CHAPTER V

DISCUSSIONS

5.1 Screening, isolation and identification of 4-chloroaniline-degrading bacteria

Three 4-chloroaniline-degrading bacteria were all isolated from soil sample of tangerine groove with a history of herbicide exposure. The properties of agricultural soil could be briefly described as loam soil. Agricultural soil was the most suitable soil samples used for screening because it has been enriched with several herbicides. This statement agrees with Radianingtyas et al. (2003) who was also successful on isolating bacterial consortium degrading 4-chloroaniline from an Indonesian agricultural soil. In this study, the agricultural soil sample was enriched with 25 ppm 4-chloroaniline to increase potential for screening and isolation of 4-chloroaniline-degrading bacteria. Similary, Dejonghe et al. (2002) isolated chloroaniline degraders using soil that was spiked with 25 ppm 3-chloroaniline, while 25 ppm 3,4-dichloroaniline treated soil was enriched for 3,4-dichloroaniline degraders. Nonetheless, natural soil could also be a source for such bacteria as well, for example Helm and Reber, (1979) isolated *Pseudomonas multivorans* strain An1 which degraded aniline from a forest soil.

In this study, gram negative bacteria, 4CA-2, 4CA-16, 4CA-17, were isolated and identified as *Acinetobacter baumannii, Pseudomonas putida* and *Klebsiella pneumoniae*, respectively. 4-Chloroaniline-degrading bacteria could degrade 25 ppm (0.2 mM) 4-chloroaniline at 30°C without aniline as an inducer. They could use 4-chloroaniline alone as a growth substrate and an inducer for the degradation pathway. Then, 4-chloroaniline-

degrading enzymes of the isolates could be induced with 4-chloroaniline alone. The results of this study were different from the previous reports regarding the induction of 4-chloroaniline degradation of others gram negative bacteria. Helm and Reber (1979) isolated *Pseudomonas multivorans* which degraded 3- and 4- chloroaniline in the presence of 2 mM aniline and 8 mM glucose (0.1% each, w/v) at 28°C. *Pseudomonas multivorans* could use 3- and 4- chloroaniline via cometabolism and could use aniline as inducer for monochloroaniline degradation. Zeyer and Kearney (1982) previously isolated *Moraxella* sp. strain G that was the first isolate that could use 4-chloroaniline (2.5 mM) as a sole source of carbon and nitrogen. However, to isolate *Moraxella* sp. strain G, 2 mM aniline was used as an inducer and stepwise replaced by 4-chloroaniline after a few weeks. The microorganism finally grew on 4-chloroaniline as a sole source of carbon and nitrogen. According to Zeyer and Kearney (1982) and studied so far, it was necessary to use aniline to induce chloroaniline degradation including 4-chloroaniline degradation during screening and isolation steps. For instance, the similar induction condition was reported by Helm et al. (1979) who isolated *Pseudomonas multivorans* strain An 1 using 2mM chloroaniline with 2 mM aniline and Surovtseva et al., (1980) who isolated *Alcaligenes faecalis* using 2 mM aniline In all cases, aniline was necessary as an inducer for 4-chloroaniline-degrading enzymes. Interestingly, the result from this study showed that 4-chloroaniline was able to act as a sole inducer and as a substrate for 4-chloroaniline degradation.

Although in this study, three isolated were gram negative bacteria, i.e. *Acinetobacter baumannii, Pseudomonas putida, Klebsiella pneumoniae*, other gram negative and gram positive bacteria have been reported as chloroaniline-metabolizing

strains, for example, *Pseudomonas* sp. strain JL2 degrading 4-chloroaniline (Latorre et al., 1984), *Brevundimonas diminuta* INMI KS-7 degrading 4-chloroaniline (Surovtseva et al., 1985), *Delftia acidovorans* CA28 degrading monochloroaniline (Loidl et al., 1990), *Comamonas testosteroni* I2 degrading 3-chloroaniline (Boon et al., 2000), and *Paracoccus denitrificans* 3CA degrading 3-chloroaniline (Surovtseva et al., 1996). *Rhodococcus* sp. An117 degraded 2- and 3-chloroaniline (1 mM) in the presence of glucose (500 mg/L) could grow on monochloroanilines via cometabolisms as the only source of nitrogen (Emtiazi *et al.*, 2001). Besides bacteria which can degrade toxic substance and fungi can also degrade monochloroaniline via cometabolisms (Schukat et al., 1983; Emtiazi et al., 2000). The result from previous report showed that *Rhodococcus* sp. An117, *Fusarium* sp. and *Rhizopus* sp. could not use chloroaniline alone as sole source of carbon and nitrogen.

Three 4-chloroaniline-degrading bacteria designated 4CA-2, 4CA-16 and 4CA-17 were identified with biochemical characterization using Manual of Clinical Microbiology (Murray *et al.*, 2003) and 16S rDNA sequence comparison as *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16), and *Klebsiella pneumoniae* (4CA-17), respectively.

Acinetobacter baumannii (4CA-2) and *Pseudomonas putida* (4CA-16) have been previously reported to involve in biodegradation of xenobic. For example, *Acinetobacter* strain was isolated as phenol, benzoate, crude oil, acetonitrile and in the removal of phosphate or heavy metals (Abdel-El-Haleem, 2003). Adaptation of *Acinetobacter calcoaceticus* was occurred when cells were grown at concentrations of aniline greater than 16 mM (Wyndham, 1986). Similarly *Pseudomonas acidovorans* CA28 was reported

that it degraded aniline and 3-chloroaniline. (Hinteregger, 1992). Previous report, *Klebsiella pneumoniae* subsp. *ozaenae* could completely convert 0.05% bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid and use the liberated ammonia as a soie nitrogen source. Interestingly, this is the first report on *Klebsiella pneumoniae* to be involved in halo aromatic degradation (Mcbride, et al., 1986).

5.2 Biodegradation of 4-chloroaniline by 4-chloroaniline-degrading bacteria

5.2.1 Degradation kinetics of 4-chloroaniline

Preliminary screening demonstrated that these three bacterial isolates (*Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16) and *Klebsiella pneumoniae* (4CA-17)) were able to grow on 25 ppm (0.2 mM) 4-chloroaniline containing mineral medium agar. They were then examined for their degradation kinetics toward 4-chloroaniline. The degradation kinetic protocol and calculation were clearly described in Chapter 3 and Appendix C, respectively. *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16) and *Klebsiella pneumoniae* (4CA-17) were grown in 25 ppm (0.2mM) 4-chloroaniline and their initial specific degradation rates calculated within the first 4 days were 8.70 ± 1.60, 13.60 ± 2.50 , and 19.00 ± 2.30 n mol(min.mg protein)$^{-1}$, respectively. Although the specific degradation rates of *Klebsiella pneumoniae* (4CA-17) was significantly higher than those of the two isolates within the first 4 days of degradation, it was found that the total 4-chloroaniline degradations after 12 days of incubation of all three isolates were comparatively similar, i.e. 61.00% ± 1.68, 59.82% ± 0.71, 62.82% ± 3.87, respectively. The possibility of incomplete 4-chloroaniline degradation (approximate 60%

degradation) suggested that further degradation of 4-chlorocatechol may be the rate-limiting step in the metabolism of 4-chloroaniline. In addition, the result of non-enzymic conversed 4-chloroaniline, or 4-chlorocatechol, to various products (Parris, 1980). For example, redox oxidation product could result in oxidative coupling of chloroanilines (Bachofer et al., 1975). This result showed that the isolates degraded 25 ppm (0.2mM) 4-chloroaniline as sole source of carbon and nitrogen and could induce 4-chloroaniline-degradation enzyme without aniline. On the other hand, previous result of Zeyer (1982) also showed that *Moraxella* sp strain G could degrade 2.5 mM 4-chloroaniline by 87.5% for 10 days. However, it was necessary to induce the degradation pathway using 2 mM aniline in the isolation step as it was found that aniline is the main inducer for 4-chloroaniline-degrading enzymes.

Theoretically, if chloroaniline was utilized through the *meta*-cleavage pathway, the product was changed to distinctly greenish yellow. In this study, the color of culture medium was not changed to distinctly greenish yellow, suggesting that 2-hydroxymuconic semialdehyde, an intermediate of *meta*-cleavage pathway was not occurred (Surovtseva *et al.*, 1980; Zeyer and Kearney, 1982). In the contrary, the previous finding revealed that filtrate culture containing 4-chloroaniline provided distinctly greenish yellow at pH 6.0 by *Moraxella* sp. strain G (Zeyer and Kearney, 1982). Furthermore, 4-chlorobenzoate could be used as substrate through *meta*-cleavage pathway by *Pseudomnas* sp. In this study, the possible result could be explained that 4-chloroaniline could not be degraded via *meta*- cleavage pathway.

The degradation kinetic was determined to evaluate substrate utilization or product formation. Product formation of 4-chloroaniline degradation was determined by

using the accumulation of chloride which could prove the ability of isolates whether or not it accomplished the degradation of 4-chloroaniline. The dechlorination was occurred via modified *ortho-* or *meta-* pathway of 4-chloroaniline degradation (Radianingtyas *et al.*, 2003). In this investigation, the total degradation of 25 ppm (0.2 mM) 4-chloroaniline was occurred at 61.00% ± 1.68, 59.82% ± 0.71, 62.82% ± 3.87 which accounted for 0.14 *m*M 4-chloroaniline degraded in each bacterium. The accumulated chloride was apparently detected at 0.14 mM, 0.13 mM, 0.14 mM using Ion-Selective electrode (ISE) and detected at 0.14 mM ± 0.01, 0.13 mM ± 0.02, 0.15 mM ± 0.01 using colorimetric procedure as described in Methodology (3.6.2) (Bergmann and Sanik, 1957). The results of both methods for chlorine determination were similar suggesting that the ratio of 4-chloroaniline to chloride formation was occurred at 1:1 and confirming that 4-chloroaniline was utilized by the three organisms. A similar observation was reported by Zeyer and Kearney (1982) who found that a complete chloride removal in *Moraxella* sp. strain G. Complete dechlorination of 2.5 mM 4-chloroaniline degradation in sp. strain G. was also occurred with the accumulation of 2.4 mM chloride.However Incomplete dechlorination was observed by Radianingtyas et al., (2003) where the ratio of chloroaniline degradation to chloride formation was 1: 0.25 (25% chloride formation). There were two possibilities for incomplete dechlorination. First, it could be due to the minor amounts formation of 5-chloro-2-hydroxymuconic acid semialdehyde, a meta-cleavage product of 4-chlorocatechol by the action of a catechol 2,3 dioxygenase, which gave a distinctive greenish yellow colour to the bacterial culture growing on 4-chloroaniline. The second possibility was the accumulation of 4-chlorocatechol, which inhibited further degradation of 4-chloroaniline. Then, 4-chloroaniline degradation

pathway of the isolates could not be degraded via meta-pathway in which the distinctive greenish yellow colour of 5-chloro-2-hydroxymuconic acid.

5.2.2 Determination of enzymes involving 4-chloroaniline degradation

The 4-chloroaniline degradation pathway was investigated using enzyme determination isolated from *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16) and *Klebsiella pneumoniae* (4CA-17) grown in the presence of 4-chloroaniline. The activity of enzymes, i.e. catechol 1,2 dioxygenase, catechol 2,3 dioxygenase and chlorocatechol 1,2 dioxygenase were determined in crude extract prepared from 4-chloroaniline grown cells and compared with these in LB grown cells. The induction of enzyme(s) responsible for degradation would clarify the degradation pathway whether it is *ortho*-cleavage pathway (Fig 2.1, Chapter2), or *meta*-cleavage pathway (Fig 2.3, chapter2 and Fig 5.1, chapter5) or modified *ortho*-cleavage pathway (Fig 2.1-2.2. Chapter2 and Fig 5.1, chapter5).

Catechol 1,2 dioxygenase is the enzyme in the *ortho*-cleavage pathways that frequently found in benzoate-, phenol-, or aniline-degrading microorganisms (Dorn and Knackmuss, 1978a and 1978b). The ability of the isolates to degrade chlorinated anilines as sole source of carbon and nitrogen derived principally from two properties, namely its broad-specificity aniline oxygenase and its modified *ortho*-cleavage pathways. A lack of one or both of these properties would be a plausible reason why several investigators failed to obtain growth of microorganisms on chlorinated anilines (Walker and Harris, 1969). Modified *ortho*-cleavage pathway detected chlorocatechol 1,2-dioxygenase which was usually involved in the degradation of chlorinated aromatics (Harwood and Parales,

1996). Catechol 2,3 dioxygenase is the enzyme in the *meta*-cleavage pathways encoded by the *xylE* gene on the TOL catabolic plasmid (Nakai, 1983). The substrate range of catechol 2,3-dioxygenase is relatively broad as this enzyme oxidizes 3-methyl-, 3-ethyl-, 4-methyl-, and 4-chlorocatechol. 3-Chloro- and 4-ethylcatechol, in contrast, are not efficiently oxidized by this enzyme (Nozaki, 1979).

In the enzymatic study of *Acinetobacter baumannii* (4CA-2), the activities of the enzyme of the modified *ortho*-cleavage pathway was high, with a maximal activity of 191.8 ± 47.5 nmole(min.mg protein)$^{-1}$ for the chlorocatechol 1,2-dioxygenase in cell free extracts of cells which was grown on a mineral medium containing 0.1% yeast extract and 25 ppm (0.2 mM) 4-chloroaniline for 2 days. The activities of the enzyme of the *ortho*-cleavage pathway was also expressed 50.6 ± 17.5 nmole(min.mg protein)$^{-1}$ for the catechol 1,2-dioxygenase in cell free extracts of cells which was grown on a mineral medium containing 0.1% yeast extract and 25 ppm (0.2 mM) 4-chloroaniline for 2 days. A. *baumannii* (4CA-2) exhibited 4-fold chlorocatechol 1,2-dioxygenase specific activity higher than that of catechol 1,2-dioxygenase specific activity. Not surprisingly, activity of the enzyme of the *meta*- cleavage pathway, catechol 2,3 dioxygenase, which was usually involved methylated aromatic degradation (Assinder and Williams, 1990) was insignificantly observed.

In the enzymatic study of *Pseudomonas putida* (4CA-16), the activities of the enzyme of the modified *ortho*-cleavage pathway was high, with a maximal activity of 205.8 ± 68.6 nmole(min.mg protein)$^{-1}$ for the chlorocatechol 1,2-dioxygenase in cell free extracts of cells which was grown on a mineral medium containing 0.1% yeast extract and 25 ppm (0.2 mM) 4-chloroaniline for 2 days. The activities of the enzyme of the *ortho*-cleavage pathway was also expressed 33.8 ± 14.6 nmole(min.mg protein)$^{-1}$ for the catechol 1,2-

dioxygenase in cell free extracts of cells which was grown on a mineral medium containing 0.1% yeast extract and 25 ppm (0.2 mM) 4-chloroaniline) for 2 days. *P. putida* (4CA-16) exhibited 6-fold chlorocatechol 1,2-dioxygenase higher than that of catechol 1,2-dioxygenase. Catechol 2,3 dioxygenase was insignificantly observed.

In the enzymatic study of *Klebsiella planticola* (4CA-17), the activities of the enzyme of the modified *ortho*-cleavage pathway was highest when was compared in all isolates. A maximal activity of 223.4 ± 35.7 nmole(min.mg protein)$^{-1}$ for the chlorocatechol 1,2-dioxygenase in cell free extracts of cells which was grown on a mineral medium containing 0.1% yeast extract and 25 ppm (0.2 mM) 4-chloroaniline for 2 days. The activities of the enzyme of the *ortho*-cleavage pathway was also expressed 41.2 ± 14.3 nmole(min.mg protein)$^{-1}$ for the catechol 1,2-dioxygenase in cell free extracts of cells which was grown on a mineral medium containing 0.1% yeast extract and 25 ppm (0.2 mM) 4-chloroaniline for 2 days. *K. pneumoniae* (4CA-17) exhibited 5-fold chlorocatechol 1,2-dioxygenase specific activity higher that that of catechol 1,2-dioxygenase. Catechol 2,3 dioxygenase activity was insignificantly observed.

It can be concluded from their inducible activities of chlorocatechol 1,2-dioxygenase for all of the isolates that they degraded 4-chloroaniline *via* a modified-*ortho* cleavage pathway. Catechol 2,3 dioxygenase was insignificantly observed. *Pseudomonas putida* GJ31 (Mars et al., 1997) probably also possessed an *ortho*-cleavage pathways for the catechol degradation, since some activities of enzymes of this route were found in crude extracts of cell grown in benzene or benzoate. However, this combination of a *meta*- and *ortho*- cleavage pathway had been observed in *Pseudomonas putida* mt-2. In this strain, the genes of *meta*-cleavage pathway were located on TOL plasmid, while the

ortho- cleavage pathway was encoded on the chromosome (Assinder and Williams, 1990). *Pseudomonas putida* GJ31 also possessed a large plasmid, and mutants of this strain which was unable to grow on chlorobenzene and toluene but could stiil grow on benzoate and benzene that was isolated. This phenotype could be explained by the loss of *meta*-cleavage pathway genes which was possibly encoded on the plasmid (Mars et al., 1997). In this study, chlorocatechol 1,2-dioxygenase were an inducible system using 4-chloroaniline as an inducer. The synthesis of the enzymes for catabolism of an aromatic substrate generally requires the presence of an inducer, which is the aromatic substrate itself (Worsey et al., 1978). On the other hand, luria bertani medium (LB) affected on the express of induction 4-chloroaniline-degrading enzyme. Specific activity of the isolate cultured on luria bertani medium supplemented with 4-chloroaniline was comparatively similar in the absence of 4-chloroaniline.

Identification of the intermediate of 4-chloroaniline degradation was determined. 4-Chlorocatechol was reported to be the intermediate in both modified *ortho*-cleavage pathway and meta- cleavage pathway as shown in Figure 5.1 (Surovtseva et al., 1980; Zeyer et al., 1985). In this study, although HPLC chromatogram results showed the reduction of the 4-chloroaniline peak, the accumulation of the intermediate products peak was not observed. The possibility might be that the unidentified compounds were transformed product into 4-chlorocatechol or non-enzymic oxidation products of 4-chlorocatechol (Parris, 1980). An example of the latter case was reported by Zeyer and Kearney (1982) who found 4,4′-dichloroazobenzene in the culture filtrate of *Moraxella* sp. strain G grown on 4-chloroaniline. Although mutants of *Moraxella* sp. strain G which

lacked chlorocatechol 1,2-dioxgenase, 50% of the radioactivity as [^{14}C]4-chlorocatechol was accumulated as [^{14}C]4-chlorocatechol (Zeyer et al., 1985).

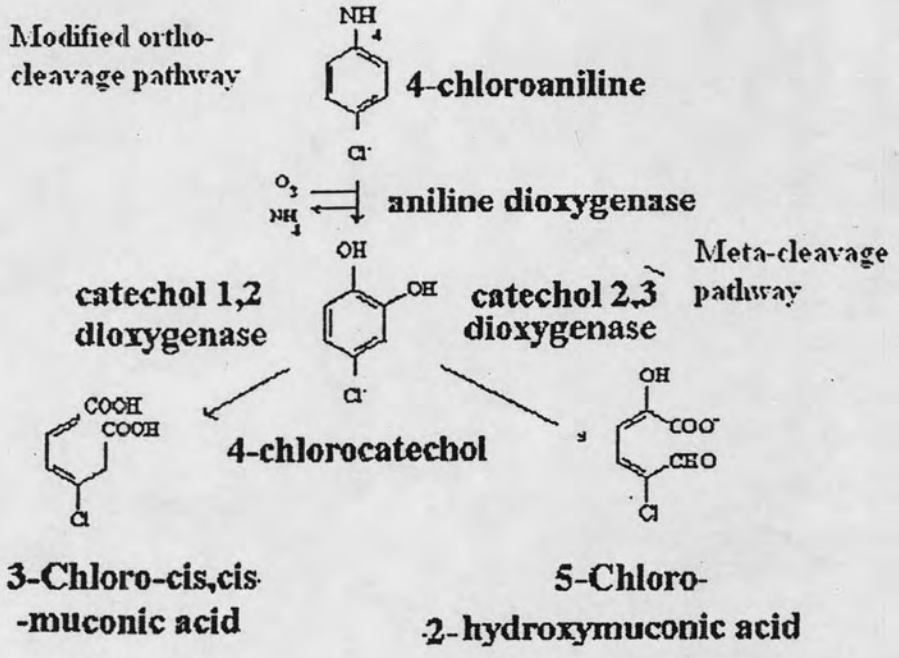

Figure 5.1 The major pathway of monochloroaniline degradation

(Surovtseva *et al.*, 1980; Zeyer *et al.*, 1985)

5.3 Role of additional carbon and/or nitrogen source for 4-chloroaniline degradation

Additional carbon and/or nitrogen source in the presence of 4-chloroaniline were optimized in order to increase the degradation rate and/or improve cell growth as well as cell degradability. For *Acinetobacter baumannii* (4CA-2), additional carbon source (4 mM, 8 mM and 18 mM citrate) or additional nitrogen source (4 mM, 8*m*M and 18 NH_4Cl) in the absence of 25 ppm (0.2 mM) 4-chloroaniline were not enough

to support the cell growth. This result was similar to that of Zeyer and Kearney (1982) who found that growth was not observed on a basal medium containing only 0.1% (37 mM) succinate without (6 mM) NH_4NO_3 in *Moraxella* sp. strain G. In this study, in the absence of 4-chloroaniline, *Acinetobacter baumannii* (4CA-2) could grow in both of carbon and nitrogen source (citrate and NH_4Cl) for cell growth. The concentration at 8 and 18 mM citrate were toxic with cell growth. Then the concentration at 8 mM, 18 mM citrate and the combination of 8 mM citrate + 8 mM NH_4Cl, 18 mM citrate + 18 mM NH_4Cl affected on growth due to citrate concentration. The possible reason could be explained as the influence of high concentration of citrate (8 mM and 18 mM) which also affected on cell growth when citrate combined with NH_4Cl at concentration 8 mM and 18 mM. In this study, the presence of NH_4Cl was not affected on cell growth or degradation. The result indicated that 4-chloroaniline was not subject to nitrogen repression of *Acinetobacter baumannii* (4CA-2). The result was similar to that of Zeyer *et al.*, (1985) who found that the induction of 4-chloroaniline degradation was similar to that in the presence of 10 mM ammonium or nitrate as additional nitrogen sources of *Moraxella* sp. strain G. In this study, the suitable carbon and/or nitrogen sources were 4 mM citrate which increased growth rate from 0.086 to 0.088 (h^{-1}), total degradation from 61% to 64%, specific degradation rate from 8.7 to 9.5 nmole(min.mg.protein)$^{-1}$ and 4 mM NH_4Cl which increased growth rate from 0.086 to 0.088 (h^{-1}), total degradation from 61% to 68%. The result corresponded with that of Dinkla and Janssen (2003) who isolated *Pseudomonas putida* mt2 and *Pseudomonas putida* WCS358 (TOL) which grew on 10 mM toluene where the addition of 5 mM citrate increased specific growth rates.

Carbon source (citrate and succinate) could be supported growth and energy (Reece, 2005) in the absence of 25 ppm (0.2 mM) 4-chloroaniline for a few day of incubation for *Pseudomonas putida* (4CA-16) after that growth curve droped. Citrate and succinate at 8 and 18 mM decreased growth rate, total degradation and specific degradation. This indicated that high concentration of additional carbon source repressed 4-chloroaniline degradation. In this study, the suitable carbon sources were 4 mM citrate which increased growth rate from 0.057 to 0.065 (h^{-1}), total degradation from 59.82% to 68.24%, specific degradation rate from 13.6 to 14.2 nmole(min.mg.protein)$^{-1}$, and 4 mM succinate which growth rate from 0.057 to 0.065 (h^{-1}), total degradation from 59.82% to 68.31%, specific degradation rate from 13.6 to 16.14 nmole(min.mg.protein)$^{-1}$rate. The result corresponded with that of Daugherty and Karel (1994) who isolated *Pseudomonas cepacia* DBO1(pRO101) which grew on 2,4-dichlorophenoxyacetic acid addition of (2 g/L) succinate which increased the cell density as well as the percentage of 2,4-dichlorophenoxyacetic acid by 95%.

In the presence of 25 ppm (0.2mM) 4-chloroaniline, additional carbon and nitrogen source as 1 mM aniline increased growth rate for 0.011 (h^{-1}), total degradation for 5.56% and specific degradation for 2.11 nmole(min.mg.protein)$^{-1}$ of *Klebsiella pneuminiae* (4CA-17). The concentration at 2 mM aniline was also increased total degradation by 4.37%. At 1 mM aniline was used as the additional carbon source to stimulated 3-chloroaniline degradation in *Rhodococcus* sp. An 117 (Schukat *et al.*, 1983). Aniline as an additional carbon and nitrogen source had ability to increase degradation and growth in *Klebsiella pneuminiae* (4CA-17). The positive effect of aniline

corresponded to that reported by Surovtseva et al. (1980) who found that the presence of aniline induced the 4-chloroaniline degradation in *Alcaligenes faecalis*.

The suitable conditions in each bacterium affected on the increase of the total degradation, the bacterial growth and the specific degradation rate for three isolates. In this study, the result showed that at high concentration of additional carbon or/and nitrogen source affected on the decrease of total degradation of 4-chloroaniline. Subsequently, the simultaneous utilization of 4-chloroaniline and other organic substrates by three isolates had significance in the biodegradation of 4-chloroaniline in the environments. If the organic substrates were present in high concentration, they could repress 4-chloroaniline degradation. Growth of the suitable conditions in each bacterium was not observed as diauxic growth. Diauxic growth was observed in two substrates when there were saturating concentrations of a good substrate; that was, one which supported a faster growth rate than the second substrate (Harder and Dijkhuizen, 1982). Furthermore, additional carbon or/and nitrogen source and 4-chloroaniline were used simultaneously; this result was similar to that of Konopka et al., (1989) who found that the mixed substrate culture on (16 mM) lactate plus a low concentration of aniline (1 mM) were not observed as diauxic growth. On the contrary, *Pseudomonas multivorans* An 1 always showed diauxic growth curves when. The dual substrate media containing aniline and various additional carbon sources at 28°C. Growth on aniline had a lag phase of nearly 10 h, whereas growth on other carbon sources started much earlier. These result suggested that, additional substrates (glutamate, succinate, pyruvate), was added the synthesis of the aniline catabolizing enzymes were completely repressed (Helm and Reber, 1979).

5.4 Effect of 4-chloroaniline concentration on growth and biodegradability of 4-chloroaniline-degrading bacteria

This experiment determined if three 4-chloroaniline-degrading bacteria were able to tolerate and to degrade 4-chloroaniline at higher concentrations in the range of 25 ppm (0.2 mM), 50 ppm (0.4 mM), 100 ppm (0.8 *m*M mM), 150 ppm (1.2 mM) and 200 (1.6 mM). All of the isolates showed the maximum total degradation of 4-chloroaniline at the concentration 25 ppm (0.2 mM). The increasing of 4-chloroaniline concentration decreased percentage of total degradation and growth rate suggesting that the toxicity of 4-chloroaniline might be affected on the activities of chlorocatechol 1,2-dioxygenase. The ability of each bacterium to degrade 4-chloroaniline at various concentrations showed in Fig 5.2, Fig 5.3 and Fig 5.4 of *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16) and *Klebsiella pneumoniae* (4CA-17), respectively. The result exhibited that 4-chloroaniline at 200 ppm (1.6 mM) inhibited growth and total degradation of all isolates. Previous report showed that the concentration of 4 mM 4-chloroaniline, growth of *Moraxella* sp. strain G was totally inhibited (Zeyer and Kearney, 1982). The medium of Zeyer and Kearney (1982) was adjusted pH 6.0. Subsequently, the specific degradation rate of *Moraxella* sp. Strain G at 3 mM 4-chloroaniline was 9.3 μmol $(min.g.protein)^{-1}$. The specific degradation rate of *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16) and *Klebsiella pneumoniae* (4CA-17) was 8.70 ± 1.60, 13.60 ± 2.50 and 19.00 ± 2.30 nm ol $(min.mg.protein)^{-1}$, respectively at 1.2 mM 4-chloroaniline which was the maximum concentration by the isolates could be survived. Therefore, pH of the medium might be one of the factors influence on growth and degradation of the organism in that pH might have an effect on the 4-chloroaniline-

degrading enzyme. The result of Radianingtyas et al. (2003) who found that bacterium consortium was able to degrade 4-chloroaniline in the presence of aniline, which was essential for bacterial growth. At 3 mM of 4-chloroaniline, it inhibited the bacterial growth. In this study, not only that aniline was not used as an inducer for 4-chloroaniline degradation, but pH adjustment was not determined, therefore this might be the reason that the bacterial isolates were able to cope with 4-chloroaniline at 1.2 mM.

The specific degradation rate within 4 days noticeably increased when 4-chloroaniline concentration was increased. Contrarily, growth rate and total degradation of all isolates decreased when 4-chloroaniline concentration also increased. The effect of 4-chloroaniline concentration on growth and total degradation of the isolates exhibited in Fig. 5.2-5.4

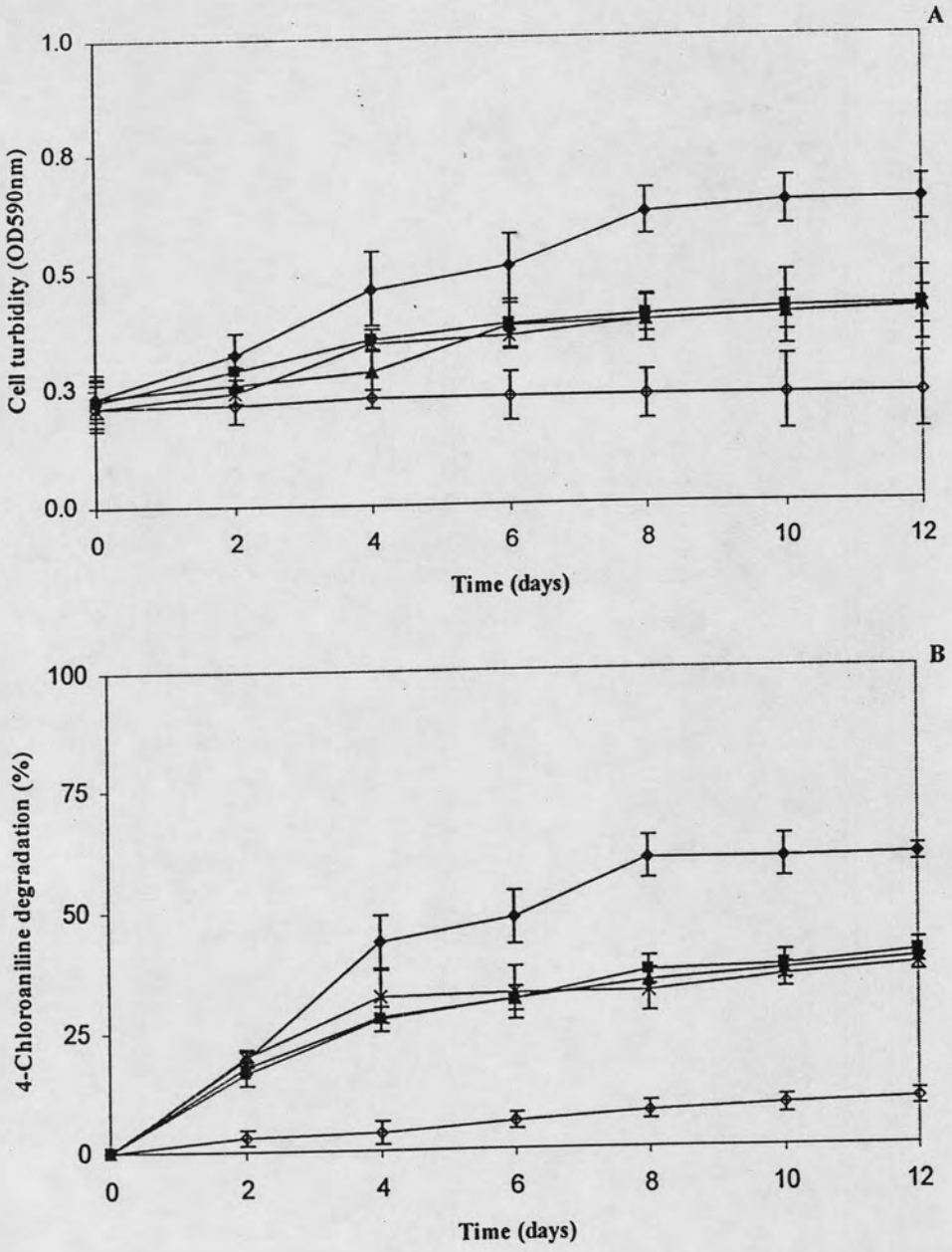

Fig 5.2 Effect of 4-chloroaniline concentration of *Acinetobacter baumannii* (4CA-2) on (A) growth and (B) total degradation in mineral medium supplemented with 25 ppm 0.2 mM (—◆—), 50 ppm (0.4 mM) (—■—), 100 ppm (0.8 mM) (—▲—), 150 ppm (1.2 mM) (—×—) and 200 ppm (1.6 mM) (—◇—). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

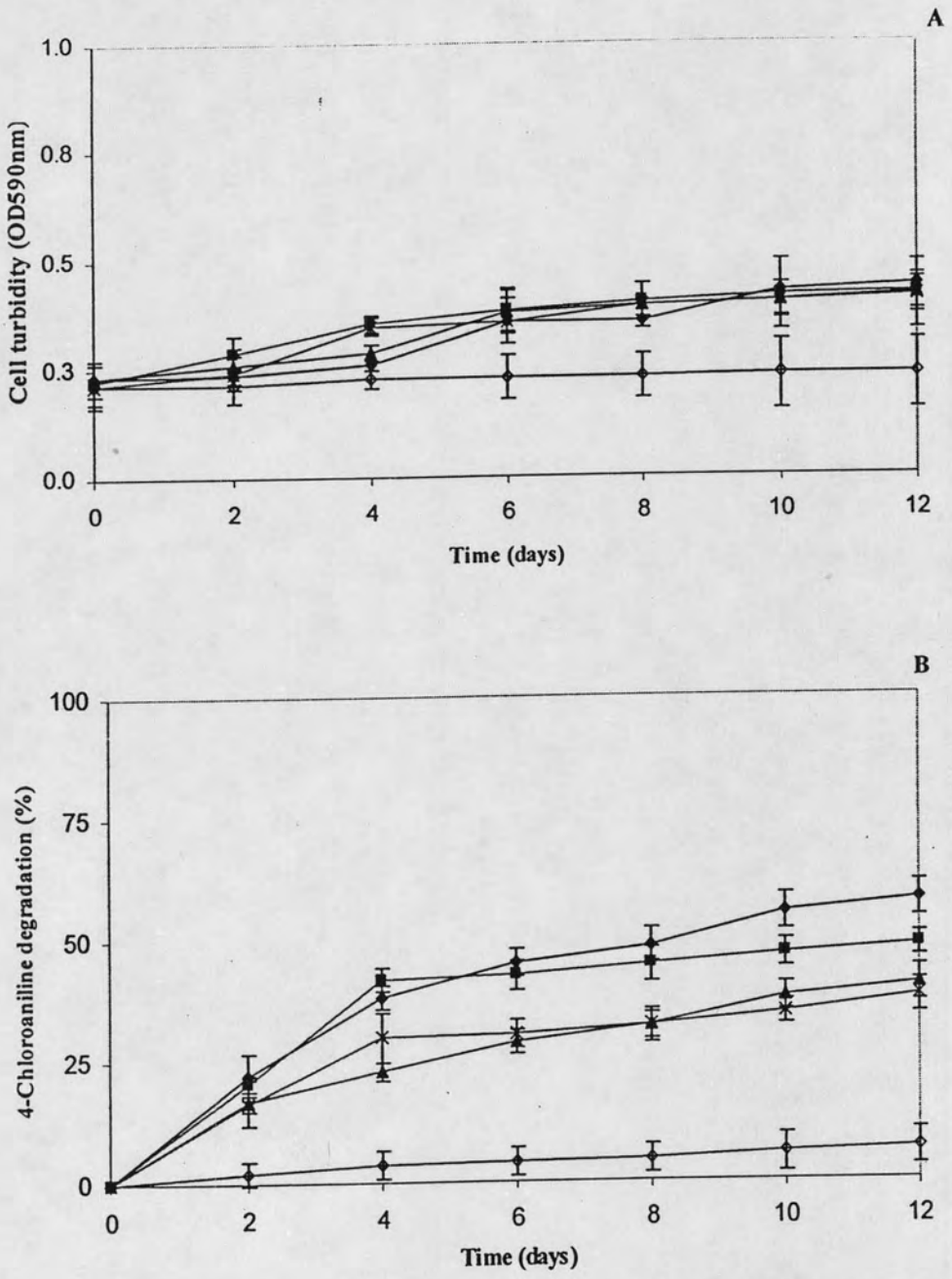

Fig 5.3 Effect of 4-chloroaniline concentration of *Pseudomonas putida* (4CA-16) on (A) growth and (B) total degradation in mineral medium supplemented with 25 ppm 0.2 mM (—◆—), 50 ppm (0.4 mM) (—■—), 100 ppm (0.8 mM) (—▲—), 150 ppm (1.2 mM) (—×—) and 200 ppm (1.6 mM) (—◇—). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

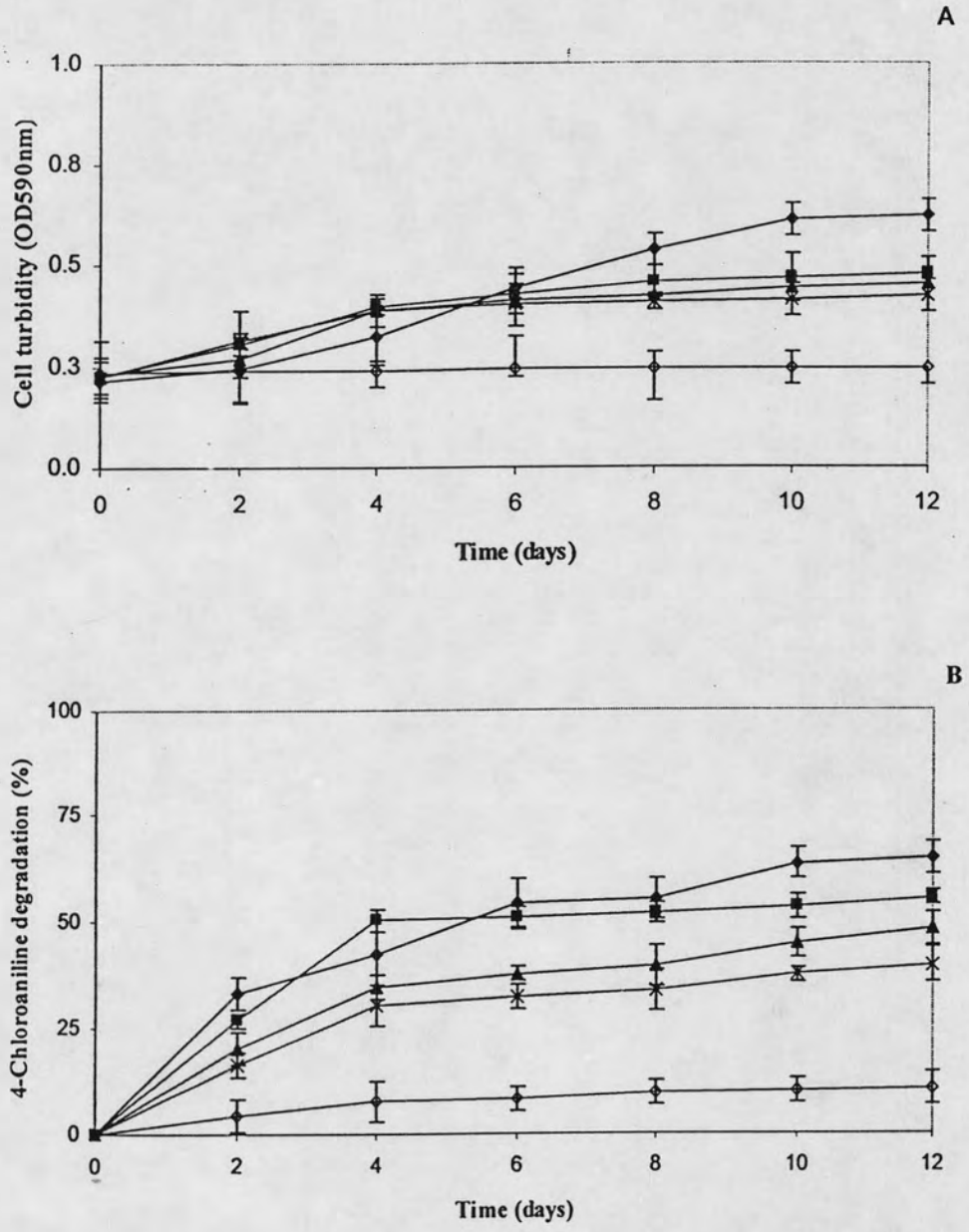

Fig 5.4 Effect of 4-chloroaniline concentration of *Klebsiella pneumoniae* (4CA-17) on (A) growth and (B) total degradation in mineral medium supplemented with 25 ppm 0.2 mM (—◆—), 50 ppm (0.4 mM) (—■—), 100 ppm (0.8 mM) (—▲—), 150 ppm (1.2 mM) (—✕—) and 200 ppm (1.6 mM) (—◇—). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

5.5 Substrate range

This experiment was to determine if the isolates were able to degrade substrate range of chloroanilines. Then, cells were subcultured into mineral medium supplied to 25ppm (0.2 mM) 2-chloroaniline, 25ppm (0.2 mM) 3-chloroaniline, 25ppm (0.2 mM) 3,4 dichloroaniline and (1 mM) aniline. In the present results showed that three 4CA-degrading bacteria could also degrade aniline and monochloroaniline (2-chloroaniline and 3-chloroaniline) although they could not degrade dichloroaniline (3,4-dichloroaniline).

Acinetobacter baumannii (4CA-2) exhibited 4- chloroaniline degradation with 61.00 ± 1.68% total degradation efficiency within 12 days of incubation. The relative total degradation, specific degradation and growth rate can be summarized in Table 5.1. It was found that *Acinetobacter baumannii* (4CA-2) showed the highest specific degradation rate within the first four days towards aniline, while it fairly utilized 2-chloroaniline, 3- chloroaniline and 4-chloroaniline, respectively. The reason for this is that aniline has less complicated chemical structure and it is less toxic than chloroanilines (Table 2.1). Due to water solubility and toxicity of 3,4-dichloroaniline (log Kow = 2.69), then, *Acinetobacter baumannii* (4CA-2) could not degrade 3,4-dichloroaniline. This result was similar to that *Moraxella* sp. strain G which could not degrade 3,4-dichloroaniline (Zeyer, 1982 and 1985).

Pseudomonas puida (4CA-16) exhibited comparatively well 4-chloroaniline degradation with 59.82 ± 0.71% total degradation efficiency within 12 days of incubation. It was interested to examine whether *P. putida* (4CA-16) could utilize other chloroanilines, i.e. 2-chloroaniline, 3-chloroaniline, 4-chloroaniline, 3,4-dichloroaniline

as well as aniline. The relative values can be summarized in Table 5.2. It was found that *P. putida* (4CA-16) showed the highest specific degradation rate within the first four days towards aniline, while it fairly utilized 4- chloroaniline, 2- chloroaniline and 3-chloroaniline, respectively. The reason for this is that aniline has less complicated chemical structure and it is less toxic than chloroanilines (Table 2.1). Interestingly, although 4-chloroaniline has been reported as the most toxic compound among other chloroanilines (Table 2.1), *P. putida* (4CA-16) could utilize 4-chloroaniline with fair rate and the total degradation could reach up to approximately 60% (Table 4.15). This result partly agrees with the previous reports of other aniline-degrading *Pseudomonas* sp. Reber et al. (1979) determined aniline and chloroaniline consumption in aniline-adapted *Pseudomonas multivorans* strain An1 by measuring the specific oxygenase oxidation rate. Their result was similar to ours in that aniline (1 and 2 mM) was consumed with comparatively highest rate 2.17 (umol.mg protein^{1}.h^{-1}). However *P. multivorans* strain An1 had comparatively poor activity towards 4-chloroaniline when compared to that of its aniline degradation.

*Klebsiella pneumoniae (*4CA-17) exhibited the maximum 4- chloroaniline degradation with 68.20 ± 3.87% total degradation efficiency within 12 days of incubation. The relative total degradation, specific degradation and growth rate can be summarized in Table 5.3. It was found that *Klebsiella pneumoniae (*4CA-17) showed the highest specific degradation rate within the first four days towards aniline, while it fairly utilized 4-chloroaniline, 3-chloroaniline and 2-chloroaniline, respectively. The reason for this is that aniline has less complicated chemical structure and it is less toxic than chloroanilines (Table 2.1). This result was corresponded with the previous study of Reber

et al. (1979) who found that the relatively high pK-value of 4-chloroaniline, which is second after aniline, might possibly favor diffusion of the molecule into the cell so that induction could occur.

Table 5.1 Relative total degradation, specific degradation and growth rate of *Acinetobacter baumannii* (4CA-2)

Substrate	**Relative Total degradation (%)**	**Relative Specific degradation rate (%)**	**Relative growth rate (%)**
Aniline	46	100	65
2CA	100	79	77
3CA	61	58	50
4CA	94	55	100
3,4DCA	14	IS	3

IS – Insignificant value

Table 5.2 Relative total degradation, specific degradation and growth rate of *Pseudomonas putida* (4CA-16)

Substrate	**Relative Total degradation (%)**	**Relative Specific degradation rate (%)**	**Relative growth rate (%)**
Aniline	57	100	37
2CA	100	43	79
3CA	61	30	49
4CA	95	48	100
3,4DCA	9	IS	11

IS – Insignificant value

Table 5.3 Relative total degradation, specific degradation and growth rate of *Klebsiella pneumoniae (*4CA-17)

Substrate	Relative Total degradation (%)	Relative Specific degradation rate (%)	Relative growth rate (%)
Aniline	67	100	56
2CA	86	31	68
3CA	95	25	47
4CA	100	51	100
3,4DCA	14	IS	2

IS – Insignificant value