Trop J Nat Prod Res, May 2025, 9(5):2341 - 2349 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Characterization of Immobilized *Endo*-Polygalacturonase PGC-AN64 from *Aspergillus niger* HO32 with Potential Biotechnological Interest

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Published online *****

ABSTRACT

This study focuses on the characterization and application of an immobilized purified *endo*-polygalacturonase PGC-AN64 from *Aspergillus niger* HO32 using sodium alginate (SA), and its combinations with guar gum (SA-Gu) or chitosan (SA-Ch) as supports. Immobilization efficiencies were 75%, 83%, and 77% for PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu, respectively. Immobilized enzymes showed enhanced thermostability and pH stability. At 80°C, the half-lives reached 8, 11, and 16 h, and residual activity at pH 5 after extended incubation (12–24 h) was maintained at approximately 56–58%, depending on the matrix. Reusability tests showed activity retention of ~50% for PGC-AN64-SA after 4 cycles, and ~64% and 51% for PGC-AN64-SA-Ch and PGC-AN64-SA-Gu, respectively, after 6 cycles. Orange juice clarification efficiency was reflected by transmittance values of 85.20±1.68% for free PGC-AN64, and ~84% for all immobilized forms. XRD, FTIR-ATR, and FE-SEM analyses confirmed structural changes upon immobilization. In addition, orange juice clarification resulted in a decrease in total soluble solids and color modifications: reduced a* values, and increased L* and b* values. These findings highlight that immobilizing PGC-AN64 on SA-based matrices improves enzyme stability, operational reusability, and retains orange juice clarification performance. The approach offers an efficient and safe enzymatic solution for agro-industrial applications, particularly in juice processing.

Keywords: *Aspergillus niger*; *Endo*-polygalacturonase; Immobilization; Reusability; Fruit juice clarification process.

Introduction

Polygalacturonases specifically hydrolyze the glycosidic bonds of pectic substrates. Due to their biological functions, these enzymes have found application in the food sector.[1,2] Polygalacturonases are divided into two main categories: *exo*-polygalacturonases and *endo*-polygalacturonases. *Exo*-polygalacturonases break down the terminal bonds of pectic molecules, gradually shortening and reducing the chain length. In contrast, *endo*-polygalacturonases randomly target all the bonds within the pectin molecules at random points. [3,4]

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Citation: Bentouhami NE, Mechri S, Moumnassi S, Idrissi Yahyaoui M, Bekkouch A, Abouloifa H, Ghabbour N, Bellaouchi R, Abousalham A, Saalaoui E, Firdaous L, Le Roes-Hill M, Jaouadi B, Asehraou A. Characterization of Immobilized *Endo*-Polygalacturonase PGC-AN64 from *Aspergillus niger* HO32 with Potential Biotechnological Interest. Trop J Nat Prod Res. 2025; 9(5): 2341 – 2349 https://doi.org/10.26538/tjnpr/v9i5.64

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Endo-polygalacturonase, in particular, is widely utilized in various industries due to its ability to maintain stability across diverse physicochemical conditions, Polygalacturonase from fungal sources can be prepared through solid-state or submerged fermentation techniques.[5,6]. generally, fungi are responsible for 90% of industrial enzyme production, mainly through submerged fermentation. This method not only aids in enzyme recovery but also provides economic benefits.[7,8] Notably, the organism most favored for the synthesis of commercial polygalacturonases, is *Aspergillus niger*, which is generally regarded as safe (GRAS).[9-12] Agricultural by-products such as banana peel, orange peel, sugarcane bagasse, and apple residue can be used as a substrate for the production of pectinolytic enzymes such as polygalacturonase.[13-17] Using orange peel as a substrate has increased pectinolytic enzyme production when applied in submerged fermentation.[7] Pectinolytic enzymes produced under submerged fermentation conditions were then subsequently applied in several biotechnological applications.[18-20]

Endo-polygalacturonases are frequently used for the clarification of beverages such as fruit juices.[21] Clarification is a key process in the juice industry, which involves breaking down pectin in juices through eco-friendly methods. This reduces juice viscosity and turbidity, enhancing the product's marketability, taste, texture, color, and overall

appearance.[22,23] In this scenario, the application of free enzymes faces significant drawbacks, such as limited reusability and low operational stability.[24] They are vulnerable to external influences and can be easily inactivated and denatured, resulting in poor thermostability, solvent tolerance, and reusability.[25,26] Immobilizing enzymes allows for efficient recovery and reusability, significantly boosting their stability across various operational conditions.[7,27,28] Various methods have been evaluated for enzyme immobilization. These include, but are not limited to physical adsorption, to microspheres of magnetic cornstarch or chitosan-tethered silica, as well as covalent immobilization on sodium alginate, magnetite nanoparticles, agar–agar, or chitosan-tethered silica [29,30,31]. Immobilizing enzymes brings numerous benefits, significantly improving the enzyme's storage stability, reusability, and heat stability. [29,32-34] However, problems frequently associated with enzyme immobilization include resistance to mass transfer, protein leaching, and enzyme denaturation.[35]

In the search for a novel thermoactive and thermostable polygalacturonase from a fungal source, suitable for juice clarification, a new *endo*-polygalacturonase (PGC-AN64) produced by *Aspergillus niger* HO32 has recently been purified and characterized.[14] Interestingly, the optimal conditions for PGC-AN64 activity were 70 °C and pH 6.5. The enzyme was stable over a wide pH range between 5-7 and a temperature range of 60-80 °C. More intriguingly, PGC-AN64 demonstrated significant substrate specificity, catalytic efficiency, and higher transmittance percentages during the juice clarification process in contrast to the two PECLYVE enzymes that are available commercially (V and CP).[14] The current study aimed to evaluate the immobilization of PGC-AN64 using sodium alginate, chitosan, and guar gum, the analysis of the immobilized beads by FTIR-ATR, XRD, and FE-SEM, as well as the various biochemical attributes, including pH stability, temperature stability, and the reusability of immobilized PGC-AN64. Furthermore, this study evaluated the biotechnological application of both the free and immobilized PGC-AN64 in orange juice clarification.

Materials and Methods

Chemicals and substrates

Citrus pectin (CP), sodium alginate (SA), chitosan (Ch), guar gum (Gu), galacturonic acid, and calcium chloride ($CaCl_2$) were acquired from Sigma-Aldrich (USA). All chemicals used in this investigation were of analytical quality and were obtained from Merck (Germany) and Sigma-Aldrich (USA).

Biological materials

The *endo*-polygalacturonase PGC-AN64 used in this study was purified from an *Aspergillus niger* HO32 fermentation (on orange fruit by-products), which was recently characterized by the authors.[14]

PGC-AN64 immobilization

The PGC-AN64 was immobilized utilizing the technique outlined by Mechri *et al* (2022).[36] with a few modifications. In brief, for the encapsulation of PGC-AN64, 1% (w/v) sodium alginate (SA) or SA in combination with 1% (w/v) chitosan (SA-Ch) or 1% (w/v) guar gum (SA-Gu) was used. For encapsulation, 300 µL of PGC-AN64 enzyme was homogenized with 700 µL of buffer solution, followed by the addition to 3 mL of 1% (w/v) SA solution previously prepared as detailed elsewhere.[36] Where the SA was combined with another support, 300 µL of the PGC-AN64 enzyme was added to the organic supports, and incubated for 2 h at 4 °C. After adding the SA, the mixture was slowly homogenized for several minutes. The resulting solution was then added dropwise into an ice-cold 2 mM $CaCl_2$ solution (using a syringe to create beads) and was incubated for 1 h at 4 °C, before being transferred to a 2 mM $CaCl_2$ solution. After decantation, the beads were carefully washed with a buffer solution (pH 6.5) to remove non-immobilized enzymes and were stored in the same buffer at 4 °C until further use.

Immobilization efficiency

Immobilization efficiency (%), specific activity (U/mg), and retention activity (%) of the immobilized PGC-AN64 were determined. Immobilization efficiency is a specific indicator of the effectiveness of the immobilization method. To determine this parameter, the amount of PGC-AN64 leached was subtracted from the total amount of PGC-AN64 initially introduced. Immobilization efficiency (IE) was calculated using equation (1).

$$IE\ (\%) = \frac{Protein\ immobilized}{Protein\ loaded} \times 100 \qquad (Eq.1)$$

Retention activity (RA) is a parameter that determines the efficacy of the immobilization method as measured by the relative increase or decrease in enzyme activity. Equation (2) was used to calculate the Retention Activity (RA).

$$RA(\%) = \frac{Specific\ activity\ of\ immobilized\ enzyme}{Specific\ activity\ of\ free\ enzyme} \times 100 \qquad (Eq.2)$$

Polygalacturonase activity assay

The PGC-AN64 activity of free and immobilized forms was determined by the 3,5-dinitrosalicylic acid reagent (DNS) method.[37] The mixture was adjusted at pH 6.5 and 70 °C for 20 min. Galacturonic acid served as the reference for determining the quantity of sugars that were reduced. The experiment was conducted in triplicate.

Protein quantification

The Bradford technique was used to estimate the protein content.[38] Briefly, 160 µL of Bradford reagent was added to 40 µL of sample solution. The mixture was incubated for 15 min at ambient temperature (25±2 °C), and the absorption was recorded at 595 nm utilizing the BioTek Gen5^{TM} microplate reader (Agilent BioTek, Fisher Scientific, USA). Bovine serum albumin (BSA) was used as the standard.

Characterization of immobilized PGC-AN64

Fourier Transform InfraRed spectroscopy (ATR-FTIR)

The immobilized PGC-AN64 was examined using the ATR-FTIR method. A Jasco 4700-ATR spectrophotometer was used to obtain the ATR-FTIR spectra of the materials with a 4 cm^{-1} resolution in the spectral region of 500–4000 cm^{-1}. The measurements were made in triplicate.

X-ray diffraction (XRD)

The phase identification of the immobilized PGC-AN64 was determined by using XRD. For this experiment, the XRD-6000 apparatus (SHIMADZU, Kyoto, Japan) was used with an angle of 10-65°, a scanning speed of 4°/s, and Cu Kα radiation (λ = 0.154 nm).[36]

Field Emission Scanning Electron Microscopy (FE-SEM)

The surface morphology of the different immobilized PGC-AN64 preparations was evaluated by FE-SEM using a HIROX SH 5500P coupled with an ESD detector (BRUKER QUANTAX). The working distance between the sample and objective lenses was set at 6 mm. Using various magnifications, the filament SEM was run at varying currents while keeping the voltage at 5 kV. X-ray energy dispersive spectroscopy was utilized to determine the elemental composition.

Thermal and pH stability of immobilized PGC-AN64

The pH stability of free and immobilized PGC-AN64 forms was determined by the incubation of PGC-AN64 for 24 h at room temperature (25±2 °C) in a buffer solution adjusted to pH 5. The thermal stability was estimated for 24 h at 80°C and pH 6.5. All tests were conducted in triplicate.

Reusability of the immobilized PGC-AN64 beads

The recycling of immobilized PGC-AN64 beads was examined by measuring the sample activity at optimal conditions during each cycle. After each cycle, the PGC-AN64 beads were retrieved and cleaned with demineralized water to eliminate reaction residues, which were then reused in subsequent cycles. The initial activity of the PGC-AN64 beads was set at 100%.

Biotechnological application of immobilized PGC-AN64 in orange juice clarification

Oranges (*Citrus sinensis*) were bought from a supermarket in Oujda, Morocco. After being cleaned with sterile distilled water, they were dried. The juice was extracted manually with a screw juice extractor and subsequently filtered using filter paper. The clarification process involved using the immobilized enzyme (PGC-AN64-SA, PGC-AN64-SA-Ch, or PGC-AN64-SA-Gu), and the outcomes were compared to those of applying free PGC-AN64. All tubes were incubated at 50 °C for 30 min. After the clarification process, the transmittance (T%) was determined at 660 nm using a BioTek Gen5TM microplate reader to assess the level of juice clarity. Additionally, color parameters (L*, a*, and b*) were measured, utilizing the Konica Minolta Chroma Meter CR-410 equipment (Konica Minolta, INC., Tokyo, Japan). The total soluble solids (TSS) were quantified and expressed in Brix (°Bx, g/100g) using a handheld refractometer (Brix Refractometer Hand-Held RHB-80). All experiments were conducted in triplicate. Equation (3) was used to determine the transmittance percentage (T%) of clarified juice.

$$T\ (\%) = Log\left(\frac{Abs}{Abc}\right) \times 100 \qquad (Eq.3)$$

Where: *Abc* and *Abs*, refer to the absorption of control and sample, respectively.

Statistical analysis

All experimental work was conducted in triplicate (n=3). The findings are displayed as mean ±SD (the standard deviation of the mean). GraphPad Prism 8, a computer-based statistical software product, was used to determine the analysis of variance (ANOVA) of the data and to compare treatment means, the Latin Square Design (LSD) at $P < 0.05$ was used.

Results and Discussion

Immobilization efficiency of PGC-AN64

The PGC-AN64 enzyme was immobilized on and within SA, SA-Ch, and SA-Gu matrices. The performance of the immobilized PGC-AN64 was assessed by evaluating the immobilization efficiency (IE), as well as apparent and retention activities (RA) (Table 1). Enzyme immobilization often encounters issues such as mass transfer resistance, protein leaching, and denaturation. The results showed a high percentage of immobilization efficiency with 75, 83, and 77% for PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu, respectively. In addition, the apparent activity was 30.34, 24.47, and 27.75 U/g of beads, and the retention activity was 46, 40, and 19%, respectively, for PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu (Table 1). The results obtained from the immobilization and characterization of the immobilized PGC-AN64 are similar to those obtained by Amin *et al* (2017), which showed a retention activity of 45.89%, and an immobilization efficiency of 71.62% for a polygalacturonase.[35] Therefore, previous studies on polygalacturonase immobilization reported that the retention activities recorded in this study are higher than those found by Rehman *et al* (2013), and lower than those reported by Ramirez *et al* (2013) and Xue *et al* (2021).[45,46,47] The drop in PGC-AN64 activity following immobilization may be attributed to the reduced enzyme concentration on the support matrix.[48] The optimal immobilization efficiency was achieved at 83.34% when SA-Ch was used, demonstrating the effectiveness of immobilization. When alginate is combined with chitosan (cationic polymer), it enhances electrostatic interactions with the enzyme, improving immobilization efficiency and reducing enzyme loss.[41] Similarly, the combination with guar gum, a hydrophilic polymer, improves mechanical properties and water retention, increasing the enzyme's functional stability.[49] These combinations also promote controlled diffusion of the substrate and products through the matrix, allowing for optimal enzymatic activity. Together, these polymeric supports provide an effective solution for pectinase immobilization, with applications in industries such as food processing and bioengineering.

ATR-FTIR spectroscopy

ATR-FTIR spectroscopy revealed that the PGC-AN64 enzyme formed complexes and adhered to the supports being studied (Fig. 1), with the greatest modifications occurring in secondary structure. A strong peak at 1639 cm^{-1} in the sodium alginate (SA) spectra is attributable to the antisymmetric COO^- group.[39] It also shows the existence of a notable peak between 3000 and 3600 cm^{-1}, which is associated with the OH-group stretching vibration.[40]

Table 1: Immobilization of the PGC-AN64 enzyme through the use of different supports

Immobilization type	Protein loaded (mg)	Protein leached (mg)	Immobilization efficiency (%)	Apparent activity of beads (U/g)	Specific activity of free enzyme (U/mg)	Specific activity of immobilized enzyme (U/mg)	Retention activity (%)
PGC-AN64-SA	2.6	0.65	75.00	30.34	1.330	613.76	46.14
PGC-AN64-SA-Ch	2.6	0.42	83.84	24.47	1.330	533.17	40.08
PGC-AN64-SA-Gu	2.6	0.58	77.69	12.68	1.330	261.76	19.68

SA = sodium alginate; SA-Ch = sodium alginate-chitosan; SA-Gu = sodium alginate-guar gum.

In addition, the spectrum of the free and immobilized enzyme revealed three significant peaks that were absent in the SA spectra. A strong peak at 1596 cm^{-1} may be attributed to the amide band.[41] The absorption at 1418 cm^{-1} may be due to the C-N stretching vibration, and the absorption peak at 1026 cm^{-1} attributed to the C-O-C group.[42,39] It also demonstrates the existence of a notable peak between 3000 and 3600 cm^{-1}, which corresponds to the OH^- group stretching vibration.[40,43] The ATR-FTIR spectroscopy results showed the peaks linked to the COO^- group in all immobilization beads shift from 1639 to 1596 cm^{-1}. This change results from these groups complexing with Na^+ ions. Consequently, new peaks emerge in SA beads when combined with Ch or Gu, and in the PGC-AN64 immobilization beads, but with varying peak lengths corresponding to the symmetrical COO^- and C-O-C groups at 1418 and 1026 cm^{-1}, respectively (Fig. 1). These new peaks likely resulted from the interaction between SA and the compounds (Ch and Gu), as well as the interaction of the supports with the PGC-AN64 enzyme during immobilization.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

XRD analysis of immobilized PGC-AN64

The XRD pattern of the beads (SA, SA-Ch, or SA-Gu) reveals a larger amorphous region, which vanishes after immobilizing PGC-AN64. As shown in Figure 2, a remarkable reduction in the amorphous portion is observed in all beads containing the enzyme compared to those without the enzyme, and revealed three peaks at 2 θ = 32°, 2θ = 45.7°, and 2θ = 56.7°.

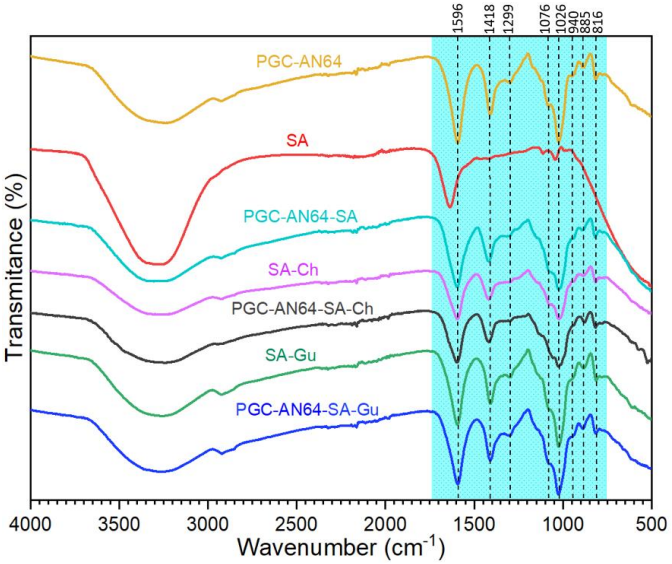

Figure 1. ATR-FTIR spectra of PGC-AN64-SA, PGC-AN64-SA-Ch, PGC-AN64-SA-Gu beads, and PGC-AN64 from *Aspergillus niger* strain HO32. PGC-AN64-SA: PGC-AN64 immobilized with sodium alginate, PGC-AN64-SA-Ch: PGC-AN64 immobilized with sodium alginate and chitosan, PGC-AN64-SA-Gu: PGC-AN64 immobilized with sodium alginate and guar gum. The ATR-FTIR profile was generated by OriginLab OriginPro software 2021b (SR1).

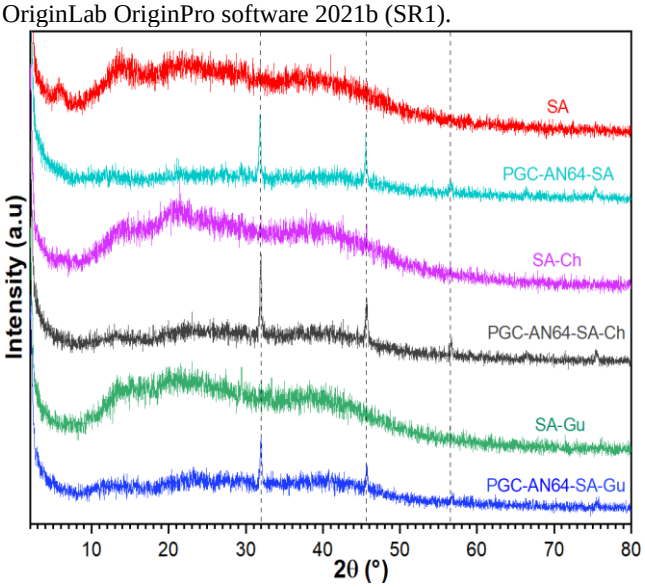

Figure 2. XRD diffractogram of PGC-AN64-SA, PGC-AN64-SA-Ch, PGC-AN64-SA-Gu beads, and PGC-AN64 from *Aspergillus niger* strain HO32. PGC-AN64-SA: PGC-AN64 immobilized with sodium alginate, PGC-AN64-SA-Ch: PGC-AN64 immobilized with sodium alginate and chitosan, PGC-AN64-SA-Gu: PGC-AN64 immobilized with sodium alginate and guar gum. The XRD diffractogram was generated utilizing Match software (version 3.10.2.173).

XRD is an analysis method utilized to get information on crystal lattice arrangements and the crystallinity degree in the microstructure of immobilized beads.[7] The ATR-FTIR results align with the XRD spectra, showing that all peaks intensified after PGC-AN64 immobilization, and the crystal structure remained intact throughout the enzyme immobilization process (Fig. 1). The XDR results indicate that the rigid structures of SA, Ch, and Gu can all be used as excellent supports for PGC-AN64 immobilization. This discovery led us to the conclusion that immobilized PGC-AN64 could easily be retrieved using external permanent magnets, thereby simplifying the reuse of the biocatalyst.

FE-SEM analysis of immobilized PGC-AN64

The microscopy images distinctly highlight the changes in surface morphologies after immobilization. The FE-SEM analysis revealed the surfaces of the beads before their complexation with the enzyme (SA, SA-Ch, and SA-Gu) and after their complexation with the enzyme (PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu) (Fig. 3). The immobilized beads, whether or not they contained PGC-AN64, exhibited dense, rough, and mesh-like structures. Figure 3 clearly shows a difference in pore structure between the beads containing the enzyme and those without the beads with the enzyme display no surface pores, unlike the beads without the enzyme. This indicates that the structure of the beads appears reinforced after immobilization, with the pores filled with the enzyme. The FE-SEM analysis of immobilized PGC-AN64 showed that the PGC-AN64 occupied pores, while those without it exhibited larger pores. While SA beads featured smooth surfaces, the structures of SA-Ch and SA-Gu beads were distinct. This does not imply that enzyme leakage occurred due to the beads' rough surface. However, research has revealed that altering the immobilized enzyme surface can stop leaks and preserve the enzyme by creating a physical barrier.[50] Furthermore, guar gum and chitosan, in our case, in the alginate beads, can inhibit the leakage and maintain the PGC-AN64 activity.

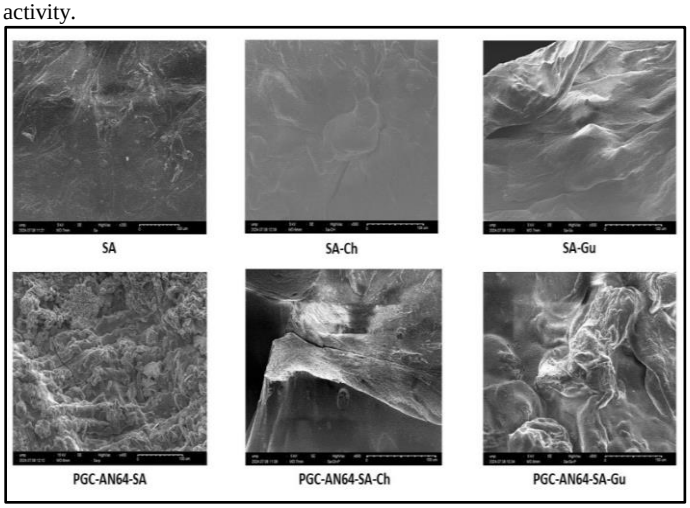

Figure 3. FE-SEM images of surface morphologies of the immobilized beads with or without PGC-AN64 from *Aspergillus niger* strain HO32. PGC-AN64-SA: PGC-AN64 immobilized with sodium alginate, PGC-AN64-SA-Ch: PGC-AN64 immobilized with sodium alginate and chitosan, PGC-AN64-SA-Gu: PGC-AN64 immobilized with sodium alginate and guar gum.

Thermal and pH stability of PGC-AN64

The thermal stability of the free and immobilized PGC-AN64 as a function of time was studied at 70 °C (pH 6.5). Enzyme thermal stability is one of the most crucial application requirements in various commercial applications that depend on the preparation strategy and stability of the biocatalyst.[3] The free PGC-AN64 lost 74% of its original activity at 80 °C after 6h of incubation, and the PGC-AN64-SA lost 50% of its initial activity at the same temperature after 8h of incubation.

However, the PGC-AN64-SA-Gu and PGC-AN64-SA-Ch lost 68 and 25.83% of their initial activities, respectively, after 14h of incubation at the same temperature.

Table 2: Enzyme treatment effect on the transmittance (T%), color parameters (L*, a*, b*), and TSS (°Brix) of orange juice by free and immobilized enzyme: PGC-AN64, PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu

	Transmittance (%)	Color			TSS (°Brix)
		L*	a*	b*	
Control	-	64.43±0.60^{c}	-5.81±0.04^{d}	40.54±0.75^{a}	12.10a±0.17^{a}
PGC-AN64	85.20±1.68^{a}	66.68a±0.38^{ab}	-3.50±0.07^{b}	15.82±0.09^{c}	11.56±0.05^{b}
PGC-AN64-SA	84.64±1.19^{a}	67.14±0.13^{a}	-3.31±0.06^{a}	13.00±0.09^{e}	11.60±0.10^{b}
PGC-AN64-SA-Ch	84.13±1.33^{a}	66.91±0.06^{b}	-3.79±0.07^{c}	16.45±0.07^{b}	11.60±0.10^{b}
PGC-AN64-SA-Gu	83.60±2.61^{a}	67.04±0.08^{ab}	-3.58±0.04^{b}	14.77±0.04^{d}	11.63±0.05^{b}

SA = sodium alginate; SA-Ch = sodium alginate-chitosan; SA-Gu = sodium alginate-guar gum. $^{a-e}$ Significant differences ($p < 0.05$) were observed between the means of each parameter in the same column with distinct lower-case letters. Values are shown with ± SEs and reflect the average of three independent replicates. Lightness (L*); green-red value (a*); blue-yellow value (b*).

PGC-AN64-SA-Ch beads exhibited greater stability with a half-life ($t_{1/2}$) of 16 h, compared to free PGC-AN64, PGC-AN64-SA, and PGC-AN64-SA-Gu beads, which had a $t_{1/2}$ of 4, 8, and 11 h, respectively (Fig. 4A). The results of stability profiles of PGC-AN64 in free and immobilized forms at pH 5 are presented in Fig. 4B. The immobilized PGC-AN64 retained activity levels of approximately 56.91, 58.21, and 56.57% after 12, 22, and 24 h of incubation for PGC-AN64-SA, PGC-AN64-SA-Gu, and PGC-AN64-SA-Ch, respectively. In contrast, the free PGC-AN64 lost 64.42% of its activity after an incubation period of 8 h. Moreover, the activity of the immobilized PGC-AN64 surpassed that of the free enzyme.

The biochemical characterization of immobilized PGC-AN64 showed that the immobilized form exhibited a notably lower inactivation rate compared to its free form. Increased thermal stability was observed for PGC-AN64-SA, PGC-AN64-SA-Gu, and PGC-AN64-SA-Ch compared to the free PGC-AN64 at 80 °C. The increased thermal stability of immobilized PGC-AN64 can be explained by the trapping of enzyme molecules within the encapsulation supports, guarding them against changes in enzyme structure at high temperatures.[51] The immobilization also increased pH stability compared to the free enzyme. Under acidic conditions, PGC-AN64 immobilization (using SA, SA-Gu, or SA-Ch) can protect the microenvironment of the enzyme (polar and ionic contacts, hydrogen bonds, etc.) due to the hydrophilic properties of these supports.[52,53] Consequently, SA combined with chitosan (SA-Ch) and guar gum (SA-Gu) acts as a protective sheath for PGC-AN64 to a greater extent than the non-combined support (SA). In conclusion, the thermal and pH stability of PGC-AN64 immobilized on food-grade supports such as alginate, chitosan, and guar gum represents a significant advantage for industrial applications, particularly in the food sector. These enhanced properties enable efficient enzyme performance under challenging conditions, extending their operational lifespan and reducing costs. Thus, these supports provide a sustainable and efficient solution tailored to the growing demands of modern industrial processes.

Recycling of the immobilized PGC-AN64 beads

The potential practical application of immobilized enzymes greatly hinges on their reusability. To evaluate the operational stability of immobilized PGC-AN64, ten successive reusability cycles were carried out using the same parameters (pH 6.5 and 70 °C). PGC-AN64-SA retained approximately 50% of its original activity following four successive cycles (Fig. 5A). Meanwhile, the operational stability of PGC-AN64-SA-Ch (Fig. 5B) and PGC-AN64-SA-Gu (Fig. 5C) was maintained at about 64% and 51% of their original activity following six successive cycles, respectively. The results demonstrated that the PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu beads have the ability to separate from the product of the reaction and can be recycled for application in subsequent reactions.

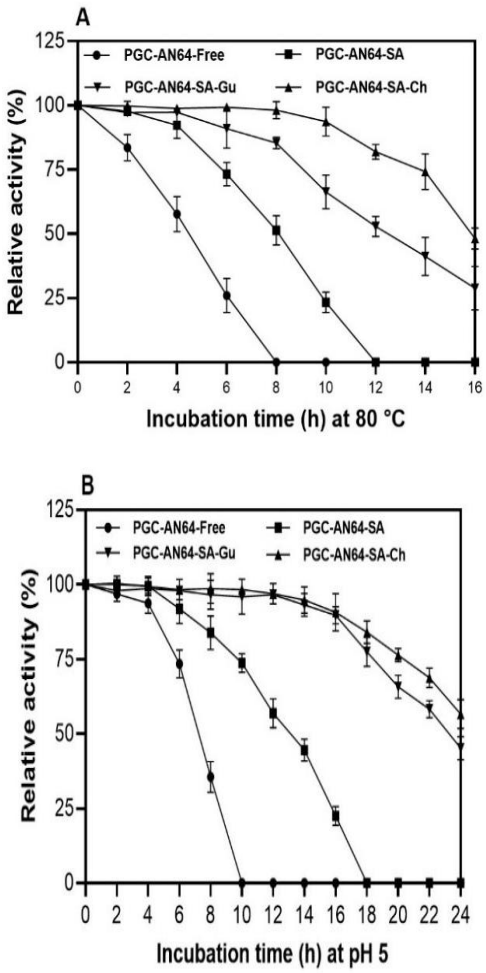

Figure 4. Thermal stability (A), and pH stability (B) of free and immobilized PGC-AN64 from *Aspergillus niger* strain HO32. PGC-AN64-SA: PGC-AN64 immobilized with sodium alginate, PGC-AN64-SA-Ch: PGC-AN64 immobilized with sodium alginate and chitosan, PGC-AN64-SA-Gu: PGC-AN64 immobilized with sodium alginate and guar gum.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

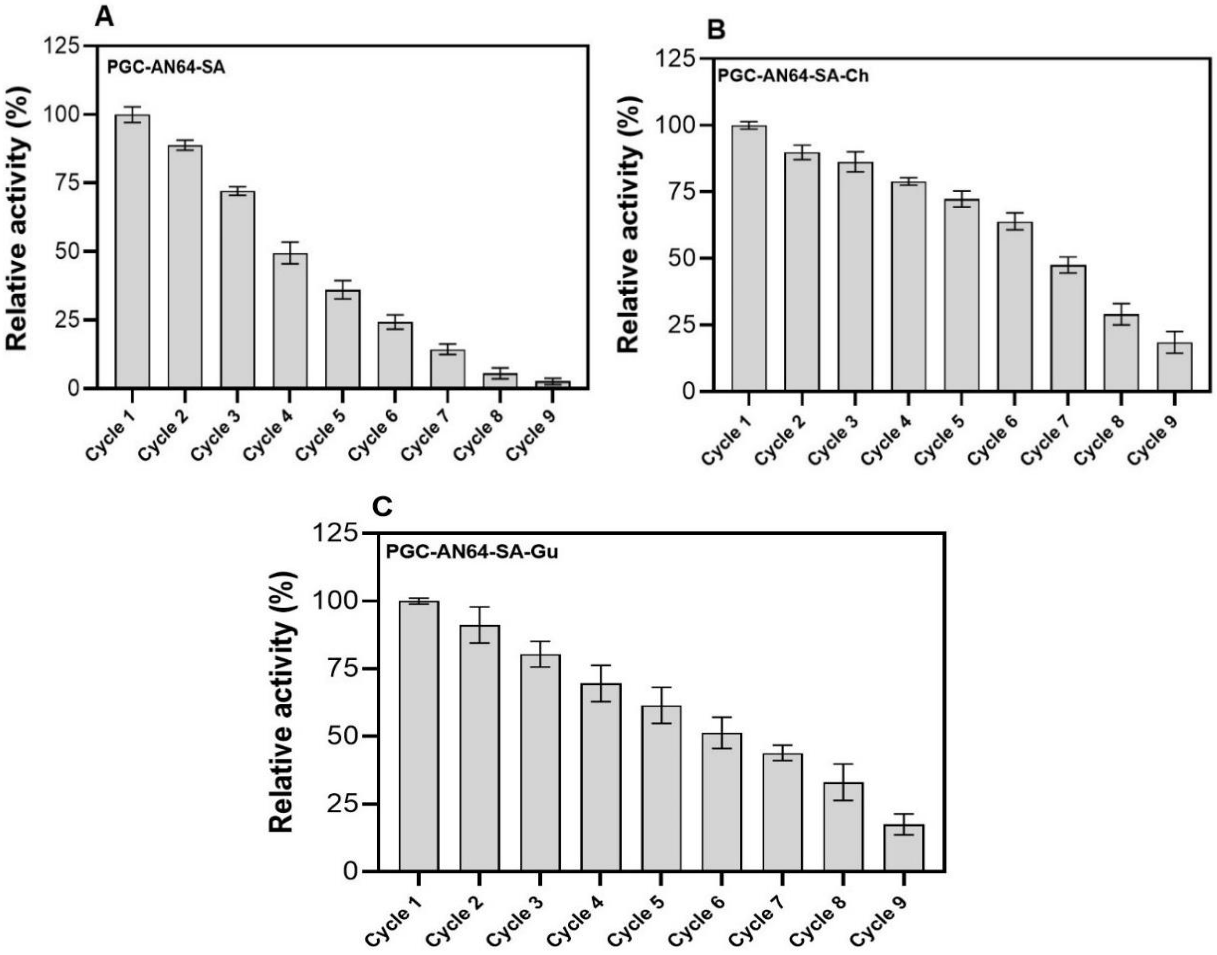

Figure 5. Reusability of immobilized PGC-AN64-SA (A), PGC-AN64-SA-Ch (B), and PGC-AN64-SA-Gu (C) tested under the same conditions (pH 6.5 and 70 °C for 20 min).

The immobilized PGC-AN64 maintained its high activity after several cycles. These results align with previous reports on the recyclability of immobilized pectinase.[3,47,54,55] which have been shown to retain much of their initial activity following several reuse cycles. The immobilized enzymes can be used economically and efficiently for a range of industrial operations.[7,56]

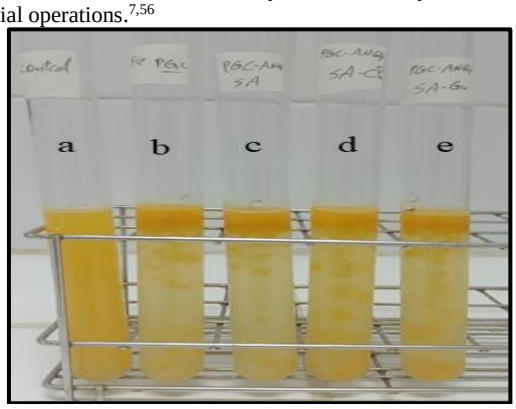

Figure 6. Clarification of orange juice at 50 °C following a 30 min incubation period (a) control; (b) free PGC-AN64; (c) PGC-AN64-SA; (d) PGC-AN64-SA-Ch; and (e) PGC-AN64-SA-Gu (SA = sodium alginate; SA-Ch = sodium alginate-chitosan; SA-Gu = sodium alginate-guar gum).

Application of immobilized PGC-AN64 in orange juice clarification

Both free and immobilized PGC-AN64 resulted in orange juice clarification at 50 °C following a 30 min period of incubation (Fig. 6 and Table 2). The results indicate that the transmittance of clarification was 85.20±1.68, 84.64±1.19, 84.13±1.33, and 84.13±1.33 for PGC-AN64, PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu, respectively (Table 2), with no significant difference ($p < 0.05$) between the treatments with free and immobilized forms. Our results are consistent with those obtained when pomegranate juice was clarified in a bioreactor containing immobilized pectinase enzymes on glass beads.[44] However, the decrease in turbidity and clarity observed in orange juice post-clarification (Fig. 6) can be attributed to the potential degradation of the pectin in the orange juice.

The color characteristics, a* (green-red value), b* (blue-yellow value), and L* (lightness), of the clarified orange juice that was treated with free and immobilized PGC-AN64, were measured after clarification at 50 °C for 30 min. The results presented in Table 2 indicate that the L* parameter increased from 64.43±0.60 to 66.68±0.38, 67.14±0.13, 66.91±0.06, and 67.04±0.08 respectively, for PGC-AN64, PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu. This increase in the L* value may be due to the result of pectin and polysaccharide hydrolysis, which improved the lightness of the juice. Therefore, a decrease in the a* value from -5.81±0.04 to -3.50±0.07, -3.31±0.06, -3.79±0.07, and -3.58b±0.04, respectively for PGC-AN64, PGC-AN64-SA, PGC-AN64-SA-Ch, PGC-AN64-SA-Gu, and an increase in the b* value from 40.54±0.75 to 15.82±0.09, 13.00±0.09, 16.45±0.07, and 14.77±0.04, respectively for PGC-AN64, PGC-AN64-SA, PGC-AN64-SA-Ch, PGC-AN64-SA-Gu, suggests that the clarified orange juice had

a lower color intensity than the control, as shown in Fig. 6. The TSS measurement (°Brix) was reduced by the clarification process with free and immobilized PGC-AN64. The °Brix was decreased from 12.10±0.17 to 11.56±0.05, 11.60±0.10, 11.60±0.10, 11.63±0.05, respectively for PGC-AN64, PGC-AN64-SA, PGC-AN64-SA-Ch, and PGC-AN64-SA-Gu (Table 2).

The results of the orange juice clarification align with previous research, which shows that enzyme activity is responsible for increasing transmittance and decreasing turbidity during the clarification process.[7,21,57] This finding is in agreement with other research, which highlighted the function of pectinases in improving grape and pomegranate juice clarity and color.[44,58] When using immobilized pectinase to clarify pomegranate juice, comparable results were observed.[44] These results are in accordance with the studies of fruit juice clarification by free and immobilized enzymes.[57,59] Furthermore, this decrease following the clarification process with both free and immobilized PGC-AN64 may be due to the release of reducing sugars and the settling of solid compounds.

Conclusion

PGC-AN64, an *endo*-polygalacturonase produced by *Aspergillus niger* strain HO32, was immobilized using sodium alginate, or sodium alginate-chitosan, or sodium alginate-guar gum. The immobilized PGC-AN64 beads were characterized using XRD, ATR-FTIR, and SEM techniques. The immobilized PGC-AN64 beads showed superior thermal stability and pH stability at 80 °C and pH 5 compared to the free enzyme. Following four continuous cycles, the immobilized enzyme maintained about 50% of its original activity for PGC-AN64-SA, while PGC-AN64-SA-Ch and PGC-AN64-SA-Gu retained about 64% and 51% after six continuous cycles, respectively. The immobilization efficiency and stability of immobilized PGC-AN64 indicated that the combined supports, particularly those combined with chitosan (SA-Ch), provided better protection for PGC-AN64 activity than the non-combined support. The immobilization process offers significant benefits for various biotechnological applications, enhancing the stability and reusability of PGC-AN64. This study highlighted the potential use of PGC-AN64 in the process of orange juice clarification. Finally, the development of multimodal enzymatic systems and the exploration of other industrial applications, such as the production of bioethanol and prebiotics from pectic oligosaccharides derived from agricultural by-products, are also being considered.

Conflicts of Interest

The authors declared no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgment

The authors acknowledge the support of the Moroccan Ministry of Higher Education, Scientific Research and Innovation, under the framework of the Multilateral Project Partenariats Hubert Curien (PHC)-Maghreb 2020 Program (FranMaghZYM 2020-2024 & code Campus France: 43791TM & code PHC: 20MAG01) and the European Union under the EU-PRIMA Project Oli4Food, and the French Embassy in Morocco for the Mobility Grant under the Doctoral Mobility Support Program in France 2023 and Program 2024.

References

1 Zafar T, Hadri S, Imran M, Asad J, Athar I, Mahmood R. Production, purification and characterization of endo-polygalacturonase using novel strain of *Bacillus pumilus* through RSM. Process Biochem. 2024; 146:188–194. doi.org/10.1016/j.procbio.2024.07.029

2 Zhong L, Wang X, Fan L, Ye X, Li Z, Cui Z, Huang Y. Characterization of an acidic pectin methylesterase from *Paenibacillus xylanexedens* and its application in fruit processing. Protein Expres Purif. 2021; 179:105798. doi.org/10.1016/j.pep.2020.105798

3 Rezaei PF, Ghanbari S, Mahdavinia G. Purification and immobilization of exo-polygalacturonase using nanomagnetic beads. Process Biochem. 2023; 132:180–190. doi.org/10.1016/j.procbio.2023.06.018

4 Satapathy S, Rout JR, Kerry RG, Thatoi H, Sahoo SL. Biochemical prospects of various microbial pectinase and pectin: an approachable concept in pharmaceutical bioprocessing. Front Nutr. 2020; 7:117. doi.org/10.3389/fnut.2020.00117

5 Arpana M, Rathore SS, Rao AS, Nair A, More SS, Fasim A. Statistical bioprocess optimization for enhanced production of a thermo alkalophilic polygalacturonase (PGase) from *Pseudomonas* sp. using solid substrate fermentation (SSF). Heliyon. 2023; 9:e16493. doi.org/10.1016/j.heliyon.2023.e16493

6 Jahan N, Shahid F, Aman A, Mujahid TY, Qader SAU. Utilization of agro waste pectin for the production of industrially important polygalacturonase. Heliyon. 2017; 3:03-30. doi.org:doi:10.1016/j.heliyon.2017.e00330

7 Núñez-Serrano A, García-Reyes RB, Solís-Pereira S, García-González A. Production and immobilization of pectinases from *Penicillium crustosum* in magnetic core-shell nanostructures for juice clarification. Int J Biol Macromol. 2024; 263:130268. doi.org/10.1016/j.ijbiomac.2024.130268

8 John J, Kaimal KS, Smith ML, Rahman PK, Chellam PV. Advances in upstream and downstream strategies of pectinase bioprocessing: a review. Int J Biol Macromol. 2020; 162:1086–1099. doi.org/10.1016/j.ijbiomac.2020.06.224

9 Blanco P, Sieiro C, Villa TG. Production of pectic enzymes in yeasts. FEMS Microbiol Lett. 1999; 175:1–9. doi.org/10.1111/j.1574-6968.1999.tb13595.x

10 Reginatto C, Rossi C, Miglioranza BG, dos Santos M, Meneghel L, da Silveira MM, Malvessi E. Pectinase production by *Aspergillus niger* LB-02-SF is influenced by the culture medium composition and the addition of the enzyme inducer after biomass growth. Process Biochem. 2017; 58:1–8. doi.org/10.1016/j.procbio.2017.04.018

11 Kant S, Vohra A, Gupta R. Purification and physicochemical properties of polygalacturonase from *Aspergillus niger* MTCC 3323. Protein Expr Purif. 2013; 87:11-16. doi.org/10.1016/j.pep.2012.09.014

12 He Y, Pan L, Wang B. Efficient over-expression and application of high-performance pectin lyase by screening *Aspergillus niger* pectin lyase gene family. Biotechnol Bioprocess Eng. 2018; 23:662-669. doi.org/10.1007/s12257-018-0387-1

13 Barman S, Sit N, Badwaik LS, Deka SC. Pectinase production by *Aspergillus niger* using banana (*Musa balbisiana*) peel as substrate and its effect on clarification of banana juice. J Food Sci Technol. 2015; 52:3579-3589. doi.org/10.1007/s13197-014-1413-8

14 Bentouhami NE, Asehraou A, Mechri S, Hasnaoui I, Moumnassi S, Yahyaoui MI, Jaouadi B. Purification and biochemical characterization of a novel thermostable *endo*-polygalacturonase from *Aspergillus niger* strain HO32 and its suitability for clarification of orange juice. Process Biochem. 2024; 145:63-73. doi.org/10.1016/j.procbio.2024.06.013

15 Kaul K, Rajauria G, Singh R. Valorization of agro-industrial waste for pectinase production and its influence on circular economy. Food Bioprod Process. 2024; 148:141-153. doi.org/10.1016/j.fbp.2024.09.008

16 Mahmoodi M, Najafpour G, Mohammadi M. Bioconversion of agroindustrial wastes to pectinases enzyme via solid state fermentation in trays and rotating drum bioreactors. Biocatal Agric Biotechnol. 2019; 21:101280. doi.org/10.1016/j.bcab.2019.101280

17 Martínez-Trujillo A, Arreguín-Rangel L, García-Rivero M, Aguilar-Osorio G. Use of fruit residues for pectinase production by *Aspergillus flavipes* FP-500 and *Aspergillus terreus* FP-370. Lett Appl Microbiol. 2011; 53:202-209. doi.org/10.1111/j.1472-765X.2011.03096.x
18 Li J, Yang M, Zhao F, Zhang Y, Han S. Efficient expression of an alkaline pectin lyase from *Bacillus licheniformis* in *Pichia pastoris*. Bioresour Bioprocess. 2024; 11:37. doi.org/10.1186/s40643-024-00752-w
19 Cheng Z, Chen D, Wang Q, Xian L, Lu B, Wei Y, Huang R. Identification of an acidic endo-polygalacturonase from *Penicillium oxalicum* CZ1028 and its broad use in major tropical and subtropical fruit juices production. J Biosci Bioeng. 2017; 123:665-672. doi.org/10.1016/j.jbiosc.2017.01.013
20 Nwokeoma CI, Arotupin DJ, Olaniyi OO, Adetuyi FC. Enhanced production, purification and characterization of pectinase from *Aspergillus flavus*. Trop J Nat Prod Res. 2021; 5:1876-1882. doi.org/10.26538/tjnpr/v5i10.28
21 Sharma N, Patel SN, Rai AK, Singh SP. Biochemical characterization of a novel acid-active endopolygalacturonase for pectin depolymerization, pectic-oligomer production, and fruit juice clarification. Int J Biol Macromol. 2024; 267:131565. doi.org/10.1016/j.ijbiomac.2024.131565
22 Carli S, Meleiro LP, Ward RJ. Biochemical and kinetic characterization of the recombinant GH28 *Stereum purpureum* endopolygalacturonase and its biotechnological application. Int J Biol Macromol. 2019; 137:469-474. doi.org/10.1016/j.ijbiomac.2019.06.165
23 Sandri IG, Fontana RC, Barfknecht DM, da Silveira MM. Clarification of fruit juices by fungal pectinases. LWT-Food Sci Technol. 2011; 44:2217-2222. doi.org/10.1016/j.lwt.2011.02.008
24 Carli S, de Campos Carneiro LAB, Ward RJ, Meleiro LP. Immobilization of a β-glucosidase and an endoglucanase in ferromagnetic nanoparticles: A study of synergistic effects. Protein Expr Purif. 2019; 160:28-35. doi.org/10.1016/j.pep.2019.03.016
25 Ji L, Chen M, Zhang W, Nian B, Hu Y. Comprehensive applications of ionic liquids in enzyme immobilization: Current status and prospects. Mol Catal. 2024; 552:113675. doi.org/10.1016/j.mcat.2023.113675
26 de Oliveira RL, Dias JL, da Silva OS, Porto TS. Immobilization of pectinase from *Aspergillus aculeatus* in alginate beads and clarification of apple and umbu juices in a packed bed reactor. Food Bioprod Process. 2018; 109:9-18. doi.org/10.1016/j.fbp.2018.02.005
27 Ramirez HL, Gómez Brizuela L, Úbeda Iranzo J, Arevalo-Villena M, Briones Pérez AI. Pectinase immobilization on a chitosan-coated chitin support. J Food Process Eng. 2016; 39:97-104. doi.org/10.1111/jfpe.12203
28 Kaushal J, Khatri M, Singh G, Arya SK. Xylanase enzyme from novel strain and its immobilization onto metal-organic framework MOF for fruit juice clarification. Biotechnol Bioprocess Eng. 2024; 29:197-210. doi.org/10.1007/s12257-024-00007-7
29 Wang B, Cheng F, Lu Y, Ge W, Zhang M, Yue B. Immobilization of pectinase from *Penicillium oxalicum* F67 onto magnetic cornstarch microspheres: Characterization and application in juice production. J Mol Catal B: Enzym. 2013; 97:137-143. doi.org/10.1016/j.molcatb.2013.07.018
30 Navarro-López DE, Bautista-Ayala AR, Rosales-De la Cruz MF, Martínez-Beltrán S, Rojas-Torres DE, Sanchez-Martinez A, López-Mena ER. Nanocatalytic performance of pectinase immobilized over in situ prepared magnetic nanoparticles. Heliyon. 2023; 9:e19-21. doi.org/10.1016/j.heliyon.2023.e19021
31 Rajdeo K, Harini T, Lavanya K, Fadnavis NW. Immobilization of pectinase on reusable polymer support for clarification of apple juice. Food Bioprod Process. 2016; 99:12-19. doi.org/10.1016/j.fbp.2016.03.004
32 Chen M, She W, Zhao X, Chen C, Zhu B, Sun Y, Yao Z. Immobilization of *Thermomyces lanuginosus* lipase in a novel polysaccharide-based hydrogel by a two-step crosslinking method and its use in the lauroylation of α-arbutin. Bioresour Bioprocess. 2024; 11:7. doi.org/10.1186/s40643-023-00721-9
33 Xie P, Lan J, Zhou J, Hu Z, Cui J, Qu G, Sun Z. Co-immobilization of amine dehydrogenase and glucose dehydrogenase for the biosynthesis of (S)-2-aminobutan-1-ol in continuous flow. Bioresour Bioprocess. 2024; 11:70-72. doi.org/10.1186/s40643-024-00786-0
34 Wei C, Zhou Y, Zhuang W, Li G, Jiang M, Zhang H. Improving the performance of immobilized β-glucosidase using a microreactor. J Biosci Bioeng. 2018; 125:377-384. doi.org/10.1016/j.jbiosc.2017.09.011
35 Amin F, Bhatti HN, Bilal M, Asgher M. Improvement of activity, thermo-stability and fruit juice clarification characteristics of fungal exo-polygalacturonase. Int J Biol Macromol. 2017; 95:974-984. doi.org/10.1016/j.ijbiomac.2016.10.086
36 Mechri S, Allala F, Bouacem K, Hasnaoui I, Gwaithan H, Chalbi TB, Jaouadi B. Preparation, characterization, immobilization, and molecular docking analysis of a novel detergent-stable subtilisin-like serine protease from *Streptomyces mutabilis* strain TN-X30. Int J Biol Macromol. 2022; 222:1326-1342. doi.org/10.1016/j.ijbiomac.2022.09.161
37 Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959; 31:426-428. doi.org/10.1021/ac60147a030
38 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254 doi.org/10.1016/0003-2697(76)90527-3
39 Ellerbrock RH, Gerke HH. FTIR spectral band shifts explained by OM–cation interactions. J Plant Nutr Soil Sci. 2021; 184:388-397. doi.org/10.1002/jpln.202100056
40 Xu H, Cao Y, Li X, Cao X, Xu Y, Qiao D, Cao Y. An electrospun chitosan-based nanofibrous membrane reactor immobilized by the non-glycosylated rPGA for hydrolysis of pectin-rich biomass. Renew Energy. 2018; 126:573-582. doi.org/10.1016/j.renene.2017.08.094
41 Almulaiky YQ, Alkabli J, El-Shishtawy RM. Improving enzyme immobilization: A new carrier-based magnetic polymer for enhanced covalent binding of laccase enzyme. Int J Biol Macromol. 2024; 8:137-362.
42 Wu Y, Meng F, Fu J, Zhou Y, Fan H, Ju X, Li L. PDA/SiO2 microspheres immobilize dual enzymes to increase temperature tolerance and catalyze the production of D-ribulose and D-xylulose from D-xylose. Mol Catal. 2024; 555:113-900. doi.org/10.1016/j.mcat.2024.113900
43 Ellerbrock R, Höhn A, Rogasik J. Functional analysis of soil organic matter as affected by long‐term manurial treatment. Eur J Soil Sci. 1999; 50:65-71. doi.org/10.1046/j.1365-2389.1999.00206.x
44 Hosseini SS, Khodaiyan F, Mousavi SM, Azimi SZ. Clarification of the pomegranate juice in a bioreactor packed by pectinase enzymes immobilized on the glass bead activated with polyaldehyde polysaccharides. LWT. 2021; 137:110500. doi.org/10.1016/j.lwt.2020.110500
45 Rehman HU, Aman A, Silipo A, Qader SAU, Molinaro A, Ansari A. Degradation of complex carbohydrate: immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 using calcium alginate as a support. Food Chem. 2013; 139:1081-1086. doi.org/10.1016/j.foodchem.2013.01.069

46 Ramirez HL, Briones AI, Úbeda J, Arevalo M. Immobilization of pectinase by adsorption on an alginate-coated chitin support. Biotecnol Apl. 2013; 30:101-104.
47 Xue L, Long J, Lu C, Li X, Xu X, Jin Z. Immobilization of polygalacturonase for the preparation of pectic oligosaccharides from mango peel wastes and assessment of their antibacterial activities. Food Biosci. 2021; 39:100837. doi.org/10.1016/j.fbio.2020.100837
48 Busto M, García-Tramontín K, Ortega N, Perez-Mateos M. Preparation and properties of an immobilized pectinlyase for the treatment of fruit juices. Bioresour Technol. 2006; 97:1477-1483. doi.org/10.1016/j.biortech.2005.06.013
49 Sharma G, Sharma S, Kumar A, Al-Muhtaseb AAH, Naushad M, Ghfar AA, Stadler FJ. Guar gum and its composites as potential materials for diverse applications: A review. Carbohydr Polym. 2018; 199:534-545.
50 Gülay S, Şanlı-Mohamed G. Immobilization of thermoalkalophilic recombinant esterase enzyme by entrapment in silicate coated Ca-alginate beads and its hydrolytic properties. Int J Biol Macromol. 2012; 50:545-551. doi.org/10.1016/j.ijbiomac.2012.01.017
51 Karataş E, Tülek A, Yildirim D, Tamtürk F, Binay B. Immobilization of Sporothrix schenckii 1099-18 exo-polygalacturonase in magnetic mesoporous silica yolk-shell spheres: Highly reusable biocatalysts for apple juice clarification. Food Biosci. 2021; 43:101324. doi.org/10.1016/j.fbio.2021.101324
52 Tapdigov SZ. The bonding nature of the chemical interaction between trypsin and chitosan based carriers in immobilization process depend on entrapped method: A review. Int J Biol Macromol. 2021; 183:1676-1696. doi.org/10.1016/j.ijbiomac.2021.05.059
53 Işik C, Arabaci G, Doğaç YI, Deveci İ, Teke MS. Synthesis and characterization of electrospun PVA/Zn2+ metal composite nanofibers for lipase immobilization with effective thermal, pH stabilities and reusability. Mater Sci Eng C. 2019; 99:1226-1235. doi.org/10.1016/j.msec.2019.02.031
54 Carli S, Salgado JCS, Meleiro LP, Ward RJ. Covalent immobilization of *Chondrostereum purpureum* endopolygalacturonase on ferromagnetic nanoparticles: catalytic properties and biotechnological application. Appl Biochem Biotechnol. 2022; 12:1-14. doi.org/10.1007/s12010-021-03688-5
55 Rehman HU, Nawaz MA, Pervez S, Jamal M, Attaullah M, Aman A, Qader SAU. Encapsulation of pectinase within polyacrylamide gel: Characterization of its catalytic properties for continuous industrial uses. Heliyon. 2020; 6:45-78. doi.org/10.1016/j.heliyon.2020.e04578
56 Oktay B, Demir S, Kayaman-Apohan N. Immobilization of pectinase on polyethyleneimine based support via spontaneous amino-yne click reaction. Food Bioprod Process. 2020; 122:159-168. doi.org/10.1016/j.fbp.2020.04.010
57 dos Santos EG, de Assis SA, Ferreira AN, de Almeida Bezerra M, de Paula SA, Valasques LM, Júnior GLV. Production, characterization and application of polygalacturonase produced by *Lentinus tigrinus* CCMB 553. Biocatal Agric Biotechnol. 2024; 58:103216. doi.org/10.1016/j.bcab.2024.103216
58 Gani G, Naik HR, Jan N, Bashir O, Hussain SZ, Rather AH, Amin T. Physicochemical and antioxidant properties of pear juice prepared through pectinase enzyme-assisted extraction from William Bartlett variety. J Food Meas Charact. 2021; 15:743-757. doi.org/10.1007/s11694-020-00676-x
59 Diano N, Grimaldi T, Bianco M, Rossi S, Gabrovska K, Yordanova G, Mita DG. Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors. J Agric Food Chem. 2008; 56:11471-11477. doi.org/10.1021/jf8019437