CHAPTER IV

RESULTS AND DISCUSSION

4.1 Soil Characterization

The physical and chemical characteristics of soil were shown in Table 4.1. This soil was classified as sandy loam. TCE background concentration in the soil was not detected.

Table 4.1 Characteristics of soil

Property	Value (Unit)
pH	8.05
EC	0.023 (mS/cm)
Organic matter	2.47 (%)
Total nitrogen	0.0896 (%)
Organic carbon	1.43 (%)
C:N ratio	15.97

4.2 Bioaugmentation of TCE

4.2.1 Degradation of TCE by Free Cell of *Rhodococcus* sp. P3 and Acclimatized Activated Sludge

After 3 weeks of incubation, the relative percentage of TCE removal in soil microcosm inoculated with acclimatized activated sludge, *Rhodococcus* sp. P3, and without inoculum were 88%, 53% and 20% (Table 4.2), respectively, indicating that mixed cultures obtained from acclimatized activated sludge and TCE degrader, *Rhodococcus* sp. P3, effectively removed TCE from soil. Immobilized *Rhodococcus* sp. P3 on corncob had a similar TCE degradation activity to immobilized acclimatized activated sludge on corncob in soil. However, immobilized *Rhodococcus* sp. P3 on corncob was the superior TCE degrader. A relative percentage of TCE removal in soil microcosm augmented with mixed cultures obtained from acclimatized activated sludge was higher than in soil microcosm augmented with *Rhodococcus* sp. P3 might due to the fact that acclimatized activated sludge contains complex microbial consortium which degrade pollutants by symbiotic associations resulting in a better degradation of pollutants (Sukesan and Watwood, 1998) than pure culture. Similar finding was

reported by Buitron and Gonzalez (1996) in which the degradation of phenol, 4-monochorophenol, 2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorrophenol by activated sludge was one to two orders of magnitude higher than pure strain isolated from activated sludge which was resulted from proliferation conditions. Proliferation of a pure strain generally requires strictly sterilized conditions and control methods, while the 2,4-DCP degrading mixed cultures could grow quickly and easily in an off-line enrichment reactor. Other advantage of mixed cultures included a possibility to be applied at the sites contaminated with various compound. For example, 2,4-DCP degrading mixed cultures could not only degrade 2,4-DCP but also degraded a wide range of other chlorophenols, including 2-chlorophenol, 4-chlorophenol, 2,4,5 chlorophenol, 2,4,6 chlorophenol and pentachlorophenol (Quan et al., 2004). This finding was confirmed by Otte et al. (1994) who revealed that the activity of pure culture is often restricted to a limited number of contaminants and is less applicable to the complex mixture of pollutants.

Table 4.2 Relative percentage of TCE removal by free and immobilized cells forms of *Rhodococcus* sp. P3 and acclimatized activated sludge in soil microcosm at day 21

Treatment	Relative percentage of TCE removal (%)[1][2]
Control	19.96±4.73
P3	53.34±1.31
AS	88.15±0.97
ICC-P3	94.26±0.07
ICO-P3	98.79±0.10
ICC-AS	94.31±0.93
ICO-AS	60.48±14.19

[1] Relative percentage of TCE removal was calculated from concentration of TCE at day 21

$$\frac{\text{Conc. of TCE at day 0- Conc. of TCE at day 21}}{\text{Concentration of TCE at day 0}} \times 100$$

[2] Value are expressed as the mean and standard deviation of three replicates.

ICC= Immobilized cell on corncob, ICO= Immobilized cell on coir, P3= *Rhodococcus* sp. P3, and AS= Acclimatized activated sludge, Control= Without inoculum

4.2.2 Degradation of TCE by Immobilized *Rhodococcus sp.* P3 and Immobilized Acclimatized Activated Sludge on Corncob and Coir

This experiment examined the TCE degradation abilities of immobilized cells. Profiles of TCE degradation by immobilized *Rhodococcus* sp. P3 and immobilized acclimatized activated sludge on corncob and coir were depicted in Figure 4.1. After 3 weeks of incubation, TCE was degraded 94% and 98% by immobilized P3 on corncob (ICC-P3) and immobilized P3 on coir (ICO-P3), respectively, which were higher than the relative percentage of TCE removal by free cells of *Rhodococcus* sp. P3 (53%). Immobilized acclimatized activated sludge on corncob (ICC-AS) showed a higher relative percentage of TCE removal than free cells of acclimatized activated sludge i.e., 94% and 88%, respectively. However, a lower relative percentage of TCE removal was found in immobilized acclimatized activated sludge on coir (60%) when compared to free cells. Results implied that immobilization technique improved the degradation of TCE comparing to free cells. A porous structure of support materials enhancing adsorption capacity and diffusion of contaminant or substrate to the immobilized cells might be responsible for this trend (Omar et al., 1990; Labana et al., 2005). Similar results were reported by Omar et al. (1990) who found that immobilized *Candida parapsilosis* on granular clay degraded 90% of the high concentration of hydrocarbons in the oily sludge, while free cells degrade only 27.5% within 3 weeks. Porosity of granular clay possible adsorbed hydrocarbons on it thus it was convenient to be used by microorganisms. In addition, granular clay might act as barrier and against toxic compounds which protect cells from the harmful environment.

Survival of microorganisms in soil after immobilization was monitored by checking number of toluene degrading bacteria. Toluene degrading bacteria produce oxygenase which is a broad-substrate enzyme that could transform TCE into less toxic compounds. Growth of toluene degrading bacteria could be used to indicate the survival and the population of TCE degrader in soil (Mu and Scow, 1994). However, it's important to note that number of toluene degraders do not always represent number of TCE degraders. Indigenous TCE degrader in soil was found to be quite high with a cell number of 6.4×10^7 CFU/g soil (Table 4.3) at day 0. Soil used in this study was collected from the area nearby an abandoned site, contaminated with TCE and other organic solvents, which is a hill slope. Therefore, there is a possibility of

TCE going to our sampling site via a runoff during a rainy season, resulting in an adaptation of microorganisms to co-metabolize TCE.

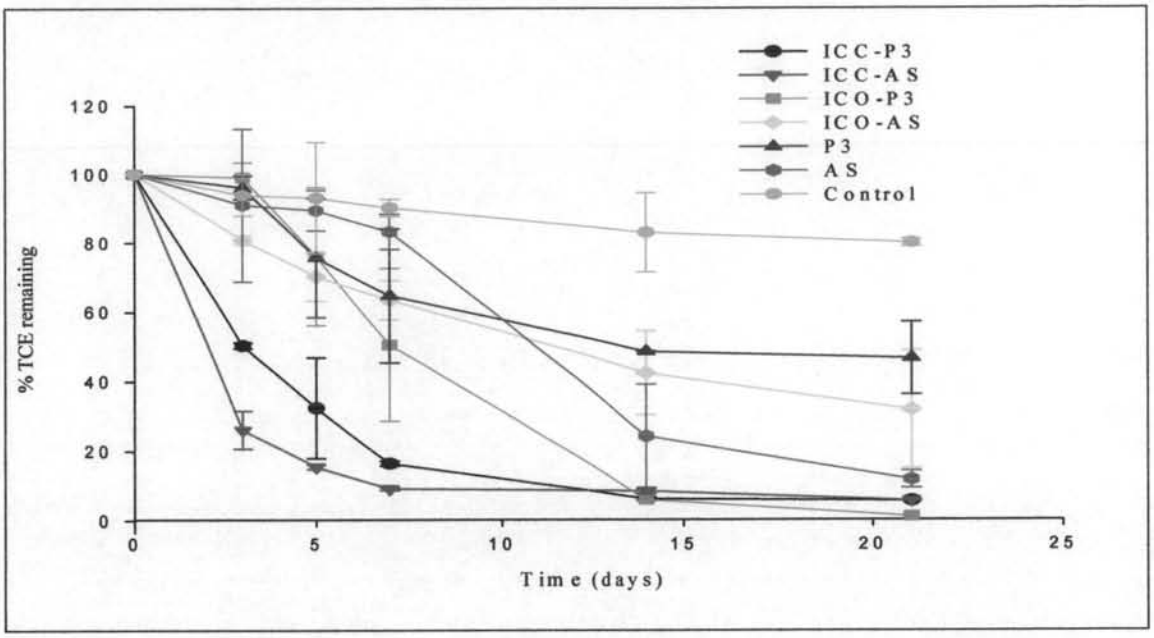

Figure 4.1 Degradation profiles of TCE in soil microcosms (ICC= Immobilized cell on corncob, ICO= Immobilized cell on coir, P3= *Rhodococcus* sp. P3, and AS= Acclimatized activated sludge, Control= Without inoculum)

Table 4.3 Cell number of free cell and immobilized cell forms of *Rhodococcus* sp. P3 and acclimatized activated sludge in soil

Time (days)	In soil ($x10^7$ CFU/g soil)						
	Control (without inoculum)	Free cell		Immobilized cell			
		P3	AS	P3		AS	
				Corncob	Coir	Corncob	Coir
0	6.40±1.04	5.77±2.12	7.57±2.14	5.43±3.00	8.30±1.23	2.48±1.97	5.67±1.96
3	1.43±0.24	11.50±5.45	5.59±2.65	20.80±2.75	20.70±9.62	25.20±2.52	9.25±3.75
5	1.73±0.86	23.90±2.65	32.00±1.41	56.90±3.81	3.70±0.10	24.30±3.06	9.67±2.19
7	8.03±3.69	51.70±3.41	13.50±4.87	153.0±21.50	9.00±3.46	25.80±2.55	11.10±1.33
14	5.27±1.01	7.30±0.42	8.20±1.13	31.60±9.12	5.70±2.75	29.00±2.55	11.60±1.42
21	6.73±3.47	5.53±0.75	5.23±1.27	38.00±1.41	7.30±1.91	75.0±18.7	15.60±4.61

Cell numbers of TCE degrader in soil, estimated by number of toluene degrading bacteria, were approximately 10^7-10^8 cells/g soil throughout 21 days of incubation. The cell numbers of TCE degraders in soils inoculated with immobilized *Rhodococcus* sp. P3 and immobilized acclimatized activated sludge on corncob and coir were higher than in soil inoculated with free cells at the end of incubation (Table 4.3). In addition, cell numbers in support material did not markedly decrease throughout the experiment (Table 4.4). These results suggested that there was a growth of immobilized cells on support materials, but the porous space in support materials was limited resulting in leakage of the cells from support materials into soil. Kumar and Das (2001) reported a similar finding in which the daughter cell produced by binary fission of the immobilized of *Enterobactor cloacae* IIT-BT 08 leaked to the culture media when there was no free space on the porous support materials. In this present work, we speculated that cells were immobilized on support materials by physical absorption due to electrostatic forces or by covalent binding between cell membrane and support materials which adsorbed cells and attached cells into their porouses. Thus, there were no barriers between cells and soil leading to a possibility of cell detachment and relocation (Kourkoutas et al., 2004). It's important to note that a scanning electron microscopy (SEM) should be conducted in order to verify this speculation. It's worth noting that advantages of immobilization by adsorption method are a simple in application and no chemical reagent was used (Iqbal and Saeed, 2005).

The efficacy of corncob and coir as support materials was evaluated. Results revealed that cell numbers in support materials did not decrease over incubation time when compared to the cell number at day 0 (Table 4.3) suggesting that immobilization enhanced the survival and stability of cells, hence both corncob and coir were suitable support materials to immobilize P3 and acclimatized activated sludge. Porosity of corncob and coir might provide air through the soil, thus prolong the survival and growth of bacteria (Omar et al., 1990). Moreover, the support materials might protect cells from predation and contaminant toxicity resulting in a better survival of immobilized cells than free cells (Elsas and Heijnen, 1990; Heijnen and van Veen, 1991).We observed that cell numbers in corncob was greater than in coir (Table 4.4). Corncob might serve as carbon source for microorganisms resulting in a better survival of immobilized cell on corncob than in immobilized cells on coir.

Since immobilized acclimatized activated sludge on corncob showed a high degradation of TCE as well as a high cell number of TCE degrader in soil microcosm and in support material, thus we further investigated the reusability of this immobilized cell.

Table 4.4 Cell number of immobilized cell forms of *Rhodococcus* sp. P3 and acclimatized activated sludge in soil in support materials

Time (days)	In support material ($x10^7$ CFU/g support material)			
	P3		AS	
	Corncob	Coir	Corncob	Coir
0	5.83±0.56	6.70±1.55	1.75±1.08	6.17±1.95
3	6.80±0.87	13.50±2.12	32.10±3.74	11.50±6.36
5	201.00±29.70	6.35±1.73	244.00±34.60	19.80±7.35
7	34.00±1.61	13.80±7.35	212.00±65.70	27.10±2.26
14	10.10±1.28	13.60±3.48	84.70±9.81	19.80±4.73
21	7.73±4.24	16.80±2.94	53.40±15.10	25.70±2.25

In order to confirm that immobilization technique is an appropriate delivery method of inoculum into soil microcosm, we examined a reusability of the immobilized cells. Results demonstrated that the immobilized cell could be reused one time, a total of two uses, with a remaining of TCE degradation ability similar to the first use (Figure 4.2) due to a soften structure of corncob after delignification. Therefore, we suggested that delignified corncob was not suitable for repetitive use. The relative percentage of TCE removal in the first use and the second use of immobilized cells at Day 21 of incubation were 94.31% and 94.96% (Figure 4.2), respectively, indicating an advantage of using immobilized cell over free cell. Number of TCE degraders in corncob decreased from $6.85x10^7$ to $3.3x\ 10^6$ CFU/g corncob after the first use (Table 4.5) which resulted from the leakage of the cells from support materials into soil. We observed that particle size of corncob was reduced from 0.5 cm to 0.3 cm after 21 days of incubation which might be resulted from a decomposition of this material by microorganisms in soil thus contributed to the leakage of the cells from corncob to soil. In addition, the cell number of TCE degrader in corncob was found to be decreased after day 7 while the cell number of

TCE degrader in soil increase (Table 4.5) confirming a possibility of cell leakage from corncob into soil.

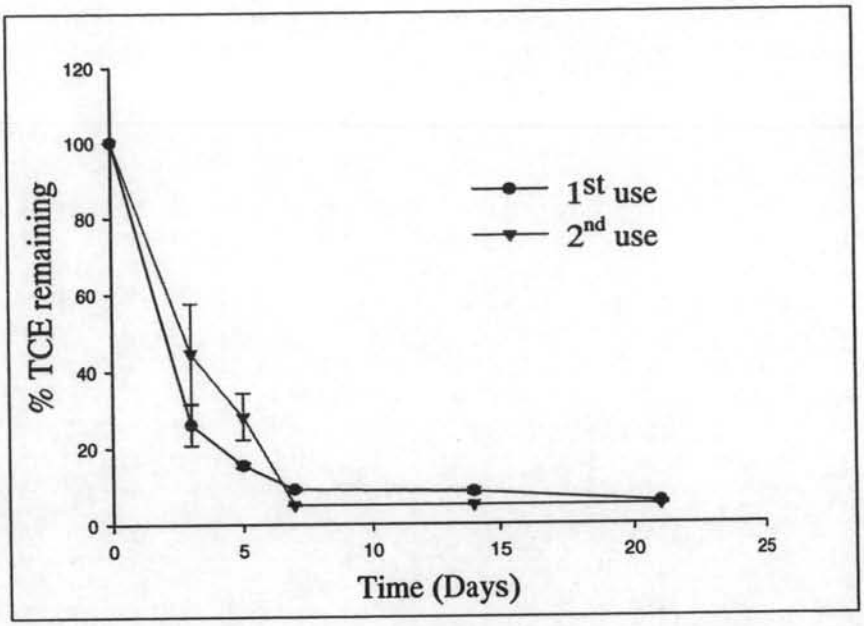

Figure 4.2 Degradation profile of TCE in soil microcosm augmented with reused immobilized acclimatized activated sludge on corncob

Table 4.5 Cell number of reused immobilized acclimatized activated sludge on corncob in soil and support material

Time (days)	Cell number of TCE degrader	
	In soil ($x10^7$ CFU/g soil)	In support material ($x10^7$ CFU/g support material)
0	6.50±1.27	6.85±1.06
3	2.63±0.38	8.30±0.85
5	11.80±2.76	10.05±0.99
7	41.00±1.13	1.23±0.16
14	37.80±1.41	0.52±0.10
21	12.20±3.32	0.33±0.13

4.3 Effect of Kaffir Lime Peel as a Potential Primary Substrate on TCE Degradation

This experiment investigated a feasibility of using kaffir lime peel as a primary substrate in aerobic degradation of TCE in soil as well as its optimal concentration. Relative percentages of TCE removal in soil microcosm at day 14 of incubation were shown in Table 4.6. Results indicated that kaffir lime peel added into the soil at a concentration of 50 mg/kg significantly induced indigenous microorganisms in soil to degrade TCE with the relative percentage TCE removal of 80.14% in comparison to soil without primary substrate (59.87%) (Table 4.6) ($P<0.05$). An addition of kaffir lime peel at a higher concentration of 100 to 250 mg/kg did not induce indigenous microorganisms to degrade TCE in which relative percentages of TCE removal (58.55 to 63.90%) were not significantly different from control ($P<0.05$) (Table 4.6). In addition, a low amount of kaffir lime peel added to soil microcosm could induce indigenous microorganisms to remove TCE better than high amount of kaffir lime peel (Table 4.6). High concentration of limonene in kaffir lime peel might be toxic to the indigenous and introduced microorganisms (Gilbert and Crowley, 1997). Results suggested that an optimal concentration of kaffir lime peel to induce TCE degradation by indigenous microorganisms in soil was at 50 mg/kg.

In comparison of the efficiency of kaffir lime peel to toluene which is a known primary substrate for inducing microorganisms to degrade TCE under aerobic condition, results indicated that TCE removal by indigenous microorganisms in soil induced by toluene at concentrations of 50 to 250 mg/kg were 52.02 to 67.42% (Table 4.6) which were lower than that in soil added with 50 mg/kg kaffir lime peel (Table 4.6) ($P<0.05$). Toluene could be used as c-source by microorganisms capable of co-metabolisms TCE by producing oxygenase enzyme which fortuitously transforming TCE to daughter compounds.

These findings suggested that kaffir lime peel at 50 mg/kg could be effectively used as primary substrate for inducing microorganisms to degrade TCE in soil and could replace the use of toluene which is considered as a toxic compound itself.

Table 4.6 Relative percentage of TCE removal in soil microcosm amended with various concentrations of kaffir lime peel after 14 days of incubation.

Treatment	Primary substrate concentration (mg/kg soil)	Relative percentage of TCE removal[1] [2]	
		Indigenous microorganisms	Immobilized acclimatized activated sludge on corncob
Sterile soil	None	52.71±2.13	
Kaffir lime peel as primary substrate	None	59.87±0.09	59.87±0.09
	50	80.14±9.60*[3]	88.82±4.65*
	100	58.55±17.09	85.52±0.61*
	150	63.90±4.91	79.43±2.63*
	250	63.12±0.68	76.26±6.85*
Toluene as primary substrate	None	59.87±0.09	59.87±0.09
	50	52.02±6.93	70.93±1.63
	100	65.02±3.40	92.34±0.17*
	150	67.42±0.61	91.47±2.37*
	250	50.52±2.13	60.69±3.63

[1] Relative percentage of TCE removal was calculated from concentration of TCE at day 14

$$\frac{\text{Conc. of TCE at day 0 - Conc. of TCE at day 14}}{\text{Concentration of TCE at day 0}} \times 100$$

[2] Value are expressed as the mean and standard deviation of three replicates.

[3] Comparisons between control (without amendment) and treatment in each block which * indicate significantly different at $P<0.05$ according to LSD test

It is important to note that there was about 53% of TCE removal in sterile soil (Table 4.6). This high percent loss of TCE might be resulted from the consequences of changes in sorptive properties of the soils caused by the autoclaving (Brigmon et al., 1998) or a volatilization of TCE from soil.

Kaffir lime peel consists of various terpene especially limonene which has a structure similar to aromatic hydrocarbons that can induce microorganisms to produce broad substrate enzymes i.e., oxygenase responsible for degrading xenobiotic pollutants (Gilbert and Crowley, 1997). Plant terpene extracts and plant materials consisting of terpenes were reported to be effectively used as primary substrate for enhancing the degradation of chlorinated hydrocarbons under aerobic condition. Advantages of the plant terpene extracts included non-priority pollutants and cost effective comparing to organic solvents which was commonly used (Harnandez et al., 1997). Limonene was used as a possible inducer for PCB biodegradation by *Pseudomonas stutzeri* (Tandlich et al., 2001). Results indicated that percentage of PCBs elimination were 30 to 88% and 35 to 92% in the presence of limonene at 10 and 20 mg/L, respectively. A component of spearmint, carvone, induced *Arthrobacter* sp. strain B1B to co-metabolize Aroclor 1242, resulting in a significant degradation of congeners about 15 % after 15 hrs (Gilbert and Crowley, 1997). In addition, several compounds structurally related to carvone, including limonene, *p*-cymene and isoprene could also induce co-metabolism of PCBs by *Arthrobacer* sp. strain B1B. Orange peel, eucalyptus, pine needles, and ivy leave directly added to the soil could induce microorganisms to completely degrade PCBs in soil within six months (Harnandez et al., 1997). Terpenes in these plants served as major substrates and were slowly released to the soil thus enhancing degradation of PCBs.

We further investigated the effect of various concentrations of kaffir lime peel on induction of microorganisms to degrade TCE in soil microcosm augmented with immobilized acclimatized activated sludge. As a concentration of kaffir lime peel increase, a TCE degradation by immobilized activated sludge decrease (Table 4.6). An optimal concentration of kaffir lime peel to induce TCE degradation in soil by immobilized acclimatized activated sludge was at 50 mg/kg with the highest relative percentage of TCE removal of 88.83% (Table 4.6). Augmentation of immobilized acclimatized activated sludge on corncob into soil increased percentage of TCE removal in comparison to indigenous microorganisms at all concentrations of kaffir lime peel (Table 4.6). Corncob might provide aeration in the soil for the prolonged

survival and growth of introduced microorganisms. Successful bioaugmentation with immobilized cell have been reported by Labana et al. (2005). They demonstrated that bioremediation of *p*-Nitrophenol (PNP) contaminated soil by augmenting with immobilized *Arthrobacter protophormiae* RKJ 100 on corncob powder resulting in complete removal of PNP within 5 days while indigenous microorganisms degraded PNP approximately 30 to 40% after 30 days.

Toluene at concentrations of 50 and 250 mg/kg soil was not able to induce indigenous microorganisms to degrade TCE in comparison to control (Table 4.6). However, there was a markedly effect of toluene on inducing immobilized acclimatized activated sludge to degrade TCE at all concentrations of toluene in comparison to indigenous microorganisms as shown in Table 4.6. Therefore, the bioaugmentation of immobilized acclimatized activated sludge could be achieved in soil microcosm when induced with toluene at concentration of 50 to 250 mg/kg soil. An addition of induced TCE-transforming bacteria could provide sufficient degradation for *in situ* remediation in the absence of indigenous TCE-degrading bacteria or oxygenase-inducing substrate (Marr et al., 1997). Success bioaugmentation of TCE was also reported by Marr et al. (1997). They investigated TCE biodegradation in small-column aquifer microcosms which repeatedly fed solutions containing 6.5 mg/L of phenol or 15 mg/L of lactate and 250 μg/L of TCE every 2 to 3 days and *Burkholderia cepacia* G4 or *Burkholderia cepacia* PR 1301 was used as inocula. Introduced microorganisms could remove TCE, approximately 120 μg/L, better than indigenous microorganisms, 100 μg/L, after 80 days. In contrast, Simon et al. (2004) found that the bioaugmentation with a large consortium of hydrocarbon degrading bacteria has failed to enhance the degradation of petroleum oil in soil because microbial population was not significantly change throughout the experiment. The augmented microorganisms introduced to the soil might compete to use the resources against indigenous microorganisms resulting in decrease survival of augmented microorganisms. In addition, the degradation products occurred during the degradation of pollutants might inhibit the growth and degradation ability of augmented microorganisms (Semprini and McCarty, 1992; Massol-Deya et al., 1997; Mars et al., 1998).

Results from this experiment indicated that an optimum concentration of kaffir lime peel and toluene as primary substrate were 50 mg/kg and 100 mg/kg, respectively. Therefore, these concentrations of kaffir lime peel and toluene were further used to investigate the effect of cassava pulp as organic amendment on TCE degradation.

4.4 Effect of Cassava Pulp as Organic Amendment on TCE Degradation

This study investigated the use of cassava pulp as organic amendment to stimulate indigenous microorganisms to degrade TCE. Kaffir lime peel at an optimal concentration of 50 mg/kg was used as primary substrate to induce indigenous microorganisms compared to toluene at an optimal concentration of 100 mg/kg. Results revealed that a degradation of TCE in soil amended with cassava pulp at all C:N ratios was significantly lower than in unamended soil (Table 4.7). An increase in C:N ratio resulted in a decrease in relative percentage of TCE removal. Relative percentages of TCE removal in soil amended with cassava pulp were in the range of 18.87 to 43.44% which was significantly lower than in unamended soil (80.14%) (Table 4.7) ($P<0.05$) suggesting that the addition of cassava pulp did not promote TCE degradation in soil when kaffir lime peel was used as primary substrate. Only Kaffir lime peel might be sufficient to induce microorganisms in soil to degrade TCE. Kaffir lime peel and cassava pulp contained carbon and nitrogen which could increase C:N ratio in the soil resulting in a decrease of TCE degradation due to an increase in sorption of TCE by kaffir lime peel and cassava pulp. Organic content in the soil can absorb the contaminant thus obstruct an attachment between microorganisms and contaminant in the soil resulting in a reduction of degradation efficiency of the contaminant by microorganisms in soil (Rahman et al., 2003). A negative effect of adding organic amendment was reported by Scelza et al. (2007). They found that degradation of phenanthrene in soil decreased from 30% to 20% when amount of compost added was increased from 0.27% to 0.83%, respectively, within 280 days. High concentrations of compost components e.g., heavy metals and salts might inhibit the indigenous and/or introduced soil microbial population as well as put the microbial population under stress.

Table 4.7 Effect of cassava pulp on stimulation of microorganisms

Treatments	Amendment	Relative percentage TCE removal[1] [2]	Microbial populations[3] (Log CFU/g soil)			Dehydrogenase activity[3] (mg INTF/kg dry soil/hr)
			TCE degrader	Bacteria	Fungi	
Kaffir lime peel 50 mg/kg	None	80.14±9.60	7.63±0.06	7.68±0.02	4.19±0.04	119.12±11.51
	20:1[4]	43.44±1.73*[5]	7.62±0.07	7.87±0.01	4.67±0.08	115.75±8.72
	30:1	30.32±14.99*	7.57±0.05	8.13±0.04*	4.59±0.04	106.66±9.04
	40:1	18.87±9.48*	7.70±0.03	7.78±0.04	4.57±0.04	77.23±2.60*
Toluene 100 mg/kg	None	65.02±3.40	7.66±0.18	8.14±0.18	4.38±0.05	111.64±0.13
	20:1	95.63±2.00*	8.61±0.01*	8.46±0.07*	4.07±0.31*	134.97±3.84*
	30:1	95.21±0.38*	8.46±0.07*	8.48±0.02*	4.29±0.08	104.09±2.97
	40:1	73.48±7.10	8.40±0.03*	8.44±0.22*	4.04±0.01*	78.09±4.56*
Without primary substrate	None	59.87±0.09	7.68±0.06	8.11±0.26	4.44±0.00	58.25±6.80
	20:1	50.00±1.99	8.20±0.01*	7.68±0.01*	4.19±0.13*	115.10±5.78*
	30:1	48.30±8.22	7.91±0.03*	7.66±0.04*	4.16±0.01*	113.58±2.48*
	40:1	49.25±12.3	7.64±0.12	7.64±0.03*	4.19±0.05*	105.05±6.30*

[1] Relative percentage of TCE removal was calculated from concentration of TCE at day 14

$$\frac{\text{Conc. of TCE at day 0- Conc. of TCE at day 14}}{\text{Concentration of TCE at day 0}} \times 100$$

[2] Value are expressed as the mean and standard deviation of three replicates

[3] Mean and standard deviation of 5 sampling times over 14 days

[4] C:N ratio

[5] Comparisons between control (without amendment) and treatment in each block which * indicate significantly different at $P<0.05$ according to LSD test

However, when 100 mg/kg of toluene was used as primary substrate in the microcosm, results showed that relative percentage of TCE removal in soil amended with cassava pulp were significantly higher than in unamended soil at C:N ratio of 20:1 and 30:1 (Table 4.7) ($P<0.05$). Microcosm amended with cassava pulp at C:N ratio of 40:1 removed 73.48% of TCE which was not significantly different from unamended soil (Table 4.7) ($P<0.05$), indicating that C:N ratio of 40:1 adjusted by cassava pulp was not suitable for stimulating TCE degradation.

A low relative percentage of TCE degradation of less than 60% (Table 4.7) was found in soil microcosm without primary substrate while a high relative percentage of TCE of 80 and 60% in microcosm were found in soil microcosms with kaffir lime peel and toluene, respectively. These results confirmed that TCE degradation in aerobic condition is a co-metabolism. A high percentage of TCE removal in microcosm without an inducer might be resulted from a volatilization of TCE from soil or sorption of TCE into soil.

Microbial populations i.e., TCE degrader, bacteria and fungi summarized as mean and standard deviation of 5 sampling times over 14 days were tabulated in Table 4.7. In the soil microcosms with kaffir lime peel as primary substrate, populations of TCE degrader, bacteria and fungi were not significantly affected by the addition of cassava pulp at all C:N ratios which indicated by the microbial population in soil amended with cassava pulp was not significantly different from control ($P<0.05$) (Table 4.7). However, the presence of toluene as primary substrate in soil microcosm resulted in significantly increase in the population of TCE degrader and bacteria amended with cassava pulp at C:N ratios of 20:1 and 30:1 in comparison to unamended soil ($P<0.05$) (Table 4.7) which coincided with a high TCE degradation in these microcosms. Number of fungal population was lower in cassava pulp amended soil than in control which suggested that fungi was not responsible for TCE degradation. As of our knowledge, the degradation of TCE by fungi has not been reported.

Dehydrogenase activity was investigated in order to evaluate the effects of cassava pulp on soil microbial activity. Results indicated that the addition of cassava pulp at the C:N ratio of 20:1 and 30:1 did not affect dehydrogenase activity in soil with the activity of 106 to 135 mg INTF/kg soil/hr, (Table 4.7). The inhibitory effects on dehydrogenase activity was evident when the soil was added with cassava pulp at the C:N ratio of 40:1 in soil microcosms added with kaffir lime peel and toluene as

primary substrates. The dehydrogenase activities of these treatments were 77 to 78 mg INTF/kg soil/hr which were significantly lower than in control (111 to 119 mg INTF/kg soil/hr) ($P<0.05$) (Table 4.7). These results were in contrast to a degradation of TCE in soil. Dehydrogenase activity represents the activity of all types of microorganisms in the soil. It did not represent only the TCE degrader in this study. It can be speculated that the added nutrients could have been inducing shifts in the metabolism of degrader, or favored the growth of other microorganisms by competing with the degraders for available nutrients thus contributed to a repression of target compound degradation (Johnson and Scow, 1999). Devinny and Chang (2000) reported similar results. They found that an increase in microbial activity was not followed by an increase in the number of degrading microorganisms and heterotrophic population. This negative relationship indicated that specialized microorganisms were adjusting to the changing substrate conditions, increasing their metabolic activity in a stressed environment, thus limiting the growth of the microbial population.

4.5 Effects of Biostimulation plus Bioaugmentation on TCE Degradation

The combination of biostimulation and bioaugmentation were investigated in this experiment. Immobilized acclimatized activated sludge on corncob were introduced as inoculum, kaffir lime peel was added as primary substrate and cassava pulp was added as organic amendment into soil microcosm. Relative percentage of TCE removal at all C:N ratios were in the range of 25.64 to 48.18% which were significantly lower than unamended soil which was 88.82% (Table 4.8) ($P<0.05$). Results indicated that the combination of adding immobilized acclimatized activated sludge and cassava pulp into the soil did not improve TCE degradation. Populations of TCE degrader and bacteria were not significantly affected by this amendment at all C:N ratios in comparison to unamended soil. However, population of fungi significantly increased in soil at C:N ratio of 30:1 and 40:1 (Table 4.8), while the TCE degradation did not increase indicating that fungi was not involved in TCE degradation in soil. Dehydrogenase activity decreased when C:N ratio increased which indicated that a high C:N ratio was not appropriate to stimulate microorganisms. In general, microbes have an average C:N:P ratio of about 10:1:0.1 within their biomass, depending on the type of microorganisms (Norris, 1994). In this study, C:N ratio might not be appropriate for the microorganisms requirement due to a

Table 4.8 Effect of combination between biostimulation and bioaugmentation on TCE degradation

Treatments	Amendment	Relative percentage of TCE removal[1] [2]	Microbial populations[3] (Log CFU/g soil)			Dehydrogenase activity[3] (mg INTF/kg dry soil/hr)
			TCE degrader	Bacteria	Fungi	
- Kaffir lime peel 50 mg/kg	None	88.82±4.65	7.73±0.09	7.84±0.09	4.00±0.11	131.05±0.09
	20:1[4]	30.66±3.44*[5]	7.72±0.08	7.87±0.02	4.14±0.01	122.51±6.84
	30:1	25.64±9.02*	7.62±0.01	7.95±0.04	4.31±0.16*	117.65±7.19*
	40:1	48.18±1.75*	7.61±0.05	8.04±0.02	4.40±0.00*	104.54±17.47*

[1] Relative percentage of TCE removal was calculated from concentration of TCE at day 14

$$\frac{\text{Conc. of TCE at day 0- Conc. of TCE at day 14} \times 100}{\text{Concentration of TCE at day 0}}$$

[2] Value are expressed as the mean and standard deviation of three replicates

[3] Mean and standard deviation of 5 sampling times over 14 days

[4] C:N ratio

[5] Comparisons between control (without amendment) and treatment in each block which * indicate significantly different at $P<0.05$ according to LSD test

high amount of carbon content in the amendments (20:1 to 40:1). Nitrogen and phosphorus were essential nutrients for microbial growth thus there should be considerations to add exogenous nitrogen and phosphorus into soil for achieving successful bioremediation. An evident of addition of N source leading to a successful biodegradation of hydrocarbon was reported by Juteau et al. (2003). They applied activated sludge as a nitrogen source in soil contaminated with hydrocarbon. This soil contained low C:N ratio of 100:2. Addition of 0.07% (w/w) sludge to the soil increased the nitrogen content up to 100:3 which stimulated degradation of alkanes and polycyclic aromatic hydrocarbons (PAHs) from 24 to 80% and 49 to 70%, respectively, in comparison to unamended soil. This can be explained by the fact that only carbon present in the soil can not be assimilated by microorganisms. Others essential nutrients are needed.