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Purification and Characterisation of Xylanase Produced by *Aspergillus niger* Isolated from Otomycosis from Diyala, Iraq

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Abstract **Background**: In the practice of otolaryngology, otomycosis is a prevalent issue. However, because many patients exhibit resistance to antifungal drugs and have a high recurrence rate, we typically face certain challenges when treating them. This study aimed to identify the fungi that cause otomycosis and how susceptible they are to the widely used antifungal medications. Moreover, to determine the primary causes of antifungal resistance. **Methods:** From March 2025 to April 2025, we performed experimental descriptive research on 50 patients who had been clinically diagnosed with otomycosis. Specimens of auditory discharge were gathered for fungal culture and direct microscopic analysis. The commonly used antifungal medications were tested for in vitro antifungal susceptibility. We examined the enzymatic activity of the isolated fungus. **Results**: Patients frequently get otomycosis, a fungal infection of the ear. Fifty samples tested positive for fungus. conducted mycologic investigations on scraping samples and debris from the external ear canals of fifty patients with otomycosis clinical diagnoses. Forty-one samples (82.0%). *Aspergillus niger* 18 (43.9% of all isolates), *Aspergillus fumigatus* 16 (39.0%) and *Candida albicans* 7 (17.1%) were the most prevalent pathogens. The highest clearing zone around the colony, where the strain's xylanolytic activity was seen, was between 12 and 7.2 cm. At 80% saturation, the dialysed enzyme precipitation produces 3.1U/mg of protein-specific activity. And Concentrations were made of the fractions having the highest specific activity (numbers 19-25). With a specific activity of 1102 U/mg, xylanase was purified 97 times. **Conclusion**: The most prevalent fungal isolates in otomycosis are *Aspergillus* and *Candida* species. The high enzymatic activity of fungal infections is responsible for their high pathogenicity. Antifungal empirical use needs to be discouraged. Ion-exchange chromatography using DEAE-cellulose provided an efficient means of isolating and concentrating xylanase with high specificity and yield. The results are consistent with other recent the continued use of this method as a standard for fungal enzyme purification.

Key Words Xylanase, Otomycosis, *Aspergillus*, Malt Extract Agar

INTRODUCTION

One of the most often described clinical manifestations of *A. niger* is otomycosis, a prevalent illness found in otolaryngology outpatient clinics. Particularly prevalent in tropical and humid areas, it is a superficial fungal infection of the external auditory canal [1]. Otomycosis, most of the time, it is referred to as an external ear canal fungal infection. Less commonly, the mastoid cavity is followed by an open mastoidectomy [2]. Common side effects include tinnitus, hearing loss, aural discharge, auditory fullness, itching and discomfort [3]. Numerous fungi were identified in otomycosis. However, Aspergillus, particularly *Aspergillus niger*, appears to be the most common causative agent [4].

The symptoms of otomycosis caused by *A. niger* include itching, discomfort, black discharge and hearing loss. Conidia of *A. niger* are easily identified under a microscope due to their black hue [5]. Remarkably, strains isolated from these diseases are often metabolically active and capable of producing a range of hydrolytic enzymes, including xylanase, an enzyme essential for the degradation of hemicellulose in plant cell walls [6]. Common risk factors for otomycosis include poor personal hygiene, minor trauma, inflammation or physical injury; swimming pools; exposure to hot, humid environments, such as those found in tropical and subtropical regions; prolonged use of antibiotics; and the use of steroid ear drops by individuals with weakened immune systems [7,8]. Many studies have been conducted

on the industrial potential of *A. niger* strains that produce xylanase. However, it is unclear if clinical isolates, especially those from otomycosis, have comparable or superior enzymatic qualities [9]. *A. niger*'s dual role as a pathogen and a biotechnological resource may be better understood if the synthesis of xylanase from these clinical strains is identified and described. Separating *A. niger* from otomycosis patients, evaluating its xylanase activity and characterising the enzyme's synthesis in several cultural contexts are the goals of this study [10].

METHODS

Isolation of *Aspergillus niger*

Selection of patients: Participants in an experimental descriptive study were patients with a clinical diagnosis of otomycosis who came to our otolaryngology outpatient clinic between March and May 2025. The examination of those patients, who had a range of complaints, including otorrhea, itching and aural pain, revealed erythema, fungal debris and creamy or blackish auditory discharge. Individuals who had recently had topical antifungal treatment were not included. 50 ear swabs from patients with otomycosis were among the clinical samples that were aseptically obtained and cultured on Sabouraud Dextrose Agar (SDA) with the addition of chloramphenicol. Plates were incubated at 28 to 30°C for five to seven days. To identify the fungus, both macroscopic and microscopic characteristics were employed.

Screening for Xylanase Production

Using xylan agar medium (1% birchwood xylan, 0.5% peptone, 0.3% yeast extract, 1.5% agar and 0.1% KH_2PO_4), a preliminary screening of xylanase activity was carried out. After fungal isolates were added to the plates, they were incubated for 48 hours at 37°C. Clear halo zones after 15 minutes of flooding with 0.1% Congo red solution and 1 M NaCl destaining indicated xylanase activity.

Submerged Fermentation for Enzyme Production

The chosen *A. niger* strain was inoculated into a xylanase production medium that contained the following ingredients (per liter): 10 g of xylan, 5 g of peptone, 5 g of yeast extract, 1 g of KH_2PO_4 and 0.5 g of $MgSO_4 \cdot 7H_2O$. The mixture was then incubated for 5 days at 30°C and 150 rpm. After centrifuging the soup for ten minutes at 10,000 rpm, the supernatant was utilised to make a crude enzyme extract.

Enzyme Activity Assay

Using the dinitrosalicylic acid (DNS) technique, xylanase activity was measured. The reaction mixture (0.5 ml of 1% birchwood xylan and 0.5 ml of crude enzyme in 50 mm of citrate buffer, pH 5.3) was incubated for 10 minutes at 50°C. At 540 nm, the amount of reducing sugars released was measured. The quantity of enzyme needed to release one µmol of xylose per minute was called one unit (U) of xylanase activity.

Protein Concentration

Protein content was estimated using the Bradford assay, with bovine serum albumin (BSA) as the standard.

Partial Purification

Crude enzyme was subjected to ammonium sulfate precipitation (80% saturation), followed by dialysis against phosphate buffer (pH 7.0). The partially purified enzyme was used for further characterization.

Characterisation of Xylanase

- **Optimum pH:** Enzyme activity was measured across pH range (3.0-9.0) using appropriate buffers
- **Optimum Temperature:** Activity was assessed at temperatures ranging from 30 °C to 70 °C
- **Thermal Stability:** Enzyme samples were pre-incubated at different temperatures for 30-60 min and assayed for residual activity
- **Effect of Metal Ions and Inhibitors:** Enzyme activity was tested in the presence of metal ions (e.g., Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}) and inhibitors (e.g., EDTA, SDS)

Microorganism and Culture Conditions

Liquid Media for Isolation and Production of Xylanase:

- Yeast Extract….0.2 g
- NaCL 0.2 g
- MgSo4… 0.02 g
- K2Hpo4 1.5 g
- Xylan… 0.5%

After dissolving all the ingredients in 90 ml and adjusting the PH to 8, the volume was increased to 100 ml and autoclaved for 15 minutes at 121 C to sanitise it.

Xylanase Medium for Semi-Quantitative Method

- Nutrient agar… 2.8 g
- Xylan …… 0.5%
- All dissolved in 100 mL DW
- PH was 8 and
- Sterilized by autoclave at 121°C for 15 minutes

Semi-Quantitative Method

The activated bacteria were grown on Xylan and then inoculated for 48 hours at 37 °C. By evaluating the diameter of colonies and the plate zone clarity around bacteria and colonies implanted with xylanase production, a semi-quantitative xylanase assay was carried out.

Xylanase Activity

Xylanase activity was measured, per Bailey *et al.* [11]. 900 µL of 1% solubilised birchwood xylan solution and 100 µL of enzyme solution were mixed in a test tube. The mixture was incubated for five minutes at 50°C in a water bath after 1.5 mL of DNS reagent was added [12]. The absorbance was

measured at 540 nm. At zero time, the reaction was halted in the control tubes. The standard graph was made using 0-500 µg of xylose and put in a UV-VIS spectrophotometer with a buffer solution. One unit of xylanase activity was defined as the amount of enzyme that produces one micromole of reducing sugar equal to xylose per minute under the given test conditions. To produce soluble xylan, birchwood xylan was mixed with 1M NaOH for six hours at room temperature, centrifuged and the resulting supernatant was freeze-dried after the alkali was neutralised with 1M HCl. The protein was estimated using the Lowry *et al.* [13] method.

Xylanase Assay

Using the technique first outlined by Murachi [14] and adapted by Senior [15], xylanase activity in the culture supernatant was ascertained as follows:

For ten minutes, casein (0.8 mL, 0.5%, PH = 8) was pre-incubated in a water bath at 37 ˚C. The quantity of enzyme that, in assay conditions, results in a 0.01 rise in absorbance at 280nm/min was designated as the unit of enzyme activity. This formula was used to calculate the protease activity:

The protease activity at 280 nm/0.001×20 min.×0.2mlisU/ml= Ab.

Using bovine serum albumin as a reference, a unit of xylanase activity was defined as the amount of enzyme that, under assay conditions, releases one milligram equivalent of peptide fragment. Units per milligram of protein are used to express specific activity [16].

Optimisation of the Enzyme:

- PH: the natural protease production was determined by using different PH 5,6,7,8
- Incubation period: the natural protease production was determined by using different incubation periods, 7, 8, 9 and 10 days
- Temperature: the influence of temperature on the production of natural proteases was studied by incubating media at different temperatures, 25, 30, 35 and 40 ˚C
- Nitrogen source: the nitrogen source of natural protease production was determined by using different sources (peptone, tryptone, casein and yeast extract)
- Inoculum size: The influence of inoculation represents (1×106) of the production of natural protease was studied by inoculation the media at different volumes,1×106,2×106,3×106and 4×106 spores/ml

Xylanase Purification

As previously mentioned, *Aspergillus niger* was grown in an aerobic medium for 48 hours at 37°C. The cells were separated by centrifugation at 12,000 × g for 10minutes and they were utilised as crude enzymes. At four degrees Celsius, the xylanase was purified. Ammonium sulfate was used to precipitate the crude xylanase at a concentration equivalent to 80% saturation. Following a 20-minute centrifugation at 15,000 × g, the precipitate was dissolved in 50 mM sodium phosphate buffer (pH 7.0) and dialysed against the same buffer before being moved to the DEAE-Sepharose column. 0-0.5 M NaCl was used for the elution. A 0.25 M NaCl gradient was used to elute the xylanase active fraction. After being equilibrated with 20 mm sodium phosphate buffer (pH 6.8) containing 1.5M ammonium sulfate, the active fractions from the DEAE-Sepharose column were mixed with the same volume of 3M ammonium sulfate. After that, they were placed on a Phenyl 5 PW column. Ammonium sulfate was applied in a linear gradient to elute the adsorbed proteins.0.2 M of the xylanase activity. Phenol 5 PW's active fraction was dialysed using a pH 7.0, 5 mm sodium phosphate buffer. A hydroxyapatite column that had been previously equilibrated with the same buffer was placed on top of the dialyzed enzyme solution. Using a sodium phosphate linear gradient that extended from 5 to 100 mm, the absorbed protein was eluted [17].

RESULTS AND DISCUSSION

Fungal Isolation and Dominant Species

The current study investigated the mycological profile of otomycosis by analysing debris and scraping samples from the external ear canals of 50 clinically diagnosed patients. Of these, 41 samples (82%) were confirmed to be positive for fungal pathogens, affirming the high prevalence of fungal infections in otic conditions, especially in tropical and subtropical climates. Among the positive samples, the predominant fungal pathogens identified were: *Aspergillus niger*(18isolates;43.9%), *Aspergillus fumigatus* (16isolates;39.0%) and Candida albicans (7 isolates; 17.1%)

The predominance of *Aspergillus* species, particularly *A. niger*, aligns with a number of prior studies. For instance, Moubasher *et al.* (2019) [17] and Al-Bedak *et al.* (2020) [18] both reported *Aspergillus niger* as the most frequent isolate in otomycosis, attributing its high frequency to its ubiquitous presence in the environment and its ability to colonies the moist, warm auditory canal effectively. *Aspergillus* fumigatus followed closely, which is also consistent with other studies that recognise it as a significant opportunistic pathogen in otic infections, especially in immune-compromised individuals [19].

The presence of *Candida albicans* in 17.1% of cases, although less frequent than *Aspergillus* species, is clinically significant. *Candida* is a known commensal organism but may become pathogenic under certain conditions, such as prolonged antibiotic or steroid use or inpatients with underlying immune suppression [20]. Comparison with Literature. The overall fungal positivity rate of 82% in our study concurs with other reports, which cite otomycosis rates ranging from 60% to 90% in patients presenting with ear canal infections [21]. Environmental factors such as humidity, poor hygiene and overuse of topical antibiotics are known contributors to the high incidence in such populations.

Our findings reinforce the widely held understanding that *Aspergillus* species are the leading cause of otomycosis

globally, especially in warmer climates. However, the noticeable occurrence of *Candida albicans* emphasises the need for clinicians to consider yeasts as potential pathogens in non-responsive cases.

Fungal Isolation and Identification

About five *Aspergillus niger* were isolated from otomycosis patients collected at a chosen study site and chosen for additional investigation after they produced transparent halos surrounding their colonies on xylan agar plates. The 12-7.2 cm zone was the highest zone of clearing surrounding the colony, Table 1 and Figure 1 demonstrating the strain's xylanolytic activity.

The highest five *A. niger* isolates from patient samples with otitis media showed clear hydrolysis zones on xylan agar plates, indicating xylanolytic activity. Among these isolates, isolates (A1, A2, A3, A4 and A5) showed the highest hydrolysis zones (12, 11.2, 9.6, 8.6, 7.2 cm), respectively, indicating that it was the most efficient xylanase producers. These results are consistent with previous findings in which A*ngier* was identified as a prolific xylanase producer due to its natural habitat and enzymatic properties [22].

Partial Putrefaction of Xylanase

Using Ammonium Sulfate Precipitation. Fractional (35-80%) ammonium sulphate saturation precipitated the culture filtrate. Proteins that precipitated in this range were utilised for purification because they exhibited the highest xylanase activity. The dialysed enzyme precipitation at 80% saturation yields 3.1 U/mg protein specific activity (Figure 2).

In the current study, partial purification of xylanase from *Aspergillus niger* was achieved using fractional ammonium sulfate saturation ranging from 35% to 80%. This classical salting-out method is widely employed in enzyme purification due to its efficiency, cost-effectiveness and ability to concentrate proteins while maintaining enzymatic activity [23].

The protein fractions precipitated within this range demonstrated significant xylanase activity, indicating that the enzyme of interest was effectively concentrated [24].

Notably, the highest xylanase activity was observed in the fraction precipitated at 80% saturation, which yielded a specific activity of 3.1U/mg protein. This level of activity suggests a successful enrichment of the enzyme relative to the total protein content. These findings are in line with previous reports where 70-80% ammonium sulfate saturation was found optimal for xylanase precipitation from filamentous fungi such as *Aspergillus* spp. [25].

The increase in specific activity following precipitation reflects partial removal of other non-enzymatic proteins and impurities. However, as a preliminary step, this method provides a foundation for further purification techniques, such as ion-exchange or affinity chromatography, to achieve higher purity and specific activity necessary for industrial or analytical applications.

Purification of Xylanase

A DEAE cellulose ion exchange column was used to further purify xylanase. The enzyme was extracted from the DEAE cellulose column at a concentration of 0.25M NaCl (Figure 2). The concentrated fractions were those with the highest specific activity (numbers 19-25). With a specific activity of 1102 U/mg, 97 xylanase purifications were accomplished (Table 2).

Table 1: Aspergillus *niger* isolates and their in-Xylanagar media incubation at 37°C for 72 hours

Isolation number	Zone of hydrolysis/cm
A1	12
A2	11.2
A3	9.6
A4	8.6
A5	7.2

Table 2: purification of xylanase from Aspergillus Niger

Sample	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Enzyme activity (u/ml)	Total activity (U/ml)	Ecific activity U/mg protein	Fold	Yield %
Crude extract	1000	1.9	1900	21.5	21500	468	1	100
Perception 80%	50	7.85	392.5	401	20050	19.6	4.5	93.26
DEAE-cellulose	5	2.55	12.75	2810	14050	4.68	97.5	65.3

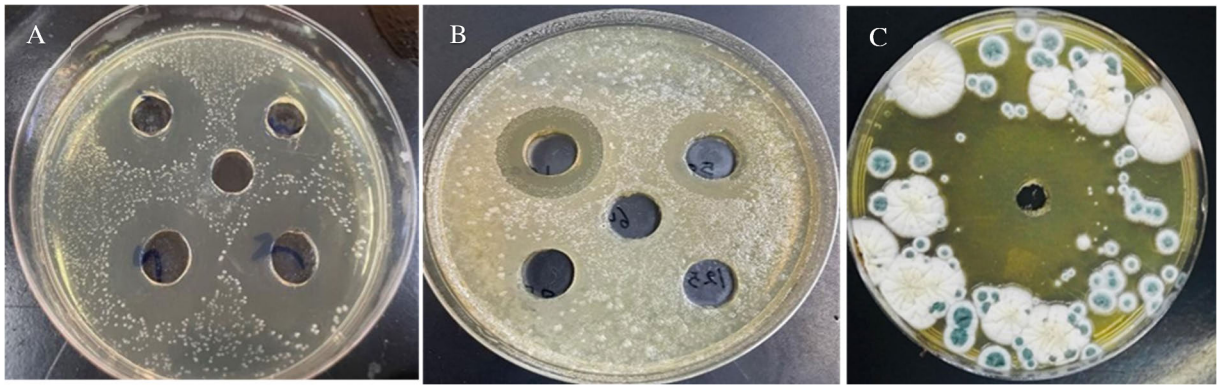


Figure 1A,B,C: *Aspergillus niger* isolates and there in-Xylan agar media incubation zone of inhibition at 37°C for 72 hours

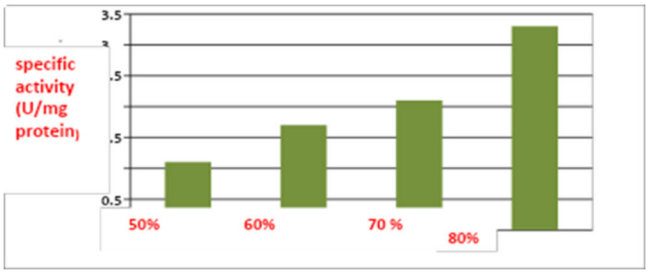


Figure 2: Specific activity of Aspergillus Niger xylanase after precipitations with ammonium sulphate

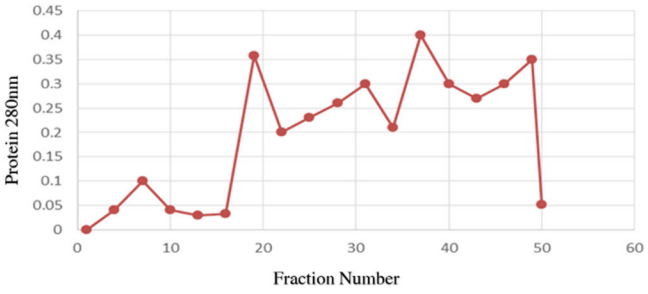


Figure 3: Elution profile of xylanase from protein concentration

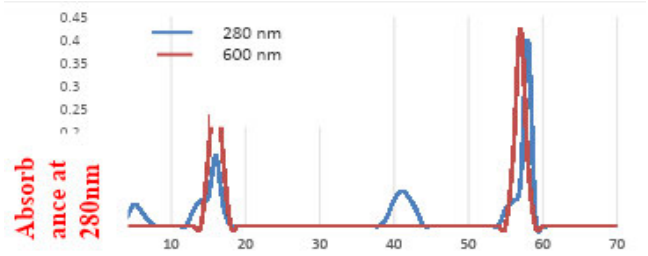


Figure 4: Elution profile of xylanase from DEAE-cellulose column chromatography

In the present study, DEAE-cellulose ion exchange chromatography was employed to purify xylanase produced by *Aspergillus niger* following ammonium sulfate precipitation. The elution was performed using a 0.25 M NaCl gradient, which successfully released the enzyme bound to the column. The active fractions (numbers 19-25) displayed the highest specific activity, which reached 1102 U/mg protein, representing a 97-fold purification compared to the crude extract.

This significant increase in enzyme purity confirms the efficiency of ion exchange chromatography for xylanase purification. DEAE-cellulose, an anion exchanger, selectively binds negatively charged proteins like xylanase at a given pH, allowing other non-target proteins to be washed out. The subsequent elution with salt gradient ensures the release of bound enzyme fractions with minimal denaturation [26]. Trichoderma viride via ion exchange, validating its robustness and reliability as a downstream purification step. Similar results have been documented in recent literature. For example, Ding *et al.* [27] purified xylanase from *Aspergillus* Tubingen is using DEAE-Sepharose, achieving over 90-fold purification and retaining high enzymatic activity and thermal stability. In another study, Uhoraningoga [28] reported a 100-fold purification of xylanase from the successful concentration of highly active xylanase fractions aligns with its isoelectric point and net charge behaviours at the buffer pH. The resulting specific activity of 1102 U/mg protein not only indicates an effective separation but also suggests suitability for industrial applications where high enzyme purity is crucial, especially in sectors such as food, pulp and paper and biofuel production.

Influence of Temperature

After three days, the xylanase production at various temperature ranges was analysed while maintaining the other fermentation constants. At 55 °C, the largest amount of protease produced was 6.2 U/ml (Figure 3).

Influence of Temperature on Xylanase Production. Temperature is a critical environmental factor influencing the metabolic activity of microorganisms and the secretion of extracellular enzymes such as xylanase. In the current study, the production of xylanase by *Aspergillus niger* was evaluated under various temperature conditions while maintaining all other fermentation parameters constant. The results revealed that the maximum xylanase activity was achieved at 55°C, yielding 6.2 U/ml after three days of incubation.

This finding suggests that the strain of *A. niger* used in this study is thermotolerant, with an optimal temperature close to the upper range of mesophilic fungi. While many *Aspergillus* species exhibit optimal xylanase production between 30-50°C, the elevated optimum observed here is consistent with reports indicating enhanced enzyme yields at higher temperatures in certain thermotolerant strains [29]. The increased enzyme activity at 55°Ccouldbeattributedto the enhanced solubility of substrates and improved enzyme secretion rates by the fungal cells [30].

However, beyond the optimum temperature, enzymatic activity typically declines due to thermal denaturation of cellular proteins and impaired fungal growth. Although this study did not report data beyond 55°C, it is reasonable to assume that further temperature elevation may lead to a drop in enzyme yield, as noted in related studies [31].

Moreover, thermostable xylanases are industrially desirable, especially in processes such as paper pulp bleaching, textile biopolishing and biofuel production, which often operate at elevated temperatures. Thus, the optimal output of xylanase at 55°C enhances the potential applicability of this *A. niger* isolate in industrial settings.

Influence of pH on Xylanase Production

At PH 7, the highest xylanase output of 5.4 U/ml was observed (Figure 4). The outcome made it very evident that the bacteria were neutrophilic. In the PH 6-8 range, *Aspergillus niger* was found to produce medium amounts of xylanase.

The pH of the growth medium plays a fundamental role in microbial enzyme production by affecting nutrient availability,

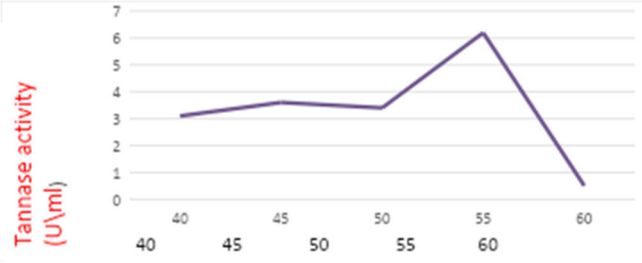


Figure 5: Effect of temperature regimes on the production of xylanase by *Aspergillus niger*

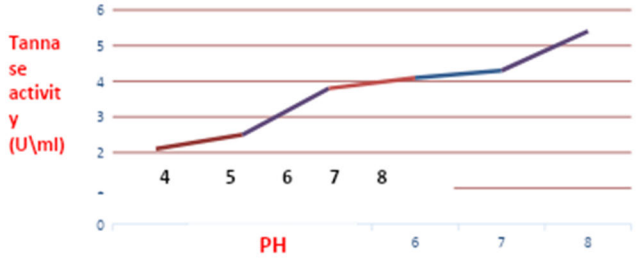


Figure 6: Effect of pH on the production of xylanase by *Aspergillus niger*

enzyme stability and the metabolic activity of the organism. In this study, *Aspergillus niger* exhibited maximum xylanase production of 5.4 U/ml at pH 7, with substantial activity maintained across a pH range of 6 to 8. These findings indicate that the strain is neutrophilic, favoring a neutral environment for optimal enzyme synthesis (Figure 5).

The results are consistent with previously reported studies, which show that many Aspergillus species, particularly Angier, produce xylanase optimally at neutral to slightly acidic pH levels. For example, Priyashini *et al.* [32] reported optimal xylanase production at pH 6.5-7.0 for *A. niger*, while Sikander *et al.* [33] observed significant enzyme activity within the pH range of 5.5 to 7.5, suggesting robust tolerance to near-neutral conditions (Figure 6).

Neutral pH may favour enzyme secretion by enhancing cell membrane integrity and facilitating efficient transport of proteins across the cell wall. Furthermore, xylanase enzymes are generally stable within this pH range, ensuring minimal degradation during production. According to Ajijolakewu *et al.* [34], maintaining pH near neutrality not only optimizes xylanase yield but also preserves its catalytic efficiency and structural stability, which are crucial for downstream industrial applications. Given the industrial relevance of xylanases in paper, textile and food industries, where operational pH often hovers around neutral, xylanase produced under these conditions is particularly suitable. This strengthens the industrial potential of the *A. niger* isolate used in this study [35,36].

CONCLUSIONS

The most prevalent fungal isolates in otomycosis are *Aspergillus* and *Candida* species. The high enzymatic activity of fungal infections is responsible for their high pathogenicity. Antifungal empirical use needs to be discouraged. Ion exchange chromatography using DEAE-cellulose provided an efficient means of isolating and concentrating xylanases with high specificity and yield. The results are consistent with other recent findings and support the continued use of this method as a standard for fungal enzyme purification.

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