11. SUMMARY AND CONCLUSIONS

11.1. Introduction

*Bacillus thuringiensis* (Bt) is a Gram-positive, sporulating and mostly soil-dwelling bacterium. Owing to the production of insecticidal crystal proteins (the Cry proteins), *Bt* is popularly known as a biopesticide. This δ-endotoxin is the active ingredient of most (90%) of the microbial insecticides produced in the world. It also resides naturally in the caterpillars of some moths and butterflies, as well as on the surface of plants. Some commercially available subspecies/varieties of *Bt* include: *Bt aizawai*, *Bt israelensis*, *Bt. kurstaki* (*Btk*), *Bt sandiego* and *Bt tenebrionis*. Bt-toxin is a mixture of endospores and Cry proteins. During sporulation, *Bt* cells produce parasporal protein inclusions juxtaposed to the endospore. These inclusions consist of one or more insecticidal protoxic proteins (insect crystal proteins or δ-endotoxin) in the form of a crystal or crystal-complex. Upon consumption by the insect, these protoxins are cleaved by protease in the insect gut to form active toxin, which kills the insect. The acidic environment created by the destruction of gut cells (by influx of ions) is utilised by the endospores for germination and subsequent attack.

Main focus of all *Bt*-based studies has been to produce the biopesticide. The δ-endotoxin found as a crystalline inclusion in the insect pathogen *Btk* has potent insecticidal activity toward lepidopteran pests including the gypsy moth, spruce budworm, and hemlock looper. Of late, there are inconclusive reports on the efficacy of *Btk* against the eriophyid coconut mite, *Aceria guererronis* syn *Eriphyes guererronis*. This pest (known as *mandari* in Malayalam) is a menace to coconut cultivation in the world, especially in Kerala and Tamilnadu.

Industrially significant extracellular amylases are neither produced nor characterised from *Bt* yet. In the process of harvesting the δ-endotoxin, supernatant containing amylases and other extracellular enzymes is left out as effluent by the *Bt* industries. If such enzymes also purified from the effluents as a by-product and
made available on a commercial level, the Bt market would become more attractive as it offers additional income. Thus, founded on solid-state fermentation, the specific objectives of this study are: (a) to produce, purify and characterise α-amylase from the culture broth/supernatant of Btk, (b) establishment of a fermentation strategy to maximising the production of Btk toxins (δ-endotoxin/Cry protein), and (c) to study the efficacy of Btk toxins (δ-endotoxin) to combat A. guerreronis (mandari), which severely damaging coconut palms and yield.

The thesis contains eleven chapters, viz., (1) introduction, (2) literature review, (3) materials and methods, (4) α-amylase production by Submerged Fermentation (SmF), (5) amylase production by Solid-State Fermentation (SSF), (6) purification and characterisation of α-amylase, (7) production of endospore, (8) production and purification of Btk toxins, (9) efficacy studies of Btk toxins to combat A. guerreronis, (10) general discussion, (11) summary and conclusions, followed by bibliography, and publications as appendix.

11.2. Summary of major findings is given below.

11.2.1. α-Amylase production by submerged/ liquid fermentation (SmF)

The standard strain Bacillus thuringiensis subsp. kurstaki was procured from Institute of Microbial Technology (IMTECH), Chandigarh (Strain designation: BA 83B and MTCC No. 868). Growth characteristics (λ = 600 nm) showed that the log phase was extended between 6 – 22 h. Under SmF, α-amylase production was accomplished by supplementing 1% (w/v) soluble starch (Merck) as supplement in the Luria-Bertani (LB) medium. 12 h seed culture (inoculum) containing about 6.2 x 10^5 cfu (colony forming units) was added per 1mL medium, and 0.5 mL samples were withdrawn for assay at 6 h intervals. α-Amylase activity in the supernatant was assayed by Dinitrosalicylic Acid (DNS) method. Maximum amylase activity in the starch supplemented medium was about 11.21 U/mL after 12 h growth, while the control showed only about 4.36 U/mL activity. When the soluble starch was replaced by various raw starch supplements [1% (w/v)], viz., banana powder, jack seed powder, potato powder, tapioca powder, taro powder and bengal gram powder; maximum activity (13.19 U/mL) was obtained with tapioca powder, while the least
activity (1.9 U/mL) was obtained with bengal gram powder supplement. Thus, the SmF study clearly suggests that some naturally available crude starches were suitable raw supplements for amylase production by \textit{Btk}.

\subsection*{11.2.2. Amylase production by Solid (or semi-solid) State Fermentation (SSF)}

Soluble starch (5, 10, 15, 20, 25 - all in \% w/v) was supplemented to the LB medium for enrichment and incubated (37 °C) in an incubator. Data showed that maximum \(\alpha\)-amylase activity (in units per gram dry substrate, U/gds) was obtained with 10\% (w/v) starch (27.51 U/gds for 5\%, 28.59 U/gds for 10\%, 28.08 U/gds for 15\%, 26.98 U/gds for 20\% and 25.79 U/gds for 25\%). For maximising the \(\alpha\)-amylase production, the LB medium was supplied with various locally available raw starch substrates such as banana powder, jack seed powder, potato powder, tapioca powder, taro powder and bengal gram powder with different concentrations (w/v), \textit{i.e.}, 5\%, 10\%, 20\%, 30\%, 40\%, 50\%, 60\%, 80\%, 100\%. Data showed increased \(\alpha\)-amylase activity (12 h cultivation) at 10\% and gradually decreased from 20\% to 100\%. Time dependence data showed an increased \(\alpha\)-amylase activity at 12 h and gradually deceasing from 18 h to 24 h. At 12 h, all the supplements induced maximum \(\alpha\)-amylase activity. Of these six raw starch supplements, 10\% (w/v) potato powder induced maximum \(\alpha\)-amylase activity (867.55 U/gds at 12 h harvest), while bengal gram yielded the least activity (4.31 U/gds).

\subsection*{11.2.3. Purification and characterisation of amylase}

From the \(\alpha\)-amylase production profiles by SmF and SSF, amylase produced by supplementing the LB medium with 10\% (w/v) potato powder (which supported maximum activity) was chosen for purification. Ammonium sulphate fractionation, dialysis, Sephadex G100 gel permeation chromatography were the methods used for purifying the enzyme. 40-60\% ammonium sulphate fraction was used for the characterisation of the enzyme. Upon characterisation, effects of pH, temperature and metal ion concentration were studied, and their optima were: 6.0 pH, 60 °C and 3 \(\mu\)M Ca\(^{2+}\), respectively. Complex chemicals like EDTA and SDS adversely affected the enzyme activity and \(\beta\)--mercaptoethanol completely abolished the activity. Under standardised assay conditions (pH 6.0, temperature 60 °C, 3 \(\mu\)M Ca\(^{2+}\), 2.5\% starch,
incubation 5 min), the crude extract showed a specific activity of 864 U/mg crude protein; 2983 U/mg by ammonium sulphate fraction and 12,751 U/mg activity by Sephadex G-100 column fraction. SDS-PAGE profile of the Sephadex G-100 column active fraction showed that the apparent molecular weight (MW) of the purified α-amylase was about 51kDa (14.75 fold purity). The $K_m$ and $V_{max}$ values were calculated as 2.9 mg/mL and 0.05335 μmol/mL/min by using the software Hyper 32.

**11.2.4. Production of endospore**

Endospore production was studied in the cultures supplemented by % (w/v) of jack seed powder, potato powder and tapioca powder. The spore production was studied for 3 days and the samples were withdrawn in 6 h intervals. Malachite-green was used for staining endospores and photographed by Image Analyser (Nikon Eclipse E 400, Towa Optical, Japan) fitted with Nikon (DXM 1200F, Japan) digital camera. Endospore production was noticed in the samples at 12 h, which attained optimum at 48 h and continued upto 72 h. Release of endospores from the cell was also optimum at 48 h in the starch supplemented media. But in the control, maximum spore release was noticed at 72 h culture. The spores showed about 1.5 – 2.0 μM length and 0.75 - 1μM breadth. Spore production was also studied by staining with acridine orange, a fluorescent dye, and photographed by Olympus fluorescent microscope fitted with Nikon digital camera (Japan). This data was in corroboration to that obtained by malachite-green staining.

**11.2.5. Production of Bt-toxin (δ-endotoxin/ crystal protein)**

*Bt*-toxin production was also studied with media supplemented by 10% (w/v) jack seed powder, potato powder and tapioca powder and samples were withdrawn at 6 h intervals. Malachite-green and safranin were used to stain crystals and photographed using Image Analyser (Nikon Eclipse E 400, Towa Optical, Japan) fitted with Nikon (DXM 1200F, Japan) digital camera. The presence of crystals was observed at 48 h to 72 h and the maximum crystals were seen at 48 h in the starch supplemented medium and progressed upto 72 h. Photomicrographs taken
by Scanning Electron Microscope (SEM) (JEOL, JWS 3000) also substantiated the above results. The shape of the crystals was cuboidal, bipyramidal, rhomboidal or spherical.

For reducing the harvesting time of crystal, the supernatant was discarded from the fermentation medium at 12 h by centrifugation (800 g for 10 min) and the pellets were incubated further to monitor the crystal production. 12 h pellet contained only vegetative bacteria and at 24 h, large amount of crystal production was observed, which was comparable to those obtained at 48 h of normal SSF. This observation was confirmed by SEM images. The crystals were purified in a discontinuous sucrose gradient. The purified crystals were analysed using 12% SDS-PAGE gel. SDS-PAGE profiled the crystal protein fractions of various MWs.

11.2.6. Efficacy studies of δ-endotoxin against mandari

Coconut buttons of about one month old were collected from the uninfected coconut palms of about 10 M height during March-May (~32 °C). For standardisation of the growth of the mites (Aceria guerreronis) in lab conditions, hundreds of coconut buttons were collected every week and cultured providing suitable temperature (30 °C) and water (for keeping the buttons alive). The control cultures showed all the three growth stages of the mites, i.e., adult nymph, eggs, first nymph and second nymph. After standardisation, adult mites (~ 2µ length) were transferred from the infected coconut palms and brushed on to the uninfected buttons under culture. About 45 mites per cm² were transferred on the top of the buttons (in the region where the cap was removed). The finely powdered Bt-toxin containing raw fermented matter was applied on the top of the buttons at different concentrations (µg/ cm²), i.e., 1.25, 1.88, 2.5, 3.13 or 3.73. The cultures were kept in an incubator at 30 °C for 10 days. In the control buttons, some mites (91%) were dead and the remaining mites grew normally and laid eggs, which underwent further developmental stages like first nymph and second nymph. But on the experimental buttons, wherein 1µg/cm² crystals was applied, about 75% of mites were dead in the second day, while in all other treatments, all mites were dead in the second day.
11.3. Conclusions

The present study suggests that *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) is a potential organism for $\alpha$-amylase production as a by-product in the process of *Btk*-toxin production by SSF, and thus for judicious industrial exploitation. Moreover, the *Btk*-toxin produced was found 100% effective to combat *A. guereronis*, a dangerous coconut mite causing great economic loss.

- $\alpha$-Amylase production was maximum, when potato powder (10% w/v) supplemented LB was used as the supplement. This medium composition was better for *Btk*-toxin production also.

- Optimum conditions for maximum $\alpha$-amylase activity were: 6.0 pH, 60 °C temperature, 3$\mu$M Ca$^{2+}$ and 2.5% starch.

- The Sephadex-G100 purification fold was 14.75.

- The $K_m$ and $V_{max}$ values were 2.9 mg/mL and 0.05335 $\mu$mol/mL/min, respectively.

- The *Bkt*-toxin production was optimum at 48 h in the 10% (w/v) potato starch supplemented medium, and size of the crystals varied from cuboidal, bipyramidal, rhomboidal to spherical; while it was maximum at 72 h in the control.

- The *Btk*-toxin production time was reduced to 24 h by the water-restricted cultivation strategy.

- Crude *Btk*-toxin is proved as an effective biopesticide to combat *A. guereronis*.

11.4. Major outcomes

- Demonstration of the production, purification and characterisation of extracellular $\alpha$-amylase from *Btk* for the first time by SSF.
Demonstration of production and purification of *Btk*-toxin from the solid-fermented matter for the first time, and

Demonstration of the efficacy of *Btk*-toxin to combat the coconut mite, *A. guereronis* – a notorious coconut pest for the first time.

11.5. Limitations of the study

In the present study, partially purified α-amylase was used for characterisation studies, compared to commercial strains of *Bacillus* moderate levels of enzyme activity were obtained. Purified enzyme has to be used to fix the exact characteristics of this enzyme, which, no doubt, would be very high.

Purified α-amylase has to be subjected to amino acid sequencing for further confirmation.

Quantification of the crystals (*Btk*-toxins) in large production vessels like fermenters has to be accomplished.

Field trial of *Btk*-toxin on the *A. guereronis* affected coconut palms has to be conducted.