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Enhanced Biosynthesis of *Laccase* and Concomitant Degradation of 2, 3-Dichlorodibenzo-p-Dioxin by Pleurotus florida

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Abstract

Objective: Laccase enzyme has proven to be an excellent catalyst for the degradation of dioxin into less toxic metabolites. In the present study, submerged culture conditions of *Pleurotus florida* were optimized by Taguchi design of experiments (DOE) for enhanced laccase production and 2, 3-Dichlorodibenzo-p-dioxin (2, 3-DCDD) degradation. **Methods/Statistical Analysis**: An orthogonal array layout of L-8 (2^7) was constructed using Qualitek-4 software with seven influential factors at two levels with "bigger is better" quality character. **Findings:** At individual levels, copper showed maximum effect and at the interactive level RPM accounted for more than 90 % of the severity index (SI) with duration. The optimized conditions: nitrogen 0.2 mM; copper 0.04 mM; pH 5.5; temperature 25ºC, inoculum size 10% w/v, RPM 120 and 30 days duration predicted an increase of laccase production by 24.4% (621.36 U to 773.04 U) and dioxin (initial concentration of 10 ppm) degradation by 20.2% (83.1% to 100%). The validation experiments confirmed an improvement of laccase production by 21.5% and exhibited complete degradation of 2, 3-DCDD. **Application**: This is the first report on parametric optimization of laccase production by *P. florida* by Taguchi DOE and its utilization for complete degradation of chlorinated dioxin molecules. The optimized process parameters can be adopted for the large-scale production of laccase for bioremediation of dioxin.

Keywords: Dioxin Degradation, Laccase Enhancement, Optimization, Taguchi Design of Experiments

1. Introduction

Polychlorinated dibenzo-p-dioxins (dioxins) have garnered attention over the past few decades from environmentalists across the globe for their toxic and recalcitrant nature[1-4]. The highly lipophilic nature of dioxins promotes bioaccumulation of this compound in several species. In humans it results in numerous health hazards like carcinogenic, mutagenic and teratogenic defects[5]. Therefore, complete degradation of dioxins into non-toxic products is the prerequisite for establishing a toxic free environment. Although several modes of degradation (physical[6], chemical[7] 3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TeCDD, biological[8]) have been studied; yet, mycodegradation by white rot fungi through the enzymatic action has remained the most preferred strategy to eliminate dioxins. White rot fungi can extensively degrade persistent organic pollutants (POPs) efficiently due to the catalytic action of its ligninolytic enzyme cascade[9]. In this regard, laccase has remained the most prominent

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exoenzyme involved in degradation of triclosan[10], chlorophenols[11], herbicide Dymron[12], sulfomethoxazole[13], Bisphenol A[14] and Polychlorinated biphenyls (PCBs)[15]. White rot fungal strains: *Phanerochaete chrysosporium*[16]; *Phlebia sp.*[17]; *Trametes sp.*[18] and *Pleurotus sp*.[19] have been extensively studied due to their potential to cleave dioxins into simpler metabolites. However, *P. florida* studied here, had an added potential of rapid growth irrespective of stringent temperature conditions unlike the normal white rot fungal strains. Thereby, making this strain highly useful for scale-up studies and in-situ bioremediation processes, where temperature cannot be controlled manually.

Laccase (EC 1.10.3.2; benzenediol: oxygen oxidoreductase) is a multicopper phenol oxidase enzyme with the ability to catalyze one electron oxidation of four aromatic molecules concomitant with the four-electron reduction of molecular oxygen into water[20]. Due to the multifaceted catalytic properties and broad substrate specificity, laccase holds potential for several industrial applications: dye decolourization of dyes in textile industries[21], Kraft bleaching in paper and pulp mill[22], ascorbic acid determination, beverage processing and sugar beet pectin gelation in food processing industries[23]. For efficient biosynthesis of laccase, optimization of media composition and culture conditions are the prerequisites that further attributes to the industrial design of the full-scale fermentation system. Conventional optimization procedures follow one factor at a time (OFAT) strategy, where one parameter is altered at a particular time, keeping all the other parameters constant. This single dimensional search is cumbersome, time-consuming and requires more experimental datasets and do not provide information regarding the mutual interaction between parameters and their cumulative effect on the process performance[24] phenol concentration and reaction time. CCD and RSM were applied to optimize the significant factors identified from PBD. The results obtained from CCD indicated that the interaction between the concentration of algae and phenol, phenol concentration and reaction time and algal concentration and reaction time affect the phenol degradation (response.

The Taguchi orthogonal array (OA) design of experiments is a multifactorial experimental design with a statistical approach to understand a system using a set of independent variables (factors) spanning over a particular region of interest (levels)[25]. The statistical design of Taguchi's OA facilitates in identifying the influence of individual factors along with mutual interactions between different variables that establish the optimal conditions for maximal process performance with a minimal number of experimental runs. Taguchi method improves process performance through Robust Technology Development (RTD) by focusing on the effects of the process characteristics variation[26]. This methodology incorporates L-8 (2^7-seven factors at two levels) OA where variables are arranged in a manner, wherein; each combination of variables at their corresponding levels between each pair of columns appears an equal number of times (pair-wise balancing property)[27].

Unlike other (DOE), Taguchi methodology incorporates statistically planned experimental sets that reduce the number of trial runs by developing a specific design of experiments, which also minimize the error in determining the values for significant parameters[20]. This method also determines the optimal level of the important controllable factors based on the concept of robustness and S/N (Signal-to-Noise ratio). Finally, Analysis of variance (ANOVA) helps in determining the effect of different variables that are statistically significant in finding the optimum level. This approach provides a simple, systematic and statistical methodology for the optimization of parameters with few experimental sets that are both economical and time saving with limited labour inputs. Several papers have reported the use of this approach for process optimization regarding numerous industrially useful enzymes like xylitol[28], alkaline protease[29], α-amylase[30], thermostable protease[31], L-asparaginase[32], tannase[33], xylanase[34] and bioethanol[25].

The present work demonstrates the optimization of submerged culture conditions for enhanced laccase production and simultaneous 2,3-DCDD degradation by white rot fungal strain *Pleurotus florida* by Taguchi DOE methodology using the L-8 (2^7) orthogonal array. The experimental sets were designed with seven factors - nitrogen, copper, pH, temperature, inoculum size, RPM, and duration of incubation at two different levels. These factors were selected based on the preliminary study (data not shown) with significant influence on laccase production and subsequent 2,3-DCDD degradation. This is the first article to report the complete degradation of 2,3-Dichlorodibenzo-p-dioxin by *P. florida* through enhanced laccase activity resulted by the optimal conditions obtained through the multifactorial Taguchi design. The process progresses through enzymatic cleavage of the

aromatic moiety and simultaneous oxidation of phenolic core into quinone metabolites. ANOVA was performed to study the variability among statistically significant parameters and it was further validated to confirm the optimized conditions.

2. Materials and Methods

2.1 Chemicals

2,3-DCDD (CAS No. 29446-15-9) was purchased from Accu Standard Inc. (New Haven, CT, USA). 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ABTS (CAS No. 30931-67-0^{N}) and Laccase were procured from Sigma-Aldrich (St. Louis, MO, USA). Potato dextrose agar (PDA), nutrient supplements were purchased from HiMedia (Mumbai, India) and all GC grade solvents used for the experimental study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

White rot fungi *Pleurotus florida* was procured from Centre for Advanced Study in Botany, Madras University, Chennai, India. The fungi were maintained on PDA slants, sub-cultured fortnightly and stored at 4°C until further use. Fungal inoculums were prepared in 250 mL Erlenmeyer flasks containing 50 mL of the modified basal medium that consisted of (g/L): glucose- 10; KH_2PO_4- 0.8; NH_4NO_3- 2; Na_2HPO_4- 0.4; $MgSO_4.7H_2O$- 0.5; yeast extract- 2; amended with trace elements solutions (g/L): $ZnSO_4.7H_2O$- 0.001 ; $FeSO_4.7H_2O$- 0.005; $CaCl_2.2H_2O$- 0.06; $CuSO_4.7H_2O$- 0.005 and $MnSO_4.7H_2O$- 0.005. The sterilized modified basal media were inoculated with five agar plugs (5 mm diameter each) excised from the edges of flourishingly growing fungal mycelium and incubated at 25°C under static cultivation for 7 days. The fungal mycelia thus obtained were homogenized under sterile conditions and used as inoculums for optimization experiments.

2.2 Enzyme Assay

After the respective incubation period (15 and 30 days), the fungal mycelia were separated using a muslin cloth and squeezed tightly to obtain maximum extrudates and was later centrifuged at 6,708 g for 20 mins at 4°C along with the culture filtrate for maximal enzyme extraction[20]. The supernatant thus obtained was used to estimate the laccase activity. Laccase activity was assessed by the oxidation of 1mmol/L ABTS in 50 mmol/L sodium acetate buffer (pH 4.5). The oxidized ABTS produces free cation radical that was spectrophotometrically monitored at 420 nm (extinction coefficient ε420 = 36,000 M^{-1} cm^{-1}). Enzyme activity was expressed in units (U = µM/min/L), defined as the number of µM of ABTS converted per minute per litre[35] 2\u2032-azinobis 3-ethyl-benzothiazoline-6-sulfonate.

2.3 Residual 2,3-DCDD Analysis

2.3.1 Extraction and Purification of Residual 2,3-DCDD

After the incubation period, concentrated sulphuric acid was added to each flask to dissolve the mycelia thoroughly and to retrieve all the dioxin residues adsorbed to the mycelia. Later, the culture filtrate was extracted twice with 20 mL of ethyl acetate (GC grade). The mycelia were thoroughly homogenized and extracted in a Soxhlet apparatus for 6-8 hrs with ethyl acetate after washing it with deionised water. The organic layers were pooled together after the water fraction was re-extracted with 20 mL of ethyl acetate and dried over anhydrous sodium sulphate to remove excess moisture. The dried organic extract was macro-concentrated under reduced pressure by purging nitrogen gas and re-suspended in ethyl acetate[36]. Further, the extracts were cleaned up using multilayer silica gel column that contained modified silica from bottom to top in the following order: 0.9g silica gel, 3g potassium hydroxide impregnated silica gel (2%), 0.9g silica gel. This was followed by 4.5g sulphuric acid-impregnated silica gel (44%), 6g sulphuric acid impregnated silica gel (22%), 0.9g silica gel, 3g silver nitrate impregnated silica gel (10%); 0.9g silica gel and 3g anhydrous sodium sulphate (Na_2SO_4)[37] polychlorinated and mixed brominated\u2013chlorinated dibenzo-p-dioxins and dibenzofurans (PBDD/DFs, PCDD/DFs and PXDD/DFs, respectively. The silica gel column was pre-eluted with n-hexane (GC grade), and the residual dioxin in the sample was eluted using 30 mL ethyl acetate (GC-MS grade) and macro-concentrated to 1mL.

2.3.2 Gas Chromatography - Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of the samples were carried out on Agilent 7890B GC system with 30m fused DB-5MS column (0.25mm inner diameter, 0.18µm film thickness) coupled with Agilent 5977A MSD mass selective detector. The GC oven temperature was programmed from 70°C

to 280°C at a rate of 10°C/min, and 2 mins hold at 100°C and 200°C. The transfer line temperature and ion source temperature were maintained at 270 and 280°C respectively. The carrier gas was Helium at a flow rate of 1ml/min and the electron impact ionization energy was set at 40 eV, with the resolution higher than 10,000 (5% valley). The inlet temperature was maintained at 270°C, and one µL of the sample was injected in splitless mode[38]. For data acquisition, selective ion mode (SIM) was used for the estimation of 2,3-DCDD using the quantifier ions- 252, 189, 126, 161 and 191. Highly productive Mass Hunter qualitative and quantitative analysis software were used for the study.

2.4 Taguchi Methodology

The process and culture media optimization for maximum laccase activity and simultaneous 2,3-DCDD degradation potential of *P. florida* were performed using Taguchi's design of experiments. The entire study was divided into four phases with multiple internal steps viz., planning, experimentation, software analysis with prediction and validation of results. Each step has a separate objective and was interconnected in a sequence to achieve the overall optimization process.

2.4.1 Planning - Design of Experiments (Phase 1)

The first step in the planning stage is to determine the most significant factors to be optimized that have a critical effect on laccase activity and simultaneous dioxin degradation by *P. florida*. Next step is to assign different levels to the factors considered for optimization within an expedient range so that the variation implicit in the process does not veil the factor's effect. Seven factors that showed significant influence on laccase activity and dioxin degradation based on initial studies was selected for the present Taguchi DOE study. The seven factors considered were nitrogen, copper, pH, temperature, inoculum size, RPM and duration at two different levels (Table 1). Taguchi provides several orthogonal arrays and corresponding linear graphs. The present study employed L-8 (2^7) orthogonal array with seven factors at two different levels of variation as shown in Table 1. In general, the full factorial approach will require 128 experimental trials for optimization process while in fractional factorial design using L-8 OA requires only 8 experimental runs.

Table 1. Selected culture conditions factors and assigned levels

Sl.No	Factor	Level 1	Level 2
1	Nitrogen (NH_4NO_3) mM	0.2	0.4
2	Copper ($CuSO_4$) mM	0.02	0.04
3	pH	5.5	6.5
4	Temperature (°C)	25	30
5	Inoculum size (%w/v)	5	10
6	RPM	100	120
7	Duration (Days)	15	30

2.4.2 Experimentation - Submerged State Fermentation (Phase 2)

Submerged state fermentation experiments were performed for maximum laccase production and 2,3-DCDD degradation by *P. florida* employing 8 experimental trials (Table 2) in combination with seven factors at selected levels (Table 1). Experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of the basal medium supplemented with nitrogen (NH_4NO_3 - 0.2 mM or 0.4 mM), copper ($CuSO_4$- 0.02 mM or 0.4 mM) with pH adjusted to 5.5 or 6.5 and inoculated with selected levels of homogenized inoculums (5% w/v or 10 % w/v). The flasks were incubated at different temperatures (25°C or 30°C) at two levels of agitation (100 RPM or 120 RPM) for 15 or 30 days. All the flasks were spiked with 2,3-DCDD (10 ppm) in N,N-Dimethylformamide (DMF) after four days of fungal incubation. After the incubation period, the entire contents of the flasks were used for enzyme estimation and residual dioxin analysis. The values presented for laccase activity (U) and dioxin degradation percentage is the average of three individual determinations (Table 2).

2.4.3 Analysis of Experimental Data and Prediction of Performance (Phase 3)

The experimental results obtained from the 8 trials for laccase activity and percentage dioxin degradation were processed in Qualitek-4 software (NutekInc., MI) with "bigger is better" quality characteristics to determine the optimal culture conditions. The software is equipped with L-4 to L-64 orthogonal arrays along with a selection of 2-63 parameters at two, three or four levels[39]. The contribution of individual factors for process optimization was presented in detail along with interaction factor pair

Table 2. L-8 orthogonal array of design of experiments with laccase activity (U) and percentage 2,3-DCDD degradation in each run. All results are represented as mean ± S.D. (n= 3 replicates and S.D. = Standard Deviation)

Exp.No.	N	C	pH	T	I	RPM	Duration	Laccase activity (U)	Percentage of 2,3-DCDD degradation (%)
1	1	1	1	1	1	1	1	613.05 ± 0.95	78 ± 0.1
2	1	1	1	2	2	2	2	671.5 ± 1.5	92.8 ± 0.2
3	1	2	2	1	1	2	2	688.5 ± 0.5	94.9 ± 0.2
4	1	2	2	2	2	1	1	642.95 ± 0.45	84.45 ± 0.15
5	2	1	2	1	2	1	2	588.65 ± 0.55	77.15 ± 0.35
6	2	1	2	2	1	2	1	477.25 ± 0.95	71.15 ± 0.75
7	2	2	1	1	2	2	1	663.35 ± 0.85	86.35 ± 0.05
8	2	2	1	2	1	1	2	625.65 ± 0.85	80.65 ± 0.45
Current grand average of performance								621.362	83.181
Expected result at optimum conditions								773.046	100

effects. Analysis of variance (ANOVA) was applied to evaluate optimal configuration of selected variables. The contribution of each factor in laccase activity and dioxin degradation was assessed by a combined index called Overall evaluation criteria (OEC). For calculating OEC, firstly the readings under individual assessments must be normalized (dimensionless), and following assumptions were made-

X1 = numeric evaluation under criteria 1
X1 ref = Highest numerical value X1 can assume
Wt1 = Relative weight of criteria 1
Then OEC was calculated as:

$$OEC = \frac{X1}{X1\ ref} \times Wt1 + \frac{X2}{X2\ ref} \times Wt2 + \ldots$$

In the present study, X1 is the laccase activity (U) observed at individual experimental runs, and the X1 ref is the highest numerical value for laccase activity. X2 represents the percentage of dioxin degraded for individual trials, and the X2 ref is the maximal numerical value for X2. Since the catalytic activity of laccase enzyme is directly proportional to the amount of dioxin degraded, thus the relative weight (Wt1 and Wt2) for both parameters was 50. All the values were fed into the software and single combined value (OEC) was calculated for each run.

2.4.4 Validation (Phase 4)

To validate the optimized methodology, submerged fermentation experiments using the optimized conditions were performed in triplicates for maximum laccase activity and dioxin degradation.

3. Results and Discussion

3.1 Statistical Optimization of Laccase Production and Dioxin Degradation by *P. florida*

Taguchi L-8 (2^7) orthogonal array-based design of experiments using seven factors at two levels resulted in an enhanced production of laccase and simultaneous increment in dioxin degradation by white rot fungi *P. florida*. Based on preliminary studies, the seven factors considered were nitrogen, copper, pH, temperature, inoculum size, RPM and duration at two different levels as shown in Table 1. The results of the 8 experimental runs revealed that the highest production of laccase (688.5 U) and maximum degradation of 2, 3-DCDD (94.9%) occurred when the conditions (3rd experimental run) were: nitrogen - 0.2 mM, copper - 0.04 mM, pH - 5.5, temperature - 25°C, inoculum size - 10%, RPM - 120 and duration time - 30 days (Table 2). Whereas, minimum average production of laccase (477.25 U) and least 2,3-DCDD degradation percentage occurred (6th experimental run) at nitrogen - 0.4 mM, copper - 0.02 mM, pH - 6.5, temperature - 30°C, inoculums size - 5%, RPM - 100 and duration time - 15 days condition.

The current grand average performance for laccase production was 621.36 U and percentage of dioxin degradation was 83.18 %. The expected result at optimum conditions exhibited a 24.41% increase in laccase produc-

tion (773.046U) and 20.21 % increase in 2,3-DCDD degradation percentage (initial concentration was 10 ppm) as shown in Table 2. The target enzyme - laccase in this study was responsible for triggering the catabolic breakdown of 2,3-DCDD into quinone and catechol metabolites by *P. florida* strain. The GC-MS analysis of the culture extract after the initial spiking of 2,3-DCDD revealed a single peak at 16.3 min and residual dioxin analysis at regular intervals exhibited complete degradation of 2,3-DCDD into intermediate metabolites with no characteristic peak at 16.3 min substantiating complete exhaustion of 2,3-DCDD after 30 days of incubation (Supplementary data). The primary mechanism involved in dioxin degradation is the oxidative ring cleavage of the central moiety into the corresponding quinone products which further reduces into respective hydroxybenzene molecules. In this study, residual 2,3-DCDD was monitored at regular intervals to keep a track on the percentage of dioxin degradation irrespective of the metabolites formed.

This study doesn't emphasize on the characterization of the metabolites as it would not affect the primary objective of this study that is process optimization. The metabolites observed at the end of the study and the reduced concentration of 2,3-DCDD indicated the oxido-reductive reactions of laccase substantiating its importance in this study. Degradation of 2,7-Dichlorodibenzo-p-dioxin by *Phanerochaete chrysosporium* was one of the earliest reports on dioxin degradation by a white rot fungus explaining the cumulative mechanism of laccase, lignin peroxidase and manganese peroxidase[40] the white-rot basidiomycete Phanerochaete chrysosporium degraded 2,7-dichlorodibenzo-p-dioxin (I. *Pleurotus* genera also have reported the production of laccase enzyme and its consecutive catalytic action on chlorinated dioxin molecules[41] which fulfils the requirements for POP soils, is incineration at high temperature. In this study, we investigated if bioaugmentation with fungal inoculum or treatment with manganese peroxidase (MnP. Since laccase holds an explicit role in determining the degradation efficiency of an organism, it was selected as the key target for enhancement in the present study. The enhanced production of laccase had a direct influence on the extent of 2,3-DCDD degraded.

3.2 Influence of Individual Factors on Laccase Production and 2,3-DCDD Degradation

Process optimization holds an indispensable role to achieve the enhanced performance of an experiment. Physical factors: pH, temperature, RPM and duration; along with nutritional supplements/media components: nitrogen and copper content have a direct influence on laccase production and dioxin degradation. Selection of the influential factors is the most important step in the process optimization for enhanced performance in submerged fermentation technique for large scale production of laccase. Several statistical methods like Plackett-Burman design[42] and response surface methodology (RSM)[43] are incorporated for optimization studies. However, the major drawback of these models is cumbersome nature with a lot of experimental runs, which is time-consuming and they do not explain the interacting factor pair effects on the criteria under study. Taguchi DOE is a fractional factorial design with a limited number of experimental runs.

In this study, incorporation of L-8 OA reduced the experimental setup from 128 trials (full factorial design) to just 8 trials (fractional factorial design). The results obtained from the runs can be applied as the consequence of the most influential factors. Submerged fermentation study incorporating the suggested experimental setup exhibited significant variation in laccase activity of *P. florida*, which directly affected the percentage of 2,3-DCDD degradation. Production of laccase evidently depended on the influence of individual factors under study. However, a factor may have an effect on the production levels either independently or in combination with other factors. Since the optimized media will have the concerned factors at different levels, it is evident that the final result attributes to the cumulative effect of these factors that can occur in different possible combinations.

The main effects of selected factors at assigned levels on laccase production by *P. florida* are shown in Table 3. The main effects represent the influences of individual factors at two different levels (L1and L2) and their difference (L2-L1) on the characteristics under study. In this case, highest laccase production (655.11U) was observed at level 2 of copper that acts as an inducer. There is an increase in laccase activity from level 1 to 2 in the case of copper, thus having the most positive influence on laccase production by *P. florida*. This was followed by the duration factor with laccase activity (643.57 U) at level 2 (30 days).

The difference calculated between the average value of each factor at level 1 and 2 (L2-L1) indicates the relative influence of the effect (Table 3). Larger difference indicated a stronger influence of the factor on the experimental parameter. In this case, the relative influence of

Table 3. Main effects of selected factors on laccase production by *Pleurotus florida*

Sl.No.	Factor	Level 1	Level 2	L2-L1
1	Nitrogen	654	588.724	-65.276
2	Copper	587.612	655.112	67.5
3	pH	643.387	599.337	-44.05
4	Temperature	638.387	604.337	-34.05
5	Inoculum size	607.112	641.612	40.5
6	RPM	617.575	625.15	7.574
7	Duration	599.15	643.575	

copper was the highest on laccase production followed by the relative influence of nitrogen. The negative symbol represents the decrease in laccase production from level 1 to 2 for nitrogen factor, thus confirming lower nitrogen condition requirement for enhancement of laccase production in *P. florida.* The least relative influence on laccase production was by the RPM factor with a value of 7.57 U (L2-L1), indicating that the change in agitation conditions did not affect the enzyme release largely (Table 3). The present study with the L-8 OA showed laccase production ranging from 477.25U to 688.5U at varied assigned levels with an average of 621.36 U (Table 2). Similarly, the percentage of 2,3-DCDD degradation ranged from 71.15% to 94.9% concomitant with the experimental runs having the highest and lowest laccase production respectively. The effect of laccase on the biotransformation of the dioxins and furans[41] which fulfils the requirements for POP soils, is incineration at high temperature. In this study, we investigated if bioaugmentation with fungal inoculum or treatment with manganese peroxidase (MnPand other recalcitrant compounds[44] by white rot fungal strains have earlier reported a positive linear relationship with the catalytic action of the enzyme[45].

In the present study, copper that acts as an inducer had the highest influence on laccase production by *P. florida* at level 2 (Table 3). Laccase has copper at its core active site; hence the additional presence of copper has an inducing effect in the media for enhanced production of the enzyme. Moreover, laccase needs copper to reach its maximum activity level as; this inducer triggers the active site of the enzyme to catalyze the oxidation of substrate[46]. The incitement of the inducer is hugely dependant on several factors such as concentration[47]; the point of addition[48] in the fermentation media during the growth phase of the organism, as an additional presence of copper, has an inductive effect on laccase production[49]. Copper was also reported to enhance laccase activity in *Trametes pubescens* when added during the initiation of exponential phase[50]. Table 3 and Table 4 showed that low nitrogen media supported increased production of laccase as well as dioxin degradation. The limited source of nitrogen induces a stress environment triggering the release of laccase from white rot fungi[51].

The combination of low nitrogen and high copper concentration has a positive effect on laccase production[52]. Supplementation of production media with an additional quantity of nitrogen lowered laccase and other fungal specific enzymes in *Pleurotus sp.*[53] and *Trametes sp.*[54]. Nitrogen limited media proved beneficial for enhanced laccase production by *Pleurotus Ostreatus* and subsequent decolourization of anthraquinone dye[51]. Hence, the optimized production media had a low nitrogen concentration to abide by the requirement of *P. florida* for extensive release of laccase and increase in the action of dioxin cleavage. Both the nutritional factors considered had a varied influence on laccase production based on their concentration in the media. Lower nitrogen enhanced laccase production and higher copper concentration elevated the enzyme activity in *P. florida*. Figure 1 represents the effect of each factor on laccase production at the two assigned levels. Nitrogen had a stronger influence at level 1 and decreased at level 2 with higher concentration. Copper which acts as an inducer, had a positive increase in laccase production from level 1 to 2. Similarly, inoculums size, RPM and duration period showed an increased laccase production at level 2. Both pH and temperature exhibited a decrease in laccase production from level 1 to 2 confirming the requirement of lower temperature and pH for proliferating fungal growth and laccase activity.

Table 4. Main effects of selected factors on dioxin degradation by *Pleurotus florida*

Sl.No.	Factor	Level 1	Level 2	L2-L1
1	Nitrogen	86.587	79.775	-6.811
2	Copper	78.824	87.537	8.714
3	pH	86.3	80.062	-6.237
4	Temperature	84.45	82.262	-2.538
5	Inoculum size	81.174	85.187	4.012
6	RPM	82.262	84.099	1.838
7	Duration	79.987	86.375	6.388

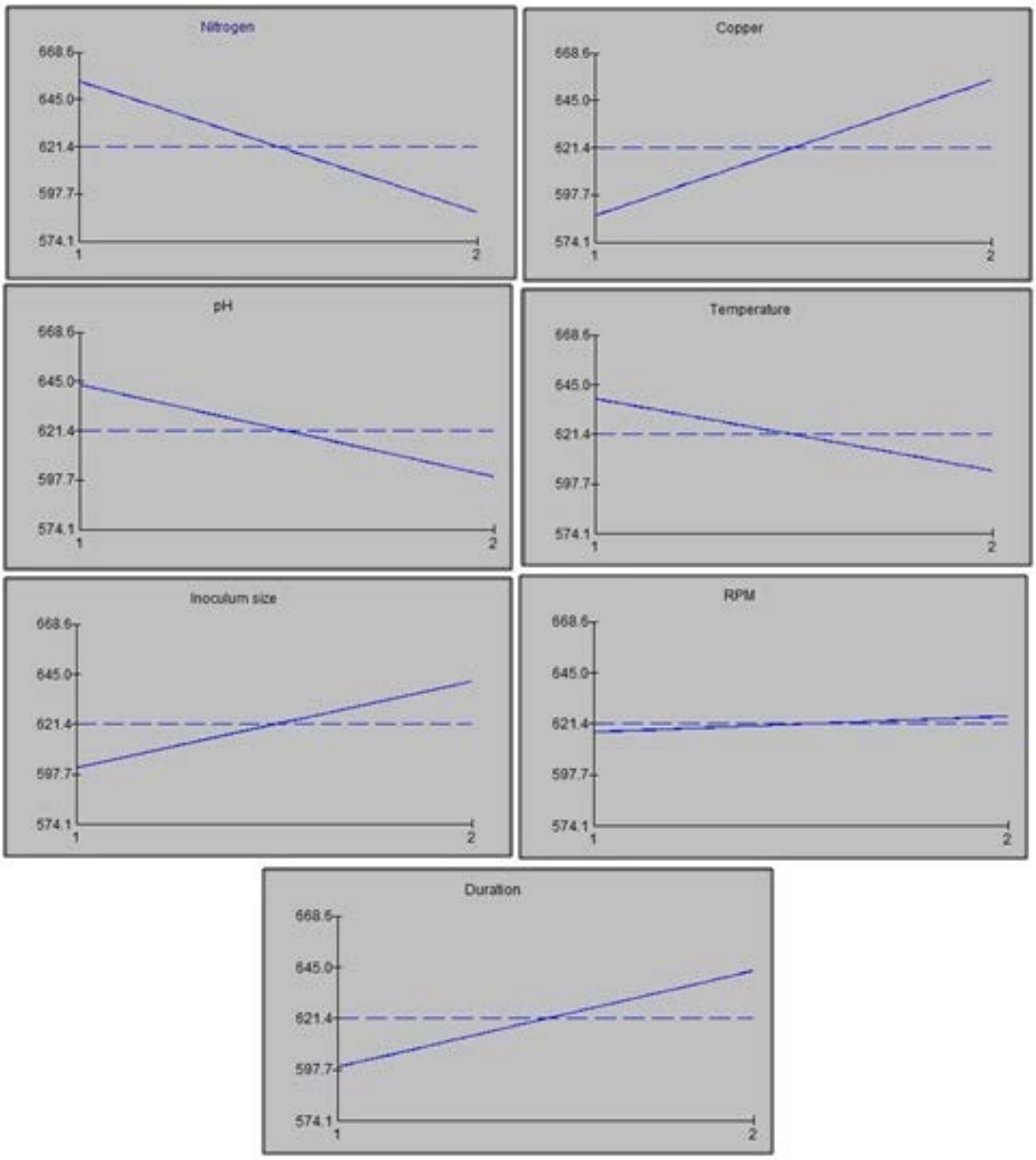

Figure 1. Individual factor performance at different levels on laccase production by *Pleurotus florida* under submerged state fermentation.

Table 4 represents the influence of individual factors on 2,3-DCDD degradation by *P. florida*. Gas chromatography-Mass spectrometry (GC-MS) analysis of 2,3-DCDD revealed the molecular ion peak at 19.9 mins on average (Supplementary data). Standard calibration graph was created using five different concentrations and showed linear correlation value of $R^2 = 0.996$ (preliminary study) and was used to estimate the residual dioxin after the incubation study. Maximum degradation percentage (87.5%) was observed at level 2 of copper, which also supported enhanced laccase production. The least relative influence on dioxin degradation (1.83) was exhibited by RPM, as an apparent change in the degradation percentage was not observed (Table 4). In case of dioxin degradation, the relative influence of nitrogen, pH, and duration had an almost similar effect, but only duration had an increase from level 1 to 2 (Table 4). Since two parameters, i.e., laccase production and 2,3-DCDD degradation percentage were considered, a cumulative effect concept was carried out to understand the influence of individual

factors as well as the interaction between factor pairs on both the parameters simultaneously.

Thus Overall Evaluation Criteria (OEC) was calculated and the results were fed into the software for analysis of the influence of variables on the combined concept (laccase production and 2,3-DCDD degradation) which is dimensionless (free from units). Among the factors studied, RPM has the least effect on laccase production as the exo-enzyme released by the mycelia is not dependent on the agitation parameter. Both solid state and submerged fermentation of *P. florida* had a similar influence on laccase production with no distinctive difference due to agitation of the media (results not shown). Lower pH (5.5) supported rapid and robust fungal growth along with enhanced laccase production in the current study. The pH is one of the most prominent factors that influences the metabolic activity of an organism, thus it plays a significant role in process optimization for fermentation experiments. The optimal pH for production of extracellular enzymes-laccase, lignin peroxidase and manganese peroxidase is acidic in the range of 4-6 in *Pleurotus florida* strain[55] xylanase, and laccase activities. An unconventional pre-culture method was established by cultivating the P. ostreatus mycelia in a solid substrate medium for an initial fungal growth phase, followed by a transition to submerged fermentation through adding a liquid culture medium. The lignocellulolytic enzymes of P. ostreatus in different fermentation methods revealed wide differences. The higher yields of endoglucanase (3152 \u00b1 139 U/L[56]. A pH 5.5 was found to be optimum for the maximal yield of laccase in submerged fermentation of *Pleurotus* 1804 strain[57]. However, in the present study, pH contributed only 14.8% compared to the duration factor; which was the third most influential parameter (15.9%) on dioxin degradation after nitrogen and copper content.

The higher duration period had a greater impact on the process performance in this study since OEC value considered showed complete degradation of 2,3-DCDD only after 30 days of incubation. Even though laccase was produced with the initiation of the exponential phase of fungal growth, yet the catalytic action of the enzyme on 2,3-DCDD was slow. The main reason for this was the low bioavailability of this enzyme in submerged fermentation media. Thus, larger duration of the incubation period guaranteed wide spread distribution of the enzyme throughout the media targeting all the available dioxin molecules. On an individual basis, temperature and inoculum size contributed almost equal influence on

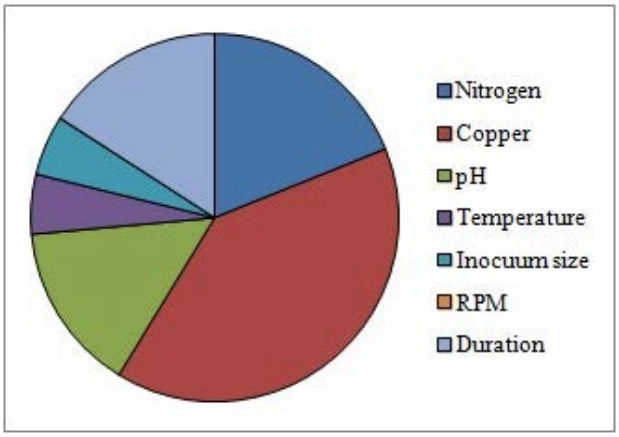

Figure 2. Relative percentage contribution of individual factors and interaction influences for *Pleurotus florida.*

the OEC value representing the cumulative result of both laccase production and 2,3-DCDD degradation. Figure 2 represents the relative percentage contribution of individual factors on laccase production by *P. florida*.

Copper exhibited higher influence at level 2 compared to level 1 on both laccase production and dioxin degradation percentage (Figure 1). Among the factors studied, RPM did not exhibit a clear difference in influential pattern on laccase enhancement (Figure 1). Low temperature supports the extensive growth of white rot fungi, as colder temperature condition promotes hyphal extensions and mycelial interconnections[58] in this fungal strains. Lower temperature condition is also a prerequisite for optimal activity of extracellular enzymes in detoxification studies by *Phanerochaete flavido-alba*[59]. Higher inoculums size results in larger biomass concentration which confirms improved laccase production due to increased hyphal extensions[60]. A multifold increase in laccase activity and biomass were observed when the ratio of inoculum size to medium composition was high for the cultivation of *Pycnoporus sp*. in a scale-up bioreactor[61]. Although the individual factors have a certain pattern of influence on laccase production, their interrelations exhibited an altogether different phenomenon.

3.3 Effect of Interaction between Factor Pairs

For a better understanding of the overall process performance, analysis of the interaction between two factors is inevitable. Regarding interacting factor pairs, RPM showed highest severity index with duration (SI - 93.03%) followed by inoculums size (SI - 90.76%) (Table 5).

Table 5. Interactions between different factors along with Severity Index (SI) % for *Pleurotus florida*

Sl.No.	Interacting factor pairs	Columns	SI (%)	Col.	Optimum
1	RPM × Duration	6 × 7	93.03	1	[1,1]
2	Inoculum size × RPM	5 × 6	90.76	3	[1,1]
3	Inoculum size × Duration	5 × 7	83.53	2	[1,2]
4	Temperature × Inoculum size	4 × 5	63.18	1	[1,1]
5	Copper × RPM	2 × 6	63.03	4	[2,2]
6	Copper × pH	2 × 3	57.63	1	[1,1]
7	Temperature × Duration	4 × 7	55.78	3	[1,1]
8	pH × Duration	3 × 7	44.21	4	[1,1]
9	Nitrogen × Copper	1 × 2	42.36	3	[1,1]
10	Temperature × RPM	4 × 6	36.96	2	[1,2]
11	Nitrogen × Inoculum size	1 × 5	36.81	4	[1,1]
12	Nitrogen × pH	1 × 3	19.69	2	[1,1]
13	Copper × Inoculum size	2 × 5	16.46	7	[2,1]
14	pH × Inoculum size	3 × 5	9.23	6	[1,1]
15	Copper × Temperature	2 × 4	8.09	6	[2,1]
16	Nitrogen × Duration	1 × 7	6.96	6	[1,1]
17	Nitrogen × RPM	1 × 6	6.26	7	[1,1]
18	pH × Temperature	3 × 4	5.11	7	[1,1]
19	Copper × Duration	2 × 7	2.84	5	[2,2]
20	pH × RPM	3 × 6	1.43	5	[1,1]
21	Nitrogen × Temperature	1 × 4	0.73	5	[1,1]

The least influential individual factor (RPM) exhibited the highest influence on OEC values. The least severity index on OEC values was contributed by the nitrogen-temperature factor pairs (SI - 0.73%). Despite being the second most influential factor, nitrogen contributed very little effect on OEC values on interaction with temperature which also contributed high impact on individual basis. The two most influential factors - nitrogen and copper contributed only 42.36% (SI) in interacting factor pair influence on process optimization. Hence, the effect of individual parameters largely varies when studied individually and in combination with other factors on media optimization for process enhancement. However, the influence of individual factors was considered more prominent in designing the optimized media conditions pertaining to enhanced laccase production and 2,3-DCDD degradation.

During submerged fermentation process, one factor interacts with one or more than one factor to give the final result in the end. Hence, it's paramount to understand the effect of such interacting factor pairs to ascertain their combinational influence on the study parameter. The level of interaction between two individual variables can be deduced from the severity index (SI) %. In Table 5, columns represent the locations to which the interacting factors are assigned. The SI % ranges from 0% to 100%, where 0% indicates parallel lines and no interaction between variables while 100% represents 90° angle between the lines (factors)[20]. The highest interaction SI 93.03% was observed in between RPM and duration on the combined

OEC effect of laccase production and dioxin degradation. This was followed by interaction between inoculums size and RPM (90.76%). It is interesting to note that RPM with the least individual impact factor on laccase production and dioxin degradation showed higher SI in combination with duration and inoculum size. On the contrary, the SI interaction between higher impact factors such as nitrogen and copper showed only 42.36%. It was evident from these observations that the effect of individual factors on laccase production and dioxin degradation had varying effects compared to their influence in combination on the cumulative parameter. Table 5 represents the possible combinations of interaction between two variables and is arranged in decreasing order of their SI between interacting factor pairs on the combined effect on laccase production and dioxin degradation by *P. florida*.

3.4 Analysis of Variance (ANOVA)

ANOVA was used to analyze the impact of individual factors on the combined effect of laccase production and 2,3-DCDD degradation through overall evaluation criteria (OEC). ANOVA is used to analyze the results obtained from Taguchi orthogonal array experiments and to determine how many variations was contributed by each factor. The percentage contribution of each factor to their respective variance (confidence limit 95%) is shown in Table 6. The statistical analysis revealed that copper (39.8%) was the most significant contributing factor for both laccase production and dioxin degradation. The next most significant factor for the study was nitrogen with 18.9%. Temperature and inoculums size (5.2% each) had similar percentage of contribution during the optimization study just like pH (14.8%) and duration (15.9%). The least contributing factor was RPM (0.01%) during this OA experimental study (Table 6). The relative percentage contribution of individual factors on the cumulative parameter of laccase production and 2,3-DCDD degradation by *P. florida* based on the ANOVA results is represented graphically in Figure 2.

3.5 Optimum Conditions and Validation

The Taguchi DOE provided the optimum conditions for each factor and their influence on maximal laccase production and dioxin degradation based on the OA of experimental runs as shown in Table 7. The expected result at optimum condition was 105.63 with the total contribution from all factors being 24.4. The maximal laccase production (773.04 U) and 2,3-DCDD degradation (100 %) (Table 2) was observed at optimal condition nitrogen - 0.2 mM, copper - 0.04 mM, pH - 5.5, temperature -25°C, inoculums size - 10%, RPM - 120 and duration - 30 days. The optimized conditions showed that copper was the most significant factor in both laccase production and dioxin degradation. The least significant factor during this experimental study was RPM. The variation reduction plot represented the performance distribution of current and improvised conditions after process optimization by Taguchi DOE (Figure 3). The experimental study predicted a 24.41% increase in laccase production, 20.21% increase in 2,3-DCDD degradation (Table 2) and a cumulative increase of 30.16% in the cumulative effect (OEC) from current a grand average performance of 81.15 to 105.63 (Table 7). Further, the validation experiments revealed an increase of laccase production by 21.34% (621.36U to 754.3U) and 20% increase in 2,3-DCDD degradation using the optimized conditions.

Table 6. Analysis of variance (ANOVA) of main effect of factors by *Pleurotus florida* on combined concept of laccase activity (U) and 2,3-DCDD degradation percentage

Sl.No.	Factor	DOF (f)	Sum of Squares (S)	Variance (V)	F-ration (F)	Pure Sum (S')	Percent P (%)
1	Nitrogen	1	327.554	327.554	-	327.554	18.93
2	Copper	1	606.043	606.043	-	606.043	39.826
3	pH	1	70.746	70.746	-	70.746	14.816
4	Temperature	1	2.745	2.745	-	2.745	5.225
5	Inoculum size	1	3.389	3.389	-	3.389	5.278
6	RPM	1	0.083	0.083	-	0.083	0.01
7	Duration	1	205.741	205.741	-	205.741	15.915
Total		7	1216.304			1216.304	

Table 7. Optimum culture conditions and their contribution

Sl.No.	Factor	Level description	Level	Contribution
1	Nitrogen	0.2	1	6.398
2	Copper	0.04	2	8.703
3	pH	5.5	1	2.973
4	Temperature	25	1	0.586
5	Inoculum size	10	2	0.651
6	RPM	10	2	0.101
7	Duration	30	2	5.071
Total contribution from all factors				24.483
Current grand average of performance				81.156
Expected result at optimum conditions				

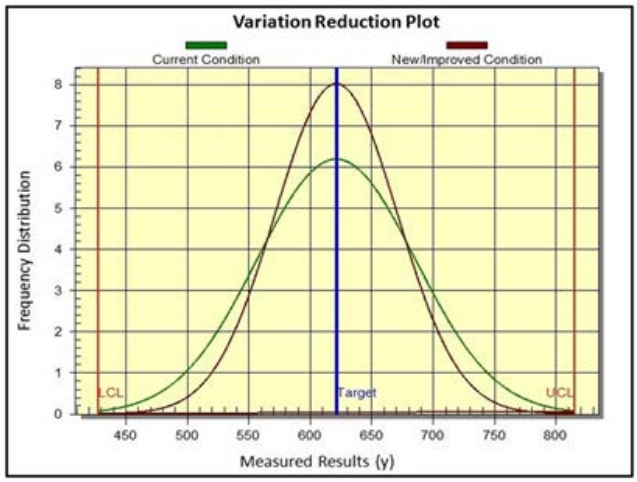

Figure 3. The variation reduction plot represents the performance distribution of current and improved condition after Taguchi DOE optimization study.

The laccase production of 754.3U and 100% degradation of 2,3-DCDD is very close to the predicted response of 773.046U and 100% respectively, thus validating Taguchi L-8 orthogonal array of the design of experiments for process performance optimization in *P. florida*. The proposed experimental methodology was validated using the optimized conditions obtained through Taguchi DOE; nitrogen 0.2 mM; copper 0.04 mM; pH 5.5; temperature 25°C, inoculum size 10 % w/v, RPM 120 and duration of 30 days (Table 7). The validation experiments resulted in an increase of laccase production by 21.5% (755 U) as predicted against an increase by 24.4% (expected result at optimum condition - 773.04 U -Table 2).

Similarly, complete degradation of 2,3-DCDD was observed in the fermentation media with optimized conditions validating the usefulness of the Taguchi approach to process optimization. Figure 3 represents variation reduction plot for understanding the performance distribution of pre-optimized conditions along with the post optimized conditions. An apparent increase in the frequency distribution can be observed in the optimized condition experiment proving an increase of laccase activity from 621.36 U to 755 U and complete (100%) degradation of 2,3-DCDD. This experiment further validated that there is a linear correlation between dioxin degradation and concentration of laccase.

4. Conclusion

Parametric optimization is the prerequisite for an incremented laccase production and 2,3-DCDD degradation in *P. florida*. Taguchi DOE proved to be an efficient statistical factorial design for optimization. From the study, copper that acts as a laccase inducer had the highest influence in the optimized media composition and physical parameter-RPM had the least influence. The validation experiments reported laccase production and dioxin degradation values similar to the predicted values by the statistical analysis, thus confirming the potential applicability of the current strategy. As mentioned earlier, this is the first report on parametric optimization of laccase production from *P. florida* by Taguchi DOE and its utilization for complete degradation of chlorinated dioxin molecules. The results obtained from the optimized conditions can be used as a prelude to designing large scale fermentation system for enhanced production of laccase. Thus, the present study formulates the applicability of the Taguchi DOE for designing large-scale experimental setup, useful for the bioremediation of dioxin contaminated matrices through enhanced laccase activity of *P. florida* strain.

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