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Kinetics and Thermodynamic Properties of Pectinase Obtained from *Trichoderma longibrachiatum* MT321074

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ABSTRACT

Thermo-instability affects enzymes' efficiency in the industry. This study explored the thermodynamic and kinetic properties of *Trichoderma longibrachiatum* MT321074 pectinase, to ascertain its potential for industrial application. *Trichoderma longibrachiatum* MT321074 was isolated from decaying mango fruits and identified using the 18S rDNA sequencing technique. The *Trichoderma longibrachiatum* MT321074 pectinase was purified 5.61 fold with a specific activity and yield of 2.86 U/mg and 17.63%. The pH and temperature optima were 5.0 and 40°C. The Michaelis constant, K_M, and maximum velocity, V_{max} were 1.0 mg/ml and 82.64 µmol/min. The enzyme retained 92%, 87%, and 72% of its original activity after 2 h at 30ºC, 40ºC, and 50ºC, respectively. At 60ºC, 70ºC and 80ºC, the enzyme lost 38%, 65%, and 73% of its activity after 120 min. The enzyme's melting temperature (T_m) was 64°C. The rates of thermal inactivation (*Kd*) were low at all temperatures tested. The activation energy of inactivation (*Ea*) was 50.30 KJ/mol^{-1}. The *D-values* and half-life ($t_{1/2}$) decreased from 3838.33 to 261.70 min and 1155.00 to 78.75 min, respectively. The enzyme had a high *Z-value* of 42°C. The Gibbs free energy (*ΔGin*) increased with a rise in temperature. High enthalpy changes (*ΔHin)* were obtained at all temperatures tested. The entropy (*ΔSin)* values were negative at all temperatures tested. In summary, the increase in Δ*Gin* accompanied by decline in *ΔSin* and Δ*Hin* values at elevated temperatures suggests that the enzyme is heat stable and thus, very suitable for the degradation of pectic substances in fruit effluents, food, and beverage products.

***Keywords*:** *Trichoderma longibrachiatum* MT321074, 18S-rDNA sequencing, Pectinase, Heat stability.

Introduction

Fruit juice is a good source of nutrients needed for human development.[1] The turbid nature caused by the presence of pectin, limits its general acceptability due to the poor appearance. Juice clarification involves removal of the pectic substance, which improves the taste, texture, appearance and its general acceptability. Different approaches, which include filtration, use of gelatin, bentonite, tannic acid, albumin, chitosan and casein, have been used to clarify fruit juice.[2] However, these clarifying agents through electrostatic interaction form insoluble flocs with pectin to form large amount of wastes and also changes the phenolic composition of the juice. The use of enzymes in clarification of fruit juice prevents the problems associated with the use of these chemical agents. Pectinases breaks down pectin present in juice into galacturonic acid units hence, improving the taste, and appearance of the juice.

Pectinases are biological catalysts that degrade pectin present in the middle lamella of the primary cell wall in vegetables and fruits of higher plants, such as mangoes and citrus fruits. Pectin degrading enzymes may be grouped into three[3] categories as follows: protopectinases, which break down the insoluble protopectin into highly polymerized soluble pectin; esterases, which catalyze the de-esterification of pectin by the elimination of methoxy esters, producing pectic acid; and depolymerases, which catalyze either the hydrolytic or non-hydrolytic cleavage of the α-(1-4)-glycosidic linkages of the pectic substances producing methyl galacturonates (for hydrolases) or unsaturated galacturonates (for lyases) with pectic substances of lower molecular weights as end products, respectively. Pectin is a polysaccharide found in plant cell walls that is composed of α-(1-4) linked D-galacturonic acid units in a linear chain as well as other branched polysaccharides.[4] The middle lamella of plants contains the highest concentrations of pectins, with a gradual decrease from the primary cell wall to the plasma membrane.[5] Pectic structures are grouped into two distinct regions: first, the smooth region, which consists of a straight homopolymer of α-(1, 4) linked D-galacturonic acid called homogalacturonan structure (HGA), and second, the branched polysaccharides of pectin called the "hairy region", which consists mainly of rhamnogalacturonan 1 (RG1), rhamnogalacturonan 11 (RG11), and xylogalacturonan (XGA)[6,7], an α-(1, 4)-linked D-galacturonic acid chain majorly substituted with β-D-xylose at the C3 position. The carboxylate groups of D-galacturonic acid are partially acetylated (at C2 and C3) and/or methylated in ester bonds and partially or wholly counteracted by K^+, Na^+, or NH_4^+ ions.[8] The main source of industrial enzymes are microorganisms dominated by 50% fungi and *yeast*, 35% bacteria, while the remaining 15% is either of animal or plant origin.[9] Pectinolytic enzymes have widespread uses in the food, textile[10], and paper-making industries. Treatment of wines, fruit, and vegetable pulps with acid pectinases degrade colloids formed by pectin[11] hence resulting in viscosity reduction, releasing wanted components from cell wall materials as well as improving yield, flavor, color, taste, and stability desired by juice and wine consumers.[12,13] Catalytic efficiency of enzymes depends on its heat stability.[14] Therefore, the use of heat-stable pectinase in industry will

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enhance its catalytic efficiency and thus increases the product yield. There is need to locally source for cheap pectinase with high thermal stability and suitable properties for industrial applications. Thus, this study is a bid to molecularly characterize pectinase-producing fungi, determine the effect of pH, temperature, pectin concentration on purified pectinase activity and the effect of heat treatment on the stability of *Trichoderma longibrachiatum MT 321074* pectinase.

Materials and Methods

Chemicals

Reagents utilized in this research were of analytical ranking and were produced by BDH Chemical Limited (England), Sigma Aldrich, Merck (Germany), May, and Baker Limited (England).

Isolation of Trichoderma longibrachiatum MT321074

Pectin depolymerizing fungi were isolated from decayed mango fruit obtained from Enugu's Mayor Market. The pure fungal strain was isolated as described by Nsude *et al*.[15] Infected mango fruit (5 g) was cut using a sterile blade and homogenized in sterile media containing 1% mango pectin, 0.02% $MgSO_4.7H_2O$, 0.14% $(NH4)_2SO_4$, 0.2% K_2HPO_4 and 1 ml of nutrient solution (comprising 1.4 mg/L $ZnSO_4.7H_2O$, 1.6 mg/L $MnSO_4.H_2O$, 2.0 mg/L $CoCl_2$, 5 g/L $FeSO_4.7H_2O$). The broth preparation was adjusted to pH of 5.0 (using 1.0 N HCl and 1.0 N NaOH) and the culture mix was incubated for 24 h at 30^{o}C. A loop full of well-stirred and homogenized liquid broth culture was streaked onto solid mango pectin agar medium, under sterilized conditions (using the flame of Bunsen burner). The plates were incubated at 30^{o}C until noticeable colonies of fungi were observed. Fungal strains from the morphological distinct colonies were purified by repetitive streaking and sub-culturing on separate plates. This process was repeated until pure fungal isolate was obtained and then identified. The fungal strains were screened for pectinase production ability using the method described by Nsude *et al*.[16]

Molecular characterization of the fungal isolate

Preparation of pure fungal isolate for DNA extraction

Trichoderma species were identified at the species level. A pure fungal isolate was inoculated into a potato dextrose agar (PDA) culture broth.[17] The culture was allowed to grow for 4-6 days, after which fungal cells were harvested for DNA extraction.

DNA Extraction

The fungal genome was extracted using a solution-based JENA Bioscience Animal and Fungi DNA Preparation Kit, following the manufacturer's guides. Fungal cells were obtained from 1000 µl of culture broth by centrifugation at 15,000 g for 1 min using a microcentrifuge. The pellet was re-suspended in a re-suspension buffer (300 µl) and 1.5 µl of proteinase K solution. The mixture was properly mixed by inverting the tubes several times and then incubated at 55°C for 1 h. Centrifugation was used to recover the cells, which were then lysed with 300 µl of lysis solution. Protein precipitation solution (100 µl) was added, and the mixture was agitated vigorously and centrifuged at 15,000 g for 3 min. The supernatant was added into a clean microcentrifuge tube containing 300 µl of isopropanol and mixed gently by inverting the tube for 1min to precipitate the DNA. The DNA was pelleted by centrifugation at 15,000 g for 1 min, washed with washing buffer (500 µl), and air-dried after discarding the washing buffer. Hydration solution (50 µl) and RNase A (1.5 µl) were then added to the DNA pellet and incubated at 37 °C for 1 hour followed by a final incubation at 65°C for 1 hour, for proper hydration of the DNA pellet.

Polymerase chain reaction (PCR)

The ITS (internal transcribed spacer) region of rDNA was amplified using the polymerase chain reaction (PCR) technique. The PCR reaction mixture contained 12.5 µl master mix (2x JENA Ruby hot start master mix), 1 µl each of forward primer ITS1-TCCGTAGGTGAAC CTGCGG, and reverse primer ITS4-TCCTCCGCTTATTGATATGC, 1 µl DNA template, and 9.5 µl sterile nuclease-free water to obtain a total reaction volume of 25 µl. PCR was carried out using an Applied Biosystems 2720 thermocycler. The reaction conditions were; initial denaturation at 95°C for 5 min, followed by denaturation at 94°C for 45 s, annealing at 55°C for 45 s, elongation at 72°C for 45 s, and a final extension at 72°C for 5 min. The PCR products were visualized on an agarose gel (2 %) containing ethidium bromide in 0.5 X Tris-borate buffer (pH 8.0) using a blue LED transilluminator. The amplicons were purified and sequenced by the Sanger sequencing method using an AB1 730XL sequencer by Inqaba Biotec, Pretoria, South Africa.

Bioinformatics analysis

The DNA sequence was subjected to three search tools to confirm identity (ID), namely: DNA Subway, direct submission to a Basic Local Alignment Search Tool (BLAST) search in GenBank, and a serial BLAST search in the curated fungal taxonomic reference database User-friendly Nordic ITS Ectomycorrhiza Database (UNITE). A phylogenetic analysis of the DNA sequence was conducted by maximum likelihood tree using Molecular Evolutionary Genetics Analysis (MEGA) version 7.

Pectinase production

Production of pectinase was carried out using a submerged fermentation process described by Nsude *et al*.[15] Ten Erlenmeyer flasks (250 ml) were added 50 ml each of cultivation medium charged with 0.1% $(NH_4)_2SO_4$, 0.1% $NH_4H_2PO_4$, 0.1% $MgSO_4.7H_2O$, and 0.5% mango pectin for pectinase production. The media were adjusted to pH 5.5 using 1.0 N HCl and 1.0 N NaOH. The flasks were covered with aluminum foil and autoclaved for 15 min, at 121^{o}C 15 psi. Three discs of freshly grown pure *Trichoderma longibrachiatum* were added into each of the flasks containing the sterile broth using a cork borer of diameter 0.8 cm. The flasks were then covered properly and placed on a rotary shaker at 150 rpm and 30^{o}C. On the 6th day of pectinase production, the crude enzyme was separated from the mycelia biomass by filtration, centrifuged at 5000 rpm for 20 min. The supernatant was used for further studies.

Pectinase assay and protein determination

Pectinase activity was determined by measuring the release of galacturonic acid groups from mango and industrial pectins, using the 3, 5 dinitrosalicylic acid (DNS) reagent assay method described by Miller[18] and described in Wang et al.[19] The reaction mix comprising 0.1 ml Mn^{2+} in distilled water, 0.5 ml of 0.5% fruit pectin in sodium acetate buffer (0.05 M, pH 5.0) and 0.5 ml of pectinase solution were incubated for 1h. An aliquot (1 ml) of DNS reagent was added and the reaction was terminated by boiling the mix for 10 min in a water bath. The reaction volume was adjusted to 4.1 ml with Rochelle salt solution (1 ml) and distilled water (1 ml). The reaction mixture was allowed to cool and the absorbance was measured at 540 nm. A unit of pectinase activity was defined as the amount of enzyme that catalyzes the liberation of one micromole of galacturonic acid per minute. The protein level of the enzyme was determined using the method of Lowry *et al*.[20], and bovine serum albumin as standard.

Purification of pectinase

Using the method of Chilaka *et al*.[21] a saturation level of 40% was chosen for mass precipitation. The precipitated enzyme was dialyzed against sodium acetate buffer (0.05 M, pH 5.0) for 12 hours with constant stirring. The buffer was changed every 6 h to remove low molecular-weight substances that may interfere with the enzyme activity. Both dialyzed and crude enzymes were centrifuged and subjected separately to gel filtration chromatography. About 10 ml of the supernatant enzyme sample (dialyzed or crude enzyme) were loaded into sephadex G 200 column (1.6 × 57 cm) equilibrated with sodium acetate buffer (pH 5.0, 0.05 M). The enzyme was eluted at a flow rate of 0.3 $ml.min^{-1}$ using the same buffer. The protein content and enzyme activity in each fraction were determined. The fractions with high activity were pooled together and used for further studies.

Effect of pH and temperature changes on pectinase activity

The pH optimum for pectinase activity was obtained using 0.05 M sodium acetate buffer pH (3.5-5.5), phosphate buffer pH (6.0-7.5), and Tris-HCl buffer pH (8.0-9.0) at intervals of 0.5. To make the pectin

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solution (0.5%), 0.5 g of pectin was dissolved in 20 ml of the appropriate buffers. The optimum pH was obtained by incubating differently 0.5 ml of enzyme and 0.5 ml of various pectin solutions for 1 h. Also, the temperature optimum was obtained by incubating 0.5 ml of enzyme with 0.5 ml of pectin solution at different temperature range (25-90^{o}C) (at an interval of 5^{o}C) for 1 h and at pH 5.0. The pectinase activity in each case was accessed as described above.

Effect of substrate concentration on pectinase activity
The effect of substrate concentration on pectinase activity was monitored using a modification of Ezike *et al*.[22] The reaction mixtures contained 0.1 ml of 0.05 M Mn^{2+} solution, graded concentration of mango pectin (1.0, 1.5, 2.0 5.0, 10.0, 18.0, and 20.0 mg/ml) in 0.05 M acetate buffer solution of pH 5.0 and 0.5 ml of the enzyme. The reaction mixture was incubated at 40°C and the enzyme activity was assayed as described above. The maximum velocity (V_{max}) and Michaelis constant (K_M) were determined from the double reciprocal plot of initial velocity values at different pectin concentrations.

Thermostability of pectinase
The pectinase solution was incubated at various temperatures ranging from 30 to 80°C (at an interval of 10°C) for 2 h. An aliquot (0.5 ml) of the enzyme solution was withdrawn every 20 min and the residual activities were determined.

Pectinase's (active site's) melting temperature (T_m)
This corresponds to the temperature when *Amin* drops down to 50% of the native activity (*A0*) from the plot of $\frac{Amin}{A0}$ Versus Kelvin temperature, *T* (°K). Where *Amin* = the pectinase activity at the minimum time of each temperature and *A0* = the initial pectinase activity at zero time.

First-order rate constants of inactivation (Kd)
At temperature range of 30-90^{o}C (interval of 10^{o}C), *Kd* (min^{-1}) was obtained from the slope of plots of natural logarithms of percentage residual activity, $lnK\left(\frac{Ares}{A0}\right)X\ 100\%$ of different temperatures against time, t (min) from the equation 1:

$$ln\left(\frac{Ares}{A0}\right) = -Kdt \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where Ares = the pectinase activity at a time, t and A0 = the initial pectinase activity at zero time.

Determination of energy of activation (Ea) of pectinase
Trichoderma longibrachiatum MT321074 pectinase was incubated at 30-70°C (at an interval of 10°C) in a water bath as described by Stauffer and Etson.[23] An aliquot (0.5 ml) of the enzyme was withdrawn and cooled to room temperature. The activity of the enzyme was assayed as explained in the method of Miller[18]. An Arrhenius plot of natural logarithms of *Kd-values* ($lnKd$) versus the reciprocal of Kelvin temperature, $1/T$ (1/°K) was done to obtain the activation energy of enzyme inactivation (*Ea*) using the Arrhenius equation 2:

$$lnKd = \frac{-Ea}{RT} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where *Ea* = activation energy of enzyme inactivation, *R* = Universal gas constant (8.314 J K^{-1} mol^{-1}), and T = Absolute or Kelvin temperature, (°K).

Decimal reduction time (D-values)
D-value is the time (in minutes) required to pre-incubate the enzyme at a given temperature to reduce its initial activities (A0) by 90% or retain its initial activities (A0) by 10%. It was calculated, using equation 3:

$$D = \frac{ln10}{Kd} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Z-value
This is the temperature increase required to reduce the *D-value* by ten-fold or one logarithmic cycle) and it was estimated from the inverse of the slope (*Z*) of the plot of *logD* versus change in temperature ΔT (°C) using equation 4:

$$Log\ D = \frac{-\Delta T}{Z} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Half-lives (t1/2)
This is the time required for the enzyme to lose half (50%) of its initial activities (A0) at different temperatures. The first-order rate constant of inactivation (*kd*) can be used to calculate half-lives ($t_{1/2}$) of the enzyme at the individual temperatures using equation 5:

$$t1/2 = \frac{ln2}{Kd} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Thermodynamic properties of pectinase
Thermodynamic parameters (changes in enthalpy, Gibbs free energy, and entropy of inactivation) of *Trichoderma logibrachiatum* pectinase were calculated as described in Saqib and Siddiqui.[24]
The change in enthalpy (*ΔHin*) of inactivation, which is the amount of heat energy (J/mol) needed for the inactivation of the enzyme was estimated using equation 6:

$$\Delta Hin = Ea - RT \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (6)$$

The Gibbs free energy (*ΔGin*) of inactivation, which is the maximum amount of energy (J/mol) available to thermodynamic process that can be converted to useful work was calculated using equation 7:

$$\Delta Gin = -RT + ln\left(\frac{Kd\ X\ h}{Kb\ X\ T}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Where *Kd* = First order rate constant of inactivation (min^{-1}), h = Planck's constant (6.63 X 10-34JS) and Kb = Boltzmann constant (1.38 X 1.38 X 10-23 J/K)
Also change in entropy (ΔSin) of inactivation, which is the amount of energy per degree (J/mol/K), needed in the transition from a native to an inactive state was calculated using equation 8.

$$\Delta Sin = \left(\frac{\Delta Hin - \Delta Gin}{T}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (8)$$

Results and Discussion

Different strains of *Trichoderma* have been shown to have pectinolytic activities (Yasin *et al*.[25] In this work, the pectinase-producing green fungus was isolated from rotten mango fruits and identified as *Trichoderma* species based on its macroscopic features (such as texture, color, nature of mycelia and/or spores produced and growth pattern) and microscopic features (such as spores, shapes, and separation). Fungal identification centered on morphology alone can be tough, particularly when learners are dealing with fungal cultures, as there are a restricted number of morphological features used for identification and thus, DNA sequence-based methods have emerged for identifying species within the broad group of the fungal family[26]. Molecular identification and phylogenetic analysis of the *Trichoderma* species confirmed it to be *Trichoderma longibrachiatum* MT3201074. The results of the agarose gel electrophoresis showed a band at approximately 700bp (lane G) compared to the standard DNA ladder of lane L (Figure 1). Gene sequencing products were represented in a text-based nucleotide sequence format called the Pearson/FASTA format (Table 1). *Trichoderma longibrachiatum* was identified based on the percentage similarity with the known species sequences in the databases. Bioinformatics analysis showed that the gene sequence of the isolate had 98.79%, 98.79%, and 100% similarities, respectively against known sequences in the databases (Table 2). The internal transcribed spacer (ITS) regions have been established to be among the markers with the utmost likelihood of accurate identifications for the mega-diverse group of fungi (Schoch *et al*.[27]

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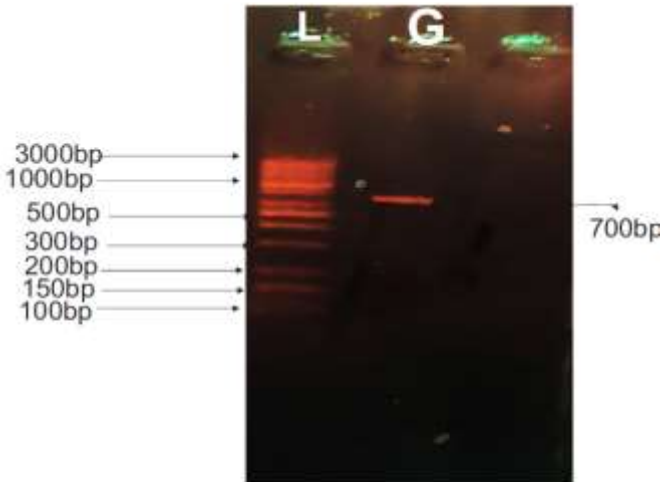

Figure 1: Agarose gel electropherogram of the amplicons

Industrial enzymes are well-known to be stable and unlikely to denature at elevated temperatures.[40] Enzymes with large *Ea* values require more thermal energy to be inactivated, and the inactivation process is so strongly temperature-dependent that the rate becomes insignificant at a lower temperature. The decimal reduction time, *(D-value*) was calculated using equation 3. This was to establish the relationship between the time of treatment and pectinase activity. The *D-values* decreased from 3838.33 to 261.70 min with increasing temperatures from 30 to 90^{o}C (Table 4). Also, half-life ($t_{1/2}$) decreased from 1155.00 to 78.75min with temperature rises from 30 to 90^{o}C (Table 4). Adedeji and Ezikiel[53] reported decreased half-life and decimal reduction time of polygalacturonase with increasing temperature. The *D-values* and $t_{1/2}$ are notable pointers of enzyme thermal stability.[56] Increased values of decimal reduction time and half-life are necessary for commercial applications of enzymes, as they guarantee improved resistance of enzymes to temperature-induced inactivation.[57] In addition, the decline in *D-values* and $t_{1/2}$ with the corresponding temperature increases indicate a faster inactivation of *Trichoderma longibrachiatum* MT 321074 pectinase at higher temperatures.[48]

Table 1: Nucleotide sequence (in FASTA format) of green fungus ITS region after gene sequencing

>HWL148_ITS-1

AACGTTACCAATCTGGTTGCCTCGGCGGGATTCTCTTGCCCCGGSYGYGTCGCAGCCCCGGATCCCATGGCGCCCGCCGGAGGACCAA
CTCCAAACTCTTTTTTTCTCTCCGTCGCGGCTCCCGTCGCGGCTCTGTTTTATTTTTGCTCTGAGCCTTTCTCGGCGACCCTAGCGGGCG
TCTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA
ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTC
AACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCCTCACCGGGCCGCCCCCGAAATACAGKGGCGGTCTCGCCGCAGC
CTCTCCTGCGCAGTAGTTTGCACACTCGCACCGGGAGCGCGGCGCGGCCACAGCCGTAAAACACCCCAAACTTCTGAAATGKTGACCT
CGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGSGGAGGA

Table 2: BLAST analysis result

Sample	DNA Subway	% Match	NCBI GenBank BLAST	% Match	UNITE	% Match
Green sample	*Trichoderma longibrachiatum*	98.79	*Trichoderma longibrachiatum*	98.79	*Trichoderma longibrachiatum*	100

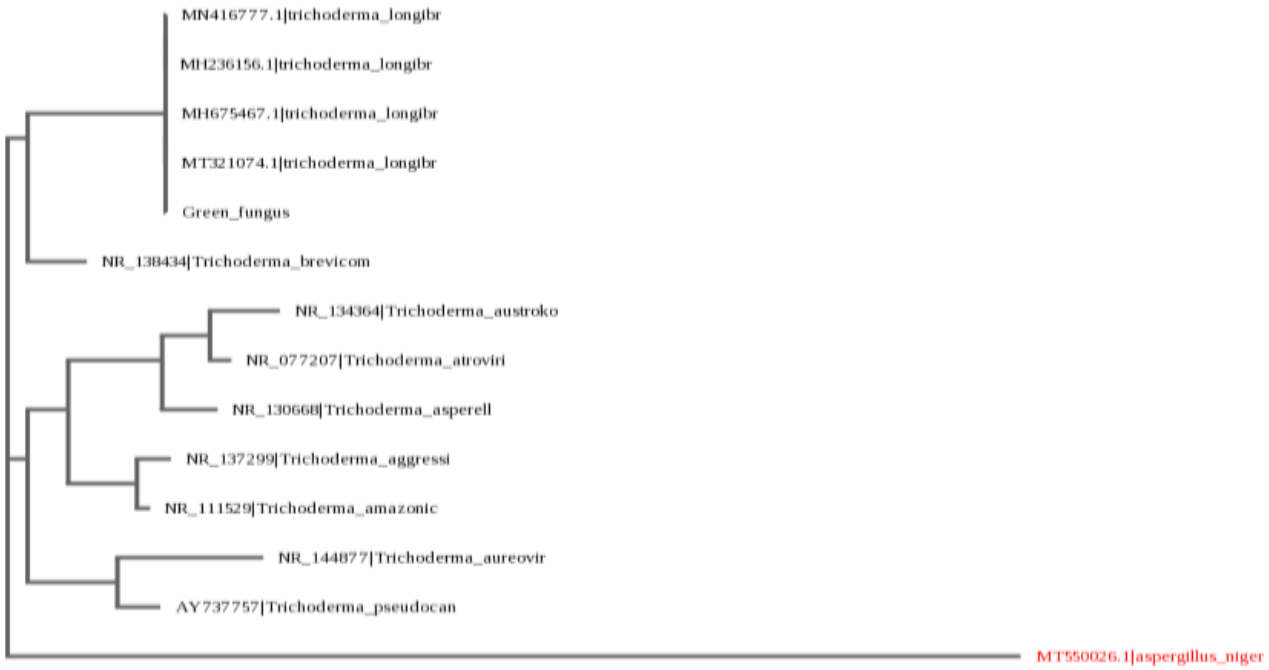

Figure 2: Maximum likelihood (ML) tree of green fungus

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Molecular phylogenetic relationships of this fungal strain (green fungus) with other strains of *Trichoderma* using a maximum likelihood tree showed that the isolated green fungus was a member of the genus *Trichoderma longibrachiatum* with an accession number of MT3201074 (Figure 2). Shahid *et al*.[28] in their studies on molecular identification of *Trichoderma longibrachiatum* 21PP reported a sharp amplified gene fragment band at approximately 700 bp on the gel having 90% sequence similarity with the 18S rRNA gene sequence of *Trichoderma* (GenBank Acc. No.: JX978542). Their result is comparable with the 98.79%, 98.79%, and 100% similarities reported in this work, using the DNA Subway, the BLAST search in GenBank, and the UNITE databases, respectively. Nearly 90% of all commercial enzymes are produced by submerged fermentation using explicitly optimized conditions where growth nutrients and microorganisms are submerged in a liquid medium with higher concentration of products, minimal effluent generation, and simpler equipment requirements.[6] The crude enzyme showed total and specific activities of 19.49 U and 0.63 U/mg (Table 3). After forty percent (40%) ammonium sulphate saturation, the precipitated enzyme had a specific activity of 0.18 U/mg and a percentage yield of 4.26%. Abdel-Mohsen *et al*.[29] precipitated *Trichoderma viride* EF-8 pectinase with specific activities of 2.80 and 2.08 U/mg protein using 50% and 70% ammonium sulfate saturation. Also, Poondla *et al*.[10] precipitated more than 90% of *Saccharomyces cerevisiae* pectinases using 20 and 80% ammonium sulfate saturation. After dialysis, the specific activity was 0.81 U/mg. Gel filtration of the dialysate yielded 4.60 % of the enzyme with a specific activity of 1.32. When the crude enzyme was directly purified using gel filtration, a purification factor of 5.61 and a specific activity of 2.86 U/mg were achieved, yielding 17.63% of the enzyme (Table 3). Siddiqui *et al*.[30] purified *Rhizomucor pusillus* polygalacturonase using Sephadex G-200 followed by Sephacryl S-100 column chromatography and achieved a purification fold of 12.34 and a percentage protein recovery of 27.06%. A rise in a specific activity is a measure of a protein's purity.[31] This current method of enzyme purification demonstrated that purification exercises can be quick, inexpensive, with a high protein yield. Therefore, the purification of crude enzyme, which exhibited the highest specific activity, protein percentage yield, and higher purity, was adopted for the entire protein purification process. The purification profile revealed one protein peak coinciding with the maximum pectinase activity (Figure 3).

Changes in pH and temperature influence pectinase activity. Generally, enzymes must be at the optimal pH and temperature at which the catalytic activity is greatest.

At pH values below or above this optimal value, the catalytic activity of the enzyme is reduced.[32] The optimal pH and temperature of pectinase obtained from *Trichoderma longibrachiatum* MT 321074 were 5.0 (Figure 4) and 40^{o}C (Figure 5), respectively. Most pectinolytic enzymes have higher enzymatic activity at pH levels ranging from 4.0 to 7.0 [33] and are produced by fungi.[34] The optimal pH obtained in this work is a suggestion of the acidic nature of the enzyme and its suitability for application under acidic conditions. Okonji *et al*.[35] presented an optimum pH of 5.0 for *A. fumigatus* pectinase. On the other hand, Adebayo *et al*.[36] reported 40^{o}C as the optimum temperature for *A. niger* and *A. flavus* pectinases. The fall in activity of pectinase beyond 40^{o}C might be a result of enzyme denaturation emanating from peptide bond hydrolysis, disulfide bond destruction, oxidation, and deamination of the amino acid side groups of pectinase.[37]

The Michaelis constant (K_m) and maximum velocity (V_{max}) of pectinase from *Trichoderma longibrachiatum* MT 321074 were 1.0 mg/ml and 82.64 µmole/min, respectively, using the Lineweaver-Burk plot (Figure 6). Amin *et al*.[38] reported K_m and V_{max} values of 16.6 mg/ml and 20 µmol/mL/min for pectinase obtained from *Penicillium notatum*. Hadri et al.[39] reported Km and Vmax values of 0.45 mg/ml and 285.7 M/min for a novel thermophilic endopolygalacturonase produced by *B. licheniformis* IEB-8. Jalil and Ibrahim[32] also reported Vmax and Km values of 1701 U/mg and 3.89 mg/mL for partially purified *Aspergillus niger* LFP-1 pectinase. The V_{max} and K_m values of pectinases differ with the type of substrate, source of enzyme, and assay protocol used.[35] The low value of K_m obtained in this work shows that the enzyme has a strong affinity mango pectin.

Most industrial enzymes are stable at elevated temperature of 40-45^{o}C.[40] Figure 7 showed the influence of reaction time at different temperatures (from 30 to 80◦C) on the activity of partially purified pectinase from *Trichoderma longibrachiatum* MT 321074. The enzyme was relatively stable between 30-50ºC, retaining 92%, 87%, and 72% of its original activity after 2 h at 30ºC, 40ºC, and 50ºC, respectively. When pre-incubated at temperatures above 50ºC, it was rapidly inactivated. Incubation at 60ºC, 70ºC and 80ºC for 120 min resulted in the loss of 38%, 65%, and 73% of the enzymes' original activity, respectively. After 15 and 120 minutes of pre-incubation, respectively, Xu et al.[41] found 39.1% residual activity below 40ºC and a complete loss of native polygalacturonase activity above 50 ºC for an endo α-1, 4-polygalacturonase (AnEPG) from *Aspergillus nidulans*. Ahmed *et al*.[42] reported suppressed enzyme activity at a temperature higher than 50^{o}C.

Also, Okonji *et al*.[35] reported pectinase thermal stability at 60°C with 100% enzyme activity for 45 min and a 50% loss in original activity after 120 min of incubation. Fungal pectinase, being an industrial enzyme are suitable for industrial uses because of their thermostability.[43] Thus, the stable temperature ranges of 30-50°C obtained in this work suggests the suitability of pectinase from *Trichoderma longibrachiatum* MT 321074 for industrial applications.

The thermal stability of an enzyme is estimated based on the distortion of the enzyme's active site via activity assays done at different temperatures. The enzyme's (active site's) melting temperature (T_m) was estimated to be 64^{o}C (Figure 8). The T_m is an inherent characteristic of an enzyme that relates to the alterations in secondary and tertiary structural organizations of proteins upon heating, leading to the unfolding of the enzyme's active site and a consequential reduction in the activity of an enzyme.[44]

Table 3: Purification table of *Trichoderma longibrachiatum* MT321074 pectinase

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude enzyme	200	19.49	30.85	0.63	1	100
(NH4)2SO4	18	0.88	4.61	0.18	0.29	4.26
Dialysis	23	1.42	2.80	0.51	0.81	7.29
Sephadex G200	25	0.90	0.68	1.32	2.10	4.60
*Crude enzyme	200	17.02	33.60	0.51	1	100
*Sephadex G200	25	3.00	1.05	2.86	5.61	17.63

*Crude enzyme = produced using shake and absorbance read at 540nm
*Sephadex G200= obtained from direct purification of "*Crude enzyme"

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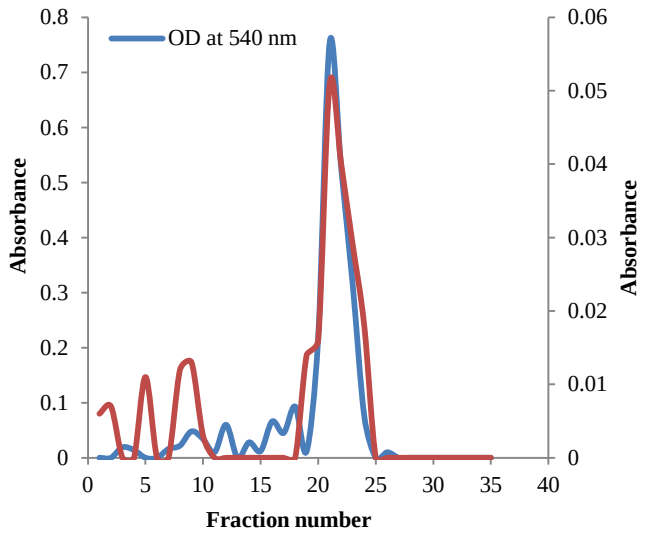

Figure 3: Gel filtration chromatogram for *Trichoderma longibrachiatum* MT321074 pectinase.

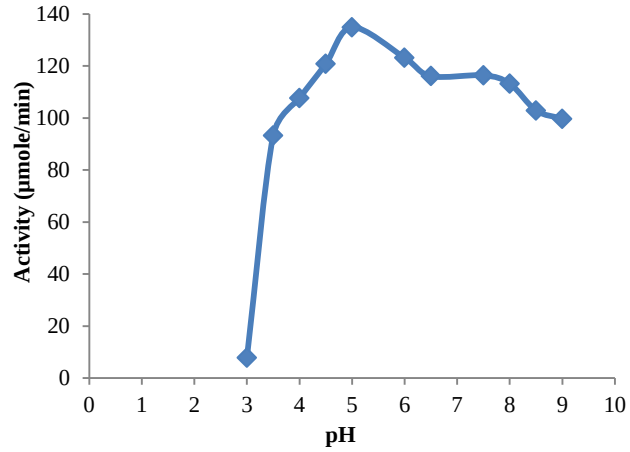

Figure 4: Effect of pH changes on the activity of pectinase from *Trichoderma longibrachiatum* MT 321074

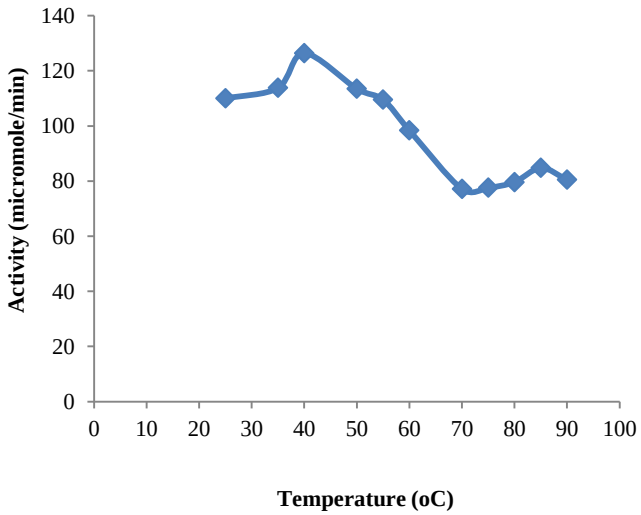

Figure 5: Effect of temperature changes on the activity of pectinase from *Trichoderma longibrachiatum* MT 321074

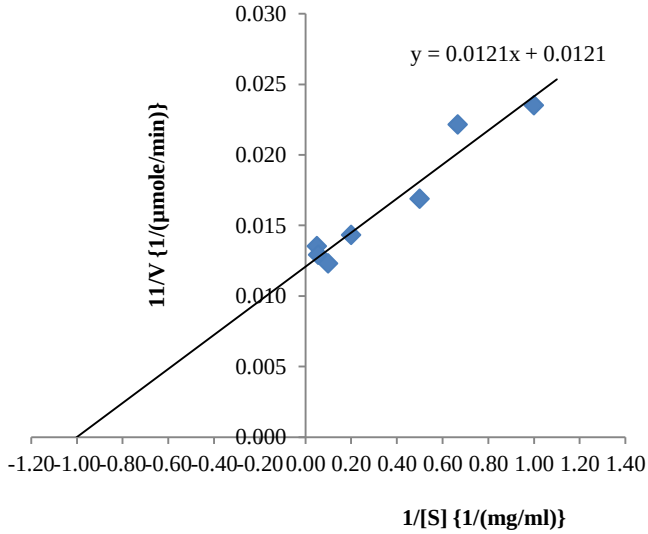

Figure 6: Lineweaver-Burk plot of *Trichoderma longibrachiatum* MT 321074 pectinase.

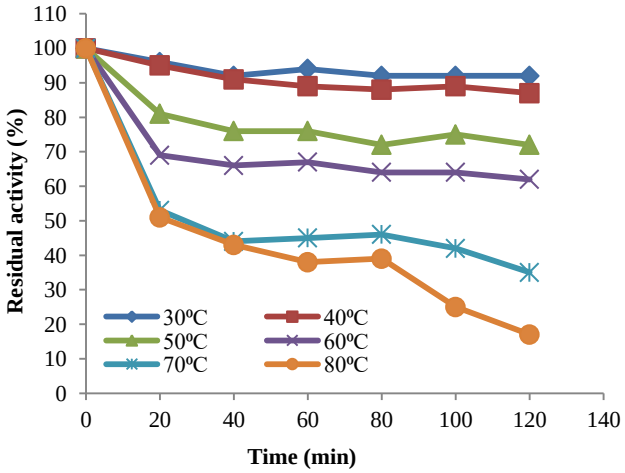

Figure 7: Effect of temperature on *Trichoderma longibrachiatum* MT321074 pectinase stability.

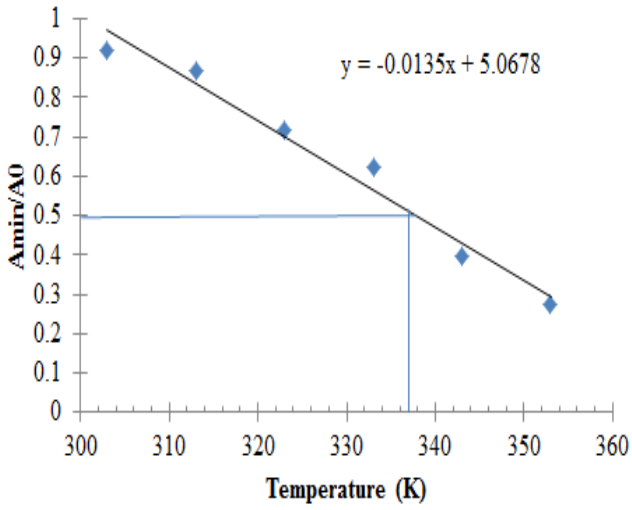

Figure 8: Determination of melting temperature (T_m) that corresponds to the temperature when Amin declines to 50% of the native activity (A_0).

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At melting temperature, 50% of the native enzyme is denatured to an inactive form. Melting temperatures (Tm) of purified -glucosidases from wild and mutant strains of *Cellulomonas biazotea* were reported by Rajoka et al.[45] to be 52°C and 60.5°C, respectively. A thermostable protein is thought to have a T_m value greater than 65°C (Christensen *et al*.[46] Thus, a high T_m value of 64°C reported in this work suggests that *Trichoderma longibrachiatum* MT 321074 pectinase might tolerate an upper temperature while maintaining its conformational structures and functionality.

Protein inactivation involves distortions in the secondary, tertiary, or quaternary structures of proteins without covalent bond breakage.[47] The graph of *lnAres/A0* versus time (min) followed the first-order kinetic pattern (Figure 9) and the rate of thermal inactivation (*Kd*) of *Trichoderma longibrachiatum* MT 321074 pectinase indicated low values between 0.0006 and 0.0088 over temperature ranges of 30-90°C (interval of 10°C) (Table 4). At 50-70°C, Sea *et al*.[48] found *Kd* values of 0.0015 to 0.0207 min1 and 0.0011 to 0.0146 min^{-1}, respectively for *Limicolaria flammea* pyrgalacturonases (PG1 and PG2). A low *Kd value* suggests improved enzyme thermostability, resulting from its ability to resist thermal denaturation. Also, the proportionate increase in *Kd* with temperature rise (Table 4) indicates a loss in pectinase stability at elevated temperatures.[49]

The activation energy of *Trichoderma longibrachiatum* MT 321074 pectinase inactivation (*Ea*) was calculated to be 50.30 KJ/mol (Figure 10) and it represents the energy barrier that must be surmounted for the enzyme to be irreversibly denatured to an irreversibly denatured state (*I*S) from the equilibrium between the native (*N*S) and denatured states (*DS*) of the enzyme (i.e., *N*S ↔ *D*S → *I*S). The Arrhenius plot was linear, suggesting that the rate and mechanism of enzyme inactivation were strongly dependent on temperature changes[50,51] and that these factors turn out to be insignificant at a lower temperature. Bustamante-Vargas *et al*.[52] reported *Ea* values of 91.63 $kJmol^{-1}$, 88.61 $kJmol^{-1,}$ and 83.55 $kJmol^{-1}$ for immobilized pectinase in rigid foam polyurethane (RFPU), free enzyme lyophilized and liquid pectinases, respectively. Adedeji and Ezikiel[53] also reported enzyme inactivation activation energies of 2.26-3.07 kJ/mol and 1.23-2.15 kJ/mol for crude and purified *Aspergillus awamori* CICC 2040 polygalacturonases, respectively. The lower activation energy of an enzyme implies an increased speed and frequency of collisions with lesser energy to form an activation complex for protein hydrolysis.[54,55] There are great demands for enzymes with a higher activation energy for enzyme inactivation (*Ea*) in industrial biotechnology.

The *Z-values* of *Trichoderma longibrachiatum* MT 321074 pectinase were estimated from the inverse of the slope of the linear regression of *logD* versus the change in temperature (Figure 11). The enzyme presented a high *Z-value* of 42°C (Table 4). The *Z-value* is normally used as one of the markers for the inactivation of quality-related enzymes. Enzymes with very low *Z-values* are always very sensitive to heat treatment[58] whereas those with higher *Z-values* are more stable to thermal energy. Thus, a high amount of heat energy and temperature increase is needed to initiate *Trichoderma longibrachiatum* MT 321074 pectinase denaturation.

Furthermore, thermodynamic parameters, such as the Gibbs free energy (Δ*Gin*), changes in enthalpy (ΔH), and entropy of enzyme thermal inactivation (Δ*Sin*) were also determined within the temperature ranges of 30-90°C (Table 4). The Gibbs free energy (*ΔGin*) increased with increasing temperature from 103.25 to 112.85 kJ/mol for *Trichoderma longibrachiatum* MT 321074 pectinase (Table 4). The *ΔGin* is the maximum amount of energy available to a thermodynamic process that can be converted into useful work. It is a more reliable parameter used in estimating and comparing the stability of different enzymes.[59] At constant temperature and pressure, the *Gin* is used to predict the direction and spontaneity of chemical reactions. Within the temperatures tested, the Gibbs free energy values reported in this study were all high and positive. The elevated Gibbs free energy values suggest that the enzyme structure resists denaturation (i.e., thermostable) upon temperature increase.[48] The positive *ΔGin* values suggest that the thermal inactivation process was not spontaneous, but required heat energy and increased temperature to overcome the energy barrier of inactivation, thereby increasing the spontaneity of the process.[60]

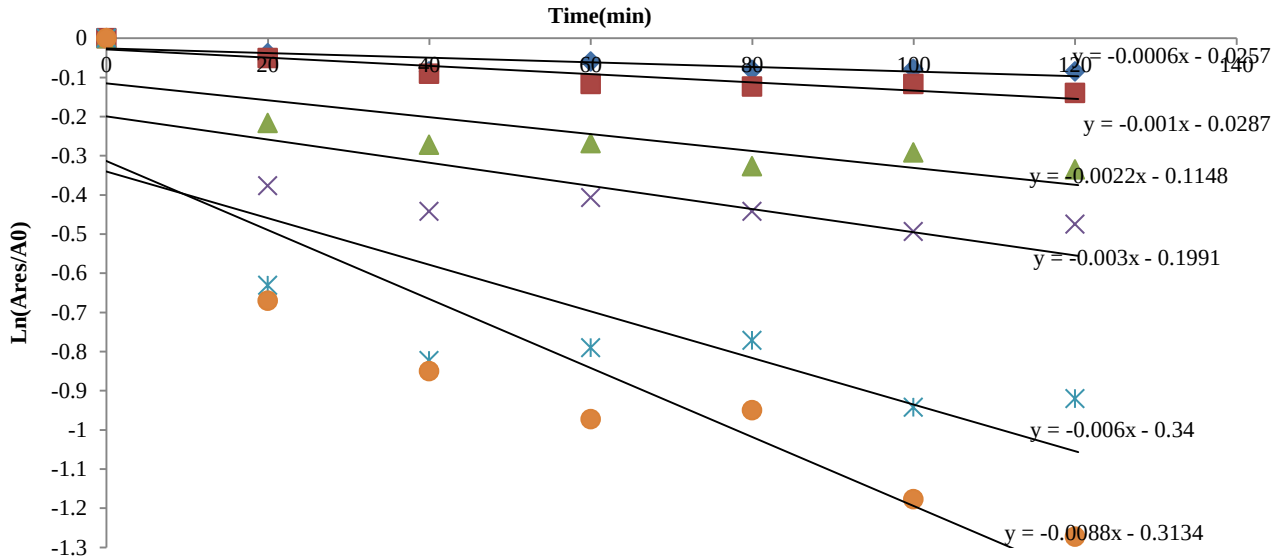

Figure 9: First-order plot to obtain the rates of thermal inactivation, *Kd*

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Table 4: Kinetic and thermodynamic factors of the irreversible thermal inactivation of *Trichoderma longibrachiatum* MT 321074 pectinase

Temp (^{o}C)	Temp (K)	*Ea* (KJ/mol)	*Kd* (min-1)	*t1/2* (min)	*D* (min)	*ΔHin* (KJ/mol)	*ΔGin* (kJ/mol)	*ΔSin* (KJ/mol/K)
30	303	50.30	0.0006	1155.00	3838.33	47.78	103.25	-0.183
40	313	50.30	0.0010	693.00	2303.00	47.70	105.41	-0.184
50	323	50.30	0.0022	315.00	1046.82	47.61	106.75	-0.183
60	333	50.30	0.0030	231.00	767.67	47.53	109.28	-0.185
70	343	50.30	0.0060	115.50	383.83	47.45	110.67	-0.184
80	353	50.30	0.0088	78.75	261.70	47.36	112.85	-0.186

Z-value = 42^{o}C.

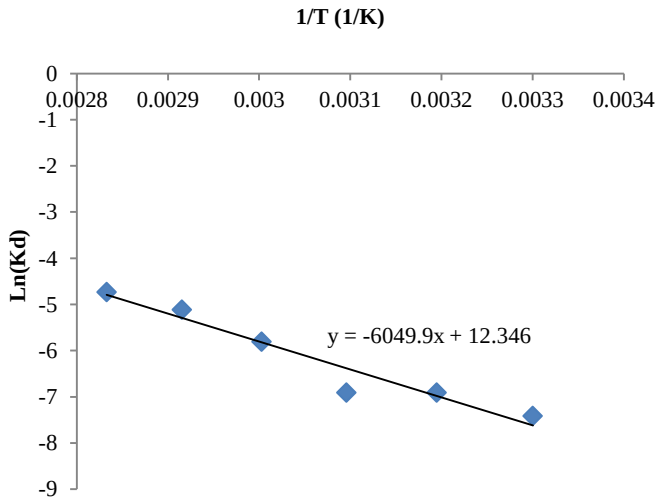

Figure 10: Arrhenius plot of the *lnKd* vs *1/T*, the slope gives the activation energy of denaturation, *Ea* (J mol^{-1}).

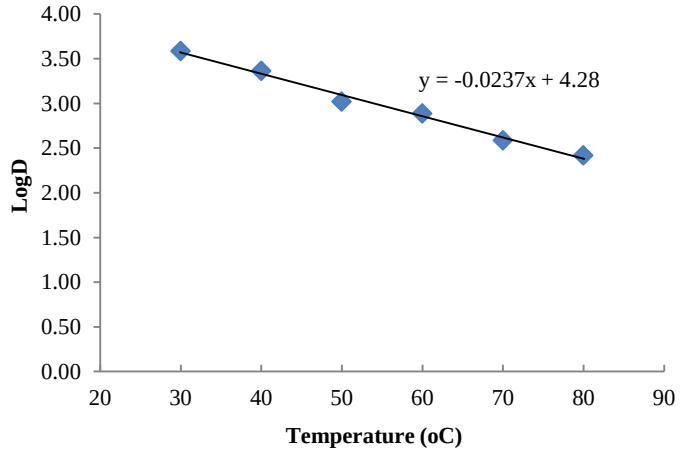

Figure 11: Plot of the *LogD* vs temperature (^{o}C), the negative inverse of the slope (*-1/S*) gives the *Z value.*

The amount of non-covalent interactions present in the natural structure of the enzyme, such as Vander Waals forces, hydrogen, electrostatic, and hydrophobic interactions, determines the change in enthalpy (Δ*Hin)* values of enzyme inactivation.[24] The value of *ΔHin* is usually used in predicting the intensity of thermal disruption of enzymes' non-covalent interactions.[61] If a protein has a high number of non-covalent interactions in its native structure, it will be very stable at high temperatures. That is to say, that higher *ΔHin* values are associated with more enzyme thermal stability. High *ΔHin* values obtained in this study were all positive in the ranges of 47.78-47.36 KJ as the temperature was increased from 30-90^{o}C (Table 4). These positive *ΔHin* values suggest that the protein inactivation process was endothermic and therefore, required a thermal energy supply to break the non-covalent interactions holding the native protein structure. The *Hin* value also decreased slightly as the temperature increased, indicating that less heat energy is required for pectinase denaturation at higher temperatures. The *ΔHin* values of the enzyme were lower than the corresponding *ΔGin* values at all temperatures of the study. According to Mansour *et* al.[62] thermostable enzymes usually have high Δ*Gin* values with corresponding low Δ*Hin* values.

The change in entropy (*ΔSin*) of enzyme inactivation is another important thermodynamic factor that determines the enzyme's thermal denaturation. It is a measure of enzyme disorderliness and randomness due to its structural disruptions during thermal denaturation.[63] The *ΔS* values obtained in this work were increasingly negative with temperature rise from 30-90^{o}C (Table 4), indicating a more ordered transition state. The Negative values of *ΔSin* imply that the enzyme was in an organized and aggregated state during inactivation[62] in which some intra and intermolecular forces were formed. Ibrahim et al.[58] attributed the negative entropies of protein thermal inactivation to more water molecules ordered at the protein interface. In summary, the increase in Δ*Gin* accompanied by decline in *ΔSin* and Δ*Hin* values at elevated temperatures suggests that *Trichoderma longibrachiatum* MT 321074 pectinase is heat stable.

Conclusion

The optimal pH and temperature for pectin hydrolysis into galacturonic acid by pectinase isolated from *Trichoderma longibrachiatum* MT 321074 were 5.0 and 40^{o}C. Studies on the effect of heat treatment on *Trichoderma longibrachiatum* MT 321074 pectinase showed an increase in Δ*Gin*, which was accompanied by a decline in *ΔSin* and Δ*Hin* values at high temperatures. This suggests that *Trichoderma longibrachiatum* MT 321074 pectinase is a heat stable enzyme suitable for galacturonic acid production, degradation of pectic substances in fruit effluent, food and beverage products. These results would assist in establishing the economic sustainability of *Trichoderma longibrachiatum* MT 321074 pectinase in industry.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

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