CHAPTER 4. DISCUSSION

4.1. Optimization of Biodegradation

Three factors, pH, temperature and nutrients mainly limit the optimization of bacterial growth as well as the biodegradation of chemical compounds. To find out the suitable conditions for biodegradation of carbaryl with selected bacterial strain, the different ranges of pH, temperatures, and different nutrients were examined during this research.

4.1.1. pH

This is one of the most important factors which limit biodegradation since bacteria are susceptible to pH. A specific pH can provide the optimum conditions for bacterial growth and biodegradation. Eight ranges of pH were tested during this study, viz. 6.0, 6.5, 6.8, 7.0, 7.2, 7.5, 8.0, and 8.5. Out of these, higher degradation was found at pH 8.5 which does not correlate to the growth curve of bacterial strain(Figure 2). Higher degradation at this pH may be influenced by the alkalinity of the medium. Aly and El-Dib(972) and Fullmer(977) found that carbaryl is susceptible to chemical hydrolysis under alkaline conditions whereas its persistence increases in acidic condition. This was found to be true by observing Figure 1 for pH 6.0. At this pH the carbaryl concentration remained constant. Although a continuous strain growth was found at pH 7.5 and 8.0, the final growth rate was lower than at pH 6.5, 6.8, and 7.2.

Whereas degradation of carbaryl was higher at pH 7.5 and 8.0. Hence, it is concluded that higher degradation of carbaryl at pH 7.5, 8.0 and, 8.5 not only by bacterial strain but also by the induction of alkaline conditions of medium. Whereas, the carbaryl degradation at pH 6.5, 6.8 and 7.0 was occurred only by bacteria.

4.1.2. Temperature

This is also one of the most important factors which limit the growth of bacteria. To find out the optimum temperature for biodegradation of carbaryl for selected bacterial strain of isolate-5, four temperatures were tested, viz. 30, 34, 37 and 41 °C. Higher degradation was found at 34 °C (Figure 3). After 48 hours incubation the remaining amount of carbaryl was 7.89 ppm, 4.00 ppm, and 6.47 ppm at 30, 34 and 37 °C, respectively. There was good correlation between the strain growth curve and carbaryl degradation at 34 and 37 °C(Figure 3). The second highest rate of degradation of carbaryl was found at 37 °C. There was no temperature induced carbaryl degradation at 37 °C. If temperature itself is responsible for chemical hydrolysis of carbaryl no relationship was found between the bacterial strain growth and the carbaryl degradation curve (Figure 3). Temperature induced carbaryl degradation was found at 41 °C. At this temperature the selected bacterial strain was unable to grow whereas continuous reduction of carbaryl was observed. After 48 hours of incubation, the carbaryl concentration was reduced from 10.0 ppm to 3.65 ppm. This may be due to the higher temperature which induced the chemical hydrolysis of carbaryl. According to Worthing and Hance(1991) carbaryl has persistence up to 70 °C and that carbaryl is totally decomposed above this and below this temperature carbaryl decomposes slowly found in this experiment.

4.1.3. Nutrients

Three nutrients were tested during this study. It was found that the bacterial strain growth in nutrient broth and in yeast-extract supplemented MM was better than in the MM medium with vitamins. In minimum mineral media a low growth was observed due to the limiting amount of carbon and nitrogen sources where 10 ppm of carbaryl acts as a sole source of carbon and nitrogen. Larkin *et al.*(986) isolated bacteria from garden soil which degraded the carbaryl, but failed to metabolize it rapidly in laboratory conditions. The same problem was faced during this research work. May be needed to consider other factors for natural conditions. In nutrient broth, increasing strain growth as well as biodegradation of carbaryl was observed. After 48 hours only 0.763 ppm of carbaryl was found in this medium (Figure 4). The higher biodegradation of carbaryl in nutrient broth may be due to higher cell density which increased the demand for more carbon and nitrogen so carbaryl was used to meet this demand.

Addition of yeast-extract to the minerals media also increased the growth rate of bacterial strain nearly as much as the nutrient broth, but lower degradation was observed. After 48 hours the remaining amount of carbaryl was 5.86 ppm in MM with yeast-extract (Figure 5), whereas it was 0.763 ppm in nutrient broth (Figure 4). After 81 hours 3.12 ppm of carbaryl was found in a yeast-extract containing MM medium. Lower degradation in this media is due to the absence of a protein source, viz. peptone. Peptone in nutrient broth may induce bacteria to secrete any enzyme which enhanced the biodegradation of carbaryl.

With addition of vitamin no significant enhancement of strain growth as well as carbaryl degradation observed. In vit. B_6 containing MM medium higher strain growth and carbaryl degradation obtained than vit. B_1 and nicotinamide containing MM(Figure 6).

4.2. Bacterial Enrichment

Enrichment for microbial adaptations for pesticides degradation have been receiving a great deal of attention in environmental microbiology. The first pesticide used for enrichment was 2,4-D about 40 years ago (Audus, 1949). During this study , the bacteria was enriched in carbaryl containing MM medium for five days to increase the biodegradation ability of bacteria. It was found that enrichment did not enhance the biodegradation of carbaryl. Using carbaryl enriched bacterial strain, the remaining amount of carbaryl found 7.01 ppm after 44 hours of incubation (Figure 7) which was a little lower than the without enriched bacteria (Figure 1, pH 6.8). It was found in the literature that enrichment enhanced the capability of biodegradation. Audus(1964) found that enrichment of microorganisms that use of certain chemical as a nutrient or carbon source caused a remarkable increase in the degradation of that chemical. No improvement of biodegradation of carbaryl may be due to the short of time of enrichment.

4.3. Cross Feeding

This study was done in presence of other two carbamate insecticides, viz. carbofuran and carbosulfan and in the presence of the intermediate metabolite of carbaryl, 1-naphthol. It was found that there was no enhancement of carbaryl degradation in the presence of carbofuran and carbosulfan. Bacterial strain growth and carbaryl degradation was lower with the addition of these two insecticides than without them(Figure 1, pH 6.8). If this selected bacterial strain of isolate-5 was able to use carbofuran and carbosulfan, the growth rate should be higher than the without them. Karns *et al.*(1986) found that resting cell suspensions of a pure bacterial isolate adapted for carbofuran degradation also rapidly degraded carbaryl. A separate addition of carbofuran to the MM medium revealed that this bacterial strain was unable to use carbofuran as a sole source of carbon and nitrogen(Table, Appendix-D). Carbaryl degradation was slightly higher in the presence of carbosulfan than with carbofuran (Figure 8 and 9).

Continuous bacterial strain growth was observed in MM medium which was supplemented with only 10.0 ppm of 1-naphthol (Figure 10). After 48 hours of incubation, the bacterial strain growth was 0.161 (A_{600}). From this experiment it was found that the selected bacterial strain in this study, *Pseudomonas* sp., was able to use 1-naphthol as a sole source of carbon and nitrogen. Walker *et al.*(1975) isolated one soil *Pseudomonad* which was able to degrade 1-naphthol.

4.4. Bacterial Mutation:

UV radiation is important for bacterial mutation because bacteria are susceptible to UV-radiation. Absorption of ultraviolet radiation with bacterial DNA caused the changing of the nucleotide sequence. This change is responsible for changing the genetic character of bacteria which can enhance or reduce biodegradation. To find a mutant the selected bacterial strain of isolate-5 was exposed to UV at different duration (10, 30, 60 and 120 seconds). It was found that UV-60 caused half of strain population to die compare to without UV exposure(Figure 11). UV-60 seconds caused the strain colony shape bigger. In addition it produced the distinct inner boundary to each bacterial colony which was not found in normal colonies (Figure 12C). Whereas, the UV-120 seconds caused most of the strain to die causing cell damaging and a change in colony shape (Figure 12D).

Biodegradation of carbaryl was tested with UV-60 seconds exposed bacterial strain. 34 UV-exposed individual colonies were tested for biodegradation and except for one colony all others degraded as the same as normal bacteria. The of exception was U3. This one degraded higher than all UV-exposures as well as normal bacteria. After 48 hours of incubation, this bacteria reduced the carbaryl concentration from 10 ppm to 5.95 ppm whereas the others reduced it to about 7.0 ppm (Table 1). First it was assumed that this one may a be mutant but, later it was found that it was not. For confirmation the U3 strain was sub-cultured four times and again the biodegradation ability was tested, but it was unable to degrade more carbaryl than the normal one (Table 6). Hence, it was concluded that the higher degradation the first time was due to the adaptation of this bacteria not by mutation. If it was a mutant it should degrade

more carbaryl during the repetition study. Another possibility may be that the DNA sequence of U3 strain was affected by UV-light temporarily which was later recovered by a DNA repair mechanism.

Table 6. Repetition of biodegradaton study with U3 strain at pH 6.8 at 37 and 34 °C.

Temperature	Sample name	Remaining carbaryl (ppm)
37 °C	Normal	5.90
	U3	6.80
34 °C	Normal	3.63
	U3	3.61

4.5. Identification of Bacteria

Identification of this selected bacterial strain of isolate-5 was done by several tests. It was found that this bacteria was motile, short rod, gram-negative and oxidase positive. It has more than one flagella. The motility was confirmed by the hanging drop technique and flagella was observed by flagella staining. From these tests it was assumed that the selected bacterial strain in this study belonged to the genus *Pseudomonas.*

More tests were done to confirm the identity of the studied bacterial strain. This bacterial strain was showed negative test for urea and phenylalanine deaminase, whereas the citrate and MacConkey utilizing tests were positive (Table 2). It was able

to reduce nitrate and could liquefy gelatin. It was non-fermentative and did not produce gas from sugars such as glucose, sucrose, arabinose, lactose, rhamnose, inositol, and xylose (Table 3). It was unable to grow in maltose. This bacterial strain produced a characteristic fluorescent pigment which is easily seen under UV. To compare all of these tests and the natural habitat of this selected bacterial strain of isolate-5 it was identified as *Pseudomonas fluorescens*(Holt et al., 1994, Faddin, 1980)

4.6. I solation of DNA

DNA was isolated from the Pseudomonas fluorescens. Generally, lysis buffer is effective to lyse the cell membrane of all gram-negative bacteria. Whereas this buffer found to be not enough strong against this bacterial strain. Hence, lysozyme was used. From this isolation about 1 mg of DNA was obtained. This DNA was analyzed in gel electrophoresis (Figure 13).