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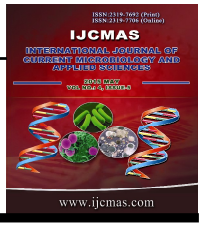

Original Research Article

Optimization of *Tremetes Versicolor* Laccase – Reverse Micelles System for the Removal of Phenolic Environmental Pollutant Bisphenol A

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Keywords

Laccase-RM system, Bisphenol A, High Performance Liquid Chromatography, Central Composite Design

A B S T R A C T

In the present study we have tried to optimize preparation of reverse micelle from *Tremetes versicolor* laccase using isooctane: AOT system for removal of Bisphenol A {2,2- bis-(4 hydroxyphenyl) propane} (BPA) by statistical optimization using central composite design method. On the basis of the various diagnostic plots and point prediction, actual concentration of different variables were found to be 150mM for AOT, 1 mM for Dimethoxy phenol and 100 µg/ml for Enzyme at pH 4.5 with 3.33 fold increase in the enzyme activity in reverse micelles as compare to aqueous system. The disappearance of Bisphenol A was monitored by High Performance Liquid Chromatography and after the incubation at 50°C for 240 minutes 84.3% elimination of 200 ppm Bisphenol A was observed which 36.81% higher than elimination by free laccase. Owing to insolubility of compounds like Bisphenol A in aqueous media, system like laccase-RM provides better alternative over aqueous enzyme system to degrade such pollutants.

Introduction

Laccase (benzenediol: oxygen: oxidoreductases, (EC. 1.10.3.2), phylogenitically old enzyme of oxidoreductase family and major secretome of many of the white rot fungi. They are capable of oxidizing diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols, polyphenols and even some inorganic compounds (Bakkiyaraj *et al.*, 2013). It has been widely used in various applications including paper manufacturing (Flory *et al.*, 2013), wood processing (Fackler *et al.*, 2008), environmental bioremediation (Ashrafi *et al.*, 2013), food industry (Dhillon *et al.*, 2012), as well as in textile engineering (Basto *et al.*, 2007).

Bisphenol A [2,2 bis (4 hydroxyphenyl) propane] is widely used in the variety of industrial and residential applications such as the synthesis of polymers including polycarbonates, epoxy resins, phenol resins, polyesters and polyacrylates. BPA has been

recognized as an Endocrine Disrupting Chemicals (EDC), thus it is necessary to assess its biodegradability or fate in the natural environment. 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 its acute toxicity towards algae, invertebrates, fish within the range of 0.04–0.4µM, as well as its mutagenic and estrogenic effects on humans within the range of 0.1–10µM (Saiyood *et al.,* 2010). In general, environmental pollutant such as BPA does not dissolve in aqueous media, owing to their high hydrophobicity, and hence non-aqueous catalysis can be employed to enhance biodegradability of phenolic environmental pollutant.

This implies that the use of organic solvents inevitably allows the degradation reaction to proceed at a high concentration of environmental pollutants in a homogenous system. However, native enzymes do not exhibit significant catalytic activities inorganic media. The reverse micellar system was then introduced to enhance the activity of laccase in organic media. This technique enables us to perform biodegradation reaction in an organic solvent facilitating degradation of hydrophobic pollutant (Michizoe *et al.,* 2001). In the present study, a reversed micellar (RM) system was optimized to entrap laccase enzyme by Central composite design followed by the oxidative removal of Bisphenol A by laccase-RM system.

Materials and Methods

Chemicals

Laccase used in the current study was purchased from SIGMA Life Science which is extracted and purified from *Trametes versicolor* (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.

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.

$$\text{Laccase activity (u/ml)} = \frac{\text{Difference in OD} \times \text{Volume in cuvette} \times 106}{\text{Aliquot of enzyme} \times \text{Extinction co-efficient} \times \text{Time}}$$

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.

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, β_0 is a constant, βi is 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).

Spectrophotometric determination of Bisphenol A 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 (Yardanova *et al.*, 2013).

Degradation of Bisphenol A using free laccase

Reaction mixture contained 5ml of Bisphenol A (stock of 120µg ⁄ ml) and 0.5 ml of laccase enzyme. At different time intervals 0.5 ml of reaction mixture was analyzed spectrophotometricaly for residual Bisphenol A as mentioned above.

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 A from the stock solution corresponding to 200 ppm of Bisphenol A in to the reaction mixture containing 150 mM of AOT 2.5 mg/ml of laccase at 50ºCand pH-5.0 for different time intervals 120,180 and 240 min.

The disappearance of Bisphenol A from the reaction mixture was monitored by high performance liquid chromatography.

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

was detected at 276 nm using UV detector at 4.91min.

Results and Discussion

Laccase activity determination:

Laccase activity for the purchased enzyme was found to be 6.17 U/mg. 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.

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

The Central Composite Design of total 21 experiments with four variables namely enzyme concentration, substrate concentration, pH and AOT concentration was used to determine the optimum conditions at which the maximum removal of Bisphenol A was achieved. The results of these experiments are shown in Table 2.

Analysis of variance (ANOVA) test on laccase activity results determined by CCD

Table 3 represents analysis of variance (ANOVA) for laccase activity results determined by CCD. 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 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 R^2 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.

On the basis of response surface plots maximum laccase activity was observed at AOT concentration of 150mM and pH 4.7, these optimized conditions were maintained to further correlate the enzyme (laccase) and substrate (Dimethoxyphenol) concentrations and laccase activity. Based on this, response surface plot shows that laccase activity increases with the increase in both enzyme (laccase) as well as substrate (DMP) concentrations. At the optimized pH value (4.7) and laccase concentration (100µg), the above response surface plots show that laccase activity increases with the decreasing concentration of substrate DMP and AOT (Figure 1a, 1b and1c).

From these response surface plots and subsequent experiments conducted it was observed that after optimization Laccase-RM system, under optimized conditions laccase activity was found to be 20.6 units/ml as compared to free laccase which was found to be 6.17 units/ml using dimethoxy phenol as a standard substrate.

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 (Figure 2).

Degradation of Bisphenol A by free laccase

The ability of free laccase (i.e. laccase in aqueous solution) to degrade Bisphenol A was detected to be 47.3% degradation (Figure 3) within 240 min. This finding is lower than that (92% degradation) reported by Okazaki et al (2002) for the degradation of substrate 1-hydroxybenzotriazole (HBT).This group used a different substrate to determine free laccase activity from the present study and hence, accurate and conclusive comparison cannot be made.

BisphenolA 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 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 (Figure 4). 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

Table.1 Central composite design (Design Expert -9.0) for Bisphenol A 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		µg	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

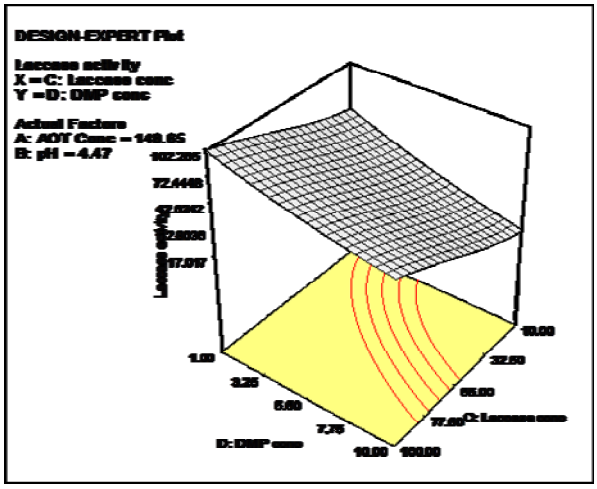
Table.2 Central composite design for preparing reverse micelles of laccase enzyme

		Factor 1	Factor 2	Factor 3	Factor 4	Response
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

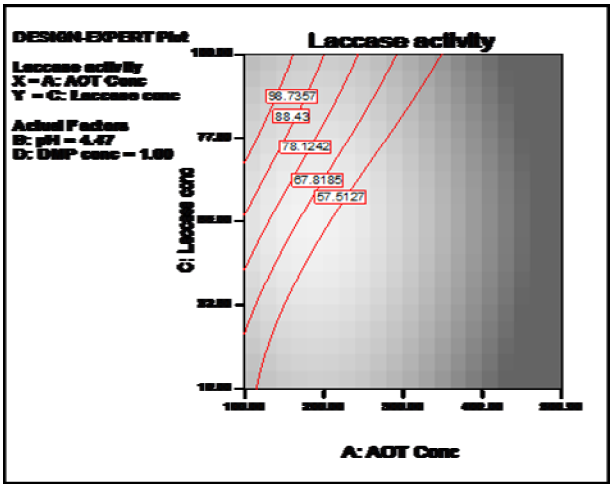
Table.3 Analysis of variance (ANOVA) for laccase activity results determined by CCD

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				

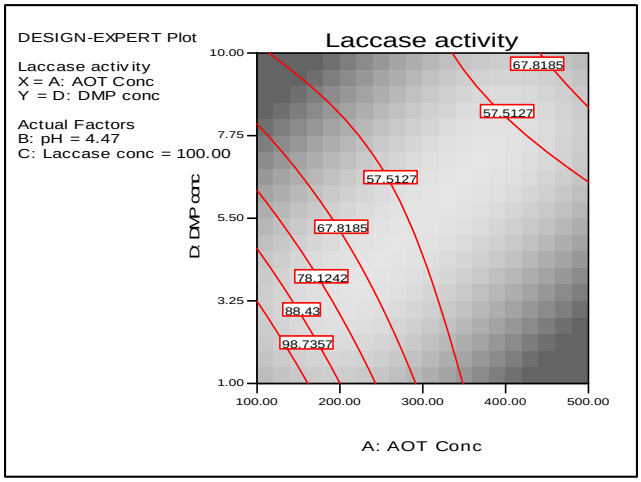
Figure.1 Response surface plot showing (a) relationship between laccase and DMP concentration, (b) Laccase and AOT concentration and (c) DMP concentration and AOT concentration



(a)



(b)



(c)

Figure.2 Standard Curve for Bisphenol A

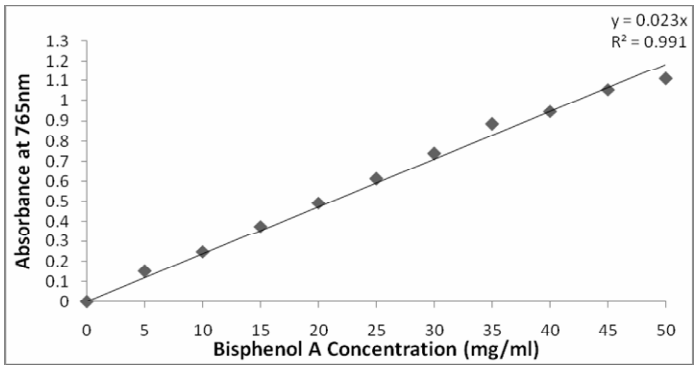


Figure.3 Percentage (%) degradation of Bisphenol A using free laccase with time course

BisphenolA degradation by Laccase/RM system in organic solvent

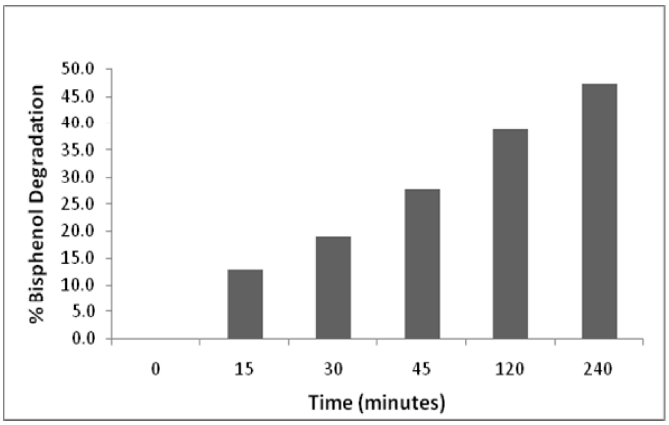
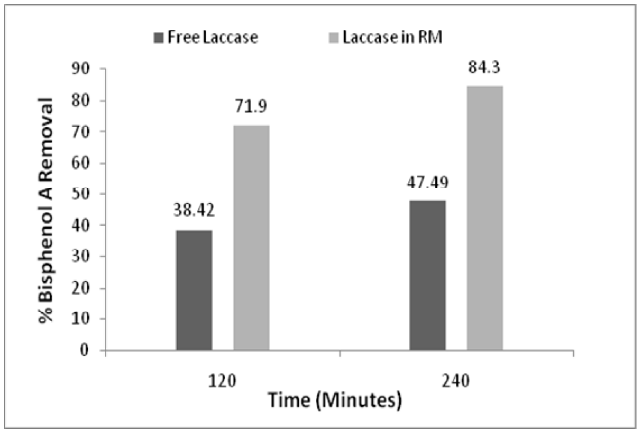


Figure.4 Comparison of Bisphenol A removal by free laccase and laccase hosted in reverse micelles



A in organic media was attempted by Michizoe *et al.* (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 and Gupte (2013) reported 91.43% elimination of Bisphenol A after 75 min in Laccase/RM system. Fouda *et al.* (2015) reported that *Aspergillus flavus* and *Aspergillus terreus* degraded 40.2% and 50% of 200 ppm of Bisphenol A on 6th day under optimized conditions as measured by HPLC. All these studies are in agreement with our results.

Laccase from *Trametes versicolor* 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 pool temperature 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.

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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