CHAPTER I

INTRODUCTION

1.1 Statement of problem

Chloroanilines have been used for a long time in the production of azo dyes and pigments, pharmaceutical, cosmetic products, insecticide and herbicides. They are also accumulated in the environment as a result of microbial degradation of various phenyl urea, acylanilide and phenylcarbamate herbicides (Kearney and Kaufman, 1969). The sources of 4-chloroaniline or *p*-chloroaniline (4-chloroaniline) come from both of the production in the industry and the intermediates of the degradation pathways from the agriculture. The 4-chloroaniline-based azo dyes and pigments are especially used for the dyeing and printing of textiles. 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). The application of pesticides and herbicides (mainly phenylurea herbicides) may lead to releases of 4-chloroaniline into soils. Monolinuron is reported to contain an average of 0.1% 4-chloroaniline. The insecticide diflubenzuron and the herbicides monolinuron, buturon, propanil, chlorofenprop-methyl, benzoylpropmethyl, chloroaniformmethane, chlorobromuron, neburon and oxidazon can release 4-chloroaniline as a degradation product (http://www.inchem.org/documents/cicads/cicads/cicad48.htm quoted from BUA 1995). In Germany, in agricultural soil, in which phenylurea herbicide was widely used, 4-chloroaniline was detected with a maximum concentration of 968 µg/kg (Boehncke et al., 2003). As a consequence of the widespread use, they are usually also detected in wastewater. Moreover, chloroanilines have been found in water resources as a consequence of the transformation of frequently used acetamide and urea herbicides (Lacorte, 1999). Anilines and chloroanilines generally tend to adsorb to soil particles (Bollag, 1978), undergo various spontaneous chemical transformations (Parris, 1980) and turn into non-extractable humic acid-like compounds (Pillai, 1982). Similarly, 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. Otherwise, 4-chloroaniline in soil was evaporated in the range of 0.11-3.65% at 25°C by heat depending on soil type and sorption capacity (Kilzer et al., 1979). However, soil sorption coefficients in a variety of soil types, determined according to the Freundlich sorption isotherm, were in the range of 1.5–50.4, with the highest levels found in soils containing the highest amounts of organic carbon (Müller-Wegener, 1982). Furthermore, 4-chloroaniline shows the low solubility and low volatility from aqueous solutions at a water depth 1 meter at 20°C. The accumulations of 4-chloroaniline from soil to cultivated plants have been shown in several experiments. Especially, 4-chloroaniline was predominantly incorporated in the roots and translocated into shoots (http://www.inchem.org/documents/cicads/cicads/cicad48.htm quoted from Kloskowski et al., 1981a).

The toxicity of chloroanilines has been clearly described. They are known to be toxic and carcinogenic to living organisms (Lyons, 1985). 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. However, the World Health Organization concludes that 4-chloroaniline is carcinogenic in both mice and rats. In human and several animal species, 4-chloroaniline can cause the formation of methanemoglobin (produced from haemoglobin in the compound no longer functioning reversibly as an oxygen carrier). Because of their toxicity and recalcitrant property as well as the rather high concentration of free chloroanilines in the environment, they 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). Although the banning of the use of 4-chloroaniline-based azo dyes will potentially reduce the amount of its contamination in the environment to some extent, 4-chloroaniline can still be highly produced and accumulated from the degradation of the herbicides (BUA, 1995)

In Thailand, the phenylurea herbicides such as diuron have been widely used in agricultural area as the one of the top ten ranks of imported herbicides (Table 1.1). Therefore, phenylurea herbicides and their metabolite residues would be accumulated in the soil.

Biodegradation, which is the use of natural, enhanced or genetically engineered organisms to degrade or transform hazardous chemical into water, carbon dioxide, biomass or other innocuous products, is a means to treat soil contaminated with 4-chloroaniline. The ability of fungi to degrade aniline and its derivatives in water during 30 days is studied by Emtiazi et al. (2001). Previous finding, the degradation of aniline and monochloroaniline has been widely studies in both of gram-positive bacteria and gram-negative bacteria. However, the degradation of 4- chloroaniline by gram-negative bacteria, namely *Klebsiella pneumoniae* has not been reported. Therefore, in the present study, the screening, isolation and characterization the 4-chloroaniline degrading bacteria were studied.

Table 1.1 Top ten rank of imported herbicides in Thailand in 2003 (Data obtained from Bangkok, Lardkrabang, Leamchabang pier by essence)

List	Common name	Quantity (kg.)	Value (baht)	Essence (kg.)
1	Glyphosate isopropylammonium	24,812,105	1,824,107,984	14,643,378
2	2,4-D	5,114,724	392,071,423	4,525,65
3	Paraquat dichloride	8,366,582	1,385,300,727	3,757,33
4	Atrazine	2,364,450	309,974,446	1,998,32
5	Ametryn	2,374,950	488,492,508	1,898,16
6	Butachlor	1,309,267	126,341,644	1,168,81
7	**Diuron**	**984,245**	**178,858,920**	**768,712**
8	Propanil	827,333	118,439,010	749,580
9	Alachlor	1,104,694	91,456,071	749,304
10	Butachlor + propanil	468,752	78,412,941	244,710

1.2 Objectives

According to toxicity of hazardous chemical in the environment, the biodegradation is one important alternatives to biotransform the hazardous chemical such as 4-chloroaniline. Therefore, in this research, the biodegradation of 4-chloroaniline were investigated. There are three objectives of this research:

1.2.1 To isolate, identify and characterize soil bacteria that has ability for 4-chloroaniline degradation.

1.2.2 To optimize the conditions for 4-chloroaniline biotransformation and to determine its degradation rate.

1.2.3 To identify the intermediate(s) of 4-chloroaniline degradation.

1.3 Hypothesis

1.3.1 Isolated bacteria from various soils in Thailand, which has been exposed to phenylurea herbicide, might have the ability to degrade 4-chloroaniline.

1.3.2 The optimum conditions and the possible degradation pathway will be beneficial for further application to remedy 4-chloroaniline contamination.

1.4 Scope of study

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

The soil bacteria, which can degrade 4-chloroaniline, were screened and isolated. The isolated bacteria were identified by biochemical characterization and comparison of 16s rDNA gene sequence.

1.4.2 Characterization of 4-chloroaniline degradation ability of the isolated bacterial strains

1.4.2.1 Factors involving bacterial growth and its 4-chloroaniline degradation

Types and concentrations of an alternative carbon source (succinate and citrate), nitrogen source (NH_4Cl and $NaNO_3$) towards growth and 4-chloroaniline degradation were investigated. Subsequently, the various concentrations of 4-chloroaniline in range of 25, 50, 100, 150 and 200 ppm were used to study on growth and degradation, respectively.

1.4.2.2 The biodegradability of the isolated 4-chloroaniline-degrading-bacteria towards others chloroanilines (substrate range)

The ability of the isolated 4-chloroaniline-degrading bacteria to degrade 2-chloroaniline, 3-chloroaniline, 3,4-dichloroaniline (0.2 mM) and (1 mM) aniline was investigated.

1.4.3 The kinetics of 4-chloroaniline biotransformation was determined

The degradation rate and the specific degradation rate were determined.

1.4.4 The intermediate(s) of 4-chloroaniline degradation was identified.

1.4.4.1 Chloride ion formation was examined to confirm the degradation of 4-chloroaniline using ion selective electrode (ISE) and colorimetric assay. The sensitivity of colorimetric assay depends upon displacement of thiocyanate ion from mercuric thiocyanate by chloride ion; in the presence of ferric ion, a highly colored ferric thiocyanate complex is formed, the color of which is stable and proportional to the original chloride ion concentration (Bergmann and Sanik, 1957).

1.4.4.2 HPLC analysis of 4-chlorocatechol accumulation

1.4.4.3 The degradation pathway (*ortho*-cleavage pathway, modified *ortho*-cleavage pathway or *meta*-cleavage pathway) was examined. The enzymatic assay was described in 3.3.2.

- To classify the *ortho*-cleavage pathway, the enzymatic assay of catechol 1,2 dioxygenase was studied (Dorn and Knackmuss, 1978b).

- To classify the modified *ortho*-cleavage pathway, the enzymatic assay of chorocatechol 1,2 dioxygenase was studied (Dorn and Knackmuss, 1978b).

- To clasified the *meta*-cleavage pathway, the enzymatic assay of catechol 2,3 dioxygenase and chlorocatechol 2,3 dioxygenase was studied. The yellow coloration in the medium was the observation of this pathway (Mars et al., 1997).

1.5 Expected result

Isolated bacteria will efficiently degrade 4-chloroaniline and the optimum factors and/or conditions for degradation will be useful for further information of application to remediate site contaminated with 4-chloroaniline.

1.6 Thesis Organization

This thesis is comprised of seven different chapters. First, this chapter provides the introduction part of this research. Second, the theoretical background and literature review are described. Third, the research methodology is explained. Afterthat, the results and discussions are demonstrated; then, these results are being concluded. Finally, the suggestion and future works is orderly described.