Research article

Statistical, physicochemical, and thermodynamic profiles of chitinase production from local agro-industrial wastes employing the honey isolate Aspergillus niger EM77

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ABSTRACT

Enzyme synthesis from local wastes has a lot of potential because it eliminates the problem of waste accumulating in conjunction while also cutting the cost of these useful products. Plackett-Burman (PBD) and central composite designs (CCD) were used to optimize the manufacturing process utilizing the honey isolate Aspergillus niger EM77, resulting in a 60-fold increase in enzyme productivity using a group of wastes comprising wheat, rice straw, and sawdust. The enzyme had its optimum activity at 60 °C, pH 5, and had high thermo-stability at 60 °C, with K_m and V_max of 0.8 mg mL^{-1} and 2083.33 μmol mL^{-1} min^{-1} respectively. The activation (E_a) and deactivation (E_d) energies of chitinase were 2.78 and 174.46 kJ mol^{-1}, respectively, with the thermodynamic constants ΔH° and ΔG° ensuring enzyme stability. As a result, Aspergillus niger EM77 chitinase has the efficiency to meet the global market demand for chitinase enzyme while also providing a significantly lower price than what is now available on the websites of specialist international companies. The production process is almost costless because it is based primarily on waste and contains traces of minerals.

1. Introduction

Waste recycling has become one of the most serious local and global issues, as its accumulation is an environmental disaster that has a negative impact on life on the planet as a whole. As a result, all governments are working to reduce pollution caused by trials to get rid of these wastes. For example, in Egypt, rice and wheat straw are the most accumulative agricultural wastes because rice and wheat represent the majority of crops cultivated by farmers and are necessary food for the majority of people. According to the US Foreign Agriculture Service (FAS), Egypt’s rice production would be around 4.3 million tons in 2020/2021. The majority of farmers burn these wastes to get rid of them, resulting in a severe pollution problem known as the black cloud, which is a gathering of pollutants. As a result, the government is working hard to build sites where these wastes can be recycled. Egypt’s Agricultural and Environment Ministries agreed to an agreement to help mitigate the effects of the black cloud. They recycled around 1.4 million tons of rice straw, which they used to make organic fertilizers and non-traditional feeds. Egypt’s Ministry of Environment, on the other hand, recycles rice straws for furniture and animal feed. Despite all of the foregoing efforts, substantial amounts of waste remain, and scientists can play an important role in turning these wastes into valuable goods such as enzymes. Indeed, numerous recent researches have shown that agricultural waste may be used to produce a variety of useful enzymes, Saleh et al. (2021) utilized Corchorus olitorius stems (molokhia stems) to make xylanase, Shehata et al. (2018) used potato shells to make chitinase, and chitosanase, and Abdel Wahab et al. (2021) used rice straw and orange peel to make β-galactosidase. Enzymes are extremely significant and have been used in a variety of industries, including pharmaceuticals, food, and biological and safe pesticides, among others. Despite the importance of enzymes and their numerous applications in various fields, one of the most significant barriers to their industrial application is their high production cost (50 units of Sigma-Aldrich chitinase cost € 693.00). Hence the significance of this study, which provides a low-cost, safe, and environmentally friendly production process that allows the environment to breathe again. Chitinase is one of the most valuable enzymes that operates on chitin (the linear polymer of β-(1–4)-linked N-acetyl-D-glucosamine, GlcNAc) and normally requires a rather high-cost production technique when it relies on chitin present in the production medium. Rice straw, wheat straw, and sawdust were employed as the main carbon and nitrogen sources for chitinase synthesis since the economic cost became the primary target for enzyme application capabilities. Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that has been extensively employed in chitin breakdown to make a high molecular weight (MW) linear
polymer of N-acetyl-D-glucosamine units, as well as in the hydrolytic processes that yield Carbonyl sulfide (COS). In terms of chitin break position, chitinases are divided into three categories: endo-chitinases [EC 3.2.1.14], exo-chitinases [EC 3.2.1.29], and N-acetylglucosaminidases [EC 3.2.1.52]. Exo-chitinases generate diacetyl-chitobiase by breaking the chain at both the reducing and non-reducing ends (GleNaC2). Chitinases can be produced by a wide range of organisms, including animals, viruses, plants, insects, bacteria, and fungi. However, filamentous fungi generate chitinases with higher activity levels and a broader antifungal spectrum (Adrangi and Faramarzi, 1993). One of the most promising chitinase producers is Aspergillus Niger (Rattanakit et al., 2007; Brzezinska, and Jankiewicz, 2012). It's worth noting that chitinous materials are abundant in nature, accounting for around 75% of the entire weight of shellfish like shrimp, crabs, and krill, which is regarded as waste. Chitinases could be used in a variety of applications, including the production of pharmaceutically valuable chito-oligosaccharides and N-acetyl β-glucosamine, the formation of single-cell protein, the control of pathogenic fungi, the recycling of chitin-containing waste (Li et al., 2005), pesticides (Fang et al., 2012), and bio-ethanol production (Deshmukh et al., 2013). Plackett-Burman (PB) and central composite designs (CCD) were employed as a two-step technique to improve and optimize chitinase production on wastes, saving time and money. The characterization of the enzyme, as well as the calculation of its kinetics and thermodynamic characteristics, provide a complete picture of the enzyme for the best use (Mostafa et al., 2018). As part of the global trend to save our planet, this research focuses on the production of chitinase using local wastes, purifying the enzyme, and estimating its physicochemical qualities, kinetics, and thermodynamic features to research the most suitable design aspects are.

The parameters that had a substantial effect (95% confidence level, Prob > F ≤ 0.05) on chitinase production were examined in the next optimization stage based on regression analysis.

2.4. Medium optimization process through experimental designs

2.4.1. Plackett-Burman design

For this study, the Plackett–Burman (PB) experimental design (Plackett and Burman, 1946) had eleven components (rice straw, wheat straw, K2HPO4, sawdust, wheat bran, chitin, glucose, soya bean, NaNO3, MnSO4, CaCl2). The experiment was designed as a 12-trial experiment using the rule R = n + 1, where R represents the number of trials and n represents the number of components. Each factor is represented at two levels, high (+1) and low (−1) as shown in (Table 1). The following first-order equation is used in the PB design:

\[
Y = \beta_0 + \sum_{i=0}^{k} B_i X_i
\]

where Y denotes the response (chitinase production), \(\beta_0\) denotes the model intercept, \(B_i\) denotes the linear coefficient, \(X_i\) denotes the independent factor level, and k denotes the number of factors. The trials were done in triplicates, with the mean chitinase production for each trial serving as the response variable. A balanced design should have equivalent standard errors, with smaller standard errors indicating a stronger design.

2.4.2. Central composite design (CCD)

The most efficient three components (glucose, wheat straw, and sawdust) were chosen for central composite design (CCD) to discover the most significant concentration following the PBD.

The selected factors were tested at five coded levels (−2, −1, 0, +1, +2), with a total of 25 trials consisting of 8 factorial design trials, 8 axial point trials, and 9 replications of the central points (Table 2). Other parameters and circumstances in the PB design were kept at the same level as in the experiment with the highest chitinase output.

The CCD's conclusions are based on the second-order polynomial equation:

\[
Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i^2 + \sum \beta_{ijk} X_i X_j
\]

where Y is the chitinase production, \(\beta_0\) represents the intercept term, \(\beta_i\) is the linear coefficients, \(\beta_{ij}\) is the quadratic coefficients, \(\beta_{ijk}\) is the interactive coefficients, and \(X_i\) and \(X_j\) represent the coded independent components.

2.5. Model validation

The model was assessed by carrying out two experimental combinations under the conditions predicted by the model and then comparing the results to the expected values.

2.6. Statistical analysis

It was carried out after the analysis of variance (ANOVA).

2.7. Partial purification

The optimized culture filtrate (crude chitinase) of A. niger was fractionated using ethanol concentrations (25–75 %), and each fraction...
Table 1. Optimization of *A. niger* EM77 chitinase production using Plackett-Burman design (PBD).

| Run | Factor A | Factor B | Factor C | Factor D | Factor E | Factor F | Factor G | Factor H | Factor J | Factor K | Factor L | Chitinase activity |
|-----|----------|----------|----------|----------|---------|---------|---------|---------|---------|---------|---------|-------------------|
|     | Rice straw | Wheat straw | K₂HPO₄ | Sawdust | Wheat bran | Glucose | Soya bean | NaNO₃ | MnSO₄ | CaCl₂ | (U/ml) |
|     | gm/lank | gm% | gm/lank | gm% |
| 1 | 2 | 5 | 0 | 5 | 5 | 0.1 | 0.5 | 5 | 0.02 | 0 | 0.01 | 2430.54 | 2446.00 |
| 2 | 2 | 5 | 0.5 | 2 | 5 | 0.5 | 0.5 | 2 | 0.01 | 0 | 0.05 | 2368.35 | 2352.89 |
| 3 | 5 | 5 | 0.5 | 2 | 2 | 0.1 | 0.5 | 2 | 0.02 | 0.001 | 0.01 | 2443.75 | 2447.52 |
| 4 | 4 | 2 | 0.5 | 2 | 5 | 0.2 | 0.2 | 5 | 0.02 | 0.001 | 0.01 | 1953.44 | 1968.23 |
| 5 | 2 | 5 | 0.1 | 5 | 2 | 0.1 | 0.2 | 5 | 0.01 | 0.001 | 0.05 | 2390.56 | 2386.11 |
| 6 | 5 | 2 | 0.2 | 5 | 5 | 0.1 | 0.2 | 2 | 0.02 | 0 | 0.05 | 2310.14 | 2313.91 |
| 7 | 5 | 2 | 0.1 | 5 | 5 | 0.5 | 0.5 | 5 | 0.01 | 0 | 0.01 | 2397.05 | 2392.60 |
| 8 | 5 | 5 | 0 | 2 | 2 | 0.5 | 0.5 | 2 | 0.02 | 0 | 0.05 | 2339.98 | 2355.44 |
| 9 | 2 | 2 | 0 | 5 | 5 | 0.5 | 0.5 | 2 | 0.02 | 0.001 | 0.05 | 2325.71 | 2330.16 |
| 10 | 5 | 5 | 0 | 5 | 5 | 0.5 | 0.5 | 2 | 0.01 | 0.001 | 0.01 | 2236.04 | 2221.25 |
| 11 | 2 | 2 | 0 | 2 | 2 | 0.1 | 0.2 | 2 | 0.01 | 0 | 0.01 | 2210.32 | 2195.53 |
| 12 | 5 | 2 | 0 | 2 | 5 | 0.1 | 0.5 | 5 | 0.01 | 0.001 | 0.05 | 2316.63 | 2312.86 |

R² = 0.99, Adjusted R² = 0.97, Predicted R² = 0.88, Adeq Precision = 24.96.

Table 2. CCD of *A. niger* EM77 chitinase production.

| Run | Factor A | Factor B | Factor C | Chitinase activity | Actual value | Predicted value |
|-----|----------|----------|----------|-------------------|-------------|-----------------|
|     | Glucose | Wheat straw | Sawdust | (U/ml) |
|     | gm/f | gm/f | gm/f |
| 1 | 0.2 (-1) | 4 (+1) | 2 (-1) | 1000.60 | 938.28 |
| 2 | 0.2 (-1) | 4 (+1) | 4 (+1) | 3200.32 | 3430.20 |
| 3 | 0.2 (-1) | 2 (-1) | 4 (+1) | 1814.13 | 1961.74 |
| 4 | 0.2 (-1) | 2 (-1) | 2 (-1) | 1018.75 | 1203.78 |
| 5 | 0.4 (+1) | 2 (-1) | 2 (-1) | 4718.87 | 4546.38 |
| 6 | 0.4 (+1) | 4 (+1) | 2 (-1) | 778.07 | 687.85 |
| 7 | 0.4 (+1) | 4 (+1) | 4 (+1) | 1628.90 | 1501.26 |
| 8 | 0.4 (+1) | 2 (-1) | 4 (+1) | 3506.12 | 3625.83 |
| 9 | 0.13 (-2) | 3 (0) | 3 (0) | 1500.23 | 1428.86 |
| 10 | 0.13 (-2) | 3 (0) | 3 (0) | 1520.62 | 1428.86 |
| 11 | 0.3 (0) | 3 (0) | 3 (0) | 2330.32 | 2199.35 |
| 12 | 0.3 (0) | 3 (0) | 3 (0) | 2252.12 | 2199.35 |
| 13 | 0.3 (0) | 3 (0) | 3 (0) | 2199.25 | 2199.35 |
| 14 | 0.3 (0) | 3 (0) | 3 (0) | 2250.62 | 2199.35 |
| 15 | 0.3 (0) | 3 (0) | 3 (0) | 2300.12 | 2199.35 |
| 16 | 0.3 (0) | 3 (0) | 3 (0) | 2350.45 | 2199.35 |
| 17 | 0.3 (0) | 3 (0) | 3 (0) | 2000.32 | 2199.35 |
| 18 | 0.3 (0) | 3 (0) | 3 (0) | 2010.66 | 2199.35 |
| 19 | 0.3 (0) | 3 (0) | 3 (0) | 2100.32 | 2199.35 |
| 20 | 0.3 (0) | 4.73 (+2) | 3 (0) | 1342.89 | 1399.94 |
| 21 | 0.3 (0) | 1.27 (-2) | 3 (0) | 3603.36 | 3469.79 |
| 22 | 0.3 (0) | 3 (0) | 1.27 (-2) | 1445.62 | 1554.46 |
| 23 | 0.3 (0) | 4.73 (+2) | 3 (0) | 3100.67 | 2915.31 |
| 24 | 0.47 (+2) | 3 (0) | 3 (0) | 2600.33 | 2653.12 |
| 25 | 0.47 (+2) | 3 (0) | 3 (0) | 2590.66 | 2653.12 |

R² = 0.99, Adjusted R² = 0.97.

2.8. Characterization of *A. niger* EM77 chitinase

The ideal temperature, pH, time, and thermal stability, among other characteristics, were determined.

2.8.1. Effect of different times on *A. niger* chitinase activity

To determine the best time for reaction, sets of an identical preparation of reaction mixtures were tested for different time intervals ranging from 30 to 150 min.

2.8.2. Optimum temperature and pH of *A. niger* chitinase

The optimal temperature of *A. niger* chitinase was established by running the assay at various temperatures (30–80 °C) for 30 min (the best reaction time calculated from the previous experiment) under the standard assay conditions. The enzyme was assayed in a 0.05 M final concentration of citrate buffer (pHs 4.0–6.0) and acetate buffer (pHs 5.0–7.0) for the pH analysis.

2.8.3. Thermal stability profile for *A. niger* chitinase

The thermostability of *A. niger* chitinase was assessed by measuring the residual enzyme activity after the enzyme had been incubated in 0.05 M acetate buffer for periods ranging from 50 to 70 °C (15–60 min). The enzyme activity of aliquots taken at different time periods was evaluated using a conventional assay.

2.8.4. Estimating the optimum substrate concentration for *A. niger* chitinase

It was calculated by running the assay with different concentrations of the typical substrate (PNP–GlcNAc) (0.125–3 mg/mL), then calculating *Kₘ* and *Vₘₐₓ* from the Lineweaver Burk plot.

2.8.5. Thermodynamic profile of *A. niger* chitinase

It has a number of parameters that were calculated as follows.

The following formulae were used to calculate the t₁/₂ and D-value (decimal reduction time).

\[ D = \frac{ln(10)}{k_d} \]

\[ t_{1/2} = \frac{ln2}{k_d} \]
The activation energy (E<sub>a</sub>) for chitinase was determined using the Arrhenius plot, and the deactivation energy (E<sub>d</sub>) was computed using a graph of the ln dissociation constant (ln k<sub>d</sub>) vs the reciprocal of the absolute temperature (K) using the equation,

\[
\text{Slope} = -\frac{E_d}{R}
\]

The following equations were used to calculate the change in enthalpy (\(\Delta H\), kJ mol\(^{-1}\))-free energy (\(\Delta G\), kJ mol\(^{-1}\)) and entropy (\(\Delta S\), J mol\(^{-1}\) K\(^{-1}\)) for thermal denaturation of chitinase,

\[
\Delta H = E_d - RT
\]

\[
\Delta G = -RT \ln(K_d\ h/K_b\ T)
\]

\[
\Delta S = \frac{\Delta H - \Delta G}{T}
\]

where T is the corresponding absolute temperature (K), R is the gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)), h is the Planck constant (6.626 \times 10^{-34} J min), and k<sub>b</sub> is the Boltzman constant (1.38 \times 10^{-23} J K\(^{-1}\)).

3. Results and discussion

3.1. Optimization process of chitinase production using multi-factorial experiments

3.1.1. PBD for chitinase production

First, a (PBD) was used to investigate the relationship between various medium ingredients and to identify the parameters that influence <i>A. niger</i> chitin productivity. The enzyme records indicated a wide range of activity from 1953.44 to 2443.75 U/mL (Table 1) while the various medium ingredients and to identify the parameters that in

| Source       | Sum of Squares | df | Mean Square | F-value | p-value | significance |
|--------------|----------------|----|-------------|---------|---------|--------------|
| Model        | 1.921E+05      | 8  | 24018.30    | 48.85   | 0.0043  | Significant  |
| A-Rice straw | 11082.02       | 1  | 11082.02    | 22.54   | 0.0177  |              |
| B-Wheat straw| 40359.88       | 1  | 40359.88    | 82.08   | 0.0028  |              |
| C-Saw dust   | 17447.53       | 1  | 17447.53    | 35.48   | 0.0095  |              |
| D-Wheat bran | 20190.86       | 1  | 20190.86    | 41.06   | 0.0077  |              |
| F-Chitin     | 19309.76       | 1  | 19309.76    | 39.27   | 0.0082  |              |
| G-Glucose    | 59017.20       | 1  | 59017.20    | 120.02  | 0.0016  |              |
| K-MnSO<sub>4</sub> | 12691.26 | 1  | 12691.26    | 25.81   | 0.0147  |              |
| L-CaCl<sub>2</sub> | 12047.90 | 1  | 12047.90    | 24.50   | 0.0158  |              |
| Residual     | 1475.16        | 3  | 491.72      |         |         |              |

The data analysis Pareto chart (Figure 1) revealed that glucose, wheat straw, and sawdust had the greatest impact on enzyme productivity, followed by CaCl<sub>2</sub> and rice straw. The addition of glucose, wheat straw, and sawdust to the chitinase production medium as additional carbon and nitrogen sources increased output and had a higher significance than chitin, wheat bran, and soya bean. Joo (2005) showed that adding glucose to chitin enhanced chitinase production in <i>S. halstedii</i>, but Narayana and Vijayalakshmi (2009) found that Streptomyces sp. ANU 6277 chitinase production was inhibited in the medium supplemented with glucose and arabinose compared to control. In addition, Sandhya et al. (2004) found that adding carbon sources (0.75 % w/v) other than colloidal chitin (1.5 % w/v) to the medium had no effect on the productivity of <i>Trichoderma harzianum</i> chitinase, whereas adding nitrogen sources (0.42 % w/v) such as peptone and tryptone to the medium had a significant effect on enzyme production. According to Zarei et al. (2010), malt extract and colloidal chitin were the best nitrogen and carbon sources, respectively.

Chitinase production was explained using the first-order polynomial equation:

Chitinase activity (U/mL) = +1992.24944 + 20.25944 Rice straw + 38.66278 wheat straw + 25.42056 saw dust – 72.34611 wheat bran – 200.57083 chitin + 467.52778 glucose – 65041.66667 MnSO<sub>4</sub> + 1584.29167 CaCl<sub>2</sub>

The high significance of the design was also emphasized from the closure between actual and predicted values (Table 1).

3.1.2. CCD for chitinase production

The second step CCD (Table 2) (second-order model) was used to estimate the ideal concentration of each ingredient based on the results of the PB design (glucose, wheat straw, and sawdust). According to the P-values, all linear variables (A, B, C) and interactions (AB, AC, BC) are significant model terms, but none of the quadratic components showed a significant effect (Table 4).

The multiple regression analysis of the experimental results, lead to the following second-order polynomial equation:

Chitinase activity (U/mL) = –9291.79990 + 46510.27549 glucose + 322.73264 wheat straw + 277.16261 saw dust – 8982.57000 glucose * wheat straw – 4196.27500 glucose * saw dust + 433.49000 wheat straw * sawdust – 5492.08178 glucose<sup>2</sup> + 79.00892 wheat straw<sup>2</sup> + 12.34892 sawdust<sup>2</sup>

The model’s R<sup>2</sup> value suggested that it could explain around 98.51 % of the fluctuations in chitinase activity, indicating that it was fit for purpose; also, the adjusted R<sup>2</sup> value (0.9725) was very close to R<sup>2</sup>, indicating model validity. The low value of CV = 7.71 % suggested the design validity, while the model F-value of 26.14 indicated the model significance.

The relationship between chitinase production and experimental levels of each variable was expanded using three-dimensional (3D) response surface graphs (Figure 2A-C), each of which displays the influence of two parameters while keeping the other at a zero level. The strong interaction between glucose and each of sawdust and wheat straw is evident in Figure 2B and C, while Figure 2A explains the interaction between sawdust and wheat straw.

The final optimized medium components are (gm/l) (rice straw, 5; wheat straw, 2; saw dust, 2; wheat bran, 2; soya bean, 2; chitin, 0.1; glucose, 0.4; and (gm%) K<sub>2</sub>HPO<sub>4</sub>, 0.1; NaNO<sub>3</sub>, 0.02; MnSO<sub>4</sub>, 0.001; CaCl<sub>2</sub>, 0.01), incubation time is 6 days.

3.2. Experimental validation of the model

The close relationship between the experimental and predicted chitinase yields (4718.87 and 4546.38 U/mL, respectively) indicated model validity, as shown by the normal plot of residuals (Fig not shown), where the data points are close to the straight line that explained the model’s effectiveness.

In the current study, the optimization method featured two-step designs, each of which played a part in improving enzyme productivity to a
high level in a short period of time. The PBD increased output by almost 58 times (2443.75 U/mL) as compared to the non-optimized one (42.39 U/mL), and the CCD (4718.87 U/mL) increased production by about two times.

According to Singh et al. (2009), Paenibacillus sp. D1 chitinase production increased by 2.56 times over the non-optimized medium utilizing statistical designs (PBD, CCD), but Pantoea dispersa chitinase production grew by 4.21 times using PBD (Gohel et al., 2006). When compared to the activity of the basal medium of Aspergillus awamori chitinase, Dukariya, and Kumar, 2021 reported a five-fold increase in Bacillus cereus chitinase production using Box-Behnken Design, while Awad et al. (2017) reported a 22-fold increase in chitinase production using Box-Behnken Design.

3.3. Partial purification of A. niger EM77 chitinase

The fractions obtained at 50 and 75 % ethanol purified 2.9 and 3.4 times, respectively, therefore they were combined for the subsequent procedure.

3.4. Characterization of A. niger EM77 chitinase

3.4.1. Estimating the optimal time for chitinase activity

The optimum period for chitinase assay was investigated (30–150 min), and it was revealed that 30 min is the best time (data not shown), with a 94 % increase over the control time (60 min).

3.4.2. Temperature and pH profiles for A. niger EM77 chitinase activity

The ideal temperature for A. niger chitinase activity was 60 °C, where the enzyme activity rose by around 14% (Figure 3A); similarly, Abdel Wahab et al. (2018) found that the conjugated form of Trichoderma longibrachiatum KT693225 exochitinase was 60 °C, whereas the native was 40 °C.

The ideal pH was investigated, and the enzyme demonstrated maximal activity at pH 5 (control) (data not shown), with a quick
decrease in activity on both the acidic and alkaline sides. Shivakumar et al. (2014), on the other hand, found that *Bacillus subtilis* JN032305 chitinase activity peaked at pH 9 and 50°C.

### 3.4.3. Estimation of kinetic parameters of *A. niger* EM77 chitinase activity

Using the Lineweaver Burk plot (Figure 3B), the enzyme's $K_m$ and $V_{max}$ values were estimated as (0.8 mg mL$^{-1}$ and 2083.33 μmol mL$^{-1}$min$^{-1}$), respectively. The low $K_m$ value demonstrates the enzyme's strong sensitivity to the substrate, it was 10 times lower than that of *T. longibrachiatum* KT693225 free form chitinase (Abdel Wahab et al., 2018) and two times lower than that of *Rhizopus stolonifer* NCIM 880 chitinase (Sonawane et al., 2016). The $V_{max}$ value of *A. niger*, on the other hand, was higher than that of *Bacillus pumilus* JUBCH08 chitinase (38.23 μmol mL$^{-1}$ min$^{-1}$) reported by Bhattacharya et al. (2016) and lower than that scored by (Abdel Wahab et al., 2018) for the free form of *T. longibrachiatum* KT693225 chitinase (4000 μmol mL$^{-1}$ min$^{-1}$).

### 3.4.4. Thermal stability profile and thermodynamic calculations of *A. niger* EM77 chitinase

The enzyme has remarkable thermal stability, retaining approximately 91% of its activity after 1 h at 60°C (Figure 3C). At (65 and 70°C), activity dropped dramatically, with the enzyme retaining only around 55 and 12% of its original activity after 1 h of incubation, respectively. Low activation energy $E_a$ (energy required to generate the activated enzyme-substrate complex) and high denaturation energy $E_d$ (energy required for denaturation) are indicators of an enzyme's thermal stability (Tayefi-Nasrabadi and Asadpour, 2008). The $E_a$ and $E_d$ for chitinase were computed as 2.78 and 174.46 kJ mol$^{-1}$ from the Arrhenius plot (Figure 3D and E), which are more promising than the $E_a$ and $E_d$ for free *T. longibrachiatum* chitinase (3.39 and 6.88 kcal mol$^{-1}$ equivalent to 14.22 & 28.87 kJ mol$^{-1}$ respectively) Abdel Wahab et al., 2018.

According to Abdel Wahab et al. (2018), the $T_{1/2}$ of *A. niger* chitinase is lengthy (1386.294 and 1155.245 min at 55 and 60°C), which is superior to that of both free and conjugated forms of *T. longibrachiatum* chitinase (316.48 and 869.10 min). Because of the low energy required to activate *A. niger* chitinase and the lengthy $T_{1/2}$, all prior findings have underlined its economic value. The D-value (time required to suppress enzyme activity by 90%) for pure chitinase was 4605.17 and 3837.64 min at 55 and 60°C, respectively, whereas Abdel Wahab et al. (2018) determined the D-value for free *T. longibrachiatum* chitinase to be 733.08 min at 60°C. The $\Delta H$ and $\Delta G$ value of chitinase (Table 5) at 70°C were 171.60 and 98.60 kJ mol$^{-1}$ respectively which reflected the high stability of the enzyme as more amount energy is required for thermal denaturation (Mostafa

### Table 5. Kinetic and Thermodynamic parameters of *A. niger* EM77 chitinase.

| Temperature (°C) | $K_m$ (mg mL$^{-1}$) | $V_{max}$ (μmol mL$^{-1}$min$^{-1}$) | $E_a$ (kJ mol$^{-1}$) | $E_d$ (kJ mol$^{-1}$) | $T_{1/2}$ (min) | $\Delta H$ (kJ mol$^{-1}$) | $\Delta G$ (kJ mol$^{-1}$) |
|------------------|---------------------|-------------------------------|---------------------|---------------------|-----------------|-------------------|-------------------|
| 55               | 0.5                 | 171.73                        | 2.78                | 174.46              | 1386.29         | 101.36            |                   |
| 60               | 0.6                 | 171.69                        | 2.78                | 174.46              | 1155.25         | 102.45            |                   |
| 65               | 2.5                 | 171.65                        | 2.78                | 174.46              | 277.26          | 100.01            |                   |
| 70               | 7                   | 171.60                        | 2.78                | 174.46              | 99.02           | 98.60             |                   |

Figure 3. (A–E) Different physicochemical, kinetic, and thermodynamic properties of *A. niger* EM77 chitinase, these figs are derived from the temperature study where the assay was done using (PNP—GlcNAc) as a substrate, acetate buffer; pH 5.0 for 30 min at different temperatures.
The free form of *Trichoderma longibrachiatum* chitinase scored lower ΔH° value (26.18 kJ mol⁻¹) at 50 °C (Abdel Wahab et al., 2018), also, Shehata et al. (2018) recorded lower values for ΔH° and ΔG° (47.95, 65.46 kJ mol⁻¹) of *A. griseoarantiacus* chitinase at 60 °C. The positive value of ΔS° (Table 5) indicates that the universe’s disorder is increasing from reactants to products, which could be due to enzyme purity, but it can also be beneficial because it signifies generating more molecules. *T. longibrachiatum* KT693225 and *A. griseoarantiacus* chitinase, on the other hand, have a negative ΔS°, indicating increased resistance to inactivation (Abdel Wahab et al., 2018; Shehata et al., 2018).

4. Conclusion

In keeping with the goal of producing enzymes from waste, chitinase was produced using a low-cost process that aids in the disposal of many agro-industrial wastes. The optimization procedure included (PBD and CCD) designs, each of which had a significant impact on increasing enzyme productivity in a short period of time. The enzyme has been shown to be extremely heat-stable. Furthermore, all thermodynamic and kinetic studies indicated that *A. niger* EM77 could be used in a wide range of pharmaceutical and industrial applications.

Declarations

**Author contribution statement**

W.A. Abdel Wahab: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mona A. Esawy: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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**Data availability statement**

Data included in article/supp. material/referenced in article.

**Declaration of interest’s statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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