*J. Agr. Sci. Tech. (2023) Vol. 25(2): 469-484*

# Optimization of the Culture Medium, Fermentation Process, and Effectiveness of a Biopesticide from an Iranian *Bacillus thuringiensis* var. *tenebrionis* (BN2)

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## ABSTRACT

**The local strain of *B. thuringiensis* var. *tenebrionis*-BN2 (Btt-BN2) was used to control the alfalfa weevil. Experimental design using Response Surface Methodology (RSM) was applied for the optimization of the culture medium and fermentation parameters in order to achieve a high level of Colony-Forming Unit (CFU) (spore $mL^{-1}$). The parameters including the amount of carbon and nitrogen sources at three levels were investigated with CFU (spore $mL^{-1}$) response for two sets of experiments. The obtained results showed that the maximum CFU (spore $mL^{-1}$) for the minimum concentration of oat (2 g $L^{-1}$), the minimum concentration of corn steep liquor (10 g $L^{-1}$), and the maximum concentration of sugarcane molasses (10 g $L^{-1}$) were equivalent to $1.4\times10^{13}$ spore $mL^{-1}$. Optimum fermentation parameters to obtain the highest value of CFU (spore $mL^{-1}$) were determined as a maximum level of pH of eight and a medium level of temperature ($28^0$C). Amazingly, optimum conditions enhanced the CFU value to $8.06\times10^{13}$ spore $mL^{-1}$, which is very significant in the Btt research. Finally, the bioassay analysis of Btt in a single system and binary system (combination of two insecticides; Matrine® and Abamectin®) at different concentrations illustrated 83% of mortality efficiency (3 ppm of Btt and 0.5 ppm Matrine) on the 3rd day of treatment and 100% efficiency almost for all combination of Btt with Matrine and/or Abamectin after the 7th day. The bioassay results showed promising environmentally friendly mortality efficiency compared to the current chemical treatments.**

**Keywords*:*** Alfalfa weevil, Biological control, Fermentation parameters, Integrated Pest Management, Mortality.

## INTRODUCTION

Microbial biopesticides have a special position in the integrated pest management strategy, where natural inhibitors of pests and other factors play an important role in preserving crops. Despite the effectiveness of chemical insecticides, it has caused environmental problems such as increased pest resistance to pesticides, unintended effects, toxicity to mammals, and pesticide residues in the food chain. Therefore, these problems have highlighted the need for biological control agents due to the limited hosts, good safety records for human and environmental health, and high public acceptance (Oberemok *et al*., 2015; Marzban *et al*., 2016). Today, many *Bacillus thuringiensis* (Bt) strains are commercially used to control invasive forest and crops insects including Lepidoptera, Coleoptera,

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[ DOI: 10.52547/jast.25.2.469 ]

and Diptera insects (Behle *et al*., 1997; Tamez-Guerra *et al*., 2000). For the first time in 1985, insecticidal activity of *B. thuringiensis* var. *tenebrionis* (Btt) on beetles of the Chrysomelidae family, including the Colorado potato beetle, was documented in Germany by Langenbruch *et al.* (1985). Hilbeck *et al*. (1998) stated that Btt is specific to the larvae of Colorado potato beetle and a limited number of beetles of the Chrysomelidae family. Ferro *et al.* (1993) demonstrated that the efficiency of Btt is related to the temperature, with the highest effect at temperatures of 28 to 33°C.

The insecticidal activity of this bacterium is due to the toxic proteins produced during sporulation. Among toxins produced by *B. thuringiensis* strains, the crystalline protein of delta-endotoxin has attracted much attention and has been used commercially for the production of biological pesticides (Eagan, 2002; Thakore, 2006; Mazid and Kalita, 2011). Delta-endotoxin is dissolved in an alkaline gastric medium after being ingested by the insect and activated enzymatically, adhering to the specific sites on the stomach lining cells and disrupting the osmotic balance of the gastric cells. As a result, gastric cells become swollen and lysed and insect death occurs within a short time. At low doses, *B. thuringiensi*s delayed the growth and emergence of the complete insect and reduced the life span and oviposition of the insects fed on the delta-endotoxins (Marzban *et al*., 2009). Factors affecting the insecticidal activity of Btt include the age of the target larvae, temperature, amount of the crystals and spores sprayed, coating mechanism on the plant surface, time and number of replications of foliar application, and sunlight activity in delta-endotoxin neutralization (Khorramvatan *et al*., 2014; Khorramvatan *et al*., 2017). This crystalline protein is completely biodegradable, safe for humans, vertebrates, and plants, and does not cause toxic residues in the environment (Lord, 2005).

The aim of biological pesticides based on *B. thuringiensis* microcapsule formulation technology is to improve the resistance to the sun and rain and to attract pests (Bartelt *et al*., 1990; Gillespie *et al*., 1994; Suchy, 1988). Since Btt is a specific biological pesticide of the elm leaf beetle, an experiment conducted over 3 weeks on elm trees showed that, by application of Btt, only 10% of the leaves were damaged, while the damage before applying Btt was 40%. When Btt was used in combination with an insect pathogenic nematode (*Steinernema*) to control elm leaf beetle, the population of invasive larvae decreased significantly and, therefore, no chemical pesticides were required (Thurston, 1998). Btt illustrated specific bio-toxins effect on Coleoptera, which is a serious pest of the elm leaf beetle. Delta endotoxin production of Btt is dependent on the amount of spore production, culture medium, pH, and temperature (Eski *et al*., 2017).

After obtaining the best bacterial strains of Btt in terms of resistance, host range, and preparation of suitable media for mass production, it is necessary to develop bacterial growth conditions, sporulation, and crystal production under fermenter conditions as the primary model for its mass production in bioreactors (Huang *et al*., 2007; Saberi *et al*., 2014). A long history of identifying Bt as a biological insecticide product and its mass production in recent decades attracts the researchers' attention to optimizing growth conditions, including pH, oxygenation rate, temperature, etc., for maximum spore and crystal production in minimum time (Sikdar *et al*., 1991). Overall, the performance of commercial Bt products in pest control has been related to enhancing the concentration of delta-endotoxins and spores in the final product. The concentration of these two parameters is strongly dependent on the combination of the culture medium and the production environment of the bioreactor/fermenter. By changing the content of the culture medium and the culture conditions, the spore and crystal production can be optimized (Holmberg *et al*., 1980). Mass production of Bt has also been performed under semi-



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continuous and continuous conditions (Yezza *et al*., 2004).

Alfalfa weevil *Hypera postica* (Gyllenhal) (Curculionidae) is the most important alfalfa pest. We know that *B. thuringiensis* alone is not able to control the alfalfa weevil population. Microbial insecticide *B. thuringiensis* var. *galleriae*, which has been recently commercialized in the United States, has been able to control up to 60% of alfalfa weevils (Shrestha *et al.*, 2018). Chemical pesticides use is the most common method of controlling this pest. Chemical pesticides, in addition to contaminating livestock feed and consequently contaminating livestock products, cause the destruction of natural enemies and pollinators, especially honey bees. By using biological and low-risk pesticides, while preserving biodiversity, the health of the community is promoted. Matrine and Abamectin insecticidal activities are lower than the popular insecticides introduced by international pesticide companies during the last few years (Cheng *et al.*, 2018). Based on the biological activities of Matrine and Abamectin, they can be used in mixture with other natural pest control agents. The repetitive application of pesticides at high doses against *H. postica* has resulted in the development of pesticide resistance and harmful effects on the natural environment. Hence, finding alternate pest control strategies, such as *B. thuringiensis* or their application in combination with other biopesticides, is of great importance to solve the above-mentioned problems.

In this study, we aimed to optimize the culture medium factors (including carbon and nitrogen sources) and the operational parameters of the fermentation process (pH and temperature) for the growth of the Btt-BN2 strain were optimized. In addition, Response Surface Methodology (RSM) was applied in order to improve the CFU response. Finally, the bioassay analysis for the effect of produced biopesticide on the alfalfa weevil will be presented.

## MATERIALS AND METHODS

### *B. thuringiensis* var. *tenebrionis* Strain and Inoculum Preparation

*Bacillus thuringiensis* var. *tenebrionis* (Btt-BN2) was isolated from the dead larvae of the elm leaf beetle and provided by the Agricultural Research, Education and Extension Organization (AREEO) in Tehran, Iran. Inoculum of Btt-BN2 was prepared by dispensing one loop of the tested organism from nutrient agar slants into 2 mL of distilled water containing 15% w/v glycerin and incubated for 72 hours at 30°C. The microtubes were kept at -80°C in Freezer (Cryo Freezer Conqueror). Aliquots (2 mL) were used to inoculate in 200 mL Erlenmeyer flasks containing 16 g NB (nutrient broth) in 200 mL distilled water and autoclaved at 121°C for 15 minutes. Then, the culture broth flasks were incubated in a shaking incubator at 30°C and 190 rpm for 24 hours.

### Insects

Alfalfa leaf weevils were collected in April 2021 using netting from Karaj and Qazvin areas, Iran, and transferred to the laboratory. After separating the larvae from the alfalfa foliage, healthy larvae that were free of disease symptoms were transferred to the vessels selected for testing, and the larvae of the third instars of the pest were used for testing.

### Growth Media

In order to develop a cost-effective medium for spore production, various agricultural wastes (sugarcane molasses, wild oat, wheat bran, and corn steep liquor) were screened as alternatives for the carbon sources of the complex medium. Table 1 shows the content of carbon and nitrogen in each of the agricultural wastes used (based



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**Table 1.** Elemental analysis of carbon sources used in this study.

| Carbon source | C (%) | N (%) | Based on the 100 g | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Carbohydrate (g) | Sugars (g) | Protein (g) | Fat (g) | P (mg) | K (mg) | Fe (mg) | Zn (mg) |
| Sugarcane Molasses (SCM) | 32.61 | 1.88 | | | 4.5 | 1.8 | 500 | 3600 | 9.5 | 1.3 |
| Wheat Bran (WB) | 41.5 | 2.78 | 64.51 | 0.41 | 15.55 | 4.25 | 1013 | 1182 | 10.6 | 7.3 |
| Wild Oat (WO) | 42.3 | 2.98 | 66.22 | 1.45 | 17.3 | 7.03 | 734 | 566 | 5.41 | 3.11 |

on elemental analysis) as well as other compounds and metals. Twenty of 100 mL shake flasks were used for spore production, each one containing different amounts of agricultural wastes, 0.1% w:v $KH_2PO_4$, 0.1% w:v $K_2HPO_4$, 0.03% w:v $MgSO_4 \cdot 7H_2O$, 0.002% w:v $ZnSO_4 \cdot 7H_2O$, 0.002% w: $FeSO_4 \cdot 7H_2O$, and 0.1% w:v $CaCO_3$ (1 g of $CaCO_3$ in 100 mL distilled water). The pH was adjusted to 7.0 before sterilization at 121°C for 15 minutes. Two sets of experiments of 20 combinations (for each set) were performed to investigate the spore formation and CFU response.

## Colony Count

Total spore counts of the biomass were determined by the spread plate method using Mueller–Hinton agar plate. Culture samples were heat-treated at 80°C for 10 minutes, serially diluted, and plated on agar plates. Plates were incubated at 30°C for 24 hours and the developing Btt colonies were counted.

Formulated Btt-BN2 (1 mL) was rehydrated with sterile $ddH_2O$ (9 mL) and serially diluted to $10^{-7}$. Viable spore counts were determined by incubating $10^7$ dilution in a water bath at 80°C for 10 minutes. An aliquot of 10 mL from the $10^7$ dilution was spread to three Mueller–Hinton agar plates. Then, it was incubated at 30°C for 24 hours and the developing Btt-BN2 colonies were counted (Amin *et al*., 1983). To determine the CFU (spore $mL^{-1}$), Equation (1) was used:

$$\text{CFU} = \frac{Colony\ formed \times Dilution\ factor}{Aliquot\ taken} \quad (1)$$

## Experimental Design for Growth Medium Optimization

The effect of independent variables on the responses was investigated by Central Composite Design (CCD) of Response Surface Methodology (RSM) by SPSS in Design-Expert software (version 12). The experiments were performed with two sets of three independent variables of three carbon sources (SCM: Sugarcane Molasses, WB: Wheat Bran, and WO: Wild Oat) and one nitrogen source (CS: Corn Steep liquor) including: (1) SCM, WB, and CS, and (2) SCM, WO, and CS at three levels of 1, -1, and 0. The range of independent variables and their levels for the first and second sets are represented in Tables 2 and 3, respectively.

The independent variables and their ranges were chosen based on the preliminary study results.

The Number of experiments (N) of the CCD was obtained from the Equation (2) as follows:

$$N = 2^k + 2 \times k + n_0 \quad (2)$$

Where, k is the number of factors/parameters and $n_0$ is the number of iterations at the central point (center of the cube). The number of experiments (20 experiments) for each set of experiments was obtained from Equation (2).

## Fermentation Process

Batch fermentation was carried out in an agitator-equipped 6 L Fermenter (FCU/PU05, Medorex, Germany) with a working volume of 5 L. The related devices



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**Table 2.** The range of independent variables for the first set.

| level | SCM Carbon source (g $L^{-1}$) | WB Carbon source (g $L^{-1}$) | CS Nitrogen source (g $L^{-1}$) |
|---|---|---|---|
| –1 | 2 | 2 | 10 |
| 0 | 6 | 6 | 30 |
| +1 | 10 | 10 | 50 |

**Table 3.** The range of independent variables for the second set.

| level | SCM Carbon source (g $L^{-1}$) | WO Carbon source (g $L^{-1}$) | CS Nitrogen source (g $L^{-1}$) |
|---|---|---|---|
| –1 | 2 | 2 | 10 |
| 0 | 6 | 6 | 30 |
| +1 | 10 | 10 | 50 |

including a control system, a cooling circulator, and an air pump were manufactured by, respectively, Circulator Co (Model; VS-190 CS) and Millipore. The fermentation was performed under completely aseptic conditions to prevent contamination during the process. The 10% (v/v) inoculation was transferred from the Erlenmeyer flask to the fermenter, which contained 3 liters of culture medium. The operational parameters were pH of 6 to 8 (adjusted by 1N $H_2SO_4$ and 1N NaOH), temperature of 26 to 30°C, and mixer speed of 250 rpm. The airflow rate was set at 1 vvm and foam production was controlled by the automatic addition of sterile antifoam solution.

## Experimental Design for Fermentation Process Optimization

The RSM and CCD were applied (as previously described in Equation 3) for two parameters of temperature and pH at three levels of +1, -1, and 0. Table 4 shows the range of these parameters at three levels.

**Table 4.** The range of independent variables for the fermentation process.

| level | Temperature ($^0$C) | pH |
|---|---|---|
| –1 | 26 | 6 |
| 0 | 28 | 7 |
| +1 | 30 | 8 |

A number of 13 experiments was obtained for the fermentation process.

## Bioassay

Experiments were conducted in a completely randomized design with four replications and 33 treatments including two concentrations of 5 and 3 ppm of Btt. Two insecticides, namely, Matrine ® and Abamectin® with concentrations of 0.05, 0.1, 0.5, 1, 1.5 and 0.1, 0.2, 0.5, 1, 1.5 ppm, respectively, were used as combining insecticide with Btt treatments. In each plastic Petri dish, 10 weevil larvae with treated small branches were placed. It should be noted that in order to delay the wilting of alfalfa branches, the ends of the branches were placed in agar. They were kept in an incubator at 26±1°C, relative humidity of 55-60%, and photoperiod of 8 hours of darkness and 16 hours of light. Larval mortality was recorded in two rounds, 3 and 7 days after treatment.

## Effect of Chemical Insecticides on the Colonization of Btt

The efficacy of the chemical insecticides on the colonization of Btt was investigated using the method described by Touhidul Islam *et al*. (2010). Culturing Btt was done in plastic Petri



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dishes (8 cm in diameter) containing 18 mL of Nutrient Agar (NA) medium (Merck, Germany) and 2 mL of the chemical solution. The insecticides Abamectin and Matrine were used. The formulated insecticides were diluted with distilled water and added to the culture medium based on the recommended field doses. The final concentrations of Abamectin and Matrine in NA medium were 0.2, 0.5, and 1 and 0.1, 0.5, and 1 ppm, respectively. The colony formation was assessed by inoculating each Petri dish with 0.5 mL of Bt spores suspension containing 100 spore $mL^{-1}$. The Petri dishes were incubated at 28°C and after 24 hours, the number of colonies in each Petri dish was recorded. There were five Petri dishes for each treatment and the experiment was replicated three times on different days. Colonization was evaluated using three controls including NA only and NA plus the chemical insecticide as negative controls to demonstrate that the growth medium and the insecticide solutions were not infested with any bacteria; and NA plus Bt spores alone as the positive control. Only the positive controls were entered in the data analysis.

### Bioassay Statistical Analysis

One-way ANOVA was performed using SPSS software (1998). The mortality was corrected by the equation: M (%)= $[(t-c)/(100-c)]\times 100$, where $M$ is corrected Mortality, $c$ is the mortality in controls and $t$ is mortality in treatments (Abbott, 1925; Duffield and Jordan, 2000). The normalization of the data was done in SPSS. Then, mean corrected mortality was compared using Duncan's test at $P< 0.05$.

## RESULTS AND DISCUSSION

### Effect of Carbon and Nitrogen Source on Culture Medium

The effect of three parameters including different media with different carbon compositions as a carbon source of agricultural wastes (sugarcane molasses, wheat bran, wild oat) and one nitrogen source of agricultural wastes (corn steep liquor) based on CFU was studied. Experimental design was the central compound method with the aim of simultaneous study of the effects of the variables and their interaction. The current research consisted of 20 experiments (for each set of variables). The coded levels of the variables in each experiment and the main variables along with the CFU (spore $mL^{-1}$) value of the laboratory results were obtained and reported in Tables 5 and 6.

### Regression Analysis

The significance of regression coefficients based on t-statistics for the main parameters, their squares, and their double interactions were determined. Tables 7 and 8 shows the coefficients and statistics for each set of experiments. The analysis of the results is based on the value of 0.05 for P-value. As shown in Table 7 for the first set of experiments (SCM-WB-CS), the squares and binary interventions of WB and CS parameters are not significant, except for SCM and CS×SCM. Therefore, in order to obtain a better model, it is necessary to eliminate the effect of insignificant parameters and consider them as a trial error, to regain the regression model.

Table 8 presents the results for the second set of experiments (SCM-WO-CS). Based on the P-value, all main factors of WO, SCM, CS, and their binary interactions have a significant effect. Therefore, it is necessary to remove meaningless factors from the model.

The results of Tables 7 and 8 show the significant parameters (for each experiment) that affect the final response of CFU. The actual model proposed for these experimental runs will be linear as shown below for both sets of experiments (Eq. 3 for SCM-WB-CS and Eq. 4 for SCM-WO-CS):

$$R_{\text{SCM-WB-CS}} \times 10^{10} = 66.5095-24.8730\ CS-40.4810\ SCM + 33.6588\ CS\times SCM \quad (3)$$



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**Table 5.** Experiment runs designed by CCD for set SCM-WB-CS.

| Run | Main parameters level | | | Response |
|---|---|---|---|---|
| | SCM | WB | CS | CFU (spore $mL^{-1}$) |
| 1 | -1 | -1 | -1 | $12.4 \times 10^{11}$ |
| 2 | -1 | +1 | -1 | $19.6 \times 10^{11}$ |
| 3 | -1 | -1 | +1 | $0.83 \times 10^{11}$ |
| 4 | -1 | +1 | +1 | $1.70 \times 10^{11}$ |
| 5 | +1 | -1 | -1 | $2.37 \times 10^{11}$ |
| 6 | +1 | +1 | -1 | $0.40 \times 10^{11}$ |
| 7 | +1 | -1 | +1 | $0.12 \times 10^{11}$ |
| 8 | +1 | +1 | +1 | $0.11 \times 10^{11}$ |
| 9 | 0 | -1 | 0 | $10.6 \times 10^{11}$ |
| 10 | 0 | +1 | 0 | $5.30 \times 10^{11}$ |
| 11 | 0 | 0 | -1 | $1.16 \times 10^{11}$ |
| 12 | 0 | 0 | +1 | $8.30 \times 10^{11}$ |
| 13 | -1 | 0 | 0 | $9.76 \times 10^{11}$ |
| 14 | +1 | 0 | 0 | $0.80 \times 10^{11}$ |
| 15 | 0 | 0 | 0 | $10.0 \times 10^{11}$ |
| 16 | 0 | 0 | 0 | $9.80 \times 10^{11}$ |
| 17 | 0 | 0 | 0 | $10.1 \times 10^{11}$ |
| 18 | 0 | 0 | 0 | $10.0 \times 10^{11}$ |
| 19 | 0 | 0 | 0 | $9.80 \times 10^{11}$ |
| 20 | 0 | 0 | 0 | $9.90 \times 10^{11}$ |

**Table 6.** Experiment runs designed by CCD for set SCM-WO-CS.

| Run | Main parameters level | | | Response |
|---|---|---|---|---|
| | SCM | WO | CS | CFU (spore $mL^{-1}$) |
| 1 | -1 | -1 | -1 | $19.8 \times 10^{11}$ |
| 2 | -1 | +1 | -1 | $1.10 \times 10^{11}$ |
| 3 | -1 | -1 | +1 | $9.60 \times 10^{11}$ |
| 4 | -1 | +1 | +1 | $0.56 \times 10^{11}$ |
| 5 | +1 | -1 | -1 | $140.6 \times 10^{11}$ |
| 6 | +1 | +1 | -1 | $1.25 \times 10^{11}$ |
| 7 | +1 | -1 | +1 | $10.9 \times 10^{11}$ |
| 8 | +1 | +1 | +1 | $0.77 \times 10^{11}$ |
| 9 | 0 | -1 | 0 | $15.4 \times 10^{11}$ |
| 10 | 0 | +1 | 0 | $0.82 \times 10^{11}$ |
| 11 | 0 | 0 | -1 | $5.40 \times 10^{11}$ |
| 12 | 0 | 0 | +1 | $1.39 \times 10^{11}$ |
| 13 | -1 | 0 | 0 | $1.48 \times 10^{11}$ |
| 14 | +1 | 0 | 0 | $1.98 \times 10^{11}$ |
| 15 | 0 | 0 | 0 | $1.58 \times 10^{11}$ |
| 16 | 0 | 0 | 0 | $1.55 \times 10^{11}$ |
| 17 | 0 | 0 | 0 | $1.58 \times 10^{11}$ |
| 18 | 0 | 0 | 0 | $1.60 \times 10^{11}$ |
| 19 | 0 | 0 | 0 | $1.56 \times 10^{11}$ |
| 20 | 0 | 0 | 0 | $1.58 \times 10^{11}$ |

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**Table 7.** Related coefficients and statistics for the complete regression model (for set SCM-WB-CS).

| Term | Coef | SE Coef | T | P- value |
|---|---|---|---|---|
| Constant | 89.7056 | 12.79 | 7.015 | 0.000 |
| WB | 0.8330 | 11.76 | 0.071 | 0.945 |
| CS | -24.8730 | 11.76 | -2.114 | 0.061 |
| SCM | -40.4810 | 11.76 | -3.441 | 0.006 |
| WB×WB | 4.0359 | 22.43 | 0.180 | 0.861 |
| CS×CS | -27.9641 | 22.43 | -1.247 | 0.241 |
| SCM×SCM | -22.4641 | 22.43 | -1.001 | 0.340 |
| WB×CS | -5.4587 | 13.15 | -0.415 | 0.687 |
| WB×SCM | -12.5587 | 13.15 | -0.955 | 0.362 |
| CS×SCM | 33.6588 | 13.15 | 2.559 | 0.028 |

**Table 8.** Related coefficients and statistics for the complete regression model (for set SCM-WO-CS).

| Term | Coef | SE Coef | T | P-value |
|---|---|---|---|---|
| Constant | -0.3141 | 5.921 | -0.053 | 0.959 |
| WO | -19.1800 | 5.447 | -3.521 | 0.006 |
| CS | -14.4930 | 5.447 | -2.661 | 0.024 |
| SCM | 12.2960 | 5.447 | 2.258 | 0.048 |
| WO×WO | 11.2577 | 10.386 | 1.084 | 0.304 |
| CS×CS | 6.5427 | 10.386 | 0.630 | 0.543 |
| SCM×SCM | 4.8777 | 10.386 | 0.470 | 0.649 |
| WO×CS | 17.3600 | 6.089 | 2.851 | 0.017 |
| WO×SCM | -15.2175 | 6.089 | -2.499 | 0.032 |
| CS×SCM | -14.9300 | 6.089 | -2.452 | 0.034 |

$$R_{SCM\text{-}WO\text{-}CS}\times 10^{11} = 11.0250 - 19.18\ WO - 14.493\ CS + 12.296\ SCM + 17.36\ WO\times CS - 15.2175\ WO\times SCM - 14.93\ CS\times SCM \quad (4)$$

Where, $R_{SCM\text{-}WB\text{-}CS}$ and $R_{SCM\text{-}WO\text{-}CS}$ are the predicted response of CFU (spore $mL^{-1}$) for SCM-WB-CS and SCM-WO-CS sets of experiments, respectively (see Tables 7 and 8). The linear model according to Equations (3) and (4) demonstrates the correlation between CFU (spore $mL^{-1}$) and the three variables. The coefficients of determination ($R^2$) of these two models are 89 and 88%, respectively.

## Effects of Agricultural Waste Sources on CFU Response for SCM-WB-CS

Figure 1 shows the contour plot of interactions for nitrogen and carbon source values of CS and SCM, respectively, and their effect on the CFU response for the first set of experiments. In each of the response diagrams, the third factor (WB) is kept at its mean level and the effect of the other two factors on the response is plotted. The response of the model was specified inside the screen, the dark color indicates the response above 120 and the light color indicates the response between 0-20. As shown, the maximum CFU response was obtained at minimum values and/or

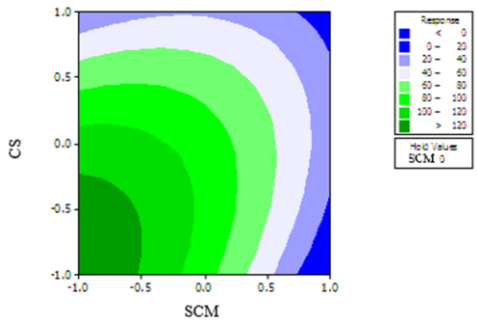


**Figure 1.** Contour plots (two-dimensional surface plots) of the effect of variables on CFU response (spore $mL^{-1}$): interaction of carbon source of SCM and nitrogen source of CS for set SCM-WB-CS.



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concentrations of CS and SCM.

Figure 2 presents a three-dimensional graph showing the simultaneous effect of the carbon content of SCM and nitrogen content of CS on the CFU value. This plot confirmed the results of the two-dimensional graph in which minimum concentrations of both carbon source from SCM and nitrogen source from CS resulted in a high response value.

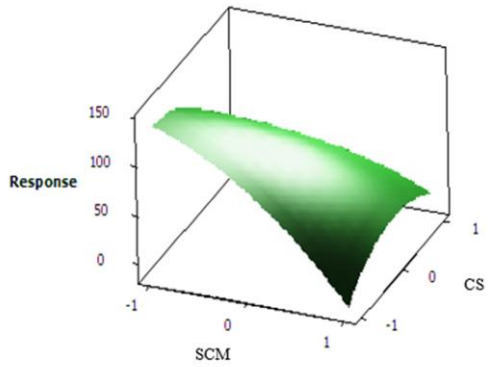


**Figure 2.** Surface plots (three-dimensional surface plots) of the model equation fitted to the data for CFU response (spore $mL^{-1}$) value based on the interaction of CS and SCM for set SCM-WB-CS.

### Effects of Agricultural Waste Sources on CFU Response for SCM-WO-CS

Figures 3 and 4 show the diagram of the contour and surface diagram, respectively, for the CFU value of SCM-WO-CS experimental runs. The answer chart shows the curvature effect of each of the indicators. The contour diagram (Figure 3) of the SCM and CS contours refers to the diagram whose vertical axis is related to SCM and whose horizontal axis is related to the CS index. The answer to the process inside the page is specified so that the dark color indicates the answer above 60 and the light color indicates the answer between 0-15. The SCM and CS contours show that the response value decreases as the SCM factor decreases. For the CS×WO contour, it shows that the response increases with decreasing CS and WO factors.

The obtained results for optimization of culture medium (Figures 1 to 4) show the interaction of carbon and nitrogen sources on each other. The results of the first experiment, which included two carbon sources of SCM and WB along with a nitrogen source of CS, showed that the highest CFU response was obtained under the conditions of minimum concentrations of SCM and CS, and a maximum concentration of WB. However, the overall results of the experimental design based on the 20 experiments showed that the concentration of WB was ineffective in the overall process of optimizing the culture medium. In other words, the amount of carbon in SCM in different experimental conditions was sufficient to achieve the maximum response of CFU (spore $mL^{-1}$).

The second experiment set used a combination of SCM, WO, and CS. The results showed that the response of CFU with a growth of about 7.5 times at minimum concentrations of WO and CS and a maximum concentration of SCM was obtained, which showed a very positive effect of WO presence in increasing CFU. The results of both experiments show that in all experiments, the best CFU response was obtained at the minimum concentration of nitrogen source. This could be explained with a look at Table 1, which illustrates that the carbon sources contained enough source of nitrogen, since they have a protein content.

The ranking of carbon sources to enhance the CFU value in the lowest concentration of the nitrogen source of CS was as follows:

WB> SCM> WO

The best response of WO can be due to its higher carbon content than other compounds (Table 1). As shown, WO has a higher amount of carbohydrates, sugar (3 times more), and fat (1.5 times more) compared to WB. In other words, the amount of carbon as well as the amount of nitrogen in this compound, while placing the used nitrogen source in its minimum concentration, makes



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this compound have adequate carbon source to get the highest amounts of CFU.

Other studies on the growth of Bt strains have shown the importance of carbon and nitrogen sources in bacterial growth. Research by Anderson and Jayaraman (2003) on the *B. thuringiensis* var. *galleriae* strain has shown that high glucose concentrations increase cell density while high concentrations of yeast cause delayed sporulation. In another study, Sarrafzadeh (2014) found that corn steep, sodium acetate, and manganese sulfate ions were highly effective on Bt-H14 strain growth. Applying the low-cost resources to reduce production costs was the main research of Chandrashekhar *et al.* (2014), which showed that the cost of production for the sv2 strain of *B. thuringiensis* with soy flour decreased by 23 times.

## Optimization of the Fermentation Process

### Statistical Analysis

Table 9 illustrates the design matrix and experimental results obtained for CFU value based on two variables in the optimization experiments (13 experiments).

### Regression analysis

Table 10 shows the coefficients and statistics for each parameter and their binary interactions. The risk level was considered as 5%, so, the analysis of the results was based on the value of 0.05 for P-value. According to the regression analysis, the pH×pH and pH×T parameters are insignificant. Therefore, in order to obtain a better model, it is necessary to eliminate these effects and consider them as a trial error, to regain the regression model.

Based on the above analysis and considering the effective parameters on response CFU, the following linear model was proposed (Equation 5):

$$R_{\text{Fermenter}} \times 10^{13} = 4.449 + 2.575\ pH + 1.034\ T - 2.389\ T \times T \quad (5)$$

**Table 9.** Experiment runs designed by CCD for the fermentation process.

| Run | Main parameters level | | Response |
|---|---|---|---|
| | Temp | pH | CFU (spore $mL^{-1}$) |
| 1 | -1 | -1 | $0.17 \times 10^{13}$ |
| 2 | -1 | +1 | $2.80 \times 10^{13}$ |
| 3 | +1 | -1 | $0.36 \times 10^{13}$ |
| 4 | +1 | +1 | $5.90 \times 10^{13}$ |
| 5 | 0 | -1 | $0.78 \times 10^{13}$ |
| 6 | 0 | +1 | $8.06 \times 10^{13}$ |
| 7 | -1 | 0 | $0.106 \times 10^{13}$ |
| 8 | +1 | 0 | $3.02 \times 10^{13}$ |
| 9 | 0 | 0 | $4.50 \times 10^{13}$ |
| 10 | 0 | 0 | $4.40 \times 10^{13}$ |
| 11 | 0 | 0 | $4.50 \times 10^{13}$ |
| 12 | 0 | 0 | $4.60 \times 10^{13}$ |
| 13 | 0 | 0 | $4.30 \times 10^{13}$ |

Where, $R_{\text{Fermenter}}$ is the predicted Response of CFU for parameters [Temperature ($^{o}$C) and pH] of Fermenter (see Table 10). The coefficient of determination ($R^2$) of this model is 94%.

### Effects of Operational Parameters of Fermenter on CFU Response

Figures 5 and 6 show the diagram of the contour (two-dimensional) and surface (three-dimensional) plots, respectively, for the interaction between the two main parameters of pH and temperature. As presented in Figure 5, the dark color indicates the response above 6 and the light color indicates the response between 0-2. The maximum level of pH and the medium level of temperature resulted in a maximum response of CFU value (spore $mL^{-1}$).

## Bioassay and Lethality Analysis

### Effect of Insecticides on Colonization of Bt

There was a significant difference in the number of colonies formed following direct exposure to the different insecticides. The



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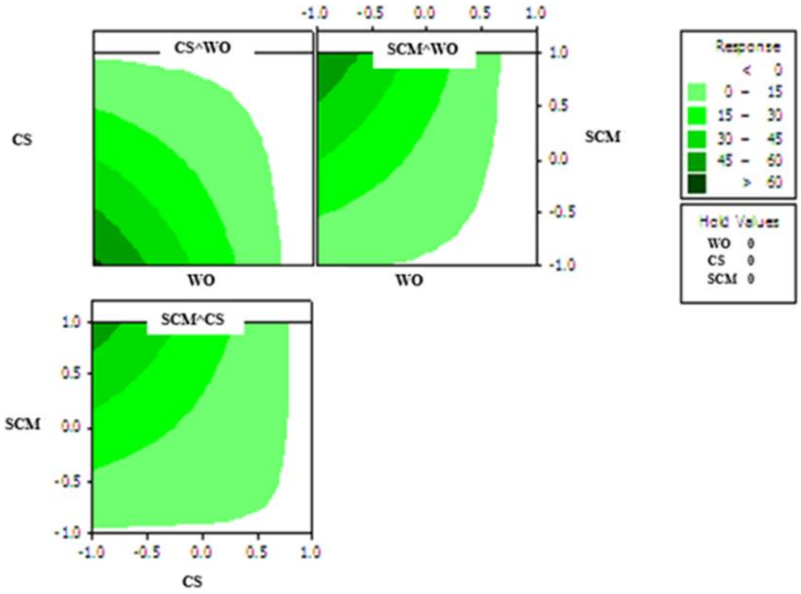


**Figure 3.** Contour plots (two-dimensional surface plots) of the effect of variables on CFU (spore $mL^{-1}$) response: interaction of carbon sources of SCM, WO, and nitrogen source of CS for set SCM-WO-CS.

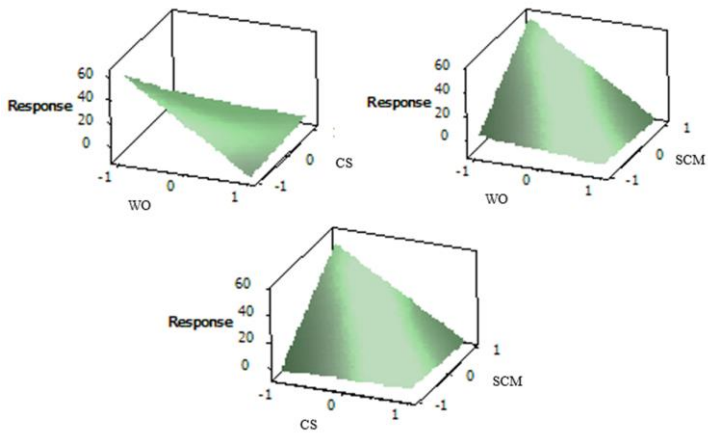


**Figure 4.** Surface plots (three-dimensional surface plots) of the model equation fitted to the data for CFU (spore $mL^{-1}$) response value based on the interaction of nitrogen source of CS and carbon sources of WO and SCM for set SCM-WO-CS.

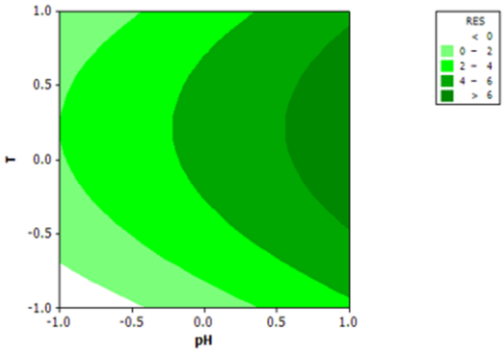


**Figure 5.** Contour plots (two-dimensional surface plots) of the effect of operational parameters of pH and temperature on CFU (spore $mL^{-1}$) response.



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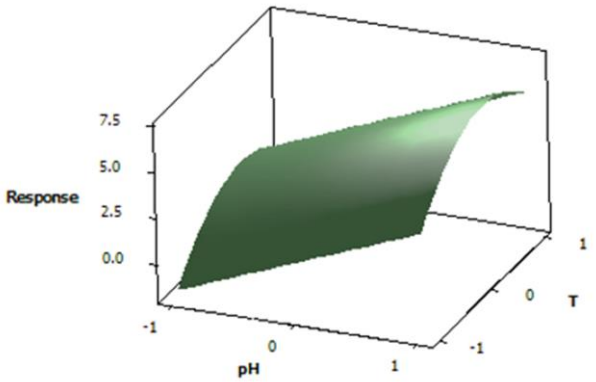


**Figure 6.** Surface plots (three-dimensional surface plots) of the model equation fitted to the data for CFU (spore $mL^{-1}$) response value based on the interaction of pH and temperature.

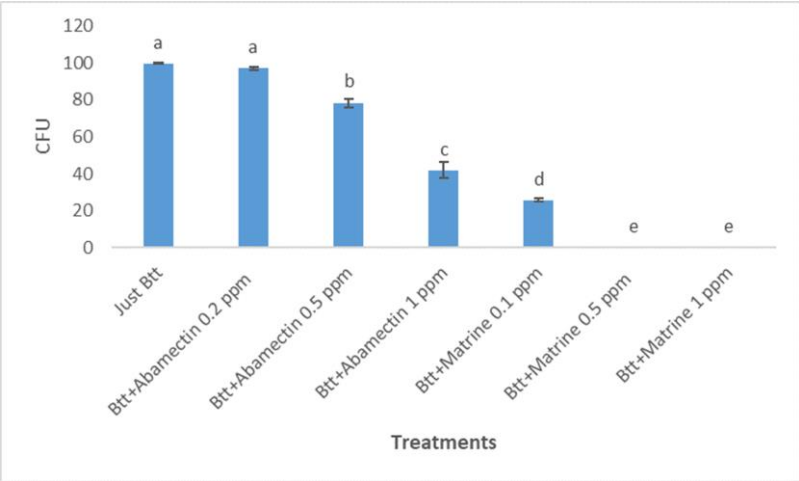


**Figure 7.** The *Bacillus thuringiensis* var. *tenebrionis* of CFU in combination with different doses of Abamectin® and Matrine ®.

percentage of spore colonies formed was highest in Abamectin 0.2 ppm (97.2%), which showed no significant variation. The lowest percentage of spore colonies was formed in Matrine 0.5 and 1 ppm (0%). In the case of Matrine 0.5 ppm and Matrine 1 ppm, no colonies formed (100% decrease) compared with the control. The results indicated that the concentrations of Matrine higher than 0.1 ppm significantly hampered Btt colonization ($F_6$= 494.73, P< 0.001) (Figure 7).

## The Efficacy of Btt, Insecticides, and Their Combination on Third Instar Larvae *H. postica*

The 3rd instar larvae of *H. postica* that were fed on the treated alfalfa lives containing different combinations of Btt-insecticides showed significant variation in terms of their mortality on the 3rd day ($F_{31}$= 3.07, P< 0.001) and 7th day ($F_{31}$= 13.67, P< 0.001). The mortality rate for most of the Btt- insecticides combinations (different concentrations) was higher than those of the treatments containing only one of the studied Btt or insecticides (Figures 8-a and -b). The treatment on the 3rd day illustrated a maximum of 83% mortality efficiency when a combination of Btt (3 ppm) and Matrine (0.5 ppm) was applied. However, after the 7th day of treatment, almost all combinations of Btt with Matrine and/or Abamectin (at different concentrations) reached 100% efficiency. Our results are also similar to the studies of some researchers. Their results demonstrated significantly higher



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**Table 10.** Regression analysis for parameters of fermenter.

| Term | Coef | SE Coef | T | P-value |
|---|---|---|---|---|
| Constant | 4.3518 | 0.3298 | 13.195 | 0.000 |
| pH | 2.5750 | 0.3243 | 7.941 | 0.000 |
| T | 1.0340 | 0.3243 | 3.189 | 0.015 |
| pH×pH | 0.3387 | 0.4779 | 0.709 | 0.501 |
| T×T | -2.5183 | 0.4779 | -5.269 | 0.001 |
| pH×T | 0.7275 | 0.3971 | 1.832 | 0.110 |

*S. exigua* mortality for the mixtures of Matrine with *Bacillus thuringiensis* when compared with the control and only Matrine treatments (Han *et al*., 2015). Moreover, different combined treatments of *Beauveria brongniartii* and Matrine showed a significant synergistic effect against *Spodoptera litura* under laboratory and semi-field conditions (Wu *et al*., 2019).

## CONCLUSIONS

In this study, the growth medium of the local strain of Btt was optimized using cost-effective agricultural wastes as carbon sources to maximize Btt production. The experimental design software of RSM-CDD was applied for all experiments. The results showed that the two carbon-rich sources of WO (with the minimum concentration of 2 g $L^{-1}$) and SCM (with the maximum concentration of 10 g $L^{-1}$) along with nitrogen source of CS (with the minimum concentration of 10 g $L^{-1}$) achieved the maximum CFU value of $1.406\times10^{13}$ spore $mL^{-1}$. These remarkable findings were enhanced in the fermentation process reaching the CFU value of $8.06\times10^{13}$ spore $mL^{-1}$ by applying the optimum operational conditions for temperature (medium level of 28$^{o}$C) and pH (maximum level of 8.0). Bioassay results showed 83% mortality efficiency (3 ppm of Btt and 0.5 ppm Matrine) on the 3$^{rd}$ day of treatment and 100% efficiency almost for all combinations of Btt with Matrine and/or Abamectin after the 7$^{th}$ day. This work provides an alternative scenario to use agricultural wastes, and the finding is relevant looking towards process development and its economy.

## ACKNOWLEDGEMENTS

The authors would like to appreciate the financial and technical support of the Biological Control Dep. at Plant Protection Research Institute in Agricultural Research, Education, and Extension Organization (AREEO).

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# بهینه سازی محیط کشت، فرایند تخمیر و اثربخشی آفت‌کش زیستی حاصل از یک سویه ایرانی *Bacillus thuringiensis* var. *tenebrionis* (BN2)

ف. صابری، ر. مرزبان، م. ارجمند، ف. پژوم شریعتی، و ا. توکلی

## چکیده

از سویه بومی *Bacillus thuringiensis* var. *tenebrionis* (Btt-BN2) برای کنترل سرخرطومی برگ یونجه استفاده شد. طراحی آزمایشی با استفاده از روش سطح پاسخ (RSM) برای بهینه‌سازی محیط کشت و پارامترهای تخمیر به منظور دستیابی به سطح بالایی از واحد تشکیل‌دهنده کلونی (CFU) ($spores.ml^{-1}$) انجام شد. پارامترها شامل مقدار منابع کربن و نیتروژن در سه سطح با پاسخ میزان واحد تشکیل‌دهنده کلونی ($CFU\ spores\ ml^{-1}$) برای دو مجموعه آزمایش بررسی شد. نتایج به‌دست‌آمده نشان داد که حداکثر CFU برای حداقل غلظت جو دوسر (۲ گرم در لیتر)، حداقل غلظت شربت ذرت (۱۰ گرم در لیتر)، و حداکثر غلظت ملاس نیشکر (۱۰ گرم در لیتر) معادل $1.4 \times 10^{13}\ spore\ ml^{-1}$ بود. پارامترهای تخمیر بهینه برای به دست آوردن بالاترین مقدار CFU در حداکثر سطح pH (معادل ۸) و سطح متوسط دما (۲۸ درجه سلسیوس) بدست آمد. مقدار CFU به میزان قابل توجهی در شرایط بهینه به $8.06 \times 10^{13}\ spore\ ml^{-1}$ افزایش یافت که در تحقیقات Btt بسیار مهم است. در نهایت، تحلیل زیست سنجی Btt-BN2 در یک سیستم واحد و سیستم دوتایی (ترکیب دو حشره کش؛ Matrine® و Abamectin®) در غلظت‌های مختلف، ۸۳ درصد تلفات را در روز سوم برای ترکیب ۳ ppm از Btt و ۰/۵ ppm از Matrine نشان داد. تلفات ۱۰۰% برای اکثر ترکیبات Btt همراه با Matrine و/یا Abamectin بعد از روز هفتم حاصل شد. نتایج زیست سنجی بیانگر کارایی و سازگاری با محیط زیست آفت کش زیستی مذکور در مقایسه با تیمارهای شیمیایی می‌باشد.



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