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Original Article

Solid Fermentation of *Bacillus thuringiensis*: Method Development

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Abstract: *Bacillus thuringiensis* (*Bt*) is a significant bioinsecticidal agent that has been mostly produced by liquid fermentation. With the increasing demand of bioinsecticides for organic agriculture, solid fermentation is preferred to enable low-cost and large-scale production of *Bt*. Therefore, in this study, we attempted to develop a solid state fermentation method to produce *Bt* by testing different solid substrates and also the effects of water percentage, fermentation time, temperature and seeding rate on the productions of biomass and parasporal crystal bodies (crystal proteins) of the *Bt* strain PAM33. Corn bran, rice bran and soybean meal are suitable to be utilized as substrates for biomass production. However, crystal proteins are only observed in solid fermentation using soybean meal as the substrate after 6 days of culture. The temperature range of 25-30 ℃ and 10% seeding rate are the most suitable conditions for solid fermentation of strain PAM33 to obtain the bacterial density of approximate 10^9 CFU/g and the highest quantity of crystal proteins. This study shows that solid fermentation of *Bt* is feasible and effective to obtain bacterial biomass and crytal proteins by using cheap and available agricultural wastes. Thus, it can be a potential approach to produce the important *Bt*-based insecticide products for use in organic agriculture.

Keywords: Bacillus thuriengiensis, solid fermentation, soybean meal, rice bran, corn bran.

1. Introduction

Increasing the application of microorganisms in biocontrol to prevent insect-pests and diseases in agricultural production is an important direction for the development of organic agriculture. More than 100 species of bacteria have been identified as anthropod pathogens that have been isolated from insects, plants, soil and water [1]. Among these, the most studied species are *Bacillus thuringiensis* (*Bt*), *B. sphaericus*, *B. cereus* and *B. popilliae.* However, *Bt* is the first microbial control agent to be studied and produced, and is still the most popular bioinsecticidal product. Today, thousands of different strains of this bacterium are known

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and a number of strains have been used to create biopesticides against many different pathogenic insects [2]. *Bt* bacteria have the ability to produce spores that are resistant to adverse environmental conditions, so bioproducts from *Bt* bacteria are often preserved in the form of spores [3]. *Bt* bacteria infect insects through toxic crytal proteins (Cyt and Cry) during sporulation and release them from the cells [4]. Therefore, the structure as well as the number of toxic crystals play a very important role in the pesticide efficiency of *Bt* strains.

Liquid fermentation is a popular method used to produce *Bt* products because of the advantages of short time, easy product purification, and simple fermentation control. However, the liquid fermentation method exposes certain disadvantages in large-scale production such as: complicated fermentation equipment, relatively low product concentration, low volumetric yield, and large amount of waste water causing adverse effects on the environment [5]. Therefore, the research and development of solid fermentation processes, which are simple and low-cost, to produce *Bt* are attracting more and more attention [6, 7]. Therefore, this study was carried out with the aim of developing a solid fermentation method for *Bt* bacteria by implementing simple pratices and using the available sources of agricultural scraps. The *Bt* strain utilized in this study is strain PAM33 that was isolated from soil and exhibited strong insecticidal activities. We studied the effects of water percentage, fermentation time, temperature and seeding rate on the productions of biomass and parasporal crystal bodies of the *Bt* strain PAM33, thereby figuring out an efficent solid fermentation method for strain PAM33.

2. Methodology

2.1. Bacterial Strain

Strain *Bacillus thuringiensis* PAM33 was used. It belongs to the microbial collection of the GREENLAB laboratory - Center for Life Science Research (CELIFE), University of Science, Vietnam National University, Hanoi.

2.2. Bacterial Density Determination

Bacterial density was determined by serial dilution and colony counting method [8]. The number of bacterial colonies was counted at 3 consecutive dilution levels, each dilution level was performed on 3 plates. The bacterial density was calculated according to the following formula:

$$N = \frac{\sum C}{n_1 Vd_1 + ... + n_i Vd_i} (CFU/ml)$$

N: Number of colony forming units (CFU) in 1 mL of sample solution; ΣC: the total number of colonies counted on the plates; n_i: number of plates counted at dilution rate i; V: volume of diluted sample solution (ml) spread on each plate; d_i: dilution rate i.

2.3. Crystal Concentration Determination

After solid fermentation, 1g of *Bt* solid culture was added to 9 mL of 0.9% NaCl, filtered through filter paper with pore size of 11 µm. Then, 1 mL of the filtrate was centrifuged at 8000 rpm for 20 min, and the precipitate was dissolved in 1 mL of 0.9% NaCl. 5 µL of the resulted solution was dropped onto a haemacytometer chamber and then heated on the flame of an alcohol lamp until dry. Toxic crystals, identified as bipyramidal bodies, were stained using Coomassie Brilliant Blue dye (100 mL dye contains 0.25 g Coomassie Brilliant Blue, 50 mL ethanol, 7 mL acetic acid and 43 mL DW) and counted under a microscope [9].

2.4. Determination of the Volumes of Water Added to Solid Fermentation Media

The *Bt* strain PAM33 were cultured in 100 mL of LB medium shaken at 200 rpm, 30 °C for 6 h. The culture broth was then diluted accordingly so that the final OD_{600} reached 0.5. Then, 10% (v/w) of the diluted culture was added to a conical flask containing sterilized solid medium with the following formula: 100 g

of rice bran, corn bran or soybean meal + 27.34 g rice husk + 6.45 g glucose + 0.125 g NaCl + 0.05 g $CaCl_2$ + 0.125 g $(NH_4)_2SO_4$ + 0, 10, 50, 100, 150 or 200 mL distilled water (DW). The bacterial densities were assessed after 24 h incubation at 30 °C.

2.5. Determination of the Suitable Substrate and the Suitable Fermentation Time

10% (v/w) of the diluted *Bt* culture broth (OD_{600} = 0.5) was added to the solid fermentation media with the above formula (containing rice bran, corn bran or soybean meal) and suitable amount of water. Bacterial densities were assessed after 12, 18, 24, 36, 42 and 48 h incubation at 30 °C.

2.6. Determination of the Suitable Fermentation Temperature

10% (v/w) of the diluted *Bt* culture broth (OD_{600} = 0.5) was added to the solid fermentation medium containing the suitable substrate determined above. Bacterial density was assessed after 24 h incubation at 10, 25, 30, 37 and 45 °C.

2.7. Determination of the Suitable Seeding Rate

The diluted *Bt* culture broth (OD_{600} = 0.5) was added to the solid fermentation medium containing the suitable substrate determined above at different seeding rates including: 5, 10, 15 and 20% (v/w). Bacterial density was assessed after 24 h incubation at 25 °C.

2.8. Determination of Suitable Substrate to Obtain the Highest Number of Crystals

10% (v/w) of the diluted *Bt* culture broth (OD_{600} = 0.5) was added to the solid fermentation media (containing rice bran, corn bran or soybean meal). Crystal count was assessed after 4 and 6 days of incubation at 25 °C.

2.9. Determination of the Suitable Seeding Rate to Obtain the Highest Number of Crystals

10% (v/w) of the diluted *Bt* culture broth (OD_{600} = 0.5) was added to the solid fermentation medium containing the suitable substrate at different seeding rates including 5, 10 and 15% (v/w). Crystal count was assessed after 4 and 6 days of incubation at 25 °C.

2.10. Statistical Analysis

The statistical significance among the data sets was assessed by the Student's t-test ($p<0.05$).

3. Results and Discussion

3.1. Effects of Water Volume Added to the Solid Fermentation Media Containing Different Substrates on Bt Biomass Production

To determine the appropriate moisture content for *Bt* solid fermentation, different volumes of water added to the media were tested on three selected inexpensive, readily available substrates including corn bran, rice bran and soybean meal. The results were evaluated based on the bacterial densities in the fermentation media after 24 h of incubation at 30 °C (Figure 1). The results showed that the highest density of *Bt* bacteria cells ($6.95x10^8$ CFU/g) was observed in the solid fermentation medium containing corn bran and 50 mL of added distilled water (DW), corresponding to 37.29 mL of DW/100 g of medium. With rice bran as the substrate, *Bt* PAM33 grew best (with its density reaching $8.65x10^8$ CFU/g) in the solid fermentation medium with 100 mL of added DW, corresponding to 74.58 mL of DW/100g of medium. With soybean meal as the substrate, the *Bt* strain grew best in the solid fermentation medium without added water (with its density reaching $9.47x10^8$ CFU/g). These results also indicated that all three tested agricultural scraps, i.e. corn bran, rice bran and soybean meal, can be utilized as substrates for *Bt* biomass production.

3.2. Effects of Different Substrates and Fermentation Times on Bt Biomass Production

Solid fermentation experiments for strain PAM33 were subsequently conducted with the three different substrates (rice bran, corn bran or soybean meal) in the media containing the

respective suitable moisture contents. The bacterial densities were assessed after 12, 18, 24, 36, 42 and 48 h. The results shown in Figure 2 indicated that all the 3 substrates have good potentials to be used for solid fermentation of *Bt* with the bacterial densities reached 10^9 CFU/g by 12 h. Among the three substrates, soybean meal is the one that resulted in the highest bacterial density ($1.28x10^9$ CFU/g) after 24 h of solid fermentation at 30 °C. Even though the differences of bacterial densities among the experimental cases with these three substrates are not significant based on Student's t-test, soybean meal was chosen for further experiments, because there is no need to add water to this substrate, hence reducing contamination. In addition, 24 h was the chosen as the harvest time point as it is the peak of the growth curves in Figure 2.

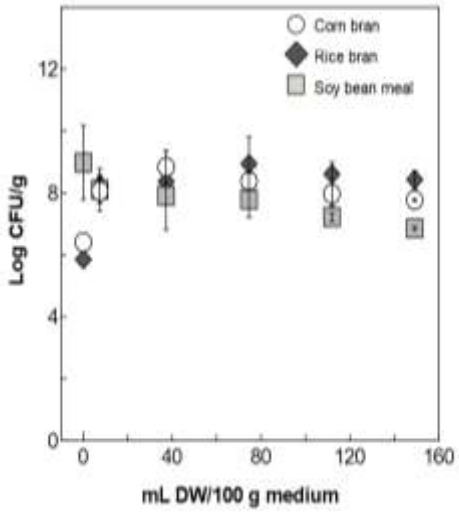

Figure 1. *Bt* PAM33 biomass densities in the solid fermentation media containing different substrates and different volumes of added water.

In a study published by Mourin et al., [10], the δ-endotoxin yield was found to be even higher when replacing glucose and peptone in the solid-state fermentation medium by 0.5% molasses and 10% soybean extract. Another study on *Bt* var. *aizawai* strain HD-133 by Morris et al., [11] has shown that the most suitable substrates among a variety of agricultural products and by-products to produce biomass and endotoxins are cotton seed meal, defatted soy flour and corn gluten meal.

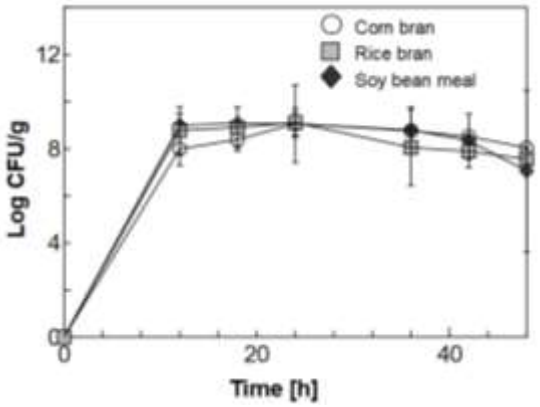

Figure 2. *Bt* PAM33 growth curve in the solid fermentation media containing different substrates.

3.3. Effects of Temperatures on Bt Biomass Production

The suitable temperature for solid fermentation of strain PAM33 was determined by testing at 10, 25, 30, 37 and 45 °C with soybean meal as the substrate. The results in Figure 3 showed that, the bacterial density reached 10^9 CFU/g at 25 and 30 °C. The densities were only $1.27x10^6$ and $1.08x10^8$ (CFU/g) at 10 °C and 37 °C, respectively. PAM33 was not able to grow at 45 °C. This result indicates that the optimal temperature range of *Bt* PAM33 is 25-30 °C.

3.4. Effects of Seeding Rate on Bt Biomass Production

Seeding rate is an important parameter in solid fermentation. Therefore, we evaluated the effects of the seeding rates of 5, 10, 15 and 20% on the growth of *Bt* in the solid fermentation medium with soybean meal as the substrate. The results in Figure 4 showed that after 24 h fermentation, the seeding rates of 10, 15 and 20 all led to high cell densities of *Bt* PAM33, at about 10^9 CFU/g. The *Bt* biomass was lowest with the seeding rate of 5%, possibly because this seeding rate is too small, causing bacteria to be unable to fully contact with the nutrients. Since the bacterial densities achieved at the

seeding rates of 10, 15 and 20% were not significantly different, the rate 10% should be used to reduce the cost.

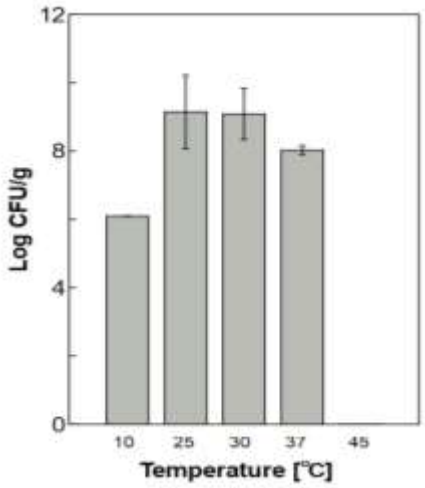

Figure 3. Effect of temperature on *Bt* PAM33 biomass production after 24 h of solid fermentation. No growth was observed at 45 °C.

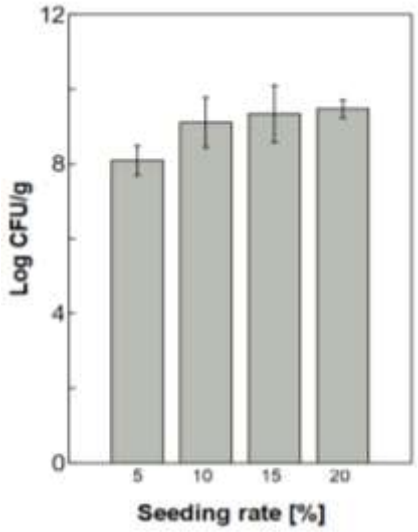

Figure 4. Effect of seeding rate on *Bt* PAM33 biomass produciton after 24 h of solid fermentation.

3.5. Effects of Substrate on the Production of Bt Crystals

Our preliminary result showed that *Bt* strain PAM33 started producing toxic crystals after 4 days shaking in LB medium and reached the highest number at day 6 (data not shown). Therfore, we investigated the abilities to produce crystal proteins of PAM33 after 4 and 6 days of solid fermentation at 25 °C using 3 substrates including corn bran, rice bran and soybean meal. The results showed that this *Bt* strain did not produce crystals after 4 days of solid fermentation on all the 3 substrates. After 6 days of fermentation, toxic crystals were detected only in soybean meal medium with an average quantity of 358 ± 42 crystals/microscopic field (Figure 5C). In the culture media containing corn bran and rice bran, only *Bt* bacteria cells and spores were detected (Figure 5A, B). Soybean meal is also chosen as the most suitable substrate (among the three studied substrates) for the biomass production of *Bt* strain PAM33 as described above. According to a previous study by Içgen et al.[12], the crystal protein biosynthesis of *Bt* bacteria is highly dependent on the nutritional factors such as carbon and nitrogen sources. Also in this study, soybean meal has been shown to be one of the best substrates for crystal production, possibly due to its balanced carbon and nitrogen contents.

3.6. Effects of Seeding Rate on the Production of Bt Crystals

The results of evaluating the influence of different seeding rates on the ability to produce crystal proteins of *Bt* PAM33 showed that under all seeding conditions, toxic crystals were not found after 4 days but only observed after 6 days of fermentation. Considering the results after 6 days of fermentation, we can see that the most suitable seeding rate for PAM33 to produce crystal proteins was 10%, at which we observed an average quantity of 360 ± 38 crystals/microscopic field (Figure 6B). With a seeding rate of 5%, only a few crystals were observed on a microscopic field from a sample taken after 6 days of fermentation (Figure 6A). This data is in line with the low biomass production of *Bt* bacteria with 5% seeding rate. Meanwhile, an average of 55 crystals/ microscopic field was observed with the sample from the fermentation with the seeding rate of 15% after 6 days (Figure 6C).

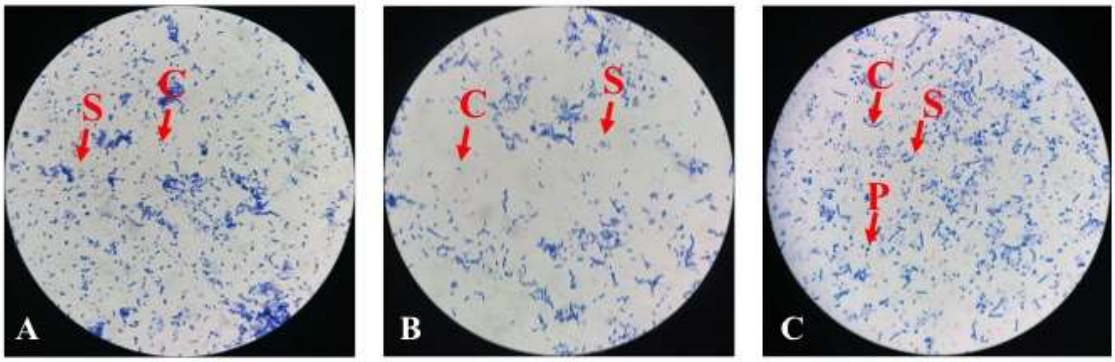

Figure 5. Coomasie Brilliant Blue staining images of *Bt* PAM33 after 6 days of solid fermentation using corn bran (**A**), rice bran (**B**) and soybean meal (**C**) as substrates. Notes in red: C: cells, S: spores, P: crystal proteins.

Together, our results suggested that 10% is the most suitable seeding rate in solid fermentation of *Bt* PAM33, for both the productions of biomass and toxic crystals. The commonly used seeding rate is also 10% in other *Bt* solid fermentation studies [13]. Thus, our data are quite correlated with published studiesPérez et al., [14] have shown that solid-state fermentation of *Bt* var. *kurstaki* HD-73 resulted in higher biomass and spore yields than submerged fermentation using the same media. These results together shed light to the applications of solid-state fermentation to produce *Bt*-based bioinsecticidal agent.

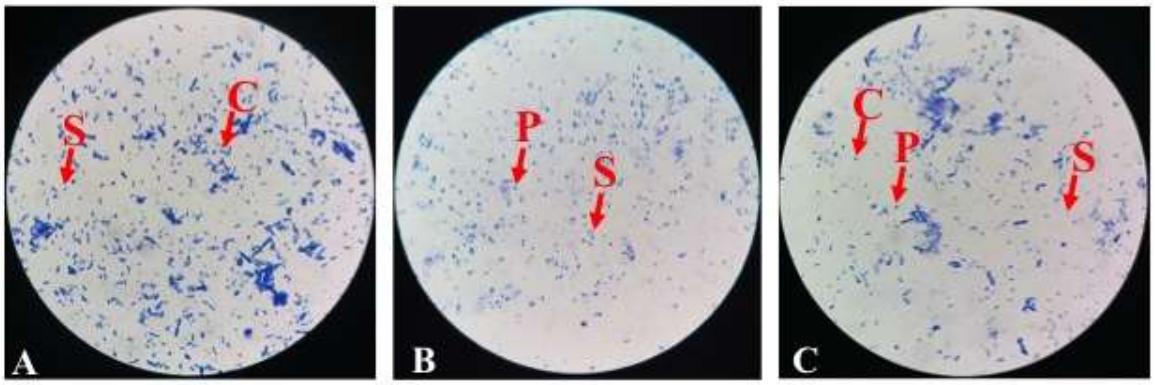

Figure 6. Coomasie Brilliant Blue staining images of *Bt* PAM33 after 6 days of solid fermentation on soybean meal as the substrate, with 5% (**A**), 10% (**B**) and 15% (**C**) seeding rates. Notes in red: C: cells, S: spores, P: crystal proteins.

4. Conclusion

This study has shown that agricultural scraps such as corn bran, rice bran, and soybean meal can be the main substrate source for biomass production of *B. thuringiensis* strain PAM33 by solid fermentation. However, among the three tested substrates, soybean meal is the only one that can promote the production of crystal proteins by this *Bt* strain. The suitable conditions for this strain to produce both biomass and crystal proteins are: temperature range of 25-30 °C and seeding rate of 10%. The fermentation time to obtain high yield of biomass is 24 h while that to obtain high number of crystal proteins is 6 days. Under such conditions, the cell density of *Bt* PAM33 could reach 10^9 CFU/g. This study shows that

solid fermentation to achieve high productivities of *Bt* bacterial biomass and crytal proteins, with agricultural wastes as substrates, is feasible and has great application potential for bioinsecticide production to serve organic agriculture.

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