2. Improvement of lignin-degrading enzymes production from *Ganoderma* sp. KU-Alk4 by medium engineering

In the basic Kirk's medium of initial pH 7.0, 1% glucose, 0.22 g/L ammonium tartrate, 0.02 mM $CuSO_4$, production of lignin-degrading enzymes by *Ganoderma* sp. KU-Alk4, started when the inducer veratryl alcohol was added to the culture in day 3 (Figure 25). The control without inducer addition showed only a typical curve of microbial growth. The onset of the secondary growth phase was on day 5 and production of lignin-degrading enzymes in particular laccase could be observed in the secondary phase. The maximal laccase production was detected on day 9. The activity against ABTS was 20 IU/mL. The titre obtained was low compared to typical reported strains, 4-100 IU/mL (Revankar and Lele, 2006).

This result was obtained in the lab scale which pH was not controlled throughout the experiment. Hence, in order to produce the lignin-degrading enzymes in large scale fermentation for industrial or environmental used, the best conditions in fermentor must be optimized. To optimize conditions changing several factors one at a time in a fermentor is unpractical because of the cost of time and materials. Therefore, to improve the production of lignin-degrading enzymes, especially laccase from *Ganoderma* sp. KU-Alk4, a Box-Behnken experimental design was applied for investigation of the relationship between substrate medium components, their concentration and the pH of the medium to optimize the production of laccase. To the best of our knowledge the optimization of the medium ingredients for laccase production by using this design has not been reported.

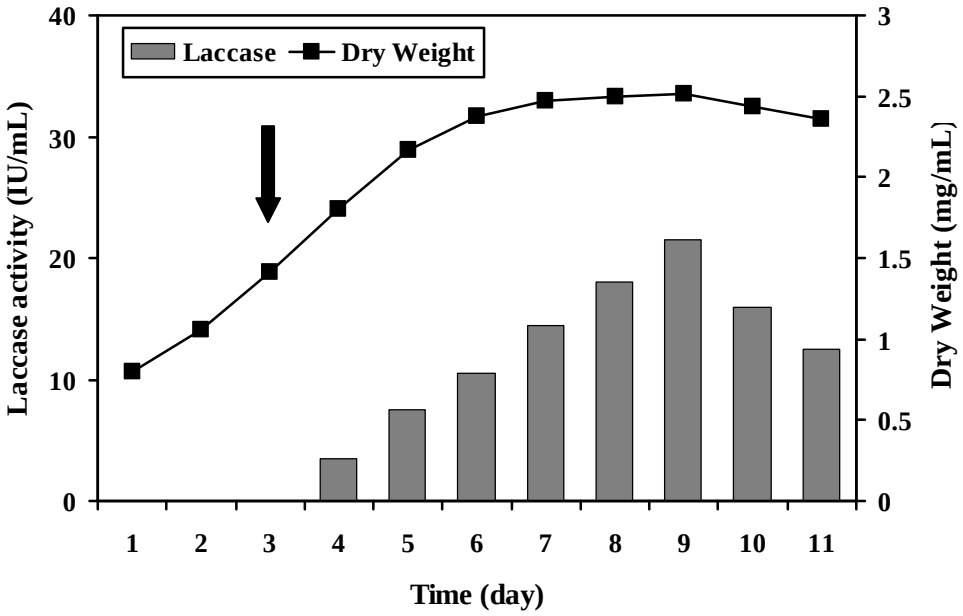

Figure 25 Laccase activity and growth curve of *Ganoderma* sp. KU-Alk4 in non-optimized Kirk's liquid medium with 10 g/L glucose and 0.22 g/L ammonium tartrate. Initial pH was 7.0 and no pH control throughout the experiment. Culture conditions: first 3 days as static culture. After 3 days, culture were shaken at 140 rpm 30ºC. Arrow indicates the addition of 0.85 mM veratryl alcohol and the starting time of shaking. ABTS was used for laccase assay.

2.1 Box-Behnken experimental design

In this experiment ABTS was used as substrate for laccase assay and the activity was defined as International Unit (IU). By the design of Box-Behnken (Table 12) followed with 66 trial experiments (Table 18), laccase production varied from 0 to 149 IU/mL in the 66 different media tested. *Ganoderma* sp. KU-Alk4 could not grow in the media in which pH was controlled at pH 8.0 throughout the experiment, therefore, no laccase activity was detected. A constant increase of laccase production was observed from the day 5 to day 13 when laccase reaches its peak of production.

The 6 best conditions were run no. 4 (25 g/L of lactose; 0.44 g/L of malt extract; 0.85 mM veratryl alcohol), run no. 20 (25 g/L of glycerol; 0.44 g/L of yeast extract; no inducer), run no. 49 (10 g/L of glucose; 0.22 g/L of yeast extract; 0.85 mM veratryl alcohol), run no. 50 (10 g/L of glycerol; 0.22 g/L of yeast extract; 0.85 mM veratryl alcohol), (run no. 51 (40 g/L of glucose; 0.22 g/L of yeast extract; 0.85 mM veratryl alcohol) and run no. 52 (40 g/L of glycerol; 0.22 g/L of yeast extract; 0.85 mM veratryl alcohol).

Time courses of laccase production of the 6 best run nos. were compared in Figure 26. In all of the media pH was controlled at pH 6.0 throughout the experiment. The best medium had 40 g/L glycerol, 0.22 g/L yeast extract and 0.85 mM veratryl alcohol and the culture pH was controlled at pH 6.0. The maximum activity obtained in day 13 was 149 IU/mL.

Table 18 Seven factors in three levels Box–Behnken design ten replications of the centre point used to design the best medium for *Ganoderma* sp. KU-Alk4.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Laccase (IU/mL)	Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Laccase (IU/mL)
1	0	0	0	-1	-1	-1	0	25.8	30	+1	-1	0	+1	0	0	0	0
2	0	0	0	+1	-1	-1	0	19.8	31	-1	+1	0	+1	0	0	0	0
3	0	0	0	-1	+1	-1	0	8.4	32	+1	+1	0	+1	0	0	0	0
4	0	0	0	+1	+1	-1	0	29.8	33	0	0	-1	-1	0	0	-1	14.4
5	0	0	0	-1	-1	+1	0	0	34	0	0	+1	-1	0	0	-1	12.2
6	0	0	0	+1	-1	+1	0	0	35	0	0	-1	+1	0	0	-1	4.9
7	0	0	0	-1	+1	+1	0	21.3	36	0	0	+1	+1	0	0	-1	3.3
8	0	0	0	+1	+1	+1	0	5	37	0	0	-1	-1	0	0	+1	0
9	-1	0	0	0	0	-1	-1	0	38	0	0	+1	-1	0	0	+1	0
10	+1	0	0	0	0	-1	-1	0	39	0	0	-1	+1	0	0	+1	0
11	-1	0	0	0	0	+1	-1	0	40	0	0	+1	+1	0	0	+1	0
12	+1	0	0	0	0	+1	-1	0	41	-1	0	-1	0	-1	0	0	0
13	-1	0	0	0	0	-1	+1	0	42	+1	0	-1	0	-1	0	0	0
14	+1	0	0	0	0	-1	+1	0	43	-1	0	+1	0	-1	0	0	0
15	-1	0	0	0	0	+1	+1	0	44	+1	0	+1	0	-1	0	0	0
16	+1	0	0	0	0	+1	+1	0	45	-1	0	-1	0	+1	0	0	0
17	0	-1	0	0	-1	0	-1	4	46	+1	0	-1	0	+1	0	0	0
18	0	+1	0	0	-1	0	-1	8.5	47	-1	0	+1	0	+1	0	0	0
19	0	-1	0	0	+1	0	-1	18.5	48	+1	0	+1	0	+1	0	0	0
20	0	+1	0	0	+1	0	-1	27.5	49	0	-1	-1	0	0	-1	0	65.1
21	0	-1	0	0	-1	0	+1	0	50	0	+1	-1	0	0	-1	0	95.2
22	0	+1	0	0	-1	0	+1	0	51	0	-1	+1	0	0	-1	0	70.7
23	0	-1	0	0	+1	0	+1	0	52	0	+1	+1	0	0	-1	0	149
24	0	+1	0	0	+1	0	+1	0	53	0	-1	-1	0	0	+1	0	15.2
25	-1	-1	0	-1	0	0	0	0	54	0	+1	-1	0	0	+1	0	15.2
26	+1	-1	0	-1	0	0	0	0	55	0	-1	+1	0	0	+1	0	19.1
27	-1	+1	0	-1	0	0	0	0	56	0	+1	+1	0	0	+1	0	13
28	+1	+1	0	-1	0	0	0	0	57-66	0	0	0	0	0	0	0	0
29	-1	-1	0	+1	0	0	0	0									

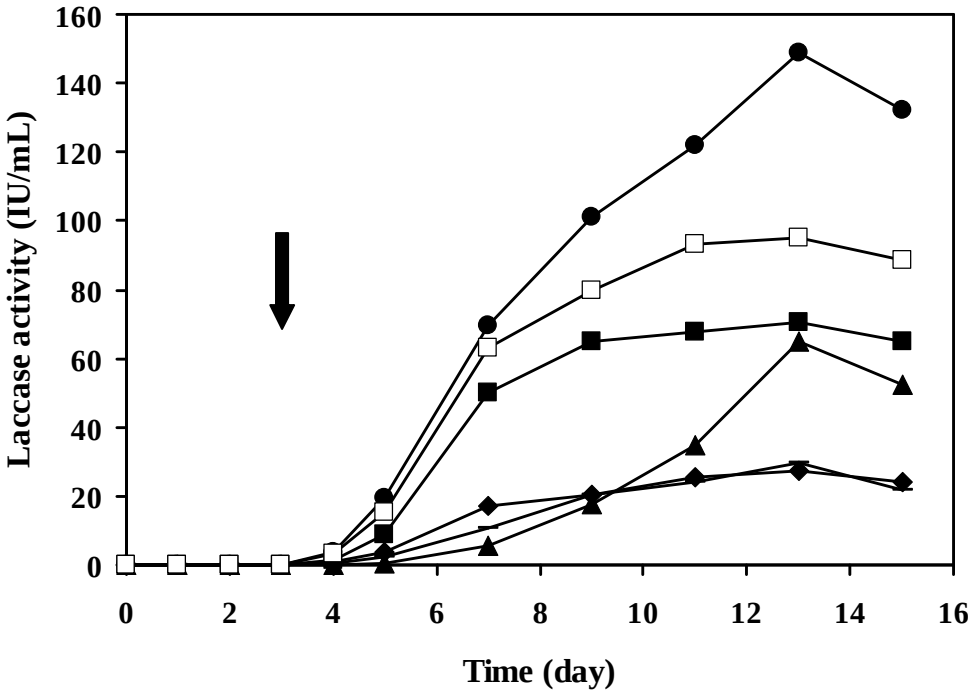

Figure 26 Laccase production by *Ganoderma* sp. KU-Alk4 at time course of fermentation in the selected optimized media by the Box-Behnken factorial design, run nos., 4 (–); 20 (♦); 49 (▲); 50 (□); 51 (■); 52 (●). Conditions of growth: static condition for 3 days and then shaken at 140 rpm at 30ºC. The medium pH 6.0 was controlled throughout the experiment. Arrow indicates the addition of inducer and the starting time of shaking.

Table 19 shows the analysis of variance (ANOVA) of the results on the peak of laccase production over the time course of the fermentation. The pH, nature and concentration of carbon sources and the nature and concentration of inducers, were highly significant (P<0.0005). Furthermore, the interaction between the sources of carbon and the type of inducer was also highly significant (P<0.00001) while the interaction between types and concentrations of carbon and that of carbon concentration and inducer were significant to the level of P<0.005. The statistical analysis shows that the carbon sources and inducer type and concentration are the most important factors for laccase production. On contrary, sources and concentrations of nitrogen were not significant for laccase production.

Table 19 The analysis of variance of the Box-Behnken experimental design for the laccase production by *Ganoderma* sp. KU-Alk4.

	F	*P*
(1) pH	35.75	0.00000***
(2) Carbon sources	69.61	0.00000***
(3) Concentration of carbon source	55.83	0.00000***
(4) Nitrogen sources	4.06	0.03754^{NS}
(5) Concentration of nitrogen source	2.11	0.15404 ^{NS}
(6) Inducer	148.61	0.00000***
(7) Concentration of inducer	12.69	0.00050**
2*3	8.56	0.00128*
2*6	77.15	0.00000***
3*6	10.96	0.00442*

Levels of statistical significance ***P<0.00001, **P<0.0005 and *P<0.005

^{NS} No significant

In order to find the optimum and statistically significant interactions between factors, a second order (quadratic) polynomial equation fitted the experimental data for laccase produced by *Ganoderma* sp. KU-Alk4 was constructed with a multiple correlation coefficient (R^2) of 0.98 (residual: 0.045, variance explained: 93%):

$$\begin{aligned}\text{Laccase production (IU/mL)} = {} & 179.4 + 67.3\,X_1^2 + 79.6\,X_2 - 89.3\,X_2^2 + 52.4\,X_3 \\ & - 81.9\,X_3^2 - 15.0\,X_4 + 22.0\,X_4^2 + 20.1\,X_5 + 14.6\,X_5^2 - 240.6\,X_6 - 105.4\,X_6^2 \\ & - 34.3\,X_7 + 38.1\,X_7^2 + 0.0\,X_1 X_2 + 0.0\,X_1 X_2^2 + 8.5\,X_1^2 X_2 - 2.4\,X_1^2 X_3 \\ & - 0.5\,X_1^2 X_4 + 11.9\,X_1^2 X_4 - 36.0\,X_1^2 X_6 - 21.8\,X_1^2 X_7 + 52.6\,X_2 X_3 \\ & - 55.5\,X_1 X_3^2 - 40.5\,X_2^2 X_3 + 5.6\,X_2 X_5 - 9.1\,X_2^2 X_5 - 143.2\,X_2 X_6 \\ & + 162.5\,X_2^2 X_6 - 16.9\,X_2 X_7 + 14.8\,X_2^2 X_7 + 0.7\,X_3 X_4 + 11.0\,X_3^2 X_4 \\ & - 72.2\,X_3 X_6 + 4.8\,X_3 X_7 + 14.0\,X_4 X_5 - 39.6\,X_4 X_6 + 23.1\,X_4 X_7 \\ & + 41.9\,X_5 X_6 - 42.0\,X_5 X_7 \qquad \text{(Eq. 2)}\end{aligned}$$

Where X is the coded value (between −1 and +1) for the factor indicated by the attached subscript. The coefficients of pH (quadratic), carbon sources (linear and quadratic), carbon source concentrations (linear and quadratic) sources of carbon (quadratic), inducer (linear and quadratic) and the interactions between carbon sources and carbon source concentrations (linear and quadratic), carbon sources (quadratic) and carbon source concentrations (linear), carbon sources (linear and quadratic) and inducer levels (linear) and the levels of sources of carbon and inducer (linear) were all statistically significant at a level of $P<0.005$. The least significant terms were included in the equation to maintain the hierarchy in the model.

Figure 27 shows the effect of carbon sources, levels and pH on the laccase activity produced under the sets of conditions and treatment levels tested. Glucose and glycerol at 10 and 40 g/L were efficient carbon sources at pH 6.0 but lactose at each concentration was not. With both effective carbon sources, the enzyme production was better with 40 g/L than 10 g/L. For all sources of carbon, including lactose, using 25 g/L resulted in very little laccase production. It is also noticeable that at pH 6.0, higher laccase production was observed using both glucose and glycerol.

When the pH was controlled at pH 4.0 and 8.0 throughout the experiment, none of the carbon source was used for enzyme production. The best carbon source, in these conditions, was 40 g/L glycerol that gave 80 IU/mL which was 45% higher than produced using 10 g/L and 100, and 78% higher than the activity obtained using 10 and 40 g/L glucose.

Figure 28 shows the effect of nitrogen sources, concentration and pH on laccase activity produced under the sets of conditions and treatment levels tested. Overall, yeast extract at 0.22 g/L produced the highest laccase activity with a mean of 50 IU/mL which was 4 times higher than that produced with ammonium tartrate and malt extract at pH 6.0. For all nitrogen sources other than yeast extract, use of 0.22 g/L resulted in a very little laccase production. At pH 4.0 and 8.0, no enzyme production was suggested with any N-source used.

Figure 29 shows the effect of inducer type and concentration at different pHs on the laccase production. Maximum laccase production (58 IU/mL) occurred when veratryl alcohol (0.85 mM) was used and at pH 6.0. Only small activities of laccase production were obtained otherwise.

Comparison of the observed versus predicted yields is shown in Figure 30. The points above or below the diagonal line represent areas of over or under prediction of the model. This showed that no significant violations of the model were found in the analysis, with 98% correlation of the model with the experimental data obtained.

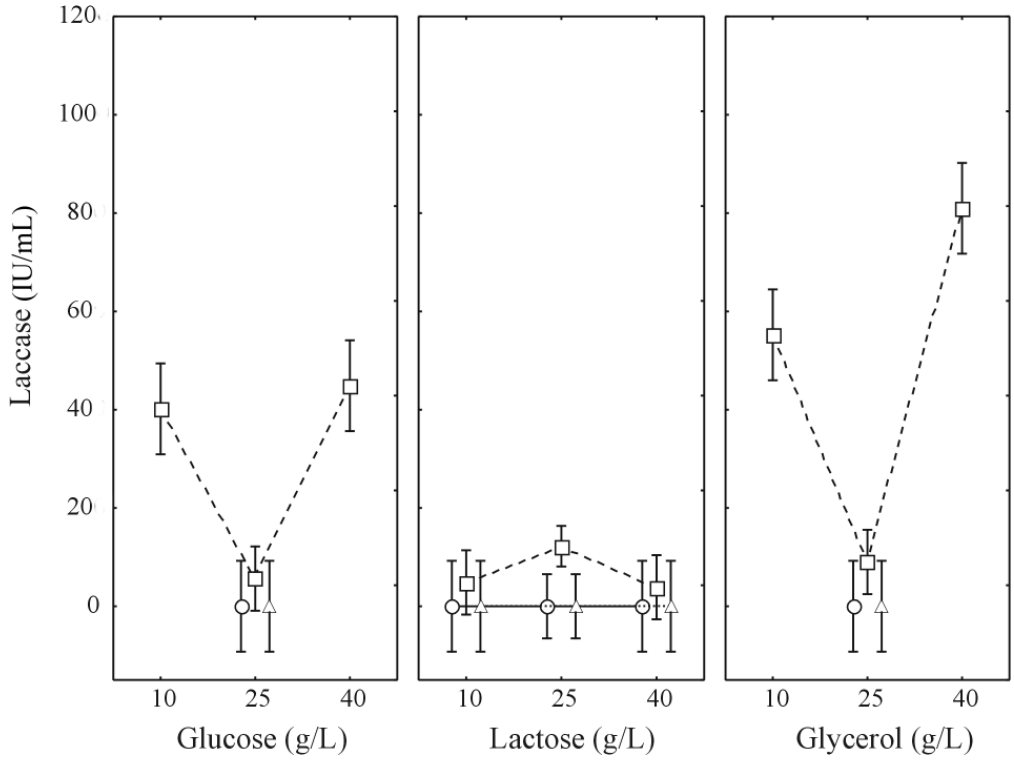

Figure 27 Graphical analysis of the relationship between carbon sources, type and concentration and the laccase produced by *Ganoderma* sp. KU-Alk4 at pH 4.0(○), 6.0(□) and 8.0(Δ). The bars represent the confidence intervals (95%) and the figure the mean per treatment.