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EXPERIMENTAL ARTICLES

Dual Production of Amylase and δ-Endotoxin by *Bacillus thuringiensis* subsp. *kurstaki* during Biphasic Fermentation[1]

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Abstract—This study examined the efficacy of a *Bacillus thuringiensis* (*Bt*) strain in producing amylase (EC 3.2.1.1) as a by-product without affecting its unique ability for producing δ-endotoxin, thus to establish a cultivation strategy for the dual production and recovery of both δ-endotoxin and amylase from the fermented medium with an industrial perspective. LB medium was individually supplemented (5 to 100%, wt/vol) with flour from six naturally available starchy stored foods (banana, Bengal gram, jack seed, potato, taro or tapioca); after initial fermentation (12 h), the supernatant in the medium obtained by centrifugation (1000 *g*, 10 min) was used for harvesting amylase and the resultant pellet was further incubated aseptically for the production of endospores and δ-endotoxin by solid-state fermentation. Maximum crude amylase activity (867 U/gram dry substrate, 12 h) was observed in potato flour-supplemented medium (10% wt/vol, 12 h), while the activity in LB control was only 4.36 U/mL. SDS-PAGE profile of the crude (supernatant), as well as partially purified (40–60% $(NH_4)_2SO_4$ fractionation) amylase showed that its apparent molecular mass was 51 kDa, which was further confirmed by native PAGE. The harvest of industrially significant extracellular amylase (probably α-amylase) produced as a byproduct during early growth phase would boost the economics of the *Bt*-based bio-industry engaged in δ-endotoxin production.

Keywords: Bacillus thuringiensis kurstaki, natural starch substrates, amylase, δ-endotoxin, PAGE

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Owing to the production of parasporal insecticidal crystal proteins, *Bacillus thuringiensis* (*Bt*) has a pivotal status in bioindustry as well as in agriculture. Literature shows that the main focus of *Bt* research is on these insecticidal proteins and allied topics [1, 2]. Production of industrially significant extracellular amylase (EC 3.2.1.1) and protease from the *Bt* culture supernatant offers high industrial potentials. A few unfocused studies showed that *Bt* secreted traces of amylase during its growth in the culture medium [3–5]. Kuppusamy and Balaraman [4] observed traces of extracellular proteolytic and amylolytic activities of *Bt* strain H14 while assessing its ability to utilize the complex carbon and nitrogen sources. Vu et al. [5] reported that *Bt* subspecies *kurstaki* (*Btk*) strain HD1 produced traces of amylase, while investigating the effect of various pH control agents, such as sodium and ammonium acetate and sulfate, upon the biopesticidal activity of the toxin. They showed that amylase production in the culture medium was an important factor, because it could boost the growth and synthesis of δ-endotoxin through hydrolysis of residual starch. Thamthiankul et al. [6] showed that *Bt* subsp. *pakistani* was toxic to *Aedes aegypti* larvae only in the presence of an extracellular chitinase. However, apart from the insecticidal toxins, the efficiency of *Bt* and its strains for the production of extracellular enzymes, which could be useful in the industry, is not documented globally with an industrial perspective.

Production of *Bt*-based insecticides involves liquid or submerged fermentation culture (SmF) of bacteria and recovery of the biologically active proteinaceous ingredients as the biopesticide [7]. Conventionally, commercial production of *Bt*-toxin has been achieved by SmF, by batch or fed-batch processes [5]. SmF has traditionally been used for the production of industrially important enzymes, because of its easiness in handling and greater control of environmental factors such as agitation, temperature and pH [8]. Of late, the utilization of agro-industrial residues as substrates for the fermentation attracted growing interests, as they are inexpensive energy-rich sources; it also partially eliminates large-scale accumulation of such residual biomass from the environment [9].

Many investigators developed the fermentation strategies for production of *Bt*-toxin using different natural substrates, like waste water sludge [10], starch industry waste water [5], extract of bird's feather or corncob [11], etc. All these formulae were enriched liquid media for SmF. In fact, solid-state fermentation (SSF) offers many advantages over the conventional SmF for the production of both primary and secondary metabolites of microbial origin [12, 13]. The SSF

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technique is mainly confined to the bioprocesses involving fungi [14]. Because of higher water activity requirements, it is believed that SSF technique is not suitable for bacterial cultures. However, successful bacterial growth by using the SSF technique is known for the natural fermentation [14–16]. Agricultural substrates like corn, wheat, sorghum and other cereal grains contain about 60 to 75% of starch hydrolysable to glucose and offer a good raw material for many fermentation processes [16]. To meet the growing demand for the microbial products in industry, it is necessary to improve the performance of the production system and thus to increase the yield without increasing its production cost.

Based upon the aforesaid background, this pioneering study evaluates how efficiently valuable amylase could be recovered from the medium in which *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) is grown, without affecting the yield of δ-endotoxin. Thus, the specific aims were the following: (a) to examine the ability of *Btk* to efficiently produce amylase as a byproduct without affecting its unique role for producing δ-endotoxin; (b) to examine the efficacy of natural stored food as a supplement to the LB medium for the dual production of amylase and δ-endotoxin; (c) to establish a cultivation strategy for the dual production of amylase and δ-endotoxin; and (d) to purify the amylase produced.

MATERIALS AND METHODS

Source of organism. The standard culture of *Bacillus thuringiensis* subsp. *kurstaki* was obtained from the Institute of Microbial Technology, Chandigarh, India (strain: BA 83B; MTCC No. 868) and maintained on the conventional Luria-Bertani (LB) medium (initial pH, 7.0).

Media and biphasic fermentation strategy. Fermentation was carried out in two ways, i.e., conventional submerged or liquid fermentation (SmF) and biphasic solid-state fermentation (SSF). The medium used in the former strategy is designated as medium 1 (M1) and the latter as medium 2 (M2). For making M1, either the standard LB medium with no further supplements or LB supplemented with soluble starch (0.5 to 15%, wt/vol) was used. M1 was autoclaved (1 atm at 121°C for 20 min) prior to inoculation and incubated at 37°C (initial pH, 7.0) with constant shaking (140 rpm) for 72 h in an environmental shaker (Scigenics Biotech, India).

For making M2, the standard LB medium was supplemented with one of the starch-containing flours, the powdered natural stored foods. The natural raw starch sources used in this study were: potato flour (PF), tapioca flour (TF), Bengal gram flour (BgF), jack seed flour (JF), taro flour (TaF) or banana flour (BF); the air-dried raw tuber or seed was ground into flour using a mixer-grinder. Their concentration in the medium was 5, 10, 20, 30, 40, 50, 60, 80 or 100% (wt/vol). M2 was autoclaved (1 atm, 121°C, 20 min) prior to inoculation and incubated at 37°C (initial pH 7.0) with constant shaking (120 rpm) in an temperature-controlled shaker (Scigenics Biotech, India). This initial liquid/semi-solid fermentation was performed for up to 12 or 18 h, depending on the time of maximum amylase production.

Analytical and bacteriological grade chemicals from Chromous (India), Genei (India), Himedia (India), Merck India Ltd., Qualigens (India) and Sigma-Aldrich (United States) were used for the study.

Inoculum. The seed culture (overnight) contained about 6.5×10^7 CFU per mL; 5 μL of this seed culture was used to inoculate 1 mL LB medium for M1 (control) or 1 mL LB used to wet the flour supplements (M2). Both SmF and SSF were carried out in Erlenmeyer flasks.

Supernatant for crude amylase and water-restricted pellet for SSF. For amylase assays, 1 mL (in the case of M1) or 0.5 g (in the case of M2) sample was withdrawn periodically at 6 h intervals. M1 was centrifuged at 9400 *g* (10 min, 4°C) in a refrigerated centrifuge (Remi, India), and the supernatant was collected for amylase assay or harvest.

For preparing the water-restricted pellet (with no free water or with only bound water), 10 g of the partially fermented M2 (after 12 or 18 h fermentation) was spun at 1000 *g* (10 min, 4°C) in a refrigerated centrifuge, the resultant pellet with no free water (termed as water-restricted pellet) was incubated (37°C) further for the production of δ-endotoxin by SSF. This supernatant was centrifuged again (9400 *g*, 10 min, 4°C) to get a clear solution for amylase assay or harvest. The first phase of M2 until the collection of the supernatant was the liquid or semisolid phase and further incubation of the pellet for sporulation and concomitant production of δ-endotoxin was SSF.

Amylase assay. Amylase was assayed by the 3,5-dinitrosalicylic acid (DNS) method of Ezeji and Bahl [17]. The amylase activity from M1 was calculated in terms of international units per mL of supernatant (U/mL) using the formula,

$$\frac{\Delta E \times Vf}{\Delta t \times \Sigma \times Vs \times d},$$

where ΔE is absorbance at 540 nm, Vf is the final volume including the DNS, Vs is the volume (mL) of the enzyme solution, t is the reaction time, Σ is the extinction coefficient of D-glucose, and d is the diameter of the cuvette. One U/mL of amylase activity is defined as the amount of protein (amylase) in one gram dry substrate (gds) required to liberate 1 μmol (0.18 mg equivalence) of reducing sugar (D-glucose) from starch per minute under the assay conditions.

For accuracy, the amylase activity of M2 was expressed in grams per dry substrate (gds), using the formula,

$$\frac{\Delta E \times Vf \times Df}{\Delta t \times \Sigma \times Vs \times gds \times d},$$

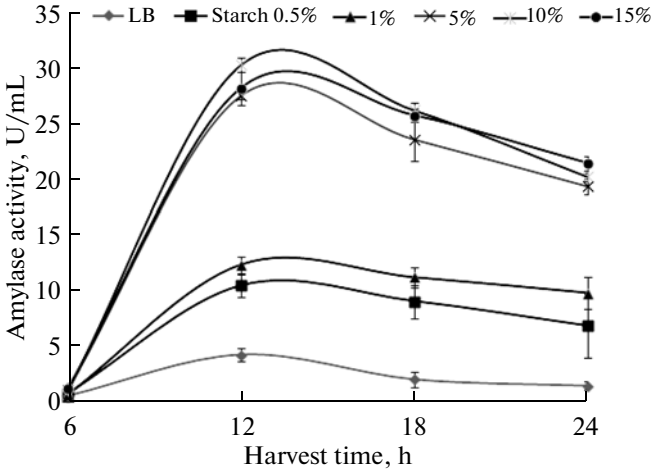

Fig. 1. Amylase activity of *Btk* in LB medium (control) on LB medium supplemented with soluble starch (0.5, 1, 5, 10, or 15% wt/vol). *Btk* was grown under SmF (140 rpm, 37°C), and amylase activity in the liquid broth was assayed in the supernatant (9400 *g*, 10 min, 4°C) at 6 h intervals (whole flask harvest).

where (in addition to the designations in the previous formula), gds (gram dry fermented substrate) is the total mass of the dried solid-fermented matter (80°C for 24 h) and *Df* is the dilution factor. Briefly, it is equivalent to the extracellular amylase present in the liquid part of 1 mL LB + supplement (natural substrate) in the medium, extracted by centrifugation after specific period of fermentation (1000 *g* for 10 min at 4°C).

Partial purification of amylase. Amylase was purified by the method of Ezeji and Bahl [17]. The strategy included ammonium sulfate fractionation, followed by dialysis using the cellulose membrane. Finely powdered ammonium sulfate was added slowly into the crude enzyme preparation up to 80% saturation (0–20, 20–40, 40–60 and 60–80%). The purity was judged by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18] using a vertical mini gel (8 × 7 cm) slab with notched glass plate system (BioTech, India). Gels of 1.5 mm thickness were prepared for the whole study.

Native PAGE. The modified method of Wisessing et al. [19] was used for the Native PAGE. Briefly, α-amylase activity was detected on 8% polyacrylamide gel impregnated with 0.5% starch. Protein was placed on the native PAGE and run at 150 V for 1 h. Then the gel was immersed in the optimum pH buffer (sodium phosphate buffer pH 6.9) for 1 h, followed by 2 min washing with distilled water. The gel was stained with Lugol's iodine (10 mM iodine in 14 mM potassium iodide) for 5 min. Excess iodine was washed off with cold distilled water and the gel was soaked in 1% acetic acid for visualization of the α-amylase activity band and recording.

Delta-endotoxin and Scanning Electron Microscopy (SEM). Samples from the water-restricted wet pellet were withdrawn at 12 h intervals resuspended in 10 mL sterile ddH_2O (4°C), and centrifuged. The pellet collection and the resuspension-centrifugation procedure were repeated four times. All centrifugation steps were at 9.400 *g* for 10 min (4°C). Finally, the pellet was collected and stored overnight at 4°C for bursting the sporangial cells to liberate endospores. This pellet was used for the analysis by SEM facilities available at the Sree Chitra Tirunal Institute of Medical Sciences and Technology, Thiruvananthapuram (JEOL, JWS 3000, United States) and also with National Institute of Technology (Hitachi, SU 6000, Japan) in Calicut.

Statistics. For accuracy, all experiments were conducted 3 to 5 times. SD is given wherever necessary. The LB control value was subtracted from the results (for amylase activity from the pellet) presented in this report.

RESULTS

Amylase production in liquid media. The primary objective of this study was to check whether *Btk* is capable of producing significant amount of extracellular amylase as a byproduct useful to industry, without affecting the production of δ-endotoxin during the course of fermentation. As a prerequisite, we assayed the control in LB medium for amylase activity, which showed a maximum activity of 4.36 U/mL at 12 h (Fig. 1). We also examined the efficacy of the commercially available soluble starch as a supplement to the LB medium in enhancing amylase production by *Btk*. Of various concentrations of commercial starch tested, LB supplemented with 10% starch showed maximum activity (28.89 U/mL) at 12 h (Fig. 1). Then we proceeded with the six natural substrates (flours) as supplement to LB.

Efficacy of natural substrates. Amylase activity was studied using six natural raw starch sources, i.e., flour from potato (PF), tapioca (TF), Bengal gram (BgF), jack seed (JF), taro (TaF) and banana (BF). LB media supplemented with these substrates were designated as M2. For making solid media, the LB medium was supplemented (wt/vol) with one of these starch flours at 5, 10, 20, 30, 40, 50, 60, 80, or 100% (wt/vol). In all cases, amylase activity was maximum at 12 h with 10% (wt/vol) concentration of starch, except for BgF (Fig. 2). M2 became more and more viscous upon increasing the concentration of the starchy supplement.

Of six natural starch sources tested, as shown in Fig. 2, maximum amylase activity (867 U/gds) was observed in PF-supplemented medium (10%) at 12 h. This was the highest activity among six natural substrates tested. TF-supplemented (10%) LB medium showed the second highest (481 U/gds at 12 h) amylase activity (Fig. 1), maximum amylase activity in the BgF-supplemented medium was 56 U/gds (18 h) at its

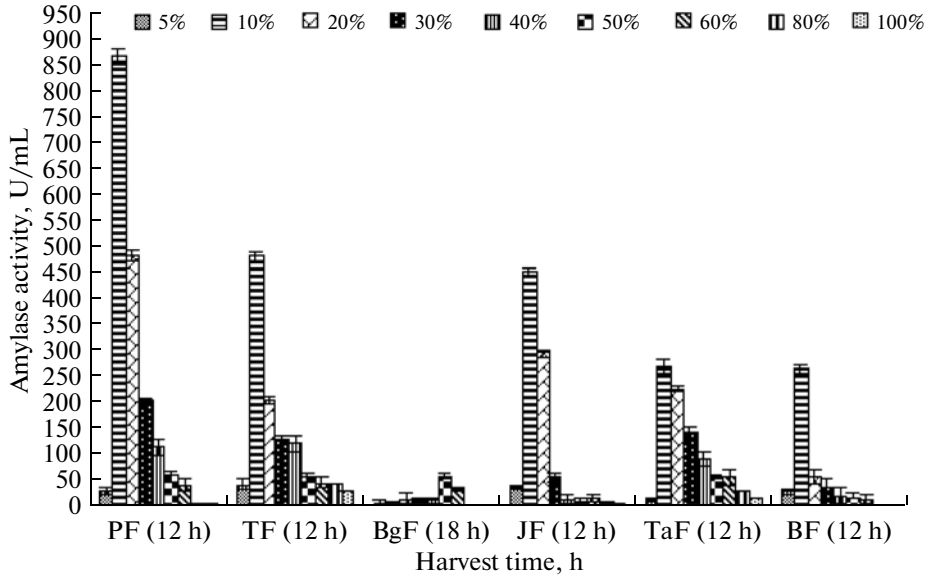

Fig. 2. Amylase activity profile upon SSF. The LB medium was supplemented with various concentrations (5, 10, 20, 30, 40, 50, 60, 80, or 100%, wt/vol) of the flour from 6 stored natural raw foods, i.e., potato flour (PF), tapioca flour (TF), Bengal gram flour (BgF), jack seed flour (JF), taro flour (TaF) or banana flour (BF). At 12 h intervals, the fermented matter (whole flask) was centrifuged (1000 *g* for 10 min) to separate the supernatant from the wet pellet. The supernatant so obtained was centrifuged again (9400 *g*, 10 min, 4°C) to obtain a clear solution containing crude amylase. The clear supernatant thus obtained was used for amylase activity assay.

50% concentration. JF-supplemented LB medium (10%) showed the amylase activity (452 U/gds) comparable to that of TF. TaF-supplemented medium (10%) showed only 269 U/gds amylase activity (at 12 h). The maximum amylase activity in the BF-supplemented medium was 266 U/gds (12 h) at 10% concentration. In fact, this was the lowest activity among the six natural substrates tested. Interestingly, out of six supplements, five natural substrates (except BgF) at 10% (wt/vol) showed maximum amylase activity, all at 12 h fermentation. The flour-supplemented solid at this stage was in the semi-solid and viscous from (Fig. 3).

Partial purification of amylase. Since PF-supplemented medium showed the highest amylase activity, we opted PF for the partial purification of amylase. Figure 4 clearly shows an amylase active fraction with an apparent MW of 51 kDa in the crude supernatant as well as the 40–60% fraction, precipitated by $(NH_4)_2SO_4$.

Production of *Btk* toxin by SSF. We also made a preliminary evaluation of the δ-endotoxin produced by *Btk* on the free water-restricted solid pellets (the pellets obtained after removing the supernatant for amylase harvesting). Interestingly, at 36 h incubation of the pellet (excluding initial 12 h fermentation prior to amylase harvesting), a large quantity of crystals and spores with a very few vegetative cells were found tangled in the solid medium as shown by SEM images (Fig. 5).

DISCUSSION

Since no specific study is available in the literature on amylases from *Bt* strains, amylase produced by *Btk* is discussed with a focus on amylases from *Bacillus* spp.

SSF holds a tremendous potential for the production of microbial enzymes, and thus it can be of special interest in those processes where the crude fermented products may be used directly as enzyme sources [16]. Bacteria such as *Bacillus amyloliquefaciens*, *B. subtilis*, and *B. licheniformis*, as well as some mycelial fungi are the major producers of amylase for the industry, especially by SmF [16, 20, 21]. Before this study no attempt has been made to produce amylase using *Bt* strains; however; the presence of amylase in the culture medium was analyzed with a view to study the efficiency of δ-endotoxin produced by *Bt* [5]. Moreover, the raw starch substrates used in this study have been rarely used for the cultivation of other bacterial or fungal species for producing various enzymes.

In the present study, we used seven starch sources: a commercially available soluble starch and six natural raw starches. Being stored food (as tubers and seeds), the natural raw starches generally offer a balanced nourishment for human consumption and thus, they should contain many growth factors apart from the major nutrient starch [15], hence these substrates were tested as supplements in LB medium for the cultivation of *Btk*. This fact is evident from the comparison of amylase activity in the media, for instance, PF-supplemented medium showed 867 U over the commercially available soluble starch (29 U), both at

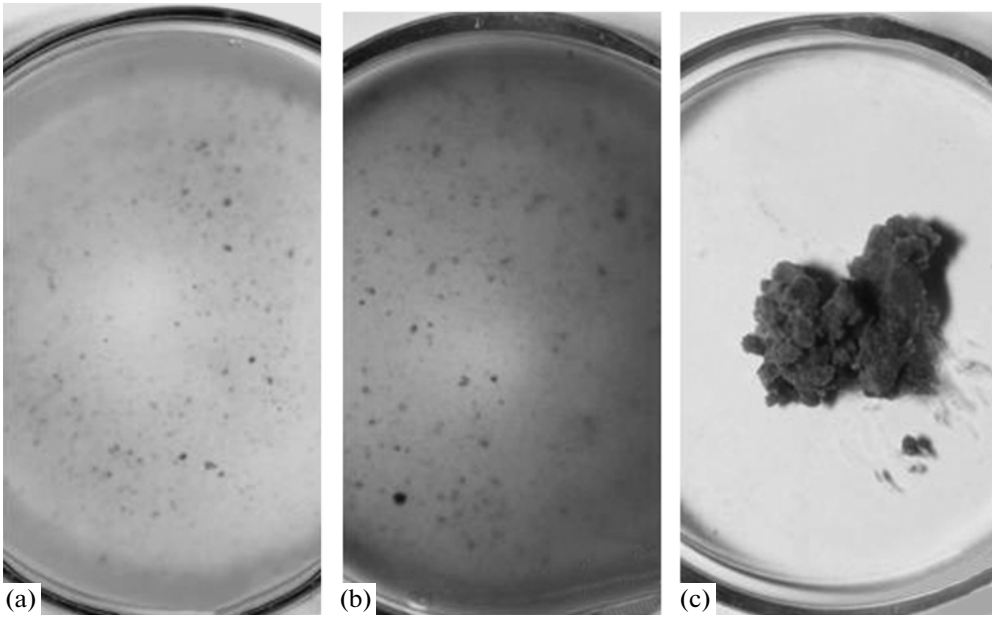

Fig. 3. Texture of LB supplemented with 10% (wt/vol) potato flour (M2). (a) Texture of the autoclaved (15 psi, 121°C, 20 min) M2 prior to incubation (125 rpm, 37°C); (b) texture of 12 h old M2 (with color change) prior to centrifugation (1000 *g* for 10 min) to separate supernatant for harvesting amylase and water-free wet solid pellet; (c) solid pellet obtained as above was incubated (37°C) at static condition for enhancing *Btk* toxin yield (image at 48 h).

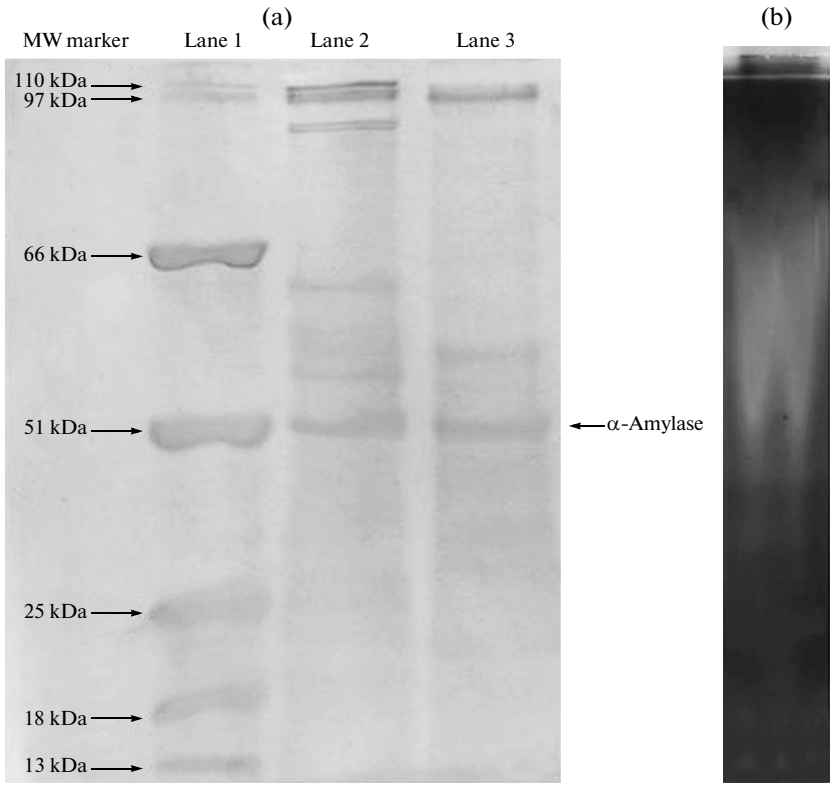

Fig. 4. Profile of partially purified amylase. (a) Non-native SDS-PAGE profile of the partially purified amylase fraction (40–60% ammonium sulfate fraction): lane 1, molecular marker, lane 2, amylase from LB supplemented with 10% soluble starch and lane 3 LB supplemented with 10% (wt/vol) potato flour; and (b) image of native PAGE profile. Amylase activity was detected on 8% polyacrylamide gel impregnated with 0.5% starch, the gel was stained with Lugol's iodine for 5 min. Excess iodine was washed off with cold distilled water and the gel was soaked in 1% acetic acid for visualizing α-amylase activity band.

10% (wt/vol) and 12 h cultivation. The production of α-amylase under SSF using various agricultural substrates was reported by several authors [16, 22, 23]. There are reports in which potato peel was used as a solid substrate in the fermentation medium which resulted in the highest amylase yield among the tested substrates [23]. Shukla and Kar [23] found that potato peel was a better substrate for SSF than wheat bran for the production of α-amylase by *B. licheniformis* and *B. subtilis*. The enzyme activity was 270 U/mL and 600 U/mL, respectively for these commercial α-amylase producers.

No report is available in the literature which documents the purification of amylase produced by *Bt* during SmF or SSF. The present study, therefore, is the first attempt of the purification of *Btk* amylase. The supernatant obtained from 10% (wt/vol) PF supplemented with LB medium after 12 h fermentation was used for the partial purification of amylase. In the present study, the purified amylase (probably α-amylase) from *Btk* showed a prominent protein band on SDS-PAGE with apparent MW of 51 kDa. This finding is in a good agreement with previous reports, such as the study of α-amylase from various *Bacillus* spp. Demirkan et al. [24] obtained α-amylase produced by *B. amyloliquefaciens* and the MW of the purified enzyme was estimated as 52 kDa according to SDS-PAGE. Faber et al. [25] reported that the MW of α-amylase from *B. licheniformis* was 55 kDa. Bernhardsdotter et al. [26] purified α-amylase from *Bacillus* sp. strain L1711 and its MW was 51 kDa. The α-amylase from *B. subtilis* WB600 was purified by NH_3SO_4 fractionation, anion exchange and gel filtration and the MW of the purified enzyme was 53 kDa. [27] It indicates that the MW of α-amylase secreted by the *Bacillus* spp. varies from 50 to 55 kDa. These results confirm our assumption that the amylase active fraction presented in this paper is α-amylase.

Another crucial factor which needs discussion is the gestation time for the production of *Btk* crystals. In control (LB medium) it took about 72 h for the maximum release of the crystals from the *Bt* cells [28], while in 10% starch-supplemented media, it took only 48 h (including 12 h initial incubation prior to enzyme harvesting) for the maximum release of the crystals from the water-restricted pellet by SSF; thus the production time was reduced by 24 h. Sporulation and crystal production are associated with the nutritional status in the medium [29]. It is expected that the raw starch supplement used in the present study (which was partially digested by the amylase secreted by *Btk*) upon initial fermentation, as well as traces of amylase available in the medium even after harvesting might offer a balanced diet for the optimum growth of *Btk*. After enzyme harvesting, the pellet was in a clogged stage with restricted air spaces. Sarrafzadeh et al. [28] found that aeration plays a crucial role in sporulation. They showed the highest rate of sporulation in the absence of oxygen and the mature spores were the only cell forms present in these conditions at the end of cultivation, while the sporulation in a significant part of the cells failed under saturated oxygenation and either mature spores or vegetative cells were present at the end of cultivation.

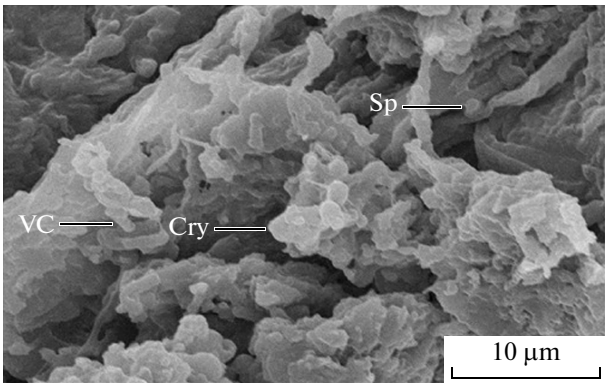

Fig. 5. The SEM image of water-free solid fermented matter (M2) obtained after SSF showing many endospores (Sp) and δ-endotoxin (Cry), together with a very few vegetative cells (Vc) tangled in the solid matter. LB was supplemented with 10% (wt/vol) potato flour and autoclaved (15 psi, 121°C, 20 min), inoculated with *Btk*; after 12 incubation (125 rpm, 37°C), the viscous semisolid fermented medium was centrifuged (1000 *g* for 10 min) to separate the water-free wet solid pellet from the supernatant (for harvesting amylase); and solid pellet obtained as above was incubated (for another 36 h at 37°C) at static condition for enhancing *Btk* toxin yield. The raw pellet was processed for the analysis by SEM.

Moreover, it is possible that the water-restricted environment also produced a critical water stress on the cells, which resulted in termination of the exponential growth phase much earlier, and, thus, maximum spores and crystals were produced 24 h earlier in the water-restricted pellets. According to Gitahy et al. [30], the optimum time for the release of *Bt* spores and crystals is 72 h; but during SSF, the stress caused by the water-restricted environment could considerably reduce the time of microbial growth [14]. Nevertheless, the *Btk*-toxin yield was not affected by SSF technique. SSF strategy adopted in the present study restricted the availability of oxygen to the cells; the stress induced by oxygen starvation initiated to end of *Btk* growth cycle at early stage after producing spores and crystals.

Engineering of cultivation strategy as described in this study is unique, reproducible, and industrially useful. The *Bt* industry normally discards the supernatant after the harvesting of δ-endotoxin. If the strategy that we demonstrated in this work is adopted for coupled production of industrially significant amylase and δ-endotoxin, the production costs could be minimized, which would in turn boost the δ-endotoxin industry further.

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REFERENCES

1. Schnepf, E., Crickmore, N., Van, R.J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., and Dean, D.H., *Bacillus thuringiensis* and its pesticidal crystal proteins, *Microbiol. Mol. Biol. Rev.*, 1998, vol. 62, pp. 775–806.
2. Soberon, M., Gill, S.S., and Bravo, A., Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells?, *Cell. Mol. Life. Sci.*, 2009, vol. 66, pp. 1337–1349.
3. Li, E. and Yousten, A., Metalloproteases from *Bacillus thuringiensis, Appl. Microbiol.*, 1975, vol. 30, pp. 354–361.
4. Kuppusamy, K. and Balaraman, K., Extra cellular hydrolytic enzyme secretion in *Bacillus thuringiensis* H14 & *B. sphaericus* and their significance in media design, *Indian. J. Med. Res.*, 1990, vol. 91, pp. 149–150.
5. Vu, K.D., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y., Batch and fed-batch fermentation of *Bacillus thuringiensis* using starch industry wastewater as fermentation substrate, *Bioprocess. Biosyst. Eng.*, 2010, vol. 33, pp. 691–700.
6. Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S., and Panbangred, W., Chitinase from *Bacillus thuringiensis* subsp. *pakistani*, *Appl. Microbiol. Biotechnol.*, 2001, vol. 56, pp. 395–401.
7. Bryant, J.E., Commercial production and formulation of *Bacillus thuringiensis, Agric Ecosys. Environ.*, 1994, vol. 49, pp. 31–35.
8. Benjamin, S. and Pandey, A., Optimization of liquid media for lipase production by *Candida rugosa, Biores. Technol.*, 1996, vol. 55, pp. 167–170.
9. Arikan, B., Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate isolate A3-15, *Biores. Technol.*, 2008, vol. 99, pp. 3071–3076.
10. Yezza, A., Tyagi, R.D., Valero, J.R., and Surampalli, R.Y., Production of *Bacillus thuringiensis*-based biopesticides in batch and fed batch cultures using wastewater sludge as a raw material, *J. Chem. Technol. Biotechnol.*, 2005, vol. 80, pp. 502–510.
11. Poopathi, S. and Archana, B., Production of *Bacillus thuringiensis* subsp. *israelensis* from agro-based product (concob), *Curr. Sci.*, 2011, vol. 101, pp. 1009–1010.
12. Benjamin, S. and Pandey, A., *Candida rugosa* lipases: Molecular biology and versatility in biotechnology, *Yeast*, 1998, vol. 14, pp. 1069–1087.
13. Pandey, A., Soccol, C.R., and Soccol, V.T., Biopotential of immobilized amylases, *Indian J. Microbiol.*, 2000, vol. 40, pp. 1–14.
14. Pandey, A., Solid-state fermentation, *Biochem. Eng. J.*, 2003, vol. 13, pp. 81–84.
15. Mussatto, S.I., Ballesteros, L.F., Martins, S., and Teixeira, J.A., Use of agro-industrial wastes in solid-state fermentation processes, in *Industrial Waste*, Show, K.Y. and Guo, X., Eds., Croatia: InTech, 2012, pp. 121–140.
16. Benjamin, S., Smitha, R.B., Jisha, V.N., Pradeep, S., Sajith, S., Sreedevi, S., Priji, P., Unni, K.N., and Sarath Josh, M.K., A monograph on amylases from *Bacillus* spp., *Adv. Biosci. Biotechnol.,* 2013, vol. 4, pp. 227–241. doi: 10.4236/abb.2013.42032
17. Ezeji, T. and Bahl, H., Purification, characterization and synergistic action of phytate resistant alpha amylase and alpha glucosidase from *Geobacillus thermodenitrificans* HRO10, *J. Biotechnol.*, 2006, vol. 125, pp. 27–38.
18. Laemmli, U., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 1970, vol. 277, pp. 680–685.
19. Wisessing, A., Engkagul, A., Wongpiyasatid, A., and Chuwongkomon, K., Purification and characterization of *C. maculatus* α-amylase, *Kasetsart. J. (Natural Science)*, 2008, vol. 42, pp. 240–244.
20. Konsula, Z. and Liakopoulou-Kyriakides, M., Hydrolysis of starches by the action of alpha amylase from *Bacillus subtilis, Process. Biochem.*, 2004, vol. 39, pp. 1745–1749.
21. Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K.M., Sukumaran, R.K., and Pandey, A., Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens, Biores. Technol.*, 2008, vol. 99, pp. 4597–4602.
22. Baysal, Z., Uyar, F., and Aytekin, C., Solid state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water, *Proc. Biochem.*, 2003, vol. 38, pp. 1665–1668.
23. Shukla, J. and Kar, R., Potato peel as a solid state substrate for thermostable α-amylase production by thermophilic *Bacillus* isolates, *World J. Microbiol. Biotechnol.*, 2006, vol. 22, pp. 417–422.
24. Demirkan, E.S., Mikami, B., Adachi, M., Higasa, T., and Utsumi, S., Alpha amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli, Proc. Biochem.*, 2005, vol. 40, pp. 2629–2646.
25. Faber, C., Hobley, T.J., Mollerup, J., Thomas, O.R.T., and Kaasgaard, S.G., Study of the solubility of a modified *Bacillus* licheniformis α-amylase around the isoelectric point, *J. Chem. Eng. Data*, 2007, vol. 52, pp. 707–713.
26. Bernhardsdotter, E.C.M.J., Ng, J.N., Garriott, O.K., and Pusey, M.L., Enzymic properties of an alkaline chelator resistant alpha amylase from an alkaliphilic *Bacillus* sp. isolate L 1711, *Proc. Biochem.*, 2005, vol. 40, pp. 2401–2408.
27. Liu, X.D. and Xu, Y., A novel raw starch digesting alpha amylase from a newly isolated *Bacillus* sp. YX-1: purification and characterization, *Biores. Technol.*, 2008, vol. 99, pp. 4315–4320.
28. Sarrafzadeh, M.H., Guiraud, J.P., Lagneau, C., Gaven, B., Carron, A., and Navarro, J., Growth, sporulation, δ-endotoxins synthesis, and toxicity during culture of *Bacillus thuringiens* H14, *Curr. Microbiol.*, 2005, vol. 51, pp. 75–81.
29. Rajalekshmi, S. and Shethna, Y.I., Spore and crystal formation in *Bacillus thuringiensis* var. *thuringiensis* during growth in cystine and cysteine, *J. Biosci.*, 1980, vol. 2, pp. 321–328.
30. Gitahy, P.M., de Souza, M.T., Monnerat, R.G., Arrigoni, E.B., and Baldani, J.I., A Brazilian *Bacillus thuringiensis* strain highly active to sugarcane borer *Diatraea saccharalis* (Lepidoptera: Crambidae), 2007, *Braz. J. Microbiol.,* 2007, vol. 38, pp. 56–58.