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A UV Tolerant Mutant of *Bacillus thuringiensis* subsp. *kurstaki* Producing Melanin

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Abstract. A UV-resistant mutant (*Bt*-m) of *Bacillus thuringiensis* subsp. *kurstaki*, producing a dark brown pigment, identified as melanin, was studied. *Bt*-m had higher larvicidity against *Heliothis armigera* than its parent. Survival of *Bt*-m spores and their insecticidal activity to irradiation at 254 nm and 366 nm were higher than those of the parent. The only toxic polypeptide produced by *Bt*-m was Cry1Ac (130 kDa); it lost *cry1Aa*, *cry2Aa*, and *cry2Ab*.

Bacillus thuringiensis is a gram-positive, aerobic, endospore forming bacterial species. It is characterized by a parasporal crystalline body which is proteinaceous in nature and possesses insecticidal properties against larvae of Lepidoptera, Coleoptera, and Diptera insects [41, 26]. The use of conventional *B. thuringiensis* as insecticides is however limited because the spores and toxins are inactivated by solar radiation [19, 22, 35, 36]. Various formulations are not sufficiently stable under field conditions and rapidly lose their biological activities [3, 8]. Attempts to protect *B. thuringiensis* toxicity from damaging UV radiation under field conditions has yielded limited success. Different formulations were developed with addition of variety of screens [9, 16, 33], but some of the UV screens used have negative impact on the environment [31]. It has recently been demonstrated that spores of *B. subtilis* are susceptible to solar radiation as well as to UV at 254 nm (major peak of UV-C) [43]. This phenomenon can be attributed to the fact that sunlight is composed of UV-B (between 290–320 nm), UV-A (320–390 nm), visible (between 390–780 nm), and infrared radiation (longer than 780 nm). The high-energy photons of UV harm cells by direct DNA damage (e.g. pyrimidine dimers, cross-linking with proteins) or by producing reactive oxygen-derived free radicals [36, 43]. Spores of *B. thuringiensis* are known to germinate, outgrow and multiply in the hemolymph of the target insect, thus contributing to larval mortality. Protection of spores from inactivation by solar radiation is anticipated to enhance toxicity [25].

Resistance to UV light of *Bacillus* spores is caused by altered conformation of DNA, high concentrations of small acid-soluble proteins (SASP) [11, 32] and low concentrations of dipicolinic acid (DPA) [17]. In addition, abundance of plasmids, surface-localized Cry protoxins, and UV-induction of bacteriophage may increase susceptibility to UV of *B. thuringiensis* spores [2, 6, 10, 14, 23]. Mutants with increased survival and residual toxicity can be isolated after successive rounds of UV exposure [25].

Several investigators have reported that production of melanin by various microorganisms protects their susceptibility to oxidative damage caused by UV and ionizing radiation by acting as a free radical trap for example [1, 37, 40]. In addition, melanin appears to compete for base- and nucleoside-damages of free radicals [21]. Liu *et al.* [29] succeeded to protect mosquitocidal activity of *B. thuringiensis* subsp. *israelensis* from UV irradiation by melanin from *Streptomycetes lividans*. These methods may eliminate the use of external UV protectant and result in stable and safe formulations.

Here, we describe a mutant of *B. thuringiensis* producing melanin. Insecticidal activities against *Heliothis armigera* and *Spodoptra littoralis* were used to determine the mutant efficacy following UV irradiation, and *cry* gene composition and expression was examined.

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Table 1. Toxicities of *Bt*-m and its parent strain *B. thuringiensis* subsp. *kurstaki*

Strain	LC_{50}, (ng)[a], against	
	Heliothis armigera	*Spodoptera littoralis*
B. thuringiensis subsp. *kurstaki*	996.1 (557.22–1627.31)[b]	1034.79 (684.32–1572)
Bt-m	622.79 (364.88–943.09)	3209.11 (1735.68–7088.58)

[a] ng of biomass in each well.
[b] 95% Fiducial limits.

Materials and Methods

Bacterial strains and growth conditions. *B. thuringiensis* subsp. *kurstaki* (*Bt*-01) was isolated from dead insect and belongs to 3a3b3c serotype (serotyped by Institute Pasteur, Paris). A strain designated *Bt*-m (serotyped by Institute Pasteur) was isolated as a brown pigment producer mutant of *Bt*-01 after repeated rounds of exposure to UV at 254 nm. Cells were grown in rotary shaker (250 rpm) at 28°C in BP medium [28]. The release of spores and crystals was visualized microscopically after 48–72 h. In both strains (*Bt*-01 and *Bt*-m) sporulation level reached 80–90%, determined as the ratio between the number of colony forming units (CFU) after heat shock (70°C for 10 min) to the number of total viable counts. Biomass was isolated by the lactose-acetone precipitation [15]. Proteins were fractionated and observed on 12% SDS-PAGE [27].

Immunological assay. Biomass obtained after centrifugation of 1 ml broth was vortexed with 500 μl of extraction buffer (EnviroLogix; Portland, ME), centrifuged, and the supernatants was analyzed for Cry1Ac by Western blot using Lateral Flow Quickstix (EnviroLogix; detection limit <10 parts 10^{-9}) [38, 39].

Pigment characterization. Cultures of *Bt*-m, grown in BP medium, were centrifuged (3000 g, 20 min), and the pigment was extracted from the supernatant by acidification [29]. The primary analyses were done as described by Shivaprasad and Page [40]. Absorption spectrum was scanned with a spectrophotometer (8451A Hewlett Packard, USA). FTIR spectra of *Bt*-m pigment and melanin (Sigma) after preparing pellet with potassium bromide were taken on Perkin-Elmer FT-IR analyser.

UV irradiation. Biomass (equivalent to 2×10^6 cells ml^{-1}, washed previously to remove any melanin from the medium) was uniformly suspended in 0.05 M phosphate buffer, pH 6.8. Samples (10 ml) of this suspension were irradiated (with either 254 nm or 366 nm) in glass petri dish (90 mm) at a constant distance from the UV source (BII Illuminator, 1 A-7 SBS Korea). The samples were frequently agitated at the time of exposure. Aliquots (100 μl) were removed from the irradiated samples at different time intervals to determine total viable count and insecticide activity. A concentration corresponding to LC_{90} before irradiation was applied in each well as explained below.

PCR analysis. Amplification of PCR products (MiniCycler, MJ Research Inc., Watertown, MA) and identification of their predicted sizes as *cry* genes were carried out using specific and universal primers, as described by Ben-Dov *et al.* [4].

Larval growth and bioassays. Newly hatched larvae (of *H. armigera* or *S. littoralis*) were distributed singly into wells (40 $\mu l/cm^2$) of a bioassay tray containing 1.5–2 ml of freshly prepared (after 2 h) diet, with the following composition, in 1 liter: wheat germ, 37.5 g; cracked wheat, 50.0 g; semolina, 31.25 g; casein, 25.0 g; ascorbic acid, 3.0 g; sorbic acid, 1.5 g; para-aminobenzoic acid, 62.5 mg; 10% formalin, 8.75 ml; linseed oil, 6.25 ml. The ingredients were blended, mixed with agar (15 g in 800 ml sterile distilled water), cooled to 70°C after boiling, blended again for 2 min, and poured into bioassay trays.

For bioassays, the biomass of the appropriate strain was sonicated (two cycles of 45 s at 4°C) in dilution buffer (0.85% NaCl and 0.01% Tween 80) and further serially diluted (twofold). One hundred microliter of each were evenly spread onto the solidified diet, and allowed to air dry for about 30–60 min before placing the larvae. Duplicate 32 larvae were used for each treatment and for controls. The bioassays were incubated at 26°C with 60–70% relative humidity, and in 16/8 h light/dark photoperiod for 5–7 days. Larval mortality was then recorded and LC_{50} and 95% Fiducial Limits (FI) were obtained using computerized program of probit analysis [12].

Results

The mutant of *Bt*-01, designated *Bt*-m, was isolated by successive rounds of UV exposure. The colonies and batch cultures of the mutant were brown-gray in color. Its larvicidity was slightly increased against *H. armigera* and decreased against *S. littoralis* compared to that of its parent, *B. thuringiensis* subsp. *kurstaki* (Table 1).

The pigment produced by *Bt*-m appeared during exponential phase (about 8–24 h) and stopped accumulating after 48 h (Fig. 1). Primary characterization indicated that the brown pigment recovered was melanin: similar to a standard melanin obtained from Sigma (M-8631), it was soluble in 1 M KOH and insoluble in organic solvents (such as acetone, chloroform, methanol, and ethanol), it bleached by NaOCl and H_2O_2, and it yielded a brown precipitate with $FeCl_3$. The UV spectrum (data not shown) and FTIR analysis of the purified pigment and the standard are compared in Fig 2.

The mutant *Bt*-m produced a polypeptide of 130 kDa (apparently the Cry1 toxin protein) but no 71 kDa Cry2Aa toxin as found in the parent (*Bt*-01) of *Bt*-m, as indicated by SDS-PAGE (Fig. 3). PCR with specific primers for *cry1* and *cry2* discovered that *Bt*-m contained the same *cry1Ac* as that of the standard strain HD-73 of *B. thuringiensis* subsp. *kurstaki* but no *cry2*. PCR with template of standard strain used, *B. thuringiensis* subsp. *kurstaki* HD-1, revealed all three *cry1A* genes (*-Aa*, *-Ab*, *-Ac*) and two *cry2* genes, *cry2Aa*, and the cryptic *cry2Ab* (Fig. 4). The parent of *Bt*-m amplified four of these genes

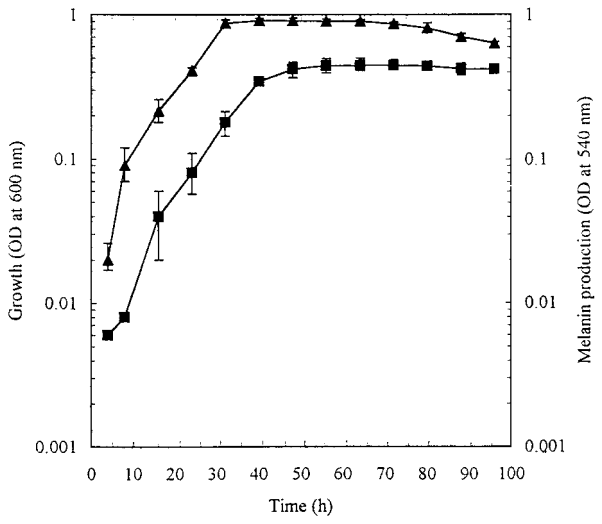

Fig. 1. Cell growth and melanin production in *Bt*-m. ▲, growth; ■, melanin production. Data are expressed as mean ± the standard error of the means, which is indicated when not within the dimension of the symbols.

but not *cry1Ab* (Fig. 4). Immunological test also indicated the presence on Cry1Ac toxin in *Bt*-01 and *Bt*-m (data not shown).

The comparative survival of the melanin-producing *Bt*-m and of its parental strain by UV irradiation at 254 and 366 nm, respectively, are shown in Figs. 5 and 6. In both wavelengths (C and A, respectively), the viability of the parent strain was inactivated much faster than of the mutant. *B. thuringiensis* subsp. *kurstaki* lost 80–90% of its colony-forming ability after 2 and 16 min irradiation, respectively, whereas in the same time *Bt*-m lost viability of 30 and 10%, only.

The relative reduction of larvicidity against *H. armigera* of UV irradiation at 254 nm and 366 nm are shown in Figs. 7 and 8. At 254 nm, the parent strain lost about 35% toxicity in 30 s while *Bt*-m lost only 5%. Irradiating the mutant for 20 min at 366 nm, resulted in a loss of 10% of its insectidal activity only, while the wild-type lost 90%.

Discussion

Sunlight-mediated inactivation of *B. thuringiensis* larvicidal preparations is believed to be due to UV damage to the spores as well as to their δ-endotoxins [19, 22, 35, 36]. For purified *B. thuringiensis* subsp. *kurstaki* HD-1 or HD-73 crystals, the 360–380 nm range of the solar spectrum (UV-A) is largely responsible for substantial photodegradation and consequent loss of toxicity, attributed to ability of chromophores to act as photosensitizers by creating highly reactive singlet oxygen species upon irradiation [36]. Certain minor free-radical reactions (such as attacking sulphur atoms and cross-linking of polypeptides) could also occur. In addition, plasmid content seems to affect UV sensitivity of *B. thuringiensis* spores [6, 10]: strains cured of plasmids are more resistant than their plasmids-containing parents and the quantity of dipicolinic acid is about twice as high in the latter [6]. However, the UV sensitivity of *B. thuringiensis* subsp. *kurstaki* strain HD-1 is similar to that of strain HD-73 despite the large difference in the number of their plasmids (11 versus 5, respectively) [6]. Our resistant strain, *Bt*-m, had lost at least three toxic genes (Fig. 4), *cry1Aa*, *cry2Aa*, and *cry2Ab*, most likely by losing the plasmid(s) carrying them; its resistance (Figs. 5 and 6) is probably a consequence of the melanin it accumulates.

Spores of *B. thuringiensis* subsp. *kurstaki* HD-1, containing protoxin polypeptides in their coat (on the expense of low-molecular-weight proteins), germinate slower [2] and are more sensitive to UV-B, than spores of *B. thuringiensis* subsp. *israelensis*, which do not [33a]. Our UV-resistant strain *Bt*-m expresses less *cry* genes (only *cry1Ac*) than its parent (Fig. 3). It may have partially restored the amount of low-molecular-weight coat proteins, as several acrystalliferous *B. thuringiensis* subsp. *kurstaki* strains do [2], thus conferring UV-resistance. In addition, *Bt*-m seems to deposit some melanin in its spores, as in certain melanin producing microorganisms [1, 20, 37, 42]: its melanin is accumulated during the sporulation process (Fig. 1).

The efficacy of a biological pesticide largely depends on its stability in the field. Viability and toxicity of *B. thuringiensis* decreases significantly following UV irradiation at wavelengths ranging from 250 to 380 nm [22, 35]. Formulations of *B. thuringiensis* and its δ-endotoxin encapsulated with starch and UV screens like congo red are not feasible; starch is easily degraded by various microorganisms, and congo red may make formulation less palatable to insect larvae and can have adverse effect on the environment because of its mutagenic nature [7]. Photoprotection of *B. thuringiensis*' mosquitocidal activity has recently been achieved with melanin produced by *Streptomyces lividans* [29]. A self-producing melanin strain of *B. thuringiensis* subsp. *kurstaki* would be more economical. Patel *et al*. have recently isolated such a strain [34], with higher larvicidity against *Plutella xylostella* and spore resistance to UV-A. We have independently isolated another mutant of *B. thuringiensis* subsp. *kurstaki* (nicknamed *Bt*-m) that produces diffusible melanin (Figs. 1 and 2), the spores of which were also several fold more resistant to UV-A than of its parent strain (Fig. 6). Resistance of our strain, examined against UV-C (254 nm) as well, was also higher than of its parent (Fig. 5).

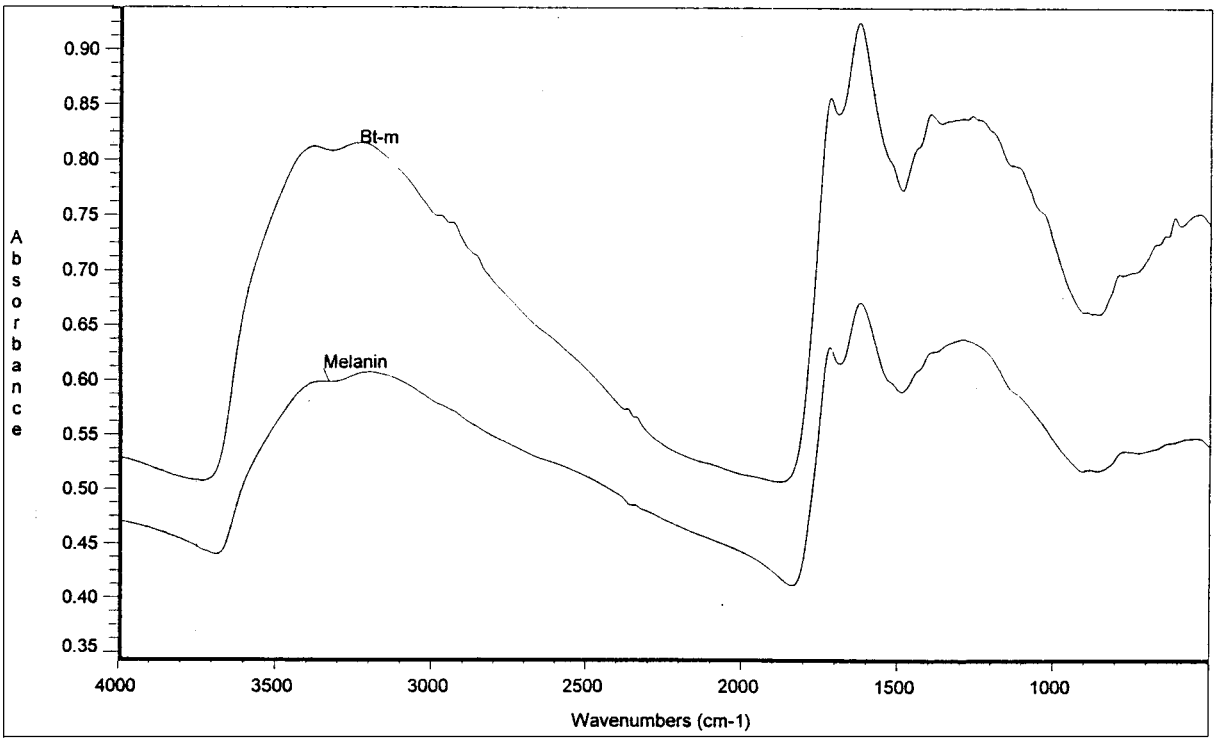

Fig. 2. FTIR spectrum of melanin extracted from *Bt*-m and standard obtained from Sigma.

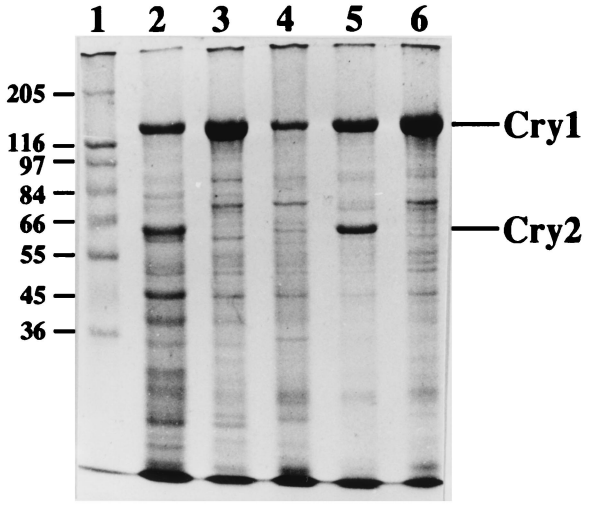

Fig. 3. Protein patterns of various *B. thuringiensis*, analyzed by 12% SDS-PAGE. Lane 1, molecular weight marker (kDa); Lane 2, *B. thuringiensis* subsp. *kurstaki* (*Bt*-01); Lane 3, *Bt*-m; Lane 4, *B. thuringiensis* subsp. *kurstaki* HD-73; Lane 5, *B. thuringiensis* subsp. *kenyae* HDB-23; Lane 6, *B. thuringiensis* subsp. *aizawai* HD-133.

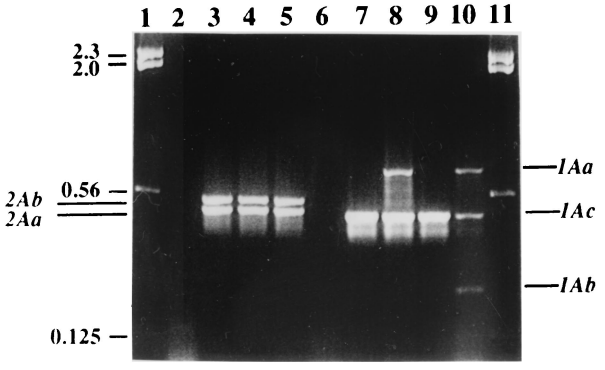

Fig. 4. Agarose gel (2.2%) electrophoresis of PCR products obtained with specific primers for *cry1* and *cry2*. Lanes 1 and 11, molecular weight markers (λ_{DNA} cleaved by *Hin*dIII), with sizes (in kb) indicated on left; lanes 2–5, respectively, DNA of (all amplified with a mixture of specific primers for *cry2*) *B. thuringiensis* subsp. *kurstaki* (melanin producing mutant *Bt*-m), *B. thuringiensis* subsp. *kurstaki* (parent; *Bt*-01), *B. thuringiensis* subsp. *kurstaki* HD-73, and *B. thuringiensis* subsp. *kurstaki* HD-1; lanes 6–10 (all amplified with a mixture of specific primers for *cry1*), respectively, negative controls (without template), DNA of *B. thuringiensis* subsp. *kurstaki* (melanin producing mutant *Bt*-m), *Bt*-01 (parent), *B. thuringiensis* subsp. *kurstaki* HD-73, and *B. thuringiensis* subsp. *kurstaki* HD-1.

Bt-m produced only Cry1Ac and its toxicity towards *H. armigera* was increased, but it lost ability to synthesized Cry2Aa and its toxicity against *S. littoralis* decreased as compared with the parent strain. This observation is concordant with the higher toxicity against *H. armigera* of *B. thuringiensis* subsp. *kurstaki* HD-73

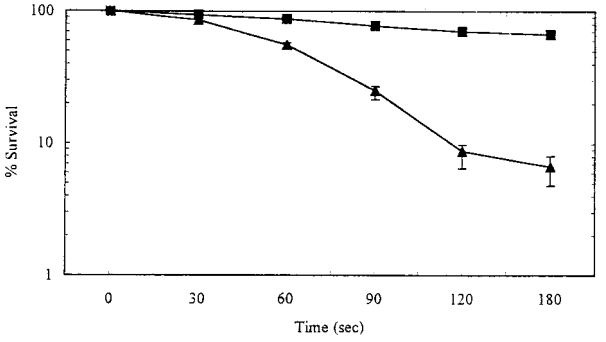

Fig. 5. Effect of UV irradiation at 254 nm on the biomass of *Bt*-01 (▲) and *Bt*-m (■). Data are expressed as mean ± the standard error of the means, which is indicated when not within the dimension of the symbols.

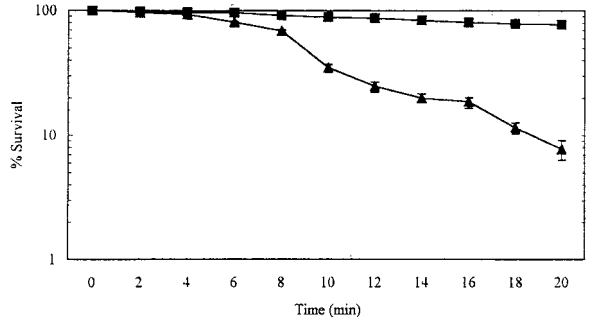

Fig. 6. Effect of UV irradiation at 366 nm on the biomass of *Bt*-01 (▲) and *Bt*-m (■). Data are expressed as mean ± the standard error of the means, which is indicated when not within the dimension of the symbols.

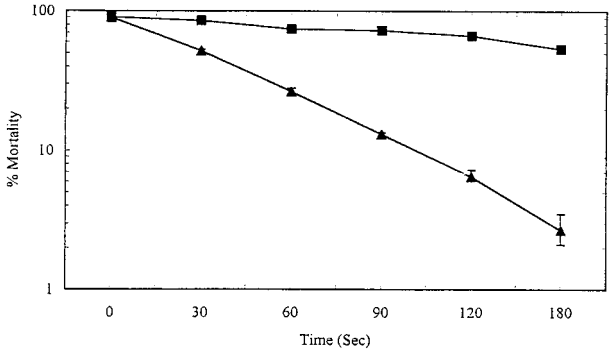

Fig. 7. Insecticidal activity of biomass of *Bt*-01 (▲) and *Bt*-m (■) after irradiating at 254 nm. Data are expressed as mean ± the standard error of the means, which is indicated when not within the dimension of the symbols.

(with Cry1Ac only) than that of *B. thuringiensis* subsp. *kurstaki* HD-1 (with two additional Cry1A polypeptides) [30]. It may explain the higher toxicity of the previously isolated melanin-producing *B. thuringiensis* subsp. *kurstaki* against *P. xylostella* than its parent [34].

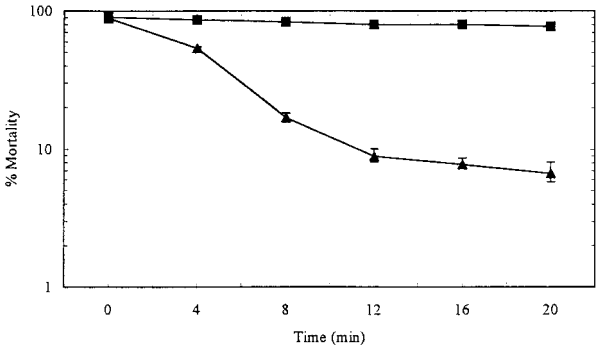

Fig. 8. Insecticidal activity of biomass of *Bt*-01 (▲) and *Bt*-m (■) after irradiating at 366 nm. Data are expressed as mean ± the standard error of the means, which is indicated when not within the dimension of the symbols.

Here, it was confirmed by PCR using specific primers that *Bt*-m had lost *cry1Aa*, *cry2Aa*, and *cry2Ab*, apparently by repeated exposure to UV-C used for its isolation. Loss of plasmids occurs both spontaneously and during exposure to curing condition in *B. thuringiensis* subsp. *thuringiensis* [18]. The decreased toxicity towards *S. littoralis* could perhaps be overcome by cloning for expression of *cry2Aa* in *Bt*-m by various approaches, as reported earlier [5]. Characterization of such mutant with increased UV resistance might contribute to develop stable formulations for field application. Being a natural product, melanin is easily biodegradable and, thus, will not pose any threat to the environment.

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