Solid-state Fermentation of Kitchen Waste for Production of *Bacillus thuringiensis*-based Bio-pesticide

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In this work, kitchen waste was used as substrate for bio-pesticide production by solid-state fermentation. It was assessed to be well suited for the growth of *Bacillus thuringiensis* in the experiments. The culture medium contents were optimized by an orthogonal test. The optimum mixture was 55.21% kitchen waste, 22.08% wheat bran, 11.04% soybean cake power, 11.04% grain hulls, and 0.63% mixed ions. In the optimized conditions, a spore count of 5.01×10^{10} CFU/g and entomotoxicity of 15200 IU/mg were obtained after 48 h fermentation, while 2.51×10^{10} CFU/g spore count and 12900 IU/mg entomotoxicity were obtained from the conventional medium. Oil and salt had few adverse effects on *Bacillus thuringiensis* growth, yield of spores, and toxicity when the concentration of oil and salt were controlled below 10% and 0.4% to 1.2%, respectively. Fermentation medium of 35 kg was successfully used to produce bio-pesticides from solid-state fermentation in a scale-up experiment. Therefore, the present study proved the feasibility of using kitchen waste for the production of bio-pesticides. It seemed to be a promising alternative to conventional media to reduce costs.

Keywords: Bacillus thuringiensis; Kitchen waste; Solid-state fermentation; Bio-pesticides

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INTRODUCTION

Kitchen waste is a kind of municipal refuse that contains high contents of moisture and organic compounds. It has been utilized as an ideal substrate for resource utilization (Wang *et al*. 2002; Lee *et al*. 2009; Wang *et al*. 2008; Mi-Hyung and Jung-Wk 2010). Kitchen waste is discharged from households, cafeterias, and restaurants, and accounts for a considerable proportion of municipal solid garbage in China (Wang *et al*. 2008). Over the last few decades, interest in uses of kitchen waste for methane fuel, hydrogen, and polyhydroxyalkanoate (Naves *et al*. 2004; Shin and Youn 2005; Du and Yu 2002) production has increased due to its relative abundance, renewable nature, and availability as an almost zero cost substrate (Yan *et al*. 2011). However, as the population's standard of living improves, the amount of kitchen waste also increases. For example, according to statistical data in 2004, over 1300 tons of kitchen waste was produced per day in Shanghai and over 1000 tons in Beijing (Wang *et al*. 2005). Therefore, the treatment of such an abundance of kitchen waste has become an important factor of urban environmental sanitation. Putting kitchen waste directly into landfills has caused various problems, such as putrid smells and leachate polluting ground and surface waters, while incineration treatment has been restricted due to its low calorific value and the cost of fuel supplements for operation (Yang and Tsai 2002; Yun *et al*. 2000). Hence, it is necessary to find new ways as soon as possible to deal with kitchen waste.

Bio-pesticides derived from *Bacillus thuringiensis* (*Bt*) are the most well-known biological agents for selective control of pest insects (Zhuang *et al*. 2011). However, the high cost of raw materials is one of the key factors hindering their development speed and scale. Recently, some cheap raw materials such as maize starch, wheat bran, rice straw, coconut water, fish meal, feather wastes, hydrolyzed sludge, and wastewater sludge have been successfully substituted for expensive media (Poopathi and Abidha 2007; Satinder *et al*. 2007; Prabakaran *et al*. 2008; Zhuang *et al*. 2011). Kitchen waste is renewable, zero-cost, and is composed of organic material that contains the necessary nutritional elements to sustain growth of microbes (Namita *et al*. 2012; Tsai *et al*. 2007). Using kitchen waste as a raw material for *Bt* production can substantially reduce the production cost of *Bt* and also make a significant contribution to sustainable waste utilization and management.

Submerged fermentation is the traditional production process of *Bt*-based bio-pesticides. However, it is quite difficult to harvest microorganisms from submerged fermentation because of the low concentration of products and complicated downstream processing (Brar *et al*. 2006). In addition, the required equipment (such as high-speed cooling centrifuge and spray dryer) is very expensive (El-Bendary 2006). Compared to conventional submerged fermentation, solid-state fermentation has the advantages of low medium cost and energy consumption, low wastewater output, and high stability of the products (Singhania *et al*. 2009; Holker and Lenz 2005). In recent years, solid-state fermentation has been applied to produce *Bt* and other microbial control agents using agricultural wastes or others as raw materials (El-Bendary 2010), but it has been rarely reported that kitchen waste can be used as a culture medium to produce *Bt*-based bio-pesticides.

The aim of the present study was to investigate the technical feasibility of using low-cost kitchen waste as substrate to support the production of *Bt*-based bio-pesticides with solid-state fermentation technology. Medium components were optimized through an orthogonal experiment. To study the effect of oil and salt on the growth and toxicity of *Bt*, the oil and salt in kitchen waste were applied to *Bt* fermentation in culture media at different concentrations. Using a sequential extraction procedure, the effect of content of oil and salt on *Bt* growth during solid-state fermentation was evaluated. Lastly, the amplificatory experiment of *Bt* fermentation was processed.

EXPERIMENTAL

Microorganisms and Inocula Preparation

Bacterial strain

This work used the bacterial strain *B. thuringiensis subsp. kurstaki* HD-1, which was preserved in our lab. The strain was subcultured and streaked on beef extract-peptone (BP) agar slants (Chang *et al*. 2007). After being incubated for 48 h at 30 ± 1 °C, they were preserved at 4 °C for future use.

Inoculum preparation

All media used for inoculum preparation were adjusted to pH 7.2 before autoclaving. A loopful of *Bt* from the BP slant was used to inoculate a 500 mL Erlenmeyer flask containing 100 mL sterilized BP medium. The flask was incubated in a reciprocating shaker (200 r/min) at 30 °C for 8 to 12 h.

Solid-state Fermentation

Kitchen waste

Kitchen waste was collected from the garbage recollecting station in Beijing and used as a raw material for *Bt* growth in solid-state fermentation. In order to prevent it from spoiling, the kitchen waste was dried and sealed in plastic bags and then stored in a freezer at -20 °C as soon as possible. The kitchen waste was ground into powder and well mixed before use. Total solids (TS), pH, total Kjeldahl nitrogen (TN), total phosphorus (TP), total crude fiber (TC), total reducing sugar (TR), and metals concentration (K, Ca, Na, Mg, Al, and Fe) were determined according to standard methods (APHA 1998).

Reactor for solid-state fermentation

Solid-state fermentation designed in the laboratory was carried out in a 15.0 L autoclavable stainless steel reactor consisting of a mesh screen pallet for medium support. Eight layers of pledget were spread out on the mesh pallet, and the air-intake fittings were located on the bottom of the reactor. In order to prevent contamination during fermentation, the air-intake fittings were attached with filtration and degerming equipment. The reactor and fermentation chamber were completely sterilized, and then the fermentation substrate was sterilized at 121 °C for 30 min and loaded onto the mesh screen pallet. After cooling, the medium was inoculated with an active 15% inoculum (wt%) of *Bt*. The fermentation temperature was 35 °C and supplied with humidified, sterile air at a flow rate of 80 mL/min. After 45 h fermentation, the solid media were collected. After being dried, they were taken for analysis.

Optimization of the Supplement Solution Using Orthogonal Array

An experimental design based on an orthogonal array (Table 1) was formulated to study the effect of different supplement components and their optimal levels for *Bt* production. The four components of the supplement solution, *i.e.*, wheat bran, soybean cake powder, grain hulls, and mixed ions (29% $MgSO_4 \cdot 7H_2O$, 29% $FeSO_4 \cdot 7H_2O$, 42% KH_2PO_4) were varied at four different levels to study their effect. The experimental design and yield values were analyzed using MINITAB 13.30 software.

Table 1. Orthogonal Array Design for Optimization of Supplement Components

Runs	A[b]	B[b]	C[b]	D[b]
1	25	0	25	2.8
2	25	25	50	4.2
3	25	50	75	5.6
4	25	75	100	7.0
5	50	0	25	5.6
6	50	25	50	7.0
7	50	50	75	2.8
8	50	75	100	4.2
9	75	0	25	7.0
10	75	25	50	5.6
11	75	50	75	4.2
12	75	75	100	2.8
13	100	0	25	4.2
14	100	25	50	2.8
15	100	50	75	7.0
16	100	75	100	5.6

The factor codes A, B, C, D refer to wheat bran, soybean cake power, grain hulls, mixed ions
[b] Unit: g/250g kitchen waste

Effects of Salt and Oil on the Growth and Toxicity of *Bt*

As is well known, kitchen waste contains salt and oil, which may affect the growth of *Bt*, so their effects were studied in this work. An active 1% volume inoculum of *Bt* was vaccinated into 300 mL Erlenmeyer flasks containing 100 mL sterilized optimized medium based on kitchen waste. Sterilized soybean oil and salt (NaCl) were added to the medium. The concentrations were 5, 10, 15, 20, 25, and 30 g/L for soybean oil, and 1, 5, 10, 15, 20, and 30 g/L for NaCl. Then the flasks were placed in a constant temperature vibrator where the speed was set to 200 rpm/min, at 30 °C for 48 h. After 48 h, the final spore count and entomotoxicity were evaluated.

Enlargement Experiment

After analyzing the results of the above experimental conditions, the amplificatory experiment of *Bt* HD-1 fermentation was processed. Enlargement experimentation can be used to determine the ability of this method and reactor to be scaled-up, and it was believed to be critical to efficient process development and expected process transfer to a manufacturing facility.

Levels of amplification for the optimized medium were 4 kg, 8 kg, 12 kg, 16 kg, 20 kg, 24 kg, 28 kg, 35 kg, 40 kg, 45 kg, and 50 kg. Then water was added to ensure that the moisture content in medium was 50%. The optimal culture period and temperature were 48 h and 35 °C. After fermentation, the products were analyzed.

Analyses

Spore count

The progress of bio-pesticide production was monitored by measuring the spore count. To determine the spore count, the culture samples were accurately weighed (10.00 g in a 250 mL Erlenmeyer flask), dissolved by 90 mL sterile water, and then agitated in a shaker at 200 rpm/min for 30 min.

The prepared samples were heat treated at 80 °C for 15 min, serially diluted, then plated on the beef extract-peptone (BP) agar plates and incubated at 30 °C for 48 h to form developed colonies. For all counts, an average of at least three replicate plates was used for each tested dilution. For enumeration, the colonies counted on the plates were between 30 and 300 CFU (colony forming units).

Bioassay

The entomotoxicity of the samples were assayed against the third instar larvae of laboratory-reared *Plutella xylostella*. Lyophilized powders of *Bt* produced in different media were prepared in sterile distilled water and applied to clean cabbage leaves, which were placed in boxes, each box containing 30 larvae. Each experiment included 6 doses of three replicates, along with the appropriate control. Larval mortality was scored after 48 h and corrected for control mortality. The test was treated as null if the mortality in the control was more than 10%. The LC_{50} represented the final dilution that killed 50% of the larval population. The results were defined as the following equation and expressed in IU/mg.

$$\text{Entomotoxicity of sample} = \frac{LC_{50}\text{of standard}}{LC_{50}\text{of sample}} \times \text{Entomotoxicity of standard} \quad (1)$$

RESULTS AND DISCUSSION

Composition Analysis of Kitchen Waste

Physico-chemical characteristics of kitchen waste are presented in Table 2. According to the results of the determination of the contents of kitchen waste and compared with the optimum proportion of all ingredients that *Bt* needs to grow, it can be concluded that the nitrogen content of kitchen waste was a little too low, and it was necessary to supplement the raw materials with a nitrogen source. At the same time, K^+, Mg^{2+}, Fe^{2+}, and other trace elements were added.

Table 2. Characteristics of Kitchen Waste

Parameter	Concentration[a] (%)
Total solids (TS)	27.52 ± 0.30
pH	5.63 ± 0.03
Total Kjeldahl nitrogen (TN)	5.21 ± 0.08
Total phosphorus (TP)	0.608 ± 0.012
Total crude fiber (TC)	1.90 ± 0.02
Total reducing sugar (TR)	7.85 ± 0.05
K	0.309 ± 0.001
Ca	1.120 ± 0.02
Na	0.641 ± 0.04
Mg	0.0852 ± 0.001
Al	0.00456 ± 0.0002
Fe	0.00263 ± 0.0001

[a] The present values are the mean ± standard deviation (SD)

Optimization Using an Orthogonal Array

The test and measurement were carried out by the orthogonal experimental method, and a detailed analysis was made on the test data by using the visual analysis and variance of analysis methods. The data are shown in Tables 3 and 4.

In the orthogonal test, *k* is the mean value of every factor and level. By comparing to *k*, the optimal level of variables can be confirmed. The parameter *R*, as defined by Eq. 2, scales the effect of variables on the results.

$$R = k_{max} - k_{min} \quad (2)$$

A high *R* value means that this variable has a strong effect on the result. The optimum scheme was $A_4B_3C_2D_1$, but this combination does not exist in Table 3, so additional experimentation was done. The spore counts were 30.8×10^{10} CFU/g; they were higher than the maximum value (30×10^{10} CFU/g) of the orthogonal test. Therefore, it was suggested to use $A_4B_3C_2D_1$ as the applied scheme. There were 100 g wheat bran, 50 g soybean cake power, 50 g grain hulls, and 2.8 g mixed ions. The fermentation medium contained 55.21% kitchen waste, 22.08% wheat bran, 11.04% soybean cake power, 11.04% grain hulls, and 0.63% mixed ions.

From Table 4, it can be seen that the factors A, C, and D had no marked effect; that is, wheat bran, grain hulls, and mixed ions had no significant impact on the number of spores, while soybean cake powder had a significant impact on the number of spores. Therefore, the best level, as determined by the orthogonal test, was taken as the applied scheme. The result was consistent with the previous result of intuitive analysis.

Table 3. Visual Analysis of the Orthogonal Array Experiments

Runs	A[b]	B[b]	C[b]	D[b]	Spore count(×10^{10})
1	1	1	1	1	7.0
2	1	2	2	2	10.3
3	1	3	3	3	7.3
4	1	4	4	4	7.6
5	2	1	2	3	1.2
6	2	2	1	4	5.3
7	2	3	4	1	18.0
8	2	4	3	2	3.0
9	3	1	3	4	4.8
10	3	2	4	3	2.3
11	3	3	1	2	8.6
12	3	4	2	1	13.5
13	4	1	4	2	2.0
14	4	2	3	1	3.0
15	4	3	2	4	30.0
16	4	4	1	3	13.0
k_1	8.050	3.750	8.475	10.375	
k_2	6.875	5.225	13.750	5.975	
k_3	7.300	15.975	4.525	5.950	
k_4	12.000	9.275	7.475	11.925	
R	5.125	12.225	9.225	5.975	

The factor codes A, B, C, D refer to wheat bran, soybean cake power, grain hulls, mixed ions
[b] Unit: g/250g kitchen waste
k is the mean value of every factor and level

Table 4. Analysis of Variance of the Orthogonal Array Experiments

Factors	Sum of square of deviations	Degree of freedom	F_{ratio}	$F_{critical\text{-}value}$
A(g)	66.082	3	1.000	5.390
B(g)	359.007	3	5.433	5.390
C(g)	177.607	3	2.688	5.390
D(g)	112.447	3	1.702	5.390
Errors	66.08	12		

The factor codes A, B, C, D refer to wheat bran, soybean cake power, grain hulls, mixed ions

Bt Production from Different Solid Culture Media

In order to study the fermented products in different media, conventional medium (CM), kitchen waste only (KW), a mixture of kitchen waste and soybean cake powder (KS), and the medium optimized by orthogonal experiments (OM) were used. The variation of pH, spore count, and entomotoxicity of *Bt* during the fermentation period are illustrated in Figs. 1 through 3.

As shown in Fig. 1, the pH in CM initially dropped before it grew steadily (same as in KS), while in KW and OM, the pH simply gradually increased. One reason why pH was different was that the CM and KS contain carbohydrates that would lead to a greater release of fatty acids and generate an initial drop in pH. After fermentation, the highest spore counts of CM, KW, KS, and OM were 2.51×10^{10}, 1.02×10^{10}, 1.58×10^{10}, and 5.01×10^{10} (Fig. 2). It can be seen that the media of CM, KW, KS, and OM were all able to support the growth of *Bt*. Compared to other previously reported substrates, kitchen waste as a cost-free substrate was best suited to produce biological pesticides. From Fig. 3, it is easy to see that the OM medium had the highest entomotoxicity yield after 48 h

fermentation, which indicated the entomotoxicity of *Bt* was related to the composition of the medium.

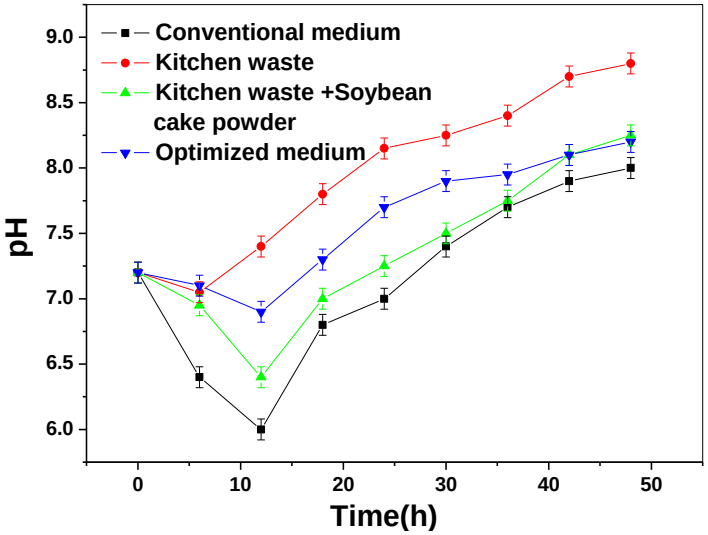

Fig. 1. Variation of pH of *Bt* during fermentation time by using different culture media

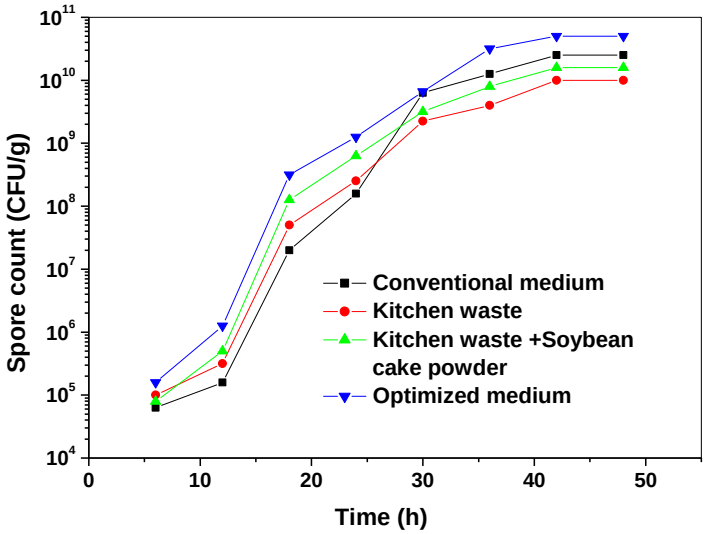

Fig. 2. Variation of spore count of *Bt* during fermentation time by using different culture media

By comparison, the KW and OM reactions yielded the lowest and highest spore counts and entomotoxicity. It has been proven that the entomotoxicity is related to the composition of the fermentation medium. It is important that the availability of biodegradable organic matter in the medium is a limiting factor for obtaining a high entomotoxicity. Kitchen waste and soybean cake powder contain more complex organic matter that was less biodegradable, which led to lower entomotoxicity than the OM medium. For this reason, kitchen waste as a complex substrate for fermentation should be added to more easily degradable compounds. In order to increase *Bt* growth and toxin yield, oxygen transfer requires more extensive research. From Fig. 4a, it can be seen that the toxic proteins (130kD, 60kD) of *Bt* parasporal crystal extracted from the optimized medium based on kitchen waste were very integrated. The morphology structure of *Bt* parasporal crystal can be clearly seen in Fig. 4b; most of the crystal proteins were subclavate or oval, and a few were standard biconical. The majority of crystal proteins

retained their structural integrity. The results demonstrated that kitchen waste used as raw material to produce *Bt*-based bio-pesticide was feasible.

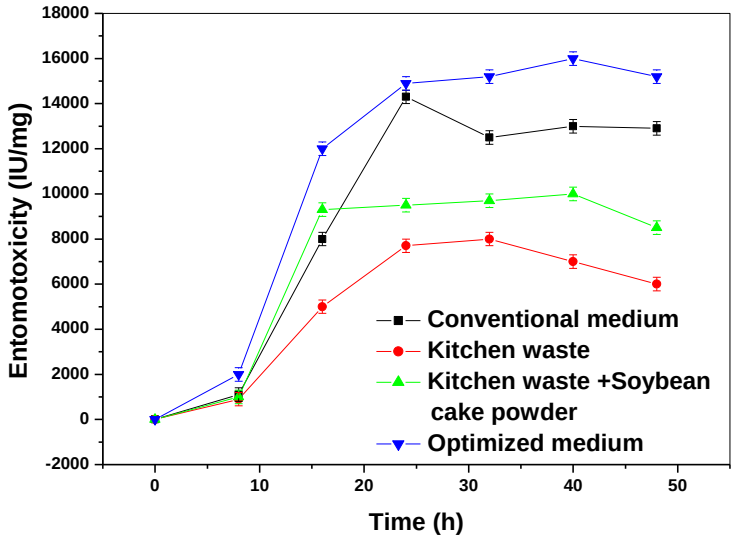

Fig. 3. Variation of entomotoxicity of *Bt* during fermentation time by using different culture media

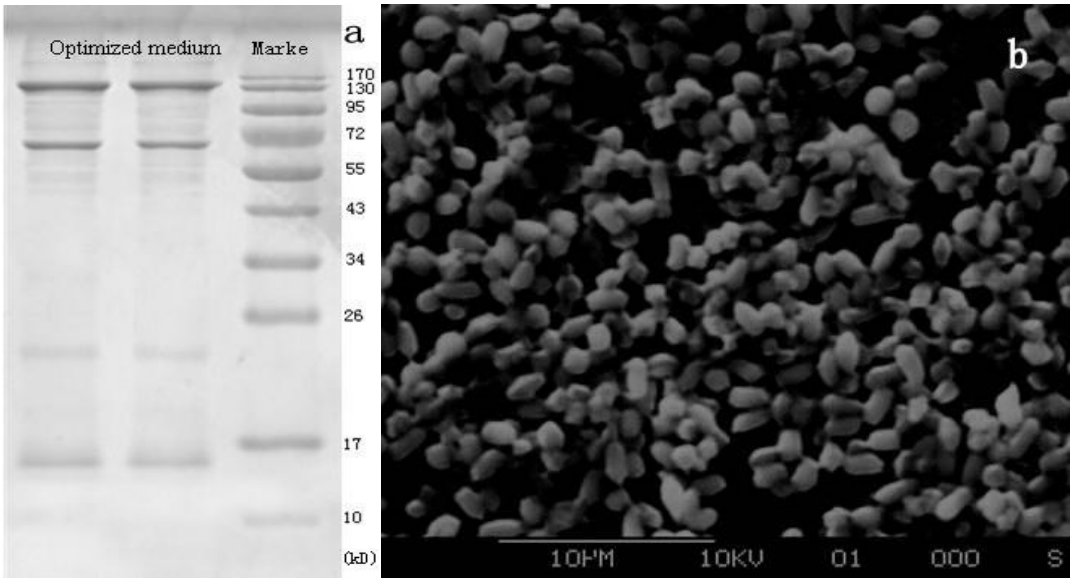

Fig. 4. Analysis of *Bt* parasporal crystal extracted from the optimized medium by SDS-PAGE (a) and SEM (b)

Effects of Salt and Oil on the Growth and Toxicity of *Bt*

Kitchen waste is rich in oil and salt, which may affect *Bt* growth, so their effects were studied in this work. For oil (soybean oil) and salt (NaCl), the concentrations applied for *Bt* growth were individually defined, based on their average concentration range found in kitchen waste. The spore count and entomotoxicity of *Bt* products under different contents of oil and salt after 48 h fermentation are shown in Fig. 5.

For oil, within the range from 5% to 10%, *Bt* was capable of growing without any obvious effect on its spore count or toxicity. But when the oil content was higher than 15%, the toxicity sharply decreased (Figs. 5a, 5b). With respect to salt content (Figs. 5c,

5d), there was no significant difference between the results at 0.4%, 0.6%, and 0.8%, or between the results at 1% and 1.2%. The spore count and entomotoxicity of *Bt* were maintained at a high level, but salt had a great impact on spore count and toxicity, even when the content was as low as 0.2%.

These results suggested that the exposure of *Bt* to high levels of oil generally led to a reduction of the total biomass yield. Because too much oil can gradually spread a slick over the solid medium that will prevent enough oxygen from getting through, the growth of *Bt* will be inhibited. Adequate oxygen is important for *Bt* growth. For this reason, the oil content is best maintained below 10%. Regarding this point, it is important to further study how to remove oil from kitchen waste. It was found that salt has an insignificant impact on the toxicity of *Bt*, that is, the content of salt in kitchen waste was suitable for the growth of *Bt*. On the whole, kitchen waste can be used as raw material to produce *Bt*-based bio-pesticides.

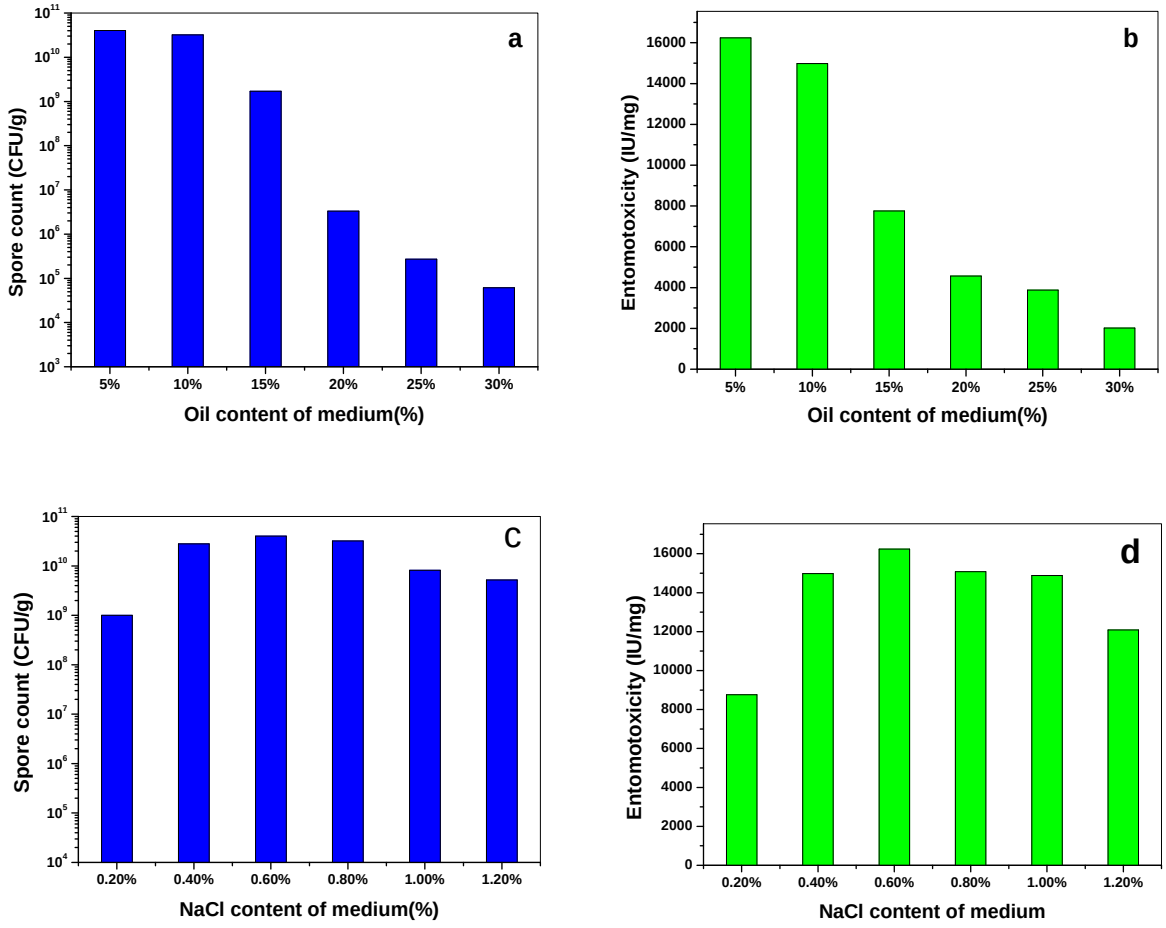

Fig. 5. The effects of oil and salt on spore count and entomotoxicity of *Bt* in optimized medium

Results of Scale-Up Experiment

The above experimental results proved that it was feasible to use kitchen waste as a raw material to produce *Bt* bio-pesticides. The scale-up experiment of *Bt* fermentation was processed by the solid-state fermentation reactor designed in our laboratory. The fermentation results are shown in Fig. 6.

It was shown that the spore count increased slightly from 4 kg of fermentation medium to 8 kg. This was because the increase of medium mass provided more nutritious substances for *Bt* growth, and the ventilation and oxygen supply were basically unaffected. Additionally, more medium increased the temperature during the fermentation process. From 8 kg to 35 kg, the spore count appeared to descend smoothly, and then from 35 kg to 50 kg, it decreased rapidly. The results of entomotoxicity tests were nearly the same as spore counts. When the fermentation medium was 35 kg, the spore count was 9.6×10^8 CFU/g, while the entomotoxicity was 10874 IU/mg. However, when the fermentation medium was 40 kg, 45 kg, and 50 kg, the spore count was only 6.4×10^6 CFU/g, 1.2×10^6 CFU/g, and 3.7×10^5 CFU/g, while the entomotoxicity was 5021 IU/mg, 4671 IU/mg, and 4600 IU/mg. At present, the entomotoxicity of commercial *Bt* production is 8000 IU/mg.

With increasing medium mass, the spore count and entomotoxicity decreased without interruption. The main reason for this result is ventilation, which can provide oxygen and decrease the temperature. *Bt* growth requires oxygen and gives off a lot of carbon dioxide and heat, which may inhibit bacterial growth. Therefore, too much culture medium can lead to poor ventilation so that carbon dioxide and heat are not removed in time and there is not enough oxygenation. This scale-up experiment can supply theoretical values for pilot-scale production in the future.

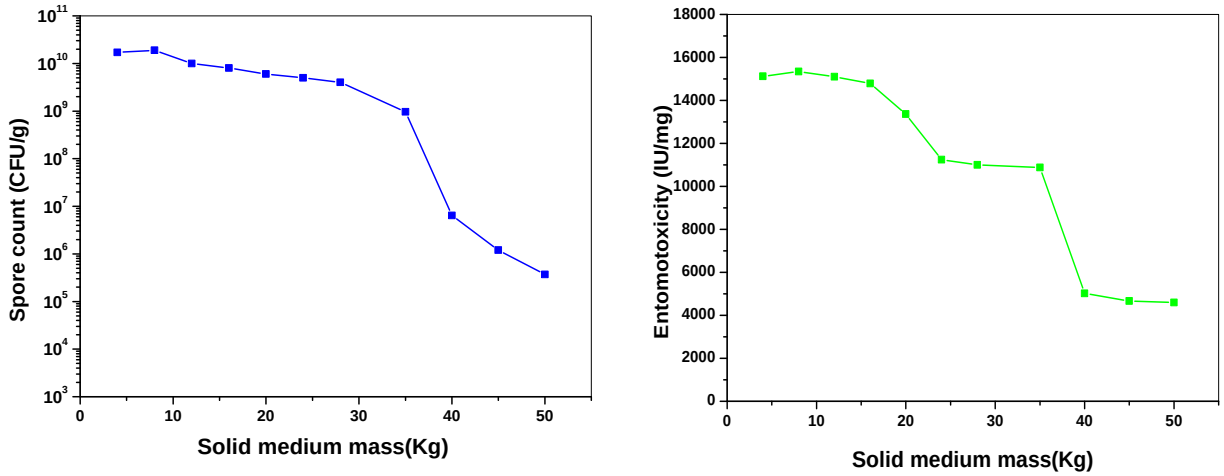

Fig. 6. The spore count and entomotoxicity in different quantities of medium

CONCLUSIONS

1. This study suggested that kitchen waste is a suitable substrate for *Bt* production using solid-state fermentation with a self-made fermentation reactor.
2. The spore count (5.01 × 1010 CFU/g) and toxicity (15200 IU/mg) were satisfactory in the optimum medium, which contained 55.21% kitchen waste, 22.08% wheat bran,

11.04% soybean cake powder, 11.04% grain hulls, and 0.63% mixed ions. Kitchen waste contains nutrients required for growth of microorganisms.

3. Oil and salt had few adverse effects on *Bacillus thuringiensis* growth, yield of spores, and toxicity when the concentration of oil and salt were below 10% and 0.4% to 1.2%, respectively.
4. Fermentation medium of 35 kg was successfully applied to produce bio-pesticides by using the solid-state fermentation method in the enlargement experiment. The use of kitchen waste as a raw material for the production of *Bt*-based bio-insecticides will minimize *Bt* production costs and reduce the quantity of kitchen waste.

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