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Laccase mediated diclofenac transformation and cytotoxicity assessment on mouse fibroblast 3T3-L1 preadipocytes

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Diclofenac is recently considered as one of the most devastating environmental pollutants, because of its biomagnification in the food chain which leads to potential harmful effects on non-targeted organisms. This study describes the optimized laccase mediated diclofenac transformation using response surface methodology (RSM) and cytotoxicity testing on mouse fibroblast 3T3-L1 preadipocytes. Three factors (laccase, syringaldehyde, reaction time) were used to optimize the diclofenac transformation. The optimum level of laccase, syringaldehyde and reaction time was found to be 1.91 U mL^{-1}, 187 μg and 51 min for diclofenac transformation (20 mg L^{-1}). The cytotoxicity assessment on mouse fibroblast 3T3-L1 preadipocytes showed that a maximum of 67.9% cell death occurred at 72 h treatment with diclofenac (200 μg mL^{-1}), while the cells treated with laccase treated diclofenac (LTD) showed less toxicity on the cells. These findings can be addressed for the removal of diclofenac toxicity.

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1 Introduction

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID), used in human and veterinary medicine.[1] Global consumption of diclofenac is about 940 tons per annum.[2] It was reported that the residual existence of diclofenac in effluent from wastewater treatment plants was found to be between 10 and 2200 ng L^{-1}, which might cause a risk to the ecosystem.[3] The diclofenac metabolism and their adverse effects to living beings have been studied extensively.[1,4–6] In humans and animals, diclofenac is transferred as hydroxylated derivatives by cytochrome P450 oxidation.[7,8] These diclofenac derivatives are excreted with urine and faeces either in the form of conjugated (glucuronides) or as nonconjugated dienes and enter into the wastewater stream, thereby affecting aquatic life.[9,10] Stepanova *et al.*[11] observed that sub-chronic exposure of carp to diclofenac had considerable toxic effects on the oxidative stress. Moreover, the biomagnification of diclofenac in the food chain leads to unpredictable adverse effects on non-target ecological species.[12] Therefore, the detoxification of diclofenac is considered to be essential before it is biomagnified into the food chain. In order to provide a remedy for this issue, environmental engineers have been trying to develop various innovative techniques for the remediation process.

Nowadays, laccase based treatment processes have received much attention in the bioremediation field.[13–16] Laccase (benzenediol oxygen oxidoreductases; EC 1.10.3.2) is a multi-copper oxidase enzyme that catalyzes single electron oxidation of four phenolic molecules with concomitant reduction of oxygen to water. The active site of the laccase enzyme includes four copper ions in which type-I (T1, one Cu atom) acts as an electron acceptor from the substrate, type-II (T2, one Cu atom) copper forms a tri-nuclear copper-cluster with type-III copper (T3, two Cu atoms), where the reduction of oxygen takes place.[17,18] In addition, laccase enzymes do not require co-substrates to facilitate the functions and use the readily available oxygen as an electron acceptor. The free radicals formed by the action of laccase bypass the steps involved in the formation of carcinogenic amines.[19] Laccase production from WRF can be easily enhanced by the addition of inducers and their substrate range can be extended to non-phenolic compounds in the presence of small molecular weight redox mediators.[20,21]

In the laccase–mediator oxidation system, laccase oxidizes the mediator, which acts as an electron transferring compound and the mediator transfers the electron from the substrate of interest. Synthetic redox mediators are commonly used in laccase mediated pollutant transformation.[22,23] However, the use of these synthetic mediators is limited by their toxic nature and high cost of exploitation. Interestingly, the free radicals

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generated from lignin-derived phenols with consequent degradation of the non-phenolic aromatic structures might overcome such limitations.[23] The use of low cost and naturally occurring eco-friendly lignin-derived phenolic compounds could facilitate the application of laccase mediated systems for bioremediation processes.

Recent studies have demonstrated that diclofenac transformation is catalyzed by laccase and reported for different rates of transformation.[24–26] These reports clearly indicate that the rate of transformation mainly depends on several factors such as diclofenac, laccase, redox mediator concentrations, pH, temperature, and reaction time. The conventional method used for the optimization process is time consuming and incomplete. Now, the application of central composite design of response surface methodology (CCD-RSM) in the effluent treatment process can result in improved degradation rate, as well as reduced process variability, time, and overall costs. In addition, the optimum operational conditions and interaction effects between the factors can be evaluated.[27] The previous study shows that efficient diclofenac transformation is catalyzed by laccase in the presence of the natural mediator syringaldehyde.[35] Even though, over certain concentrations of syringaldehyde caused a slight enzyme denaturation; however, there is no optimization study on diclofenac transformation catalyzed by laccase in the presence of syringaldehyde.

Therefore in this study, the most important parameters involved in the diclofenac transformation such as laccase and syringaldehyde concentration, also the reaction time was optimized under standard assay conditions using response surface methodology (RSM). In order to check the efficacy of the model, a validation experiment was performed with optimized results. In addition, to understand the cytotoxicity of diclofenac and laccase treated diclofenac (LTD), the experiment was performed in mouse fibroblast 3T3-L1 preadipocytes. This could be perhaps the first report for the optimization of laccase mediated diclofenac transformation and the assessment of its cytotoxicity in mouse fibroblast 3T3-L1 preadipocytes.

2 Materials and methods

2.1 Chemicals

2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), diclofenac sodium, Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin–streptomycin, syringaldehyde, and 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma-Aldrich. Mouse fibroblast 3T3-L1 preadipocytes were obtained from ATCC, USA. All the chemicals and solvents used in the experiment were of analytical grade. The molecular structure of diclofenac and syringaldehyde are shown in Fig. 1.

2.2 Laccase source and assay

In this study, *Pleurotus florida* NCIM 1243 laccase was used as the biocatalyst and was prepared as described by Palvannan and Sathishkumar.[20] Laccase activity was determined as per the method of Wolfenden and Wilson[28] using ABTS as the substrate.

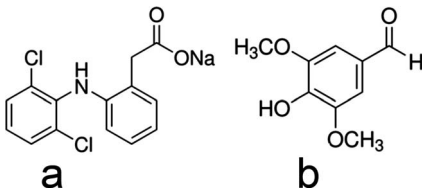

Fig. 1 Molecular structure of (a) diclofenac and (b) syringaldehyde.

The assay mixture consisted of ABTS (1 mM), sodium acetate buffer (100 mM; pH 5.5), and laccase enzyme. Assay mixtures were placed in a 96-well polypropylene microplate, and incubated at 30 °C in an orbital shaker. The increase in absorbance of the assay mixture was measured at 420 nm ($\varepsilon_{420} = 36.0\ mM^{-1}\ cm^{-1}$) in a microplate reader (Biotek, USA) for 2 min intervals, until the end of the reaction. The activity was expressed as enzymatic units per volume and one unit (1 U) of activity was defined as the amount of laccase that oxidized 1 μmol of ABTS per minute.

2.3 Response surface methodology

The diclofenac transformation was optimized using RSM. Three independent factors of laccase (A), syringaldehyde (B), and reaction time (C) were used at five levels (−1.68, −1, 0, +1, +1.68), a second order central composite experimental design was followed. The range and levels of these parameters are shown in Table 1. The experimental range for the independent factors in the coded (x_i) and actual (X_i) levels are given in Table 2. A set of 20 runs were designed in order to optimize the independent factors and six replicates at the center point were performed to provide information on the variation of responses about the average and residual variance. For statistical calculations, the factor X_i was coded as x_i according to eqn (1):

$$x_i = \frac{(X_i - X_0)}{\delta X} \quad (1)$$

where x_i is the coded value, X_i is the actual value, X_0 is the center point value and δX is the step change between the factor levels. The diclofenac transformation (%) was considered as the response (dependent factors). In order to show the relationship between dependent and independent factors, the response was fitted with a second order polynomial model. The system behavior of the model was explained using eqn (2):

$$\hat{Y} = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (2)$$

where $\hat{Y}$ is the predicted response (percentage of diclofenac transformation), β_0 is a constant, β_i is the linear coefficient term, β_{ii} is the quadratic coefficient term, and β_{ij} is the interaction coefficient.

2.3.1 Software and data analysis. The Design-Expert 8.0.7.1 (Stat-Ease, Inc., Minneapolis, USA) was used to analyze the RSM response in order to determine the analysis of variance (ANOVA), three dimensional (3D) response surface plot, interactive effects of the factors, and their corresponding optimum levels.

Table 1 Factors investigated in the experimental design

Factors	Code	Range of variables and level					Step change value (ΔZ_i)
		−2	−1	0	+1	+2	
Laccase (U mL^{-1})	A	0.66	1	1.5	2	2.34	0.5
Syringaldehyde (μM)	B	23.87	75	150	225	276.13	75
Reaction time (min)	C	6.36	20	40	60	73.64	20

2.4 Diclofenac transformation

Diclofenac sodium (1000 mg L^{-1}) stock solution was prepared in 50% methanol (v/v), and further appropriate dilutions were made for the transformation experiments. The transformation experiments were carried out in a test tube containing 10 mL of reaction mixture with laccase and syringaldehyde (as per CCD design mentioned in Table 2) at a fixed concentration of diclofenac (20 mg L^{-1}) in sodium acetate buffer (100 mM; pH 4.5) at 30 °C, 50 rpm under dark conditions. As per CCD design, the reaction time of each run was maintained and the reaction was stopped by acidification with H_2SO_4. All the experiments were performed in duplicate and the reported values are the average of at least three experiments.

2.4.1 Diclofenac transformation analysis in HPLC. Diclofenac transformation was assessed using HPLC. An HP 1200 (Agilent, USA) liquid chromatography equipped with 2.1 μm × 150 mm Eclipse C18 capillary column, particle size 3.5 μm (Agilent, USA) was used to determine the diclofenac transformation. The percentage of diclofenac transformation is determined by the following eqn (3):

$$D = \frac{(A_i - A_f)}{A_i} \times 100 \quad (3)$$

where D is the percentage of diclofenac transformation, A_i is the total area under the plot, and A_f is the final area under the plot.

2.5 Cytotoxicity study on cell culture and MTT assay

The mouse fibroblast 3T3-L1 preadipocytes were cultured in a 75 cm^2 flask with RPMI-1640 supplemented with 10% FBS, 10 units of penicillin and 10 μg mL^{-1} streptomycin was maintained in an incubator at 5% CO_2 and 37 °C. The cells were harvested and seeded in a 96-well cell culture plate at a density of 5×10^4 cells per well. A concentration of 200 μg mL^{-1} for diclofenac and LTD were added to the cells and further incubated for 24, 48, and 72 h. After treatment, 10 μL MTT (5 mg mL^{-1}) was added to each well and incubated for 4 h. The supernatant was discarded and 100 μL of isopropanol was added to each of the wells and the plate was read using a plate reader at 598 nm. The cell viability was calculated by the following eqn (4):

Table 2 Full factorial CCD design matrix[a]

Std	Coded variables			Response – diclofenac degradation (%)		
	A – laccase (U mL^{-1})	B – syringaldehyde (μM)	C – reaction time (h)	Actual data (Y_j)	Predicted data ($\hat{Y}_j$)	Residual value ($Y_j - \hat{Y}_j$)
1	−1[2.5]	−1[200]	−1[60]	39 ± 3.2	32	7
2	+1[7.5]	−1[200]	−1[60]	37 ± 2.6	38	−1
3	−1[2.5]	+1[400]	−1[60]	32 ± 1.8	30	2
4	+1[7.5]	+1[400]	−1[60]	66 ± 4.0	64	2
5	−1[2.5]	−1[200]	+1[120]	66 ± 2.7	67	−1
6	+1[7.5]	−1[200]	+1[120]	83 ± 4.1	84	−1
7	−1[2.5]	+1[400]	+1[120]	61 ± 2.5	59	2
8	+1[7.5]	+1[400]	+1[120]	100 ± 0	106	−6
9	−1.68[0.5]	0[300]	0[90]	31 ± 3.7	36	−5
10	+1.68[10]	0[300]	0[90]	84 ± 4.1	79	5
11	0[5]	−1.68[100]	0[90]	56 ± 3.3	58	−2
12	0[5]	+1.68[500]	0[90]	75 ± 3.8	73	2
13	0[5]	0[300]	−1.68[30]	24 ± 3.4	30	−6
14	0[5]	0[300]	+1.68[150]	97 ± 2.2	92	5
15	0[5]	0[300]	0[90]	80 ± 2.1	79	1
16	0[5]	0[300]	0[90]	80 ± 2.1	79	1
17	0[5]	0[300]	0[90]	80 ± 2.1	79	1
18	0[5]	0[300]	0[90]	80 ± 2.1	79	1
19	0[5]	0[300]	0[90]	80 ± 2.1	79	1
20	0[5]	0[300]	0[90]	80 ± 2.1	79	1

[a] Data are given as mean ± S.D., $n = 3$.

$$V(\%) = \frac{A_t}{A_c} \times 100 \quad (4)$$

where V is cell viability (%), A_t is absorption of the test, and A_c is absorption of the control and the results are provided as means ± SD values. The level of significance between samples was calculated using SPSS statistics 20 software.

3 Results and discussion

3.1 Diclofenac transformation optimization using RSM

The physico-chemical properties such as pH, temperature, substrate, enzyme and redox-mediator concentrations, and reaction time plays a vital role in every biochemical transformation. Generally, the redox potential and optimum pH are dependent not only on the laccase properties but also on the properties of the target substrate.[26] Therefore, in the first series of experiments, the optimum pH for diclofenac transformation and stability nature of laccase was evaluated in order to elucidate the most favorable reaction conditions for subsequent removal experiments. Fig. 2a shows the optimum pH for the laccase catalyzed diclofenac transformation. The maximum percentage of diclofenac transformation was achieved from 4 to 5.5 with an optimum at pH 4.5. Similarly, Lloret *et al.*[24] obtained the maximum diclofenac degradation at pH 4.5 with commercial laccase from *Myceliophthora thermophila*. The stability results showed that the half-life of initial activity retained up to 5 h at pH 4.5 (Fig. 2b). In the case of high acidic and near neutral pH conditions the diclofenac transformation decreased. This drop of reactivity at alkaline pH is probably due to the combination of hydroxide ion inhibition and it's binding with the T2/T3 site of laccase and moreover the decrease in the reduction potential of oxygen would certainly lead to a reduction of reaction rates.[29] The decrement of reactivity at high acid pH is mainly due to the presence of ionizable side-chains in the tertiary structure of the enzyme.[24] Nevertheless, the enzyme can be denatured at extreme pH because of the conformational changes.[30] Consequently, the diclofenac transformation experiment was carried out at 4.5 pH.

Previously, it was found that syringaldehyde had an immediate effect on laccase mediated diclofenac transformation.[31] In the laccase–syringaldehyde oxidation system; laccase oxidizes the syringaldehyde, which acts as an electron transferring compound and the syringaldehyde transfers the electron from the substrate of interest (diclofenac). However, syringaldehyde might give a negative effect over certain concentrations by inhibiting the laccase activity.[24] Therefore, optimization of laccase mediated diclofenac transformation is considered important for scaling-up. In this study, laccase and syringaldehyde concentrations and their reaction time were selected as factors with fixed level of diclofenac concentration for diclofenac transformation optimization. Table 2 shows the design matrix together with both factor level and obtained experimental response value. Diclofenac transformation percentage was found in the range between 24 and 100%, respectively. The RSM experimental response was analyzed with Design-Expert software so as to obtain an empirical model for the best response. The experimental and predicted diclofenac transformation (%) is shown in Table 2. Actual values correspond to the measured response data for a particular run and the predicted values were evaluated from the model. A quadratic model was used to explain the mathematical relationship between the independent factors and dependent response.

The significance and adequacy of the models were justified with variance (ANOVA) and the results were analyzed using ANOVA as shown in Table 3. These results indicate that the model is highly significant, as it is evident from the Fisher's F-test with a very low probability value of $[(P \text{ model} > F) = 0.0001]$. The predicted R^2 value (0.856) was in reasonable agreement with the adjusted R^2 value (0.965). The model consistency fit can be expressed by the coefficient of regression R^2 and was found to be 0.9817; this data representing 98.17 percentage variability in the response could be explained by this model. This implies that R^2 values of the model had advocated a high correlation between the experimental and predicated response, indicating

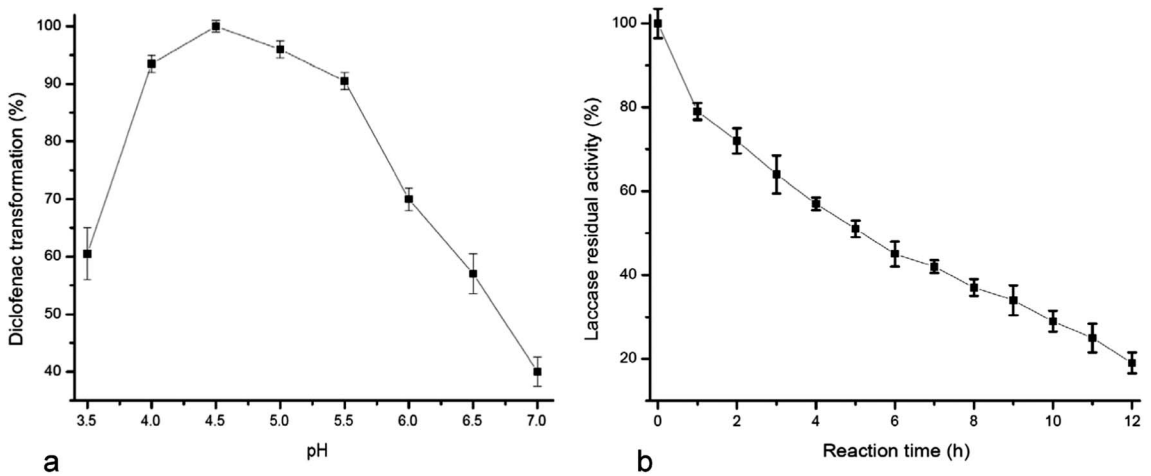

Fig. 2 Effect of pH on (a) diclofenac transformation and (b) laccase stability. The reaction was performed with diclofenac (20 mg L^{-1}), laccase (2.5 U L^{-1}) and syringaldehyde (250 μM) at 30 °C, 50 rpm.

Table 3 ANOVA results for the response surface quadratic model[a]

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	Prob. > *F*	
Model	9641	9	1071	60	<0.0001	Significant
A	2298	1	2297	128	<0.0001	Significant
B	319	1	319	18	0.0018	Significant
C	4903	1	4903	272	<0.0001	Significant
AB	421	1	421	23	0.0007	Significant
AC	72	1	72	4	0.0734	
BC	13	1	13	1	0.4241	
A^2	889	1	889	49	<0.0001	Significant
B^2	364	1	364	20	0.0011	Significant
C^2	665	1	665	37	0.0001	Significant
Residual	180	10	18			
Lack of fit	180	5	36			
Pure error	0	5	0			
Cor total	9821	19				

[a] Std. dev. – 4.242; mean – 66.55; C.V.% – 6.374; PRESS – 1405; R^2 – 0.9817; adj R^2 – 0.965; pred R^2 – 0.856; adeq. precision – 25.71.

that the predicted values for diclofenac transformation percent would be closer to its actual value. From the *P*-values, it was found that linear terms of laccase (*A*), syringaldehyde (*B*), reaction time (*C*), quadratic terms of laccase (A^2), syringaldehyde (B^2), reaction time (C^2), interaction between laccase and syringaldehyde (*AB*) were significant model terms for the diclofenac transformation. In addition, a relatively low coefficient variation value (CV = 6.374) proved higher consistency of the experiments for diclofenac transformation.[32]

The overall second-order polynomial equation represents the mathematical model relating to the percent of diclofenac transformation with independent factors as seen from eqn (5):

$$Y = 79.98 + 12.97A + 4.83B + 18.95C - 7.85A - 5.03B - 6.79C^2 + 7.25AB + 3.00AC - 1.25BC \quad (5)$$

where *Y* is diclofenac transformation (%), *A* is laccase concentration (U mL^{-1}), *B* is syringaldehyde concentration (μM), and *C* is reaction time (min). The interaction effects of selected factors on diclofenac transformation are shown in Fig. 3(a–c). The three-dimensional (3D) plot was constructed by plotting the factors of the central point which affects the percent of diclofenac transformation (laccase (1.5 U L^{-1}), syringaldehyde (150 μM) and reaction time (40 min). The influence of laccase and syringaldehyde on the response at a fixed reaction time 1.5 U L^{-1} is illustrated in Fig. 3a. Moreover, the increase in both laccase and syringaldehyde concentration yielded an increase in the response surface. The response value reached its maximum level at 1.76 U L^{-1} for laccase and 1.82 μM for syringaldehyde, respectively. This result also showed that increasing laccase and syringaldehyde concentration does not increase the diclofenac transformation beyond the above mentioned level. In general, the reaction rate increases with increase in either substrate or enzyme concentration up to a certain level and the reaction rate reaches a plateau and maintains a constant level after saturation.[33]

The effect of laccase and reaction time on the response at fixed syringaldehyde concentration of 150 μM is shown in Fig. 3b. The 3D plot revealed that the percentage of diclofenac transformation has increased with the increase in laccase concentration and decreased in reaction time. The maximum level of transformation was obtained at 1.65 U L^{-1} of laccase concentration with 45 min of reaction time. This result clearly indicated that the increasing level of laccase enzyme concentration could transfer diclofenac within a shorter reaction time. The effect of syringaldehyde and reaction time at a fixed laccase concentration of 40 min is shown in Fig. 3c. This result confirms that the percent of diclofenac transformation decreased with increase in syringaldehyde and increased with increase in reaction time. The maximum diclofenac transformation obtained for the concentration of syringaldehyde and reaction time was found to be 160 μM and 42 min. It can be observed that the percent of transformation decreased with increase in concentration of syringaldehyde even during the extended reaction time. This result shows that syringaldehyde might be inhibiting the laccase activity over a certain amount of concentration. Lloret *et al.*[24] reported that the presence of syringaldehyde at concentrations higher than the optimum level might cause remarkable enzyme inactivation. Consequently, the current study also confirms the significance of syringaldehyde concentration optimization for diclofenac transformation which is catalysed by laccase and also coincides with the findings of Lloret *et al.*[24] The predicted maximum diclofenac transformation derived from RSM regression was obtained with laccase, syringaldehyde, and reaction time of 1.91 U mL^{-1}, 187 μ, and 51 min, respectively.

To check the model efficiency, laccase mediated diclofenac transformation was performed under an optimized level of factors (laccase, syringaldehyde and reaction time) obtained from this study. The results of Fig. 4 show that complete transformation of diclofenac is possible. This result also suggests that the actual percent of transformation in the optimized conditions are close to the predicted value and the model was once again proven to be adequate. Marco-Urrea[34] observed 95% diclofenac transformation (40 mg L^{-1}) with laccase alone (2000 U L^{-1}) in pH 4.5 at 4½ h. Lloret *et al.*[24] reported complete transformation of diclofenac (5 mg L^{-1}) with 2000 U L^{-1} laccase

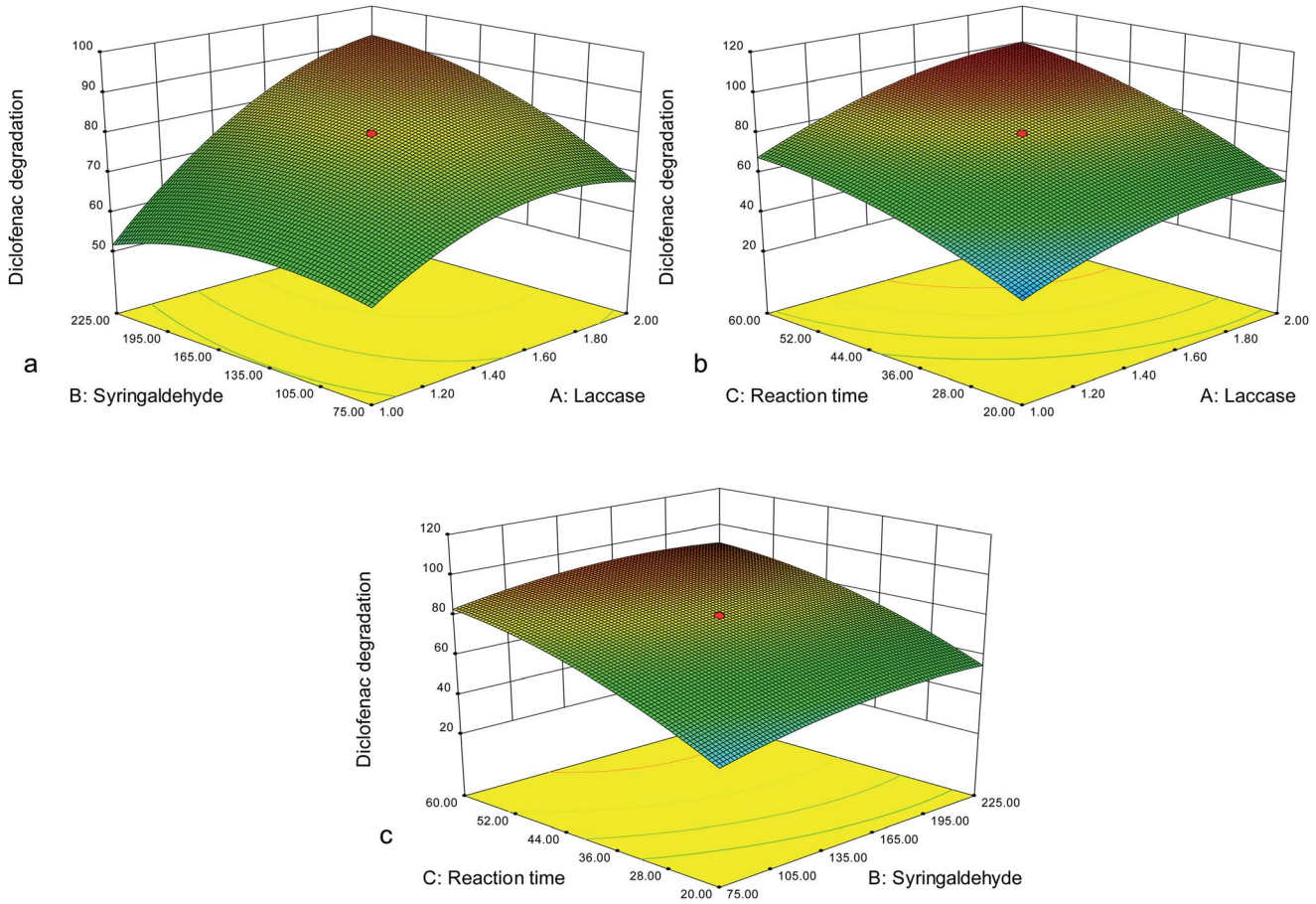

Fig. 3 3D plot of the interactions between factors for laccase mediated diclofenac degradation. Interaction effect between (a) laccase and syringaldehyde, (b) laccase and reaction time, (c) syringaldehyde and reaction time.

and 1 mM syringaldehyde with pH 4 at 1 h. The complete transformation of diclofenac (10 μg L^{-1}) was obtained with 2000 U L^{-1} laccase in the presence of 1-hydroxybenzotriazole or ABTS with pH 4.5 at 3 h by Tran *et al.*[35] The optimization results of this study clearly show that the maximum percentage of diclofenac transformation was achieved with the minimum concentration of laccase and syringaldehyde at a shorter reaction time.

The chromatograms of diclofenac and LTD samples are illustrated in Fig. 4a and b, which are obtained from HPLC analysis. The diclofenac peak was well-resolved with a retention time of 5.831 min (Fig. 4a). In the case of LTD, a peak which appeared at 5.831 min completely disappeared and new diclofenac transformed peaks appeared at the retention times of 3.379, 2.554, 2.124, 1.930, 1.814, 1.656, 1.572, and 1.183, as shown in Fig. 4b. On-going efforts are currently focused on the documentation of transformation by-products aiming to elucidate the complete laccase mediated transformation of diclofenac. Even though, diclofenac transformation mechanisms and their metabolic products were discussed with available literature data. Recently, Lloret *et al.*[36] detected product I decarboxylated compound, [2-[(2,6-dichlorophenyl)amino]phenyl]methanol as a laccase mediated diclofenac transformation by-product. In addition, they hypothetically proposed 2,6-dichloro-*N*-*o*-tolylbenzenamine as product II. Marco-Urrea *et al.*[34] reported that the laccase-catalyzed transformation of diclofenac leads to the formation of 4-(2,6-dichlorophenylamino)-1,3-benzenedimethanol, and two hydroxylated metabolites, namely 4′-hydroxydiclofenac and 5-hydroxydiclofenac were elucidated as transformation intermediates in fungal culture (*Trametes versicolor*) spiked with diclofenac. Soufan *et al.*[37] investigated the reactivity and fate of diclofenac during water chlorination and identified three major derivatives like decarboxy-DCF (Product I), chlorodecarboxy-DCF (Product II), and chloro-DCF (Product III). Martínez *et al.*[38] observed eight metabolites from diclofenac photo-degradation, mainly corresponding to the photocyclisation (2-(8-chloro-9*H*-carbazol-1-yl)acetic acid, 1-chloro-8-methyl-9*H*-carbazole), decarboxylation (2,6-dichloro-*N*-*o*-tolylbenzenamine) and dehalogenation. Huguet *et al.*[39] studied diclofenac elimination by means of fast chemical oxidation on natural manganese oxide in a column reactor and observed that diclofenac transformation by-products such as 5-hydroxydiclofenac and 5-iminoquinone with the mechanism of decarboxylation, iminoquinone formation, and dimerization. Michael *et al.*[40] reported that diclofenac degradation by (sono)photocatalysis, mainly proceeds with the oxidation and hydroxylation reactions occurring between chloroaniline and phenylacetic acid. However, still the detailed study on laccase mediated diclofenac transformation pathway is not elucidated. Therefore,

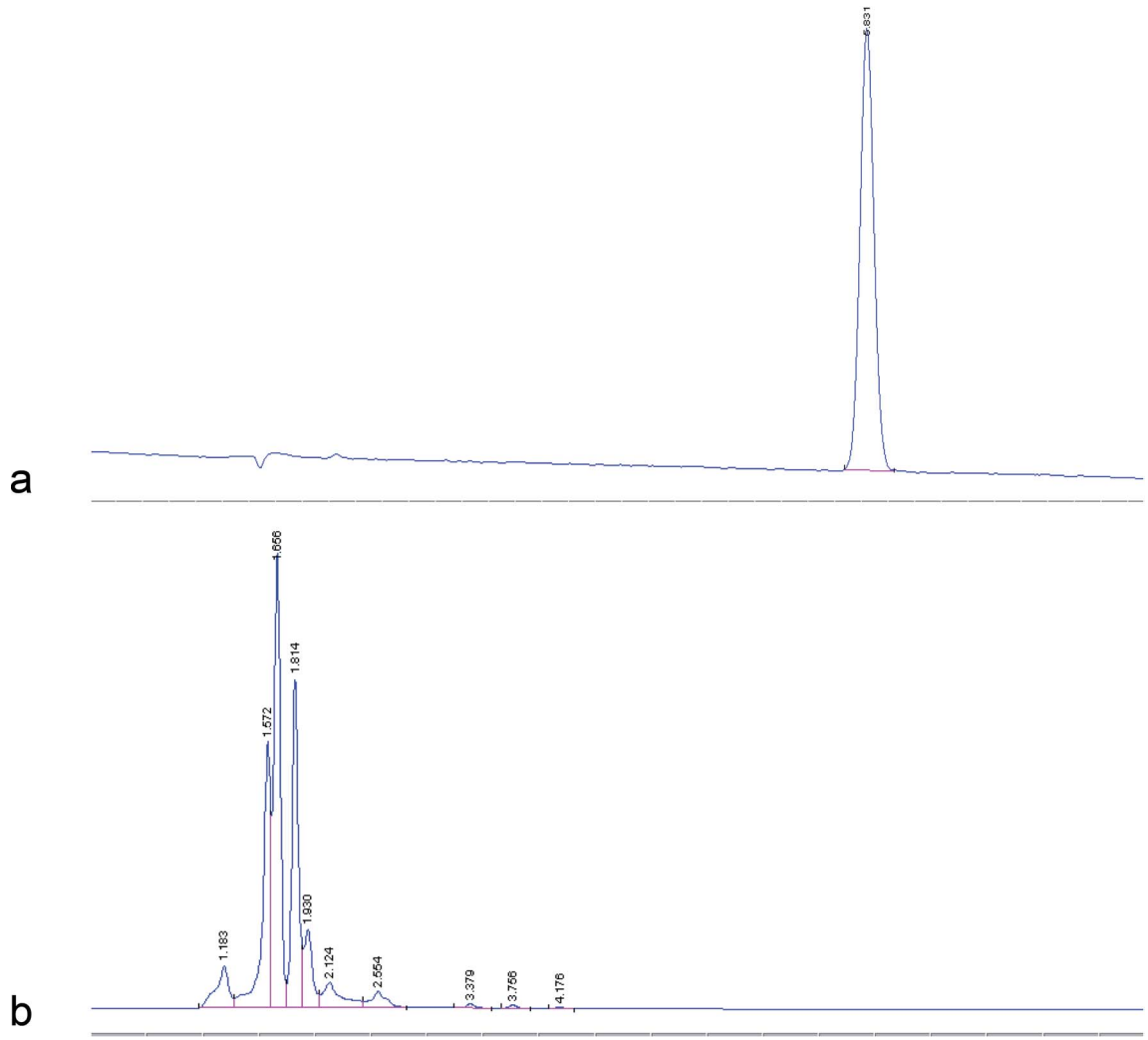

Fig. 4 HPLC chromatogram of (a) diclofenac and (b) laccase treated diclofenac (LTD).

further research is needed for understanding the complete mechanism and pathway elucidation for the laccase mediated diclofenac transformation processes.

3.2 Cytotoxicity of diclofenac and LTD on mouse 3T3-L1 preadipocytes

Diclofenac is found to be toxic for liver and kidney cells of both humans and animals. It is directly involved in causing renal failure, gastro intestinal toxicity and idiosyncratic hepatic injury.[41,42] The residues of veterinary diclofenac are the major causes of death in the vultures of India and Nepal.[43] To assess and compare the toxicity of LTD, mouse 3T3-L1 preadipocytes were cultured and treated with both diclofenac and LTD. The cell viability was used to assess the toxicity induced from both the samples. Effects of diclofenac and LTD were tested on mouse 3T3-L1 preadipocytes. As the drugs were dissolved in water, no vehicle control was used. The cells treated with 200 μg mL^{-1} diclofenac induced cell death. A decrease in cell viability of 61.6, 64.7 and 67.9% was observed at 24, 48 and 72 h, respectively (Fig. 5). Recent reports show that, among four different pharmaceutical compounds (atenolol, carbamazepine, diclofenac and gemfibrozil), diclofenac has the elevated cytotoxic effect on zebra mussel cell line, even at μg level.[44] The findings of the current study also confirm the cytotoxicity of the diclofenac drug towards the mouse 3T3-L1 preadipocytes. A decrease of 14.6, 16.9 and 23.3% cell viability was observed when the cells were treated with LTD for 24, 48 and 72 h. This result suggests a low level of apoptotic cell death was induced by LTD when compared to diclofenac induced cell death in mouse fibroblast cells. This is considered to be the first report on the toxicity of diclofenac drug and LTD on mouse fibroblast cells.

Finally, the result of cytotoxicity studies shows that diclofenac induced cell death; whereas, laccase treated diclofenac did not induce considerable cell death. This shows that contamination of diclofenac in the water system may induce health disorders among the consumers. Generally, different kinds of treatment methods (biological, chemical, and physical) are involved in the water treatment plants for water purification. This proposed laccase mediated treatment system will reduce the toxicity of diclofenac and further treatment systems like absorbance process involved in the water treatment plant will

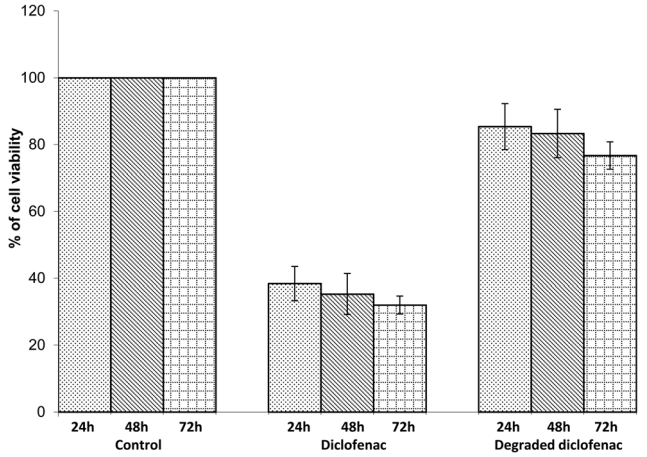

Fig. 5 Effect of diclofenac and LTD on mouse 3T3-L1 preadipocytes. Treatment of 200 μg mL^{-1} diclofenac induced cell death in mouse cells in a time-dependent manner and the data were significant at $p < 0.05$. Whereas the cells treated with LTD showed a slight decrease in cell viability, which shows that LTD is less toxic to the cells. Values are represented as mean ± SD, * denotes $p < 0.05$ significant from control.

remove the degraded compounds. Therefore, in this way detoxification of diclofenac may help in water purification.

4 Conclusions

In this study, the cytotoxic effect of diclofenac on mouse 3T3-L1 preadipocytes was confirmed. The cytotoxicity of diclofenac was completely removed after laccase treatment in the presence of syringaldehyde under optimized conditions. This environmental friendly strategy can provide a solution and insight for removing diclofenac toxicity.

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References

1 H. Islas-Flores, L. M. Gómez-Oliván, M. Galar-Martínez, A. Colín-Cruz, N. Neri-Cruz and S. García-Medina, *Ecotoxicol. Environ. Saf.*, 2013, **92**, 32–38.
2 Y. Zhang, S. U. Geissen and C. Gal, *Chemosphere*, 2008, **73**, 1151–1161.
3 E. Vulliet, C. Cren-Olive and M. F. Grenier-Loustalot, *Environ. Chem. Lett.*, 2011, **9**, 103–144.
4 R. Triebskorn, H. Casper, V. Scheil and J. Schwaiger, *Anal. Bioanal. Chem.*, 2007, **387**, 1405–1416.
5 J. S. van Leeuwen, G. Vredenburg, S. Dragovic, T. F. J. Tjong, J. C. Vos and N. P. E. Vermeulen, *Toxicol. Lett.*, 2011, **200**, 162–168.
6 C. E. Cagliyan, M. Balli, K. Tekin, S. Turkmen and İ. H. Tanboga, *J. Cardiol. Cases*, 2013, **8**, 17–19.
7 S. Shen, M. R. Marchick, M. R. Davis, G. A. Doss and L. Pohl, *Chem. Res. Toxicol.*, 1999, **12**, 214–222.
8 R. W. Sparidans, J. S. Lagas, A. H. Schinkel, J. H. Schellens and J. H. Beijnen, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2008, **872**, 77–82.
9 D. Stülten, M. Lamshöft, S. Zühlke and M. Spiteller, *J. Pharm. Biomed. Anal.*, 2008, **47**, 371–376.
10 M. Letzel, G. Metzner and T. Letzel, *Environ. Int.*, 2009, **35**, 363–368.
11 S. Stepanova, E. Praskova, L. Chromcova, L. Plhalova, M. Prokes, J. Blahov and Z. Svobodova, *Environ. Toxicol. Pharmacol.*, 2013, **35**, 454–460.
12 V. Naidoo and G. E. Swan, *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.*, 2009, **149**, 269–274.
13 P. Sathishkumar, S. Kamala-Kannan, M. Cho, J. S. Kim, T. Hadibarata, M. R. Salim and B. T. Oh, *J. Mol. Catal. B: Enzym.*, 2014, **100**, 111–120.
14 K. Poonkuzhali, P. Sathishkumar, R. Boopathy and T. Palvannan, *Carbohydr. Polym.*, 2011, **85**, 341–348.
15 P. Sathishkumar and T. Palvannan, *Journal of Environmental Treatment Techniques*, 2013, **1**, 27–34.
16 K. Balan, P. Sathishkumar and T. Palvannan, *J. Taiwan Inst. Chem. Eng.*, 2012, **43**, 776–782.
17 P. Baldrian, *FEMS Microbiol. Rev.*, 2006, **30**, 215–242.
18 F. Thurston, *Microbiology*, 1994, **140**, 19–26.
19 M. Chivukula and V. Renganathan, *Appl. Environ. Microbiol.*, 1995, **61**, 4374–4377.
20 T. Palvannan and P. Sathishkumar, *J. Basic Microbiol.*, 2010, **50**, 325–335.
21 P. Sathishkumar, K. Murugesan and T. Palvannan, *J. Basic Microbiol.*, 2010, **50**, 360–367.
22 P. Sathishkumar, K. Balan, T. Palvannan, S. Kamala-Kannan, B.-T. Oh and S. Rodríguez-Couto, *Clean: Soil, Air, Water*, 2013, **41**, 665–672.
23 K. Murugesan, I. H. Yang, Y. M. Kim, J. R. Jeon and Y. S. Chang, *Appl. Microbiol. Biotechnol.*, 2009, **82**, 341–350.
24 L. Lloret, G. Eibes, T. A. Lú-Chau, M. T. Moreira, G. Feijoo and J. M. Lema, *Biochem. Eng. J.*, 2010, **51**, 124–131.
25 P. Sathishkumar, J. C. Chae, A. R. Unnithan, T. Palvannan, H. Y. Kim, K. J. Lee, M. Cho, S. Kamala-Kannan and B. T. Oh, *Enzyme Microb. Technol.*, 2012, **51**, 113–118.
26 J. Margot, J. Maillard, L. Rossi, D. A. Barry and C. Holliger, *New Biotechnol.*, 2013, **30**, 803–813.
27 D. C. Shrama and T. Satyanarayan, *Bioresour. Technol.*, 2006, **97**, 727–733.
28 B. S. Wolfenden and R. L. Wilson, *J. Chem. Soc., Perkin Trans. 2*, 1982, 805–812.
29 F. Xu, *J. Biol. Chem.*, 1997, **272**, 924–928.
30 S. Kurniawati and J. A. Nicell, *Enzyme Microb. Technol.*, 2007, **41**, 353–361.
31 T. Hata, H. Shintate, S. Kawai, H. Okamura and T. Nishida, *J. Hazard. Mater.*, 2010, **181**, 1175–1178.
32 M. Amini, H. Younesi, N. Bahramifar, A. A. Z. Lorestani, F. Ghorbani, A. Daneshi and M. Sharifzadeh, *J. Hazard. Mater.*, 2008, **154**, 694–702.

33 R. O. Cristovao, A. P. M. Tavares, A. S. Ribeiro, J. M. Loureiro, R. A. R. Boaventura and E. A. Macedo, *Bioresour. Technol.*, 2008, **99**, 4768–4774.
34 E. Marco-Urrea, M. Pérez-Trujillo, C. Cruz-Morató, G. Caminal and T. Vicent, *J. Hazard. Mater.*, 2010, **176**, 836–842.
35 N. H. Tran, T. Urase and O. Kusakabe, *J. Water Environ. Technol.*, 2010, **8**, 125–140.
36 L. Lloret, G. Eibes, M. T. Moreira, G. Feijoo and J. M. Lema, *J. Mol. Catal. B: Enzym.*, 2013, **97**, 233–242.
37 M. Soufan, M. Deborde and B. Legube, *Water Res.*, 2012, **46**, 3377–3386.
38 C. Martínez, M. L. Canle, M. I. Fernández, J. A. Santaballa and J. Faria, *Appl. Catal., B*, 2011, **107**, 110–118.
39 M. Huguet, M. Deborde, S. Papot and H. Gallard, *Water Res.*, 2013, **47**, 5400–5408.
40 I. Michael, A. Achilleos, D. Lambropoulou, V. Osorio Torrens, S. Pérez, M. Petrovi, D. Barceló and D. Fatta-Kassinos, *Appl. Catal., B*, 2014, **147**, 1015–1027.
41 A. Yano, S. Higuchi, K. Tsuneyama, T. Fukami, M. Nakajima and T. Yokoi, *Toxicology*, 2012, **293**, 107–114.
42 L. E. Ng, B. Halliwell and K. P. Wong, *Biochem. Biophys. Res. Commun.*, 2008, **369**, 873–877.
43 V. Prakash, M. C. Bishwakarma, A. Chaudhary, R. Cuthbert, R. Dave, M. Kulkarni, S. Kumar, K. Paudel, S. Ranade, R. Shringarpure and R. E. Green, *PLoS One*, 2012, **7**, e49118.
44 M. Parolini, B. Quinn, A. Binelli and A. Provini, *Chemosphere*, 2011, **84**, 91–100.