

Effects of Process Parameters on the Activities of Enzymes by Different Species of *Aspergillus* in Crude Polluted Soil Sites

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Authors' contributions

This work was carried out in collaboration among all authors. Author JMM designed the study and wrote the first draft of the manuscript. Author AIO wrote the protocol. Author CICO managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The effects of temperature, pH and incubation time on amylase, protease and cellulase activity by different species of *Aspergillus* in crude oil polluted soil sites in Nigeria were studied. Incubation period, Optimal pH values and temperatures for the enzymes produced by the different micro-organisms were determined. The production of amylase by *A. fumigatus* and *A. niger* isolated from crude oil polluted sites showed that both fungi had their peaks on the first day of incubation for amylase, protease and cellulase. *A. niger* showed higher stability across a range of pH (3-6) and temperature (25-70°C) changes in all the enzyme activities. Further experiments are recommended to purify the secreted enzymes and stability studies will be performed to enhance the application of enzyme to commercial level.

Keywords: *Aspergillus*; enzymes; incubation time; pollution.

1. INTRODUCTION

The biodiversity of microorganisms in underdeveloped countries, is becoming increasing due to extreme environments. The increasing number of hot days had modified micro-organisms to develop various adaptations mechanism, enabling them to survive the harsh effects of extreme climates [1]. Adaptive mechanisms utilized by the micro-organisms include modifications in amino acid sequence, hydrogen bonding patterns, electrostatic interactions, disulfide bonds, and metal binding ability, resulting in superior conformational structure. The increasing interest in thermostable tolerant microorganism for industrial use is due to their novel, multifold applications and resistance to harsh industrial conditions. They are capable of catalyzing a range of novel and important reactions in both aqueous and nonaqueous media. Therefore, the enzymes present a novel area for future researches on the study of thermostable organic solvent, as well as bacteria-producing enzymes and factors affecting their production [2].

Fungi diversity is characterized by thermotolerance, the expression of cell wall components, the presence of a capsule and the secretion of enzymes [3]. Fungi show an array of broad spectrum of enzymes, with propensity of degrading various components of host tissues [4]. Temperature has been described as one the most important parameters in determining the rate and effectiveness of bioremediation. It has been reported that the range of temperature favourable for micro-organisms population and degradation of crude oil is 30°C and 40°C [5]. Low temperature has been found to result in slower degradation rates, possibly because it increases the viscosity of oil, resulting in low drifting capacity which in turn creates a low surface area for degradation. pH affects solubility and consequently the availability of many constituents of soil which can affect biological ability [6]. Biodegradation is affected negatively by conditions of pH, and therefore most laboratory-based biodegradation studies are conducted at pH range near neutral [6].

2. MATERIAL AND METHODS

2.1 Location

The area selected for the study included Ukwa West Local Government Area in Abia State, Nigeria, with the headquarters in the town of Dike Ikpe. It has an area of 271km² and a population

of 88, 555 as at the 2006 census. The Local Government is the only crude oil producing area in Abia State. Its Oil producing communities include Owaza, Uzuaku, Umuokwor, Umuahala and Umuorie [7].

2.2 Spectrophotometer Assay of the Enzymes Produced by the Fungi

For the enzyme assay, the method used by [8] was adopted. A volume of 100ml of the synthetic medium was prepared in six (6) different 250ml Erlenmeyer flask using soluble starch as sole carbon source. The flasks were autoclaved for 10 min at 121 °C and allowed to cool. About 5mm mycelia plug of the organisms, *Aspergillus niger*, *A. fumigatus*, *A. terreus* were inoculated into each of the flasks. The reaction mixture contained 0.5 ml of each of the enzymes source was measured and put into six separate test tubes. 0.05 ml citrate buffer with 0.5ml of 1% starch was added into the test tubes and mixed properly. The seventh test tube that contained only distilled water served as the blank. The test tubes were then placed in water bath at 30°C for 30minutes. The reaction was stopped by the addition of 1ml of 3, 5 – Dinitrosalicylic acid (DNSA) reagent into 7 test tubes. It was afterwards boiled for 10minutes and cooled in water for colour stabilization. The absorbance was read using a spectrophotometer at 625 nm wavelength to ascertain the enzyme activity. The starch activity was measured using a calibration curve of glucose. One (1) unit of starch was defined by the amount of enzyme that released 1µmol of glucose per minutes.

The same preparation was made using skim milk, Carboxymethylcellulose (CMC) and groundnut oil as sole carbon sources. Weights of 5mm mycelia plugs of the organisms were all inoculated respectively. The flasks were incubated at 25°C for a period of 9 days under static condition. Enzyme activity was assessed at 3, 6 and 9 day intervals using cell-free culture filtrate of each organism. Distilled water was used as blank and D-glucose as standard. Reducing sugar was estimated using spectrophotometer at 540 nm for cellulase activity. The experiment was conducted in triplicate to increase the reliability of the results so to generate the means with the standard deviation.

2.3 Effects of Incubation time on Amylase Activity

The method used by [9] was employed for the experiment [9]. 1 ml of each of the culture filtrate

was harvested at 24-hour interval for 5 days and pipetted into six test tubes. 1 ml 0.1 M acetate phosphate buffer (pH 6.5) was added. A blank of 2ml of the enzyme extract that has been boiled for 20mins was set up. One percent Starch solution was added to the experimental tubes. The flasks were placed in a water bath at 40°C for 30mins. The reaction was stopped by addition of 2ml of 3, 5-Dinitrosalicylic Acid (DNSA) and was boiled for 5mins. It was allowed to cool, and 2ml of distilled water was added. The absorbance was read at 625nm using the spectrophotometer.

2.4 Effects of Incubation Time on Cellulase Production

The method used by [10] was employed for the experiment [10]. A volume of 2ml of culture filtrate from the incubated flask of the culture broth was taken every 24 hrs for 5 days. The broth was subjected to centrifugation at 6000rpm for 15mins. The cell-free supernatant of the liquid culture was then used as the enzyme source. 0.5ml of enzyme source was taken in a tube and added with 0.5ml of 0.05 M citrate buffer (pH 4.8) and 0.5ml of 1% Carboxymethylcellulose (CMC) was added. The reaction mixture was incubated at 30°C for 30mins and was stopped by the addition of 1ml of 3,5-Dinitrosalicylic Acid (DNSA) reagent and was boiled for 5mins. It was allowed to cool, then 2ml of distilled water was added. The absorbance was read at 540nm using the spectrophotometer. Boiled enzyme extract from the control flask served as blank and D-glucose served as standard. Enzyme activity was defined as milligram (mg) of glucose released per mins, per ml of culture filtrate.

2.5 Effects of Incubation Time on Protease Activity

The method used by [10] was employed for the experiment [10]. 1% casein and 1% skim milk were used as substrate. 1.0ml of enzyme sample was added and the mixture was incubated at 30°C for one hour. The reaction was stopped by the addition of 5ml of 5% Trichloroacetic Acid (TCA) to precipitate the protein. The mixture was centrifuged, and 1.0ml of supernatant was mixed with 5ml of alkaline reagent (one ml of in NaOH), to make the content alkaline. After 10mins, 0.5ml of folin phenol reagent were added to the test tube and then mixed. The blue colour produced

was measured at 700nm after 30mins. A unit of protease activity was defined as the amount of Enzyme required to produce an increase of 0.1m optical density at 700nm.

2.6 Effects of Incubation Temperature on Cellulase Production

The method used by [8] was employed for the experiment [8]. One ml of the crude enzyme for each isolate was collected separately, and 1ml of 0.05 Sodium citrate buffer (pH 4.8) was added to the crude enzyme. 1 ml of 1% CMC was added, and then incubated. DNSA was added to stop the reaction and was heated for 10mins. After cooling, the absorbance was read at 540nm using the spectrophotometer.

2.7 Effects of Incubation Temperature on Lipase Activity

For temperature stability and activity, the reaction mixture included One ml of crude extract, 10ml of 0.1M phosphate buffer (pH 6.5), 2mls of groundnut oil. The mixture was incubated at temperatures ranging from 25, 30, 40, 50, 60 and 70°C for 1 hour. The enzyme mixture was immersed in an ice bath for 30 mins. Activities were assessed under standard conditions by titration as described by [8].

2.8 Data Analysis

The statistical analyses were performed using the JMP, version 13.0. Studentized t-test was performed to compare the expression of enzyme activity at different times and temperatures in the aspergillus species.

3. RESULTS AND DISCUSSION

3.1 Effects of Incubation Time on Amylase Activity

Fig. 1 shows the effects of incubation time on amylase activity. The production of amylase by *A. fumigatus* and *A. niger* isolated from crude oil polluted sites showed that both fungi had their peaks on the first day of incubation with *A. fumigatus* having 123.64 $\mu\text{mol/ml}$, while that of *A. niger* was 122.96 $\mu\text{mol/ml}$. The peak declined on the 3rd day with *A. fumigatus* having 116.45 $\mu\text{mol/ml}$ and *A. niger* having 115.55 $\mu\text{mol/ml}$. The continued up to 9th day of incubation, with *A. fumigatus* having 51.96 $\mu\text{mol/ml}$ and *A. niger* having 48.8 $\mu\text{mol/ml}$.

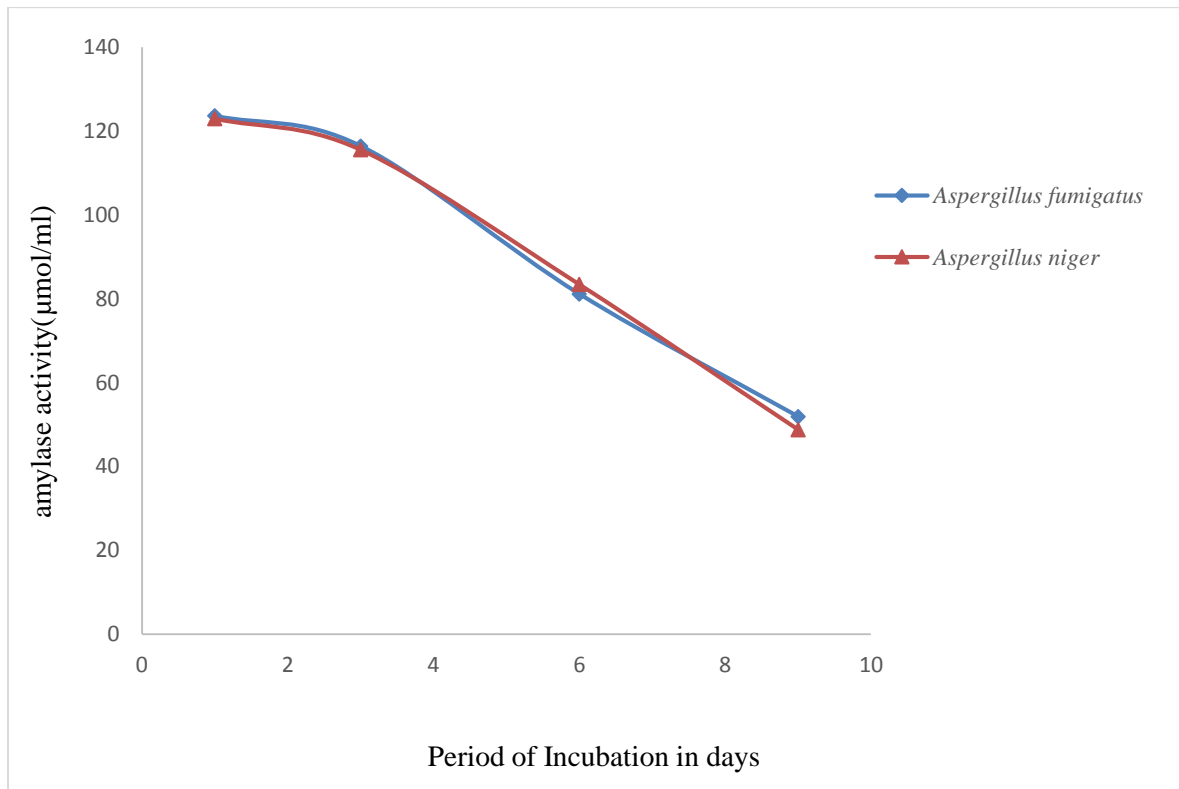


Fig. 1. Effects of Incubation Time on Amylase Activity

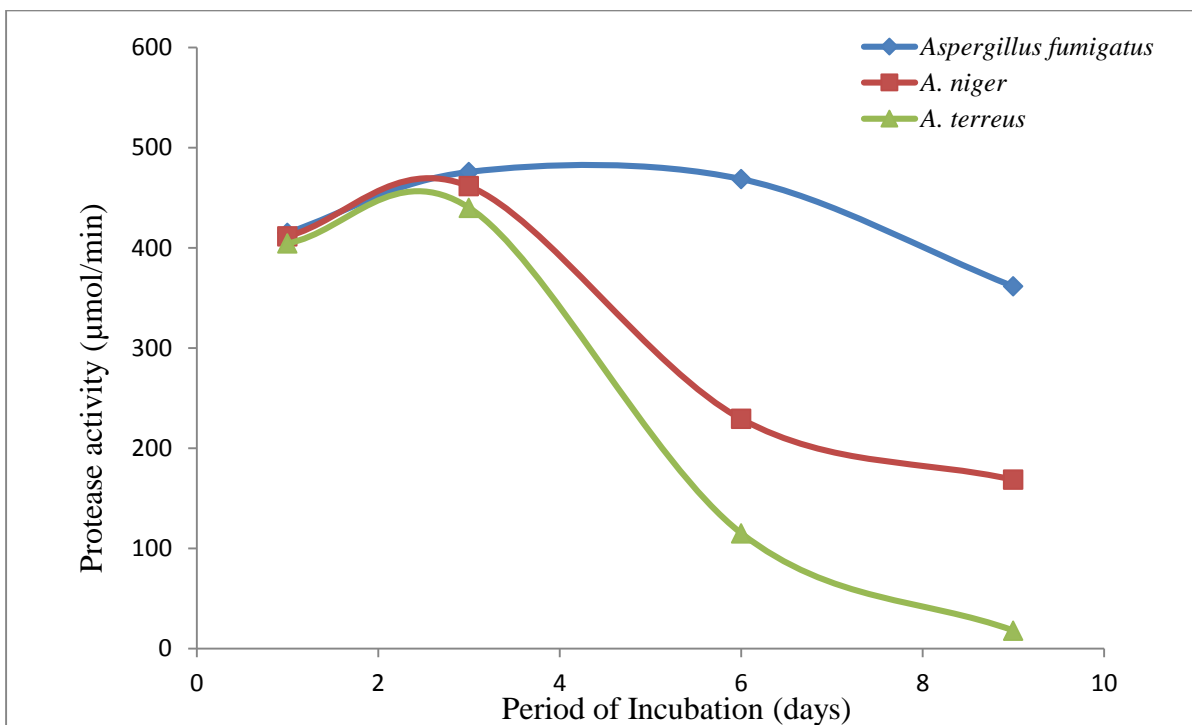


Fig. 2. Effects of incubation time on protease activity

3.2 Effects of Incubation Time on Protease Activity

It was observed that protease production increase steadily at 24hr interval, and highest on the third day after which enzyme production declined from all the test organisms. *Aspergillus terrus*, *A. fumigatus* and *A. niger* had their peak enzymes production of 475.8 μ mol/ml, 461.5 μ mol/ml and 440.0 μ mol/ml respectively, showing that there were significant differences ($p < 0.05$) on enzyme production among the test fungi as shown in Fig. 2.

3.3 Effects of Incubation time on Cellulase Activity

There was a linear decline in cellulase activity as incubation time increases in *Aspergillus fumigatus* and *Aspergillus niger* as shown in Fig. 3.

3.4 Effects of Incubation Time on Lipase Activity

Incubation time had a significant effect on enzyme production. The lipase enzyme from both *A. niger* and *A. fumigatus* showed the peak

action after 3days incubation. The rate of lipase activity in *A. niger* started showed increase from the 3rd day through the 6th day of incubation and 9th day (100 μ mol/ml, 128 μ mol/ml and 151 μ mol/ml respectively). The enzyme production peak for *A. fumigatus* increased on the 3rd day and then declined (100 μ mol/ml 77 and 60 μ mol/ml respectively) which reveals that the carbon source favoured the growth of *A. niger* more than *A. fumigatus* as shown in Fig. 4.

3.5 Effects of Incubation Temperature on Amylase Activity

Fig. 5 shows that amylase activity was affected by incubation temperature in the two test fungi. Amylase activity in the two fungi reached peak at 50°C. *A. niger* peaked at 41.62 μ mol/ml and declined at 60°C with enzyme activity of 26.33 μ mol/ml. *A. fumigatus* had amylase production of 20.27 μ mol/ml at 50°C. The results therefore revealed that there were increases in the enzyme activity at temperature range of 30-50°C. As for *A. niger*, there was drastic increase in the amylase activity up to 50°C. However, there was a decline in amylase activity from this fungus beyond 50°C. This attribute could be exploited in industrial activity that require a wide temperature range of 25, 30, 40 and 50°C, respectively.

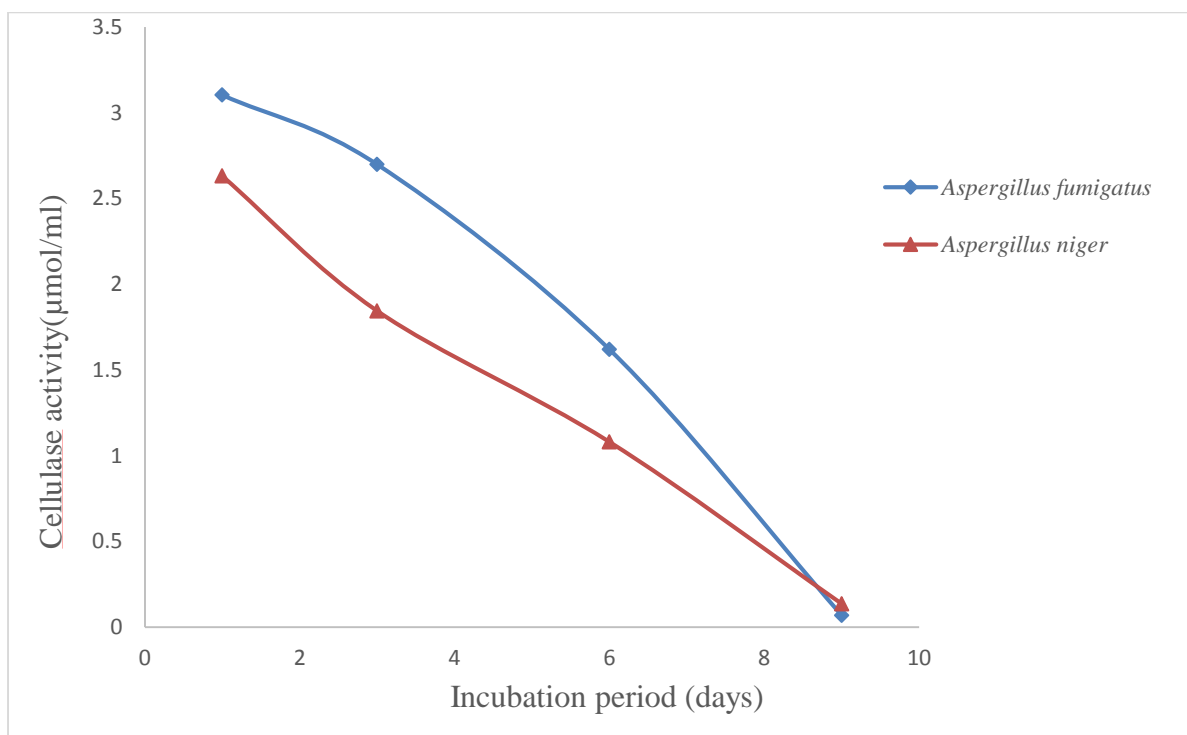


Fig. 3. Effects of Incubation period on Cellulase Activity

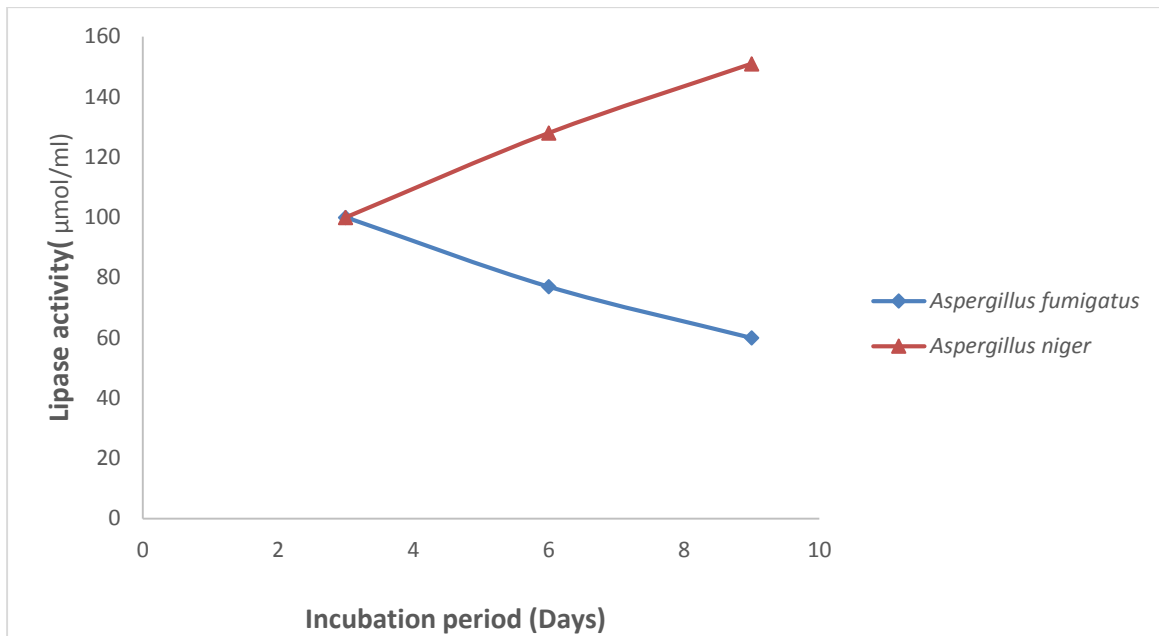


Fig. 4. Effects of incubation time on lipase activity

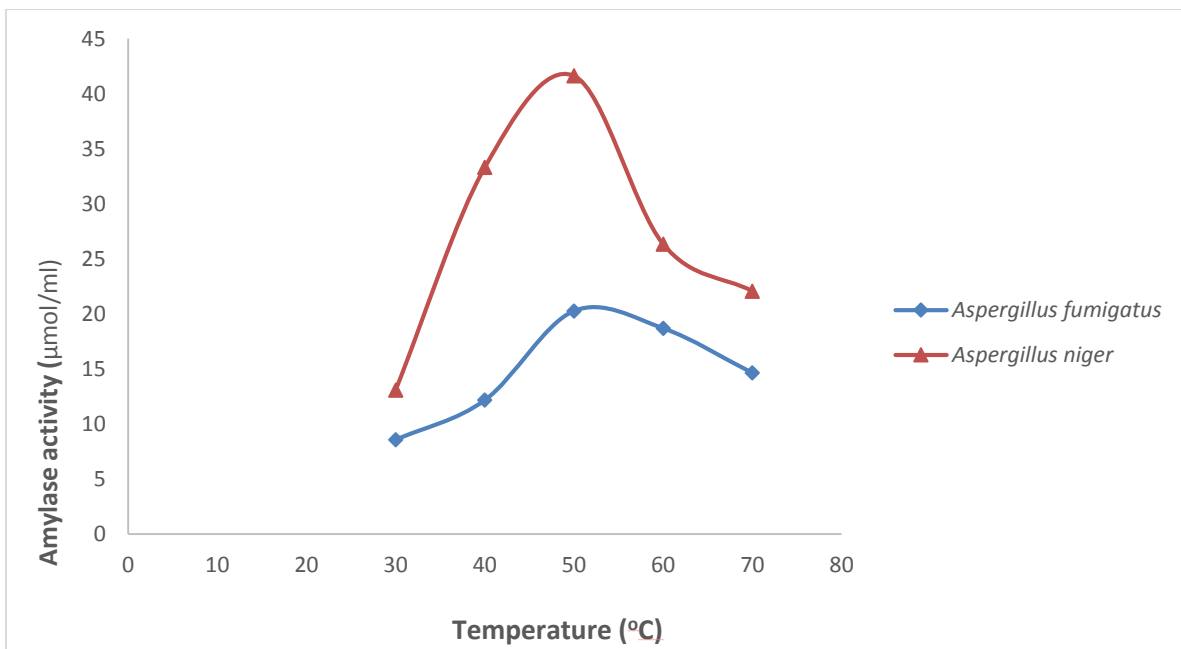


Fig. 5. The effects of temperature on amylase activity

3.6 Effects of Different Incubation Temperatures on Cellulase Production

Fig. 6 shows the effects of different incubation temperatures on enzyme production at 30°C. *A. niger* had its peak production of 23.82 µmol/ml while *A. fumigatus* has its peak production (21.46

µmol/ml) at 25°C. *A. fumigatus* activity declined at 40°C, producing 16.72 µmol/ml which led to the decrease of cellulase activity while as *A. niger* was at increase. At 50°C *A. fumigatus* declined to 11.05 µmol/ml while *A. niger* recorded 22.77 µmol/ml to which indicate that *A. niger* was at increase at different incubation period which led to increase in cellulase activity.

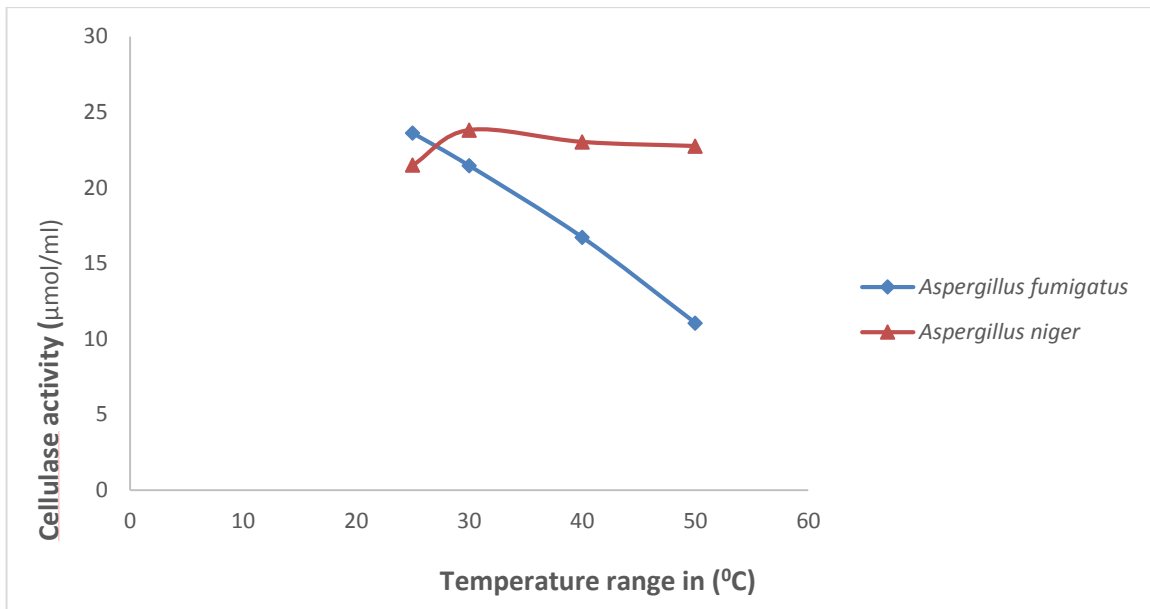


Fig. 6. The effects of different incubation temperatures on cellulase production

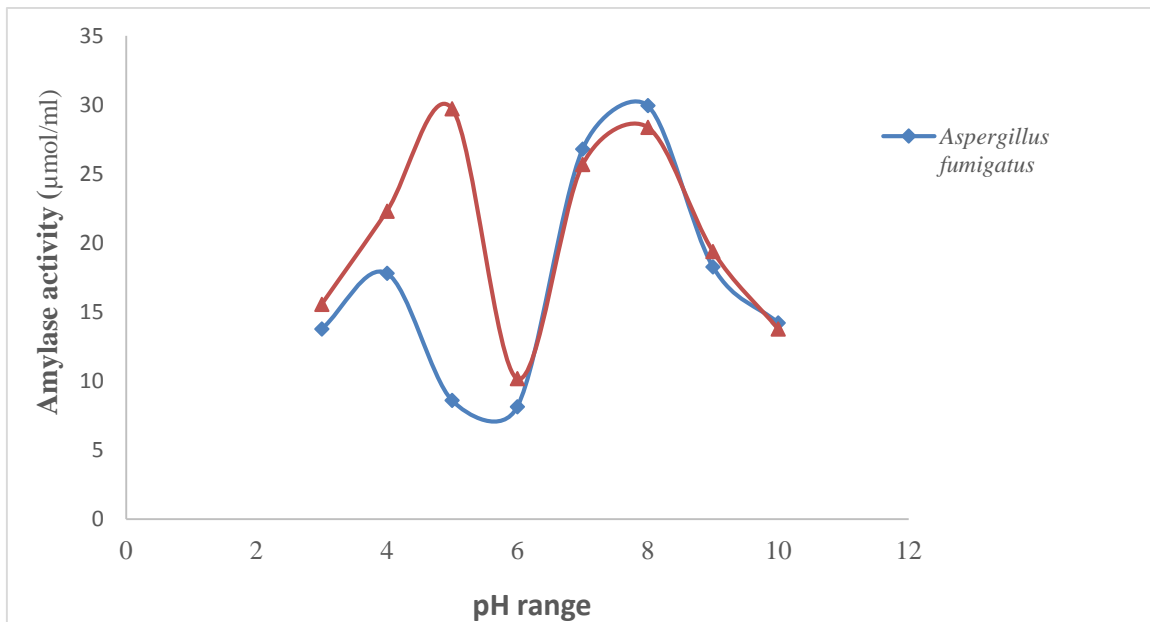


Fig. 7. The effects of pH on amylase activity

3.7 Effects of pH on Amylase Activity

The results showed that pH range affected amylase activity as presented in Fig. 7. The activities of the amylase from *A. fumigatus* and *A. niger* were 29.43 µmol/ml at pH 8 and 29.70 µmol/ml at pH 5.0 respectively. This result suggests that the enzyme would be useful in processes that require wide range of pH range from acidic to neutral range and vice versa.

3.8 Effects of pH on Lipase Activity

Effects of pH on lipase production showed that *A. niger* had its peak production of 58 µmol/ml at pH 6, while *A. fumigatus* had its peak of production of 50 µmol/ml at pH 7 as shown in Fig. 8. Lipase activities decreased on *A. niger* and it performed best on acidic environment than *A. fumigatus* that performed best at pH 7.0 which is neutral level.

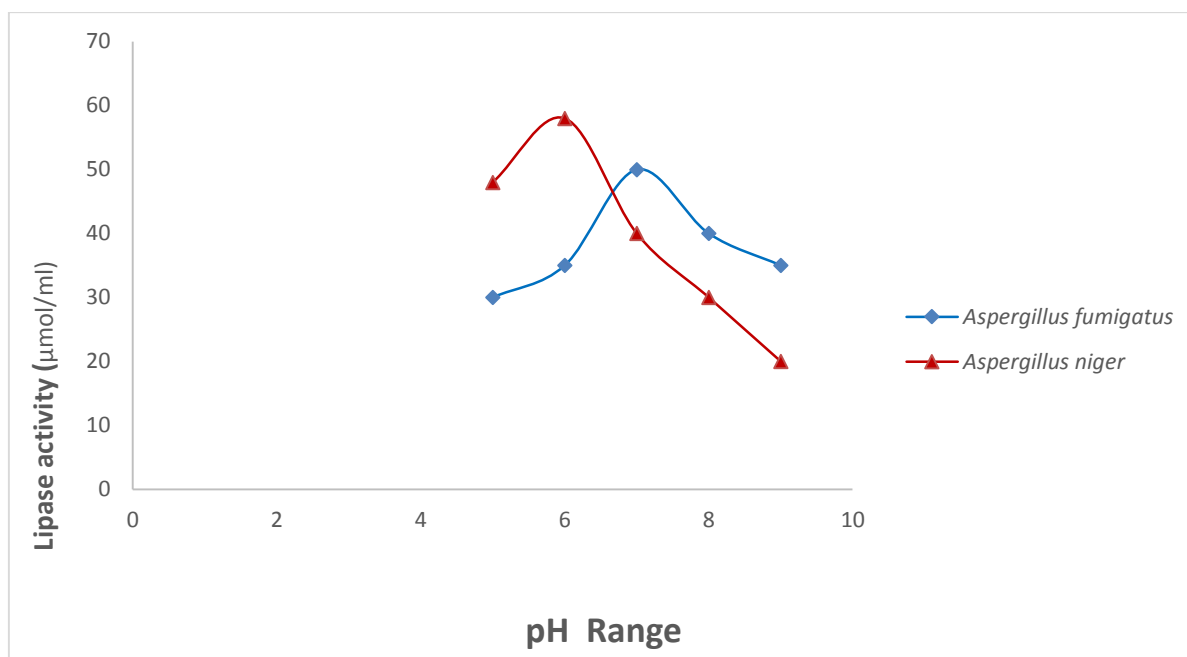


Fig. 8. The effects of pH on lipase activity

It is widely accepted that abiotic factors such as pH and temperature can have a strong influence on enzymatic processes, as well as cell membrane permeability [10]. Lipases can metabolise well in a wide range of pH and temperature conditions, and bacterial lipases are most commonly effective in an alkaline medium [10]. Maximum lipase production in the current study was obtained between pH 6 and 7 (Figure 8), consistent with the review by [11]. As shown in Figure 7, very low lipase production was detected at acidic (pH 4.0 and 5.0) and alkaline conditions (pH 9.0). As shown in Figure 8, amylase activity was low at pH 3 (acidic) and 10 (alkaline). Rapid changes in pH may modify enzymatic changes in amylase and lipase production [12]. The *Aspergillus* isolate in this study could be employed as a source of enzymes for use in specific industrial processes. *Aspergillus niger* showed higher stability across different temperature gradients as compared to *aspergillus fumigatus*. This agrees with the assertion of [13] that *Aspergillus niger* showed higher resilience at different temperature gradients, though higher temperature eventually denatures the enzyme [14]. Maximum amylase, protease and cellulase production in the current study was obtained after 9 days incubation, with the rate subsequently declining in two *aspergillus* species, most likely due to the depletion of nutrients available to the cells in the production medium [15]. Plausible biotechnological

applications of high temperature, high enzyme activity strains in the processing of volatile substances due to their ability to reduce processing temperature, thereby bring down energy costs [16]. *Aspergillus fumigatus* was generally sensitive to higher protein in this study and easily broken down. This agrees with the works of several researchers that at higher temperatures enzyme are easily broken down since they are made from protein molecules [17,18].

4. CONCLUSIONS

Based on the results of this study, it can be concluded that production of amylase by *A. fumigatus* and *A. niger* isolated from crude oil polluted sites showed that both fungi had their peaks on the first day of incubation for amylase, protease and cellulase. *A. niger* showed higher stability to pH and temperature changes in all the enzyme activities. The results are significant as they indicate that the enzyme will be active even under the presence of high temperatures and pH, thereby favoring its use under harsh conditions of textile wet processing.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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