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Production and Characterisation of Peroxidase from *Aspergillus terrens* Isolated from Water Sample in Wukari, Taraba State, Nigeria

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Abstract

The use of biocatalysts like enzymes in the development of environmentally sustainable processes has been reported as an ecofriendly approach for the current bioeconomy. This research aimed at producing and characterizing peroxidase from *Aspergillus terrens* isolated from water sample in Wukari, Taraba State, Nigeria. Fungi species from surface water sample obtained in Federal University Wukari, Taraba Nigeria were isolated through serial dilution of peptone water and was cultured in a room temperature in different petri dishes using Potato Dextrose Agar (PDA). A portion of the fungi growth chosen from the best growth of cultured water sample were cut and transferred into the 250 Erlenmeyer flask each containing an acceptable proportion of phosphate buffer, pH 6.0. The screening for peroxidase production was aseptically performed on a plate by inoculating the fermented medium with azur B dye. The appearance of a clear zone indicated a positive result for peroxidase activity. The incubation of the peroxidase enzyme was conducted over eleven days, with the highest enzyme yield observed on the eighth day. The results obtained in this study showed that the effect of pH on peroxidase activity was studied, revealing that the optimum pH for enzyme activity was 7.5. Peroxidase activity was lowest at pH 1. Temperature variation studies showed that the optimum temperature for peroxidase activity was 30°C. Peroxidase activity changed as substrate concentration was varied. Peroxidase activity was revealed to be highest at substrate concentration 1.9 mM. The kinetic parameters for peroxidase were investigated to be: $V_{max} = 10.57082$; $K_m = 0.244186$. The evidence presented in this research has shown that peroxidase can be produced in mass by using of white rot fungi from/around our surroundings and can be explored as a cheap source of peroxidase for industrial and biotechnological purposes. In conclusion, producing peroxidase from local isolates presents an eco-friendly and affordable alternative for industrial applications, contributing to environmental sustainability and public health.

Keywords: *Aspergillus terrens,* Peroxidase, Water, Fermentation, Enzyme

INTRODUCTION

Development of environmentally sustainable processes is a challengeable task for the current bioeconomy. In this direction, the use of biocatalysts, enzymes, in various processes is considered as an ecofriendly approach (Timothy *et al.,* 2022). The stability, activity and specificity of enzymes are the fundamental parameters that are required to develop enzymes for their optimal applications in various industrial processes (Pandey *et al.,* 2017). The implementation of alternative solutions to reduce the quantity of waste discharged into the environment has become a necessity. In this context, enzymatic transformation aroused a growing interest in the design of innovative processes known as "eco-process", offered an interesting option to succeed chemical catalysts and switch to biological catalysts capable to convert very complex molecules under moderate conditions

(Shakerian *et al.,* 2020, Stadlmair *et al.*, 2018). Peroxidases embody and assume this role of biocatalyst exploited for environmental (Debing *et al.,* 2006).

Fungi like *Aspergillus niger, Fusarium culmorum,* and P*enicillium* are able to produce a great deal of enzymes and metabolites through solid-state fermentation (SSF). Fungi are the source of about 60% of the enzymes sold in stores (Yohanna *et al.,* 2023). Additionally, yeasts work well with SSF. Even in low-water environments, filamentous fungi and yeasts can thrive. Enzyme production by SSF has also been utilized by several bacterial species, including *Bacillus thuringiensis*, *Pseudomonas*sp., and *Bacillus subtilis (*Umaru *et al.,* 2024). Microbes are frequently utilized to create proteases because of their versatility, simplicity of genetic modification, and fast development rate (Timothy *et al.,* 2022). Proteases are typically produced by fungal species such as *Aspergillus, Penicillium*, and Rhizopus because they are harmless. Heat-stable proteases, produced by certain microbes (Martins *et al.,* 2024), are enzymes that remain active at elevated temperatures. Enzymes may be able to perform their biological function at greater temperatures or be stabilized by the presence of certain metal ions.

Peroxidases (EC 1.11.1.7) are heme proteins and contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as their prosthetic group. They have a molecular weight ranging from 30,000 to 150,000 Da (Fahmy *et al.,* 2008). They are a group of oxidoreductases that catalyse the reduction of peroxides, such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds (Pandey *et al.,* 2017). Peroxidases have been classified into two, on the basis of presence or absence of heme (i.e. heme and non-heme peroxidases) (Pandey *et al.,* 2017). This group of enzymes are versatile, widely distributed in nature and they can be found in bacteria, fungi, algae, plants and animals and useful biological tools. A report from PeroxiBase database captured that > 80% of known peroxidases are heme-containing while the remaining 20% code for non-heme peroxidases (Jayani *et al.,* 2005) such as thiol-peroxidase, alkylhydroperoxidase, and NADH-peroxidase, constituting only a small proportion (Yohanna *et al.,* 2023). Two superfamilies of heme peroxidases are peroxidasecyclooxygenase superfamily (PCOXS) and the peroxidasecatalase superfamily (PCATS) (Falade *et al.,* 2017). The peroxidases of PCOXS superfamily exclusively contain animal peroxidases which have been suggested to be involved in the innate immunity, defense responses etc. (Dick *et al.,* 2008). The PCATS is the most intensively studied superfamily of non-animal heme peroxidases (Pandey *et al.,* 2017).

Peroxidases have been reported to have wide industrial and biotechnological applications (Falade*, et al.* ,2017). A number of industrial applications of peroxidases have been reported in the area of agriculture, analytical, environmental, medical sectors etc. Peroxidases have been used in bioremediation of contaminating environmental pollutants such as phenols, delignification in paper and pulp industry, diagnosis kit development, immunoassay, organic and polymer synthesis as well as in and biosensor technology. They are also used for developing convenient and quick methods for the determination and quantification of hydrogen peroxide in both the biological and industrial samples (Adams, 1997). Additional applications of peroxidases include determination of extent of lipid peroxidation in meat food products, in polymerization and precipitation of aqueous phenols as well as in de-colorization of industrial effluents (Biz *et al.,* 2014). Little or no work has been done on production of peroxidase using *Aspergillus terrens* isolated from water samples This research therefore, underscores the feasibility of harnessing local microbial resources for biotechnological advancements.

MATERIALS AND METHODS

Study Area

The present study was carried out in Federal University Wukari Central Research Laboratory, Wukari, Taraba state, Nigeria, from the period of December 2023 to March 2024.

Materials and Chemical Reagents

All chemicals used in the study were of analytical grade and were obtained directly from a local commercial vendor in Makurdi, Benue State, Nigeria. These chemicals include potato dextrose agar, potato dextrose broth, Czapek Dox agar, Glucose, peptone, Yeast extract, H_2PO_4, Na_2HPO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, ammonium sulfate, sodium malonate buffer (pH-4.5), NaCl, trichloroacetic acid, Folin Ciocalteu's phenol reagent, sodium carbonate, ethanol, sodium acetate buffer. Materials, apparatuses and equipment used in this study were Water sample, conical flasks, 250 ml beakers, petri dish, cotton wool, syringe, aluminum foil paper, distilled water, pressure pot, spatula, scissors, 250 Erlenmeyer flask, test tube rack, test tubes, weighing balance, muslin bag, freezer, autoclave, spectrophotometer, water bath, among others.

Isolation/Culture of Fungi

Fungi species from surface water sample obtained in Federal University Wukari, Taraba Nigeria were isolated through serial dilution of peptone water and was cultured in a room temperature in different petri dishes using Potato Dextrose Agar (PDA). Fungi species were isolated after noting the highest growth the sample which was proceeded for screening to confirm the presence of ligninolytic enzyme.

Screening Assay for Ligninolytic Enzyme

A portion of the fungi growth chosen from the best growth of cultured water sample were cut and transferred into the 250 Erlenmeyer flask each containing an acceptable proportion of phosphate buffer, pH 6.0. Potato Dextrose Agar (PDA) (Fluka) and Yeast Extract Agar (YEA) were used to isolate fungi. Guaiacol and syringaldazine and the production of ligninolytic enzymes was observed as a colorless halo around fungi growth (Khatri *et al.,* 2015).

Solid State Fermentation (SSF)

Solid-state fermentation was carried out in 250ml conical flask containing 10g of substrates with 10 ml of salt solution (g/l), KNO_3 2.0, $MgSO_4$.$7H_2O$ 0.5, K_2HPO_4 1.0, $ZnSO_4$.$7H_2O$ 0.437, $FeSO_4.7H_2O$ 1.116, $MnSO_4$.$7H_2O$ 0.203, pH 7.0 and the mixture was autoclaved at 121^oC for 20 min. After sterilization, the flasks were inoculated with 1.0 ml of spore solution (106 spores/ ml) and incubated at 30^oC for seven days in an incubator. At the end of fermentation, cultures were extracted with 100ml of distilled water by shaking for 2 hours. The filtrate obtained was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was used as crude enzyme extract and little quantity was taken for enzyme activity and determination of protein content.

Ammonium Sulphate Precipitation

To ensure thorough precipitation, an ammonium sulphate solution was used for the partial purification using the culture supernatant and equilibrated at 4 ^0C for 30 min. Ammonium sulfates with different concentrations (35, 40, 45, 50, 55, 60, 65, 70, 75 and 80%) were used. Ammonium sulfate was used for salting out process as it can be fully dissolved into water resulting in high ionic power as described by Garg et al. (2016). At high ionic concentration, added salt can remove hydration water from proteins resulting in protein coagulation. The concentration at which peroxidase assay revealed minimum absorbance was selected as an optimum concentration because of maximum protein coagulation at that concentration. The precipitates were extracted by centrifugation at 10,000g for 10 min at 4 ^{0}C, then re-dissolved in 2.5 ml sodium acetate buffer (25 mM, pH 5.5), and the protein content and enzyme activity were measured at each step. The active fractions were precipitated at a saturation of 80% ammonium sulphate.

Culture Conditions for Ligninolytic Enzyme Production

With slight adjustment, isolated fungal strains were maintained on 2% (w/v) MEA or PDA slants at 4ºC and the fungi were activated at 26ºC. The mycelium was harvested with sterile 0.9% NaCl solution and then inoculated into 100 ml 2% malt extract broth or potato dextrose broth in 250 ml Erlenmeyer flasks at 26ºC. In both stages, incubation time depends on the fungal strains. After the growth period, pellets were inoculated into 250 ml Erlenmeyer flasks containing medium and incubated at 26ºC. In order to optimize enzyme production, 3 different media and were used. Each media was centrifuged after incubation without agitation and the supernatant of the culture was used for enzymatic assays. All enzymatic analysis was carried out in duplicate (Yohanna *et al.,* 2023). The media were composed of the following;

M1(Media one): 10 g/l glucose, 0.025 g/l yeast extract, 0.05 g/l $MgSO_4.7H_2O$, 1.0 g/l $NH_4H_2PO_4$ and adjusted at pH 4.5.

M2 (Media two): 10 g/l glucose, 5.0 g/l yeast extract, 0.22 g/l ammonium tartarate, 2 g/l KH_2PO_4, 0.05 g/l $MgSO_4.7H_2O$, 0.1 g/l $CaCl_2.2H_2O$, 0.5 g/l KCl, 0.2 g/l Thiamin, 10 ml/l trace element solution and adjusted at pH 4.5.

M3 (Media three): 10 g/l glucose, 0.22 g/l ammonium tartarate, 0.2 g/l KH_2PO_4, 0.05 g/l $MgSO_4.7H_2O$, 0.01 g/l $CaCl_2.2H_2O$, 0.001 g/l Thiamin, 10 ml/l trace element solution, 10 ml/l 10% Tween solution, 1.5 mM veratryl alcohol (VA) and adjusted at pH 4.5

Peroxidase Activity Assay

Peroxidase assay was done in triplicate using the method described by Chowdhury et al. (2017) with some modifications. The change in absorbance at 470nm due to the activation of guaiacol to tetraguaiacol in the presence of H_2O_2 and the enzyme extract at 28°C was monitored using Spectrophotometer after every 30 seconds for 3min. The standard assay solution contained 2.7 ml sodium acetate buffer, pH 5.5, 0.1 ml of 0.2% guaiacol and 0.1ml of suitably diluted enzyme extract in a total of 3ml. The absorbance was read at 470 nm using Spectrophotometer after 30 seconds for 3 min. A unit enzyme activity was defined as the amount of enzyme that gives an absorbance change of 0.1AU/min at 28°C.

Protein Determination

For the determination of protein, 0.2 ml of BSA working standard was poured in 5 test tubes and made up to 1ml using distilled water. The test tube with 1 ml of distilled water served as blank. 4.5 ml of reagent I was added and the mixture was incubated for 10 minutes. After incubation, 0.5 ml of reagent II was then added and incubated for 30 minutes. The absorbance was measured at 660 nm and a standard graph was plotted. The amount of protein present in the given sample was then estimated from the standard graph.

Peroxidase Characterization

Effect of pH on Peroxidase Activity

The effect of pH on Peroxidase activity was determined at 25°C using sodium acetate buffer (0.1M) with a pH range of 4.0-5.5, Sodium phosphate buffer (0.1M) with pH of range from 6.0-7.5, and Tris- HCL buffer (0.1M) with a pH range 8.0-9.0. Tubes containing 2.7ml of the respective buffers, 0.1ml of guiuacol, 0.1ml of H_2O_2 and 0.1ml of the enzyme were mixed. All the tubes were further incubated at 30°C for 10 minutes, after which the activity of the enzyme was assayed. The residual activity was then determined using guaiacol as the substrate for the assay method.

Effect of Temperature on Peroxidase Activity

Determination of the optimum temperature for peroxidase activity was carried out using 0.1M of sodium acetate buffer over temperatures ranging from 25°C to 50°C at 5°C intervals, taking 1ml of the crude enzyme into a test tube in a water bath and the temperature at which the enzyme expressed maximum activity was taken.

Effect of Substrate Concentration on Peroxidase Activity

The following substrate concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8% were used to determine the effect on the activity of Peroxidase using guiuacol.

Kinetic Parameter Determination

The kinetic parameters (K_m and V_{max}) of peroxidase was determined by the double reciprocal plot. The concentration varied from 1 to 8mg/ml and the initial reaction velocities were used.

RESULTS

Peroxidase Screening Assay

The screening for peroxidase production was aseptically performed on a plate by inoculating the fermented medium with azur B dye. The appearance of a clear zone indicated a positive result for peroxidase activity, as shown in Figure 1 and 2.

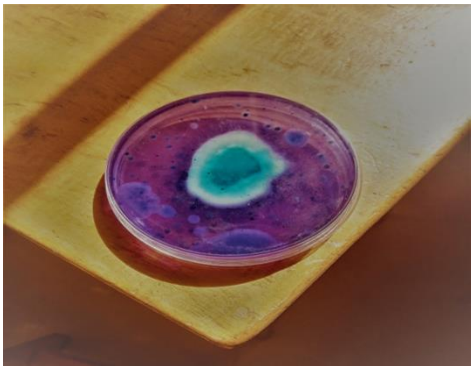

Figure 1. Screened plate showing the clear zone area for peroxidase activity.

Figure 2. A picture of *Aspergillus terrens* on a screened plate

Peroxidase Production under Solid State Fermentation (SSF)

The incubation of the peroxidase enzyme was conducted over eleven days, with the highest enzyme yield observed on the eighth day, as depicted in Figure 3. Peroxidase production was monitored over twelve days of fermentation. Maximum peroxidase and protein production from *Aspergillus terrens* occurred on the eighth day, which was then utilized for mass production.

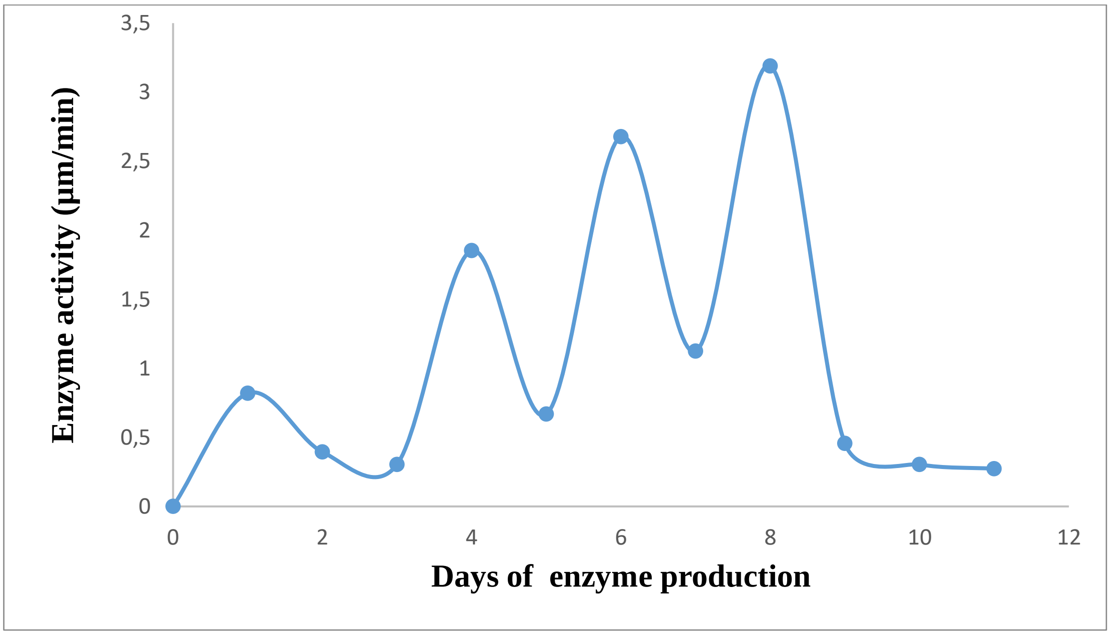

Figure 3. Production of peroxidase under Solid State Fermentation (SSF)

Protein Determination

The amount of protein present in the given sample was estimated from the standard graph presented below (Figure 4). Peroxidase activity was found to be highest on day 8.

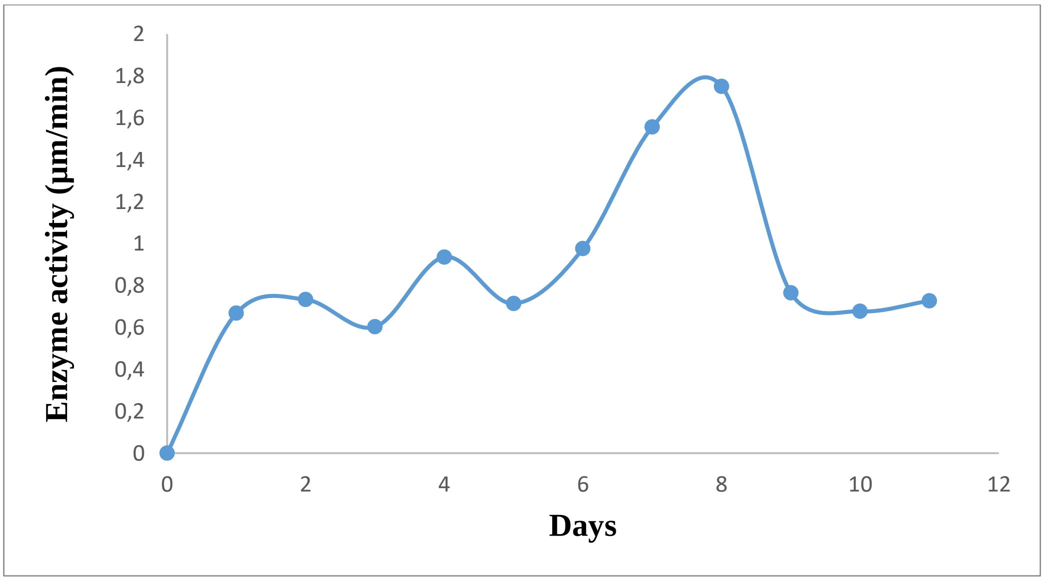

Figure 4. Protein determination plot

Effect of pH Change on Peroxidase Activity

The effect of pH on peroxidase activity was studied, revealing that the optimum pH for enzyme activity was 7.5. Peroxidase activity was lowest at pH 1. There was increase in the activity of the enzyme as the pH increased from 1 to 4. Upon peaking at pH 7.5 with an enzyme activity of 3.5 µm/min, there was sharp decline in the activity of peroxidase as the pH increased from 7.6 to 9 (Figure 5).

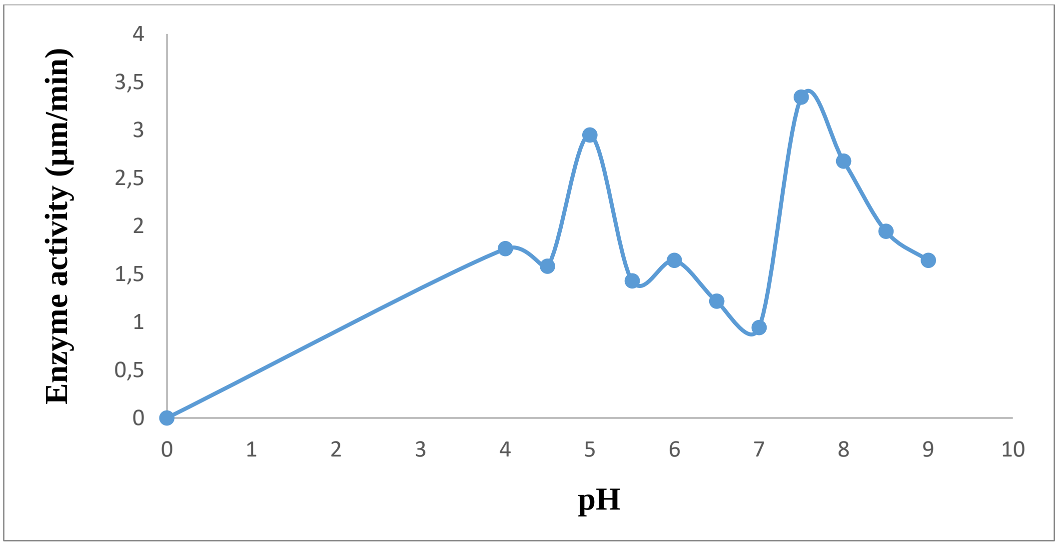

Figure 5. Effect of pH change on peroxidase activity

Effect of Temperature Change on Peroxidase Activity

Temperature variation studies showed that the optimum temperature for peroxidase activity was 30°C. As temperature increased from 1°C to 20°C, peroxidase activity also increased. However, from 38°C, there was a sharp decrease in peroxidase activity, such that at 40°C, peroxidase activity was reduced to 0.5 µm/min. Again, peroxidase activity began to increase beyond 40°C and peaked at 50°C, giving an activity of 5.5 µm/min (Figure 6).

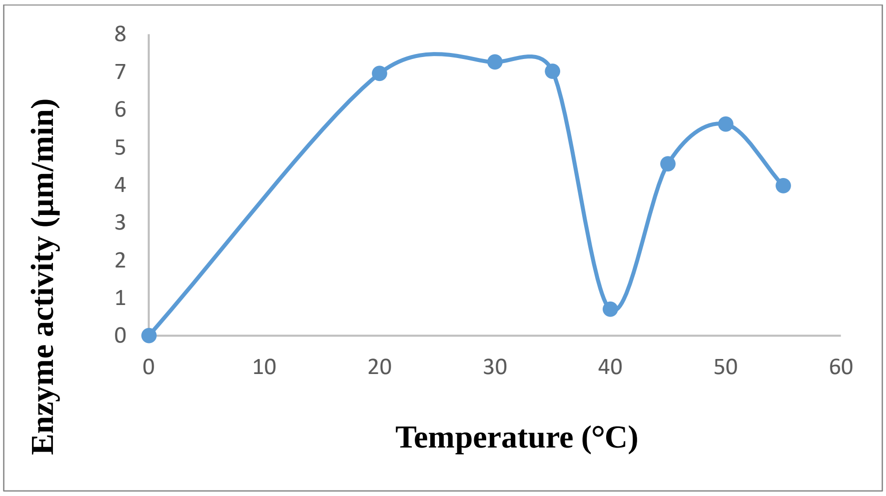

Figure 6. Effect of temperature change on peroxidase activity

Effect of Substrate Concentration on Peroxidase Activity

Peroxidase activity changed as substrate concentration was varied. Peroxidase activity was revealed to be highest at substrate concentration 1.9 mM as presented in Figure 7. Peroxidase activity was seen to increase as substrate concentration increase. However, at 0.3 mM, there was a sharp decline in peroxidase activity which increased subsequently as substrate concentration increased beyond 0.5mM (Figure 7).

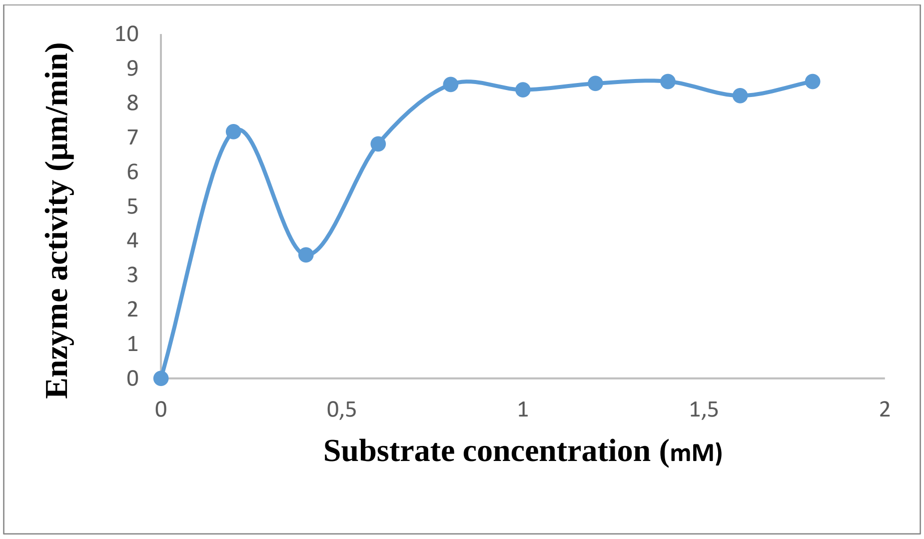

Figure 7. Effect of substrate concentration on peroxidase activity

Determination of Kinetic Parameters

In this study, the kinetic parameters of peroxidase were investigated. The kinetic data and reciprocal plot (Lineweaver-Burk plot) for peroxidase is presented below (Figure 8). Substrate concentration effects were analyzed to determine the enzyme kinetics. The kinetic parameters were found to be: $V_{max} = 10.57082$; $K_m = 0.244186$.

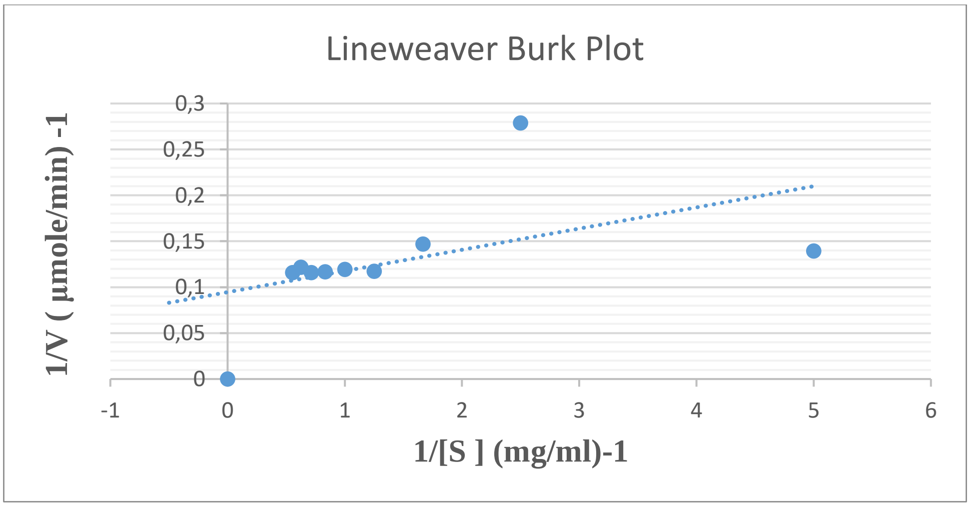

Figure 8. Lineweaver-Burk plot used for determination of peroxidase kinetic parameters.

$V_{max} = 10.57082$; $K_m = 0.244186$.

DISCUSSION

Peroxidases are a group of oxidoreductases that catalyse the reduction of peroxides, such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds. Development of environmentally sustainable processes is a challengeable task for the current bioeconomy.

In the present study, enzyme production was gradually increased with the passage of time and highest enzyme activity was obtained on 8th day of incubation. It was also observed that prolonged incubation decreased the enzyme activity. However, the growth of the microorganism was not significantly affected (Yohanna *et al.,* 2023).

Peroxidase activity was highest at pH 7.5, showing that the enzyme is more active in an alkaline medium (Figure 5). This result is contrary to the report by Yu and Xu (2018) that showed maximum enzyme activity at pH 5.0. However, it is somewhat in tandem with the findings of Yohanna et al. (2023) who observed that the optimum pH for protease was in a different study was 8.0. This disparity may be as a result of change in shape and charge properties of the enzyme and substrate. pH change causes alteration of the ionization state of amino acids residue in a protein which leads to alteration of the ionic bonds which determine the tertiary structure and charge properties of the protein. This ultimately results in enzyme inactivation or altered substrate recognition.

Temperature is known to have great effect on protease activity as it may reduce, give stable activity or express maximum activity of the enzyme (Yohanna *et al.,* 2023). The effect of temperature on peroxidase activity in this study was assayed by subjecting the enzyme to different temperature values. According to the result presented in Figure 6, peroxidase activity at temperature 30^0C was maximum. However, there was a sharp decrease in protease activity at 40^0C. This may be as a result of enzyme denaturation. This result is tandem with the findings of Yandri et al. (2008) who worked on a different enzyme however, and reported that the optimum temperature of protease isolated from *Enterococcus faecalis* was 30^0C. Enzymes are known to be sensitive to temperature changes, and the change in activity of any particular enzyme to such conditions is a distinguishing characteristic of such enzyme (Yohanna *et al.,* 2023).

Protease showed maximum activity at 1.9mM substrate concentration and began to decrease at 0.3mM substrate concentration (Figure 7). This result is not in tandem with the findings of Gupta et al. (2002) who reported that protease from *Lactobacillus acidophilus* had maximum activity at 2 casein concentration. Matta et al. (1994) obtained a slightly lesser value of 1.2mM for protease produced by *Bacillus subtilis* in their study on the effect of casein concentration on protease activity by the bacterium.

The kinetic parameters (K_m and V_{max}) of peroxidase were calculated by taking the double reciprocal of the plot. K_m and V_{max} of peroxidase obtained were 10.57082 and 0.244186 respectively (Figure 8). Yu and Xu (2018) reported a K_m of 1.0 mg/mL and a V_{max} of 85 U/mg protein for pectinase isolated from *P. chrysogenum*. Siddiqui et al. (2012) reported a K_m value of 0.22 mg/mL for polygalacturonase from *Rhizomucor pusillus* isolated from decomposing orange peels. K_m values less than 0.15 and up to 5.0 mg/mL (<0.15-5.0 mg/mL) and specific activities 8.8-7000 U/mg were reported for some fungal pectinases by Sharma and Giridhar (2011). The findings by these researchers differ from the values obtained for k_m and V_{max} in this study.

CONCLUSION

The evidence presented in this research has shown that peroxidase can be produced in mass by using of white rot fungi from/around our surroundings and can be explored as a cheap source of peroxidase for industrial and biotechnological purposes.

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