ENHANCED POLYGALACTURONASE BIOSYNTHESIS: TAILORING FERMENTATION CONDITIONS USING *ASPERGILLUS NIGER* FS001

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ABSTRACT

A significant challenge in contemporary biotechnology is the efficient extraction of valuable by-products from agri-food industry waste, necessitating the development of advanced and sustainable biotechnological methods to maximize resource recovery while minimizing environmental impact. Pectinase is crucial in this process because it breaks down complex polysaccharides like pectin, enabling the efficient recovery of important compounds such as polyphenols, dietary fibers, and bioactive substances from fruit and vegetable processing waste. *Aspergillus* species are prevalent fungi found in high numbers globally, and they are essential in the synthesis of polygalacturonase (PG), which is an enzyme responsible for degrading cell wall. The current study was focused on the exploitation of pectin for the production of polygalacturonase enzymes, through submerged fermentation using indigenous fungal strain *Aspergillus niger*-FS001. The optimization of PG production was performed by adopting the approach of taking on one variable sequentially. The five varied fermentation media were analyzed for the maximal production of the enzyme. Among all media, newly designed medium-M5 containing 1% (w/v) pectin yielded the highest enzyme activity. Additionally, the impact of varied factors has also been conducted, which includes temperature, duration of incubation, pH and sources of carbon as well as nitrogen. The most favorable enzyme production level was reached after 120 hours at a temperature of 30°C and pH 5.5. Using pectin served as carbon source, whereas yeast extract, peptone and ammonium sulphate worked as nitrogen sources that contributed achieving maximum polygalacturonase production (1461 U/mL). The current study was highly effective in achieving highest economic output of polygalacturonase by *Aspergillus niger* FS001 using economical and locally sourced substrate. Moreover, a simple, low-cost, industrially significant medium formulation was also developed.

Keywords: *Aspergillus niger*, pectin, polygalacturonase, submerged fermentation.

1. INTRODUCTION

Pectin-degrading enzymes, collectively known as pectinolytic enzymes, are widely synthesized by *Aspergillus niger* and broadly used in the food industry (Abdullah *et al*., 2018; Madika *et al*., 2020). Pectinases are universally utilized in the industrial processing of fruits and vegetables, contributing to the reduction of juice thickness and stream-lining the procedures of extraction, softening, liquefaction and clarification (Naidu and Panda, 1999; Ezugwu *et al*., 2023). Polygalacturonase (PG) is an enzyme that hydrolytically breaks down the glycosidic linkages within pectins whereas pectinesterase (PE) is involved in hydrolysis of ester bonds within pectins, making it a pectolytic enzyme (Satapathy *et al*., 2020). To achieve the hydrolysis of the pectin molecule, the combined action of both enzymes is essential (Taragano and Pilosof, 1999).

Various fermentation processes widely utilize *Aspergillus niger* strains to synthesize pectin enzymes (Ahmed *et al*., 2021). Pectinases are synthesized through pectin or from several of the derivative forms (Solís Pereira *et al*., 1993; Ahmed *et al*., 2021). The composition of the medium as well as fermentation conditions can affect cell growth, sporulation and enzyme synthesis (Maldonado and Callieri, 1989; Costa *et al*., 2007). Generally, these enzymes are manufactured through either solid-state or submerged fermentation processes. Secretory enzymes are generally produced in smaller quantities by submerged fermentation, while solid fermentation is not susceptible to automation (Mojsov, 2010).

Improved fermentation conditions played a significant role in the industrial utilization of pectinolytic enzymes, as well as to enhance the production of extracellular enzymes in liquid culture using economical carbon sources such as agricultural waste biomass (Almulaiky *et al*., 2020; Afzia *et al*., 2024). It has been documented that employing a

finely-tuned medium composition can lead to an increase in enzyme concentration (Mojsov, 2010; Riaz *et al*., 2023).

The objective of this research was to investigate the effects of varying medium component concentrations on the synthesis of pectinolytic enzymes, with the aim of optimizing conditions for maximum enzyme production and fungal growth. Key factors such as pectin concentration, fermentation time, pH, and temperature were systematically evaluated for their impact on polygalacturonase production by *Aspergillus* species under submerged fermentation. This study involved adjusting one factor at a time while maintain a constant for the others. This approach doesn't illustrate how the variables interact with each other.

2. MATERIALS AND METHODS

2.1. Sample Source and Collection

Fungal strains were isolated from native agri-food waste, including orange, lemon, onion, wheat bran, grapes, peach, grapefruit, and apple, collected from local markets and processing units in Karachi. These wastes were chosen for their abundance and suitability as substrates for fungal growth.

2.2. Identification and Screening of Organisms

For screening, ten selected fungal isolates were cultured on pectin agar plates, and pectinolytic activity was detected using a plate assay method. Among these isolates, two strains exhibited clear halo zones around their colonies after staining with iodine-potassium iodide solution. Further identification of these strains was carried out by examining the structural and biochemical traits characteristic of *Aspergillus* species (Phutela *et al*., 2005).

2.3. Characterization of Fungi

After colony identification, the microscopic characteristics of the fungi were observed by Lacto Phenol Cotton Blue (LPCB) staining technique (Leck, 1999).

2.4. Culture Maintenance

Fungal strains that were isolated were kept preserved on Czapek-Dox Agar slants at 4°C by sub-culturing every alternate month.

2.5. Fermentation Medium

Production of polygalacturonase by these strains was further analyzed by submerged fermentation technique in a medium composed of citrus pectin (1.0%), glucose (2.5%), yeast extract (0.5%), peptone (0.1%), potassium dihydrogen phosphate (0.4%), ammonium sulfate (0.4%) and magnesium sulfate (0.2%). The pH was adjusted to 5.5 for the medium prior to sterilization at 115°C for the duration of 20 minutes.

2.6. Enzyme Assay for Polygalacturonase Activity

The enzyme activity was quantified by colorimetric technique using citrus pectin serving as a substrate and galacturonic acid monohydrate as the standard. Partially purified enzyme (0.1 ml) was mixed with 1.0 ml of substrate (1.0% citrus pectin dissolved in 50 mM phosphate buffer, pH 6.0) and incubated at 37°C for 30 minutes. The concentration of reducing sugar released in the form of galacturonic acid was assessed by DNS method (Miller, 1959). One unit of enzyme activity is defined as "*the amount of polygalacturonase that releases 1 µM of galacturonic acid per minute under specific conditions (pH 6.0 and 37°C)*".

2.7. Estimation of Total Protein

Bovine serum albumin (BSA) was used as a standard (Lowry *et al*., 1951) to determine the total protein.

2.8. Optimization of Fermentation Conditions for Polygalacturonase Production

Fermentation conditions essential for the maximum yield of pectinase from *Aspergillus niger* FS001 were optimized using a one-variable-at-a-time approach. All experimental procedures were performed in pairs.

2.8.1. Selection of Different Media for Polygalacturonase Production

Various previously reported media were studied for the synthesis of polygalacturonase from *Aspergillus niger* FS001. These media included medium-1 (Banu *et al*., 2010), medium-2 (Martos *et al*., 2009), medium-3 (Angelova *et al*., 1998) and medium-4 (Patil and Dayanand, 2006b), while medium-5 was the newly designed medium. Fungal culture was incubated at the temperature of 30°C for 5 days in each medium and the cells were collected by

centrifugation at 4000 rpm for the duration of 30 minutes at 4°C. The clear supernatant containing extracellular polygalacturonase enzyme was preserved at -18°C for further use for the enzyme activity.

2.8.2. Effect of Substrate (Pectin) Concentration on Polygalacturonase Production

The impact of substrate concentration on production of enzyme by fungal isolate was studied through different concentration of citrus pectin ranging from 0.5, 1.0, 1.5 and 2.0% in fermentation medium.

2.8.3. Effect of Yeast Extract and Peptone Concentration on Polygalacturonase Production

The effect of yeast extract and peptone concentration on enzyme production by fungal isolate was analyzed simultaneously by using different concentrations of each ranging from 0.0, 0.1, 0.25, 0.5, 1.0 and 2.0% respectively in fermentation medium.

2.8.4. Effect of Incubation Time on Polygalacturonase Production

The effect of fermentation time on enzyme production was determined by growing the fungal isolate for time intervals of 72, 96, 120, 144 and 168 hours respectively at 30°C.

2.8.5. Effect of pH and Temperature on Polygalacturonase Production

The effect of pH and temperature on polygalacturonase production was analyzed simultaneously at variable pH ranging from 4.0, 5.0, 5.5, 6.0, 7.0, and 8.0 as well as by incubating the fungal isolate at various temperatures at 20°C, 30°C, 40°C and 50°C respectively for 120 hours (5 days).

RESULTS AND DISCUSSION

Pectinolytic organisms collected from fruits and vegetables peel wastes were discovered to generate a variety of pectinolytic enzymes, collectively referred to as pectinases. Selected ten fungal strains isolated from indigenous waste sources are given in Table **1**.

Table 1. Fungal Strains Isolated from Native Waste Sources.

S. No.	Fungal Strain	Source
1	FS001	Orange
2	FS002	Lemon
3	FS003	Orange
4	FS004	Onion
5	FS005	Wheat Bran
6	FS006	Grapes
7	FS007	Peach
8	FS008	Grapefruit
9	FS009	Apple
10	FS010	Onion

These strains were further identified based on the morphological observations and biochemical profiling typical of *Aspergillus* species (Phutela *et al*., 2005; Madika *et al*., 2020). Among the isolates, *Aspergillus niger* FS001 was selected due to the highest production of enzyme (Khan *et al*., 2014). The morphological features of this strain are presented in Table **2**. The morphological characteristics observed under LPCB staining were consistent with those previously described by Khan *et al*. (2014).

Similar to the physicochemical conditions required for optimal production of fungi, chemical constitution of the medium varies greatly among microorganisms. Most of the factors influencing the production of fungal enzymes, particularly substrate, pH and temperature have been widely reported (Schäfer *et al*., 2020; Mat Jalil *et al*., 2023; Bentouhami *et al*., 2024).

Five different media compositions were evaluated to determine the most suitable formulation for optimal pectinase production. The first four media were selected based on previously reported formulations, while the fifth was a newly developed medium. The formulation details of medium-5 are given in Table **3**. Medium-5 was designed

by modifying individual components of the first four media, one at a time, to assess their influence on enzyme production based on nutritional variations (Fig. **1**).

Table 2. Morphological Characteristics of *Aspergillus niger* FS001.

Characteristics	***Aspergillus niger* FS001**
Size of the Colony	400-3000 µm
Stipes Color	Slightly brown
Surface	Smooth walled
Vesical Serration	Biseriate large size
Metula Covering	Entirely
Shape	Globose
Conidia Surface	Very rough irregular

Table 3. Chemical Composition of Medium-5.

Components	**Quantity (g/L)**
Pectin	10
Yeast extract	2.5
Peptone	2.5
$(NH_4)_2SO_4$	2
KH_2PO_4	2
K_2HPO_4	2
$CaCl_2$	0.1

pH 5.0; Sterilization at 121°C for 15 minutes.

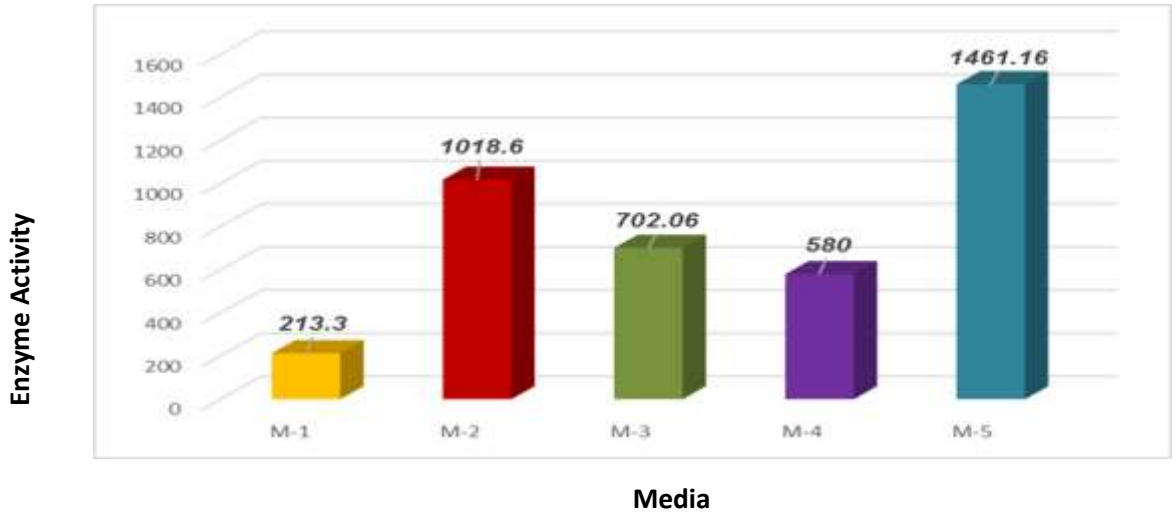

Fig. 1. Selection of media for the hyper-production of pectinase enzyme.

The optimization of culture conditions for maximum production of pectinase was performed by conventional methods (one variable at a time approach) (Khan *et al*., 2012; Mat Jalil *et al*., 2023).

Carbon sources are known to affect the synthesis of pectinase. As noted, the presence of pectin induced pectinase synthesis (Boakye *et al*., 2024). The concentration dependent effect of pectin on enzyme production was investigated and the obtained results demonstrated optimal enzyme yield at 1.0 gm% substrate concentration (Fig. **2**). Similar finding has been reported earlier as well (Palaniyappan *et al*., 2009; Khan *et al*., 2012).

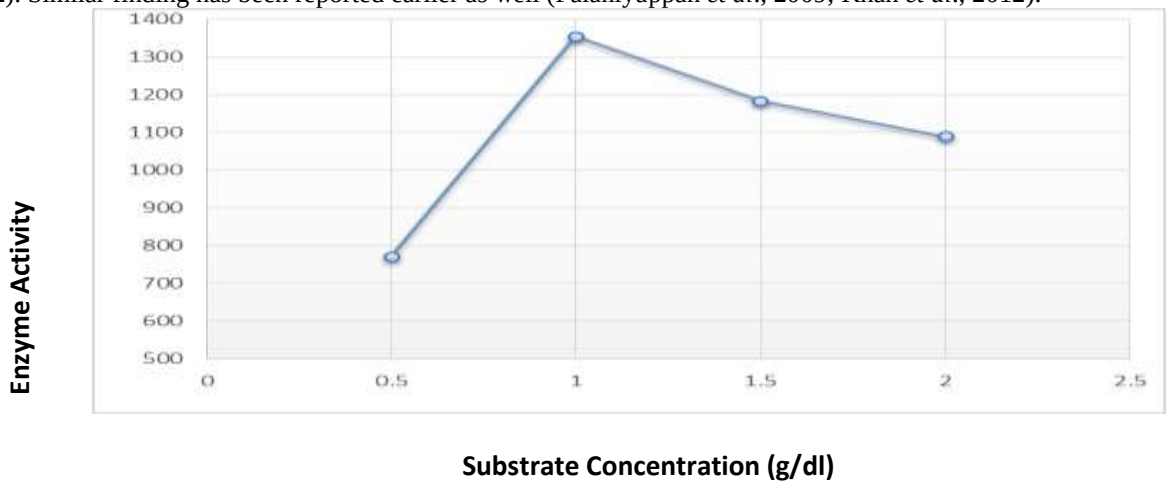

Fig. 2. Effect of substrate concentration on hyper-production of pectinase enzyme.

Yeast extract was observed to enhance pectinase production (Mat Jalil *et al*., 2023). The results showed maximum enzyme production at 1.0% yeast concentration (Fig. **3**). It has also been reported that yeast extract demonstrated the highest amount of pectinase among the nitrogen sources tested for production (Chaudhri and Suneetha, 2012; Schäfer *et al*., 2020). The obtained results showed maximum enzyme production at 0.5% peptone concentration (Fig. **4**).

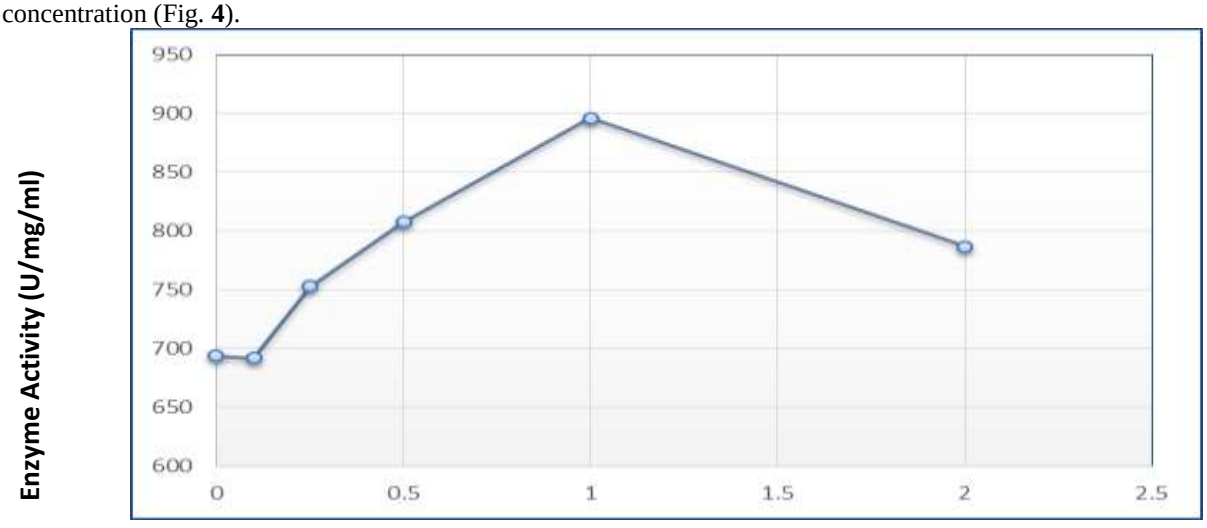

Fig. 3. Effect of yeast concentration on hyper-production of pectinase enzyme.

Culture media flasks were incubated for 72, 96, 120, 144, and 168 hours respectively at 30°C, and pectinase production was determined. Maximum enzyme production was obtained after 120 hours of incubation; however, enzyme activity declined thereafter (Fig. **5**). The longer incubation time required by strain FS001 compared to other fungi may be related to its distinct growth and metabolic characteristics, including potentially slower biomass accumulation and enzyme secretion dynamics under the conditions tested. Such strain-specific variation in incubation time is common among fungi and depends on multiple factors such as nutrient utilization, oxygen availability, and enzyme induction mechanisms (Schäfer *et al*., 2020; Bentouhami *et al*., 2024). Previous studies have reported diverse incubation periods for maximal pectinase production, ranging from 48 hours in *Aspergillus*

flavus (Acuna-Argulles *et al*., 1995) to 6 days for *Moniliella* sp. (Solbak *et al*., 2005), with some fungi achieving peak activity at 72 hours in submerged fermentation (Patil and Dayanand, 2006a). The aerobic nature of fungi, requiring sufficient oxygen supply for secondary metabolite production, further influences enzyme yields (Palaniyappan *et al*., 2009). More recently, Mangrio *et al*. (2023) reported that strain-specific environmental adaptation significantly impacts incubation time for optimal enzyme production, supporting the variability observed in our study. Therefore, the 120-hour incubation observed for FS001 reflects its unique physiological response under the given culture conditions.

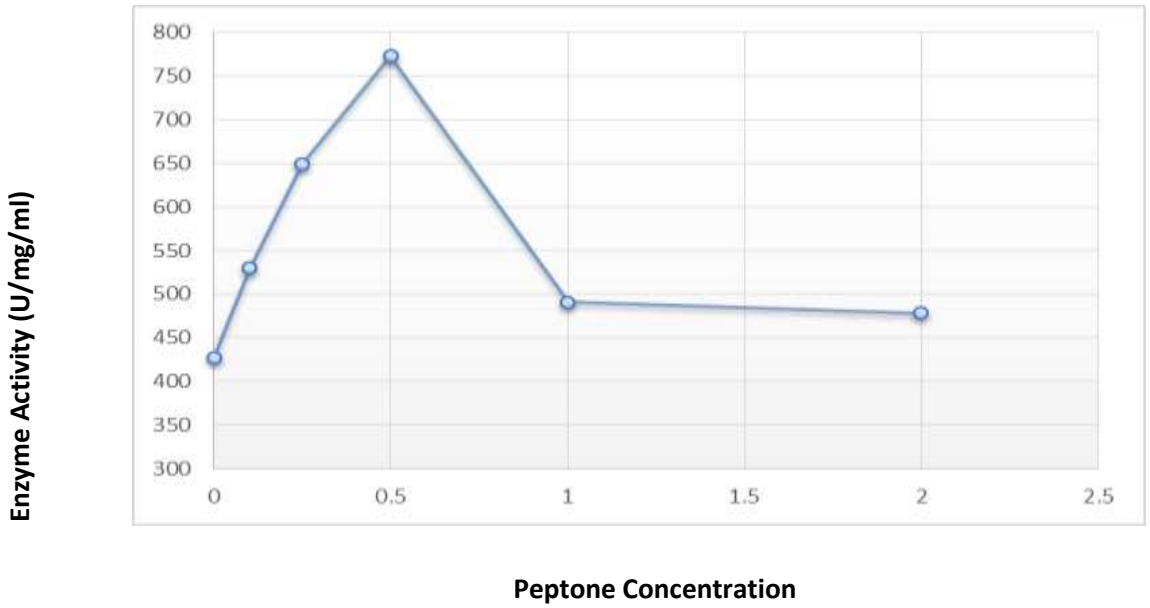

Fig. 4. Effect of peptone concentration on hyper-production of pectinase enzyme.

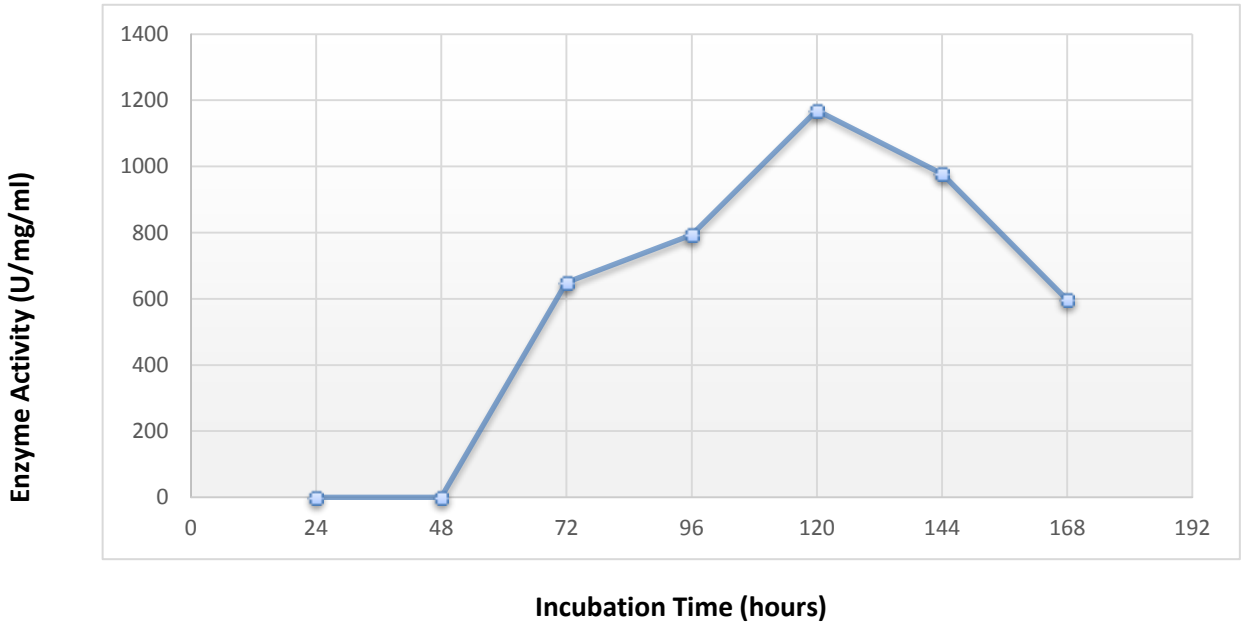

Fig. 5. Effect of incubation time on hyper-production of pectinase enzyme.

Among the physical parameters, temperature played an important role in enzyme production. Culture media flasks with pH 5.5 were incubated at temperatures ranging from 20°C to 50°C for 120 hours (5 days), and pectinase production was determined. The results showed maximum enzyme production at 30°C (Fig. 7). This optimum

temperature is consistent with findings from other *Aspergillus* strains, where maximum pectinase production generally occurs between 28°C and 35°C. For example, *Aspergillus niger* demonstrated optimal pectinase activity at 30°C (Abdullah *et al*., 2018), while *Aspergillus oryzae* showed peak production around 32°C (Koser *et al*., 2014). Rajendran *et al*. (2011) reported an optimum temperature range for enhanced pectinase activity between 27°C and 47°C. In contrast, *Penicillium chrysogenum* exhibited maximum enzyme productivity at 37°C, with peak polygalacturonase production reported at 35°C (Banu *et al*., 2010). These variations in optimal temperature reflect differences among fungal species. The aerobic nature of fungi further influences enzyme yields by requiring sufficient oxygen supply for secondary metabolite production (Palaniyappan *et al*., 2009; Bentouhami *et al*., 2024).

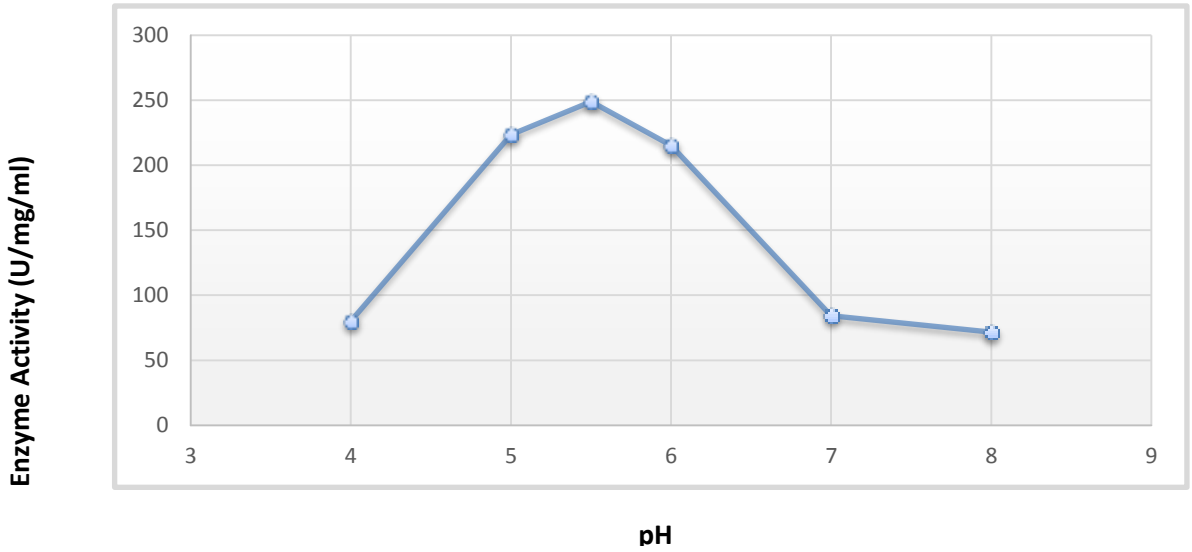

Fig. 6. Effect of pH on hyper-production of pectinase enzyme.

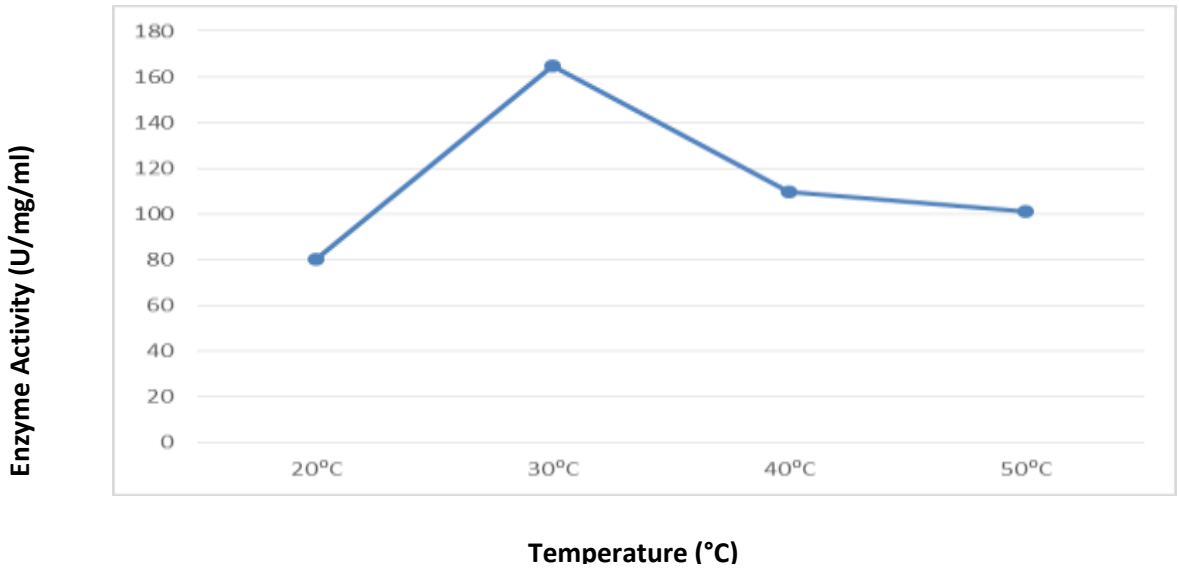

Fig. 7. Effect of temperature on hyper-production of pectinase enzyme.

CONCLUSION

This study demonstrates that *Aspergillus niger* FS001 is a high-yielding strain for polygalacturonase production under submerged fermentation. Using a newly designed, optimized medium (M5) composed primarily of 1% pectin and yeast, adjusted to pH 5.5, we achieved maximum enzyme yield at 30°C after 120 hours of incubation. This

simple and cost-effective fermentation system offers significant potential for industrial-scale polygalacturonase production, highlighting the promising commercial applicability of the enzyme produced by *A. niger* FS001.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DISCLAIMER

The content of this article has been adapted from the first author's Ph.D thesis.

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