Preliminary Study on the Effect of Culture Medium on the Number and Size of Endospores of *Bacillus megaterium*

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Abstract

Bacillus megaterium, an effective bacterial antagonist against *Rhizoctonia solani,* was cultured in the media prepared from cheap agricultural substrates. Number of bacterium and size of endospores of *B. megaterium* were assessed after culturing the bacterium in the broth extracted from potato *(Solanum tabersosum),* cassava root *(Manihot esculenta Crantz),* sweet potato *(Impomoea batatasil* (L.) Poir), rice *(Oryza sativa),* brown rice *(O. sativa),* sticky rice *(O. sativa),* and Job's tears *(Coix lachryma jobi* L.). The highest number of endospores of *B. megaterium* was obtained when the bacterium was cultured in broth medium prepared from brown rice. However, after 3 months storage, number of the bacterium cultured in cassava root remained highest. Bacterium cultured in broth medium prepared from sticky rice provided the highest width of endospores. Nevertheless, types of substrates used for broth medium preparation had no effect on length of the endospores.

Keywords : *Bacillus megaterium,* endospore production

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Introduction

Sheath blight of rice caused by *Rhizoctonia solani* is one of the most destructive rice diseases worldwide (Ou, 1985). Sheath blight control relies primarily on chemical fungicide. There are no reliable rice resistant varieties. Disease control using biological control agents is thus offer an alternative for sheath blight control (Cook, 1993). There were many reports in using bacterial antagonists for controlling sheath blight disease of rice (Mew and Rosales, 1986; Vasantha Devi et al., 1989; Gnanamanikam and Mew, 1990; Gnanamanickam et al., 1992). However, most of the studies have been carried out using fresh cells of bacterial antagonist. This form of bacterial inoculum is suitable for research purpose, but may not be practical for use by the farmers. As a result, some recent studies have been done to devise suitable bacterial formulations and test their efficacy in controlling sheath blight in both greenhouse and field conditions (Kanjanamaneesathian et al., 1998; Kusongwiriyawong et al., 1999; Pengnoo et al., 2000; Wiwattanapatapee et al., 2004a; Wiwatttanapatapee et al., 2004b).

Formulation of bacterial antagonist is the process in which fresh cells of bacterial antagonist were incorporated into the materials that have desired characteristics. The result is the finishing bacterial product that contains suitable number of living bacterium. Furthermore, the product is required to remain effective to suppress disease after passing the formulation process, and can be stored in a period up to 24 months without special treatment. To obtain this goal, the formulation process should be started by incorporating quite high number of bacterial cells into other materials. In addition, resistant structure of the bacterial cells such as endospores should also be used as an initial inoculum in the formulation process. This is because both quantity (number of bacterial cells) and quality (type of bacterial cells) used in the formulation process may influence the quality of the finishing product. For this reason, the study to find suitable substrates to use for medium preparation for bacterial cultivation is required.

Substrates should be abundant, not expensive and able to support growth of the bacterium.

In Thailand, agricultural produces are readily available and can be utilized as a substrate for producing biomass of bacterial antagonists. In this study, we investigated the possibility of using sweet potato (*Impomoea batatasil* (L.) Poir), cassava root (*Manihot esculenta Crantz*), rice, brown rice and sticky rice (*Oryza sativa* L.) and Job's tear (*Coix lachryma jobi* L.) to replace potato (*Solanum taberosum*) as a substrate to culture *Bacillus megaterium.* This study is necessary because potato is comparatively expensive than other produces and may not be suitable for using as a substrate to produce bacterial antagonist for commercial purpose.

In this preliminary investigation, both number of bacterial cells and size of endospores of this bacterium were assessed after culturing and after 3 months storage at room temperature.

Materials and Methods

Bacillus megaterium used in this experiment

B. megaterium was isolated from paddy rice soil collected from Satun province in the South of Thailand (Kanjanamaneesathian et al., 1998). Biochemical and physiological properties of *Bacillus* sp. was determined and the bacterium was identified as *B. megaterium* (Pengnoo et al., 2000). *B. megaterium* produced heat stable antibiotics which could suppress mycelial growth of *R. solani* (Pengnoo et al., 2000). This bacterium had been used in formulation study and its efficacy to suppress sheath blight disease of the product had been tested in both greenhouse and field conditions (Wiwattanapatapee et al., 2004a and Wiwattanapatapee et al., 2004b).

Medium preparation for bacterium cultivation

Potato Dextrose Broth (PDB) was prepared using 200 g of potato, 20 g of dextrose and 1000 ml of distilled water. Potato was

boiled in 500 ml of distilled water for 30 min. After this, the supernatant was obtained and 20 g of dextrose was added. Distilled water was added to this mixture until the volume was at 1000 ml. This broth was autoclaved for 20 min and ready for use for bacterium cultivation when cooled. Broth preparation using other substrates was also made in a similar manner but the same amount of sweet potato, cassava root, rice, brown rice, sticky rice or Job's tear were used to substitute potato. Estimate cost of preparing broth media using these agricultural materials as nutrient sources was also calculated. Time required for heating each substrate before the solid part was discarded and the broth is ready for cultivating the bacterium was also measured.

Cultivation of bacterium in broth media

Single colony of B. megaterium cultured on PDA for 24 h was transferred to flasks containing 100 ml of each broth media prepared as described above. These flasks were incubated at 35-37 ˚C for 4 days on the laboratory bench. After 4 days of incubation, the flasks were heated in water bath at 65 ˚C for 10 min to kill vegetative cells of the bacterium. These broths were then centrifuged at 3,000 rpm for 10 min and the endospores suspended in the reduced volume of the media were stored in the refrigerator for enumeration.

Enumeration of *B. megaterium* endospores after cultivation

Endospores of *B. megaterium* were enumerated after 4 days of cultivation and after 3 months storage at 10 ˚C. The enumeration was carried out on PDA using drop plate technique (Zuberer, 1994). The plates were incubated at room temperature (26-32 ˚C) for 1 day after which colony-forming units were counted. The value of viable bacterium (CFC/ml) was calculated from the average of 4 replications (4 drops) per dilution.

Measurement of *B. megaterium* endospores

Endospores of *B. megaterium* suspended in the broth media prepared from each substrate were mounted on slide. The measurement of bacterial endospores was carried out using ocular micrometer mounted stereo-zoom microscope. Both width and length (micron) of the bacterial endospore was assessed using 50 endospores taken from each broth media. Data of the width and length of the endospores were subjected to one way analysis of variance and compared with Duncan's Multiple Range Test (DMRT) at $P<0.05$ and $P<0.01$using SAS computer package program (SAS Institute Inc., Cary, NC, USA).

Results

Estimate cost of broth medium preparation

Estimate cost for preparing broth media using various substrates was calculated (Table 1). Broth medium prepared from cassava root was the cheapest, followed by those prepared from rice, sticky rice, and sweet potato, while broth medium prepared from Job's tear was the most expensive, followed by that prepared from brown rice, and potato (Table 1).

Table 1 Estimate cost of preparing broth media using various agricultural produces

Types of substrate	Time required for heating substrate (min)	Total cost (baht/L)
Potato	85	49.2
Sweet potato	102	28.0
Cassava root	83	19.9
Rice	60	23.0
Brown rice	80	59.4
Sticky rice	50	24.4
Job's tear	164	70.4

Enumeration of *B. megaterium* endospores after cultivation

B. megaterium cultured in broth prepared from brown rice had the highest number of bacterium after 4 days of cultivation, followed by that cultured in broth prepared from potato, cassava root, sticky rice, rice, Job's tear, and sweet potato (Table 2). After 3 months storage, however, *B. megaterium* cultured in broth prepared from brown rice had declined greatest, followed by the bacterium cultured in broth prepared from potato. However, the number of *B. megaterium* cultured in broth prepared from cassava root remained highest (Table 2). Number of bacterium cultured in other materials, such as sweet potato, cassava root, rice, sticky rice, and Job's tear, remained stable or slightly increased after 3 months storage (Table 2).

Table 2 Number of endospores of *Bacillus megaterium* cultured in broth prepared from various agricultural produces 4 days after cultivation and 3 months after storage

Types of substrate	Number of *B. megaterium* (Log. number/ml)	
	4 days	90 days
Potato	19.18b	16.44e
Sweet potato	16.05f	17.04c
Cassava root	17.84c	17.40b
Rice	16.52e	16.62d
Brown rice	20.56a	18.61a
Sticky rice	16.67d	17.05c
Job's tear	16.15f	16.44e
C.V. (%)	0.31	0.29

Means in each column with difference letters are significantly different at $P<0.05$ by DMRT.

Measurement of *B. megaterium* endospores

Width of bacterium cultured in broth prepared from sticky rice was greater than that of bacterium cultured in broth prepared from cassava root and brown rice (Table 3). Length of bacterium cultured in broth prepared from potato, sweet potato, rice, sticky rice and Job's tear had no statistical difference (Table 3).

Table 3 Size of endospores of *Bacillus megaterium* cultured in broth prepared from various agricultural produces

Types of substrate	Width (µ)	Length (µ)
Potato	0.85ab	1.97
Sweet potato	0.87ab	1.95
Cassava root	0.76b	1.99
Rice	0.85ab	1.97
Brown rice	0.81b	1.97
Sticky rice	0.95a	1.97
Job's tear	0.85ab	1.95
C.V.(%)	17.71	6.39

Means in each column with difference letters are significantly different at $P<0.05$ by DMRT.

Discussion

Development of a suitable medium using inexpensive, readily available agricultural by-products with the appropriate nutrient balance is one of the major steps after effective biological control agent has been selected. These by-products include molasses and brewer's yeast (Lumsden and Lewis, 1988).

In Thailand, molasses had been used for producing biomass of *B. subtilis* to control the rice blast and rice sheath blight diseases. Molasses as carbon source in conjunction with ammonium sulphate as nitrogen source was suitable for culturing *B. subtilis* strain NSRS 89-24. This bacterial strain still remained capable of

producing antibiotics effective in inhibiting both *Pyricularia grisea* and *Rhizoctonia solani.* However, although molasses is generally consistent in composition but there may be problems in biomass formation if there are major disparities in batches of this substrate (Lumsden and Lewis, 1988). This characteristic may have a negative effect of utilizing this by-product for bacterial cultivation. For this reason, agricultural materials can be a better choice because nutrients extracted from these substrates are less likely to vary drastically between batches.

In this study, we found that all the selected substrates supported the growth of *B. megaterium* very well. However, cassava root was more preferable than other materials because the cost of cassava broth was quite lower than that of other materials. Nevertheless, time required for heating this substrate before it was ready for use was quite long, but it was not longer than that required for heating potato and sweet potato.

The number of *B. megaterium* cultured in broth prepared from cassava root was quite high after cultivation in the broth for 4 days and 3 months storage (Table 2). This level of inoculum was sufficient for use in the formulation process. Size of endospores of *B. megaterium* cultured in broth prepared from cassava root was as large as that cultured in broth prepared from potato, sweet potato, rice, brown rice, and Job's tear (Table 3). For successful production of biological control agent, sufficient biomass containing adequate amounts of effective propagules must be obtained. For fungal biological control agents, the products were effective in disease control when formulation was prepared using chlamydospore, a resistant and survival propagules of the fungus (Lumsden and Lewis, 1988). Chlamydospores also allowed greater proliferation of the fungal biological control agents in soil than the formulations containing conidia (Lewis and Papavizas, 1984). Endospores of the bacterium are better than vegetative cells in being used for the formulation preparation because endospores are the site where antibiotics have been produced (Wiwattanapatapee

et al., 2004a). The end result is that the finishing product contains not only effective bacterial propagules, but also has the potential to store for a reasonable period after formulation.

In conclusion, cassava root has potential to use as a substrate for broth preparation for bacterial cultivation. The number of vegetative cells and size of endospores of *B. megaterium* obtained from broth prepared from cassava root are similar to those obtained from broth prepared from other materials, except sticky rice (Table 2 and 3). However, it costs a lot less than other materials (Table 1).

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