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ORIGINAL PAPER

A new biopesticide from a local *Bacillus thuringiensis* var. *tenebrionis* (Xd3) against alder leaf beetle (Coleoptera: Chrysomelidae)

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Abstract Use of chemical pesticides in agriculture harms humans, non-target organisms and environments, and causes increase resistance against chemicals. In order to develop an effective bio-pesticide against coleopterans, particularly against *Agelastica alni* (Coleoptera: Chrysomelidae) which is one of the serious pests of alder leaf and hazelnut, we tested the insecticidal effect of 21 *Bacillus* isolates against the larvae and adults of the pest. *Bacillus thuringiensis* var. *tenebrionis*-Xd3 (*Btt*-Xd3) showed the highest insecticidal effect based on screening tests. For toxin protein production and high sporulation of Xd3, the most suitable medium, pH and temperature conditions were determined as nutrient broth medium enriched with salts, pH 7 and 30 °C, respectively. Sporulated *Btt*-Xd3 in nutrient broth medium enriched with salts transferred to fermentation medium containing soybean flour, glucose and salts. After fermentation, the mixture was dried in a spray dryer, and spore count of the powder product was determined as 1.6×10^{10} c.f.u. g^{-1}. Moisture content, suspensibility and wettability of the formulation were determined as 8.3, 86% and 21 s, respectively. Lethal concentrations (LC_{50}) of formulated *Btt*-Xd3 were determined as 0.15×10^{5} c.f.u. ml^{-1} for larvae at laboratory conditions. LC_{50} values were also determined as 0.45×10^{6} c.f.u. ml^{-1} at the field condition on larval stage. Our results showed that a new bio-pesticide developed from *B. thuringiensis tenebrionis* (Xd3) (*Btt*-Xd3) may be valuable as a biological control agent for coleopteran pests.

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Keywords Bio-pesticide · *Bacillus thuringiensis* · Spray drying · *Agelastica alni*

Introduction

Plant protection chemicals started to be used in the middle of the nineteenth century. Organophosphates, organochlorides, carbamates, pyrethroids and formamides have been powerfully used to control pests (Oberemok et al. 2015). Despite their efficiency, conventional insecticides have caused ecological problems such as the development of resistance, nontarget effects, mammalian toxicity, and accumulation of pesticide residues in the food chain. Therefore, these problems have highlighted the need for alternative biological control agents.

The entomopathogenic bacterium *Bacillus thuringiensis* (*Bt*) is one of the most promising biological control agents for pest management. *Bt* is a gram positive spore forming bacterium producing crystalline proteins called delta-endotoxins which are used commercially in insect control in the agriculture and forestry (Eagan 2002; Thakore 2006; Mazid and Kalita 2011). These crystalline proteins are harmless to humans, vertebrates and plants, are completely biodegradable and cause no toxic residual products to accumulate in the environment.

The first record of *Bt* application to control insects in Hungary at the end of 1920, and in Yugoslavia at the beginning of 1930s, it was applied to control the European corn borer (Lord 2005). The first commercial *Bt* product was produced in 1938 by Libec in France, but the product was used only for a very short time due to World War II, and then in the USA in the 1950s (Nester et al. 2002). Today several isolates of the *Bt* are commercially used with

actively against the members of Lepidoptera, Coleoptera, and Diptera nowadays.

The target of formulation technology is to produce dried *Bt* based bio-pesticides with improved sunlight persistence, rain fastness (Behle et al. 1997; Tamez-Guerra et al. 2000) and palatability (Bartelt et al. 1990; Gillespie et al. 1994).

Chrysomelidae family includes important leaf-eater pests. The alder leaf beetle, *Agelastica alni* (Coleoptera: Chrysomelidae) is one of them, and it is also most serious pests of hazelnut and alder trees distributed in Europe, the Caucasuses, Siberia, north-eastern Kazakhstan and Turkey (Suchy 1988; Baur et al. 1991; Urban 1999). However, there is no application to control this pest which damages the hazelnut and alder leaves during spring and summer (Sezen et al. 2004).

The aim of this study is to develop a new and effective bio-pesticide from local *Bt* isolates for controlling *A. alni* and the other members of chrysomelids, and to determine efficiency of the new product on *A. alni* both in laboratory and field conditions.

Materials and methods

Insect collection

Agelastica alni larvae and adults used in this study were collected from infested fields in the vicinity of Trabzon. Collected larvae and adults were put into sterile plastic boxes with perforated covers and transferred to laboratory. Larvae and adults were feed with fresh alder leafs at 28 ± 2 °C and 60 ± 5% relative humidity under a 12:12 h light:dark cycle, until screening tests were performed.

Screening test

Twenty-one *Bacillus* isolates, isolated and characterized in previous studies were used for screening tests (Table 1). Isolates were inoculated into nutrient broth medium, enriched with salts, and incubated at 30 °C for 72 h. At the end of the incubation period, spore–crystal mixture was obtained by centrifugation at 6000×*g* for 10 min. After that, the pellet was washed twice in 30 ml of sterile distilled water and centrifuged at 6000×*g* for 10 min. The pellet was resuspended in 5 ml of sterile PBS (phosphate buffer solution) and used in screening tests. Sterile PBS was used in control group.

Thirty-third instar larvae fed with fresh alder leafs contaminated with bacterial suspension of each isolate containing 10^9 c.f.u. ml^{-1} and kept at 28 ± 2 °C and 60 ± 5% relative humidity under a 12:12 h light:dark cycle (Ben-Dov et al. 1995). Larval mortality was recorded every 24 h. Bioassays were repeated three times on different durations.

Table 1 Bacterial isolates used in screening test

Isolates	Code	Source	References
Bacillus thuringiensis subsp. *kurstaki*	MnD	*Malacosoma neustria*	Kati et al. (2005)
Bacillus thuringiensis subsp. *kurstaki*	BnBt	*Balaninus nucum*	Sezen and Demirbag (1999)
Bacillus thuringiensis subsp. *morrisoni*	Tp6	*Thaumetopoea pityocampa*	Ince et al. (2008)
Bacillus thuringiensis	Tp7	*Thaumetopoea pityocampa*	Ince et al. (2008)
Bacillus thuringiensis subsp. *morrisoni*	Tp14	*Thaumetopoea pityocampa*	Ince et al. (2008)
Bacillus subtilis	Tp4	*Thaumetopoea pityocampa*	Ince et al. (2008)
Bacillus pumilus	Tp11	*Thaumetopoea pityocampa*	Ince et al. (2008)
Bacillus thuringiensis	Ag14	*Agriotes lineatus*	Danismazoglu et al. (2012)
Bacillus thuringiensis	Ag18	*Agriotes lineatus*	Danismazoglu et al. (2012)
Bacillus safensis	Cq1	*Cimbex quadrimaculatus*	Çakici et al. (2015)
Bacillus safensis	Sn8	*Sesamia nonagrioides*	Eski et al. (2015)
Bacillus thuringiensis	Sn10	*Sesamia nonagrioides*	Eski et al. (2015)
Bacillus megaterium	Xd2	*Xyleborus dispar*	Sezen et al. (2008a, b)
Bacillus thuringiensis subsp. *tenebrionis*	Xd3	*Xyleborus dispar*	Sezen et al. (2008a, b)
Bacillus gibsonii	Gg7	*Gryllotalpa gryllotalpa*	Sezen et al. (2013)
Bacillus circulans	Ar1	*Anoplus roboris*	Demir et al. (2002)
Bacillus polymyxa	Ar2	*Anoplus roboris*	Demir et al. (2002)
Bacillus weihenstephanensis	Mm7	*Melolontha melolontha*	Sezen et al. (2007)
Bacillus sphaericus	Mm5	*Melolontha melolontha*	Sezen et al. (2007)
Bacillus thuringiensis	Mm2	*Melolontha melolontha*	Sezen et al. (2007)
Bacillus thuringiensis subsp. *morrisoni*	Dm2	*Dendroctonus micans*	Yilmaz et al. (2006)

Selection of bacterial strain for production of biopesticide

At the end of screening tests, *B. thuringiensis* var. *tenebrionis*-Xd3 (*Btt*-Xd3) was selected for bio-pesticide production since it had the highest mortality on both larvae and adults of *A. alni*. This strain was isolated from European shot-hole borer, *Xyleborus dispar* Fabricius (Coleoptera: Scolytidae) and had significantly high insecticidal activity against some coleopteran pests in the laboratory conditions (Sezen et al. 2008a, b). Also, cloning, characterization and expression of *cry3Aa* gene of *Btt*-Xd3 was performed in earlier studies (Mert Tatar 2008).

Growth conditions of *Btt*-Xd3

In order to determine an optimum medium for Xd3, it was inoculated ($OD_{600}=0.05$) in tryptic soy broth, nutrient broth and luria bertani broth enriched with salts and incubated at 30 °C. Optical density of the culture was measured with spectrophotometer at OD_{600}. In addition, optimum pH and temperature of bacterium were determined in nutrient broth medium enriched with salts (El-Bendary 2006).

To improve spore and crystal production before fermentation, bacterium was grown in nutrient broth medium containing 0.1% wt:vol K_2HPO_4, 0.1% wt:vol KH_2PO_4, 0.03% wt:vol $MgSO_4{\cdot}7H_2O$, 0.002% wt:vol $FeSO_4{\cdot}7H_2O$, 0.002% wt:vol $ZnSO_4{\cdot}7H_2O$ and 0.1% wt:vol $CaCO_3$ (Sigma–Aldrich) at optimum growth condition during 24 h.

Fermentation process

Fermentation was carried out in a laboratory scale fermenter (5 l, Biostat A, Sartorius, Italy). The sporulated bacteria was transferred to fermentation medium consisting of 2.5% wt:vol soybean flour, 2.5% wt:vol glucose, 0.3% wt:vol KH_2PO_4, 0.3% wt:vol K_2HPO_4, and 0.4% wt:vol $MgSO_4$. The fermentation parameters were applied as 30 °C, pH 7, 10% inoculum, 1 vol air/vol media/min of aeration, and 200 rpm of agitation. Fermentation process was terminated when the medium contains 90% free spores and crystals and 10% vegetative cells (approximately 72 h later). Spores and crystals were collected via centrifugation at 6000×*g*, and 0.5% potassium sorbate was added as preservative. Spore–crystal mixture was stored at 4 °C until spray drying (Tyagi et al. 2002).

Formulation of bacterial sample

Gelatinized starch was prepared by suspending 10 g potato starch in 100 ml sterile distilled water, boiled to obtain clear gel, and then cooled to room temperature. The other ingredients (10% wt:wt sucrose, 1% vol:vol sunflower seed oil, 38% wt:wt potato starch, 20% wt:wt milk powder, 5% vol:vol tween20, 10% wt:wt silica fume, 2% vol:vol polyvinyl alcohol, 1% vol:vol antifoam solution and 10% wt:wt spore–crystal mixture) were slowly added to the starch gel while stirring. The suspension was mixed until exactly homogeneous (Teera-Arunsiri et al. 2003).

Spray drying conditions

A laboratory spray dryer (SD-Basic, Lab Plant, UK) was used for spray drying. The experiment was carried out at inlet and outlet air temperatures of 120 and 60 °C, respectively (Teera-Arunsiri et al. 2003). The suspension was stirred by a mixer during the experiment.

Physical (suspensibility, wettability, moisture content) and biological (viable spore count and toxicity bioassays) assays were performed after spray drying.

Physical characteristics of product

Moisture contents

Moisture content was calculated by heating the spray-dried powder. Ten grams of spray-dried powder in a 50 ml capacity beaker, in triplicate was held at 130 °C in an oven for 24 h and then the final weight was recorded. Results were presented as percentage on dry matter.

Suspensibility

Suspensibility of spray-dried powder was calculated by a procedure described earlier (Lisansky et al. 1993). Three gram powder was added to 97 ml distilled water in a 100 ml measuring cylinder. The cylinder was inverted for 30 times, and 5 ml sample was taken from the top 10 ml, dried to constant weight at 105 °C in a hot air oven. The test was repeated three times. The suspensibility was reported as percentage.

Wettability

A sample of the powder (0.1 g) was poured uniformly and quickly in the baker including 100 ml of standard hard water. The stop-watch was started concurrently. The time was taken when the whole powder was completely submerged in to the water. The experiment was repeated five times. The average was calculated, and results were reported as percentage (Lisansky et al. 1993).

Biological characteristics of product

Spore count

Total spore counts of the biomass before drying and after drying were determined by spread plate method using mueller–hinton agar plate. Formulated *Btt*-Xd3 (1 g) was rehydrated with sterile ddH_2O (99 ml). After the powder rehydrated exactly, sample was serially diluted to 10^{-7}. Viable spore counts were determined by incubating 10^{-7} dilution in a water bath at 80 °C for 10 min. An aliquot of 0.1 ml from the 10^7 dilution was spread to mueller–hinton agar plate. Then, it was incubated at 30 °C for 48 h and the developing *Btt*-Xd3 colonies were counted (Amin et al. 1983).

Toxicity bioassays

Determining the insecticidal activities of unformulated and formulated *Btt*-Xd3, both mixtures were separately tested against larvae of *A. alni* on laboratory and field conditions. Suspensions of unformulated and formulated *Btt*-Xd3 were prepared in sterile distilled water, respectively and adjusted from 10^3 to 10^9 c.f.u ml^{-1} and each concentration was applied to leaf of alder in disposable cups. Thirty-third instar larvae were placed on the contaminated leafs. All tests were conducted at 30 °C and 60% relative humidity on a 12:12 photoperiod. Sterile PBS was used in control group. The experiment was performed three times on different days. Larval mortality was scored up to 10 days after infection to check pathogenicity and corrected for control mortality, using Abbott's formula (Abbott 1987). The data were subjected to ANOVA and subsequently to least significant difference (LSD) multiple comparison tests to compare test concentrations with each other and the control group. The lethal concentrations (LC_{50} and LC_{95}) were estimated by probit regression analysis (Finney 1971). Statistical analyses were performed using SPSS 20.0 software.

Results

In order to find a potential bio-pesticide agent against *A. alni*, 21 local *Bacillus* isolates have been tested. Among tested isolates, *B. thuringiensis* var. *tenebrionis*-Xd3 (*Btt*-Xd3) displayed the highest mortality against both larvae and adults of *A. alni* (Figs. 1, 2). It showed 90 and 87% insecticidal activity with 10^9 c.f.u. ml^{-1} against the larvae and adults of the pest, at the end of 10 days, respectively. Based on these results, *Btt*-Xd3 was determined the

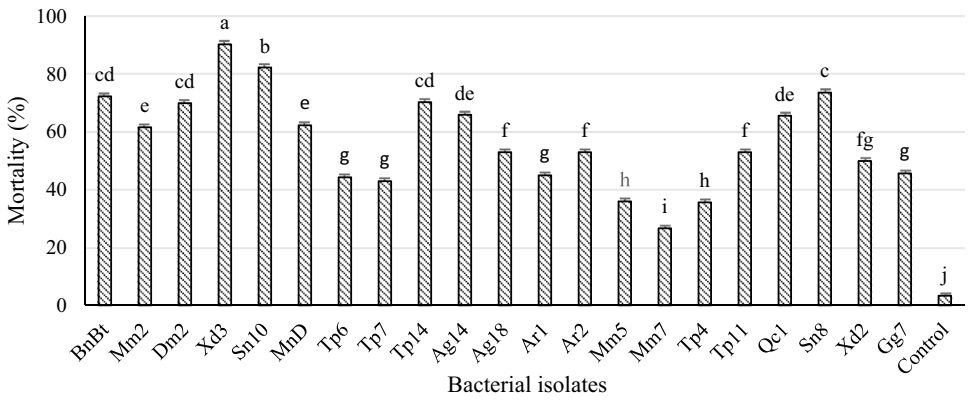

Fig. 1 Insecticidal effects of *Bacillus* isolates against *A. alni* larvae 10 days after treatment on laboratory condition. Different *lower case letters* represent statistically significant differences amongst mortalities according to the LSD multiple comparison test ($P < 0.05$). *Bars* show standard error. Control: phosphate buffer solution (PBS)

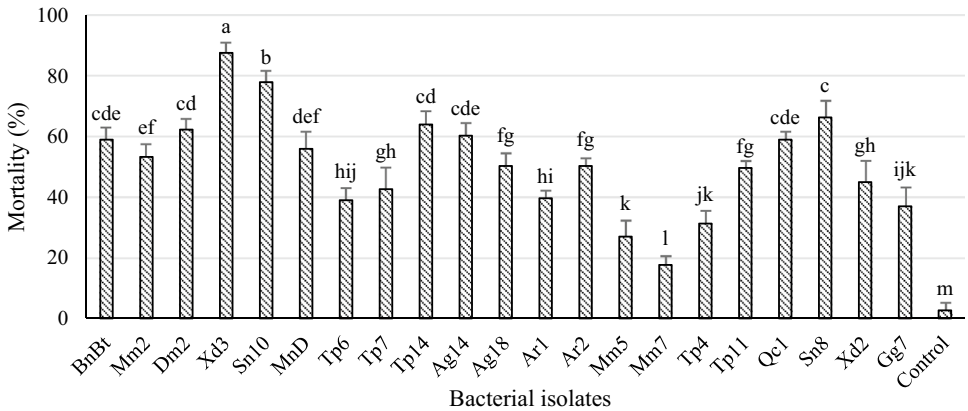

Fig. 2 Insecticidal effects of *Bacillus* isolates against *A. alni* adults 10 days after treatment on laboratory condition. Different *lower case letters* represent statistically significant differences amongst mortalities according to the LSD multiple comparison test ($P < 0.05$). *Bars* show standard error. Control: phosphate buffer solution (PBS)

most appropriate bacterium for bio-pesticide production, therefore, the next studies have been continued with this bacterium.

Nutrient broth medium enriched with salts was determined as the suitable medium for the toxin protein production and the high sporulation (Fig. 3). Optimal growth conditions of *Btt*-Xd3 were also detected as pH 7 and 30 °C (Figs. 4, 5). After fermentation, spore of the biomass was counted as 1.8×10^{11} c.f.u. ml^{-1}. After spray drying at inlet and outlet temperature of 120/60 °C spore count was also determined as 1.6×10^{10} c.f.u. ml^{-1}. In addition, formulated *Btt*-Xd3 powder recovery was about 81.6%. This reduction comes from the adhesion of powder to drier chamber wall. Moisture content, suspensibility and wettability of the formulation were measured as 8.3, 86% and 21 s, respectively (Table 2).

We tested the insecticidal activity of unformulated and formulated *Btt*-Xd3 against the larvae of *A. alni* in laboratory condition. While formulated *Btt*-Xd3 powders had 94% mortality, unformulated *Btt*-Xd3 had 82% mortality with 10^9 c.f.u. ml^{-1} concentration on the larvae of the pest (Fig. 6). Lethal concentrations (LC_{50} and LC_{95}) were determined as 0.15×10^5 and 1.5×10^{10} c.f.u. ml^{-1}, respectively, for formulated *Btt*-Xd3 powder. LC_{50} and LC_{95} of unformulated *Btt*-Xd3 were also determined as 0.21×10^5 and 1.9×10^{10} c.f.u. ml^{-1}, respectively (Table 3).

We also tested unformulated and formulated *Btt*-Xd3 against the larvae of the pest in the field conditions. Formulated *Btt*-Xd3 was displayed 88% mortality, unformulated *Btt*-Xd3 had 68% mortality with 10^9 c.f.u. ml^{-1} concentration (Fig. 7). Lethal concentrations (LC_{50} and LC_{95}) were determined as 0.45×10^6 and 7.2×10^{10} c.f.u. ml^{-1} for formulated *Btt*-Xd3, respectively. Lethal concentrations of unformulated *Btt*-Xd3 were also determined as 0.23×10^7 and 4.9×10^{12} c.f.u. ml^{-1}, respectively (Table 4).

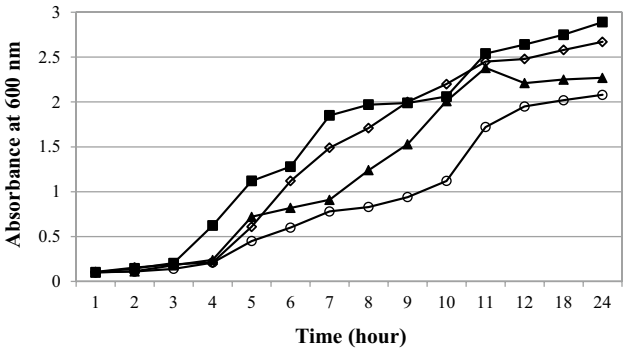

Fig. 4 Growth of *Btt*-Xd3 at 28 °C (*open diamond*), 30 °C (*filled square*), 34 °C (*filled triangle*) and 37 °C (*open circle*) in nutrient broth enriched with salts

Discussion

Scientists all over the world have been trying to develop effective *Bt* based bio-pesticide by using inexpensive and easily procurable raw material as medium and investigating new bacterial isolates in order to have better commercial *Bt* based products. Spores and crystals of *Bt* producing during the sporulation phase adversely affected by environmental conditions (Ignoffo 1992). So that various methods have been developed to improve sunlight, persistence and rainfastness (Tamez-Guerra et al. 1996, 2000). Spray drying is the most commonly used method to protect from sunlight and rain. However, temperatures of spray dryer decrease the number of viable spores during the spray drying. According to Teera-Arunsiri et al. (2003), the optimum outlet and inlet temperature for spray drying were determined as 60–85 and 120–180 °C, respectively. Prabakaran and Hoti (2008) found that the optimum outlet temperature for spray drying to be 70 °C and an inlet temperature of 160 °C and they observed that when the inlet temperature was increased, the

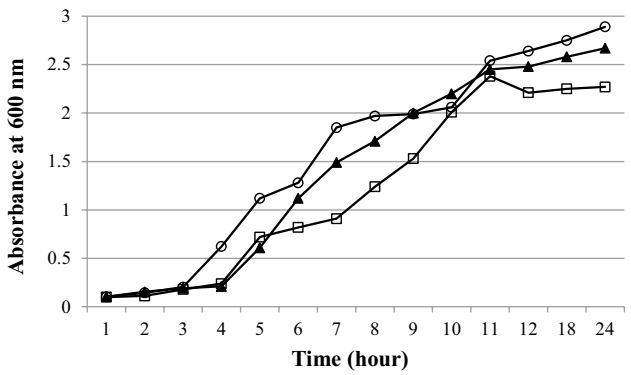

Fig. 3 Growth of *Btt*-Xd3 in nutrient broth + salts (*open circle*), luria–bertani broth + salts (*open square*), and tryptic soy broth + salts (*filled triangle*)

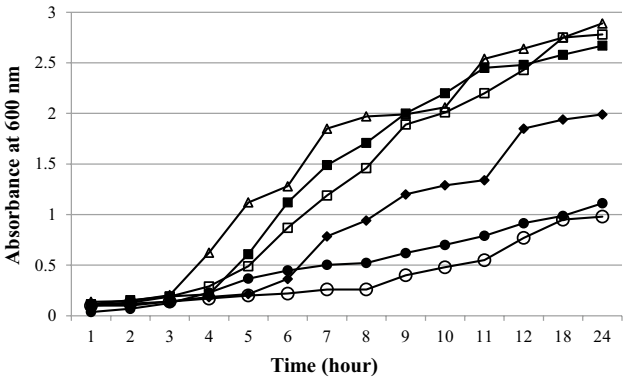

Fig. 5 Growth of *Btt*-Xd3 at pH 5 (*filled circle*), pH 6 (*open square*), pH 7 (*open triangle*), pH 8 (*filled square*), pH 9 (*filled diamond*), and pH 10 (*open circle*) in nutrient broth enriched with salts

Table 2 Physical characteristics of formulated *Btt*-Xd3 product

Product	Spore count (c.f.u. g^{-1})	Moisture content (%)	Suspensibility (%)	Wettability (s)
Formulated *Btt*-Xd3	$1.6\times10^{10}\pm1.42$	8.3 ± 0.49	86 ± 1.52	21 ± 2.51
Desired characteristics	1×10^{10}	6–8	>60	<30

Mean ± SD

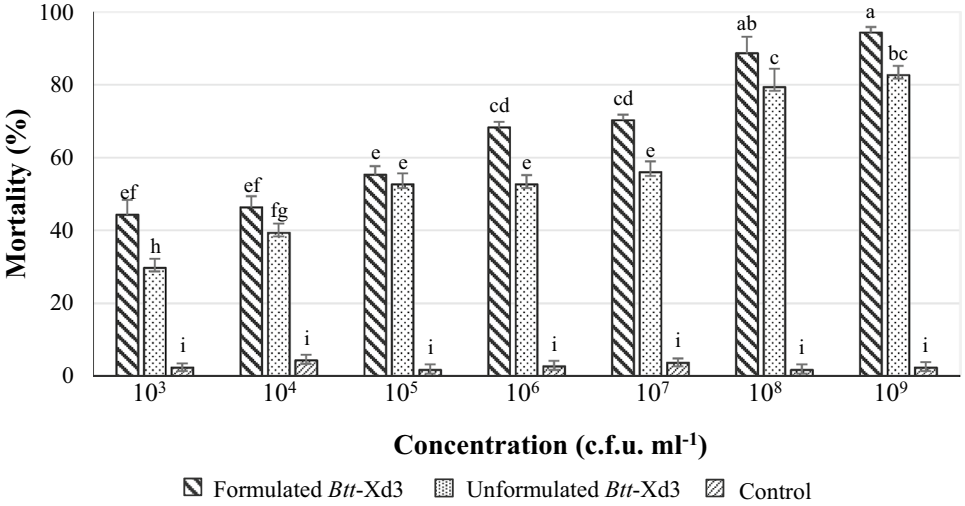

Fig. 6 Insecticidal effects of formulated and unformulated *Btt*-Xd3 against *A. alni* larvae 10 days after treatment on laboratory condition. Different *lower case letters* represent statistically significant differences amongst mortalities according to the LSD multiple comparison test ($P<0.05$). *Bars* show standard error. Formulated *Btt*-Xd3: formulated and spray-dried spore–crystal mixture, Unformulated *Btt*-Xd3: Unformulated and nonspray-dried spore–crystal mixture, Control: phosphate buffer solution (PBS)

Table 3 LC_{50} and LC_{95} (c.f.u. ml^{-1}) values of formulated and unformulated *Btt*-Xd3 against *A. alni* larvae in laboratory condition

Product	LC_{50} (*FL*, %95)	Slope ± *SE*	LC_{95} (*FL*, 95%)	*df*	X^2
Formulated *Btt*-Xd3	0.15×10^5 (0.016×10^5–0.76×10^5)a	8.9 ± 0.168	1.5×10^{10} b	5	8.64
Unformulated *Btt*-Xd3	0.21×10^5 ($0.052\times10^5-0.88\times10^5$)a	8.4 ± 0.174	1.9×10^{10} b	5	9.26

Lowercase letters (a and b) represent statistical differences among mortalities according to LSD multiple comparison test ($P<0.05$). *FL* fiducial limit, *SE* standard error, *df* degree of freedom, X^2 Chi square, *formulated Btt-Xd3* formulated and spray-dried spore–crystal mixture, *unformulated Btt-Xd3* unformulated and nonspray-dried spore–crystal mixture

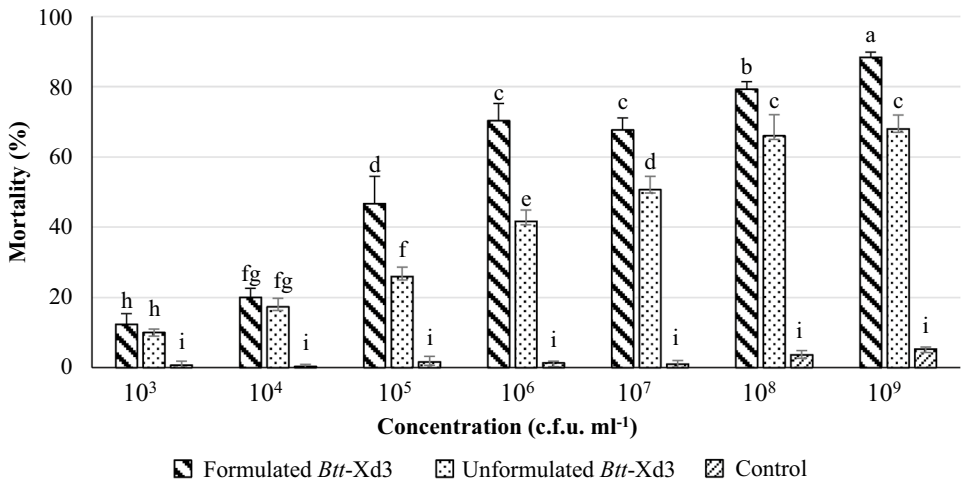

Fig. 7 Insecticidal effects of formulated and unformulated *Btt*-Xd3 against *A. alni* larvae 10 days after treatment on field condition. Different *lower case letters* represent statistically significant differences amongst mortalities according to the LSD multiple comparison test ($P<0.05$). *Bars* show standard error. Formulated *Btt*-Xd3: formulated and spray-dried spore–crystal mixture, Unformulated *Btt*-Xd3: Unformulated and nonspray-dried spore–crystal mixture, Control: phosphate buffer solution (PBS)

Table 4 LC_{50} and LC_{95} (c.f.u. ml^{-1}) values of formulated and unformulated *Btt*-Xd3 against *A. alni* larvae in field condition

Product	LC_{50} (*FL*, 95%)	Slope ± *SE*	LC_{95} (*FL*, 95%)	*df*	X^2
Formulated *Btt*-Xd3	0.45×10^6 (0.093×10^6–1.7×10^6) a	13.3 ± 0.174	7.2×10^{10} c	5	18.3
Unformulated *Btt*-Xd3	0.23×10^7 (0.142×10^7–0.82×10^8) b	11.7 ± 0.168	4.9×10^{12} d	5	11.6

Lowercase letters (a, b, c, and d) represent statistical differences among mortalities according to LSD multiple comparison test ($P<0.05$). *FL* fiducial limit, *SE* standard error, *df* degree of freedom, X^2 Chi square, *formulated Btt-Xd3* formulated and spray-dried spore–crystal mixture, *unformulated Btt-Xd3* unformulated and nonspray-dried spore–crystal mixture

larvicidal activity decreased. In this study, inlet and outlet temperature of spray drier were adjusted to 120 and 60 °C for maximum viable spore, respectively. Total spore counts of the biomass before drying and after drying were determined as 1.8×10^{11} and 1.6×10^{10} c.f.u. g^{-1}, respectively. After spray drying, the number of the viable spores of formulations was decreased because of heat damage of spray drier. Spray dryer conditions also directly affect the moisture content of formulations. Optimum moisture contents of *Bt* formulations should be 5–8%. Also it has been shown that formulations that have moisture content lower than 5% significantly decrease the insecticidal activity. In addition, a moisture content of 6–8% incites the longest life of formulations (Lisansky et al. 1993). Moisture of formulated *Btt*-Xd3 powder was determined as 8.3%.

Natural ingredients such as corn starch, flour, and gluten have been used as adjuvants to enhance the residual activity of *Bt* in response to rain and solar exposure. We used potato starch for the encapsulation and the protection of *Btt*-Xd3 during spray drying. Starch and sucrose were added to prevent *Btt*-Xd3 spores and crystals from solar degradation (McGuire and Shasha 1990; McGuire et al. 1996; Behle et al. 1997). However, addition of starch and sucrose enhanced the physical characteristics of the formulation (Teera-Arunsiri et al. 2003). To improve the physical properties, some ingredients were also added to formulation. Milk powder, tween20, vegetable oil, silica fume, and polyvinyl alcohol were used to improve wettability and suspensibility of formulation. In this study, the suspensibility and wettability of the formulation were 86% and 21 s, respectively.

Determination of bacterial flora of *A. alni* and insecticidal activity of flora members was firstly performed by Sezen et al. (2004). According to study the highest insecticidal effect determined on larvae and adults of pest within 7 days were 70 and 56% with *Pseudomonas fluorescens*, respectively. Sezen and Demirbag (2006) also reported that *Bacillus sphaericus* Ar4 and *Bt* Mm2 had 90% mortality against larvae of *A. alni*. In our study, *Btt*-Xd3 similarly showed high mortality (90%) against the larvae of the pest. Apart from these, there are no studies about biological control of this pest.

*Bacillus thuringi*ensis var. *tenebrionis* and *Bt* var. *san diego* are toxic to beetles by highly specific toxins. M-One, a commercial product including *Bt* var. *san diego* was registered for use against larvae of colorado potato beetle and elm leaf beetle. Novodor is also formulation of *Bt* var. *tenebrionis*, it's treatment has been shown to reduce feeding damage of Colorado potato larvae (Ferro et al. 1993; Hilbeck et al. 1998; Nault et al. 2000; Chu et al. 2006). In this study, *B. thuringiensis*-Xd3 (*Btt*-Xd3) showed considerable mortality effect against the larvae with 90%. This mortality rate is due to the similar feeding behavior with Colorado potato beetle.

There was no statistically significant difference in the LC_{50} values of the unformulated and formulated *Btt*-Xd3 against *A. alni* in laboratory conditions. Minor variation between mortality of unformulated and formulated *Btt*-Xd3 is probably because of feeding stimulant effects of sucrose (Bartelt et al. 1990; Horton et al. 2012). According to Allsopp (1992), sucrose and fructose produced the strongest feeding response in 14 different sugars. Although LC_{50} values of unformulated and formulated *Btt*-Xd3 are statistically not different in laboratory conditions, it showed difference in the field conditions (Table 3). This difference presumably arose from short residual effect of unformulated *Btt*-Xd3 in the field conditions. Solar radiation is the most ruinous environmental factor that affects the stability of crystals and spores (Pozsgay et al. 1987; Pusztai et al. 1991).

In summary, the fermentation of *Btt*-Xd3 was performed in a laboratory scale fermenter. The culture medium was concentrated and spray dried. The spray drying conditions were optimized to have the maximum viability of spores. The concentrated culture medium was formulated with natural ingredients. The mixture was then spray dried under the optimum conditions. The formulation was also optimized based on physical and biological properties of the formulated *Btt*-Xd3. According to results, this *Btt*-Xd3 based bio-pesticide may be registered for *A. alni* management and also can be tested against other coleopterans. So, it will minimize the use of quite toxic chemical insecticides, contribute to control insects that have developed resistance to chemical insecticides and will protect environment from pollution problems which are the main concerns at present.

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