Original Research Paper

Production and Characterization of Hydrocarbon Degrading Peroxidase from *Rhizopus* and *Saccharomyces* Species

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Abstract: The production, characterization and partial purification of hydrocarbon degrading peroxidase from di-culture of *Rhizopus* and *Saccharomyces* species was assessed. The fungi were isolated from hydrocarbon polluted soil and identified using standard microbiological and biochemical techniques. Fermentation was carried out for enzyme production and the extracellular extract was precipitated with 50% ammonium sulphate salts and further purified by gel filtration\using sephadex G100 gel and by dialysis. The enzyme activity and stability was assayed against varied pH and temperature. Four genera of fungi which included *Saccharomyces*, *Penicillium*, *Rhizopus* and *Aspergillus species* were isolated from the crude oil polluted soil of which *Saccharomyces* sp. and *Rhizopus* sp. produced peroxidase. Maximum enzyme production occurred on day 8. The enzyme activity was optimum at a pH of 4.5 and temperature of 50°C. The Km and V_{MAX} were 1.8 mg/mL and 20.3 μmol/min respectively, against the 5 mM of O-dianisidine substrate. Stability studies showed that the peroxidase was stable in the presence of Ca^{2+}, Mg^{2+} and Co^{2+}, with Ca^{2+} showing the best stability. In conclusion, peroxidase was partially purified from di-culture of *Saccharomyces and Rhizopus* species isolated from crude oil polluted soil. The presence of this enzyme in crude oil polluted soil may suggest its implication in the degradation of crude oil. Further studies need to be done to assess the potential of this enzyme in the degradation of crude oil.

Keywords: Peroxidase, *Rhizopus* sp., *Saccharomyces* sp., pH and Temperature Stability

Introduction

Microorganisms have been considered as treasure of useful enzymes. Microorganisms possess specific enzyme systems that enable them to degrade and utilize hydrocarbons as their sole carbon and energy sources (Al-sayegh *et al*., 2016). Fungal isolates from polluted soil are able to use hydrocarbon present in that area as substrates for growth. This can be achieved probably by releasing extracellular enzymes and acids which are capable of breaking down the recalcitrant hydrocarbon molecules into simpler forms or products that can be absorbed for the growth and nutrition of fungi. Enzymes are biological catalysts that facilitate the transformation of substrates into products by providing favorable conditions that lower the activation energy of the reaction. The functional diversity of these enzymes and their specificity in reactions has led to increased interest in the production process and field application studies of enzymes (Nair and Jaayachandran, 2017). Peroxidases are oxidative enzymes with the capacity to oxidize several recalcitrant compounds. They are characterized by various biotechnological potentials spanning different industries including textiles, paper and pulp, chemicals, water and cosmetics etc. (Draelos, 2015; Taboada-Puig *et al*., 2015). They are ubiquitous enzymes that catalyse the oxidation of lignin and other phenolic compounds at the expense of hydrogen peroxide (H_2O_2) in the presence of a mediator. These peroxidases can be

heam or non-heam proteins (Chandrakant and Shwetha, 2011). The super-family of haem peroxidases from plants, fungi and bacteria are a group of enzymes that utilize hydrogen peroxide to oxidize a second (reducing) substrate, often an aromatic oxygen donor. Peroxidases catalyze various oxidative reactions in which electrons are transferred to peroxide species (often H_2O_2) and substrate molecules are oxidized. These enzymes have been found in all living organisms, involved in a variety of biological processes (Krainer and Glieder, 2015).

The detoxification of toxic organic compounds by various bacteria and fungi through oxidative coupling is mediated by oxidoreductases such as peroxidase. During such oxidation-reduction reactions, the contaminants are reduced to harmless or less harmful compounds. Hamid *et al.* (2016) reported in his review, that peroxidase are involved in the manufacturing of many aromatic complexes, elimination of phenolics complexes from waste water and peroxides from foods, beverage and industrial wastes. However, its isolated form, peroxidases are among the most useful enzymes in biotechnology, since several industrial processes can be performed by this kind of enzyme, such as soil detoxification and bioremediation of waste waters contaminated with phenols, cresols and chlorinated phenols (Pinto *et al.*, 2015).

Various studies have shown that peroxidases from various sources especially microbial sources have remarkable properties in the degradation of varying concentration of crude oil (Ehiosun and Usman, 2018; Feltrin *et al.*, 2017a; 2017b; Neelam and Shamsher, 2013) and detoxification of polluted environment based on their ability to catalyze the reduction of peroxides and the oxidation of a variety of organic and inorganic compounds. However, little is known on the characteristics of peroxidase produced from fungi species especially from hydrocarbon populated soils. This study was aimed to produce, purify and characterize peroxidase isolated from fungi species obtained from crude oil polluted soil.

Materials and Methods

Sampling of the Crude Oil Spilled Site

Soil samples were collected from crude oil contaminated soil around crude effluent disposal of Onne, Eleme Local Government Area (LGA), River State Nigeria. They were put into a clean aseptic container and transferred to the laboratory.

Isolation and Identification of Rhizopus and Saccharomyces Spp. from the Crude Oil Polluted Soil

Fungal isolates from crude oil contaminated soil were obtained according to the method described by Ezeonu *et al.* (2013). Serial dilution of soil sample was done and inoculated on Sabaround Dextrose Agar (SDA)

media prepared according to the manufacturer's description. The inoculation plates were incubated at 30°C for 3-4 days for colony growth. Colonies of different hydrocarbon utilizing fungi and hydrocarbon degrading fungi were randomly picked and pure isolates were obtained following repeated sub-culturing on SDA. The spores of the isolated fungus were harvested and, aseptically transferred to the SDA slants and incubated at 30°C for 4 days. The three day old pure cultures were morphologically examined and identified by microscopy according to the method of Ezeonu *et al.* (2013) and the features and micrographs were related to "Atlas of mycology" by Barnett and Hunter (1972). The fungal isolates were further identified and characterized using biochemical techniques as described by Cappuccino and Sherman (2004) with some modifications. The total heterotrophic biomass from both the nutrient media and the mineral salt agar was counted from the grown media plate as follow:

Colony forming unit per gram (CFU/g) = colony observed x dilution factor × volume of inoculum

Production of Peroxidase from the Isolates

A wire loopful of the inoculum was incubated for 3 days at room temperature in a basal mineral medium supplemented with O-dianisidine (standard substrate) prepared in 3% methanol and 0.2 m of phosphate buffer (pH 6.0). Peroxidase was produced from the di-culture of fungal isolates using submerged fermentation technique according to Ezeonu *et al.* (2013). Hundred mililiter (100 mL) of sterile culture medium optimized for hydrocarbon degrading peroxidase with various optimized nutrients (2g of $C_6H_{12}O_6$, 0.4g of $CaCO_3$, 0.5g $FeSO_4$, 0.1g $AgNO_3$) at favorable physical conditions (incubation temperature, (25-40°C), pH (3-5)) was used for the enzyme production in a 250 mL Erlenmayer sterile flask. The flask was stoppered with aluminum foil and autoclaved at 121°C for 15 min. Two discs of the respective fungal isolates were added in the medium using a cork borer of diameter 10 mm and the culture was incubated for 14 days at 30°C. Each day, the filtrate was assayed for peroxidase activity and extracellular protein concentration. The day of peak activity of the peroxidase was chosen for mass production of the enzyme from the respective fungal isolates.

Enzyme Assay

The supernatant was collected and the mixture was filtered using Whatman No. 1 filter paper. The filtrate was centrifuged at 3500 rpm for 15 min and supernatant was considered as the crude enzyme. Peroxidase activity was assayed using the method of (Mclellan and Robinson, 1987; Eze *et al.*, 2010). The change in absorbance at 460 nm due to the oxidation of o-dianisidine in the presence of hydrogen peroxide and

enzyme extract at 30°C was determined using Jenway 6405 UV/VIS Spectrophotometer. The standard assay solution contained 0.3 mL of 0.1% o-dianisidine, 0.2 mL of hydrogen peroxidase, 2.4 mL of sodium phosphate buffer pH 6.5 and 0.1 mL of enzyme extract in total of 3.0 mL. One unit of enzyme activity was defined as the amount of enzyme that gave an absorbance change. = 0.1/min at 30°C. The readings were taken every 30 s for 5 min.

Protein concentration of the crude extract was quantified at 750 nm according to the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Partial Purification of the Crude Extract

The crude extract was purified following three fold purification method according to Ertan *et al.* (2012)

Ammonium Sulphate Precipitation

Peroxidase was precipitated by gently stirring of the crude enzyme with 20-90% saturation of solid ammonium sulphate ((NH$_4$)$_2$SO$_4$) with increasing concentration of 10% in each tube. The ammonium sulphate crude enzyme solutions were allowed to stand at cold temperature of 4°C for 30 h till the supernatant could be gently decanted off. The test tubes were centrifuged at 3500 rpm for 20 min. Precipitates from the individual percentage ammonium sulphate saturations were re-dissolved respectively in equal volumes of 0.1 M sodium phosphate buffer pH 6.5. Peroxidase activity of the precipitates was assayed to determine the percentage ammonium sulphate saturation that precipitated enzyme with maximum activity. To the remaining volume of the enzyme extract, 75.52 g of (NH$_4$)$_2$SO$_4$ was added slowly to the enzyme extract in a beaker until it became 70% saturation. The pellet holding major peroxidase activity was suspended in 0.1 M sodium phosphate buffer (pH 6.5) and the enzyme activity and protein content were determined.

Dialysis

The supernatant was discarded and precipitated extract was transferred in a dialysis bag suspended in a beaker containing ice pack and 0.01 M sodium phosphate buffer (pH 6.5) and kept for 48 h. The buffer was continuously stirred, changed after every 12 h and the total volume of the dialysed protein was measured and recorded. The enzyme activity and protein contents of the dialyzed extract was determined at 460 nm using o-dianisidine, as substrate and 750 nm for protein determination using bovine serum albumin (BSA) respectively. The dialyzed protein was further purified using Sephadex G-75 by column chromatography.

Gel Filtration

Ten gram of Sephadex G-75 was soaked in 0.1 M sodium phosphate buffer (pH 6.5) for 12 h. The prepared gel was gently poured in a standing column tube (50 cm by 2.5 cm). The packed gel was equilibrated with 0.1 M sodium phosphate buffer solution (pH 6.5) and 30 mL of the enzyme solution was introduced into the column. A total of 70 fractions were collected in 5 mL fraction tubes at a flow rate of 5 mL per 20 min. The protein concentration of each fraction was determined using a spectrophotometer at wavelength of 280 nm. Peroxidase activity of each fraction was also assayed using the spectrophotometer at 460 nm. The active fractions were pooled and stored at-4°C.

Characterization of the Peroxidase

The enzyme characterization was done via determination of optimum parameters below according to the method of Eze *et al.* (2010).

Determination of Optimum pH

The activity of peroxidase was assayed at various pH ranging between 4.0 and 9.0 for 24 h at 35°C using the following buffers: Sodium-acetate buffer (0.1 M, pH 4-5.5); sodium phosphate buffer (0.1 M, pH 6.0-7.5) and Tris- HCl buffer (0.1 M, pH 8-9.0). The residual activity was then determined using o-dianisidine as the reducing substrate for the assay.

Determination of Optimal Temperature

The optimum temperature was determined by incubating the enzyme at its optimal pH at various temperatures ranging from 30 to 70°C in a water bath. Thereafter, the residual activity of the partially purified peroxidase was determined after incubating the enzyme at the various temperature ranges for 4 h in phosphate buffer at 6.0. After heat treatment, the enzyme solution was cooled and the residual activity assayed under standard assay conditions.

Determination of Kinetic Parameters

The Michaelis-Menten kinetic parameters Km and Vmax were determined by measuring the peroxidase activity using various concentrations of O-dianisidine complex as substrate in the presence of hydrogen peroxide and a suitable amount of purified enzyme. They were mixed and incubated to determine the effect of the substrate. The parameter values were obtained by curve fitting of the reciprocal plot of reaction rates versus substrate concentrations using the Line-weaver bulk plot transformation of Michealis-Menten rate equation.

Stabilization Studies of the Purified Enzyme

The purified extract was incubated with various concentrations (0.01-0.05 M) of each divalent metal ions (Ca^{2+}, Mg^{2+}, CO^{2+} and Pb^{2+}) according to the method of Singh *et al.* (2012). At varying time interval, an aliquot was drawn from the incubated sample and assayed for residual activity as follows:

$$\% \ Residual \ activity \ \frac{At}{Ao} \times 100$$

where, *At* is activity at present while *Ao* is activity at time 0. The % residual activity was plotted against different time interval of incubation.

Statistical Analysis

The average values of duplicate experiments were taken. The results were presented in tables and figures. Graphs were plotted using Microsoft excel worksheet 2010 model.

Results

Microbial Isolation and Identification

The soil samples from the crude oil polluted soil had heterotrophic and hydrocarbon utilizing fungi with CFU/g of 6.9×10^8 and 1.15×10^7 while that of the uncontaminated soil (control) was 2.58×10^8 and 5.5×10^2 respectively. The polluted site also had the highest percentage of heterotrophs that are hydrocarbon utilizing. A total of four fungi isolates, belonging to three fungal families were identified. They included *Saccharomyces*, *Penicillium*, *Rhizopus* and *Aspergillus* species.

Production of Peroxidase from the Isolates

The organisms of interest, *Rhizopus* and *Saccharomyces* spp. tested positive for the peroxidase production with a distinct yellow coloration in the presence of the standard substrate O-dianisidine. A 14 day co-cultures of *Rhizopus* and *Saccharomyces* spp. showed a rise in the peroxidase activity with a peak on day 8 after which there was a decrease till day 14 as shown in Fig. 1.

Purification of the Crude Peroxidase

The crude peroxidase enzyme precipitated efficiently when 70% of the ammonium sulphate salt was used. The precipitated pellet showed the greatest peroxidase activity compared to the supernatant which precipitated when 20% ammonium sulphate was used (Fig. 2). Further purification by size exclusion gel chromatography using Sephadex G-75 (Fig. 3) showed that the specific activity (U/mg) of peroxidase and protein concentration were high between fractions 20-28 and 40-45. Fractions 25 and 45 which showed the greatest peroxidase activity were used for the characterization studies. Peroxidase activity increased sequentially in each of the three purification methods (size exclusion chromatography > dialysis > ammonium sulphate precipitation).

Characterization of the Purified Enzyme
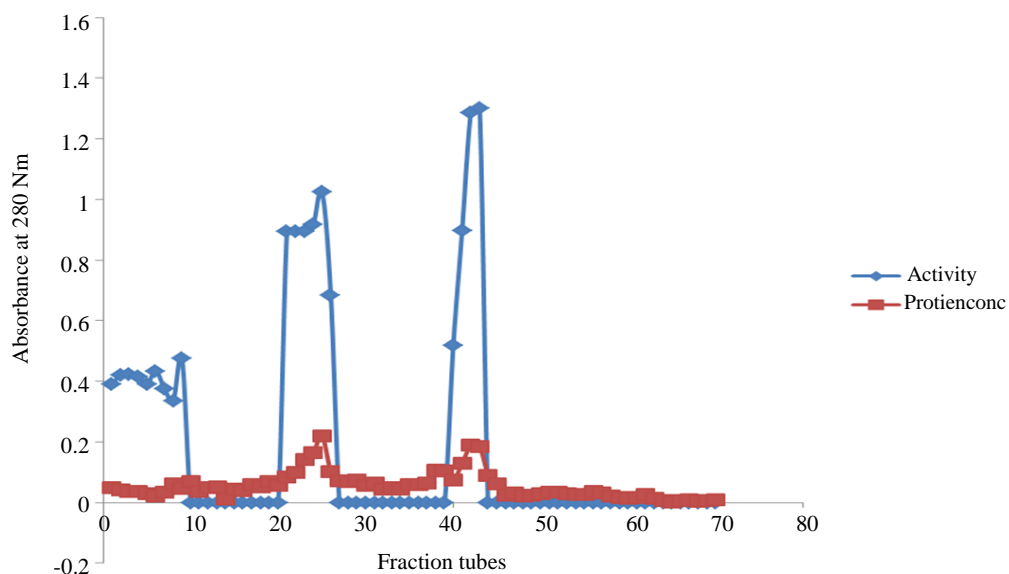
Optimum pH

Peroxidase was active over a broad pH range of 3.5-8.0 with an optimum activity at pH 4.5 as shown in Fig. 4. However, the control showed maximum activity at pH 6.2. More so, the peroxidase was relatively stable at a pH range between 4.0 and 5.5 as the enzyme residual activity remained high between 65 and 76% at this pH range (4.0-4.5) after 180 min of pre-incubation of the enzyme at various pH. The enzyme activity decreased at higher pH. The optimum pH for the test sample was used for the stability studies.
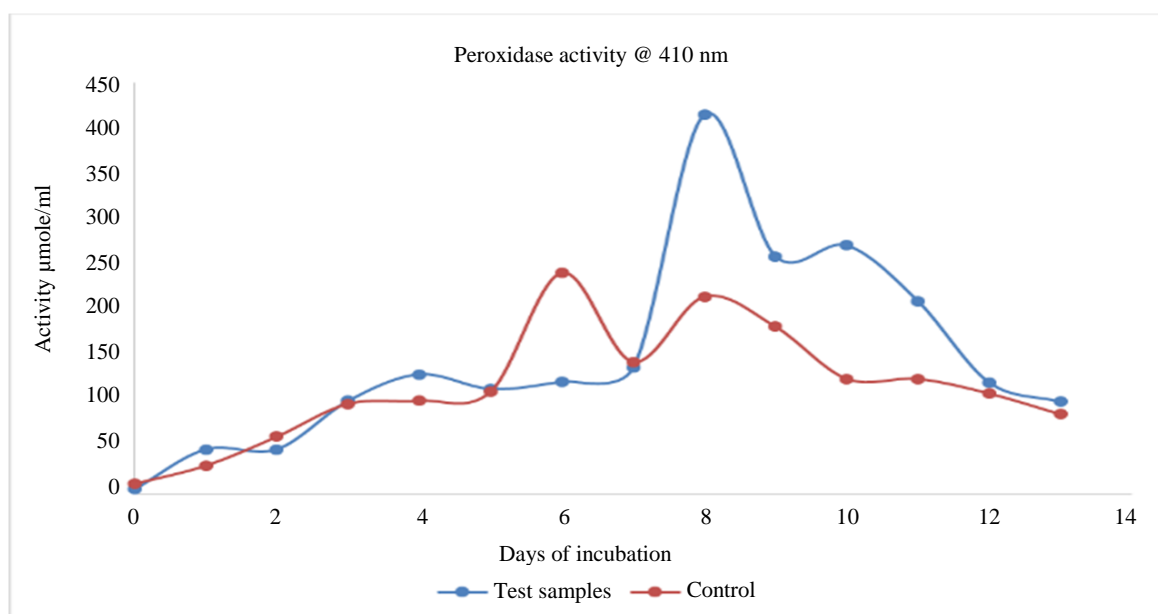
Fig. 1: Effect of incubation days on the enzyme activity of the peroxidase produced from the di-cultures of *Saccharomyces* sp. and *Rhizopus* sp
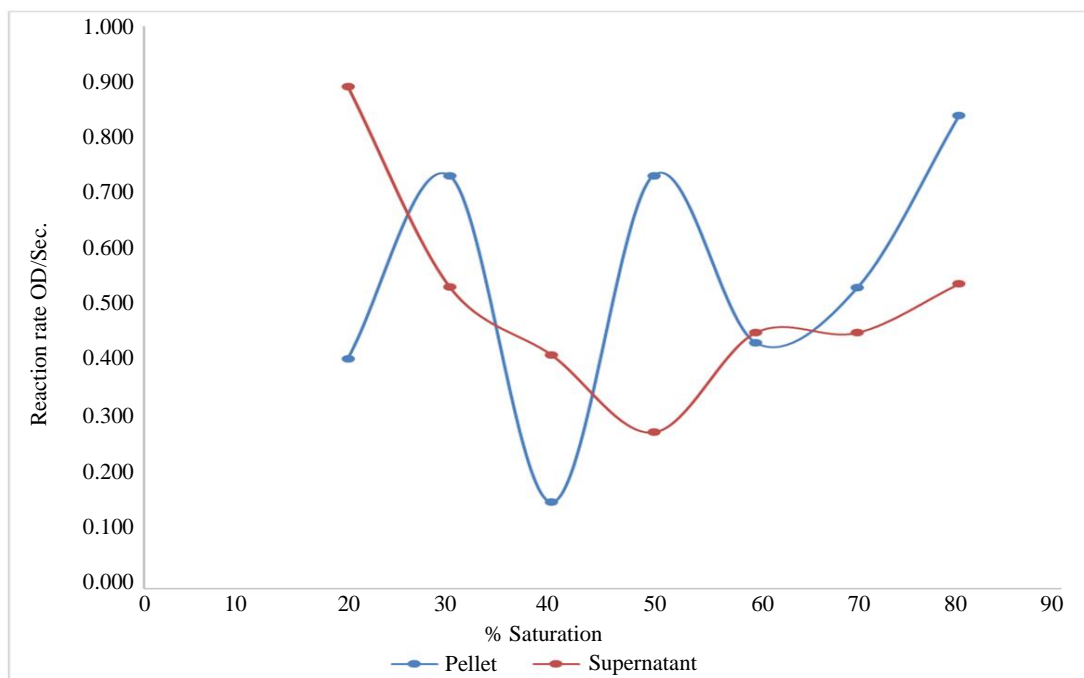
Fig. 2: Ammonium sulphate precipitation profile of the peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp.

Fig. 3: Gel elution profile of the protein extracts produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp

Optimum Temperature

Thermo-stability analysis revealed that peroxidase had maximum stability at temperature of 60°C as 58% activity was retained after 180 min of pre-incubation at this temperature as shown in Fig. 5. The enzyme activity examined at different temperatures showed an optimum activity at 50°C while the control showed optimum activity at 40°C. The result illustrated in Fig 5 showed an increase in peroxidase activity with the increase in temperature and reached the maximum at 50°C, then it began to decline with increasing temperature until reached about 40% of its activity at 70°C for the test sample. The enzyme activity began to decline after the optimum temperature. The optimum temperature for the test sample was used for the stability study.
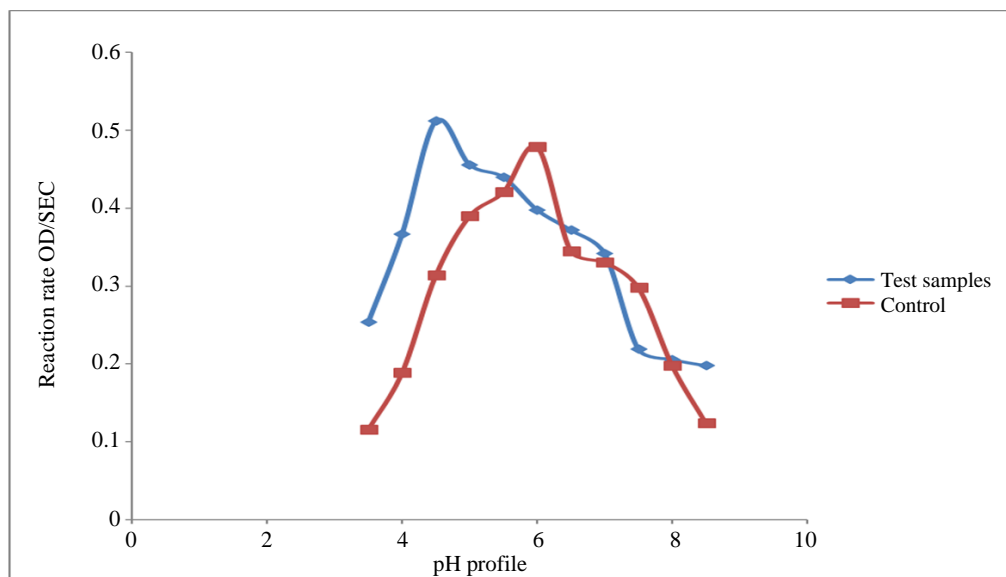
Fig. 4: Effect of pH profile on the activity of the peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp
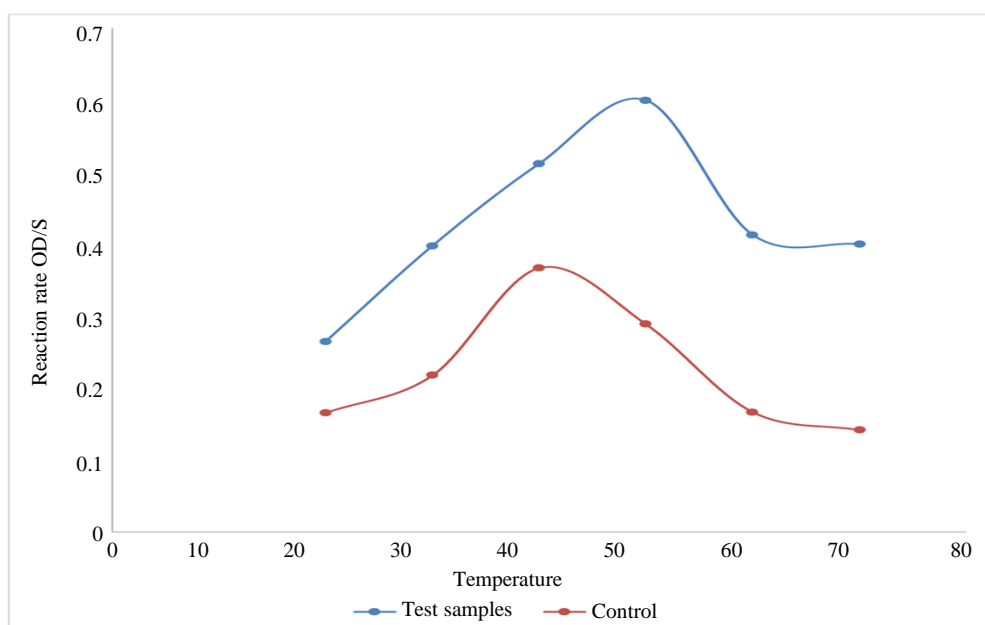

Fig. 5: Effect of varying temperature ranges on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* sp

Enzyme Kinetic Parameters

The activity of peroxidase increased with corresponding increase in substrate concentration until a saturation point of 5 mM for the O-dianisidine as shown in a Michaelis-Menten curve in Fig. 6. A Lineweaver-Bulk plot of the initial velocity using o-dianisidine substrate in Fig. 7 showed the peroxidase to have a Km of 1.8 mM and Vmax of 20 µ/ml/min.

Stability Studies on the Purified Enzyme Extract

Stability study at pH 4.5 showed $CaCl_2$, $MgSO_4$ and $CoCl_2$ to stabilize the enzyme activity of peroxidase. $CaCl_2$ showed the best stability of the enzyme as up to 90-95% of the enzyme activity was retained at 0.02 M $CaCl_2$ at pH 5.0-5.5 while 78-88% of the activity was retained at pH 3.5-4.5. The stabilization in activity

increased with increase in the concentration of the metal salts with 0.05 M concentrations of $CaCl_2$ conferring the highest stability. Other metal ions ($MgSO_4$ and $CoCl_2$) followed the same trend. Alternatively, $Pb^{2+}$ at all concentrations inhibited the enzyme activity of peroxidase as the enzyme lost about 83-95% of its activity with $PbCl_2$ at concentrations of 0.02-0.03 M (Fig. 8). Also at 50°C, the peroxidase activity was greatest when $CaCl_2$ was used. Thus, $CaCl_2$ showed the greatest stability of the enzyme activity at 50°C (Fig. 9).
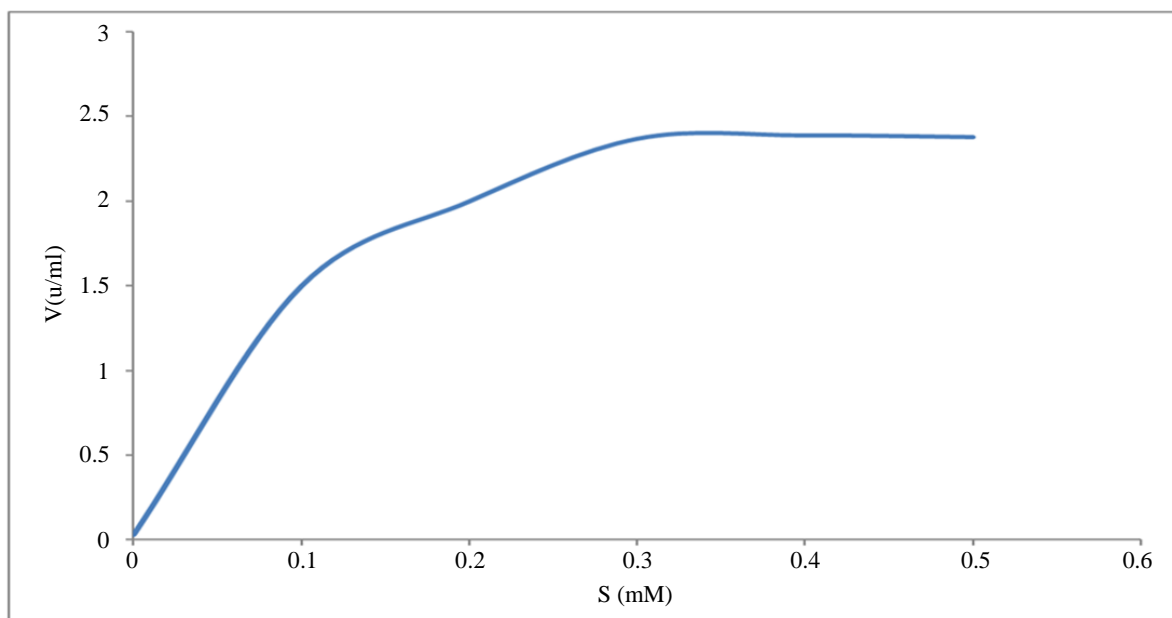
Fig. 6: Michaelis-Menten curve on the effect of o-dianisidine on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp
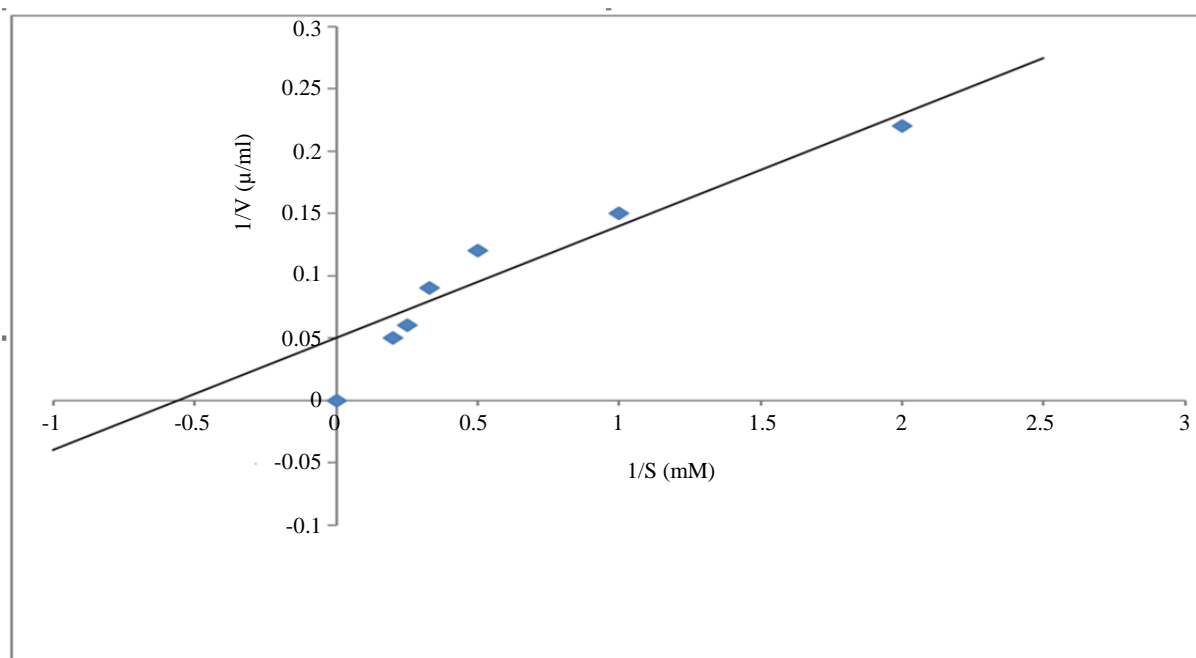
Fig. 7: Lineweaver-burke double reciprocal curve on the effect of o-dianisidine on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* spp
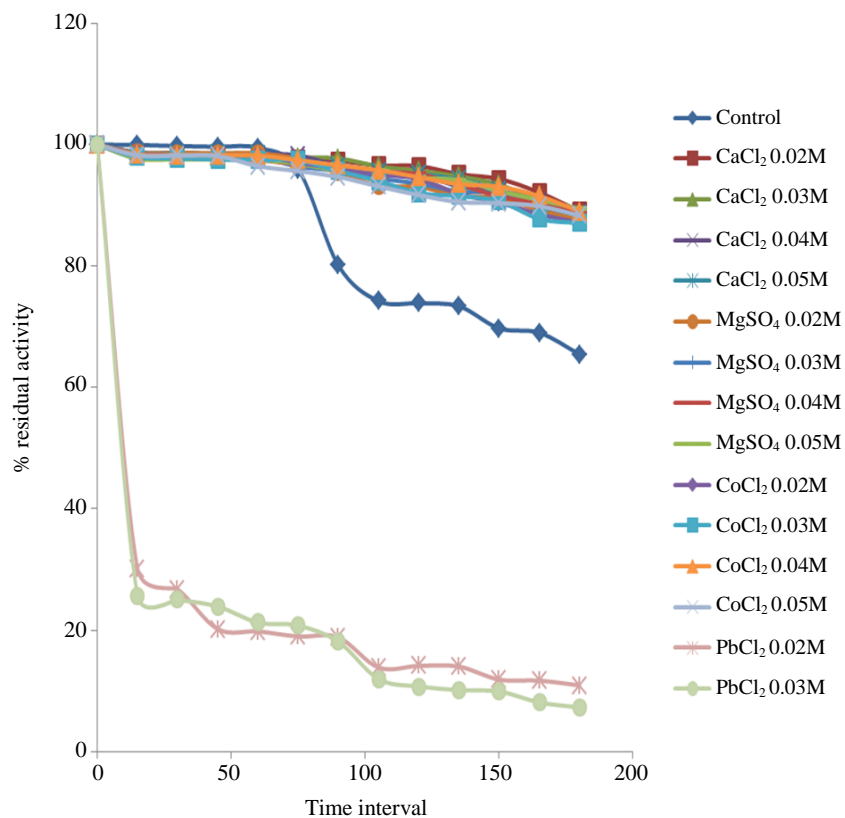
Fig. 8: pH 4.5 stability study on the activity of the peroxidase produced from the di-culture of *Rhizopus* and *Saccharomyces* sp. incubated for 180 min
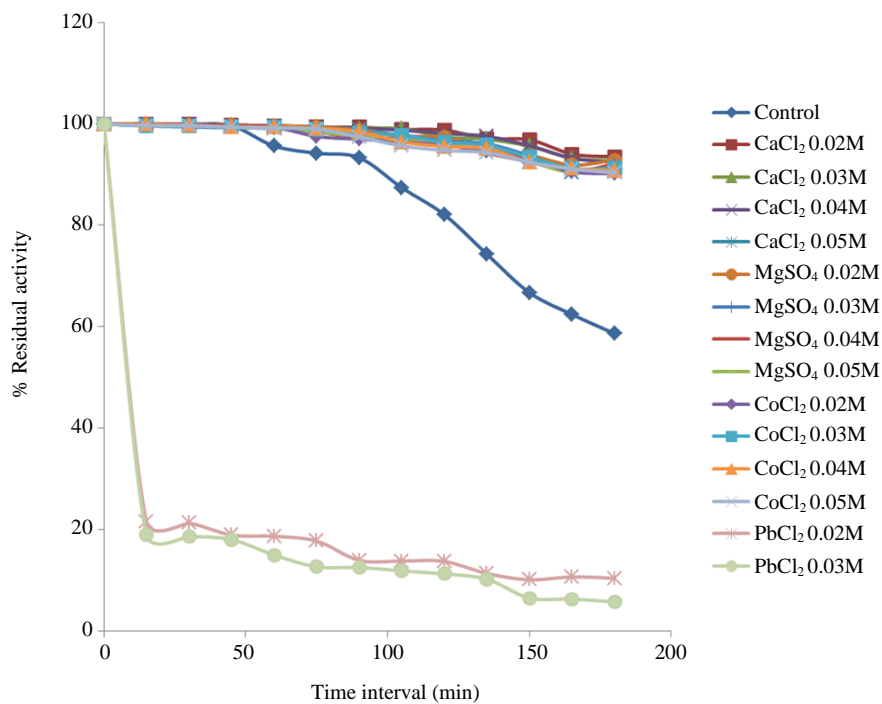
Fig. 9: Temperature 50°C stability study on peroxidase produced from the di-culture of *Rhizopus* sp. and *Saccharomyces* sp. incubated for 180 min

Discussion

Fungus is considered as an efficient candidate for potential degradation of hydrocarbons. Hundreds of different species of fungi inhabit the soil, especially near the soil surface where aerobic conditions prevail. Such fungi are active in degrading a wide variety of biological materials present in the soil (Saranraj and Stella, 2013). In this study, crude oil polluted soil contained heterotrophic and hydrocarbon utilizing fungi. This finding suggests that the polluted site may be actively receiving hydrocarbons which in turn increased microbial population. This study concurs with that of Chikere and Azubuike (2014) which showed high level of heterotrophic fungi count than hydrocarbon utilizing fungi confirming that the site was chronically polluted with hydrocarbons. Four fungi isolates which included *Saccharomyces, Penicillium, Rhizopus and Aspergillus species* were isolated. Previous studies have confirmed the presence of these fungi species in crude oil populated soil (Rahman *et al*., 2002; Brooijmans *et al*., 2009; Balaji *et al*., 2013).

Peroxidase activity was confirmed in *Rhizopus* and *Saccharomyces* spp. cultures which showed a characteristic yellow coloration in the presence of the standard substrate, O-dianisidine. Crude enzyme was produced from the fungal di-culture and the enzyme activity was greatest at day 8 of the incubation period. The crude enzyme was partially purified with 70% saturation of ammonium sulphate which led to increased activity of the enzyme. Similar result was observed by Idesa and Getachew (2018) who reported 80% saturation of Ammonium sulphate precipitation of peroxidase from plant sources. However, this result is comparable to the report of Hamid *et al*. (2016) whose peroxidase extracted from wheat bran showed that peroxidase was precipitated efficiently with ammonium sulfate in saturation ratio of 70%. Upon dialysis of the precipitated enzyme, unwanted salt and proteins were removed. Following further purification by gel filtration, the enzyme activity increased at each purification step. This is also similar to the findings of Hamid *et al*. (2016) who reported a purification fold of 2.4, 3.4 and 5.1 with specific activities of 730 U/ml on a three-purification step using ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography.

Characterization and stabilization of purified enzymes under controlled optimum condition (pH and temperature) are key physiological factors to be considered for maximum enzyme activity and for applications in industries and the environment (Singh *et al*., 2014). The partially purified peroxidase showed a maximum activity at pH 4.5 and was relatively stable in the pH range between 4.0 and 5.5. This result is similar to that of Osuji *et al*. (2014) which showed peroxidase extracted from garlic to have an optimum pH of 5.0 with

the highest percentage decolorization of dye at pH 4.5 and was stable at pH between 4.0 and 6.0. Also, another study by Bhatti *et al*. (2012) has shown peroxidase from *Raphanus sativus* to have an optimum activity at pH 4.0. The findings from this study agrees with findings of Pinto *et al*. (2015) who observed a broad range of pH 4.5 up to 7.5 for umbu peroxidase and its activity maintained at 70% levels. The pH curve also showed a bell-like shape distribution suggesting an involvement of two amino acid residues in catalysis as reported by Pinto *et al*. (2015).

Peroxidase when examined at different temperature ranges showed maximum activity at 50°C. The thermo-stability of the peroxidase dropped significantly at 70°C suggesting that temperature above that range can lead to denaturation and inactivation of the enzyme. Hamid *et al*. (2016) observed the same trend of optimum temperature for peroxidase activity purified from wheat bran, which showed an increase in peroxidase activity with the increase in temperature. This is comparable with the report of Osuji *et al*. (2014) and Bhatti *et al*. (2012) which reported peroxidase to have an optimum activity at 55°C. Chilaka *et al*. (2010) and Eze *et al*. (2010) reported a thermostable peroxidase from an oil bean plant with optimal temperature of 70°C. Nwagu and Okolo (2011) reported an optimum temperature of 70°C, 55°C and 50°C respectively for peroxidase from *Candida, Mucor* and *Aspergillus* respectively. Dayanand *et al*. (2011) reported an optimum temperature of 40°C and optimum pH of 3.0 in bacterial peroxidase isolated from strain of *Pseudomonas* sp. SUK1.

The effect of the substrate concentration on the peroxidase activity increased with increase in substrate concentration until a saturation point of 5 mM for O-dianisidine indicating that the active sites were saturated with the substrate. The Km (1.8 mM) for this study was similar to that of Kim and Lee (2005) who reported Km value of 1.1 8 mM for o-dianisidine and 1.27 mM for hydrogen peroxide. However, the Vmax (20 u/ml/min) was greater as compared to that of Kim and Lee with Vmax 0.032 u/ml/min for o-dianisidine and 0.138 u/ml/min for hydrogen peroxide, for peroxidase from *Raphanus sativus*. Pinto *et al*. (2015) reported that peroxidases oxidize several different substrates in the presence of hydrogen peroxide. Peroxidase showed high affinity and catalytic efficiency towards the O-dianisidine substrate.

The stability studies showed $CaCl_2$ to best stabilize the enzyme. Adefila *et al*. (2012) reported similarly in their work that Ca^{2+} and Mn^{2+} were potent activators of α-amylase. Pinto *et al*. (2015) reported a stimulatory effect of Ca^{2+} on peroxidase present in *Xilopodium* exsudates of umbu plants, with Mg^{2+} having similar effect but at a lower concentration than that of Ca^{2+}, whereas Mn^{2+} displayed inhibitory effect. However, Pb^{2+} inhibited the

activity of the enzyme. Singh *et al.* (2014) have also reported an inhibitory effect by Hg^{2+}, Pb^{2+} and Cu^{2+} and attributed this inhibition by these ions on the enzyme activity to the presence of indole amino acids in the enzyme and also a reflection of competition between exogenous and protein associated cations. In the presence of $CaCl_2$, the enzyme retained up to 87-89% activity at temperature range 30°C-40°C and 90-93% activity at temperature range of 50°C-60°C and 89-90% at 70°C. It was observed that the activity of the enzyme increased as the metal ions concentrations increased in all metal salts. $MgSO_4$ showed a promising strength in thermo-stability of the enzyme while $CoCl_2$ followed suit. In the presence of Pb^{2+}, the enzyme lost 90-95% activity in all the temperature ranges. These findings is in accordance with that of Adefila *et al.* (2012) and Singh *et al.* (2014) respectively which stated that Pb^{2+} and other heavy metals (Cu^{2+}, Hg^{2+} and Zn^{2+}) are like poison to enzymes and thus can inhibit their activity. These results confirms that the effect of metal ions on peroxidase activity is highly variable over enzymes from different sources.

Conclusion

This finding showed that an appreciable amount of peroxidase was produced and partially purified from a di-culture of *Rhizopus* and *Saccharomyces* spp. isolated from crude oil polluted soil. The characteristics of peroxidase produced was able to show activity over a wide range of pH (3.5-8.0) and temperature (20°C-70°C) with optimum activity at pH 4.5 and temperature 50°C and was best stabilized by $CaCl_2$. Therefore, the peroxidase exhibit high thermostable properties and has proven to be an environmentally stable enzyme which can be employed as a molecular tool for biotechnological process. Peroxidases among other vital biotechnological applications are useful tools in environmental monitoring. They are relatively cheaper to produce and do not require much augmentation and bio-stimulation in the presence of recalcitrant. Therefore peroxidase from the di-culture of *Rhizopus* and *Saccharomyces* spp. can be produced on large scale for industrial and eco toxicological relevance. The three-step purification and stability process indicates its strong capability for bioremediation processes. However, further studies are necessary to ascertain its effectiveness in the biodegradation of hydrocarbon contents in a petroleum polluted soil.

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Author's Contributions

Ferdinand U. Paschaline: Conceived and designed the experiments, performed the experiment and processed the data, analyzed the data and wrote the manuscript.

Onyeocha O. Ignatius: Supervised the research, analyzed the data, interpreted the data and revised the manuscript.

Ezeji E. Uchechukwu: Co-supervised the research and revised the manuscript.

Ukwandu C. D. Nnamdi: Analyzed the research design and methodology, interpreted the data.

Engwa A. Godwill: Co-supervised and revised the manuscript.

Oparaji E. Henry: Guided the experimental design, performed the experiment and processed the data.

Ethics

Authors declared no ethical issues that may arise after the publication of this manuscript.

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