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# Optimization of pectinase immobilization on grafted alginate-agar gel beads by $2^4$ full factorial CCD and thermodynamic profiling for evaluating of operational covalent immobilization

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ABSTRACT

Pectinase produced by a honey derived from the fungus *Aspergillus awamori* KX943614 was covalently immobilized onto gel beads made of alginate and agar. Polyethyleneimine, glutaraldehyde, loading time and enzyme's units were optimized by $2^4$ full factorial central composite design (CCD). The immobilization process increased the optimal working pH for the free pectinase from 5 to a broader range of pH 4.5–5.5 and the optimum operational temperature from 55 °C to a higher temperature, of 60 °C, which is favored to reduce the enzyme's microbial contamination. The thermodynamics studies showed a thermal stability enhancement against high temperature for the immobilized formula. Moreover, an increase in half-lives and D-values was achieved. The thermodynamic studies proved that immobilization of pectinase made a remarkable increase in enthalpy and free energy because of enzyme stability enhancement. The reusability test revealed that 60% of pectinase's original activity was retained after 8 successive cycles. This gel formula may be convenient for immobilization of other industrial enzymes.



## 1. Introduction

Enzymes, the eco-friendly catalysts, are used in many industrial manufacturing processes and are attracting a growing number of researchers to investigate them [1,2]. The difficulty of recovering enzymes and their products which limited their reusability and their low operational stability are considered the main reasons for the unaffordable and limited uses of enzymes in different industrial applications.

Enzyme immobilization offers a solution to overcome these shortcomings. One of the most effective techniques is the immobilization onto a solid carrier which stabilizes the enzymes under different operational conditions. This immobilization will also allow for reusability of enzymes in multiple cycles and for easy recovery [3].

Among the different immobilization methods, the formation of covalent bonds on the surface of solid supports can be promising approach in stabilizing enzymes and preventing their leaching out and sequential leakage, the latter being the cause of enzymes' loss and poor operational stability [4–6]. However, covalent bonding can enhance the catalytic activity by stabilization of the active conformation, or affect the active center of enzymes and decrease its enzymatic activity and efficiency [7,8] Therefore, development of novel and more convenient methods for immobilization of enzymes is still critically needed.

Pectinolytic enzymes are extensively used in food processing and juice manufacturing. They are employed in retting and degumming of fiber crops [9], in plant pathology [10] and in protoplast fusion technology [11]. Furthermore, they are extensively used in the pretreatment of wastewater from fruit juice industries, in maceration, liquefaction and extraction of vegetable tissues [12] and in oil extraction [13]. Due to the macerating effect of pectinase, it is widely used in paper and pulp industry [10]. Recently, technologies for the improvement of juice quality have attracted a considerable attention [14]. One of the major hurdles in fruit juice processing is the pressing process as the freshly pressed fruit juices have a cloudy and turbid appearance due to the colloidal dispersion of pectin (0.9–1.5%) present in the forms of disrupted cell materials and cell wall of the fruit [15]. Pectinase is mostly used to improve the fruit juice quality and to decrease cloudiness [16]. Immobilized pectinase is a more convenient formula for industrial uses especially in apple juice clarification due to its operational stability [17].

Response surface method (RSM) is a collection of mathematical and statistical techniques used for designing experiments, building models

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and searching for the optimum conditions. RSM is a convenient method for developing the region that fits the operation specification [18].

In this work we report a non-hazardous, eco-friendly and efficient strategy for immobilization of pectinase which can be compatible with the food industries. Pectinase immobilization process on alginate-agar gel generated beads which were optimized by $2^4$ full-factorial central composite design. Thermodynamics studies of both free and immobilized enzyme were conducted to evaluate the efficiency of the generated polymer formula.

## 2. Materials and methods

All experiments were carried out in triplicate and data are means ± SD (n = 3).

### 2.1. Chemicals

Sodium alginate (Alg) was obtained from Fluka. Agar was purchased from Sigma, Polyethyleneimine (PEI) (MW: 423), Cat # 468533, was obtained from Aldrich and, Pectin was obtained from Sigma. Crude pectinase was prepared in our laboratory. Other chemicals were of Analar or equivalent quality. Innotech Encapsulator, model IE-50, was purchased from Innotech Encapsulator in Switzerland.

### 2.2. Microorganism and its maintenance media

The honey isolate *Aspergillus awamori* KX943614, which was used in this study as a source for pectinase enzyme, was isolated and identified in our laboratory [19].The fungal isolate was grown on potato dextrose agar medium (PDA) at 27 °C and preserved at −80 °C in 50% (v/v) glycerol. Safety of *Aspergillus awamori* KX943614 was estimated by determining its ability to produce aflatoxins and ochratoxin using HPLC according to Ben Fredj et al. [20].

### 2.3. Cultivation conditions and enzyme preparation

Five grams of oranges peel placed in 250 ml Erlenmeyer flasks were moistened with 10 ml of the following medium (g/L): 2.0; $NaNO_3$, 1.0; $K_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$ and 0.5 KCl at pH 6.0. Then, they were autoclaved for 20 min at 121 °C and cooled to room temperature before inoculation. Each flask received 2 ml inoculum containing ($10^6$ spores/ml) of 5 days old culture which was prepared by inoculating a fungal slant in 50 ml of Czapek's Dox broth medium. The inoculated flasks were incubated for 5 days at 30 °C. After this period, 100 ml of distilled water were added for enzyme extraction purpose and left in a rotary shaker at 200 rpm for 30 min. The spore suspension was filtered through a cloth followed by centrifugation for 15 min at 5000 rpm and 4 °C. The supernatant was used as the crude enzyme. Crude pectinase was further partially purified using 50–70% ethanol and the precipitated enzyme was used for immobilization during the study.

### 2.4. Determination of enzyme activity and protein content

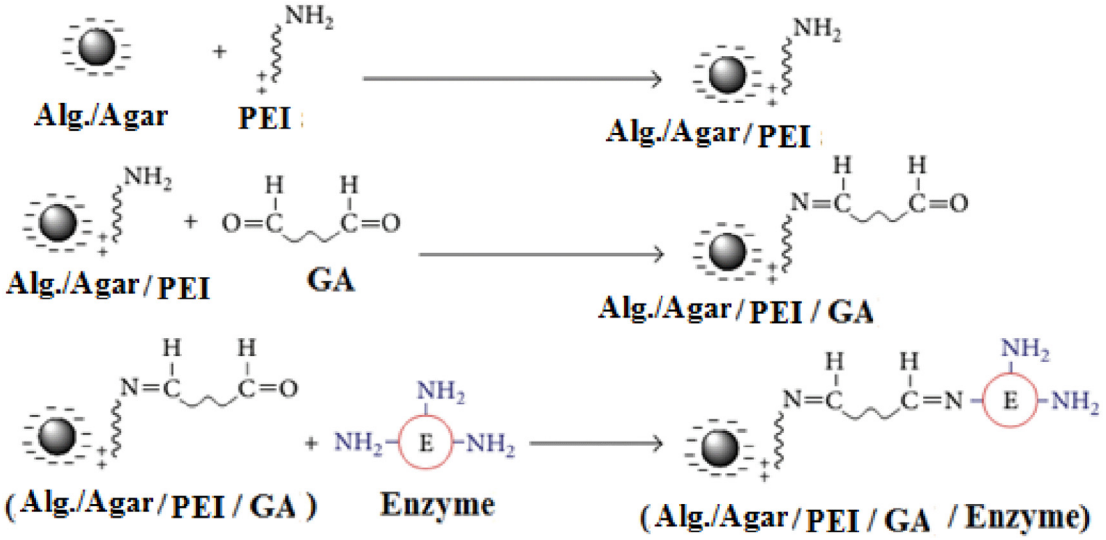
Pectinase activity was determined using Nelson's method [21]. A volume of 0.05 ml of the partially purified pectinase or (1 g immobilized enzyme) was added to 0.2 ml of pectin (1% (w/v) in 0.05 M acetate buffer, pH 5) and the reaction mixture was incubated at 50 °C for 10 min. Somogyi's copper reagent was added and the color absorbance was read at 520 nm. One unit of pectinase (U) was defined as the amount of enzyme that releases 1 μmol of galacturonic acid per min under the assay conditions. Soluble proteins were determined according to [22] and expressed as mg/ml.

### 2.5. Preparation and grafting of alginate/agar beads

For gel beads formation, sodium alginate (alg) and agar were mixed together in a ratio of 1:1 to reach a final concentration of 4% (w/v). The alg-agar solution was dropped through a nozzle of 300 μm using the Innotech Encapsulator in a hardening solution containing 2% (w/v) $CaCl_2$ ($Ca^{2+}$). The generated beads were soaked for 3 h in PEI solution. The un-reacted PEI was then removed from the beads by successive washing with distilled water. After washing, aminated gel beads were soaked in glutaraldehyde (GA) solution for 3 h to incorporate the new functionality, aldehyde group. Then, the gel beads were washed with distilled water to remove un-reacted GA [23]. The solutions of both PEI and GA were prepared according to the appropriate concentrations. After that, the activated gel beads were ready for the step of covalent immobilization of enzyme as shown in Scheme 1.

### 2.6. Enzyme immobilization

One milliliter of the partially purified pectinase was mixed with 1 g of the prepared grafted alginate carrier. The mixture was left for the desired time at 4 °C. At the end of incubation period gel beads were washed twice with distilled water and used for pectinase assay.




**Scheme 1.** Schematic representation of alg-agar gel activation and enzyme immobilization.