COMPLETE MICROBIAL DEGRADATION OF ALDICARB

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ABSTRACT

COMPLETE MICROBIAL DEGRADATION OF ALDICARB

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Microbial degradation appears to be the most important approach in handling recalcitrant compounds (e.g. pesticides) in soil.

In the present study, aldicarb (AS: the active ingredient of Temik®) [2-methyl-2-(methylthio) propionaldehyde *O*-(methyl-carbamoyl) oxime], was chosen as a model pesticide to understand complete microbial degradation.

Reversed-phase HPLC accompanied with UV detection was used for the rapid and sensitive determination of aldicarb and its metabolic byproducts.

A facultative methylotrophic bacteria, capable of utilizing aldicarb as the sole carbon and energy source, had been previously isolated (Asghari, 1993) through enrichment from soils with a history of Temik application. The isolate hydrolysed aldicarb into aldicarb oxime (AS oxime), methylamine and CO_2. Furthermore, AS oxime accumulated in the medium as an end-product as verified by HPLC, TLC, spectrophotometric and NMR analysis.

The kinetic studies with the enzyme responsible for aldicarb hydrolysis (also other N-methylcarbamates) was performed in cell-free crude extracts. The V_{max} and K_m values for the enzyme with AS as substrate were 0.290 μmol.(min.mg protein)$^{-1}$, 2.06 mM respectively. The results showed that the enzyme is exclusively intracellular.

A number of bacteria capable of utilizing aldicarb oxime as the sole source of nitrogen, were isolated through enrichment from soils with a history of Temik application. Among the new isolates, *Methylobacterium mesophilicum*, utilized 100 ppm aldicarb oxime completely in 8-10 days period and a bacterial consortium, having this particular bacteria along with *Psudomonas putida* utilized higher amount of aldicarb oxime in much shorter period of time (4-5 days).

The activity of the AS oxime hydrolyzing enzyme was determined in crude extracts (prepared by ultrasonication and enzymatic digestion) of *Methylobacterium mesophilicum* grown in PYE- AS oxime. Activity was detected in the pellet of cell-free crude extract (cell debris) implying that the enzyme was membrane-

bound by origin.

Enzyme activity studies, performed in PYE grown cells with and without AS oxime (100 ppm), indicated that AS oxime hydolysing enzyme was of inducible nature.

When two bacteria (the facultative methylotrophic isolate, degrading aldicarb to aldicarb oxime and the *Methylobacterium mesophilicum*, that degraded aldicarb oxime) were ultimately brought together; AS was shown to be completely degraded. As a result, no traces of AS and its metabolic byproducts (AS oxime) remained in the culture medium. This was also confirmed by experiments employing crude extracts of both the isolates.

Keywords: Aldicarb, Biodegradation, Aldicarb oxime, Carbofuran, Pesticides, Soil.

ÖZ

ALDİKARBIN TAM MİKROBİYAL DEGRADASYONU

ASGHARI, Noshin

Doktora, Biyoteknoloji

Tez Yöneticisi: Prof. Dr. N. Gürdal ALAEDDİNOĞLU

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Mikrobiyal yıkım topraktaki yıkıma dirençli bileşiklerin (pestisid gibi) ortadan kaldırılmasında en önemli yaklaşım gibi görünmektedir.

Bu çalışmada, tam mikrobiyal yıkımın anlaşılması için model pestisid olarak aldikarb (AS:Temik®'in aktif bileşeni) [2-metil-2-(metiltio) propionaldehid *O*-(metil-karbamoil) oksim] seçilmiştir.

Aldikarbın ve metabolik ürünlerinin hızlı ve duyarlı bir şekilde belirlenmesinde için UV ışınının ölçümüne dayanan ters-faz HPLC sistemi kullanıldı.

Temik uygulanmış toprak örneklerinden, zenginleştirme yöntemiyle, aldicarbı tek karbon ve enerji kaynağı olarak kullanabilen fakültatif metilotrof bir bakteri önceden izole edilmişti (Asghari, 1993). Aldikarbın bu isolat tarafından hidrolizi sonucunda aldikarb oksim, metilamin ve CO_2 ortaya çıkmaktadır. Ayrıca HPLC, TLC, spektrofotometrik ve NMR analizlerinde de doğrulandığı üzere AS oksim besiyerinde son ürün olarak biriktirmektedir.

Ham özütlerde, aldikarb substrat olarak kullandığında, enzimin V_{max} ve K_m değerleri sırasıyla 0.290 μmol.(dak mg protein)$^{-1}$ ve 2.06 mM olarak belirlenmiştir. Sonuçlar, enzimin kesinlikle hücre içinde bulunduğunu göstermiştir.

Temik uygulanmış topraklardan, zenginleştirme yöntemiyle, aldicarb oksimi tek azot kaynağı olarak kullanabilen bazı bakteriler izole edilmiştir. Yeni izolatlardan birisi olan *Methylobacterium mesophilicum*, 100 ppm aldikarb oksimi 8-10 gün içerisinde tamamen kullanmıştır. Bu bakterinin *Pseudomonas putida* ile birlikte bulunduğu bir karışık kültür ise çok daha kısa sürede (4-5 gün), daha yüksek miktarda aldikarb oksimi kullanmıştır.

AS oksimi hidrolize eden enzimin aktivitesi, PYE'de üretilmiş *mesophilicum*'un hücresiz ham özütlerinde (ultrasonikasyon ve enzimatik parçalama ile hazırlanmış) tesbit edilmiştir. Ham özütün hücre yıkıntısında aktivite saptanaması, enzimin membrana bağlı bir yapı olduğuna işaret etmektedir.

AS oksim içeren (100ppm) ve içermeyen PYE besiyerinde üretilmiş olan hücrelerde yapılan enzim aktivite çalışmaları, AS oksimi hidroliz eden enzimin teşvik edilebilir bir yapıya sahip olduğunu göstermiştir.

Bu iki bakteri (aldikarbı, aldikarb oksime parçalayan fakültatif methylotroph izolat ve aldikarb oksimi parçalayan *mesophilicum*) bir araya getirildiğinde AS'nin tamamen parçalandığı gösterilmiştir. Sonuç olarak kültür ortamında AS ve metabolik yan ürünü (AS oksim) tamamen tüketilmiştir. Bu olay aynı zamanda her iki izolatın ham özütleri kullanılarak yapılan deneylerle de gösterilmiştir.

Anahtar kelimeler: Aldikarb, Biyoloji yıkım, Aldikarb oksim, Karbofuran, Pestisid, Toprak.

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TAS	: Temik aldicarb
AS Oxime	: Aldicarb oxime
AS	: Aldicarb
ASO	: Aldicarb sulfoxide
ASO_2	: Aldicarb sulfone
EAS	: Solvent Extracted aldicarb
TLC	: Thin layer chromatography
HPLC	: High performance liquid chromatography
MM	: Minimal medium
MM-AS	: Minimal Medium Supplemented with aldicarb
MM-EAS	: Minimal medium supplemented with extracted aldicarb
MM-AS oxime	: Minimal Medium Supplemented with aldicarb
TTR	: Total toxic residues

O.D. : Optical density

λ_{max} : Maximum absorption wavelength

UV : Ultra violet

MA : Methylamine

TES : Trace element solution

PYE : Peptone yeast extract medium

CHAPTER I

INTRODUCTION

Biotechnological degradation is concerned with the protection of the environment by removal of pollutants. Although the concentrations of organic chemicals on the surface of earth are supposed to be maintained constant with the integrated activities (e.g. biosynthesis and biodegradation) of plants, animals and microorganisms today, the world is facing a great deal of pollution with industrial chemicals that do not readily participate in the global cycles of carbon, nitrogen or sulfur (Haas 1983; Dow and Whittenburt, 1980). Such compounds cause problems of disposal and may, if they escape containment, lead to adverse effects on the environment. Chemicals exhibiting transitory or permanent accumulation have been termed as "pollutants" or "environmental pollutants" to stress their undesired effects on the environment.

Microorganisms play a crucial role in the natural cycles of elements such as carbon, nitrogen, phosphorus, and sulfur in the biosphere (Brock and Madigan, 1988). The catabolic abilities of present day microorganisms are the result of some four billion years of evolution, during which microorganisms have been presented with a profusion

of natural organic compounds of widely differing complexity and biological recalcitrance. The ability of microorganisms to change the structure or completely mineralize an environmental organic compound is termed biodegradation. Detergents, dyestuffs, pesticides and organic solvents are among the synthetic xenobiotic challenges presented to microbial catabolism.

1.1. Xenobiotics

Any structural feature of a chemical precluding or retarding its attack by microbes will lead to its accumulation in the environment i.e. to persistence or recalcitrance of the compound. Recalcitrance of a molecule may be caused by insolubility or it may be due to novel chemical structures, to which microorganisms have not been exposed during their evolutionary history, such as unusual substitutions I such as with chlorine and other halogens, unusual bonds or bond sequences (such as in tertiary and quaternary carbon atoms), highly condensed aromatic rings (Wilson and Jones, 1993), and excessive molecular size (in the case of polyethylene and other plastics). In the latter case the compound is a xenobiotic. However, Hutzinger and Verkamp (1981) pointed out that the term xenobiotic should not be reserved for the compounds with structural features foreign to life but should be used for all compounds (both organic and inorganic), that are released to the environment by the action of man and thereby occurring in a concentration that is higher than natural and cause undesirable effects.

Some of these compounds may be purposefully released, and are designed to be beneficial to humanity (e.g. pesticides). Alternatively, they may be accidentally released into the environment, as wastes or residues, from industrial manufacturing and processing of fossil fuels. Xenobiotics, particularly pesticides are often applied directly to soil to control organisms considered deleterious to agricultural crops, live stocks and humans.

An important branch of microbiological research is concerned with the fate of xenobiotics in nature. This is an area of great importance for assessing the environmental impact of chemicals. Once in the environment, xenobiotics are subject to photochemical, chemical, and biological effects capable of causing transformations in the compound's chemical structure. Biological, Chemical, and environmental factors affecting the fate of chemicals in the environment are shown in Table 1.1.

When exposed to xenobiotic compounds, various segments of the soil microbial community are affected to different extents (Figure 1.1). The degree to which a xenobiotic affects microbial activities is largely dependent on the chemical, its dosage, and the particular physiochemical parameters of the environment, such as soil type, temperature, water content, pH, method of application, redox potential (E_h) and other factors (Vallaeys et al, 1997).

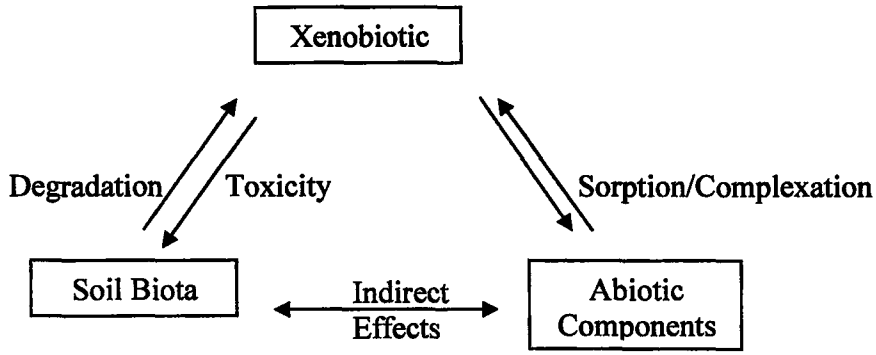


Figure 1.1. Interactions of xenobiotic chemicals with soil

Table 1.1. Factors Controlling the Fate of Xenobiotics in the Environment

Factors	Consequences
Chemical factors	
Molecular weight or size	Limited active transport
Polymeric nature	Extracellular metabolism required
Aromaticity	Oxygen-requiring enzymes (in aerobic environment)
Halogen substitution	Lack of dehalogenating enzymes
Solubility	Competitive partitioning
Toxicity	Enzyme inhibition, cell damage
Environmental factors	
Dissolved oxygen	O_2-sensitive and O_2-requiring enzymes
Temperature	Mesophilic temperature optimum
pH	Narrow pH optimum
Dissolved carbon	Concentration-dependence of organic/pollutant complexes for growth
Particulates, surfaces	Sorptive competition for substrate
Light	Photochemical enhancement
Nutrient and trace elements	Limitations on growth and enzyme synthesis
Biological factors	
Enzyme ubiquity	Low frequency of degradative species
Enzyme specificity	Analogous substrates not metabolized
Plasmid-encoded enzymes	Low frequency of degradative species
Enzyme regulation	Repression of catabolic enzyme synthesis, Required acclamation or induction
Competition	Extinction of low density populations
Habitat selection	Lack of establishment of degradative populations

Since soil (Brussaard, 1997) serves as the main repository for many of these chemicals, it has a major role in determining their ultimate fate. Abiotic and biotic processes can transform the chemical compound, thus altering its chemical state and, subsequently, its toxicity and reactivity. The most versatile and active systems that affect the modification of xenobiotics in soil are biotic. Table 1.2 lists some of the processes that affect the behavior of xenobiotic in soil. The conversion of such compounds into carbon dioxide, water and mineral elements or at least to some harmless substances is, ideally, what is generally desired by one or more members of the microbial ecosystem. However, intermediate transformation products, which can become toxic pollutants in their own right, can sometimes be formed (Charpalamadugu and Chaudry, 1991).

Biological and non-biological processes may work together to degrade xenobiotics. In nature it is difficult to distinguish between the two modes of degradation in most cases. Though some reactions are clearly non-biological, such as photolysis, others, such as hydrolysis, can be either non-biological or biologically maintained. Examples of reactions that can transform xenobiotics in the environment are shown in Table 1.3.

Whether or not a xenobiotic is adsorbed, absorbed, activated, persistent, short-lived, mobile, stationary, or will eventually constitute a residue problem, may depend upon its transformation by soil microorganisms. The microbial metabolism of xenobiotics can be classified as in Table 1.4.

Table 1.2. Processes in soil that affect the behavior of xenobiotics

Process	Governing Factors
Hydrolysis	pH
Microbial transformation	Presence of degradative enzymes; appropriate environment conditions
Volatilization	Equilibrium vapor pressure
Oxidation-reduction	E_h (Redox potential)
Leaching	Solubility
Adsorption	Partition coefficient pKa of adsorbate; types of adsorbents available; solubility
Bioconcentration	Partition coefficient; pKa of adsorbate

Table 1.3. Reactions that transform chemicals in the environment (Chapalamadugu and Chaudhry, 1992)

Category	Example	Category	Examples
Photolysis	Aldrin	Sulphur oxidation	Aldicarb
Hydrolysis	Diazinon	Reduction	DDT
Oxidation	2,4-D	Oxime metabolism	Aldicarb
Dehalogenation	Chlorophenols	Ester cleavage	Malathion
Deamination	Aniline	C-N cleavage	Alachlor
Decarboxylation	Bifenox	C-S cleavage	Benthiocarb
Methyl oxidation	Isopropylnaphtha	C-Hg cleavage	Ethylmercury
Hydroxylation	Dicamba	S-N cleavage	Oryzalin

The rate of microbial decomposition of a chemical in soil and in liquid medium is mediated by three factors; 1) the availability of the chemical to the microorganisms or enzyme system that can degrade it, 2) the quantity of these microorganisms or enzyme systems and, 3) the activity level of these microorganisms or enzyme systems. The availability of a chemical to a microbial population in soil and liquid medium is determined by the physical properties of that chemical. The

chemical's structure and its resulting solubility in water, dissolution rate, and adsorption/desorption characteristics in soil are properties that determine availability. Biodegradation rates of available organic substrates have been shown to be directly related to microbial biomass and its activity. Environmental factors such as pH, temperature, soil moisture level, and soil composition are important regulators for both, the microbial activity, and the degradation rate of chemical.

Table 1.4. General classification of the microbial metabolism of xenobiotics (adopted from Cork and Kruger, 1991)

Reaction type	Description
Enzymatic	Incident metabolism:xenobiotic does not serve as energy source. Metabolism by generally available enzymes. Metabolism due to generally present broad-spectrum enzymes (hydrolyases, oxidases, etc.) Analog-induced metabolism (co-metabolism): Metabolism by enzymes utilizing substrates structurally similar to pesticides. Catabolism: Xenobiotics serves as an energy source. Xenobiotic or part of the molecule is readily available source of energy for microbes. Xenobiotic is not readily utilized; some specific enzymes must be induced. Detoxification metabolism. Metabolism by resistant microbes.
Non-enzymatic	Participation in photochemical reactions. Contribution through pH changes. Contribution through production of inorganic and organic reactants. Contribution through production of cofactors.

1.2. Biodegradation and Bioremediation

In bioremediation, the major approach is the use of microorganisms in the removal of environmental pollutants. The same approach could be used in the removal of residual pesticides from the fields to decrease water and soil contamination (Kazumi et al, 1995; Gutnick, 1994).

The role of soil microorganisms in the degradation of pesticides has been appreciated for several decades (Watanabe and Baker, 2000; and Watanabe, 2001). Continuous introduction of synthetic chemicals into the environment from agricultural and industrial sources has lead to emergence of microorganisms with the ability to degrade some of these compounds. The expanding field of biotechnology has also helped research programs to generate specially designed protocols for detoxification of these compounds present in the environment.

The realization that at least some recalcitrant compounds become readily biodegradable in soil and water, has led to renewed interest in biodegradation as a means of removing problem pollutants (and presumably xenobiotics) (Cook et al., 1983; Charpalamadugu and Chaudry, 1991; Chaudry and Ali, 1988). The rate, extent, and pathway of degradation of synthetic chemicals are biologically controlled.

Mineralization or ultimate biodegradation of organic molecule in water and soil is almost always a consequence of microbial activity (Robertson and Alexander,

1992). Microbes are able to degrade a wide variety of chemical from simple polysaccharides, amino acids, proteins, lipids, etc. to more complex materials such as plant residues, waxes, and rubbers. Some important degradative bacteria that occur in water and soil environment are described in Table 1.5.

Table 1.5.Classification of degradative bacteria that occur in water and soil (adopted from Cork and Kruger, 1991)

Description	Family	Genus
Gram-negative aerobic rods and cocci	*Pseudomonaceae* *Monadaceae* *Azotobacteraceae* *Rhizobiaceae* *Methylococcaceae* *Neisseriaceae*	*Pseudomonas, Xanthomonas* *Azotobacter* *Rhizobium, Agrobacterium* *Methylomonas,* *Methylococcus* *Moraxella, Acinetobacter* *Alcaligenes, Flavobacterium*
Facultative anaerobic Gram-negative rods	*Enterobacteriaceae* *Vibrionaceae*	*Escherichia,Enterobacter,* *Serratia, Proteus* *Aeromonas*
Endospore-forming Gram-positive rods and cocci	*Bacillaceae*	*Bacillus*

For microorganisms, to be able to degrade a given substance they should be pre-exposed to that substance, i.e. a sort of adaptation to substrate generally involves adaptation to the intermediates in the breakdown pathway related either to the parent compounds, or to its metabolites. Thus, it is apparent that cross-adaptation of soil

microorganisms to pesticides of similar structure can occur. A pollution adapted to degrade one pesticide may give rise to metabolites that induce enzymes in a different fraction of the soil micro flora, resulting in co-adaptation of several species, which may degrade different pesticides. These adaptations of microbial populations are known to occur in soil (Vallaeys et al, 1997).

1. 2.1. Enhanced degradation of pesticides

The mineralization of many of the organic compounds by microorganisms is often preceded by an acclimation (or adaptation) period which is the time interval during which biodegradation is not detected. The acclimation time required for a microbial population to degrade a chemical can be influenced by the rate and frequency of exposure to that chemical. This phenomenon has been termed enhanced degradation (Cork and Kruger, 1991). For pesticides, enhanced degradation is defined as the phenomenon whereby a soil-applied pesticide is rapidly degraded by a population of adapted microorganisms, induced by prior pesticides treatment (Aharonson and Katan, 1993).

High or low concentrations of a chemical may increase the acclimation period. High concentrations of the chemical may be toxic or inhibitory to the microbial population present. At low concentrations of the compound the long acclimation may be the result of slow growth of the mineralizing organism or inadequate inducing concentration of the substrate (Wiggins and Alexander, 1988).

There are two schools of thought on the mechanism by which microorganisms in soil develop enhanced degradation. One is as a consequence of repeated applications of pesticide, where an increase in the number of microorganisms capable of degrading the chemical result in more rapid degradation of the chemical compared to untreated soil. The other hypothesis is that repeated applications of a pesticide result in an increase in the enzyme activity but not community size, specifically toward the degradation of the pesticide (Trabue et al., 2001).

The result of this enhanced degradation (Suett et al., 1993) may be a failure of the pesticide to adequately control the target pest due to dramatically decreased persistence. In contrast, soil-incorporated pesticides must, for economic, as well as biological reasons, persist for sufficient time in the soil milieu to be effective in controlling their target pests, even though they are exposed to biologically active microflora. Pesticide catabolism by adapted soil microorganism is microbially beneficial, in that the pesticide or hydrolysis product serve as a microbial carbon, energy, or nutrient source. Enhanced degradation is, at its core, a pesticide/microbe interaction.

Enhanced degradation has been reported for a number of insecticides (Rackeand Coats, 1988 a; Suett and Jukes, 1988; Read, 1987; Smelt et al., 1987; Wilde and Mize, 1984), herbicides (Gray and Joo, 1985), and fungicides (Racke and Coats, 1988b). Clearly, the problem of enhanced degradation is more difficult to resolve than it

is to avoid. It therefore seems essential that, if optimum performance is to be achieved and maintained, appropriate rotation of crops and pesticides should be observed.

Numerous studies have presented evidence of cross-enhancement of degradation within and between different groups of compounds. Most of these have indicated that self-enhancement causes faster degradation than cross-enhancement. Therefore, the potential implications of cross-enhancement should also be considered when strategies to minimize the development of enhanced degradation are being devised (Suett, and Jukes, 1988).

The enhanced degradation of pesticides in soil following their repeated application is, in general, desirable because of the reduced environmental impact. This particularly true for compounds having foliar action such as the hormone related herbicide "2, 4-Dichlorophenoxyacetic acid" which do not exert their herbicidal effect in soil and ground water. Conversely, soil-incorporated pesticides such as carbamate and organophosphate insecticides must of necessity persist for at least several weeks in order to be effective in controlling their target. Any enhancement of the degradation of these compounds in soil reduces their concentration below an active threshold and results in reduced efficacy of the chemicals.

These problems lead to significant crop losses for the farmers growers (Knight and Norton, 1989). The solutions to the problems suggested to date are based on chemical control of the soil microflora using partial soil sterilants or more specific

microbial inhibitors, and use of cultural remedies such as crop and pesticide rotation which are either prophylactic or avoid the use of chemicals which are rapidly degraded in particular soils.

Although cross-adaptation for enhanced degradation exists within carbamate insecticide class (Cotterill and Owen, 1989), structural similarity may play a role in modifying the expression of enhanced degradation in soil. It is possible that both inherent and induced soil properties such as soil pH, moisture, organic matter content and temperature may play a role in modulating the extent to which carbamate cross-adaptations are expressed (Racke and Coats, 1988a).

Reports of single pesticide treatments inducing enhanced degradation are widespread and demonstrated for iprodione (Walker, 1987), carbofuran (Suett, 1986; Felsot et al., 1982; Karns et al., 1986; Veukateswarlu and Sethurathan, 1985), mephosfolan and aldicarb (AS) (Suett and Jukes, 1988). The duration of enhanced degradation is different for different chemicals. Iprodione-enhanced degradation has been found to endure for at least 2 years whereas the number for carbofuran is 5 years.

Pesticide transformation is affected by specific microbial enzymes. One important group of those enzymes is the hydrolytic enzymes, these enzymes are generally tolerant of considerable modifications in their substrates; this is particularly true for esterases, hydrolases, hydroxylases, and recorded for many hydratases, aldolases and decarboxylases. These enzymes typically modify structures but exposure

of substrate analogues to one, two or even more such enzymes in microorganisms may not still necessarily confer any advantage upon the organism unless they are either acting as detoxification agents or are coordinated in metabolic sequences to provide energy for biosynthesis of the cellular constituents of the organisms (Cain and Heat, 1991).

Agricultural uses of pesticides often result in accumulation of these chemicals in groundwater, lakes, streams, and estuaries. Contamination may occur either as a result of the pesticide out of the root zone in to groundwater by run-off into surface water (non-point source), or through improper handling of the pesticide waste and its leakage at a storage facility (point source). Farmers, commercial applicators, and industry from excess or unused aqueous pesticide solutions can generate the waste. It must be pointed out that although the concentrations of the chemicals required to cause acute toxicity in humans would generally be higher than those found in the environment, the recalcitrance of the compound in local areas might still be a potential danger over time (Chapalamadugu and Chaudry, 1991).

Microbial degradation of pesticides in the soil is natural process. It is one of the main mechanisms in detoxification of the pesticide as well as responsible for their removal from the environment. Processes that cause a rapid breakdown of pesticide contribute to maintaining a safe environment. The rate, extent and mode of degradation of pesticides are biologically controlled. In certain cases, the pesticide may only be

partially degraded to one or more toxic products introducing, in turn, further contamination to the environment.

The most common agent responsible for biodegradation is the soil microflora (Brussaard, 1997). Although there are a few fungal species represented for the biodegradation there are no examples with algae and protozoa (Avidov et al, 1988). This is probably due to fact that the common enrichment procedures for the isolation of microorganisms were designed for the isolation of versatile heterotrophic bacteria.

1.2.2. Enrichment Cultures

The range of microorganisms and the spectrum of their activities are enormous, and the problem is to obtain the organism(s) with the required degradative capacity, assuming such organism(s) exists. One approach as to solve this problem is the application of so called "enrichment cultures". The enrichment procedure, a classical microbiological technique, has been used successfully to isolate various microbes with degradative potential. The theory of running an enrichment culture is startlingly simple. The compound to be degraded is supplied as the growth-limiting factor (usually as the sole carbon and energy source). Only the organism (s) with necessary degradative ability will grow significantly under these conditions and will eventually out-number other organisms present at the onset of the experiment.

As a basis for successful enrichment, four factors are usually taken into consideration; growth conditions, media, the size of inoculum, and the analytical methods. Microorganisms structurally consist of the elements C, O, N, H, P, and S, (varying in that order from somewhere near 50 to 1 percent of dry weight) as well as ions (e.g. Mg^{++}, K^{+}) and trace elements; these components must be supplied in the growth medium (Cook and Hutter, 1981). Growth limitation, as required for a successful enrichment, is easily achieved by introducing a given compound that can be utilized by one or a group of microorganisms but not by the others. Among the major constituents of cell, neither O nor H comes exclusively from the substrate, so that neither is utilized as a limiting factor for the growth. C, P, N and S, on the other hand, are the best known elements to do growth limitations since each of these elements can be supplied from a compound (say pesticide, petroleum etc.) to be used as the substrate (Pipke and Amrhein, 1988; Smith and Kelly, 1988).

The inoculum is the source of the microorganisms with the degradative ability for a given compound and the choice is thus important. Usually successful enrichments are inoculated with samples collected from locations pre-exposed to the compound to be degraded.

Having arranged the initial factors (growth media, inoculum preparation etc.) the advantages and disadvantages of batch wise and continuous culture conditions are viewed by the experimenter and decision made accordingly.

Batch wise culturing can be simply run in test tubes or flasks of appropriate volumes allowing many individual experiments to be set up with little effort and expense. The batch culture is very advantageous as minute quantities of the compounds can be studied. It is disadvantageous, however, if the compounds are poorly soluble or highly toxic to the microorganisms because the substrate is at highest concentration when inoculum provides fewest organisms. This can be overcome by the "fed-batch" technique when addition small amounts of substrate are added at successive intervals that in the culture is progressively metabolizes.

In continuous cultures, highly selective pressures can be exerted upon the compounds desired to be utilized. Continuous culture further allows the use of low concentrations when the chemical is sparingly soluble or toxic. The disadvantages lie in the amount of monitoring equipment required per culture and in that a correspondingly smaller number of experiments can be handled.

In almost all microbiological studies, the first parameter to be measured is the growth yield. An essential complement to the growth yield is an adequate and sensitive method for specific determination of the substrate. The proof of substrate disappearance coupled with a measurable microbial growth is strong evidence for biodegradation (Cook et al, 1978; Stanlake and Finn, 1982).

1.2.3. Mixed Cultures in the detoxification of hazardous waste

The potential advantage for use of mixed cultures over pure cultures in the degradation of toxic compounds in hazardous wastes is becoming apparent, especially when we know that traditionally most waste-treatment systems use mixed cultures (e.g. activated sludge systems, anaerobic digesters) (Ottinger and Blumentha, 1974). Mixed cultures are particularly important when the emphasis is placed on complete mineralization of toxic organics to CO_2 (under aerobic conditions) or to CO_2 and either CH_4, H_2S, or N_2 (under anaerobic conditions). Many pure-culture studies have shown that toxic intermediates accumulate during biodegradation, because a single organism may not have the ability to completely mineralize the xenobiotic.

Biodegradation studies essentially start with developing mixed microbial cultures capable of detoxifying a toxic chemical by employing enrichment and selection techniques. Microbial biochemists and geneticists work with pure cultures in order to understand the basic biochemical mechanisms and pathways of microbial detoxification, which are not well known. As a follow-up of such studies, several purified enzymes (e.g. oxygenases or dehalogenases) (Hardman and Slater, 1981) and their corresponding genes have been described.

One of the key aspects of mixed cultures and microbial communities is the interrelationships between different species in respect to their degradative capabilities toward toxic chemicals. This information is important in establishing the catabolic

reaction sequence of a toxicant and the synergistic role of individual microbial species or group in facilitating the rate of xenobiotic biodegradation (Singh et al., 1993).

1.2.3.1. Ecological Role of Microbial Communities

Suitable techniques are now available to isolate and characterize stable microbial communities. It still remains an open question whether such isolated microbial communities perform differently in their natural habitats. It has been demonstrated that mixed cultures more effectively detoxify a chemical when compared to individual members of the mixed population.

1.2.3.2. Types of Communities

In spite of their distinctive properties, special locations, and metabolic activities, the growth abilities of different microorganisms overlap. The basic biological mechanisms of mixed culture growth and activity are still not understood.

The different types of microbial communities with special emphasis on the "tightly" interacting communities where biodetoxification can be achieved through complete mineralization of xenobiotic by sequential catabolic activities of two or more microorganisms. Studies with these kinds of communities can allow elucidation of basic mechanisms for biodegradation and the role of microbial compounds in the overall process of biodetoxification. Efforts have been made to classify various types of

microbial communities. At least seven different types of microbial communities have been described based on various metabolic interactions; (1) provision of specific nutrients, (2) removal of growth-inhibitory products, (3) modification of individual organisms' basic growth parameters, (4) combined (concerted) metabolic attack, (5) co-metabolism, (6) hydrogen (electron) transfer, and (7) presence of more than one primary substrate utilizer.

1.2.3.2.1. Biodegradative function of microbial Communities

The first three types of microbial communities based on metabolic interaction are of importance for degradation of simple organic compounds, whereas the last four catagories include microbial association involved in the catabolism of more complex organic compounds and xenobiotics. As an example of the first category, a stable community containing a *Nocardia* sp. and a *Pseudomonad* was isolated from enrichments on cyclohexane. From this two member microbial community, the *Nocardia* sp. could oxidize cyclohexane alone but it did not grow unless the pseudomonad was also present indicating that *Nocardia* sp., required growth factors, particularly biotin.

A four member community growing on methane that was used for single-cell protein production was isolated, representing the second type of interaction. Methanol inhibited the methane-oxidizing pseudomonad unless it was consumed by *Hyphomicrobium* sp., present in the community. The other two members of the

community were *Flavobacterium* sp. and *Acinetobacter* sp. Removal of inhibitory products is also an important characteristic of communities involved in sulfate reduction and sulfide oxidation.

A three-member community was developed from enrichments on orcinol (3,5-dihydroxy toluene) which included a *Pseudomonas* sp. that degraded orcinol. The other two members of the community *Brevibacterium linens* and *Curtobacterium* sp. did not grow on orcinol unless the primary degrader *Pseudomonas* sp. was present (Spiers et al., 2000). This represents the third category of microbial community based on the modification of an individual organism's basic growth parameters.

The fourth category of microbial community based on a combined or concerted metabolic attack is extremely important in the degradation of xenobiotics. The component organisms may not separately have the capacity to transform or detoxify the toxic chemical, whereas collectively the microbial community can completely mineralize the compound. This is probably because none of the individual species has a complete set of enzyme systems or genetic information for degradation of a xenobiotic. A multimember community was isolated by growth on the herbicide Dalapon. It was shown that the rate of Dalapon degradation was over 20 percent greater than the combined rates of the individual organisms.

A mixed microbial community isolated from soil was shown to degrade styrene intermediates such as phenylethanol and phenylacetic acid were detected in

mixed cultures. Phenylacetic acid was further metabolized by well-known mechanisms by an organism of the community which was unable to grow directly on styrene.

The fifth type of microbial community is based on co-metabolism. In general, many microorganisms growing on one substrate may be able to transform or degrade a different co-substrate in a reaction or sequence of reactions that is not directly associated with the organism's energy generation, carbon assimilation, and biosynthesis or growth. Co-metabolism is not clearly understood and its importance in nature has probably been underestimated because of the difficulties in designing appropriate enrichment and selection procedures. Co-metabolism was formerly termed co-oxidation, but the concept also includes reactions not necessarily involved in catabolism of the growth substrate. It is important to note that in a microbial community one organism generates a compound which it can not use itself but which can be used as a metabolite for other organisms in the community. If these organisms can not use the initial co-metabolic substrate, then potential exists for an interacting microbial community.

The sixth type community is based on interspecies electron (H_2 or formate) or other nutrient transfer. These communities function on the principle that under anaerobic condition organisms require a sink to dispose of excess reducing equivalents. One of the classical examples of this type of community came with the discovery of the closely associated two-member methanogenic community *Methanobacillus omelianski*. This community comprised the "S organism" which oxidized ethanol to acetate and

hydrogen, and methanogen *Methanobacterium* strain MOII which used hydrogen to reduce CO_2 to methane. This association permitted continuous ethanol metabolism, as the methanogen present prevented accumulation of inhibitory levels of hydrogen. Similar communities have been isolated from anaerobic environments. In other anaerobic mixed cultures, syntrophs degrade alcohols and fatty acids into acetate and formate which are then degraded by methanogens.

The last class of microbial communities is based on the presence of more than one primary utilizer. Many continuous culture enrichments have resulted in stable communities that contain more than one species capable of growing on the sole carbon and energy source provided. These communities are different from combined metabolism communities since the substrate can be completely metabolized by each of the primary utilizers. Often secondary organisms which are unable to metabolize the primary substrates are members of this stable community. The presence of several primary organisms and some secondary organisms indicates that interactions must exist between these two in order to stabilize free competition and prevent culture wash out. Such communities have been isolated on the herbicide Dalapon and benzoic acid. It was shown that Dalapon was degraded at much higher rates by the community than by pure cultures of the primary organisms, which may account for the success of the whole community.

One study (Singh et al, 1993) reports on the synergistic interaction between two bacterial isolates in the degradation of carbofuran. The dominant bacteria

Pseudomonas sp. and *Arthrobacter* sp. were isolated from the standing water of carbofuran-retreated Azollaplot. *Arthrobacter* sp. hydrolyzed carbofuran added to the mineral salts medium as a sole source of carbon and nitrogen while no degradation occurred with *Pseudomonas* sp. Interestingly, when the medium containing carbofuran was inoculated with both *Arthrobacter* sp. and *Pseudomonas* sp. a synergistic increase in its hydrolysis and subsequent release of CO_2, from the side chain was noticed. This synergistic interaction was better expressed at 25°C than 35°C. Likewise, related carbamates, carbaryl, bendiocarb and carbofuran were more rapidly degraded in the combined presence of both bacterial isolates. Most of the studies on the degradation of pesticides by microorganisms have been confined to individual cultures while pesticides, applied to natural ecosystems, are subjected to attack by diverse microorganisms.

1.2.3.3. Detoxification by Aerobic Mixed Cultures

Over the last few decades enormous quantities of industrial chemicals have been released into the environment. Microorganisms are essentially capable of catabolizing any organic molecule that structurally resembles a natural product. However, in recent years, microbes have encountered numerous novel compounds rarely found in nature but which are released into biosphere by human activities. Fortunately, microorganisms collectively exhibit a remarkable ability to degrade a wide range of synthetic chemicals employing enzymatic systems that have evolved over 4 billion years for the catabolism of naturally occurring compounds. The evidence that

these organisms will degrade many anthropogenic compounds can be attributed to their relaxed enzyme specificity. However, there are many synthetic chemicals of recent origin that are recalcitrant to microbial attack. This is particularly true of halogenated compounds (Kocabıyık, 1989). In terms of geological or evolutionary time scale, microorganisms have not had enough time to evolve the necessary enzymes to degrade such chemicals. However, considering the microbial ability to evolve catabolic systems for degradation of organic compounds, it is likely that many environmental toxic pollutants can be destroyed (Pollis et al, 1998).

Although the potential role and application of a mixed microbial community in the biodetoxification of xenobiotics is apparent, most bioaugmentation process has used pure cultures. In many cases the performance of a pure culture in the degradation of a toxic organic xenobiotic compound is very poor when compared to that of an adapted mixed culture. Apparently, individual microbes do not have the genetic information to code for all the enzymes required for biodegradation because they have not been exposed to xenobiotics long enough to expect the evolution of a complete catabolic pathway. Therefore, it is thought that genetic manipulation to construct a complete metabolic pathway in one organism from different organisms by forced genetic transfer over a short period of time (directed evolution) with strong selective pressure may provide a "superbug" which can effectively meet the toxicity and environmental challenge to detoxify a given xenobiotic. Since these superbugs would still have to survive and compete in the environment as a mixed culture, it would seen

that emphasis should be placed on the design, feeding, and use of "super mixed cultures" to ensure the job is done right.

1.3. Carbamate pesticides

2, 4-Dichlorophenoxy acetic acid (Sinton et al., 1986), carbamates (such as aldicarb, carbaryl and carbofuran), and trifuralin are the three major pesticides currently in use in Turkey.

Carbamate pesticides are of great significance in pest control and are increasingly used instead of recalcitrant organochlorin and organophosphorous pesticides, due to their broad spectrum of biological activity. The toxicity of carbamate insecticides is due, as for organophosphorus compounds, to the inhibition of the enzyme acethylcholine esterase, but with carbamate the inhibition is reversible, so they are less toxic to mammals (Lifshitz et al., 1997; Matitiahu, 1997). Among them, the N-methylcarbamates (NMCs) are a group of about 15 active compounds that constitute a family of important insecticides which cover a wide range of utilization in the treatment of seed, soil or crop.

Methylcarbamates (Hassal, 1983) are related to the naturally occurring carbamate alkaloid, physostigmine, which was isolated from Calabar Bean, physostigma venenosum (Balfour), in 1864 (Casida, 1963). Because of its

its pronounced cholinergic action, physostigmine has been used for many years to treat glaucoma and myasthenia gravis in humans.

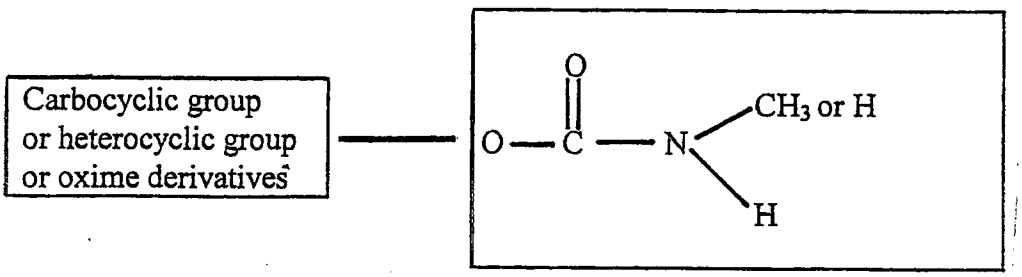


Figure 1.2. General structure of carbamate pesticides

Gisin and Metcalf and co-workers were the first to describe the insecticidal properties of methylcarbamates. Their research, stimulating subsequent chemical synthesis, has produced several methylcarbamates, which have attained considerable importance as commercial insecticides (Kearney and Kaufman, 1969).

Carbamate pesticides show a broad spectrum of biological activity; they are used as herbicides (such as chlorpropham, butylate, barban), fungicides (such as maneb, thiram, benomy), and foliar and soil-administered insecticides (such as pirimicarb, methiocarb, oxamyl, carbaryl, carbofuran, and aldicarb) in which either the amino or carboxyl groups are derivatized.

There are three major subgroups for carbamates:

Subgroup 1. Comprises aryl N-methylcarbamate esters of phenols-that is, compounds with a hydroxyl group attached directly to a phenyl or naphtyl ring. Three members of the subgroup are listed in Table 1.6. and the formula of carbaryl, as type example, is given in Figure 1.3.

Subgroup 2. Similarly comprises N-methyl- and N-dimethylcarbamate esters of heterocyclic phenols, and carbafuran (Figure 1.3) is an example.

Subgroup 3. Contains oxime derivatives of aldehydes (e.g. aldicarb, Figure, 1.3.) and the closely related thiohydroximidates. In the oximes, the OH group has been carbamylated.

Compounds in subgroup 3 illustrate most clearly the way in which carbamates were designed specifically to resemble acetylcholine and yet to remain sufficiently lipophilic to penetrate to their site of action in insects (Hassal, 1990). Registered compounds belonging to this series include methomyl and aldicarb; the latter arose from a synthetic attempt to structurally mimic acetylcholine.

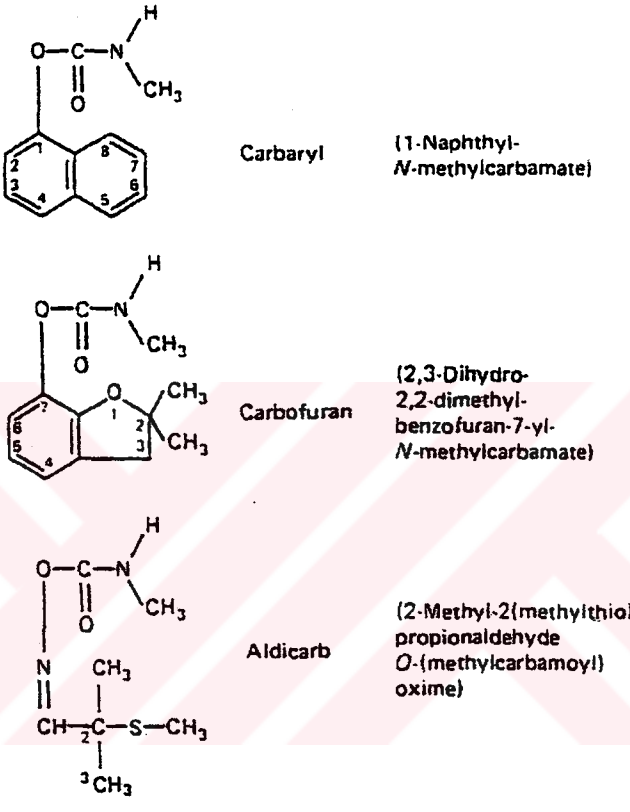


Figure 1.3. One important member of each of the carbamate insecticide subgroups.

The three most important members of this subgroup are aldicarb, methomyl and oxamyl (Gerstl, 1984; Harvey et al, 1978). All three are very toxic to higher animals (Table 1.6).

Carbamates, in spite of their large scale production, have the attraction of limited persistence in the environment even in the abiotic conditions, because the stability of the carbamate residue is particularly pH dependent. Carbamates slowly hydrolyze chemically in neutral and mildly acidic aqueous surroundings, but in the presence of alkali, decomposition occurs rapidly (Sahoo, 1990).

Table 1.6. Physicochemical and biological characteristics of some carbamate insectisides

Substance (subgroup)	Route of systemic action	Oral LD_{50} toxicity to rats[a] (mg/kg)	Vapour pressure at 25-30°C (mmHg)	Uses, other than as an insecticide	Solubility in water at 25-30°C[a] (ppm)
Carbaryl (1)	None	700	$3x10^{-3}$	Earthworm killer; fruit thinning	40
Propoxur (1)	None	90	-	-	1000
Methiocarb (1)	None	130	-	Molluscicide	-
Carbofuran (1)	By root uptake	11	$1x10^{-5}$	(Mematicidal at high dosage)	700
Primicarb (2)	By leaves or roots but persistence very low	~100	$3x10^{-5}$	-	2700
Aldicarb (3)	By root uptake	1	$1x10^{-4}$	Nematicide	6000
Oxamyl (3)	By root uptake	5	-	Nematicide; millipede killer	-
Methomyl (3)	By leaf uptake	21	$5x10^{-5}$	-	58000

[a] Data from commercial literature, from Kuhr and Dorough, 1976 and from Matsumara, 1975. Adapted from Hassall, 1990.

Biological degradation of N-methylcarbamate (NMC) insecticides to ineffective concentrations has been documented in numerous reports of "problem or aggressive" soils (Vink, 1997). The mechanism of enhanced N-methylcarbamate insecticide degradation by microorganisms in such soils is unknown (Derbyshire et al., 1987; Smelt et al., 1983).

The development of accelerated biodegradation of carbamates, resulting in reduced field performance of the pesticide, is of particular concern for carbamate insecticides such as carbaryl (Racke and Coats, 1988a; Charpalamadugu and Chaudry, 1991), aldicarb (Suett and Jukes, 1988) and carbofuran (Chaudry and Ali, 1988) which are used against several soil-borne pests of root crops. (Table 1.7).

Carbamates such as AS, carbofuran and carbaryl lose their pesticide activity upon hydrolysis of the methylcarbamate moiety so soil bacteria need to produce only a carbamate hydrolase to render the molecule biologically inactive, and to leave the molecular structure otherwise intact. Enhanced degradation of a pesticide therefore, does not necessarily require its complete mineralization. The fact that bacteria often effect mineralization stems from the knowledge that, they have evolved or acquired the genes coding for the enzyme of peripheral catabolic pathways that channel these molecules into central metabolism. The source of this genetic material is, in many instances, plasmid-born (Cain and Head, 1991; Boronin, 1991; Chaudhry and Huang, 1988; Desaint, 2000; Feng et al., 1997).

Table 1.7. Selected microbial species capable of degrading carbamates

Compound	Microorganism	References
Carbofuran	*Arthrobacter sp.*	Ramanad, et al., 1998
	Actinomycetes	Williams, et al.,1976
	Azospirillum lipoferum	Venkateswarlu, et al.,1984
	Streptomyces ssp.	
	Achromobacter sp.	Felsot, et al., 1981
	Micrococcus sp.	
	Pseudomonas sp.	
	Arthrobacter sp.	Karns, et al.,1986
	Bacillus sp.	
	Nocordia sp.	
Carbaryl	*Pseudomonas spp.*	Larkin, et al.,1986
	Rhodococcus sp.	
	Bacillus sp.	Karns, et al., 1986
	Micrococcus sp.	
	Several fungal and bacterial isolates	Rodrigues, et al., 1977
AS	*Achromobacter sp.*	Karns, et al., 1986
	Pseudomonas sp.	Chaudry, et al., 1988
	Nocardia sp.	
	Arthrobacter sp.	
EPTC	*Pseudomonas sp.*	
	Alcaligenes sp.	
	Micrococus sp.	
	Arthrobacter sp.	
	Rhodococcus sp.	Dick, et al., 1990
	Flavobacterium sp.	Mueller, et al., 1988
	Penicillium spp.	
	Bacillus sp	

The most common reaction mechanism evolved by microorganisms to degrade carbamate insecticides is the hydrolysis process through esterases (Matsumura and Boush, 1968). The oxidative processes, which are important in higher animals in degrading these compounds, are less frequently observed. This is probably because of

the lack of defined mixed function oxidase systems in microorganisms (Edwards, 1973).

1.3.1. N-methylcarbamate hydrolyzing enzymes

As was described for the organophosphate insecticides, the main detoxification routes of carbamate insecticides are hydrolysis. The hydrolysis by carboxyesterase being most effective detoxification route. (Sogorb and Vilanova, 2002).

In general hydrolytic enzymes which figure predominantly in pesticide transformations are generally esterases, hydrolases, and hydroxylases (Golovieva et al. 1990) and recorded for many hydratases, aldolases, and decarboxylases (Dagley, 1978). Enzymatic hydrolysis of carbamates is brought about by the enzymes of two kinds, namely estrases and amidases. Esterases attack the bond on the side of the carbonyl group attached to the oxygen atom, whereas amidases attack the bond on the side attached to the nitrogen atom. N-methyl carbamates are primarily used as insecticides and the hydrolysis of an N-methylcarbamate insecticide at either position totally destroys the insecticidal activity of the compound (Hassal, 1990) (see Figure, 1.6).

In the first description of a microbial enzyme that degraded N-methylcarbamate insecticides (Derbyshire et al, 1987), the enzyme was isolated from *Achromobacter* sp. WMIII which degrades N-methylcarbamates isolated (Karns et al, 1986).The products of carbofuran hydrolysis by this enzyme were found to be the 7-

phenol of carbofuran and methylamine. Further studies on the purified enzyme showed that the enzyme was a hydrolase that required no cofactors. The lack of any cofactor requirement for this enzyme indicated that the reaction proceeded by the direct hydrolysis of the pesticide as opposed to hydroxylation.

1.3.2. Environmental contamination caused by carbamate pesticides

Use of pesticides in the production of agricultural commodities has increased with the worldwide demand for food and fibre, which in turn increased the potential for contamination of terrestrial and aquatic environments by agricultural chemicals. In recent years, several pesticides have been reported to contaminate groundwater (Cohen, 1996; Chaudry and Ali, 1988; Cohen et al, 1984; U.S. EPA, 1987). The widespread use of organic pesticides and herbicides is one of the possible sources of groundwater contamination. Aldicarb residues ranging from 1 to 500 ng/l have been found in ground water in Arizona, California, Florida, Maine, Missouri, New York, North Carolina, Oregon, Virginia, Washington, and Wisconsin, as a result of agricultural practices, whereas the recommended maximum contamination level is only 7 ng/l (Baier and Robbins, 1982; Rothschild et al, 1982; Russel and Tammara, 1995; Sebae, 1993). Similarly, carbofuran has also been found in ground water in New York and Wisconsin at 1-to 50-ppb levels (EPA data).

Recent finding that cite the presence of pesticides in drinking water supplies illustrate the fact that some fraction of pesticides applied to a particular location can be transported off site and into surface water (Koplin et al, 1996). Specifically, the carbamate pesticide, carbofuran has been found in surface water that serves as a drinking water source for Sacramento CA). Given the potential human and wildlife health risks associated with toxic pesticides in surface water (Nicosia et al, 1991), it is important to determine the probability that a certain chemical will persist in the environment by examining the various reactions of the molecule.

Pesticide residues have been detected in vegetable and fruits that were sprayed with these chemicals for insect control. Three outbreaks of illnesses associated with aldicarb sulfoxide-contaminated watermelons and cucumbers in California and one in Nebraska have been reported (Goldman, 1990). Although the concentration of these chemicals required causing acute toxicity in humans would generally be higher than those found in the contaminated environment, conditions present in the human gut favor the formation of N-nitrosocarbamates (Rickard and Dorough, 1984). The potential for the formation of such mutagens in the gut cautions that carbamate contamination might pose health problems. Therefore, it is important to investigate the enzymology and biochemistry of microbial degradation of these pesticides to help design strategies for detoxifying pollutants in the environment (Chapalamadugu and Caudry, 1992).

1.3.3. Aldicarb

AS is an oxime N-methyl carbamate pesticide introduced under the trade name of Temik by union Carbide Corporation in 1967 (Figure 1.4). It has been used on tobacco, sugar, beets, sugar cane, potatoes, and peanuts for the control of aphids, thrips, mealy bugs, white flies, mites, and nematodes (Chapalamadugu and Caudry, 1992). The systemic properties of AS enabled it to be used, to good effect, for the indirect control of viral diseases and against phytophagous nematodes. It can in fact, act as an insecticide, nematocide, acaricide, as well as being a growth regulator (Hassall, 1990).

AS is one of the most potentially toxic substances currently used in the crop protection with an oral lethal dose (LD_{50}) of about 1 mg/kg body weight in rats (Table 1.8). It is usually formulated, for reasons of safety, as granules. Its solubility in water is about 0.6% (w/v) at room temperature that is much higher than most soil-administered pesticides and the compound is considered to be sufficiently soluble in soil (Chapalamadugu and Caudry, 1992) to be readily leached out to enable it to be used systematically in plants.

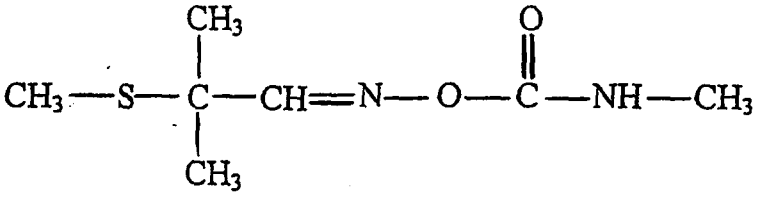


Figure1.4. Chemical structure of Aldicarb

Table 1.8. Some physical properties of aldicarb

Common name	Pesticide Movement Rating	Soil Half-life (days)	Water Solubility (mg/l)	Sorption Coefficient (soil Koc)
Aldicarb	High	30	6000	30

AS illustrates most clearly the way in which carbamates were designed specifically to resemble acetylcholine, and yet to remain sufficiently lipophilic to penetrate to their side of action in insects. This similarity of structure to that of acetylcholine probably helps to explain the very high toxicity of aldicarb to both mammals and insects (Hassal, 1990), and act by inhibiting acetylcholinestrase, an enzyme vital to the functioning of the nervous system, involved in the transmission of

nerve impulses (Chapalamadugu and Caudry, 1992; Hassal, 1990) in the target organisms. (Kuhr and Dorough, 1976). The metabolism of Temik aldicarb (TAS) has been studied in plants (Bull 1968; Bull et al, 1970), animals (Pelekis and Krishnan, 1997; Wei et al, 1997; Kallander, 1997; Bull and Coppedge, 1967), and soil (Dai et al, 2001; Ou et. al., 1985, 1986; Jones, 1976; Bull et al, 1970; Andrews et al, 1971) disregarding microbial composition of the soil.

Daily administration of aldicarb in male Sprague-Dawley rats at various dosage levels for 21 days revealed 1) 0.1 mg/kg, nontoxic dose; 2) 0.2 mg/kg, moderately toxic dose; and 3) 0.4 mg/kg severely toxic dose, inhibition of acetylcholinesterase (AchE) in discrete brain regions and diaphragm muscle was dose dependent (Gupta and Kadel, 1991; Dourson et al, 1997) .

The soil half-life is a measure of the persistence of a pesticide in soil (Chapman, 1994). Pesticides can be categorized on the basis of their half- life as non-persistent, degrading to half the original concentration in less than 30 days, moderately persistent, degrading to half the original concentration in 30 to 100 days, or persistent, taking longer than 100 days to degrade to half the original concentration. A typical soil half-life value is an approximation and may vary greatly because persistence is sensitive to variations in site, soil, and climate.

The sorption coefficient (K_{oc}) describes the tendency of pesticide to bind to soil particles. Sorption retards movement, and may also increase persistence because the

pesticide is protected from degradation. The higher the K_{oc}, the greater the sorption potential. K_{oc} is derived from laboratory data. Many soil and pesticide factors may influence the actual sorption of a pesticide to soil (Bosatta and Agren, 1997).

The GUS or Groundwater Ubiquity Score is an empirically derived value that rates pesticide persistence (half life) and sorption in soil (sorption coefficient, Koc). The GUS may be used to rank pesticides for their potential to move toward groundwater. GUS=log10 (half-life) x [4-log10 (Koc)].

The pesticide movement rating is derived from the GUS. Movement rating range from extremely low to very high. Pesticides with a GUS less than 0.1 are considered to have an extremely low potential to move toward groundwater. Values of 1.0-2.0 are low, 2.0-3.0 are moderate, 3.0-4.0 are high, and values greater than 4.0 have a very high potential to move toward groundwater.

Water solubility describes the amount of pesticide that will dissolve in a known amount of water. Most of the values reported were determined at room temperature (20° C or 25° C). The higher the solubility value, the more soluble the pesticide. Highly soluble pesticides are more likely to be removed from the soil by runoff or by moving below the root zone with excess water.

1.3.3.1. Fate of the aldicarb

The disappearance of AS is accounted for by photodegradation, evaporation, leaching, chemical decomposition, and biotic intervention although, to varying degrees in different soils (Caux et al, 1996; Vink, 1997).

Several studies have shown that a major degradative pathway of AS in soils is the rapid oxidation of the parent compound to AS sulfoxide (ASO), followed by a slower oxidation to AS sulfone (ASO_2), or by hydrolysis to ASO oxime (Union Carbide, 1983). Additional work has suggested that AS oxime is degraded further to the corresponding nitriles (Andrews et al, 1971). Since all of the carbamoyl oximes (AS, ASO, and ASO_2, sometimes called the total toxic residue, "TTR") retain a high mammalian toxicity, hydrolysis step represents the major detoxification mechanism (Figure 1.5).

Several studies have discussed the degradation of AS, ASO, and ASO_2 in abiotic, reagent grade water solution. The investigators reported that alkaline hydrolysis is an important reaction mechanism in solution and that acid hydrolysis occurs at a lower rate. Degradation of ASO and ASO_2 was significantly affected by temperature and the reaction followed an Arhenieus relationship. Also, several investigators have reported that increase in the ionic strength significantly decrease the hydrolysis rate of the carbamate pesticides (Fukoto et. al, 1967, Lemley and Zhong, 1984).

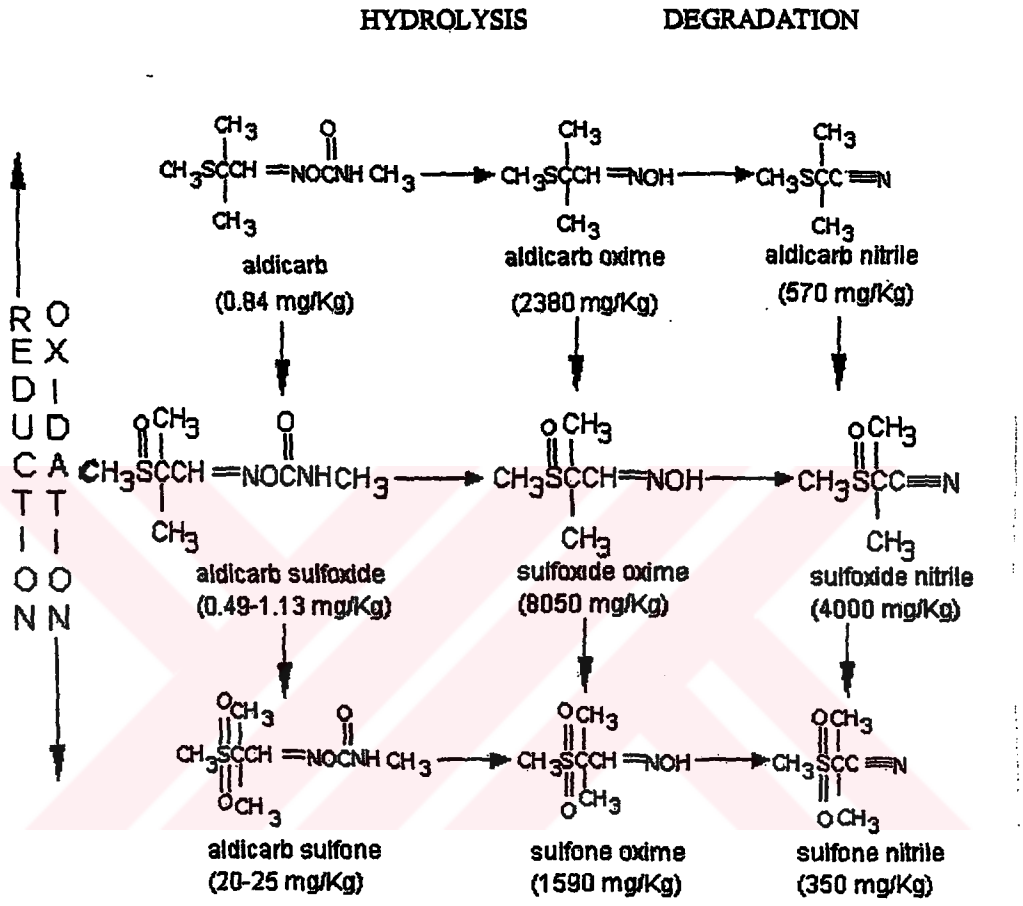


Figure 1.5. Degradative pathways of AS. Values in parenthesis are acute oral LD_{50} values for rats (after Union Carbide, 1983)

Although microbial degradation of AS is poorly understood, it has been shown to be oxidized to ASO and ASO_2, and hydrolyzed to AS oxime and nitrile (Ou, et al.,1985; Lemley and Zhong, 1984). Bank and Tyrrel, (1984) investigated acidic and alkaline hydrolysis of AS. At high pH values above 7.0, AS oxime, dimethyl urea, methyl amine and carbon dioxide were found to be the major products (Figure 1.6.). But

in acidic hydrolysis, at pH value below 5, an unusual acid-catalyzed reaction leading principally to AS nitrile and methylamine occurs.

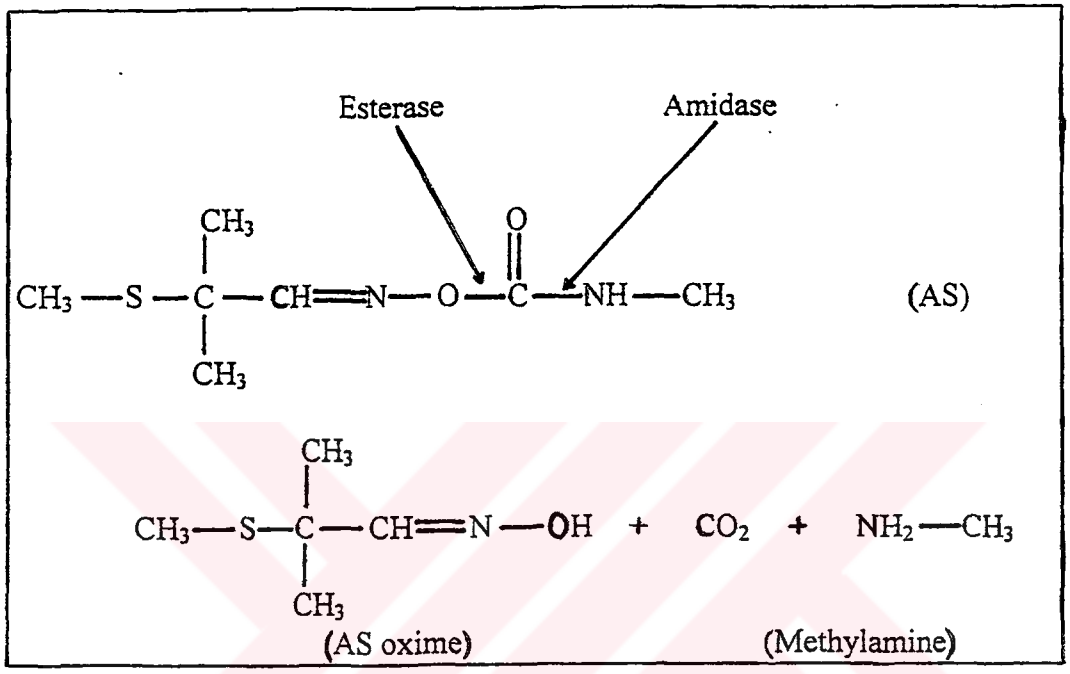


Figure 1.6. Possible enzymatic hydrolysis of AS (Bank and Tyrell, 1984)

In near neutral or alkaline soils, AS can more readily broken down by microorganisms to metabolites of lower toxicity (oximes and then to nitriles) and finally mineralized to CO_2. Under such conditions, toxic residues are less likely to be leached into ground water or well water (Read, 1987).

Attempts have been made to isolate microorganisms responsible for AS degradation in soil. Several microorganisms, such as isolates of *Fusarium* and

Penicillium, and isolates of *Arthrobacter*, *Pseudomonas, Nocardia, Achromobacter*, and *Bacillus* have been isolated from AS-treated soil (Read, 1987). The fungi slowly metabolized AS. Whereas a consortium of isolates collectively degraded the pesticide rapidly through collective action. To date, there have been no reports involving single isolates that can degrade AS, carbaryl, and carbofuran or their toxic metabolites completely. The degradation of AS was concentration dependent. Concentrations of pesticide higher than 800 and 5000 ppm inhibited bacterial and fungal growth, respectively (Chapalamadugu and Caudry, 1992).

The effect of ionic strength on the hydrolysis rates of AS was also surprising. Although the phosphate buffer concentration was increased 10 fold the hydrolysis rates were not changed. In the presence of high ionic concentrations, microorganisms (pH 6.8) or in the presence of ground limestone (pH 7.0 – 7.4) AS degraded rapidly to AS nitrile under anaerobic condition. In anaerobic ground water pH 8.2 AS degraded to AS oxime. These results contrast with AS degradation rates observed in other studies, but can be ascribed to high concentration of microbes.

The hydrolysis of AS under anaerobic conditions stimulated metanogenesis, as methylamine, a metabolite of AS, was utilized as a source of C1 units energy by methanogenic bacteria. Also the hydrolysis of N-methylcarbamates under aerobic conditions by methylotrophs was previously reported (Topp et al., 1993).This methylotrophic organism was able to utilize the methylamine product of the hydrolysis as a sole carbon and nitrogen source (Large and Haywood, 1981; Devries et al., 1990).

The characterization of C1 compound (methylamine) assimilation (Peel and Quayle, 1961; Halicigil, 2001) is complex but also required for the complete understanding of N-methylcarbamate pesticide biodegradation as their hydrolysis is the major detoxification route in nature.

One study (Khandaker and Young, 2000) reports on the kinetics of AS degradation under methanogenic conditions using batch reactors containing acclimated and unacclimated cultures under controlled conditions. Culture acclimation was accomplished by exposing anaerobic microorganisms maintained in a semi-batch reactor to low concentrations of aldicarb. Results of the kinetic studies showed that in an anaerobic system aldicarb is converted to aldicarb nitrile by the hydrolytic pathway. Analysis of the hydrolysis/dehydration rate constants showed that anaerobic cultures enhanced the rate of conversion of aldicarb by 4-fold for acclimated cultures and by 2-fold for unacclimated cultures compared to the rate of abiotic hydrolysis. Only the acclimated cultures were able to further mineralize the reaction intermediate aldicarb nitrile.

Heavy usage of AS together with the small amount of clay, silt, and organic matter in the sandy soil, a shallow water table, high solubility in water, weak adsorption by soil, and other factors may create the potential for ground-water contamination (Munster, 1995; Bailey and Love, 1999; Cova et al, 1990). Although at relatively low concentration, still is widespread problem (Bromilow et al, 1986; Rothschild et al, 1982; Cova et al, 1990). One possible approach to solving the contamination problem is

through microbial degradation (biodegradation), which is well-known to affect the persistence of most pesticides applied to the soil (Gottschalk and Knackmuss, 1993; Materon et al, 1987). Recent studies demonstrate that the danger of contamination is real and that the routes, through which chemical residues can reach ground waters, are complex depending on various internal and external factors.

Once in the aquifer some of the degradative pathways available in surface soils, such as photolysis by sunlight are no longer available and some, such as biolysis by soil microbes, are less likely. The major degradation process remaining is hydrolysis, and is unlikely without oxidative pathways.

An enzyme, carbufuran hydrolase, capable of hydrolyzing several N-methyl carbamate insecticides including carbaryl, carbofuran and AS has been purified (Chapalamadugu and Caudry, 1991; Karns and Tomasek, 1991).The optimum pH was broad (9.0-10.5) and the optimum temperature was between 45° and 53°C (Derbyshire, 1987). Chaudhry and Ali (1988) have reported that hydrolase activity is greater in isolates that utilize carbofuran as the sole source of carbon than in those that utilize it as the sole source of nitrogen. The hydrolase activity was increased with an increase in pH (maximum at pH 9.0), and temperature (maximum at 45°C) (Hayatsu et al., 2001). The degradative enzymes of these isolates are rather nonspecific, as far as the basic molecular structure of the target compound is concerned, and are specific to the carbamate moiety only. Enzymes with the capability to hydrolyse these compounds are

not still well characterized, and it is not clear if the enzymes that degrade these insecticides are interrelated (Sogorb and Vilanova, 2002).

A carbaryl hydrolase was purified to homogeneity from *Arthrobacter* sp. RC100 by protamine sulphate treatment, ammonium sulphate precipitation, and hydrophobic, anion-exchange, and gel filtration chromatographies. The hydrolase activity was strongly inhibited by DIFP, PMSF, Hg^{+2} and paraoxon but not by EDTA. The optimum pH and temperature for the enzyme activity were 9.0 and 50° C, respectively (Hayatsu et al., 2001).

1.3.3.2.. Analysis of AS and its derivatives

Investigation of residues of carbamates is often a delicate problem. A host of methods has been developed in the last few years for their determination. Most of these methods are based on gas or liquid chromatography (MacGarvey, 1993; Mora et al., 1995; Rouberty and Fournier, 1996). High performance liquid chromatography (HPLC) offers a simple, sensitive and rapid method for determination of AS, and its derivatives. When combined with multiple-wavelength UV detection and an appropriate pre-concentration step, this method, in principle can be applied to the routine monitoring of AS and its soil derivatives in water at concentrations less than 1 mg/L. UV absorbance has been used as a detection technique in HPLC determination of N-methylcarbamate pesticides, but Liquid chromatography (LC) is the favored technique for determination of NMCs as a class. Since the carbamates are somewhat polar and

thermally unstable, analysis by the more traditional gas chromatographic methods used for most pesticide residues is difficult. (Santos Delgado et al, 2001; Lawrence and Leduce, 1978; Krause, 1979; Miles and Delfine, 1984, 1985; Chaput, 1988; Trehy and Yost, 1984; Munster, 1995)

UV absorbance has been the most commonly used detection method in LC determination of NMC insecticides (Valenzuela et al, 1999; Balinova, 1998; McGarvey, 1993; Lawrence and Leduce, 1978; Rouberty, 1996; Cochrani, 1982; Wang, 1993; Spalik, 1982; Lin and Cooper, 1987; Miles and Delfino, 1984), probably due its wide applicability and consequent presence in most LC systems (Kramer, 1996). But this technique is subject to interferences of other co-extractives taken from the sample and also to the lack of sensitivity for some compounds at below 205 nm. In this respect, the development of diode array detectors has been the most important advance in HPLC quantification of such compounds, since they provide an opportunity to explore all wavelengths in the UV range and choose the monitoring wavelength which maximizes instrumental sensitivity (Nunes et al., 2000).

Post-column reactions with fluorescence detection methods for NMC analysis (Abad, 1999; Krause, 1979; Yang and Smetena, 1994; Sabala et al, 1997; Moye and Scherer, 1977) as the one described in the pesticide Analytical Manual of the Food and Drug Administration (FDA) and adapted by Chiron, (1993) offer a degree of selectivity and sensitivity, often an order magnitude or more over that offered by UV. This method was further validated; satisfactory recoveries were demonstrated to below

50 ppb. Post-column derivatisation affords low detection limits, and optimization has yielded determination limits of 5-50 ppb. Although the sample preparation step of this fully automated LC-fluorescence (FL) method has involved solid-phase extraction (SPE) (Johnson et al, 1997; Stuart et al, 1997; Koeber and Niessner, 1996) clean-up that is fast and provides a based-routine method for carbamate analysis in crude extracts, the main disadvantage of this analytical methodology is still the lack of confirmation of positive responses.

In the chromatographic field, several new methodologies for pesticide analysis are increasingly being applied. These include immunoaffinity chromatography, LC-MS with variable interfaces (Crescenzi, 1997), especially using atmospheric pressure chemical ionization devices and have undoubtedly contributed to increasing the separation efficiency and improving the sensitivities of the analytical methods (Nunes et al, 2000).

1.4. Soil decontamination

Tests in the laboratory and under outdoor conditions indicate that spills and accidents leading to comparatively high concentrations of xenobiotics in soil may be treated by the addition of specialized cultures. Inoculation with pure cultures degrading the contaminants shortens the time necessary for building up a population with the right biodegradation potential. It was shown to be effective for accelerating the degradation of parathion, 2,4,5- trichlorophenoxy-acetic acid, and pentachlorophenol. In the latter

case, the addition of 10 pentachlorophenol-utilizing *Artrobacter* cells per gram of dry soil reduced the half-life of the contaminant from 2 weeks to less than one day.

The enhancement of the biodegradation process at spilling sites is of great importance for preventing the distribution and dilution of pollutants, once xenobiotic pollutants have reached subsurface environments, they endanger aquifers. Their removal then becomes extremely difficult and costly. To control leaching and runoff, large investments for hydro-geological analysis, for drilling and for pumping out contaminated groundwater are necessary. Alternatively, physical removal and placement of contaminated soil in landfills are practiced in-situ biodegradation of pollutants is therefore of great economical.

1.5. Aim of the study

As a class, the N-methylcarbamates are some of the most widely used pesticides in agriculture. The ratio of total pesticide usage versus carbamate is presented in Figure 1.7.

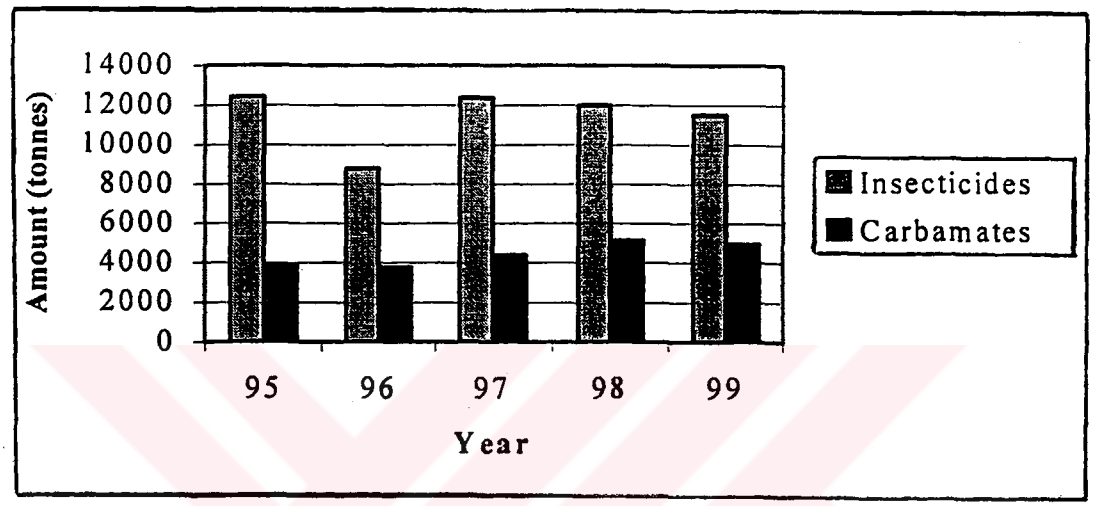


Figure 1.7. Total insecticide usage versus carbamate usage in Turkey (Data fom Turkish Ministry of Agriculture, personal contact, 2001)

The tremendous diversity in the type of chemicals to be detoxified mandated that many different disposal techniques are considered. Physical methods of incineration, entrapment, and burial were developed, as were chemical methods involving oxidation, reduction, and hydrolysis (Ottinger and Blumenthal, 1974). The use of biological treatment systems, persisted and this technology is being improved. An important aspect of earlier pesticide metabolism studies helped to develop a new approach to biological pesticide waste treatment technology.

The heavy use of AS creates problems locally in southern Turkey since, in this region AS has been used as the major pesticide against cotton pests. The present study therefore aims at complete microbial degradation of AS that would rely on development of biological treatment systems. Bacterial degradation is the method of choice because of the relative ease of growth, characterization and manipulation of bacterial cultures. Moreover, biological treatment is both cheaper and regarded as safe when compared to chemical and physical treatment methods.

CHAPTER II

MATERIAL AND METHODS

2.1 Materials

2.1.1 Chemicals

Analytical grade aldicarb [2-methyl-2-(methylthio) propionaldehyde O-(methylcarbamoyl) oxime)]; aldicarb sulphoxide [2-methyl-2-(methylsulfinyl) propionaldehyde O-(methylcarbamoyl) oxime); aldicarb sulfone [2-methyl-2-(methylsulphonyl) propionaldehyde O-(methylcarbamoyl) oxime) were purchased from Riedel-de Haen (Germany). Aldicarb oxime [2-methyl-2-(methylthio) propanal oxime] was purchased from Dr. Ehrenstorfer. Stock solutions were prepared by adding accurately weighed quantities of the analyte to varying volumes of the solvents like distilled water, chloroform, or methanol : water (50:50 by volume) as necessary. Stock solutions (4 g/L = 4000 ppm) of these compounds were periodically prepared, filter sterilized and stored in a dark bottle at 4°C.

Temik ®, a commercial and granular formulation of aldicarb, containing 10-15% (w/v) active ingradient (i.e. aldicarb), was kindly supplied by Rhone Polence (Istanbul). Stock solutions of temik (TAS) was prepared adding 20 gr of the granular product to 300 ml of distilled water to give a concentration of about 10,000 ppm. The mixture was stirred periodically for 48 hrs and then filter sterilized through 0.45 μm millipore filter to obtain a clear solution which was stored in a dark bottle at 4°C. Aldicarb has a water solubility of 6 g/l and therefore the filtered solution was assumed to contain 6000 ppm of the parent compound (aldicarb). Water used throughout the study was distilled, and ultra purified by Satin 9000 purification unit. All other chemicals used in this study are given in Appendix A.

2.1.2. Media and reagents

The composition of media and reagents used in this study are given in Appendix B.

2.2. Methods

2.2.1. Analytical methods used for detection of aldicarb and its metabolic product(s)

An essential complement to growth measurement is an adequate and preferably sensitive method of specific determination of the substrate. The proof of substrate disappearance by employing a detection method, with relevant controls,

coupled to quantification of growth is a strong evidence for biodegradation. The detection methods used throughout the study was as follows :

2.2.1.1. Spectrophotometric detection of aldicarb and its derivatives

Serial dilutions of the standard stock solutions of analytical grade aldicarb, aldicarb oxime, aldicarb sulfoxide, and aldicarb sulfone were prepared, using different solvents, such as water: methanol, chloroform, MM, and distilled water.

The absorption spectra of Temik, analytical grade aldicarb and its derivatives were scanned using Shimadzu UV-2100S spectrophotometer to determine the maximum absorption wavelengths (λ_{max}) of the corresponding compounds.

Calibration curves were constructed by plotting absorption (OD) values measured at λ_{max} versus concentration. All readings were made against a blank of the corresponding solvents.

2.2.1.2. Detection of aldicarb and its derivatives using TLC

TLC was employed to both seperate and identify AS, AS oxime, ASO and ASO_2. Samples for TLC were prepared by dissolving known quantities of the test compounds in different organic solvents. Aliquots (10-20 µl) of the samples were spotted on precoated TLC plates (silica-60 F_{254}, Merck) by micropipette.The spots were

dried by a dryer. Seperation was performed in a glass tank which contained the developing solvent to a depth of about 1.5 cm, with a glass plate over the top of the tank. This was allowed to stand for at least an hour to ensure the saturation with the appropriate solvent vapore (equilibrium). The chromatogram was run until the running solvent reached just the top of the plate.

The various solvents used for TLC analyses were as follows;

System A; benzene :dioxane (1:1 v/v)

System B; chloroform : n-hexane : ethyl acetate : dioxane (5:1:1:1 v/v)

System C; chloroform : n-hexane : ethyl acetate : ethanol (5:1:1:1 v/v)

System D; diethylether : n-hexane : ethanol (5:1:1 v/v)

System E; acetonitrile : water (4:1 v/v)

System F; chloroform : methanol (10:0.1 v/v)

System G; dichloromethane : methanol (10:0.1 v/v)

The chromatograms were visualized through exposure to UV light, or after staining with iodine and the R_f values of the compounds were determined.

Identification of the spots was based primarily on coincidence of R_f (relative front) values of the unknown samples with those of the authentic analytical grade compounds.

2.2.1.3. Detection of aldicarb and its derivatives using HPLC:

In the present study isocratic, reversed-phase HPLC (Wang et al.,1993; Miles and Delfino, 1984; Lin and Cooper, 1987) combined with UV detection (Shimadzu, C-R4A or SPD 10A) was employed for analysis. This may offer a simple, sensitive, and rapid method for the detection of AS, and its metabolic byproducts.

The solvent used as the mobile phase was methanol:water (40:60, or 50:50), with a flow rate of 1 ml/min. Cyanopropyl-silica (6mm x 15cm long, shimadzu Shim-pack CLC series; 4.6mm Ø x 25 cm long, Higgins analytical) columns were used for seperation .

Detection of samples were performed at their maximum absorption. The λ_{max} for AS and AS oxime were 246.5nm and 237 nm, respectively. But in order to be able to detect AS and its derivatives (AS oxime, ASO, and ASO_2) at the same time, a λ_{max} of 214 nm was employed.

Prior to analysis, the column was equilibrated with degassed mobile phase (degassing was done through helium gas substitution and continous Knauer Degasser Unit, or ultrasonic sound degasser) until stabilization of the base line.

Qualitative and quantitative analysis of AS and its metabolic byproducts were determined by the direct injection of 20 µl from the appropriate solutions.

Initially, the experimental growth medium was screened for the presence of any interfering medium ingradients, that could co-elute with any of the tested standards. Analytical grade aldicarb and its derivatives were used as references throughout the study. AS and AS oxime were then analysed and compared in terms of their retention times.

During the growth of bacteria in selective media, samples were withdrawn periodically from the cultures and microfuged at 10,000 rpm for 15 minutes at ambient temperature and then the clear, bacteria-free supernatents were collected and maintained at 4°C until analyses.

An HPLC calibration curve was constructed for analytical grade aldicarb at 246.5 nm, and aldicarb oxime at 237nm, as a standard. Amount of aldicarb and aldicarb oxime in culture medium was calculated from the constructed calibration curves. Analytical grade aldicarb (AS), aldicarb oxime (AS oxime), aldicarb sulfoxide (ASO), and aldicarb sulfone(ASO_2) could be readily seperated by reversed-phase HPLC.

2.2.2. Purification of Temik

Although bacteria could grow well in liquid and solidified Temik supplemented media, there was no detectable change in the amount of aldicarb as verified by HPLC and TLC analyses.

Temik was purified for its active ingradient aldicarb in two steps involving the extraction of TAS by an organic solvent followed by silica type I (sigma) column chromatography.

2.2.2.1. Extraction of Temik

Two different extraction procedures were performed using chloroform and diethyl ether.

In the first procedure, TAS was added to chloroform (1:10 w/v) in a dark glass bottle and occasionally mixed for a day. The extracts were filtered through 0.2 μm membrane filters and then the extracted material was collected by the complete evaporation of chloroform, under reduced pressure in a rotary evaporator at 30°C .

In the second procedure, TAS was added to diethylether (1:10 w/v) in a dark glass bottle and occasionally mixed for 2 hours. The extracts were filtered through 0.2 μm membrane filters and was left at -20°C for 18hr. The AS crystals formed by saturation were seperated, depending upon decrease in the temperature, from the organic solvent by filtration through a filter paper. The organic solvent remaining in in the crystals was removed under reduced pressure, using a rotary evaporator at 30°C.

2.2.2.2. Column chromatography

The extracted temik (EAS) were dissolved in chloroform:methanol (10:0.1), and the solution was applied to a silica gel type-I (sigma) column (1cm ID). The column was packed with silica type I and was pre-conditioned with enough eluting solvent. Fractions (2ml) were collected and analysed by spectrophotometer (Section 2.2.1.1), TLC (Section 2.2.1.2), and HPLC-UV detection (Section 2.2.1.3), as described above.

2.2.3. Isolation of extracted TAS utilizing bacteria

Bacterial isolates capable of degradading aldicarb as a sole source of carbon and energy were isolated from soil employing the enrichment method (Hardman et al., 1981; Chaudry, 1988).

2.2.3.1. Characterization of the AS ultizing isolate

Characterization tests were employed as in the Bergey's Manual of Determinative Bacteriology (1993).

2.2.3.2. Determination of biodegradative potential of the bacterial isolate

In order to follow the AS degradation potential of the isolated bacteria several criteria were selected as indications of AS degradation. The proof of substrate

disappearance by employing a detection method, with relevant controls, coupled to quantification of growth was assumed as a strong evidence for biodegradation.

2.2.3.2.1.Growth in MM-EAS medium

Bacterial growth in minimal salts medium supplemented with EAS (MM-EAS) as sole source carbon and energy is the primary evidence for the utilization of the respective substrate.

The isolated colonies were tested for their ability to grow in liquid culture. For this reason, a starter culture was prepared; bacteria were grown to the exponential growth phase at 30°C in 50 ml MMII supplemented with EAS (200ppm) and yeast extract (0.01%) with agitation (150 rpm). The cultures were asceptically harvested by centrifugation at 10,000 rpm for 15 minutes using a Sorvall centrifuge, washed twice with MMII to eliminate residual nutrients (yeast extract), and resuspended in 1ml of the same medium. This bacterial suspension was introduced to 100 ml MMII-EAS (200ppm) in 250 ml Erlenmeyer flasks. The batch cultures were grown at 30°C in MMII-EAS (200ppm) operating at 200rpm for 2-4 days.

Two sets of control flasks were run under the same conditions; one was composed of inoculated MMII (lacking EAS) as a control, and the other was uninoculated MMII-EAS (200ppm) for the determination of substrate stability.

The ability of isolates to grow on EAS as a sole source carbon and energy was determined by measuring the optical density of the cultures at 500 nm.

While bacterial isolates were grown in selective medium, 1ml samples were withdrawn at predetermined intervals for HPLC analyses.

2.2.3.2.2. Detection of AS utilization by the isolate

2.2.3.2.2.1. Spectrophotometric detection of bacterial EAS utilization

The possibility of measuring the decrease in the AS concentration, as it is consumed by the isolate, spectrophotometrically was investigated. Samples were withdrawn from the cultures, processed as described in Section 2.2.1.1. and their absorptions were measured at 246.5 nm.

2.2.3.2.2.2. Detection of bacterial AS utilization by HPLC

Aliquots were collected from the cultures as described above, and the HPLC analyses were carried out as given in Section 2.2.1.3. Controls for substrate stability was also examined.

2.2.4. Studies on the AS hydrolyzing enzyme

In this part of the study, the nature of the AS hydrolyzing enzyme was investigated.

2.2.4.1. Determination of enzymatic activity in post-culture supernatent

The bacterial cells are grown to the log phase in peptone yeast extract supplemented by 400 ppm AS (PYE-AS) and the bacterial growth was collected by centrifugation at 10,000 rpm for 10 min. 10ml of post-culture supernatant were filtered, and incubated with 200 ppm AS at 30°C for several hours. Aliquots of the reaction mixture were withdrawn at one hour intervals and analyzed by HPLC.

2.2.4.2. AS degradation studies in crude cell-free extract

A 1 L culture, grown for 18 h to A_{500}= 0.65 on PYE medium containing 2mM (400 ppm) aldicarb, was harvested and washed three times prior to cell lysis. 3 g of thawed and frozen cells were suspended in 3 ml of lysis buffer containing 50 mM Tris-HCL buffer (pH 7.5), 5% (v/v) glycerol, 1mM DTT and 1mM PMSF. The suspension was incubated at 4°C for 1 h after the addition 30 mg/ml lysozyme. The incubation was extended for 30 min after addition of 10mM $MgCl_2$ and 0.77 Kunits of DNAase I to remove DNA from the extract. To remove the cell debris, the extract was centrifuged at 27,000 g for 30 min. The supernatent was used as the cell-free crude

extract for aldicarb hydrolase assay.

2.2.4.2.1.The aldicarb hydrolase assay

The aldicarb hydrolase assay was carried out in 30 ml volume tubes in a total volume of 5 ml. The reaction mixture contained 50mM Tris-HCL buffer (pH 7.5), 4 μmole of aldicarb, and 0.5 ml of crude extract. The incubation was carried out at 30°C with shaking at 200 $rev.min^{-1}$ by a rotary shaker. Aliquots (0.9 ml) were withdrawn at intervals and centrifuged. The reaction was stopped by the addition 0.1 ml trichloroacetic acid (TCA) and analyzed for the residual aldicarb by HPLC.

2.2.4.2.2. Effect of TCA on AS concentration and stability

To examine the stability of the residual aldicarb in the samples treated with TCA, samples standards were prepared in 50 mM Tris-HCL buffer (pH 7.5) with different concentration of aldicarb, treated with water or TCA as in the experimental tests. Samples were withdrawn, from the TCA- treated and non-treated samples, analyzed by HPLC for residual aldicarb and the concentrations plotted against time.

2.2.5. Chemical production of aldicarb oxime from AS

AS oxime preparation by alkalene hydrolysis:

According to the study of Bank and Tyrrel (1984), treatment of AS with KOH leads to the rapid hydrolysis and production of the corresponding oxime (aldicarb oxime) and methylamine and CO_2 (see Figure 1.6).

For stock prepration of AS oxime, AS with a final concentration of 4000 ppm (20 mM) was treated with KOH (final concentration of 1M), incubated o/n and the stock were neutralized by HCl.

To detect the time period required for the conversion of AS to AS oxime, samples were taken at intervals and analyzed by HPLC.

2.2.6. Identification of AS metabolic by-products after bacterial treatment

The characterization of the compound accumulating after bacterial treatment is of extreme importance for the understanding the fate of AS utilization. The AS metabolic by-product was compared with analytical grade ASO, ASO_2 , and AS oxime through spectrophotometric, TLC and HLPC analysis.

2.2.6.1. The spectrophotometric analyses of AS oxime and the bacterial AS by-product

The spectrophotometric analyses were carried out by the determination of the λ_{max} values of AS derivatives in the ranges of 190-300 nm.

2.2.6.2. TLC analysis of AS oxime and the bacterial AS by-product

AS oxime and AS by-product were analyzed as described in Section 2.2.1.2.

2.2.6.3. HPLC analyses of AS oxime and the bacterial AS by-product

AS oxime and AS by-product were analyzed as described in Section 2.2.1.3.

2.2.6.4. NMR analyses of AS oxime and the bacterial AS by-product

Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were recorded with a Bruker GmbH DPW-400 MHz high performance digital FT-NMR spectrometer by using $CDCl_3$ as a solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts for ^{1}H-NMR are as in parts per million (Î´) downfield from an internal standard TMS. Spin multiplicities are mentioned as: s (singlet), d (doublet), dd (doublets of doublet), t (triplet), q (quarted), and m (multiplet).

2.2.7. Isolation of aldicarb oxime utilizing bacteria

Although aldicarb oxime is less toxic (Read, 1987; Ou et al.,1985; Lemley and Zhong, 1983; Miles and Delfino, 1985) than parent compound; it is still toxic and hence must be removed from the medium. Studies on the biodegradation of xenobiotics

stress utilization of the compounds as carbon sources, whereas other elements have received little attention as nutrient sources such as nitrogen and sulfur.

Therefore, the present study concentrated on furthur enrichment cultures in the hope of isolation of bacteria with the ability to completely utilize aldicarb oxime.

2.2.7.1. Collecting soil samples

Surface and subsurface soil samples from nine different cotton fields were collected in southern Turkey by the end of harvesting season with a history of Temik applications. Soil samples were sieved to remove debris before applying enrichment.

2.2.7.2. Enrichment of soil bacteria

Bacterial isolates capable of degradading aldicarb oxime were isolated from soil employing the enrichment methods (Cook and Hutter, 1981; Cook and Hutter, 1982).

2.2.7.2.1. Nitrogen Enrichment

Soil samples (20 g) were suspended in 100 ml of "buffered salts" solution (MMI) (see appendix B for the description of the media), shaken at 30°C for 1 hr, and

allowed to settle for 30 min. The supernatant was passed through a Whatman no.1 filter, and the organisms in the filtrate were used as inoculum.

Enrichment cultures were nonsterile, containing MMI suplemented with 50-200 ppm aldicarb oxime (as the sole source of nitrogen for growth), 1% w/v glucose, 5mM succinate, 10mM glycerol and 0.1 ml/ml of soil extracts as inoculum. The substrate was added to the sterile MMI as a filter-sterilized stock solution. The inoculated vials were incubated at 30°C on an orbital shaker operating at 200 rpm.

Parallel to the enrichments, two sets of controls were run under the same conditions; one was composed of inoculated MMI (lacking aldicarb oxime) as a growth control, and the other was uninoculated medium for the determination of substrate stability.

Samples were withdrown periodically from the batch cultures. they were then centrifuged and filtered before HPLC analyses.

2.2.7.2.2. Sulfur Enrichment

The same procedure given in Section 2.2.7.2.1. was repeated but, instead of MMI, MMIII (see appendix B for the description of the media) were used throughout the enrichment. In addition, AS oxime (50-100 ppm) were used as a sole source of sulfur, instead of nitrogen, for growth.

2.2.7.3. Selection of AS utilizing bacterial consortium

All enrichments were subcultured into homologous medium. Cultures indicating growth (estimated visually) as compared with growth control were considered as "positive enrichments".

Bacterial characterization tests were employed for the pure isolates in nitrogen-enriched bacterial consortium, by ID 32 GN system which is an automatic identification system using standardized and miniaturized assimilation tests with a specially adapted database.

2.2.8. Optimization studies on the nitrogen-enriched bacterial consortium

Preliminary attempts to isolate pure culture of the most efficient nitrogen-enriched bacterial consortium was unsuccessful and none of the pure isolates was able to utilize aldicarb oxime. Therefore, bacterial consortium were used in the following experiments.

Alterations in cultural conditions played an important role in the optimization of degradation rates for aldicarb oxime. The effect of AS oxime concentration on the biodegradative potential of bacterial consortium was examined by supplementing the MMI with 100, 200, 300, 400, and 600 ppm of AS oxime as an sole source of nitrogen for growth.

In order to determine the effect of pH on bacterial growth and AS oxime consumption, pH values of 5.5, 6.5, 7.2, and 8.0 were selected.

The effect of temperature on bacterial growth and hence on degradation of AS oxime was examined by subjecting the growth cultures to various incubation temperatures such as 20°C, 30°C, and 36°C while keeping all other parameters constant.

2.2.9. Detection of methylamine by 2,4-dinitrofluorobenzene

The method devised by Dubin (1960) was used for the detection and determination of volatile methylamine. 0.05 ml of neutralized methylamine solution (containing 10-150 μ-equivalents of free amino groups) was poured into a test tube, containing 0.05 ml of reagent C, and incubated for 10 min at 65°C. At the end of incubation, 1.0 ml of reagent D was added to the mixture, and the whole sample was analysed spectrophotometrically at 360 nm. (see Appendix for description of the reagents)

2.2.9.1.Construction of calibration curve for MA

The absorption spectrum of MA in the range 200-450 nm was measured and the λ_{max} value was determined.

Different concentration of methylamine (MA) were prepared and processed,

using the predetermined λ_{max} value. A standard curve was then constructed by plotting the absorbance of the sample measured against the concentration of MA.

2.2.9.2. Analysis of the MA-containing samples

Samples were analysed as described in Section 2.2.9. and corresponding concentrations were calculated using the standard MA curve.

2.2.10. Purification of the AS oxime stock from methylamine

As AS oxime was not the only nitrogen source in the growth medium, it causes also the outgrowth of the methylamine-utilizing bacteria in the enrichment culture.

2.2.10.1. Solvent extraction

Two different solvent systems were used; using chloroform, and dichloromethane.

AS oxime stock solution was added to chloroform or dichloromethane and occasionally mixed. The extraction was repeated three times. The extracted materials were collected by the complete evaporation of solvents, under reduced pressure in a

rotary evaporator at 30°C. Samples were then taken and analyzed for the existance of methylamine as described in Section 2.2.9.

2.2.10.2. Microbial removal of methylamine

In order to demethylaminate the growth medium, the bacterial suspension of *Pseudomonas putida*, isolated from the nitrogen-enriched bacterial consortium, was introduced to 100 ml MMI-AS oxime (100ppm) in 250 ml Erlenmeyer flasks. The batch culture was grown at 30°C operating at 200 rpm for 1 day. Samples were taken to detect the existance of methylamine (as described in Section 2.2.9) and AS oxime by HPLC. The culture was harvested by centrifugation at 10,000 rpm for 15 minutes using a Sorvall centrifuge.

The supernatent was supplemented with MMI components (KP buffer, glucose, Mg_2SO_4, and TES; see Appendix B for description), filter-sterilized and used as methylamine free MMI-AS oxime (100ppm) (MAFMM).

2.2.11. Physiological studies involving the microbial consortium

In order to characterize the interrelations of bacterial isolates present in bacterial consortium, different combinations of the isolates were constructed. The samples were taken periodically for HPLC analysis.

It was shown that the predominant ones for the degradation of aldicarb oxime were *Pseudomonas putida* and *Methylobacterium mesophilicum.*

2.2.12. Studies on the *Pseudomonas putida* isolate

2.2.12.1. Bacteriological tests on solidified media

Effect of different amino acids supplementation (auxonography) on bacterial growth and on the biodegradative potential of the *Pseudomonas putida*, was investigated by spotting MMI-AS oxime agar plates with crystals of the amino acids.

The particular amino acids that enhanced the AS oxime utilization and growth (as revealed by auxonography) were selected and supplied to MMI-AS oxime liquid culture. The samples were taken periodically and analysed for HPLC analysis .

2.2.13. Studies on the *Methylobacterium mesophilicum*

2.2.13.1. Determination of biodegradative potential of the bacterial isolate

In order to follow the AS oxime degradation potential of the isolated bacteria several criteria were selected as indications of AS oxime degradation. The proof of substrate disappearance by employing a detection method coupled to quantification of growth was measured as a strong evidence for biodegradation.

2.2.13.1.1.Growth studies

Bacterial growth in minimal salts medium supplemented with AS oxime (MM-AS oxime medium) as the sole source of nitrogen is the primary evidence for the utilization of the respective substrate.

The starter cultures were prepared as follows; bacteria were grown to the exponential growth phase at 30°C in 50 ml of MMI (for nitrogen-enriched bacterial isolates) supplemented with AS oxime (100ppm) and yeast extract (0.02%) with agitation (180 rpm). The cultures were asceptically harvested by centrifugation at 10,000 rpm for 15 minutes using a Sorvall centrifuge, washed twice with MMI to eliminate residual nutrients (yeast extract), and resuspended in 1ml of the same medium. This bacterial suspension was used as inoculum in all experiments.

Two sets of control flasks were run under the same conditions; one was composed of inoculated MMI (lacking AS oxime) as a control, and the other was uninoculated MM-AS oxime (100 ppm) for the determination of substrate stability.

The ability of isolates to grow on AS oxime as a sole source nitrogen for growth was determined by performing the viable cell count by spread plating.

While bacterial isolates were grown in selective medium, 1ml samples were withdrawn at predetermined intervals for HPLC analyses.

2.2.13.1.1.1. Growth in methylamine free medium

Methylamine free MMI-AS oxime (100 ppm) was prepared by using solvent-extracted AS oxime stock solution, and/or microbial removal of methylamine (MA) by *Pseudomonas putida* as described in Section 2.2.9.1. and 2.2.9.2. respectively. Samples taken and analysed for the existance of MA. In the latter, upon complete utilization of MA by *Pseudomonas putida* (20 hrs), the culture centrifuged at 10,000rpm for 10 min. The supernatant containing AS oxime, as verified by HPLC, supplemented with the MMI components, filter-sterilized and used as methylamine free MMI-AS oxime (100 ppm) medium. *Methylobacterium mesophilicum* was inoculated into the methylamine free medium. Growth and degradation was followed and analysed by HPLC.

2.2.13.2. Optimization studies

In order to achieve the highest degradation rate, the cultural conditions involving pesticides concentration, pH, and temperature were studied.

The effect of AS oxime concentration on the biodegradative potential of bacterial consortium was examined by supplementing the MMI with 100, 200, 400, and 600 ppm of AS oxime as an sole source of nitrogen for growth.

In order to determine the effect of pH on AS oxime consumption, pH values of 5.5, 6.5, 7.2, and 8.0 were selected.

The effect of temperature was examined by subjecting the growth cultures to various incubation temperatures such as RT (25°C) , 30°C, and 40°C while keeping all other parameters constant.

In order to detect the nutritional requirements, MMI-AS oxime growth medium was supplemented with yeast extract (0.02%), peptone (0.02%), tryptone (0.02%), or vitamine solution (1%) and compared with MMI-AS oxime growth medium without supplements.

2.2.14. AS oxime as a sole source of carbon and nitrogen

AS oxime biodegradation were detected in glucose-free MMI-AS oxime (as the sole source of carbon and nitrogen) and in MMI-AS oxime (the sole source of nitrogen) containing glucose. Samples were taken periodically for HPLC analyses.

2.2.15. Bacterial Utilization of AS oxime in a rich medium

In order to detect the effect of rich media on AS oxime utilization *Methylobacterium mesophilicum* was inoculated in pepton-yeast extract (PYE), nutrient broth, and LB containing 100 ppm AS oxime. The bacterial culture were grown at 30°C

on a rotary shaker (200rpm) for 4 days. The samples were taken periodically, and analyzed by HPLC.

The effect of AS oxime concentration on the biodegradative potential of bacterial isolate was examined by supplementing the MMI with 50, 100, 200, 400, and 600 ppm of AS oxime. The bacterial culture were grown at 30°C on a rotary shaker (200rpm) for 10 days.The samples were taken periodically, and analyzed by HPLC.

2.2.16. Studies on the AS oxime hydrolyzing enzyme

In this part of the study, the activity of the AS oxime hydrolyzing enzyme was determined in cell-free crude extracts of PYE-grown cells.

2.2.16.1. Effect of TCA on AS oxime concentration and stability

To examine the stability of the residual aldicarb oxime in the samples treated with trichloroacetic acid (TCA), samples standards were prepared in 100 mM KP buffer (pH 7.0), treated with water or TCA as in the experimental tests. Samples were withdrawn, from the TCA- treated and non-treated samples, analyzed by HPLC for residual aldicarb and the concentrations plotted against time.

in a total volume of 1 ml. The reaction mixtures had the ratio of 1:1 cell-free crude extract:AS oxime-buffer (for the supernatent fraction of the extract), using 100 mM KP buffer (pH 7.0) containing 1mM 2-mercaptoethanol and AS oxime (2800 ppm) stock solution. The reaction mixture for the cell debris (the pellet of the cell-free crude extract, obtained from 1L of the bacterial culture) was prepared by dissolving the pellet in 2 ml of 100 mM KP buffer (pH 7.0) containing 1mM 2-mercaptoethanol and the reaction was started by addition of AS oxime.

Reaction mixtures containing changing concentration of AS oxime was prepared. The incubation was carried out at 30°C with shaking at 200 $rev.min^{-1}$ in a shaking water bath. Aliquots were withdrawn at intervals, centrifuged and analyzed for the residual aldicarb oxime by HPLC.

2.2.16.3.2. Optimization studies

AS oxime concentration in the range of 25-200 ppm were incubated with cell debris (the pellet of crude cell-free extract).

The pH values in the range of 6.0-8.0 were examined. Aliquots of the reaction mixture were withdrawn periodically and analyzed by HPLC.

2.2.16.3.3. Induction studies in crude extract

Two sets of bacterial culture were prepared, in which the only difference is existance of AS oxime.

In the first set, bacterial culture were grown in PYE-AS oxime (100ppm) medium. The culture was grown to the late log phase at 30°C. After centrifugation at 10,000 rpm for 15 min in a Sorvall centrifuge, the pellet was treated as described in Section 2.2.16.3. AS oxime enzyme assay was performed as described in Section 2.2.16.3.1.

In the second set, the bacterial cells were grown to the late log phase in PYE at 30°C, without supplementation of AS oxime, to detect the inducing effect of the substrate (AS oxime), and treated as described in the first set.

2.2.16.4. Extraction and solubilization of membrane proteins

Our results indicated that AS oxime hydrolysing enzyme is a membrane-bound insoluble protein, therefore further effords focused on the solubilization and extraction of the responsible enzyme from the membrane. Different methods were followed as listed bellow.

2.2.16.4.1.Enzyme extraction by osmotic shock

Thawed and frozed bacterial cells (see Section 2.2.16.3.) were suspended (gram wet weight per 2.5 ml) in 100mM KP (pH 7.0) containing 1mM 2-mercaptoethanol. Then EDTA (final concentration 1.22%) and sucrose (final concentration 20%) were added using stock solutions. After 10 min of stirring in an ice bath, the treated cells were recovered by centrifugation at 4°C and immediately resuspended in a suitable volume of cold distilled water to recreate the original concentration. The mixture was stirred at 4°C for 10 min and the supernatent was removed by centrifugation at 10,000 rpm for 15 min (Ertan, 1995; Rodriguez et al., 1992).

2.2.16.4.2. Enzyme extraction by chloroform shock

Two different procedure were followed:

In the first one, 0.33 ml of chloroform was added to the 0.4 g of thawed and frozed bacterial cells (see Section 2.2.16.3.) which suspended in 1 ml of 100 mM KP (pH 7.0) containing 1mM 2-mercaptoethanol. The mixture was incubated at room temperature for one hour by slightly shaking (Ames et al, 1984; Ertan, 1995).

In the second one, thawed and frozed bacterial cells were sonicated (see Section 2.2.16.3.). The resulting cell debris (pellet of cell-free crude extract) was

suspended with 0.5 ml of chloroform/methanol (2:1 v/v) and shaken for 15 min at room temperature. After centrifugation at 10,000 rpm for 15 min at 4°C, the solvent was removed and pellet was suspended to two further extraction cycles with chloroform/methanol. Finally, the pellet was suspended in 100mM KP (pH 7.0) containing 1mM 2-mercaptoethanol (Orazio, D.M, *et al.*, 2001).

2.2.16.4.3. Enzyme extraction by Triton-X 100 treatment

Two different procedure were followed:

In the first one, thawed and frozed bacterial cells (see Section 2.2.16.3.) were sonicated (see Section 2.2.16.3). The resulting cell debris (pellet of cell-free crude extract) was suspended in 0.1 ml of 100mM KP (pH 7.0) containing 1mM 2-mercaptoethanol , 0.1mM EDTA /100mM NaCl / 1% Triton X-100 and kept on ice for 1 h. Finally, the mixture was centrifuged at 10,000 rpm for 15 min at 4°C (Orazio, D.M, *et al.*, 2001).

In the second one, higher concentration of the Triton X-100 was used. Thawed and frozed bacterial cells were sonicated. The resulting cell debris was suspended in 800 µl of solubilization buffer [100mM KP (pH 7.0) containing 1mM 2-mercaptoethanol, 1.25 % glycerol, 1.25 mM EDTA]. While stirring at 4°C, enzyme was extracted with 200 µl of Triton X-100 (using 20 % solution) which was added stepwise (10 x 20 µl). After stirring on ice for 1 h, the solubilizate was centrifuged for 30 min at

10,000rpm. The supernatent (1 ml) was transferred to a fresh tube. The pellet was resuspended again in 800 µl of solubilization buffer and the solubilization procedure was repeated once. Finally, the mixture was centrifuged at 10,000 rpm for 15 min at 4°C.

2.2.16.4.4. Enzyme extraction by lysozyme treatment

Thawed and frozed bacterial cells were sonicated. The resulting cell debris was suspended in 1 ml of 50 mM Tris-Hcl (pH 7.2) containing 170 mM NaCl, 16mM $MgCl_2$, lysozyme (0.5 mg/ml), ribonuclease A (0.77 Kunitz units/ml), and deoxyribonuclease I (27 Kunitz units/ml). Stirring was continued for 1 h on ice. The solution was incubated overnight. Finally, the mixture was centrifuged at 10,000 rpm for 15 min at 4°C.

2.2.16.4.5. Enzyme extraction by combination of Triton-X 100 and lysozyme

The same procedure as described in Section 2.2.16.4.4. was repeated. But this time 10 mg/ml of lysozyme was used. Triton-X 100 (1%) was also added to the mixture. Stirring was continued for 1 h on an ice (Novella et al., 1994).

2.2.16.4.6. Enzyme extraction by EDTA treatment

Thawed and frozed bacterial cells were sonicated. The resulting cell debris was suspended in 10 mM EDTA-100mM KP (pH 7.0) containing 1mM 2-mercaptoethanol. The cell suspension was kept on ice for one hour and then centrifuged at 10,000 rpm for 15 min.

2.2.17. Construction of a bacterial consortium for complete microbial degradation of aldicarb

A two-membered bacterial consortium were constructed consisting of facultative methylotrophic isolate and *Methylobacterium mesophilicum*, degrading aldicarb to aldicarb oxime and aldicarb oxime alone, respectively. Two different procedure were followed:

In the first procedure, facultative methylotrophic isolate was inoculated to the MMII-EAS medium. Upon complete conversion of aldicarb to aldicarb oxime (as detected by HPLC), the culture medium was supplemented with MMI components and inoculated with *Methylobacterium mesophilicum* isolate.

In the second procedure, both bacterial isolates (facultative methylotrophic and *Methylobacterium mesophilicum* isolates) were inoculated to MMII-EAS growth

medium at the start of the experiment.The bacterial culture were grown at 30°C on a rotary shaker (200rpm).

2.2.18. In vitro utilization of aldicarb by cell-free crude extract

Crude extracts were prepared from each of the bacterial isolates as described earlier. The cell-free crude extract of facultative methylotrophic isolate was added into the reaction mixture tube containing aldicarb as the substrate (see Section 2.2.4.2.1). Aliquots of the reaction mixture were withdrawn periodically and analyzed by HPLC. The HPLC analysis indicated that AS was completely converted to its metabolic byproduct, that is AS oxime. In the second part of the experiment, the cell debris (pellet of cell-free crude extract) of *Methylobacterium mesophilicum* was incorporated into the same reaction mixture tube. Samples were taken periodically and analysed by HPLC.

CHAPTER III

RESULT AND DISCUSSION

3.1. Isolation of bacterial strains capable of utilizing aldicarb as the sole source of carbon and energy

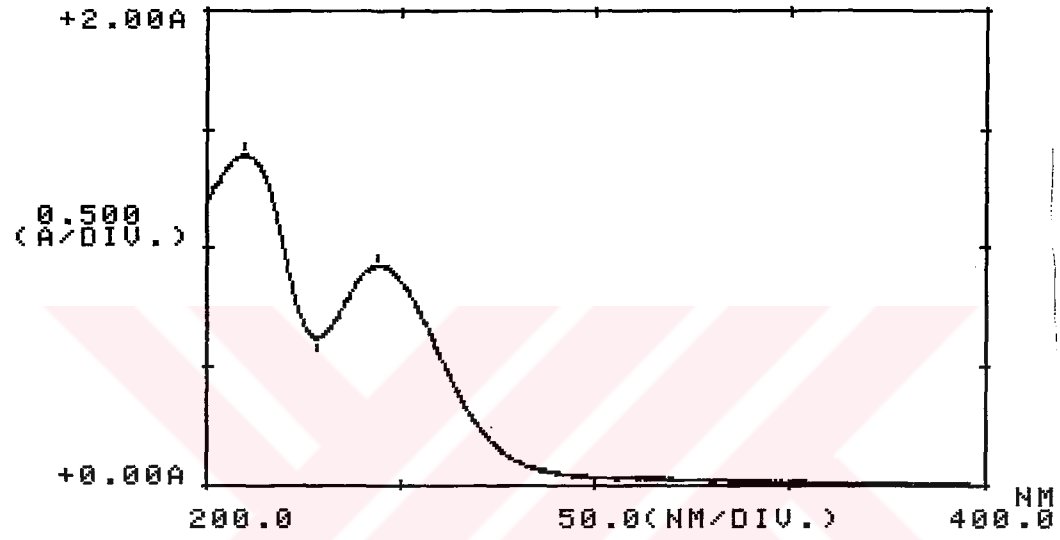
Preliminary enrichment experiments (Asghari, 1993) led to the isolation of bacterial strains in pure form. The selection was based on the ability of the isolates to grow on MMII-AS plates, in which aldicarb (AS) was the only carbon and energy source.

3.2. Detection of AS and its derivatives

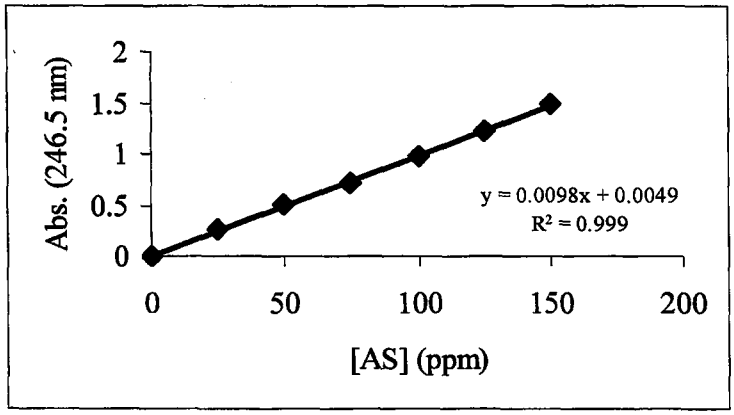
3.2.1. Spectrophotometric detection of AS and AS oxime

The UV spectrum of analytical grade AS and AS oxime, and their calibration curves was linear over the range examined as illustrated in Figures 3.1-3.4. Spectrophotometric data of the pesticide (AS) and its metabolic by product (AS oxime) are listed in Table 3.1. The λ_{max} values (Table 3.2) were to be determined for the

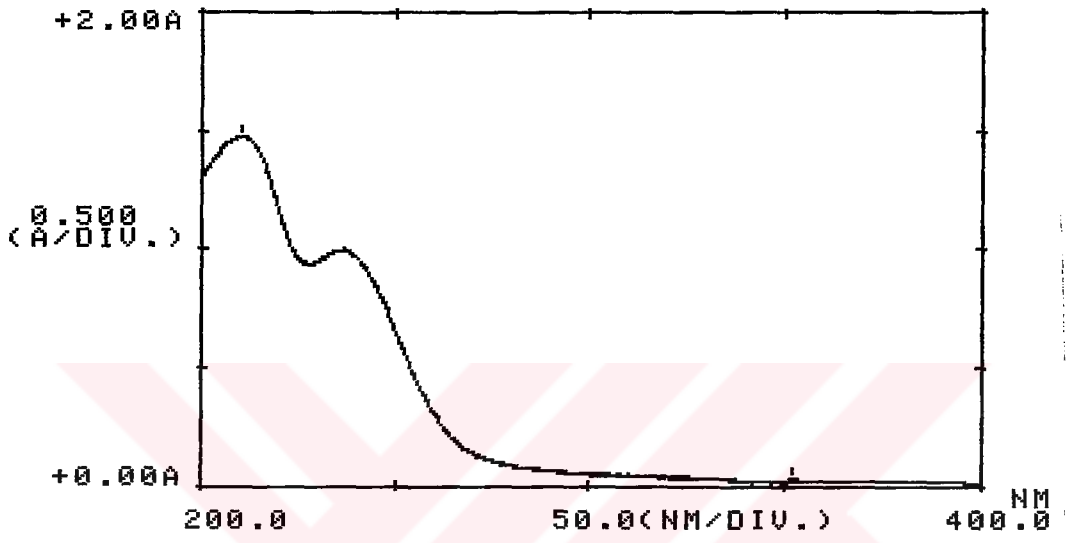
are listed in Table 3.1. The λ_{max} values (Table 3.2) were to be determined for the qualitative and quantitative analysis of these compounds by HPLC equipped with an UV/Vis Spectrophotometric detector.



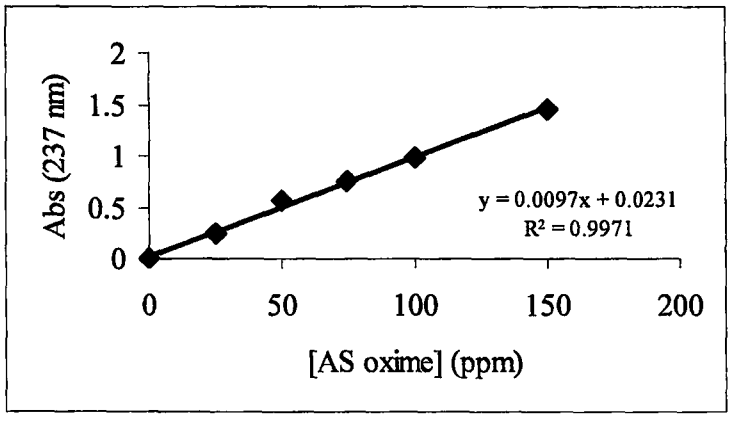
Figures 3.1. The UV spectrum of analytical grade aldicarb



Figures 3.2. The calibration curve for analytical grade aldicarb (λ_{max} : 246.5 nm)



Figures 3.3. The UV spectrum of analytical grade aldicarb oxime



Figures 3.4. The calibration curve for analytical grade aldicarb oxime (λ_{max} : 237 nm)

In spite of linearity of AS and AS oxime calibration curves, the routine monitoring of AS and AS oxime was not possible because of overlapping occured in their UV-spectra in the growth media after utilization of the parent compound. The spectrophotometric detection of AS and AS oxime only helped in the detection of their λ_{max} values, and the construction of relevant calibration curves. This phenomenon was reported by Miles and Delfino (1984). Therefore, the spectrophotometric detection was not a reliable method in AS biodegradation studies.

Table 3.1. Spectrophotometric data of the pesticide (AS) and its metabolic by product (AS oxime)

Pesticides	Peak Data
	λ_{max} (nm)
AS	246.5 205
AS oxime	237.1 207.5

The λ_{max} values chosen to use in HPLC studies are given in Table 3.2.

Table 3.2. The λ_{max} values of AS and AS oxime

Pesticides	λ_{max} (nm)
AS	246.5
AS oxime	237

3.2.2. Detection of AS and its derivatives by HPLC

High-performance liquid chromatography (HPLC) offers a simple, sensitive and rapid method for determination of aldicarb and its metabolic byproducts. In this study it has been demonstrated that reverse-phase HPLC equipped with UV detection is a reliable method for the rapid and sensitive determination of AS, AS oxime, ASO, ASO_2, AS nitrile and other metabolic byproducts. In about less than 10 min, AS and its derivatives could be unambiguously seperated through a single run. The retention times of the AS and AS oxime in the corresponding systems were determined and listed in Table 3.3.

Table 3.3. The retention times of the compound in the corresponding systems

Column	Retention Times (min)	
	AS	AS oxime
CN-silica (Shimatzu Shim-pack; 6mm Ø x 15cm long)	6.3	5.6
CN-silica (Higgines Analytical; 4.6mm Ø x 25 cm long)	5.0	4.7
C 18 (Higgines Analytical; 4.6mm Ø x 25 cm long)	8.89	8.51
C 8 (Shimatzu Shim-pack; 6mm Ø x 15cm long)	8.2	7.3

The detection of AS and its derivatives was simultaneously recorded as 214 nm. A chromatogram from a mixture of AS and its metabolites seperated isocratically with a mobile phase of water:methanol (60:40 v/v) on a CN silica stationary phase at 214 nm with a flow rate of 1 ml/min, is presented in Figure 3.5.

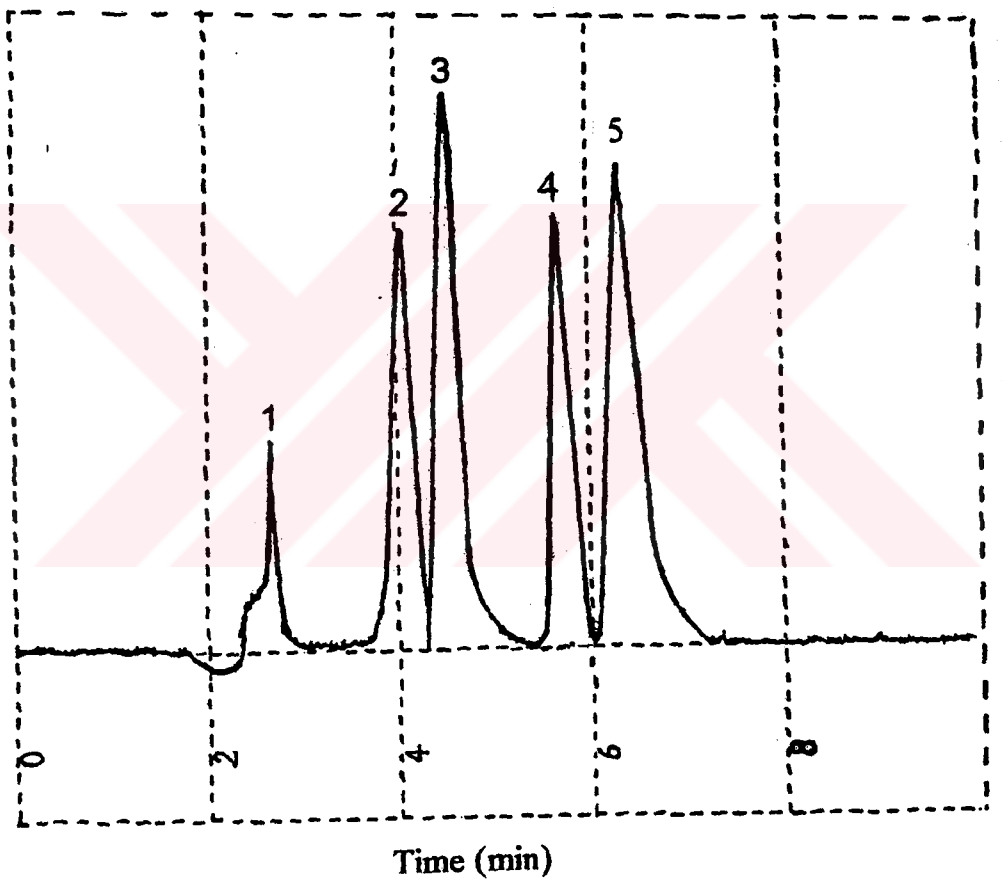


Figure 3.5. The chromatogram from a mixture of AS and its metabolites seperated isocratically with a mobile phase of water:methanol (60:40 v/v) on a CN silica (Shimadzu; 6mm x 15cm long) stationary phase at 214nm with a flow rate of 1 ml/min. 1: MM (2.5), 2:ASO (4.099), 3:ASO_2 (4.55), 4:AS oxime (5.6), 5:AS (6.3)

In order to verify the biodegradative potentials of the isolated bacteria, batch cultures were prepared and subjected to HPLC analysis. By this way any detectable loss in AS or AS oxime could be directly attributed to the bacterial utilization.

3.2.2.1. Construction of standard curves

The standard curves are given in Figures 3.6., and 3.7. The results showed that the peak areas vs. concentrations of the compound were linear in the measured concentration range (25 ppm-200 ppm).

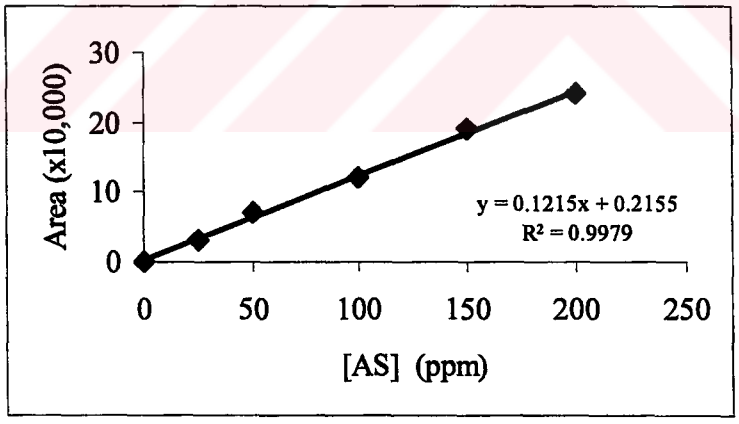


Figure 3.6. The calibration curve of AS seperated isocratically with a mobile phase of water:methanol (50:50 v/v) on a CN silica stationary phase at 246.5 nm with a flow rate of 1 ml/min.

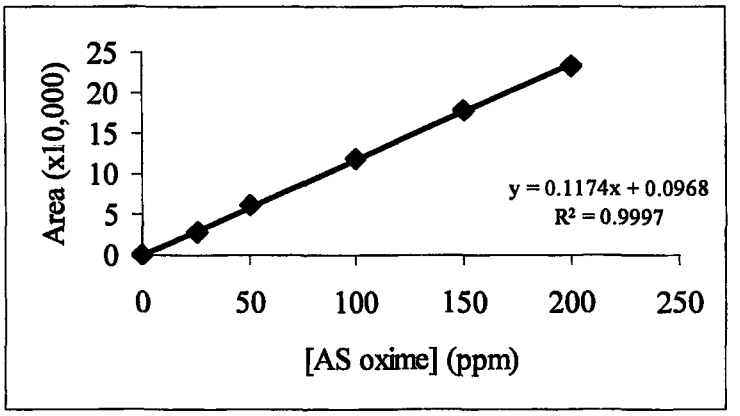


Figure 3.7. The calibration curve of AS oxime seperated isocratically with HPLC and mobile phase of water:methanol (50:50 v/v) on a CN silica stationary phase (Higgines Analytical; 4.6mm Ø x 25cm long) at 237 nm with a flow rate of 1 ml/min.

3.2.3. Detection of AS and its derivatives by TLC

The relative front (R_f) values of AS, TAS, Chloroform-extracted TAS, and its derivatives are given in Table 3.4. and Table 3.6.

Table 3.4. R_f (relative front) values of analytical grade AS, TAS, and Chloroform-extracted TAS in TLC employing different solvent systems

Compound	Solvent Systems and Relative front values (R_f)						
	A	B	C	D	E	F	G
Analytical grade AS	0.79	0.84	0.89	0.9	0.9	0.41	0.22
TAS	0.79	0.84	0.89	0.9	0.9	0.41	0.22
Aldicarb in chloroform extracted TAS	0.79	0.84	0.89	0.9	0.9	0.41	0.22
Inpurity in chloroform extracted TAS	0	0.09	0	0.12	0	0.043	0

3.3. Isolation of AS utilizing bacteria

An enrichment culture was obtained, which could quantitatively degrade 200 ppm AS within 4 days, as verified by HPLC (Asghari, N., 1993). For this reason, each colony was grown in MMII supplemented with 200 ppm AS to check their growth and biodegradative potential. HPLC analysis showed that all of the isolates had the ability to degrade AS with different biodegradation rate. All of the isolates converted AS to the same metabolite.

3.3.1. Characterization of the most efficient bacterial isolate

The preliminary characterization studies showed that the selected bacteria were aerobic, gram negative, slightly curved rods that occured singly and in pairs. Colonies on MMII agar plates supplemented with 2 mM AS (400ppm) were circular, and approximately 0.5 mm in diameter and slightly grayish white and translucent after incubation at 30° C for 4 days. Incubation in PYE agar medium for 2 days, however, resulted yellowish white and translucent colonies, about 3 mm in diameter

According to Bergey's Manual of Systematic Bacteriology (1994), single-carbon compound (C_1) utilization is an important criterion that makes the identification easier. The ability of the isolate to grow on different carbon sources including C_1 compounds shows that the isolate is a facultative methylotroph.

3.3.2. Determination of AS utilization by HPLC in MMII

Examination of samples collected periodically from minimal growth medium by HPLC indicated that AS concentration decreased with a concomittant accumulation of the new metabolic byproduct of AS and increase in culture turbidity. No traces of AS could be detected in the growth medium at the end of incubation period. (Figure 3.8 and 3.9).

To check whether the new AS metabolite was biologically but not spontanously generated, uninoculated control samples were also included in similar conditions and analysed. The results indicated no detectable degradation or transformation of AS (the same concentration as inoculated growth media) in the uninoculated cultures, thus implying the involvement of microbial process in the transformation of AS.

The results showed that the highest degradation rate were achieved when the microbial isolate were grown in MMII with 400 ppm (2mM) EAS (solvent-extracted AS) at 30°C in an alkaline pH (Figure 3.8).

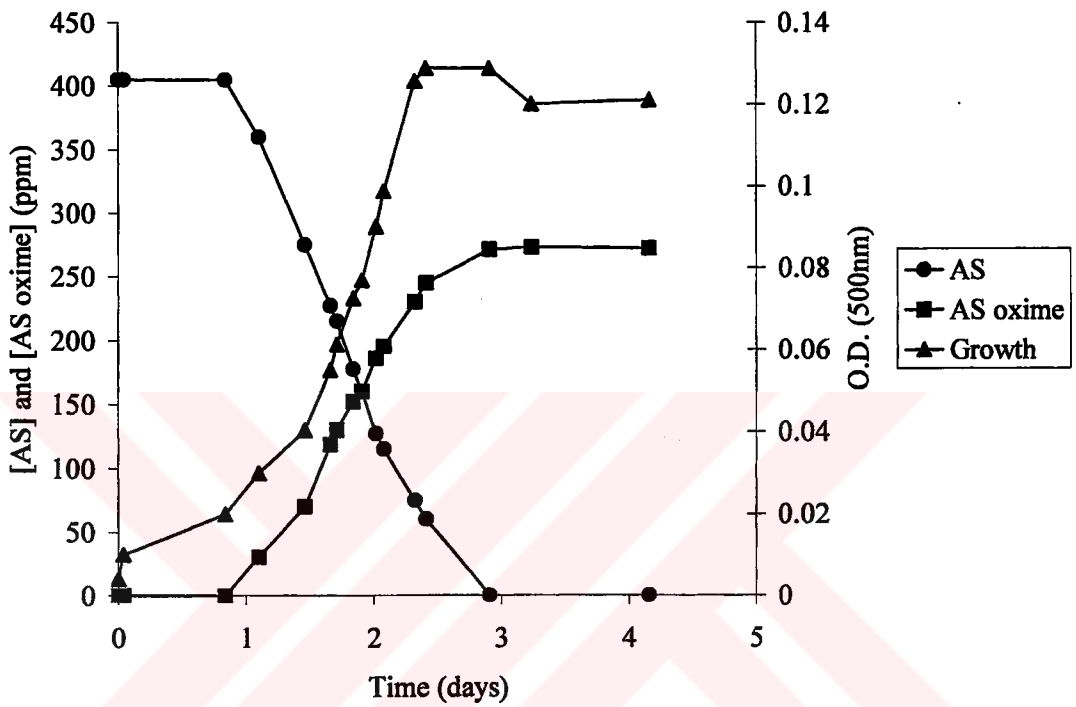


Figure 3.8. The biodegradation of AS (400 ppm) in MMII vs. growth by the bacterial isolate

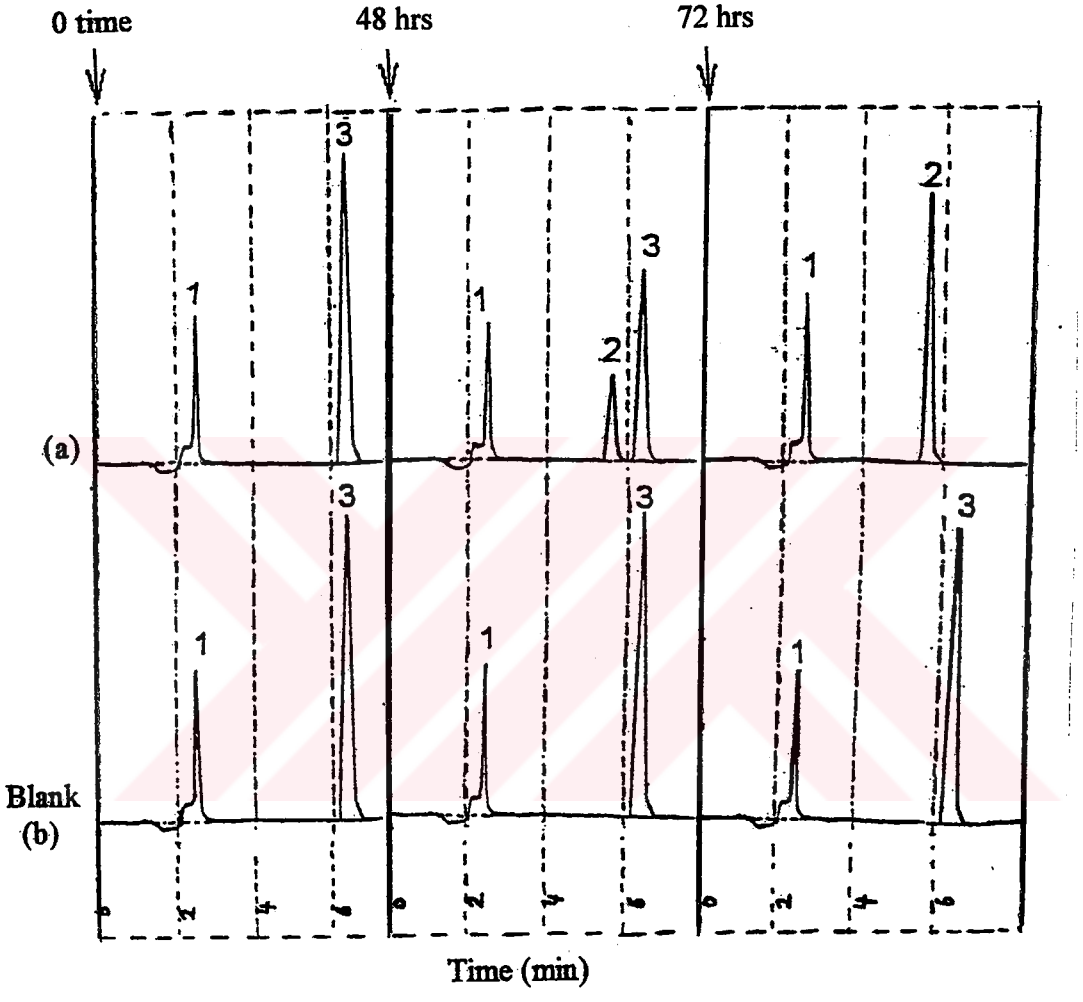


Figure 3.9. EAS loss profile. The HPLC chromatograms of a) EAS degradation in the growth medium, b) Uninoculated control (MMII-EAS) using Cyanopropyl-silica column (shimadzu Shim-pack; 6mm x 15cm long). 1:MMII, 2:Newly-produced AS metabolic byproduct, and 3:AS.

3.4. Studies on the aldicarb hydrolysing enzyme

The HPLC analysis showed that no enzymatic activity could be detected in the post-culture supernatent of the growth media as there was no AS breakdown. Cell-free crude extract, however, showed a high level of AS degrading enzyme activity. This results indicated that the enzyme system(s) responsible for AS biodegradation is exclusively intracellular.

The quantitative conversion of AS to its oxime derivative and to methylamine suggested that the organism contained a carbamate hydrolysing enzyme activity. Such activity was found in peptone yeast extract (PYE)-grown cell extracts or methylamine-grown cell extracts (Figure 3.10.). Extracts catalysed the degradation of AS (4mM) in Tris-HCL buffer (pH 7.5) at 30°C. The specific activities of the both of the extracts were similar and the total cell yields from the PYE-grown cells were higher than that of methylamine-grown cells. No activity was observed in boiled extracts. The rate of the reaction was dependent on the amount of crude extract used.

As AS is acid and base labile, the rate of AS decomposition in samples containing TCA were checked. The AS stability vs. time in such system is presented in Figure 3.11. As seen, there was no detectable loss of AS in 2 h with all the samples analysed. All the acid-treated samples were analyzed within two hours.

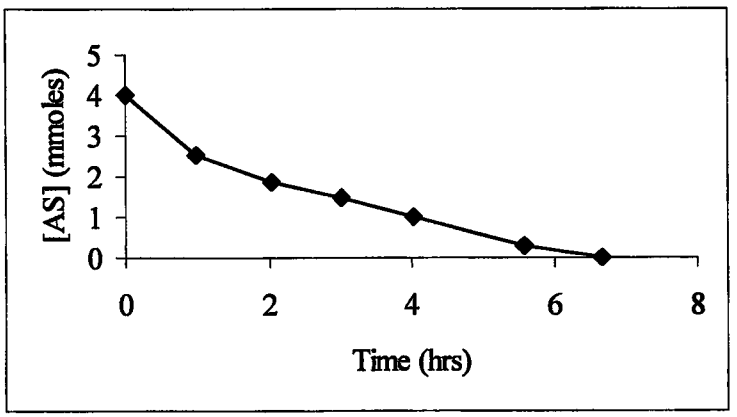


Figure 3.10. AS (The N-methyl carbamate) hydrolysing enzyme activity by PYE-grown cell extracts in 100mM Tris-HCL buffer (pH 7.5) at 30°C.

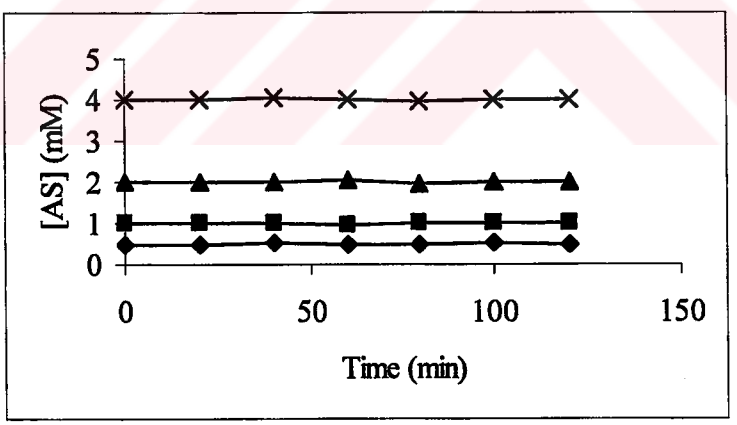


Figure 3.11. The time-dependent stability of AS in TCA acidified samples of crude cell-free extract measured by HPLC (AS in 50 mM Tris-HCL buffer (pH 7.5) containing10^{-1} diluted saturated TCA).

3.4.1. The determination of V_{max}, and K_m vales for N-methylcarbamate (AS) hydrolysing enzyme

The V_{max}, K_m values for the enzyme with AS as substrate were 0.290 μmole.(min.mg protein)$^{-1}$, and 2.06 mM, respectively (Figure 3.12). It appears that inhibition of the enzyme by its substrate AS at concentrations above 4 mM was observed.

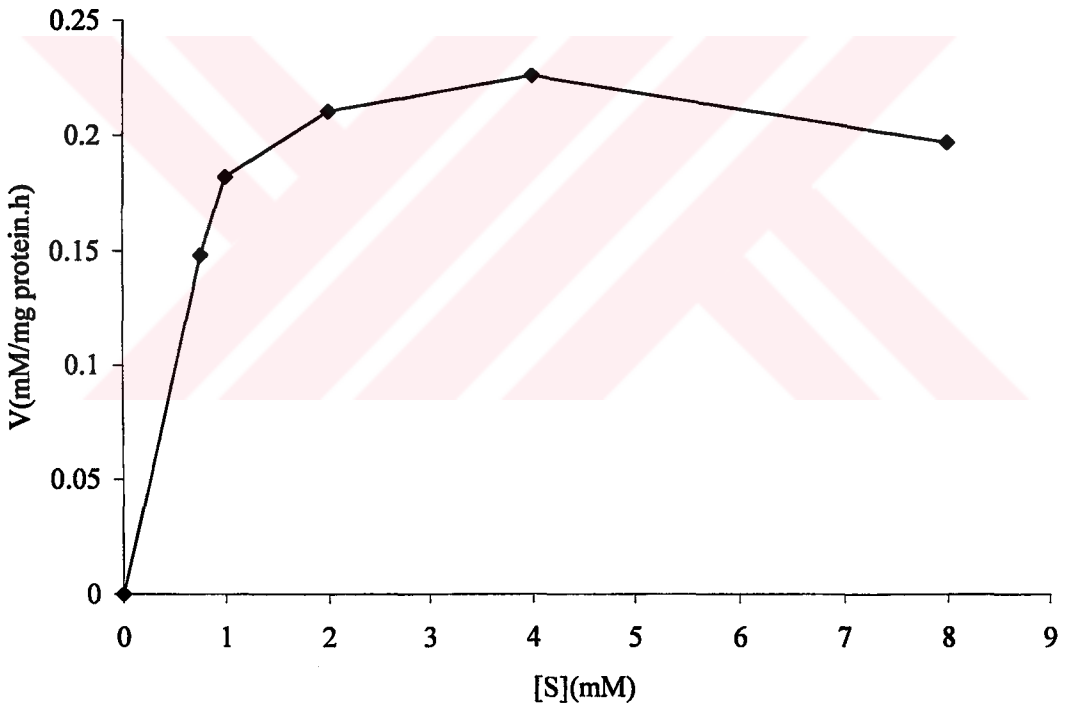


Figure 3.12. The V vs. [S] plot of N-methyl carbamate (AS) hydrolyzing enzyme

3.5. The identification of AS metabolic by-product after bacterial utilization

The characterization of compound that is accumulating after bacterial treatment is of extreme importance for understanding the fate of AS utilization. The AS metabolic by-product was compared with analytical grade ASO, ASO_2 and AS oxime by using spectrophotometric, TLC, HLPC, and NMR analysis.

3.5.1. Spectrophotometric detection of AS and its metabolic by-product

The λ_{max} value of AS byproduct was compared to that of the readily available derivatives of AS (Table 3.5) and found to be the same as AS oxime.

Table 3.5. UV absorption maxima (190-300nm) for AS and its derivatives

Compound	Maximum wavelength (nm)	Secondary peak (nm)
Aldicarb	205	246.5
Aldicarb sulfoxide	195	245
Aldicarb sulfone	201	
Aldicarb oxime	207.5	237
Bacterial AS by-product	207.5	237

3.5.2. Detection of AS and its derivatives by TLC

The R_f values of AS oxime and AS by-product were proved to be the same in two different solvent system (Table 3.6.).

Table 3.6. The comparison of the R_f values of AS derivatives and bacterial AS by-product

Compound	Systems and Relative front values (R_f)	
	A	B
AS oxime	0.88	0.85
ASO	0.14	0.39
ASO_2	0.44	0.29
AS utilization by-product	0.88	0.85

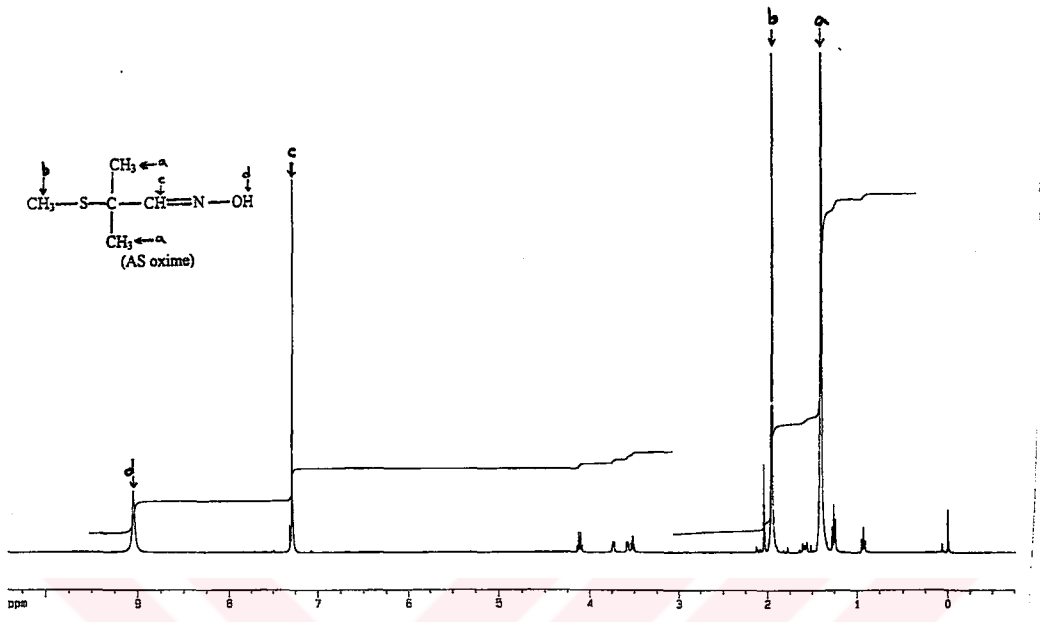
3.5.3. Detection of AS and its derivatives by HPLC

Both the AS by-product and AS oxime have shown the same retention times upon HPLC analysis (4.7 min; using Cyanopropyl-silica, Higgines Analytical; 4.6mm x 25 cm long).

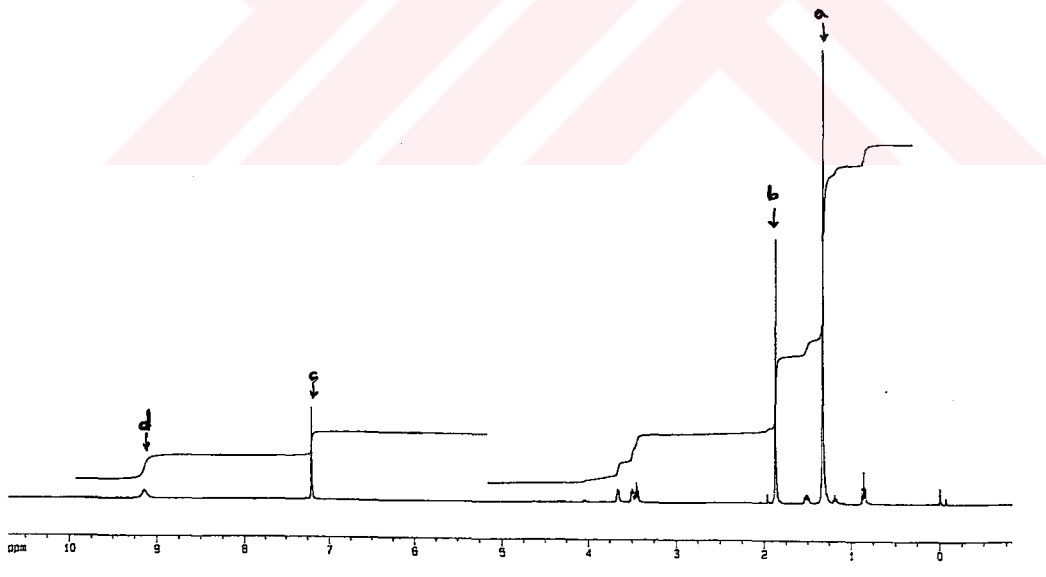
Under the light of these findings, bacterial by-product was assumed to be AS oxime (this was further supported by NMR analysis).

3.5.4. NMR analysis

NMR analysis has shown that both the newly-produced bacterial byproduct and the compound produced through chemical treatment of AS (see Section 2.2.5.) are the same compounds that points out to AS oxime. (Figure 3.13. and Figure 3.14.)

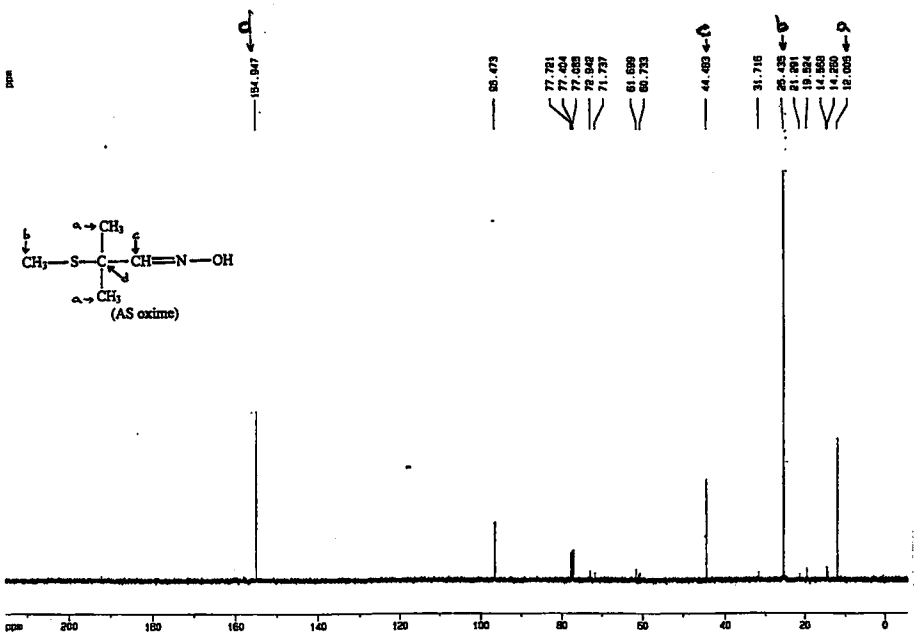


(a) ^{1}H-NMR spectrum of AS oxime

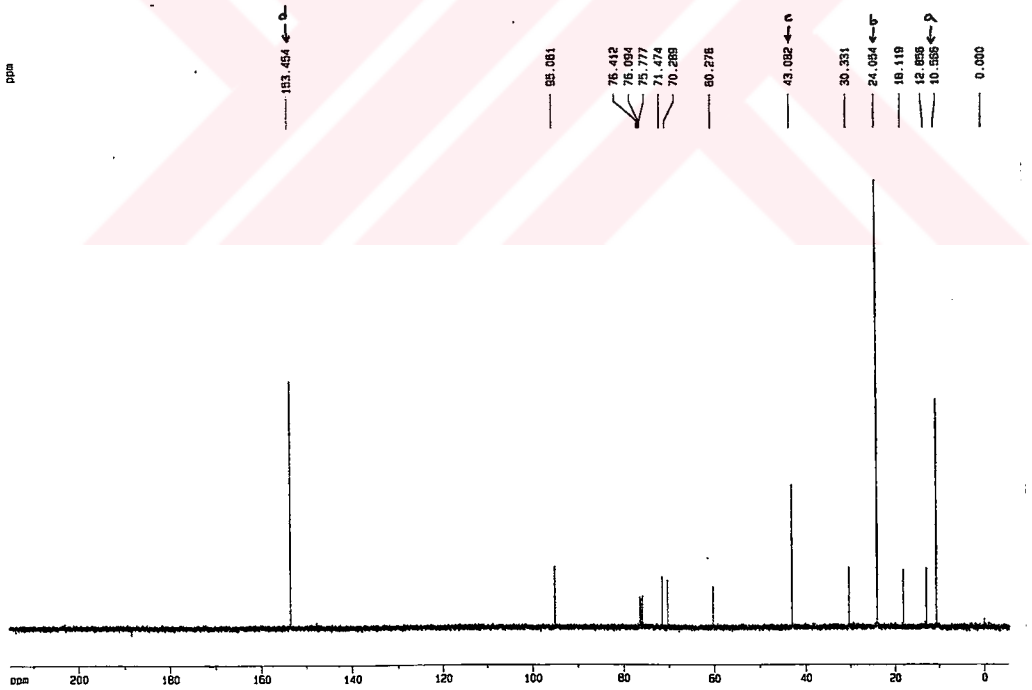


(b) ^{1}H-NMR spectrum of AS metabolic byproduct

Figure 3.13. ^{1}H-NMR ($CDCl_3$ 400MHz) spectra of (a) AS oxime, compared with the (b) AS metabolic by-product. For the ^{1}H NMR ($CDCl_3$, 400 MHz) the labeled assigned peaks are as follows (in ppm): a, 1.407 (s,6H); b, 1.957 (s,3H); c, 7.314 (s,1H); d, 9.050 (s,1H).



(a) ^{13}C--NMR spectrum of AS oxime



(b) ^{13}C-NMR spectrum of AS metabolic byproduct

Figure 3.14. ^{13}C-NMR ($CDCl_3$ 100MHz) spectra of (a) AS oxime, compared with (b) the AS metabolic by-product. For the ^{13}C NMR the labeled assigned peaks are as follows (in ppm): a, 12.0; b, 25.4; c, 44.5; d, 154.9.

3.6. Isolation of AS oxime utilizing bacteria

Although aldicarb oxime is less toxic (Read, 1987; Ou et al.,1985; Lemley and Zhong, 1983; Miles and Delfino, 1985) than the parent compound it is still desirable for an efficient biodegradation to remove this compund completely from the environment. Our effords, at this point were therefore concentrated on isolation of new bacterial species from soil samples with a history of Temik application.

In the biodegradation studies of xenobiotics, isolation of degradative organism may be very time-consuming. Therefore, the enrichment step is of primary importance in obtaining "good-degrader" strains.

After enrichments by AS oxime, a bacterial consortia capable of utilizing AS oxime either as the sole source of sulfur or nitrogen for growth were isolated. Sulfur-enriched bacterial consortium, to that respect, was discarded as their degradation rate was very low as compared to nitrogen-enriched bacterial consortia.

A variety of bacteria were isolated from nitrogen-enriched cultures. The bacteria were purified and each was tested for their ability to grow in selective liquid medium. First attempts to isolate pure cultures were unsuccessful and none of the pure isolates was able to utilize aldicarb oxime alone. It was therefore necessary to work with the most efficient mixture of bacteria (a consortium) that utilized aldicarb oxime completely within 3 days (Figure 3.15).

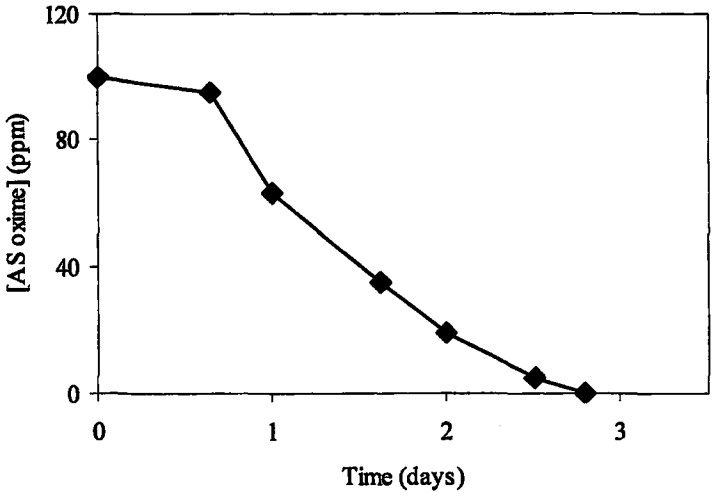


Figure 3. 15. Biodegradation of AS oxime by nitrogen-enriched bacterial consortium in optimized condition

3.7. Characterization of the most efficient AS oxime ultizing bacterial consortium

Characterization tests were employed for the pure isolates by ID 32 GN system. Classification was made to their generic level, on the basis of gram stain, catalase reaction, oxidase activity, and 32 different carbohydrate substrate tests (Bergey's Manual of Systematic Bacteriology) .

The bacterial consortium composed of predominantly *Pseudomonas putida*, *Agrobacterium radiobacter*, *Citrobacter koseri*, and *Methylobacterium mesophilicum* (that belongs to the pink-pigmented facultative methylotrophic bacteria) strains.

3.8. Optimization studies on the bacterial consortium

Alterations in cultural conditions played an important role in the optimization of growth and biodegradation rate for aldicarb oxime.

The results indicated that the most efficient degradation efficiency could be obtained when bacterial consortium were grown in MMI suplemented with AS oxime (100 ppm) at 30° C under neutral conditions within 3 days (see Figure 3.15).

3.8.1. Effect of pH on biodegradation of AS oxime

In order to determine the effect of pH on bacterial growth and AS oxime consumption, pH values of 5.5, 6.5, 7.2, and 8.0 were selected. The pH studies indicated that the optimum pH for AS oxime degradation is at pH:7.2 (Figure 3.16).

Control flasks containing uninoculated MMI-AS oxime (100 ppm) showed that AS oxime was stable at pH values examined throughout the experiment.

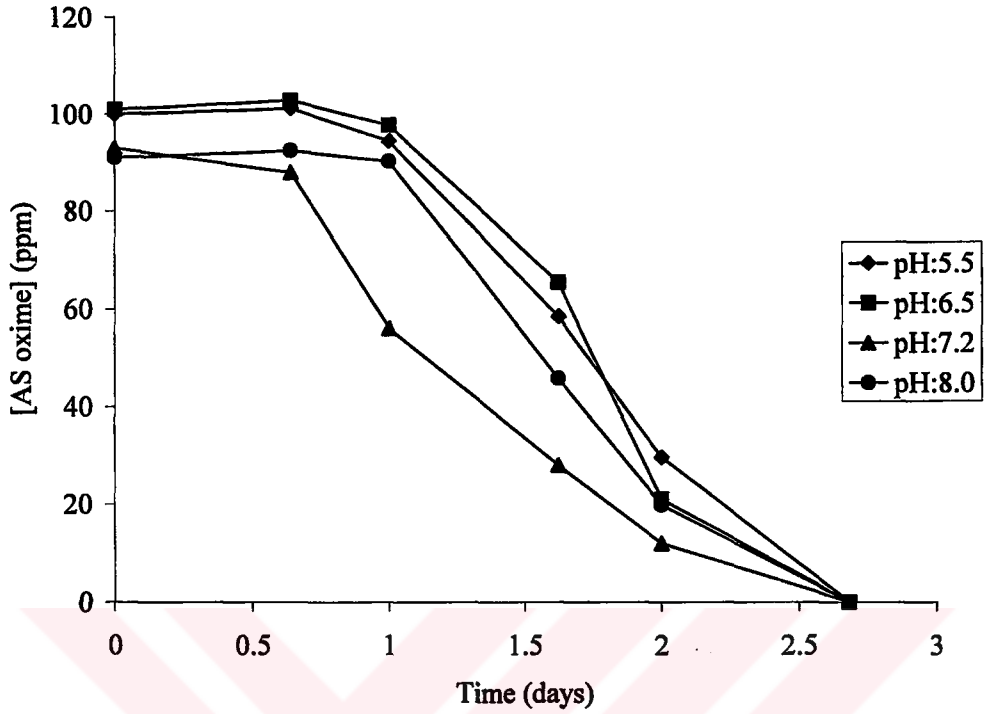


Figure 3.16. The effect of pH on biodegradation rate of mixed bacterial culture

3.8.2. Effect of temperature on biodegradation of AS oxime

To optimize the temperature, three temperatures (20°, 30°, 36°C) were studied as incubation temperatures. The highest degradation rate was detected at 30°C-36°C (Figure 3.17).

Control flasks containing uninoculated MMI-AS oxime (100ppm) showed that AS oxime was stable at different temperature values examined.

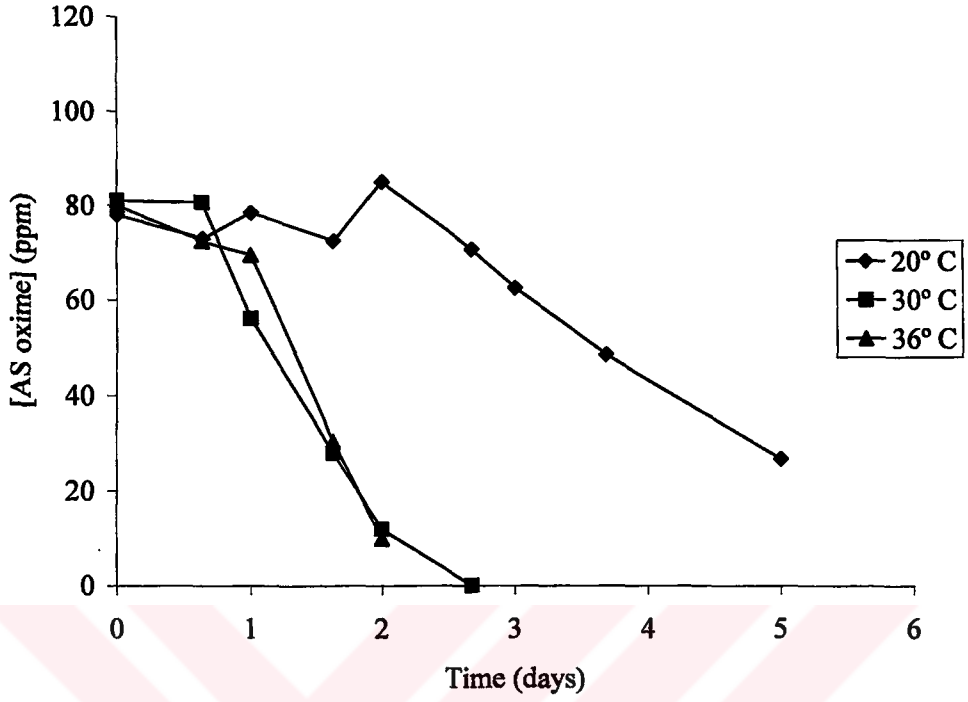


Figure 3.17. The effect of temperatures on biodegradation rate of mixed bacterial culture

3.8.3. Effect of concentration on the AS oxime biodegradation

100, 200, 300, 400, and 600 ppm of AS oxime as an sole source of nitrogen for growth were examined. The results showed that the bacterial consortium utilized AS oxime efficiently up to 400 ppm at 30° C within 8 days depending on the concentration tested. As seen, the lag period increased as the concentration of AS oxime increased. Above 400 ppm concentration, there was an inhibition on growth and degradation. At 600ppm no growth and degradation was detected for 7 days and a long lag period was observed (Figure 3.18).

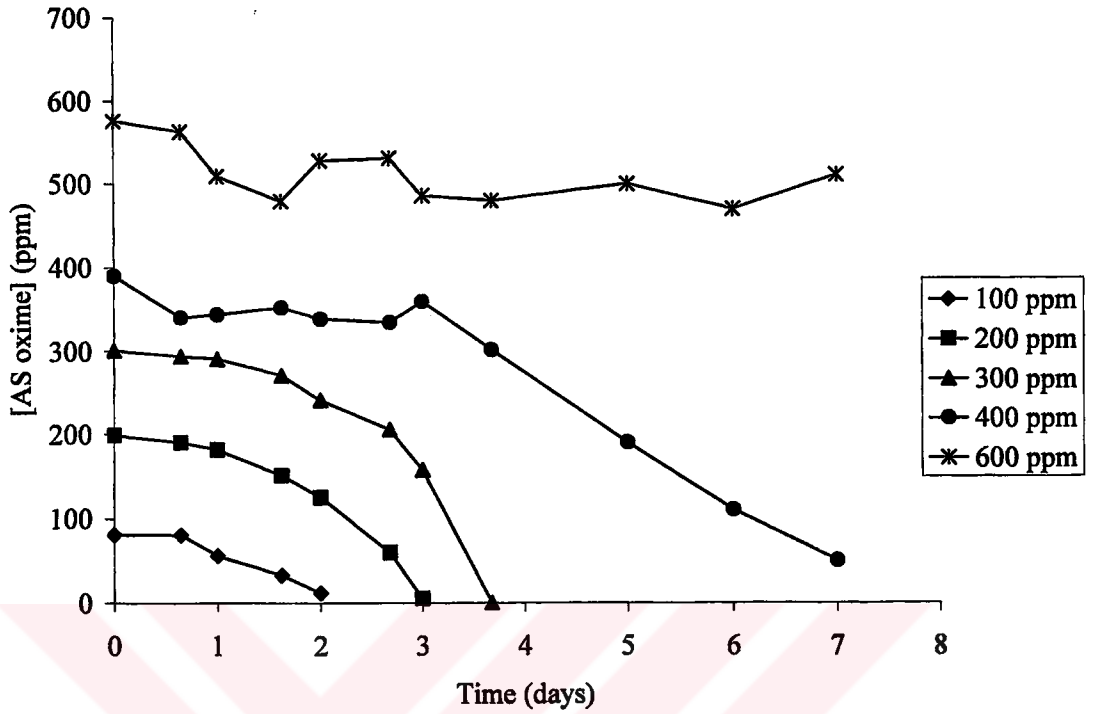


Figure 3.18. The effect of AS oxime concentration on biodegradation rate of mixed bacterial culture

During examining the effect of AS oxime concentration on the biodegradative potential of bacterial consortium, it was shown that, the higher the concentration of the aldicarb oxime, the more growth would be seen in bacterial culture without any degradation of the aldicarb oxime. This would imply that another compound rather than AS oxime supported the growth of bacterial consortium. This was indeed the case. During the preparation of AS oxime, the chemical method adapted from Bank and Tyrrel (1984) relied on alkalene treatment of aldicarb, treatment of AS with KOH leads to the rapid hydrolysis and production of the corresponding oxime (aldicarb oxime) and methylamine (MA) and CO_2. During AS oxime production,

methylamine was expected to evaporate completely. However, in our case this was not true (please see below) as methylamine was detected in the stock solution.

3.9. Detection of methylamine (MA) by 2,4-dinitrofluorobenzene

The method devised by Dubin (1960) was used for the detection and determination of methylamine.

3.9.1. Construction of calibration curve for MA

The λ_{max} value of analytical grade methylamine-dinitrofluorobenzene complex was determined at 360 nm. The calibration curve of analytical grade methylamine is presented in Figure 3.19. The calibration curve was linear over the range examined.

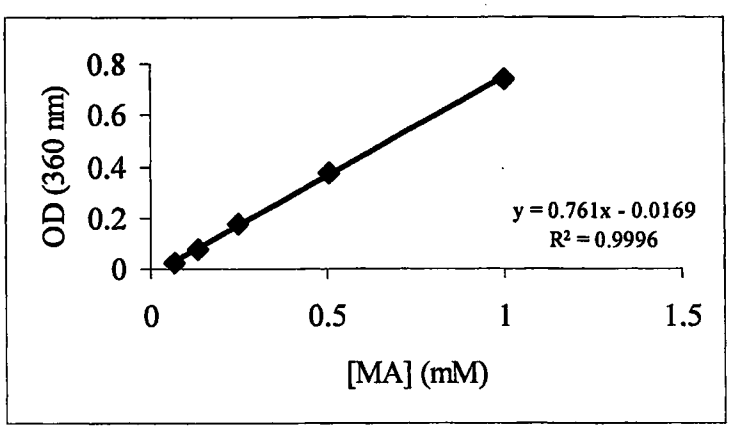


Figure 3.19.The calibration curve for methylamine-dinitrofluorobenzene complex at 360nm

3.9.2. Analysis of the MA-containing samples

MA were detected in samples taken from the stock solution of AS oxime and growth medium having different concentration of AS oxime, by using the standard MA curve.

The results showed that most of the methylamine remained in the aldicarb oxime stock solution. In view of this finding, it was decided that the growth media (MMI-AS oxime) prepared with different concentrations of AS oxime had already been contaminated with methylamine prior to their use. It was then made clear that the presence of methylamine interferred with the initial enrichment cultures in which AS oxime was not the only nitrogen source in the growth medium.

3.10. Purification of the AS oxime stock from methylamine

3.10.1. Solvent extraction

Two different solvent systems were used; using chloroform, and dichloromethane.

AS oxime was extracted by using both extraction systems (chloroform or dichloromethane) as verified by HPLC and TLC using solvent A and B solvent systems.

It was also shown that the extracted materials had no methylamine as verified by the method described in Section 2.2.9.

It is important to note that the extraction must repeated for several times, as in the first run of the extraction only a small proportion of the AS oxime pass from water to the organic solvent fraction.

3.10.2. Microbial removal of methylamine

No methylamine was detected in the samples (as verified by the method described in Section 2.2.9) taken in the first day of growth of the *Pseudomonas putida* in MMI-AS oxime (100ppm) indicating that methylamine was utilized by the isolate. HPLC analysis also confirmed that AS oxime remained in the growth medium (Figure 3. 20).

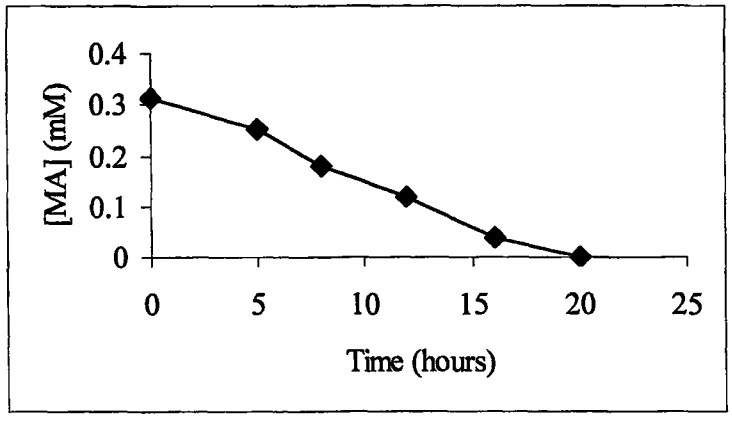


Figure 3.20. The loss of methylamine in MMI-AS oxime by *Pseudomonas putida*

3.11. Physiological studies involving the microbial consortium

One of the key aspects in studying mixed cultures is the interrelationships between different species in respect to their degradative capabilities toward toxic chemicals. This information is important in establishing the catabolic reaction sequence of a toxicant and the synergistic role of individual microbial species or group in facilitating the rate of xenobiotic biodegradation.

In order to characterize the interrelations of bacterial isolates in bacterial consortium, different combinations of the isolates present in the population were constructed. The samples were taken periodically for HPLC analysis.

It was shown that the predominant isolates for the degradation of aldicarb oxime were *Pseudomonas putida* and *Methylobacterium mesophilicum*. This two-membered bacterial consortium could utilize AS oxime efficiently in 4-5 days at 30°C. (see Figure 3.21, the curve related to the two-membered bactereial consortium).

3.11.1. Studies on the *Pseudomonas putida*

Biodegradation studies essentially start with developing mixed microbial cultures capable of detoxifying a toxic chemical by employing enrichment and selection techniques. Microbial biochemists and geneticists work with pure cultures in order to understand the basic biochemical mechanisms and pathways of microbial deoxification,

which are not well known. As a follow-up of such studies, several purified enzymes (e.g. oxygenases or dehalogenases) and their corresponding genes have been described. Therefore, our attempts focused on finding a "good degrader" in pure form.

3.11.1.1. Bacteriological tests on solidified media

As *Pseudomonas putida* failed to grow and degrade aldicarb oxime in MMI-AS oxime (100 ppm) alone, it was assumed that the isolate might be nutritionally deficient, and therefore was subjected to auxonography.

Detection of improved bacterial growth around the aminoacid crystals was taken as a measure for aminoacid deficiency for the isolate.

L-histidine, L-arginine and L-threonine appeared to be the most prominant amino acids enhancing growth of the isolate to a large extent.

As studies showed that *Pseudomonas putida* wasnot a "good degrader" in pure form, our further studies focused on the *Methylobacterium mesophilicum* isolate (mentioned in the following section).

3.11.2. Growth studies on the *Methylobacterium mesophilicum*

The results has shown that *Methylobacterium mesophilicum* alone could still

utilize 100 ppm aldicarb oxime completely but in much longer period of time (in 8-11 days) (Figure 3.22, and 3.23) as compared to the two-membered bacterial consortium (Figure 3.21). With the latter, AS oxime was completely eliminated from the growth medium within a much shorter (4-5 days) incubation period (Figure 3.21).

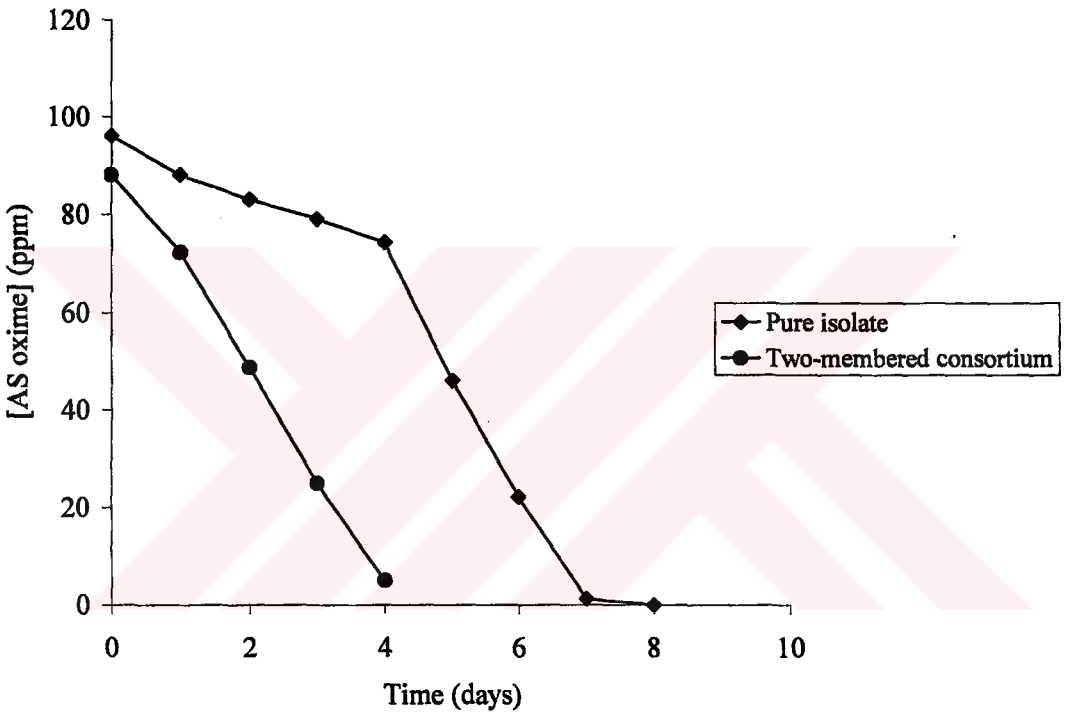


Figure 3.21. Biodegradation of a single isolate of *Methylobacterium mesophilicum* vs two-membered community

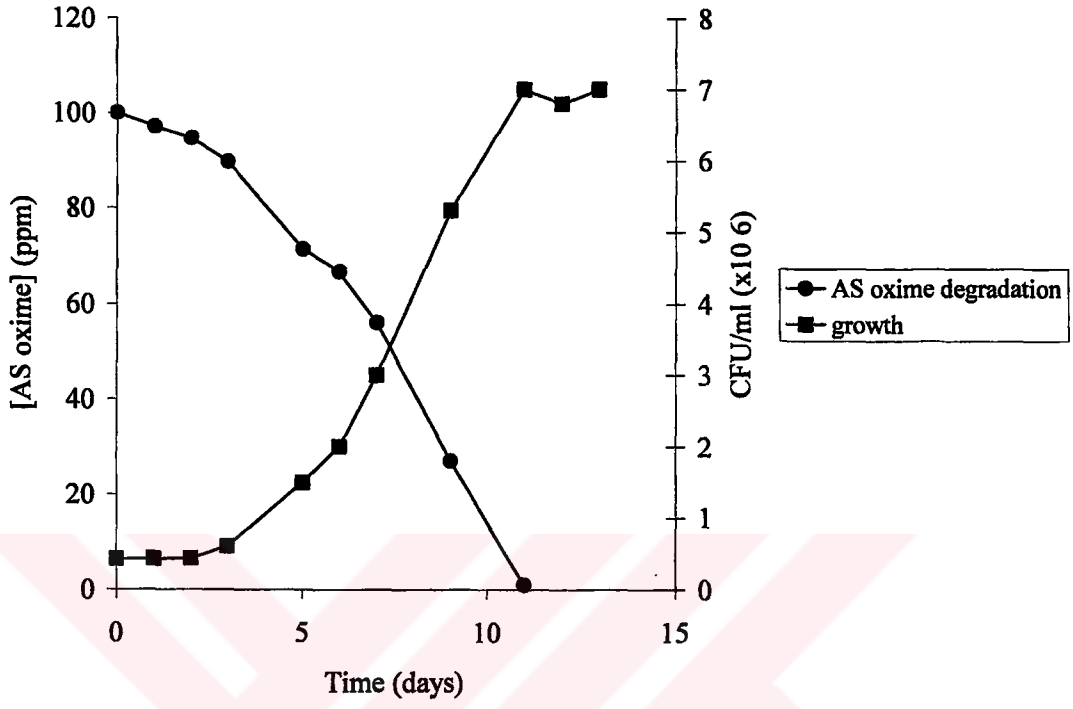


Figure 3.22.The biodegradation of AS oxime in MMI (100 ppm) vs. growth by the bacterial isolate (*Methylobacterium mesophilicum*)

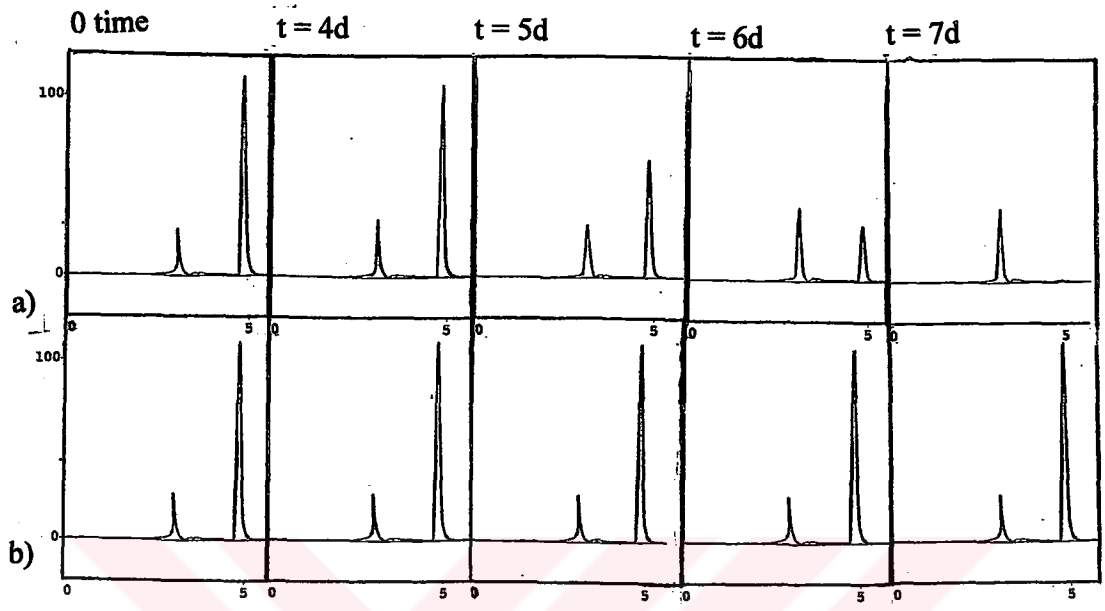


Figure 3.23. Biodegradation profile of AS oxime growth by the bacterial isolate (*Methylobacterium mesophilicum*). The HPLC chromatograms of a) AS oxime biodegradation in the minimal growth medium b) blank (uninoculated MMI-AS oxime medium), using CN silica column (Higgines Analytical; 4.6mm Ø x 25 cm long), 1:MMI, 2:AS oxime.

Microbial biochemists and geneticists work with pure cultures in order to understand the basic biochemical mechanisms and pathways of microbial detoxification, which are not well known. As a follow-up of such studies, several purified enzymes and their corresponding genes have been described.

In spite of the higher degradation rate of bacteial consortium compared to the pure isolate of *Methylobacterium mesophilicum*, our further studies focused on the pure isolate of *Methylobacterium mesophilicu*m in order to to investigate the enzymology and biochemistry of microbial degradation

3.12. Optimization studies on the *Methylobacterium mesophilicum* isolate

3.12.1. Effect of concentration on the AS oxime biodegradation in MMI-AS oxime

AS oxime in the range of 100-600 ppm was used to determine the optimum concentration for bacterial growth and biodegradation. The results showed that the bacterial isolate utilized AS oxime (100 ppm) at 30°C within 8-10 days. Above 100 ppm concentration, the biodegradation rate was much lower, taking about 15-30 days. As AS oxime concentration increased, an increased lag-period was observed and, at 600 ppm growth and degradation was retarded (Figure 3.24).

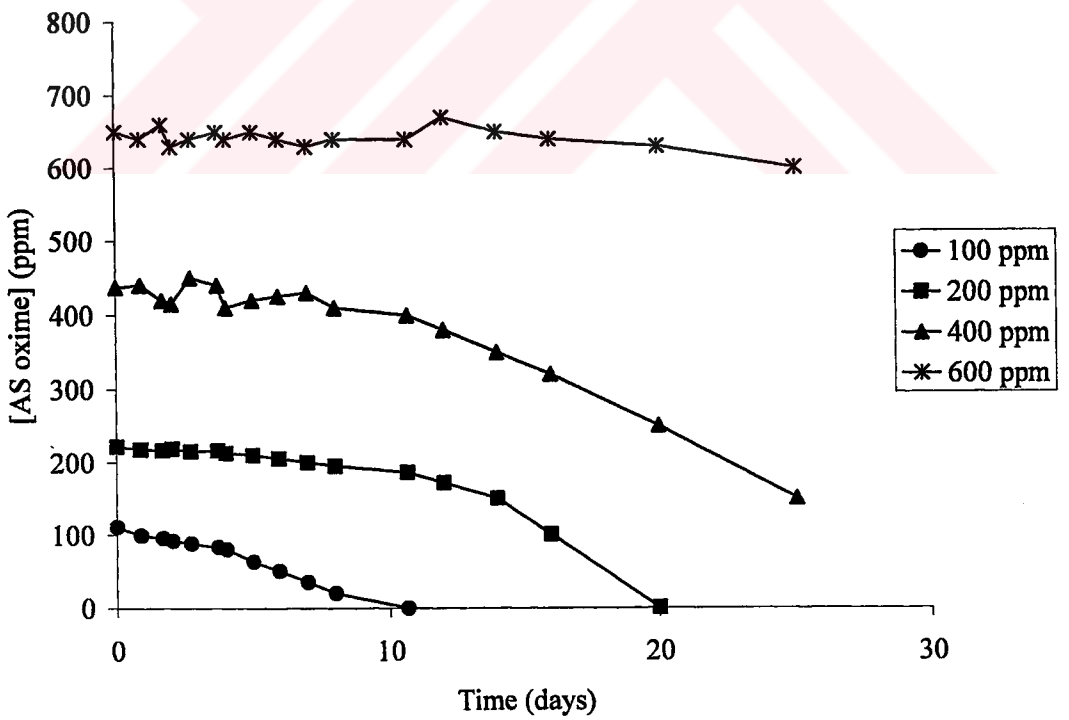


Figure 3.24. The effect of AS oxime concentration on biodegradation rate by the pure isolate of *Methylobacterium mesophilicum* in MMI-AS oxime

3.12.2. Effect of pH

In order to determine the effect of pH on bacterial growth and AS oxime consumption, pH values covering acidic and alkaline regions (ranging between 5.5-8) were selected.

The pH studies indicated that the optimum pH for AS oxime degradation was around 7.0 (Figure 3.25).

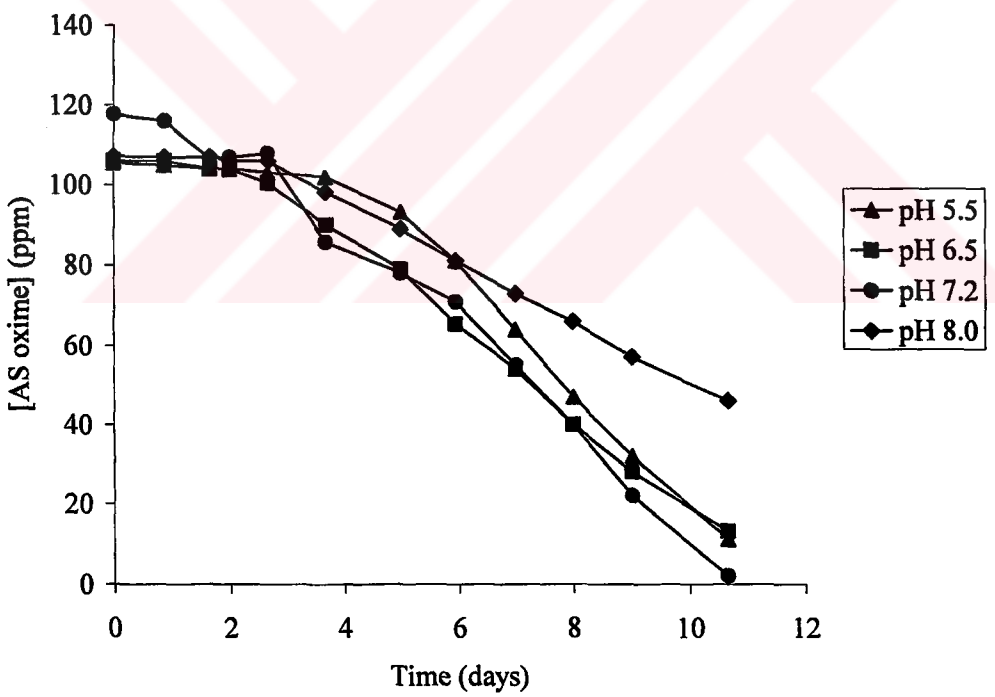


Figure 3.25. The effect of pH on As oxime biodegradation of *Methylobacterium mesophilicum*

Control flasks containing uninoculated MMI-AS oxime (100ppm) showed that AS oxime was stable at pH values examined during incubation period.

3.12.3. Effect of temperature on biodegradation of AS oxime

To test the effect of temperature, three temperatures of 25° (room temperature; RT), 30°, 40°C were chosen. The highest degradation rate appeared to be around 25°-30°C. (Figure 3.26)

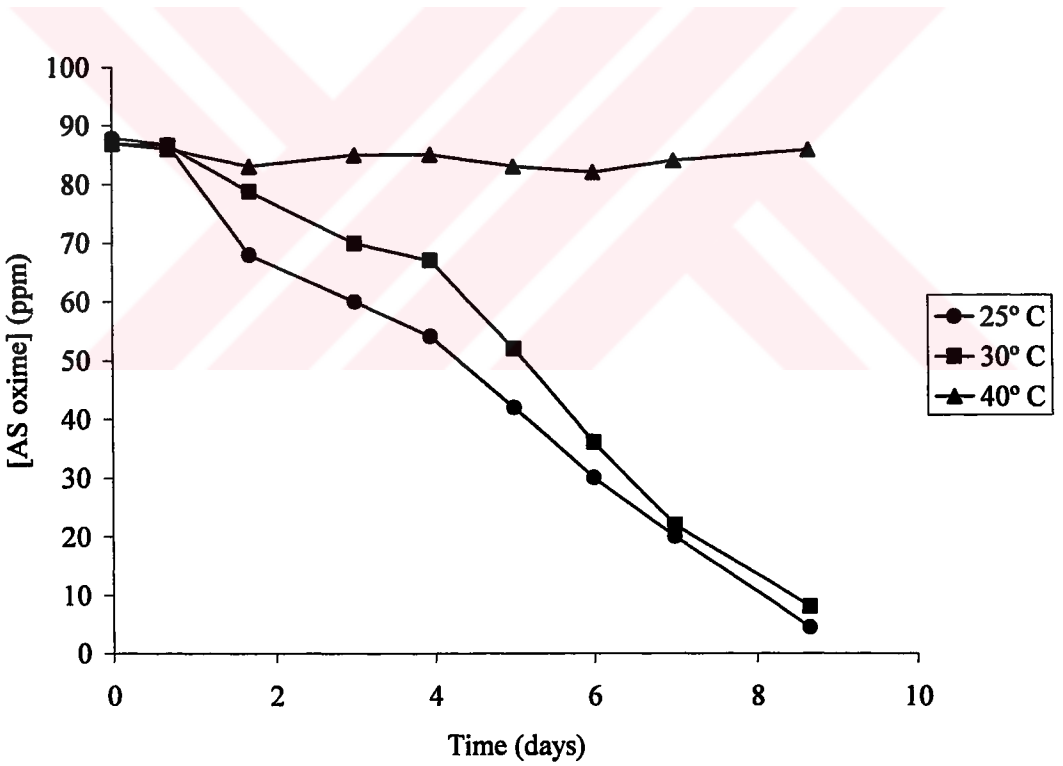


Figure 3.26. The effect of temperatures on AS oxime biodegradation of *Methylobacterium mesophilicum*

Control flasks containing uninoculated MMI-AS oxime (100ppm) showed that AS oxime was stable at different temperature values employed.

3.12.4. Effect of additional nutrients

In order to detect the effect of growth supplements, MMI-AS oxime (see Appendix B for composition) growth medium was supplemented with yeast extract (0.02%), peptone (0.02%), tryptone (0.02%), or vitamine solution (1%).

AS oxime was utilized in a shorter period of time when MMI-AS oxime culture medium was supplied with any of the four. However, it was more pronounced with yeast extract addition. The cultures supplied with yeast extract could utilize 100ppm within 4-5 days (Figures 3.27).

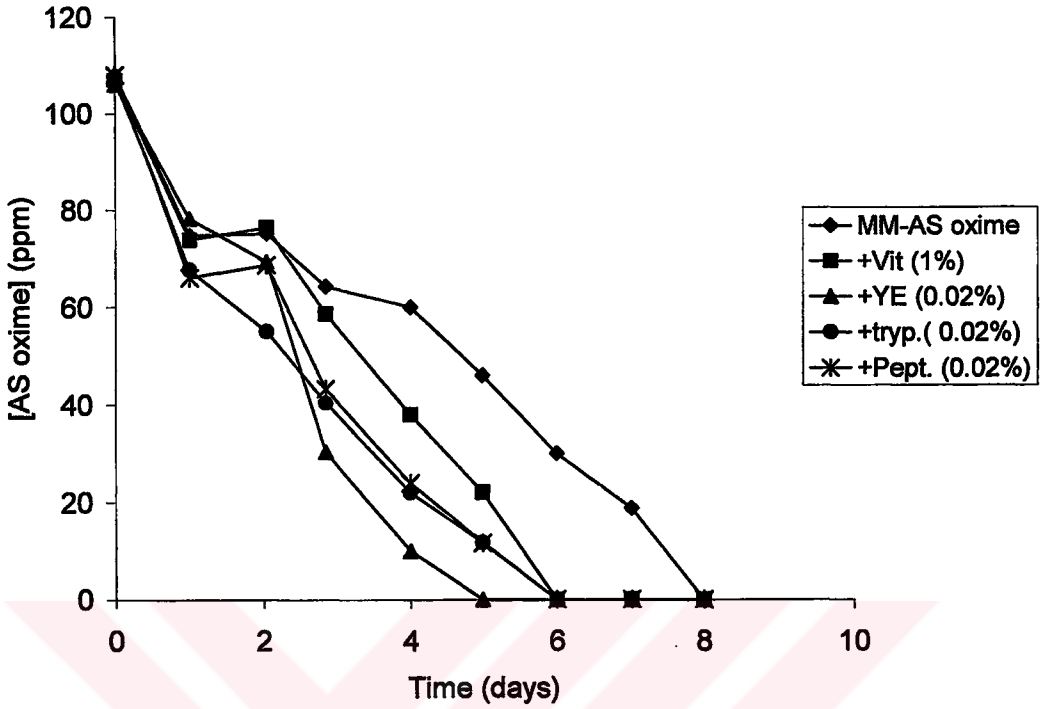


Figure 3.27. Effect of additional nutrients on biodegradation of AS oxime by *Methylobacterium mesophilicum*

3.13. AS oxime as a sole source of carbon and nitrogen

AS oxime biodegradation were detected in glucose-free MMI-AS oxime (as the sole source of carbon and nitrogen) and in MMI-AS oxime (as the sole source of nitrogen) containing glucose (1%). The bacterial isolate could also utilize AS oxime as a sole source of carbon and nitrogen but within a slight longer period of time (Figure 3.28).

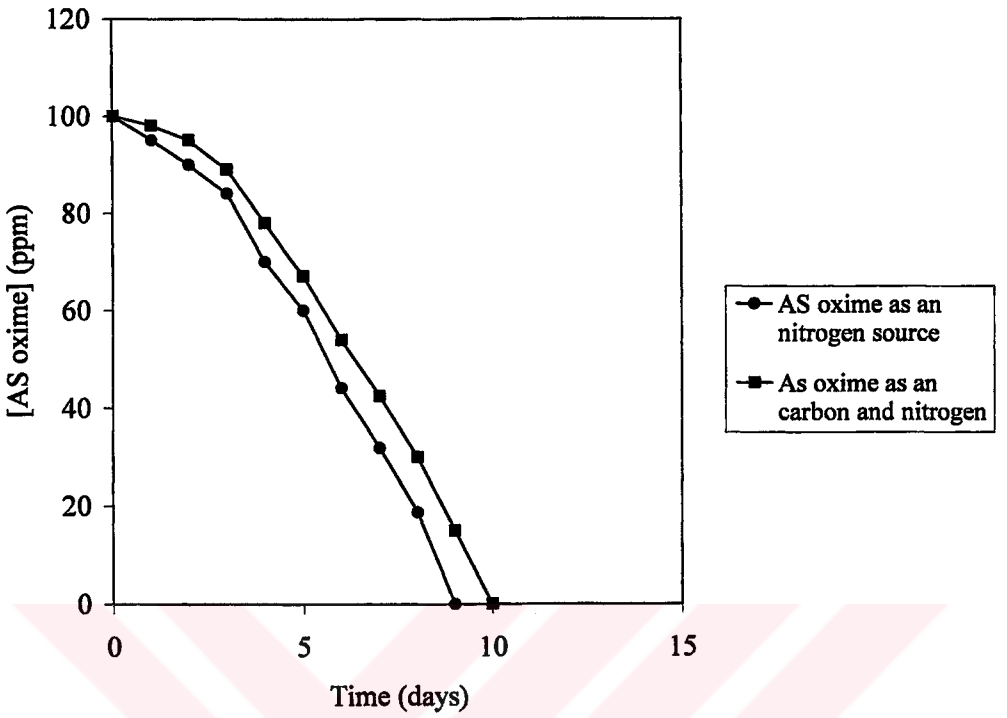


Figure 3.28. Biodegradation of AS oxime as a sole source of carbon and nitrogen vs sole source of nitrogen by *Methylobacterium mesophilicum* isolate

3.14. Bacterial utilization of AS oxime in a rich medium

AS oxime utilization was assayed in pepton-yeast extract (PYE), Lauria broth (LB), or nutrient broth (NB) mediums containing 100 ppm AS oxime. As seen in Figure 3.29, AS oxime was utilized efficiently in all media, with the best degradation being in PYE.

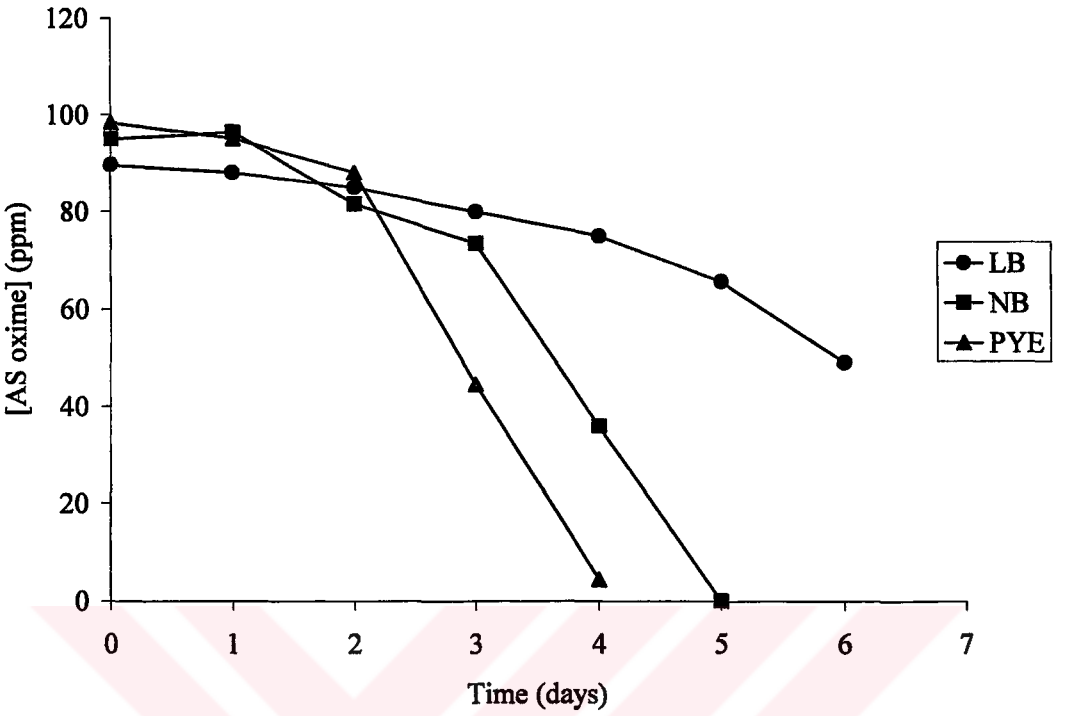


Figure 3.29. Biodegradation of *Methylobacterium mesophilicum* in different rich media; LB, NB, PYE.

As seen in Figure 3.30, AS oxime (100 ppm) was utilized within 4 days in PYE medium. The bacterial isolate first utilized the available nutrients of PYE (first two days) with no appreciable utilization of AS oxime.

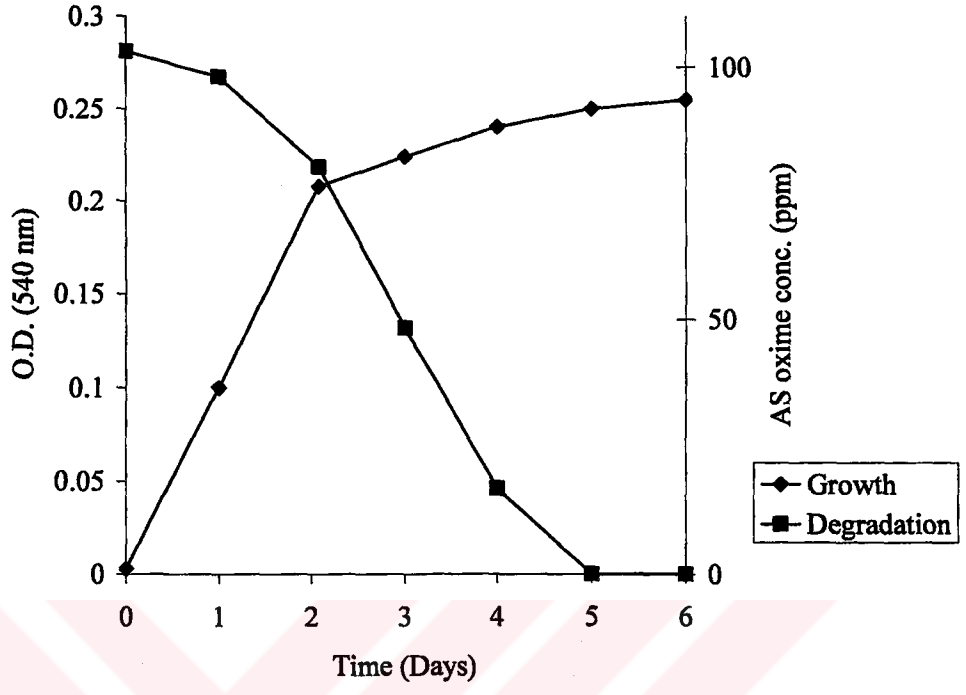


Figure 3.30. Growth of *Methylobacterium mesophilicum* isolate versus degradation of AS oxime in PYE

3.14.1. The effect AS oxime concentration in a rich medium

AS oxime in the range of 50-600 ppm was used to determine the optimum concentration for bacterial growth and biodegradation in rich medium. The results indicated that the bacterial isolate utilized AS oxime (50 and 100 ppm) completely within 4-5 days at 30°C, and 200 ppm AS oxime within 6-7 days. Above 400 ppm concentration, there was an inhibition on growth and degradation and long lag period could be seen. At 600ppm no growth and degradation was detected during incubation period (Figure 3.31).

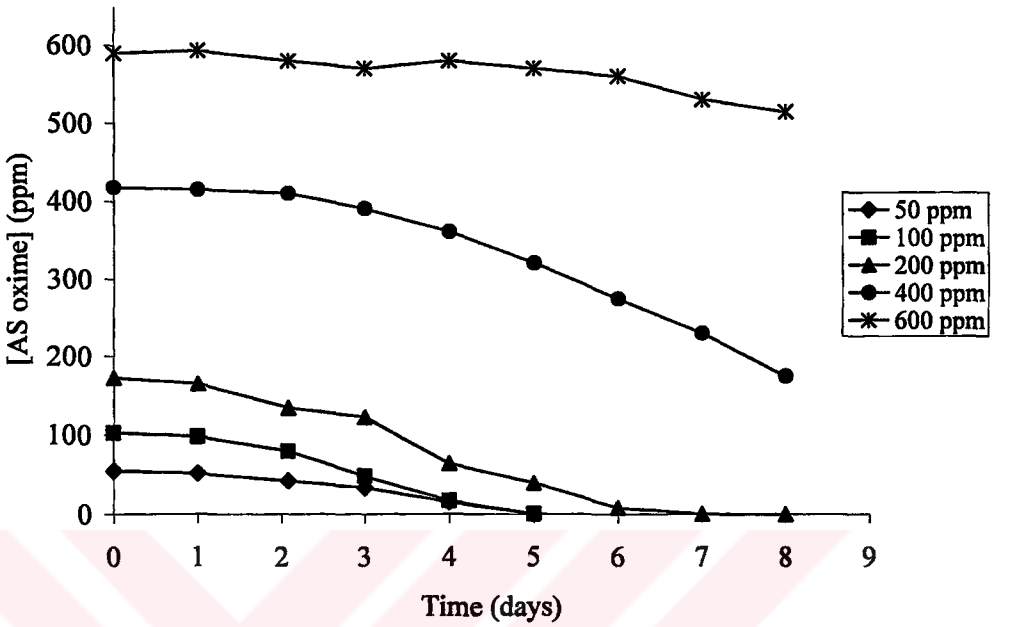


Figure 3.31. The effect of AS oxime concentration in a PYE medium by *Methylobacterium mesophilicum* isolate

3.15. Studies on the AS oxime hydrolyzing enzyme

In this part of the study, the activity of the AS oxime hydrolyzing enzyme was determined in whole and cell-free crude extracts of PYE-grown cells. Having detected no enzymatic activity in the post-culture supernatent, it was decided that the enzyme responsible for AS oxime hydrolysis was not extracellular. The forecoming studies involving enzymology were therefore performed with crude cell-free extracts.

3.15.1. Effect of TCA on AS oxime concentration and stability

The AS oxime decomposition in samples containing TCA were checked. The AS oxime stability vs. time in such system is presented in Figure 3.32. As seen, TCA has obvious effect on AS oxime. There is a detectable loss of AS oxime in a very short period of time. About 50% of AS oxime was decomposed upon addition of TCA concentrations higher than 5%. It has also an adverse effect on column performance (causing broadening and shouldering of the AS oxime peak), in spite of neutralization performed before analysis.

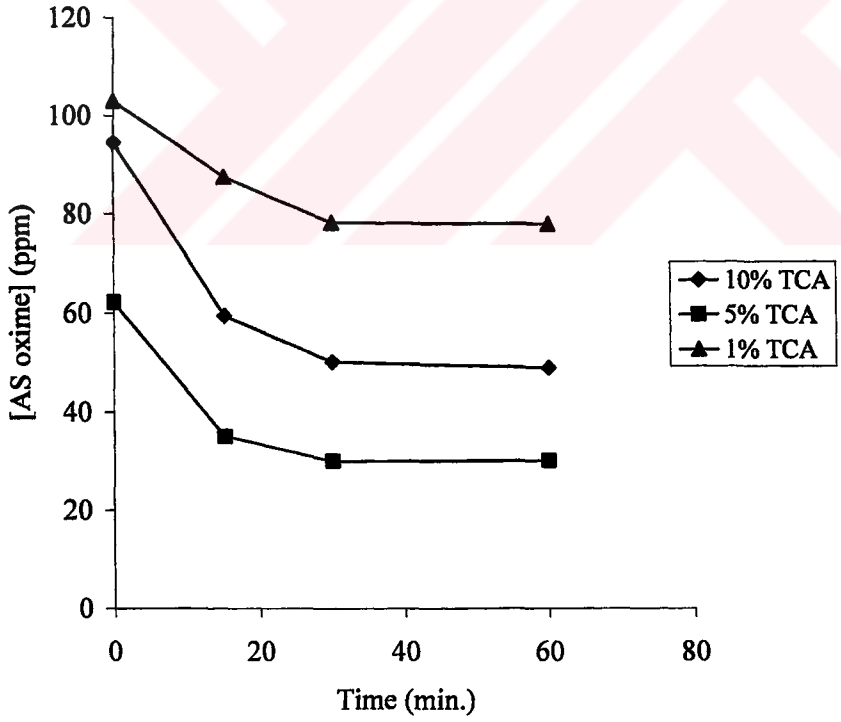


Figure 3.32. The time-dependent stability of AS oxime in TCA acidified samples measured by HPLC (AS oxime in KP buffer containing different concentration of TCA).

3.15.2. AS oxime degradation studies in crude cell-free extract

3.15.2.1. By enzymatic digestion and ultrasonication

After either enzymatic treatment or ultrasonication of the whole cell preparations, both the supernatant (crude cell-free extract), and the pellet fraction (cell debris) were tested separately for determining enzymatic activity. No activity was detected in the cell-free crude extract implying that the enzyme was not of cytoplasmic nature. This was further verified as activity was present only in the pellet of cell-free crude extract (i.e. cell debris), that referred to a membrane-bound enzyme of origin (Figure 3.33). The rate of the reaction was dependent on the amount of cell debris used. No activity was observed in boiled extracts.

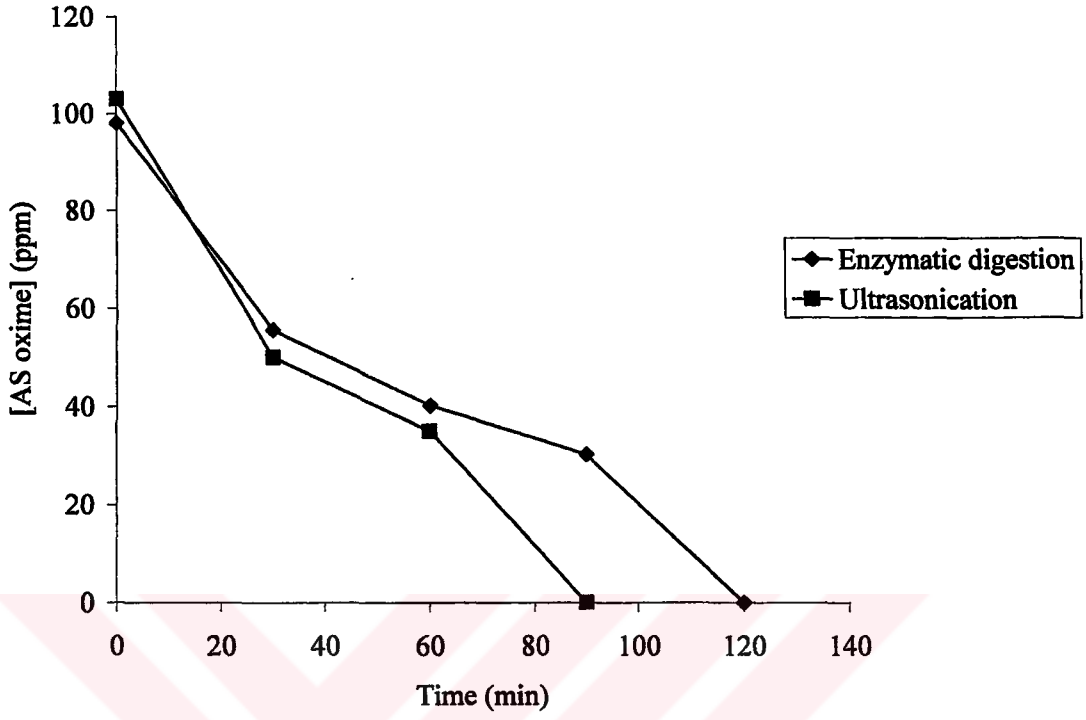


Figure 3.33. The AS oxime hydrolysing enzyme activity by PYE-grown cell extract by enzymatic digestion and by ultrasonication in 100mM KP (pH:7.0) at 30°C.

3.15.2.2. Optimization studies

The optimization studies through the AS oxime concentration, and the pH on the enzyme activity were as follows;

3.15.2.2.1. The effect of AS oxime concentration

25-100 ppm of AS oxime could be completely degraded by hydrolyzing enzyme in a very short period of time (2 hrs). 150 ppm AS oxime is also degraded within a approximately 4 hrs (Figure 3.34).

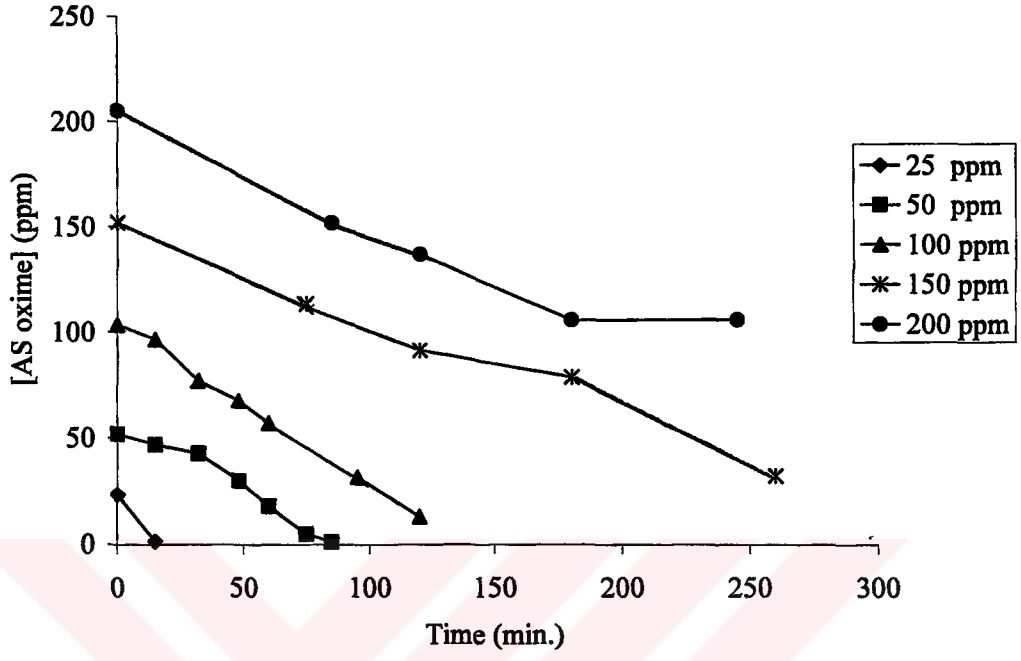


Figure 3.34. Effect of concentration on the enzyme activity of the cell debris (pellet of crude cell-free extract) of *Methylobacterium mesophilicum* isolate prepared by sonication

In order to evaluate the kinetic properties of AS oxime hydrolysing enzyme, varying concentrations of AS oxime were examined. Reaction mixtures containing changing concentration of AS oxime (ranging from 10-200 ppm) were prepared. All other parameters were kept constant. The results has shown that the reaction rates is not proportional to the substrate (AS oxime) concentration and nearly independent of AS oxime. Therefore it appears that the enzyme do not obey Michaelis-Menten kinetics. But this behaviour may also due to the membrane-bound nature of the enzyme.

3.15.2.2.2. The effect of pH

The pH studies indicated that the best for the enzyme activity is around neutral pH values (Figure 3.35).

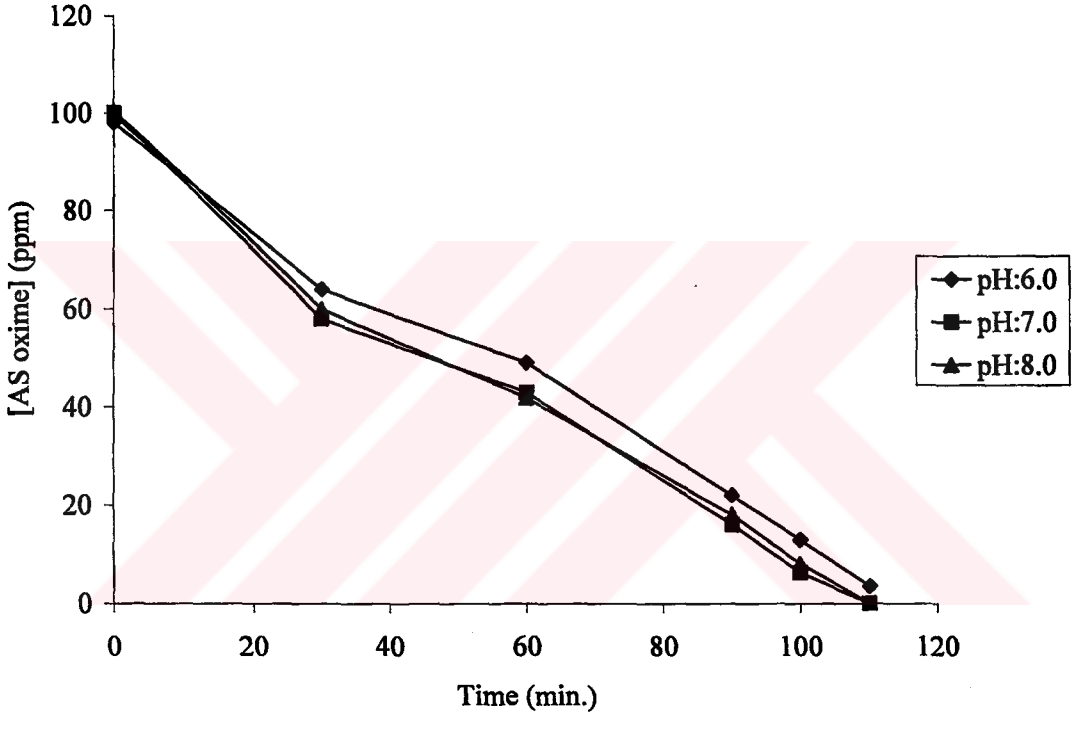


Figure 3.35. The effect of pH on the enzyme activity of the cell debris (pellet of crude cell-free extract) of *Methylobacterium mesophilicum* isolate prepared by sonication

3.15.2.2.3. Induction studies

Two batches of PYE-grown cells (with and without 100 ppm AS oxime were used for the preparation of cell debris (pellet of cell-free crude extract). No activity

was detected in the preparation that had not been exposed to AS oxime.This was an evidence clearly indicating that the enzyme was of inducable nature (Figure 3.36).

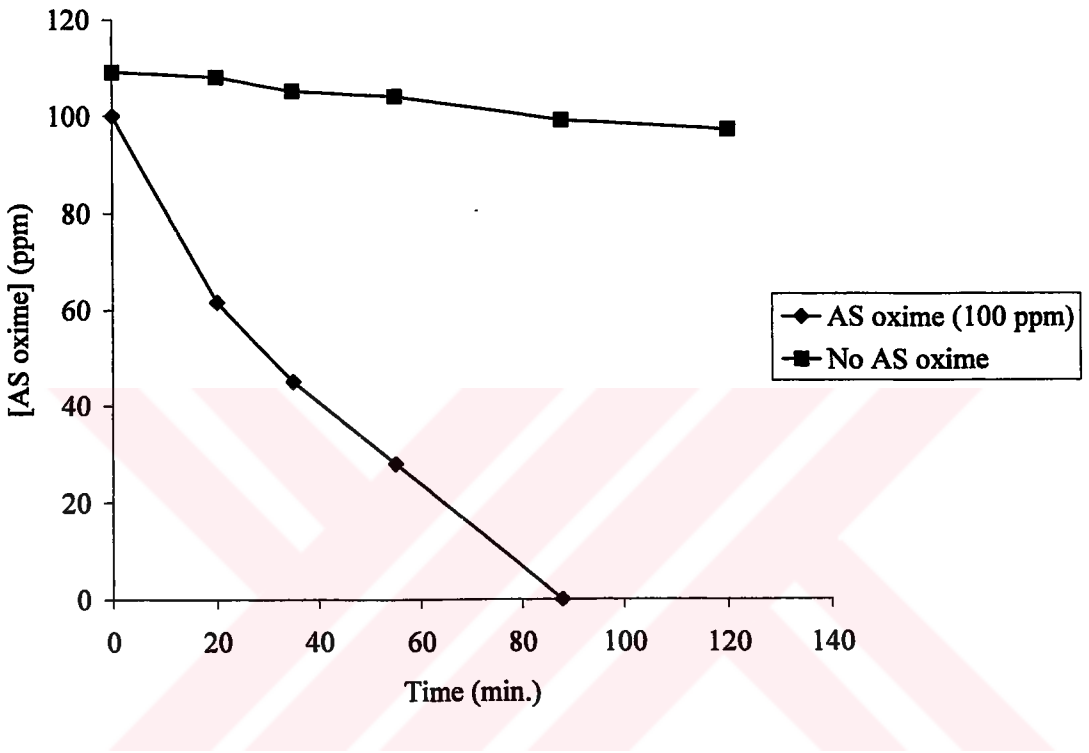


Figure 3.36. Effect of AS oxime as an inducer on the enzyme activity of the cell debris of *Methylobacterium mesophilicum* isolate prepared by sonication

3.15.2.2.4. Failing effords of extraction and solubilization of membrane proteins

Upon treatments of cell debris by triton X-100 (1%), lysozyme (1mg/ml), and EDTA (10 mM), the enzyme activity was detected only in the pellet of the cell-free crude extract (cell debris) implying that the enzyme was still bound to the membrane and the chemical used in an efford to detach the enzyme failed to do so. A more

vigorous method was therefore needed to free the enzyme from the membrane.

After Triton-X 100 (final concentration of 4%), treatment with a combination of lysozyme (10mg/ml) and triton X-100 (1%), chloroform extraction, and osmotic shock, no activity was detected in the pellet and supernatent fractions. Two possibilities could explain the situation, a more probable answer was that the enzyme was solubilized away from their normal cellular milieu with a lost activity. The other possibility was that the enzyme was not at all solubilized but applications had some adverse effect on the enzyme leading to loss of activity.

3.15.3. Complete degradation of aldicarb by pure isolates

A two-membered bacterial consortium composed of facultative methylotrophic isolate degrading aldicarb to aldicarb oxime, and *Methylobacterium mesophilicum*, degrading aldicarb oxime was constructed and introduced to MMII-AS medium. Facultative methylotrophic isolate was first inoculated to the MMII-EAS medium. Upon complete conversion of aldicarb to aldicarb oxime (as detected by HPLC), the culture medium was supplemented with MMI components and inoculated with *Methylobacterium mesophilicum* isolate (see Section 2.2.17). As a result, the metabolic by-product of AS, which is aldicarb oxime, has completely degraded (Figure 3.37).

This bacterial consortium could completely eliminate AS. As a result, no traces of AS and its metabolic byproduct (AS oxime) remained in the culture medium as varified by HPLC (Figure 3.37).

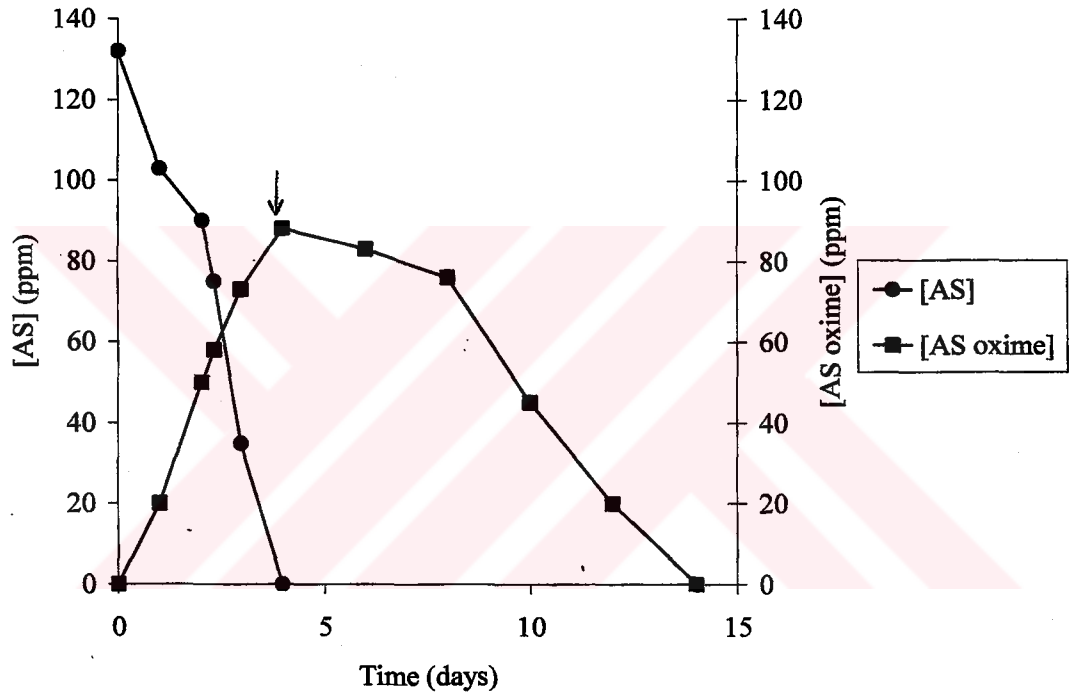


Figure 3.37. Complete degradation of aldicarb by two-membered bacterial consortium, arrow shows the time of inoculation of *Methylobacterium mesophilicum* isolate to the minimal growth medium.

3.15.4. Complete degradation of aldicarb by crude extracts of two different isolates

It was shown that crude extracts prepared from each of the bacterial isolates could also completely degrade AS. The crude cell-free extract of facultative methylotrophic isolate was added into the reaction mixture tube containing aldicarb as the substrate (see Section 2.2.18). The HPLC analysis indicated that AS was completely converted to its metabolic byproduct, that is AS oxime. In the second part of the experiment, cell debris (the pellet of cell-free crude extract) of *Methylobacterium mesophilicum* was incorporated into the same reaction mixture tube.

The HPLC analysis indicated that crude cell-free extract of the facultative methylotrophic isolate degrade AS to AS oxime and cell debris (pellet of crude cell-free extract) of the *Methylobacterium mesophilicum* isolate degrade produced AS metabolic byproduct (AS oxime). As a result, no traces of AS and its metabolic byproducts (AS oxime) remained in the reaction mixture as detected by HPLC. (Figure 3.38).

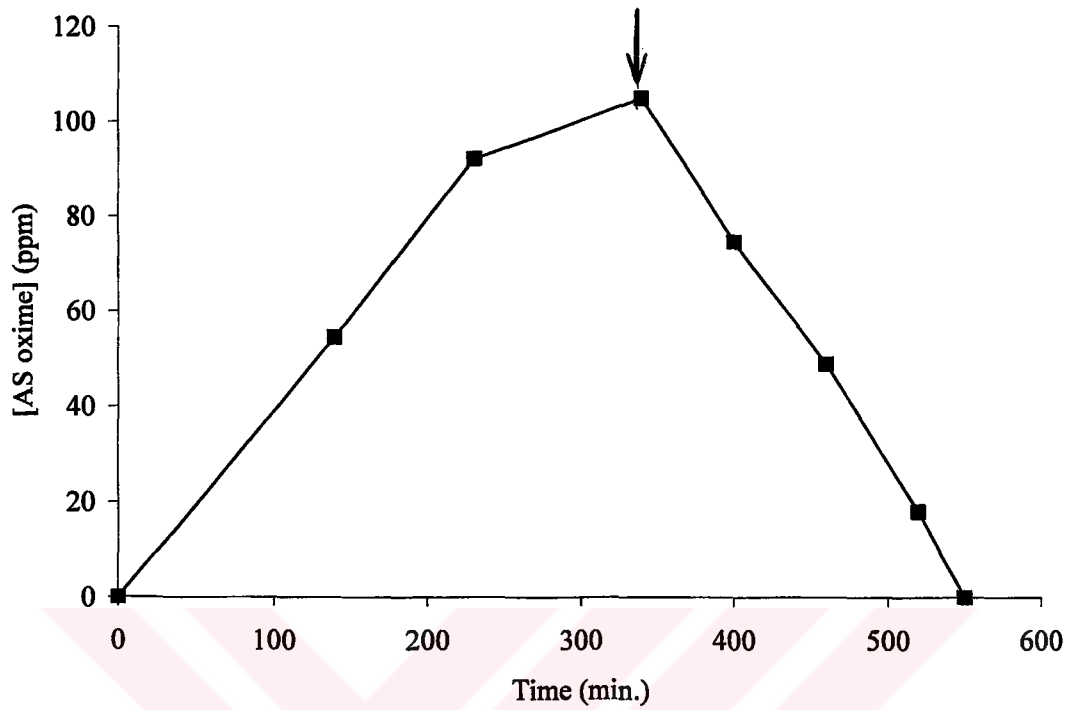


Figure 3.38. Complete degradation of aldicarb by crude extract of two different isolates, the arrow indicate the time of addition of the *Methylobacterium mesophilicum* cell debris (pellet of cell-free crude extract) to the reaction mixture

CHAPTER IV

CONCLUSIONS

In the present study, aldicarb (AS: the active ingredient of Temik®) [2-methyl-2-(methylthio) propionaldehyde O-(methyl-carbamoyl) oxime], was chosen as a model pesticide to understand complete microbial degradation.

A facultative methylotrophic bacteria, capable of utilizing aldicarb as the sole carbon and energy source, isolated through enrichment from soils with a history of Temik application. The results showed that the highest degradation rates were achieved when the microbial isolate were grown in MMII with 400 ppm (2mM) AS at 30°C in an alkaline pH.

AS was found to be hydrolysed into AS oxime, methylamine and CO_2. The former was accumulated in the medium as an end-product and were not metabolized any further.

Although aldicarb oxime is less toxic than parent compound; it is still toxic and cause undesirable effects on the environment.

A bacterial consortium was isolated from soil samples with previous history to AS. It could completely utilize 100 ppm AS oxime as an sole nitrogen source within 3 days. It was shown that the predominant isolates for the degradation of aldicarb oxime were *Pseudomonas putida* and *Methylobacterium mesophilicum*. This two-membered bacterial consortium could utilize AS oxime efficiently in 4-5 days at 30°C.

The *Methylobacterium mesophilicum* could utilize 100 ppm aldicarb oxime completely as an sole nitrogen source in a long period of time (in 8-10 days) and with very slow growth and degradation, as compared to the two-membered bacterial consortium which were more efficient in utilizing higher amount of aldicarb oxime in shorter period of time. The bacterial isolate could utilize AS oxime also as a sole source of carbon and nitrogen.

In order to increase the rate of degradation of AS oxime by the pure isolate, it was necessary to manipulate certain cultural parameters which in turn, would result in optimized growth, and better degradation rates. A higher degradation rate was seen when YE (0.02 %) supplied as growth factor to the MMI-AS oxime culture medium of *Methylobacterium mesophilicum*. Under these conditions, it could utilize 100 ppm in shorter period of time (4-5 days).

Effect of different growth media on degradation rate was studied. AS oxime utilization was assayed in pepton-yeast extract (PYE) containing 100 ppm AS oxime. AS oxime (100 ppm) was utilized within 4 days in PYE medium by *Methylobacterium*

mesophilicum. Therefore it could be concluded that growth in PYE has an inducing effect on the biodegradation of AS oxime.

The activity of the AS oxime hydrolyzing enzyme was determined in crude extracts of PYE-grown cells of *Methylobacterium mesophilicum*. Crude extracts were prepared by ultrasonication and enzymatic digestion. There is no activity detected in the cell-free crude extract (supernatent) therefore the enzyme is not cytoplasmic. The activity is only detected in pellet of the cell-free crude extract (cell debris) of PYE-grown cells implying the membrane-bound nature of the enzyme.

Enzyme activity in PYE grown cells with and without AS oxime (100 ppm) to detect the inducing effect of the substrate (AS oxime) were followed. There is no activity detected when the substrate is absent in the culture medium, indicating the inducing effect of the substrate (AS oxime). Therefore it is concluded that AS oxime hydolysing enzyme is an inducing enzyme not a constitutive one.

To achieve ultimate biodegradation of AS (the aim of the study), a two-member bacterial consortium consisting of a facultative methylotrophic isolate and a *Methylobacterium mesophilicum* were tested together. As a result, no traces of AS and its metabolic byproduct (AS oxime) remained in the culture medium indicating that the bacterial mixture could completely eliminate AS

It was shown that the crude extracts prepared from facultative methylotrophic isolate and *Methylobacterium mesophilicum* could also completely degrade AS.

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APPENDIX A

CHEMICALS AND SUPPLIERS

CHEMICAL	SUPPLIER
Temik®	Rhone Poulence
Aldicarb	Riedel-de Haen
Aldicarb sulfoxide	Riedel-de Haen
Aldicarb sulfone	Riedel-de Haen
Aldicarb oxime	Dr.Ehrenstorfer
Nutrient broth	Difco
Yeast Extract	Difco
Peptone	Difco
Tryptone	Difco
Agar	Difco
Glucose	Merck
HCl	Merck
Methanol (HPLC grade)	Merck
Diethylether	Merck
Acetone	Merck
Chloroform	Merck

Ethanol	Merck
Benzene	Merck
Dioxane	Merck
n-Hexane	Merck
Ethyl acetate	Merck
Acetonitrile	Merck
Dicholoromethane	Merck
Phenol	Merck
2,4-Dinitroflorobenzene	Sigma
Dimethyl formamide	Sigma
Trizma Base	Sigma
Dipotassium hydrogen phosphate	Sigma
Potassium dihydrogen phosphate	Sigma
Magnesium chloride	Sigma
Magnesium sulfate	Sigma
Ammonium sulfate	Sigma
Sodium Acetate	Sigma
Sodium chloride	Sigma
Sodium hydroxide	Sigma
Sodium hydroxide	Sigma
Potassium tetraborate	Sigma
Succinic acid	Sigma
Acetic Acid	Merck

Triton X-100	Sigma
2-mercaptoethanol	Merck
Lysozyme	Sigma
Ribonoclease A	Sigma
Deoxyribionuclease I	Sigma
SDS	Merck
Glycerol	Sigma
Methylamine	Sigma
Trichloroacetic acid	Sigma

APPENDIX B

COMPOSITION AND PREPARATION OF CULTURE MEDIA

B1. Minimal Medium I (MMI) ("buffered salts" solution, used for nitrogen-enriched culture medium)

10mM potassium phosphate buffer, (pH:7.3), containing 0.25 mM $MgSo_4$, glucose* (1%), and 1ml TES* (see B5).

Sterilization was done by autoclaving at 121° C for 15 minutes.

*Glucose and TES were to MMI as an filtere sterilized solution.

Wherever it was necessary to employ any of the MM in solid form, this was achieved by addition of 1.5% (w/v) agar prior sterilization.

B2. Minimal Medium II (MMII, used for carbon-enriched culture medium)

Component	g/L
$MgSo_4 .7H_2O$	0.2
$(NH_4)_2SO_4$	0.5
K_2HPO_4	0.5

KH_2PO_4	1.5
Trace element solution	10 ml
Agar*	15

The final pH of the medium was adjusted to 7.1 prior to sterilization. Sterilization was done by autoclaving at 121° C for 15 minutes.

*It is used for solidification when required.

B3. Minimal Medium III (MMIII) ("buffered salts" solution, , used for sulfur-enriched culture medium)

10mM potassium phosphate buffer, (pH:7.3), containing 25 mM NH_4Cl, 0.25mM $MgCl_2$, glucose* (1%), and 1ml TES* (see B5).

Sterilization was done by autoclaving at 121° C for 15 minutes.

*Glucose and TES were to MMI as an filtere sterilized solution.

Wherever it was necessary to employ any of the MM in solid form, this was achieved by addition of 1.5% (w/v) agar prior sterilization.

B4. Trace element solution (used for carbon-enriched culture medium)

Component	g/L
Sodium EDTA. $2H_2O$	12

$CaCl_2$	10
$ZnSO_4 .7H_2O$	0.4
$MnSO_4 . 4 H_2O$	0.4
$CuSO_4 .5 H_2O$	0.1
Conc. H_2SO_4	0.5
$Na_2 SO_4$	10.0
$Na_2Mo_4 . 2H_2O$	0.1
$FeSO_4 . 7H_2O$	2.0

The mixture was autoclaved at 121° C for 15 minutes to be maintained as a stock solution at room temperature.

B5. Trace element solution(used for sulfur and nitrogen-enriched culture medium)

Component	g/L
H_3BO_3	0.3
$CaCl_2 .6H2O$	0.2
$ZnSO_4 .7H_2O$	0.1
$MnCl_2 . 4 H_2O$	0.03
$Na_2MoO_4 . 2H_2O$	0.03
$Ni_2 SO_4. 7H_2O$	0.028
$CuSO_4 . 5 H_2O$	0.01

pH : 7.2

The mixture was autoclaved at 121° C for 15 minutes to be maintained as a stock solution at room temperature.

B.6. Nutrient Agar Medium

Component	g/L
Nutrient broth	1.3
Agar	1.5

PH:7

Sterilized at 121° C for 15 minutes

B 7. LB.Broth

Component	g/L
Casaminoacids	10.0
Yeast Extract	5.0
NaCl	7.0

PH: 7.2

Sterilized at 121° C for 15 minutes.

B8. Peptone yeast extract (PYE)

Component	g/L
Peptone	3.0
Yeast Extract	3.0
Agar*	15

pH: 7-7.2

Sterilized at 121° C for 15 minutes.

*It is used for solidification when required.

APPENDIX C

COMPOSITION AND PREPARATION OF BUFFERS AND REAGENTS

C.1 Dinitrofloro benzene (DNFB) assay for determination of methylamine.

Reagent A-Reagent A is prepared by addition of 0.65 ml of DNFB into 50ml acetone.

Regent B- 0.066M sodium tetraborate ($Na_2B_4O_7$. $10H_2O$). Reagent B is prepared by disolving 25g sodium tetraborate ($Na_2B_4O_7$. $10H_2O$) in 1L of H_2O.

Reagent C- Reagent C is prepared by diluting reagent A in reagent B in 1 to 10 ratio.

Reagent D- dilution of concentrated HCL in dimethyl formamide (1 : 100)

VITA

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PUBLICATIONS

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N.Asghari, F.A. Sharif, M.Y. Özden, and N.G. Alaeddinoğlu, 1993. "Temik'in (Aldicarb) Mikrobiyal degradasyonu", Kükem Dergisi, Vol.16, 2: 26-27.

ATTENDED SEMINARS

12th ECCO (European Culture Collections' Organization), International Meeting, June 1993, Istanbul, Turkey, Poster Presentation

GRANT

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