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Characterization of Locally Isolated Strains of *Bacillus* and Their Evaluation as Potential Biocides Against House Fly, *Musca domestica*

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Abstract.- Soil samples (n=500) collected from different areas of Pakistan were screened for various species of *Bacillus* by Gram staining, spore staining and by a number of biochemical tests. Seven identified species *i.e. B. thuringiensis, B. sphaericus, B. brevis, B. firmus, B. coagulans, B. stearothermophilus* and *B. megaterium* were then administered to the house flies, *Musca domestica* to ascertain and evaluate their toxic effects. *B. thuringiensis* and *B. sphaericus* were found to be the most toxic isolates against houseflies and were then selected to study their growth conditions with specific objective of enhancing their toxin production.

Key words: *Bacillus*, bioinsecticide, endotoxin protein, house fly.

INTRODUCTION

The chemical insecticides are being extensively used in agricultural countries like Pakistan. The residues of these toxic compounds persist in the environment and food stuffs, which not only contaminate the environment but also result in the development of resistance against these chemicals in the target organisms (Chatterjee *et al*., 1986; Hoellinger *et al*., 1987; Olcay and Keriman, 1987; Hardy, 1987; Miellet, 1988; Van Den Bercken and Henk, 1988; Sharma and Agarwal, 1988; Kawano *et al*., 1988; Zahida and Masud, 1988). The three main groups of insecticides *i.e*., organo-phosphorus (OP), organochlorinated (OC) and pyrethroids, have proven to be toxic, both to target as well as non-target organisms (Edwards *et al*., 1987; Khillare and Waghs, 1988; Reddy and Bashamohideen, 1989; Shakoori *at al*., 1990). According to US World Resource Institute and International Federation Control Meeting held in Brussels, pesticides cause about 400,000 illnesses and 20,000 deaths every year (Bhatti *et al*., 1993).

Inspite of the deleterious effects of all these insecticides, tons of these insecticides are being poured into the field every year as these are the easiest short cut to increase agricultural production. Keeping in view the hazards of chemical compounds attention have been focussed on the development of alternate and relatively safe means to control harmful insects. One of the strategies involves the use of micro-organisms, especially bacteria, against insects (Mittal *et al*., 1993; Pietrantonio and Gill, 1992; Orduz *et al*., 1992). Among microbial biological control, bacteria belonging to Genus *Bacillus* have been implicated as potential insecticide (Lambert and Peferoen, 1992; Priest, 1992; Vadlamudi *et al*., 1993; Harcourt *et al*., 1996) because endotoxin proteins of different species of *Bacillus* have several advantages over the prevalent chemical insecticide, regarding hazard to human beings and non-target organisms (Bauer and Gameron, 1995).

Various *Bacillus* species, particularly *Bacillus thuringiensis* (*B.t*.) are being used for the biological control of a great variety of insects like mosquitoes (Rosso and Delecluse, 1997), *Helicoverpa zea, Spodoptera frugiperda, Diatraea graridiosella* and *Diatraea saccharalis* (Bohorova *et al*., 1996), *Glossina morsitans* (Omolo *et al*., 1997), etc. Recent efforts suggest the use of ICP of *B.t.* in combination with endochitinase (Regev *et al*., 1996) or with insecticide, pyrethroid (Migranov and Poskryakov, 1996) to increase its insecticidal effect.

These days, a wide range of bacteria-based products especially of *B.t.* (Sundaram *et al*., 1994; Hou, 1997) and *B. sphaericus* (Mittal *et al*., 1993) are in use for the control of most of the agricultural pests. No comprehensive effort has been made in Pakistan to look for microorganisms with biocidal activity. This is an attempt to look for different species of *Bacillus* with their potential biocidal

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activity against house flies, *Musca domestica*.

MATERIALS AND METHODS

Isolation and identification of different species of Bacillus

Soil samples were collected from different parts of Pakistan in sterilized bottles. These samples were suspended in normal saline, serially diluted, and aliquots transferred to Luria Bertani (LB) agar plates (prepared by dissolving 10 g NaCl, 10 g tryptone, 5ÿg yeast extract and 15 g agar in 1 L distilled water). The plates were incubated at 37°C for 24 hours. The isolates were then characterized by Gram staining, spore staining, and a number of biochemical tests, *i.e.* catalase test, Voges-Proskauer test, motility test, test for acid production from glucose, xylose, mannitol and arabinose, nitrate reduction test, indole test, tyrosine decomposition test, citrate utilization test, hydrolysis of casein and starch, growth at Sabouraud dextrose agar, phenylalanine deamination test, growth at 65°C, growth in 7% NaCl and 0.001% lysozyme, production of intracellular protein crystal, as recommended in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The details of all these tests are given in Benson (1994) and Collee *et al.* (1989).

Characterization of isolated strains of Bacillus

Different isolated species of *Bacillus* were further characterized by their interaction with antibodies raised against them in domesticated rabbits. Formation of precipitate helped in identifying different strains of bacterial isolates. For this purpose bacterial isolates were cultured in 20 ml LB Broth medium (prepared by dissolving 0.2 g NaCl, 0.1 g tryptone and 0.05 g yeast extract in 20 ml distilled water) in a conical flask for 24 h at 37°C. The culture was centrifuged at 6000 rpm, 4°C for 10 min. The cell pellet was resuspended in 10 ml sterile saline solution (0.85%). To break clumps of bacterial pellet, the suspension was shaken with sterile beads (n=70; diameter=2 mm) for 15 min on Gyromixer. The suspension was then filtered through Whatman filter paper (pore size, 0.22 µm). The filtered suspension (0.5 ml) was inoculated in 10 ml of LB medium or thioglycollate medium and incubated for 48 hours at 37°C. If no turbidity developed in the test medium, then the cells were ready to be injected in the rabbit. Each rabbit was given i.v. a dose of 0.1 ml ($1x10^8$ cells), followed by a second dose of 0.2 ml ($1x10^9$ cells) after an interval of one week. A control experiment was also run simultaneously in which water was injected i.v. After 14 days of last injection, the rabbit was bled and blood serum was separated as antisera. The antisera were tested for the formation of precipitates against the specific bacterial isolates, against which antibodies were raised.

Toxicity of Bacillus *sp. against houseflies*

Large scale growth of bacterial isolates

Each bacterial isolate was grown in glass jar fermenter (Eyde, Tokyo) for large scale production. Pure bacterial culture inoculum was added in 1500 ml autoclaved medium (glucose 1%, peptone 0.5%, potassium dihydrogen phosphate 1%, magnesium sulphate .025%, beef extract 0.25%, yeast extract 0.1% dissolved in 100 ml distilled water, pH 7-7.3) in a sterilized fermenter and allowed to incubate with shaking at 37°C for 48 hours. The bacterial culture was centrifuged at 4,000 rpm for 15 minutes at 4°C and the pellet dried at 37°C. The dried pellet was used for toxicity studies on housefly.

Preparation of house fly culture

A master culture of houseflies was prepared by taking male and female flies in 1:2 ratio in a glass jar containing diet (prepared by dissolving 0.5 g yeast powder, 100 g flour, one big spoon of molasses, 3 g of sodium benzoate, 0.2 g agar in 400 ml distilled water). After achieving 2 or 3 generations, 10 male and 20 female flies were introduced in wide mouthed sterilized glass bottles with cotton soaked in sugared milk at the bottom and sterilized tissue paper at the top. Eggs were laid by the flies in the furrows, grooves and corners of the tissue paper.

Procedure adopted

Fifty house fly eggs from above culture bottles were transferred in each of two sets, control and treated, comprising of 3 sterilized glass bottles each with 70 g diet in slant forms. In experimental jars 2.5 ml of 20% bacterial suspension (prepared in autoclaved distilled water) was uniformly mixed, whereas the control jars were without bacterial

mass. The total number of adult flies emerged in each bottle at 26±3°C after 10 days, were counted to ascertain the mortality of flies due to bacilli. To test the bacterial toxicity, the dead larvae were homogenized in autoclaved distilled water and streaked on LB agar medium. The plates were incubated at 37°C for 24 hours. The bacterial colonies were examined under the microscope and characterized biochemically.

Determination of optimum growth conditions

Bacterial strains showing most significant toxicity against houseflies were selected for determination of optimum growth conditions.

For determination of optimum temperature, 4 sets each of 3 test tubes were used for this study. In each tube 5 ml of LB medium was inoculated with 100 µl of log phase growing bacterial cells and incubated at 20, 30, 37 and 40°C for overnight. The bacterial growth was assessed by measuring absorbance at 600 nm, and by determining cell count with the help of haemocytometer.

For ascertaining optimum pH, LB liquid medium was adjusted at various pH viz. 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 in 3 sets of test tubes. The cultures were inoculated with log phase growing bacterial cells (100 µl) and incubated at 37°C for 10 hours. The cells were counted and optical density (O.D) of culture was taken at 600nm.

The optimum inoculum size was determined by inoculating LB liquid medium (6 ml) in different sets of glass tubes with 5% (300 µl), 10% (600 µl), 20% (1200 µl) and 40% (2400 µl) log phase bacterial culture in shaking water bath at 37°C. After 10 hours, OD was taken at 600 nm on UV spectrophotometer and bacterial cells were counted by haemocytometer counting chamber.

Determination of growth curve

For preparation of growth curves 24 test tubes for each strain were prepared with LB broth medium and inoculated with (10%) inoculum of log phase bacterial culture. The tubes were incubated in shaking water bath at 37°C. Every hour the tubes were taken out, cells were counted and O.D. recorded at 600ÿnm. Growth curves were prepared by plotting graph between time of incubation and O.D., and time of incubation and cell count.

RESULTS

Out of 500 soil samples collected from all over the Pakistan, 7 species of *Bacillus* were identified *i.e*., *B. megaterium, B. coagulans, B. firmus, B. thuringiensis, B. sphaericus, B. brevis, B. stearothermophilus.* For confirmation of various strains of *Bacillus* spp, the antisera raised against these bacterial isolates in rabbits, were used for bacterial precipitation. Result showed that there was only one strain of *B. firmus, B. stearothermophilus* and *B. sphaericus* because all these strains showed positive reaction with their respective antisera. The other four bacterial isolates *B. brevis, B. megaterium, B. thuringiensis* and *B. coagulans* showed two strains each, as some of these isolates did not give positive reaction with the antisera.

Toxicity of bacterial isolates

The isolated strains of *Bacillus* were used in toxicity experiment against house flies, *Musca domestic*. Among 7 isolates of *Bacillus, B.t.* and *B. sphaericus* were found to be the most toxic isolates, causing, respectively, 34% and 33% mortality of the houseflies. The houseflies were also found to be susceptible to *B. megaterium, B. firmus* and *B. brevis* which showed 30%, 27% and 26% toxicity, respectively (Table I). The dead larvae were then examined for the presence of bacteria, which these insects were fed on. All the dead larval had the specific bacteria.

Growth conditions and growth curve

Bacillus species *i.e., B.t.* and *B. sphaericus* showing maximum toxicity against domestic house flies were selected to study their optimum growth conditions just to enhance the production of toxic compound in these isolates. To make the comparison between two different strains of *B.t.*, both strains were selected to study the growth conditions. For all these strains the optimum pH was 7 and optimum temperature was 37°C. The inoculum size of 10% of the volume (for *B.t.* 1 and *B. sphaericus*) and 20% of the volume (for *B.t.* 2) was found to be the most optimum for bacterial growth (Fig. 1).

Fig. 2 shows growth curves of all the three bacterial isolates. These are typical growth curves.

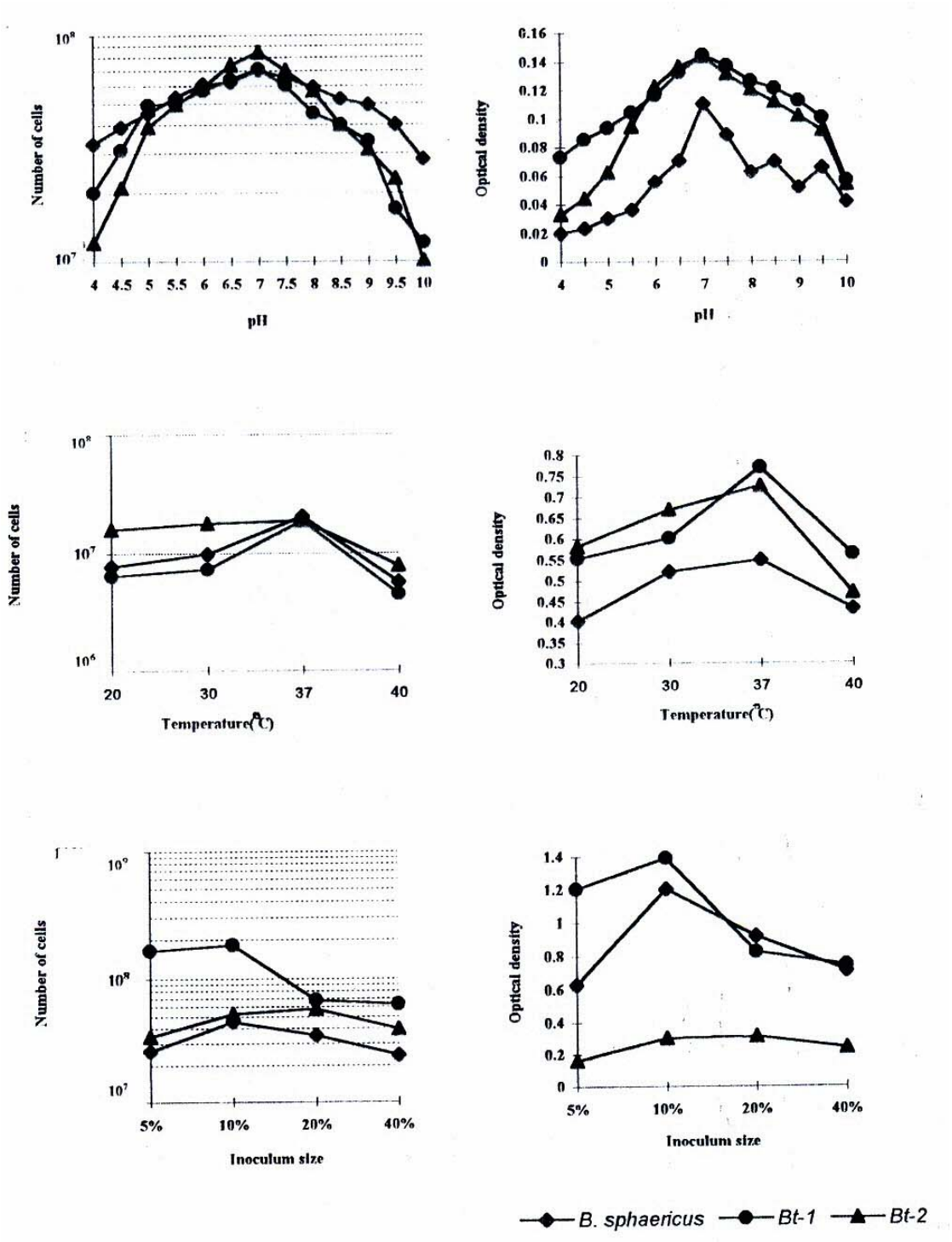

Fig. 1. Optimum growth conditions of isolated strains of *Bacillus*. The figure shows effect of pH (top panel), temperature (middle panel) and inoculum size (botton panel) on the growth of *B. sphaericus* (□), *B.t.1* (•) and *B.t. 2* (▲), which is represented in terms of optical density (O.D.) as well as number of cells.

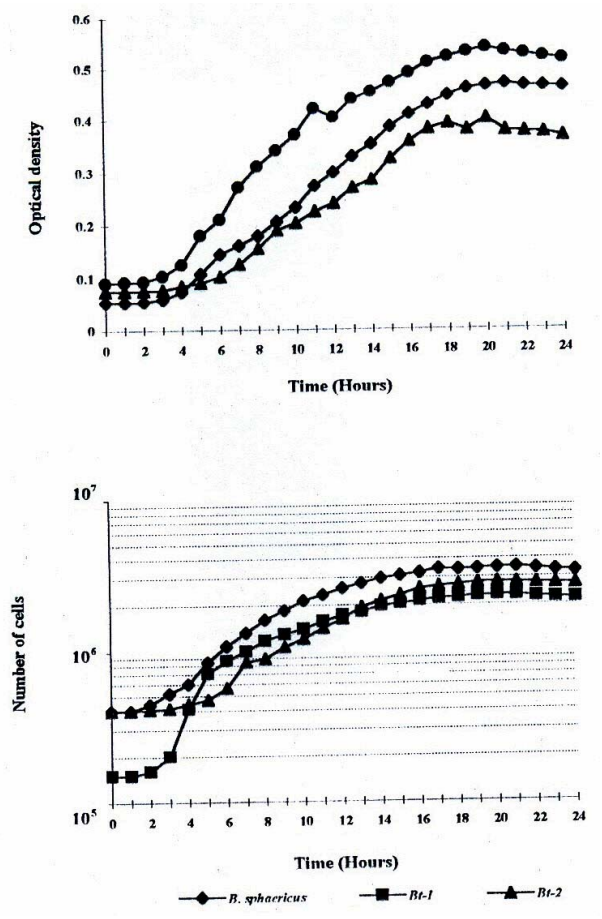

Fig. 2. Growth curve of isolated strains of *Bacillus. B. sphaericus* (□), *B.t.1* (•) and B.t.2 (▲).

Table I.- Percentage mortality after exposure of houseflies, *Musca domestica*, eggs in a feed containing bacterial pellets

Name of organisms	Control (n=150)	Treated (n=150)
Bacillus sphaericus	4±0.6	32.6±0.88**
Bacillus thuringiensis	5.3±0.88	34±1.16**
Bacillus firmus	7.6±0.88	27.3±1.45**
Bacillus brevis	5.3±0.88	26.3±1.76**
Bacillus megaterium	4±0.58	29.6±2.03**
Bacillus coagulans	4.3±0.67	16±2.08
Bacillus stearothermophilus	6.6±0.67	26±2.35*

Mean±SEM; Student's `t' test; * P<0.05, ** P<0.01.

DISCUSSION

Due to harmful effects of insecticides, there is considerable interest in the use of biological pesticides to control a wide range of pest. Several products based on varieties of *B.t.* are preferred over the chemicals for the control of agricultural and forestry pests and also insect vectors of disease (Watkinson, 1994). *B.t.* is gram positive bacterium, the biocidal activity of which mainly resides in a parasporal protein inclusion body or crystal (Prieto-Samsonov *et al*., 1997). These insecticidal crystal proteins synthesized by the *Bacillus* are the active ingredient of various environmental-friendly insecticides that are (i) highly compatible with natural enemies and other non-target organisms due to narrow host specificity, (ii) harmless to vertebrates, (iii) biodegradable in the environment and (iv) highly amenable to genetic engineering.

B.t. is used as bioinsecticide against a wide spectrum of insects. Various strains of diamond moth, *Plutella xylostella* were found to be susceptible to *B.t.* products (Hou, 1997). Similarly Colorado beetle, *Spodoptera litura* (Grigor-Eva *et al*., 1994; Asano and Hori, 1995), potato tuber moth (Escriche *et al*., 1994), *Culex quinquefasciatus* (Smith *et al*., 1995) etc. were also sensitive to *B.t.* There is a need to look for more strains of *Bacillus* that may be highly toxic and can be used as bioinsecticide.

Present study shows the toxicity of *Bacillus* species against houseflies *i.e. Musca domestica*, *Bacillus thuringiensis* (34%) and *B. sphaericus* (32.6%) are found to be the most toxic. But in addition to *B.t.* and *B. sphaericus*, other strains also show significant results *i.e., B. megaterium* (29.6%), *B. firmus* (27.3%) and *B. brevis* (26.3%). Grigor-Eva *et al.* (1994) and Kuznetsova *et al.* (1995) described the toxicity of endotoxin protein of *B.t.* against domestic fly (Diptera). Hodgman *et al.* (1993) discovered *B.t.* isolate which was toxic to the common housefly (*Musca domestica*). Crystal delta endotoxin purified from this isolate killed 50% of *Musca* larvae at concentration of 10.2 µg/ml, while no beta-endotoxin was detected.

All *Bacillus* strains were isolated from soil samples. The low frequency of isolates is due to the

fact that dust from mills and soils as well as insects from nature were more successful sources of *Bacillus* than soil samples (Chaujaux *et al*., 1997). This emphasizes the diversity of biotopes where the Bacillus is encountered.

Bhattacharya (1993) investigated the correlation, if any, between sporulation and the production of parasporal insecticidal crystal protein (delta-endotoxin) in *B.t. var israelensis*. He found that toxicity was exhibited in both cases either the experiment was performed only with asporogenous strains or with acrystalliferous asporogenous strains.

Toxicity against houseflies, in the present study was tested by introducing bacterial isolates in the artificial diet of houseflies. Robacker *et al*. (1996) also used the same method of exposing insects to *B.t.* pellets. They used pellets of *B.t.* against adult Mexican fruit fly and observed 65-80% mortality.

Briefly, several factors make the local production of *Bacillus* highly appropriate for pest control in developing countries. *Bacillus* can be cheaply produced on a wide variety of low cost, organic substrates.

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