WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Production, Partial Purification and Characterization of Peroxidase Using *Aspergillus Terreus Under* Submerged Fermentation

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ABSTRACT

Background and Objective: Peroxidases (EC number 1.11.1.x) represents a large family of oxidoreductases known to catalyze the oxidation of a variety of inorganic and organic substrates using H_2O_2. These enzymes are widely distributed in nature and are found in both eukaryotic and prokaryotic organisms. They are involved in biological processes such as the breakdown of toxins, heavy metals detoxification and hormone regulation. Peroxidases are widely distributed in plants, animals and microorganisms, where they protect the cells against the effects of oxidative stress and cell damage due to H_2O_2. Bacteria and fungi are the most frequently used microorganisms for industrial enzyme production. This is due to the fact that microbial sources are cheaper, their enzyme production and secretion systems are well-know and more controlled, hence they are preferred over other enzyme sources such as animal or plant. The aim of this study is isolation, partial purification and characterization of peroxidase from *Aspergillus terreus* under submerged fermentation. Materials and Methods: Fermentation filtrate was estimated for peroxidase activity, guaiacol was used as a substrate found out that the broth showed peroxidase activity. Results: The optimal PH and temperature of the enzyme were 6.0 and 25^{o}C respectively. The enzyme was more stable at alkaline pH than at the acidic and it retained 80% of the activity at 30^{o}C for 60 minutes. All these data suggest that the selected strain of *Aspergillus terreus* can significantly produce peroxidase enzyme from the rice bran substrate. Details on the microbial production of the enzyme as well as industrial applications including the effects of various environmental parameters were carried out. Conclusion: The result obtained indicates that rice bran can be successfully used in the production of peroxidase under submerged fermentation varying the physiochemical parameters such as incubation period, temperature and pH.

KEYWORDS: peroxidase, enzyme, guaiacol, *Aspergillus terreus,* fermentation, temperature

Introduction

Enzyme metabolism is a fundamental biological process that is crucial for the survival of all species. Their specific function is to catalyze chemical reactions Raja *et al.,* (2011). Some examples of enzyme includes; Peroxidase, amylase, cellulose, chitinase, dehydrogenase, phosphatase, protease and many more. Peroxidase is an enzyme found in a wide variety of organisms, from plants to humans to bacteria. Peroxidases are classified as oxidoreductases and are the second largest class of enzymes applied in biotechnological processes. These enzymes are used to catalyze various oxidative reactions using hydrogen peroxide and other substrates as electron donors. Its function is to break down hydrogen peroxide (H_2O_2), which is one of the toxins produced as a by-product of using oxygen for respiration Whitson (2020). Peroxidases, is a key antioxidant enzymes that is widely distributed in nature and catalyze oxidation of various electron donor substrates concomitant with the decomposition of H_2O_2 Pandey *et al.,* (2017).

They are isolated from various sources such as plants, animals and microbes. Peroxidase enzymes have versatile applications in bioenergy, bioremediation, dye decolorization, humic acid degradation, paper and pulp, and textile industries. Besides, Peroxidases from different sources have unique abilities to degrade a broad range of environmental pollutants such as petroleum hydrocarbons, dioxins, industrial dye effluents, herbicides and pesticides. Ironically, unlike most biological catalysts, the function of peroxidases varies according to their source. For instance, manganese peroxidase (MnP) of fungal origin is widely used for depolymerization and demethylation of lignin and bleaching of pulp, horseradish peroxidase of plant origin is used for removal of phenols and aromatic amines from waste waters. Microbial enzymes are believed to be more stable than enzymes of plant or animal origin. Thus, making microbially-derived peroxidases a sought-after biocatalysts for versatile industrial and environmental applications. Soil enzymes are a group of enzymes found in soil. They are excreted by soil microbes such as fungi, bacteria and archaea, and play a key role in decomposing soil organic matter into humus, in the process releasing nutrients essential for the growth of plants.

Fungi elaborate a wide variety of Peroxidase enzymes than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients (Haq *et al*., 2006). Several species of strains including fungi (*Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporiumkeratinophilum, Fusariumgraminarum,*

Penicilliumgriseofulvin, Scedosporiumapiosermum) and bacteria (Bacillus licheniformis, Bacillus firmus, Bacillus alcalophilus, Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus subtilis, Bacillus thuringiensis) are reported to produce peroxidase (Ellaiah *et al.,* 2002). A microbe is commonly used to produce peroxidase due to its feasibility in genetic manipulation, rapid growth rate, and wide biochemical diversity. Fungal species like *Aspergillus, Penicillium,* and *Rhizopus* are also generally used for peroxidase production as they are considered safe. In the production of peroxidase, it has been shown to be inducible and was affected by the nature of the substrate used in fermentation. Therefore, the choice of an appropriate inducing substrate is of great importance. The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of a microbial population. The most important among these are the medium, incubation temperature and pH. The pH of the fermentation medium is reported to have substantial effect on the production of proteases (AlShehri, 2004). It can affect growth of the microorganisms either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces.

Economically, submerged fermentation offers many advantages including superior volumetric productivity, use of simpler machinery, and use of inexpensive substrates, simpler downstream processing, and lower energy requirements when compared with Solid state fermentation (Paranthaman *et al.,* 2009). Another important environmental factor is the incubation temperature, which is important to the production of proteases by microorganisms. Higher temperature is found to have some adverse effects on metabolic activities of microorganisms producing peroxidase enzymes.

Practically all the fermentation processes were based on the concepts and theory of SSF as it played a very important role for humankind, mainly for food and beverages, both in western and eastern countries. In recent decades, SSF employing microorganisms for the production of biomolecules and has been applied in various industries and sectors, including textile, pharmaceutical, food, biochemical and bioenergy, and others (Pandy, 2003; Soccol *et al.,* 2017 and Farinas *et al.,* 2015). Submerged fermentation can be utilized and applied in a controlled way to produce value-added products, for example, enzymes (Soccol *et al.,* 2017).

Fungi such as *Aspergillus niger, Fusariumculmorum,* and *Penicillium* can excrete significant amounts of enzymes and metabolites through SmF as this technique can stimulate their natural habitat. There are approximately 60% of commercially available enzymes produced from fungi. Yeasts are also suitable for SmF. Filamentous fungi and yeasts are able to grow in an environment with low water activity. Some species of bacteria, such as *Bacillus thuringiensis, Pseudomonas sp.,* and *Bacillus subtilis,* have also been employed to produce the enzyme through SmF (Singhania *et al.,* 2009; Soccol *et al.,* 2017 and Meghavarnam *et al.,* 2017). Different carbon sources such as rice bran and cassava bagasse have been studied for the induction and biosynthesis of peroxidase. However, rice bran is reported as a superior carbon source for the production of peroxidase by *Aspergillus terreus.* The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling (Singh *et al.,* 2009). Major impediments to the exploitation of commercial enzymes are their yield, stability, specificity and the cost of production. New enzymes, for use in commercial applications with desirable biochemical and physiochemical characteristics and low production cost is the focus of most research (Kabili, 2007).

Submerged fermentation (SF) was chosen for the present research because it has been reported to be of more grated productivity than that of solid state fermentation.

Waste is any unwanted material intentionally thrown away for disposal. However, certain wastes may eventually become resources valuable to others once they are removed from the waste stream (Wiebe, 2003). Waste products arise from our ways of life and they are generated at every stage of process of production and development. Solid waste is used to describe non-liquid waste material arising from domestic, trade, commercial and public services. One of the most critical problems of developing countries is improper management of vast amount of wastes generated by various anthropogenic activities. More challenging is the unsafe disposal of these wastes into the ambient environment. Water bodies especially freshwater reservoirs are the most affected. This has often rendered these natural resources unsuitable for both primary and/or secondary usage (Fakayode, 2005). Wastes entering water bodies are both in solid and liquid forms. They are mostly derived from industrial, agricultural and domestic activities. The resultant effects of this on public health and the environment are usually great in magnitude (Osibanjo *et al.,* 2011). Edible foods are also wasted when cultural or individual preferences, sometimes the food is undesirable. Both edible and inedible foods may be considered garbage and therefore wasted. Edible foods are considered inedible when their quality deteriorates due to microbial contamination until they become unhealthy or noxious. Deterioration of food occurs from microbial contamination or rotting as a result of overproduction, storage problems, or improper preparation (Osibanjo *et al.,* 2011).

Owing to the increase in population and the ever growing development, there has been an increase in pollution especially environmental pollution and air pollution from various sources such as water waste and sewages, from manufacturing industries, pesticides, herbicides, etc. These pollutants has led to the damage and killing of normal Flora found in the soil for the plant growth and lignin degradation and also resulting to health risk thus, the need to seek a way for the remediation of our environment and natural resources. The use of enzymes in bioremediation has been advocated to be very effective and peroxidase has being considered for its several applications in bioremediation. Peroxidases from different sources which has unique abilities to degrade a broad range of environmental pollutants and its excellent option in bioremediation, the challenge remains the high cost of commercial enzymes. Providing a cheaper source of peroxidase from locally sourced agro waste is being considered alongside the optimization of few culture conditions. The aim of this study is to optimize a few culture conditions for the production of manganese peroxidase from fungi by submerged fermentation. The objective is to isolate fungi from soil and water samples, morphological identification of the fungi, screen for manganese peroxidase, produce manganese peroxidase by submerged fermentation, and characterization of manganese peroxidase in terms of temperature and pH.

MATERIALS AND METHODS

Sample Collection and Preparation

The soil and water samples were collected from three different riverine locations of Katsina-Ala (Benue State, Nigeria), Gindin Dorowa and Ibi in Taraba state, Nigeria. The soil samples were air dried and grinded and homogenization obtained, while the water sample was refrigerated at low temperature.

Enrichment and Isolation of Fungi in Media supplied with GP Source, Nitrogen and Phosphorus Sources

Pure sample were obtained by repeated subculture of the distinct fungal colonies. The culture is sub-cultured and maintained on potato dextrose agar plates grown at 27º C and stored at 4 ºC (Rajesh *et al*., 2016).

Screening for Fungi

Screening for Manganese Peroxidase was done by inoculating fungal mycelia on PDA medium supplemented with azure B dye as described by (XU *et a*l., 2015) with some modifications. After 24 days culture, the MnP producing strains were confirmed by a halo to colony ratio of the strain.

Morphological Identification

The isolated fungi was identified based on method by Sneath and Halt; 1986. Fungi isolated was identified based on cellular morphology and growth condition and the culture isolated was further harvested after 3 to 7 days of age and microscopical characters was assessed after staining with lacto phenol cotton blue (Fawole and Oso 1988).

Fermentation

Submerged fermentation (SmF) was carried out using the method described by Yogesh *et al*,. 2009. The filtrates was analysed daily for peroxidase activity (Roy *et al.,* 2018).

Manganese Peroxidase Assay.

Manganese Peroxidase activity was assayed by Rao and Tien (1995) at 470nm.

Determination of Crude Protein

Crude Protein determination was carried out by the method described by Lowry (1959).

RESULTS:

4.1 Screening of Peroxidase enzyme

a **b**

Fig. 1. Growth and morphology of *Aspergillus terreus*. A; Growth of *Aspergillus terreus* on peroxidase selected medium containing 0.6% guaiacol. B; Growth of Aspergillus *terreus* on Potato dextrose agar medium.

Fermentation

Result for incubation period from 11 days fermentation of Manganese peroxidase, day 9 gave the highest yield this comes after an appreciable yield record on the 9th day of fermentation.

Table 1: Effect of incubation

Days of incubation	**Activity** (μmolmin^{-1})
1	**0.85**
2	**0.85**
3	**0.67**
4	**1.15**
5	**0.85**
6	**1.10**
7	**2.61**
8	**5.28**
9	**0.55**
10	**0.43**
11	**0.18**

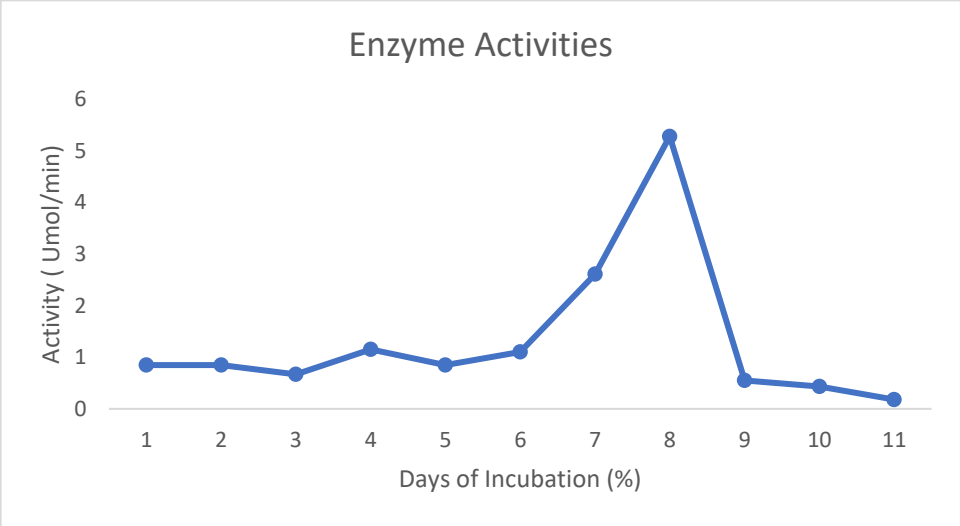

Fig. 2 Effect of incubation day on the Peroxidase enzyme activity

The above figure shows the result on the effect of incubation days on peroxidase activity. The highest enzyme activity was obtained on the 8th day.

Effect of pH on Manganese Peroxidase Activity

Varying the pH level showed an effect on peroxidase activity as shown in figure 4.3 below. Optimum pH for enzyme activity was found at pH 6.0 while the minimum activity was found to be at pH 9.0.

Table 2: Effect of pH on peroxidase enzyme activity

pH	**Activity** (μmolmin^{-1})
4.0	**0.98**
4.5	**1.21**
5.0	**0.85**
5.5	**0.43**
6.0	**1.34**
6.5	**0.61**
7.0	**0.31**
7.5	**1.16**
8.0	**0.16**
8.5	**0.49**
9.0	**0.22**

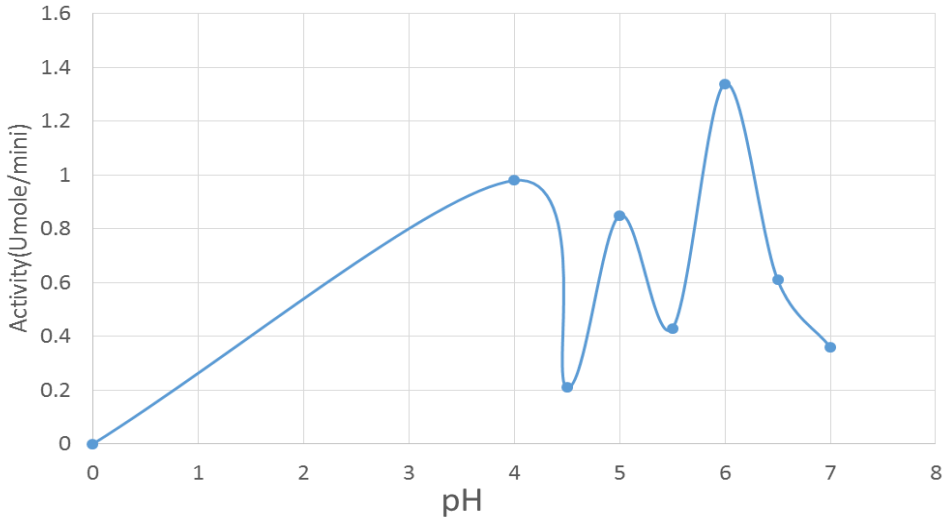

Fig. 3 Effect of pH on peroxidase enzyme activity

Effect of Substrate Concentrations

Change in substrate concentrations showed an effect on Manganese Peroxidase activity. The result revealed that optimum concentration of Manganese peroxidase activity is at 1.4% while the minimum activity was found to be at 0.4.

Table 3: Effect of Substrate Concentrations on peroxidase enzyme

Substrate Concentration (%)	**Activity** ($\mu molmin^{-1}$)
0.2	**7.1672**
0.4	**3.8569**
0.6	**6.6812**
0.8	**8.4338**
1.0	**8.3819**
1.2	**8.5641**
1.4	**8.6249**
1.6	**8.2119**
1.8	**8.6249**

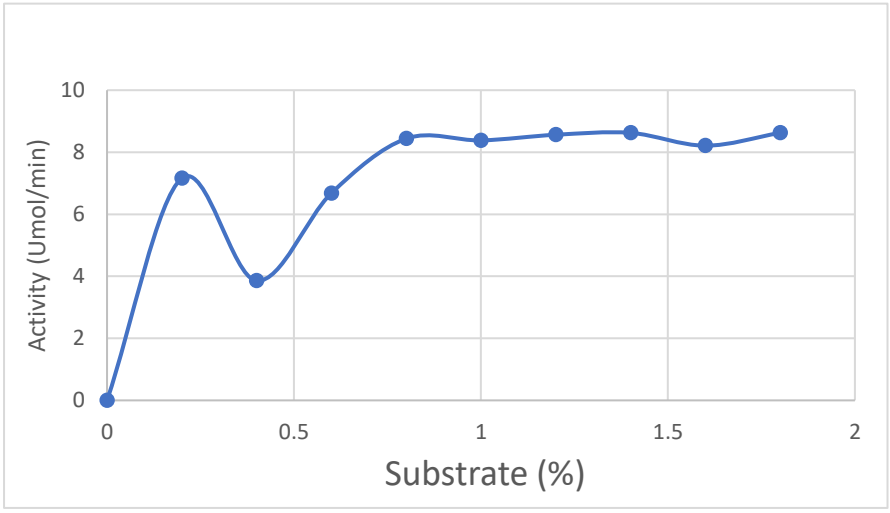

Fig. 4 Effect of Substrate Concentrations on Peroxidase enzyme

Effect of Temperature on Peroxidase enzyme

Change in temperature showed an effect on peroxidase activity as shown in figure 4.5 below, optimum temperature for enzyme activity was found to be at 25C while the minimum activity at 40^0C.

Table 4: Effect of Temperature on Peroxidase enzyme activity

Temperature	**Activity** (μmolmin^{-1})
25	**6.25**
30	**3.21**
35	**1.82**
40	**1.15**
45	**3.71**
50	**3.89**
55	**2.13**

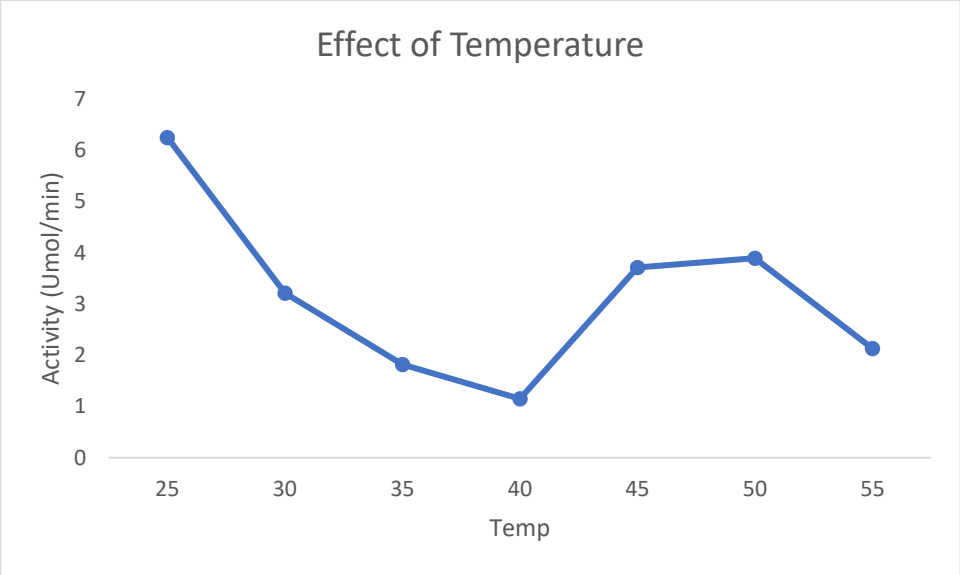

Fig. 5 Effect of Temperature on Peroxidase enzyme activity.

Table 5: Determination of Kinetic Parameters

1/V	**1/S**
5	**0.1395**
2.5	**0.2593**
1.7	**0.1498**
1.25	**0.1186**
1	**0.1193**
0.83	**0.1168**
0.71	**0.1259**
0.625	**0.1218**
0.5	**0.1159**

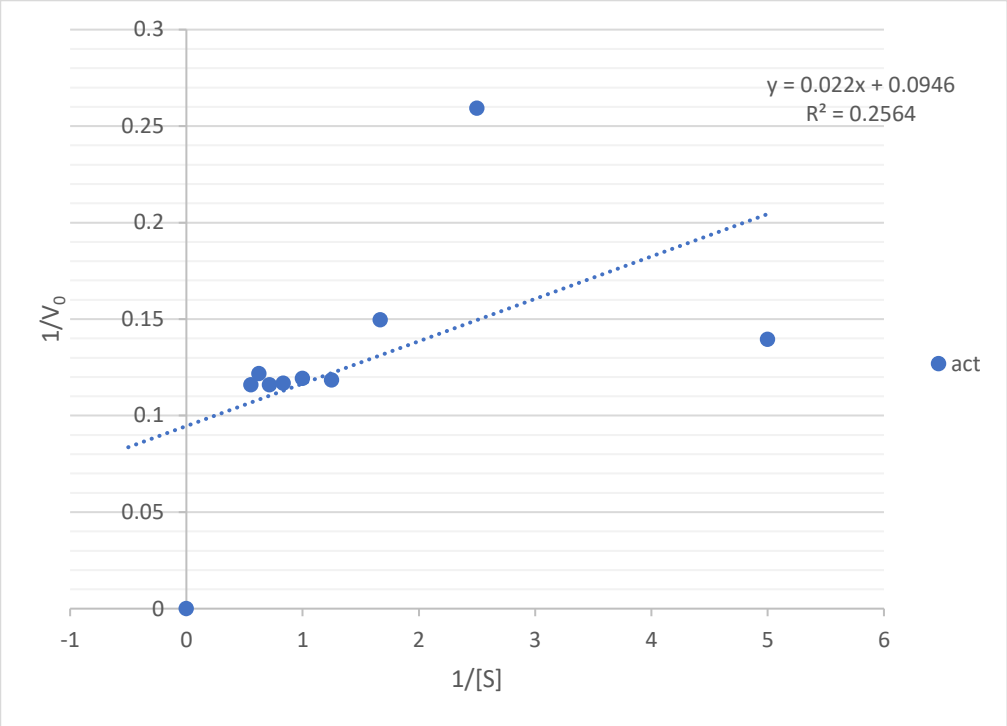

Fig. 6 Line weaver-Bulk plot

DISCUSSION:

The fermentation medium was inoculated with the fungal strain and incubated for various time intervals (1-11 days) in contrast to what was reported by Jarvinen *et al., (2012)* that the MnP peak timing and maximal activity varied between 7–21 days from *Bjerkandera sp* and in accordance to Nguyen *et al*., (2017) who reported that MnP activity was highest after 9 days culture on rice straw medium. The enzyme production was gradually increased with the passage of time and highest enzyme activity was obtained on 9th day of incubation. It was also observed that prolonged incubation decreased the enzyme activity. However, the growth of the microorganism was not significantly affected.

For the optimization of incubation period, the culture was grown in a flask. The flask was harvested after every 24 h, and was analysed to determine the enzyme activity. Activities of Manganese peroxidase produced by *Aspergillus terreus* with varying incubation periods are given in figure 2. Highest enzymatic activity was recorded at 216h (5.28) followed by 72 h (0.415). The results reveal that there was difference among different times of incubation. The duration of incubation for different substrates in SmF is different and may range from 3 to 10 days depending on the type of fungi. So our results are in significant accordance with international standards.

Result representated in Figure 3, there was higher peroxidase activity at pH 6.0 and slightly increase at pH 9.0 which is similar to what was reported by Nguyen *et al.,* (2017) who observed that Manganese Peroxidase (MnP) displayed high activity in an acidic medium with a pH around 4.0. This may be as a result of change in shape and charge properties of the enzyme and substrate. pH changes causes alteration of the ionization state of amino acids residue in a protein which leads to alteration of the ionic bonds which determine the tertiary structure and charge properties of the protein. These ultimately result in enzyme inactivation or altered substrate recognition.

From the result presented in figure 4, enzyme activity varies with different substrate concentrations of guaiacol. At 1.0% of the substrate, peroxidase has the highest activity and lowest at 0.2%, hence, peroxidase activity increases with increase in the concentration of the substrate.

According to the result presented in Figure 5, there was increase in enzyme activity at temperature 25^0C, and peroxidase activity was lower at temperature 30^0C, and sharply decreased at 40^0C. The optimal temperature of the presently studied is in accordance with MnP from *P.chrysosporium* of temperature 20^0C and drastically increased to 35^0C as reported by Saravanakumar *et el., (2013) This* may be due to enzyme denaturation.

Aspergillus terreus peroxidase showed its highest enzymatic activity of 6.25 U/ml at 25°C (optimum temperature). In the experiment on the effect of pH on enzyme activity, the highest activity of 1.31 U/ml was observed at pH 6.0 peroxidase from the fungal isolate (*Aspergillus terreus*) in the experiment designed to test its temperature and pH dependence. This is in agreement with the values reported by Ismat (2012) who recorded optimum pH of purified peroxidase oxidation of veratryl alcohol as 6.8. These are in contrast to previous studies by Asgher *et al*. (2007) who reported optimum pH of 4.0, for free lignin peroxidase using veratryl alcohol as substrate. However this value was slightly lower than the findings by Ismat, (2012) who recorded optimum temperature of 45^0C for purified lignin peroxidase oxidation of veratryl alcohol. This result is also in contrast to the previous studies reported by Nguyen *et al.* (2017) of optimal pH of Phanerochaete chrysoporium to be 2.4-4.0 having lower values.

It is clear that the results obtained from the present work should be interpreted within the context of the nature of enzyme sources used. It is quite likely that the enzymes would behave more like the ones reported in the literature if they were purified. The result obtained from this study indicates that *Aspergillus terreus* are peroxidase producers. This microorganism can be used as potential source for the commercial production of this enzyme for application in different industries such as food, paper, textiles, cosmetics biotechnology and chemical industries. In order to fully explore its ligninolytic potential, the peroxidases from these sources need to be purified and characterized. Full sequencing and characterization of the genes for the isolated peroxidase will open the way for its modification in order to optimize the production of enzymes for local industries.

CONCLUSION

Peroxidase enzyme was isolated and characterized from *Aspergillus terreus* using rice bran as a carbon source. The result obtained indicates that rice bran can be successfully used in the production of peroxidase under submerged fermentation. Varying the physiochemical parameters such as incubation period, temperature and pH to improve enzyme production *Aspergillus terreus* exhibited optimum peroxidase production at an acidic pH, optimum temperature was 25^0C and optimum substrate concentration was 1.0%.

Significance Statement

This study shows that peroxidase can be produced on large scale using rice bran as carbon source for *Aspergillus terreus* in commercial applications of peroxidase and bioremediation purposes.

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