WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 7, 295-302. Conference Article ISSN 2277– 7105

A PRELIMINARY STUDY ON MOSQUITOCIDAL ACTIVITY AND EXTRACELLULAR PROTEASE PRODUCTION BY BACTERIAL ISOLATES FROM SOIL

Sumitha J.[1]* and Akila M. P.[2]

[1]Assistant Professor, Department of Microbiology, JBAS College for Women, Teynampet, Chennai-600 018, Tamil Nadu, India.

[2]Vector Control Research Centre, Pondicherry, Tamilnadu, India.

Article Received on
12 Feb. 2018,

Revised on 05 March 2018,
Accepted on 26 March 2018

DOI: 10.20959/wjpr20187-11617

***Corresponding Author**
Sumitha J.
Assistant Professor, Department of Microbiology, JBAS College for Women, Teynampet, Chennai-600 018, Tamil Nadu, India.

ABSTRACT

The insects have traditionally been fought with chemical pesticides with their disadvantages like long term toxicity, environmental pollution and food chain accumulation. Their lethal effects also fairly non-specific are killing beneficial predators that naturally control the pest population. Bio-insecticides were formulated to overcome the disadvantages of chemical pesticides. Biological control is one of the main components of IPM (Indian Pest Management) and there has been greater awareness to utilize the insect pathogens in IPM systems in recent years. Though commercial formulations are not used widely at present in India, studies carried out in various parts of our country reveal their potential use. But both in bacterial isolates from soil and in their mosquitocidal activity not too many studies have been carried out and there is a need to take up systematic surveys to identify naturally occurring insects' pathogens under varied agro-ecological conditions. Proteolytic enzymes are ubiquitous in microorganisms and the extracellular proteases are probably the most widespread of all microbial secreted enzymes. These enzymes are simple to detect and are often synthesized in high yield. They have therefore been extensively studied and their molecular properties are understood in detail. Microbial proteases are produced from high yielding strains by fermentations under controlled conditions in surface or submerged culture. The enzymes are produced extracellularly and recovery involves separation of the cell free liquor by filtration or centrifugation. This study has tried to exploit the diverse potential of bacteria isolated from soil sample by studying its mosquitocidal activity by determining the LC_{50} and LC_{90} of the potential isolates against larva

of *Culex quinquefasciatus* as well as studying the extracellular protease production by them. Out of 48 bacteria isolated from soil samples, only one showed remarkable larvicidal activity, marked as BII. And among 5 bacteria isolates tested for proteases production all showed proteases production with one species (BI) showing highest production.

KEYWORDS: Proteases, Culex Quinquefasciatus, Mosquiticidal Property.

1. INTRODUCTION

The insects have traditionally been fought with chemicals pesticides with their disadvantages like long term toxicity, environmental pollution and food chain accumulation. Their lethal effects also fairly nonspecific, killing beneficial predators, that naturally control the pest population. Bio-insecticides were formulated to overcome the disadvantages of chemical pesticides.

Biological control is one of the main components of IPM (Indian Pest Management) and their greater awareness to utilize the insect pathogens in IPM system in recent years. Though commercial formulations are not used widely at present in India, studies carried out in various parts of the country reveal their potential use. There have been very few studies on 'bacterial isolates from soil: and their mosquitocidal activity' and there is a need to take up systematic surveys to identify naturally occurring insect pathogens under varied agro-ecological conditions.

Most of the bacterial agents used for insect control are spore forming, rod shaped in the genus Bacillus. They occur commonly in soils and most insecticidal strains have been isolated in soil samples. Among the available control agents for mosquitoes, *Bacillus thuringiensis* var *isrealensis*, *Bacillus sphearicus* have shown great promise. They are highly toxic to mosquito larvae.

Out of more than 2000 different enzymes that have been described in the literature (enzyme Nomenclature, 1979), only a few have as many conceivable biotechnological applications as proteolytic enzymes. Proteolytic enzymes account for nearly 60% of the industrial enzyme marker. Proteases of commercial importance are produced from microbial, animal and plant sources.

Proteolytic enzymes are ubiquitous in microorganism and the extracellular proteases are probably the most widespread of all microbial, secreted enzymes. These enzymes are simple

to detect and are often synthesised in high yield. They have therefore been extensively studied and their molecular properties are understood in detail. Microbial proteases are grouped into three classes, serine, metallo and acid proteases which have alkaline neutral and acid pH optima, respectively. Serine and metallo proteases are produced commercially from Bacillus strain while the acid proteases are prepared from fungi, notably Mucor strain.

Microbial proteases are produced from high yielding strains by fermentation under controlled conditions in surface or submerged culture. The enzymes are produced extracellular and recovery involves separation of the cell free liquor by filtration or centrifugation. Depending on the product and the degree of purity required, further purification of might involves steps such as concentration, precipitation and stabilization.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria from Soil Sample

3.1.1 Media Preparation

Nutrient agar medium was prepared and steerlised by autoclaving.After sterilisation, 15-20ml of the medium was poured into sterile petri dishes so as to get a uniform thickness of 4-5mm.

3.1.2 Collection of Soil Sample and Treatment

A total of five soil samples were collected viz.,S1,S2,S3,S4 and S5 from various places in Villupuram, Tamilnadu, India and used for isolation and preliminary characterisation.

2.1.3 Primary Treatment of the Soil Sample

Each soil sample (1g) was emptied in a tube containing 10ml of sterile distilled water.The tubes were then placed in the shaker for 1hr ,from each tube, 0.5ml of the supernatant was transferred to a tube containing 4.5ml of sterile distilled water and serially diluted.

From each of these dilutions, 0.1ml was taken and spread plate was done.The inoculated plates incubated and examined for any growth after 24hours & 48 hours.The isolated colonies were then picked up and transferred to nutrient agar slants.

The slants were then incubated and stored at 40c for further use.

2.2 Testing of Bacteria Isolated from Soil Samples

A loopful of culture taken from nutrient agar slants and were inoculated into 10ml sterile NYSM (Nutrient Yeast Salt Medium) broth in conical flask and kept in a shaker for 72hours

to ensure proper aeration.after incubation, the cultures were tested for activity by performing bioassay using third install larvae of *Culex.quinquefasciatus.*

Disposable paper cups containing 100ml of water for this each of 10ml Culex larva were introduced into disposable paper cups.Then 1ml of the whole culture from the NYSM broth was added to this.The mortality was assayed by counting the number of live larvae after 24hour of exposure.

2.3 Determination of lc50 and lc90 of Potential Isolate Against Larve of culex. Quinquefasciatus

A loopful culture (B II) taken from nutrient agar slants was inoculated into 10ml NYSM broth in a conical flask and kept in a shaker at 370c for 6 hrs.After incubation, the cultures were transferred to a fresh 250ml NYSM broth and kept in a shaker for 48hrs at the same temperature.The whole culture was obtained like this against third install of *Culex.quinquefasciatus* as the Standard method (WHO) for bioassay. Gram Stain and spore stain preparations were made and examined under microscope.

2.4 For Bioassay

250ml of chlorine free tap water was taken in disposable paper cups 50 third install larvae were introduced.different concentrations of the whole culture ranging from 50 µl to 800 µl were added to the bioassay cups.After 24 hr of exposure the live larvee were counted and mortality was determined using counted with control mortality with Abbots Formula and LC50 and LC 90 were calculated by Probit regression Analysis.

2.5 Protease Assay

The bacterial isolate from the soil samples, five were randomly selected and used for production of protease.Four isolates tested belongs to Bacillus species and one was gram negative bacterium.All the bacterial isolates were inoculated into NYSM broth and incubated at 300c,250rpm for 72hrs.After the incubation period the cultures were centrifuged at 8000 rpm, for 20 min at 40c.The culture filtrates were used for the enzyme assay using the method Li and Yousten (1975).

2.6 Estimation of Protein

The protein concentration of the culture filtrate at 68th hr of growth was measured because protease production was high at this particular point of growth.Protein was estimated by BCA

method using Genei Kit.Protein assay based on bicinchoninic acid is a most sensitive and detergent compatible method for the colorimetric detection and quantitation of total proteins.The BCA working Reagent(BWR)was prepared and was stable in a closed container at room temperature for a day.The test was done at a sample to BWR ratio (1:10).The tubes were incubated at 370c for 30 minutes.After the incubation the tubes were cooled to room temperature and the absorb was measured at 562nm.

3. RESULT AND DISCUSSION

3.1 Isolation of Bacteria from Soil samples

48 bacterial isolates were obtained from 5 soil samples. These isolates were screened for mosquitocidal activity and extracellular protease (enzyme) production.

3.2 Testing of Bacteria Isolated from Soil samples

Out of 48 bacteria isolated from the soil samples only 1 showed remarkable larvicidal activity. This bacterial isolate was designated as (BII) and used for further experiments.

3.3 Determination of LC50 & LC90 of Potential Bacterial Isolate against Larvae of *Culex. Quinquefastiacus:* The bacterial isolate designated as (BII) was identified as a gram positive, aerobic spore forming bacteria of the Genus Bacillus. It forms terminal bulging spore. Table 1 shows the percentage mortality at different concentrations and LC 50 and LC 90 values. The dose requirements of the culture filtrate to kill 50% of larvae was 479 µl. This dose may be reduced if the cells are separated by centrifugation. Activity of this bacterial culture against other species and stages of mosquitoes are to be studied.

Table. 1: LC 50 and LC 90 for the Larvicidal Bacillus sp when tested against the Third instar larvae of Cx. *Quinquefasciatus.*

Dose (in µl)	Percentage mortality	LC 50	LC 90
50	5	479 **µl**	3931 **µl**
100	20		
200	36		
400	45		
600	48		
800	60		
1000	72		

3.4 Protease Assay of the Culture Filtrate of the Bacterial Isolates: Among the 5 bacteria isolates tested for protease production, two Bacillus species (BII & BV) found higher protease production (2700 & 2440 units/dl) than the other two Bacillus species (BIII & BIV)

(2420 & 220 units/dl). Highest enzyme production was observed with the (BI) gram negative bacteria (3340 units/dl).

Table. 2: Protease production for 5 randomly selected bacterial isolates.

Samples	Protease production (units/dl)
BI	3340
BII	2700
BIII	2420
BIV	220
BV	2440

3.5 Extra cellular proteolytic enzyme secretion in different hours by bacteria isolate

Extra cellular protease production by the bacterium was started from 20th (880 units/dl) hour of incubation and showed an increasing trend till 24 hours (1960 units/dl). Then it showed decreasing trend till 48 hours (1600 units/dl). However, there was a sudden increase in the enzyme production at 52nd hour. Following this the production was again reduced to 1420 units/dl at 64th hour but reached a range of maximum levels of 2200-2400 units/dl around the 68th hour of growth. However the protease activity was found to fall sharply thereafter.

Table. 3: Extracellular proteolytic enzyme secretion in different hour by bacteria isolate.

Hours	Protease Production (units/dl)
4	Negative value
8	460
12	Negative value
16	380
20	880
24	1960
28	940
32	1360
36	960
40	1060
44	1600
48	1600
52	2240
56	1900
60	1740
64	1420
68	2440
72	1940

3.6 Estimation of Protein

The amount of protein present in the culture filtrate of the gram negative bacterium was found to be 1476 µg/ml.

4. CONCLUSION

There is evidence for development of resistance to any bacterial toxin, as soon as its mode of action implies only one toxin, or toxins with identical mode of action (binding on the same receptor). This microbial insecticide has therefore to be used in a reasonable way in integrated control program. Monitoring of the susceptibility of the treated mosquito populations before and during treatments is necessary. Other measures to be taken are to multiply the control methods and/or the insecticides. Th identification of toxin, may give good tools to identify other mechanisms of resistance, in order to predict and reduce resistance.

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