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Biochemical and molecular characterization of *Bacillus* spp. isolated from insects

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Abstract
In the present study two isolates of bacteria from insect cadavers were characterized by 16S rRNA, protein profiling, biochemical, antibiotic sensitivity and evaluated for insecticidal activity against *Aphis gossypii*. Isolates VKK-AC1, VKK-SL1 identified as *Bacillus thuringiensis (Bt*), *B. subtilis* (*Bs*) respectively through 16S rRNA sequencing. SDS–PAGE analysis reveals that two isolates shows protein pattern range of 20-245 kDa. Both the strains were positive to catalase, nitrate, citrate, glucose, argenine and negative reaction with Voges-proskauers. *Bt* VKK-AC1, *Bs* VKK-SL1 and one reference *Bt* strain (*Btk* HD1) were sensitive to antibiotics which act on protein synthesis *viz.*, streptomycin, tetracycline and resistant to ampicillin, pencillin G. Toxicity studies revealed that isolates *Bt* VKK-AC1 and HD1 were most effective in trypsinized form whereas *Bs* VKK-SL1 was found to be effective in pre-solubilized form (90% mortality). The present study shows that potential of *B. subtilis* besides *Bt* as biocontrol agent for successful management of insect pests.

Keywords: 16S rRNA gene, SDS-PAGE, biochemical, antibiotic sensitivity, *Bacillus thuringiensis*, *B. subtilis*

1. Introduction
Insects are the most diverse group of animals with over a million of different species found almost in every habitat [1]. They are inescapably associated with an extremely large variety of microrganisms due to their widespread distribution. Bacteria is one of them, the major species of bacteria with mechanisms to infect and kill healthy insects are spore forming bacilli. Many different *Bacillus* species have been isolated from dead or living insects. The *Bacillus* species commonly recognized as insect pathogens, like *B. popilliae*, *B. lentimorbus*, *B. larvae*, *B. thuringiensis*, and some strains of *B. sphaericus* [2]. For these species, the haemolymph of insect larvae is an excellent nutritional environment for bacterial proliferation, and sometimes for sporulation [3]. The ubiquity and diversity of these bacteria in nature, an unusual resistance of their spores to physical and chemical agents, production of antibiotics, the toxicity of their spores and protein crystals to various insects have given them impetus for unceasing interests in these bacteria for more than a century. During sporulation many *Bacillus* strains produce crystal proteins that have insecticidal actions. At present *Bacillus thuringienis (Bt)*, is the only microbial insecticide in widespread use but with the development of resistance in some insects, there is increased research on other *Bacillus* spp for pest control management.
Bacillus spp. is gram positive bacterium, ubiquitous, spore-forming bacterium. In *Bt*, δ endotoxin is expressed during sporulation and is divided into the crystal (Cry) and cytolytic (Cyt) toxins. The Cry toxins found to be active against specific insect orders *viz.*, Lepidoptera, Diptera, Hymenoptera and Coleoptera while the Cyt toxins are toxic to Diptera [4]. The alkaline pH of the lepidopteran mid gut in combination with mid gut proteases leads to activation of the protoxin to toxin. The disruption of the gut epithelium by binding of toxin to receptors in the gut epithelial cells as a result feeding cessation occurs leading to starvation and finally death of the insect. However, a decade back Broderick and co-workers revealed that the enterobacteria that normally reside in the insect mid gut is responsible for the insecticidal activity of *Bt* [5].
B. sphaericus is another member of the insecticidal toxin producing species of the *Bacillus* genus which is toxic to against mosquito larvae and is part of the *B. subtilis* group [6]. The mosquitocidal activity is due to the action of two types of toxins, the highly active binary toxins BinA/BinB within spore crystals and the Mtx toxins [7]. Upon ingestion by the target insect, the binary toxins are solubilised, activated by proteases, bind to specific receptors that leads to pore formation in target cells followed by disruption of the mosquito gut epithelium [8].

Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using colony/cell morphology, gram staining, as well as physiological, biochemical and nutritional features that resembled *Bacillus* spp. The versatile physiology of *Bacillus* spp. requires biochemical tests for their identification [9]. 16S rRNA gene sequence analysis has proved to be of evident for phylogenetic analysis of bacteria [10]. *Bacillus* species may be divided into five or six groups (groups I–VI), based on 16S rRNA phylogeny or phenotypic features respectively [11]. Pathogenicity among *Bacillus* spp. is however mainly a feature of organisms belonging to the *B. cereus* group, a subgroup of the *B. subtilis* (*Bs*) group (group II) within the *Bacillus* genus. The insecticidal activity of *B. subtilis* against *Helicoverpa armigera*, *Earias vitella*, *Pectinophora gossypiella* (Kalia *et al.*, unpublished) and aphids [12] is emphasized by the fact that they do produce toxins. Thus, *B. subtilis* is of research interest to understand its physiological diversity, genetic relatedness with other *Bacillus* spp. and the possible presence of insecticidal factors. The present study dealt with the identification of two *Bacillus* spp. using 16S rRNA gene sequencing and their comparative characterization by insecticidal activity, protein profiling, biochemical, nutritional features and antibiotic sensitivity assays.

Material and Methods

Bacterial isolates

Two *samples viz.*, VKK-SL1 and VKK-AC1 were isolated from larvae of *Spodoptera litura* and *Aphis craccivora* [13] respectively. *Bacillus thuringiensis* var. *kurstaki* strains HD-1, (obtained from Pasteur Institute, Paris) was used as reference strain for this study.

Molecular Characterization of *Bacillus* isolates through 16S rRNA gene

Molecular characterization of these isolates was done by analyzing 16S rRNA gene sequence for further confirmation of *Bacillus* spp. The 16S rRNA gene of two isolates (VKK-SL1 and VKK-AC1)) were PCR amplified using universal primers *viz.*, 27 Forward: 5' AGAGTTTGATCCTGGCTCAG 3', 1492 Reverse: 5' TACGGCTACCTTGTTACGACTT 3' designed by Lane [14]. All PCR reactions were carried out in 50 µl reaction volumes. DNA template (~50 ng) was mixed with reaction mixture of 5.0µl consisting of Taq assay buffer (10x), 2 µl dNTPs (10 mM), 1 µl of each primer (100 ng), 0.2 µl Taq DNA polymerase (3 U/µl) and make up the volume up to 50 µl with PCR grade water. The reactions were placed in a thermocycler (Genepro, BIOER). An initial denaturation step was applied for 5 min at 94 ºC and followed by denaturation for 30 sec at 94 ºC, annealing for 30 sec at 55 ºC then extension for 1.30 min at 72 ºC. Thirty-five cycles were carried out with final extension step for 5 min at 72 ºC. The PCR product was loaded on 1.0 % agarose gel along with 500 bp DNA ladder. Gels were visualized in a gel documentation system (Alphaimager™).

Subsequently, ~1.3 kb PCR products (Fig.1) were purified using a PCR purification kit (Qiagen, Germany) and sequenced by Amnion Biosciences Pvt. Ltd, Bengaluru.

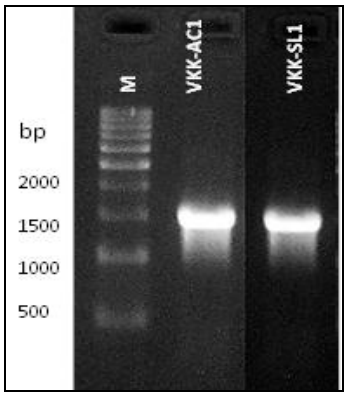

Fig 1: PCR amplified product from VKK-AC1 and VKK-SL1 isolates for 16s rRNA gene along with 500 bp marker

The sequences were subjected to homology search using BLAST programme of the National Centre for Biotechnology Information (NCBI). Based on the homology index the bacteria were identified and phylogenetic tree was constructed using NCBI: https://blast.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?request=page&blastRID=VMTFX2C301R&queryID =gb|KT714051.1.

Toxicity studies

Two native *Bacillus* isolates *viz.*, *Bacillus thuringiensis* strain VKK-AC1 (*Bt* VKK-AC1) and *Bacillus subtilis* strain VKK-SL1(*Bs* VKK-SL1) and one reference *Bt* strain (*Btk* HD-1) were evaluated for its bioactivity against adults of cotton aphids. The feeding assays were carried by diet incorporation method at single concentration (10 µg $g^{-1}$of diet) in three different forms *viz.*, pre-solubilized form (spore crystal), solubilized form (pre-toxin form), trypsinized form (toxin form) on the basis of total protein concentration [15]. Each container served as one replicate, with three replications per treatment. Ten adults were released on the treated diet per replication and fed for four days. All the bioassays were performed with their respective buffer based controls under controlled conditions of 18 ± 2 °C, 70 ± 10% RH, and 16:8 L: D against adult aphids. Mortality data was recorded after every 24 h till 96 h. Per cent mortality was calculated on 4th day of bioassay.

Protein Profiling by SDS –PAGE

The protein profiles of *Bt* spore-crystal toxins were studied by SDS-PAGE according to the discontinuous system of Laemmli [16]. Samples were run on 10 % SDS Polyacrylamide gel and gel analysis done using Alphaimager™ Documentation system and analysed [12].

Biochemical characterization of *Bacillus* isolates

Biochemical characterization of *Bacillus* isolates was carried out by using standard HiBacillus™ Identifications kit and KB009 HiCarbohydrate™ Kit. The main principle of this test is change in pH and substrate utilization after incubating, which exhibited as a visual colour change in the media. The

HiBacillus™ Identifications kit consists of 12 tests for identification of *Bacillus* species like major carbohydrates assimilation, catalase, nitrate reduction and Voges-Proskauer's. Whereas, KB009 HiCarbohydrate™ Kit comprises of 35 exclusively carbohydrate utilization tests. 50 µl of inoculum which is grown over night on nutrient broth was added to each well and incubated for 24 hrs at 37 ºC. 48 h old culture grown on nutrient agar was used for Catalase test. 3% hydrogen peroxide was added to the well containing loop full of bacterial culture and formation of gas bubbles treated as a score for catalase positive. In case of VogesProskauers test 1-2% of Barritt reagent A added followed by Barritt reagent B. Nitrate reduction test performed by adding 1-2 drops of sulphanilic acid followed by1-2 drops N, N-Dimethyl-1-Napthylamine.

Antibiotic susceptibility

A total of 33 antibiotics with a different mode of action *viz.*, inhibition of protein synthesis (9), bacterial metabolism (2), DNA synthesis (3), cell wall inhibitors (19) were used for susceptibility test. In order to characterize *Bacillus* isolates, antibiotic susceptibility tests were performed including reference strain HD-1 by the standard disc-diffusion method on Muller Hinton agar with antibiotic disc (Hi-Media). The cultures were grown in nutrient broth overnight and different antibiotic discs with concentrations as per manufacturer's protocol were placed on the plates inoculated with bacteria and allowed to incubate for 24 hours at 30 ºC after which zone of inhibition was observed where antibiotics impeded the bacterial growth.

Results

Molecular characterization of *Bacillus* isolates with 16S rRNA gene

Bacillus isolates VKK-AC1 and VKK- SL1 were characterized using 16S rRNA gene sequencing for identification of *Bacillus* species. PCR amplified products were sequenced and compared with the 16S rRNA gene sequences submitted in the GenBank Database by using Neighbor joining method. The 16S rRNA gene sequences of isolate VKK-AC1 showed 99% similarity with sequences of *B. thuringiensis* and identified as *B. thuringiensis* strain (Fig 2). Whereas, VKK-SL1 isolate was identified as *B. subtilis* (Fig 3). Sequence of VKK-AC1 submitted to GenBank (NCBI) [Acc. No.KT714051.1] and sequence of VKK-SL1 is yet to be submitted.

Efficacy of *B thuringiensis* and *B. subtilis* strains against *Aphis gossypii*

Bs VKK-SL1 was found to be most effective with 90% mortality in pre-solubilized form followed by *Bt* VKK-AC1 (53.3% mortality) and *Btk* HD-1 (30% mortality)(Table 1). While in solubilized form mortality was ranged from 30 to 53.33% but was found to be significantly different. Nevertheless, toxicity of *Bt* VKK -AC1 was found to be at par in both pre as well as solubilized form. In trypsinized form, strains of *B. thuringiensis viz.*, *Btk* HD-1 (80% mortality) and *Bt* VKK-AC1(90% mortality) were found to be better than *B. subtilis* i.e. *Bs* VKK-SL1 (50% mortality). There was increased trend of toxicity found in all three forms of *B. thuringiensis* in both reference strain (*Btk* HD-1) and native isolate (*Bt* VKK -AC1). This may be due to the conversion of spore crystal complex (pre-solubilized) to pre-toxin (Solubilized form) to toxin form (Trypsinized form). Native isolate *Bt* VKK-AC1 was found to be significantly different and effective than reference strain *Btk* HD-1 and *B. subtilis* in both solubilized as well as trypsinized form. Yet *B. subtilis* isolate VKK-SL1 was found to be effective in pre-solubilized form.

Table 1: Toxicity of *Bacillus thuringiensis viz.*, reference strain *Btk* HD-1 & *Bt* VKK-AC1 and *B. subtilis* strain *Bs* VKK-SL1 in pre solubilised, solubilised and trypsinized form against adults of *Aphis gossypii*

S. No	*Bt* Strains ID	Corrected per cent mortality*		
		Pre-solubilized	Solubilized	Trypsinized
1	*Btk*HD-1	30.00^{c}	46.66^{b}	80.00^{b}
2	VKK -AC1	53.33^{b}	53.33^{a}	90.00^{a}
3	VKK -SL1	90.00^{a}	30.00^{c}	50.00^{c}

*Numeral in same column followed by different alphabets is significantly different at 5% level

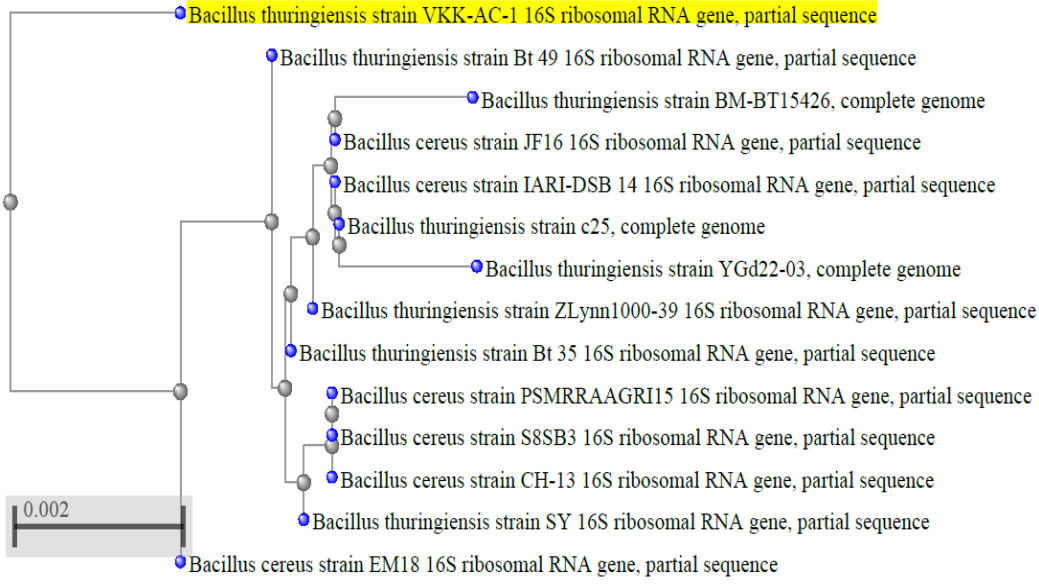

Fig 2: Phylogenetic analysis of VKK-AC1 based on 16S r RNA gene sequence

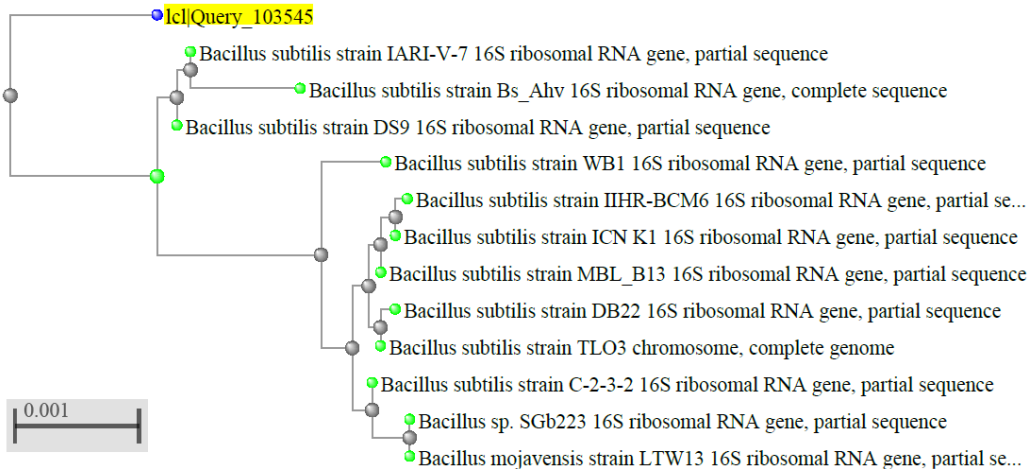

Fig 3: Phylogenetic analysis of VKK-SL1 based on 16S rRNA gene sequence

Characterization of *Bacillus* isolates protein by SDS-PAGE:

Protein profile characterization of *Bt* VKK-AC1 and *Bs* VKK-SL1 by SDS–PAGE showed banding pattern ranging from 39-105 and 47-245 kDa in presolubilized form, whereas 22-94 kDa and 27-110 kDa in solubilized form respectively (Fig 4). In case of trypsinized form, in *Bt* as well as in *Bs* strains showed 2 bands each just in the range of 60-65 kDa. However in *Bs* VKK-SL1 protein bands of <60 kDA were also present.

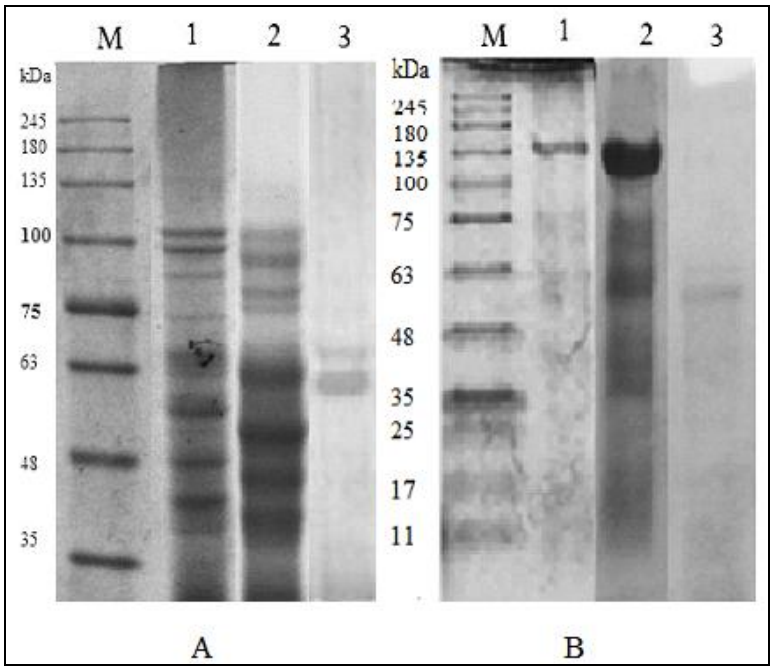

Fig 4: A. Protein profile of *Bacillus thuringiensis* strains VKK-AC1: Lane M-Marker, Lane1-Pre-solubilized form, Lane2- Solubilized form, Lane3-Trypsinized form

B. Protein profile of *Bacillus subtilis* strains VKK-SL1: Lane M-Marker, Lane1-Pre-solubilized form, Lane2- Solubilized form, Lane3-Trypsinized form

Biochemical characterization

Two native *Bacillus* isolates *viz.*, *Bt* VKK-AC1 & *Bs* VKK-SL1 and one reference strain *Btk* HD-1 screened for biochemical test using KB013 HiBacillus identification kit and KB009 HiCarbohydrate™ Kit (Table 2 & 3). The results indicated that strains *Bt* VKK-AC1 and *Bs* VKK-SL1 showed similar positive reaction to nitrate reduction by converting nitrate to nitrite. Catalase test was positive for all strains by generating oxygen when treated with H_2O_2. Regarding utilization of citrate as carbon source all the strains effectively utilized citrate and showed good growth. Both the isolates showed similar positive reaction to aregnine, glucose, maltose, dextrose, esculin hydrolysis and trehalose when compared with reference strain HD1. All the three strains showed negative reaction with voges-proskauers, ONPG, mannitol, arabinose.

Table 2: Biochemical characterization of *Bacilus* isolates using KB013 HiBacillus identification kit

S. No	Test	*Btk* HD-1	*Bt* VKK-AC1	*Bs* VKK-SL1
1	Malonate	-	-	+
2	Voges-Proskauer's	-	-	-
3	Citrate	+	+	+

4	ONPG	-	-	-
5	Nitrate reduction	+	+	+
6	Catalase	+	+	+
7	Arginine	+	+	+
8	Sucrose	-	-	+
9	Mannitol	-	-	-
10	Glucose	+	+	+
11	Arabinose	-	-	-
12	Trehalose	+	+	+

-Not present; + Present

However, *Bs* VKK-SL1 alone showed positive response to malonate and sucrose utilization. The three strains responded similarly for few tests when both *Bacillus* identification and carbohydrate kits were used. *Btk* HD1 alone shown positive reaction to cellobiose and salicin moreover *Bt* VKK-AC1 showed positive reaction for inulin and sodium gluconate. The two *Bacillus* strains VKK-AC1 and HD-1 showed positive reaction to glycerol and showing similar pattern of biochemical properties as both are *Bt* strains and confirming results of 16S rRNA gene sequencing. Whereas strain *Bs* VKK-SL1, *Btk* HD-1 showed positive reaction to fructose. The variable response of two *Bacillus* isolates in utilization of carbohydrates and other biochemical tests made them diverse strains with HD-1.

Antibiotic susceptibility

Antibiotic susceptibility study reveals that two native *Bacillus* isolates *viz.*, *Bt* VKK-AC1 & *Bs* VKK-SL1 and *Btk* HD-1 were highly susceptible to nine antibiotics which act on protein synthesis *viz.*, streptomycin, tetracycline, erythromycin, gentamycin, clindamycin, clarithomycin, linezolid, chloramphenicol, doxycycline and three antibiotics which inhibit DNA synthesis *viz.*, ciprofloxacin, nalidixic acid, levofloxacin (Table 4). All the three strains were showing resistance to ampicillin, pencillin G, amoxyclav, ceftazidime, fostomycin, cefepime as all of them are inhibiting cell wall formation. Isolate *Btk* HD-1 and *Bt* VKK-AC1 showed resistance to co-trimoxazole which is an inhibitor of bacterial metabolism and cell wall inhibitors like cefuroxime, clavulanic acid, piperacillin, cephalothin, oxacillin. *Bs* VKK-SL1 alone showed resistance to cefotaxime, which is a cell wall inhibitor.

Table 3: Carbohydrate utilization by different isolate was determined by using KB009 HiCarbohydrate^{TM} Kit

S. No	Isolates / Test	*Btk* HD-1	*Bt* VKK-AC1	*Bs* VKK-SL1
1.	Lactose	-	-	-
2.	Xylose	-	-	-
3.	Maltose	+	+	+
4.	Fructose	+	-	+
5.	Dextrose	+	+	+
6.	Galactose	-	-	-
7.	Raffinose	-	-	-
8.	Trehalose	+	+	+
9.	Melibiose	-	-	-
10.	Sucrose	-	-	+
11.	L-arabinose	-	-	-
12.	Mannose	-	-	-
13.	Inulin	-	+	-
14.	Sodium gluconate	-	+	-
15.	Glycerol	+	+	-
16.	Salicin	+	-	-
17.	Dulcitol	-	-	-
18.	Inositol	-	-	-
19.	Sorbitol	-	-	-
20.	Mannitol	-	-	-
21.	Adonitol	-	-	-
22.	Arabitol	-	-	-
23.	Erythritol	-	-	-
24.	**α** Methyl-D-mannoside	-	-	-
25.	Rhamnose	-	-	-
26.	Cellobiose	+	-	-
27.	Melezitose	-	-	-
28.	**α** Methyl-D-Mannoside	-	-	-
29.	Xylitol	-	-	-
30.	ONPG	-	-	-
31.	Esculin Hydrolysis	+	+	+
32.	D-Arabinose	-	-	-
33.	Citrate utilization	+	+	+
34.	Malonate	-	-	+
35.	Sorbose	-	-	-

-Not present; + Present

Table 4: Antibiotic susceptibility of three *Bacillus* strains

S. No.	Antibiotics	Mode of action	*Btk* HD-1	*Bt* VKK-AC1	*Bs* VKK-SL1
1	Gentamycin	Protein Synthesis	S	S	S
2	Clarithomycin	Protein Synthesis	S	S	S
3	Streptomycin	Protein Synthesis	S	S	S
4	Linezolid	Protein Synthesis	S	S	S
5	Erythromycin	Protein Synthesis	S	S	S
6	Tetracycline	Protein Synthesis	S	S	S
7	Clindamycin	Protein Synthesis	S	S	S
8	Chloramphenicol	Protein Synthesis	S	S	S
9	Doxycycline	Protein Synthesis	S	S	S
10	Co- trimoxazole	Bacterial metabolism	R	R	S
11	Nitrofurantoin	Bacterial metabolism	S	S	S
12	Ciprofloxacin	DNA synthesis	S	S	S
13	Nalidixic acid	DNA synthesis	S	S	S
14	Levofloxacin	DNA synthesis	S	S	S
15	Ampicilin	Cell wall	R	R	R
16	Cephalothin	Cell wall	R	R	S
17	Oxacillin	Cell wall	R	R	S
18	Ampicillin / sulbactum	Cell wall	R	R	S
19	Cefotaxime	Cell wall	S	S	R
20	Ceftriaxone	Cell wall	S	S	S
21	Fostomycin	Cell wall	R	R	R
22	Vancomycin	Cell wall	S	S	S
23	Penicilin G	Cell wall	R	R	R
24	Amoxyclav	Cell wall	R	R	R
25	Teicoplanin	Cell wall	S	S	S
26	Cefuroxime	Cell wall	R	R	S
27	Cefoxitin	Cell wall	S	S	S
28	Ticarcillin/ clavulanic acid	Cell wall	R	R	S
29	Imipenem	Cell wall	S	S	S
30	Cefepime	Cell wall	R	R	R
31	Piperacillin/ Tazobactum	Cell wall	R	R	S
32	Methicillin	Cell wall	S	S	S
33	Ceftazidime	Cell wall	R	R	R

Discussion

Conventional methods based on biochemical and phenotypic techniques for the identification of aerobic Gram-positive spore bearing bacilli is the most common method used in the laboratory. However, due to the similarities among closely related species, species identification is sometimes difficult. 16S rRNA gene sequencing remains the standard approach in most of the cases, although it is not always practical for routine use due to its high cost. In the present study the *Bt* VKK-AC1, *Bs* VKK-SL1 were 99 % identical to the 16S rRNA gene sequence of several *B. thuringiensis* and *B. subtilis* strains submitted in GenBank database respectively. Hence, VKK-AC1 is considered as strain of *B. thuringiensis* and VKK-SL1 as *B. subtilis*. Similarly, 16S rRNA gene sequencing confirmed that twelve of 18 isolates collected from soil samples of different parts of Karnataka as *B. thuringiensis* [17]. Earlier studies on 16S rRNA gene sequences of *B. thuringiensis*, *B. anthracis* and *B. mycoides* helped to consider all these three species as subspecies of *B. cereus* [10]. Based on the 16S rRNA gene sequence analysis, 5 groups have been identified within the genus *Bacillus*, of which the group 1 (*B. subtilis* group) comprises of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* [18].

In the present study native isolates *Bt* VKK-AC1 was found to be significantly different and effective than reference strain *Btk* HD-1 and *Bs* VKK-SL1 in both solubilized as well as trypsinized form. Yet *B. subtilis* strain VKK-SL1 was found to be effective in pre-solubilized form than trypsinized form against cotton aphid, this shows the presence of toxin other than δ endotoxin such as coat protein [19]. 80%, 70% and 100% mortality reported when *H. armigera* larvae treated with suspensions of *B. subtilis*, *B. thuringiensis*, *Bs/Bt* mixture respectively. Significant low LC50 values reported in *Bt/Bs* mixture than *Bt* and *Bs* individually [20]. 40 strains of *B. thuringiensis* isolated from corpses of Hemiptera and evaluated their activity against *Myzus persicae*, out of 40 strains, seventeen strains were significantly different from the control, causing mortality ranged from 64.4 to 88.9% at 10 ng/µL total protein concentration, and from 71.1 to 91.1% at 100 ng/µL total protein concentration [21]. Moreover, our results were positively correlated with earlier reports that the trypsinized form of *Bt* strains was effective against pea aphid [22].

Simplest way to compare protein profiles of *Bacillus* spp is SDS-PAGE analysis. In the present study protein profile analysis of pre-solubilized *Bt* strains showed the banding patterns of 20 - >245 kDa in *Bs* VKK-SL1 and 27-110 kDa in *Bt* VKK-AC1. Correspondingly to our findings, a protein with a molecular weight of about 230 kDa was obtained when the crystal is dissolved under controlled conditions without reducing agents [23]. *Bt* strains are grouped into three main protein groups based on protein profiles *viz*., group I (28 -58 kDa), group II (60- 80 kDa) and group III (125-150 kDa) [24]. Biochemical characterization of two native isolates showed positive reaction to nitrate reduction, catalase, citrate, argenine, glucose, maltose, dextrose, esculin hydrolysis and trehalose when compared with reference strain *Btk* HD-1. *Bt* strains collected from high altitude mountains, forests, horticultural plantations also showed similar response [25]. *B. thuringiensis* strains showed positive reaction to nitrate, catalase, starch, casein hydrolysis [26-28]. *B. thuringiensis* strains isolated from soils of Kolhapur, Maharashtra were also

shown positive response to catalase, nitrate, starch, Voges–Proskauer [29]. In contrary to this in the present study *Bacillus* isolates showed negative response to Voges-Proskauer and positive reaction to argenine. These results are positively correlated with earlier reports when *Bt* stains isolated from different sources like grains, soils from sudan [30]. *Bs* VKK-SL1 alone showed positive reaction to malonate and sucrose and showed similar pattern of sucrose positivity like *B. subtilis*. The three strains are showing variable performance in utilization of carbohydrates and other biochemical properties due to diversity among the strains.

Antibiotic studies also revealed that all strains showed variation in susceptibility to antibiotics. Three strains showed susceptibility to the antibiotics which interfere protein synthesis and DNA synthesis whereas, showing resistance to cell wall inhibitors *viz.*, ampicillin, pencillin G. *Btk* HD-1 and *Bt* VKK- AC1 were resistance to co-trimoxazole and can be grouped under a similar category. Strain HD-1 and other soil isolated strains from mountains, forests areas showed resistance to ampicillin and co-trimoxazole [25]. *Bt* isolates were resistant to ampicillin, pencillin, amoxicillin, oxacillin which are susceptible to other bacterial isolates [31]. Strains SBT-21, SBT-22, SBT-62, HD-1 isolated from soils of different parts of Karnataka were highly effective against white grub. These four strains reported as ampicillin resistance and highly susceptible to other most commonly used antibiotics like streptomycin, tetracycline, vancomysin, kanamycin [17]. Similar pattern of results was observed in the present study. There is a wide spread distribution of ampicillin resistance in *B. thuringiensis* strains [32].

Thus biochemical and antibiotic susceptibility studies can be considered as preliminary keys to check the variability in the different *Bacillus* isolates. Morphological, biochemical and antibiotic resistance tests proved that both *Bt* VKK-AC1 and *Bs* VKK-SL1 were aerobic and *Bacillus* species. A variable response in some biochemical tests confirms that these two belong to two different species. Molecular biological methods like nucleic acid analysis, protein patterns have great importance due to rapid identification of bacteria [33]. Species-specific 16S rRNA gene sequencing is an effective tool for rapid identification and distinguishes closely related species.

The presence of low amounts of toxin-activating proteolytic activity in the aphid gut lumen is likely another limiting factor for *Bt* toxicity against aphids [34-35]. The high insecticidal activity of *Bs* VKK-SL1 in pre-solubilized form against cotton aphid in the present study clearly indicates that low amount of toxin activating proteolytic activity in the aphid gut lumen is not a limiting factor. Hence, *B. subtilis* can be considered as a potential microbial insecticide against aphids and can become an alternative to *B. thuringiensis* for effective management of insect pests. Further studies are needed to elucidate the mode of action of *Bacillus* species other than *Bt* to incorporate as a potential biocontrol agent in IPM Programmes.

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