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Isolation and Characterization of *Bacillus* from Some Warehouses in Trabzon

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Abstract

In order to identify *Bacillus* strains with new toxin combinations, 26 bacterial isolate belonging to *Bacillus* sp. were isolated from warehouses in Trabzon. Firstly, colonial and cellular characteristics, then, physiological features and biochemical properties of these isolates were analyzed by the light microscopy, manual tests and API kit, respectively. For the molecular characterization, 16S rDNA sequence and *cry* gene contents were detected. As a consequence of characterization, the isolates were identified as *Bacillus thuringiensis*, *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. atrophaeus*, *B. megaterium* and *Lysinibacillus sphaericus.* It was also recorded that the isolates Bg5 have a *cry*1 gene, B2, B3 and N6 have a *cry*3 gene. The toxic effects of the isolates were determined by the bioassay using larvae of *Plodi ainterpunctella* (Indianmeal moth), *Ephestia kuehniella* (Mediterranean flour moth) and adults of *Sitophilus granarius* (Wheat weevil). As a results of the bioassay, the highest insecticidal effects were 100% with Bg5 (*Bacillus thuringiensis*) against the larvae of *P. interpunctella* and 63,3% with B4 (*B. subtilis*) against the adult of *S. granarius*. Especially, Bg5 has the highest insecticidal effect may be valuable as a microbial control agent for lepidopteran warehouse pests.

Keywords: *B. thuringiensis*, *cry* gene, microbial control, warehouse, pests

INTRODUCTION

The genus of *Bacillus* is a spore-forming and Gram + bacteria that used mostly in biological control. Especially, *B. thuringiensis*, *B. sphaericus*, and *B. popilliae* species are used against the larvae of various pests. In addition, they originate the basis of microbiological insecticide [1]. In these days and future, the using Bt products on pest control, the screening different new Bt isolates, the detecting toxic effect of its *in vivo*/in laboratory, the improving application methods and the developing different formulations will be so important [2]. *B. thuringiensis* is ubiquitous in natural environments and is readily isolated from soil [3], [4], [5], and [6], warehouses [5], [7], and [8], leaf surfaces of broad leaf trees, conifers and grasses [5] and [9] and insect habitats [5], [6], [10], [11], [12], and [13]. Among these environments, warehouses provide an ideal environment for the creation of strains with new toxin combinations and long-term survival [7] and [14]. They harbor different subspecies *B. thuringiensis* to those isolated from soil [14], and are a particularly good source of *B. thuringiensis* [6]. Crystal proteins from many *B. thuringiensis* strains are toxic to lepidopteran pests and *B. thuringiensis* formulations are widely used to control lepidopteran pests [15].

Here, we report on the isolation and identification of the bacteria from warehouses in Trabzon. We tested the insecticidal activities of these bacterial isolates against the lepidopteran pests.

MATERIALS AND METHODS

Sample Collection

The samples were collected from warehouses, animal feed mills, and grain processing facilities by scraping about 5 g of material into sterile plastic bag and stored at 4 °C. The collected samples included the variety of residue materials present within the site: settled grain dusts, stored products, insect webbings, and insect cadavars.

Isolation of Bacillus Species

Approximately 1 g of each samples were suspended in 10 ml of nutrient broth, vortexed vigorously and incubated in test tube at 30 °C for 4 h, then pasteurized at 80°C for 10-15 min as described by Traver et al. (1987) [17]. Samples were plated on nutrient agar. The plates were incubated at 30°C for 48 h, and examined for colony morphology and the presence of spores by light microscopy. All spore-forming colonies were subcultured on L-agar [17] and maintained for further investigation.

Characterization of Bacteria

Physiological and Biochemical Tests

Bacterial cultures were identified by various tests, such as utilization of organic compounds, spore formation, NaCl tolerance, optimum temperature, motility test, starch hydrolysis, catalase test, and oxidase test. Five sets of nutrient broth were prepared containing 5%, 9%, 11%, 13%, and 15% NaCl, respectively. Semisolid motility test

medium may be used to detect motility. When motile organisms are stabbed into soft agar, they swim away from the stab line. Catalase activity was determined by the productioof bubbles from 3% (v/v) H_2O_2, and oxidase activity was determined using 1% (w/v) N,N,N',N'-tetramethyl-p phenylenediamine. Biochemical features of the bacterial isolates were determined using API 20E and API 50CH (bioMerieux) strips.

Molecular Characterization

16S rDNA Gene Sequencing

DNA was extracted from the bacterial isolates using Promega- Wizard Plus SV Minipreps DNA Purification System Kit and stored at +4 °C until use. The amplification and sequencing of the nearly complete 16S rDNA gene was performed according to the methods which has been already described (Ben-Dov, 1997). PCR amplification of 16S rDNA genes of bacterial isolates was performed with the following universal primers; UNI16S-L: 5'-ATTCTAGAGTTTGATCATGGCTCA-3'; UNI16S-R: 5'-ATGGTACCGTGTGTGACGG-3' [16]. PCR amplification was performed by using BioRad Thermal Cycler. PCR reactions and amplification were carried out as described before [18]. PCR products were analyzed by electrophoresis in 1% agarose gel. The gel was then examined in aGel Logic; Kodak. Amplified 16S rDNA gene fragments were cloned into pGEM-T Easy Vector (Promega) and transformed to *Escherichia coli* DH10β and JM101 strains. Sequencing of the 16S rDNA genes were performed by Macrogen Inc. (Amsterdam, Netherlands). No standardized guidelines exist for defining a bacterial species based on 16S [19], although Stackebrandt and Goebel (1994) [20] have suggested that less than 97% 16S identity definitively denotes separate species. So, the sequences obtained were used to perform BLAST searches [21] using the NCBI GenBank database. Comparison of approximately 1,400 bp fragments of 16S rDNA gene sequences of each isolates with other 16S rDNA sequences in the NCBI GenBank database [21] were performed and after comparison, species that shared a similarity between 97-100% were recorded for further identification. 16S rRNA gene sequences of Ld1-6 have been deposited in GenBank under accession number HQ132731, HQ132732, HQ659186, GU187010, HQ132733 and HQ132734, respectively.

Detecting of cry Gene Contents

In this work, universal primers were used for the detection of subgroups of *cry* genes. Primers are *cry*1 (forward, 5'-CATGATTCATGCGGCAGATAAAC-3'; reverse, 5'- TTGTGACACTTCTGCTTCCCATT-3'), *cry*2 (forward, 5'-GTTATTCTTAATGCAGATGAATGGG-3'; reverse, 5'-CGGATAAAATAATCTGGGAAATAG T-3'), *cry*3 (forward, 5'-CGT TAT CGC AGA GAG ATG ACA TTA AC-3'; reverse, 5'-CATCTGTTGTTTCTGGAGGCAAT-3') and *cry* 4 (forward, 5'-GCATATGATGTAGCGAAACAAGCC-3'; reverse, 5'-GCGTGACATACCCATTTCCAGGTCC-3') [18]. Each experiment was associated with negative (without DNA template) and positive (with *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *tenebrionis* and *B. thuringiensis* subsp. *israelensis*) controls.

Phylogenetic Analyses

16S rDNAwas aligned using the multiple alignment program, CLUSTAL W program [22]. Bootstrap analysis based on 1000 replicates was also conducted in order to obtain confidence levels for the branches [23]. The phylogenetic trees were constructed using the programs MEGA5.

Bioassays

To prepare the sporulated culture, all isolates were cultured in 5 ml nutrient broth mediumat 30°C for 72 h (for sporulation). After incubation, the bacterial density was measured at OD_{600}. Spore-forming bacteria were incubated in a nutrient broth medium respectively. And then, all these bacteria were tested against 3. instar larvae of *Ephestia kuehniella* ZELLER (Mediterranean Flour Moth) (Lepidoptera: Pyralidae) and *Plodia interpunctella* HUBNER (Indianmeal moth) (Lepidoptera: Pyralidae), and adults of *Sitophilus granarius* HUSTACHE (Wheat weevil) (Coleoptera: Curculionidae) at $1.8x10^9$ CFU/ml dose within ten days [24] and [25]. Also *B. thuringiensis* subsp. *tenebrionis* (MmBt) [26], *B. thuringiensis* subsp. *tenebrionis* (Xd3) [12], and *B. thuringiensis* subsp. *kurstaki* (BnBt) [27] strains were kindly provided as positive control by the Microbiology Laboratory at Department of Biology, Karadeniz Technical University, Trabzon/Turkey.

Toxicity of Bacillus Isolates against Lepidopteran and Coleopteran Pests

Indianmeal moth, *Plodia interpunctella* HUBNER, Mediterranean Flour Moth, *Ephestia kuehniella* ZELLER, and Wheat weevil, *Sitophilus granarius* HUSTACHE are the major lepidopteran and coleopteran pests of stored products in Turkey, and were obtained as laboratory colony from Faculties of Agriculture, Ankara University, University of Urmia, and Gaziosmanpasa University, respectively. The preparations were bioassayed with ¼ dried figs for the larvae of *P. interpunctella*, 1 gr of flour for the larvae of *E. kuehniella*, and 1 gr of wheat for the adults of *S. granarius*. Bioassays with larvae of *P. interpunctella* were performed with the bacteria applied on the diet. The diets were placed into individual sterilized glass containers. 30 third instar larvae were placed on the diet in containers. Containers were kept at 28±2°C and 60% Relative Humidity on a 12:12 h photo regime, with the diet without bacteria changed after eating. The mortalities of nymphs were recorded every 24 h and all dead larvae were removed from containers. Sterilized water was used in bioassay as negative control agent. Mortality was recorded 10 days after initiation of the treatment. Bioassays were repeated 5 times for each insect. All tests were repeated 3 times at differet times. Means were analyzed using one-way analysis of variance (ANOVA) and compared by least significant difference (LSD) test [28].

RESULTS

In this study, we isolated 26 bacteria from warehouses (including samples of wheat, peas, lentil, rice, chickpea, and hazelnut). These isolates were named as Bg 1-5 (wheat), B 1-5 (peas), N 1-10 (chickpea), and F 1-6 (hazelnut). The dust from lentil and rice did not contain any spore-forming bacteria. Total of these isolates were examined for morphology, spore formation, and motility (Table 1). Based on all tests, we were able to identify all isolated bacteria, to at least the genus level, as *Bacillus* sp. (Bg1, Bg4, B5, N2, F2, F3), *B. thuringiensis* (Bg2, Bg5, B3, N6, N9), *B. pumilus* (Bg3, N1, N3, N7, F1, F5 ve F6), *B. subtilis* (B1, B4, N5), *B. amyloliquefaciens* (N4), *B. licheniformis* (N8), *B. atrophaeus* (N10), *B. megaterium* (F4) and *Lysinibacillus sphaericus* (B2).

Table 1. The morphological characteristics of bacterial isolates from warehouse.

Isolates	Colony Colour	Shape of Colony	Gram Stain	Spore Stain	Motility
Bg1	Transparent	Wavy	+	+	-
Bg2	Transparent	Fimbriated	+	+	+
Bg3	White	Wavy	+	+	-
Bg4	Cream	Round	+	+	
Bg5	Transparent	Fimbriated	+	+	+
B1	Cream	Fimbriated	+	+	+
B2	Transparent	Round	+	+	-
B3	Cream	Round	+	+	-
B4	Cream	Round	+	+	-
B5	Transparent	Wavy	+	+	+
N1	Cream	Round	+	+	-
N2	Transparent	Wavy	+	+	+
N3	Cream	Round	+	+	-
N4	Transparent	Wavy	+	+	W+
N5	Cream	Wavy	+	+	-
N6	Cream	Fimbriated	+	+	+
N7	Cream	Fimbriated	+	+	-
N8	Transparent	Fimbriated	+	+	W+
N9	Cream	Fimbriated	+	+	+
N10	Transparent	Wavy	+	+	-
F1	Light-Yellow	Wavy	+	+	W+
F2	Cream	Wavy	+	+	-
F3	Cream	Round	+	+	-
F4	Light-Yellow	Wavy	+	+	-
F5	Cream	Wavy	+	+	-
F6	Cream	Wavy	+	+	-

W: Weak

Physiological and Biochemical Tests

According to results, we observed that these isolates were able to grow easily in alkaline and saline medium and optimum 30°C (Table 2). In order to determine production of organic compounds, starch hydrolysis, catalase test, and oxidase test were performed. We came to a conclusion that Bg1, Bg2, Bg5, B1, B4, N2, N4, N5, N6, N8, and N9 isolates were produce starch hydrolayse, all isolates were produce catalase and Bg2, N2, N6, F3, F4 isolates were not produce oxidase (Table 3). In addition to these test we used API test kit and 46% of isolates were detected by software of API (Table 4).

Molecular Characterization

In addition to the results of numerical tests and API test systems, the results from 16S rDNA gene sequences were also used for the molecular characterization of bacterial isolates. A total of 1,400 nucleotides of the 16S rDNA from 26 bacterial isolates were aligned and compared to sequences of related bacteria in GenBank (Table 5).The phylogenetic tree for isolates were constructed using the maximum parsimony method (Figure 1). The results of 16S rDNA and phylogenetic tree support each other.

We used a method based on the polymerase chain reaction (PCR) to allow rapid and highly sensitive determination of the *cry* gene content of bacterial isolates. DNA amplification was carried out using universal primers (*cry*1, *cry*2, *cry*3 and *cry*4). Of the 26 bacterial isolates detected *cry* gene contents, Bg5 has *cry*1 gene (Figure 2). Fragments with the expected sizes of about 272 bp corresponding to *cry*1 were amplified with DNA from Bg5 isolate [18]. Bg5 isolate that contained *cry*1 was similar to the reference strain, ***B. thuringiensis*** subsp. ***kurstaki*** (4D1). B2, B3, and N6 have *cry*3 genes (Figure 2). Fragments with the expected sizes of about 589-604 bp corresponding to *cry*3 were amplified with DNA from these isolates [18]. They contained *cry*3 gene was similar to the reference strain, *Bacillus thuringiensis* subsp. *tenebrionis* (BTS1).

Bioassay

It was observed that bacterial isolates except Bg5 and BnBt did not have highly mortality on all the tests (Figure 3, 4, 5). Of the 26 species of bacteria tested against the larvae of *P. interpunctella*, Bg5 only caused 100% mortality on larvae (Figure 3). Of these, a significant mortality (63.3%) was only found in adults fed with *S. granarius* (B4). B4 showed more or less 100% more mortality than MmBt and Xd3 having *cry*3 gene. In addition, it was determined that the death did not ocur in control groups during 10 days.

Table 2. Physiological characteristics of bacteria

Isolates	pH					NaCl (%)					Temperature (°C)			
	5	**6**	**9**	**11**	**12**	**5**	**9**	**11**	**13**	**15**	**20**	**30**	**37**	**45**
Bg1	+	+	+	-	-	+	-	-	-	-	+	+	+	+
Bg2	+	+	+	+	+	+	-	-	-	-	+	+	+	-
Bg3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bg4	+	+	+	+	+	+	-	-	-	-	-	+	+	-
Bg5	+	+	+	+	+	-	-	-	-	-	W+	+	+	-
B1	-	-	+	+	-	+	+	-	-	-	-	+	+	+
B2	-	-	+	+	-	-	-	-	-	-	+	+	+	-
B3	-	+	+	+	+	+	-	-	-	-	-	+	+	+
B4	-	+	+	+	-	+	-	-	-	-	-	+	+	+
B5	-	-	+	+	-	+	-	-	-	-	W+	+	+	+
N1	-	+	+	+	+	+	+	-	-	-	-	+	+	+
N2	-	-	+	+	-	+	-	-	-	-	-	+	+	+
N3	-	+	+	+	-	+	+	+	-	-	W+	+	+	+
N4	-	+	+	+	+	+	-	-	-	-	W+	+	+	+
N5	+	+	+	+	-	+	+	-	-	-	-	+	+	+
N6	-	+	+	+	+	+	-	-	-	-	W+	+	+	-
N7	+	+	+	+	+	+	+	+	-	-	-	+	+	+
N8	-	+	+	+	-	+	-	-	-	-	-	+	+	+
N9	-	+	+	+	-	+	-	-	-	-	+	+	+	-
N10	-	+	+	+	-	+	+	-	-	-	W+	+	+	+
F1	-	+	+	+	-	+	+	+	+	-	W+	+	+	-
F2	-	+	+	+	+	+	+	+	-	-	-	+	+	+
F3	-	+	+	+	-	+	+	-	-	-	W+	+	+	+
F4	-	+	+	+	-	+	+	-	-	-	-	+	+	+
F5	+	+	+	+	-	+	+	+	-	-	-	+	+	+
F6	-	+	+	+	+	+	+	-	-	-	-	+	+	+

W: Weak

Table 3. Biochemical characteristics of bacteria

Isolates	Biochemical Tests		
	Starch Hydrolysis	Catalase	Oxidase
Bg1	+	+	W+
Bg2	+	+	-
Bg3	-	+	W+
Bg4	-	+	+
Bg5	+	+	W+
B1	+	+	W+
B2	-	+	+
B3	-	+	+
B4	+	+	+
B5	-	+	+
N1	-	+	W+
N2	+	+	-
N3	-	+	W+
N4	+	+	+
N5	+	+	W+
N6	+	+	-
N7	-	+	+
N8	+	+	+
N9	+	+	W+
N10	-	+	W+
F1	-	+	+
F2	-	+	+
F3	-	+	-
F4	-	+	-
F5	-	+	+
F6	-	+	+

W: Weak

Table 4. Results of API of Bacteria Isolates

Isolates	Name of Bacteria	Results of API (%)
Bg1	Not Determined	-
Bg2	*Bacillus cereus*	58.0
Bg3	*Bacillus pumilus*	99.9
Bg4	Not Determined	-
Bg5	Not Determined	-
B1	*Bacillus licheniformis*	99.9
B2	*Bacillus laterosporus*	46.1
B3	*Bacillus pumilus*	99.9
B4	*Bacillus licheniformis*	94.8
B5	Not Determined	-
N1	*Bacillus pumilus*	99.9
N2	Not Determined	-
N3	*Bacillus pumilus*	99.9
N4	Not Determined	-
N5	*Bacillus amyloliquefaciens*	99.6
N6	*Bacillus mycoides*	79.4
N7	*Bacillus pumilus*	99.9
N8	*Bacillus licheniformis*	99.9
N9	*Bacillus mycoides*	79.4
N10	Not Determined	-
F1	*Bacillus pumilus*	99.6
F2	*Bacillus licheniformis*	90.9
F3	*Bacillus licheniformis*	99.2
F4	*Bacillus megaterium*	94.7
F5	*Bacillus pumilus*	99.9
F6	*Bacillus pumilus*	99.9

Table 5. IsolatesPercentage of 16S rRNA gene similarity

Isolates	Species	Rate of similarities (%)	Accession Number
Bg1	*Bacillus amyloliquefaciens* *Bacillus subtilis subsp. subtilis* *Bacillus subtilis subsp. spizizenii* *Bacillus velezensis* *Bacillus methylotropicus*	99 99 99 99 99	NC_014551 AJ831841 NR_024931 EF433407 EU194897
Bg2	*Bacillus thuringiensis* *Bacillus anthracis* *Bacillus weihenstephanensis* *Bacillus mycoides* *Bacillus pseudomycoides*	99 99 98 97 97	AB592540 AB190217 AB592543 AB592538 AF013121
Bg3	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	99 99 99 99 99	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
Bg4	*Bacillus vireti* *Bacillus novalis* *Bacillus drentensis* *Bacillus bataviensis* *Bacillus soli*	98 98 98 98 98	NBRC102452T AJ542512 AJ542506.1 AJ542508.1 AJ542513.1
Bg5	*Bacillus thuringiensis* *Bacillus anthracis* *Bacillus weihenstephanensis* *Bacillus mycoides* *Bacillus pseudomycoides*	99 99 99 98 98	AB592540 AB190217 AB592543 AB592538 AF013121
B1	*Bacillus amyloliquefaciens* *Bacillus vallismortis* *Bacillus aerius* *Bacillus sonorensis* *Bacillus subtilis subsp. subtilis*	99 98 98 97 97	NC_014551 EU138463 NR_042338 NR_025130 AJ831841
B2	*Lysinibacillus sphaericus* *Lysinibacillus fusiformis* *Lysinibacillus xylanilyticus* *Lysinibacillus boronitolerans*	99 99 98 97	AB271742 AB271743 FJ477040 AB199591
B3	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	99 99 99 99 99	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
B4	*Bacillus amyloliquefaciens* *Bacillus aerius* *Bacillus sonorensis* *Bacillus subtilis subsp. subtilis* *Bacillus subtilis subsp. spizizenii*	99 98 97 97 97	NC_014551 NR_042338 NR_025130 AJ831841 NR_024931
B5	*Bacillus licheniformis* *Bacillus sonorensis* *Bacillus mojavensis* *Bacillus aerius* *Bacillus amyloliquefaciens*	99 99 98 98 98	CP000002 NR_025130 EU138460 NR_042338 NC_014551
N1	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	99 99 99 99 99	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
N2	*Bacillus licheniformis* *Bacillus sonorensis* *Bacillus aerius* *Bacillus mojavensis* *Bacillus subtilis subsp. subtilis*	99 99 99 98 98	CP000002 NR_025130 NR_042338 EU138460 AB598736
N3	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	98 98 98 98 98	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
N4	*Bacillus licheniformis* *Bacillus sonorensis* *Bacillus aerius* *Bacillus mojavensis* *Bacillus subtilis subsp. subtilis*	99 99 99 98 98	CP000002 NR_025130 NR_042338 EU138460 AB598736
N5	*Bacillus amyloliquefaciens* *Bacillus subtilis subsp. subtilis* *Bacillus subtilis subsp. spizizenii* *Bacillus atrophaeus* *Bacillus methylotropicus*	99 99 99 99 99	NC_014551 AJ831841 NR_024931 EU138516 EU194897
N6	*Bacillus thuringiensis* *Bacillus anthracis* *Bacillus weihenstephanensis* *Bacillus mycoides*	99 99 99 97	AB592540 AB190217 AB592543 AB592538
N7	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	99 99 99 99 99	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
N9	*Bacillus thuringiensis* *Bacillus anthracis* *Bacillus weihenstephanensis* *Bacillus mycoides* *Bacillus pseudomycoides*	99 99 99 97 97	AB592540 AB190217 AB592543 AB592538 AF013121
N10	*Bacillus vallismortis* *Bacillus atrophaeus* *Bacillus amyloliquefaciens* *Bacillus subtilis subsp. subtilis* *Bacillus subtilis subsp. spizizenii*	99 99 98 98 98	AB021198 EU138516 NC_014551 AJ831841 NR_024931
F1	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	99 99 99 99 99	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
F2	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	98 98 98 98 98	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
F3	*Bacillus megaterium* *Bacillus aryabhattai*	99 99	AB271751 EF114313
F4	*Bacillus megaterium* *Bacillus aryabhattai*	99 99	AB271751 EF114313
F5	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	99 99 99 99 99	NR_043242 AF234854 AJ831842 AJ831844 AJ831841
F6	*Bacillus pumilus* *Bacillus safensis* *Bacillus altitudinis* *Bacillus aerophilus* *Bacillus stratosphericus*	99 99 99 99 99	NR_043242 AF234854 AJ831842 AJ831844 AJ831841

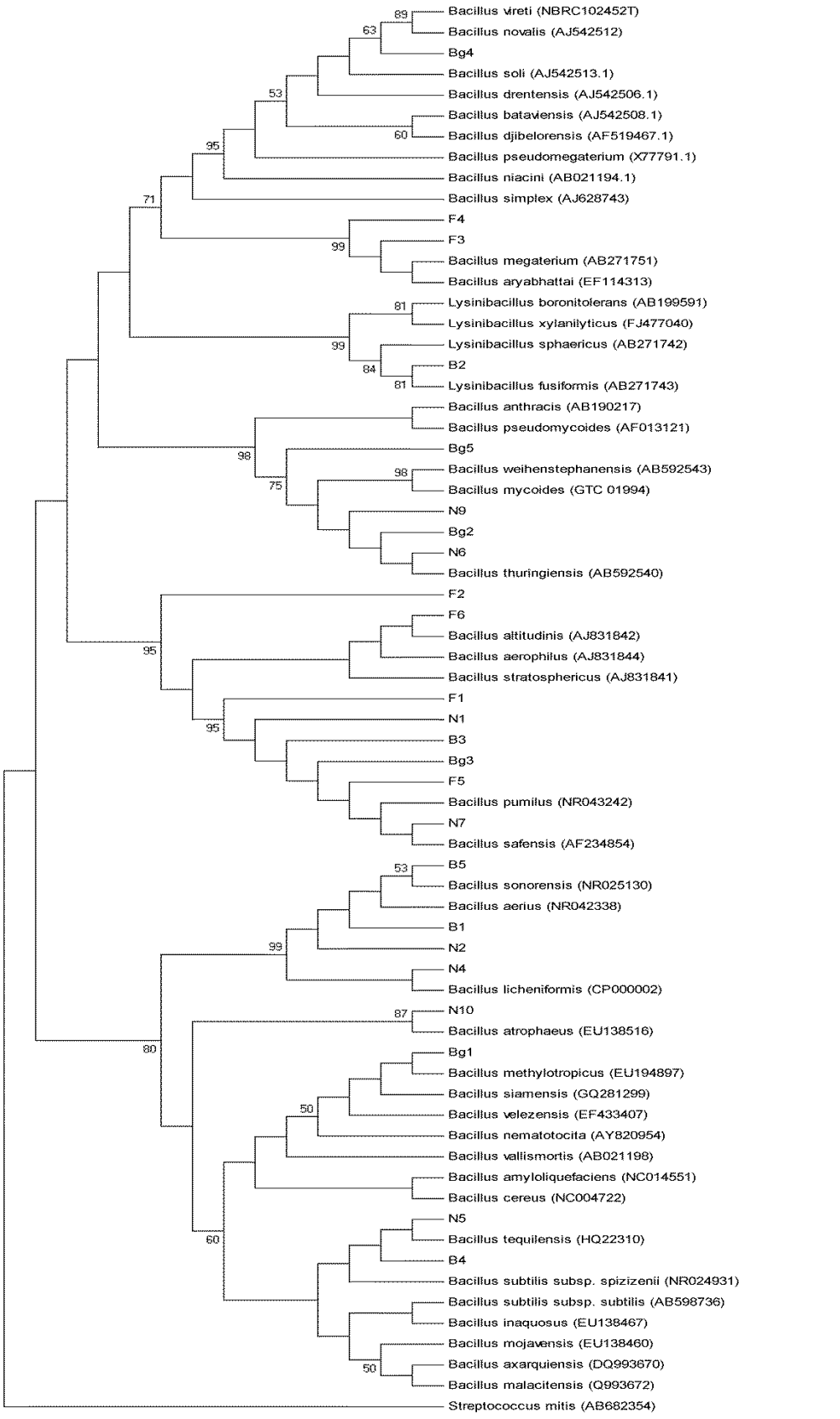

Figure 1. Maximum parsimony tree based on 16S rDNA gene sequences of bacterial isolates

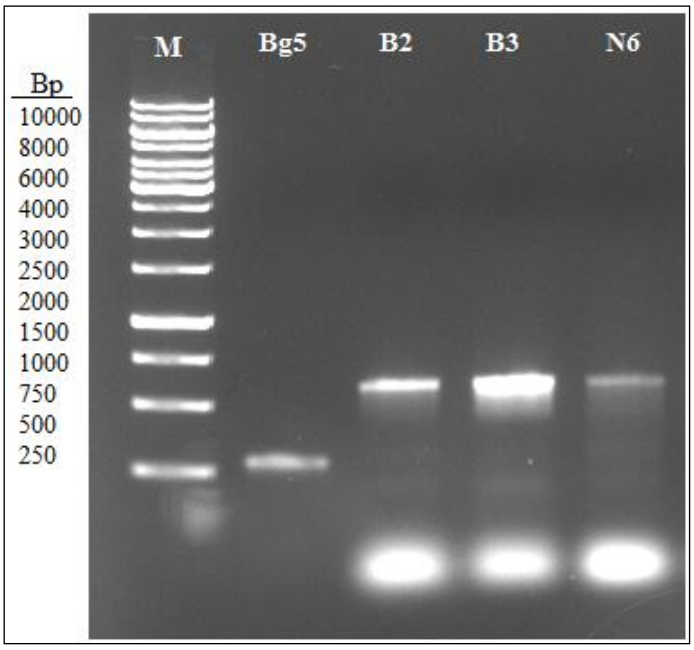

Figure 2. Agorose gel electrophoresis analysis of PCR products obtained by using the *cry*1, *cry*2, *cry*3, and *cry*4 general primers pairs. Lanes M, Marker (1000bp DNA Ladder), Bg5, ~ 277 bp (*cry*1), B2; B3; N6, ~ 600 bp (*cry*3).

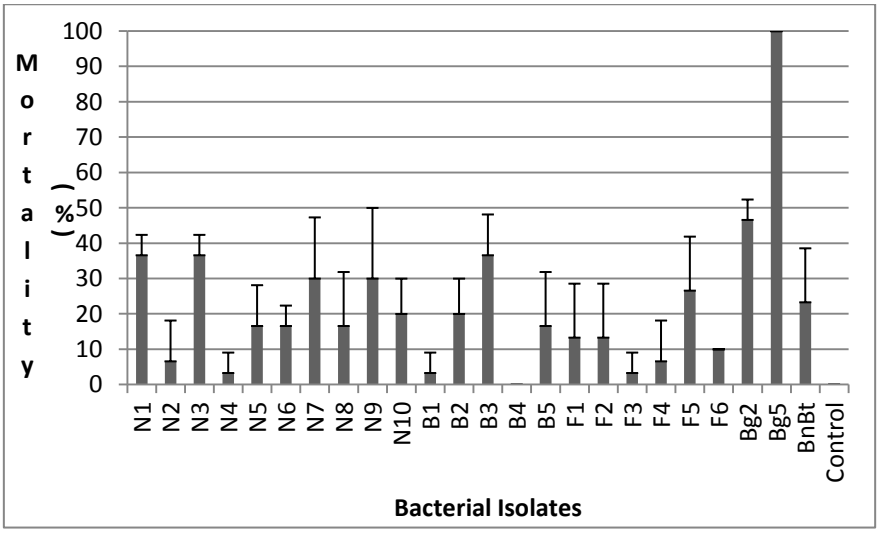

Figure 3. The insecticidal effects of the bacterial isolates on *Plodia interpunctella* larvae.

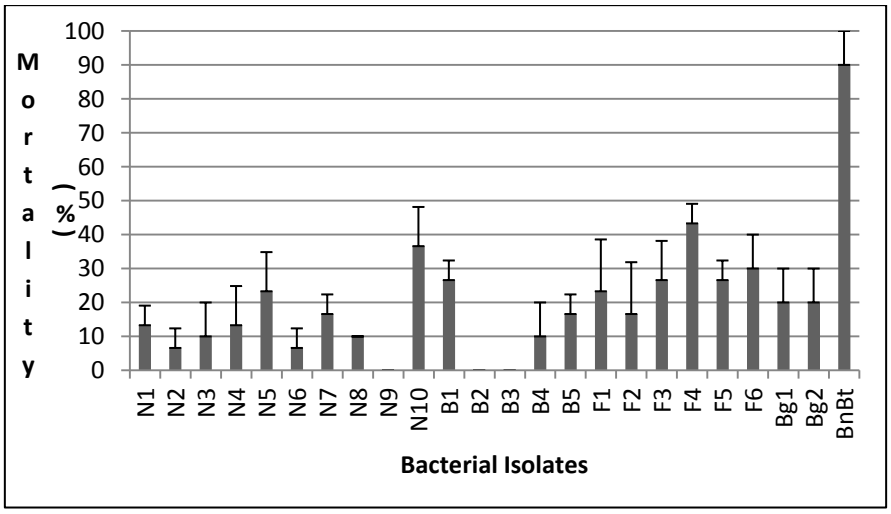

Figure 4. The insecticidal effects of the bacterial isolates on *Ephestia kuehniella* larvae.

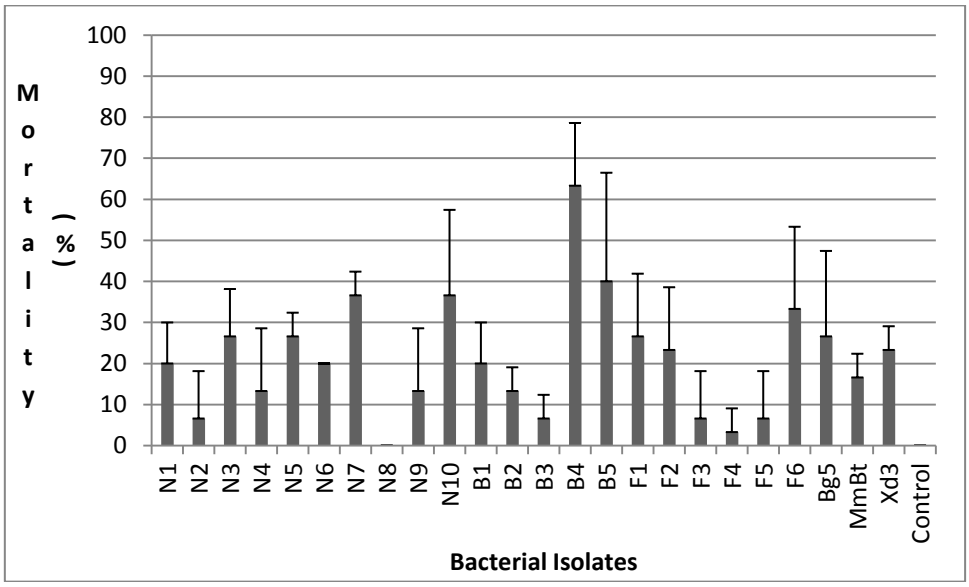

Figure 5. The insecticidal effects of the bacterial isolates on *Sitophilus granarius* adults.

DISCUSSION

In the present, it is prefer to biological agent instead of chemicals in biological control for the damage of insects. *Bacillus* is the most favorite for using against insects. One of the best ways is to utilize the entomopathogens of harmful insect for the purpose of biological control. Therefore, the properties of bacteria which are newly isolated must be determined for safety and effectiveness as biological control agent. To date, although there have been many biological control studies especially on insects, there has been limited information on the isolation and characterization of bacteria from warehousesas as potential biological control agents. Hongyu et al. (2000) [29] reported that the reason for more abundant *B. thuringiensis* in grain storage facilities compared with soil is still not known, but several factors may provide for enrichment of *B. thuringiensis* within a stored-product environment and those *B. thuringiensis* isolates had different toxicity levels to insects tested, causing from 0 to 100 % mortality. Most isolates from warehouses were toxic to lepidopteran pests but not toxic to coleopteran pests and mosquitoes at dosage used. Lack of highly toxic strains is still a limiting factor for *B. thuringiensis* in controlling noctuid and coleopteran pests. In addition to, as a reason of several strains that were highly toxic to insect will be that insects usually are not sensitive to the known *B. thuringiensis* strains [29]. There has recently been an increasing interest in finding more pathogenic and safer bacterial isolate against hazardous insects.

Isolate Bg1 was determined as *Bacillus* sp., because there are some properties/test the same with *Bacillus amyloliquefaciens*, *B. vallismortis*, and *B. tequilensis* for example D-Ribose, D-Xylose, D-Mannitol, D-Sorbitol, NO_2, oxidase, gelatin, starch, citrate and urea. In addition, 16S rDNA tests showed that this bacterial isolate was 99% similar to *Bacillus amyloliquefaciens*, *B. vallismortis*, *B. methylotropicus*, *B. tequilensis* and *B. mojavensis.*

Isolates Bg2 and N9 were determined as *Bacillus thuringiensis*, because 16S rDNA tests showed that Bg2 was 99% similar to *Bacillus thuringiensis, B. anthracis*, and 98% *B. weihenstephanensis.* Similarly, N9 was 99% similar to *B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis* and 97% *B. mycoides*. For Bg2 and N9 are motile, these isolates are not *B. anthracis*. Can be differentiated from B. *thuringiensis* by the presence of the 16S rDNA signature sequence ^{1003}TCTAGAGATAGA [30]. Bg2 and N9 do not include this signature sequence and with the result of the API50 CHB software these isolates were determined as *B. thuringiensis*.

Bg3, N1, N3, N7, F1, F5 and F6 were 99 % similar to *Bacillus pumilus* according to the results of 16S rDNA tests and this result was supported with the result of the API50 CHB software. When 16S rDNA sequences of these isolates were aligned using BLAST to each other, it was showed that they were 100 % similar but only F1 was 80 %. In regard to results Bg3, N1, N3, N7, F1, F5 and F6 were determined as *B. pumilus*. Also, comparing with the biochemical tests on API50CHB Bg3 and N1 are same strain, N3, N7, F1, F5, and F6 are other strains related to *B. pumilus* (Table 6). Therefore, these 8 isolates were recorded as same species.

Table 6. Comparision of *B. pumilus* isolatesby API50CHB

Tests	N3	N7	F1	F5	F6
Arbutin	-	+	+	+	+
D-Glukoz	+	+	+	+	+
D-Tagatoz	+	+	+	-	+
D-Laktoz	-	-	-	+	-
D-Ksiloz	+	+	+	+	-
D-Galaktoz	-	+	+	+	-
D-Maltoz	-	+	+	+	-
D-Turanoz	-	+	+	+	-
N-asetilglukoz amin	-	+	-	+	+
VP	-	-	+	-	-
Amigdalin	-	+	+	+	+

Isolate Bg4 was determined as *Bacillus* sp., because the biochemical and 16S rDNA tests showed that this bacterial isolate was 98% similar to *B. bataviensis*, *B. drentensis*, *B. soli*, *B. novalis* and *B. djibelorensis*. There are some properties/test the same with *B. bataviensis*, *B. drentensis*,

B. soli, *B. novalis,* for example D-Glucose, L-Rhamnose, Inositol, L-Ornithine, Sodium Thiosulfate, NO_2.

Isolate Bg5 was identified as *Bacillus thuringiensis*, because biochemical and 16S rDNA tests showed that this bacterial isolate was 99 % similar to *B. thuringiensis* and it was include nearly 277 bp *cry*1 gene [18] and [27]. The most common *cry* genes found in nature belong to *cry*1 gene group [31]. Ben-Dov et al. (1997) [18], Bravo et al. (1998) [32], and Wang et al.(2003) [33] have reported *cry*1 genes were the most frequent in their collections.

It was determined that as a result of 16S rDNA sequence, B1, B4, and N5 are similar to 99% *B. amyloliquefaciens* and 97% *B. subtilis*. *Bacillus amyloliquefaciens*is responsible for much of the world production of α-amylase and protease. It's close affinity with *Bacillus subtilis* has long been recognized, and the organism has been given subspecies status as *B. subtilis* subsp. *amyloliquefuciens* [34] or has been included in *B. subtilis* as a variant that produces copious quantities of extracellular enzymes [35]. Thus, *Bacillus amyloliquefaciens*is closely related to *B. subtilis* and the other two species which compose the *B. subtilis* group, *Bacillus licheniformis* and *Bacillus pumilus.* These organisms share many common properties, and few characteristics have been found by which they can be discriminated [35]**.** Indeed, *B. amyloliquefaciens*is phenotypically is similar to *B. subtilis* that it is not possible to separate these organisms solely on the basis of classical tests [35], [36], [37], and [38], and for this reason that *B. amyloliquefaciens* was not included as a separate species on the Approved Lists [39]. However, there is now a body of evidence that suggests that the name *B. amyloliquefuciens* should be revised. It has been shown that *B. amyloliquefaciens* and *B. subtilis* can be differentiated by using a number of techniques. Moreover, there is a need for this name in the enzyme industry to avoid confusion with *B. subtilis,* which differs metabolically and secretes different enzymes [40]. *B. amyloliquefaciens* strains can be distinguished from *B. subtilis* strains by the inability of most strains to hydrolyze DNA and pectin, the failure of the organisms to produce acid from inulin [41], and the formation in most *B. amyloliquefaciens*strains of long chains of cells. Therefore, it was recorded that B1, B4, and N5 are *B. subtilis*. However, some difference on biochemical test showed that these isolates are different strain (Table 7).

Table 7. Comparision of *B. subtilis*isolatesby API50CHB

Testler	B1	B4	N5
D-Tagatoz	+	-	-
D-Melibioz	-	+	+
L-Sorboz	+	-	-
L-Ramnoz	+	-	-
Dulsitol	+	-	-
D-Galaktoz	+	-	-
L-Arabinoz	+	-	-
L-Ksiloz	-	+	-
Eritrol	-	+	-
D-Turanoz	+	-	+
L-Arjinin	+	+	-
VP	+	-	-
H_2S	+	-	-

It was determined that as a result of 16S rDNA sequence, B2 are similar to *Lysinibacillus sphaericus* and *L. fusiformis*. In contrast to the type species of the genus *Bacillus*, the strains contained peptidoglycan with lysine, aspartic acid, alanine and glutamic acid [42]. Therefore, B2 was recorded as *Lysinibacillus* sp.

Isolate B3 was determined as *Bacillus thuringiensis*, because it was include nearly 600 bp *cry*3 gene [12] and [27].

There are some properties/test the same with *B. licheniformis*, *B. mojavensis,* and *B. amyloliquefaciens* with B5 for example D-mannitol, glucose, D-xylose, citrate, starch, and NO_2. Also, 16S rDNA tests showed that this bacterial isolate was 99 % similar to *B. licheniformis*, *B. sonorensis*, 98 % similar to *B. mojavensis, amyloliquefaciens*, *B. tequilensis*. Due to these properties, B5 was determined as *Bacillus* sp.

Isolate N2 was determined as *Bacillus* sp., because biochemical and 16S rDNA tests showed that this bacterial isolate was 99 % similar to *B. axarquensis* and *B. sonorensis*.

Isolate N4 was determined that it was 99% similar to *B. licheniformis* and 98% *B. subtilis* and *B. amyloliquefaciens*. When it was evaluated as characteristic property producing acid from inulin, it was showed that N4 did not produce acid from inulin like *B. amyloliquefaciens* [41]. So, N4 was recorded as *B. amyloliquefaciens.*

Isolate N6 was determined as *Bacillus thuringiensis*, because biochemical and 16S rDNA tests showed that this bacterial isolate was 99% similar to *B. thuringiensis* and it was include nearly 600 bp *cry*3 gene [17] and [36].

Meadows et al. (1992) [7] and Hongyu et al. (2000) [29] reported that stored product samples are rich in *B. thuringiensis* strains. Meadows et al. (1992) [7] also suggested that *B. thuringiensis* multiplied in the cadavers of insects that have been killed by the *B. thuringiensis* toxins, and these cadavers were ingested by birds and mammals who spread spores in their feces. However, in our study, among the stored product samples, only five *B. thuringiensis* strains were isolated. Meadows et al. (1992) [7] isolated *B. thuringiensis* from 78 % of the settled grain dust samples. This indicates that grain is not as good source as the others for *B. thuringiensis*. This may be related to climate and geographic conditions. In addition, Hongyu et al. (2000) [29] and Bernhard et al. (1997) [43] reported that *B. thuringiensis* is more abundant in stored product environments than in soil.

According to API50CHB software, isolate N8 was determined that it was similar to 99% *Bacillus licheniformis*. So, we decided that N8 was *B. licheniformis.*

It was determined that as a result of 16S rDNA sequence, N10 was similar to 99% *B. atrophaeus* and *B. vallismortis* and 98% *B. velezensis*, *B. tequilensis*, *B. mojavensis*, *B. malacitensis*, and *B. axarquensis*. *B. axarquensis*as members of a *B. subtilis* is known producing soluble black pigment [44]. It was known that N10 produce soluble black pigment like *B. axarquensis*, so N10 was recorded as *B. axarquensis.*

As a result of 16S rDNA sequence, it was determined that F2 is similar to 98% B. *safensis* and *B. pumilus* and of API50CHB software F2 is similar to 90,9 % *B. licheniformis*. Therefore, F2 was recorded as *Bacillus* sp.

According to results of 16S rDNA sequence, it was determined that, F3 is similar to 99% *B. megaterium* and *B. aryabhattai* and of API50CHB software F3 is similar to 99,2% *B. licheniformis*. Therefore, F3 was recorded as *Bacillus* sp.

As a result of 16S rDNA sequence and API50CHB software, it was determined that is similar to 99% *B. megaterium*.

On the second part of this study, we did bioassays for our bacterial isolates against the larvae of *Ephestia kuehniella* and *Plodia interpunctella* and the adults of *Sitophilus granarius* that they are major pest of stored products in warehouses in Trabzon. In this test, all isolates had different toxicity levels to insects tested, caused from 0 to 100% mortality. The highest insecticidal activity of the bacterial isolates on the larvae of *E. kuehniella* was %90 for *B. thuringiensis* (BnBt, positive control) and 43,3% for *B. megaterium* (F4). On the literaure, there is no study any insecticidal activity with *B. megaterium*. Bg5 (*B. thuringiensis*) caused 100% mortality against the larvae of *P. interpunctella* on the second day and so we think that this isolate may be important as biological control agent against *P. interpunctella* is important pest in warehouses. In the future, this isolate having *cry*1 gene may replace with chemical used in warehouses. The highest insecticidal activity of the bacterial isolates on the adults of *S. granarius* was 63,3% for *B. subtilis* (B4). It is obviously interesting that B4 has more insecticidal effect than bacterial isolates which are include *cry*3 gene (B2, B3 and N6) and MmBt and Xd3 (used as positive control).

Consequently, the results indicate that Bg5 (*B. thuringiensis*) for the *P. interpunctella* and BnBt for the *E. kuehniella* may be valuable as potential biological control agents. We suggest to develope *B. thuringiensis* (Bg5 and BnBt) as biopesticide. None of these bacteria are human pathogenic. These two bacterial isolates are well defined at species. Future studies will be conducted with the aim of finding a better microbial control agent against these hazardous insect using this pesticide or other newly improved pesticides. The present study has contributed significantly to the literature on the bacterial isolates were isolated from warehouses.

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