As. J. Food Ag-Ind. **2009**, *Special Issue*, S145-S151

GO ORGANIC 2009 BANGKOK, THAILAND

Asian Journal of Food and Agro-Industry
ISSN 1906-3040
Available online at www.ajofai.info

Efficacy of *Xenorhabdus* sp. (X1) mutant on mushroom mite (*Luciaphorus* sp.)

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This article was originally presented at the International Symposium "GoOrganic2009", Bangkok, Thailand, August 2009.

Abstract

Xenorhabdus sp. (X1), a bacterium symbiotic to *Steinernema siamkayai* nematode, has been used as a biological control agent against certain pests. Since the bacterium X1 were originally screened from natural locations have been found to have low efficacy, a protocol for strain improvement was thus required to generate the strain suitable for production at industrial levels. This study aims to use ultraviolet (UV) radiation for improving X1 strain and to determine the efficacy of derived UV-irradiated mutants against mushroom mite (*Luciaphorus* sp.). UV radiation for 15-120 seconds resulted in 9 mutants which, on skim milk agar, could produce clear zones at sizes equivalent to wild type. The cell-free supernatant, derived from RP58 mutant cell culture being grown for 48 hour to be 1×10^8 cells/mL, has been found to be toxic to the female mites and, within 3 days, resulted in 92.49% mortality which was higher than those derived from the wild type (87.49%). In conclusion, UV radiation can be beneficial for improving the efficacy of X1 strain. The RP58 mutant may also be further improved by other mutagens for enhancing its consistent virulence to mushroom mite.

Keyword: Strain improvement, Ultraviolet radiation, *Xenorhabdus* sp.

Introduction

Xenorhabdus sp. is a gram negative bacterium associated with *Steinernema siamkayai* nematode. This symbiotic bacteria have been shown to be effective against a wide range of agriculturally damaging pests such as diamondback moth *Plutella xylostella* L.(DBM) which is a significant pest of cruciferous crops (Mahar et al., 2004) and *Luciaphorus* sp. which is considered to be the most destructive pest of mushroom cultivation in Thailand (Bussaman et al., 2004). Both cell suspension and cell-free supernatant of symbiotic bacteria can be used for controlling the fire ant (*Solenopsis invicta*) and the beet armyworm (*Spodoptera exigua*) (Mahar et al., 2004) and may be useful as alternatives to chemical insecticides for pest control (Kaya and Gaugler, 1993). When the bacterium invades into the insect's hemococe, it being to multiply and causes septicemia, which ultimately kills the infected insect (Dunphy and Webster, 1988; Park and Kim, 2000).
Bussaman et al. (2006) showed that cell-free supernatants of *X. nematophila* (Thai) and *Photorhabdus luminescens* subsp. *laumondii* (strain GPS 12) were virulent against *Luciaphorus* sp., resulting in 70 and 95% mortality of the female mite, within 48 hrs, respectively. However the parental bacteria, which were originally screened from natural locations, have been found to has poor efficacy, a protocol for strain improvement was thus required to generate the mutants suitable for production at industrial levels (Ghribi et al., 2004). Conventionally, the improvement of bacterial strains can be achieved by mutation, selection, or genetic recombination. The process of mutation can be succeeded by treating such bacteria with a variety of physical actions or chemical agents (so called mutagens). Most mutagenic agents are capable of causing some damages to the DNA by deletion, addition, transversion or substitution of bases or by breakage of the DNA (Parekh et al., 2000). As a consequence, this random mutagenesis could affect numerous pathways of the cell machinery. Ghribi et al. (2004) reported that *Bacillus thuringiensis* subsp. *kurstaki* mutant BNS3 which was treated by classical mutagenesis using ultraviolet radiation could produce bioinsecticides (delta-endotoxin) as much as 59% higher than the wild type.

The objective of this study is to improve *Xenorhabdus* sp. (X1) by classical mutagenesis using ultraviolet radiation for enhancing its biocontrol efficacy specific for mushroom mite (*Luciaphorus* sp.).

Materials and Methods

Bacteria, mushroom and mite cultures

Symbiotic bacteria (*Xenorhabdus* sp., X1) were isolated from the surface-sterilized infective juveniles of *Steinnernema siamkayai* nematode obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives as previously described by Kaya and Stock (1997). The genus and species of isolated bacteria were identified using the following protocols. Firstly the bacteria were smeared onto nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) medium (Lacey, 1997), then sealed and incubated in the dark at 28 °C for 24 hrs. The colonies were characterized by morphology and color to determine primary phase characteristics. Phase I colonies were picked up and individually grown in 100 mL flasks containing 25 mL Luria Bertani broth (LB broth, Sigma Ltd., Missouri, USA) (Lacey, 1997) which were placed in an incubator shaker (200

rpm) for 48 hrs in the dark at 28 °C until the bacterial concentration reached 1×10^8 cells/mL. Bacterial stock was kept in 50% glycerol at -80 °C for long term storage.

Mushroom mycelia (*Lentinus squarrosulus*), obtained from the Mushroom Researchers and Growers Society of Thailand, was sub-cultured using 90 mm plastic Petri dishes containing Potato Dextrose Agar (PDA, Sigma Ltd., Missouri, USA) and incubated at 25 °C in the dark. The mushroom mycelium was then inoculated into a sawdust and sorghum grain mixture to generate fresh spawn (Bussaman, 2005).

Luciaphorus sp. mites were isolated from *Lentinus squarrosulus* basidiocarps and composts which were previously obtained from Rapeephan mushroom farm in Khon Kaen province in the northeast of Thailand. A pair of male and female mites were placed in each glass bottle containing *Lentinus squarrosulus* spawn and kept at 28 °C for reproduction. These mites were used in all of the experiments from these bottles.

Ultraviolet mutagenesis and screening

Xenorhabdus sp. (X1) was grown in the dark at 28 °C for 15 hrs (mid-log phase). The bacterial culture was diluted to be 10^2-10^3 cells/mL and 0.1 mL of the bacterial cell suspension were then spread on the LB agar and incubated at 28 °C in the dark for 10-12 hrs. The culture of X1 was exposed to UV radiation using a 30 W lamp at wavelength of 254 nm from a distance of 30 cm for various time intervals (0, 15, 30, 45, 60, 75, 90, 105 and 120 seconds). UV-irrdiated cultures were incubated at 28 °C for 24 hrs. The number of colonies were counted to determine the survival rates.

The survived bacteria were investigated for their protease activity by picking up the survival colonies from LB agar and placing them on the skim milk agar and following by incubation at 28 °C in the dark for 24 hrs. The isolated bacteria capable of producing clear zones on skim milk agar as large as the wild type (X1) does were chosen and their morphology were identified by Gram's staining and culturing using nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) medium.

Toxicity of bacterial cell-free supernatants on mite

The effects of cell-free supernatants from bacterial culture on the mortality of *Luciaphorus* sp. were studied using the Petri dishes containing mushroom mycelia, as previously described by Bussaman et al. (2006). Cell-free supernatant from bacterial culture was prepared by filtering the 48 h bacterial cultures (1×10^8 cells/mL) through a 0.22-mm Millipore filter. One hundred adult female mites were transferred to each 50 mm plastic Petri dish which containing mushroom mycelia growing on PDA. Then 500 µL of cell - free supernatant was spread onto the mite-infested mushroom mycelia. An equal volume of LB broth was used as a negative control. Each bacterial strain was tested in quadruplicates. All Petri dishes were covered and placed in a growth chamber at 28 °C and 80% RH in the dark. Mite mortality was monitored every 24 hrs for 5 days. This experiment was repeated twice.

Statistical analyses

As there were no differences of mite mortality being observed among the repeated experiments data from the two repeats were thus pooled and used for statistical analyze. All data were subjected to analysis of variance (ANOVA) using the general linear models procedure (SAS Institute, Cary, NC, USA). The data of the percentages of mite mortality

were arcsine transformed before analysis. The menns of mortality rates of all treatments were compared and determined using the LSD test at $p \leq 0.05$.

Results and Discussion

Cell viability after UV-irradiation and selection of mutant

After the UV treatment at various time periods, the survival *Xenorhabdus* sp. (X1) bacteria (Fig. 1) were transferred to the skim milk agar for screening of their ability to produce protease. The survival rates of the mutants were ranged between 70.55-77.15 % with the note that when time increased the survival rates were steady because the bacteria may be fully adapted to UV radiation. The totals of 1,157 mutants were tested using skim milk agar and only 9 mutants were found to produce clear zones as large as the X1 (Fig. 2). Gram's staining of these nine mutants showed that they were short-rod, gram-negative bacteria. When these mutants were streaked on NBTA medium, they formed cocci and smooth colony as the wild type (X1).

Effect of cell-free supernatant of mutants on female mites

The cell-free supernatant of nine mutants have been shown to be significantly effective against the female mites ($p \leq 0.05$), when compared with negative control (LB broth) (Fig. 3). For all treatments the mite mortality rates gradually increased and remained steady after 3 days. The cell-free supernatant of Mutant RP58 resulted in mite mortality at levels higher than the wild type (X1) and the other mutants. This RP58 Mutant caused 84.16% mite mortality at 24 hr 88.33% at 48 hr and 92.49% at 72 hr, whereas the wild type X1 caused 84.99% and 87.49% mite mortality at 48 and 72 hr, respectively. The cell-free supernatant of all mutants resulted in maximum mortality of the female mites at day 3. These were no death of the female mites in the control treatment group (LB). In the last day of treatment the propragite (miticide) was shown to resulted in 100% death of the female mites.

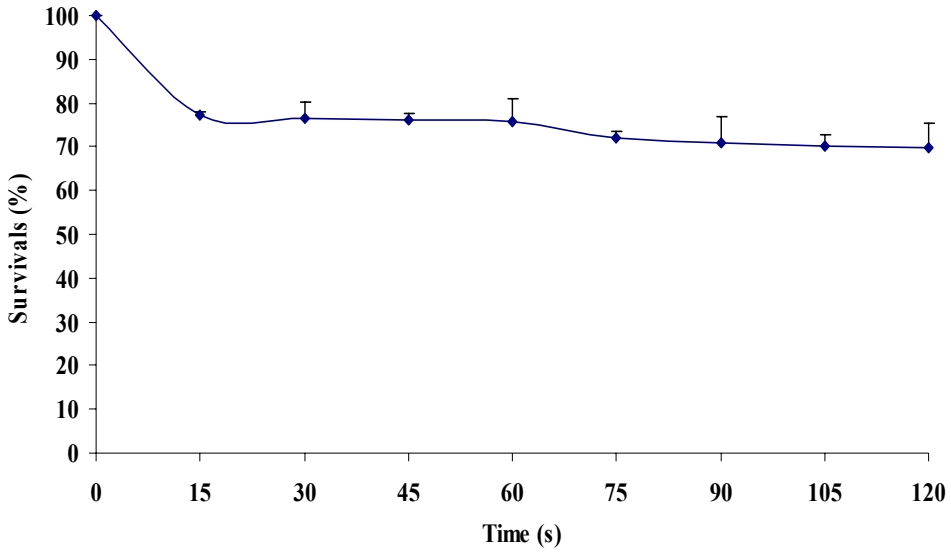

Figure 1. The survival curves of *Xenorhabdus* sp. (X1) from strain mutagenesis by UV radiation.

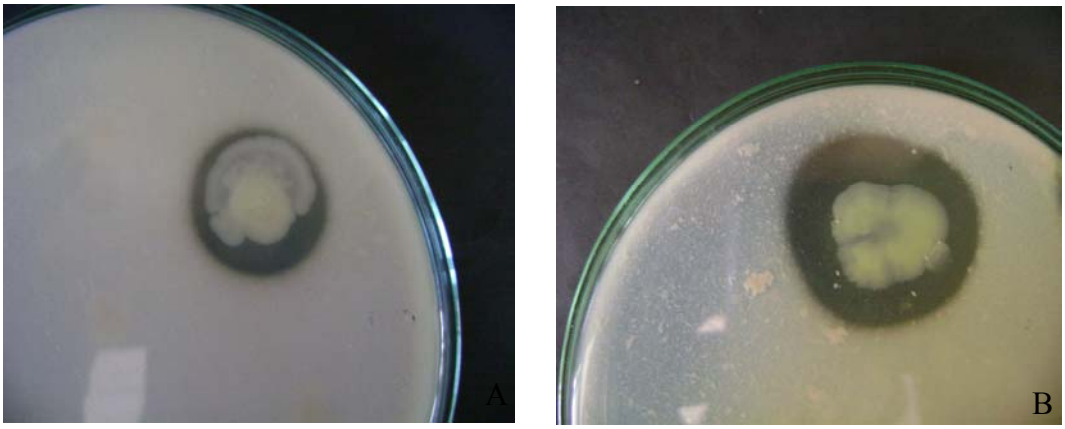

Figure 2. Generation of clear zones on skim milk agar by the wild type (A) and the mutant RP58 (B).

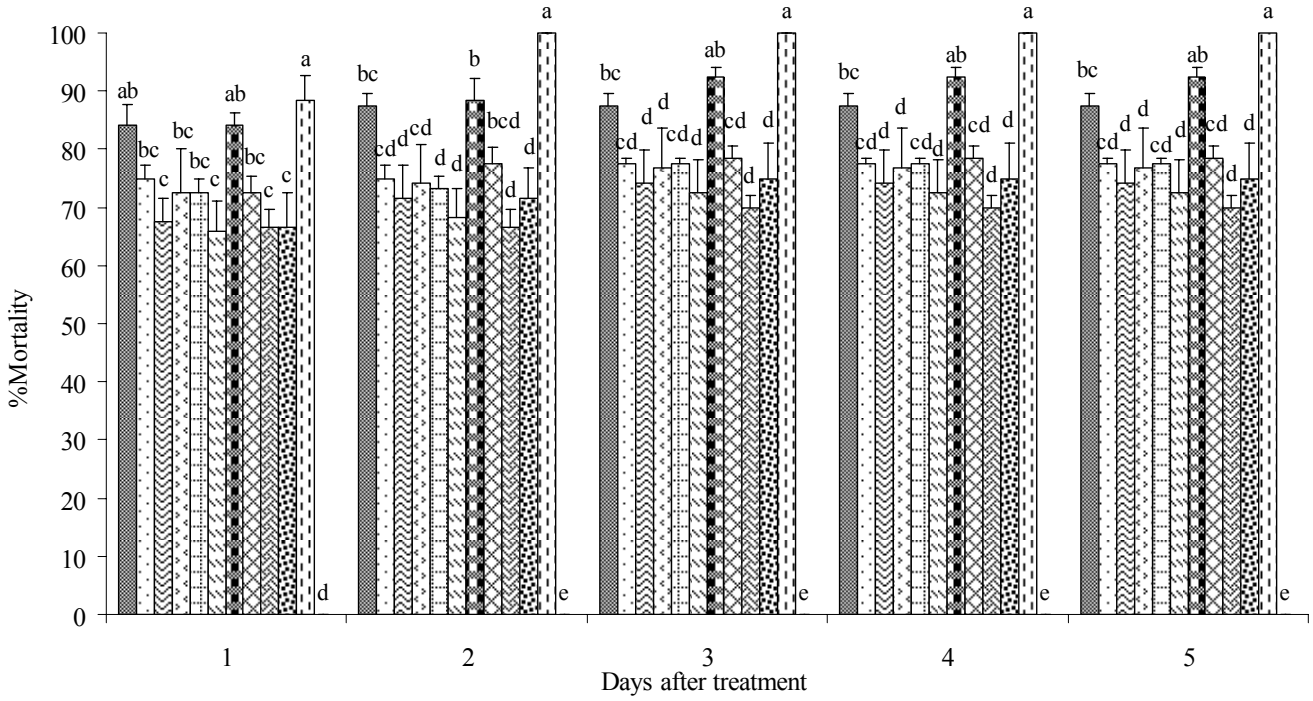

Figure 3. The mortality rates of *L. perniciosus* female after being treated with the cell –free supernatant of *Xenorhabdus* sp. (X1) and mutants at concentrations 1×10^8 cells/mL and kept at 28±2 °C, 80% RH in the dark for 5 days, ( ) X1, ( ) RP12, ( ) RP49, ( ) RP51, ( ) RP52, ( ) RP54, ( ) RP58, ( ) RP71, ( ) RP133, ( ) RP141, ( ) Propargite, ( ) LB broth. Bar (mean ± SE) in the same and between the time intervals with the same letter (s) are not significantly different, according to LSD test.

Bussaman et al. (2006) showed that the cell-free supernatant of *X. nematophila* (Thai) and *Photorhabdus luminescens* subsp. *laumondii* (strain GPS 12) were virulent to *Luciaphorus* sp., resulting in 70 and 95% mortality of the female mites, respectively at 48 hr after treatment. The Mutant RP58 was shown to be toxic to the female mites at levels higher than the wild type (X1). The UV radiation is one of the physical mutagens which can cause damages to the DNA via transversion, deletetion, frame shift and transition from GC→ AT (Parekh et al., 2000), and if it damages the regions required for controlling DNA translation it may generate mutants which may excessively produce enzymes or protein toxins. Vaidya et al. (2003) reported that the *Alcaligenes xylosoxydans* mutant (UV28) which derived from ultraviolet radiation could produce chitinase enzyme at levels higher than the wild type by 2.3 fold. This results agree with the report of Gohel et al. (2004) that found that the *Pantoea dispersa* mutant (UV19) obtained by UV mutagenesis could produce chitinolytic enzyme at levels higher than the wild type by 1.23 fold, and this mutant could also inhibit the growth of fungal plant pathogen (*Fusarium* sp. and *Macrophomena phaseolina*) more effective than the wild type.

Conclusions

Ultraviolet radiation has shown to be beneficial for improving the efficacy of *Xenorhabdus* sp.(X1) for controlling mites, and the obtained information can be used for bacterial strain development for industrial scales. In the future, the Mutant RP58 may be further improved by other mutagens for enhancing its efficacy and consistent virulence to mushroom mite.

Acknowledgements

We would like to thank Assoc. Prof. Sunha Panichajayakul, the owner of Rapeephan mushroom farm, for providing the mushroom mites. We would like to express our sincere thank for Dr. Praweena Rattanasena of Mahasarakham University, Thailand for reviewing the manuscript. We also thank Mrs. Vatcharee Somsook of the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand for providing entomopathogenic nematodes for this study. This work was financially supported by the Thailand Research Fund (TRF), grant numbers MRG-WII 525S080 and Mahasarakham University, Thailand.

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