

Bacillus thuringiensis Entomotoxicity Activity in Wastewater Sludge-Culture Medium towards *Bactrocera dorsalis* and their Histopathological Assessment

(Aktiviti Entomotoksisiti *Bacillus thuringiensis* dalam Medium Air Sisa Kultur Enap Cemar ke arah *Bactrocera dorsalis* dan Penilaian Histopatologinya)

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ABSTRACT

This study investigates the production of biopesticide based on Bacillus thuringiensis activity in culture media supplemented with semi-solid wastewater sludge as one of the raw ingredient. A series of testing using mixture of sludge and source of protein as B. thuringiensis growth media were carried out and selection of media was based on viable spore count. The entomotoxicity test of B. thuringiensis was carried out against larvae of fruit fly using diet incorporation method. Further impact of entomotoxicity was observed based on histology deformities on columnar epithelial cell and goblet cell of the midgut. A mixture of sludge with 60% wheat bran produced up to 1.64×10^{10} CFU/mL of viable spore count within 10 days of incubation. Based on entomotoxicity test, incorporation of 12 mL of semi-solid wastewater sludge-culture media into fruit fly artificial diet caused the highest fruit fly mortality at 64.8%. The value of semi-solid wastewater sludge-culture media concentration for LC_{50} was determined at 8.43%. Effect of entomotoxicity can be seen started from 3rd instar larvae where histopathological studies showed that up to 10% of columnar epithelial cells in the intestine were swollen and severe reduction of goblet cell's size. Thus, it decreases the survivality of the fruit fly larvae. The present study indicated that semi-solid wastewater sludge has the potential to enhance B. thuringiensis entomotoxicity activity.

Keywords: diet; goblet cell; histopathology; lumen; semi solid; wheat bran

ABSTRAK

Penyelidikan ini mengkaji pengeluaran biopestisid berdasarkan aktiviti Bacillus thuringiensis dalam kultur media yang ditambah dengan enap cemar air sisa separa pepejal sebagai salah satu bahan mentah. Siri ujian menggunakan campuran enap cemar dan sumber protein seperti media pertumbuhan B. thuringiensis telah dijalankan dan pemilihan media adalah berdasarkan kiraan spora berdaya maju. Ujian entomotoksisiti B. thuringiensis telah dijalankan terhadap larva lalat buah menggunakan kaedah penggabungan diet. Satu kesan daripada entomotoksisiti diperhatikan berdasarkan histologi kecacatan sel epitelium turus dan sel goblet daripada perut tengah ini. Campuran enap cemar dengan bran gandum 60% dihasilkan sehingga 1.64×10^{10} CFU/mL kiraan spora berdaya maju dalam tempoh 10 hari dari inkubator. Berdasarkan ujian entomotoksisiti, penubuhan 12 mL media kultur air sisa enap cemar separa pepejal ke dalam diet tiruan lalat buah disebabkan kematian lalat buah yang tertinggi sebanyak 64.8%. Nilai kepekatan kultur media separa pepejal buangan enap cemar PL_{50} telah ditentukan pada 8.43%. Kesan entomotoksisiti boleh dilihat bermula daripada tiga instar larva dengan kajian histopatologi menunjukkan bahawa sehingga 10% daripada sel-sel epitelium turus dalam usus telah bengkak dan pengurangan saiz sel goblet yang ketara. Oleh itu, ia mengurangkan kemandirian untuk larva lalat buah. Kajian ini menunjukkan bahawa air sisa enap cemar separa pepejal mempunyai potensi untuk meningkatkan aktiviti entomotoksisiti B. thuringiensis.

Kata kunci: Dedak gandum; diet; histopatologi; lumen; sel goblet; separa pepejal

INTRODUCTION

The presence of pests, which have a significant impact on crop and human health are control using pesticides. Most of the commercial pesticides work by altering the feeding behaviour or olfactory capacity, which lead to reduction in percentage of adult survivor (Desneux et al. 2006). Though the use of pesticides generally reduces the occurrence of pest, it does not guarantee the decrease in crop loses where more than 400 insect species have developed resistant towards chemical pesticide (Pimentel 2009; Tirado Montiel et al. 2001).

In the current trend of agriculture, the objective of pest control is to reduce the occurrence of pests and diseases to a point that they do not seriously damaging the crops without changing the nature's balance. This can be achieved with integrated pest management including biological control and the use of biopesticide (Li & Yu 2012). Biopesticides are based on living microbes, organic and the most positive factor it is biodegradable, less dangerous on non-target pests, generally host specific and preventive (Gupta & Dikshit 2010). There are a few biopesticides based on compounds produced by the microbes such as *Bacillus*

thuringiensis, which kills insect via their toxic proteins and not by infective actions (Glare et al. 2012). However, several disadvantages of biopesticides such as lacking in efficiency, inconsistent field performance and high costs have raised many questions among the end user.

Bacillus thuringiensis, a gram-positive bacterium has been widely used as microbial insecticide to control many pests via secretion of entomotoxin (Lachhab et al. 2001). Entomotoxicity or biopesticidal activity of *B. thuringiensis* is usually closely related to delta-endotoxin and spore concentration in the final products (Vu et al. 2009). The use of *B. thuringiensis* is frequently challenged by the cost of raw materials for *B. thuringiensis* entomotoxin production. Therefore, to encourage the commercial production of *B. thuringiensis* biopesticides, utilization of less expensive material is advisable and several raw materials (industrial and agricultural by products) have been tested as alternative culture media for entomotoxin production (Brar et al. 2006; Tirado-Montiel et al. 2001).

One of the promising sources of cheap material is utilization of wastewater. The exploitation of sewage sludge for entomotoxin production by *B. thuringiensis* and application to agricultural crops and forests for pest control seems to be fully compatible with current sludge disposal practices (Tirado-Montiel et al. 2001). Wastewater such as semi solid sludge contained nutrients like carbon, nitrogen, phosphorus and other nutrients that are required by *B. thuringiensis* for their growth and sporulation (Lachhab et al. 2001). Several studies have been conducted on the production of biopesticides from sludge by *B. thuringiensis* through manipulation on nutrient composition of sludges (Brar et al. 2005; Tirado-Montiel et al. 2001). The degree of entomotoxicity of the crystal spore complex of *B. thuringiensis* is much depend on the concentration and type of carbon sources in the medium employing different sludge solids concentration during fermentation, which could vary the entomotoxicity yield (Keshavarzi et al. 2005).

In this study, semi-solid wastewater sludge was used as culture medium for production of entomotoxin by *B. thuringiensis*. The semi-solid wastewater sludge with different treatments was used as an alternative medium for *B. thuringiensis* growth and sporulation. We attempted to identify the type of semi-solid wastewater sludge preparation in order to produce high numbers of *B. thuringiensis* spore counts. We also examined any deformities caused by *B. thuringiensis* culture on semi-solid wastewater sludge on fruit fly's gastrointestinal system.

MATERIALS AND METHODS

PREPARATION OF BACTERIA STOCK CULTURE, SLUDGE AND SLUDGE-CULTURE MEDIA

A single colony of *B. thuringiensis* ATCC 10792 (obtained from Laboratory of Plant Systematic and Microbes Collection, Department of Biology, Faculty of Science,

Universiti Putra Malaysia) was used and cultivated into sterilized nutrient broth prior 16 hours of incubation ($30\pm 0.5^\circ\text{C}$; 150 rpm). Semi-solid wastewater sludge samples were collected from a sludge pool located at Recycle Energy Semenyih, Selangor. Determination of total solids, suspended solids, volatile solids and volatile suspended solids were carried out as described in Clesceri et al. (1999). The sludge sample was sterilized ($121\pm 0.5^\circ\text{C}$; 15 min) before use.

Sludge-culture medium was prepared in three different mixture: 100% semi-solid wastewater sludge; 60% wheat bran with 40% semi-solid wastewater sludge; and 60% paddy straw powder with 40% semi-solid wastewater sludge, before the initial pH been adjusted to 7.0. All sludge-culture media were inoculated with *B. thuringiensis* and incubated for 10 days ($30\pm 0.5^\circ\text{C}$; 150 rpm). The samples were collected every 24 h to determine the viability of the (CFU/mL) using heat shock treatment at $75\pm 0.5^\circ\text{C}$ for 10 min (Tirado-Montiel et al. 2001). The highest viable spores concentration was selected for entomotoxicity test.

BIOASSAY FOR ENTOMOTOXICITY AND LC_{50} DETERMINATION

The entomotoxicity of *B. thuringiensis* was determined against fruit fly (*Bactrocera dorsalis*) by using the diet incorporation method (Vidyarthi et al. 2002). Three mL of 60% wheat bran with 40% semi-solid wastewater sludge mixture inoculated with *B. thuringiensis* were mixed with an artificial diet and placed in vial. The artificial diet was mixed with a series of semi-solid wastewater sludge culture media inoculated with *B. thuringiensis* concentrations (1.5, 3.0, 6.0, 9.0 and 12.0 mL), respectively. Two types of control treatment were prepared: An artificial diet was mixed with un-inoculated semi-solid wastewater sludge culture media (absence of *B. thuringiensis*) to eradicate the effect of the sludge and mixture of an artificial diet and distilled water. Twenty five of 2nd instar fruit fly larvae were placed in each vial and left to feed for 10 days at $25\pm 0.5^\circ\text{C}$ and their mortality was monitored daily. The LC_{50} value was determine by plotting a graph of probit against \log_{10} concentration.

HISTOPATHOLOGICAL ANALYSIS

The experiment was set-up as described in bioassay for Entomotoxicity and LC_{50} determination section except for the artificial diet was mixed with $\approx LC_{50}$ value of semi-solid wastewater sludge culture media inoculated with *B. thuringiensis*. The control culture (mixture of an artificial diet and distilled water) was used to observe the effect of entomotoxicity. Observations of deformities on lumen, cytoplasm and goblet cell were performed on dead 3rd instar larvae.

STATISTICAL ANALYSIS

All the data collected was analysed using ANOVA where the confidence level was set at 95%. Any significance

difference ($p < 0.05$) was analyzed using Tukey test. The mortality rate of the larvae for LC_{50} was analyzed using one-way ANOVA. The data was analyzed using SPSS program version 16.0.

RESULTS AND DISCUSSION

Bacillus thuringiensis is a gram positive and a spore forming bacteria that are known to affect a variety of insect in the orders Diptera, Lepidoptera and Coleoptera due to their insecticidal properties (Brar et al. 2006). Tirado-Montiel et al. (2001) reported that wastewater sludge is a good alternative media used for the growth of *B. thuringiensis* as the content of the sludge helps to produce certain important metabolic product like endotoxin. Composition of total solids, suspended solids, volatile solids and volatile suspended solids is shown in Table 1.

After undergoing heat and cold treatment, the number of spores was calculated for 10 days. A mixture of 40% semi-solid wastewater sludge-culture and 60% wheat bran

had produced the highest value of spore count after 10 days of incubation (Figure 1). Viable spore count increased steadily within 8 days before sudden increase from 2.7×10^9 CFU/mL to 1.64×10^{10} CFU/mL at day 10. The addition of wheat bran into the culture medium provides significant improvement on *B. thuringiensis* growth and entomotoxin activity. Wheat bran contained up to 13% of protein as well as 71% of carbohydrate (Devi et al. 2005) thus offer higher carbon and nitrogen source which are essential for the growth in microbial cultivation (Anderson & Jayaraman 2013). Furthermore, formation of parasporal crystals, that consists of 95% protein and 5% carbohydrate as component of spore delta-endotoxin complexes require a media that rich in protein (Keshavarzi et al. 2005; Salama et al. 1983). However, semi solid wastewater sludge-culture media supplemented with 60% wheat bran produced less number of viable spore concentration compared to commercial media due to the difference in protein content. Most of the commercial media is the by-product of oil extraction from soybeans which contained up to 44% protein (Vitcosque et al. 2012). Even though superior in viable spore count can be achieved by using commercial media, the cost of production is more expensive than the wastewater (Yezza et al. 2006) where the raw material cost alone may comprise more than 70% of the overall production cost (Poopathi & Archana 2012).

A trial to use 100% semi solid wastewater sludge as culture medium does not promote good yield of spore concentration as the spore produced was only 2.00×10^8 CFU/mL per day. Lack of viable spore concentration in this medium may be due to viscosity of the sludge. Viscosity of semi-solid wastewater caused low oxygen transfer into the culture media and also leads to high osmotic pressure where finally combination of both factors gave negative effect on the nutrient transfer across the *B. thuringiensis* (Brar et al. 2005).

TABLE 1. Composition of semi-solid waste water sludge

Parameter	Semi-solid waste water sludge
Total solid	5.02 ± 0.31 g/L
Total volatile solids	2.76 ± 1.5 g/L
Total fixed solids	3.66 ± 1.89 g/L
Total carbon	2.0%
Total nitrogen	10.1 ppm
Total phosphorus	0.18 ppm
Potassium	96.2 ppm
Calcium	16.09 ppm
Magnesium	20.76 ppm
Copper	0
Ferum	4.58 ppm
Manganese	0.13 ppm
Zinc	0.59 ppm

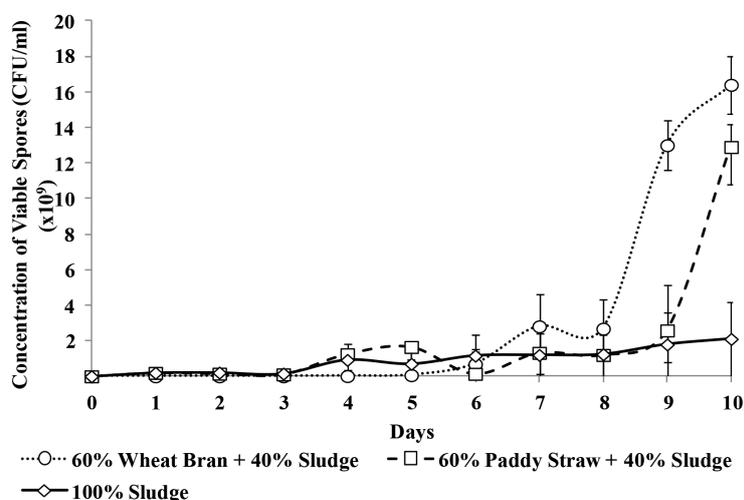


FIGURE 1. Concentration of *Bacillus thuringiensis* viable spores obtained from three types of culture media after 10 days of incubation

A comparative study to assess entomotoxicity activity of *B. thuringiensis* spore on *B. dorsalis* using six different concentrations of semi-solid wastewater sludge-culture media supplemented with 60% wheat bran showed encouraging results (Figure 2) with the highest mortality of 64.8% was recorded using 12 mL semi-solid wastewater sludge-culture media and only 9.5% of mortality observed in both types of control. The results indicated that the entomotoxicity activity was more pronounced in the presence of *B. thuringiensis* spores. The sporulation of *B. thuringiensis* produce Cry proteins, endotoxin and β -exotoxin that are toxic to a limited number of target insect larvae and lead to epithelial cell disruption, decrease in pH of midgut lumen and increase in pH of haemolymph leading to rapid gut paralysis (Carlberg 1986; Gringorten 2001; Santos et al. 2009).

The value of LC_{50} was determined using probit graph and indicated that LC_{50} can be achieved at 8.43 mg/L. Even though further test using LC_{50} concentration was only able to produce 43.2% mortality rates in the population, this value is not significantly different to mortality caused by 9 mL of semi-solid wastewater sludge-culture media. However, the LC_{50} value for any entomotoxicity may vary in different stages of pest development where toxicity against larvae and adults did not always coexist in the same concentration of pesticides. Earlier reports stated that perhaps it is caused by one or combination of these factors: Different types of gene that responsible for toxicity in these two developmental stages of the insect (Alberola et al. 1999); differences in modes of feeding larvae and adults; where with the filter-feeding mode, the concentration of delta endotoxin delivered to larvae is amplified (Karamanlindou et al. 1991); or differences in chemical compound where other endotoxin may present;

of which in this case, *B. thuringiensis* also secrete beta-exotoxin known as thuringiensin (Carlberg 1986).

Comparison of lumen structure between two treatments showed that the lumen structure in larvae became swollen and large cytoplasmic spaces can be seen in most part of the intestine (Figures 3(a) and 3(b)). In the early stage of 3rd instar, around 10% of columnar epithelial cell begun to swell and the destruction of these cells increased with time. By the end of the 3rd instar stage, most of these cells were affected and many have been sloughed off to the midgut lumen. In some cases, the lumen was separated from peritrophic membrane. The entomotoxin produced by *B. thuringiensis* is able to cause cytolytic effect on the epithelial cell causing the cell to swell and disrupted (Ryser et al. 1990). An expansion of epithelial cells later caused separation of the lumen from the peritrophic membrane, which is responsible for mucus secretion and significant drop of mucus lumen will happen (Nu Hung et al. 2000). Separation of the lumen from peritrophic membrane will also rapture the cell and allow *B. thuringiensis* spore to germinate in a nearly neutral environment inside the larvae gut and later lead to larvae death (Whalon & Wingerd 2003).

Further histological investigation on intestine layer revealed the reduction of goblet cell's size. Goblet cell is one of the components in peritrophic membrane to produce mucus to ease the movement of the food particle along the gut (Nu Hung et al. 2000). The result of feeding trails showed that goblet cell in control diet was in the range of normal size of which the cells are packed in neat and tidy arrangement (Figure 3(c)) while goblet size in semi-solid sludge treated diet has shrunk, scattered and separated from each other (Figure 3(d)). Similar observations have been reported previously where shrunk goblet cells

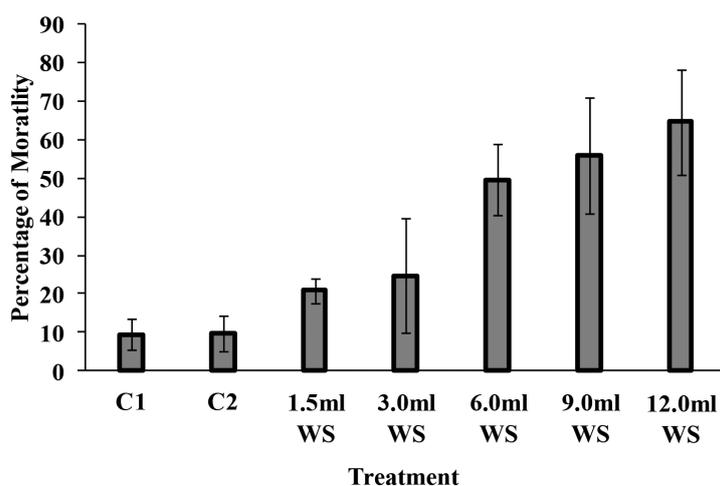


FIGURE 2. Percentage of mortality of 2nd instar fruit fly larvae after 10 days of feeding with different concentrations of semi-solid wastewater sludge culture media (60% wheat bran and 40% sludge). C1: Mixture of an artificial diet was mixed with un-inoculated semi-solid wastewater sludge culture media; C2: mixture of an artificial diet and distilled water; WS: Semi-solid wastewater sludge culture media inoculated with *B. thuringiensis*

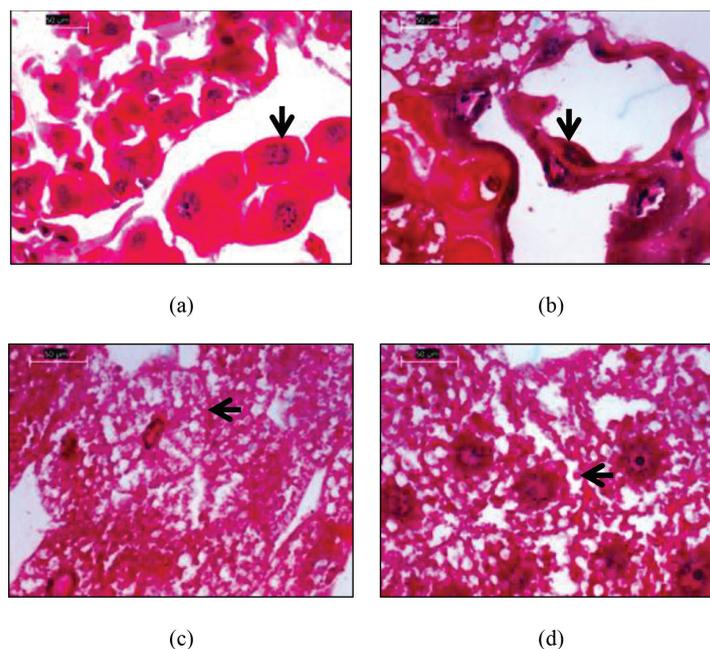


FIGURE 3. The histological structure of cells in 3rd instar fruit fly larvae (a) epithelial cell in control diet; (b) epithelial cell in diet mixed with 9 mL semi-solid wastewater sludge; (c) goblet cell in control diet and (d) goblet cell in diet mixed with 9 mL semi-solid wastewater sludge

causing wider inter-cellular spaces and leaky columnar cells (Pandey et al. 2009). In the case of *B. thuringiensis* entomotoxicity, the Cry protein has bound to the peritrophic membrane and disrupt K^+ pump in goblet cell leading to a rapid disruption of K^+ transport across the cells (Lane et al. 1989). As a result, goblet cell became shrivel due to the changes in intracellular pH and Ca^{2+} and inability to maintain the homeostasis stage.

Even though there are many reports of Cry entomotoxins, most of the reports highlighted their effect on pesticide, whereas the effect of Cry entomotoxins on mammals is almost zero. McClintock et al. (1995) has proven that Cry protein can be digested (90% in 2 min) by stomach acidification and by the absence of specific binding sites for Cry toxin in mammal intestine. Therefore, in theory any usage of Cry protein based pesticide on vegetable for human consumption will not have or have only minor impact on humans. Nonetheless, further studies on this with other toxic protein or allergic protein should be conducted to ensure the safety of the biopesticides.

CONCLUSION

The results of the presented study indicated the potential of semi-solid wastewater sludge as an alternative raw material for the production of *Bacillus thuringiensis*-based biopesticides but supplementary with other source of protein is needed. Mixture of 60% wheat bran and 40% sludge in culture media able to produce the highest spore count, 1.64×10^{10} CFU/mL with significant biopesticides activity towards fruit fly. In summary, the entomotoxin in semi-solid wastewater sludge-culture media has an ability

to reduce the survival of fruit fly and adult emergence significantly even at larvae stage. Abnormality can be seen in cell surrounding the intestine wall especially the goblet cell, thus reduced the chances of fruit fly survival.

ACKNOWLEDGEMENTS

The authors would like to thank Universiti Putra Malaysia for the financial support through RUGS 6 Grant No. 05-02-2170RU and all staff of the Plant Systematic and Microbe Laboratory, Biology Department, Universiti Putra Malaysia for all their effortless contributions.

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Received: 1 April 2014

Accepted: 30 October 2015