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Exploring Agricultural Potentiality of *Serratia entomophila* AB_2: Dual Property of Biopesticide and Biofertilizer

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Research Article

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ABSTRACT

Aims: In an attempt to explore novel agents for application in agriculture, the strain *Serratia entomophila* AB_2 was investigated.
Study design: Pesticidal, Fertilizing and Toxicological effects.
Place and Duration of Study: Department of Botany (Microbiology Unit), Visva-Bharati, Santiniketan, West Bengal, India and M/S Ajay Biotech (India) Ltd., Pune, India, between December 2006 to August 2007.
Methodology: Pesticidal activity of the isolate *Serratia entomophila* strain AB_2 was tested against 5 day old larvae of *Heliothis armigera*, *Spodoptera litura*, and *Plutella xylostella* through feeding assay. Fertilizing capacity of the strain was determined by inorganic phosphate and zinc solubilizing experiments. Conventional T-tests were performed to check effect against certainr rhizospheric organisms (*Azotobactor chroococcum* NCIM 2452, *Rhizobium japonicum* NCIM 2746, *Azospirillum brasiliensis* NCIM 5135, *Erwinia amylovora* MDVB). In animal toxicity experiments, adult male Swiss albino mice (body wt ≈ 25 g) were used to find LD_{50} within the experimental doses and male Sprague Dawley strain of Swiss albino rat (body wt ≈ 110 g) were used to find out the effect of feed inoculation treatment in liver and blood as general target of intoxication through standard thiobarbituric acid reactive substance (TBARS) and serum glutamic pyruvic transaminase (SGPT) assay.
Results: The mortality rate of, lepidopteron larvae tested was determined between 89.5 to 94.3% while LC_{50} value estimated between $0.44x10^5$ to $1.44x10^5$ CFU (colony forming unit) mL^{-1} through probit analysis. As a part of fertilizing activity, *S. entomophila* AB_2 was found to solubilize phosphorus and zinc in *in-vitro* condition. In cross reactivity study with other rhizospheric bacteria, the isolate proved as non reactive. No mortality was recorded with

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Swiss albino mice and other toxicological data resulted from the experiment with Sprague Dawley strain of Swiss albino rat were found insignificant.
Conclusion: The isolate *S. entomophila* AB_2 showed its potential in *in vitro* conditions for both pesticidal and fertilizing activity. The data generated in this study show much promise of *S. entomophila* AB_2 for its field application in integrated crop management (ICM). These predictors, however, need further work for field validation.

Keywords: Serratia entomophila; ICM; biopesticide; biofertilizer; toxicity.

1. INTRODUCTION

Insects and other pests cost billions of dollars to farmers, in terms of maintenance of quality and quantity of the product, and are added to the cost of production (Ignacimuthu and Jayaraj, 2003). In developing countries, the use of chemical pesticide is a common practice mainly for its efficacy and cost effectiveness. Only diamondback moth (*Plutella xylostella*) a lepidopteron pest infecting cruciferous crops, cause worldwide loss of $1 billion per annum (Verkerk and Wright, 1996). In India, the use of chemical pesticides is a common practice particularly to control lepidopteron pests of Noctuidae, such as *Spodoptera litura* (cutworm)*, Heliothis armigera* (pod borer), and of Plutellidae such as *Plutella xylostella* (Khan and Law, 2005). Usually, chemical pesticides are toxic to non-target species including human, also the recurring use may lead to the development of resistance property, becomes an insect variant (Magaro and Edelson, 1990). Cultivation methods, like crop rotation for avoidance of the target host and by stimulating the growth of adventitious root system, can partially address the resistance problem (Smith et al., 2000; Bailey et al., 2009). Biological control offers better alternative to synthetic chemical pesticides, because biopesticides (both live organisms and compounds of organic origin) are target specific, easy biodegradability, having less self-life and user friendly for sustainable agriculture (Ferré, 2002; Sayyed and Patel, 2011).

Among Gram positive biocontrolling agents, popularity enjoys by spore forming soil bacterium *Bacillus thuringiensis* (Bt) (Bizzarri et al., 2008; Porcar et al., 2008) while among Gram negative, popularity enjoys by *Serratia entomophila*, a non spore former (Inglis and Lawrence, 2001; O'Callaghan and Gerard, 2005). *B. thuringiensis* is often used for controlling Lepidoptera, Diptera, Coleoptera, and Hymenoptera (Bravo et al., 2007). Whereas, strains of *S. entomophila* were effective natural biocontrolling agent for the grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae), a major pasture pest of New Zealand (Grimont et al., 1977; Jackson et al., 1992). Biocontrolling of different insect genera including *Anomala*, *Costelytra*, and *Phyllophaga* by *S. entomophila* are also known (Nunez-Valdez et al., 2008). Chitinase, from *S. macrescens*, is in use to control plant diseases (Ordentlich et al., 1998; Soiuza et al., 2005).

Microbial inoculant as biofertilizer is the other important component of ICM that help to nourish the crop plants by increasing availability of soil nutrients to plants (Gyaneshwar et al., 2002). Biofertilizers mainly act in three different ways, either fixing atmospheric nitrogen, or provide plant growth regulators (PGR), while others solubilize and translocate water insoluble soil nutrients including macro- (P & K), and micro- (Zn & Cu) elements. Soil pH either alkaline or acidic, decreases phosphorus availability to plants. Phosphate solubilizing microorganisms (PSM) solubilize insoluble phosphate to inorganic phosphorus to act as

natural buffering system (Narula et al., 2000). Blue green algae (BGA), *Rhizobium*, *Azotobacter*, *Azospirillum* are in use as biofertilizers since long (Deubel and Merbach, 2005; Jeenie et al., 2011). While the insecticidal property of *S. entomophila* is known, but its nutrient solubilizing or fertilizing potential is yet to be explored.

It is observed that the farmers usually overlook the field and do not use pesticide until there is heavy infestation. In such condition, the biological pesticide fails to produce an instant knockout effect, hence, enhances the possibility to accept the challenge by using chemical pesticides, leaving enough residue in the soil environment. Usage of multidimensional activity of the organisms will help in encouraging the farmers for sustainable and environment friendly agriculture. But major constrain is their availability for practical use.

The present study is to address the bottle neck by using the novel strain *S. entomophila* AB_2 in agricultural practice.

2. MATERIALS AND METHODS

2.1 Bacterial Culture Used

The working isolate, *S. entomophila* AB_2 (Chattopadhyay et al., 2011) was maintained as 20% glycerol stocks at -20˚C in Luria-Bertani broth (HiMedia, India). The culture size was scaled up to 1 L in Erlenmeyer flasks using modified Schlegel's medium (Sarma et al., 2009). Cultivation was carried out for 24 h at 32˚C in an orbital shaker (120 rpm, Scigenics Biotech, India).

Pseudomonas putida NCIM 2650 used as positive control in phosphate solubilization experiment was maintained in Luria-Bertani agar medium (HiMedia, India). *Azotobactor chroococcum* NCIM 2452, *Rhizobium japonicum* NCIM 2746 and *Azospirillum brasiliensis* NCIM 5135 used as beneficial rhizospheric microorganisms for cross-reactivity study, maintained in their respective recommended agar medium (HiMedia, India). However, *Erwinia amylovora* MDVB 121 (laboratory isolate) was used as harmful rhizospheric microorganism and maintained in Soya-agar medium (HiMedia, India).

2.2 Insect Pests Used

Test pests were, the common lepidopteron insect larvae (*Heliothis armigera*, *Spodoptera litura*, and *Plutella xylostella*), reared at the facility of M/S Ajay Biotech (India) Ltd., Pune, India. Pest larvae were maintained on commercial diet (IM002, Hi-Media, India) separately, at constant ambient temperature (25±1℃) and RH of 60±5%, with a photoperiod of 16:8 h (Light:Dark) (Chenchaiah and Bhattacharya, 2005). In order to obtain homogeneous mass of the test insect pests, the larvae were reared till emergence of adults. After rearing for two generations, the 5-day old larvae were used for bioassay tests.

2.3 Bioassay Test

The test broth culture (1 L) of *S. entomophila* AB_2 was centrifuged to pellet down the bacterial cells as in clear supernatant solutions. The pellet washed thrice with sterile phosphate buffer solution (PBS) and finally suspended in PBS to use as bacterial stock ($1.0x10^{18}$ CFU mL^{-1}). The bacterial stock was serially diluted upto $1.0x10^{2}$ CFU mL^{-1} by

thorough mixing with commercial diet (IM002, Hi-Media, India) at 32℃ and after solidification used as feed for treating pest larvae.

Experimental larvae (5-day old) were distributed into seven batches to treat with 3 g of feed having different inoculant load ($0.95x10^2$ to $0.95x10^8$ CFU mL^{-1}) for 24 h and observed for feeding. Each experimental batch contained 30 larvae. Each larva was kept in separate vial. In each case, untreated larvae were maintained as negative control to check the mortality otherwise. After 24 h larvae were transferred to fresh set of vials and maintained under commercial diet only. At every 12 h interval, upto 96 h the cumulative rate of larval mortality were recorded, to work out LC_{50} value. Larvae were considered dead when it stopped movement for at least one minute or the larval body turned to red.

2.4 Inorganic Nutrient Solubilization

To understand the solubilizing property of the working isolate, water insoluble calcium tri-phosphate and zinc carbonate were attempted in minimal medium (Katiyar and Goel, 2003). The property ascertained by measuring the clearing zone diameter, in plate diffusion technique. Also the commercial phosphate solubilizer, *Pseudomonas putida* NCIM 2650, was used as positive control in this experiment.

2.5 Cross Reactivity Test

To understand the environmental load impact, the isolate was cross reacted with test bacteria by coculture technique, in which, *Azotobactor chroococcum* NCIM 2452, *Rhizobium japonicum* NCIM 2746 and *Azospirillum brasiliensis* NCIM 5135 used as standard beneficial rhizospheric organisms, and *Erwinia amylovora* MDVB 121, as a non-benificial rhizospheric organism. Conventional T-tests were performed by streaking the rhizospheric test organisms against *S. entomophila* AB_2 at 90º on nutrient agar plate (Lee et al., 2008). After incubation at 32˚C for 24 h, growth inhibition, if any, at the junction of test organisms was recorded to determine antagonistic effect.

2.6 Mammalian Toxicity Test

In animal toxicity experiment, adult male Swiss albino mice (body wt ≈ 25 g) were used to find LD_{50} within the experimental doses and male Sprague Dawley strain of Swiss albino rat (body wt ≈ 110 g) were used to find out the effect of feed inoculation treatment in liver and blood as general target of intoxication. Animals were acclimatized and quarantined for 2 weeks before experimentation (Zhou et al., 2000). While performing the experiment, the regulations laid down by the Institutional Ethical Committee (IEC) were strictly followed.

The mice were distributed in twelve groups (including one control set) treated with different CFU of log_{10} dilutions (from $1.0x10^{18}$ CFU mL^{-1} and serially graded upto $1.0x10^8$ CFU mL^{-1}) in sterile PBS, except the control set in force feeding trial. The treatment was carried out for seven days, consecutively and the mice were maintained on normal diet upto 10 weeks to observe long-term effect. Test mice were observed twice a day for the appearance of any clinical sign or mortality.

Only the three higher doses were selected for experimentation with rats and were distributed in four groups (including control set) having ten number in each group. On day 7, microsome enriched fraction isolated from homogenate. The magnitude of lipid peroxidation was

estimated following standard thioburbituric acid reactive substance (TBARS) assay (Zhong et al., 1990). To detect liver damage, if any, caused by formulated feed consumption, serum glutamic pyruvic transaminase (SGPT) assay from blood samples was performed, using kit following manufacturer guideline (Span diagnostic). The control sets were treated only with sterile PBS.

2.7 Statistical Analysis

Each experiment was performed in triplicates maintaing standard laboratory conditions and the results expressed as mean ± standard error. For bioefficacy study data were analyzed using single factor of completely randomaized design with different concentrations of the experimental isolate. For mortality studies, the original data were checked by Abbott's formula (Abbott, 1925), transferred into arcsin and percentage values to interprete through probit analysis (Finney, 1971).

3. RESULTS AND DISCUSSION

3.1 Bioefficacy Test

Lepidopteron larvae challenged with isolate-supplemented diet, were observed to stop feeding within 8-10 h after consumption. But results varied with different concentration of supplementation. Progress of the disease was very distinct as the color of the insects gradually turned brown; the darkened elementary canal became distinctly visible from the ventral side and succumbed to death by leaching of body fluid. Among the experimental doses, maximum mortality was found at 72 h of ingestion of formulation with $0.95x10^7$ CFU mL^{-1} (Fig. 1) and, mortality rate was 94.3%, 92.7% and 89.5% for *Heliothis ermigera*, *Spodoptera litura* and *Plutella xylostella*, respectively (Fig. 2). The results showed the estimated LC_{50} value of *S. entomophila* AB_2 for 5-day-old larvae of *H. armigera* as $0.44x10^5$ CFU mL^{-1}; for *S. litura* as $0.95x10^5$ CFU mL^{-1}; and for *P. xyostella* as $1.44x10^5$ CFU mL^{-1} (Table 1).

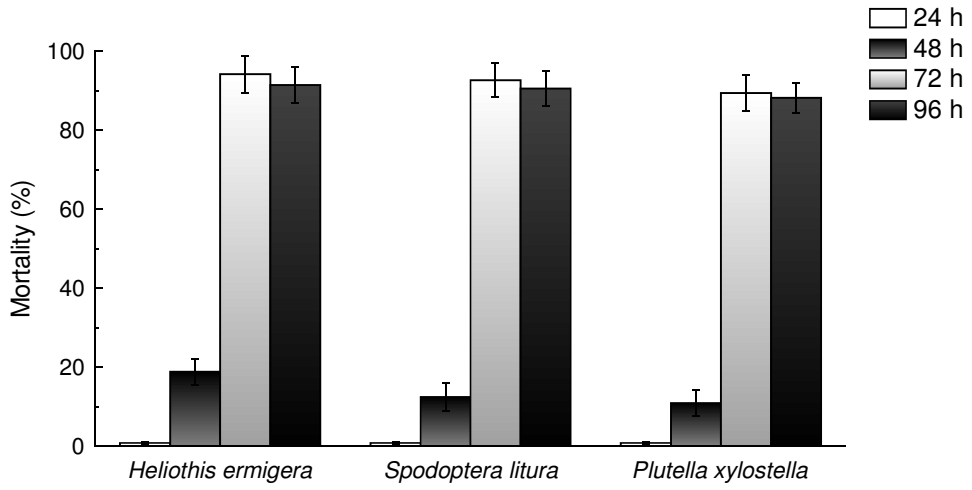

Fig. 1: Effect of time period on insecticidal activity of *S. entomophila* AB_2 against the larvae of *H. armigera*, *S. litura,* and *P. xylostela in vivo*.

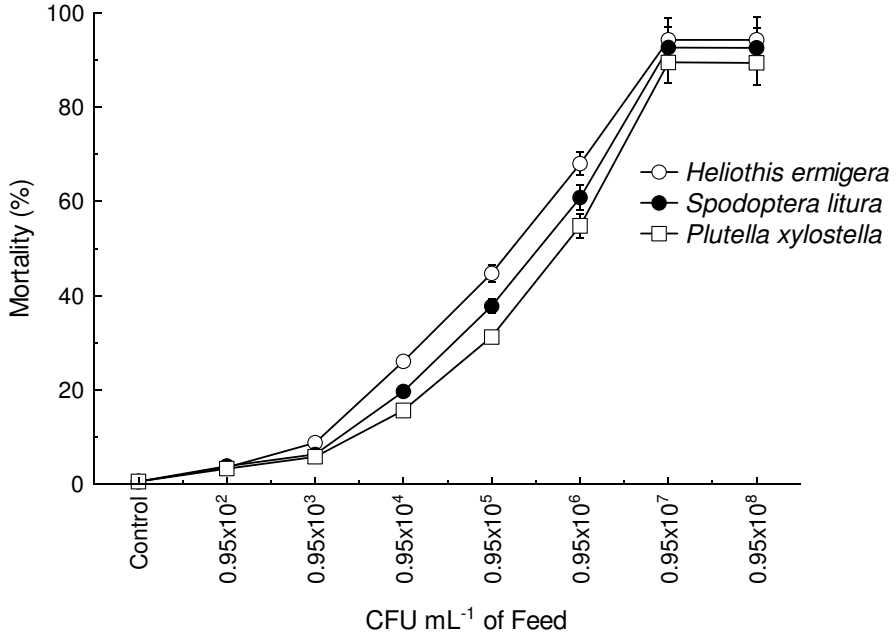

Fig. 2: Bioefficacy of *S. entomophila* AB2 against lepidopteron pests: *H. armigera*, *S. litura, P. xylostella*

Pesticidal activity for its general acceptability was verified against different lepidopteron pests. Bioefficacy of the isolate appeared quite high (≥ 90% of test pests) having a low LC_{50} value (≤ $1.5x10^5$ CFU mL^{-1}). Red or pink biotype strains of *Serratia* sp. are highly lethal to insect pests (Grimont et al., 1977). The isolate AB_2 infected larvae became brown, indicating complete infection. The Lepidopteron pests encompass a broad spectrum of plant taxa including cruciferous crops, such as cabbage, cauliflower and mustard. Several workers observed the resistance of diamondback moth against the pesticidal agents like, *B. thuringiensis* (Tabashnik et al., 1998; Heckel et al., 2007).

Table 1: Effect of *S. entomophila* AB_2 (LC_{50}) on mortality of insect larvae

Median of applied dose (CFU mL^{-1} of Feed)	Mortality (%) at 72 h[a]			LC_{50} (CFU mL^{-1} of Feed) (95% confidential level)[b]		
	H. ermigera	***S. litura***	***P. xylostella***	***H. ermigera***	***S. litura***	***P. xylostella***
1.5 X 10^6	42.50	39.24	36.25	0.44 x 10^5 (2.75 X 10^5 & 0.33 X 10^5)	0.95 X 10^5 (0.41 X 10^5 & 0.14 X 10^5)	1.44 x 10^5 (2.75 X 10^5 & 0.14 X 10^5)

[a]*Mortality rates for overall seven dose represents as average value*
[b]*Calculated through probit analysis on mortality (%) after 72 h of feed ingestion (upper and lower fiducial limits within bracket)*

3.2 Inorganic Nutrient Solubilization

Nutrient solubilization activity of the isolate was tested with agar plate diffusion technique determined by color change, from opaque to yellow, surrounding the colony due to lowering of pH (Table 2). The ratio between diameters of the colony and halo zone was used to evaluate the nutrient solubilizing activity. It was further observed that the isolate AB_2 was able to produce halo zone on plates containing $Ca_3(PO_4)_2$ and $ZnCO_3$, while *Pseudomonas putida* NCIM 2650 (positive control) was able to solubilize $Ca_3(PO_4)_2$ only.

Table 2: Nutrient solubilization activities(P&Zn) of *S. entomophila* AB_2

Treatment	Minimal agar media with $Ca_3(PO_4)_2$			Minimal agar media with $ZnCO_3$		
	z[a] (mm)	n[b] (mm)	z/n[c]	z (mm)[a]	n (mm)[b]	z/n[c]
P. putida NCIM 2650	23.00	10.00	2.30	-	-	-
S. entomophila AB_2	29.75	12.75	2.33	33.75	18.50	1.82

[a]*Diameter of halo zone;* [b]*Diameter of colony;* [c]*Ratio between halo zone and colony diameter*

There is an increasing trend to use microorganisms having macro- and micro-nutrient solubilizing property for ICM (Stamford et al., 2007), with increased availability of phosphorus and zinc to support plant growth (Katiyar and Goel, 2003). The working isolate *S. entomophilla* AB_2 can be exploited for nutrient acquisition for P &Zn. It showed notably better solubilizing property when compared with *P. putida* NCIM 2650, a commercial phosphate solubilizer.

3.3 Cross Reactivity Test

While understanding the ability of the isolate for maintenance of soil microenvironment through cross reactivity test, it did not show growth retarding effect between the isolate and the test rhizospheric organisms (Fig. 3). The isolate found indifferent towards symbiotic (*Rhizobium japonicum*) and free living (*Azotobactor chroococcum*, *Azospirillum brasiliensis*) nitrogen fixing bacteria, as well as to bacterial plant pathogen (*Erwinia amylovora*).

Bacterium-bacterium antagonism is not uncommon. Bacterial antagonistic interactions were examined among marine pelagic bacteria through Burkholder agar diffusion assay (Long and Azam, 2001). Microbial antagonism from Arctic soil was also demonstrated (Prasad et al., 2011). However, Roberts et al. (2005) reported an increased biocontrol with the help of certain combinations of *Trichoderma virens*, *Serratia marcescens* and *Burkholderia* spp. using antagonism with soil borne pathogens *Rhizoctonia solani* and *Pythimum ultimum* on cucumber plants. But in the present study *S. entomophila* AB_2 was not found to show any antagonism with beneficial (*Pseudomonas putida*, *Azotobactor chroococcum*, *Azospirillum brasiliensis*, *Rhizobium japonica*) or pathogenic (*Erwinia amylovora*) rhizospheric bacteria.

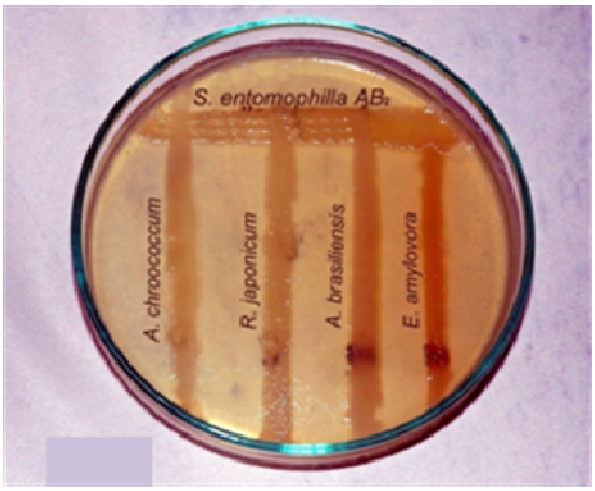

Fig. 3: Cross reactivity of *S. entomophila* AB_2 against test bacteria (*Pseudomonas putida*, *Azotobactor chroococcum*, *Azospirillum brasiliensis*, *Rhizobium japonica*, *Erwinia amylovora*) following conventional T-test.

3.4 Toxicity Tests

Feeding trials with mice failed to trigger mortality within the range of test doses ($1.0x10^8$ to $1.0x10^{18}$ CFU mL^{-1}) and did not induce even clinical symptoms of toxicity either immediately or in post feeding period (Table 3). In rats TBARS assay showed the lipid peroxidation magnitude of the liver was within threshold limit, supported by SGPT assay of blood samples, providing further the evidence of normal liver function (no damage) even after oral administration of bacterial dose of $1.0x10^{18}$ CFU mL^{-1} (Fig. 4).

Table 3: Feeding Effect of *S. entomophila* AB_2 on body weight of Swiss Albino mice

Bacterial load (CFU mL^{-1})	Change of body weight according to duration of feeding (in week)[a]					
	0[b]	2	4	6	8	10
Control	25.0±0.5	27.2±0.4	28.7±0.4	30.0±0.4	31.2±0.5	32.0±0.4
$0.95X10^8$	25.2±0.5	27.4±0.3	28.9±0.3	30.5±0.4	31.6±0.5	32.4±0.4
$0.95X10^9$	25.1±0.5	27.1±0.3	28.5±0.6	29.7±0.4	30.9±0.3	31.7±0.5
$0.95X10^{10}$	25.2±0.5	27.4±0.3	28.9±0.3	30.5±0.4	31.6±0.5	32.4±0.4
$0.95X10^{11}$	25.2±0.5	27.4±0.3	28.9±0.3	30.5±0.4	31.6±0.5	32.3±0.4
$0.95X10^{12}$	25.2±0.5	27.4±0.3	28.9±0.3	30.5±0.4	31.6±0.5	32.4±0.4
$0.95X10^{13}$	25.0±0.5	27.2±0.4	28.7±0.4	30.0±0.4	31.3±0.3	32.0±0.4
$0.95X10^{14}$	25.0±0.5	27.2±0.4	28.7±0.4	30.0±0.4	31.2±0.5	32.0±0.4
$0.95X10^{15}$	25.0±0.5	27.2±0.4	28.5±0.6	29.7±0.4	31.2±0.5	32.5±0.4
$0.95X10^{16}$	25.0±0.5	27.2±0.4	28.7±0.4	30.0±0.4	31.2±0.5	32.0±0.4
$0.95X10^{17}$	24.8±0.2	26.9±0.3	28.7±0.4	30.0±0.4	31.2±0.5	32.2±0.4
$0.95X10^{18}$	24.8±0.2	26.9±0.3	28.6±0.5	30.7±0.4	31.3±0.3	32.1±0.5

[a] *Values presented as the mean ± SD;* [b] *Initial body weight*

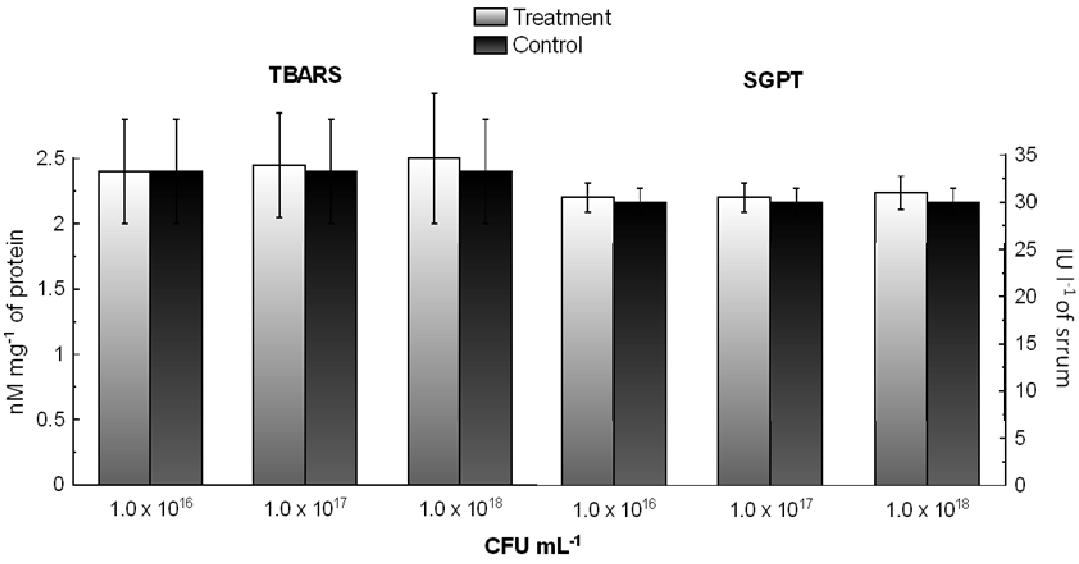

Fig. 4: Effect of *S. entomophila* AB_2 on TBARS and SGPT activities for Mammalian toxicity in adult male Sprague Dawley strain of Swiss albino rat

The bacterial strain, *S. entomophilla* AB_2 possesses suitable criteria for its use as a biocontroling agent against moth larvae, but animal toxicity may limits its practice (Stock et al., 2003; Tao et al., 2006). However, the experimental results indicate no antagonistic effect with test rhizospheric bacteria and practically no mammalian toxicity has been observed with high experimental dose of oral administration of experimental mice and rats. Therefore, it appears that the *S. entomophilla* AB_2 may be potential for controlling lepidopteron pests.

4. CONCLUSIONS

To minimize inorganic input in soil environment through commercial cultivation, pest control by natural means is the alternative way out for any field management. Thus, the use of organics becomes inevitable for ICM practice, especially for production of organic foods. Though biopesticides are available in the market but the increasing trend of resistance by common pests and horizontal gene transfer sometimes raises serious restriction in their use. Moreover, there is no isolate available that can play the role of biopesticide as well as biofertilizer, to make ICM practice more effective.

The study undertaken suggests that the isolate *S. entomophila* AB_2 is having dual property to act as pesticide, as well as fertilizer. In the present scenario, when most of the pest species of *Heliothis*, *Spodoptera*, and *Plutella* have evolved resistance against different *Cry* genes of Bt. The present study ensures a new bacterial pesticide of non-transgenic origin that also facilitates nutrient availability and resulting non toxic to mammals becomes an ecofriendly component for ICM. Alternatively, search for new isolates having multidimensional use in agriculture or atleast having novel pesticidal genes would be the best possible attempt to control insect pests. Thus, the isolate *S. entomophila* AB_2 carries enough promise for its exploitation as sound ecofriendly alternative of chemical pesticide and fertilizer, as well.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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