

Optimization of *Aspergillus niger* NRC1ami pectinase using citrus peel pectin, purification, and thermodynamic characterization of the free and modified enzyme

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Abstract

Enzyme cost and stability are the main problems facing industrial applications. Consequently, *Aspergillus niger* NRC1ami was isolated from rotten orange and recorded a promising pectinase activity (13.8 U/ml). The enzyme was optimized using citrus peel pectin as the sole carbon source and recorded (40 U/ml). It was purified by two steps purifications and recorded 632 purification folds. The pure enzyme showed 14.7% carbohydrate content and consists of 15 amino acids. Glutamic acid was the major (22%) followed by leucine (10.67%) and threonine was the minor (2.70%). *A. niger* NRC1ami pectinase was conjugated by covalent coupling to sodium periodate (NaIO_4) activated polysaccharides. Galactomannan showed the highest recovered activity (85%) and 94.34% reduction in viscosity. The optimum temperature for the pure enzyme shifted from 40°C to 45°C after the conjugation process. Also, the free enzyme showed its optimum activity at pH 5 compared to pH 4, 5 in the conjugated form case. The thermal stability of the free enzyme greatly improved after the modification process. The conjugated process reduced the activation energy to 36%, prolonged the enzyme half-life 5.6-fold at 60°C, and increase the deactivation energy (E_d) by about 19% in comparison to the free form. The D value of the conjugated enzyme increased to 13.2-fold at 50°C in compared to the free form. Gibbs's free energy (ΔG) of the enzyme increased after the modification process, while the enthalpy (ΔH) and entropy (ΔS) decreased. Na^+ and Zn^{2+} had a stimulating effect on both enzyme forms.

Statement Of Novelty

The process of modifying enzymes plays a major role in improving the properties of the enzyme and its ability to withstand difficult conditions such as high temperatures. The modification process using polysaccharides is considered one of the safest and preferred industrial methods. Until this moment, the process of modifying the pectinase using polysaccharides and correlating it with thermodynamic parameters is still rare. Despite this is of great importance in the industrial field application. This study tried to optimize *A. niger* NRC1ami pectinase using the citrus peel. The enzyme was purified and modified in different polysaccharides. The modification in galactomannan recorded the highest activity. A comparison studied between the physiological and the thermodynamic properties of the two forms was done.

Introduction

Pectinases are heterogeneous groups of enzymes catalyzing the degradation of the cell wall pectin component in plants and are produced by various fungi including *Aspergillus* sp. [1] *Penicillium* sp. [2], [3]. They are an essential class of enzymes according to their important uses in industries fields, such as: wine, paper, and food for fruits processing, vegetables, tea, and coffee. Pectinase enzymes that could work at high temperatures and suitable pHs conditions speed up the previous processes. Speed of processing shows an economic gain in industrial and commercial applications [4]. To further purify the enzyme, a combination of one or more techniques is applied, viz. affinity chromatography (AC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and gel filtration

chromatography [5]. The necessity of the use of citrus peel to develop a bioeconomy and to decrease its negative environmental impacts is assessed. Citrus peel is considered one of the most important wastes in biochemical processes through the bioconversion of this waste to fermentative products [6].

Enzymatic modification is the most potential technology to change their molecular structure to obtain desired properties, because of its advantages over chemical modification. Recently much attention has been focused on the chemical modification of pectinases and their catalytic performance by various researchers [7]. **Chiba and Kobayashi** [8] reported in the chemical modification of pectinase by 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and diethylpyrocarbonate (DEP) caused a loss of most of the enzyme activity, but the substrate binding ability was not impaired. Modified galactomannan by enzyme has tremendous potential to obtain desired properties because of its advantages over chemical modification [9]. Chemical modification of an enzyme may be used to change the overall properties of the enzyme surface or for the modification of key residues [7]. The most essential enzymes information comes from their kinetic and thermodynamic parameters. The thermodynamic studies are considered a good way that could explain the enzyme unfolding and denaturation process. The use of these combined parameters through correct mathematical tools could predict their behavior under conditions not tested experimentally [10], [11].

The results in this study showed that. *A.niger* NRC1ami was successfully optimized, purified, and showed the galactomannan superiority in chemical modification compared to all the used activated polysaccharides, it showed the highest retained specific activity and the highest recovered activity and reduction viscosity. The properties of the free and conjugated enzymes confirmed that the conjugation process succeeded to improve the *Aspergillus niger* NRC1amipectinase to great instant. The thermodynamic parameters of the modified pectinase for thermo-inactivation suggested a predominating mechanism of reversible un-folding, which was responsible for lower sensitivity to heating than free purified pectinase D value of the conjugated enzyme increased to 13.2-fold at 50°C in compared to the free form. Gibbs's free energy (ΔG) of the enzyme increased after the modification process, while the enthalpy (ΔH) and entropy (ΔS) decreased. Na^+ and Zn^{2+} had a stimulating effect on both the free and conjugated forms.

Materials And Methods

Fungal isolation and identification

The fungus was isolated from Egyptian orange fruit (Bousorra), purchased from the local market in Cairo city. Small parts of orange rut were gently shaken in a 250 ml flask containing 100 ml sterilized distilled water for 15 minutes on a mechanical wrist action, then serial dilutions were used. One ml of the desired dilution was transferred aseptically over the surface of Petri-dish containing an appropriate amount of potato dextrose medium and was spread and streaked by needle with streak method (3 ways method) so that the dilution suspension was dispersed above agar surface. After incubation within 4-7 days at 30°C, one colony was picked up by sterilized needle and re-cultivated on potato- dextrose medium (glucose, 20

g/L, agar, 15g/L, and potatoes infusion, 500 ml). The cultivation was repeated 3 times. The purity of the colony was examined under the visible microscope. The isolate was identified as *Aspergillus niger*NRC1ami by Cairo University, Micro Analytical Center, Cairo, Egypt. Potato dextrose agar was used for the fungal culture isolation, maintenance, and stock cultures.

Production medium and Inoculum preparation

The basal medium used for physiological studies of fungal strains had the following composition (g/L): Pectin from citrus peel, 15.0; KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 and KNO_3 , 2.5. The pH was adjusted to 5 before sterilization. The cultivation was done by adding 5 ml of sterile production medium to each of four slants (4 days old) scratched with a sterile needle. One ml of spore suspension was spread on Petri-dish containing an adequate amount of solidified potato-dextrose medium. After incubation at 30°C usually for 4 days, the cultivated medium was cut into equal discs with a sterilized cork borer and two disks were transferred to 250 ml Erlenmeyer flask each containing 50 ml of the culture broth in triplicate (number of replicates) and sterilized for 20 min at 121°C (1.5 atm.). The flasks were then incubated at 30°C on a rotator shaker at 150 rpm. After incubation for 4 days, the culture broth from each flask was filtered off to separate the mycelium from the culture filtrate. The latter was then centrifuged in a cooling centrifuge (JANEKTZKI, K 70, Germany). The clear culture filtrate was taken in which protein content and enzyme assays were determined.

Determination of mycotoxins

Cultivation of isolate A and purification of the crude extracts was done according to mycotoxin extraction [13].

Enzyme assays

The pectinase activity was determined using 1% (w/v) citrus pectin as substrate product of Fluka company Switzerland (0.3 ml of enzyme + 0.7 ml substrate for a free enzyme for 15 min at 40 °C. The liberated galacturonic acid content was determined by the Somogyi method [13].

One unit (U) of pectinase activity was defined as the amount of enzyme-producing 1 μmole galacturonic acid per min at 40°C and pH 4 (assay conditions).

Enzyme optimization parameters

Each experiment was done under optimized conditions. The effect of different incubation temperatures (20-55°C), incubation periods (24–168 h), different carbon sources (0.75 g/flask) such as (Pectin, citrus peel pectin, soluble starch, lemon peel, Guava (peel and pulp), orange peel, Garlic scale, onion scale),

different nitrogen sources were substituted at (2g/L) nitrogen such as (KNO₃, Beef, peptone, Casein, yeast extract, baker's yeast, AlCl₃, (NH₄)₂SO₄).

Viscosity measurement

Citrus pectin solution (1%) dissolved in 0.2M acetate buffer, pH 4 was used as the substrate. To 10 ml of the substrate solution, 1.5 ml of the enzyme sample was added, the reaction mixture was incubated at 40°C for 20 min in a water bath. At the end of the incubation period, 5 ml of the reaction mixture were pipetted into an Ostwald viscometer and the time of flow was recorded. Control tests were also run employing the enzyme sample which had been heated at boiling for 10 minutes. The percentage decrease in relative viscosity was expressed according to the following formula [14]:

$$A-B/A \times 100$$

Where:

A= Rate of flow in records of the heated enzyme in seconds (blank).

B= Rate of flow in records of the active enzyme in seconds.

Fractional precipitation by ethanol

A known volume (v/v %) of cold ethanol was added slowly to the ice-cold enzyme solution until the required concentration of ethanol was reached. After removing the precipitated fraction by centrifugation in a refrigerated centrifuge, further ethanol was added to the supernatant fluid and the process was repeated until the final concentration of ethanol was reached (90%). The enzyme fractions obtained at 30, 50, 70, and 90% concentrations of ethanol were dried over anhydrous calcium chloride under reduced pressure at room temperature and weighed. Each enzyme fraction was assayed for pectinases activity and protein content.

Purification of the fungal pectinases

Chromatography on DEAE-Sephadex A-50 column

A column of DEAE-Sephadex A-50 (2 x 30 cm) equilibrated with 0.02 M acetate buffer (pH 4) was used for chromatographic purification. The elution was performed at room temperature with 0-1 M sodium chloride gradient dissolved in 0.02 M acetate buffer, pH 4. 5 ml fractions were collected with a flow rate of 67 ml/ h from the top of the column by gravity feed (siphoning) system. Pectinases and protein contents were determined. Polyacrylamide gel disc electrophoresis was carried out according to Laemmli [15].

Determination of the total carbohydrate of the pure enzyme

The total carbohydrate content of the pure enzyme was determined after hydrolysis by the phenol-sulfuric acid method [16]. One milliliter of 5% phenol solution was added to 1 ml of the diluted enzyme (1:5) and 5 ml concentrated H₂SO₄ was added rapidly to the mixture, shaken, and set aside for 10 min at room temperature, then at 20–30°C (in a water bath) for 20 min. Thereafter, the color density was measured at 490 nm

Molecular weight determination of the pure enzyme

The molecular weight of the purified pectinases was determined by using SDS-gel electrophoresis. The protein standard used was Ferment as marker page Ruler TM plus pre-stained protein ladder SM1811.

The logarithmic molecular weight of the protein standard is plotted against the rate of flow which represents the distance migrated by the protein divided by the total distance migrated by the dye and the curve can be used to determine molecular weights of unknown proteins.

Amino acid composition of the purified pectinases

The pure enzyme samples were analyzed by high-performance amino acid analyzer LC3000 Eppendorf Germany in the lab of HPLC and amino acid analysis, National Research Center, using acid hydrolysis with hydrochloric acid and sample volume of 50 µl.

Chemical modification of the purified pectinase

Chemical modification of the pectinase was achieved according to Ben Ammar *et al.* [17].

Oxidation of polysaccharide

One gram of polysaccharide (agar, soluble starch, galactomannan, dextran 70.000, dextran 250.000, dextran 275.000, xylan and CMC) was dissolved into 40 ml 0.25M periodate solution and allowed to stand at 37°C for 6 h. After that, 1.2 ml ethylene glycol was added and allowed to react for 1 h. The reaction mixture was dialyzed against water overnight and then lyophilized [17].

Preparation of conjugated pectinases

The pure pectinase (0.1 g) was mixed with 0.5 of oxidized polysaccharide in acetate buffer (0.02M, pH 4) at 4°C for 1 h. After that, the conjugated enzyme was precipitated at 40 % ethanol. The precipitate was redissolved in acetate buffer (0.02M, pH 4) and used.

Comparison between the free and the conjugated enzyme properties

Optimum pH and temperature of the reaction

The optimum pH was determined in the range of pHs 4-10. The optimum temperature 30-70 °C was tested at the optimum pH and the enzyme assay was done as described above. E_a was calculated from the slope of the Arrhenius plot of $1000/T$ versus \ln [levansucrase relative activity] ($E_a = -\text{slope} \times R$), where R (Gas constant) = $8.314 \text{ J} \cdot \text{K}^{-1} \text{ mol}^{-1}$.

pH stability at 45°C and thermal stability at pH 4

The pH stability of the free and chemically modified enzymes was examined after pre-incubating the enzyme samples at 45°C from 30 to 120 minutes at different pH values, followed by adjusting the pH to the value of the standard assay system. Then the residual activity was assayed under the standard conditions. For thermal stability determination, the enzyme samples were incubated with acetate buffer (0.2 M, pH 4.0) at the design temperature (30-70°C) from 30 to 120 minutes and the residual activity was assayed under the standard conditions.

The K_d was estimated by regression plot of:

\log relative activity versus time (min). The $T_{1/2}$ and D -values for the pure and the conjugated pectinase was determined as followed:

$$T_{1/2} = \ln 2 / K_d$$

$$D\text{-value} = \ln 10 / K_d$$

The temperature rises necessary to reduce D -value by one logarithmic cycle. z value was calculated from the slope of the graph between $\log D$ versus T (°C) using the equation:

$$\text{Slope} = -1/z$$

The activation energy (E_d) for Purified and conjugated enzyme levansucrase denaturation was determined by a plot of \log denaturation rate constants ($\ln K_d$) versus reciprocal of the absolute temperature (K) using the following Eq.:

$$\text{Slope} = -E_d/R$$

The change in enthalpy (ΔH° , $\text{KJ} \cdot \text{mol}^{-1}$), free energy (ΔG° , $\text{KJ} \cdot \text{mol}^{-1}$) and entropy. (ΔS° , $\text{J} \cdot \text{mol}^{-1} \text{ K}^{-1}$) for thermal denaturation of levansucrase were determined using the following Eqs:

$$\Delta H = E_d - RT$$

$$\Delta G = -RT \ln (K_d \cdot h / K_b \cdot T)$$

$$\Delta S = \Delta H - \Delta G / T$$

Effect of different salts on pectinases activity

Some metal ions ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{SO}_4$, ZnSO_4 , Na_2SO_4 , Mn_2SO_4 , CaCl_2 , NaCl , CuSO_4 , $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, FeCl_3 , EDTA disodium salt dehydrated, Ascorbic acid, KI/I_2 , and HgCl_2) each was added separately to the culture medium at 0.02M final concentration.

Results

Isolate A was isolated from rotten orange. It was detected as a good pectinase producer and identified as *Aspergillus niger* NRC1ami. The results appeared the absence of mycotoxins types (alpha-, ochra and zeralenon). This result assured the fungus's safety.

Enzyme optimization

A. niger pectinase optimization was done to evaluate the best pH and temperature, incubation period, carbon, and nitrogen sources (Table 1). The most favorable conditions for enzyme production were 30°C, pH5, and 96 h. Several wastes and cheap materials were used as carbon sources as shown in Table (1). Citrus peel pectin was detected as the best carbon source since it was recorded (23.88 U/ml), Followed by (orange peel) (20.89 U/ml). The best nitrogen source for pectinase production was baker's yeast and recorded (40.78 U/ml).

Purification of pectinases by chromatography on DEAE-Sephadex A-50 column

In this experiment, the pectinase was partially purified by fractional precipitation with ethanol. The fraction precipitated at 50% showed the highest specific activity (155.16 U/mg) and purification fold (5.3). It was introduced to a column of DEAE-Sephadex A-50. Stepwise elution was performed at room temperature with NaCl at different molarities (0.1 – 1 M) dissolved in 0.02 M acetate buffer pH 5.0. The fractions were collected, and their enzyme activity and protein content were determined.

The results (Fig.1A) indicated that the first 11 fractions were devoid of any protein or pectinase activity. The activity appeared in one peak covered by fractions 21-30 which had 92.23 % total recovered activity. Fractions no 26 had the most potent pectinase activity (424 U/ml) recording 17.85 % recovered activity and 861 fold purification related to the culture filtrate (Table 2), respectively. The fractions with maximum

pectinase activity (25 - 27) were pooled and lyophilized and subjected to SDS-Polyacrylamide gel electrophoresis to test its purity. As shown in Fig. (1B) the purified pectinase had a molecular weight of 36 KDa and showed a single band, indicating its purity and homogeneity.

Chemical modification of pectinase enzymes

The present series of experiments was undertaken to modify the *Aspergillus niger* NRC1 amipectinase by glycosylation (immobilization to water-soluble carrier). Glycosylation of the enzyme was achieved by covalent coupling to sodium periodate (NaIO_4) activated polysaccharides such as soluble starch, agar, galactomannan, xylan, CMC, dextran (MW: 70,000-200,000 and 275,000).

The results (Table 3) indicated that the conjugated enzyme with galactomannan showed the highest retained specific activity (29225U/mg protein) and the highest recovered activity (85%) and 94.34% reduction in viscosity, followed by agar which recorded 78.69% recovered activity. Comparatively, the conjugated enzyme with dextran (MW: 185,000) showed the lowest retained specific activity (21.46 U/mg protein) and 54.58% reduction in viscosity. Thus, *A. niger* NRC1 amipectinases conjugated to galactomannan was used throughout this study.

Properties of the free and galactomannan conjugated pectinases

Since the glycosylated enzyme with galactomannan. Therefore, the properties of the enzyme-conjugated with galactomannan will be studied.

Effect of reaction time on the activity of free and conjugated pectinases by *A. niger* NRC1 ami

The activities of the free and conjugated pectinases were assayed at various incubation times (5- 60 min). Both the free and conjugated enzymes had the maximum activity after 15 min. with 100 % relative activity and the reduction in viscosity was 92.12% and 94.34%, respectively. Increasing or decreasing the period of the incubation time decreased the enzyme activity (data not shown).

Effect of reaction temperature on the activity of free and conjugated pectinases by *A. niger* NRC1 ami

The activities of the free and conjugated pectinases were assayed at various temperatures (30- 70°C). The free enzyme had an optimum temperature at 40°C whereas that of the conjugated enzyme was 45 °C

(Fig 2A). The Arrhenius plot result showed that E_a recorded $14.585 \text{ KJ.mol}^{-1}$ and $10.705 \text{ KJ.mol}^{-1}$ for the free and conjugated enzyme respectively Fig. (2B).

Effect of reaction pH on the activity of free and conjugated pectinases by *A. niger* NRC1ami

The effect of the reaction pH of the free and conjugated pectinases was studied (pH 3.0 – 10.0). The results (Fig. 3) indicated that the free enzyme had an optimum pH of 4.0. On the other hand, the pH was shifted to 5.0 in the case of conjugated form.

Thermal stability of the free and conjugated pectinases by *A. niger* NRC1ami

The rates of heat inactivation of the free and conjugated pectinases were investigated at temperatures of 40, 45, 50, 60, and 70 °C. The data (Fig. 4a, b) showed that the conjugated enzyme showed promising thermostability compared to the free enzyme. For example, the conjugated enzyme retained 44.67% of its original activity when heated for 120 min. at 60°C, whereas at this temperature the free enzyme completely lost its activity. The Deactivation rate constants (K_d) were evaluated from Fig. (5 c, d) at 45 °C, 50 °C, 55 and 60 °C. The deactivation energy evaluation (E_d) (Fig. 6) recorded 2.1 KJ.mol^{-1} , 2.5 KJ.mol^{-1} for PP and PF, respectively. The conjugation process increased the half-life at different temperatures compared to the free form. For instance, the half-life was prolonged from 105 to 1380 minutes at 50°C, also the D value increased 13.50 -fold after the conjugation process. Gibbs's free energy (ΔG) showed a noticeable increase in ΔG after the modification process. For instance, at 45 °C ΔG increased from 88.8 to 90.70 kJ/mol and at 60 °C it was elevated from 85.97 to 90.34 kJ/mol. On contrary, ΔG and ΔS of the modified form decreased in comparison to the free form (Table 4).

pH stability of the free and conjugated pectinases by *A. niger* NRC1ami

In the present experiment, the pure or the conjugated enzyme was incubated with buffers that have different pH values. This experiment was done at 40 °C in the absence of the substrate and for different time intervals from 30 to 120 min. Thereafter, the activity was determined under the standard conditions. The results (Table 5) showed that as the pH increases, the enzyme activity decrease. This result was more pronounced with the pure than the conjugated enzyme.

Effect of various metal salts and compounds on the activity of free and conjugated pectinases by *A. niger*

NRC1ami

In this experiment, the free and conjugated enzymes were incubated with metal salts with a concentration (0.02M) for 15 minutes at 45°C then the substrate was added and incubated at the optimum conditions for each enzyme. The control was assayed without added metal salts.

The results indicated that Hg^{2+} , Al^{3+} , Fe^{3+} , and I^{3+} had inhibitory effects on both free and conjugated enzyme activity. On the other hand, NH_4^+ , Na^+ , Zn^{2+} , Mn^{2+} , and ascorbic acid had a stimulating effect on the activity of both free and conjugated enzymes where the relative activity equal 115%, 135%, 130%, 110%, 120% for the free enzyme and 123%, 146%, 133%, 116%, 122% respectively. EDTA, Ca^{2+} had a non-noticeable stimulating effect on free the enzymes (105%, 104%) respectively, but the stimulatory effect was more observed on the conjugated enzyme (112%, 110%) respectively. Mg^{2+} had o effect on enzyme activities for both enzymes. Cu^{2+} had an inhibitory effect on both free and conjugated enzymes, as well as coagulation, appeared, it completely inhibited pectinase activity (data not shown). Fe^{3+} , I^{3+} and Hg^{2+} had adverse effect in enzyme activity of both enzyme forms (71%, 55%, 27% for the free enzyme respectively) but the conjugated form was more tolerant to Fe^{+3} and I^{+3} (90%, 68%, respectively) and more sensitive to Hg^{+2} (22%).

Discussion

The *isolate A* cultivated under submerged culture was detected as an efficient pectinase producer. No kind of mycotoxins was noticed in the isolate. The isolate safety is an important parameter to continue this study. This is due to the wide use of pectinases in the food industry. The isolate was identified as *Aspergillus niger* NRC1ami. Previously, many authors reported *Aspergillus niger* as good pectinase producers [18],[19]. Also, Heerd et al. [20] reported that the highest endo-pectinase activities were obtained by *A. sojae* ATCC20235. Similarly, Sarvamangala and Dayan [21] reported the production of endo-pectinase and exo-pectinase by *Aspergillus niger* in submerged culture (SMF).

The physiological properties studies influenced to great instant in enzyme productivity. From this finding, some physiological studies were achieved on *Aspergillus niger* NRC1ami. The results indicated that the most favorable temperature and pH for *Aspergillus niger* NRC1ami pectinase was 30 °C and pH5. A similar result was reported by Palaniyappan et al. [22]. It was reported that the maximum productivity of *Aspergillus niger* (MTCC:281) pectinase was at pH 5.5 and 30 °C after 72h. On the other hand, Patil et al. (2010) [23] found that the optimum conditions for *Penicillium* sp pectinase were at 35 °C, pH 6 after 72 h. It was reported that the range of enzymes that a microorganism produces, whether as free enzymes or as part of a complex, reflects the preferred carbon source as well as the microorganism's strategy for accessing this carbon source [24]. The results indicated that the most suitable substrate (carbon source) for pectinase production was citrus peel under submerged culture. Looking for inexpensive media for active pectinases production aiming to their use in juice and wines industries was reported by many authors [2]. Also, the utilization of agricultural by-products as low-cost substrates for microbial enzyme

production resulted in an economical and promising process [20]. On the other hand, **El-Enshasy [25]** deduced that the mandarin peel pectin used as the substrate for pectinase production would promote enzyme production.

Finally, **Et al.et al. (2012)** said lemon peel pomace is an attractive agro-industrial residue and alternative to produce high levels of pectinolytic enzymes, considering the adaptation of microorganisms. The effect of different nitrogen sources in *Aspergillus niger* NRC1ami was studied. The results indicated that the most promising nitrogen source for pectinase production was baker's yeast since the enzyme productivity was increased more than twofold. Previously many authors reported in baker's yeast role in enzyme activation [26], [27]. It was reported that yeast extract (YE), peptone and ammonium chloride were found to enhance *Bacillus* sp. pectinases production up to 24% [28].

Most of the enzyme properties are clearly and reliably revealed only with purified enzymes. The *Aspergillus niger* NRC1ami pectinase was eluted as one band by using stepwise elution and DEAE Sephadex A-50. The most promising fractions showed 16.35% recovered activity accompanied with 18523 specific activity and 632 purification fold. This result is superior to the result obtained by polygalacturonase from *Aspergillus niger* (SA6) where the purification fold was 9- with a yield of 0.18% and specific activity of 246 $\mu\text{mole/ml/mg}$ [29].

The molecular mass of the *Aspergillus niger* NRC1ami pectinase was estimated to be 38 KDa. Similar was this result it was reported that the molecular mass of the enzyme was estimated as 38 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [30]. This mass is like those of pectin methyl Esterases previously isolated from bacteria, fungi, and plants [30].

Also, the carbohydrate content of the enzyme was evaluated to be 14%. This result indicated the glycoprotein nature of *Aspergillus niger* NRC1ami pectinase. The amino acid composition referred to the superiority of the glutamic acid followed by leucine in enzyme structure where they represented about 34%.

Enzymatic modification is the most potential technology to change their molecular structure to obtain desired properties, because of its advantages over chemical modification. Recently much attention has been focused on the chemical modification of pectinases and their catalytic performance by various researchers [7]. **Chiba and Kobayashi [8]** studied the chemical modification of pectinase by 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) and diethylpyrocarbonate (DEP) caused a loss of most of the enzyme activity, but the substrate binding ability was not impaired. The results in this study referred to the galactomannan superiority in chemical modification compared to all the used polysaccharides, it showed the highest retained specific activity and the highest recovered activity and reduction viscosity. Modified galactomannan by enzyme has tremendous potential to obtain desired properties because of its advantages over chemical modification [9]. The properties of the free and conjugated enzyme confirmed that the conjugation process improved the *Aspergillus niger* NRC1ami stability to a great instant. The results showed that the optimum pH and temperature were shifted by the chemical modification.

Chemical modification of an enzyme may be used to change the overall properties of the enzyme surface or for the modification of key residues [10].

The optimum temperature for the pure pectinase recorded that 40 °C was the best while the conjugation process shifted it to 45°C. Also, the conjugation process reduced the amount of energy needed to activate the enzyme (E_a). This result referred to the economic validity of the process. The activation energy (E_a) is considered an important parameter to determine the enzyme cost [12]. The results showed that chemical modification by galactomannan had a pronounced effect on the thermal and pH stability of the enzyme. This result could be explained that such cross-linking occurs between different structural elements of a protein, it will typically enhance structural rigidity and therefore increase protein stability concerning agents that induce conformational changes (such as chaotropic agents or heat) [31]. The E_d increased to some extent after the conjugation process. It is worth mentioning that high E_d values are greatly demanded in industrial enzyme applications because this referred to the high thermostability [32]. Also, the enzyme half-life and D values greatly improved. The same result was obtained by *Aspergillus awamori* KX943614 immobilized on grafted alginate-agar gel beads where the immobilization process increased the half-life and the D-values [12]. The negative values of entropy ΔS° kJ mol⁻¹ for both the free and modified forms mean that process is an in-order state, where the modified was in a more orderly state in comparison to the free enzyme confirms the dramatic reduction of disorder degree in the disruption of enzyme structure [33] expected by enzyme modification. In this study ΔH for both enzyme forms were lower than ΔG , also ΔH of the modified form was lower than the free forms at all examined temperatures. It was mentioned that the enzyme stabilization is parallel by a reduction in the values of ΔS^* (entropy of deactivation) and ΔH^* (enthalpy of deactivation) [34]. The results showed that ΔG^*d for the modified form was always higher than that for the free form, which means that, despite a more favorable entropic contribution, the enzyme molecules bound to the outer surface of galactomannan were high protected than the free ones against the adverse surrounding environment conditions [35]. The raise of Gibbs free energy (ΔG) after the modification process indicated the decrease in the enzyme thermal unfolding [36]. Na₂SO₄, ZnSO₄, ascorbic acid, MnSO₄ activate the enzyme activity with varying degrees. EDTA, CaCl₂, NaCl slightly affect the enzyme activity.

Conclusion

*Aspergillus niger*NRC1amipectinase was optimized using citrus peel pectin to reduce the medium cost. The enzyme was successfully purified through two purification steps. The enzyme was modified by covalent coupling to activated polysaccharides. Among all the used polysaccharides the modification in galactomannan recorded the best-recovered activity and viscosity reduction. The properties and the thermodynamic characterization of the free and modified were studied. They confirmed that the modification process improved the enzyme stability to great instant and recommended *Aspergillus niger*NRC1ami pectinase to be used in the industrial field.

Declarations

Acknowledgment

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Declarations Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Competing Interests

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Author statement

Author contribution

Amira A. Gamal: Performed the practical work, Zeinat Kamel: Analyzed and interpreted the data, Mona A Esawy: designed the work, write the manuscript and putting it in the final form. All authors interpreted of results, drafted manuscript preparation, and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analysed during the current study are not publicly available

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Tables

Table (1)

The effect of different environmental and physiological parameters in *A. niger* NRC1ami pectinase (continue)

Temperature °C	20	25	30	37	40	45	50	55
Pectinase activity (U/ml)	07.53 ± 1.87	08.92 ± 1.61	13.58 ± 0.54	06.21 ± 0.81	04.95 ± 0.98	03.55 ± 1.43	02.33 ± 1.25	1.30± 0.85
Viscosity reduction (%)	19.21	25.22	39.1	30.22	22.26	17.82	12.23	6.33
pHs	4	5*	6	7	8	9	10	-
Pectinase activity (U/ml)	12.76 ± 0.63	13.77 ± 0.42	11.16 ± 1.41	09.46 ± 1.5	08.49 ± 0.85	07.60 ± 0.74	06.72 ± 0.83	-
Viscosity reduction (%)	35.00	40.10	32.00	27.85	24.85	20.50	17.30	-
Incubation period (h.)	24	48	72	96	120	144	168	-
Pectinase activity (U/ml)	04.33 ± 0.85	07.91 ± 0.29	11.66 ± 0.31	14.04 ± 0.15	10.69 ± 0.61	02.10 ± 0.51	0	-
Viscosity reduction (%)	07.50	10.20	28.10	45.40	17.50	12.70	-	-

Table (2)

A summary of *A. niger* NRC1ami pectinase purification steps

Purification steps	Total protein of fraction (mg)	Recovered protein of fraction (%)	Total pectinase activity (U)	Recovered activity (%)	Specific activity (U/mg protein)	Fold purification
Culture filtrate	155.69	100	4557.0	100	29.27	1
Ethanol fraction at 50%	24.58	15.79	3813.75	83.69	155.16	5.3
DEAE-Sephadex A-50 0.1 M–0.6 M NaCl	0.021	0.27	389	16.35	18523	632

Table (3)

Covalent coupling of *A. niger* NRC1ami pectinase to activated polysaccharides

Activated polysaccharide	Coupled enzyme		Specific activity of conjugated enzyme (U/mg protein)	Recovered activity (%)	Reduction of viscosity (%)
	Protein (mg)	Activity (U/ml)			
Control	2.10	12006	25213	100	92.12
Dextran (MW: 70,000)	1.03	17895	18432	37.52	59.10
Dextran (MW: 200,000-275,000)	1.05	14438	15160	31.54	56.02
Dextran (MW: 185,000)	0.95	8239	7827	14.70	54.58
Xylane	1.09	19857	21645	46.91	61.54
Galactomannan	1.47	19880	29225	85.00	94.34
Soluble starch	1.10	21015	23177	51.01	65.74
Agar	1.44	19087	27485	78.69	85.22
CMC*	1.03	15387	15849	32.21	56.84

Table (4)

Kinetic and thermodynamic parameters of native and conjugated *A. niger* NRC1ami pectinase

Kinetic and thermodynamic parameters	Free form	Conjugated form
K_d (min⁻¹)		0.0005
45 °C	0.001	0.0005
50 °C	0.0058	0.0019
55 °C	0.0082	0.0028
60 °C	0.0136	0.0055
70 °C		
T_{1/2} (min)		1380
45 °C	639	1380
50 °C	120	363
55 °C	85	246
60 °C	51	125
70 °C	-	
D-values (min)		
45 °C	2302	4600
50 °C	396	4600
55 °C	397	1210
60 °C	147	821
70 °C		418
E_d (KJ.mol⁻¹)	2.10-	2.50
ΔG° (KJ.mol⁻¹)		
45 °C	88.88	90.7
50 °C	85.6	92.18
55 °C	86.02	90.01
60 °C	85.97	90.34
ΔH° (KJ.mol⁻¹)		8.94
45 °C	11.93	8.89
50 °C	11.89	8.85
55 °C	11.85	8.81
60 °C	11.81	
ΔS° (KJ.mol⁻¹)		
45 °C	-268	-271
50 °C	-253	-256
55 °C	-226	-253
60 °C	-222	-232

Table (5)

pH stability of native and galactomannan- conjugated pectinases by *A. niger* NRC1ami

pH value	Relative activity (%)							
	Free enzyme				Conjugated enzyme			
	Exposure time (min)							
	30	60	90	120	30	60	90	120
Control*	100 ^a	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	97.08	61.22	55.39	29.15	100	97.54	95.88	90.65
6	72.89	54.52	30.61	21.28	88.12	80.33	78.55	75.50
7	30.61	26.24	24.78	19.53	75.20	70.54	65.43	62.31
8	21.87	21.28	19.83	17.78	66.25	62.20	59.30	52.22
9	20.04	19.83	18.37	7.85	56.23	52.43	49.57	41.49

Figures

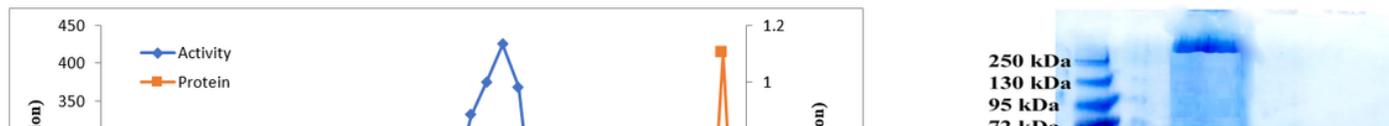


Figure 1

A, B Purification of partially purified *A. niger* NRC1ami pectinase by stepwise elution (chromatography) on DEAE-Sephadex A-50 (**A**). SDS-polyacrylamide gel electrophoresis of the purified Pectinases (**B**)

M: marker

1: Culture filtrate

2: partially pure enzyme (Ethanol fraction at 50%)

3: column fraction 26 (pure enzyme)

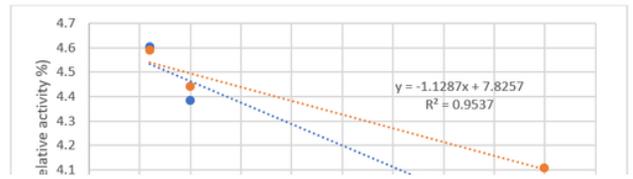
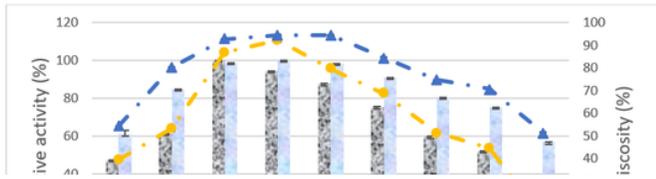


Figure 2

A, B, Temperature profile for free and conjugated pectinase (A), Arrhenius plot to calculate the activation energy of pectinase (Ea) (B).

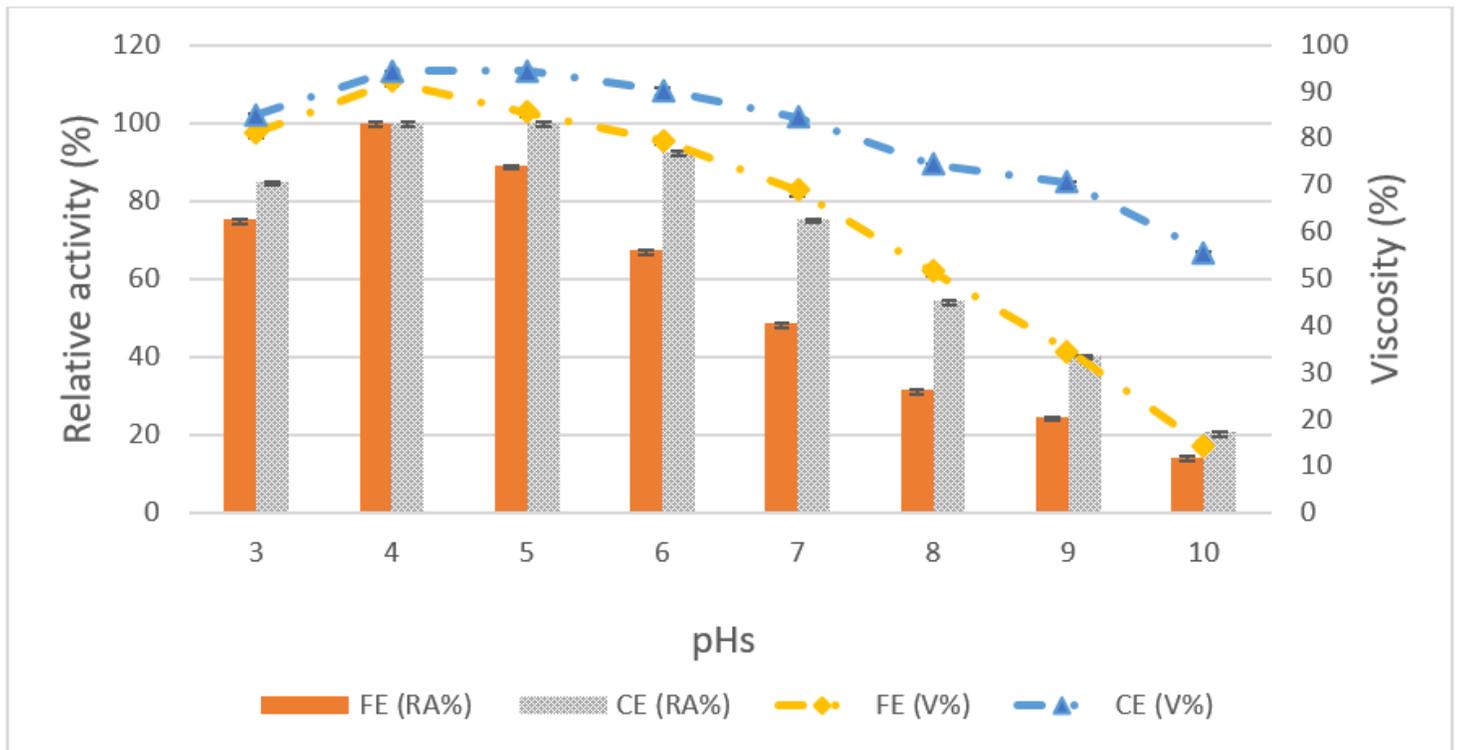


Figure 3

pH profile for free and conjugated pectinase (C)

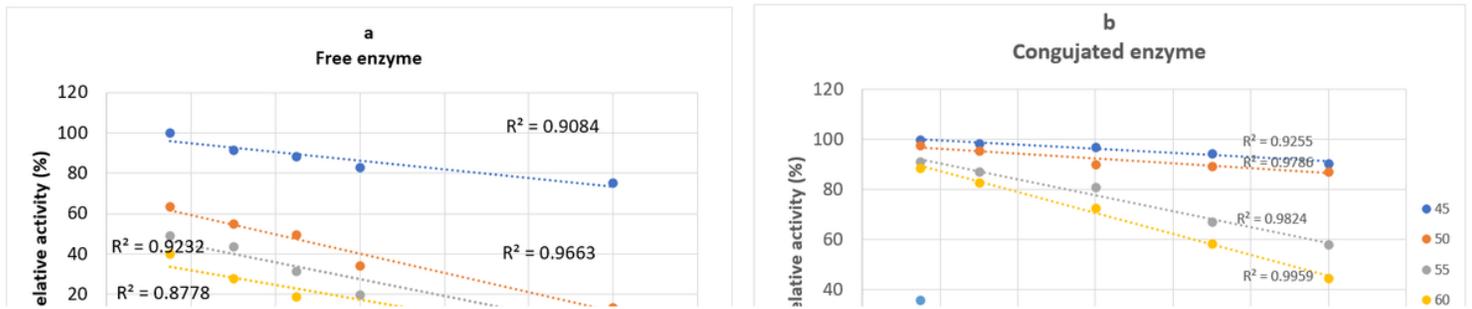


Figure 4

a, b Thermal stability of the free and modified *Aspergillus niger* amylpectinases (**a,b**), .

Figure 5

c, d First order of thermal deactivation of *A. niger* NRC1 amylpectinase free © and conjugated amylpectinase (**d**).

Figure 6

Arrhenius plot to calculate activation energy for denaturation (E_d).

Supplementary Files

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