Islamic University- Gaza
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Biological Science

الجامعة الإسلامية بغـزة
عمادة الدراسات العليا
كلية العلوم
قسم العلوم الحياتية

Isolation and Characterization of Methamidophos Biodegrading Microorganisms from Soil

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Submitted in partial fulfillment of the reqirements for the degree of master of biological sciences in microbiology.

May, 2010

(قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ)

البقرة 32

DEDICATION

I dedicated this thesis to my mother soul, to my father, to my brothers and my sisters who always supported my career and helped me to achieve this degree.

Isolation and Characterization of Methamidophos Biodegrading Microorganisms from Soil

Abstract

Pesticides are important production inputs for many agricultural supplies, and decisions concerning their use require balancing factors between their economic benefits and the protection of the environment and human health. Microorganisms play key roles in the biodegradation of pesticide-contaminated sites. Methamidophos is one of the most widely used organophosphorus insecticides usually detectable in the environment. Methamidophos is considered as one of the most toxic insecticides; it can cause acute toxic effects by the inhibition of acetylcholinesterase enzyme activity in nervous tissue and can significantly damage peoples' health. Methamidophos was often detected in high concentrations in the ecosystem and thus lead to some direct and potential adverse effects on environmental safety. However, few studies of the microbial degradation of methamidophos have been carried out. For this project, we proposed a model for methamidophos biodegradation by bacteria grown in soil samples sites in agricultural area which had a history of methamidophos exposure. The pesticides Methamidophos was used in a minimal salt medium as a sole source of carbon inorder to isolate and characterize the methamidophos degraders. Strains of *Neisseria lactamica, Micrococcus lylae and Bacillus sphaericus* were isolated from Methamidophos contaminated soil and identified as mehamidophos

degraders and were found to use methamidophos as a sole carbon source. The presented data offers useful information about the biodegradation mechanism of methamidophos using microorganisms and can be used in the bidegradation of pesticide-contaminated sites.

Key words

Methamidophos, acetylcholinesterase enzyme , biodegradation, pesticide, *Neisseria , Micrococcus and Bacillus.*

عزل وتشخيص كائنات حية دقيقة من التربة قادرة علي تكسير الميثاميدوفس وهى المادة الفعالة في المبيد الحشري التمارون

الملخص

تعتبر المبيدات الحشرية منتج هام يستخدم في الأغراض الزراعية والقرار باستخدام المبيدات الحشرية يتطلب موازنة بين الفوائد الاقتصادية من ناحية وحماية البيئة و صحة الإنسان من ناحية أخري. الكائنات الحية الدقيقة تلعب دورا مهما في التقليل من خطورة المبيدات الحشرية، ويعتبر الميثاميدوفس من احد المبيدات الحشرية الفسفورية العضوية الأكثر استعمالا وعادة ما تكون موجودة في البيئة ، ويعتبر الميثاميدوفس احد المبيدات الأكثر سمية حيث انه يؤدي إلي تثبيط نشاط أنزيم الاستيل كولنستيرز في الأنسجة العصبية ومن ثم يسبب ضررا كبيرا لصحة الإنسان ، وغالبا ما يتم الكشف عن الميثاميدوفس بتركيز عالي في النظام البيئي حيث أدي ذلك إلي تأثيرات ضارة علي سلامة البيئة، و هناك القليل من الدراسات التي أجريت علي التكسيرالحيوي للميثاميدوفس عن طريق الكائنات الحية الدقيقة ، ولذلك فان هدف البحث هو استخدام البكتيريا التي تنمو في التربة لتكسير الميثاميدوفس عن طريق البكتيريا التي تنمو في التربة المعدة لأغراض زراعية والتي تتعرض للميثاميدوفس و قد تم استخدام الميثاميدوفس في وسط ملحي قليل كمصدر وحيد للكربون من اجل عزل وتشخيص مكسرات الميثاميدوفس ومن ثم قمنا بعزل ثلاث سلالات من البكتيريا من التربة التي تتعرض للميثاميدوفس حيث تم تشخيصها والتعرف عليها بناء علي الاختبارات الشكلية والميكروسكوبية و الكيمياء الحيوية وهي

Neisseria, Micrococcus and Bacillus .

وتعطي هذه النتائج معلومات مفيدة عن عملية تكسير الميثاميدوفس باستخدام الكائنات الحية الدقيقة حيث يمكن استخدامها في معالجة الأماكن الملوثة بالمبيدات الحشرية وكذلك تستخدم في محطات معالجة مياه المجاري.

كلمات مفتاحية:

الميثاميدوفس – أنزيم الاستيل كولنستيرز–التكسير الحيوي– المبيد الحشري

Acknowledegement

I thank to Allah whose blessings have enabled me to accomplish my thesis work successfully.

I take this opportunity to thank Dr. Tarek El Bashiti Department of Biology and Biotechnology, chairmen of the supervisory committee, for his supports, encouragements, and guidance. No words can express my sincere and deep sense of reverence for him.

I wish to express my gratefulness to Dr. Atef Masad for their supports and guidance during my project work.

I am highly thankful to my lab. Staff, Mohammed Ouda, Youssief Abu Selmeya and Ashraf Al Shafei for their help during project course.

The whole contribution for my achievements goes to my father, brothers and sisters who always stood by me during thick, thin and providing me the moral support and resources to finish my work. Their unwavering faith in me has been a source of constant inspiration for me.

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ABBREVIATIONS

Abbreviation	Full form
AchE	Acetylcholinesterase
AS	Ammonium salt
AOP's	Advanced Oxidation Processes
AS	Arsenic compound
BaP	Benzo[a] pyrene
BH	Bombay high
BP	Bipyridylium derivative
C	carbon
Ca	Carbamate
CO	Coumarin derivative
CU	Copper compound
DMABA	dimethylaminobenzaldehyde
DMPT	Dimethyl phosphorothioate
DO	Dissolved oxygen
F	fructose
FQPA	Food Quality Protection Act
G	glucose
GC/MS	gas chromatography/ mass spectrometry
HG	Mercury compound
IMViC	Indole, Methyl Red, Voges-Proskauer, and Citrate
L	lactose
M	maltose
Ma	mannitol
MF	Melamine formaldehyde
MMA	Mal-monocarboxylic acid
MOF	Modified oxidation-fermentation
MPN	Most probable number
MR	Methyl red
MSMN	Mineral salts medium supplemented with nitrogen

N	Nitrogen
n	Test not applicable
NP	Nitrophenol derivative
OC	Organochlorine compound
OP	Organophosphorus compound
opd	Organophosphorus degrading
OT	Organotin compound
P	Phosphorus
PAA	Phenoxyacetic acid derivative
PAHs	polycyclic aromatic hydrocarbons
PY	Pyrethroid
PZ	Pyrazole
S	Sucrose
SEM	Scanning electronic microscope
SEME	Soil extract medium
T	Triazine derivative
TC	Thiocarbamate
4,5,6-TCG	4,5,6-Trichloroguaiacol
UN	Unite Nuation
USA	United States of America
V	Variable
VP	Voges- proskauer
WHO	World Health Organization

Abbreviation	Full form
FQPA	Food Quality Protection Act
WHO	World Health Organization
UN	Unite Nuation
AS	Arsenic compound
BP	Bipyridylium derivative
C	Carbamate
CO	Coumarin derivative
CU	Copper compound
HG	Mercury compound
NP	Nitrophenol derivative
OC	Organochlorine compound
OP	Organophosphorus compound
OT	Organotin compound
PAA	Phenoxyacetic acid derivative
PZ	Pyrazole
PY	Pyrethroid
T	Triazine derivative
TC	Thiocarbamate
USA	United States of America
AchE	acetylcholinesterase
AOP's	Advanced Oxidation Processes
opd	organophosphorus degrading
DMPT	dimethyl phosphorothioate
MPN	most probable number
MSMN	Mineral salts medium supplemented with nitrogen
SEM	soil extract medium

BH	bombay high
MF	melamine formaldehyde
DO	dissolved oxygen
PAHs	polycyclic aromatic hydrocarbons
BaP	benzo[a] pyrene
SEM	scanning electronic microscope
4,5,6-TCG	4,5,6-Trichloroguaiacol
GC/MS	gas chromatography/ mass spectrometry
MMA	mal-monocarboxylic acid
C	carbon
N	nitrogen
P	phosphorus
G	glucose
M	maltose
S	sucrose
F	fructose
Ma	mannitol
L	lactose
AS	ammonium salt
V	variable
n	test not applicable
MR	methyl red
VP	voges- proskauer
IMViC	Indole, Methyl Red, Voges-Proskauer, and Citrate
DMABA	dimethylaminobenzaldehyde
MOF	modified oxidation-fermentation

CHAPTER 1
INTRODUCTION

1.1 Pesticides

The chemical pesticides have successfully been used to control plant diseases due to their quick and effective management. However their continual and random use will have harmful effects on human health and environment. The deliberated application and intensive use of pesticides in the environment has been leading to the contamination of air, soil, surface and ground water, and living organisms including humans (1,2). Pesticides are important production inputs for many agricultural commodities, and decisions concerning their use necessitate balancing tradeoffs between their economic benefits and the protection of the environment and human health. The passage of the Food Quality Protection Act (FQPA) by United States of America (USA) Congress represents a significant change in pesticide policy that could have large impacts on both pesticide users and the rest of society. The FQPA focuses on protecting children and other sections of the population from the aggregate effects of pesticides with common mechanisms of toxicity. New safety standards target the organophosphate and carbonate insecticide classes of pesticides and could restrict or cancel several pesticide uses (3).

Some pesticides have been shown to be suitable carbon sources for microorganism development In such process, the rapid biodegradation induces its use in much larger amounts than those expected due to partial degradation do(4). Pesticides are widely found in the aquatic environment: in water surfaces, ground water and rainwater (5). The World Health Organization (WHO) recommended the classification of pesticides by hazard which approved on the 28th World Health Assembly in 1975 and has since then it gained a wide acceptance globally. When it was published in the WHO Chronicle, 29, 397-401 (1975), an annex, which was not part of the classification, illustrated its use by

listing examples of classification of some pesticidal active ingredients and their formulations. Later suggestions were made by Member States and pesticide registration authorities that further guidance should be given on the classification of individual pesticides. Guidelines were first issued in 1978, and have since been revised and reissued at 2-3-year intervals. Chemical type is also a determinant of the UN numbering system. These chemicals classifications are included only for convenience, and do not represent a recommendation of the WHO as to the way in which the pesticides should be classified. It should be understood that some pesticides may fall into more than one type (6).

1.2 Types of Pesticides

Arsenic compound (AS)
Bipyridylium derivative (BP)
Carbamate (Ca)
Coumarin derivative (CO)
Copper compound (CU)
Mercury compound (HG)
Nitrophenol derivative (NP)
Organochlorine compound (OC)
Organophosphorus compound (OP)
Organotin compound (OT)
Phenoxyacetic acid derivative (PAA)
Pyrazole (PZ)
Pyrethroid (PY)
Triazine derivative (T)
Thiocarbamate (TC) (6).

1.3 Safe Use of Pesticides

When it is necessary to use insecticides to protect your garden, use them wisely and safely. The following tips will help you make better use of insecticides. Inspect the entire garden at least weekly to monitor insect's number and activity.

Pay particular attention to leaves underside where mites, whiteflies, aphids and insect eggs may present. If a treatment is applied an infestation starts first, insects numbers can be maintained at lower levels much more easily, and with smaller amounts of chemicals (7).

At the present time, the most widely used pesticides belong to the organophosphorus group. In the USA alone over 40 million kilos of Organophosphorus pesticides (OPs) are applied annually (8). On the other hand more than 250 metric tons of formulated pesticides, are used annually in the Gaza Strip (9). OPs are frequently used in agriculture in the Gaza Strip and could be an important source of poisoning. Most occupational exposures to pesticides occur from skin absorption, although inhalation may be an important route of exposure during pesticide manufacture and application; the gastrointestinal tract and eyes may also be affected (10).

According to WHO report, two million people suffering from pesticide poisonings with about 40,000 deaths per year (11). OP are the most widely used insecticides, accounting an estimation of 34% of world-wide insecticide sales(12). The wide use of OPs has created numerous problems, including the pollution of the environment especially the environmental pollution (13). OPs are toxic substances that exert their toxic effect by acetylcholinesterase (AchE) inhibition (14). OPs such as Parathion, Methyl parathion, and Methamidophos are a group of highly toxic agricultural chemicals widely used in plant protection. As these pesticides cause enormous damage to non-target organisms (15).

1.4 Environmental Fate

Different pathways of organophosphates decomposition have been reported such as hydrolysis, photolytic oxidation, microbial transformations and other biological processes have been reported recently (16). Once in the environment, pesticides are subject to photochemical, chemical, and biological effects capable of causing transformations in the chemical structure compound's. Biological and

non biological processes work together to degrade herbicides. In nature it is difficult to distinguish between the two modes of degradation in most cases. Though some reactions are clearly nonbiological, such as photolysis, others, such as hydrolysis, can be either nonbiological or biologically mediated. Examples of reactions that can transform herbicides in the environment are shown in Table 1.1 (17).

Table 1.1 Reaction that can transform chemicals in the environment (17).

Category	Example	Reference
Photolysis	Aldrin	Matsumura (1982)
Hydrolysis	Diazlnon	Matsumura (1982)
Oxidation	Dichlorophenoxyacetic acid (2,4-D)	Sandmann and Loos(1988)
Dehalogenation	Chlorophenols	Steiert et al. (1987)
Deamination	Aniline	Zeyer et al. (1985)
Decarboxylation	Bifenox	Leather and Foy (1977)
Methyl oxidation	Isopropylnaphtha	Yoshida and Kojima (1978)
Hydroxylation	Dicamba	Smith (1971)
Sulfur oxidation	Aldicarb	Andrews et al. (1971)
Reduction	DDT	Pfaender and Alexander (1972)
Oxime metabolism	Aldicarb	Jones (1976)
Ester cleavage	Malathion	Paris et al (1975)
C-N cleavage	Alachlor	Tiedje and Hagedron (1975)
C-S cleavage	Benthiocarb	Ishikawa et al. (1976)
C-Hg cleavage	Ethylmercury	Kimura and Miller (1975)
S-N cleavage	Oryzalin	Golab et al. (1975)

1.4.1 Photodegradation Processes

Photochemistry of this class of compounds (mainly the pesticides) started with direct photolysis in organic solutions, and other solutions used for the application of these pesticides. Afterwards the studies have been oriented in aqueous solution with environmental concerns. The understanding of photodegradation processes is complementary to biological ones, and both processes can

contribute to the fate of benzothiazoles in the environment. However, at the moment the photodegradation is more in relation with related to the Advanced Oxidation Processes (AOP's) of these compounds in water for applications in wastewater treatment plants(18).

1.4.2 Biodegradation

Controlled persistence and biodegradation of pesticides in soil are highly desirable for reducing contamination and protecting our food, fiber, and environment (19). Microbes are able to degrade a wide variety of chemicals, from simple polysaccharides, amino acids, proteins, lipids, etc. and to more complex material such as plant residues, waxes, and rubbers. Some important degradative bacteria that occur in water and soil environments are described in (Table 1.2) (17).

Table 1.2 Classifications of derivative bacteria that occur in water and soil (17).

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

Pseudomonas strains are extremely common and are often the predominant members of the populations selected from natural sources such as soil, polluted waters, and sediments, for their ability to grow on single compounds as sole carbon sources. *Pseudomonas* strains are facultative anaerobes and some can use nitrate as a terminal acceptor for a limited number of substrates. Aerobically grown *Pseudomonads* can be described as Gram-negative unicellular rods (17).

The role of soil microorganisms in the persistence of agricultural pesticides has been the subject of two areas of study. The first is the capacity of rapid elimination of highly persistent or toxic chemicals. The second is reducing pesticids efficiency attributed to enhanced biodegradation, particularly of chemicals applied under a continuous cropping program (14). Catabolism and detoxification metabolism occur when a soil microorganism uses the pesticide as a carbon and energy source. Their degradation has received considerable attention from soil microbiologists. Several OPs degrading bacteria have been isolated from agricultural soils in diverse geographical regions. Most of these bacteria possess a novel triesterase often referred to as organophosphorus acid anhydrase and encoded by the highly conserved organophosphate degradation (opd) gene, localized either on dissimilar indigenous plasmids or on the chromosome (19).

Accelerated Microbial degradation of OPs is of particular interest because of their high mammalian toxicity and widespread use. Accelerated microbial degradation of pesticides has been observed in widely separated geographical zones such as Europe, the United States, New Zealand, Australia, India, Israel and Costa-Rica. Microbial degradation of pesticides applied to soil is the principle mechanism which prevents the accumulation of these chemicals in the environment (20).

Table 1.3 Organophosphorus pesticides (OPs) (21).

OPs	chemical structures of the organophosphorus
Acephate	[0,Sdimethyl- phosphoroamidothioate]
Chlorpyrifos	[O,O-diethyl-O-(3,5,6-trichloro-2-pyridylphosphoro-thioate)]
Malathion	[O,O-dimethyl-S-(ethyl-1,2-dicarboethoxy) phosphorodithioate]
Methamidophos	[O,S-dimethylphosphoramidothioate]
Parathion-methyl	[O,Odimethyl O-(4-nitrophenyl) phosphorothioate]

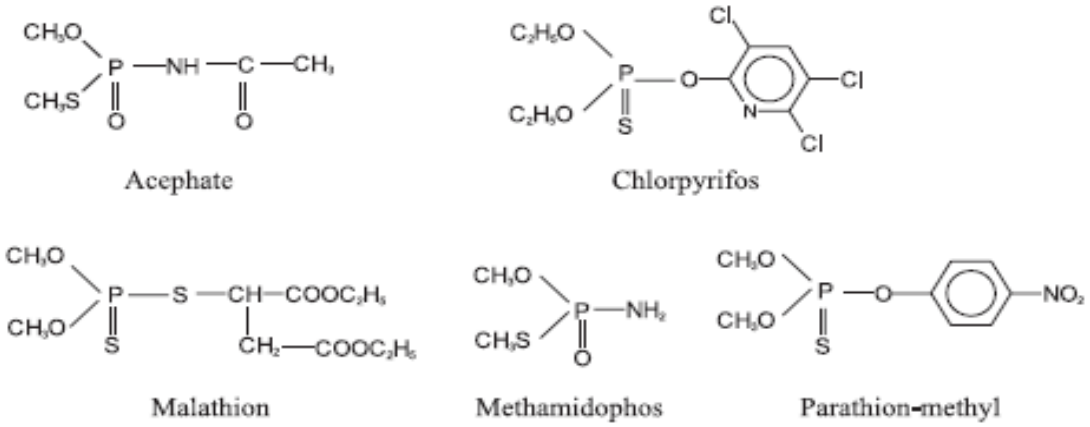


Figure 1.1 The chemical structures of the organophosphorus pesticides (21).

1.6 Methamidophos:

1.6.1 Chemical History

Methamidophos (Fig.1.2) is an organophosphate insecticide, was first registered in the United States in 1972 and used for cotton, potatoes, and numerous other crops(22).

Methamidophos is an organophosphate pesticide used to control a wide range of insects through direct contact and the systemic inhibition of insects cholinesterase. The insecticide methamidophos Common Name:

Methamidophos, Tamaron, Monitor, SRA 5172, Bayer 71 628, RE9006, Filitox, Tamanox, Tam, Patrole, Metamidofos Estrella; 60 WSC:Methedrin 60; Morithion;Red Star Alloran.

IUPAC Name: O,S-Dimethyl Phosporamidothioate

Chemical Formula: $C_2H_8NO_2PS$

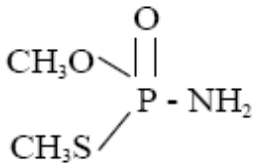


Figure 1. 2 Chemical structure of methamidophos (23).

Table 1.4 Physical properties of methamidophos (23).

Property	Data
Form	Crystalline solid, with off-white color and pungent odor
Molecular Weight	141.1 g/mol
Solubility in Water	Highly soluble
Melting Point	37-39^{0}C (technical) 44.5^{0}C (pure)
Physical Form	Yellowish to colorless crystals (technical). Colorless crystals (pure)
Vapor Pressure	3× 10^{-4} mm Hg at 30^{0}C (pure)
Relative Density	1.3 (water =1)

Methamidophos is readily soluble in alcohol, ketone and aliphatic chlorinated hydrocarbons; sparingly soluble in ether, and practically insoluble in petroleum ether.

1.6.2 Methamidophos Use

Methamidophos is a systemic pesticide and acaricied with contact and stomach action. It is absorbed by the roots and leaves and used to control chewing and sucking insects, and spider mites on ornamentals, hops, potatoes, maize, stone fruit, brassicas, vines, cotton, head lettuce, and many other crops. Methamidophos is marketed mainly as the water-soluble concentrate in various concentration (23). Methamidophos is commonly used in Gaza Strip (9).

1.6.3 Human Health Effects

Methamidophos is known to cause inhibition of acetylcholinesterase enzyme (AchE) activity and plasma cholinesterase in nervous tissue. The inhibition of cholinesterase enzyme leads to the accumulation of Acetylcholine in nerve synapses, the motor end-plate and in the central nervous system with desensitization of cholinergic receptors and signs of poisoning can be observed.

1.6.4 Environmental Fate of Methamidophos

Aerobic soil metabolism is the main degradative process for methamidophos. A half life of 14 hours was observed in a sandy loam soil at greater than currently registered application rates. The main intermediate degradate O-desmethyl methamidophos was produced, which itself has a half life of less than days, and is rapidly metabolized by soil microorganisms to carbon dioxide and microbial biomass. As a result, methamidophos was often detected in high concentrations in the ecosystem and thus lead to some direct and potential adverse effects on environmental safety. Another major degradate, S-dimethyl phosphorothioate (DMPT) is also produced. DMPT rapidly degradates in soil having a half life of less than 4 days. No information is available regarding the anaerobic soil metabolism of methamidophos (23).

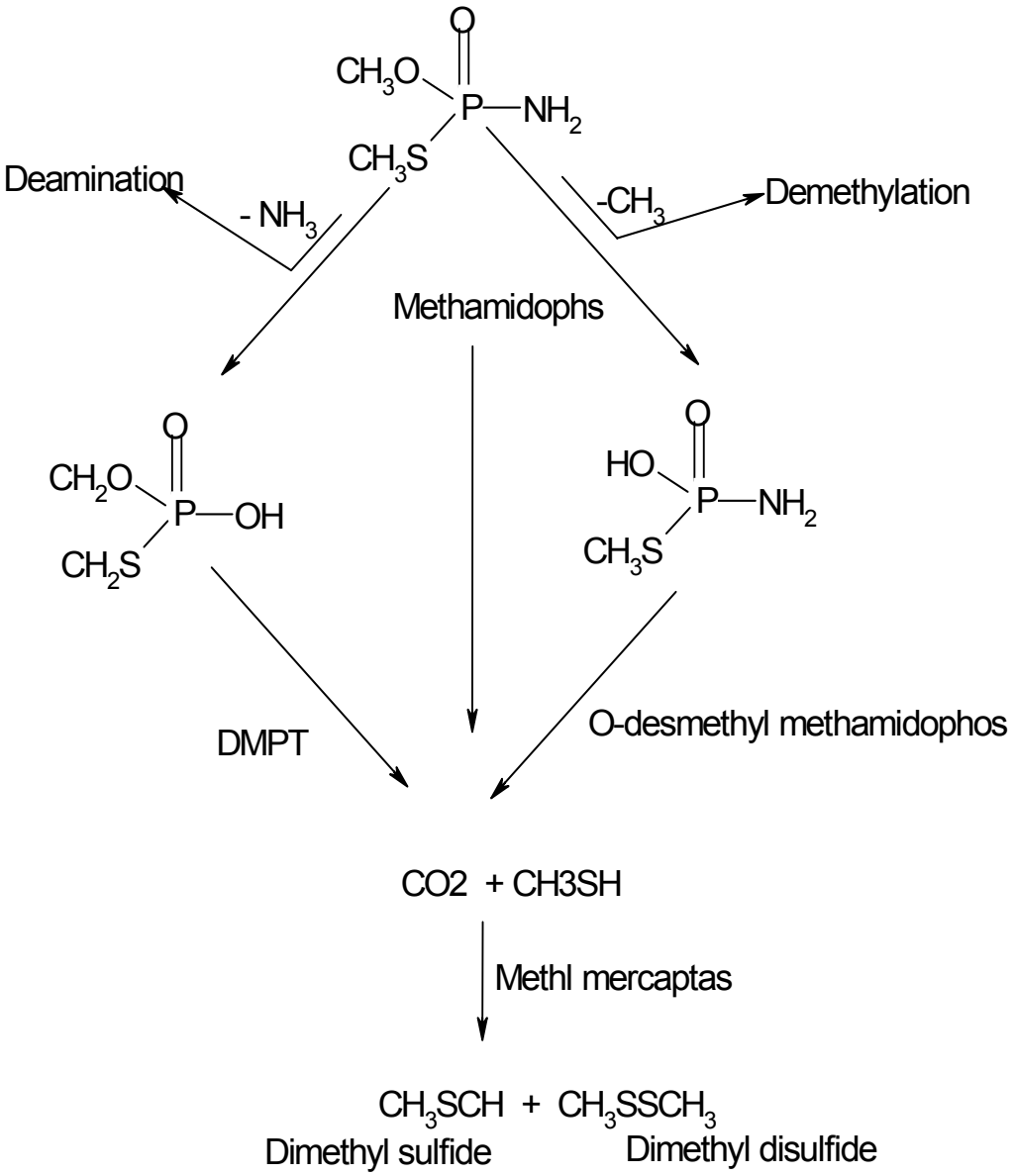


Figure 1.3 Proposed pathways for the degradation of methamidophos during soil metabolism (24).

1.7 Aim of Study

The aim of this study was to isolate and characterize indigenous strains of bacteria that are capable to degrade methamidophos.

1.8 Significance of the Study

1-Methamidophos is a broad-spectrum organophosphate insecticide with uses on many crops (11).

2- Methamidophos is known to cause inhibition of AChE activity and plasma cholinesterase in nervous tissue in insects and mammals (23).

3-Methamidophos is highly toxic to bees and other beneficial non-target insects (25).

4- To find microorganisms capable to degrade methamidophos accumalted in soil will be high significance (4).

1.8.1 Specific Objectives

1- Enrichment & isolation of methamidophos degrading organisms from soil.

2-Identification & Characterization of the isolates by morphological & biochemical tests.

3-Acclimatization of the isolates and selection of the strain with high biodegradability.

CHAPTER 2
LITERATURE REVIEW

2.1 Microorganisms

2.1.1 *Neisseria lactamica*

Table 2.1 Scientific classification of *Neisseria Lactamica* (26)

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Beta Proteobacteria
Order	Neisseriales
Family	Neisseriaceae
Genus	*Neisseria*
Species	*Lactamica*
Neissria Lactamica	

N. lactamica was described by Hollis et al. in 1969 and as *N. meningococcoides* by Berger in 1970**.** *Neisseria lactamica* is a gram-negative diplococcus bacterium. It is strictly a commensal species of the nasopharynx. Uniquely among the *Neisseria* they are able to produce β-D-galactosidase and ferment lactose. This species is most commonly carried by young children. There is an inverse relationship between colonisation by *N. lactamica* and *Neisseria meningitidis* (27) .

Strains of *N. lactamica* had not been described in studies previous to before 1969 because acid production from lactose was not used as a differential test for the identification of *Neisseria* spp. Prior, to the identification of these species, strains of *N. lactamica* would have been identified as *N. meningitides*. *N. lactamica* strains are colistin-resistant, grow on gonococcal selective medium, and are characterized by the ability to produce acid from glucose, maltose, and lactose, and by the ability to produce beta-galactosidase. Among the *Neisseria*

species, *N. lactamica* is one of the easiest species to identify; it is the only species that can produce acid from lactose and produces beta-galactosidase. Thus, with a rare exception (one known, unpublished report of a lactose-negative strain), this species should not be misidentified (28)

16S ribosomal DNA sequence analysis, chromosomal DNA-DNA hybridiziation studies, and representational difference analysis clearly support the separation of the two species *N. lactamica* which is pathogenic, and *Neisseria meningitidis*, which is a facultative pathogen (29).

Neisseria lactamica is similar to *Neisseria* meningitidis in many respects but differs in its relative lack of virulence and ability to utilize lactose. The commensal *Neisseria*, unlike pathogenic strains, rarely grows on media selective for meningococci. *N. lactamica* and *N. polysaccharea* and occasionally other species, allow to carry out prevalence studies to be carried mainly in children (30).

2.1.2 *Micrococcus lylae*

Table 2.2 Scientific classification of *Micrococcus lylae (31)*

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales
Family	Micrococcaceae
Genus	*Micrococcus*
Species	*Lylae*
Micrococcus lylae	

The genus *Micrococcus* was first described by Cohn (1872). The description of the genus has been revised several times, aerobic, catalase-positive, Gram-

positive cocci into two groups. Strains fermenting glucose were placed into group 1 and were later described as members of the genus *Staphylococcus.* Those utilizing glucose oxidatively or not at all, were placed in group 2 (the genus *Micrococcus*). Proposed a classification into tow groups based on the G-C content of the genomic DNA. Strains with a G-C content within the range 30±7±36±4 mol% were classified in the genus *Staphylococcus*, whereas it was proposed that strains with a G-C content within the range 66±3±73±3 mol% belongs to the genus *Micrococcus*. Subsequently, the genus *Micrococcus* was extended to include the species *Micrococcus lylae*, *Micrococcus kristinae*, *Micrococcus nishinomiyaensis*, *Micrococcus sedentarius* and *Micrococcus halobius*. (32 and 33)

2.1.3 Bacillus sphaericus

Table 2.3 Scientific classification of *Bacillus sphaericus* (34)

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species	*sphaericus*
Bacillus sphaericus	

Bacillus sphaericus is a gram-positive, mesophilic, strictly aerobic, round spore former saprophytic bacterium (35) . The spore size is around 0 .5-1.0 µm. *B. sphaericus* is found commonly throughout the world in soil and aquatic environments. A rapid decline in spore viability, on the other hand, is observed during storage under acidic conditions. These bacteria are also characterized by negative reaction to most of the traditional phenotypical tests used for the

classification and identification of bacilli. This largely arises from their obligately aerobic physiology and inability to use sugars as source of carbon and energy.

Although *Bacillus sphaericus* has been studied by a number of workers since its original description by Neide in 1904. Many strains of B. *sphaericus* are also urease positive. The possibility of human pathogenicity among strains of B. *sphaericus* has been raised by reports of Allen , Wilkinson and Farrar. Pathogenicity for mosquito larvae although it is unknown how widespread either human or mosquito larval pathogenicity is among strains of the species (36).

2.1.3.1 Toxins Produced by *B. sphaericus*

The entomocidal *B. sphaericus* strains synthesize two types of toxins, binary toxin (Btx) and mosquitocidal toxin (Mtx). Many high-toxicity strains synthesize both Btx and the Mtx, while others synthesize only the binary toxin. Low-toxicity strains synthesize either none of the two toxins or only the Mtx. Both toxins differ in their compositions and time of synthesis. There is also evidence for presence of additional toxin genes encoding novel type of mosquitocidal toxins of 31.8 kDa and 35.8 kDa. Mosquitocidal toxin (Mtx) is unrelated to Btx. It is synthesizeduring vegetative growth phase and is proteolytically degraded as the cells enter the stationary phase. Three types of Mtx toxins have been described, Mtxl, Mtx2 and Mtx3 with molecular mass of 100, 30.8 and 35 kDa respectively. These toxins do not display any similarity to each other to the crystal proteins or any other insecticidal proteins (37).

2.2 Biodegradation of Pesticides

1- Isolated, characterizated 2,4-dichlophenoxyacetic acid degrading was investigated by Brajesh K. Singh and Allan Walker. The first micro-organism that could degrade organophosphorus compounds was isolated in 1973 and identified as *Flavobacterium species*. and microbial degradation of organophosphorus

compounds were reviewed and the list of all isolated organisms that can degrade different pesticides are listed in the table 2.4 (12).

2- A constructed microbial consortium for biodegradation of the organophosphorus insecticide parathion was investigated by E. S. Gilbert and A. W. Walker · J. D. Keasling A consortium comprised of two engineered microorganisms was assembled for biodegradation of the organophosphate insecticide parathion. *Escherichia coli SD2* harbored two plasmids, one encoding a gene for parathion hydrolase and a second carrying a green fluorescent protein marker. *Pseudomonas putida KT2440 pSB337* contained a p-nitrophenol-inducible plasmid-borne operon encoding the genes for p-nitrophenol mineralization. The co-culture effectively hydrolyzed 500 mM parathion (146 mg L^{-1}) and prevented the accumulation of p-nitrophenol in suspended culture. Kinetic analyses were conducted to characterize the growth and substrate utilization of the consortium members. Parathion hydrolysis by *E. coli SD2* followed Michaelis–Menten kinetics. p-Nitrophenol mineralization by P. putida *KT2440 pSB337* exhibited substrate-inhibition kinetics. The growth of both strains was inhibited by increasing concentrations of p-nitrophenol, with *E. coli SD2* completely inhibited by 600 mM p-nitrophenol (83 mg L^{-1}) and *P. putida KT2440 pSB337* inhibited by 1,000 mM p-nitrophenol (139 mg L^{-1}). Cultivation of the consortium as a biofilm indicated that the two species could cohabit as a population of attached cells. Analysis by confocal microscopy showed that the biofilm was predominantly comprised of *P. putida KT2440 pSB337* and that the distribution of *E. coli SD2* within the biofilm was heterogeneous. The use of biofilms for the construction of degradative consortia may prove beneficial (38)

3- Biodegradation and Utilization of OPs by *Cyanobacteria* was investigated by G. Subramanian et al. Ten strains of filamentous-heterocystous cyanobacteria were screened for their growth in and tolerance of upto 50ppm of the organophosphorus pesticides, monocrotophos and malathion (39).

4- Isolation, characterization and growth response of pesticides degrading bacteria was investigated by S. Jilani and M.Altaf Khan. They isolated bacterial strain from soil using enrichment technique and identified as *Pseudomonas sp*. by microscopic examination and biochemical tests. Growth curve experiments showed that *Pseudomonas* strain was able to grow in nutrient medium containing malathion (35-220 mg L^{-1}), methamidophos (80-320 mg L^{-1}), cartap (60-120 mg L^{-1}) and cypermethrin (40-125 mg L^{-1}) pesticide. These data indicate that the isolated *Pseudomonas* strain can be used as a microorganism for the bioremediation of pesticide contaminated soil or water (11).

5- Isolation and characterization of 2,4- Dichlorophenoxyacetic Acid (2,4-D) Degrading organisms from Soil in Jordan Valley was investigated by Amjad B. Khalil . The pesticide (2,4-D) degrading bacteria were isolated from soil samples collected from selected sites in Jordan Valley,which had a history of 2,4-D exposure. The herbicide 2,4-D was used in a minimal salt medium as a sole source of carbon to isolate and enumerate the 2,4-D- theaders by most probable –number (MPN) method . One site had previous history of exposure to 2,4-D found to contain 88 2,4-D– degraders /g soil suspension; other sites were not exposed to 2,4-D and showed much lower number of 2,4-D degraders ranging from 0.5-2.1 degraders /g soil suspension. One of the seven isolates was found to degrade 2,4-D entirely in 45 h and it was the most potent isolate. It was identified and found to be *Pseudomonas putida*. It grew and degraded 500 ppm of 2,4-D optimally at pH 6.5 and 30˚ C (40).

6- Degradation of methamidophos by *Saccharomyces rouxii WY-3* was investigated by Chinese groups.They isolated a strain of Yeast WY-3 from wastewater samples. It is capable of utilizing methamidophos as sole nitrogen and phosphorus sources, and also capable of utilizing methylamine, ethylamine and ammonium sulfate as nitrogen sources except nitrate and hydroxylamine. The yeast could grow in medium containing 60% glucose and was identified as *Saccharomyces rouxii WY-3* (41).

7- Non-specific biodegradation of the OPs, cadusafos and ethoprophos, by two bacterial isolates was investigated by Dimitrios G. et al. An enrichment culture technique was used for the isolation of microorganisms responsible for the enhanced biodegradation of the nematicide cadusafos in soils from a potato monoculture area in Northern Greece. Mineral salts medium supplemented with nitrogen (MSMN), where cadusafos (10 mg L^{-1}) was the sole carbon source, and soil extract medium (SEM) were used for the isolation of cadusafos-degrading bacteria. Two pure bacterial cultures, named CadI and CadII, were isolated and subsequently characterized by sequencing of 16S rRNA genes. Isolate CadI showed 97.4% similarity to the 16S rRNA gene of a *Flavobacterium* strain, unlike CadII which showed 99.7% similarity to the 16S rRNA gene of a *Sphingomonas paucimobilis*. Both isolates rapidly metabolized cadusafos in MSMN and SEM within 48 h with concurrent population growth. This is the first report for the isolation and characterization of soil bacteria with the ability to rapidly degrade cadusafos and use it as a carbon source. Degradation of cadusafos by both isolates was accelerated when MSMN was supplemented with glucose (42).

8- Towards efficient crude oil degradation by a mixed bacterial consortium was investigated by K.S.M. Rahman et al. A laboratory study was undertaken to assess the optimal conditions for biodegradation of Bombay High (BH) crude oil. Among 130 oil degrading bacterial cultures isolated from oil contaminated soil samples, *Micrococcus sp*. *GS2-22*, *Corynebacterium sp. GS5- 66, Flavobacterium sp. DS5-73*, *Bacillus sp*. *DS6-86 and Pseudomonas sp*. *DS10-129* were selected for the study based on the effciency of crude oil utilisation. A mixed bacterial consortium prepared using the above strains was also employed. Individual bacterial cultures showed less growth and degradation than the mixed bacterial consortium. At 1% crude oil concentration, the mixed bacterial consortium degraded a maximum of 78% of BH crude oil. This was followed by 66% by *Pseudomonas sp.* DS10-129, 59% by *Bacillus sp. DS6-86,* 49% by *Micrococcus sp*. *GS2-22*, 43% by *Corynebacterium sp*. *GS5-66* and 41% by

Flavobacterium sp. *DS5- 73*. The percentage of degradation by the mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1% to 10%. Temperature of 30^0C and pH 7.5 were found to be optimal for maximum biodegradation (43).

9- Biodegradation of melamine formaldehyde by *Micrococcus sp*. Strain MF-1 isolated from aminoplastic wastewater effluent was investigated by Wael S. El-Sayeda et al.Novel bacterial strain MF-1, able to grow using melamine formaldehyde (MF) resin as main carbon and nitrogen source, is described and characterized. Strain MF-1 was isolated from wastewater effuent of an aminoplastic industrial plant. Growth rate kinetics of the strain on MF showed a maximum specific growth rate (μ_{max}) of 0.83 cells mL^{-1} h^{-1} and K_s of 7.18 cells mL^{-1}. Mineralization of MF by strain MF-1 was confirmed by the decrease in dissolved oxygen (DO), release of ammonia, and detection of intermediate metabolites during biodegradation. Melamine, cyanuric acid, and biuret were detected as intermediate metabolites in the culture, biodegradation of MF by strain MF-1 proceeds via successive deamination reactions of melamine to cyanuric acid, which is hydrolyzed to biuret and .nally to NH_3 and CO_2. Based on 16S-rDNA sequence analysis, strain MF-1 had a similarity of 97% to *Micrococcus sp. MN 8.1d*. However, the high bootstrap value obtained in the phylogenetic analyses suggests that this is a novel strain (44) .

10- Biodegradation of benzo[a]pyrene in soil by *Mucor* sp. SF06 and *I*. *SB02* co-immobilized on vermiculite was investigated by SU Dan and et al.Two indigenous microorganisms, *Bacillus sp. SB02* and *Mucor sp*. *SF06*, capable of degradmg polycyclic aromatic hydrocarbons (PAHs) were co-immobilized on vermiculite by physical adsorption and used to degrade benzo[a] pyrene *(BaP).* The characteristics of BaP degradation by both h e and co-immobilized microorganism were then investigated and compared. The removal rate using the immobilized bacterial-fungal mixed consortium was higher than that of the fireely mobile mixed consortium. 95.3% of BaP was degraded using the co-immobilized

system within 42 d, which was remarkably higher than the removal rate of that by the free strains. The optimal amount of inoculated co-immobilized system for BaP degradation was 2%. The immobilized bacterial-bgal mixed consortium also showed better water stability than the fiee strains. Kinetics of BaP biodegradation by co-immobilized SF06 and SB02 were also studied. The results demonstrated that BaP degradation could be well described by a zero-order reaction rate equation when the initial BaP concentration was in the range of 10-200 mg/kg. The scanning electronic microscope (SEM) analysis showed that the co-immobilized microstructure was suitable for the growth of SF06 and SB02. The mass transmission process of co-immobilized system in soil is discussed. The results demonstrate the potential for employing the bacterial-fungal mixed consortium, co-immobilized on vermiculite, for *in* situ bioremediation of BaP (45).

11-Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India was investigated by Kishore Das and Ashis K. Mukherjee. The efficiency of *Bacillus subtilis DM-04* and *Pseudomonas aeruginosa M* and *NM* strains isolated from a petroleum contaminated soil sample from North-East India was compared for the biodegradation of crude petroleum-oil hydrocarbons in soil and shake flask study. These bacterial strains could utilize crude petroleum-oil hydrocarbons as sole source of carbon and energy. Bioaugmentation of TPH contaminated microcosm with *P. aeruginosa M and NM* consortia and *B. subtilis* strain showed a significant reduction of TPH levels in treated soil as compared to control soil at the end of experiment (120 d). *P. aeruginosa* strains were more efficient than *B. subtilis* strain in reducing the TPH content from the medium. The plate count technique indicated expressive growth and biosurfactant production by exogenously seeded bacteria in crude petroleum-oil rich soil. The results showed that *B. subtilis DM-04* and *P. aeruginosa M* and *NM* strains could be effective for in situ bioremediation (46).

12-Isolation, biodegradation ability and molecular detection of hydrocarbon degrading bacteria in petroleum samples from a Brazilian offshore basin was investigated by Suzan Pantaroto de Vasconcellos a,b, et al The detection of microorganisms with potential for biodeterioration and biodegradation in petroleum .eldsfildr is of great relevance, since these organisms may be related to a decrease in petroleum quality in the reservoirs or damage in the production facilities. In this sense, petroleum formation water and oil samples were collected from the Campos Basin, Brazil, with the aim of isolating microorganisms and evaluating their ability to degrade distinct classes of hydrocarbon biomarkers (9,10-dihydrophenanthrene, phytane, nonadecanoic acid and 5a-cholestane). Twenty eight bacterial isolates were recovered and identified by sequencing their 16S rRNA genes. Biodegradation assays revealed that bacterial metabolism of hydrocarbons occurred through reactions based on oxidation, carbon–carbon bond cleavage and generation of new bonds or by the physical incorporation of hydrocarbons into microbial cell walls. Based on the biodegradation results, selective PCR-based systems were developed for direct detection in petroleum samples of bacterial groups of interest, namely *Bacillus spp., Micrococcus spp.,* Achromobacter xylosoxidans, *Dietzia spp*. and *Bacillus pumilus*. Primer sets targeting 16S rRNA genes were designed and their specifcity was confirmed in silico (i.e. computational analysis) and in PCR reactions using DNA from reference strains as positive and negative controls. Total DNA from oil was purifed and the amplifcation tests revealed the presence of the target bacteria in the samples, unraveling a signifcant potential for petroleum deterioration in the reservoirs sampled, once proper conditions are present for hydrocarbon degradation. The application of molecular methods for rapid detection of specific microorganisms in environmental samples would be valuable as a supporting tool for the evaluation of oil quality in production reservoirs (47).

13- Identification and molecular characterization of a *Bacillus subtilis IS13* strain involved in the biodegradation of 4,5,6-trichloroguaiacol was investigated by C.W.S. Andretta a, et al. Henriques 4,5,6-Trichloroguaiacol (4,5,6-TCG) is a

recalcitrant organochlorine compound produced during pulp bleaching and a potential environmental hazard in paper mill effiuents. Here the identification by biochemical tests and molecular biological analysis, using 16S ribotyping, of a 4,5,6-TCG-degrading bacterium, identified as a strain of *Bacillus subtilis* that is most closely related according to the phylogenetic analysis to *B. subtilis* strain Lactipan (alignment score 99%). Biodegradation of 4,5,6-TCG by this organism in a mineral salts medium was shown to occur only when the inoculum was composed of cells in the stationary phase of growth and to be accelerated by an additional carbon source, such as glucose, sucrose, glycerol or molasses. An additional nitrogen source (as ammonium sulfate) did not affect the rate of 4,5,6-TGC removal. No plasmids were detected in the bacterial cells. This is the first strain of *B. subtilis* which degrades chlorophenols and shows that 4,5,6-TCG is not degraded by cometabolism and that the gene encoding this characteristic is probably located on the chromosome. The lack of requirement for additional nitrogen source, the ability to enhance biodegradation by adding cheap carbon sources such as molasses, and the fact the trait is likely to be stable since it is encoded on the cell chromosome, are all characteristics that make the organism an attractive possibility for treatment of wastes and environments polluted with organochlorine compounds (48).

14- Biodegradation of pyridine by the new bacterial isolates *S. putrefaciens* and *B. sphaericus* was investigated by Anil Kumar Mathur ,C.B. et al. Roy In this study, two bacterial strains capable of utilizing pyridine as a sole carbon source were isolated from biofilters. Based on the biochemical test, the organisms were identified as *Shewanella putrefaciens* and *Bacillus sphaericus*. In liquid cultures, *S. putrefaciens* and *B. sphaericus* degraded pyridine quite effectively up to 500 mg L^{-1}. *S. putrefaciens* degrades 500 mg L^{-1} of pyridine completely within 140 h, whereas the *B. sphaericus* degrades 500 mg L^{-1} of pyridine only nearly 75% and takes a longer duration of 150 h. *S. putrefaciens* used pyridine as sole carbon and energy source better than *B. sphaericus* (49).

15- Biodegradation and Detoxification of Malathion by of *Bacillus Thuringiensis MOS-5* was investigated by Zeinat Kamal M. et al. Efficiency of a strain of *B. thuringiensis MOS-5 (Bt),* isolated from agricultural waste water near Berket El-Sabaa Egypt contaminated with organophosphorus insecticide, for degradation of malathion was investigated. It was able to utilize malathion as a sole carbon and energy source and to degrade it cometabolically. In a minimal salt medium supplied with 250 mg L^{-1} malathion either alone or incombination with glucose or yeast extract MOS-5 caused 99.32% reduction in malathion after 30 days. Addition of glucose (5 g L^{-1}) and yeast extract (0.5 g L^{-1}) increased the growth rate 104 and 105 fold, respectively, compared to malathion alone. Results of HPLC, gas chromatography/ mass spectrometry (GC/MS) and infrared spectroscopic analysis revealed that one malathion- derived compound mal-monocarboxylic acid (MMA) was producted after three days. Two additional malathion derivatives, mal-dicarboxylic acid (MDA) and unidentified mal-x were detected after 7 days. MMA and MDA were the major degrading compounds. Esterase activity involved in malathion degradation was also determined in culture filtrate of MOS-5. Results indicated that esterase activity was two folds more in the presence of yeast extract compared to glucose. These results indicate that Bt MOS-5 may consider as highly potential candidate in the biodegradation of organophosphorus in contaminated soil (50).

16-Biodegradation of organochlorine pesticides by bacteria grown in microniches of the porous structure of green bean coffee was investigated by B.E. Barraga et al. In this paper, the authors propose a model for DDT biodegradation by bacteria grown in microniches created in the porous structure of green bean coffee. Five bacteria isolated from coffee beans, identified as *Pseudomonas aeruginosa, P. putida, Stenotrophomonas maltophilia, Flavimonas oryzihabitans, and Morganella morganii. P. aeruginosa and F. oryzihabitans*, were selected for pesticide degradation (51).

Table 2.4 Microorganisms isolated for degradation of organophosphorus compound (12).

Compound	Microorganisms	Mode of Degration	Reference
Chloropyrifos	*Enterobacter sp.*	Catabolic (C, P)	Singh et al. (2003)
	Flavobacterium sp ATCC27551	Co-metabolic	Mallick et al. (1999)
	Pseudomonas diminuta	Co-metabolic	Serdar et al.(1982)
	Micrococcus sp.	Co-metabolic	Guha et al. (1997)
	Fungi		
	Phanerochete chrysossporium	Catabolic (C)	Bumpus et al. (1993)
	Hypholama fascicularae	ND	Bending et al. (2002)
	Coriolus versicolor	ND	Bending et al. (2002)
	Aspergillus sp.	Catabolic (P)	Obojska et al. (2002)
	Tricnoderma harizanum	Catabolic (P)	Omar (1998)
	Pencillium brevicompactum	Catabolic (P)	Omar (1998)
Parathion	*Bacteria*		
	Flavobacterium sp.	Co- metabolic	Sethunathan &Yoshida (1973)
	Pseudomonas diminuta	Co- metabolic	Serdar et al. (1982)
	Pseudomonas stutzeri	Co- metabolic	Daughton & Hsieh (1977)
	Arthrobacter spp.	Co- metabolic	Neison et al. (1982)
	Agrobacterium radiobacter	Co- metabolic	Horne et al. (2002 b)
	Bacillus spp.	Co- metabolic	Neison et al. (1982)
	Pseudomonas sp.	Catabolic (C, N)	Siddaramappa et al. (1973)
	Pseudomonas spp.	Catabolic (P)	Rosenberg& Alexander (1979)

Table 2.4 continued

Parathion	*Arthrobacter sp.*	Catabolic (C)	Nelson et al.(1982)
	Xanthomonas sp.	Catabolic (C)	Rosenberg& Alexander (1979)
Methyl parathion			
	Pseudomonas sp.	Co- metabolic	Chaudry et al. (1988)
	Bacillus sp.	Co- metabolic	Sharmila et al. (1989)
	Plesimonas spM6	Co- metabolic	Zhongli et al. (2001)
	Pseudomonas putida	Catabolic (C)	Rani &Lalitha-Kumari(1994)
	Pseudomonas sp. A3	Catabolic (C, N)	Zhongli et al. (2002)
	Pseudomonas sp. WBC	Catabolic (C, N)	Yali et al. (2002)
	Flavobacterium balustinum	Catabolic (C)	Somara &Siddavattam (1995)
Glyphosate	Bacteria		
	Pseudomonas ssp.	Catabolic (P)	Kertesz et al. (1994 a)
	Alcaligene sp.	Catabolic (P)	Tolbot et al. (1984)
	Bacillus megaterium 2BLW	Catabolic (P)	Quinn et al. (1989)
	Rhizobium sp.	Catabolic (P)	Liu et al. (1991)
	Agrobacterium sp.	Catabolic (P)	Wacket et al. (1987)
	Arthobacter sp.GLP	Catabolic (P)	Pipke et al. (1987)
	Arthobacter atrocyaneus	Catabolic (P)	Pike & Amrhein(1988)
	. Geobacillus caldoxylosilyticus T20	Catabolic (P)	Obojska et al. (2002)
	Flavobacterium sp	Catabolic (P)	Balthazor & Hallas (1986)
	Fungi		
	Penicillium citrium	Co- metabolic	Pothuluri et al. (1998)
	Penicillium natatum	Catabolic (P)	Pothuluri et al. (1992)
	Penicillium chrysogenum	Catabolic (N)	Klimek et al. (2001)

Table 2.4 continued

	Trichoderma viridae	Catabolic (P)	Zboinska et al. (1992b)
	Scopulariopsis spand	Catabolic (P)	Zboinska et al. (1992b)
	Aspergillus niger	Catabolic (P)	Zboinska et al. (1992b)
	Alternaria alternata	Catabolic (N)	Lipok et al. (2003)
Coumaphos	Bacteria		
	Nocardiodes simplex NRRL B24074	Co- metabolic	Mulbry (2000)
	Agrobacterium radiobacter P230	Co- metabolic	Horne et al. (2002b)
	Pseudomonas monteilli	Co- metabolic	Horne et al. (2002b)
	Flavobacteriumn sp.	Co- metabolic	Adhya et al. (1981)
	Pseudomonas diminuta	Co- metabolic	Serdar et al.(1982)
	Nocardia strain B-1	Catabolic (C)	Mulbry (1992)
Monocrotophos	Bacteria		
	Pseudomonas ssp.	Catabolic (C)	Bhadbhade et al. (2002b)
	Bacillus spp.	Catabolic (C)	Rangaswamy & Venkateswaralu (1992)
	Arthrobacter spp.	Catabolic (C)	Bhadbhade et al. (2002b)
	Pseudomonas mendocina	Catabolic (C)	Bhadbhade et al. (2002a)
	Bacillus megaterium	Catabolic (C)	Bhadbhade et al. (2002b)
	Arthrobacter atrocyaneus	Catabolic (C)	Bhadbhade et al. (2002b)
	Pseudomonas aeruginose F10B	Catabolic (P)	Singh &Singh (2003)
	Clavibacter michiganense SBL11		Singh &Singh (2003)
Fenitrothion	Bacteria		
	Flavobacterium sp.	Co- metabolic	Adhya et al. (1981)

Table 2.4 continued

	Arthrobacter aurescenes TW17	Catabolic (C)	Ohshiro et al. (1996)
	Burkholderia sp.NF100	Catabolic (C)	Hayatsu et al. (2000)
Diazinon			
	Flavobacterium sp.	Catabolic (P)	Sethunathan & Yoshida (1973)
	Pseudomonas spp.	Co- metabolic	Roseunberg & Alexander (1979)
	Arthrobacter spp.	Co- metabolic	Barik et al. (1979)
Chemical warfare agents			
G Agent	*Pseudomonas diminuta*	Co- metabolic	Mulbry & Rainina (1998)
	Altermonas spp.	Co- metabolic	DeFrank et al. (1998)
V Agent	*Pseudomonas diminuta*	Co- metabolic	Mulbry & Rainina (1998)
	Pleurotus ostreatus (fungus)	Co- metabolic	Yang et al. (1990)

Symbol in brackets after mode of degradation represents the type of nutrient that the pesticide provides to degrading microorganisms. C, carbon; N, nitrogen; P, phosphorus.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1 Apparatus

The Apparatus that were Used are Listed in Table

Table 3.1. List of the apparatus used in this work

Apparatus	Manufactures
Vortex mixer	Labret's VX-1OO (USA)
Incubator	Memmert (Oxford)
Shaker	Memmert (Oxford)
Autoclave	Tuttnauer (USA)
Refrigerator	UGUR (Turkey)
Light microscope	Olympus ch20 PLMS 200 (USA)
spectrophotometer	Haytona (USA)
Digital camera	Hp (China)
Safety capinet	Walker(USA)
Hot plate	Scientific company (China)

3.1.2 Stains

Table 3.2. List of the stains used in this work

Stain	Manufactures
Gram stain kit	Hi Media Company
Malachite green stain	

3.1.3 Reagents

Reagents	Manufactures
Sterile distilled water	Islamic University Lab
Normal saline	
Methamidophos	Bayer Crop Science
Hydrogen peroxide (3%)	Hi Media Company
Glycerol	
Ethanol	
Ammonium Dihydrogen phosphate	
Dipotassum Hydrogen Phosphate	
Dipotassium Hydrogen Phosphate	
Kovac's reagent	Hi Media Company
Phenol red	

3.1.4 Culture Media

Details of the Culture media used are summarized in table 3.3

Table 3.3 Suppliers for culture media

Nutrient agar medium	Hi Media Company
Nutrient broth	
Simmons citrate agar medium	
Methyl red –voges proskaur medium	
Starch agar medium	
Fermentation sugar (glucose , maltose, sucrose, fructose, mannitol l& lactose)	Hi Media Company

3.1.4.1 Minimal Salt Medium Preperaration

Minimal salt medium was prepared as follow :

Sodium Chlorid	5g
Magnesium Sulfate	0.2g
Ammonium Dihydrogen phosphate	1g
Dipotassum Hydrogen Phosphate	1g

Then the medium was bolied on hot plate and the agar and methamidophos were added and the culture medium was autoclaved at 121^0C for 15 min (40).

3.1.4.2 Modified Oxidation-Fermentation Medium (MOF)

MOF was used to detect *Neisseria spp* by carbohydrate utilization, the basal MOF medium was prepared as follows

0.2% Protease Peptone
0.5% NaCl
0.03% Dipotassium Hhydrogen Phosphate
0.03% agar,
and 0.25 ml of 0.017% phenol red solution per liter.

These ingredients were dissolved and sterilized at 121°C for 15 min. Filter-Sterilized carbohydrate solution was added at a final concentration of 1% (vol/vol), the pH was adjusted aseptically to 7.2, and the medium was dispensed in 12- by 75-mm sterile test tubes at the rate of 2.5 ml/tube.

3.1.5 Special Equipments

Inoculating needle

Aluminum paper

Cotton

Filter paper

Tissue paper

Parafilm

Plastic droppers

Plastic Petri plates

Plastic droppers

Plastic tubes

Sterile cotton

Magnetic stirrer

Labels

3.2 METHODOLOGY

3.2.1 Isolation of Pesticide Degrading Bacteria

Soil samples were collected from agricultural sites which had a history of methamidophos exposure from Bet lahya north Gaza strip Palestine.

The steps of isolation was as follow:

1- A wet unsieved soil (2-5 g) sample from the upper 10 cm of the soil samples were taken from the agricultural site and were kept in plastic bag at ambient temperature until processing. The bag was kept open to prevent an increase of their temperature and the subsequent death of bacteria (29).

2- samples were inoculated into 250 ml of wastewater in a 500 ml Erlenmeyer flask containing 100 mg／L methamidophos.

3-The flask was incubated on a shaker operating at 240 r.p.m for several days at ambient temperature (25 ˚C).

4- At daily intervals one loopfull of enrichment culture medium from the flask was streaked onto nutrient agar plates supplemented with different concentration of methamidophos pesticide and basal salt medium supplemented with methamidophpos at 35˚C for 24 h.

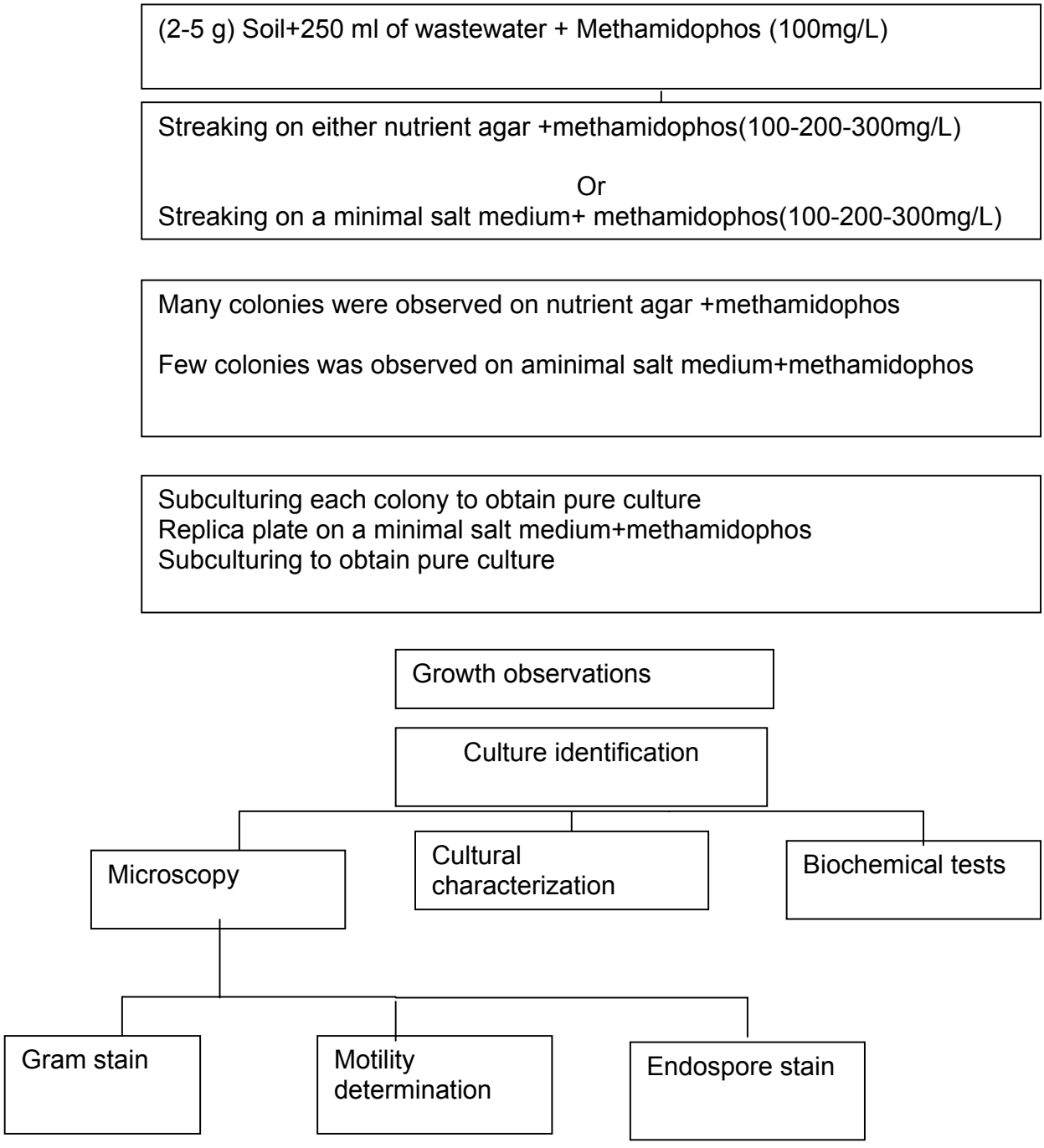
5- Individual colonies were sub-cultured into nutrient agar plates containing methamidophos until pure cultures were isolated.

6-Bacterial isolates that could

handle high concentration of pesticide were subjected to morphological and biochemical tests (11and 40)

The steps of isolation was as follow:

Figure 3.1 Flow chart for the isolation and identification of methamidophos degrading bacterial culture



3.2.2 Gram-Negative Cocci

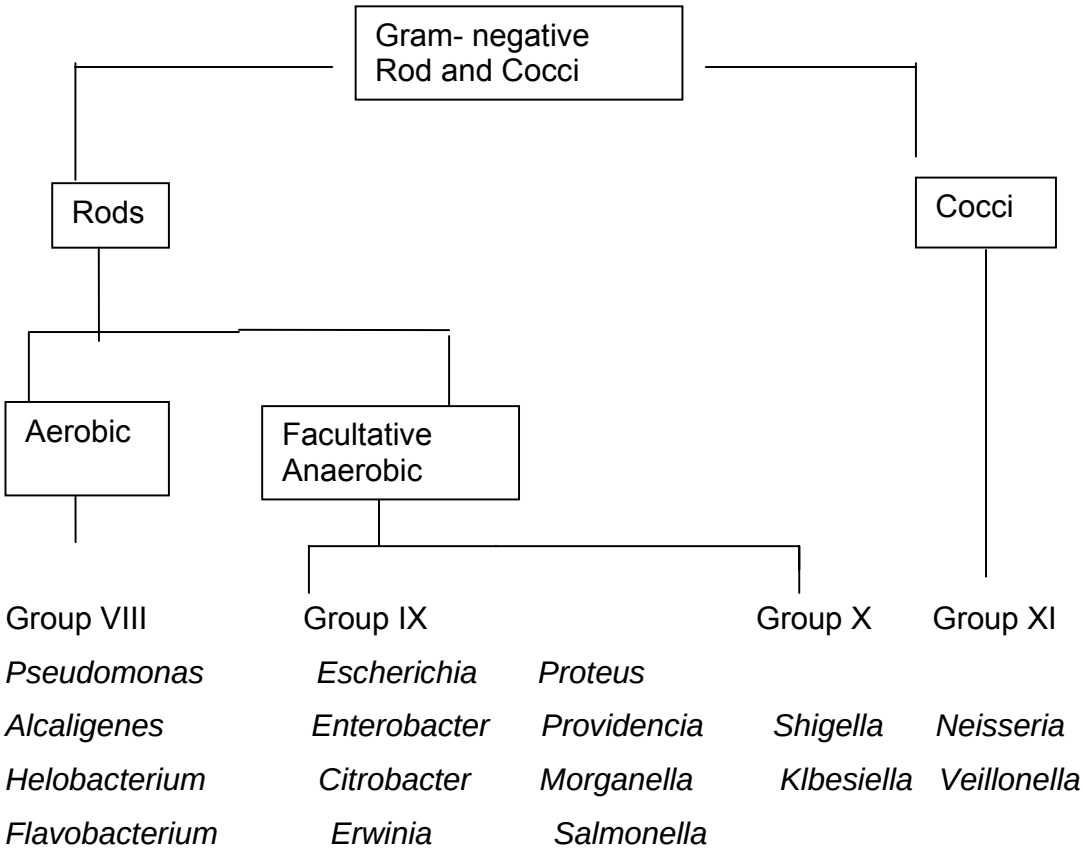


Figure 3. 2 The outline of gram –negative rods and gram –negative cocci separations according to, "Bergey's Manual of Systematic Bacteriology" (52).

For genus differentiation

Group XI genera are morphologically quite similar, but physiologically quite different.

All *Neisseria* species were oxidase- and catalse-positive.

All *Veillonella*-species were oxidase- and catalse-negative (52).

3.2.3 Sugar Utilization of *Neisseria spp*

Table 3.4 Selected biochemical tests for the differentiation between *Neisseria spp.* (53).

Species	Acid from						Nitrate reduction	Polysaccharide from sucrose
	G	M	S	F	Ma	L	-	+
N. animalis	-	-	+	-	-	-	+	-
N. canis	-	-	-	-	-	-	+	-
N. caviae	-	-	-	-	-	-	-	-
N. cinerea	-	-	-	-	-	-	-	-
N. cuniculi	-	-	-	-	-	-	-	+
N. denitrificans	+	-	+	+	+	-	-	-
N.elongata	-	-	-	-	-	-	-	-
N. flava	+	+	-	+	-	-	-	+
N. flavescens	-	-	-	-	-	-	-	-
N. gonorrhoeae	+	-	-	-	-	-	-	-
N. lactamica	+	+	-	-	-	+	-	-
N. meningitidis	+	+	-	-	-	-	-	-
N. mucosa	+	+	+	+	-		+	+
N. ovis	-	-	-	-	-	-	+	+
N. perflava	+	+	+	+	-	-	-	+
N. sicca	+	+	+	+	-	-	-	+
N. subflava	+	+	-	-	-	-	-	-

Key of bbreviations: G, glucose; M, maltose; S, sucrose; F, fructose; Ma, mannitol; L, lactose (53).

3.2.4 Gram-Positive Cocci

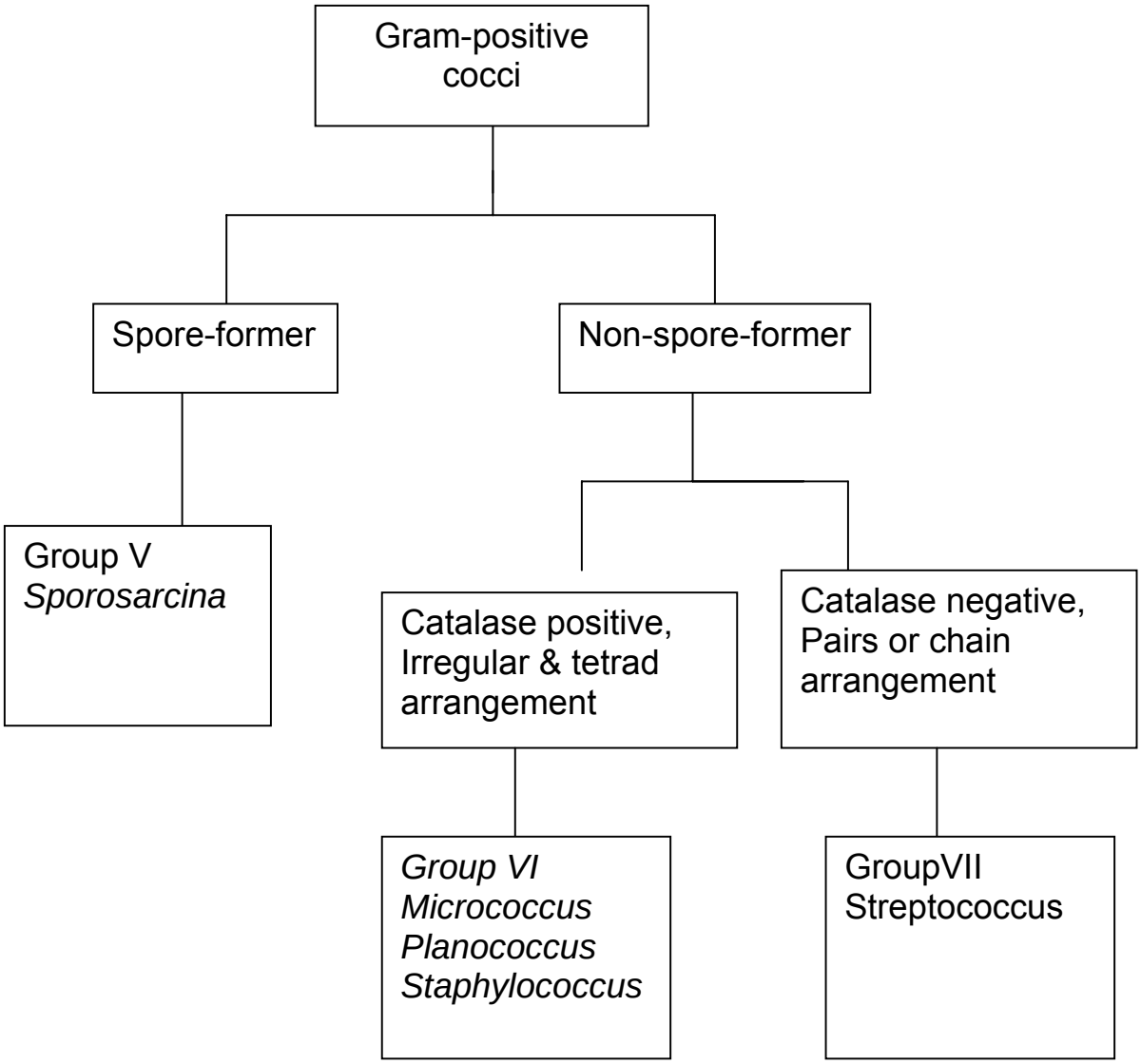


Figure 3.3 The outline of gram-positive cocci separation according to, "Bergey's Manual of Systematic Bacteriology" (52).

Table 3.5 Comparison of the phenotypic and the physiological properties of the members of the genus *Micrococcus* (54)

Characteristic	*M. luteus*	*M. lylae*	*M. antarcticus*
Optimal growth temp.	37	37	16.8
Hydrolysis of Tween:			
20	-	-	+
40	-	-	+
80	-	-	+
Urease	**+**	**+**	**-**
Starch hydrolysis	-	-	+
Indole formation	-	-	+
Nitrate reduction	-	-	+
Voges-Proskauer reaction	-	-	+
Methyl red test	**+**	**-**	**+**
Qninone system	MK -8, MK -8(H_2)	MK -8(H_2)	MK -8, MK -8(H_2)
Cellular fatty acids	S, A, I	A, I	anteise-$C_{15:0'}$ iso- $C_{15:0}$
Cell wall amino sugar	Mannosamine	Galactosamine	Mannosamine
G+C content (mol %)	70.0	69	66.4

Key of abbreviations:S, straight –chain saturated. A, anteiso-methyl branched. I, iso-methyl-branched.

3.2.5 The Gram- Positive Rods

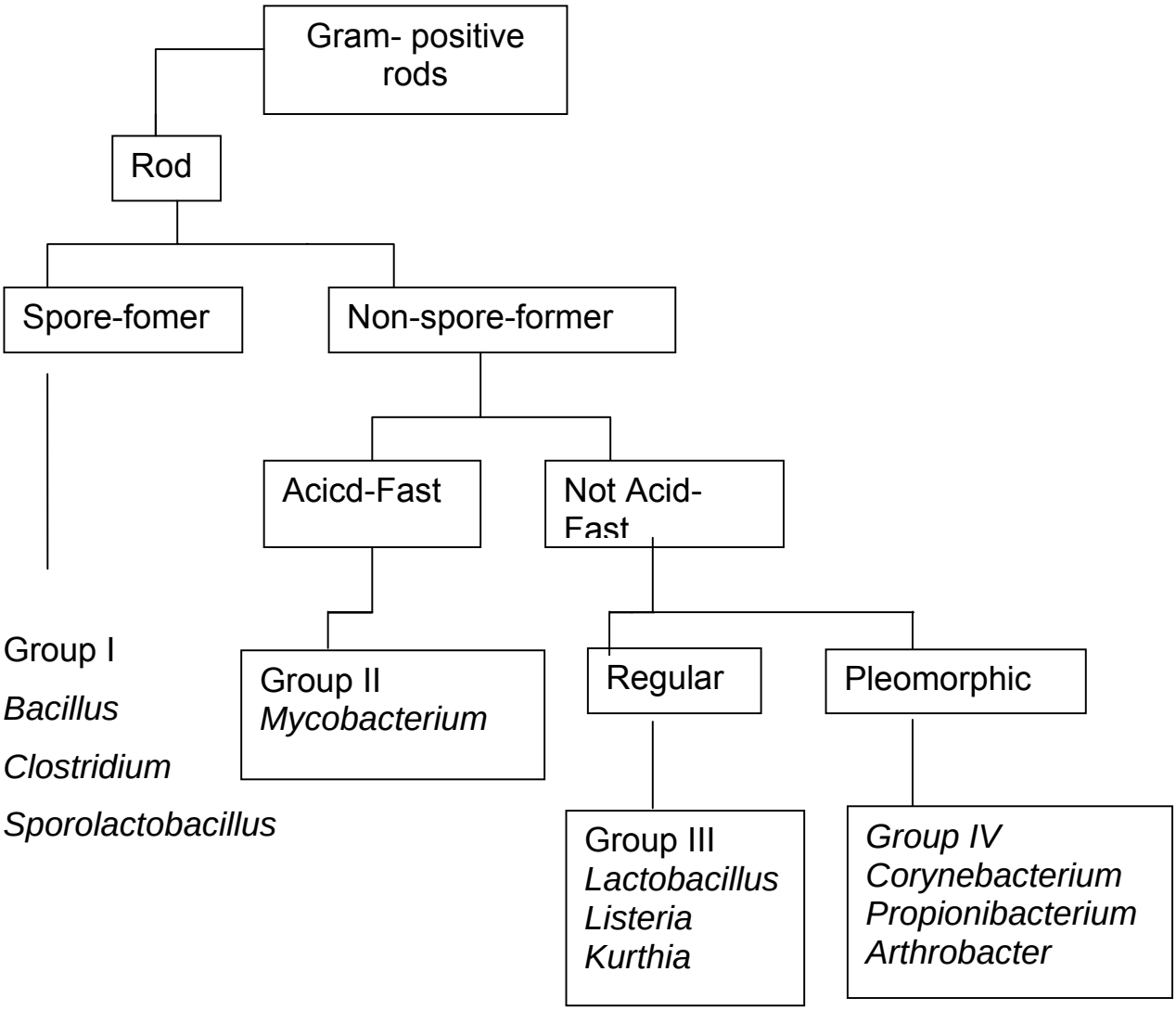


Figure 3.4 The Gram positive rods bacteria according to, "Bergey's Manual of Systematic Bacteriology" (52).

species	Molilty	Catalase production	Parasporal bodies	Lipid globules in protoplasm	Lecilnovitellin reaction	Citrate utilization	Anaerobic growth	V-P reation	PH in V-P medium 60	Growth at 50^{0}C	Growth at 60^{0}C	Growth in 7%Nacl	Acid from AS glucose	Acid +gas from AS glucose	Nitrate reduction	Casein hydrolysid	Starch hydrolysis	Propionate uilization
Morphologic group 1																		
B megaterium	V	+	-	+	-	+	-	-	V	-	-	+	+	-	V	+	+	n
B cereus	+	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	n
B cereus subsp mycoides	-	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	n
B anthracis	-	+	-	+	+	V	+	+	+	-	-	+	+	--	+	+	+	n
B thuringiensis	+	+	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	n
B licheniformis	+	+	-	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+
B subtilis	+	+	-	-	-	+	-	+	V	V	-	+	+	-	+	+	+	-
B pumilus	+	+	-	-	-	+	-	+	+	V	-	+	+	-	-	+	+	-
B firmus	v	+	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-
B coagulans	+	+	-		-	V	+	+	+	+	V	+	+	-	V	V	+	-
Morphologic group 2																		
B polymyxa	+	+	-	-	-	-	+	+	V	-	-	-	+	+	+	+	+	n
B macerans	+	+	-	-	-	V	+	-	+	+	-	-	+	+	+	-	+	n
B circulans	V	+	-	-	-	V	V	-	+	V	-	V	+	-	V	V	+	n
B stearothermophilus	+	V	-	-	-	-	-	-	+	+	-	-	+	-	-	V	+	n
B alvei	+	+	-	-	-	-	+	+	+	-	+	-	+	-	+	+	+	n
B laterosporus	+	+	-	-	+	-	+	-	+	-		-	+	-	V	+	-	n

B brevis	+	+	-		-	V	-	-	-	V	V	-	+	-	+	+	-	n
Morphologic group 3				-														
B sphaericus	+	+	-		-	V	-	-	-	-	-	V	+	-	-	V	-	n

Table 3.6 The basic characteristics which have been used for the identification of selected *Bacillus species* (55).

Key of abbreviations: V-P, Voges-Proskaure, AS ammonium salt; +,v, variable; n, test not applicable; (+), under colony which must be scraped off see positive reaction.

3. 3 Morphological Characterization of Isolated Strains

3.3.1 Staining Procedure

3.3.1.1 Gram Stain

This staining procedure, developed in 1884 by the Danish physician Christian Gram, is the most important procedure in microbiology. It separates most bacteria into two groups: the gram-positive bacteria, which stain blue, and the gram-negative bacteria, which stain red. The Gram stain involves the following four-step procedure:

1-The crystal violet dye stains all cells blue/purple.

2-The iodine solution (a mordant) is added to form a crystal violet–iodine complex; all cells continue to appear blue.

3-The organic solvent, such as acetone or ethanol, extracts the blue dye complex from the lipid-rich, thin-walled gram-negative bacteria to a greater degree than from the lipid-poor, thick-walled gram-positive bacteria. The gram-negative organisms appear colorless; the gram-positive bacteria remain blue.

4-The red dye safranin stains the decolorized gram-negative cells red/pink; the gram-positive bacteria remain blue (56).

3.3.1.2 The Procedure of Gram Stain

1- Slides with heat-fixed smears were prepared

2- The smears were covered with crystal violet and let stand for *20 seconds.*

3- The stain was briefly washed off, using a wash bottle of distilled water. Drain off excess water.

4-The smears were covered with Gram's iodine solution and let stand for *one* minute.

4. The Gram's iodine was poured off and the smears were flooded with 95% ethyl alcohol for 10 to 20 seconds*.*

This step is critical. Thick smears required more time than thin ones*.*

5- Alcohol action was stopped by rinsing slides with water from the washing bottle for few seconds.
6- The smears were covered with safranin for *20* seconds.
7- Slides were washed gently using running water for ~~a~~ few seconds, blotted and dried with filter paper, and air-dry.
8- The smears were examined using light microscope oil immersion lens (56).

3.3. 2 Endospore Stain

The spore stain is a differential stain used to detect the presence and location of spores in bacterial cells. Only a few genera produce spores. Among them are the genera Bacillus and clostridium.

3.3.2.1 Principle of the Endospore Stain

An endospore is a dormant form of the bacterium that allows it to survive poor environmental condition. Spores are resistant to heat and chemicals because of a tough outer covering made of the protein keratin. The keratin also resistant staining, so extreme measures must be taken to stain the spore. In the Schaeffer-Fulton method
1 – Cells and spores prior to staining are transparent.
2- After staining with malachite green, cells and spores are green. Heat is used to force the stain into spore, if present.
3- Docolorization with water removes stain from cells, but not spores.
4- Safranin is used a counterstain cells (57).

3.3.3 Motility Determination

3.3.3.1. Hanging Drop Slides

Most bacterial microscopic preparations result in death of the microorganisms due to heat fixing and staining.

3.3.3.1.2 Principle of the Hanging Drop Slides

A hanging drop preparation allows longer observation of the specimen since it doesn't dry out as quickly. A thin ring of petroleum jelly was applied to the four edges on one side of a cover glass. A drop of water was then placed in the center of the cover glass and living microbes were transferred into it. A depression microscope slide is carefully placed over the cover glass in such a way that drop was received into depression and was undisturbed. The petroleum jelly causes the cover glass to stick to the slide. The preparation may then picked up, inverted so the cover glass was on top, and placed under the microscope for examination (57).

3.3.3.2. Motility Test

This test is used to detect bacteria motility.

3.3.3.2.1 Principle of the Motility Test Medium

Motility test medium is a semisolid medium designed to detect bacterial motility. Its agar concentration was reduced from the typical 1.5% to 0.4%-- just enough to maintain its form while allowing movement of motile bacteria. It is inoculated by stabbing with a straight transfer needle. Motility is detectable as diffuse growth radiating from the central stab line (52).

3.3.4 Biochemical Characterization

3.3.4.1 Catalase Test

This test is used to identify organisms that produce the enzyme catalase. It is most commonly used to differentiate members of catalase-positive *Micrococcaeae* from the catalase-negative *Streptococcacea.*

3.3.4.1.1 Principle of Catalase Test

Aerobic and facultatively anaerobic bacteria produce enzymes capable of detoxifying these compounds. Superoxide dismutase catalyzes conversion of superoxide radicals to hydrogen peroxide. Catalase converts hydrogen peroxide

into water and gaseous oxygen. Bacteria that produced catalase can be easily detected using typical store grade hydrogen peroxide. When hydrogen peroxide is added to catalase-positive culture, oxygen gas bubbles form immediately (57)

3.3.4.2 Oxidase Test

This test is used to identify organisms that produce the respiratory enzyme cytochrome oxidase. Among its many uses is the presumptive identification of the oxidase-positive *Neisseria*.

3.3.4.2.1 Principle of Oxidase Test

The Oxidase test was performed using a chromogenic reducing agent called tetrmethyl-*p*-phenylenediamine to detect bacteria that produce cytochrome oxidase enzyme. Chromogenic reducing agents are chemicals that change or produce color as they become oxidized. In the oxidase test, reagent was added directly to bacterial growth on solid media or (more conveniently) colony was transferred to paper saturated with reagent. Within seconds the reagent, which acts as an artifical electron donor, would change color if oxidized cytochrome c oxidase is present (57).

3.3.4.3 Citrate Test

The citrate utilization test is used to determine the ability of an organism to use citrate as its sole carbon source.

3.3.4.3.1 Principle of Citrate Test

Simmon`s citrate agar is defined medium that contains sodium citrate as the sole carbon source and ammonium phosphate as the sole nitrogen source. Bromthymol blue dye, which was green at pH 6.9 and blue at pH 7.6, is added as an indicator. Bacteria that survive in the medium and utilized the citrate also convert the ammonium phosphate to ammonia (NH_3) and ammonium hydroxide (NH_4OH), both of which tend to alkalinize the agar. As the pH goes up, the medium changes from green to blue (57).

3.3.4. 4 Starch Test

This test is used to differentiate bacteria based on their ability to hydrolyze starch with the enzyme α –amylase or oligo-1,6-glucosidase. It aids in differentiation of species from the genera *Corynebacterium*, *Clostridium*, *Bacillus*, *Fusobacterium*, and members of *Enterococcus.*

3.3.4.4.1 The Principal of Starch Test

Starch agar is a simple plated medium of beef extract, soluble starch and agar. When organisms that produce α-amylase and oligo-1,6-glucosidase are grown on starch agar they hydrolyzed the starch in the medium surrounding the bacterial growth. Because both the starch and its sugar subunits were soluble (clear) in the medium, the reagent iodine was used to detect the presence or absence of starch in the vicinity around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth (57).

3.3.4.5 Nitrate Reduction Test

This test is used to detect the ability of an organism to reduce nitrate to nitrite, nitrogen gas, or other nitrogenous compound.

3.3.4.5.1 The Principle of Nitrate Reduction Test

Nitrate broth is an undefined medium of beef extract, peptone, and potassium nitrate (KNO_3). An inverted Durham tube was placed in each broth to trap a portion of any gas produced. In contrast to many differential media, no color indicators are included. The color reactions obtained in nitrate broth take place as a result of reactions between metabolic products and reagents added after incubation. Before a broth can be tested for nitrate reductase activity (nitrate reduction to nitrite), it must be examined for evidence of denitrification. This was simply a visual inspection for the presence of gas in the Durham tube. If there is a gas in the Durham tube, the organism was organism is known not to be a

fermenter (as evidenced by a fermentation test) the test was complete. Denitrification has taken place (57).

3.3.4.6- Methyl Red (MR) Test

The methyl red test is a component of the IMViC battery of tests (Indole, M R, Voges-Proskauer, and Citrate) used to differentiate *the Enterobacteriaceae*. It identifies bacterial ability to produce stable acid end products by the mean of a mixed-acid glucose fermentation.

3.3.4.6.1 The Principle of Methyl Red Test

The MR test is designed to detect organisms capable of overcoming the buffer and lowering pH. Few enterics can do this by performing what is called a mixed acid fermentation. The acids produced by these microorganisms tend to be stable wherease acids produced by other organisms tend to be quickly converted to more neutral products. It was for this reason that media inoculated for the MR test is incubated for 5 days.

After incubation, an aliquot of broth is removed and methyl red indicator is added. Methyl red was red at pH 4.4 and yellow at pH 6.2. Between these two pH values it is various shades of orange. Red color was the only true indication of a positive result; orange broth was considered negative or inconclusive. Yellow was negative(57).

3.3.4.7 Voges -Proskauer (V-P)Test

The V-P Test is a component of the IMViC battery of tests (Indole, M R, V-P and Citrate) used to differentiate the *Enterobacteriaceae*. It identifies organisms able to produce acetoin from the degradation of glucose during a 2,3- butanediol fermentation.

3.3.4.7.1 The Principle of V-P Test

The V-P test is designed for organisms that are able to ferment glucose but quickly convert their acid products to acetoin an 2,3-butanediol. Addition of VP

reagents to the medium oxidizes the acetoin to diacetly, which in turn reacts with guanidine nuclei from peptone to produce a red color. No color change is negative. Copper color was a result of interaction between the reagents and should not be confused with the true red color of a positive result (57).

3.3.4. 8 Urease Test

This test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease.

3.3.4.8.1 The Principle of Urease Test

Urea ager is formulated to differenate rapid urease positive organisms from slower urease positive and urase negative bacteria. It urea, peptone, potassium phosphate, glucose, and phenol red. Peptone and glucose provide essential nutrients for a broad range of bacteria. Potassium phosphate was a mild buffer used to resist alkalinization of the medium from peptone metabolism. Phenol red, which is yellow or orange below Ph 8.4 and red or pink above, is included as an indicator(57).

3.3.4.9 Indole Test

The indole test identifies bacteria capable of prouducing indole using the enzyme tryptophanase. The indole test is one component of the IMViC battery of tests used to differentiate the Enterobacteriaceae.

3.3.4.9.1 The Principle of Indole Test

The indole test, as it appeared in this manual, is performed using sulfief-indole motility media (SIM) medium. Indole production in the medium was made possible by the presence of tryptophan. Bacteria possessing the enzyme tryptophanase could hydrolyze tryptophan to pyruvic acid, ammonia (by deamination), and indole. The hydrolysis of tryptophan in SIM medium can be detected by addition of Kovacs' reagent after a perioed of incubation. Kovacs' reagent contains dimethylaminobenzaldehyde (DMABA) and HCl dissolve in

amyl alcohol. When a few drops of Kovacs' reagent are added to tube, DMABA reacts with any indole present and produces a quinoidal compound that turns the reagent layer a cherry red color. The formation of red color in the indicates a positive reaction and the presence of tryptophanase. No red color is indole-negative (57).

3.3.4.10 Casease Test

The casease test is used to identify bacteria capable of hydrolyzing casein with the enzyme casease.

3.3.4.10.1 The Principle of Casease Test

The presence of casease could easily be detected with the test medium, Milk agar. Milk agar was undefined medium containing peptone, beef extract and casein. When plated milk agar was inoculated with a casease-positive organism, secreted casease would diffuse into the medium around the colonies and create a zone of clearing where the casein had been hydrolyzed. Casease-negative organisms do not secrete casease and, thus, do not produce clear zones around the growth (57).

3.3.4.11 Suger Utilization for *Neisseria spp*

MOF for detection of acid production from carbohydrates by *Neisseria spp*.

3.3.4.11.1 The Principle of Suger Utilization for *Neisseria spp*

A MOF was developed as a practical medium for highly sensitive and specific detection of acid production from carbohydrates by Neisseria spp. Filter-sterilized carbohydrate solutions were added to a final (MOF) medium concentration of 1% (vol/vol), the pH was adjusted aseptically to 7.2, and the medium was dispensed in 2.5-ml volumes in 12- by 75-mm sterile tubes laboratory strains were tested concurrently in MOF media containing glucose, maltose, sucrose, mannitol, and lactose. Concurrently, strains were tested for polysaccharide production. The heavy inoculum was removed from a pure subculture with a single wipe of a cotton applicator, which was then stabbed with the MOF medium along the side

of the tube and then rotated against the side of the tube as it was withdrawn to express a very dense inoculum.The acid reaction patterns obtained for these strains in MOF medium agreed with (Table 3.4). All acid reactions in MOF media were compared with the phenol red color standards to make a semiquantitative estimate of the acid detected in these media. For reactions in MOF medium all strains produced sufficient acid to lower the pH to 6.8, The reactions for tests in MOF medium are incorporated in (Table 4.2) (53).

3.3.4.12 Turbidity Estimation of Bacterial Numbers

Estimating turbidity was a practical way of monitoring bacterial growth. As bacteria multiply in liquid medium, the medium become turbid, or cloudy with cells. This instrument used to measure turbidity was a spectrophotometer.

3.3.4.12.1 The Principle of the Turbidity Estimation of Bacterial Numbers

In the spectrophotometer, a beam of light is transmitted through a bacterial suspension to a light-sensitive detector. As a bacterial numbers increase,less light will reach the detector . This change of light will register on the instrument`s scale as the percentage of transmission. Also printed on the instrument`s scale is a logarithmic expression called the absorbance (sometimes called optical density (OD); a value derived from percentage of transmission may also reported. The absorbance was used to plot bacterial growth (56).

3.3.4.12.2 The Procedure of Turbidity Estimation of Bacterial Numbers

The isolated bacterial culture was enumerated with and without adding methamadiophos. Aliquot (2.5 ml) of 24 h culture grown in nutrient broth was inoculated into 25 ml nutrient broth flask containing different concentration of methamadiophos(100-200-300) mg/L and tested their ability to degrade supplemental substrate (the methamadiophos). Control flasks of equal volume of nutrient broth medium containing culture but no pesticide the zero dose (control).

Growth of the isolate was determined by bacterial number enumeration immediately after inocculation and at o , 2, 24, and 48 h later. Turbidity was measured by spectrophotometer at OD 660 nm.

3.3.4.11 Maintenance of Pesticide Degrading Bacterial Culture:

The isolated pure bacterial strain was streaked on nutrient agar slant and slants containing 57 mg/L methamidophos pesticide. After incubation at 35^0C for 24 h, the culture will be maintained at 4^0C. The bacterial culture was subculture every three months (11).

CHAPTER 4
RESULTS

4.1 Isolation and Characterization of the Methamidophos-Degrading Strains

Three different bacterial strains were isolated from the longterm methamidophos-polluted soil samples by the enrichment culture technique as follows:

1. *Neisseria lactamica*
2. *Micrococcus lyale*
3. *Bacillus sphaericus*

All isolates were tested for their methamidophos-degrading capacities.

4.1.1 *Neisseria lactamica*

One strain designated as *Neisseria lactamica* (Tables 4.1 and 4.2), which could completely degrade 100 mg L^{-1} methamidophos in 24 h in MSM medium, was selected. Colonies of *Neisseria* l*actamica* on MSM agar are raised, entire, white and 0.5-1 mm in diameter after 24 h of incubation at 35°C. The morphology of *Neisseria lactamica* cells is cocci-shaped. It is non-spore-forming, Gram-negative diplococci. *Neisseria lactamica* showed positive reactions for catalase, oxidase and showed negative reactions for nitrate reduction. It was able to metabolize glucose, maltose and lactose. It did not metabolize sucrose and mannitol .

4.1.1.1 Morphological and Biochemical Characteristics of the Isolated Strain

Results of microscopic and biochemical tests for *Neisseria lactamica* are shown in tables 4.1 and 4.2.

Table 4.1 Morphological and microscopic characteristics of *Neisseria lactamica*

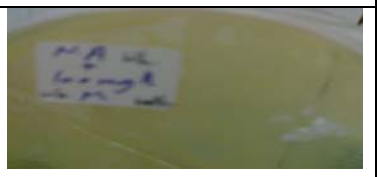
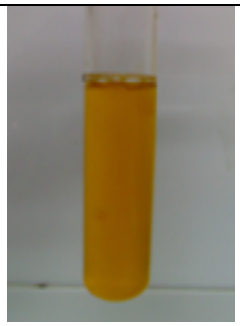
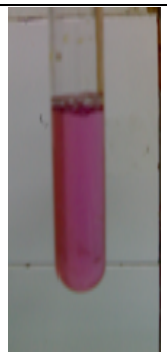
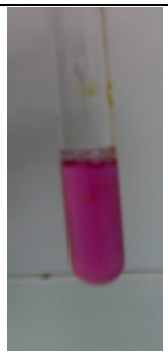
Test	Result	
Colour of colony	white	
Shape	Pairs	
Motility	Negative A negative result shows growth only along the stab line.	
Gram stain	Gram negative diplococcus	

Table 4.2 Biochemical characteristics of *Neisseria lactamica*

Test	Result	
Catalase slide test	Positive The catalase slide test in which visible bubble production indicates a positive result.	

Oxidase test on Bactrial Growth	Positive Oxidase-positive will produce a purple-blue color immediately.	
Acid production		
Lactose	Positive Yellow color in the medium indicates a positive result.	
Maltose	Positive Orange color in the medium indicates a positive result.	
Glucose	Positive Orange color in the medium Indicates a positive result.	

Sucrose	Negative Pink color in the medium indicates a negative result.	
Mannitol	Negative Pink color in the medium indicates a negative result.	
Nitrate reduction	Negative The medium was colorless after addition of reagents nitrite absence; the medium turns pink after the addition of a small amount of zinc powder the result was negative.	

4.1.1.2 Growth Kinetics of *Neisseria lactamica* at Temp 30ºC by Using Different Concentrations of Methamidophos.

The growth kinetics of *Neisseria lactamica* in nutrient broth at 30 ºC was conducted to evaluate the potential degradation of isolated strain when exposed to different concentration of methamidophos pesticides. A control sample was conducted without adding methamidophos. It is seen from Table 4.3 and Fig 4.1, that the growth rate of *Neisseria lactamica* decreased gradually by increasing the concentration of methamidophos. The bacterial growth at 0 hr was minimal and then started to increase gradually and reach the maximum growth rate after 2 hrs as shown in table 4.3 and figure 4.1. After 27 hrs the bacterial growth was significantly decreased .

4.1.1.3 Growth Kinetics of *Neisseria lactamica* at Temp 35ºC by Using Different Concentrations of Methamidophos.

The growth kinetics of *Neisseria lactamica* in nutrient broth at 35 ºC was conducted to evaluate the potential degradation of isolated strain when exposed to different concentration of methamidophos pesticides. A control sample was conducted without adding methamidophos. It is seen from Table 4.4 and Fig 4.2, that the growth rate of *Neisseria lactamica* decreased gradually by increasing the concentration of methamidophos. The bacterial growth at 0 hr was minimal and then started to increase gradually and reach the maximum growth rate after 2 hrs as shown in table 4.4 and figure 4.2. After 24 hrs the bacterial growth was significantly decreased .

Table 4.3 Growth knetics at Temp 30ºC by using different concentrations of methamidophos.

Time	control	100 mg/L of Methamidophos	200 mg/L of Methamidophos	300 mg/L of Methamidophos
Zero time	0.541	0.426	0.381	0.334
2 hr	0.732	0.531	0.453	0.271
24 hr	1.28	1.32	1.30	1.14
48 hr	1.45	1.37	1.25	1.22

Table 4.4 Growth kinetics at temp 35ºC by using different concentrations of methamidophos.

Time	Control	100 mg/L of Methamidophos	200 mg/L of Methamidophos	300 mg/L of Methamidophos
Zero time	0.255	0.275	0.202	0.121
2 hr	0.685	0.634	0.706	0.543
24 hr	1.323	1.180	1.136	1.064
48 hr	0.896	0.710	1.059	0.615

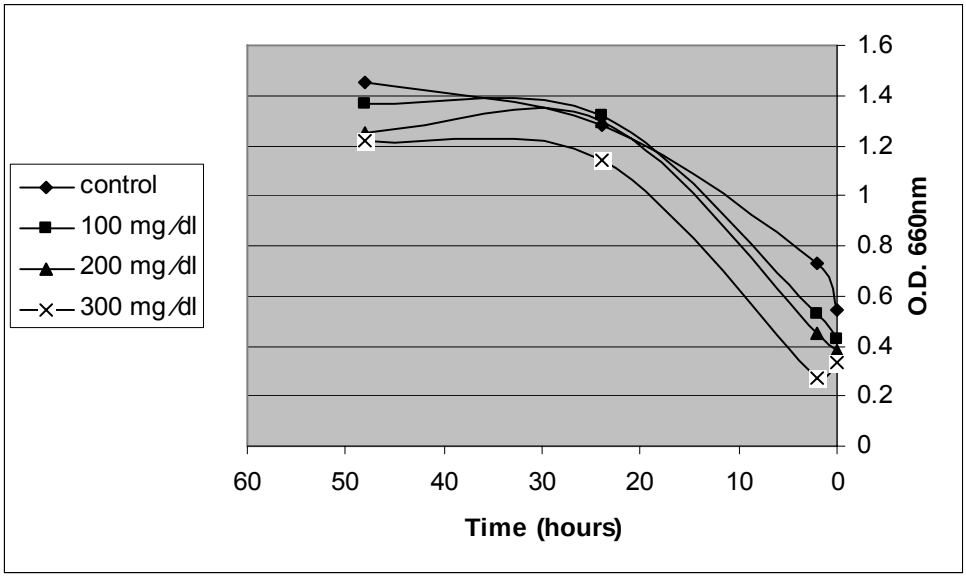


Figure 4.1 Growth kinetics at temp 30ºC. by using different concentrations of methamidophos.

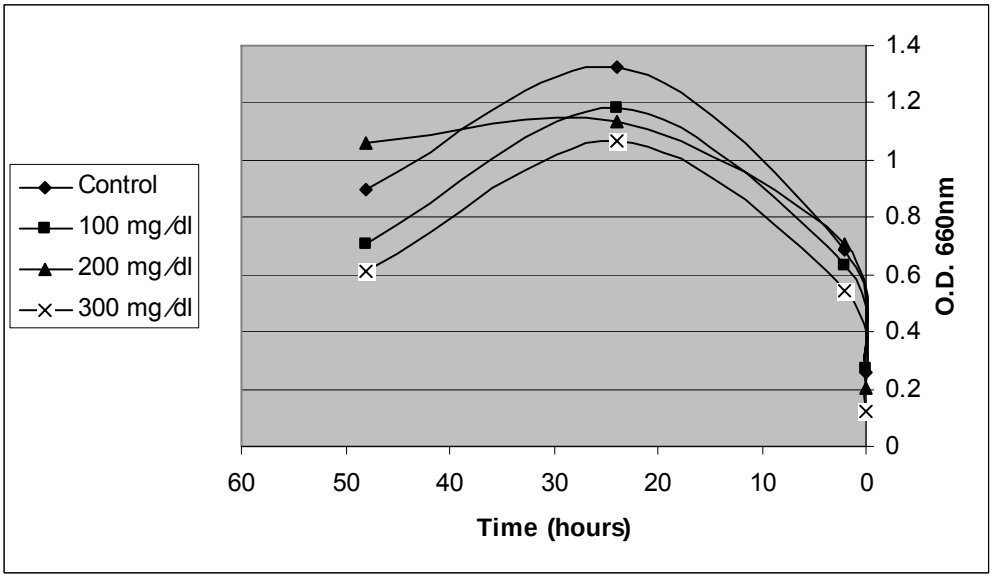


Figure 4.2 Growth kinetics at temp 35ºC. by using different concentrations of methamidophos.

4.1.2 *Micrococcus lyale*

One strain designated as *Micrococcus lyale* (table 4.5 and 4.6), which could completely degrade 100 mg L^{-1} methamidophos in 24 h in MSM medium, was selected. Colonies of *Micrococcus lyale* on MSM agar are raised, entire, yellow and O.5-1.2 mm in diameter after 24 h of incubation at 35°C. The morphology of *Micrococcus lyale* cells is tetrad -shaped. It is non-spore-forming, Gram- positive cocci. *Micrococcus lyale* showed positive reactions for oxidase and urease and showed negative reactions for starch hydrolysis , indole formation, nitrate reduction, MR test and V-P test.

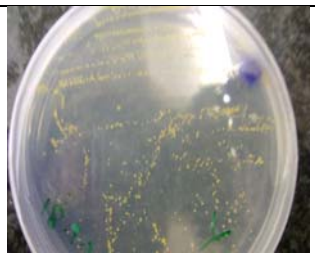
4.1.2.1 Morphological and Biochemical Characteristics of the Isolated Strain

Results of microscopic and biochemical tests for *Micrococcus lyale* are shown in tables 4.5 and 4.6.

Table 4.5 Morphological and microscopic characteristics of *Micrococcus lyale*

Test	**Result**	
Colour of colonies	Yellow	
Gram stain	Positive cocci.	
Shape	Tetrad	
Motility	Negative A negative result shows growth only along the stab line.	

Table 4.6 Biochemical characteristics of *Micrococcus lyale*

Oxidase disc	Positive Oxidase-positive will produce a purple-blue color immediately .	
Catalase	Positive The catalase slide test in which visible bubble production indicates a positive result.	
Urease test	Positive Pink color in the medium indicates a positive result.	
Starch hydrolysis	Negative No clear zone surrounding the growth.	

Indole formation	Negative No red color was indol negative.	
Nitrate test	Negative The medium was colorless after addition of reagents nitrite absence; the medium turns pink after the addition of a small amount of zinc powder the result was negative.	
V R test	Negative No color change was negative.	
M R test	Negative Yellow color was negative resut.	

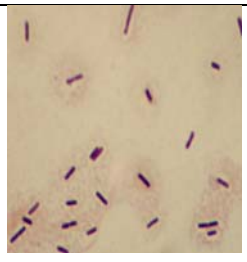
4.1.3 *Bacillus sphaericus*

One strain designated as *Bacillus sphaericus* (table 4.7 and 4.8), which could completely degrade 100 mg L^{-1} methamidophos in 24 h in MSM medium, was selected. Colonies of *Bacillus sphaericus* on MSM agar are raised, circular, orange and 0.5-1.5 mm in diameter after 24 h of incubation at 35°C. colonies have orange color, rod -shaped , spore-forming, gram- positive. *Bacillus sphaericus* showed positive reactions for catalase, oxidase and citrate. and showed negative reactions for VP test ,nitrate reduction, casein test and ,starch hydrolysis

4.1.3.1 Morphological and Biochemical Characteristics of the Isolated Strain

Results of microscopic and biochemical tests for *Bacillus sphaericus* are shown in tables 4.7 and 4.8.

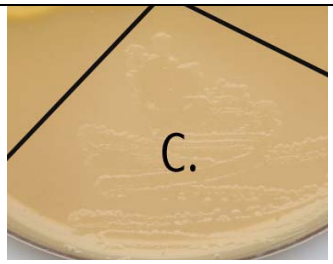
Table 4.7 Morphological and microscopic characteristics of *Bacillus sphaericus*

Test		Result
Colour of colony	Orange	
Gram stain	Gram positive rod	
Spore stain	Spore former	

Motility test	Positive A Positive result shows growth diffuse radiating from the central stab line.	

Table 4.8 Biochemical characteristics of *Bacillus spaeericus*.

Test	Result	
Catalase test	Positive The catalase slide test in which visible bubble production indicates a positive result.	
Citrate test	Positive Conversion of the medium to blue was a positive citrate test result.	
VP	Negative No color change was negative result.	

Nitrate reduction	Negative The medium was colorless after addition of reagents nitrite absence; the medium turns pink after the addition of a small amount of zinc powder the result was negative.	
Casein hydrolysis	Negative No a clear zone surrounding the growth.	
Starch hydrolysis	Negative No clear zone surrounding the growth.	

CHAPTER 5
DISCUSSION

Microorganisms play key roles in the biodegradation of pesticide-contaminated sites. However, few studies of the microbial degradation of methamidophos have been carried out worldwide and this study is the first study at Gaza Strip. In the present study, a high efficient methamidophos-degrading strains were isolated.The bacterial culture capable of degrading methamidophos was isolated from soil using enrichment technique, with varying concentration of methamidophos in the medium, the soil samples were collected from agricultural sites which had a history of methamidophos exposure from Beit lahiya north Gaza Strip Palestine. Three different bacterial strains were isolated from the long-term methamidophos-polluted soil samples by the enrichment culture technique.

5.1*Neisseria lactamica*

On the basis of the morphological, and biochemical characteristics present in Table 4.1 and 4.2 and the “Bergey’s Manual of Systematic Bacteriology” (52).
, the bacterial isolate was identified as a member of the genus *Neisseria. Neisseria* species which found oxidase and catalse-positive. *Neisseria lactamica* produced acid from lactose and found to grow well in MSM and to use methamidophos as its primary carbon source of energy.

5.1.1 Growth kinetics of *Neisseria lactamica* at 30ºC Using Different Concentrations of Methamidophos

The potential degradation of different concentrations of methamidophos by the *Neisseria lactamica* was evaluated by measuring its growth kinetics in nutrient broth at 30 ºC and 35 ºC compared with the control. Table 4.3 and Fig 4.1, shows that the growth rate of *Neisseria lactamica* at 30 ºC decreased gradually by increasing the concentration of methamidophos from 100 mg L^{-1} and 200 mg $L^{-1}$to 300 mg L^{-1}. The bacterial growth at 0 hr was minimal at all methamidophos

concentrations used and then started to increase gradually and reach the maximum growth rate after 2 hrs as shown in table 4.3 and figure 4.1, growth rate at 100 mg and 200 mg L^{-1} methamidophs was higher than 300 mg which reflects the ability of this strain to adapt this level of methamidophos toxicity. After 27 hrs the bacterial growth was significantly decreased at all methamidophos concentrations.

It is seen from Table 4.4 and Fig 4.2 that the growth rate of *Neisseria lactamica* at 35 ºC decreased gradually by increasing the concentration of methamidophos. The bacterial growth at 0 hr was minimal and then started to increase gradually and reach its maximum growth rate after 2 hrs as shown in table 4.4 and figure 4.2. After 24 hrs the bacterial growth was significantly decreased. Methmidophos degradation and bacterial growth was higher at 30 ºC than at 35 ºC which indicates than the optimum *Neisseria lactamica growth rate and* Methmidophos degradation is 30 ºC.

5.1.2 Growth kinetics of *Neisseria lactamica* in nutrient broth supplemented with methamidophos:

Commercial grade methamidophos in the range (100-200-300 mg L^{-1}) was used to determine the growth response of bacterial isolate. The stimulatory and inhibitory responses of *Neisseria lactamica* were observed when exposed to various concentrations of methamidophos. The growth pattern are shown in Fig. 4.1 and recorded in Table 4.3, significant reduction in the bacterial growth was observed after 24hrs of incubation at 300mg L^{-1} methamidophos dose proved to decrease bacterial isolate growth rate reversely. When compared with the control test (growth without pesticide), the growth pattern of *Neisseria lactamica* in the medium containing 100-200 mg L^{-1} methamidophos was very much similar during 24 h of incubation, the bacterial growth remains the same after 24 hrs where as at 48 hrs the bacterial growth, was observed. The results of the analysis indicate that methamidophos concentration in the range of 100 mg L^{-1} to 200 mg L^{-1} stimulated the growth of isolates, however a marked reduction in bacterial growth

at 48 h was noted when 300 mg L^{-1} mg L^{-1} was used, this indicates that bacterial metabolism rate was suppressed and the growth rate thus decreased which significantly decrease the rate of methamidophos at 300 mg L^{-1}

5.2 *Micrococcus lylae*

On the basis of the morphological, and biochemical characteristics present in Table 4.5 and 4.6 and the "Bergey's Manual of Systematic Bacteriology" (52). the bacterial isolates were identified as a member of the genus *Micrococcus, Planococcus or Staphlocccus*.species

On the basis of morphological, colonies yellow pigment colour on MSM+ 1OO mg /L of methamidophos according to gram stain the isolated stain was gram – positive cocci tetrad arrangment and I used MacConkey agar to ensure this result which was negative .The isolated strain was gram positive and I used endosopre stain, the isolated strain was non spore- formering bacteria. For genus differentiation

5.2.1 *Micrococcus species*

Has sphere colonies shape, occurring as singles, pairs, irregular clusters, tetrads, or cubical packets. Usually non-motile strict aerobes (one species is facultative anaerobic) catalase- and oxidase-positive. Most species produce carotenoid pigments.

5.2.2 *Planococcus species*

Colonies have sphere shape, occurring singly, pairs, groups of three cells and occasionally in tetrads. Although cells are generally gram positive, they may be gram-variable and motile. Catalase and Gelatinase-positive. Carbohydrates not attacked and can not hydrolyze starch or reduce nitrate.

5.2.3 *Staphylococcus*

Spheres, occurring as singles, pairs, and irregular clusters. Nonmotile. Facultative anaerobes. Usually catalase-positive (52). According to (table 4.6) the bacterial isolate was positive with Catalase- and oxidase and the bacterial isolate was identified as a member of the genus *Micrococcus* according to (figure 3.3) "Bergey's Manual of Systematic Bacteriology" (52).For species differentiation see (table 3.5) starch hydrolysis, indol formation, nitrate reduction V-P, MR were negative result except the urease test was positive result. The bacterial isolate was identified as a member of the species *lylae.* MR was discriminated between *Micrococcus lylae and Micrococcus luteus*, MR negative result with *Micrococcus lylae*. The isolated strain was *Micrococcus lylae*. Characterization studies of the isolate from these experiments, as well as of those by other researchers, indicate that bacteria belonging to the genus *Micrococcus* are gram-positive cocci and Catalase positive able to degrade organophosphours compounds (table 2.4), crude oil, melamine formaldehyde and hydrocarbon (12, 40, 41, and 44). *Micrococcus lylae* can grow well in simple minimal medium and used methamidophos as its carbon source of energy.

5.3 *Bacillus spaeericus*

On the basis of morphological, cultural orange pigment colour on nutrient agar according to gram stain are gram –positive rod and I used MacConkey agar to ensure this result negative.The isolate strain is gram positive and I used endosopre stain, the The isolate strain was non spore- formering bacteria **.**
On the basis of biochemical characteristics (figure 3.4) the bacterial isolate was identified as a member of the genus *Bacillus, Clostridum and Sporolactobacillus* for genus differentiation.

5.3.1 *Bacillus* Although most of these organisms are aerobic, some are facultative anaerobes. Catalase is usually produced. For comparative characteristics of the species in this genus refer to Table (3.6).

5.3.2 *Clostridium* While most of members of this genus are strict anaerobes, some may grow in the presence of oxygen. Catalase is not usually produced.

5.3.3 *Sporolactobacillus*

Microaerophilic and catalase negative. Nitrates are not reduced and indole is not formed. Spore formation occurs very infrequently (1% of cells) (52).

On the basis of morphological, cultural and biochemical characteristics (table 4.7) and (table 4.8) the bacterial isolate was identified as a member of the genus *Bacillus* according to "Bergey's Manual of Systematic Bacteriology" (52). Characterization studies of the isolate from these experiments, as well as of those by other researchers, indicate that bacteria belonging to the genus *Bacillus* are gram-positive bacilli, able to degrade organophours compounds (table2.4),benzo[a]pyrene, crude petroleum-oil, hydrocarbon and pyridine. (12,42,44, & 49). *Bacillus spaeericus.* could grow well in simple minimal medium with mthamidophos as carbon and energy source.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

CONCLUSION

1-It can be concluded that the experimental system described here is suitable for measurements of the degradation of pesticides and other in mixed bacteria cultures originating from soil.

2-Microbial biodegradation of pesticides applied to soil is the principle mechanism which prevents the accumulation of these chemicals in the environment.

3-Our results provide an indication that microorganisms are capable of degrading pesticide methamidophos.

4-The development of strategies to prevent environmental contamination (e.g. reduction of pesticide application, utilization of less toxic substances), and to clean-up contaminated sites is important. In contrast to what happens with industrial effluents, the implementation of agricultural wastewater treatment.

6-One of the major disadvantages of the use of pesticides is the contamination of air, waters, soils and even the trophic chain with xenobiotic compounds or their partial degradation products .

RECOMMENDATION

1-It is known that conventional wastewater treatment plants do not remove all the pollutants, especially the persistent pesitcides

2- it is recommended to use *Neisseria lactamica, Micrococcus lylae and Bacillus sphaericus* in the bidegradation of pesticides

3-Enhanced biodegradation of pesticides in the Environment by microorganisms.

4-We strongly recommend to use the agriculture ministry this technique of degradation by using these strains after optimization experiments.

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