Scientific Research

Solid-State Fermentation for the Concomitant Production of δ-Endotoxin and Endospore from *Bacillus thuringiensis* subsp. *kurstaki*

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Abstract

Water stress and limited aeration imparted by solid-state fermentation (SSF) were reported as crucial factors for the enhancement of endospore production by *Bacillus thuringiensis* (*Bt*); and thus, more δ-endotoxin could be produced concomitantly with reduced time. Therefore, *Bt* subsp. *kurstaki* (*Btk*) was employed in the present study to evaluate its efficiency for the concomitant production of endospores and δ-endotoxin in LB medium supplemented with various naturally available agricultural products, *i.e.*, flours of soybean, Bengal gram or jack seed at various concentrations (10%, 20%, 30%, 40%, 50%, 60%, 80% or 100%, all w/v). After 12 h fermentation, the supernatant in it was centrifuged off aseptically to obtain solid substrate for subsequent SSF. Of them, soybean (30%) supplemented medium was the best for the enhanced production of endospore and δ-crystals. The maximum yield of endospores during solid-state fermentation was observed 48 h, *i.e.*, compared to submerged fermentation in LB, it was 24 h less gestation period. In control sample, the endospores achieved the maximum length (1.10 ± 0.13 μm) and diameter (0.63 ± 0.07 μm) at 72 h; while in soybean supplemented medium, the maximum length (2.10 ± 0.16 μm) and diameter (1.63 ± 0.16 μm) were at 48 h and 72 h, respectively. Upon staining, acridine orange specifically stained the endospores; malachite green-saffranin stained both δ-crystals and endospores; and coomassie brilliant blue specifically stained δ-endotoxin. Briefly, normal gestation period or harvest time for *Btk* is 72 h, which could be reduced to 48 h, if SSF is employed as demonstrated in this study.

Keywords

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1. Introduction

Bacteria of the gram positive genus *Bacilli* include a variety of commercially important species responsible for the production of a range of products including enzymes, antibodies and insecticides. *Bacillus thuringiensis* Berliner (*Bt*) is a ubiquitous Gram-positive and sporulating bacterium producing insecticidal crystal proteins (the δ-endotoxin) juxtaposed to the endospores in the bacterium (sporangium) during the stationary phase of its growth cycle [1]. The toxicity of *Bt* is due to its capacity for the production of a crystalline protein (the δ-endotoxin) concomitantly with endospore; and this observation led to the development of bioinsecticides, based on *Bt* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera [2]. *Bt* isolates are also found active against nematodes, mites and protozoa [3].

The term solid-sate fermentation (SSF) denotes cultivation of microorganisms on solid, moist substrates in the absence of a free aqueous phase (water). SSF has been developed in eastern countries over many centuries for the large scale production of microbial products including primary and secondary metabolites [4]. Conventionally, commercial production of *Bt* toxin has been achieved by submerged or liquid fermentation (SmF), or by batch or fed-batch process [5], but advantages of SSF for the production of both primary and secondary metabolites of microbial origin have well been appreciated by many investigators [6] [7]. Compared to SmF, SSF received more attention recently, as it uses simpler fermentation medium and requires a smaller space, easier to aerate, lower waste water output, lower energy requirement and contamination, higher productivity, extended stability of the products and low production costs [4].

Staining is the conventional method for distinguishing endospore from δ-crystals, and also for monitoring the time-dependent production pattern. Schichnes *et al.* [8] developed a quick and simple technique for the fluorescent staining of bacterial endospores. This technique was developed using *B. subtilis* endospores. Sharma and Prasad [9] used the *Bt* strains and proposed the use of aqueous acridine orange to differentiate non-viable (lemon green) and viable (orange red) spores in the culture. Rampersad *et al*. [10] used a parasporal body staining, *i.e.* coomassie brilliant blue. Based up on this background, the objective of the present study was to evaluate the visually and numerically concomitant production pattern of endospore and δ-endotoxin by *Btk* in the Luria-Bertani (LB) medium supplemented with, naturally available agricultural products (flours) such as soybean, Bengal gram or jack seed.

2. Materials and Methods

Source of organism and medium: The standard strain of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) was procured from IMTECH, Chandigarh (Strain designation: BA 83B, MTCC number: 868), maintained in LB medium. The seed culture (12 h old) was streaked on the LB-agar medium in the petri-dish. For the preparation of overnight seed culture (12 h), single colony from LB-agar plate was inoculated in to the LB broth, 50 µL seed culture was used to inoculate 10 mL sterilized production medium, which was equivalent to 6.5×10^7 cfu per 1 mL medium. For SmF, the medium was incubated at 37˚C with constant shaking (150 rpm, and initial pH 7.0) in a temperature-controlled shaker (Orbitek, India). For SSF, the samples were incubated in an oven (Technicho, India) at 37˚C.

2.1. Production of Endospore and δ-Endotoxin by SmF

The production of spore and crystal in the medium was monitored by supplementing the LB medium with various naturally available stored plant foods, *i.e.* soybean flour, and Bengal gram flour and jack seed flour (1% w/v 12 h to 72 h).

2.2. Production of Endospore and δ-Endotoxin by SSF

Spore and crystal production was done with various raw substrates as supplements. The LB medium was supplemented with flour of soybean, Bengal gram or jack seed at different concentrations of w/v (1%, 5%, 10%, 20% 30%, 40%, 50%, 60%, 80% and 100%). The supplements were mixed well, autoclaved and incubated at 37˚C in an incubator. For maximization of crystal and spore production, the fermented matter at 12 h was centrifuged at 800 g for 10 min in a refrigerated centrifuge. This step was to remove free supernatant from the fermented matter, so as to shift the cultivation phase to SSF. The pellets were collected aseptically and incubated further at 37˚C.

2.3. Preparation of Sample for Staining

Raw pellet at different time intervals was used for analysis by staining. For this, the culture was washed in sterile distilled water and after thorough mixing (5 min), the supernatant was removed by centrifugation (9400 g, 10 min, 4˚C). This process was repeated trice, and the pellet so obtained was used for staining and spore counting.

2.4. Malachite-Green Staining

Malachite-green staining technique was used for spore staining [11]. Bacterial smears were prepared in the usual manner using standard sterile techniques. The smear was allowed to air dry and, heat-fixed at 60˚C in a hot air oven. Smears were flooded with malachite-green and placed on a warm hot plate allowing the preparation to steam for 10 min, cool, and wash under running tap water; then counter stained with safranin for 1 min; washed with running tap water and air-dried. The slides were observed under the binocular microscope (100× magnification).

2.5. Acridine-Orange Staining

The acridine-orange staining technique was used as demonstrated by Laflamme [12]. Ten μL of 0.1 μg/mL acridine orange staining solution was applied to the area of smear and covered with a cover-slip.

2.6. Coomassie Brilliant Blue (CBB) Staining

The crystal toxin (δ-toxin) was stained with CBB solution (0.25% CBB, 50% ethanol, and 7% acetic acid) for 3 min, washed in tap water, and visualized by the Image Analyser fitted with Nikon digital camera.

2.7. Measurement of Spore Size

The length and diameter of the endospores were measured using an ocular and stage micrometer attached to Olympus CX 21 binocular microscope. Hundred μL of the serially diluted sample was spread on nutrient agar plate, and incubated at 37˚C for 12 h in an incubator.

2.8. Visualization and Endospores

The spores stained by malachite-green were visualized by the Image Analyser fitted with Nikon digital camera. The spores stained with acridine orange [12] were visualized using Olympus fluorescent microscope.

3. Results

This study makes a comparison of the concomitant production of endospore and δ-endotoxin by *Btk* in LB (control) against 30% (w/v) soybean flour supplemented LB, 30% (w/v) Bengal gram flour supplemented LB and 10% (w/v) jack seed flour supplemented LB. These specific media combinations were chosen based on the best performance of the flour-supplemented media in terms of the production of endospore and δ-endotoxin. For enhancing the production free water was removed from the medium at 12 h by centrifuging at 800 g for 10 min in a refrigerated centrifuge and the pellets were collected and incubated further at 37˚C. Microscope-aided spore counting and staining techniques were used to demonstrate the results.

Three staining techniques were used, *viz.*, malachite green-safranin, acridine orange and coomassie brilliant blue; the former two were specific for visualizing endospores and the latter was for δ-endotoxin. As shown in **Figure 1**, concomitant production of endospore and δ-endotoxin is presented in 4 vertical panels with 3 rows indicating harvest time (24, 48 and 72 h). Many trials were made for monitoring the growth patterns of *Btk* in different media combinations comprising different percentages (1%, 10%, 20%, 30%, 40%, 50%, 60%, 80% and 100%) of flours in LB. For simplicity of data presentation, only the best results out of various percentages of soybean flour, Bengal gram flour and jack seed flour supplements are shown in the **Figure 1**. First panel represents LB (control), second panel represents 30% soybean flour supplemented LB, third panel represents 10% jack seed flour supplemented LB, and the fourth panel represents Bengal gram flour supplemented LB media. Horizontal panels represent harvest time. Acridine orange specifically stain endospore, and malachite green was

best staining technique for both crystal and endospore, but crystals clearly stained with coomassie brilliant blue.

3.1. Size of Endospores

For cross-comparison, the length and diameter of endospores (only LB control and 30% soybean flour supplemented media) were also measured (Table 1). The size of endospores harvested at 24 h time intervals from the soybean flour-supplemented LB medium was compared with that of LB control. The control sample showed that the endospores achieved the maximum length (1.10 ± 0.13 µm) and diameter (0.63 ± 0.07 µm) at 72 h. However, in soybean supplemented medium, the maximum length (2.10 ± 0.16 µm) and diameter (1.63 ± 0.16 µm) were achieved at 48 h and 72 h. It further showed that the length and diameter of endospores formed in soybean flour supplemented medium were almost double the size of endospores produced in the control. Moreover, the endospores achieved maximum size in about 48 h in soybean supplemented medium, as against 72 h in LB control. It clearly indicates the advantage of solid-state fermentation, this was also true in the case of the number of endospores and vegetative cells, *i.e.*, as seen in soybean flour supplemented media.

3.2. Production Pattern of Endospore and δ-Endotoxin at 24 h

At 24 h, almost all cells in soybean supplemented medium contained endospores. A few cells started rupturing to release the endospores. Size of the spore was much bigger in samples with higher concentration of soybean flour (20%, 30%, 40%, 50%, 60%, 80% and 100%), and number of spores was higher in 30% soybean flour supplemented medium; and the number of parasporal bodies (crystals) was equal to that of the endospores, because both are produced concomitantly (Figure 1). The results obtained from Bengal gram (30%) supplemented LB medium was comparable to the data obtained from soybean flour supplemented medium, but number of endospore production was less than that of the pattern in the latter. Compare to the soybean flour and Bengal gram flour supplemented media, jack seed flour supplemented medium showed different pattern of results; *i.e.*, endospore was not that much bigger in size, and number of released endospores are less in number. However, in control, most of the cells were intact with endospores, and size of endospore was smaller than that in flours supplemented media (Figure 1).

3.3. Endospore and δ-Endotoxin Production Pattern at 48 h

The 48 h samples contained higher number of endospores in all the samples, except control. At this stage, most of the sporangia were lysed and the endospores were exposed, highest numbers of endospores were noticed in soybean flour supplemented medium, parasporal bodies or crystals were clearly observed in this stage as darkly stained bodies with safranin, while dark-blue with CBB staining (Figure 1). The number and size of endospores and crystals obtained from Bengal gram flour supplemented medium were similar to that of soybean flour supplemented medium, and in jack seed flour supplemented medium, the size and number of endospores and crystals were smaller and lesser, respectively than the other two supplements. In soybean supplemented medium, the maximum size and exposed endospore and crystals were seen at 48 h and 72 h, respectively, which were much higher in size and number than those obtained in the LB medium.

3.4. Endospore and δ-Endotoxin Production Pattern at 72 h

At 72 h, release of spores and crystals was more or less complete in all the natural flour supplemented media. In fact, the release pattern of endospores and crystals in LB medium at 72 h was similar to those obtained at 48 h cultivation in soybean flour supplemented medium, as clearly seen in both malachite green-safranin and CBB

Table 1. Time-dependent variations in the average length and diameter of endospores (µm) produced in LB (control) and 30% soybean flour supplemented LB.

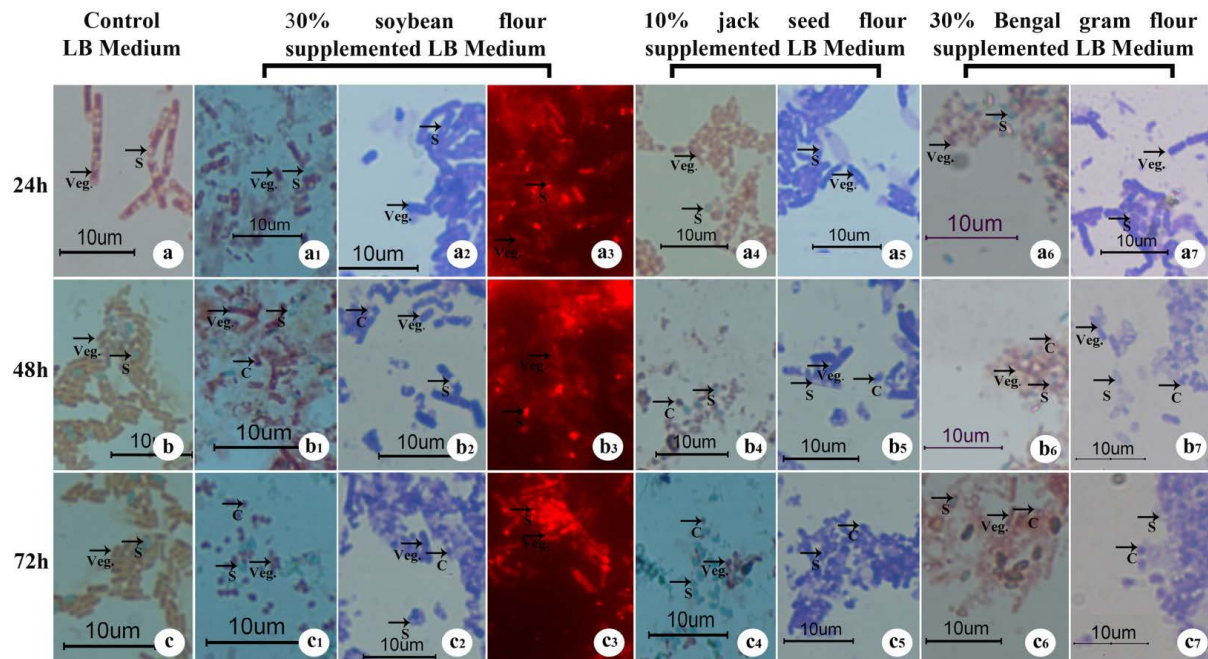| Sample	Harvest Time					
	24 h		48 h		72 h	
	Diameter	Length	Diameter	Length	Diameter	Length
LB	0.25 ± 0.05	0.75 ± 0.15	0.55 ± 0.09	0.75 ± 0.11	0.63 ± 0.07	1.10 ± 0.13
30% Soybean Flour + LB	0.87 ± 0.07	1.25 ± 0.13	1.34 ± 0.25	2.10 ± 0.16	1.63 ± 0.16	2.10 ± 0.28

Figure 1. Pattern comprising frequency of vegetative cells, endospores and, δ-endotoxin. Time dependent (24, 48 and 72) vegetative cells, endospores and, δ-endotoxin production pattern of *Btk* in LB control against 30% soybean flour supplemented LB and 10% jack seed supplemented LB and 30% Bengal gram supplemented LB. The patterns of *Btk* growth and concomitant production of endospore and endotoxin are presented in 4 vertical panels, first panel shows LB control, second panel shows 30% soybean supplemented LB, third panal shows 10% jack seed flour and fourth panel shows 30% Bengal gram supplemented LB. The staining pattern also marked on the top of each column, as Malachite Green-Saffranin (MG-S), Acridine Orange (AO), Coomassie Brilliant Blue (CBB). MG-S and AO specifically stain endospores and CBB for δ-endotoxin. Production pattern of vegetative cells (Vg) spores (S) and crystals (C) are evident from figure.

stained samples (**Figure 1**). At this stage release of endospores was almost complete in all the flour supplemented media, while in LB medium many intact sporangia could be seen.

4. Discussion

Bacillus spp. are known to produce endospores [2]. Sporulation process is induced by starvation or adverse environmental conditions indicating the end of their life cycle; which is controlled by many factors—such as unfavorable environmental conditions like desiccation, heat, and ultraviolet radiations and unavailability of carbon and nitrogen sources [13]. These spores are metabolically dormant and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals [14]. Many important by-products (solvents, antibiotics, enzymes, insecticides, *etc*.) are produced by spore-forming bacteria. Typically, the maximum rate of synthesis of these products occurs during the sporulation process [15].

Malachite-green, a low cost stain is commonly used for staining spores, which specifically stains endospores in to greenish-blue, thus facilitate fast visual screening for endospores and crystals. In the present study, malachite-green was used as the primary stain for staining the endospores; the vegetative cells were pinkish upon counter staining with safranin and crystals also darkly stained with safranin. Hamouda *et al*. [16] stained endospores of *Bt* at different stages of germination with malachite green and safranin, and found that with malachite-green, spores were stained greenish-blue and vegetative cells took pink coloration by safranin. Chilcott *et al*. [17] studied the endospore production pattern of *Bt* serotypes and found that cells and spores of all *Bt* serotypes appeared phase light under phase-contrast microscopy, while the crystals appeared phase dark.

Schichnes *et al*. [11] developed a quick and simple fluorescent staining (acridine orange) technique for endospores produced by *B. subtilis*, and this stain was used to differentiate viable and non-viable spores [12]. Fadel and Sabour [18] cultivated four strains of *Bt* on locally available sugar cane molasses for monitoring the production profile of bioinsecticide.

Bacteria produce endospores normally at the senescence of their growth phase [12] [19], mainly due to the unavailability of carbon and nitrogen sources [13]. Sporulation time and size of endospore and the rupturing of sporangial wall are important aspects to be considered, because δ-endotoxin production is concomitant with sporulation. In the present study, development of sporangia was seen even at 12 h (especially in flour supplemented media), and rupturing of sporangia started in the natural flour supplemented samples from 24 h cultivation onwards. In natural flour supplemented media, most of the spores were released at 48 h cultivation, but in LB control, similar trend was observed after 72 h cultivation. General trend was that *Bt* strains take 3 - 5 days for the maximum production of toxins under the normal culture conditions by SmF. However, Chestukhina *et al.* [20] showed that *Bt* produced copious amounts of spores and crystals at 48 h. The mature spores were eventually liberated by lysis of the mother cell (sporangia). The entire process of lysis takes place over a period of 6 - 7 h and requires the temporal regulation of more than 50 unique genes [20].

Since sporulation and germination in *Bacilli* are dependent on the nutritional status of the organism [21], a study on the nutritional requirement of *Bt* is important for delineating the control mechanisms which regulates the formations of spore and parasporal crystal. Certain amino acids support growth, sporulation and crystal formations in *Bt* [21]. In the present study, soybean and Bengal gram flour supplemented media showed the highest number of spores with much enhanced size and shape. In this enriched media, sporulation starts at 12 h onwards, similar result was noticed in the industrially used medium TSB (Tryptocase Soy Broth) and in the sludge; the sludge achieved the highest number of spores was ISWWTP (industrial sludge waste water treatment plant) with 2.16×10^8 cfu/mL [22]. Variables like physical and chemical constitution, total solid concentration, available oxygen, initial volume of inoculum, or temperature are common to limit bacterial growth. In this batch fermentation, ideal parameters for *Bt* growth were used [23]-[26].

In the present study, we proposed SSF for the maximum yield of endospore and endotoxin without agitation; we suppose this is the reason for the decrease in production time. The highest yield obtained should be due to the anaerobic condition and water stresses developed in the solid medium during fermentation. Similar results were previously reported with other strains of *Bacillus* and other subspecies of *Bt*. The toxicity (due to δ-endotoxin and endospore) was about four times higher under non-aerated conditions [27]. Sarrafzadeh and Navarro [28] observed the highest concentration of spores being 100% mature under anaerobic conditions for *Bt* strain H14. Das and Danker [29] showed that anoxic and water stresses favoured production of spores and crystals at an early stage of growth. Wang *et al*. [30] disclose gene expression and regulatory mechanisms associated with spores and parasporal crystal formation based on transcriptomic and proteomic data of a *Bt* strain.

5. Conclusions

Briefly, in this study we showed that SSF facilitates the maximum yield of endospore and δ-endotoxin without agitation at decreased production time; we assume that it was due to the anoxic condition and water stress prevailed in the solid medium.

The maximum yield of endospore concomitantly δ-endotoxin during SSF was observed in 30% soybean flour at 48 h, *i.e.* with 24 h less gestation period, thus also reducing production cost. Maximum spore size observed in LB was at 72 h and that of SSF was at 48 h.

Acknowledgements

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