EVALUATION OF THE TOXICITY OF 3,4,5-TRICHLOROCATECHOL ON *STREPTOMYCES ALBUS* AND ITS BIODEGRADATION

Jidon Janaun[1], Awang Bono[1] and Ho Coy Choke[2]

[1] *School of Engineering and Information Technology,*
Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia.
[2]*School of Science and Technology,*
Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia

ABSTRACT. *Although the amount of pulp and paper mill effluent is relatively low compared to other industries like rubber and palm oil, but they pose greater danger because it contains chlorinated organic compounds beside other conventional pollutants such as COD, BOD, TSS and many more. Chlorinated organic compounds are toxic to aquatic lives, resistant to biodegradation and also bioaccumulate. They harm human indirectly through contamination in the food chains. These chronic problems force scientists around the globe to find for their remedy. In this study, the biodegradation of particular 3,4,5-trichlorocatechol was evaluated. The work was started with the evaluation of the toxicity of 3,4,5-trichlorocatechol against the growth of Streptomyces albus strain K1. The results showed that at concentration of 100 mg/l, 3,4,5-trichlorocatechol inhibited the growth of S. albus on oatmeal media. These results also indicated that the culture of S. albus may have the potential to biodegrade 3,4,5-trichlorocatechol. In the subsequent biodegradability study, it showed that the culture of Streptomyces albus was able to remove 21.9% of 725 mg/l 3,4,5-trichlorocatechol. Using Monod formulation for the bacterial growth and utilization of the sequencing batch reactor, kinetics of degradation were obtained as: the specific growth rate,* $\mu_m = 0.31\ d^{-1}$; *the half saturation constant,* $K_s = 12.58$ *mg TrCC/l; the decay constant,* $k_d = 0.0026\ d^{-1}$;*and yield,* $Y = 0.57$ *mg TSS/mg 3,4,5-trichlorocatechol.*

KEYWORDS. 3,4,5-Trichlorocatechol, Adsorption, biodegradation, Monod formulation, *Streptomyces albus* strain K1,.

INTRODUCTION

The presence of xenobiotic compounds in the environment attracts attention because of their persistence and their potential to harm aquatic lives (Lindstrom and Mohamed, 1988). For instance, 3,4,5-trichlorocatechol and other chlorinated phenolic compounds are produced mainly from the pulp and paper industry (Springer, 1986) as a result of chlorination of lignin in the bleaching process (O'Connor and Voss, 1992). In particular, 3,4,5-trichlorocatechol is reported to have 25h-LC_{50}: 2.9 – 3.9 mg/l (*Daphnia magna*) and the threshold toxic concentration: 250 mg/l (Zebra fish embryo/larvae) (Material Safety Data Sheet, 2000). 3,4,5-Trichlorocatechol and other pollutants from pulp and paper mill effluent also pollutes the receiving sediment (Eriksson *et al,* 1996). Chlorinated organic compounds bound to sediments and suspended particulate matters are not necessarily unavailable to the aquatic biota. Trichlorocatechol with other chlorinated phenolic compounds have been accumulated by clams, oysters, shrimps, crabs, and fish (Beak Associates Consulting (BC) Ltd, 1990). Organochlorine have been found in benthic invertebrates, which live and/or feed on sediments in waters receiving effluents from pulp mills using chlorine bleaching (Landner *et al,* 1977; Wesen, 1988). Fish feed on these contaminated benthic invertebrates, thereby accumulating organochlorine. These are then further accumulated by fish-eating birds (Rannug *et a,.* 1981). As a result of these activities, human are exposed to these toxic pollutants by consuming them indirectly through the food chains.

In this regard, intensive studies have been done to find a system, which can remove 3,4,5-trichlorocatechol. A study by Allard *et al,* (1992) found a stable enrichment cultures of anaerobic-bacteria were able to dechlorinate 3,4,5-trichlorocatechol to produce either 3,5-dichlorocatechol or 3,4-dichlorocatechol. However, this incomplete mineralization needs to be further investigated to obtain a system that can completely biodegrade them. In the preliminary study (Janaun *et al,* 2003), *Streptomyces albus* strain K1 indicated the ability to metabolize 3,4,5-trichlorocatechol. This present study, thorough investigation was done to evaluate the toxic effect of 3,4,5-trichlorocatechol on *S. albus* strain K1 growth and also its subsequent biodegradation. Biodegradation was evaluated based on its kinetic parameters.

Biodegradation kinetic

The sequencing batch process without sludge wasting can be represented as in the diagram in Figure 1.

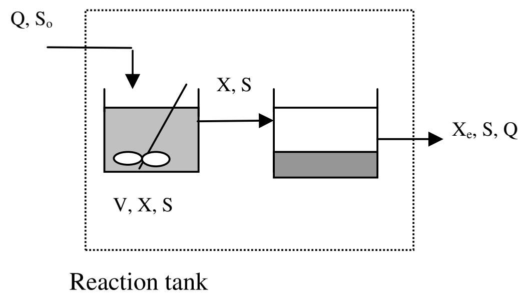


Figure 1: Schematic diagram of sequencing batch process

Representation of reaction tank and the settling tank is made separately in order to ease the mass balance. In an actual situation, the reaction and settling processes occur in the same tank. V stands for occupied reactor volume; X and S the biomass and substrate concentrations respectively and Q the flow rate. Subscript 'o' refers to feed and 'e' to effluent. The kinetic parameters for the process, that is the maximum specific growth rate, μ_m, the half saturation constant, K_s, the death constant, k_d, and the yield, Y, can be obtained as below:

Mass balance across system for biomass:

$$V\frac{dx}{dt} = \mu XV - k_d XV - QX_e \;\;............ (1)$$

At steady state, $\frac{dx}{dt} = 0$,

$$\mu = k_d + \frac{QX_e}{VX}$$

Where, the term $\frac{VX}{QX_e}$ represents the biomass residence time (BRT) of the system. Hence,

$$\therefore \mu = \frac{1}{BRT} + k_d \;\;........................ (2)$$

The Monod formulation for bacterial growth gives the growth rate as dependent on a limiting substrate concentration according to the following expression:

$$\mu = \frac{\mu_m S}{K_s + S} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

By substituting equation (2) into equation (3)
gives :

$$\therefore \frac{1}{S} \cdot \frac{K_s}{\mu_m} + \frac{1}{\mu_m} = \frac{BRT}{1 + BRT.k_d} \ldots\ldots (4)$$

Equation (4) shows that the effluent substrate concentration is independent of influent concentration. According to Shamat and Maier (1980) this is generally valid provided that there are no inhibition effects. For example, in anaerobic contact processes, certain non-kinetic factors will limit the loading rate; these factors are nutrient availability, toxicity, pH control and efficiency of solid-liquid separation.

Mass balance across system for substrate

$$V\frac{ds}{dt} = QS_o - \frac{\mu VX}{Y} - QS \ldots\ldots\ldots\ldots (5)$$

At steady state $\frac{ds}{dt} = 0$, thus

$$\therefore \mu = \frac{YQ(S_o - S)}{VX}$$

By substituting for μ from Equation (2);

$$\frac{1}{BRT} + k_d = \frac{YQ(S_o - S)}{VX}$$

$$\therefore \frac{1}{BRT} \cdot \frac{1}{Y} + \frac{k_d}{Y} = \frac{Q(S_o - S)}{VX} \ldots\ldots\ldots\ldots (6)$$

The basic requirements for the determination of the kinetic parameters: Y, k_d, μ_m, and K_s above are that the reactor must be operated at various biomass residence times and effluent substrate concentrations. This can be achieved by operating the reactor with different feed concentrations and/or by varying the effluent withdrawal volume.

MATERIAL AND METHODS

Strain.

Streptomyces albus strain K1 (Kaken Chem. Co. 80614, ATCC 21838) is a derivative from original strain, JCM4703. The strain was maintained on slants of oatmeal agar at 4 ^oC.

Media

Oatmeal agar was prepared as described by Shirling and Gottlieb (1966). Two types of growth media were used: Seed preparation and fermentation media. Seed preparation medium contain (g/l): Yeast extract, 10; soluble starch, 2; oatmeal (Quacker), 20, in a 125 ml Erlenmeyer flask, while fermentation medium contain (g/l): $(NH_4)_2SO_4$, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 1;

NaCl, 1; $CaCO_3$, 2; Trace solutions, 1 ml and pH 7.0 ~ 7.4 (Trace solution as described by Shirling and Gottlieb (1966)).

Bioassay of TrCC concentration

Interaction between *S. albus* K1 and 3,4,5-trichlorocatechol was studied by assaying various 3,4,5-trichlorocatechol concentration against the growth of *S. albus* K1 on oatmeal agar. Volume of oatmeal agar used throughout the experiments was made constant to 35 ml. 3,4,5-Trichlorocatechol solutions were prepared for 100, 300, 500, 1000, 1500 and 2000 mg/l concentration in separate universal bottles. *N,N*-dimethylformamide was used as solvent (3,4,5-trichlorocatechol at 95-99% purity was purchased from Cansyn Chemical Corp., USA). Inoculum (200 µl) from the seed preparation culture was used to inoculate the oatmeal agar. The inoculum was spread uniformly all over the oatmeal surface using a spreader. 3,4,5-Trichlorocatechol solution (35 µl) was dropped onto a paper disc sized 6mm diameter (the paper discs were prepared from Whatman paper No. 3 using a hole punch). These paper discs were then carefully shifted onto the top of the inoculated oatmeal agar surface. Every test was repeated three times to observe consistency. All plates were stored at 4 ^oC overnight prior to incubation at 28 ^oC for four days.

Biodegradation in batch cultivation

Investigation of the biodegradation of 3,4,5-trichlorocatechol was performed in batch cultivation. Three different systems were incorporated: A, B, and C containing 25 ml seed preparation medium. Flask A and B were inoculated with *S. albus* and pre-grown for 4 days, while Flask C was left uninoculated and stored at 4 ^oC while pending for the cultivation in Flask A and B to complete. Upon completion, Flask B was autoclaved at 121 ^oC for 20 minutes to kill all cells in the system. 3,4,5-Trichlorocatechol was then added into Flask A, B, and C to 725 mg/l concentration. All flasks were then operated at 28 ^oC and 200 rpm. Samples (150 µl) were taken from each flask at set intervals and centrifuged at 7,000 rpm for 10 minutes. The supernatant then assayed on the growth of *S. albus* K1 on oatmeal agar. The systems can be notified as: Flask A: Living cells + 725 mg/l 3,4,5-trichlorocatechol; Flask B: Dead cells + 725 mg/l 3,4,5-trichlorocatechol; Flask C: Fresh medium + 725 mg/l 3,4,5-trichlorocatechol.

The Sequencing Batch Process

The apparatus used as a reactor was a 2000 ml conical flask. Prior to operation, the culture of *S. albus* K1 was pre-grown in the reactor. The flask was filled with 1000 ml of inorganic salts (Table 1) with 500 mg/l glucose as the initial carbon source. Its mouth was plugged with cotton wool to prevent contamination from the air while allowing aeration. Prior to inoculation, these reactors were autoclaved at 121 ^oC for twenty minutes, to kill all unwanted microbes. The fermentation solutions was then inoculated with 100 % of pellet obtained through centrifugation of a 25 ml culture of pre-grown *S. albus* K1 at 7000 rpm for ten minutes. The cultivation was carried out at room temperature (26 $\pm$ 3 ^oC), aeration and agitation by a magnetic-stirrer (Barnstead/Thermolyne) for seven days.

Table 1: Inorganic salts in the feed

Component	Concentration, mg/l
$(NH_4)_2SO_4$	2,000
K_2HPO_4	1,000
$MgSO_4.7H_2O$	1,000
NaCl	1,000
Trace solution*	1 ml

pH	7.0 ~ 7.4

* Trace solution is as described by Shirling and Gottlieb (1966)

Operation

Operations were carried out with biomass residence time (BRT) which ranged from 4 to 8 days. The feedstock contained various 3,4,5-trichlorocatechol concentrations ranging from 40 to 75 mg/l with and without glucose as a co-substrate source. The daily fill and draw process was carried out until eight consecutive steady state conditions for 3,4,5-trichlorocatechol and biomass content had been achieved. Subsequent schedules were conducted as soon as the previous schedule was completed.

Analysis

3,4,5-Trichlorocatechol was detected using UV-Vis spectrophotometer (GBC scientific instrument) at 295 nm. Meanwhile, biomass concentration was analyzed by gravimetrically measuring the total suspended solids (TSS) dried at 103 – 105 ^{o}C according to Standard Method (APHA, 1985).

RESULTS AND DISCUSSION

Bioassay of 3,4,5-trichlorocatechol toxicity

Growth of *S. albus* strain K1 on oatmeal agar was sensitive to the concentration of 3,4,5-trichlorocatechol. The relation of the size of inhibition against the concentration of 3,4,5-trichlorocatechol is shown in Figure 2. The diameter of inhibition was shown to increase as the concentration of 3,4,5-trichlorocatechol increases. This indicates that at concentrations 100 mg/l and above; 3,4,5-trichlorocatechol inhibit the growth of *S. albus* strain K1. The sensitivity of *S. albus* K1's growth in the presence of 3,4,5-trichlorocatechol is an indication of its toxicity. This lays high possibility of *S. albus* K1 culture to biodegrade 3,4,5-trichlorocatechol at concentrations lower than 100 mg/l. This concentration however may not be accurate as there might be small effects of oxidization of 3,4,5-trichlorocatechol by ferrous ion used as a trace element in the fermentation medium.

Biodegradation in batch cultivation

Results from the bioassay of samples taken from every flask are shown in Figure 3. It shows that there were reduction of the diameters of inhibition observed in Flasks A, B and C. These indicate that the concentration of 3,4,5-trichlorocatechol in the samples of Flasks A, B and C was reduced. The inhibition in Flask A disappeared within 24 hours of operation. However, a decrease in inhibition area was also observed in Flask B and C even though those flasks were sterile. Meanwhile, a decrease in inhibition area in Flask B and C became stagnant after 50 hours of operation, where the minimum reduction in Flask B was lower than in Flask C. The difference observed in the decrease of the inhibition area among the three systems is mostly due to the number of suspended particles in the flasks. Number of particles in Flask B was higher than in Flask C. The content of Flask A and B was the same except for the nature of the cells, where the cells in Flask A were living cells while Flask B held dead cells. The most probable reason for the total disappearance of the inhibition area observed in Flask A was due to the activity of *S. albus* K1. Reduction in Flask B and C was mostly attributable to the adsorption of 3,4,5-trichlorocatechol onto suspended particles in both flasks. By comparing between Flask A and B

within 24 hours of operation, it can be calculated that 78.1% of the 3,4,5-trichlorocatechol reduction was due to adsorption, and 21.9% was due to biodegradation. These results confirmed the observation by Remberger *et al,* (1993) where more than 70% of trichlorocatechol in a naturally contaminated sediment is contained in particulate material.

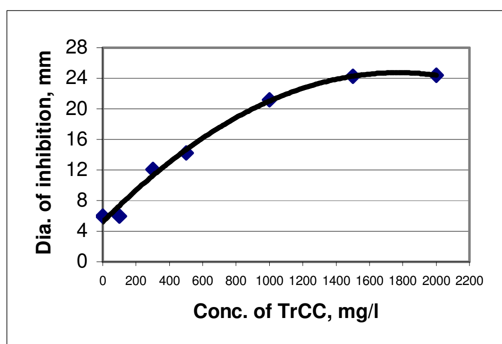


Figure 2: Profile of dependence of the diameter of inhibition on 3,4,5-trichlorocatechol concentration

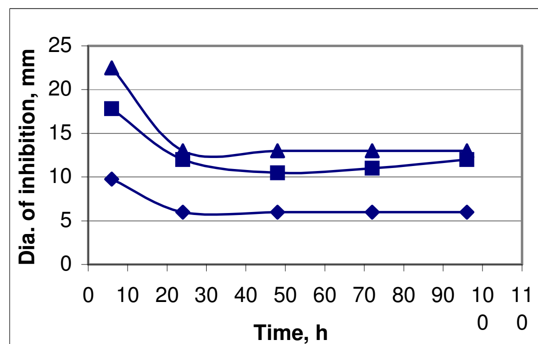


Figure 3: Profile of 3,4,5-trichlorocatechol decrease in the different systems: ◆, Flask A; ■, Flask B; ▲, Flask C.

This evidence of disappearance of inhibition shows that *S. albus* K1 might have degraded 3,4,5-trichlorocatechol even though in a lesser amount. The mechanism of transformation may go as aerobic pathway process where it favours ring cleavage as shown in Figure 4 and 5 (Tiedje *et al,* 1969; Reineke, 1984), even though dechlorination may also have occurred.

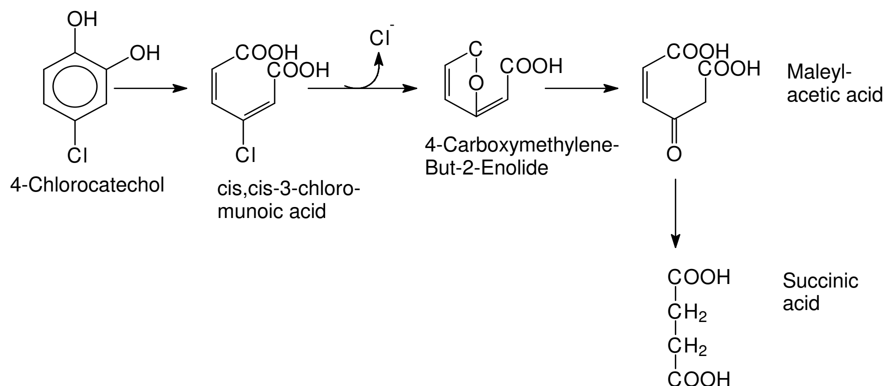


Figure 4: Metabolism of 4-chlorocatechol by *Arthrobacter* sp. (Tiedje *et al,* 1969)

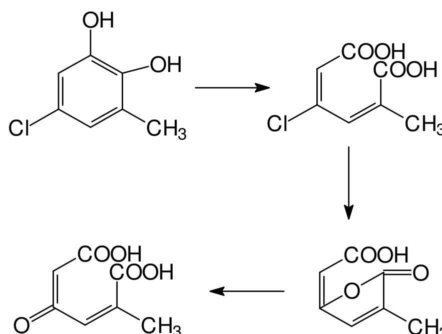


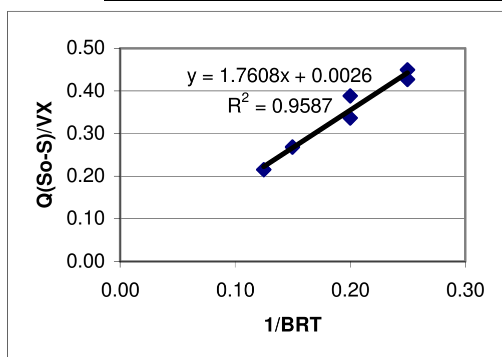
Figure 5: Aerobic degradation of chlorinated catechol, 3-methyl-5-chlorocatechol (Reineke, 1984)

Biodegradation kinetic

he average results of all the operating schedules are given in Table 2. From biomass balance for a process with no direct wasting from reactor, the BRT is given by VX/QX_e. Equation (6) was used to obtain k_d and Y while equation (4) was used to obtain μ_m and K_s. The lines of best fit obtained by linear regression for the two plots are given in Figure 6 and 7.

Table 2: The steady state data of the sequencing batch operation

Operation Schedule No	S_o (mg/l)	S (mg/l)	X_e (mg TSS/1)	BRT (d)	Q (1/d)	V (1)
1	75	47.64	16.00	4.00	0.25	1
2	75	51.50	13.96	5.00	0.20	1
3	50	31.81	10.13	4.00	0.25	1
4	50	19.95	15.50	5.00	0.20	1
5	40	10.73	16.38	6.67	0.15	1
6	40	10.67	17.00	8.00	0.13	1



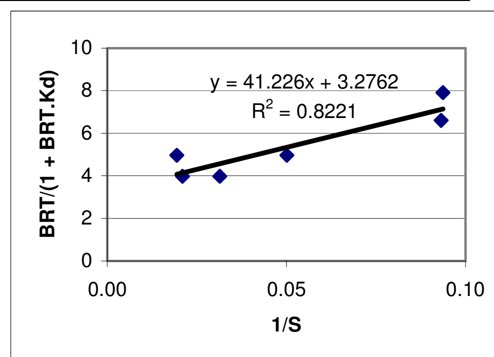


Figure 6: Plot for obtaining Y and k_d

Figure 7: Plot for obtaining μ_m and K_s.

The r values obtained were 0.9587 and 0.8221 respectively, indicating a fair correlation. The kinetic parameters obtained are given in Table 3. With the biodegradation parameters obtained for this process, using equation (2), the BRT_{min} was estimated to be 3.25 days.

Table 3: Degradation parameters for the sequencing batch process

Parameters	Values
μ_m	0.31 d^{-1}
K_s	12.58 mg TrCC/l
Y	0.57 mg TSS/mg TrCC
k_d	0.0026 d^{-1}
BRT_{min}	3.25 d

Fate of metabolites of 3,4,5-trichlorocatechol biodegradation

From this study, it was observed that the growth of *Streptomyces albus* strain K1 in the presence of 3,4,5-trichlorocatechol is possible, even though the growth might be caused by the presence of glucose. This is because glucose is simpler compared to the complex structure of 3,4,5-trichlorocatechol. The observed reduction of 3,4,5-trichlorocatechol in the sample partly

indicated its biodegradation as well as adsorption on to suspended particles. This needs to be studied further.

Examination of the metabolites of 3,4,5-trichlorocatechol degradation was not conducted in this study. However, according to Allard *et al.* (1991), 3,4,5-trichlorocatechol can be dechlorinated. This observation however, differs from aerobic biodegradation, which normally occurs through the elimination of halide after an ortho cleavage of the halocatechols (Tiedje *et al* 1969; Reineke 1984). Therefore, the biodegradation pathway of 3,4,5-trichlorocatechol mostly follows the aerobic biodegradation mechanism.

CONCLUSION

This study shows that *Streptomyces albus* strain K1 has the potential to degrade 3,4,5-trichlorocatechol at concentrations lower than 100 mg/l. This study also confirms that 3,4,5-trichlorocatechol has a high affinity for and can be adsorbed onto particulate material. The high value of K_s (12.58 mg TrCC/l) indicates that 3,4,5-trichlorocatechol is resistant to biodegradation. Therefore, further studies should be done before this system can be introduced to the pulp and paper effluent treatment. It can be expected that a consortium of microorganisms with the inoculation of *Streptomyces albus* stain K1 will give better biodegradation. A study also needs to be thoroughly conducted to investigate the fate of the biodegradation product, as well as the biodegradation mechanism. After all, besides the contribution to the removal of chloroorganic compounds, this study contributes to the bioassay of 3,4,5-trichlorocatechol.

ACKNOWLEDGEMENT

The authors owed to University Malaysia Sabah for funding this research through Research Grant No. 9/2000 and Peter Kuok Han Tung for supplying the strain.

REFERENCES

Allard, A. S., Hynning, P. A., Lindren, C., Remberger, M., Neilson, A. H. 1992. Role of sulfate concentration in dechlorination of 3,4,5-trichlorocatechol by stable enrichment cultures grown with coumarin and flavanone glycones and aglycones. *Applied and Environmental Microbiology* **58**: 961-968

American Public Health Association. 1985. *Standard Methods For The Examination of Water and wastewater*, 16th Edition. American Water Works Association & Water Pollution Control Federation. Washington, D.C.

Beak Associates Consulting (BC) Ltd. 1990. Baseline organoclorine monitoringprogram, prepared for: Canadian Pacific Forest Products Limited – Gold River Mill, Project No. 14-163-01-01.

Eriksson, A. K., Sundelin, B., Broman, D., Naf, T. 1996. Reproduction effect on *Monoporeia affanis* of HPLC-factionated extracts of sediments from pulp mill receipient. In *Environmental fate and effects of pulp and paper mill effluents* (Servos, M. R., Munkittrick, K. R., Carey, J. H., Van Der Kraak, G. J., eds) St. Lucie Press, Delray Beach.

Landner, L., Lindstrom, K., Karlsson, M., Nordin, J., Sorensen, L. 1977. Bioaccumulation of fish of chlorinate phenols from kraft pulp mill bleachery effluents. *Bull Environ Contam Toxicology* **18**: 663-673.

Janaun, J., Ho, C. C., Rakmi, A. R. and Bono, A. 2003. Wastewater Treatment of Pulp and Paper Effluents: Biodegradation of Chlorocatechol. *Proceedings of The 3rd International Conference on Strategic Technologies*. Faculty of Engineering, Universiti Kebangsaan Malaysia.

Lindstrom, K., Mohamed, M. 1988. Selective removal of chlorinated organics from kraft mill total effluents in aerated lagoons. *Nordic Pulp and Paper Research Journal* **3**: 26-33.

O'Connor, B. I., and Voss, R. H. 1992. A new perspective (sorption/desorption) on the question of chlorolignin degradation to chlorinated phenolics. *Environ Sci Tech* **26**: 556-560.

Rannug, U., Jenssen, D., Ramel, C., Erikkson, E., Kringstd, K. 1981. Mutagenic effects of effluents from chlorine bleaching of pulp. *Journal of toxicology and environmental health* **7**: 33-47.

Reineke, W. 1984. Microbial degradation of halogenated aromatic compounds. In *Microbial degradaion of organic compounds* (Gibson, D. T., ed).Marcel Dekker, New York.

Remberger, M., Hynning, P., Neilson, A. H. 1993. Release of chlorocatechols from a contaminated sediment. *Environ Sci Tech* **0**: 158-164.

Shamat, N. A., and Maier, W. J. 1980. Kinetics of biodegradation of chlorinated organics. *J. Water Pollut. Control Fed.* **52**: 2158-2166.

Shirling, E. B., and Gottlieb, D. 1966. Methods for characterization of *Streptomyces* species. *International Journal Systematic Bacteriology* **16**: 313-340.

Springer, A. M. 1986. *Industrial Environmental Control Pulp and Paper Industry*. John Wiley and Sons, Inc. New York.

Tiedje, J. M., Duxburg, J. M., Alexander, M., and Dawson, J. E. 1969. 2,4-D Metabolism: Pathway of degradation of chlorocatechol by *Arthrobacter* sp. *Journal of Agriculture and Food Chemistry* **17**: 1021-1026.

Voss, R. H. 1983. Chlorinated neutral organics in biologically treated bleached kraft mill effluents. *Environ Sci Tech*. **17**: 530-537.

Wesen, C. 1988. Chemical characterization of chlorinated lignin derivatives in organism, sediment and air. *Wat Sci Tech* **20**: 185-188.