CHAPTER 1. INTRODUCTION

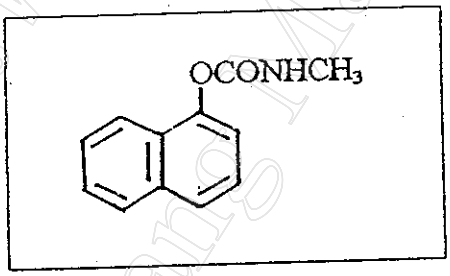
1.1. Pesticides and Its Impact on the Environment

Pesticides are used everywhere in the world in modern agricultural practices. They have an important role for more food production. Due to increasing food demand with increasing population, usage of pesticides has increased tremendously. Although pesticides usage has a significant role in agricultural sectors, excessive and unwise use of pesticides are now creating serious environmental problems. Pesticides are not only threatening the biodiversity of plants and animals, but also have direct impact on human health. These problems are mainly caused by pesticides which have long persistence in the environment.

Most pesticides applied are not totally consumed by pests. Excess organophosphate and carbamate pesticides are degraded rapidly into simpler non-toxic substances. However, organochlorine compounds generally persist longer in the environment (Edward, 1981; Eto, 1977, Nicholson, 1959). A very slow degradative process is done by soil microorganism. This microbial degradation is influenced by several factors such as temperature, nature of the soil, organic matter content, extent of cultivation, etc. Usually the degraded products are also toxic and persist in the environment. Shortly after application, these poisons enter plants and animals in the area and biomagnification increases in the food chain. These chemicals then moves out of the treated area with migratory birds and fishes, and spreads all around. Among the organochlorides, dieldrin is about 40-50 times as toxic as DDT, aldrin is still more toxic, and endrin is the most toxic of all, being about 5 times as poisonous as dieldrin. The destructive power of endrin is well known, a few drops of which can kill all fishes in a large pond. These compounds are usually absorbed by plants from the soil and accumulate in fruits, food-grains, etc. and inhibit nitrification by killing soil bacteria (Grozdyev *et al.*, 1983).

The WHO estimates that about 500,000 pesticides poisoning cases occur annually in the world and that about 1 per cent are fatal (i.e. 5000 deaths/year). It is believed that most of the tumors and cancers are originating from the use of pesticides (Chatwal et al., 1989). 12-31 ppm DDT have been found in humans in the Indian sub-continent, 11 ppm in USA, 5.2 ppm in France, 2.3 ppm in Germany, and 2.2 ppm in England (Ahmed, 1985).

1.2. Carbaryl and Environment



Carbaryl

Carbaryl (1 -naphthyl N -methyl carbamate) was first synthesized in 1953 and commercially introduced in 1958 as a broad-spectrum contact insecticide with systemic properties (Hayes and Laws, 1991). The empirical formula of carbaryl is $C_{12}H_{11}NO_2$ and has a molecular weight of 201.20. It is a white to light tan solid with a mild phenolic odour. It has a melting point of 142 °C and a vapour pressure of less than 4 X10^{-5} mm Hg at 26 °C. Up to 40 ppm of carbaryl is soluble in water at 30 °C. Carbaryl is moderately soluble in most polar organic solvents, such as dimethylformamide, dimethyl sulfoxide, and acetone. It is slightly soluble in hexane, benzene, and methanol, and about 5 per cent soluble in petroleum oils. Carbaryl is stable under normal conditions, but it is hydrolyzed rapidly at pH 10 or above (Worthing and Hance, 1991).

Carbaryl is used widely to control over 150 major pests on more than 120 crops as well as control of pests of domestic animals (Hayes and Laws, 1991). It is effective against many insects which can not be controled with organochlorides or organophosphates (Goewie and Hogendoorn, 1987). Carbaryl has a short persistence in the environment as other carbamate insecticides. It has a low acute mammalian toxicity because it is degraded more rapidly in the environment (Klaassen *et al.*, 1986).

To study the resistance of carbaryl, three different doses of the insecticide was tilled into the top 6 inches of sandy loam soil. All three doses were dissipated with almost equal half-life of 8-days and it totally disappeared within 40 days. However, when it was applied in higher concentration in carrots plots (5 to 16 gm/running meter), high persistence was observed for up to 82 days (Kuhr and Dorough, 1976).

The fate and persistence of carbaryl was studied in the Nile River of Egypt. During this study, 40 mg of carbaryl was added to 10 liters of river water. It was found that the carbaryl disappeared within one week. Another study found that continuous enrichment of fresh water with carbaryl increased the capability of microorganisms, not only for the degradation of carbaryl, but also its hydrolysis products. Using fourth times enriched microorganisms caused the dissipation of carbaryl within one day, no recoverable 1 naphthol was found (Kuhr and Dorough, 1976).

Recent studies revealed that carbamate insecticides are not free from toxicological effects. Sub-chronic neurotoxicity has been reported after long-term exposure to carbaryl which was used as a household insecticide (Branch and Jacqz, 1986). Some studies indicated that carbaryl may be a viral enhancer and teratogen (Bushway, 1981). Hence, in the United States, the status of carbaryl has been changed to a higher risk category.

1.3. Biodegradation of Pesticides and Its Importance

Most pesticides used for insect and weed control are applied to the soil. These are mainly reduced from soil by microbiological metabolism. Biodegradation, a term usually used in reference to microbial metabolism of natural and synthetic chemicals in soil and water, is needed to reduce the accumulation of pesticides residues in the environment. Without biodegradation, the environmental sinks would be rapidly filled with all kinds of chemical contaminants.

During the late 1950's and throughout the 1960's great concern was expressed about the accumulation of chlorinated hydrocarbon and cyclodiene insecticides in the biosphere. From the mid-1970's and throughout the 1980's these persistent insecticides have gradually been phased out by regulations and replaced by highly toxic, but biodegradable, insecticides (Felsot, 1989). These biodegradable pesticides are reducing some environmental hazards.

Since the classical demonstration of microbial breakdown of the hormonal herbicide 2,4-D, it has become well established that the major, or frequently the only, means of degradation for several pesticides in the environment is microbial (Audus, 1951). In biological reactions essentially involving constitutive and adaptive enzyme systems, step-wise degradation of pesticide molecules is accomplished, often with the formation of inorganic end products. In non-biological reactions the degradation is less extensive and far from complete.

Microbial involvement in pesticide degradation is generally established by the following experiments (Sethunathan *et al.*, 1982):

1. More rapid degradation in non-sterile than in sterile soil samples,
2. An initial lag in the degradation followed by a more rapid loss attributed to the adaptation of microorganisms,
3. The initial lag becoming shorter with successive additions of pesticides due to the enrichment of pesticide-degrading microorganisms,
4. Evolution of $^{14}CO_2$ from ring-^{14}C-labeled pesticides applied to biological systems, and
5. Final and conclusive demonstration of the degradation of pesticides in isolated cultures of microorganisms.

In using one or more of these methods, microbial participation has been demonstrated with some degree of confidence in the degradation of some pesticides in soils.

1.4. Improvement of Microorganisms for Higher Biodegradation of Pesticides

There are many methods available for the improvement of microorganisms for higher biodegradation of pesticides. Mutagenesis is one of the major methods to improve the productivity of a target strain. For a specific gene, the frequency of a spontaneous mutation is very low and would be expected to occur 1 in 10^5 - 10^6 nuclei. Considering the whole genome, such spontaneous mutations are much less, although they provide the basis for the overall genetic variation found in different isolates of bacteria.

The low frequency of spontaneous mutation of individual genes is clearly not adequate to meet the constant demands for improved strains for biodegradation or bioremediation of residual compunds in the environment. To meet this demand, it is necessary to induce mutations using a mutagenic agent which takes either a physical or chemical form. Under optimal conditions a mutagen will increase the mutation frequency of a particular gene by at least one order of magnitude.

N-methyl-N-nitro-N-nitrosoguanidine (NTG) is widely used as a mutagen and is an effective carcinogen. Other chemicals are used for this purpose the glucose analogue, 2-deoxy-D-glucose (DG), and dimethyl succinate (DMS), an analogue of the TCA cycle intermeidate, succinic acid. A physical method of UV-irridiation is also used for the production of mutant strains.

The biochemical genetics and molecular biology of microorganisms are very important for better understanding the enhanced biodegradation of pesticides (Kearney and Kellog, 1985). Serder *et al.* (1982) found that the plasmids, which are extrachromosomal pieces of DNA, carry the genome for synthesizing organophosphate-degrading enzymes. Plasmid-assisted molecular breeding has been used to develop a microbe capable of degrading 2,4,5-T. A strain of *Pseudomonas* sp. has been manipulated to completely mineralize dichloronaphtalene (Cork and Krueger, 1989).

1.5. Literature Review: Biodegradation of Carbaryl and Carbamate Insecticides

Johnson and Stansburry (1965) found that carbaryl has a half-life of seven days in soil. Lichtenstein *et al.* (1970) observed that carbaryl was hydrolyzed to 1-Naphthol not only chemically, but also with a variety of microorganisms.

Bollag and Liu (1970) found that the hydrolysis of carbaryl to 1-Naphthol was accelerated by a fungus, *Fusarium solani*, and by a gram-negative coccus and a gram-positive rod-like bacterium. Liu and Bollag (1971) found that during degradation of carbaryl with the fungus *Gliocladium roseum* to N-hydroxymethyl carbamate and to two derivatives (4- and 5-hydroxy carbaryl) where the naphthalene ring is hydroxylated. Eight other species of fungi proved to be capable of hydroxyling carbaryl in these three positions on the molecule (Bollag and Liu, 1972).

Kezano *et al.* (1972) found that *Aspergillus terreus* produced 1-naphthol from hydrolysis of carbaryl through the formation of 1-naphthylcarbamate (desmethylcarbaryl) and hydroxymethyl carbaryl. This naphthol was quickly metabolized to CO_2 by *Fusarium solani* and by a gram-negative coccus. They also found that a species of *Pseudomonas* which produces coumarin and three other metabolites in the process of conversion of 1-naphthol to CO_2. They also found that 38 % of carbon of carbaryl was converted to CO_2 during biodegradation within 30 days.

Aly and El-Dib (1972) and Fullmer (1977) found that carbaryl and carbofuran were susceptible to chemical hydrolysis under alkaline conditions similar to several organophosphate pesticides. However, in neutral and acid soils capable of attaining of these pesticides were observed near neutral pH.

It has been found that heat treatment of flooded soils prior to their incubation with insecticides increased the persistence of both carbofuran (Venkateswarlu *et al.*, 1977) and carbaryl (Venkateswarlu *et al.*, 1980), although appreciable loss of these insecticides occurred also from heat-sterilized soils, possibly by chemical means. Venkateswarlu (1979) found that flooded soils harbor microorganisms, bacteria and actinomycete which are capable of degrading carbofuran and carbaryl.

Venkateswarlu *et al.* (1980) also studied the metabolism of both carbofuran and carbaryl in a yeast-extract-supplemented mineral medium by *Pseudomonas* sp., isolated from a flooded soil amended with carbofuran. The evidence of microbial degradation of carbaryl was more conclusive from the study of Sud *et al.* (1972) who found that a strain of *Achromobacter* sp. utilized carbaryl as a sole carbon source with concomitant formation of 1-naphthol, hydroquinone, catechol and pyruvate.

Kearny *et al.* (1969) found that the persistence of pesticides in aerobic systems generally follows the following order: chlorinated hydrocarbons > organophosphates =

carbamates. However, this generalization may not always be applicable to anaerobic systems because of the susceptibility of several chlorinated hydrocarbon pesticides to anaerobic biodegradation.

Venkateswarlu and Sethunathan (1979) found that the carbofuran phenol was the major product of carbofuran metabolism in flooded soils which accumulated under continued anaerobiosis. Concomitantly, evolution of $^{14}CO_2$ from the aromatic ring in carbofuran was almost negligible, accounting for less than 0.9 per cent of the ring-^{14}C even after 40 days of flooding as compared to 27 per cent released from the carbonyl-^{14}C. They also found that the carbofuran degraded much faster in the presence of rice straw in flooded soils. According to Seiver *et al.* (1978) carbamate pesticides are rapidly hydrolyzed under alkaline conditions in natural ecosystems, whereas they persist in acid soils (Venkateswarlu *et al.*, 1977).

Williams *et al.* (1976) found that the remaining amount of carbofuran in non-sterile soil decreased to less than 60 percent of the amount of originally present after eight weeks of incubation but in sterile soil no measurable loss occured. From this study it was evident that in a soil containing high levels of actinomycete rapid degradation of carbaryl would be expected.

Venkateswarlu *et al.* (1977) isolated a bacterium from flooded soils of rice fields which could degrade carbofuran within 40 days in pure culture, but repeated applications of carbofuran to flooded soil did not cause rapid proliferation of microorganisms capable of decomposing carbofuran.

Rajagopal *et al.* (1984) found that enriched cultures of bacteria from flooded soils previously treated with carbofuran rapidly degraded carbofuran when it was the sole source of carbon and nitrogen. Rajagopal *et al.* (1986) also found that the hydrolysis product of carbofuran, carbofuran phenol, enhanced the biodegradation in flooded soils and that the pretreatment of flooded soil with the hydrolysis product of carbaryl, 1-naphthol, induced enhanced biodegradation of carbaryl.

Ramanand *et al.* (1988) found that low temperatures (6 °C) inhibited the carbofuran-adaptation of microflora in a flooded soil, but incubation at 36 °C was optimal for microbial adaptation. Larkin and Day (1986) isolated bacteria from garden soils, but paradoxically failed to metabolize carbaryl rapidly. Karns *et al.* (1986) found that resting cell suspensions of a pure bacterial isolate adapted for carbofuran degradation also rapidly degraded carbaryl and propoxur.

1.6. Research Scope and Objectives

Considering the importance of biodegradable pesticides for the environment, this research aims to study the biodegradation of a widely used insecticide, carbaryl, with pure bacterial culture which was isolated from the Royal Pang Da Agricultural Station in northern Thailand.

The scope of this research is as follows,

1. Optimization of Bacterial Degradation of Carbaryl

For optimization of biodegradation of carbaryl, different nutrients were added to the minimum mineral (MM) media. The effect of temperature and pH were considered for the optimization of carbaryl degradation. The rate of degradation of carbaryl was measured by HPLC, whereas the growth rate by spectrophotometry was done at different intervals.

2. Cross-feeding

This study was done to find out effects of other carbamate pesticides (e.g. carbosulfan and carbofuran) for biodegradation of carbaryl.

3. Bacterial Mutation

This was done by UV-radiation to produce a suitable mutant for enhancing biodegradation of carbaryl.

4. DNA Preparation

This is designed to establish a method for isolation and preparation of DNA from both normal and mutant bacteria.

5. Identification of Bacteria

Identification was done by several biochemical tests.