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PEROXIDASE AND ITS APPLICATIONS

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ABSTRACT

Peroxidases also known as haem proteins are versatile commercially important biocatalysts that catalyse the reductive cleavage of hydrogen peroxide by an electron donor. They have been divided into several structural classes and are produce by a number of microorganisms, plants and animals. Peroxidases catalyze a variety of reactions in the presence of peroxide(s) such as hydrogen peroxide. Reduction of peroxidases at the expense of electron donating substrates makes peroxidases useful in a number of biotechnological applications. Peroxidases have potential to decrease environmental pollution by bioremediation of wastewater containing phenols, cresols and chlorinated phenols, for bio-pulping and decolourization of synthetic textile azo-dyes. They are also used in analytical applications in diagnostic kits for quantification of cholesterol, glucose, uric acid, lactose etc., in the fine chemical and pharmaceutical industries, and it is most common enzyme used for labeling an antibody in Enzyme linked immunosorbent assay (ELISA). ELISA based assays are useful in diagnosis of respiratory, cardiovascular, periodontal disease and to detect toxins, pathogens, cancer risk in bladder and prostrate and many other analytes.

INTRODUCTION

Peroxidases (EC 1.11.1.7) are oxido-reductases that catalyse the reduction of peroxides, such as hydrogen peroxide (H_2O_2) and the oxidation of a variety of organic and inorganic compounds (Hamid and Rehman, 2009). Specifically, peroxidase activity involves donating electrons that bind to other substrates such as ferricynides and ascobate, in order to break them into harmless components. Peroxidases degrade H_2O_2, a naturally occurring by-product of oxygen metabolism in the body. As a result, these substances are converted into water and oxygen (Kobayashi *et al*., 1987). By the early 1900s, as yet unknown enzyme at work in human body were labeled as 'catalases' while the simultaneous observation that plants and animals utilized polyphenols to degrade H_2O_2 lead to the term 'peroxidases' (Paul, 1987). Peroxidases play an important role in protection of plant leaves from salt-induced oxidative damage (Tayefi-Nasrabadi *et al.,* 2010)**.** The well-known and best studied peroxidase is horseradish peroxidase (HRP) Welinder, 1979) but somehow bacterial peroxidases have attracted comparatively more attention than plant peroxidases (Tuncer *et al.*, 2009). Many peroxidases have been reported from different sources (Table 1) with their vital role in different fields/ industries. The mechanism of peroxidase catalyzed reaction is depicted in the following general equation;

$$AH_2 + H_2O_2 \rightarrow A + 2H_2O$$

Peroxidases are heme proteins and contain iron (III) protoporphyrin IX (ferriprotoprophyrin IX), as the prosthetic group. They have a molecular weight ranging from 30 to 150 kDa and may be divided into mammalian and plant peroxidases (Welinder, 1992). Peroxidases catalyse the oxidation of a wide variety of substrates, using H_2O_2 or other peroxides. The catalytic cycle involves distinct intermediate enzyme forms (Wang 1995; Chung *et al.,* 1997; Mantha *et al.,* 2002). Peroxidase activity has been identified in plants, microorganisms and animals, where these play important roles. In plants they participate in the lignifications process (Johnsy and Kaviyarasan, 2011) as well as in the mechanism of defense in physically damaged or infected tissues (Biles and Martin, 1993). Peroxidases are versatile biocatalyst with an ever increasing number of applications (Colona *et al*., 1999; Veitch and Smith 2001). An exhaustive screening of the *Pleurotus ostreatus* has been performed to search for heme peroxidases in a white-rot fungus, which could be useful for different biotechnological applications (Ruiz-Dueñas *et al*., 2011).

Table 1: Microbial peroxidases and their applications.

S. No.	Microbial source	Organism	Peroxidase type	Use(s)	Reference(s)
1.	Bacteria	*Bacillus sphaericus*	Intracellular peroxidase	Use for the decomposition of pollutants, as biosensors.	Anke *et al.*, 2001
2.	Actinomycetes	*Streptomyces* sp.	Liginin peroxidase ,molecular weight 45.2 kDa,	Use for the production of animal feedstock and raw materials for the chemical, agricultural, and paper industries.	Jeon *et al.,2002*
3.	Fungus	*Phanerochaete chrysosporium*.	Liginin peroxidase and Manganese peroxidase	Biodegradation of dyes	Chai Chu Chia, 2008
4.	Fungus	*Dichomitus squalens*	Manganese dependent peroxidase , molar mass = 50 k Da	Dye decolorization	Sulsa *et al.,2008*
5.	Cynobacteria	*Anabaena* sp.	heme-dependent peroxidase ,molecular weight = 53-kDa	Dye decolorization	Henry *et al.*, 2009
6.	Fungus	*Phanerochaete chrysosporium*	Liginin peroxidase	sewage-treatment	Md Zahangir *et al*.,2009
7.	Bacteria	*Bacillus* sp.	Liginin peroxidase and Manganese peroxidase	Textile dye degradation	Vishal *et al*., 2009
8.	Actinomycetes	*Streptomyces* sp.	Liginin peroxidase	Use of the enzyme in the treatment of effluents from chemically bleached pulp; pulp and paper mill effluents.	Munir.,2009
9.	Bacteria	*Acinetobacter calcoaceticus*	Liginin peroxidase, molecular weight=55–65 kDa	Decolorization of textile dyes	Gajanan *et al.*, 2009
10.	Bacteria	*Bacillus pumilus* and *Paenibacillus* sp.	manganese peroxidase, molecular weight=25 kDa and 40 kDa	Used in Paper–pulp industry for lignin degradation and dye decolorization.	Patrícia *et al.*, 2009
11.	Fungus	Coprinopsis cinerea	Versatile peroxidase	in the bleaching plant of paper pulp mills,	Francisco *at al.*,2009
12.	Bateria (Actenomycete)	*Thermobifida fusca*	Amonomeric, heme containing, thermostable, and Tatdependently exported peroxidase.	Dye decolorization	Edwin *et al.*, 2010
13.	Bateria (Actenomycete)	*Thermobifida fusca*	Amonomeric, heme containing, thermostable, and Tatdependently exported peroxidase.	Dye decolorization	Edwin *et al.*, 2010
14.	Fungi	white rot fungus strain	manganese peroxidase	Bioremediation of waste water, biopulping, biobleaching and bio-ethanol production.	Shinya *et al., 2010*
15.	Bacteria	*Bacillus subtilis*	Liginin peroxidase	Biodegradation of toxic chemicals, pulp, paper processing, and in the textile industry.	Renugadevi R *et al*.,2011
16.	Bacteria	*Citrobacter* sp.	Manganese peroxidase	Used in Paper–pulp industry for liginin degradation	Ram and Amar, 2011
17.	Fungus	*Candida krusei*	Liginin peroxidase	Dye decolorization	Charumathi and Nilanjana, 2011
18.	Bacteria	*Psuedomonas* sp.	Manganese peroxidase	Dye decolorization	Lin-Na *et al*., 2011

Peroxidases are also widely used in clinical biochemistry and enzyme immunoassay and for the colorimetric measurement of biological materials (Vamos-Vigyazo 1981). Some novel applications of peroxidases include treatment of waste-water containing phenolic compounds, synthesis of various aromatic chemicals and removal of peroxide from materials such as foodstuffs and industrial wastes (Agostini *et al*., 2002). Peroxidases have potential for bioremediation of wastewater contaminated with phenols, cresols and chlorinated phenols, for bio-pulping, bio-bleaching in paper industry and textile-dye degradation (Dutt *et al.,* 2010). Reduction of peroxide at the expense of electron donating substrate makes peroxidases useful in a number of industrial and practical analytical applications in diagnostic kits, such as quantification of uric acid, glucose, cholesterol, lactose and it is also the most common enzyme used for labeling an antibody in immunoassays. Peroxidase is probably the well-suited enzyme for the formation of conjugated antibodies, which are used in ELISA test, due to its ability to yield chromogenic product at low concentration, and in part to its relative good temperature stability (Krell, 1991).

1. Applications and peroxidase biocatalysis in management of environmental pollutants

1.1 Decolorization of synthetic dyes

Dye wastes represent one of the most problematic groups of pollutants, considered as xenobiotics that are not easily biodegradable (Ong *et al*., 2011). These dyes are mostly used in textile dyeing, paper printing, and colour photography and as additive in petroleum products. When these synthetic dyes are discharged into industrial effluents they cause environmental pollution. 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 (Tien *et al*., 1988; Munoz *et al*., 1997). These oxidases and peroxidases have been reported as excellent oxidant agents to degrade dyes (Heinfling *et al*., 1988; Kirby *et al.*, 1995). Several bacterial peroxidases have been used for decolorization 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. AO7 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 (Ng *et al*., 2010). Decolorization of different azo dyes by *Phanerochaete chrysosporium* RP 78 under optimized conditions was studied (Ghasemi *et al*., 2010) by reaction mechanism via azo dye (Fig. 1.).

A purified peroxidase produced by *Geotrichum candidum* Dec I, was involved in decolorization of dyes (Kim and Shoda, 1999). Peroxidase was produced under aerobic conditions as a secondary metabolite in the stationary phase. *Bacillus* sp. VUS isolated from textile effluent contaminated soil showed capability for degrading a variety of dyes (Dawkar *et al.,* 2008). Purified forms of lignin peroxidase have been found to oxidize recalcitrant xenobiotic compounds such as polycyclic aromatic hydrocarbons, chlorophenols and other dyes (Paszczynski and Crawford, 1991). The production of ligninolytic peroxidases directly oxidizing aromatic compounds has been described in fungi (Paszczynski and Crawford, 1991; Jeries and Viikari, 1996). Other peroxidases were detected in microorganisms responsible for the biodegradation of industrial dyes (Dey *et al*., 1994) together with lignin peroxidase (Pomar *et al*., 2002). An edible macroscopic fungi *Pleurotus ostreatus* produced an extracellular peroxidase that can 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%; Shin and Kim, 1997). HRP 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). Peroxidases purified from plants have also been used for the degradation of dyes. Purified peroxidase of *Saccharum spontaneum* leaf could degrade a variety of dyes ranging from the 70% to 100% in 1 h (Hernandez *et al*., 1998). In contrast, chlorophenols are transformed *in vitro* to polychlorinated dibenzo-p-dioxins and dibenzofurans by peroxidase-catalyzed oxidations. This has been demonstrated with bovine lactoperoxidase, HRP (Wittsiepe *et al*., 1999) and myeloperoxidase (Oberg *et al*., 1990) with 3,4,5- and 2,4,5-trichlorophenol. In the azo dyes transformation mediated by lignin peroxidase from *Pseudomonas chrysosporium,* a cleavage of azo bond was observed resulting in the production of a quinine and sulfophenyl hydroperoxides (Chivukula *et al.,* 1995) as mentioned in the proposed mechanism (Fig. 1.). Peroxidase produced by *Pseudomonas sp.* was used in the biodegradation of Malachite green via a proposed mechanism (Fig. 2.).

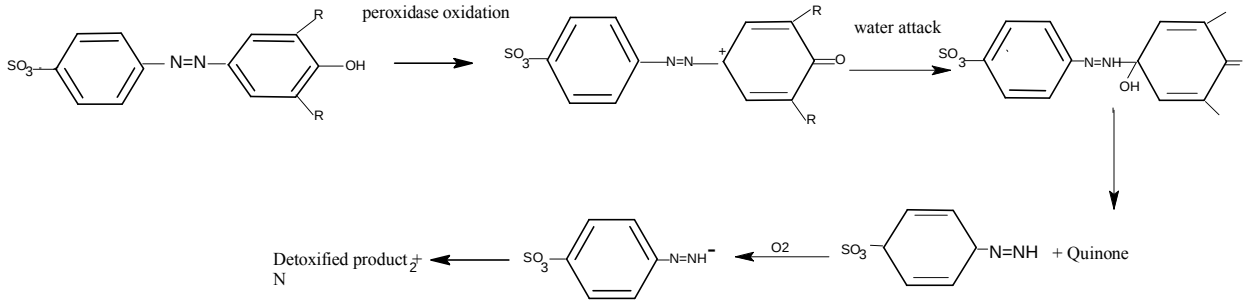

Fig.1. Proposed mechanism of azo dye decolorization by peroxidase

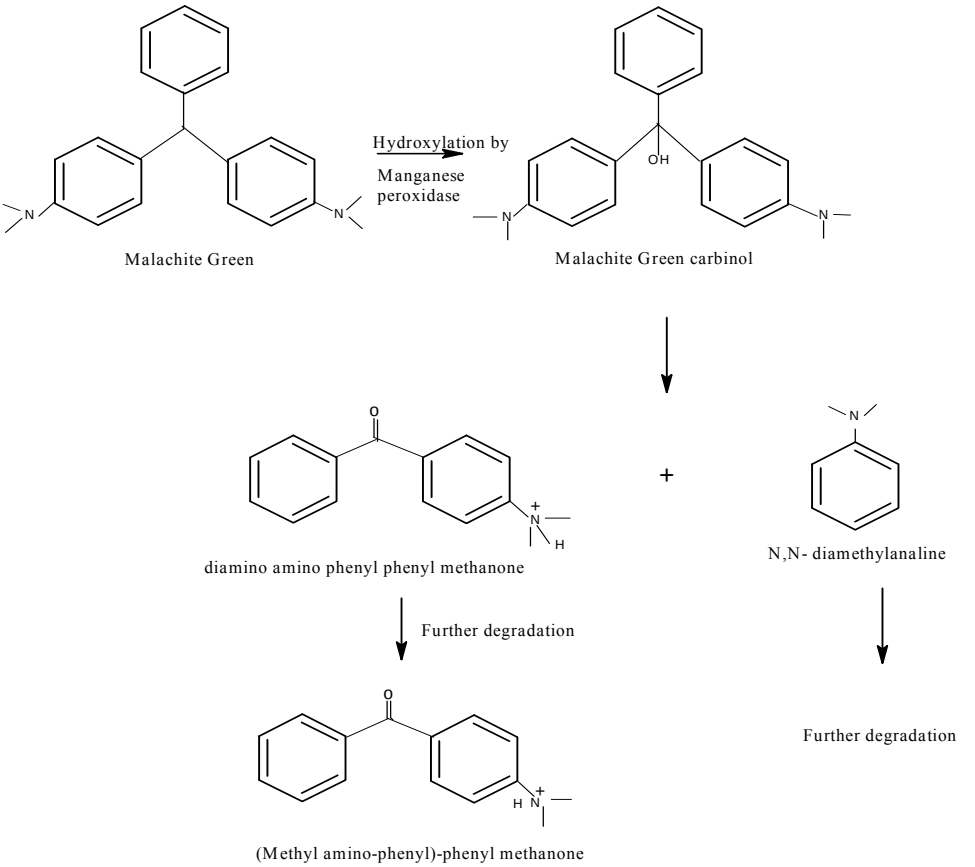

Fig. 2. The proposed biodegradation pathways of Malachite green by *Psedomonas* sp. strain DY 1 under shaking conditions based on the result of LC-MS and GC-MS.

1.2 Removal of phenolic contaminants and related compounds (Bioremediation of waste water)

Peroxidases have been applied to the bioremediation of waste waters contaminated with phenols, cresols and chlorinated phenols (Hamid and Rehman, 2009). Aromatic compounds, including phenols and aromatic amines, constitute one of the major classes of pollutants. They are found in the waste waters 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 (Nicell *et al*., 1993). Phenols and halogenated phenols are known to be toxic and also, some of them are hazardous carcinogens that can accumulate in the food-chain (Nicell, 1993). Peroxidases comprise an important class of enzymes able to catalyze the oxidative coupling reactions of a broad range of phenolic compounds (Job and Dunford 1976; Nannipieri and Bollag, 1991; Bollag, 1992). Liginin peroxidase from *Phanerochaete chrysosporium* (Reuttimann and Lamar 1996; Hammel and Tardone, 1988), HRP (Samokyszyn *et al*., 1995), myeloperoxidase, lactoperoxidase, microperoxidase-8, a versatile peroxidase from *Bjerkandera adusta*, and chloroperoxidase from *Caldariomyces fumago* (Longoria *et al.,* 2008) were able to transform pentachlorophenol to tetrachloro-1,4-benzoquinone by an oxidative dehalogenation in the presence of H_2O_2.

1.3 Removal of endocrine disruptive chemicals (EDCs)

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 (Cabana and Agathos, 2007). They are widely dispersed in the environment, but they can mainly be found in wastewater effluents. Several works reported the EDC oxidation by manganese peroxidase. Using 10 U/ml of manganese peroxidase from *Pleurotus ostreatus*, 0.4 mM bisphenol A was eliminated in 1 h (Huang and Weber, 2005). Peroxidases also helpful in removal or degradation of other potent environmental pollutants such as chloroanilines and polycyclic aromatic hydrocarbons: (Renner, 1980).

1. 4 Degradation of pesticides, polychlorinated biphenyls (PAHs)

Pesticides include a broad range of substances most commonly used to control insects, weeds, and fungi. Pesticide exposure is associated with chronic health problems or health symptoms

such as respiratory problems, memory disorders, dermatologic conditions, cancer, depression, neurologic deficits, miscarriages and birth defects (McCauley *et al*., 2006). Peroxidases extracted from some fungal species have great potential to transform several pesticides into harmless form(s). Transformation of organophosphorus pesticides by white-rot fungi has been studied (Jauregui *et al*., 2003) and transformation of several organophosphorus pesticides by the chloroperoxidase from *Caldariomyces fumago* has been reported. PAHs are composed of two or more fused aromatic rings and are components of crude oil, creosote and coal (Harayama 1997). Most of the contamination by PAHs had originated from the extensive use of fossil fuels as energy sources. Peroxidases and phenoloxidases can act on specific PAH's by transforming them to less toxic or products easier to degrade. PAHs are oxidized by peroxidases such as lignin peroxidase (Weber *et al.,* 2008) and manganese peroxidase (Harford-Cross *et al*., 2000). In spite of their versatility and potential use in environmental processes, peroxidases are not applied at large scale yet. Diverse challenges, such as stability, redox potential and the production of large amounts, should be addressed in order to apply peroxidases in the pollutant transformation.

2. Application in analysis and diagnostic kits

Due to the HRP ability to yield chromogenic products at low concentrations and its relatively good stability, it is well-suited for the preparation of enzyme conjugated antibodies and application in diagnostic kits. In analytical application(s), the enzyme must be present in saturating amounts to make sure that the H_2O_2 produced in the test is stoichiometrically converted into a colored substance (Krell, 1991). Various peroxidase isoenzymes were purified from roots and hairy-roots cultures of turnip (*Brassica napus*; Agostini *et al*., 2002). They developed a diagnostic test kit for determination of uric acid. The assay was based on the following reactions:

Uric acid $+O_2 + 2H_2O$ Uricase $\rightarrow$ Aallantoin $+ H_2O_2 + CO_2$

H_2O_2 + 4-aminophenazone + phenol peroxidase $\rightarrow$ p-(Benzoquinone) monoiminephenazone

HRP is the most commonly used enzyme for practical analytical applications. However, peroxidases from other sources appear to be a good alternative as substitutes for HRP (Carlos *et al.,* 2004). Peroxidase isoenzyme from turnip hairy roots could be used as a reagent for clinical diagnosis, as part of a kit where H_2O_2 is generated. The concentration of turnip peroxidase giving a linear response with time and increasing uric acid concentration was 30 mM. Analysis of uric

acid in human serum from ten different patients using either the kit containing turnip peroxidase or a commercially available kit, gave the same results (Hamid and Rehman 2009). Peroxidase was used in clinical strategies for detecting and treating thyroid disorders by serum-based immunoassay (Carole, 2010) and to detect several autoimmune diseases in human body. An autoimmune disease occurs when the body's immune system begins to attack its own antigens. A hallmark is the production of high-affinity auto-antibodies. Different techniques have been used to develop specific tests for auto-antibody detection including enzyme immunoassays in which peroxidase is used to labeling an antibody (Ilza, 2010).

3. ELISA

The first enzyme immunoassay method was introduced in 1972 and since that time several different forms of enzyme immunoassays have been developed, which were simple, high-throughput analyses and could be automated and standardized (Baloch *et al*., 2003; Spencer *et al.,* 2005). HRP is probably the most common enzyme used as a reporter (enzyme-labeled antibody) in enzyme immunoassays. HRP-containing ELISA kits, which have found applications in food control, in diagnostic microbiology and as disposable amperometric immuno-sensors, and of peroxidase-containing bio-electrosensors that improve the simpler colorimetric biosensors, are usually employed in medical diagnostic test kits (Green *et al*., 2004). The widespread application of ELISAs for analytical purposes is due to the extremely high selectivity and affinity of antibody molecules to their corresponding antigens. Since the two reaction partners are usually chromatographically and electrochemically inert, an enzyme (mainly HRP in the case of peroxidases) is used for labeling of either the antigen or the antibody, allowing both the detection of the antigen and the amplification of the primary signal. An ELISA tests in which peroxidase is used for labeling an antibody, have been developed for screening monoclonal antibodies against mycotoxins (Kawamura *et al*., 1989). Mycotoxins are dangerous by-products of several species of fungi, such as *Aspergillus* and *Pencillium* sp. (ochratoxins), and *Fusarium* sp. (T-2 toxins, trichothecenes) amongst others. They are known to be hepatotoxic, nephrotoxic, teratogenic and mutagenic to a wide variety of mammalian species (Clarke *et al*., 1993).

Several other enzyme immunoassays using peroxidase as reporter enzyme have been developed to detect toxins, pathogens, and other analytes. Such as ELISA for detection of Hepatitis-E virus using human serum as product by (Zhuang *et al*., 2001) and alkaline phosphatase ELISA using

milk as a product in competitive indirect ELISA by (Vega-Warner *et al.,* 2000). A type of non-competitive ELISA has been developed for detection of Gossypol using Cotton seed as product (Wang and Plhak, 2000).

Schistosomiasis continues to be a public health problem in many tropical and subtropical countries. Improving the diagnostic tools for surveillance and monitoring in areas that have reached elimination level will help hasten the possible elimination of this disease. A study developed ELISA through the use of recombinant proteins such as thioredoxin peroxidase-1 and four tandem repeat proteins. Cut off values were calculated using 38 serum samples from healthy Japanese volunteers. Sera from 35 schistosomiasis-confirmed patients, four cured from the disease by chemotherapy, and 15 endemic negative controls were used to assess these antigens. SjTPx-1 and Sj7TR both had 85.71% sensitivity. Furthermore, these antigens were also tested against human sera positive for other parasitic infections and showed no or very minimal cross-reaction. These results suggested the use of potential defined antigens for development of an accurate diagnostic test for schistosomiasis (Jose, 2011). A novel HRP immunoassay for the diagnosis of chronic *Chlamydia pneumoniae* infection has been reported. The test is based on the quantification of chlamydial lipopolysaccharide in human serum utilizing lipopolysaccharide-binding protein as a capture molecule and HRP/O-phenylenediamide for chromatographic detection (Tiirola *et al.,* 2006). Usefulness of ELISA using peroxidase as conjugated protein in diagnosis of human prion disease has been reported (Yuki, 2011, Fig. 3.). By using Maize, wheat, rye, barley as products a useful ELISA has been developed in order to detect T-2 toxin having detection limit of 50 ng/g (Sibanda *et al*, 2000). HRP was used as conjugated antigen detecting antibody in development of sandwich and dot-ELISA to detect circulating *fasciola* antigen in serum sample collecting from a total of 154 sheeps (Tarek *et al,* 2011).

HRP is used as an enzyme conjugate in secondary antibodies in ELISAs. Therefore, the HRP will bind to a primary antibody, which binds to the target protein (the target protein is the protein that one intends to detect). The HRP will then bind to a substrate to yield a signal that leads to detection; HRP catalyzes a reaction with the substrate that causes the color change (Fig. 3)

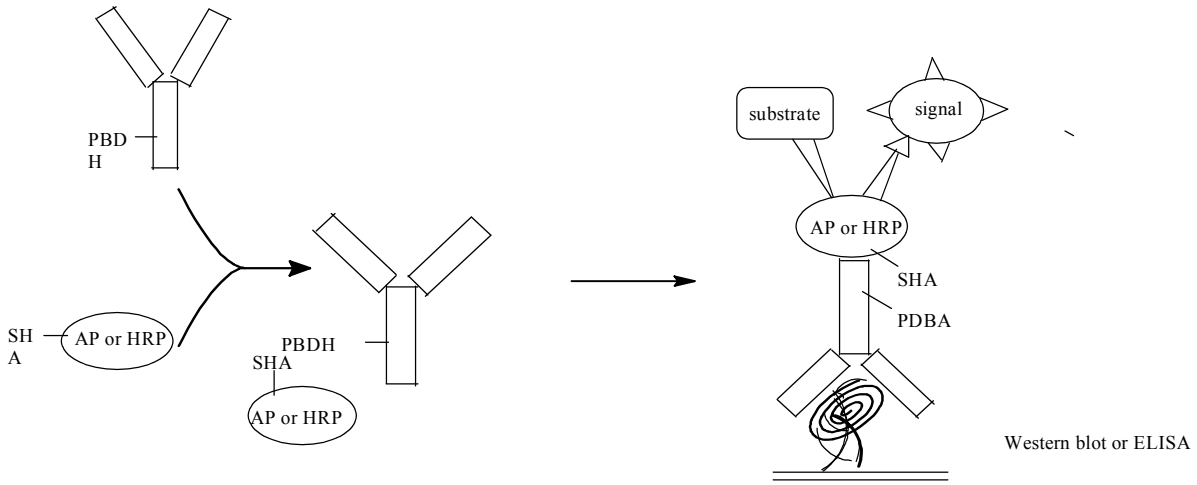

Fig.3. How HRP might work in ELISA (or Western Blot)

In the above example, the primary antibody was modified with phenyldiboronic acid in order to increase its binding properties to the HRP (or AP, alkaline phosphatase). The key point to understand in this diagram is that HRP binds to the primary antibody and when the substrate binds to HRP, a signal is elicited (Schutz *et al*, 1997).

4. Applications in paper pulp industry

Biopulping is a process where extracellular enzymes (hydrolytic and oxidative) produced by a white-rot fungus remain adsorbed on the wood chips thus leading to degradation of lignin (De *et al.,* 2004).The pulping by products cause serious environmental problem due to its high pollution load. In order to degrade these by products, many potential fungal and bacterial strains can be applied. Two bacterial stains *Citrobacter freundii* (FJ581026) and *Citrobacter* sp. (FJ581023) were applied in axenic and mixed condition for degradation of black liquor (pulping by product). The optimum activity of lignin degrading enzyme was noted at 96 h and characterized as manganese peroxidase by SDS-PAGE analysis (Chandra and Abhishek, 2011). Ligninolytic haem peroxidases were able to break down the main linkage types in lignin due to their high redox-potential and specialized catalytic mechanisms (Martinez, 2002, 2007; Hammel and Cullen, 2008). White-rot fungi attacked lignin and simultaneously degraded wood components to carbon dioxide and water (Arana *et al*., 2002). Direct use of microorganisms for breaking down of lignocellulosic materials had many drawbacks, including degradation of cellulose fibers (Jimenez *et al.,* 1997) and long reaction times, extending to several days (Katagiri *et al*., 1995).

5. Organic and polymer synthesis

HRP has been used to polymerize phenolic and aromatic amine compounds, while new types of aromatic polymers have been synthesized in water and in water-miscible organic solvents (Oguchi *et al.*, 1999). HRP catalysed the oxidative coupling of methyl-sinapate with a syringyl lignin model compound 1-(4-hydroxy-3, 5-dimethoxyphenyl) ethanol in the presence of H_2O_2. The main product was a novel spiro-cyclohexadienone, which was important in understanding the biosynthesis of lignin in the cell walls of woody plants by oxidative cross-coupling of different phenolic precursors (Setala *et al.*, 1999). Polyaniline is one the most extensively investigated conducting polymers because of its high environmental stability and promising electronic properties. Currently, polyaniline is synthesized by oxidizing monomer aniline under strongly acidic conditions and low temperature using ammonium persulfate as the initiator of radical polymerization (Rannou *et al*., 1998). In nature, peroxidases have good stability at low pH, making them a good alternative for polymerizing aniline under acidic conditions. Using an anionic peroxidase purified from the African oil palm tree, an enzymatic synthesis of the polyelectrolyte complex of polyaniline and also sulfonated polystyrene has been developed (Sakharov *et al*., 2003). In the presence of H_2O_2, peroxidase catalyzes the oxidation of phenols that eventually give rise to high molecular weight polymers (Nicell and Wright, 1997). This characteristic could be used as an attractive alternative to the conventional formaldehyde method used for the production of lignin-containing phenolic resins. The production of conducting polymers has remarkable interest because of their wide range of applications including anticorrosive protection, optical display, light-emitting diodes etc (Raitman *et al*., 2002).

6. Deodorization of swine manure

The HRP could be used as an enzymatic source in the deodorization of swine slurry (Govere *et al*., 2007). Odorant compounds such as phenols, indoles, volatile fatty acids, ammonia, hydrogen sulfide and mercaptans are either initially present in manure or result from anaerobic transformation of animal wastes (Hobbs *et al*., 1995; Zahn *et al*., 1997). Elevated odour level in confinement buildings can reduce livestock growth rates, thereby increasing the outbreaks of infections and adversely affecting farm workers (Hardwick, 1985). Treatments, such as dietary management, intense aeration or zone treatment and the application of manure additives have been used to decrease or eliminate odorous compounds (Hobbs *et al*., 1995; Wu *et al*., 1999;

McCrory and Hobbs, 2001). HRP has been proven an effective alternative for deodorization of manures. Minced horseradish with calcium peroxide reduced the concentration of phenol by 70% and for VFAs by 45%. A 100% reduction in the concentration of phenolic odorants without reoccurrence within 72 h was achieved by using HRP (Govere *et al*., 2005).

7. Peroxidase biosensors

Peroxidase has great potential in the field that comprising electrochemical biosensors. Peroxidase- based electrodes have had widespread use in analytical systems for determination of H_2O_2 and organic hydroperoxides (Jia *et al*., 2002). When co-immobilized with a H_2O_2 producing enzyme, they may be exploited for determination of glucose, alcohols, glutamate and, choline (Ruzgas *et al*., 1996).The importance of determination of H_2O_2 lies in the fact that H_2O_2 plays an important role in clinical, chemical, biological, environmental and many other fields (Tripathi *et al*., 2006; Shi *et al*., 2007).Techniques for detecting H_2O_2 include spectrofluorimetry (Pa'zdzioch-Czochra and Widenska, 2002), chemiluminescence (Hanaoka *et al*., 2001), electrochemical methods (Qiu *et al*., 2007; Zhou *et al*., 2005) and so on. Among these procedures, amperometric enzyme-based biosensors have received considerable attention, because they offer improved sensitivity, extended dynamic range and rapid response time (Liu *et al*., 2000). H_2O_2 are considered the mediators of the biochemistry of cellular pathology and maybe involved in the etiology of aging and progressive neurodegenerative diseases, such as Parkinson's disease (Halliwell and Gutteridge, 1984). Due to its crucial role in neurochemistry, determination of the concentration of H_2O_2 has been a considerable interesting research field. Among those, electrochemical methods have proved to be significantly advantageous to the biosciences due to their direct, real-time measurements, and capability for practical applications. A novel myoglobin-based electrochemical biosensor was developed. It is based on a nanocomposite prepared from multi-walled carbon nanotubes that were coated with ceria nanoparticles. UV-vis and electrochemical measurements displayed that the nanocomposite provides a biocompatible matrix for the immobilization of myoglobin (Mb) and also facilitates direct electron transfer between its active center and the surface of the electrode (Jian-Ding *et al*., 2010). A novel third generation biosensor for H_2O_2 was constructed by cross-linking HRP onto an electrode modified with multiwall carbon nanotubes (MWNTs). Glutaraldehyde and bovine serum albumin were used to cross-link HRP and the MWNTs. Factors influencing the

performance of the biosensor were studied in detail. The biosensor exhibited a fast amperometric response (2.0 s) to H_2O_2 which was linear in the concentration range from 9.5 mM to 0.95 µM (Xu *et al*., 2011). Selected examples of biosensors based on peroxidase and the purpose of their development are pyrolytic graphite electrodes made of HRP-immobilized in TiO_2 films with potential in electrochemistry and bio-electrocatalysis (Zhang *et al*., 2004), Direct electron transfer (ET) between the electrode and the heme group of HRP. Study of the kinetics of electron transfer (Lindgren *et al*., 2001), Graphite-teflon-peroxidase composite electrodes for amperometric detection of 18 phenolic compounds (Serra *et al*., 2001), Cellobiose dehydrogenase (CDH), and peroxidase biosensors for phenolic and diphenolic quantitation. The CDH biosensor could detect 5 mM of diphenolic compounds (Lindgren *et al*., 2000a).

Use of rotating-disc electrodes has been reported to test different plant peroxidase sources suitable for biosensor applications. Direct electeron transfer (ET) was hindered by glycosylation, while a low isoelectric point enhanced it. Sweet potato peroxidase gave the lowest detection limit (40 mM) for H_2O_2 (Lindgren *et al.*, 2000b). Compared with other analytical techniques, electrochemical enzyme biosensors had the advantage of high selectivity of the biological recognition elements and high sensitivity of the electrochemical transduction process. In this respect, a novel immobilization platform was developed by synergistically using ZnO crystals and nano-sized gold particles as HRP-loading material (Zhang *et al*., 2009).

8. Fungal peroxidases for biofuel production

Considering energy sources, human society has dramatically increased the use of fossil fuels in the past 50 years in a way that the most successful economies are large consumers of oil. However, geopolitical factors related to security of oil supply, high oil prices and serious environmental concerns, prompted by global warming; the use of petrol for transportation accounts for one-third of greenhouse gas emissions (Wyman, 1996) have led to a push towards decreased consumption. Indeed, the world's strongest economies are deeply committed to the development of technologies aiming at the use of renewable sources of energy. Within this agenda, the substitution of liquid fuel gasoline by renewable ethanol is of foremost importance. Biomass hydrolysis, *i.e*. the depolymerization of the biomass polysaccharides to fermentable sugars to produce ethanol and other biofuels, must be performed via environmentally friendly and economically feasible technologies (Lynd *et al.,* 2005). The enzyme based application was

advantageous over chemical treatments due to its higher conversion efficiency, the absence of substrate loss due to chemical modifications and the use of more moderate and non-corrosive physical-chemical operating conditions, such as lower reaction temperatures, 'neutral pH' and the use of biodegradable and nontoxic reagents. Nevertheless, biomass degradation is a highly complex multi-enzymatic process (Soderstrom *et al*., 2003).

Ethanol and other biofuels produced from lignocellulosic biomass represent a renewable, more carbon-balanced alternative to both fossil fuels and corn-derived or sugar-cane-derived ethanol. Unfortunately the presence of lignin in plant cell walls impedes the breakdown of cell wall polysaccharides to simple sugars and subsequent conversion of these sugars to useable fuel. To achieve an optimal biological conversion of lignocellulosic biomass to biofuel, lignin must be physically removed from plant tissue before saccharification (Weng *et al*., 2008). One of the most common fates of lignin in nature is to be metabolized by lignin peroxidases, manganese peroxidases and closely-related enzymes of white rot basidiomycetes (Chen and Dixon, 2007; Hammel and Cullen, 2008). These organisms are responsible for initiating the depolymerisation of lignin and are able to oxidise lignin to carbon dioxide (Weng *et al*., 2008). Peroxidases have potential in deigning enzymatic biofuel cells which are attractive for a number of special applications, such as disposable implantable power suppliers for medical sensor-transmitters and drug delivery; they offer practical advantages of using abundant organic raw materials as biofuels for clean and sustainable energy production. A hybrid biofuel cell formed by the battery type Zn anode and the bio-cathode, comprising HRP-immobilized on graphite and utilizing H_2O_2 as an oxidizer, was considered. When coupled to the H_2O_2-producing glucose oxidizing enzymes glucose oxidase or pyranose oxidase, the HRP bio-cathode was functional in the presence of glucose (Gomez *et al*., 2010).

9. Prospective applications of peroxidases

9.1. Pharmaceutical industries

Heme peroxidases have the potential to be widely used as catalysts in fine chemical preparations. This is because they are enzymes capable of performing a wide variety of oxidation reactions, ranging from radical coupling reactions, to oxygen-atom insertion into substrates, to several types of halogenation processes (Aehle, 2007). This delocalization of the radical on the phenol nucleus accounts for the product composition, since the radical coupling can occur through an

ortho-ortho process, giving rise to an 0-biphenyl adduct, or through an ortho-para process, forming the so called Pummerer's ketone, a pharmacophoric synthon (Valenti *et al*., 2006). All peroxidases can be employed for these reactions, but HRP is usually preferred with respect to other peroxidases due to its higher availability and to its broad specificity. Protein polysaccharide cross-linking gives rise to protein conjugates with modified properties, such as an enhanced stability to heat, organic solvents, and proteolysis, which could bear biotechnological applications, but could also be used for preparation of gels, foams, and colloids. The peroxidase-catalyzed oxidation of ortho phenylenendiamine by hydrogen peroxide can be used for the synthesis of diaminophenazine, the starting material for the preparation of many dyes. Synthesis of neurotrophic americanol A and isoamericanol by HRP-catalyzed oxidative coupling of caffeic acid (Matsumoto *et al*.,1999). Peroxidase oxidation of dopa and dopamine that produced a series of compounds such as dopachrome and dopaminechrome, respectively could be further evolve to compounds related to melanin pigments (Prota, 1992).

Several examples of halohydrin formation from styrene derivatives and saccharides catalyzed by CPO are reviewed by Adam and coworkers (Adam, 1999). Formation of bromohydrin derivatives of some saccharides can be of interest for the preparation of bioactive compounds (Fu *et al*., 1992). The problems connected with the high cost and low stability of peroxidases, which limits their potential applications in processes of industrial interest, could be possibly overcome, at least in part, by the use of heme-peptide complexes as small-size peroxidase analogs. Using micro-peroxidases, the heme-peptide complexes obtained from proteolytic degradation of cyt c, as starting point for the preparation of complexes with improved activity and controlled substrate selectivity (Lombardi *et al.,* 2001; Casella *et al.,* 2000; Dallacosta., 2003, 2004).When these problems are overcome, it is expected that number of technological applications of these versatile enzymes will experience a significant increase over a period of time.

CONCLUSIONS

In spite of versatility of peroxidases, till now a few industrial applications of peroxidases, in most cases are still at a prospective level as is the case of use of peroxidase(s) as bleaching-agent(s)/ additive(s) in detergents and in the paper and pulp industry, for degradation of electron-rich aromatic residues in waste waters, for antimicrobial applications, and for the synthesis of phenolic resins. The possible reasons for the limited application(s) of peroxidase are low water

solubility of the substrates of interest. Peroxidase activity is also greatly affected by elevated temperature, limiting its application to processes at relatively low temperatures and inactivation of peroxidases by peroxides through oxidation. The main limitation has probably to do with the chemistry these enzymes catalyze, which involves as an essential substrate a strong oxidant such as H_2O_2. The scale-up of processes requiring this reagent inevitably leads to a relatively rapid reduction in the performance of peroxidases, which are enzymes of considerable cost. In spite of its low operational stability at narrow temperature and pH range, peroxidase can be an efficient biocatalyst for the production of industrially important compounds. Some of the practical limitations to the use of peroxidases could be overcome by site-directed mutation. Therefore, a mutated peroxidase produced from a microbial source with improvements in its stability and activity, could be useful in a number of industrial applications.

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