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Isolation and Characterization of Indigenous Endotoxin-Forming *Bacillus* sp. with Insecticidal Activity from Northern Iranian Soil

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ABSTRACT

The main goal of the current study was to isolate and characterize endospore-forming soil bacteria that produce parasporal crystalline proteins against larvae of Lepidoptera insects during stationary phase of growth. Two different methods were applied for the isolation of spore-forming strains. Polymerase Chain Reaction (PCR) was used for the characterization of *cry* gene content of the isolated strains and 16S rRNA sequencing was carried out to identify bacterial strains. Efficiency of the isolates as insecticide was evaluated in a bioassay experiment using insect's larvae. Regarding presence of parasporal inclusion crystals and SDS-PAGE patterns, 10 bacterial strains were isolated from about 200 soil samples. Analysis of crystal-spore mixtures with SDS-PAGE showed a broad range of proteins with molecular weight between 11-230 kDa. Data from PCR analysis indicated that only two isolates (RIPI6 and RIPI18) may contain *cry3* gene. Isolate RIPI21 and reference strain (*Bacillus thuringiensis* subsp. kurstaki) were positive for *cry1* gene. *16S rRNA* gene sequences of all isolates showed at least 96% sequence match with *B. thuringiensis* strains deposited in the GenBank. The results of bioassay experiments showed the efficacy of strains RIPI7, 10 and 22 on killing of larvae of both *Anagasta kuehniella* Zeller and *Plutella xylostella* Curt. It is concluded that there are some bacterial candidates for biological control of major agricultural pests in north of Iran.

Keywords: *Bacillus thuringiensis*, Cry protein, Lepidoptera, Parasporal crystal, Pesticidal activity.

INTRODUCTION

Widespread usage of synthetic chemical pesticides has led to various problems ranging from environmental pollution to a wide spectrum of human health hazards, such as neurological diseases, cancers, and immune system disorders (Bravo *et al*., 2011). Biological control as an important component of Integrated Pest Management (IPM) programs may have a significant impact on biodiversity, ecosystem sustainability, and food safety. *Bacillus thuringiensis* (Bt) is known to be an important microbial entomopathogen for the biological control of many agricultural insect pests and disease vectors (Santos *et al*., 2010; Unalmis *et al*., 2015).

Insecticidal toxins (in the form of crystalline proteins), which are produced by *Bt* species, are specific against insects and in most cases are safe to vertebrates and beneficial arthropods in the ecosystems (Roh *et al*., 2007). Crystalline parasporal proteins of *Bt* when ingested orally by insect larvae are solubilized and activated by proteases in the juices of insect's midgut in an alkaline environment (pH 8.0-10.5) to produce toxic proteins called δ-endotoxin (Bravo *et al*., 2007). Binding of active toxins to specific receptors on the epithelium of the midgut

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(Hofmann *et al*., 1988; Yi *et al*., 1996) will result in the pore formation, cell lysis, paralysis and, eventually, death of the larvae (Aronson *et al*., 1999).

Thanks to their safety, the *Bt* products were commercialized firstly in the late 1930 (Nester *et al*., 2002). It is expected that application of bacterial toxins in pest control programs will result in a healthier environment, improvement of human health, and production of safer foods. In recent years, many researchers around the world have focused their studies on the isolation and characterization of new toxins of *Bt* or other bacterial strains for the control of a diverse range of agricultural pests (Ibarra *et al*., 2003; Armengol *et al*., 2007; Kati *et al*., 2007; Jouzani *et al*., 2008; Seifinejad *et al*., 2008; Nazarian *et al*., 2009; Reddy *et al*., 2012; Salekjalali *et al*., 2012; Khojand *et al*., 2013; Kutasi *et al*., 2016). There is a great diversity in *Bt* toxins and more than 300 pesticidal Crystal protein (Cry and Cyt), genes located mainly on plasmids, have been cloned and sequenced (Crickmore *et al*., 2016). Crystalline proteins of *Bt* strains, which are produce during the sporulation process, are toxic to a wide variety of insect species among the orders of Lepidoptera, Diptera and Coleoptera (Lacey *et al*., 2001). Insects from these orders cause serious damages to economically important crops (Vidyarthi *et al*., 2002) and some of those are also considered as important vectors (mechanical or biological) for infectious agents in animals (Rodhain, 2015). Some reports about *Bt* showed that it is possible to find new bacterial isolates against protozoa, nematodes, and mites (Marvier *et al*., 2007; Jouzani *et al*., 2008) in addition to other order of insects such as Mallophage, Hymenoptera, Homoptera and Orthoptera (Feitelson, 1993).

Isolation of novel *Bt* strains with toxic effects on a target insect group by identification of genes encoding Cry or Cyt toxins (Porcar and Juarez-Perez, 2003; Federici, 2006) has some technical limitations due to this fact that the primers are designed against known genes. As a result, implementation of bioassay approaches and SDS-PAGE analysis of parasporal proteins are inevitable techniques in the finding of new and more effective *Bt* isolates to fight against key agricultural pests (Baig and Mehnaz, 2010).

In the current study, our focus was isolation and characterization of indigenous *Bt* strains with insecticidal activity against *Anagasta kuehniella* Zeller and *Plutella xylostella* Curt larvae from northern Iranian soils. *Anagasta kuehniella* from the order of Lepidoptera also known as Mediterranean flour moth or mill moth is a common pest of dry grains especially cereals and is found all over the world. *P. xylostella* (diamondback moth, cabbage moth) with a worldwide spread is one of the most important pests of cruciferous crops including broccoli, cabbage, Brussels sprouts etc. These pests cause many damages to agricultural products each year and, therefore, their biological control is of great benefits to agriculture industry and human societies.

MATERIALS AND METHODS

Chemicals

Trypton, yeast extract, iodine, agar, malachite green and crystal violet were purchased from Merck (Darmstadt, Germany). Methylene blue, coomassie brilliant blue, ethidium bromide, sodium acetate, Tris-base, agarose, acrylamide, bis-acrylamide, TEMED, EDTA and ammonium persulfate were obtained from Sigma-Aldrich Co. (St, Louis, Missouri, United States). Pre-stained protein molecular weight marker and DNA molecular weight ladder were purchased from Fermentas (Massachusetts, United States). Reference strain *Bt*. subsp. kurstaki was obtained from Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology (IROST), Iran. All other chemicals and reagents were purchased from Merck.

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Soil Sample Collection

Soil samples were collected from north of Iran in a region distributed between 35.95-37.50° N and 52.38-55.03° E (Figure 1). The sampling locations were from areas with no history of the use of *Bt*-based microbial insecticides. Soil samples (approximately 100 g) were collected at 10 points at a depth of 2-5 cm and stored at 4°C until use.

Isolation of Spore-Forming Bacteria

Soil samples were processed using two methods. In the first approach, samples (10 g) were mixed with 10 mL of saline solution (0.85% w/v NaCl) according to the WHO *Bt* isolating procedure (Ferreira da silva *et al*., 2002). After vortexing vigorously for 2 min, one mL of this mixture was incubated at 80°C for 12 minutes and then ice-cooled for 5 minutes. Finally, serially diluted samples (10^{-1}-10^{-3}) were cultured at 30°C on Luria-Bertani (LB)-agar plates (10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar in 950 mL of deionized water, pH 7.2) using spread plate method. In the second approach, soil samples were preheated according to Santana method (Santana *et al*., 2008). Briefly, soil samples (5 g) were wrapped in aluminum foil and incubated for 7 hours at 80°C in a dry oven before performing WHO procedure. *Bacillus*-like colonies (rough, white and spread out over the plate) were subcultured on new LB-agar plates and incubated at 30°C for 2-5 days to obtain pure cultures. After storage of the plates for 2 weeks at 4°C, smears of *Bacillus* isolates were prepared and examined for the presence of spore and parasporal crystals under light microscope (B380 series, Optika, Italy).

For visualization of spores, two different staining methods, namely, methylene blue and malachite green (Bartholomew and Mittwer, 1950) staining were performed. In staining with methylene blue, unstained spores may be seen within methylene blue-stained bacterial cells. Treatment of bacterial

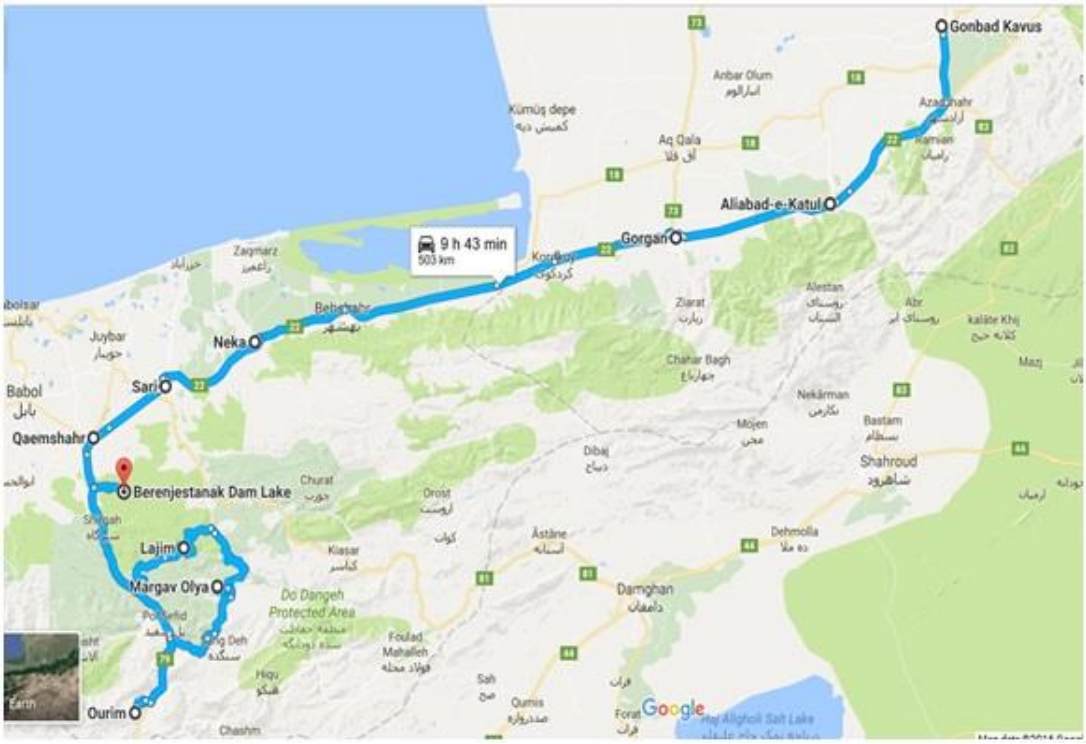

Figure 1. Soil sampling locations (10 points) which included Gonbad Kavus, Aliabad-e-katul, Gorgan, Neka, Sari, Qaemshahr, Berenjestanak, Lajim, Margav Olya and Ourim.

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cells with malachite green and steam heat followed by washing with water results in appearance of green spores in unstained cells which can be counterstained with the red dye safranin.

To stain the parasporal bodies, coomassie brilliant blue was used as described by Rampersad *et al*. (2002). Gram staining and KOH test (Gregersen, 1978) were also performed on the isolated strains. Reference strain *Bt*. subsp. kurstaki (PTCC 1494) was used as the positive control in our study.

SDS-PAGE Analysis of Parasporal Crystalline Proteins

A single colony from each bacterial isolate was cultured into T3 sporulation medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 mol sodium phosphate and 5 mg $MnCl_2$ in 1 liter of distilled water, pH 6.8) at 37°C for 5 days with continuous shaking at 200 rpm. After sporulation, spore-crystal mixtures were collected by centrifugation at 10,000×g for 5 minutes. Pellets were washed three times with ice-cold 1 M NaCl solution and finally with distilled water. The resulting pellet was resuspended in the sample buffer of SDS-PAGE (Tris-base 0.178 g, SDS 1 g, bromophenol blue 1 mg, 2-mercaptoethanol 2.5 mL and glycerol 5 mL in 25 mL of deionized water, pH 6.8) and boiled for 5 minutes. After centrifugation at 10,000×g for 10 minutes, the obtained supernatant was analyzed electrophoretically using SDS-PAGE with a 12% resolving gel according to Laemmli method (Laemmli and Favre, 1970). SDS-PAGE gels were stained with coomassie brilliant blue R-250. Molecular weights of the resolved protein bands were determined with respect to standard pre-stained protein size marker after documentation of the gel slab with Syngene Gel-Doc system (Synoptics group, England).

DNA Extraction, PCR Amplification and Gene Sequencing

Total DNA from bacterial isolates was obtained from 6 mL of overnight grown bacterial culture in LB using a Gram-Positive DNA extraction kit (CinnaPure™ DNA KIT, Cinna Gen, Iran) following the supplier's instruction. Also, a rapid freezing-boiling approach according to Bravo *et al*. (1998) was used for bacterial DNA extraction. Briefly, a loop of bacterial cells in deionized water (0.1 mL) was frozen at -70°C for 20 minutes and then boiled for 10 minutes. The resulting cell lysate was centrifuged at 10,000 rpm for 20 seconds and then 15 µL of supernatant was used as DNA template in the PCR. Genes of *16S rRNA* and *cry* (cry1, 2, 3 and 4) were amplified by PCR using indicated primers (Ben-Dov *et al*., 1997; Bravo *et al*., 1998) (Table 1) and approximately 500 ng of total bacterial DNA. PCR mixture (100 µL) contained 10X PCR buffer (10 µL), 10 mM

Table 1. Characteristics of primers used in the current study.

Target gene	Primer	Sequence
16S rDNA	16s-9F	*5′-AGAGTTTGATCCTGGCTCAG-3′*
	16s-1492R	*5′-GGTTACCTTGTTACGACTT-3′*
Cry1	Un1-r	*5′-TTGTGACACTTCTGCTTCCCATT-3′*
	Un1-d	*5′-CATGATTCATGCGGCAGATAAAC-3′*
Cry2	Un2-r	*5′-CGG ATAAAATAATCTGGGAAATAGT-3′*
	Un2-d	*5′-GTTATTCTTAATGCAGATGAATGGG-3′*
Cry3	Un3-r	*5′-CATCTGTTGTTTCTGGAGGCAAT-3′*
	Un3-d	*5′-CGTTATCGCAGAGAGATGACATTAAC-3′*
Cry4	Un4-d	5′-GCATATGATGTAGCGAAACAAGCC-3′
	Un4-r	5′-GCGTGACATACCCATTTCCAGGTCC-3′

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dNTP (2 µL), 10 µM primer (5 µL), 50 mM $MgCl_2$ (3 µL), Taq polymerase (0.7 µL) and bacterial DNA. Final volume of the PCR mixture was adjusted to 100 µL with enough deionized water. PCR was initiated by a denaturing step at 94°C for 4 minutes followed by 30 cycle of 94°C for 45 seconds (denaturing), 54-57°C for 30 seconds (annealing) and 72°C for 90 seconds (extension). The PCR products were checked by electrophoresis on a 1% (w/v) agarose gel in Tris-borate buffer. Gels were stained using ethidium bromide (Sambrook, 1989) and the bands of interest were excised and eluted from the gel using a gel extraction kit (Qiagen, Germany). PCR-amplified DNA fragments were sequenced using the same sets of primers that were used for amplification. The obtained sequences were analyzed with Blast software (blastn suite) at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The most similar sequences were aligned with CLUSTALW 2.0.12 program and phylogenetic tree was constructed with MEGA 6.0 software using the Bootstrapped neighbor-joining method to analyze evolutionary relationship among isolates.

Bioassay with Bacterial Isolates and Insect's Larvae

Bacterial isolates with parasporal bodies were inoculated in 500-mL flasks with 100 mL of T3 sporulation medium and incubated at 35°C under shaking at 200 rpm for 10 days. During incubation period, formation of spore-crystal bodies was monitored using light microscope. To remove soluble exotoxins, bacterial cultures were centrifuged at 5000 rpm and pellets (containing spore and parasporal crystal bodies) were washed twice with phosphate buffer (50 mM, pH 7.4). Pellet fractions were resuspended in 5 mL of distilled water and kept at 4°C until bioassay. Number of spores in the preparations was counted using neubauer chamber.

Bioassay with *A. kuehniella* Larvae

Spore-crystal preparations were examined for their toxicity against the second larval stage of *A. kuehniella*. Briefly, diet of larvae was prepared by adding different amounts of bacterial preparations (2×10^8, 2×10^9 and 2×10^{10} spores) to one gram of larvae food (70% wheat flour, 23% wheat bran and 7% yeast). The prepared diet was dried and spread in the culture plates. Fifteen larvae (second-instars) were added to each plate and then plates were incubated in a humidified chamber at 25°C for 14 days. Mortality was monitored daily by counting dead larvae. Larvae in the negative control groups were fed on a diet without bacterial contamination. Diet of larvae in the positive control group was supplemented with *Bt.* subsp. kurstaki (PTCC 1494). All experiments were performed in triplicate. The bioassay data were recorded after 7 and 14 days and cumulative mean percent mortality of larvae was calculated.

Bioassay with *P. xylostella* Larvae

After catching butterflies from their environment, they were kept in a hand-made container of plexiglas and net cloth. To increase oviposition rate, they were fed with honey solution. To provide a surface for oviposition, leaves of red cabbage were placed in the containers. Oviposition container was placed in a humidity chamber set at 25°C, 50% RH, and a photoperiod of 12: 12-hour (L: D). After observation of white eggs on the leaves of red cabbage, fresh leaves of organic Kohlrabi reared in the laboratory were added to the containers. After few days, larvae in the second stage were seen in the containers. These larvae were separated and fed on fresh leaves impregnated with the bacterial spore-crystal preparations. Bioassay was performed as described in section 2.6.1 and analysis was performed by counting dead larvae.

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Statistical Analysis

All treatments were performed in triplicate and data presented as mean±*SD* (standard deviation). Bioassay data were subjected to student t-test analysis using SPSS 13.0 for windows and *P* value of < 0.05 (in comparison to untreated control group) was considered as statistically significant.

RESULTS AND DISCUSSION

Selection and Isolation of Bacterial Strains Producing Parasporal Crystalline Inclusions

New bacterial isolates with higher crystal toxicity and broader spectrum of activity are needed for production of commercial pesticides. The main goal of the current study was searching for promising bacterial candidates against insects of the order Lepidoptera by analyzing crystalline protein patterns and by characterization of *cry* gene contents. Two hundred diverse soil samples were collected from a region in the north of Iran (Figure 1). Soil samples were used as the source of *Bacillus* bacteria capable to produce parasporal crystalline proteins. Our selection approaches resulted in the isolation of 10 spore-forming *Bacillus*-like colonies that were streaked on LB plates to obtain pure cultures. Selected isolates were grown in liquid T3 medium and rechecked for the ability to produce spore and parasporal bodies using phase-contrast microscope and by specific staining approaches. SDS-PAGE analysis was used to compare protein profile of spore-crystal preparations. In Figure 2, electrophoretic pattern of spore-crystal suspensions from 10 selected isolates is shown. The *B. thuringiensis* subsp. kurstaki is included in the analysis as a crystalline protein-producing control. As seen, each isolate has produced a characteristic protein profile, while some degree of similarity is seen among isolates. Analysis of the crystal-spore mixtures of the isolates with SDS-PAGE showed proteins with molecular

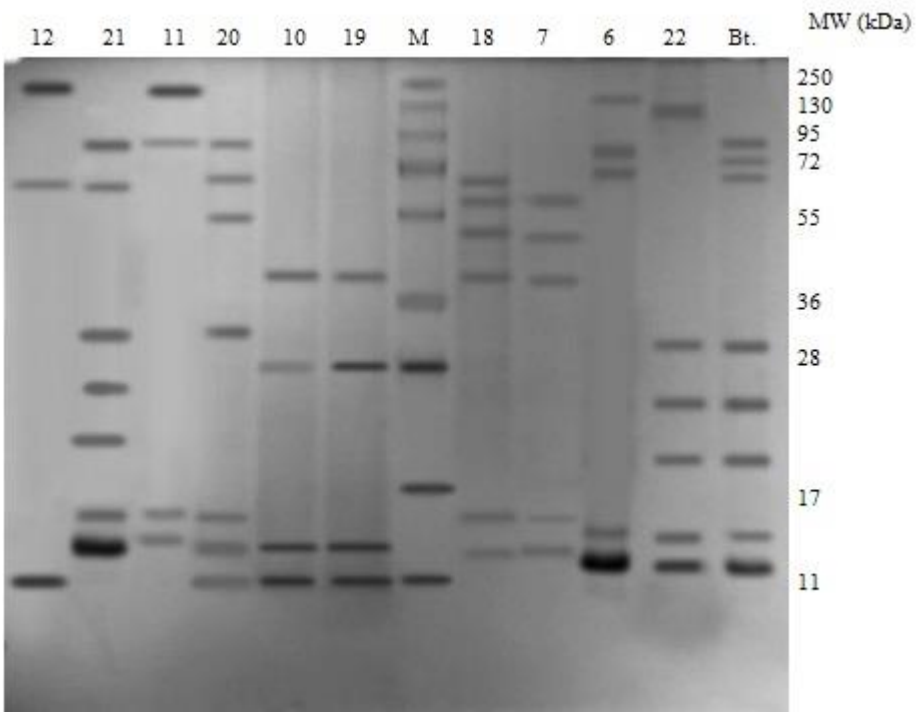

Figure 2. SDS-PAGE profile of spore-crystal mixture of isolates. Line M, protein molecular weight marker and line BT, *Bacillus thuringiensis* subsp. kurstaki (PTCC 1494). Indigenous isolates are shown with number.

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weights between 11-230 kDa. The molecular weights of the major protein bands that were visualized by coomassie-blue staining are presented in Table 2. Two distinct regions, a region with molecular weights between 10-20 kDa and another between 40-90 kDa, are seen in SDS-PAGE profile of spore-crystal mixtures of the isolates. In all of the isolates, except RIPI12, a protein band with a molecular weight of about 12 kDa is common (Figure 2). Based on the reports by Höfte and whitely (1989), products of *cry1*, *cry2*, *cry3*, *cry7/8* and *cry9* genes are protoxins with molecular weights of 130-150, 65-70, 75, 130 and 130-140 kDa, respectively. Some Cry proteins such as Cry23, Cry34 and Cry37 have molecular weights in the range of 10-30 kDa (Arrieta *et al*., 2004). Based on the data gathered from the related literatures, predicted Cry proteins in our bacterial isolates are presented in Table 2. It must be stated that molecular weights of Cytolytic (Cyt) proteins of *Bt* strains is about 28-30 kDa. Thus, expression of Cyt proteins in isolates RIPI 20, 21, 22 and also *Bt.* subsp. kurstaki is probable. Because of post-transcriptional and post-translational regulations of *cry* gene products, it is possible that *Bt* strains with the same type of *cry* genes exhibit different protein profile. Also, it is noteworthy that each *cry* gene group may

Table 2. Molecular weight of spore-crystal mixture proteins visualized in coomassie blue-stained SDS-PAGE and their predicted corresponding Cry proteins.

Protein band (kDa)	Bacterial isolates										
	Bt	RIPI 6	RIPI 7	RIPI 10	RIPI 11	RIPI 12	RIPI 18	RIPI 19	RIPI 20	RIPI 21	RIPI 22
11	*			*		*		*	*		
15		*									*
16	*	*	*	*	*		*	*	*	*	*
17			*		*		*		*	*	
19	*										*
20										*	
22	*										*
23										*	
25				*				*			
26	*										*
28									*	*	
35			*	*			*	*			
43			*				*				
48									*		
52			*				*				
58										*	
60						*					
62	*						*		*		
65		*									
74	*										
80		*									
85					*					*	
90	*								*		
134											*
178		*									
230					*	*					
Predicted Cry Protein	2, 3 34,37	3, 34 37	35,34 37	34,37	3, 22 34,37	34,37	2, 35 34, 37	34,37	2, 35 34,37	3, 22 34,37	1,7 8,9 34 37

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contain different subtypes with some degree of differences in characteristics of protein products. Therefore, observation of dissimilar protein profiles in different strains with the same *cry* gene content e.g. Cry1 and Cry2 in *Bt.* subsp. kurstaki, is probable. Difference in the protein profiles suggest diversity of *cry* genes and probability of finding new Cry proteins with novel and distinct insecticidal activity to protect crops from insects.

The presence of *cry* genes as an indicator of the insecticidal activity in 10 isolates was investigated by PCR using universal primers for *cry1-4* genes (Un1-Un4, Table 1). Total DNA was extracted from isolates and used as template for PCR analysis of *cry* genes. Expected PCR products for *cry1*, *cry2*, *cry3* and *cry4* genes are fragments with 274-277, 689-701, 589-604 and 430-439 bp, respectively (Ben-Dov *et al*., 1997). In the current study, PCR analysis indicated that of the 10 isolates, only isolates RIPI6 and RIPI18 may contain *cry3* gene (Figure 3). Isolate RIPI21 and also *Bt*. subsp. kurstaki were positive by PCR for *cry1* gene. Other isolates did not contain any of the examined *cry1* to *4* genes. Presence of *cry1* gene is an indicator of the pesticide activity against insects from order Lepidoptera and Cry3 proteins are marker of insecticidal activity against Coleoptera (Bravo *et al*., 1998; Crickmore *et al*., 2016). The isolates, with no cry amplification products, may contain other classes of *cry* genes, especially *cry34, 35, 37* and genes of *cyt* family based on a prediction from their crystalline protein profiles.

Identification of Parasporal Inclusion-Producing Strains

16S rRNA sequencing is a reliable method for the identification of bacteria in a reasonable time. Total DNA from each isolate with parasporal inclusion body was amplified in the presence of 9F and 1541R primers (Nakagawa *et al*., 2002). The amplified products were resolved in agarose gel and, following staining with ethidium bromide, were visualized in a gel documentation system. As shown in Figure 4, each isolate gave an electrophoretic band

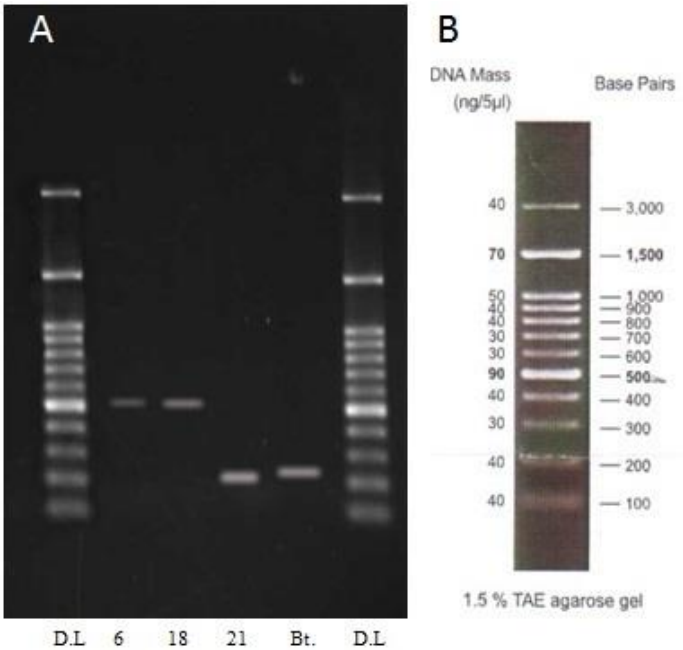

Figure 3. (A) Agarose gel electrophoresis of PCR products for *cry3* (Lanes 2 and 3 from left) and *cry1* (Lanes 4 and 5 from left) genes. Lane DL: 100-3,000 bp DNA ladder, Lane 2: Isolate RIPI6, Lane 3: Iisolate RIPI18, Lane 4: Isolate RIPI21 and Lane 5: *Bt.* subsp. kurstaki. (B) Agarose gel electrophoretic profile of 100-3,000 bp DNA ladder.

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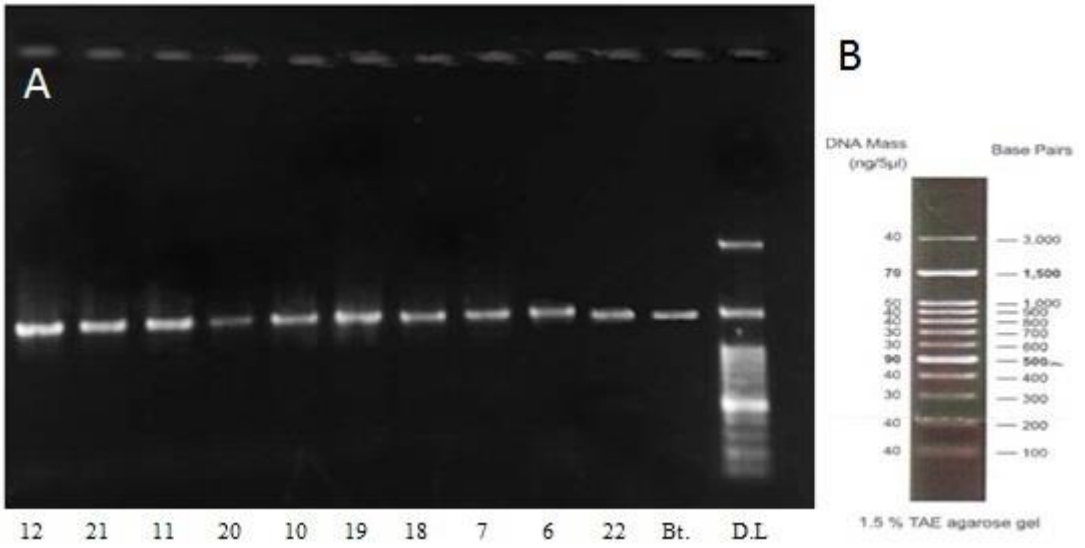

Figure 4. (A) Agarose gel electrophoresis of 16S rRNA PCR products amplified using 9F and 1541R primers. Lane DL: DNA ladder, Lane Bt: *Bt.* subsp. kurstaki, Lanes 1-10 (from left): Isolates RIPI12, 21, 11, 20, 10, 19, 18, 7, 6 and 22, respectively. (B) Agarose gel electrophoretic profile of 100-3,000 bp DNA ladder.

with a size of about 1,500 bp. PCR products of *16S rRNA* genes were sequenced in a commercial sequencing company using ABI 3100 automated DNA sequencer (Applied Biosystem). The obtained *16S rRNA* gene sequences were compared with sequences already submitted in the GenBank database using the standard BLAST tool provided by NCBI (www.ncbi.nlm.nih.gov). Based on the BLAST results (Table 3), the 16S rDNA sequences of all isolates showed at least 96% sequence match with *Bt* strains deposited in the GenBank. After alignment of the most similar sequences with CLUSTALW, phylogenetic tree was depicted. As seen in Figure 5, the phylogenetic tree displays distribution of 10 isolated strains into two major A and B groups. Group A includes isolates RIPI7, 10, 18, 19, 20, 21 and 22 and Group B includes isolates RIPI6, 11 and 12. There were two clusters in Group A. Isolates RIPI10 and RIPI22 along with reference strain of *Bt*. subsp. kurstaki were in the cluster I and other members of Group A were within cluster II. In the cluster II, strains RIPI19, 20 and RIPI7, 18 are more closely related, whereas RIPI10 and RIPI21 are the most divergent.

Insecticidal Bioassay

The insecticidal activities of 10 different crystal-spore mixtures from isolated environmental samples along with crystal-spore preparation from references strain *Bt.* subsp. kurstaki (PTCC 1494) were evaluated against *A. kuehniella* and *P. xylostella* larvae. The bioassay data were recorded after 7 and 14 days and cumulative mean percent mortality of larvae was calculated and presented in Table 4 as mean±*SD*. Only at a dose of 2×10^{10} spores g^{-1} food a significant and repeatable insecticidal activity was observed. At 7^{th} day, cumulative mean percent mortality of *A. kuehniella* larvae was in the range of 27±4% (isolate RIPI6) to 76±10% (isolate RIPI22). Mortality of larvae was increased during a treatment period of 14 days and ranged from 76±4% (isolate RIPI18) to 97±4% (isolate RIPI10).

The bioassay data for the isolated strains against *P. xylostella* after a 7-day period of treatment showed that RIPI20 and RIPI22 are the most toxic strains against larvae.

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Table 3. Results of BLAST search of 16S rRNA sequences of isolated strains with those deposited in the GenBank database.

Strains	Closest relevant	Similarity (%)
Bt. Subsp. kurstaki	*Bacillus thuringiensis* strain 262AG1 16S ribosomal RNA gene	99%
RIPI22	*Bacillus subtilis* strain SAN15 *16S ribosomal RNA* gene	99%
	Bacillus thuringiensis 16S ribosomal RNA gene	98%
RIPI6	*Bacillus thuringiensis 16S ribosomal RNA* gene	99%
	Bacillus subtilis strain SAN15 *16S ribosomal RNA* gene	99%
RIPI7	*Bacillus* sp. 24B *16S ribosomal RNA* gene	98%
	Bacillus thuringiensis strain VKK-GJ-4 *16S ribosomal RNA* gene	97%
RIPI18	*Bacillus* sp. FFANFB-8 16S ribosomal RNA gene	98%
	Bacillus thuringiensis strainFayB1 *16S ribosomal RNA* gene	96%
RIPI19	*Bacillus thuringiensis* strain VKK-GJ-4 *16S ribosomal RNA* gene	98%
	Bacillus sp. 24B *16S ribosomal RNA* gene	99%
RIPI10	*Bacillus subtilis* strain cjp-3 *16S ribosomal RNA* gene	99%
	Bacillus thuringiensis strain FayB1 *16S ribosomal RNA* gene	99%
RIPI20	*Bacillus thuringiensis* strain JeSa1 *16S ribosomal RNA* gene	98%
	Bacillus sp. 24B *16S ribosomal RNA* gene	99%
RIPI11	*Bacillus* sp. FFANFB-9 *16S ribosomal RNA* gene	98%
	Bacillus thuringiensis strain WB27 *16S ribosomal RNA* gene	97%
RIPI21	*Bacillus thuringiensis* strain FayB1 *16S ribosomal RNA* gene	96%
	Bacillus sp. FFANFB-1 *16S ribosomal RNA* gene	98%
RIPI12	*Bacillus* sp. KP071r *16S ribosomal RNA* gene	99%
	Bacillus thuringiensis strain WB27 *16S ribosomal RNA* gene	98%

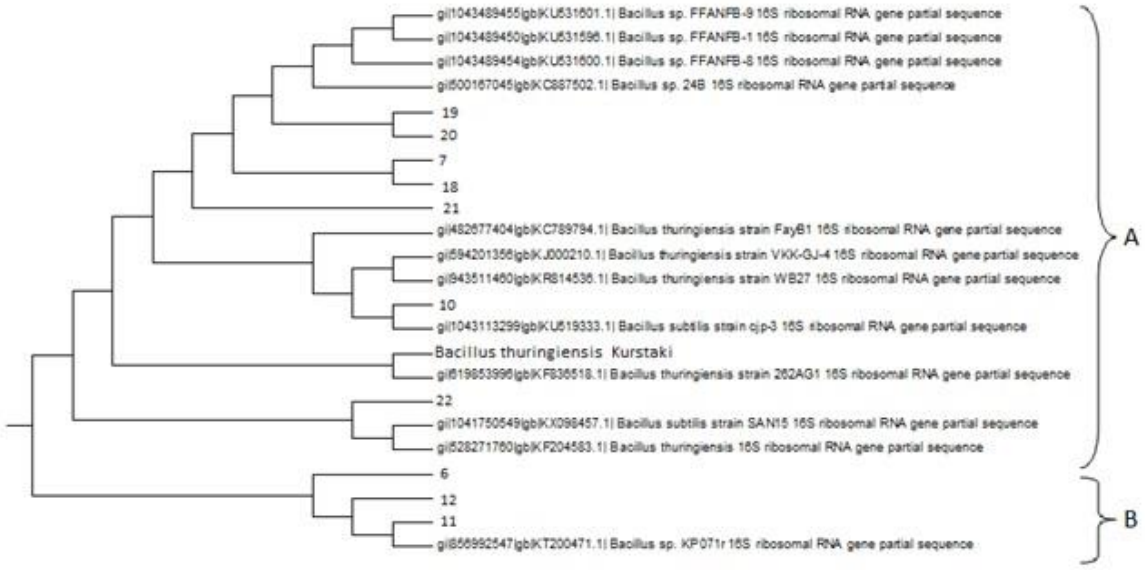

Figure 5. Phylogenetic tree of 10 isolated strains constructed from *16S rRNA* gene sequences,

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Table 4. Efficiency of the crystal-spore mixture from isolated strains and *Bt.* subsp. kurstaki reference strain against *A. kuehniella* and *P. xylostella* larvae

Strains	*A. kuehniella* mortality (%)		*P. xylostella* mortality (%)	
	7- day	14-day	7-day	14-day
Bt. subsp. kurstaki	35± 3.5	89± 14	29± 10	100
RIPI6	27± 4	84± 16	12± 4*	66± 5.3
RIPI7	28±10	91± 10	72± 21	100
RIPI10	31± 4	97± 4	85± 14	100
RIPI11	47±14	86± 17	9± 3.6*	66± 5.3
RIPI12	55± 10	80± 11	7±5*	35± 3.5
RIPI18	52± 4	76± 4	36± 12	86± 17
RIPI19	33± 13	80± 11	40± 5	100
RIPI20	41± 5	86± 17	100	100
RIPI21	37± 10	80± 11	45± 14	100
RIPI22	76± 10	90± 10	100	100

* No significant difference in comparison to untreated control group (P> 0.05).

RIPI6, 11 and 12 were the least effective strains against *P. xylostella* larvae. Again, only at a dose of 2×10^{10} spores g^{-1} food, a significant insecticidal response was seen. Extending treatment period to 14 days resulted in higher mortality such that cumulative mean percent mortality of larvae ranged from 35± 3.5% (RIPI12) to 100% (RIPI7, 10, 19, 20, 21, 22 and *Bt*. subsp. kurstaki reference strain).

In conclusion, we reported here a number of *Bt* bacterial strains active against *P. xylostella* and *A. kuehniella* larvae in the soil samples collected from North of Iran. Among isolated strains, RIPI22 had a two-fold higher activity against *A. kuehniella* than control strain *Bt*. subsp. kurstaki in 7-day experiments. Also, insecticidal activity of RIPI22 against *P. xylostella* was three-fold higher compared to the control strain. Strains such as RIPI22 and to a lesser extent RIPI10 and RIPI20 are candidate of choice for biological control of major agricultural pests in the indicated regions.

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جداسازی و تعیین خصوصیات باسیلوس های تولید کننده اندوتوکسین های حشره کش از خاک شمال ایران

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چکیده

هدف اصلی از انجام این مطالعه، جداسازی و تعیین خصوصیات آن دسته از باکتری های اسپورزای خاک می باشد که طی فاز سکون می توانند پروتئین های کریستالی پارا-اسپورال علیه لارو فلس بالان تولید کنند. دو روش مختلف برای جداسازی سویه های اسپورزا مورد استفاده قرار گرفت. از واکنش زنجیره ای پلیمراز (PCR) برای شناسایی محتوای ژن های cry در سویه های جداسازی شده و از روش تعیین ترادف 16S rRNA برای شناسایی سویه های باکتریایی استفاده شد. کارایی سویه ها بعنوان حشره کش در یک آزمون زیست سنجی با استفاده از لارو حشرات انجام شد. با توجه به وجود کریستال های ذخیره ای و الگوهای الکتروفورزی (SDS-PAGE) ، ده سویه باکتریایی از ۲۰۰ نمونه خاک جداسازی شد. آنالیز مخلوط های کریستال-اسپور با کمک SDS-PAGE طیف وسیعی از پروتئین ها با وزن مولکولی بین ۱۱ تا ۲۳۰ کیلودالتون را نشان داد. داده های آنالیز PCR نشان داد که فقط دو سویه RIPI6 و RIPI18 واجد ژن cry3 می باشند. همچنین، فقط سویه های RIPI21 و شاهد (باسیلوس تورنجیینسیس کورستاکی) واجد ژن cry1 بودند. ترادف یابی ژن های 16S rRNA تمام سویه های جداسازی شده وجود یک انطباق حداقل ۹۶ درصدی با سویه های باسیلوس تورنجیینسیس ثبت شده در بانک ژنی را نشان داد. نتایج آزمون های زیست سنجی نشان دهنده کارایی سویه های RIPI7, 10 , 22 در کشتن لاروهای *Anagasta kuehniella* و *Plutella xylostella*بود. به طور خلاصه می توان گفت که برای کنترل بیولوژیک آفات گیاهی در شمال ایران می توان برخی از گزینه های باکتریایی را نیز مورد توجه قرار داد.

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