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Characteristics of Luffa sponge-immobilized pectinase from *Aspergillus niger* FTR 002 and its use in the continuous clarification of orange juice in packed-bed column bioreactor

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Abstract

Background and Objective One of the major challenges of linking enzymes to insoluble support matrices is the use of non-naturally occurring support matrices that are expensive and not readily accessible, which most times are not easy to handle with the process needing several steps. Therefore, naturally-occurring materials that are cheap, available and need a little or no modifications with the potentials to act as support matrices for enzyme immobilization were investigated.

Material and Methods: In this study, coconut fibers, pseudo stems of banana and plaster of Paris luffa sponges as appropriate carriers for the immobilization of pectinase produced by *Aspegillus niger* FTR 002 were investigated and purified using chromatographic techniques, physical adsorption and covalent bonding with glutaraldehyde. Characteristics of native and immobilized enzyme preparations were studied and then used in the clarification of juice using packed-bed column reactor and standard procedures.

Results and Conclusion: Of the four materials in this study, sponge included the highest immobilization rate. Free and sponge-immobilized pectinase preparations were characterized and results recorded a temperature optimum of 50 °C for the two enzyme preparations. A shift in pH optima from 6.0 to 5.0 by the pectinase was observed after immobilization on sponge. The K_m and V_{max} of the free and immobilized pectinases included 2.33 mM and 0.018 µM/min and 1.612 mM and 0.019 µM/min respectively. Reusability studies showed that the immobilized enzyme included approximately 55% of its initial activity after 15 repeated cycles in batch operation. Orange juice clarification using sponge-immobilized pectinase using packed-bed column reactor at a flow rate of 0.5 ml/min showed that the immobilized catalyst included 40% of its clarification capacity after 80 h of continuous operation. These findings indicate that the pectinase immobilized on sponge includes promising potentials as a clarifying catalyst in fruit-juice industries.

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1. Introduction

Pectinases (EC number: 3.2.1.15) are industrially important enzymes as they break down pectin, which is a polysaccharide within the cell walls of plants through hydrolysis, trans elimination and de-esterification reactions [1-3]. These pectinases have significantly been interested due to their versatile uses in foods, textiles, beverages, pulps and papers, plant fiber processing, teas, coffees, oil extractions, treatments of pectin-containing industrial waste-waters and biofuel industries [4]. In food industries, pectinases are extensively used in fruit juice extraction and clarification, where presence of pectin is not desirable due to high turbidity of the pectin-containing extracts and formation of sediments during storage [5-6]. Furthermore, use of pectinase in clarification of juices reportedly enhances efficiency of the juice production in as well as helping in improvements of the juice appearance and stability and

quality characteristics of the final products [6]. Although industrially important, use of free pectinases in juice clarification is reported to include challenges linked to costs, low stabilities and problems with downstream processing. One of the strategies to overcome these limitations and optimize juice clarification processes with pectinases includes immobilization of free pectinases onto appropriate carrier molecules using stablished protocols [5,7-9]. These immobilization protocols enhance characteristics of the pectinases, including activity, stability, selectivity, specificity and resistance to inactivation by chemicals and other adverse industrial environments.

Immobilization strategies involving the attachment of free pectinases on appropriate carrier molecules, including anion exchange St-DVB macroporous base resins, glass beads, sodium alginate beads, microporous polyacrylamide, magnetic nanoparticles (MNPs), microspheres, porous hydroxyapatite/calcium alginate composite beads, nanoporous activated carbon, chitosan magnetic particles and CLEA smart magnets with or without crosslinking with agents, have been reported as great potentials in juice clarification [2,3,5,9,10-12]. One of the problems of major immobili-zation protocols is the use of synthetic molecules that are expensive, which ultimately increase the costs of industrial processes. Furthermore, enzyme linking with these synthetic materials sometimes leads to changes in the chemical characteristics of the enzymes, resulting from the several steps [2,13-15]. To overcome these problems, naturally-occurring (with a little or no modifications), cheap available materials with potentials to act as support matrices for the enzyme immobilization have been investigated in the current authors' laboratories, who previously reported successful immobilization of inulinase on kaolin clay [16]. The present study reported successful immobilization of pectinase from *Aspergillus niger* FTR 002 on luffa sponges and described characteristics of the native and immobilized pectinases. Furthermore, the sponge-immobilized pectinase was assessed as an appropriate catalyst for use in clarification of juices using fixed-bed reactor (FBR). Available cost-effective sponges with their excellent protein retention capacity are expected to play critical roles in decreasing production costs of immobilized pectinases. This favorable combination includes significant potentials for large-scale industrial uses.

2. Materials and Methods

Sample collection

Coconut (*Cocus nucifera*) shafts were purchased from coconut processing outlet in Bodija Market, Ibadan, Nigeria. Pseudo stems of banana (*Musa* sp.) were purchased from Teaching and Research Farm, University of Ibadan, Nigeria. Plaster of Paris (POP) fibers were provided by the local suppliers within Ibadan Metropolis, Nigeria. Luffa sponges were purchased from Bodija Market, Ibadan, Nigeria. All other chemicals were supplied by the local distributers and included analytical grades.

Microorganism used and cultural condition

The *A. niger* FTR 002 was provided by the Culture Collection of the Department of Microbiology, University of Ibadan, Nigeria. It was previously isolated from spoilt oranges and its sequences were deposited in GenBank with the accession number of OP231019. This was preserved on pectin agar slants containing pectin, 3 g l^{-1}; $(NH_4)_2SO_4$, 0.33 g/l; K_2HPO_4, 0.1 g/l; $MgSO_4.5H_2O$, 0.005 g l^{-1}; KCl, 0.005 g/l; and $FeSO_4.5H_2O$, 0.01 g/l.

Pectinase production and assay

An aliquot of 100 ml of sterile pectin media (as previously described without agar) in 250 ml Erlenmeyer's flasks was inoculated with 1 ml of spore suspension (containing 1.0 × 10^6 spores) of a 5-day-old *A. niger* FTR 002 on potato dextrose agar (PDA) and then incubated at 35 □C for 3 d at 150 rpm using shaker incubator (New Brunswick Scientific, Edison, USA). After incubation, the whole content was centrifuged (Bioevopeak, CFGR-B580, Shandong, China) at 1,073 g for 20 min and the supernatant was used as the crude pectinase. Pectinolytic activity was assessed using DNS method [17] by adding 1 ml of the crude enzyme to 1% (w v^{-1}) citrus pectin in sodium acetate (0.1 M, pH 4.0) and incubating at 50 °C for 10 min. After the incubation, reaction mixture was supplemented with 1 ml of freshly prepared DNS reagent and incubated at 100 °C for 5 min using water bath. Solution was allowed to cool down using ice water bath, followed by the addition of 3 ml of distilled water (DW). The resulting solution absorbance was measured at 540 nm using Jenway spectrophotometer (Jenway, UK). Estimation of pectinase activity was carried out based on the quantity of galacturonic acid released during the hydrolysis of citrus pectin, utilizing galacturonic acid monohydrate as the standard in concentrations ranging 0.2-10.0 mg ml^{-1}. One unit of pectinase activity was defined as the quantity of enzyme needed to release 1 µmol of galacturonic acid per minute under standard conditions.

Pectinase purification

Purification of pectinase included a series of steps of cold acetone precipitation and then dialysis by tubing and gel chromatography with Sephadex G-50 Column (2.5 × 100 cm) based on the methodology by Manisavagan and Oh [17].

Immobilization of pectinase

Activation of the coconut fibers (obtained by removing the shaft of coconut), pseudo-stems of banana, luffa sponge and POP fibers was carried out by washing them using acetone with gentle agitation for 30 min and then overnight drying them at 60 °C using oven [16].

Immobilization by adsorption

Two grams of each activated sample (carrier material) were mixed with 20 ml of pectinase enzyme (50-100 U) in 100 ml of 0.1 M sodium acetate (pH 4.0) using beaker. Mixture was

gently stirred and left to set for 24 h. Then, supernatant was carefully removed and the immobilized pectinase was collected by filtration using Whatman filter paper no. 1 (Whatman, USA). This was dried at room temperature (RT) for 24 h [16].

Immobilization by covalent method

Briefly, 100 ml of 0.1 M sodium acetate buffer (pH 4.0) and 8 ml of 2% (w v^{-1}) glutaraldehyde were added to 2 g of each sample of dried coconut fibers, pseudo stems of banana, luffa sponge and POP fibers and mixed gently for 1 h at 25 °C before filtration by Whatman filter papers no. 1 (Whatman, USA). Support matrix (recovered by filtration) was rinsed with DW and dried at RT until reached a consistent weight. Then, each support matrix was gently agitated for 2 h at 4 °C using 20 ml of pectinase (50-150 U of enzyme) and 100 ml of 0.2 M sodium acetate buffer (pH 4.0). Immobilized pectinase was recovered by filtration using Whatman filter papers no. 1 (Whatman, USA) and carefully rinsed three times with DW before drying on the filter papers at RT. Adsorbed pectinase was desorbed by gently rinsing in 100 ml of 0.5 M NaCl in 0.1 sodium acetate buffer (pH 4.0) for 30 min using magnetic stirrer. Immobilized pectinase was stored at 4 °C until use [16].

Assessment of the immobilization extent

Extent of the pectinase immobilization (quantity of pectinase attached to the carrier materials) was investigated by assessing protein contents [18] of the filtrates (as the unattached pectinase) using centrifugation after each round of washing of the carrier molecules with DW. Protein contents of the immobilized enzymes were estimated by subtracting the protein content of the enzymatic solution from the protein content of the total filtrate. Equation 1 was used to calculate the extent of immobilization:

Quantity of pectinase immobilized (%) = Activity of immobilized enzyme / activity of free enzyme × 100 Equation 1

Optimization of immobilization rate

Optimization of the quantity of pectinase attached to the carrier matrix was investigated as follows.

Contact time

Effects of various contact times on immobilization rates by the support materials were assessed based on a modified method of Saxena et al. [19]. An aliquot of 20 ml of pectinase solution (50-100 U) with 2 g of the fibers and 2% glutaraldehyde was transferred into a shaking water bath (4 °C, 100 rpm) for contact times of 30 min to 2 h. After cross-linking, immobilized rate was calculated as described previously.

Support concentration

This was assessed based on a modified method of Saxena et al. [19]. Pectinase solution (2 ml) and various support weights (0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 g), each with 2% glutaraldehyde, were incubated at 4 °C for 2 h at 100 rpm using shaking water bath; after which, immobilized rate was assessed as previously described.

Characteristics of the immobilized and free pectinases

Effects of temperature and pH on the activity and stability of free and immobilized pectinases

Effects of temperature on free and immobilized pectinase activities were investigated based on a method by Madu et al. [20]. Enzymatic assessment was carried out in 0.1 M sodium acetate buffer (pH 4.2) at various temperatures (25-70 °C) for 10 min using water bath. Reactions were assessed against a blank solution. Similarly, effects of pH on free and immobilized pectinase activities were investigated using reaction mixtures of 1% pectin in appropriate sodium acetate buffer with pH ranging 3.0-6.0. Thermal stabilities of free and immobile pectinases were further investigated by assessing residual enzyme activities at 55 °C for 1 h with 10-min intervals. Moreover, pH stability was assessed by investigating residual activities with 10-min intervals during incubation at pH 5.0 for 1 h.

Kinetic parameters

Kinetic constants of V_{max} and K_m were estimated using citrus pectin (Sigma-Aldrich, USA) as a substrate. This was carried out by recording rates of reaction at various concentrations of citrus pectin (0.5-20 mg ml^{-1}) and data from the experiments fitted into Michaelis-Menten model using GraphPad Prism 6 (GraphPad, San Diego, California, USA).

Reusability of the immobilized pectinase

Reusability of the immobilized pectinase was assessed using batch pectin hydrolysis cycles. One gram of the immobilized pectinase was added to 10 ml of 1% pectin solution and pectin hydrolysis was proceeded for 20 min as a cycle. After each round of hydrolysis, immobilized pectinase was rinsed with repeated changes of sterile DW and immersed in the fresh substrate. Quantity of the reducing sugar released was estimated using DNS method as previously described.

Use of immobilized pectinase in clarification of the orange juice using packed-bed reactor

Packed-bed reactor

The packed-bed column reactor (PBCR) included a double-walled glassware cylinder with an inside diameter of 1 cm and a length of 20 cm. The inner chamber was packed with 25 cm^3 of the immobilized pectinase while water of controlled temperature (50 °C) was recirculated in the outer chamber of the doubled-walled apparatus. Orange juice was pumped from the system bottom and exited from the top based on a method by Azimi et al. (2021).

Effects of the flow rate and residence time on juice clarification

Flow rates of the orange juice varied 0.1-5 ml/min at 50 °C while residence time of the orange juice in the PBCR column was calculated as follows.

Residence time (min) = V/F Equation 2

Where, V was the reactor volume (cm^3) and F was the flow rate (ml min^{-1}).

Operational stability of the PBCR

Operational stability of the immobilized pectinase for the clarification of orange juice was assessed within continuous use. Samples were periodically achieved from the PBCR at every 10 h and clarification proportions were calculated using Equation 3. Turbidity of the orange juice was assessed at 860 nm using Jenway spectrophotometer (Jenway, UK) and the clarification capacity of the PBCR was calculated as follows.

Clarification (%) = Initial turbidity - final turbidity / initial turbidity × 100 Equation 3

Use of free and immobilized pectinases in clarification of the orange juice in batch process

For clarification using luffa sponge-immobilized pectinase, 1 g of the immobilized pectinase was added to 10 ml of orange juice solution and incubated at 50 °C. Clarification process was proceeded for 30 min. After 30 min, immobilized pectinase was separated by filtration and the quantity of reducing sugar released in the clarified juice sample was calculated using DNS method as previously described. For the clarification of orange juice using free pectinase, 1 ml of pectinase enzyme (containing 5-10 U) in 100 ml of 0.1 M sodium acetate (pH 4.0) was added to 10 ml of orange juice solution and incubated at 50 °C for 30 min. After incubation, quantity of reducing sugar released was assessed as described previously.

Physicochemical parameters of the various juice samples

Physicochemical characteristics of the clarified orange juice were investigated as described later and then compared with those of raw juice samples. Total titratable acidity was assessed by titration of samples with potassium hydroxide (0.1 N) using phenolphthalein indicator and pH was calculated using pH meter. Digital refractometer (Brix Meter, Bioevopeak, RFT-A670/RFT-A630, China) was used to investigate total soluble solids (TSS) of the samples. The total reducing sugar in clarified and raw samples was investigated using DNS method by Miller [21]. Turbidity was measured at 860 nm using Jenway spectrophotometer (Jenway, UK) as previously described. Presence of pectin in orange juices before and after clarification was investigated by the addition of acidified ethanol to the juice samples at a ratio of 2:1 (v v^{-1}) and the samples were mixed upside down three times and then set for 15 min at RT. Formation of gel indicated the presence of pectin in juice samples.

Statistical analysis

All experiments were carried out in triplicate and results were reported as mean values ±SD (standard deviation). Data were used in one-way analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$ using SPSS software v.20 (IBM, USA) [22].

3. Results and Discussion

Pectinase immobilization

In this study, luffa sponges, POP fibers, coconut fibers and pseudo stems of bananas as cheap and available materials were assessed for their suitability in the immobilization of pectinase using physical adsorption technique and covalent bonding (using glutaraldehyde) methods (Table 1). Results showed that for all the materials under investigation, quantity of immobilized pectinase ranged 54-84% and immobileization by covalent bonding using glutaraldehyde was higher than that of physical adsorption (Table1). The low pectinase immobilization observed with physical adsorption could be a result of inefficient binding affinity between the support matrices and the pectinase enzyme since adsorption technique depends on non-specific interactions between the immobilization matrices and the target enzyme [23-25]. Furthermore, physical adsorption is usually based on relatively weak reversible interactions between the matrices and the enzyme; hence, enzyme could leach out from the support. Differences in the quantity of immobilized pectinase attached to various materials could be attributed to the differences in protein retention as immobilization capacity has been suggested as a function of the surface area as well as pore volume of the carrier molecule [2,16]. Furthermore, protein-carrier interaction during immobilization has been reported to affect immobilization [15].

In this study, immobilization rates by various carrier supports are similar to those for various synthetic carrier molecules [15,26,27]. Results from this study could be advantageous since these materials are cheaper, non-toxic, physiologically inert, widely available and highly accessible organic materials. Moreover, their use could be a supportive scaffolding decreasing enzymatic manufacturing costs by via use of the appropriate immobilization techniques. From the results of immobilization rate presented in Table 1, immobilization of pectinase from *A. niger* FTR 002 onto luffa sponge with glutaraldehyde through covalent methods was selected for further investigation. Optimization of the immobilization pectinase on sponges was investigated regarding effects of various contact times and sponge concentrations on the immobilization rates. Results showed that contact time of 2 h and sponge concentration of 2 g were optimum for a maximum immobilization rate of 88% in this study (Tables 2 and 3). Concentration of 2 g of sponge supporting a maximum immobilization rate of 88% could be due to the fact that this concentration provided the optimum surface area for the pectinase attachment.

Table 1. Capacity of various materials in immobilization of pectinase produced by *Aspergillus niger* FTR 002

Carrier molecules	Immobilization method/Percentage immobilization (%)	
	Adsorption	Crosslinking with GA
POP fiber	*54 ± 0.02[b]	62 ± 0.05[c]
Luffa sponge	56 ± 0.23[b]	84 ± 0.03[a]
Banana pseudo-stem	43 ± 0.11[c]	54 ±0.21[d]
Coconut fibre	70 ± 0.08[a]	82 ± 0.06[b]

Data are means of three replicates. *Means with different letters within each column differ significantly ($p \leq 0.05$)

Table 2. Effects of contact time on capacity of luffa sponge in immobilization of pectinase produced by *Aspergillus niger* FTR 002

Contact time (mins)	Immobilization rate (%)
30	*60 ± 0.01[f]
60	68.8 ± 0.22[d]
90	75 ± 0.12[b]
120	88 ± 0.08[a]
150	71 ± 0.11[c]
180	64 ±0.06[e]

Data are means of three replicates. *Means with different letters within each column differ significantly ($p \leq 0.05$)

Table 3. Effects of various concentrations of luffa sponge on its capacity in immobilization of pectinase produced by *Aspergillus niger* FTR 002

Concentration of luffa sponge (w v^{-1})	Capacity (%)
0.4	*58 ±0.04[d]
0.8	65 ± 0.18[e]
1.2	85 ± 0.02[b]
1.6	86 ± 0.06[b]
2.0	88 ±0.03[a]
2.4	82 ± 0.03[c]

Data are means of three replicates. *Means with different letters within each column differ significantly ($p \leq 0.05$)

Immobilization has been reported to affect activities and characteristics of the enzymes due to conformational effects, microenvironment and diffusional or mass transfer effects [2,9,13-15]. In this study, an enhanced relative activity was seen after immobilization of pectinase on sponge covalently attached with glutaraldehyde. Enhanced relative activity of the enzyme after immobilization has been reported by several authors [10,14,15] and might be connected to the multipoint covalent bonds between the enzyme and the carrier molecule, which protects the pectinase active site from distortion caused by heat [28]. Furthermore, immobilization has been reported to decrease inhibition effects of a substrate resulting in enhanced activities of the immobilized enzyme, compared to the free enzyme [25,29].

Figures 1a and 1b illustrate effects of temperature on the activity and stability of free and sponge-immobilized pectinases. Findings revealed that free and immobilized pectinase preparations included their peak activities at 50 °C (Fig. 1a). Compared with the free pectinase, sponge preserved 76% of its activity after incubation at 50 °C for 6 h while the free pectinase preserved 30% of its activity under similar conditions (Fig. 1b). Enhanced thermal stability after immobilization has previously been reported and this might be as a result of additional mechanical supports for the enzyme as provided by the sponge [9,15,30]. Moreover, enhanced thermal stability might be attributed to the crosslinking of the pectinase with the luffa sponge at multiple points. This crosslinking created a further rigid spatial configuration of the immobilized enzyme, making it resistant to unfolding when exposed to heat; thereby, preserving its stability at elevated temperatures [5,9,24,29]. The present findings include significant advantages when considering use of pectinase at increased temperatures. Use of pectinases at high temperature is particularly important as pectin has been reported to include decreased viscosity at increased temperatures. This decreased viscosity results in increases in the substrate mobility inside support pores; thus, favoring interactions with enzymes located inside the pores of the support matrices [8]. Furthermore, this decreases chances of microbial contaminations as most microbial contaminations are produced by mesophilic microorganisms [16].

Effects of pH on the activity and stability of free and immobilized pectinase preparations are present in Fig. 2b. The highest pH of 5 and 6 were reported for sponge-immobilized pectinase and the free pectinase, respectively. Hence, shifts in optimum pH were seen towards the acidic

region after immobilization on sponge (Fig. 2a). Earlier reports have indicated shifts in the optimum pH towards the acidic range for enzymes after immobilization and these shifts were possibly due to secondary interactions between the enzyme and the support system with the crosslinking agent, which might result in distribution of negative charges in the support system [2,15,31]. Results of the pH stability of the two pectinase preparations showed that while the free pectinase preserved 40% (pH 6) of its activity after 6 h of incubation, the sponged-immobilized pectinase retained 76% (pH 5) of its activity at a similar incubation time (Fig. 2b). A better stability of the pectinase after immobilization in this study has previously been reported by Dal Magro et al. [24]. The enhanced stability could be linked to the immobilized enzyme resistance to alterations in ionization states of the surrounding microenvironment close to its active sites. This resistance typically delays substrate binding and affects enzyme activity during fluctuations in pH.

Estimated kinetic parameters

Estimated kinetic parameters of the two pectinase preparations were investigated at 50 °C and pH of 5 and 6 for the immobilized and free pectinase, respectively, using pectin substrate. Results respectively revealed K_m and V_{max} of 2.33 mM and 0.018 mM min for the free enzyme while K_m and V_{max} of 1.612 mM and 0.019 µM/min were reported for the immobilized enzyme, respectively. The smaller K_m of 1.612 mM for the immobilized enzyme suggested that a smaller concentration of substrate was needed to reach half of the maximum reaction rate, compared to the free enzyme. The V_{max} of 0.019 µM/min for the immobilized enzyme suggested a mildly greater maximum reaction rate, compared to the free enzyme. From these results, immobilized enzyme with a smaller K_m could include a greater affinity for the pectin substrate, needing a smaller concentration for the optimal reaction rate. Additionally, a mildly greater Vmax could suggest that it included a greater potential for catalyzing the breakdown of pectin at its maximum capacity [16]. Immobilization of the enzyme have been reported to produce changes in kinetic parameters of the enzymes after immobilization [14,15]. These alterations could be ascribed to conformational changes occurring close the enzyme active sites due to the close proximity between the enzyme and the support. Such proximity could lead to changes in substrate affinity, allosteric characteristics and subunit dissociation [8,14].

Reusability of the immobilized pectinase in batch system

Reusability studies are important because it creates insights into the potential capacity of enzymes in industrial uses since this directly affect economic feasibility of a bioprocess. Investigation of the reusability potential of the sponge-immobilized pectinase and its results are present in Fig. 3. Results showed that the immobilized enzyme preserved approximately 100% of its initial activity after four repeated cycles in batch operation. However, gradual decreases in the initial activity were seen with activities of approximately 97 and 84% after Cycles 6 and 8, respectively. Azimi et al. [5] reported a nearly 75% activity for pectinase immobilized on glass beads. De-Oliveira et al. [15] reported that pectinase immobilized by entrapment techniques lost nearly 70% of its initial activity after eight cycles of repeated uses. Generally, variations have been reported in the residual activity of immobilized pectinases after repeated uses in batch operation for pectin hydrolysis [10,32]. This variation could be associated to the nature of the enzyme and types of procedures used for enzyme immobilization [5]. Additionally, decreases in activity of the immobilized pectinase following repeated uses might be resulted from conformational changes in the enzyme and detachment of pectinase from the carrier surface. These decreases in activity could be linked to diffusional limitations of the reacting species. However, certain structural variations of the immobilized enzymes on supports prepared using various protocols cannot be ruled out, including enzyme distortions [5,33].

Juice clarification in sponge-immobilized packed-bed column reactor

Effects of the flow rate on juice clarification

Effects of luffa sponge-immobilized pectinase were assessed for the continuous clarification of orange juice in PBCR at various flow rates (Table 4). Based on the results, no significant decreases were seen in clarification capacity as the flow rate increased. At a flow rate of 0.5 ml/min, clarification capacity was reported as 89%. When the flow rate was doubled to 1.0 ml min^{-1}, clarification capacity decreased to 83%. This suggested that increased flow rate negatively affected the performance of the luffa sponge-immobilized pectinase, resulting in a smaller efficiency of clarification. Furthermore, decreases in clarification capacity were further reported at a greater flow rate of 3.0 ml min^{-1}, where the capacity significantly decreased to 30%. This substantial decrease in performance indicated that the immobilized pectinase was less effective at higher flow rates, leading to a poorer clarification. This lower clarification at a higher flow rate could be a result of the fact that at higher flow rates, contact time between the orange juice and the immobilized pectinase decreased, which might limit the immobilized pectinase ability to break down pectin and clarify the juice effectively. Similar results have previously been reported as this has been attributed to the substantial quantity of biocatalyst present in the PBCR per reaction volume [5,8,15]. Based on these results, a flow rate of 0.5 ml per minute was selected to investigate operational stability of the PBCR.

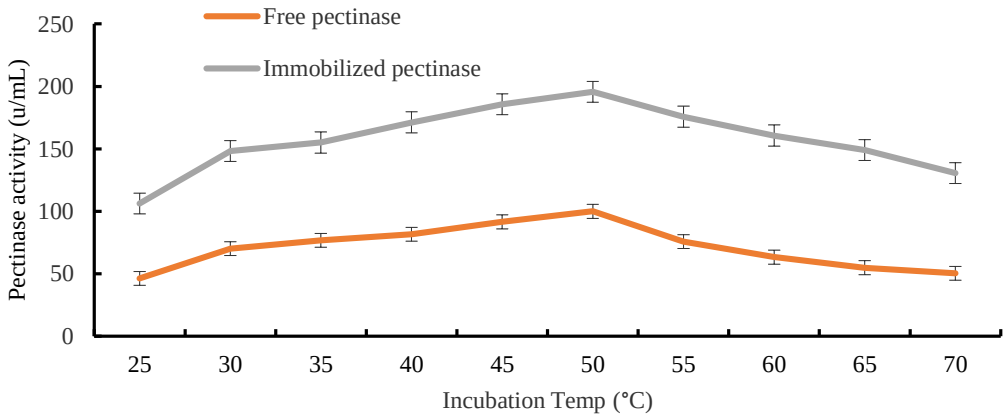

Figure 1a. Effects of various temperatures on the activity of luffa sponge-immobilized and free pectinases from *Aspergillus niger* FTR 002

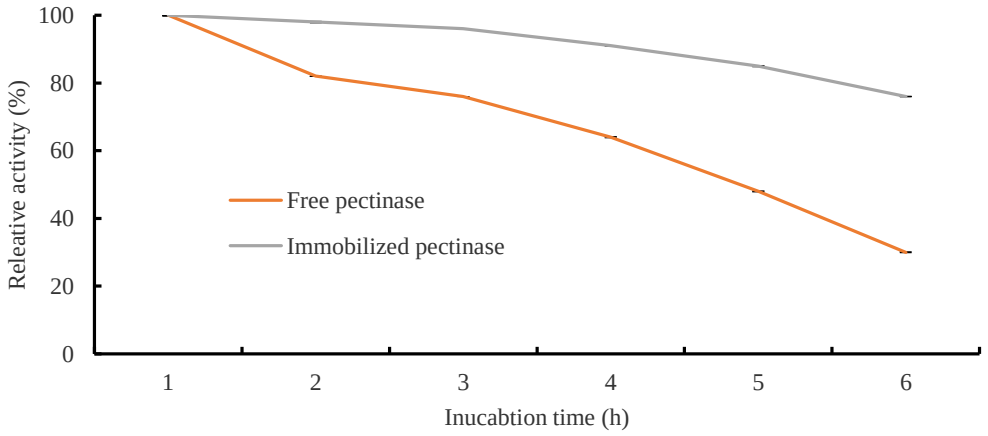

Figure 1b. Thermal stabilities of luffa sponge-immobilized and free pectinases produced by *Aspergillus niger* FTR 002. Incubation was carried out at 50 °C

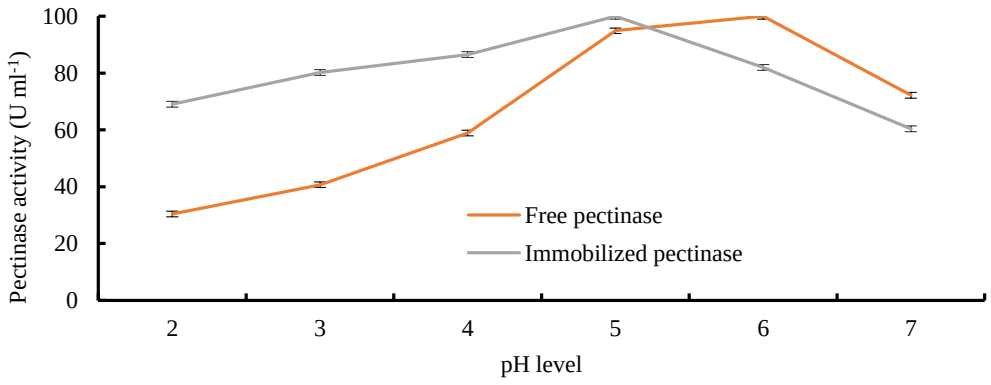

Figure 2a. Effects of various pH levels on the activity of free and luffa sponge-immobilized pectinases produced by *Aspergillus niger* FTR 002

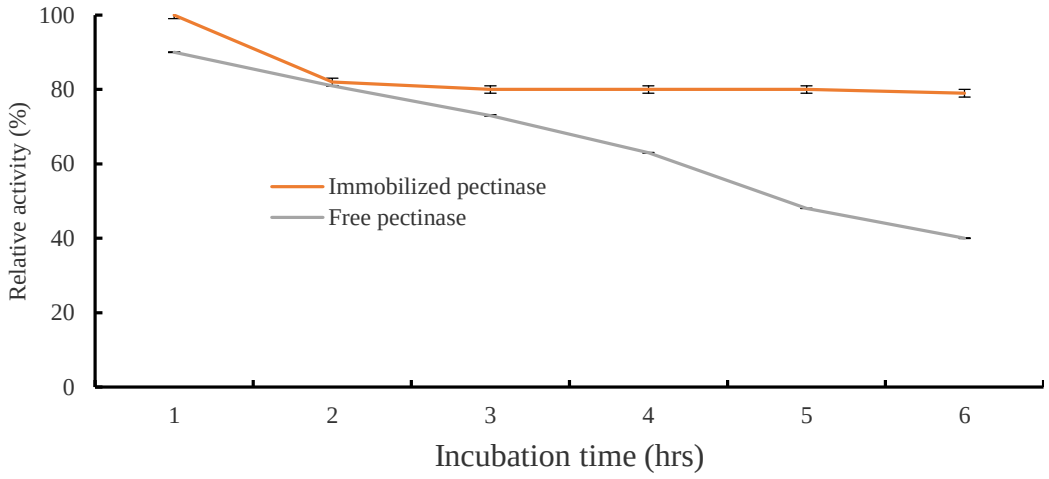

Figure 2b. The pH stability of luffa sponge-immobilized and free pectinases produced by *Aspergillus niger* FTR 002 at pH 5 and 6, repectively

Table 4. Effects of flow rate and residence time on the clarification capacity of luffa sponge-immobilized pectinase using packed-bed column reactor

Flow rate (ml min^{-1})	Residence time (min)	Clarification capacity (%)
0.5	15.7	*89 ± 0.02^{a}
1.0	7.82	83 ± 0.08^{b}
1.5	5.23	80 ± 0.05^{c}
2.0	3.9	46± 0.08^{d}
2.5	3.14	36 ± 0.21^{e}
3.0	2.61	30 ± 0.12^{e}

Data are means of three replicates. *Means with different letters within each column differ significantly ($p \leq 0.05$)

Results of the operational stability of the PBCR in juice clarification are shown in Fig. 4. Results showed linear decreases in clarification capacity of the luffa sponge-immobilized pectinase during 80 h of operation with 40% clarification capacity at Hour 80. During this 80 h of operation, steady increases in accumulation of the juice particles were seen in the PBCR. These findings were similar to those by Dal Magro et al. [8], who also reported linear decreases in the catalytic capacity within 54 h of operation. They detected increases in accumulation of juice particles inside the reactor over time, leading to similar results. Although accumulation of the juice particles is not desirable, periodic washing of the PBCR to remove the accumulated juice particles has been suggested by Azimi et al. (2021) as a way of increasing its efficiency.

Physicochemical characteristics of the orange juice samples

Investigation results of the physicochemical characteristics of the raw and pectinase-treated (using free and immobilized pectinase samples) are illustrated in Table 5.

Table 5. Physicochemical characteristics of the juices clarified with free and luffa sponge-immobilized pectinases

	Raw Juice	Clarified		
		*FP	LSIPB	LSIPC
Pectin	Present	Not detected	Not detected	Not detected
TTA	*0.34±0.12^{c}	0.51±0.02^{a}	0.41±0.06^{b}	0.44±0.11^{b}
pH	3.8±0.11^{a}	3.7±0.21^{b}	3.7±0.42^{b}	3.7±0.08^{b}
TSS	7.8±0.02^{a}	5.2±0.11^{d}	6.0±0.05^{b}	6.2±0.08^{c}
%Transmittance	0.85±0.06^{c}	6.5±0.05^{b}	8.0±0.03^{a}	8.0±0.07^{a}
Reducing sugar conc (mol ml^{-1})	3.84±0.09^{d}	4.35±0.11^{c}	6.82±0.22^{b}	7.35±0.04^{a}

Data are means of three replicates. *Means with different letters within each row differ significantly ($p \leq 0.05$). FP- free pectinase, LSIPB and sponge-immobilized pectinase in batch process, LSIPC and sponge-immobilized pectinase in continuous process

As shown in the table, increases ($p \leq 0.05$) in total titratable acidity (TTA) were recorded with the clarification of orange juice by free and immobilized pectinases while mild decreases in pH were recorded. Similar results have previously been documented in the clarification of various juice samples. These were linked to the liberation of organic acids, particularly galacturonic acid, resulting from the pectin hydrolysis by pectinase enzymes [5,15,34]. Moreover, decreases ($p \leq 0.05$) in total suspended solids (TSS) of the juice were reported after clarification of the orange juices. These decreases in TSS have previously been reported and suggested due to the effective removal of suspended solid particle in the juice which contributed to the TSS assessment [34]. Results of the investigating sugar concentrations of the treated and untreated raw juices revealed increases in sugar concentration of the juice after enzymatic treatments (Table 5). Similar results have previously been reported by [35,36]. Increases in sugar concentrations were linked to the possible releases of these sugars by pectin hydrolysis [2].

Physicochemical investigation of the raw and pectinase-treated orange juices revealed increases in the transmittance after treatment of the raw juice with the immobilize pectinase (Table 5). Diano et al. [37] reported increases in juice transmittance following a 2-h clarification treatment with pectinase immobilized on activated alginate-montmorillonite beads. They described that increases in transmittance with free pectinase were mildly higher than that with the immobilized pectinase. Increases in percentage transmit-ttance in the present study were similar to those in studies by Azimi et al. [5] and Deng et al. [36] when grape juices were clarified with pectinase immobilized on glass beads and calcium alginate microspheres, respectively. Increases in the proportion of transmittance in this study suggested that the luffa sponge-immobilized pectinase could be a potential candidate for the effective clarification of orange juice samples. Results of the investigation of pectin in raw and treated juice samples revealed that pectin was not detected in the enzyme treated samples after clarification of juice sample with free and immobilized pectinases (Table 5). Similar results were reported by Deng et al. [36] and Azimi et al. [5]. This could be due to the complete hydrolysis of the pectic polysaccharides by the enzyme. These findings indicated that batch and continuous methods using free and immobilized pectinases included promising results for use in the clarification of orange juices.

4. Conclusion

In this study, purified pectinases from *A. niger* FTR 002 were successfully immobilized on luffa sponges using covalent boding with glutaraldehyde as a cross-linking agent. Characteristics of the native and immobilized pectinases were investigated and then used in the clarification of orange juices. To the best of the authors' knowledge, this is the first study reporting pectinase immobilization using luffa sponge as carrier. The temperature optimum of 50 °C, pH of 5.0 and K_m and V_{max} of 1.612 mM and 0.019 µM/min respectively with orange clarification capacity of 40% after an 80-h of continuous operation in PBCR suggests that luffa sponge-immobilized pectinase includes a great potential use as a biocatalyst in clarification of orange juices.

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6. Conflict of Interest

The authors declare no conflict interest.

References

1. Singh A, Varghese LM, Battan B, Patra AK, Mandhan RP, Mahajan R. Eco-friendly scouring of ramie fibers using crude xylano-pectinolytic enzymes for textile purpose. Environ. Sci Pollut Res. 2020; 27: 6701-6710. https://doi.org/10.1007/s11356-019-07424-9

2. Hosseini SS, Khodaiyan F, Mousavi SM, Azimi SZ. Continuous clarification of barberry juice with pectinase immobilised by oxidised polysaccharides. Food Technol Biotechnol. 2021; 59(2): 174-184. https://doi.org/10.17113/ftb.59.02.21.6976

3. Maroufi LY, Rashidi M, Tabibiazar M, Mohammadi M, Pezeshki A, Ghorbani M. Recent advances of macromolecular hydrogels for enzyme immobilization in the food products. Adv Pharm Bull. 2022; 12(2): 309-318. https://doi.org/10.34172/apb.2022.043

4. Rehman H, Baloch AH, Nawaz MA. Pectinase: immobilization and applications. A review Trends Pept Protein Sci. 2021; 6:1-16. https://doi.org/10.22037/tpps.v6i.33871

5. Azimi SZ, Hosseini SS, Khodaiyan F. Continuous clarification of grape juice using a packed bed bioreactor including pectinase enzyme immobilized on glass beads. Food Biosci. 2021; 40: 100877. https://doi.org/10.1016/j.fbio.2021.100877

6. Setegn H, Abate A. Pectinase from microorganisms and its industrial applications. Sci World J. 2022:1-15. https://doi.org/10.1155/2022/1881305

7. Nouri M, Khodaiyan F. Magnetic biocatalysts of pectinase: Synthesis by macromolecular cross-linker for application in apple juice clarification. Food Technol Biotechnol. 2020; 58(4): 391-401. https://doi.org/10.17113/ftb.58.04.20.6737

8. Dal Magro L, Pessoa JPS, Klein MP, Fernandez-Lafuente R, Rodrigues RC. Enzymatic clarification of orange juice in continuous bed reactors: Fluidized-bed versus packed-bed reactor. Catal. Today. 2021; 362:184-191. https://doi.org/10.1016/j.cattod.2020.02.003

9. Behram T, Pervez S, Nawaz MA, Ahmad S, Jan AU, Ur Rehman HU, Ahmad S, Khan NM, Khan FA. Development of pectinase based nanocatalyst by immobilization of pectinase on magnetic iron oxide nanoparticles using glutaraldehyde as crosslinking agent. Molecules. 2023; 28(1): 404. https://doi.org/10.3390/molecules28010404

10. Qi D, Gao M, Li X, Lin J. Immobilization of pectinase onto porous hydroxyapatite/calcium alginate composite beads for improved performance of recycle. Ame Chem Soc. 2020; 5: 20062-20069.

11. Mahesh M, Arivizhivendhan KV, Maharaja P, Boopathy R, Hamsavathani V, Sekaran G. Production, purification and immobilization of pectinase from *Aspergillus ibericus* onto functionalized nanoporous activated carbon (FNAC) and its application on treatment of pectin containing wastewater. J Mol. Catal B: Enzym. 2016; 133: 43-54. https://doi.org/10.1016/j.molcatb.2016.07.012

12. Dal Magro L, Hertz PF, Fernandez-Lafuente R, Klein MP, Rodrigues RC. Preparation and characterization of a Combi-CLEAs from pectinases and cellulases: A potential biocatalyst for grape juice clarification. Royal Society Advance Chem. 2016; 6(32): 27242-27251. https://doi.org/10.1021/acsomega.0c01625

13. Hassan SS, Williams GA, Jaiswal AK. Computational modelling approach for the optimization of apple juice clarification using immobilized pectinase and xylanase enzymes. Curr. Res Nutr Food Sci. 2020; 3: 243-255. https://doi.org/10.1016/j.crfs.2020.09.003

14. Tasgin E, Babagil A, Nadaroglu H, Allegretti PE. Immobilization of purified pectin lyase from *Acinetobacter calcoaceticus* onto magnetic carboxymethyl cellulose nanoparticles and its usability in food industry. J Chem. 2020:1-12. https://doi.org/10.1155/2020/4791408.

15. 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. https://doi.org/10.1016/j.fbp.2018.02.005

16. Garuba EO, Onilude AA. Immobilization of thermostable exo-inulinase from mutant thermophilic *Aspergillus tamarii*-U4 using kaolin clay and its application in inulin hydrolysis. J Genet Eng Biotechnol. 2018; 16(2): 341-346. https://doi.org/10.1016/j.jgeb.2018.03.009

17. Manivasagan P, Oh J. Production of a novel fucoidanase for the green synthesis of gold nanoparticles by *Streptomyces* sp. and Its cytotoxic effect on heLa cells. Mar Drugs. 2015; 13: 6818-6837. https://doi.org/10.3390/md13116818

18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent, J Biol Chem. 1951; 193(1): 265-275.

19. Saxena S, Shukla S, Thakur A, Gupta R. Immobilization of polygalacturonase from *Aspergillus niger* onto activated polyethylene and its application in apple juice clarification. Acta Microbiol Immunol Hung. 2008; 58(1): 351-355. https://doi.org/10.1556/amicr.55.2008.1.3

20. Madu JO, Nkem T, Emuebie OR, Isreal JE, Kayode AF. Physicochemical factors influencing pectinolytic enzyme produced by Bacillus licheniformis under submerged fermentation. Nat Sci. 2014; 12(8):110-116.

21. Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 1959; 31:426-428. http://dx.doi.org/10.1021/ ac60147a030

22. IBM SPSS, V. 20, IBM Corp., Armonk, NY, USA; 2020. Available from: https://www.ibm.com/products/spss-statistics

23. Soozanipour A, Taheri-Kafrani A, Barkhori M, Nasrollahzadeh M. Preparation of a stable and robust nanobiocatalyst by efficiently immobilizing of pectinase onto cyanuric chloride-functionalized chitosan grafted magnetic nanoparticles. J Colloid Interface Sci. 2019; 536: 261-270.

24. Dal Magro L, de Mouraa KS, Backes BE, de Menezes EW, Benvenutti EV, Sabrina Nicolodic S, Kleind MP, Fernandez-Lafuentee R, Rodrigues RC. Immobilization of pectinase on chitosan-magnetic particles: Influence of particle preparation protocol on enzyme properties for fruit juice clarification. Biotechnol Rep. 2019; 24: e00373. https://doi.org/10.1016/j.btre.2019.e00373

25. Basso A, Serban S. Industrial applications of immobilized enzymes-A review. Molecular Catalysis. 2019; 479: 110607. https://doi.org/10.1016/j.jcis.2018.10.053

26. Ramirez HL, Briones AI, Ubeda J, Arevalo M. Immobilization of pectinase by adsorption on an alginate-coated chitin support. Biotecnol Apl. 2013; 30:101-104.

27. Nawaz A, Javed R, Munir M, Hanif M, Mukhtar H. Extraction, purification and industrial applications of pectinase: a review. J Biotechnol Biores. 2018; 1(1) 1-6.

28. Modenez AI, Sastre DE, Moraes CF, Marques Netto CG. Influence of glutaraldehyde cross-linking modes on the recyclability of immobilized lipase B from *Candida antarctica* for transesterification of soy bean oil. Molecules 2018; 23(9): 2230. https://doi.org/10.3390/molecules23092230

29. Holyavka MG, Goncharova SS, Sorokin AV, Lavlinskaya MS, Redko YA, Faizullin DA, Baidamshina DR, Zuev YF, Kondratyev MS, Kayumov AR, Artyukhov VG. Novel biocatalysts based on bromelain immobilized on functionalized chitosans and research on their structural features. Polymers. 2022; 14(23):5110. https://doi.org/10.3390/polym14235110

30. Kadam AA, Shinde SK, Ghodake GS, Saratale GD, Saratale RG, Sharma B, Hyun S, Sung J-K. Chitosan-grafted halloysite nanotubes-Fe3O4 composite for laccase-immobilization and sulfamethoxazole-degradation. Polymers. 2020; 12: 222. https://doi.org/10.3390/polym12102221

31. Hosseini SS, Khodaiyan F, Kazemi M, Najari Z. Optimization and characterization of pectin extracted from sour orange peel by ultrasound assisted method. Int J Biol Macromol. 2019; 125: 621-629. https://doi.org/10.1016/j.ijbiomac.2018.12.096

32. Sahin S, Ozmen I. Immobilization of pectinase on Zr-treated pumice for fruit juice industry. J Food Process Preserv. 2020; 44 (8): e14661 https://doi.org/10.1111/jfpp.14661

33. Liu C, Zhang L, Tan L, Liu Y, Tian W, Ma L. Immobilized crosslinked pectinase preparation on porous ZSM-5 zeolites as reusable biocatalysts for ultra-efficient hydrolysis of β-glycosidic bonds. Front Chem. 2021; 9: 677868. https://doi.org/10.3389/fchem.2021.677868

34. Bilal M, Asgher M, Iqbal HMN, Hu H, Zhang X. Delignification and fruit juice clarification properties of alginate-chitosan-immobilized ligninolytic cocktail. LWT Food Sci Technol. 2017; 80: 348-54. https://doi.org/10.1016/j.lwt.2017.02.040

35. Ramirez HL, Gomez Brizuela L, Ubeda Iranzo J, Arevalo-Villena M, Briones Perez AI. Pectinase immobilization on a

chitosan-coated chitin support. J Food Process Eng. 2016; 39(1):97-104. https://doi.org/10.1111/jfpe.12203

36. Deng Z, Wang F, Zhou B, Li J, Li B, Liang, H. Immobilization of pectinases into calcium alginate microspheres for fruit juice application. Food Hydrocoll. 2019; 89:691-699. https://doi.org/10.1016/j.foodhyd.2018.11.031

37. Diano N, Grimaldi T, Bianco M, Rossi S, Gabrovska K, Yordanova G, Godjevargova T, Grano V, Nicolucci C, Mita L, Bencivenga U, Canciglia P, Mita DG. Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors. J Agric Food Chem. 2008; 56:11471-11477. https://pubs.acs.org/doi/10.1021/jf8019437

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خواص پکتیناز *آسپرژیلوس نایجر* FTR 002تثبیت شده بر اسفنج لوفا و کاربرد آن در شفاف سازی پیوسته آب پرتقال در بیوراکتور ستونی پر شده با بستر

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چکیده

سابقه و هدف: یکی از چالش‌های اصلی جفت کردن آنزیم‌ها به ماتریس‌های پشتیبان نامحلول، استفاده از ماتریس‌های پشتیبانی غیرطبیعی است که گران هستند و به آسانی در دسترس و قابل استفاده نیستند زیرا اغلب اوقات به انجام فرآیندی چند مرحله‌ای نیاز دارد. در نتیجه، موضوع بررسی، مواد طبیعی با پتانسیل کارکرد به‌عنوان ماتریس پشتیبان برای تثبیت آنزیم که ارزان، قابل دسترس بدون نیاز به تغییرات یا تغییرات کمی لازم داشته باشد، بررسی شده‌اند.

مواد و روش ها: در این مطالعه سعی شد از الیاف نارگیل، ساقه کاذب موز، گچ پاریس و اسفنج لوفا به‌عنوان حامل‌های مناسب برای تثبیت پکتیناز تولید شده توسط *آسپرژیلوس نایجر* FTR 002استفاده و خالص‌سازی با روش‌های کروماتوگرافی، با استفاده از جذب فیزیکی و پیوند کووالانسی با استفاده از گلوتارالد انجام شود. خواص آنزیم‌های بومی و تثبیت شده مورد مطالعه قرار گرفت و سپس در شفاف سازی آب میوه در یک بیوراکتور ستونی پر شده با بستر (Packed Bed Column Bioreactor) با استفاده از روش های استاندارد مورد استفاده قرار گرفت.

یافته‌ها و نتیجه‌گیری: از چهار ماده مورد بررسی، اسفنج لوفا بالاترین بازده تثبیت را داشت. پس از آماده سازی پکتیناز آزاد و تثبیت شده بر اسفنج لوفا مشخص شد دمای بهینه برای هر دو آماده سازی آنزیمی ۵۰ درجه سلسیوس می‌باشد با این حال، پس از تثبیت پکتیناز بر اسفنج لوفا، pH بهینه از ۶/۰ به ۵/۰ تغییر کرد. K_m و V_{max} پکتینازهای آزاد و تثبیت شده به‌ترتیب mM ۲/۳۳، µM/min ۰/۰۱۸ و mM ۱/۶۱۲، µM/min ۰/۰۱۹ بود. مطالعات قابلیت استفاده مجدد نشان داد که در عملیات ناپیوسته، آنزیم تثبیت شده تقریباً ۵۵ درصد از فعالیت اولیه خود را پس از پانزده تکرار چرخه حفظ کرد. شفاف‌سازی آب پرتقال با استفاده از پکتیناز تثبیت شده بر اسفنج لوفا در بیوراکتور ستونی پر شده با بستر (Packed Bed Column Bioreactor) با سرعت جریان ml/min ۰/۵ نشان داد که کاتالیزور تثبیت شده ۴۰ درصد از ظرفیت شفاف‌سازی خود را پس از ۸۰ ساعت عملیات پیوسته حفظ می‌کند. این یافته‌ها نشان می‌دهد که پکتیناز تثبیت‌شده بر اسفنج لوفا به‌عنوان یک کاتالیزور شفاف کننده در صنعت آب میوه توانایی امیدوارکننده‌ای دارد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- تثبیت آنزیم
- پکتیناز
- شفاف‌سازی آب‌میوه
- ضایعات کشاورزی

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