FINAL REPORT OF

MINOR RESEARCH PPROJECT ENTITLED

BIOREMEDIATION OF PHENOLIC ENVIRONMENTAL POLLUTANTS USING FUNGAL METALLOENZYMES BY NON AQUEOUS APPROACH

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SUBMITTED

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1.1Introduction

Owing to the global increase in water pollution and aquatic ecosystem imbalance andthe increased public awareness, more attention has been given to thedevelopment of environmental friendly measures. Among several chemical pollutants, phenolic compounds are the major pollutants which can be eliminated by the enzymes capable of oxidizing them. In this context, two groups of enzymes are of primary importance in biotechnology, environmental protection and bioanalysis fields. They are (i) plant and fungal peroxidases and (ii) multicopper mono and/or polyphenol oxidases (tyrosinase, laccase). These enzymes have been proposed for use in waste water treatment, soil remediation, pulp bioleaching, polycyclic aromatic hydrocarbons (PAHs) and other xenobiotic degradation and as biosensors (Rodakiewicz-Nowak, 2000). In comparison to other oxidoreductases, for example the peroxidase whichrequires H_2O_2 in its catalytic process, laccase only uses oxygen for the oxidation of its reduced state (Moreira et al., 2001).

1.2 Laccase

Laccases, the oxidizing enzymes, arefrom an interesting group of multi copper enzymes which possess an ability to oxidize both phenolic and non-phenolic lignin related compounds and highly recalcitrant environmental pollutants with concomitant reduction of molecular oxygen to water (Figure 1).Several organic compounds which contain hydroxyl, acid or amino groups can act like substrates for these enzymes (Torres-pacheco, Octavio, & Guanajuato, 2006).

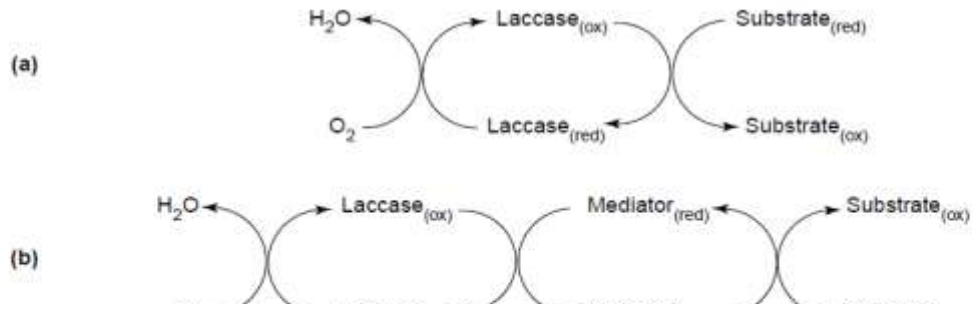


Figure 1Laccase-catalyzed redox cycles. Schematic representation of laccase mediated substrates oxidation in the absence (a) or in the presence (b and c) of chemical mediators(Riva, 2006).

1.2.1 Sources of laccase

Laccase is extensively present in higher plants, fungi and insects as well as bacteria.After their discovery in 1883 from the Japanese tree *Rhusvernicifera*, laccases have been found to be widely distributed among plants, where they are involved in the synthesis of lignin and in the wounding response. Three probable functions of laccases in fungi are described as: pigment formation, lignin degradation and detoxification (Kunamneni, Plou, Ballesteros, & Alcalde, 2008).

Laccaseshave been identified in several plants and trees including cabbages, turnips, beets, apples, potatoes, mango, pine and various other vegetables.Laccase is also found to be present in insects of various genera that include*Bombyx,Drosophilia,Musca,Papilio*and*Tenebrio*.Common fungi known to possess laccaseinclude*Ascomycetes*, *Deuteromycetes*, and *Besidiomycetes*.Most common laccase producers are the wood rotting fungi *Trametesversicolo,Trameteshirsute,Trametesvillosa, Cerena maxima* etc.On the contrary, laccase activity has been reported only in few bacterial strains including*Azospirillum,Lipoferum, Streptomyces griseus*and*Bacillus subtillis*(Madhavi & Lele, 2009).

Laccaseshave earned much attention recently owing to their diverse applications, such as delignification of lignocellulosics,cross linking of polysaccharides,bioremediation applications, waste detoxification, textile dye transformation, food technology uses,medical care related applications and biosensor and analytical applications (Madhavi & Lele, 2009).

1.2.2 Mechanism of laccase enzyme

The active site of the enzyme is constituted by four copper atoms associated at three types of sites, designed as T1, T2, and T3 sites. The T1 site is responsible for the substrate oxidation occurs whereas in the T2 and T3 sites mainly function in the reduction of molecular oxygen to water. The redox potential of the T1 site is characteristic for each particular laccase(Medina et al., 2013).

The laccase enzyme oxidizes substrates by removing one electron per timeand generates free radicals which can be polymerized. The enzyme storeselectrons of individual oxidation reactions and in its totally reduced statecontains a total of four electrons, thus, the enzyme can transfer these electronsto molecular oxygen to form water (Kunamneni et al., 2008).

The laccase molecule, as an active holoenzyme form, is a dimeric or tetrameric glycoprotein, usually containing -per monomer- four copper (Cu) atoms bound to three redox sites (Type 1, Type 2 and Type 3 Cu pair.The molecular mass of the monomer ranges from about 50 to 100 kDa with acidic isoelectric point around pH 4.0.An important feature is the high level of glycosylation (with covalently linked carbohydrate moieties ranging from 10-50% of the total weight, depending on the species or the heterologous host), which may contribute to the high stability of the enzyme(Riva, 2006).

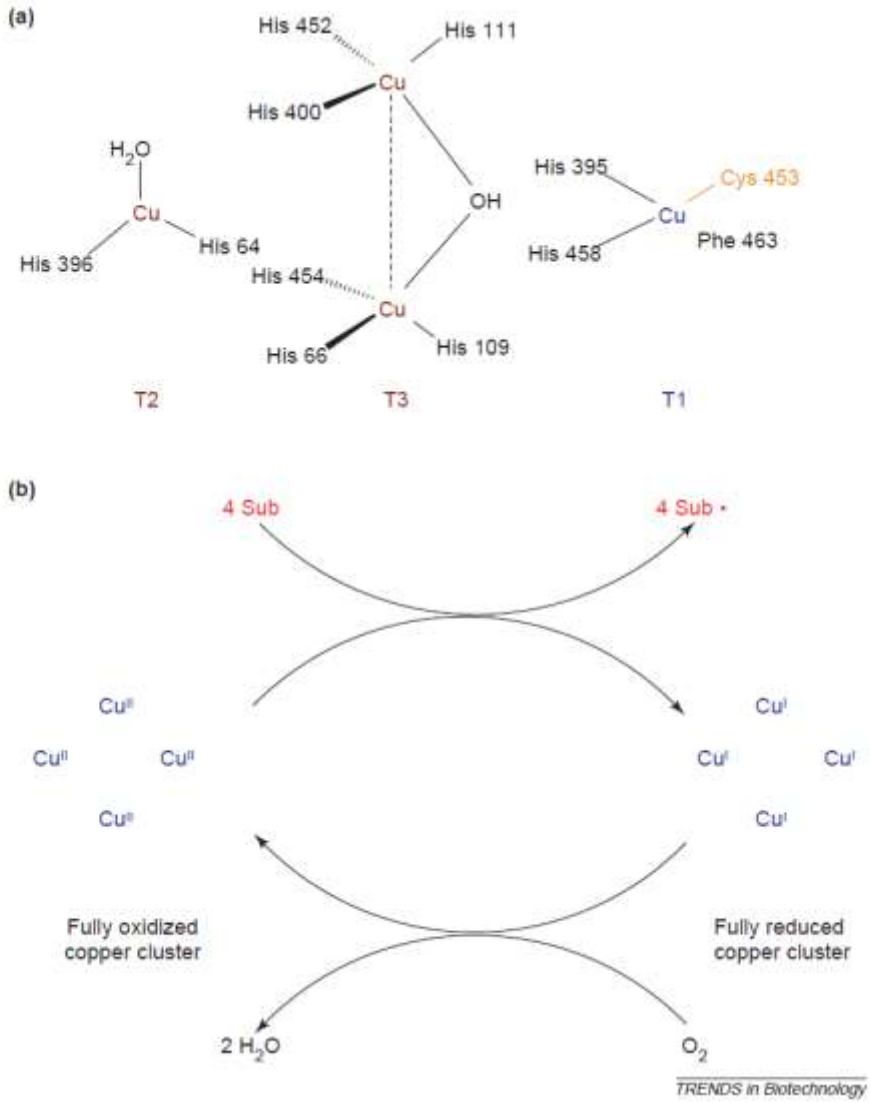


Figure 2Laccases active-site structure and catalytic cycle.(a) Catalytic cluster of the laccase from Trametesversicolor made of four copper atoms at Type 1 (T1), Type 2 (T2) and Type 3 (T3) sites. (b) Laccase catalytic cycle producing two molecules of water from the reduction of one molecule of oxygen(Riva, 2006).

As shown in the figure 2, Copper T1 is the primary electron acceptor, and at least one oftwo electrons required for reducing the site T3 comes from this particular site. Therefore, the reaction rate of Cu T1 is the limiting step of the total reaction rate. The copper T2 is necessary for the aerobic oxidationof reduced site T3 and additionally it allows site T3 to act like a two electron acceptor(Torres-pacheco et al., 2006). The role of Cu T2 site is maybe such that itparticipates in the transference of one of the electrons required to reduce site T3.In the catalytic mechanism of laccase enzyme, it has been suggested thatthe type 2 copper stabilizes an intermediary in the reduction of O_2 to H_2O;this indicates that type 2 copper is part of the O_2 reduction site in the enzyme(Torres-pacheco et al., 2006). It has been suggested that enzyme inhibition at an elevated pH value, isdue to the formation of a copper T2-OH- complex. This copper ion cannotallow the reduction of site T3 until the OH- has been dissociated or turned into a water molecule(Torres-pacheco et al., 2006). At low pH values, one of the water molecules formed in the reduced enzyme reoxidation seems to be united to site Cu T2.Fungic laccase studies show that water is an interchangeable binder for the T2 site(Torres-pacheco et al., 2006).

1.2.3 Functions of laccase

Among many diverse functions of laccases here are described some important ones. Laccase plays a role in the morphogenesis and differentiation of sporulating and resting structure in basidiomycetes as well as lignin biodegradation of wood in the white-rot fungi. Laccase is responsible for pigment formation in mycelia and fruiting bodies,improves cell to cell adhesion,assists in the formation of rhizomorphs and is also responsible for the formation of polyphenolicglue that binds hyphae together.Various plant pathogens also produce extracellular laccases that enable the fungus to overcome the immune response of the host.Laccase also facilitates the detoxification of the plant tissue via the oxidation of antifungal phenols or deactivation of phytoalexins.Recent studies on the physiological function oflaccase include those on plant cell biosynthesis,phytopathogenesis,wood material degradation and humification,insectsclerotization,bacterial melanization(Madhavi & Lele, 2009).

1.2.4. Applications of laccase

Owing to such above mentioned diverse functions of laccase enzymes, there are wide ranging applications in various fields. Figure 3 and table 1 on the next page summarizes the major applications of laccase enzymes in various industries.

Table 1: Specific applications of laccase enzymes in different fields. **(Information taken from (Kunamneni et al., 1998))**

Industries	Application of Laccase
Paper and Pulp Industry	To obtain brighter pulp and low lignin.
Textile Industry	To improve the whiteness in conventional bleaching of cotton.
Food Industry	As an O2-scavengers for better food packing.
Bioremediation	Laccases may be applied to degrade various substances such as undesirable contaminants, by products, or discarded materials.
Pharmaceutical Sector	Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents.
Nanobiotechnology	Laccase can be applied as biosensors or bio reporters.
Ethanol Production	To improve the production of fuel ethanol from renewable raw materials.
Wine clarification	Laccase immobilized was used successfully to remove phenols from white grape must.

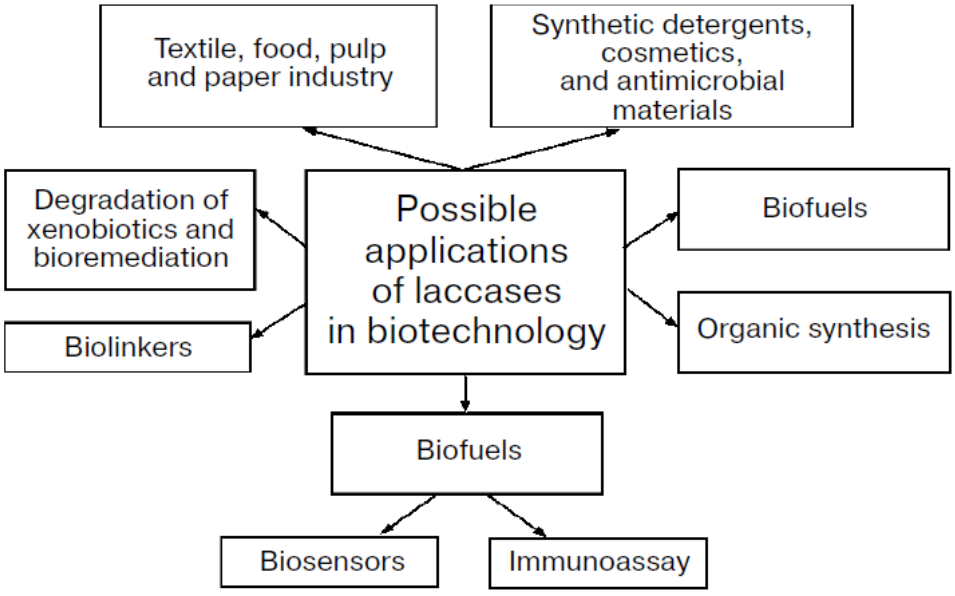


Figure 3:Chart showing wide-spread applications of laccases in biotechnology sector**(Morozova, Shumakovich, Gorbacheva, Shleev, & Yaropolov, 2007)**

1.3 Aromatic hydrocarbons (Bisphenol A)

Bisphenol A (**BPA**) is a man-made carbon-based synthetic compound with the chemical formula $(CH_3)_2C$ $(C_6H_4OH)_2$ belonging to the group of diphenylmethane derivatives and bisphenol.Bisphenol A [BPA, 2, 2-bis (4hydroxyphenyl) propane] has been used widely as an intermediate for the production of epoxyphenolic resins, polycarbonates, polyacrylates, plastics, food-drink packaging coating and other specialty chemicals. It has also been used as an inert ingredient in pesticides, antioxidant, flame retardant, and rubber chemicals. Due to its mass production and widespread use, the environmental release and contamination of BPA have been found through permitted discharges of industrial wastewater treatment systems, sewage sludge, and leachate from waste plastic in landfills. BPA has become one of the major toxic environmental pollutants of concern due to BPA's acute toxicitytowards algae, invertebrates, fish within the range of 0.04–0.4μM, as well as its mutagenic and estrogenic effects on humanswithin the range of 0.1–10μM (Saiyood, Vangnai, Thiravetyan, & Inthorn, 2010)

1.3.1 Background

BPA was first synthesized in 1891 by A.P.Dianin(Alessio, 2012).During the 1930s BPA was investigated for estrogenic activity along with other synthetic compounds.In the 1940's BPA was introduced in the plastics industry as a widely used primary raw material. BPA is used in the production of polycarbonate plastics, epoxyresins, lacquer coatings, and in dental composites and sealants ((Viñas & Watson, 2013)(Alessio, 2012)). Many of the plastics made from BPA are used in common consumer products such as toys, drinkingcontainers, eyeglass lenses, medical equipment and electronics, and thermographic and pressure-sensitive papers, such as those used for sales receipts.With the many uses of BPA in consumer products it has become one of the highest volumeworldwide.[(Alessio, 2012)] The production pathway of BPA is shown in figure 4 on the next page.

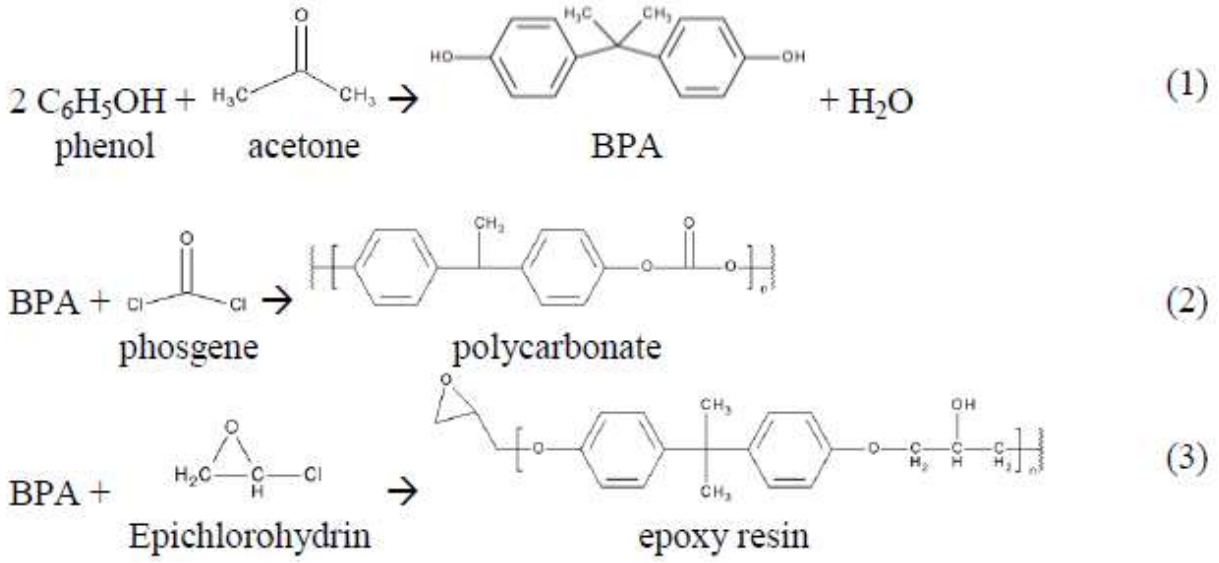


Figure 4.Equations showing production pathway of BisphenolA (BPA).

In this figure 4, Eqn. (1) shows the condensation of phenol and acetone to produce BPA. This isusually performed in the presence of an acid catalyst, hydrochloric acid, or with the useof cation exchange resins. The production of polycarbonate plastics accounts forapproximately 65% of the consumption of BPA, Eqn. (2). Epoxy resins are the second largest use for BPA accounting for about 30% of the consumption, Eqn. (3) (Alessio, 2012).

1.3.2 Occurrence ofBisphenol A in the environment

BPA can enter the natural environment's water sources, air, and soil during manufacturing and processing. The presence of BPA in the natural environment may have an effect on both wildlife and the ecology(Huang et al., 2012)(Hecker & Hollert, 2011) The widespread uses of BPA in consumer products provide another path for entering the environment. BPA has been found to leach into the environment from landfills in which polyvinylchloride products and thermo graphic paper is often a source of unreacted BPA. The decomposition of polycarbonate and epoxy resins may also be releasing BPA to the environment(Alessio, 2012)(Asakura, Matsuto, & Tanaka, 2004)(Cousins, Staples, Kleˇ, & Mackay, 2002) Aside from BPA entering the natural environment, humans have direct exposure to BPA through the use of polycarbonates and epoxy resins used in food storage containers.

1.3.3 Presence of Bisphenol A in food and consumer products

Food and beverage cans often have an epoxy resin coating to protect the food andprevent interaction with the metal. Epoxy resins are suitable as they are resistant to manysolvents and can bond to a metal substrate (Alessio, 2012). The production of such epoxy resins usesBPA diglycidyl ether (BADGE) which is produced from a reaction of BPA withepichlorohydrin, Eqn. (3). Residues of unreacted BPA present in BADGE can migrateinto food. Additionally, non-crossed linked residues of BADGE in the can coating canmigrate into the food which can be accelerated at elevated temperatures(Cao et al., 2011).

1.3.4 Human exposure to Bisphenol A through the environment

Several studies suggest that the intake of BPA is greater for young childrencompared to adults.(Alessio, 2012) Due to their rapid physical development, higher respiratory and metabolic rates, and their activities, children are more vulnerable to exposure and intake.

1.3.5 Bisphenol A - a potential health risk

Endocrine disrupting chemicals (EDCs) are naturally occurring compounds or man-made chemicals that act like hormones in the endocrine system and disrupt the physiologic function of endogenous hormones. Bisphenol A (BPA), known as one of EDCs since 1936 (Dodds, 1936), has aroused the public concerns. The potential adverse effects of BPA on human health and reproductive biology include breast and prostate cancer, sperm count reduction, abnormal penile/urethra development in males, early sexual maturation in females, neurobehavioral problems, prevalence of obesity, type 2 diabetes and immunodeficiency (Gong *et al*, 2009),(Chhaya & Gupte, 2013)

1.3.6 Solution to the problem

The removal of BPA from wastewater is important in the protection of the ecological environment and human health. A number of methods such as sonochemical degradation (Inoue et al., 2008), ozonation(Irmak, Erbatur, & Akgerman, 2005), chemical oxidation (YOSHIDA Mitsuru,ONO Hiroshi, 2001), enzymatic oxidation (Fukuda, Uchida, Takashima, & Uwajima, 2001), photooxidation(Zhou, Wu, Deng, & Xiang, 2004), solvent extraction (Fan et al., 2008), membrane filtration (Bing-zhi, Lin, & Nai-yun, 2008),phytoremediation (Nakajima, Oshima, Edmonds, & Morita, 2004), and biodegradation ((Kang & Kondo, 2002), have been employed to eliminate Bisphenol A from wastewater.

1.4 Reverse micelles

As Bisphenol A does not dissolve in aqueous media owing to its high hydrophobicity, organic solvents are required to dissolve it. This implies that the use of organic solvents inevitably allows the degradation reaction to proceed at a high concentration of environmental pollutants and in a homogenous system. However, native enzymes do not exhibit significant catalytic activities in organic media. Thus the reverse micellar system was introduced to enhance the activity of laccase in organic media. This technique enables us to perform biodegradation reaction in an organic solvent and degradation of hydrophobic pollutant is facilitated (Michizoe *et al*, 2001). Reverse micelles consist of three components: amphiphilic surfactant molecules, water and non-polar organic solvent. The polar heads of the surfactant molecules are directed towards the interior of a water containing sphere, whereas the aliphatic tails are oriented towards the non-polar organic phase (Figure 5). The water structure within the reverse micelles may resemble that of water adjacent to biological membranes (Boicelli & Conn, 1982).

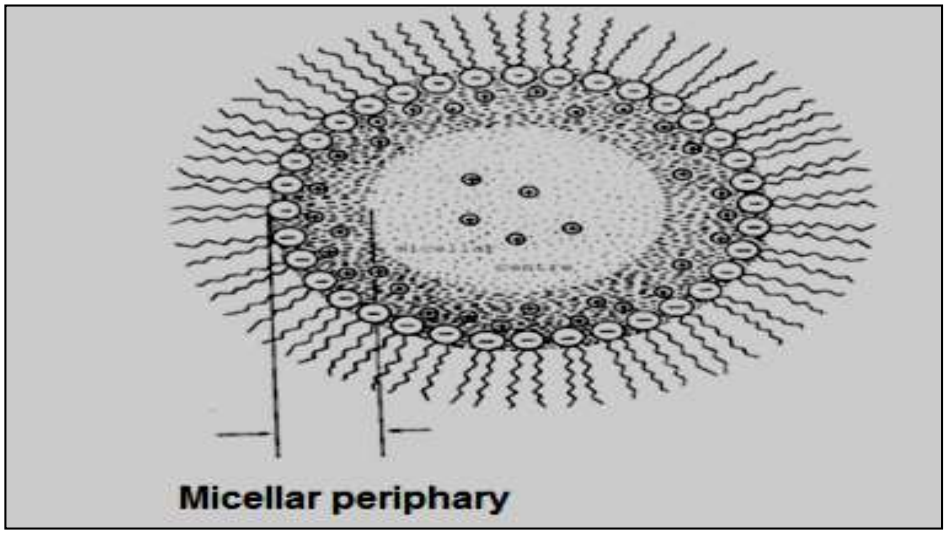


Figure 5.schematic presentation of an AOT reverse micelle showing the different regions of the reverse micellar interior, water is bound in the micellar periphery and water is free (= bulk water) in the micellarcentre (El Seoud, 1984)

There are three major factors which influence enzyme activity in reverse micellar systems.

1. The geometrical factor is the most important for enzyme regulation in micellar systems. The highest enzyme activity is attained under the condition when the size and shape of enzyme molecule is similar to that of micellar matrix (Levashov, 1992).
2. The hydration ratio W_0 = [H_20]/[Surfactant] influences the size of the inner cavity and hence the compressibility of biocatalyst in the reverse micelles. Increase in water leads to an increase in micellar size, increase in water and surfactant simultaneously leads to an increase in micelle concentration, while increase in surfactant concentration decreases micellar size (Figure 6).
3. Type of solvent used for the preparation of reverse micells influences both the water activity and compressibility of protein in the water pool. Since non polar solvents reduce the product inhibition, confined the biocatalyst to the aqueous phase and increases its thermostability in contrast to polar organic solvents are prefered for the preparation of reverse micelles (Ruckenstein, E. Karpe, 1990)(Valdez & Hue, 2001)

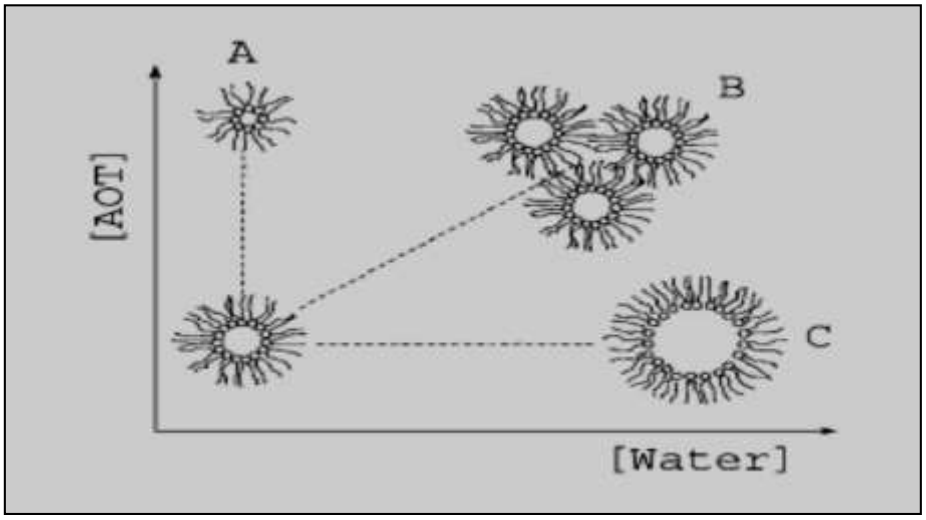


Figure 6.Effect of water content and surfactant on the size of AOT reverse micelles. (A) Increase in AOT concentration (B) Incrase in water and AOT concentration at a fixed ratio (C) Increase in water concentration. (Adapted from (Bru, Sanchez-ferrer, & Garcia-carmona, 1995))

Electrolytes and proteins solubilised in the water pools of reverse micelles are exchanged between reverse micelles. The possible mechanisms proposed for these solubilisate exchange processes are shown in Figure 7. Mechanism A involves the transient fusion of two reverse micelles to form a short-lived 'dimer droplet' the solubilised molecules can then redistribute by

diffusion during the finite lifetime of this dimer, the 'dimer droplet' splits into two reverse micelles. Mechanism B involves the diffusion of the exchanging molecules through the surfactant bilayer formed at the point of contact of non-fusing reverse micelles Mechanism C involves migration of the solubilisates through the oil/solvent phase (Luisi, P.L. Giomini, M. Pileni, M.P. Robinson, 1988)(Fletcherj, Howet, & Robinson, 1987)

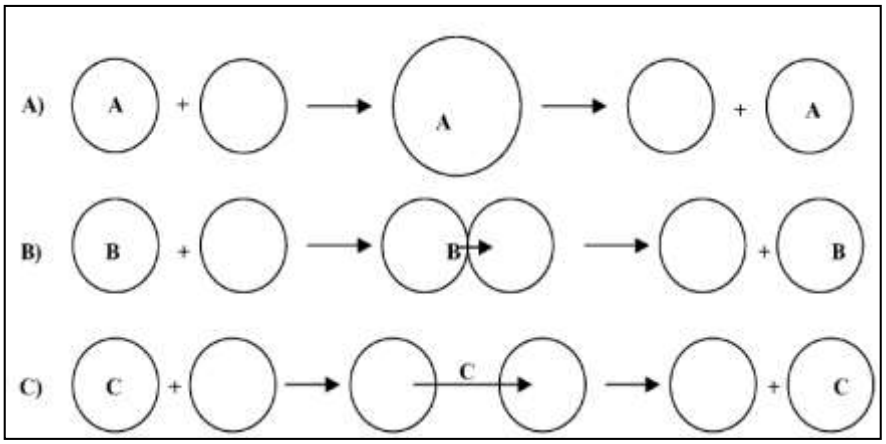


Figure 7: Schematic presentation of solubilisate exchange between reverse micelles. (Adapted from Adriana, 2003)

Reverse micelles possess some macroscopic properties that make them an ideal system for enzymological studies. Reverse micellar solution is thermodynamically stable and optically transparent, and large amount of host molecules can be accomodated without disturbing their macroscopic properties.These properties have permitted the normal use of the techniques of CD, polarization and time resolved fluorescence, phosphorescence, UV visible spectroscopy and dynamic light scattering spectroscopy (Bru et al., 1995)

Reverse micelles have various applications such as enzymatically catalysed organic phase synthesis (Okazaki, Michizoe, & Goto, 2002), extraction of proteins by phase transfer methods (Marcozzi, Correa, & Luis, 1991), preparation of monodisperse colloidal particles and medical applications (Wu et al., 2001).

In the present study, a reversed micellar (RM) system was applied to laccase to maintain its catalytic activity in organic media, and then the oxidative degradation of phenolic environmental pollutant, Bisphenol A in organic media catalyzed by laccase/RM system has been investigated. Optimal preparation and operation conditions to activate the enzyme in organic media were also examined. Furthermore, attempts were also made to measure the extent of BPA degradation.

1.5 Aims and objectives of the current study

Based on the above described basic information and background, the main aims of the present study are as below:

1. **Use of response surface methodology to optimize oxidation of aromatic hydrocarbons using laccase entrapped in reverse micelles by Central composite design**
2. **Estimation of Bisphenol A by Folin-Ciocalteau method**
3. **Degradation of Bisphenol A using free laccase enzyme and its estimation by Folin-Ciocalteau method**
4. **Degradation of Bisphenol A using laccase entrapped in reverse micelles (laccase/RM) system and its detection by HPLC**
5. **Statistical comparison between the respective activity of free laccase and laccase entrapped in reverse micelles by t-test**

2.Materials and methods

2.1 Chemicals

Laccase used in the current study was purchased from SIGMA Life Science which is extracted and purified from *Trametesversicolor* (12.9 u ⁄ mg) ; 2,2-bis-(4 hydroxyphenyl) propane (Bisphenol A) was procured from LobaChemie (Mumbai),M.W.: 228.29; Bis (2-ethylhexyl) sulfosuccinate sodium salt (AOT) was purchased from Fluka Chemical Ltd.(Finland); 2, 6 dimethoxyphenol (2,6 DMP) was purchased from SIGMA-M.w.154.16 ; Organic solvents used in this work were of analytical grade and were purchased from Merck Chemicals (Mumbai, India); All other chemicals used were of analytical grade and of highest purity available.

2.2 Analytical methods

2.2.1 Laccase activity determination

Laccase activity was determined by measuring the oxidation of 2, 6 DMP with laccase enzyme dissolved in sodium acetate buffer (pH-5.0).Increase in absorbance for 3 min was measured spectrophotometrically (Shimadzu Corp. 01846) at 469 nm (ε =27,500 $cm^{-1}M^{-1}$). One unit of enzyme was defined as amount of enzyme that oxidized 1µM of substrate per minute.

$$\textbf{Laccase activity (u/ml)} = \frac{\textbf{Difference in OD} \times \textbf{Volume in cuvette} \times 10^6}{\textbf{Aliquot of enzyme} \times \textbf{Extinctionco-efficient} \times \textbf{Time}}$$

2.2.2 Preparation and reaction of reverse micelle system with laccase

A reversed micellar solution containing laccase was prepared by direct injection of 40µl of purified laccase in aqueous solution pre-pared in 100 mM of sodium acetate buffer pH-5.0 in to 150 mM of AOT in isooctane. The mixture was vortexed till it became optically transparent. 40 µl of 2,6 dimethoxy phenol, 1.0 mM used as a model substrate was injected in to a micellar solution and vortexed for 3 min. Laccase activity in reverse micelles was determined by measuring the oxidation of 2,6 DMP. Increase in absorbance for 3 min was measured spectrophotometrically (Shimadzu Corp. 01846) at 469 nm (ε =27,500 $cm^{-1}M^{-1}$). One unit of enzyme was defined as amount of enzyme that oxidized 1µM of substrate per minute.

2.2.3 Response Surface Methodology (RSM) for optimization of the screened components

Response Surface Methodology was used to optimize the screened components for DMP oxidation using central composite design(CCD).The behavior of the system was demonstrated by the following quadratic equation.

$Y= \beta o+\Sigma\beta ixi+\Sigma\beta ijxixj +\Sigma\beta iixi$

Where Y is predicted response,β0 is a constant, βiis the linear co-efficient,βii is squared coefficient,βij is the cross product co-efficient,xi is the dimensionless coded value of (X_i).The above equation was solved by using the software Design-Expert(Version 9.0 ,State ease inc.,USA).A 2^5 factorial design with five replicates at the centre point with a total no of 21 trials were employed.

Table 1 Central composite design(Design Expert -9.0) for aromatic hydrocarbon oxidation by laccase enzyme entrapped in reverse micelles

		Factor 1	Factor 2	Factor 3	Factor 4
Std	Run	A: AOT conc.	B: pH	C: Laccase conc.	D: DMP conc.
		mM		mcg	mM
19	1	300.00	6.50	55.00	5.50
16	2	300.00	6.50	55.00	13.07
4	3	100.00	9.00	10.00	10.00
12	4	300.00	10.70	55.00	5.50
7	5	100.00	9.00	100.00	10.00
14	6	300.00	6.50	130.68	5.50
13	7	300.00	6.50	-20.68	5.50
21	8	300.00	6.50	55.00	5.50
1	9	500.00	9.00	100.00	1.00
18	10	300.00	6.50	55.00	5.50
8	11	100.00	4.00	10.00	1.00
5	12	500.00	4.00	10.00	10.00
6	13	100.00	4.00	100.00	1.00
20	14	300.00	6.50	55.00	5.50
17	15	300.00	6.50	55.00	5.50
11	16	300.00	2.30	55.00	5.50
9	17	-36.36	6.50	55.00	5.50
10	18	636.36	6.50	55.00	5.50

2	19	500.00	9.00	10.00	1.00
3	20	500.00	4.00	100.00	10.00
15	21	300.00	6.50	55.00	-2.07

2.2.4 Spectrophotometric determination of Bisphenol A was done by Folin-Ciocalteau method

From stock solution of BisphenolA (50µg/ml) different aliquots were used giving final concentration 5µg to 50µg; 3 ml of 15% Na_2CO_3solution and 0.5 ml of the reagent were added to each one. The preparation was heated for five minutes in a 50°C thermostatic water bath. After cooling at room temperature, absorbance was measured at 765 nm.

2.2.5 Degradation of Bisphenol A using freelaccase

Reaction mixture contained 5ml of Bisphenol A (stock of 120µg/ml) and 0.5 ml of laccase enzyme.At different time interval 0.5 ml of reaction mixture was taken, 2.5 ml of 15%Na_2CO_3 and 0.5 ml of the Folin-Ciocalteau reagent were added.The reaction mixture was incubated for five minutes in a 50ºCthermostatic water bath.After cooling at room temperature,absorbance was measured at 765 nm.

2.2.6 BPA degradation by laccase/RM system in organic media

The stock solution of Bisphenol A (5 mg/ml) was prepared by dissolving it in alcohol and further dissolved in isooctane. The oxidative reaction was initiated by injecting 40 µl of Bisphenol Afrom the stock solution corresponding to 200 ppm of Bisphenol A in to the reaction mixture containing 150 mM of AOT 2.5 mg/mlof laccase at 50ºCand pH-5.0 for different time intervals 120,180 and 240 min. The disappearance of Bisphenol A fromthe reaction mixture was monitored by high performance liquidchromatography.

2.2.7 Monitoring disappearance of Bisphenol A from the reaction mixture by HPLC:

After the reaction was started, aliquots of the reaction mixturewere periodically withdrawn and filtered through Millex®-LG filter with 0.2 µm pore size (Millipore, Billerica, MA, USA) for thequantitative analysis by HPLC using an intersile ODS 3–5 µm column(4 mm × 250 mm) with a linear gradient of 30% acetonitrile (Iso-cratic for 5 min) to 90% acetonitrile (10–20 min) in water containing0.1% phosphoric acid at a flow rate of 1 ml/min. Bisphenol A wasdetected at 276 nm using UV detector at 4.91min.

3. Results and Discussion

3.1 Laccase activity determination: Laccase activity for the purchased enzyme was found to be 6.17 U/mg which was slightly less than the claimed value of manufacturer(which was reported to be 12.9U/mg); this variation may be due to variation in lab instruments and chemicals used for analysis or loss of enzyme activity during transportation from manufacturer to the customer.

3.2 Reaction of reverse micelles with laccase enzyme: Laccase entrapped in reverse micelles has unit activity of 11.7 U/mg which was almost 90% more activity found for laccase in aqueous medium.

3.3 Optimization of variables such as enzyme and substrate concentration, pHand AOT concentration using Central Composite Design (CCD)

The Central Composite Design was used to determine the optimum conditions at which the maximum degradation was achieved.For this, a total of 21 experiments with four variables namely enzyme concentration, substrate concentration, pH and AOT concentration were used and results are shown in table 3.

Table 3.Central composite design for aromatic hydrocarbon oxidation by laccase enzyme entrapped in reverse micelles:

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:AOT (mM)	B:pH	C:Laccase unit / ml	D:DMP (mM)	Laccase Units/ml
19	1	300.00	6.50	55.00	5.50	0.6
16	2	300.00	6.50	55.00	13.07	10.9
4	3	100.00	9.00	10.00	10.00	32.7
12	4	300.00	10.70	55.00	5.50	20
7	5	100.00	9.00	100.00	10.00	33.3
14	6	300.00	6.50	130.68	5.50	30
13	7	300.00	6.50	-20.68	5.50	15.2
21	8	300.00	6.50	55.00	5.50	19.1
1	9	500.00	9.00	100.00	1.00	12.1
18	10	300.00	6.50	55.00	5.50	9.4
8	11	100.00	4.00	10.00	1.00	61.5
5	12	500.00	4.00	10.00	10.00	9.1
6	13	100.00	4.00	100.00	1.00	123.9
20	14	300.00	6.50	55.00	5.50	27.6
17	15	300.00	6.50	55.00	5.50	25.8

11	16	300.00	2.30	55.00	5.50	36.1
9	17	-36.36	6.50	55.00	5.50	76.7
10	18	636.36	6.50	55.00	5.50	24.2
2	19	500.00	9.00	10.00	1.00	34.5
3	20	500.00	4.00	100.00	10.00	85.2
15	21	300.00	6.50	55.00	-2.07	49.1

3.4 Analysis of variance (ANOVA)test onlaccase activity results determined by CCD

Table 4 represents the experimental design matrix for CCD along with the experimental results for aromatic hydrocarbon degradation. The experimental values for regression coefficient were obtained by quadratic polynomial equation, where only significant coefficients ($P < 0.05$) were considered. The smaller P-values indicate the higher significance of the corresponding coefficient. The insignificant coefficients were not omitted from the equations, since it was a hierarchical model. The predicted responses for Aromatic hydrocarbon degradation were obtained as follows:

Final equation in terms of actual factors:

Unit activity = + 97.74 - 0.35* AOT conc. - 2.74* pH + 1.07* Laccase conc. - 11.85* DMP conc. * AOT conc. * pH - 1.29 x 10^{-4}* AOT conc. * Laccase conc. + 0.018 * AOT conc. * DMP conc. - 0.18* pH * Laccase conc. - 0.16 * pH * DMP conc. + 0.023* Laccase conc. * DMP conc. + 3.53 x 10^{-4} * AOT conc.^{2} + 0.99* pH^2 + 2.12 x 10^{-3}* Laccase conc.^{2} + 0.34* DMP conc.^{2}

The model F-Value of 6.62 implies the model is significant. There is only a 1.43% chance that an F-Value this large could occur due to noise.Values of "Prob> F" less than 0.0500 indicate model terms are significant.In this case A,C, BC, A^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 2.18 implies the Lack of Fit is not significant relative to the pure error. There is a 22.94% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- the model to fit.

The statistical significances of the quadratic model for the experimental responses were evaluated by the analysis of variance (ANOVA). According to the ANOVA results (figure 8), the model was significant with an F-test of a very low probability value ($P > F) < 0.0001$.The goodness of fit for the model was expressed by the coefficient of determination R2 indicates and the values were found to be 0.9392.The values of R2 indicate that the experimental values in agreement with the predicted values and also suggested that the model is suitable and

predictable. The lack of fit F-values 2.18 for the Aromatic hydrocarbons degradation were not significant relative to pure error. These large values could occur due to noise. The purpose of statistical analysis is to determine which experimental factors generate signals, which are large in comparison to noise. The adequate precision value measures Signals to noise ratio and ratio greater than 4.0 is desirable. In the present study the value of this ratio was higher for production and suggested that the poly nominal quadratic model can be used to navigate the design space and further optimization.

Table 4:Analysis of variance (ANOVA) for laccase activity results determined by CCD

ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob> F**	
Model	16753.23	14	1196.66	6.62	0.0143	significant
A-AOT concen.	*1378.13*	*1*	*1378.13*	*7.62*	*0.0328*	
B-pH	*129.61*	*1*	*129.61*	*0.72*	*0.4297*	
C-Enzyme conc.	*1467.97*	*1*	*1467.97*	*8.12*	*0.0292*	
D-substrate conc.	*729.62*	*1*	*729.62*	*4.03*	*0.0913*	
AB	*19.00*	*1*	*19.00*	*0.11*	*0.7568*	
AC	*10.81*	*1*	*10.81*	*0.060*	*0.8150*	
AD	*859.05*	*1*	*859.05*	*4.75*	*0.0721*	
BC	*3212.01*	*1*	*3212.01*	*17.76*	*0.0056*	
BD	*10.69*	*1*	*10.69*	*0.059*	*0.8160*	
CD	*168.36*	*1*	*168.36*	*0.93*	*0.3719*	
A^2	*2987.18*	*1*	*2987.18*	*16.52*	*0.0066*	
B^2	*577.92*	*1*	*577.92*	*3.20*	*0.1241*	
C^2	*275.26*	*1*	*275.26*	*1.52*	*0.2634*	
D^2	*713.16*	*1*	*713.16*	*3.94*	*0.0943*	
Residual	1085.06	6	180.84			
Lack of Fit	*565.38*	*2*	*282.69*	*2.18*	*0.2294*	*not significant*
Pure Error	*519.68*	*4*	*129.92*			
Cor Total	17838.29	20				

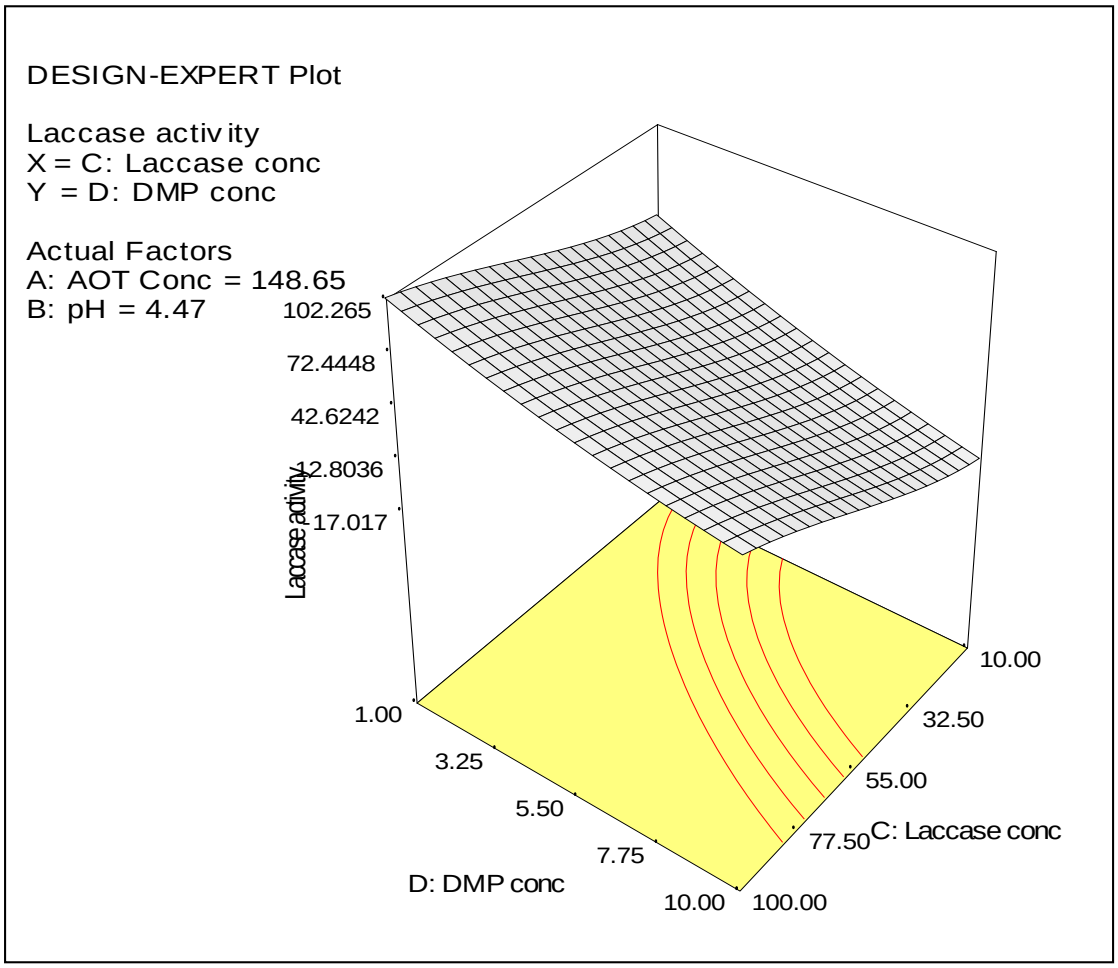


Figure 8: Design expert plot for DMP concentration v/s laccase concentration

Since, maximum laccase activity was observed at AOT concentration of 150mMand pH 4.5, theseoptimized conditions were maintained to further correlate the enzyme (laccase) and substrate (DMP) concentrations and laccase activity. Based on this, the above design expert plot at AOT concentration148.65mM and pH 4.47 shows thatlaccase activity increases with the increase in both enzyme (laccase) as well as substrate (DMP) concentrations.

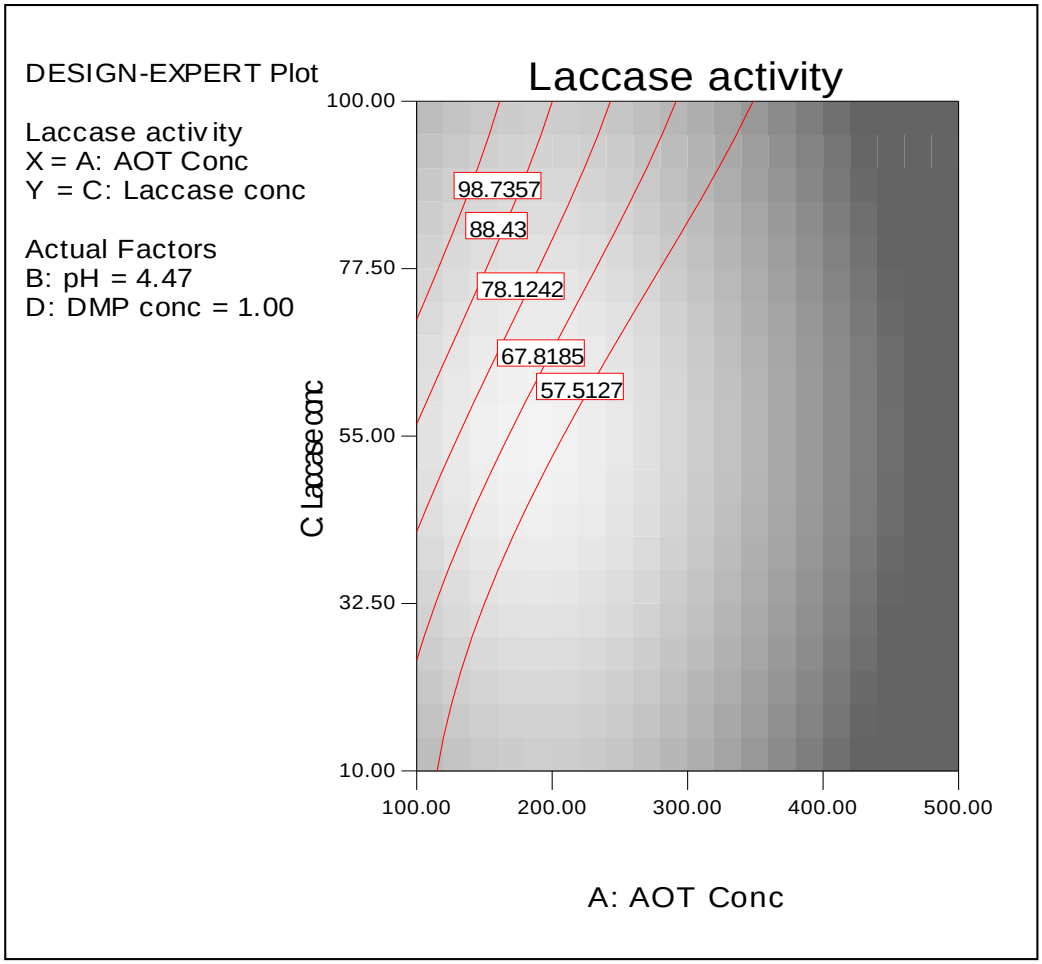


Figure 9:Design expert plot for laccase concentration vs. AOT concentration

Similarly, maximum laccase activity was observed at DMP concentration of 1mM and hence, this optimized condition was used for correlation between laccase and AOT concentration and laccase activity. Based on this, the above design expert plot at DMP concentration of 1mM and pH 4.47 shows that laccase activity increases with increase in laccaseconcentration and decrease in AOT concentration.

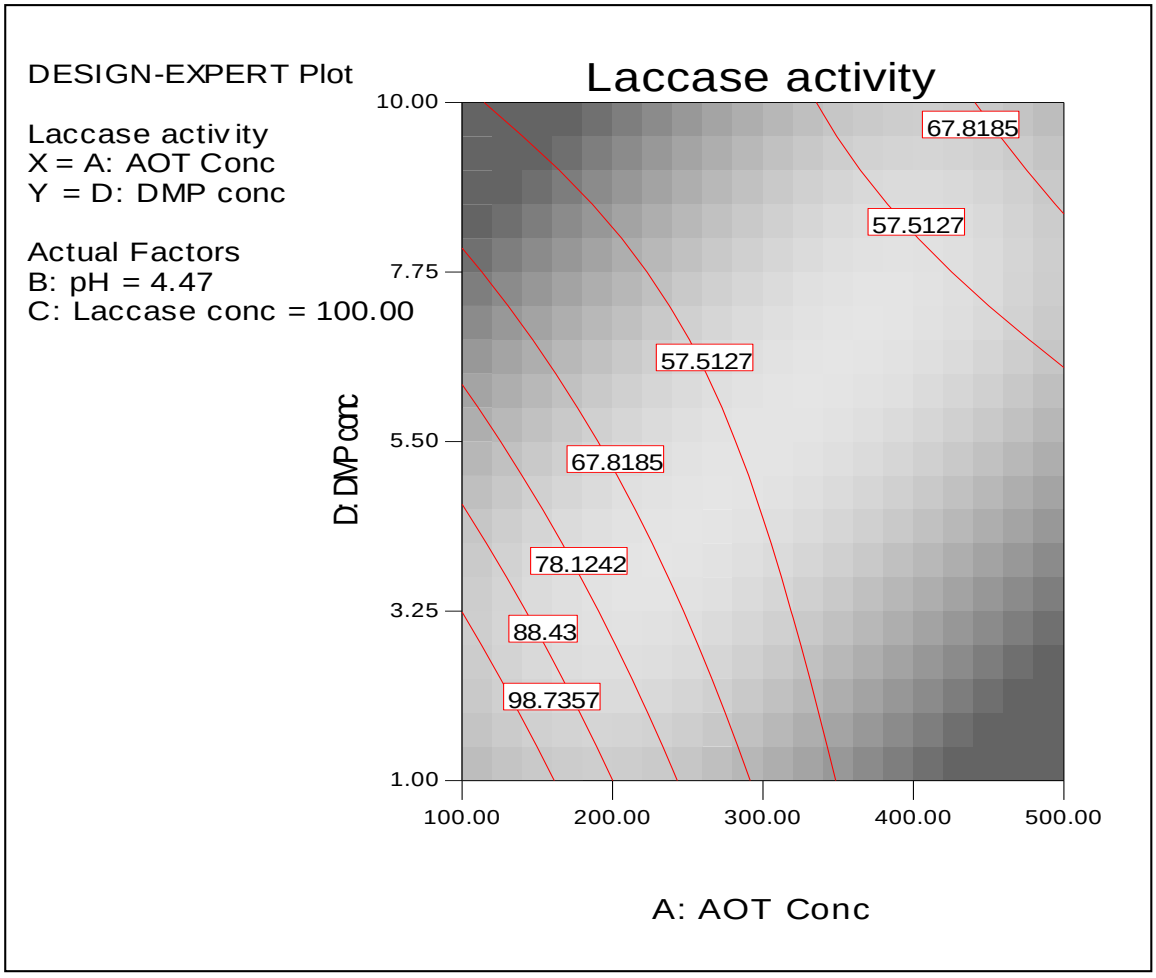


Figure 10:Design expert plot for DMP concentration Vs. AOT concentration

At the optimized pH value (4.7) and laccase concentration (100µg), the above design expert plot shows that laccase activity increases with the decreasing concentration ofsubstrate DMP and AOT. Inverse correlation between AOT concentration and laccase activity is in accordance with previous result (figure 9), but inverse correlation between DMP (substrate) concentration and laccase activity is contradictory from the above result (figure 8).

3.5Estimation of Bisphenol A by Folin-Ciocalteu method

3.5.1Reaction mechanism of Folin–Ciocalteu reagent

The Folin-Ciocalteau reagent, which is a mixture of tungstates and molybdates, works on the mechanism ofoxidation–reduction reaction. The method strongly relies on the reduction of the mixtureheteropolyphosphotungsates–molybdates by the phenolic compound which results in the formation of blue coloredchromogen. The phenolic compounds react with Folin-Ciocalteau reagent only under basic conditions adjusted bysodium carbonate solution. Under Basic conditions it has been observed that the phenolic compound undergoesdissociation to form a phenolate anion which reduces the Folin-Ciocalteau reagent i.e. the mixture of tungstates andmolybdates rendering a blue colored solution. The color intensity of the formed blue chromogen can be measuredby the absorbance readings using a spectrophotometer(Sinica, Jadhav, Kareparamban, Nikam, & Kadam, 2012).

(Lez, N, & Rudyk, 2003) reported that theestimation of phenolic compounds provided best results withFolin-Ciocalteau method as compared to other methods. Therefore, in present study Folin-Ciocalteau method was used and the results are shown in table 5.

Table 5:Estimation of Bisphenol A by Folin-Ciocalteau method

Bisphenol A Conc. (µg/ml)	OD at 765nm
0	0
5	0.152
10	0.249
15	0.379
20	0.494
25	0.614
30	0.738
35	0.885
40	0.948
45	1.056
50	1.109

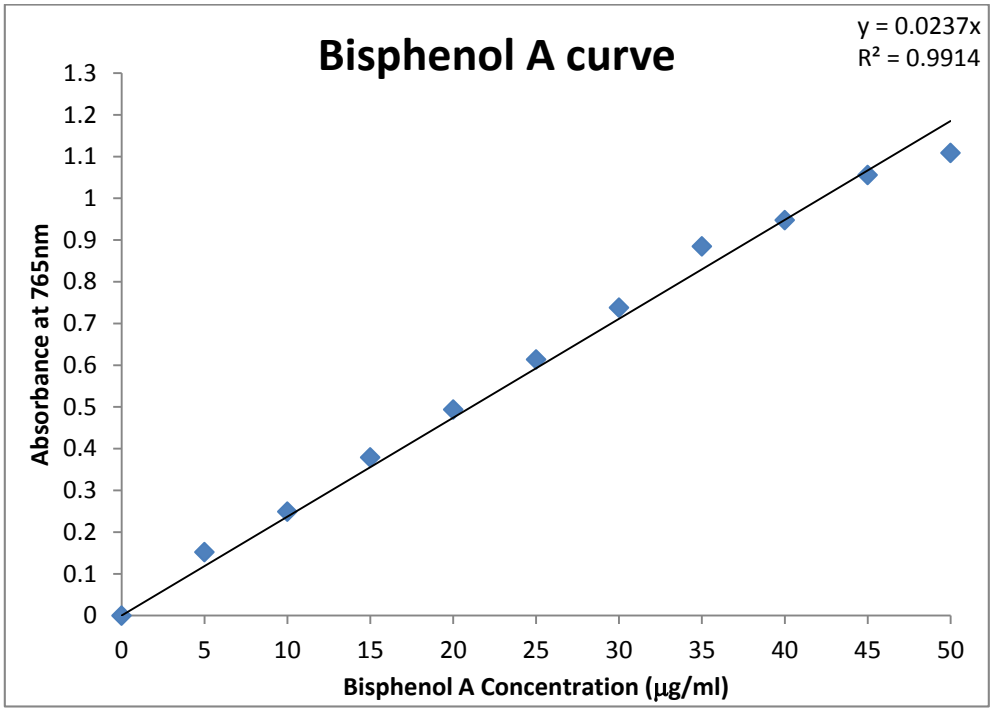


Figure 11:Estimation of Bisphenol A by Folin-Ciocalteau method

A linear relationship was obtained, when a graph was plotted for Bisphenol A concentration (µg/ml) v/s absorbance at 765nm,with a correlation coefficient value r^2 = 0.991 and the linear regression equation was y=0.023x.

3.6 Degradation of Bisphenol Aby free laccase

The ability of free laccase (i.e. laccase in aqueous solution) to degrade Bisphenol A was detected to be 47.3% degradation (shown in the Table 6) within 240 min. This finding is lower than that (92% degradation) reported by Okazaki et al. for the degradation of substrate 1-hydroxybenzotriazole (HBT).This group useda different substrate to determine free laccase activity from the present study and hence, accurate and conclusive comparison cannot be made. However, a possible explanation for low Bisphenol A degradation in this study may bedue to low laccase activity determined compared to the value claimed by manufacturer.

Table 6:Percentage (%) degradation of Bisphenol A using free laccase

Time (min)	OD at 765nm	Bisphenol A Conc. (µg/ml)	Percentage (%) degradation
0	1.172	102	0.0
15	1.021	89	12.9
30	0.949	83	19.0
45	0.846	74	27.8
120	0.718	62	38.7
240	0.618	54	47.3

The graph shown below depicts the percentage degradation of Bisphenol A proportionally increasing with time.

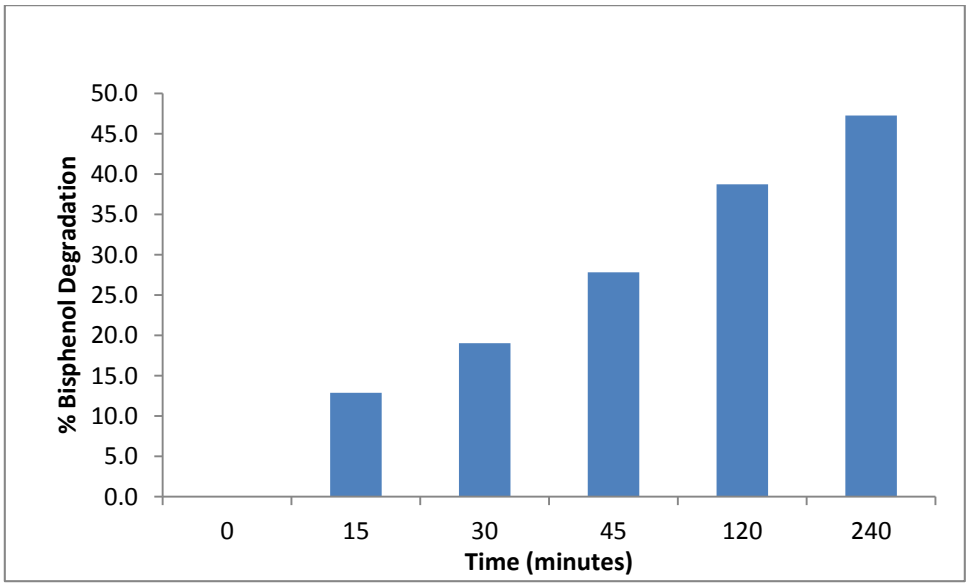


Figure 12: Percentage (%) degradation of Bisphenol A using free laccase with time course

3.7BisphenolA degradation by Laccase/RM system in organic solvent

The removal of BisphenolA by the laccase/RM System was pursued under the optimized conditions (Laccase concentration 100mM, pH 4.5 and incubation temperature 50ºC) For 0,120, 180 and 240 min. The time course of the removal of Bisphenol A from the reaction mixture is shown in Table 7. The disappearance of Bisphenol A was monitored using High Performance Liquid Chromatography (HPLC). It was found that after the incubation at 50ºC for 240 min 84.3% elimination of 200 ppm Bisphenol A was observed. Efficiency of Degradation of Bisphenol A was found to be less in aqueous media than organic media.

The results clearly indicated that BPA disappearance was due to the catalytic behavior of laccase hosted in the RM system. Laccase in the RM system maintains the active conformation because water layer and a surfactant shell surround the enzyme effectively which protects it from the inactivation caused by the bulk organic phase. By using reversed micellar system either laccase or Bisphenol A is soluble in the reaction medium, which may bring about better access of hydrophobic substrates to the enzyme active site(Chhaya & Gupte, 2013).

Many researchers have attempted oxidative degradation of Bisphenol A in aqueous and organic media with and without mediator system. Laccase mediator degradation of phenolic environmental pollutant Bisphenol A in organic media was attempted by (Michizoe, Goto, & Furusaki, 2001) who reported complete elimination of Bisphenol A after 3 h of incubation. While (Okazaki et al., 2002) reported 92% elimination in the presence of 1-hydroxybenzotriazole (HBT) as a mediator using laccase in reverse micelles. (Chhaya & Gupte, 2013) reported 91.43% elimination of Bisphenol A after 75 min in Laccase/RM system.

Highest Bisphenol A degradation ability by laccase in RM was found to be 84.3% with 240 min of incubation. This is low when compared with reports of Chhaya et.al. andMichzoe et al. who observed maximum Bisphenol A degradation 91.43% degradation in 75 minand 100% degradation in 3 h respectively which is higher than those obtained in the present study.

A possible explanation for low Bisphenol A degradation in our study could be due to low laccase activity and smaller size of laccase particles entrapped in RM system which can be correlated with the study done using central composite design (Table 2) for AOT optimization

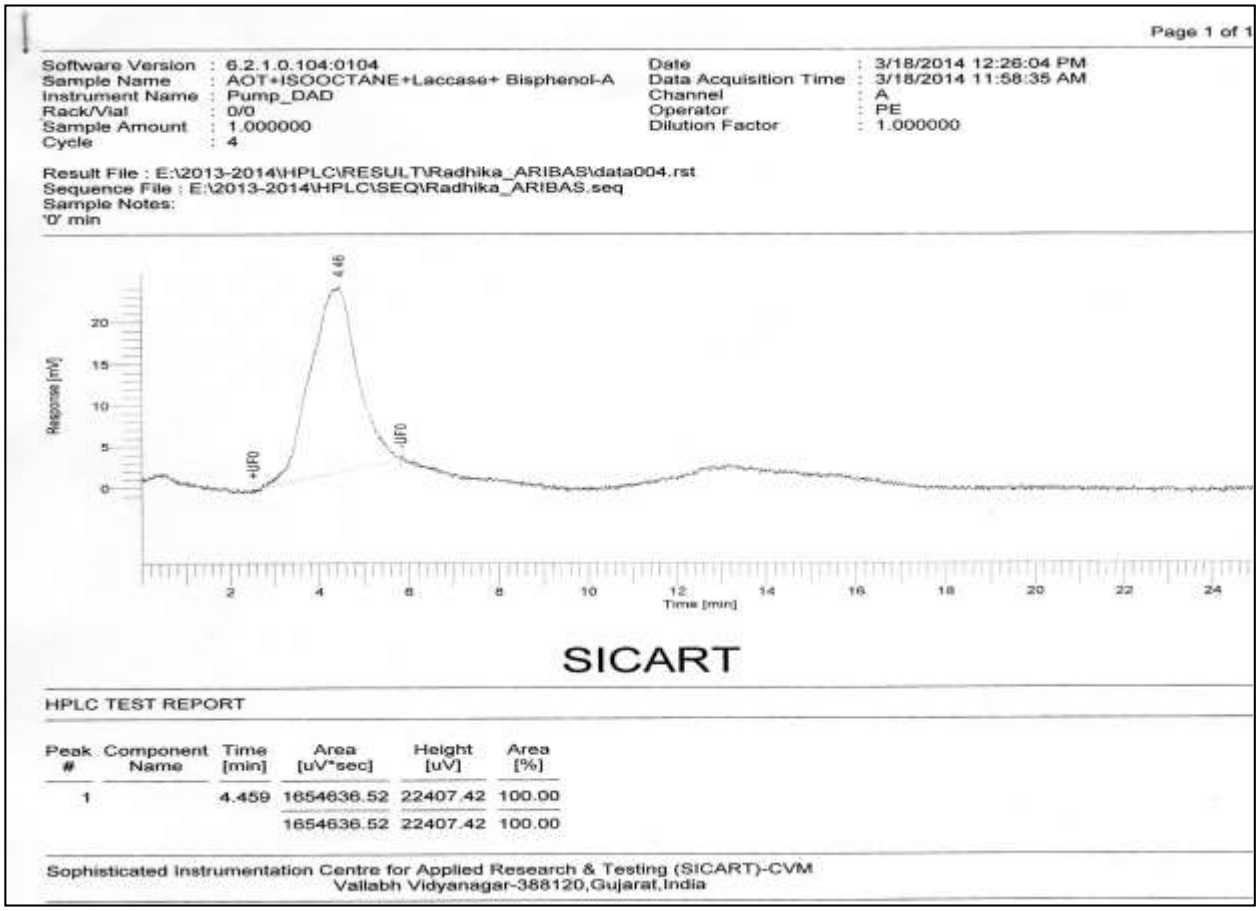
Table 7:BPA degradation by Laccase/RM system in organic solvent

Time (min)	BPA (µg/ml) Concentration	BPA (µg/ml) degradation	% BPA degradation
0	200	0	0
120	56	144	71.9
180	45	155	77.3
240	31	179	84.3

3.8 Monitoring disappearance of Bisphenol A from the Reaction Mixture by HPLC:

The HPLC analysis for degradation of Bisphenol A using enzyme in reverse micelles Figure no 13 shows the retention time of Bisphenol A is 4.46 min.The concentration of Bisphenol A at 0 min of incubation 200ppm which was degraded to the concentration of 31µg/ml .

FIGURE 13:HPLC chromatogram of BPA degradation by Laccase in RM at 0 min



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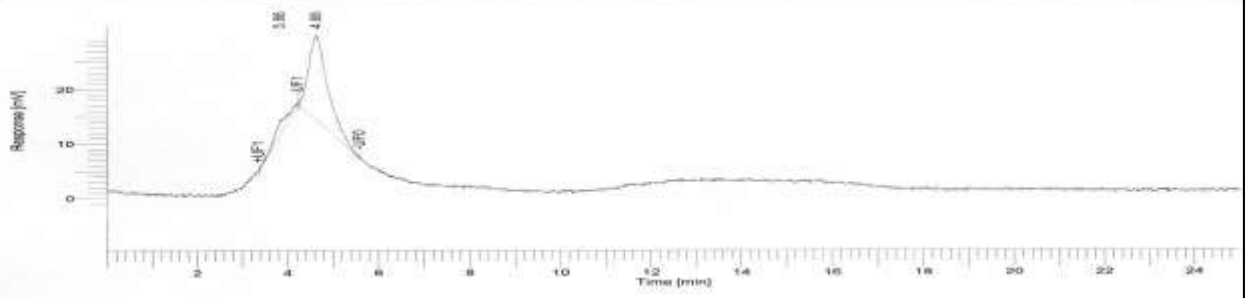
Sample Name	: AOT+ISOOCTANE+Laccase+ Bisphenol-A	Data Acquisition Time	: 3/18/2014 1:52:23 PM
Instrument Name	: Pump_DAD	Channel	: A
Rack/Vial	: 0/0	Operator	: PE
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 2		

Result File : E:\2013-2014\HPLC\RESULT\Radhika_ARIBAS\data007.rst
Sequence File : E:\2013-2014\HPLC\SEQ\Radhika_ARIBAS.seq
Sample Notes:
'4' hrs



SICART

HPLC TEST REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]
1		3.857	52594.40	2633.57	10.15
2		4.684	465677.23	15750.20	89.85
			518271.64	18383.76	100.00

Sophisticated Instrumentation Centre for Applied Research & Testing (SICART)-CVM
Vallabh Vidyanagar-388120,Gujarat,India

Software Version	: 6.2.1.0.104:0104	Date	: 3/18/2014 1:50:59 PM
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Instrument Name	: Pump_DAD	Channel	: A
Rack/Vial	: 0/0	Operator	: PE
Sample Amount	: 1.000000	Dilution Factor	: 1.000000
Cycle	: 1		

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Sample Notes:
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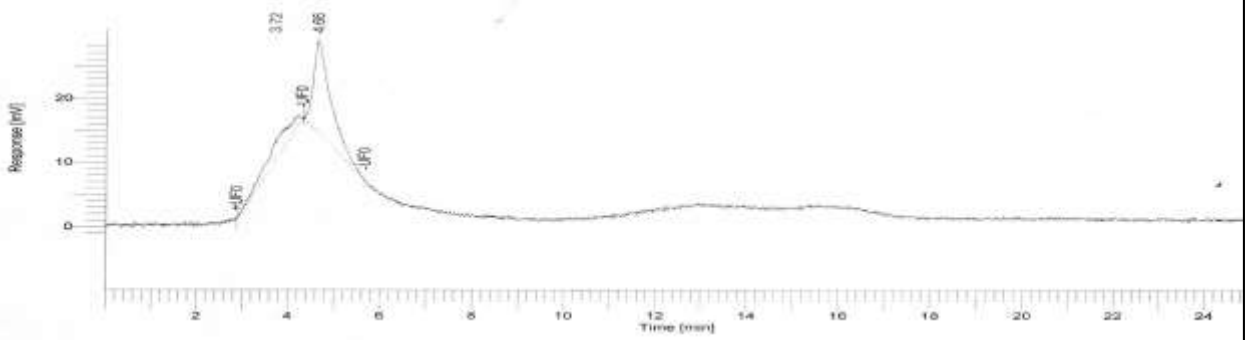

SICART

HPLC TEST REPORT

Peak #	Component Name	Time [min]	Area [uV*sec]	Height [uV]	Area [%]
1		3.718	135204.52	3190.94	26.51
2		4.664	374873.46	14223.16	73.49
			510077.89	17414.10	100.00

Sophisticated Instrumentation Centre for Applied Research & Testing (SICART)-CVM
Vallabh Vidyanagar-388120,Gujarat,India

Figure14:HPLC chromatogram of BPA degradation by laccase in RM at 180 min

3.9 Comparative study of percentage (%) BPA degradation in free laccase and laccase entrapped in reverse micelles

As compared in table 8, efficiency of degradation of Bisphenol A was found to be less in aqueous media than organic media.

Table 8:Comparative study of % BPA degradation in free laccase and laccase entrapped in reverse micelles

	% BPA degradation	
Time (min)	Free Laccase	Laccase in RM
0	0	0
120	38.42	71.9
240	47.49	84.3

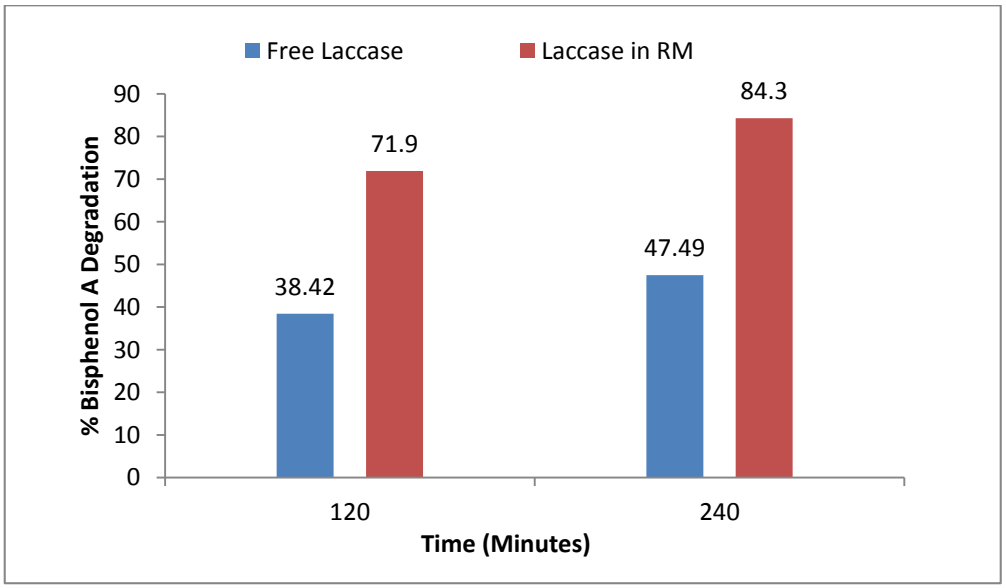

Figure 15:Graphical representation of comparative study of % BPA degradation by free laccase and laccase entrapped in reverse micelles

Table 9:Laccase activity in reverse micelles in unoptimized and optimized conditions

Laccase activity in Aqueous (u/ml)	Laccase activity in Reverse Micelles (u/ml)	
	Unoptimized	Optimized
7.2	12.1	22.1
5.2	11.3	19.1
6.1		

Table 10: Anova: Single Factor

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
Free Laccase	3	18.5	6.17	1.00
UnoptimizedLaccase in RM	2	23.4	11.70	0.32
optimized Laccase in RM	2	41.2	20.60	4.50

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	250.07	2	125.03	73.26	0.0007	6.94
Within Groups	6.83	4	1.71			
Total	256.89	6				

Table 11: t-Test for Laccase in aqueous and solvent media

t-Test: Two-Sample Assuming Unequal Variances

	Free Laccase	*Laccase in RM*
Mean	6.16667	11.7
Variance	1.00333	0.32
Observations	3	2

Hypothesized Mean Difference	0	
Df	3	
t Stat	-7.86915	
P(T<=t) two-tail	0.00428	
t Critical two-tail	3.18245	

ANOVA FOR SINGLE FACTOR

Our observed value F_{obs}. 73.26 is greater than F_{crit}6.94. Between the groups there is a greater variability than the within groups variability.These three groups namely Free Laccase,Laccase in reverse micelles for unoptimized condition,Laccase in reverse micelles for optimized conditions are not from the same populations having identical means andwhich means they are significantly different from one another.

t-Test:Two-Sample Assuming Unequal Variances

From the result of t-Test,laccase entrapped in reverse micelles found to be significantly different in their activity as comparedto the laccase in aqueous phase which is also evident from the p-value ($P < 0.05$).

CONCLUSION

Laccase from *Trametesversicolor* entrapped in the reversed micellar system effectively catalyzes the oxidation reaction of Bisphenol A in isooctane: AOT: Laccase ternary system in the absence of a mediator. Laccase in the RM system exhibited a high and stable enzymatic activity, and better catalytic efficiency than laccase in aqueous media. The activity of the laccase/RM system strongly influenced by the pH of water poolstemperature of the reaction mixture and also on the hydration degree of surfactant (W_o). The laccase in the RM system prepared at pH 4.5, with a protein concentration of 100µg in 150 mM AOT in isooctane exhibited the highest enzymatic activity at 50ºC.Under optimized conditions, the laccase/RM system was capable of degrading Bisphenol A with high efficiency. It could be concluded that the laccase hosted in reverse micelles was found to be an efficient system for the oxidative degradation of hydrophobic phenols, which might be due to better solubility of either enzyme or substrate in organic media conferring greater stability and catalytic efficiency.

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