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Statistical optimization of pectinases from thermophilic *Aspergillus fumigatus* BT-4 employing response surface methodology through submerged fermentation using agricultural wastes

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Abstract

Background In this study, thermophilic pectinase-producing strains were isolated. Among all the isolates, strain No. 4 was identified as *Aspergillus fumigatus* BT-4 based on its morphology and 18 S rDNA analysis. This strain was employed to screen various fermentation media to enhance pectinase production. Pectinases are crucial enzymes with significant industrial applications, particularly in the food and textile industries. Identifying efficient pectinase producers and optimizing their production processes are essential for improving industrial applications.

Results Maximum pectinase production was observed using 1% grapefruit peel in M5 media. Shake flask kinetics demonstrated the highest values of specific rate constant (qp), specific growth rate (μ), product yield coefficient ($Y_{p/x}$), volumetric rate of product formation (Q_p), and biomass formation (Q_x) after 72 h of incubation. Furthermore, Optimization of fermentation components via Response Surface Methodology (RSM) improved pectinase production by 50%, showcasing the effectiveness of factorial and central composite designs in fine-tuning parameters. The use of agricultural waste (grapefruit peel) significantly reduced production costs, offering an economically viable substrate alternative. The pectinase enzyme was purified through ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography, resulting in a 2.3-fold purification. The molecular weight of the purified enzyme was determined to be 48 kDa. Enzyme kinetics, determined using a Lineweaver-Burk plot at various pectin concentrations, showed a V_{max} of 32.7 UmL^{-1} and a K_m of 0.3 mg mL^{-1}. Thermodynamic parameters, including activation energy (Ea), enthalpy (ΔH), and entropy (ΔS), were measured at 41.74 $kJmol^{-1}$, 39.53 $kJmol^{-1}$, and 46.9 $kJmol^{-1}$, respectively.

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Conclusions The study successfully isolated and identified *Aspergillus fumigatus* BT-4 as a potent thermophilic pectinase producer. Optimization of the fermentation process using 1% grapefruit peel in M5 media significantly enhanced pectinase production. Using grapefruit peel as an agricultural waste in pectinase production reduces costs by eliminating the need for expensive raw materials and utilizing a low-cost, sustainable, and locally available substrate. This approach also minimizes waste disposal expenses, making the process more economical. The enzyme was effectively purified, and its kinetic and thermodynamic properties were thoroughly characterized, revealing its potential for industrial applications. The comprehensive analysis of production kinetics and optimization strategies provides a robust foundation for scaling up pectinase production, contributing to more efficient and cost-effective industrial processes.

Keywords Coefficient, Kinetics, Pectinases, *A. fumigatus*, Volumetric rate

Background

Pectinases are hydrolases that break down pectin-based compounds present in plant biomass. They have been classified based on their mode of action on galacturan backbone, substrate specificity, or their target site [1]. Among pectinases, Polygalacturonases are able to hydrolyze glycosidic linkages found in pectic acid and can be categorized as endo-, exo-polygalacturonases. Endo-polygalacturonases randomly cut α-1-4 glycosidic bonds of polygalacturonic acid, present inside the chain, whereby exo-polygalacturonases cleave the hydrolytic bond inside backbone from non-reducing ends. Endo-polygalacturonases produce oligogalacturonic acids, while exo-polygalacturonases generate unsaturated mono- or di-galacturonic acids [2]. This heterogeneous enzyme class has wide applications in food industries and is routinely used for juice clarifications, pretreatment of grape juice for wine making, tomato pulp extraction, oil processing and tea fermentation. Pectinases are also employed in the textile sector for fibers degumming [3]. Fungi excrete enzymes to break down complex substances present in their surroundings. They are industrially important microorganisms for producing enzymes such as cellulases, xylanases, ligninases, pectinases, etc [4]. Though some fungi secrete substantially higher amounts of lignocellulolytic enzymes during submerged fermentation, solid-state fermentation can still be used to produce enzymes [5]. Submerged fermentation has proven to be a useful system for enzyme production at large scale as it is easy to upscale and optimize using various bioprocessing approaches viz., fed batch or continuous mode of nutrition.

Thermophilic pectinases are enzymes derived from heat-loving microorganisms, which remain active at higher temperatures. Their importance lies in their ability to accelerate juice clarification and fruit processing at elevated temperatures, improving efficiency and reducing energy consumption. Unlike mesophilic enzymes, thermophilic pectinases maintain stability at low pH and high temperatures, making them ideal for industrial applications such as juice clarification, wine production, and waste treatment. Their robust nature offers significant advantages in terms of process optimization and product quality [6].

Thermophilic fungi have the tendency to grow at temperatures exceeding 40 °C. Various thermophilic fungi play an eminent role in decomposing organic matter piles and are pathogens to humans and animals both [7]. Most of studies reporting pectinases production by fungi make use of species isolated from mesophilic environment [3]. There are scarce reports covering pectinase production using thermophilic fungi. Nevertheless, enzyme production using mesophilic microorganisms is on the rise, owing to their biochemical and physiochemical characteristics and stability [8, 9]. However, some fungal species belonging to *Chaetomium*, *Dactylomyces*, *Malbrenchea*, *Taloromyces*, *Scytalidium*, *Myceliophthora*, *Thermoascus*, *Paelomyces Stibella*, *Thermomyces*, *Thermomucor*, *Sporotrichum*, *Rhizomucor*, *Acrophiolophora*, and *Thielavia* have been described to synthesize pectinases [10]. Hence, the exploitation of thermophilic microorganisms for synthesizing thermostable enzymes imperative to industries can be very useful for the latest biotechnological interventions.

The use of agro-industrial by-products like wheat bran, grapefruit peel, and other fruit residues in pectinase production significantly contributes to the circular economy by transforming waste into valuable resources. This process not only reduces the environmental impact of agro-waste, which often ends up in landfills, but also promotes resource efficiency, particularly in the food and agriculture sectors. With millions of tons of fruit and vegetable by-products generated annually, valorizing these materials helps minimize waste accumulation, lower disposal costs, and reduce methane emissions from landfills. Additionally, this approach supports several United Nations Sustainable Development Goals (SDGs), such as SDG 12 (Responsible Consumption and Production), SDG 9 (Industry, Innovation, and Infrastructure), and SDG 11 (Sustainable Cities and Communities), by encouraging sustainable industrial practices, reducing ecological footprints, and improving waste management. By repurposing these by-products, industries can reduce their reliance on synthetic materials, promoting more

sustainable and circular production cycles while advancing the goals of resource efficiency, food security, and environmental sustainability.

The concept of valorizing waste into valuable products is an integral part of sustainable waste management and resource recovery. The pectinase production, utilizing agro-industrial by-products, particularly fruit peels such as orange and lemon, significantly contributes to waste minimization by diverting these organic residues from landfills. According to estimates, food waste and agro-industrial by-products contribute to a substantial portion of landfill mass, with fruit waste alone representing about 50–60% of total organic waste in some regions [11]. By converting these waste materials into valuable bioproducts like pectinases, we not only reduce landfill waste but also decrease the need for synthetic chemicals in various industries. This approach aligns with the principles of the circular economy, wherein waste materials are transformed into resources for other processes, promoting both environmental sustainability and economic efficiency. Furthermore, valorizing these by-products can lead to significant reductions in carbon footprint and environmental impact. For example, substituting synthetic carbon sources with fruit peels in enzyme production helps lower the overall environmental impact of biotechnological processes, reducing the reliance on fossil-derived materials and energy. Thus, the use of fruit peel waste for pectinase production offers a win-win scenario: it mitigates waste management challenges while fostering sustainable industrial practices [12].

Using agro-waste like fruit peels and wheat bran for pectinase production offers significant economic and environmental benefits. These low-cost materials, often discarded by the agriculture and food industries, are a cheaper alternative to traditional fermentation media like molasses or glucose. Agro-waste can cost as little as $0.05–$0.10 per kilogram, compared to $0.25–$0.50 for refined sugars, making enzyme production more affordable at a commercial scale. This practice reduces waste, lowers production costs, and promotes resource efficiency, supporting sustainability goals. It also creates new revenue opportunities, reduces landfill waste, and contributes to a circular economy [11, 13].

Fermentation medium is responsible for an enormous segment of production cost. Economically suitable carbon and nitrogen sources for pectinase production may include inexpensive carbon-containing by-products such as wheat bran, sunflower seeds, peanut hulls, soybean extract and other agro-industrial wastes, particularly the fruit peels of sweet orange and lemon [1]. Many obstacles are faced when evaluating the growth determining factors in solid-state fermentation such as cellular growth determination in the case of structurally and nutritionally complex substrates such as any agro-industrial by-product [14]. Kinetic models provide significant mathematical depictions of the biological processes aiding in their better understanding and upscaling. They allow for the optimization of fermentation processes in a convalescent manner by taking into consideration the combined effects of all the significant variables [15].

The conditions of fermentation and the substrate's composition greatly affect the microorganism's ability to produce pectinase. Statistical methods have been widely used in the previous few decades to optimize industrial processes and lower production costs. Finding the important variables influencing the synthesis of the enzymes and figuring out their ideal concentrations are often the first steps in the optimization process. However, the One-Factor-At-a-Time (OFAT) strategy for optimizing culture conditions and medium has certain limitations regarding the accuracy of the interactions between the variables or factors. For the purpose of studying the interaction between components, statistically based methodologies thus offer more advantageous and economical options. The Plackett-Burman Design (PBD) and Response Surface Methodology (RSM) have been successfully integrated in several studies to optimize the levels of significant variables. This approach is especially effective when multiple significant variables (typically two or three) require further optimization [16].

Recent advances in pectinase production have highlighted the potential of thermophilic fungi, particularly *Aspergillus fumigatus*, for sustainable biotechnological applications. *Aspergillus fumigatus* BT-4 was chosen for this study due to its robust pectinase-producing capability at elevated temperatures, making it ideal for industrial applications. The strain's ability to efficiently utilize agricultural waste as a substrate for pectinase production offers a cost-effective and environmentally friendly approach, aligning with the growing demand for green biotechnological solutions. This study aims to explore its potential in optimizing pectinase production for industrial use. The production of pectinase was statistically optimized through the application of RSM. In this regard, the PBD helps in determining the most significant factors involved in an experiment and screens out insignificant ones. Furthermore, Central Composite Design (CCD) generates a 2nd order quadratic model to assess responses in certain regions. This strategy helps in improving the pectinase yield in less time with reduced medium costs. This research study also presents the enzyme characterization and kinetics of pectinases produced by the novel thermophilic *Aspergillus fumigatus* BT-4.

Methods

Isolation and screening of pectinolytic fungi

In this study, a total of ten thermophilic pectinolytic fungal strains were isolated from different sources including 10 cm deep soil, compost and industrial waste piles such as agricultural waste piles (e.g., leftover crop residues and fruit peels), sugarcane bagasse piles (from sugar mills) from different areas of Punjab by serial dilution method on petri plates containing Pectinase Screening Agar Medium (PSAM) g/L of pectin 1, KH_2PO_4 0.2, NH_4Cl 0.3, $MgSO_4$ 0.01 and agar 25 [17]. Following inoculation, the plates were incubated at 50 °C for 3–4 days to facilitate fungal growth. The colonies exhibiting larger zones of pectin hydrolysis were selected and subsequently identified based on their microscopic and macroscopic characteristics. The selected strain was identified using the 18s rDNA sequencing technique. Phylogenetic and molecular evolutionary analyses were performed using MEGA software (version x).

Inoculum preparation

The spore or conidial inoculum were prepared by adding 10 mL of sterilized distilled water in a 3 to 4 days old fungal culture on a slant. The spores/conidia were dispersed in distilled water by scratching the fungal culture with a sterile inoculation loop and the test tubes were shaken vigorously to make homogenized suspension [18].

Shake flask fermentation

Submerged fermentation studies were conducted by inoculating 1×10^4 spores/mL of fungal culture into a 50 mL media volume. Seven media (g/L) were screened for the synthesis of pectinases by *Aspergillus fumigatus* BT-4 (Table 1). The carbon source in the selected optimized medium was then substituted with various fruit peel wastes such as lemon peel, grapefruit peel, orange peel and banana peel in order to make the pectinase production more economical.

Table 1 Screening media for pectinase production

Media	Components
Medium 1	$KHPO_4$. $3H_2O$ 1; sucrose 10 and Czapek concentrate 10 mL. Czapek concentrate contained (g/100 mL), $NaNO_3$ 30; KCl 5; $MgSO_4.7H_2O$ 5; $FeSO_4.7H_2O$ 0.1 [19].
Medium 2	$(NH_4)_2SO_4$ 10, $MgSO_4{\cdot}7H_2O$ 10, $NH_4H_2PO_4$ 10, orange bagasse 20, distilled water 1000 mL [20].
Medium 3	$(NH_4)_2SO_4$ 10, $MgSO_4{\cdot}7H_2O$ 10, $NH_4H_2PO_4$ 10, citrus pectin 20, distilled water 1000 mL [21].
Medium 4	$(NH_4)_2SO_4$ 10, $MgSO_4{\cdot}7H_2O$ 10, $NH_4H_2PO_4$ 10, a mixture of wheat bran 10 and orange bagasse 10 (1:1), distilled water 1000 mL [20].
Medium 5	$(NH_4)_2SO_4$ 10, $MgSO_4{\cdot}7H_2O$ 10, $NH_4H_2PO_4$ 10, wheat bran 20, distilled water 1000 mL [20].
Medium 6	Lemon peel 10 (ground and autoclaved), $(NH_4)_2SO_4$ 5, $MgSO_4{\cdot}7H_2O$ 6, KH_2PO_4 2, and $FeSO_4{\cdot}7H_2O$ 1, distilled water 1000 mL [22].
Medium 7	Na_2HPO_4 2, KH_2PO_4 4, $FeSO_4.7H_2O$ 0.2, $MnSO_4.7H_2O$ 0.007, $CaCl_2$ 0.01, $(NH_4)_2SO_4$ 2, H_3BO_3 0.01, pectin 15, distilled water 1000 mL [23].

Experimental design

This software design expert version 12 facilitated the application of the Plackett-Burman design for initial screening, followed by a rotatable Central Composite Design (CCD) for further optimization of significant factors. The Plackett-Burman design was employed to identify the key medium components that significantly influence PGase production (Table 2). The three significant factors chosen based on PDB results were then evaluated via central composite design for further optimization studies (Table 3).

The quadratic model generated by CCD representing the relationship between independent variables for enzyme production is as follows:

$$\begin{aligned} Y =& b_0 + b_1A + b_2B + b_3C + \ b_{12}\mathrm{AB} \\ & - b_{13}AC - b_{23}BC - b_{11}\mathrm{A}^2 - b_{22}B^2 - \ b_{33}C^2 \end{aligned} \quad (1)$$

Where Y is the response (PGase activity), b's are the regression coefficients, A, B and C are temperature, pH and inoculum age, respectively. Optimal conditions for PGase production by *A. fumigatus* BT-4 were estimated using regression analysis of CCD. The actual responses were then assessed in relation to the predicted ones to estimate the precision of this methodology.

Enzyme assay

Pectinase activity was assessed using Okafor's [24] method. The reaction mixture included 1 mL of crude enzyme and 1 mL of 1% citrus pectin in sodium acetate buffer (pH 5.5), incubated at 50 °C for 30 min. After incubation, 1 mL of DNS reagent was added, followed by heating in a boiling water bath for 5 min and dilution with 7 mL of distilled water. A blank was prepared using distilled water instead of enzyme. Reducing sugars were quantified at 546 nm, using galacturonic acid as a standard. One unit of enzyme activity (U) was defined as the amount of enzyme needed to release 1 µmol of galacturonic acid under these conditions [25].

Dry cell weight measurement

This parameter was studied according to the method of Ali et al. [4]. The fermented broth was filtered, and the resulting cell mass was washed. The cell mass was then placed in a preweighed Petri dish and oven-dried at 105 °C for 2 h. After drying, the Petri dish was weighed again. The dry cell mass (DCM) was calculated by subtracting the initial weight of the Petri dish from its final weight, expressed in grams per liter (g/L).

Table 2 Plackett Burman design for the optimization of medium using seven independent variables

Factor	Name	Low level	High lvel
A	Temperature (° C)	40	50
B	pH	4	8
C	Volume of fermentation medium (mL)	25	125
D	Inoculum size (mL)	2	5
E	Peptone (%)	1	6
F	Tween 80 (%)	0.1	0.5
G	Inoculum age (days)	1	4

Table 3 Levels of three factors for central composite design

Factor	Name	Low level	High level
A	Temperature (° C)	40	50
B	pH	1	5
C	Inoculum age (days)	1	5

Protein determination

Total protein content was determined using the Bradford assay [26].

Kinetic studies

Kinetic studies for batch fermentations were carried out following [27]. Specific growth rate i.e., μ (h^{-1}) and volumetric rates such as product formation Q_p ($UL^{-1}h^{-1}$) and biomass formation Q*x* ($g\ L^{-1}h^{-1}$) were determined. However, Product yield co-efficient $Y_{p/x}$ and Specific rate constant was estimated by the following equations:

$$Y_{p/x} = dp/dx \tag{2}$$

$$qp = \mu \times Y_{p/x} \tag{3}$$

Enzyme purification steps of PGase

Flasks were removed from the rotating shaker after the specific incubation time. The crude extract was centrifuged at 4000 rpm for 20 min, followed by partial purification using ammonium sulfate precipitation (0–90% saturation). The resulting extract was then dialyzed against 0.05 M citrate buffer (pH 4.4) overnight, with multiple buffer changes (three to four) to remove any excess salt and was dissolved in the same buffer. Anion exchange chromatography was performed on the concentrated crude enzyme, 0.4 mL of the concentrated enzyme was added to a DEAE Sephadex column (19 cm × 0.7 cm) that had been washed and equilibrated with 0.05 M citrate buffer (pH 4.4). Following equilibration, 2 mL of the sample was then collected after the enzyme was eluted using the same buffer. Following that, the active fraction was recovered for the subsequent purification procedure. The Sephadex-100 column, which had been pre-equilibrated with 0.05 M citrate buffer (pH 4.4), was then loaded with the active fractions from the previous step. The same buffer was used to elute the fractions at a flow rate of 0.41 milliliters per minute. Enzyme activity was measured in the fractions from each purification stage. After every purification step, active peak fractions were collected. The enzyme activity, along with the total protein, was measured in all the elution [28].

Molecular weight determination

The purity and molecular weight of PGase were assessed using SDS-PAGE gel electrophoresis, employing a 12% resolving gel and a 5% stacking gel [28].

Enzyme characterization of PGase

The enzyme was characterized for appropriate temperature and hydrogen ion concentration variations. The enzyme activity was measured at varying pH 3–6, whereas the effect of temperature was evaluated at 40 to 100 ° C.

Michaelis-Menten constant (km) and vmax values

Kinetic constants such as *Km* and *V*max were estimated by documenting the reaction velocity at different concentrations (0.2–1.4% (w/v) of pectin substrate. Michaelis Menten's equation was converted into the Lineweaver-Burk equation. The values of Km and Vmax were derived from the Lineweaver Burk plot's slope and intercept between 1/[S] and 1/V [29].

Thermodynamic parameters

The enthalpy change (ΔH) reflects the energy input needed to activate the enzyme at a given temperature, whereas entropy (ΔS) was calculated using standard thermodynamic equations. Furthermore, the Activation energy (Ea) was estimated using the Arrhenius method, as previously described by [30].

$$Ea = -\ slope\ *\ R \tag{4}$$

$$\Delta H = Ea - RT \tag{5}$$

$$\Delta S = LnVmax/T = Ln(KB/h) + \Delta S/R - \Delta H/R.1/T \tag{6}$$

Where ΔH=Change in enthalpy; Ea=activation energy of inactivation; R=universal gas constant; T=absolute temperature (K).

h=Plank's constant (6. 63×10^{-34} m^2.kg/s); KB=Boltzman constant (1.3807×10^{-23} J/K). ΔS=Change in entropy.

Table 4 Screening of thermophilic fungi for the production of PGase

Serial No.	Fungal isolates	Pectinase activity (U/mL)
1	*Aspergillus flavus* BT-1	1.76 ± 0.03
2	*Talaromyces thermophiles* BT-2	1.52 ± 0.01
3	*Thermomyces dupontii* BT-3	1.32 ± 0.05
4	*Aspergillus fumigatus* BT-4	**2.84 ± 0.01**

Results and discussion

Isolation and screening of high pectinase potential thermophilic fungal strain

Finding new fungal strains that can produce the desired product is a crucial step in the fermentation process as it enables the discovery of new and potentially more efficient producers of the target compound [31]. During the process of producing and optimising enzymes, a huge number of fungal strains were isolated and screened. For a variety of reasons, it became crucial to comprehend their features. Fungal identification is often based on morphological and biochemical characteristics; nevertheless, distinguishing between closely related species requires in-depth phenotypic analysis and molecular identification [32]. Ten thermophilic fungal strains were isolated (Table S1) from different samples, such as industrial waste piles, soil and compost from different cities of Punjab, through the serial dilution method [33]. Primary screening of the fungal strains was carried out using a Pectinase Screening Agar Medium (PSAM). The colonies producing bigger zones of pectin hydrolysis on the PSAM plates with higher pectinolytic activity were selected (Fig S1) and subjected to secondary screening at 50° C for 3 days under submerged fermentation.

Morphological and molecular identification

Among all the isolates, four thermophilic fungal colonies showing higher titers of PGase production (Table 4) were selected and identified by their morphological characteristics. Two colonies, coded BT-1 and BT-2 were isolated from soil whereas BT-3 and BT-4 were isolated from waste piles and compost, respectively. BT-1 showed powdery masses of yellowish-green spores with white margins and morphologically identified as *Aspergillus flavus* (Fig. 1). Colony morphology of isolate BT-2 exhibited a greyish central region with a distinctive brush-like penicillus structure, leading to its identification as *Talaromyces* thermophiles based on characteristic morphological features. (Fig. 2). The colonies possessing round margins and are white and green or pale green from the interior are identified as *Thermomyces dupontii* BT-3 (Fig. 3). BT-4 showed velvety colonies with a flat surface. The colonies were smokey green or greyish green in the centre, possessed a whitish margin, and were identified as *Aspergillus fumigatus* (Fig. 4). Among all the thermophilic strains, *A. fumigatus* BT-4 produced the highest titers of pectinase and was molecularly identified through 18 S rDNA sequencing. The 18 S rDNA coding technique, renowned for its slow evolutionary rate, was employed to reconstruct the evolutionary history of organisms. DNA extraction, amplification, and sequencing were performed to obtain the 18 S rDNA sequences.

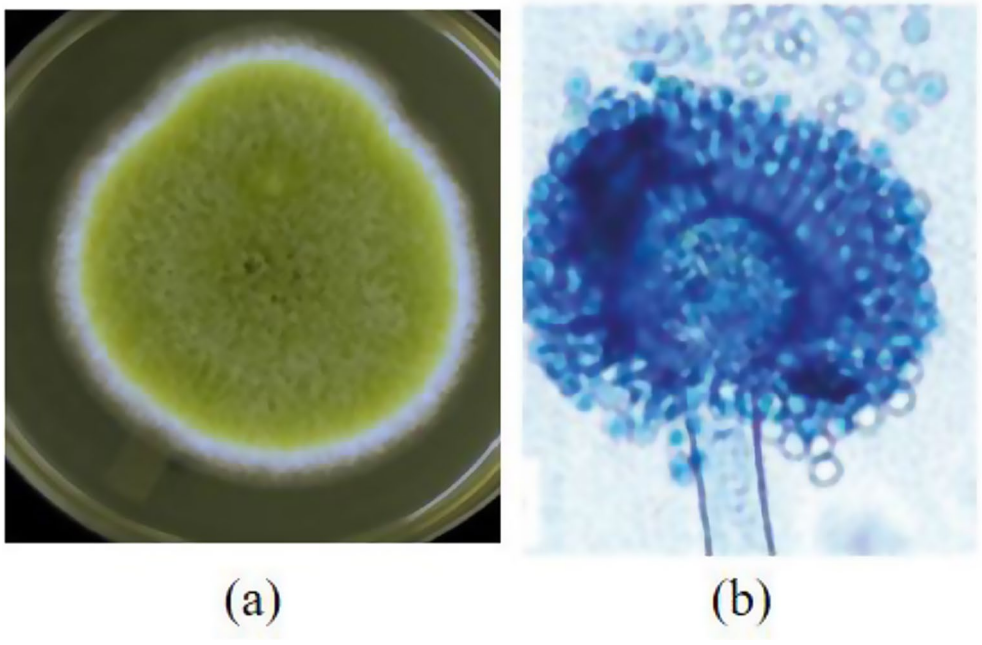

Fig. 1 (**a**) Colony morphology of *A. flavus* (**b**) Microscopic structure

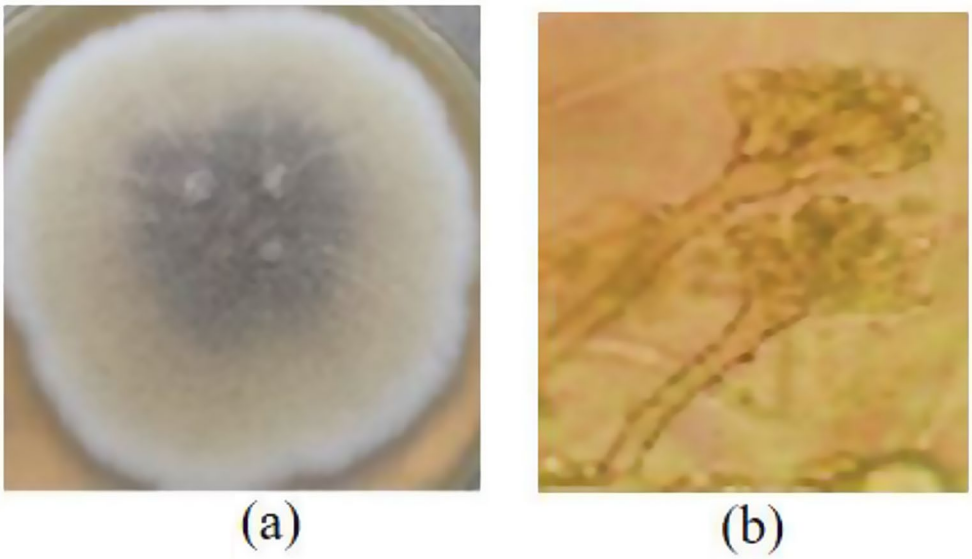

Fig. 2 (**a**) Colony morphology of *Talaromyces thermophiles* (**b**) Microscopic structure

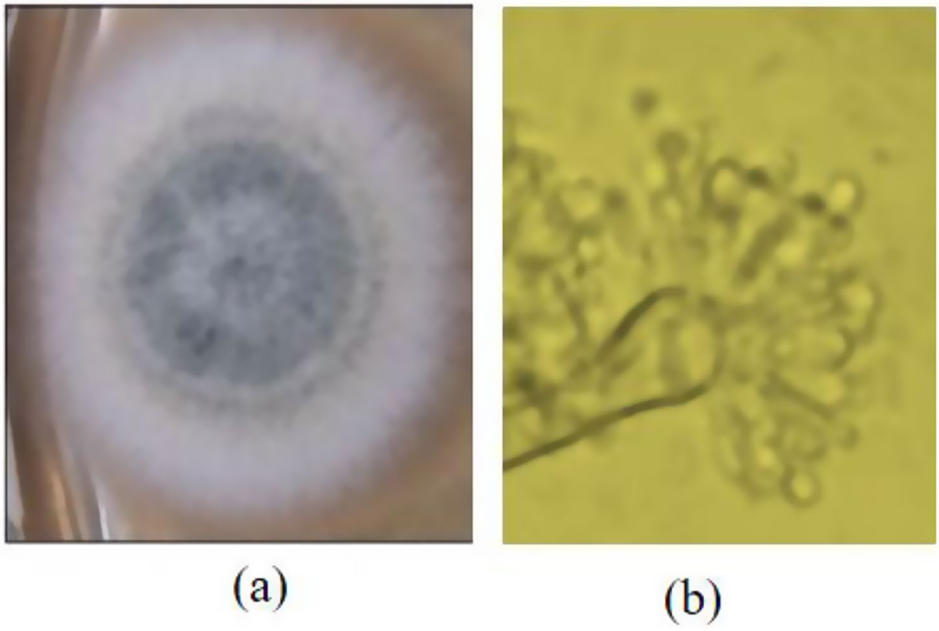

Fig. 3 (**a**) Colony morphology of *Thermomyces dupontii* (**b**) Microscopic structure

The ITS region (5.8 S rDNA) was amplified using the universal primers ITS-1 (F): TCCGTAGGTGAACCTG CGG and ITS-4 (R): TCCTCCGCTTATTGATATGC. The PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. A final extension was carried out at 72 °C for 5 min [34]. The 548 bp sequence was subjected to a BLAST search against the GenBank database to identify its microbial origin and was assigned accession number PQ569976. The resulting sequences were then used to construct a phylogenetic tree using the neighbour-joining method, providing insight into the evolutionary relationships between the query sequence and its closest relatives (Fig. 5). A BLAST search yielded a perfect match (100% similarity) with the *A. fumigatus* sequence, confirming the strain's identity as *A. fumigatus*.

Fermentation studies

Seven media were screened for pectinase synthesis by *A. fumigatus* BT-4, and M5 medium exhibited the highest PGase production of 4.18 U/mL with total protein content of 0.1 mg/mL and dry cell mass of 5.12 g/l (Fig. 6a).

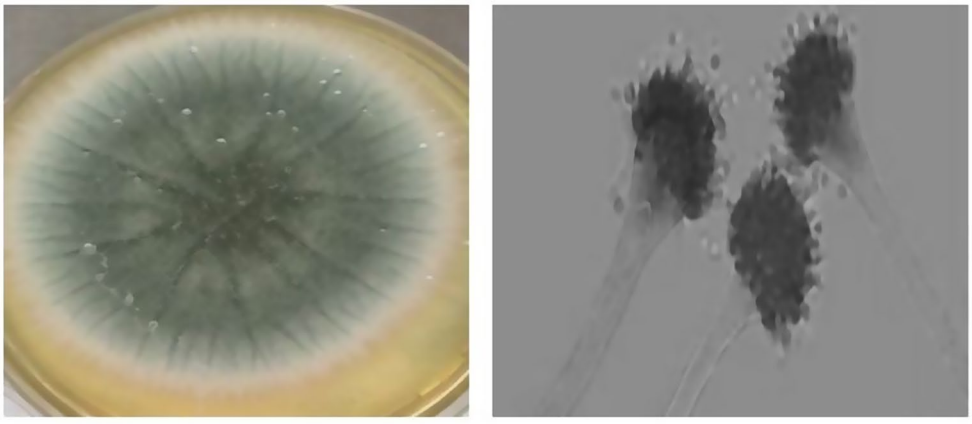

Fig. 4 (**a**) Colony Morphology of *A. fumigatus* (**b**) Microscopic Structure

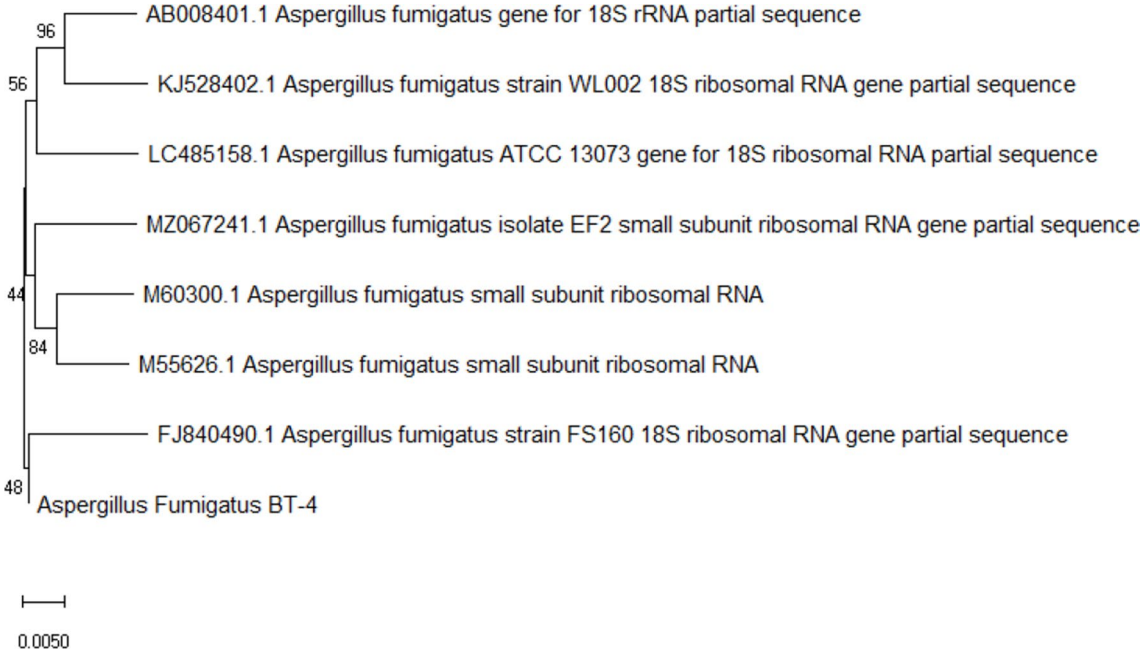

Fig. 5 Phylogenetic analysis of *Aspergillus fumigatus*

While M3 medium exhibited lowest enzyme activity i.e., 1.01 U/mL, 0.05 mg/mL of total protein, while dry cell mass was 2.70 g/L. The choice of carbon source is imperative to the production of pectinases [35]. Wheat bran is rich in protein content and other nutrients like manganese and vitamins. Effective microbial production of pectinases using wheat bran as the best carbon source in submerged fermentation systems has also been reported by other scientists [36]. M5 medium also contained ammonium sulphate as an inorganic nitrogen source that had a greater influence on the synthesis of pectinase enzymes under SmF. Metal ions act as activators and their addition to the cultivation medium increases the production of enzyme [5]. After 72 h, carbon and nitrogen sources started to deplete due to the extensive utilization of these nutrients by the fungus for biomass and product synthesis [37].

Subsequently, the impact of different fermentation media on the volumetric rate of product formation (Q_p), biomass formation (Q_x) and product yield coefficient ($Y_{p/x}$) was also studied. M5 medium exhibited maximum (Q_p, 0.05 U/l/h), and (Q_x, 0.07 g cell mass$L^{-1}h^{-1}$) values as well as 0.8 Yp/x biomass formation (Fig. 6b). Whereas, the lowest volumetric rate and product yield were obtained by M3 medium.

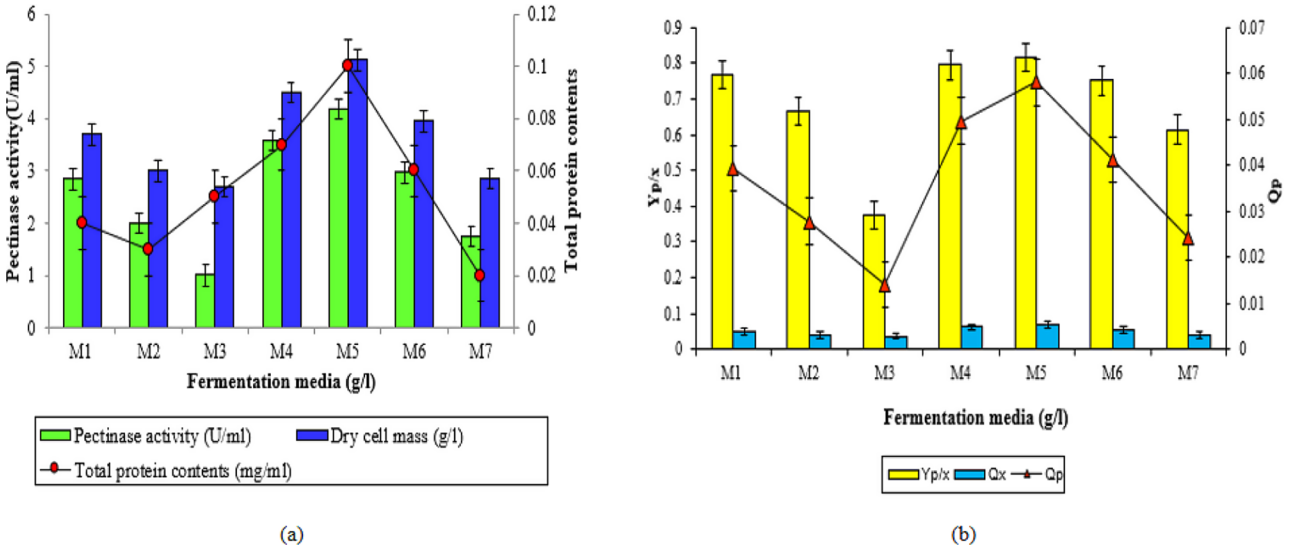

Fig. 6 Screening of fermentation media (**a**) PGase activity, dry cell weight, total protein content (**b**) Volumetric rate (Q_p, Q_x), product yield coefficient ($Y_{p/x}$)

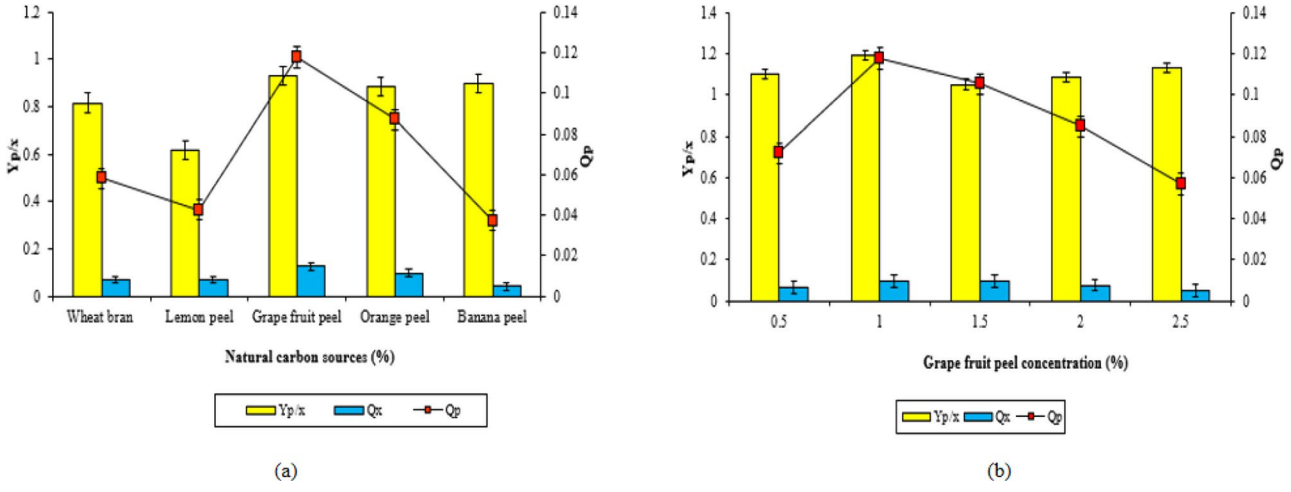

Fig. 7 (**a**) Effect of different fruit residues used as carbon sources on PGase activity exhibited by *A. fumigatus* BT-4 (**b**) Impact of various concentrations of grapefruit peels on volumetric rate and product yield coefficient on PGase activity exhibited by *A. fumigatus* BT-4

PGase production was then evaluated relative to the effect of replacing wheat bran in fermentation media with other natural carbon sources such as lemon peel, grapefruit peel, orange peel and banana peel (Fig. 7a). Among these carbon sources, grapefruit peel was the most efficacious at replacing wheat bran since it exhibited highest enzyme activity of 8.48 U/mL, total protein content of 0.17 mg/mL and dry cell mass of 9.11 g/L. Fruit residues can be considered as suitable and economical substrates for the production of fungal pectinases as they can be easily fermented by various fungi owing to their chemical composition and low moisture availability. Pectinases are routinely being produced via the valorization of pectin-rich waste materials such as apple pomace, grape skin, lemon peels and tamarind kernels at the industrial scale [38]. Moreover, grapefruit peel pectin also had vital sugars such as rhamnose, arabinose and xylose, essential for the fungal growth to increase the enzyme synthesis [39].

Grapefruit peel was also shown to have the highest volumetric rates (Q_p =0.9 UL^{-1}/h^{-1}) and (Q_x = 0.17 $g^{-1}L^{-1}h^{-1}$) and product yield coefficient ($Y_{p/x}$ =0.12) of all the other natural carbon sources for pectinase synthesis (Fig. 7b). Submerged fermentation was then carried out using various concentrations of 0.5 to 3.0% for the optimized amount of substrate. Grapefruit peel used at 1% concentration gave the maximum enzyme activity and total protein content. Moreover, maximum volumetric rates (Q_p =1.19 $UL^{-1}h^{-1}$), and (Q_x= 0.12 cell $massL^{-1}h^{-1}$) and product yield coefficient $Y^{p/x}$=0.09 were noticed.

Table 5 Placket Burman design of medium components for PGase production

Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Response
	A	B	C	D	E	F	G	Pectinase activity(U/mL)
1	40	4	25	2	1	0.1	1	3.6
2	50	4	125	5	1	0.5	4	14
3	40	4	125	2	6	0.5	1	4.6
4	40	8	125	2	6	0.5	4	11.2
5	50	4	25	2	6	0.1	4	12
6	40	4	25	5	1	0.5	4	9.2
7	50	8	25	2	1	0.5	1	8.8
8	50	8	125	2	1	0.1	4	13
9	50	4	125	5	6	0.1	1	6.3
10	50	8	25	5	6	0.5	1	7.4
11	40	8	25	5	6	0.1	4	10.3
12	40	8	125	5	1	0.1	1	5.4

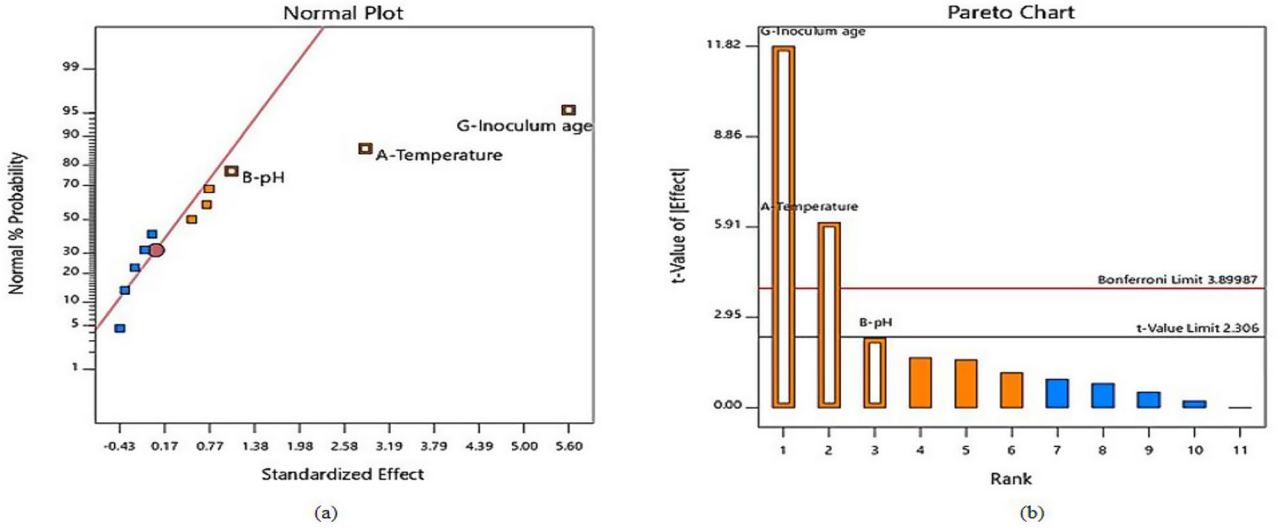

Fig. 8 (**a**) Normal plot (**b**) Pareto chart of Placket Burman design showing effects of variables on PGase activity

Response surface methodology

Experimental variables for the Plackett-Burman Design (PBD) and Central Composite Design (CCD) were selected based on previous research demonstrating their impact on enzyme production. Parameters including pH, temperature, substrate concentration, and incubation duration were chosen for their established impact on microbial proliferation and enzyme production, as evidenced in analogous research involving *Aspergillus* species. Once the significant factors were identified through PBD, the CCD was employed to fine-tune the values of these variables and establish an optimal set of conditions. CCD provides a second-order quadratic model, which allows the investigation of interactions between variables and their impact on pectinase production.

The combined effect of key medium components on pectinase production was evaluated using Plackett Burman Design. In this regard, 12 experimental runs were carried out at varying combinations of high and low values of seven significant parameters (Table 5). According to the RSM model, the maximum pectinase production was witnessed at 14.0 U/mL (Run no 2). In the current investigation, results from the Pareto chart of the Plackett, Burman Design revealed that inoculum age with a t-value of 11.82 has a major effect on pectinase production. This was followed by temperature (t-value: 5.91) and pH (t-value: 2.306), being the other two most significant factors affecting enzyme activity (Fig. 8).

In line with the Placket Burman Design, further optimization studies were based on the three most significant independent variables i.e., (A1) Temperature, (B2) pH and (G7) Inoculum age. In this regard, the combined effect of temperature, pH and inoculum age on pectinase activity was elucidated using a Central Composite Design (Table 6). The maximum enzyme activity (24 U/mL) was shown at temperature (50°C), pH (5.0) and 3 days old fungal inoculum. Regression analysis was performed, and the resulting quadratic model was evaluated by ANOVA.

Table 6 Central composite design of the most significant medium components for PGase production

Run	Factor 1	Factor 2	Factor 3	Response 1
	A: Temperature	B: pH	C: inoculum age	Pectinase activity
1	45	3	3	22
2	45	3	6.4641	20
3	53.6603	3	3	15
4	40	5	1	16
5	45	3	3	22
6	36.3397	3	3	15
7	40	1	1	5
8	50	1	5	9
9	45	3	0.464102	16
10	50	5	3	24
11	40	1	5	14
12	45	0.464102	3	9
13	50	1	1	10
14	45	3	3	23
15	50	5	1	19
16	45	6.4641	3	17
17	40	5	5	20

All the insignificant factors were excluded and the fitted equation (in terms of coded values) for pectinase synthesis (Y) was given as:

$$\begin{aligned}\text{PGase activity (Y)} =& 22.37 + 0.3294\text{A} + 4.74\text{B} \\ &+ 1.75\text{C} + 0.5765\text{AB} - 1.74\text{AC} - 0.4889\ \text{BC} \\ &- 2.28\text{A}^2 - 4.26\ \text{B}^2 - 1.91\text{C}^2\end{aligned} \quad (7)$$

Where, Y = predicted enzyme production, A = temperature, B = pH, C = inoculum age.

As per the analysis of variance (Table 7), the F-value of 21.72 suggested the model terms to be significant and upholding. The pectinase activity can be explained using the above model. The 'insignificant' Lack of Fit F-value of 9.70 also validates the association of the three independent variables as per the derived equation to regulate the pectinase production. The R-squared value of 0.9654 also represented a high degree of correlation between experimental and predicted values. The predicted value of R^2 was calculated to be 0.7231, which was quite close to the adjusted R^2 of 0.9210, i.e., the difference was less than 0.2. Adequate precision measured the signal-to-noise ratio. A ratio greater than 4 was desirable. The results showed the ratio of 14.220, which indicates an adequate signal. Therefore, this model could be used to navigate the design space.

Table 8 indicates coefficients in terms of coded factors. The coefficient estimate signified the projected variation in the response variable in relation to per unit change in one particular independent variable, keeping all the other factors constant. In an orthogonal design, the intercept represents the overall mean response across all experimental runs, while the coefficients serve as adjustments to this average, reflecting the specific effects of each factor setting. The positive coefficient specified the direct relationship between the independent and dependent variable i.e., for each unit increase in temperature, pH and inoculum size value, the pectinase activity also increased. Likewise, the negative coefficient estimate value predicted that the independent and dependent variables are inversely proportional to each other. Variance inflation factors (VIFs) exceeding 1 revealed the presence of multicollinearity, with higher VIF values indicating a more severe correlation between factors. Basically, VIFs less than 10 are generally accepted.

The linear normal probability plot of the residuals indicated that the error terms are normally distributed (Fig. S2a). The scatter plot of residuals (y axis) and predictors (x axis) showed that the residual data was equally scattered resulting in horizontal band around the x axis (Fig. S2b). It indicated that the variance was independent of

Table 7 Analysis of Variance for quadratic model of PGase activity of *A. Fumigatus* BT-4

Source	Sum of squares	df	Mean square	F-value	*p*-value	
Model	470.22	9	52.25	21.72	0.0003	Significant
A-Temperature	1.45	1	1.45	0.6016	0.4634	
B-pH	253.69	1	253.69	105.45	< 0.0001	
C-inoculum age	31.69	1	31.69	13.17	0.0084	
AB	2.45	1	2.45	1.02	0.3469	
AC	18.57	1	18.57	7.72	0.0274	
BC	1.47	1	1.47	0.6102	0.4603	
A^2	67.39	1	67.39	28.01	0.0011	
B^2	161.64	1	161.64	67.19	< 0.0001	
C^2	33.29	1	33.29	13.84	0.0075	
Residual	16.84	7	2.41			
Lack of fit	16.17	5	3.23	9.70	0.0961	Not significant
Pure error	0.6667	2	0.3333			
Cor total	487.06	16				

Model F-value = 21.72, R-squared = 0.9654, Adjusted R- squared = 0.9210, Predicted R-squared 0.7231, Adequate precision ratio = 14.2196

Table 8 Regression coefficient estimates and variance inflation factors for quadratic model of pectinase activity of *A. Fumigatus* BT-4

Factor	Coefficient estimate	df	Standard error	95% CI Low	95% CI High	VIF
Intercept	22.37	1	0.8405	20.38	24.36	
A-temperature	0.3294	1	0.4247	-0.6749	1.33	1.05
B-pH	4.74	1	0.4612	3.65	5.83	1.11
C-inoculum age	1.75	1	0.4830	0.6109	2.90	1.12
AB	0.5765	1	0.5718	-0.7755	1.93	1.09
AC	-1.74	1	0.6258	-3.22	-0.2590	1.13
BC	-0.4889	1	0.6258	-1.97	0.9910	1.13
A^2	-2.28	1	0.4300	-3.29	-1.26	1.11
B^2	-4.26	1	0.5193	-5.48	-3.03	1.15
C^2	-1.91	1	0.5143	-3.13	-0.6971	1.17

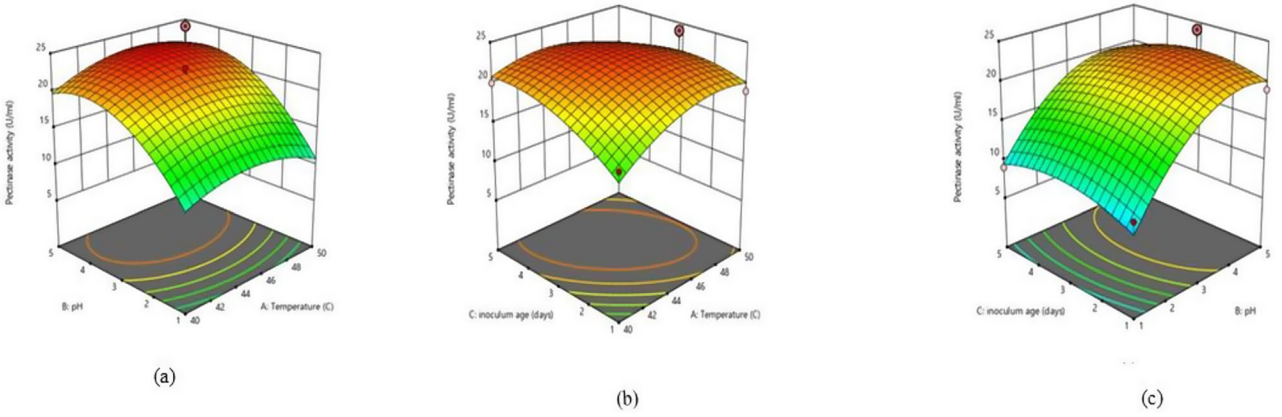

Fig. 9 Response surface plot and contour plot showing the effects (**a**) temperature and pH (**b**) temperature and inoculum age (**c**) pH and inoculum age on enzyme activity exhibited by *A. fumigatus* BT-4

the value of pectinase production, thus supporting the adequacy of the least square fit [19].

The relationship between the temperature, pH and inoculum age to regulate the pectinase synthesis is represented by the response surface curve and contour plots in Fig. 9. The shape of the contour plots gives information about the interaction of independent variables to regulate the response variable. Significant interactions between the variables are represented by the elliptical curves while less significant interactions are shown by the circular contours. The extent of interactions can be inferred by the linear responses: less linear response corresponds to more interactive variables. The surface plots reveal the synergistic effects of temperature, inoculum age and pH on enzyme production. Figure 9a showed the interaction between temperature and pH while keeping inoculum age constant, whereas Fig. 9b showed the effect of Temperature and inoculum age on pectinase activity while keeping the pH at a constant point, and Fig. 9c showed the effect of pH and inoculum age (days) on enzyme activity, where the temperature remained at a constant level. With further increase in optimum conditions, a reduction in pectinases was witnessed. This could be the reason that temperature has a significant role in enzyme production, so a slight change in temperature may affect the physiological activity and growth regulation of the microorganisms [40]. On the other hand, pH has a great effect on the growth of microorganisms and the osmotic pressure of the cell. It is also reported that maximal pectinase production has been obtained from different fungal strains within the acidic pH range [41]. While inoculum age also plays a critical role in enzyme activity due to prolonged cultural time, inhibition of microbial growth and metabolic activity occurs, which ultimately lowers the enzyme production [42].

Enzyme purification

The purification process began with ammonium sulfate fractionation, followed by gel filtration and ion-exchange chromatography, resulting in a significant yield (44.04%) and a 2.3-fold purification (Table 9). The purified pectinase enzyme was then analyzed using SDS-PAGE electrophoresis, confirming its homogeneity and purity. The result of the current study indicated that the molecular weight of purified enzyme from *A. fumigatus* was 48 kDa (Fig. 10). In previous studies, molecular mass by *Aspergillus flavus* was estimated as 42 kDa [43].

Table 9 Purification steps of PGase production by *Aspergillus fumigatus* BT-4

Sr. No	Purification Steps for Pectinase	Total enzyme activity (IU)	Total protein content (mg/mL)	Specific activity (U/mg)	Purification fold	% Yield
1	Crude	2400	76.4	31.4	1	100
2	Ammonium sulphate precipitation	1518	42	36.1	1.15	63.25
4	Dialysis	975	24	40.6	1.29	64.22
5	Ion-exchange chromatography	529	11	48.0	1.53	54.25
6	Gel-Filtration	233	3.2	72.8	2.31	44.04

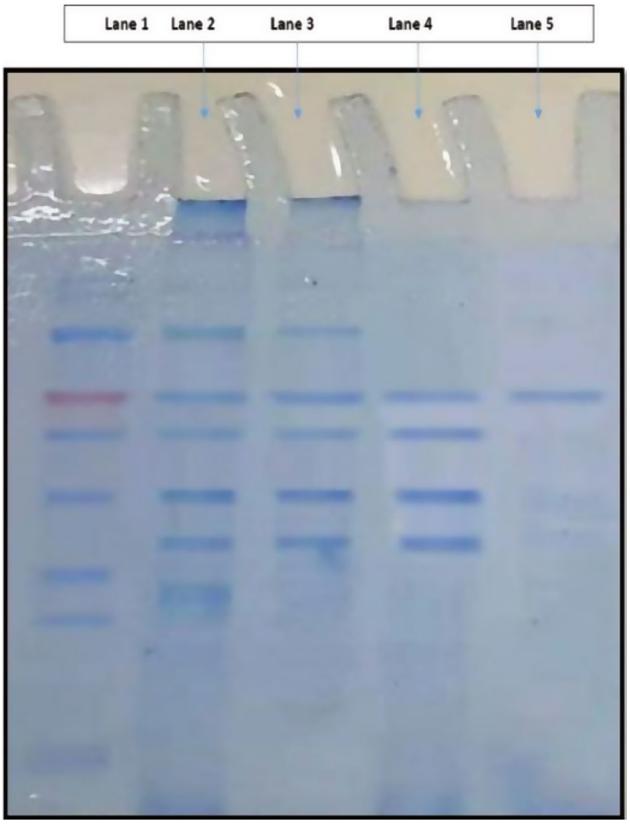

Fig. 10 SDS-PAGE analysis of purified enzyme. Lane 1 marker, Lane 2 and 3 Crude extract, Lane 4 Ion exchange Lane 5, Gel filtration

Characterization and kinetic studies

The influence of temperature on pectinase activity was evaluated by incubating the reaction mixture at a series of temperatures spanning 40–100 °C enabling the determination of optimal temperature conditions for enzyme activity. The maximal temperature that showed higher titers of PG activity for *A. fumigatus* BT-4 was exhibited at 50 °C (Fig. 11a). Present results are in line with Alana et al. [44] who mentioned 50 °C for PG activity by *P. italicum*. Results from Fig. 11b indicated that the pectinase activity of *A. fumigatus* BT-4 was found to be stable between 4 and 5 pH, and the highest activity was achieved at pH 5.0. Martin [20] mentioned that maximum PG activity was produced in the pH range of 3–8 from *Penicillium* sp. The kinetic parameter of the pectinase enzyme from *A. fumigatus* BT-4 was also estimated.

The PGase enzyme from *A. fumigatus* BT-4 showed a Vmax of 32.7 U/mL and Km of 0.3% (Fig. 12). Figure 12a represents linear Lineweaver-Burk Plot that is a linear transformation of the data, where 1/V is plotted against 1/[S]. This linear form helps in estimating Km (from the x-intercept) and Vmax (from the y-intercept).

The Fig. 12b non-linear Michaelis-Menten Plot shows the hyperbolic relationship between substrate concentration [S] and reaction velocity (v). The curve demonstrates enzyme saturation as substrate concentration increases, approaching Vmax.

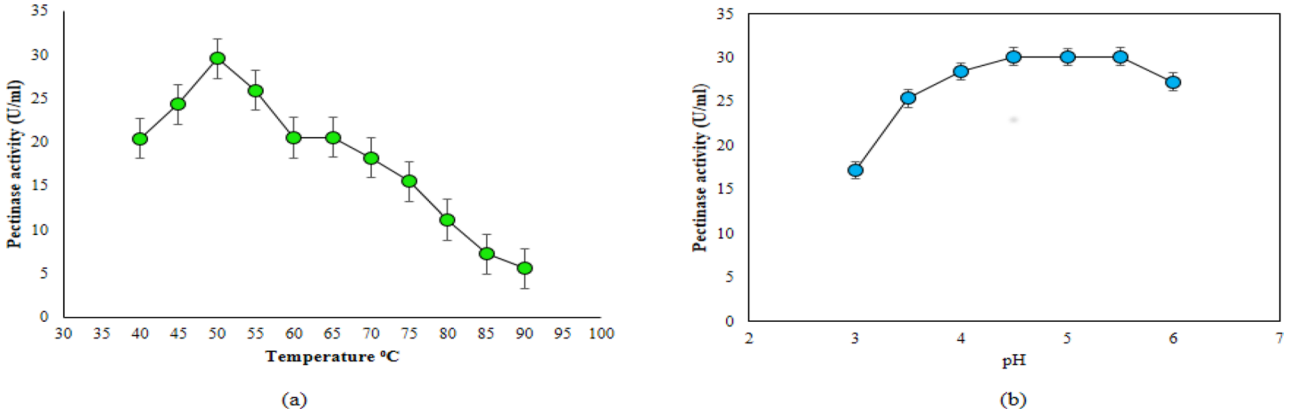

Fig. 11 Effect of (**a**) Temperature and (**b**) pH on PGase activity

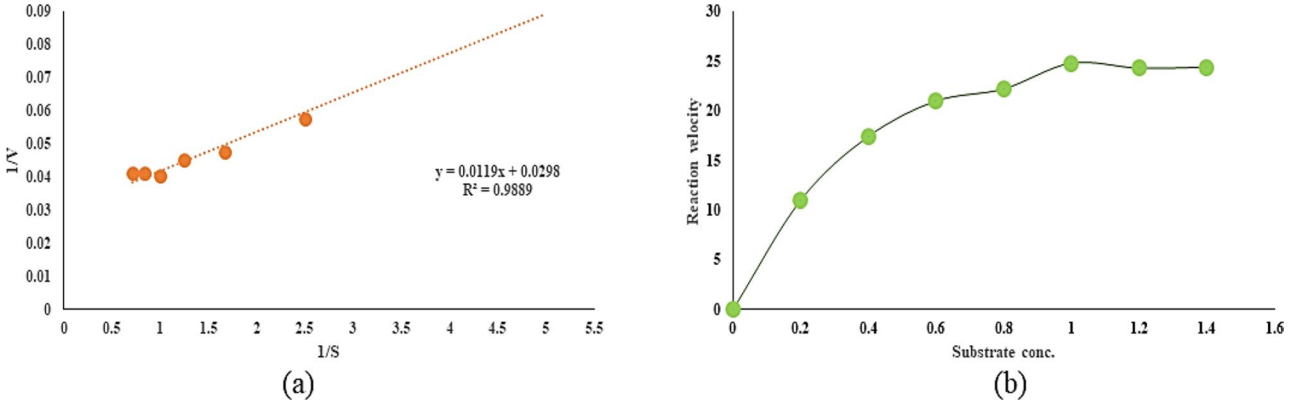

Fig. 12 Determination Km and Vmax (**a**) Linear Lineweaver-Burk Plot (**b**) Non-linear Michaelis-Menten Plot

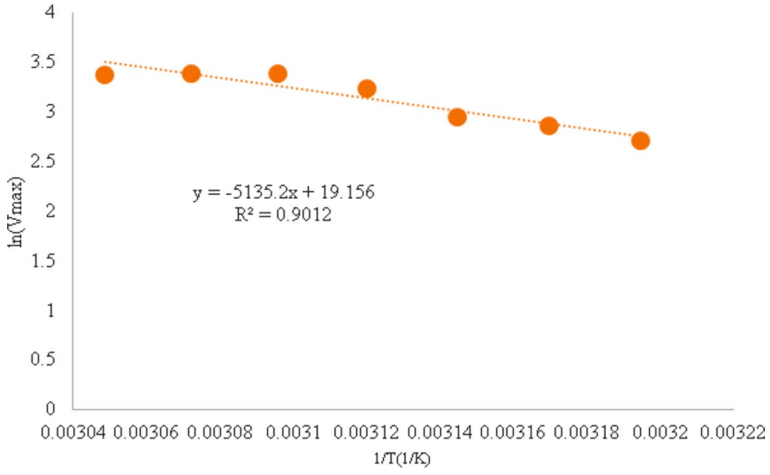

Fig. 13 Estimation of E_a and ΔH on the enzyme by Arrhenius plot

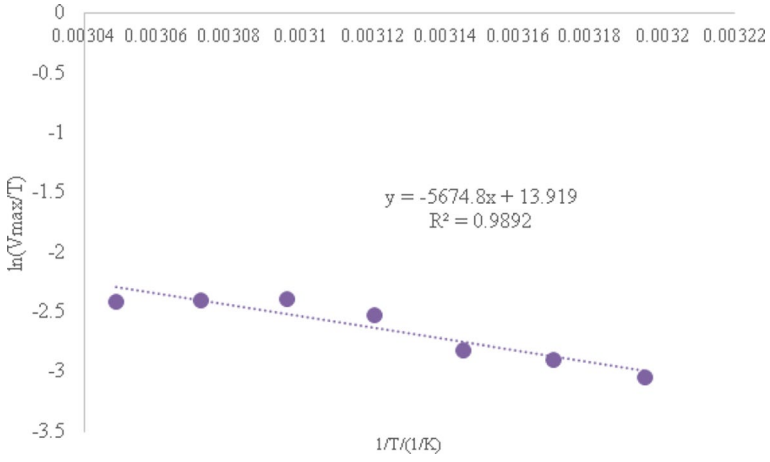

Fig. 14 Determination of entropy change (ΔS)

Thermodynamic studies

The values of thermodynamic parameters were calculated from Figs. 13 and 14. The activation energy Ea ΔH* (enthalpy of deactivation) and ΔS* (entropy of deactivation) were 41.74 kJ/ mol, 39.53 kJ/ mol and 46.9 kJ/ mol, respectively. Estimation of activation energy involves determining the energy required for a reaction to occur by analyzing the rate of reaction at different temperatures. This is often done using the Arrhenius equation, where the activation energy is derived from the slope of

a plot of the natural logarithm of reaction rate versus the inverse of temperature. The enthalpy (ΔH) change measures the number of bonds broken during inactivation; it is therefore interpreted that kinetically PG of *A. fumigatus* BT-4 is favorably good for the conversion of pectin into galacturonic acid and the entropy (ΔS) change indicates the net enzyme and solvent disorder.

Conclusions

The study concludes that thermophilic *A. fumigatus* BT-4 is suitable for high pectinase production through submerged fermentation. Statistical tools like Plackett-Burman design and Central Composite Design (CCD) in Response Surface Methodology (RSM) were employed to optimise fermentation components, resulting in improved enzyme yield, enhanced fermentation efficiency, reduced production costs, and increased product quality. This approach enables the production of high-quality pectinase more efficiently, making the process more effective and cost-effective. Additionally, using agricultural by-products as substrates offers a cost-effective and sustainable approach, reducing production costs and environmental impact. This study highlights the potential of thermophilic fungi, RSM, and agricultural by-products for efficient and economical pectinase production, making it a viable option for industrial applications. Thermophilic fungi are a valuable resource for enzyme production, and RSM effectively optimises fermentation conditions. Future work could focus on scaling up production, genetically enhancing thermophilic strains for better yield and stability, testing pectinase in relevant industrial applications, and further optimizing substrate compositions with different agricultural residues. These findings underscore the promise of thermophilic fungi, RSM, and agro-industrial by-products to advance sustainable enzyme production on an industrial scale.

Supplementary Information

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Supplementary Material 1

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Author contributions
IA and SS performed the formal analysis, KN, AK and MI conceptualized the research, II helped in writing and editing the manuscript, while RA and XC supervised the research.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The deposited data can be found at NCBI having accession number PQ569976.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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