3. Properties of the crude enzymes

The enzyme of *Ganoderma* sp. KU-Alk4 was produced in the liquid Kirk's medium with initial pH equal to 8.0 and no pH controlled during the production. Optimal condition for the laccase production as described in part 1.2 was employed. Only laccase were produced under such condition by *Ganoderma* sp. KU-Alk4. The crude enzyme from 1 to 4% glucose cultures showed differences in the laccase isozymes produced. Therefore, catalytic characteristics of both crude enzymes were compared.

3.1 Optimum pH and pH stability

Optimum pH and pH stability of the crude laccase from *Ganoderma* sp. KU-Alk4 cultured in pH 8.0 Kirk's medium with 1% glucose (G1%) were compared to those of 4% glucose (G4%) medium (Figure 32). Optimum pH of G1% and G4% crude enzyme was at 3.5 and gradually decreased. However, the difference of G1% with G4% crude enzyme was that activity of the G1% crude enzyme was more rapidly decreased. At pH 4.0, 5.0 and 6.0, activity of G4% exhibited 77, 34 and 9%, respectively, while those of the G1% crude enzyme was only 67, 16 and 4% active.

Both crude enzymes were stable for 1 h in alkaline region up to pH 10.0. Relative activity of the G1% crude enzyme of *Ganoderma* sp. KU-Alk4 was highly stable at alkaline pH, 8.0-9.0 as well as the G4% crude enzyme that their activities remained 100% at pH 8.0-9.0. At pH 10.0, The G1% crude enzyme was still 100% stable while the G4% one was 95%. The activity of both crude enzymes were more sensitive at lower pH, 7.0-2.0. Both slightly decreased about 15 to 20%, when the enzymes were kept at lower pH for 1 h (Figure 32). The relative activities remained at pH 2.0-6.0 were approx. 80-85%. The stability of the crude enzyme of *Ganoderma* sp. KU-Alk4 grown with 1% glucose was slightly more stable to pH that that of the crude enzyme obtained from 4% glucose medium.

3.2 Optimum temperature and temperature stability

Optimum temperature for the G4% crude enzyme to exhibit its activity in 1 min was an extremely high temperature at 90ºC that was at higher temperature than the G1% crude enzyme which its optimum temperature was 37ºC (Figure 33). For the temperature stability, the G4% crude enzyme was 100% stable at high temperature up to 55ºC for 1 h. The activity could remained upto 90% after incubated at 60ºC for 1 h. At 65, 75, 85ºC, its activity remained 78, 47 and 16%, respectively. No activity remained at its optimum temperature, 90ºC, when the enzyme was at such high temperature for 1 h. On the otherhand, the G1% crude enzyme was 100% stable upto 45ºC. At 55, 65 and 75ºC, the activity remained 89, 60 and 22%, respectively. No activity remained at 80ºC. The crude enzyme of *Ganoderma* sp. KU-Alk4 grown with 4% glucose was more stable to high temperature than that of the culture grown with 1% glucose.

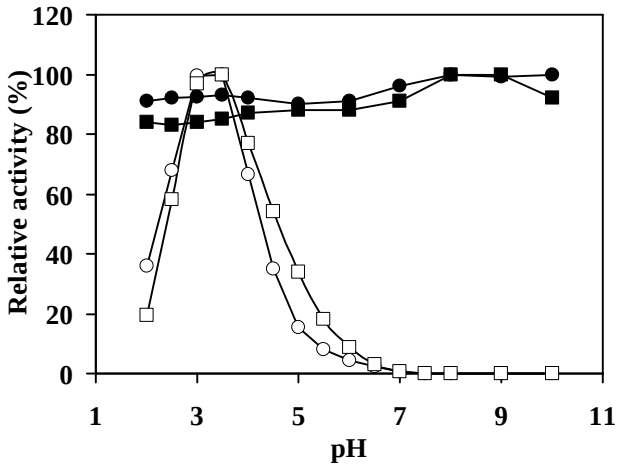

Figure 32 Optimum pH of crude laccase from *Ganoderma* sp. KU-Alk4 cultured in pH 8.0 Kirk's medium with 1% glucose (o), 4% glucose (□) and pH stability at 1 h for crude enzyme of 1% glucose (●), 4% glucose (■).

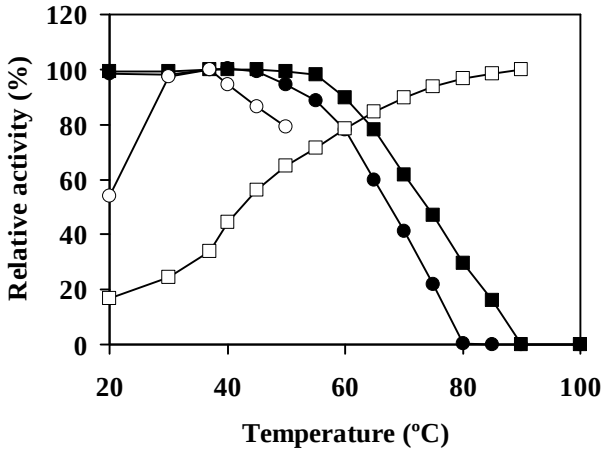

Figure 33 Optimum temperature of crude laccase from *Ganoderma* sp. KU-Alk4 cultured in pH 8.0 Kirk's medium with 1% glucose (o), 4% glucose (□) and temperature stability at 1 h for crude enzyme of 1% glucose (●), 4% glucose (■).

4. Biotechnological use of the laccase from *Ganoderma* sp. KU-Alk4

4.1 Dye decolorization by using crude enzymes

The ability of laccase (180 IU) from G1% and G4% culture to decolorize 12 dyes of varies structure (25 mg/L) were tested in 2.7 mL of 50 mM malonate buffer pH 3.5 for 6 h (Figure 34). The indigoid dye, Indigo Carmine, was the dye most effectively decolorized by crude laccases from *Ganoderma* sp. KU-Alk4 with 100% colour removal after 6 h. Indigo Carmine was rapidly decolorized by laccases from several fungi such as *Panus rudis* (Zhang *et al*., 2006), *Trametes versicolor* (Moldes *et al*., 2004) and *Pycnoporus cinnabarinus* (Schliephake *et al*., 2000). However, the maximum decolorization obtained by those fungi was 12, 91 and 83%, respectively.

Crude enzyme, G1% showed more effective decolorization than G4%. The triphenylmethane dye, Malachite Green, was decolorized by G1% by 60% colour removal, while that by G4% was 43% in 6 h. Complete decolorization of Malachite Green was observed at 24 h. In the case of Bromophenol blue and Crystal Violet, G1% caused 44 and 22% decolorization in 6 h, respectively. G4% showed 41% decolorization of Bromophenol blue, while Crystal Violet was decolorized 13% in 6 h. Complete decolorization of Bromophenol blue by both crude enzymes was observed at 24 h. On the other hand, decolorization of Crystal Violet by both crude enzymes was terminated after 24 h with 60% decolorization. Compared with the use of fungus that produce laccase, decolorization of the triphenylmethane dyes was also successful but in the different rate of decolorization when using *Pycnoporus sanguineus*. Complete decolorization of Malachite Green and Bromophenol blue, was observed in 12 days, while that of Crystal Violet was 80% (Pointing and Vrijmoed, 2000). Therefore, the direct use of crude laccase from *Ganoderma* sp. KU-Alk4 was more effective in saving time than using the fungal culture.

The decolorization in 6 h of azo dyes, Congo Red and Direct Blue 15, by G1% was 43%. There was only 7% decolorization of Direct Red 23 by G1%. The decolorization of Congo Red, Direct Blue 15 and Direct Red 23 by G4% were 39, 38 and 5%, respectively. Though, Direct Yellow 12 has been reported to be easily decolorized by *Phanerochaete chrysosporium* (Paszczynski and Crawford, 1991), it was negligible to crude laccase of *Ganoderma* sp. KU-Alk4. Reactive Red 4 and Reactive Yellow 2 were not decolorized by the crude enzymes. It has been reported that azo dyes are recalcitrant to decolorization and could only be decolorized to a limited extent (Nyanhongo *et al*., 2002) but crude laccase of *Ganoderma* sp. KU-Alk4 could efficiently degrade Congo Red, Direct Blue 15 and Direct Red 23.

In recent years, dye decolorization studies have centered on *Phanerochete chrysosporium* (Bumpus and Brock, 1988; Capalash and Sharma, 1992; Ollikka *et al*., 1993). There are only two reports on dye decolorization by *Ganoderma* sp. cells (Asgher *et al*., 2006; Revankar and Lele, 2006) and no reports on using enzymes of those fungi directly to decolorize dyes. This is the first report on direct use of the crude laccase of *Ganoderma* sp. for dye decolorization.

4.2 Dye decolorization using immobilized laccase entrapped in copper alginate

Immobilization enhances stability and allows reuse of the enzymes including laccase (Peralta-Zamora *et al*., 2003; Couto *et al*., 2004; Delanoy *et al*., 2005). Entrapment in alginate beads is one of the simplest methods of enzyme immobilization (Kierstan and Bucke, 1977) but in some circumstances the low physical stability of the beads in the presence of chelating agents can be problematical. Large pore size alginate beads allow low molecular weight substrates and products to diffuse easily but may also allow enzyme leakage. Selection of immobilization conditions is essential to design a system appropriate to each particular purpose and enzyme.

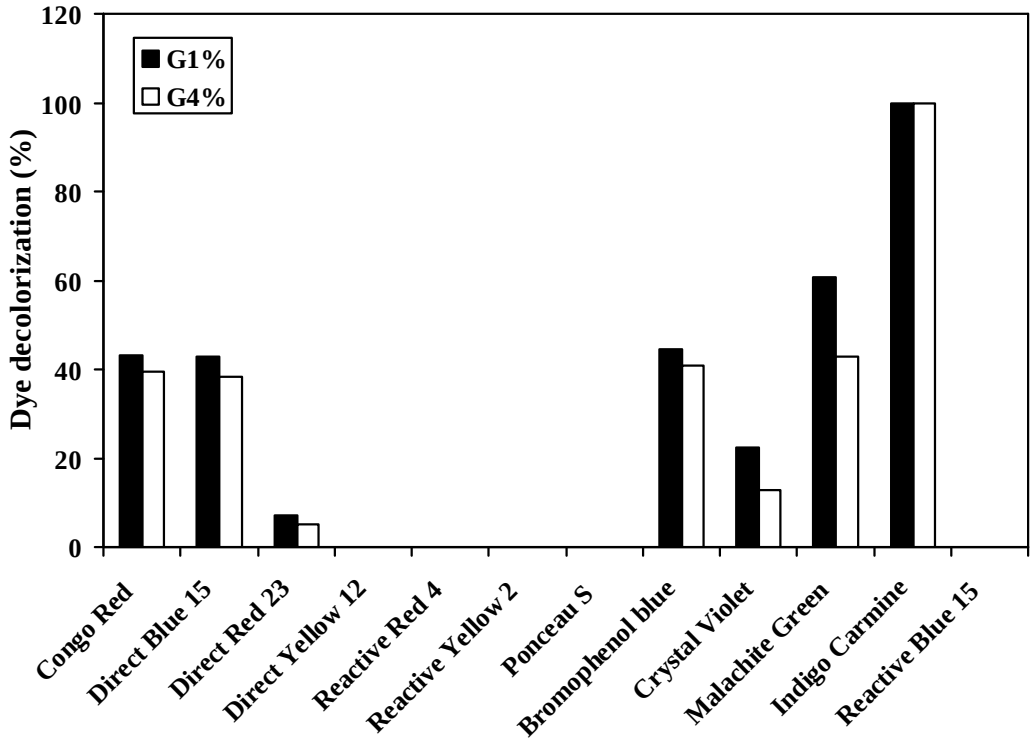

Figure 34 Decolorization of various dyes by the crude enzymes of *Ganoderma* sp. KU-Alk4 cultivated in Kirk's medium with 1% (G1%) and 4% glucose (G4%). Crude enzyme = 180 IU, dye = 25 mg/L dissolved in 50 mM malonate buffer pH 3.5 of total volume = 3 mL.

Laccase is a copper-dependent enzyme and the enzyme immobilized in copper alginate is likely to retain more activity than laccase immobilized using other methods. Laccase of *Pleurotus ostreatus* was successfully entrapped in copper alginate beads and decolorized Remazol Brilliant Blue R efficiently (Palmieri *et al*., 2005). However, optimization of laccase entrapment in copper alginate beads has not been fully examined. Hence, effects of immobilization conditions using copper-alginate laccase on activity and dye decolorization were examined using both statistical design and experimental work. Latin Square Experimental Design was used to optimize the immobilization conditions of laccase from *Ganoderma* sp. KU-Alk4. Since the free laccase of *Ganoderma* sp. KU-Alk4 was effective in decolorizing Indigo Carmine, the immobilized enzyme systems were used to decolorize Indigo Carmine. Indigo Carmine is one of the major dyes contaminating natural environments. The factors concerned in developing a satisfactory immobilized laccase preparation were the composition and concentration of alginate and the concentrations of $CuSO_4$, the cross-linking agent. Use of aluminium ions to increase the physical strength of the copper alginate beads was also investigated.

4.2.1 Decolorization of Indigo Carmine by free laccase of *Ganoderma* sp. KU-Alk4

A typical profile of Indigo Carmine decolorization by the free laccase at 25ºC is shown in Figure 35. In 3 days of incubation 45 IU enzyme with 25 mg/L dye in total volumn of 3 mL distilled water, 100% dye was decolorized. After the second addition of fresh dye into the enzyme reaction mixture, only 49% of decolorization was observed after 5 days. Total amount of dye removed was 37.2 mg/L over 12 days.

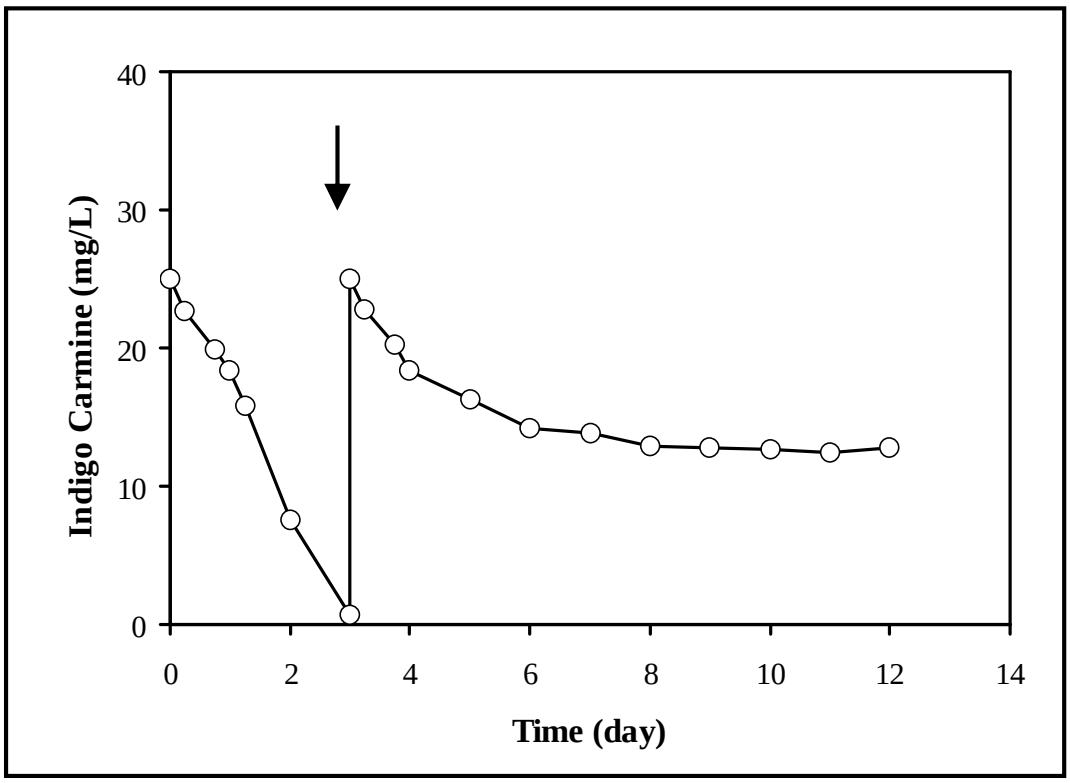

Figure 35 Typical Indigo Carmine dye decolorization by free laccase of *Ganoderma* sp. KU-Alk4 at 25ºC. Arrow indicates the addition of fresh dye solution. Values are the average of 3 independent experiments and the maximal mean deviation is ± 5% of values.

4.2.2 Effect of cross-linking agents on efficiency of the enzyme

Different kinds of alginate bead were used to entrap laccase from *Ganoderma* sp. KU-Alk4 using a standard immobilization procedure (Figure 36). With $CaCl_2$, the cross linking agent most commonly used, only 67% of the immobilization yield was obtained. Cu-Al alginate beads gave the highest immobilization yield followed by Cu-alginate beads and Ca-alginate beads, respectively. The activity of *Ganoderma* sp. KU-Alk4 laccase was increased 1.15 times by the addition of 0.15 M $CuSO_4$ and 1.25 times with the combination of $CuSO_4$ and $AlCl_3$, each at 0.075 M.

$CuSO_4$, 0.15 M, could stimulate the activity of *Ganoderma* sp. KU-Alk4 to 40% which 140% residual activity was obtained using 0.15 M $CuSO_4$ for 2 days (Figure 37). In the presence of 0.15 M $CuSO_4$ 124% activity remained even after incubation for 5 days. Stimulation of laccase activity upon the addition of Cu^{2+}-ion (50 mM) is also reported in *Pleurotus ostreatus* (Baldrian *et al*., 2002). Laccase is a copper enzyme that has 4 copper (II) ions which are distributed among the enzyme binding sites and play important role in the catalytic mechanism of laccase (Duran *et al*., 2002). Therefore, the copper ions used to form beads enhanced the activity of laccase of *Ganoderma* sp. KU-Alk4.

$CuSO_4$ and $AlCl_3$, each of 0.075 M when used as cross-linking agents, gave even higher activity to that with 0.15 M $CuSO_4$ (Figure 36). The trivalent cation, Al^{3+}, could improve gel strength and reduce pore size of beads. Laccase of *Trametes villosa* immobilized by adsorption on $Al(OH_3)$ showed enhanced activity as well as stability (Ahn *et al*., 2007). However, beads produced using 0.15 M $AlCl_3$, exhibited only 58% of the initial activity of *Ganoderma* sp. KU-Alk4 laccase (Figure 36).

Laccase of *Ganoderma* sp. KU-Alk4, though, showed the highest activity at zero time when immobilized in the Cu-Al alginate gel but its activity was not stable in alginate beads produced using 0.15 M $AlCl_3$ or 0.075 M $AlCl_3$ in

combination with $CuSO_4$ (Figure 37). The activity of the free laccase of *Ganoderma* sp. KU-Alk4 was inhibited by 0.15 M $AlCl_3$ 55, 20 and less than 10% of initial activity remaining after 1, 2 and 5 days, respectively. With 0.075 M $AlCl_3$ in combination with $CuSO_4$ at the same concentration, 80% of initial enzyme activity was retained after 1 day and activity gradually decreased to retain only 30% in 5 days. This indicated that Al^{3+}, but not Cu^{2+}, caused the progressive inactivation of *Ganoderma* sp. KU-Alk4 laccase. Therefore, use of Al^{3+} with laccase of *Ganoderma* sp. KU-Alk4 gave the highest immobilization yield but was not appropriate for the retention of enzyme activity. This is the first time, to our knowledge, that inhibition of laccase activity by aluminium ions has been demonstrated.

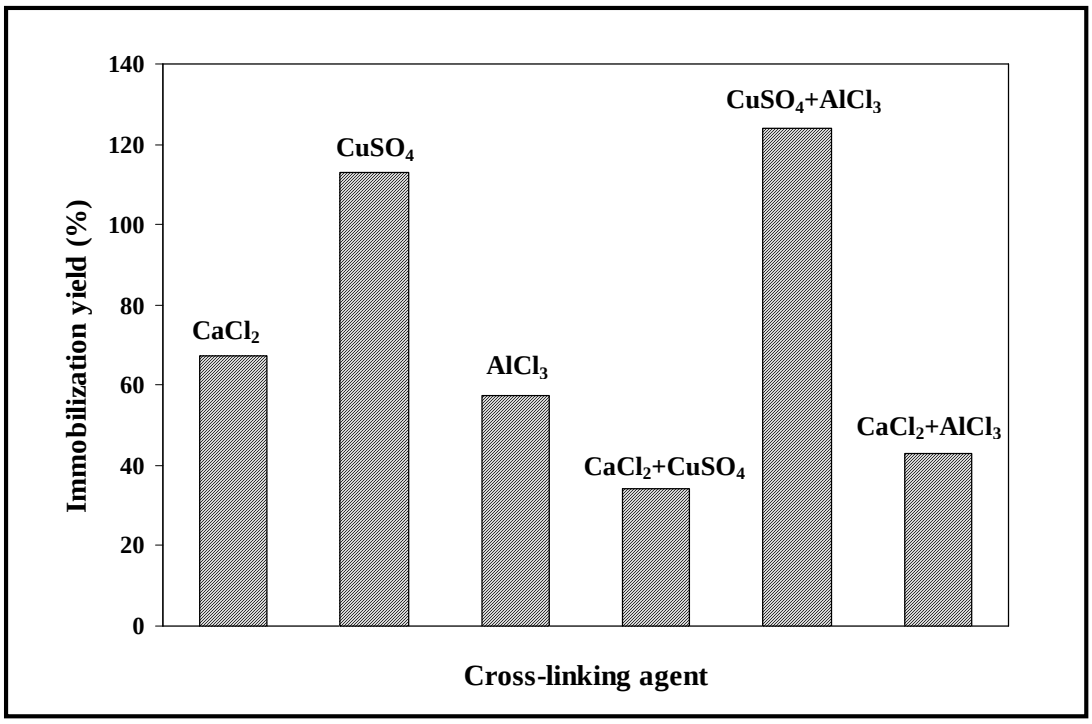

Figure 36 Activity of the immobilization laccase that entrapped in alginate bead with different kinds of cross linking agents. Standard immobilization conditions: 3.0% sodium alginate type A (BDH Chemicals) and 0.15 M cross-linking agent. Values are the average of 3 independent experiments and the maximal mean deviation is ± 5% of values.

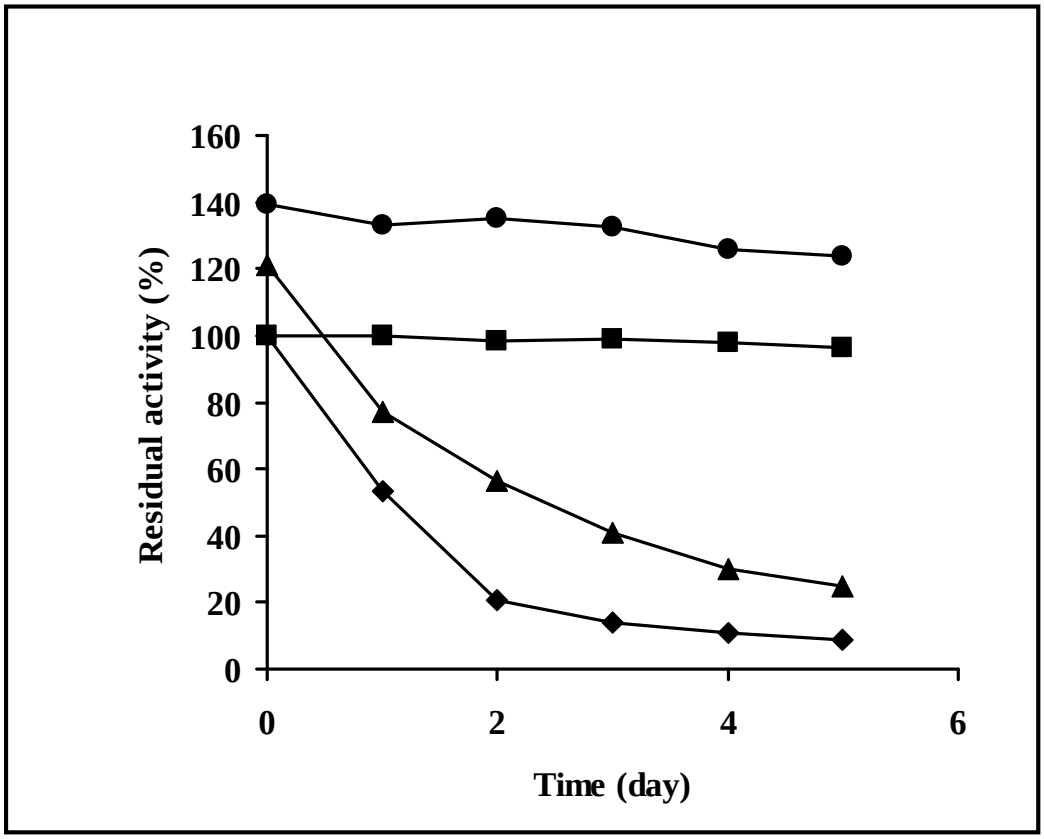

Figure 37 Stability of free laccase of *Ganoderma* sp. KU-Alk4 in the presence of 0.15 M of the different cations, $CuSO_4$ (●), $AlCl_3$ (◆), and $CuSO_4$:$AlCl_3$ (ratio = 1:1,▲) incubated at 200 rpm, room temperature (25ºC ± 2). Control was that without addition of any cations (■).Values are the average of 3 independent experiments and the maximal mean deviation is ± 5% of values.

4.2.3 Effect of immobilization conditions on residual activity of laccase

Optimization of laccase entrapped in Cu-alginate bead was studied using Latin Square Design. Table 21 shows data on the effect of three variables on the residual activity and Indigo Carmine decolorization. The 3 variables were alginate type (F_1), alginate concentration (F_2) and $CuSO_4$ concentration (F_3).

Analysis of variance (ANOVA) of the main factors affecting immobilization yield showed that the most significant factor was the concentration of cross-linking agent or gel inducer ($P<0.05$) followed by alginate composition ($P<0.1$) while polymer concentration was not significant ($P>0.1$) (Table 22)**.**

The maximum immobilization yield of entrapped *Ganoderma* sp. KU-Alk4 laccases was 146.8% (Table 21, treatment 6) which higher than that was prepared with a standard immobilization procedure. The yield is also higher than that obtained on immobilization of the laccase of *Pleurotus ostreatus* (Palmieri *et al*., 1994, 2005).

The maximum immobilization yield was obtained with the 50:50 mixture of alginates types A and B at 3.0% (w/v) alginate concentration and 0.075 M $CuSO_4$ (Figure 38). Increasing the concentration of $CuSO_4$ had a negative effect on immobilization yield. Increasing of the alginate or $CuSO_4$ concentration limited the substrate transfer into the alginate bead (Knezevic *et al*., 2002). In the immobilization of *Candida rugosa* lipase, increasing the alginate concentration decreased immobilization yield but increasing the concentration of the cross-linking agent, $CaCl_2$, had little effect (Won *et al*., 2005). Results of Latin Square studies suggested the conditions that gave the highest immobilization yield of *Ganoderma* sp. KU-Alk4 laccase would be 3% alginate in 50:50 % w/w of type A:type B and 0.075 M $CuSO_4$ that gave the enzyme activity 3 IU/bead.

Table 21 The Coded and Latin Square Experimental Design.

Treatment	Factors			Immobilization yield (%)	Dye decolorization[a] (%)	Total dye removed[b] (mg/L)
	F_1	F_2	F_3			
1	-1	-1	-1	109.9	53.9	95.3
2	-1	0	0	114.3	63.7	123.5
3	-1	+1	+1	74.6	58.2	112.6
4	0	-1	0	85.2	50.3	55.8
5	0	0	+1	122.1	52.8	118.6
6	0	+1	-1	146.8	57.4	123.0
7	+1	-1	+1	109.7	53.0	77.8
8	+1	0	-1	117.4	45.8	65.5
9	+1	+1	0	120.5	46.1	99.8

F_1 = alginate type, F_2 = alginate concentration, F_3 = $CuSO_4$ concentration

[a] Reaction time: 6 h.

[b] Total amount of dye removed over 7 days (total dye supplemented = 150 mg/L)

Table 22 Analysis of variance of the effects of co-polymer type (F_1), concentrations of polymer (F_2) and gel inducer (F_3) on immobilized yield of laccase of *Ganoderma* sp. KU-Alk4.

Key	SS	df	MS	F	*p*-value
F_1	1603.3	2	801.7	2.659	0.095*
F_2	1150.0	2	575.0	1.902	0.175
F_3	2247.0	2	1123.5	3.716	0.042**
Residual	6046.2	20	302.3		

Key: SS, sum of squares; df, degree of freedom; MS, mean square.

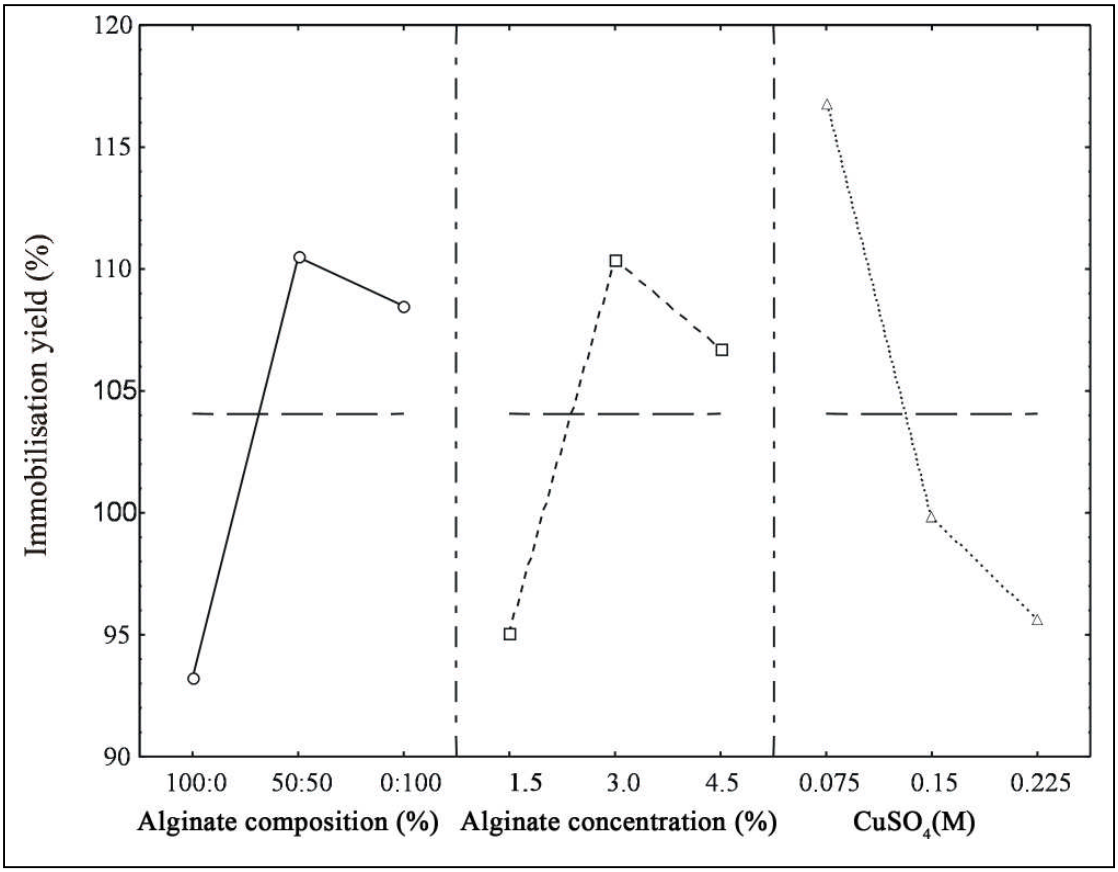

Figure 38 Effect of alginate composition (○), alginate concentration (□) and $CuSO_4$ concentration (Δ) on immobilization yield of laccase of *Ganoderma* sp. KU-Alk4.

4.2.4 Effect of immobilization conditions on Indigo Carmine decolorization

After 12 h of Indigo Carmine decolorization, results of ANOVA showed that the key statistically significant (P<0.001) treatment that affected dye decolorization was alginate composition, while concentrations of alginate and $CuSO_4$ agent were not significant (Table 23). Figure 39 showed that the use of alginate of low mannuronate content (type A), favoured dye decolorization more than those of higher mannuronate content such as 50:50 of type A:type B or 100% type B. Changing the alginate concentration, whatever the alginate composition, did not alter the extent of dye decolorization. The higher percent decolorization was obtained when the concentration of $CuSO_4$ increased from 0.075 to 0.225 M, plausibly because the Cu^{2+} ion improved the stability of the laccase. This occurred with the laccases of *Chalara paradoxa* CH32 and *Trametes villosa* (Robles *et al*., 2002; Brandi *et al*., 2006). However, the effect of alginate concentration was very small on dye decolorization over the test range of 1.5-4.5%. The optimal condition that resulted in the fastest decolorization of Indigo Carmine was at 3% alginate A with 0.225 M $CuSO_4$.

Table 23 Analysis of variance of the effect of co-polymer type (F_1) and concentrations of polymer (F_2) and gel inducer (F_3) on Indigo Carmine decolorization by immobilized laccase of *Ganoderma* sp. KU-Alk4

Key	SS	df	MS	F	*p*-value
F1	478.9	2	239.4	10.050	0.001**
F2	15.5	2	7.7	0.324	0.727
F3	23.5	2	11.7	0.493	0.618
Residual	476.5	20	23.8		

For key to treatments, see Table 1. Key: SS, sum of squares; df, degree of freedom; MS, mean square

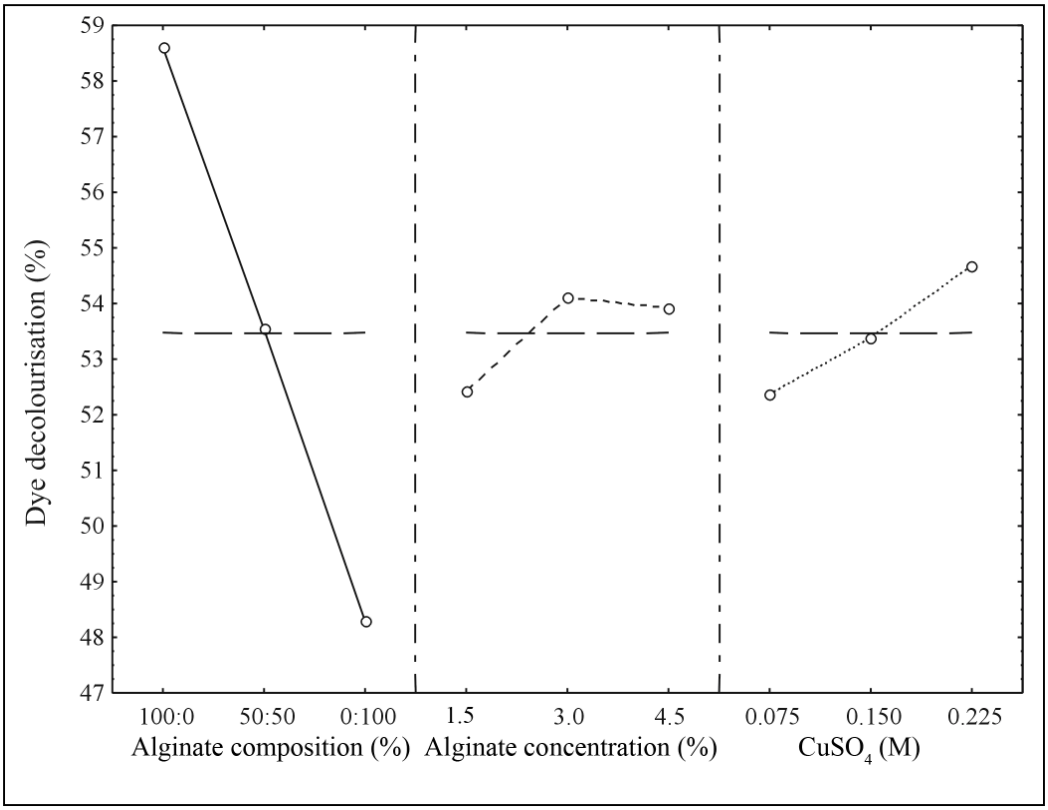

Figure 39 Effect of alginate composition (○), alginate concentration (□) and $CuSO_4$ concentration (Δ) on Indigo Carmine decolorization by immobilized laccase of *Ganoderma* sp. KU-Alk4.

4.2.5 Effect of immobilization conditions on repeated batch decolorization

If the immobilized laccase is to find commercial use it must retain its activity over several reuses. In order to carry out a continuous process for further implementation in industry, the effects of immobilization conditions on Indigo Carmine decolorization were further checked by comparing the performance of the immobilized preparations in repeated batches. Each experiment ran for 7 days. Results of ANOVA (Table 24) showed that the most significant factor in repeated batch was the concentration of alginate ($P<0.001$) followed by the alginate composition ($P< 0.05$). Concentration of the cross-linking agent was not significant. Alginate type A was still the best enzyme entrapment material for dye decolorization (Figure 40). This confirms that the "tightest" alginate gels, produced using the highest concentration of the alginate richest in guluronate residues is the most effective entrapment material for long-term use of this immobilised laccase, plausibly because there is minimal leakage of enzyme from the beads.

Table 24 Analysis of variance of the effect of co-polymer type (F_1) and concentrations of polymer (F_2) and gel inducer (F_3) on repeat sequencing batch decolorization.

Key	SS	df	MS	F	*p*-value
F1	3967.3	2	1983.6	5.638	0.011*
F2	6101.0	2	3051.5	8.673	0.002**
F3	518.0	2	259.0	0.736	0.492
Residual	7036.9	20	351.9		

Key: SS, sum of squares; df, degree of freedom; MS, mean square

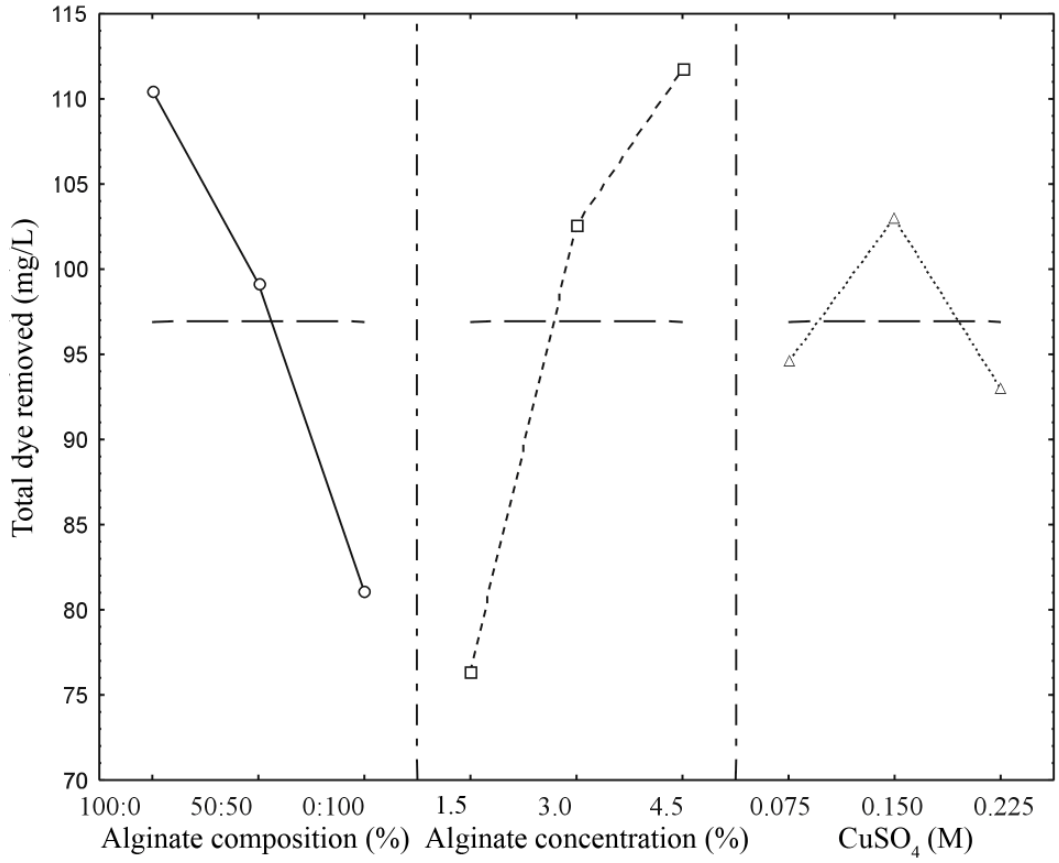

Figure 40 Effect of alginate composition (○), alginate concentration (□) and $CuSO_4$ concentration (Δ) on repeat sequencing batch of Indigo Carmine decolorization by immobilized laccase of *Ganoderma* sp. KU-Alk4.

4.2.6 Performance of immobilized laccase of *Ganoderma* sp. KU-Alk4 at the optimal conditions in Indigo Carmine decolorization

Data from repeated batch experiment was used to find the optimal conditions in dye decolorization. A contour diagram plotted by using polynomial equation summarized the optimal condition in relation to composition and concentration of alginate and concentration of cross-linking agent (Figure 41).

To confirm the optimal condition, a set of 5-replicated experiments in using the immobilized laccase with 3.6% w/v alginate type A and 0.15 M $CuSO_4$ to decolorize Indigo Carmine in repeated batch was run (Figure 42). Total activity of the immobilized laccase was 45 IU. Indigo Carmine, 25 mg/L, dissolved in 3 mL distilled water was used in each batch with 200 rpm shaking rate. Decolorization efficiency towards Indigo Carmine of the immobilized laccase remained 100% completed over 6 cycles, one day for each cycle. After that, the time needed to achieve 100% decolorization increased. At the 8^{th} repeated batch, after 12 days, only 64% of the dye was degraded. During the experiment, neither adsorption of dye to the gel nor significant enzyme release was detected. At the end of 12 days incubation, total amount of removed dye in the repeated batch system by immobilized laccase was 216 mg/L which was 5.4 times more than achieved when using the free enzyme system.

Our results strongly support the use of Cu-alginate laccase based on an optimized immobilized system for Indigo Carmine decolorization. The optimization of the immobilized system not only improved total amount of dye removed under repeated batch but also reduced each batch operation time over that achieved in previous work with free systems. Enzymes are generally more effective in buffered systems but our immobilized laccase showed effective decolorization without buffering giving considerable savings in time and cost.

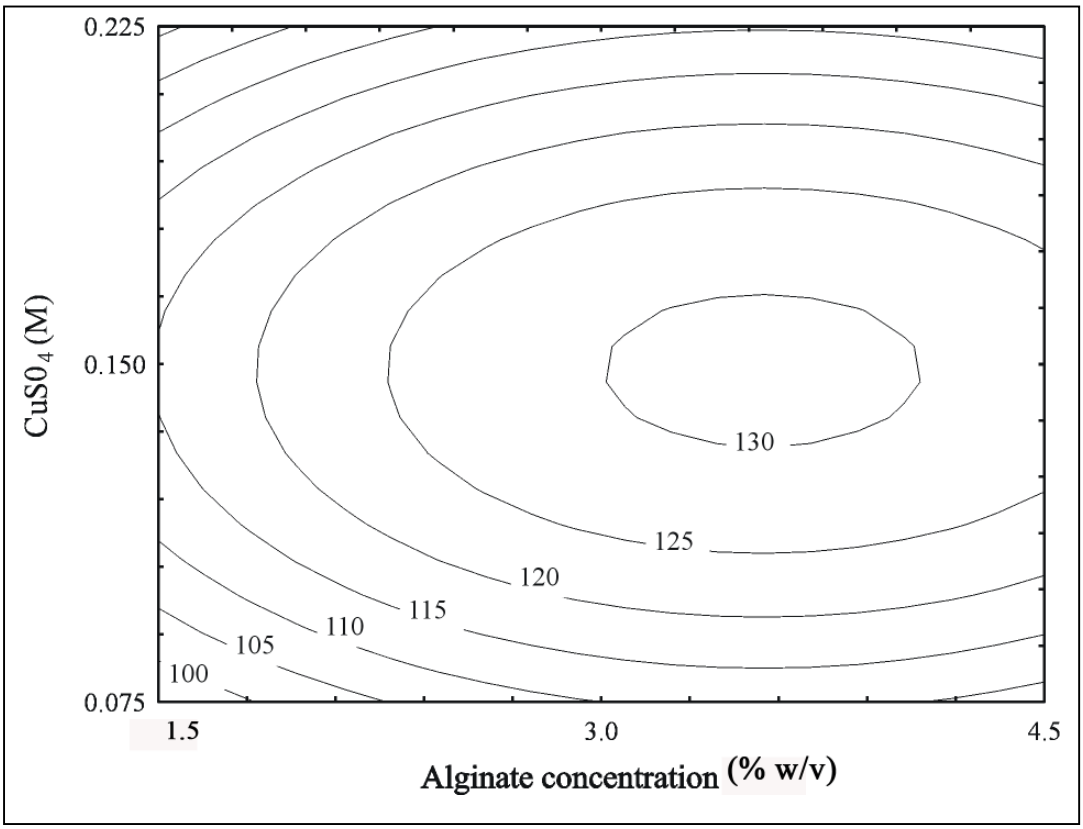

Figure 41 Contour diagram of total Indigo Carmine removed affected by alginate concentration and $CuSO_4$ concentration at alginate type A using Latin Square Design.

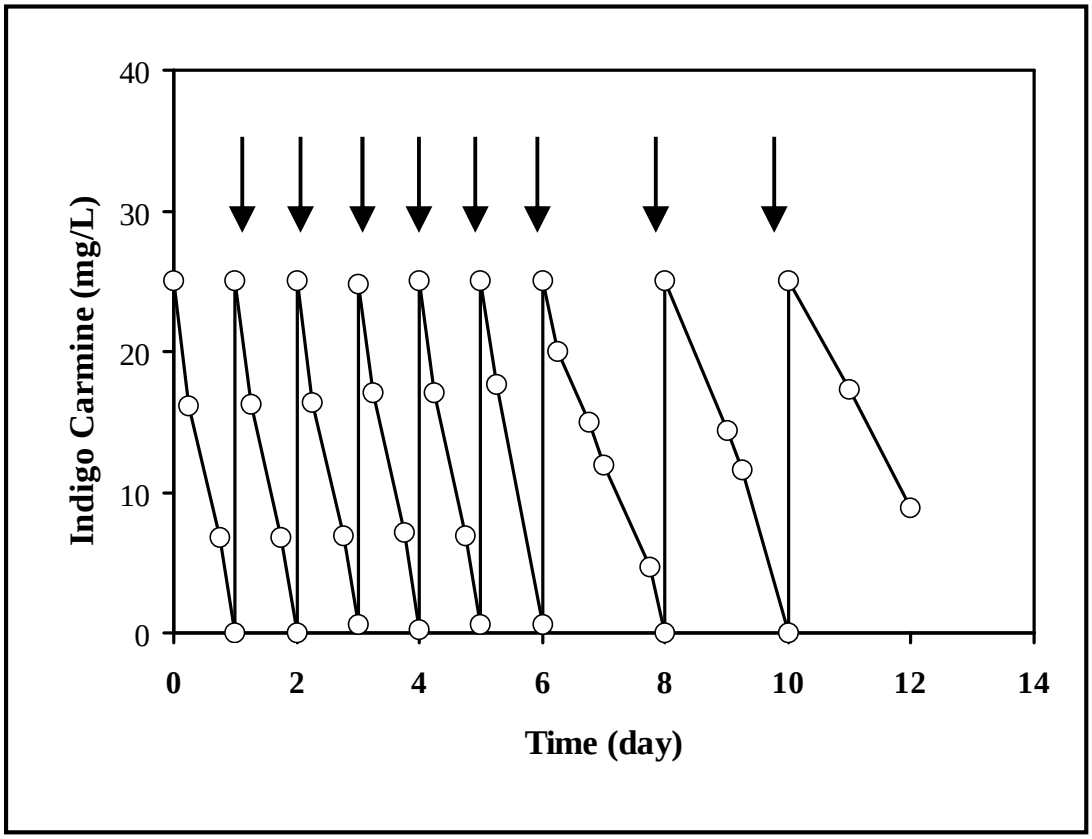

Figure 42 Confirmatory run of the best immobilization treatment: alginate A at 3.6% w/v and 0.15 M $CuSO_4$ as cross-linking agent. Arrows indicate the addition of the fresh dye solution, 25 mg/L dye in 2.7 mL distilled water. Total activity of the immobilized laccase was 45 IU. Values are the average of 5 independent experiments and the maximal mean deviation is ± 5% of values.

Palmieri *et al*. (2005) reported that 70% Remazol Brilliant Blue R decolorization was achieved by using Cu-alginate laccase of *Pleurotus ostreatus* prepared with 3% sodium alginate type B and 0.15 M $CuSO_4$. The dye decolorization was still efficient even after 10 cycles when a pH 4.5 buffer was added to the reaction, however, this is not easily achievable, the main reason being the leaking of laccase at the beginning of process that resulted from the large amount of enzyme use for immobilization.

The beads of *Ganoderma* sp. KU-Alk4 laccase made of type A alginate showed no enzyme leakage occurring during 6 days of dye decolorization. The use of 0.15 M $CuSO_4$ enhanced the enzyme activity and stability, allowing small amounts of enzyme to be used to produce effective beads. Moreover, the immobilization enzyme reaction against Indigo Carmine was successfully achieved in water for 10 cycles and 100% of the dye was diminished. The Latin Square Design approach to process optimization thus proved effective in developing a dye decolorization system using the immobilized laccase of *Ganoderma* sp. KU-Alk4.

4.3 Using a 5-litre airlift bioreactor for Indigo Carmine decolorization by copper alginate immobilized laccase of *Ganoderma* sp. KU-Alk4

The immobilized enzyme entrapped in copper alginate beads was prepared according to the optimal condition suggested by Latin Square Design to decolorize Indigo Carmine in a 5 L-airlift bioreactor. The optimal condition was 3.6% w/v BDH alginate and 0.15 M $CuSO_4$. The immobilized laccase of 6 x 10^4 IU, 400 mL were added to decolorize 3.6 L of 25 mg/L Indigo Carmine dissolved in distilled water. Air flow rate was fixed at 10 L air/4 L reaction mixture/min to ensure a good circulation of Cu-alginate beads within airlift bioreactor expected good degradation of dye. The beads were used over 20 days in airlift bioreactor. In these condition, decolorization efficiency towards Indigo Carmine of the immobilized laccase remained 100% completed over 4 cycles, four days for each cycle (Figure 43). At the 5^{th} repeated batch, after 20 days, only 20% of the dye was degraded. At the end of 20 days incubation, total amount of removed dye of the repeated batch system in airlift

bioreactor by the immobilized laccase was 104 mg/L which was 2.1 times less than achieved using the immobilized enzyme in 3 mL-shaking system. Moreover, the time needed to achieve 100% decolorization increased 2 times when compared to that of 3 mL-experiment.

During the experiment, neither adsorption of dye to the gel nor significant enzyme release was detected. In the dye-decolorization system no buffer was used so there is no degradation of the alginate gels by chelation of copper during use. The decolorization efficiency of two-replicated bioreactors were similar. Using airlift bioreactor with the optimized immobilized system not only decreased total amount of dye removed under repeated batch but also increased each batch operation time over that achieved in previous work with shaking system. According to catalytic mechanism of laccase utilizes molecule oxygen as oxidant so this would be occurred from unsuitable of oxygen transfer in the airlift bioreactor.

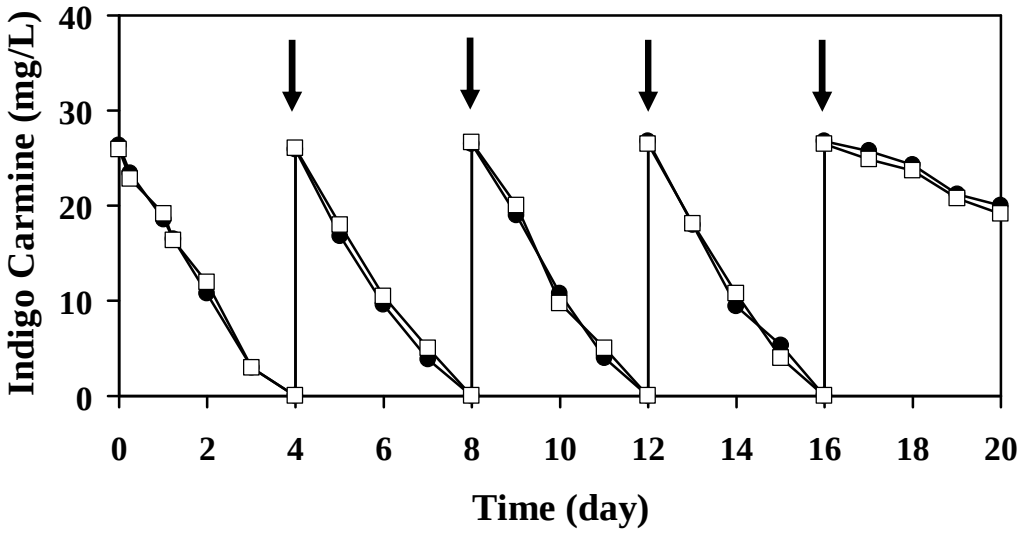

Figure 43 Indigo Carmine decolorization in 5 L-airlift bioreactor using immobilized laccase of *Ganoderma* sp. KU-Alk4 entrapped in Cu-alginate bead. The immobilized laccase of 6 x 10^4 IU were added to decolorize of 25 mg/L Indigo Carmine dissolved in 4 L distilled water. An airflow rate was controlled at 10 L air/4 L reaction mixture/min. Arrows indicate the addition of 3.6 L of 25 mg/L fresh dye solution.

Optimization of oxygen transfer rate affected to the efficiency of the immobilized laccase in Indigo Carmine decolorization was examined. The air flow rates were adjusted between 0-10 L air/4 L reaction mixture/min. Increase in dissolved oxygen (DO) in the reactor fluid was measured. The dye decolorization was followed for 20 days.

When air flow was omitted from bioreactor, decolorization efficiency towards Indigo Carmine of the immobilized laccase remained 100% completed over 9 cycles, two days for each cycle (Figure 44A). After 20 days, only 35% of the dye was degraded. At the end of 20 days incubation, total amount of removed dye of the repeated batch system in the airlift bioreactor by the immobilized laccase was 237 mg/L which was 1.1 times more than achieved using the immobilized enzyme in 3 mL-shaking system and 2.3 times better than that obtained from the reactor with 10 L air/4 L reaction mixture/min oxygen flow rate was used.

When air flow rate was adjusted to 4 L air/4 L reaction mixture/min, decolorization efficiency was 100% completed over 14 cycles (Figure 44B). The complete dye decolorization rate of the first 10 days was one day for each cycle. Decolorization time exponentially increased after the 10^{th} cycle to 2 days. Four cycles of complete decolorization, though 2 days-each, could be obtained until 20 days. Total amount of removed dye at the end of 20 days incubation was 350 mg/L which was 1.6 times more than that of 3 mL-shaking system and 3.4 times better than that when 10 L/min oxygen flow rate was used.

At flow rate of 7 L air/4 L reaction mixture/min, decolorization efficiency was 100% completed over 6 cycles (Figure 44C). The first 4 cycles were two days of each cycle. After that decolorization time expanded to four days for the other cycles. At the last cycle, the 7^{th}, the dye was 80% decolorized. Total amount of removed dye at the end of 20 days incubation was 169 mg/l which was 1.3 times more than that of 3 mL-shaking system and 1.6 times better than that when 10 L/min oxygen flow rate was used.