



Investigation of Seasonal Dynamism of Peroxidase from Strains of *Aspergillus* sp.

O. Ezenwelu, Chijioke ^a, A. Oladejo Afeez ^a,
M. Okeke, Chisom. ^a and E. H. Oparaji ^{b*}

^a Department of Applied Biochemistry, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

^b Department of Biochemistry, State University of Medical and Applied Sciences, Enugu State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Peroxidases A and B production were carried out from *Aspergillus tamari* and *fumigates* respectively 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 ($\Omega^{-1} \text{cm}^{-1}$), 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 was 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. Molecular tests (18S rDNA.) were used to identify the

*Corresponding author: E-mail: emeka.oparaji65@yahoo.com;

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pure isolates of *Aspergillia*. Studies on the effect of the incubation period on the production of peroxidase from strains of *Aspergillus tamarrii* sp. and *Aspergillus* showed that the highest peroxidase (A and B) activity representing peroxidase from dry and wet conditions were obtained on the day 6th and 5th of the fermentation time figure peroxidase A activity peaked at pH 5 while that of peroxidase B peaked at pH 6.0; proteins with highest peroxidase activity was peak precipitated at 60% and 80% saturation of the salt for peroxidase A and B, respectively. The gel chromatogram showed single almost superimposed peaks of enzyme activity for peroxidase A and B respectively. Peroxidase A and B activity peaked at pH 4.5 and 5.0. Optimum temperature for the enzyme activity was at 50 and 60°C respectively. Km and V max of 3.45mM and 280 µmole/min; 2.44mM and 305µmole/min were extrapolated from the reciprocal curve of Lineweaver-burke at various concentrations of 2,6 DMP for peroxidase A. 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 represents the first order; Peroxidase B maintained greater stability than A at its optimum pH and pH 7. 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 A and B at their respective optimum temperatures (50 and 60°C) and at 70°C showed a biphasic stability curve of peroxidase A but single phase in peroxidase B. Peroxidase B maintained greater stability than A at its optimum temperature and at 70°C. The enzymes maintained greater than 50% of their activity after 60 min of incubation. Stability curve of peroxidase A and B at 70°C showed a maximum activity of the enzymes after 30 min of incubation. However, Peroxidase B maintained greater stability than A after 60 min of incubation. Peroxidase B maintained 57.89% of its activity after 60 min while peroxidase A from fig 19 showed a residual activity of 41.2%.

Keywords: Peroxidases; aspergillus; proteins; catalysis; physicochemical.

1. 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 [1]. As stated by Opwis et al. [2] generally, they are very integralas cost-efficient and environmentally sensitive biological adduct(s) for chemical processes in the industries Viz: biotechnological and other alike.

Radical scavenging enzymes such as peroxidases are “house-keeping” biomolecules 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 [3-7]. Zhang et al. [8] reported that peroxidases generally catalyze several chemical processes involving oxygen-transfer between hydrogen peroxide and othersuperoxides where they act as charge chelators and another form of substrates such as xenobiotics and phenolics through deoxygenation of O₂ from H₂O₂ [9-12].

Peroxidases are diverse in ubiquity and distributed in plants, animals and

microorganisms, where they shield vital cells/organelles against the lethality of oxidative stress and the resultant on destruction of the sited cells/organelle accruing from liberation of H₂O₂(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, indigestibility, 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 [13,14-16].

Wide range of utility, diverse in nature and reactions catalyzed by peroxidases, have over the time announced their potentiality as an integral biological catalyst for future generational biotechnological advancement in a related field of bioremediation, textile synthetic dye decolourization, polymer synthesis, paper and pulp industry, in development of biosensor and diagnosis kits [17,18,19].

However, microbial peroxidase catalytic efficiencies are metabolically affected by *in situ* physiologic conditions of their producing organisms; stabilization potentials of these

proteins within the organisms are seen being repressed when posed with harsh environmental conditions and in the presence of ecologically implicated recalcitrant (Valero, 2010) [20]. 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 [21,22-24]. Peroxidases are extracellular proteins in this filamentous fungus and foster the *In vivo* stabilization of the fungus over time especially at unfavourable condition(s) [25,26]. *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 serves the sole purpose of the present study aiming to produce wide stable peroxidase with high catalytic efficiency for future advancement.

2. MATERIALS AND METHODS

2.1 Materials

The following designated establishments were the purchasing companies for the chemicals, reagents, apparatus and equipments utilized for the present study and they include: B&H-Aldrich, Bristol, May and Baker, Merck, Pyrex and B and T trimeline, Jenway.

2.2 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 *Aspergillus* was collected from a petroleum hydrocarbon spilt site located at Agbada front terminal in Rivers state as described by Ezenwelu et al. (2022) [27]. Collections each sampling period was conducted at exactly 6:00 am at three respective points in the mark area each at a distant of 9m from the other. The collected homogenized soil was pooled together in an aseptic 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 was 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.

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

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 cork 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 (et al., 2016).

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

Treatment	Temperature (°C)	Time (Min)
Pre-denaturatio	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 *folinciocateau* 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 enzyme production. Each conical flasks optimized for peroxidase production contains the following: 1 % (NH₄)₂SO₄, 0.4 %

K₂HPO₄, 1 % glucose, 0.01 % sodium acetate, 0.1% di-ammonium citrate, 0.05 % MgSO₄.7H₂O and 0.2 % FeSO₄.4H₂O. 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).

2.3 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°C 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 were was further purified through column chromatography using the sephadex G-100 as described by Ezenwelu et al. [27].

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.

2.4 Enzymatic Properties of Purified Peroxidase

Effect of pH on Peroxidase Activity: Purified peroxidase optimum pH was determined as described by Eze et al. [28]. 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. [28]. 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 (K_m and V_{max})

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 [29]. 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:

$$\% \text{ residual activity} = 100 \times \frac{At}{Ao}$$

Whereas At= Activity at a given treatment time
Ao= Activity at no treatment time

2.5 Statistical Analysis

Data obtained shall be expressed as mean \pm 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 analysed using the Microsoft excel program.

3. 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 ($\Omega^{-1} \text{ Cm}^{-1}$), 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).

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 where as 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 safranin 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). *Aspergillustamarrii* T5 was identified after the genomic sequencing with ascribed NCBI accession number of KR149638 as shown in the evolutionary relatedness tress (fig. 3).

Table 2. Physicochemical properties of the soil sample

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

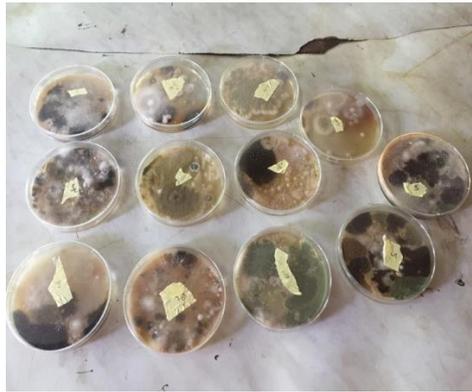


Fig. 1. Soil microbial load from the respective soil

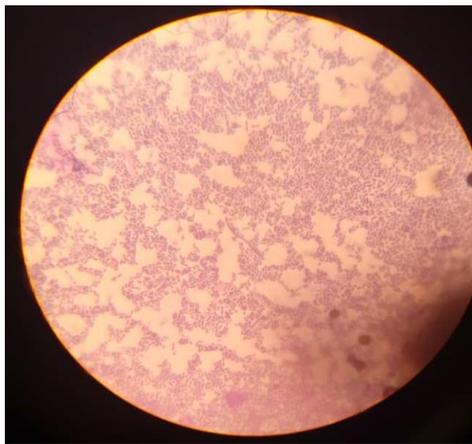


Plate 1. The micrograph of the fungi suspensions under an objective magnification of x40

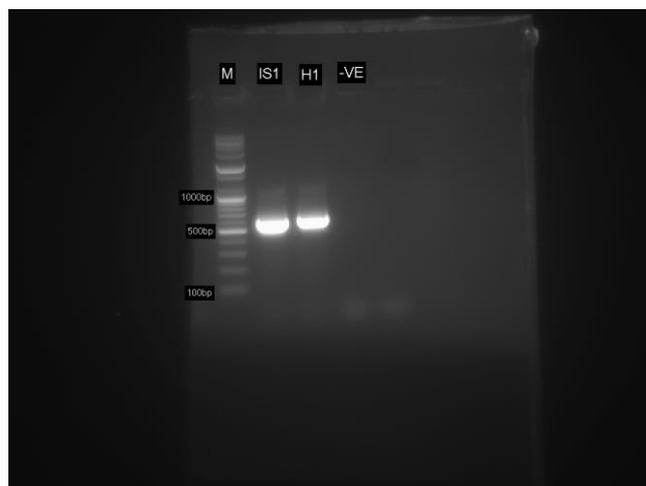


Fig. 2 Electrophoretogram of the amplified genomic DNA viewed on a UV trans-illuminator

Studies on effect of incubation period on the production of peroxidase from strains of *Aspergillus tamarrii* sp. and *Aspergillus* showed that the highest peroxidase (A and B) activity representing peroxidase from dry and wet

conditions were obtained on the day 6th and 5th of the fermentation time (Fig 5). However peroxidase activity was optimum with peroxidase B produced from *Aspergillus* isolated from petroleum spilled soil during wet condition.

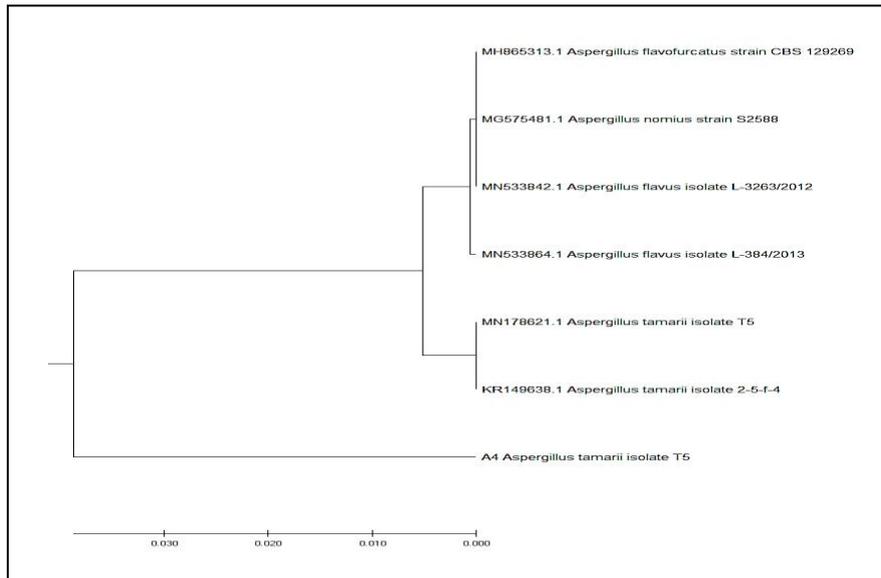


Fig 3 Phylogenetic evolutionary relatedness of strain of *Aspergillustamaris* obtained using NCBI BLAST tools

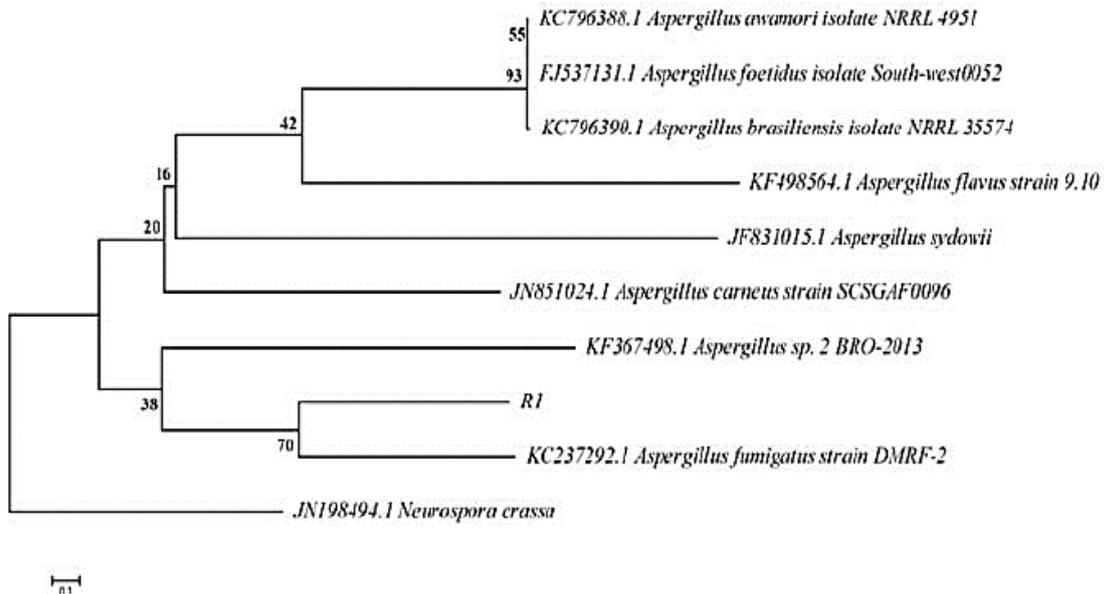


Fig 4 Phylogenetic evolutionary relatedness of strain of *Aspergillusfumigatus*obtained using NCBI BLAST tools

Fig 6 below shows the effect of pH on the production of peroxidase A and B from *Aspergillus tamaris* and *Aspergillus*, respectively. From the fig. peroxidase A activity peaked at pH 5 while that of peroxidase B peaked at pH 6.0 after 5 and 6 days of incubation respectively.

Precipitation of the crude peroxidases using ammonium sulphate from fig. 7 and 8 showed proteins with highest peroxidase activity

was peak precipitated at 60% and 80% saturation of the salt for peroxidase A and B, respectively.

Fig 9 shows the gel chromatogram of the respective peroxidases (A and B) carried out using sephadex G-100 at pH 6 and 5 respectively. The chromatogram showed single almost superimposed peaks of enzyme activity for peroxidase A and B respectively. From the

table 3 below peroxidase A and B were purified upto 2.89 and 3.11 folds while specific activity increased upto 344.78 and 576.38 U/mg respectively

Kinetic properties of the peroxidases from strains of *Aspergillus tamari* and *Aspergillus* showed the following enzymatic properties: Peroxidase A and

B activity peaked at pH 4.5 and 5.0. optimum temperature for the enzyme activity were at 50 and 60°C respectively. Km and V max of 3.45mM and 280 µmole/min; 2.44mM and 305µmole/min were extrapolated from the reciprocal curve of Lineweaver-burke at various concentrations of 2,6 DMP for peroxidase A and B respectively.

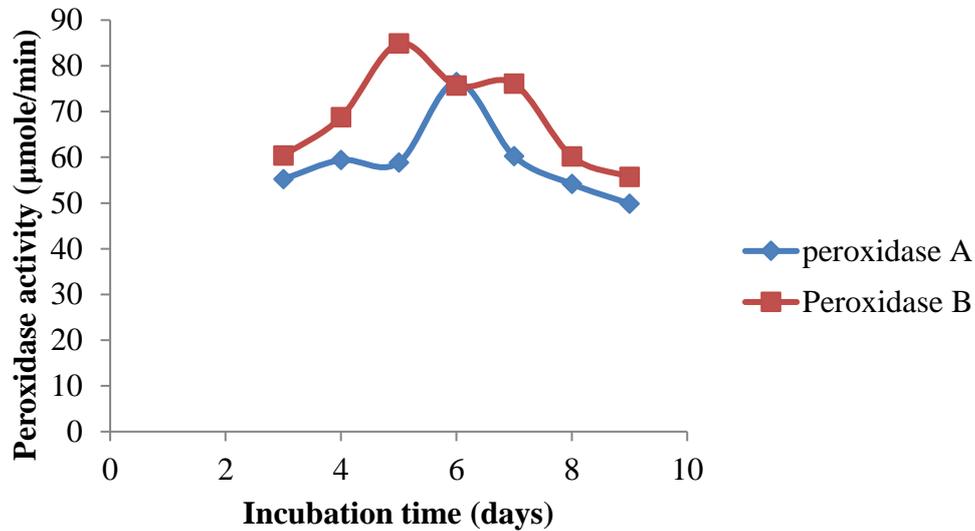


Fig 5. Effect of incubation time on peroxidase A and B production from *Aspergillus tamari* and *Aspergillus* respectively

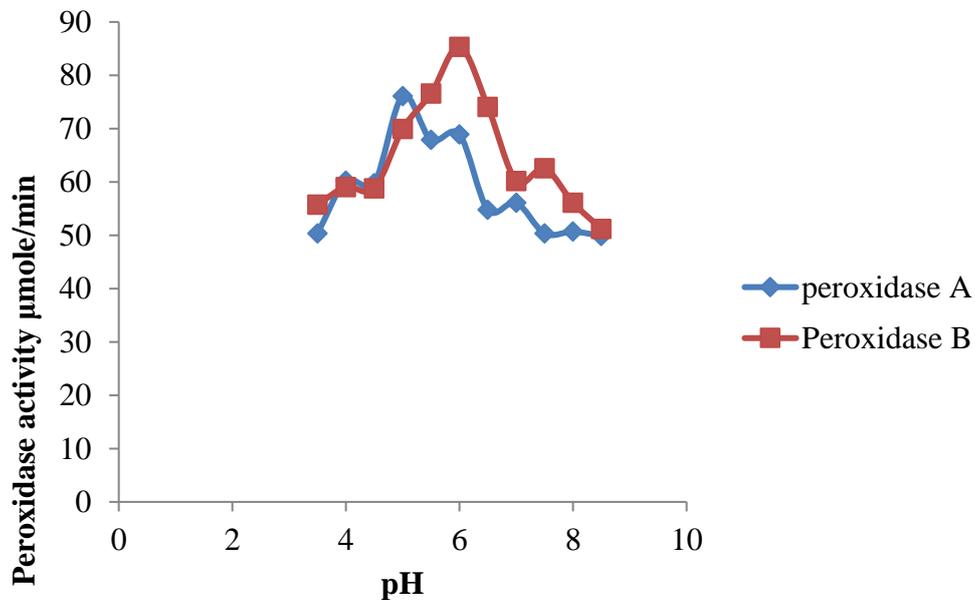


Fig 6. Effect of pH on peroxidase A and B production from *Aspergillus tamari* and *Aspergillus* respectively after 6 and 5 days of fermentation incubation

Table 3. Purification table peroxidase A and B from *Aspergillus tamari* and *Aspergillus fumigates*, respectively

	Volume (ml)		Total protein		Total activity (U/ml)		Specific activity (U/mg)		Purification folds		Percentage yield	
	A	B	A	B	A	B	A	B	A	B	A	B
Crude peroxidase	1000	1000	590	720	76500	99220	129.66	137.81	1	1	100	100
NH ₄ (SO ₄) ₂	250	250	77.5	87.5	15125	19550	195.20	223.42	1.51	1.62	19.76	19.71
Dialysis	100	100	20.1	26.7	6239	7798	211.3	274.52	1.94	2.11	6.71	7.2
Gel filtration	50	50	5.41	5.78	3076	4238	344.78	536.7	2.89	3.11	3.1	3.76

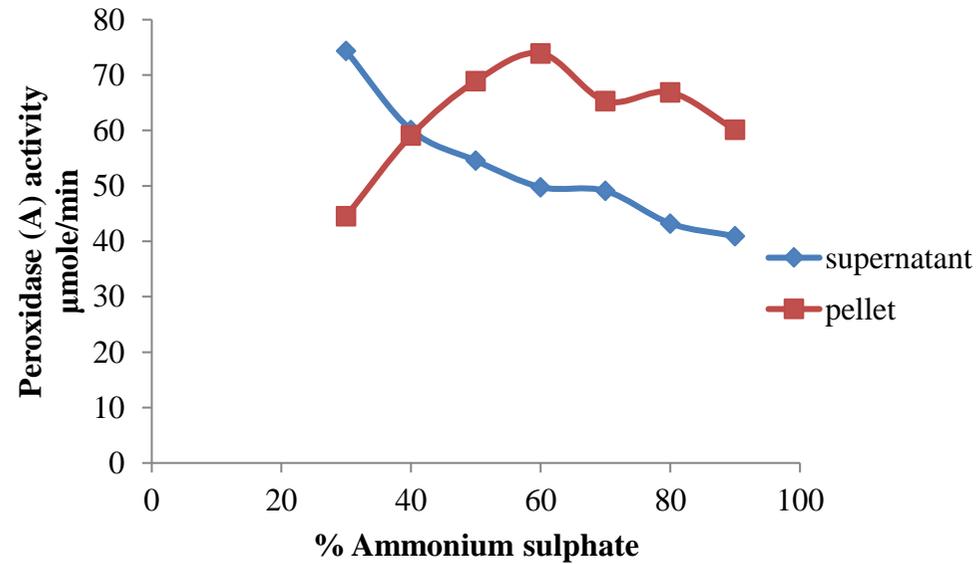


Fig. 7. Ammonium sulphate precipitation of peroxidase A produced from *Aspergillus tamari* after 6 days of fermentation at pH 5.0.

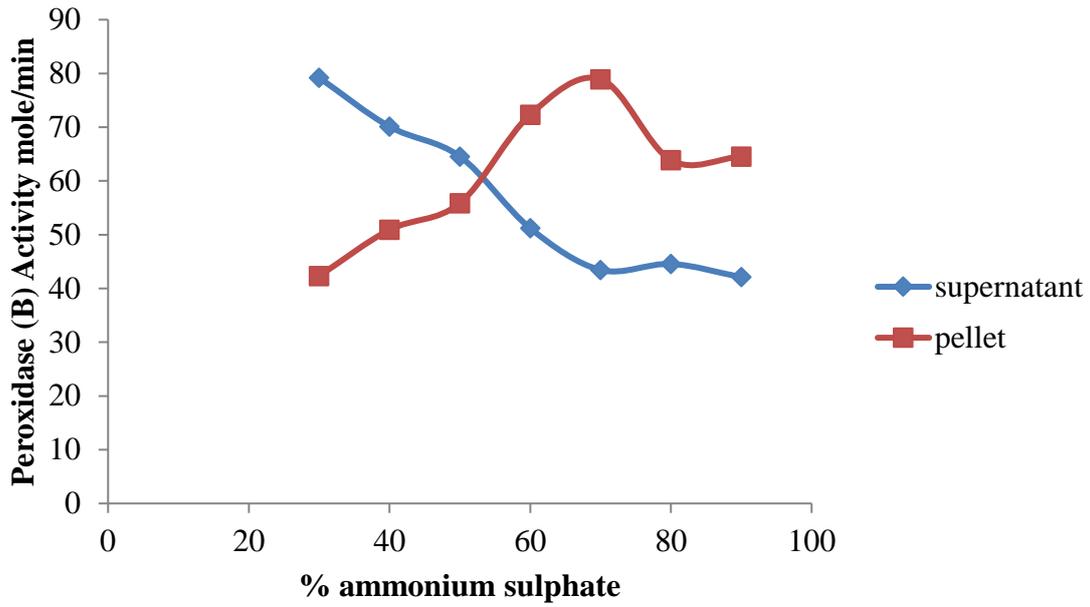


Fig. 8. Ammonium sulphate precipitation of peroxidase B produced from *Aspergillus tamari* after 5 days of fermentation at pH 6.0

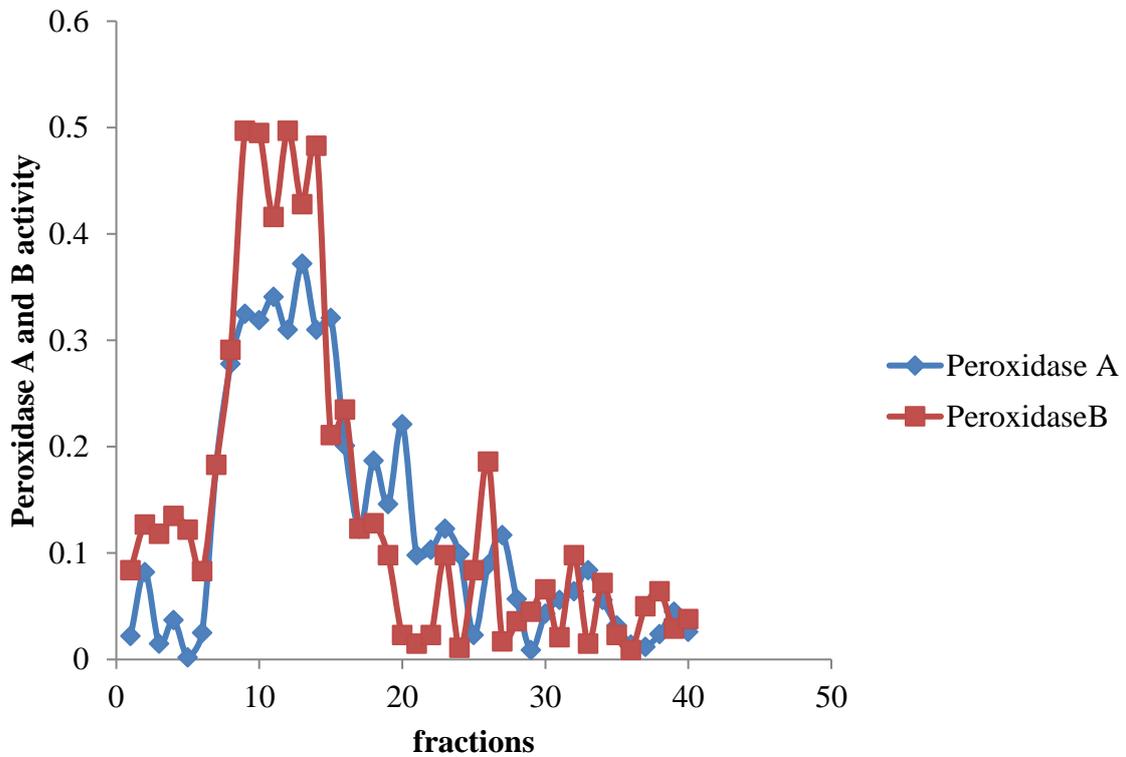


Fig. 9. Chromatogram of peroxidases (A and B) using sephadex G-100 with eluting solvent of pH 6 and 5 respectively

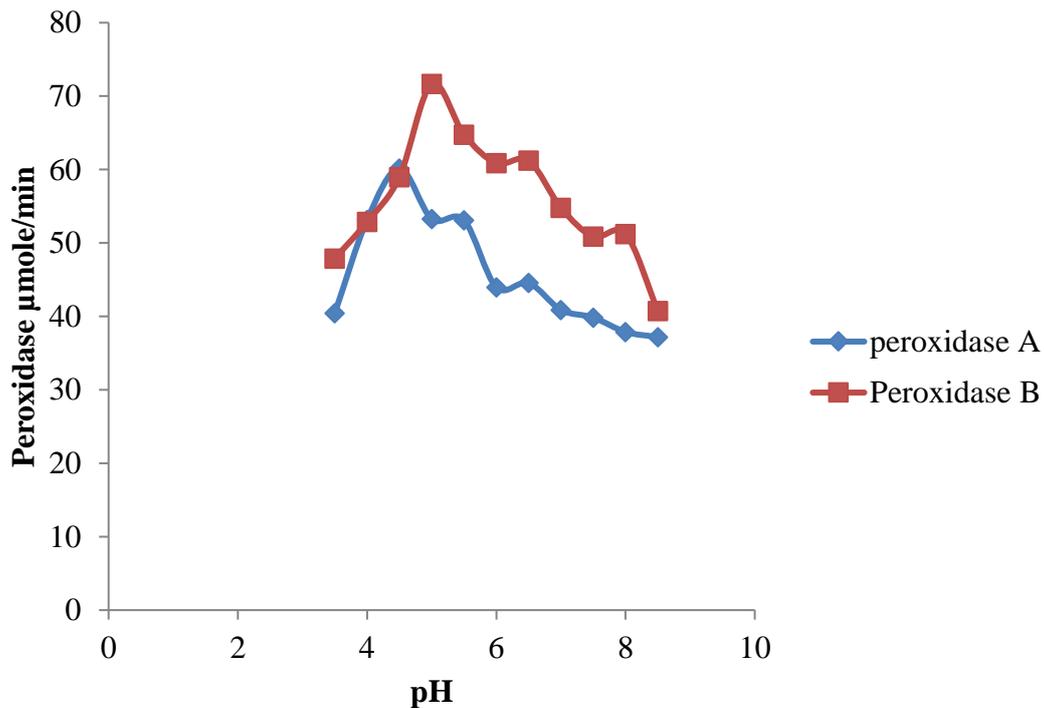


Fig. 10. Effect of pH on peroxidase A and B activity produced from *Aspergillus* sp. and *Aspergillus* after 6 and 5 days of incubation

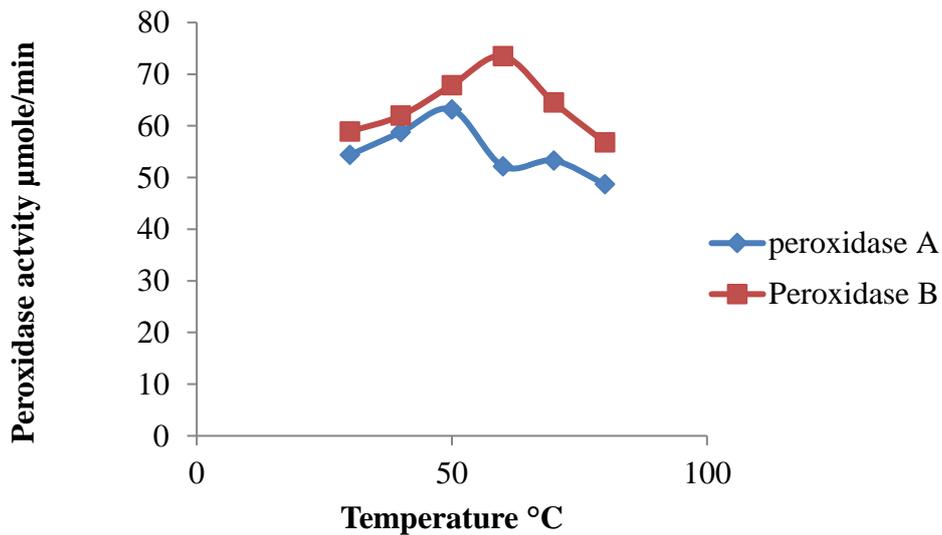


Fig. 11. Effect of teperature on peroxidase A and B activity produced from *Aspergillus* sp. and *Aspergillus* after 6 and 5 days of incubation

Effect of stabilizing metals and stability studies of peroxidase A and B.

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 13 and 14).

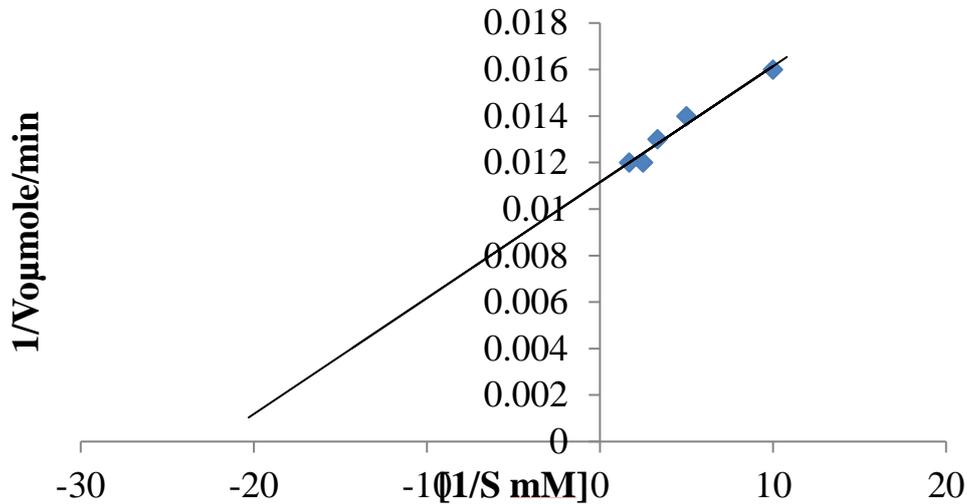


Fig. 12. Linearweaverburke plots of peroxidase A produced from *Aspergillus tamarireseptively*

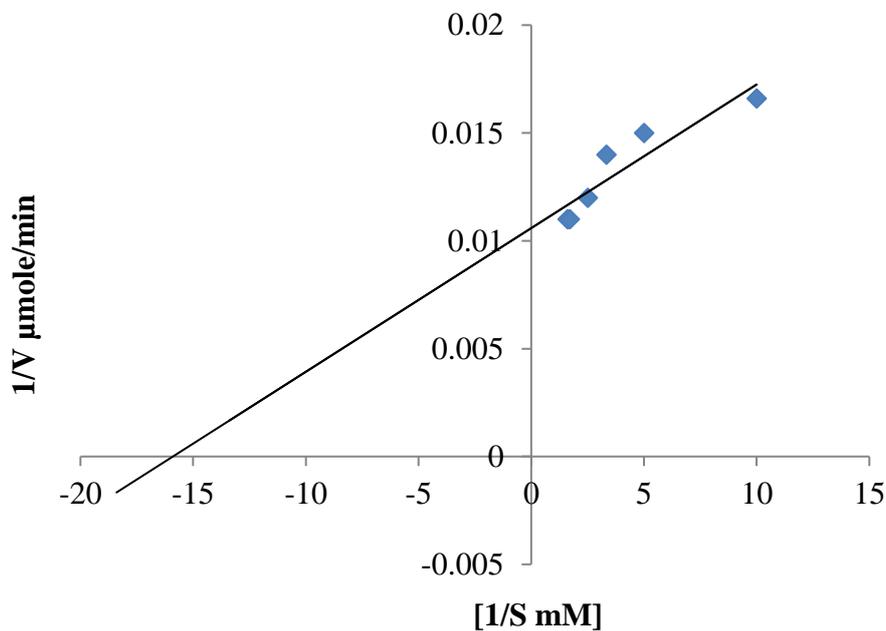


Fig. 13. Linearweaver-Burke plots of peroxidase B produced from *Aspergillus tamarireseptively*

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 16,17, 18, 19 and 20 below. The stability curve was single biphasic which represent the first order; Peroxidase B maintained greater stability than A at its optimum pH and pH 7. 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. Peroxidase B was affected at the basic pH as the enzyme maintained less than 40% of its activity. Thermal stability of peroxidase A and B as shown in fig 18 and 19 at their respective optimum temperatures (50 and 60°C) and at 70°C showed a biphasic stability curve of peroxidase A but single phase in peroxidase B. Peroxidase B maintained greater stability than A

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

incubation. However Peroxidase B maintained greater stability than A after 60 min of incubation. Peroxidase B maintained 57.89% of its activity after 60 min while peroxidase A from fig 19 showed a residual activity of 41.2%.

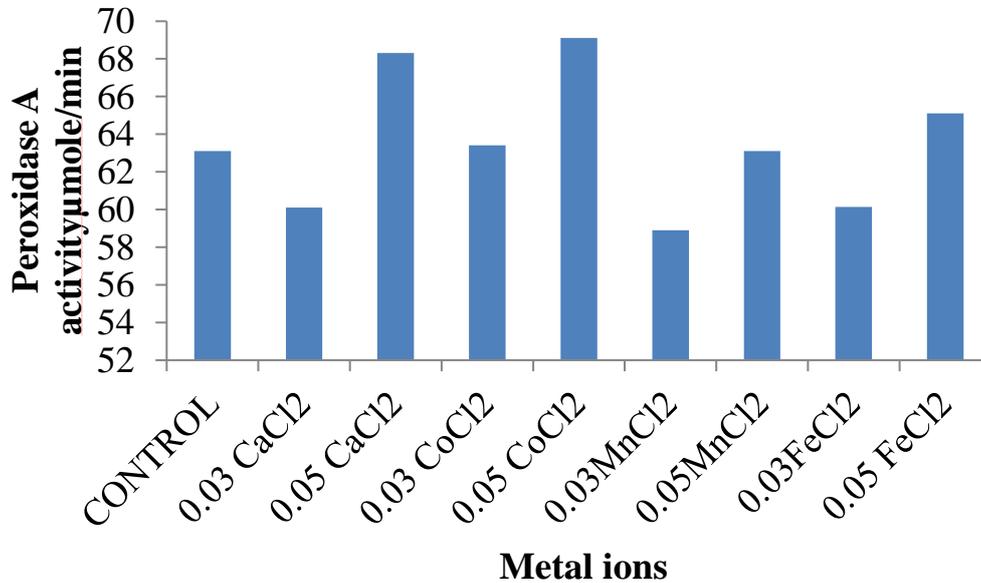


Fig. 14. Effect of stabilizing metals on peroxidase A activity produced from *Aspergillus* after 6 days of incubation

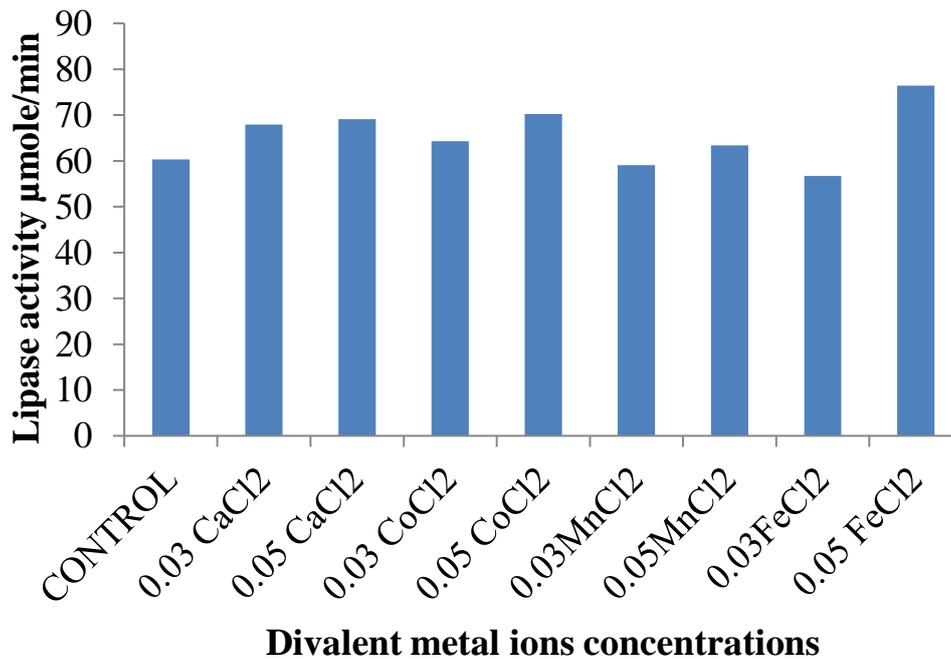


Fig. 15. Effect of stabilizing metals on peroxidase B activity produced from *Aspergillus* sp. after 5 days of incubation

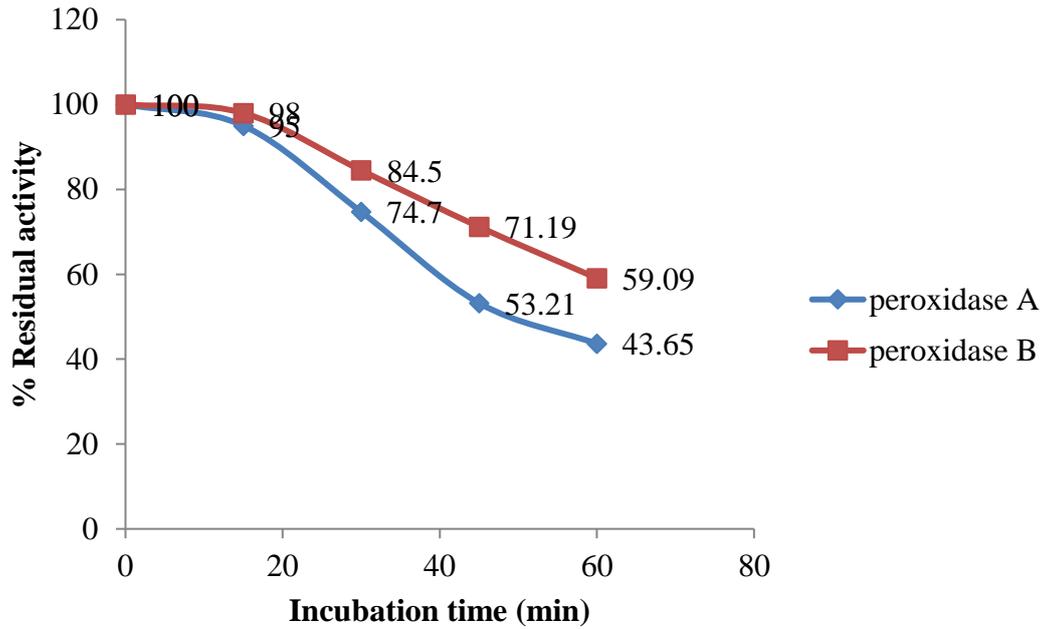


Fig 16. Stability curve of peroxidase A and B at pH 4.5 and 5.0 respectively

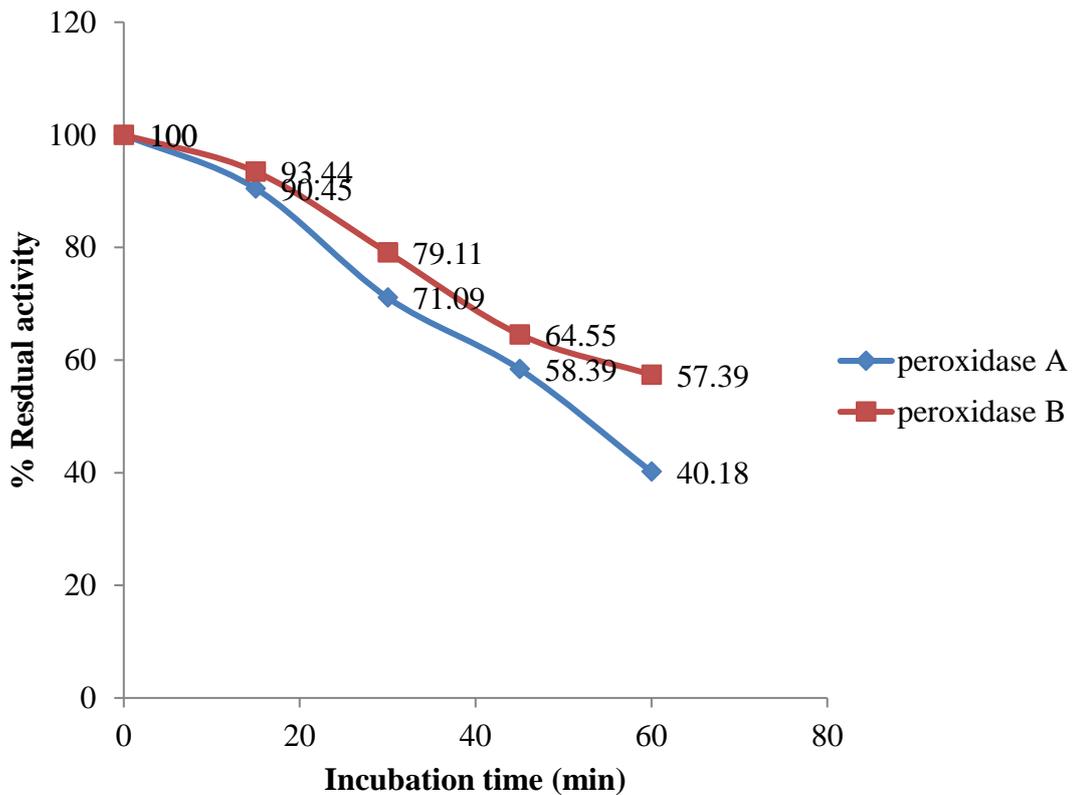


Fig. 17. Stability curve of peroxidase A and B at pH 7.0 respectively

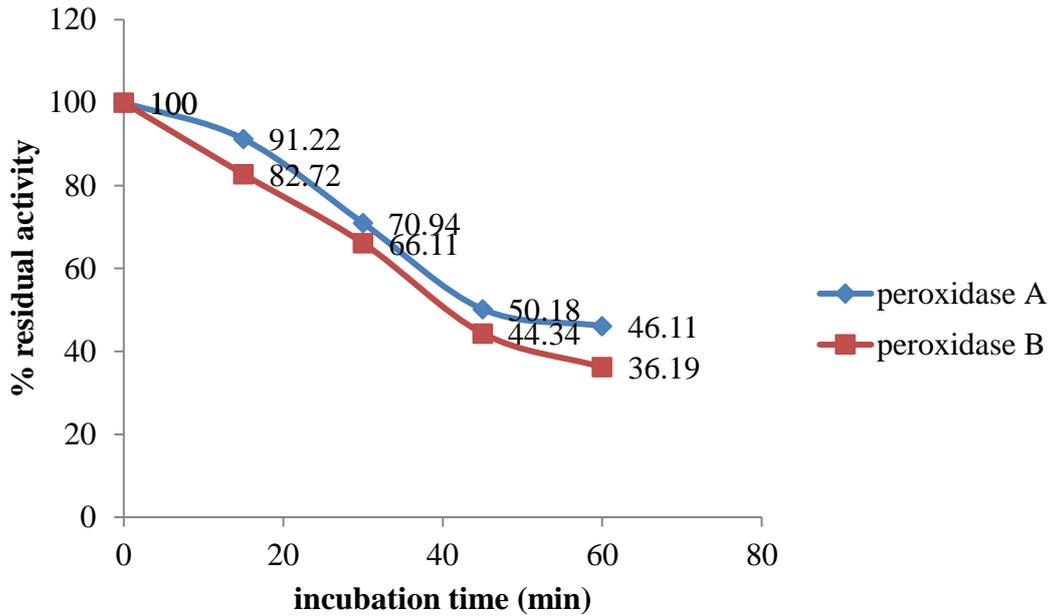


Fig. 18. Stability curve of peroxidase A and B at pH 8.5 respectively

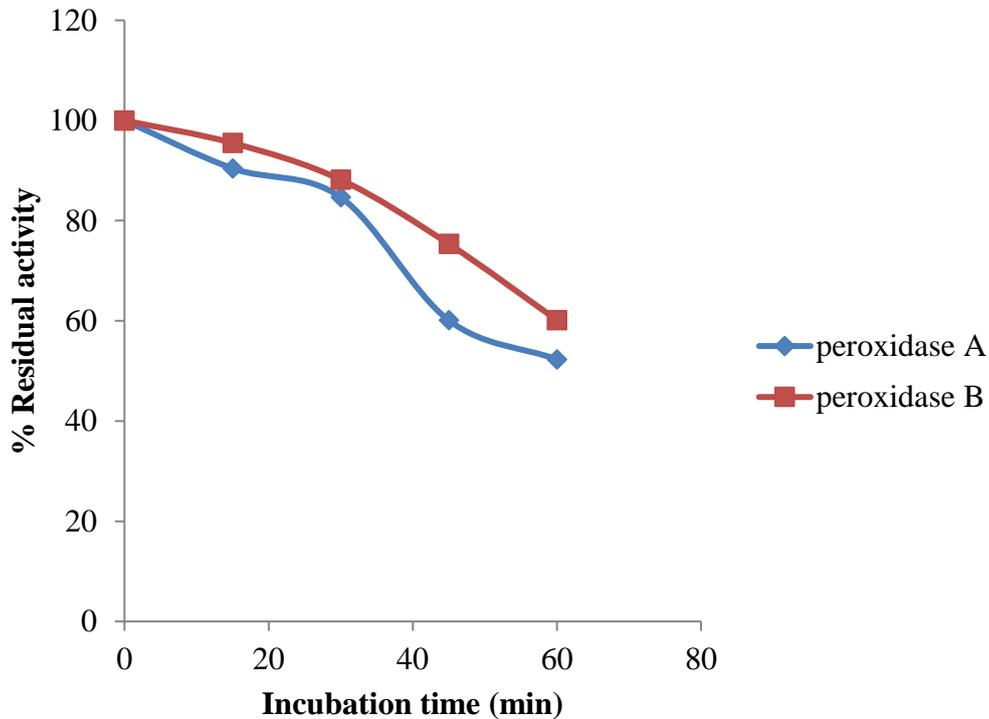


Fig. 19 stability curve of peroxidase A and B at 50 and 60°C, respectively

4. 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

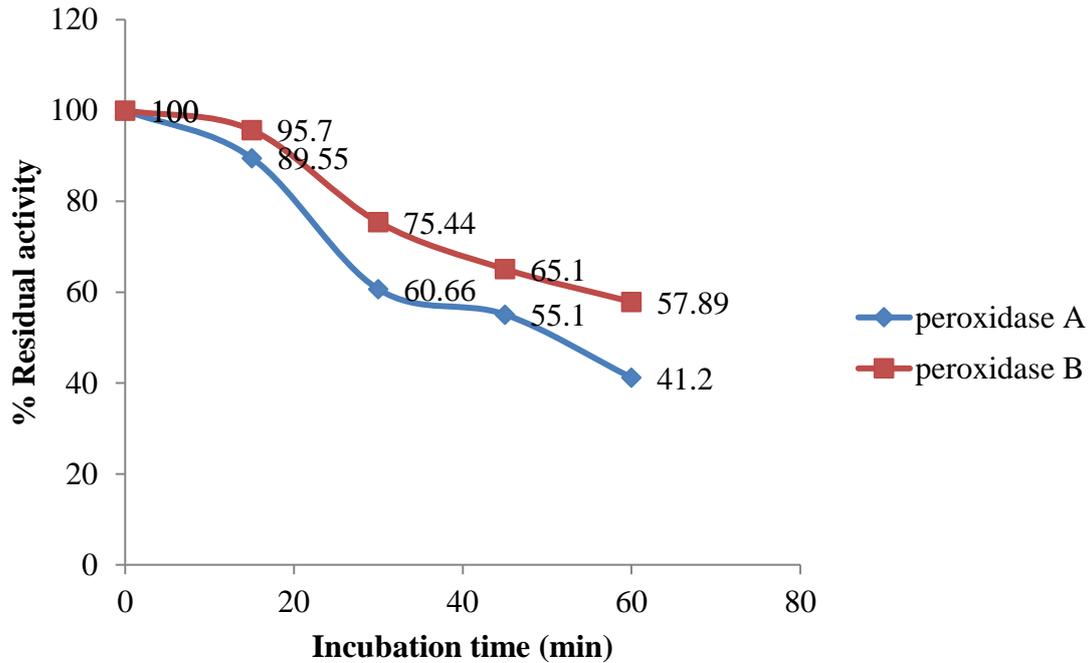


Fig. 20. Stability curve of peroxidase A and B at 70°C

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 exosecretory proteins by their housing microorganisms, among other physiologic conditions conditioning their catalytic rates, it has being 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 deepend in culture broth with fungus from the wet sampled soil. The screened *Aspergillia* were confirmed *Aspergillus*

tamari and *Aspergillus fumugatus* 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 ($\Omega^{-1} \text{Cm}^{-1}$), 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. [27] 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 give ecological niche over the time and contributes to the high $[\text{H}^+]$ of the medium, however the acidity decreases when there are influx of solvations by fluids eg. Water [20]. 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. [30] 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* and *fumigates* 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. [26] reported day 5 for peroxidase production from *Nonomuraea gerzanensis* growing on alkali lignin. Difference in the days of peroxidase A and B production can be likened to the peroxidase producing *Aspergillus* sp typed difference. Peak production of peroxidase A and B were recorded at pH 5 and 6 respectively. Adewale and Adekunle, [31] 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* and *fumigatus* was carried out through ammonium sulphate precipitation and gel filtration respectively. Proteins with highest peroxidase activity were precipitated at 60 and 80% ammonium sulphate for peroxidase A and B respectively. As reported by Oparaji et al. [32] 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 help 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 A and B respectively.

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 235.65cm³. A phase peak of peroxidase A and B activity were 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. [33], they stated that there appears to be a relationship between dialysis of enzymes usually after ammonium sulphate 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 and B were purified upto 2.89 and 3.11 folds while specific activity increased upto 344.78 and 576.38 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 (invivo/invtro), 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 and B produced from *Aspergillus tamari* and *fumigatus* was 4.5 and 5.0 respectively. Peroxidase A and B activity peaked at 50 and 60°C respectively. Chanwun et al. [34] reported an optimum peroxidase activity from *Heceabrasiliensis* at 50°C. Dragana et al. [35] 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 and 2.44mM. 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 [33]. 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 A and B during catalysis shows V_{max} of 280 $\mu\text{mole}/\text{min}$ and 305 $\mu\text{mole}/\text{min}$ respectively.

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 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. [17] 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 spinned electron shell metals (especially transition metals) give more regulatory and activation effect in every enzyme/non enzymatic catalyzed reactions 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; 5.0) respectively, 7.0 and 8.5 as shown in fig 15,16 and 17 below. The stability curve was single biphasic which represent the first order; Peroxidase B maintained greater stability than A at its optimum pH and pH 7. 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. Peroxidase B was affected at the basic pH as the enzyme maintained less than 40% of its activity. Thermal stability of peroxidase A and B as shown in fig 18 and 19 at their respective optimum temperatures (50 and 60°C) and at 70°C showed a biphasic stability curve of peroxidase A with the initial phase occurring very fast and thereafter there was a noticeable retardation after 60min of incubation. Peroxidase B maintained greater stability than A at its optimum temperature and at 70°C. The enzymes maintained greater than 50% of their activity after 60 min of incubation. Stability curve of peroxidase A and B at 70°C showed a maximum activity of the enzymes after 30 min of incubation. However Peroxidase B maintained greater stability than A after 60 min of incubation. Peroxidase B maintained 57.89% of its activity after 60 min while peroxidase A from fig 19 showed a residual activity of 41.2%. the findings is in correlation with the results from Eze [36] who reported the stability and thermodynamics of peroxidase from oil bean seeds; he stated that peroxidase from oil bean seeds maintained upto 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.

5. 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 encourageable catalytic activity. The present study have shown the kinetics of the proteins from *Aspergillus tamari* and fumigates sampled from petroleum hydrocarbon spilled soil at respective seasons. The study has exposed the impact of physiologic season variations on microbial proliferations which goes inline with the stabilizing proteins like peroxidases. Peak activity of peroxidase B than A from the study is a guide for protein chemist and folks biotechnologist on plausible docking of organisms at respective seasons for optimum production of proteins with peak enzymatic activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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