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Biosynthesis of Zinc Oxide Nanoparticles and Optimization of Enzymes from Endophytic Fungi

J. Valli*[1], K. Kanimozhi[1], V. Ambikapathy[2], A. Panneerselvam[2], and N. Thajuddin[3]

*[1]*PG and Research Department of Microbiology, A.V.V.M Sri Pushpam College (Autonomous), Poondi - 613 503, Thanjavur (Dt), Tamil Nadu, India (Affiliated to Bharathidasan University, Tiruchirappalli -24).*

[2]*Department of Botany and Microbiology, A.V.V.M Sri Pushpam College (Autonomous), Poondi - 613 503, Thanjavur (Dt), Tamil Nadu, India (Affiliated to Bharathidasan University, Tiruchirappalli -24).*

[3]*B. S. Abdur Rahman Crescent Institute of Science &Technology, (Deemed to be University), Vandalur, Chennai - 600 048, India.*

**Corresponding author Email: josphin.valli@gmail.com*

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ABSTRACT

Endophytes are recognized as potential sources of novel secondary metabolites, including enzymes and drugs, with applications in medicine, agriculture and textile industry. There is an increasing demand for novel enzymes such as protease, in the industrial sector that can effectively operate across diverse conditions. In the present study, the plant samples were collected from marine environment of Kodiyakarai, Nagapattinam District, Tamil Nadu, India. Fungal species were isolated by plating method, in 50% marine plant containing potato dextrose agar medium. Selective two fungal strains (*Aspergillus niger* and *A. terreus*) were chosen in the secondary screening of protease and lipase enzymes. The lyophilized growth media obtained after fungal fermentation were analysed for two enzyme production was optimized by assessing the effects of temperature, pH, carbon source and nitrogen source on activity. *A. niger* and *A. terreus* showed the greatest protease activity in a wide range of pH (5-9) and showed the lowest lipase activity range from pH (5-9) at two fungi. The broadest activity between 9 and 30^{o}C was observed at pH 7 suggesting a neutral in protease. Overall, the optimum conditions were 35^{o}C and pH 7 with a maximum specific activity value of *A. niger* and *A. terreus*. In minimum specific activity value of lipase in *A. niger* and *A. terreus*. In biosynthesized (ZnO NPs) characterization using UV-Vis spectrophotometer, The maximum absorption peak was observed in the UV-Vis spectra of zinc oxide nanoparticles at 350-600 nm The bio synthesis of (ZnO NPs) nano materials were performed with comparison of control and concentration 2.5 mM was treated samples revealed well-dispersed nanoparticles ZnONPs. The characteristics demonstrated by these fungal endophytes showed that it is a potential source of a protease enzyme with particular application in the cotton industry. However, further studies of the tolerance to higher temperatures and pH will indicate whether the enzyme is suitable for biomedical application.

Keywords; Endophytic fungi, Optimization, Zinc oxide nanoparticles, UV-Vis spectrophotometer

INTRODUCTION

Endophytic fungi are characterized as fungi that reside within the intercellular or intracellular spaces of healthy plant tissues, exhibiting no apparent signs of disease. Microorganisms residing within plants, known as microbial endophytes, are widely regarded as promising reservoirs of unique natural compounds or bioactive secondary metabolites (Joseph and Priya, 2011). Encompassing a broad spectrum of biological activities, including antibiotics, immune suppressants, anticancer compounds and antioxidants (Zhang *et al.*, 2006). With a wide range of biological functions which include antibiotics, immuno suppressants, anticancer agents and antioxidants this encompasses a diverse array of activities. A diverse range of proteins can be hydrolyzed by protease (Genckal and Tari, 2006). Proteases are utilized in various industries such as detergent, meat processing, protein brewing, photography, leather production, and dairy (Anwar and Saleemuddin, 1998).

Protease are classified into six prominent groups such as serine protease, cysteine (or) sulphydral protease, aspartic protease, metalloprotease, threonine protease and glutamic acid protease (Son and Kim, 2002) and based on their optimum pH activity they are classified as acid, neutral and alkaline protease (Asoodeh and Abadi, 2012). Filamentous fungi are widely utilized among the microorganisms employed

for protease production due to their ability to thrive on diverse substrates and generate a wide range of enzymes. These compounds possess promising prospects in the fields of medicine, agriculture, and industry (Joseph and Priya, 2011; Miles *et al.*, 2012).

Lipase activity and production depend up on the microbial strain and composition of the fermentation medium (Cihangir and Sarikaya, 2004). Continuous demand for highly active enzymes with appropriate properties has encouraged the research for the new sources of lipase. The composition of the growth medium, cultivation conditions, pH, temperature and the kind of carbon and nitrogen sources greatly affect the production of lipase. The production of pharmaceuticals from medicinal plants or trees, on the other hand, major challenges (Mani *et al.*, 2023). This practice poses a serious threat to the environment as it leads to the endangerment and extinction of specific plant species. Microorganisms, particularly fungi, have emerged as a new source for the manufacturing of therapeutics (Chen *et al.*, 2022)

In 2000, the global market for industrial enzymes was estimated and continues to increase by 5-10% annually (Nevalainen and Te'o, 2003). Proteases serve as crucial enzymes in a multitude of industries, including detergents, leather, food, pharmaceuticals, and bioremediation processes (Najafi *et al.*, 2005). Extracellular proteases play a crucial role in the physiological process of breaking down large proteins into smaller molecules, facilitating their absorption by the cell. On the other hand, intracellular proteases are essential for regulating metabolism, as highlighted by (Rao *et al.*, 1998).The selection of enzyme sources is contingent up on factors such as the accessibility of sources, the expense of source materials, and the simplicity of retrieval (Nirmal *et al.*, 2011). Microorganisms are the favored origins of enzymes in industrial applications due to their swift proliferation and economical production expenses in contrast to enzymes derived from flora and fauna (Laxman *et al.*, 2005). During the downstream processing of protease production from microbes, the fungal proteases that are secreted are preferred over those derived from bacteria due to the less complex cell removal process involved. (Shankar *et al.*, 2011; Nirmal *et al.*, 2011). Furthermore, their wide range of pH activity and broad substrate specificity (Rani *et al.*, 2012) provide numerous benefits, making them suitable for various industrial applications, including the leather industry. Furthermore, the use of these nanoparticles has been showed in medication distribution and sensing applications (Mouhaned *et al.*, 2024)

Fungal enzymes hold great significance in agriculture, industry, and human health due to their remarkable stability at high temperatures and extreme pH levels, surpassing the enzymes obtained from plants and animals (Maria *et al.*, 2005). Industrial enzymes often have to operate at temperatures that are either higher or lower than the usual range, depending on the specific demands of the process they are engaged in thermostable enzymes play a vital role in various industries such as pulp and paper manufacturing, baking, and brewing. On the other hand, cold-tolerant enzymes find their application in the production of dairy products, cosmetics, detergents, and as bioremediation enzymes (Peterson *et al.*, 2009). As a result, environmental screening initiatives are employed to discover enzymes from diverse habitats, aiming to express them in exceptionally efficient production hosts. Nanotechnology coupled with basic sciences offer a wide spectral solution to the major environmental challenges (Kumar *et al.*, 2020). Fungus such as *Pichia kudriavzevii*, *Aspergillus aeneus*, *Fusarium solani* and *Alternaria alternata* were effectively explored for the synthesis of ZnO NPs. Marine endophytic fungi from seaweed have an inexhaustible source for biologically novel compounds that created the considerable interest and curiosity among researchers. These metabolites are still unexplored into various biological applications.

The objective of our work was to optimization of protease production, according to temperature, pH, carbon sources and nitrogen sources, from an endophytic fungus isolated from different marine associated plant. The plant was selected as a source of endophytes as it has an established ethnobotanical history and is of great importance to the aboriginal people of Australia due to its medicinal properties (Richmond and Ghisalberti, 1994). They establish unique relationships with the plant species ranging from symbiosis to latent pathogens (Fatima Bhadra *et al.*, 2022). This will highlight the need for more

research on fungi growing at alkaline pH and their biotechnological potential (Pawar *et al.,* 2023).

MATERIALS AND METHODS

Sample collection

The medicinal plant samples were collected seasonally from coastal areas of Kodiyakarai at Nagapattinam (Dt).The healthy mature plant samples were collected and kept in sterilized polythene bags, sealed and transported to the laboratory, sterilized every time with 70% alcohol. At each station 5 to 7 samples were collected randomly and were pooled together.

Optimization of protease and lipase enzyme production

Effect of pH

The pH of each flask were altered in 5, 6, 7, 8, and 9 in different flasks using 1N HCl and 1N NaOH and sterilized. The cultures are inoculated and incubated at particular temperature on protease production using the method (Oyeleke *et al.,* 2010).

Effect of Temperature° C

The effect of temperature on activity of protease, lipase produced by *A. niger* and *A. terreus* was studied by taking various temperatures ranges like 20, 25, 30, 35, and 40 °C. The optimization media were inoculated with the test samples at different temperatures and the protease assay was done after 24 hrs (Oyeleke *et al.,* 2014).

Effect of Incubation period

The incubation period on protease, lipase production was determined by different time intervals of 26, 32, 38, 42, and 48 hours incubated. The enzyme activity was carried out by the method of (Ali and Vadhale, 2013).

Effect of Carbon sources

The different carbon sources were altered and introduced by *A.niger* and *A.flavus* individually and protease, lipase production was investigated. The carbon sources for the production medium was replaced with 10.0 g were added. Furthermore, for carbon sources optimization, different concentration of the best carbon source (Dextrose, glucose, mannitol, starch and sucrose) were used for the protease and lipase production. This was carried out using the method.of (Karthikeyan *et al.,* 2014).

Effect of Nitrogen sources

The nitrogen sources influenced and inoculated *A. niger* and *A. flavus* individually was determined for protease, lipase production and optimized the nitrogen source, different concentrations of Ammonium nitrate, beef extract, peptone, potassium nitrate and yeast extract was used and produced protease and lipase enzymes respectively (Karthikeyan *et al.,* 2014).

Effect of Iron sources

About 50ml of marine water samples were tested for iron was adjusted to pH with HCl of NH_3 solution. The column was filled in test solution the optimum pH 7 and the test sample passed through the column at a flow rate of about 1ml min^{-1}. The adsorbed metals on the absorbance in 572 were then eluted with 5ml of 2mol/1 hydrochloric acid solution for iron (Sattar and Rahman, 1987)

BioSynthesis of Zinc oxide nanoparticles

The synthesis of zinc nanoparticles, 10ml of crude extract was added to 150 ml in a conical flask that containing 90 ml of a solution of 1mM zinc oxide. The mixture was again incubated at 60˚C in the dark while being stirred at intervals of a different time interval. Over the period of 24 hours, the resulting reduction in zinc ions (Zn^{+}) was periodically monitored. The reaction mixture's colour changed from light yellow to pale yellow and finally to dark brown after 4 hours of incubation, indicating the formation of ZnO NPs (Gurunathan *et al.,* 2013).

Characterization techniques

UV-Vis spectroscopy analysis

UV-Vis spectral analysis was done using an Elico UV-Vis spectrophotometer. After diluting of a small aliquot of the sample into distilled water, the UV-Vis spectrum of the reaction medium was measured at 72 hours to monitor the reduction of pure Zn^{+} ions (Devika *et al.,* 2012).

RESULT AND DISCUSSION

Screening of enzyme from marine endophytic fungi

The isolated fungi from marine plant include *Acremonium lutyri, A. carbonarius, A. candidus, A. conicus,A terreus, A. fischeri, A. flavus, A. fumigatus, A. niger, A. nidulans, A. konji, A. ochraceus, A. restrictus, A. tamarii, Curvularia geniculata,*

C.lunata, Drechslera oryzae, Fusarium falcatum, Humicola brevis, H. nigrescens, Helminthosporium oryzae, Mucor hiemalis , Nigrospora sphaerica, Neurospora sp. and *Penicillum citrinum* confirmed to be recorded accurately. The screening and optimization of enzymes such as amylase, protease, lipase and cellulase from selected potential fungi such as *Aspergillus niger, A. flavus, A. terreus* and *A. fumigatus.*

The fungi provides a fascinating and almost endless source of biological diversity which is a rich sources for exploitation. Coastal region of India are dispersed in tropical as well as subtropical condition. Marine fungi have a significant impact on the nutrient regeneration cycle by acting as decomposers of deceased and decaying organic matter in estuaries. Until recently, there has been limited knowledge regarding the fungi that are associated with the marine organisms inhabiting the Indian coastal line.

In the present study, totally 25 fungal species were isolated from marine plant samples of Kodiyakarai, Nagapattinam (Dt), TamilNadu, India. The previous report has revealed (Kathiresan *et al.,* 2006) the (*T.viride*) produced chitinolytic enzymes have been detected and purified. In our study, concentration of protein in the enzyme samples were determined with reference to standard Bovine serum albumin (Sattar *et al.,* 2019). Four different previously isolated *Aspergillus* strains were screened quantitatively and qualitatively for extracellular protease enzyme production. Among them *A. niger* was capable to release high quantity of protease into the fermentation medium (Reda *et al.,* 2021). The quantification of amylase and cellulase activities from *A. niger* ASP2 was analysed in the supernatant by the DNS method. The result demonstrated in the ability of *A. niger* respectively. ASP2 to produce amylase and cellulase enzymes with various activities. The enzyme were optimized with *A. niger*, *A. flavus* and *A. terreus* for the production of protease. The maximum production of protease was estimated in *A. niger* at 35 ºC.

In the current study the comparison of protease and lipase activities from *A. niger* and *A. terreus* was determined in the supernatant by the DNS method. The ability of *A. niger* to produce protease and lipase enzymes with activities. The production and optimization of enzyme *A. niger* and *A. terreus* for the production of two enzyme. The maximum production of protease and lipase was observed at 30 ºC temperature respectively.

The previous report indicated a discrepancy in the *Aspergillus brasiliensis* strain BCW2 at pH 9 (Sattar *et al.,* 2019) and impact of pH on protease activity was assessed across a range of pH values specifically 4, 6, and 8 with *A.niger* demonstrated its highest protease production at pH 8, resulting in an optimal yield. In this study pH on protease and lipase activity was assessed across a range of pH values specifically 5, 6, 7, 8, and 9. *A. niger* demonstrated its highest protease production at pH 7, resulting in an optimal yield of 9.18±2.25 IU/mL and lowest lipase production at pH 5 was recorded at *A. niger* 1.14±0.06 IU/mL **(Figure 1)**.The purification and characterization of protease derived from *A. niger* would greatly contribute to numerous industrial biotechnological and environmental applications.

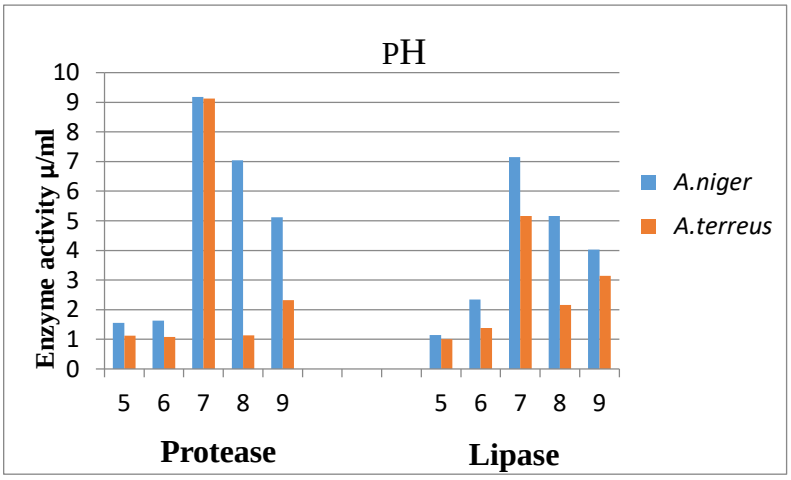

Figure 1: Optimization of pH for protease and lipase enzyme production

The influence of temperature on the activity of protease was determined at 35 ºC. Similar kind of result was recorded (Oyeleke *et al.*, 2010). The optimum enzyme activity of purified protease was at 35 °C (Reda *et al.*, 2021). In present investigation, influence of temperature on the enzyme activity of protease and lipase was determined at 30°C and maximum activity of protease (9.06±2.08 IU/mL) and minimum activity of lipase at 6.17±1.46 IU/mL. **(Figure 2)** had similar observations as temperature of 30 °C.

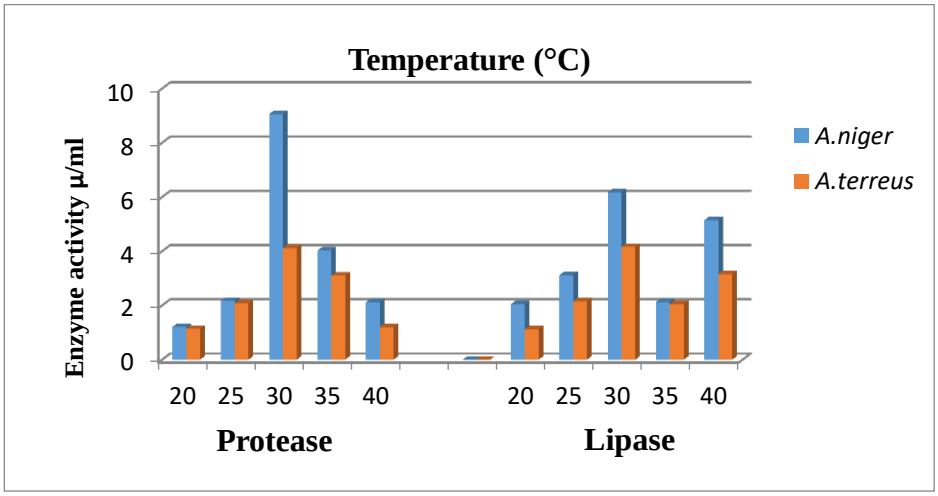

Figure 2: Optimization of temperature for protease and lipase enzyme production

The present study focused the incubation period for protease production by *A. niger* was optimum at 48 h with protease (18.4±0.63 IU/mL) and lipase production of *A. niger* was optimum at 6.04±1.23 IU/mL **(Figure 3)**. Deviation in the incubation period was recorded at 120 h (Chimbekujw and Ja'afaru Adeyemo, 2020). The yield of protease when increased in the incubation but time reduced the enzyme activity.

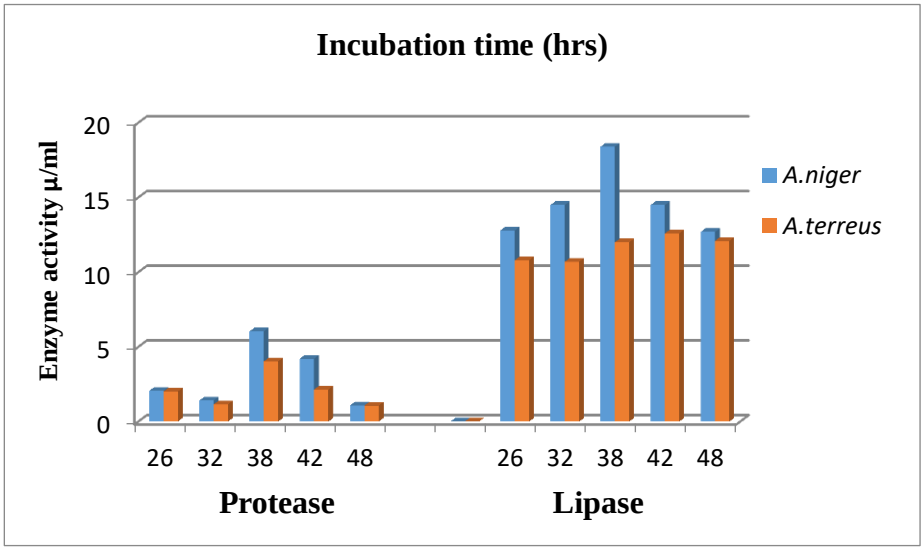

Figure 3: Optimization of incubation time for protease and lipase enzyme production

The present study of organic carbon sources such as dextrose, glucose, mannitol, starch and sucrose were evaluated for their effect on protease production and sucrose was high (23.4±1.30 U/mL) and lipase production was lowest amount of sucrose a (18.2±0.31 IU/mL) were respectively **(Figure 4)**.The highest effect of protease activity in *A.niger* supported optimal protease production with protease

activity of 10% concentration. The least amount of protease produced was by dextrose (17.4±0.93 IU/mL) respectively. Vast difference in the yield was recoded by (Sattar *et al.,* 2019).when organic carbon substrates were used as supplement.For their effect on protease production by *A. niger*. The least amount of protease produced was by *A. terreus*.

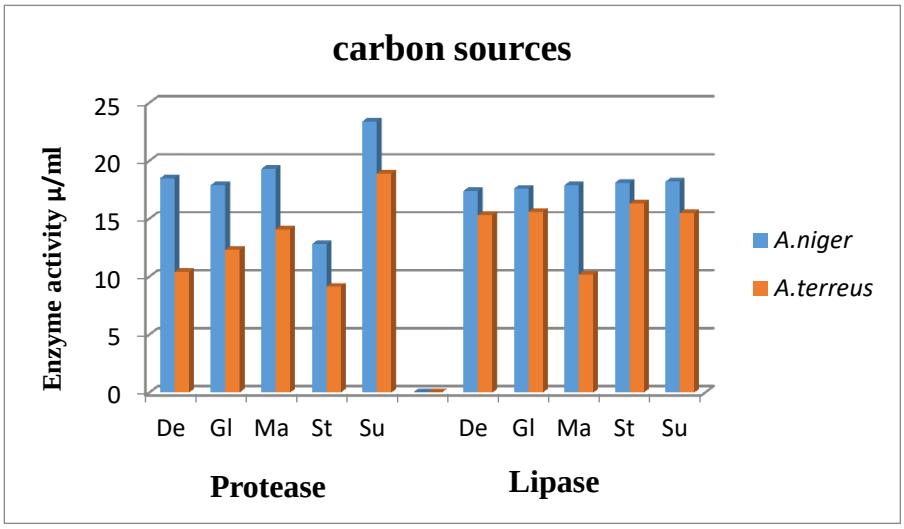

Figure 4: Optimization of nutrient carbon source for protease and lipase enzyme production

In previous study six different nitrogen sources were tested for protease production by *Aspergillus brasiliensis.* Maximum protease production was observed with yeast extract of 2% concentration (Sattar *et al.,* 2019). Nitrogen sources were tested in this study for protease and lipase production by *A. niger* maximum protease production was observed in ammonium nitrate with the activity of (20.2±6.20 IU/mL) at 50 mg/g concentration **(Figure 5)**. Contrary to the present study, five different nitrogen sources were tested for lipase production by *A.niger* Maximum lipase production was observed with yeast extract of 20.2±3.96 U/mL at 2% substrate concentration.

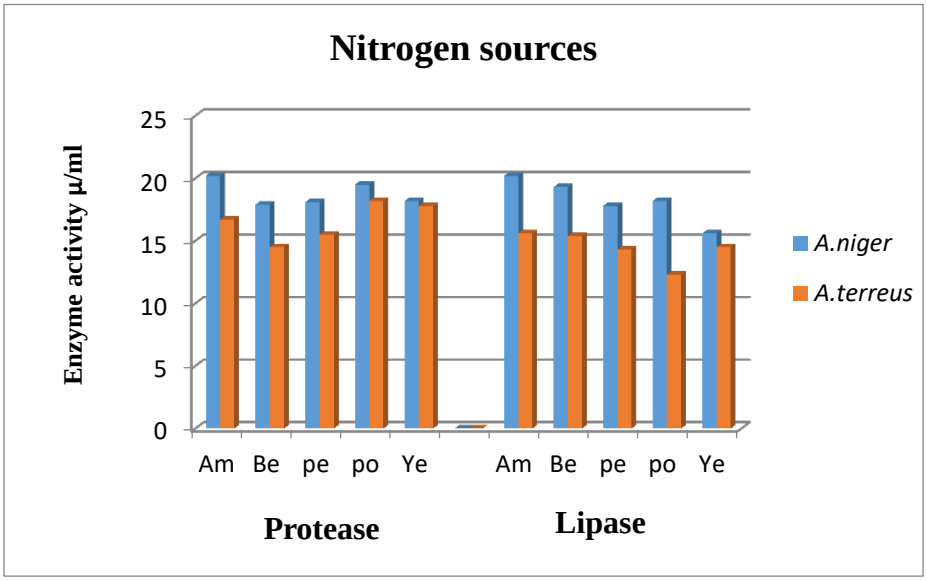

Figure 5: Optimization of nutrient nitrogen source for protease and lipase enzyme production

The nutrient of phosphorous for protease production by *A. niger* was excellent activities and followed by minimum protease production was recorded respectively. The effect of potassium on protease production by *A.niger* was optimized followed by low protease production. This result is in tandem with

the earlier reports (Chimbekujw and Ja'afaru Adeyemo, 2020). In the present investigation iron sources for protease production by *A. niger* was optimized at 100 mg/g with protease activities of (22.5±1.36 IU/mL) and followed by less protease production was recorded at 20 mg/g in 12.3±0.37 IU/mL **(Figure 6)**. The effect of iron sources on lipase production by *A. niger* was optimized at 100 mg/g with lipase activities of (7.11±1.30 IU/mL) and followed by less lipase production in 20 mg/g in (1.09±0.05 IU/mL) recorded respectively.

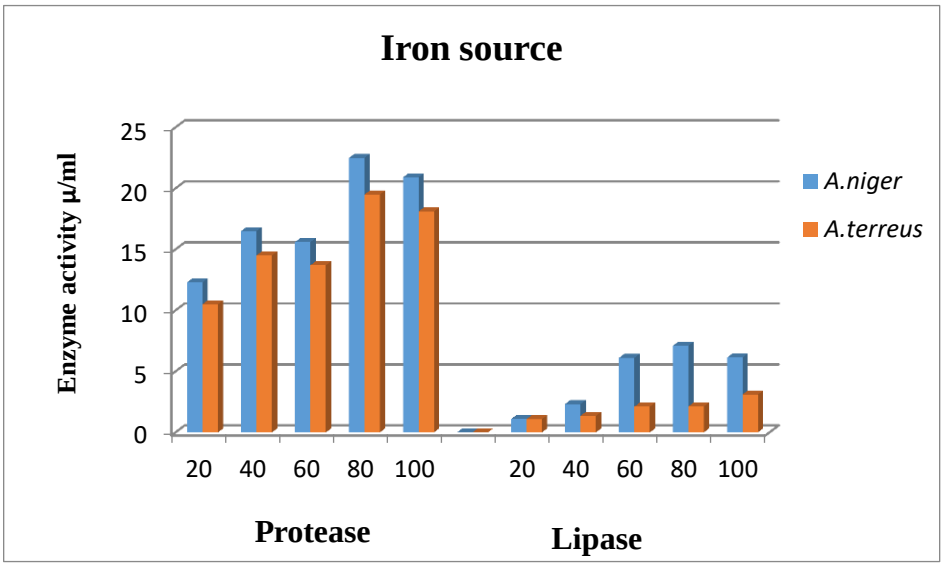

Figure 6: Optimization of nutrient iron source for protease and lipase enzyme production

Characterization of biosynthesized ZnO NPs from Endophytic fungi

The maximum absorption peak of ZnO NPs synthesized was 380 nm, demonstrating the synthesis of ZnO in a nano while it was 236 nm in fungal aqueous extract. ZnO in a nano form has shorter absorption wavelengths than normal ZnO. The UV absorbance bands indicated the surface plasmon resonance (SPR) of the formed nanoparticles at the same absorbance with minimum shifts according to the shape and size of the nanoparticles. This finding is consistent with the previous results. The fungus-produced nanoparticles were white and suspended in distilled water (Alotaibi *et al.*, 2022).

In the current study, the biosynthesis of zinc nanoparticles is the colour of the solution changes from yellow to ruby-brown and finally to dark brown for crude extract of endophytic fungi (*A. niger* and *A. terreus*) respectively as shown in **Figure 7 and 8**. The fungal extract are constant for 1g and after addition of five concentration of zinc oxide (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM) are including control (without zinc oxide). It was observed that the color of the solution turned from dark brown after 48h of the reaction, which indicated the formation and stability of the reduced zinc nanoparticles in the colloidal solution were monitored. The UV-vis spectra showed absorbance at 350 to 600nm, shows increased absorbance in various concentration (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, and 2.5 mM) and the zinc nanoparticles surface plasmon resonance is where the peaks were detected. The biosynthesized ZnO NPs from the *A. niger* shows the maximum absorbance reaches 0.516 in control, 0.721 in 0.5 mM, 2.756 in 1.0 mM, 0.643 in 1.5 Mm, 0.711 in 2.0 Mm, 0.620 in 2.5 Mm at the highest peak of 400 nm and *A. terreus* 0.510 in control, 0.621 in 0.5mM, 0.718 in 1.0mM, 0.611 in 1.5Mm, 0.701 in 2.0Mm and 0.716 in 2.5 Mm at the highest peak of 450nm. The concentration 1.0 mM are highest peak in all wavelength at absorbance process were analyzed. The spectra also clearly show the increase in zinc solution intensity with time, which is a sign that there are increasingly ZnO NPs forming in the solution. The highest values were recorded in *A. niger* is synthesized zinc nanoparticles from potential fungi **(Figure 7 and 8)** So, the further analysis will be compared with the control and higher concentration obtained previously.

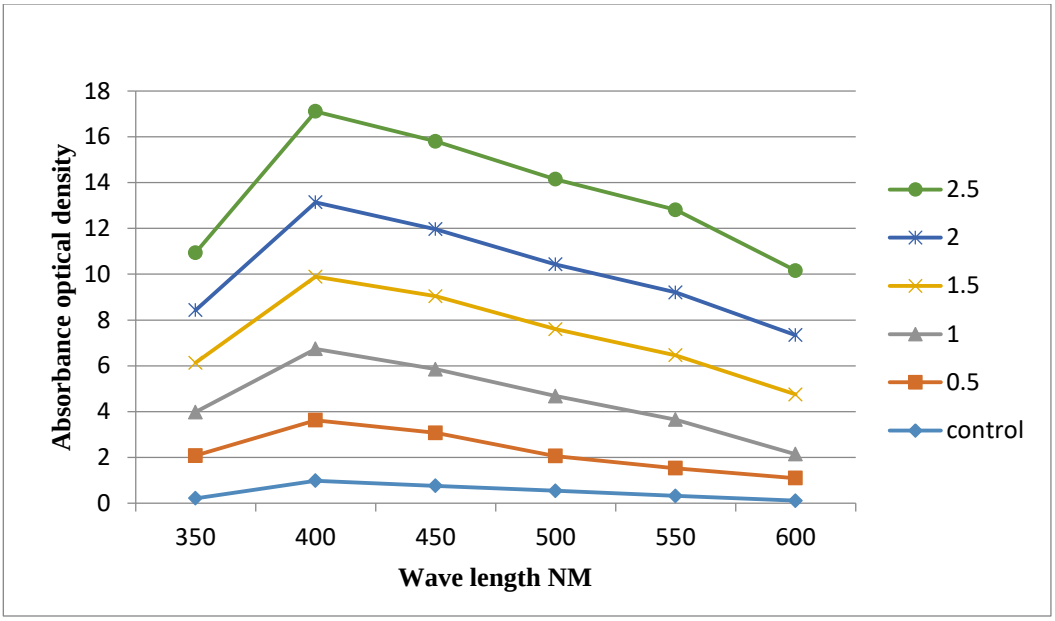

Figure 7: UV – Vis spectra of zinc nanoparticles (ZnO NPs) synthesized using *A.niger*

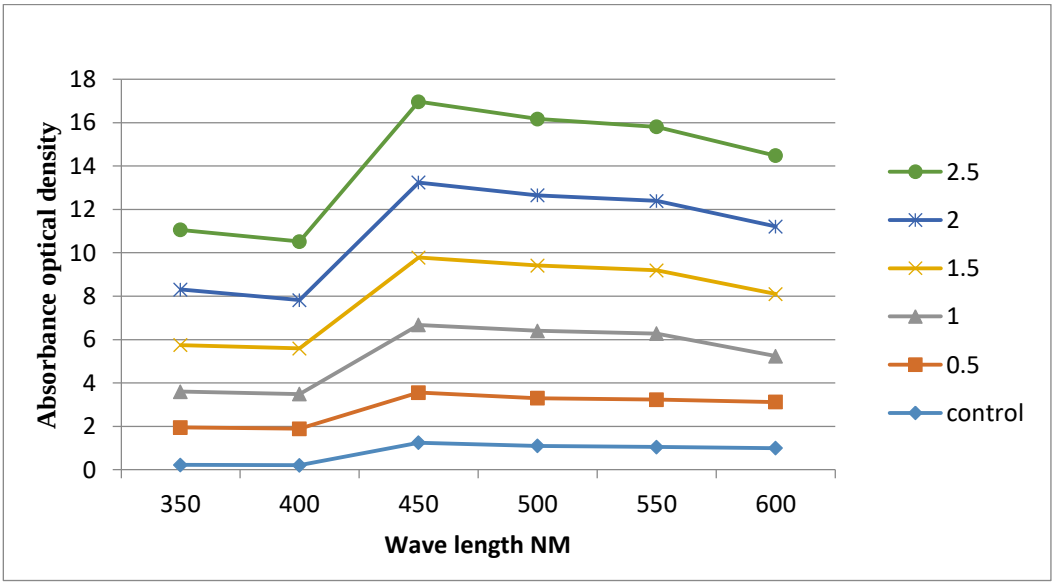

Figure 8: UV – Vis spectra of zinc nanoparticles (ZnO NPs) synthesized using *A.terreus*

CONCLUSION

The present study focused on optimization and production of protease and lipase enzyme in *A.niger*, *A. terreus*. Production of Protease by *A. niger* was optimized at pH 7, temperature of 30 °C and sucrose as carbon sources, were excellent production for enzyme, The nitrogen source like ammonium nitrate were recorded the incubation period of 38 hrs and 80 mg/g of iron sources. It concluded that possible to treat infectious diseases caused by pathogenic microorganisms using zinc nanoparticles (ZnO NPs) could find applications in administering drugs, and their application has been extended to include cancer diagnosis and treatment. This study provides the cost effective technology for enzymes protease using preparation for orthopaedic band aid cloth.

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