sour fermented and vinegary (Ogbulie *et al.,* 2007). Palm wine is a substrate for microbial growth. Fermentation starts after the sap is collected and within an hour (or) two, becomes reasonably high in alcoholic (up to 4 %) content. If fermentation persists for more than a day, it turns into vinegar.

The super-family of haem peroxidases from plants, fungi and bacteria are haem proteins that catalyzed the oxidation of various electrons donor substrates (phenols, aromatic amines) at the expense of hydrogen peroxide (Welinder, 1992). The haem peroxidases are ubiquitous with diverse functions. They are classified into three groups based on their amino acid sequences (Welinder, 1976). The class one is the intracellular peroxidases, cytochrome *c* peroxidase, ascorbate peroxidase, and gene-duplicated bacterial catalase-peroxidase. Class two contains secretory enzymes such as manganese peroxidase and lignin peroxidase. Class three consists of secretory plant peroxidases (Welinder, 1992). There are interests in these classes of peroxidase, to establish their physiological roles and for possible industrial and analytical applications. Important areas where peroxidase could have application are in wastewater treatment (Duran and Esposito, 2000); soil remediation (Machado *et al*., 1996) as indicators for reactive oxygen species formed during food processing (Cluck *et al*., 1996) and as catalysts for de-lignification of paper pulp (Archibald *et al*., 1997) and the synthesis of polyelectrolyte complexes (Sakharov *et al.,* 2003). Although peroxidases are found in various plant tissues, horseradish (*Amoracia rusticana*) roots are the old source for commercial production of peroxidases.

Almost all strains of *S. cerevisiae* can grow aerobically on glucose, maltose, and trehalose but are unable to grow on lactose and cellobiose. Galactose, glucose and fructose are shown to be best fermenting sugars (Boekhout and Robert, 2003). The ability of yeasts to use different sugars can differ depending on whether they are grown aerobically or anaerobically. They use ammonia and urea as nitrogen source, but unable to use nitrate (Jansma and David, 1999). They can also use most amino acids, small peptides, and nitrogen bases as nitrogen sources. Histidine, glycine, cystine, and lysine are, not readily used. *S. cerevisiae* does not excrete proteases, so extracellular protein cannot be metabolized (Longo *et al*., 2012). Yeasts also have a requirement for phosphorus, which is assimilated as a dihydrogen phosphate ion, and sulfur, which can be assimilated as a sulfate ion or as organic sulfur compounds such as the amino acids methionine and cysteine. In order to harness the biochemical functions of *S. cerevisiae,* this study was designed to isolate the peroxidase with partial purification and characterizing the peroxidase from *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Collection of palm wine

Fresh palm wine was purchased from Owerre Obukpa market in Nsukka Local Government Area of Enugu State, Nigeria.

2.2. Isolation of Saccharomyces cerevisiae *from palm wine*

Saccharomyces cerevisiae was isolated from palm wine according to the method described by Ezeonu *et al*. (2013). A 5 ml of the fresh palm wine was dissolved in 50 ml of distilled water in a clean conical flask. It was shaken vigorously and made as the stock culture. From the stock culture, ten-fold serial dilutions were drawn out - 10^{-4} to 10^{-6} dilutions were plated out into plates with media.

2.3. Inoculations of dilute palm wine on plates and sub-culturing

With the 10^{-4} to 10^{-6}-fold serial dilutions, inoculations were done on the prepared sabouraud dextrose agar plates using a 1 ml insulin syringe and a glass rod spreader close to a bunsen flame. Streaks were made from each side of the plate, marking an initial point, with sterilization of the wire loop in a bunsen flame after each side has been completed. The inoculated plates were incubated for 3-4 days at 37 ^{o}C. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure fungal cultures were obtained.

2.4. Storage of the Saccharomyces cerevisiae *isolates*

Pure fungal isolates were maintained on sabouraud dextrose agar (SDA) slants as stock cultures in bijou bottles. SDA media were prepared according to the manufacturer's description and poured inside the bijou bottles. The medium was autoclaved at 121 ^{o}C for 15 min. It was allowed to cool to about 45 ^{o}C and the bijou bottles were slanted slightly and allowed to gel. The fungal culture was inoculated into the bijou bottles and was then incubated at normal room temperature for 3-4 days to check for sterility.

2.5. Identification of Saccharomyces cerevisiae

The identification of *Saccharomyces cerevisiae* was carried out by relating features and the micrographs to "Atlas of mycology" by Barnett and Hunter (1972). The three-day old pure cultures were used in preparing microscopic slides. A colony of the mycelia was smeared on the slide and a drop of lactophenol blue was added to it. A cover slip was placed over it and the slide was examined under the light microscope at x 40 magnification.

2.6. Mass production of crude enzyme

After the 12-day pilot SmF studies, the 8th day had the peak of peroxidase activity and was chosen for mass production of the enzyme from the respective *Saccharomyces cerevisiae* isolates. Harvesting was carried out on each day of enzyme activity. 250 ml Erlenmeyer flasks numbering 6 were used to produce a litre of the enzyme. The harvested enzyme was filtered with Whatman No. 1 filter paper and kept at 4 °C.

2.7. Determination of peroxidase Activity with o-dianisidine substrate

Peroxidase activity was assayed using the modified method of Mclellan and Robinson (1987) and 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 monitored using Jenway 6405 UV / VIS spectrophotometer. The standard assay solution contained 0.3 ml of 1 % o-dianisidine, 0.2 ml of hydrogen peroxide, 2.4 ml of sodium phosphate buffer pH 6.0 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 sec for 5 min.

The variables below were calculated as shown using the method of Segel (1993).

$$Reaction\ rate = \frac{Change\ in\ absorbance(OD)\ at\ 460\ mm}{Time\ interval}$$

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

where $Total\ units = (Unit/ml)\ x\ Total\ volume\ of\ enzyme$

$$Percentage\ yield\ or\ recovery = \frac{Total\ unit\ of\ \ purified\ enzyme}{specific\ activty\ of\ \ crude\ enzyme} \times 100$$

$$Purification\ \ fold = \frac{Specific\ activity\ of\ \ the\ \ purified\ enzyme}{specific\ activty\ of\ \ crude\ enzyme}$$

2.8. Determination of protein

Protein content of the enzyme was determined by the method of Lowry *et al.* (1951), using Bovine Serum Albumin as standard.

2.9. Partial purification of crude enzyme

Eight test tubes were used to form an ammonium sulfate precipitation profile. Peroxidases were precipitated by gentle stirring at 20-90% saturation of solid ammonium sulfate at 10% of each test tube. The ammonium sulfate-crude enzyme solutions were allowed for 30 h at 4 °C to cool, while the supernatant was gently decanted. The test tubes were centrifuged at 4000 rpm, for 20 min. Precipitates from the individual percentage ammonium sulfate saturations were re-dissolved in equal volumes of 2.4 ml of 0.05 M sodium phosphate buffer pH 6.0. Peroxidase activities of the precipitates were assayed to determine the percentage ammonium sulfate saturation that precipitated enzyme with the highest activity.

2.10. Ammonium sulfate precipitation of peroxidase

A liter of crude enzyme was used; seventy percent (70%) ammonium sulfate saturation was suitable for mass precipitation of the crude enzyme. Ammonium sulfate precipitation (at 70 % saturation) was carried out by

dissolving gently 436 g of the salt in the filtrate and stirring gently till the salt was completely dissolved. The precipitate was re-dissolved in 40 ml of 0.05 M phosphate buffer (pH 6.0) after centrifugation and kept for further studies.

2.11. Gel filtration of peroxidase using sephadex G -100

A packed gel is equilibrated with 0.05 M sodium phosphate buffer solution (pH 6.0) and 20 ml of the precipitated protein was introduced into the column. 80 fractions were collected using 5 ml fraction tubes at a flow rate of 5 ml per 15 min. The protein concentration of each fraction was monitored using a spectrophotometer at 760 nm. The peroxidase activity of each fraction was also monitored spectrophotometrically at 460 nm. The active fractions were pooled into two (2) fractions (A and B) and stored in a freezer.

2.12. Characterization of enzyme

The activity of the peroxidase was examined within the pH range of 4.0-9.0 by using four different buffers systems: Sodium-acetate (0.05 M, pH 4–5.5), sodium phosphate (0.05 M, pH 6.0–7.5), and Tris – HCl (0.05 M, pH 8.0–9.0). The peroxidase activity was determined using 2.4 ml of 0.05 M of each of the buffer (sodium-acetate, sodium phosphate and tris HCl) at their different pH ranges. 0.2 ml of hydrogen peroxide (H_2O_2), 0.1 ml of enzyme, and 0.3 ml of 1% o-dianisidine, o-dianisidine was used as the reducing substrate and read at 460 nm for 5 min at 30 s. the following properties were used to characterized the enzymes: Effect of time of submerged fermentation on the production of peroxidase from *Saccharomyces cerevisiae,* optimum temperature, optimum pH, effect of graded concentrations of hydrogen peroxide (H_2O_2) on peroxidase activity, effect of graded concentrations of o-dianisidine on peroxidase activity, and Determination of K_m and $V_{max.}$

3. Results and Discussion

The isolation and production of peroxidase from *Saccharomyces cerevisiae* in palm wine was carried out in twelve-day submerged fermentation and characterized. Day eight (8) was found suitable for mass production of the crude enzyme. The crude enzyme had 0.751 U / mg specific activity. The crude enzyme was purified using ammonium sulfate followed by gel filtration on sephadex G-100. A 70 % ammonium sulfate precipitation had the highest peroxidase activity. Precipitation occurred due to protein hydration and hydrophilic interaction with water molecules. However, the addition of ammonium sulfate salt makes the molecules to be more attracted to the salt than to the protein. This antagonism for hydration favors the salt, due to the interaction between the proteins. This gives rise to aggregation and salting out of the proteins (precipitation). The purification of peroxidase from *Saccharomyces cerevisiae* on a two-step purification process of ammonium sulfate precipitation and gel filtration yielded enzyme with 0.657-fold of purification and specific activity of 0.494 U / mg when ammonium sulfate was used.

The elution profile of the gel filtration gave two peaks of the peroxidase (fractions A and B). The both fractions were found to be peroxidase of different isoforms thereby possessing different properties. This could be as a result of a slight difference in the amino acid sequence or size of the protein molecule. In this research, fraction "A" will be discussed. A purification fold –2.470, and specific activity of 1.855 U / mg were obtained for fraction A of the gel filtration. There was a reduction in peroxidase activity after ammonium sulfate precipitation and an increased in activity after gel filtration for fraction "A". One can infer that successive purification step will eliminate interfering protein and allow enzyme to develop affinity for its substrate to increase its activity. Yihong *et al*. (2012) reported 17.92 purification fold for peroxidase from lettuce stems when three-step purification of ammonium sulfate precipitation, G-100 filtration and concanavalin A affinity chromatography was used. Also, Melda *et al*. (2010) reported a 9.7 purification fold of peroxidase using three-step purification - ammonium sulfate precipitation, dialysis, and CM Sephadex ion exchange chromatography on peroxidase from Turkish black radish. On a four-step purification process on Sephadex G-25, ammonium sulfate precipitation, DEAE Sepharose, and concanavalin A Sepharose, peroxidase from horseradish was purified 2692 fold. The low purification fold from this result was because of two-step purification.

It has been established that pH influences enzyme activity, pH causes the changes in the ionization states of the protein and substrate (Gawlik-Dziki *et al*., 2008). In an acidic solution, a basic moiety such as nitrogen would be protonated. If the environment becomes too basic, the acid groups would be deprotonated. This would alter the electrical attractions between polar groups. Every enzyme has an optimum pH range separate from the pH that inhibits the enzyme functionality. Peroxidase from *Saccharomyces cerevisiae* has an optimum

pH of 6.5 for fraction A. The peroxidase activity was found to be pH-dependent with complete inactivation at pH greater than or equal to pH 7.0 (Figure 1). The drop in activity was sharper in the alkaline range than the acidic range. Mamounata *et al*. (2011) had reported optimum pH for four different sources of peroxidase, pH 5.5 to 6.5 for *Allium sativum,* pH 6.0 for *Ipomoea batatas,* pH 5.0 for *Raphanus sativus* and pH 3.5 to 4.0 for *Sorghum bicolor.* Optimum pH range 6.5-7.5 has been reported previously by Khalil-Ur-Rehaman *et al*. (1999) for peroxidases from different vegetables. In the cases of horseradish peroxidase and soyabeans peroxidase, the optimum pHs were 8.5 and 7.2 (Bowell *et al*., 2002). This result is dependable with the findings of Majed and Mohammad (2005), who reported that haem-peroxidase from palm leaves, is stable over a broad pH range with optimum pH at pH 6.0-7.0. Kim and Lee (2005) reported optimum pH of 5.0 from cauliflower bud peroxidase when guaiacol was used as substrate, pH 4.0 when catechol was used and pH of 7.5 with pyrogallol and 4-methyl catechol as substrates. An optimum pH - 6.5 for fraction A of peroxidase implies that maintaining the pH of the enzyme within these pH ranges would prolong the shelf life of the palm wine during oxidative stress.

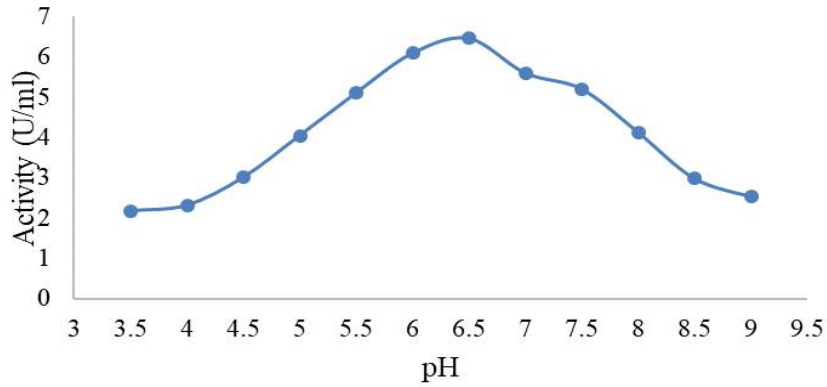

Figure 1: Effect of pH on peroxidase activity (fraction A)

Peroxidase is thought to be the most heat-stable enzyme, because microbial peroxidases are glycosylated proteins. It was observed that peroxidase from *Saccharomyces cerevisiae* has an optimum temperature of 30 °C for fraction A. It had minimum activity at 90 °C, which was attributed to thermal inactivation of the peroxidase. This was foreseen due to an increase in kinetic energy. The aggregating kinetic energy led to an increase in the number of collisions between the enzyme and the substrate to form the enzyme-substrate complex, and increased the product. However, at temperatures beyond 30 °C to 40 °C, peroxidase activity decreased and this was ascribed to the partial denaturation of the enzyme. Denaturation of an enzyme can change the conformation and makes it difficult for the substrate to bind the active site. Optimum temperature 30 °C correlated with Civello *et al*. (1995) who reported maximum enzyme activity at 30 °C of peroxidase from strawberry fruit. Mamounata *et al*. (2011) also reported the optimum temperature of four different sources of peroxidase—40 °C for *Allium sativum* and *Sorghum bicolor,* 30 °C for *Ipomoea batatas* and *Raphanus sativu* (which corroborate this result) (Figure 2). The differences in optimum temperature may be due to the differences in reducing substrate

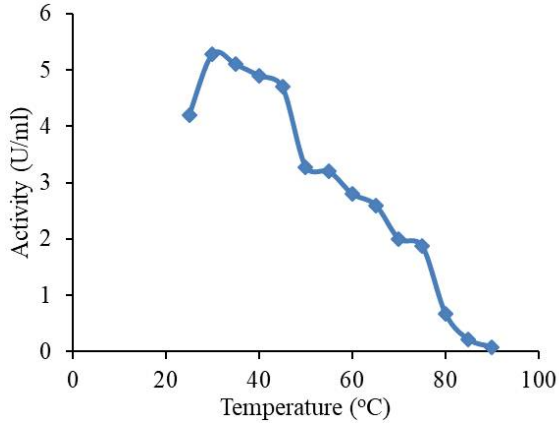

Figure 2: Effect of temperature on peroxidase activity (fraction A)

used for the assay. Peroxidase from *Saccharomyces cerevisiae* with optimum temperatures 30 °C and 40 °C could improve the durability of palm wine during oxidative stress.

The increase in enzyme activity as a result of increase in substrate concentration could be due to the availability of substrate that bound to the enzyme active sites by increasing the enzyme catalytic efficiency find (Figure 3). Further addition of substrate may cause a reduction in the activity due to lack of free active sites. The determinations of kinetic parameters help to elucidating the efficiency of the enzyme. The Michaelis-Menten kinetic measures peroxidase velocity (V) with various substrate concentrations [S], (Figure 5). It was evaluated using Lineweaver-Burk plot, that correlated to the equation $1/V_O = K_m/V_{max}.[S] + 1/V/_{max,}$ (Figures 4 and 6). The effect of the substrates (hydrogen peroxide and o-dianisidine) concentrations on peroxidase activity showed that the activity of peroxidase increased with corresponding increase in substrates concentration to a saturation point –0.4 mM for fraction A. The effect of different concentrations of hydrogen peroxide on peroxidase activity gave 0.4 mM for fraction A. The effect of different concentrations of o-dianisidine on peroxidase activity, indicate that the active sites were saturated with the substrates. The K_m value was 0.2 mM. The different concentration of o-dianisidine was used and 0.0833 mM for fraction A was obtained. The V_{max} –10 U / min for o-dianisidine and 8.333 U / min for hydrogen peroxide were obtained. Kim and Lee (2005) reported the K_m values of 1.18 mM for o-dianisidine and 1.27 mM for hydrogen peroxide, with V_{max} values 0.032 U / min for o-dianisidine and 0.138 U / min for hydrogen peroxide, for peroxidase from *Raphanus sativus*. Melda *et al.*

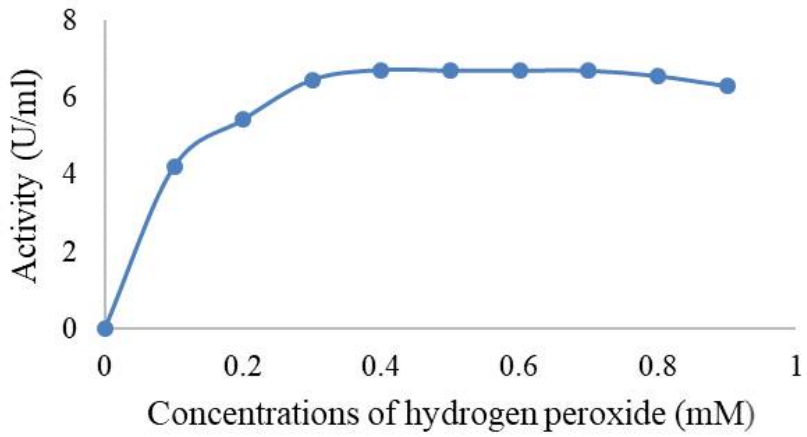

Figure 3: Michaelis-Menten plot of peroxidase activity using graded concentrations of hydrogen peroxide (fraction A)

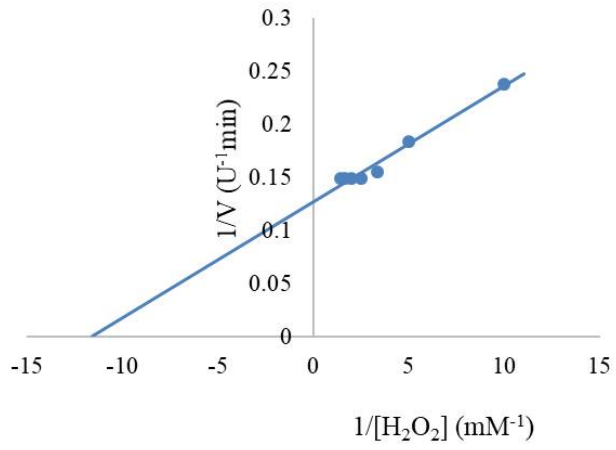

Figure 4: Lineweaver-Burk plot for peroxidase activity with graded concentrations of hydrogen peroxide (fraction A)

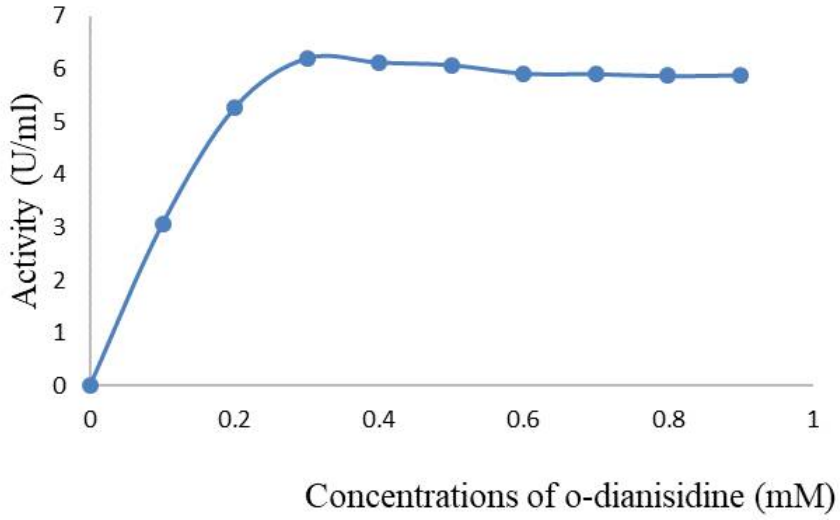

Figure 5: Michaelis-Menten plot of peroxidase activity with concentrations of o-dianisidine (fraction A)

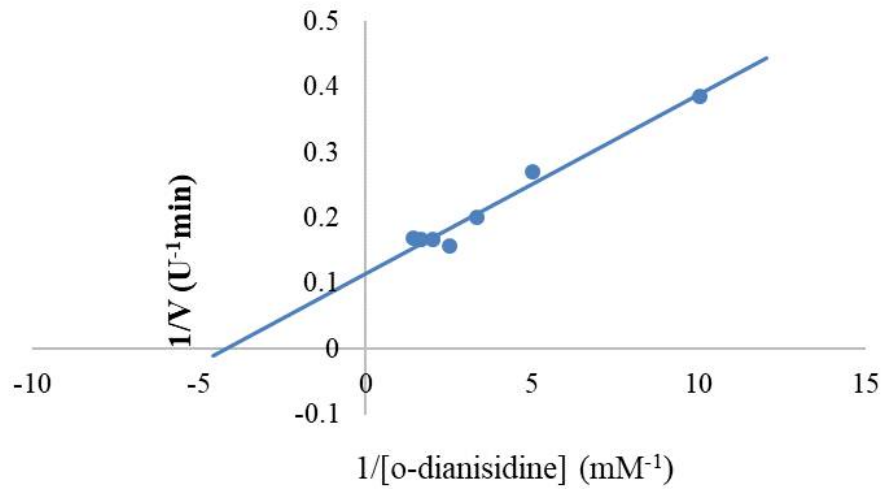

Figure 6: Lineweaver-Burk plot for peroxidase activity with graded concentrations of o-dianisidine (fraction A)

(2010) reported that peroxidase from *Raphanus sativus* had K_m –0.036 mM for guaiacol and 0.0084 mM for hydrogen peroxide, with V_{max} - 3512.23 U/min and 38728.17 U/min respectively. By applying Michaelis-Menten concept, it is known that a small K_m indicates that the enzyme requires small quantity of substrate to become saturated. Thus, the maximum velocity is reached at moderately low substrate concentration while a large K_m indicates the need for high substrate concentrations to achieve reaction velocity. From these reports, it is evident that the nature of different reducing substrates - o-dianisidine and hydrogen peroxide affected the peroxidase K_m and V_{max}.

4. Conclusion

Based on the characteristics of the peroxidase, *Saccharomyces cerevisiae* can withstand oxidative stress that occurs during prolonged fermentation. However, the maintenance of a particular temperature and pH ranges for peroxidase will sustain the shelf life and storage of the palm wine.

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