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EVALUATION OF DIFFERENT CEREAL BASED MEDIA FOR THE PRODUCTION OF *BACILLUS THURINGIENSIS* AND THEIR VIRULENCE AGAINST *SPODOPTERA LITURA* (FABRICIUS)

JASDEEP KAUR AND NEELAM JOSHI*
Department of Microbiology and Department of Entomology
Punjab Agricultural University, Ludhiana -141 004, Punjab, INDIA
e-mail: neelamjoshi_01@pau.edu

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***Corresponding author**

ABSTRACT
Bacillus thuringiensis is one of the most exploited microbial biocontrol agent used for control of many crop pests. Inexpensive and potential medium is required for its mass production which maintains its viability and virulence. So, in present study different cereal based liquid media viz rice, wheat, sorghum and maize were evaluated for selection of potential medium for production of *B. thuringiensis*. Five potential *B. thuringiensis* isolates including two procured isolates (*B. thuringiensis* MTCC 868 and MTCC 4715) and three native isolates (Bt-4, Bt-10 and PAU Bt) were evaluated on these media and among all, wheat based medium was best and was significantly better than others. Influence of selected additives at their selected concentration for persistence of *B. thuringiensis* spores in wheat based medium was maximum after fifteen days and was at par with spore count upto sixty days. Laboratory bioassay against the second instar larvae of *S litura* and untreated control recorded maximum mortality in MTCC 868 at higher concentration and was significantly better than other treatments.

INTRODUCTION

Biological pesticide is one of the promising alternative method over conventional chemical pesticides, which offers less or no harm to the environment and biota (Veloorvalappil *et al.,* 2013). *Bacillus thuringiensis* is entomopathogenic rod shaped sporulating bacteria which has unique property of producing an insecticidal protein crystal next to spore at time of sporulation and is pathogenic to many crop pests (Martin and Travers 1989). *B. thuringiensis* can utilize different nutrients for growth and sporulation. Carbon source as glucose, nitrogen sources as peptone or yeast extract and salts like ammonium sulfate and mineral salts (Dulmage, 1970). Metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} are essential for its growth and potassium ions are necessary for the production of *B. thuringiensis* crystals (Wakisaka *et al.,* 1982 and Foda *et al.,* 1985). In laboratory synthetic media with all these elements are generally used. However, at industrial scale, the synthetic and expensive substrates are usually replaced by agro-industrial by-products to reduce its cost of mass production (Mounsef *et al.,* 2014). Therefore, for mass production of *B. thuringiensis* need for cheap and potential medium is required which supports spore and toxin production and maintains viability and virulence of bacteria.

Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae) commonly known as Tobacco caterpillar is an important polyphagus pest in Asia. It is a serious pest of important crops such as cotton, groundnut, chilly, tobacco, cole crops and pulses which causes serious economic losses based on crop stage and its infestation level in the field (Kumar and Regupathy, 2001). The young caterpillars of *S. litura* feed voraciously on leaves, defoliating the plants making insecticidal application mandatory for the cultivation of crops (Choudary *et al.,* 2014). The extensive and indiscriminate use of pesticides has led to the development of resistance in insects to most frequently used chemicals (Sarkar *et al.,* 2015). Among entomo pathogenic bacteria, *B. thuringiensis* Berliner is the most successful microbial pesticides and is commercially used as biocontrol agent against many crop pests (Mathew *et al.,* 2014). Therefore, there is a need for the development of formulations and optimization for its utilization as biopesticides to management of crop pest. So, present study was conducted to evaluate different cereal based liquid media for production of *B. thrungiensis* which maintains its viability and virulence.

MATERIALS AND METHODS

Cultures

Five potential *B. thuringiensis* isolates were used in present study. These were two standard *B. thuringiensis* isolates (MTCC 868, MTCC 4715) procured from *Institute of Microbial Technology, Chandigarh and three native Bacillus isolates (Bt-4, Bt-10 and PAU Bt) previously isolated in Department of Entomology. The *Bacillus* isolates were maintained on Luria Agar slants. The stock culture was maintained at 4°C, until

used.

Media preparation

Different cereal grains viz rice, wheat, maize and sorghum were finely grinded and media was prepared @ 2.5 per cent (w/v) as per methodology of Shojaaddini *et al.,* 2010. Medium "A" consisted of sorghum flour, "B" consisted of rice flour, "C" consisted of wheat flour and, "D" consisted of maize flour. All ingredients of media were soaked in distilled water for 1 hour. The ingredients of all media were dissolved in distilled water and filtered through a plastic strainer (mesh size 100 ìm) to remove all the insoluble solid particles. To each litre of the above media, 10 ml of stock salt solution (20.3 g $MgCl_2$, 10.2 g $CaCl_2$, and 1.0 g $MnCl_2$ per litre of distilled water) was added, according to the method by Prabakaran and Balaraman, 2006. The media was sterilized by autoclaving at 121 ºC for 15-20 mins at 15 lbs/inc^2.

***Bacillus thuringiensis* production**

First-stage seed culture was prepared by inoculating 10 ml of Luria broth with one loop-full of *B. thuringiensis* inoculum and incubating it in orbital shaker-incubator at 30±2°C for 24 hour at 200 rpm. Second-stage seed culture was prepared by transferring 10 ml of first stage seed culture into 190 ml of the luria medium in a 500 ml Erlenmeyer flask and incubating in a shaker-incubator at 30±2°C, at 200 rpm for 48 hours (Shojaaddini *et al.,* 2010). Second stage seed culture was inoculated in 1000 ml Erlenmeyer flasks, containing 500 ml of the above four culture media (A, B, C and D). These flasks were then incubated at 30±2°C at 200 rpm in a shaker-incubator for 72 hours. The samples taken from each flask was heat treated at 80ºC for 15 min, serially diluted, and inoculated and incubated onto Luria agar plates at 30±2°C for 48 hours. The *B. thuringiensis* colonies were counted and expressed as colony forming units (CFU) per ml.

Evaluation of additives on persistence of *Bacillus thuringiensis* in formulation

The selected wheat based media was inoculated with *B. thuringiensis* for 72 hours and supplemented with selected additives at their selected concentrations viz glycerol (1%), methyl-p-hydroxybenzoate (1%), triton x 100 (0.1%) and boric acid (1%) for persistence of *B. thuringiensis* spores in the medium as per methodology of Bibi *et al.,* 2013. The medium was stored in dark at room temperature .The spore count of *B. thuringiensis* was recorded fortnightly.

Insect rearing and bioassay

Spodoptera litura larvae and pupae were collected from crop fields and reared in laboratory on castor leaf. The second instar larvae were used for the laboratory bioassay by leaf dip method (Kamel *et al.,* 2010). There were six treatments and three replication per treatment with ten larvae per replicate. These treatments were two procured *B. thuringiensis* isolates (MTCC 4715, MTCC 868) and three local *Bacillus* isolates (Bt-4, Bt-10 and PAU Bt), at different concentrations (1.0, 1.5, and 2.0%) along with untreated control The leaves were dipped for 5-10 seconds in solution of different concentrations of formulations, air dried and then placed individually in plastic vials. Ten larvae were introduced in each vial. The larvae were allowed to feed on treated leaves and were observed daily for their cumulative mortality up to ten days.

RESULTS AND DISCUSSION

Selection of potential medium for *B. thuringiensis* production is an important aspect of process development as *B. thuringiensis* can utilize different carbon and nitrogen source for its growth. Dhingra and Chaudhary, 2009 used different low-cost agro-industrial based carbon sources such as potato extract, corn starch, wheat flour, sugarcane molasses, barley flour and soluble starch to develop low-cost *B. thuringiensis* formulations for the management of *Helicoverpa armigera* and reported that potato extract supported the highest biomass followed by wheat and was comparable to Luria broth medium. Sabbour *et al.,* 2012 evaluated different additives to improve the potency of the formulation of *B. thuringiensis* var. *Kurstaki* (HD-234), and to increase its persistence. They reported that the persistence of the modified *B. thuringiensis* var. *kurstaki* (HD-234) formulation suspension against *Phthorimaea operculella* was 5.3, 6.9, 5.6 and 4.0 folds more stable than those of the commercial preparations of DipelÂ®2X, AgerinÂ®, HD-1-S-1980 and *B. thuringiensis* var. *kurstaki* (HD-234). The bioefficacy of liquid formulation of *B. thuringiensis* against *H. armigera* using five different types of stabilizers viz glycerol, groundnut oil, mustard oil, mineral oil and sunflower oil were evaluated by Dhingra, 2012. He reported maximum persistence of *B. thuringiensis* cells in liquid formulation with glycerol as additive upto two months when formulation was stored at room temperature. Several scientists also reported virulence of *Bacillus* isolates against *S. litura*. Haggag and Yousef, 2010 found that all *B. thuringiensis* strains, as well as, reference strain had the toxicity to insect larvae, where the percentages of mortality were in the range of 47.5 per cent to cent per cent and 25 per cent to cent per cent against first and second instars larvae, respectively.

Table 1: Spore count of *Bacillus* isolates on different cereal based media

Medium	Colony forming units (1×10^8 CFU/ml) of *Bacillus* isolates (Mean*±S.E.**)					Mean
	MTCC 868	MTCC 4715	Bt-10	Bt-4	PAU Bt	
Medium A	20.67±1.76	16.67±2.40	18.67±1.45	15.33±2.03	16.67±1.45	17.60
Medium B	14.00±1.53	16.00±2.08	6.67±1.76	12.33±1.45	9.33±0.88	11.67
Medium C	30.67±1.45	29.33±1.76	27.00±2.89	28.67±2.60	24.67±1.86	28.07
Medium D	22.33±2.60	21.67±2.03	11.33±2.03	23.67±2.03	12.00±2.08	18.20
Mean	21.92	20.92	15.92	20.00	15.67	
CD (p=0.05)	CD (5%) Substrates = 2.41 CD (5%) Isolates = 2.70 CD (5%) Substrates × Isolates = 5.39					

*Mean = Mean of three replications **S.E. = Standard error

Table 2: Spore count of *Bacillus* isolates with selected additives at different time intervals

Days of Incubation	Colony forming units (1×10^8 CFU/ml) of *Bacillus* isolates (Mean* ± S.E.**)					Mean	Per cent colony reduction over control
	MTCC 868	MTCC 4715	Bt-10	Bt-4	PAU Bt		
Control	30.67 ± 1.45	29.33 ± 1.76	27.00 ± 2.89	28.67 ± 2.60	24.67 ± 1.86	28.07	
15	29.67 ± 1.45	28.33 ± 1.45	26.67 ± 1.45	28.00 ± 1.73	24.33 ± 1.45	27.40	2.39
30	29.33 ± 1.67	26.00 ± 1.53	25.33 ± 1.76	27.33 ± 1.76	23.67 ± 1.45	26.33	6.19
45	28.33 ± 2.19	25.33 ± 1.45	25.33 ± 1.45	26.33 ± 1.76	23.00 ± 1.53	25.67	8.55
60	26.33 ± 1.76	23.00 ± 1.53	23.33 ± 1.76	25.67 ± 2.03	21.33 ± 1.45	23.93	14.75
Mean	28.42	25.67	25.17	26.83	23.08		
CD (p = 0.05)	CD (5%) Days of incubation = 2.10 CD (5%) Isolates = 2.35 CD (5%) Days of incubation × Isolates = Non-Significant						

*Mean = Mean of three replications **S.E. = Standard Error

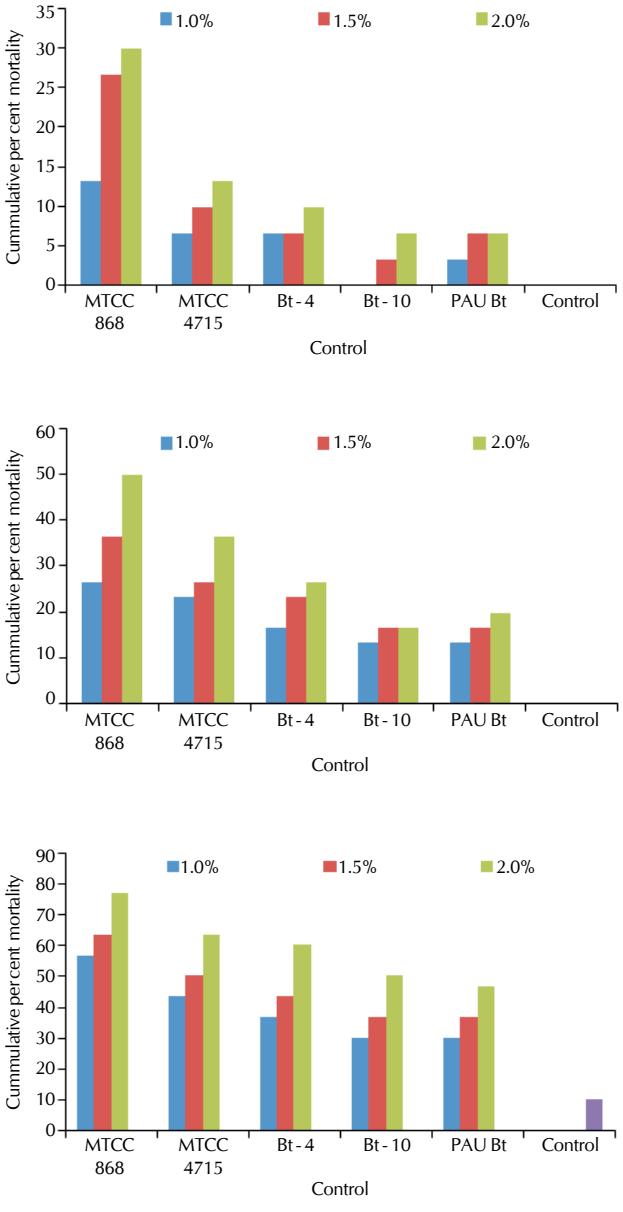

Figure 1: Cumulative per cent mortality on different days

Donovan *et al*, 2013 worked on *B. thuringiensis* isolates viz B-21365, B-21366 and B-21367 and reported that diet containing spores and delta endotoxin of *B. thuringiensis* isolates was active against the larvae of Coleoptera, including the red flour beetle, *T. castaneum* and the Japanese beetle, *Popillia japonica*. Ahmad and Shakoori, 2013 reported that forty eight *Bacillus* isolates obtained from seventy two soil samples gave positive tests specific for *Bacillus thuringiensis*. They further reported that three isolates (MS-SBS-*Bt*1, MS-SBS-*Bt*2 and MS-SBS-*Bt*3) were found positive for cry1Ca, and two isolates (MS-SBS-*Bt*4 and MS-SBS-*Bt*5) were found positive for cry1Cb genes. Their bioassays studies showed that MS-SBS-*Bt*1 was most toxic to *S. litura* and *Musca domestica* when compared to *Bacillus thuringiensis* strain HD 139 and HD29 respectively taken as control.

In the present study, four different cereal based liquid media: sorghum, rice wheat and maize were prepared for growth and persistence of *Bacillus* cells was monitored. Wheat based liquid media supported maximum *Bacillus* cell growth (28.07×10^8 CFU/ml) followed by sorghum and maize (Table 1). The selected wheat based liquid media supplemented with four additives at their selected concentrations viz boric acid (1%), glycerol (1%), methyl-p-hydroxybenzoate (1%) and triton x 100 (0.1%) recorded maximum mean spore count (27.40×10^8 CFU / ml) after fifteen days and were at par till sixty days (Table 2). Further percent colony reduction over control was minimum (2.39%) at fifteen days of storage at room temperature.

The insecticidal potential of different *B. thuringiensis* isolates were evaluated under laboratory conditions against second instar larvae of *S. litura* by feeding treated castor leaf at different concentrations (1.0, 1.5 and 2.0%) and cumulative percent mortality was recorded. *Maximum cumulative per cent mortality was recorded in procured B. thuringiensis isolate MTCC 868 at higher (2.0%) on fifth, seventh and tenth day of treatment (Fig 1).* After ten days of treatment maximum cumulative per cent mortality (76.67 %) was recorded in *B. thuringiensis* MTCC 868 and was significantly better than all other treatments.

The results of present study indicate that wheat based medium supplemented with selected additives at their selected concentration was best for persistence of *Bacillus* spores and *B. thuringiensis* isolates, MTCC 868 was most pathogenic

against second instar larvae of *S. litura*.

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