Research Article

Preliminary Batch Cultures on the Recombinant Strain of *Bacillus megaterium* for Mosquito Larvicidal Toxin Production

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Abstract

The spontaneous mutant of *Bacillus megaterium* pWH1520TK recombinant strain harboring a 93 kDa fusion binary toxin gene of mosquito larvicidal activity, has been preliminarily studied in batch cultures. The fermentation medium was Luria Bertaini medium supplemented with 20 μg/ml of tetracycline and 0.5 % xylose addition to induce expression of the toxin gene. Maximum dry weight and maximum toxin produced have been found at 20 h and 24 h, respectively. The toxin levels stayed high over 60 h, and decreased remarkably at 72 h, perhaps indicating some protease activity. Bioassays were also carried out to confirm mosquito larvicidal activity of the batch fermentation samples. LD_{50} values were in a range between 2.86-3.11 x 10^3 cells/ml, indicating a down mutation to low toxicity and instability of toxin production, when compared to the LD_{50} value of 2.31 x 10^2 cells/ml for the original mutant. The results show that the recombinant strain of *B. megaterium*, with further studies on its gene harboring stability and toxin optimization, could be a promising host expressing mosquito larvicidal toxin.

Keywords: fusion binary toxin gene; batch fermentation; recombinant strain; *Bacillus megaterium*; mosquito larvicidal activity

1. Introduction

Among various insect pathogenic bacteria, *Bacillus thuringiensis* subsp. *israelensis* (Bti) and *Lysinibacillus sphaericus* (Ls, formerly and well known as *Bacillus sphaericus*) are the most promising agents because of their high toxicities to mosquito larvae and the potential for manipulating their toxin genes. At present several commercial formulations of Bti has been used worldwide for mosquito control for over 30 years, while Ls commercials have been available within the past few years (Berry, 2012). Comparison to the Bti-based larvicide formulations, the Ls-based formulations possess the ability to survive in treated habitats or polluted water, and their toxicity appears to

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persist for a longer time. In addition, Ls spores can recycle in *Culex* spp. larvae, as the capability of its spores to germinate and propagate in the larval midgut. However, an emergence of Ls resistance has been reported significantly in some field populations (Park *et al*., 2010).

The Ls strains can produce various types of proteins that account for insecticidal properties. In the most toxicity larvicidal strains, i.e. Ls 2362, 1593 and 2297, the paracrystalline binary (Bin) toxin has been shown to be synthesized during sporulation. The Bin toxin protein comprises of two components, BinA (370 amino acids, 42 kDa) and BinB (448 amino acids, 51 kDa protein) (Baumann *et al*., 1988). Blockages at early stages of sporulation affect no accumulation of crystal toxins (Charles *et al.*, 1988). In the low toxicity strains, the mosquitocidal toxins (Mtx family) have been demonstrated throughout the vegetative growth (Thanabalu *et al*., 1991). Besides the other two-component crystal toxin (Cry48/Cry49) has been discovered on sporulation in those strains which overcome Bin resistance or slowly developed resistance (Pei *et al.*, 2002; Jones *et al.*, 2007). Lately, a novel insecticidal protein active against cockroaches and caterpillars, sphaericolysin, has been found in the strain of Ls strain lacking *bin* and *mtx* genes (Nishiwaki *et al.*, 2007).

As Bin toxin is mainly responsible for mosquito larvicidal activity, studies on Bin proteins and *bin* genes have been done extensively. BinA is proposed to be essential for toxicity, while BinB has been reported to involved as a component to the specific binding receptor at midgut brush border membrane (BBMF) of the larvae. Upon the binding to the receptor, an oligomeric binary toxin form (BinA2BinB2) was suggested to play role in pore formation. Conformational changed in BinA was observed in a weak interaction of BinA and BinB oligomeric formation in the solution (Kale *et al*., 2013). N-terminal region of BinB has been suggested to be essential in both the receptor recognition and the membrane interaction (Singkhamanan *et al*., 2013) Mechanism of resistance to binary toxin in mosquito was shown to be associated with the loss of the BBMF bound receptors (Silva-Filha *et al*., 2004)

Since Ls strains have some potential limitations as a mosquito control agent related to a need for expensive growth media, sedimentation in the natural environment and UV toxin inactivation, the genes encoding Bin toxins and Mtx toxins have been cloned and transferred into a number of alternative hosts, such as *B. subtilis* (Baumann and Baumann, 1989; Broadwell *et al.*, 1990a), cyanobacteria (Sangthongpitag *et al.*, 1997), *Caulobacter* spp. (Thanabalu *et al.*, 1992), *Ancylobacter* spp. (Yap *et al.*, 1994), *Asticcacaulis* spp. (Liu *et al.*, 1996) and *E. coli* (Shamugavelu *et al*., 1997; Hire *et al*., 2009). The results of the toxin gene expression and toxicity of the recombinant strains have shown relatively low levels, compared with naturally occurring toxic parent Ls strains. The most toxic recombinant strains

with the expression of toxicity comparable to Ls 2362 have been *Asticcacaulis excentricus* and *B. subtilis*.

The gram-positive bacterium *Bacillus megaterium* (Bm), a simple soil bacterium with large size, has been employed commercially for more than 50 years to produce a wide range of enzymes and vitamins, i.e. α-amylase, β-amylase, glucose dehydrogenase, chitosanasen, neutral protease, oxetanocin, penicillin amidase, steroid hydrolases and vitamin B_{12}. The strain of Bm has a number of attractive features, viz. a wide distribution ecologically, ability to grow on a variety of inexpensive media, a low intrinsic protease activity, readily secreted proteins into the growth medium, as well as maintaining structural and segregational stability of plasmids (Vary, 1994; Vary *et al*., 2007). Bm has been used to express a number of heterologous genes with high yields and without degradation (Rygus and Hillen, 1991; Wittchen and Meinhardt, 1995). Several recombinant shuttle vectors carrying various inducible promoters and combinations of affinity tags for one-step protein purification have been reported (Malten *et al*., 2006). Moreover, It has also been used as a model system for the systematic evaluation of single and multiple directed molecular and different metabolic engineering strategies (Biedendieck *et al*., 2010). Recently, production of heterologous exoproteins by *B. megaterium* was systematically optimized and further improved with high yield (Yang *et al*., 2006; Stammen *et al*., 2010)

Since the recombinant Bm pWH1520TK encoding fusion Bin toxin whose expression is regulated by xylose has been constructed (England *et al.*, 1997), an obtained spontaneous mutant by using antibiotic gradient plate was selected for a preliminary batch culture study in this paper, as a conduct to trial an alternative host for larvicidal toxin production.

2. Materials and Methods

2.1 The spontaneous mutants of the recombinant strain of *B. megaterium*

The original plasmid pWH1520 with ampicillin resistance (Ap^r) and tetracycline resistance (Tc^r) markers (Rygus and Hillen, 1991) was introduced into *B. megaterium* to produce xylose regulated high-level expression of non-native proteins. England *et al.* (1997) constructed derived plasmid pWH1520TK encoding the 93-kDa fusion binary toxin gene of mosquito larvicidal activity from Ls (Broadwell *et al.*, 1990b) with a downstream kanamycin (Km) resistance gene, and propagated in *B. megaterium* WH320 (Rygus *et al.*, 1991). Since the Km resistant gene is assigned downstream of the toxin gene, by means of using Km gradient plates containing 0.5% xylose, the spontaneous mutants of *B. megaterium* pWH1520TK cultures with high Km resistant together with enhanced expression of the toxin were selected for further studies.

2.2 Batch fermentation

Seed cultures of the selected mutants of *B. megaterium* pWH1520TK were

grown in 250 ml flasks, on a rotary shaker (280 rpm) at 37 °C overnight. The seed medium was Luria Bertaini (LB) broth supplemented with 20 μg/ml tetracycline (Tc) and 0.5 % xylose. Batch fermentation was performed in a 2 litre glass LH fermentor (Model 502D) equipped with controls for pH 7.0 (7.0±0.1) and temperature at 37 °C (37±0.5 °C), using the same medium as that for the seed culture. Aeration was kept at 700 rpm with air supply 1 vvm and DO ≥ 20 % air saturation. Samples were withdrawn every 3-5 h for determination of biomass concentration, cell counts, Western blot analysis followed by semi-quantitative fusion toxin determination, and bioassay evaluations.

2.3 Determination of biomass concentrations

Biomass concentrations were determined by the dry weight cell method. Samples, 3 ml volume, were pipetted into preweighed glass tubes and centrifuged in a bench top centrifuge for 10 min at high speed. The cell pellet was washed twice with RO water and put in the oven at 105 °C for 24 h. After cooling, the tubes with dried biomass were reweighed and dry weight cell (DWC) was calculated as mg/ml or g/l.

2.4 Determination of cell counts

Samples were diluted to give a final absorbance at 600 nm (OD_{600}) within the range of 0.1-0.2. A drop of 10% chloroform solution was added to prevent further growth of cells. A diluted cell suspension was dispersed (to eliminate clumping) in an ice bath by a Branson Sonifier® Cell Disruptor B15 equipped with a tapered microtip (1/8 inch) for 40 sec at outputs 10 W (50 % duty). Cells were counted microscopically using a Weber Thoma bacterial counting chamber under phase contrast microscopy (Olympus BHJ) at 400x magnification.

2.5 Western blot and semi-quantitative analysis for fusion toxin levels

Unlike Ls 2362 that produces a crystal toxin, the spontaneous mutant has been demonstrated to produce amorphous inclusion bodies of binary toxin inside the cells (Jiaviriyaboonya *et al*., 1998a). To determine toxin level in the mutant, cells were lysed and assayed for toxin levels by Western blot analysis. An aliquot of the sample, 1 ml, was pipetted into an Eppendorf tube and centrifuged by Eppendorf centrifuge (Vision VS-15,000, Brushless D.C. motor centrifuge) at 10,000 rpm for 5 min. Cell pellets were washed twice with RO water and 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.1 % Triton X100 and 500 μg/ml egg white lysozyme) was added. Digestion was carried out at 37 °C for 10 min and 100 μl sample buffer was added. SDS-PAGE followed by Western blot analysis was performed to estimate toxin band quantitatively throughout the samples collected from the fermenters.

SDS-PAGE (SDS-polyacrylamide gel electrophoresis) was done in a Mini-Protein II vertical electrophoresis cell (Bio-Rad). The slab gels with either 10 or 12 % separating gels and 4 % stacking gel were cast as modified from the previously described method (Laemmli, 1970). Electrophoresis was carried out at a

constant voltage at 200 V (Power-Pac 300, Bio-Rad). The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane in transfer buffer in a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad). Running conditions were at a constant voltage of 100 V for 1 h. The nitrocellulose membrane was removed, immersed in TBS (Tris buffer saline, 20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and incubated in blocking solution (3 % gelatin in TBS) for 1 h. After washing the membrane with TTBS (Tween Tris buffer saline, 0.05 % Tween 20 in TBS), the mouse anti-binary toxin antibody (Osborne, 1995) was diluted (1/2,000) with antibody buffer (1 % gelatin in TTBS) and added to the membrane. The primary antibody was incubated for 1-2 hours. The unbound primary antibody was washed out twice with TTBS. The secondary antibody, goat anti-mouse IgG (H+L) alkaline phosphatase conjugate (GAM-AP, Bio-Rad), 16 µl, was diluted in 50 ml of antibody buffer. The membrane was incubated with secondary antibody solution for another hour. After removal of the unbound secondary antibody by washing twice with TTBS and once with TBS, the final color was developed by an alkaline phosphatase conjugate substrate kit (Bio-Rad). Color development was stopped by washing twice with Milli-Q water. The membrane was dried for 1 h and stored in laminate coating. To semi-quantify the larvicidal toxin activity, the fusion binary toxin band (MW ~ 100 kDa) was scanned by Scanmaster 3+ (Howtek) and analyzed by a computer with Bio Image®: Whole Band Analyzer software (Millipore). One unit of toxin is defined arbitrarily as 1 unit of peak area quantified by scanning.

2.6 Bioassay

Bioassays were performed by using third instar larvae of *Culex quinquefasciatus* at 25 °C. Ten third instar larvae were counted and transferred to 100 ml of RO water in 125 ml plastic containers. Various concentrations of cell suspension samples were added, with each dilution done in duplicate. The larvae were fed 100 µl of 5 % baker's yeast suspension once a day. The number of dead larvae was counted after 48 h. The procedure was repeated 3 times for each set of data, with new batches of larvae. Negative controls were carried out by the same protocol but no sample additions. The dose to cause 50 % mortality (LD_{50}) was calculated by the probit analysis method of Finney (1971). Chi-square analysis with a significance level at $p = 0.05$ and 5 cycles was selected.

3. Results and Discussion

Since the Km resistance gene was located downstream of the fusion toxin gene, such spontaneous mutants with enhanced expression of the toxin was anticipated. The most toxic strain, designated T2 (with LD_{50} value of 2.3×10^2 cells/ml), was selected for this study. It was about 8-12 times more toxic than *B. megaterium* pWH1520TK (LD_{50} value of 3.0×10^3 cells/ml) and *L. sphaericus* 2362 (LD_{50} value of 1.6×10^3 cells/ml). Previous study by TEM (transmission electron

microscope) followed by immuno-staining (Jiaviriyaboonya *et al*., 1998a) has demonstrated amorphous inclusions (presumably of toxin proteins and/or the Km resistance protein) in T2. There was not any obvious structural features characteristic of crystal toxins. Growth at 37 °C produced more inclusions than at 30 °C (England *et al*., 1997).

Preliminary batch fermentation studies on T2 were conducted in LB medium supplemented with 20 μg/ml tetracycline and 0.5 % xylose in a controlled fermentor. Maximum dry cell weight and cell count were found at 20 h, and no further significant changes occurred up to 72 h (Figure 1). These relatively constant values indicated that low cell lysis occurred, perhaps due to lower protease activity in Bm cells. Maximum specific growth rate (μ_m) and maximum DCW of T2 were 0.28 h^{-1} and 2.13 g/l, respectively. Slow growth characteristics are typical for recombinant strains because of the need for antibiotics in the medium for plasmid maintenance, inducer requirements for expression of heterologous proteins, and also the metabolic burden involved in the high level production of such proteins. It was evident also that spores of different Bm strains were morphologically distinct. For example strain QM B1551 has a thick, outer spore coat and an exosporium while strain KM has a normal coat, no outer coat and no exosporium (Vary, 1994). In the present study, unlike Ls and other *Bacillus* strains those display an obvious morphological difference between spores and vegetative cells, T2 did not show any apparent spore coat or exosporium. In addition, as the cell morphology of T2 changed to a rounded form, this resulted in difficulty in distinguishing between spore and vegetative cells by simple phase contrast microscopy when counting cells. Therefore, the cell count values in this study refer to total cell counts.

Since T2 has been shown to produce amorphous inclusion bodies of fusion toxin inside the cells, the protocol using alkaline extraction of Bin toxin for a sandwich enzyme-linked immunosorbent assay (ELISA) (Jiaviriyaboonya *et al*., 1998b) was no longer practical. To determine the toxin levels in the spontaneous mutants, cell lysis followed by SDS-PAGE and Western blot analysis was carried out. A fusion binary toxin protein band with molecular weight of 93 kDa was observed following 0.5 % xylose induction. Optimization a time course with various lysozyme concentrations for cell lysis was done. Finally lysates digested by 500 μg/ml of lysozyme with 10 min incubation at 37 °C was chosen for the cell lysis protocol.

Various samples withdrawn at different times in batch fermentation were subjected to the protocol, followed by Western blot analysis. Time course toxin productions were estimated quantitatively as arbitrary units by scanning toxin protein bands from the blots (Figure 2). Maximum toxin level was found at 20-24 h and the level remained high over 60 h (Figure 1). It decreased significantly at 72 h, perhaps indicating some protease activity. As toxin

levels were relatively compared the band intensity in the same blot, there were some limitations of the semi-quantitative Western blot estimation related to color fading and a difficulty in comparison between runs. Even bioassays involve a time consuming procedure, and are not practical for monitoring activity of toxin throughout the whole fermentation, they are still needed to confirm bioactivity. Thus bioassays were carried out to determine larvicidal activity at 7, 13, 22, 30 and 36 h. Cells from the batch culture at 7 and 13 h showed LD_{50} values of 2.09 x 10^4 and 6.02 x 10^3 cells/ml, respectively, indicating increasing toxin formation. LD_{50} values were not significantly changed from 22 to 36 h, with a range between 2.86-3.11 x 10^3 cells/ml. These values are 10-fold higher than the initially measured values (2.31 x 10^2 cells/ml). It is possible that the metabolic burden of high levels of toxin synthesis in T2 strain resulted in strain instability and decreased expression, such that toxicity was reduced significantly.

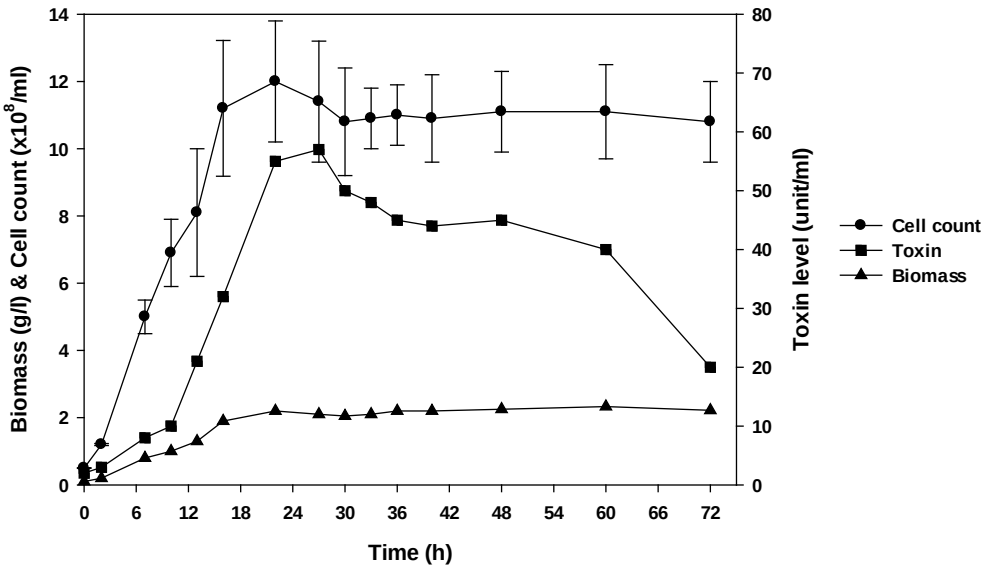

Figure 1 Growth curves and toxin levels estimated by toxin scanning from Western blot, in batch fermentation established in LB medium containing 20 μg/ml Tc and 0.5 % xylose (T = 37 °C, pH = 7.0)

The major problems in exploiting heterologous hosts encoding the larvicidal toxin genes are low expression levels of toxin and low stability of the recombinant strains. Among various heterologous hosts expressing larvicidal toxin genes (based on LD_{50} values expressed as cells/ml) the most toxic recombinant strains with the expression levels of toxicity comparable to Ls 2362 were those of *Asticcacaulis excentricus*. Based on LD_{50}

values expressed as dry weight/ml, the highest toxicities strains were demonstrated in *B. subtilis* BD104 (Broadwell *et al.*, 1990b), *E. coli* GM2199 (Šebo *et al.*, 1990) and an *E. coli* MS16 clone (Shamugavelu *et al.*, 1997), with LD_{50} values of 6, 10 and 19-24 ng/ml, respectively. This compares with Ls 2362 with an LD_{50} of 14-18 ng/ml (Baumann *et al.*, 1991).

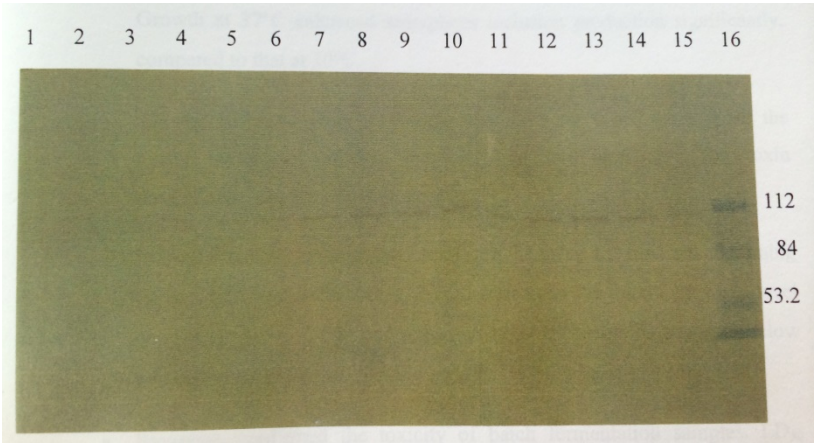

Figure 2 Western blots of cell lysates from batch fermentation of T2 sampling at different times after subjecting to cell lysis, 1-15: lysates of samples collected at 0, 4, 7, 10, 13, 16, 22, 27, 30, 33, 36, 40, 48, 60 and 72 h, respectively, 16: prestained marker proteins, 112, 84 and 53.2 kDa, respectively.

In the present study, the spontaneous mutant of Bm pWH1520TK selected from Km gradient plates, was found initially to have an LD_{50} of 2.31 x 10^2 cells/ml and finally increased up to an 3.11 x 10^3 cells/ml after preliminary batch fermentation, indicating unstable high level expression of the toxin gene. Later studies in batch fermentation in the absence of Km have shown a change of LD_{50} values of the mutant back to the original values before mutant selection on Km gradient condition. Low stability of recombinant mosquito larvicidal strains has been generally reported in other strains under non-selective conditions. This is probably due to plasmid loss. Stability under non-selective conditions is a challenge to study since selection conditions would not exist in the natural environments of mosquito breeding sites. Moreover, it is also indicated that the use of plant-associated microorganisms expressing insecticidal toxin genes under field conditions may be a more practicable approach with an extended stability.

Preliminary batch fermentation studies with T2 showed, by means of SDS-PAGE and Western blot analysis, that the fusion toxin gene was expressed at relatively high levels for at least 60 h with no apparent degradation of the fusion gene product. For definitive analysis, a quantitative and more reliable method is

needed to monitor toxin production during fermentation, since some difficulties arise in comparing the intensity of different blots during scanning. Alternatively, to obtain more accurate results for comparison with other reported data, a purification protocol for recovery of toxin protein from cell lysates prior to determination of toxin level by the developed ELISA technique is suggested.

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