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# Characterization of Novel *Bacillus thuringiensis* Isolated from *Attacus atlas* and Its Growth Kinetics in the Cultivation Media of Tofu Whey for Bioinsecticide Production

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**Abstract**
*Bacillus thuringiensis* that produces endotoxins, can serve as active ingredients of biopesticide. *Bacillus thuringiensis* that previsiously have been isolated from carrion worm of *Attacus atlas*. The characteristics of *Bacillus thuringiensis* isolates isolated from carrion worm of *Attacus atlas* from Bogor-Indonesia are gram-positive, motile, punctiform, white and slimy, long and round vegetative cells with a length of 1720 nm and a width of 836 nm. The protein crystals yielded are spherical, with a diameter of 770 nm. Genomic of DNA amplified using PCR with universal primers for 16S rRNA showed maximum similarity of 95% with a few lines of *Bacillus thuringiensis*. Philogenetikc tree reconstruction shows the isolates are in the same cluster with *Bacillus thuringiensis*. The kinetics growth of *Bacillus thuringiensis* on tofu whey as media results in parameter N-max 10.51 log CFU/mL, P-max 8.67 log CFU/mL, $\mu_N$-max 0.052 $h^{-1}$, and $(S_0\text{-St})/S_0$ 27.57%. Toxicity calculation on second instars larvae of *Aedes aegepty* from the cultivation broth at the 24$^{th}$ hour yielded $LC_{50}$ values of 0.7 mg/mL and product potency of 2142 IU/mg.
**Keywords:** *Bacillus thuringiensis,* isolation, characteristics, kinetics, toxicity

## 1. Introduction
Various types of *Bacillus thuringiensis* species isolates are well known as sources for bioinsecticide. *Bacillus thuringiensis* is Gram-positive and rod-shaped bacterium, and able to live in facultative anaerobic (Dulmage *et al.*1990). *Bacillus thuringiensis* has many subspecies beneficial to overcome the lepidoptera, coleopteran or diptera (Federici *et al.,* 2010).
The development of local bioinsecticide product are still considerably potential, particularly the use of the active ingredient of *Bacillus thuringiensis*. This is supported by the abundant and affordable local strains and materials for agro-based medium. One of those is tofu whey, that is waste generated out by tofu factories. The utilization of the tofu whey is still very limited and it is more likely dumped into the river which lead to river pollution. Tofu whey contains carbon, nitrogen and minerals that can be used for cell medium, while the Ca content in the effluent can be used to induce the formation of *Bacillus thuringiensis* spores.
The different geographical conditions depict genetic differences and toxicity of *Bacillus thuringiensis*. From every habitat there is a possibility of obtaining new isolates with more effective and potential toxicity. Therefore a lot of research has been done to get local *Bacillus thuringiensis* strains from different environments and different origins such as from soil, grain, dust, animal feces, straw and the trunks of trees, as well as different locations, with a view to acquire a new strain of *Bacillus thuringiensis* with high toxicity and wider target insects (Apaydin 2004; Patel *et al.* 2009).
Commercial products with active ingredients that are produced by *Bacillus thuringiensis* are already widely used, however the study in order to isolate and identify *Bacillus thuringiensis* is still underway, because there are still many pests that cannot be controlled by using the existing toxins. So far, the insecticide products used are imported ones. As bioinsecticide is biological product, there is a possibility of not being in accordance with conditions in Indonesia. Thus, it is necessary to find potential local isolates. The use of local isolates will also reduce the cost of production because it does not have to pay a license fee. In addition, new strains are also required to provide an alternative when insect pests' resistance against particular *Bacillus thuringiensis* strains emerges.
The isolation was using the carrion worm of *Attacus atlas*, because the caterpillars have a large size, do not die when eating the leaves which have the nature to kill insects. Therefore, the active substance produced by the bacterium is expected not only effective to kill the caterpillar but also other insects.
This study aimed to isolate and characterize the bacteria from carrion worm *Attacus atlas*, to study the kinetics growth of these isolates on tofu whey as media and to examine its toxicity to larvae of *Aedes aegepty*.

## 2. Materials and Methods
Carrion worm of *Attacus atlas* was obtained from Bogor, Indonesia. Larvae *of Aedes aegepty* from Pest Control Studies Unit, Faculty of Veterinary Medicine, Bogor Agricultural University was used toxicity test. Tofu whey

 

(traditional tofu industry) and nutrient broth (Merck Millipore) was used as media cultivation.

*2. 1 Isolation of Bacteria*

Isolation method used is a modification of the method done by Xavier *et al.* (2007). Intestinal fluid of carrion worm of *Attacus atlas* was introduced into a 250 ml conical flask containing distilled water. The sample was homogenized on an orbital shaker at 250 rpm. One mililitre aliquot was taken from the sample and subjected to heat shock at 80 °C for 15 minutes. The sample was than serially diluted and plated onto nutrient agar. The plates were incubated at 30 $^{o}$C for 2 days. Appeared colonies are transferred to sporulation medium *Bacillus thuringiensis* and isolated.

*2. 2 Morphological Characterization*

Microscopic characterization observed were colony form, color, shape of the cell, spore forms, crystalline form, the size of the cell, spore size, and the size of the crystal. Cell shape, form of spores, crystal shape and size were observed using a scanning electron microscope (Phenom proX).

*2. 3 Identification of Bacteria*

Bacterial DNA was extracted by cetyl trimethyl ammonium bromide (CTAB) following the method of Sambrook and Russell (2001). The next stage was, the result of the DNA isolation was electrophoresed on 1% agarose gel for 45 minutes. After the migration process was complete, agarose gel was soaked in EtBr (Ethidium Bromide) for 20 minutes, then soaked in distilled water for 10 minutes. In the end, the agarose gel was observed on transilluminlator UV exposure, and documented using Geldoc.

The bacteria were amplified in 16S rRNA region using the forward primer 63F: 5'CAG GCC TAA CAC ATG CAA GTC'3 and reversed 1387R: 5'GGG CGG WGT GTA CAA GGC'3 (Marchesi *et al.* 1998). Conditions Polymerase Chain Reaction (PCR) was set as follows: pre-PCR 94 $^{o}$C for 5 minutes, followed by cycles of denaturation 94 $^{o}$C for 30 seconds, annealing 50 $^{o}$C for 30 seconds, elongation at 72 $^{o}$C for 1.5 minutes, which was repeated 30 cycles, and post-PCR at 72 $^{o}$C for 5 minutes, followed by 15 $^{o}$C for 20 minutes.

The sequence of DNA constructing the 16S was analyzed by using a DNA sequencer ABI Prism Model 310 version 3.7. Further sequencing results were analyzed and the homology was determined with species that have existed in Gen Bank using the program Clustal W Bioedit and the Basic Local Alignment Search Tools (BLAST) with the aid of the NCBI site (http://www.nebi.nlm.nih.gov/ blast) (Altschul *et al.* 1997).

The phylogenetic tree was developed by applying the MEGA 5.05 program (Tamura *et al.* 2011). The sequenced DNA and the DNA sequences from various species existing in the Gene Bank which was going to be analyzed were saved as FASTA files using Bioedit software. Later, the file was opened by applying the MEGA 5.05 program, then the data were aligned by selecting Align ClustalW. The kinship analysis was done to the aligned data analyzed kinship aligned by selecting data and phylogenetic analysis based on neighbor-joining tree (NJT).

*2. 4 Cultivation Process*

The tofu whey was characterized in advance by analyzing carbon, nitrogen, moisture, ash, fat, protein content (AOAC, 2005) and minerals (Fe, Mg, Mn, Zn, and Ca)(APHA, 2005). Characterization of materials was required for the formulation of media.

*Bacillus thuringiensis* cultivation process was done with batch system in 250 ml erlenmeyer flasks at 28-32$^{o}$C, initial medium pH of 6.8-7.2, a medium volume of 50 ml. After the media had been sterilized by autoclave and cooled, the media later were inoculated with 10% starter (v/v), the speed of agitation was 150 rpm. The growth was observed at specified intervals, ie at 0, 4, 8, 12, 16, 20 and 24 h. The cultivation media were tofu whey (TW), tofu whey plus urea (the urea addition was formulated according to the appropriate C/N ratio of nutrient broth concentration of 8 g / liter) (TWU) and nutrient broth concentration of 8 g/liter (NB).

*2. 5 Parameter Analysis*

The measurement of pH, total carbohydrate content was done during the cultivation process (Masuko *et al*., 2005). The measurement of cell growth used the TPC (Total Plate Count), and the measurement of spore was conducted by determining the number of live spores (Viable Spore Count). The parameters measured to determine the efficiency and productivity of the production process were the number of cells (N), number of live spores (P), the amount of substrate consumed by looking at the levels of carbohydrate (S), the maximum specific growth rate ($\mu_{max}$), substrate utilization efficiency ( S/So) (Wang *et al.* 1979).

The toxicity of the bioinsecticide was determined from the 24-hour cultivation, using a bioassay method conducted by Rahayuningsih (2003) in testing there was as much as 1 ml of cultivation liquid in 10 ml of distilled water which then performed serial dilutions. A total of 10 larvae second instar *Aedes aegepty* were inserted in the tube. The count of the dead larvae amount was performed after 24 hours. The $LC_{50}$ value was determined by using Probit Quant program analysis. Potency of samples were calculated by comparing them to the commercial product belong to Vektobac as standard. According to Dulmage *et al* (1990) the potency bioinsecticide is calculated by the formula below:

$$\text{Potency of sample} = \frac{\text{LC50 standar}}{\text{LC 50 sampel}} \text{ x standard potency } (\text{IU/mg})$$

 

## 3. Results and Discussion

### *3.1 Characteristic*

Bacteria isolated from carrion worm of *Attacus atlas* was only one type isolate. Its characteristics are punctiform, white and slimy. Vegetative cell shows long and round shape, with a length of 1720 nm and a width of 826 nm, more than one flagella in every vegetative cell, the crystalline proteins are round. The crystal diameter is approximately 753 nm and the spores diameter is approximately 654 nm. The observations result using SEM is shown in Figure 1.

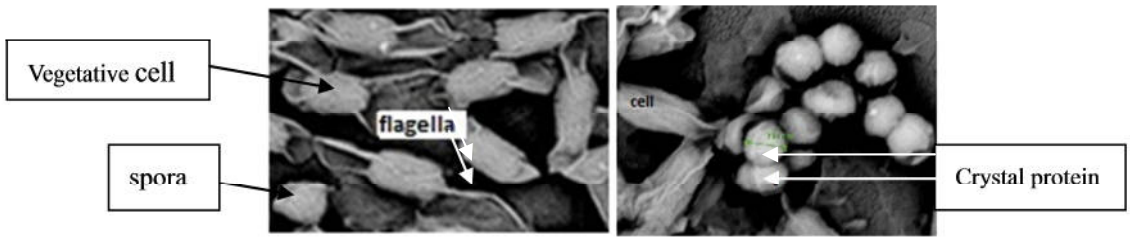


Figure 1. Morphology of cells

Gram staining resulted in blue color showing the bacteria is Gram-positive one. This is consistent with the results of the reconstruction of the philogenetic tree. There isolates showed one cluster with gram-positive bacteria and different cluster with gram-negative bacteria (Figure 3.).

### *3.2 Identification of Bacteria*

Genomic DNA was isolated from *Bacillus* derived from carrion worm of *Attacus atlas*. DNA was isolated by the method of *cetyl trimethyl ammonium bromide (*CTAB) method according to Sambrook and Russell (2001). Subsequent genomic DNA was amplified using universal primers 16S bacterial, obtained sequences long at 1321 bp (Figure 2).

Having been analyzed by BLAST, based on the nucleotide sequence, it was known that these isolates sequence had a 95% similarity with 16S RNA of several lines ribosoma *Bacillus* (Table 1). These isolates had maximum identicality value <97% with E-value 0.0, so that the isolates are thought to be novel species (Stackebrandt and Goebel, 1994), with similarities colony and microscopic morphology, as well as the proximity of the 16S rRNA gene sequences were approaching the species.

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AGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCA
TAAGACTGGGATAACTCCGGGAAACCGGGGGCTAATTCCGGAATACTTTTTGAACTTCT
TGTTTAAAAATGAAAGGGCGGTTTCGGCTGTCATTTAGGGAGGGCCCCGGTTCGCCATTA
GTTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGT
CGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGG
TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGT
GAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGA
GGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGG
CGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCC
GCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
AGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTT
CTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGGTTAAGTCCCCCAACGAGCGCAACCCTTGATCTTATTTGCCATCATTTAATTTTG
GCACTCTAAGGTGACTGCCGGTGACAAACCGAAGAAAAGGTGGGGATGACGTCAAATAAT
CATGGCCCCTTATGACCTGGGTTAACACCGTGCTCCAATGGACGGTTCAAAAGACTTCAA
AACCCCCAGGTGGAGCTAATCTCTAAAAACCGTTCTCCTTTTCCAATTGTAAGGTCGC
AACTCGCCCCACTGAAGCTTGGAATCCCTTTTAATCCCGGA
```

Figure 2. Nucleotide sequence of the DNA fragment of the 16S rRNA

 

Table 1. Results of BLAST 16S rRNA gene sequences of new isolates

| **Comparison Strain (*Database GenBank*)** | **Maximum Identicality** | **Accession** |
|---|---|---|
| *Bacillus thuringiensis* Bt 407 | 95 % | NR.102506.1 |
| *Bacillus thuringiensis* strain IAM 12077 | 95 % | NR 043403.1 |
| *Bacillus mycoides* strain 273 | 95 % | NR 036880.1 |
| *Bacillus weihenstephanensis* KBAB4 strain KBAB4 | 95 % | NR 074926.1 |

The results of phylogenetic tree construction applying NJT method with bootstrap 1000x also showed the consistency that the isolates are novel species. The isolates were in the same cluster as *B.thuringiensis* 407 Bt, *B. thuringiensis* strain IAM 12077, *B. mycoides* strain 273, *B. weihenstephanensis* KBAB4 KBAB4 strains, This isolate is in genus *Bacillus*. Seen on a phylogenetic tree that is separated from the cluster of Pseudomonas aeruginosa (Gram negative bacteria) (Figure 3).

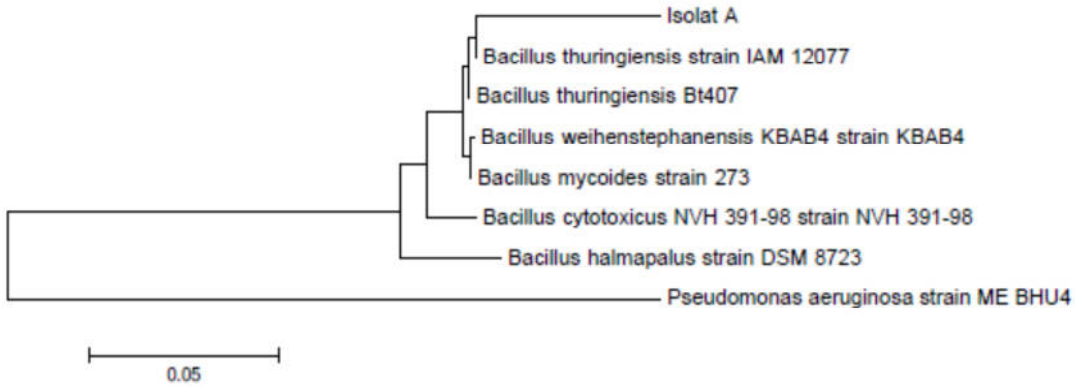


Figure 3. Phylogenetic tree of 16S rRNA gene sample isolates (isolate A)

### *3.3 Characterization of Media*

Media is one of the most influential factors on the cultivation of *Bacillus thuringiensis* in generating δ--endotoxin. To grow and form spores, *Bacillus thuringiensis* requires carbon, nitrogen and minerals (Ca, Fe, Mn, Mg and Zn) sources. In the production of protein crystals, calcium takes a significant role. Cultivation of *Bacillus thuringiensis* in media containing calcium salts produced more increasing protein crystals (Foda et al. 1985). In this study, a commercial nutrient broth medium (nutrient-rich media) is used as a comparison. The results of the analysis of the chemical composition of the medium used for cultivation is presented in Table 2.

Table 2. The results of chemical analysis of nutrient broth and tofu whey

| Component | Nutrient Broth (8g/L) | Tofu whey |
|---|---|---|
| Water (%) | 99.28 | 99.20 |
| Ash (%) | 0.09 | 0.20 |
| Nitrogen (%) | 0.06 | 0.05 |
| Carbon (%) | 0.08 | 0.27 |
| Calcium (ppm) | 17.12 | 249 |
| Iron (ppm) | 0.48 | 5.30 |
| Mangan (ppm) | 0.24 | 0.02 |
| Magnesium (ppm) | 0.72 | 31.00 |
| Zing (ppm) | 5.12 | 2.40 |

The ratio of carbon and nitrogen in nutrient broth was 1.3: 1, while tofu whey containing carbon and nitrogen at the ratio of 5.4: 1. Lack of nitrogen can be made up by adding urea (Stanbury and Whitaker, 1984). While the minerals contained in tofu whey are quite complete as necessary for the production of δ-endotoxin like media compositions performed by Dulmage *et al.* (1990).

### *3.4 Cultivation Kinetics*

The growth and product formation during the cultivation process were observed through changes of cultivation liquid pH, measurement of cell amount, and measurement of spore formation as well as substrate consumption based on total carbohydrate as seen in Figure 4. In general *Bacillus thuringiensis* grew well in the media developed, as seen on the growth of *Bacillus thuringiensis* and the duration of cultivation which shows a positive correlation the longer cultivation time up to 24 hours, the growth of *Bacillus thuringiensis* measured by

 

TPC and VSC also increased.

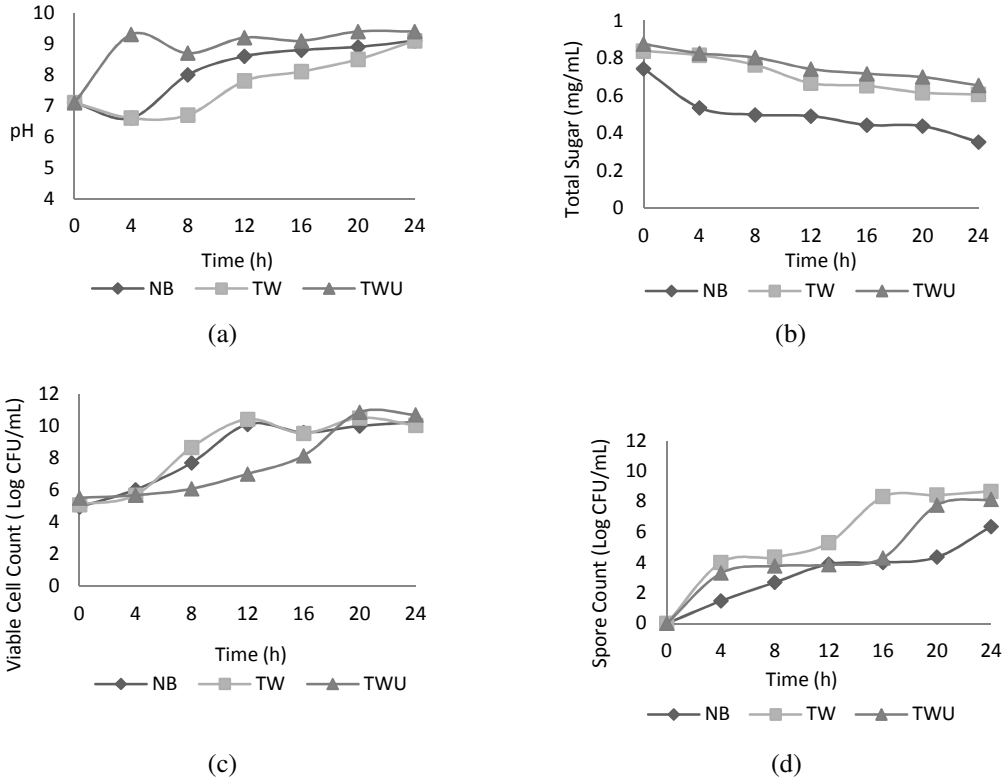


Figure 4. Evolution of pH, Total Sugar, Total Viable Cell Count, and Viable Spore Count over Cultivation Time by using Media Nutrient Broth, Tofu Whey and Mix Tofu Whey and Urea

Changes in pH during cultivation are described in Figure 4a. The pH in TWU media showed a striking increase caused by the decomposition of urea into ammonia due to metabolis process, as *Bacillus thuringiensis* has urease (Sneath, 1986) so that the pH rose to 9.3. This condition resulted in a medium condition which is not optimum for the growth of *Bacillus thuringiensis*. The optimum pH condition for *Bt.* growth according to Benhard and Utz (1993) is 5.5-8.5, so that cell growth is slower than that in NB and TW media. In the other hand, in NB and TW media, during the cell exponential phase, *Bacillus thuringiensis* cells consume carbohydrates to produce organic acids such as lactic acid, pyruvic acid and acetic acid, causing the pH drops.

Figure 4b was changes of carbohydrate consumed during the cultivation process described. Visible reduction in carbohydrate occuring in NB media is faster than that in TW and TWU media because the NB media containing that can be easily consumed by microorganisms. While in the TW and TWU some part of carbohydrate is in the form of oligosaccharide sugars so that it needs to be decomposed first by the amylase into monosaccharides to be able to consume by *Bacillus thuringiensis*.

Consecutively, the highest numbers of cells in nutrient broth medium (NB), tofu whey (TW) and tofu whey urea (TWU) are 10.25 log CFU/mL, 10.51 log CFU/mL and 10.86 log CFU/mL. In addition, the highest amount of spores in a row for NB media, TW and TWU are 6.36 log CFU/mL, 8.67 log CFU/mL and 8.14 log CFU/mL. The data showed the best cell growth is in TWU compared to TW and NB media. This may indicate the cells grow well in TWU as it contains carbon and nitrogen with equal ratio to NB, but with higher mineral content. As for higher spore production on TW and TWU media, it is because the media have conducive mineral content to produce spores especially the presence of high calcium compared to that of the NB.

The calculation result of kinetics cultivation is attached in Table 3. In nutrient broth medium, the efficiency of substrate($\Delta$ $S/S_0$ ) of 52.55 % shows how much carbohydrate that is utilized. In tofu whey medium, the efficiency value of substrate utilization ($\Delta$ S/S0) was 27.57%. Moreover, in tofu whey plus urea medium, the efficiency value of substrate utilization is ($\Delta$S/S0) 25.14%. The efficiency value of substrate utilization was still not optimal. To improve the efficiency of substrate utilization, proper mixing processes need to be considered because the transfer would affect the substrate distribution evenly, resulting in a better metabolism.

 

Table 3. *Bacillus thuringiensis* cultivation kinetics parameters on nutrient broth medium, tofu whey and tofu whey urea

| Parameter | Nutrient Broth | Tofu whey | Tofu whey + Urea |
|---|---|---|---|
| N-max (LogCFU/mL) | 10.25 | 10.51 | 10.86 |
| P-max (LogCFU/mL) | 6.36 | 8.67 | 8.14 |
| $\mu_N$-max ($h^{-1}$) | 0.045 | 0.052 | 0.025 |
| $(S_0\text{-St})/S_0$ (%) | 52.55 | 27.57 | 25.14 |

Table 3. shows that the NB medium produces spores lower than TW media and TWU. According to Pearson and Ward (1988) medium containing high protein like NB can inhibit sporulation due to the influence of nitrogen catabolite repression.

*3.5 Bioinsecticide Toxicity Test*

The product toxicity against larvae of *Aedes aegepty* is presented in Table 4. The toxicity test showed the δ-endotoxin was in line with the formation of spores. It can be seen that the media (tofu whey) that produces the highest number of spores also has the highest toxicity values, whereas the nutrient broth with lower spores also has low toxicity values. According to Devi *et al.* (2005), not only cultivation conditions and isolates used, but also the media will affect the toxin production.

Toxicity product against the target insect is highly dependent on the amount of crystal protein (δ-endotoxin) produced during the sporulation process takes place. It was also in accordance with those reported by Morris and Converse (1996) that the number of viable spore count is not always linearly correlated with toxicity. One of the factors that determined the high toxicity is the potential of the crystal protein crystals which can be seen from the composition of proteins.

Table 4. The influence of the media type of toxicity

| Medium | $LC_{50}$ ( g/mL) | Potency (IU/mg) |
|---|---|---|
| Nutrient broth | 2.38 | 63 |
| Tofu Whey | 0.07 | 2142 |
| Tofu whey + urea | 0.97 | 155 |
| Standard | 0.01 | 15000 |

## 4. Conclusion

A new isolate was obtained from the isolation of carrion worm of *Attacus atlas*. This isolate is *Bacillus thuringiensis.* It has the characteristic was the shape of colony punctiform, white and slimy, more than one flagella in every vegetative cell. The bacterium produces a spherical crystalline protein.

The growth of cells on medium TW similar to the commercial medium (NB) and achieve max faster than TWU medium. TW medium produces the highest spore and ability of toxicity better than NB and TWU media. Tofu whey can be used as a growing medium for *Bacillus thuringiensis* to produce bioinsectiside. Cultivation broth containing cells, spores and *Bacillus thuringiensis* proteins has potency as bioinsecticide to eradicate *Aedes aegepty.*

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