

Chapter 4

*Optimization of
Laccase production*

by

Cerrena unicolor

MTCC 5159



ABSTRACT

To maximize laccase production, *Cerrena unicolor* MTCC 5159 was subjected to medium optimization. For laccase production, it was found that carbon and inorganic nitrogen act antagonistically with each other. A combination of low inorganic nitrogen and high carbon levels, favour biomass and laccase production. However, organic nitrogen at much higher concentrations also supported good laccase production. Buffering action of citrate-phosphate buffer is required to decrease the temporal variation in laccase production. Inducible laccases are produced in the idiophase only after addition of an appropriate inducer. Known inducers such as CuSO_4 induced maximum laccase activity however comparable activity had been obtained with 1 % raw textile effluent B which is economically lucrative. A single addition of inducer effectively aids in laccase induction, this is especially effective if added after sufficient growth has occurred. It has been observed that for growth of the fungus, pure sodium chloride cannot replace seawater and that oxygenation enhances laccase production. Addition of a surfactant such as Tween 80 at 0.25 % after sufficient growth had occurred positively impacted both biomass and laccase production. However at higher Tween 80 concentrations, laccase specific activity decreased. The surface to volume ratio of the culture vessel employed is critical for biomass and laccase production.

4.1 INTRODUCTION

Various factors influence laccase production, they include nutrients such as carbon, nitrogen, microelements and inducers. They also include cultivation conditions such as stationary or shake conditions, surface to volume ratio, pH of medium, temperature, light and oxygenation (Wesenberg *et al.*, 2003). Xavier *et al.*, (2001) have shown that laccase hyper-production was not dependent on high biomass yields since laccases are generally produced during the idiophase, in fungi.

D'Souza-Ticlo *et al.*, (2006) have shown that *Cerrena unicolor* MTCC 5159 under stationary conditions along with aeration produce a high titer of lignin-degrading enzymes. Fungi prefer acidic pH for growth, generally between 4.5 and 6; laccases are produced in abundance in this range. It has been found that maximal laccase production occurs when cultures are incubated at approximately 30°C in the dark (Thurston, 1994). Although high concentrations of an easily assimilable carbon source such as glucose have a positive impact on biomass accumulation, it showed an inhibitory effect or at least a delay in laccase production (Eggert *et al.*, 1996a). High levels of easily assimilable carbon source favour constitutive production of laccase but repress its induction (Bollag & Leonowicz, 1984).

The general consensus is that a high carbon to nitrogen ratio is required for laccase production. Laccase production occurs during secondary metabolic phase of fungi and is often triggered by nitrogen depletion (Keyser *et al.*, 1978). However, it was found that some strains were non-responsive to varying nitrogen concentrations (Leatham & Kirk, 1983) whereas, Buswell *et al.* (1995) have not only shown that laccases could be produced earlier with high nitrogen concentrations but also that these changes did not affect the biomass yield in anyway. The type of nitrogen also affects the types of laccase isozymes induced (D'Souza-Ticlo *et al.*, 2006). Hence, the nutritional requirements of the fungal strain under investigation were studied in detail before initiating medium optimization. Determination of the contribution of a particular nutrient without interference from other nutrients present in the medium can be achieved using mathematical modeling.

Main effects and interactions of the factors that play fundamental roles in the medium optimization for a particular response such as biomass or enzyme production can

be understood by applying statistical methods. Although several methods are found in literature, only a few such as the Plackett Burman design and response surface methods have been used in biotechnological processes (Mundra *et al.*, 2007; Soni *et al.*, 2007).

The medium components critical to either response requires to be first identified before the optimization of varying levels/concentrations of the components. This is done by screening a large number of medium components for their relative significance in affecting a particular response. Screening is generally done when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or nearly optimal responses. According to the classical method, screening procedure for each category of sources will consume a lot of time as it is done for one category at a time while keeping the other parameters/components constant. Experimental design techniques present a more logical alternative to the one-factor-at-a-time approach for medium improvement.

All possible process factors, expected to be crucial in experimental design are identified by consulting relevant literature. Once the list of factors has been made, the settings for each factor are determined. In a screening experiment, two settings corresponding to two different concentrations of the same medium component are chosen.

The screening design contains only a sub unit of all possible factor-setting combinations, resulting in fewer required combinations which is ultimately economical and time saving. The Plackett-Burman design is a type of two-level screening design which allows for the study of 'n' number of factors with 'n+1' + '2' Trials (Plackett & Burman, 1946). Moreover, the design is orthogonal in nature, thereby the effects of each component worked out are pure in nature and are not confounded with interactions among components for further optimization.

In the Plackett-Burman design to make the factors computationally equivalent, low and high factor settings corresponding to the low and high concentrations of a particular medium component are coded as -1 and +1, respectively. Each factor is tested equal number of times at its low and high settings. Because of this equal allocation within each factor, balance exists between each and every pair of factors throughout the design. Equal allocation and balance characterize all two-level designs and make statistically designed experiments complete and efficient from the stand point of resources used and information gained.

The screening method of Plackett-Burman frees the researcher from keeping ‘other factors’ at constant levels. The large number of components considered for experimentation is varied simultaneously but at different levels (as per a chosen design and ranges selected). The effects of individual components as well as their significance are conveniently computed mathematically:

$$Y = \beta_0 + \sum \beta_i X_i (i=1, \dots, k)$$

Where Y is the estimated response; β_0 the linear intercept; β_{i-k} are the linear regression coefficients; X_{i-k} are the experimental variables (i.e. medium components)

The coefficients obtained from this equation, give information about the impact of each factor individually on the response measured. A number of tools may be used to help assess the significance of each factor. These include p values, normal plots and pareto charts. The most common means of assessing significance is the ‘ p ’ value.

The p value is the probability that the magnitude of a parameter estimate is due to random process variability. A low p value indicates a “real” or significant effect and provides a base line for determining the relative criticality of the variables tested with respect to the response required. Out of these, generally the most effective components with positive effects are selected for further study, while those showing large negative effects may be dropped for all further experiments. This however is left to the discretion of the investigator depending upon the response studied. Therefore, dual purpose is served with such a systematic approach.

Once the critical factors have been identified via screening, one can then proceed to the optimization stage of experimental design. Information from screening experiments must first be used to fix certain factor settings and possibly revise the design space of the critical factors. All non-critical factors i.e. those that will not be tested further, must be fixed at a constant setting. Typically, the investigator will need to select either middle level of the factor setting range or level at which the factor provides the best response in light of individual effects and interactions observed earlier.

After the critical components have been identified via screening, their concentrations are required to be optimized to achieve maximum or minimum response as per the requirement. One way to achieve this is via a statistically designed optimized

study such as the Central Composite Design (CCD). This also confirms or rejects previous effects and interactions obtained from the screening experiment. The design can consist of two to five variables (Myers & Carter, 1973).

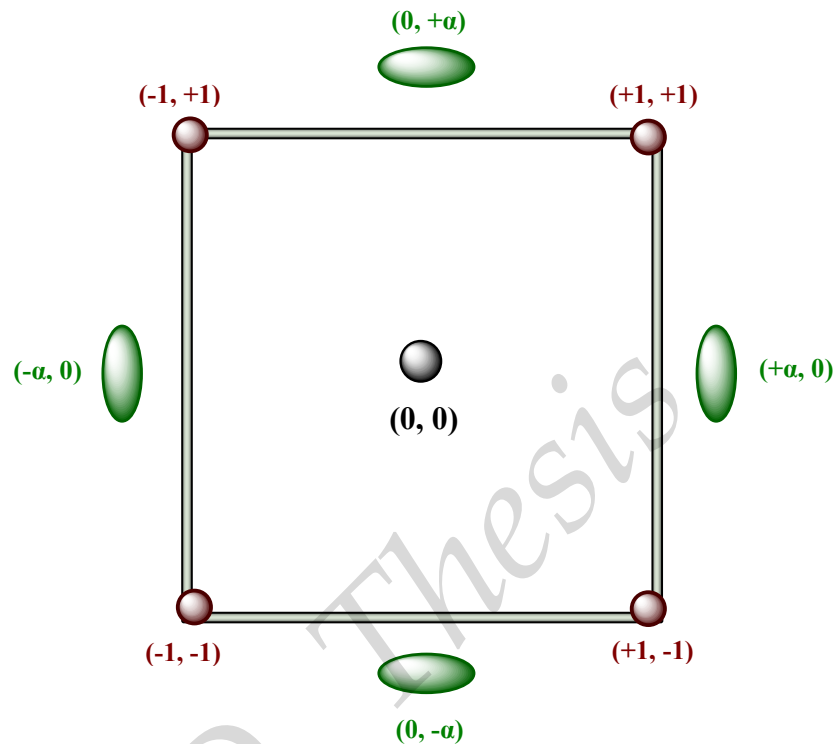


Fig. 1: Central Composite Design with two variables

In a Two Variable Composite design, variables can be presented as the two axis of a square (X_1, X_2). One end of the axis represents the high level and the other as the low level of the variable. Further, the center of the axis is presented as central level of variables according to the Central Composite design (Fig. 1). Complete randomization can be achieved by assuming all the levels as equally important and the central value as the optimum for maximum or minimum of response, as per the two experimental set-ups. Boundary limits of the design are considered as two levels. This requires a minimum of 12 experiments described as four corner points (high-low level coordination), 4 face / star points (boundary limits) and one center point (optimum level) of the square to achieve the goal of regression and estimation of error within experiments, the center point has to be repeated. The experimental data is then processed to fit the following second order polynomial equation: $Y = \beta_0 + (\beta_1 * A) + (\beta_2 * B) + (\beta_{11} * A^2) + (\beta_{22} * B^2) + (\beta_{12} * A * B)$

Where A and B are the experimental variables, β_0 is the constant coefficient, β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients whereas β_{12} is second order interaction coefficient.

Validation of the model developed is then carried out by comparison between the experimental and calculated values of response observed. If the calculated values fall close to the experimental value, the model is said to be fitted well. To validate a model, the lack of fit should be less than 50 % of the experiments.

After determining the critical nutrients which affect laccase production through screening experiments such as the Plackett Burman design and then optimizing their concentrations for the particular fungal strain being investigated, laccase production can be further enhanced by the inclusion of inducers. Many of these compounds resemble lignin molecules or other phenolic chemicals (Farnet *et al.*, 1999). They include aromatic compounds such as xyloidine, ferulic acid, guaiacol etc and a heavy metal such as copper (D'Souza *et al.*, 2006). These inducers activate response element sites in the promoter regions of laccase genes (Faraco *et al.*, 2003) and are known to increase the laccase titer ~400 times over the non-induced cultures (D'Souza *et al.*, 2006). These compounds can also affect the metabolism or growth rate hence indirectly, laccase production. White-rot fungi are very diverse in their response to laccase inducers (Eggert *et al.*, 1996a) and a large number of inducers have to be screened for their laccase-inducing ability with a particular fungus.

4.2 OBJECTIVES

The aim of this study was to determine the medium components critical to laccase production, optimize their concentrations and cultivation conditions that support high laccase yields from the selected hyper-secretory fungus, *Cerrena unicolor* MTCC 5159. The selected strain was evaluated for laccase production using different media, inducers and incubation conditions to support laccase production. Initial work was focused on the isolation, screening and identification of a laccase-producing marine-derived fungus (Chapter 2). Confirmation of the choice of hyper-secretory fungal strain was based on characteristics of the laccase produced by the selected hyper-secretory strain (Chapter 3).

4.3 MATERIALS AND METHODS

4.3.1. MICROORGANISM, CULTURE CONDITIONS AND RESPONSES

Cerrena unicolor MTCC 5159 was raised for 6 days in a medium identical to the one used in the subsequent experiment. Under sterile conditions, it was rinsed with DW then homogenized for 5 sec. The mycelial suspension was used as inoculum at 5 % v/v. The cultures were raised in 250 mL Erlenmeyer flasks with 20 mL total volume of medium i.e. surface to volume ratio of 2.5 and incubated at 30°C in the dark under static conditions. Various responses in terms of biomass accumulation, laccase activity, protein concentration and specific activity were determined, details of which are given in [Appendix 8.2.1.5](#), [8.2.2.1.1](#), [8.2.1.4](#) and [8.2.3.4](#) respectively. In all experiments, the biomass obtained on any particular day had been subtracted from the initial biomass used as inoculum.

4.3.2 Effect of synthetic and organic media

Three types of media were employed viz. a synthetic medium such as low nitrogen (LN) medium, an organic low nitrogen medium such as Boyd and Kohlmeyer (B&K) medium and a nutritionally rich high nitrogen organic medium such as Malt extract broth (MEB). The composition for LN, B&K and MEB are given in [Appendix 8.1.1 - 8.1.3](#) respectively. Laccase and its specific activity were determined every 3 days after inoculation until day 21.

4.3.3 LACCASE PRODUCTION MEDIUM

The laccase production medium always contained a mineral salts basal medium consisting of 5 % (v/v) basal salts solution ([Appendix 8.1.1.1](#)), 10 % (v/v) trace elements solution ([Appendix 8.1.1.2](#)) and 1 mL of 0.005 % (w/v) thiamine. The pH was adjusted to 4.5 using citrate-phosphate buffer and the final volume was made using distilled water. To this basal medium, glucose, ammonium chloride, copper sulphate, Tween 80 and sodium chloride were added at varying concentrations as per the requirement of the experiment.

4.3.4 Effect of glucose and citrate

To determine the type of interaction between the designated carbon source, glucose and citrate from the citrate-phosphate buffer since both contain assimilable carbon; three sets, G, C and B were prepared. In all sets, 0.2044 % ammonium chloride, 0.1 % NaCl, 0.05 % Tween 80 and 0.0375 M CuSO₄ were added to the mineral salts base. In addition to the above medium, in set ‘G’ only glucose at 4.5 %, in ‘C’ only citrate at 0.2 % and in ‘B’ both glucose and citrate at 4.5 and 0.2 % respectively were present.

4.3.5 Screening of significant components using Plackett Burman design

Screening of medium components was carried out using the Plackett-Burman design to determine their effect on biomass and laccase production. The five components used included glucose (carbon), ammonium chloride (nitrogen), CuSO₄ (inducer), Tween 80 (surfactant) and sodium chloride (salt). Low and high settings of each of these components were used to prepare a combination of eight Trials of production medium, whose compositions are specified in Table 1. Inoculation was carried out using 5 % (v/v) of the inoculum and the day of inoculation was considered as day 0. An additional set of experiment was carried out with a Cu spike (0.375 mM) on day 6. Both sets of experiments were monitored every 3 days from the day 0 to 15.

Table 1: Distribution of the low and high setting for five variables with the coded and uncoded values in eight experimental Trials of the Plackett-Burman design.

Trial	Variables									
	Coded					Uncoded				
	Glucose (%)	NH ₄ Cl (%)	CuSO ₄ (mM)	Tween 80 (%)	NaCl (%)	Glucose (%)	NH ₄ Cl (%)	CuSO ₄ (mM)	Tween 80 (%)	NaCl (%)
A	-	-	-	-	+	0.9	0.2044	0.0375	0.05	1
B	-	-	+	+	-	0.9	0.2044	0.375	0.25	0.1
C	-	+	-	+	-	0.9	1.022	0.0375	0.25	0.1
D	-	+	+	-	+	0.9	1.022	0.375	0.05	1
E	+	-	-	+	+	4.5	0.2044	0.0375	0.25	1
F	+	-	+	-	-	4.5	0.2044	0.375	0.05	0.1
G	+	+	-	-	-	4.5	1.022	0.0375	0.05	0.1
H	+	+	+	+	+	4.5	1.022	0.375	0.25	1

4.3.6 Optimization of medium components using Central Composite design

Glucose and ammonium chloride were used for medium optimization. The concentrations of glucose and ammonium chloride corresponding to the various coded levels including details elucidating the experimental design are given in [Table 2](#).

Table 2: Central Composite design matrix for the experimental design with the coded and uncoded values chosen for the experimental variables.

Trial	Variables			
	Coded		Uncoded	
	Glucose (%)	NH ₄ Cl (%)	Glucose (%)	NH ₄ Cl (%)
1	-1	-1	1.25	0.25
2	-1	+1	1.25	0.75
3	+1	-1	3.75	0.25
4	+1	+1	3.75	0.75
5	0	0	2.5	0.5
6	- α	0	0.7	0.5
7	+ α	0	4.3	0.5
8	0	- α	2.5	0.14
9	0	+ α	2.5	0.86
10	0	0	2.5	0.5
11	0	0	2.5	0.5
12	0	0	2.5	0.5

Each Trial contains different combinations of glucose and ammonium chloride. Copper sulphate at 0.375 mM, was added on day 6. Tween 80 was excluded from the medium. Laccase and biomass production were determined on day 12.

4.3.7 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Screening of the significance of medium components for biomass and laccase production was carried out by the Plackett Burman design. [Table 1](#) represents the low and high

settings for the five components under testing. A linear approach is considered sufficient for screening for the effect of these components.

$$Y = \beta_0 + \sum \beta_i X_i (i = 1, \dots, k)$$

Where Y is the estimated biomass or laccase production and β_i is the regression coefficient.

The Central Composite design (CCD) was used, to study the interactions between glucose and ammonium chloride as medium components. The design of the experiment along with the coded and uncoded variables for the two experimental factors presented in Table 2 is based on the results obtained from the screening experiment (Table 3A & B and Table 4A & B). The predicted and experimental values for laccase and biomass production obtained from the CCD experiment are given in Table 5. Various measurements for each experimental set were carried out in triplicates. Each experimental set was carried out in duplicate. The yields reported are the mean of the duplicates. The relationship of the independent variables (A & B); and their response (Y) estimated biomass or laccase production, were calculated by the second order polynomial equation, given below:

$$Y = \beta_0 + (\beta_1 * A) + (\beta_2 * B) + (\beta_{11} * A^2) + (\beta_{22} * B^2) + (\beta_{12} * A * B)$$

Where β_0 is the constant coefficient, β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients whereas β_{12} is second order interaction coefficient.

The statistical analysis using multiple regressions and MANOVA were performed with Statistica v 6.0 (StatSoft, Tulsa, Okla) software package.

4.3.8 Impact of addition of CuSO₄ and Tween 80

Production medium with 3.75 % glucose, 0.25 % ammonium chloride and 0.1 % NaCl was employed. To this, 0.375 mM CuSO₄ was added along with varying Tween 80 concentrations (0 % - 0.5 %) on day 6. Laccase and biomass production were estimated on day 12.

4.3.9 Effect of surface to volume ratio

Metabolite production is known to be enhanced under stationary conditions where the fungal mycelium is in maximum contact with the atmospheric surface above the medium. To test this, the following experiment was performed. The production medium containing 3 % glucose and 0.1 % NaCl with either 0.1 or 0.2 % ammonium chloride were tested along with varying flask capacities. The flask sizes ranged from 100 to 2000 mL capacity Erlenmeyer flasks. The volume of the production medium was maintained at 1/5th flask capacity. On day 6, 0.375 mM CuSO₄ was added along with 0.25 % Tween 80 and laccase and biomass production were measured on day 12.

4.3.10 Effect of oxygenation

The cultures were raised in 2000 mL capacity Erlenmeyer flasks in production medium containing 3 % glucose, 0.1 % ammonium chloride and 0.1 % NaCl at 1/5th flask capacity. Two identical experimental sets were maintained. In one set, the flasks (in triplicates) were oxygenated under sterile conditions with pure oxygen for 1 min using tygon tubing and a Pasteur pipette. Oxygenation was carried out every 3 days including day 0 until the measurement of response. The other set was not oxygenated. On day 6, in both the sets, 0.375 mM CuSO₄ was added along with 0.25 % Tween 80. Laccase and biomass production were measured on day 12 for both the sets.

4.3.11 Effect of inducers

In order to enhance laccase production, various known inducers at 1 mM such as *p*-anisidine, catechol, guaiacol, ferulic acid, vanillic acid, veratryl alcohol and 2,5 dimethyl aniline were used. A model lignin compound such as indulin SS at 0.25 mg mL⁻¹, 2 mM CuSO₄ and a combination of 2 mM CuSO₄ and 1 mM guaiacol were also used. Besides the known inducers, industrial effluents such as textile effluents A and B (TeA and TeB), molasses spent wash (MSW) and black liquor (BL) were also screened for laccase-inducing properties at 1 % final concentration. The details of TeA, TeB, MSW and BL

are given in [Appendix 8.4.1 - 8.4.4](#) respectively. The inducers were added on day 3 to B & K medium prepared in 25 ppt seawater.

4.3.12 Effect of various carbon sources

Various carbon sources at 1 % final concentration were tested for laccase production using a synthetic LN medium ([Appendix 8.1.1](#)) prepared in distilled water, without inclusion of an inducer. The carbon sources included monosaccharide hexoses such as glucose, fructose, galactose and rhamnose; monosaccharide pentoses such as ribose, arabinose and xylose; disaccharides such as trehalose, maltose, cellobiose, sucrose, lactose and melibiose; trisaccharides such as raffinose; sugar-alcohols such as inositol, mannitol, sorbitol and glycerol and the polysaccharide, starch. Glucosamine although containing nitrogen was also included as a carbon source. Laccase was estimated on day 12. The mean values from triplicate experiments were used for comparing the effect of various inducers.

4.4 RESULTS

Low nitrogen (LN) medium is a completely synthetic medium whereas B&K and MEB are complex organic media. The effect of synthetic and complex media on laccase production was determined (Fig. 2A).

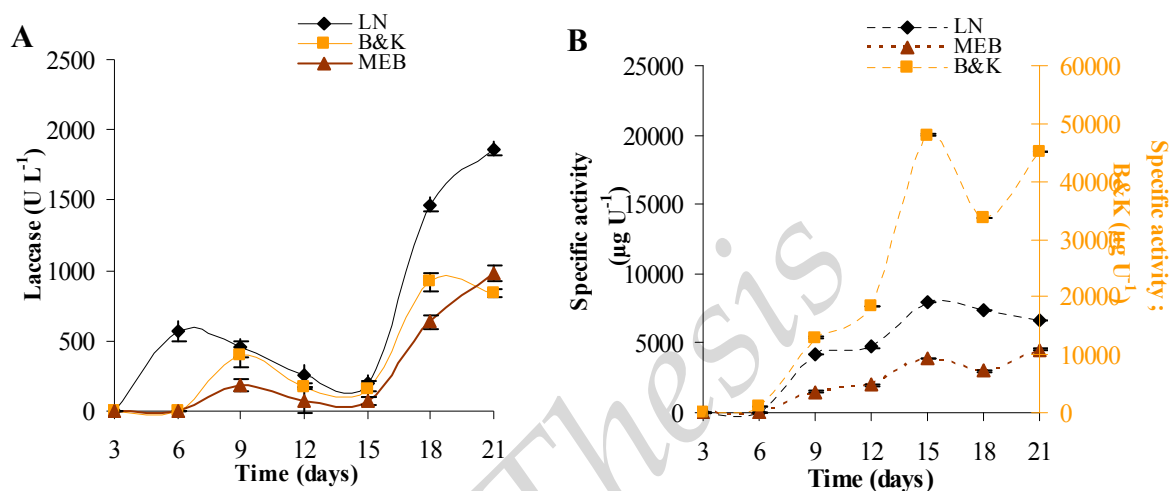


Fig. 2: Effect of medium types on (A) Laccase production and (B) Laccase specific activity.

The percent total nitrogen in LN, MEB and B&K are 0.0933 %, 3.256 % and 4.22 % respectively from calculations and carbon-nitrogen-sulfur (CNS) analysis. For all the sampling days, LN supported better laccase production than either B&K or MEB. However, maximum laccase specific activity was observed in B&K followed by LN and then MEB (Fig. 2B).

Low nitrogen medium was chosen to determine the effect of individual medium components on laccase production since it was a synthetic medium and supported maximum laccase production. This was performed using the Plackett Burman design, a statistical screening method that requires the medium components (in this case) to be independent of each other in affecting laccase production.

However, the designated carbon source, glucose and the buffer, citrate-phosphate, both contain carbon. Hence it was required to determine whether citrate could

be used as a buffer. Figures 3A and 3B show that both glucose and citrate served as independent sources of carbon for both laccase and biomass production.

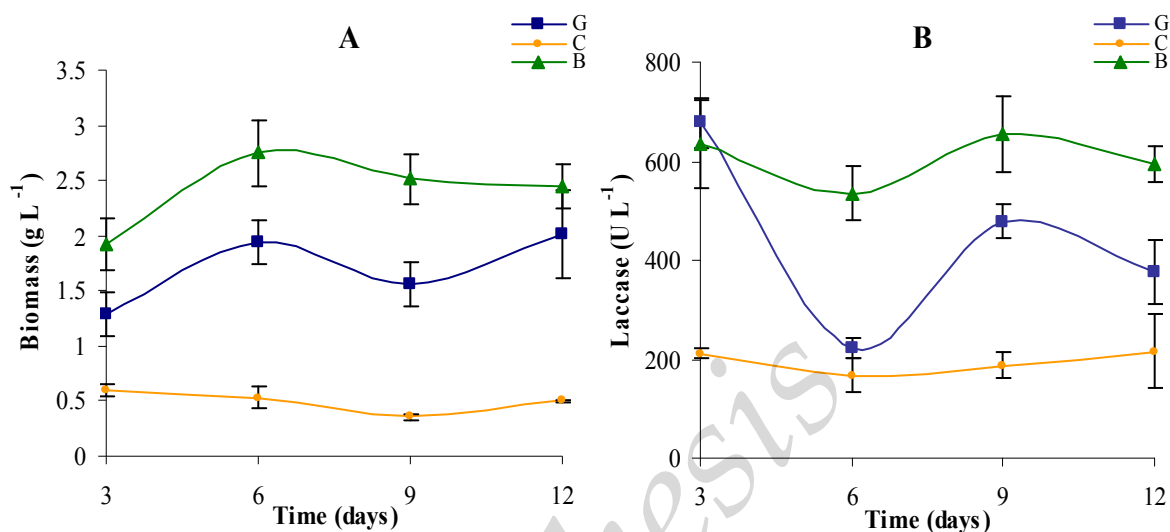


Fig. 3: Effect of glucose and citrate on production of (A) Biomass and (B) Laccase.

Here G = glucose only; C = citrate only and B = glucose and citrate both.

When only citrate was used, maximum laccase and biomass values were attained by day 3 and a low but stable biomass and laccase production was observed throughout. Whereas when only glucose was present, there was a large temporal variation in laccase production. When both glucose and citrate were present together, the effect was additive on biomass whereas for laccase besides an improvement in titer, temporal variation also decreased. No adverse interaction, between glucose and citrate for either laccase or biomass production was found. Thus, citrate-phosphate was used in the medium as a buffer and maintained at a constant level in all further experiments.

The screening of medium components for their significance was carried out using the Plackett Burman design. The experimental values of the Plackett-Burman sets determined at various sampling intervals for laccase and biomass are given in Table 3A. The effect on production of biomass and laccase due to a spike of 0.375 mM CuSO₄ on day 6 is presented in Table 3B.

Table 3A: Laccase and biomass production at 3-day sampling intervals, based on the eight Trials of the Plackett-Burman design.

Trial	Day 3		Day 6		Day 9		Day 12		Day 15	
	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)
A	0.96	807	1.21	901	1.30	888	1.08	48	1.70	3
B	0.48	17	2.59	2926	3.37	2358	3.36	1393	2.44	760
C	0.57	809	1.00	1651	1.25	792	1.32	3	1.55	220
D	0.38	8	0.51	33	0.62	908	0.34	3	1.15	84
E	0.89	6	1.85	1122	2.60	805	2.52	1070	2.12	156
F	1.00	10	2.52	1815	3.68	1744	3.47	1787	2.56	662
G	1.08	802	0.93	541	1.61	178	1.49	178	1.80	122
H	0.30	3	1.17	253	1.79	825	1.75	624	1.72	434

The medium composition of Trials A to H, are explained in Table 1. Here CuSO₄ was added only at the time of inoculation (day 0). This set of eight Trials is collectively referred to as the ‘*unspiked*’ set. Here laccase and biomass were measured every 3 days from day 3 to 15.

Table 3B: Laccase and biomass production at 3-day sampling intervals, based on the eight Trials of the Plackett-Burman design after the CuSO₄ spike on day 6.

Trial	Day 9		Day 12		Day 15	
	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)
A'	0.93	502	1.21	254	1.50	145
B'	3.62	320	3.12	74	2.41	67
C'	1.08	288	1.10	82	1.24	72
D'	0.11	301	0.36	88	0.76	73
E'	2.44	302	2.26	62	2.09	65
F'	3.64	110	3.51	91	2.62	71
G'	1.15	100	1.62	102	1.55	82
H'	1.58	67	1.43	54	1.37	45

The medium composition of Trials A' - H' is identical to Trials A - H except besides day 0, 0.375 mM CuSO₄ was also added each of the Trials on day 6. This set of eight Trials is collectively referred to as the ‘*spiked*’ set. Here laccase and biomass were measured every 3 days from day 9 to 15.

In majority of the cases, Trial B showed greater laccase titer than other Trials (Table 3A & 3B).

Table 4A: Degree of positive and negative effects of medium components on biomass production according to the Plackett-Burman design.

Variables	Sampling Interval (Days)														
	3			6			9			12			15		
	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level
<i>Intercept</i>	1.250	0.016	0.0000	1.916	0.016	0.0000	2.310	0.060	0.0000	2.107	0.092	0.0000	2.142	0.152	0.0000
Glucose	0.061	0.003	0.0000	0.081	0.003	0.0000	0.217	0.010	0.0000	0.217	0.016	0.0000	0.095	0.026	0.0043
NH₄Cl	-0.305	0.012	0.0000	-1.393	0.012	0.0000	-1.739	0.045	0.0000	-1.692	0.069	0.0000	-0.794	0.113	0.0000
CuSO₄	-0.994	0.029	0.0000	1.327	0.030	0.0000	1.999	0.108	0.0000	1.854	0.167	0.0000	0.525	0.275	0.0849
Tween 80	-1.469	0.048	0.0000	1.798	0.050	0.0000	2.242	0.182	0.0000	3.213	0.282	0.0000	0.780	0.464	0.1235
NaCl	-0.172	0.011	0.0000	-0.639	0.011	0.0000	-1.002	0.041	0.0000	-1.096	0.063	0.0000	-0.465	0.103	0.0011
	Adjusted r ² = 0.9958			Adjusted r ² = 0.999			Adjusted r ² = 0.9952			Adjusted r ² = 0.9890			Adjusted r ² = 0.84900		
	S.E.E.: 0.0193			S.E.E.: 0.020			S.E.E.: 0.0729			S.E.E.: 0.1129			S.E.E.: 0.18545		
Variables	Sampling Interval (Days)														
	A spike of 0.375 mM CuSO ₄ was introduced on day 6.														
B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	
<i>Intercept</i>	2.126	0.092	0.0000	2.364	0.160	0.0000	2.141	0.1604	0.0000	2.141	0.1604	0.0000	2.141	0.1604	0.0000
Glucose	0.213	0.016	0.0000	0.211	0.027	0.0000	0.119	0.0272	0.0014	0.119	0.0272	0.0014	0.119	0.0272	0.0014
NH₄Cl	-2.053	0.069	0.0000	-1.710	0.120	0.0000	-1.130	0.1197	0.0000	-1.130	0.1197	0.0000	-1.130	0.1197	0.0000
CuSO₄	2.481	0.167	0.0000	1.653	0.290	0.0002	0.578	0.2900	0.0741	0.578	0.2900	0.0741	0.578	0.2900	0.0741
Tween 80	3.605	0.282	0.0000	1.502	0.489	0.0118	0.840	0.4894	0.1170	0.840	0.4894	0.1170	0.840	0.4894	0.1170
NaCl	-1.231	0.063	0.0000	-1.137	0.109	0.0000	-0.583	0.1088	0.0003	-0.583	0.1088	0.0003	-0.583	0.1088	0.0003
	Adjusted r ² = 0.9919			Adjusted r ² = 0.96483			Adjusted r ² = 0.90251			Adjusted r ² = 0.90251			Adjusted r ² = 0.90251		
	S.E.E.: 0.1129			S.E.E.: 0.19546			S.E.E.: 0.19576			S.E.E.: 0.19576			S.E.E.: 0.19576		

Main effects (B) = biomass production (g L⁻¹); Std. Err. Of B = standard error of B for uncoded values; p-level = 0.05 % and S.E.E = Standard Error of Estimate.

Table 4B: Degree of positive and negative effects of medium components on laccase production according to the Plackett-Burman design.

Variables	Sampling Interval (Days)																				
	3			6			9			12			15								
	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level						
<i>Intercept</i>	952	146	0.000	2375	30	0.000	1471	26	0.000	540	86	0.000	198	37	0.000						
Glucose	-57	25	0.044	-124	5	0.000	-97	4	0.000	154	15	0.000	21	6	0.007						
NH₄Cl	239	109	0.053	-1310	23	0.000	-946	19	0.000	-1067	64	0.000	-221	28	0.000						
CuSO₄	-1768	264	0.000	602	55	0.000	2350	46	0.000	1858	155	0.000	1067	67	0.000						
Tween 80	-991	446	0.051	3329	92	0.000	1329	78	0.000	1343	262	0.000	873	113	0.000						
NaCl	-226	99	0.046	-1284	20	0.000	-457	17	0.000	-449	58	0.000	-302	25	0.000						
	Adjusted r ² = 0.7997			Adjusted r ² = 0.998			Adjusted r ² = 0.998			Adjusted r ² = 0.9761			Adjusted r ² = 0.9725								
	S.E.E.: 178.5200			S.E.E.: 36.884			S.E.E.: 31.245			S.E.E.: 104.7401			S.E.E.: 45.1227								
Variables	Sampling Interval (Days)																				
	A spike of 0.375 mM CuSO ₄ was introduced on day 6.												9			12			15		
													B	Std. Err. of B	p-level	B	Std. Err. of B	p-level	B	Std. Err. of B	p-level
<i>Intercept</i>													507	9.5	0.000	228	29	0.000	143	10	0.000
Glucose													-58	1.6	0.000	-13	5	0.024	-7	2	0.003
NH₄Cl													-146	7.1	0.000	-47	22	0.054	-23	7	0.010
CuSO₄													-292	17.1	0.000	-143	53	0.022	-80	18	0.001
Tween 80													-45	28.9	0.151	-329	89	0.004	-153	30	0.000
NaCl													98	6.4	0.000	30	20	0.155	10	7	0.161
													Adjusted r ² = 0.9956			Adjusted r ² = 0.66959			Adjusted r ² = 0.82375		
													S.E.E.: 11.5611			S.E.E.: 35.46043			S.E.E.: 11.88795		

Main effects (B) = laccase production (U L⁻¹); Std. Err. Of B = standard error of B for uncoded values; p-level = 0.05 % and S.E.E = Standard Error of Estimate.

Glucose has a positive impact on biomass production throughout. With the exception day 3, CuSO₄ and Tween 80 also had a positive impact on biomass however NH₄Cl and NaCl had a large negative impact throughout (Table 4A).

From the CuSO₄-spiking experiment in majority of the cases, it was observed that CuSO₄-spiking increased the magnitude of impact that the medium components had on biomass production. However, the deviation between the spiked and the unspiked set was not very large.

For laccase production, most of the medium components had a negative impact on day 3. The negative impact of glucose was reversed by day 12. Except for day 3, the impact of NH₄Cl was negative throughout and its maximum negative impact coincided with maximum laccase production on day 6 (Table 4A). When higher amounts of glucose and NH₄Cl were present simultaneously, laccase production was delayed. After a very high negative impact on day 3, the impact of CuSO₄ turned positive and remained considerably high for the remaining period. Tween 80 followed a similar trend to CuSO₄ but with a higher magnitude of positive impact and its maximum positive impact also coincided with maximum laccase production on day 6. Sodium chloride had a negative impact on laccase production and on day 6 its negative impact increased 6 fold which coincided with maximum laccase production.

The CuSO₄ spike on day 6 did not yield the expected spurt in laccase production. In fact, the laccase titer levels had drastically decreased (Table 3B) in such a way that the additional CuSO₄ spiking overshadowed the impact of all other medium components. From Table 4B, it is observed that sodium chloride had a negative impact on laccase production but CuSO₄ spiking resulted in a reversal of this impact to positive. From Table 3A it is evident that laccase production is at its maximum, 6 days after the addition of CuSO₄. For all further experiments, a single addition of 0.375 mM CuSO₄ on day 6 was carried out and laccase and biomass production was recorded 6 days after that i.e. on day 12. Since NaCl had a negative impact on both laccase and biomass production and was thus maintained at a constant low level.

Glucose and NH₄Cl as carbon and nitrogen source are essential for biomass accumulation yet both these components if present together at higher concentrations, negatively affected biomass and laccase. The concentration of both components required further optimization. The Central Composite design (CCD) was employed to achieve this.

The experimental values of the CCD are given in Table 5. These values were fitted in a second order polynomial equation. The values of regression coefficients were calculated and the fitted equations (using uncoded values) for predicting biomass (Y_B) and laccase (Y_L) production were;

$$Y_B = 1.575 + (0.5211*A) + (-0.1586*B) + (-0.05917*A*A) + (0.1224* B*B) + (-0.2988*A*B)$$

$$Y_L = 115 + (276*A) + (-364*B) + (10*A*A) + (1358* B*B) + (-634*A*B)$$

Where A, is glucose concentration and B, is NH_4Cl concentration.

Predicted values were generated using the above equation are also presented in Table 5. Irrespective of whether biomass or laccase was concerned, glucose and NH_4Cl interacted antagonistically with each other. Further, for biomass, the linear coefficient of glucose was strongly positive whereas its quadratic coefficient was slightly negative. On the other hand, the interaction of glucose with NH_4Cl was negative. The quadratic coefficient of NH_4Cl for biomass, indicates that biomass accumulation is not very dependant on NH_4Cl concentration and only low concentrations of NH_4Cl appeared favourable for laccase production.

Table 5: Central Composite design matrix for the experimental design with predicted and experimental results for biomass and laccase production.

Trial	Experimental values		Predicted values	
	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)	Biomass (g L ⁻¹)	Laccase (U L ⁻¹)
1	2.0	374	2.01	271
2	1.56	254	1.80	372
3	2.62	917	2.38	690
4	2.24	135	1.81	-2
5	1.93	282	2.09	233
6	1.87	156	1.76	249
7	2.43	153	2.03	281
8	2.26	416	2.38	621
9	1.79	325	1.82	196
10	2.0	271	2.09	233
11	1.9	286	2.09	233
12	1.89	243	2.09	233

A surface plot for both biomass and laccase production elucidating the interaction between glucose and NH_4Cl was plotted using the above equations (Fig. 4A & B). For laccase production (Fig. 4B), glucose and NH_4Cl act antagonistically with each other, hence the inverted bell shaped curve and the low r^2 values.

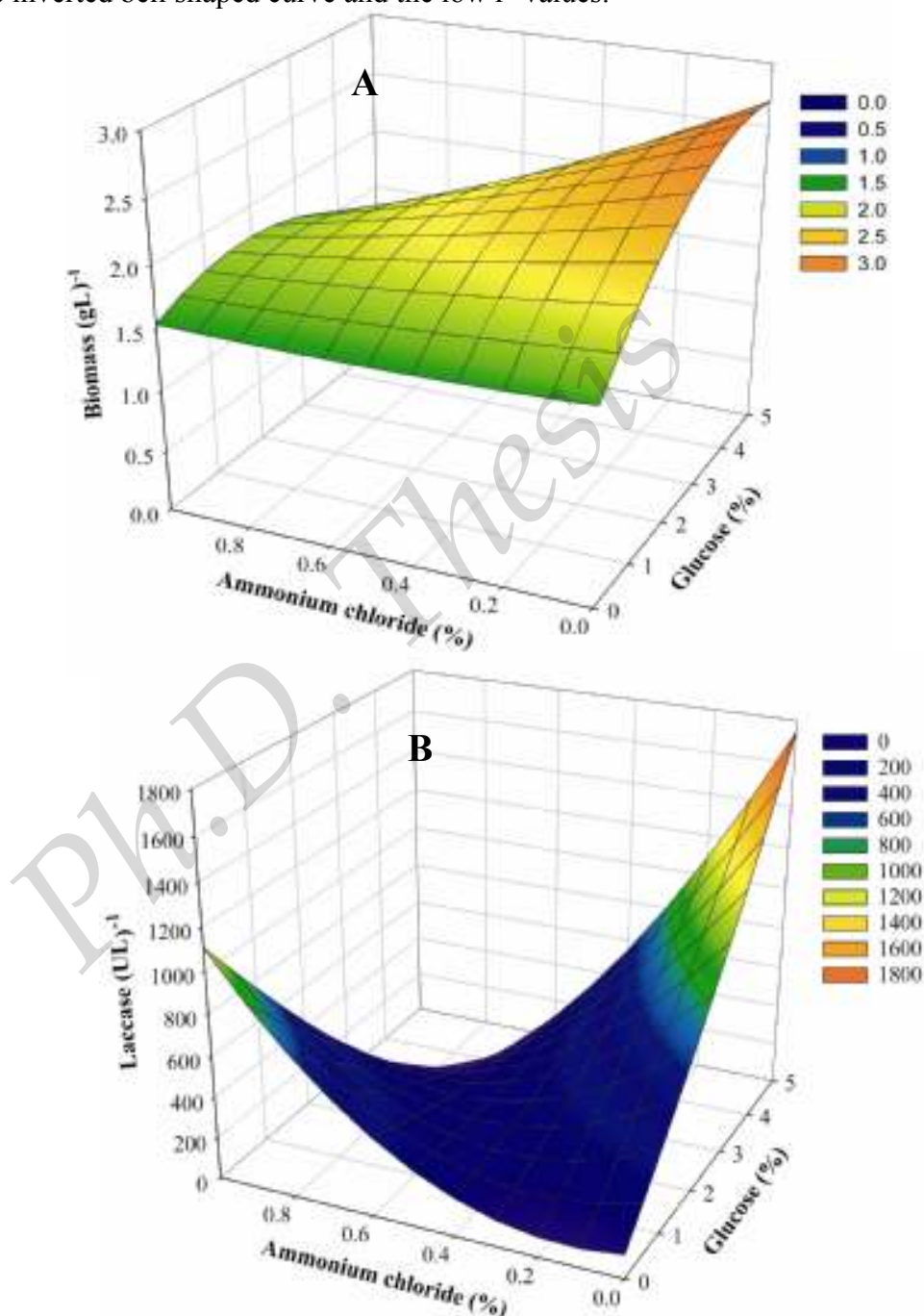


Fig. 4: Surface plots of production of (A) Biomass and (B) Laccase, illustrating the interaction between glucose and ammonium chloride.

A medium containing either a combination of high NH_4Cl and low glucose or a combination of low NH_4Cl but high glucose concentration would support greater laccase production. However only high glucose favours biomass build up which is required for laccase production. Hence a medium containing high glucose and low NH_4Cl concentration would support both biomass and laccase production. From these results, it appears that for laccase production, the concentration of NH_4Cl is more critical than glucose and merits further investigation.

Tween 80 was omitted from the experimental set up in CCD as its concentration was determined independently (Fig. 5).

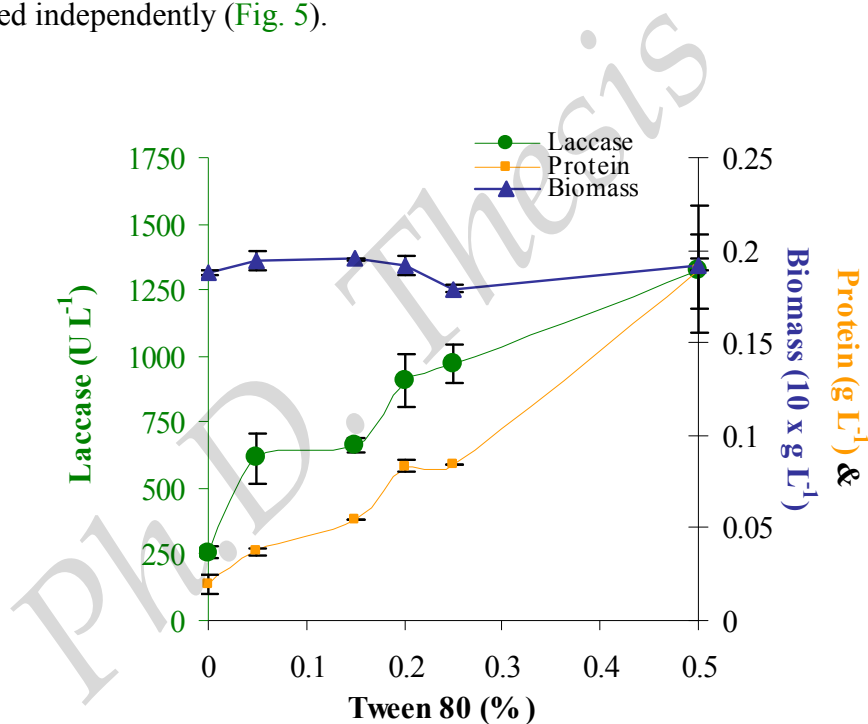


Fig. 5: Effect of varying concentrations of Tween 80 on laccase, protein and biomass production.

Increasing Tween 80 concentrations did not significantly affect biomass production. However, both laccase and protein production were roughly proportional to Tween 80 concentration. Also beyond 0.25 % although laccase concentration increased, its specific activity decreased significantly. Thus 0.25 % Tween 80 was selected for further work.

The effect of surface to volume ratio on biomass, laccase, protein and specific activity are presented in Fig. 6A-D.

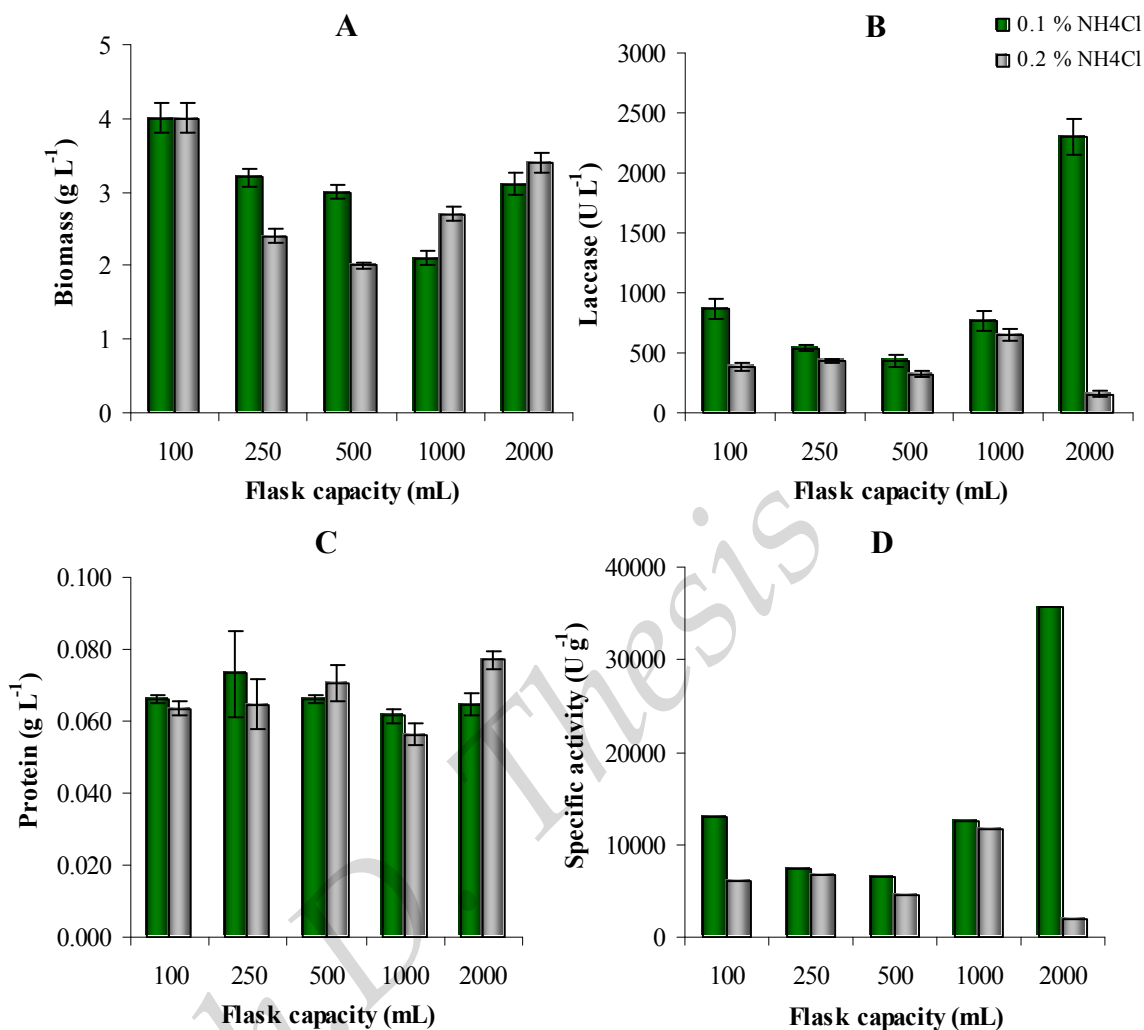


Fig. 6: Effect of surface to volume ratio on (A) Biomass; (B) Laccase; (C) Protein and (D) Specific activity.

The surface to volume ratios of the 100, 250, 500, 1000 and 2000 mL capacity flasks were 1.59, 1.11, 0.87, 0.68 and 0.54 respectively. The surface to volume ratio is roughly inversely proportional to flask capacity. At surface to volume ratios up to 0.87, 0.1 % NH_4Cl supported better biomass production whereas, 0.2 % NH_4Cl supported better biomass production below 0.87-surface to volume ratio (Fig. 6A).

In all the flasks, 0.1 % NH_4Cl supported greater laccase production than 0.2 % . Increasing surface to volume ratios have been reported to favour laccase production however the 1000 and 2000 mL capacity flasks proved to be exceptions. Especially the 2000 mL flask with the least surface to volume ratio, supported maximum laccase activity

at 0.1 % NH_4Cl whereas at 0.2 %, it yielded the minimum (Fig. 6B). With 0.1 % NH_4Cl laccase specific activity increased only below 0.87-surface to volume ratio (Fig. 6D).

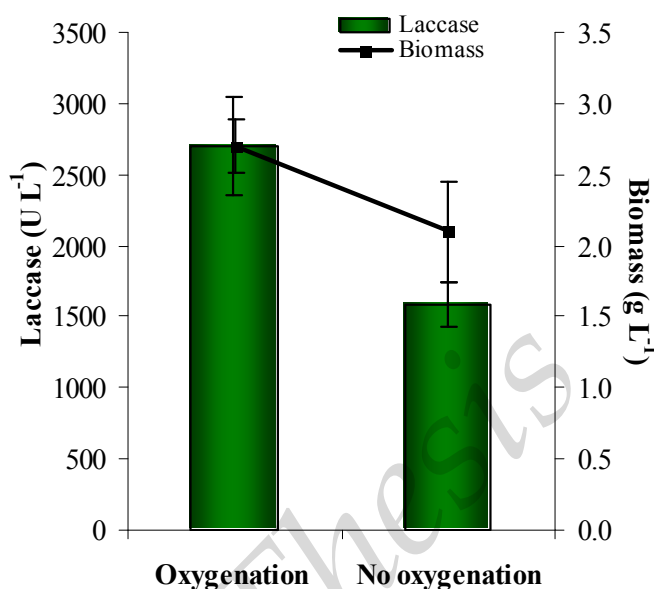


Fig. 7: Effect of oxygenation on laccase and biomass production.

From Fig. 7 it is clear that oxygenation had a positive effect on both laccase and biomass production compared to the unoxxygenated culture.

The effect of different known inducers on laccase and laccase specific activity are presented in Fig. 8 A₁ - B₁ and Fig. 8 A₂ - B₂, respectively. Among the aromatic inducers; ferulic acid and guaiacol were the most successful in laccase-induction, ferulic acid also supported high specific activity. Among the various known inducers used, CuSO_4 induced the maximum laccase. However the combination of an aromatic inducer such as guaiacol and copper supported consistently better laccase production (Fig. 8 B₁). The effect of industrial effluents on laccase induction and laccase specific activity are presented in Fig. 8 C₁ & C₂, respectively. Among the effluents, 1 % textile effluent B (TeB) induced the highest laccase activity (Fig. 8 C₁) the other effluents stimulated a moderate increase in laccase production.

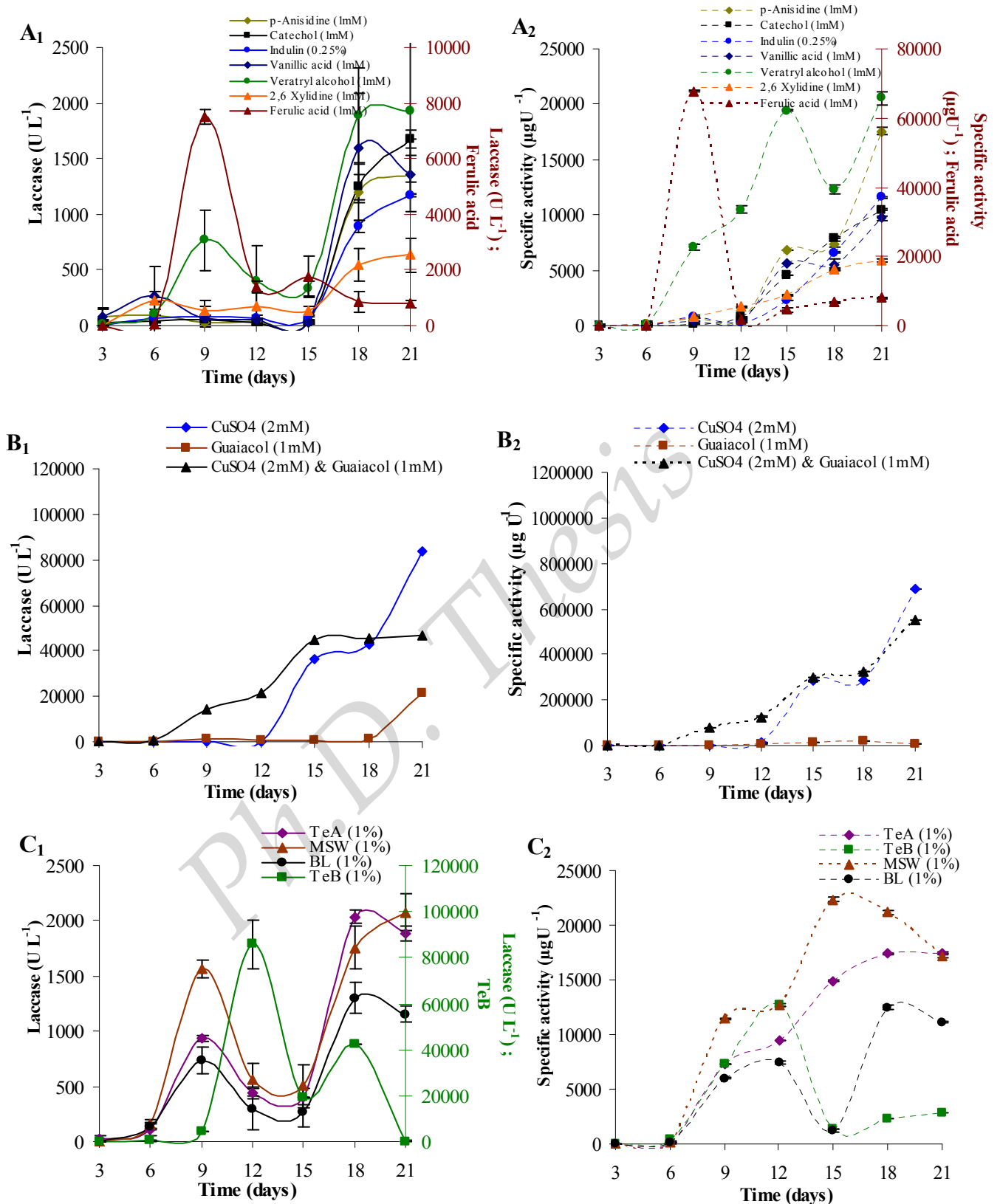


Fig. 8: Effect of different inducers on (A₁-C₁) Laccase production and (A₂-C₂) Specific activity.

The maximum laccase titer induced by either CuSO_4 or TeB were similar, however TeB was able to induce the same laccase titer 9 days earlier than CuSO_4 .

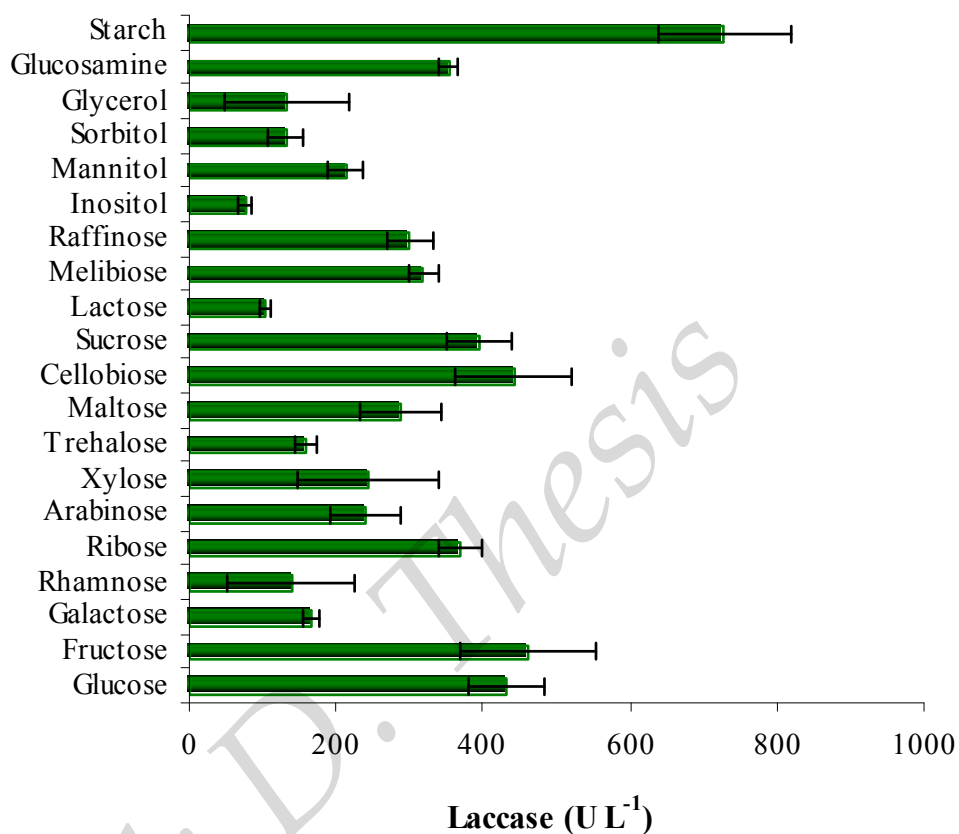


Fig. 9: Effect of different carbon sources on laccase production.

Among the carbon sources tested (Fig. 9), the polysaccharide starch, supported maximum laccase production. Monosaccharide hexoses such as fructose and glucose; disaccharides such as cellobiose and sucrose were also good carbon sources.

4.4 DISCUSSION

The cultures were incubated in the dark at 30°C, since the darkness has been shown to aid in laccase production (Thurston, 1994). Fungi have been cultivated at temperatures between 25° and 30°C for optimal laccase production. Temperatures higher than that have been known to reduce the activity of ligninolytic enzymes (Arora & Gill, 2000; Fåhraeus & Reinhammar, 1967; Lang *et al.*, 2000; Vasconcelos *et al.*, 2000).

As compared to either B&K or MEB, the low nitrogen content of LN most satisfactorily mimicked the nitrogen depletion which is known to trigger laccase production, when the fungus reaches the idiophase (Keyser *et al.*, 1978). Since LN is a completely synthetic medium, the effect of individual components could be estimated accurately. It supported maximum laccase activity as well and hence was chosen to determine the effect of individual medium components for its production.

Thurston, (1994) states that when fungi are grown in a medium at which the pH is optimal for growth (~pH 5) laccase would be produced in excess. Little information is available on the influence of pH on fungal laccase production and most studies indicate that pH levels are set between pH 4.5 and 6 prior to inoculation but not controlled during most cultivations (Arora & Gill, 2000; Fåhraeus & Reinhammar, 1967; Lang *et al.*, 2000; Vasconcelos *et al.*, 2000). In the present work too, the medium was initially set at pH 4.5, using citrate phosphate buffer. The amount of buffer required to set the medium pH to 4.5 contained about 0.2 % citrate whereas glucose was present at 4.5 %. The growth of this fungus was not determined using citrate at 4.5 % as was done with glucose. The effect of citrate was examined whether it serves as an additional carbon source when supplied solely through the buffer. Both served as independent carbon sources for laccase and biomass production. However due to the very low concentration of citrate, whatever biomass production could have been produced, was accumulated by day 3 itself, which itself was minimal. After which, there was no further biomass build-up i.e. the fungus reached its idiophase. When both glucose and citrate were present together, no adverse interaction was observed in fact, the effect was additive on biomass.

When citrate was the sole carbon source, the fungus went into stationary phase by day 3 itself since laccase is known to be produced in this phase, hardly any variation was observed in laccase production and the low laccase titer was probably due

to the low biomass that had been produced. The large variation in laccase titer observed only when glucose was present was probably due to the lack of buffering action that the citrate-phosphate buffer provided. When both glucose and citrate were present together, no adverse interaction was observed. In fact, there was a more consistent production of laccase. However, the percentage of carbon that was contributed individually by either carbon source, to the build up of biomass was not included in this study and merits further investigation. The addition of a basal level of citrate decreased but did not eliminate the temporal variability in laccase titer. Hence, the laccase titer was estimated every 3 days to determine the day at which maximum production was achieved.

The large negative coefficient of ammonium chloride and sodium chloride suggests that higher concentrations of either component would drastically and negatively affect biomass synthesis. However, it appears that inorganic salts affect biomass accumulation. Whereas the level of positive impact of glucose, copper sulphate and Tween 80 on biomass accumulation, reflects their criticality.

[Palmieri et al. \(2000\)](#) found that the addition of 0.15 mM copper sulphate to the cultivation media could result in a fifty-fold increase in laccase activity compared to the basal medium. Here, the copper sulphate was added to the medium on the day of inoculation itself. To avoid possible toxic effects of high levels of copper ([Baldrian, 2003](#); [Eggert et al., 1996](#)), in the present investigation its maximum concentration used was 0.375 mM. In spite of this, its presence at the time of inoculation resulted in a delay in biomass accumulation however, this impact was later reversed. The same trend was noticed with Tween 80. Prolonged incubation periods often lead to concentration of medium components due to moisture evaporation, this leads to increased concentrations of the other components present in the medium to such an extent that precipitation of critical components could occur or result in adverse interactions between components. The presence of Tween 80 probably decreases the evaporation of moisture, which could account for the very high positive impact it had on the production of biomass.

Overall, however the deviation in biomass between the spiked and the unspiked sets was not very large probably since sufficient growth had already occurred. Therefore, the effect of copper toxicity on biomass was not very evident.

All the medium components including glucose had a negative impact on laccase production on day 3, which got reversed later. This delay in laccase production in

the presence of increased amounts of glucose has also been previously observed by [Monteiro and De Carvalho \(1998\)](#). These findings are in concurrence with the earlier hypothesis that easily assimilable components allow for the constitutive production of laccase, but repress its induction ([Bollag & Leonowicz, 1984](#)) This view was later experimentally endorsed when starch, the only polysaccharide from all the carbon sources tested supported maximum laccase production ([Fig. 9](#)). The laccase titer in the presence of starch was roughly double than any of the mono- and disaccharides. Also, the presence of both constitutive and inducible laccase isozymes have been previously demonstrated in this fungus when grown in varying nitrogen sources ([D'Souza-Ticlo *et al.*, 2006](#)).

The nitrogen sources here included inorganic nitrogen such as potassium nitrate, amino acids such as glycine and glutamic acid and complex organic source such as beef extract and cornsteep liquor. All these nitrogen sources supported the production of a 47 kDa constitutive laccase, whereas glycine, beef extract and cornsteep liquor induced the production of a 55 kDa laccase, in addition to the constitutive laccase. An alternative method to avoid the delay in laccase production would be to use a carbon source that is not very easily assimilable so that instead of a constant but minimal level of constitutive laccase, an inducible laccase at high titer values is produced.

The impact of ammonium chloride an inorganic nitrogen source, was decidedly negative throughout. Its maximum negative impact coincided with maximum laccase production on day 6, indicating that the concentration of nitrogen is especially critical for laccase production.

Higher amounts of glucose and ammonium chloride significantly affected laccase by delaying its production. This may be due to the increased log phase of fungus resulting in a delayed production of laccase which is normally produced in the idiophase of the fungus.

After an initial highly negative impact on laccase production, the impact of both copper sulphate and Tween 80 became highly positive and were the highest among the components tested. Some of compounds affect the metabolism or growth rate while others, trigger laccase production ([Froehner & Eriksson, 1974](#); [Lee *et al.*, 1999](#)). It seems that at the concentrations used here, copper sulphate and Tween 80 affected biomass as well as laccase production. Heavy metals especially copper are well known laccase inducers and [Faraco *et al.*, \(2003\)](#) have experimentally proven that the promoter regions

of laccase genes contain various recognition sites that are specific for heavy metals, which induce laccase production. Heavy metals especially copper are also known to dictate the kind of laccases produced, the presence of copper favours the production of inducible laccases over constitutive ones (Robene-Soustrade & Lung-Escarmant, 1997). Apparently, in this medium setup the inducible enzymes are dominant due to the presence of copper. Surprisingly the second addition of copper sulphate led to a dramatic decrease in laccase production.

Sodium chloride, throughout had a negative impact on laccase production and copper sulphate spiking dramatically decreased this impact and turned it to positive. This lowering of the negative impact of sodium chloride after the second addition of copper sulphate was probably due to the drastic decrease in the overall laccase titer. Lower laccase titer values would result in a lowered impact rather than due to a positive interaction between copper sulphate and sodium chloride.

The overall positive impact of Tween 80 on laccase accumulation was probably due to either of the following reasons or a combination thereof ; (1) the surfactant property of Tween 80 which emulsifies the fungal membrane aiding in the release of cell membrane-associated laccases as well as increased secretion of the normal extracellular laccases. (2) The decrease in amount of evaporation of moisture due to the presence of Tween 80, which otherwise would have led to concentration of medium components during prolonged incubation periods and thus increasing adverse interactions between components or the precipitation of critical components.

The initial negative impact of the first addition of copper sulphate on laccase production suggests that either copper has an initial toxic effect on biomass accumulation or merely that the major laccases produced in this medium are not constitutive but of the inducible type and thus the mandatory lag before enzyme production begins. Strangely, an additional spike of copper on day 6 did not yield the expected spurt in laccase production considering that sufficient biomass had accumulated to offset any toxicity resulting from copper addition. In fact, the laccase production had drastically decreased in such a way that the additional copper sulphate spiking overshadowed the effect of all other medium components. Thus, copper sulphate was effective in increasing the laccase titer but when its concentration was increased by 0.375 mM, it had an adverse effect. Thus at 0.375 mM concentration (Trial B), copper sulphate positively affected laccase

production. However, the upper limit of copper sulphate concentration requires careful fine tuning. It is hypothesized that the initial addition of copper sulphate is welcomed by the fungus since it is used in biomass accumulation. Metals are required by fungi for their normal growth but more importantly in laccase synthesis as four copper ions are required per molecule of laccase, for it to be fully functional. However, the second addition of copper contrary to the assumption that sufficient biomass had accumulated to offset copper toxicity, adversely affected laccase production. Whether this toxicity was a universal effect or not cannot be ascertained since sufficient growth had already occurred, however laccase production was negatively affected. Either the synthesis of laccase was affected or merely its activity was affected while assaying due to the inhibiting and prolonged presence of copper. This requires further investigation and would put to rest the questions thrown up by the above observations. Thus, a single addition of copper is preferred. However, since its addition at the time of inoculation results in a delay, if the delay in laccase production was a function of toxicity on growth and not because laccase is generally produced in the idiophase, then a one time of addition of copper sulphate after sufficient growth has occurred would be ideal. This would overcome the toxic effect of copper on biomass yet retain its positive impact on laccase production.

Glucose and ammonium chloride as carbon and nitrogen source are essential and their concentrations critical for biomass and laccase production. The effect of the interactions between these two medium components on biomass and laccase production was interesting.

Irrespective of the whether biomass or laccase was concerned, glucose and ammonium chloride interacted antagonistically with each other. However, the degree of the antagonistic interaction with reference to biomass was negligible as compared to laccase. The linear and quadratic coefficients for biomass indicate that its accumulation was favoured in the presence of increased amounts of glucose but was almost independent of ammonium chloride concentration. Only low concentrations of ammonium chloride appeared to favour laccase production. The inverted bell shaped curve and the low r^2 values of the surface plot for laccase production further emphasizes the antagonistic interaction of glucose and ammonium chloride. These low r^2 values are a result of the antagonistic interaction between these two components and not a reflection

of poor experimental reliability, since for biomass, high r^2 values indicate that there was minimal error in experimental set up.

Since these two components interact antagonistically with respect to laccase production, a medium containing either a combination of high ammonium chloride and low glucose levels or a combination of low ammonium chloride and high glucose levels would be ideal for laccase production. Even though laccase production is known to increase during the stationary phase of growth or in secondary metabolism (Bourbonnais *et al.*, 1995), for laccase production to occur a critical biomass accumulation is required, which is only favoured at higher glucose concentrations. Thus, a combination of high glucose but low ammonium chloride concentration would yield sufficient biomass to produce laccase in an appreciable titer. However, the negative quadratic coefficient of glucose for biomass production and the positive but low value for laccase production, limits the maximum concentration of glucose that can be used, above which biomass and laccase would be detrimentally affected. This has been experimentally proven in laccase-producing fungal strains where excessive concentrations of glucose had an inhibitory effect on laccase production (Eggert *et al.*, 1996). It has also been experimentally proven that laccase production is highly dependent on the conditions of cultivation of the fungus (Heinzkill *et al.*, 1998) and media supporting high biomass does not necessarily support high laccase yields (Xavier *et al.*, 2001). This probably acts as a sort of check on uncontrolled laccase production which would probably have been harmful for the fungus since active growth requires the presence of metabolites and enzymes other than laccase, whose production may have not been possible if the entire cell machinery had been routed to laccase production.

Tween 80 concentrations did not significantly affect biomass production whereas there was approximately a linear correlation between laccase and Tween 80, the same relation was present with total protein. This medium was designed to selectively produce laccase and not the other lignin-degrading enzymes which this fungus is also known to secrete (as observed in Chapter 2). At lower concentrations of Tween 80, laccase was the major protein secreted but beyond 0.25 %, the medium became increasingly frothy due to its surfactant properties and the specific activity decreased greatly.

It is a well known fact that maximum metabolite production occurs under stationary conditions where the fungal mycelium is in maximum contact with the atmospheric surface above the medium and especially under higher oxygen tensions. This was experimentally confirmed since the oxygenated culture produced greater biomass as well as laccase than the unoxxygenated culture.

The experimental values of both the concentrations of ammonium chloride were lower than the predicted values since the equations for biomass and laccase were formulated using data obtained with a surface to volume ratio of 2.5, whereas in this case, the surface to volume ratio was only 1.1 less than half, emphasizing the role of surface to volume ratio in fungal metabolite production.

Surface to volume ratios above and equal to 0.87, included the 100, 200 and 500 mL capacity flasks. As the capacity suggests, these flasks were smaller in size than the 1000 and 2000 mL capacity flasks and hence the total biomass produced in these would be limited by their size. Thus, 0.1 % ammonium chloride was more than sufficient for supporting biomass production. Whereas for surface area to volume ratios below 0.87 i.e. the 1000 and 2000 mL capacity flasks, the total surface area was larger and accordingly the total biomass production that could be achieved, for which ammonium chloride at 0.2 % was required to support better biomass production. It seems that biomass production decreases, with decrease in surface to volume ratio up till 0.87. Below 0.87, the biomass production increases with further decrease in surface to volume ratio. It seems that below 0.87, the total surface area provided to the fungus gains more importance than the actual surface to volume ratio as seen in the 1000 and 2000 mL flasks.

The 2000 mL capacity flask with the least surface to volume ratio, supported maximum laccase production. This is probably since the 2000 mL capacity flask had maximum surface area than all the other flasks and thus produced maximum total biomass. The 0.1 % ammonium chloride mimicked the low nitrogen conditions required to induce laccase production. Thus, maximum total biomass and low nitrogen conditions, favoured laccase production, which was the highest among the varying ammonium chloride concentrations and flask capacities.

It has been experimentally proven that an organic nitrogen fared better in inducing laccase production than inorganic nitrogen (Hou *et al.*, 2004). It has also been

found that some fungal strains can be non responsive to varying nitrogen concentrations in terms of ligninolytic activity (Leatham & Kirk, 1983). Heinzkill *et al.* (1998) and Buswell *et al.* (1995) reported a higher yield of laccase in nitrogen-rich media rather than the nitrogen-limited media usually employed for laccase induction although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. Thus, a high carbon to nitrogen ratio cannot be a universal pre-requisite for high laccase production, rather the ratio is strain-dependant as was observed by Buswell *et al.* (1995).

Whether this fungal strain has a strict requirement for low nitrogen concentrations for maximum laccase production to occur or whether this rigid requirement for low concentrations is a result of the inorganic nature of the nitrogen source needs further study. B&K contains forty times more nitrogen than in the organic form and glucose, less than one fourth present in the LN medium. According to the CCD results, the composition of the B&K medium, with its low carbon to nitrogen ratio should support very low laccase production. However, this medium was also supplemented with several aromatic compounds, an inorganic salt such as copper sulphate and some industrial effluents which also acted as laccase-inducers. Laccase was estimated every 3 days, since the growth conditions were very different from those maintained during the screening and optimization studies and the day at which maximum laccase production occurred may not be the same.

This is the first report wherein high amount of laccase (921 U L^{-1}) is reported to be produced by marine-derived fungus when grown in seawater medium with peptone as a nitrogen source, without any inducer. Biomass production in this medium was rapid in organic medium and could easily sustain the addition of 2 mM copper sulphate even on day 3 which resulted in ~ 100 -fold increase in laccase production (83619 U L^{-1}). Besides, addition of aromatic compounds such as *p*-anisidine, catechol, guaiacol, ferulic acid, vanillic acid and veratryl alcohol also induced laccase. This is also the first report where a 1 % textile effluent B (TeB) increased laccase production by ~ 100 -fold. Other effluents such as black liquor from paper and pulp mill and molasses spent wash from alcohol distillery also induced laccase but to a lesser extent. The effluents acted as inducers of laccase were also simultaneously decolourized. These by-products probably further induced laccase. Textile effluent B had an acidic pH of 2.5

whereas textile effluent A had a pH of 8.9 this was probably the reason for the much lower laccase induction and activity.

From the laccase titers obtained, it is apparent that the presence of inducers and nitrogen in the organic form offset the negative effects of the high nitrogen to carbon ratio. A batch culture of this fungus in B&K medium with 1 % textile effluent could be routinely used to obtain laccase activity of $\sim 80 \text{ U mL}^{-1}$. This is much higher than those reported in terrestrial fungi recently (Jordaan & Leukes, 2003; Niku-Paavola et al., 2004; Nyanhongo et al., 2002; Wesenberg et al., 2003).

Although the fungus showed maximum biomass and laccase production in the seawater medium at 25 ppt as seen in Chapter 2, addition of sodium chloride had an inhibitory effect as seen in the screening experiments. Seawater with its other constituents supports growth and apparently unlike sodium chloride. Therefore, for the growth such a marine-derived fungus, sodium chloride may not be able to replace the requirement of seawater. Although sodium chloride at such low concentrations does not affect laccase activity as seen in the previous chapter, it does adversely affect its production. This adverse effect could also be due to the combined stress of the synthetic nature of the medium, which contained only inorganic salts since it is a known fact that fungi have a preference for organic substrates (Hou et al., 2004). The level of tolerance of this fungus to sodium chloride concentrations in organic and inorganic media requires to be investigated further.

Thus, a high carbon to low nitrogen ratio or vice versa may not be the main criteria in maximizing laccase production. Rather the overall conditions such as the kind of form of nitrogen present, time of addition of inducers, presence of seawater rather than pure sodium chloride and especially the presence and concentration of inducers are equally important for laccase production in this fungus.