Optimization of Lignin Peroxidase, Manganese Peroxidase, and Lac Production from *Ganoderma lucidum* Under Solid State Fermentation of Pineapple Leaf

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This study was undertaken to isolate ligninase-producing white-rot fungi for use in the extraction of fibre from pineapple leaf agriwaste. Fifteen fungal strains were isolated from dead tree trunks and leaf litter. Ligninolytic enzymes (lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac)), were produced by solid-state fermentation (SSF) using pineapple leaves as the substrate. Of the isolated strains, the one showing maximum production of ligninolytic enzymes was identified to be *Ganoderma lucidum* by 18S ribotyping. Single parameter optimization and response surface methodology of different process variables were carried out for enzyme production. Incubation period, agitation, and Tween-80 were identified to be the most significant variables through Plackett-Burman design. These variables were further optimized by Box-Behnken design. The overall maximum yield of ligninolytic enzymes was achieved by experimental analysis under these optimal conditions. Quantitative lignin analysis of pineapple leaves by Klason lignin method showed significant degradation of lignin by *Ganoderma lucidum* under SSF.

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INTRODUCTION

Pineapple (*Ananas comosus*) leaves contain 2.5 to 3.5% of textile grade fibre (Paul *et al.* 1998), but an assured supply to the processing industry is a challenge. Pineapple is cultivated in India on approximately 87,200 hectares of land; roughly 600,000 tons of pineapple leaf fibre (PALF) can be extracted from post-harvest leaf waste (Doraiswami and Chellamani 1993). In Kerala, India, pineapple is cultivated on 12,500 hectares of land. Pineapple farms are present in Ernakulam, Kottayam, Pathanamthitta, Idduki, and Kozhikode districts. Vazhakulam, which lies east of Muvattupuzha in the Ernakulam district, is famous as Pineapple City, since it is the biggest pineapple market in Asia. At present, pineapple cultivation in Kerala generates employment of about 4.5 million man-days in farming, transportation, warehousing, and retailing (Joy 2010).

Lignin is present in the plant cell wall to give structural support, impermeability, and resistance against microbial attack and oxidative stress (Sanchez 2009). It is linked to both hemicellulose and cellulose, forming a physical seal that is an impenetrable barrier in the plant cell wall. Although lignin resists attack by most microorganisms,

basidiomycetes white-rot fungi are able to degrade lignin efficiently (Wong 2009) as they produce various extracellular ligninolytic enzymes, such as laccase (Lac, EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1.13), and lignin peroxidase (LiP, EC 1.11.1.14) (Nagai *et al.* 2007). Due to their ability to remove lignin, there is considerable interest in the applications of these fungi in bio-pulping for paper, textile, and chemical industries. Ligninolytic enzymes could replace the conventional chemical processes of several industries (Blanchette 1991).

Solid-state fermentation (SSF) is defined as a process in which micro-organisms are grown on solid substrates in the absence of free water (Lagemaat and Pyle 2001). The application of the ligninolytic enzymes for lignin degradation in industrial bioprocesses requires the utilisation of an efficient production system (Robinson *et al.* 2001). SSF is an attractive technique for enzyme production because it presents many advantages, especially for fungal cultivation. In SSF, the productivity per reactor or fermenter volume is much higher compared with that of submerged culture (Grajek 1987). Also, the operation cost is lower because of the use of simple machines that use less energy (Roche *et al.* 1994). Utilisation of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilised residues (Robinson *et al.* 2001).

Ganoderma, a polyporoid fungus of the order Polyporales, has a worldwide distribution. It has taken an important place in various studies during the last few decades. *Ganoderma lucidum* is a fungus famous as a tonic in traditional Asian medicines because of its properties and its production of numerous biologically active compounds (Jo *et al.* 2011; Berovic *et al.* 2003). Recent investigations have found that *Ganoderma* contains natural chemicals called triterpenoids, which possess important anti-cancer properties (Harhaji Trajkovic *et al.* 2009). *Ganoderma lucidum* produces three extracellular ligninolytic enzymes (D'Souza *et al.* 1999; Silva *et al.* 2005), which have been reported to be produced on different lignocellulosic substrates under SSF (Asgher *et al.* 2010).

The present study aims to improve the production of ligninolytic enzymes by *Ganoderma lucidum* under SSF using pineapple leaf as substrate through optimization of different process parameters. This study also tested the use of these enzymes for the degradation of lignin during fibre extraction from pineapple leaf agriwaste.

EXPERIMENTAL

Materials and Methods

Organism and growth medium

Several strains of basidiomycete fungi were isolated from dead and decaying wood and leaf litter from several locations in Kerala. The strains were cultured on potato dextrose agar (PDA) in petri plates and grown on test tube slants at 27 °C for 3 days. The slants were stored at 4 °C and sub-cultured every 4 weeks.

Genomic DNA extraction protocol

The DNA extraction was carried out using mycelium grown on PDA plates, by the method described by Rogers and Bendich (1994).

DNA amplification

Ribosomal DNA primers known to be conserved among fungal taxa were used to amplify the internal transcribed spacer (ITS) region. The Primers ITS1 and ITS4 were used (Sigma-Aldrich, USA). The sequences of the primers were as follows: ITS1(forward primer): 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4(reverse primer): 5'- TCC TCC GCT TAT TGA TAT GC-3' (White *et al.* 1991). Amplification was performed in 20 μL of reaction mixture containing 1 μL of DNA template, 2 μL 10X PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP (dATP, dTTP, dGTP, and dCTP), 0.4 μM of both primers, 1 unit Taq polymerase (Sigma-Aldrich, USA), and double-distilled water. Bio-Rad MJ Mini Thermal Cycler was used to run the Polymerase Chain Reaction (PCR). The PCR started with denaturation for 2 min at 95 °C. This was followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 2 min at 55°C and extension for 1 min at 72°C. The final step of extension was then carried out for 10 min at 72 °C before it was maintained at 10 °C. The PCR product was run on 2% agarose gel, stained with ethidium bromide (EtBr), visualized under UV light, and photographed using gel documentation system (SynGene). Sequencing of PCR product was performed on an ABI 3730XL DNA sequencer.

Preparation of substrate and inoculum

Pineapple leaf was selected as the substrate for SSF. Pineapple leaves were cut to a length of 2 inches and were autoclaved at 121 °C for 20 min. The moisture content was determined to be 90% by drying to constant weight at 110 °C in a hot-air oven. Fungal inoculum was prepared from spores harvested from test tube slants after 3 days cultivation with sterile distilled water. The spore suspension was passed through a 0.5 mm sieve to eliminate mycelia, and the spore concentration was estimated by direct microscopic counting using hemocytometer (Raimbault and Alazard 1980). The final concentration of the spore suspension was adjusted to 5×10^7 spore/mL. The inoculum was added to 250 mL Erlenmeyer flasks containing 10 g autoclaved pineapple leaves.

Enzyme activity assays

Enzyme was extracted by adding 50 mL of 0.1 M citrate buffer of pH 5 to the culture. The crude enzyme was filtered through Whatman No. 1 filter paper and the culture supernatant was harvested by centrifugation at 8000 rpm for 10 minutes.

Lignin peroxidase activity was estimated by the method of Tien and Kirk (1983). The assay is based on the oxidation of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) to veratraldehyde in the presence of H_2O_2; the increase in absorbance at 310 nm is monitored. Reaction mixture contains 0.25 mL of enzyme solution, 0.25 mL of 1 mM veratryl alcohol, 0.2 mM H_2O_2, and 0.5 mL of 0.1 M citrate buffer.

MnPase activity was assayed using phenol red as substrate (Kuwahara *et al.* 1984) by measuring optical density spectrophotometrically at 610 nm. The reaction mixture consists of enzyme extract (250 μL), 0.1% phenol red (50 μL), 250 mM sodium lactate (100 μL), 2 mM manganese sulphate (25 μL), 0.5% BSA (100 μL), 0.2 mM H_2O_2 (25 μL) in 0.1 M citrate buffer pH 5 (0.5 mL).

Lac activity was determined via the oxidation of 2,2'-azino-bis(3 ethylbenzthiazoline)-6-sulfonate (ABTS) (Papinutti *et al.* 2003). The reaction mixture

consists of 0.1 mL of 0.3 mM ABTS, 300 μL citrate buffer and 0.6 mL enzyme. ABTS oxidation was monitored by increase in absorbance at 420 nm.

All ligninolytic enzyme activity is expressed as IU/mL. An international unit (IU) is defined as the amount of enzyme activity which will catalyse the transformation of 1 micromole of the substrate per minute under standard conditions.

Process parameter optimization

Single parameter optimization was employed to assess the effect of each parameter on enzyme activity: inoculum (1 to 7 mL), temperature (23 to 33 °C), pH (3 to 8), incubation period (24 to 216 hrs), and different organic and inorganic nitrogen sources at 1% (w/v) (peptone, yeast extract, beef extract, urea, nutrient agar, ammonium sulphate, and ammonium nitrate), carbon sources at 1% (w/v) (glucose, sucrose, starch, maltose, and lactose), agitation (0 to 200 rpm), Tween-80 (0.1 to 0.5 mM), SDS (0.1 to 0.5 mM), pineapple leaf weight (5 to 25 g), and pineapple leaf length (0.5 to 1 inch).

Response surface was plotted using the statistical software package Design Expert® 8.0 (Stat-Ease, Inc., Minneapolis, MN) (Mishra and Kumar 2007; Arockiasamy *et al.* 2008; Bhattacharya and Banerjee 2008). The randomized factorial design of the experiment consisted of eleven factor variables (incubation period, inoculum, temperature, pH, pineapple leaf weight, pineapple leaf size, sucrose, agitation, Tween-80, SDS, and beef extract).

Chemical analysis

The cellulose, hemicelluloses, lignin, and pectin content of the control and fungal-treated leaf samples were evaluated after incubation of *Ganoderma lucidum* in SSF for 120 hrs with pineapple leaf under standardized conditions obtained from single parameter optimization.

A quantitative analysis of lignin degradation was done by estimation of Klason lignin (Theander and Westerlund 1986). In this method, hydrolysis of plant cell walls by sulphuric acid (70%) dissolves all carbohyrate components other than the insoluble lignin. The residual material is thus considered to be lignin. Determination of acid-insoluble (Klason) lignin was carried out for both control and fungal-treated pineapple leaf substrates after SSF.

Cellulose estimation was done by Anthrone method (Sadasivam and Manickam 2005). In this method 3 mL acetic/nitric reagent was added to a known amount (0.5 or 1 g) of the sample. To 1 mL of the sample (control and fungal-treated), 10 mL of anthrone reagent was added and mixed well in a test tube. The tubes were heated in a boiling water bath for 10 min. These were cooled and the colour developed was measured at 630 nm. A blank with anthrone reagent and distilled water was also tested.

Hemicellulose estimation of both control as well as fungal-treated sample is done by refluxing the samples with neutral detergent solution and acid detergent solution separately to remove the water-solubles and minerals other than the fibrous components. The left-out material is weighed after filtration and expressed as neutral detergent fibre (NDF) and acid detergent fibre (ADF). The difference between NDF and ADF is the hemicellulose content of the samples (Sadasivam and Manickam 2005).

Pectin content is estimated by gravimetric method (Sadasivam and Manickam 2005). Pectin content of both control and fungal-treated sample is weighed after precipitating it as calcium pectate by the addition of calcium chloride to an acid solution.

RESULTS AND DISCUSSION

Fifteen strains of fungi were isolated and maintained on PDA plates. From a preliminary screening for ligninase activity, the strain showing highest activity was selected for isolation of DNA. PCR amplifications of total genomic DNA of the fungus using primer pair ITS1–ITS4 (White *et al.* 1991) produced a PCR product of about 600 bp (base pairs) (Fig. 1). The PCR product was sequenced (GenBank Accession Number: JQ040846). The fungus was identified to be *Ganoderma lucidum* as it showed 100% homology with existing sequences under the same name in NCBI GenBank.
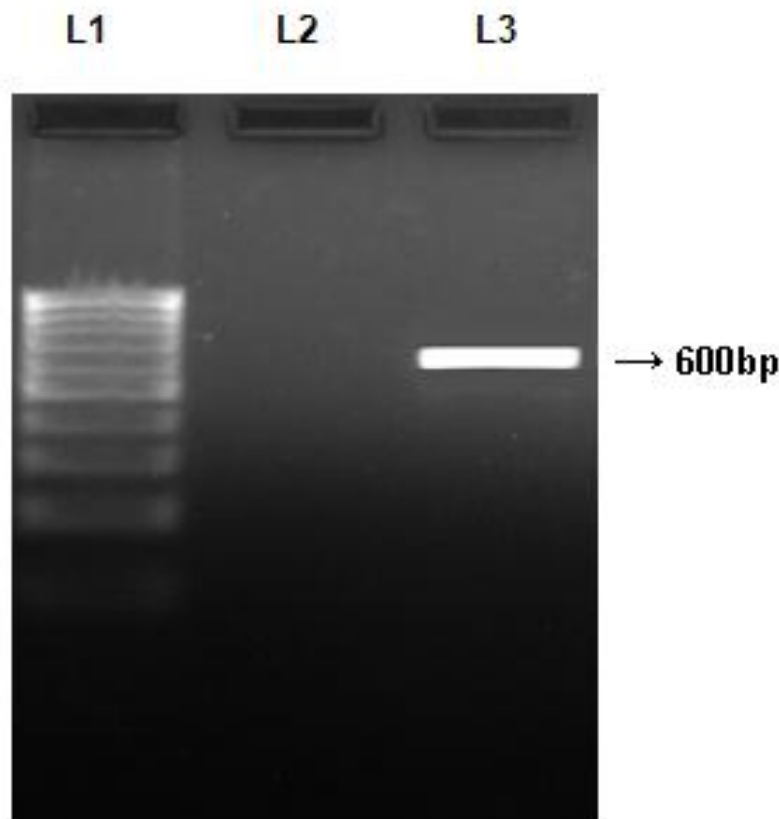
Fig. 1. Result of PCR amplification of the ITS region of the fungus using the primer pairs IT1–IT4; L1: 100 bp DNA ladder (Chromous Biotech), L2: Negative control of the PCR reaction mixture without adding the DNA, L3: PCR reaction mixture with DNA of the sample to be identified.

### Factors Affecting Enzyme Activity: Single Factor Optimization

To evaluate the effect of inoculum volume on ligninolytic enzyme production by *Ganoderma lucidum*, an inoculum range of 1 to 7 mL of spore suspension from 3 day old culture was added to different flasks. SSF was carried out for a period of 24 hrs and maximum enzyme production (LiP 1126.3 IU/mL, MnP 658.3 IU/mL, and Lac 317.8 IU/mL) was observed when an inoculum volume of 4 mL was added, as shown in Fig. 2A. Poor ligninase activity was observed with further increase in inoculum volume. Effect of incubation period on enzyme activity of *Ganoderma lucidum* was checked by

incubating them at a temperature range of 24-192 hrs with 4 mL inoculum volume. The ligninase production was at a maximum after 120 hrs of incubation (LiP 1203.7 IU/mL, MnP 650.8 IU/mL, and Lac 320.6 IU/mL), as seen in Fig. 2B which gradually decreased with increasing incubation periods. SSF was then carried out at different temperatures ranging from 23 to 33 °C and the enzyme samples were extracted after 120 hrs of fermentation. The organism exhibited better ligninolytic enzyme production at 27 °C (LiP 1236.2 IU/mL, MnP 677.4 IU/mL, and Lac 310.7 IU/mL) as shown in Fig. 2C which was found to decrease with increase in temperature. The effect of initial pH on enzyme activity was determined by adjusting pH of the moistening solution with 0.1 M citrate-phosphate buffer from pH 3 to pH 8. Fermentation was carried out for 120 hrs and maximum ligninolytic activity (LiP 1312.8 IU/mL, MnP 667.8 IU/mL, and Lac 330.7 IU/mL) was observed at pH 5.0 as shown in Fig. 2D. Ligninolytic activity was found to decrease by further increasing the pH range.

The effect of nutrient sources on ligninase activity was checked by the addition of various carbon and nitrogen sources. Different carbon sources tested included glucose, sucrose, starch, maltose, and lactose at 1% (w/w) (Fig. 2E). Incorporation of glucose and sucrose as carbon source enhanced enzyme yield compared to control. Sucrose (1% w/w) promoted maximal yield (LiP 2098.3 IU/mL, MnP 798.8 IU/mL, and Lac 402.3 IU/mL) after 120 hrs incubation. Other carbon sources tested showed decreased enzyme activity. Different nitrogen sources (peptone, yeast extract, beef extract, ammonium sulphate, ammonium nitrate, and urea) at 1% (w/w) were also tested for enhancing ligninase activity (Fig. 2F). Among the different nitrogen sources tested, beef extract (1% w/v) was the best nitrogen source to produce maximum activity of all three ligninases enzymes compared to control (LiP 2280.6 IU/mL, MnP 810.9 IU/mL, and Lac 425.8 IU/mL) after 120 hrs incubation.

In order to evaluate the effect of agitation on ligninolytic enzyme production, an agitation range of 0 to 200 rpm was provided under 120 hrs of incubation (Fig. 2G). The enzyme activity was found to be at a maximum at 100 rpm (LiP 2589.9 IU/mL, MnP 770.8 IU/mL, and Lac 450.5 IU/mL), which was then found to decrease with increasing the agitation. Effect of different concentrations of Tween-80 and sodium dodecyl sulphate (SDS) was investigated on ligninases biosynthesis in optimum fermentation medium. It was observed that both Tween-80 and SDS stimulated enzyme production in SSF when used in lower concentrations. Tween-80 produced maximum enzyme activity at 0.3 mM (LiP 2690.5 IU/mL, MnP 890.6 IU/mL, and Lac 490.2 IU/mL). SDS was found to produce maximum activity at 0.1 mM (LiP 2853.00 IU/mL, MnP 899.1 IU/mL, and Lac 495.3 IU/mL). Higher concentrations of surfactants, especially SDS, were found inhibitory to enzyme production by *Ganoderma lucidum* (Figs. 2H & 2I).

The effect of the amount of substrate on enzyme activity was also evaluated. Pineapple leaf weight and pineapple leaf length was varied from 5 to 25 g and 0.5 to 5 inches, respectively (Figs. 2J & 2K). The ligninolytic activity was found to be maximum with 10 g pineapple leaf as substrate (LiP 2875.7 IU/mL, MnP 826.6 IU/mL, and Lac 426.7 IU/mL). The enzyme activity was also found to be at a maximum with pineapple leaf with a length of 2 inches (LiP 2902.6 IU/mL, MnP 835.8 IU/mL, and Lac 436.3 IU/mL).
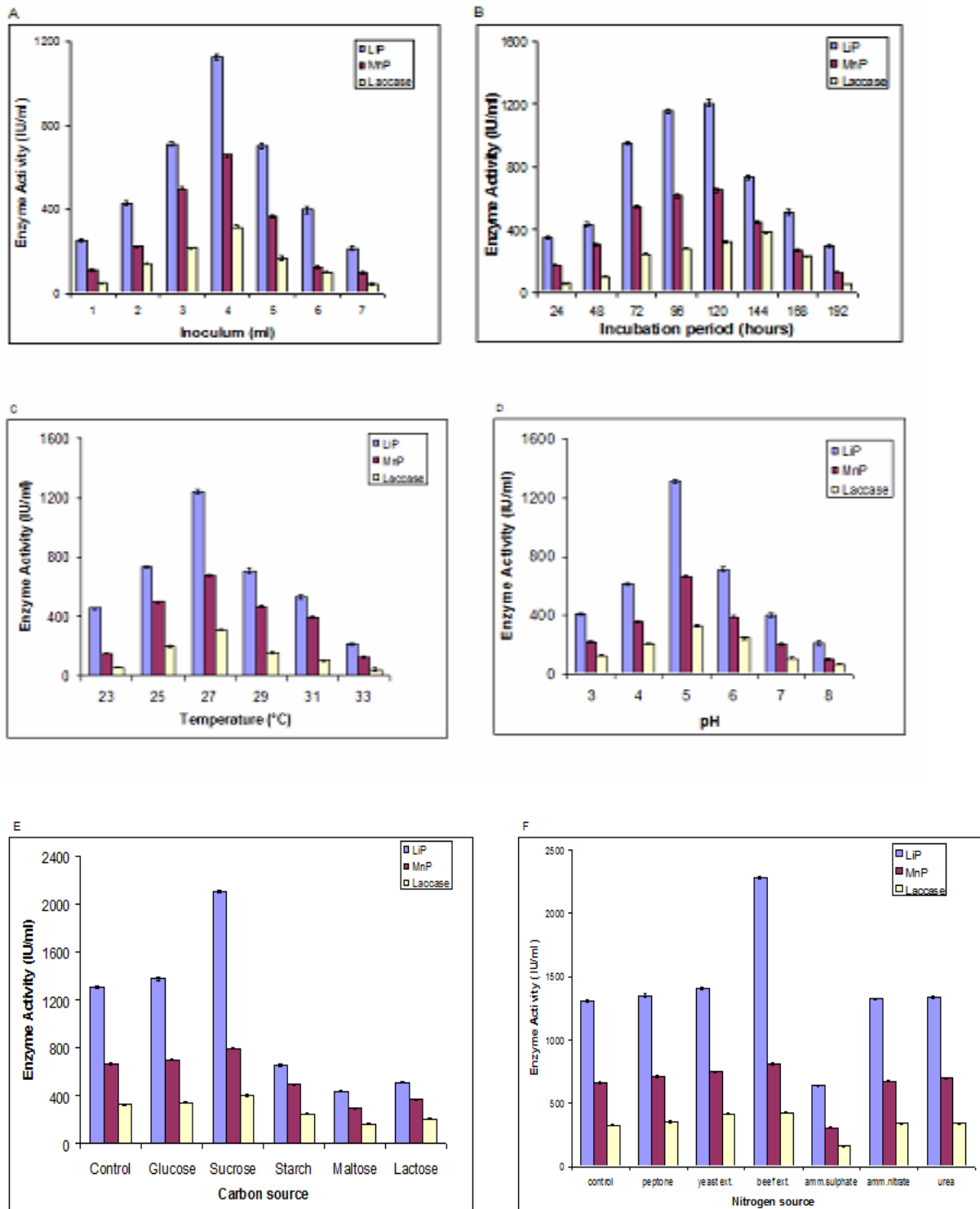
Fig. 2. Effect of A) Inoculum, B) Incubation period, C) Temperature, D) pH, E) Carbon source, and F) Nitrogen source on ligninolytic activity of *Ganoderma lucidum*. (Error bars represent $\pm$ standard deviation from a triplicate average)
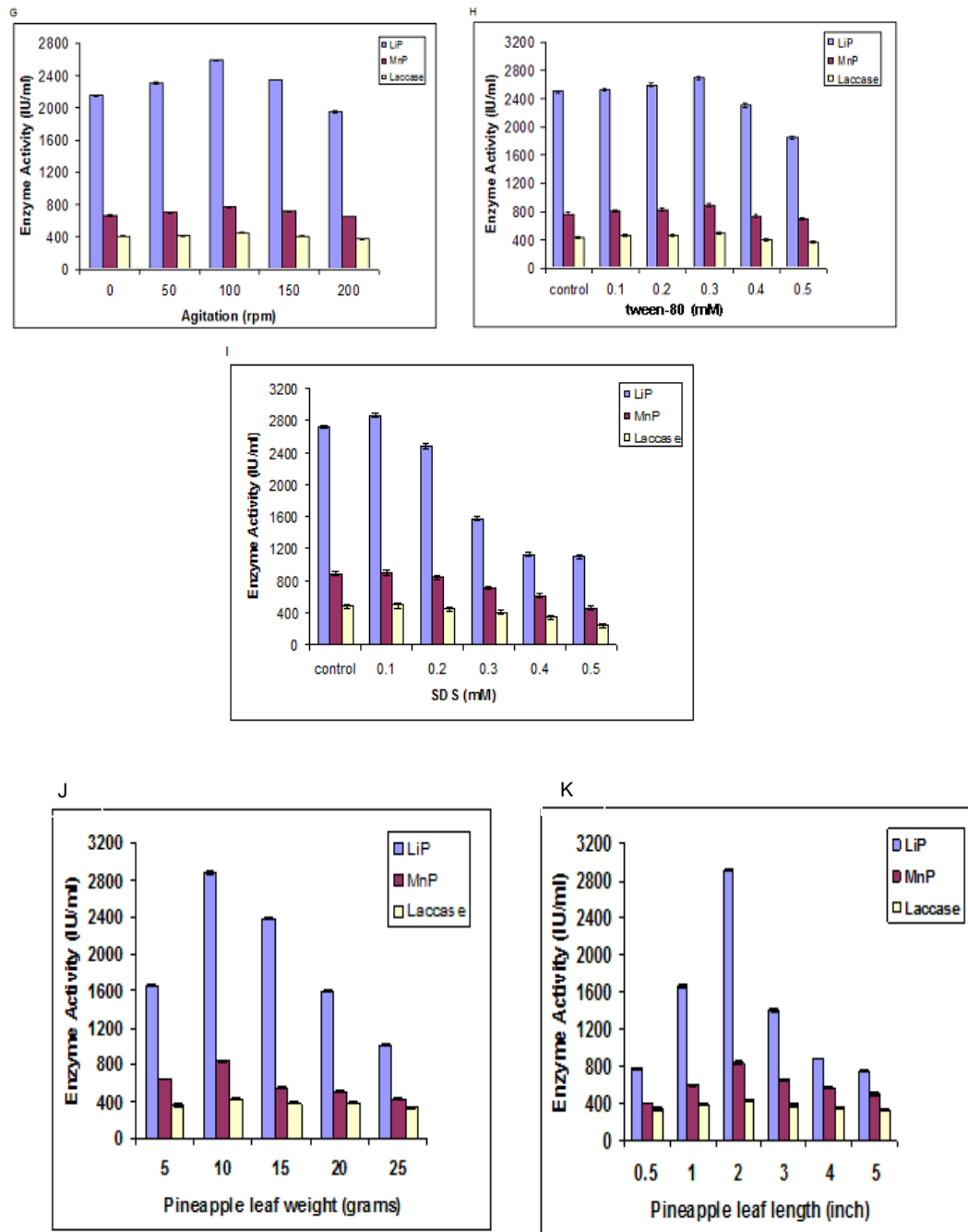
Fig. 2 (cont.). Effect of G) Agitation, H) Tween-80, I) SDS, J) Pineapple leaf weight, and K) Pineapple leaf length on ligninolytic activity of *Ganoderma lucidum*. (Error bars represent $\pm$ standard deviation from a triplicate average)

Medium Optimization Using the Plackett–Burman Design

From our preliminary experiments, different factors affecting the production of ligninolytic enzymes LiP, MnP, and Lac had been identified as being incubation period, inoculum, temperature, pH, pineapple leaf weight, pineapple leaf size, sucrose, agitation, Tween-80, SDS, and beef extract. The Plackett-Burman design (PBD) was adopted as it

aims to select the most important variables in the system that influences overall enzyme productivity (Plackett and Burman 1946). The effects of different medium components on the volumetric activity of LiP, MnP, and Lac were evaluated using a 11 x 12 PBD as detailed in Table 1.

Table 1. Plackett-Burman Design for Medium Optimization, High, and Low Levels of Independent Variables used in Trials

Experimental Variables	Factor setting	
	High	Low
Incubation period (hrs.)	24	192
Inoculum (mL)	1	7
Temperature (°C)	25	31
pH	4	6
Pineapple leaf weight (grams)	5	25
Pineapple leaf length (inch)	0.5	5
Sucrose (%)	5	25
Agitation (rpm)	0	200
Tween-80 (mM)	0.1	0.5
SDS (mM)	0.1	0.5
Beef Extract (%)	5	25

For medium optimization, 12 different trials were made with process variables and LiP, MnP, and Lac activity was measured. The row in the Table 2 represents the 12 different trials and each column represents a different variable.

Table 2. Plackett-Burman Designs for Medium Optimization, High (+), and Low (-) Levels and Measured Response

Run	Variables											Activity (IU/ml)		
	A	B	C	D	E	F	G	H	I	J	K	LiP	MnP	Lac
1	–	+	+	–	+	+	+	–	–	–	+	2540.96	823.48	435.57
2	+	–	–	–	+	_	+	+	–	+	+	2561.88	837.65	459.35
3	+	+	+	–	–	–	+	–	+	+	–	2602.50	898.87	497.78
4	+	+	–	+	+	+	–	–	–	+	–	2574.67	844.55	464.22
5	+	+	–	–	–	+	–	+	+	–	+	2606.87	900.78	502.77
6	–	+	–	+	+	–	+	+	+	–	–	2520.34	810.22	414.94
7	+	–	+	+	–	+	+	+	–	–	–	2529.43	817.11	419.47
8	–	–	+	–	+	+	–	+	+	+	–	2525.45	812.75	416.44
9	–	+	+	+	–	–	–	+	–	+	+	2492.49	798.29	391.77
10	–	–	–	–	–	–	–	–	–	–	–	2511.33	808.77	406.22
11	+	–	+	+	+	–	–	–	+	–	+	2597.77	895.88	492.49
12	–	–	+	+	–	+	+	–	+	+	+	2590.55	891.60	489.77
Incubation period (A), inoculum (B), temperature (C), pH (D), pineapple leaf weight (E), pineapple leaf size (F), sucrose (G), agitation (H), Tween-80 (I), SDS (J), and beef extract (K).														

From the PBD analysis, incubation period, agitation, and Tween-80 were found to significantly affect the volumetric activity of LiP, MnP, and Lac as compared to other variables. The order of the impact of these components on the LiP, MnP, and Lac activity was: incubation period > Tween-80 > agitation > pineapple leaf length > beef extract for LiP; Tween-80 > incubation period > agitation > beef extract > pineapple leaf weight for MnP; and Lac was incubation period > Tween-80 > agitation > beef extract > temperature for Lac.

Optimization of Process Parameters by Response Surface Methodology (RSM)

Box–Behnken Design

After determining the significant factors for LiP, MnP, and Lac enzyme production, the three most significant factors were selected for optimization by using response surface methodology (RSM). In the present study, Box-Behnken experimental design (BBD) was chosen for finding out the relationship between the response function (LiP, MnP, and Lac activity) and the three selected variables (incubation period, agitation, and Tween-80) designated as X_1, X_2, and X_3. Each variable for a desired response are represented at two levels namely, "high" and "low" (Table 3). Each selected variable was analyzed at these two levels in a total of 46 runs (Table 4). The behavior of the system is explained by the following quadratic model equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

Equation (1)

where Y is the predicted response; β_0 is the mode constant; X_1, X_2, X_3, X_4, and X_5 are the independent variables; β_1, β_2, β_3, β_4, and β_5 are the linear coefficients; β_{12}, β_{13}, β_{15}, β_{23}, β_{24}, β_{25}, β_{35}, β_{35}, and β_{45} are the cross product coefficients, and $\beta_{11,}$ $\beta_{22,}$ $\beta_{33,}$ $\beta_{44,}$ and β_{55} are the quadratic coefficients (Kwak 2005).

Table 3. The Level of Variables Chosen for Box-Behnken Design

Experimental Variables	Coded level	
	Low	High
Incubation period (hrs.)	24	192
Agitation (rpm)	0	200
Tween-80 (mM)	0.1	0.5

Table 4. Box-Behnken Design Matrix for Optimization of 3 Parameters for LiP, MnP, and Lac Production By _Ganoderma Lucidum_ In Solid State Fermentation

Run	Incubation period (hrs.)	Agitation (rpm)	Tween-80 (mM)	LiP activity (IU/mL) predicted	LiP activity (IU/mL) observed	MnP activity (IU/mL) predicted	MnP activity (IU/mL) observed	Lac activity (IU/mL) predicted	Lac activity (IU/mL) observed
1	192.00	100	0.50	2896.40	2998.99	892.08	895.58	497.98	498.98
2	192.00	0	0.30	2847.92	2948.14	845.52	845.05	460.75	461.78
3	108.00	200	0.10	2693.72	2796.54	694.59	697.62	290.21	292.25
4	108.00	100	0.30	2747.23	2835.43	756.72	745.92	363.56	353.97
5	108.00	100	0.30	2731.80	2857.35	742.07	759.22	349.48	367.76
6	108.00	100	0.30	2755.48	2841.66	764.55	768.39	370.43	379.90
7	24.00	0	0.30	2667.84	2771.39	681.79	681.19	293.48	292.23
8	108.00	0	0.50	2716.97	2814.15	718.63	715.60	321.15	319.11
9	24.00	200	0.30	2747.00	2846.77	751.42	751.89	360.58	359.54
10	192.00	100	0.10	2797.99	2898.73	780.06	776.43	389.31	386.03
11	192.00	200	0.30	2836.77	2933.22	838.95	839.55	448.30	449.55
12	24.00	100	0.50	2720.32	2819.58	710.01	713.64	320.08	323.36
13	108.00	100	0.30	2750.07	2808.44	759.52	709.47	366.42	315.99
14	108.00	200	0.50	2807.91	2908.87	811.13	807.03	408.15	405.9
15	108.00	100	0.30	2731.80	2907.45	742.07	814.59	349.48	414.5
16	24.00	100	0.10	2704.22	2801.63	710.87	707.37	312.22	311.22
17	108.00	0	0.10	2716.65	2815.69	724.02	728.12	322.56	324.81

Based on BBD analysis, the second-order polynomial expressions for LiP, MnP, and Lac activity were obtained as the equations given below:

$$\text{LiP activity (IU/mL)} = 2750.07 + 67.46X_1 + 17.00X_2 + 28.62X_3 - 22.58X_1X_2 + 20.58X_1X_3 + 28.47X_2X_3 + 35.37X_1^2 - 10.55X_2^2 - 5.70X_3^2 \qquad \text{Equation (2)}$$

$$\text{MnP activity (IU/mL)} = 759.52 + 62.82X_1 + 15.77X_2 + 27.79X_3 - 19.05X_1X_2 + 28.22X_1X_3 + 30.48X_2X_3 + 28.03X_1^2 - 8.13X_2^2 - 14.30X_3^2 \qquad \text{Equation (3)}$$

$$\text{Lac activity (IU/mL)} = 366.42 + 63.75X_1 + 13.66X_2 + 29.13X_3 - 19.89X_1X_2 + 25.20X_1X_3 + 29.84X_2X_3 + 34.37X_1^2 - 10.01X_2^2 - 20.89X_3^2 \qquad \text{Equation (4)}$$

To validate the statistical results and the model equation, ANOVA was conducted as shown in Table 5, 6, and 7. The _F_-value is a measure of variation of the data about the mean. Generally, the calculated _F_-value should be several times greater than the tabulated value, if the model is a good prediction of their experimental results, and the estimated factors effects are real (Datta and Bannerjee 2004). The _P_-value serves as a tool for checking the significance of each of the coefficients. The variables with low probability levels contribute to the model, whereas the others can be neglected and eliminated from the model. Values of _P_ >_F_ less than 0.0500 indicate model terms are significant (Khuri and Cornell 1993). In addition, the coefficients of determination, R-squared gives information about the goodness of fit of the model. For a good statistical model, the R-squared value should be close to unity. A value of R-squared greater than 0.75 means that

more than 75 percent of the variation in the response variable can be explained by explanatory variable and it indicates the aptness of the model (Chauhan and Gupta 2004). In the present study, high F-values and non significant lack-of-fit for LiP, MnP, and Lac indicated that model was a good fit. The R-squared for LiP, MnP, and Lac were 0.9144, 0.8986, and 0.9135, respectively, which further ensures a satisfactory adjustment of the quadratic model to the experimental data. The predicted R-squared is also in reasonable agreement with the adjusted R-squared value.

Table 5. Analysis of Variance for Lip Production by *Ganoderma lucidum* using Box-Behnken Design Matrix

Source	Sum of squares	Degree of freedom	Mean square	F-value	$P>F$	R-square
Model	57917.56	9	6435.28	8.31	0.0054	0.9144
Lack of fit	57.57	3	19.19	0.014	0.9973	
Pure Error	5363.58	4	1340.89			
R-Squared (predicted) = 0.8531; R-Squared(adjusted) = 0.8044						

Table 6. Analysis of Variance for Mnp Production by *Ganoderma lucidum* using Box-Behnken Design Matrix

Source	Sum of squares	Degree of freedom	Mean square	F-value	$P>F$	R-square
Model	52318.37	9	5813.15	6.89	0.0093	0.8986
Lack of fit	104.06	3	34.69	0.024	0.9942	
Pure Error	5801.43	4	1450.36			
R-Squared (predicted) = 0.8157; R-Squared(adjusted) = 0.7682						

**Table 7.** Analysis of Variance for Lac Production by *Ganoderma lucidum* using Box-Behnken Design Matrix

Source	Sum of squares	Degree of freedom	Mean square	F-value	P>F	R-square
Model	55350.52	9	6150.06	8.21	0.0056	0.9135
Lack of fit	47.23	3	15.74	0.012	0.9979	
Pure Error	5193.38	4	1298.34			
R-Squared (predicted) = 0.8536; R-Squared(adjusted) = 0.8023						

From the BBD analysis, the optimal values obtained were 0.5mM Tween-80, 191.41 hrs incubation period and 199.31 rpm agitation for LiP, 0.49mM Tween-80, 191.36 hrs incubation period and 190.39 rpm agitation for MnP, and 0.5 mM Tween-80, 191.45 hrs incubation period and 152.96 rpm agitation for Lac. At these optimal conditions, the predicted volumetric activity of LiP, MnP, and Lac was 3007.23 IU/mL, 907.53 IU/mL, 506.68 IU/mL, respectively. To determine the validity of the predicted values, the experiments were performed at the above optimal conditions. The volumetric activity of LiP, MnP, and Lac from the experiments was 2885.59 ± 65.2 IU/mL, 889.71 ± 46.6 IU/mL, and 472.31 ± 41.2 IU/mL, respectively; the predicted values were in good agreement with the experimental values.

The response surface plots of incubation period, agitation, and Tween-80 (Figs. 3, 4, and 5) revealed that the volumetric activity of LiP, MnP, and Lac was strongly dependent on the three parameters selected from the Box Behnken design. The activity of the ligninolytic enzymes was found to increase when one parameter was fixed at its optimal value and the other two parameters were increased. For instance, when agitation was fixed at its optimal value, an increase in incubation period or Tween-80 elicited a concomitant increase in LiP, MnP, and Lac activity.
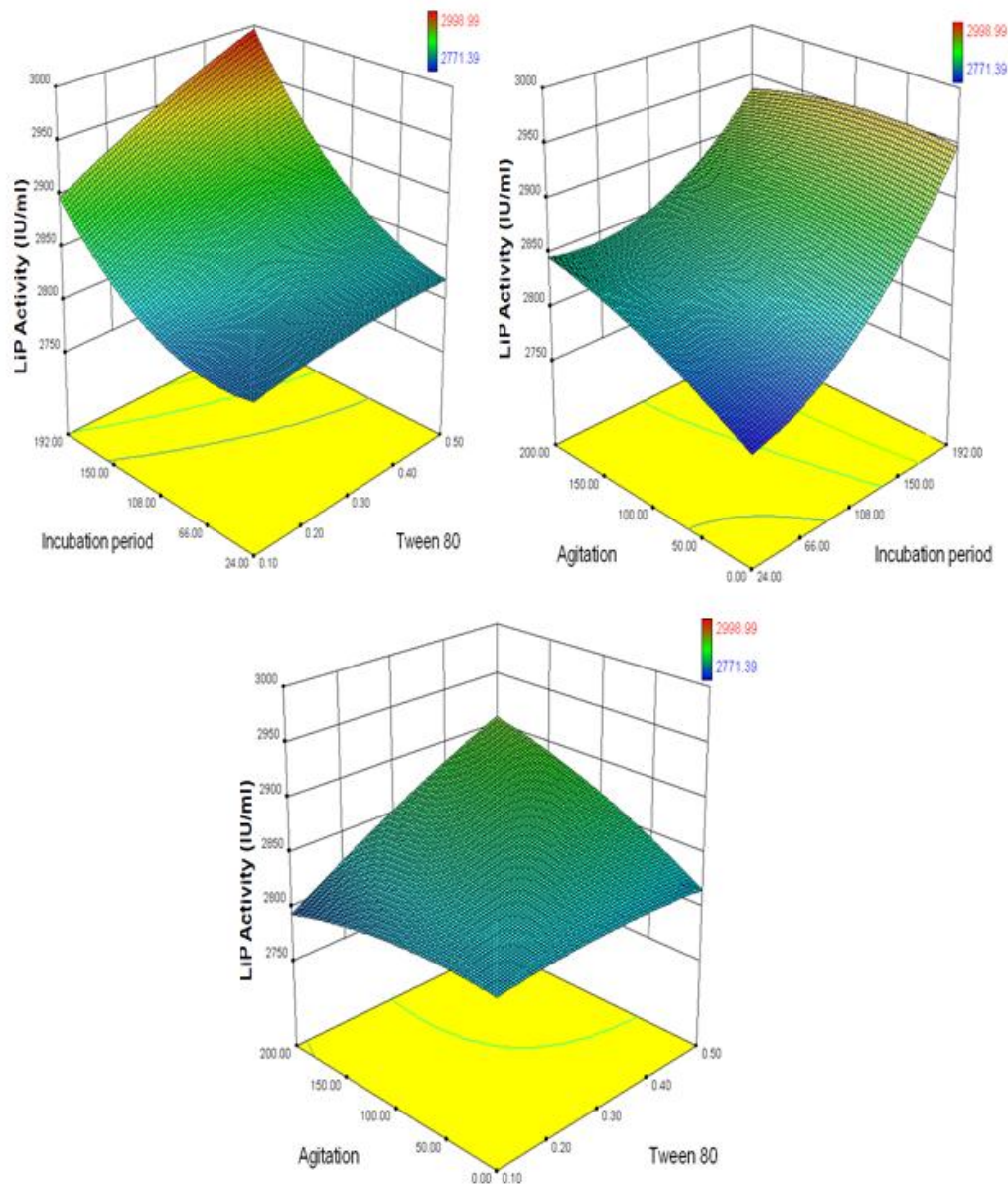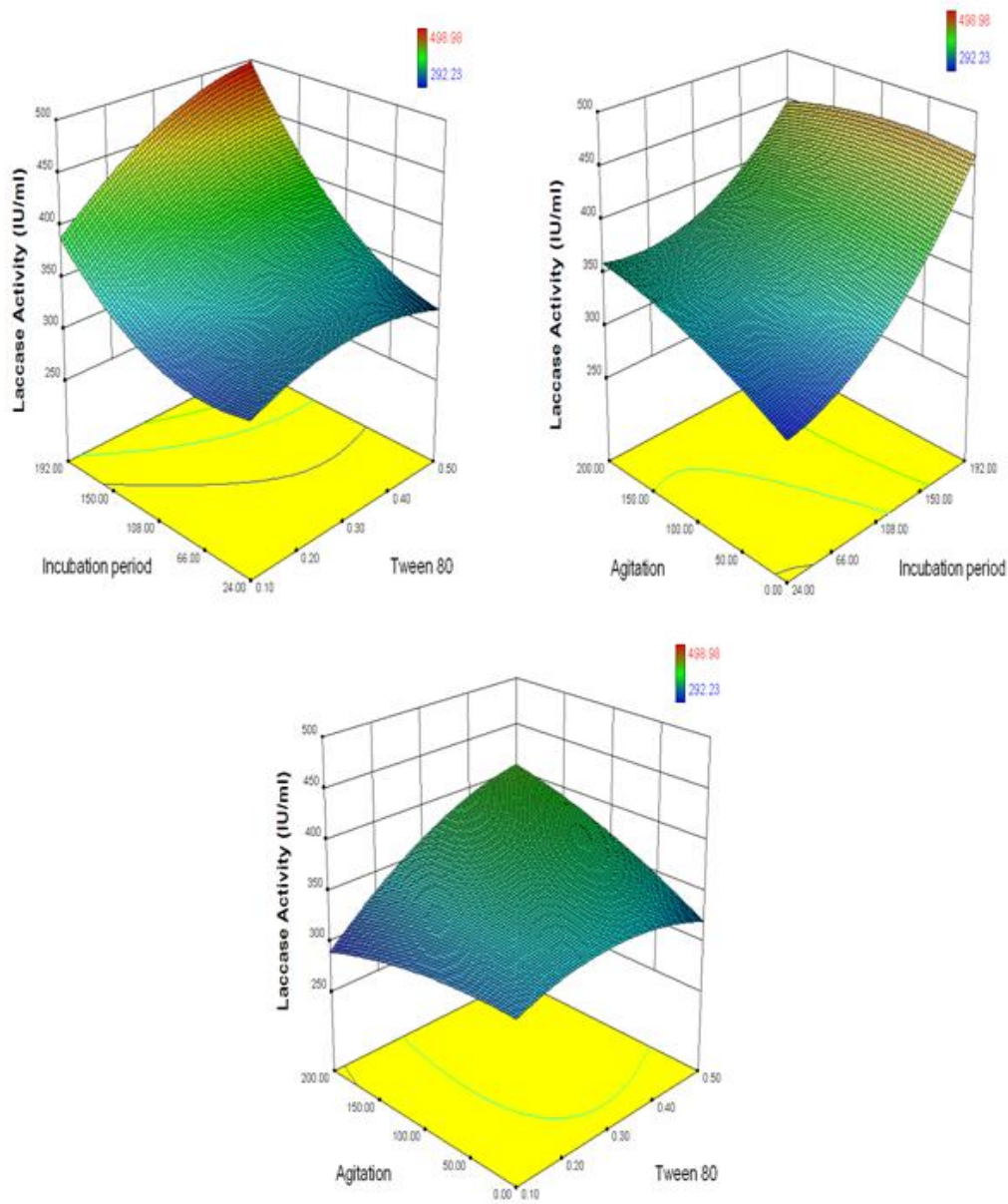
Fig. 3. Response surface plots of the interaction between three selected variables and the LiP activity of *Ganoderma lucidum*: agitation and incubation period with a fixed concentration of Tween-80 at 0.3 mM, agitation, and Tween-80 with a fixed concentration of incubation period at 108 hrs, incubation period and Tween-80 with a fixed concentration of agitation at 100 rpm.

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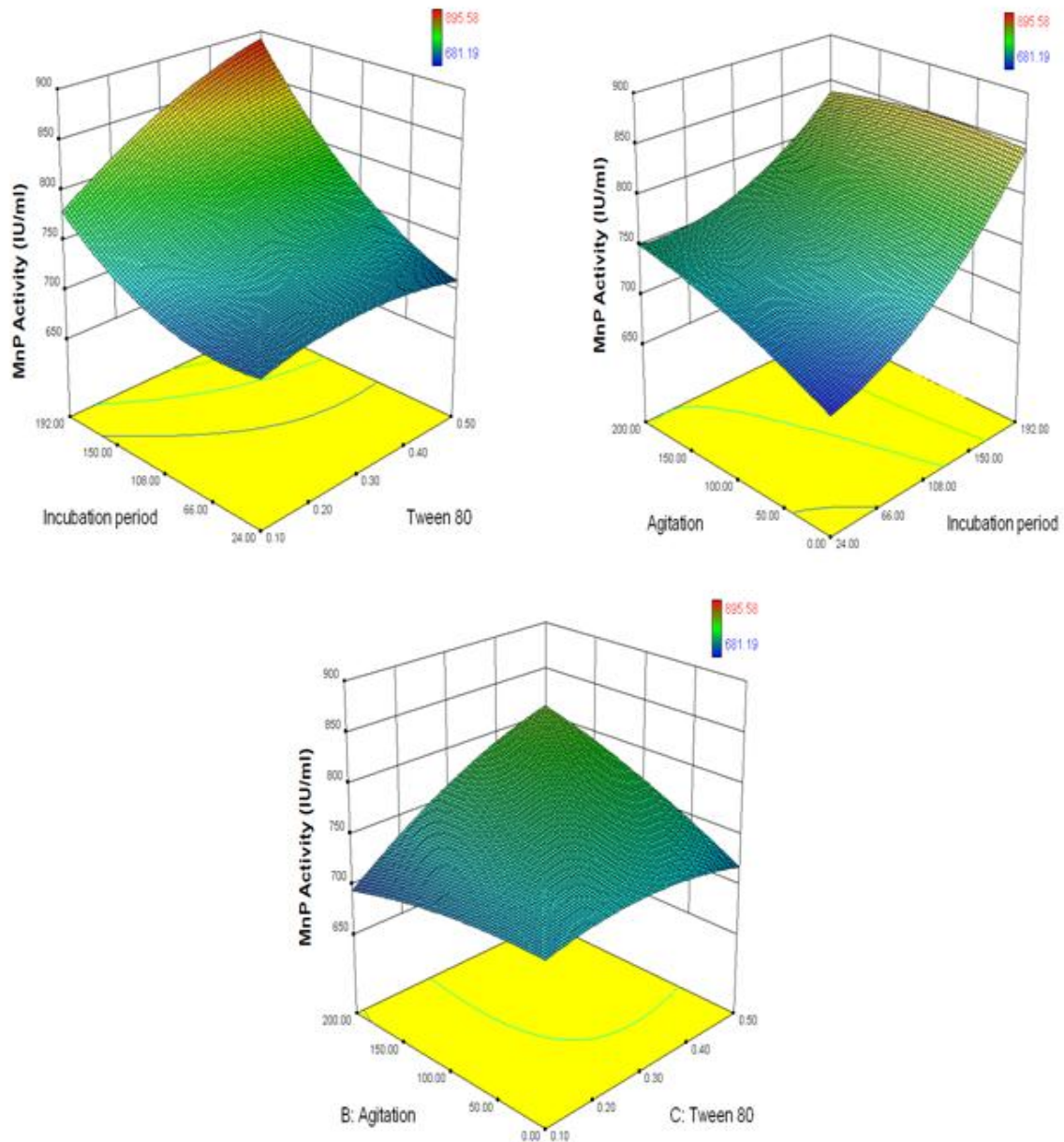
Fig. 4. Response surface plots of the interaction between three selected variables and the MnP activity of *Ganoderma lucidum*: agitation and incubation period with a fixed concentration of Tween-80 at 0.3 mM, agitation, and Tween-80 with a fixed concentration of incubation period at 108 hrs incubation period and Tween-80 with a fixed concentration of agitation at 100 rpm.

Fig. 5. Response surface plots of the interaction between three selected variables and the Lac activity of *Ganoderma lucidum*: agitation and incubation period with a fixed concentration of Tween-80 at 0.3 mM, agitation, and Tween-80 with a fixed concentration of incubation period at 108 hrs, incubation period and Tween-80 with a fixed concentration of agitation at 100 rpm.

Ligninolytic enzymes have been reported to be produced by several basidiomycetous fungi including *Ganoderma lucidum* (D'Souza *et al.* 1999; Silva *et al.* 2005). As summarized in Table 8, enzyme production depends apparently on the fungal species as well as the substrate used for SSF. For instance, Asgher *et al.* (2010) observed that enzyme production using a strain of *Ganoderma lucidum* was dependent on the substrate, being higher in rice straw than in wheat straw, corn stover, banana stalk, and sugarcane bagasse. To the best of our knowledge, this is the first report of the pineapple leaf substrate for ligninase enzyme production. As can be seen from Table 8, LiP and Lac

production was higher in *Ganoderma lucidum* with pineapple leaf substrate than other agriwaste substrates. However, this LiP and Lac production is lower than that reported from *Phanerochaete chrysosporium* by Nakamura *et al.* (1997) and *Pleurotus ostreatus* by Mazumder *et al.* (2009). Also, the *Ganoderma lucidum* used in this study produced lower amounts of MnP than other reported strains.

Optimization of ligninolytic enzyme production by SSF from different organisms has been achieved by various workers (Bak *et al.* 2009; Kumar and Mishra 2011) using the Box-Behnken experimental design. The present study is the first report of the culture conditions for LiP, MnP, and Lac production from *Ganoderma lucidum* on pineapple leaf substrate using the Box-Behnken design. The study indicates that the three major factors influencing enzyme production are incubation period, agitation, and surfactant (Tween-80) concentration. It has been reported that time period of incubation influences maximum ligninolytic enzyme production in white-rot fungi depending on the genetic variation among the strains, as well as nature and composition of the substrates used (Heinzkill *et al.* 1998; Giardina *et al.* 2000; Patel *et al.* 2009). Surfactants have been found to enhance microbial growth in SSF by promoting the penetration of water into the solid substrate matrix leading to an increase in exposed surface area (Asgher *et al.* 2006). However, increasing the surfactant concentration can also have inhibitory effect in ligninolytic enzyme production on *Ganoderma lucidum* (Asgher *et al.* 2010). Providing appropriate agitation conditions is a key factor in SSF for enzyme production and it largely depends on the resistance of the fungus to mechanical agitation (Rodriguez, Couto and Sanroman 2005).

Table 8. Comparison of Lip, Mnp, and Lac Production of this Study with Previous Studies

Organism	Substrates	Enzyme activities (IU/mL)			Reference
Ganoderma lucidum	Rice straw	2185±1.7	1972±1.4	338.4±2.8	Asgher *et al.* 2010
Ganoderma lucidum	Corn cobs	2807	ND	ND	Mehboob *et al.* 2011
Ganoderma lucidum	Wheat straw	1201.1	1131	100.4	Asgher *et al.* 2010
Ganoderma lucidum	Corn stove	466.7	347.4	20.1	Asgher *et al.* 2010
Ganoderma lucidum	Banana stalk	1193.5	1022.5	249.7	Asgher *et al.* 2010
Ganoderma lucidum	Sugarcane bagasse	486.7	416.8	192.1	Asgher *et al.* 2010
Ganoderma lucidum	Pineapple leaves	2885.59±65.2	889.71±46.6	472.31±41.2	This study
Schizophyllum commune IBL-06	Banana stalks	2688	3637	331	Irshad and Asgher 2011
Pleurotus ostreatus	Polyurethane foam	ND	ND	3×10^5	Mazumder *et al.* 2009
Phanerochaete chrysosporium	Polyurethane foam	3800	ND	ND	Nakamura *et al.* 1997
Phanerochaete chrysosporium	Rice straw	ND	2630.8 ± 89.2	ND	Bak *et al.* 2009
White-rot fungus (WRF-1)	Cyanobacterial bio-mass, groundnut shell	ND	ND	384.15	Kumar and Mishra 2011

Lignin Degradation

Pineapple leaves are currently an underutilized agriwaste (Abdul Khalil *et al.* 2006). Although a rich source of fibres suitable for textile and paper industries, the extraction of fibres is reported to be uneconomical (Banik *et al.* 2011). In our own experience, extraction of fibres by alkali digestion has been unsatisfactory as the thick insoluble leaf cuticle contributes to the high content of shives. Microbial retting for fibre extraction has been suggested as an environmentally friendly and economical alternative to chemical extraction (Maciel *et al.* 2010). Lignin degradation by white-rot fungi in relation to various biotechnical applications such as bio-pulping, bio-bleaching, treating of pulp mill effluents, and soil bio-remediation has been studied (Akhtar *et al.* 1992, 1998; Lamar *et al.* 1992; Messner and Srebotnik 1994), and many species of white-rot fungi have been found to degrade lignin efficiently (Hatakka 1994).

In the present study, the chemical composition of the pineapple leaf fibre (PALF) obtained after 120 hrs incubation with *Ganoderma lucidum* indicated that the ligninolytic enzymes produced by the fungus were effective in reducing the lignin content in the leaves. The Klason lignin estimation revealed significant lignin degradation in the sample treated with *Ganoderma lucidum* as compared to the untreated control. There was an approximate 15% decrease in the amount of lignin in the fungal-treated sample after 120 hrs incubation under SSF. Pectin degradation was also seen in the control and treated sample. However, there was not much difference in the cellulose and hemicelluloses content of both the control and treated sample (Table 9).

Table 9. Chemical Composition of Pineapple Leaf Before and After Fungal Treatment

Components	Control Sample (%)	Treated Sample (%)
Cellulose	60.28	58.8
Hemicellulose	2.93	2.87
Pectin	2.6	1.2
Lignin	32.4	17.1

CONCLUSIONS

1. This study reported the isolation of ligninase-producing-basidiomycete fungus *Ganoderma lucidum* and culture of the same by solid state fermentation using pineapple leaf agriwaste as substrate.

2. Enhanced production of lignin peroxidase, as well as Lac from *Ganoderma lucidum,* was achieved under SSF through optimization of different process parameters by Box–Behnken experimental design.

3. Degradation of lignin in fungal-treated pineapple leaf sample was confirmed through a quantitative method.

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