Chapter 4

Results and Discussion

This chapter discusses and analyses the effects of pertinent factors, such as: pH, co-substrate concentration and contact time, on the removal efficiency of color, BOD, COD of different types of leachate in batch experiment by white rot fungi. It also investigates and evaluates the feasibility of leachate treatment with the fungi immobilized on PUF at the optimum conditions in continuous experiment.

4.1 Leachate characteristics

The characteristics of 4 types of leachate collected during different time periods and from different location used in this study were compared with the industrial effluent standards of Thailand and are shown in Table 4.1. Leachate 1 was collected from the large stabilization pond in May 2006. Leachate 2 was collected from a pipe as discharged from landfill to the stabilization pond in December 2006. Leachate 3 was collected from the garbage truck directly in Nonthaburi district in December 2006. Leachate 4 was collected from a pipe as discharged from landfill to the stabilization pond in March 2008. Leachate 4* was the leachate 4 which was kept for one year in 4ºC storage room.

Table 4.1 Landfill leachate characteristics

Parameters	Unit	Industrial Effluent Standard (PCD, 2006)	Leachate 1	Leachate 2	Leachate 3	Leachate 4	Leachate 4*
1. pH	-	5.5-9.0	8.31	5.33	3.85	5.7	7.9
2. SS	mg/L	<50 (not exceed 150)	376	5,526	5,467	16,584	-
3. Color	ADMI	No standard	600	900	600	3196	2074
4. BOD_5	mg/L	<20 (not exceed 60)	2,100	5,600	625	48,900	38,100
5. COD	mg/L	<120 (not exceed 400)	4,870	34,560	1,728	96,512	69,580
6. BOD/COD			0.43	0.16	0.36	0.6	0.54
7. Ammonia	mg/L	TKN <100 (not exceed 200)	1,542	182.1	216	20	1,568 (TKN = 3,432 mg/L)
8. Total heavy metal concentration							
- Cd	mg/L	<0.03	0.023	1.20	0.1	0.05	-
- Cr (Total)	mg/L	<0.25	0.56	31.50	22.5	0.17	-
- Pb	mg/L	<0.2	0.012	2.50	0.05	0.02	-
- Hg	mg/L	<0.005	ND	1.60	0.05	0.02	-
- Ni	mg/L	<1.0	0.264	ND	0.01	0.1	-

Note: ND = Not Detectable

The fresh and stabilized leachate was defined based on the pH, SS, BOD/COD, and ammonia concentration. In fresh leachate from young landfill (the acid-phase landfill), the concentrations of organic compounds (BOD and COD) are very high whereas in old leachate from old landfill (the methanogenic-phase landfill), the levels of organic matter are substantially lower (Tsilogeorgis et al., 2008). According to Amokrane (1997), the fresh leachate has the pH less than 6.5, BOD:COD ratio about 0.5, low ammonia whereas the intermediate stabilized leachate has the pH around 5-10, BOD:COD ratio of about 0.1-0.5 and higher ammonia.

It can be observed from Table 4.1 that the pH of the leachate 1 and leachate 4* (stabilized) is higher than the fresh leachate (leachate 2, 3, and 4). In addition, the leachate samples displayed high concentrations of contaminants. The organic strength of leachate varies in the different samples collected with leachate 4 being the highest. In all cases, COD values are higher than BOD. Analysis shows that leachate 1 and 4* are stabilized with high ammonia concentration and high pH as compared to leachate 2, 3, and 4 which seem to be fresh with low ammonia concentration, lower pH and very high suspended solids. For leachate 4*, it is stabilized as can be seen from BOD and COD reduction and the ammonia content is increased from 20 mg/L to 1,568 mg/L and cause the pH to be basic. However, the BOD:COD ratio of 0.16 of leachate 2 corresponded to a partly stabilized leachate (0.2) according to Tatsi et al., 2003. For leachate 3 and 4, they are fresh leachate because of the acidic pH, very high suspended solids, and high BOD/COD ratio as well as low ammonia content (Amokrane et. al., 1997).

The color of leachate 1 was brown and the color of leachate 2 and 4 were black and leachate 3 was dark-yellowish. The heavy metal concentration (Cr) in leachate 1 is beyond the standards. For leachate 2, Cd, Cr, Pb and Hg exceeded the standards, particularly; Cr concentration was very high with the maximum of 31.5 mg/L, exceeding the Industrial Effluent Standard which is 0.25 mg/L. For leachate 3, the Cd, Cr and Hg concentration exceeded standard. For leachate 4, only Cd and Hg exceeded standard. This may be due to the mixing of solid waste with some toxic waste and also due to low pH which increases the solubility of heavy metals. Higher Pb concentrations in the leachate 2 may be due to that Pb had higher leaching rate than the other metals by the toxicity characteristic leaching procedure (TCLP), since lead acetate is one of the most soluble lead compounds (44.2 g/100 ml H_2O) (Wei et al., 1998 and Ioannidis and Zouboulis, 2003). Moreover, Pb solubility increases at lower (acidic) pH values (Ioannidis and Zouboulis, 2003). In addition, these may be due to the difference of the incoming raw waste and seasonal variation. Overall, it can be seen that five types of leachate varied widely in terms of organic content and ammonia.

Characteristics of leachate collected from Ram-Indra Transfer Station

The characteristics of leachate from Ram-Indra Transfer station is shown in the Table 4.2 below.

Table 4.2 Characteristics of leachate from Ram-Indra Transfer station, Bangkok, Thailand

Parameters	Unit	Value
pH	-	4.33
BOD	mg/L	37,000
COD	mg/L	416,000
NH3-N	mg/L	0.24
Color	ADMI	250
TKN	mg/L	0.7

The leachate characteristics indicated that the leachate was not as fresh as expected. BOD and COD value is extremely high and ammonia nitrogen was very low. Due to very high value of COD compared to other leachate, the leachate collected from Ram-Indra was not used for further study.

4.2 BOD and COD of co-substrate

While previous studies have shown a need for a carbon source (Swamy and Ramsay, 1999b), it is of interest to find the carbon source as the co-substrate which coincides with the growth rate of the fungi as well as the decoloration rate. BOD and COD of the co-substrates used in this study (glucose, corn starch, cassava and bagasse) are shown in Figure 4.1.

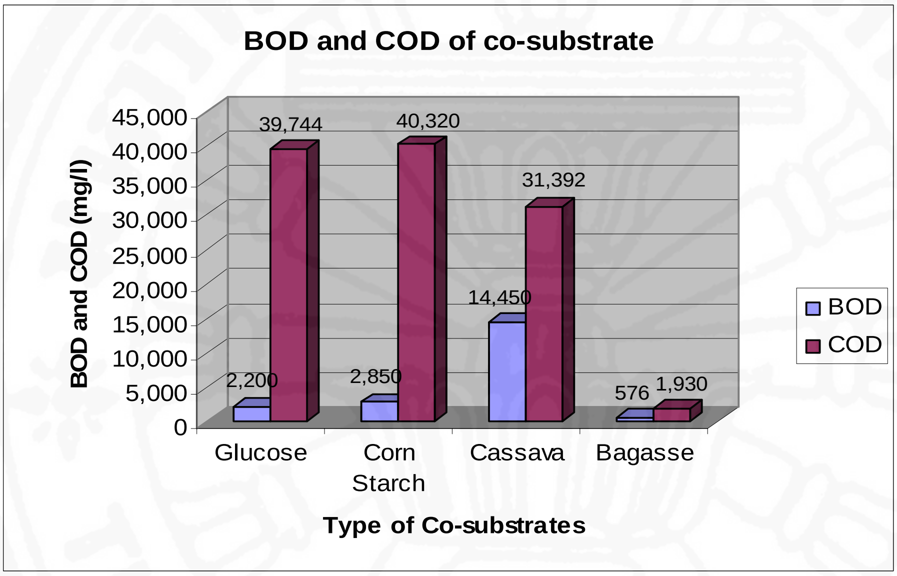


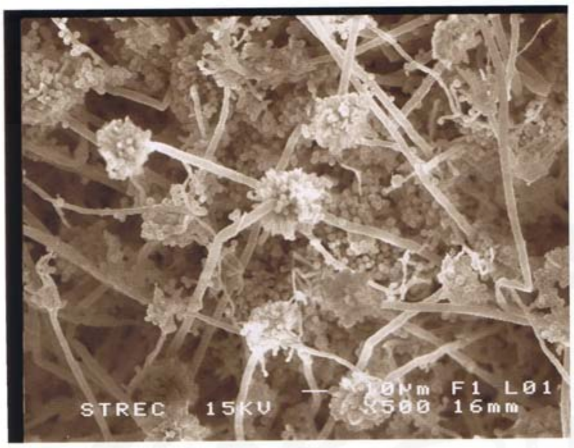
Figure 4.1 BOD and COD of co-substrates

The procedure to find BOD and COD of the co-substrates is to start with drying, grinding and dissolving them in the water. These are then filtered to get the clear water and follow the same procedure as stated in Standard Method.

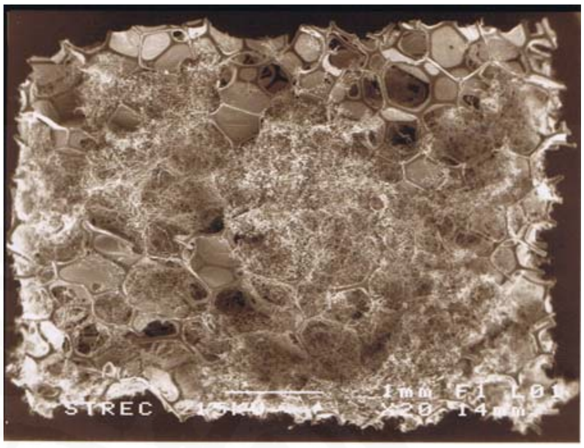
From Figure 4.1, it can be seen that the BOD values (organic content) are in the order from high to low of cassava>corn starch>glucose>bagasse, while COD value as corn starch>glucose>cassava>bagasse. COD value of glucose and corn starch is almost similar while BOD value of cassava is extremely high compare to glucose and corn starch. Bagasse has the lowest carbon content. However, bagasse was not used for further study because it was found to have lowest removal efficiency among the different co-substrates used in this study.

4.3 Structure of *T.versicolor* BCC 8725 by SEM

The Scanning Electron Microscope (SEM) study was done to investigate the structure of *T.versicolor* BCC 8725. The mycelia immobilized PUF cubes SEM images are shown in Figure 4.2 (a) and (b).



(a)



(b)

Figure 4.2 (a) Spore-producing structure of *T.versicolor* BCC 8725 **(b)** *T.versicolor* BCC 8725 immobilized on PUF

Figure 4.2 (a) shows spore-producing structure of *T. versicolor* BCC 8725 and Figure 4.2 (b) shows *T.versicolor* BCC 8725 immobilized on PUF. Experiments were conducted both with mobilized and immobilized fungi. Higher yield of biomass was observed in case of immobilization. The higher yield of biomass growth in immobilized fungi compare to mobilized maybe attributed to the fact that PUF cubes allows the contact area between cells and oxygen to be increased without the effect of shear stress. The increase in surface area of fungal biomass of PUF cubes tends to reduce the mass transfer limitations compared to free mycelia, which increases easy access to the substrate utilization. In addition, oxygen is necessary for the production of ligninolytic enzymes by white rot fungi. It has been reported that immobilization of fungal cells using PUF could stably maintain the production of various enzymes at levels higher than achieved with suspended or pellet forms (Lapadatescu et al., 1997; Nakamura et al., 1999; Kim and Shoda, 1999). Removal efficiency in this study was also found higher in case of immobilized fungi compared to pellet form or mobilized fungi (results shown in section 4.4.3.5).

4.4 Batch experiment

Batch experiments were conducted for treatment of all types of leachate with different co-substrates (glucose, corn starch, cassava) and concentrations (1, 2, 3 g/L) by *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 to determine the optimum condition. Experiments were conducted with filtered leachate to remove suspended solids and the biomass growth on PUF can occur efficiently as well. Color, BOD and COD removal were measured after filtration.

4.4.1 Leachate 1

4.4.1.1 Biomass growth of fungi

Biomass growth of both fungi in leachate 1 was studied and the results are shown in Figure 4.3.

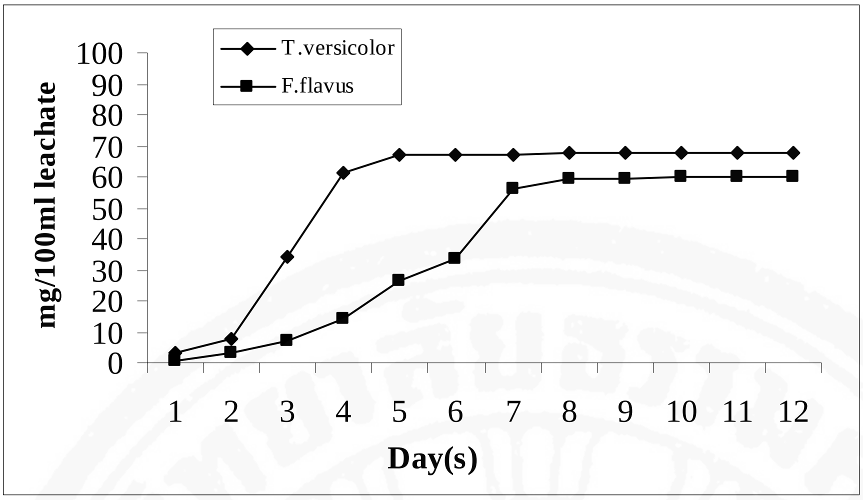


Figure 4.3 Biomass growth of *T.versicolor* BCC 8725 *vs. F.flavus* BCC 17421 of leachate 1

It can be seen from Figure 4.3 that *T.versicolor* BCC 8725 growth sharply increased on the 2nd day to 4th day and then become almost constant whereas the *F.flavus* BCC 17421 gradually increased from the beginning and tend to become constant from the 9th day. After 12 days, the biomass of *F.flavus* BCC 17421 was still lower than *T.versicolor* BCC 8725. Thus, the overall growth of *T.versicolor* BCC 8725 is higher than *F.flavus* BCC 17421.

4.4.1.2 Effect of pH on color removal

The effect of pH on color removal without co-substrate addition by *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 in leachate 1 are shown in Figure 4.4 and 4.5, respectively.

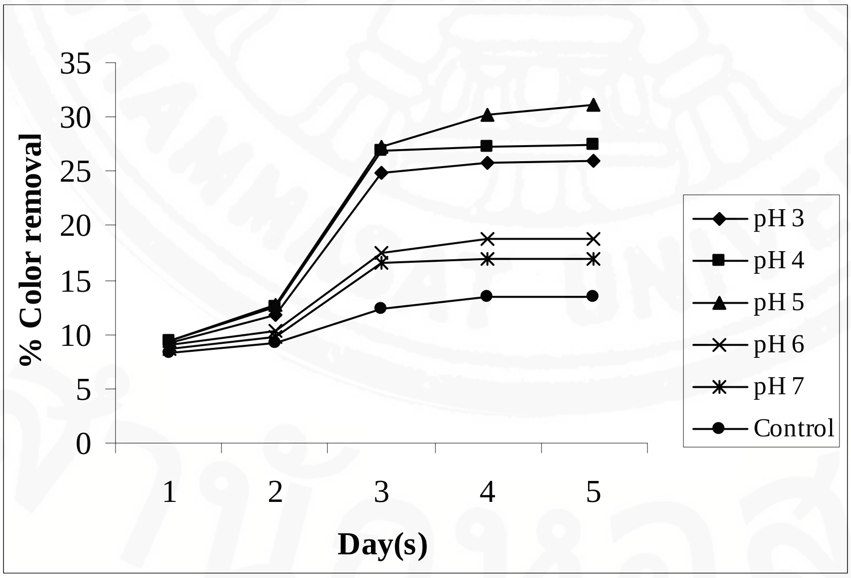


Figure 4.4 Effect of pH on color removal by *T.versicolor* BCC 8725 in leachate 1 without co-substrate

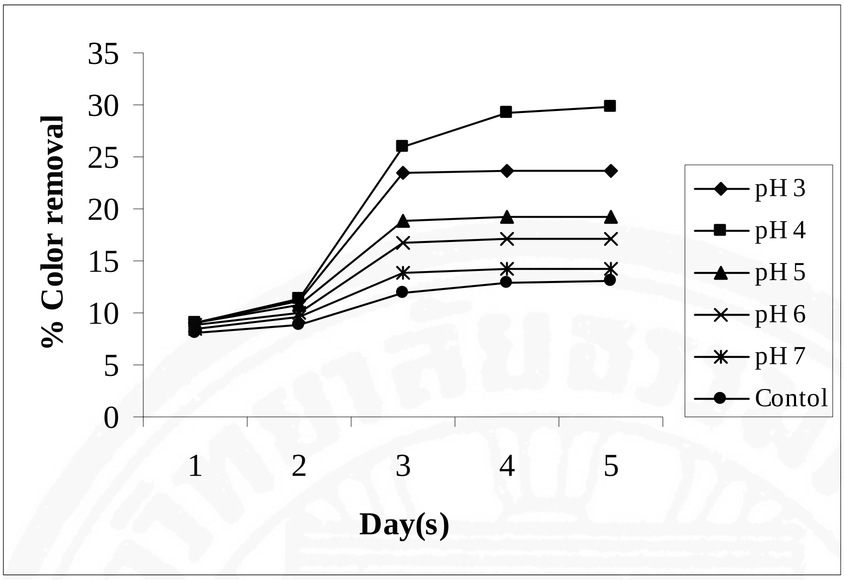


Figure 4.5 Effect of pH on color removal by *F.flavus* BCC 17421 in leachate 1 without co-substrate

The experiment was carried out until the color removal was constant. The optimum pH where maximum color removal was observed, was found to 5 for *T.versicolor* BCC 8725 and 4 for *F.flavus* BCC 17421, respectively. *T.versicolor* BCC 8725 seemed to have faster activity than *F.flavus* BCC 17421. The removal rate increased significantly on the 2nd and 3rd day for *T.versiclor* BCC 8725 and *F.flavus* BCC 17421 as depicted in Figure 4.4 and Figure 4.5. However, *T.versicolor* BCC 8725 can decolorize greater than *F.flavus* BCC 17421 which is 31.04% and 29.86%, respectively without adding any co-substrate.

4.4.1.3 Effect of co-substrates and contact time on color removal

Color removal by *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 at different co-substrates concentrations of glucose, cassava, and corn starch and one without co-substrate are shown in Figure 4.6 and 4.7, respectively.

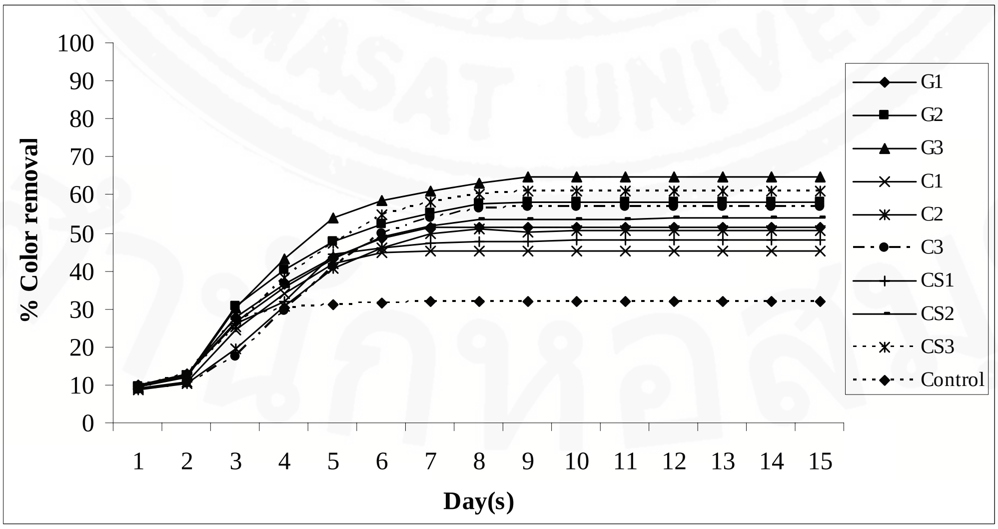


Figure 4.6 Color removal of leachate 1 by immobilized *T.versicolor* BCC 8725 at different co-substrate concentration at optimum pH of 5 in 15 days (Note: G = Glucose; CS = Corn Starch; C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

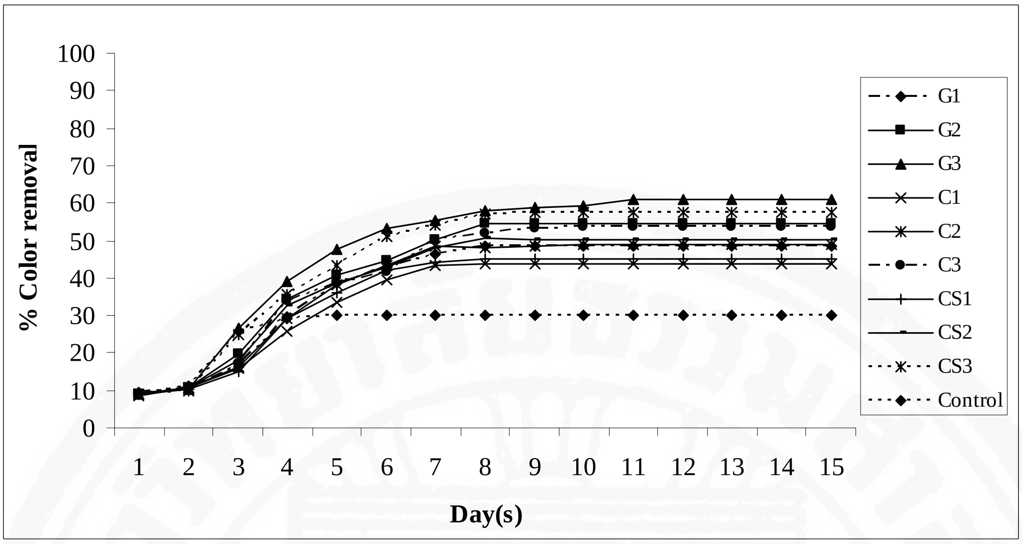


Figure 4.7 Color removal of leachate 1 by immobilized *F.flavus* BCC 17421 at different co-substrate concentration at optimum pH of 4 in 15 days (Note: G = Glucose; CS = Corn Starch; C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

It can be seen from Figure 4.6 and 4.7 that by adding certain co-substrates to the culture medium, especially glucose, cassava and corn starch, fungi activities were considerably enhanced. Among the three co-substrates used, the highest to lowest efficiency for color removal is glucose, corn starch, cassava and without co-substrate, respectively.

Higher concentration (3 g/L) has the slower removal in the early stage and then tends to increase sharply and have longer removal than the lower concentration. The color removal increased from the 3rd to 10th day and then became almost constant for *T.versicolor* BCC 8725. Similar pattern was observed for *F.flavus* BCC 17421. However, it can be seen that *T.versicolor* BCC 8725 can decolorize approximately 4% more than *F.flavus* BCC 17421 (glucose 3 g/L). Removal was found to be about 33% and 31% higher when comparing the results with and without 3 g/L glucose by *T.versicolor* BCC 8725 and *F.flavus* BCC 17421, respectively. Thus, presence of co-substrate is important.

It can be concluded that co-substrates; glucose, cassava, and corn starch are important for the fungi activity and helped in increased color removal. However, *T.versicolor* BCC 8725 was more effective than *F.flavus* BCC 17421 based on the percentage of color removal within the same time period. These results show that the leachate is degraded to different extent depending on the extracellular enzyme and fungi activity. Moreover, each fungi differ in terms of its ability of color removal by presence of the different types of co-substrates and different concentration to treat leachate. Not all co-substrate experimented in this study gave the same results.

4.4.1.4 Leachate characteristics after treatment

After leachate treatment with fungi at optimum pH of 5 with different types of co-substrates at 3 g/L in 15 days contact time, the leachate was analyzed for its color, BOD and COD and compared with before the fungal treatment. The results of leachate characteristics in terms of color, BOD and COD after treatment by *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 are shown in Figure 4.8-4.10.

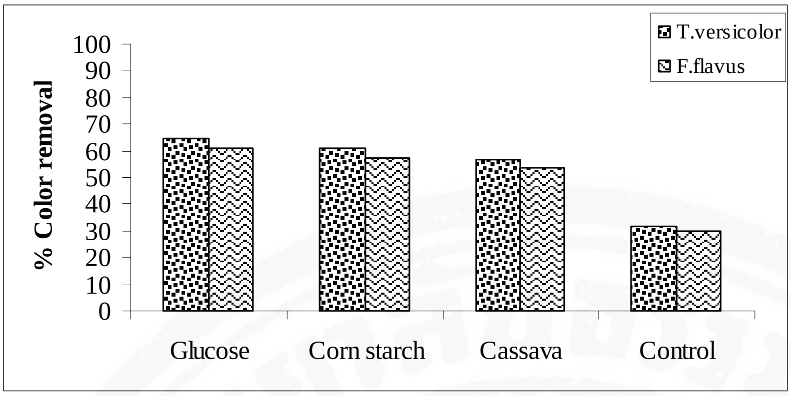


Figure 4.8 Percent color removal of *T.versicolor* BCC 8725 vs. *F.flavus* BCC 17421 in leachate 1

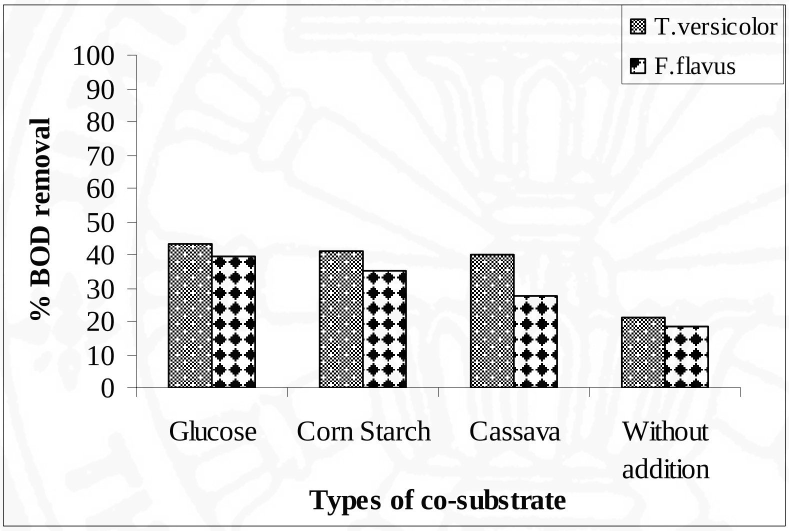


Figure 4.9 Percent BOD removal of *T.versicolor* BCC 8725 vs. *F.flavus* BCC 17421 in leachate 1

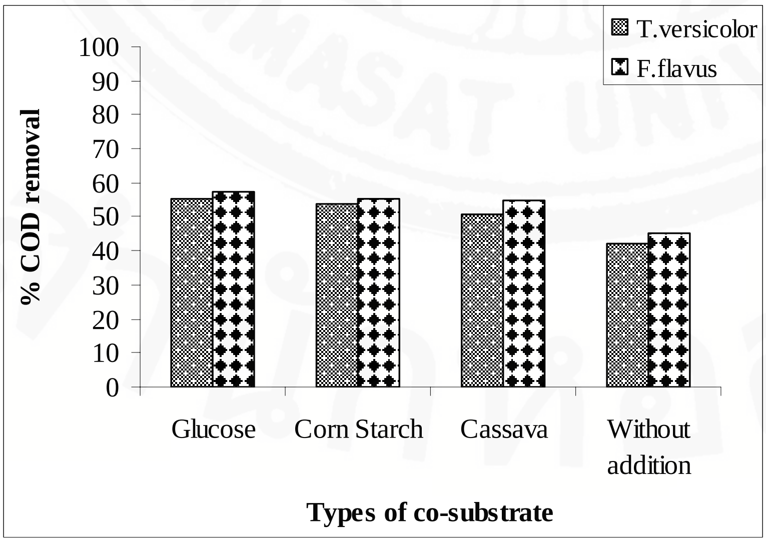


Figure 4.10 Percent COD Removal of *T.versicolor* BCC 8725 vs. *F.flavus* BCC 17421 in leachate 1

Both types of fungi can reduce different amount of color, BOD and COD depending on the types of co-substrate. The color removal of 64.91%, 60.93%, 56.98%, and 31.98% by *T.versicolor* BCC 8725 and 60.96%, 57.32%, 53.6%, and 29.94% by *F.flavus* BCC 17421, by using glucose, corn starch and cassava, respectively, can be obtained (Figure 4.8). For *T.versicolor* BCC 8725, BOD removal of 43.0%, 40.0%, 41.0% and 20.9% at 3 g/L of glucose, cassava, corn starch and without co-substrates, respectively as shown in Figure 4.9. COD decreased 57.2%, 54.6%, 55.3% and 45.1% at 3 g/L of glucose, cassava, corn starch and without co-substrates, respectively as shown in Figure 4.10. For *F.flavus* BCC 17421, BOD removal of 39.2%, 27.6%, 35.0%, and 18.3%, at 3 g/L of glucose, cassava, corn starch and without co-substrates, respectively. COD decreased 55.5%, 50.8%, 53.6% and 42.2% at 3 g/L of glucose, cassava, corn starch and without co-substrates, respectively. This shows that *T.versicolor* BCC 8725 is better in removal of color and BOD but *F.flavus* BCC 17421 is better in COD removal in leachate 1. This indicates that fungi are good in degradation of organic compounds that are readily and not readily biodegradable. Fungi cannot remove only color, but they can also help in removing BOD and COD in leachate.

4.4.2 Leachate 2

4.4.2.1 Biomass growth of fungi

As the fungi need to be supplied with an external carbon source (Cerniglia, 1997), biomass growth of fungi in leachate was studied by varying different types of co-substrates at 1-3 g/L and without co-substrate addition. Fungi followed a typical pattern of growth in response to the carbon source in the environment. Typically, a single unit of fungus would grow rapidly at the beginning. The exponential phase is followed by the stationary phase. Similar growth patterns were observed in the study done by other researchers (Carlile and Watkinson, 1994). After that, as the organic matter in the form of nutrients such as glucose became depleted, growth of fungi did not increase much. The fungi consume and grow readily on available carbon sources at the initial stages of growth and then produce secondary metabolites and extracellular enzymes for biodegradation in the presence of low concentration of nitrogen (Gramss and Ziegenhagen, 1999). Figure 4.11 and 4.12 show biomass growth of *T.versicolor* BCC 8725 and *F.flavus* BCC 17421, respectively in leachate 2 at varying co-substrate concentration.

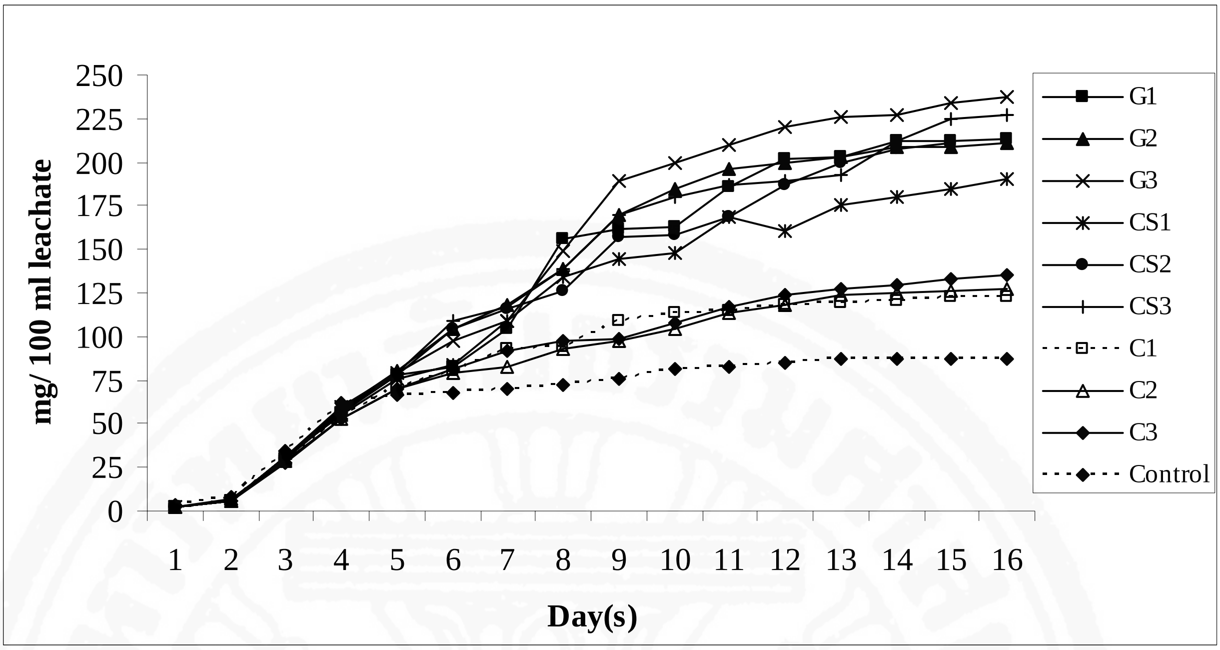


Figure 4.11 Biomass growth of *T. versicolor* in leachate 2 at varying co-substrate concentration (Note: G = Glucose; CS = Corn Starch; C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

From Figure 4.11, it can be seen that the biomass growth was strongly influenced by the concentration and type of co-substrates. At the end of the experiment, the biomass growth of *T. versicolor* BCC 8725 with glucose, corn starch, cassava and without addition of co-substrate was 0.2378, 0.2267, 0.1348 and 0.0877g wet weight, respectively. Glucose is the most favorable co-substrate. It is a monomeric sugar that fungi can easily transport into cells and use as energy. Cassava is a complex substrate and fungi had to produce an enzyme to degrade them into monomer first before transporting into cells. Fungi need high energy to produce enzymes and secrete extracellular enzymes to degrade complex organics. This indicates that the growth of fungi not only depends on concentration but also on the type of co-substrate. Some co-substrate can be readily utilized by fungi (like glucose) resulting in higher biomass growth compared to others.

F. flavus BCC 17421 required more time to grow as compared to *T. versicolor* BCC 8725. The fungi grew almost at the same rate with different types of co-substrate until the 5^{th} day. However, glucose, corn starch, cassava and without addition were still the most to least favorable co-substrates (same as in *T.versicolor* BCC 8725*)* after 15 days time period.

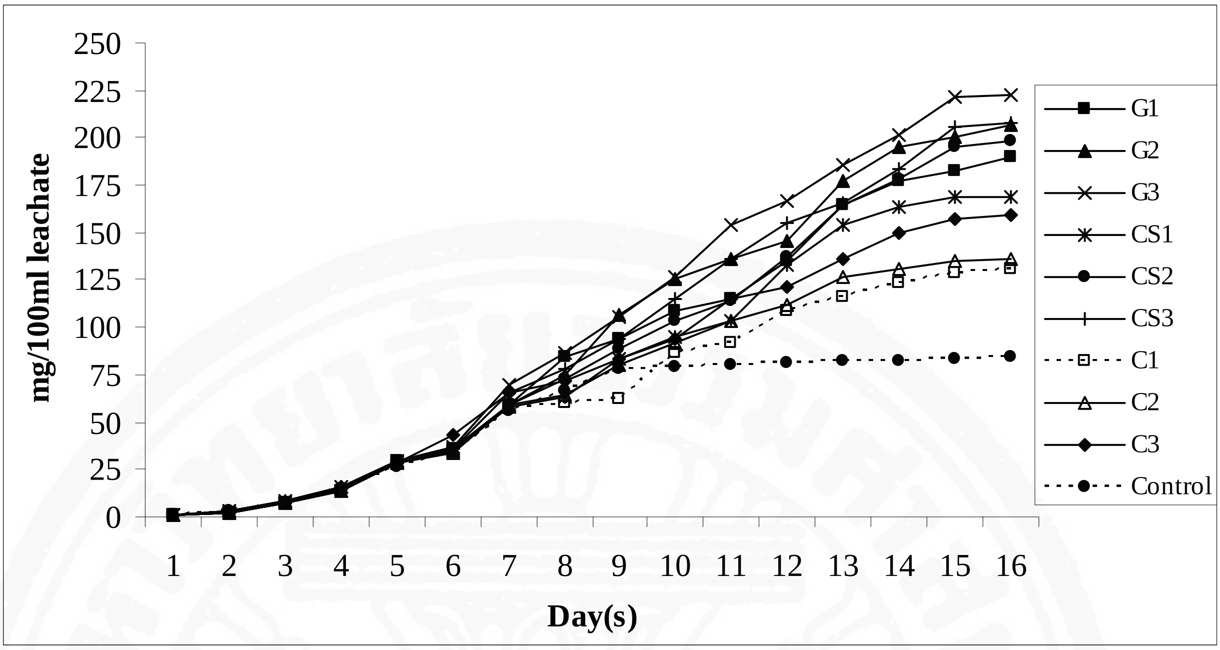


Figure 4.12 Biomass growth of *F.flavus* BCC 17421 in leachate 2 at varying co-substrate concentration (Note: G = Glucose, CS = Corn Starch, C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

At the end of the experiment, the biomass growth of *F. flavus* BCC 17421 with glucose, corn starch, cassava, and without co-substrate addition was 0.2223, 0.2075, 0.1598 and 0.0841g wet weight, respectively (Figure 4.12). Higher growth with co-substrates indicates that additional carbon sources serves as a source of food for fungi. However, the fungi can also grow in the leachate without co-substrate indicating that organic compounds present in the leachate can support the growth but the growth is very low as compared with co-substrate.

4.4.2.2 Effect of pH on color removal

The results of color removal by *T. versicolor* BCC 8725 and *F. flavus* BCC 17421 at different pH levels are shown in Figure 4.13 and 4.14 below. To find the effect of pH, 100 ml of leachate was stirred at 150 rpm with ten pieces of PUF covered with immobilized fungi for 1-5 days at different pH levels.

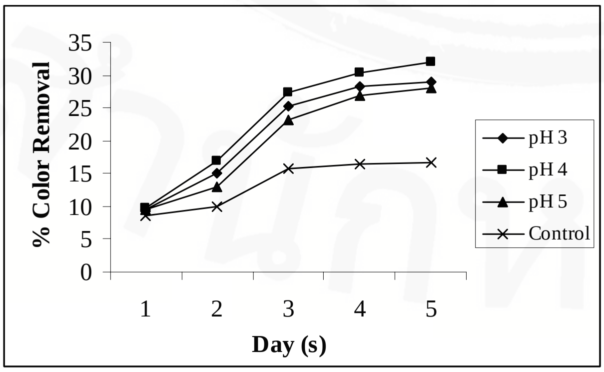


Figure 4.13 Effect of pH on color removal of leachate 2 by *T.versicolor* BCC 8725

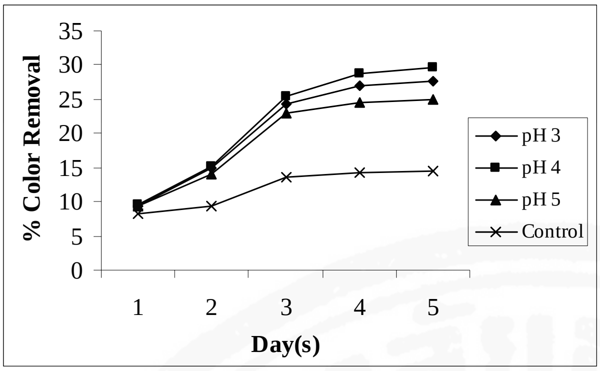


Figure 4.14 Effect of pH on color removal of leachate 2 by *F.flavus* BCC 17421

From Figures 4.13 and 4.14, it can be seen that the optimum pH for both fungi is 4 as maximum color removal was observed at that pH. Similar results were reported by other authors when treating textile wastewater by *T. versicolor* (Kapdan et al., 2000). It can be seen that pH can affect decolorization and fungi favors acidic pH for decolorization. The experiments were continued until the color removal was constant. Very little change in pH was observed during the experiment, although no buffer was used. *T. versicolor* BCC 8725 seemed to have better color removal than *F. flavus* BCC 17421.

4.4.2.3 Effect of co-substrate and contact time on color removal

After obtaining the optimum pH where maximum color removal was observed for both types of fungi in leachate 2, the optimum co-substrate was found by controlling the optimum pH. Three types of co-substrates were used; (1) glucose, (2) cassava, (3) corn starch and one set without co-substrate was done to compare in the time course of 15 days. The color removal was checked and recorded everyday. The results of color removal by *Trametes versicolor* BCC 8725 and *Flavodon flavus* BCC 17421 for both types of leachate are shown in Figure 4.15-4.16.

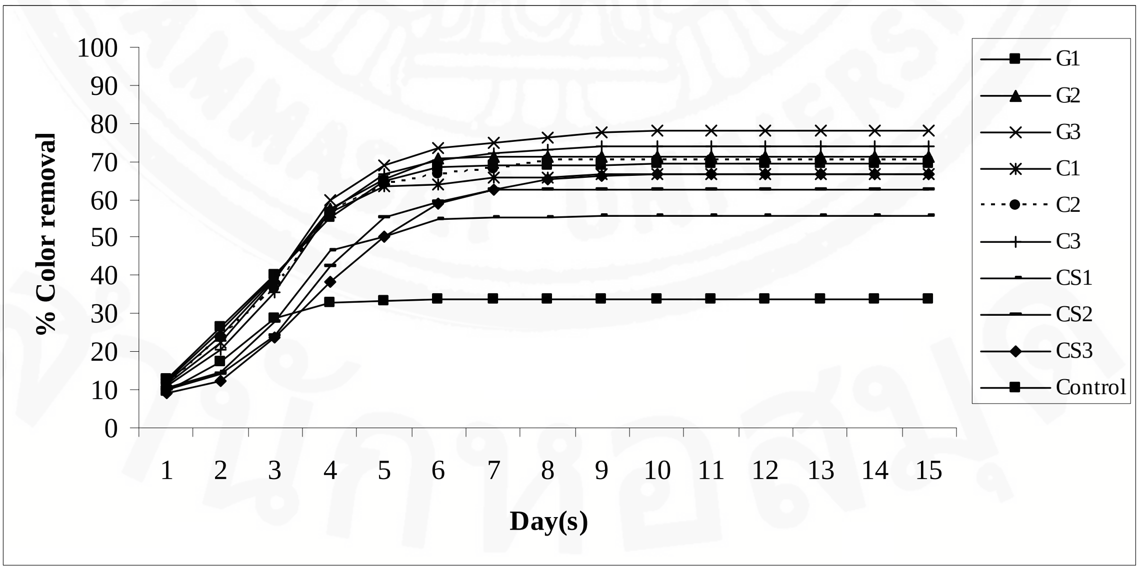


Figure 4.15 Color removal of leachate 2 by *T. versicolor* BCC 8725 at different co-substrate at optimum pH of 4 in 15 days (Note: G = Glucose, CS = Corn Starch, C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

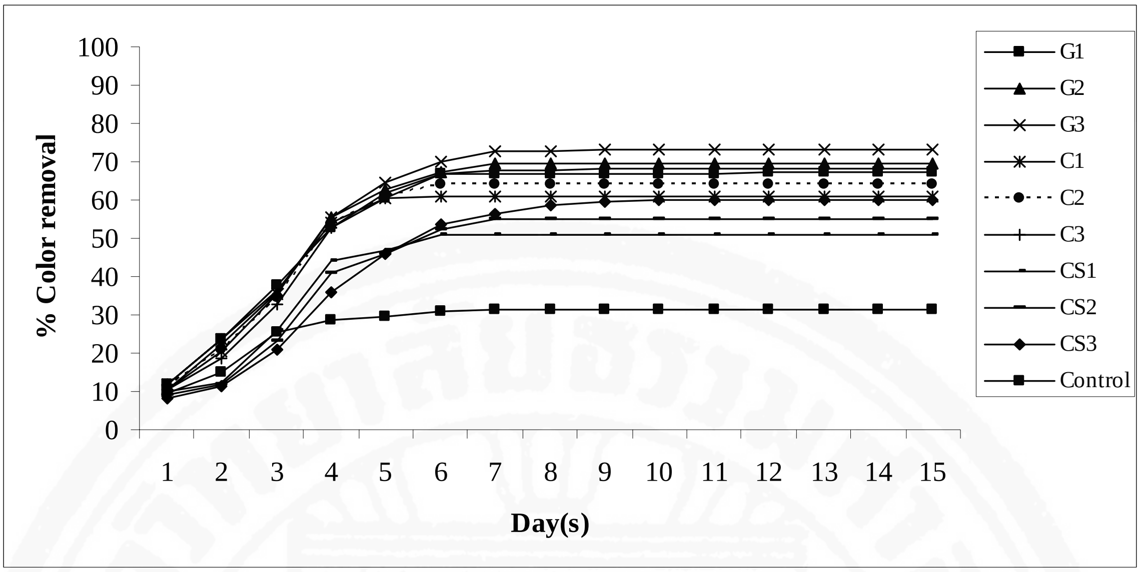


Figure 4.16 Color removal of leachate 2 by *F. flavus* BCC 17421 at different co-substrate at optimum pH of 4 in 15 days (Note: G = Glucose, CS = Corn Starch, C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

It can be seen that the color removal increase with time initially and then becomes almost constant after 7-8 days. It is clearly visible from the figures that the presence of co-substrate greatly influences the decolorization by both types of fungi.

It was known that the presence of carbon source triggers ligninolytic activity in white rot fungi and is required for pollutant degradation (Reddy, 1995). Many researchers have found that the decolorative metabolism requires the presence of carbohydrates (Martin and Manzanares, 1994) and an additional carbon source could fuel the decolorization process (Knapp, 1997, Swamy et al., 1999, Swamy and Ramsay, 1999b). As the addition of a carbon source such as glucose can help in decolorization and degradation of organic compounds, this study also focused on different types of co-substrates at different concentrations to see the color removal efficiency by two fungi.

It was found that by adding glucose, cassava and corn starch to the culture medium, fungi activities were considerably enhanced (Figure 4.15 and 4.16). Among 3 types of co-substrate used, the highest to lowest color removal efficiency was observed for glucose, corn starch, cassava and without addition, respectively. Ashnish et.al (1995) also found that by using *T.versicolor* to treat the pulp mill wastewater, the maximum color removal was found to be 80% and 65% by using glucose and corn starch as co-substrate, respectively. *T. versicolor* BCC 8725 and *F. flavus* BCC 17421 could decolorize the maximum of 78% and 73% in leachate at optimum condition (glucose 3 g/L, pH 4, HRT 15 days), respectively. The values were nearly 43% higher than those obtained in the culture without addition of co-substrates. *T. versicolor* BCC 8725 was more effective than *F. flavus* BCC 17421 in terms of the percentage of color removal within the same period. Higher concentration of co-substrate (3 g/L) had a faster removal rate in the initial stages as compared to the lower concentration. The decoloration rate became almost stable after the 8^{th} day. This enhanced rate of decolorization was most likely due to an increase in biomass and/or the production of enzymes involved in decoloration. For no co-substrate addition, no further decolorization was observed after the 5^{th} day for both fungi. This shows that the fungi require some source of carbon for growth. Thus, co-substrate is very important for biomass growth which coincides with the decoloration efficiency.

4.4.2.4 Leachate characteristics after treatment

The characteristic of leachate 2 in terms of BOD and COD removal at optimum condition by both types of fungi is shown in Figure 4.17 and 4.18.

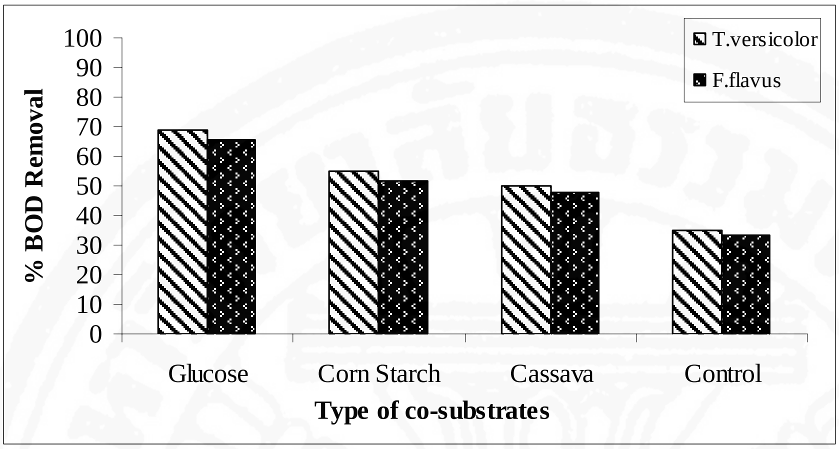


Figure 4.17 BOD removal of leachate 2 by *T.versicolor* BCC 8725

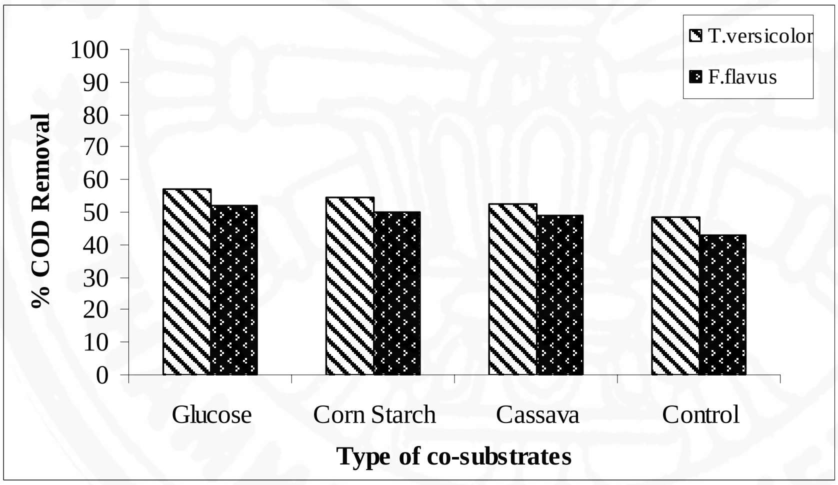


Figure 4.18 COD removal of leachate 2 by *F.flavus* BCC 17421

Figure 4.17-4.18 depicts BOD and COD removal of different types of leachate at optimum condition by *T.versicolor* BCC 8725 and *F.flavus* BCC 17421. Both types of fungi can reduce not only color but also BOD and COD proportional to co-substrate concentration. This is due to the degradation capabilities of enzymes secreted by fungi for lignin and other organic compounds degradation. The maximum BOD and COD reduction of leachate 2 of 69% and 57% by *T.versicolor* BCC 8725; 66% and 52% by *F.flavus* BCC 17421 could be obtained with glucose 3g/L at the optimum condition.

4.4.2.5 Effect of carbon source concentration

As glucose was the best co-substrate among the three co-substrates studied, batch experiments were also conducted at higher concentration (4-5 g/L) to see the color removal and the results are shown in Figure 4.19.

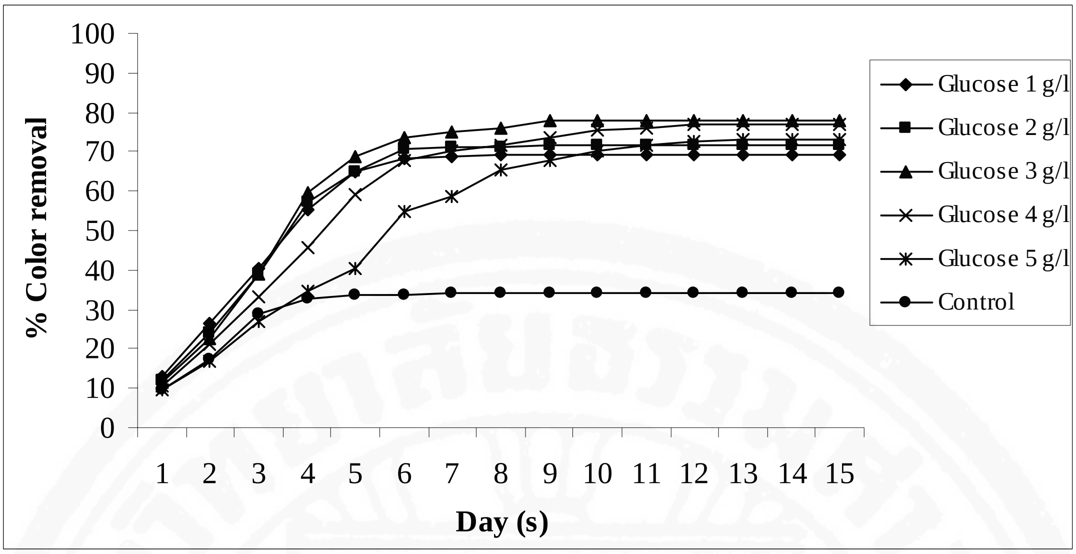


Figure 4.19 Effect of glucose on color removal in leachate 2

From Figure 4.19, it was found that higher doses of glucose did not enhance the color removal efficiency. The color removal rates at the glucose 4 and 5 g/L are 77% and 73%. So, under the conditions tested in this study, it was essential to dose adequate carbon source and excessive glucose was not necessary. Excessive glucose feeding could have negative effects on the process such as increases in BOD and COD in the effluent, which is an environmental problem (Blanquez et al., 2007).

In addition, BOD and COD removal at glucose 4 g/L was 56.07% and 47.28%, respectively. BOD and COD at glucose 5 g/L is 50.32% and 42.40%, respectively. The COD removal efficiency increased by only 3% as glucose concentration increased from 3 g/L to 5 g/L. There was also no significant increase in BOD and COD removal efficiency with the increase in co-substrate concentration. As reported by Elisa et al. (1991), the removal of color from kraft mill wastewater was significant only at an appropriate range of carbon concentration. Excessive carbon source resulted in a decline in enzyme activity, therefore reduced the removal efficiency of COD from effluent. This demonstrates that it was essential to dose adequate carbon source, but the presence of excessive carbon source did not enhance or even inhibit the degradation.

Although, the exact role of glucose is not known, it may play a role in decoloration by acting as the substrate for glucose oxidase (Machida and Nakanashi, 1994) to generate H_2O_2 needed for peroxidase activity or it may produce small organic acids which complex with Mn^{3+} generated by manganese peroxidase. Possible roles for glucose in decoloration include (i) the generation of H_2O_2 required for extracellular peroxidase activity and/or (ii) the generation of Mn^{3+} complexing agents necessary for MnP activity.

4.4.3 Leachate 3

4.4.3.1 Effect of pH on color removal

The effects of pH on color removal in leachate 3 by both fungi are shown in Figure 4.20 and 4.21.

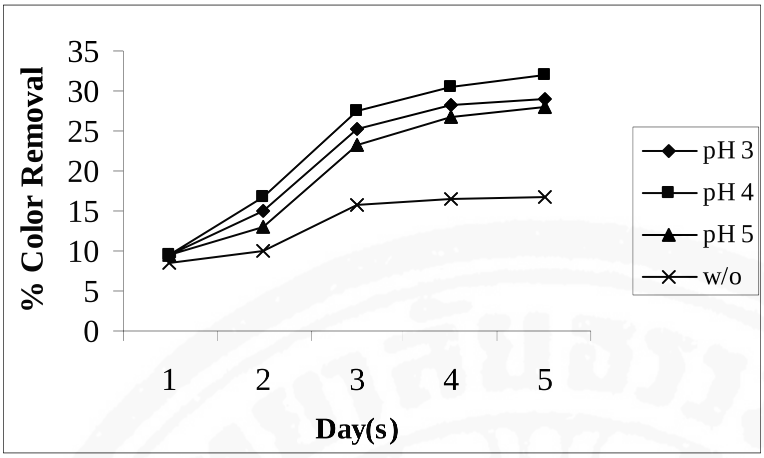


Figure 4.20 Effect of pH on color removal by *T.versicolor* BCC 8725 in leachate 3

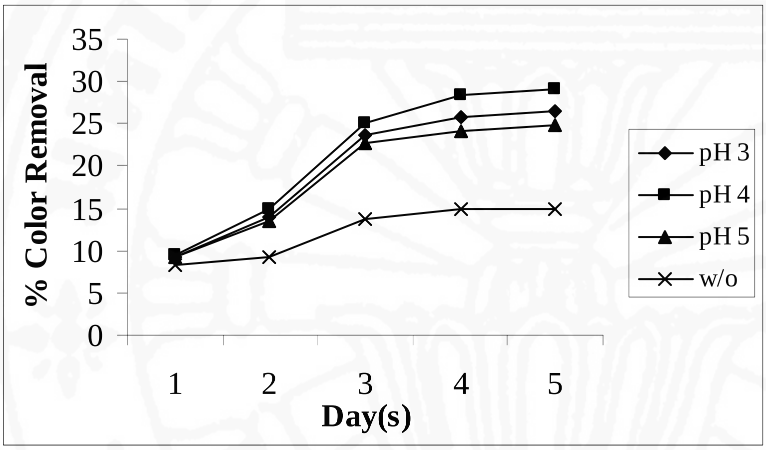


Figure 4.21 Effect of pH on color removal by *F.flavus* BCC 17421 in leachate 3

From Figure 4.20-4.21, it can be seen that the optimum pH of both fungi from both types of leachate are at 4 (the same which were found in leachate 2). The experiment was done until the color removal was constant. When pH was periodically measured, little change was found during decolorization even though no buffer was used. *T.versicolor* BCC 8725 seems to have faster activity than *F.flavus* BCC 17421. The removal rate increases on the 2^{nd} and 3^{rd} day.

4.4.3.2 Effect of co-substrates and contact time on color removal

Color removal for *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 of leachate 3 at different types of co-substrates and concentration of glucose, cassava, and corn starch and one without co-substrate are shown in Figure 4.22 and 4.23.

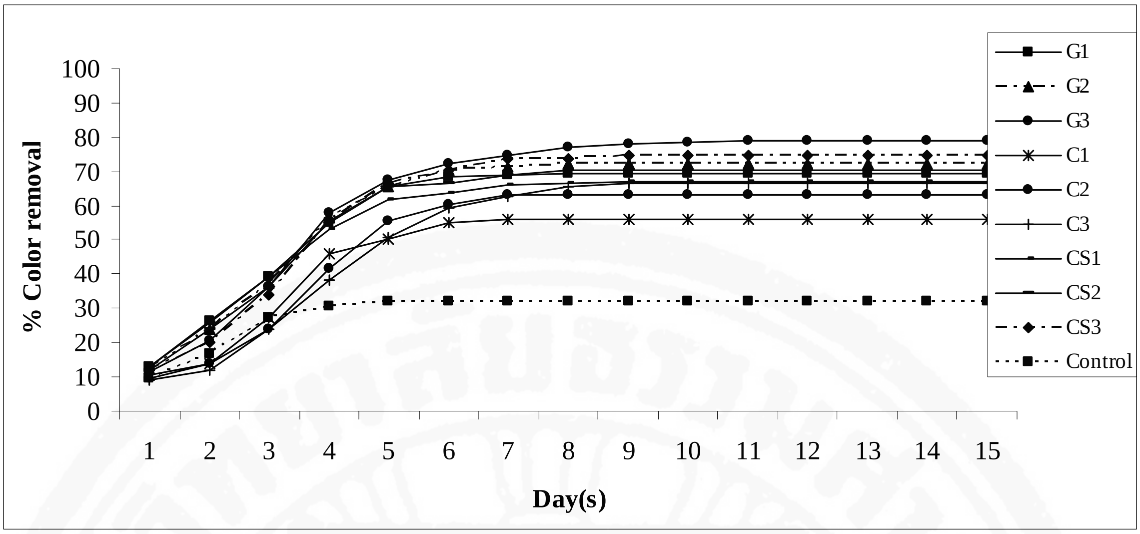


Figure 4.22 Color removal of leachate 3 by *T.versicolor* BCC 8725 at different co-substrate at optimum pH of 4 in 15 days (Note: G = Glucose; CS = Corn Starch; C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

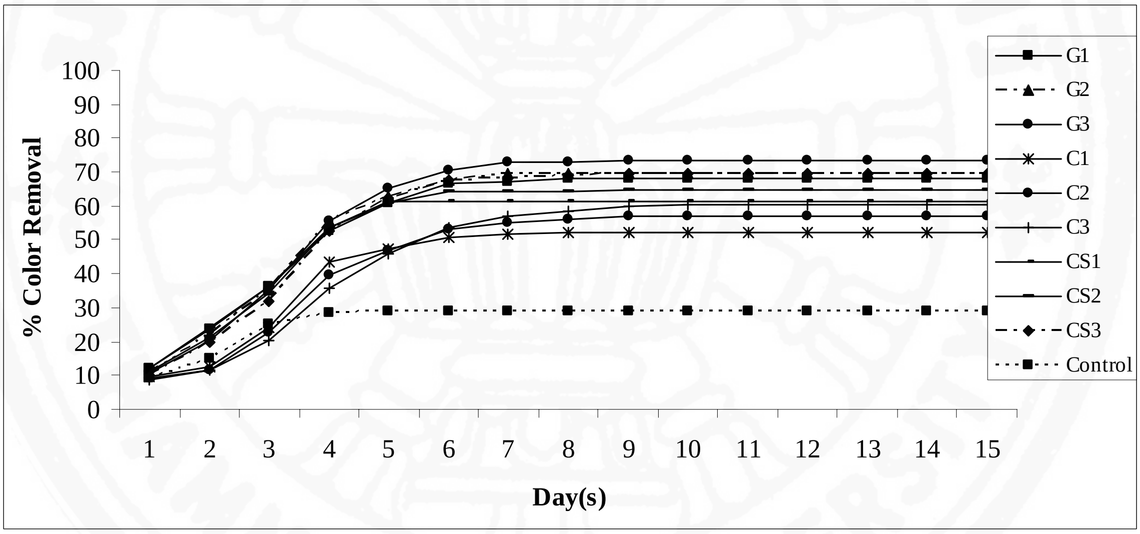


Figure 4.23 Color removal of leachate 3 by *F.flavus* BCC 17421 at different co-substrate at optimum pH of 4 in 15 days (Note: G = Glucose; CS = Corn Starch; C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

From Figure 4.22-4.23, it can be seen that by adding certain co-substrates to the culture medium, especially glucose, cassava and corn starch, fungi activities were considerably enhanced. Among 3 types of co-substrate used, the highest to lowest color removal efficiency for color removal is glucose, corn starch, cassava and without addition, respectively. *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 can decolorize the maximum of 78.79% and 73.20%, respectively in leachate 3.

As can be seen from the figures, higher concentration (3 g/L) have the slower removal in the early stage and tend to increase sharply and longer removal than the lower concentration. The decoloration rate declined at lower co-substrate concentration. The decrease in decoloration rate maybe due to co-substrate depletion and the exhaustion of other essential nutrients or trace elements.

This enhanced rate of decolorization was most likely due to an increase in biomass and/or the production of enzymes involved in decoloration. As mentioned earlier that glucose is monomeric sugar while cassava is more complex sugar so the fungi take times to degrade and decolorize so the fungi in leachate with cassava will have slower activity than glucose.

4.4.3.3 Leachate characteristics after treatment

After treatment by both types of fungi at the optimum condition (optimum pH of 4 and co-substrate concentration at 3 g/L), leachate 3 was analyzed for its BOD and COD in order to compare with the leachate before treatment and the results are shown in Figure 4.24-4.25.

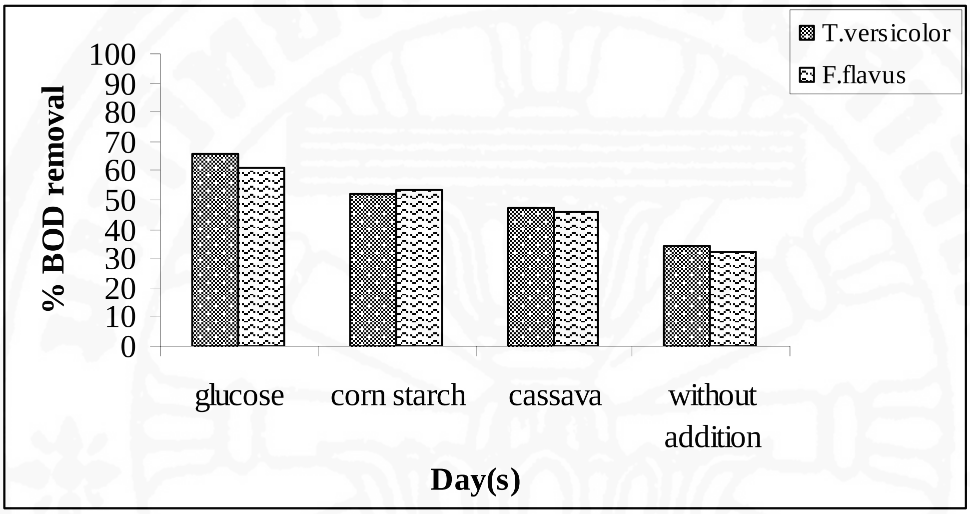


Figure 4.24 BOD removal of leachate 3 at 3 g/L of co-substrates

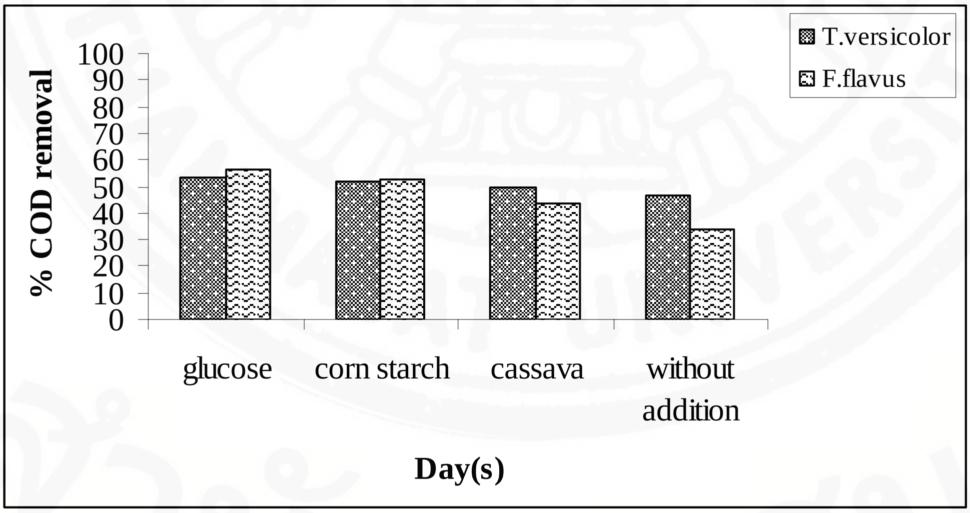


Figure 4.25 COD removal of leachate 3 at 3 g/L of co-substrates

BOD and COD removal of leachate 3 at optimum condition by *T.versicolor* BCC 8725 and *F.flavus* BCC 17421 are depicted in Figure 4.24-4.25. Both types of fungi can reduce not only color but also BOD and COD proportional to co-substrate concentration. The maximum BOD and COD reduction is achieved at 65% and 53% by *T.versicolor* BCC 8725; 61% and 56% by *F.flavus* BCC 17421, respectively. It is about 31% more BOD removal with glucose 3 g/L as co-substrate by *T.versicolor* BCC 8725.

As *T.versicolor* BCC 8725 had higher removal performance in terms of color, BOD and COD (in most cases) than *F.flavus* BCC 17421, *F.flavus* BCC 17421 was not selected for further experiments.

4.4.3.4 Biomass growth of mobilized fungi

Biomass growth of mobilized fungi in leachate 3 was studied with varying types of co-substrates at 3 g/L and is shown in Figure 4.26.

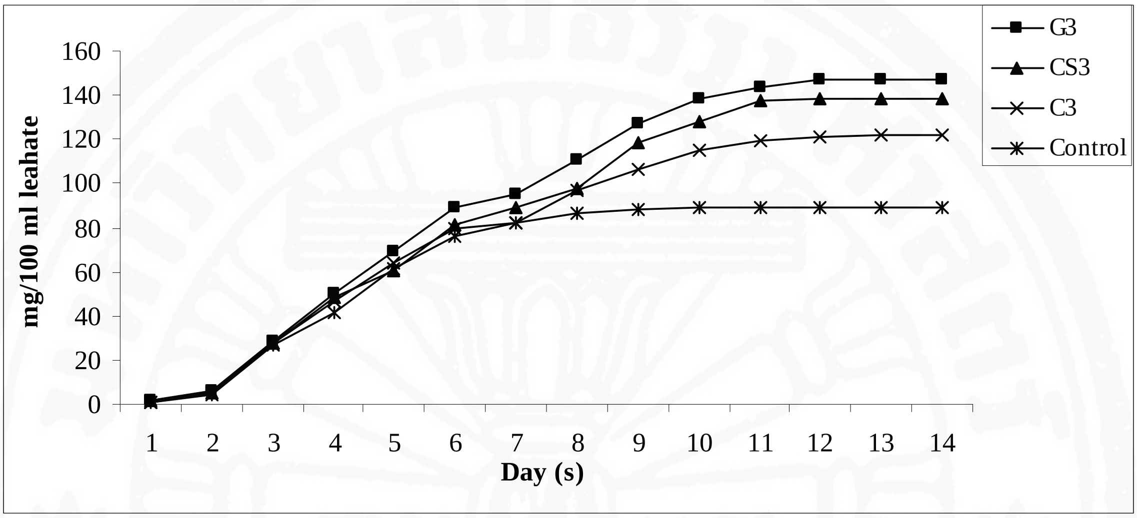


Figure 4.26 Biomass growth of mobilized *T.versicolor* BCC 8725 in leachate 3 (Note: G = Glucose; CS = Corn Starch; C = Cassava and control = without co-substrate)

Co-substrates (glucose, corn starch and cassava) at different concentrations were added to leachate to supply carbon. The highest biomass growth was obtained with the glucose. The biomass growth with glucose is about 0.06g more than without co-substrate addition. The biomass growth pattern with mobilized fungi was found to be similar to the growth with immobilized fungi.

4.4.3.5 Color removal of mobilized fungi

After the biomass growths of mobilized *T.versicolor* BCC 8725 on leachate from garbage truck were studied, the batch experiments on decolorization were conducted for all types of co-substrates at 3 g/L for 10 days and one set without addition of co-substrate and compared with immobilized fungi results for 10 days. The color removal was checked everyday. The results of decolorization of mobilized *T.versicolor* BCC 8725 on leachate from garbage truck are shown in Figure 4.27.

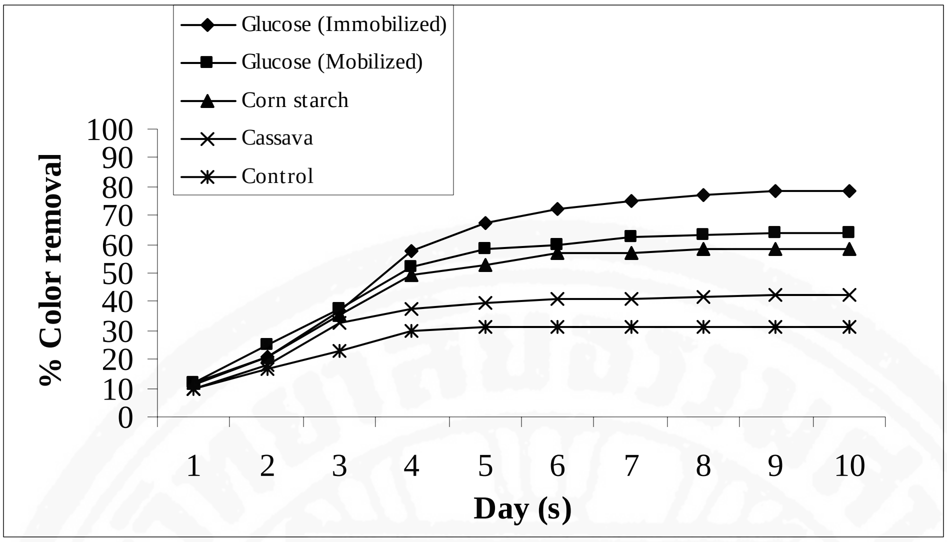


Figure 4.27 Color removal of mobilized *T.versicolor* BCC 8725 using leachate 3

It was found that with 3 g/L of co-substrates, the mobilized *T.versicolor* BCC 8725 can decolorize the leachate to a maximum level of 63.38% by using glucose whereas corn starch, cassava and no addition removed high to low level respectively. Glucose additions (3 g/L) stimulated decolorization by *T.versicolor* BCC 8725 from 31.29 % (no glucose addition) up to 63.38%.

Comparison of mobilized and immobilized *T.versicolor* BCC 8725 on PUF with glucose 3 g/L for 10 days was also conducted (Figure 4.27). Almost similar percentage of decolorization was achieved within the 1st to 3rd day using mobilized or immobilized fungus with glucose 3 g/L but later on the fungus immobilized on PUF remain viable for at least 15 days (data from the previous experiment). After the 4th day, the decolorization efficiency of immobilized fungi with glucose continuously increases until day 10 and reach maximum color removal at 78.79% under the same condition whereas the efficiency slowly stable after the 5th day and tend to stop since the 8th day for the mobilized fungi with glucose, corn starch, cassava and without co-substrate addition. About 16% higher color removal was observed with immobilized fungi in 10 days. This showed that the immobilized has better decolorization efficiency than mobilized one. It has been reported that immobilization of fungal cells using PUF could stably maintain the production of various enzymes at levels higher than achieved with suspended or pellets forms (Lapadatescu et al., 1997, Nakamura et al., 1999, Kim and Shoda, 1999). Similar results were obtained in this study. The advantages of using immobilized microorganisms for pollutant degradation are well-known (Cassidy et al., 1996). Besides being economically cheaper and easier to handle, the immobilized fungus is reusable for several batches. It was found that the immobilized fungi on PUF (79%) gave better decolorization efficiency than mobilized fungi (64% results below) when using leachate with 3 g/L of glucose collected from garbage trucks in Nonthaburi district of Thailand. In addition, immobilized fungi could be used effectively for at least three consecutive cycles for decolorization.

The porous nature of polyurethane foam may help in better diffusion of oxygen. The fact that the fungus remains viable in foam for longer time makes the process easier to handle as well. *Phanerochaete chrysosporium* immobilized in alginate beads can decolorize diluted MSW by 60% on day 8 (Fahy et al., 1997). On the contrary, *F.flavus* BCC 17421 immobilized in foam cubes removed about 60% of the color even by day 5 when 10%

Molasse Spent Wash was used (Raghukumar et al., 2004). Therefore, polyurethane foam is ideally suited media for immobilization of *T.versicolor* BCC 8725.

Besides decolorization, the effect on BOD and COD removal were also examined. The initial BOD and COD for leachate from garbage truck are 625 and 1,728 mg/L, respectively. After the treatment by *T.versicolor* BCC 8725, it can remove BOD and COD to 187.37 and 774.83 mg/L or 70.02% and 55.16%, respectively. Even organic loading is low but the BOD and COD are still not completely removed. This might be due to the generation of high concentration of intermediate compounds (mainly carboxylic acid) that cannot be further oxidized by hydroxyl radicals and consequently are accumulated and remained.

4.4.3.6 Reuse of immobilized fungi

Same batch of immobilized fungus was used for treatment of different batches of leachate. The experiments were done on leachate 3 by varying the co-substrates: glucose, corn starch and cassava at 3 g/L concentration to see the color removal by reusing the same fungi, *T.versicolor* BCC 8725. The results are shown in Figure 4.28.

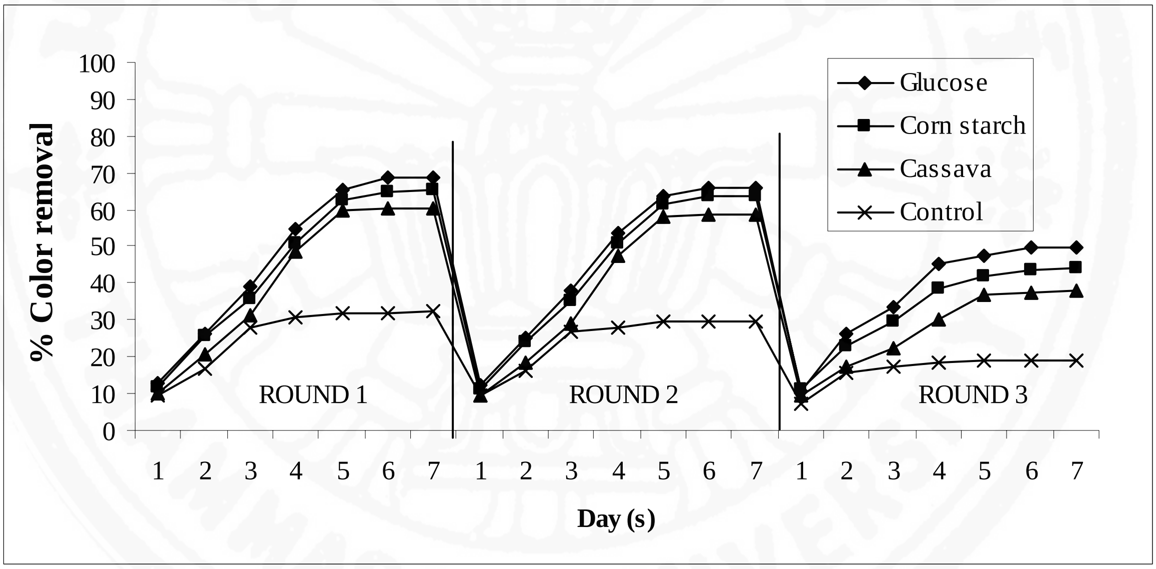


Figure 4.28 Reuse of immobilized *T.versicolor* BCC 8725 in leachate 3

The same batch of immobilized fungus could be used effectively for three consecutive cycles of decolorization of fresh leachate (Figure 4.28). The decolorization of the first and second cycle is very similar (slightly different) whereas the third cycle is about 20% lower than the first cycle. The maximum amount of color removal was observed at the end of the first and second cycle of treatment, where 68.5% and 66% of decolorization was evident and no initial lag phase was observed as reported by other authors (Sayadi and Ellouz, 1992).

For no addition of co-substrate one, the decolorization was only 22.3%. A dramatic decline in the decolorizing capability was observed during a subsequent fourth cycle (data not shown) indicating that fungi could be used for 3 cycles of leachate treatment (each 7 days). However, glucose was found to be the best among co-substrates used following by corn starch, cassava and no addition, respectively. The most readily usable carbon source by white rot fungi is glucose (Kapdan et. al., 2000).

These results indicated that repeated use of the immobilized fungal cells was possible for stable decolorization. On the other hand, when fungus in pellet form was subjected to repeated used, the decolorization efficiency decreased from about 50 to 10-30% over the course of 3 cycles under similar conditions (data not shown) (Masanori et al., 2000).

4.4.4 Leachate 4

4.4.4.1 Biomass growth of fungi on PDB

The pH was adjusted to be 4 for *T.versicolor* BCC 8725 as it was the optimum pH found from leachate 2 and leachate 3. The results of the biomass growth of immobilized *T.versicolor* BCC 8725 in Potato Dextrose Broth (PDB) with different types of co-substrate are shown in Figure 4.29.

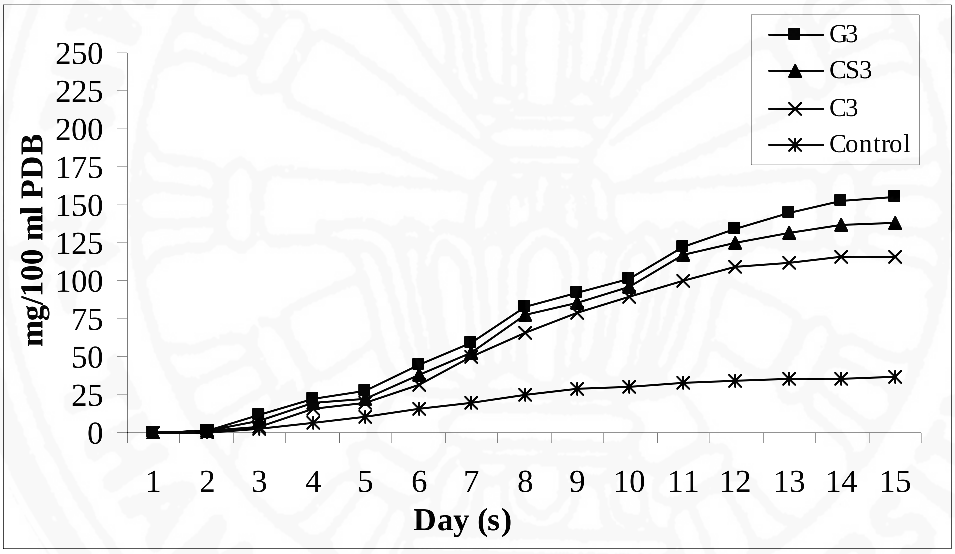


Figure 4.29 Biomass growth of immobilized *T.versicolor* BCC 8725 in PDB with different types of co-substrate at 3 g/L (Note: G = Glucose; CS = Corn Starch; C = Cassava and control = without co-substrate)

PDB was normally used as food for fungi in mycelial suspension. From Figure 4.29, it can be seen that the fungi growth was considerably enhanced by adding certain co-substrate to the culture medium, especially glucose, cassava and corn starch. Without co-substrate (in pure PDB), the growth of fungi after 7 days was comparatively lower than with co-substrates. Among three types of co-substrates used, glucose was found to be the best.

4.4.4.2 Biomass growth of fungi on leachate 4

The results of the biomass growth of immobilized *T.versicolor* BCC 8725 in leachate 4 with different types of co-substrate are shown in Figure 4.30.

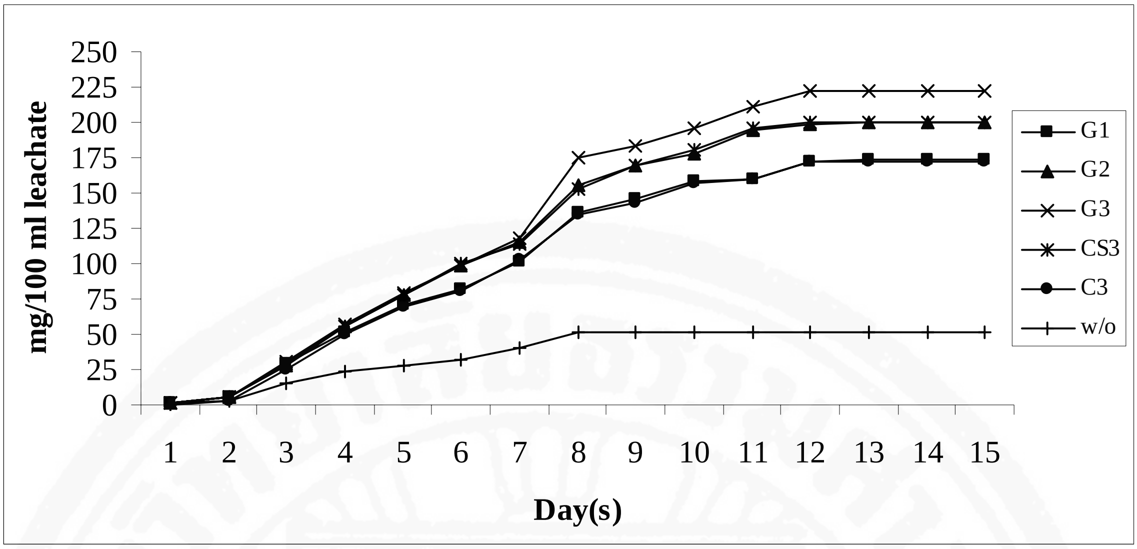


Figure 4.30 Biomass growth of immobilized *T.versicolor* BCC 8725 in leachate 4 (Note: G = Glucose; CS = Corn Starch; C = Cassava at 1, 2, 3 g/L and control = without co-substrate)

From Figure 4.30, highest biomass growth was obtained using glucose as a co-substrate at 3 g/L. With co-substrate addition in all concentration, the biomass growth is about the same on the 1st to 4th day, after that the biomass growth of glucose at 3 g/L can grow more than other types of co-substrate. Without co-substrate the fungi can also grow in the leachate indicating that organic compounds present in the leachate can support the growth. However, the growth substantially increased after addition of co-substrate.

From the comparison between the biomass growth in PDB and leachate 4, it can be seen that the biomass growth in leachate is higher than in PDB in same time period (15 days). This suggests that leachate contain nutrients that can lead to better growth of fungi compare to PDB, however this might depend on the leachate characteristics.

4.4.4.3 Effect of co-substrate on color, BOD and COD removal of leachate 4

Color removal of leachate 4 by *T.versicolor* BCC 8725 at different co-substrate at optimum pH of 4 in 15 days is shown in Figure 4.31**.**

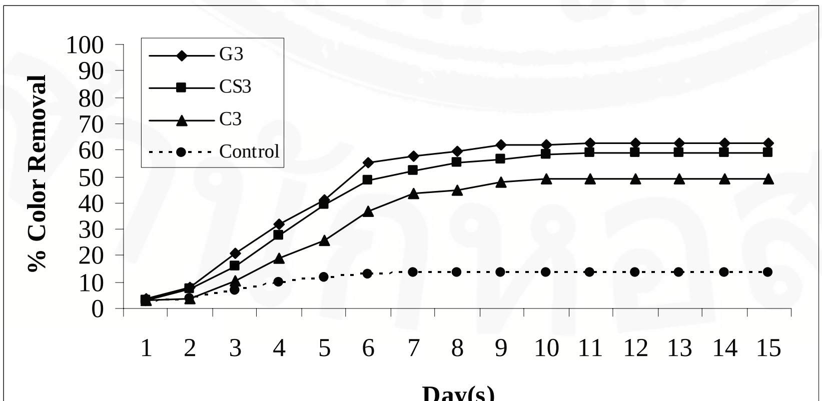


Figure 4.31 Color removal of leachate 4 by immobilized *T.versicolor* BCC 8725 (Note: G = Glucose; CS = Corn Starch; C = Cassava at 3 g/L and control = without co-substrate)

From the Figure 4.31, it can be seen that the removal efficiency generally increased with increase in contact time and then it tends to be constant with a longer period. It was noticed that the removal efficiency increased from approximately 4%-62% when the contact time was increased from the 1st to 10th day. Beyond that, the removal efficiency tends to be constant. By adding certain types of co-substrates to the culture medium, especially glucose, cassava and corn starch, fungi activities were considerably enhanced. For no co-substrate addition, no further decolorization was observed after the 5th day. *T.versicolor* BCC 8725 could decolorize the maximum of 62.59% using leachate 4. Addition of glucose at 3 g/L represents nearly 48.85% higher than those obtained in the culture without addition of co-substrates.

Biomass growth coincides with the color removal efficiency which is different according to the types of leachate. Generally, the color removal is low in the early stage and tends to increase sharply. This enhanced rate of decolorization was most likely due to an increase in biomass and or the production of enzymes involved in the decoloration. After that the decolorization became constant. Thus, co-substrate is very important for biomass growth which coincides with the production of extracellular enzymes for the decolorization.

4.4.4.4 Leachate characteristics after treatment

The characteristic of leachate 4 in terms of BOD and COD removal at optimum condition by both types of fungi is shown in Table 4.3.

Table 4.3 Percent BOD and COD removal of leachate 4 after treatment at optimum condition by *T.versicolor* BCC 8725

Type of Co-substrate	Day 5		Day 10		Day 15	
	BOD	**COD**	**BOD**	**COD**	**BOD**	**COD**
Glucose (1 g/L)	33.05	35.28	39.84	41.02	35.16	41.12
Glucose (2 g/L)	32.86	33.86	43.51	46.23	43.20	46.64
Glucose (3 g/L)	32.14	34.95	46.48	48.76	50.84	51.30
Corn Starch (3 g/L)	29.25	31.76	37.11	41.82	40.96	46.61
Cassava (3 g/L)	18.96	22.43	27.05	30.97	33.12	36.73
Without addition	10.54	12.46	17.73	19.94	18.21	21.55

The BOD and COD removal for glucose 1 and 2 g/L occur fast in the beginning period and tend to be stable after the 10th day. At glucose at 3 g/L, the BOD and COD removal are the same in the beginning period and can still have more removal after the 10th to 15th day. For corn starch at 3 g/L, the fungi have slower removal of BOD and COD at the beginning but finally it can have the removal efficiency almost the same as in glucose at 2 g/L. For cassava, the fungi have less and slower BOD and COD removal compare to the other co-substrate. Highest BOD and COD removal was 52.84% and 55.30%, respectively, at glucose 3 g/L which is 34.63% and 33.75% better than no co-substrate addition on the 15th day (Table 4.3). Although BOD and COD itself in the raw leachate and glucose addition can contribute much higher BOD, the final BOD and COD value reduced to the value less than the initial BOD and COD in the raw leachate. This means that the fungi utilize the glucose, which serves as food for growth, and fungi could easily transport it into cell and use it as energy to produce enzyme to degrade pollutant. However, with no addition of co-substrate, the fungi can still remove BOD and COD which is about 18.21% and 21.55%, respectively. This indicated that the fungi could utilize the complex organic content in the leachate as well but glucose being simple sugar can be used by fungi easily.

The mg removal of BOD and COD per mg of biomass for leachate as discharged from pipe to the pond (leachate 2 and leachate 4) was calculated and compared the removal efficiency in batch experiment with 3 g/L of glucose within 15 days. The mg BOD and COD removed per mg biomass for leachate 2 is 15.63 and 78.12, respectively. For leachate 4, the mg BOD and COD removed per mg of biomass is 109.48 and 187.84, respectively. So, the removal efficiency in leachate 2 is much less than leachate 4. Although leachate 2 is low strength leachate but high ammonia concentration (BOD = 5,600 mg/L, COD = 34,560 mg/L, ammonia = 182 mg/), the fungi still have the less removal efficiency as compared to leachate 4 which is very high strength leachate but lower ammonia concentration (BOD = 48,900 mg/L, COD = 96,512 mg/L, ammonia = 32 mg/L). This indicates that ammonia strongly affects the treatment efficiency by *T.versicolor* BCC 8725.

4.4.5 Leachate 4*

4.4.5.1 Biomass growth of leachate 4*

The biomass growth of *T.versicolor* BCC 8725 in leachate 4* at pH 4 with 3 g/L of glucose and 4-day immobilization is shown in Figure 4.32.

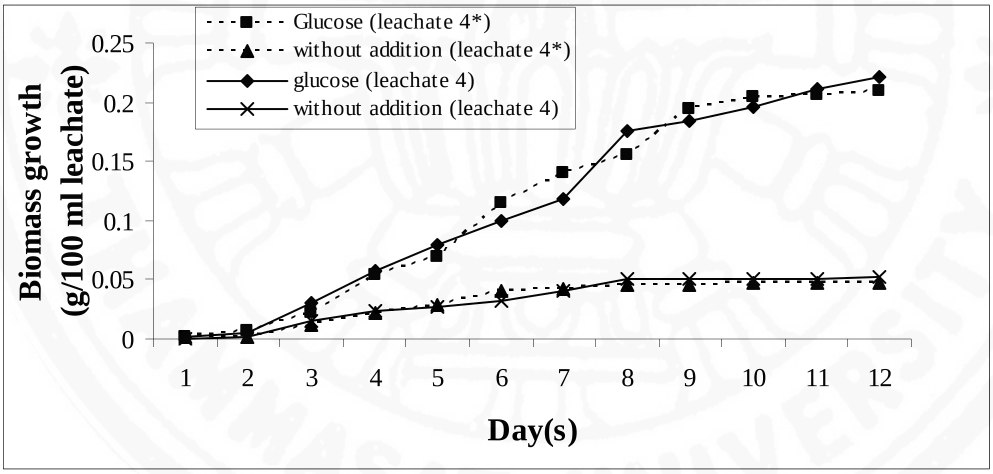


Figure 4.32 Biomass growths with and without glucose addition of leachate 4*

Compare the biomass growth of leachate 4 and 4*, it was found that the growth in leachate 4* is almost same on the same day (the 12th day) after storage. The reason may be because the leachate is already stabilized and there is more ammonia content, which possibly limits the growth of fungi, though, the organic content in terms of BOD and COD are lesser. The growth in leachate 4* is about 0.013g and 0.0042g for leachate with and without glucose, respectively, less than leachate 4.

4.4.5.2 Effect of co-substrate on color removal of leachate 4*

Figure 4.33 shows the color removal of leachate 4* with and without addition of glucose for the time course of 12 days.

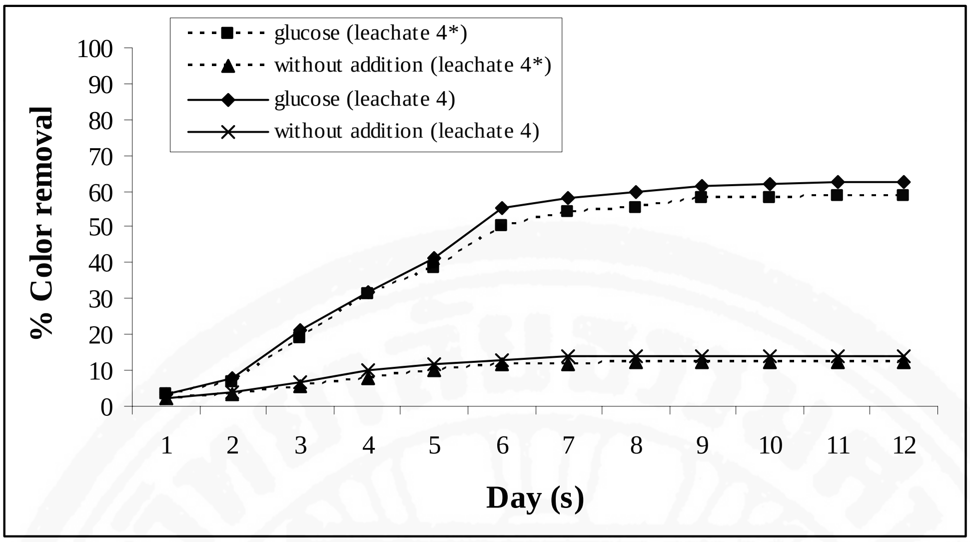


Figure 4.33 Color removal of leachate 4* by *T.versicolor* BCC 8725

From Figure 4.33, it can be seen that higher color removal was obtained in leachate with glucose. The results of color removal of leachate 4* were compared with leachate 4. As the biomass growth is slightly lesser in leachate 4*, the color removal is also slightly less. The maximum color removal in the leachate 4* (the 12th day) with and without glucose addition is 3.84% and 1.53%, respectively less than leachate 4.

4.4.5.3 Leachate characteristics after treatment

The comparison of BOD and COD removal of leachate 4* without and with glucose before and after treatment is shown in Figure 4.34.

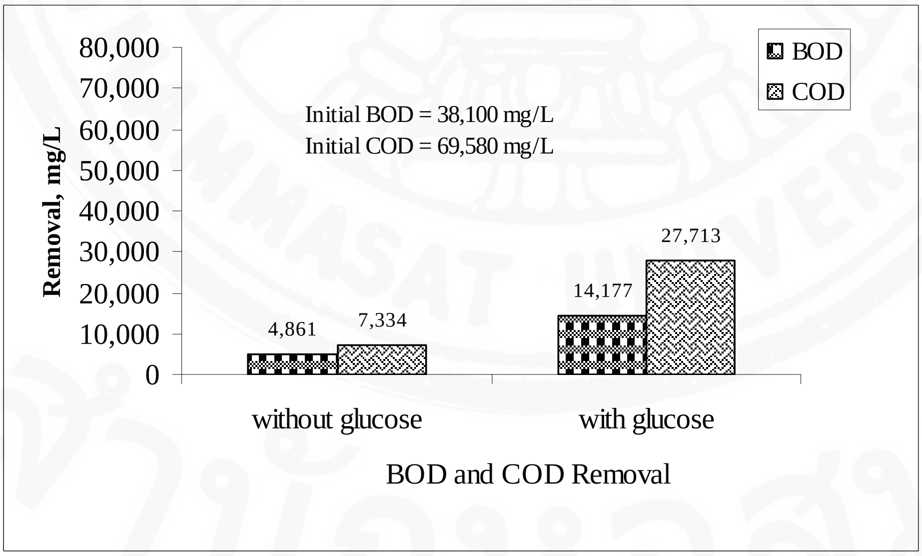


Figure 4.34 BOD and COD removal of leachate 4* in batch experiment

BOD removal is about 12.76% and 37.21% and COD is about 10.54% and 39.83% for without and with glucose, respectively. BOD is about 3.27% and 1.97% and COD is about 0.93% and 3.4% less than leachate 4 without and with glucose, respectively.

4.4.5.4 Enzyme analysis (Unit/L)

In order to find correlation between decolorization and enzymes, activities of enzymes released by *T.versicolor* BCC 8725 were analyzed. Other researchers have also found the production of enzymes (especially LiP, MnP, laccase) in decolorization process using white rot fungi, *T.versicolor* (Muhammad et al., 2008; Amaral et al, 2004; Fulya et al., 2009; Daljit et al., 2002). Thus, in this study, the relationship between the color removal efficiency and the enzyme activities of *T.versicolor* BCC 8725 without and with glucose were carried out and the results are shown in Figure 4.35 (a) and (b).

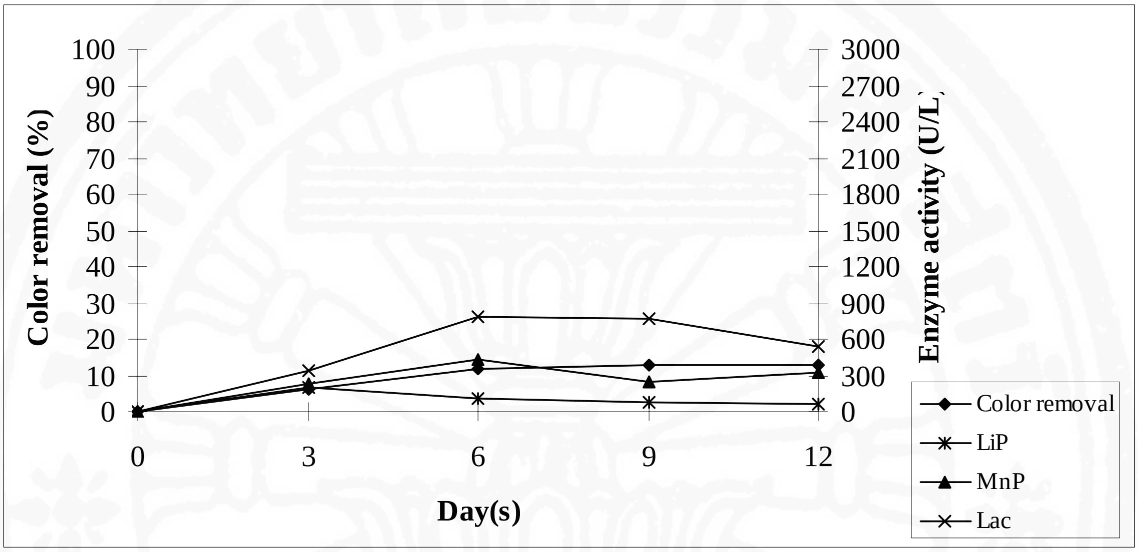


Figure 4.35(a) Extracellular enzymes activity and leachate decolorization by *T.versicolor* BCC 8725 without glucose (Note: LiP = Lignin peroxidase, MnP = Manganese peroxidase, Lac = Laccase)

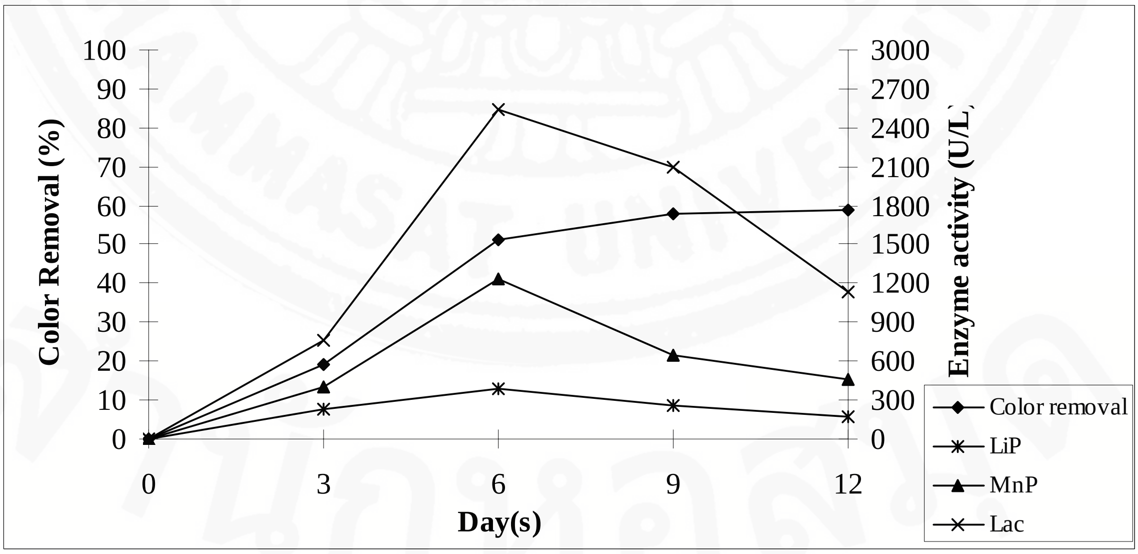


Figure 4.35(b) Extracellular enzymes activity and leachate decolorization by *T.versicolor* BCC 8725 with glucose (Note: LiP = Lignin peroxidase, MnP = Manganese peroxidase, Lac = Laccase)

The production of enzyme by *T.versicolor* BCC 8725 was determined as a function of time at the optimum condition. From Figure 4.32, it can be seen that the biomass growth steadily increased and reached the highest on the 12th day but the maximum enzyme released were on the 6th day. Therefore, it can be concluded that the enzyme activity is not growth-associated as compared to the biomass growth. Although in absence of glucose, the enzymes were also detected but the presence of glucose was necessary for the color removal by *T.versicolor* BCC 8725. All enzymes produced by *T.versicolor* BCC 8725 had higher enzyme activity in leachate with glucose.

T.versicolor BCC 8725 was found to produce LiP, MnP and laccase and helped in degradation. Lignin peroxidase (LiP) is a heme containing glycoprotein secreted during secondary metabolism in a response to nitrogen limitation (Tien and Kirk, 1983b; Shrivastava et al., 2005). LiP is a strong oxidizer capable of catalyzing the oxidation of phenols and aromatic amines, chromatic ethers and polycyclic chromatic hydrocarbons (Collins et al., 1997). Manganese peroxidases (MnP) secreted by most white rot fungi are also glycosylated, heme containing enzymes that functionally require H_2O_2 (Jensen et al., 1996). Laccase is a blue multicopper system that may interact directly with phenolic components of lignin and in the presence of a mediator compound can react with a wide range of substrates (Wells et al., 2006).

It was found that laccase was the main enzyme which was produced at the highest level by *T.versicolor* BCC 8725. Pointing (2001) observed that purified laccases, LiPs and MnPs are able to decolorize dyes of different chemical structure. The fungi was able to decolorize 58.5% of the leachate with the peak LiP, MnP and laccase activity of 193, 437 and 781 U/L (Figure 4.35 (a)) and 384, 1,241, 2,534 U/L (Figure 4.35b)). This indicated that enzyme formation increased when adding glucose as the co-substrate leading to higher decolorization also as compared to without glucose. The co-susbtrate (glucose) has the effect on the amount of enzyme produced. Levin et al. (2005) found that MnP production was enhanced by higher glucose concentration.

Laccase was seen to increase gradually as the percentage of color removal increased. This suggested that laccase was the main enzyme contributing to the decolorization of the leachate (Figure 4.35 (a) and (b)). The three enzymes reached the peak in the 6th day on the leachate both with and without glucose. The maximum color removal also occurred by 9th day and then slow decolorization occurred. LiP activity was slightly rose towards day 6 and drop slightly towards the end of incubation period where most of the color were broken down and decolorized. No further decolorization occurred after the 9th day, with approximately 60% and 13%, respectively for leachate with and without glucose. For MnP in leachate without glucose, the enzyme gradually increased and has two peaks on the 6th and 12th day. Laccase activity during the peak period without glucose was just one-third of the laccase produced with the presence of glucose. The function of laccases is to detoxify highly reactive aromatic compounds by polymerizing, repolymerization, demethylation, or quinine formation them (Thurston, 1994).

According to the biomass growth and color removal, the color removal tended to be constant after the 8th day while the biomass growth was still gradually increased. In terms of enzyme activity and the color removal, the enzyme activity reached the peak on the 6th day and dropped after that whereas the color removal still increased slowly until the 12th day (Figure 4.33). This can indicate that when the enzymes were produced maximally, the decolorization was highest. This is in agreement with Mtui and Nakamura (2008). These results indicated that color removal rates were proportional to the enzyme activity. However, assistance in color removal of the leachate may be due to other enzymes presented which were not assayed. The assayed enzyme activities still maintained high levels at the end of degradation.

Relatively high enzyme activities can be obtained only when nitrogen sources are limited (Wu et al., 2005). The enzymes involved in decoloration in white rot fungi may be inhibited in N-rich cultures (Swamy and Ramsay, 1999; Kapdan et al., 2000). It might be predicted that more enzyme production, therefore higher color removal, could be obtained in the leachate with lower ammonia and organic loading. However, the enzyme analysis was done only in one set in leachate 4*.

4.4.6 Comparison of various types of leachate used in this study

Effect of ammonia and organic loading in terms of color, BOD and COD removal with and without glucose from batch experiment is shown in Figure 4.36.

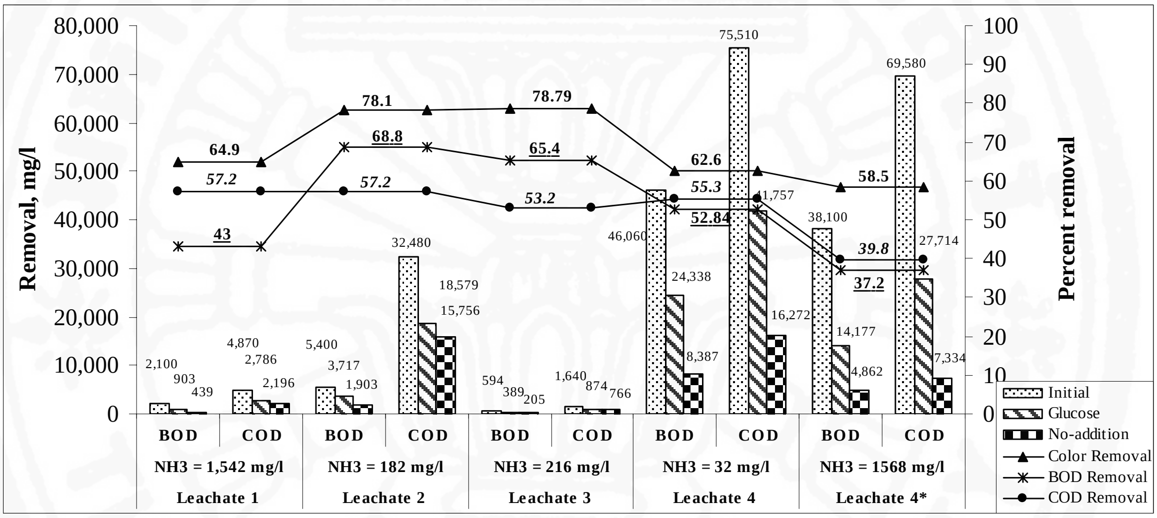


Figure 4.36 Comparison of color, BOD and COD removal with the initial value with glucose and without glucose for various types of leachate used

From Figure 4.36, it can be seen that leachate 1 and leachate 4* (analyzed before enzyme analysis) have the highest concentration of ammonia of 1,542 mg/L and 1,568 mg/L, respectively. With approximately the same amount of ammonia in leachate 1 and 4*, the organic loading of leachate 1 is much lower than leachate 4*. The BOD and COD removal of leachate 1 and 4* is 43%, 57% and 37% and 40%, respectively (with glucose). Color removal in leachate 1 and 4* is 65% and 59%, respectively with glucose. So, leachate 1 has better removal efficiency than leachate 4* in terms of color, BOD and COD. This shows that the organic loading has an effect on removal efficiency.

When comparing, leachate 4 and leachate 4*, the organic loading is roughly the same (BOD = 46,060 and 38,100 mg/L) but the ammonia content is 32 mg/L and 1,568 mg/L, respectively. It can be seen that the color removal with glucose is 62% and 58% for leachate 4 and leachate 4*. The BOD and COD removal with glucose is 46% and 49% and 37% and 40%, respectively for leachate 4 and leachate 4*. So, the removal efficiency of leachate 4 is better than leachate 4*. This indicates that the ammonia content has an effect on removal efficiency as well. Previous studies have shown that ammonia is toxic to the fungi (Keenan et al., 1984) and is the main factor that inhibits the growth of fungi and a high level of nitrogen can inhibit the degradation (Kirk et al., 1978, Kapdan et al., 2000).

Consequently, the amount of biomass and the enzyme released are limited reducing the decolorization efficiency. Therefore, the nitrogen content and organic loading are very important factors and might have effect on decoloration rate. The difference in the removal efficiency is due to the different initial organic loading, and also ammonia concentration.

The removal of color, BOD and COD in leachate 2 is about the same as in leachate 3. Although the organic loading in leachate 3 is much lower but the fungi treatment was still not complete. As leachate from garbage truck did not show much removal of color, BOD and COD by fungi, it was not used for further experiments. Garbage truck sample is very fresh leachate and is not a good representative as compared to that from the landfill.

The mg removal of BOD and COD per mg of biomass for leachate from the stabilization pond (leachate 1) is 13.35 and 41.21, respectively which is the lowest efficiency among the leachate studied. The reason might be because leachate 1 is stabilized (pH 8.3) with very high ammonia concentration (BOD = 2,100 mg/L, COD = 4,870 mg/L, ammonia = 1,542 mg/L and low concentration of heavy metal). In case of leachate as discharged from pipe to the pond (leachate 2, leachate 4 and leachate 4*), the mg BOD and COD removed per mg biomass for leachate 2 is 15.63 and 78.12, respectively and for leachate 4 is 109.48 and 187.84, respectively. So, the removal efficiency for leachate 2 is much less than leachate 4. Although leachate 2 is low strength leachate but has higher ammonia concentration and high concentration of heavy metal (BOD = 5,600 mg/L, COD = 34,560 mg/L, ammonia = 182 mg/L) than leachate 4, the fungi still have the less removal efficiency as compared to leachate 4 which is very high strength leachate but low ammonia concentration (BOD = 48,900 mg/L, COD = 96,512 mg/L, ammonia = 32 mg/L). This indicates that ammonia and heavy metal might have affected the treatment efficiency. For leachate 4*, the mg BOD and COD removed per mg of biomass is 67.8 and 132.6, respectively which is less than leachate 4. The reason might be because leachate 4* is high strength leachate and has very high ammonia concentration as compared to leachate 4, and thus the removal efficiency is lower than leachate 4. Leachate 4 showed highest removal efficiency among all the leachate used in this study although it is very high strength leachate but it contains low ammonia and low concentration of heavy metal. Thus, the factors affecting the removal efficiency by *T.versicolor* BCC 8725 might be the ammonia concentration and the heavy metal concentration. Similar factors affecting the treatment efficiency by white rot fungi are reported by other researchers (Kirk et al., 1978; Kapdan et al., 2000; Gadd, 1993; Baldrian, 2003, Vallee and Ulmer, 1972; Yetis, 1998).

4.5 Continuous experiment

4.5.1 Leachate 2

4.5.1.1 Characteristics of leachate 2 before and after dilution

The leachate characteristics were analyzed before running continuous experiment and are shown in Table 4.4.

Table 4.4 Characteristics of leachate 2 after dilution

Parameters	Unit	Before storage	After storage (No dilution)	Dilute 5 times	Dilute 10 times
pH	-	5.33	6.12	7.01	7.03
BOD	mg/L	5,600	4,880	1,020	560
COD	mg/L	34,560	18,400	4,140	1,920
NH_3-N	mg/L	182	230	42	20
Color	ADMI	900	900	185	94
OLR	gBOD/L.d		0.9	0.2	0.1

The leachate characteristics indicated that the leachate was stabilized because the pH increased, the BOD and COD decreased, the ammonia concentration increased and the color was about the same.

4.5.1.2 Effect of organic loading

The organic loading was calculated to determine decolorization, BOD and COD removal efficiency at different organic loading rate.

Organic Loading Rate per volume = (Flow rate*BOD)/volume
= (0.4 ml/min * 4,880 mg/L)/3000 ml
= 0.937 gBOD/L/d

Dilute 5 times, BOD = 915 mg/L = (0.4 ml/min * 1,020 mg/L)/3000 ml
= 0.196 gBOD/L/d

Dilute 10 times, BOD = 560 mg/L = (0.4 ml/min * 560 mg/L)/3000 ml
= 0.107 gBOD/L/d

Organic Loading Rate per surface area = (Flow rate*BOD)/surface area
= (0.4 ml/min * 4,880)/ (800*1cm^2)
= 3.514 mgBOD/cm^2/L/d

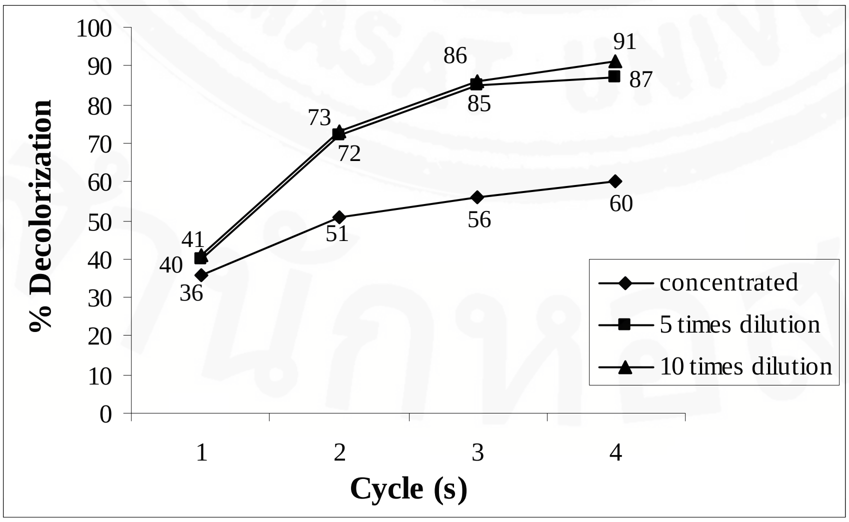


Figure 4.37 Effect of organic loading (leachate 2) on decolorization using *T.versicolor* BCC 8725 with glucose 3 g/L and air addition

Effect of organic loading on color removal is shown in the Figure 4.37. From the figure, it can be seen that the decolorization is increasing, especially, sharply increased from the first to the second cycle. The decolorization of concentrated, 5-time dilution and 10-time dilution were almost the same (36-41%) at the first cycle. It was found that, with 10 times dilution and the organic loading rate of 0.0001 kgBOD/L/d, the fungi could decolorize 91% whereas in the concentrated leachate with the organic loading rate of 0.0009 kgBOD/L/d, decolorization was only 60%. About 31%, higher decolorizationas is observed in 10-time dilution as compared to the concentrated leachate. For 5-times dilution, the decolorization was about the same as in 10-time dilution leachate so 10-times dilution leachate is not economically sound. So, 5-time dilution leachate was selected and used in further experiment. Thus, leachate dilution has the effect on decolorization. Livernoche et al. (1983), using *T.versicolor* BCC 8725 had shown that in undiluted liquor the color decreased by 65% after 3 days, whereas a 90% color reduction was achieved in the diluted liquor. When comparing leachate with and without air addition, not significant difference in result was observed (with air (91%) and without air addition (89%) at 10 times dilution at the same condition.

4.5.1.3 Biomass Growth of fungi

Biomass growth of fungi in the reactor was studied to determine if the biomass growth relate to the decolorization efficiency. The biomass growth was done for the concentrated, 5 times dilution and 10 times dilution leachate and was observed at day 1 and day 20 for both with glucose 3 g/L and without addition. The results are shown in the Figure 4.38.

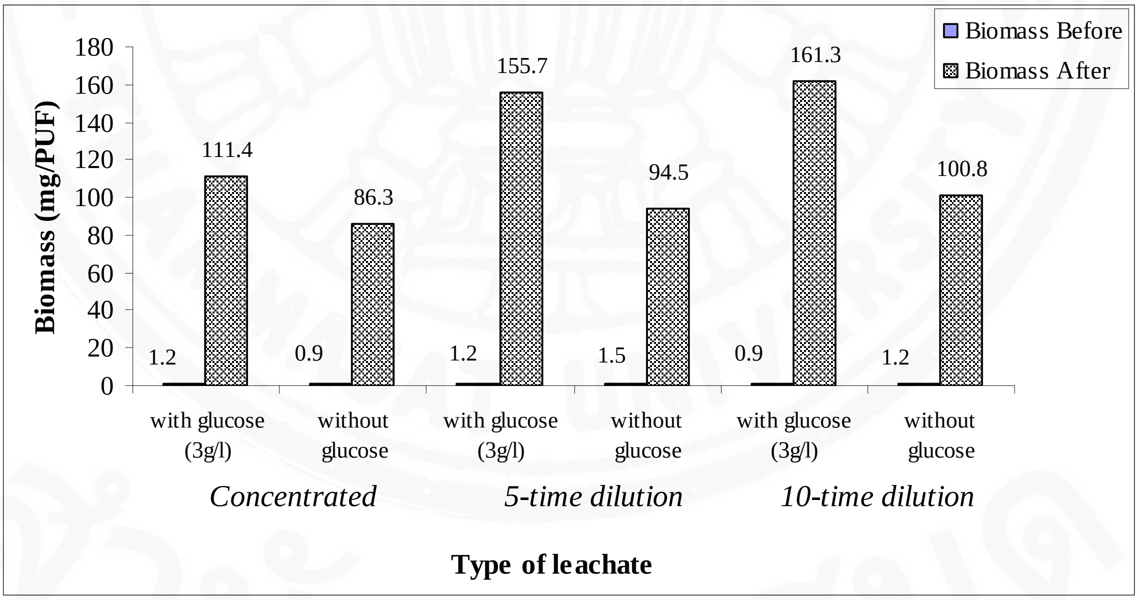


Figure 4.38 Biomass growth of *T.versicolor* BCC 8725 at different dilution in leachate 2

The same fungi were used for all 4 cycles. Figure 4.38 shows the biomass growth on the first day and the last day of with and without glucose concentration at different dilution. The biomass growth is related to the decolorization, BOD and COD removal efficiency. The highest biomass growth is found in 10-time diluted leachate and the lowest biomass growth was found in concentrated leachate. It was about 0.16g (0.1613g and 0.0009g) different from the beginning to the end (20^{th} day) in 10 times dilution leachate with glucose.

4.5.1.4 Heavy metal analysis

Heavy metal concentration was studied at the leachate after treatment and was shown in Table 4.5.

Table 4.5 Heavy metal concentration after treatment of leachate 2

Heavy metal (mg/L)	Standard	Before storage	After storage	After treatment in continuous experiment		
				Concentrated	5-time dilution	10-time dilution
Cd	<0.03	1.20	0.80	0.83	0.82	0.78
Cr	<0.25	31.50	12.64	12.63	12.4	12.4
Pb	<0.2	2.50	1.29	1.25	1.14	1.12
Hg	<0.005	1.60	0.92	0.91	0.90	0.90
Ni	<1.0	ND	ND	ND	ND	ND

It can be seen from Table 4.5 that though the leachate was kept in 4ºC room, the heavy metal concentration has decreased may be due to precipitation and reaction that occur with time because the leachate was partly stabilized. However, the concentration of heavy metal is still excess the standard. After treatment, the heavy metal concentration still presents in excess. This shows that the presence of heavy metal did not affect much on the activity of extracellular enzymes. Among the white rot fungi species tested by Palmans et al. (1995), *T.versicolor* BCC 8725 was found to be resistant to metals (Cd, Zn, Ni, Co, Cr, Mo, Pb, Hg, and Sn). *T.versicolor* BCC 8725 were able to decolorize Poly-R-478 in the presence of heavy metals. *T.versicolor* BCC 8725 effectively decolorized the Ni-containing Reactive blue 38 and Cu-containing Reactive blue 15. *T.versicolor* BCC 8725 showed adsorptive capacities from Pb>Ni>Cr>Cd>Cu (Yetis et al., 1998).

Some heavy metals are essential for the fungal metabolism whereas others have no known biological role. Both essential and nonessential heavy metals are toxic for fungi when present in excess. The metals necessary for fungal growth include copper, iron, manganese, molybdenum, zinc, and nickel. Nonessential metals commonly encountered include chromium, cadmium, lead, mercury and silver (Gadd, 1993). Cd and Hg are in general the most toxic metals for all white rot fungi (Baldrian, 2003). Heavy metals in general are potent inhibitors of enzymatic reactions (Vallee and Ulmer, 1972).

4.5.2 Leachate 4

Batch reactor is easy to use in the laboratory study but it is less convenient to use in industrial application because it cannot give the accurate scale-up data for flow reactor. In practice, continuous operation is very essential for industrial scale to design the desired technical systems as it provides reliable data on the fungi capacity from the first cycle to subsequent cycles and acceptable flow rate as well as hydraulic retention time.

4.5.2.1 Effect of organic loading and degree of immobilization with and without co-substrate on removal efficiency

Organic loading rate is one of the factors influencing the removal efficiency. The organic loading rates of concentrated and 5-times diluted leachate without glucose were 6.52 and 1.3 g BOD/L/d, respectively. Organic loading rate per unit surface area of PUF of concentrated leachate without glucose was 24.45 mgBOD/cm^2/L/d. As the treatment by *T.versicolor* BCC 8725 is dependent on pH, the pH of leachate was adjusted to 4 which were found to be optimum during the batch experiments. The optimum dose of glucose as observed from the batch experiments, 3 g/L, was used as co-substrate.

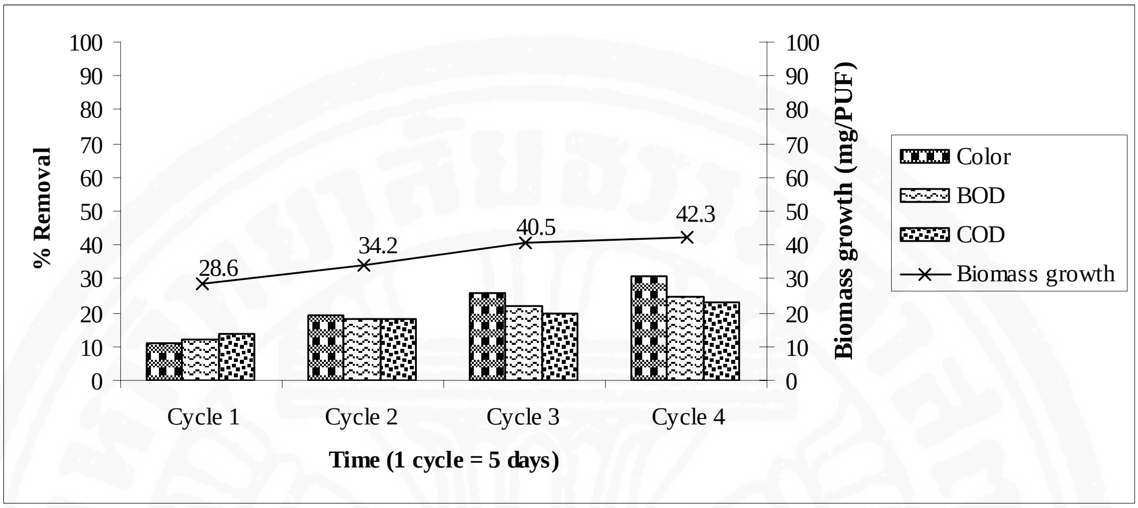


Figure 4.39 Percent removal and respective biomass growth in different cycles using concentrated leachate without glucose (4-day immobilization)

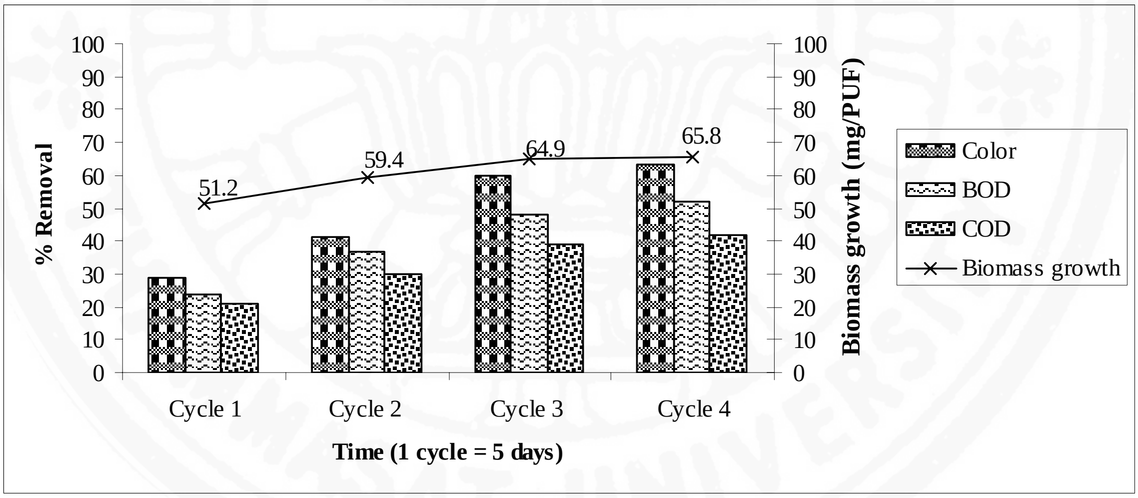


Figure 4.40 Percent removal and respective biomass growth in different cycles using concentrated leachate with glucose (4-day immobilization)

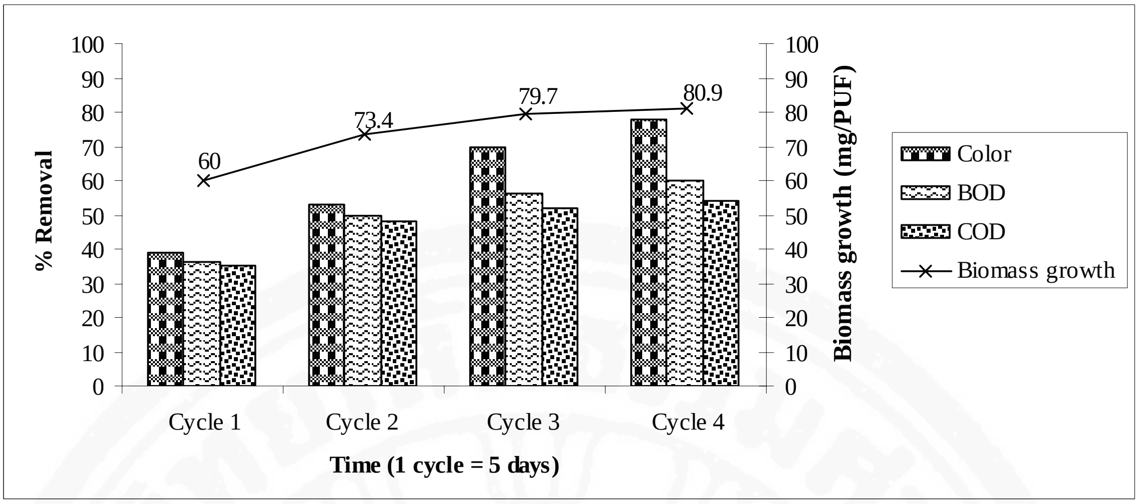


Figure 4.41 Percent removal and respective biomass growth in different cycles using 5-times diluted leachate with glucose (4-day immobilization)

Figure 4.39-4.41 showed the importance of co-substrate (with and without glucose addition) and the effect of organic loading (concentrated and 5-times diluted leachate) on the biomass growth as well as the degree of immobilization. It was assumed that biomass growth on PUF was uniform. Based on this, Figure 4.39-4.41 shows the biomass growth at each cycle after 4 days initial immobilization in PDB and Figure 4.42-4.43 showed biomass growth at each cycle with 15 days initial immobilization on PUF. The biomass growth generally increases with the time, especially between cycle 1 and 2, and after that a slower increase was observed. About 15 mg higher biomass growth per PUF was observed in 5-time diluted leachate than in concentrated leachate at the last cycle. Highest biomass growth (80.2 mg/PUF) was observed in 5-time diluted leachate using glucose as co-substrate with 15-day immobilization of fungi, while the lowest growth was in concentrated leachate without glucose with 4-day immobilization.

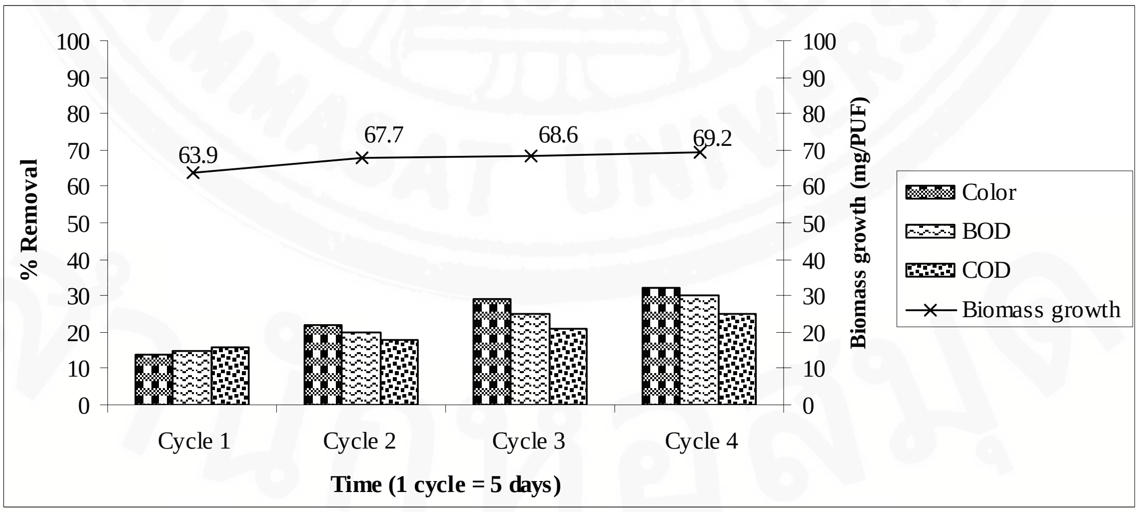


Figure 4.42 Percent removal and respective biomass growth in different cycles using concentrated leachate without glucose (15-day immobilization)

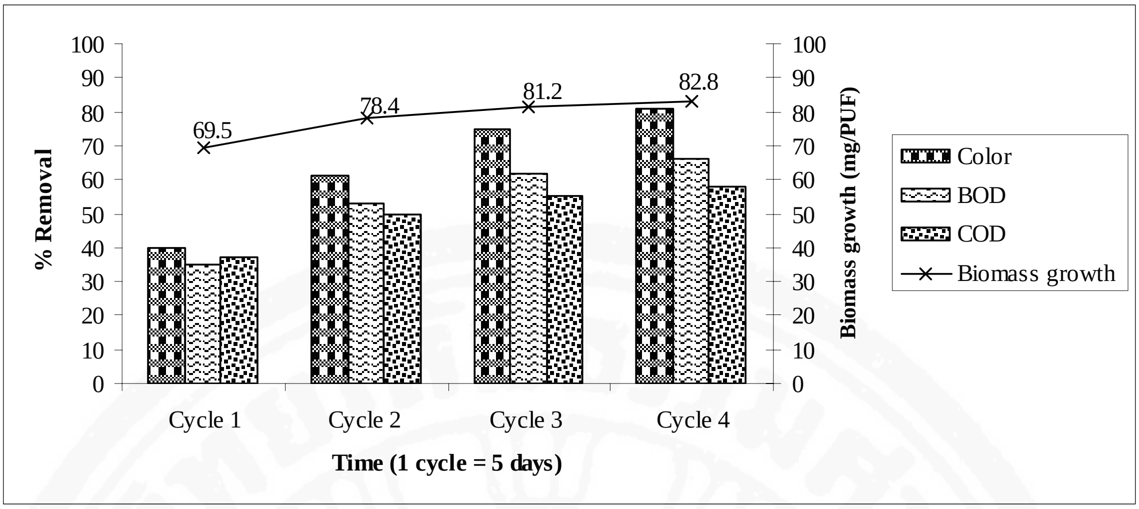


Figure 4.43 Percent removal and respective biomass growth in different cycles using 5-times diluted leachate with glucose (15-day immobilization)

It is also observed that the percent removal increases with the number of cycles as the fungi get acclimatized to the leachate and more biomass growth occurs. When glucose was added, the biomass growth was high (almost double) which also resulted in higher color, BOD and COD removal. The fungi utilized glucose as food and gave better removal efficiency as compared to samples without glucose addition. The biomass growth coincided with the color, BOD and COD removal. About 32% higher decolorization was observed when glucose (3 g/L) was added in the concentrated leachate. From Figure 4.42, it can be seen that even though the initial biomass growth was high due to 15-day immobilization, still not much removal was observed because no co-substrate was added. Also, the amount of biomass did not increase much in the four cycles. However, it can be seen that removal increases when glucose is added (Figure 4.40-4.41) which also enhances biomass growth. These results show that co-substrate plays an important role and is required for degradation of pollutants when using white rot fungi as well as for biomass growth.

Fungi have higher color removal efficiency (15%) in diluted leachate than in concentrated leachate both with and without co-substrate (4-day immobilization). This indicates that fungi can work better at lower organic loading; however, the fungi is still able to treat concentrated leachate. The recycling of the treated effluent further diluted raw leachate. The advantage of the recycle process is that it can treat the wastewater at very high concentration although extra energy is required for recycling. The color removal was low at the early stage for concentrated leachate because the fungi took time to acclimatize into the new environment and then started degrading the pollutants. After that, the color removal tends to increase sharply. The decolorization slowly increased after the third cycle. Although the leachate is very concentrated, the fungi can still work effectively. This shows that white rot fungi can degrade the organic compounds with time and can be used for treatment of wastewater with high BOD and COD.

The initial biomass growth in continuous experiment was higher when the fungi were immobilized on PUF for 15 days rather than 4 days, but the removal efficiency is not high initially and increases significantly after the second cycle. This shows that fungi need time to get acclimatized and perform better once acclimatized to the leachate. Removal efficiency increases after the second cycle and gets better until the last day. The percentage of color removal between cycle 3 and 4 was not much and thus the experiment was stopped after the fourth cycle.

4.5.2.2 Comparison of color, BOD and COD removal after treatment at various conditions

The comparison of color, BOD and COD removal after treatment (in cycle 4) at various conditions are shown in Figure 4.44.

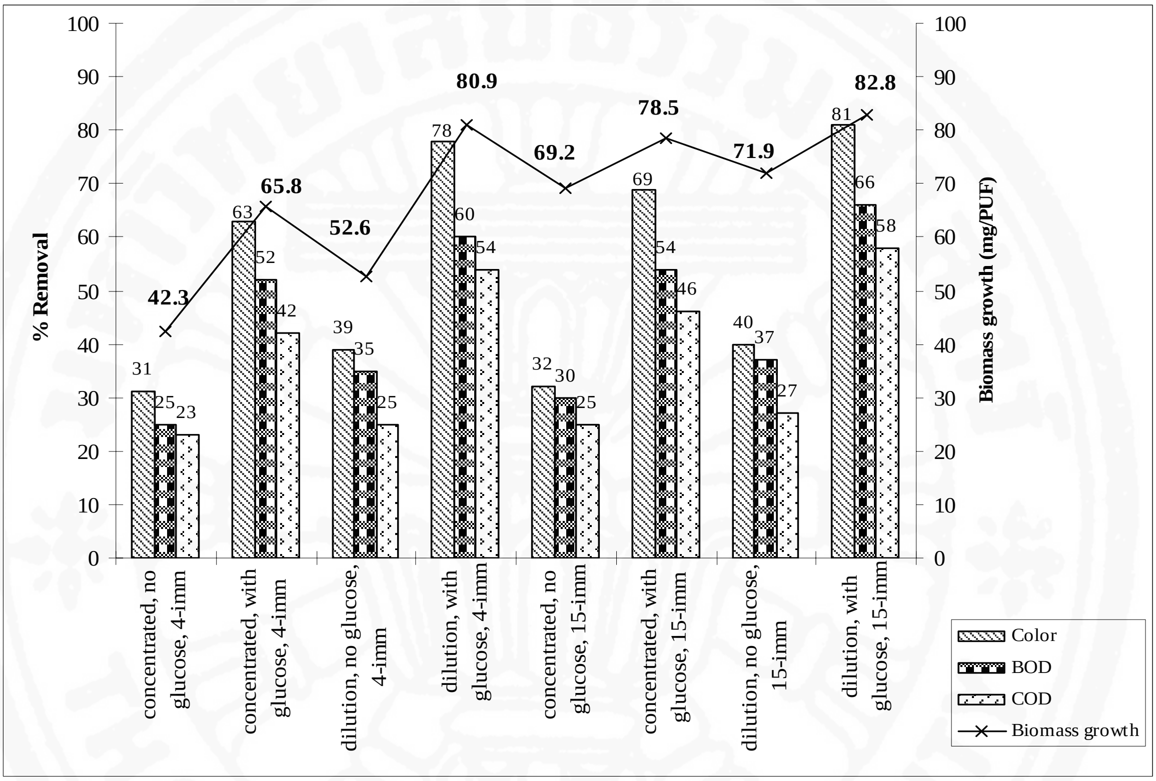


Figure 4.44 Comparison of color, BOD and COD removal and biomass growth in cycle 4 at various conditions

It can be seen that removal for all three parameters is low when no glucose was added as co-substrate, even though the BOD and COD of the leachate is high and this can act as a source of food for fungi. When glucose is added, removal efficiency increases significantly. This clearly indicates that the fungi utilizes readily available glucose as a source of energy for biomass growth, and starts degrading the pollutants. Pollutants are removed mostly through degradation by enzymes released by fungi. To some extent, pollutants may be removed by adsorption as well.

In general, it can be seen that biomass growth and removal efficiency are related. When biomass growth is higher, removal efficiency is higher also. However, in one set of experiments where fungi was immobilized for 15 days in PDB, which led to higher biomass growth (as compared to 4-day immobilization), still not much removal was observed as glucose was not added as co-substrate. This also strongly supports that fungi required glucose as co-substrate, which is an easily accessible source of energy for fungi.

Dilution of leachate reduced organic loading and when such leachate was used, higher removal efficiency (15% higher color removal) was observed with glucose as co-substrate and with 4 days initial immobilization. This shows that organic loading affects the removal efficiency. Dilution of leachate did not significantly increase the removal efficiency when glucose was not added.

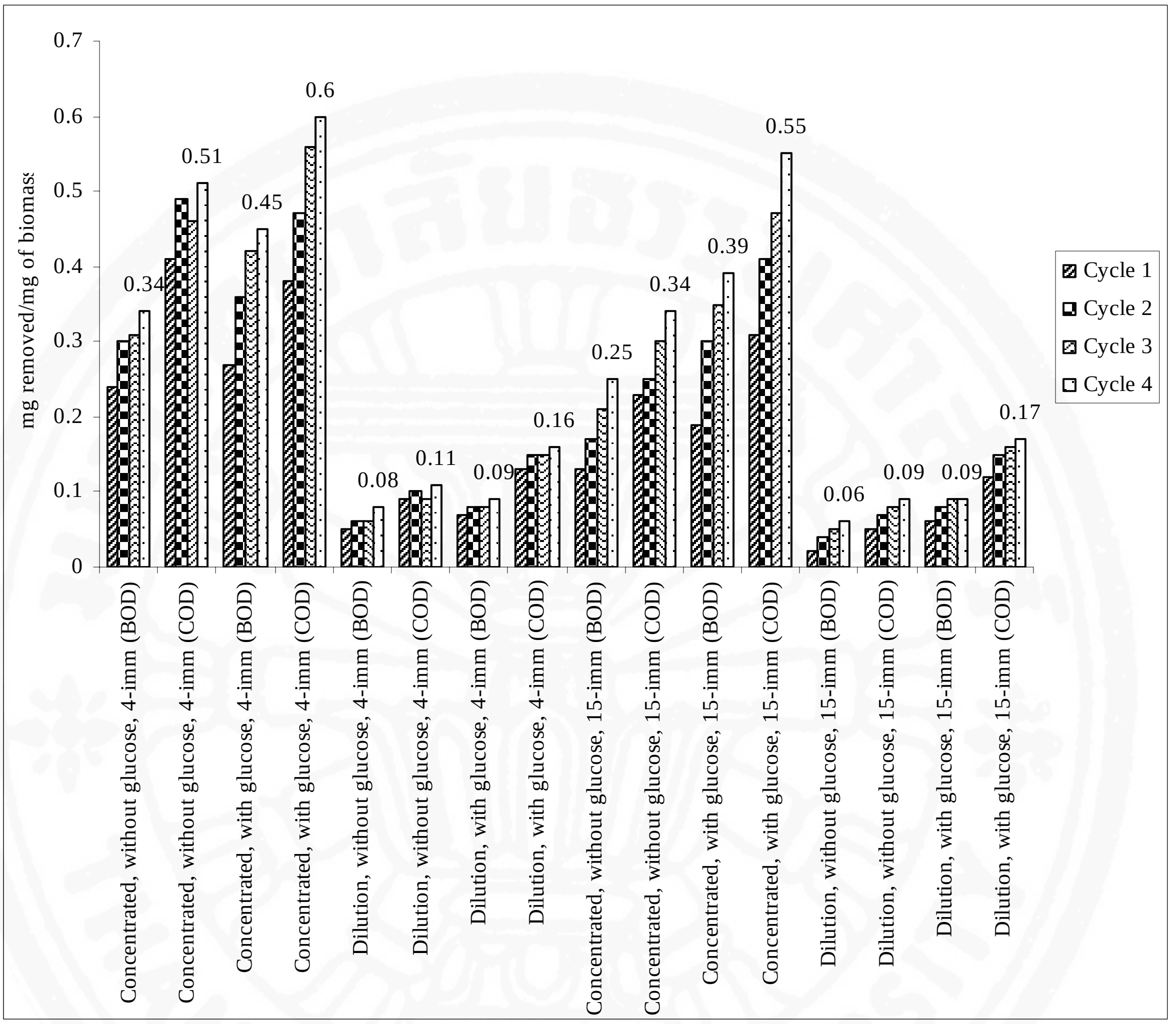


Figure 4.45 The ratio of mg of BOD and COD removed per unit of biomass

Figure 4.45 showed the ratio of mg of BOD and COD removed per unit of biomass in continuous experiment in various conditions. Based on the assumption that the glucose was used for the growth of fungi and did not contribute to the BOD and COD of the leachate and the biomass of fungi is uniformly grown on PUF, mg removal of BOD and COD per mg of biomass was calculated. It is interesting to note that the COD removal per unit of biomass is higher than BOD for all cases. It can also be seen that when the leachate is diluted 5 times, the mg/mg removal of BOD and COD is much less than that of the concentrated leachate. Removal efficiency was slightly better when the fungi were immobilized for 4 days as compared to 15 days immobilization. Although the organic loading is less after dilution, the mg /mg removal is much less after dilution as compared to the concentrated leachate. A higher percent of removal was observed when overall removal efficiency was calculated (Figure 4.44) for all three parameters after dilution, but the fungi is not utilized to its full potential. Thus, it is better to treat the concentrated leachate with fungi followed by another treatment process. It can be seen that the ratio of the BOD and COD removed to the biomass growth in continuous experiment in the four cycles keep increasing with each cycle. This demonstrates that a continuous experiment may need longer time to reach the stable point.

As the pH of the treated leachate is acidic, lime should be added to adjust the pH before discharging to the environment.