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Extraction, Partial Purification and Characterization of *Inulinase* Produced from *Aspergillus niger*

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ABSTRACT

Inulinase, an enzyme that hydrolyzes inulin into fructose, holds significant potential for applications in the food and pharmaceutical industries. This study focuses on the extraction and purification of *inulinase* produced by *Aspergillus niger* isolated from onion peel soil using solid-state fermentation. The enzyme was purified through a two-step process, yielding a specific activity of 1400 U/mg proteins, a purification fold of 56, and an enzymatic yield of 28%. The partially purified inulinase exhibited an optimum pH for activity of 6. Enzyme stability was observed in the pH range of 5.0 to 7.0, and the maximum activity occurred at 45°C, with thermal stability between 15°C and 40°C. These findings highlight *A. niger* as a promising source for *inulinase* production, with onion peel soil serving as a sustainable resource for fungal isolation. The results emphasize the potential of solid-state fermentation for large-scale enzyme production, and further investigation into enzyme kinetics and substrate specificity is suggested for future biotechnological applications.

Keywords: *Inulinase, Aspergillus niger, extraction and purification*

INTRODUCTION

Inulinases are enzymes that specifically target and break down the β-(2, 1) linkages found in β-fructans, such as inulin. These enzymes are classified into two categories: endo-inulinases (EC 3.2.1.7) and exo-inulinases (EC 3.2.1.80). The distinction lies in their mode of action-exo-inulinases hydrolyze the terminal β-(2, 1) fructofuranosidic bonds, releasing monosaccharides, while endo-inulinases cleave the internal linkages, generating inulooligosaccharides.

Inulinases are produced by a variety of fungal species, and *Aspergillus* is among the most significant genera in this regard. *Aspergillus niger*, a well-known soil saprobe, is particularly important due to its production of a range of hydrolytic and oxidative enzymes that are involved in the degradation of plant lignocellulose. These enzymes, including inulinase, have broad applications in the biotechnology industry. In fact, *Aspergillus niger* is a key microorganism used in fermentation processes to generate valuable enzymes for industrial purposes.

Inulinase production, specifically by fermentation, has been widely explored as a means to manufacture enzymes for various industrial applications. The two primary methods of fermentation used for enzyme production are submerged fermentation and solid-state fermentation (SSF). Solid-state fermentation has gained significant attention, especially for the production of enzymes like lipases, proteases, and inulinases, due to its various advantages. These benefits include the use of low-cost agricultural residues as substrates, higher productivities, lower energy requirements, reduced wastewater production, and the extended stability of the enzymes. As a result, SSF is particularly attractive from both an economic and environmental perspective.

When considering the extraction of inulinase from *Aspergillus niger* grown on substrates like onion peel, which is a waste product commonly found in agriculture, the process becomes even more beneficial. The use of onion peel as a substrate for solid-state fermentation offers an environmentally friendly approach, utilizing agricultural waste that would otherwise go unused. Moreover, onion peel is rich in fructans, making it a suitable source for inulinase production. This method not only adds value to agricultural waste but also makes the enzyme production process more sustainable and cost-effective.

In summary, the production of inulinase from *Aspergillus niger* using onion peel as a substrate through solid-state fermentation is an efficient and sustainable method. It aligns with the growing demand for cost-effective, eco-friendly solutions in enzyme production while also supporting the reduction of agricultural waste.

Materials and Methods

Chemicals and Media

Sterilized cotton swabs moistened with a glucose-chloramphenicol solution were used to swab onion surfaces and inoculate Pal and APRM agar plates, which were then incubated to isolate *A. niger*. Both media successfully supported the isolation and morphological examination of *A. niger* from onions. In the present study, *Aspergillus niger* was cultured on Potato Dextrose Agar (PDA), which was obtained from Hi-Media. DEAE-cellulose columns and Sephadex G-150, essential for

enzyme purification processes, were sourced from Pharmacia Fine Chemicals. For protein estimation and enzyme assays,

Coomassie Brilliant Blue and bovine serum albumin (BSA) were procured from Sigma Co. In addition to these, other chemicals used throughout the study were purchased from BDH Chemicals, ensuring the availability of necessary reagents for enzyme analysis and biochemical procedures.

Inulinase Production

The *Aspergillus niger* strain was initially cultured on PDA to obtain a pure fungal culture. To assess its capacity to produce inulinase, the fungal isolate was transferred into a fermentation medium consisting of a mixture of sugar cane and corn steep liquor in a 5:5 (v/w) ratio. This composition was chosen for its potential to support optimal fungal growth and enzyme production. The medium was adjusted to an initial pH of 5.0, which is typically ideal for the activity of *Aspergillus niger* enzymes. The fermentation process was carried out at a controlled temperature of 30°C for a period of 96 hours under static conditions to facilitate maximum inulinase production.

Inulinase Extraction

After the 96-hour fermentation period, the inulinase enzyme was extracted from the culture medium. For extraction, 50 ml of sodium acetate buffer solution (0.1 M, pH 4.8) was added to each fermentation flask. The buffer solution was chosen to maintain the optimal pH for enzyme extraction, enhancing the stability of inulinase. The flasks were then incubated in a shaker at 30°C with a shaking speed of 150 rpm for 30 minutes, ensuring thorough mixing and efficient enzyme release from the fungal biomass into the surrounding medium.

Following incubation, the enzyme extract was filtered using Whatman filter paper No. 1 to remove any solid particulate matter and fungal residues. The resulting clear filtrate, which contained the crude enzyme, was collected for subsequent analysis. The enzyme activity in the crude extract was measured using standard assay techniques, and the protein concentration was determined through the Bradford method using BSA as the standard. These assays provided the quantitative data necessary to evaluate the inulinase production efficiency and protein yield from the *Aspergillus niger* under the given fermentation conditions.

Estimation of Inulinase Activity and Concentration

Inulinase activity was determined in the solutions obtained after enzyme extraction, following the method described by Miller (7). This method relies on the estimation of reducing sugars released from inulin upon hydrolysis by inulinase. The substrate concentration used was 1% inulin in sodium acetate buffer (pH 5.0). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μM of fructose per minute under standard assay conditions. Protein concentration was measured using the Bradford method (8), which involves the use of BSA as a standard curve and Coomassie Brilliant Blue G-250 dye. The protein concentration was then determined by measuring the absorbance at 595 nm.

Partial Purification of Inulinase

Inulinase Purification by Ion Exchange Chromatography, DEA E-cellulose was prepared as described by Whitaker (9) for ion exchange chromatography. A 10 ml sample of the inulinase concentrate, obtained using sucrose, was applied to a DEAE-cellulose column (16 × 1.5 cm). The sample was carefully added using a pasture pipette, and the column was washed with 0.005 M phosphate buffer at pH 7.0. The proteins were then eluted with a phosphate buffer at pH 7.0 and a sodium chloride gradient ranging from 0.1 to 1 M. The elution process was carried out at a flow rate of 45 ml/hour, with 3 ml fractions being collected. The protein content in each fraction was monitored by measuring the absorbance at 280 nm. Enzyme activity was estimated in each fraction. Fractions that exhibited enzyme activity were pooled and concentrated using sucrose.

Inulinase Purification by Gel Filtration Chromatography

Sephadex G-150 was prepared and packed according to the manufacturer's instructions (Pharmacia, Sweden). Nine milliliters of the enzyme solution was carefully applied to a Sephadex G-150 column (37 × 1.5 cm) using a pasture pipette. The protein sample was eluted using 0.2 M phosphate buffer (pH 7.0), and 3 ml fractions were collected throughout the process. The protein content of each fraction was determined spectrophotometrically by measuring absorbance at 280 nm. The peaks were identified by plotting absorbance versus fraction number, and both protein concentration and inulinase activity were assessed for each fraction.

Inulinase Characterization

Optimum pH for Inulinase Activity

A 1% inulin substrate solution was prepared in buffers with varying pH values (ranging from pH 3.5 to pH 8). Inulinase activity was measured at each pH level, and the relationship between enzyme activity and pH was plotted to determine the optimum pH for inulinase activity.

Optimum pH for Enzyme Stability

To assess enzyme stability, 2 ml of enzyme solution was mixed with 2 ml of buffers prepared at different pH values (ranging from pH 3 to pH 10) in separate test tubes. The test tubes were incubated in a water bath at 50°C for 30 minutes and then rapidly cooled in an ice bath. After cooling, enzyme activity was measured for each condition. The relationship between remaining enzyme activity (as a percentage of the initial activity) and pH was plotted to determine the pH range in which inulinase remained most stable.

Optimum Temperature for Inulinase Activity

A 0.9 ml aliquot of the substrate solution (1% inulin) was mixed with 0.1 ml of enzyme solution and incubated in a water bath for 15 minutes at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65,70 and 75°C). After incubation, enzyme activity was measured for each temperature condition. The relationship between enzyme activity and temperature was plotted to identify the optimum temperature for inulinase activity.

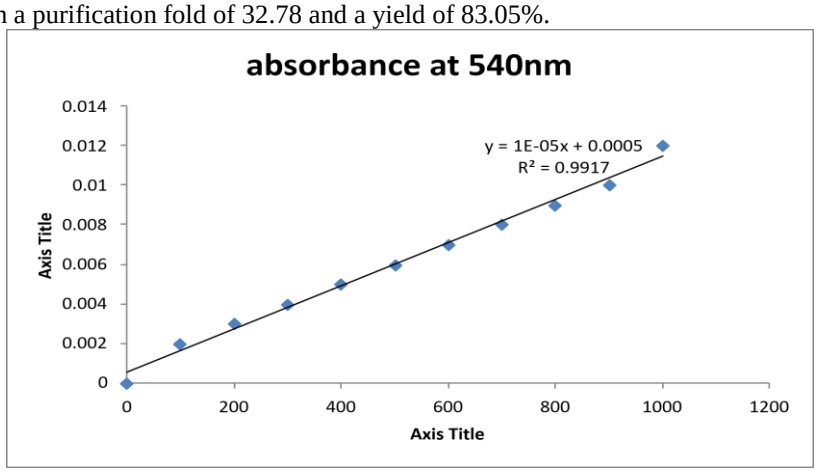
Thermal Stability of Inulinase

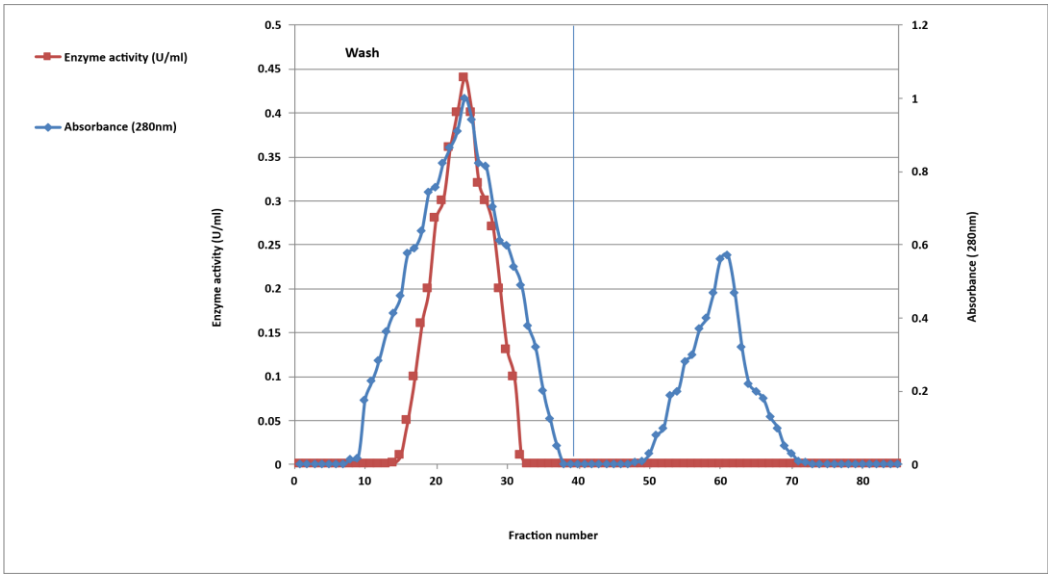
One milliliter of partially purified inulinase was incubated in a water bath at various temperatures (15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80°C) for 30 minutes. After incubation, the test tubes containing the enzyme were immediately transferred to an ice bath to cool. The enzyme activity was then measured, and a plot of remaining activity (%) versus temperature was created to assess the thermal stability of the enzyme.

Results and Discussion Purification of Inulinase

Ion Exchange Chromatography

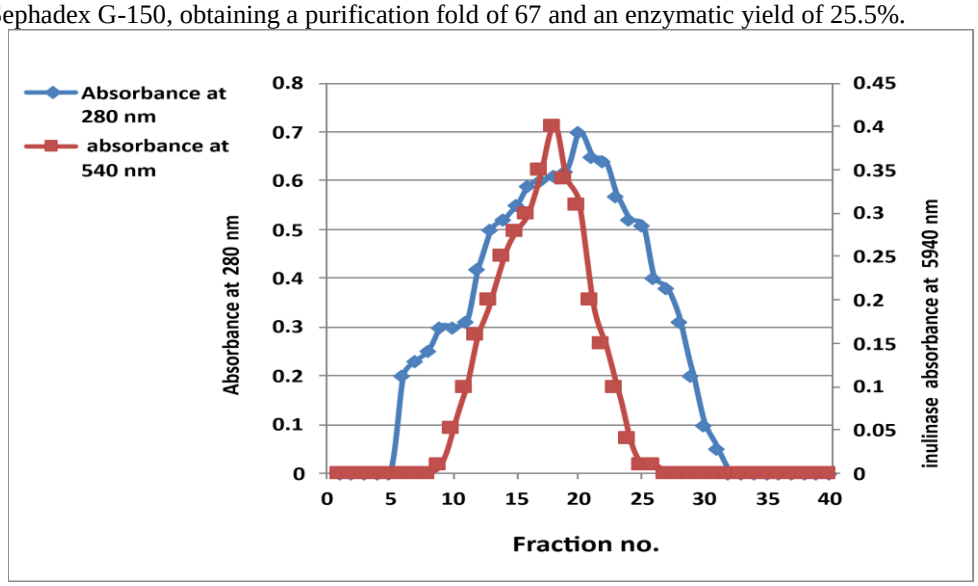
Ion exchange chromatography was performed on the crude enzyme after concentration with sucrose, using DEAE-cellulose as the ion exchanger in the presence of a 0.02 M phosphate buffer at pH 7.0. The chromatography results revealed two protein peaks during the wash step, with only one peak corresponding to enzyme activity found in fractions 14-31. In the elution step, one peak was observed, but didn't show enzyme activity with only protein in fractions 47-73. The purification fold achieved in this step was 16, with a yield of 32%, resulting in a specific activity of 400 U/mg (Table 2, Figure 1). Ion exchange chromatography has been used in several studies for inulinase purification from fungi. For instance, Ertan et al. (12) utilized DEAE-cellulose to purify inulinase from *Rhizoctonia solani*, achieving a purification fold of 5.21 and a yield of 17.93%. Similarly, inulinase was purified from *Alternaria alternata* using DEAE-cellulose, resulting in a purification fold of 65.89 and an enzymatic yield of 11.56% (13). Souza-Motta et al. (14) employed DEAE-52 to purify inulinase from *Aspergillus niveus*, with a purification fold of 32.78 and a yield of 83.05%.





Gel Filtration Chromatography

The gel filtration step was performed following ion exchange chromatography. During this stage, three protein peaks were separated, as shown in Figure 2. After evaluating the enzymatic activity of each peak, two peaks found in fractions 14-24 exhibited enzyme activity. These active fractions were pooled together, and the solution was concentrated to 9 ml using sucrose. After the gel filtration step, the specific activity reached 1400 U/mg, the purification fold was 56, and the enzymatic yield was 28% (Table 1). Sephadex G-150 gel was selected for this process because it allows the passage of proteins with molecular weights ranging from 5 to 300 kDa, which covers the typical molecular weight range of inulinases purified from different *Aspergillus niger* isolates. Singh & Gill (15) reported that the molecular weights of inulinases range from 28 to 300 kDa. Sephadex G-150 has been widely used in inulinase purification studies. For example, Kochhar et al. (16) used Sephadex G-150 to purify inulinase from *Aspergillus versicolor*, achieving a purification fold of 50. Chen et al. (17) also used Sephadex G-150, obtaining a purification fold of 67 and an enzymatic yield of 25.5%.



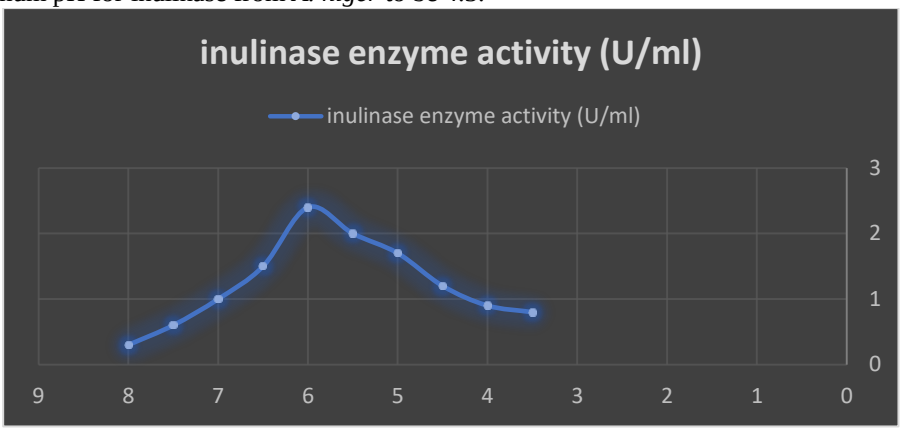
Characterization of Inulinase from Local Isolate *A. niger*

Optimum pH for Enzyme Activity

The effect of pH on inulinase activity was investigated, and the results shown in Figure 5 indicate that the optimum pH for inulinase activity is 6. Activity decreased when the pH was below or above this value. pH influences the structure and function of enzymes by affecting the ionization state of acidic and basic amino acids. Acidic amino acids contain carboxyl functional groups in their side chains, while basic amino acids have amine groups. Changes in the ionization state of these amino acids can disrupt the ionic bonds that maintain the enzyme's three-dimensional shape, potentially altering its function or rendering the enzyme inactive. Moreover, pH changes may not only affect the enzyme's shape but could also

modify the substrate's structure or properties, preventing it from binding to the enzyme's active site or undergoing catalysis (Chesworth et al., 21).

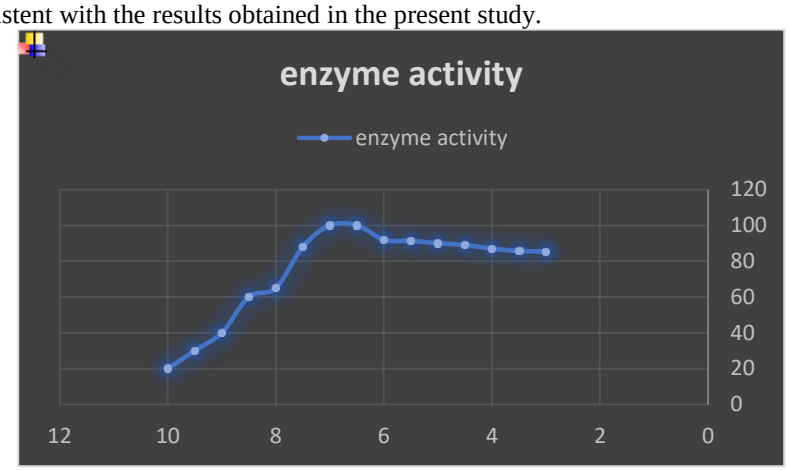
Arand et al. (22) reported that the optimum pH for inulinase activity from *A. awamorii* was 6, while Derycke & Vandamme (23) found the optimum pH for inulinase from *A. niger* to be 4.3.



Enzyme Stability at Different pH

The stability of the enzyme at different pH levels was also studied, as it plays a crucial role in determining the optimal conditions for enzyme purification and storage. The results, shown in Figure 6, revealed that inulinase remained stable within the pH range of 5.0 to 7.0. However, enzyme activity was significantly reduced at acidic pH values below 4 and at basic pH values between 9 and 10. The decrease in enzymatic activity under extreme acidic and basic conditions could be attributed to alterations in the enzyme's tertiary structure, as well as changes in the ionic state of the active site and the substrate (24, 25).

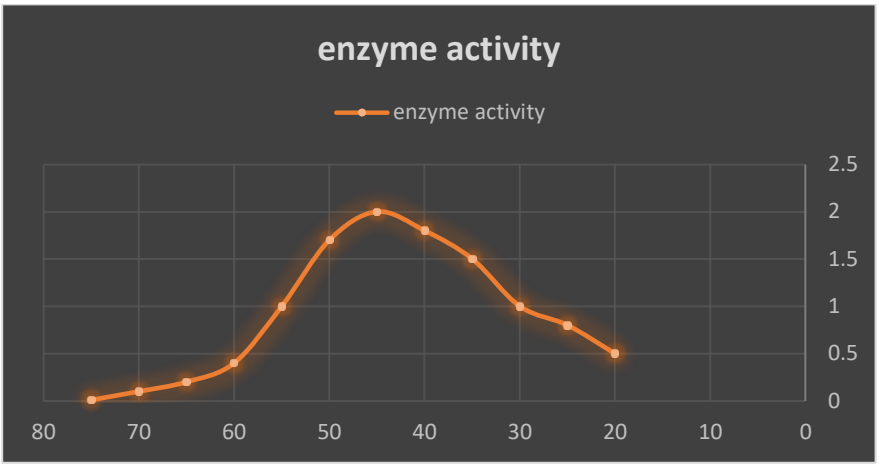
Nakamura et al. (18) conducted a study to determine the pH stability of inulinase isozymes and found that the *A. niger* strain PII isozyme was stable in the pH range of 5–7, while the PIII isozyme remained stable between pH 5 and 7. Similarly, Skowronek & Fidurek (26) found that the *A. niger* 20 OSM endo-inulinase was stable in the pH range of 5–7. These findings are consistent with the results obtained in the present study.



Optimum Temperature for Inulinase Activity

The optimum temperature for inulinase activity was investigated by incubating the enzyme with the substrate at temperatures ranging from 20°C to 75°C for 15 minutes. The results indicated that the optimum temperature for inulinase activity from *Aspergillus niger* was 45°C, where the enzyme exhibited the highest activity of 2.00 U/ml (Figure 7). Activity declined both above and below this temperature, and the enzyme activity was completely lost at 75°C. The results showed an increase in reaction rate as the temperature rose until it reached 45°C, after which the rate began to decrease at temperatures above 50°C. This could be attributed to the increased collisions between enzymatic molecules and the substrate, as the kinetic energy of the molecules increased. However, the reduction in enzyme activity at temperatures above 50°C is likely due to protein denaturation and alterations to the active sites, which impair enzyme function (27).

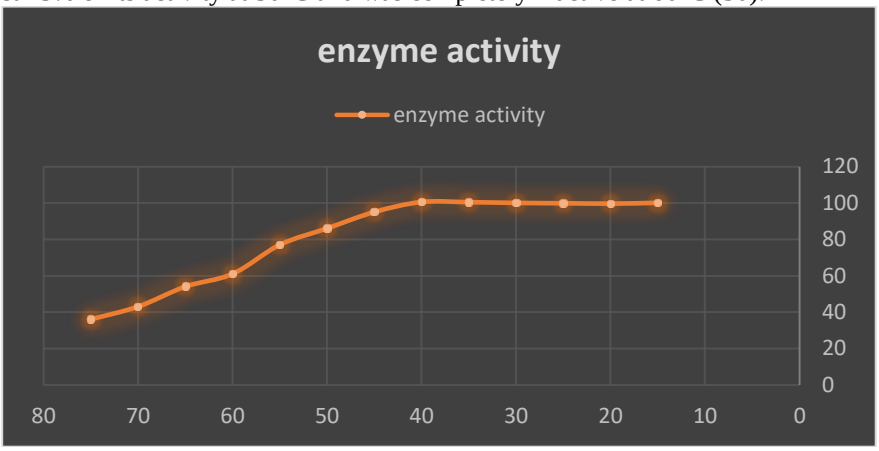
These findings are consistent with those of Rouwenhorst et al. (28), who also observed maximum activity between 40°C and 50°C.



Thermal Stability of Inulinase

When inulinase from *A. niger* was incubated at various temperatures ranging from 15°C to 75°C for 30 minutes, the enzyme remained stable between 15°C and 40°C. However, activity began to decline as the temperature increased and was completely lost at 75°C (Figure 8). This reduction in enzymatic activity could be attributed to the thermal effects on the enzyme's structure, leading to its denaturation (29). Elevated temperatures may disrupt the bonds that stabilize the protein's secondary and tertiary structures, causing denaturation (21).

Studies on the thermal effects on enzymatic activity have reported varying results. Nakamura et al. (18) found that inulinase isozymes PII and PIII from *Aspergillus niger* Strain 12 maintained their activity when incubated at 50°C and 60°C for 30 minutes, but lost activity at 70°C and 80°C. In contrast, studies on the thermal stability of inulinase from *Chrysosporium pannorium* showed that the enzyme remained stable when incubated for 30 minutes at temperatures up to 45°C. However, it lost 15% of its activity at 50°C and was completely inactive at 60°C (30).



Step	Volume (ml)	activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification folds	Yield (%)
Crude	100	5	0.2	25	500	1	100
Dialysis	35	19.4	0.26	74.61	679	2.984	135.8
On exchange chromatography DEA E-cellulose	10	16	0.04	400	160	16	32
Gel filtration chromatography Sephadex G-100	10	14	0.01	1400	140	56	28

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