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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF NATIVE ISOLATES OF *BACILLUS THURINGIENSIS* FROM SOIL

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ABSTRACT

The present investigation was for the isolation and characterization of *B. thuringiensis* strains from different habitats based on the morphology, staining properties and biochemical characters. A total of 20 soil samples were collected from different localities across the Nanded region to isolate native *Bacillus thuringiensis* (BT) strains. Sodium acetate selection heat-pasteurization treatment methods were used for *B thuringiensis* isolation. Identification of isolates was accomplished on the basis of morphological characteristics of colonies and various biochemical tests like oxidase, catalase test, starch hydrolysis, aesculine hydrolysis, levan production, arginine hydrolysis, casein hydrolysis, gelatine liquafication, acid production from carbohydrate, VP test, nitrate reduction test etc**.** These spores were cultured and further characterized through colony shape and colour.

Key words: *Bacillus thuringiensis,* Isolation, Biochemical.

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INTRODUCTION

The competition for crops between human and insects is as old as agriculture. The use of chemical substances to control pests was started in the mid-18 century. Insecticides were some inorganic and organic compounds. Many of these chemicals are also being used today. Certain properties made these chemicals useful, such as long residual action and toxicity to a wide spectrum of organisms. However, chemical pesticide applications have caused many environmental problems including insect resistance, toxicity to humans (Gillis, 2008). Biocontrol is the better option than chemical pesticides in order to control insect pests in insect management. *Bacillus thuringiensis* (Bt) is a naturally occurring bacterium, commonly found in soils, that has the ability to infect and kill certain insects. Because of this property, *Bacillus thuringiensis* has been developed for insect control, using the bacteria as the active ingredient in some insecticides. *Bacillus thuringiensis* is a biological pathogen or biological control agent marketed as a microbial insecticide (Randhawa *et al.,* 2009). *B. thuringiensis* is a member of the genus *Bacillus* and like the other members of the taxon has the ability to form endospores that are resistant to inactivation by heat, desiccation and organic solvents. The spore formation of the organism varies from terminal to sub terminal in sporangia that are not swollen, therefore, *B. thuringiensis* resembles other *Bacillus* species in morphology and shape (Ramalakshmi and Udayasuriyan 2010, Martin 2010).

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The organism is a gram-positive and facultative anaerobe. The shape of the cells of the organism is rod. The width of the rod varies 3-5 µm in size when grown in standard liquid media. *Bacillus thuringiensis* is a major microorganism, which shows entamo pathogenic activity. The organism *Bacillus thuringiensis* is a ubiquitous, gram-positive and spore-forming bacterium that forms parasporal crystals (delta-endotoxin) during the stationary phase of its growth cycle. Its insecticidal activity depends on parasporal crystals encoded by *cry* genes and this insecticidal activity varies according to insect type. Natural isolates of *B. thuringiensis* have been used as a biological pesticide since the 1950 century for the control of certain insect species among the orders Lepidoptera, Coloeptera and Diptera. The genes of *B. thuringiensis* coding parasporal crystals are also a key source for transgenic expression which provides pest resistance in plants. This feature makes *B. thuringiensis* the most important biopesticide on the world market. In 1995, worldwide sales of *B. thuringiensis* based insecticides were estimated at $90 million representing about 2% of the total global insecticide market. The initial purpose of this research was the collection of *B. thuringiensis* strain from Nanded region.

MATERIALS AND METHODS

Sample collection

Twenty Soil samples were collected from different regions of Nanded i.e. field soil samples from Limbgaon, Maralak, Kavtha, Ardhapur, taroda, Hadco, Vishnupuri, Yeshwant

college botanical garden, Science college botanical garden and MGM's college botanical garden. Soil samples were taken 2-5cm below the surface and were stored in sterilized plastic bag at 4°C.

Isolation of *B. thuringiensis* from the sample

The sodium acetate/ heat treatment method was applied to isolate *B.thuringiensis* from samples. Approximately, 0.5gm of each soil sample was suspended in test tubes containing 10 ml LB broth with concentration of sodium acetate 0.25M. Next, suspensions were vortexed vigorously and incubated overnight at 37 °C in a shaking incubator. Afterwards, the samples were heated for 10 minute at 80 °C in order to kill vegetative bacterial cells and to eliminate non-spore forming bacterial cells. Following heat treatment, the samples were placed on LB agar plates, which were incubated overnight at 35 °C. (Randhawa *et al* 2009). Finally, bacterial colonies were separated by their colony morphology. The colonies, which showed *B. thuringiensis*-like colony morphology were rough, white and spread out over the plate.

Characterization based on staining

The Gram's stain test: A very small inoculum of bacteria was smeared onto a clean slide using an inoculation loop. The sample was diluted with a drop of sterile water and allowed to air dry. The specimen was heat-fixed by passing the slide through an open flame. The slide was stained with crystal violet for 1 minute and rinsed with sterile water. The slides were then stained with Gram's iodine (1% iodine, 2% potassium iodide in water) for 1 minute to fix the dye and then rinsed with sterile water. Excess stain was removed with 95% ethanol and then rinsed with sterile water. Specimens were counterstained with Safranin for 1 minute, rinsed with water and then air dried. Slides were viewed using light microscopy under oil immersion (Provine & Gardner, 1974; Bergey's Manual of Systematic Bacteriology, 1986).

Endospore stain (Schaeffer-Fulton staining method)

A small inoculums of bacteria was smeared onto a clean slide using an inoculation loop and diluted with a drop of sterile water. Once dry the slides were flooded with Malachite green (made by dissolving 5.0g in distilled water, made up to 100ml) and immediately steamed over a water bath for 5 minutes. Once cooled the slides were rinsed with sterile water. The slides were then counterstained with Safranin O (made by dissolving 0.5 grams Safranin O powder in distilled water, made up to 100ml) for 2 minutes and then rinsed with sterile water. Once the slides had dried, the specimens were viewed under a compound microscope with oil immersion (Mormak & Casida, 1985; Bergey's Manual of Systematic Bacteriology, 1986).

Biochemical characterization of *Bacillus thuringiensis*

Catalase test: The test involved adding hydrogen peroxide to each sample of bacteria. A small sample of 48 hour cultures was smeared onto a clean slide. A drop of 10% hydrogen peroxide was alloquoted onto the bacterial smear and observed using light microscopy. The presence of bubbles indicated the ability to break down hydrogen peroxide into water and oxygen (Bergey's Manual of Systematic Bacteriology, 1986); (Reagents and Tests, in Bailey & Scott's Diagnostic Microbiology, 1978).

Oxidase test: The bacterial suspension is prepared in the saline and one drop of suspension is put in the oxidase is which contain NNN'N – treatment P–phenylene amine dihydro chloride. The color changes and if takes longer upto 60 seconds it is weakly positive.

Aesculin hydrolysis: Aesculin media containing peptone 10gm, Aesculin 1g, Ferric citrate 0.5g, agar 15gm in one litre of distilled water was prepared slants and culture of organism's streak on it and incubated for 2-5 days. After incubation dark color is developed the slant recorded as test positive

Gelatine Liquefaction: The media of gelatine liquefaction containing beef extract 39, peptone 5g, gelatin 120g in one liter of and dispense into 10 ml per test tube and autoclaved at 121 ºc for 15 minutes and cools the test tube without slanting. The media were stab inoculated by using organisms grown on YDC media and incubated at 20 ºc. After 3, 7 and 15 days of incubation. The tubes were observed for gelatin liquefaction for this the tubes are kept at 5 ºc for 30 minutes and gently tipped immediately. The medium that flows readily as the tubes are gently tipped was taken as a positive for gelatin liquefaction.

Starch hydrolysis: The nutrient agar mediums of starch hydrolysis contain starch soluble 20g; peptone, 5 g; Beef extract 3g; agar, 15g. In one liter distilled water and the PH of the medium is 7. Autoclaves the media at 121ºc for 15 minutes and prepare the starch agar plates. Streak the starch agar plates with cultured organism and incubate for 2-7 days. After incubation flood the plate present in the media stains blue or black and clear zone is observe around the bacterial colony is recorded at test positive.

Levan production (utilization of sucrose)

The media for levan production contain nutrient agar with 5% sucrose. Streak the plate with cultured organism and incubate for 3-5 days. Levan is produced when colonies are convert white cloned and mucoid.

Arginine hydrolysis reaction

The media for Arginine hydrolysis contain Peptone - 1.0gm, Nacl - 5gm, K_2HPO_4 -0.3gm L.arginine -10gm, Phenolred - 0.01gm, Agar -15.0gm, Distilled water – 1000ml, pH – 7.2 Inoculate was streaked on media for positive reaction colour changes from yellow to red/pink colour.

Acid Production from Carbohydrates

The dyes medium C for the acid production from carbohydrates containing $NH_4H_2PO_4$, 0.59;K_2HPO_4, 0.5 gm ; $MgSO_4 * 7H_2O$, 0.029; yeast extract,1.0 g; Bromo- cresol purple (1.5% in ethanol) 0.7ml and agar 12g adjust the PH of the medium 6.8, 5g of carbon source dissolved in water and filter- sterilized. Autoclaves the above dyes's medium at 121 ºc for 15 min after autoclaving cool the medium 50 ºc and add the carbon source previously sterilized. Mix the media and prepare slants. Streak the culture on media and incubate the colour change from purple to yellow recorded as acid production

RESULTS AND DISCUSSION

The ten isolates of *Bacillus thuringiensis* obtained from different soil region of Nanded. they were identified as morphologically characterized by studying the different characters such as colony colour, colony elevation, colony margin, colony shape and colony surface (Fig. 1.).

Fig.1- Isolated colonies of native isolates of B.*thuringiensis*

Morphological characters as flat, circular, creamy white colour colony with irregular margin and wavy surface. BT strains were isolated from soil samples by using sodium acetate and heat treatment method. Colonies obtained from soil sample were tested for identification by various biochemical tests. The characterizations of Bt isolates was carried out for primarily screened by using gram staining, Endospore staining.

Table 1.Characterization based on Biochemical test of *Bacillus thuringiensis*

Biochemical test	Isolated Organism Given In Slant Number									
	A	B	C	D	E	F	G	H	I	J
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+
Gelatine Liquefaction	+	+	+	+	+	+	+	+	+	+
Levan production	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	_	_	_	_	_	_	_	_	_	_
Acid production Test	+	+	+	+	-	-	-	+	+	+
Ceatalase test	+	+	+	+	+	+	+	+	+	+

-: Negative, +W : weakly Positive, +: positive

Biochemical characterization was carried out by various method like Catalase test, Oxidase test, aesculin hydrolysis, Gelatin liquefaction, Starch hydrolysis, Levan production, Arginine hydrolysis and Acid production (Table-1). Soil samples were collected from different regions of Nanded for the isolation of *Bacillus thuringiensis,* using method by Randhawa et al (2009). The isolates were initially characterized on the basis of morphological characterization Kati et al., 2007, Sarita Agrahari et al., 2008 also have characterized *B thuringiensis* isolates on morphological basis. The isolates were further confirmed by gram staining and endospore staining. The biochemical characterization of Bt isolates was carried out for Catalase test, Oxidase test, aesculin hydrolysis, Gelatin liquefaction, Starch hydrolysis, Levan production, Arginine hydrolysis and acid production. Our findings are very well similar and supported by Wafula *et al* (2014) to characterize B *thuringiensis* isolates from soil sample. In conclusion, this study has given a insight to isolate new *Bacillus thuringiensis* from native soil sample.

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