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## RESEARCH ARTICLE

### ISOLATION, CHARACTERIZATION AND INSECTICIDAL POTENTIAL OF *BACILLUS SUBTILIS* AND *STAPHYLOCOCCUS VITULINUS* AGAINST *SPODOPTERA LITURA*

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#### ABSTRACT

*Spodoptera litura* Fab. is one of the most destructive pest of cotton. Damage caused in spite of various control methods, the discovery of new entomopathogenic bacterial species and isolates bearing insecticidal traits against novel targets is needed in the near future. In this study, isolation, characterization of *Staphylococcus vitulinus* and *Bacillus subtilis* was assessed for insecticidal activity against *Spodoptera litura*. Bacterial isolation were performed from dead and alive larvae of *Spodoptera litura*, bacterial isolates were characterized based on their morphological and biochemical characteristics. The application of concentration were conducted with five different bacterial concentrations ( $8.5 \times 10^7$  cfu/ml,  $12.2 \times 10^7$  cfu/ml,  $16 \times 10^7$  cfu/ml,  $20.2 \times 10^7$  cfu/ml and  $24.4 \times 10^7$  cfu/ml) of the active cells and for insecticidal activity 1 ml of bacterial suspension were performed on the third instar larvae of *S. litura* with the leaves of *Ricinus communis*. The sub lethal and median lethal dose of *Staphylococcus vitulinus* was  $LD_{10} = 87.06$  cfu/ml and  $LD_{50} = 215.5$  cfu/ml at 96 hrs and for *Bacillus subtilis* was  $LD_{10} = 31.61$  cfu/ml and  $LD_{50} = 113.9$  cfu/ml at 96 hrs against third instar larvae. Result revealed that, the mortality increase with increase in concentration of bacterial populations. The highest mortality of *Spodoptera litura* is found by bacterial strain *Staphylococcus vitulinus* at  $24.4 \times 10^7$  cfu/ml and for *Bacillus subtilis* at  $20.2 \times 10^7$  cfu/ml. Stastical variance, 95% confidence limits and regression equations are presented.

**Key words:** *Staphylococcus vitulinus*, *Bacillus subtilis* and *Spodoptera litura*.

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#### INTRODUCTION

*Spodoptera litura* is serious polyphagous pest of various economically important crops such as cotton, soybean, groundnut, chilli, tobacco, castor, bhendi, jawar, maize, cabbage and pulses etc. It was found to cause 26 - 100 % yield loss in ground nut (Dhir *et al.*, 1992). Loss of major crops due to insect pest varies between 10 and 30% (Ferry *et al.*, 2004). India is basically an agro-based country where more than 80% of Indian population depends on agriculture. Insects are known to cause significant damage to crops and affect agricultural productivity. The body of this pest is light brown in colour while fore wings are greyish brown with white markings and hind wings with a brown border. The life cycle of *Spodoptera litura* is consisting of egg, larva, pupa and adult. The eggs are laid on leaves in clusters and the egg masses are covered with buff coloured hairs obtained from the mother's body. The caterpillars cause the damage by feeding on tender leaves, shoots and fruits at night. It is extremely destructive pest of cultivated plants. The majority of the insect pests are controlled by using chemical insecticides that harm the environment, humans and many beneficial organisms. Thus, it is pivotal and well accepted that other control methods such as physical prevention, cultural actions and organic and

environmentally friendly compounds are essential for integrated pest management programmes. The bioinsecticides are the biochemical pesticides that are naturally occurring substances that control pests by nontoxic mechanisms. Bioinsecticides are living organisms or their products (phytochemicals, microbial products) or byproducts which can be used for the management of pests that are injurious to plants. Biopesticides based on pathogenic microorganisms specific to a target pest offer an ecologically sound and effective solution to pest problems. They pose less threat to the environment and to human health. The most widely known microbial pesticides are varieties of the bacterium *Bacillus thuringiensis* or Bt which can control certain insects in various fields. In the last 50 years, microbial control of pests and plant diseases showed an amazing development associated with pronounced good results under optimized laboratory conditions. Thus we need to understand the important concepts required to produce reliable, effective, and safe entomopathogens for insect pest control. In the present study, we focused on the determination of bacterial species isolated and characterized based on morphological and biochemical characteristics for insecticidal activity against *S. litura*.

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## MATERIALS AND METHODS

### Insect Culture

The eggs of *S. litura* (NBAIL-MP-NOC-02: *S. litura*) were purchase from National bureau of Agriculture Insect Resources Bangalore and maintained in the laboratory. The III instar larvae were used for these experiments.

### Isolation of bacteria

Bacterial isolation was performed on dead and living larvae separately. Healthy and dead larvae were separated based on microscopic examination, distinguishing between living larvae that showed general disease symptoms. Twenty living and dead larvae were surface sterilized with 70% ethanol and washed three times with sterile distilled water and homogenized in nutrient broth media by using a glass tissue grinder. Suspensions were diluted and 0.1ml suspension was placed on nutrient agar. Plates were incubated at 30°C for 2-3 days. After the incubation period, the plates were examined and bacterial colonies were selected. The colonies determined were purified by subculture on the plates (Crecchio *et al.*, 2001).

### Inoculum and culture conditions

The inoculum was prepared as following: one isolated colony was dispensed in 3 ml of Liquid Broth medium and incubated overnight at 37°C. Aliquots (0.2 ml) were used to inoculate 250 ml Erlenmeyer flasks containing 50 ml Liquid Broth medium and incubated in a rotatory shaker at 200 rpm and 37°C, From the larval extracts, 10, 25, and 50 µL were placed on nutrient agar and incubated at 30 °C for 2–3 days. The remaining mixtures were incubated at 30 °C for 3–4 h to increase the number of bacteria that had low concentrations. From these mixtures, 10, 25, and 50 µL were also placed on nutrient agar and incubated at 30 °C for 2–3 days. Isolates were distinguished based on colony color and morphology. Pure cultures of the bacterial colonies were prepared and stocked in 0.9% biological saline solution in Laboratory (Filiz, *at el* 2014). Bacterial cultures were identified according to their morphology, nutritional features, and biochemical, physiological and molecular characteristics.

### Identification of bacterial isolates

Bacterial isolates were identified by utilization of organic compounds, spore formation, Gram staining, and upon confirmation of the bacterial gram nature, same bacterial strain was identified by using bioMerieux, vitek2 compact.

### Insecticidal bioassay

Third instar larvae of *S. litura* were used for the insecticidal assay of bacterial isolated species are *Staphylococcus vitulinus* and *Bacillus subtilis*. The concentration application experiments were conducted with bacterial isolates with various population of the active cells in colony forming unit (CFU) (Table 5 and 6). One milliliter of bacterial suspensions of different concentrations ( $8.5 \times 10^7$  cfu/ml,  $12.2 \times 10^7$  cfu/ml,  $16 \times 10^7$  cfu/ml,  $20.2 \times 10^7$  cfu/ml and  $24.4 \times 10^7$  cfu/ml) were saturated onto lettuce leaves (approximately 10 cm<sup>2</sup>) of *Ricinus communis* and placed in individual plastic boxes released 10 third instar larvae for 96 hrs of each experiment. Three replications were conducted. The percent mortality was calculated after 96h and the observed data was subjected to probit analysis (Finney 1947; Busvine 1971).

## RESULTS

In this study, *Staphylococcus vitulinus* and *Bacillus subtilis* bacterias from the surface of dead and live larvae of *S. litura* was isolated and characterized. It was found gram positive. The colony colour of isolates was white cream and had the shape of cocci and gram positive rods. Some morphological and biochemical characteristics of *Staphylococcus vitulinus* and *bacillus subtilis* are summarized in Table – 2. The bacterial isolate was identified by using bioMerieux, vitek2 compact (Table -3 and 4). The isolated and identified bacteria, *Staphylococcus vitulinus* and *bacillus subtilis* was tested against third instar larvae of *S. litura*.

The total percent mortality was observed after 96h and corrected mortality was calculated by using Abbott's formula and the results are presented. The results showed that the mortality increases with increase in concentration at all doses. The results of the probit analysis for the estimation of LD<sub>10</sub>, LD<sub>50</sub>, variance, 95% confidence limits and regression equation at 96h for the mortality of third instar larvae of *S. litura* are presented in Table – 5 and 6. The highest mortality (60%) was found at  $24.4 \times 10^7$  cfu/ml at 96 hrs for *Staphylococcus vitulinus* and (100%) was found at  $20.0 \times 10^7$  for *Bacillus subtilis* of third instar larvae. (Table-4, 5 and Figure-1, 2 and 3). The sub lethal and median lethal dose of *Staphylococcus vitulinus* was LD<sub>10</sub> = 87.06 cfu/ml and LD<sub>50</sub> = 215.5 cfu/ml at 96 hrs and for *Bacillus subtilis* was LD<sub>10</sub> = 31.61 cfu/ml and LD<sub>50</sub> = 113.9 cfu/ml at 96 hrs against third instar larvae of *S. litura*. Among the various estimate of regression based probit analysis, the  $\chi^2$  values for the regression coefficients showed homogeneity to the data.

Table 1. *Staphylococcus* and *Bacillus* species and their host used in this study.

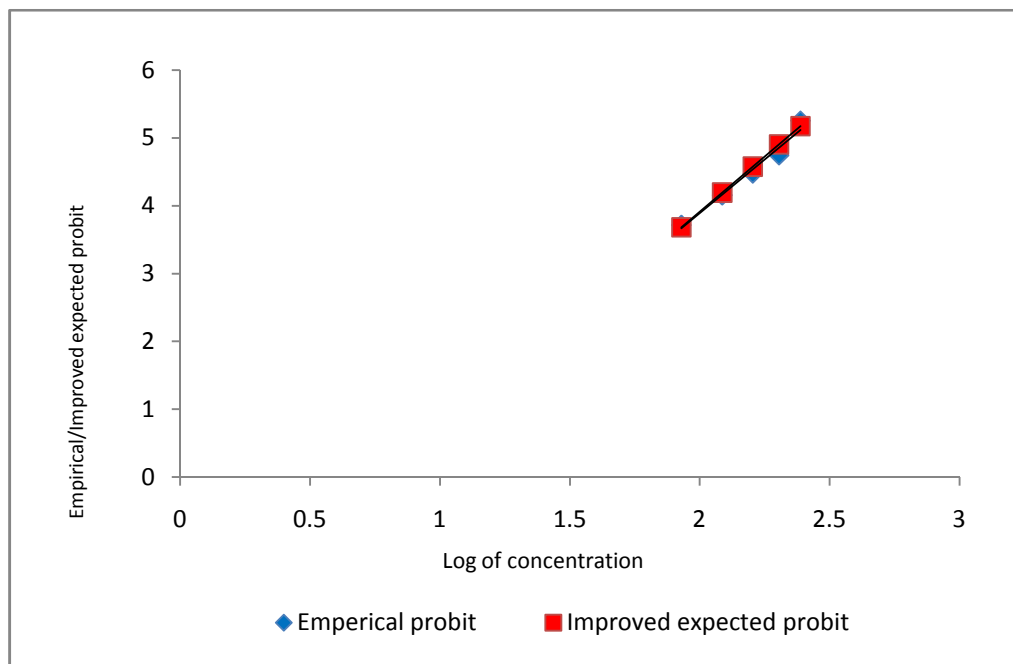
Species	Host	Source
Staphylococcus	<i>Spodoptera litura</i>	Larvae
Bacillus	<i>Spodoptera litura</i>	Larvae

Table 2. Morphological characteristics of *Staphylococcus vitulinus* and *Bacillus Subtilis*

Isolated sample	Bacterial strains	Colony Color	Shape of Colony	Shape of Bacteria	Gram stain	Spore stain	Source	Growth in Nutrient Broth
S-1	<i>Staphylococcus vitulinus</i>	White	Round	Cocci	+	-	Dead larvae	Turbid
S-4	<i>Bacillus Subtilis</i>	White	Round	Rod	+	+	Dead larvae	Turbid

**Table 3. The biochemical characteristics of *Staphylococcus vitulinus* isolated from *S. litura* larvae based on conventional and vitek 2 compact Biomerix identification system**

Well	Test	Mnemonic	Result
2	D-AMYGDALIN	AMY	-
4	PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C	PIPLC	-
5	d-XYLOSE	dXYL	-
8	ARGININE DIHYDROLASE 1	ADH1	-
9	BETA GALACTOSIDASE	BGAL	-
11	ALPHA GLUCOCIDASE	AGLU	-
13	Ala-Phe-Pro ARYLAMIDASE	APPA	-
14	CYCLODEXTRIN	CDEX	-
15	L-Aspartate ARYLAMIDASE	AspA	-
16	BETA GALACTOPYRANOSIDASE	BGAR	-
17	ALPHA-MANNOSIDASE	AMAN	-
19	PHOPHATASE	PHOS	-
20	Leucine ARYLAMIDASE	LeuA	-
23	L- Proline ARYLAMIDASE	ProA	-
24	BETA GLUCURONIDASE	BGURr	-
25	ALPHA-GALACTOCIDASE	AGAL	-
26	L- Pyrrolidonyl- ARYLAMIDASE	PyrA	-
27	BETA-GLUCURONIDASE	BGUR	-
28	Alanine ARYLAMIDASE	AlaA	-
29	Tyrosine ARYLAMIDASE	TyrA	-
30	D-SORBITOL	Dsor	-
31	URASE	URE	-
32	POLYMXIN B RESISTANCE	POLYB	-
37	G-GALACTOSE	Dgal	-
38	D-RIBOSE	dRIB	-
39	L-LACTATEalkalization	ILATk	-
42	LACTOSE	LAC	-
44	N-ACETYL-DGLUCOSAMINE	NAG	-
45	D-MALTOSE	dMAL	-
46	BACITRACIN RESISTANCE	BACI	-
47	NOVOBIOCINE RESISTANCE	NOVO	-
50	GROWTH IN 6.5% NaCl	NC6.5	-
52	D-MANNITOL	dMAN	-
53	D-MANNOSE	dMAN	-
54	METHYL-B-D-GLUCOPYRANOSIDE	MBdG	-
56	PULLULAN	PUL	-
57	D-RAFFINOSE	Draf	-
58	0/129 RESISTANCE (comp.vibrio.)	0129R	-
59	SALICIN	SAL	-
60	SACCHAROSE/SUCROSE	SAC	-
62	D-TREHALOSE	dTRE	-
63	ARGININE DIHYDOROLASE 2	ADH2s	-
64	OPTOCHIN RESISTANCE	OPTO	-

**Fig. 1. Regression and provisional lines for *Spodoptera litura* exposed to bacterial strain, *Staphylococcus vitulinus* after 96h**

**Table 4. The biochemical characteristics of *Bacillus subtilis* isolated from *S. litura* larvae based on conventional and vitek 2 compact Biomerix identification system**

Well	Test	Mnemonic	Result
1	BETA-XYLOSIDASE	BXYL	+
3	L-Lysine ARYLAMIDASE	LysA	-
4	L-Aspartate ARYLAMIDASE	Asp-A	-
5	Leucine ARYLAMIDASE	LeuA	-
7	Phenylalanine ARYLAMIDASE	PheA	+
8	L-Proline ARYLAMIDASE	ProA	-
9	BETA GALACTOSIDASE	BGAL	-
10	L-Pyrolydonyl ARYLAMIDASE	PyrA	+
11	ALPHA-GALACTOSIDASE	AGAL	+
12	Alanine ARYLAMIDASE	AlaA	-
13	Tyrosine ARYLAMIDASE	TyrA	+
14	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	-
15	Ala-Phe-Pro ARYLAMIDASE	APPA	+
18	CYCLODEXTRINE	CDEX	-
19	D-GALACTOSE	dGAL	-
21	GLYCOGEN	GLYG	-
22	myo-INOSITOL	INO	-
24	METHYL-A-D-GLUCOPYRANOSIDE acidification	MdG	+
25	ELLMAN	ELLM	-
26	METHYL-D-XYLOSIDE	MdX	-
27	ALPHA-MANNOSIDASE	AMAN	-
29	MALTOTRIOSE	MTE	+
30	Glycine ARYLAMIDASE	GlyA	-
31	D-MANNITOL	dMAN	+
32	D-MANNOSE	dMNE	-
34	D-MELEZITOSE	dMLZ	-
36	N-ACETYL-D-GLUCOSAMINE	NAG	-
37	PALATINOSE	PLE	+
39	L-RHAMNOSE	IRHA	-
41	BETA-GLUCOSIDASE	BGLU	+
43	BETA-MANNOSIDASE	BMAN	-
50	PHOSPHORYL CHOLINE	PHC	-
53	PYRUVATE	PVATE	-
54	ALPHA-GLUCOSIDASE	AGLU	-
56	D-TAGATOSE	Dtag	-
58	D-TREHALOSE	dTRE	+
59	INULIN	INU	-
60	D-GLUCOSE	dGLU	+
61	D-RIBOSE	dRIB	+
62	PUTRESCINE assimilation	PSCNa	-
63	GROWTH in 6.5% NaCl	NaCl 6.5%	-

**Table 5. Effect of *Staphylococcus vitulinus* bacterial populations on percent mortality of *Spodoptera litura* after 96 hrs**

Sr. no.	Bacterial population cfu/ml	No. of insects exposed	Percent mortality at 96 hrs
1	85	10	10
2	122	10	20
3	160	10	30
4	202	10	40
5	244	10	60

**Table 6. Effect of *Bacillus subtilis* bacterial populations on percent mortality of *Spodoptera litura* after 96 hrs**

Sr. no.	Bacterial population cfu/ml	No. of insects exposed	Percent mortality at 96 hrs
1	42	10	20
2	85	10	30
3	122	10	50
4	160	10	70
5	202	10	100

**Table-7. LD<sub>10</sub>, LD<sub>50</sub> values with variance, 95% confidence limits and probit analysis parameters of *Bacillus subtilis* and *Staphylococcus vitulinus* after 96h of treatment against larvae of *Spodoptera litura***

Isolated Bacterial strain	LD <sub>10</sub> CFU	LD <sub>50</sub> CFU	Variance	95% CL		Regression equations	$\chi^2$ (degree of freedom)
				Lower	Upper		
<i>Staphylococcus vitulinus</i>	87.06	215.5	0.003857	2.2118	2.4552	Y= 3.2350x - 2.5955	0.2275 (2)
<i>Bacillus subtilis</i>	31.61	113.9	0.008478	1.8758	2.2368	Y= 2.3032x + 0.2639	0.6591 (2)

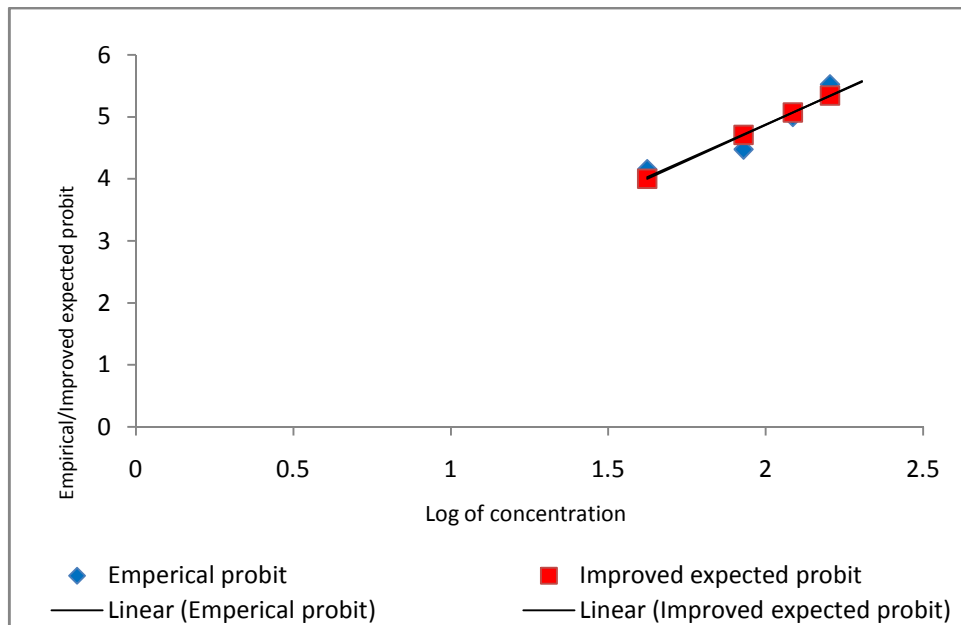


Fig. 2. Regression and provisional lines for *Spodoptera litura* exposed to bacterial strain, *Bacillus subtilis* after 96h.

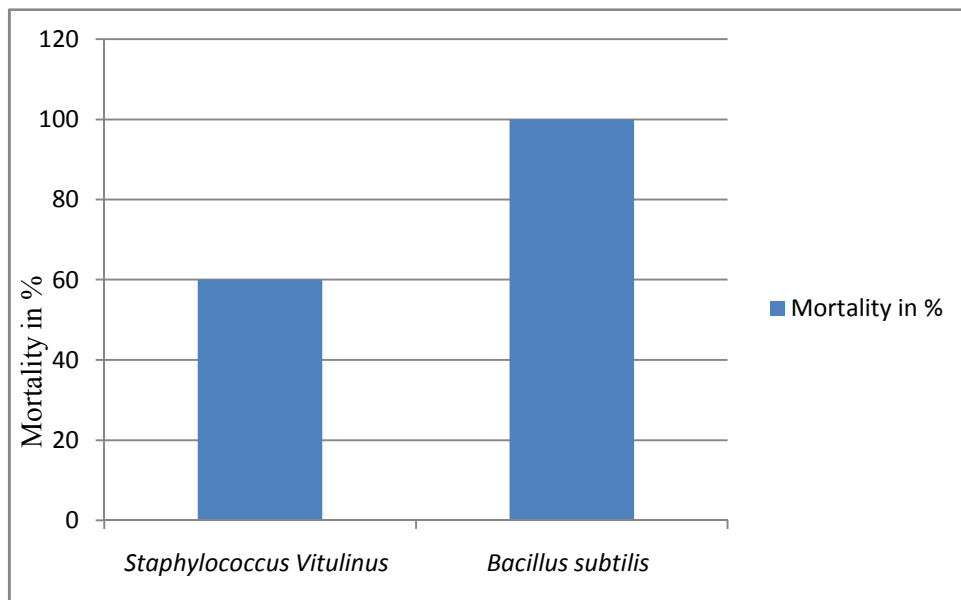


Fig. 3. Insecticidal potential of isolated bacteria, *Staphylococcus vitulinus* and *Bacillus subtilis* against, *S. litura*

## DISCUSSION

*Spodoptera litura* is one of severe agricultural pest. Entomopathogenic bacteria are of great importance in the control of insect pests. In the presented study we isolated bacteria from the surface of dead and living larvae of *Spodoptera litura*. The isolated bacteria were purified, cultured and identified. We also tested their insecticidal potential of isolated bacteria, against *S. litura*. The isolated bacteria, *Staphylococcus vitulinus* was identified from the sample. These bacterial species are the first report from *S. litura*. The members of the genera *Staphylococcus* and *Bacillus* were isolated most commonly from the pest insect. These genera include entomopathogenic bacterial species isolated from insects (Yaman *et al.* 1999, 2000, 2002, 2005, 2010; Kuzina *et al.* 2001; Darriet and Hougard 2002; Ertürk *et al.* 2008; Manimegalai and Shanmugam 2013). The members of *Staphylococcus*, *S. aureus* and *S. sciuri* were isolated from the pest's population in Samsun.

Different *Staphylococcus* species have been isolated from insects (Yaman *et al.* 2002; Nagaraju *et al.* 2012; Kati and Kati 2013; Manimegalai and Shanmugam 2013). *S. aureus* isolated from termites and from mulberry silkworm, *Bombyx mori* (Nagaraju *et al.*, 2012; Manimegalai and Shanmugam 2013). Kati and Kati (2013) isolated *S. sciuri* from *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae). Podgwaite *et al.* (2013) found that *S. sciuri* is the most common isolate associated with adults of the Asian long horned beetle (Coleoptera: Cerambycidae). Kalane and Pardeshi (2018) studied the larvicidal effect of *Staphylococcus vitulinus* against *Spodoptera litura*. In this study, *Staphylococcus vitulinus* and *Bacillus subtilis* showed promising biocontrol effect against *Spodoptera litura* without targeting susceptible stage of the pest during field application. Bioassay experiments showed that the isolated bacteria *Staphylococcus vitulinus* and *Bacillus subtilis* have insecticidal potential and their LD<sub>10</sub> and LD<sub>50</sub> against the larval instars of *S. litura* are 87.06 CFU and 215.5 CFU respectively. There are a few studies on using living microorganisms for the control of harmful insects (Sidor and

Jodal 1986; Vriesen and Keller 1994; Ziemnicka 2007). The study needs further investigation to find out active ingredients responsible for insecticidal properties against *Spodoptera litura*.

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