INVESTIGATION ON THE GENUS BACILLUS IN THE CONTROL OF MOSQUITOES

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Abdel-Wahab Ali Ahmed

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ABSTRACT

Screening programme to isolate strains of the genus *Bacillus* for their potential use in the control of mosquitoes was done. Over 90 spore-forming bacillus strains were examined. Six isolates (AA-2, AA-6, AA-10, AA-11, AA-71, and AA-82) have shown killing ability of which four (AA-2, AA-11, AA-71, and AA-82) were found to be effective. Biochemical and morphological characterizations have revealed similarity between these isolates and the well known mosquito pathogens (*B.thuringiensis* and *B.sphaericus*).

The effect of temperature and aeration supply on biomass production was studied. Isolates were found to differently respond to temperature. Isolate AA-2 was found to grow well at 30^{0}C, AA-11 has been found to have a wide range of temperature (23-40^{0}C), AA-71 has shown inverse relation with temperature, whereas isolate AA-82 demonstrated critical decrease in growth with increase in temperature and 30^{0}C was found to be optimal. Dry biomass was found to increase with the increase in aeration levels.

The efficacy of the isolates was tested on different larval stages of mosquito. LC_{50} that ranged from 43-170 µg/ml was obtained. No significant difference has been observed in sensitivity of different larval stages of mosquito to different bacteria at a significance level of 1% except isolate AA-11. Efficacy of the isolates under natural conditions was also tested. Some strains (eg. AA-82) have shown good results.

Further confirmatory characterization is needed as well as investigation for optimal conditions to increase toxin yield.

I. INTRODUCTION

Mosquitoes are the world's primary vectors of human and domestic animal diseases. They transmit a number of diseases such as malaria, filariasis, Rift valley fever, yellow fever and encephalitis.

Malaria has been one of the major public health problems found in over hundred countries in the world. About 270 million people are infected and 110 million cases occur every year of which more than 90 million are in Africa south of the Sahara (WHO, 1991).

Attempts to control mosquitoes using chemical insecticides such as DDT and other organophosphate chemicals have failed due to resistance (WHO, 1976). Besides chemicals are expensive, create environmental problems and affects the health of man and his domestic animals.

In recent years the need for environmentally safe pesticides has encouraged the use of microbial pathogens as insecticides which are thought to have considerable environmental and other advantages over chemical agents (Smith & Couche, 1991). Studies have shown that bacteria (Berliner, 1915 cited in Couch & Ross, 1980; Kellen *et al.*, 1965), viruses (Chapman *et al.*, 1966) and fungi (Jaronski & Axtell, 1982) have promising insecticidal role.

Among bacteria the spore-forming forms of the genus *Bacillus* have shown to produce crystalline proteins during the course of sporulation. The proteins have been shown to kill many susceptible insect larvae. Many strains of

B.thuringiensis are capable of producing the crystals. *B.thuringiensis* strains have been used over three decades as bioinsecticide to control agricultural pests and , more recently, insect vectors of human and animal diseases (Hofte & Whitely, 1989).

The species, *B. sphaericus* (Kellen *et al.*, 1965), was found to contain strains that are highly mosquito larvicidal. These strains have shown persistence, recycling under natural condition (Hertlein *et al.*, 1979) and to be host specific (Singer, 1980a). These characters offer the advantage of being good candidate for better control of mosquito larvae. However, lack of new strains that have an ability to keep in contact with the target animal, long lasting larvicidal effect and easy to be grown on available agroindustrial by-products, has limited the wide use of these organisms (Travers *et al.*, 1987).

It is, therefore, evident that search for new strains with different target spectra will augment the current commercial bioinsecticides. The objective of this work is to identify strains of *Bacillus* spp from different sources in Ethiopia that can have the potential use in the control of mosquitoes vectors of malaria, and study their characteristic features.

1.1. The genus Bacillus Cohn

The major species of bacteria with mechanism to infect and kill healthy insects are spore-forming bacilli.

1.1.1. *General Description*

The genus *Bacillus* includes 34 species which share the common property of production of resistant endospore while growing in the presence of oxygen. They are rod-shaped, sometimes in chain and the sporangia do not differ from the vegetative cell except when bulged by spores larger than the cell diameter. The sporangia are spindle shaped when the spores are central and wedged or drum-stick shaped when the spores are terminal. Species of *Bacillus* may be motile and move by means of peritrichous flagella or they may be non-motile, Gram-positive, Gram-variable or Gram-negative (Gordon, 1952). Some species of *Bacillus* usually occur in the rough stage forming a pellicle on broth, whereas other species are smooth and the rough stage is rarely seen. Most species decompose proteins with the production of ammonia. Carbohydrates are generally fermented with the production of acids and a few also produce visible gas. Some are catalase positive. They may be aerobic or facultatively anaerobic. Maximum temperature for growth vary greatly not only between species but also between strains of the same species

Variation in other characters also frequently occur within species. Bacilli are mostly saprophytes commonly found in soil while a few are parasites or pathogens of insect and other animals (Gordon, 1952).

1.1.2. *Life Cycle*

Unlike most other bacteria, *Bacillus* species are potentially able to form resting cells after the end of exponential cell growth or when their vegetative cells are transferred from a rich to a poor medium. Resting cells are formed intercellular and are therefore designated as endospores.

The formation of endospores is a multiphasic process which is similar in all *Bacillus* strains. These are: stage 0-vegetative cell growth; stage I-preseptation, the DNA forms an axial filament; stage II-septation; stage III-engulfment of the forespore, the membrane of the developing spore becomes completely detached from that of the mother cell and surrounds the spore protoplast; stage IV-cortex formation starts; stage V-spore coats are synthesized; stage VI-development of refractivity and heat resistance, spore maturation, and stage VII-lysis of the sporangium and liberation of the mature spore (Claus &Berkeley, 1986).

The transformation of a dormant endospore into a vegetative cell usually involves three sequential processes which are known as activation, germination and outgrowth. Activation is the process responsible for the breaking of dormancy. It can be achieved by heat treatment or aging even at low temperature. Germination is the change of an activated endospore from the dormant to a metabolically active state, which can take place within a minute under suitable condition of pH value and ionic strength of the medium. Outgrowth is the development of a vegetative cell

from a germinated endospore. After germination is completed, the young vegetative cell emerges and divides. (Claus & Berkeley, 1986).

1.2. Potential members of the genus Bacillus as mosquito pathogens

Most species of the genus *Bacillus* are saprophytes occurring in soil, fresh water and sea water. Some are pathogenic to insect including mosquitoes. The inclusion in the spore of some species of bacillus bacteria have been found to be toxic to larvae of certain insects specially those belonging to the orders Lepidoptera, Coleoptera and Diptera(Aronson *et al.*, 1986).

1.2.1 *Perspective of the genus Bacillus*

A few species of the genus *Bacillus* exhibit the unique ability of being primary pathogen of insects. These bioinsecticides comprise two groups depending on the mode of action: the first group includes bacilli that kill their host by invasion and multiplication inside the host tissue , they include *Bacillus larvae* White, *B. lentimorbus* Dutky and *B.popilliae* Dutky. The second comprises those that kill their host by means of toxin produced outside the host (production of substance toxic upon ingestion), and they include *Bacillus thuringiensis* Berliner and *B.sphaericus* Neide. The latter two are well known pathogens of mosquito larvae. For these species of

bacteria the hemolymph of insect larvae is an excellent nutritional environment enhancing proliferation and sometimes sporulation (Davidson, 1984).

Bacillus thuringiensis and *B.sphaericus* are known to produce protoxins during the course of sporulation. The proteins are deposited as parasporal inclusion and, in some cases, are found on the surface of the spore. When the inclusion or spore is ingested by susceptible larvae, the protoxin is solubilized in the alkaline environment of the midgut. The midgut of these larvae also contain protease enzyme necessary to convert the protoxins to toxins and perhaps contain receptors on the surface of midgut epithelial cells to which the toxin bind to initiate their activity (Aronson *et al.*, 1986).

The use of these bacteria for practical pest control has advantages. The most important advantage is the production of stable spores which are readily formulated for use in conventional pest control application and which are remarkably safe for human, other mammals and non-target fauna. A major problem with the use of these bioagents for control of mosquito larvae, however, is the requirement for formulations which keep the spores and inclusions in the feeding zone of the target pest in aquatic habitat (Davidson *et al.*, 1984).

1.2.2 *Bacillus thuringiensis Berliner*

1.2.2 A. *General description*

The bacteria are rods, 1.0 to 1.2 by 3.0 to 5.0 micron, with square ends. Spore size varies between 1.0 to 1.5 microns. Sporangia are ellipsoidal, central or paracentral and thin walled. Sporangia are not definitely swollen. Spores tend to lie obliquely in the sporangium. After aging, a knob of protoplasm remains at each end (Claus & Berkeley, 1986).

1.2.2 B. *Historical background*

The earliest isolation of spore-forming bacterium from insects was by Ishiwata in Japan in the year 1901 (Couch & Ross, 1980). He isolated the bacterium "sotto bacillum" which was known as sotto disease bacillus. At about the same time in Thuringia, Germany, Berliner, in the year 1915, independently isolated a similar organism from diseased larvae of Mediterranean flour moth, *Anagasta kachniella*. This species was named as *Bacillus thuringiensis* (Couch & Ross, 1980). Angus (1954) had confirmed the relationship between pathogenicity and the crystals. In the middle and late 1950 , *B.thuringiensis* products have continued to be commercially available in the market and have been used as a bioinsecticide to control agricultural pests such as Cabbage worm,

Pieris rapae; Diamondback moth, *Plutella xylostella*; Cabbage looper, *Trichoplusia ni*; and Alfalfa caterpillar, *Colias eurytheme*. Moreover, it had been used against wide range of lepidopterous defoliators in cotton, soybeans, forests, grapes, tobaccos, nuts and vegetables (Couch & Ross, 1980).

1.2.2 C. *Potency of Bacillus thuringiensis*

Up to recently, 13 serotypes of *B. thuringiensis* have been identified showing a high pathogenicity to larvae of Lepidoptera; only two or three serotypes had some effectiveness against young larvae of susceptible species e.g.Alfalfa caterpillar. In contrast, *B.thuringiensis* serotype H-14 does not demonstrate useful activity against the larval stage of Lepidoptera but is highly pathogenic to mosquito larvae (Ignoffo *et al.*, 1980). *B. thuringiensis* serotype H-14, was isolated from a soil sample taken from known temporary mosquito breeding site in Israel (Gorlberg & Margalit, 1977). De Barjac (1978) cited in Abdel-Hameed et al. (1990a) identified the bacterium as a new serotype H-14, and designated the variety *israelensis*.

Results of laboratory evaluations (WHO, 1979) showed that *Aedes* and *Culex* are more sensitive than *Anopheles* larvae to this serotype H-14. This is due to the surface feeding behavior of the *Anopheles*

mosquito larvae than to some innate resistance of these animals (Lacey & Fedrici, 1979; Lahkin-Tsror *et al.*, 1983).*B.thuringiensis* (H-14) has been tested for toxicity and pathogenicity in mice, rats and rabbits. It was found to be safe against all these non-target vertebrates(WHO, 1979).

The crystal toxin is effective against mosquito larvae such as ***Aedes aegypti***, ***A.triseriatus***, ***Culex tarsalis*** and ***Culex pipiens***. LC_{50} of pure crystal is reported to be at the nanogram levels,0.2-20 ng/ml(Tryell *et al.*, 1979). In contrast, larvae of *Anopheles* species are 50-100 times less sensitive when they are exposed to the crystal toxin. Variation in sensitivity to the bacterium was also recorded between strains of the same epecies of mosquitoes (WHO, 1979).

The narrow spectrum of the activity of the delta-endotoxin of *B.thuringiensis* (H-14) is probably due to the absence, in most invertebrates, of enzymatic systems to transform this protoxin into a potent toxin. Moreover, the differences in pH of the larval gut, which affect the solubilization of the protein, and the presence of specific toxin-binding sites (receptors) in the gut of different species could also be counted for the limited host range of the bacterial crystals (Hofte & Whitely, 1989). It has been documented by Van-Rie *et al.* (1990) that most *B.thuringiensis* toxins contain a mixture of structurally different insecticidal crystal proteins

and each of them may contribute to the insecticidal spectrum of the strain.*B.thuringiensis* (H-14) was found to rarely induce epizootics except where insects are confined to crowded areas. Because of this, it has been hypothesized that the association of *B. thuringiensis* and insects may be serendipitous or at least uncommon (Martin & Travers, 1989) and therefore has been suggested that the normal habitat of *B.thuringiensis* is the soil.

Environmental conditions have some influences upon the activity of the bacterium. Temperature has a moderate influence on the effectiveness of the bacterium, mainly, due to a high metabolic and feeding activity of the mosquito larvae at higher temperature (WHO, 1979). Salinity of the water, up to 20g/L Nacl, did not seem to have any effect on the efficacy of the organism (Ignoffo *et al.*, 1981). Nevertheless, organic and inorganic matters affect the activity of the bacterium against mosquito larvae as they get adsorbed to them and hence rendered unavailable to the target organism. Mud was found to drastically decrease the larvicidal activity of the organism. Results of field experiments showed that *B.thuringiensis* (H-14) is very active and has a rapid and specific action against mosquitoes even in harsh environment such as salt marches (Purcell, 1981).

Unlike classical biological agents, which are characterized by their ability to recycle in the host

and to suppress host population over time, B.thuringiensis H-14, is unable to establish high lasting infections among natural mosquito populations (Van-Essen & Hembree, 1982). However, Christoph (1985) reported that germination and growth of *B.thuringiensis* in the larval gut of *Aedes aegypti* was photographically documented. In an experiment with ***Aedes aegypti*** and ***A.vexans,*** as test mosquitoes, Christoph (1985) found that the number of spores per gut increased to the maximum in the first 40-140 minutes of exposure and decreased in the subsequent times. Twenty four hours after the death of the larvae vegetative cells were found in cadavers.

It is evident that the major disadvantage associated with the use of *B.thurigiensis* (H-14) in the control of mosquitoes is the rapid disappearance of the larvicidal effect which lasts only for three days (Van-Essen & Hembree, 1982). The adsorption of the bacterium onto soil particles in the water and other factors such as high density of the formulations that result in the rapid settling down of the crystals may be accounted for the disappearance (Van-Essen & Hembree, 1982) .

1.2.2 D. *Mode of Action of B.thuringiensis*

Among the many potentially toxic materials produced by ***B.thuringiensis,*** beta-exotoxin and delta-

endotoxin are generally regarded most significant in pathogenicity to insects (Aronson *et al.*, 1986).

The delta endotoxin was found to be safe against non-target animals while beta-exotoxin has shown toxicity to some vertebrates and therefore it was excluded from all strains used for commercial production of insecticidal formulations for the control of mosquito (WHO, 1979).

Heimpel and Angus (1959), cited in Wayne (1966), have classified Lepidopteran larvae into three types based on their susceptibility to crystalline delta-endotoxin, bacterial spores or mixture of the two. Type I larvae are killed by preparations of crystalline delta-endotoxin alone and spores of bacterium do not increase toxicity; type II larvae are susceptible to endotoxin but the effect is enhanced by presence of spores; type III insects are only killed by spore-endotoxin mixture.

The delta-endotoxin of several strains of *Bacillus thuringiensis* occurs as bipyramidal parasporal inclusion, the so called crystals. It has been called a protoxin in the sense that it must be dissolved before it becomes effective as a toxin (Fast & Donaghue, 1971). This protoxin composes of a single subunit of approximately 134 Kilo Daltons (KDa) molecular weight. It rapidly converts to smaller toxic subunits (53-78 KDa.) in high pH and suitable enzyme (protease) in the gut of susceptible host

(Tryell *et al.*, 1979, 1981).

Upon the ingestion of the crystalline protein by the ever most susceptible insects, silkworm larvae, cessation of feeding is observed within a few minutes. This is followed by a general paralysis of larvae within 1-7 hours, the insect is usually dead within 24 hours. That was demonstrated by Heimpel and Angus in 1959 who followed by x-ray observation the movement of barium sulfate in the gut of a number of susceptible Lepidoptera.

Studies on the mode of action showed that the toxin altered the selective permeability of the silkworm gut to glucose and to carbonate ions (Fast & Angus, 1965). Louloudes and Heimpel (1969) confirmed these results and extended them to show that acetate and leucine also appear in the hemolymph more slowly in insects intoxicated by delta-endotoxin.

The mid-gut pH of most susceptible larvae is too alkaline to allow spore germination but is suitable for dissolution and activation of protoxin, thus most susceptible insects fall into type I. In some cases, mid-gut pH may be closer to neutrality, allowing germination, or the activation of endotoxin.

As shown by Heimpel and Angus in 1959, the first sign of poisoning following ingestion of crystalline endotoxin by type I insects is paralysis of the gut and mouth parts which leads to a cessation of feeding. Subsequent to the onset of gut paralysis the following

are observed: swelling of microvilli on the lumenal surface of midgut epithelial cells are followed by swelling of the cells themselves. Changes in endoplasmic reticulum and mitochondria take place. Ions and glucose transport and oxygen uptake is disrupted and is accompanied by the loss of adenosine triphosphate from midgut cells. Eventually the cells of the midgut are separated from the basement membrane and bursting of separated cells in the midgut lumenal is seen (Aronson *et al.*, 1986). Disruption of mid-gut structure and function lead to ion and pH imbalances in the hemolymph which result in total body paralysis and death.

1.2.3 *Bacillus sphaericus Neide*

1.2.3 A. *General description.*

The bacteria are rods, 0.6 to 1.0 by 1.0 to 7.0 micron, with ends rounded or pointed and occurring singly or in short chains. They are motile, Gram-variable and often Gram-negative with Gram-positive granules. The spores are 0.7 to 1.2 micron in diameter, round in shape. The sporulation variable and best seen on soybean agar. The sporangia are definitely swollen and usually drum-stick-shaped. Their habitat is widely distributed in nature (Gordon, 1952).

1.2.3 B. *Potential of Bacillus sphaericus*

Bacillus sphaericus is an ubiquitous spore-forming microorganism which is very commonly found in soil and aquatic systems. It was first isolated in the year 1965 from 4th instar larvae of *Culiseta incidens* in California, U.S.A, (Kellen *et al.*, 1965). Some strains of *B.sphaericus* produce a parasporal crystal which is proteinaceous in nature and toxic to the larvae of mosquito species that are responsible for the transmission of human and animal diseases (Yousten *et al.*, 1984). All those strains of *B.sphaericus* showing pathogenicity to mosquito have been isolated from dead insects (Davidson, 1984). The insecticidal *B.sphaericus* strains develop full toxicity only during sporulation (Myers *et al.*, 1979; Yousten & Davidson, 1982). The process needs considerable amount of oxygen to be completed as *Bacillus sphaericus* is a highly aerobic organism. The toxin produced by insecticidal strains has shown to be concentrated in the crystal-like inclusion formed alongside the spore. It has been indicated that the larvicidal activity is due to a cell-bound toxin that is not released into the culture.(Singer, 1974). Davidson & Myers (1981) have reported that the toxin may be primarily in the spore itself and /or in the parasporal inclusion that is present in sporulated cells of the most toxic strains. Sporulation is,

therefore, an important factor in the maximization of larvicidal activity of *B.sphaeriucs* strains.

Spores of *B.sphaericus*, like those of most bacilli, have an ability to survive period of desiccation, cold or high temperature that might reach up to 30°C (Davidson, 1984). Because spores of *B. sphaericus* are more dense than water, they tend to settle down rapidly from the larval feeding zone e.g. *Anophelese* spp. at water surface (Davidson *et al.* 1984).

Bacillus sphaericus was found to be very host specific and has neither toxicity for non-target organisms nor apparent perturbation of the environment. Species of *Culex* are very susceptible to this bacterium. *Anopheles* species have moderate sensitivity, while most species of *Aedes* are quite insensitive to it (Ramoska & Lacey, 1979). Moreover, it has been shown that mosquito larvae exhibit decrease susceptibility to *B.sphaericus* with increasing age. Wright *et al.*, (1981) reported that the first instar larvae of *Culex pipiens* L. are 2-5 fold more susceptible than the fourth instar larvae. Furthermore, The ability of *B.sphaericus* to recycle in aquatic system, particularly in those containing moderate to high organic matter, is documented (Hertlein *et al.*, 1979). In contrast to this potential of persistence and recycling under the above mentioned conditions it is reported that some strains

of *B.sphaericus* do not show significant persistence or recycling ability in clear water (Mulla *et al.*, 1984; Davidson *et al.*, 1984).

1.2.3 C. *Mode of Action of B.sphaericus*

Once in the gut of the larvae, the bacterium is digested in a peritrophic membrane. As the *B.sphaericus* cells are digested, a component of the bacterial cell wall is released. This toxic material find its way through the peritrophic membrane to sensitive cells lining of the lumen of the larval midgut.

The first evidence of death of the larvae occurs between 8-12 hours. Partial feeding inhibition may occur as early as 10 minutes. Swelling of the mid-gut can be seen within 30 minutes followed by body tremors, irregular heart rhythm, shuddering, sluggishness and finally death. Only after death of the larvae do the non- digested *B.sphaericus* cell grow out in the tissue of the dead larva (Singer, 1980b).

2. MATERIALS AND METHODS

2.1. *Collection of materials*

Soil samples and dead adults mosquitoes were collected from different places and habitats in Ethiopia.

2.2. *Preparation of sample suspension*

About 4-5 grams of soil sample were placed in a beaker and 20 ml of sterile water added and the beaker heated in water bath for 10 minutes at 80^0C .

Dead mosquito adults were rinsed twice with alcohol and twice with sterile water to sterilize the surface area. The mosquitoes were then homogenized manually by a clean glass rod. The homogenate was finally heated in water at 80^0C for 10 minutes.

2.3. *Isolation of spore-forming bacteria*

About 0.5 ml of each suspension of each sample material was serially diluted(10^{-3}) and then aseptically pipetted onto the surface of nutrient agar plates. The suspension was spread evenly and the plates were incubated at 30^0C for 24-48 hours. Colonies which looked morphologically different were selected and the pure cultures were maintained on agar slants for further test.

2.4. *Selective isolation of Bacillus thuringiensis*

A modified method of Travers *et al.* (1987) to selectively

isolate *Bacillus thuringiensis* was used. A soil sample and dead mosquito adult material (after being homogenized) were incubated at 30°C for four hours by shaking(120rpm) in a semi-synthetic medium (grams/litre; Tryptone 10.0, yeast extract 5.0, NaCl 5.0 and sodium acetate 5.0). About 0.5 ml of the sample material was diluted at a rate of 1:100 (0.5 ml of the sample in 99.5 ml of sterile water) and then aseptically pipetted, spread evenly on the same solidified medium. The agar plates were incubated at 30°C for 24-48 hours. Colonies showing morphological variations were selected and maintained in pure culture on nutrient agar slants.

2.5. *Selective isolation of Bacillus sphaericus*

A medium described by Weiser (1991) to selectively isolate *Bacillus sphaericus* was used. It constituted of (gram per litre); Peptone 3.0, beef extract 5.0, yeast extract 0.5, MnCl 0.08, MgCL 0.07 and agar 15.0.

The soil samples and dead mosquito adult homogenate were each diluted(10^{-3}) and aseptically plated onto the surface of the same medium. Plates were incubated at 30°C for 24-48 hours. Representative colonies were selected and cultured on nutrient agar and kept for further evaluation.

2.6. *Cultivation conditions*

A loopful of bacterial culture of the representative

colonies grown on agar slants was used to inoculate 50 ml of sterile nutrient broth found in 250 ml Erlemeyer flask. The flasks were placed on a rotary shaker (GFL-3020) at 120 rpm and at room temperature until sporulation and cell lysis were essentially completed. Sporulation was monitored by using either a light microscope or phase contrast microscope.

2.7. *Larvicidal tests*

Toxicity tests against culicine mosquitoes were made using early instar larvae. Assay was carried out in 250 ml beaker containing 100 ml of distilled water and 15 mosquito larvae. About 3-6 ml of the bacterial culture was introduced in each beaker. Mortality rate was recorded after 24 and 48 hours of incubation at room temperature (death of larva a was verified by touching it with a bacteriological needle; if it responded then it was alive or dead otherwise). To confirm the death of larvae, they were transferred to another beaker containing sterile water and kept there for 24 hours to observe recovery. A beaker to which no bacterial culture was added served as a control. Isolates causing mortality were retained for further investigation.

2.8. *Biochemical and morphological characterization of isolates*

The following biochemical tests were conducted in accordance with Bergey's Manual of Systematic Bacteriology

(Claus & Berkeley, 1986): Production of catalase, Voges-Proskauer reaction, Voges-Proskauer broth, growth in sodium chloride, growth at pH 5.7 and 6.8, acid from carbohydrate, utilization of propionate, utilization of citrate, production of indole,
dihydroxyacetone production, egg-yolk lecithinase reaction, deamination of phenylalanine, hydrolysis of tyrosine, hydrolysis of casein, hydrolysis of starch, liquification of gelatine and growth at different temperatures. In addition, morphological characterisation such as spore and sporangium shape was done.

2.9. *Investigation on culture conditions for optimal growth*

2.9.1 *Media components*

The following media were used:

medium M1 (MB) (Kalfon *et al.*1983)

KH_2PO_4	6.80
$MgSO_4.7H_2O$	0.30
$MnSO_4.4H_2O$	0.02
$ZnSO_4.7H_2O$	0.02
$Fe_2(SO_4)_3$	0.02
$CaCl_2$	0.20
Tryptone	10.00
Yeast extract	2.00
Dextrose	15.00 gram/Litre
pH	7.0

medium M2 (Scheerrer *et al.*, 1973)

$(NH_4)_2SO_4$	2.00	
K_2HPO_4	0.50	
$MgSO_4$	0.20	
$MnSO_4$	0.05	
$CaCl_2$	0.05	
Yeast extract	2.00	
Glucose	6.00	gram/Litre
pH	7.3	

medium M3 (NYSM) (Yousten et al., 1984)

Nutrient broth		
Yeast extract	0.50	gram/Litre
MnCl	$5X10^{-5}$	M
CaCl	$7X10^{-4}$	M
MgCl	$1X10^{-3}$	M
pH	7.0	

medium M4 (modified) Scheerrer *et al.*, 1973

$(NH_4)_2SO_4$	4.70	
$MgSO_4.7H_2O$	0.50	
$CaCl_2$	0.05	
K_2HPO_4	1.50	
kH_2PO_4	1.50	
Glucose	6.00	
Yeast extract	3.00	gram/Litre
pH	7.3	

medium M6 (Dulmage *et al.*, 1970)

K_2HPO_4	1.00	
kH_2PO_4	1.00	
Corn starch	5.00	
Yeast extract	2.00	
Dextrose	5.00	
Tryptone	10.00	gram/Litr
pH	7.0	

medium M7 (modified) Dulmage *et al.*, 1970

$MgSO_4.7H_2O$	0.30	gram/Litre
$Fe_2(SO_4)_3$	0.02	
$ZnSO_4.7H_2O$	0.02	
$CaCO_3$	1.00	
Yeast extract	2.00	
Dextrose	15.00	
Ground nut	20.00	
pH	7.0	

2.9.2 *Effect of aeration*

Four flasks, each containing 50 ml of the medium, were inoculated with a loopful of the bacterial culture (grown in nutrient broth for 24 hours). One set of the flasks was placed on a rotary shaker having a speed of 150 rpm and the other set were placed on another rotary shaker having a speed of 120 rpm. All cultures were then incubated for 75

hours. Dry biomass at each shaking speed were recorded. Dry biomass was detremined as follows: For each isolate, four flasks each containing 50 ml of the medium were inoculated with a loopful of the bacterium from broth culture 24 hours old. The whole cultures were centrifuged at 5000 rpm for 30 minutes. The supernatant was discarded and the residue was dried in an oven at 55°C (75-80 hours) until a constant weight was attained for each biomass.The total dry weight of biomass of each isolate per litre of culture was, accordingly, determined. Sprulation was monitored by light microscope

2.9.3 *Effect of temperature on biomass production*

For each isolate four flasks, each of which contains 50 ml of the medium (M1 in case of isolates AA-2, AA-11 and AA-71; M7 in case of isolate AA-82), were inoculated with a loopful of the bacterium from young broth culture. The flasks were placed on a rotary shaker at 150 rpm. They were incubated under four different temperatures (23°C, 30°C, 35°C, 40°C). Cultures were incubated until the full growth of the bacterium was achieved (45-50 hours). The final pH levels of the culture were recorded and adjusted to neutrality. The whole cultures were centrifuged at 5000 rpm for 30 minutes. The supernatant was discarded and the residue washed and centrifuged two more times and then dried in an oven at 55°C for 75-80). The total dry weight of biomass of each isolate

per litre of culture was determined.

2.10. *Efficacy of the isolates on different stages of mosquito larvae*

2.10.1 *Culture preparation*

Four flasks, each containing 50 ml of nutrient broth, were inoculated with a loopful of a 24 hours old culture. The cultures were grown on a rotary shaker at 150 rpm. About 2% by volume of these cultures were used to inoculate the fermentation flasks. The fermentation flasks contained medium M1 to be used for isolate AA-2, AA-11 and AA-71. For isolate AA-82, medium A7 was used as a fermentation medium. The fermentation flasks were placed on a rotary shaker at 150 rpm and at 23^0C for 75 hours until 70-85% of the sporulation was achieved.

2.10.2 *Bioassay*

A series of volumes (0.5, 1.0, 1.5, 2.0 and 2.5 ml) of each culture of different isolate were added separately into 250-ml beakers. 50 ml of distilled water and 10 larvae were added to each beaker. A beaker to which no culture was added has been used as a control.

Fed mosguitoes were brought from households and incubated at 26^0C till hatching. The ages of larvae were recorded. For each volume two replicates were made. Larvae of culicine spp

of 6-,8- and 11-days-old were used in the bioassay.

2.10.3 *Determination of dry biomass of isolates*

Fermentation flasks, each containing 50 ml of the culture of each isolate, were shaken as described earlier and the cultures were centrifuged at 5000 rpm for 30 minutes. The residues washed with sterile water and centrifuged for three times. The residues were then dried in an oven at 55^0C for 75-80 hours. The average dry weight of material obtained in this way was used in the determination of the amount of dry biomass added into each of the volume applied in the bioassay.

2.11. *Activity of the isolates on larvae in stagnant water*

Natural breeding sites of mosquitoes were chosen in Arat Kilo campus of the Addis Ababa University to test larvicidal activity in natural stagnant water. A medium (M1 for strain AA-2, AA-11 and AA-71; M7 for strain AA82) of 300 ml volume in different flasks were inoculated with bacterial culture that was grown in nutrient broth for 24 hours. The flasks were placed on a rotary shaker (GFL-3020) at 120 rpm at room temperature for 70-75 hours. 200-250 ml of culture was adjusted to pH 7.0 and applied into stagnant water. Natural death among larval population was estimated.

3. RESULTS

3.1.Screening

A total of 97 spore-forming bacillus strains were isolated from 16 soil samples and 5 dead mosquitoes. Of the 97 isolates six bacillus strains (AA-2, AA-6, AA-10, AA-11, AA-71 and AA-82) have shown high toxicity level to mosquito larvae (Table 1).

Strain AA-71 was isolated by the selective procedure of Travers *et al.*(1987). The strains AA-71 and AA-10 were isolated from a soil sample taken from the University campus while strain AA-6 was isolated from a soil sample collected from Mennagesha state forest (Ethiopia). Strains AA-2, AA-11 and AA-82 were isolated from dead mosquitoes.

3.2.Morphological and Biochemical Characterization

Four isolates (AA-6, AA-10, AA-71 and AA-82) out of the six isolates showed a typical *Bacillus* morphology. The isolates have large ellipsoidal spores and were found to have non-swollen sporangium. The other two isolate (AA-2 and AA-11) have round spores (Table 2).

All of the six isolates were found to be catalase positive. They grow at 30 to 50^{0}C and in 2 - 5% NaCl. They don't grow at temperature below 5^{0}C or above 65^{0}C. The isolates showed variable characters or responses under other condition or treatments (Table 2).

Table 1. Screening for larvicidal activity of the strains.

Isolate code	%larval mortality at:	
	24 hr.	48 hr.
AA-2	70	100
AA-6	100	100
AA-10	100	100
AA-11	70	100
AA-71	70	100
AA-82	80	100

Table 2. Biochemical and morphological characteristics of the isolates

Characteristics	Isolates						Ref. Bact.	
	AA-2	AA-6	AA-10	AA-11	AA-71	AA-82	BT*	BS*
ellipsoidal spore	-	+	+	-	+	+	+	-
spore round	+	-	-	+	-	-	-	+
sporangium swollen	-	-	-	-	-	-	-	+
catalase	+	+	+	+	+	+	+	+
pH in V-P broth								
<6	+	-	-	+	+	+	+	-
>7	+	+	+	+	+	+	-	+
acid from:								
D-glucose	+	+	+	+	-	+	+	-
L-arabinose	+	-	-	-	-	-	-	-
D-xylose	-	-	-	-	+	-	-	-
D-mannitol	+	+	+	+	+	-	-	-
hydrolysis of :								
casein	+	+	+	+	-	+	+	d
gelatine	+	+	+	+	+	+	+	d
starch	-	+	+	+	+	+	+	-
Utilization of								
propionate	+	-	+	+	-	-	ND	ND
degradation of citrate	-	-	-	ND	+	+	+	d
degradation of								
tyrosine	-	+	+	-	-	-	d	_
Production of								
indole	-	-	-	-	-	-	-	-

continued

Table 2. continued

deamination of								
phenylalanine	-	-	-	-	-	-	-	+
egg-yolk lecithinase	+	+	+	+	-	+	d	-
formation of								
dihdroxyacetone	+	+	+	ND	+	+	ND	-
growth in NaCl								
2%	+	+	+	+	+	+	+	ND
5%	+	+	+	+	+	+	+	d
7%	+	-	+	+	+	+	+	d
10%	+	-	-	+	+	-	ND	-
growth at pH								
5.7 broth	+	+	-	+	+	+	+	d
6.8	+	+	+	+	+	+	+	d
Voges-Proskauer reactions	-	-	-	-	-	-	d	-
growth at								
5°C	-	-	-	-	-	-	-	-
10°C	ND	ND	ND	ND	ND	ND	d	+
30°C	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	d
50°C	+	+	+	+	+	+	-	-
55°C	+	-	-	+	+	+	-	-
65°C	-	-	-	-	-	-	-	-

* Data obtained from Bergey's Manual of Systematic Bacteriology. (Claus & Berkeley 1986)
d = 11-89% positive.
ND = No data available.
BT = *Bacillus thuringiensis*
BS = ***Bacillus sphaericus***

3.3. Culture conditions for optimal growth

3.3.1 *Media components*

A modified medium (M1) which was suggested by Kalfon *et al.* (1983) as a valuable medium to increase sporulation for *Bacillus thuringiensis* and other *Bacillus* species was also found useful for some of the six isolates (Table 3).

All isolates showed variable toxicity level, especially after exposure for 48 hours when large volumes of culture (14 ml) were used. When smaller volumes of culture(5.0 ml) were used only a few of the isolates (AA-2, AA-11 and AA-71) showed consistent toxicity at 48 hours. When even lower volumes (1.0 ml) were used only isolate AA-2, AA-11 and AA-71 have shown some toxicity and isolate AA-2 was found to be the most toxic of all (Table 3). The investigation for an optimal medium for larvicidal activity showed variable results (Table 4).

From this investigation it was found that M1 could be considered as a suitable medium for the growth of larvicidal bacilli (AA-2, AA-11 and AA-71) and production of the toxic substance, while medium M7 was found to be suitable for the growth of isolate AA-82. Based on this two media (M1 and M7) the percent larval mortality at different volumes of culture was determined (Table 5).

Table 3. Percent larval mortality (%LM) at 24 and 48 hours when the isolate cultures that were grown on medium M1 were used at different volumes (ml).

Isolates	%LM(14ml)		%LM(5.0 ml)		%LM(1.0 ml)		%LM(0.5 ml)	
	24	48	24	48	24	48	24	48
AA-2	90	100	70	100	30	100	-	20
AA-6	35	90	-	-	-	-	-	-
AA-10	50	80	-	-	-	-	-	-
AA-11	90	100	70	100	30	70	20	20
AA-71	100	100	50	100	-	60	-	-
AA-82	70	100	30	80	-	-	-	-

Table 4. Percent larval mortality (%LM) at 24 and 48 hours when the isolate cultures that were grown on different media(M1-M7) were used at a rate of 1.0 ml

isolates	%LM(M1)		%LM(M2)		%LM(M3)		%LM(M4)		%LM(M6)		%LM(M7)	
	24	48	24	48	24	48	24	48	24	48	24	48
AA-2	30	100	-	-	-	-	-	-	-	-	-	-
AA-6	-	-	-	-	-	-	-	-	-	-	-	-
AA-10	-	-	-	-	-	-	-	-	-	-	-	-
AA-11	30	70	-	-	-	-	-	-	-	-	-	-
AA-71	-	60	-	-	-	-	-	-	-	-	-	20
AA-82	-	-	-	10	-	10	10	20	-	-	60	80

- Zero larval mortality.

Table 5. Percent larval mortality (%LM) obtained at 24 and 48 hours when cultures of isolates grown on medium M1 and medium M7 were used at different volumes.

isolate code	medium used	volumes of cult. used (ml)	%LM at: 24 h.	48 h.
AA-2	M1	0.5	10	10
	=	1.0	30	70
	=	1.5	40	80
	=	2.0	90	100
AA-11	M1	0.5	20	20
	=	1.0	30	40
	=	1.5	40	70
	=	2.0	70	90
AA-71	M1	0.5	0	0
	=	1.0	10	40
	=	1.5	10	70
	=	2.0	10	100
AA-82	M7	0.5	10	20
	=	1.5	70	90
	=	1.0	60	90

3.3.2. *Effect of aeration*

The dry biomass weight and sporulation were found to differ under different aeration rates of 120 rpm and 150 rpm (Figures 1 and 2). The difference was found to be 2.2%, 11.7%, 2.3% and 4.5% for strains AA-82, AA-71, AA-11 and AA-2 respectively.

3.3.3. *Effect of temperature on biomass production*

Growth of isolates based on dry biomass was found to vary under four different temperatures (23^{0}C, 30^{0}C, 35^{0}C and 40^{0}C) (Figure 3). Isolate AA-2 was shown to produce the highest biomass at a temperature of 30°C. Isolate AA-11 has been found to have a wide range of temperature. The growth was almost constant under different temperatures. Isolate AA-71 demonstrated decreasing production of biomass with an increasing in temperature. Isolate AA-82 has an optimal growth temperature of 30^{0}C.

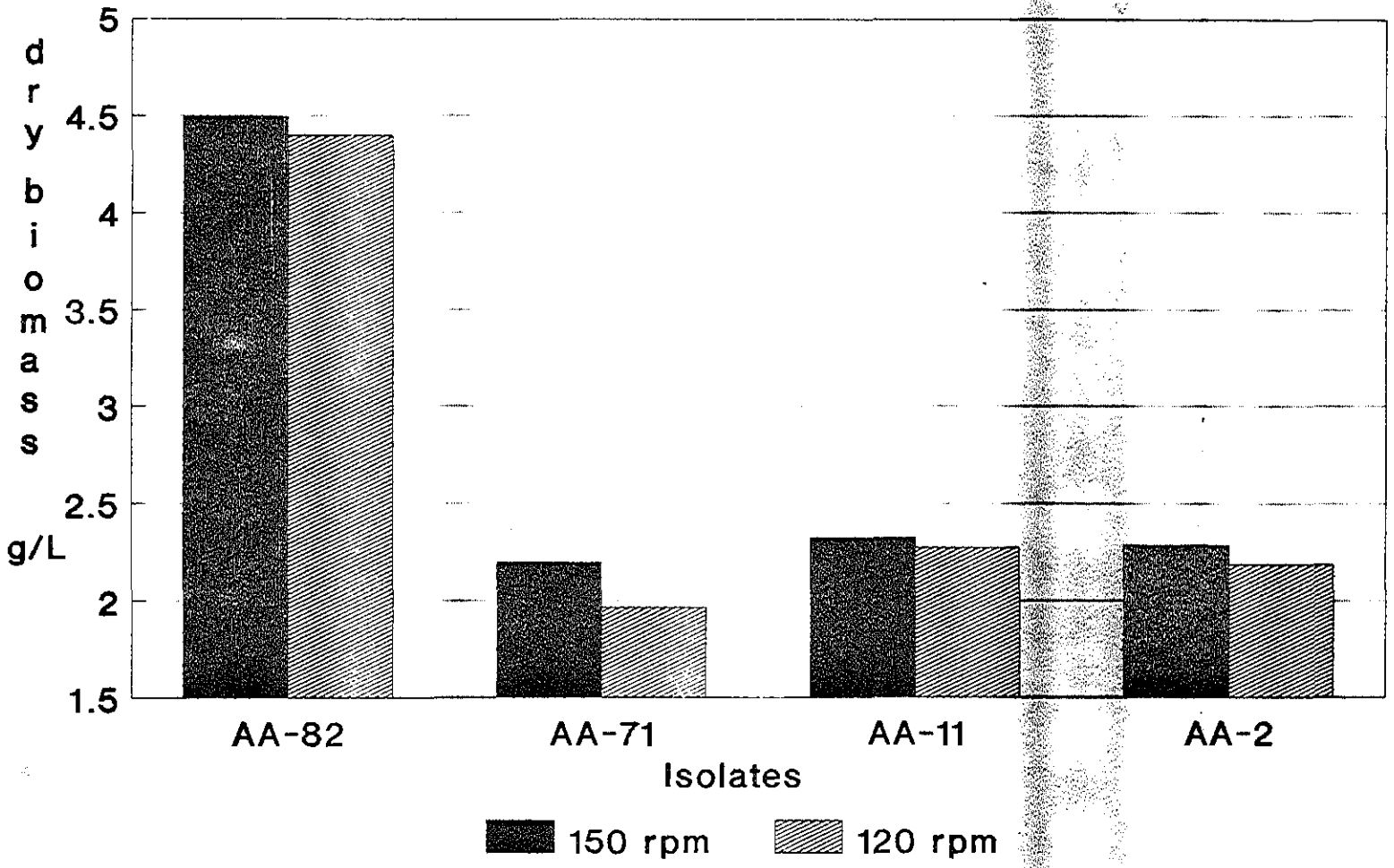

Figure 1. Effect of aeration levels on biomass production of the isolates.

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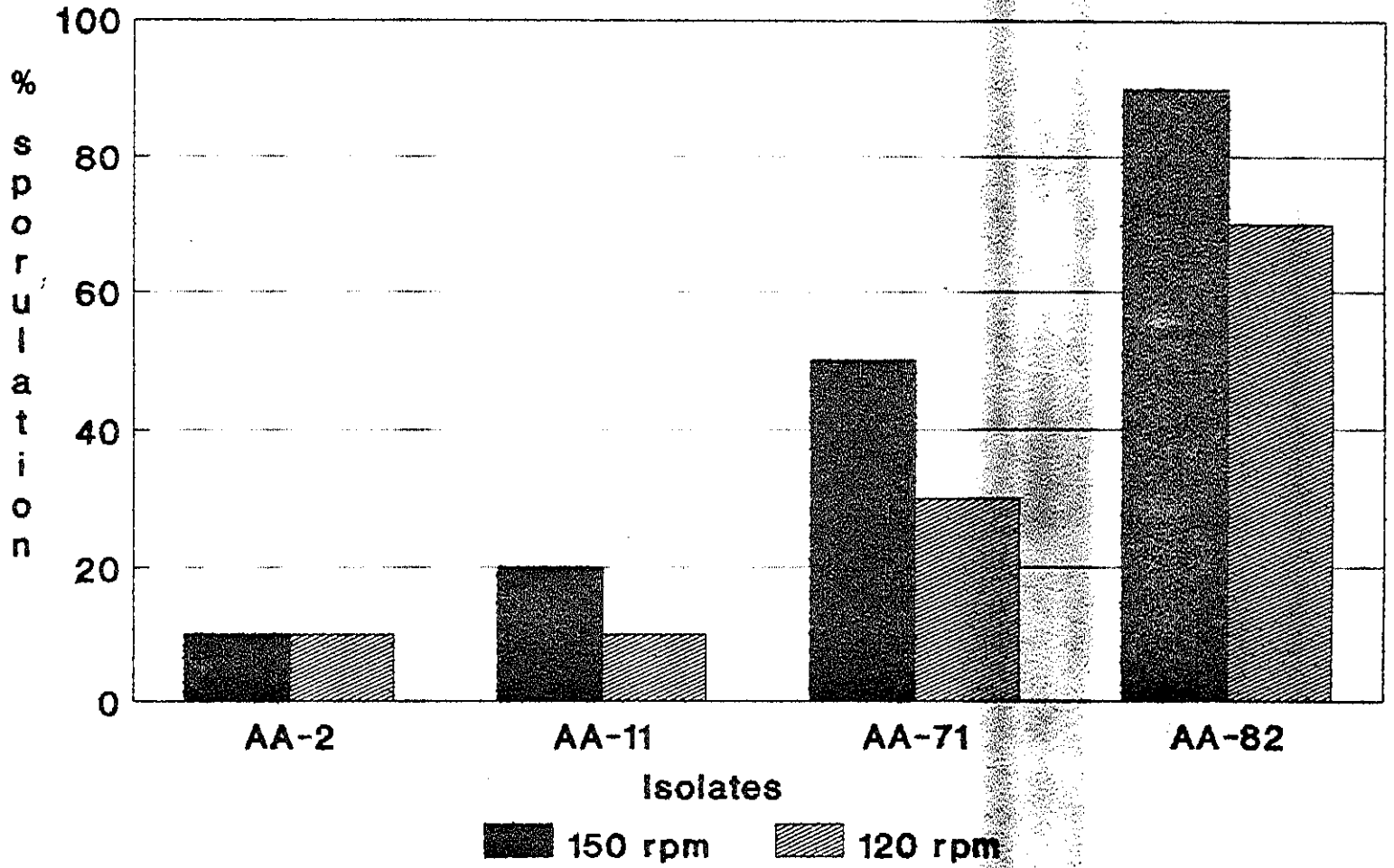

Figure 2. Effect of aeration levels on sporulation of the isolates.

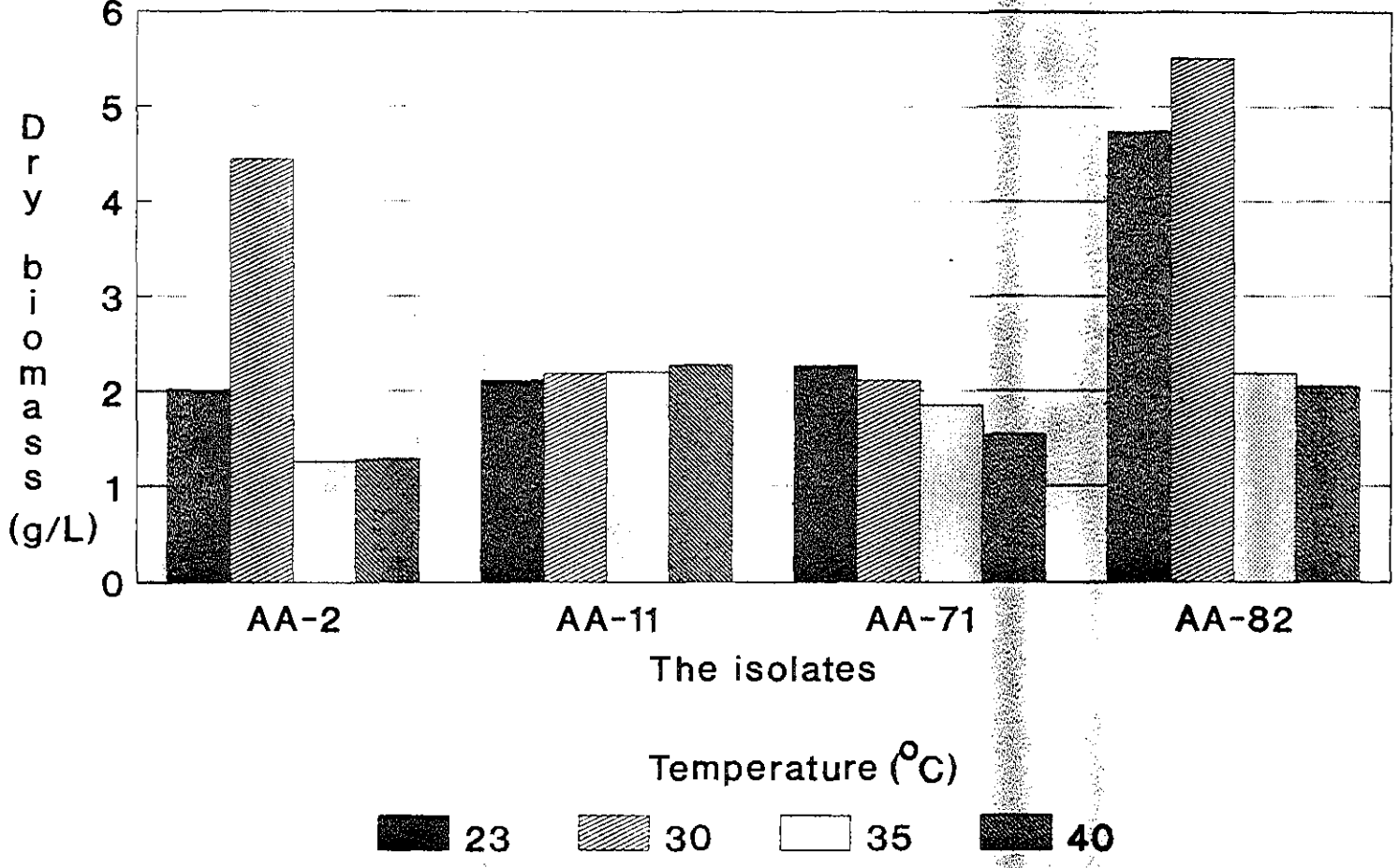

Figure 3. Effect of temperature on growth of the isolates.

3.4.Efficacy of the isolates on different stages of mosquito larvae

The minimum lethal dose that kills 50% of the population (LC_{50}) was determined using the Finney's Probit for Quantal Data which calculates LC_{50} and the effective dose (ED_{50}). Isolate AA-2 had LC_{50} of 100.573 µg/ml on 8-days old larvae of culicine mosquito after 24 hours of incubation. The isolate was not effective at lower concentrations on the other two larval stages (6-days and 11-days old) of culicines. The LC_{50} of the isolate after 48 hours of incubation on different larvae of culicine mosquito was found to be 67.521 µg/ml, 56.955 µg/ml and 97.582 µg/ml for 6-days, 8-days and 11-days old larval stages respectively (Table 6).

Isolate AA-11 had LC_{50} of 66.943 µg/ml, 43.019 µg/ml and 135.31 µg/ml on 6-days, 8-days and 11-days old larval stages, respectively (Table 7).

Isolate AA-71 was found to have LC_{50} of 95.334 µg/ml, 66.69 µg/ml and 10.270 µg/ml for 6-days,8-days and 11-days old larval stages of *Culex* spp., respectively, while it was found to have LC_{50} of 89.996 µg/ml for 8-days old larval stage of *Aedes aegypti* (Table 8).

Isolate AA-82 had LC_{50} of 144.264 µg/ml, 101.409 µg/ml and 170.158 µg/ml on 6-days, 8-days and 11-days old larvae of *Culex* spp., respectively. For *Aedes aegypti* (8-days old larvae), the LC_{50} was found to be 96.082 µg/ml (Table 9).

Table 6. Lethal concentration (LC_{50}) of isolate AA-2 on different larval stages of culicine mosquitoes after 48 hours.

dry biomass (µg/ml)	avg.%larval mortality at: 48hr	LC_{50} (µg/ml)	95% confid.limt Lower	Upper
A				
20.16	15	67.521	59.824	77.685
40.32	30			
60.48	35			
80.64	60			
100.8	70			
B				
20.16	0	56.955	53.189	60.723
40.32	25			
60.48	60			
80.64	75			
100.8	90			
C				
20.16	0	97.582	88.425	111.526
40.32	10			
60.48	20			
80.64	35			
100.8	60			

A = 6-days old;
B = 8-days old);
C = 11-days old.

Table 7. Lethal concentration (LC_{50}) of isolate AA-11 on different larval stages of culicine mosquitoes at 24 hours.

dry biomass (µg/ml)	avg.%larval mortality at: 24hr	48hr	LC_{50} (µg/ml)	95% confidence limit Lower	Upper
A					
21.6	0	10	66.937	62.987	70.979
42.32	10	35			
63.48	50	80			
84.64	70	85			
105.0	85	100			
B					
21.16	5	10	43.019	39.013	46.989
42.32	65	65			
63.48	80	85			
84.64	80	90			
105.0	85	100			
C					
21.16	10	10	135.308	108.602	197.729
42.32	10	15			
63.48	25	25			
84.64	30	35			
105.0	50	70			

A = 6-days old;
B = 8-days old;
C = 11-day old;

Table 8. Lethal concentration (LC_{50}) of isolate AA-71 on different larval stages of culicine mosquitoes at 24 hours.

dry biomass (µg/ml)	avg.%larval mortality at: 24hr	48hr	LC_{50} (µg/ml)	95% confidence limit Lower	Upper
A					
22.68	0	5	95.334	86.562	107.810
45.36	15	30			
68.04	40	35			
90.72	40	60			
113.4	60	75			
B					
22.68	0	0	66.690	61.872	71.863
45.36	25	25			
68.04	60	60			
90.72	65	75			
113.4	80	90			
C					
22.68	5	5	102.796	91.917	119.486
45.36	15	20			
68.04	20	30			
90.72	40	60			
113.4	65	65			
D					
22.68	0	25	89.996	84.293	96.834
45.36	0	40			
68.04	40	55			
90.72	50	80			
113.4	70	100			

A = Cx.sp.(6-days old);
B = Cx.sp.(8-days old);
C = Cx.sp.(11-days old);
D = *Aedes aegypti*(8-days old).

Table 9. Lethal concentration (LC_{50}) of isolate AA-82 on different larval stages of culicine mosquitesat 24 hours.

dry biomass (µg/ml)	avg.%larval mortality at: 24hr.	48hr.	LC_{50} (µg/ml)	95% confid. limit. Lower	Upper
A					
47.36	0	0	144.264	136.274	152.047
94.72	20	25			
142.08	25	30			
189.44	80	100			
236.80	100	100			
B					
47.36	20	20	101.461	85.238	117.328
94.72	60	60			
142.08	65	70			
189.44	65	70			
236.80	70	90			
C					
47.36	5	20	170.158	156.687	186.546
94.72	15	35			
142.08	30	70			
189.44	65	85			
236.80	70	90			
D					
47.36	20	45	96.082	83.008	108.659
94.72	55	60			
142.08	70	75			
189.44	75	75			
236.80	75	95			

A = Cx.sp.(6-days old);
B = Cx.sp.(8-days old);
C = Cx.sp.(11-days old);
D = *Aedes aegypti*(8-days old)

The degree of difference in susceptibility of larvae of each stage to different bacterium was statistically tested (Table 10). No significant difference was found among different larval stages to strains AA-2, AA-71 and AA-82. A decrease in susceptibility as age increased was seen from the ingestion of strain AA-11 by different larval stages of mosquitoes.

3.5.Efficacy of the isolates on larvae in stagnant natural water

Application of culture of the isolates on stagnant water (artificial concrete ponds) showed different larvicidal effectiveness after a period of 2 days of treatment. Isolate caused approximately 40% mortality, isolate AA-11 30% mortality, isolate AA-71 40% mortality while isolate AA-82 was found to be the most toxic showing 70% larval mortality (Table 11).

Table 10. Differences in the LC_{50} of different isolates on different larval stages (A, B, C) of culicine mosquitoes at different significance levels(1%, 5%, and 10%).

Isolate code	larval Stages	Calculated t_v# values	Tabulated t_v# values at: 1%	5%	10%
AA-2	**A-B**	**0.4687 ***	**3.36**	**2.31**	**1.86**
	B-C	**1.3357 ***			
	A-C	**1.8020 ***			
AA-11	A-B	1.0093 *	,,	,,	,,
	B-C	2.8851 **			
	A-C	3.8158 ***			
AA-71	A-B	1.1249 *	,,	,,	,,
	B-C	1.4180 *			
	A-C	0.2931 *			
AA-82	A-B	0.8900 *	,,	,,	,,
	B-C	1.2981 *			
	A-C	0.4884 *			

\# t-test at v degree of freedom
A culicine larvae 6-days old
B culicine larvae 8-days old
C culicine larvae 11-days old
* not significant at all levels
** significant at level 5%
*** significant at level 1%

Table 11. Larvicidal effectiveness shown by the isolates when applied into natural stagnant water

Isolates	period of incbation	%morality rate
AA-2	48 hours	40
AA-11	,,	30
AA-71	,,	**40**
AA-82	,,	70

4. DISCUSSION

Bacteria antagonistic to insects including mosquitoes were found in association with soil (Gorlberg & Margalit, 1977; Kalfon *et al.*,1983; Martin & Travers, 1989).

In this study spore-forming bacillus strains were isolated from various samples taken from different habitats.

Biochemical tests (Table 2) which were conducted according to procedures described in Bergey's Manual of Systematic Bacteriology (Claus & Berkeley, 1986) revealed how variable the toxic isolates were. From the tests conducted, the isolates showed (Figure 4) various similarities to the known mosquito pathogen, *B.thuirngiensis* and *B.sphaericus*, as well as to the other insect pathogens, *B.larvae*, *B.lentimorbus* and *B.popilliae*. However, our results of identification would have been more reliable if we had used reference type strains for the biochemical confirmatory work.

A complex of factors (medium composition, pH of the culture, temperature, aeration levels and the strain of the bacterium were found to be determining factors to the production of the bacterial toxic substances against the mosquitoes (Foda *et al*,.1985; Scherrer *et al.*, 1973; Pearson & Ward 1988; Avignone-Rossa *et al.*, 1990 and Abdel-Hameed *et al.* 1990b).

In our study the investigation of suitable media for the production of toxin protein from the isolates was

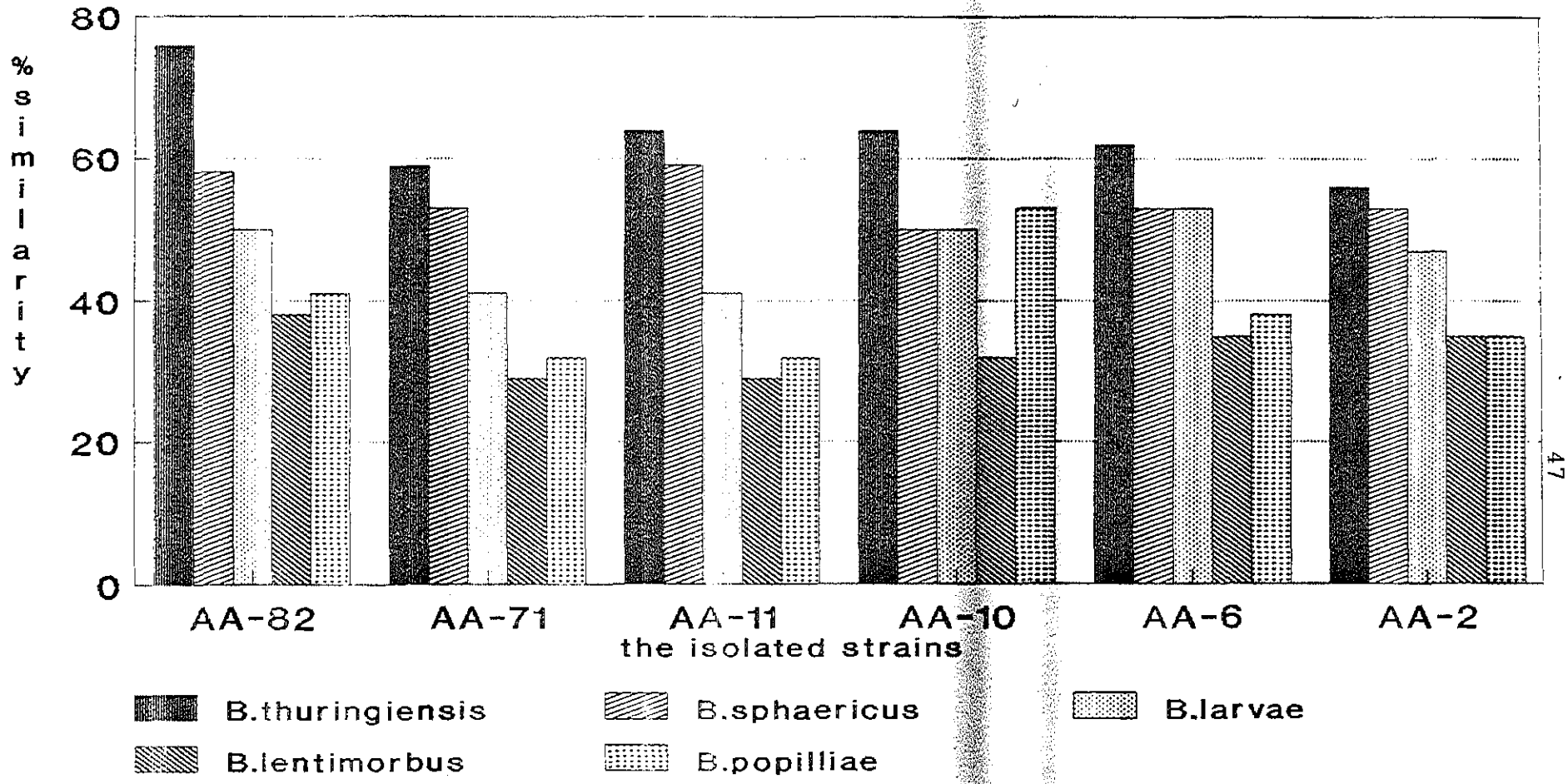

figure 4. Similarity of the isolated bacteria to the most potent insect pathogens in the genus Bacillus.

mainly based on the use of defined media employed by previous workers with slight modifications. Due to the fact that all media can't perform very well in small shake-flask fermentation (WHO, 1990), we were not able to investigate new media for our isolates. However, some of the used media (M1 and M7) were found to be useful for some of the isolated bacteria.

Medium M1 was found to be the most suitable one for the better growth of isolates A-2, AA-11 and AA-71 (Table 3 and Table 4). In this medium (M1) the ratio of carbohydrate to nitrogen is as high as 15:2. A larvicidal rate of 60-100% was achieved with medium M1 when a volume of 1.0 ml of culture was used throughout the test. This is possibly due to the fact that basic compounds produced from the utilization of nitrogenous compounds was encountered by the acid produced from the assimilation of carbohydrates rendering the pH level of the medium suitable for the growth of the isolate.

Medium M3, which has been found useful for the growth of *B.sphaericus* (Yousten *et al.*1984) was not found to be optimal in the case of our isolates (Table 4) and this may, probably, be due to the amount of carbohydrate in the medium . Medium M2 contains both inorganic and organic nitrogen sources, NH_4SO_4 and yeast extract, respectively, and the ratio of carbohydrate to nitrogen is as low as 3:2. No larvicidal effects were observed from isolates when they were grown on this medium. This could be attributed to the effect of both

the ratios of carbohydrate to nitrogen and to organic and inorganic substance (Avignone-Rossa *et al.*, 1990). Medium M4 and Medium M6 also contain low ratios of carbohydrate to nitrogen (2:1 and 5:2 respectively) and no larvicidal effect has been observed from the isolates when they were grown on both media. Though Medium M7 has a high carbohydrate to nitrogen ratio (>15:2), it was found not to be triggering the growth of isolates AA-2, AA-6, AA-10, AA-11 and AA-71, while it did for isolate AA-82 (Table 4). This may be, again, due to the effect of pH level of the culture which favours the growth only of isolate AA-82. A partially defatted ground nuts was used in this medium. This result highlights the possible use of natural substrates in the production of the isolated strains.

Aeration has been found by many authors (Yousten & Walis, 1987; Freedman, 1970; Pearson & Ward, 1988 and WHO, 1990) to be very essential and limiting factor in the processes of bacterial growth and subsequent sporulation .

In our study an aeration rate of 120 rpm (GFL-3020) has been used. It is assumed that the isolates would have shown even better killing ability if higher aeration was provided to the cultures. From the result obtained it was found that the dry weight of biomass was lower compared to the same parameter when obtained under an aeration rate of 150 rpm (Figure 1). This results go in line with the fact that for several species of the genus *Bacillus*

growth and subsequent toxin production are highly related to oxygen supply (Avignone-Rossa *et al.*, 1992).

From the present study, it has become clear that if fermentation process has been studied under good aeration supply levels, (as we were using low rates of aeration 120-150 rpm in small shake flasks 250 ml) better result could have been obtained,from these isolates.

The effect of temperature on the production of biomass and consequently the toxicity levels of bacterial crystal has been reported (Yousten *et al.* 1984; Pearson & Ward, 1988).

In this study four different temperatures, (23^{0}C, 30^{0}C, 35^{0}C and 40^{0}C), were investigated to determine the optimal temperature range of the isolates. Isolate AA-2 was found to grow very well at a temperature of 30^{0}C. The growth at a temperature of 35^{0}C or 40^{0}C resulted in 72% decrease of biomass to that produced at 30^{0}C, while a temperature below 30^{0}C has shown 55% reduction in growth. Isolate AA-11 has shown an almost consistent pattern of growth at all temperatures investigated. This isolate has shown widest range of optimal temperatures (23-40^0). This characteristic might be useful in the utilization of this strain for a wide range of habitats where temperatures are highly variable. Isolate AA-71 has shown to have a different pattern of growth and biomass production. As the temperature increases, the biomass production decreases. The highest growth was observed under room temperature (23^{0}C). Isolate AA-82 was found

to produce the highest biomass at a temperature of 30^0C, and an increase of temperature beyond that level resulted in 60-65% reduction of growth compared to that produced at 30^0C(Figure 3).

From this investigation it could be observed that each isolate has a different pattern of growth under different temperatures. Each of these strains needs to be studied for the possible use in the corresponding environments. The optimal temperature for high biomass production was found to be 30^0C for isolates AA-2 and AA-82, 23-21^0C (room temp.) for isolate AA-71 while all temperatures were found to be optimal for isolate AA-11.

The effect of temperature on the pH of the final culture of isolates AA-2 and AA-82 was indirect. Whenever the growth rate was high, the pH level was found to be low (Figure 5). This is possibly due to the fact that the bacterium was utilizing the carbohydrate substrate found in the medium as most of *Bacillus* species are able to decompose hexose sugars and produce acid.

The approach of using LC_{50} based on dry biomass to evaluate bacterial toxicity has been used previously (Myers & Yousten, 1981; Tryell *et al.*,1979). The result obtained during the present study is quite promising. The LC_{50} in all assays ranged from 43-170 µg/ml.

Comparing the result of this study with that obtained by Tryell *et al.* (1979) and Abdel-Hameed *et al.* (1990a) (370mg/ml and 957mg/ml respectively), one could see that the present isolates (AA-2, AA-11, AA-71

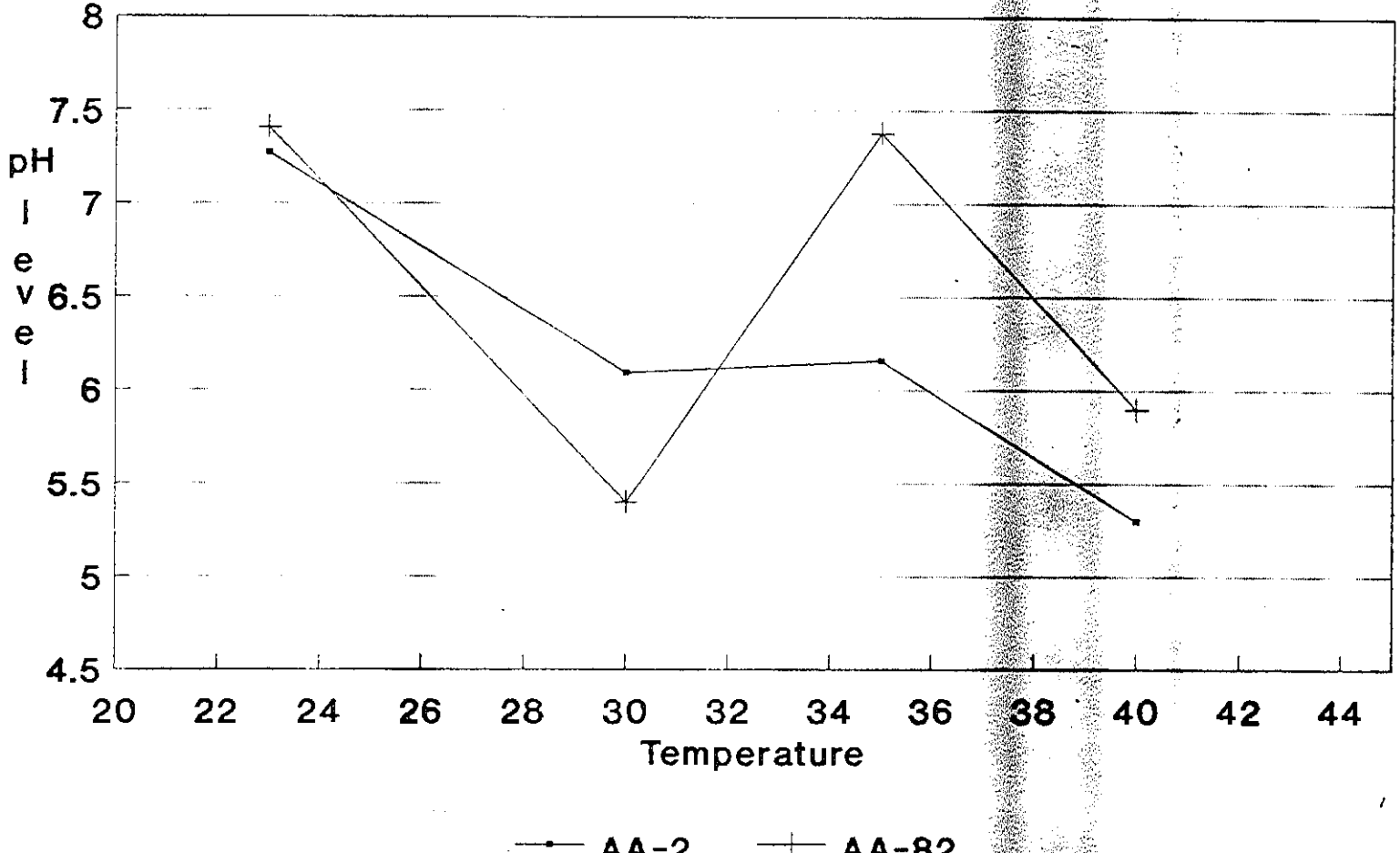

Figure 5. Effect of temperature on pH levels of the cultures of isolates

and AA-82) are quite promising although the media used were different.

In the present study, apparently, a difference that ranged between 5-100 micrograms of LC_{50} of different isolates on different larval stages of culicine mosquitoes was seen. Statistically (Table 10), there were no significant differences found among different larva stages at a significant level of 1%, 5%, and 10%. this finding goes in line with the result of (Singer) cited in (Wraight *et al.*, 1981) and with that of Mulligan *et al.*(1978). Isolate AA-11 has shown significantly different toxicity levels on 8-days larval stage at a significant level of 5% and on 11-days larval stage at a significant level of 1%. This result agrees with that of Wraight *et al.*(1981) and that of Ramoska *et al.*(1977). Accordingly, it could be said that different culicine larvae have shown no difference in susceptibility to isolates AA-2, AA-71 and AA-82, whereas a decrease in susceptibility by age was seen in the culicine larvae upon ingestion of isolate AA-11. From this result it has become clear that isolate AA-2, AA-71 and AA-82 could be used to control both early and late larval stages of mosquitoes. This feature, from cost-effectiveness point of view, is advantageous over isolate AA-11 which could be used effectively only on early larval stages.

In the attempt to evaluate the efficacy of the isolated strains under natural conditions, cultures of strains were applied into stagnant water. Although the

condition under which the strains were applied is different from those in the laboratory as the stagnant water contains different fauna, mud, organic and inorganic matters in addition to the difference in pH, strains (specially AA-82) have shown promising results (Table 11). This result provides a preliminary information on the possible use of the larvicidal *Bacillus* spp isolated in this work for practical mosquito control.

5. CONCLUSIONS AND RECOMMENDATIONS

The screening for effective bioinsecticides has resulted in four promising strains of *Bacillus* spp.

Strain AA-82 is very similar to *Bacillus thuringiensis*. The strain has shown killing ability under natural condition (natural stagnant water). In addition, the isolate is effective both for early and late larval stage of mosquitoes. This strain can be a promising bioinsecticidal candidate.

Since isolate AA-71 is effective at natural lower temperatures (20-23^{0}C), it could be utilized in relatively cold areas. Isolate AA-2 on the other hand has high temperature optima (up to 40^{0}C). The strain may be useful under various temperature regimes.

It is recommended that optimal nutritional and physical conditions for better production of toxic principles be investigated further .

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