

**BIOCHEMICAL AND KINETIC STUDIES OF FUNGAL GLUCOSE OXIDASE USING
PHASEOLUS VULGARIS PEROXIDASE AS A COUPLING ENZYME**

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TITLE PAGE**BIOCHEMICAL AND KINETIC STUDIES OF FUNGAL GLUCOSE OXIDASE USING
PHASEOLUS VULGARIS PEROXIDASE AS A COUPLING ENZYME**

**A DISSERTATION SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY,
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REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (MSc.) DEGREE IN
BIOCHEMISTRY (ENZYMOLGY AND PROTEIN CHEMISTRY)**

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CERTIFICATION

Eje, Ozoemena Emmanuel, a postgraduate student with Registration Number, PG/MSc/17/01794 in the Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, , has satisfactorily completed the requirements for the course work and research for the award of Masters of Science (M.Sc) degree in Biochemistry (Enzymology and Protein Chemistry). The work embodied in this dissertation is original and has not been submitted in part or full for any other diploma or degree of this or any other university.

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DEDICATION

This work is dedicated to my brother, Eje, Ifeanyi Ezekiel

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My gratitude goes to the Almighty God who gives me wisdom and directs my path by his immeasurable grace even in the course of this project work.

I remain grateful to my supervisor and academic god, Professor F. C. Chilaka who has been a father-figure in guiding and piloting this research. Indeed, your intellectual and technical contributions during the course of this work would not have been possible if not for your readiness to constructively guide and direct me. Honestly, I could not have wished for a better supervisor.

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.

ABSTRACT

Glucose oxidase (GOD) was produced from *Aspergillus fumigatus* by submerged fermentation system. To monitor the kinetics of GOD, peroxidase is used as coupling enzyme. This research was carried out to isolate and purify peroxidase from low cost, *P. vulgaris* (green beans) in order to use it for GOD assay which could be a robust and cost effective approach. Peroxidase and GOD activities in crude extract were measured spectrophotometrically. The supernatant was assayed for peroxidase activity, 35.5 U/mL with protein content of 0.82 mg/mL. The crude peroxidase was partially purified to the level of gel filtration chromatography (SG-200). With 80 % ammonium sulfate saturation, it was found suitable to precipitate peroxidase with enzyme activity of 26.9 U/mL and protein content of 0.44 mg/mL. After gel filtration, the specific activity was 143.6 U/mg with a purification fold of 3.3 for green beans peroxidase (gPOD). Peroxidase extracted from green beans was used as a coupled enzyme for fungal glucose oxidase. Glucose oxidase which was purified through ammonium sulphate precipitation (90 %), dialysis, and gel filtration chromatography (Sephadex G-200) showed a specific activity of 67.5 U/mg proteins with 7.7 degree of purification and 1.2 % yield. Glucose oxidase was characterized using partially purified peroxidase, gPOD as coupled enzyme based on the pH effect on enzyme activity, temperature and effect of substrate concentration. Maximum enzyme activity (50.43 U/mL) was obtained at pH 5.5 and was considered optimum pH of GOD; glucose oxidase showed highest activity (34.72 U/mL) at 45 °C, hence the optimum temperature. Moreover, the substrate (glucose) concentration effect was carried out and the Michaelis-Menten constant, K_m and maximum velocity, V_{max} obtained from Line-Weaver-Burk plot were 5.66 mM and 16.67 U/mL, respectively. It is more evident that peroxidase is the most heat stable enzyme; therefore, it is concluded that it may be potentially useful for coupling with GOD and other industrial purposes.

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LISTS OF ABBREVIATIONS

GOD	Glucose Oxidase
POD	Peroxidase
gPOD	Green beans peroxidase

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CHAPTER ONE

INTRODUCTION

Enzymes are catalysts that speed up chemical reactions which take place in living organisms. They do this by lowering the activation energy of reactions without being unchanged. They differ from the inorganic catalysts by being specific to its substrate and operate in very mild circumstances. Since enzymes are proteins, they are all affected from the factors that denature proteins such as: pH, temperature and inhibitors. Glucose oxidase (GOD, EC: 1.1.3.4), which was discovered in 1928 by Muller in *Aspergillus niger* and *Penicillium glaucum*, catalyzes the oxidation of β -D-glucose to D-glucono-1,5-lactone and hydrogen peroxide in aerobic conditions. It does this in the presence of molecular oxygen and to a lesser degree could oxidize mannose and galactose. Ten (10) years later, Franke *et al.* (1939) reported a purer and therefore 80 times more active glucose oxidase than that of Müller. They showed that oxygen was reduced to hydrogen peroxide and that indophenol could be used as hydrogen acceptors. Their research revealed that this enzyme is a flavoprotein. In the 40s, Coulthard and his colleagues (1945) were looking for the substance responsible for the antibacterial properties of *Penicillium notatum* in presence of glucose and they obtained a highly purified enzyme initially named penicillin A and later renamed notatin, in order to avoid the confusion with penicillin discovered by Fleming. It was demonstrated that notatin was only effective in presence of glucose and O₂ and was identical to the enzyme discovered by Müller. Its bactericidal action was attributed to H₂O₂ produced during the oxidation of glucose.

In parallel, two research groups also identified an antibacterial substance from the culture media of *P. notatum*. Van Bruggen *et al.* (1943) named it penicillin B and was considered different from notatin because penicillin B could oxidize glucose, mannose, and galactose while Coulthard and his friends reported that notatin could only oxidize glucose. In 1943, Kocholaty (1942) named Penatin a newly identified enzyme. While the researchers recognized the probable similarity between penatin and notatin, they doubted it could be identical with penicillin B. Finally, the same year, Birkinshaw and his colleagues demonstrated that notatin, penatin, and penicillin B were the same enzymes identical to the glucose oxidase (GOD) initially identified by

Müller. The extensive work of Keilin later confirmed it and identified in 1946 the prosthetic group as an alloxazine adenine dinucleotide. Further characterization of this enzyme and its properties showed that GOD was highly specific to D-glucose rather than L-glucose.

Glucose oxidase from *Aspergillus fumigatus* is a dimer consisting of 2 equal subunits with a molecular mass of 80 kDa each. Each subunit contains one flavin adenine dinucleotide (FAD) moiety and one iron (Fe). The enzyme is a glycoprotein containing ~16 % neutral sugar and 2 % amino sugars. It also contains 3 cysteine residues and 8 potential sites for N-linked glycosylation. Glucose oxidase is capable of oxidizing D-aldoheptoses, monodeoxy-D-glucoses, and methyl-D-glucoses at varying rates. The pH optimum for glucose oxidase is 5.5, while it has a broad activity range of pH 4-7. Glucose oxidase is specific for β -D-glucose with a K_m of 33-110 mM. Glucose oxidase does not require any activators, but it is inhibited by Ag^+ , Hg^{2+} , Cu^{2+} , phenylmercuric acetate, and p-chloromercuribenzoate. It is not inhibited by the non-metallic SH reagents: N-ethylmaleimide, iodoacetate, and iodoacetamide. Glucose oxidase can be utilized in the enzymatic determination of D-glucose in solution. As glucose oxidase oxidizes β -D-glucose to D-gluconolactone and hydrogen peroxide, horseradish peroxidase is often used as the coupling enzyme for glucose determination. Although glucose oxidase is specific for β -D-glucose, solutions of D-glucose can be quantified as α -D-glucose will mutarotate to β -D-glucose as the β -D-glucose is consumed by the enzymatic reaction.

1.1 Enzymes that Oxidize Glucose

There are four types of enzyme that oxidize glucose as a principal substrate:

- (1) Glucose dehydrogenases
- (2) Quinoprotein glucose dehydrogenases
- (3) Glucose I-oxidases
- (4) Glucose 2-oxidases

Glucose dehydrogenases (Metzger *et al.*, 1964, 1965) and quinoprotein glucose dehydrogenases (Hauge, 1964) are both specific for β -D-glucose and have a high turnover. However, the former requires a soluble cofactor and the latter is relatively unstable. Although biosensors based on

these enzymes have been constructed these drawbacks have precluded their widespread use. Glucose 2-oxidases oxidize glucose to glucosone. In the presence of dioxygen (O₂) the other product is hydrogen peroxide. However, they also oxidize other carbohydrates like xylose and gluconolactone. This lack of specificity rules out the widespread use of this group of enzymes in biosensors for glucose, however they can be used to determine the total amount of monosaccharides present in a sample (Olsson *et al.*, 1990)

1.1.1 The structure of glucose oxidase

Structurally, GOD is a holoenzyme consisting of two identical 80 kDa subunits at the active site that contains flavin adenine dinucleotide (FAD as a cofactor). A comparison of the primary structure of GOD with that of other enzymes shows that it is closely related (26 % identical) to the alcohol oxidase of *Hansenula polymorpha*. To a lesser extent it is also similar to a large number of other flavoenzymes. In particular, the N-terminal region is similar to regions of p-hydroxybenzoate hydroxylase and glutathione reductase that are involved in binding the AMP moiety of FAD. This suggests that the N-terminal region of GOD also has this function (Frederick *et al.*, 1990). The dimer containing two disulphide bridges has been suggested to link the two subunits together which act as a redox carrier in catalysis. This suggestion, however varied with the observation that the dimer can be dissociated into its monomers by the detergent sodium dodecyl sulphate (SDS) (Jones *et al.*, 1982). In practice there appears to be one disulphide bridge per monomer. These are located towards the end of the primary sequence and are not involved in maintaining the dimeric structure of the enzyme (O'Malley and Weaver, 1972). There is also one free cysteine per monomer which is unlikely to be involved in catalysis (Frederick *et al.*, 1990). Little is known about the secondary structure of the enzyme, however, it has been reported that 16 % of it is in the form of α -helix (Nakanishi *et al.*, 1984).

Glucose oxidase has been described as a branched glycoprotein belonging to the family of glucose/methanol/choline (GMC) oxidoreductase, which incorporates numerous other industrially imperative catalysts, particularly in the field of diagnostics such as cholesterol oxidase, choline oxidase, methanol oxidase, alcohol oxidase, amino acid oxidase, and pyranose oxidase (Ferri *et al.*, 2011). These GMC oxidoreductase family share a homologous structural backbone, including an adenine-dinucleotide-phosphate binding bab-fold close to their amino

terminus and five other segments of conserved sequences dispersed throughout their primary sequences (Horaguchi *et al.*, 2012). Most researchers have confirmed that GOD contains 10-17 % carbohydrate while certain group argued and proposed that there are six different forms of GOD each with different carbohydrate content (Hayashi and Nakamura, 1981). The latter group has proposed that the carbohydrate content ranges from 8 % to 12 % and that these differences account for variations in the isoelectric point (pI) between 3.9 and 4.3. Elsewhere the pI has been reported as 4.2 (Swoboda and Massey, 1965; Tsuge *et al.*, 1984), which indicates that at physiological pH GOD is a very anionic enzyme which enables polyamines like putrescine to inhibit it by altering the ionic environment of amino acids involved in catalysis (Voet and Andersen, 1984). There are potentially eight sites per monomer at which the protein and carbohydrate parts of the enzyme could be joined together. At least two of these are glycosylated by *A. niger* (Frederick *et al.*, 1990). The carbohydrate content of the enzyme consists mainly of D-mannose (about 14 % of the enzyme by mass), D-glucosamine (2.3 %) and D-galactose (0.3 %) (Pazur *et al.*, 1963). It has been suggested that glucose is also present (Nakamura *et al.*, 1976) but other researchers have failed to confirm this (Pazur *et al.*, 1963; Tsuge *et al.*, 1975). As mentioned earlier, the carbohydrate moiety appears to be in the form of a branched polysaccharide that partly surrounds the protein core and not involved in catalysis, but its partial removal with periodate reduces the enzyme's stability (Degani and Heller, 1988). Conversely, the increase in carbohydrate content increases the stability of the protein when the enzyme is cloned in yeast (De Baetselier *et al.*, 1991). Hence, the GOD's unique physical properties: resistance to precipitation by trichloroacetic acid; failure to precipitate at ammonium sulphate concentrations below 80 %; very high solubility in water and resistance to proteases may be ascribed to its carbohydrate shield (Swoboda and Massey, 1965). However, this shield also seems to act as a barrier to the transfer of electrons between the enzyme and an electrode in amperometric biosensor (Degani and Heller, 1988), but this barrier can be surmounted by perturbing the carbohydrate shell using an immobilization technique.

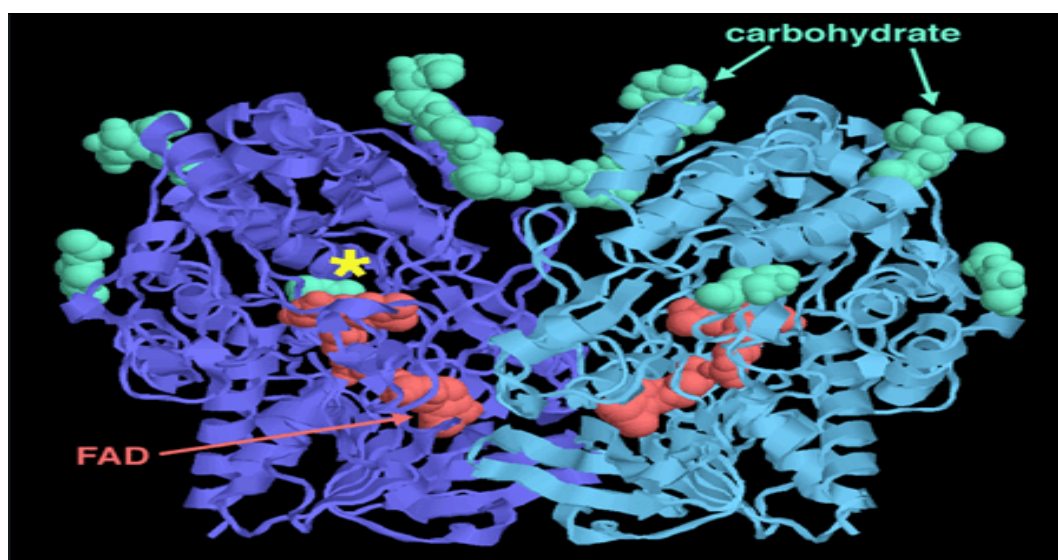


Fig.1. Structure of GOD

(Source: Shaikh and Ratna, 2015)

1.1.2 General reaction catalyzed by glucose oxidase

Glucose oxidase from *Aspergillus fumigatus* (GOD; E.C. 1.1.3.4) is a homodimeric flavin-dependent oxidoreductase enzyme that catalyzes the oxidation of β -D-glucose by a ping-pong bi-bi mechanism. In the reductive half reaction which occurs in a concerted manner, proton and hydride are transferred from glucose C1-atom to His516 and N5-atom of flavin adenine dinucleotide (FAD) respectively and gluconolactone is produced. In the oxidative half-reaction, the FADH_2 formed due to the hydride transfer in the reductive half reaction reduces the natural co-substrate, atmospheric oxygen to hydrogen peroxide in two single electron transfer steps. Both end products break down spontaneously and catalytically, specifically, D-glucono- δ -lactone is subsequently hydrolyzed slowly by lactonase to D-gluconic acid (GA), while the generated H_2O_2 is broken down to O_2 and H_2O by catalase or peroxidase. However, to monitor the kinetics of this reaction, peroxidase utilizes reduced organic compound (chromogenic substrate e.g o-dianisidine) forming water and oxidized o-dianisidine which can be monitored spectrophotometrically; hence the rate of oxidized chromogen is a measure of GOD in the solution (Leskovac *et al.*, 2005).

The overall reaction is given below:

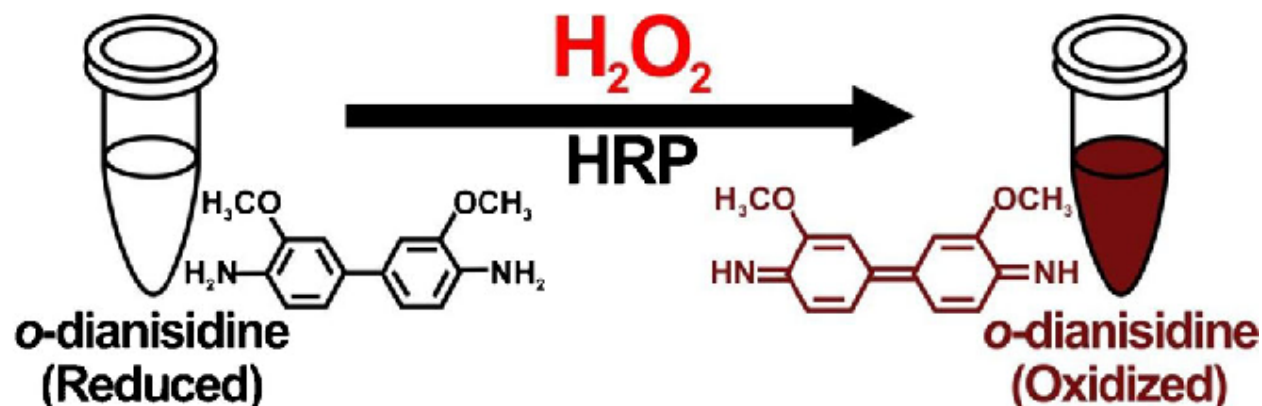
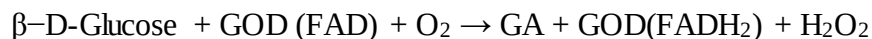
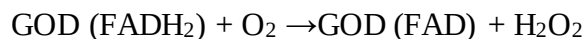


Figure 2: Reaction sequence and mechanism of catalysis of glucose oxidase and peroxidase
Sources: (Reyes *et al.*, 2014; Dubey *et al.*, 2017)

1.1.3 Sources of GOD and their Physiological Roles

Glucose oxidase can be obtained from a large number of different sources, including red algae, citrus fruits, insects, bacteria, plants, animals, and fungi. The regular capacity of GOD in these organic frameworks is to act primarily as an antibacterial and antifungal specialist through the generation of H_2O_2 . Among these sources, fungi have the largest amount and industrially fungal sources have been preferred since the early 1950s (Fiedurek and Gromada, 1997a).

For decades, fungi have been thoroughly researched for GOD production as cell factories due to their magnificent capacities to use an assortment of carbon sources and to accumulate a large proportion of natural GOD under stressed conditions (Bankar *et al.*, 2009b; Zehra *et al.*, 2015). Because GOD from fungi has a broad spectrum application, it must be stable at the higher temperature and for a longer duration for economic use. However, the mechanisms of GOD accumulation by different fungi are not fully understood, even though many successful attempts have been made to improve and optimize fungal GOD production by using genetic modifications and other approaches. The filamentous fungi *Aspergillus* and *Penicillium* serve as industrial producers of GOD at a large scale (Wong *et al.*, 2008; Bankar *et al.*, 2009b), among which *Aspergillus niger* is the most ordinarily used for the industrial yield of GOD.

1.1.4 Potential Fungal Sources of Glucose Oxidase

The most common fungal sources of the enzyme GOD from the genus *Aspergillus* are *A. niger* (Liu *et al.*, 2001), *A. tubingensis* (Kriaa and Kammoun, 2016), *A. flavus* (Bhat *et al.*, 2013), *A. terreus* (Anas *et al.*, 2012), *A. oryzae* (Gunasundari, 2014), *A. carbonarius* (Yang *et al.*, 2014), and *A. nidulans* (Witteveen *et al.*, 1990), while those from *Penicillium* are *P. amagasakiense* (Todde *et al.*, 2014), *P. variable* (Crognalea *et al.*, 2008), *P. chrysogenum* (Konishi *et al.*, 2013), *P. notatum* (Nandy, 2016), *P. funiculosum* (Esmailpour *et al.*, 2014), and *P. adametzii* (Mikhailova *et al.*, 2007). Many other species of *Penicillium* have also been reported to produce GOD, such as *P. pinophilum* (Rando *et al.*, 1997), *P. canescens* (Simpson, 2006), *P. fellutanum* (Manivannan and Kathiresan, 2007), *P. glaucum* (Müller, 1928), and *Penicillium vitale* (Dolgi *et al.*, 1977). Other reported fungal species include *Talaromyces flavus* (Kim *et al.*, 1990), *Phanerochaete chrysosporium* (Zhao and Janse, 1996), *Alternaria alternata* (Caridis *et al.*, 1991), *Pleurotus ostreatus* (Shin *et al.*, 1993), *Pycnoporus cinnabarinus* (Levasseur *et al.*, 2014), *Rhizopus stolonifer* (Guimarães *et al.*, 2006), and *Flavodon flavus* (Raghukumar *et al.*, 2004).

1.2 Characteristics of Glucose oxidase

The molecular weight of GOD ranges from approximately 150 to 175 kDa (Kalisz *et al.*, 1997). Glucose oxidase is highly specific for the β -anomer of D-glucose, while α -anomer does not appear to be a suitable substrate. Inhibitors of GOD include pchloromecuribenzoate, Ag^+ , Hg^{2+} , Cu^{2+} , hydroxylamine, hydrazine, phenylhydrazine, dimedone and sodium bisulphate. The overall carbohydrate content of GOD was found to be 16 % for *A. niger* and 11 % for *P. amagasakiense*. The amino acid content of the two enzymes revealed that the *A. niger* GOD contained more histidine, arginine and tyrosine and less lysine and phenylalanine than the *P. amagasakiense* GOD. The optimum pH ranges for GOD from *A. niger* and *P. amagasakiense* were shown to be 3.5–6.5 and 4.0–5.5, respectively. It is evident that GOD from *A. niger* has a broader pH range than that from *P. amagasakiense* GOD (Nakamura and Fujiki, 1968).

1.2.1 Analysis of Glucose oxidase Activity

Most researchers use an assay method for determination of GOD activity. This is based on the principle that GOD oxidizes β -D-glucose in the presence of molecular oxygen to β -D-glucono- δ -

lactone and H_2O_2 . The H_2O_2 is then utilized to oxidize a reduced chromogenic substrate in a secondary reaction with horseradish peroxidase (HRP) with a resultant colour change that can be monitored spectrophotometrically. Two of the chromogenic substrates used for the GOD reaction are: 2, 2'-Azino-di-[3-ethylbenzthiazolin- sulfonate] (ABTS) and o-dianisidine (Bergmeyer *et al.*, 1974). The former forms a greenish-blue oxidized product while the latter forms a quinoneimine dye that is measured spectrophotometrically at 420 nm 500 nm respectively.

A new assay for GOD using Fourier transform infrared (FTIR) spectroscopy, was developed by Karmali *et al.* (2004), who concluded that the method was useful to study the kinetic properties of GOD since the substrate and product of the reaction absorbs at different frequencies. Distinct advantages over coupled assays were the speed of assay, requirement of smaller amounts of substrate and enzyme, and the feasibility of following the reaction by quantifying δ -gluconolactone.

1.2.2 Mechanism of Catalysis of Glucose oxidase

A general mechanism for the oxidation of glucose catalyzed by glucose oxidase has been proposed to proceed in two half reactions, the reductive and the oxidative half reactions (Wolfahrt *et al.*, 1999). In the reductive half reaction, two electrons and two protons are transferred from the glucose to the flavin as stated earlier. This can occur in two ways; the first involved a nucleophilic addition by the 1-OH group of the glucose to the C-4a position of FAD. This step is followed by proton abstraction from C-1 of the glucose and would be assisted by general base catalysis (Gibson *et al.*, 1964). The imidazole ring of His516 and His538 are potential proton acceptor in this reductive half reaction hence, act as general base towards the C1-OH group of the glucose substrate (proton acceptor). Moreover, Asp514 and Arg4518 are critical amino acids for the binding of glucose, although the side chains of the residues Asp424 and Arg512 are also involved in the substrate binding while Arg512 and Asp424 are part of the fixed protein environment. The second is a hydride transfer from glucose C-1 to flavin N-5 and Arg224 promotes electron/proton transfer from glucose to the FAD. The hydride transfer is thought to be regulated by dissociation between Glu412 and His559. The electrostatic interaction between these residues controls the pH and therefore reactivity of the active site, although they

are not directly involved in the catalytic mechanism. As the proton is removed, the oxygen partial negative charge drives the hydride transfer to the N5 of FAD resulting in FADH₂. The conversion of FAD to FADH₂ does not change the amount of α -helix present in the protein outer shell, but reorganizes a fraction of random coil to β -sheet structure. A ketone is formed at the C1 of glucose as a result forming glucono- δ -lactone- the first product which hydrolysis to gluconic acid (GA).

In the oxidative half reaction, these two protons and two electrons transferred to the co-enzyme, FAD are transferred from the enzyme to molecular oxygen which yields hydrogen peroxide, H₂O₂ in the process. The H₂O₂ which is generated as a by-product in the equation below is a good oxidizing agent and in the presence peroxidase and reducing agent such as o-dianisidine (3, 3'-Dimethoxybenzidine), it is catalytically reduced to water (Hanušová *et al.*, 2013).

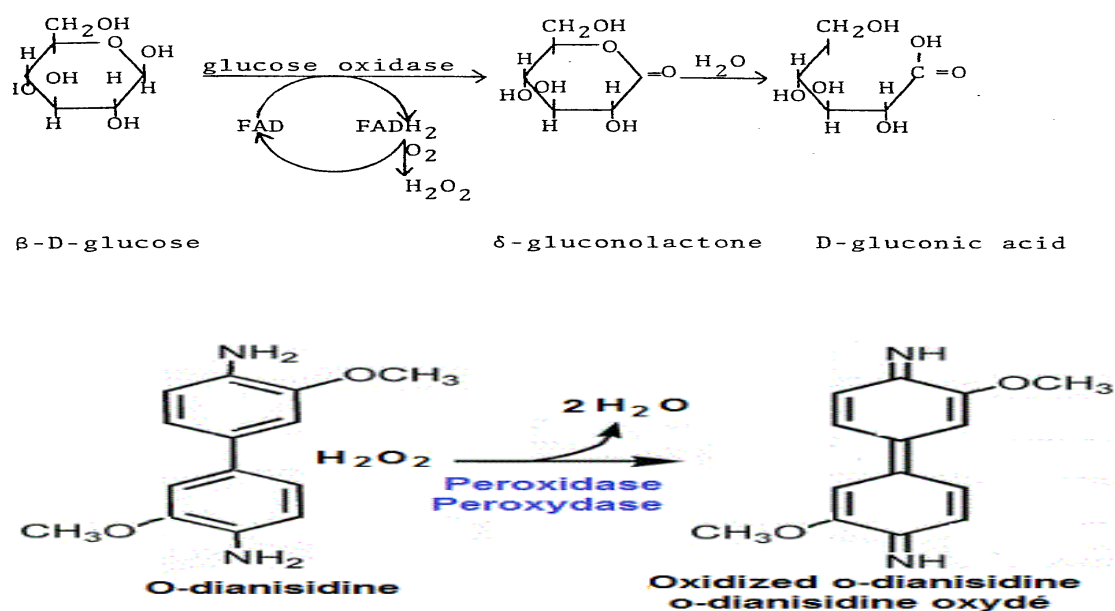


Fig.3. Mechanism of Catalysis of GOD and POD
 Source: sciencedirect.com

The oxidized form of o-dianisidine absorbs strongly at 430 nm or 460 nm, thus, by monitoring the absorbance, the kinetics of glucose oxidase catalyzed oxidation of D-glucose can be readily studied (Holmes, 1975).

1.3 Strategies for Improving GOD Production

For commercial applications, the production of GOD should be cost-effective and economical. Concerning these facts, researchers are putting their continuous efforts in opening and finding various facts and strategies. The large-scale application of GOD through naturally-occurring wild-type fungal strains has been hampered by low fermentation capacity, complicated purification process, and low efficiency. Further, impairments in the application of GOD are associated with an impurity in resources of GOD, which, include CAT, cellulase, and amylase (Frederick *et al.*, 1990).

It is also important to note that during production, GOD can be inactivated by H_2O_2 by oxidizing key methionine residues in the active sites. This occurrence results in its accumulation during the oxidation reaction followed by pH reduction, which may accompany the breakdown of D-glucono- β -lactone to GA, as well as inhibits enzyme production. It is by and large acknowledged that the appropriateness of a protein for industrial purposes relies upon its high turnover number, heat-stability, and dependability in different media (Klibanov, 1983). Although the expression of GOD is not high in its native state, engineering technologies have improved the capacity for large-scale GOD production. Studies of the enhancement of production and properties of GOD are still receiving a great deal of attention, presumably because of the current and extensive applications of this enzyme. The practical perspectives on the use of GOD includes its high catalytic activity and substrate affinity as well as high stability (Bhatti and Saleem, 2009). Therefore, the generation of ameliorated versions of GOD is an important biotechnological objective. As an effective biotechnological prospective, several strategies already available for efficient production of GOD are discussed below.

1.3.1 Solid-State and Submerged Fermentation Strategy

Production of GOD by fungi is commonly performed by solid-state fermentation (SSF) and submerged fermentation (SmF) (Mirón *et al.*, 2002, 2010). Submerged fermentation has been found to be more effective at producing GOD due to easier control of environmental factors involved in this technique when compared to SSF. Recently, Kriaa *et al.* (2016) assessed the

variability of GOD by *A. tubingensis* CTM507 based on titers measured under both SmF and SSF in association with growth and substrate-consumption and suggested that comparatively better results on fungal growth were obtained using SmF, whereas SSF represented significantly higher GOD activity (170 U/mL) as compared to SmF (43.73 U/mL). These findings increased the understanding of the phenomena responsible for the better ability of SSF than SmF to produce functional biomolecules. The studies also explained an SSF process for the low-cost production of important enzymes with attractive properties for commercial applications. These research findings confirmed that *A. tubingensis* was able to exhibit remarkable GOD activity when culture conditions were optimum as compared to other strains of organism. However, these traditional methods for the production of GOD have reached their limit. Accordingly, different strategies to overcome these limitations and enhance their production have been explored, including recombination, immobilization, mutagenesis and screening (Zia *et al.*, 2010).

1.3.2 Factors Affecting Glucose oxidase Production

1.3.2.1 Effect of carbon source on GOD production

The carbon sources used for GOD production ranged from simple C-3 such as glycerol up to a complex carbon source such as starch. Glucose is mainly used in all cultivation media with a concentration ranging from 40 to 80 g/L. However, in the industrial scale, sucrose or corn steep liquor can be used in case of bulk production of GOD in non-purified form which is utilized in gluconic acid production, food preservation and other non-analytical purposes.

During microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but is also used in the synthesis of polysaccharide and as energy source. The rate at which carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. Rapidly metabolized sugars are often associated with high productivity of growth-associated products or primary metabolites (Stanbury *et al.*, 1997). Hatzinikolaou and Macris (1995) observed that *A. niger* grew on all the carbon sources that they tested, but significant levels of GOD were only obtained using glucose, sucrose and molasses. They also stated that glucose (pure or as a product of sucrose hydrolysis) was the principal inducer for the transcription of the GOD gene.

1.3.2.2 Effect of Nitrogen Source on GOD Production

Also, different organic and inorganic nitrogen sources added to the cultivation medium enhanced production. Hatzinikolaou and Macris (1995) revealed that peptone or polypeptone with a concentration ranging from 3 to 20 g/L or yeast extract were the main common organic nitrogen sources. On the other hand, sodium nitrate was mainly used as inorganic nitrogen source for GOD production followed by ammonium dihydrogen phosphate. Ammonia has been used for pH control and its salts such as ammonium sulphate usually produces acidic conditions as the ammonium ion gets utilized and the free acid is liberated. On the other hand, nitrates normally cause an alkaline drift as they get metabolized. Hatzinikolaou and his colleague also found that peptone concentration had a marked effect on the total GOD production. With sucrose and molasses as carbon sources, maximum GOD activity was achieved at 1–2 % and 0.2–0.3 % peptone, respectively.

1.3.2.3 Effect of Calcium Carbonate on GOD Production

Petrucchioli *et al.* (1995) found that the addition of calcium carbonate to growth medium in shake flasks and in fermenters prevented pH drop due to gluconic acid production during *A. niger* cultivation for GOD production. Rogalski *et al.* (1988) showed that the synthesis of GOD was sensitively influenced by increasing concentrations of calcium carbonate (0 – 4.5 %) with maximal GOD activity at 3.5 %. Hatzinikolaou and Macris (1995) reported further that calcium carbonate is a potent and strong inducer of GOD in *A. niger* and obtained optimum GOD production at 4 and 5 % calcium carbonate supplementation in medium containing sucrose and molasses respectively. Bankar *et al.* (2008) also found that 3 % of CaCO₃ was found to be optimum for highest GOD production. Hatzinikolaou *et al.* (1996) grew *A. niger* in optimized cultivation media and demonstrated that the activity of the glycolytic enzyme, glucose-6-phosphate isomerase, was higher in growth media without calcium carbonate, while that of GOD and catalase (CAT) were quite low. Inclusion of calcium carbonate increased the GOD and CAT activities with a simultaneous decrease in the glucose-6-phosphate isomerase activity. They suggested that the addition of calcium carbonate in the growth media might cause a metabolic shift from glycolysis to the pentose phosphate pathway, thereby increasing GOD levels. Addition of calcium carbonate in the growth medium caused changes in GOD, CAT, 6-phosphofructokinase (6-PFK) and glucose-6-phosphate dehydrogenase (G-6-PDH) activities.

The 6-Phosphofructokinase is a key regulatory enzyme of Embden–Meyerhof–Parnas (EMP) pathway in most living cells (Liu *et al.*, 2001). Liu *et al.* (2001) further showed that cells grown in media without calcium carbonate produced high levels of 6-PFK and low amounts of G-6-PDH, GOD and CAT. Addition of calcium carbonate to the growth medium was found to increase the production of GOD and CAT, and decrease the synthesis of 6-PFK. Therefore, this observation might indicate that the inclusion of calcium carbonate is accompanied by a metabolic shift from the glycolytic pathway (EMP) to direct oxidation of glucose by GOD (Bankar *et al.*, 2009).

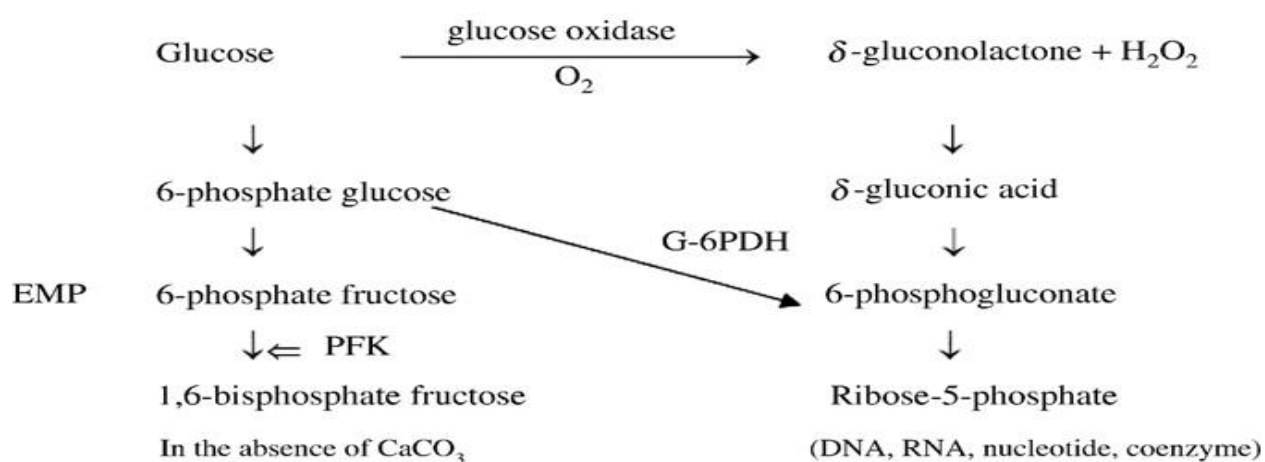


Figure 4: Metabolic pathway of glucose in the absence and presence of CaCO₃ by *Aspergillus niger*.

Source: Bankar *et al.* (2009)

1.3.2.4 Effect of Aeration and Agitation

Aeration and agitation are both therefore important factors for aerobic fermentation processes. Aeration of growing aerobic cultures fulfills the requirements of oxygen supply and also removes gaseous waste products. Oxygen for growth and production in fungal cultures is ensured by the aeration and agitation of the mycelial culture (Zetelaki and Vas, 1968). Filamentous fungi grow as dense aggregated mycelial mats resulting in decreased accessibility of oxygen to the actively growing cells. These problems are overcome by agitation of broth cultures (Zetelaki,

1970), the use of H_2O_2 as the sole oxygen source and the use of pure oxygen over air (Fiedurek and Gromada, 2000). Agitation increases the efficiency of aeration by forcing the supplied air bubbles to disintegrate into smaller bubbles resulting in an increased interface between the gas and the liquid (Zetelaki and Vas, 1968). Increased agitator speed corresponds to an increase in the concentration of dissolved oxygen resulting in a faster growth rate and increased GOD production (Zetelaki and Vas, 1968; Zetelaki, 1970).

1.3.2.5 Effect of culture pH on GOD Production

In microbial production of enzymes, culture pH can have profound effects on both the growth rate of microbes and the synthesis of enzymes. The appropriate pH for maximum production of the GOD can differ from that for optimum growth. The hydrogen ion concentration (pH) of the growth medium plays an important role by inducing conformational change in the organism and in enzyme secretion. It affects nutrient solubility and uptake, enzyme activity, cell membrane structure, byproduct formation and redox reactions (Banker *et al.*, 2009). The pH change observed during the growth of organism also affects product stability in the medium. Most of the strains used for the productions of GOD have an optimum pH between 6.0 and 7.0 for growth and enzyme production. This is maintained by the buffering capacity of CaCO_3 and phosphates.

1.3.2.6 Effect of Growth Temperature

The effect of temperature on GOD production is related to the growth of the organism. Most literatures have reported the temperature range of 25–37 °C for GOD production by mesophilic fungi. Optimum yields of GOD were achieved at 27–37 °C for *A. niger*. Caridis *et al.* (1991) studied the simultaneous production of GOD and CAT by *Alternaria alternate*, and revealed that GOD had its optimum temperature at 32.3 °C and CAT at 18.1 °C. Hatzinikolaou and Macris (1995) examined the effect of growth temperature on total GOD production at 22.5 to 32 °C, and found 27.5 °C as the optimum.

1.4 Characterization of Glucose Oxidase

1.4.1 Substrate Specificity

Enzymes have high level of catalytic efficiency, often far superior to chemical catalysts, and a high degree of *specificity* that allows them to discriminate not only between reactions but also

between substrates (*substrate specificity*), similar parts of molecules (*regiospecificity*) and between optical isomers (*stereospecificity*). These specificities warrant that the catalyzed reaction is not perturbed by side reactions, resulting in the production of one-wanted end-products, whereas production of undesirable by-products is eliminated. GOD is highly specific for β -D-glucose and show only marginal activities with other sugars. Using the reaction rate of glucose as reference (100 %), only 2-deoxy-D-glucose (20–30 %), 4-O-methyl-D-glucose (15 %) and 6-deoxy-D-glucose (10 %) are oxidized by glucose oxidase from *A. niger* at a significant rate (Leskovac *et al.*, 2005). The other sugars such as maltose and fructose were oxidized at lower rate while galactose and arabinose were not oxidized by GOD from *A. niger*. β -D-glucose gets oxidized in the presence of molecular oxygen at C-1 position to δ -glucono-1, 5-lactone, which is, in turn is spontaneously hydrolyzed to D-gluconic acid. These structural features and the regioselectivity of the reaction allow a clear distinction between GOD and pyranose oxidase, which is a homotetramer, and oxidizes D-glucose at the C-2 position (Danneel *et al.*, 1993; Hatzinikolaou *et al.*, 1996; Pluschkell *et al.*, 1996).

1.4.2 pH Optima and Stability

The change in pH affects the ionization of essential active-site amino acid residues, which could be involved in substrate binding and catalysis. The pH plays an important role in maintaining the proper conformation of an enzyme. Most proteins are only active within a narrow pH range, usually in the range of 5–9 (Bankar *et al.*, 2009). The pH optima of GOD vary from 5.0 to 7.0; glucose oxidase from most fungi and yeast have pH optima in the acidic to neutral range such as *A. niger* and *P. chrysogenum* which show 5.0 to 6.0 as the optimum pH (Bhatti *et al.*, 2006). In contrast, the GOD obtained from *P. funiculosum* 433 and *P. canescens* show slightly alkaline pH optima of 6 to 8.6 (Sukhacheva *et al.*, 2004).

1.4.3 Optimum Temperature and Stability

Enzymes are known to be sensitive to changes in temperature. The relationship between reaction rate of an enzyme and temperature is exponential. For every 10 °C rise in temperature, the rate of an enzyme reaction doubles to Q_{10} and enzymes display maximal activity at a temperature known as the optimum temperature of the enzyme (Bankar *et al.*, 2009). The lowest optimum

temperature range for GOD is reported to be 25–30 °C from *P. funiculosum* 433 (Sukhacheva *et al.*, 2004) and the highest to be 40–60 °C from *A. niger* and *P. amagasakiense* ATCC 28686 (Kalisz *et al.*, 1997); Bhatti *et al.* (2006) also reported the pH optimum for *A. niger* to be 40 °C.

1.5 Applications of Glucose Oxidase in Various Sectors

Since its discovery in 1928, glucose oxidase (GOD) is still one of the most studied enzymes worldwide because of its various industrial applications. GOD as a hydrogen peroxide producing enzyme is commonly used in the textile industry for bleaching and in food industry for preservation. In the last decade's logical gates amperometric biosensors and biofuel cells were constructed using GOD. For example, glucose oxidase has been used for textile bleaching, in the food and baking industry or for the production of gluconic acid. The most well-known application of glucose oxidase is its use in daily and continuous glucose sensors for diabetes management. Glucose oxidase may also find applications in glucose/O₂ biofuel cell or self-power sensors. Glucose oxidase (β -D-glucose: oxygen 1 oxidoreductase, EC 1.1.3.4) is a flavoenzyme that catalyses the oxidation of β -D-glucose to gluconic acid using molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide (Bankar *et al.*, 2009). The resulting hydrogen peroxide is an environmentally acceptable oxidizing agent that can be used, for example, in textile industry to promote the whiteness of fabrics (Tzanov *et al.*, 2002) and treat strongly coloured wastewaters. The latter are conventionally treated by application of membranes, physico-chemical and advanced oxidizing processes (Hung *et al.*, 2005).

Further, the most remarkable and novel application of GOD has been found in the biosensors and biofuels in recent years as a useful tool for outlining new and more proficient modern procedures. In addition, the GOD combined with cross-linked enzyme aggregates of HRP (horseradish peroxidase) are used in various sectors of food and pharmaceutical industries for catalyzing cascade chemical reactions (Zhang *et al.*, 2016; Nguyen and Yang, 2017). Recently, the GOD modified with hydrophilic polymers via *in-situ* polymerization methodology showed higher enzyme bioactivity. This efficient technique for the surface modification of enzyme can be applied for the amelioration of other biomolecules, and could envision broad application in

varied areas such as biomedicine, food industry, and biotechnology (Xu *et al.*, 2017a). Thus, widespread application and increasing demand of GOD in different industries (chemical, pharmaceutical, food, beverages, clinical chemistry, biotechnology, medical diagnostics, environmental conservation, energy, textile, and other industries) have led to the need to produce the enzyme extensively (Bankar *et al.*, 2009b). However, utilization of enzymes is frequently restricted by their quick inactivation at extreme temperature, pH or the presence of surfactants upon exposure to elevated temperatures.

1.5.1 Applications in Various Food Sectors

1.5.2 In Baking Industry

Glucose oxidase is an efficient oxidant for the production of bread with improved quality and extended loaf volume in the baking industry (Rasiah *et al.*, 2005; Wong *et al.*, 2008; Steffolani *et al.*, 2010). Hydrogen peroxide produced by GOD yields more elastic and viscous dough. In addition, Vemulapalli and Hoseney (1998) reported the drying effects of GOD on the dough, which were mediated by the gel-forming ability of water-soluble pentosans, reduction in sulfhydryl content, and the increase of viscosity in the water-soluble dough. Also, GOD has been found to display protein cross-linking in the dough (Rasiah *et al.*, 2005). Glucose oxidase improves the quality of bread and strengthening of wheat dough when used as an additive (Bonet *et al.*, 2006).

Glucose oxidase also enhances the viscoelasticity of dough (Kouassi-Koffi *et al.*, 2014). Specifically, Kouassi-Koffi *et al.* (2016) assessed the effects of wheat dough viscoelasticity by adding GOD to predict final bread quality. However, enzymes must be added with care since undesirable effects can be caused by excessive enzymes. Glucose oxidase, along with lipase also enhances the quality and shelf-life of pan bread (El-Rashidy *et al.*, 2015). Dagdelen and Gocmen (2007) studied the effects of GOD along with ascorbic acid and hemicellulase on bread quality and dough rheology and found that bread quality is mainly dependent on original wheat flour quality, while dough rheology depended on the amount of enzyme. The combination of GOD, α -amylase, and xylanase on dough properties and bread quality were also studied by Steffolani *et al.* (2012). Kerman *et al.* (2014) further investigated the strengthening properties of wheat dough and quality enhancing parameters of wheat bread in response to the addition of GOD along with

ascorbic acid. Similarly, da Silva *et al.* (2016) verified the performance of xylanase and its interaction with GOD and ascorbic acid on the quality of whole wheat bread. Furthermore, the addition of basal additives as well as ascorbic acid (32 %), α -amylase (4.2 %), and GOD (63.8 %) to wheat flour, reduced crumb firmness and chewiness, as well as improved adhesion, elasticity, cohesion, and bread volume specifically (Kriaa *et al.*, 2016). Recently, the synergistic effects of amyloglucosidase, GOD and hemicellulase utilization on the rheological behavior of dough and quality characteristics of bread were studied by Altinel and Ünal (2017). Decamps *et al.* (2013) revealed the molecular mechanism of dough and bread stability improvement by the pyranose oxidase from *Trametes multicolor* and GOD by *A. niger* during the cross-linking of gluten protein and arabinoxylan by the formation of H_2O_2 . Aprodu and Banu (2015) studied the effects of Psyllium, pea fiber, oat bran, water, and GOD on rheology and baking properties of gluten-free bread made from maize, and it was suggested that GOD has significant ability to improve the specific bread volume for all types of fibers.

1.5.3 In Beverage Industry

Glucose oxidase plays a novel role in the manufacturing of beverages because it is used to diminish the low alcohol substances of wine by eliminating the residual glucose that would otherwise be converted into alcohol through anaerobic fermentative processes. Moreover, the H_2O_2 produced during chemical processes imparts a bacteriocidal effect on acidic corrosive and lactic corrosive microbes amid the fermentative procedures. This process must be conducted by adding GOD prior to fermentation as GOD utilizes a portion of the glucose presents, making it inaccessible for liquor aging, bringing about wine with decreased alcohols and simultaneous generation of H_2O_2 that reduces the growth of fermentative microorganisms. The H_2O_2 produced could easily be removed from the system using CAT, which breaks it into molecular oxygen and water. The bacteriocidal effect of H_2O_2 reduces the addition of other chemical preservatives in the wine (Malherbe *et al.*, 2003). It has been well-demonstrated that pre-treatment of grape juice by GOD/CAT enzyme system can reduce alcohol fermentation potency significantly through conversion of available glucose to GA. Similarly, the technical feasibility of GOD/CAT enzyme system was investigated as an alternative to decrease the glucose concentration and eventually production of reduced red wine (Valencia *et al.*, 2017). Glucose oxidase has shown significant

efficacy on determining glucose content in body fluid and effectively removes oxygen and residual glucose from beverages (Yildiz *et al.*, 2005). Lopes *et al.* (2012) developed a biosensor composed of GOD and immobilized HRP, which efficiently determined glucose content in beverage samples such as orange juice, as well as energetic and sport drinks. Glucose oxidase bound emulsified nano-particles of bovine serum albumin (BSA) along with chymotrypsin results in soft drinks/non-alcoholic beverages being free from turbidity and opalescence while maintaining their pH as acid regulators (Sharma, 2012). Further, Mason *et al.* (2016) demonstrated the potential role of a novel glucose electrochemical biosensor based on the immobilization of GOD into a nylon nano-fibrous membrane (NFM) during analysis of glucose in commercial beverages and monitoring of the brewing process for making beer.

1.5.4 Use for Production of Dry Egg Powder

Glucose oxidase has been used effectively to remove remaining glucose and oxygen from foods to extend their shelf-life (Zia *et al.*, 2012). The reaction of protein amino group and reducing sugars is known as non-enzymatic Maillard browning, which results in the formation of unwanted flavor and undesirable browning in dried egg powder, suggesting prior removal of glucose content from the liquid egg before its drying (Sisak *et al.*, 2006). Removal of glucose provides dried egg powder a prolonged shelf-life and increased microbial tolerance. Also, production of H_2O_2 by Maillard reaction helps to destroy unwanted microbes normally found in liquid egg (Dobbenie *et al.*, 1995), while it can later be evacuated utilizing a moment catalyst, CAT, which changes over H_2O_2 to oxygen and water (El-Hariri *et al.*, 2015). The GOD/CAT system is utilized to remove glucose during egg-powder production for use in the baking industry, which prevents browning during dehydration brought about by the Maillard response, and to give slight improvements to the crumb properties of bread and croissants (Rasiah *et al.*, 2005). Increasing browning mediated by the Maillard reaction is also a noteworthy aspect causing harmful effects to eggs and potato products. Application of GOD may provide sustainable results to reduce unwanted browning in the process (Low *et al.*, 1989; Chilaka *et al.*, 2002).

1.5.4.1 Applications in Various Pharma/Medicine Sectors

1.5.4.2 Use of Gluconic Acid (GA) Production in the Food/Pharma Industry

One of the major applications of the GOD catalyzed reaction is the production of GA and its derivative salts. Gluconic acid was found to play extensive roles in different sectors of food industries and utilized as a causticity controller, raising specialist, colour stabilizer, an antioxidant, and chelating agent in bread, feeds, beverages, and so on (Brookes *et al.*, 2005). In dairy industries, GA is used for the cheese curd formation, improvement of heat stability of milk, prevention of milk stone, and cleaning of aluminum cans. However, GA is most widely used as acidulant, sequestrant as well as potential anti-oxidant in various industries (Golikova *et al.*, 2017). In the pharmaceutical based industries, the metal derived Na, Ca, Zn, and Fe salts of GA is widely used in the synthesis of important drugs including sodium, calcium or ferrumgluconates, and glucono- δ -lactone which have diverse industrial applications (Ramachandran *et al.*, 2006; Golikova *et al.*, 2017). Sodium gluconate has great potential to chelate metal ions and can be used to remove bitterness from food stuff (Costa *et al.*, 2015; Pal *et al.*, 2016). Pharmaceutical application of calcium gluconate has been confirmed for the treatment of calcium associated deficiencies (Khurshid *et al.*, 2013), whereas for the treatment of common cold, wound healing, and zinc deficiency associated with delayed sexual maturation, infection susceptibility, mental lethargy, and skin rashes have been well treated using zinc gluconate.

Gluconic acid can be produced through biochemical, electrochemical, bio-electrochemical, and fermentative processes, although the latter are preferred for GA production as other approaches are expensive and less productive (Wong *et al.*, 2008; Pal *et al.*, 2016). It has been reported that GA production through GOD catalyzed reaction is highly dependent on the substrates used, oxygen concentration and temperature (Ramachandran *et al.*, 2006; Khurshid *et al.*, 2013). Moreover, the catalytic efficiency for the conversion of glucose to GA is highly dependent on the stability of GOD. Recently, it has been demonstrated that enzymatic cross-linking with GLU modified on inorganic support (SiO₂) system provides most active and stable system that causes the maximum (up to 85 %) yield of GA (Golikova *et al.*, 2017). Several studies have been conducted to optimize the production of GA. For example, Purane *et al.* (2011) optimized parameters such as glucose concentration, inoculum density and inoculum age for GA

production using *P. chrysogenum* 724 and found that the maximum amount of GA produced (31.16 g/L) was reported at a glucose concentration of 100 g/L. Ping *et al.* (2016) demonstrated the effects of oxygen supply on intracellular flux distribution for enhanced production of sodium gluconate by *A. niger* and reported that the higher oxygen concentration was required for enhanced synthesis of GA. Further, Ramezani *et al.* (2013) reported the effects of hydrodynamic properties on kinetics parameters to achieve higher GA production by using GOD. It was found that the increased oxygen gas velocity resulted in the increasing rate of glucose oxidation reaction because of the higher transformation of oxygen from a gas to a liquid state. Optimization of mass transfer characteristics and operating condition during GA production with immobilized GOD showed that a bubble column reactor had better mass transfer properties because it provided higher GA production under low GOD activity relative to other reactors. Recently, many researchers have investigated GA production using substrates through multienzymatic steps.

Mafra *et al.* (2015) developed a method for GA production using sucrose as the substrate that was catalyzed by a multi-enzymatic system, including invertase, GOD, and CAT in an air-lift reactor. Similarly, Silva *et al.* (2011) reported the production of GA using sucrose and multi-enzymatic components in batch and membrane continuous reactors. Gluconic acid production through fermentative processes also depends on morphological parameters of the fermenting organism used. Indeed, it was reported that the dispersed pattern of the mycelia morphology of *A. niger* resulted in increased GA production rather than pellet morphology (Lu *et al.*, 2015). Gluconate production through fermentative processes is the most widely accepted technique and the most common challenge to these methods is downstream processing (separation and purification). However, these hurdles can be resolved by membrane-based separation (Pal *et al.*, 2016).

1.5.4.3 Use as an Antioxidant/Preservative Agent

Many food products contain oxygen, which promotes bacterial growth. In canned/bottled/package food, it is important to maintain an anaerobic condition, thus, removal of oxygen is essential in packaged food products (Kirk *et al.*, 2002). Karimi *et al.* (2012b)

studied the removal of dissolved oxygen from water through the reduction of glucose, catalyzed by GOD and CAT enzymes using substrate concentration of 65.5 mM L^{-1} , and glucose oxidase activity 252 U/L and found efficient removal of oxygen dissolved in water under optimal conditions (pH 5.6, $T = 40^\circ\text{C}$) which measured to be 98.2 % in 30 seconds. Moreover, GOD could be utilized for the removal of oxygen from the top of bottled beverages such as wine and beer to maintain the taste and flavour (Labuza and Breene, 1989; Wong *et al.*, 2008).

Non-enzymatic browning of processed fruits and tomato puree can be controlled using the GOD/CAT system during storage. Food deterioration and rotting of high-fat types, for example, mayonnaise and mixed greens dressing are associated with lipid peroxidation (Isaksen and Adler-Nissen, 1997), where the application of the GOD/CAT system has potential to retard lipid peroxidation during storage (Bankar *et al.*, 2009b). The GOD/CAT system has the oxygen scavenging ability, thereby making oxygen unavailable for lipid metabolism during oxidation of glucose (Isaksen and Adler-Nissen, 1997). The overall GOD catalytic reaction consumes two glucose molecules and an oxygen molecule, resulting in the production of two GA molecules. During the reaction, the consumption of oxygen allows GOD to be used as a strong antioxidant and scavenger of oxygen, thus facilitating its application as a food preservative due to stabilizing effect. Additionally, GOD has been effectively used in the food system as a strong stabilizer due to its oxygen removing ability and prevents colour and flavour loss in a variety of beverages including canned fish, beer, soft and energetic drinks (Crueger and Crueger, 1990; Bhat *et al.*, 2013). Glucose oxidase can also be used instead of potassium bromate as an oxidizing agent in bread making (Moore and Chen, 2006).

1.5.4 .4 Use in Oral Care

Glucose oxidase and lactoperoxidase have been used in oral health-care products due to their antimicrobial potential (Güneri *et al.*, 2011). The H_2O_2 produced by GOD functions as a valuable bactericide. *Streptococcus mutans*, which inhabits the oral cavity and causes tooth-decay, is carried by almost every human being. The ability of GOD to kill *S. mutans* can be improved by enzymatic fusion using heavy-chain antibodies (Etemadzadeh *et al.*, 1985). Hill *et al.* (1997) reported that GOD and/or GOD with HRP encapsulated reactive liposomes were found to exhibit

antibacterial effects against tooth decay bacteria in saliva, suggesting their potential efficacy in oral hygiene. Senol *et al.* (2005) also reported the antibacterial activities of oral care products containing GOD against ventilator-associated pneumonia pathogens.

1.5.4.5 Use as an Antimicrobial Agent

Glucose oxidase has antimicrobial activity against different foodborne pathogens. It has shown enormous potential to inhibit the growth of various foodborne pathogens, including *Clostridium perfringens*, *Campylobacter jejuni*, *Salmonella infantis*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Cichello, 2015). Further, covalently immobilized GOD on biorientated polypropylene films was found to inhibit the growth of *E. coli* and *B. subtilis* (Vartiainen *et al.*, 2005). Murray *et al.* (1997) reported inhibitory effects of culture filtrates of transformed *T. flavus* against *Verticillium dahliae* *in vitro* and found that the GOD secreted from *T. flavus* dramatically inhibited the microsclerotial and hyphal growth of the large proportions of *V. dahliae*. Malherbe *et al.* (2003) reported that the *S. cerevisiae* transformants harbouring the GOD gene from *A. niger* exhibited antimicrobial efficacy in plate culture assay against lactic acid and acetic acid producing bacteria. Zia *et al.* (2013) also observed the antibacterial activity of GOD produced by *A. niger*. Glucose oxidase produced by *P. chrysogenum* showed antifungal activity against different fungal pathogens (Leiter *et al.*, 2004). Glucose oxidase and its products such as H₂O₂ and GA showed *in vitro* antimicrobial activity against *Paenibacillus larvae* ATCC9545 (Sagona *et al.*, 2015). Application of edible antimicrobial films has been approved to enhance the shelf-life of food products by releasing enough amounts of antimicrobial substances on the surface of food products. The antimicrobial activity of the edible films containing antimicrobial agents, nisin (N), and/or GOD, into the matrix of whey protein isolate (WPI) films (which is a liquid that separates from milk during cheese production) was assessed against *Brochothrix thermosphacta* (NCIB-10018), *Listeria innocua* (ATCC-33090), *E. coli* (JM-101), and *Enterococcus faecalis* (MXVK-22). The greatest antibacterial activity was observed in WPI films containing only GOD (Murillo-Martínez *et al.*, 2013). The polyamide and ionomer films with immobilized GOD inhibited the growth of bacteria such as *E. coli* CNCTC 6859, *Pseudomonas fluorescens* CNCTC 5793, *Lactobacillus helveticus* CH-1, *Listeria ivanovii* CCM 5884 and *L. innocua* CCM 4030 on agar media (Hanušová *et al.*, 2013). Recently, a new photodynamic glucose-based antimicrobial

system encapsulating GOD, HRP, and BRET (bioluminescence resonance energy transfer) was developed for the inactivation of various bacterial and fungal pathogens through the network of organic and inorganic materials (Yuan *et al.*, 2015).

1.6 Use as Biosensors in Medicine Industry

Biosensors are widely used in the food industry, monitoring of environmental hazards, and clinical applications. Glucose oxidase has been widely employed in glucose-based biosensors because of its high selectivity for glucose and functionality under extreme temperature, pH, and ionic resistance. Glucose biosensors for diabetic blood monitoring are very convenient, reliable, rapid, and accurate. Many studies have been conducted to develop sophisticated advanced technologies, and better alternatives such as point sample tests, and the continuous glucose monitor (CGM) are being developed (Wang and Lee, 2015; Sode *et al.*, 2016). The CGM sensor has shown a significant role in diabetes as an alternative means while measuring blood glucose level and provides an alarming node on events associated with blood glucose metabolism (Wang and Lee, 2015). Further, in recent years, the drawbacks and limitations associated with glucose biosensors have been improved using advanced approaches such as electrodes, membranes, enzyme immobilization, and nanocomposite film modified electrodes.

The glucose biosensor system works on the concept of catalyzation of β -D-glucose oxidation by the immobilized GOD using molecular oxygen, resulting in the production of GA and H_2O_2 . Amperometric glucose biosensors can be divided into three generations based on their operative principles. In the first generation biosensors, the concentration of oxygen/ H_2O_2 is measured through an appropriate electrode and used as an indicator for glucose monitoring. The chemical reaction leading to oxidation of glucose causes depletion of oxygen or production of H_2O_2 . The involvement of other redox species was the major problem associated with this generation. In second generation biosensors, mediators are involved in the backward and forward flux of electrons between the enzyme and electrodes. This generation of biosensors had a low turnover rate and reduced proximity between the electrodes. Hence, these biosensors suffered from redox interferences (mostly oxygen). However, in the greatly advanced third generation biosensors, a method of direct electron transfer from enzyme to electrode was developed through “wired”

relay centers (Wong *et al.*, 2008) by immobilizing enzymes within the thin films with different modifications. The direct electron transfer process could be achieved through several modifications and the biosensors developed based on such modified electrodes have been found to have good reproducibility, selectivity, and stability for glucose oxidations. Velmurugan *et al.* (2015) reported the direct electron transfer reaction of GOD at gold nanoparticles-electro activated graphite/screen printed carbon electrode (AuNPs-EGr/SPCE).

The method of immobilization of GOD is considered a pivotal factor for the development of highly stable glucose biosensors with long-term operational life. The various methods by which GOD is incorporated into a biosensor include absorption, covalent attachment, cross linking and microencapsulation (inert membrane entrapping enzyme into the transducer surface). Moreover, such immobilization could be achieved through one or in combination with others. Hong *et al.* (2016) developed biosensors based on GOD immobilized on graphene oxide (GRO) through various preparative approaches including enzyme adsorption (EA), enzyme-adsorption and cross-linking (EAC), and enzyme adsorption, precipitation and cross-linking (EAPC). One of the most important aspects of such GOD based immobilization is the greater loading of enzymes for the efficient functioning which makes the overall system stable and selective in the form of cross-linking precipitated GOD aggregates. It has been reported that the problem of enzyme absorption on GOD based biosensors could be improved by using silicalite modified electrodes. The response of glutaraldehyde (GLU) cross-linked along with GOD adsorption on the silicalite-modified electrode (SME; GOD-SME-GLU) was found to be much more sensitive and reproducible than alone GOD-GLU based biosensors and have been employed in determining the concentration of glucose in juicy nectars and fruits (Dudchenko *et al.*, 2016). Recently, carbon nano-chips (CNCs) have been used to modify the glassy carbon electrode for immobilizing GOD with the help of chitosan. These GODs/CNCs based system have increased the electrochemical response when used in GOD based bioelectronics devices (Kang *et al.*, 2017). The properties of the immobilized enzyme in biosensors depend on both the enzyme and the supportive material involved (Ang *et al.*, 2015). The two major limitations that restrict the immobilization of GOD on solid electrodes include inadequate electrical communications between the active sites of GOD and the surface of electrodes including enzyme leaching.

A huge range of electrode substrates has been used recently to overcome these problems which include metal-based nano-particles, carbon nano-tubes (CNTs), mesoporous silica, polymers, and sol-gels. However, the electrocatalytic activities of GOD toward the oxidation of H_2O_2 in GOD based amperometric biosensors has been improved through the use of multilayered reduced graphene oxides (MRGO) sheets (Hossain and Park, 2017). In one such type of amperometric biosensor, GOD was immobilized onto MRGO sheets, and decorated with platinum and gold flower-like nanoparticles (PtAuNPs) modified Au substrate electrode. It has been found that fabricated MRGO/PtAuNPs modified hybrid electrode revealed higher electrolytic oxidation of H_2O_2 (Hossain and Park, 2017). Apart from these methods, the use of CNTs is considered a notable advancement in biosensing for the construction of glucose sensors due to their ability to promote the reactions of electron transfer of biologically significant biomolecules. Low-site-density based nano-electrodes aligning CNTs have been used for the detection of glucose (Lin *et al.*, 2004), which showed good signal-to-noise ratio and detection limits. The use of nano-materials in biosensors and bioelectronic devices has provided a new platform for efficient glucose monitoring due to their improved response time, high sensitivities, low detection limits, wide range linearity, and low power requirements. Recently, the real-time monitoring of glucose concentrations in GOD based biosensors has been achieved through the use of plasmonic nanoparticles/nanorods (Xu *et al.*, 2017b). Wang *et al.* (2016) recently reported optimization and characterization of covalent immobilization of GOD using multi-walled carbon nano-tubes (MWCNTs) to maximize the loading of GOD, thereby, increasing the longevity of electric power or sensing signals.

1.6.1 Use for Inducing Resistance

Glucose oxidase also plays an important role in the induction of defense responses in plants. Transgenic *Trichoderma atroviride* incorporating multiple copies of a GOD-encoding gene from *A. niger* was able to produce H_2O_2 after infection by fungal pathogens and showed higher antimicrobial activity against fungal pathogens as well as induced systemic resistance in plants (Brunner *et al.*, 2005). Involvement of H_2O_2 during the plant resistance to bacterial disease agent was also revealed in Arabidopsis plant challenged by transconjugants of *Pseudomonas syringae* Pv. *phaseolicola* expressing the avirulence genes *avrPpiA* and *avrPphB* matching the RPM1 and

RPS5 resistance genes (Soylu *et al.*, 2005). The application of genetic engineering tools has provided sustainable results in GOD expression in plants with increasing resistance to plants from bacterial infections (Wu *et al.*, 1995).

Further, Maruthasalam *et al.* (2010) reported that fungal GOD expression in transgenic tobacco plants provides them resistance from the cold by activating antioxidative defense system. Endogenous H₂O₂ levels of tobacco plants (*Nicotiana tabaccum L. cv. SR1*) were enhanced by constitutively expressing a GOD gene isolated from *A. niger*, and transgenic tobacco plants exhibited resistance to leaf spot fungal disease and bacterial wilt disease (Selvakumar *et al.*, 2013). Similarly, the GOD gene from *A. niger* was inserted into potato plants, which resulted in leaves and tubers that produced high amounts of H₂O₂ constitutively and acquired resistance to the bacterium *Pectobacterium carotovorum* sub sp. *carotovorum* and the fungi *Phytophthora infestans* and *V. dahliae* (Bastas, 2014). Kachroo *et al.* (2003) also reported that GOD-overexpressing transgenic rice plants showed enhanced resistance to both *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae*. Moreover, GOD isolated from *A. tubingensis* CTM 507 was found to have reduced spore formation, mycelial cord induction and mycelical vacuolization of pathogenic *Fusarium solani*. Hence, *A. tubingensis* CTM507 suppressed the pathogenic attack and manifestation of disease in tomato (Kriiaa *et al.*, 2015).

1.7 Applications in Textile and Energy Production Sectors

1.7.1 Use for Enzymatic Bleaching in the Textile Industry

Bleaching provides decolourization of natural pigments with a pure white appearance of the fibers. Glucose oxidase has proven to be effective in the production of H₂O₂ for bleaching in the textile industry, the most effective bleaching agent of industrial significance (Bankar *et al.*, 2009b; Soares *et al.*, 2011). Hydrogen peroxide produced by GOD decolorizes paprika dye effluent and malachite green. Moreover, the GOD application to the bleaching of textiles during upstream resizing and bio-scouring processes has shown promising results with the additional release of glucose (Buschle-Diller *et al.*, 2002). The immobilization of GOD enzyme for the generation of H₂O₂ and its optimization has significantly affected the processing of bleaching in textiles. Tzanov *et al.* (2002) suggested the use of covalently-immobilized GOD on alumina and

glass underpins for increased re-usage efficiency of enzymatic bleaching in textiles. The stability of GOD has been enhanced by a few immobilization procedures on different blankings (Godjevergova *et al.*, 2006). Recently, Aber *et al.* (2016) utilized a bio-fenton procedure for the decolourization of a dye solution with *in situ* production of H_2O_2 by enzymatically catalyzed oxidation of glucose. They developed the optimal decolourization conditions by immobilizing GOD on magnetite nano-particles (Fe_3O_4) and reported that the best decolourization was achieved at a temperature of 10 °C, pH of 6, GOD/support ratio of 1,800 U/g and time of 2.5 h. Under these conditions, they found that 450 U of GOD immobilized/grams of magnetite and that this system could be efficiently used for the oxidation of glucose and *in situ* generation of H_2O_2 for the removal of acid yellow 12. Farooq *et al.* (2013) compared conventional bleaching with GOD catalyzed bleaching of knitted cotton fabric and found that enzymatic bleaching led to better whiteness and mechanical properties such as tensile strength and tear strength. Furthermore, H_2O_2 produced by GOD was shown to be a significant alternative to the most extensively used commercial H_2O_2 , in the textile processing industries. Moreover, Tzanov *et al.* (2001) found that the whiteness index of fabrics increased in response to a high concentration of hydrogen peroxide followed by a significant decrease in glucose concentration. However, the initial high concentration of glucose may cause discoloration of fabrics due to the presence of residual glucose. This problem can be overcome by using an excessive amount of GOD with an increased incubation time (Saravanan *et al.*, 2010). Other important aspects of enzymatic processing in textiles are that the H_2O_2 generated during bleaching produces a comparable effect to scoured woven cotton fabric, while the GA produced acts as a chelator for metal ions, removing the need for use of an additional stabilizing agent (Tzanov *et al.*, 2002). Additionally, the simultaneous application of GOD with peroxidases in the decoloration process improves bleaching of natural fibers (Opwis *et al.*, 2008).

1.7.2 Use in Improving Biofuel Production

Biofuel cells (BFCs) use either enzymes or whole cell organisms as a biocatalyst to generate power directly from fuel substrates (glucose and ethanol). These enzyme-based systems are considered better alternatives for the development of future implantable devices (Sode *et al.*, 2016). During the last decade, the rapid progress in enzyme-based BFCs allowed the

development of membrane/compartment-less devices for miniaturization and use in implantable devices such as insulin pumps and glucose sensors in artificial pancreata and pacemakers (Falk *et al.*, 2014). The design for manufacturing BFC is adjusted in such a way so that one electrode consisting of electro-conductive material is modified by a biocatalyst (enzymes) for specialized oxidation and reduction reactions. In one approach to the construction of BFCs, the catalytic reactions occurring at the anode are either mediated by GOD or glucose dehydrogenase or coupled with reduction reactions at the cathode mediated by di-oxygen reducing enzymes such as laccases, bilirubin oxidase or cytochrome oxidase (Barrière *et al.*, 2006; Figure 5). More recently, efforts have been made to improve the catalytic efficiency of this system several folds by co-immobilization of GOD with other enzymes such as CAT. Christwardana *et al.* (2017) developed membrane less glucose biofuel cells (GBFCs) system for enhancing the power generation of membrane-less BFCs using GOD-CAT co-immobilized catalyst (CNT/PEI/(GOD-CAT) and reported that the system has increased the biocatalytic efficiency of GBFCs due to some synergistic mechanisms including removal of harmful H_2O_2 moiety by CAT and the simultaneous activation of GOD based desirable reactions.

Most of the output voltage and current signals in a typical BFC depends on the concentration of fuel, hence, enzyme-based BFCs could serve as an alternative means of an enzyme sensor system. Recently, there has been a great deal of efforts toward the development of bio-electrochemical devices based on unique enzymes. The presence of electron transfer subunits or domain in these enzymes imparts specificity to directly transfer electrons to the electrodes during a bio-catalytic reaction (Tsugawa *et al.*, 2012). The sensitivity and conversion efficiency of BFCs are highly determined by efficient electron transfer occurring at the enzyme active center and electrode interface. However, these critical factors of sensitivity and conversion efficiency lead to difficulty in GOD catalysis based BFCs because the redox center inside this enzyme is buried inside the structure, a long way from any feasible electrode binding site (Sode *et al.*, 2016). This problem can be resolved by using artificial electron acceptors and mediators or by precisely modifying the electrode surface with nano-scale conductive materials. The mediator assists in this electron transfer reaction through a mediator electron transfer (MET) mechanism by using little redox dynamic particles/polymers as electron bearers (arbiters) to/from one electrode to another or bio-catalytic site (Barton *et al.*, 2004; Figure 6). These mediators can be

polymerized specifically onto the surface of electrodes or co-immobilized with GOD to facilitate the rate of electron transfer by several-fold. Recently, a novel method entrapping cross-linked aggregates of GOD within a graphitized mesoporous carbon (GMC) network has been reported for the production of GOD nano-composites with an ability to provide the maximum rate of electron transfer and high electrical conductivity (Garcia-Perez *et al.*, 2016). In contrast, the application of CNT immobilized GOD has given promising results (Ivnitski *et al.*, 2007). Nano-carbon function has shown perfect compatibility with other biological and chemical approaches with enhanced enzymatic functionality in implanted BFCs. Babadi *et al.* (2016) found that they could generate biocatalyst either by direct transfer of electrons or redox mediators for electron transfer.

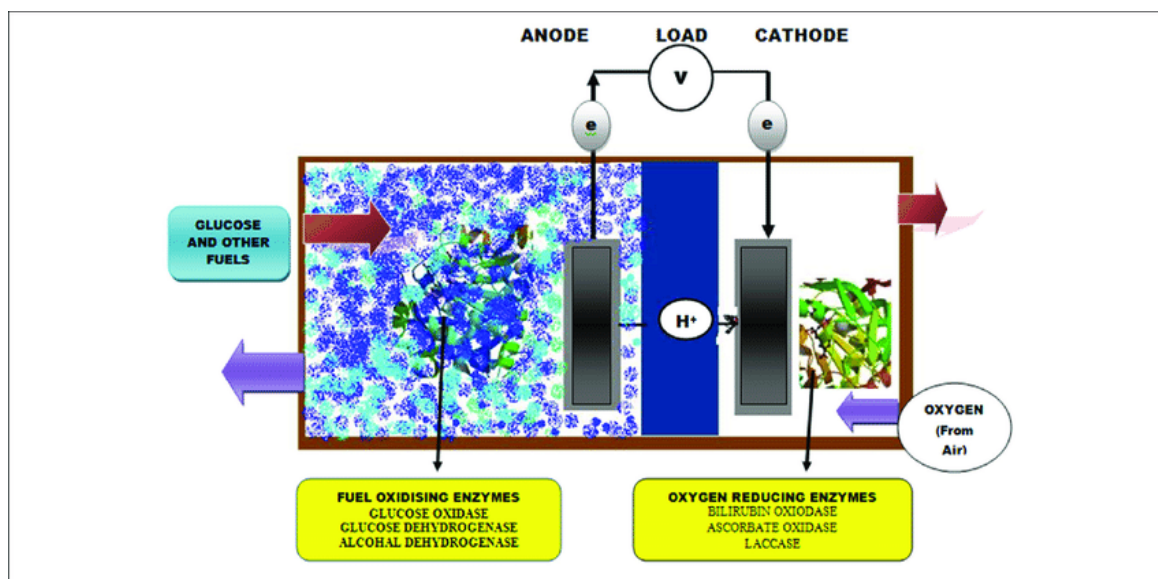


Fig. 5: Generalized diagram of typical enzymatic biofuel cell (EBC) with associated components

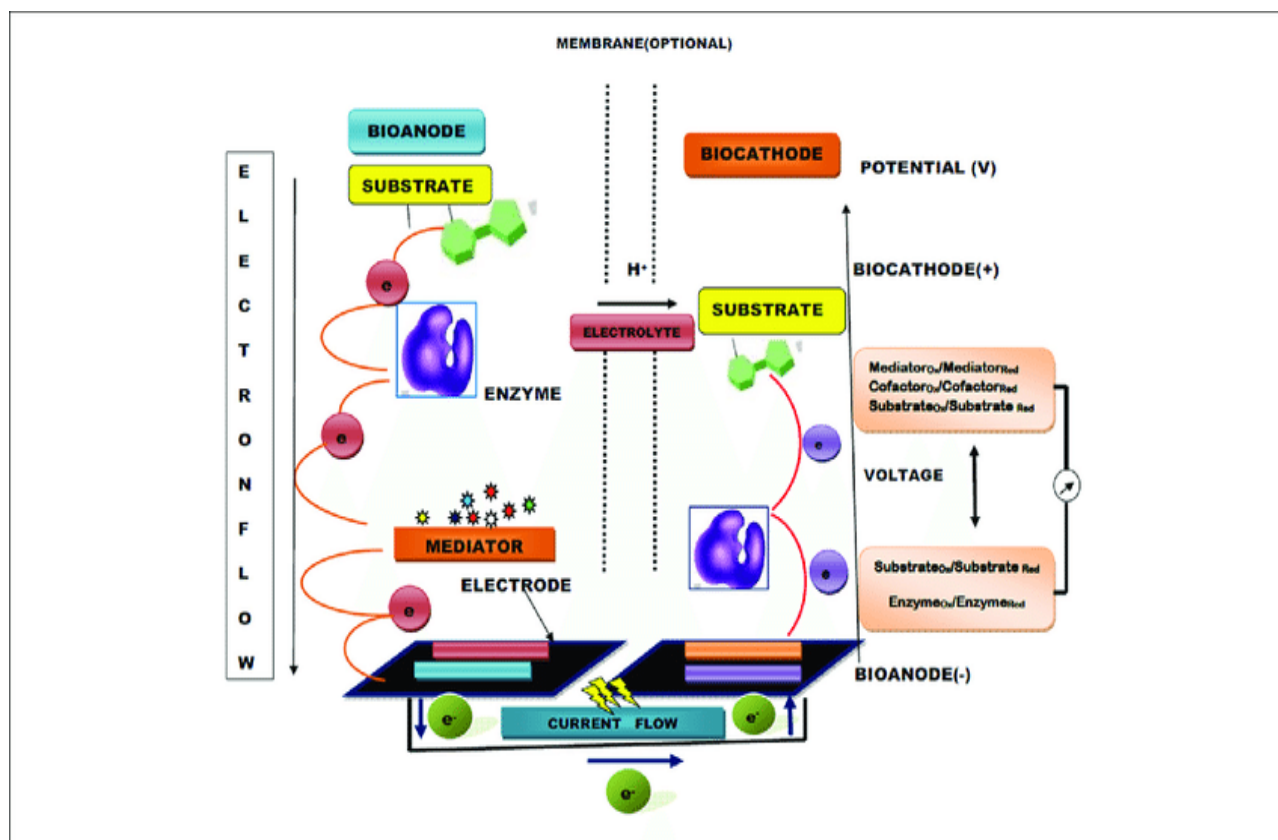


Fig. 6: Generalized diagram of an enzymatic biofuel cell (EBC) with a mediator bioanode and direct electron transfer (DET) based biocathode (mediators are involved in fluxing electron flow between enzyme and electrode).

The use of immobilization facilitates retention of biomass in reactor geometry, enabling their economic reuse and development of the continuous process. The technique also improves stability and prevents product contamination, paving the way for use of crude enzyme preparations such as whole cells in the bioprocessing. However, limitations such as lower power supply and reduced theoretical voltage (limited by the redox potential of cofactors and/or mediators employed in the anode and cathode) of a single BFC make it inadequate for providing power to any biomedical devices. Therefore, biocapacitors have been developed using charge pumps connected to fuel cells. This novel approach has generated high voltage with sufficient temporary currents to operate an electric device without changing the design and construction of the EFC (Sode *et al.*, 2016). The limited life expectancy of BFCs could be enhanced using

technologies that favor enzyme stability, whereas power supply could be resolved by improving catalytic efficiency using directed evolution or other protein engineering methods.

1.7.3 Other Miscellaneous Applications

In addition to the above-mentioned benefits, GOD could be used in some miscellaneous applications. For example, GOD has been used in several immunoassays and staining techniques and shown to have ability on the removal of an excess level of glucose. From the geochemical prospects, GOD can be used for the preparation of leaching solutions, since both H_2O_2 and GA produced by GOD have a significant role in leaching (Wong *et al.*, 2008). In medical sectors, GOD is widely employed in the monitoring of diabetic patients to measure their blood glucose activities using finger-prick blood samples. In addition, GOD induces several apoptosis characteristics of mitochondrial dysfunction, accumulation of Bax and release of cytochrome C in mitochondria, accompanied by activation of caspase-9 and caspase-3 (Yu *et al.*, 2016). Moreover, GOD was also reported to induce cellular senescence in immortal renal cells through integrin-linked kinase (ILK) by downregulating Klotho gene expression, an aging-related kidney-secreted hormone with antioxidant properties (Troyano-Suárez *et al.*, 2015). Apart from these applications, it has been reported that the enzymatic activity of the white rot fungi *P. chrysosporium* is very effective in the presence of GOD and could be used in the biodegradation of lignin, recalcitrant pollutants, pulping and bleaching treatments (Ansari *et al.*, 2016). Wang *et al.* (2016) developed a chemiluminescence (CL) amplification platform based on hollow structural calcium carbonate (HCC)/lucigenin and GOD (HLG) film and found that the GOD immobilized in the confined space of HCC particles exhibited improved biocatalysis. The visual CL bio-platform showed outstanding performance with high selectivity, a wide linear range and a low detection limit for sensing trace glucose. Recently, Nascimento *et al.* (2016) suggested the use of single cell “glucose nanosensors” as nano-pipettes for tracing out cancer cells from normal cells. These nano-pipettes functioned as specifically developed nano-sensors to measure the glucose level in single cells with temporal and spatial resolutions. The covalently immobilized GOD at the sensor tip interacted with glucose forming GA, which was measured as the change in impedance due to the drop in the pH. Bandodkar *et al.* (2016) suggested the use of wearable chemical sensors and showed their promising effects in continuous monitoring of the user’s

health and fitness. Diets supplemented with GOD have been reported to enhance growth performance, increase the contents of growth and development-related hormones and improve the fecal microflora of growing piglets (Tang *et al.*, 2016). These changes might be attributed to the functional activities of GOD in the gastrointestinal tract (GIT), which utilizes O_2 and produces H_2O_2 and GA. Using a similar approach, Tang *et al.* (2013) found that the contents of serum related hormones, intestinal health and growth performance of piglets can be effectively improved by a diet supplemented with 100 g/t GOD.

1.8 Peroxidase

Peroxidases (PODs, E.C. 1.11.1.7) are oxidoreductase haemoproteins that are widely distributed in the plant kingdom (Lin *et al.*, 2004), microorganisms and animal tissues where they catalyze the reduction of hydrogen peroxide (H_2O_2) to water, rendering it harmless. They are glycoproteins with diverse physiological functions and catalyze the oxidation of numerous organic compounds including phenols, aromatic amines, indoles and sulfonates using hydrogen peroxide or its derivatives as the oxidant (Tzika *et al.*, 2008). Hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atom. Hydrogen peroxide being a common end product of oxidative metabolism is a strong oxidizing agent, when allowed to accumulate could prove toxic and may lead to the impairment of metabolic activities in living cells of organism. Peroxidase is one of the key enzymes directly involved in controlling plant differentiation and development, hormone regulation, indole acetic degradation, lignin biosynthesis defence mechanisms-participates in the protection of plant tissues from damage and infection by pathogenic microorganisms (Serrano-Martínez *et al.*, 2008). Moreover, many heme-containing proteins like haemoglobin, catalase and cytochrome P-450 also possess peroxidase activity to some extent. Peroxidases can catalyze the oxidation of a wide variety of substances through a reaction with hydrogen peroxide.

The major source of commercially available peroxidase is the roots of horseradish (*Armoracia rusticana*) (HRP). However, availability of peroxidases with higher stability and different specificity may allow the improvement of its industrial applications. Some research are carried out with the aim of peroxidase production from other plant sources (Yu *et al.*, 2016)

1.8.1 Classification of Peroxidases

Peroxidases are ubiquitous enzyme which belong to the oxidoreductases class of enzyme and are divided into three classes which differ in molecular weight and in absorption spectra. They include: (i) Ferriprotoporphyrin peroxidases which are brown in nature and their main sources are plants, animals and microorganisms, (ii) Verdoperoxidases which are green in nature and are gotten from animals and (iii) flavoprotein peroxidases having FAD as prosthetic group and found in animals and microorganisms (Burnette, 1977).

1.8.2 Sources of Peroxidase

These enzymes are produced by a variety of sources including plants, animals, and microbes. Microbial sources such as bacteria (*Bacillus sphaericus*, *Bacillus subtilis*, *Pseudomonas* sp., *Citrobacter* sp.), Cyanobacteria (*Anabaena* sp.), fungi (*Candida krusei*, *Coprinopsis cinerea*, *Phanerochaete chrysosporium*), actinomycetes (*Streptomyces* sp., *Thermobifida fusca*), and yeast are used in decomposition of pollutants, production of animal feedstock, and raw materials for the chemical, agricultural, paper industries, textile dye degradation, paper-pulp industry for lignin degradation, dye decolorization, sewage treatment, and also as biosensors Marco *et al.* (2009).

Plants are the rich sources of peroxidases and primarily found in roots and sprouts of higher plants. The rich plant sources of peroxidases are potato tuber, horse radish and beet, while it is also present in higher plants like turnip, sweet potato, tomato, sour lime, soybean, carrot, wheat, pears, apricot, bananas, date and sap of fig tree (Reed, 1975), horseradish, papaya (*Carica papaya*), banana (*Musa paradisiacal*), green beans (*P. vulgaris*) as shown in fig 7 and bare (*Acorus calamus*). Horse radish roots are used as a traditional source of peroxidase for commercial purpose. Researchers are still investigating for new peroxidases of elevated stability and properties suitable for different biotechnological, biomedical and other application, e.g., spring cabbage peroxidase was suggested as a potential tool in biocatalysis and bio-electro catalysis (Belcarz *et al.*, 2007). Peroxidase obtained from horseradish (HRP) is extensively used in diagnostic kits, in ELISA for labeling an antibody, synthesis of various aromatic chemicals, and removal of peroxides from materials such as foodstuff and industrial wastes (Table 1).

Naturally thermostable peroxidases have been described and include peroxidase from leaves of royal palm tree, spring cabbage peroxidase.



Fig 7: Diagram of *P.vulgaris*

Source: (pinterest.com. retrieved on 2nd November, 2019)

1.8.3 Characteristics of Peroxidase(s)

Peroxidases are oxidoreductases that catalyze a variety of reactions such as reduction of peroxides such as hydrogen peroxide and oxidation of a variety of organic and inorganic compounds. These are haeme proteins and contain iron (III) protoporphyrin IX as the prosthetic group. They have a molecular weight ranging from 30 to 150 kDa. The term peroxidase represents a group of specific enzymes, such as NADH peroxidase (EC 1.11.1.1), glutathione peroxidase (EC 1.11.1.9), and iodine peroxidase (EC 1.11.1.8), as well as a variety of nonspecific enzymes that are simply known as peroxidases.

1.9 Applications and Peroxidase Biocatalysis in Management of Environmental Pollutants

Peroxidases are versatile biocatalyst with an ever increasing number of industrial applications (Colonna *et al.*, 1999). Peroxidase can be an efficient biocatalyst for the production of

industrially relevant compounds (Hames and Hooper, 2010). Lignin peroxidase and manganese peroxidase have significant role in lignin degradation which ultimately reduces the effort in bioethanol production from agricultural wastes (Mohanty and Abdullahi, 2016). They also have possibilities for applications clinically, in diagnostic kits, antibody labeling, wastewater treatment and in food and paper industries (Bansal and Kwanwar, 2013).

1.9.1 Decolourization of Synthetic Dyes.

Dye wastes represent one of the most problematic groups of pollutants considered as xenobiotics that are not easily biodegradable (Ong, 2011). These dyes are mostly used in textile dyeing, paper printing, colour photography, and as additive in petroleum products. When these synthetic dyes are discharged into industrial effluents they cause environmental pollution. Textile industries play a vital role in the economic increases in India. Water is one of the major products of nature used enormously by human beings, and it is not unnatural that any growing community generates enormous wastewater or sewage. To achieve the biodegradation of environmentally hazardous compounds, white rot fungi appear as a valuable alternative. The capability of oxidation is based on the ability of white rot fungi to produce oxidative enzymes such as laccase, manganese peroxidase, and lignin peroxidase. These oxidases and peroxidases have been reported as excellent oxidant agents to degrade dyes (Gopi *et al.*, 2012).

Several bacterial peroxidases have been used for decolourization of synthetic textile dyes. Removal of chromate Cr (VI) and azo dye Acid Orange 7 (AO7) using *Brevibacterium casei* under nutrient-limiting conditions has been studied. Acid Orange 7 was used as an electron donor by the reduction enzyme of *Brevibacterium casei* for the reduction of Cr (VI). The reduced chromate Cr (III) complexed with the oxidized AO7 formed a purple intermediate. *Bacillus* sp. VUS isolated from textile effluent contaminated soil showed capability for degrading a variety of dyes. The production of ligninolytic peroxidases directly oxidizing aromatic compounds has been described in fungi. Other peroxidases were detected in microorganisms responsible for the biodegradation of industrial dyes together with lignin peroxidase. An edible macroscopic fungi *Pleurotus ostreatus* produced an extracellular peroxidase that can also decolorize remazol brilliant blue and other structurally different groups including triarylmethane, heterocyclic azo,

and polymeric dyes. Bromophenol blue was decolorized best (98%), while methylene blue and toluidine blue O were least decolorized 10%. Horse radish peroxidase was found to degrade industrially important azo dyes such as remazol blue. This dye contains at least one aromatic group in its structure making it a possible substrate of HRP (Bhunia *et al*, 2002). The dyeing and bleaching unit's contaminants seeping into the ground have polluted the ground water rendering it unsuitable for consumption (Table 1).

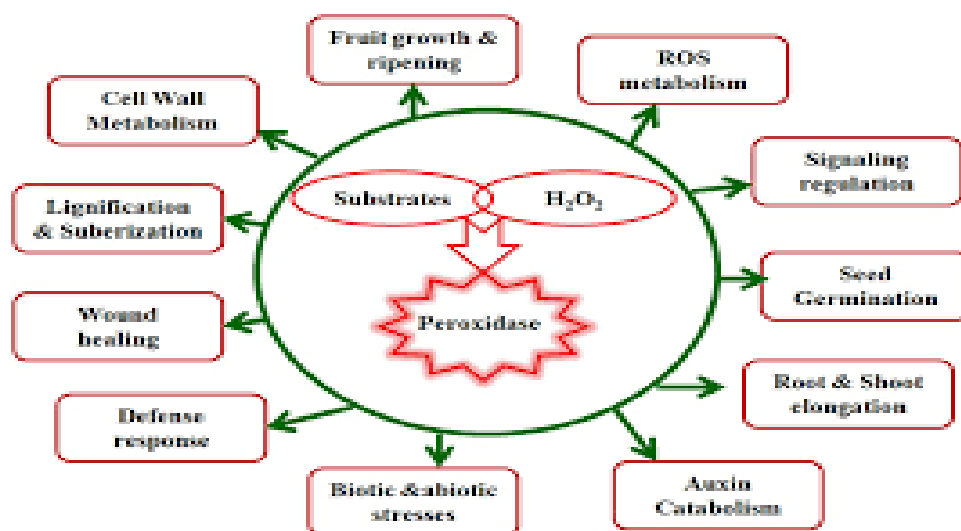


Table 1: Peroxidase activities

(Source: longdom.org)

1.9.2 Bioremediation of Wastewater: Removal of Phenolic Contaminants and Related Compounds.

Industrial pollution has been a major factor causing environmental degradation, thus, affecting the water and its quality and human health are directly issues of great concern. Improved quality and increased quantity of water would bring forth health benefits since safe water eliminates the infective agents associated with water-borne diseases; availability of greater quantity of water can improve health by allowing improved personal hygiene. Water pollution could result from the release industrial waste products into lakes, rivers, and other water bodies which make marine unsafe. Peroxidases have been applied to the bioremediation of wastewaters contaminated with phenols, cresols, and chlorinated phenols (Gopi *et al.*, 2012). Aromatic compounds including phenols and aromatic amines constitute one of the major classes of

pollutants. They are found in the wastewaters of a wide variety of industries, including coal conversion, petroleum refining, resins and plastics, wood preservation, metal coating, dyes and other chemicals, textiles, mining and dressing, and pulp and paper industries. Phenols and halogenated phenols present in textile industries processed water are known to be toxic and also some of them are hazardous carcinogens that can accumulate in the food chain (Huber and Carré, 2012).

Peroxidases comprise an important class of enzymes able to catalyze the oxidative coupling reactions of a broad range of these phenolic compounds. Lignin peroxidase from *Phanerochaete chrysosporium*, HRP, myeloperoxidase, lactoperoxidase, microperoxidase-8, a versatile peroxidase from *Bjerkandera adusta*, and chloroperoxidase from *Caldariomyces fumago* were able to transform pentachlorophenol to tetrachloro-1,4-benzoquinone by an oxidative dehalogenation in the presence of H_2O_2 (Longoria *et al.*, 2008). Two electron oxidation of that extracellular peroxidase by H_2O_2 yields Compound I which undergoes two consecutive one-electron reduction steps by oxidizing Mn^{2+} into Mn^{3+} that in turn oxidizes phenolic compounds. Many toxic aromatic and aliphatic compounds occur in wastewater of a number of industries. Among these, phenol is the most common aromatic pollutant and is also found in contaminated drinking water. Phenol can be toxic when present at an elevated level and is known to be carcinogenic. It has an effect on health even at low concentration. A laboratory phenol was treated with turnip root enzyme (peroxidase) extract in the presence of H_2O_2 as an oxidant to form corresponding free radicals. Free radicals polymerize to form substances that are less soluble in water. The precipitates were removed by centrifugation and residual phenol was estimated (Mamatha *et al.*, 2012). The results showed that turnip root enzyme extract degraded phenol more efficiently. Another versatile peroxidase produced by *P. eryngii* and *P. ostreatus* oxidized Mn^{2+} into Mn^{3+} similar to the action of MnP, and also high redox potential aromatic compounds, as LiP does, had broad specificity and oxidized non-phenolic compound.

1.9.3 Removal of Endocrine Disruptive Chemicals

Endocrine disruptive chemicals (EDCs) are a group of compounds that due to their chemical structure are able to act as agonists or antagonists of hormones. They can disturb the synthesis, secretion, transport, binding, action, and elimination of the endogenous hormones, which are

responsible for maintaining homeostasis, reproduction, development, and integrity in living organisms and their progeny. They are widely dispersed in the environment but are mainly found in wastewater effluents. Several classes of oxidative enzymes have shown promise for efficient removal of EDCs that are resistant to conventional wastewater treatments. Although the kinetics of reactions between individual EDCs and selected oxidative enzymes such as HRP are well documented in the literature, there has been little investigation of reactions with EDC mixtures (Zheng and Olosi, 2011). Research works reported that EDC can be oxidized by manganese peroxidase. Using 10 U/mL of manganese peroxidase from *Pleurotus ostreatus*, 0.4 mM bisphenol was eliminated in 1 h. Peroxidases are also helpful in removal or degradation of other potent environmental pollutants such as chloroanilines and polycyclic aromatic hydrocarbons (Huang and Weber, 2005).

1.9.4 Degradation of Polychlorinated Biphenyls Pesticides

Pesticides include a broad range of substances most commonly used to control insects, weeds, and fungi. Pesticide exposure in human is associated with chronic health problems or health symptoms such as respiratory problems, memory disorders, dermatologic conditions, cancer, depression, neurologic defects, miscarriages, and birth defects (McCauley *et al.*, 2006). Biological decomposition of pesticides is the most important and effective way to remove these compounds from the environment. Microorganisms have the ability to interact, both chemically and physically, with substances leading to structural changes or complete degradation of the target molecule (Raymond *et al.*, 2001). Peroxidases extracted from some fungal species show great potential to transform several organophosphorus pesticides into harmless form(s). Polyaromatic hydrocarbons (PAHs) composed of two or more fused aromatic rings and are components of crude oil, creosote, and coal. Most of the contamination by PAHs had originated from the extensive use of fossil fuels as energy sources. Peroxidases and phenol oxidases can act on specific PAH's by transforming them to less toxic or products easier to degrade. Polyaromatic hydrocarbons are oxidized by peroxidases such as lignin peroxidase and manganese peroxidase (Weber *et al.*, 2008).

1.9.5 Degradation of Chlorinated Alkanes and Alkenes

Contamination of soils and aquifers by the aliphatic halocarbons trichloroethylene (TCE) and perchloroethylene (PCE) widely used as degreasing solvents is a serious environmental pollution problem. Trichloroethylene is subject to *in vitro* reductive dehalogenation catalyzed by LiP of *P. chrysosporium* in the presence of tertiary alcohol, H₂O₂, and EDTA (or oxalate) leading to the production of the corresponding reduced chlorinated radicals. One strain of bacterium IM-4 capable of degrading imazethapyr (IMZT) was isolated from the IMZT-contaminated soil. This strain also showed the capability to degrade other imidazolinone herbicides such as imazapyr, imazapic, and imazamox (Huang *et al.*, 2009). Extracellular hydroxyl radicals produced by *T. versicolor*, via quinone redox cycling, were also shown to catalyze degradation of PCE and TCE. Trichloroethylene is mineralized by *P. chrysosporium* cultures grown aerobically (Marco-Urrea and Reddy, 2012).

1.9.6 Degradation of Phenoxy Alkanoic and Triazine herbicides

The most commonly used broad leaf herbicides around the world are 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). 2, 4-dichlorophenoxyacetic acid and perhaps 2,4,5-T are a component of Agent Orange that was widely used as a defoliant. 2,4-dichlorophenoxyacetic acid is quite susceptible to bacterial degradation and generally does not persist for long in the environment. On the other hand, 2,4,5-T is relatively more resistant to microbial degradation and tends to persist in the environment. It has been blamed for serious illnesses in many veterans of Vietnam War, where they got exposed to Agent Orange that was used as a defoliant. These were also reported to be mutagenic agents and thus very toxic to humans. Ligninolytic peroxidases of *P. chrysosporium* and *Dichomitus qualens* were involved in the degradation of chlorinated phenolic intermediates of 2,4-D and 2,4,5-T. These results were based on the increased degradation of ring-labeled and side chain-labeled 2,4,5-T and 2,4-D by *D. Squalens* on addition of Mn²⁺ (a known inducer of MnP) to the medium and on increased degradation by *P. chrysosporium* in nitrogen limited medium (in which production of both LiP and MnP is induced). Atrazine is a commonly used triazine herbicide and is degraded by a number of white rot fungi produced laccases and peroxidase (Bending *et al.*, 2002).

1.9.7 Degradation of Chlorinated Dioxins

Polychlorinated dibenzodioxins (PCDDs) are a group of highly toxic environmental pollutants that are confirmed human carcinogens and tend to bioaccumulate in humans and animals due to their lipophilic properties. Polychlorinated dibenzodioxins (PCDD) and polychlorinated dibenzofurans (PCDF) have been shown to be degraded by several species of white rot fungi suggesting the possible involvement of LiP and MnP. A fungus *P. sordida* produced MnP but no LiP; and crude MnP showed degradation of the dioxins (Kasai *et al.*, 2010).

1.9.8 Degradation of Chlorinated Insecticides

Lindane (c isomer of hexachlorocyclohexane) was a widely used pesticide in the past, and an estimated 600,000 tons of lindane were produced globally between the year 1950 and 2000. There is a global ban on the use of lindane now due to its environmental persistence as a pollutant. *P. chrysosporium* cultured under ligninolytic conditions was reported to partially mineralize lindane in liquid cultures and in a corn cob amended soils inoculated with *P. chrysosporium* but lindane degradation was not observed *in vitro* using purified LiP and MnP from *P. chrysosporium*. 1,1,1-trichloro-2,2-bis [4-chlorophenyl] ethane) (DDT), the first of the chlorinated organic insecticides, was used quite heavily after World War II. High levels of DDT found in agricultural soils are of deep concern, because they present serious threats to food security and human health. The white rot fungi *P. chrysosporium*, *P. ostreatus*, *T. versicolor*, and *Phellinus weirii* have been shown to mineralize DDT (Purnomo *et al.*, 2010).

1.10 Peroxidase as Biosensors

Biosensors have been defined as analytical devices which tightly combine biorecognition elements with physical transducers for detection of the target compound. Biosensors can be useful, for example, for the continuous monitoring of a contaminated area. They may also present advantageous analytical features, such as high specificity and sensitivity (inherent in the particular biological recognition bioassay). Hydrogen peroxide is considered the mediator of the biochemistry of cellular pathology and may be involved in the etiology of aging and progressive neurodegenerative diseases such as Parkinson's disease. Due to its crucial role in neurochemistry, determination of the concentration of H₂O₂ has been a considerable interesting

research field. Electrochemical methods based on peroxidases biosensors have proved to be significantly advantageous to the biosciences due to their direct real-time measurements and capability for practical applications (Song *et al.*, 2011). A novel third generation biosensor for hydrogen peroxide was constructed by cross-linking HRP onto an electrode modified with multiwall carbon nanotubes. A novel myoglobin-based electrochemical biosensor based on a nanocomposite prepared from multiwalled carbon nanotubes that were coated with ceria nanoparticles has been developed (Qiu *et al.*, 2010). At the same time, biosensors offer the possibility of determining not only specific chemicals but also their biological effects, such as toxicity, cytotoxicity, genotoxicity, or endocrine disrupting effects, i.e. relevant information that in some occasions is more meaningful than the chemical composition. Enzymatic biosensors are based on the selective inhibition of specific enzymes by different classes of compounds, with the decrease in activity of the immobilized enzyme in the presence of the target analyte as the parameter that is frequently used for quantification (Xu *et al.*, 2011).

Another application of whole-cell biosensors is the determination of the biological oxygen demand (BOD). Pesticides (herbicides, fungicides, and insecticides) are widely used in the agriculture and industry around the world due to their high insecticidal activity. Biosensors are potentially useful as they detected pesticides quickly and have been active in the research area for some years. Another valuable HRP-based biosensor was developed in which polyvinyl pyrrolidone (PVP) nanofibers were spun with incorporation of the enzyme HRP. Scanning electron microscopy (SEM) of the spun nanofibers was used to confirm the non-woven structure which had an average diameter of 34 nm. The HRP containing fibers were tested for their change in activity following electrospinning and during storage. A colorimetric assay was used to characterize the activity of HRP by reaction with the nanofiber mats in a microtiter plate and monitoring the change in absorption over time (Dai *et al.*, 2012). Rapid and sensitive detection methods are of utmost importance for the identification of pathogens related to health and safety. Peroxidase used in development of a nucleic acid sequence-based lateral flow assay which achieves a low limit of detection using chemiluminescence and enzymatic signal amplification (Wang *et al.*, 2012).

1.10.1 Use in Pulp-Paper Industries

The pulping by products (black liquor) and pulp mill wastewater cause serious environmental problem due to its high pollution load. Solving the pulp and paper industries' environmental problems is essential to maintaining the forest industry and accommodating the changing economic needs of forest communities (Khalid *et al.*, 2009). Pulp manufacturing in pulp paper industries involve two main processes, that is, wood digestion and bleaching. In the process of wood digestion, wood chips are cooked in the solution of sodium hydroxide and sodium sulphate at elevated temperature and pressure to break chips into fiber mass. The chemical reaction with wood fibers dissolves all the depository materials which are hard to degrade, and these derivatives are washed away from the fiber during washing and dewatering process. Various extracts during washing include mainly lignins, cellulose, phenolics, resins, fatty acids, and tannins mixed together to make a dark black viscous alkaline waste known as black liquor. The alkaline effluent consists only in 10 % – 15 % of total wastewater but contributes in almost above 90 % – 95 % of the total pollution load in terms of high pH, BOD, chemical oxygen demand (COD), and color which makes it significantly toxic to the environment. Hence, the adequate treatment of black liquor prior to its discharge into environment is warranted. The biological methods for the treatment of black liquor involving the use of fungi, bacteria, algae, and enzymes as single step treatment or in combination with other physical and chemical methods seem to be more economical and ecofriendly. Among biological methods tried so far, most of the literature is confined to a few genera of white rot fungi because of their nonspecific extracellular enzymatic system (LiP, MnP, and Laccase) involved in lignin biodegradation (Hammel and Cullen, 2008).

A relatively recent alternative to chemical degradation of phenols is the use of class III peroxidases (plant peroxidases). Peroxidases (EC 1. 11. 1. 7) catalyze the oxidation of phenols to corresponding radicals in the presence of hydrogen peroxide, or in the presence of dioxygen. Heme-peroxidases: chloroperoxidase (*Caldariomyces fumago*), lignin peroxidase (*Phanerochaete chrysosporium*), manganese peroxidase (*Coriolopsis gallica*) and horseradish peroxidase (HRP) use hydrogen peroxide or other organic peroxides as electron acceptors to oxidize a wide range of reducing substrates. Horseradish peroxidase is efficient in the polymerization of phenol, aniline and their derivatives even in the presence of large amounts of

pollutants, and is known to be active in a number of organic solvents or aqueous solvent mixtures (Puiu *et al.*, 2008). It is generally accepted that the HRP-catalyzed oxidation of phenols or aromatic amines, with hydrogen peroxide follows a ‘ping-pong’ mechanism.

1.11 Rationale for the Study

The quest to find a better and cheaper source of peroxidase that could be utilized as a coupled enzyme with glucose oxidase for local industries to help reduce heavy importation of oleo-chemicals, ensure eco-friendly industrial practices and ultimately improve the national economy is the force behind the present study.

1.12 Aim and Objectives of the Study

1.12.1 Aim of the Study

The aim of this study was to produce and characterize fungal glucose oxidase from *Aspergillus fumigatus* and assay its activity using a peroxidase extracted from *P. vulgaris* (green bean seed).

1.12.2 Specific Objectives of the Study

The specific objectives of this study were to:

- Extract peroxidase from green beans (*Phaseolus vulgaris*)
- Partially purify peroxidase (POD) via ammonium sulphate precipitation, dialysis and gel chromatography.
- Subculture to obtain a pure culture of *A. fumigatus*.
- Produce extracellular glucose oxidase by submerged fermentation.
- Partially purify glucose oxidase (GOD) via ammonium sulphate precipitation, dialysis and gel chromatography.
- Determine the effect of temperature on activity of glucose oxidase
- Determine the effect of pH on the activity of glucose oxidase
- Determine the effect of substrate concentration on the activity of glucose oxidase

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

All chemicals and reagents used in this present study were of analytical grade and were obtained from Sigma Co. St Louis, MO 63103 USA and they include: Horse radish peroxidase, glucose, o-dianisidine dihydrochloride, folin-ciocalteau phenol, peptone, ethanol, bovine serum albumin (BSA), Tris – hydrochloric acid (Tris-HCl), sodium acetate, potato dextrose agar, disodium hydrogen phosphate, sodium dihydrogen phosphate, copper sulphate, magnesium sulphate, diammonium hydrogen phosphate, potassium dihydrogen phosphate, calcium carbonate.

2.1.2 Equipment and Apparatus

The equipment and apparatus used in this study were obtained from the laboratory unit of Department of Biochemistry, University of Nigeria, Nsukka and they included:

Stainless Steel Thermostatic Water Bath	SSY-H, China
pH meter	Searchtech Model PHS-3C, U.K
Weighing balance	B2404-5 mettler Toledo, Switzerland
Orbital shaker	MKV-300, LH Fermentation LTD, U.K
Spectrophotometer	Jenway 6405, England
Centrifuge	Model 800, China
Magnetic stirrer	B.Bran Scientific and Instrument Company, England
Water bath	DK-420 WOM, China

2.2 Methods

2.2.1 Collection of Sample

Matured fresh green bean seeds were purchased from Ogige Market in Nsukka Senatorial Zone of Enugu State, Nigeria.

2.2.2 Collection of Microorganism

Microorganism was collected from microbial culture of Enzymology and Protein Chemistry Unit, Department of Biochemistry, University of Nigeria, Nsukka.

2.2.3 Extraction of Peroxidase

Peroxidase was extracted from green bean seeds using a slightly modified method of Eze *et al.* (2010). A known quantity (100 g) of green bean seeds was weighed and homogenized in an electronic blender (Binatone, Model No: BLG-402, China) for 20 minutes in 500 mL ice-cold 0.1 M phosphate buffer of pH 7.0. The extracts were filtered through a four-layered cheesecloth and centrifuged at 4,000 rpm for 15 min to remove all cell debris. The supernatant was separated and stored at -10 °C in a deep freezer as the crude enzyme extract.

2.2.4 Peroxidase Assay

Peroxidase activity assay was carried out according to the method described by Eze *et al.* (2010). The reaction mixture included 200 µL of 0.1 % *o*-dianisidine, 200 µL of 0.3 % hydrogen peroxide, and 2.5 mL of 0.1 M sodium phosphate buffer pH 7.0 and 100 µL enzyme extract in total volume of 3.0 mL. 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 at room temperature using Jenway 6405 UV/VIS spectrophotometer immediately the enzyme was introduced into the reaction mix. Increase in absorbance up to 2 minutes at 15 seconds interval starting from zero showed the presence of gPOD and the activity was estimated by the change in optical density per minutes as a result of *o*-dianisidine oxidation. The enzyme activity was calculated following the formula below:

$$\text{Activity (U/mL)} = \frac{\Delta \text{ Absorbance per minutes} \times \text{Total reaction volume (mL)} \times 1000}{\text{Absorptivity of } o\text{-dianisidine (11.1 mMcm}^{-1}) \times \text{Volume of enzyme used (mL)}}$$

2.2.5 Preparation of Culture Medium

Potato dextrose agar (PDA) medium was used to isolate the fungi. Potato dextrose agar (3.9 % w/v) medium was prepared by dissolving 3.9 g of PDA in 100 mL of distilled water contained in conical flask. The pH of the medium was adjusted to 5.6 and autoclaved at 121°C, 15 psi for 15

minutes. After sterilization, the molten PDA medium (45-50 °C) was poured into sterile petri-dishes and allowed to gel at room temperature.

2.2.6 Preculture of *A. fumigatus*

Pure culture of the organism was isolated from the stock culture plate and inoculated into the freshly prepared PDA and kept at room temperature for 72 hrs for growth of fresh moulds of *A.fumigatus*.

2.2.7 Production of Glucose oxidase

Extracellular GOD was produced under submerged fermentation system in 250 mL with 100 mL fermentation medium working volumes. The media comprised (% w/v): glucose, 80; peptone, 3; NH₄NO₃, 0.388; KH₂PO₄, 0.188; MgSO₄.7H₂O, 0.156, and CaCO₃, 35. Autoclave (Model: YX-280A, Lifecare Medical Limited) was employed to effect sterilization at 121 °C for 15 psi and cooled to 30 °C. The flaks were inoculated with in (Fiedurek and Szczodrak, 1995). 100 mL of sterilized broth in 250 ml conical flasks was inoculated with six pure culture agar plugs of *A. fumigatus* using 1 cm diameter cork-borer. After inoculation, the flasks containing the mixture were incubated at 30 °C in an MK V orbital shaker operating at 150 rpm. The fermented mycelium biomass after 7 days were harvested, filtered and centrifuged at 15000 rpm for 15 min (Kim *et al.*, 1990). The supernatant constituted the crude enzyme, GOD. The filtrate was then assayed for extracellular GOD activity as described below.

2.2.8 Glucose oxidase Assay

Activity assay for GOD was carried out according to a method described by Bergmeyer *et al.* (1974) using the substrates glucose, hydrogen peroxide as co-substrate and *o*-dianisidine as a chromogenic substrate. Reagents A, B, C, D and E were prepared for the assay. Reagent A: 0.05 M sodium acetate buffer (pH 5.5); reagent B: 10 mL 0.5 % w/v *o*-dianisidine solution dissolved in methanol and 90 mL of reagent A; reagent C: 10 mL 10 % w/v β-D-glucose solution; reagent D: 48 mL solution B and 10 mL of reagent C; reagent E: solution of SG-200 partially purified *Phaseolus vulgaris* peroxidase (gPOD). The reaction mix contained 2.9 mL reagent D, 0.1 mL GOD and finally 0.1 mL gPOD solution to initiate the reaction. Absorbance was recorded every

15 sec for 2 mins using Jenway 6405 UV-Visible spectrophotometer at 500 nm. The enzyme activity was calculated following the formula below:

Calculations

$$\text{Activity (U/mL)} = \frac{(\Delta A_{500\text{nm}} / \text{minute}) \times \text{Total volume reaction mixture (mL)} \times \text{dilution factor}}{\text{Molar extinction coefficient} \times \text{volume of enzyme used} \times 1 \text{ cm path-length}}$$

Dilution factor = 10

Total volume of assay = 3.1 mL

Molar extinction coefficient of oxidized o-dianisidine at 500 nm = 7.5 mMcm^{-1}

Volume of enzyme used = 0.1 mL

Unit definition

One unit of GOD will oxidize 1.0 μmole of β -D-glucose into D-gluconolactone and H_2O_2 per minute in the pH of 5.5 (equivalent to an O_2 uptake of 22.4 μl per minute).

2.2.9 Protein Assay

The protein content of GOD and gPOD were carried as described by Lowry *et al.* (1951) using different concentrations of 2 mg/mL bovine serum albumin (BSA) for the standard curve (0.2 g BSA was dissolved in 100 mL of distilled water and used as stock solution). An aliquot of the protein (0.1 mL) was added to 0.9 mL distilled water followed by 5 mL alkaline solution of reagent E (Appendix II). A 0.5 mL 50 % v/v in deionized water oxidizing Folin reagent (Solution C) was added after 10 minutes. The absorbance was measured at 750 nm after 30 minutes of incubation at room temperature.

2.2.9.1 Purification of Green Beans Peroxidase and Glucose oxidase

2.2.9.2 Ammonium Sulphate Precipitation

Ammonium sulphate profile of peroxidase (gPOD) and GOD were carried out using different weight of ammonium salt with equivalent percentages of 20 % (0.53 g), 30 % (0.82 g), 40 % (1.10 g), 50 % (1.46 g), 60 % (1.81 g), 70 % (2.18 g), 80 % (2.58 g), 90 % (3.02 g) and finally 100 % (3.46 g) saturation as described by Chilaka *et al.* (2002). These equivalent masses of the salt were weighed respectively into 5 mL of the enzyme(s) in a 20 mL capacity tube. The suspension was stirred using a glass rod and kept in the fridge (4 °C) for 24 hrs. The solution was

centrifuged at 4,000 rpm for 20 min to separate the supernatant and the pellet. Activity assays were carried out and eighty percent (80 %) and Ninety percent (90 %) ammonium sulphate saturation were suitable for concentration of the proteins, gPOD and GOD respectively. Each of the pellets after concentration was redissolved in 1 mL of the assay buffer.

2.2.9.3 Dialysis

The precipitated gPOD and GOD were dialyzed against 0.01 M sodium phosphate buffer, pH 7.0 and 0.01 M sodium acetate buffer pH 5.5 respectively for 12 h with change of buffer twice at 6 h according to the method described by Chilaka *et al.* (2002). An aliquot (10 mL) each of the proteins were into the dialysis membranes. The dialysis membranes or bags were tighten end-to-end with a string extending 10 cm at both ends. The membranes with the protein contents were immersed into the respective buffers in an ice bath. After 6 hours, the buffers were discarded and replaced by fresh buffer in each case.

2.2.9.4 Gel Filtration Chromatography

The matrix pore size, sephadex-G-200 column (2 × 70 cm) was used for desalting as described by Chilaka *et al.* (2002). The gel was soaked overnight in buffer for 24 hrs and was carefully packed to 50 cm bed height. After 12 hours, 10 mL each of the dialysed gPOD and GOD enzymes were introduced into different tightly packed columns and eluted with 0.1 M phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.5) respectively. 50 fractions each of 5 mL were collected for both gPOD and GOD. Activity and protein assay were also carried and the peaks with the highest gPOD and GOD activities were pooled and stored at -10 °C in deep freezer until characterization.

2.3 Characterization of Partially Purified Glucose oxidase

2.3.1 Effect of pH

The pH studies on the GOD activity was carried out as described by Simpson *et al.* (2006). Different buffer solutions, 0.05 M sodium acetate buffer (pH 3.0-5.5), 0.05 M sodium phosphate buffer (pH 6.0-7.5) and 0.05 M Tris-HCl buffer (pH 8.0-9.0) at 0.5 intervals were prepared by multiplying the concentration with the molecular weight of each salt to obtain the mass

concentration (in g/L). The masses in each case were weighed and dissolved in 800 mL distilled water; using glass rod to stir for complete dissolution. A pH meter was used to measure the pH value of the dissolved salt and the acid equivalents in each case was used to adjust to a desired pH value, then made up to 1L with distilled water. In each assay procedure, these varied pH buffer solutions were used for activity assay after incubation of the enzyme at room temperature for 10 minutes.

2.3.2 Effect of Temperature

The effect of temperature on GOD activity was determined at different temperature ranges (30-80 °C) at 5 °C interval using a temperature-controlled water-bath at optimum pH, as described by Simpson *et al.* (2006). Glucose oxidase and solution D (Appendix II) was incubated at each varied temperature for 10 minutes in a water-bath. The reaction was initiated immediately by drawing an aliquot (0.1 mL) of gPOD into the reaction mix. The increase in absorbance was monitored for 2 minutes at 15 seconds interval from zero seconds.

2.3.3 Effect of Substrate Concentration

The effect of different substrate concentrations on GOD was determined as described by Sandalli *et al.* (2014) using glucose solutions of various concentration (0.5 – 10 % w/v) at pH 5.5. The concentration of glucose was increased two-folds for the preparation of reagent C (Appendix II) while other reagents concentrations were kept constant. Activity assay was performed at optimum conditions of pH and temperature in each case. The maximum velocity (V_{max}) and the Michaelis constant (K_m) were obtained from Lineweaver-Burk plot of initial velocity data.

CHAPTER THREE

RESULTS

3.1 Pure-culture of *Aspergillus fumigatus*

Plate 1 showed the pure culture of *A. fumigatus* spores after seven days of inoculation



Plate 1: Pure culture of *A. fumigatus*

3.2 Ammonium Sulphate Profile of Peroxidase

Fig 8 showed ammonium sulphate precipitation curve of green bean peroxidase (gPOD) at different percentage of $(\text{NH}_4)_2\text{SO}_4$ salt saturation. Highest activity was achieved at eighty percent (80 %) $(\text{NH}_4)_2\text{SO}_4$ saturation, hence suitable percentage to salt out and concentrate the protein of interest. The precipitate obtained after concentration was re-dissolved in 1 mL 100 mM phosphate buffer, pH 7.0 and dialyzed against the same buffer of lower concentration for 12 h with two changes.

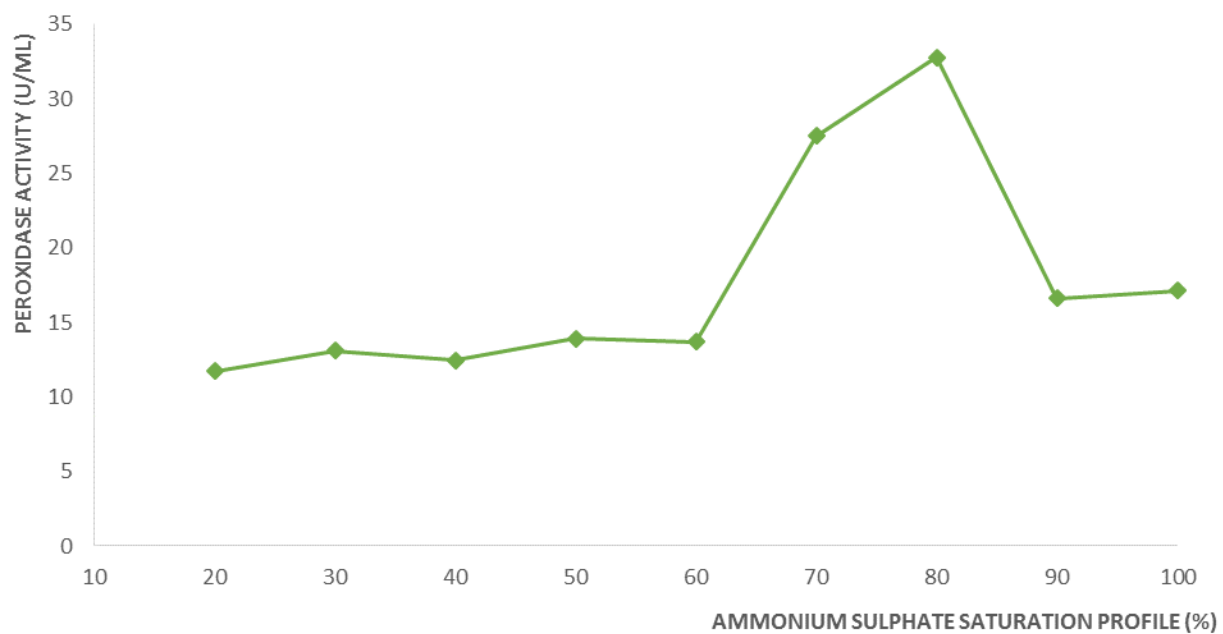


Figure 8: Ammonium sulphate profile of *P.vulgaris* peroxidase

3.3 Ammonium Sulphate Profile of Glucose oxidase

Fig 9 showed ammonium sulphate precipitation curve of *A. fumigatus* glucose oxidase (GOD) at different percentage $(\text{NH}_4)_2\text{SO}_4$ salt saturation. Ninety percent (90 %) $(\text{NH}_4)_2\text{SO}_4$ saturation showed highest activity, hence suitable percentage to salt out GOD. The precipitate obtained after concentration was re-dissolved in 1.0 mL 50 mM sodium acetate buffer, pH 5.5 and dialyzed against the same buffer of lower strength for 12 h with two changes.

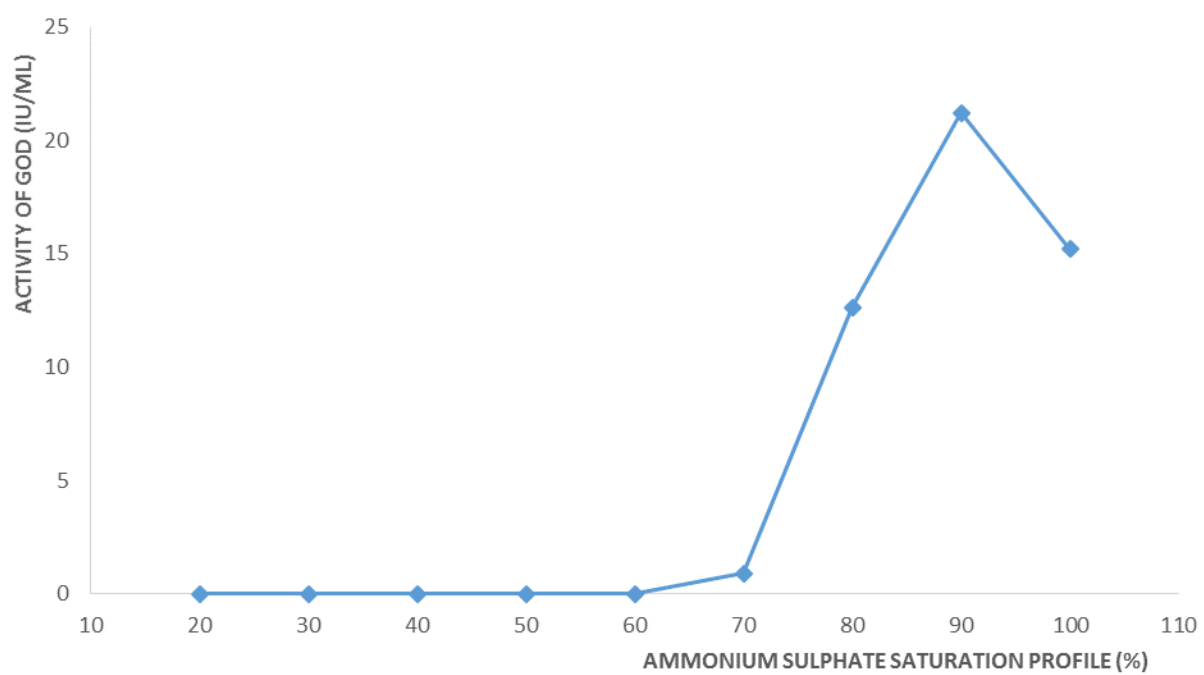


Figure 9: Ammonium sulphate profile of glucose oxidase

3.4 Molecular Exclusion Chromatography of *P. vulgaris* (green bean seed) POD

Fig. 9 showed the elution profile or gel filtration chromatogram of *P. vulgaris* peroxidase on sephadex G-200 column (2 × 70 cm) and 0.1 M phosphate buffer (pH 7.0) elution buffer. 10 mL of the dialysed gPOD was introduced into the column and 50 fractions (5 mL each) were collected at a flow rate of 15 mL/h. The protein concentration of each fraction was assayed at 280 nm and activity assayed was also carried out at 460 nm.

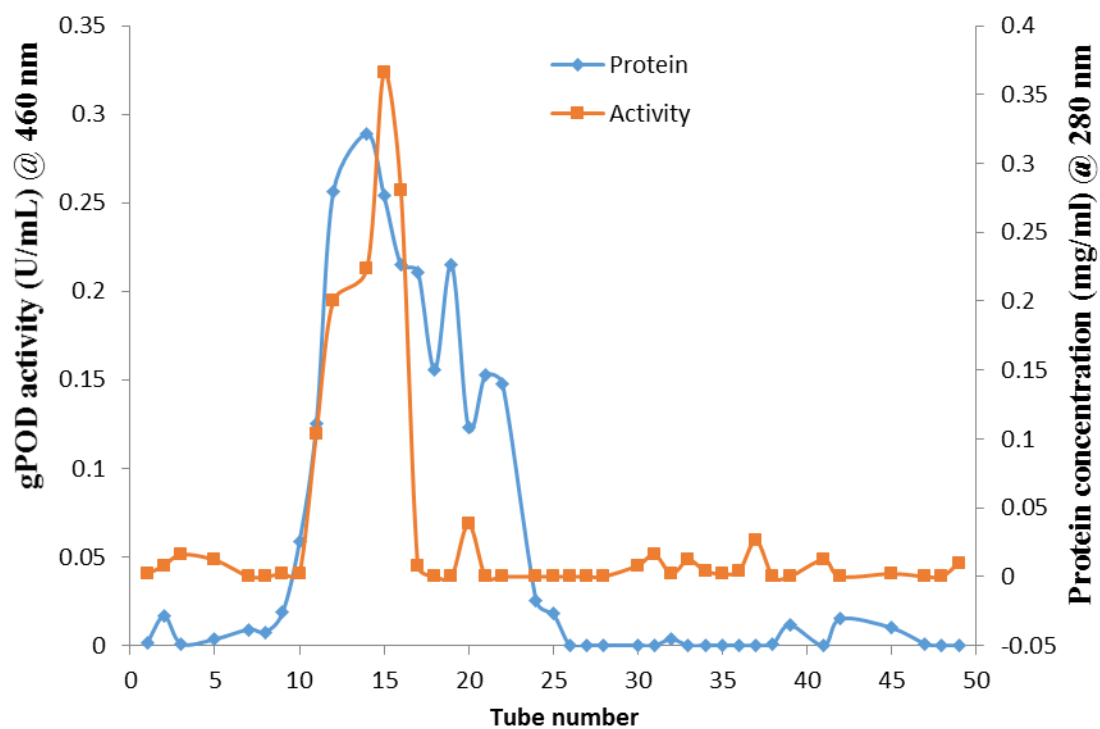


Figure 10: Elution profile of *P. vulgaris* peroxidase

3.6 Molecular Exclusion Chromatography of fungal GOD

Fig. 11 showed the elution profile of fungal glucose oxidase gel filtration chromatography carried on SG-200 column (2 × 70 cm) in 0.05 M sodium acetate, pH 5.5 elution buffer. An aliquot (10 mL) of GOD obtained from dialysis was introduced into the column and eluted with the same buffer of the same strength. A total of 50 fractions, 5 ml each were collected at a flow rate of 15 ml/h. The activity and protein assay was carried out. Fraction 36 to 39 showed the maximum activity and were pooled together and used for characterization.

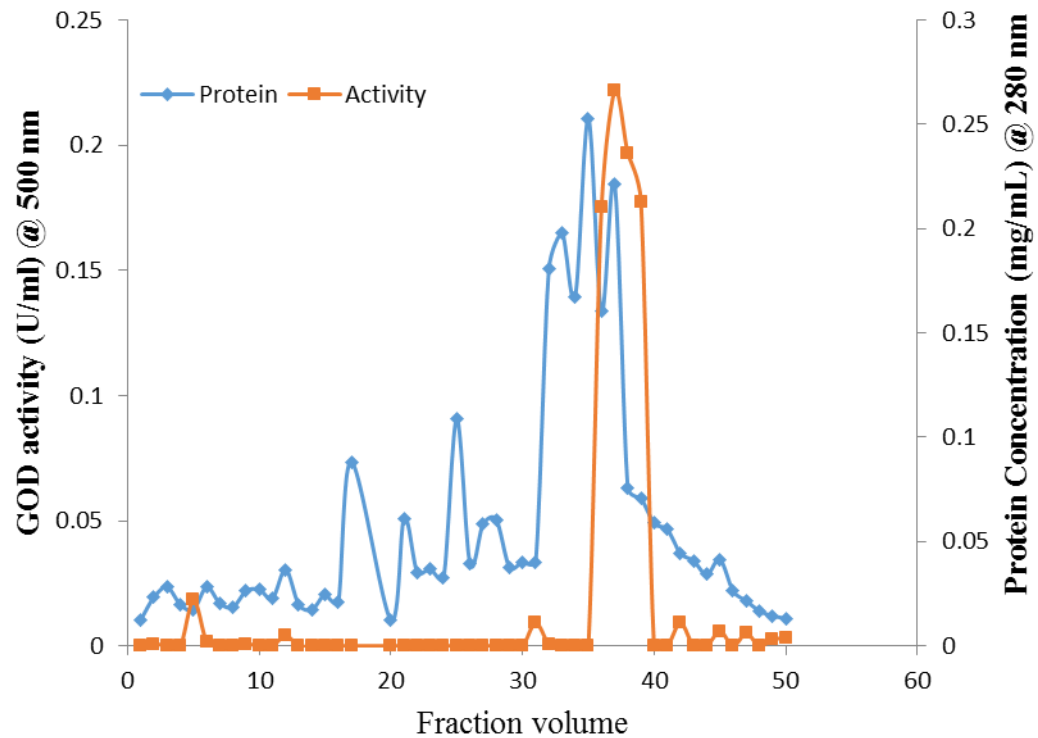


Figure 11: Elution profile of glucose oxidase

3.7 The Purification Table of *P. vulgaris* Peroxidase

Table 2 showed the purification summary of *P. vulgaris* (green bean seed) peroxidase (gPOD). Peroxidase activity decreased with protein concentration at each purification step. However, specific activity increased with purification fold until dialysis. This could be due to over dilution of the protein during dialysis. The activity of gPOD retained decreased progressively at each step of purification as shown below.

Table 2: Summary of *P. vulgaris* (green bean seed) peroxidase (gPOD) purification

Purification steps	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total activity (U/ml)	Specific Activity (U/mg)	Purification Fold	% Yield
Crude enzyme	500	0.82	410	35.5	17750	43.3	1.0	100
(NH ₄) ₂ SO ₄ (80%)	300	0.44	132	26.9	8070	61.1	1.4	45
Dialysis	25	0.12	3	22.2	555	185.0	4.3	3.1
Gel Filtration	20	0.11	2.2	15.8	316	143.6	3.3	1.7

3.8 The Purification Table of Glucose oxidase

Table 3 showed the summary of purification of *A. fumigatus* glucose oxidase (GOD). Activity decreased with decrease in protein concentration at each level of purification step. However, increase in specific activity corresponds with increase purification fold (activity retained).

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Table 3: Summary of *A.fumigatus* glucose oxidase (GOD) purification

Purification steps	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total activity (U/ml)	Specific Activity (U/mg)	Purification Fold	% Yield
Crude enzyme	1000	1.98	1980	17.36	17360	8.77	1.0	100.0
(NH ₄) ₂ SO ₄ (90%)	300	0.71	213	13.35	4005	18.80	2.1	23.0
Dialysis	20	0.27	5.4	19.39	388	71.85	8.2	2.2
Gel Filtration	20	0.16	3.2	10.79	216	67.50	7.7	1.2

3.8.1 Effect of pH on GOD Activity.

Fig. 12 showed pH optimum of 5.5 as determined by incubating the enzyme in a wide pH range (3.0 – 9.0) at 25 °C. Increase in pH from 3.0 to 5.5 was accompanied with increase in GOD activity beyond or below which the activity decreased making pH 5.5 the optimum.

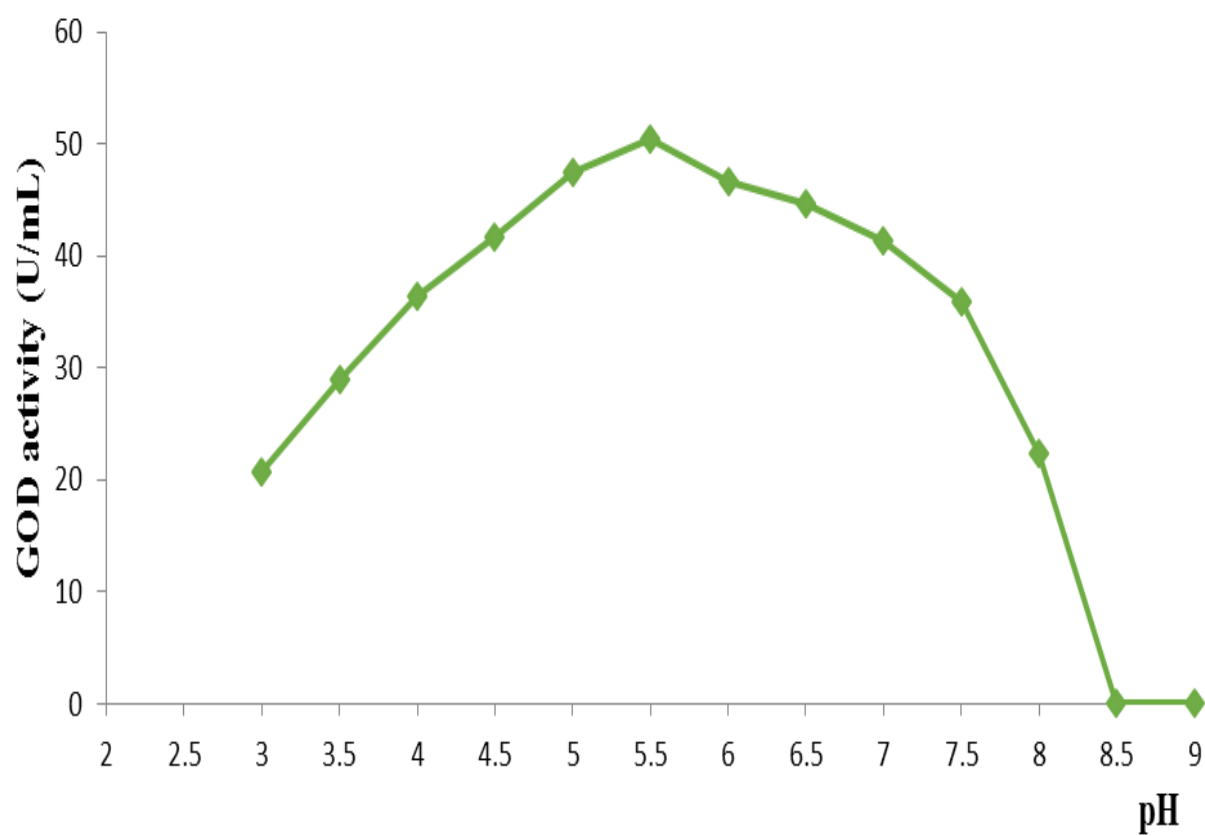


Figure 12: Effect of pH on GOD activity

3.8.2 Effect of Temperature on GOD Activity

Fig. 13 showed 45 °C as the temperature optimum of GOD. Activity increased with temperature from 30 °C to 45 °C beyond which the activity decreased making 45 °C the optimum temperature. The increase in GOD activity was as a result of increase in collision between the substrate and enzyme active site, whereas the decrease might be due to subunit dissociation, denaturation or change in conformation (3-D structure) of the enzyme.

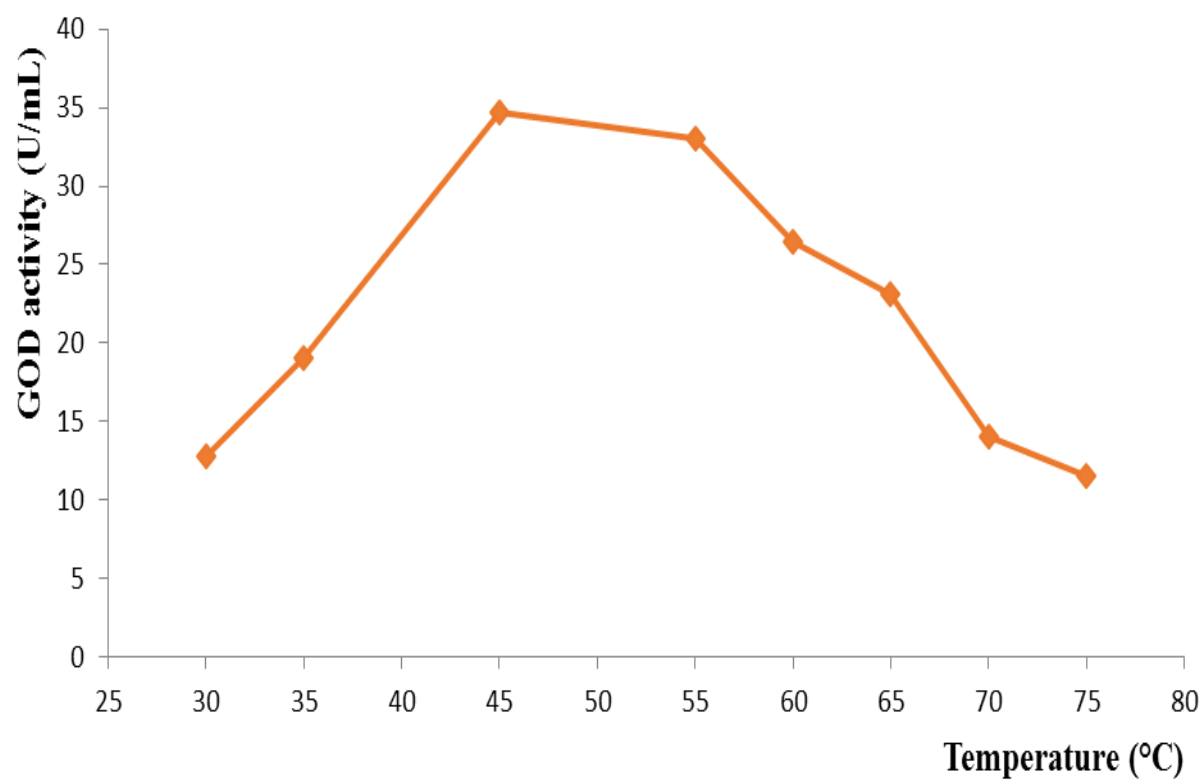


Figure 13: Effect of temperature on GOD activity

3.8.3 Effect of Substrate Concentration on GOD Activity

Fig. 14 showed that at low concentration of the substrate, GOD activity increased almost linearly with increase in substrate concentration [glucose] indicating a first order kinetics where increase in activity is dependent on substrate concentration. However, at higher substrate concentration, activity decreased progressively with increase in substrate concentration and eventually levelled off to almost a constant value indicating a zero order kinetics in which further increase in glucose concentration does not affect the reaction rate. This may be due to active site saturation with substrate; hence, the reaction obeyed saturation kinetics.

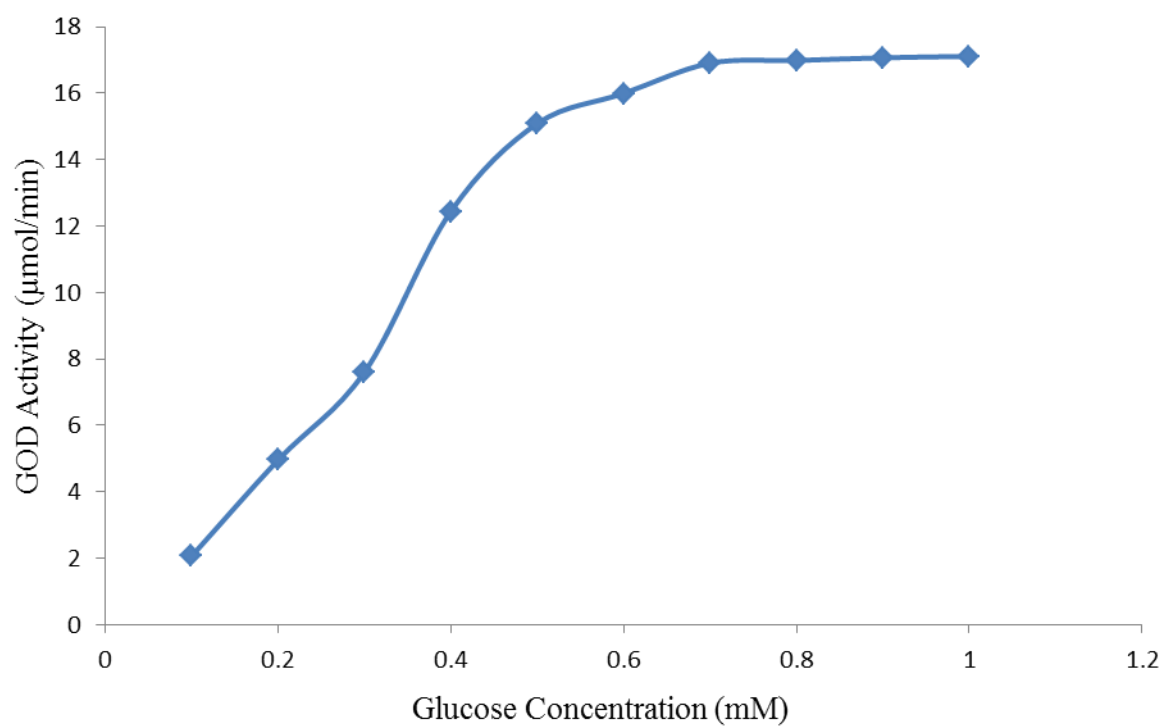


Figure 14: Michaelis-Menten direct linear plot of V against [S]

Figure 15: Determination of the Kinetic Parameters

The K_m and V_{max} values as determined from Lineweaver-Burk plots were 5.66 mM and 16.67 U/mL respectively. The Michaelis-Menten constant (K_m) is the substrate concentration at half the maximum velocity (V_{max}) and the dissociation constant of the enzyme-substrate complex. When the $K_m \ll V_{max}$, it indicates that GOD require little substrate to reach V_{max} showing higher affinity of the enzyme to the substrate. However, if the $K_m \gg V_{max}$, it means the GOD requires high amount of the substrate to attain V_{max} signifying lower affinity of the enzyme to the substrate.

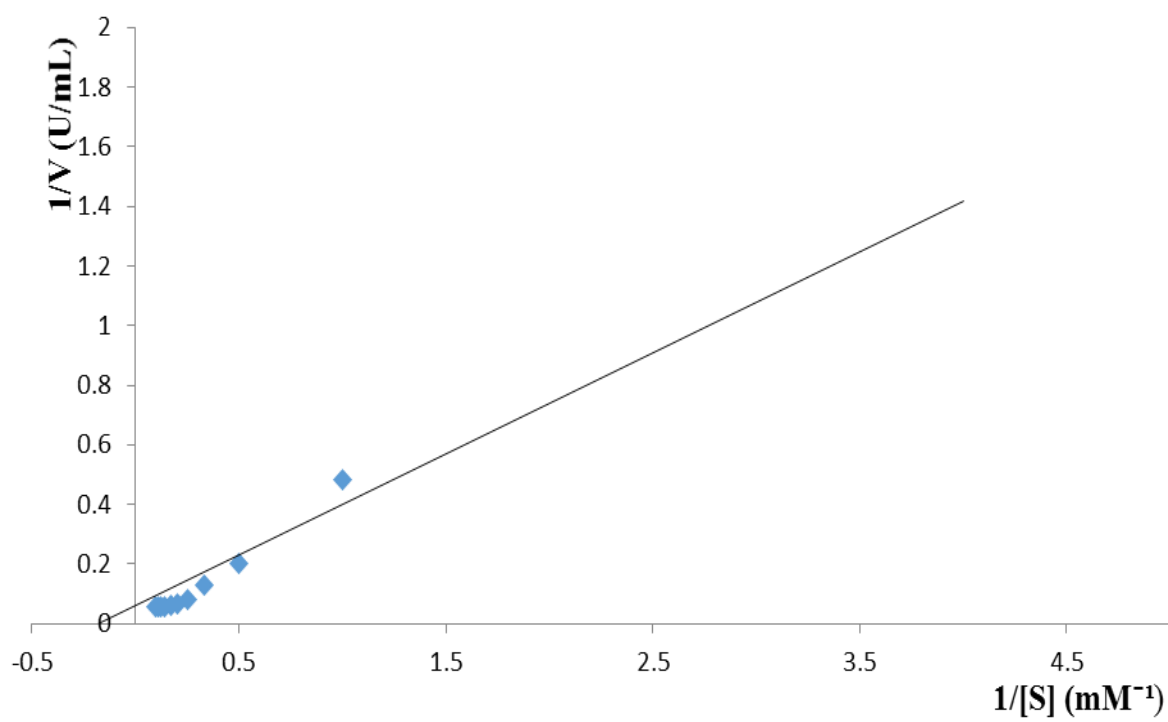


Fig. 15: Lineweaver-Burk plot

CHAPTER FOUR

DISCUSSION

Results of this study revealed that green bean seed is a good source of peroxidase. Peroxidase purified from green bean seed has various industrial as well as pharmaceutical applications. For instance, glucose oxidase used for enzymatic determination of glucose level in the blood fluid oxidizes β -D-glucose to D-gluconolactate and hydrogen peroxide with peroxidase acting as the coupling enzyme in the presence of a chromogenic substrate (o-dianisidine). In this work, peroxidase activity from green bean seed was 35.5 U/mL which has similarity with the work of Muhammad *et al.* (2011) who followed the same method of extraction at neutral (pH 7.0) and obtained 23.6 U/mL. The activity is higher when compared with the work of Ahmad *et al.* (2019) who reported 13.178 U/mL on crude sample of broccoli peroxidase. As the activity of peroxidase increases, it provides defense mechanism to plants.

Eighty percent (80 %) saturation of ammonium sulfate salt was suitable to precipitate the protein from the solution and is in agreement with the result of Ahmad *et al.* (2019) who reported that 80 % $(\text{NH}_4)_2\text{SO}_4$ salt could precipitate maximum amount of total protein in the crude sample. This report is higher compare to the work of Jos'e *et al.* (2013) who showed that 70 % saturation as the optimum for fractional precipitation of AvocPOD from avocado crude extract with specific activity of 2.83 U/mg proteins and 18.3 % yield. After ammonium sulfate precipitation and dialysis, the gPOD activity was reduced to 26.9 U/mL and 22.2 U/mL which are lower compared to the result of Ahmad *et al.* (2019) who reported 60.06 U/mL 53.20 U/mL respectively. The specific activity of the precipitated gPOD showed 61.1 U/mg proteins and 45 % yield which is in tandem with the result obtained by Ahmad and his colleagues. However, these results agreed with those obtained for peroxidase from other plant sources using high ammonium sulfate precipitation (Muhammad *et al.*, 2011) where peroxidase from orange and apple seeds got purified up to 30.64 and 8.34 fold with specific activities of 18.16 and 9.20 U/mg proteins, respectively. Bruemmer *et al.* (1976) partially purified peroxidase from fresh orange juices by using ammonium sulfate precipitation which increased activities to different fold in each variety. Kermasha and Metche, (1988) purified apple seed peroxidase with solid ammonium sulfate which increased its activity by a factor of 3. Civello *et al.* (1995) also used ammonium sulfate

precipitation method for partial purification of peroxidase from strawberry fruit. They reported 2.37 purification fold of the straw berry fruit peroxidase which is higher compare to present study. Hui *et al.* (2006) reported 12.8 % yield on the crude extract of apple seeds that was purified by ammonium sulfate precipitation which is lower when compared with the present study that reported 45 % yield. Muhammed *et al.* (2013) also reported 1.16 and 4.14 purification folds in apple and orange seeds, respectively which is close to the present study result.

Ammonium sulfate precipitation leads to partial purification of enzyme which is further purified through dialysis. The 80 % $(\text{NH}_4)_2\text{SO}_4$ precipitate was concentrated, dialyzed against 10 mM phosphate buffer, pH 7.0. The specific activity of dialyzed gPOD was 185 U/mg proteins with protein content of 0.12 mg/mL. This is higher compared to Ahmad *et al.* (2019) who reported 44.3367 U/mg proteins; however, the protein content (1.2 mg/mL) is higher compared to the present studies.

The dialyzed gPOD was applied to the gel filtration chromatography column (2×70) containing gel with a pore size SG-200, 100 mM of phosphate buffer was used as the eluting buffer. The specific activity of the purified gPOD increased to 143.6 U/mg proteins after gel filtration with purification fold of 3.3 and 1.7 % yield. This is in tandem with the work of Ahmad *et al.* (2019) who reported 72.83 purification fold and 1.5 % yield from broccoli stem peroxidase but lower compared to the work of Deepa and Arumughan (2002) who reported purification fold of 429 and 54 % yield. Muhammad *et al.* (2011) also reported 30.64 and 8.34 folds with specific activities of 18.16 and 9.20 U/mg proteins, respectively from orange and apple seeds peroxidases. The results were higher compared to 3.3 fold with 143.6 U/mg proteins obtained from the present study.

The crude GOD produced from *Aspergillus fumigatus* in a submerged fermentation system after 7 days had a specific activity of 8.77 U/mg proteins. This was higher compared to 5.7 U/mg proteins reported by Hatzinikolaou and Macris (1995) for GOD from *A. niger* BTL after 6 days.

A complete precipitation of *A. fumigatus* GOD was suitable at 90 % ammonium sulfate. This is close to the report of Muhammad *et al.* (2007) who observed maximum precipitation at 85 % ammonium sulfate. Moreover, Sukhacheva *et al.* (2004) subjected the *P. funiculosum*-433

glucose oxidase to 80 % saturation and found specific activity of 18 U/mg proteins, 94 % yield and 1.6 purification fold. The result of their work agreed with that obtained from *A. fumigatus* GOD in the present study. However, the present study had lower yield but higher purification fold than Muhammad *et al.* (2007).

After gel filtration, the specific activity of 67.5 U/mg proteins was obtained with 7.7 purification fold and 1.2 % yield. The purification fold of this present study is in tandem with Bhatti *et al.* (2006) who reported 7.5 purification fold on *A. niger* GOD but 28.43 % yield which is higher than that reported in the present study.

Glucose oxidase (GOD) was characterized using the partially purified peroxidase (gPOD) as coupling enzyme with various parameters such as, pH effect on enzyme activity, temperature and effect of substrate concentration. The results showed that GOD exhibited high activity in a pH range spanning 5.0-6.0, with the pH optimum at 5.5 after which the activity was found to decline above and or below pH 5.5 which indicates that the reaction rate increased with increase in pH of the medium but as the pH becomes higher the rate decreased. The result of this study agreed with Bhatti *et al.* (2006) who reported pH 5.5 as the optimum for *Aspergillus niger* NFCCP glucose oxidase, Muhammed *et al.* (2007) who reported pH range of 4–8 from *Aspergillus niger* UAF-1 glucose oxidase while exhibiting maximum activity at pH 5.5 and Zia *et al.* (2007) who reported optimum of pH 5.5 on *Aspergillus sp.* glucose oxidase; however, this optimum pH is lower compared to pH 6.5 reported by Odebunmi and Owalude (2007). Moreso, Weibel and Bright (1971) also argued that kinetic study at different pH values has established that as the pH of the reaction medium increases, catalytic activity of glucose oxidase decreases which has been attributed to the protonation of the active site histidine in the FAD molecule resulting results in a reduction of the kinetic barrier for the electron transfer to the oxygen. Thus at low pH, glucose oxidase provides a rigid and polar environment of low polarizability which reduces the intrinsic barrier of the charge transfer to oxygen. Thus, change in pH affects the ionization of essential active-site amino acid residues, which are involved in substrate binding and catalysis, i.e., in the breakdown of substrate into products. Some ionizable residues may be located on the periphery of the active site, commonly known as nonessential residues. The ionization of these residues may cause distortion of active site cleft and, hence, indirectly affect the enzyme activity. This

could be the reason why the pH curve took this trend. The results of the present study are also comparable to those of Weibel and Bright (1971) who reported that GOD assayed in the pH range of 4.0–7.0, has optimum pH of 5.5. A pH optimum of 5.6 has previously been reported by Swoboda and Massey (1965) for GOD from *A. niger* and Rose *et al.* (2019) reported that *A. niger* NFCCP GOD was better than that from *Penicillium pinophilum*, which was unstable in the pH range of 2.0–4.0 and above 7.0. A recombinant glucose oxidase (rGOD) produced by Meng *et al.* (2013) and Belyad *et al.* (2018) reported optimum pH of 6.0 and 7.0 respectively for *Aspergillus niger* both in *Pichia pastoris*.

The *A. fumigatus* glucose oxidase catalyzed oxidation reaction was studied at different temperatures from 30 °C to 80 °C. Increase in temperature from 30 °C to 45 °C was accompanied with increase in GOD activity beyond which the activity decreased making 45 °C the optimum temperature. The optimum temperature obtained in the study is in line with the reported data of Bhatti and Saleem (2009) for GOD from an *Aspergillus sp.* In addition, *P. variable* P16 recombinant GOD also displayed optimum temperature at 45 °C and its native at 55 °C, while GOD from *P. funiculosum* 433 and *P. canescens* Tt42 had slightly lower optimal temperature at 30 and 25–30 °C (Ramani *et al.* 2012). This increase in GOD activity with temperature could be as a result of increase in collision between the substrate and enzyme active sites-the collision theory (molecules that have acquired more energy as a result of their vibration), whereas the decrease might be due to denaturation of the GOD, subunit dissociation or change in the conformation (3-D structure) of GOD. Glucose oxidase from *P. pinophilum* could also be rapidly deactivated at 40 °C by increasing the pH to 7.0 or decreasing it to 4.0. The present study showed that GOD activity from *A. fumigatus* was deactivated after 45 °C which is however higher compare to Swoboda and Massey (1965); Bahtti *et al.* (2006); Muhammad *et al.* (2007) and Zia *et al.* (2007) reported temperature optimum at 40 °C from *A. niger*. Glucose oxidase being a dimeric enzyme, its concentration might affect its stability, suggesting that the dissociation of subunits could be playing a key role in its inactivation as observed for other multimeric enzymes. The thermal denaturation of this dimeric enzyme is accompanied by the disruption of noncovalent linkages, including hydrophobic interactions, leading to subunit dissociation.

In another set of experiments, the reaction velocities were determined at different concentrations of the substrate (glucose) while maintaining constant concentration of glucose oxidase. The reaction velocities increased with substrate concentration to a certain level and tends to level off with a constant value. A plot of the reaction rate against substrate concentration showed two regions. First, at a low concentration of the substrate, velocity (V) increased almost linearly with substrate concentration [S] showing first order kinetics within this range. On the other hand, at high glucose concentration, the rate of increase in activity decreased progressively with concentration and it eventually levelled off to a constant value that is independent of further increase in glucose concentration. The reaction rate tends towards zero order at this point indicating that the active sites have been filled up or saturated with the substrate, obeying the saturation kinetics (Bright and Porter, 1975; Odebunmi and Owalude, 2007). Meanwhile, a double reciprocal plot of $1/V$ versus $1/[S]$ is a linear graph used to estimate the important kinetic parameters: K_m of 5.66 mM and V_{max} of 16.67 U/mL. The results of the present study kinetic constants are in agreement with the work of Owalude, (2004) hence, the substrate showed higher affinity to the *A. fumigatus* GOD.

4.2 Conclusion

The results of this research revealed that locally extracted peroxidase from *P. vulgaris* (green bean seed) could be utilized as a coupling enzyme with *Aspergillus fumigatus* glucose oxidase, hence providing a robust, cheaper and cost-effective source of peroxidase rather than total dependent on commercially produced horseradish POD.

4.3 Suggestions for Further Studies

1. The biochemical and molecular characterization of the *P.vulgaris* peroxidase should be carried out.
2. The thermostability and thermoactivity studies of *P.vulgaris* peroxidase for possible industrial application should be looked into.
3. The inhibitory studies should be carried out for diagnostic purposes.
4. Effect of different organic solvents should be studies for possible environmental remediation strategies.

5. The study on electrospinning using *P.vulgaris* peroxidase on nanofiber should be encouraged.

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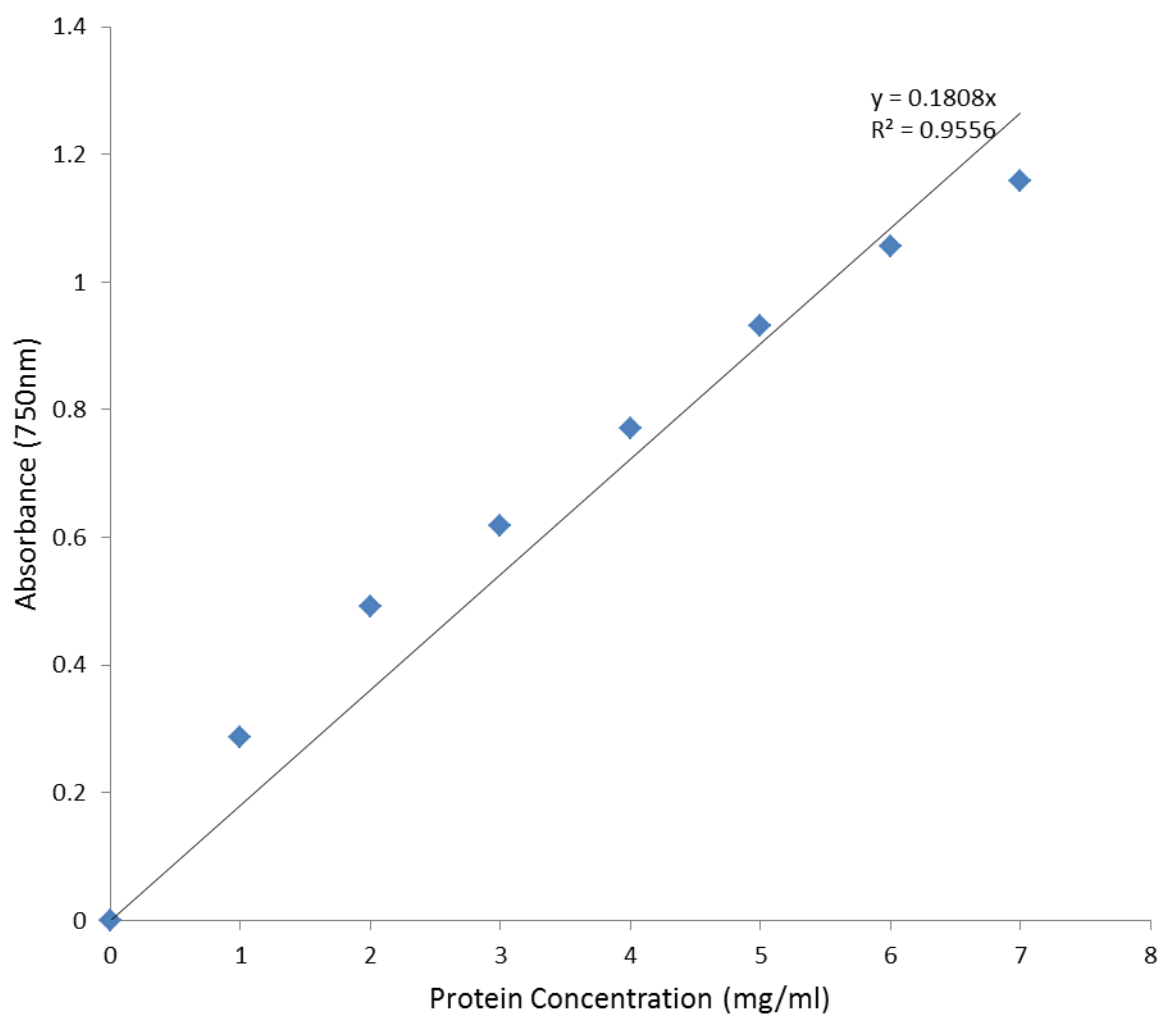
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APPENDICES

APPENDIX I

Appendix 1: Protein Standard Curve, Using 2 mg/ml Bovine Serum Albumin (BSA)



APPENDIX II

Procedure for POD Assay for Activity

Preparation of substrate solution

- (1) 0.1 % w/v *o*-Dianisidine: This was prepared by weighing 0.1 gram of *o*-dianisidine into 100 mL of methanol and filtered with Whatman No 1 filter paper to get clearer solution (w/v, gram solute/ 100mL solution).
- (2) 0.3 % v/v H₂O₂ the stock H₂O₂ is 30 %. That means that 1 mL of H₂O₂ was dissolved in 100 mL of distilled H₂O.
- (3) 0.1 M phosphate buffer, p H 7.0. The buffer was prepared by multiplying the required concentration with the molecular weight of disodium hydrogen phosphate. The required gram was measured and dissolved into 1000 mL distilled water, then using phosphoric acid and pH meter to adjust to the desired pH.

The reaction mix is 2.5 mL buffer, 0.2 mL *o*-dianisidine, 0.2 mL H₂O₂ and 1mL of the enzyme.

Procedure for GOD Assay for Activity

A: Sodium acetate buffer (50 mM), pH 5.5. This is prepared by 4.1 grams of sodium acetate into 100 mL of distilled water. Acetic acid (1:1 dilution) was used to adjust to the desired pH using syringe to drop the acetic acid while stirring for uniform circulation.

B: *o*-Dianisidine solution. 50 mg (0.05 g) of *o*-dianisidine dihydrochloride was dissolved in 10.0 mL of methanol and 90 mL reagent A was added.

C: β -D-glucose (10 % w/v) substrate solution was prepared using distilled water by dissolving 10 grams of the substrate in 100 mL of distilled water or 1 gram into 10 mL of distilled to serve as solution C.

D: Reaction cocktail was prepared by *o*-dianisidine 0.5 % (w/v) and 1.72 % (w/v) of glucose solution. (The combination of 24 mL of reagent B and 5 mL of reagent C were mixed and adjusted the pH to 5.5. This solution was freshly prepared i.e., 48 mL of reagent B was added to reagent C to serve as reagent D.

E: Peroxidase solution from green beans (gPOD)

Preparation of the Component Reagents for Protein Determination

Solution A: An alkaline sodium carbonate (Na_2CO_3) was prepared by dissolving 2g of Na_2CO_3 in 100 mL of 0.1M NaOH (0.4g of sodium hydroxide pellets were dissolved in 100 mL of distilled water).

Solution B: A copper tetraoxosulphate IV - sodium potassium tartarate solution was prepared by dissolving 0.5g of CuSO_4 in 1g of sodium potassium tatarate, all in 100 mL of distilled water. It was prepared fresh by mixing stock solution, and so was done whenever required.

Solution C: Folin-Ciocalteau phenol reagent was made by diluting the commercial reagent with water in a ratio of 1:1 on the day of use.

Solution D: Standard protein (Bovine Serum Albumin, BSA) solution.

Solution E: Freshly prepared alkaline solution was made by mixing 50 mL of solutions A and 1mL of solution B.