Rafidain Journal of Science
https://rsci.mosuljournals.com
Vol. 33, No. 1, pp. 49-67, 2024

Studies on Kinetic Properties of *Aspergillus* Producing Peroxidase from Petroleum Hydrocarbon Spilled Soil

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p-ISSN: 1608-9391
e -ISSN: 2664-2786

Article information

Received: 25 /10/2023
Revised:15/1/2024
Accepted: 25/1/2024

DOI: 10.33899/rjs.2024.182828

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ABSTRACT

Peroxidases production was carried out from *Aspergillus tamari* isolated from petroleum hydrocarbon spilled soil. Physicochemical properties of the respective soil showed pH of 4.45 and 6.5 for soils from point 1 and II respectively and higher conductivity of 613 and 1013 (Ω^{-1} $Cm^{-1)}$, respectively when compared with the control sample. Dissolved mineral of Cl^-, SO_4, K, Ca, Mg in the respective soil samples from the petroleum spilled sites were significantly high when compared with the control experiment except for soil sample I which showed a relative low phosphate concentration of 1.23 mg/g in the presence of the control experiment, respectively. TOC and TOM contents were 87.91, 119.04; 108.13 and 146.42 mg/g for soil sample I, and III, respectively. In all the tested parameters, the experimented soils were significantly high than the control soil sample. Molecular tests (18s rDNA.) was used to identify the pure isolate of fungus as *Aspergillus tamarii*. Studies on effect of incubation period on the production of peroxidase from strains of *Aspergillus tamarrii* sp. showed that the highest peroxidase activity was obtained on the day 6^{th} of the fermentation time; Peroxidase activity peaked at pH 5. Protein with highest peroxidase activity was peak precipitated at 60% saturation of the salt. The gel chro-matogram showed single almost superimposed peaks of enzyme activity. Purified peroxidase activity peaked at pH 4.5. Optimum temperature for the enzyme activity was at 50 °C. Km and V max of 3.45mM and 280 µmole/min was extrapolated from the reciprocal curve of Lineweaver-burke at various concentrations of 2,6 DMP. Fe, Ca, Co and Mn selected as their notable impact in the active site of peroxidase guided the selected were assayed in the presence of the enzymes, respectively. The stability curve obtained for the peroxidases was single biphasic which represent the first order; the enzymes maintained greater than 50% of their activity after 30 min of incubation as activity progressively decreased upto to 40% after 60 min of incubation. Thermal stability of peroxidase at its optimum temperature (50) and at 70°C showed a biphasic stability curve. The enzymes maintained greater than 50% of their activity after 60 min of incubation.

Keywords: peroxidases, *Aspergillus*, proteins, catalysis, physicochemical.

INTRODUCTION

Biological catalysts foster less adiabatically the transformation of substrates into products and in thereof providing suitable physiologic condition that lower the activation energy (i.e the required minimum amount of energy) of the reaction pathway (Karigar and Rao, 2011). As stated by Opwis *et al*. (2016) generally, they are very integral as cost-efficient and environmentally sensitive biological adduct(s) for chemical processes in the industries which include: biotechnological and other alike.

Radical scavenging enzymes such as peroxidases are "house-keeping" bio molecules produced extracellularly or as part of a cell membrane by a variety of microorganisms ranging from yeast (non-filamentous fungi) bacteria and filamentous fungi with wide range of applications. Zhang *et al.* (2018) reported that peroxidases generally catalyze several chemical processes involving oxygen-transfer between hydrogen peroxide and other superoxides where they act as charge chelators and other form of substrates such as xenobiotics and phenolics through deoxygenation of O^{-}_{2} from H_2O_2.

Peroxidases are diverse in ubiquity distributed in plants, animals and microorganisms, where they shield vital cells/ organelles against the lethality of oxidative stress and resultant on destruction of the sited cells/ organelle accruing from liberation of H_2O_2 (Adewale and Adekunle, 2018). Recently, there are aroused noticeable concerns in organismal peroxidases; these interests are because of their numerous catalytic advantages including: their ability to display a high level of biocompatibility, biodegradation, biodigestibility, less technicality in preparation and stability over a wide range of physicochemical condition(s): temperature, pH and salt concentration when compared to peroxidases from other sources (Chen *et al*., 2007).

Wide range of utility, diverse in nature and reactions catalyzed by peroxidases, have over the time announce their potentiality as an integral biological catalyst for future generational biotechnological advancement in related field of bioremediation, textile synthetic dye decolorization, polymer synthesis, paper and pulp industry, in development of biosensor and diagnosis kits (Pandey *et al*., 2017).

However, microbial peroxidase catalytic efficiencies are catabolically affected by *insitu* physiologic conditions of their producing organisms; stabilization potentials of these proteins within the organisms are seen being repressed when posed to harsh environmental conditions and in the presence of ecological implicated recalcitrant (Valero, 2010). These eco-dynamic conditions have contributed to the repressed utility of the enzyme especially in bio-monitoring, remediation and other field of industrial processing.

Aspergillus, a filamentous fungus of *Zygomycete* class is a noted producer of peroxidases, they are widely distributed in the soil and at contaminated sites of refuse dumps and recently widely distributed in petroleum hydrocarbon spilled areas (Kijpornyongpan *et al*., 2022). Peroxidases are extracellular proteins in this filamentous fungus and foster the invivo stabilization of the fungus over time especially at unfavorable condition(s) (Colpa *et al*., 2014; Casciello *et al*., 2017). *Aspergillus* from petroleum hydrocarbon polluted site can serve as prolific producer of high catalytic peroxidase when seasonally assessed to extrapolate the condition at which the activity peaked. To this serve the sole purpose of the present study aiming to produce wide stable peroxidase with high catalytic efficiency for future advancement.

MATERIALS AND METHODS

Materials

The following designated establishments were the purchasing companies for the chemicals, reagents, apparatus and equipment's utilized for the present study and they include: BdH-Aldrich, Bristol, May and Baker, Merck, Pyrex and B and T trimline, Jenway.

METHODS

Experimental Duration

Sampling was conducted twice in twelve months ranging from March 2022 to April 2023.

Soil sample collection

Soil for isolation of strains of *Apsergillus* was collected from a petroleum hydrocarbon spilled site located at Agbada front terminal in River's state as described by Ezenwelu *et al*. (2022). Collections each sampling period was conducted at exactly 6'oo am at three respective points in the mark area each at distant of 9m from each other. The collected homogenized soil was pooled together in an asceptic container for laboratory analysis.

Preparation of working solutions

Buffer salts which include: Sodium acetate, sodium mono hydrogen phosphate and Tris-HCl of 0.1 M concentrations, respectively were prepared accordingly and adjusted to respective pH values using their respective conjugate acids and conjugate base.

Determination of physicochemical properties of the respective soil

Categorically, soils sampled at the various periods of the sampling duration were classified A and B representing wet and dry seasons respectively. Physicochemical properties of the respective soils were carried out as stated in the compendium of ATSDR (2010). Properties such as: pH, conductivity, mineral ions, total oxidizable carbons and organic matter were determined.

Microbial isolations

Fungi isolation from the respective soil samples were carried out experimentally as described by Ezeonu *et al*. (2013) using basic culture dependent technique. Serially diluted soil samples were inoculated on prepared Potato Dextrose Agar media. Grown cultures of the media (after three days) were observed under the microscope (X40). Basic biochemical techniques were used to confirm the filamentous cells as *Aspergillus* sp.

Emulsification Properties of the Identified Isolate

Strains of *Aspergillus* isolated from the petroleum spilled site were evaluated in the presence of different oils to determine their emulsification properties. Various screening tests including: Oil spreading and emulsification capacity index were carried out as described by Morikawa *et al.* (2000) and Cooper and Goldenberg (1987), respectively.

$$\text{E24} = Height\ of\ emulsion \div total\ height\ \times 100$$

Evaluation of Identified *Aspergillus sp* for Exo Secretion of Peroxidase

Identified *Aspergillus* sp. was subjected to evaluation for peroxidase production capability; this was carried out in a nutrient broth supplemented for phenolic substrate (2, 6 DMP) as described by Arora *et al*. (2010). Fungal cells were aseptically inoculated into the broth using corn borer of 2mm in diameter. The inoculated broth was incubated at 37°C for 3 days.

Molecular Identification of *Aspergillus* sp.

Genomic DNA (gDNA) from the internal transcribed spacer regions of the *Aspergillus* sp. were obtained respectively using the QIA amp DNA Mini Kit. The 18S rDNA gene was amplified by RT-PCR (the conditions for the amplification stated below) using the forward (5'-GGTTTGATCATGGTCAG-3') and reverse (5'-AGTTACCTTGTTACGACT-3') primers. The amplified DNA sequence was compared to the Gen Bank database of National Center for Biotechnology Information (NCBI) using the BLAST program (Kumar *et al*., 2016).

Table 1: Conditions for Amplification of the Bacteria Genome using RT- PCR

Treatment	Temperature (°C)	Time (Min)
Pre-denaturation	90	7
Denaturation	94	1
Annealing	52	1
Elongation	71	7
Final elongation	72	7

Determination of Peroxidase Activity

Hydroxyl (OH^-) radical scavenging activity of peroxidase was monitored during the assay process. This was carried out as described by De jong *et al*. (1992) using 2,6 di methoxy phenol (DMP) at pH 5.0 as the substrate in the presence of hydrogen peroxide. Spectrophotometric readings were taken for every 30 seconds for 5 minutes.

$$\text{Reaction rate} = \frac{\text{Change in absorbance (OD) at 468nm}}{\text{Time interval}}$$

$$\text{Specific activity (Unit/ml)} = \frac{\text{Reaction rate}}{\text{Protein concentration}}$$

Absorbance was taken at 468 nm using the UV-VIS spectrophotometer.

Extracellular protein determination

The total protein content of peroxidase was determined as described by Lowry *et al*. (1951); bovine serum albumin (BSA) served as the reference protein during the assay process. Assay solution prepared was incubated with phenolic reagent *folin ciocateau* after incubation. Absorbance was taken at 750 nm.

Fermentation system for peroxidase production.

Solid state fermentation technique was employed for peroxidase production from *Aspergillus* as described by Silva *et al*. (2012). Rice husks collected from milling centers were treated and utilized as the fermentation support system during the enzyme production. Each conical flasks optimized for peroxidase production contain the following: 1 % $(NH_4)_2S0_4$, 0.4 % K_2HPO_4, 1 % glucose, 0.01 % sodium acetate, 0.1% di-ammonium citrate, 0.05 % $MgSO_4.7\ H_2O$ and 0.2 % $FeSO_4.4H_2O$. The whole setups were sterilized at 121°C/ 15psi for 20 minutes using the electronic autoclave Production parameters such as: Incubation time and pH were optimized during the production process as described by Silva *et al*. (2012).

Purification of peroxidase produced from *Aspergillus* sp.

Precipitation of crude protein from the solution

Protein precipitation was from the solution was carried out using ammonium sulphate described by Allam *et al*. (2016). Precipitation was carried out using 20-90% of the solid precipitating salt at pH 5.0 at 10% intervals. Enzyme solution with the salt was incubated at cold temperature of 4^oC for 24 hrs. Peroxidase activity was assayed in both the precipitates and the supernatants simultaneously as described in the section above.

Gel Filtration of the Precipitation

Precipitated extracts were dialyzed for 12hrs using the dialysis bag; thereafter the dialysate was further purified through column chromatography using the sephadex G-100 as described by Ezenwelu *et al*. (2022).

Packed sample in the column containing the separating gel were eluted using the working buffer while samples were collected using the sample bottle. Enzyme activity was assayed as described in the section above using the UV-VIS spectrophotometer.

Enzymatic Properties of Purified Peroxidase

Effect of pH on Peroxidase Activity

Purified peroxidase optimum pH was determined as described by Eze *et al*. (2010). Peroxidase was incubated at pH 3.5-8.5 in the range of 0.5 units. Peroxidase activity was determined as stated above.

Effect of Temperature on Peroxidase Activity

Purified peroxidase optimum temperature was determined as described by Eze *et al*. (2010). Peroxidase was incubated at its optimum pH at varying temperatures of 30-80°C in the range of 10 units using the water bath. Peroxidase activity was determined as stated above.

Determination of Peroxidase Kinetic Constants

Catalytic constants (Km and Vmax) of the peroxidases produced from the respective *Aspergillus* sp. were extrapolated from the reciprocal curve of Lineweaver-burke plot at inverse concentration of the 2,6 DMP.

Effect of Stabilizing Metals on Peroxidase Activity

Activity of the peroxidases was monitored in the presence of four selected metals (Ca, Fe, Co and Mn) of double valency with 0.03M concentration. This was carried out as described by Riodan (2010).

Stability Studies of the Enzyme

Peroxidase stability at respective incubation pH and temperatures were monitored for 60 min as described by Nogale and Lopez (2005). Aliquots were drawn after 15 min and thereafter, the residual activity of the peroxidase after the respective pre-incubation time was assayed as described above.

Percentage residual activity is deducted from equation one below:

% residual activity: $100x\frac{At}{A0}$

whereas At= Activity at a given treatment time

Ao= Activity at no treatment time

Statistical Analysis

Data obtained shall be expressed as mean ± SD and tests of statistical significance will be carried out using two-way analysis of variance (ANOVA). Mean values with $p < 0.05$ i.e 95% confidence interval were considered as significant. Other statistical variables are analyzed using the Microsoft excel program.

RESULTS AND DISCUSSION

Studies on the physicochemical properties of the surrounding soils around the petroleum spilled sites as shown in table 2 showed pH of 4.45 and 6.5 for soils from point 1 and II respectively and higher conductivity of 613 and 1013 (Ω^{-1} Cm-1), respectively when compared with the control sample. Dissolved mineral of Cl^-, SO_4, K, Ca, Mg in the respective soil samples from the petroleum spilled sites were significantly high when compared with the control experiment except for soil sample I which showed a relative low phosphate concentration of 1,23 in the presence of the control experiment, respectively. TOC and TOM contents were 87.91, 119.04; 108.13 and 146.42 mg/g for soil sample I, and III, respectively. In all the tested parameters, the experimented soils were significantly high than the control soil sample (Table 2).

Table 2: Physicochemical properties of the soil sample

Physiochemical parameters	Control sample	S1	S2
pH	7.6±0.01	4.45±0.24	6.3±0.015
Soil conductivity	610±0.25	613±0.24	1033±0.52
Chloride ion (mg/g)	433±0.11	1002.21±0.43	1031.32±0.4
Phosphorous (mg/g)	1.78±0.05	2.31±0.15	2.28±0.018
Magnesium (mg/g)	6.27±0.45	13.05±0.36	09.44±0.01
Potassium (mg/g)	7.22±0.21	7.68±0.24	14.17±0.64
Calcium (mg/g)	18.23±0.41	34.55±0.55	34.76±0.53
Total organic carbon(mg/g)	10.45±0.2	87.91±0.52	119.04±0.31
Total organic matter(mg/g)	12.85±0.5	108.13±0.23	146.42±0.54

N=2 NOTE each sampled water were drawn at approx. 9m apart from the corresponding water point.

Where S1: soil sample point I from Nov to April

S2: soil sample point 2 from June to oc

Basic morphological features of the isolates observed on the culture plates as shown in Fig. (1). Organismal growth pattern was observed from the respective media whereas observable morphology was observed from the cultured organisms.

The micrograph of the bacteria suspensions under an objective magnification of x40, Micrograph of the fungi suspension in a red stained background using safranine dye showed motile, filamentous fungi with multi hyphael walls (Plate 1). Strains of *Aspergillus* and *Mucor* were seen much in abundance.

Molecular tests (18s rDNA.) was used to identify the pure isolates of *Aspergillus* Electrophoretogram of the amplified genome of *Aspergillus* using RT-PCR showed a typical base-pair of a fungal strain with 550 bp from the ladder DNA segment Fig. (2). *Aspergillus tamarrii T5* was identified after the genomic sequencing with ascribed NCBI accession number of KR149638 as shown in the evolutionary relatedness tress Fig. (3).

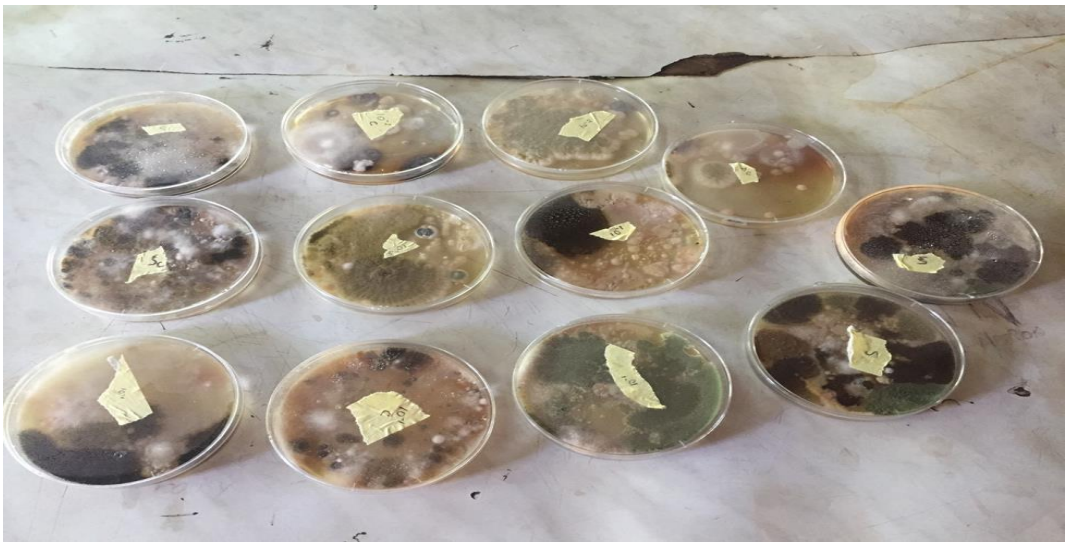

Fig. 1: Soil microbial load from the respective soil

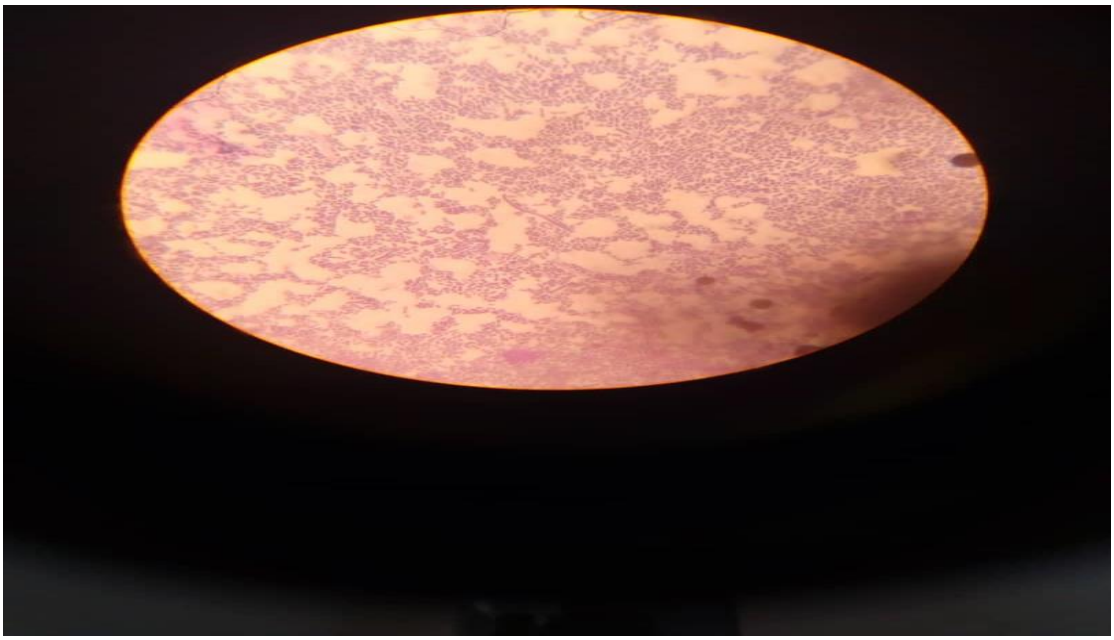

Plate 1: The micrograph of *Aspergillus tamarii* suspensions under an objective magnification of x40.

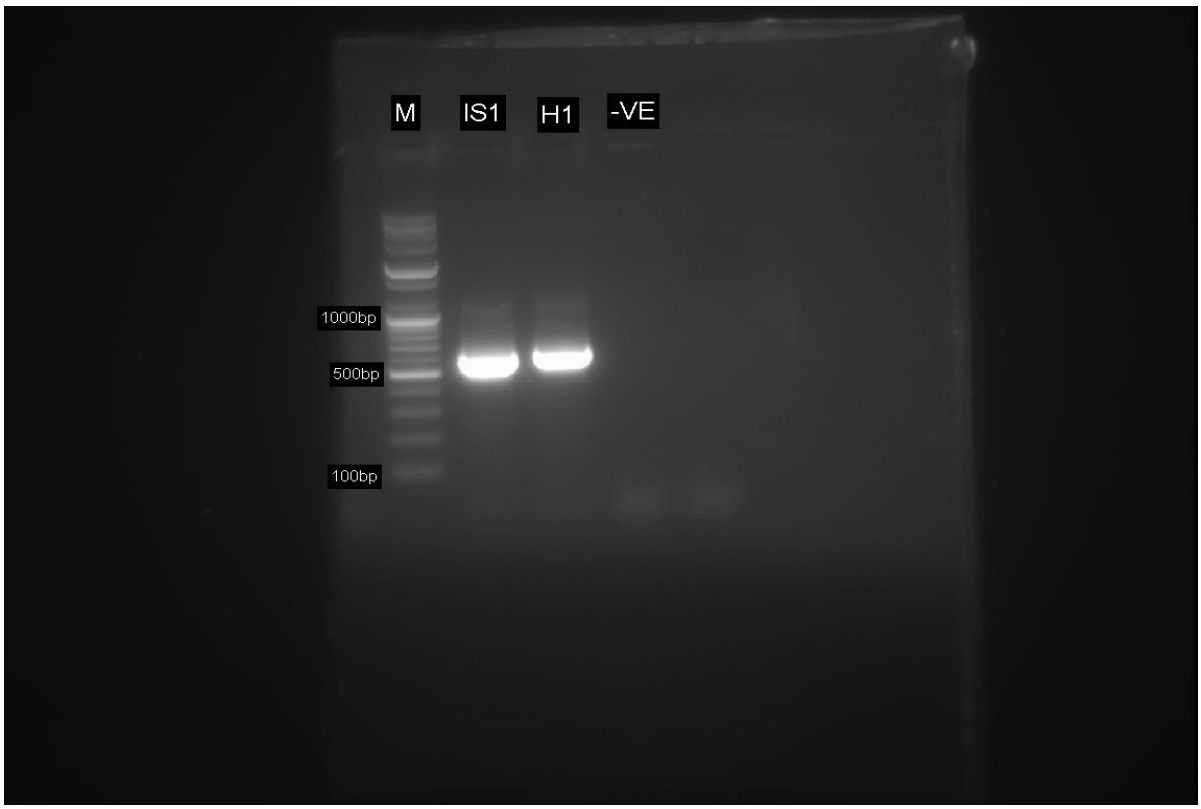

Fig. 2: Electrophoretogram of the amplified genomic DNA viewed on a UV trans-illuminator

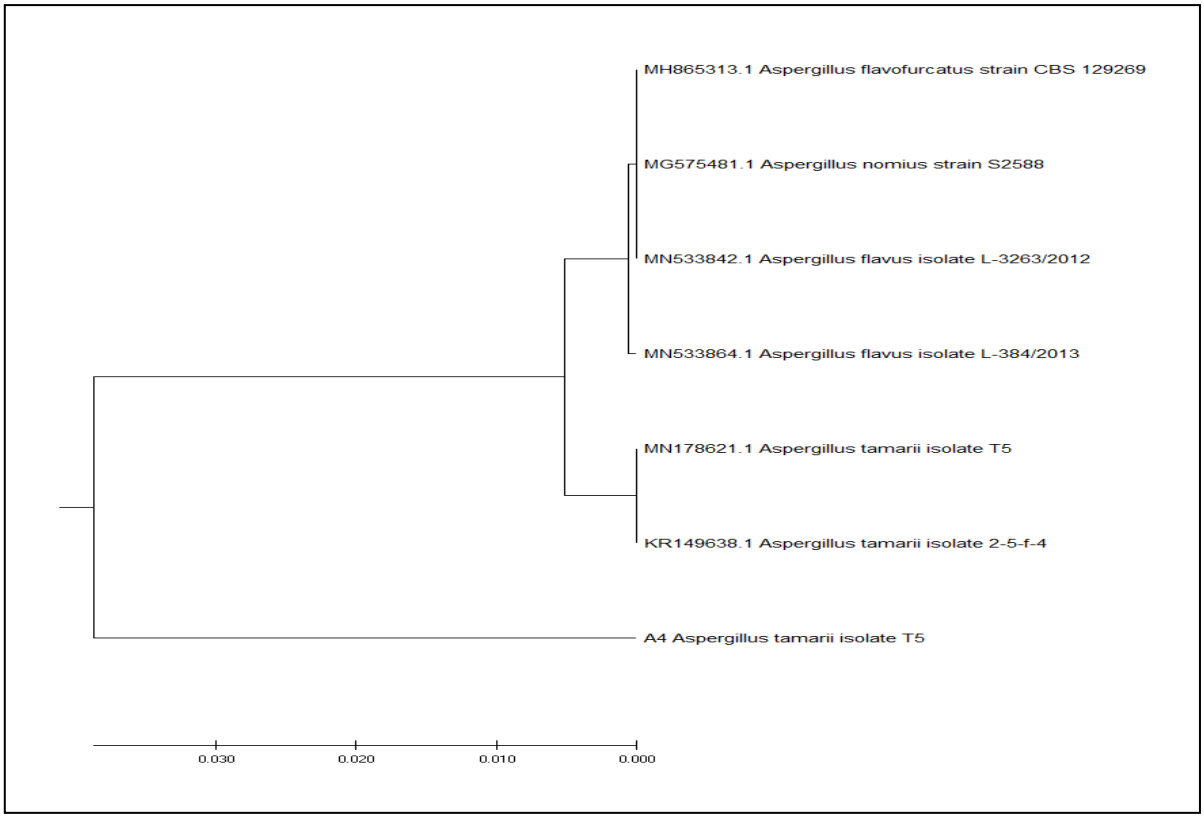

Fig. 3: Phylogenic evolutionary relatedness of strain of *Aspergillus tamarii* obtained using NCBI BLAST tools.

Studies on effect of incubation period on the production of peroxidase from strains of *Aspergillus tamarrii* sp. showed that the highest peroxidase activity was obtained on the day 6th of the fermentation time Fig. (2).

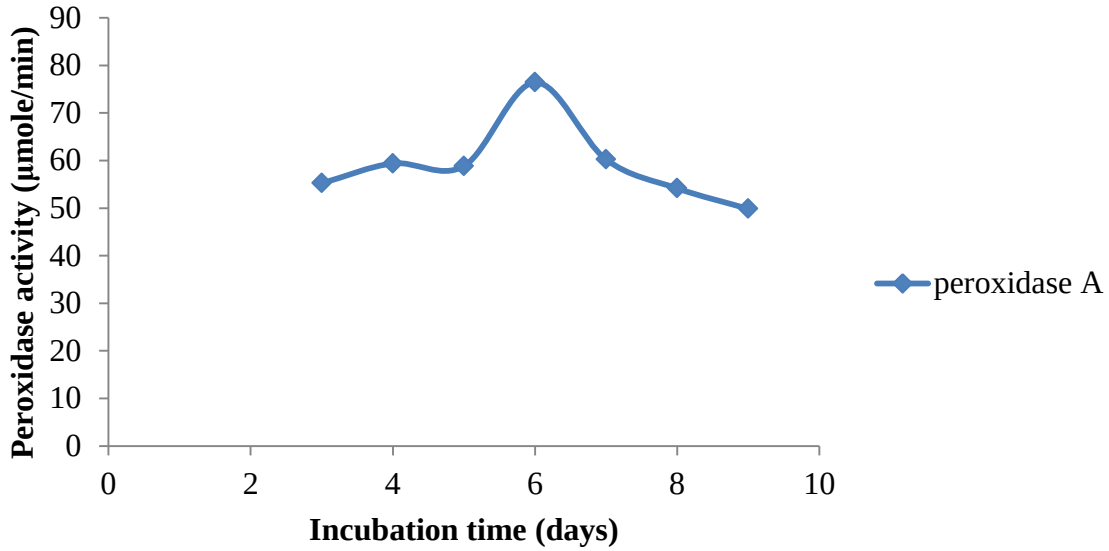

Fig. 4: Effect of incubation time on peroxidase production from *Aspergillus tamari*

Fig. (5) below shows the effect of pH on the production of peroxidase from *Aspergillus tamarii*. From the figure peroxidase activity peaked at pH 5 after 5 days of incubation.

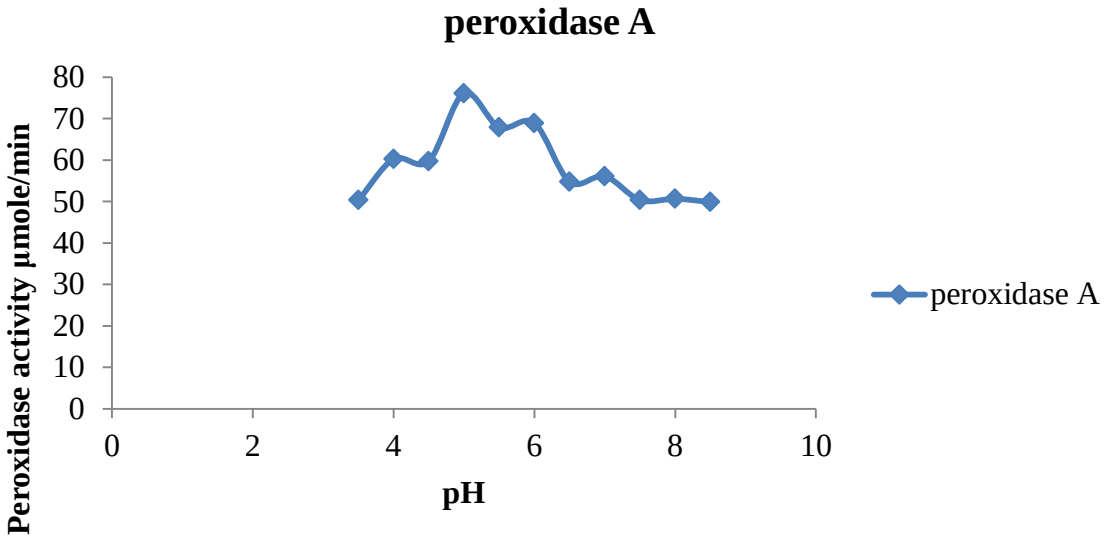

Fig. 5: Effect of pH on peroxidase production from *Aspergillu tamarii* after 5 days of fermentation incubation

Precipitation of the crude peroxidases using ammonium sulphate from Fig. (6) showed proteins with highest peroxidase activity was peak precipitated at 60% saturation of the salt.

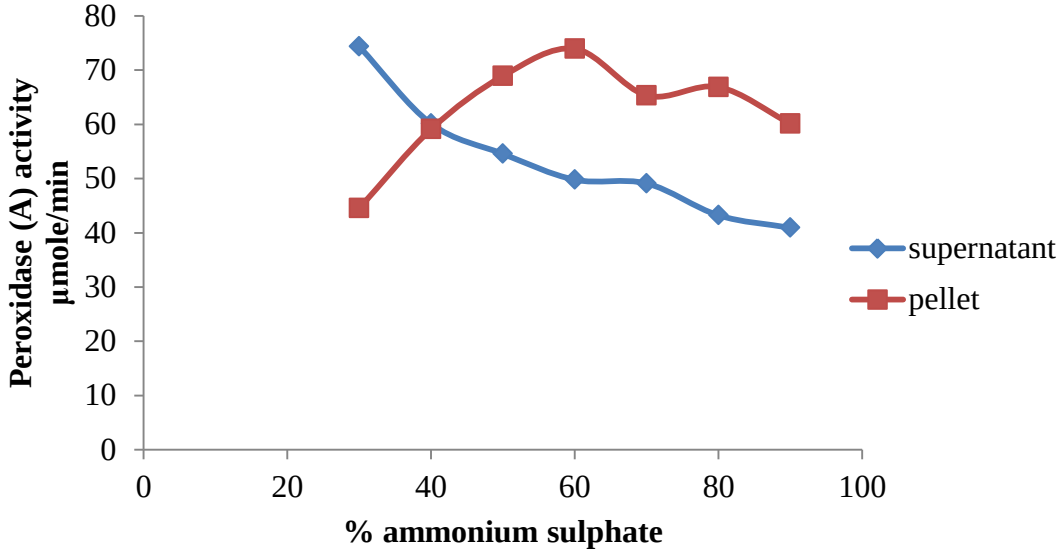

Fig. 6: Ammonium sulphate precipitation of peroxidase produced from *Aspergillus tamari* after 6 days of fermentation at pH 5.0.

Fig. (7) shows the gel chromatogram of peroxidase carried out using Sephadex G-100 at pH 6. The chromatogram showed single almost superimposed peaks of enzyme activity for peroxidase. From the table four below peroxidase A were purified up to 2.89 folds while specific activity increased up to 344.78 U/mg.

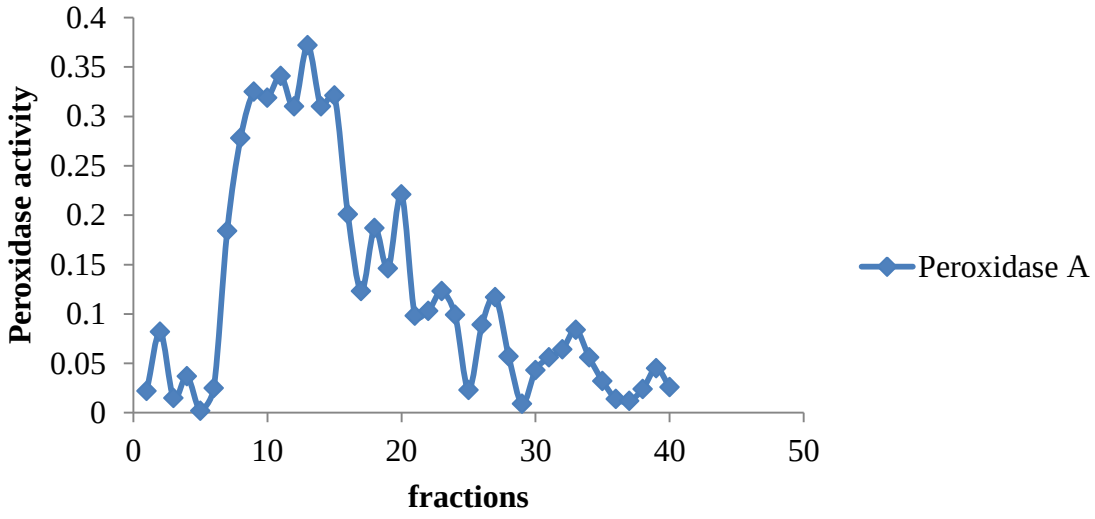

Fig. 7: Chromatogram of peroxidases using sephadex G-100 with eluting solvent of pH 6.

Table 2: Purification table peroxidase from *Aspergillus tamarii*

	Volume (ml)		Total protein		Total activity (U/ml)		Specific activity (U/mg)		Purification folds		Percentage yield	
Crude peroxidase	1000	1000	590		76500		129.66		1		100	
$NH_4(SO_4)_2$	250	250	77.5		15125		195.20		1.51		19.76	
Dialysis	100	100	20.1		6239		211.3		1.94		6.71	
Gel filtration	50	50	5.41		3076		344.78		2.89		3.1	

Kinetic properties of the peroxidases from strains of *Aspergillus tamari* showed the following enzymatic properties: Peroxidase activity peaked at pH 4.5. Optimum temperature for the enzyme activity was at 50°C respectively. Km and V max of 3.45mM and 280 µmole/min were extrapolated from the reciprocal curve of Line weaver-burke at various concentrations of 2,6 DMP for peroxidase.

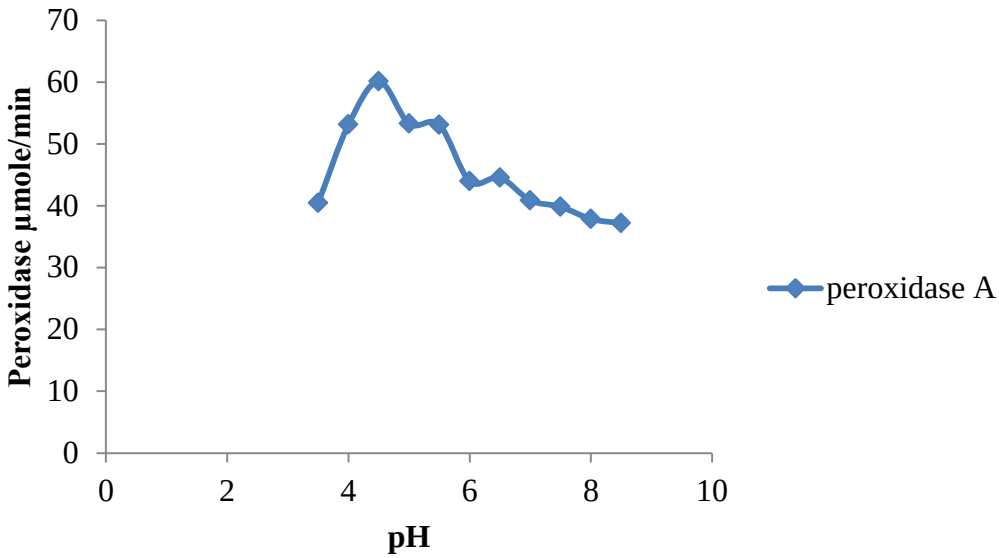

Fig. 8: Effect of pH on peroxidase A and B activity produced from *Aspergillus* sp. after 6 days of incubation

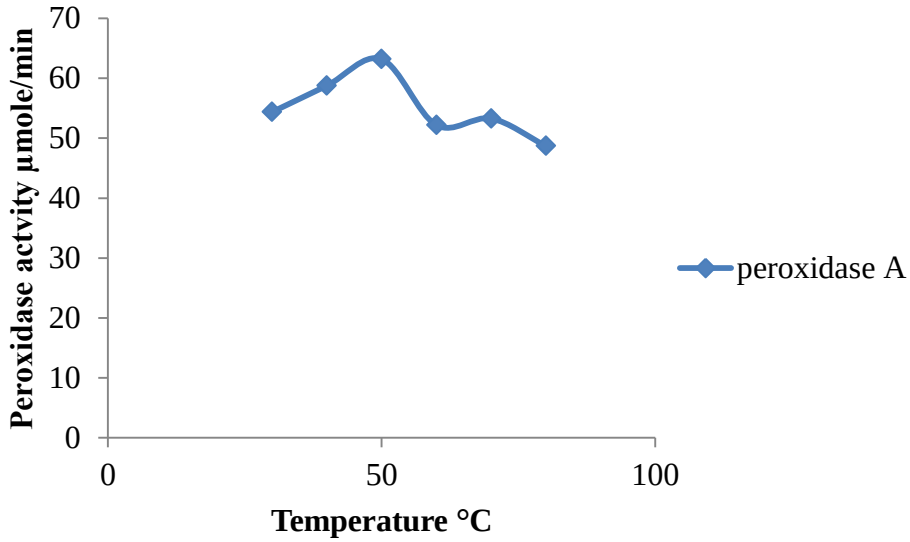

Fig. 9: Effect of temperature on peroxidase activity produced from *Aspergillus* sp. after 6 days of incubation

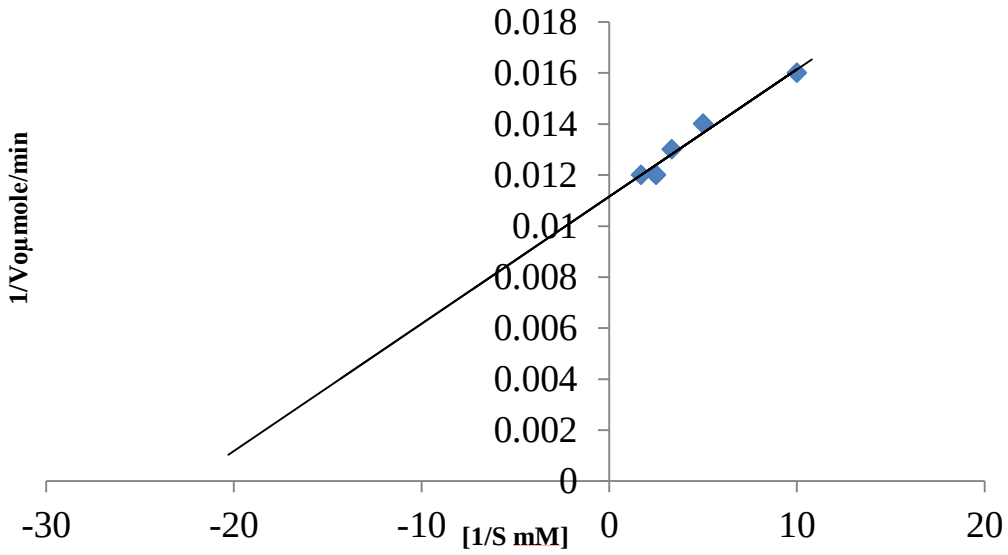

Fig. 10: Line weaver-burke plots of peroxidase produced from *Aspergillus tamari* respectively

Effect of stabilizing metals and stability studies of peroxidase.

Stabilizing metals: Fe, Ca, Co and Mn selected as their notable impact in the active site of peroxidase guided the selected were assayed in the presence of the enzymes, respectively. The stabilizing metal showed power of chelating in the presence of the control experiment. However, Co was seen very stabilizing than other assayed stabilizing metals Fig. (11).

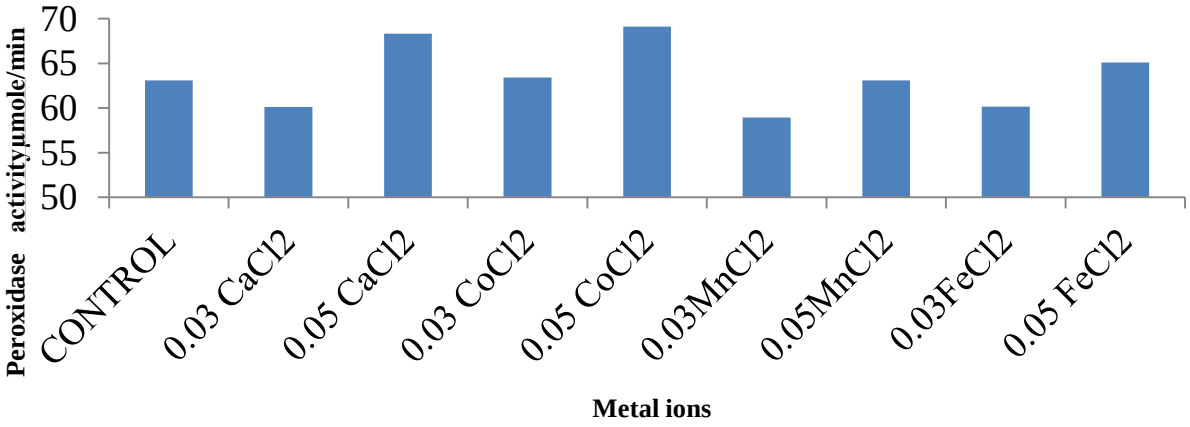

Fig. 11: Effect of stabilizing metals on peroxidase A activity produced from *Aspergillus* after 6 days of incubation

Stability studies of the peroxidases monitored at their respective optimum pH (4.5; 5.0) respectively, 7.0 and 8.5 as shown in Fig. (12, 13 and 14 below). The stability curve was single biphasic which represent the first order. The enzymes maintained greater than 50% of their activity after 30 min of incubation as activity progressively decreased up to 40% after 60 min of incubation. Thermal stability of peroxidase at their respective optimum temperatures (50°C) and at 70°C showed a biphasic stability curve of the peroxidase. Fig. (15 and 16).

The enzymes maintained greater than 50% of their activity after 60 min of incubation. Stability curve of peroxidase at 70°C showed a maximum activity of the enzymes after 30 min of incubation.

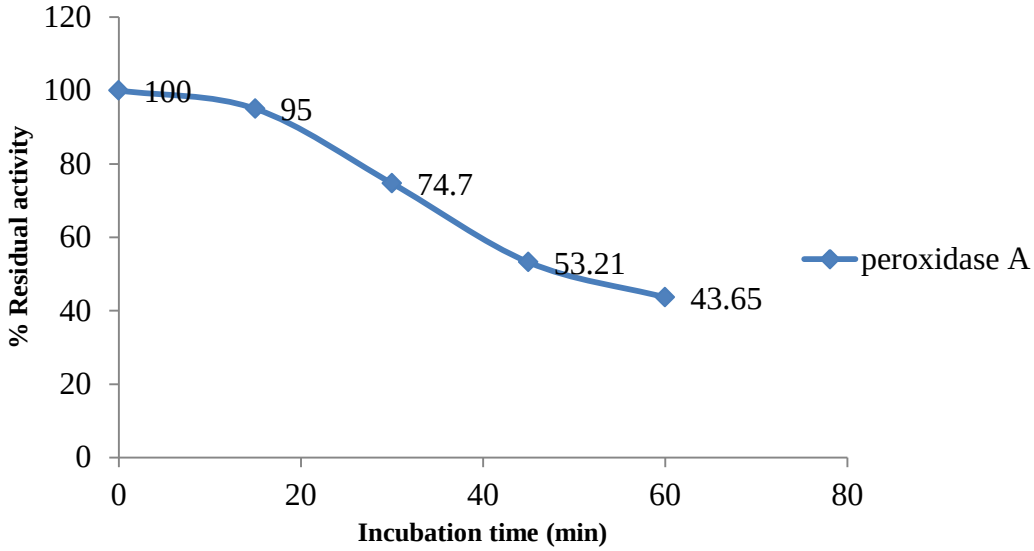

Fig. 12: Stability curve of peroxidase at pH 4.5 respectively

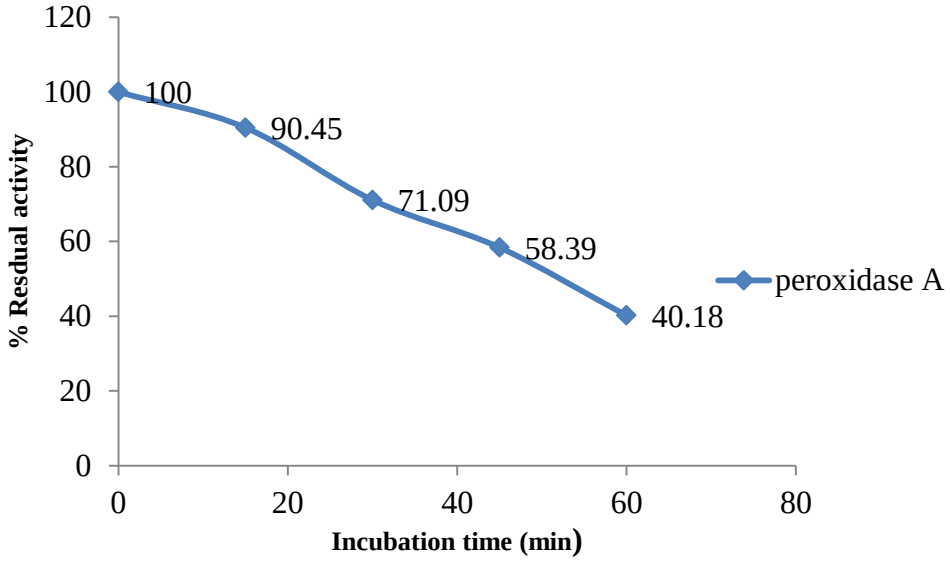

Fig. 13: Stability curve of peroxidase at pH 7.0 respectively

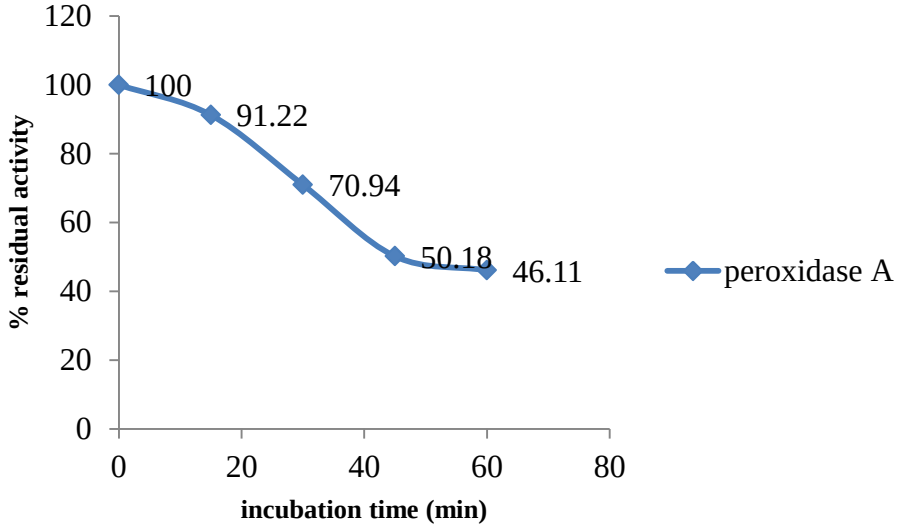

Fig. 14: Stability curve of peroxidase at pH 8.5 respectively.

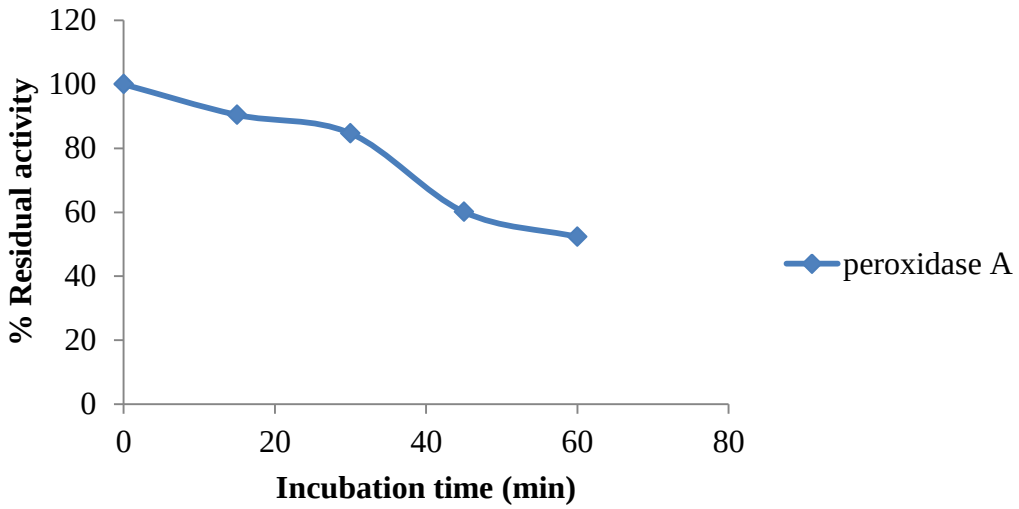

Fig. 15: Stability curve of peroxidase at 50°C

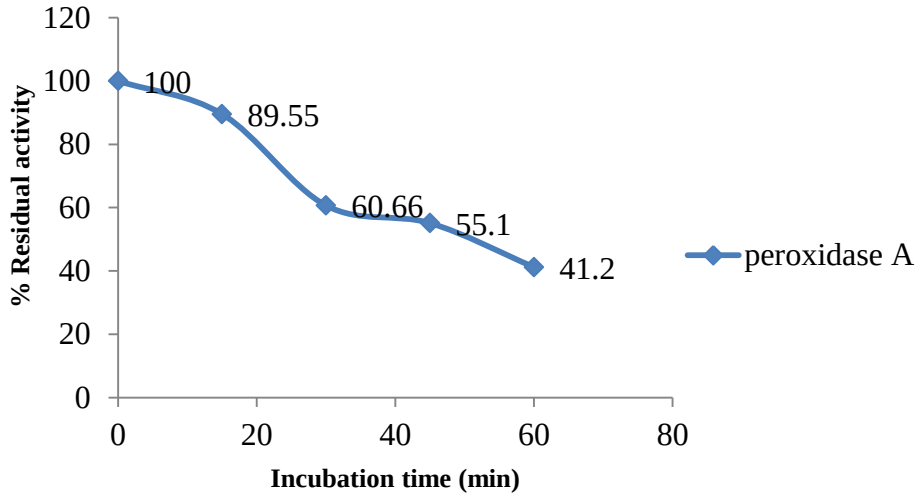

Fig. 16: Stability curve of peroxidase at 70°C

Discussion of findings

Peroxidases are integral housekeeping biocatalysts; very indispensable for future biotechnological advancement in multifarious fields. Hurdles in the utilization of the enzymes generally especially from microorganisms are solely low yield of enzyme specific activity (i.e. activity per proteins) (De jong *et al*., 1992). These compromises limit the greater potentials of microbial enzymes in revolutionizing the various aspect of livelihood. Most peroxidases are execratory proteins by their housing microorganisms, among other physiologic conditions conditioning their catalytic rates, it has been envisaged though without empirical that seasonal variances can modulate the secretion of highly active and specific peroxidase from producing organisms to which the present study under-look.

Sampling of soil for isolation of the strains of peroxidase producing fungi was carried out seasonally across a total period of 40 weeks. Strains of grey filamentous fungi were ubiquitous among the fungi isolates. These as stated by Vallero (2010) that most fungi (filamentous class of zygomycetes) and bacteria strains of *Pseudomonas* are hydrocarbonolistic and are predominant at petroleum hydrocarbon spilled site. *Aspergillus* sp was confirmed after standard biochemical and microbiology test. Screening of the isolate for peroxidase producing ability carried out in the

presence of 2,6 DMP confirmed the isolated potentials with grey yellow coloration after three days of incubation. However, the coloration intensity was depended in culture broth with fungus from the wet sampled soil. The screened *Aspergillus* were confirmed *Aspergillus tamari* after culture independent molecular test.

Physicochemical properties of the soils around the petroleum spilled sites showed pH of 4.45 and 6.5 for soils from point 1 (dry) and II (wet) respectively and higher conductivity of 613 and 1013 (Ω^{-1} Cm-1), respectively when compared with the control sample. The relative lower pH and higher conductivity of the respective soils from the petroleum hydrocarbon spilled soil compared to the control experiment can be attributed to the nature of the contaminant in the soil such as oil and other recalcitrant which may contain higher acidic contents (oleic, benzoic acids) as stated in the proceedings of the ASTDR (2009). However, soil sample during the dry season showed a higher hydrogen ion concentration than the correspondence from wet season. Ezenwelu *et al*. (2022) in their study on physicochemical properties of soil samples from Mgbuka artisanal mechanic workshop reported a low pH of 5.0 in the respective Mgbuka soils. They stated that the acidity decreases upon incident of rainfall. It should be recorded that certain acidic recalcitrant persist within a given ecological niche over the time and contributes to the high [H^+] of the medium, however the acidity decreases when there is influx of solvation's by fluids e.g. Water (Valero, 2010). Dissolved mineral of Cl^-, SO_4, K, Ca, Mg in the respective soil samples from the petroleum spilled sites were significantly high when compared with the control experiment except for soil sample I (dry) which showed a relative low phosphate concentration of 1,23 in the presence of the control experiment, respectively. TOC and TOM contents were 87.91, 119.04; 108.13 and 146.42 mg/g for soil sample I, and II, respectively. In all the tested parameters, the experimented soils were significantly high than the control soil sample. Mbachu *et al*. (2016) stated that total organic carbon and organic matter content of a medium reveal the carbon catenation oxidizable in the sampled area and organic matter show the degradable composite of the oxidizable carbon. The two enlisted components revealed the presence of carbon in an ecosystem. They reported a TOC and TOM of 196.71 and 241.95 mg/g Total petroleum hydrocarbon content was 2123.410 mg/g.

Effect of incubation days on peroxidase production from *Aspergillus tamari* showed an exoscretory proteins from the respective peroxidases. Peroxidase A activity peaked on day 6 while peroxidase B recorded day 5 as the peak day of production. Casciello *et al*. (2017) reported day 5 for peroxidase production from *Nonomuraea gerenzanensis* growing on alkali lignin. Peak production of peroxidase was recorded at pH 5. Adewale and Adekunle, (2018) in their research on properties of peroxidase from white and red cultivars of kolanut stated that peroxidases preferentially require low pH medium for their peak production. Their findings correlate with the evidence of the present study as they stated pH 4.5 as the peak production pH of their peroxidase.

Purification of the produced peroxidases from *Aspergillus tamari* was carried out through ammonium sulphate precipitation and gel filtration respectively. Proteins with highest peroxidase activity were precipitated at 60% ammonium sulphate for peroxidase. As reported by Oparaji *et al*. (2022) ammonium sulphate precipitation works on the principle of common ion effects, differential precipitation of molecules at different saturation of the precipitating agents is due to difference in the distribution of hydrophobic and hydrophilic sub groups within a compound. It is evident from the present study that peroxidase B required high ammonium sulphate saturation revealing the hydrophilic nature of the protein.

Salt removal from precipitated macromolecules is considered an essential step in protein purifications as it helps in stabilization of desired protein seen evidently in the activity after desalting. Desalting of precipitated proteins through dialysis is done on the principle of Reverse osmosis. The internal surrounding of the precipitate is lowered in concentration (hypotonic) to enable easy exchange of salts with the outside environment until no other salt will be available for exchange (saturation point). The precipitated protein was de-salted for 12 hours using dialysis bag (with pore size of 2mm) in an ice pack container with buffer exchange after six hours of the dialysis. After dialysis specific activity was found to be 425.17 U/mg and for peroxidase.

Size exclusion chromatography was used for further purification of the protein to various molecular sizes and weight. This was done using sephadex G-100 packed into a column of bed height 75 cm and column volume of 235.65cm^3. A phase peak of peroxidase A activity was obtained from the chromatogram. Subtle peaks were obtained from the chromatograms for peroxidase A and B but not significant. As reported by Chilaka *et al*. (2002), they stated that there appears to be a relationship between dialysis of enzymes usually after ammonium sulfate precipitation and the presence of isoenzymes, they went further to state that when dialysis is replaced by gel filtration in enzyme purification, isoenzymes were lost out.

Multiple peaks of the enzyme activity could be attributed to ionic scrambling encouraged by dialysis and this leads to formation of aggregates with incorrect ionic bond pairs. Such aggregates express ionic heterogeneity on any column chromatography (ion exchange and size exclusion). peroxidase A was purified up to 2.89 folds while specific activity increased up to 344.78 U/mg respectively.

Variations in pH largely affect the ionization constants of functional groups in a protein thus having an overall effect on the enzyme structures and its functionality due to destabilization of the linking bonds that helps in maintenance of the enzyme active structures. Temperature represents the heat (enthalpy change) content of a chemical system. For most chemical reactions (*in vivo/in vitro*), every 10°C rise in temperature results to double increase of the process. Heat content of a system increases the kinetic energy of atoms and molecules of the system thus resulting in an increase in the number of effective collisions of the therein particles. Increase in catalysis by enzymes in a biological system is mediated by effective collision between an enzyme's desired substrate(s) at its active site (Anosike, 2002). Optimum pH for peroxidase A produced from *Aspergillus tamari* was 4.5. Peroxidase activity peaked at 50°C; Chanwun *et al*. (2013) reported an optimum peroxidase activity from *Hecea brasiliensis at* 50°C. Dragana *et al*. (2017) reported a moderate thermo-tolerant peroxidase of peak activity at 70°C.

Kinetic constants (K_m and V_{max}) of peroxidase A and B determined during the study at various concentrations of 2,6 DMP showed K_m of 3.45mM. K_m which is the substrate concentration at half the maximum velocity during enzyme catalyzed reaction shows the affinity of the enzyme to its available substrates (Chilaka *et al.,* 2002).

It is shown that the higher the K_m value for each enzyme catalyzed reaction the lower the affinity of the enzyme to its available substrate and vice-versa. Velocity maximal (V_{max}) which shows the catalytic efficiency (rate of turnover) of peroxidases during catalysis shows V_{max} of 280 μmole/min μmole/min.

Metal ions generally play important roles in the biological function of many enzymes (both clinical and industrial implicated ones) (Riordan, 1977).

The various modes of metal-protein interaction include metal-, ligand-, and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis's acids or structural regulators (chelators) (Riordan, 1977). Divalent metal ions (double cationic charged) are mostly implicated in enzyme active sites where they are directly involved in catalysis, maintenance of structural compatibility of the enzyme or in affinity binding of enzyme substrates together with the arrays of amino acids making up the protein (Adalberto *et al.,* 2010). Stabilizing metals: Fe, Ca, Co and Mn selected as their notable impact in the active site of peroxidase guided the selected were assayed in the presence of the enzymes, respectively. The stabilizing metal showed power of chelating in the presence of the control experiment. However, Co was seen very stabilizing than other assayed stabilizing metals. Pandey *et al.* (2017) stated that certain divalent metal such as Fe, Co are cofactors to peroxidase activity as they responsible for catalytic and structural compatibility of peroxidases.

Riordan, 1977 reported that differential effect of the metals on enzyme activity is attributed to spinning of electrons to the valence shells of the metals. He went further to state that paramagnetic spined electron shell metals (especially transition metals) give more regulatory and activation effect

in every enzyme/non enzymatic catalyzed reaction with respect to their ease to donate and accept (chelation power) electrons, radicals and charges during the mediated reactions.

Stability studies of the peroxidases monitored at their respective optimum pH (4.5) respectively, 7.0 and 8.5. The stability curve was single biphasic which represent the first order.

The enzymes maintained greater than 50% of their activity after 30 min of incubation as activity progressively decreased up to 40% after 60 min of incubation. Thermal stability of peroxidase at their respective optimum temperature and at 70°C showed a biphasic stability curve of peroxidase with the initial phase occurring very fast and thereafter there was a noticeable retardation after 60min of incubation. The enzymes maintained greater than 50% of their activity after 60 min of incubation. Stability curve of peroxidase at 70°C showed a maximum activity of the enzymes after 30 min of incubation. Peroxidase activity as shown maintained a residual activity of 41.2%. The findings are in correlation with the results from Eze (2013) who reported the stability and thermodynamics of peroxidase from oil bean seeds; he stated that peroxidase from oil bean seeds maintained up to 50% of their activity after 30min of incubation reportedly a slower initial denaturation rate at 60°C. Biphasic curves as they reported is evidently from the isoenzymes present in the enzyme solution.

CONCLUSION

Peroxidases are evidently indispensable proteins with wide application in various biotechnological endeavors. Turn offs in the application of the enzymes is due to low yield from sources and non-encourage able catalytic activity. The present study has shown the kinetics of the proteins from *Aspergillus tamari* sampled from petroleum hydrocarbon spilled soil at respective seasons. The study has exposed the impact of physiologic season variations on microbial proliferations which goes in line with the stabilizing proteins like peroxidases.

Funding information

The present ecotoxicology and protein engineering research is solely sponsored by Dr. Ezenwelu C.O of department of Applied Biochemistry, NNamdi Azikiwe University, Awka Anambra state, Nigeria.

Appreciation

The research laboratory group of Ezenwelu C.O. is very thankful to the Inqaba biotech center Fort-Hare South-Africa for fostering the molecular typing of their microbial isolates. We are grateful.

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دراسة الخواص الحركية لفطر الرشاشيات المنتج للبيروكسيديز والمعزول من التربة الملوثة بالهيدروكربونات البترولية

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الملخص

تم إنتاج البيروكسيديز من *Aspergillus tamari* المعزول من التربة الملوثة بالهيدروكربونات النفطية. أظهرت الخواص الفيزيائية والكيميائية للتربة ان درجة حموضة كانت 4.45 و6.5 للترب المأخوذة من الموقع 1 و2 على التوالي وبلغت التوصيلة أعلى 613 و1013 (Ω-1 سم−1)، لكلا الموقعين على التوالي عند مقارنتها بعينة السيطرة. كانت المعادن المذابة من Cl وSO_4 و K و Ca و Mg في عينات التربة المعزولة من المواقع الملوثة بالنفط مرتفعة بشكل ملحوظ عند مقارنتها بعينة السيطرة باستثناء عينة التربة 1 والتي أظهرت تركيزًا منخفضًا نسبيًا للفوسفات قدره 1.23 ملغم/غم.

بلغت محتويات TOC و TOM،87.91 و119.04؛ 108.13 و146.42 ملغم/غم لعينتي التربة الأولى والثالثة على التوالي. حيث كانت جميع عينات التربة المختبرة عالية بشكل ملحوظ مقارنة بعينة التربة الضابطة. تم استخدام الاختبارات الجزيئية (18S rDNA) للتعرف على عائدية العزلة النقية للفطر *Aspergillus tamarii*. أظهرت نتائج دراسات تأثير فترة الحضانة على إنتاج البيروكسيديز من سلالات فطر *Aspergillus tamarrii* أن أعلى نشاط للبيروكسيديز تم الحصول عليه في اليوم السادس من وقت التخمير. بلغ نشاط البيروكسيديز ذروته عند الرقم الهيدروجيني 5. وكان البروتين ذو أعلى نشاط للبيروكسيديز قد وصل إلى ذروته عند تشبع 60٪ من الملح. بلغ نشاط البيروكسيديز المنقى ذروته عند الرقم الهيدروجيني 4.5. كانت درجة الحرارة المثلى لنشاط الإنزيم عند 50 درجة مئوية. تم استقراء Km و Vmax 3.45 ملي مولر و280 مايكرومول/ دقيقة من المنحنى المتبادل لـ Lineweaver-burke بتركيزات مختلفة تبلغ DMP 2,6. تم اختبار الحديد والكالسيوم والكوبالت والمنغنيز كعوامل مساعدة تدخل في الموقع النشط للبيروكسيديز وتم تقييمهم في وجود الإنزيمات، على التوالي. كان منحنى الاستقرار الذي تم الحصول عليه للبيروكسيديز ثنائي الطور والذي يمثل الدرجة الأولى؛ وحافظت الإنزيمات على أكثر من 50% من نشاطها بعد 30 دقيقة من الحضانة، حيث انخفض النشاط تدريجيًا حتى 40% بعد 60 دقيقة من الحضانة. أظهر الثبات الحراري للبيروكسيديز عند درجة الحرارة المثلى (50) وعند 70 درجة مئوية منحنى ثبات ثنائي الطور. حافظت الإنزيمات على أكثر من 50% من نشاطها بعد 60 دقيقة من الحضانة.

الكلمات الدالة: انزيمات البيروكسيديز، فطر الاسبيرجيلس، البروتينات، انزيمات الكاتاليز، الفيزوكيميائية.