Isolation and Geographical Diversity of *Bacillus thuringiensis* from Some Soils in Iran

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Abstract*— *Bacillus thuringiensis is a spore-forming bacterium showing the unusual ability to produce endogenous crystals during sporulation that are toxic for some pest insects. This work was performed to study the Isolation Bacillus genus and biochemical Identification of *B. thuringiensis* and it`s ecological distribution from some Iranian soils and also introducing soil as a strength source of this bacterium. Using a standard isolation method with specific medium, *B. thuringiensis* was isolated from 23 out of 30 soil samples collected in the Soil and Water Research Institute (SWRI) of Iran. The bacillus population percentage mean of samples` total bacteria population was (44.44%) and *B.thuringiensis* population percentage mean of total bacteria population was 3.45%. Maximum and minimum B.T. population logarithms were 4.78 and 0 for sample number 2759 and 7 samples had no B.T. population, respectively. Using Muller Hilton agar for total bacteria population and Hicrome Bacillus Agar for bacillus genus population and then biochemical Identification of bacillus isolates showed a great biodiversity among soil samples, which were distributed among different species, including *megaterium*, *polymixa* and *cereus* etc. We found variable percentages of *B.thuringiensis* isolates among soil samples and knew about soil as a valuable source of this bacterium to use in biological pesticides and insecticides because of their high ability against pests and insects.

***Keywords*—** *Bacillus thuringiensis*, Insecticides, Geographical diversity.

I. INTRODUCTION

BACILLUS Thuringiensis (Berliner) is a gram-positive spore-forming bacterium which has the unusual property of producing a parasporal protein crystal (delta-endotoxin) which is toxic (Cry proteins) for some pest insects. Because of the insecticidal activity, which represents an alternative strategy to chemical insecticides, a number of strains has been isolated and used to control pest of agricultural which is called bio control and also they have medical importance [1]. Bio control agents kill their host or make them powerless and to some extent act as special to some groups. Most of the commercial species belong to genus Bacillus and most of products in market are made by using *bacillus thuringiensis* [18]. Up to previous years, existence of subsidized fertilizers and pesticides caused irregular use of CHEMICAL PESTICIDES and fertilizers by farmers and in recent years, the poor quality of imported pesticides have caused environmental problems. Also failing to measure agricultural products pollution to these materials has threaten public health. Therefore biological fertilizers and insecticides are the best option to prevent human and environment from these harmful materials. Since biological control involves the use of organisms which are a natural part of environment, is environmental friendly.

To this end, there has been much effort in many countries to isolate new strains with increased potency against target pest insects and a wider host range. An accessible and reliable resource of micro organisms including *B.thuringiensis* is soil which contains a wide range of different types of micro organisms, such as Bacteria, Fungi and Algae. *B.thuringiensis* is one of the bacillus genus species. Phonotypical, Biochemical and Molecular methods are used to differentiation of micro organisms into genus, species and subspecies. At least 69 serotypes and 80 subspecies are numbered and registered at the International Entomopathogenic Bacillus Centre (IEBC) collection at Institute Pasteur, Paris, France [2]. Although there is some correlation between subspecies and pathogenicity, at the moment it is known that strains of different subspecies may be toxic to insect of various orders, and even that strains from the same subspecies show differences in toxicity. The toxicity spectrum of *B.thuringiensis* subspecies is determined by the different delta-endotoxin genes (cry genes) carried by their strains, and by the encoded proteins (Cry proteins) [3]; thus knowing more about different resources of this bacteria and their population is a good basis to study insecticidal activity of *B.thuringiensis* and is an important component of studying *B.thuringiensis* resources.

It has been suggested by some authors that even if *B.thuringiensis* is a ubiquitous distributed organism, the normal habitat of this bacterium is in the soil, and many efforts for isolating novel *B.thuringiensis* from soils of several countries from the five continents has been performed [4,5,6,7]. Today, several tens of thousands of isolates, probably more than 50,000 are distributed among various private and public collections, and a reconsidered to be potential ‘‘reservoirs’’ for novel insecticidal toxins [8]. To be able to estimate the risk of releasing any microorganism into the environment it is important to understand the way that it interacts with its surroundings and the other biota. In the case of *B.thuringiensis*, there has been extensive study of its

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toxicology and safety, but there has been only limited research on its role in the environment. This is the case for Iran, where surveying new *B.thuringiensis* strains and their Cry proteins has not been reported but in other countries, such as Spain, has been done [9,10], which is indicating a rich diversity of serotypes (H-serotypes) and insecticidal activities. But the ecological distribution of this bacterium in the soil remained unexplored. Iran has a heterogeneous territory with different types of soil that begins with Caspian Sea in North and ends to Persian Gulf in south with different climates which gives unique geographical features and abundant biological resources to ecologically study the distribution of this organism in the soil. The objective of this work was to study the Isolation and ecological distribution of B.thuringiensis spores from the Iranian territory using a standard isolation method with specific medium in order to understand about soil ability to be as source of this bacteria because of its` insecticidal activity against insect species of different orders.

II. Methods and Materials

1 –When serial dilution of soil samples suspensions inoculated to bacillus agar plates from last three dilutions within 3 repetitions were done, cultivated plates were kept into the incubator for 24 hours. Then bacteria colonies with similar color and type have been counted and typical colonies have been isolated. Colony counting has been done for each soil sample separately. All steps of diagnostic tests were done based A color Atlas of Bacillus species [11].

2- 49 g of γ-radiated HiCrome bacillus agar media (Himedia M1651) was poured into the flask containing 1 liter sterile distilled water under Laminar and aseptic condition. Then the flask was heated to be resolved smoothly. After cooling, the media was distributed into the plates under aseptic condition. After becoming solid and putting the plates into the incubator over night to control pollution, the non-polluted plates were ready to be used.

Fig. 1 Different bacillus species grow in different colony shapes on Hicrom media

3 – To serial dilution, 10 g of soil was poured into the flask containing 90cc sterile distilled water and was shacked for half an hour on the Shaker. Then 1cc of soil suspension was transferred into the tubes containing 9cc sterile distilled water using sterile pipette, then the tube was shacked very well. 1cc of this tube was transferred into the next tube which contains 9cc of sterile distilled water using sterile pipette. This was continued up to 10^{-8} dilution. Finally, 0.1cc of the last three dilutions was transferred into the bacillus agar plates using sterile pipettes with 3 repetitions under aseptic condition.

4- Because of total bacteria counting, 24 gram of Muller Hilton Agar media was poured into the flask containing 1 liter distilled water. Then, the flask was put into the autoclave at 115°c for 25 minutes in order to be sterile. After media cooling, it was poured into the plates under laminar and sterile condition. Plates were kept for 24 hours in order to be sure that plates are not polluted. Then plates were inoculated from last three dilutions of serial dilution for each soil sample suspension separately. After an overnight incubation all colonies have been counted [12].

5- In order to Gram staining, after preparing slides from 24 hours cultures, drying and fixing it, Gram stain was performed. Slides were observed under the microscope. Cells which were stained blue were considered as positive.

6 - For VP test, 14 grams of MR-VP media was poured into the flasks containing 1 liter distilled water. After dissolving, 15 cc of the media were transferred into the test tubes, and then they have been put into the autoclave at a temperature of 115 °c for 15 minutes. The desired bacteria were transferred into the tubes under Laminar and aseptic condition. Then they have been incubated at 30 ° for 7 days. 6 drops of 5% α-naphtol solution was poured into the tubes and tubes were shacked very well and finally two drops of KOH 40% solution were poured into the tubes and they have been put into the incubator for 5 hours. Tubes that changed color to red, considered as positive.

7- Growth in 7 percent salt was done by adding 20 g of the nutrient agar media into the flask containing 1 liter distilled water, and also 70 g of NaCl was added into the flask simultaneously. Then the flask was put into the autoclave for 25 minutes to be sterile. After sterilization, and cooling the sterile media was poured into the palates under the laminar and sterile condition.

8- In order to test bacteria ability to grow under the anaerobic condition, 20 g of the Nutrient Agar media was added into the flask containing 1 liter distilled water. After dissolving, the flask was put into the autoclave for 25 minutes to be sterile. After sterilization, the media was poured into the sterile tubes (15×1.5 cm). Then tubes were left to cool and be solid and over night incubator to be sure that tubes are not polluted. Then tubes were be inoculated by straight wire through the appropriate depth of the media. After inoculation, sterile liquid paraffin was poured into the tube in order to have anaerobic condition. Tubes were kept into the incubator for 7 days. If the bacteria could grow in anaerobic conditions, growth at the depth of the tube was visible and considered as positive otherwise it was negative.

(a) (b)

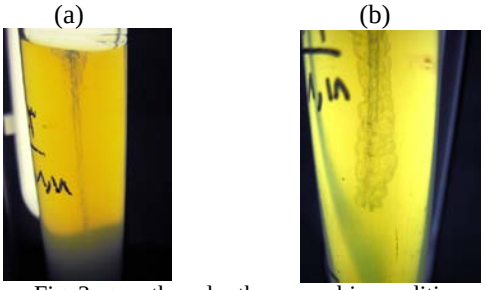

Fig. 2 growth under the anaerobic condition
(a) negative (b) Positive

9- For the growth at 50°c, desired plates were kept in incubator adjusted to 50°c (temperature) for 24 hours.
10- Malachite green slide staining method was used for parasporal bodies staining. Slides were observed under the microscope and the existence of parasporal bodies was investigated.

III. Results

A total of 23 out of 30 collected samples yielded *B. thuringiensis* (chart2). Of these, the higher percentage of samples with *B. thuringiensis* was observed in soil sample no.2756(chart1), very similar to the one observed in the samples 6335 and 6336 (chart1), while the lower was found in the soil sample no.760 just like samples 3155 and 7770(chart1). 7 samples showed *B. thuringiensis* population less than 103 (bacteria/10 gram soil) including samples no. 7774, 7771, 7767, 6338, 6331, 6334, 3256. *B. thuringiensis* population was variable depending on the territory where the samples were taken.

TABLE I
Results Of Biochemical Identification tests

Bact-eria no.	Bacteria name	V.P	Growth in 7% salt	Growth in anaerobic condition	Growth at 50 °	Parasporal bodies
1-5	*B.t*	+	+	+	-	+
2-3	*B.t*	+	+	+	-	+
3-4	*B.t*	+	+	+	-	+
4-4	*B.t*	+	+	+	-	+
8-10	*B.t*	+	+	+	-	+
9-6	*B.t*	+	+	+	-	+
10-7	*B.t*	+	+	+	-	+
12-6	*B.t*	+	+	+	-	+
13-8	*B.t*	+	+	+	-	+
14-4	*B.t*	+	+	+	-	+
15-7	*B.t*	+	+	+	-	+
16-4	*B.t*	+	+	+	-	+
17-4	*B.t*	+	+	+	-	+
19-9	*B.t*	+	+	+	-	+
20-5	*B.t*	+	+	+	-	+
21-8	*B.t*	+	+	+	-	+
24-9	*B.t*	+	+	+	-	+
25-6	*B.t*	+	+	+	-	+
26-4	*B.t*	+	+	+	-	+
27-7	*B.t*	+	+	+	-	+
28-6	*B.t*	+	+	+	-	+
29-10	*B.t*	+	+	+	-	+
30-9	*B.t*	+	+	+	-	+

Hicrome Bacillus agar medium (Himeida M1651) was a selective medium for bacillus genus which only different bacillus species could grow on it in different types of colony phonotype. Thus, different species could be counted, isolated and identified during different process. The data of colony counting was analyzed by SASS software in order to find out if there is any difference between samples or not (Table II).

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TABLE II
ANOVA Table

Sources of variation	degree of freedom	Sum of squares	Mean Square	Pr>F
Treatment(soil)	21	11.36	0.54**	0.0001
Error	37	1.04	0.028	
Total	58	12.40		
CV = 4.04				

** Significant at one percent

After an overnight incubating all Muller Hilton and Bacillus agar plates have been counted. And their results are mentioned in fig. 1. In some cases, the amounts of bacillus count were more than the amounts of total bacteria count. Bacillus agar media is a specific media for bacillus genus and it is clear that all bacillus species can grow on it. In the other hand, on the Muller Hilton agar media, all kinds of bacteria can grow and there should be competition on nutrients among all bacteria. Therefore, this reason causes some reduction in bacillus count on Muller Hilton Agar media.

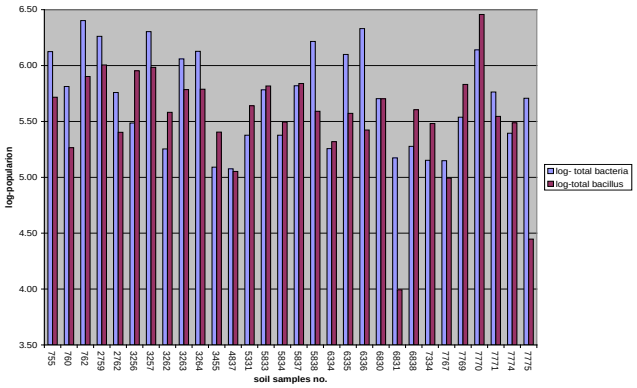

Fig. 1 Total bacteria and total bacillus population frequency in soil samples

After counting the all bacillus population on Bacillus Agar media, similar colonies were isolated and were counted separately. Each isolate went through the biochemical tests in order to identification. Finally, isolates population which have been resulted in *Bacillus thuringiensis*, were collected. Their populations are shown in fig. 2.

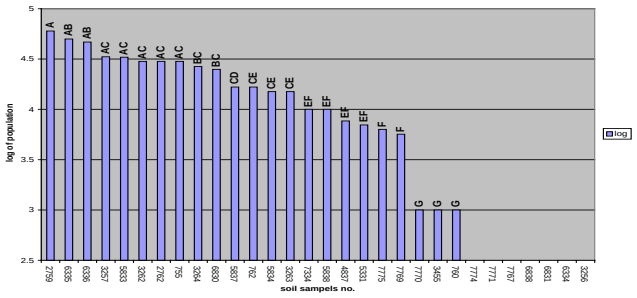

Fig. 2 comparing average population of *Bacillus thuringiensis* in soil samples

Bacillus thuringiensis population

* Samples with at least one common word do not have significant difference.

As it is showed obviously in the chart, maximum population belongs to the soil sample no. 2759 and minimum population belongs to soil sample no. 760. Also it is better to say that soil

samples no. 7774,7771,7767,6338,6331,6334,3256 have no *Bacillus thuringiensis* population of less than 103

One of the goals of this research was to gain results which show the percentage of bacillus in total bacteria. And also it was reachable that what percentage of bacillus is *Bacillus thuringiensis* or what percentage of total bacteria was *Bacillus thuringiensis*. These results are shown tableⅢ.

TABLE Ⅲ

soil numbers	mean of total bacteria	total bacillus	mean of B.t.	B.t.% in total bacteria	B.t.% in total bacillus
755	1333333.3	521000	30000	2.25	5.75815
760	650000	184166	1000	0.153846154	0.54298
762	2526666.6	798333	16666.6	0.659630607	2.08768
2759	1826666.6	1011666	60000	3.284671533	5.93081
2762	573333.3	252666	30000	5.23255814	11.8733
3256	306000	897666.666	0	0	0
3257	2010000	963000	33333.3	1.658374793	3.46140
3262	179333.3	381333	30000	16.72862454	7.86714
3263	1146666.6	609666	15000	1.308139535	2.46036
3264	1340000	614000	26666.6	1.990049751	4.34310
3455	123333.3	253666	1000	0.810810811	0.39421
4837	119000	113000	7666.66	6.442577031	6.78466
5331	238000	437000	7000	2.941176471	1.60183
5833	606666.6	656500	33000	5.43956044	5.02665
5834	238000	311666.666	15000	6.302521008	4.81283
5837	660000	690666	16666.6	2.525252525	2.41313
5838	1643333.3	390000	10000	0.60851927	2.56410
6334	180666.6	208666	0	0	0
6335	1256666.6	373333	50000	3.978779841	13.3928
6336	2140000	265000	46666.6	2.180685358	17.6100
6830	505000	504666	25000	4.95049505	4.95377
6831	149333.3	9833	0	0	0
6838	189333.3	402500	0	0	0
7334	142000	303000	10000	7.042253521	3.30033
7767	140666.6	98333.3333	0	0	0
7769	345000	678000	5666.66	1.642512077	0.83579
7770	1383333.	2865500	1000	0.072289157	0.03489
7771	580000	351333.333	0	0	0
7774	248000	307333	0	0	0
7775	510000	28000	6333.33	1.241830065	22.6190

TABLE Ⅳ

Mean frequency of %bacillus in total bacteria *	44.04
mean frequency of % B.t in total bacteria	3.45
mean frequency of % B.t in bacillus	5.68

* As mentioned before in some samples bacillus population was more than total bacteria population because of using bacillus agar media. These samples have not been involved in percentage calculation.

TABLE Ⅴ
SOIL CHEMICAL PROPERTIES

Soil	P-mg/kg	K-mg/kg	Fe-mg/kg	Zn-mg/kg	Mn-mg/kg	%O.C
755	28.2	374	8.44	5.34	13.08	1.46
760	10.1	255	81.6	1.24	42.9	2.07
762	20.7	246	14.02	0.72	19.22	1.36
2759	23.2	294	36.42	0.4	31.3	1.33
2762	28.1	51	78.4	7.46	7.46	2.45
3255	27.3	401	4.44	0.4	6.74	0.95
3256	9.1	154	2.88	3.04	8.1	0.71
3257	10.9	286	4.06	0.78	6.3	1.03
3262	8.8	60	2.26	0.86	6.02	0.44
3263	44.1	322	5.5	0.22	10	1.39
3264	3.7	140	4.48	1.06	6.02	1.29
4837	11.8	100	61.2	0.8	16.9	1.58
5331	13.3	96	168.5	2.16	11.36	3.40
5833	6.1	328	1.7	0.5	5.8	1.52
5834	13.4	291	2.14	0.5	3.4	0.98
5837	15.4	346	1.88	0.36	1.38	1.52
5838	3.7	299	3.02	0.48	2.64	1.46
6334	6.7	261	2.12	0.78	2.56	0.72
6335	16.2	226	2.68	0.42	1	0.60
6336	36.1	352	2.56	0.32	1.44	1.08
6830	10.7	306	4.32	0.96	11.82	1.79
6831	1.3	102	2.86	0.2	1.24	0.14
6838	12.1	363	5.18	0.32	1.72	1.81
7334		176	7.48	0.22	10.04	0.68
7767	4.4	102	58.98	0.6	80	2.83
7769	11.0	237	3.54	2.86	2.64	1.16
7770	11.4	109	19.82	0.52	3.58	1.12
7771	120.0	515	19.7	8.94	5.96	2.87
7774	9.8	195	12.88	4.82	5.28	1.38
7775	5.6	118	29.14	0.28	11.18	2.09

TABLE Ⅵ
CORRELATIONS

		mean	P	K	Fe	Zn	Mn	Cu	O.C.
mean	Pearson Correlation	1	-.08	-.05	.07	-.21	-.15	.23	-.10
	Sig. (2-tailed)		.66	.79	.69	.26	.41	.22	.58
	N	29	28	29	29	29	29	29	29
**. Correlation is significant at the 0.01 level (2-tailed).									
*. Correlation is significant at the 0.05 level (2-tailed).									

IV. DISCUSSION

This experiment was done on randomly selected soil samples of Soil and Water Research Institute` s chemistry department. All data was analyzed in the completely randomized design using SASS software and comparison of means table was resulted. Eventually the bacteria *B.thuringiensis* population differentiation in some soils of the Iran was resulted. The results indicate how the population distribution of the bacteria *B.thuringiensis* is and which soil has the highest population and which has lowest. And it was gained that 62% of randomly selected soil samples have *B.thuringiensis* spores. In this research, not only the comparison of all bacillus population with total bacteria population is available, but also the comparisons of *B.thuringiensis* population with total bacillus population and total bacteria population are available. Also the correlations between *B.thuringiensis* and soil chemical properties have been resulted.

Our results indicate that the Iranian soils are very rich in diversity of *B thuringiensis* species. We have found variable percentages of samples with *B.thuringiensis* percentage in total bacteria obviously depending on their origin, 16.73% in soil sample no. 5331 and 0% in soils samples no. 7774,7771,7767,6338,6331,6334,3256, respectively. We have

found no correlation between the percentage of soil with *B. thuringiensis* and the soil chemical and physical properties. In contrast, it is difficult to explain the difference between the percentages of samples with *B.thuringiensis* , since under a geological point of view because of numerous parameters which influence bacteria population in soil, such as climate, minimum and maximum temperature soil type, type of cultivation, type of agricultural practices, microorganism interactions and so and so far. These results could be explained as a combination of a local evolution to some extent related to the distribution of *B.thuringiensis* spore and a movement across the world by some factors like erosion.

This study revealed that *B.thuringiensis* flora in Iranian soils is heterogeneous consisting of at different subspecies out of a total of 80 known subspecies [3]. Our results indicate that Iranian soils were very rich in *B.thuringiensis* as indicated by the fact that it has been obtained from the different geographical areas of our study, and even by the fact that we have detected it together with some others bacillus species in single soil samples. The environmental adaptation of these bacteria has been confirmed by other works around the world, which have obtained it from dust and olive oil storage facilities [13] and from dead insects [14]. Almost, most of the researchers have not isolated *B.thuringiensis* from soil [15]. Although, some researchers have done isolation from soil but they have used L-serine as minimal medium supplement for *B.thuringiensis* isolation [16].

Using microorganisms such as fungi, bacteria and viruses in making insecticides is common. These microorganisms causes disease in insects and leads to their death and sometimes leads to stop their feeding, or reduce it and ultimately leads to minimize crops damage. Therefore, the use of biological pesticides and insecticides is always important. But the requirement of preparation and use of these products is having a collection of biological control bacteria or bacteria with the ability to control and often with maximum specificity is irresistible and also need to be known that how much of this bacterial population there is in native soils and environment. The search for *B.thuringiensis* strains producing endotoxins active against Orthoptera and Dictyoptera has and is being pursued by a number of research institutes and commercial companies, but the results of such large-scale screenings if any remain unknown. Particularly, in intensive screening trials specifically directed at finding *B.thuringiensis* strains pathogenic to locusts, some of the investigators found any isolates active against Locusta migratoria (L.) and Schistocerca gregaria (Forskal) [17]. Other researchers' results strongly support the insecticidal activity of three strains against the Mediterranean or Moroccan locust D.maroccanus.

This work shows that the soil is a very important source of *B.thuringiensis* strains providing a large genetic resource for its use in the development of bio insecticides to control insect pests that have not previously reported to be susceptible to *B.thuringiensis*. The ecological aspects of the distribution of species in the soil could contribute to a better understanding of the role of *B.thuringiensis* in the environment.

ACKNOWLEDGMENT

The authors are supported by Soil and Water research Institute of Iran (SWRI) and Science and Research Branch, Islamic Azad University, Tehran, Iran and all of the tests were done in the biology laboratory of SWRI.

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