CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Chloroanilines

Chlorinated anilines at the 2, 3 and 4 (*ortho, meta*, and *para*) positions called 2-chloroaniline, 3-chloroaniline and 4-chloroaniline, respectively. The summary of physical and chemical properties of chloroanilines and aniline is shown in Table 2.1.

2.2 4-Chloroanilines

4-chloroaniline is also known as parachloroaniline (PCA, *p*-chloroaniline), 1-chloro-4-aminobenzene, 4-chloro-1-aminobenzene and 4-chloroaminobenzene. Its IUPAC name is 1-amino-4-chlorobenzene.

2.2.1 Properties

Properties of aniline and chloroaniline are shown in Table 2.2.1.

Table 2.1 Physical and chemical properties of 4-chloroaniline (http://www.inchem.org/documents/cicads/cicads/cicad48.htm)

Property	Aniline	2-Chloroaniline	3-Chloroaniline	4-Chloroaniline	3,4-Dichloroaniline
Structure					
Formula	C_6H_7N	C_6H_6ClN	C_6H_6ClN	C_6H_6ClN	$C_6H_5Cl_2N$
Molecular weight	93.12	127.57	127.57	127.57	162.02
Color	6.15	Colorless	Colorless to red	Colorless	Brown
Melting point	6.15°C	-2°C	-11°C	Between 69-73°C	70-72.5°C
Boiling point	184.4°C	208-210°C	232°C	232°C	272°C
Water solubility	34g/litre at 20°C	5g/litre at 20°C	6g/litre at 20°C	2.6g/litre at 20°C	Insoluble
Vapour pressure	40 Pa at 20°C	50 Pa at 20°C	9 Pa at 20°C	between 1.4 and 2.1 Pa at 20°C	1.3 Pa at 20°C
Henry's law constant	19.25 Pa.m^3/mol at 25°C	0.54 Pa.m^3/mol at 25°C	0.1 Pa.m^3/mol at 25°C	0.1 Pa.m^3/mol at 20°C	0.05 Pa.m^3/mol at 25°C
Oral rat LD_{50}	250 mg/kg	256 mg/kg	880 mg/kg	310 mg/kg	648 mg/kg
Kow	-	-	1.88	1.83	2.69

2.2.2 Usage

Chloroanilines are widely used in industry production and agriculture. In the industry, chloroanilines have been used in the production of azo dyes, pigments, pharmaceutical and cosmetic products. In the agriculture, chloroanilines which are intermediates of microbial degradation of various phenyl urea, acylanilide, and phenylcarbamate herbicides (Kearney and Kaufman, 1975) are also accumulated in the environment. In 1985, 6.1 tons of monochloroanilines (sum of 2-, 3-, and 4-chloroaniline), coming completely from industrial processes, were estimated to release to the river Rhine (http://www.inchem.org/documents/cicads/cicads/cicad48.htm quoted from IAWR, 1998). In 1988, about 65% of the global annual production was processed to pesticides (Boehncke et al., 2003). In Germany, in 1990, about 7.5% was used as dye precursors, 20% as intermediates in the cosmetics industry, and 60% as pesticide intermediates. The use for the remaining 12.5% of the production quantity was not specified (http://www.inchem.org/documents/cicads/cicads/cicad48.htm quoted from BUA, 1995). Moreover, in Germany, agricultural soil in which phenylurea herbicide was used; 4CA was detected with a maximum concentration of 968 μg/kg (Boehncke et al., 2003).

2.2.3 Sources, Persistance and Environmental Fate

There are no known natural sources of 4-chloroaniline. Anthropogenic sources, in 1995, combined Western European and Japanese 4-chloroaniline production is given as 3,000-3,300 tones. In India and China, other 800-1,300 tones/year are produced. In 1991, the annual US production quantity was estimated to be 45-450 tones (IARC, 1993). In soil, 4-chloroaniline has been shown to rapidly combine chemically with soil component, particularly in the presence of high amounts

of organic material and/or clay under low pH levels and partially be mineralized by chemical and biological action. Moreover, it can be evaporated from the range of 0.11%-3.65% from soil, depending on soil type and sorption capacity (Kilzer et al., 1979). The *n*- octanol/water partition coefficient measured by high performance liquid chromatographic (HPLC) standard method is 1.83 (Garst and Wilson, 1984). Then *n*-octanol/water partition coefficient indicated that 4-chloroaniline might be stayed in water phase and might not be stayed in aquatic organism (Boehncke et al., 2003). Partition coefficient between water and soil media is calculated as 1.15 from the equation as $K_{oc} = 0.63 \times K_{ow}$ (Lagraga et al., 1994).

4-Chloroaniline released into hydrosphere may be the result from the use of pesticides that contain residual 4-chloroaniline and/or form 4-chloroaniline as a degradation product. In some laboratory experiments in the anaerobic water layer over aquarium soil and in water samples of pasture, both being treated with diflubenzuron, 4-chloroaniline concentrations of 0.1 to about 4 μg/litre were detected a few days after the application (Schaefer et al., 1980).

2.2.4 Potential health Effect

According to the studies with mice and rats, the prominent toxic effect is methaemoglobin formation. 4-Chloroaniline is a more potent and faster methaemoglobin inducer than aniline. 4-Chloroaniline also exhibits a nephrotoxic and hepatotoxic potential. 4-chloroaniline is a carcinogenic in male rats, with the induction of unusual and rate tumors of the spleen. 4-Chloroaniline causes methaemoglobinaemia and is metabolized similarly in humans and experimental animals (http://www.inchem.org/documents/cicads/cicads/cicad48.htm). The International Agency for Research on Cancer (IARC, 1993) has classified 4-

chloroaniline in Group2B (possibly carcinogenic to humans) based on inadequate evidence in humans and sufficient evidence in experimental animals.

2.2.5 Regulations

Because of their toxicity and recalcitrant property as well as the rather high concentration of free chloroanilines in the environment, chloroaniline are considered as the environmental pollutants (Latorre et al., 1984). Consequently, according to the widely use and their toxicity, they are subject to legislative control by the Priority Pollutant List of the U.S. Environmental Protection Agency (Federal register, 1979) and eventually the marketing and the use of products containing 4-chloroaniline-based azo dyes were recently banned by the European Union (EU) (EC, 2000).

2.3 Remediation of toxic substance

Nowadays, the resource in the world show, in greater or lesser degree, our carelessness and negligence in using of toxic substance, the problem of toxic substance increases and persists in the environment. Remediation is the treatment to cleanup hazardous substance. The treatment technologies are categorized into biological, chemical and physical processes. Each technology is generally applicable to treating specific substance.

(1) Biological treatment

Biological treatment is the process of utilizing naturally occurring living organisms to degrade, stabilize or destroy organic contaminants. Toxic substance provide energy and carbon source for their microorganisms, which may be

indigenous or imported. Advantages and disadvantages of biological treatment are shown in Figure 2.1.

Table 2.2 Advantages and disadvantages of biological treatment (Lagraga et al., 1984)

Advantages	Disadvantages
1.Low cost	1.Limit to those compounds that are biodegradable
2.Non-complicated technology	2.Often highly specific
3.Complete destruction of a wide variety of contaminants	3.Products may be more persistent or toxic than the parent compound

(2) Chemical treatment

Chemical treatments alter the structure of substance constituents, which is toxic to render them less hazardous than their original form such as chemical reduction-oxidation and solvent extraction, especially for related chloroaniline; for example, 4-chloroaniline converted to 4-alkylanilines using photochemical (Coppo et al., 2001). The objectives in using chemicals and chemical reactions are to immobilize, mobilize for extraction, or detoxify the contaminants (Lagraga et al., 1984)

(3) Physical treatment

Physical treatment is the manipulation of the physical properties of the wastes in order to immobilize them, detoxify them, or renders them less harmful such as stabilization/solidification and air stripping, etc. Physical treatment often produces residues that require further treatment prior to disposal (Lagraga et al., 1984)

2.4 Fundamental of biodegradation

Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds (Alexander, 1994). The capacities of aerobic microorganisms are of particular relevance for the biodegradation of such compounds and are exemplarily described with reference to the degradation of aliphatic and aromatics hydrocarbons as well as their chlorinated derivatives. The most rapid and complete degradation of the majority of pollutants is brought about under aerobic conditions (Riser-Robert, 1998). Enzymatic key reactions of aerobic biodegradation are oxidations catalyzed by oxygenase in bacteria (Alexander, 1994).

2.4.1 General properties of oxygenases

Oxygenases that catalyze the incorporation of both atoms of dioxygen in their substrates are known as dioxygenases which are two types of dioxygenases, aromatic-ring dioxygenases and aromatic-ring-cleavage dioxygenases.

2.4.1.1 The aromatic-ring dioxygenases

The aromatic-ring dioxygenases incorporate two hydroxyl groups on the aromatic ring at the expense of dioxygen and NAD(P)H, e.g.:

$$C_6H_5COOH + O_2 + NADH + H^+ \longrightarrow C_6H_5(COOH)(OH)(H)(OH) + NAD^+$$

2.4.1.2 The aromatic-ring-cleavage dioxygenases

The aromatic-ring-cleavage dioxygenases open the aromatic ring by incorporating two atoms of dioxygen into substrates, e.g.:

OH OH + O_2 → COOH COOH (Intradiol cleavage)

OH OH + O_2 → OH COOH CHO (extradiol cleavage)

Nearly all bacterial pathways for the degradation of aromatic compounds transform initial substrates intermediates that carry two or more hydroxyl groups on the aromatic ring. Ring fission is catalyzed by dioxygenase and termed *ortho*-cleavage when it occurs between the hydroxyl groups (intradiol cleavage) and *meta*-cleavage when it occurs adjacent to one of the hydroxyls (extradiol cleavage) (Harayama and Kok, 1992).

(1) Extradiol Cleavage Enzymes

The first aromatic-ring-cleavage dioxygenase for which the primary structure was determined catechol 2,3-dioxygenase encoded by the *xyl* gene on the TOL catabolic plasmid (Nakai et al, 1983) and chlorocatechol 2,3-dioxygenase encoded this dioxygenase (*cbzE*) preceded by a gene (*cbzT*) potentially encoding a ferredoxin. The *cbzT* gene product was overproduced in *Escherichia coli* and purified in recombinant form. Two homologous proteins (CdoT and AtdS) encoded by genes identified in strains degrading nitrobenzene and aniline, respectively (Tropel, 2002). Catechol 2,3-dioxygenase contains Fe (II) as a cofactor (Nakai et al, 1990). The

reaction product, 2-hydroxymuconic semialdehyde (or a substituted derivative), is yellow. 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). While most bacteria that degrade chlorobenzene transform this compound into chlorocatechol and metabolize it further through the modified *ortho* pathway, *Pseudomonas putida* GJ31 was found to use the meta-cleavage pathway (Mars et al., 1997). Strain GJ31 was able to grow on a mixture of toluene and chorobenzene due to an a typical extradiol dioxygenase showing exceptional resistance to inactivation by methyl- and chlorocatechols (Kaschabek et al., 1998). The gene encoding this dioxygenase, *cbzE*, was found to be adjacent to the *cbzT* gene which encodes a putative *xylT* analogue (Mars et al., 1999).

(2) Intradiol Cleavage Enzymes

Contrast to the extradiol cleavage enzymes, intradiol-cleavage enzvmes are catechol 1,2-dioxygenase and protocatechuate 3,4- dioxygenase. Catechol 1,2-dioxygenase exhibits little or no activity towards chlorocatechols and is also called catechol dioxygenase I in order to distinguish it from chlorocatechol 1,2-dioxygenase (catechol dioxygenase II) found in degradative pathways for chlorinated aromatic compounds. The latter enzyme exhibits broader substrate specificity and cleaves both catechol and chololcatechols. Type II catechol 1,2-dioxygenases have increased specificity for halogenated substrates. Only subtle differences in enzymatic activities have been found between type II catechol 1,2-dioxygenases. The type I and type II enzymes show a global sequence similarity (Broderick and O'Halloran, 1991 and Van-der-Meer et al., 1991). Modified *ortho*-cleavage pathways included enzymes that are closely related to those of *ortho*-pathways but that have evolved to handle

chlorinated substrates (Figure 2.1). These pathways appear to be used primarily for the dissimilation of chlorinated catechols generated from the metabolism of chlorobenzoates, chlorobenzenes, and chlorophenoxyacetate. The modified *ortho*-cleavage pathway of 4-chloroaniline actively on catabolic plasmid was shown in Figure 2.2 (Zeyer, 1985).

(3) Difference between extradiol and intradiol cleavage enzymes

Meta-fission pathway enzymes differ from those of the *ortho*-pathway in their ability to catalyze the degradation of methylated catecholic of methylated catecholic substrate, and thus they have been well studied in correction with the degradation of methylated aromatic hydrocarbon such as toluene and xylene. Meta-cleavage pathways specifying the degradation of phenol, toluene, and naphthalene have been described that are plasmid encoded. Because they contribute to the degradation of environmental pollutants, the *meta*-cleavage pathway and modified *ortho*-cleavage pathway has been the subject of number of recent review (Assinder, 1990 and Schlomann, 1994).

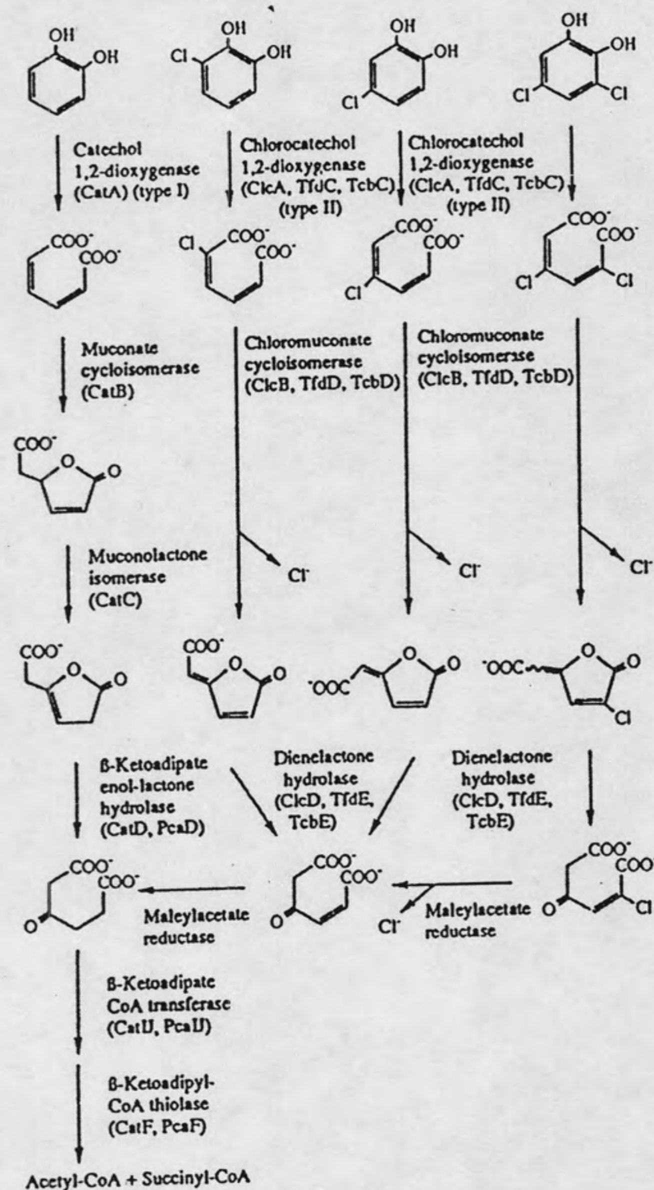

Figure 2.1 Comparison of the *ortho-* and modified *ortho-* pathways

(Schlomann, 1994)

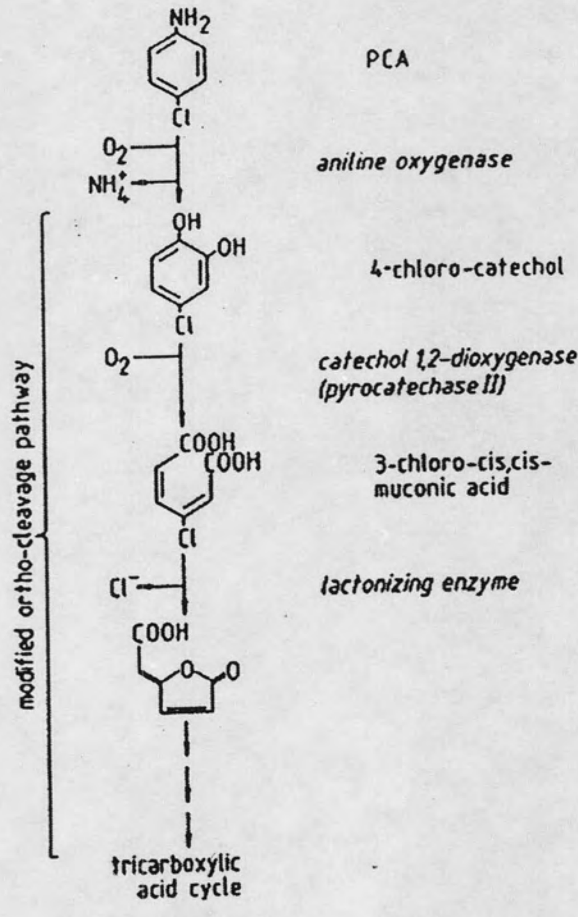

Figure 2.2 Pathway of 4-chloroaniline degradation in *Morazella* sp. strain G

(Zeyer, 1985)

catechol
O_2
catechol-2,3-dioxygenase
2-hydroxymuconic semialdehyde
H_2O
HCOOH formic acid
2-oxopent-4-eneoate
H_2O
4-hydroxy-2-oxo-valeric acid
4-hydroxy-2-oxovalerate aldolase
acetaldehyde
pyruvate
NADH + H^+
CoASH NAD^+
acetyl-CoA
CoASH
CO_2
acetyl-CoA
TCA cycle
TCA cycle

Figure 2.3 *Meta*-cleavage pathway of catechol catabolism (Wrenn, 1998)

2.5 Bioremediation of 4-chloroaniline

2.5.1 4-Chloroaniline-degrading bacteria

Chloroaniline is persistent in the environment. Therefore, many efforts have been undertaken to isolate bacteria capable of degrading chlorinated anilines. *Moraxella* sp.strain G (Zeyer, 1982) was the first strain isolated that could use 4-chloroaniline as a sole source of carbon and nitrogen. Later, more chloroaniline-metabolizing strains were isolated, such as *Pseudomonas* sp. strain JL2 (Latorre et al, 1984), *Brevundimonas diminuta* INMI KS-7 (Surovtseva et al, 1985), *Delftia acidovorans* CA28 (Loidl et al, 1990), *Delftia acidovorans* BN3.1 (Brunsbach and Reineke, 1993), *Comamonas testosteroni* I2 (Boon et al, 2000), *Aquaspirillum* sp. strain 2C and *Paracoccus denitrificans* 3CA (Surovtseva et al, 1996).

In this study, bacteria capable to degrade chloroaniline or aniline was formerly screened and isolated before applying in other tests.

Helm et al. (1979) isolated *Pseudomonas multivorans* strain An1 which grew on mineral salt media, pH 7.0, containing 0.1% (w/v) aniline as a sole carbon and nitrogen source and which degraded ^{14}C-labeled 3-chloroaniline and 4-chloroaniline through 4-chlorocatechol to $^{14}CO_2$ in presence of aniline and glucose at 28°C. *P. multivorans* strain An1 used aniline but not chloroanilines as the sole of carbon and energy for growth. The first intermediates in the metabolism of chloroanilines were chlorocatechols. 3-Chlorocatechol accumulated during growth of the organism in the presence of 2-chloroaniline, whereas 4-chlorocatechol was an intermediate metabolite of 3-chloroaniline and 4-chloroaniline. The accumulation of chlorocatechols and their non-biological oxidation products in the continuous cultures suggested, however, that cleavage of chlorocatechols might be the rate-limiting step in the metabolism.

Surovtseva et al. (1979) isolated a bacterium identified as *Alcaligenes faecalis* which degraded 3-chloroaniline and 4- chloroaniline in presence of aniline to 4-chlorocatechol and further to 2-hydroxy-5-chloromuconic semialdehyde suggesting a degradation *via meta*-cleavage pathway. The presence of aniline in the medium was essential for both strains, because the 4-chlorocatechol-degrading enzymes were not induced by the addition of chlorinated anilines alone.

Zeyer (1982) isolated *Moraxella* sp. strain G from the chemostat containing a constant volume (200ml) of basal medium in the presence of 2 mM aniline as a sole source of carbon and nitrogen. Aniline was stepwise replaced by 4-chloroaniline after a few weeks, before the microorganism finally grew on 4-chloroaniline as a sole source of carbon and nitrogen at 28°C. Balance studies with ^{14}C-ring-labeled 4-chloroaniline revealed that 64% of the carbon of 4-chloroaniline was released as CO_2 and 14% was associated with the biomass. The degradation of 4-chloroaniline to CO_2 was not subject to cometabolism and the enzymes involved in the metabolism of 4-chloroaniline were inducible system by 4-chloroaniline. Therefore, it was unlikely that strain G was a constitutive mutant of an aniline-degrading strain, despite the fact that the strain was isolated from a chemostat culture originally enriched on aniline. For the ring substituents, 60% of the nitrogen and 96% of the chlorine of 4-chloroaniline accumulated in the medium as ammonium and chloride, respectively.

2.5.2 Enzymes involving the 4-chloroaniline degradation

Dorn and Knackmuss (1978a, b) started that the normal *ortho*-cleavage pathway that is frequently found in benzoate-, phenol-, or aniline-degrading microorganism is generally inefficient at processing chlorinated analogs due to

narrow substrate specificity of the catabolic enzymes. Chlorocatechol 1,2-dioxygenase, key enzymes of the modified *ortho*-cleavage pathway, exhibited higher activities and affinities toward chlorinated catechols than catechol 1,2-dioxygenase toward ordinary *ortho*-cleavage pathway.

Surovtseva et al. (1980) isolated *Brevundimonas diminuta* INMI KS-7 that degraded aniline through the *meta*-cleavage pathway. *Meta*-cleavage pathway leads to the formation of nonproductive (dead end) or highly reactive (suicide) metabolite as 2-hydroxymuconic semialdehyde.

However, later Don and Pemberton (1981) suggested productive metabolism of chlorinated catechols via a modified *ortho*-cleavage pathway in *Alcaligenes* strain. The enzymes of this route are characterized by broad substrate specificities and high affinities for chlorinated substrates.

Zeyer (1982) reported that the catabolic enzymes involved in the metabolism of aniline and 4-chloroaniline in *Moraxella* sp. strain G are not constitutive, but inducible by 4-chloroaniline. Moreover, it can be assumed that 4-chlorocatechol is an intermediate in the degradation of 4-chloroaniline by *Moraxella* sp. strain G. This assumption is supported by the fact that resting cells of *Moraxella* sp. strain G pregrown on 4-chloroaniline were simultaneously adapted to aniline, 4-chloroaniline, catechol and 4-chlorocatechol but not to 4-chloronitrobenzene and 4-chlorophenol. Direct evidence, however accumulation of 4-chlorocatechol in a culture growing on 4-chloroaniline cannot be expected. Zeyer (1985) found that *Moraxella* sp. strain G would therefore also seem to possess a normal *ortho*-cleavage pathway. The ability of *Moraxella* sp. strain G to degrade halogenated anilines as sole sources of carbon and nitrogen derives principally from two properties, namely its broad-specificity aniline oxygenase which converts substituted anilines to the corresponding

catechols (and which is inducible by various anilines), and its modified *ortho*-cleavage pathway. A lack of one or both of these properties in an aniline-degrading organism would be a plausible reason why several investigators failed to obtain growth of such microorganisms on halogenated anilines.

Maltseva et al. (1994) reported that chlorocatechol 1,2 dioxgenase from *Rhodococcus erythropolis* 1CP was different from gram-negative strains which had a very broad substrate tolerance. The *Rhodococcus* enzyme was relatively more specific and had a distinct preference for 4-substituted catechols. *R. erythropolis* 1CP was grown in a 10-l fermenter using repeated additions of 0.2 mM 4-chlorophenol as a carbon source. Specific activity of cell-free extract of chlorocatechol 1,2 dioxgenase was 0.058 μmol/min.mg protein which was exhibited in Table 2.3.

Mars et al. (1997) reported that *Pseudomonas putida* GJ31 grown on various aromatic substrates, including chlorobenzene. The enzymes of the modified *ortho*-cleavage pathway were never present, while the enzymes of the *meta*-cleavage pathway were detected in cultures. This indicated that chloroaromatics and methylaromatics were both converted *via* the *meta* -cleavage pathway.

Specific activities of enzymes in 4-chloroaniline degradation in comparison with other reports exhibited in Table 2.3.

Table 2.3 Specific activities of 4-chloroaniline-degrading enzymes pregrown on various substrates in comparison with other reports

Source and strain	Pregrown on	Catechol 1,2 dioxygenase (nmole/min.mg protein)		Catechol 2,3 dioxygenase (nmole/min.mg protein)	
		Catechol	4CA	Catechol	4CA
1. Radianingtyas et al., 2003 (consortium) *Chryseobacterium indologenes* SB1 *Comamonas testosterone* SB2 *Pseudomonas corrugate* SB4 *Sknotrophomonas maltophilia* SB5	1 mM Ani + 1 mM 4CA 1 mM Aniline	156 64	17.2 13	ND ND	ND ND
2. Dorn & Knaekmuss, 1978 *Pseudomonas* SPB13	Benzoate 3-chlorobenzoate	177 640	20 172	ND ND	ND ND
3. Latorre et al., 1984 *Pseudomonas* sp. JJL1	Aniline 2-metylalanine	22 1	ND ND	ND ND	ND ND
4. Zeyer et al., 1985 *Movaxella* sp. Strain G	Basal medium + 3 mM 4CA	200	260	ND	ND
5. Maltseva et al., 1994 *Rhodococcus erythropolis* ICP	0.2 mM 4-chlorophenol	-	58	ND	ND
6. Mars et al., 1997 *Pseudomonas putida* GJ31	Acetate Succinate Chlorobenzene Toluene Chlorobenzene + toluene Benzene Benzoate	8 ND 67 4 4 111 115	ND ND 2 2 1 1 3	203 429 343 268 934 452 164	ND ND ND ND ND ND ND

ND = Not determined

2.5.3 Kinetics and 4-chloroaniline biodegradation

In the study of Zeyer (1982), *Moraxella* sp. strain G was cultured in medium that consisted of basal medium (pH 7.0) supplemented with 2.5 mM [^{14}C] aniline or 2.5 mM [^{14}C] 4-chloroaniline as a sole carbon and nitrogen. Less than 2% of the aniline and 4-chloroaniline initially added to the culture medium was recovered after 10 days. The initial radioactivity in the culture medium (about 3000 dpm/ml) was considered as 100%. About 44% of the radioactivity of [^{14}C] aniline and 64% of the radioactivity of [^{14}C] 4-chloroaniline were evolved as $^{14}CO_2$. Resting cell of strain G that had been pregrown on 4-chloroaniline were able to degrade 4-chloroaniline and 4-chlorocatechol but not 4-chlorophenol or 4-chloronitrobenzene. The specific degradation rate was calculated by expressing the rate of the disappearance (μmol/min) a particular substrate on a protein basis. The initial specific degradation rates were 9.3 μmol (min.g protein)$^{-1}$ for 4-chloroaniline and 12.1 μmol (min.g protein)$^{-1}$ for aniline. Later, 4-chlorocatechol was confirmed as intermediate in 4-chloroaniline degradation in *Moraxella* sp. strain G (Zeyer, 1985). Preparation of strain G mutant would accumulate such intermediates. 4-Chlorocatechol prevents its quantitative isolation and identification. [^{14}C] 4-Chloroaniline and mutants of strain G which lack catechol 1,2dioxygenase, accumulation some 50% of the radioactivity as [^{14}C] 4-chlorocatechol.

Helia (2002) studied that batch cultures of bacterial consortium (*Chryseobacterium indologenes* SB1, *Comamonas testosteroni* SB2, *Pseudomonas corrugate* SB4 and *Stenotrophomonas maltophelia* SB5) was able to degrade 1 mM 4-chloroaniline in the presence of 1 mM aniline. Aniline was almost completely removed after 312 hour incubation, while 4-chloroaniline remained detectable in the culture. However, despite 100% disappearance of 4-chloroaniline at the end of

observation period (1152 hour), dechlorination stopped at a chloride concentration of 0.25 mM. There are two possible reasons for this complete dechlorination. First, it could be due to the formation of minor amounts of 5-chloro-2-hydroxymuconic semialdehyde, a *meta*-cleavage product of 4-chlorocatechol by the action of a catechol 2,3-dioxygenase, which gives a distinctive greenish yellow color to the bacterial cultures growing on 4-chloroaniline. The second possibility is the accumulation of 4-chlorocatechol, which can inhibit further degradation of 4-chloroaniline.

2.5.4 The optimum conditions of 4-chloroaniline transformation

In the study of Zeyer (1982), degradation of 4-chloroaniline was measured as a function of the pH of the medium and of the concentration of 4-chloroaniline. The highest CO_2 evolution within 5 days occurred at pH 6.0. 4-Chloroaniline up to 3 mM was rapidly degraded to CO_2 at pH 6.0. At a concentration of 4 mM 4-chloroaniline, growth was totally inhibited and no significant CO_2 evolution could be observed. The generation time of *Moraxella* sp. strain G growing on 3 mM 4-chloroaniline at pH 6.0 was found to be 14.6 hr. The generation time on 3 mM aniline at pH 6.0 was 13.4 hr. *Moraxella* sp. strain G was unable to use glucose or sucrose as sole sources of carbon, but grew well on succinate or citrate.

2.6 Brief background on bacteria related to this study

In this experiment, three 4chloroaniline-degrading bacteria were identified as *Acinetobacter baumannii, Pseudomonas putida* and *Klebsiella pneuminiae*, respectively. Therefore, information of previous studies of three strains in biodegradation was useful.

2.6.1 *Acinetobacter*

Species of *Acinetobacter* have been attracting in both environmental and biotechnological applications. Some strains of this genus are known to be involved in biodegradation of a number of different pollutants such as biphenyl and chlorinated biphenyl, amino acids (aniline), phenol, benzoate, crude oil, acetonitrile, and in the removal of phosphate or heavy metals (Abdel-El-Haleem, 2003). *Acinetobacter* strains are also well represented among fermentable bacteria for the production of a number of extra-and-intracellular economic products such as lipases, proteases, cyanophycine, bioemulsifiers and several kinds of biopolymers (Abdel-El-Haleem, 2003). Other xenobiotic compounds such as toluene (Zilli et al., 2001), 4-hydroxybenzoate (Allende et al., 2000), 2-chloro-N-isopropylacetanilide (Martin et al., 1999), 4-hydroxymandelic and 4-hydroxy-3-methoxymandelic acids (Rusansky et al., 1987), benzoic and p-hydroxybenzoic (Delneri et al., 1987), 4-chlorobenzoate (Adriaens and Focht, 1991) and 3-chlorobenzoic acid (Zaitsev and Baskunov, 1985) can be metabolized to their corresponding benzoates by various *Acinetobacter* strains.

2.6.3 *Pseudomonas*

Pseudomonas strain grew aerobically on minimal media containing 8 mM aniline with a doubling time of 2 h at 30°C. Concentrations of aniline as low as 50 nM were metabolized. Cells grew as fast on aniline as on nonaromatic substrates such as lactate. The aromatic ring was cleaved via the *meta* pathway. Catechol 2,3-oxygenase activity was induced by aniline, even in cultures containing alternative carbon sources such as lactate. Cultures grown on a mixture of aniline and lactate mineralized aniline in the presence of the second substrate (Konopka, 1989). *Pseudomonas acidovorans* CA28 degraded aniline and 3-chloroaniline via *ortho-*

cleavage pathway and modified *ortho*-cleavage pathway, respectively. (Hinteregger, 1992).

2.6.3 *Klebsiella*

Klebsiella planticola strain DSZ can degrade the herbicide siamazine (SZ). Strain DSZ is metabolically diverse and grows on a wide range of s-triazine and aromatic compounds. DSZ cells grown in liquid medium with SZ (in 10 mM ethanol) as carbon source mineralized 71.6% of 0.025 mM SZ with a yield of 4.6 μg cell dry weight $mmol^{-1}$ carbon (Sanchez, 2004). *Klebsiella oxytoca* strain can grows on phenol as the only source of carbon and energy (Wagner, 1999).

2.7 Bioremediation of contaminated site that polluted with aniline and chlorinated aniline

Lyons, et al. (1984) studied the mechanism and pathways of aniline elimination from aquatic environments at 37°C. Fate of aniline was comprehensively evaluated by use of unpolluted and polluted pond water as model environments. Evaporation plus auto oxidation proved to be minor elimination mechanisms. Instantaneous binding to humic components of a 0.1% sewage inoculums removed 1-4%. A substantial portion of the degraded aniline was mineralized to CO_2 within 1-week period, and microbial biomass was formed as a result of aniline utilization. Biodegradation of aniline in pond water was accelerated by the sewage sludge inoculums. The principle biodegradation pathway for aniline in pond water with or without sewage sludge inoculums appears to involve dioxygenase attack, resulting in oxidative deamination to catechol. Catechol itself was not detected, but this is a notoriously difficult intermediate to isolate. Detection of muconic, beta-ketoadipic,

levulinic, and succinic acids clearly delineated the pathway that leads through the tricarboxylic acid cycle to the ultimate release of aniline as CO_2.

Brunsbach and Reineke (1993) studied degradation of 4-chloroaniline in soil slurry by specialized organisms. At 30°C, the degradation of chloroanilines by indigenous soil populations in soil slurries was observed when soil slurry was freshly contaminated or pre-contaminated to allow binding of chloroanilines to the soil matrix. Within 6 weeks, 3-chloroaniline and 3,4-dichloroaniline (each 2 mM) were degraded more rapidly than 4-chloroaniline and 2-chloroaniline, due to stronger adsorption of 4-chloroaniline and greater resistance of 2-chloroaniline. The addition of various supplemented such as buffer, mineral salts and acetate only slightly influenced the degradation of chloroanilines by the indigenous soil populations. The mineralization was drastically enhanced when laboratory-selected chloroaniline-degraders (8×10^6 cells/g) such as *Pseudomonas acidovorans* strain BN3.1 were supplemented to the soil slurries.

The ability of fungi to degrade aniline and its derivatives in water is studied by Emtiazi et al. (2000). Some of these fungi were obtained from activated sludge by enrichment technique. Among the 10 studied fungi, *Fusarium* sp. and *Rhizopus* sp. utilized only 2-chloroaniline and 3-chloroaniline as nitrogen source in the presence of glucose, with production of catechol, ammonium and chloride. The utilization of 2-chloroaniline was better than 3-chloroaniline, by the *Fusarium* sp. and *Rhizopus* sp.

Bioremediation techniques of aniline and chloroanilines were concluded in Table 2.4.

Table 2.4 Bioremediation of aniline and 4-chloroaniline in soil and water

Waste	Substrate	Bioremediation		
		Natural attenuation	**Bioaugmentation**	**Biostimulation**
Soil	Aniline	-	-	-
	2-Chloroaniline	Indigenous soil populations degrade at 2 mM 2CA after a lag phase of 15 days with a rate of 0.4 mM/month (Brunsbach and Reineke, 1993).	-	-
	3-Chloroaniline	Indigenous soil populations degrade 2 mM 3CA 50% at 30°C (50% Cl^- elimination) (Brunsbach and Reineke, 1993).	*Pseudomonas acidovorans* strain BN3.1 degraded 86% 2mM 3CA within 24 h (complete Cl^- elimination) (Brunsbach and Reineke, 1993).	-
	4-Chloroaniline	Indigenous soil populations degrade 2 mM 4CA 50% at 30°C (50% Cl^- elimination) (Brunsbach and Reineke, 1993).	-	-
	3,4-Dichloroaniline	Indigenous soil populations degrade 2 mM 3,4-DCA 50% (50% Cl^- elimination) (Brunsbach and Reineke, 1993).	-	-

	Substrate	Bioremediation		
		Natural attenuation	Bioaugmentation	Biostimulation
Water	Aniline	The degradation in the absence of sewage sludge was 90 % in 14 days in pond water (Lyons et al., 1985)	The degradation in the presence of sewage sludge was 100 % in 14 days in pond water (Lyons et al., 1985).	-
	2-Chloroaniline	-	-	*Fusarium* sp. and *Rhizopus* sp. utilized 2CA as nitrogen source in the presence of glucose (Emtiazi et al., 2000).
	3-Chloroaniline	The degradation in the absence of sewage sludge was 7 % in 14 days in pond water (Lyons et al., 1985).	-The degradation in the presence of sewage sludge was 9 % in 14 days in pond water (Lyons et al., 1985). -*Comamonas testosteroni* I2 degraded 1.2 mM 3CA 100% within 82h in activated sludge (Boon et al., 2000).	*Fusarium* sp. and *Rhizopus* sp. utilized 2CA as nitrogen source in the presence of glucose (Emtiazi et al., 2000).

Waste	Substrate	Bioremediation		
		Natural attenuation	**Bioaugmentation**	**Biostimulation**
Soil	4-Chloroaniline	The degradation in the absence of sewage sludge was 4 % in 14 days in pond water (Lyons et al., 1985).	The degradation in the presence of sewage sludge was 7 % in 14 days in pond water (Lyons et al., 1985).	-
	3,4-Dichloroaniline	The degradation in the absence of sewage sludge was 3 % in 14 days in pond water (Lyons et al., 1985).	The degradation in the presence of sewage sludge was 6 % in 14 days in pond water (Lyons et al., 1985).	-