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FERMENTATION OF A NEW *Bacillus thuringiensis* STRAIN

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SUMMARY

A new *Bacillus thuringiensis* strain, 00-50-5, exhibited an unusually high activity against banded sunflower moth (BSM) *Cochylis hospes*, which is an economically important pest of cultivated sunflowers in North America. The strain was fermented with a modified commercial medium in a bioreactor. This was a successful trial for production of 00-50-5 on a larger scale. After fermentation, the yield of dried powder was 5.8 g/l broth. In the laboratory assays, the LC_{50} (lethal concentration required to kill 50% of targets) of the dried powder was 0.5 µg/ml 3 d after exposure against four instars of BSM. Some practically useful conditions and parameters for the fermentation operating procedure and methods for harvesting were determined.

Key words: fermentation, *Bacillus thuringiensis*, sunflower

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive and spore forming bacterium capable of producing crystals during sporulation. The major characteristics of the bacterium is the production of crystals which contain pesticidal crystal proteins (Höfte and Whiteley, 1989; Schnepf *et al.*, 1988). Bt-based products make up more than 90% of all biological pesticides sold in the world today. Thousands of Bt strains have isolated from nature, but few of them have been commercialized. The active ingredients in most Bt commercial formulations are based on Bt toxins of serovar *kurstaki*, *thuringiensis* and *aizawai* (Glare and O'Callaghan, 2000). Each Bt strain or crystal toxin has a different pesticidal activity spectrum (Bai *et al.*, 1993).

Banded sunflower moth (BSM), *Cochylis hospes* Walsingham, is an important economic pest of cultivated sunflower, *Helianthus annuus* L., in North America (Charlet *et al.*, 1997). Some Bt commercial products, including Cutlass AF (Ecogen) and Javelin WG (Sandoz), were effective against first-instar BSM larvae (Jyoti

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and Brewer, 1999). However, Bt *serovar kurstaki* toxin was not effective against BSM larvae (Barker, 1998; Bai *et al.*, 2001).

Recently, we have isolated several new entomopathogens, including bacteria and fungi from sunflower plants and sunflower insect cadavers. In laboratory assays, they showed activity against sunflower beetles and moths. A crystal-producing strain, 00-50-5, exhibited an unusually high activity against BSM larvae (Bai *et al.*, 2001).

Before a new Bt product is successfully registered in the United States and other countries, attention has to be paid to safety, mass production, storage, formulation, standardization and application technology. The purpose of this study was to establish fermentation conditions for practical scale up production of Bt 00-50-5.

MATERIALS AND METHODS

Bacterial cultures

Bt strain 00-50-5 was isolated from sunflower head extract (Bai *et al.*, 2001). A single colony of 00-50-5 was inoculated and grown on nutrient agar (NA, Difco) as the culture medium in a petri dishes for 24 h at 30°C. The colonies were inoculated into 500 ml flasks containing 100 ml nutrient broth (NB, Difco) as culture medium for 8 h at 30°C on an incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ) running at 250 rpm. Then the cultures in the mid-logarithmic growth phase and containing approximately 10^8 cells/ml were transferred into either 400 ml NB or 2 x NB in 4 l flasks for 48 to 72 h at 30°C on the same shaker running at 250 rpm until most of the spores and crystals were released. The ratio of spores and crystals in the Bt broth was determined. The mixture of Bt spores, crystals and vegetative cells was harvested by centrifugation (Sorvall RC5 centrifuge) of the broth for 20 min at 12,210 g at 4°C. The pellet was frozen in dry ice and lyophilized. Crystal morphology of the isolate was examined by phase-contrast microscopy and scanning electron microscopy.

Scaled up fermentation with commercial medium

A single colony of strain 00-50-5 was inoculated and grown on a NA medium in two petri-dishes for 24 h at 30°C. The colonies were inoculated into 500 ml flasks containing 50 ml NB for 8 h at 30°C on a rotary shaker running at 330 rpm. Then the cultures were transferred into 400 ml NB in 4 l flasks under cultured for 16 h at 30°C on a large rotary shaker running at 225 rpm. The flask cultures then were inoculated into a NBS 75 l bioreactor containing 54 l broth of commercial medium (CM) for 27 h at 30°C until most of spores and crystals were realized. Total culture broth was 55 l. CM was design as a culture medium for 00-50-5 fermentation (Table 1) based on a medium used to produce Bt HD-1 and modified from a medium described by others (Lisansky *et al.*, 1993). Some parameters for the con-

trol of operation during the fermentation are listed in Table 2. The fermentation was terminated when the ratio of spores to crystals was <1. Quality was determined by microscopic examination using a phase light microscope with oil immersion objective (x 100) after staining. Bt broth was cooled to 20°C and then harvested with a semi-continuous-flow centrifuge (Pennwalt Corporation, Warminster, PAA). The speed of the pump was 2 to 3 l per min. A wet pellet was formed and collected, frozen in liquid N_2 and shipped in dry ice. Finally, the wet pellet was stored at -80°C or lyophilized.

Table 1: Ingredients of a commercial medium (CM) used to produce *Bacillus thuringiensis* strain 00-50-5 in a 75 l bioreactor

1.	Tryptone	10 g/l
	Powder corn starch	5 g/l
	Yeast extract	2 g/l
	KH_2PO_4	0.37 g/l
	K_2HPO_4	1.6 g/l
	Mazu DF 204 (anti-form)	5.2 ml/52 l
	d H_2O	52 l, sterilized
2.	Glucose	5 g/l
	d H_2O	0.5 l, autoclaved
3.	Trace mineral metals	
	$Mg_2SO_4.7H_20$	0.3 g/l
	$MnSO_4.H_2O$	0.1 g/l
	$FeSO_4.7H_2O$	0.001 g/l
	$ZnSO_4.7H_2O$	0.01 g/l
	d H_2O	0.5 l, autoclaved
Mix 1, 2 and 3. After inopculaion, total broth volume in the fermenter was 55 l		

Laboratory assays

Rearing of BSM was conducted at 26°C, 55% R.H., and 15:9 L:D cycle in environmental chambers. The insects were originally collected from a field in North Dakota and the colonies maintained in the laboratory for several years. Larvae were fed on an artificial diet (Barker, 1988). The detailed methods for laboratory assays were described previously (Bai *et al.*, 2001). The mortalities were recorded, pooled, and calculated with LeOra software (1987).

RESULTS

Isolate 00-50-5 contained both spores and crystals that were realized from the rod-shaped vegetative cells. Therefore, it was identified as a Bt strain based on the crystal formation (Heimple and Angus, 1958). Analysis of the strain proteins by SDS-PAGE also confirmed that 00-50-5 produced a 60 kDa toxin protein and a 27 kDa protease (C. Bai, unpublished data).

In the laboratory culture, when 2x NB was used for growing 00-50-5, the maximum absorbance of the broth at 660 nm was 2.3. After culture with either NB or 2x NB in the laboratory, the culture broth 00-50-5 consisted of 48% crystals, 48% spores and 4% vegetative cells. Examination with a scanning electron microscope showed that the 00-50-5 crystals were multiform shaped, including small bipyramidal, tetragonal and incomplete.

Table 2: Operating conditions and parameters of *Bacillus thuringiensis* strain 00-50-5 fermentation in a 75 l NBS bioreactor[1]

	Age (hour)	pH	Dissolved oxygen (%)	Agitation (rpm)	Flow meter (l/m)	Air pressure	Temperature (°C)	Oxygen (l/m)	Absorbance at 660 nm	Volume (l)
Setup		7.00	>25	400	55	5.0	30.0	0.0		55
Record	0	7.00	100	400	55	5.0	30.0	0.0	0.31	55
	27	7.02	98.8	400	55	5.0	30.0	0.0	18.5	55

[1]The following parameters, including age (h), pH, dissolved oxygen (%), agitation, flow meter (l/m), air pressure, temperature, oxygen (l/m), absorbance and volume were recorded. Agitation was fixed at 400 rpm, flow meter was 55.0 l/m, air pressure was 5.0, temperature was 30.0°C and oxygen was zero. Dissolved oxygen in the broth was controlled and maintained at more than 25% during the fermentation. The pH was adjusted and maintained at 7.00. After sterilization, the pH was adjusted with 5 M sodium hydroxide or sulfuric acid automatically. The ratios of total number of spores/crystals/ vegetative cells in the broth were also calculated.

In total, 27 h were required for the fermentation of 00-50-5 in the bioreactor under controlled conditions (Table 2). The absorbance at 660 nm for Bt broth was 0.30 at o h and increased very fast after 4 to 10 h from 1.3 to 21.6 (maximum). From 14 to 18 h, the absorbance was stable. Afterwards it slightly decreased to 18.8 at 14 h when CM was used as medium for Bt 00-50-5 fermentation. The time for the logarithmic growth phase was within 10 h. When the fermentation was terminated after 27 h, the absorbance was 18.5 (Table 2; Figure 1A).

Fermentation was controlled so that dissolved oxygen in the Bt broth were more than 25%, which is required for Bt growth. Initially, it was 100.0% for 0 h and recorded as 32.0% after 10 h, indicating the amount of dissolved oxygen consumption was increased very quickly during this period. Finally, it was recorded as 98,8% at 27h, showing that the bacteria consumed very little dissolved oxygen at the time (Table 2; Figure 1B). Bt crystals started forming after approximately 20 h. Only 15% crystals (% = total number of crystals x 100/total number of spores + total number of crystals + total number of vegetative cells) were present in the broth after 22 h. Afterwards, the crystals were 33% for 24h and 48% for 26 h, respectively. The broth contained 42% spores, 50% crystals and 8% vegetative cells after fermentation for 27 h with CM in the bioreactor (Figure 1C). Microscopic examination of the fermentation broth showed the presence of only Bt spores, crystals and vegetative cells and no contamination by other bacterium or bacterial phage, indicating that the colonies (seeds) of the bacteria for inoculation were pure

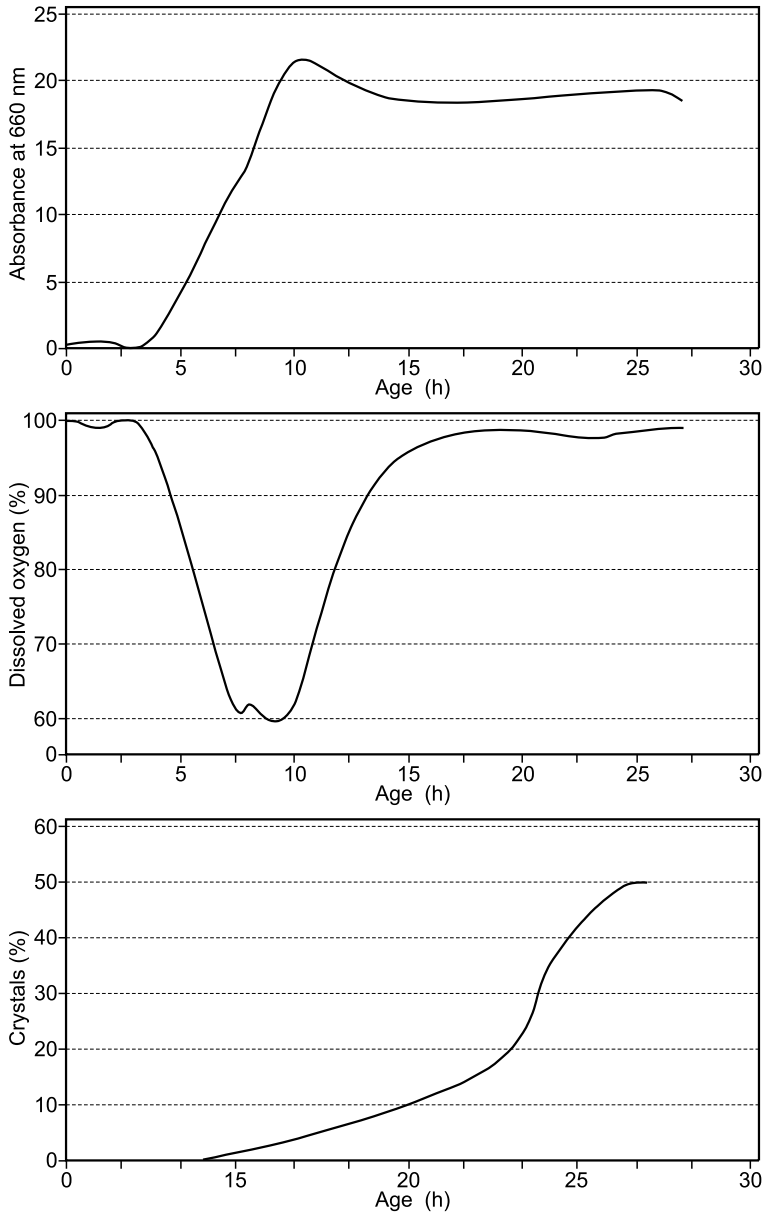

Figure 1: Changes of parameters in the broth during the fermentation of Bacillus thuringiensis strain 00-50-5 in a 75 l NBS bioreactor.
***a)** absorbance at 600 nm*
***b)** dissolved oxygen (%) in the broth*
***c)** crystals (%): total number of crystals x 100/total number of spores + total number of crystals + total number of vegetative cells*

and the operation conditions used for fermentation were workable (Table 2). The examination showed that the Bt broth contained mainly released spores and crystals, demonstration that the CM used to produce 00-50-5 could supply enough nutrients for bacterial growth and cell lysis to release crystals during the fermentation.

After harvesting by the semi-continuous-flow centrifugation, the wet pellets were composed of 44% spores, 45% crystals and 11% vegetative cells. Microscopic examination indicated a few spores and some crystals present in the supernatant, especially small crystals. However, the weight of small crystals and spores in the supernatant was less that 10% when compared with the pellets that had been centrifuged at high speed (10,810 *g*, 20 min) in a Sorvall RC5C centrifuge. The total weight of a wet pellet was approximately 1.6 kg and the dried weight in total was about 320 g. The yield of the dried powder was about 5.8 g/l broth. After the dried powder was suspended in water, the color was white.

In the laboratory assays, the LC_{50} (lethal concentration required to kill 50% of targets) was determined with fourth-instar larvae of the BSM. After inoculating Bt on the surface of the artificial diet pieces, the LC_{50} value was 0.5 µg/ml 3 d after exposure, indicating that the new Bt is very toxic to BSM larvae.

DISCUSSION

The insecticidal activities of commercial Bt products are based on Bt strains. Bt products, e.g. Dipel and Thuricide, are formulated from Bt *kurstaki* HD-1 standard strain (Glare and O'Callaghan, 2000). The *kurstaki* toxins are effective to many lepidopteran species, but not to BSM (Bai *et al.*, 2001). Thus, a new effective strain 00-50-5 was isolated in the laboratory and produced in a bioprocessing center with a 75 l bioreactor for use in a medium-scale trial. Bt strains are usually cultured without difficulty and we are now engaged in a study of optimum culture conditions for Bt 00-50-5 fermentation. The study demonstrated that we have selected a suitable commercial medium and capable conditions for the growth of 00-50-5 in the bioreactor. This was a successful trail for eventual commercial production of 00-50-5 on a large scale.

Corn starch was used in the medium as an inexpensive carbon source and for the purpose of UV protection, but it caused considerable foaming during sterilization. Therefore, appropriate amounts of anti-foaming agent had to be added into the bioreactor in order to control the operation.

The product quality not only relied on fermentation, but also relied on the method of harvest. Continuous centrifugation has the advantage of less expense and speed of harvest. However, in this study some small crystals of 00-50-5 were lost due to harvesting by the semi-continuous-flow centrifuge because the machine we used was unable to generate sufficient centrifugal force (10,810 *g*), as judged by its unclear supernatant. As a results some crystals remained in the supernatant. This

is particularly the case with the Bt strains that produce small crystals. In this situation, other methods of harvest should be considered, such as the use of chemical agents for precipitation or spray drying (Lisansky *et al.*, 1993).

For the majority of Bt strains, the crystals contain most of the pesticidal toxins. Therefore, it is critical to control the ratio of the crystals to the spores and vegetative cells during the Bt fermentation. If the ratio reaches 50% or just more than that, it is time for terminating the fermentation. At this time, the structure of the Bt crystals is strong. If the harvest time is too late, the structure of the crystals becomes weak: therefore, it may lead to crystal dissolution or hydrolysis by the bacterial protease produced by the Bt itself, and perhaps become soluble toxins. In that case, a method called isoelectric point (PI)-precipitation should be introduced for the harvest.

Usually, pH curves for Bt in a fermenter show a distinctive pH pattern during the growth cycle. In the initial part of the pH-curve these is a slight drop. Afterwards there is a rise, reaching to pH 9 before the termination of fermentation. In this study, the pH was controlled and maintained at 7.0. Initially two phosphate salts were added to the broth for buffering. Only a little alkali and acid were consumed in order to adjust the pH to 7.0 during the fermentation. As recorded, the pH was controlled in a range of 6.97 to 7.11 and the bacteria grew very fast under such conditions as reflected by absorbance values in the broth.

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FERMENTACION DE LA VARIEDAD NUEVA *Bacillus thuringiensis*

RESUMEN

La variedad nueva *Bacillus thuringiensis*, 00-50-5, mostro una eficacia inopinadamente alta contra la polilla del girasol (BSM), *Cochylis hospes*, que es un parasito economicamente importante en America del Norte. La variedad fue fermentada por medio de la solucion comercial modificada, en el bioreactor. Eso fue una prueba con exito para la produccion de la variedad 00-50-5 en cantidades mas grandes. Despues de la fermentacion, el rendimiento del polvo seco fue de 5.8 g/l de caldo. En las condiciones de laboratorio, LC_{50} (concentracion letal necesaria para matar 50% de insectos apuntados) de polvo fue de 0,5 μg/ml 3 dias despues de la exposicion al efecto de cuatro estadios de metarmofosis de BSM. Fueron comprobados las condiciones y los parametros practicamente utiles para el procedimiento de fermentacion y los metodos de recoger el polvo.

FERMENTATION DE LA NOUVELLE RACE *Bacillus thuringiensis*

RÉSUMÉ

La nouvelle race *Bacillus thuringiensis*, 00-50-5 a montré une efficacité inattendue contre l'insecte nuisible au tournesol (BSM), *Cochylis hospes*, ce qui est d'un grande importance économique en Amérique du Nord. La race a été fermentée à l'aide d'une solution commerciale modifiée, en bioréacteur. Cet essai a été probant pour la production de la race 00-50-5 en grandes quantités. Après la fermentation, le rendement de poudre sèche était de 5,8 g/l de suspension pâteuse. Dans les conditions de laboratoire, quatre pré-chrysalides BSM ont été exposées à 0,5 μg/ml de LC_{50} (concentration létale nécessaire pour éliminer 50% des insectes visés) de poudre sèche et l'effet de la préparation a été constaté trois jours plus tard. Des conditions et des paramètres d'utilisation pratique ont été établis pour la procédure de fermentation et la méthode de collecte de la poudre.