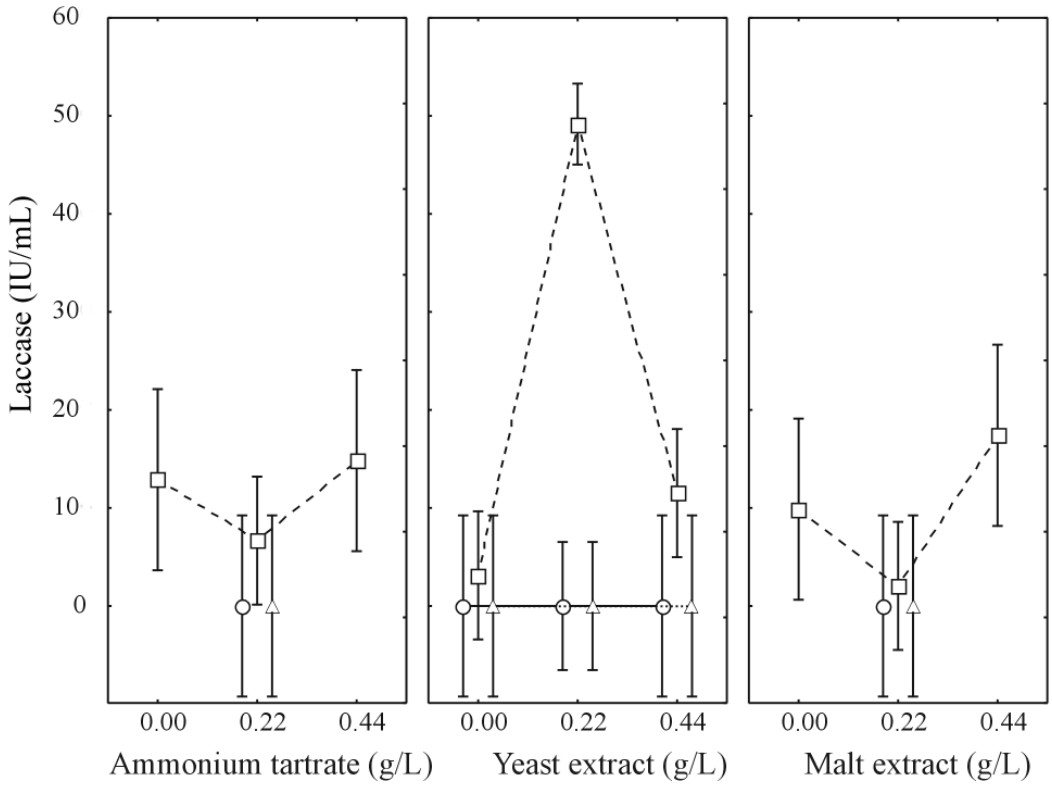

Figure 28 Graphical analysis of the relationship between nitrogen type and concentration and the laccase produced by *Ganoderma* sp. strain KU-Alk4 at pH 4.0(○), 6.0(□) and 8.0(Δ). The bars represent the confidence intervals (95%) and the figure the mean per treatment.

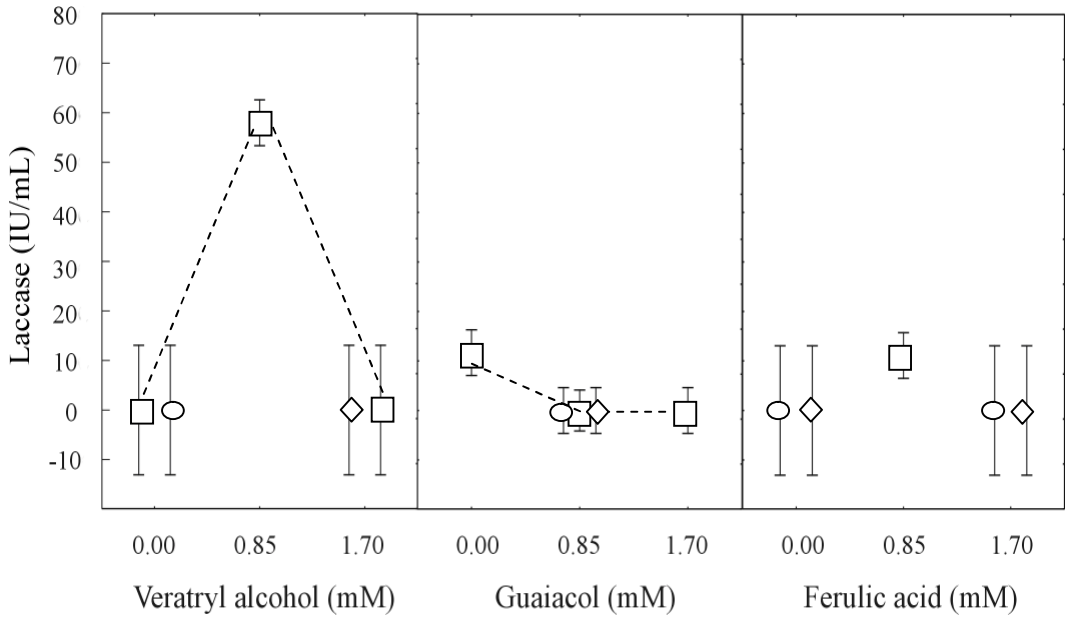

Figure 29 Graphical analysis of the relationship between inducer (mM), veratryl alcohol, guaiacol and ferulic acid and concentration and the laccase produced by *Ganoderma* sp. KU-Alk4 at pH 4.0(○), 6.0(□) and 8.0(Δ). The bars represent the confidence intervals (95%) and the figure the mean per treatment.

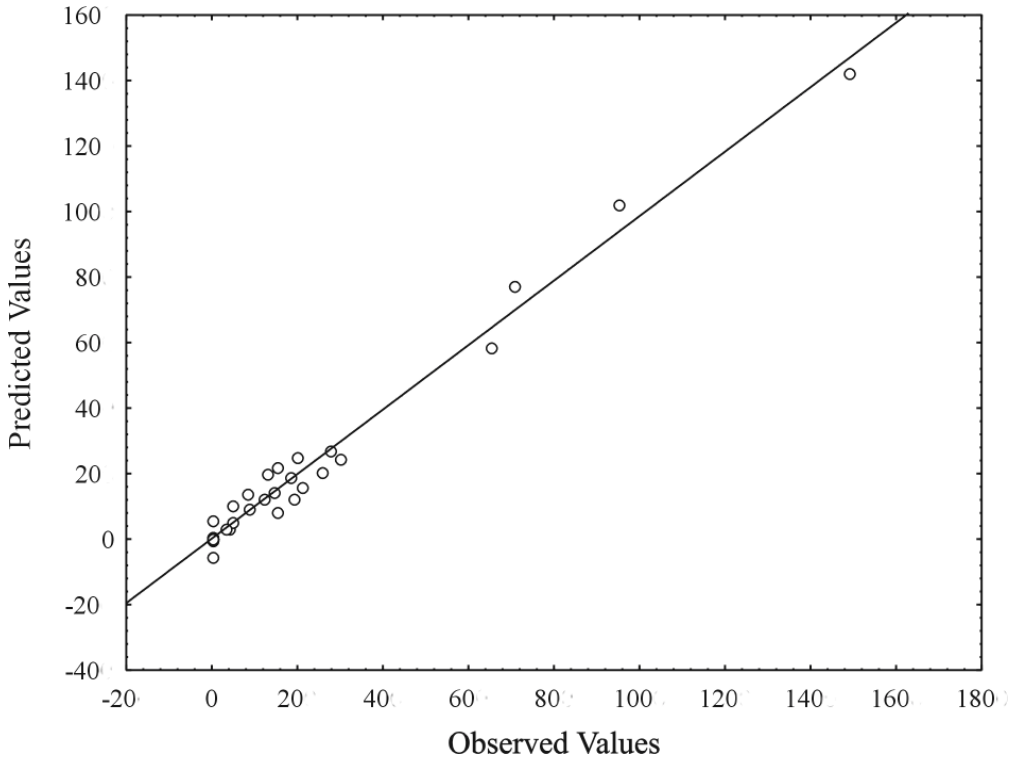

Figure 30 Observation and prediction of laccase activity of *Ganoderma* sp. KU-Alk4 calculated with the model.

2.2 Experiments followed the optimum conditions designed by Box-Behnken

Optimal condition for laccase production by *Ganoderma* sp. KU-Alk4 suggested by Box-Behnken design was glycerol (40 g/L) as carbon source and yeast extract (0.22 g/L) as nitrogen source with veratryl alcohol (0.85 mM) as inducer and the medium pH was controlled at pH 6.0 throughout the experiment. To confirm the optimal conditions forecasted for the production of laccase by Box-Behnken design, a set of five replicates using the optimal combination of substrates and concentrations were used. The highest activity of laccase obtained from *Ganoderma* sp. KU-Alk4 in these experiments was as high as 240 IU/mL (Figure 31) which was 12 times higher than the non-optimized medium. It was found that the more active culture resulted the higher enzyme activity, too. This experiment was robust with high reproducibility shown by the small error bars.

The medium engineering that was designed statistically in this study considered 7 factors with 3 levels on laccase production of *Ganoderma* sp. KU-Alk4. To achieve the results obtained in this study using a full factorial design would have required $3^7 \times 3$ replicates experiments taking into account all the variables involved. By using Box-Behnken design, a significantly smaller combination of factors and levels could be used for effectively examining the effect of interacting factors on laccase production. Thus, only a limited number of experiments (66) were suggested. Optimal medium composition and condition were found that represented a 12 times increase in titer compared to the non-optimized medium. The laccase activity of *Ganoderma* sp. KU-Alk4 achieved in this experimental lab obtained 240 IU/mL, represented a significant improvement and demonstrating success in medium engineering using statistical design of Box-Behnken.

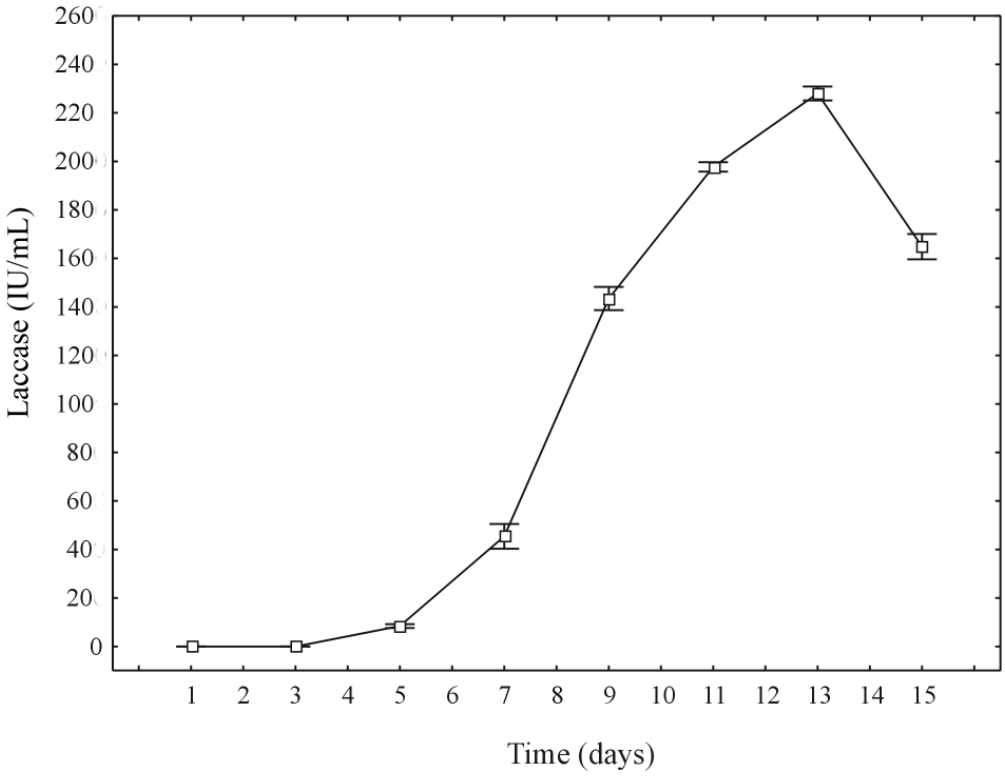

Figure 31 Confirmatory runs using the best medium: glycerol (40 g/L), yeast extracts (0.22 g/L) and veratryl alcohol (0.85 mM) at pH 6.0. Bars represent the mean and ± standard error of the five confirmed experiments.

In this study, laccase was produced under a variety of selected culture conditions to investigate their effects on the amount of laccase. Results led us to consider on the medium pH that the culture pH that was controlled throughout the experiment had significant effect on the fungal growth and laccase production, which resulted to the selection of carbon source in both types and concentration.

We also realize that the other factors such as dissolved oxygen which were not controlled in shake flasks and copper, would influence laccase production by the fungus, and may increase the titre values beyond those predicted by the model. However, to study on the effect of dissolved oxygen, a controllable fermenter would need to be used. For example, Eggert *et al*. (1996) using a 100-L fermenter to culture *Pycnoporus cinnabarinus* succeeded in doubling the laccase titres over those obtained in shake flasks. Laccase production from *Trametes versicolor* was increased 20-fold by ethanol, however, was comparable to that with veratryl alcohol (Lee *et al*., 1999). Addition of copper, a micronutrient that has key role as metal activator in fungal laccase, enhanced laccase production 30-fold with *Trametes pubescens* (Galhaup *et al*., 2001) and 2-fold with *Ganoderma* sp. *WR-1* (Revankar and Lele, 2006).

Optimum conditions for laccase production appeared to be different to those previously reported of the other fungi. Clearly, the most obvious difference is that the results of ANOVA which showed carbon sources and inducer types were the 2 most important factors for laccase production in *Ganoderma* sp. KU-Alk4. Sources and concentrations of nitrogen were the least important nutrient factor. In most of the lignin-degrading fungi, C:N ratio is a factor that influences laccase production. Nitrogen limitation usually stimulates the production of laccase in *Pycnoporus cinnabarinus* (Eggert *et al*., 1996) and *Botryosphaeria* sp. (Vasconcelos *et al*., 2000). In contrast, high levels of laccase were observed when *Ganoderma lucidum* (D'souza *et al*., 1999), *Cyathus stercoreus* (Sethuraman *et al*., 1999) and *Ceriporiopsis subvermispora* (Lobos *et al*., 1994) were grown in media with high nitrogen. Our results demonstrated that the addition of sufficient organic nitrogen in the form of yeast extract is suitable for laccase production by *Ganoderma* sp. KU-Alk4.

In general in fungi, substrates such as glucose that were efficiently and rapidly utilized by the organism resulting in high level of laccase activity (Galhaup *et al*., 2002; Nyanhongo *et al*., 2002), but laccase production by *Ganoderma* sp. KU-Alk4 was found to be optimal with glycerol as carbon source, though it was consumed more slowly than glucose.

Various aromatic compounds such as veratryl alcohol are able to induce laccase production (Arora and Gill, 2001; Dekker and Barbosa, 2001). The most widely reported inducer of laccase production is 2,5-xylidine (Galhaup and Haltrich, 2001; Galhaup *et al*., 2002; Rancano *et al*., 2003; Revankar and Lele, 2006). However, Lee *et al*. (1999) reported a doubling of laccase production by *Trametes versicolor* when veratryl alcohol was used instead of 2,5-xylidine. This is consistent with our observation that in *Ganoderma* sp. KU-Alk4, only veratryl alcohol, among a number of compounds tested, showed effective stimulation of laccase production. Ferulic acid and guaiacol enhance laccase production in *Pycnoporus cinnabarinus* (Herpoël *et al*., 2000), *Phlebia radiata* and *Daedalea flavida* (Arora and Gill, 2001). These compounds slowed growth of *Ganoderma* sp. KU-Alk4 and did not enhance laccase production, suggesting that they are toxic to the fungus.

By medium engineering with combination of all factors, we could increase laccase production of *Ganoderma* sp. KU-Alk4, by 12-fold to that of non-optimized medium. From an economic point of view, the most important parameters in screening and optimization of media are time and cost. The strategy used here demonstrates advantages in comparison with traditional methods and allows the development of a mathematical model that predicts where the optimum is likely to be located.

This is the first report on optimization of the medium ingredients for laccase production of *Ganoderma* sp. by using Box-Behnken design. The laccase activities produced by *Ganoderma* sp. KU-Alk4 in optimum conditions as designed were significantly higher than those produced by most of the fungi at similar conditions. However, laccase production by *Ganoderma* sp. KU-Alk4 cultivated in pH 8.0 medium that pH was not controlled throughout the experiment was 1.4 times higher than that produced in the medium that pH was controlled at 6.0 throughout the experiment as designed. Therefore, the enzyme of *Ganoderma* sp. KU-Alk4 produced in the liquid Kirk's medium with initial pH 8.0 and no pH controlled was used for further study.

Table 20 Comparison of laccase production by *Ganoderma* sp. KU-Alk4 with some reference fungi.

Strain	Inducer	Concentration (mM)	Activity (IU/mL)	Reference
Ganoderma sp. KU-Alk4	Veratryl alcohol	0.85	240	Present work using Box-Behnken design
			327.5	Present work using pH 8.0 medium
Trametes pubescens	Gallic acid	1	350	Galhaup *et al*., 2002
	2,5-Xylidine	1	275	
	$CuSO_4$	2	325	
Trametes versicolor	Veratryl alcohol	1	80	Lee *et al*., 1999
	2,5-Xylidine	1	30	
Trametes versicolor	2,5-Xylidine	1	10.9	Bollag and Leonowicz, 1984
Coriolus hirsutus	Syringaldazine	0.1	50	Koroljova-Skorobogat'ko *et al*., 1998
Trametes pubscens	2,5-Xylidine	1	8	Galhaup and Haltrich, 2001
Trametes versicolor	2,5-Xylidine	1	1.5	Rancano *et al*., 2003
Trametes multicolor	$CuSO_4$	1	18	Hess *et al*., 2002
Ganoderma sp. *WR-1*	2,5-Xylidine	0.8	692	Revankar and Lele, 2006
	$CuSO_4$	1	410	

Modified from Revankar and Lele (2006).

Note: In all the above cases one unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min at 25ºC.