Investigation of low-cost media for *Bacillus thuringiensis* subspecies *israelensis*

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Abstract. *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) has been used as a mosquito control agent in agricultural field and residences. The production of *Bti* by conventional medium was expensive. The objective of this study was to investigate low-cost media for *Bti*'s production with a cost analysis of the medium formulated. Soybean Casein Digest Medium (TSB) was used as a conventional medium to compare with Saberi medium at room temperature (approximately 21.5˚C) on static and agitation (by obituary shaker at 100 rpm). Saberi medium was also modified to vary the percentage of nitrogen and carbon source to observe the growth rate and sporulation, compared to the Saberi medium. The growth of *Bti* in TSB with agitation for 24 hours yielded the highest growth rate. Sporulation of *Bti* in TSB with agitation at room temperature was 6 days which was faster than *Bti* in the Saberi medium. Modified Saberi nitrogen source and carbon source medium could not support the growth of *Bti* due to an imbalance in the percentage of nitrogen source and carbon source. Determination of sugar utilization showed sugar in the medium was utilized mostly in the exponential phase of *Bti*, from 3 hours to 6 hours. The price of TSB was 76.80 Thai baht per liter while Saberi medium's cost was 20.43 Thai baht per liter. In this study, the Saberi medium may not be able to replace TSB in terms of growth rate and period of sporulation. Further study will be needed to optimize the media that use agro-industrial wastes as ingredients following the concept of circular economy and sustainability.

Keyword. *Bti*, Bioinsecticide, Low-cost medium

1 Introduction

Mosquitoes impact human health and good living. They are the vector of transmitting diseases, such as Malaria and Dengue fever. Thailand is a country with tropical rainforest, Asian tiger mosquito (*Aedes albopictus*) is commonly found as a carrier of Chikungunya fever [1]. The solution to mosquito elimination may end up using insecticide, which may contain volatile chemicals that have adverse effects on human health and the environment. An example of a dangerous insecticide is DDT, dichloro-diphenyl-trichloroethane, which was banned in many countries. Other classes of chemical insecticides, such as organophosphates and carbamate were used instead of using DDT [2]. Environmental and health concerns have led to the alternative environmentally friendly bioinsecticide

Bacillus thuringiensis subspecies *israelensis* (*Bti*) has been used in many countries to control the population of mosquitoes [3]. Many studies demonstrated that *Bti* is environmentally safe and target-specific bioinsecticide. *Bti* targeted three main insect larvae: mosquitoes, black flies, and fungus gnats [3]. Protoxins of *Bti* is produced in the stationary phase when endospore formation takes place. After ingestion of toxins, protoxin in form of crystals, including Cry (crystal) and Cyt (cytolytic) toxins that are solubilized in the alkaline condition in the larva's gut, which is activated by midgut protease. Cry toxins bind to a protein receptor on the outer membrane of gut cells [4], while Cyt toxins bind to the phospholipids [5]. The function of the gut is disrupted, causing larval death from starvation [4]

The *Bti* production for mosquito control using laboratory media is expensive. Previous work by Saberi (2020) [6] optimized the culture media for the production of *B. thuringiensis* var. *tenebrionis*-BN1 using molasses from sugar beet and sugar cane as a carbon source while using corn steep liquor as a nitrogen source. The maximum CFU of 10^7 spore/ml was achieved with the amount of carbon source above 11% and nitrogen source less than 2% [6]. For *Bt,* the media that support the growth and sporulation consists of: glucose, 5 g/L; glycerol, 1 g/L; sodium acetate, 0 g/L; $(NH_4)_2SO_4$, 1 g/L; corn steep liquor, 2 mL/L; yeast extract, 1 g/L; hydrolyzed casein, 1 g/L; $CaCl_2$, 20 mg/L; $MgSO_4$, 50 mg/L; $MnSO_4$ 100 mg/L provided the growth 6.4 x 10^{-7} cell/mL and 99% of sporulation [10].

In this study, the use of sugar cane molasses and corn steep liquor which are agro-industrial waste were explored. These wastes are used in many industries, such as supplements of livestock's feed, to reduce the production cost [11]. The compositions of sugar cane molasses and corn steep liquor provide carbon and nitrogen source which can be used to promote bacterial growth. Hence, the use of both sugar cane molasses and corn steep liquor to formulate media for the production of *BTi* will enhance the circle of material usage following the concept of circular economy and sustainability.

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2 Materials and methods

2.1 Determination of kinetic growth of *Bacillus thuringiensis* subspecies *israelensis* in conventional medium

Soyabean Casein Digest medium TSB (HIMEDIA) was prepared for 300 mL per batch in triplicate. The *Bti* was subcultured in modified TSB [6] overnight as inoculum. Three percent (v/v) of *Bti* inoculum was added to the TSB and incubated at room temperature (approximately 21.5˚C) under shaking (100 rpm) and static conditions (Figure 1). The optical density was measured using UNICO 1200 spectrophotometer at wavelength 600 nm in triplicate to observed growth. The development of the endospore in *Bti* was observed by the standard endospore staining technique.

2.2 Preparation of Saberi medium and modified Saberi media

The production medium was prepared according to the formula from Saberi *et. al.* (2020) [6] and called as Saberi medium (Table 1). Corn steep liquor, NQ. QCA 3051/21, was sponsored by Friendship Corn Starch Company Limited. The solid content in the corn steep liquor was 38.05%. The protein content in the corn steep liquor was 48.46% analyzed by the Kjeldahl method. Sugar cane molasses was purchased from New Friend Farm Company Limited. Saberi medium was modified into 2 formulas: Modified Saberi Carbon Source (MSC) contains 8% (v/v) of sugarcane molasses and Modified Saberi Nitrogen Source (MSN) contains 3% (v/v) of corn steep liquor. Inoculum medium, Saberi medium and modified Saberi media were autoclaved for 10 minutes at 115˚C. The media were prepared in batches of 350 ml.

Table 1: Compositions of Saberi medium and modified Saberi media.

Media	Compositions of media				
	Sugar cane molasses (v/v)	Corn steep liquor (v/v)	$MgSO_4$ (w/v)	KH_2PO_2 (w/v)	K_2HPO_4 (w/v)
Saberi medium (SM) [6]	12.5% (5.33%w/v sugar)	1.5% (0.73%w/v protein)	0.3%	0.3%	0.4%
Modified Saberi carbon source (MSC)	8% (3.41%w/v sugar)	1.5% (0.73%w/v protein)	0.3%	0.3%	0.4%
Modified Saberi nitrogen source (MSN)	12.5% (5.33%w/v sugar)	3% (1.45%w/v protein)	0.3%	0.3%	0.4%

2.3 Culturing in Saberi medium and modified Saberi media

Bti was cultured overnight on the orbital shaker at 30˚C 100 rpm in the modified TSB [6] as inoculum culture. The 3% (v/v) of overnight *Bti* was inoculated in each 350 mL of the media (Figure 1). After the inoculum culture was added to the batch, 1 mL of the media was collected for growth measurement using OD at 600 nm and 9 mL from each treatment were collected from each time point (0, 1, 2, 3, 4, 5, 6 and 24 hour) for further analysis.

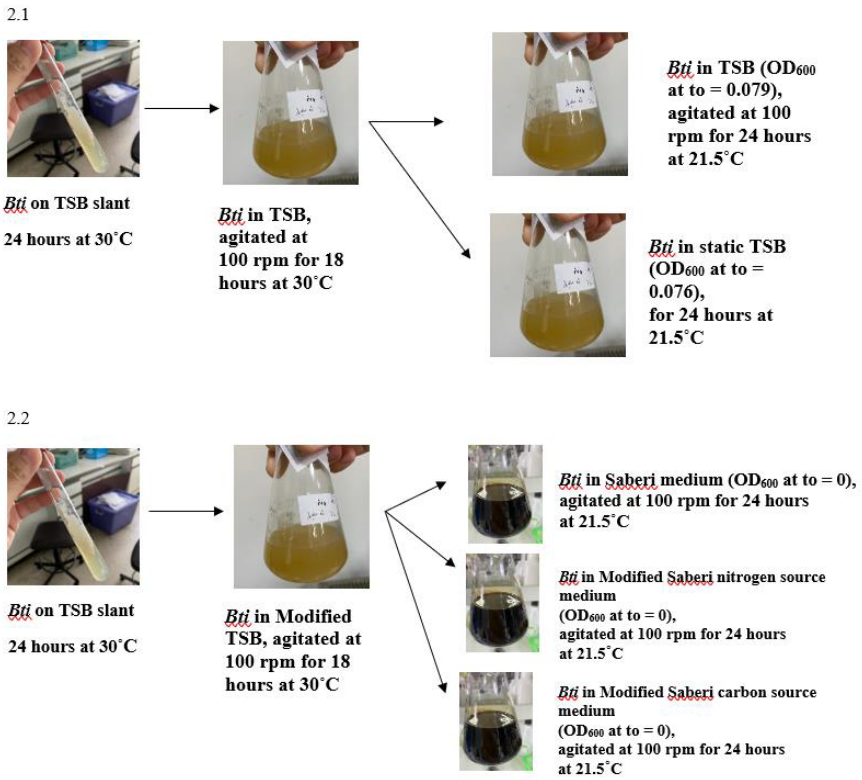

Figure 1 Schematic diagram of this experiment related to sections 2.1 and 2.2

2.4 Determination of growth kinetic of *Bacillus thuringiensis* subspecies *israelensis* in Saberi medium and modified Saberi media

For the growth of the bacteria, 1 mL of sample collected in 2.3 from each timepoint was centrifugated at 8000 rpm for 2 minutes. The supernatant was discarded; the pellet was washed with distilled water and vortexed until the mixtures became homogenized. The washing step was repeated before the mixtures were observed using a spectrometer at 600 nm. The growth curve was constructed and used to compare doubling time and specific growth rate using the following equation (eq 1-2).

Doubling time:

$$t_b = ln\ (2)/B \quad (1)$$

Specific growth rate:

$$\mu = [2.303(\log OD_2 - \log OD_1)/(t_1 - t_2)] \quad (2)$$

2.5 Determination of sugar utilization of *Bacillus thuringiensis* subspecies *israelensis* in Saberi medium and modified Saberi media

The sample was collected and centrifuged to remove solid particles and bacteria cells at 4,000 rpm for 5 minutes. The supernatant was kept at -20˚C for the sugar analysis. For reducing sugar, samples were analyzed using the dinitrosalicylic colorimetric method (DNS) in duplicate. 1 mL of the sample was diluted in 100 mL of distilled water. 3 mL of DNS reagent were added to 3 mL of supernatant in a lightly capped test tube. The mixture was heated at 90˚C for 15 minutes until the red brown color was developed. 1 mL of 40% (w/v) potassium sodium tartrate solution was added to stabilize the color. After cooling to room temperature in a cold-water bath, the absorbance was observed at 575 nm.

For non-reducing sugar, the Association of Official Agricultural Chemists (AOAC) Official method 923.09/2012 Invert Sugar in Sugar and Syrups was used for the preparation of reagents. AOAC Official Method 968.28/2012 [7]. Total Sugar in Molasses as Invert Sugar was used to analyze total sugar content as invert in the sample at each time point (0, 1, 2, 3, 4, 5, 6 and 24 hours). 8 mL of centrifugated sample was used for the total invert sugar analysis. Sugarcane molasses was also analyzed.

3 Results

The growth of *Bti* in TSB with agitation (100 rpm), at room temperature for 24 hours yielded the highest growth of *Bti* compared to other conditions and media (Figure 2). Growth of *Bti* in Saberi medium showed a higher growth rate compared to TSB without agitation at room temperature for 24 hours. When the percentage of nitrogen and carbon source from the Saberi medium was changed in the modified Saberi media, both MSC and MSN, the growth rate of *Bti* were lowered.

Reduction of reducing sugar was observed during the growth of *Bti* in TSB with and without agitation (Figure 3). The amount of reducing sugar in SM, MSC and MSN media however showed a constant level throughout the course of growth. Total sugar as inverted sugar was determined by AOAC Official Method 968.28/2012 in the SM, MSC and MSN during the growth of *Bti*. After 24 hours total sugar in SM and MSC reduced from 12.13% of sugar as inverted sugar to 5.68%. While in MSN, the total sugar was not reduced (Figure 4).

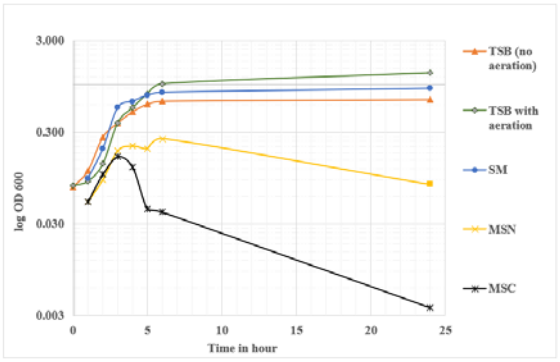

Figure 2. Growth of *B. thuringiensis* subspecies *israelensis* in TSB without agitation, TSB, Saberi medium (SM), modified Saberi carbon source (MSC) media, and modified Saberi nitrogen source (MSN) media with agitation (100 rpm) at room temperature (approximately 21.5˚C), for 24 hours.

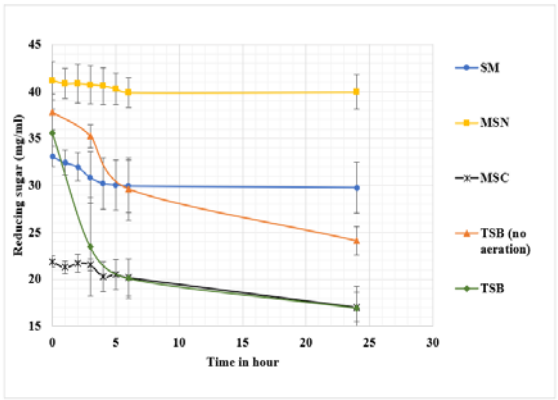

Figure 3. Reducing sugar during growth of *B. thuringiensis* subspecies *israelensis* in TSB without agitation, TSB, Saberi medium (SM), modified Saberi carbon source (MSC) media, and modified Saberi nitrogen source (MSN) media with agitation (100 rpm) at room temperature, for 24 hours.

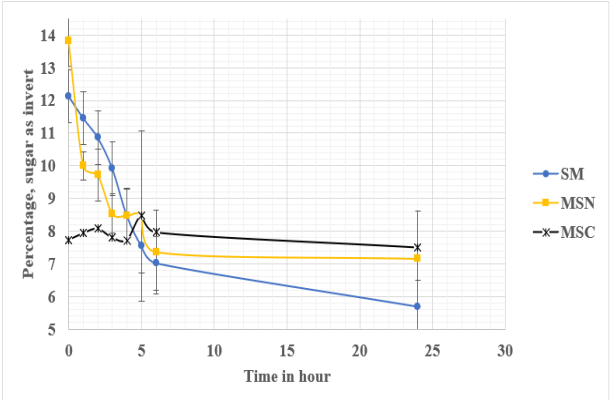

Figure 4. Non-reducing Sugar in Saberi medium (SM) and modified Saberi media (MSN, MSC) with agitation (100 rpm) at 21˚C, room temperature, for 24 hours after additional *B. thuringiensis* subspecies *israelensis*.

4 Discussion

Growth of *Bacillus thuringiensis* subspecies *israelensis* was observed in the TSB medium with and without agitation. The growth rate and reducing sugar utilization were significantly faster when agitation was provided. This result confirmed the requirement of agitation as previously described [6]. The sporulation was also observed in the population of *Bti* that was grown in TSB and found that sporulation was faster in the TSB with agitation (6 days) (Table 2). As the sporulation was associated with the production of the *Bti* protoxin [4,5,6], the media that stimulate the sporulation in a shorter time would be more economically feasible. Hence, the agitation condition was further used to examine the growth and sporulation of *Bti* in the Saberi medium.

Table 2. Kinetic growth of *B. thuringiensis* subspecies *israelensis* in different types of media and conditions.

Types of media	Agitation (rpm)	Doubling time (td) (hour)	Specific growth rate (μ) ($hour^{-1}$)	Sporulation (day)
TSB	-	3.04	0.419	16
TSB	100	1.46	0.440	8
Saberi medium	100	2.20	0.336	14
Modified Saberi nitrogen source	100	3.25	0.214	> 14
Modified Saberi carbon source	100	-	-	> 14

In previous work, different types of carbon sources and nitrogen sources were tested to formulate production mediums that have lower costs [6, 8]. These substrates include sugar beet and sugar cane molasses, sucrose, and potato. The sugar cane molasses and corn steep liquor were chosen to be used in this experiment due to the cost and availability of the raw materials in Thailand.

The Saberi media was chosen as described previously [6], and the amount of carbon source (sugar cane molasses) and nitrogen source (corn steep liquor) were varied to investigate the effect of these nutritional sources on the growth and sporulation of *Bti*.

Growth of *Bti* in TSB with agitation at room temperature for 24 hours shows the highest growth rate compared to all media tested. The doubling time of *Bti* on TSB with agitation (1.46 hours), was faster than the doubling time of *Bti* on TSB without agitation (3.04 hours) (Table 1). Growth of *Bti* in Saberi medium showed the doubling time of 2.20 hours, faster than *Bti* on TSB in static conditions. The Saberi medium consists of 12-14% (w/v) of sucrose, the bacteria can use this non-reducing sugar for growth. The sporulation of *Bti* that grew in the Saberi medium took place after 14 days. Modified Saberi carbon source (MSC) medium was formulated based on the utilization of total sugar demonstrated in the growth with Saberi medium. The total sugar as inverted sugar was reduced from 12.13% to 7.83% in the MSC medium. However, the MSC medium could not sustain the growth of *Bti* as the growth rate was reduced after 6 hours (Figure 2). Modified Saberi nitrogen source (MSN) medium consisting of 3% (v/v) of corn steep liquor was formulated and able to support the growth of *Bti* for a short period. The highest growth was reached within 6 hours. The increased percentage of nitrogen source in the modified Saberi nitrogen source medium could not sustained the growth of *Bti* which was corresponded to previous study in *B. thuringiensis* var. *tenebrionis*-BN1 [6]. In addition, the sporulation could not be observed in the *Bti* that grew in MSC or MSN medium within 14 days. The effect on the growth demonstrated in MSC and MSN medium may be caused by imbalanced carbon and nitrogen ratios. A carbon per nitrogen ratio of 7:1 is effective to support the growth of *Bti* and yields a high rate of Cry production about 150 g/L [9]. The development of the endospore on *Bti* and the doubling time is related. If the media are optimum for *Bti*, *Bti* will reach the stationary faster and have a shorter doubling time and shorter development of endospore.

DNS method was used to analyze reducing sugar to address sugar utilization of *Bti* in different media. The reducing sugar in TSB was utilized by the growth observed (Figure 2 and 3). *Bti* in TSB with agitation utilized reducing sugar from 35.61 mg/mL to 16.96 mg/mL while *Bti* in static TSB utilized from 37.80 mg/mL to 24.09 mg/mL with 24 hours. The reducing sugar observed from the growth of *Bti* in SM, MSC and MSN medium did not change over time, while the growth was observed hence indicating utilization of the other carbon source. Total sugar in SM, MSC and MSN was analyzed to monitor the utilization of non-reducing sugar (Figure 4). As *Bti* was able to utilize sucrose [8], the utilization of total sugar correlated with the growth of the *Bti*.

The cost of the medium was analyzed in which the TSB medium cost 76.80 Thai baht per liter, while the Saberi medium cost 20.42 Thai baht per liter. However, when considering the growth rate and the sporulation time, TSB despite the higher cost still has advantage of shorter operation time. Further optimization is needed to formulate a medium that is more feasible economically. Different carbon and nitrogen sources may be explored. In addition, other parameter like temperature, pH and agitation will also need to be optimized.

5 Conclusion

The Saberi medium (SM) formulated with 12.5% (v/v) sugar cane molasses and 1.5% corn steep liquor with supplementary was able to use for the production of *Bacillus thuringiensis* subspecies *israelensis* (*Bti*) when grown at room temperature (21.5˚C) with agitation (100 rpm on rotary shaker) with 14 days to sporulate. Despite the cheaper cost of 20.42 Thai baht per liter of SM compared to 76.80 Thai baht per liter of TSB, further optimization is needed to formulate a more feasible

media for the production of *Bti* as bioinsecticide. The utilization of agro-industrial wastes as the main ingredients of the production media would increase materials recycling ability that meets the concept of circular economy and sustainability.

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