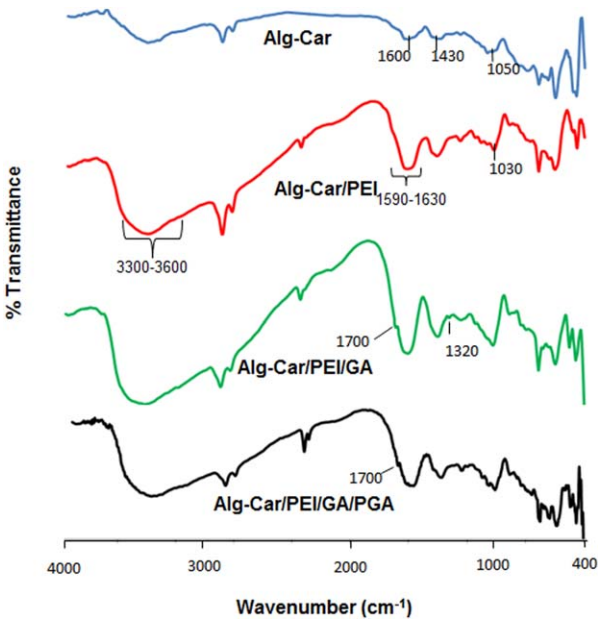


**Figure 4.** FTIR bands of Alg–Car, Alg–Car/PEI, Alg–Car/PEI/GA, and Alg–Car/PEI/GA/PGA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

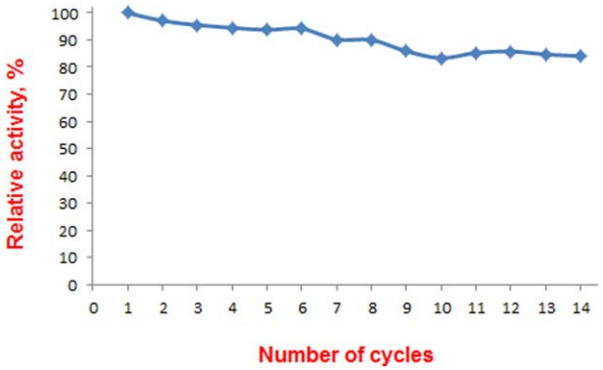


**Figure 5.** Reusability of iPGA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

composite PEI/$SiO_2$ support retained 87.5% of its initial activity after 15 runs.[23] PGA covalently immobilized onto grafted Alg beads maintained almost 80% of its initial activity after it was used 15 times.[14] It was noteworthy that the decrease in the enzyme activity by the 14th run might have been due to the inactivation of the enzyme due to continuous use.[25]

### Evaluation of the Factors Affecting iPGA

The results of the screening Plackett–Burman experiments (Table II) exhibited a wide variation of 30-fold in the amount of iPGA from 11.9 to 354.16 U/g of beads. This variation reflected the importance of optimizing the immobilization process to attain a higher enzyme immobilization.

One of the most important graphical representations offered by Design Expert to assess the significance of the tested factors is the Pareto chart (Figure 6), in which, the *t* values (The *t*-value of all the factors is the value above which the factor(s) is significant and below it, the varriable(s) is insignificant.) of the effects of all of the screened factors are plotted and ranked in descending order.

The Pareto chart (Figure 6) showed that out of the 16 tested factors, only 3 factors (Table I) belonging to group III were, in fact, significant. These three factors exceeded the *t* value limit, and this indicated their significance at a probability level of less than 0.05. On the other hand, the remaining 13 factors were proven to be insignificant. Accordingly, they did not need to be further optimized, and it was enough to follow Design Expert's recommendations concerning the use of each factor in its high (+) or low (−) level, depending on the kind of effect it offered. Factors with positive effects increased the amount of iPGA as they increased. Consequently, it would be better to use them in their high level (+). On the contrary, factors with a negative effect caused the amount of iPGA to decrease as they increased and so should be used in their low (−) level.

It was noteworthy that the insignificant factors included all of the factors dealing with the formation and activation of the gel beads. Meanwhile, all of the significant factors were from the enzyme immobilization conditions (*N*, *Q*, and *P*), which were characteristic of each enzyme. Hence, for future work on any other enzyme, the optimization of only these three significant and enzyme-distinctive factors is recommended.

The analysis of variance (ANOVA; Table III) and the model equation [eq. (1)] for the amount of iPGA were calculated with only the three significant factors taken into account. Meanwhile, all of the other 13 insignificant factors were used in the calculation of the residual, which was used to estimate the experimental error. ANOVA provided evidence that the model, *N*, *P*, and *Q* were significant at a probability level of less than 0.05:

$$\text{Amount of iPGA (U/g)} = 167.82 - 45.40N + 36.39P + 37.93Q \quad (1)$$

**Group I Factors.** The group I factors that exhibited positive effects (Figure 6 and Table I) on the amount of iPGA were the nozzle diameter, the Alg percentage, and the $CaCl_2$ soaking time with the gel beads. On the other hand, the Car percentage and the $CaCl_2$ concentration offered negative effects. Thus, the recommended levels at which these factors should be used were

- Nozzle with a 700-$\mu$m diameter.
- 1% w/v Car.
- 2% w/v Alg.

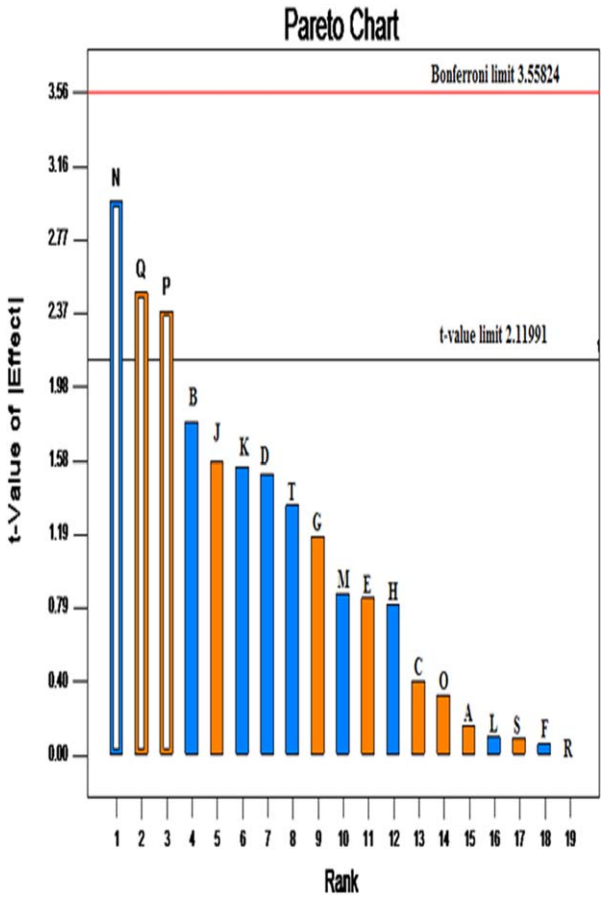


**Figure 6.** Pareto chart showing the *t* values of the effects of all of the screened factors. For factor identification (A–T), see Table I. In the online figure, orange indicates positive effects, and blue indicates negative effects. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Table III.** ANOVA Results of the Amount of iPGA from the PBD

| | | | | | *p* value | |
|---|---|---|---|---|---|---|
| Source | Sum of squares | *df* | Mean square | *F* | *p* > *F* | *p* |
| Model | 96,474.61 | 3 | 32,158.20 | 6.89 | 0.0034 | Significant |
| *N* | 41,217.19 | 1 | 41,217.19 | 8.84 | 0.0090 | Significant |
| *P* | 26,490.37 | 1 | 26,490.37 | 5.68 | 0.0299 | Significant |
| *Q* | 28,767.05 | 1 | 28,767.05 | 6.17 | 0.0245 | Significant |
| Residual | 74,641.58 | 16 | 4665.10 | | | |
| Correlation total | 171,116.19 | 19 | | | | |

*df*, degrees of freedom.

- 1% w/v $CaCl_2$.
- 6 h of soaking time of $CaCl_2$ with the gel beads.

The use of such a low concentration of $CaCl_2$ (1%) resulted in the formation of a loose ionic network between the calcium ions (cations) and the anions of both Alg (—$COO^-$) and Car (—$OSO_3^-$). This loose ionic network gave rise to rough and porous gel beads that allowed for the immobilization of a large amount of PGA on their surface and in their pores. Moreover, when this low concentration of $CaCl_2$ was used, a relatively long period was required to form an ionic network, even a loose one. Hence, the 6-h soaking period was preferred to the 1-h period.

To account for the selection of 2% w/v Alg/1% w/v Car as the optimum bead composition formula, SEM images showing the surface morphologies of the lyophilized 1.5% w/v Alg beads, 2.5% w/v Car beads, and 2% w/v Alg/1% w/v Car beads before and after they were soaked with the working citrate–phosphate buffer were investigated (Figure 7).

Beads made with only 1.5% w/v Alg were porous and offered a rough surface [Figure 7(a)]. When these Alg beads were soaked with working citrate–phosphate buffer, they were greatly disrupted. Not only did their surface become much rougher and corrugated, but the disruption was so great that the beads collapsed and shrank in size. This was obvious from the decreased size acquired by the buffer-treated Alg beads as compared to the untreated beads (Figure 8). The collapsing of the citrate–phosphate-treated Alg beads was also evident by the appearance of collapsed shrunken pores [Figure 7(b)]. This could be attributed to the fact that Alg gel actually forms due to the ionic network between cations (here $Ca^{2+}$) and anions of Alg (—$COO^-$). The citrate and phosphate ions of the buffer bonded to these calcium ions, and this led to the disruption of the Alg gel structure.[6]

On the other hand, the formation of the Car gel involved the conformational transition of its molecules and was hardly affected by the $Ca^{2+}$ chelation process.[9,10] Consequently, the relatively smooth surface [Figure 7(c)] of the Car beads acquired only few corrugations when the beads were soaked with the working citrate–phosphate buffer [Figure 7(d)]. This slightly corrugated surface was not big enough to enable the immobilization of a large amount of the enzyme.

The Alg–Car beads offered a porous and corrugated surface [Figure 7(e)]. These surface corrugations were significantly increased when the beads were used with the citrate–phosphate buffer [Figure 7(f)] because of the high Alg content (2% w/v). Meanwhile, the integrity of the Alg–Car beads was not affected because of the presence of Car. Car, being hardly affected by the $Ca^{2+}$ chelation process, maintained the integrity of the beads, even at the low concentration of just 1% w/v. Hence, 2% w/v Alg and 1% w/v Car produced porous highly corrugated beads that offered a large surface area to immobilize the enzyme. Nevertheless, the beads were mechanically stable enough to allow their use in common working buffers. It is worth mentioning that upon when Alg was used alone at a 1.5% w/v concentration[15,26] or at a 2.5% w/v concentration,[14] the beads required a higher concentration of $CaCl_2$ (2%, w/v). As the only gel-forming mechanism available was the formation of the ionic network between the carboxylate groups of the Alg and the $Ca^{2+}$ ions.

**Group II Factors.** Design Expert's recommendations (Figure 6 and Table I) concerning the group II factors were

- 1% v/v PEI.
- PEI solution with a pH of 8.42.
- A 10-min PEI soaking time.
- 5% v/v GA.
- GA solution with a pH of 4.
- A 30-min GA soaking time.
- A 2.5°C GA soaking temperature.

The use of a 1% v/v PEI solution for only 10 min during the first phase of the activation process implied that the introduction of lots of functional groups to the gel beads was not favored. PGA is a bulky molecule (≈89 KDa).[27] The binding of too many of these bulky PGA molecules to abundant, closely placed functional groups resulted in steric hindrance. This steric hindrance decreased the enzyme's activity substantially. The need for a lower number of functional groups could also have been the reason that Design Expert recommended the use of a PEI solution with a pH of 8.42 rather than 6.62. At pH 8.42, PEI would have had fewer protonated amines than at pH 6.62. These protonated amines, whose number increased as the PEI pH decreased,[13] were, in fact, responsible for the ionic interaction between PEI and the negatively charged Alg–Car gel beads. Accordingly, fewer protonated amino groups would have eventually led to fewer functional groups. Danial et al.[15] employed a

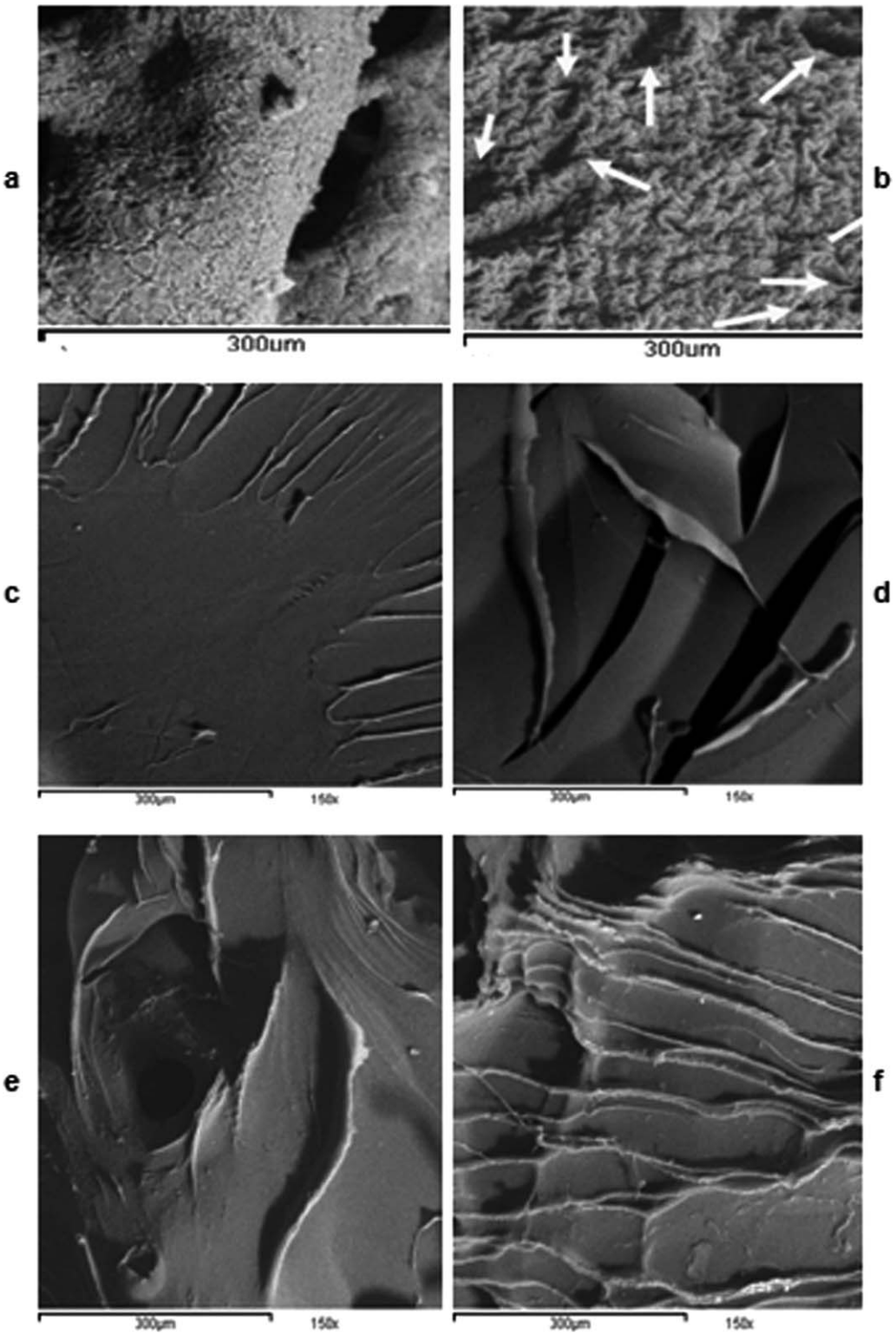


**Figure 7.** SEM photographs of the lyophilized bead surface morphologies: (a) 1.5% w/v Alg beads, (b) Alg beads after soaking in 0.05*M* citrate–phosphate buffer, (c) 2.5% w/v Car beads, (d) Car beads after soaking in 0.05*M* citrate–phosphate buffer, (e) 2% w/v Alg/1% w/v Car beads, and (f) Alg/Car beads after soaking in 0.05*M* citrate–phosphate buffer. The arrows show the collapsed pores.

PEI solution with a pH of 8 during the activation of Alg beads that were used to covalently immobilize the bulky (≈80 KDa)[28] inulinase enzyme.

During the second phase of the activation process, it was recommended that the beads be soaked in a 5% v/v GA solution for a short period of just 30 min. It is known that GA crosslinks

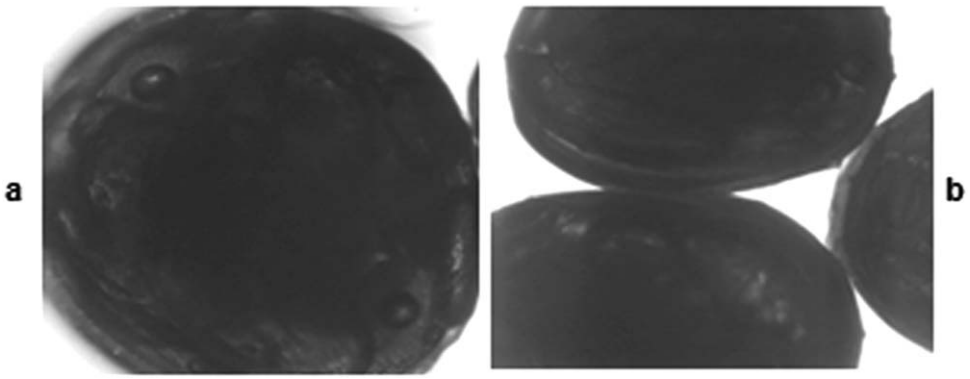


**Figure 8.** Optical and polarized microscopy photographs of the lyophilized beads magnified 4×: (a) 1.5% w/v Alg beads and (b) Alg beads after soaking in 0.05*M* citrate–phosphate.

PEI's free amino groups only.[13] These PEI free amino groups would suffer from protonation upon incubation with a GA solution of a low pH (whether 6.5 or 4), and this would render them unavailable for the reaction with GA. Thus, excess GA (5%) should be used to allow for fast crosslinking between GA and PEI before the protonation of the PEI's amino groups, and a 30-min soaking period would be enough to do so. A 5% v/v GA solution was also used during the immobilization of PGA onto glutamate-pillared-layered double hydroxides. The 5% v/v GA solution was used to crosslink the amino groups of the glutamate ions.[22]

**Group III Factors.** With regard to the group III factors, which included all of the significant factors (Figure 6 and Table I), it was shown that the loading buffer pH exerted a significant negative effect on the amount of iPGA. This was attributed to the isoelectric point (pI) of PGA. The pI values of the PGAs, isolated from different sources, were reported to be between pH 6 and pH 6.8.[23,29,30] Accordingly, at pH 4.7, which was lower than the pI, PGA was present in its cationic form. This cationic form was easily drawn to the vicinity of the anionic Alg–Car polymer by electrostatic attraction forces; this facilitated the covalent immobilization of PGA. On the other hand, PGA existed as an anion at pH 7. The anionic PGA was repelled from the vicinity of the anionic polymer, and this impeded the immobilization process. This situation was reversed when PGA was covalently immobilized onto the positively charged composite PEI/$SiO_2$ support.[23] pH 7.92 was favored to pH 5, as the negatively charged PGA was required to efficiently approach the positively charged carrier.[23]

The increase in *Q* from 0.5 to 18 h caused the amount of iPGA to increase significantly (positive effect; Figure 6 and Table I). Similar findings were reported by Elnashar et al.,[14] the extension of *Q* from 0.5 to 16 h brought about a 29-fold increase in the amount of PGA immobilized onto grafted Alg beads. This relatively long period was actually needed for the gel to reach its maximum swelling. Hence, this allowed more PGA to penetrate into the gel's pores and be immobilized inside the gel and on its surface.[14] Furthermore, PGA required a long immobilization time to allow its diffusion into the pores of the gel because of its large molecular weight (≈89 KDa).[27]

The PGA concentration was also proven to have a significant positive effect on the amount of iPGA (Figure 6 and Table I). It is worth mentioning that the positive effect exerted by the loading buffer molarity on iPGA was the only insignificant effect in group III. On the contrary, when PGA was immobilized onto the grafted Alg beads,[14] the amount iPGA exhibited a 4.7-fold increase with the increase in the buffer molarity from 5 to 50 m*M*.

## CONCLUSIONS

Within the factors' ranges tested in PBD, only three factors significantly ($p < 0.05$) affected the iPGA. *Q* with the gel beads and *P* offered significant positive effects, whereas *N* exerted a significantly negative effect. These three significant factors were characteristic of the enzyme being immobilized and not of the gel beads. Consequently, it is recommended that researchers only investigate these three significant enzyme distinctive factors when optimizing the immobilization of any enzyme onto Alg–Car beads. Moreover, we concluded that the combination of Alg and Car was favorable over the use of either polymer alone. The Alg–Car beads offered a large porous surface that enabled the beads to immobilize a large amount of PGA (up to 354.16 U/g of beads). Nevertheless, the beads maintained their integrity because of their Car content. The Alg–Car gel beads were recommended to be hardened by soaking in a 1% $CaCl_2$ solution for 6 h, whereas the beads' activation needed to be accomplished through soaking in a 1% PEI solution (pH 8.42) for 10 min followed by their immersion in a 5% GA solution (pH 4) for 30 min.

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